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Dissertação de Mestrado

Atividade mutagênica em solos sob a influência de rejeitos de carvão

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Dissertação apresentada ao Programa de Pós-Graduação em Ecologia, do Instituto de Biociências da Universidade Federal do Rio Grande do Sul, como parte dos requisitos para obtenção do título de Mestre em Ecologia.

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Dedicatória

Dedico esta obra aos seres procarióticos e demais
organismos indistinguíveis aos olhos humanos
que auxiliam os “eucariontes superiores” nos
avanços da ciência

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Resumo

O solo é um compartimento ambiental altamente complexo e, em razão do crescimento populacional acelerado e da intensa atividade industrial e agrícola, tem sofrido com a contaminação de substâncias de origens diversas. Entre as principais atividades que contribuem para a perturbação da qualidade dos solos estão as atividades de geração de energia pela queima de combustíveis fósseis, como o carvão mineral. Dentre as substâncias presentes nos rejeitos do carvão queimado em usinas termelétricas estão compostos orgânicos e metais pesados que interagem com o material genético, produzindo mutações e acarretando prejuízos em nível de organismo (como as neoplasias), mas também em níveis maiores de organização biológica (como a perda de diversidade genética em populações). Estudos de avaliação do potencial mutagênico em amostras de solo são escassos, principalmente investigando a ação de mutágenos de origem inorgânica. Além disso, estudos de mutagênese em solos sob a influência de rejeitos de carvão têm sido pouco abordados na literatura. Desta forma, os objetivos deste trabalho foram: (i) testar um protocolo para avaliação de atividade mutagênica em extratos inorgânicos e orgânicos de solo; (ii) avaliar a presença e o perfil dos compostos mutagênicos em solos sob a influência de cinzas de carvão e (iii) investigar as rotas ambientais para dispersão dos compostos mutagênicos na área de estudo. Para atingir estes objetivos, processos de extração de compostos orgânicos e inorgânicos de solo foram testados quanto a sua eficiência em extrair da amostra compostos potencialmente mutagênicos frente a linhagem de *Salmonella typhimurium* que detecta erro no quadro de leitura (TA98). Os protocolos mais eficientes foram utilizados para os testes com diferentes amostras de solo sob a influência de rejeitos de carvão utilizando diversas linhagens, na ausência e presença de ativação metabólica (fração S9 mix). Os resultados de mutagênese associados aos resultados da caracterização química de compostos orgânicos e metais indicaram que os contaminantes presentes nas cinzas de carvão podem se dispersar com facilidade para áreas adjacentes. Essa abordagem de estudo permitiu relacionar a presença de

determinadas classes de compostos com diferentes danos no DNA e inferir distintas rotas ambientais de dispersão para esses compostos. A realização deste trabalho ressalta a importância de estudos em matrizes ambientais complexas, em especial solos contaminados, que buscam integrar os dados de mutagênese e utilizá-los sob uma perspectiva ecológica.

Palavras-chave: Rejeitos de carvão, Ensaio *Salmonella*/microssoma; amostras de solo; metais pesados; compostos orgânicos.

Abstract

Soil is a highly complex environmental compartment that has suffered contamination by substances from multiple sources mainly due to fast population growth and intense industrial and agricultural activity. Among the main activities that affect soil quality are power generation activities that use fossil fuels, such as mineral coal. Among the several compounds present in coal ashes that are employed in coal-fired power plants are substances that interact with the genetic material, causing mutations and/or damage at the individual level (like neoplasias) but also at higher levels of biological organization (like loss of genetic diversity in populations). There are few studies on the mutagenic potential of soil samples, especially those that investigate the action of mutagens from inorganic sources. Moreover, studies of mutagenesis in soils under the influence of coal-fired power plants have rarely been reported in literature. Thus, this work aimed at: (i) testing a protocol for the evaluation of mutagenic activity in inorganic and organic extracts from soil samples; (ii) evaluating the presence and the profile of mutagenic compounds in soils under the influence of coal ashes and (iii) investigating environmental routes of dispersion of mutagenic compounds in the study area. In order to achieve these aims, extraction processes for inorganic and organic compounds were tested concerning their efficiency to extract compounds potentially mutagenic to the specific *Salmonella typhimurium* strain that detects frameshift mutagens (TA98). The most efficient protocols were further utilized for the tests with different soil samples under the influence of coal-fired power plant using several strains, in absence and presence of metabolic activation (S9 mix fraction). Mutagenesis results associated with the chemical characterization of the organic compounds and metals indicated that contaminants present in coal ashes can be easily dispersed to adjacent areas. This approach allowed relating the presence of certain classes of compounds to specific damages in DNA and inferring distinct dispersion routes for these compounds. This work highlights the importance of

studies concerning complex environmental matrices, specially contaminated soils, which seek the integration of mutagenesis data and their use from an ecological perspective.

Keywords: Coal wastes; *Salmonella*/microsome assay; soil samples; heavy metals; organic compounds.

Sumário

1. Revisão de literatura

1.1 Qualidade ecológica do solo

O solo pode ser definido como uma camada de intensa atividade química e biológica, localizada sobre a rocha-matriz, constituindo-se de minerais, matéria orgânica, água, ar, raízes de plantas e microrganismos. Ainda que o uso principal do solo seja a produção de alimentos, sua importância não se restringe à agricultura, agindo como um filtro para várias substâncias, um meio para degradação de resíduos e uma fonte importante de nutrientes e gases através da atividade biológica (STENBERG, 1999; DORAN; ZEISS, 2000).

O aumento populacional, o desenvolvimento industrial acelerado e o uso intensivo dos recursos naturais pela agropecuária e mineração têm contribuído para o despejo desenfreado de resíduos sólidos e líquidos, os quais aumentam em grande escala o número de áreas contaminadas, acentuando desta forma, os problemas ambientais (GILMORE, 2001; ATLAS; BARTHA, 2002). Soma-se a esse fato, a potencialidade de deposição de contaminantes provenientes da liberação de compostos na atmosfera (EDENHARNER et al., 2000). A contaminação dos solos é para Gilmore (2001) a maior barreira para o desenvolvimento sustentável, uma vez que esse problema afeta drasticamente a "saúde" do ecossistema e age de forma direta na perda de diversidade biológica.

O solo, do ponto de vista de retenção de poluentes, difere de outros compartimentos ambientais na medida em que inexiste um deslocamento contínuo, como no caso da circulação atmosférica e da água de superfície, podendo acarretar o aumento do tempo de permanência dos contaminantes em nível local. Além disso, o solo funciona como um filtro e como o receptáculo final de grande parte dos rejeitos do planeta, necessitando, assim, de cuidados especiais em seu uso (SIQUEIRA et al., 1994; SISSINO; MOREIRA, 1996; STENBERG, 1999; COSTA; COSTA, 2004). Entre os principais resíduos contaminantes do solo estão os metais pesados e os compostos orgânicos.

Os metais possuem uma origem freqüentemente natural, através da dissolução de rochas e minerais e são encontrados em pequenas concentrações no ambiente. No entanto, o ciclo natural desses elementos tem sido modificado pelo aumento exagerado das atividades industriais e de mineração, sendo as cinzas provenientes da queima de carvão, o descarte de produtos comerciais, a decomposição do lixo e esgoto e o uso de fertilizantes as principais fontes de contaminação no solo (SIQUEIRA et al., 1994; SCHNOOR, 1996; GILMORE, 2001; SILVA-JÚNIOR; VARGAS, 2007).

Metais pesados são aqueles elementos com número atômico entre 21 (Escândio) e 84 (Polônio) de origem tanto natural quanto antropogênica. Esses metais são tóxicos para os organismos dependendo de suas concentrações e espécies químicas. Ainda são considerados como metais pesados o Alumínio (número atômico 13) e os semi-metais Selênio e Arsênio (números atômicos 33 e 34, respectivamente) (SCHNOOR, 1996).

Esses poluentes não são biodegradáveis e em determinados ecossistemas, dependendo de suas concentrações e dos fatores bióticos e abióticos atuantes, são naturalmente incorporados (DUARTE, 2001). Entretanto, algumas dessas substâncias podem ser acumuladas através da teia trófica, resultando em um desequilíbrio do ambiente, através de alterações nas propriedades físico-químicas, no metabolismo do solo e na diversidade biológica (SILVA-JÚNIOR, 2006).

Em meio aquoso, os íons metálicos podem se ligar às partículas orgânicas ou inorgânicas como, por exemplo, radical hidroxila, partículas de argila, detritos orgânicos e microrganismos. Este fenômeno é conhecido como complexação ou adsorção. De maneira geral, a adsorção é potencializada pelo aumento do pH do meio (SCHNOOR, 1996). Para cada metal, existe um valor de pH acima do qual, esses elementos metálicos se precipitam como hidróxidos ou sais básicos, esse valor é denominado pH de hidrólise (TEÓDULO, 2003).

Os compostos orgânicos de ação tóxica são geralmente de natureza antropogênica e persistentes no ambiente, destacando-se entre esses os hidrocarbonetos policíclicos aromáticos

(HPAs), compostos heterocíclicos e aminas aromáticas (WHITE; CLAXTON, 2004). A crescente contaminação por poluentes de origem orgânica é influenciada principalmente pelo uso de produtos agrícolas (herbicidas, fertilizantes e inseticidas), de combustíveis fósseis e pelo despejo inadequado de resíduos industriais (WESP et al., 2000; ESTEVE-NUNEZ et al., 2001; GILMORE, 2001).

A deposição indiscriminada e o acúmulo dessas substâncias podem formar sítios contaminados, sendo que pesquisas com objetivo de diagnóstico ambiental, monitoramento e recuperação de solos expostos a contaminantes devem ser priorizadas. Nesses estudos, muitas são as ferramentas utilizadas para medir alterações do ecossistema, sendo estas chamadas de *indicadores de qualidade do solo*. Lanna (2002) divide esses indicadores em dois grupos distintos: *inerentes* (parâmetros físico-químicos) e *dinâmicos* (parâmetros biológicos). Esses últimos despontam como importantes descritores da qualidade do solo, por serem altamente sensíveis a mínimas alterações ambientais (STENBERG, 1999).

Dentre os indicadores biológicos de qualidade do solo, os parâmetros microbiológicos são os mais utilizados em estudos de avaliação/diagnóstico e monitoramento de solos contaminados. Isto se deve a atividades fundamentais exercidas por estes microrganismos na degradação e ciclagem da matéria orgânica, na capacidade em responder rapidamente a mudanças ambientais e pelo fato da atividade microbiana refletir a soma de fatores reguladores da degradação e transformação de nutrientes (STENBERG, 1999). Entre os principais parâmetros microbiológicos estão: a biomassa microbiana total, a taxa de respiração basal, a fixação de nitrogênio por bactérias, a atividade da desidrogenase e de outras enzimas, a taxa de colonização micorrizal e a diversidade microbiana (STENBERG, 1999; FILIP, 2002; ZILI et al.,2002).

1.2 Biomarcadores de Genotoxicidade

Dentro do grupo de descritores de origem biológica os biomarcadores têm se destacado no diagnóstico e monitoramento ambiental. Estes são definidos como uma medida eficaz que pode refletir a interação entre um sistema biológico e um agente ambiental (químico, físico ou biológico), evidenciada como alterações bioquímicas, celulares, histológicas, fisiológicas ou comportamentais (VAN DER OOST et al., 2003).

A utilização de biomarcadores pode fornecer informações sobre os efeitos biológicos de poluentes sem necessitar quantificá-los no ambiente. Existem alguns critérios para identificar bons biomarcadores entre eles: (i) a predileção por respostas em nível celular, pois se sabe que estas são descritores biológicos mais universais; (ii) devem possuir alta sensibilidade a mínimas alterações ambientais e respostas biológicas precoces para prever danos ao meio ambiente e à saúde humana; (iii) os ensaios devem ser rápidos, baratos e de fácil realização; (iv) os controles devem ser bem definidos para se distinguir facilmente entre variações dentro dos ensaios e alterações ambientais causadas por agentes estressores; (v) as respostas devem estar relacionadas à dosagem e ao tempo de exposição e por fim; (vi) sempre que possível deve-se dar preferência para técnicas não-invasivas e não destrutivas para possibilitar estudos com espécies e populações que estejam em perigo de extinção ou que tenham naturalmente reduzido tamanho populacional.

Os biomarcadores são classificados em três tipos a depender da resposta: *biomarcadores de exposição* - detectam ou medem substâncias, seus metabólitos ou ainda os produtos da interação entre o agente xenobiótico e alguma molécula ou célula-alvo que é medida em um compartimento dentro do organismo; *biomarcadores de efeito* - inclui medidas bioquímicas, fisiológicas ou outras alterações dentro de tecidos ou fluidos corpóreos de um organismo que podem ser reconhecidos ou associados com possíveis ou estabelecidos danos à saúde e; *biomarcadores de suscetibilidade* - indicam uma habilidade inerente ou adquirida de um organismo para responder frente à exposição de um xenobiótico específico, incluindo fatores

genéticos e mudanças nos receptores que alterem a suscetibilidade de um organismo (VAN DER OOST et al., 2003).

