Biomonitoramento de populações humanas através de avaliação de genotoxicidade em áreas sujeitas a risco ecotoxicológico

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Dedico este trabalho aos meus queridos pais, Nanci e Dilson, e ao meu irmão e grande primatólogo Thiago que sempre me apoiaram e acreditaram em mim, mais do que eu mesma o fiz!

"... la primavera ya no es anunciada por la vuelta de los pájaros y las madrugadas son extrañamente silenciosas, donde antes habían sido llenadas por la belleza del canto de los pájaros. Este repentino silenciamiento del canto de los pájaros, esta eliminación del color y la belleza que proporcionan a nuestro mundo se ha dado de forma rápida, insidiosa y ha pasado desapercibida para aquellos cuyas comunidades aún no se han visto afectadas."

Rachel L. Carson
(*Primavera Silenciosa*, 1962)

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Resumo

A emissão de uma grande diversidade de substâncias através dos efluentes industriais gasosos, líquidos ou sólidos acarreta que uma gama de compostos seja introduzida no ambiente atmosférico e aquático de meios urbanos. A exposição a substâncias genotóxicas no ambiente oferece freqüentemente um grande risco à saúde humana. Com essa preocupação, o presente trabalho avaliou a exposição das pessoas à qualidade do ar atmosférico e à da água para abastecimento público de duas cidades urbanas do Rio Grande do Sul (Brasil), que recebem diferentes contribuições antrópicas (cidades-alvo), comparando-as com uma cidade menos impactada - cidade referência. Os extratos orgânicos da água tratada para abastecimento público e das amostras de ar foram testados quanto a mutagenicidade através do ensaio *Salmonella*/microssoma (linhagens TA98 e TA100 para água e ar, com e sem fração de metabolização e, ainda para ar, YG1021 e YG1024) – marcador de mutagenicidade. As amostras de ar ainda foram analisadas por *HPLC* para a caracterização de HPAs e algumas amostras também por *HRGC-HRMS* para a composição dos *PCDD/F*s. Para o biomonitoramento humano foram avaliados através do ensaio do cometa e do micronúcleo (MN), em linfócitos e células da mucosa bucal, respectivamente, as pessoas que viviam nas três cidades – biomarcador de efeito. Análises de polimorfismos genéticos (*CYP1A1, GSTM1* e *GSTT1*) foram utilizados como biomarcadores de susceptibilidade. As duas cidades-alvo apresentaram resultados significativos quanto a mutagenicidade da água de abastecimento apresentando todos os resultados positivos, principalmente para mutação de substituição de pares de bases (TA100), indicando a presença de compostos como THMs e MX. Valores de PTS atmosféricos apresentaram-se em algumas amostragens acima dos limites da legislação brasileira (CONAMA, 1990). Os HPAs encontrados em maior quantidade foram indeno(1,2,3-c,d)pireno e benzo(ghi)perileno, sendo que em uma das amostras a análise dos *PCDD/F*s, mostrou valores que superaram em dez vezes a outra amostra analisada. As duas cidades alvo apresentaram altas respostas mutagênicas nas amostras de ar tanto por mutágenos de ação direta, como indireta e também altas concentrações de nitro-HPAs. Estes resultados sinalizaram para a necessidade de uma revisão da legislação para material particulado atmosférico, já que dentro dos atuais parâmetros, grande quantidade de compostos mutagênicos foram encontrados. Para os indivíduos estudados

quanto aos biomarcadores de efeito, tanto os linfócitos analisados pelo ensaio do cometa quanto às células da mucosa bucal pelo ensaio do MN não apresentaram resultados significativos, sendo que apenas uma cidade alvo apresentou quanto ao ensaio do cometa. Como a maioria dos indivíduos *CYP1A1, GSTM1* e *GSTT1* apresentaram genótipos prevalentes, pode estar ocorrendo uma eficiência no sistema de detoxificação garantidos pela presença desses genótipos. Este trabalho mostrou a importância de estudos ambientais que relacionem marcadores de mutagenicidade com biomarcadores de efeito e de susceptibilidade, para que haja uma maior compreensão dos efeitos da exposição humana diária à agentes genotóxicos ambientais.

Palavras chave: Biomonitoramento humano, água de abastecimento, material particulado atmosférico, HPAs, *PCDD/F*s, teste *Salmonella*/microssoma, ensaio do cometa, ensaio do micronúcleo, polimorfismos.

Abstract

The emission of a great substances amount through gaseous, liquid and solid industrial effluents have been introduced many kinds of chemical compounds in aquatic and atmospheric environments. The environmental exposure to genotoxic substances often brings great risk to human health. Therefore, the aim of this work was make an avaliation of people exposition to atmospheric air and water supply quality in two urban cities in Rio Grande do Sul State (Brazil) which were under influence of different anthropogenic sources (target cities), compared to a less intense influenced urban city (reference city). The organic extracts from supply water and airborne samples were tested to mutagenicity using the *Salmonella*/microsome assay (TA98 and TA100 strains for water and airborne samples, with and without metabolization fraction, and also, YG1021 and YG1024 strains for airborne) – mutagenicity marker. Moreover, the airborne samples were analysed by HPLC to PAHs composition and by HRGC-HRMS to PCDD/Fs composition. Human biomonitoring was evaluated by samples of lymphocytes and bucal mucosa cells from individuals living in this three cities using the comet and the micronuclei (MN) assays, respectively - effect biomarkers. Analysis of gene polymorphisms (*CYP1A1, GSTM1* and *GSTT1*) was taken as susceptibility biomarkers. Both target cities showed significative results for the water mutagenicity with mutagenic responses for all samples, mainly to base-pair substitution mutation (TA100 strain), denoting the presence of THMs and MX compounds. The TSP concentrations were above the Brazilian Legislation and World Health Organization regulation limits few times. The PAHs detected in greater amout were indeno(1,2,3-c,d)pyrene and benzo(ghi)perylene and one of the samples overcame ten times the values of PCDD/Fs compared to the other analysed. The two target cities showed high mutagenicity response in all airborne samples, both by direct and indirect mutagens, besides high concentrations of nitro-PHAs. Actually, these results indicate a law revision necessity, because great amount of mutagenic compounds were assessed in smaller concentration that those present in current legal parameters. The analysed subjects for effect biomarkers, both limphocytes tested by comet assay and buccal mucosa cells tested by MN, did not show significant results, only one city show increased primary DNA damage detected through comet assay. As the majority of subjects *CYP1A1, GSTM1* e *GSTT1* showed prevalent genotype, it maybe occuring an

efficient system detoxification warranted by these genotypes presence. This work has shown the importance of environmental studies that relate mutagenicity markers with effect and susceptibility biomarkers, thus a greater understanding about environmental genotoxic agents, which people are exposed daily, will be done carefully.

Keywords: Human biomonitoring, supply water, airborne particulate matter, PAHs, PCDD/Fs, *Salmonella*/microsome assay, comet assay, micronuclei assay, polimorphisms.

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Abreviações

BaA	-benzo(a)antraceno
BaP	-benzo(a)pireno
BbF	-benzo(b)fluoranteno
BghiP	-benzo(ghi)perileno
BjF	-benzo(j)fluoranteno
BkF	- benzo(k)fluoranteno
CONAMA	-Conselho Nacional do Meio Ambiente
dBahA	-dibenzo(ah)antraceno
DCM	-diclorometano
DMSO	-dimetilsulfóxido
FEPAM	-Fundação Estadual de Proteção Ambiental
HPA	-hidrocarboneto policíclico aromático
IP	-indeno(1,2,3-c,d)pireno
MERCOSUL	-Mercado Comum do Sul
MOE	-massa orgânica extraída
MN	-micronúcleo
Mt	-Montenegro
Nitro-HPA	-nitroderivado de hidrocarboneto policíclico aromático
PM_{10}	-partículas menores ou iguais a 10μm
PTS	-partículas totais suspensas
SA	-Santo Antônio da Patrulha
S9	-fração de metabolização
CYPs	-enzymes of P450 cytochrome
DNA	-deoxyribonucleic acid
EOM	-extractable organic matter
EPA	-Environmental Protection Agency -USA
GSTs	-Glutathione S-transferases genes
HPLC	-high-performance liquid chromatography
IARC	-International Agency for Research on Cancer
MI	-mutagenic index
HRGC-HRMS	-high resolution gas chromatography/ high resolution mass
MX	-3-Chloro-4 -(dichloromethyl)-5-hydroxy-2(5H)-furanone
PAH	-Polycyclic aromatic hydrocarbons
PCDD/F	-polychlorinated dibenzo-p-dioxins/ dibenzofurans
PCR	-Polymerase Chain Reaction
RNA	-desoxyribonucleic acid
SCGE	-single-cell gel electroforesis
TI	-tail intensity
TM	-tail moment
TSP	-total suspended particles
THM	-trihalomethanes

SUMÁRIO

1 INTRODUÇÃO

Nos últimos anos, o homem vem aumentando expressivamente o descarte de seus dejetos, seja no solo, na água ou na atmosfera. Estas ações possibilitam que diferentes grupos de produtos químicos sejam liberados no ambiente, gerando estresse ambiental que pode afetar a atual e as próximas gerações de todos os seres vivos, incluindo a espécie humana.

Entre os efeitos em longo prazo, vem sendo estudada a toxicidade genética destas misturas complexas de substâncias e seu impacto no ecossistema e na saúde humana. Compostos químicos estranhos a um organismo tanto de origem natural ou antrópica (xenobióticos), podem interagir com o DNA afetando sua estrutura e função, lesando processos de reparo celular, gerando alterações em estruturas cromossômicas ou ainda outras modificações celulares que levam inclusive à apoptose (morte celular programada). Efeitos deste tipo caracterizam a ocorrência de lesões por toxicidade ao material genético (Pinto & Felzenszwalb, 2003).

A emissão de uma grande diversidade de substâncias através dos efluentes industriais gasosos, líquidos ou sólidos acarreta que uma gama de compostos seja introduzida no ambiente atmosférico e aquático. A contaminação do compartimento atmosférico tem sido um dos maiores problemas ambientais, sendo esta exposição considerada pela Organização Mundial de Saúde um risco à saúde humana, pois pode ocasionar infecções respiratórias, cânceres, doenças respiratórias crônicas e cardiovasculares.

Entre os indicadores mais importantes de contaminação atmosférica estão o ozônio, óxidos de nitrogênio, monóxido de carbono e as partículas em suspensão, recebendo as últimas especial atenção devido aos efeitos tóxicos que

causam nos seres vivos, diminuindo ainda a radiação solar e a visibilidade. As partículas em suspensão, de material sólido ou líquido, ficam no ar na forma de poeira, neblina, aerossol, fumaça ou fuligem e possuem tamanho menor que 100 micra (partículas totais em suspensão – PTS) (Pedrozo, 2004). Segundo De Martinis et al. (1999) essas partículas oferecem um risco significativo à saúde humana devido a sua habilidade de penetrarem e se depositarem em regiões traqueobronquial e alveolar do aparelho respiratório.

O tamanho das partículas influencia sua absorção no corpo humano. Partículas com um diâmetro aerodinâmico menor que 10μm (PM_{10}) e 2,5μm ($PM_{2,5}$) representam um grande risco à saúde humana, pois podem se depositar em diferentes partes do aparelho respiratório (Claxton et al. 2004).

No Brasil, valores de PTS e PM_{10} são regulamentados pelo Conselho Nacional de Meio Ambiente (CONAMA, 1990) como medidas de poluição ambiental, sendo definidos padrões primário e secundário (Vargas, 2003). Padrões primários de qualidade de ar são concentrações de poluentes que se ultrapassadas, poderão afetar a saúde da população (240μg/m^3 para PTS e 150μg/m^3 para PM_{10}) e os padrões secundários são as concentrações previstas que acarretariam o mínimo de efeito sobre o bem-estar da população e dano a fauna e flora (150μg/m^3 para PTS e 150μg/m^3 para PM_{10}).

Na matéria particulada atmosférica pode se associar matéria orgânica contendo vários compostos, os quais podem ser absorvidos também pelo aparelho respiratório. Hidrocarbonetos policíclicos aromáticos (HPAs) estão entre esses compostos e recebem especial atenção devido a potencialidade carcinogênica, sendo alguns classificados pela Agência Internacional para Estudos do Câncer (IARC) como apresentando propriedades mutagênicos e/ou carcinogênicos (IARC, 2005).

Os HPAs são formados através de pirólise ou combustão incompleta de matéria orgânica que contenha carbono e hidrogênio. As fontes geradoras dessas combustões incluem veículos automotores, processos industriais, sistema de aquecimento doméstico, incineração de lixo, fumaça do cigarro e também fontes naturais, como a queima em florestas e erupções vulcânicas (Peltonen & Kuljukka, 1995).

A presença de determinado HPA pode indicar sua possível origem no ambiente. O benzo(a)antraceno (BaA) é emitido por veículos movidos a gasolina e diesel e, devido a seu baixo peso molecular, é muito fotorreativo e de fácil dispersão; benzo(b)fluoranteno (BbF) e benzo(k)fluoranteno (BkF) já foram encontrados em regiões destinadas a cana-de-açúcar, plantio que necessita de queima para sua retirada; indeno(1,2,3-c,d)pireno (IP) e benzo(ghi)perileno (BghiP) possuem os maiores pesos moleculares, sendo muito encontrados no compartimento atmosférico e considerados marcadores de emissões automotivas. (Franco, 2001; Yunker et al., 2002, Fernandes et al, 2002; Rehwagen et al. 2005; Barra et al. 2007; Dejean et al., 2008). O benzo(a)pireno (BaP) pode ser encontrado em amostras de queima de carvão e é classificado como carcinogênico (IARC, 2005), sendo dos HPAs o indicador de contaminação utilizado a mais tempo.

Os HPAs são lipossolúveis e rapidamente absorvidos pelos pulmões, intestinos e pele de animais experimentais. Esses compostos são rapidamente absorvidos pelo trato respiratório, sendo que maiores níveis podem ser encontrados no fígado (Meire et al., 2007). Uma importante propriedade dos HPAs é sua conversão metabólica, ativando produtos intermediários eletrofílicos que podem se ligar covalentemente a alvos nucleofílicos, como DNA, RNA e proteínas, formando adutos de DNA e induzindo mutações. A super família do

Citocromo P450 é o conjunto de enzimas responsável pelo metabolismo dos HPAs. Essas enzimas realizam uma oxidação enzimática originando diol-óxidos, seguida de hidrólise com a formação de dióis. Esses dióis sofrem novamente ação do citocromo P450, transformando-se em diol-épóxidos. Os metabolitos finais são os compostos considerados carcinogênicos e mutagênicos, devido ao seu alto poder de se ligar covalentemente ao grupo 2-amino na guanina da fita dupla de DNA (Meire et al. 2007, Boström et al., 2002).

Hidrocarbonetos aromáticos nitropolicíclicos (nitro-HPAs) surgem quando os HPAs formados pela combustão incompleta, combinam-se com óxidos nitrogenados, produzidos pela combustão do nitrogênio sob condições de alta temperatura (Kakimoto et al., 2002). Nitro-HPAs podem ser encontrados nas partículas suspensas atmosféricas provenientes de fontes diretas como exaustão a diesel e gasolina (De Marini et al., 2004). Os nitro-HPAs recebem especial atenção devido a sua persistência no ambiente, propriedades mutagênicas elevadas e carcinogênicas de certos compostos comparados com os HPAs. Os nitro-derivados são responsáveis pelas respostas mutagênicas encontradas em compostos de maior polaridade, estando associado a maior mutagenicidade sob ação direta do mutágeno (que não necessita de metabolização) (Bamford et al., 2003).

Entre os compostos de grande persistência no ambiente e de origem antropogênica estão os dibenzo-p-dioxinas policloradas (*PCDD*) e dibenzo-p-furanos policlorados (*PCDF*), mais conhecidos como dioxinas e furanos e que se referem a um conjunto de mais de 200 compostos orgânicos distintos (*PCDD* com 75 congêneres e *PCDF* com 135) (Coutinho et al., 2004). São formados por combustão incompleta de matéria orgânica que envolva altas temperaturas e cloro, sendo emitidos em níveis elevados através dos processos de refino do

petróleo, emissão de automóveis, incineração de resíduos, fundição de metais, clareamento de papel por cloro entre outros. São os compostos mais estudados e também os mais tóxicos produzidos pelo homem (Pujadas et al., 2001; Assunção et al., 2005).

No entanto, apenas sete congêneres de dioxinas e dez de furanos apresentam efeitos tóxicos, sendo 2,3,7,8-tetraclorodibenzo-p-dioxina o congênere de maior toxicidade. Os efeitos tóxicos dos *PCDD/F*s estão principalmente associados a carcinogenicidade, a disrupção endócrina e ao desenvolvimento de processos neurotóxicos, como deficiência na audição e cognição (IARC, 1997).

A água para abastecimento público é um dos meios pelos quais contaminantes ambientais depositados em recursos hídricos podem ser ingeridos pelo ser humano. Apesar do processo de tratamento, os compostos encontrados no local de captação influenciam muito na qualidade dessa água final (IARC, 1991; Pereira et al., 2007).

Além disso, o tratamento da água para consumo por cloração eleva o nível de genotoxinas. O cloro usado durante o processo de desinfecção da água pode contribuir com a presença de substâncias mutagênicas não voláteis na água tratada. Essas substâncias induzem, preferentemente, danos diretos na molécula de DNA, por substituição de pares de bases detectadas pelo teste de Ames (Cheh et al., 1980; Meier, 1988).

Meier et al. (1983) referem, em extensa revisão sobre água tratada, que a cloração dos ácidos húmicos e fúlvicos, componentes naturais do húmus do solo e de águas superficiais, resultam na formação de trihalometanos (THMs) e uma variedade de produtos orgânicos, clorados ou não. A formação dos THMs recebe

atenção especial devido ao efeitos carcinogênicos desses compostos – classificados como Grupo 2B, possíveis carcinógenos humanos (IARC, 1997).

Outro produto formado durante a desinfecção da água e por influência dos ácidos húmicos e fúlvicos é o 3-cloro-4-(diclorometil)-5-hidroxi-2(5*H*)-furanona (MX), composto responsável por mais de 60% da atividade mutagênica observada em amostras de água tratada e classificado também no grupo 2B da IARC (De Marini et al. 1995; IARC, 2004). MX pode causar dano no DNA (incluindo síntese de DNA não programada) e reagir diretamente com a glutationa nas reações de catálise das glutationas *S*-transferase (GSTs), responsável por inibir a mutagenicidade induzida por MX (Meier et al., 1988).

Uma gama de compostos orgânicos semi-voláteis e não-voláteis de natureza polar e apolar, extraídos pelas resinas *Amberlite* XAD, têm evidenciado potencialidade tóxica e genotóxica em diversos tipos de ensaios, sendo diagnosticados como mutagênicos no ensaio *Salmonella*/microssoma. Entre estas substâncias mutagênicas estão relacionadas as usualmente descritas como sub-produtos do processo de cloração, o MX e ácidos halogenados. Estes compostos são eficientemente extraídos pelas resinas XAD_4, mas somente quando a amostra é acidificada a pH 2,0 (Kummrow et al., 2003).

Portanto, compostos gerados durante o processo de tratamento da água podem se constituir em uma fonte adicional de contaminação por substâncias com atividade mutagênica. Somam-se a esse fato, a crescente contaminação de mananciais hídricos destinados ao abastecimento público por compostos químicos de origem antrópica, com potencialidade genotóxica reconhecida, fornecendo uma provável contribuição adicional na elevação do nível de exposição da população humana.

A utilização de metodologias que permitem a identificação precoce de agentes genotóxicos e seus efeitos no ambiente possibilita definir estratégias de controle preventivas e saneadoras, visando minimizar as possíveis conseqüências ao ecossistema e à saúde humana (Vargas, 2003).

O teste *Salmonella*/microssoma, que mede alterações genéticas em nível molecular, tem sido escolhido como metodologia básica para estudos do potencial genotóxico de amostras ambientais, devido ao amplo uso e contínuo aprimoramento. O seu emprego é aconselhado por organizações internacionais para o diagnóstico da presença de genotoxinas em mananciais hídricos, sedimentos, compartimento atmosférico e em águas para abastecimento público (Federal Register, USA, 1989; OECD, 1997; FEPAM/ PADCT/ FINEP, 1997, 2003).

O mecanismo básico do ensaio *Salmonella*/microssoma (teste de Ames) consiste em determinar mudanças na molécula de DNA, causadas por mutações reversas para prototrofia, utilizando linhagens padronizadas de *Salmonella typhimurium*. Permite avaliar a atividade mutagênica e o potencial carcinogênico de diversas classes de compostos químicos. As linhagens indicadoras apresentam diferentes mutações no *operon* da histidina, selecionadas para detectar substâncias que causam substituição de pares de bases ou deslocamento no quadro de leitura (Maron & Ames, 1983).

Estudos de avaliação da atividade mutagênica utilizando o ensaio *Salmonella*/microssoma, como bioindicador ambiental para mananciais hídricos e compartimento atmosférico, foram realizados em diversos estados do Brasil, destacando-se os desenvolvidos no Rio Grande do Sul e em São Paulo.

A análise da atividade mutagênica de extratos orgânicos de material particulado atmosférico foi realizada no Rio Grande do Sul, em área sob influência

industrial petroquímica e urbana. Foi possível observar variações na atividade mutagênica, caracterizando contribuições de fontes diferentes e áreas com maior ou menor impacto quanto à presença de agentes genotóxicos. Em Porto Alegre a maior contribuição foi observada em regiões sujeitas à influência de veículos automotores. Na área industrial foi possível observar valores elevados provavelmente relacionados com a contribuição da indústria acompanhando a dispersão dos poluentes no ambiente, segundo a direção preferencial dos ventos (Vargas et al., 1998; Ducatti & Vargas, 2003; Vargas, 2003).

Os trabalhos relatados no Estado de São Paulo revelaram atividade mutagênica em água bruta, tratada e corpos de água receptores de efluentes industriais (Martins et al., 1982; Sanchez et al., 1988; Valent et al., 1993). No Rio Grande do Sul, pesquisas realizadas em amostras de efluentes industriais, água e sedimento em área sob influência petroquímica do Rio Caí e Arroio Bom Jardim, além de água bruta e tratada destinada ao abastecimento público, nestas mesmas regiões, mostraram a sensibilidade deste ensaio como bioindicador (Vargas et al., 1993; 1995; FEPAM/ PADCT/ FINEP, 1997, 2003; Horn, et al., 2004; Pereira et al., 2007). A validação deste teste ainda pode ser evidenciada através de estudos em mananciais hídricos delimitando áreas com influência de metais pesados (Vargas et al., 2001; Tagliari et al., 2004) e dejetos urbanos (Cardozo et al., 2005).

Portanto, a realização de monitoramentos em diferentes compartimentos ambientais, utilizando ensaios de avaliação de dano genotóxico, se constitui numa importante ferramenta na prevenção, conservação e uso sustentável do ambiente. Possibilitam a detecção precoce de substâncias com capacidade reativa ao material genético, que podem causar danos cumulativos acarretando alterações em diferentes organismos.

A exposição humana a agentes mutagênicos presentes no compartimento atmosférico e em corpos hídricos, principalmente quando na água destinada ao abastecimento público, caracterizam uma exposição constante da população humana, podendo acarretar conseqüências à saúde em médio e longo prazo.

São bem conhecidos os efeitos à saúde humana dos agentes genotóxicos em diferentes etapas do processo de carcinogênese, em malformações congênitas, além de sua contribuição para várias doenças degenerativas. Oliveira et al. (2002) avaliaram possíveis riscos reprodutivos em estudo caso-controle nas populações de Montenegro e Triunfo em região próxima ao Complexo Petroquímico no Rio Grande do Sul. Considerando o local de residência materno durante a gestação, como parâmetro de exposição, observaram que os recém-nascidos próximos à região do complexo e na área de direção preferencial de ventos apresentavam baixo peso ao nascimento. No entanto, quando outras variáveis foram adicionadas à análise estatística (fumo, doenças crônicas e idade materna), esta influência geográfica perdeu a significância estatística. Apesar da resposta observada, os autores recomendam este fator, baixo peso ao nascimento, como um parâmetro de controle para áreas impactadas.

A seleção de metodologias sensíveis para biomonitoramento humano é uma estratégia importante na definição de riscos populacionais à exposição de contaminantes ambientais. O monitoramento utilizando metodologias genéticas em populações expostas a potenciais mutagênicos e/ou carcinogênicos é um eficiente sistema de advertência para doenças genéticas ou cânceres (Kassie et al., 2000). Entre os vários ensaios que têm sido investigados, o teste do cometa em sangue periférico ou a avaliação de micronúcleos em esfregaço bucal são técnicas rápidas, simples e sensíveis para medir lesões genômicas precoces.

No teste do cometa, um pequeno número de células suspensas em gel de agarose é aplicado sobre lâmina previamente gelatinizada; o DNA é lisado, passando em seguida por eletroforese e coloração com corante DNA-específico (Rojas et al., 1999). O grau de dano no DNA pode ser observado através do tamanho da “cauda” obtida após a eletroforese, isto é, a distância de migração da molécula indicando a quantidade de quebras das fitas de DNA presente em determinada célula (Singh, 2000). Este teste detecta ainda lesões em sítios álcali-lábeis e reparo incompleto dos sítios de excisão, podendo ser aplicado a qualquer organismo eucariótico (Singh et al., 1988).

Vários trabalhos têm evidenciado a aplicação deste ensaio em biomonitoramento humano. Garaj-Vrhovac & Zeljezic (2000), por exemplo, analisaram a formação de cometas em linfócitos de amostras sanguíneas de trabalhadores de uma indústria produtora de pesticidas. Linfócitos dos trabalhadores expostos ocupacionalmente à pesticidas manifestaram um elevado índice de dano no DNA, comparado com grupo não exposto, comprovando a eficiência do ensaio que evidenciou algumas possíveis ações de reparo no DNA.

Embora o ensaio do cometa mostre sensibilidade no biomonitoramento ocupacional, poucos trabalhos estudam pessoas que não são expostas ocupacionalmente.

As técnicas de avaliação citogenética expressam eventos cumulativos em exposições crônicas, enquanto que o ensaio do cometa pode fornecer informações sobre recentes níveis de exposição à substâncias genotóxicas, em que parte dos danos ainda são passíveis de reparo. Entretanto, a combinação deste ensaio com um teste que permite medir danos mutagênicos já estabelecidos, como o teste do micronúcleo (MN), possibilita definir uma ampla

gama de danos genotóxicos e uma avaliação de risco mais precisa (Laffon et al., 2002).

O teste do micronúcleo (MN) é um ensaio freqüentemente utilizado em biomonitoramento humano. Micronúcleos são definidos como massas de cromatina citoplasmáticas, redondas ou ovais que são visíveis microscopicamente e estão próximas ao núcleo (Schmid, 1975). Eles são o resultado de mitoses anormais e consistem de cromossomos acêntricos, fragmentos de cromátides ou cromossomos anormais. A avaliação do número de MN pode ser usada como uma estratégia na identificação de danos em células animais ou humanas, as quais estão expostas a uma substância mutagênica ou carcinogênica (Montero et al., 2006). A formação do MN é, portanto, induzida por substâncias que causam quebras cromossômicas (substâncias clastogênicas) bem como por agentes que afetam o aparato do fuso mitótico (substâncias aneugênicas) (Majer et al., 2001).

Comparado a outros ensaios de genotoxicidade este procedimento apresenta muitas vantagens: (*i*) é um teste simples e rápido, em que as células podem ser obtidas facilmente, sem precisarem ser cultivadas; (*ii*) a metodologia está bem definida e os eventos de MN podem facilmente ser identificados; (*iii*) as células podem ser fixadas e estocadas por grandes períodos de tempo; (*iv*) ao contrário das medidas de troca de cromátides irmãs e a maioria das aberrações cromossômicas estruturais, MN são encontrados na interfase do ciclo celular; (*v*) a simplicidade de coletar amostras por métodos não evasivos, tornando o teste aplicável a um grande número de amostras (Heddle, 1973; Mateuca et al. 2006).

Muitos trabalhos utilizam o teste do MN para o estudo da prevenção e investigação de células cancerosas. Bloching et al. (2000) estudaram células escamosas de pacientes com carcinoma de cabeça e pescoço, além de pacientes

com leucemia, observando uma alta contagem de micronúcleos, como um sinal de danos citogenéticos de células da mucosa oral.

Coronas (2008) avaliou a exposição de pessoas que residiam e/ou trabalhavam próximos a uma indústria petroquímica do Rio Grande do Sul. Apesar de não observar diferenças entre as pessoas estudadas com as de uma cidade referência através do teste do MN em células da mucosa bucal, o ensaio do cometa com linfócitos mostrou-se sensível na detecção de danos ao DNA das pessoas da cidade estudada.

Entretanto, a resposta de cada indivíduo aos genotóxicos ambientais pode ser diferenciada por diversos fatores como idade, estilo de vida, hábitos alimentares, etílicos e ao fumo. Somado a esses fatores, existe ainda a resposta genotípica individual, propriedades herdadas responsáveis pelo metabolismo de xenobióticos e reparo do DNA, influenciando significativamente a susceptibilidade ou resistência de um indivíduo aos impactos diversos na saúde (Novotna et al., 2007).

O processo de biotransformação (alterações enzimáticas de xenobióticos) se divide em duas fases. As enzimas da Fase I introduzem novos grupos funcionais, como –OH, -SH, -NH_3, aos xenobióticos - esta catálise é realizada pelas enzimas do citocromo P450 (CYPs), embora muitas oxidases, reductases e desidrogenases também possam participar. Esses intermediários são então conjugados com ligantes endógenos na Fase II, aumentando a hidrofilia do composto e facilitando assim sua excreção. Entre as enzimas envolvidas na Fase II estão as glutationas *S*-transferase (GSTs), *N*-acetil tranferases (NATs), hidrolases hipóxido e metiltransferases. As reações das duas fases são catalizadas por enzimas coletivamente conhecidas como enzimas metabolizadoras de xenobióticos, que apesar de serem mais abundantes no

fígado, muitos outros tecidos também possuem esta atividade. Um balanço entre as enzimas das duas fases é geralmente necessário para promover a eficiência da detoxificação e eliminação dos xenobióticos, protegendo assim o corpo contra as injúrias causadas por exposições a diversos compostos (Kelada et al., 2003).

Heuser et al. (2007) estudaram os efeitos genotóxicos de trabalhadores expostos ocupacionalmente a solventes em uma fábrica no Rio Grande do Sul, utilizando os ensaios do cometa e do micronúcleo, como biomarcadores de exposição e analisando genes responsáveis pela metabolização de xenobióticos, como biomarcadores de susceptibilidade. Os autores observaram uma associação entre o aumento de danos no DNA desses trabalhadores com os polimorfismos genéticos individuais.

Estudos populacionais que identifiquem efeitos precoces desses contaminantes ambientais na qualidade de vida e na saúde da população humana são necessários para a definição de medidas preventivas. Com essa preocupação, o presente estudo teve como objetivos (i) definir uma população de local no estado do Rio Grande do Sul que possa ser utilizado como referência quanto a qualidade ambiental para genotoxinas; (ii) avaliar a presença de atividade mutagênica em extratos de compostos orgânicos no material particulado do ar e em águas destinadas ao abastecimento público em áreas de referência e de risco para a exposição à agentes genotóxicos; (iii) caracterizar quimicamente essas amostras ambientais relacionando com suas respostas mutagênicas; (iv) Avaliar a sensibilidade dos ensaios cometa em linfócitos humanos de sangue periférico e de micronúcleo em células epiteliais da mucosa bucal como marcadores de exposição a agentes mutagênicos de forma crônica através da poluição atmosférica e da água de abastecimento público; (v) caracterizar cada

população de estudo quanto a freqüência de genes responsáveis pelas duas fases de detoxificação.

Considerando esses objetivos, essa tese está dividida em quatro capíltulos/artigos de acordo com diferentes tipos de avaliações de genotoxicidade e exposições a xenobióticos.

O primeiro artigo “Evaluation of mutagenic activity in supply water at three sites in the state of Rio Grande do Sul, Brazil” (publicado na revista *Mutation Research*, volume 629, p. 71-80, 2007) além de definir uma cidade referência para essa tese, avaliou a água de abastecimento desse local e da cidade alvo (Montenegro-RS) desse estudo.

O segundo artigo “Comparative mutagenic study of urban airborne particulate matter in areas with different antropogenic influences and PAHs levels” avaliou durante seis meses a qualidade atmosférica de Montenegro (RS) comparada com a cidade já definida como referência (Santo Antônio da Patrulha-RS) quanto à presença de agentes mutagênicos na água de abastecimento. Para esse artigo foram avaliadas a mutagênese de amostras de ar e sua caracterização quanto aos HPAs.

O terceiro artigo ”Genetic monitoring of human populations living in urban cities expostas a diferentes contaminantes in Southern of Brazil” avaliou nas mesmas cidades do primeiro e do segundo artigo a exposição de pessoas que vivem nesses locais, e recebem ambientalmente o ar circulante. Amostras de ar foram avaliadas quanto a sua mutagenicidade, caracterização dos HPAs e avaliação concomitante de parâmetros genotóxicos de pessoas expostas no mesmo período dessa amostragem ambiental.

O quarto artigo “Human biomonitoring in an urban environment under influence of industrial contaminants in Southern Brazil” avaliou a outra cidade alvo deste estudo (Rio Grande-RS) comparado com a cidade de referência, Santo Antônio da Patrulha (RS) quanto à exposição de contaminantes ambientais em pessoas que vivem nesses locais, tanto ao ar circulante, quanto à água tratada para abastecimento público. Amostras de ar e de água tratada foram avaliadas quanto a sua mutagenicidade, caracterização dos HPAs e *PCDD/F*s do ar, concomitantes a avaliação de parâmetros genotóxicos nas pessoas expostas no mesmo período dessa amostragem ambiental.

2 MATERIAL E MÉTODOS

2.1 Áreas de estudo

Este estudo foi realizado em três áreas de trabalho. Duas definidas como de risco para exposição a agentes genotóxicos gerados por diferentes contribuições antrópicas, constituindo-se as populações alvo. A terceira área de estudo foi definida como de referência, localizada fora da pluma de dispersão de poluentes atmosféricos de origem industrial e água para abastecimento público avaliada quanto à qualidade através do nível de genotoxinas, constituindo-se a população de referência.

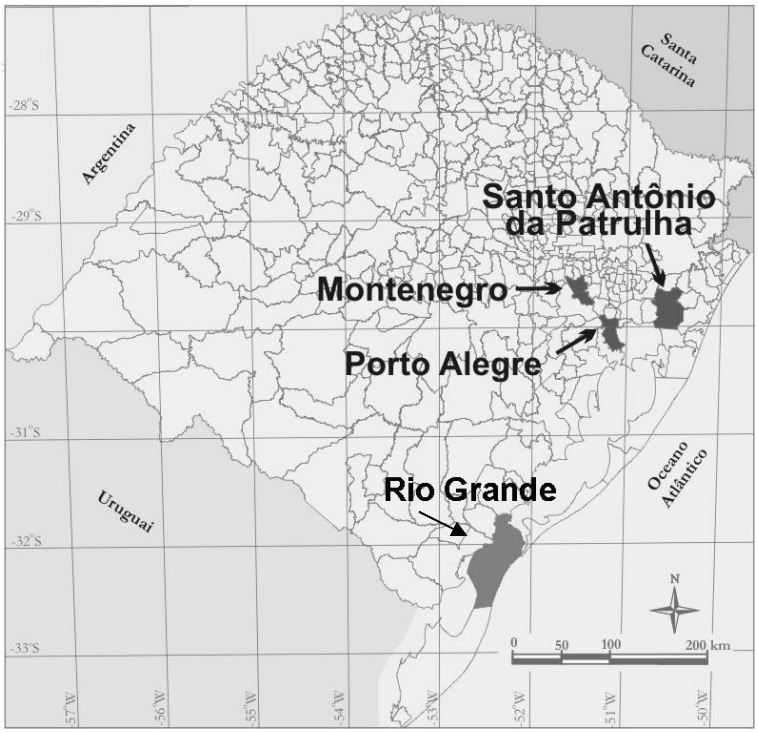

Figura 1. Cidades de estudo no Rio Grande do Sul em relação a capital Porto Alegre.