Um grupo de ferramentas atualmente utilizadas na avaliação e monitoramento de ambientes potencialmente contaminados são os biomarcadores de genotoxicidade que permitem detectar mutações em nível cromossômico e gênico, presença de aductos de DNA, produtos de excisão de DNA e alterações no sistema de reparo. Embora exista um grande número de ensaios de genotoxicidade, poucos têm sido utilizados com amostras de solo (WHITE; CLAXTON, 2004), sendo os principais: *Salmonella*/microssoma (BROOKS et al., 1998; HUGHES et al., 1998; TSUKATANI et al., 2002; WATANABE et al., 2005), SOS cromoteste e *umu* teste (PLAZA et al., 2005), ensaios cometa e micronúcleo em larvas de anfíbio (MOUCHET et al., 2006), ensaio de micronúcleo em *Tradescantia* (KNASMÜLLER et al. 1998; MONARCA et al. 2002) e alterações cromossômicas em *Allium cepa* (KOVALCHUK et al., 1998).

Esses biomarcadores de genotoxicidade surgem como uma alternativa promissora dentre os indicadores de qualidade do solo, uma vez que atuam na previsão de danos precoces ao conteúdo genético dos seres vivos. Segundo Vargas et al. (2007), o acúmulo de mutações em células somáticas e germinativas pode causar redução de populações naturais e ainda, o contato com esses agentes genotóxicos pode promover efeitos danosos à saúde humana, dentre eles o câncer. Ademais, esses marcadores podem ser úteis na investigação de misturas ambientais complexas, prevendo assim, os efeitos biológicos dos contaminantes, sem a necessidade de quantificá-los (WHITE; CLAXTON, 2004).

1.3 Genotoxicidade em Áreas de Resíduos de Carvão

Embora o Brasil possua grandes reservas de carvão mineral e se utilize dessa matriz para a produção de energia, principalmente na região Sul, sabe-se que os rejeitos de carvão causam prejuízos à saúde humana e ao meio ambiente, sejam esses provenientes da mineração ou da queima em usinas termelétricas (CELIK et al., 2007). Como forma de atenuar os prejuízos ambientais do setor termelétrico no país, alguns estudos têm sido propostos para a incorporação da variável ambiental no planejamento desse setor no país (MEDEIROS, 2003) e na avaliação da eco-eficiência de diferentes matrizes utilizadas como combustível para a geração de energia termelétrica, incluindo o carvão mineral (MICHELINI, 2005).

O Estado do Rio Grande do Sul detém aproximadamente 90% das mais de 30 milhões de toneladas das reservas de carvão do Brasil (NEVES; CHAVES, 2000), sendo que até hoje, em inúmeras cidades do Estado, a extração e manufatura do carvão possuem importante papel na geração de emprego e renda para as populações. Devido a importância deste recurso para a economia do Estado e a preocupação em fazer um diagnóstico incluindo aspectos sociais, econômicos e ambientais foram elaboradas duas importantes obras de referência para este tema: *Carvão e Meio Ambiente* (UFRGS, 2000) e *Meio Ambiente e Carvão: Impactos da exploração e utilização* (TEIXEIRA; PIRES, 2002), sendo que a primeira obra concentra seus estudos na Microrregião Carbonífera do Baixo Jacuí enquanto a segunda aborda aspectos da região de Candiota.

Analisando estudos realizados, em todo o mundo, focados em saúde humana verificam-se principalmente pesquisas voltadas para trabalhadores ocupacionalmente expostos a rejeitos de carvão utilizando diferentes marcadores biológicos. Sram et al. (1985) estudaram a ocorrência de anormalidades cromossômicas em trabalhadores da mineração do carvão e ao comparar com um grupo controle constataram o aumento de injúrias genéticas no grupo-alvo. Em outro estudo, foram avaliados diversos parâmetros de risco genético em trabalhadores que tiveram uma

diminuição dos níveis de exposição às cinzas leves e constatou-se a diminuição da ocorrência de danos genéticos avaliados pela freqüência de troca de cromátides irmãs e de mutações no gene *hprt*, pela indução de micronúcleos e pela excreção de mutágenos pela urina através do ensaio com *Salmonella typhimurium* (STERIUM et al., 1993). Por fim, Celik et al. (2007) estudaram a ocorrência de danos citogenéticos em trabalhadores de usinas termelétricas medidos pela análise de aberrações cromossômicas, poliploidia, troca de cromátides irmãs e indução de micronúcleos em linfócitos, verificando aumentos significativos de danos em trabalhadores expostos em relação ao grupo controle.

Efeitos genotóxicos de solos contaminados por carvão foram avaliados submetendo-os a dieta de ratos e avaliando a presença de lesões microscópicas, alterações enzimáticas e presença de aductos de DNA (BORDELON et al., 2000). Além disto, alguns estudos mostraram atividade mutagênica em cinzas leves de carvão provenientes de usinas termelétricas através do ensaio com *Salmonella typhimurium* (FISHER et al., 1978; 1979). No Brasil, trabalhos utilizando biomarcadores de genotoxicidade em áreas de rejeitos de carvão são escassos (SILVA et al., 2000a; 2000b; 2000c). Nesses, foram avaliados danos ao DNA de roedores encontrados apenas nas planícies arenosas da América do Sul, através do ensaio cometa e indução de micronúcleos. A ausência de estudos utilizando o ensaio *Salmonella*/microssoma em áreas sob influência deste tipo de resíduo revela a necessidade da utilização de bioensaios padronizados (como o *Salmonella*/microssoma) que facilitam comparações entre resultados obtidos em diversas partes do mundo.

1.4 Ensaio *Salmonella*/microssoma em amostras de solo

O teste de mutagenicidade com *Salmonella typhimurium*, também conhecido como ensaio *Salmonella*/microssoma ou Teste de Ames é a metodologia mais utilizada para detectar substâncias com potencial genotóxico, sendo empregado em laboratórios de vários países de forma padronizada (CHEN; WHITE, 2004; WHITE; CLAXTON, 2004; OHE et al., 2004; CLAXTON et al., 2004). Os protocolos iniciais e as primeiras tentativas de padronização desse ensaio datam da década de 1970 com os trabalhos de (AMES, 1971; AMES, 1972; AMES et al., 1973; AMES et al., 1975). Os primeiros estudos utilizando este bioensaio buscavam associar os resultados à carcinogenicidade potencial de compostos mutagênicos (AMES, 1972; AMES et al., 1973). Mais recentemente, o ensaio tem sido utilizado para fins de avaliação ambiental, particularmente para análises de misturas complexas em amostras de água, ar, sedimento e solo (ver revisões, CHEN; WHITE, 2004; WHITE; CLAXTON, 2004; OHE et al., 2004; CLAXTON et al., 2004).

White e Claxton (2004) ressaltam que avaliar a contaminação por substâncias genotóxicas no solo não é uma tarefa fácil. As dificuldades inerentes desta investigação não são exclusivas desse tipo de amostra, sendo comuns no estudo de misturas ambientais complexas tais como águas superficiais e subterrâneas, efluentes industriais, sedimentos e material particulado de ar. O grande número de substâncias agressivas, potencialmente presentes num sítio contaminado, pode dificultar uma caracterização química, sendo que este tipo de análise apresenta respostas limitadas para prever a toxicidade e a genotoxicidade de misturas complexas. Visando superar essas dificuldades, muitos pesquisadores recomendam uma estratégia que busca medir efeitos biológicos e caracterização química de forma associada, num esforço para identificar as principais classes de compostos biologicamente ativos presentes na mistura. Assim, tem sido recomendado medir, através de marcadores de genotoxicidade, a mutagênese potencial da mistura ambiental ou de extratos diferenciais de uma mesma amostra e, em uma segunda etapa, realizar a caracterização química das frações mutagênicas (BRACK, 2003).

Ensaios de mutagenicidade (incluindo o ensaio *Salmonella*/microssoma ou Teste de Ames) têm sido amplamente utilizados para avaliar riscos de contaminação do solo por certos agentes químicos, destacando-se os compostos orgânicos e metais pesados, permitindo assim, identificar substâncias com impacto potencial à saúde humana e ao meio ambiente (MORTELMANS; ZEIGER, 2000). Em uma recente revisão de literatura, esse ensaio foi recomendado para avaliação do potencial de mutagenicidade de amostras de solo com diferentes tipos de contaminação antrópica (WHITE; CLAXTON, 2004).

Edenharder et al. (2000) estudaram a variação sazonal da atividade mutagênica de amostras de solos florestais e de agricultura da Alemanha através do ensaio *Salmonella*/microssoma. Verificaram que a maior fonte dos mutágenos do solo era a atmosfera e que estes mutágenos podem sofrer transformações para formas não-mutagênicas pela ação dos microrganismos do solo. Esse mesmo ensaio foi utilizado para avaliar a genotoxicidade de amostras de solo contaminados por creosoto após diferentes processos de biorremediação: *bioslurry* (lama biológica), *biopile* (empilhamento biológico), *compost* (compostagem) e *landfarming* ("fazenda de lodo"). Nesses estudos foi constatada, a partir da utilização de linhagens específicas de *Salmonella typhimurium*, a presença de nitro-hidrocarbonetos em amostras de solo submetidas a dois dos processos de biorremediação (*bioslurry* e *biopile*), sendo que essa condição parece estar relacionada à transformação microbiana no solo (HUGHES et al., 1998), ainda que a concentração dos hidrocarbonetos policíclicos aromáticos (HPAs) tenha sofrido redução em todos os processos de biorremediação (BROOKS et al., 1998).

Diversos outros trabalhos foram realizados na Ásia para avaliar a atividade mutagênica de compostos presentes nos extratos orgânicos de solos sob a influência automotiva (TSUKATANI et al., 2002; WATANABE et al., 2005), áreas residenciais (WATANABE et al., 1998; 2000; 2005) e agriculturais (ALEEM; MALIK, 2003). Na Europa, alguns estudos foram realizados em solos da Alemanha contaminados por trinitrotolueno e hexógeno (BERTHE-CORTI et al., 1998),

hidrocarbonetos policíclicos aromáticos (HPAs) e óleos minerais (EHRLICHMANN et al., 2000), além de solos sob influência automotiva (WESP et al., 2000); solos italianos contaminados por hidrocarbonetos policíclicos aromáticos e metais pesados (MONARCA et al., 2002) e solos contaminados por cinzas de incineração de resíduos sólidos municipais na França (MOUCHET et al., 2006).

No Brasil, os estudos de genotoxicidade em amostras de solo utilizando o ensaio *Salmonella*/microssoma são escassos, contando de um único trabalho com extratos orgânicos de solos sob influência automotiva no litoral do Rio Grande do Sul (SILVEIRA, 2002). Essa escassez de estudos no país revela a necessidade de elaboração de protocolos para extração de amostras de solo visando padronizar as condições para avaliação da atividade mutagênica, através do ensaio *Salmonella*/microssoma.

1.5 Área de estudo

O trabalho foi realizado na área de influência de uma usina termelétrica localizada na Microrregião Carbonífera do Baixo Jacuí. Dentro do recorte da área de estudo selecionada para realização deste trabalho, foram definidos locais de amostragem para avaliar os potenciais impactos residuais no solo gerados pela queima do carvão fóssil e disposição de suas cinzas em uma unidade termelétrica de baixo potencial energético. A área onde a usina está inserida é moderadamente afastada de grandes centros urbanos (aproximadamente 70 Km de Porto Alegre), às margens de um importante rio da região (Rio Jacuí) que, a jusante do empreendimento une suas águas com outros rios do Estado (Gravataí, Sinos e Caí) formando o Delta do Jacuí e, por conseguinte, o Lago Guaíba. Desta forma, os contaminantes potencialmente lixiviados do solo nessa área e carreados até o rio, podem somar-se aos poluentes de outras áreas causando prejuízos de forma indireta a uma grande população.

A hipótese do estudo foi avaliar se os impactos dos rejeitos de carvão a curtas distâncias podem estar causando danos ao ambiente próximo com influências na população que vive mais associada com a área (vila de funcionários, comunidades ribeirinhas e fazendas da região).

Em toda a área de influência da usina termelétrica tem sido realizado um amplo monitoramento que inclui organismos terrestres e aquáticos, testes de toxicidade, além de parâmetros de qualidade de águas subterrâneas, investigação nas águas superficiais e nos sedimentos do rio Jacuí, dentro da área de influência do empreendimento (FEPAM, 2005).

Assim, as coletas de amostras de solo foram realizadas em locais estratégicos para prever a dispersão dos contaminantes para áreas adjacentes e ainda relevantes para estimar danos à saúde humana e ao meio ambiente. O foco central de amostragem foi um depósito de cinzas pesadas em processo de recuperação. O local foi alvo da deposição de cinzas recém-queimadas na usina termelétrica durante algumas décadas, mas que recentemente, em função de exigências do órgão ambiental, encontra-se em processo de recuperação ambiental. Além de coletas de solo

neste depósito, amostras foram retiradas de áreas abaixo (mata ciliar do rio Jacuí e campo úmido pastejado) e acima do nível do depósito (área de recreação infantil).

2. Objetivos

O principal objetivo desta pesquisa foi:

- Avaliar o uso do ensaio *Salmonella*/microssoma como indicador precoce de qualidade dos solos, com aplicação no biomonitoramento de sítios contaminados no país.

Sendo os objetivos específicos:

- Implementar um protocolo para avaliação de atividade mutagênica em extratos inorgânicos e orgânicos de amostras de solo, através do ensaio *Salmonella*/microssoma;
- Verificar o potencial mutagênico de amostras de solo coletadas em áreas sob a influência de cinzas de carvão;
- Identificar a presença e a potencialidade mutagênica de compostos orgânicos oriundos da queima incompleta do carvão e de metais presentes nas cinzas;
- Associar atividade mutagênica dos extratos com a caracterização dos principais grupos de compostos presentes nas amostras e;
- Avaliar as principais rotas ambientais de transferência de contaminantes mutagênicos na área de estudo.

3. Artigos científicos

Os resultados obtidos com a realização deste trabalho perimtiram a elaboração de dois artigos científicos.

O primeiro, "*Extraction parameters to use in the mutagenicity assay of soil samples*", investiga a eficiência de procedimentos de extração de substâncias orgânicas e inorgânicas em amostras de solo. Trata-se de um trabalho de alta relevância, uma vez que estudos de mutagenicidade em solo são escassos. Além disso, também são raros trabalhos que utilizam frações inorgânicas de extratos para investigação mutagênica.

O segundo, "Routes *of mutagenic compounds in contaminated soil by coal wastes*", traça um perfil da mutagenicidade em amostras de solo sob a influência de uma usina termelétrica a carvão, considerando frações orgânicas e inorgânicas do solo e utilizando linhagens básicas e outras com sensibilidade diferenciada para detecção de grupos de compostos específicos, como metais pesados, nitroarenos e aminas aromáticas. Ainda, o trabalho analisa as rotas ambientais de dispersão dos compostos mutagênicos que provocam diferentes danos ao conteúdo genético.

Extraction parameters to use in the mutagenicity assay of soil samples

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Abstract

This study aimed to investigate parameters of chemical extraction to diagnose mutagenicity in soil samples. In order to evaluate the extraction efficiency of inorganic mutagens, besides the chemical analysis of metals, the *Salmonella*/microsome assay was performed in the preincubation and microsuspension procedures, using two solvents, in two extraction methodologies. The efficiency of two organic compound extraction methods was compared by qualitative analysis using CG/MS in Scan mode. The results of the analysis of inorganic extracts defined mutagenicity only in the acidic extracts of soil that were shaken, in the microsuspension assay, both in the presence and absence of metabolic activation. The other conditions tested presented higher cytotoxicity. As to the organic compounds, Accelerated Solvent Extraction (ASE) proved more effective that extraction using ultrasound (sonication). This study will help the implement of extraction parameters to evaluate the presence of mutagenic substances in soil samples, both of inorganic and organic origin.

Keywords: Heavy metals; Organic compounds; Mutagenicity; Soil samples; *Salmonella*/microsome assay; Sonication; ASE extraction; Acidic extracts.

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1. Introduction

Population growth, fast industrial development and intensive use of natural resources by agriculture, livestock and mining have contributed to the uncontained discharge of solid and liquid wastes, causing a large scale increase in the number of contaminated areas, making environmental problems worse (Gilmore, 2001; Atlas and Bartha, 2002). Toxic compounds from contaminated soils may have serious effects on human health, since as the population may come into direct contact with the soil itself or inhale contaminants, ingest them through percolated water, or also through foods (Watanabe and Hirayama, 2001; van de Wiele *et al*., 2004).

The diagnosis of contaminated soils to evaluate potential risks to human health requires appropriate tools, and most studies are based on physicochemical analyses. White and Claxton (2004) emphasize, however, that evaluating soil contamination by genotoxic substances is not an easy task, and since the great number of aggressive substances potentially present at a site may render it difficult to perform chemical characterization. Furthermore, this analysis does not predict biological effects, antagonistic or synergistic actions of organisms in these mixtures of contaminants.

The use of short-term mutagenicity bioassays is an important tool to detect compounds with a carcinogenic potential. The *Salmonella*/microsome assay is the most used one-assay to detect substances with a genotoxic potential and in considered as an early marker of chemical substance carcinogenicity. It has been widely used to diagnose the presence of mutagenic substances in environmental samples, such as water (Ohe *et al*., 2004), air (Claxton *et al.*, 2004 and sediment (Chen and White, 2004), and there are fewer studies with soil samples (White and Claxton, 2004).

Most genotoxicity assays in soil samples, including *Salmonella*/microsome, require the implementation of chemical substance extraction, and the results of these researches depend

basically on the method used to prepare the samples. This stage allows access to several fractions of the sample, which will lead to significant differences in the genotoxic responses.

Many extraction techniques have been used to diagnose the mutagenicity potential of chemical compounds using organic, aqueous and acidic solvents, besides different processes such as shaking, sonication, Soxhlet and ASE (Watanabe and Hirayama, 2001). However, many of the works using the *Salmonella*/microsome assay with soil samples stop at the organic fraction, giving little or no value to test the effect of inorganic substances such as heavy metals.

White and Claxton (2004), in their review on mutagenesis in soils, to calculate the geometric mean of mutagenicity of different type of soils with the *Salmonella*/microsome assay, used only data with extracts of the organic portion of the sample, and did not take aqueous extracts into account. Several other studies report the use of organic solvents alone, in mixtures, or even in sequential procedures. Dichloromethane (DCM), an organic solvent, is more constantly used (Hughes *et al*., 1998), but also others solvents and mixtures are used in mutagenicity studies as DCM/Acetone (Courty et al., 2004) DMSO/ethanol (Berthe-Corti *et al*., 1998), acetonitrile, methanol (Edenharder *et al*., 2000) and hexane/acetone (Monarca *et al*., 2002).

For mutagenicity studies based on inorganic extracts, almost all works used aqueous extractions (Berthe-Corti *et al*., 1998; Ehrlichmann *et al*., 2000; Courty *et al*., 2004). Among the results presented in these studies, there is a prevalence of negative mutagenic responses, suggesting that water is unuseful as an extractor solvent.

Besides the nature of the solvent, the type of equipment is important for greater process efficiency (Courty *et al*., 2004). Watanabe and Hirayama (2001) in their review on genotoxicity in soil remark that soil leachates are generally processed by mechanical shaking of samples with organic or aqueous solvents, but that the ultra-sound and Soxhlet extractor have also been used. Extractions performed based on ASE for organic substances have not been much used, although

they have well-known advantages compared to the other procedures, such as short processing time and lower expenditures on solvent (Graham *et al*., 2006).

The *Salmonella*/microsome assay has been used in soil sample tests with different protocols: plate incorporation (Hughes *et al*., 1998), preincubation (Tsukatani *et al.*, 2002; Watanabe *et al*., 2005; Watanabe *et al.*, 2008) and microsuspension assays (Courty *et al*., 2004). Although the latter procedure is mentioned in the literature as the most sensitive one, positive responses were obtained for all three methods. These responses may be related to the high degree of contamination of the soils studied and, therefore, tests using soil samples that have a moderate or low degree of contamination may provide a better evaluation of the sensitivity of the procedures involving this bioassay.

Thus, the study aimed at defining a few parameters to perform a *Salmonella*/microsome assay on soil samples, such as the inorganic extraction solvents, extraction apparatus to inorganic and organic compounds and assay protocol.

2. Material and methods

2.1 Soil samples

The soil samples were collected from areas under the influence of the coal-fired power plant, located in the Rio Grande do Sul state, southern Brazil. The sampling sites selected were areas under the influence of a coal-fired power plant (L1- bottom ash deposit undergoing a recovery process; L2- riparian forest; L3- field area and L4- urban site).

2.2 Collection of Surface Soil

Soil samples were collected between January and February of 2007. The sampling area was demarcated using a GPS and collection was performed at 15 random points, at a 20 cm depth with a spade, and gravel, stones and plant materials were removed. The 15 subsamples

were homogenized in order to form a composite sample of each sampling area. About 500 g to 1 kg of surface soil was collected using stainless spatulas, placed in a glass container protected from light, and maintained at 4 ºC until the samples were prepared. This period did not exceed 180 days.

2.3 Sample preparation

In the laboratory, collected soils were dried at room temperature for up to 2 days, protected from light, mechanically broken up, screened through a 2-mm sieve with stainless material and stored immediately at 4 ºC, protected from light until extraction.

2.4 Inorganic Extraction

2.4.1 Shaking

Soil samples were stirred (115rpm) at 20 ºC for 24 h with a solution of 5.7 ml of acetic acid p.a. and 64.3 ml of sodium hydroxide 1.0 M p.a. prepared in 1000 ml of deionized water (pH 4.93±0.05 - soil: solvent, 1:2, g/ml) according to Brazilian standards (NBR 10005) (ABNT, 2004) or distillated water (in the same procedure) and then centrifuged at 13,000 x g for 15 minutes at 4 ºC, filtered (0.45µm Millipore), divided into aliquots and stored at 4 ºC for up to 24 h for mutagenicity assessment.

2.4.2 Sonication

Soil samples (25 g) were extracted by sonication using a solution of 5.7 ml of acetic acid p.a. and 64.3 ml of sodium hydroxide 1.0 M p.a. prepared in 1000 ml of deionized water (pH 4.93±0.05) or distillated water, 25 ml per 10 minutes (two cycles, totaling 50 ml). The pre-filtered extracts were passed through the glass-wool and then centrifuged at 13,000 x g for 15

minutes at 4 ºC, filtered (0.45 µm Millipore), divided into aliquots and stored at 4 ºC for up to 24 h for mutagenicity assessment.

2.4.3 Quantification of metals

The quantification of metals in the acidic extracts (Mn, Fe, Al, Zn, Cu, Cd, Pb, Cr and Ni) was performed by inductively coupled plasma optical emission spectrometer (ICP-OES), with the following detection limits in mg/l: Cu-0.004; Zn-0.02; Fe-0.04; Mn-0.03; Al-0.08; Cd-0.002; Cr-0.004; Ni-0.002; Pb-0.01. The determinations of the metals were performed in the Soil Laboratory, Universidade Federal do Rio Grande do Sul, Brazil, according to a methodology recommended by EPA (EPA, 2007a).

2.5 Organic Extraction

2.5.1 Sonication

Soil samples (25 g) were extracted by sonication using dichloromethane (200 ml) per 10 minutes (two cycles, totaling 400 ml). The pre-filtered extracts were passed through a chromatography column with a filter plate containing sodium sulfate and celite, concentrated in rotavapor, and then the extracts were sent immediately to the chromatograph.

2.5.2 Accelerated Solvent Extraction (ASE)

Soil samples (10g) were subjected to extraction via ASE, based on the EPA 3545 method (EPA, 2007b) with the following extraction conditions: dichloromethane as a solvent extractor; 1500 psi system pressure; oven temperature: 100 ºC; static time: 5 minutes; flush volume: 60%; bleed time: 60 seconds. Organic extracts were concentrated in rotavapor and the extracts were also sent immediately for a reading in the chromatograph.

2.5.3 Qualitative analysis of organic compound

The organic extracts were reduced to 1 ml for further injection using gas chromatograph with a mass spectrometry detector coupled (GC / MS Shimadzu QP5050A) in Scan mode. The chromatography conditions followed Nascimento Filho *et al.* (2001), with alterations in the injection mode (1/50 to 1/10). The organic compounds were analyzed in the Environmental Chemistry Laboratory, at the Chemistry Institute, Universidade Federal do Rio Grande do Sul, Brazil.

2.6 Mutagenicity assay

The extracts submitted to inorganic extraction, both acidic and aqueous, were tested using the *Salmonella*/microsome assay in two procedures: preincubation (Maron and Ames, 1983) and microsuspension (Kado *et al.,* 1983). The *Salmonella typhimurium* strains TA98, TA97a and TA100 were used. Strains TA98 and TA97a detect frameshift mutagens, where TA97a is more sensitive to the action of heavy metals (Pagano and Zeiger, 1992) and TA100 detects base-pair substitution mutagens. The assays were performed in the absence and presence of a microsomal fraction activated by the polychlorinated biphenyl mixture, Aroclor 1254 (purchased in a liophilized form from Moltox, USA), with added cofactors (S9 mix), it prepared according to the procedures described in Maron and Ames (1983).

For each sample, the acidic and aqueous extracts were analyzed, at six concentrations: 25, 50, 75, 100, 150 and 200 mg equivalent of soil in the extracts for the microsuspension procedures and up to five dosages for the preincubation procedure: 250, 500, 750, 1000 and 2000 mg equivalent of soil. All the concentrations were performed in duplicate and negative controls (100 µl of liquid nutrient medium and the solvent used: 100 µl of acid solution or distillated 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 according to strain and treatment used, were added to the assays. Overnight cultures were incubated with the samples at the different concentrations, and 0.1 M phosphate buffer in the absence of metabolic activation or S9-mix in the presence of metabolic activation, for 20 minutes (preincubation) or 90 minutes (microsuspension) at 37 ºC. The content was shaken with surface agar containing traces of histidine and biotin and placed in Petri plates containing minimum medium. The plates were incubated at 37 ºC for 48 h (preincubation) or 72 h (microsuspension) and then the number of revertant colonies per plate was determined.

In parallel to the mutagenicity assay, the cell survival test was performed, spreading suspension (bacterial culture + S9 mix or 0.1 M phosphate buffer + solvent or extract) diluted to a concentration of 1-2x10² cells in enriched medium (Nutrient Agar), for 72 h at 37 °C. The samples were considered cytotoxic when at least in one dosage cell survival was equal or smaller than 60% of the negative control with the solvent used.