2.1.1 Área alvo do primeiro estudo: Montenegro/RS

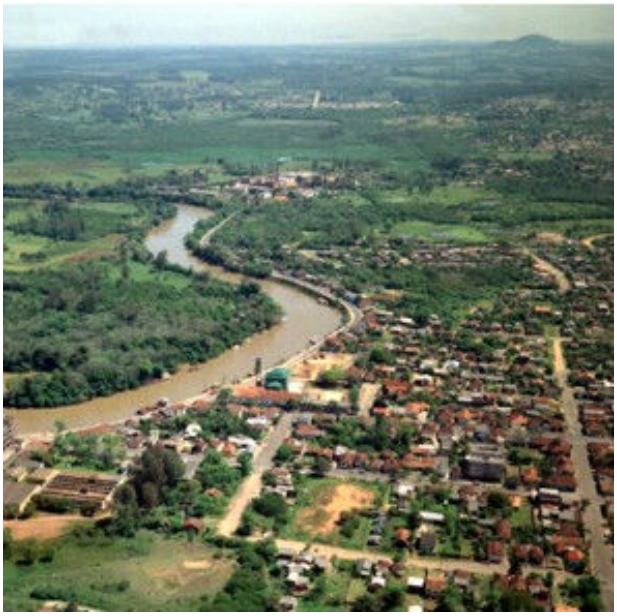

Figura 2. Município de Montenegro, Rio Grande do Sul.
(Fonte: http://www.lionsclubemontenegro.com.br/new/)

Montenegro (29°40,27'S e 51°27,35'W) possui área de 420km^2, com 60 mil habitantes e está localizado no quadrante de distribuição atmosférica principal que recebe influência do III Pólo Petroquímico do Sul dentro de região diagnosticada como de possível risco ecotoxicológico gerado pela presença de misturas de compostos químicos com atividade tóxica e genotóxica. Este diagnóstico foi realizado utilizando diversas abordagens incluindo avaliações químicas e biológicas em amostras de mananciais hídricos, água para abastecimento público, além de estudos de avaliação de genotoxicidade em material particulado do ar associado a distribuição atmosférica da região (Vargas et al.,1993; 1995; FEPAM/ PADCT/FINEP, 1997; Ducatti et al., 2001; Vargas, 2003; Vargas et al., 2008).

O Pólo Petroquímico está localizado entre os municípios de Montenegro, Triunfo e Canoas, com uma área de 14.600ha, situando-se à 22km do município de Montenegro, sendo que esta cidade situa-se a 60km de Porto Alegre.

2.1.2 Área alvo do segundo estudo: Rio Grande/RS

Figura 3. Município de Rio Grande, Rio Grande do Sul.
(Fonte: www.skyscrapercity.com/showthread.php?t=159606)

Rio Grande (32°02,98'S e 52°07,54'W), cidade mais antiga do Rio Grande do Sul, possui uma área de 2.814km², com aproximadamente 196 mil habitantes (IBGE, 2008), e esta a cerca de 300Km de Porto Alegre, no extremo sul do estado.

A cidade se localiza na Península do Estuário da Lagoa dos Patos (maior lagoa do Brasil, com 10.360km^2) próxima à desembocadura do Oceano Atlântico. Possui um complexo industrial-portuário onde se destacam as fábricas de fertilizantes, tratamento de madeira, produção de alimentos, pescados, soja, petroquímica e extração e refino de óleo vegetal. A indústria do petróleo está presente com uma refinaria, postos de abastecimento, terminais marítimos de transporte e estocagem de petróleo e derivados. A cidade de Rio Grande apresenta também importante atividade portuária para a região dentro do MERCOSUL, com um terminal marítimo e um conjunto portuário (possui o segundo maior porto do Brasil). Com isso, a região se torna vulnerável à ocorrência de acidentes e a poluição crônica por petróleo (Medeiros et al. 2005).

2.1.3 Área referência: Santo Antônio da Patrulha/RS

Figura 4. Município de Santo Antônio da Patrulha, Rio Grande do Sul.

(Fonte: http://www.santoantoniodapatrulha.rs.gov.br/)

A localização da área de referência foi definida como descrito no primeiro capítulo deste trabalho como a cidade de Santo Antônio da Patrulha (29°46,29'S e 50°35,15W), RS. A cidade possui área territorial de 1.069 km^2, tem aproximadamente 38 mil habitantes e distante de Porto Alegre 76Km.

Localiza-se em área livre da pluma de dispersão atmosférica dos contaminantes das áreas alvo, longe de grandes centros urbanos e industriais. A cidade possui pequenas propriedades rurais agrícolas e/ou pecuárias, além de se destacar no estado como principal produtora de cachaça.

2.2 Estudo da presença de genotoxinas em amostras ambientais

2.2.1 Estudo da água destinada ao abastecimento público

2.2.1.1 Coleta de amostras de água

As amostras foram coletadas pela equipe de Amostragem da FEPAM de acordo com Standart Methods (1992) para o exame de águas e efluentes. Os

volumes de água destinados à concentração (40 litros) foram acondicionados a 4°C e processados imediatamente.

2.2.1.2 Preparação de amostras

As extrações de compostos orgânicos a partir de grandes volumes de amostras de água (40L) foram realizadas por adsorção em resinas *Amberlite* XAD_4 segundo normas recomendadas pela Environmental Protection Agency – EPA, USA (1985). O reservatório com a amostra foi conectado a uma coluna cromatográfica contendo a resina (1mL de resina por litro de amostra) previamente lavada com metanol e tratada com os solventes metanol, éter etílico, diclorometano e água ultra-pura na proporção de 1:1:2:6.

Após a passagem da água pela coluna (vazão de 100mL/min), o excesso retido na resina foi retirado com auxílio de bomba de vácuo. A eluição dos compostos orgânicos em pH natural foi realizada utilizando 1mL de metanol e 4mL de diclorometano por mL de resina, extraindo compostos moderadamente polares a apolares. Para a extração ácida foram utilizados 1mL de metanol e 4mL de etilo acetato por mL de resina, resultando na extração de compostos polares.

Os eluatos foram recolhidos em *erlenmeyer*, sendo então, concentrados em aparelho de rota-vapor, até 10mL. Os extratos foram guardados em frascos graduados, com tampa e ao abrigo da luz, sendo a massa orgânica extraída determinada em balança eletrônica analítica.

Este procedimento foi realizado na Divisão de Química da FEPAM.

2.2.2 Estudo de amostras de material particulado do ar

Foram avaliadas amostras de material particulado atmosférico, coletadas por amostradores de grande volume, em locais na área de estudo das populações

alvo (continuidade de monitoramento já existente) e de referência. Estes filtros foram amostrados pela equipe de monitoramento de ar da FEPAM. Os filtros contendo material particulado de ar foram agrupados (em *pools*) segundo os objetivos de cada um dos estudos e submetidos à extração seqüencial por solventes orgânicos pela técnica de ultra-som. Através deste método (FEPAM/ PADCT/FINEP, 1997; Vargas et al., 1988) foram obtidos extratos com afinidade pelo solvente DCM (diclorometano - extrai frações moderadamente polares) .

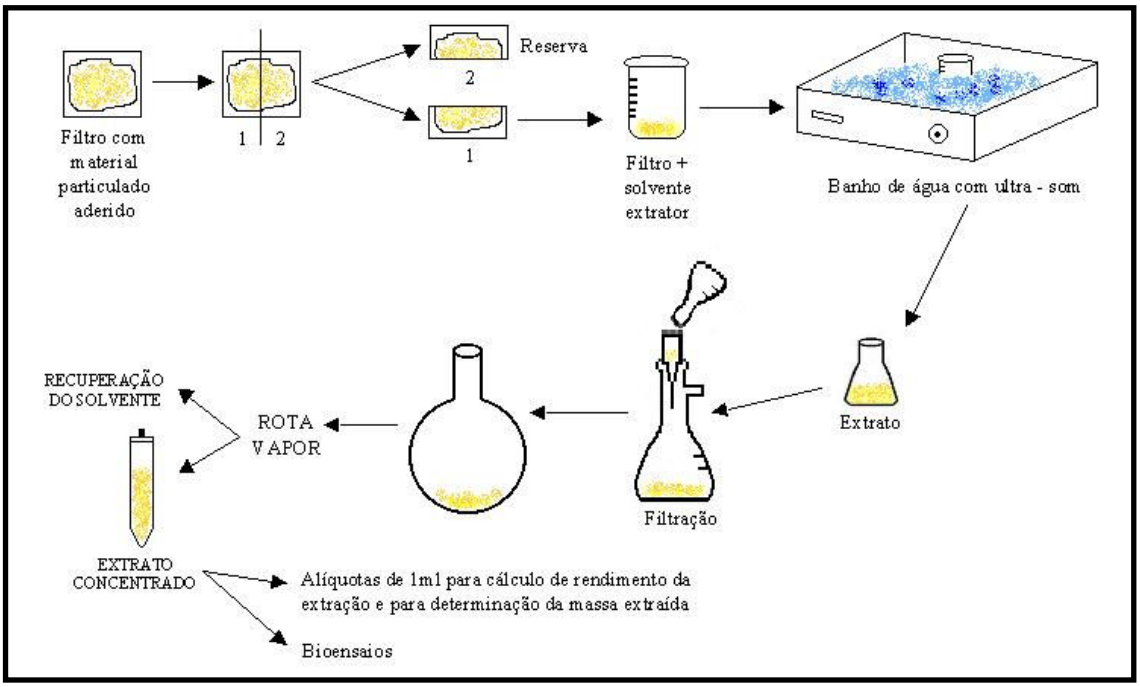

Figura 5. Extração de compostos orgânicos de filtros com material particulado atmosférico.

A obtenção dessas frações, assim como a extração dos compostos orgânicos da água tratada ficou sob responsabilidade da química Jocelita A. V. Rocha, pertencente ao grupo de pesquisa da FEPAM e foi realizada na Divisão de Química da FEPAM.

Figura 6. Amostradores de ar em Santo Antônio da Patrulha/RS.

2.2.2.1 Análise da composição química dos extratos de ar

Os extratos de ar obtidos pelo método de extração acima citado, foram submetidos à cromatografia para obtenção de oito HPAs de interesse ambiental (carcinógenos, possíveis ou prováveis carcinógenos) (IARC, 2005).

Os HPAs foram analisados através de cromatografia líquida de alta resolução (*HPLC*) em cromatógrafo Alliance 2690 (Waters) equipado com detector de fluorescência. Foi utilizado uma coluna ultra rápida de 5cm, de longitude específica para esta família de compostos (SUPERCOSIL LC-PAHS, 5cm x 4,6mm, 3µm), que permite separar em resolução adequada e no tempo de 12 minutos os HPAs da Diretiva Européia (2004/107/CE). Esta diretiva propõe o controle de sete compostos HPAs: benzo(a)antraceno, benzo(b)fluoranteno, benzo(j)fluoranteno, benzo(k)fluoranteno, benzo(a)pireno, dibenzo(a,h)antraceno e indeno(1,2,3-c,d)pireno, especificando valor objetivo apenas para BaP (1 ng/m^3).

O escaneamento feito pelo detector fluorescente foi programado para caracterizar oito HPAs específicos, como mostrados na Tabela 1.

Tabela 1. Classificação IARC (2005) dos oito HPAs de estudo.

Grupo IARC (2005)	Definição do Grupoo	HPA	Abreviação	Número de anéis aromáticos	Peso molecular (g . mol^{-1})
1	carcinógeno	Benzo(a)pireno	BaP	5	252
2A	provável carcinógeno	Dibenzo(ah)antraceno	dBahA	5	278
2B		Benzo(a)antraceno	BaA	4	228
		Benzo(b)fluoranteno	BbF	5	252
	possível carcinógeno	Benzo(j)fluoranteno	BjF	5	252
		Benzo(k)fluoranteno	BkF	5	252
		Indeno(1,2,3-cd)pireno	IP	6	276
3	não carcinógeno	Benzo(ghi)perileno	BghiP	6	276

As condições cromatográficas estão especificadas na Tabela 2.

Tabela 2. Condições cromatográficas.

Injeção	*Método 1* (Grupo): volume de injeção 10µL *Método 2* (BjF/IP): volume de injeção 20µL			
Detecção	*Fluorescência*	Método 1 (Grupo)	BaA, BbF, BkF, BaP, dBahA, BghiP	$\lambda_{excitação}$: 296 nm $\lambda_{emissão}$: 405 nm
		Método 2 BjF, IP	BjF	Do início ao minuto 10 $\lambda_{excitação}$: 318nm / $\lambda_{emissão}$: 510 nm
			IP	Do minuto 10 ao final $\lambda_{excitação}$: 270 nm / $\lambda_{emissão}$: 490 nm
Temperatura	28ºC			
Fluxo	1 mL/min			
Fase móvil	Água / acetonitrilo			
Método Gradiente	0 -1.5 min:	40% água/60% acetonitrilo		
	1.5 - 9 min:	gradiente linear		
	9 - 12 min:	100% acetonitrilo		
	12 - 18 min:	volta-se às condições iniciais e estabiliza-se		

Todas as amostras foram injetadas em duplicata. A quantificação foi feita por padrão externo. Para cada um dos HPAs de interesse foi calculado seu fator de resposta com um padrão de concentração próximo ao obtido pela amostra.

Esta etapa do trabalho foi realizada no Laboratório de Cromatografia, pertencente ao Institut Quimic Sarria, Universitat Ramon Llull (Barcelona, Espanha) sob a supervisão do Dr. Lluís R. Comellas e do Dr. Francesc Broto.

Em dois *pools* de Rio Grande foi possível a caracterização dos compostos *PCDD/F*s presentes. Para esta metodologia, os filtros foram reunidos em *pools* e submetidos à técnica de extração por soxhlet com tolueno durante 48h. Os extratos orgânicos obtidos foram submetidos a um processo de purificação e analisados por cromatografia *HRGC-HRMS,* seguindo o método UNE-EN 1948. As amostras foram analisadas em coluna capilar DB-5 (60m, 0,25mm, 0,25µm) e depois em DB-DIOXIN (60m, 0,25mm, 0.25µm), para encontrar os congêneres 2,3,7,8 que não saíram na primeira coluna. Este procedimento foi realizado no Laboratório de Meio Ambiente, pertencente ao Institut Quimic Sarria, Universitat Ramon Llull (Barcelona, Espanha) sob a supervisão do Dr. Jordi Diaz e sua equipe.

2.2.3 Método de microssuspensão - Teste de Kado

Para avaliar a atividade mutagênica dos compostos orgânicos extraídos das amostras ambientais foi empregado o ensaio *Salmonella*/microssoma pelo método de microssuspensão que mede atividade mutagênica, utilizando diferentes linhagens da bactéria *Salmonella typhimurium* associadas a um sistema de metabolização de mamíferos *in vitro*. Este procedimento difere do método clássico uma vez que as diferentes concentrações do extrato a testar foram expostas a 10 vezes mais bactérias crescidas por um período de uma noite (10^9 cel/tubo de incubação) e 5 vezes menos enzimas de ativação (100µL). A preparação da bateria de testes foi realizada em banho de gelo com tempo de pré-incubação de 90 minutos. Após, os tubos foram semeados com agar de

superfície de modo usual, sendo a leitura das colônias revertentes realizada após 72 horas de incubação. Este método apresenta sensibilidade de cinco a dez vezes maior quando comparado ao teste de Ames clássico, possibilitando a utilização de menores volumes de amostra, sendo adequado para o diagnóstico de amostras ambientais escassas (Maron & Ames, 1983; Kado et al., 1986; EPA, 1993).

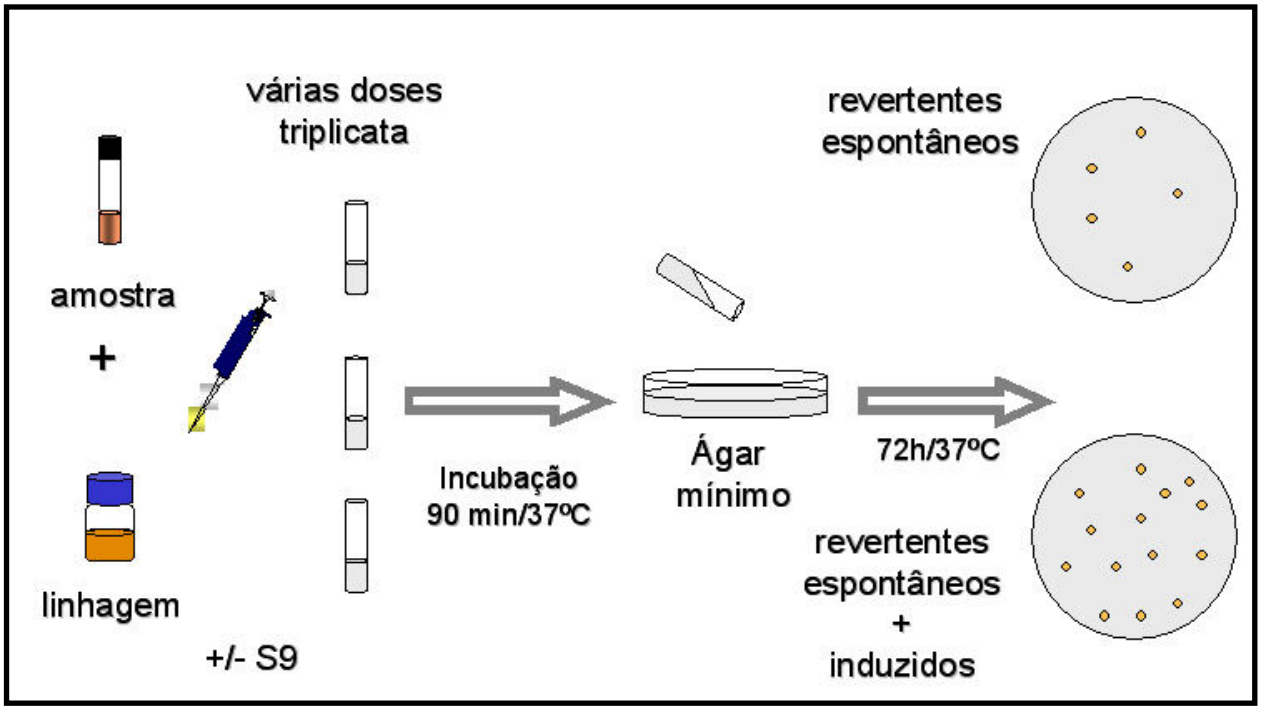

Figura 7. Procedimento do ensaio *Salmonella*/microssoma pelo método de microssuspensão.

As linhagens de *Salmonella typhimurium* apresentam diferentes mutações introduzidas no *operon* da histidina permitindo detectar os tipos clássicos de danos genéticos moleculares. Foram utilizadas neste trabalho as linhagens TA98, que detecta mutagênicos que provocam deslocamento no quadro de leitura do DNA e TA100, que detecta mutagênicos que causam substituição de pares de bases. Estas linhagens foram periodicamente testadas quanto a outros marcadores genéticos específicos que conferem maior sensibilidade ao ensaio: *(i)* mutação rfa, que confere perda parcial da barreira de lipopolissacarídeos da

parede bacteriana, facilitando a difusão de moléculas grandes para a célula; *(ii)* deleção do gene *uvrB*, um dos responsáveis pelo sistema de reparo por excisão permitindo que um maior número de lesões sejam reparadas por mecanismos sujeitos a erro, elevando assim a sensibilidade na detecção de mutágenos; *(iii)* plasmídio pKM101, que contém o gene *mucAB*, responsável pelo aumento do sistema de reparo do tipo "passível de erro" (aumenta tanto a mutagênese induzida como a espontânea), tendo como marcador a resistência a ampicilina (Maron & Ames, 1983; Umbuzeiro & Vargas, 2003).

Na avaliação da atividade genotóxica de material particulado de ar ainda foram utilizadas as linhagens YGs (YG1021 e YG1024), caracterizadas como sensíveis à presença de nitrocompostos. No plasmídio pYG216 está inserido o gene da nitroredutase clássica e no pYG219 o da O-acetiltransferase, conferindo a elevada atividade enzimática e, conseqüentemente, a maior sensibilidade para nitrocompostos como nitroarenos ou aminas aromáticas. Dessa forma, frente a essas substâncias, as linhagens YGs apresentam respostas muito mais elevadas quando comparadas com as parentais (Watanabe et al, 1989; 1990; Umbuzeiro & Vargas, 2003)

Visando a determinação do nível de citotoxidez, a amostra e a cultura bacteriana, após incubação por 90 minutos, foram diluídas com tampão fosfato (pH 7,4) até uma concentração de 1-2x10^3 células/mL. Posteriormente, foram semeadas e incubadas em meio rico completo por 72 horas a 37°C, obtendo-se o resultado da viabilidade celular através do número de colônias sobreviventes/placa. O percentual de sobrevivência foi calculado pela razão entre as médias de colônias sobreviventes/placa frente à amostra em relação às observadas frente ao controle negativo do ensaio (Vargas et al., 1988; 1993).

Os testes foram realizados na presença e ausência de um sistema de ativação metabólica *in vitro*, a fração microssomal – hepática S9 (adquirida da MOLTOX, USA). Esta era composta por um homogenato de células de fígado de rato *Sprague-Dawley,* pré-tratado com a mistura bifenil policlorinada (Aroclor 1254), que induz um aumento de enzimas (P450) neste órgão. A fração S9 foi acrescida de co-fatores adequados, fração S9 mix, necessitando de condições de pH específico para que as reações de metabolização pudessem ocorrer (Maron & Ames, 1983; Umbuzeiro & Vargas, 2003).

Para a análise estatística, as curvas dose-respostas, obtidas no teste de microssuspensão de Kado foram avaliadas pelo programa SALANAL (*Salmonella* Assay Analysis, versão 1.0 of Research Triangle Institute, RTP, Carolina do Norte, USA) Este programa permitiu avaliar a relação dose-resposta de cada ensaio e seu enquadramento em diferentes modelos de regressão. Através desta análise pode ser obtido o cálculo e a significância estatística de: análise de variância entre as médias do número de revertentes nas diferentes doses testadas (teste F); ajuste ao modelo mais aceitável e o cálculo da positividade da curva dose-resposta. A inclinação da reta na parte linear da curva dose-resposta forneceu a estimativa do número de revertentes induzidos por unidade de amostra testada. A amostra foi considerada positiva quando se observou um valor de mutagênese de pelo menos duas vezes o observado no controle negativo com curva dose-resposta reproduzível e significante pela análise de regressão.

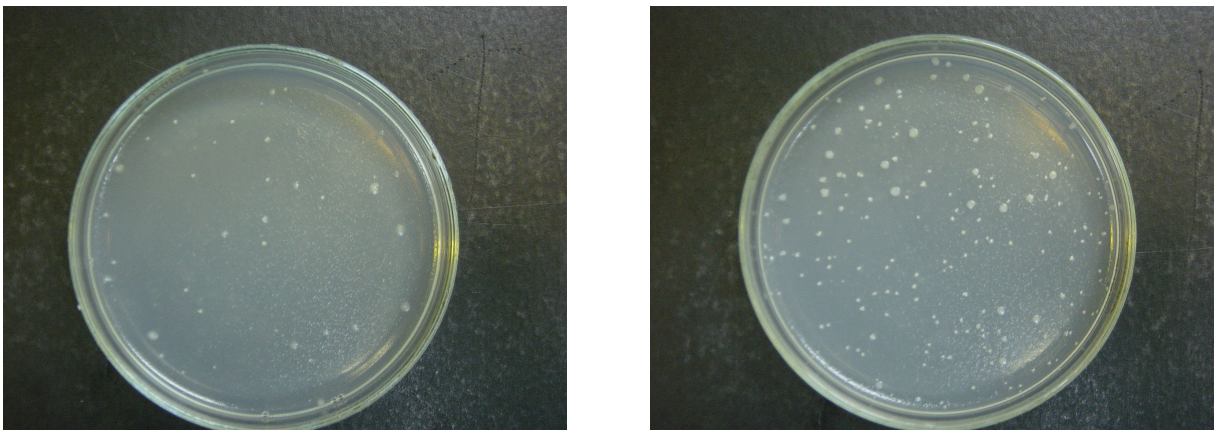

Figura 8. Resposta apresentada pelo ensaio *Salmonella*/microssoma pelo método de microssuspensão. Resposta negativa e induzida de mutagenicidade, respectivamente (Foto de Rubem C. Horn).

Os ensaios descritos nesse item foram realizados na Divisão de Biologia, no Laboratório de Análises Moleculares e Mutagênese Ambiental da FEPAM.

2.3 Biomonitoramento humano

Foram avaliados indivíduos adultos do sexo masculino, entre 18 e 40 anos de idade, amostrados nas áreas alvo e na de referência de forma que permanecessem a maior parte do dia no local de estudo, não trabalhassem com insumos agrícolas e, preferentemente, não fossem fumantes.

Esse estudo foi aprovado pela Comissão Nacional de Ética em Pesquisa, sob o Proc. Nº 23078.200270/04-17, em 23 de junho de 2004 (CONEP, Brasília).

Primeiramente foi realizado o contato com a população dos municípios (cidades alvo e de referência), através das Secretarias de Vigilância Ambiental em Saúde do Estado do Rio Grande do Sul e das Secretarias de Saúde Municipais. Inicialmente foram explanados os objetivos do trabalho, as justificativas de escolha dos municípios alvo e de referência e solicitada permissão para amostragem com auxílio dos profissionais de saúde do município.

No momento de realização do trabalho foi efetuado contato pessoal com cada doador que recebeu informações sobre os objetivos da pesquisa e o formulário para assinatura do termo de consentimento com descrição do estudo (Apêndice). Cada indivíduo respondeu a um questionário padrão, com informações gerais (Apêndice) sobre: história social, hábitos alimentares, tabagismo, dependência química, uso de medicamentos entre outros fatores, que facilitassem o entendimento dos futuros resultados obtidos.

2.3.1 Coleta de material

2.3.1.1 Coleta de sangue

As coletas de sangue de cada indivíduo foram realizadas através de punção intravenosa com agulhas e seringas descartáveis estéreis. De cada indivíduo foram coletados cerca de 4mL de sangue, o qual foi imediatamente colocado em tubos estéreis contendo 1,75mL de solução anti-coagulante-ACD, rotulados, enviados para o laboratório e mantidos a 4ºC até o momento do processamento.

2.3.1.2 Esfregaço de células da mucosa bucal

As células da mucosa oral foram obtidas através da raspagem do lado interno da bochecha com escova de polietileno. As amostras foram transferidas para solução salina fisiológica (0,9%), e transportadas ao laboratório sob refrigeração ao abrigo de luz.

2.3.2 Ensaio do Cometa

O estudo foi realizado com amostras de sangue periférico de cada indivíduo (2mL), sendo realizada a coleta de sangue das populações alvo e referência, com posterior isolamento de linfócitos em laboratório. Este isolamento foi realizado por centrifugação sobre um gradiente de densidade de Ficol. Após a obtenção dos linfócitos, foi realizado o teste de viabilidade celular com diacetato de fluoresceina com o objetivo de averiguar a integridade das células a serem utilizadas e esteve sempre superior a 90%.

Neste trabalho foi utilizada a versão alcalina do teste do cometa descrita por Singh et al. (1988), com modificações de Speit & Hartmann (1999). A eletroforese ocorreu em pH superior a 13 permitindo que danos no DNA, provenientes de vários processos biológicos, como quebras de cadeia simples de DNA ou lesões a sítios álcali-lábeis ou ainda reparo incompleto dos sítios de excisão, fossem evidenciados (Speit & Hartmann, 1999).

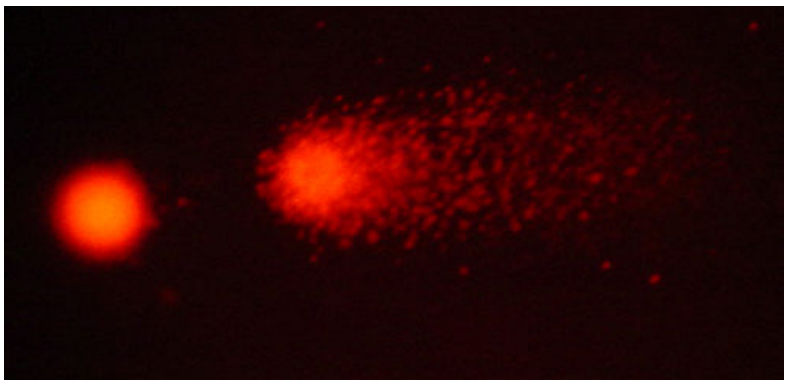

Figura 9. Célula sem dano acompanhada de célula com danos no DNA (aparência de um cometa).

Neste teste, lâminas previamente gelatinizadas com agarose de ponto de fusão normal, recebem uma mistura de linfócitos com agarose de baixo ponto de

fusão, sendo cobertas por lamínula e permanecendo em geladeira por 10 minutos. Após as lamínulas serem retiradas, as lâminas são levadas à solução de lise, onde permanecem por no mínimo 1 hora. Depois deste processo, as lâminas são colocadas em cuba horizontal de eletroforese, contendo tampão de pH alcalino, permanecendo em repouso por 20 minutos, sendo a seguir submetidas à eletroforese por mais 20 minutos. Durante o processo eletroforético, fragmentos de moléculas de DNA se movem mais rápido do que moléculas intactas, resultando na formação de "um cometa com cauda". Segundo Banu et al. (2001), o tamanho da cauda obtida indica a quantidade de quebras das fitas de DNA presentes em determinada célula. Após a eletroforese, as lâminas retiradas da cuba passam por tampão de neutralização durante 15 minutos. Quando secas, são fixadas em metanol absoluto por 10 minutos e, após passarem por novo período de secagem, são estocadas sob refrigeração a 4^{o}C. No momento da análise, são coradas com brometo de etídio.

A análise citológica foi realizada em microscópio de fluorescência conectado a uma câmera com o sistema de imagem Comet Assay 2.2, Perceptive Instruments (Suffolk, UK), que permitiu avaliar parâmetros como o tamanho e a intensidade da cauda, entre outros.

Linfócitos coletados sempre de uma mesma pessoa foram utilizados para controle positivo e negativo da corrida de eletroforese. Para o controle positivo, os linfócitos foram tratados com peróxido de hidrogênio (100mM) por 5 min no gelo.

A análise foi realizada em aumento de 400x, selecionando-se 50 imagens de núcleos não sobrepostos. O cálculo do conteúdo de DNA na imagem foi realizado por esse programa que considera a soma de intensidade da luz de todas as menores unidades de imagem existentes na área selecionada (Belpaeme et al., 1996). Podem ser feitas análises quantitativas do comprimento

dos danos no DNA e para o presente estudo foram considerados dois tipos de avaliações: a intensidade de DNA na “cauda” do cometa (*tail intensity*) e o produto de DNA na “cauda” pelo total de DNA do centro de gravidade dessa (*tail moment*).

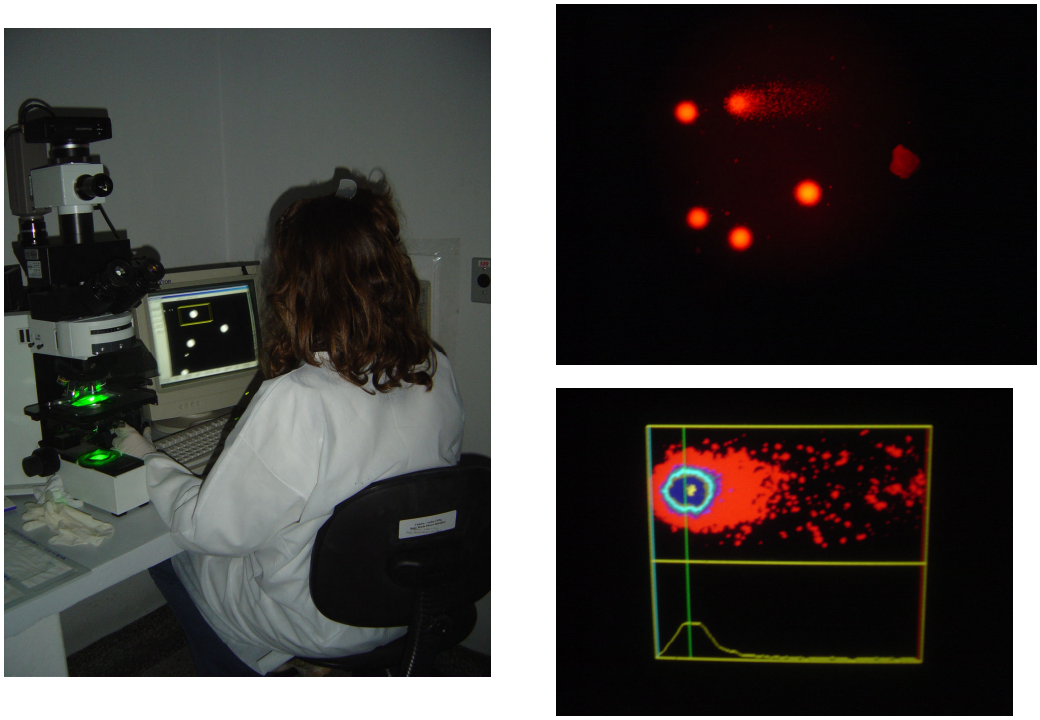

Figura 10. Análise por imagem do ensaio do cometa.

2.3.3 Teste do Micronúcleo

As células da mucosa oral em solução salina fisiológica (0,9%), foram centrifugadas a 1000 rpm por 5 minutos, sendo após fixadas com metanol e ácido acético (3:1). Para a coloração foi utilizado o reativo de Schiffs, na identificação do DNA do núcleo e do MN, seguido pelo reagente contrastante com Fast Green que delineia o citoplasma celular.

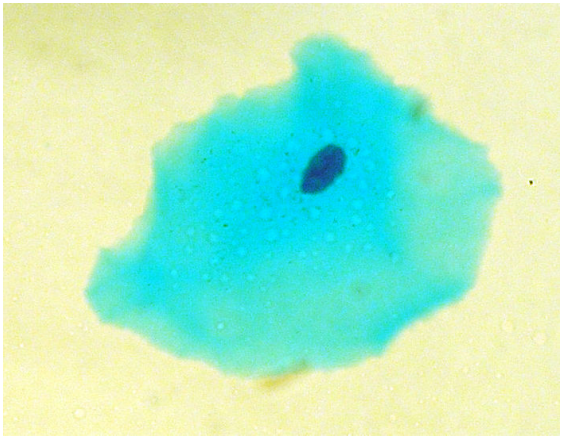

Figura 11. Célula da mucosa oral corada com *Schiffs/Fast Green*.

Foram analisadas 1000 células por indivíduo, com objetiva de alta resolução (400-1000), considerando-se o número de micronúcleo (MN) entre elas. Essa análise foi realizada por dois analisadores.

Para definição de um MN foi utilizado o critério de Tolbert et al. (1992): MN foram contados quando (*i*) a intensidade da cromatina e o padrão da coloração foram similares ao núcleo principal; (*ii*) as bordas foram identificáveis claramente indicando a presença de uma membrana nuclear; (*iii*) foram circulares e separados do núcleo principal; (*iv*) foram encontrados no mesmo plano óptico do núcleo principal; (*v*) e quando estavam dentro do mesmo citoplasma com o núcleo principal.

Os testes Kruskall Wallis, teste t de Student ou teste U, não–paramétrico de Mann-Whiteney, foram utilizados para avaliar a significância estatística dos resultados obtidos nos diferentes ensaios. Comparações entre as populações alvos e a referência foram realizadas correlacionando os dados do ensaio de micronúcleo e de cometa através de Correlação de Pearson com significância de 0,05, utilizando o programa estatístico *SPSS/PC*.

Diferenças entre os valores dos parâmetros *tail intensity* e *tail moment* foram avaliadas por análise de variância utilizando modelos hierárquicos como

descrito por Lovell & Omori (2008). A média dos valores de dano no DNA foi comparada utilizando teste t de Student para os possíveis fatores de confusão (*SPSS* para Windows statistical package, versão 13 e Proc Mixed-Statistical Analysis System (SAS) versão 9.1).

Os ensaios do cometa e do micronúcleo foram realizados na Divisão de Biologia, no Laboratório de Análises Moleculares e Mutagênese Ambiental da FEPAM. As análises do ensaio do cometa foram avaliadas no laboratório TOXICAN, pertencente ao Departamento de Patologia da Faculdade de Medicina – UNESP de Botucatu em São Paulo, sob a supervisão da Dra. Daisy M. F. Salvadori.

2.3.4 Genes de metabolização

A outra parte do sangue coletado (2mL) foi utilizada para a extração do DNA por dois métodos: extração através de kit (Kit GFX Genomic Blood DNA Purification – Amersham Pharmacia, CASRN. 27960301) e através do método *salting-out* (Miller at al., 1988). Este procedimento foi realizado em duas fases, pois como o kit não teve seu funcionamento efetivo, o método tradicional foi utilizado posteriormente.

Para a extração pelo método *salting-out*, a amostra de sangue foi centrifugada a 2500rpm e o plasma foi aspirado, como descrito em Guembarovski (2007). Foi adicionada solução de lise de hemácias (solução A: 0,32M de sucrose; 10mM de tris-HCl pH-7,5; 5 mM de MgCl2 1% de Triton X-100) e a amostra foi mantida no gelo por 30min. A amostra foi centrifugada a 2500 rpm por 15 min e o sobrenadante descartado. O procedimento foi repetido, o sobrenadante foi retirado e as células ressuspendidas em solução de lise de leucócitos (solução B:

10mM de tris-HCl, pH-7,5; 5mM de NaCl; 2mM de EDTA, pH8,0). Foram adicionados SDS a 20% (duodecil sulfato de sódio) e proteinase K (2mg de proteinase K / mL de SDS a 2%) e manteve-se a 37ºC durante a noite. Foi acrescentado NaCl a 6M e a amostra foi centrifugada a 2500rpm por 15 minutos. Foi adicionado etanol 100% e o DNA foi removido, submetido à ação de etanol 70% e ressuspenso em TE (10mM de tris-HCl pH 8,0 e 1mM de EDTA).

Foram avaliados três genes de metabolização: *CYP1A1*, pertencente a Fase I e *GSTM1* e *GSTT1*, pertencentes a Fase II de metabolização.

Para o gene *CYP1A1*, que é altamente polimórfico, foi estudada uma variante alélica. Através do uso da enzima de restrição *MspI,* foi detectada uma variante originada pela transição T-C (*3801T>C*), resultando no alelo polimórfico denominado *CYP1A1*2A* (Walker, 1996). Esta variante resulta em um aumento da atividade catalítica da enzima. Isto pode conferir uma suscetibilidade aumentada ao câncer por promover uma maior ativação de procarcinógenos na fase I da biotransformação (Goto et al., 1996; Fontana et al.,1998; Guembarovski, 2007).

Entre as enzimas envolvidas na Fase II estão as glutationas *S*-transferase (GSTs), sendo os mais conhecidos desta superfamília os genes *GSTM1* e *GSTT*. O gene *GSTM1*, localizado no cromossomo 1, é polimórfico possuindo dois alelos funcionais (*GSTM1*A* e *GSTM1*B)* e um alelo com atividade nula por deleção (*GSTM1*0),* sendo que os alelos funcionais possuem a mesma eficácia de detoxificação (Widersten et al., 1991). O gene *GSTT1*, localizado no cromossomo 22, assim como o *GSTM1*, é polimórfico, podendo apresentar fenótipo nulo por deleção.

A técnica de Reação em cadeia da Polimerase (PCR) foi utilizada para a detecção das variantes alélicas polimórficas dos genes *CYP1A1, GSTM1* e *GSTT*.

Para a análise da presença/ausência em homozigose dos genes *GSTM1* e *GSTT1* foi feita uma reação de co-amplificação em cadeia baseada no protocolo de PCR *Multiplex* de Abdel-Rahman et al. (1996), modificado por Guembarovski (2007), nas seguintes condições:

- tampão da enzima (20mM de tris-HCl pH 8,4; 50mM de KCl);

-2mM de $MgCl_2$;

-100 ng/μL de cada iniciador;

-1,25U de Taq DNA polimerase;

-2 mM de dNTPs;

-100ng de DNA genômico total;

- água ultra-pura estéril para completar o volume final.

A ausência de amplificação *GSTM1* (215pb) ou *GSTT1* (480pb), na presença de controle interno, indicou os respectivos genótipos nulos para cada gene, ou para ambos.

Os fragmentos foram amplificados no termociclador PTC-100 (MJ *Research, Inc*) e submetidos à eletroforese (3V/mL). Os produtos foram visualizados em gel de agarose 2% e corados com brometo de etídeo (10mg/mL) e 5μL de tampão (15mL de ficol, 20mL de água e 0,0125g de azul de bromofenol), para a visualização da corrida.

Foi utilizado um DNA padrão de 50 ou 100pb diluído (10μL de marcador de DNA, 70μL de TE, 20μL de tampão da amostra) para a verificação do tamanho dos produtos amplificados.

Para análise do polimorfismo *3801T>C* do gene *CYP1A1*, a reação de PCR foi realizada baseada no protocolo de Carstensen et al.(1993), modificado por Guembarovski (2007):

- 20pMol dos iniciadores;

- 25mM de dNTPs;

- 2,5U de Taq DNA polimerase;

- tampão de PCR (200mM de Tris-HCL, pH 8,0; 500mM de HCL 10x concentrado);

- 50mM de $MgCl_2$;

- 100ng de DNA genômico total;

- água estéril para completar o volume final.

A reação de clivagem com a enzima de restrição *MspI* foi realizada misturando 10µL do produto de PCR, 1U de enzima e tampão. Água ultra-pura estéril foi utilizada para completar o volume final. Os fragmentos foram amplificados e em seguida permaneceram a 37°C durante cerca de 12 horas. Os fragmentos clivados foram submetidos à eletroforese em gel de agarose 3,0% e visualizados de acordo com o descrito para os genes *GSTM1* e *GSTT1.*

Os oligonucleotídeos iniciadores originam um produto de amplificação de 340pb referente ao genótipo homozigoto T/T, o qual não sofre ação da enzima de restrição *MspI,* originando uma única banda de 340pb. O genótipo homozigoto C/C possui sítios para a enzima de restrição em ambos os alelos, gerando duas bandas de 200pb e 140pb. O genótipo heterozigoto T/C corresponde às três bandas de 340, 200 e 140pb, onde apenas um dos alelos é clivado (Carstensen et al., 1993; Guembarovski, 2007).

Para a análise estatística, o teste *t* de Student foi utilizado para comparar as médias de idade entre as populações alvo e a população referência e o teste do Qui-Quadrado de homogeneidade para verificar a distribuição dos sexos entre os grupos, na verificação da adequação do pareamento. Para o gene *CYP1A1*, no qual os três genótipos foram identificados (T/T, T/C e C/C), a significância

estatística das diferenças das freqüências genotípicas entre pacientes e controles foi estimada a partir de uma tabela de contingência 3x2.

A OR (razão de chances ou *Odds Ratios*) foi calculada como abaixo descrito, sendo A, B, C, D, E e F as freqüências absolutas obtidas:

OR T/T= 1,0 (adotado como referência)

OR T/C= (bxd)/(axe)

OR C/C= (cxd)/(axf)

Para os genes *GSTM1* e *GSTT1*, nos quais os heterozigotos não foram identificados, a significância estatística das diferenças das freqüências genotípicas entre as populações alvo e a referência foi estimada a partir de uma tabela de contingência 2x2.

A OR (razão de chances ou *Odds Ratios*) foi calculada como abaixo descrito, sendo A, B, C e D as freqüências absolutas obtidas:

OR= AxD/BxC

A análise conjunta de genótipos considerados de risco foi realizada através de tabelas de contingência 2x2 considerando-se combinações genotípicas de Fase I e II (T/C+C/C e *GSTM1* e/ou *GSTT1* nulos) e apenas de fase II (*GSTM1* e *GSTT1* nulos). O intervalo de confiança de 95% foi obtido pelo programa DPP Braile Biomédica (www.braile.com.br).

As análises dos genes, bem como a extração do DNA foram realizadas no Laboratório de Mutagênese Ambiental da UEL (Londrina-PR), sob a supervisão da Dra.Ilce Mara S. Cólus e sua equipe.

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Evaluation of mutagenic activity in supply water at three sites in the state of Rio Grande do Sul, Brazil

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Abstract

In this study, the *Salmonella*/microsome assay, using the microsuspension method, was utilized to evaluate water for public supply at three sites in Rio Grande do Sul. The first site selected was in an area under industrial influence and the others were in non-industrial reference areas. Based on 40 L samples of raw water and/or after conventional treatment, compounds were extracted with XAD4 resins using natural and acidic pH, and the extracts were analyzed in the TA98 and TA100 strains with and without S9. Raw water extracts in the industrial region induced 27.4 revertants/L (rev/L) for TA100 + S9 up to 226.3 rev/L for TA100 − S9, both for acidic pH extracts. After conventional treatment the responses varied from 20.6 rev/L (TA98 − S9) for natural pH extracts to 755.5 rev/L (TA98 − S9) for acidic pH extracts. For acidic extracts obtained from reference site samples, the response, with metabolic activation only, ranged from negative to minimal. Direct mutagenic responses in acidic extracts may be elevated in treated extracts, compared with raw water extracts, influenced by the presence of by-products of the chlorination process. However, the mutagenicity observed in the treated water extracts in the industrial area increased and reflected mainly the combination of directly and indirectly acting compounds in the source waters, that are heavily influenced by anthropogenic factors.

Keywords: Drinking water; Mutagenicity; *Salmonella*/microsome assay; Mutagenicity of organic compounds; Water quality

1. Introduction

Contamination of water used for public supply is considered a major risk factor for human health. These sources contain volatile, semi-volatile and non-volatile organic pollutants derived from agricultural, industrial and urban contaminants, many of them with known toxic and genotoxic effects. Several studies have examined the major contribution of contaminants to the genotoxic activity of aquatic environments evaluated by assays with organisms at different trophic levels, especially the *Salmonella*/microsome assay [1–9].

In addition to this pollution, there is evidence that the chlorine disinfection method traditionally used for drink-

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ing water treatment may generate mutagenic by-products by interaction with organic compounds that occur naturally in these sources, such as humic and fulvic acids which are part of the natural soil humus [10–12].

Several studies have examined drinking water mutagenicity, and the known or suspected content of chlorination products or contaminant substances present in raw water arising from anthropic processes [13–18]. These studies used mainly the *Salmonella*/microsome assay, recommended as a basic method for environmental sample studies because of its widespread use and continuous improvement [19–23].

Thus, a number of polar and apolar semi-volatile and non-volatile organic compounds extracted by Amberlite XAD resins, have been identified as mutagenic in the *Salmonella*/microsome, including MX and halogenated acids. These compounds are efficiently extracted with the XAD4 resins when the sample is acidified at pH 2 and in the presence of natural organic products such as humic and fulvic acids [12]. DeMarini et al. [24] found that XAD extracts of drinking water treated by different disinfection methods were 2–8 times more mutagenic in *Salmonella* (−S9) TA100 than in the TA98 strain. It is estimated that MX accounted for approximately 20% of the mutagenicity of the extracts. Studies in *Salmonella* showed that the water extracts and MX produced similar mutation spectra and suggest that MX and MX-like compounds (possibly halogenated aromatics) account for much of the activity of the non-volatile organics in drinking water. A number of compounds from specific chemical groups such as hydrocarbon compounds, halogenated alkanic organics, halogenated aromatic compound and trihalomethanes (THMs), which are by-products of chlorination during water treatment [25,26], are regulated by Brazilian legislation on Freshwater [27] and Water Supply [28].

In Rio Grande do Sul, Brazil, studies performed by our research group identified the presence of mutagenic compounds in the water and sediment of river basins in areas under the influence of a petrochemical industry [1–3,5], and in the leather and footwear manufacturing area [6]. Mutagenic activity was also observed in regions with a wide variety of industries [29] and in areas subject to different levels of urbanization [7]. The use of water from these rivers and streams, according to current law [28] should preserve the public water supply after conventional treatment including flocculation, filtration and chlorination. They also form the Guaiba Lake, which is the main source of drinking water for the metropolitan region of Porto Alegre, the capital of the state of Rio Grande do Sul.

The aim of this study was to evaluate the mutagenicity of water used for public supply at three sites in the state of Rio Grande do Sul, Brazil.

2. Materials and methods

2.1. Sampling sites

Three water treatment plant sites were chosen in three regions of the state of Rio Grande do Sul, Brazil. One of them was in an area of industrial influence, Site1, and two were in areas chosen as reference sites: Site2 which supplies a district of Porto Alegre, and Site3, outside the area of industrial influence (Fig. 1).

Site1: raw and conventionally treated water samples were collected in November 2000. The raw samples were collected from two areas in the river (Raw1 and Raw2) in a region located in the Caí river basin directly influenced by urban and industrial wastes from the meatpacking, beverage and tannin extraction industries. This region is upstream from the area that was influenced most by wastes from a petrochemical industry complex (34 km, Raw1 and 42 km, Raw2). It is in the main atmospheric dispersion quadrant with a history of mutagenic activity in water resources and in atmospheric particulates [1–3,5,30–32]. The sample of treated water (TreatedA) was obtained from a mixture of waters from sites Raw1 and Raw2 that supply a municipality with approximately 55,000 inhabitants. In November 2004, a second sample of treated water (TreatedB) was obtained at the same water treatment plant. However, during this period the type of intake changed, and about 80% was derived from Raw2.

Site2: raw and conventionally treated water samples were collected in June 2003. They were obtained from the Guaiba lake that supplies a Porto Alegre neighborhood with approximately 2500 inhabitants, from a water treatment plant located at the southernmost end of the municipality, inside a biological reserve, 30 km from the downtown area.

Site3: treated water samples were collected in October 2003, from two water treatment plants that supply a small municipality with approximately 38,000 inhabitants, 76 km from the state capital, in an area without the atmospheric dispersion plume of the large urban and industrial centers in the state. Raw water for the treatment plants comes from two different sources. The first (TreatedC) was from an area located in the Sinos river basin close to the headwaters, with small farms and livestock and a history of good environmental quality. The second municipal water treatment plant (TreatedD) is supplied by groundwater wells, 200–250 m deep. The waters from both intakes are treated with chlorine–fluorine (0.90 mg/L), and TreatedC receives conventional treatment.

2.2. Sampling

Water samples from Sites1 and 3 were collected by the Rio Grande do Sul State Foundation for the Environment Protection

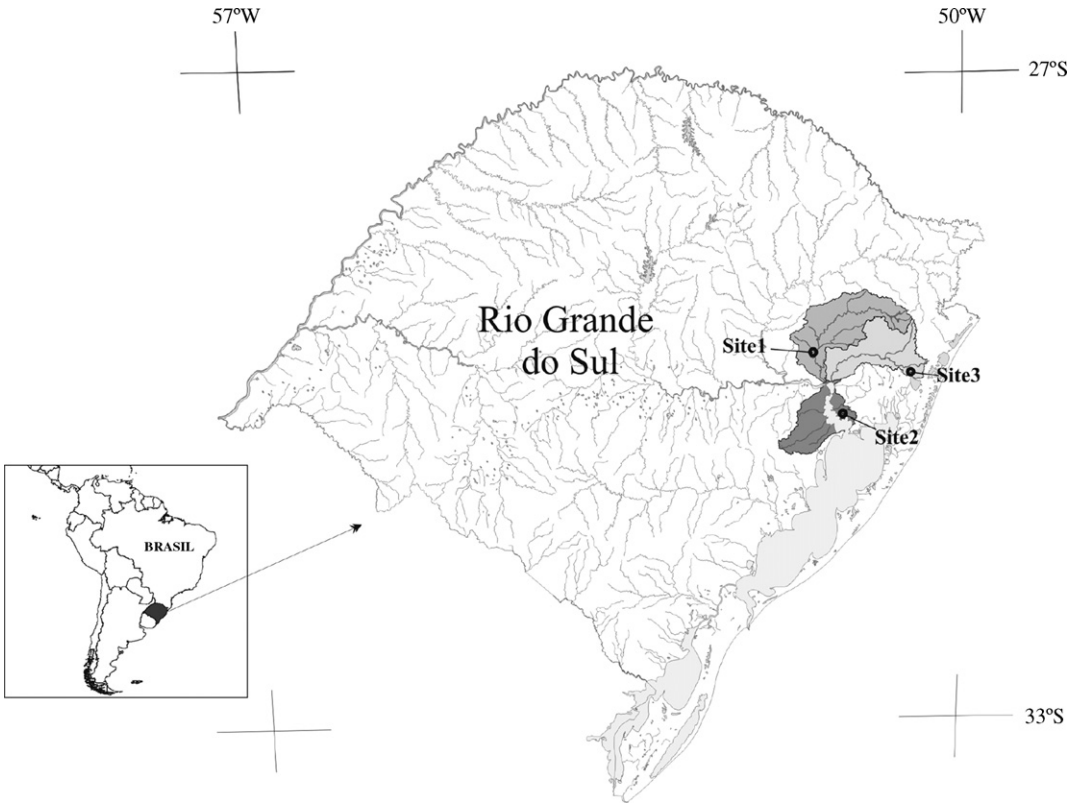

Fig. 1. Location of the sampling sites. Site1, located in the Caí river basin; Site2, Guaiba lake; Site3, Sinos river basin.

(FEPAM) team and from Site2 by the Porto Alegre Department of Water and Sewers (DMAE). Water samples were collected after and/or before conventional water supply treatment. Approximately 40 L of water were collected and stored at 4 °C for up to 10 days, the time needed for the different stages of the organic compound extraction process. The water is treated according to the quality standards established by Brazilian law [28].

2.3. Organic compound extraction

Organic compound extraction from the water samples (42 L in the sample collection of November 2000 and 40 L in the other collections) before or after conventional treatment was performed sequentially by adsorption in Amberlite XAD4-type resins, under natural pH conditions (neutral-basic) or acidified by adding HCl up to pH 2.0. The resin was placed in a chromatographic column, at ratio of 1 mL of resin/liter of sample, washed with methanol and treated using the following solvents: methanol (CASRN, 67-56-1), ethylic ether (CASRN 60-29-7), dichloromethane (DCM, CASRN, 75-09-2) and ultra pure water at a proportion of 1:1:2:3 (November 2000 extraction) or 1:1:2:6 (for the other samples). Next this was connected to the sample container, and system flow was defined for 100 mL/min. After the water passed through the chromatographic column, the excess retained in the resin was removed using a vacuum pump. Elution of organic compounds in natural pH was performed using 1 mL of methanol and 3 mL of dichloromethane per milliliter of resin (November 2000) or 4 mL (for the other sampling periods), extracting moderately polar to non-polar compounds. To elute the acid extraction, 1 mL of methanol and 3 mL (November 2000) or 1:4 (for the other sampling periods) of ethyl acetate (CASRN 141-78-6) were used per milliliter of resin, resulting in the extraction of polar compounds. The eluates were stored in a desiccator with super-dry sílica, protected from light for 3–4 days, in order to evaporate the water remaining in the sample. After this stage, the eluates were concentrated in a Rotary Evaporator until a 10 mL volume (In November 2000 the resuspension values varied from 9 to 14 mL), stored (−18 °C) in graduated flasks with a cover, protected from light and the organic mass extracted was determined in an analytic electronic scale. All the solvents used in the extraction process were pesticide grade (Merck) At the time of the mutagenic evaluation assay, the volume of extract needed was transferred to a test tube, with gaseous nitrogen reduced to dryness, and the extract was resuspended in spectrophotometric grade dimethylsulfoxide (DMSO, CASRN. 67-68-5) solvent (Riedel-de Haën) at the volume appropriate to the assay [7,33].

2.4. Salmonella/microsome assay

2.4.1. Mutagenic response

The organic extracts were tested for mutagenic and cytotoxic activity using the *Salmonella*/microsome assay by the microsuspension method [34], with TA98 and TA100 strains to measure frameshift and base-pair substitution mutations. A dose–response curve for 6 or 7 doses was analyzed corresponding to volumes of approximately 30–1000 mL of the initial sample/plate [35]. All assays were carried out at least in duplicate.

The organic extracts obtained from the treated water sample were analyzed in the presence or absence of a S9 microsomal fraction activated by the polychlorinated biphenyl mixture, Aroclor 1254 (purchased in a liophilized form from Moltox, USA), with added cofactors [35,36]. Negative (sterile distilled water) and positive (sodium azide—SAZ, CASRN. 26628-22-8, Merck do Brasil; 4-nitroquinoline oxide—4NQO, CASRN. 56-57-5; and 2-aminofluorene—2AF, CASRN. 153-78-6 from Sigma Chemical Company, St. Louis, MO) controls were included in each assay.

2.4.1.1. Data analysis. The sample was considered positive when a mutagenesis value of at least twice the negative value, a significant ANOVA ($p < 0.05$) and a positive dose–response ($p < 0.05$) were observed. The response was considered indicative when only one of these criteria was observed. The results obtained in the different assays were analyzed using the Salmonel program [37], as described in Vargas et al. [2]. In the present study, the use of linear regression or the Bernstein model was defined, which allows the elimination of doses analyzing the linear portion of the dose–response curve [38]. The result was expressed as revertants per liter of water sampled.

2.4.2. Cytotoxic response

The sample was considered cytotoxic when the percentage of surviving cells was less than 60% of the colonies compared with the negative control in at least one dose [2].

3. Results

3.1. Characteristic of organic extracts

Table 1 shows the characteristics of the organic extracts obtained from the different samples for extracted organic matter (EOM), with a natural pH, moderately polar to non-polar compounds and acidic pH, polar compounds.

For Site1 higher EOM values could be observed for extracts obtained in natural and acidic pH at site Raw1 compared with Raw2. However, for the TreatedA sample, low EOM values were observed for both types of extract. Except for TreatedB extracts from Site1 and the Treated extract from Site2, EOM values were generally higher in natural pH extracts compared to acidic extracts.

3.2. Mutagenicity results

The mutagenic activities of water samples from the different water treatment plants at three sites were studied using the *Salmonella*/microsome assay, TA98 and TA100 strains in the presence/absence of S9mix.

Tables 2 and 3 present, respectively, the mutagenesis indices (ratio between the number of his^+ induced in the sample and the number of his^+ observed in the negative control) and potency in revertants per liter (indicative and positive samples for mutagenicity), observed for raw and treated waters extracted with XAD4 at a natural pH and acidic pH at the three sites sampled: Site1 (2000 and 2004) and Site2 and Site3 (2003). The raw water sample extracts (natural or acidic pH) from the 2000 sampling showed mutagenic activity or an indicative response, sometimes with almost spontaneous mutagenesis indices for the different conditions or strains analyzed. The most meaningful responses were observed in sample Raw1 and especially the TreatedA sample. The TreatedB sample (2004) presented an indicative mutagenic response

Table 1
Characteristics of organic extracts analyzed

Water plants	Samples	Date	EOM *N* (μg/L)	EOM *A* (μg/L)
Site1	Raw1	November 2000	2228.7	1414.3
	Raw2	November 2000	266.7	151.1
	TreatedA	November 2000	165.9	141.3
	TreatedB	November 2004	262.5	577.5
Site2	Raw	June 2003	885.0	P
	Treated	June 2003	642.5	1405.0
Site3	TreatedC	October 2003	800.0	525.0
	TreatedD	October 2003	1147.5	1015.0

EOM, extracted organic material; *N*, extract in natural pH; *A*, extract in acidic pH; P, insufficient material for confirmatory weight test.

Table 2
Mutagenic index (MI) for each dose tested at three water treatment plant sites with TA98 and TA100 strains for the raw and treated waters extracted with XAD4 in natural pH (N) and acidic pH (A)

Water plants	Year	Dose (milliliter equiv/plate)	TA98				TA100			
			−S9		+S9		−S9		+S9	
			N MI	A MI	N MI	A MI	N MI	A MI	N MI	A MI
Site 1										
Raw1	2000	5	1.8	1.3	2.3	1.0	1.4	1.0	1.1	1.0
		10	1.6	1.5	1.9	1.1	1.7	1.0	1.1	1.0
		50	1.8	1.7	1.7	1.2	1.6	1.1	1.1	1.0
		100	2.1	2.0	2.0	1.2	1.5	1.2	1.2	1.0
		200	1.6	1.8	1.5	1.5	1.4	1.3	1.1	1.1
		500	1.6	1.9	1.2	1.8	1.5	1.7	1.1	1.1
		1000	1.6	1.9	1.0	2.5	1.4	2.5	1.2	1.2
Raw2	2000	5	1.5	1.2	1.5	1.1	1.3	1.1	1.2	1.2
		10	1.0	1.0	0.9	1.0	0.9	1.0	1.3	1.2
		50	1.1	1.1	0.9	1.1	0.9	0.8	1.1	1.5
		100	1.0	1.2	0.9	1.0	0.8	0.9	1.2	1.3
		200	1.1	1.0	1.0	0.9	–	1.0	1.2	1.3
		500	1.0	1.6	1.0	1.0	2.1	0.9	1.1	1.5
		1000	0.9	1.0	1.1	1.1	–	0.6	1.4	1.4
TreatedA	2000	5	1.6	2.1	1.1	1.3	1.5	1.2	1.0	1.0
		10	1.7	2.4	1.1	1.5	1.4	1.2	1.0	1.0
		50	1.6	2.7	1.3	1.4	1.1	1.2	1.0	1.0
		100	1.6	2.5	1.6	1.4	1.1	1.1	1.0	1.1
		200	1.5	2.2	1.7	1.5	1.1	0.9	1.1	1.1
		500	1.5	1.8	–	1.6	1.2	0.7	1.2	1.3
		1000	–	1.6	1.6	1.6	1.1	0.8	1.4	1.6
TreatedB	2004	31.25	1.1	1.1	0.8	0.8	1.0	1.1	1.0	0.9
		62.5	1.5	1.0	0.7	0.8	1.0	1.0	0.9	0.9
		125	1.2	1.4	0.9	0.8	1.0	1.0	1.1	0.7
		250	1.1	1.1	0.9	0.7	0.9	1.1	0.6	0.6
		500	1.3	1.2	0.9	0.6	0.7	1.2	1.1	0.6
		1000	1.5	1.0	0.9	0.8	0.5	–	1.1	0.9
Site 2										
Raw	2003	31.25	1.3	1.2	NT	NT	0.8	0.8	NT	NT
		62.5	1.0	1.0			0.7	0.9		
		125	1.2	1.1			0.7	0.9		
		250	1.4	1.0			0.7	1.0		
		500	1.5	1.2			0.8	0.8		
		1000	0.8	1.1			0.8	0.8		
Treated	2003	31.25	1.0	1.3	1.4	1.3	0.9	1.5	0.9	1.0
		62.5	0.9	0.9	1.3	1.1	1.3	1.2	1.0	1.1
		125	0.7	0.9	1.7	1.1	1.2	1.2	1.0	1.2
		250	1.1	0.8	1.2	1.4	1.3	1.3	0.9	1.2
		500	0.8	0.7	1.0	1.3	1.1	1.1	0.9	1.6
		1000	0.9	1.1	1.1	1.4	1.0	1.0	1.0	1.8
Site 3										
TreatedC	2003	31.25	0.8	0.8	1.4	1.5	1.6	1.1	0.8	0.8
		62.5	0.8	0.8	1.2	1.4	1.5	1.0	0.8	0.7
		125	0.7	0.8	1.2	1.5	1.5	1.5	1.0	0.8
		250	0.9	1.0	1.7	1.3	1.3	0.6	1.1	0.8
		500	0.9	0.7	1.8	1.3	1.5	1.2	0.7	1.1
		1000	0.7	0.3	1.3	1.3	0.5	1.4	0.6	1.0
TreatedD	2003	31.25	0.9	0.9	1.4	1.7	1.3	0.9	0.9	1.1
		62.5	0.9	0.8	1.1	1.1	1.2	1.2	0.8	1.1
		125	0.8	1.1	1.1	0.7	1.2	1.1	1.1	0.9
		250	1.0	0.8	1.2	0.9	1.0	1.0	1.2	1.0
		500	1.0	0.9	1.2	1.1	1.2	1.0	0.8	1.2
		1000	0.7	1.0	1.1	0.8	1.1	0.5	1.1	0.9

−S9, +S9 absence and presence of S9mix fraction; MI, N°. of his$^+$ induced in the sample/N° of his$^+$ in the negative control; NT, not tested. Negative control (0,5 μL DMSO/plate): 53.7 ± 21.44 (TA98 − S9), 54.9 ± 22.94 (TA98 + S9), 258.3 ± 173.93 (TA100 − S9), 182.6 ± 109.50 (TA100 + S9); Positive control: 4NQO (0.5 μg/plate; TA98 − S9) 120.9 ± 59.96; AZS (5 μg/plate; TA100 − S9) 660.3 ± 317.67; 2AF (10 μg/plate; 439.4 ± 317.32, TA98 + S9; 1043.1 ± 464.7, TA100 + S9). The sample was considered positive when a mutagenesis value at least twice that in the negative control was observed significant ANOVA ($p < 0.05$) and positive dose–response effect ($p < 0.05$). The response was considered indicative when only one of these criteria was seen. The complete data are in the Project report [32].

Table 3
Summary of results obtained for the raw and treated water samples from the three water plants sites by potency in revertants per liter (rev/L) of sampled water

Water plants	Samples	TA98				TA100			
		−S9		+S9		−S9		+S9	
		N (rev/L)	*A* (rev/L)	*N* (rev/L)	*A* (rev/L)	*N* (rev/L)	*A* (rev/L)	*N* (rev/L)	*A* (rev/L)
Site1	Raw1 2000	49.6	49.5	212.5	65.3	–	226.3	–	27.4
	Raw2 2000	–	30.8	–	–	215.0	–	78.4	124.1
	∑Raw	49.6	80.3	212.5	65.3	215.0	226.3	78.4	151.5
	TreatedA 2000	53.9	755.5	21.6	24.2	–	–	40.7	149.9
	TreatedB 2004	20.6	–	–	–	–	176.4	–	–
Site2	Raw 2003	–	–	NT	NT	–	–	NT	NT
	Treated 2003	–	–	–	–	–	–	–	128.9
Site3	TreatedB 2003	–	–	–	–	–	–	–	–
	TreatedD 2003	–	–	–	–	–	–	–	–

N, Natural pH extract; *A*, acidic pH extract; (–), mutagenic activity not detected; NT, not tested; responses presented for significant ANOVA ($p < 0.05$) and positive dose–response effect ($p < 0.05$), according to the Salmonel program [37].

for TA100 without S9 in the acidic pH extract and for TA98 without S9 in the natural pH extract. At Site2 only the extract of treated water presented an indicative mutagenic response for TA100 with S9 in the acidic pH extract. At Site3 the responses were negative.

Examining all the data for the three sites studied (Table 3) it was clear that mutagenic activity occurred at Site1 during 2000 (Raw1, Raw2 and TreatedA). Comparing the total potencies of the raw samples and of treated water in revertants per liter in this sampling, an eleva-

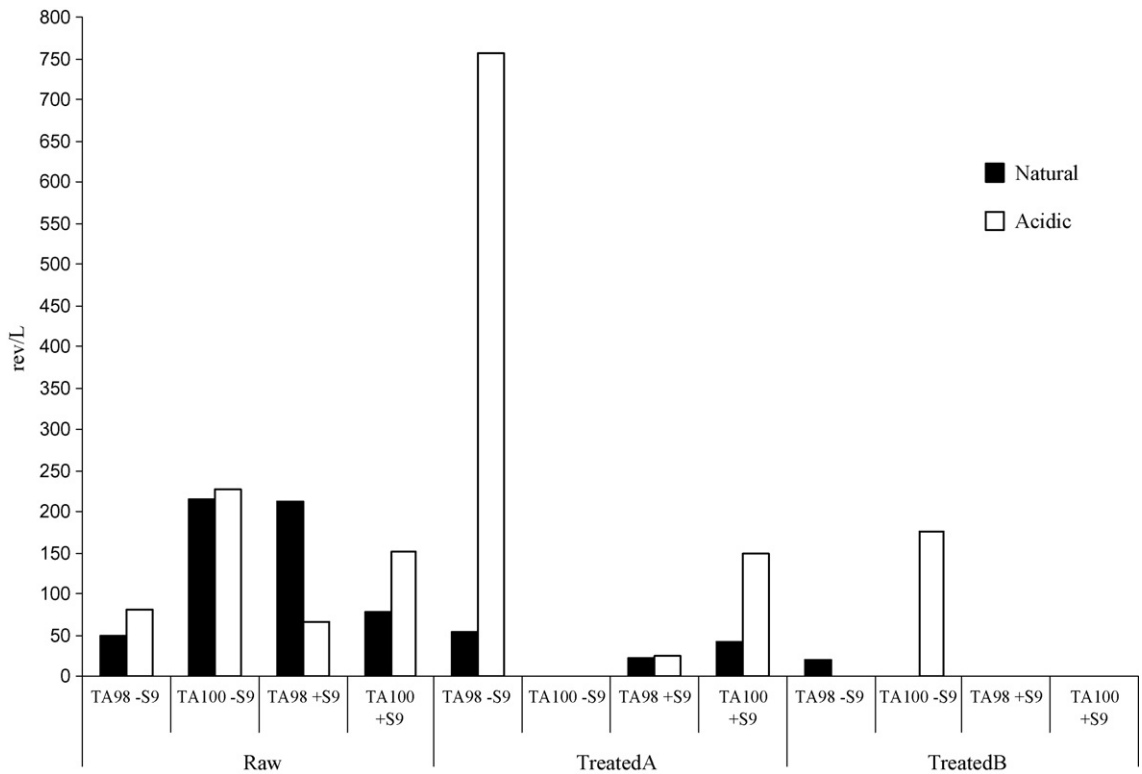

Fig. 2. Summary of results obtained for the raw and treated waters extracted with XAD4 in natural and acidic pH from the Site1 by potency in revertants per liter (rev/L).

tion in the potency of responses in the TreatedA sample for strain TA98 − S9 was seen in extracts in acid pH (755.5 rev/L), and in the others there was a reduction of most mutagenic responses. This reduction was more important for base-pair substitution without S9.

Fig. 2 shows the total potencies (natural and acidic pH) observed for the Raw1 and 2 samples together, TreatedA and TreatedB. An elevation of mutagenic activity was seen in the TreatedA sample for both extracts in assay TA98 − S9, 9.4-fold greater in the acid extract than in the raw water extract (80.3 rev/L raw water to 755.5 rev/L TreatedA). For the other assays, a reduction was found that varied from 100% in TA100 − S9 acid and natural extracts to practically no reduction for TA98 − S9 in natural pH and TA100 + S9 in acid pH.

Analysis of cell survival at the three sites studied (data not shown) did not show cytotoxicity for the extracts in natural pH and acidic pH in the presence and absence of S9 mix.

4. Discussion

This study showed increased mutagenicity in extracts of treated water samples obtained from sources that are heavily influenced by anthropogenic factors. The most significant responses were observed at Site1, collected from river areas that had suffered the impact of wastes from different industries such as meatpacking, beverages, tannin extraction, tanneries and petrochemicals in addition to those produced by the urban environment. The presence of organic mutagens and carcinogens in chlorinated drinking water as the result of industrial and agricultural contamination or untreated domestic sewage, waste-water treatment plants, air contaminants from combustion emissions, natural products of the water sources or products formed during water treatment or distribution [7,12,15,24,39] has been recognized in the literature.

Chlorine use during the water disinfection process may account for the presence of non-volatile mutagens in treated drinking water. Most of these by-products are direct mutagens extracted mainly at acidic pH and detected with TA98 and TA100 without S9, and it is even more mutagenic in TA100 strain [24]. These authors also showed that the spectra induced by two chlorinated waters obtained from two rivers were significantly different, highlighting the effect of different water sources [24]. Thus, part of the responses observed in the water treated acid extracts at Site1 (mainly at TreatedB TA100 − S9), may be caused by substances generated as by-products during the chlorination process. However, the negative response observed for TreatedA acid extract in TA100 − S9 is associated with a 9.4-fold greater increase of this extract in TA98 − S9 than the raw water extract, is not expected as a response to a by-product of the chlorination process.