2.7 Analysis of the mutagenicity assay results

Data for mutagenic activities were calculated from the analysis of the linear portion of the dose-response curve and expressed as the value of the slope (number of revertants/g soil equivalent), as described by Bernstein *et al.* (1982). This slope is an estimate of the mutagenic potency of each soil sample. Additionally, data for strain TA98 were expressed in revertants per plate. Statistical analyses were run with Salanal software (*Salmonella* Assay Analysis) version 1.0 of Research Triangle Institute, RTP, North Caroline, USA.

3. Results

3.1 Inorganic Extracts

As a first analysis, riparian forest soil samples (L2) were submitted to different extraction processes and some metals were identified from these extracts (Table 1). The comparison among the methods employed shows great differences in the concentrations of the major soil constituents (Zn, Mn, Fe and Al) as well as minor elements (Ni, Cd, Cr, Pb and Cu). Therefore, an analysis based exclusively on the chemical composition would not be a useful tool to determine the most suitable extraction method to detect mutagenic contaminants. It may be essential to use short-term mutagenicity bioassays such as the well-known *Salmonella*/microsome assay, when seeking extraction parameters for the detection of substances with a mutagenic character. In addiction, the chemical characterization of the metals would be important and useful to verify specific aspects found in the bioassays.

The riparian forest soil samples (L2) were also chosen to perform the *Salmonella*/microsome assay with the same acidic and aqueous extracts using initially strain TA98. Table 2 showed the mutagenicity results in both liquid-incubation procedures, preincubation and microsuspension, using extracts submitted to shaking and sonication extractions, in the presence and absence of S9 mix. Predominantly, there were no responses to mutagenicity, except in the case for the results of the acidic extracts performed by shaking in the microsuspension assay, both in the absence and presence of S9 mix.

The presence of cytotoxic effects in samples may lead to underestimating the mutagenic responses. Therefore, samples without the presence of cytotoxic substances are preferred in studies of environmental mutagenesis. Cytotoxicity data are shown in the Table 3, under all conditions tested. The extracts used in the microsuspension assays showed lower cytotoxicity compared with preincubation assays. Further, shaken extracts were less cytotoxic compared with sonicated extracts, and acidic extracts showed less cytotoxicity when they were compared with aqueous extracts.

The acidic extracts submitted to extraction by shaking presented the second largest total concentration of metals, and the highest concentration of manganese, zinc and nickel (Table 1), besides positive responses for mutagenicity (Table 2), and absence of cytotoxicity (Table 3). These extracts were selected to test the mutagenic potential in two other strains, TA100 and TA97a, the latter being cited in the literature as sensitive to heavy metals (Pagano and Zeiger, 1992). In order to compare results, these strains were also tested against the aqueous extract submitted to shaking, where the latter showed high concentrations of aluminum, copper, chromium, cadmium and nickel, and also absence of cytotoxicity.

The acidic extracts showed mutagenic activity in TA97a, both in the presence and absence of S9 mix, while the aqueous extracts presented this activity only in the presence of S9 mix and at a lower potency. On the other hand, a slightly greater amount of mutagens that cause base pair substitution was found in the aqueous extract in the presence of the S9 mix (Figure 1).

3.2 Organic extracts

To assess the efficiency of this type of extraction a qualitative analysis of the organic compounds present in the extracts submitted to sonication and to accelerated solvent extraction (ASE) was performed. The comparison was based on extracts from four sampling areas under the influence of the coal bottom-ash (Table 4). In general, for the four sampling areas, the presence of 15 compounds was identified in the ASE extracts and 9 compounds for the ultrasound extracts. There was a more marked difference when the four sampling areas were compared separately: L1 (ASE - 13 compounds; ultrasound - 3 compounds), L2 (ASE - 8 compounds; ultrasound - 3 compounds), L3 (ASE - 13 compounds; ultrasound - 4 compounds) and L4 (ASE - 7 compounds; ultrasound - 1 compound).

ASE was also more efficient in the extraction of markers that can be employed in areas under the influence of fossil fuels, namely aliphatic hydrocarbons. The latter were identified in

the following extracts submitted to ASE: L1 (C14, C19, C20, C21, C22, C23, C24, C25 and C26); L2 (C19, C22, C24 and C25); L3 (C14, C19, C20, C21, C22, C23, C24, C25 and C26) and L4 (C23, C24, C25 and C26); while in the extracts submitted to sonication these compounds were identified in L1 (C20 and C26), in L3 (C25) and in the extracts from L2 and L4 no aliphatic hydrocarbons were identified.

4. Discussion

Although soil contamination is one the major problems of society, mutagenicity assays on soil samples have not been studied much compared with other environmental compartments. In this study, we examined a few parameter extractions to be used in mutagenicity assays of soil samples. Short–term mutagenicity bioassays are useful in this approach, because the chemical analysis is mostly inconclusive. However, the use of chemical analyses in environmental approaches is very important, since the profile of compounds to which the environment is susceptible can be shown.

4.1 Inorganic Extract

In the inorganic fraction of the soil samples, heavy metals are a class of priority compounds in the definition of probable contaminants of anthropic origin, including mutagenic compounds. These chemical elements may occur in natural environments at low concentrations and are essential to living beings, but in excessive amounts they may be toxic, or else powerful mutagenic, carcinogenic and teratogenic agents (Monarca *et al*., 2002; Knasmüller *et al*., 1998; Vargas *et al*., 2001). The comparison of methodologies for inorganic compound extraction in the present study identified the largest total concentration of metals in aqueous extracts submitted to extraction by ultrasound, followed by acid extracts prepared by shaking. This high concentration of metals in the first extract is probably due to the high concentration of iron, a condition which

is not found in the others. However, the profile of the different chemical dosages did not allow clear identification of the best procedure to extract metals from the sample. Besides, differentiated pH conditions may generate different types of metals, making it difficult to forecast the biological effects of this complex mixture (Tack and Verloo, 1995).

As a second stage of methodological selection, the *Salmonella*/microsome assay was performed in the preincubation and microsuspension procedures, in the different extracts that were chemically analyzed. The microsuspension assay showed some advantages over the preincubation assay. This condition may be related to high sensitivity in detecting the presence of mutagenic substances. Furthermore, there are many studies that prefer this protocol because it requires smaller sample volumes than other protocols (Gichner *et al*., 1998; Courty *et al*., 2004; Tagliari *et al*., 2004).

The highest mutagenic results were obtained with the shaker extracts compared to sonicated extracts. This response may be related to the longer duration of contact between soil and solvent which should yield a better extraction. The ultrasonic apparatus is cited in literature as efficient for the mutagenic organic extraction (Tsukatani *et al*., 2002; Tagliari *et al*., 2004; Watanabe *et al*., 2005). However, although this condition of inorganic extracts was employed in a few studies (Berthe-Corti *et al*., 1998) it did not show the same efficiency.

The positive results were obtained in acidic extraction by shaker. Comparative studies with acidic and aqueous extracts obtained in a similar procedure, may be related to increased bioavailability of the metals in acidic conditions (Tack and Verloo, 1995; Pueyo *et al.*, 2002). It is known that generally metals dissociate easily in acidic pH and can reach toxic forms. It should be stressed that the pH value of the acidic extracts was similar to the soil samples (pH 4.93 and pH 5.0, respectively).

The presence of cytotoxic effects in environmental samples may underestimate the mutagenic responses. Therefore, samples without the presence of cytotoxic substances are

preferred in studies of environmental mutagenesis. Cytotoxicity appears to be associated with the type of extract and extraction procedure. The explanation for this condition may be related to the chemical constitution of the different extracts. The decreasing order of cytotoxicity among the extracts analyzed in both preincubation and microsuspension assays was: Aqueous extract / Ultrasound > Acid extract / Ultrasound > Aqueous extract / Shaker > Acid extract / Shaker. This order of cytotoxicity appears to be strongly related to the iron concentration in the extracts (Table 1), suggesting that this metal may be an important element for the cytotoxic activity in these samples. Although iron is reported in the literature as an agent of mutagenic action in the presence of metabolic activation, the concentrations in the aqueous extract submitted to ultrasound must be lethal for the test-organism (Brusick *et al*., 1976).

In the current study, the results of the cytotoxicity assay were the opposite of the mutagenicity results, where the microsuspension assay, acidic extract and extraction by shaker produced small amounts of toxicity. The cytotoxic effects were more marked in extracts submitted to the preincubation protocol, probably due to the larger volumes of sample used in this protocol.

The tests performed with additional strains helped confirm the choice of the best procedure among those used in the study. The best response for mutagenic activity with the strain sensitive to heavy metals, TA97a, was also seen in the acidic extracts submitted to shaking. This response corroborates the conclusion that this extract has the most bioavailable and bioreactive types of metal, although it does not have the largest concentration of metals. This set of results then shows the acidic extraction performed under the process of mechanical shaking, as an effective method to extract mutagenic compounds of inorganic origin in the type of soil investigated.

4.2 Organic extract

Extraction is usually the first step in analytical procedures applied to the determination of organic compounds in solid matrices. Parameters such as type of solvent and extraction procedures can directly influence the results of tests. The solvent properties define the fraction of compounds that will be extracted, while the extraction method influences the efficiency of compound extraction. Dichloromethane is an organic solvent that is widely used in studies of mutagenic evaluation, extracting the fraction of compounds that are moderately polar. This solvent was chosen to compare the extraction procedures in the present study, since this fraction separates various potentially mutagenic substances.

The two procedures analyzed were sonication and ASE extraction. The first is a simple method that uses mechanical energy produced by low frequency waves, widely used in soil mutagenesis studies (Watanabe and Hirayama, 2001). The second is a more recent method, but it has already been used successfully in extracting organic compounds (Graham *et al*., 2006). It is based on extraction under subcritical conditions of pressure and temperature in closed cells. Other advantages are extraction time, low solvent consumption and low cost per extraction (Khan *et al*., 2005).

The comparison of these two methods showed better results for the soils in the present study, using the ASE technique. Other studies also mention the efficacy of this extraction method (Graham *et al*., 2006; Khan *et al*., 2005; Courty *et al*. 2004). The greater number of compounds identified, besides a more complete selection of hydrocarbons from fossil fuels, provides sufficient information to highlight the importance of this method in the investigation of compounds with a mutagenic potential in soil samples.

5. Conclusion

The answers obtained in these comparative experiments indicated strategies to investigate mutagenic activity in contaminated soils, in both inorganic and organic extracts. The prior

definition of efficiency of extraction methodologies allows greater confidence in the investigation of the mutagenicity of difference typologies of contaminated soils. It also helps elevate the reliability of the *Salmonella*/microsome assay as an early biomarker of the presence of bioavailable substances in a complex matrix as is the case of soil and its dispersion routes in the environment, in areas where there is high anthropic contamination.

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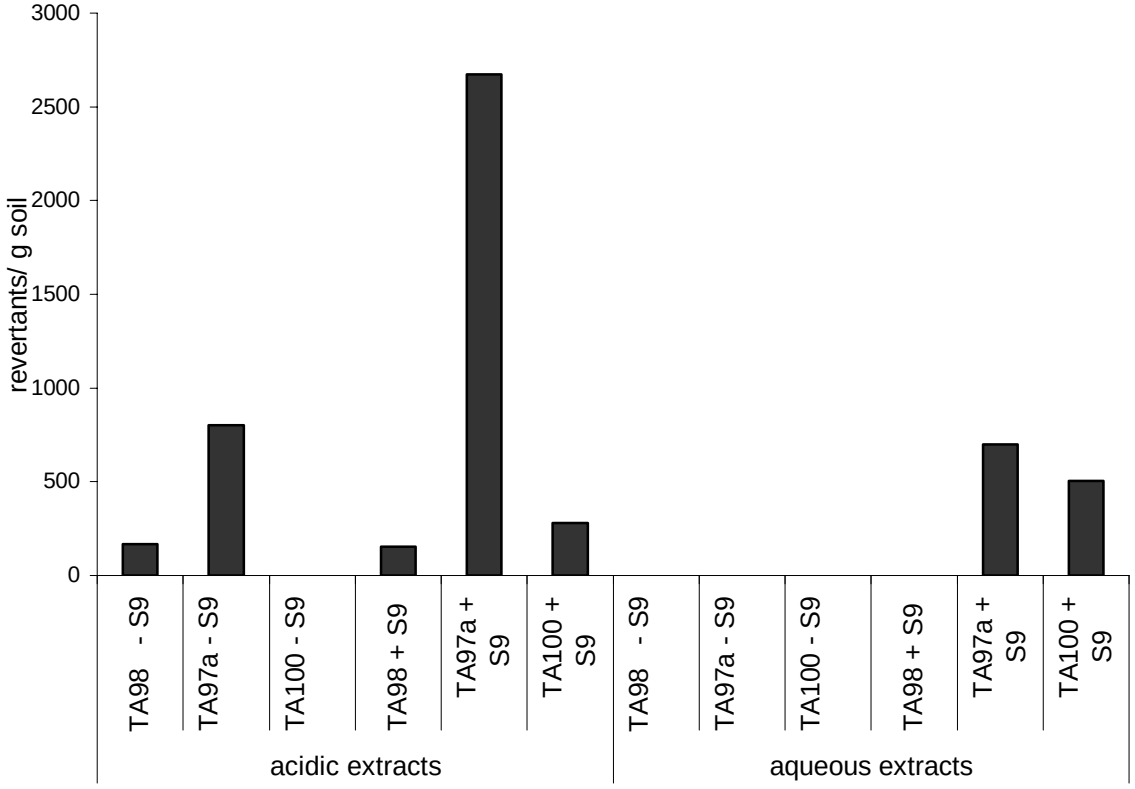

FIGURE 1. Mutagenic potency in revertants per gram of soil equivalent to strains TA97a, TA98 and TA100 in the presence (+) and absence (-) of the S9 mix in the acidic and aqueous extracts of the soil samples submitted to shaked extraction.

Negative controls (revertants per plate): 41±20.03, 66.62±14.02 (TA98 –S9, +S9); 178±33.76, 172±21.16 (TA97a –S9, + S9); 225±60.30, 216±42.36 (TA100 –S9, +S9); positive controls: 4NQO 483±159.52, 2NF 515±0.7 (TA98 –S9, +S9); 4NQO 841±148.53, 2NF 543±162.77 (TA97a –S9, +S9); AZD 985±309.84, 2NF 1,224±407.29 (TA100 –S9, +S9).

TABLE 1.

Concentration of nine metals in acidic and aqueous extracts submitted to two extraction equipment[1]

Metal	Sonication		Shaker	
	Aqueus extract	Acidic extract	Aqueus extract	Acidic extract
Copper	0.008	ND	0.020	0.002
Zinc	ND	0.054	0.486	0.496
Iron	2.972	1.082	0.112	0.222
Manganese	0.298	0.098	0.942	1.080
Aluminum	3.968	1.460	4.288	4.240
Cadmium	0.006	ND	0.010	0.002
Chromium	0.026	0.002	0.030	0.014
Nickel	0.024	0.032	0.046	0.080
Lead	0.096	0.140	ND	0.114
Total concentration	**7.398**	**2.868**	**5.934**	**6.250**

[1] mg/kg soil equivalent; ND – not detected.

TABLE 2.

Mutagenicity of acidic and aqueous extracts of soil samples from riparian forest (L2) through *Salmonella*/microsome assay using strain TA98 in the preincubation and microsuspension assays

Dose[1]	Sonication				Shaker			
	Acidic		Aqueous		Acidic		Aqueous	
	Preincubation assay							
	Without S9	With S9	Without S9	With S9	Without S9	With S9	Without S9	With S9
0[2]	26.0 ± 2.8[3]	26.0 ± 5.7	17.7 ± 6.5	32.0 ± 2.8	25.5 ± 12.0	25.0 ± 2.8	17.7 ± 6.5	34.0 ± 2.8
250	-	19.0 ± 2.8	18.5 ± 3.5	16.5 ± 0.7	18.0 ± 4.2	32.0 ± 4.2	17.0 ± 4.2	26.5 ± 0.7
500	18.5 ± 6.4	30.5 ± 2.1	14.5 ± 4.9	22.5 ± 3.5	18.0 ± 4.2	30.0 ± 5.7	12.0 ± 1.4	33.0 ± 5.7
750	21.5 ± 6.4	19.5 ± 2.1	19.0 ± 1.4	28-0 ± 2.8	22.5 ± 2.1	36.0 ± 11.3	13.5 ± 2.1	33.0 ± 0.0
1000	25.0 ± 8.5	23.5 ± 4.9	11.0 ± 0.0	27.5 ± 2.1	22.5 ± 11.6	31.0 ± 11.3	20.5 ± 2.1	32.5 ± 4.9
2000	NT	NT	14.5 ± 0.7	NT	NT	NT	10.5 ± 3.5	NT
	Microsuspension assay							
0[2]	35.0 ± 1.4[3]	67.0 ± 2.1	30.0 ± 4.2	69.5 ± 3.5	**30.5 ± 2.1**	**73.5 ± 14.8**	30.0 ± 4.2	85.0 ± 0.0
25	26.0 ± 0.0	44.5 ± 4.9	23.5 ± 0.7	58.5 ± 9.2	**18.5 ± 2.1**	**82.0 ± 7.1**	35.0 ± 0.0	92.5 ± 3.5

TABLE 2. (continued)

Dose	Sonication				Shaker			
	Acidic		Aqueous		Acidic		Aqueous	
	Microsuspension assay							
	Without S9	With S9	Without S9	With S9	Without S9	With S9	Without S9	With S9
50	36.0 ± 0.0	64.5 ± 21.9	19.0 ± 0.0	70.5 ± 7.8	**36.0 ± 1.4**	**87.0 ± 2.8**	27.0 ± 0.0	90.0 ± 11.3
75	33.0 ± 0.0	59.5 ± 13.4	26.0 ± 0.0	57.5 ± 3.5	**34.0 ± 7.1**	**89.5 ± 7.8**	43.0 ± 0.0	75.0 ± 9.9
100	29.0 ± 0.0	-	23.0 ± 0.0	56.0 ± 2.8	**43.0 ± 4.2**	**91.5 ± 0.7**	33.0 ± 0.0	85.5 ± 14.8
150	29.0 ± 0.0	59.5 ± 21.9	27.0 ± 0.0	62.0 ± 15.6	**61.5 ± 4.9**	**101.0 ± 4.2**	29.0 ± 0.0	90.0 ± 7.1
200	29.5 ± 3.5	53.5 ± 6.4	24.3 ± 3.5	55.0 ± 5.7	**20.0 ± 5.7**	**104.5 ± 0.7**	39.5 ± 7.8	106.5 ± 2.1

[1]Dose in mg equivalent/ plate; [2] dosage that shows the negative controls values; [3] number of revertants/ plate ± standard deviation; NT – not tested; columns in bold represents significantly dose-response curves positive control: 4NQO (TA98−S9) 235.5±50.81; 2AF (TA98 + S9) 543±162.77.

TABLE 3.

Cytotoxicity of acidic and aqueous extracts of soil samples from riparian forest (L2) through *Salmonella*/microsome using strain TA98 in the preincubation and microsuspension assays

Dose[1]	Pre-incubation assay							
	Sonication				Shaker			
	Acidic		Aqueous		Acidic		Aqueous	
	Without S9	With S9	Without S9	With S9	Without S9	With S9	Without S9	With S9
0	100%[2]	100%	100%	100%	100%	100%	100%	100%
250	76%	57%	100%	100%	70%	100%	100%	100%
500	76%	95%	100%	100%	92%	100%	77%	100%
750	15%	38%	100%	100%	100%	100%	60%	100%
1000	92%	68%	95%	100%	100%	97%	76%	100%
2000	NT	NT	58%	NT	NT	NT	57%	NT
	Microsuspension assay							
0	100%[2]	100%	100%	100%	100%	100%	100%	100%

TABLE 3. (continued)

Dose	Sonication				Shaker			
	Acidic		Aqueous		Acidic		Aqueous	
	Microspension assay							
	Without S9	With S9	Without S9	With S9	Without S9	With S9	Without S9	With S9
25	80%	71.90%	100%	38%	82%	73%	100%	98%
50	100%	65%	100%	-	100%	81%	-	100%
75	100%	-	100%	79%	87%	80%	97%	100%
100	100%	-	74%	46%	100%	61%	100%	67%
150	100%	-	100%	80%	94%	79%	100%	86%
200	100%	-	-	32%	80%	61%	100%	-

[1]Dose in mg equivalent/ plate; [2]cytotoxic samples when cell survival is equal or smaller than 60% of the negative control with the solvent used; NT- not tested.

TABLE 4.

Organic compounds from soil samples extracted by two extraction equipment

Compounds	L1		L2		L3		L4	
	sonication	ASE[1]	sonication	ASE	sonication	ASE	Sonication	ASE
Isobutyl phthalate		x	x	x		X		x
2-ethyl hexyl phthalate	x	x			x	X		x
di-octyl phthalate		x	x	x	x	X		x
di-isopentyl phthalate			x					
n-nonadecane (C19)		x		x		X		
Eicosane (C20)	x	x				X		
Heneicosane (C21)		x				X		
Docosane (C22)		x		x		X		
Tricosane (C23)		x				X		x
Tetracosane (C24)		x		x		X		x
Pentacosane (C25)		x		x	x	X		x
Hexacosane (C26)	x	x				X		x
2-pentadecanone, 6,10,14 - trimethyl				x				
n-tetradecane (C14)		x				X		
Hexadecanoic acid				x				
Bis-(octyl-phenyl)amine		x				X		
Decanoic acid 2-monoglyceride					x			
9,12-octadecadienal							x	

[1] ASE – Accelerated Solvent Extraction

3.2 Routes of mutagenic compounds in contaminated soil by coal wastes

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Abstract

The mutagenicity of acidic and organic extracts of surface soil under the influence of a coal-fired power plant was evaluated by *Salmonella*/microsome assay using strains TA98, TA100, TA97a in the absence and presence of exogenous metabolic systems (S9 mix). Additionally, strains YG1041 and YG1042 (sensitive to nitroderivatives) were used in organic extracts. In general, the responses were higher in the organic extracts in the presence of S9 mix. The comparison between strains TA98 and TA100 and their derived strains YG1041 and YG1042, respectively, allowed the detection of the presence of nitro-aromatic compounds in some sampling areas, which was confirmed by chemical analysis. The interpretation of the set of mutagenesis data suggests that there are two important mutagenic compound dispersion routes in the area of study: frameshift mutagens were dispersed predominantly by runoff and leaching, while base-pair substitution mutagens were dispersed mainly by the atmosphere. This mutagenic damage might be attributed to the effects of several substances detected in the area, such as aliphatic hydrocarbons and the metals aluminum, cadmium, lead and iron.

Keywords: *Salmonella*/microsome assay; coal-fired power plant; aliphatic hydrocarbons; heavy metals

1. Introduction

The deposition of ashes from burning coal in coal-fired power plants in the soil, and their release into the air compartment are major routes of environmental contamination by mutagens, such as heavy metals and organic compounds from incomplete coal-burning [1]. These wastes have negative impacts on the ecosystem and also damage human health when they are improperly deposited [2,3].

Some studies show that coal residues from mining activities, power plant firing and final disposal of tailings could cause genetic damage. Celik et al. [3] observed that the mean frequencies of chromosomal aberrations, polyploidy, sister-chromatid exchanges and micronuclei were more significantly increased in occupationally exposed workers than in the control group. Another study also found a greater frequency of aberrant cells in open-cast miners than in the control group [4]. In addition, Fisher et al. [5] showed mutagenic activity through a *Salmonella*/microsome assay in coal fly ash from a coal-fired power plant but found that ash samples collected from hoppers of an electrostatic precipitator in the plant were not mutagenic.

Several studies investigated mutagenic activity in different types of soil: agricultural, under automotive or industrial influence and bioremediated soils. Mutagen behavior is variable, they may stay in place or move, enter other environmental compartments or bioaccumulate. In areas under the influence of coal tailings, the behavior studied most involved transferring compounds from the air compartment to the biota. There are few studies using genotoxicity assays in soils contaminated by coal ash [6-12], and even less using the *Salmonella*/microsome assay [13,14]. Finally, no work using this bioassay has been done on soils under the influence of coal ash in coal-fired power plants.

The mutagenicity results of environmentally complex mixtures depend, largely, on the method used to prepare the samples [15]. Aqueous, acidic and/or organic extractions have been

performed in soil sample studies, using shaking, sonication, Soxhlet and ASE (Accelerated Solvent Extraction). In addition, the latter technique is effective in extraction procedures [16,17].

The *Salmonella*/microsome assay is the most commonly used bioassay to perform mutagenic evaluation in different environmental matrices: water supply, industrial effluent, sediment and interstitial water [18-22], air [23-25] and soil [26,27]. Strains TA98 and TA100 are the most used in environmental mutagenesis studies, however, there are few works on mutegenesis in the soil samples using strains with different sensitivities to some contaminants such as: nitroarenes, aromatic amines, oxidative mutagens, alkylating agents, heavy metals, polycyclic aromatic hydrocarbons and quinines [27].

Therefore, the aim of this research was to evaluate the mutagenic potential of soil samples adjacent to a coal-fired power plant and of a coal bottom ash deposit in a recovery process through *Salmonella*/microsome assay, using different extraction techniques (acidic and organic), basic strains and others with special sensitivities to certain groups of compounds, to estimate ecological and human health impacts caused by the release of coal wastes.

2. Materials and Methods

2.1 Sampling areas

Soil samples were collected from areas under the influence of a coal-fired power plant, located in the state of Rio Grande do Sul, southern Brazil. The four sampling sites (Figure 1) were chosen to clarify the routes of mutagenic compounds present in the coal wastes. They were: a bottom ash deposit that has been undergoing a recovery process for the last three years, but which was used for five decades (deposit); two areas at a level below the deposit (approximately 10 meters below it) which may undergo the influence of leachate from the latter. They are: an area of riparian forest at Jacuí river (riparian forest) and an old agricultural area, used currently

as grazing for cattle (field area), both of these areas are adjacent to the deposit, 20 and 65 meters from it, respectively. Finally, a recreation area for children (recreation area), located at a higher level than the deposit (approximately 20 meters above), without any leaching from the soil, but on the main route of atmospheric dispersion of the particulate materials. As an area of reference (area of reference) samples of soil were collected from a riparian forest on the opposite bank of Jacuí river, before the plant area and also off the main route of atmospheric dispersion (Figure 2).