This evidence added to other mutagenic activity responses detected at Site1 for TA98 and TA100 with S9 probably reflects the combination of direct and indirect-acting compounds in the source water [11,12,24,39–42]. Thus, it was also possible to observe a reduction in the potency of mutagenic activity (measured in rev/L) in the treated sample for (i) acidic extract for TA98 + S9 assay from 65.3 to 24.2 and for TA100 + S9 the values of raw water (151.5) and treated water (149.9) remained similar and (ii) natural extracts for TA98 + S9 assay from 212.5 to 21.6, for TA100 + S9 from 78.4 to 40.7 and for TA98 − S9 the values remained similar from 49.6 (Raw1) to 53.9.

Park et al. [15] studying the basic fraction of raw and treated (chlorine) water samples obtained from seven water treatment plants in five different cities in Korea, observed different responses for each area investigated. According to the authors, genotoxic contaminants of industrial and/or agricultural origin may appear in the source waters and contribute substantially to the observed genotoxic activity of the finished drinking waters. They also stress the fact that the addition of the S9 fraction reduced the mutagenic response of the assays. This relationship was observed in the present study for TA98 strain (Tables 2 and 3).

It should be considered that the raw samples evaluated in this study at Site1 were collected from river areas, which have a known history of mutagenic activity with a higher frequency and elevation of the response for direct-acting frameshift mutagens [1,3,5,6,30–32]. In these studies, in the area under petrochemical influence, 67% of responses for direct mutagens were observed, 40% in TA98 and 27% in TA100. On the other hand, the values for promutagens were less frequent (33%), but the answer went in the same direction [2]. Further, studies of airborne particulate matter made it possible to define the presence of direct-acting frameshift mutagens, mainly nitro and dinitro compounds, in the industrial area and their dispersion in the prevailing direction of winds up to 7, 9 and 23 km from the source [30]. There is also evidence that extracts of moderately polar organic compounds of sediment samples showed a predominant response for direct frameshift mutagens, both in the petrochemical area [5] and in the region studied which lies as far as 20 km upstream, influenced by industries such as tanneries [6]. The responses were less frequent for promutagens, but they were present for frameshift in the first area and for base-pair substitution mutagens in

the second [2,6]. Chemical tests performed in November 2000, at the same time as this study (unpublished data), identified in river raw water organic compounds such as (Raw1) fluoranthene (0.01 μg/L) and naphtalene (0.1 μg/L) (Raw2) fenantrene (0.01 μg/L) and naphtalene (0.05 μg/L), besides low concentrations of dioxins and furans.

It should also be considered that part of the pollution generated in industrial and urban areas is also due to surface runoff of storm water, increasing the complexity of interactions in river raw waters. Several studies have shown that storm water quality is not better than the effluent of a secondary treatment system for domestic waste-water and it depends on several factors, such as frequency of urban cleaning, rainfall intensity, temporal and spatial distribution, time of year and type of use of the urban area [7,44,45].

Hamester et al. [46], studying the public water supply and raw water involved in this intake in Rio Grande do Sul, related the presence of THMs to the precursor organic matter and residual chlorine in the sample. They observed that the highest organic matter concentration found in raw water in a year corresponded to the time of year when the highest amounts of THMs were found in the water supply. In the results observed in this study, the largest organic mass extracted was obtained from the Raw1 sample, which also presented higher values of mutagenic activity that were reflected in the response of TreatedA.

The decreased responses observed in the 2004 sampling (TreatedB) compared to 2000 (TreatedA) show two aspects: (i) the change in the 2004 sampling procedure in which about 80% of the raw water intake was performed at the site with a lower mutagenic response in 2000 (Raw2); (ii) the industrial control measures implemented by the environmental agencies during this period led to improved drinking water quality. The mutagenic response observed for TreatedB in assay TA100 − S9 for extracts obtained in acidic pH may have been due by-products generated in the chlorination process. However, the response observed for the extracts in natural pH for TA98 − S9, supports the complexity of interactions caused by mutagenic compounds found in raw water.

The pattern of mutagenesis detectable in the *Salmonella* assay is often different between contaminated sources and many chlorinated drinking waters [12]. Loper [43] has suggested that microsomal activation-dependent mutagenesis probably originates from industrial or agricultural sources.

The results obtained at the reference sites for this study show that mutagenesis in treated water depends, mainly, on raw water quality. This evidence supports the need to choose regions with little interference from anthropogenic activities as a source of drinking water to ensure water quality and less interference from these compounds in the natural products of the environment during the treatment process.

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4. Artigo 2

Comparative mutagenic study of urban airborne particulate matter in areas with different antropogenic influences and PAHs levels

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Abstract

Urban areas are the focus and also the target of air pollution from different sources of emission. Thus, the atmospheric compartment receives different and distinct chemical substances many of which, or their mixtures, are known genotoxic agents affecting human health. The purpose of this study was to perform the evaluation of atmospheric quality in the urban area of a city in Rio Grande do Sul State (Brazil) which is under the influence of different anthropogenic sources (Site 1), compared to a city with less intense urban influence (Site 2). For this study was quantified PAHs of environmental interest and the *Salmonella*/microsome assay was used as a mutagenicity biomarker. The organic compounds extracted of airborne particulate matter were analyzed by HPLC to detect the 8 specific PAHs of environmental interest (carcinogen and possible or probable human carcinogens). The organic extracts were also tested for mutagenic and cytotoxic activity using the *Salmonella*/microsome assay (TA98, TA100, YG1021 and YG1024 strains), in the presence or absence of a S9 microsomal fraction. At Site 1, benzo(ghi)perylene and indeno(1,2,3-c,d)pyrene were found at higher concentrations and the mutagenicity varied from 1.0±0.25rev/µg (TA98-S9) to 5.2±045 rev/µg (TA98+S9). Site 2, also showed BghiP and IP in larger amounts and the mutagenic responses were from 0.6±0.20rev/µg (TA98+S9) to 3.7±0.24rev/µg (TA98+S9), was been observed some negative results. The Site 2 presented low levels of PAHs and mutagenicity, confirming the possibility of representing reference values for other urban and industrialized areas. The occurrence of BghiP and IP together and in a larger amount, may be related to vehicular emissions. Both PAHs and nitroaromatic compounds contributed to the airborne mutagenicity.

Keywords: *urban airborne particulate matter, mutagenicity, PAHs,* Salmonella*/microsome assay.*

1. Introduction

The growing urbanization of many countries in Latin America has caused a great increase in the levels of air pollution. Many of these pollutants are mutagenic compounds. This atmospheric contamination does not only affect large cities since, according to Claxton et al. (2004), a mutagen in the air may present a much higher potential after transport, and possibly undergo photochemical reactions when close to the source of emission.

Urban areas are the focus and also the target of air pollution from different sources of emission. Thus, the atmospheric compartment receives different and distinct chemical substances many of which, or their mixtures, are known genotoxic agents affecting human health. Studies to evaluate the effects of human exposure to these substances are difficult to perform since the quantification and qualification of these substances is very complex, and they may react and form new compounds.

Polycyclic aromatic hydrocarbons (PAHs), formed from the incomplete combustion of organic matter containing carbon and hydrogen, are introduced into the environment from several sources. However, according to Reisen and Arey (2005), in urban areas the main source is exhaust gases from gasoline combustion engines, especially diesel.

Many organic air pollutants are adsorbed on the surface of air particulates (Zhao et al., 2002), and some of the components such as PAHs are a global issue because they can be transported over long distances through the atmosphere (Barra et al., 2007). Certain PAHs are classified according to IARC (2005) as

carcinogenic (Group 1), or probable (Group 2A) or possible (Group 2B) human carcinogens, besides the fact that many of them accumulate in the food chain.

Mutagenicity assays are increasingly used to evaluate the carcinogenic potential of complex atmospheric samples, and one of the often used assays is the *Salmonella*/microsome assay (Ames test) (Claxton and Woodall, 2007).

Some studies of atmospheric pollution associating the mutagenic activities of particulate matter and PAHs are documented for several cities in the world, as reported in the Reviews of Claxton et al (2004) and Lewtas (2007). However, few experiments have been performed in Brazil. In Rio Grande do Sul State studies on atmospheric pollution either investigate mutagenic activity (Vargas et al., 1998; Vargas, 2003; Ducatti e Vargas, 2003; Coronas et al., 2008) or perform chemical analyses (Dallarosa et al 2005; Mirlean and Roisenberg, 2006).

Therefore, the purpose of the present study was to perform a 6-month evaluation of atmospheric quality in the urban area of a city in Rio Grande do Sul State (Brazil) which is under the influence of different anthropogenic sources, compared to a city with less intense urban influence. For this study the *Salmonella*/microsome assay was used as a mutagenicity biomarker, besides quantifying 8 designated PAHs of environmental interest (carcinogen and possible or probable human carcinogens).

2. Material and methods

2.1. Sampling sites

Airborne particulate matter was collected in two cities of Rio Grande do Sul (southern Brazil), shown in Figure 1:

- Site 1 (Montenegro): This city has approximately 56,000 inhabitants (29°40.27'S and 51°27.35'W). The municipality covers an area of 420km^2 and it lies 55Km from the capital, Porto Alegre. The main activity of this city is trade. It suffers the influence of several types of anthropogenic activities in close-by regions, such as tanneries, metallurgical and textile industries, viticulture and others. It is located in the main atmospheric dispersion quadrant of a petrochemical industrial complex region (22Km distant), with a long history of mutagenic activity in atmospheric particulates and water resources (Vargas et al., 1988; Vargas, 2003; Pereira et al., 2007; Coronas et al., 2008; Vargas et al, 2008).

- Site 2 (Santo Antônio da Patrulha): a city of approximately 38,000 inhabitants, with a territory of 1,069km^2 lying 76Km from the capital (29°46.29'S and 50°35.15W). A commercial city, area without dispersion atmospheric plume of the large urban and industrial centers in the state, with small rural agricultural and/or cattle raising properties and the presence of distilleries (*alambiques*) (with small areas where cane burning occurs).

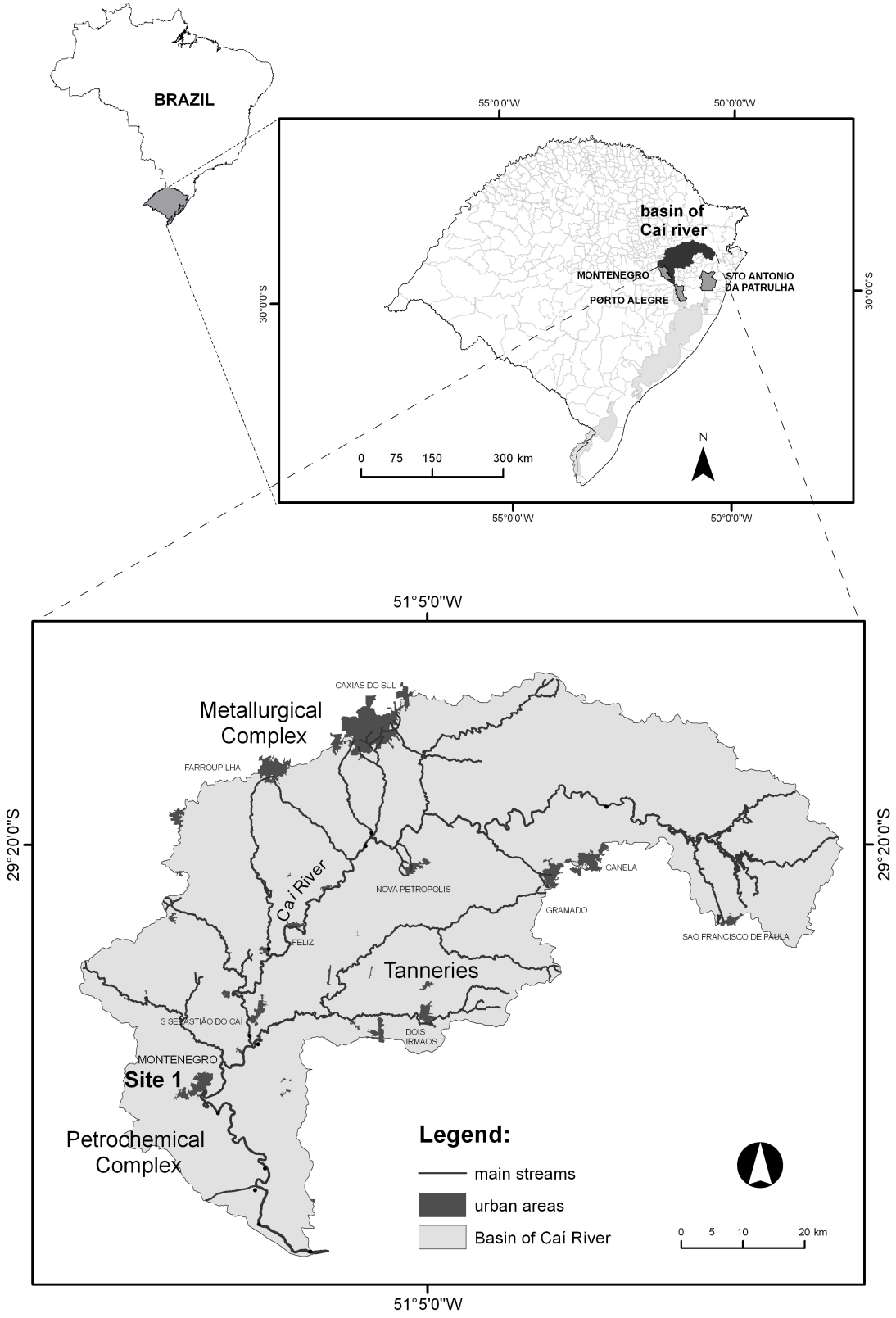

Figure 1. Location of the sampling sites: Montenegro (Site 1), Santo Antônio da Patrulha (Site 2) and Porto Alegre (capital of Rio Grande do Sul State). Detail for Basin of Caí river – Site 1.

2.2 Sampling procedure

Sampling was performed during the period from September 2004 to January 2005, and March 2005 in both cities. The data on climate variation were provided by the 8th Meteorology District of the National Institute of Meteorology (8°DISME – Porto Alegre/INMET, Brazil) and they are shown in Table 1.

Table 1.Climatic variables of Site 1 (Mt) and Site 2 (SA) during the collection period (September 2004 to January 2005 and March 2005).

Month/year	Day of filter collection	Day of variable collection	VARIABLE *					
			Temperature (°C)		*Precipitation (mm)*		*Humidity (%)*	
			Mt	*SA*	*Mt*	*SA*	*Mt*	*SA*
September 2004	05, 11, 17, 23, 29	10/sep	19.9	20.1	8.6	19.2	79	79
		20/sep	16.8	17.1	59.7	66.9	84	81
		30/sep	19.1	18.5	117.9	107.2	82	82
		Mean ± SD	**18.6±1.61**	**18.6±1.50**	**62.1±54.69**	**64.4±44.05**	**81.7±2.52**	**80.7±1.53**
October 2004	05, 11, 17, 23	10/oct	17.5	17.7	0	2.8	74	71
		20/oct	19.8	16.6	67.0	54.5	75	74
		31/oct	19.0	19.1	63.0	18.9	74	69
		Mean ± SD	**18.8±1.17**	**17.8±1.25**	**43.3±37.6**	**25.4±26.46**	**74.3±0.58**	**71.3±2.25**
November 2004	10, 16, 22, 28	10/nov	21.1	20.8	74.6	40.2	77	77
		20/nov	19.9	19.8	98.4	70.3	77	74
		30/nov	23.0	22.8	29.1	7.1	74	72
		Mean ± SD	**21.3±1.56**	**21.3±1.53**	**67.4±35.21**	**39.2±31.61**	**76.0±1.73**	**74.3±2.52**
December 2004	04, 10, 16, 22, 28	10/dec	23.3	23.1	27.9	15.3	77	75
		20/dec	23.2	22.9	15.7	18.4	72	69
		31/dec	23.4	23.3	0	0	66	64
		Mean ± SD	**23.3±0.10**	**23.1±0.20**	**14.5±13.99**	**11.2±9.85**	**71.7±5.51**	**69.3±5.51**
January 2005	03, 09, 15, 21, 27	10/jan	26.8	26.6	17.6	11.7	73	75
		20/jan	24.5	24.8	30.5	17.0	68	67
		31/jan	25.0	25.0	1.9	0.1	66	63
		Mean ± SD	**25.4±1.21**	**25.5±0.99**	**16.7±14.32**	**9.6±8.64**	**69.0±3.61**	**68.3±6.11**
March 2005	10, 28	10/mar	24.8	24.9	0.2	6.2	67	63
		20/mar	24.0	24.0	77.1	78.9	78	77
		31/mar	22.4	22.7	54.2	56.2	84	79
		Mean ± SD	**23.7±1.22**	**23.9±1.11**	**43.8±39.48**	**47.1±37.19**	**76.3±8.62**	**73.0±8.72**

Source: 8th Meteorology District of the National Institute of Meteorology (8°DISME – Porto Alegre/INMET), Brazil.
* Mean value compensated obtained on the day the variable was collected .

Samples of suspended particulate matter were collected on fiberglass filters (AP 40-810, 20cm x 25cm Millipore) using a high-volume sample (General Metal Works Inc.) operated at a flow rate of 1.3 to 1.5 m^3/min for 24h, every 6 days. The filters were weighed and stabilized before and after sampling (45% humidity) for total suspended particles (TSP) expressed in units of μg/m^3 of sampled air (ABNT, 1988).

2.3. Extraction of organic compounds

Half of each filter containing particulate matter was pooled to obtain monthly samples and submitted to extraction of the organic compounds. The compounds were extracted by sonification, with dichloromethane (DCM, CASRN. 75-09-2) – extracts moderately polar to polar compounds. The volume was reduced in a rotary evaporator at 40°C to approximately 12mL. This was followed by evaporation under a gentle stream of nitrogen gas until almost dryness. The percentage of extractable organic matter (EOM) was calculated and the mass obtained was compared to the volume of air sampled (EOM in μg/m^3), a calculation that allows a comparison between pools with different quantities of filters – Table 2.

Table 2. Characteristics of samples of the airborne particulate matter at Site 1 (Mt) and Site 2 (SA).

	Pool	Month of sampling	Number of filters	Total air volume (m^3 per filter)	TSP per filter ($\mu g/m^3$)	Total TSP ($\mu g/m^3$)	EOM ($\mu g/m^3$)
Site 1	Mt1	September 2004	5	12089	12, 13, 21, 71, 111	228	1.3649
	Mt2	October 2004	4	9493	13, 39, 54, 70	176	1.1262
	Mt3	November 2004	4	8759	21, 37, 40, 47	145	1.12
	Mt4	December 2004	5	10490	25, 46, 47, 77, 85	280	0.771
	Mt5	January 2005	5	10808	50, 53, 55, 63, 68	289	1.258
	Mt6	March 2005	2	4348	29, 51	80	0.782
Site 2	SA1	September 2004	3	7294	22, 23, 60	105	0.3784
	SA2	October 2004	3	7409	17, 22, 34	73	0.135
	SA3	November 2004	3	7365	18, 19, 64	101	0.1995
	SA4	December 2004	6	14527	14, 19, 22, 28, 41, 48	172	0.534
	SA5	January 2005	3	7227	28, 34, 43	105	0.542
	SA6	March 2005	4	10033	17, 18, 39, 64	138	0.359

TSP: total suspended particles; EOM: extractable organic material

The organic compounds extracted by this methodology were resuspended with dimethyl sulfoxide (DMSO, CASRN. 67-68-5) for the mutagenic assay and with acetonitrile (Merck; CASRN 1.00030.2500) for chemical analyses.

2.4. Analysis of Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs were analyzed by high-performance liquid chromatography (HPLC) in an Alliance 2690 Waters chromatograph equipped with a fluorescence detector. An ultra-rapid 5cm column was used, with a specific longitude for this family of compounds (SUPERCOSIL LC-PAHs, 5cm x 4.6mm, 3µm), which allows separating the PAHs of the European Directive (2004/107/CE) with an appropriate resolution, and in 12 minutes. This directive proposes to control 7 compounds of the PAHs family: benzo(a)anthracene, benzo(b)fluoranthene, benzo(j)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(ah)anthracene e indeno(1,2,3-c,d)pyrene, specifying an objective value only for BaP (1ng/m^3).

The scanning fluorescent detector was programmed to detect the 8 specific PAHs of environmental interest, according to IARC (2005), as shown in Table 3.

Table 3: IARC classification (2005) of the 8 PAHs in the study.

Group IARC (2005)	Definition of Group	PAH	Abbreviation	Number of aromatic rings	Molecular weight (g . mol^{-1})
Group 1	Carcinogenic	Benzo(a)pyrene	BaP	5	252
Group 2A	probably carcinogenic	Dibenzo(ah)anthracene	dBahA	5	278
Group2B		Benzo(a)anthracene	BaA	4	228
		Benzo(b)fluoranthene	BbF	5	252
	possibly carcinogenic	Benzo(j)fluoranthene	BjF	5	252
		Benzo(k)fluoranthene	BkF	5	252
		Indeno(1,2,3-cd)pyrene	IP	6	276
Group 3	non carcinogenic	Benzo(ghi)perylene	BghiP	6	276

The chromatographic conditions are specified in Table 4.

Table 4. Chromatographic conditions.

Injection	*Method 1* (GROUP): injection volume 10µL *Method 2* (BjF/IP): injection volume 20µL			
Detection	*Fluorescence*	Method 1 (GROUP)	BaA, BbF, BkF, BaP, dBahA, BghiP	$\lambda_{excitation}$: 296 nm $\lambda_{emission}$: 405 nm
		Method 2 BjF, IP	BjF	From the beginning to minute 10 $\lambda_{excitation}$: 318nm / $\lambda_{emission}$: 510 nm
			IP	From minute10 to the end $\lambda_{excitation}$: 270 nm / $\lambda_{emission}$: 490 nm
Temperature	28ºC			
Flux	1 mL/min			
Mobile phase	Water / Acetonitrile			
Gradient Method	0 -1.5 min:	40% water/60% acetonitrile		
	1.5 - 9 min:	linear gradient		
	9 - 12 min:	100% acetonitrile		
	12 - 18 min:	return to initial and stabilization		

All samples were injected in duplicate. Quantification is done by an external standard. For each PAH of interest, the response factor was calculated with a concentration standard close to that obtained by the sample.

Reagents: Acetonitrile HPLC (MERCK; ref: 1.00030.2500); Dichloromethane (Romil ref: H-202); Benzo(j)fluorantene (Lab. Dr. Ehrenstorfer, ref: C20575000); set of PAHs: PAH-mixture 610/525/550 in Methanol (CHEM SERVICE, ref: PPH-10M; approximate richness 99%).

2.5. Salmonella*/microsome assay*

Mutagenic response

The organic extracts were tested for mutagenic and cytotoxic activity using the *Salmonella*/microsome assay by the microsuspension method (Kado, 1986), with TA98 to measure frameshift mutations and with TA100 to measure base pair substitution.

Strains derived from TA98 were used to identify the presence of nitro compounds developed by Watanabe (1989, 1990) that resulted in increased responses to nitrated-PAHs nitroreductase over-expressing strains, since they contain plasmids with additional genes coded for enzymes that metabolized nitro and amino derivatives of PAHs to mutagenic forms. Strains YG1021 and YG1024 present higher nitroreductase and O-acetyltranferase activity, respectively. Strain YG1021 is more sensitive to the nitro derivatives and strain YG1024 is more sensitive to nitro and amino derivatives of PAHs.

A dose-response curve for 6 doses was analyzed corresponding to 1.25, 2.50, 5.0, 10.0, 20.0 and 40 µg/plate. All assays were carried out at least in duplicate. The organic extracts obtained were analyzed in the presence or absence of a S9 microsomal fraction activated by the polychlorinated biphenyl

mixture, Aroclor 1254 (purchased in a liophilized form from Moltox, USA), with added cofactors (Maron and Ames, 1983; Umbuzeiro and Vargas, 2003). Negative (5μL of dimethylsulfoxide, DMSO) and positive (TA98: 0,5μg/plate 4-nitroquinoline oxide - 4NQO, CASRN. 56-57-5; TA100: sodium azide - SAZ, CASRN. 26628-22-8; YGs: 0,15μg/plate 2-nitrofluorene-2NF, CASRN. 607-57-8, Merck do Brasil) controls were included in each assay.

Cytotoxic response

For this assay, the solution containing the sample and the bacterial culture of TA98 (100-200 cells) was plated on nutrient agar plates and incubated at 37°C. A reading of the surviving colonies was taken 72h later (Vargas et al., 1988).

2.6. Data analysis

a) Mutagenicity: the sample was considered positive when a mutagenesis value of at least twice the negative value, a significant ANOVA ($p < 0.05$) and a positive dose–response ($p < 0.05$) were observed. The response was considered indicative when only one of these criteria was observed. The results obtained in the different assays were analyzed using the SALANAL program. In the present study, the use of linear regression or the Bernstein model was defined, which allows the elimination of doses analyzing the linear portion of the dose–response curve (Bernstein, 1982). Positive and indicative results were considered as presenting significant mutagenesis. The results were expressed as revertants/μg of extract (rev/μg) and number of revertants per unit mass of particles and revertants/m^3 (rev/m^3).

Cytotoxic response: The sample was considered cytotoxic when the percentage of surviving cells was less than 60% of the colonies compared with the negative control in at least one dose.

b) The values obtained with the eight PAHs were correlated in each city with the climatic variables and also with the mutagenic analyses, using SPSS/PC statistical software package, through the Pearson correlation.

3. Results

The climatic data are shown in Table 1. During the sampling period, Site 1 presented a small media of temperature variation (18.6 to 25.4°C), the same occurring at Site 2 (17.8 to 25.5°C). As to precipitation, December was the month with least (14.5mm at Site 1 and 11.2mm at Site 2) and November the most rainfall (67.4mm) at Site 1 and September (64.4mm) at Site 2. The mean air humidity varied from 69.0% to 81.7% at Site 1 and 68.3 to 80.7% at Site 2.

The amounts of filters on the pools evaluated varied from 2 to 6, and the variation of the total air volume was 4348 (Mt6) to 14527m^3 (SA5). The TSP values found during the sampling periods are below the level of current Brazilian legislation (CONAMA, 1990). The amount of organic matter extracted ranged from 0.78 to 1.36$\mu g/m^3$ at Site 1 and from 0.13 to 0.54$\mu g/m^3$ at Site 2 (Table 2).

Table 5 shows the quantification of the eight PAHs analyzed. The month of March presented the highest amount of total PAHs (7.84ng/m^3) at Site 1, and for Site 2 it was in September (0.61ng/m^3).

Table 5. Quantification of PAHs at Site 1 (Mt) and at Site 2 (SA) obtained from September 2004 to January 2005 and March 2005. Average concentrations are expressed as ng/m^3.

	Sample	Month	PAHs								
			BaA	BbF	BkF	BaP	dBahA	BghiP	BjF	IP	TOTAL
Site 1	**Mt1**	Sep. 04	0.20	1.04	0.47	0.68	0.29	1.61	0.12	1.92	6.34
	Mt2	Oct. 04	0.10	0.39	0.18	0.19	0.10	0.58	0.07	0.12	1.72
	Mt3	Nov. 04	0.05	0.32	0.14	0.15	0.16	0.73	0.06	1.80	3.41
	Mt4	Dec. 04	0.02	0.18	0.08	0.11	0.07	0.38	0.03	0.08	0.96
	Mt5	Jan. 05	0.06	0.48	0.22	0.30	0.17	1.12	0.09	0.23	2.67
	Mt6	Mar. 05	0.12	0.89	0.45	0.64	0.37	2.40	0.11	2.85	7.84
Site 2	**SA1**	Sep. 04	0.02	0.13	0.05	0.05	0.06	0.22	0.02	0.06	0.61
	SA2	Oct. 04					NT				
	SA3	Nov. 04	0.02	0.07	0.04	0.02	0.03	0.13	0.01	0.19	0.50
	SA4	Dec. 04	0.01	0.06	0.03	0.04	0.04	0.17	0.01	0.05	0.41
	SA5	Jan. 05	0	0.03	0.02	0.01	0.02	0.07	0.01	0.02	0.18
	SA6	Mar. 05	0	0.05	0.02	0.02	0.02	0.09	0	0.13	0.34

See Table 3 for PAHs abreviation; NT: No tested sample (insufficient amount of sample).

The correlation with the climatic data and the PAHs was only significant for the humidity variable and BaA at Site 1 (r_p=0.822, $p \leq 0.05$). On the other hand, at Site 2, BbF and BkF were significant with temperature (r_p= -0.922 and r_p= -0.986, $p \leq 0.05$, respectively) and humidity (r_p=0.879 and r_p=0.906, $p \leq 0.05$, respectively), and BaA with temperature (r_p= -0.959, $p \leq 0.05$). Also at this site, the sum of the PAHs showed negative correlation with temperature (r_p= -0.970, $p \leq 0.01$).

Table 6 shows the results of PAHs in percentages. At Site 1, BghiP and IP was found at higher concentrations in the majority of samples. On the other hand BjF (in Mt1, Mt2 and Mt6) and BaA (Mt3, Mt4 and Mt5) were the least found PAHs. Site 2, also showed BghiP and IP in larger amounts, that were SA1, SA4 and SA5 with BghiP and SA3 and SA6 with IP. And as to the smaller amounts of PAHs obtained, both sites also agree: BJF for SA1, SA3 and SA6 and BaA for SA4 and SA5.

Table 6. Percentage of the quantification of PAHs at Site 1 (Mt) and Site 2 (SA) obtained for sampling. Average concentrations are expressed as %.

	Sample	Month	PAHs							
			BaA	BbF	BkF	BaP	dBahA	BghiP	BjF	IP
Site 1	**Mt1**	Sep. 04	3.22	16.41	7.48	10.79	4.50	25.44	1.84	30.32
	Mt2	Oct. 04	5.54	22.52	10.24	11.13	5.76	33.59	3.97	7.25
	Mt3	Nov. 04	1.38	9.31	4.12	4.53	4.73	21.38	1.79	52.76
	Mt4	Dec. 04	2.03	18.29	8.51	11.53	7.45	40.03	3.39	8.76
	Mt5	Jan. 05	2.18	17.86	8.32	11.36	6.36	41.92	3.27	8.72
	Mt6	Mar. 05	1.50	11.37	5.74	8.22	4.74	30.65	1.36	36.42
Site 2	**SA1**	Sep. 04	3.87	20.83	8.61	8.45	9.68	35.27	3.57	9.72
	SA2	Oct. 04	NT							
	SA3	Nov. 04	3.40	13.42	7.15	4.60	5.50	26.58	1.78	37.57
	SA4	Dec. 04	1.94	14.06	6.81	9.09	10.85	42.25	2.87	12.14
	SA5	Jan. 05	1.73	19.37	9.48	7.94	8.77	39.37	3.19	10.15
	SA6	Mar. 05	1.47	14.11	6.68	4.88	5.96	28.11	1.36	37.44

See Table 3 for PAHs abreviation; NT: No tested sample.(insufficient amount of sample).

At Site 1, mutagenicity varied from 1.0±0.25rev/µg (Mt4, TA98-S9) to 5.2±045 rev/µg (Mt6, TA98+S9), presenting only a negative response (Mt4, TA98+S9) (Table 7). As to the strains that identified nitro compounds, the response varied from 2.7±0.74rev/µg (Mt3, YG1021) to 53.8±2.03rev/µg (Mt2, YG1024). At site 2, the mutagenic responses were from 0.6±0.20rev/µg (SA5 and SA6, TA98+S9) to 3.7±0.24rev/µg (SA2, TA98+S9) and the highest value in the specific strains for nitrocompounds was observed at SA1 for YG1021 (62.3±11.34rev/µg). Negative results were seen at Santo Antonio (SA5 and SA6, TA98-S9).

Table 7. Mutagenicity (rev/µg) observed in 6 months of sampling at Site 1 (Mt) and at Site 2 (SA). The values are mean ± SE.

	Pool	TA98-S9	TA98+S9	YG1021	YG1024
Site 1	Mt1	1.1±0.51	2.1±0.73	6.8±0.60	20.2±1.00
	Mt2	2.8±2.61	2.1±0.59	10.2±1.36	53.8±2.03
	Mt3	3.5±0.58	2.1±0.60	2.7±0.74	49.1±6.45
	Mt4	1.0±0.25	-	20.0±2.15	5.6±0.67
	Mt5	2.1±0.36	1.0±0.43	36.9±5.36	14.8±1.65
	Mt6	4.0±0.44	5.2±0.45	19.6±2.13	38.5±3.38
Site 2	SA1	0.9±0.35	1.2±0.25	62.3±11.34	16.5±0.90
	SA2	a	3.7±0.24	NT	NT
	SA3	0.7±0.15	1.6±0.32	4.0±0.53	4.8±0.92
	SA4	1.3±0.30	0.7±0.19	1.6±0.72	8.5±1.11
	SA5	-	0.6±0.20	b	b
	SA6	-	0.6±0.20	b	b

$p \leq 0.05$; (-) did not present mutagenic activity; NT= No tested sample; (a) without statistical model; (b) The specific strains are only used when TA98-S9 presents a positive response for mutagenicity.

Negative control (DMSO): 53.8±9.31 (TA98-S9), 44.9±15.84rev/pl (TA98+S9), 205.8±175.45 (YG1021), 112.5±13.44 (YG1024), positive control: 4NQO- 229.7±75.06 (TA98-S9), 2AF- 646.8±184.69 (TA98+S9), 2NF- 2128.2±486.24 (YG1021), 2719.8±619.29 (YG1024)- values in revertants per plate.

It was not possible to perform all assays at Site 2, because the amount of samples was not sufficient. Since the sample from this site had a small quantity of EOM, the amount was insufficient to perform some mutagenicity assays and for PAHs analysis (SA2).

Figure 2 shows mutagenicity in rev/m^3 of the two cities studied. Site 1 presented the variation from 0.8±0.19rev/m^3 (Mt4, TA98-S9) to 4.1±0.35rev/m^3 (Mt6, TA98+S9); Site 2 varied from 0.1±0.03rev/m^3 (SA3, TA98-S9) to 0.7±0.16rev/m^3 (SA4, TA98-S9).

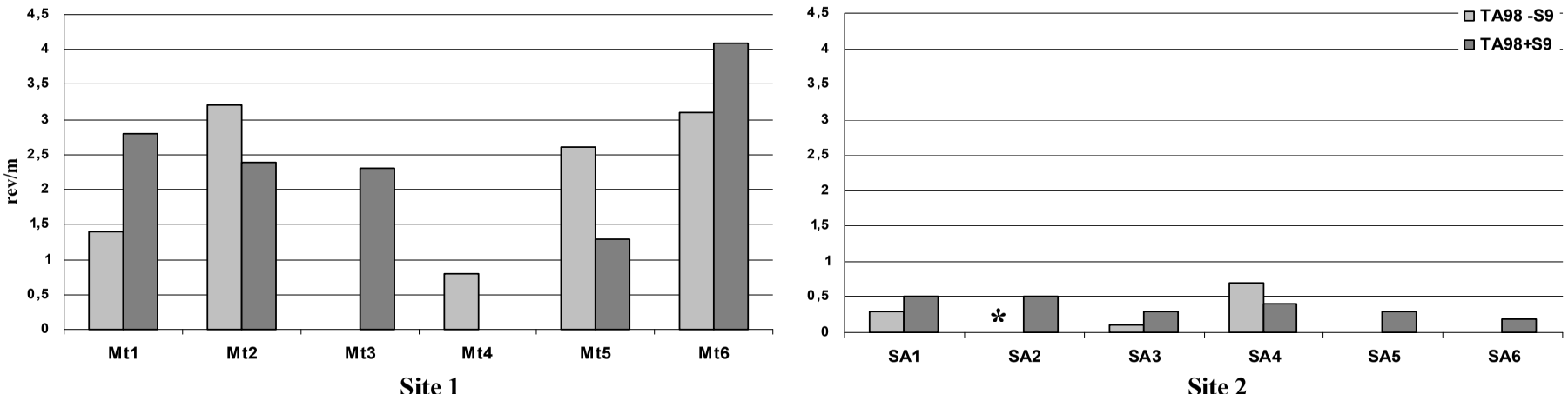

Figure 2. Mutagenic activity shown at Site 1(Mt) and at Site 2 (SA) with strain TA98, in the presence (+S9) and absence (-S9) of a liver metabolization fraction. Averages are expressed as rev/m^3. (*) SA2, TA98-S9: without a statistical model.