2.2 Collection of Surface Soil

Soil samples from sampling areas were collected between January and February 2007, while the soil samples of reference were collected in November 2007. Sampling areas were demarcated using GPS and collected at each of 15 random points, at a depth of 20 cm with a spade, and gravel, stones and plant materials were removed. The 15 subsamples were homogenized in order to form a composite sample for each sampling area. About 500 g to 1 kg of surface soil was collected using stainless spatulas, placed in a glass container protected from light, and maintained at 4ºC until samples preparation. This period did not exceed 180 days.

2.3 Sample preparation

In the laboratory, the collected soils were dried at room temperature for up to 2 days, protected from light, mechanically broken up, screened through a 2-mm sieve with stainless material and stored immediately at 4ºC protected from light until extraction.

2.4 Extraction procedures

2.4.1 Acidic Extraction

Soil samples were stirred (115rpm) at 20ºC for 24 h with a solution of 5.7 ml of acetic acid p.a. and 64.3 ml of sodium hydroxide 1.0 M p.a. prepared in 1000 ml of deionized water

(pH 4.93±0.05; soil: solvent, 1:2, g/ml), according to Brazilian standards (NBR 10005) [28] and then centrifuged to 13,000 x g for 15 minutes at 4ºC, filtered (0.45µm), divided into aliquots and stored at 4ºC, in the dark for up to 24 h for mutagenicity assessment. The pH of the extraction solution was 4.85, close to pH of the soil (5.0).

2.4.2 Organic Extraction

Soil samples (10g) were subjected to extraction via ASE (Accelerated Solvent Extraction), based on method 3545 of EPA [29] with the following extraction conditions: dichloromethane as a solvent extractor; 1500 psi system pressure; oven temperature: 100ºC; static time: 5 minutes; flush volume: 60%; bleed time: 60 seconds. Organic extracts were concentrated in rotavapor and stored in the dark for up to 30 days until biological assays were performed.

2.5 Quantification of metals

The quantification of metals in the acid extracts (Mn, Fe, Al, Zn, Cu, Cd, Pb, Cr and Ni) was performed by inductively coupled plasma optical emission spectrometer (ICP-OES), with the following detection limits in mg/l: Cu-0.004; Zn-0.02; Fe-0.04; Mn-0.03; Al-0.08; Cd-0.002; Cr-0.004; Ni-0.002; Pb-0.01. The determinations of the metals were performed in the Soil Laboratory, Universidade Federal do Rio Grande do Sul.

2.6 Qualitative analysis of organic compounds

The organic extracts were reduced to 1 ml for further injection using gas chromatograph with a mass spectrometry detector coupled to the gas chromatographer (GC/MS Shimadzu QP5050A) in Scan mode. The chromatography conditions were according to Nascimento Filho et al [30], with alterations in the injection mode (1/50 to 1/10). The organic compounds were

analyzed in the Environmental Chemistry Laboratory, at the Chemistry Institute, Universidade Federal do Rio Grande do Sul.

2.7 Mutagenicity assay

The mutagenicity assay used in the current study was the *Salmonella*/microsome assay in the microsuspension procedure [31], with strains TA97a and TA98, which detect frameshift mutagens, where the last is more sensitive to heavy metal mutagens [32]; TA100 strain, which detects base-pair substitution mutagens [33]; strains YG1041 (derived from TA98) YG1042 (derived from TA100). These later strains have elevated levels of nitroreductase and O-acetyltransferase activity and they are extremely sensitive to the mutagenicity of both nitroarenes and/or aromatic amines [34]. The assays with the TA strains were performed in the absence and presence of a S9 mammalian microsomal fraction activated by the polychlorinated biphenyl mixture, Aroclor 1254 (purchased in a liophilized form from Moltox, USA), with added cofactors (S9 mix), while tests with YG strains were performed in the absence of S9 mix.

For each sample, acidic and organic extracts were analyzed to investigate mutagenesis for heavy metals and organic compounds, respectively, at six concentrations: 25, 50, 75, 100, 150 and 200 mg equivalent of the soil acidic extracts and 10, 20, 40, 80, 120 and 160 mg equivalent of the soil organic extracts. All concentrations were performed in duplicate and negative (100 µl of nutrient broth and solvent; 100 µl of acid solution or 5µl of dimethylsulfoxide) and positive (sodium azide - SAZ, CASRN. 26628-22-8, Merck do Brasil; 4-nitroquinoline oxide - 4NQO, CASRN. 56-57-5; 2-nitrofluorene – 2NF, CASRN. 607-57-8 and 2-aminofluorene - 2AF, CASRN. 153-78-6 from Sigma Chemical Company, St. Louis, MO) controls were added in the tests, according to the strain and treatment used. The overnight cultures (37ºC) were concentrated ten times, incubated with the samples at different concentrations with adding of 0.1 M phosphate buffer (in the absence of S9mix) or S9 mix (in the presence of S9 fraction) for 90 minutes at

37ºC. The content of each tube was agitated with top agar containing traces of histidine and biotin and then spread out in a Petri plate containing 30 ml of minimal agar. The plates were then incubated at 37°C for 72h and the number of revertant colonies per plate was then determined.

In parallel to the mutagenicity assay, the cell survival test was performed, spreading suspension (bacterial culture + S9 mix or 0.1 M phosphate buffer + solvent or extract) diluted to a concentration of 1-2x10 ² cells in enriched medium (Nutrient Agar), for 72h at 37°C. The samples were considered cytotoxic when at one dosage at least cell survival was equal or smaller than 60% of the negative control with the solvent used.

2.8 Analysis of the mutagenicity assay results

Data for mutagenic activities were calculated from the analysis of the linear portion of the dose-response curve and expressed as the value of the slope (number of revertants/g soil equivalent), as described by Bernstein et al. [35]. This slope is an estimate of mutagenic potency of each soil sample. Statistical analyses were run with Salanal Software (Salmonella Assay Analysis) version 1.0 of Research Triangle Institute, RTP, North Caroline, USA.

3. Results

Results of mutagenic activity (number of revertants/g soil equivalent) in the acidic and organic extracts from soil samples under the influence of coal wastes are summarized in Tables 1 and 2. In general, the mutagenic potential in acidic extracts (Table 1) was lower that the responses in organic extracts (Table 2) and the mutagenic potential in the presence of S9 fraction were higher in most extracts.

Frameshift mutations were observed in acidic extracts of whole areas under the influence of coal, using strain TA98 with S9 fraction. In the absence of S9 mix, only the riparian forest soil showed mutagenic activity. Strain TA97a showed higher contribution of mutagenic compounds

in soil samples from riparian forests, and still lower contributions in deposit samples. Extracts of the deposit and the field area showed cytotoxicity associated with frameshift mutagenesis induction, in the presence of S9 mix. The base-pair substitution mutation was higher in soil samples from the recreation area, located above the level of deposit, in the absence and presence of S9 mix. At this site, despite cytotoxicity, direct action mutagens were detected. Base pair substitution events were also detected in riparian forest, but at a low potency. Acidic extracts from the reference area had only negative mutagenic responses, for all strains in the absence and presence of S9mix (Table 1).

The results of organic extracts were predominantly positive. All samples showed mutagenic induction and presented higher mutagenic activity with the presence of S9 mix. The riparian forest presented high values for TA98, while the deposit and field area presented high values for TA100 and TA97a, respectively. On the other hand, there was cytotoxic activity in field and recreation areas and also in the deposit, but only in the absence of S9 mix. In the reference area, cytotoxicity was present under both assay conditions (Table 2).

Comparison between TA98 and TA100 strains and their derived strains YG1041 and YG1042, respectively, allowed the evaluation of the contribution of nitrocompounds like nitroarenes and aromatic amines in the mutagenic activity from environmental samples. The mutagenic potential, expressed as revertants per gram soil equivalent, in the YG1041 strain were higher than the parental strain (TA98 in absence of S9 mix), in all sampling areas, except the reference area, and the ratio between YG1041 and TA98 ranged from 1.7 (recreation area) to 9.9 times (deposit) (Figure 3). In the YG1042 strain, the increase of the mutagenic potential was found only in samples of the deposit and the field area, reaching increases of 2.1 and 1.9 times, respectively, compared with results in TA100 strain, in the absence of S9 mix (Figure 4).

Figures 5 and 6 summarize the mutagenicity of compounds as base-pair substitution (TA100) and frameshift mutagens (TA98 and TA97a), where the mutagenic potency was added

up using all strains (TA98, TA97a and TA100), in the absence and presence of S9 mix, in both types of extraction (acidic and organic). The sampling areas could be listed in descending order of mutagenicity as follows: Recreation area > Deposit > Field area > Riparian forest > Reference area (base-pair substitution mutagens) and Riparian forest > Field area > Recreation area > Deposit > Reference area (frameshift mutagens).

Table 3 shows the organic compounds identified by the GC/MS in the extracts from soil samples. The results indicate a strong contribution from Total Petroleum Hydrocarbons (THPs) in the extracts of the areas under the influence of the coal-fired power plant, besides the presence of phtalates, compounds that are strongly associated with human occupation. In the extracts from the deposit and the field area an aromatic amine was also identified, while in the extracts from the riparian forest and also the reference area, different carboxylic acids were identified, tipical of forested areas. Additionally, in the reference area a compound of the amide functional group was identified.

The determination of the metals in the acidic soil extracts is presented in Table 4. The results show great variation in the concentration of some metals between the sampling areas, the main difference being between the site of reference and the deposit where the manganese concentration is more than 100 times higher than in the former. The highest concentration of each metal was always detected in 3 of the sampling areas: riparian forest (Cr and Cd), recreation area (Fe, Al, Cr and Pb) and reference area (Zn, Mn, Cr, Cu and Ni). Nevertheless, no Fe was detected in the field and reference area, Cr in the field area and Pb in the deposit and reference site. It should be emphasized that some metals such as Mn, Fe, Al and Zn are larger soil constituents and are commonly present in high concentrations.

4. Discussion

Soil is an environmental compartment with intense chemical and biological activity. Since it is at the interface between the atmosphere, biosphere and hydrosphere, it acts as a final receptacle of most of the planet wastes [36, 37]. Although this compartment is highly affected by contamination with genotoxic substances, few studies have been performed to investigate the mutagenic potential of soils that are contaminated, or where contamination is suspected, using short-term mutagenicity bioassays. Studies focusing on this context are relevant to include well-established assays in environmental assessments and biomonitoring [27].

The presence of complex environmental mixtures in contaminated soils may determine genotoxicity, including mixtures such as coal-burning and coal-fired power plant wastes. The state of Rio Grande do Sul, in southern Brazil, has approximately 90% of the more than 30 million tons of coal reserves in the country, and the area where the power plant in this study is located has approximately 20% of these reserves [38]. The plant investigated has operated since 1953 and its capacity is 20MW. Until 2002 the fly ash and bottom ashes were deposited in an area on site, without taking any special steps for waste disposal. In 2002, a project began to be implemented to remediate the bottom ash deposit and a broad study was started to diagnose and monitor the area, besides placing the newly-burnt ashes in the trenches of the mines from which coal was extracted.

Coal combustion wastes from power plants have shown genotoxic effects in many assays [39-42]. This study investigates the action of coal wastes in soil emphasizing their environmental routes, using different strains in *Salmonella*/microsome assays in association with chemical analysis. A broad genotoxicity spectrum of genotoxicity results in soil samples has been reported in the literature, both in terms of negative genotoxicity [43,44] and positive genotoxicity [45-47]. These results indicate the variety of responses of this type of complex environmental matrix.

The review by White and Claxton [27] presents mean mutagenicity values for organic soil extracts observed in the *Salmonella*/microsome assay for the TA98 and TA100 strains

(Table 5). For purposes of comparison, in this study, although the sampling sites are under the influence of wastes from the industrial area, the mean values were selected according to the predominant soil use at each sampling site studied. The samples from riparian forest, field and recreation areas were compared to the means for rural soils presented in the review, and the deposit was compared to the means of industrial soils. Mutagenicity values higher than the geometric means presented for the rural areas were found in the samples from the riparian forest and field area, except for the results for TA100 in the absence of metabolic activation in the riparian forest and recreation area. The soil samples from the deposit presented values lower than the means for industrial soils, except from the TA100 strain in the absence of metabolic activation.

The values presented in review by White and Claxton [27] can be taken as world references for mean values observed in different types of land occupation, and they are therefore a useful tool to investigate environmental quality. However, for a better comparison of the results obtained, a soil was used which had been collected in a region close to the area of study, with a similar typology, but located both outside the main plume of dispersion of atmospheric particles and of the release of soil from the area outside the power plant. Moreover, the mean values presented in the review by White and Claxton [27] refer to mutagenicity in organic extracts, and do not take into account responses in acidic extracts. Another limitation of the means used as an international reference in this review is that only strains TA98 and TA100 were utilized. As an example of this limitation we must observe the results of the recreation area, where all means for TA98 and TA100 remained below the values of reference, while the mutagenicity detected by strain TA97a in the presence of S9 mix was considerably higher.

In the organic extracts of soils under the influence of coal wastes, the mutagenesis values were higher than the results found in the reference soil in all strains except TA98, in the presence of S9 mix, in which only the mutagenic activity of riparian forest extract was higher than the

values of reference area (Table 2). In the acidic extracts, all the mutagenic responses in the reference soil were negative. This high number of negative responses in the reference soils allows it to be considered the natural background for the area.