The insufficient number of samples also prevented performing all assays using strain TA100. Only Mt1, Mt2, Mt3 e SA1, SA2 were tested with and without the metabolization fraction using this strain. The responses at Site 1 ranged from 3.0±0.44rev/m^3 (Mt2, TA100-S9) to 4.5±1.17rev/m^3 (Mt3, TA100-S9), Mt1 and Mt3 (TA100+S9) being non-mutagenic. At Site 2, of the two months tested only one was positive (SA2, TA100+S9 0.1±0.03rev/m^3) – data not shown.

The total contribution of each of PAHs at the sites, and their correlation with the mutagenic responses, can be seen in Figure 3. At site 1, IP, followed by BghiP, and at Site 2 , BghiP are more abundant. At both sites BaA and BjF are present in a

smaller concentration. The correlation between the 8 PAHs with the mutagenic responses showed a significant response to $p \leq 0.05$ for TA98+S9 (rev/µg) with dBahA (rp=0.830), BghiP (rp=0.837) and IP (rp=0.828) at Site 1 and TA98+S9 (rev/m^3) with dBahA (rp=0.943), BghiP (rp=0.896) and BjF (rp=0.930) at Site 2.

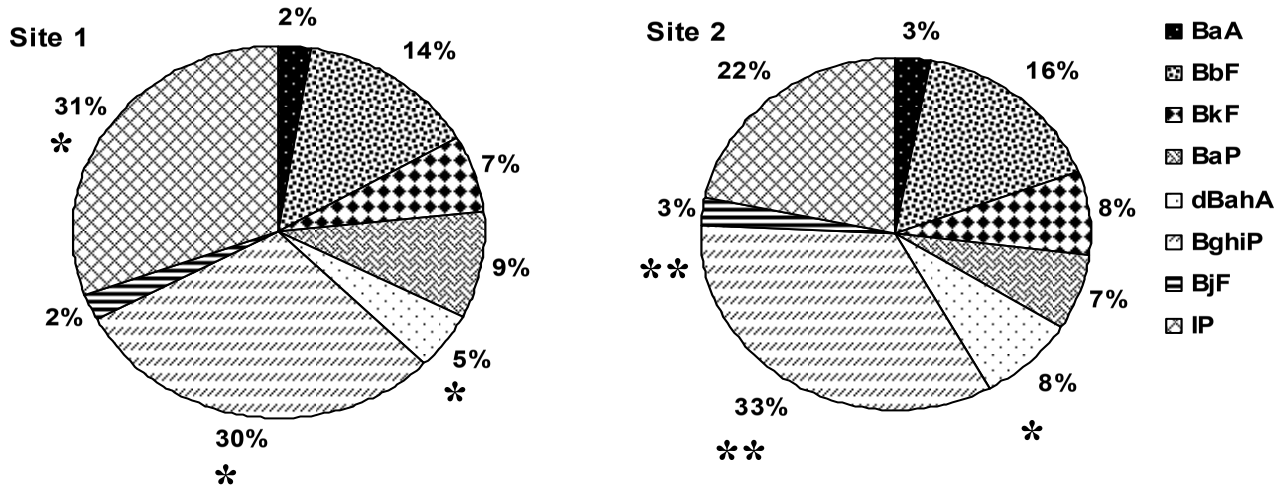

Figure 3. Composition of total PAHs (%) found at Site 1 and 2..

Correlations with $p \leq 0.05$: (*) between PAHs and TA98+S9 (rev/µg); (**) between PAHs and TA98+S9 (rev/m^3) – see the text for correlations.

As to cytotoxicity, no sample presented positive results.

4. Discussion

The present study investigated the mutagenic activity of particulate matter in the urban area of two cities, one of them (Site 1) located in a highly industrialized region, and the other in a region without an industrialized area, presenting only limited vehicular traffic (Site 2). The 6-month comparative study attempted to define Site 2 as an area of reference for studies investigating genotoxic atmospheric contaminants.

The determination of the total suspended particulate matter (TSP) in the air with a 24h sampling time is one of the quality evaluation measures that FEPAM (State Foundation for Environmental Protection) monitors in the state. The TSP shows levels below the regulation limits of the World Health Organization (150μg/m^3) and also of the Brazilian Legislation (150-240μg/m^3) (WHO, 2000; CONAMA, 1990). The TSP and EOM did not agree during the sampled months and there was no clear variation during the seasons studied.

During the sampling period, both municipalities presented little climate variation, and there was only a small difference among cities. According to Braga et al. (2005), the climate of Rio Grande do Sul State is influenced by cold air masses migrating from polar regions. The seasons are defined and rainfall is well distributed throughout the year – winter (June to September) is the rainiest season.

The climatic variables, climate and temperature, varied according to the quantities of PAHs found only at Site 2, and the variables and PAHs were highest In September and lowest in January.

Humidity and temperature are determinant conditions for PAHs dispersion. One of the positive correlations performed between the climatic data and PAHs,

indicated a significance between BaA and the humidity variable. BaA, indicating vehicular emission due to gasoline and diesel (Barra et al, 2007), is a low molecular weight PAH, one of the most photoreactive and easily dispersed (Yunker et al., 2002) – possibly a greater effect of humidity due to the adsorption of this compound in the particulate matter. However, it was this PAH that also appeared in the smallest amount at both sites.

BbF and BkF also presented a positive correlation with the temperature and humidity in the Site 2. But at this site, these compounds were not found in large amounts, and may be present because the region also has farms where sugar-cane was planted for the distilleries. Franco (2001), working in a region where there were large sugar cane plantations (crops that require burning), observed BbF and BbK as the main PAHs found.

At this site one of the most plentiful PAHs found was IP. This compound may be attributed to wood burning emissions. Vasconcelos et al. (2003) attributed the presence of IP in the study region to sugar cane burning. However, just as with the other PAHs, the levels of IP found at Site 2 are low, compared to values in cities with intensive sugar cane planting.

As to mutagenicity, this site presented low mutagenic activities, when these were not negative. The detectable mutagenic responses were obtained with the metabolizaiton fraction, and may be an indication of the participation of PAHs, since these compounds need to be metabolized to react to nucleophilic groups of cellular molecules (Meire et al.,2007). Sample SA2 presented substances with significant reactivity differing from the others at Site 2, but which did not result in significant values when related to atmospheric dispersion in rev/m^3.

Site 2 presented high values of nitro derivates only at a single event. This isolated fact shows that, despite the low mutagenic activity shown in the parental

strain (TA98), the great contribution of this mutagenicity is due to the nitro compounds.

However, this site presented low levels of PAHs and mutagenicity, confirming the possibility of representing reference values for other urban and industrialized areas. Pereira et al. (2007) studied the mutagenicity of the water supply at this site, which showed that it had not suffered any impact, and can be used as a city of reference for studies in Rio Grande do Sul State.

The limited amount of extract in some samples made it impossible to perform all assays. However, priority was given to tests with strain TA98 which is more sensitive to define the mutagenicity of air samples (De Martinis et al., 1999; Zhao et al., 2002; Umbuzeiro et al., 2008). Strain TA98 is the strain of choice because it has a low spontaneous background while being responsive to PAHs, especially when exogenous activation was used, and PAHs are found in combustion products (Claxton et al., 2004).

More representative values of PAHs were found at Site 1, especially in the months of September (Mt1) and March (Mt6) (Table 5). March also had the highest mutagenicity value, both with and without metabolization fraction. This major response is directly associated with the greater amount of PAHs found during this month.

The PAHs found in larger amounts at this site were BghiP and IP, the higher molecular weight PAH species. The occurrence of these two PAHs together and in a larger amount, may be related to vehicular emissions (Fernandes et al, 2002). However, the use of pure ethanol and a mixture of approximately 80% gasoline/20% ethanol in the Brazilian vehicular fleet results in different PAH profiles from those found in other urban regions around the world and there is no study of PAH emitted directly from ethanol and gasohol motor vehicular exhausts.

According to Vasconcelos et al. (2003), proposals of the PAH emission sources in airborne particles in Brazil are still quite limited.

Since PAHs in the cells must be mainly metabolized to be active in nuclear material, higher mutagenic responses presented in the absence of the metabolization fraction, may be due to the presence of nitro derivatives. In samples Mt2, Mt3 and Mt5 higher mutagenic responses occur in TA98-S9 than in TA98+S9 and the subsequent elevated response of nitro-specific strains. Cassoni et al. (2004) also observed this pattern of response and thought that it could be due to the nitro-PAHs usually present in diesel emissions. In four of the six samples studied at Site 1 greater sensitivity was observed for strain YG1024, more sensitive to nitro and amino derivatives of PAHs. Previous studies performed in the area of the petrochemical complex that influences Site 1 showed similar responses (FEPAM/PADCT/FINEP, 1997; 2003; Vargas, 2003; Coronas et al., 2008). Nitro-PAHs are formed both during the combustion process and through the atmosferic reactions of PAHs and nitrogen oxides, and are among the most potent bacterial direct-acting mutagens. Besides being located in the main quadrant of atmospheric dispersion of a petrochemical complex, there is a highway with heavy traffic of diesel trucks at Site 1

According to Barra et al. (2007), in Brazil, the main sources of urban PAHs are vehicular emissions, domestic heating, waste combustion, oil refineries and aluminium production. High urban PAH levels in Brazil can be explained by the high traffic levels, transport from industries located in suburban areas and a high level of total suspended particles. In this study in particular, the sum of the industrial sources at Site 1 may have a preponderant effect.

Most of the studies on air pollutants are performed during the coldest months of the year, since generally higher concentrations of all compounds were

observed in winter. Rehwagen et al. (2005), for instance, observed at La Plata City (Argentina) that the concentration of total PAHs in winter was more than five times higher than in summer. On the other hand, the results obtained from Coronas et al. (2008), who studied the mutagenicity of airborne particulate matter in a city of Rio Grande do Sul State (Brazil), close to Site 1, did not observe a clear variation during the seasons studied. Studies during the warmest periods of the year are extremely relevant, since according to Lewtas (2007), several types of DNA damage showed seasonal variation. PAH adduct levels and DNA strand breaks increased significantly in the summer period. Therefore an ideal study would be to monitor both the compounds and their effects by mutagenicity throughout the year.

Both PAHs and nitroaromatic compounds contributed to the airborne mutagenicity. However, specifically measured PAHs accounted for only a very small portion (<5%) of the total mutagenicity detected. This is because the air contains a large number of toxicants about little or nothing is known (Claxton and Woodall, 2007). Therefore, a large number of studies that evaluate the mutagenic potential of urban airborne particulate matter, especially in intensely industrialized areas, associated with the quantification of their compounds, such as PAHs, are extremely important, because they must receive more attention from the regulatory authorities and decisions makers.

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5. Artigo 3

Genetic monitoring of human populations living in urban cities exposed to different contaminants in Southern Brazil

Artigo a ser submetido para *Environmental Pollution*

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Abstract

Human exposure to the urban environment involves several hazardous airborne substances emitted by different types of sources. Microparticles found in the air may be associated with organic matter which contains several compounds, such as PAHs and nitro-PAHs, and may be a significant risk to human health, possibly leading to mutations and cancers. This study associated genotoxicity assays to evaluate human exposure with the atmospheric air of two urban areas in southern Brazil, which received different atmospheric contributions. The first site selected (Site 1) was under urban industrial influence and the other was a non-industrial reference area (Site 2). The organic extracts of airborme particulate matter were tested for mutagenicity with the *Salmonella*/microsome assay (TA98, TA100, YG1021 and YG1024 strains) and analyzed by HPLC for PAHs composition. Samples of lymphocytes and buccal mucosa cells of people residing in these two cities were evaluated using the comet and micronuclei assay (MN), respectively, analyzed as biomarkers of effect, and polymorphisms in genes *CYP1A1, GSTM1* and *GSTT1* were used as suscetibility biomarkers. Concentrations of the individual PAHs ranged from benzo(a)anthracene (0.01ng/m^3) to benzo(ghi)perylene (5.08ng/m^3). As to mutagenicity analysis, Site 1 presented all the mutagenic responses varying from 3.2±1.22rev/m^3 (TA98-S9) to 32.6±2.05rev/m^3 (TA98+S9) and Site 2 ranged from negative to minimal responses. Site 1 presented a high quantity of nitro and amino derivatives of PAHs, 56.0±3.68rev/µg being the highest value (YG1024 strain). The MN frequencies and the level of DNA damage measured by the comet assay were very low for both groups and no difference was observed between groups. Although Site 1 presented high mutagenic responses in the air samples, high amounts of PAHs, healthy people exposed to this environment did not show representative damage in their genetic material, presenting prevalent genotypes for polymorphics genes.

Keywords: total suspended particulate, human environmental exposure, mutagenicity, polycyclic aromatic hydrocarbons

1. Introduction

Human exposure to a variety of environmental chemicals is currently not limited only to individuals who are occupationally exposed or live in industrialized regions. Life in the urban environment exposes individuals to several hazardous substances which can be conveyed through the air, emitted mainly by the increasing vehicular traffic, and can also be transported from regions with intense industrial activity.

Environmental air pollution is associated with an increased incidence of morbidity and mortality. Among diseases caused by air pollution are allergy, respiratory diseases, cancer and immunodeficiencies (Faust et al., 2004; Vargas, 2003). In direct relationship with genotoxicity assays, Walker et al. (1982) showed a significant correlation between the *Salmonella* mutagenicity of airborne particulate organics and lung cancer mortality rates, but not with mortality rates for other diseases.

Environmentally low-exposure situations are common, and although the risk for the individuals is low, health effects on population can be large because of the high number of exposed individuals (Moller, 2006). Genetic monitoring studies help detect damage and possible environmental genotoxicants, and may contribute to the early detection of a carcinogenesis process.

Microparticles found in the air can become associated with organic matter which contains several compounds such as the polycyclic aromatic hidrocarbons (PAHs), and are a significant risk to human health, because they have the ability to penetrate and deposit in tracheobranchial and alveolar areas of the respiratory tract, and are thus responsible for causing mutations and cancers. According to Karahail (1999), the presence of irritating and genotoxic substances in particulate matter constituents is considered to have significant health implications. Many

PAHs have been identified as cancer-inducing chemicals for animals and/or humans.

Exposure to a mixture of chemical compounds can cause a significant increase in DNA damage levels. According to Garaj-Vrhovac and Zeljezic (2000), DNA damage revealed by the comet assay could originate from DNA single-strand breaks, repair of DNA double-strand breaks, DNA adduct, DNA-DNA and DNA-protein cross-links. This assay, also called the single-cell gel electroforesis (SCGE) assay, is a rapid and sensitive method for the detection of DNA damage (strand breaks and alkali-sites) in individual cells, induced by a variety of genotoxic agents.

The comet assay in peripheral lymphocytes and micronuclei assay in exfoliated buccal cells allow the identification of genotoxins in environmental and occupational biomonitoring and DNA repair studies, and also they are a complementary tool for clinical research (Faust et al., 2004; Fenech et al., 1999). The comet assay, which supplies information on recent levels of exposure to genotoxic substances that can still be repaired, together with the micronuclei (MN) assay, a test which allows measuring already established mutagenic damage, according to Laffon et al. (2002), allows the detection of a broad range of genotoxic damage and a more precise risk evaluation. Many studies have shown that the MN assay, especially the analysis of MN in buccal cells, is a sensitive method for monitoring genetic damage in human populations (Heuser et al., 2007; Karahalil, 1999; Majer et al., 2001).

Additional parameters that can influence MN and comet frequencies are inherited genetic polymorphisms in genes responsible for the metabolic activation and detoxification of clastogens (Mateuca et al., 2006). The inactivation of xenobiotics allows the inhibition of genotoxic and cytotoxic events that could cause

much damage to an organism, including cancers. The metabolic activation and inactivation enzymes of potential genotoxic agents are classified in two groups: Phase I enzymes, such as CYP1A1, are involved in activation of carcinogenic substances like PAHs, while Phase II enzymes, such as enzymes coded for a polymorphic family of Glutathione S-transferases genes (*GSTs*), deactivate carcinogens by conjugating them and facilitating excretion (Vineis, 2004).

Most studies that investigate the occurrence of genetic damage in human populations are performed by evaluating the occupational exposure, but few study possible damages due to daily exposure in an urban environment. This study proposes to associate genotoxicity assays to evaluate human exposure to particulate matters of urban areas that receive different atmospheric contributions.

In this study, we aimed: (1) to evaluate the mutagenicity of air samples (*Salmonella*/microssome assay) of two urban cities with different anthropic contributions, (2) to quantify 8 PAHs in these samples (3) to investigate the genotoxic and mutagenic effects (comet and micronucleus assay, respectively) on the health of people exposed to airborne matter (4) to associate these genotoxic responses with the influence of metabolization genes *CYP1A1, GSTT1* and *GSTM1*.

2. Material and methods

2.1 Sampling sites

This study was performed in two cities of Rio Grande do Sul State (southern Brazil) (Figure 1).

- Site 1: the town of Montenegro with approximately 56 000 inhabitants, and an area of 420km^2, 55Km from Porto Alegre, the state capital (29°40.27'S and 51°27.35'W). It is located in a region influenced by different types of anthropogenic sources from neighboring towns, such as a tannery center, metallurgical and textile industries, viticulture and others, and it lies in the main atmospheric dispersion quadrant from a petrochemical industry complex (22km from the complex), a region with a long history of mutagenic activity in atmospheric particulates and in water resources (Coronas et al., 2008; Pereira et al., 2007, 2008; Vargas, 2003; Vargas et al., 2008).

- Site 2: the town of Santo Antônio da Patrulha, with approximately 38 000 inhabitants, and an area of 1,069 km^2, 76Km from the state capital (29°46.29'S and 50°35.15W). A commercial city it lies in an area without the dispersion of atmospheric plume of the large urban and industrial centers in the state, with small agricultural rural and/or livestock breeding properties. This town was considered a reference for this study (Pereira et al.,2007, 2008).

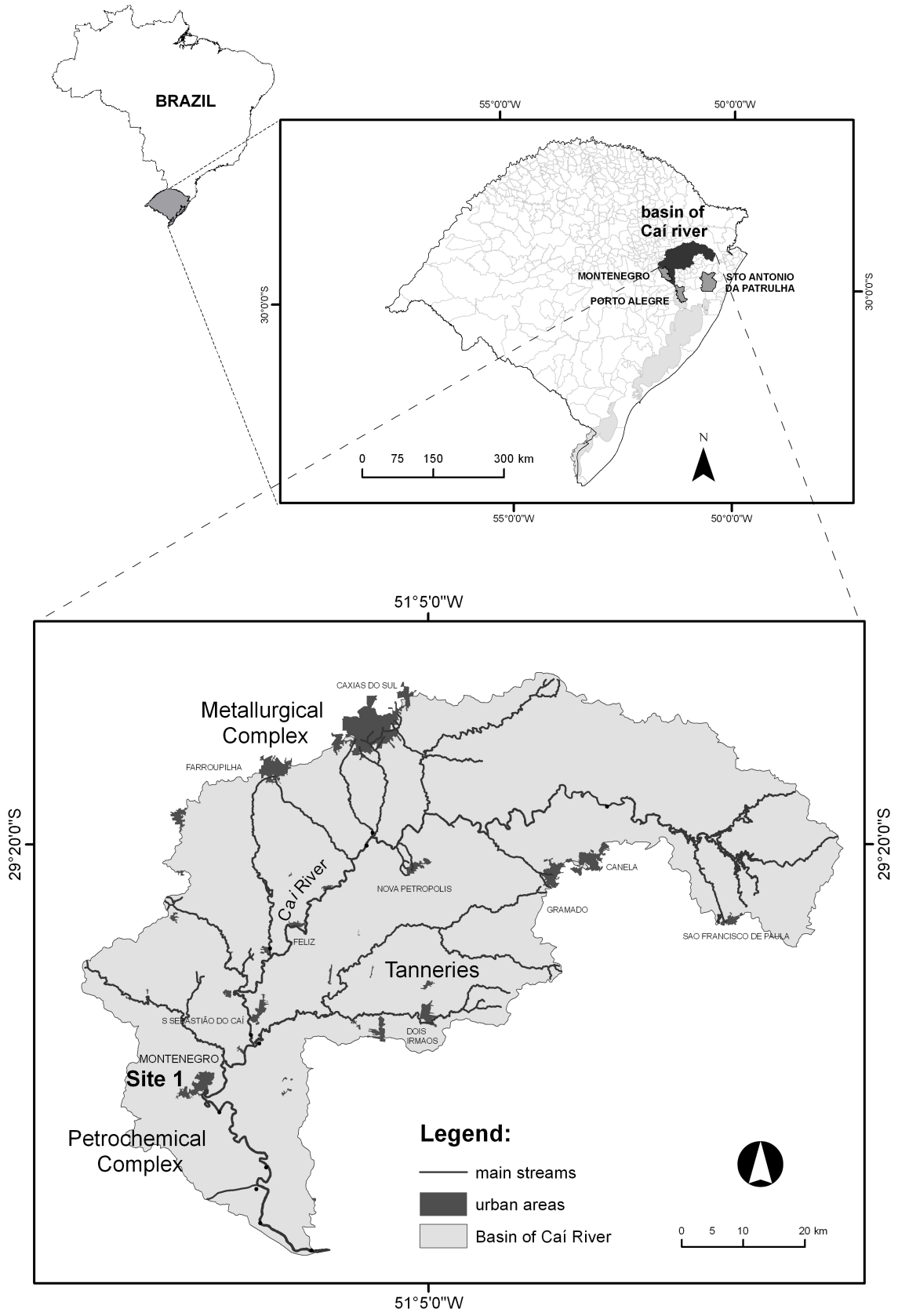

Figure 1. Location of the sampling sites: Montenegro (Site 1), Santo Antônio da Patrulha (Site 2) and Porto Alegre (capital of Rio Grande do Sul State); Basin of Caí river – Site 1.

2.2 Air Sampling procedure

Air samples were collected at the same time as the human biomonitoring and the collections occurred in August, October and November 2004, and in April 2005 at Site 1 and September to November 2004 and January and March to May 2005 at Site 2.

Samples of suspended particles matter were collected on fiberglass filters (AP 40-810, 20cm x 25cm Millipore) using high-volume samples (General Metal Works Inc.) operated at a flow rate of 1.3 to 1.5 m^3/min for 24h, every 6 days, for two consecutive weeks (one filter sampled on the same day as the human monitoring was performed and the other six days before this collection). The filters were weighed and stabilized before and after sampling (45% humidity) for total suspended particles (TSP) expressed in units of μg/m^3 of sampled air (ABNT, 1988).

2.3 Extraction of organic compounds

Half of each filter containing particulate matter was grouped two by two and submitted to extraction of the organic compounds. The compounds were extracted how described in Pereira et al (2008).

2.4 Analysis of Polycyclic Aromatic Hydrocarbons (PAHs)

The organic compounds extracted were resuspended with acetonitrile and the PAHs were analyzed by high-performance liquid chromatography (HPLC) in an Alliance 2690 chromatograph (Waters) equipped with a fluorescence detector. An ultra rapid 5cm column was used, with a specific longitude for this family of compounds (SUPERCOSIL LC-PAHs, 5cm x 4,6mm, 3μm), which allows separating the PAHs of the European Directive (2004/107/CE) with adequate

resolution and in a time of 12 minutes. This directive proposes to control 7 compounds of the PAH family: benzo(a)anthracene (BaA), benzo(b)fluoranthene (BbF), benzo(j)fluoranthene (BjF), benzo(k)fluoranthene (BkF), benzo(a)pyrene (BaP), dibenzo(ah)anthracene (dBahA) and indeno(1,2,3-c,d)pyrene (IP), specifying the maximum value only for BaP (1.0ng/m^3 for PM10 in 24h).

The scanning fluorescence detector was programmed to detect the 8 specific PAHs of environmental interest (IARC, 2005), the seven of the European Directive and also benzo(ghi)perylene (BghiP). The chromatographic conditions, reagents and data anlysis were the same used in Pereira et al. (2008).

2.5 *Salmonella*/microsome assay

2.5.1 Mutagenic response

The organic extracts were resuspended with DMSO and tested for mutagenic and cytotoxic activity using the *Salmonella*/microsome assay by the microsuspension method (Kado et al., 1986), with TA98 to measure frameshift mutations and with TA100 to measure base pair substitution. Nitro-specific strains derived from TA98 were used: strain YG1021 is more sensitive to the nitro derivatives and strain YG1024 is more sensitive to nitro and amino derivatives of PAHs (Watanabe et al., 1989; 1990).

The procedures of assay were described in previous paper (Pereira et al., 2008).

2.6 Human Biomonitoring

This study was aproved by the Brazilian National Ethics Committee on Research (CONEP, No 23078.200270/04-17, 23/06/2004).

Adult, healthy, male volunteers between the ages of 18 and 40 years, who remained most of the day, did not work with agricultural inputs and preferably non-smokers were selected for evaluation at Site 1 and Site 2.

Initially the sample exclusion factors were researched (high exposure to radiation, dietary and excessive alcohol habits, and health status such as the use of prescription medicines). Each individual signed an Informed Consent Form and answered a standard questionnaire with general information on: social history, dietary habits, smoking, chemical addiction, use of medications and other factors (Carrano and Natarajan, 1988).

2.6.1 Micronucleus assay

Exfoliated buccal cells were collected from each individual by scraping the cheek mucosa with a moist wooden spatula. The cells were transfered to a tube containing saline solution, centrifuged (2000G) three times, fixed in 3:1 methanol/acetic and dropped onto pre-cleaned slides, air-dried and stained with Feulgen/Fast Green method. The cells were examined under light miscroscope (1000x) to determine the micronucleus frequency. Two thousand cells (1000 cells from each slide and for each reader) were analyzed. Micronuclei were scored according to the criteria described by Tolbert et al. (1992).

The alkaline version of the comet assay was performed according to Singh et al. (1988) and Tice et al. (2000). Isolated lymphocytes (10μL) were immediately added to 120μL of 0.5% low-melting-point agarose at 37°C and layered onto precoated slides with 1.5% normal-melting-point agarose in duplicate. The slides were immersed in 1% lysing solution (2.5M NaCl, 100mM EDTA, 10mM buffer Tris-HCl, pH 10.0, 1% sodium sarcosinate with 1% Triton X-100, 10% DMSO) for 1 hour.

After submission to electrophoresis (under alkaline buffer solution, pH>13), they were fixed in absolute alcohol and stored until the moment of analysis. Lymphocytes collected always from the same person were used as a positive and negative control of the electrophoresis run. For positive control, the lymphocytes were processed *in vitro* by hydrogen peroxide at 100mM for 5 minutes in ice. The slides were stained with 100µl of ethidium bromide (2µg/mL) and analyzed on a fluorescence microscope (Olympus) connected to a camera with an image-analysis system (Comet Assay II, Perceptive Instruments, Suffolk, Haverhill, UK) under a magnification of 400x. A total number of 50 cells (comets) per slide were analyzed (Speit and Hartmann, 1999). Two parameters were taken into account in order to estimate DNA damage: tail moment (DNA product contained by the tail X florescence intensity) and the intensity of the comet tail (% of migrated DNA).

Data Analysis: Comparisons between Site 1 and reference Site 2 groups were performed correlating the micronucleus and comet assay data by Pearson Correlation significant at the level of 0.05 using SPSS/PC statistical software package.

Differences in tail intensity and tail moment values between groups were evaluated by analysis of variance using hierarchical models, where the various factors are nested in a specific order ("nucleoids" are nested inside the subject and the subject inside the town) (Lovell and Omori, 2008). The mean values of DNA damage were compared using Student's t test for confounding factors. Statistical evaluations were conducted using the SPSS for Windows statistical package, version 13 and Proc Mixed -Statistical Analysis System (SAS) version 9.1.

2.6.3 Metabolization genes

The other part of collected blood (2mL) was utilized for DNA extraction by a kit extraction (Kit GFX Genomic Blood DNA Purification – Amersham Pharmacia, CASRN. 27960301) and a salting-out method (Miller at al., 1988).

2.6.3.1 CYP1A1 (3801T>C) polymorphism.

A PCR-RFLP method was used to determine polymorphism 3801T>C (Carstensen et al., 1993): 100ng/μL of DNA were amplified in a total volume of 25μL reaction mixture containing 20mM Tris-HCl, 50mM KCl, 2.5mM $MgCl_2$, 2mM of each deoxynucleotide triphosphate, 100ng/μL of each primer and 2U of *Taq* DNA polymerase (Invitrogen). PCR was carried out in a PXE 0,2 Thermal Cycler (Thermo Electron Corporation) thermocycler. The PCR conditions were set as 94°C for 5 minutes, 30 1-minute cycles of 1 minute at 94°C, 1 minute at 56°C and 1.5 minutes at 72°C, followed by 2 minutes at 72°C. The amplified fragments (10μL) were digested with 15U of *MspI* enzyme (MGM Assessoria Biológica). The digestion products were separated on 3% agarose gel at 60V for 2 hours and viewed under UV light after staining with ethidium bromide.

2.6.3.2 GSTM1/GSTT1 null polymorphisms

Genotypes of GSTM1/GSTT1 genes were studied using Multiplex-PCR (Abdel-Rahman et al., 1996) with modifications: 100ng/μL of DNA were amplified in a total volume of 25μL reaction mixture containing 20mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$ 2,2mM of each deoxynucleotide triphosphate, 100ng/μL of each primer and 1U of *Taq* DNA Polymerase (Invitrogen). PCR was carried out in an PXE 0,2 Thermal Cycler (Thermo Electron Corporation) thermocycler. The PCR conditions were set as 94°C for 5 minutes, 30 cycles of 1 minute at 94°C, 1 minute

at 59°C and 30 seconds at 72°C, followed by 5 minutes at 72°C. After the amplification, PCR products were subjected to electrophoresis on a 2% agarose gel at 80V for 1.5 hours and viewed under UV light after staining with ethidium bromide.

Statistical analysis: Statistical analyses were performed using Student's *t*-test to compare the mean age between Site 1 (exposed group) and Site 2 (control group). The comparison of the gene frequencies observed in the population of Site 1 and of Site 2, the control, was performed using contingency tables to calculate the odds ratios (OR) with a confidence interval (CI) of 95%, in an association study. For the *CYP1A1* gene, for which the three genotypes were identified, a 3x2 contingency table was constructed taking the T/T genotype as reference (OR=1.0) to determine the OR value for T/C and C/C genotypes. For *GSTM1* and *GSTT1* genes (heterozygous not identified), the OR value was estimated by a 2X2 contingency table.

3. Results

3.1 Airborne monitoring

Air sampling occurred concurrently with human biomonitoring, and it was about five periods at Site 1 (Mt1 to Mt5) and 6 at Site 2 (SA1 to SA6). However, at Site 1 sampling Mt1 was performed only for human monitoring, on July 27, 2004.

The air filter data, as well as the mean of the climate conditions during the sampling period are in Table 1. At Site 1 the highest concentration of TSP (154μg/m^3) and EOM (3.96μg/m^3) was found at Mt3. Mt5 presented the lowest concentration of TSP (84μg/m^3) and Mt4 of EOM (1.08μg/m^3). At Site 2 much lower values were found, SA4 (104μg/m^3) and SA2 (0.98μg/m^3) presented the highest concentrations of TSP and EOM, respectively, and SA6 (28μg/m^3) and

SA1 (0.41µg/m^3) the lowest. The values found for TSP are below the limits determined by Brazilian legislation (CONAMA, 1990).

Table 1. Characteristics of the samples of air particulate matter and means of climatic conditions at Site 1 (Mt) and Site 2 (SA).

	Sample	Date of filter collection and human monitoring[a]	Total air volume (m^3 - ∑ of the filters)	TSP per filter[b] (µg/m^3)	Total TSP (µg/m^3)	EOM (µg/m^3)	Climatic variables [c]		
							Temperature (°C)	Humidity (%)	Precipitation (mm)
Site 1[d]	Mt2	6 and 12/8/2004	4639	42; 60	102	3.88	14.4	81,0	44.0
	Mt3	18 and 24/8/2004	4605	34; 120	154	3.96	16.9	83,0	18.5
	Mt4	29/10 and 6/11/2004	4418	11; 76	87	1.08	20.1	75.5	68.8
	Mt5	9 and 15/4/2005	3500	41; 43	84	1.66	21.4	86.0	67.4
Site 2	SA1	3/9/2004[e]	2448	35	35	0.41	20.1	79.0	19.2
	SA2	9 and 14/10/2004	4848	14; 49	63	0.98	17.7	71.0	2.8
	SA3	20 and 26/11/2004	5125	17; 39	56	0.46	21.3	73.0	38.7
	SA4	25 and 31/01/2005	1700	30; 74	104	0.71	24.9	65.0	8.6
	SA5	1 and 7/4/2005	1700	14; 48	62	0.70	22.6	80.0	87.4
	SA6	13 and 19/5/2005	1500	9; 19	28	0.66	19.6	85.0	70.9

TSP: total suspended particles; EOM: extractable organic material
(a) Human monitoring always on the second week of filter collection;
(b)CONAMA (1990): sampling of 24h - primary standard 240µg/m^3, secondary standard 150µg/m^3;
(c)Source: 8th District of Meteorology do Instituto Nacional de Meteorologia (8°DISME – Porto Alegre/INMET), Brazil (mean value obtained during the period when the variable was collected);
(d) Sample Mt1, collected 27/07/2004, does not present data on air filters, only data from human biomonitoring;
(e)pool with only a single filter.

The climate variation data were provided by the 8th District of Meteorology of the National Institute of Meteorology (8° Distrito de Meteorologia do Instituto Nacional de Meteorologia) (8°DISME – Porto Alegre/INMET, Brazil). The climatic conditions at Site 1 and Site 2 varied, respectively from 14.4(Mt2) to 21.4°C(Mt5) and from 17.7(SA2) to 24.9°C(SA4) for temperature; humidity from 81(Mt2) to 86%(Mt5) and from 65(SA4) to 85%(SA6); precipitation from 18.5(Mt3) to 68.8mm(Mt4) and from 2.8(SA2) to 87.4mm(SA5) (Table 1).

Table 2 shows the amount found of the 8 PAHs analyzed. In most samplings, hydrocarbon found in the largest amount at Site 1 was IP, followed by BghiP and BbF. However, in Mt3, a sample with the greatest amount of PAHs observed, BghiP (5.08ng/m^3) was found at a higher value, followed by BbF (2.98ng/m^3) and BaP (2.57ng/m^3). At this site, the total PAHs varied from 2.90(Mt5) to 17.54ng/m^3(Mt3). At site 2 small amounts of PAHs were found that

varied from 0.27(SA3) to 0.65 ng/m^3(SA4). The largest amount of PAHs found at this site were BghiP (SA2 and SA4), IP (SA5 and SA6) and BjF (SA3).

Table 2. Quantification of PAHs at Site 1 (Mt) and at Site 2 (SA) obtained during the sampling months . Average concentration is expressed as ng/m^3.

	Sample	Month	BaA	BbF	BkF	BaP	dBahA	BghiP	BjF	IP	TOTAL-PAHs
Site 1 [a]	**Mt2**	Ago.04	0.42	2.35	0.98	1.81	0.50	3.79	1.32	4.95	16.12
	Mt3	Ago.04	0.63	2.98	1.34	2.57	0.97	5.08	1.50	2.48	17.54
	Mt4	Oct/Nov.04	0.09	0.50	0.23	0.32	0.12	0.74	0.06	1.08	3.13
	Mt5	Apr.05	0.03	0.30	0.13	0.19	0.18	0.91	0.03	1.12	2.90
Site 2 [b]	**SA2**	Oct.04	0.03	0.11	0.05	0.06	0.04	0.17	0.02	0.04	0.51
	SA3	Nov.04	0.01	0.03	0.01	0.02	0.02	0.06	0.08	0.05	0.27
	SA4	Jan.05	0.02	0.11	0.05	0.07	0.08	0.23	0.02	0.06	0.65
	SA5	Mar/Apr.05	0	0.02	0.01	0.01	0.02	0.05	0.01	0.20	0.31
	SA6	May.05	0.01	0.03	0.02	0.02	0.03	0.16	0	0.26	0.53

See text for PAHs abreviation;
(a) Sample Mt1, collected in July, 2004, does not present data on air filters, only on human biomonitoring
(b) Sample SA1, collected in September, 2004, did not present sufficient samples to quantify the PAHs. .

The PAHs quantified at Site 1 presented a negative correlation with the precipitation (BaA rp= -0.983; BbF rp= -0.962; BkF rp= -0.976 and BghiP rp= -0.978 a p≤ 0.05 and dBahA rp= -0.997 to p≤ 0.01). Among the PAHs at Site 2, IP presented a positive correlation with humidity (rp=0.900, a p≤ 0.05) and BaA, BkF and BaP presented a negative correlation with precipitation (rp= -0.894, rp= -0.916 and rp= -0.899, respectively, with p≤ 0,05).

As to the analysis of mutagenicity shown in Figure 2, Site 1 presented for TA98-S9 the variation from 3.2±1.22(Mt3) to 18.5±4.16rev/m^3(Mt2) and for TA98+S9, from 3.2±0.48(Mt4) to 32.6±2.05rev/m^3(Mt3). Samples Mt2 and Mt3 presented the highest mutagenic responses (in TA98-S9 for Mt2 and in TA98+S9 for Mt2 and Mt3) and also the highest values of TSP, EOM and total PAHs.

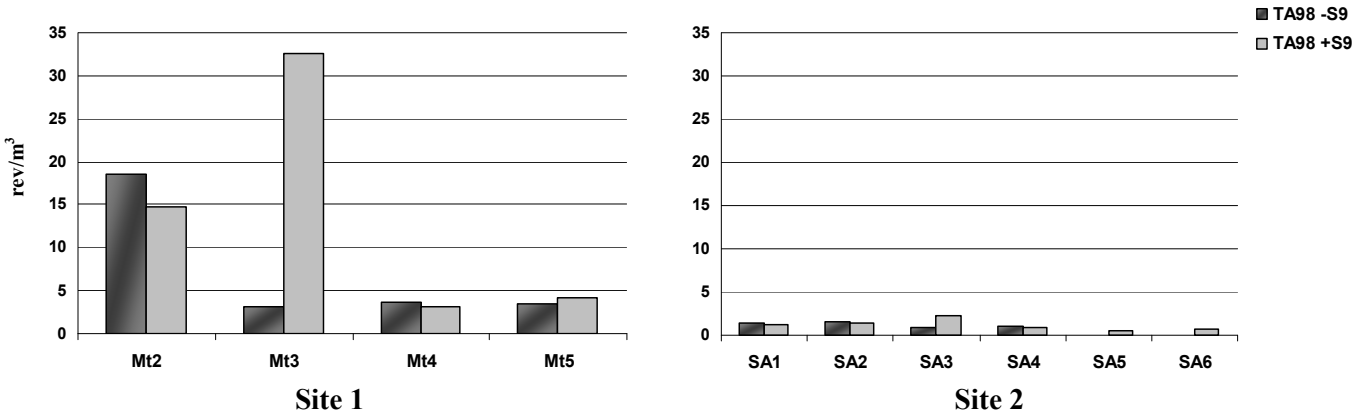

Figure 2. Mutagenic activity shown at Site 1 (Mt) and at Site 2 (SA) with strain TA98, in the absence (-S9) and presence (+S9) of a fraction of hepatic metabolization. Averages are expressed as rev/m^3. Sample Mt1 does not show air filter data, only data on human biomonitoring.
Negative control (DMSO): 63.2±14.93 (TA98-S9), (2AF) 41.5±13.95 (TA98+S9); Positive controls: (4NQO) 132.8±50.85 (TA98-S9), (2AF) 562.5±159.67 (TA98+S9) – values in revertants/plate. The values are mean ± standard deviation.

At Site 2, the responses were much lower, ranging from 0.8±0.18(SA3) to 1.5±0.32rev/m^3(SA2) in TA98-S9 and in 0.6±0.15(SA5) to 2.3±0.36rev/m^3(SA3) in TA98+S9. Negative responses to mutagenicity were seen in SA5 and SA6 for TA98-S9.

The strains identifying nitrocompound indicated in all samples from Site 1 a higher prevalence of nitro and amino derivatives of PAHs, identified by strain YG1024, 56.0±3.68rev/µg(Mt4) being the highest value (Table 3). At Site 2, on the other hand, this prevalence was observed only at SA2 and SA4. At SA1 however, there were more nitrocompounds (12.9±1.43rev/µg, YG1021) and at SA3 a mixture of nitrocompounds (2.2±0.35rev/µg, YG1021) with nitrocompounds and/or aromatic amines (2.3±0.49rev/µg, YG1024).

Table 3. Mutagenic activity of strains identifying nitrocompounds (YGs). Averages are expressed as rev/µg.

	Sample	TA98-S9	YG1021	YG1024
Site 1	Mt2	4.8±1.07	7.7±0.93	30.3±2.08
	Mt3	0.8±0.31	13.0±1.40	15.2±3.12
	Mt4	3.4±0.76	9.9±1.09	56.0±3.68
	Mt5	2.1±0.39	14.0±9.92	18.6±2.20
Site 2	SA1	3.3±0.73	12.9±1.43	8.2±0.52
	SA2	1.5±0.32	2.0±0.31	6.6±0.56
	SA3	1.7±0.39	2.2±0.35	2.3±0.49
	SA4	1.4±0.30	3.3±1.06	13.2±2.17
	SA5	-	a	a
	SA6	-	a	a

$p \leq 0,05$; (-) did not present mutagenic activity; Sample Mt1 does not present air filter data, only data from human biomonitoring.
Negative control (DMSO): 63.2±14.93 (TA98-S9), 94±47.16 (YG1021), 53.4±42.94 (YG1024); positive controls: (4NQO) 132.8±50.85 (TA98-S9), (2NF) 2067.8±418.19 (YG1021) and 4427.0±953.36 (YG1024)-values in revertants per plate.
The values are mean ± standard deviation.
(a) The specific strains are only used when TA98-S9 presents a positive response for mutagenicity.

Since some samples (especially those from Site 2) had a small amount of extracted organic matter (EOM), it was impossible to perform all tests with strain TA100, and also the analysis of PAHs at SA1. Only Mt2, Mt3, Mt4 and SA2, SA3 were tested with and without the metabolization fraction with this strain. The responses at Site 1 varied for TA100- S9, from 1.2±0.57rev/m^3 (Mt4) to 12.1±5.71rev/m^3 (Mt2), Mt3 being non-mutagenic, and from 2.8±1.09rev/m^3 (Mt4) to 8.2±1.61rev/m^3 (Mt3) for TA100+S9, presenting Mt2 4.6±1.51rev/m^3. At Site 2 the two samples tested presented negative responses for mutagenicity – data not shown.

In the two cities, the 8 PAHs were correlated with the mutagenic responses and at Site 1, positive correlations were seen for TA98+S9 with dBahA (r_p=0.998 $p \leq 0.01$), BaP (r_p=0.955 $p \leq 0.05$) and BghiP (r_p=0.950 $p \leq 0.05$); at Site 2, a

positive correlation was seen only between TA98+S9 and BjF (rp=0.959 p ≤ 0.01) (Figure 3).

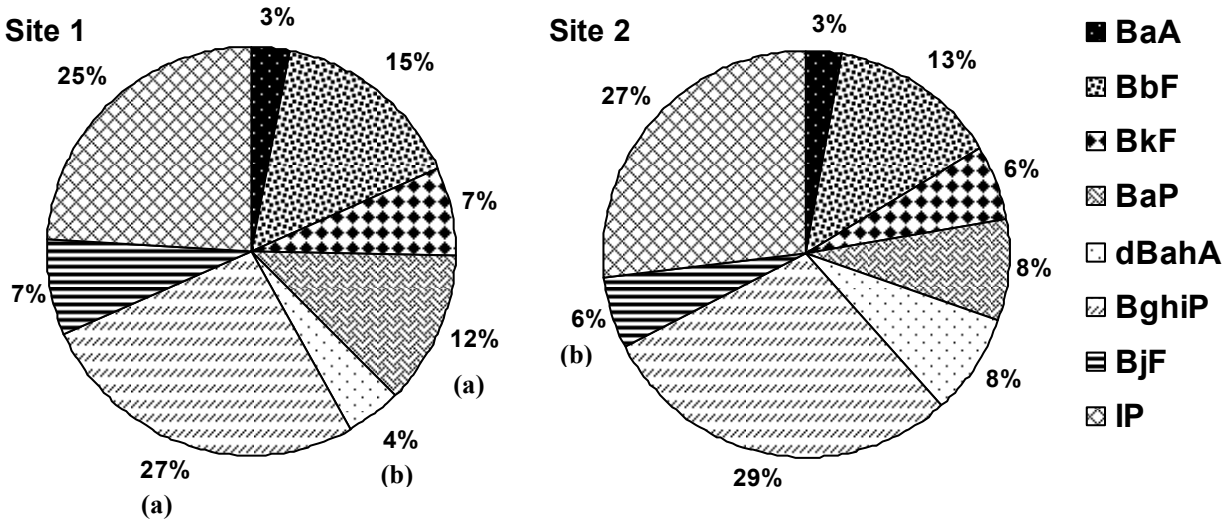

Figure 3. Total percentage of PAHs (%) found at Site 1 and at Site 2.
Pearson correlations PAHS and TA98 with significance for: (a) $p<0.05$; (b) $p<0.01$.

In the cytotoxicity assays, no sample presented positive results.

3.2 Human Biomonitoring

Evaluation of the individuals was performed concurrently with the second week of air sample collection.

The main characteristics of the studied groups are shown in Table 4. None of these groups showed a statistically significant difference between the life style and personal factors, both for the compared data of each city, and between cities.

Table 4. Main characteristic of the studied populations.

Subjects	**Site 1** (Exposed group)	**Site 2** (Reference group)
Number	24	35
Age - Mean ± SD (years)	25.3±6.03	26.2±6.92
Range (min-max)	(18-35)	(18-41)
Time they have lived at the site - Mean ± SD (years)	21.4±8.73	20.5±10.22
Range of months (min-max)	(12-420)	(2-444)
Smoking (%)		
Non smokers	95.8	97.2
Current smokers	4.2	2.8
Passive smokers [a]	58.3	63.9
Exposed to X-ray (%) [b]	37.5	36.1
Alcohol drinking status (%)		
Non drinkers	29.2	8.0
Non habitual drinkers (less than 3 times a week)	25.0	75.0
Habitual drinkers (more than 4 times a week)	45.8	16.7

(a) individuals who belong to the non-smoker and current smoker groups, who live with other smokers; (b) X-ray for diagnosis (dental or medical): number of people exposed less than 2 months from the collection of biological samples.

The data of the parameters analyzed with comet assay and MN assay are shown in Table 5. Through analysis of variance using hierarchical models it was observed that two sites did not show significant differences in the level of primary damage to the DNA of lymphocytes and micronuclei in cells exfoliated from the buccal mucosa.

Table 5. DNA damage (tail intensity - TI and tail moment - TM) in peripheral lymphocytes and micronucleated cells in buccal mucosa of individuals living at Site 1 (accompanying the air sampling from Mt1 to Mt5) and Site 2 (reference, SA1 a SA6).

	Sample	Subject no.	Tail Intensity		Tail Moment		Micronucleus (No. MN in 2000 cells)
			Mean	Median	Mean	Median	
Site 1	Mt1	1	4.54	2.63	0.32	0.19	0
		2	5.59	4.47	0.41	0.33	0
		3	5.96	6.73	0.44	0.50	0
	Mt2	4	4.08	3.70	0.32	0.26	0
		5	4.34	2.25	0.30	0.15	0
		6	6.62	4.20	0.64	0.33	0
	Mt3	7	3.45	1.95	0.32	0.12	0
		8	5.01	3.80	0.41	0.30	0
		9	3.25	1.39	0.26	0.08	0
		10	4.21	3.43	0.30	0.22	0
		11	4.55	3.25	0.33	0.23	0
	Mt4	12	11.90	8.53	1.36	0.71	0
		13	7.37	7.08	0.71	0.56	0
		14	7.47	5.91	0.63	0.43	0
		15	7.44	7.27	0.66	0.60	0
		16	6.73	6.32	0.54	0.45	0
		17	8.51	6.73	0.87	0.58	0
	Mt5	18	14.40	10.13	2.70	0.77	2
		19	11.01	3.74	2.24	0.45	3
		20	8.19	5.50	1.14	0.73	0
		21	8.11	1.39	2.04	0.13	0
		22	10.05	5.77	1.91	0.54	0
		23	3.92	0.37	0.59	0.05	0
		24	4.26	1.33	0.70	0.14	0
	Mean ± S.D.		6.7±2.90	4.5±2.52	0.8±0.70	0.4±0.22	0.10
Site 2	SA1	30	8.04	7.44	0.72	0.68	0
		31	10.49	9.91	0.90	0.84	0
		32	15.43	13.45	1.63	1.47	0
		33	4.85	4.48	0.34	0.26	0
	SA2	34	5.53	3.73	0.59	0.37	0
		35	4.58	3.40	0.44	0.29	0
		36	3.40	2.44	0.29	0.19	0
		37	2.70	2.41	0.22	0.15	0
		38	3.64	2.48	0.35	0.21	0
		39	2.43	1.45	0.20	0.10	0
	SA3	40	9.12	7.20	1.26	0.84	0
		41	6.21	4.28	0.69	0.30	0
		42	8.17	6.92	0.78	0.58	0
		43	7.60	4.96	0.77	0.32	0
		44	12.23	9.82	1.53	0.86	0
		45	4.17	3.39	0.33	0.25	0
		46	3.12	1.76	0.31	0.12	0
	SA4	47	9.61	9.32	1.25	0.85	0
		48	6.36	3.97	0.71	0.30	0
		49	7.52	5.19	0.84	0.40	0
		50	4.10	0.90	0.52	0.09	0
		51	7.61	4.32	0.96	0.36	0
	SA5	52	4.96	2.83	0.44	0.20	0
		53	6.70	3.22	0.86	0.25	1
		54	5.47	3.57	0.45	0.25	1
		55	8.07	5.02	1.03	0.47	0
		56	8.87	5.98	0.96	0.42	0
		57	8.62	4.76	1.10	0.44	0
		58	3.34	1.22	0.29	0.08	0
		59	6.13	3.45	0.60	0.27	0
		60	5.37	3.18	0.56	0.25	0
	SA6	61	6.75	3.15	0.79	0.22	1
		62	5.60	1.56	0.56	0.10	0
		63	5.31	1.21	0.69	0.13	1
		64	3.85	1.37	0.36	0.06	0
	Mean ± S.D.		6.5±2.81	4.4±2.88	0.7±0.36	0.4±0.30	0.06

Mean ± S.D.: positive control H_2O_2: 19.5±11.72 (TI), 4.1±3.04 (TM).

Table 6 presented the effect of age on DNA damage as detected by the comet assay. Data show no significant interference of age in either of the two groups.

Table 6. Mean values (mean±S.D.) obtained in the comet assay, evaluating the effect of the age of groups at Site 1 and Site 2

	Subject	Comet Assay	
		Tail Intensity	Tail Moment
Site 1	Total (n=24)	6.7±2.90	0.8±0.70
(exposed)	Age ≥ 28 (n=9)	6.7±2.24	0.7±0.52
	Age < 28 (n=15)	6.7±3.32	0.9±0.80
Site 2	Total (n=35)	6.5±2.81	0.7±0.36
(reference)	Age ≥ 28 (n=12)	6.1±2.44	0.7±0.37
	Age < 28 (n=23)	6.6±3.03	0.7±0.36

The effect of time of residence in each city is shown in Table 7. This analysis also shows that there are no differences between the groups and residence time.

Table 7. Mean values (mean±S.D.) obtained in the comet assay, evaluating the time of residence of groups at Site 1 and Site 2.

	Subject	Comet Assay	
		Tail Intensity	Tail Moment
Site 1	Total (n=24)	6.7±2.90	0.8±0.70
(exposed)	From 2 to 240 months (n=13)	6.7±3.55	0.8±0.79
	From 241 to 480 months (n=11)	6.7±2.06	0.8±0.61
Site 2	Total (n=35)	6.5±2.81	0.7±0.36
(reference)	From 2 to 240 months (n=18)	6.8±3.00	0.7±0.35
	From 241 to 480 months (n=17)	6.1±2.64	0.7±0.38

Table 8 shows a summary of the environmental and human mutagenesis data, which were correlated with each other using the Spearman Coefficient. At Site 1 a positive correlation was seen between the tail intensity and tail moment parameters with MN (r_s=0.4551 and r_s=0.478, respectively a $p<0.05$). At Site 2, a positive correlation was found between nitrocompounds (YG1021) and the tail moment parameter (r_s=1.000 $p<0.05$).

Table 8. Summary responses of mutagenicity presented by the Comet, MN and Ames assays at Site 1 (accompanying the air sampling from Mt1 to Mt5) and Site 2 (reference, SA1 to SA6).

	Sample	Human biomonitoring			Airborne monitoring			
		Mean ± SD		Micronucleus[a]	TA98 +S9		Nitrocompounds (rev/µg)	
		Mean TI[a,b,c]	Mean TM[a,b,c]		(rev/µg)[b]	(rev/m³)	YG1021[c]	YG1024
Site 1[a,b]	**Mt1**	5.4±0.73	0.4±0.06	0			NT	
	Mt2	5.0±1.40	0.4±0.19	0	3.8±0.52	14.7±2.00	7.7±0.93	30.3±2.08
	Mt3	4.1±0.05	0.3±1.04	0	8.2±0.51	32.6±2.05	13.0±1.40	15.2±3.12
	Mt4	8.2±0.74	0.8±0.90	0	3.0±0.44	3.2±0.48	9.9±1.09	56.0±3.68
	Mt5	8.6±3.71	1.6±0.81	0.36	2.5±0.32	4.1±0.53	14.0±9.92	18.6±2.20
Site 2[c]	**SA1**	9.7±4.46	0.9±0.54	0	2.9±0.53	1.2±0.22	12.9±1.43	8.2±0.52
	SA2	3.7±1.17	0.3±0.14	0	1.3±0.22	1.3±0.21	2.0±0.31	6.6±0.56
	SA3	7.2±3.08	0.8±0.45	0	5.1±0.80	2.3±0.36	2.2±0.35	2.3±0.49
	SA4	7.0±2.02	0.9±0.27	0	1.3±0.23	0.9±0.16	3.3±1.06	13.2±2.17
	SA5	6.4±1.85	0.7±0.29	0.11	0.8±0.21	0.6±0.15	-	-
	SA6	5.4±1.19	0.6±0.19	0.25	1.1±0.43	0.7±0.28	-	-

TI: Tail Intensity, TM: Tail Moment; Micronucleus: number of MN/1000; NT: no tested sample; (-) did not show mutagenic activity;
Spearman correlation (a) $p< 0.05$, (b) negative at $p< 0.01$, (c) $p< 0.01$.

As to analysis within each group studied, only Site 1 presented significant differences between the samples, and both for tail intensity and for tail moment the samples Mt3, Mt4 and Mt5 are different from each other and from the others.

Genotypings of *CYP1A1, GSTM1* and *GSTT1* were obtained for volunteers from each site. However, it was not possible to extract DNA from all individuals because the extraction kit, the first method used, malfunctioned. With the remaining blood samples, DNA extraction by a salting-out method was possible for gene *CYP1A1* (7 volunteers from Site 1 and 22 from Site 2) and for the *GST*s (with samples from 11 and 29 volunteers for each respective site).

For gene *CYP1A1*, at Site 1 and Site 2, respectively, the frequencies of 85.71 (n=6) and 45.45% (n=10) for T/T homozygous were observed; 14.29% (n=1) and 54.55% (n=12) for T/C heterozygous, and none for the C/C homozygous.

The GSTM1 genotype frequencies were 63.64 (n=7) and 44.83% (n=13) for non-null, 36.36 (n=4) and 55.17% (n=16) for null, Site 1 and Site 2 respectively. GSTT1 at Site 1 presented 100% (n=11) frequencies of non-null and Site 2, 72.41% (n=21) for non-null and 27.59% (n=8) for null.

The association between the exposed group (Site 1) and the control group (Site 2) indicated absence of positive or negative associations for *CYP1A1* (OR=0.14 – CI95%=0.01-1.35) and *GSTM1* (OR=0.44 – IC95%=0.11-1.82). For *GSTT1* the association was negative (null=0). The values obtained agree with reference values for the state of Rio Grande do Sul, and is sometimes below these (GTT1 null for Site 1 and CYP1A1 for Site 1 and Site 2) (Table 9).

Table 9: Distribution of the *CYP1A1, GSTM1* and *GSTT1* genotypes in the Site 1 and the Site 2.

Genotypes	**Site 1 (%)**	**Site 2 (%)**	**OR**	**CI 95%**
CYP1A1	N=7	N=22	0.14	0.01-1.35
T/T	6 (85.71)	10 (45.45)		
T/C	1 (14.29)	12 (54.55)		
C/C	ND	ND		
GSTM1	N=11	N=29	0.44	0.11-1.82
Non-null	7 (63.64)	13 (44.83)		
Null	4 (36.36)	16 (55.17)		
GSTT1	N=11	N=29	a	a
Non-null	11 (100)	21 (72.41)		
Null	0 (0)	8 (27.59)		

Values of reference for Rio Grande do Sul State

	Euro-Brasilians [b] (N=90)	Afro-Brasilians [c] (N=100)
CYP1A1 C/C	5.6	8.0
GSTM1 null	50.0	34.0
GSTT1 null	21.1	28.0

OR: odds ratios; CI 95%: confidence interval 95%; N: number of subjects; GST: non-null (wild-type homozigous or heterozygous), null (homozygous gene deletion); CYP1A1: T/T e T/C (wild-type homozygous or heterozygous), C/C (homozygous gene deletion); (a) without OR (null = 0); ND: not detected.
Comparing them with frequencies of the rare homozygous (*CYP1A1* C/C) and null genotypes *GSTM1* and *GSTT1*(%) of the Euro-Brazilian and Afro-Brazilian populations of the state of Rio Grande do Sul, (b) Gaspar et al. (2004); (c) Kvitko et al. (2006).

4. Discussion

Site 1, which is under the influence of areas with a history of great mutagenic activity in different environmental compartments (Coronas et al., 2008; Horn et al., 2004; Lemos et al, 2008; Pereira et al, 2007; Vargas, 2003; Vargas et

al., 1988; 2008), was investigated in this study regarding their possible atmospheric contamination. Due to its critical location, the State Foundation for Environmental Protection (FEPAM) has an air monitoring station at the site, and uses it every 6 days.

With this, the objective of this study was to evaluate the mutagenic effect of organic compounds of grouped air filters for two consecutive weeks. The second week is done at the same time as human biomonitoring. This study aimed at evaluating the exposure of people passively exposed during two weeks to the atmospheric air of this site.

Site 2 was confirmed as in previous studies (Pereira et al, 2007; 2008), as a site that can be used as a reference, with low mutagenic activity values, sometimes negative, and a low concentration of PAHs.

Brazilian law (CONAMA, 1990) and the international health agency (WHO, 2000) foresee limits for the quantity of TSP in the atmospheric compartment. However, this criterion and these values should be reviewed, since the organic matter contained in this particulate, the main mutagenic component of the sample, does not vary according to the amount of particulate found.

The PAH levels are higher during the winter season than during the summer. This is because of higher emission from combustion sources, more frequent periods with less-efficient atmospheric missing, and increased residence time in the air due to decreased degradation of PAHs (Bostrom et al., 2002). At Site 1, the amount of EOM, PAHs and the mutagenic response are remarkably larger during the colder season. However, the climate variation during the sampling period was smaller, compared to climatic variation of climate studies in countries of the Northern Hemisphere, indicating the influence of other factors, not only climate, on these samples.

The influence of rainfall on particle concentration was observed, in the inverse relationship of greater precipitation with a smaller number of particles. The rain cleans the particles in the atmosphere, but this does not mean that there is no further risk of exposure to contaminants associated with the particles that remain in the environment (Amador-Muñoz et al., 2001).

PAHs associated with the airborne particles change significantly with their emission sources. The concentration ratios of several PAHs have often been used in order to deduce the origins and behavior of environmental PAHs (Dallarosa et al., 2008; Fernandes et al., 2002; Kakimoto et al., 2002; Li and Kamens, 1993; Yunker et al, 2002;). The BghiP/IP ratio distinguishes emission from different engines types. Rehwagen et al. (2005) described a ratio over the range of 3.5-3.8 for petrol engines and a ratio of 1.1-1.2 for diesel engines. Li and Kamens (1993) and De Martinis et al. (2002) still described a ratio of 0.8 for wood combustion. Compared with this, the results presented here indicate that Site 1 presented lower ratios in almost all samples, indicating the influence of wood combustion. Only for Mt3 the ratio indicated a mixture of both engine types. Site 2 has several types of response depending on the sampling: influence of petrol engines (SA2 and SA4), diesel engines (SA3), and wood combustion (SA5 and SA6). However, the utilization of these ratios must be considered very carefully, since according to Mastral et al. (2003), different sources of categories have been found to provide similar or overlapping fingerprints. Also, no matter how much these ratios can be applied to pools of samples, according to Yunker M. (personal communication), the pool gives an average ratio and the information from individual samples may be lost

In general, the samples from Site 1 presented larger quantities of IP, BghiP and BbF. These PAHs are typical of vehicular emissions and have already been

observed in other samplings at the site (Pereira et al., 2008). Dallarosa et al. (2008) also found BghiP as the most abundant of the 14 PAHs identified in other regions located in urban areas with heavy traffic, in Rio Grande do Sul State.

However, in one of the samplings, Mt3 which presented a greater amount of PAHs and mutagenic activity, besides the fact that BghiP is the most found PAH, BbF and BaP were also outstanding. BaP is a PAH of pyrolytic origin which can be detected in coals samples and can be found in coal-burning stoves (Santos et al., 2004). Twenty-two km from Site 1 is the Southern Petrochemical Complex, whose preferential wind plume is the direction of this site. This complex burns coal for energy generation, besides being responsible for thirty percent of petrochemical production in Brazil. In 1997, 1,9 million tons of raw petrochemical material was produced including hydrocarbons and volatile organic compounds (VOCs). In 1999 the plant was expanded and its production has increased to 3.0 million of tons per year (Oliveira et al., 2002). These larger quantities of specific PAHs may have been found due to emissions from the complex, added to low precipitation at the sampling time, since in Brazil PAHs emissions were estimated at 467-6,607 t/yr (UNEP, 2002).

Differently from other samplings, Mt3 presented BaP in its composition. This hydrocarbon has been used historically as an indicator of organic PAHs. In the last IARC (2005) list it was classified as belonging to Group 1- carcinogenic to man. Cytochrome P450 is the set of enzymes responsible for the BaP metabolism. This enzyme performs enzymatic oxidation giving rise to benzo(a)pyrene-7,8-diol-oxide, followed by hydrolysis with formation of benzo(a)pyrene-7,8-diol. These diols once again suffer the action of cytochrome P450 and become benzo(a)pyrene-7,8-diol-9,10-epoxide. This final metabolite of BaP and the formation of other diol-epoxides by similar processes are the compounds considered carcinogenic and mutagenic,

due to their high power of linking by co-valence to the 2-amine group in guanine in the double strand of DNA (Bostrom et al., 2002; Meire et al., 2007).

Brazil does not have limit-values for the quantity of BaP, but the European Directive (2004/107/CE) has proposed a maximum permissible risk level of 1ng/m^3 for these PAH in ambient air. In two samples from Site 1, Mt2 and Mt3, values much above this limit were observed. Even if the values of this study are obtained from grouped filters and not only for a single filter, the amount of these PAHs and others found is worrisome, and should be better investigated in the region, including low precipitation periods, since there is a strong correlation of PAHs with this variable.

The mutagenicity of the air samples (rev/m^3) presented with the metabolization fraction varied at Site 1 according to the variation of EOM, ie., greater in MT3, followed by Mt2, Mt5 and Mt4. The dependence of the mutagenicity of the air pollutants upon the concentration in the atmosphere of organic compounds on suspended particles was also observed by Zwozdziak et al (2001). The authors analyzed the mutagenicity of atmospheric particulates for one year, and they obtained higher responses in winter. In fact, in colder periods they found higher values of PAHs, and the presence of BaP only at these times. Sample Mt3 also presented a greater quantity of PAHs, and this mutagenicity was attributed to these compounds, since without the metabolization fraction this sample was the one that presented the lowest mutagenicity values.

However, not all samples presented a higher mutagenic response, with a metabolization fraction; samples Mt2, Mt4, SA1, SA2 and SA4 were more mutagenic without fraction S9. This emphasizes that there are not only compounds that need metabolization, such as PAHs, mutagens at these sites. According to Du Four et al. (2005) when the mutagenicity of 10 mutagenic PAHs

was calculated and compared to the observed mutagenicity, only about 3% of the indirect activity could be explained. Therefore, most of the mutagenic activity is due to other compounds, thus emphasizing the importance of monitoring efforts (Claxton and Woodall, 2007).

The samples presented nitrated-polyclic aromatic hydrocarbons (nitro-PAHs) with the predominance of nitroarenes and/or aromatic amines, identified by the mutagenic activity by YG1024 strain in all samples of Site 1. The acetyltransferase present in YG1024 activated many compounds (nitroarene-type and/or aromatic amine-type) present at low concentrations that were not activated by the normal levels of nitroreductase present in TA98. The Mt4 sample presented a great quantity of these compounds, and much less PAHs. Site 2 presented a mixture of these compounds, and the presence of nitrocompounds (YG1024) found in half the samples. Nitro-PAHs may be present in ambient atmospheric particles from direct sources, such as diesel and gasoline exhaust (De Marini et al., 2004). The increased attention to nitro-PAHs, particularly in the environmental analytical community, is due to their persistence in the environment and higher mutagenic ($2x10^5$ times) and carcinogenic (10 times) properties of certain compounds compared to PAHs (Bamford et al., 2003).

The two assays used to evaluate the effect of human exposure to the atmospheric genotoxic agents indicate that, despite heavy levels of PAHs at Site 1, and confirmed mutagenic activity in the air samples, no statistically significant effect was seen in this population.

This study sought to evaluate homogeneous populations eliminating the confounding factors as much as possible. Several studies have reported that MN frequencies increase with age in adults, and women have higher MN frequencies than men (Mateuca et al., 2006). The same is observed through the comet assay

(Moller, 2006). With this concern, the study evaluated the genetic damage only in men from a certain age group, who were not occupationally exposed, and there were no significant differences between the volunteers and the confounding factors (Table 6).

DNA damage can be seen as a biomarker of exposure to genotoxic agents and as an index of a biologically effective dose. In risk assessment, the comet assay can help in hazard identification (Dusinka and Collins, 2008).

Faust et al. (2004) discuss a few studies that examined the genetic effects resulting from environmental air pollution and report that there are studies in which there are no differences in the comet values for subjects who live in highly polluted districts and control districts. In this study, individuals at Site 1 did not present significant differences from the control individuals. (Site 2). However, this assay complemented by other rmutagenic assays, such as the micronucleus test, is an important tool to identify genotoxic effects of environmental samples.

The MN assay is a technique that has been adopted by numerous laboratories, since MN induction is considered an effective biomarker of diseases and processes associated with induction of DNA damage. In this study, no presence of micronuclei was observed in the buccal muccosa of most individuals, and the MN frequencies found were similar to the reference values reported for a healthy population – 1 to 3 per 1000 cells for exfoliated cells (Fenech et al., 1999).

Several studies show that DNA damage as well as repair appear to be modulated by interactions between environmental and genetic factors. The increase of environmentally induced diseases, including several types of cancer, is associated with various exposures and host factors, including differences in carcinogen metabolism. Just as the metabolization process of BaP, many carcinogenic compounds require metabolic activation before they can react with

cellular macromolecules. According to Hietanen et al. (1997), the concerted action of these enzymes may be crucial in determining the final biological effect(s) of a xenobiotic chemical.

However, responses to environmental factors often depend on specific genetic polymorphisms. In the Review by Dusinka and Collins (2008), several occupational biomonitoring studies have shown an association between DNA damage and polymorphisms. Specific gene changes (homozygous recessive for CYP1A1, for instance or “null” genotype – gene deletion) could allow possible environmental exposures to result in elevated DNA damage.

In this study, the CYP1A1, GSTT1 and GSTM1 genotype frequencies found for both sites were as expected for the Rio Grande do Sul State population as shown by Heuser et al. (2007); Kvikto et al. (2006). It is expected that in urban environments with a high level of air pollution, subjects have been associated with a significant increase in cytogenetic endpoint incidence. However, in some European studies, usually no associating have been found (Carere et al., 2002; Leopardi et al., 2003). Novotna et al. (2007) attribute this fact to the individual response to environmental genotoxicants that may be modified by numerous factors, like age, gender, life-style, smoking and diet. However, the genotype significantly influences the susceptibility or resistance of a subject to adverse health impacts.

Heuser et al. (2007) studied footwear manufacture workers in Rio Grande do Sul State and besides observing frequencies of polymorphisms that are very similar to those found in this study, they also did not find any differences in the MN frequency in lymphocytes between control and footwear-workers.

The main focus of this study was to identify possible damage to the genetic material of people exposed environmentally to mutagenic substances due to

different industrial sources or even from urban traffic. Although Site 1 has presented high mutagenic responses in the air samples, high quantities of PAHs (in some samples above the legal level), and the presence of potential mutagens in the supply water observed in a previous study (Pereira et al., 2007), healthy people exposed to this environment did not present representative data in the genetic material. The values observed in the study were low compared to the study of people exposed occupationally. Constant doses of compounds can make the population undergo an adaptive process, and it is impossible to find significant genotoxic or mutagenic damages in the populations exposed. However, the evaluation of environmental compartments and genetic damage in populations exposed is necessary to monitor possible damages.

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6. Artigo 4

Human biomonitoring in an urban environment under influence of industrial contaminants in Southern Brazil

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Abstract

BACKGROUND: Besides the problems of a growing urban-industrial city, Rio Grande (Southern Brazil) has an industrial complex including fertilizers, foodstuffs and petrochemicals, which are located next to inhabited areas that suffer the direct impact of emissions from these industries, and thus receives different types and levels of contamination

OBJECTIVES: To investigate environmental samples (public water supply and atmospheric particulate matter) from the city of Rio Grande to evaluate the impact of urban and industrial pollution, affecting human genetic material, and to compare these results to data from a city already defined as a reference.

METHODS: The organic extracts of water treated for public supply and the organic extracts of airborme particulate matter (EOM) were tested for mutagenicity with the *Salmonella*/microsome assay (TA98 and TA100 strains for water, and TA98, YG1021 and YG1024 strains for airborne samples). The air samples were also analyzed by HPLC for the PAHs composition and by HRGC-HRMS for the PCDD/Fs composition. Samples of lymphocytes and buccal mucosa cells from individuals living in these two cities were evaluated using the comet and the micronuclei (MN) assays, respectively, as biomarkers of effect. Analysis of gene polymorphisms (*CYP1A1, GSTM1* and *GSTT1*) were used as susceptibility biomarkers.

RESULTS: Rio Grande presented significant results as to the mutagenicity of the water supply. All results were positive, mainly for the base-pair mutation (TA100), indicating the presence of THMs and MX compounds. The TSP shows one level above the regulation limits of the Brazilian Legislation and also of the World Health Organization The PAHs found in larger amounts were indeno(1,2,3-c,d)pyrene (3.09ng/m^3) and benzo(ghi)perylene (1.60ng/m^3) and the sum of PCDD/Fs was

107.0 I-TEQ fg/m^3. As to airborne sample mutagenicity, all the samples showed positive responses, ranging from 0.55±0.14 to 24.7±2.08 rev/m^3 (TA98-S9). Concerning the nitro-PAHs, the EOM samples presented a greater quantity of nitroarenes and/or aromatic amine-type (YG1024 strain), and the maximum amount found was 38.2±2.91rev/µg. Results of the comet in peripheral lymphocytes showed higher level of primary DNA damage (tail moment) in individuals from Rio Grande than in the controls. However, no mutagenic effect (MN) was detected in buccal mucosa cells. Most *CYP1A1* and *GSTT1* individuals presented prevalent genotypes.

CONCLUSIONS: Pollution has an impact on an urban-industrialized city, affecting healthy people exposed to different types of compounds, whether it be through the water supply or the airborne particulate matter

Keywords: Human biomonitoring, water supply, airborne particles, PAH, PCDD/F, *Salmonella*/microsome, comet assay, micronucleus, polymorphisms.