As to the sensitivity of the *Salmonella typhimurium* strains used in the study, the mutagenic indexes per plate observed for the TA98 strain were higher than the other strains used (data not shown). This strain is reported in the literature as being more sensitive to soil mutagens compared with the other TAs strains [15,26,48-50]. However, higher values of mutagenic potential in number of revertants per gram of dry soil were observed for the TA97a and TA100 strains. This high mutagenic potential at some of the study sites must be related to the presence of base-pair substitution compounds [33] and to the sensitivity of TA97a for heavy metals that cause frameshift mutation [32].

Differentiated responses were detected in the different strains depending on the extraction procedure used. The use of more than one type of solvent helped the environmental diagnosis of the area, since it allowed the identification of compounds of a different nature, complementing the analysis of genotoxicity. An organic extract was prepared from the soil samples, using as solvent dichlorometane, which is efficient at extracting moderately polar compounds with a mutagenic potential [15,50] and also an acidic extract which is more effective than the aqueous extracts commonly used in studies of this kind [17].

The sum total of the mutagenic activity in the two types of extraction observed in strains that detect frameshift (TA97a and TA98) and base pair substitution (TA100) mutations was used for a better diagnosis of the mutagenic potential at the areas of study. The points were chosen to identify possible routes of the mutagenic compounds: leaching from the deposit to areas below, such as riparian forest and field area, or the atmospheric dispersion of compounds released by the plant smokestacks to the recreation area, a site of preferential dispersion of the particulate material.

The compounds that cause different damages to the DNA clearly took different environmental routes (Figures 5 and 6). Mutagens that cause base pair substitution were preferentially dispersed in the atmosphere, since the recreation area had a higher mutagenic potential with a different mutagenicity profile from the other sampling sites including the deposit. The influence of atmospheric dispersion on mutagenic compound contamination in soil samples has already been described by Edenharder et al. [26] and Wesp et al. [50]. The frameshift mutagens appear to have a preferential dispersion through leaching in the soil or runoff, and the areas located below the plant presented the highest mutagenic potential which was even higher than the deposit. Leaching in the soil and in solid wastes and run-off are major genotoxic contamination routes which may affect human health and the environment, due to the contamination of groundwater, which is an important source of drinking water [44,52-54].

The diagnosis of the presence of nitroarenes and aromatic amines (Figures 3 and 4) based on the study with the specific strains YG1041 and YG1042, showed similarities in the profile of mutagenic responses between deposit and field area, but always with values higher than the former. The increment in the mutagenic potential between parental strains and those derived at these two points was higher than at the other study sites. This high sensitivity in the detection of nitroderivates observed in the mutagenic assays corroborates the results of the chemical assays, where the presence of nitroderivates of the aromatic amine type was identified at these two study sites (Table 3).

This stage of integration of data obtained from studies on mutagenesis and chemical tests is important to trace the genotoxicity profile of the mixture of contaminants, compared with the probable and main groups of substances present in the area. A few studies were performed to investigate the mutagenicity of pure chemicals such as heavy metals [54] and also isolated organic compounds using techniques for fractionation into complex environmental matrices

[48,55,56]. Other studies attempted to associate the results of mutagenesis with the profile of chemical compounds identified in environmental samples [18,45,46].

Since the availability of the metals is closely associated with acid values of pH, acid extraction allows the evaluation of biological effects (including mutagenesis) of these compounds. In this study, although mutagenicity is associated with the whole environmental mixture (in the case of inorganic extracts, metals and other low molecular weight substances), direct relations between some metals and mutagenesis can be taken into account. It should be stressed that the pH value of the acidic extracts was similar to the soil samples (pH 4.93 and pH 5.0, respectively).

In order to identify the metals of greatest interest in the study, we consider that the concentrations of these elements at the reference area expressed only negative mutagenesis results, and therefore we may assume that metal concentrations below those established at the site of reference would not cause mutagenic events in any strain. Based on this approach, the most relevant metals for mutagenic activity detected in soils under the influence of coal wastes were iron, aluminum, lead and cadmium (Table 4). Although manganese appears at a high concentration at the reference area, it does not have any relationship with mutagenic activity detected by the *Salmonella*/microsome assay [57].

Iron, due to is high concentration in the recreation area compared to the other areas, in association with lead may have been responsible for the high mutagenic potential reached with the TA100 strain in the absence of S9 mix. Mutagenic activity of lead salts, in the absence of S9 mix, has already been reported in previous studies, but, however there are studies that affirm the potential of iron to cause mutagenic induction in the presence of metabolic activation, results in the absence of a metabolization fraction, like ours results, are inconclusive in the literature [58,59]. Aluminum, which presented higher concentrations at sites under the influence of wastes, may have been the main agent of mutagenic action detected by TA98 in the presence of S9 mix. Furthermore, its high concentrations in riparian forest and in the recreation area may have

contributed to the mutagenic potential found in TA100 in the presence of S9 mix. Studies that affirm the mutagenic potential of aluminum considering TA100 in the presence of S9 mix has already been described previously [60]. Similarly, the high concentration of cadmium in the riparian forest and deposit, compared to the other areas, may have influenced the frameshift mutation in the TA97a in presence of the S9 mix, however, in the literature, only mutagenic results in the absence of S9 mix are showed to this metal [59,61].

In the case of organic extracts, the presence of base pair substitution mutagens appears to be related to the amount of aliphatic hydrocarbons at each sampling site, since phthalates detected in the soil samples are not mutagenic for TA100 [62]. The deposit and field area, which present a higher number of hydrocarbons (9) showed higher mutagenesis potencies, while the riparian forest and the recreation area have a small number of these compounds (4) and less mutagenic activity in TA100. At the site of reference, with negative mutagenesis, no presence of aliphatic hydrocarbons is known. The compounds present only in the deposit and field area are eicosane, heneicosane, n-tetradecane, besides bis-(octyl-phenyl)amine. Although the present results suggest that these compounds may be related to the high rate of mutation of the TA100 strain, previous studies emphasize negative results in assays using eicosane, bis-(octyl-phenyl)amine [63] and n-tetradecane [64] for strain TA100, in the presence and absence of S9 mix.

The association between chemical data and frameshift mutation becomes more complex, both because of the greater number of strains and because of the number of compounds involved. Moreover, the analyses to identify organic compounds that are of interest in the area are qualitative and do not define their concentration in the extract. However, since most of the frameshift mutation results were positive, it is suggested that several compounds present in the soil extracts are associated with this type of mutation.

On the other hand, the absence of mutagenic activity in the extracts of the recreation area using strain TA97a in the absence of S9 mix, indicates that compounds that are not present in this extract (but are present in the other samples) cause frameshift mutation in TA97a, namely: n-nonadecane; heneicosane; docosane; 2-pentadecanone, 6,10,14–trimethyl; n-tetradecane and hexadecanoic acid. Compounds such a bis-(octyl-phenyl)amine and eicosane were previously studied to detect mutagenicity with strain TA97a and presented negative results [63].

It should be mentioned that the organic or inorganic extracts used are a complex mixture, although simpler than the original one. However it is useful to point out the presence of positive mutagenic responses in leached extracts, using the *Salmonella*/microsome assay, since positive results for inorganic extracts are not usual in the literature [15,49,53].

The prevalence of positive mutagenic responses in soils under the influence of coal characterizes the environment that is being studied as highly susceptible to the entry of contaminants from the industrial area investigated, and especially the stage of the remediation process of the ash deposit studied. This focus on work associating high sensitivity for certain classes of compounds and specifically oriented chemical dosages is effective to define measures following up the area recovery. It allows predicting the dispersion behavior of each contaminant connected to knowledge regarding the potential biological damage to organisms, and ecological damage to the environment. The work strategy discussed helped define the dispersion routes of the compounds to the area of influence, and the hazard of changes to the fauna and flora of the adjacent ecosystems, besides problems related to human exposure, since coal-fired power plants are usually located in highly populated centers.

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Table 1. Mutagenicity and cytotoxicity of acidic extracts from surface soil under the influence of coal wastes

Área	TA98		TA97a		TA100		Cytotoxicity	
	-S9 mix	+S9 mix	-S9 mix	+S9 mix	-S9 mix	+S9 mix	-S9 mix	+S9 mix
Reference area	0[1]	0	0	0	0	0	-[2]	-
Deposit	0	350[1]	0	494	0	0	+[2]	+
Riparian forest	166	152	802	2672	0	280	-	-
Field area	0	222	0	0	0	0	-	+
Recreation area	0	374	0	0	1558	1000	+	-

[1] number of revertants/g soil equivalent; 0, non significant response

[2] – non cytotoxic,+ cytotoxic samples - in the presence of at least one dosage, cell survival is less than 60% of the negative control with the solvent used.

Negative control (rev/plate ± standard deviation) (100µl acid solution/plate) –S9mix: 42.1±20.03 (TA98), 178.67±33.76 (TA97a), 223.5±13.43 (TA100); +S9mix: 66.62±10.04 (TA98), 172.75±27.16 (TA97a), 216.17±42,38 (TA100); Positive control –S9mix: 4NQO (0.5µg/plate); 255.37±93.70 (TA98), 841±148.53 (TA97a); AZS (5µg/plate) 985.08±309.84 (TA100); +S9mix: 2AF (10µg/plate) 543±162.77 (TA98), 515.5±0.71 (TA97a) and 1224±407.29 (TA100).

Table 2. Mutagenicity and cytotoxicity of organic extracts from surface soil under the influence of coal wastes

Area	TA98		YG1041	TA100		YG1042	TA97a		Cytotoxicity	
	-S9 mix	+S9 mix	-S9 mix	-S9 mix	+S9 mix	-S9 mix	-S9 mix	+S9 mix	-S9 mix	+S9 mix
Reference area	0[1]	562[1]	0	0	0	0	0	0	+[2]	+
Deposit	233	291	2316	967	1448	2023	379	427	+	-[2]
Riparian forest	241	788	499	0	349	0	384	551	-	-
Field area	134	159	884	421	465	793	956	3238	+	-
Recreation area	329	170	548	66	247	0	0	1981	+	-

[1] number of revertants/g soil equivalent; 0, non significant response

[2] + cytotoxic, – non cytotoxic samples - in the presence of at least one dosage, cell survival is less than 60% of the negative control with the solvent used. Negative control (rev/plate ± standard deviation (5µl DMSO/plate) –S9mix: 46.5±8.98 (TA98), 63.25±10.75 (YG1041), 168.33±9.29 (TA100), 138.33±33.35 (YG1042), 151.6±22.43 (TA97a); +S9mix: 64.17±9.26 (TA98), 192.5±47.57 (TA100), 148.71±27.53 (TA97a); Positive control –S9mix: 4NQO (0.5µg/plate) 255.37±93.70 (TA98) and 841±148.53 (TA97a); AZS (5µg/plate) 985.08±309.84 (TA100), 2NF (0.15µg/plate) 975.6±201.19 (YG1041) and 599.2±114.43 (YG1042); +S9mix: 2AF (10µg/plate) 543±162.77 (TA98), 515.5±0.71 (TA97a) and 1224±407.29 (TA100).

Table 3. Qualitative analysis of organic compound extracted from surface soil under the influence of coal wastes

Compounds	Reference	Deposit	Riparian forest	Field area	Recreation area
Isobutyl phthalate		x	x	x	x
2-ethyl hexyl phthalate		x		x	x
di-octyl phthalate		x	x	x	x
n-nonadecane (C19)		x	x	x	
Eicosane (C20)		x		x	
C21		x		x	
Docosane (C22)		x	x	x	
Tricosane (C23)		x		x	x
C24		x	x	x	x
Pentacosane (C25)		x	x	x	x
Hexacosane (C26)		x		x	x
2-pentadecanone, 6,10,14 - trimethyl			x		
n-tetradecane		x		x	
4-octen-3-one	x				
Hexadecanoic acid			x		
Propanoic acid	x				
Dimethylformamide	x				
Bis-(octyl-phenyl)amine		x		x	

Table 4.Total metal concentration in soil samples under the influence of coal wastes[1]

	Reference area	Deposit	Riparian forest	Field area	Recreation area
Zinc	0.640	0.180	0.460	0.320	0.600
Iron	ND	0.220	0.440	ND	1.420
Manganese	22.400	0.200	0.780	2.200	0.280
Aluminum	1.360	2.680	5.060	1.920	6.240
Chromium	0.020	0.010	0.020	ND	0.020
Copper	0.138	0.064	0.118	0.080	0.076
Lead	ND	ND	0.020	0.020	0.040
Cadmium	0.008	0.014	0.036	0.006	0.014
Níckel	0.058	0.012	0.056	0.014	0.038

[1] mg/kg soil equivalent. ND – not detected.