1. Introduction

Urban growth and increased industrial activity generally depend on the increased use of fossil fuels and their derivatives. Rio Grande, a city in Southern Brazil, is outstanding among the growing cities, because it has a major complex of industries, such as fertilizers, foodstuffs and petrochemicals, next to inhabited areas that suffer the direct impact of emissions from these industries.

The emission of a large diversity of substances through the gaseous, liquid or solid effluents introduces a range of compounds into the atmospheric and aquatic environment. The exposure to genotoxic substances in the environment is frequently viewed as a major risk factor to human health, and also induces increased DNA damage (Binkova et al. 1996).

The water used for public supply in places that suffer this impact, for instance, may be a major source of xenobiotic intake, because despite being treated, the water intake site is very important, since the treatment process may generate new compounds that are aggressive to genetic material (IARC, 1991, 2004; Meier 1988; Pereira et al. 2007).

Vehicular traffic is major cause of increased contaminants in the environment. In Brazil, vehicular emissions and oil refinement are the main sources of urban PAHs (polycyclic aromatic hidrocarbons) (Fernandes et al. 2002). These compounds are formed by the incomplete combustion of organic matter.

PAHs, carbon monoxide and suspended particles are among the most important atmospheric contaminants, and the latter are especially important due to the toxic effects they have on human beings (Amador-Muñoz et al. 2001; Bostrom et al. 2002), since depending on the particle size, it may be deposited in a certain part of the respiratory tract. However, more important than the quantity of

breathable suspended particles, is the amount of extractable organic matter associated with them, that have higher levels of mutagenicity.

The city of Rio Grande also has significant shipping activity for the region within MERCOSUL, with an ocean terminal and a port facility (the second major port of Brazil). The city also has an oil industry, working with refinement, storage, transport and distribution of fuels. Thus the region is vulnerable to accidents and chronic oil pollution (Medeiros et al. 2005).

Besides PAHs, high levels of compounds such as dioxins and furans formed by incomplete combustion of organic matter containing chlorine, are emitted from petroleum refining processes and vehicle emissions, for example, and are ubiquitous pollutants of great concern due to their mutagenic and carcinogenic effects.

Among the effects of complex mixtures of substances, whether it be in the air and/or water, the genetic toxicity of these compounds is being studied as well as their impact on the ecosystem and on human health. Compounds of natural or anthropic origin may interact with DNA affecting its structure and function, damaging cell repair processes, generating changes in chromosomal structures or other cell modifications which may even lead to apoptosis. Effects of this kind characterize cell modifications by toxicity to the genetic material (Pinto and Felzenszwalb 2003).

There are many studies investigating the effect of pollution on the fauna and flora in Rio Grande, and the quantification of pollutants, but no study which evaluates the genotoxic potential of environmental samples and their possible damage to the DNA of the human population. Several types of methodologies in the field of genetic toxicology allow the early identification of genotoxic agents in an attempt to define the preventive and corrective control strategies, trying to

minimize or avoid possible consequences to the ecosystem and to human health (Vargas 2003). A few examples of methodologies may be seen in this study, such as the assays: (1) *Salmonella*/microsome assay: a basic methodology for studies of the genotoxic potential of environmental samples, which may diagnose the presence of genotoxins in water sources (Vargas et al. 1993; Cardozo et al. 2005; Vargas et al. 2008), sediment (Tagliari et al. 2004), soil (Silva-Junior and Vargas 2007), water for public supply (Pereira et al. 2007) and atmospheric compartment (Coronas et al. 2008; Vargas 2003); (2) Micronucleus assay: most widely *in vivo* assay used to detect agents which cause chromosomal breaks and abnormal chromosomal segregation, and is most used in occupational exposure (Bonassi et al. 2003; Karahalil et al. 1999; Majer et al. 2001; Mateuca et al. 2006), but little so far in environmental exposure; (3) Comet assay: an assay that can be used with any eukaryotic cell to detect genomic lesions which may or not be repaired; also used in human biomonitoring studies (Faust et al. 2004; Fortoul et al. 2004; Moller 2006; Sharma et al. 2007); (4) metabolization genes: they detoxify xenobiotics, and research which looks at the prevalence of polymorphisms in these genes and exposure to environmental factors may favor the interpretation of the exposure and carcinogenesis mechanisms (Kelada et al. 2003; Norppa 2003; Vineis 2004; Anderson 2006).

In the present study, environmental samples (public water supply and atmospheric particulates) in the city of Rio Grande were investigated to evaluate the impact of urban and industrial pollution, affecting the human genetic material.

2. Material and Methods

Sampling of water supply, atmospheric air and human biomonitoring occurred during the same period, from July to September 2006 (winter).

The data on climate variation were provided by the 8th Meteorology District of the National Institute of Meteorology (8°DISME – Porto Alegre/INMET, Brazil).

2.1 Sampling site

Rio Grande, the oldest city in the state of Rio Grande do Sul, has around 196 thousand inhabitants (IBGE, 2008), an area of 2,814 km² and is located a little more than 300km from Porto Alegre (capital of state) in the extreme South of state (32°02,98' S and 52°07,54' W).

The region has a subtropical or warm temperate climate, with strong oceanic influence and rainfalls regularly distributed throughout the year – mean pluviometric precipitation around 1200mm. The warmest month is January with a mean temperature of 22°C, and the coldest month is July with a mean of 13°C.

The city of Rio Grande, located on a peninsula in the Patos Lagoon estuary (the largest lagoon in South America with 10,360km^2), near its mouth into the Atlantic Ocean, has a industrial-port complex where there are mainly fertilizer plants, treatment of wood, food production, fish, soy beans, petrochemicals, besides extraction and refining of vegetal oil.

Since the purpose of this study was to evaluate possible damage to the genetic material as a result of exposure to the urban environment under industrial influence, the sampling areas is located in the main atmospheric dispersion quadrant from the most contaminated zone of the city, i.e., the industrial district and the port area (Figure 1).

Reference city - Santo Antônio da Patrulha, another city of Rio Grande do Sul (29°46,29'S and 50°35,15'W), was used as reference and the results confirming it as a reference were shown in Pereira et al. (2007, 2008a, 2008b).

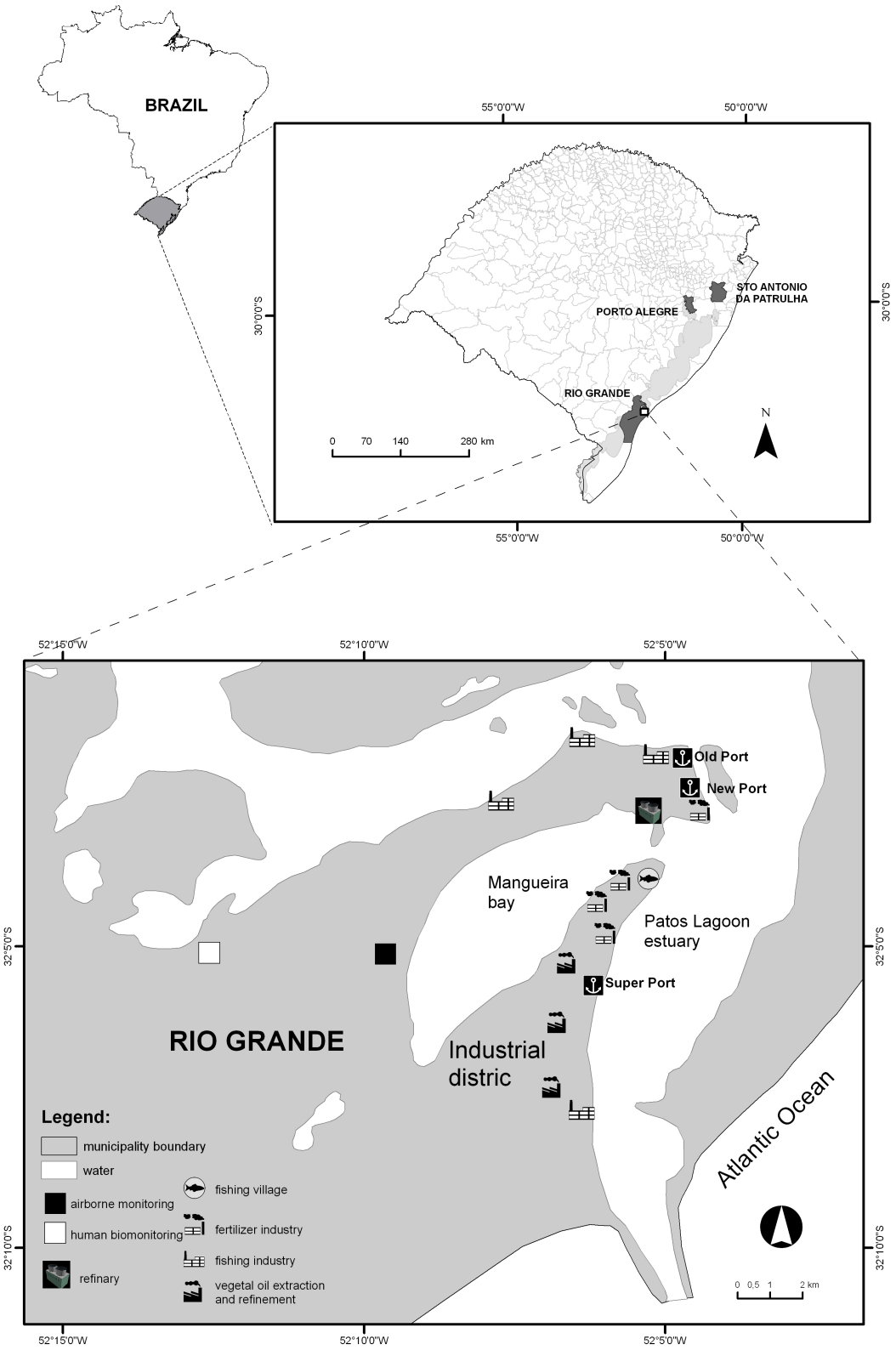

Figure 1. Location of the city of Rio Grande, the reference city (Santo Antônio da Patrulha) and the state capital (Porto Alegre). Below, Rio Grande and the monitoring points.

2.2 Environmental sample

2.2.1 Water supply sampling

Water samples from Rio Grande were collected before conventional treatment for water supply by the Rio Grande do Sul State Foundation for the Environment (FEPAM) sampling team (September 15, 2006).

The intake source of this treatment plant is divided into three points: São Gonçalo canal (located in a nearby city, Pelotas), Mirim Lagoon (the second largest lagoon in Brazil, it lies between Brazil and Uruguay), and a small amount from a stream near the treatment plant in Rio Grande.

Forty liters of water were collected and stored at 4°C for up to ten days, the time needed to perform the different stages of the organic compound extraction process. The water was treated according to the quality standards established by Brazilian law (Brasil 2004).

Organic compound extraction from the water sample was performed sequentially by adsorption in Amberlite XAD_4 type resins, under natural pH conditions (neutral-basic) and acidified by adding HCl up to pH 2.0, as described in Pereira et al. (2007)

At the time of the mutagenic evaluation assay, the necessary volume of extract was transferred to a test tube, with gaseous nitrogen reduced to dryness, and the extract was resuspended in spectrophotomeric grade dimethylsulfoxide (DMSO) solvent (Riedel-de Haën) at the volume appropriate to the assay (EPA, 1985 – modified; Cardozo et al., 2005).

2.2.2 Airborne particulate matter sampling

Airborne particulate matter (TSP) samples were collected on fiberglass filters (AP 40-810, 20cm x 25cm Millipore) using a high-volume sampler (General

Metal Works Inc.) operated at a flow rate of 1.3 to 1.5 m^3/min for 24h. The samples were collected from July to September 2006. The air samplings were divided into two types (1) pools of air filters from the months of July and September, performed to characterize the environment (1E and 2E samples); (2) pools of two air filters corresponding to a week of human monitoring and 6 days before it (1H, 2H and 3H samples). The filters were weighed and stabilized before and after sampling (45% humidity) for total suspended particles (TSP) expressed in units of μg/m^3 of sampled air (ABNT 1988).

Extraction of organic compounds: half of each filter was used for the extraction of the organic compounds, pooled monthly or two by two, depending on the type of sampling (E or H sampling). Each pool (sample) was submitted to extraction by sonication with dichloromethane (DCM, CASRN. 75-09-2) as described in Vargas et al. (1998).

The procedure of assay were described in previou paper (Pereira et al., 2008a).

2.2.2.1 Analysis of Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs were analyzed by high-performance liquid chromatography (HPLC) in an Alliance 2690 Waters chromatograph equipped with a fluorescence detector. An ultra-rapid 5cm column was used, with a specific longitude for this family of compounds (SUPERCOSIL LC-PAHS, 5cm x 4.6mm, 3μm), which allows separating the PAHs of the European Directive (2004/107/CE) with an appropriate resolution, and in 12 minutes. This directive proposes to control 7 compounds of the PAHs family: benzo(a)anthracene (BaA), benzo(b)fluoranthene (BbF), benzo(j)fluoranthene (BjF), benzo(k)fluoranthene (BkF), benzo(a)pyrene (BaP), dibenzo(ah)anthracene (dBahA) and indeno(1,2,3-c,d)pyrene (IP), specifying an

objective value only for BaP (1ng/m^3). The scanning fluorescent detector was programmed to detect these 7 specific PAHS of environmental interest (IARC, 2005) and also benzo(ghi)perylene (BghiP).

The chromatographic conditions, reagents and data analysis were the same used in Pereira et al. (2008a).

2.2.2.2 Analysis of dioxins and furans

In two of the samples it was possible to quantify polychlorinated dibenzo-p-dioxins (PCDD) and polychlorinated dibenzo-p-furans (PCDF). For this analysis, the other half of the filters were used to extract the organic compounds using a method different from the one described above. Filters of two samplings that follow the human biomonitoring were gathered in pools and submitted to the soxhlet extraction technique with toluene for 48 h.

The organic extracts obtained were submitted to a purification process and analyzed by HRGC-HRMS chromatography. The determination was performed in a DB-5 (60m, 0.25mm, 0.25µm) capillary column and in a DB-DIOXIN (60m x 0.25mm i.d., 0.25µm) capillary column, both from J&W Scientific (Folsom, CA, USA). The latter was used to separate those 2,3,7,8-congeners that were not resolved on the DB-5 column. The samples were injected (1µL) in splitless mode (1min) with an A200S autosampler (Thermo Instruments, Spain) into an injector at 280°C. Carrier gas helium with 240kPa inlet pressure. The temperature programme was 150°C (held for 1min), increased at 30ºC/min to 200ºC, increased at 3ºC/min to 235ºC (held for 10min) and increased at 6ºC/min to 300ºC (held for 20 min). Detection was performed by high resolution mass spectrometry (Micromass, Manchester UK) operating in electron ionisation mode and at 10,000 resolution power. Ionisation energy was set at 35eV. Two ions of each chlorination

homologue were recorded. Quantification were performed by the isotopic dilution method.

The data obtained were not correlated with the other results, since they were intended to trace a profile of the PCDD/Fs found in two samples from Rio Grande within the sampling period.

2.2.3 Salmonella/microsome assay

The organic extracts obtained from water supply and from airborne sampling were assayed for mutagenicity and cytotoxicity through the *Salmonella*/microsome assay (Maron and Ames 1983), in the microsuspension method (Kado et al. 1986).

For each sample *Salmonella typhimurium* TA98 strain (frameshift mutations) was used, with and without metabolic activation (S9 mix fraction). For the airborne sampling also the derivative strains YG1021 (nitroreductase -overproducing) and YG1024 (*O*-acetyltransferase -overproduction) (Watanabe et al. 1989, 1990) were used. Besides TA98, the TA100 strain (base-pair substitution mutations) was used for the water supply sample with and without S9 mix fraction.

The procedure of assay and the data analysis were described in previous paper (Pereira et al., 2008a).

2.3 Human Biomonitoring

This study was aproved by the Brazilian National Ethics Committee on Research (CONEP, No 23078.200270/04-17, 23/06/2004).

Adult males, healthy volunteers selected between the ages of 18 and 40 were evaluated. They remained most of the day close to the place where the air

sampler was, they did not work with agricultural inputs, nor in the factories in the region, and were preferably non-smokers.

Initially the exclusion factors of the sample were researched (high exposure to radiation, dietary and alcohol excessive habits, and health status such as the use of prescription medicines). Each individual signed an Informed Consent Form and answered a standard questionnaire with general information on eating habits, smoking, chemical dependence, use of medications and other factors (Carrano and Natarajan 1988).

2.3.1 Micronucleus assay

Exfoliated buccal cells were collected from each individual by scraping the cheek mucosa with a moist wooden spatula. The cells were transferred to a tube containing saline solution, centrifuged (2000G) three times, fixed in 3:1 methanol/acetic and dropped onto pre-cleaned slides, air-dried and stained using the Feulgen/Fast Green method. The cells were examined under light miscroscope (1000x) to determine the micronucleus frequency. Two thousand cells (1000 cells from each slide and for each reader) were analysed. Micronuclei were scored according to the criteria described by Tolbert et al. (1992).

2.3.2 Comet assay

About 4mL of blood were collected from each individual by intravenous puncture. The blood was kept on ice and protected from light until the time of processing.

Part of the blood collected (2mL), was used to isolate lymphocytes. This was done under a Ficoll density gradient. Cell viability was performed with fluorescein diacetate and was always above 90%.

The alkaline version of the comet assay was performed according to Singh et al. (1988) and Tice et al. (2000), and the method and data analysis was described in Pereira et al. (2008b).

2.3.3 Metabolization genes

The other part of the collected blood (2mL) was utilized for DNA extraction by a kit extraction (Kit GFX Genomic Blood DNA Purification – Amersham Pharmacia, CASRN. 27960301) and a salting-out method (Miller at al. 1988).

Polymorphisms of metabolic genes (*CYP1A1, GSTM1* and *GSTT1*) were determined by PCR (polymerase chain reaction) and RFLP (restriction fragment lengh polymorphisms) method and statistical analysis as described previously (Pereira et al. 2008b).

3 Results

Water supply sampling

Table 1 shows the characteristics of the organic extracts obtained from the city of Rio Grande for extracted organic matter (EOM), with a natural pH, moderately polar to non-polar compounds and acidic pH, polar compounds and it also shows the mutagenic activities of the samples that were studied using the *Salmonella*/microsome assay, TA98 and TA100 strains in the presence/absence of S9mix.

Table 1.Results obtained for treated water samples from the city of Rio Grande, compared to the reference city. Average in revertants per liter (rev/L) of sampled water.

Sample (extract)	EOM (µg/L)	Mutagenic activity			
		TA98 (rev/L)		TA100 (rev/L)	
		- S9	+ S9	- S9	+ S9
Rio Grande					
N	980.0	100.2±23.66 *	47.1±13.17	268.1±32.04	158.2±25.71
A	1215.0	259.6±47.40 *	135.3±18.92	1088.4±50.19	913.4±83.14
Reference city [1]					
N	1147.5	-	-	-	-
A	1015.0	-	-	-	-

EOM, extracted organic matter; A, acidic pH extract; N, natural pH extract; (–), mutagenic activity not detected; (*), presence of citotoxic activity; (1) Pereira et al. (2007).

Responses presented for significant ANOVA ($p<0.05$) and positive dose–response effect ($p < 0.05$), according to the Salmonel program.

Negative control (DMSO): 45.6±11.46 (TA98-S9), 59.2±6.08 (TA98+S9), 226.9±44.41 (TA100-S9), 198.1±21.85 (TA100+S9); positive control: 4NQO 120.9±59.96 (TA98-S9), 2AF 475.0±50.28 (TA98+S9), AZS 878.9±309.15 (TA100-S9), 2AF 1043.1±464.72 (TA100+S9) – values in revertants per plate.

In Rio Grande higher EOM values were observed for extracts obtained in acidic pH (1215.0µg/L). The values obtained for natural pH extracts were higher in the reference city (1147.5µg/L) than in Rio Grande (980.0µg/L).

The Rio Grande water sample presented a mutagenic response for TA100 and TA98, with and without S9 in the acidic and natural pH extract. For the two strains the highest mutagenic responses occurred in the absence of the S9 fraction. At the reference city all responses were negative (Pereira et al., 2007). Analysis of cell survival (data not shown) showed cytotoxicity for the extracts in natural and acidic pH in the absence of S9 mix in TA98 for the city of Rio Grande.

Airborne sampling

The data of filter sampling, the amount of extractable organic material (EOM) and climate variation during the period are shown in Table 2.

Table 2. Characteristics of samples of the airborne particulate matter and climatic variables of Rio Grande city during the collection period (July to October 2006).

Sample	Day of filter collection (Month)	Number of filters	TSP per filter (µg/m³)	Total TSP (µg/m³)	EOM (µg/m³)	Variable *		
						Temperature (°C)	Humidity (%)	Precipitation (mm)
1E	4;10;16;21;28 (July)	5	11; 29; 40; 67; 119	266	1.05	15.7	85.7	16.6
2E	2; 8; 14 (September) 2 (October)	4	17; 40; 56; 69	182	0.71	13.6	77.7	31.7
1H	3; 9 (August)	2	29; 32	61	0.68	13.4	87.0	21.1
2H	15; 21 (August)	2	61; 69	130	2.85	13.5	84.0	87.4
3H	21; 26 (September)	2	53; 327	380	0.45	15.9	79.0	87.4
Reference city[1]	9; 14 (October 2004)	2	14; 49	63	0.98	17.7	71.0	2.8

TSP: total suspended particles; EOM: extractable organic material.

(E) Samples for environmental characterization, (H) samples that follow human biomonitoring. (1) data in Pereira et al. (2008b); (*)Mean value compensated obtained on the day the variable was collected; Source of variables: 8th Meteorology District of the National Institute of Meteorology (8°DISME – Porto Alegre/INMET), Brazil.

During the sampling period, the city of Rio Grande presented a small temperature variation (13.4 to 15.9°C). The mean air humidity varied from 77.7 to 87.0%. As to precipitation, July was the month with least (16.6mm) and the second fortnight of August and September the most rainfall (87.4mm).

The amounts of filters on the environmental pools (1E and 2E) evaluated were 5 and 4 filters, respectively, and for all filters that followed human biomonitoring the pools were formed by 2 filters (1H, 2H, 3H and Reference city). For the all samples, the total suspended particle (TSP) values found during the sampling periods varied from 11 to 327 – this last value is above the current Brazilian legislation (CONAMA, 1990) and World Health Organization (WHO,

2000) levels - 150 to 240μg/m^3 and 120μg/m^3, respectively). The amount of organic matter extracted ranged from 0.45 to 2.85μg/m^3 (Table 2).

Table 3 shows the quantification of the eight PAHs analyzed. The 2H sample presented the highest amount of total PAHs (9.85ng/m^3), and IP was the hydrocarbon found in the highest quantity (3.09 ng/m^3). IP was the most often found hydrocarbon in human biomonitoring and BghiP in environmental monitoring. BaP presented the amount of 1.08ng/m^3 in the 2H sample surpassing the limit determined by the European Directive.

Table 3. Quantification of PAHs in Rio Grande obtained from July to October 2006. Average concentrations are expressed as ng/m^3.

Sample	Month 2006	BaA	BbF	BkF	BaP	dBahA	BghiP	BjF	IP	Total PAHs
1E	July	0.05	0.20	0.08	0.10	0.05	0.28	0.01	0.12	0.89
2E	Sep/Oct	0.02	0.09	0.03	0.04	0.02	0.12	0.01	0.03	0.38
1H	August	0.05	0.25	0.10	0.10	0.07	0.35	0.02	0.49	1.44
2H	August	0.62	1.66	0.65	1.08	0.33	1.60	0.85	3.09	9.85
3H	September	0.02	0.06	0.02	0.03	0.03	0.12	0.03	0.19	0.50
Reference city[1]	October 2004	0.03	0.11	0.05	0.06	0.04	0.17	0.02	0.04	0.51

See text for PAH abbreviation.(E) Samples for environmental characterization, (H) samples that follow human biomonitoring; (1) data in Pereira et al. (2008b).

The correlation with the climatic data and the PAHs was only significant for the precipitation variable and BjF and IP (rs= 0.90, $p \leq 0.05$ for both).

Figure 2 presents the total contribution of each PAH in Rio Grande and the reference city. In the two cities BbF, BghiP and IP are the most found hydrocarbons. However, IPH is the PAH most found in Rio Grande (30%) and BghiP in the reference city (29%). In Rio Grande, the amount of BaA was double (6%) that found in the reference city (3%).

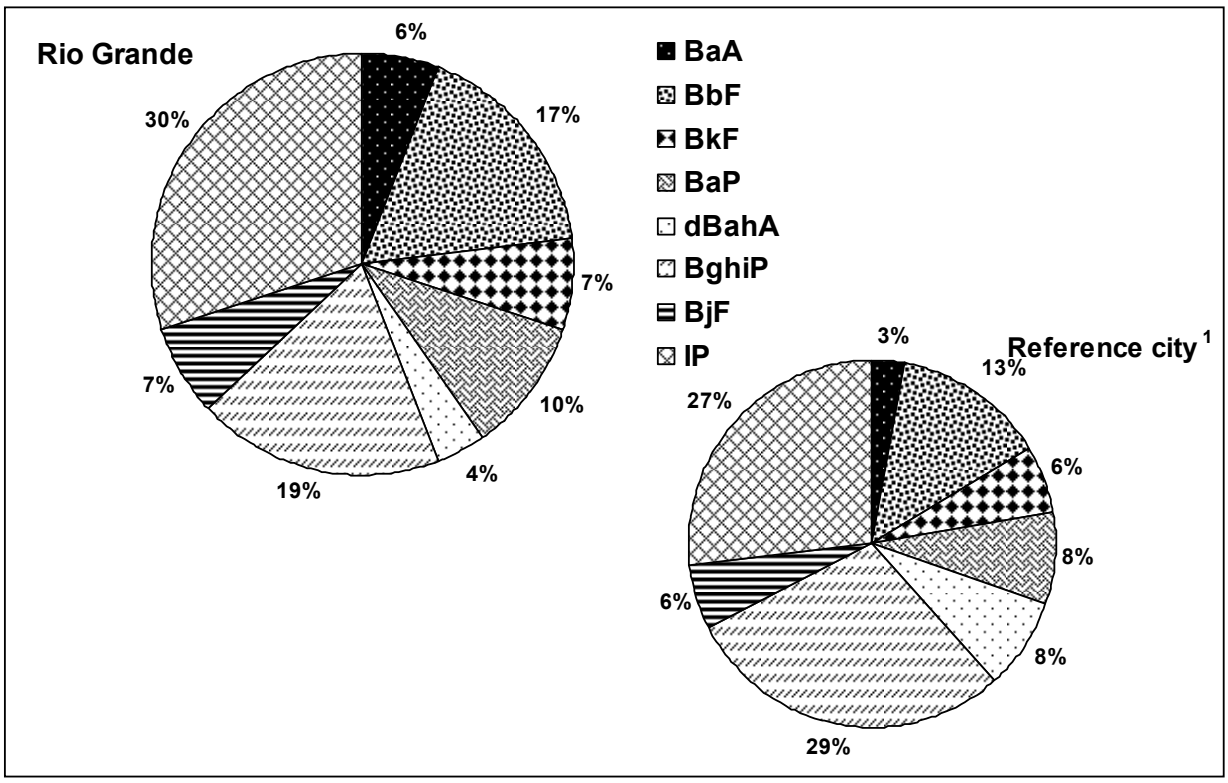

Figure 2. Composition of total PAHs (%) found in Rio Grande and the reference city. (1) Pereira et al. (2008b)

It was possible to perform the quantification of PCDD/D congeners (Table 4) in two samples only in Rio Grande. In a 1H sample the sum of these compounds presented a value ten times higher compared to the 3H sample. These data were not correlated with others; they are only used to trace a profile of the PCDD/F composition in two months, in Rio Grande.

Table 4. *PCDD/F* congeners sampled in Rio Grande in August (1H) and September (3H) 2006.

PCDD/F congeners	Concentration (I-TEQ fg/m^3) Sample	
	1H	3H
Dioxins		
2,3,7,8-TCDD	1.77	<LD
1,2,3,7,8-PeCDD	16.0	1.02
1,2,3,4,7,8-HxCDD	17.5	0.3
1,2,3,6,7,8-HxCDD	11.2	0.55
1,2,3,7,8,9-HxCDD	15.0	0.35
1,2,3,4,6,7,8-HpCDD	22.8	1.38
OCDD	3.96	1.79
Total dioxins	88.1	9.53
Furans		
2,3,7,8-TCDF	0.35	0.23
1,2,3,7,8-PeCDF	0.35	0.08
2,3,4,7,8-PeCDF	8.51	1.89
1,2,3,4,7,8-HxCDF	3.26	0.55
1,2,3,6,7,8-HxCDF	1.94	0.34
2,3,4,6,7,8-HxCDF	2.83	0.67
1,2,3,7,8,9-HxCDF	0.34	<LD
1,2,3,4,6,7,8-HpCDF	0.85	0.24
1,2,3,4,7,8,9-HpCDF	0.16	0.04
OCDF	0.08	0.09
Total furans	18.7	4.14
∑PCDD/Fs	107.0	10.7

<LD: below the detection limit.

In the city of Rio Grande the mutagenicity varied from 0.55±0.14rev/m^3 (3H) to 24.7±2.08rev/m^3 (2H) without the metabolization fraction (S9 fraction) and from 0.2±0.20 rev/m^3 (2E) to 13.4±2.37rev/m^3 (2H) with S9 fraction (Figure 3).

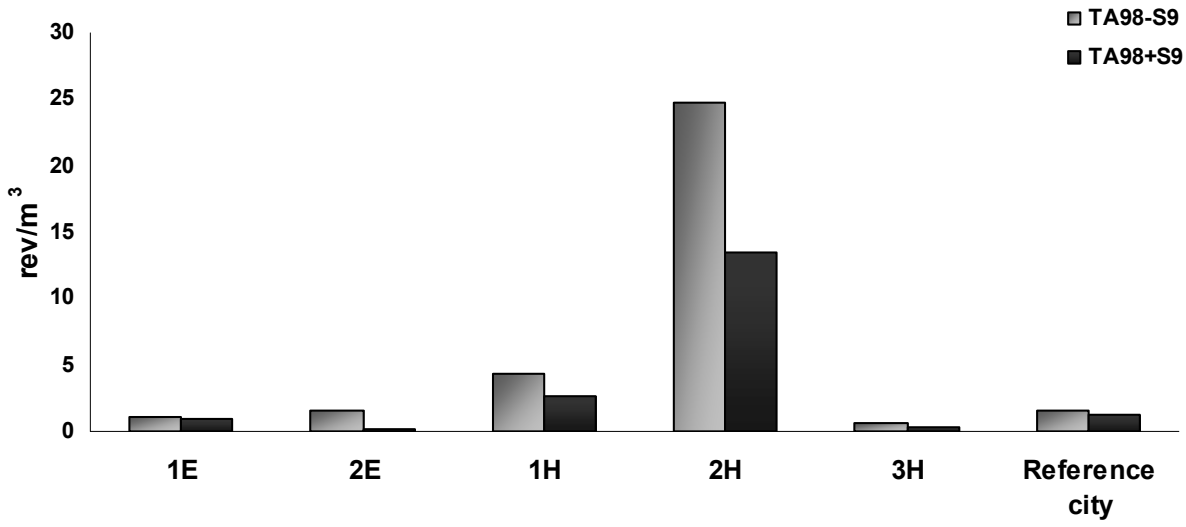

Figure 3. Mutagenic activity of airborne samples shown in Rio Grande and reference city with strain TA98, in absence (-S9) and presence (+S9) of a liver metabolization fraction. Averages are expressed as rev/m^3, $p \leq 0.05$.

Negative control (DMSO): 48.7±3.90 (TA98-S9), 54.0±17.80 (TA98+S9); positive control: 4NQO 450.2±41.35 (TA98-S9), 2AF 381.2±30.40 (TA98+S9) – values in revertants/plate.

As to the strains that identified nitro compounds the highest values were observed at 1H: 34.8±4.07rev/µg (YG1021) and 38.2±2.91rev/µg (YG1024), the composition of nitro-PAHs being a mixture of nitrocompounds (YG1021) with nitrocompounds and/or aromatic amines (YG1024) (Table 5), since the difference in mutagenicity between the two strains is very small.

Table 5. Mutagenicity (rev/µg) of airborne samples observed in Rio Grande and the reference city, expressed for the nitrocompounds strains. The values are mean ± SE.

Sample	TA98-S9	YG1021	YG1024
1E	1.0±0.36	11.7±1.08	16.6±1.57
2E	2.3±0.36	17.7±0.80	19.2±1.10
1H	6.3±0.64	34.8±4.07	38.2±2.91
2H	8.7±0.73	30.1±1.28	34.0±1.82
3H	1.2±0.32	8.3±2.48	9.1±0.57
Reference city	1.5±0.32	2.0±0.31	6.6±0.56

Negative control (DMSO): 48.7±3.90 (TA98-S9), 94.7±47.16 (YG1021), 39.5 (YG1024), positive control: 4NQO 450.2±41.35 (TA98-S9), 2NF 5839 (YG1021), 6360 (YG1024).

The correlation between the 8 PAHs and the mutagenic responses showed significant response for TA98+S9 (rev/m^3) with all PAHs (BaA rp=0.99; BbF e BbK rp=0.996; BaP rp=0.991; dBahA rp=0.998; BghiP rs=0.997; BjF rs=0.984; IP=0.997, p≤0.01).

As to cytotoxicity, the 2E sample presented cytotoxicity at the intermediary doses and 3H presented it at the highest dosage..

Human Biomonitoring

The main characteristics of the studied groups are shown in Table 6. None of these groups showed a statistically significant difference between the life style and personal factors, both for the data compared for each city and between cities.

Table 6. Main characteristics of studied groups

Subjects	Rio Grande	Reference city
Number	30	35
Age - Mean ± SD (years)	24.0±4.41	26.2±6.92
Range (min-max)	(18-40)	(18-41)
Time living at the site - Mean ± SD (years)	10.8±12.10	20.5±10.22
Range of months (min-max)	(5-480)	(2-444)
Smoking (%)		
Non smokers	93.3	97.2
Current smokers	6.7	2.8
Passive smokers [1]	63.3	63.9
Exposed to X-ray (%) [2]	20.0	36.1
Alcohol drinking status (%)		
Non drinkers	23.3	8.3
Non habitual drinkers (more than 3 times a week)	46.7	75.0
Habitual drinkers (more than 4 times a week)	30.0	16.7

(1) individuals belonging to the non smokers and current smokers groups who are in company with other smokers; (2) X-ray for diagnosis (dental or medical): number of people exposed less than 2 months from the collection of biological samples.

The data of the comet assay and MN assay parameters analyzed are shown in Table 7. By analysis of variance using hierarchical models, significant differences were found between Rio Grande and the reference city, in comet, as to the tail moment parameter ($p \leq 0.0119$).

Table 7. Mean of DNA damage (tail intensity - TI and tail moment - TM) in peripheral lymphocytes and number of micronucleated cells in buccal mucosa of individuals living in Rio Grande.

Sample	Subject no.	Tail Intensity	Tail Moment	Micronucleus (no. MN in 2000 cells)
1H	1	3.77	1.08	0
	2	1.99	0.29	0
	3	2.93	0.71	1
	4	0.90	0.13	4
	5	3.89	0.59	1
2H	6	6.23	0.96	0
	7	3.94	0.49	0
	8	4.00	0.66	0
	9	7.32	1.77	0
	10	5.20	1.01	0
	11	3.75	0.67	0
	12	2.23	0.26	1
	13	14.97	4.54	0
	14	8.02	1.97	0
3H	15	5.16	1.36	0
	16	15.34	4.42	0
	17	6.05	1.27	0
	18	5.87	1.12	1
	19	6.89	1.30	1
	20	3.50	0.55	1
	21	5.53	0.96	0
	22	2.99	0.43	2
	23	6.37	1.21	0
	24	4.01	0.58	0
	25	2.66	0.50	0
	26	8.82	1.84	1
	27	3.68	0.65	0
	28	7.37	1.27	0
	29	5.46	0.92	0
	30	4.69	1.01	0
Mean ± SD		**5.5±3.23**	**1.2±1.01***	**0.22**
Reference city [1] Mean ± SD		**6.5±2.81**	**0.7±0.36***	**0.06**

Mean ± S.D., positive control H_2O_2: 19.5±11.72 (TI), 4.1±3.04 (TM);

(1) Complete values in Pereira et al. (2008b); (*) $p \leq 0.0119$

Table 8 presented the effect of age on DNA damage as detected by the comet assay. Data show no significant interference of age in either of the two groups.

Table 8. Mean values (mean±S.D.) obtained in the comet assay, evaluating the effect of age on the subjects from Rio Grande and the reference city.

City	Subject	Comet Assay	
		Tail Intensity	Tail Moment
Rio Grande	Total (n=30)	5.5±3.23	1.2±1.01
	Age ≥ 28 (n=3)	6.5±1.12	1.4±0.38
	Age < 28 (n=27)	5.3±3.38	1.1±1.06
Reference	Total (n=35)	6.5±2.81	0.7±0.36
	Age ≥ 28 (n=12)	6.1±2.44	0.7±0.37
	Age < 28 (n=23)	6.6±3.03	0.7±0.36

Table 9 shows a summary of the air and human mutagenesis data. No correlation was found between the two monitorings.

Table 9. Summary of the mutagenicity responses presented by Comet, MN and Ames assay in Rio Grande compared with the reference city.

Sample	Human biomonitoring			Airborne monitoring			
	Mean ± SD		MN	TA98 +S9		Nitrocompounds (rev/µg)	
	Mean TI	Mean TM		(rev/µg)	(rev/m^3)	YG1021	YG1024
1H	2.7±1.26	0.6±0.37	0.6	4.0±0.51	2.7±0.35	34.8±4.07	38.2±2.91
2H	6.2±3.78	1.4±1.32	0.05	4.7±0.83	13.4±2.37	30.1±1.28	34.0±1.82
3H	5.9±3.02	1.2±0.94	0.19	0.6±0.11	0.3±0.05	8.3±2.48	9.1±0.57
Reference city [1]	6.5±2.81	0.7±0.36	0.06	0.8 to 5.1	0.6 to 2.3	2.0 to 12.9	2.3 to 13.2

Parameters of Comet assay: TI, Tail Intensity; TM, Tail Moment; MN (micronucleus): number of MN/1000;

(1) Values presented in Pereira et al. (2008b).

The genotypes of *CYP1A1, GSTM1* and *GSTT1* were obtained for volunteers from each site. However it was not possible to extract DNA from all individuals because the first methodology used malfunctioned (a commercial kit for DNA extraction). With the remaining blood samples, it was possible to extract DNA

by a salting-out method for gene CYP1A1 (14 volunteers from Rio Grande and 22 from the reference city) and for genes GSTM1 and GSTT1 (with samples from 28 and 29 volunteers for each site). For gene *CYP1A1*, in Rio Grande and in the reference city, respectively, the frequencies of 35.71% (n=5) and 45.45% (n=10) of homozygous T/T were observed; 64.29% (n=9) and 54.55% (n=12) of heterozygous T/C and none for the rare homozygous gene C/C.

The *GSTM1* genotype frequencies were 42.86% (n=12) and 44.83% (n=13) for non-null, 57.14% (n=16) and 55.17% (n=16) for null, Rio Grande and reference city respectively. GSTT1 presented frequencies of non-null in Rio Grande 71.43% (n=20) and the reference city, 72.41% (n=21) and for null 28.57% (n=8) and 27.59% (n=8), respectively. The frequencies observed in the two populations were very close to the reference ones for the state of Rio Grande do Sul.

The OR analysis with a 95% confidence interval of the exposed group (Rio Grande) and control group (reference city) indicated the absence of positive or negative association between the polymorphic allele variants of the 3 genes studied for environmental exposure: *CYP1A1* (OR=1.50 – CI95%=0.38-5.95), *GSTM1* (OR=1.02 – CI95%=0.36-2.88) and *GSTT1* (OR=0.93 – CI95%=0.30-2.90) (Table 10).

Table 10: Distribution of the *CYP1A1, GSTM1, GSTT1* genotypes in Rio Grande and the reference city.

Genotypes	Rio Grande (%)	Reference city (%)	OR	CI 95%
CYP1A1	N=14	N=22	1.50	0.38-5.95
T/T	5 (35.71)	10 (45.45)		
T/C	9 (64.29)	12 (54.55)		
C/C	ND	ND		
GSTM1	N=28	N=29	1.02	0.36-2.88
Non-null	12 (42.86)	13 (44.83)		
Null	16 (57.14)	16 (55.17)		
GSTT1	N=28	N=29	0.93	0.30-2.90
Non-null	20 (71.43)	21 (72.41)		
Null	8 (28.57)	8 (27.59)		
Values of reference for Rio Grande do Sul State				
	Euro-brasilians (N=90) [1]	Afro-brasilians (N=100) [2]		
CYP1A1 C/C	5.6	8.0		
GSTM1 null	50.0	34.0		
GSTT1 null	21.1	28.0		

OR: odds ratio; IC 95%: confidence interval 95%; N: number of subjects; GST: non-null (wild-type homozygous or heterozygous), null (homozygous gene deletion); CYP1A1: T/T e T/C (wild-type homozygous or heterozygous), C/C (homozygous gene deletion); ND: none detected. Comparisons them with frequencies of the rare homozygous (*CYP1A1* C/C) and null genotypes *GSTM1* and *GSTT1*(%) of the Euro-brazilian and African-brazilian populations of the state of Rio Grande do Sul. (1) Gaspar et al. (2004); (2) Kvitko et al. (2006).

4. Discussion

The inhabitants of Rio Grande, an urban-industrial city which receives several industrial contributions, were investigated for mutagenicity and genotoxicity of their genetic material, considering the presence of different genotoxic compounds in the environmental samples.

Water for public supply is one of the mediums in which environmental contaminants dispersed in a city and deposited in water resources may be

ingested by human beings. Rio Grande presented very significant results as to the mutagenicity of the water supply, with all the results positive, especially for base-pair mutation (TA100). The organic mass extracted from the sample of treated water from Rio Grande was as much as 4 times greater than that presented in the reference city which, on the contrary of Rio Grande, did not present any mutagenic response in the Ames assay (Pereira et al., 2007).

Besides the chemical compounds present at the point of intake in the river for the water treatment plant, products such as trihalomethanes (THMs may be formed during the treatment process). Chlorine use during water disinfection process may account for the presence of non-volatile mutagens in treated drinking water. Most of these by-products are direct mutagens extracted mainly at acidic pH and detected with TA98 and TA100 without the metabolization fraction (S9) (De Marini et al. 1995), as seen in this study.

The formation of THMs is of great concern because of their carcinogenic effects on humans, since they are classified as possible human carcinogens (Group 2B) (IARC 2004). Aquatic humic substances, comprising fulvic and humic acids, are the main precursors of THMs and these substances account for about half of the dissolved organic matter in natural water supplies (Imai et al. 2003).

However, the vast majority of the mutagenic activity of chlorinated drinking water identified is due to acidic semivolatile and nonvolatile chlorinated organics of the type that can be adsorbed to XAD resins (IARC 2004). Elevated responses in TA100 without S9, besides indicating the presence of THMs, show 3-Chloro-4 -(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX), a compound that has been shown to account for as much as 60% of the mutagenic activity of some samples of chlorinated drinking water and classified as possibly carcinogenic to humans (Group 2B) (De Marini et al. 1995; IARC 2004). MX is also a by-product during the

disinfection of water containing humic substances using chlorine, chlorine dioxide or chloramines. MX causes DNA damage (including unscheduled DNA synthesis) besides reacting directly with glutathione and the reactions catalysed by *GST*s (Brunborg et al. 1991; Chang et al. 1991).

Besides these compounds, chemical characterization allowed the identification of the presence of many substance possibly from domestic waste waters (such as oleic acid and methyl hexadecanoate) and also from industrial processes (biphenyl, naphthalene and anthracene), and the two sources are responsible for contaminating this water (dates not showed). Another study that showed aquatic contamination in the region, with great influence of domestic sewage was that of Mirlean et al (2003). They studied the concentration of mercury due to the accidental discharge of about 12,000 t of sulfuric acid into the harbor waters in 1998, and they observed that domestic groups of effluents are a more intense source of Hg pollution than industrial groups.

Several studies have described the contamination in this county from various sources and in different compartments. Working with water contamination, for instance, Mirlean et al. (2005) investigated the chemical composition of groundwater as a result of the impact of industrial emissions, and they observed that the quality of water in the industrial district of the town was not acceptable for human consumption, and the production of fertilizers was the main factor responsible for this contamination.

Another problem caused by fertilizer factories in the region is fluoride pollution, which raises the environmental pollution caused by fluorine, both in the industrial and in the urban area. Severe damage to vegetation and animals (bovine fluorosis) has been recorded. Mirlean et al. (2002), observed that in rainwater at places which were further away from the factories, this compound

reached values that were many times higher than the background content. Both the treatment plant where water was collected for this study and the air sampler and the people studied were in the city at a site was located in the main atmospheric dispersion quadrant from the most contaminated zone of the city and, as shown in this study, they did receive fluorine besides other contaminants.

Besides being mutagenic the water sample also was cytotoxic, both in the acid and in the basic extract. Data on mutagenic activity were also observed in the water supply in Montenegro, another urban city of the state of Rio Grande do Sul, also showing the presence of possible mutagens formed in water treatment. However their mutagenicity values were lower, and there was no citotoxicity. (Pereira et al. 2007). The genotoxicity responses obtained in the sample of the water that supplies the city of Rio Grande (resulting both from compounds present in raw water and formed using the water treatment process), added to their clinical characterization, indicate that although not all the water intake occurs in Rio Grande, it is improper for drinking water. These data corroborate the studies by Mirlean and Roisenberg (2005) that indicated that the water in Rio Grande is not fit for drinking.

Water resources contamination is mainly due to airborne pollution, which carries compounds to this compartment. And, as with water, also for the air it is difficult to regulate airborne pollutants, since air contains a large number of toxicants about which little or nothing is known (Claxton and Woodall 2007).

The determination of the total suspended particulate matter (TSP) in the air with a 24h sampling time is one of the quality evaluation measures that FEPAM (State Foundation for Environmental Protection) monitors in the state. The TSP showed levels below the regulation limits in Brazilian legislation and also of the World Health Organization (CONAMA 1990; WHO 2000) for most of the samples.

However, sample 3H showed a value above the limits allowed by law. The TSP and EOM concentrations did not show correspondence during the sampled months, and this sample 3H had the highest TSP value and the lowest EOM, even lower than in the reference city.

For this study, the evaluation of the air samples was performed during the coldest period of the year, since in this period there is the highest concentration of pollutants in the atmosphere due to the large number of emissions sources, besides constant atmospheric conditions (Prajapati and Tripathi 2008), lower photolytic and thermal decomposition during cold months (Mastral et al. 2003), heavier traffic and increased fuel consumption (Dallarosa et al. 2008). The city of Rio Grande, especially, is located near the cyclogenetic region on the Uruguayan coast, and is affected by the extratropical cyclones, with passing cold air masses in winter (Reboita et al. 2006). Precipitation was the only climatic variable that presented a positive correlation with two PAHs analyzed, IP and BJF. However, the sampling period was constant regarding climatic variables, with only two extremes between precipitation, which explains the correlation between the two PAHs and this variable.

IP was the hydrocarbon found in the largest amounts in the sum total of PAHs. This PAH is considered one of the hydrocarbons that are markers of automobile pollution (Dejean et al. 2008). Municipal statistics indicate that although the city is small one in every 3 inhabitants has a car. The quantity of IP found in the sample with the highest concentration of PAHs (3.09ng/m^3 at 2H) was greater than that found in other studies in Brazil, such as in São Paulo (0.49 to 0.8ng/m^3), in Niterói-Rio de Janeiro (0.76 to 0.86ng/m^3) or even studies in the state of Rio Grande do Sul , such as in Porto Alegre (0.815ng/m^3) and Candiota (0.296ng/m^3) (Dallarosa et al. 2005a, 2005b; De Martinis et al. 2002; Netto et al.

2002; Vasconcelos et al. 2003). However, Bourotte et al. (2005), in a study performed in São Paulo in winter obtained a value above that found in our study (4.71ng/m^3).

Furthermore, the second most found PAH, BghiP identifies traffic as the main source of urban PAH emission (Rehwagen et al. 2005). The ratios of concentration of several PAH compounds have often been used in order to deduce the origins and behavior of environmental PAHs. These two PAHs have been reported amongst the most stable compounds of the PAHs series (Fernandes et al. 2002) and were found in larger amounts. The use of the BghiP/IP ratio that is used to distinguish emission from different engine types, indicated that the samples were divided as to their origin: the environmental monitoring samples sometimes were from a mixture of diesel and gasoline (2.33, 1E), at other times they were due to gasoline (4.0, 2E); all the human biomonitoring samples were from combustion emissions (< 8) (Li and Kamens 1993).

Another item that may identify contamination in the city by traffic emissions is the BaA/BaP ratio, which has been used to identify combustion processes (De Martinis et al. 2002). In all the samples presented here the ratio (0.5) agreed with that attributed to gasoline exhaust. However, as already discussed in Pereira et al (2008b), these ratios are weakened when used in pools of samples, especially with the environmental monitoring samples which have more than 4 filters in their pools.

A small amount of BaA was present but the total of this hydrocarbon in Rio Grande was double that found in the reference city. PAHs of pyrolitic origin such as BaA have been detected in coal samples and are emitted by vehicles using gasoline and diesel (Barra et al. 2007; Santos et al. 2004).

BaP, another PAH of pyrolitic origin, is classified according to the International Agency for Research on Cancer as a carcinogen, belonging to Group 1 (IARC 2005).The European directive (2004/107/CE) has proposed a maximum permissible risk level of 1ng/m^3 BaP in ambient air, based on the carcinogenic potential of inhaled particulate PAHs (calculated for 24h in PM10 fraction). The 2H sample presented a quantity higher than the one proposed. Although BaP is considered the most carcinogenic PAH, this compound has a very short half-life of 5.3h under simulated sunlight conditions, because it undergoes photochemical degradation in the environment and the PAHs carcinogenicity could be underestimated under given conditions if only this compound is considered as representative of carcinogens (Mastral et al. 2003).

As to the analysis of PCDD/F only two samples that followed human biomonitoring were tested, and the 1H sample presented ten times more compounds that the 3H sample. “Dioxins and furans” refer to a set of more than 200 different organic compounds (PCDD with 75 congeners and PCDF with 135). However, only seven congeners of dioxins and ten of furans presented toxic effects, the most toxic congener being 2,3,7,8-tetrachlorodibenzo-p-dioxin. These pollutants are introduced into the environment unintentionally as byproducts of many processes that involve high temperatures and chlorine, such as the incineration of wastes, processes involving in oil refining, metal foundries, paper bleaching using chlorine, and others, and they are the most studied compounds and also the most toxic produced by man (Pujadas et al. 2001; Assunção et al. 2005).

Brazilian legislation does not yet include indexes for PCDD/F and the European one only limits it in certain foods. However, the value obtained in 1H (∑PCDD/F 107.0fg I-TEQ/m^3) is comparable to studies in industrial cities and cities

with high traffic emissions such as in winter in the cities of São Paulo (47.0 to 751fg I-TEQ/m^3), Tarragona (8.25 to 72.7fg I-TEQ/m^3) and Lisbon (100.0fg I-TEQ/m^3) (Assunção et al. 2005; Coutinho et al. 2007; Gasser et al. 2008). However, it should be highlighted that the sources of PCDD/F in the Northern Hemisphere are different from those of the Southern Hemisphere, and what predominates is pesticide production, municipal incineration of wastes and accidental fires in buildings, vehicles, etc. in the North, and burning domestic waste outdoors in the South.

The value of PAHs was not high as that of PCDD/F in the 1H sample indicating a prevalence of dioxins. This high value of PCDD/F may be associated with sudden strong atmospheric emissions from the oil refinery, which sometimes occur in Rio Grande.

As to airborne sample mutagenicity, all the samples showed positive responses, the highest being obtained without the metabolization fraction. The 2H sample presented the highest mutagenic response, both with and without the metabolization fraction. This sample also presented the highest amount of PAHs, but since these compounds need to be metabolized to express their mutagenic potential, a higher response would be expected from the tests with the metabolization fraction. Therefore, this higher response without the S9 fraction indicates that besides PAHs there are other compounds responsible for this high result. This city has a long history of atmospheric impact due to dust and particulate metals caused by port and industrial activities, especially attributed to the industries of fertilizers, oil refinery and food (Mirlean et al. 2005; Vanz et al. 2003;).

The 1H sample, the second largest mutagenic response of the samples, reinforces this profile of the presence of various contaminants since, although the

sample was not the highest, it presented a large amount of PCDD/Fs and the highest values of nitro-PAHs, especially nitroarenes and/or aromatic amine-type, identified by the YG1024 strain. Nitro-PAHs are the compounds responsible for the higher mutagenicity of the more polar compounds, and these compounds are associated with S9-independent mutagenicity (De Martinis et al. 2002), attributing to nitrocompounds the high mutagenic activity without metabolic activation presented in this study.

Nitrogen-containing compounds are usually associated with combustion sources, but are also produced when PAHs react with nitrogen and sunlight (Zwozdziak et al. 2001). Aromatic amines are associated with manufacturing processes, but two aromatic amines are documented as urban air contaminants (Claxton et al. 2004). Wada et al. (2001) reported a mutagenicity presented in the samples of polluted air in the city of Nagasaki with automobiles as a main source of atmospheric nitro-PAHs. The presence of nitrocompounds may also be due to vehicular traffic (De Marini et al. 2004) in the city of Rio Grande, since the presence of some PAHs corroborates this origin.

The 2E and 3H samples did not present such high mutagenicity responses, which may be explained by the fact that the two samples were cytotoxic. The highest S-9 dependent mutagenic responses were presented by the samples that follow human biomonitoring (1H e 2H). These samples also presented high levels of PAHs, nitro-PAHs and PCDD/Fs, which may have caused genotoxic damage to the people exposed to these compounds (Reyes et al. 2000).

As to human biomonitoring, it was not possible to analyze the correlation between the three groups sampled (1H, 2H and 3H) since there are few individuals in the samples, and it is more appropriate to analyze the population of Rio Grande as a whole.

In the comet assay, the tail intensity parameters did not present a statistical difference between the Rio Grande group and that of the reference city. However, as to the tail moment parameter, there was a difference between the two cities ($p \leq 0.0119$). This greater response obtained in Rio Grande could be due to the different genotoxic compounds (MX, THMs, PAHs, nitro-PAHs, PCDD/Fs etc.) found in this city.

The population of Rio Grande is exposed not only to the industrial, petrochemical and port complexes close to the city, but also to the problems caused by urbanization, such as vehicular emissions. Sharma et al. (2007) studied genotoxic effects on the Copenhagen population exposed to different types of airborne contamination, and attributed DNA damage to them, caused by vehicle emissions. Pereira et al. (2008b) studied an urban city that was under the influence of several kinds of anthropogenic sources and, even if the authors have identified a high mutagenic response in the samples of airborne particulate matter (Ames test) and a large amount of PAHs also attributed mainly to vehicle emissions, the people exposed to these compounds did not present genotoxic damage identified by the comet assay and MN assay. Coronas et al. (2008) evaluated the airborne samples and people residing in another urban city of the state of Rio Grande do Sul, that were under the influence of an oil refinery, and found significant results for the comet assay (10.04±7.13 for TI and 2.53±2.28 for TM) compared to the same reference city in this study. The authors also did not find significant responses for the number of MN.

The frequency of micronuclei (MN) was found to be small and can be compared to the reference values (Fenech et al. 1999). The MN assay was applied to cells of the oral mucosa, and the comet assay to the blood cells, which are more sensitive due to systemic exposure. It should also be emphasized that

the comet assay indicates the occurrence of genomic lesions, which can be corrected, and the MN assay indicates a mutagenic process.

The presence of important mutagenic activity responses in the environmental samples using the *Salmonella*/microsome assay identifies the fact that the population is exposed to active organic stressors, with a high human mutagenic and carcinogenic potential. However, there was a difference in the response to the effect of these contaminants measured by the comet and micronucleus assay in the individuals analyzed. In this analysis the differences in the concentration of stressors and the complexity of the test systems must be considered. In the environmental samples the organic matter concentrated by a simple system was tested. The system is simple but specialized in the safe determination of the presence of hazardous compounds which place the integrity of the genetic material at risk. On the other hand, the exposure of individuals is related to the dilution of this sample in an *in vivo* exposure for a complex human organism with specialized defenses. As to the differences in specific sensitivity of the two assays used, Abou Chakra et al. (2007) evaluated the organic extracts of urban airborne particulate matter and concluded that the same sample extracts showed more significant responses using the Ames test than the comet assay. The authors attributed this to the fact that the Ames test might provide more accurate information on the genotoxicity potency of the organic fraction of airborne particles.

However, the individual response to environmental genotoxicants may be modified by numerous confounding factors including the genotype, inherited properties responsible for the metabolism of xenobiotics and DNA damage repair, significantly influences the susceptibility or resistance of a subject to adverse health impacts (Georgiadis et al., 2004). The individual response to environmental

exposure may be significantly modified by genotype. *CPY1A1* plays an important role in the metabolism of PAHs converting procarcinogens, including PAHs and aromatic amines into final carcinogens (Bartsch et al. 2000, Hietanen et al. 1997). Neither of the populations analyzed in the present study presented the allele variant that is rare for this gene (C/C), and both of them also presented a large part of the *GSTT1* individuals with a prevalent genotype, which may be important indicators of the maintenance of genomic integrity. The low frequency of mutagenesis in the buccal mucosa cells may indicate that in these individuals there is an efficiency in the detoxification system, ensured by the presence of these genotypes which are responsible for the function of metabolizing and excreting carcinogens.

In conclusion, it was demonstrated that pollution has an impact on a city, affecting healthy people exposed to different types of compounds, whether it be through the water supply or the airborne particulate matter. This study is an important tool to understand the genotoxic effects caused by different types of compounds, in different environmental compartments which may be found in an urban-industrial city and affect the integrity of the genetic material of people exposed to this environment.

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7 Considerações finais

Estudos populacionais que identifiquem efeitos precoces de contaminantes ambientais na qualidade de vida e na saúde da população humana são necessários para a definição de medidas preventivas.

O estudo de metodologias sensíveis para a detecção da presença de substâncias tóxicas ao material genético nos diferentes compartimentos ambientais tem sido desenvolvido na área de pesquisa da FEPAM (Fundação Estadual de Proteção Ambiental), com os primeiros trabalhos publicados em 1988. Os pesquisas realizadas até hoje resultaram no diagnóstico de áreas de risco quanto à presença de substâncias de ação genotóxica, sendo que ações de controle já foram incorporadas na FEPAM, como Órgão de Proteção Ambiental do Estado do Rio Grande do Sul.

Tendo em vista as limitações dos estudos epidemiológicos em definir danos precoces, justifica-se a busca de metodologias mais sensíveis como marcadores de exposição a agentes potencialmente cancerígenos e que afetam o desenvolvimento no período fetal, além de outras manifestações clínicas crônicas.

A maioria dos estudos realiza o monitoramento do compartimento atmosférico utilizando quase que exclusivamente parâmetros físicos e químicos. Raros são os trabalhos que consideram esses parâmetros associados a bioensaios os quais identificam a presença de compostos químicos perigosos por seu efeito nos seres vivos ou ainda a exposição crônica em determinado ambiente e especialmente efeitos genotóxicos (Vargas, 2003; Cassoni et al., 2004).

Portanto, o presente estudo foi abrangente buscando compreender melhor quantitativamente e qualitativamente os contaminantes, sua presença no ambiente e seus efeitos sobre o material genético dos seres vivos.

Para isso, foram selecionadas como áreas de estudo duas cidades do Rio Grande do Sul, Montenegro e Rio Grande, regiões urbanas que recebem diferentes tipos de contaminações antropogênicas, comparando-as com uma cidade referência, Santo Antônio da Patrulha.

Extratos orgânicos de amostras ambientais, obtidos a partir de material particulado atmosférico e de água tratada destinada ao abastecimento público, dessas cidades foram avaliados quanto ao potencial mutagênico.

Através do emprego de linhagens específicas de *Salmonella typhimurium*, foi possível identificar as contribuições de nitro-HPAs nas amostras de ar, podendo traçar um perfil da influência desses compostos nas amostras.

Pôde ser realizado um paralelo das respostas mutagênicas encontradas tanto nas amostras de ar quanto nas de água de abastecimento com a caracterização química dessas amostras, identificando HPAs e dioxinas do ar, bem como compostos presentes na água de abastecimento.

Utilizando estes marcadores para o ambiente foi possível caracterizar que a população das duas cidades apresentam risco de exposição à agentes mutagênicos ambientais. Os estudos confirmaram o município de Santo Antônio da Patrulha como referência para exposição a agentes mutagênicos ambientais no estado do Rio Grande do Sul.

Ficou evidente que os padrões de qualidade de ar vigentes na legislação brasileira (CONAMA, 1990), devem ser revistos quanto aos seus níveis primários e secundários de exposição, já que muitas amostras que se enquadravam abaixo do limite legislado, apresentaram alto potencial de atividade mutagênica.

Os biomarcadores genéticos para estudo em biomonitoramento humano permitem identificar tanto uma exposição crônica a compostos genotóxicos ambientais como a um evento ocasional de poluição que possa ocorrer. Nesse estudo, apenas o ensaio do cometa quando realizado com a população de Rio Grande mostrou a ocorrência de danos no DNA em linfócitos. Essas respostas podem ser devidas a uma possível manutenção da integridade genômica dos indivíduos analisados. Apesar das várias injúrias que essas populações estão expostas, como os compostos mutagênicos encontrados nas amostras de ar e de água para abastecimento público, a investigação de polimorfismos genéticos para os genes de metabolização mostrou que a maioria dos indivíduos possuía genótipos prevalentes quanto aos genes identificados, indicando uma possível eficiência no sistema de detoxificação de xenobióticos.

O modelo de biomonitoramento ambiental proposto por este trabalho se confirmou como efetivo para a avaliação de susbtâncias genotóxicas presentes no ambiente, podendo ser utilizado em cidades com diferentes tipos contribuições antrópicas.

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APÊNDICES

TERMO DE CONSENTIMENTO INFORMADO

A pesquisa “Biomonitoramento de populações humanas através de avaliação de genotoxicidade em área sujeita a risco ecotoxicológico", tem como objetivo avaliar sinais iniciais para detectar efeitos da poluição ambiental na saúde humana. Estes sinais podem ser observados através de testes (que avaliam danos nas células) e entrevista individual (informações sobre alimentação, uso de medicamentos, profissão, moradia).

As análises serão realizadas no Laboratório de Biologia da Fundação Estadual de Proteção Ambiental, FEPAM, em trabalho conjunto com o Curso de Pós-graduação em Ecologia da Universidade Federal do Rio Grande do Sul, UFRGS. Todos os resultados ficarão sob a total responsabilidade dos pesquisadores deste laboratório. A identidade de cada voluntário será mantida em sigilo e você poderá a qualquer momento desistir da sua participação, sem que isto leve a qualquer prejuízo.

O desconforto que você passará será mínimo, estando basicamente relacionados à coleta de sangue (4mL), que implica em uma sensação dolorosa temporária na região da coleta, havendo possibilidade de formação de um pequeno hematoma na região. A coleta de sangue é feita com material limpo, esterilizado e descartável (usado para cada pessoa), sem risco de transmitir AIDS ou outra doença qualquer. Será realizada, também, coleta de células bucais de forma suave, sem dor. Não há risco na coleta destes exames.

O resultado destes exames não apresenta finalidade individual, mas servirá para avaliar a sensibilidade da população de seu município para os poluentes ambientais. O sangue coletado será utilizado para avaliar se há danos no seu material genético (DNA) e para extração do DNA, a fim de verificar sua sensibilidade à exposição a alguns agentes tóxicos e para estudos que esclareçam o papel genético no aumento de risco a danos no DNA.

Os pesquisadores envolvidos no Projeto garantem a você o direito a qualquer pergunta e/ou esclarecimentos mais específicos dos procedimentos realizados.

Esta pesquisa, na qual você será voluntário, poderá trazer grande benefício para a população humana, possibilitando selecionar testes que

identifiquem os efeitos iniciais de substâncias perigosas aos organismos antes que ocorram problemas graves de saúde.

Eu, _____

portador da CI_____, residente em _____- RS, fui informado dos objetivos específicos e da justificativa desta pesquisa, de forma clara e detalhada. Recebi informações sobre cada procedimento no qual estarei envolvido e do desconforto, tanto quanto dos benefícios esperados. Todas as minhas dúvidas foram respondidas com clareza e sei que poderei solicitar novos esclarecimentos a qualquer momento. Além disso, sei que as informações obtidas durante o estudo serão fornecidas e que terei liberdade de retirar meu consentimento de participação na pesquisa.

Os pesquisadores garantiram que as informações geradas terão caráter confidencial.

Caso tiver novas perguntas sobre este estudo, posso chamar os pesquisadores integrantes da equipe de pesquisa do Laboratório de Biologia da FEPAM pelo telefone (51) 3334-6765.

Declaro ainda que recebi cópia do presente Termo de Consentimento.

Data ___/___/_____

_____

_____

Assinatura do voluntário

Vera Maria Ferrão Vargas

Pesquisadora Responsável

QUESTIONÁRIO

Data ___/___/_____

I – Identificação

N° de registro:

_____

Nome:

_____

Identidade:

_____

Profissão:

_____

Data de nascimento: ___ / ___ / _____

Idade: _____

Endereço:

_____

CEP: _____-____Telefone para contato:

_____

Fatores de exclusão da amostra: residir e permanecer maior parte do dia no local de estudo; não trabalhar com insumos agrícolas; preferentemente não fumar.

N° de registro do voluntário no estudo: _____

II – História Social

- Escolaridade:

( ) não alfabetizado ( ) ensino médio incompleto ( ) ensino superior incompleto
( ) ensino fundamental incompleto ( ) ensino médio completo ( ) ensino superior completo
( ) ensino fundamental completo

- Tempo de residência na área do município:

_____

- Trabalha: ( ) Sim ( ) Não Trabalho atual:

_____

Local de trabalho: _____

Tempo de serviço: _____ Tipo:

_____

Data das últimas férias: _____

Ausentou-se do município nos últimos 12 meses por um periódico de 1 semana no mínimo?

( ) sim ( ) não

Trabalho anterior: _____ Tempo de serviço: _____ Tipo: _____

- Renda familiar média: Em Reais: R$_____

( ) menos de 1 salário mínimo ( ) de 1 a 3 salários ( ) de 3 a 5 salários
( ) de 5 a 10 salários ( ) mais de 10 salários ( ) não sabe ( ) não informou

- Tipo de casa: ( ) alvenaria ( ) madeira ( )pau-a-pique ( ) outros

Número de cômodos na casa: _____

- De onde vem a água que bebem (abastecimento de água da residência)?

( ) encanada (CORSAN) ( ) poço artesiano ( ) bombeada de arroio ( ) poço comum
( ) abastecido por carro-pipa ( ) cacimbas e/ou nascentes ( ) bica pública
( ) não sabe ( ) não informou ( ) outros

- Qual o destino do esgoto:

(**)** esgoto público encanado (cloacal) ( ) valo direto e/ou arroio ( ) fossa e/ou sumidouro
( ) não sabe ( ) não informou ( ) outros

III – Hábitos

- Fuma? ( ) Sim ( ) Não Há quanto tempo (meses) _____

Quantos cigarros por dia _____

Tipo: ( ) cachimbo ( ) charuto ( ) palha ( ) papel com filtro ( ) outros:

_____

- Já fumou? ( ) Sim ( ) Não Há quanto deixou de fumar (meses)? _____

Quantos cigarros por dia _____

Durante quanto tempo fumou (meses)?_____

Tipo: ( ) cachimbo ( ) charuto ( ) palha ( ) papel com filtro ( ) outros: _____

- Convive diariamente com fumante (s) – fumante passivo? ( ) Sim ( ) Não
- Bebe chimarrão? ( ) Sim ( ) Não Quantas cuias por dia? _____

Quantos dias da semana bebe? ( )1 a 2 dias ( )3 a 5 dias ( ) todos os dias

- Consome bebida alcoólica? ( ) Sim ( ) Não ( ) eventualmente Há quanto tempo (meses)? _____

Tipo: ( ) cachaça ( ) cerveja ( )whisky ( ) vodka ( ) vinho ( ) outras: _____

Quantidade por dia (número de copos por dia)? _____

- Já bebeu? ( ) Sim ( ) Não Há quanto deixou (meses)? _____

Quantidade (número de copos por dia): _____

Tipo: ( ) cachaça ( ) cerveja ( )whisky ( ) vodka ( ) vinho ( ) outras: _____

- Já usou drogas? ( ) Sim ( ) Não Tipo:_____ Quantidade: _____

Por quanto tempo (meses)? _____ Consome ainda? ( ) sim ( ) não

- Já foi exposto à radiação? ( ) Sim ( ) Não Número de raio-X: _____

Quantas vezes foi exposto nos últimos 12 meses? _____

- Usa algum tipo de medicamento? ( ) Sim ( ) Não

Quais: ( ) Antibiótico ( ) Vitamina ( ) Antiinflamatório ( ) Xarope ( ) Outros: _____

Freqüência por dia: _____

- Usou algum tipo de medicamento nos últimos 12 meses? ( ) Sim ( ) Não

Quais: ( ) Antibiótico ( ) Vitamina ( ) Antiinflamatório ( ) Xarope ( ) Outros: _____

Freqüência por dia: _____

Há quanto tempo deixou? _____

- Fez alguma cirurgia no último ano? ( ) Sim ( ) Não

Data: _____ Tipo: _____

- Alimenta-se de:

Alimentos construtores: ( ) carne ( ) peixe ( ) frango ( ) ovos

( ) leite e derivados (queijo e iogurte) ( ) feijão ou lentilha ( ) grão-de-bico ou soja.

Alimentos reguladores: ( ) verduras ( ) frutas ( ) legumes

Carboidratos: ( ) pão ( ) macarrão ( ) cereais-arroz, milho ( ) doces ( ) batata ( ) mandioca

ANEXOS

Normas *ENVIRONMENTAL POLLUTION*

Guide for Authors

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Preparing Tables and Figures

Tables. Each table must begin on a separate page. Tables should be numbered with Arabic numerals, followed by a brief title (not to exceed 25 words). When setting up tables, do not use table layout format; type tables as text and use tabs to align the columns. Tables should contain no more than three layers of column headings, and the entire table should fit on one journal page or less. Tables that are more than one page may be published online as Supplemental Material. A column heading must be provided for each column. Rather than placing additional column heads in the middle of a table, a new table should be created. List abbreviations and definitions under each table. General footnotes to tables should be indicated by lowercase superscript letters beginning with "a" for each table. Footnotes indicating statistical significance

should be identified in the following order: asterisks (*, **), number signs (#, ##), and daggers (†, ††). Type footnotes directly after the abbreviations beginning on the next line.

Figures. Figure legends should be typed on one page using Microsoft Word; this page should precede the figures. Graphics must fit standard letter size paper (8.5 × 11 inches or smaller), and resolution should be at least 300 dpi. All letters, numbers, and lines should be clearly legible and easy to differentiate. Provide a key defining representational elements (e.g., dotted/dashed lines, symbols, asterisks, error bars) for each figure. All axes should be clearly labeled, giving both the measure and the unit of measurement where applicable. No lines of demarcation or measurement should appear inside the graph itself, only on the axes. Consistency among terms and styles used in figures is desirable. Photomicrographs should include a scale bar in each image, and the length should be specified in the typed figure legend (e.g., bar = 10 μm). Multiple panels within a figure should fit on one page. If you have questions or problems, contact the journal (EHPmanuscripts@niehs.nih.gov).

Image integrity. Adjusting the image for brightness and contrast is acceptable if it is applied to the entire image. Background data of gels and blots must not be removed. The final image must accurately represent the original data.

S**upple**m**ent**a**l Material**

EHP welcomes material suitable for inclusion as online documentation, such as kinetic studies, background material, and supporting tables, figures, videos, etc. Materials and Methods should be briefly described in the manuscript, but intricate details, including tables or figures, should be included in Supplemental Material. Supplemental Material should be uploaded as a separate, preferably single, pdffile and designated as such. Questions regarding supplemental materials should be e-mailed to EHPManuscripts@niehs.nih.gov.