Table 5. Geometric mean *Salmonella* mutagenicity values (modified by White and Claxton [27])

Strains	*Use of soil*		
	Rural	**Urban/suburban**	**Industrial**
TA98 without S9 mix	57±6[1]	430±100	770±180
TA98 with S9 mix	60±5	470±50	950±170
TA100 without S9 mix	120±10	260±30	130±130
TA100 with S9 mix	96±10	460±40	3180±1460

[1] number of revertants/g soil equivalent ± standard deviation

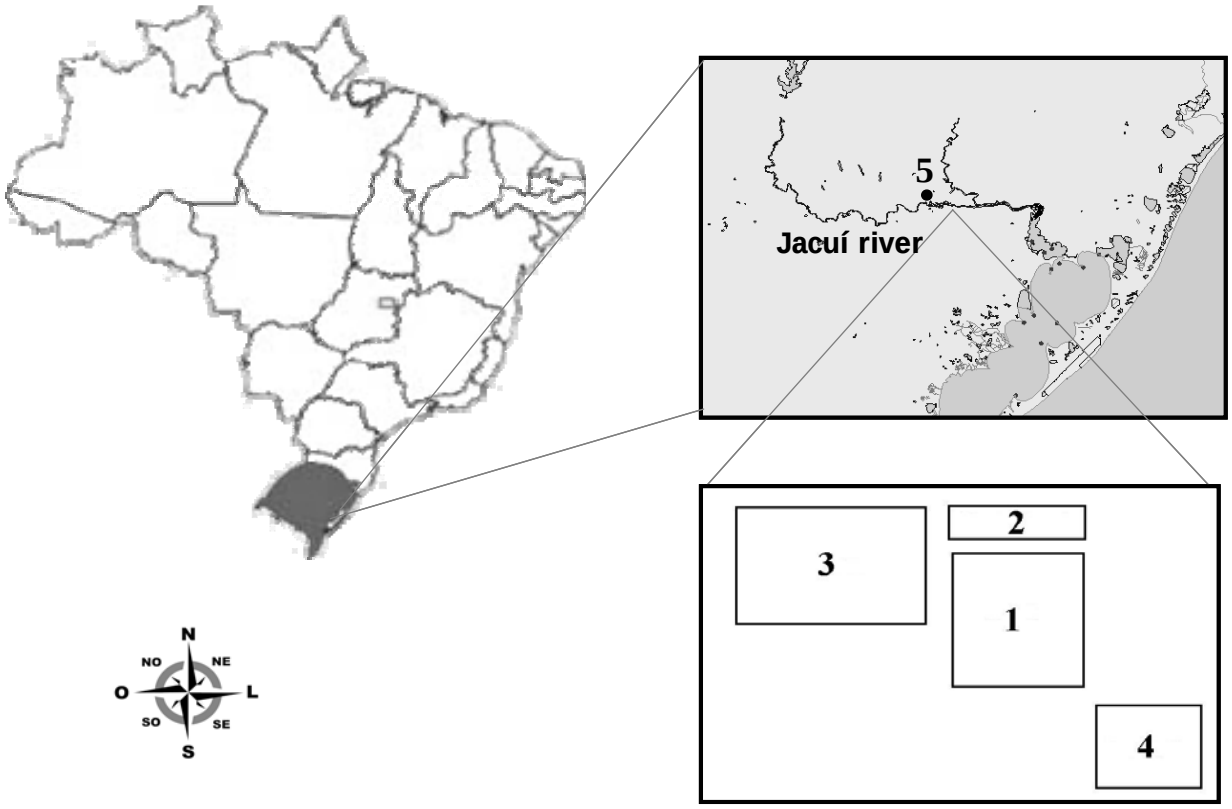

Figure 1. Site of the area of study. Sampling areas: 1 - deposit; 2 – riparian forest and 3 – field area, two areas at a level approximately 10 meters below the deposit; 4 – recreation area, approximately 20 meters above it and 5 – reference area, on the opposite bank of Jacuí river

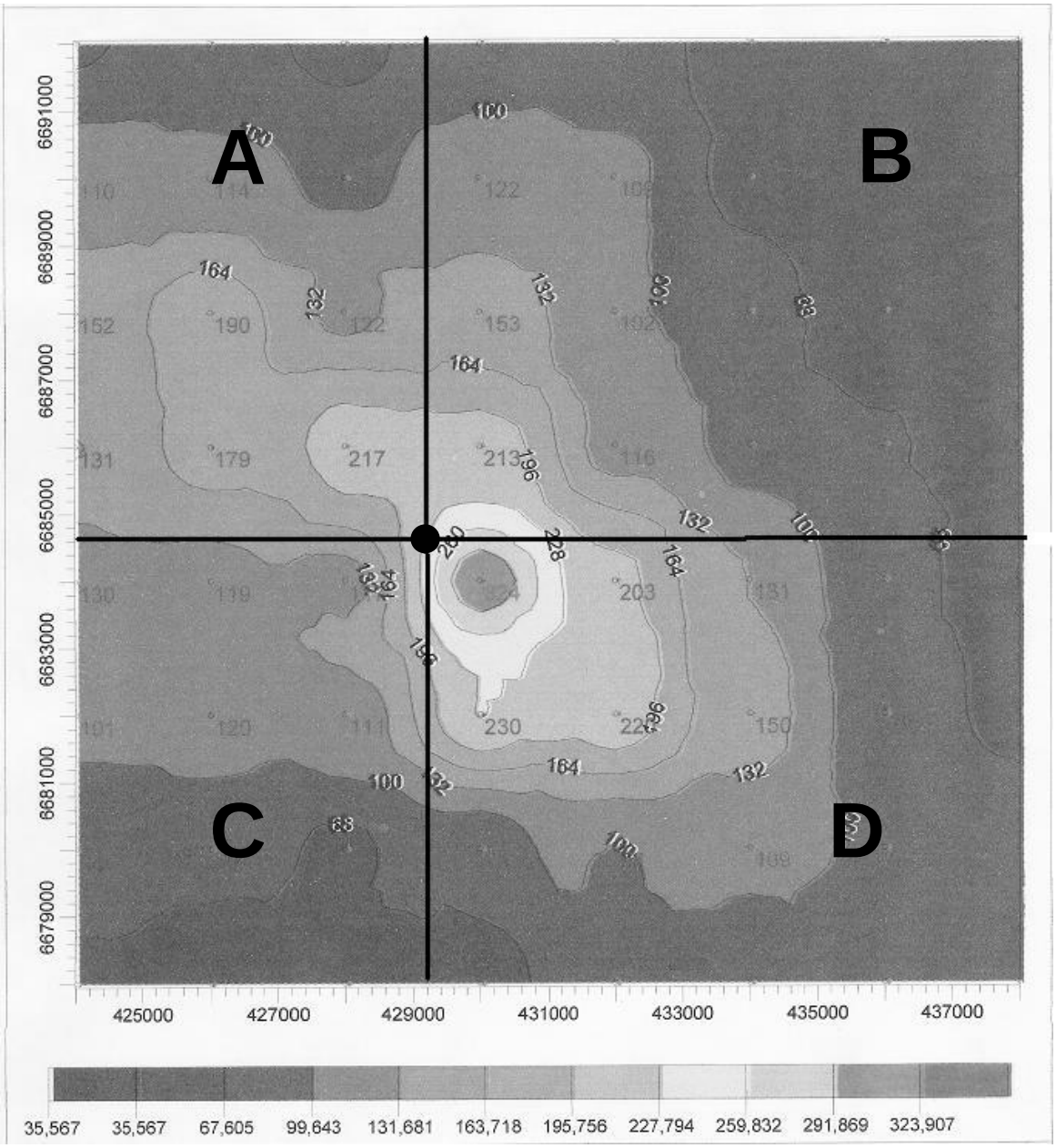

Figure 2. Atmospheric dispersion model of particulate matter in the area of study. Black circle indicate the smokestack of the coal-fired power plant. The sampling areas are distributed in the quadrants, such as: deposit, riparian forest and field area at the Quadrant A; recreation area at the Quadrant D. The major area of dispersion of particulate materials is the Quadrant D.

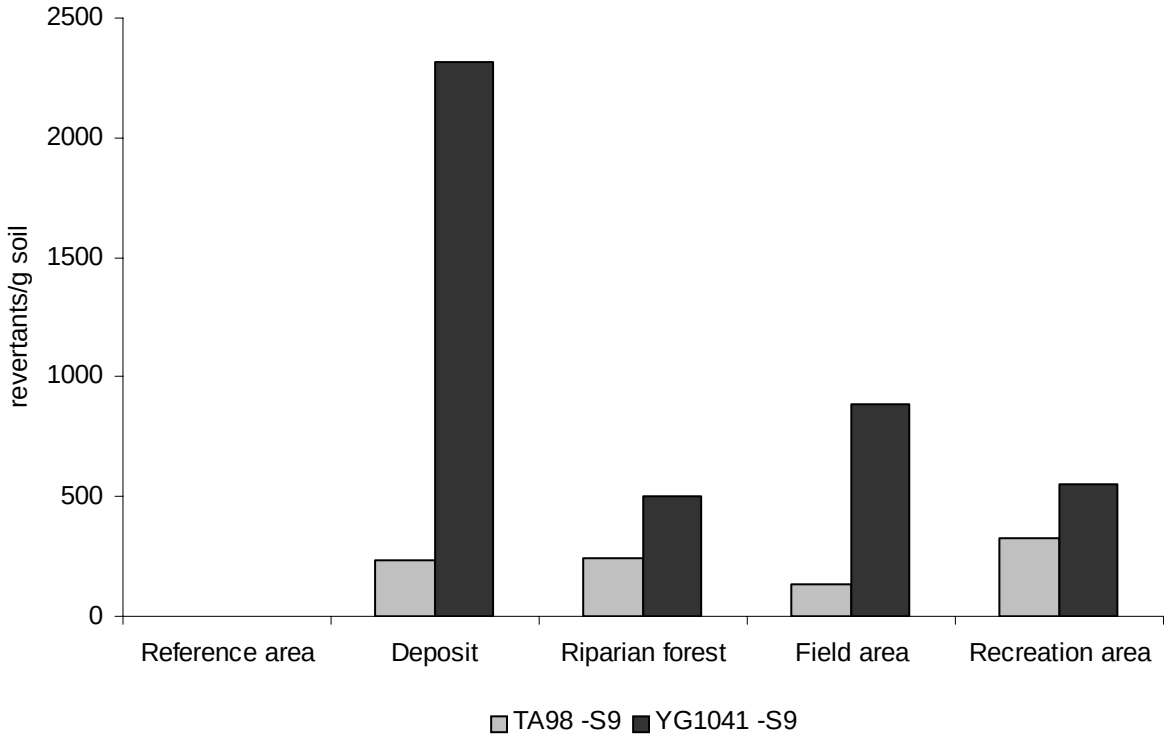

Figure 3. Mutagenic potency in revertants per gram of soil equivalent to TA98 and YG1041 strains in the organic extracts from the investigated soils. -S9 refers to assays in absence of S9 mix.

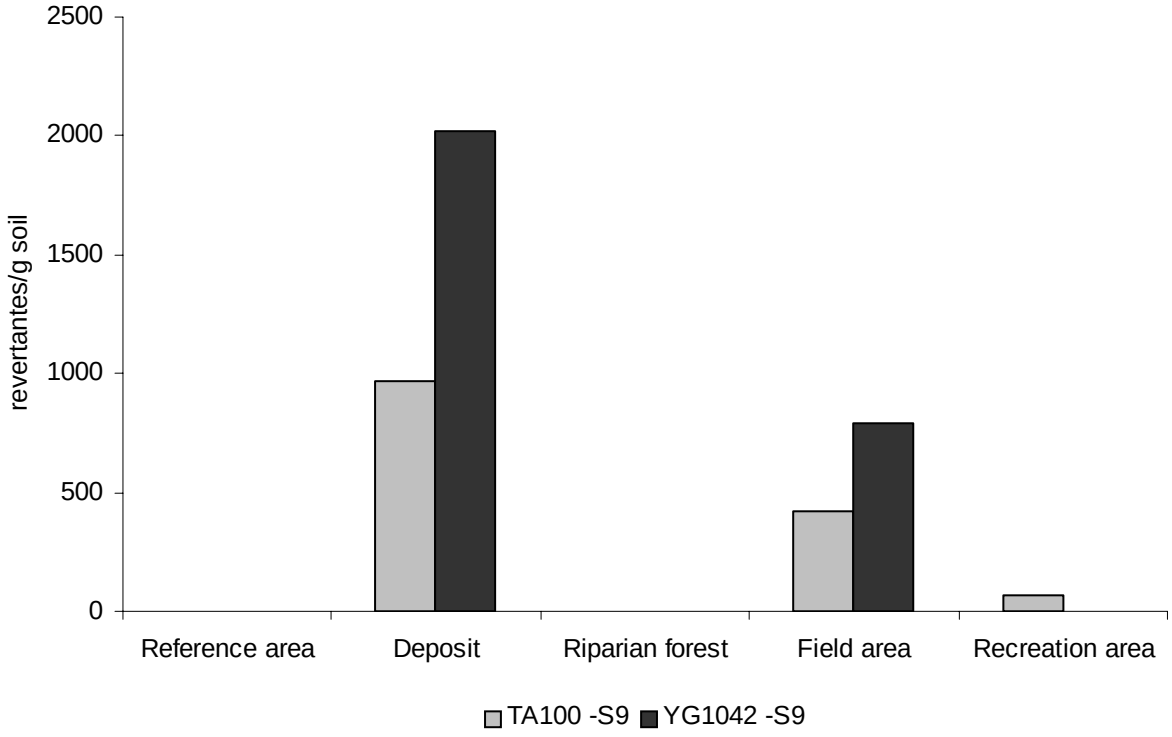

Figure 4. Mutagenic potency in revertants per gram of soil equivalent to TA100 and YG1042 strains in the organic extracts from the investigated soils. -S9 refers to assays in absence of S9 mix.

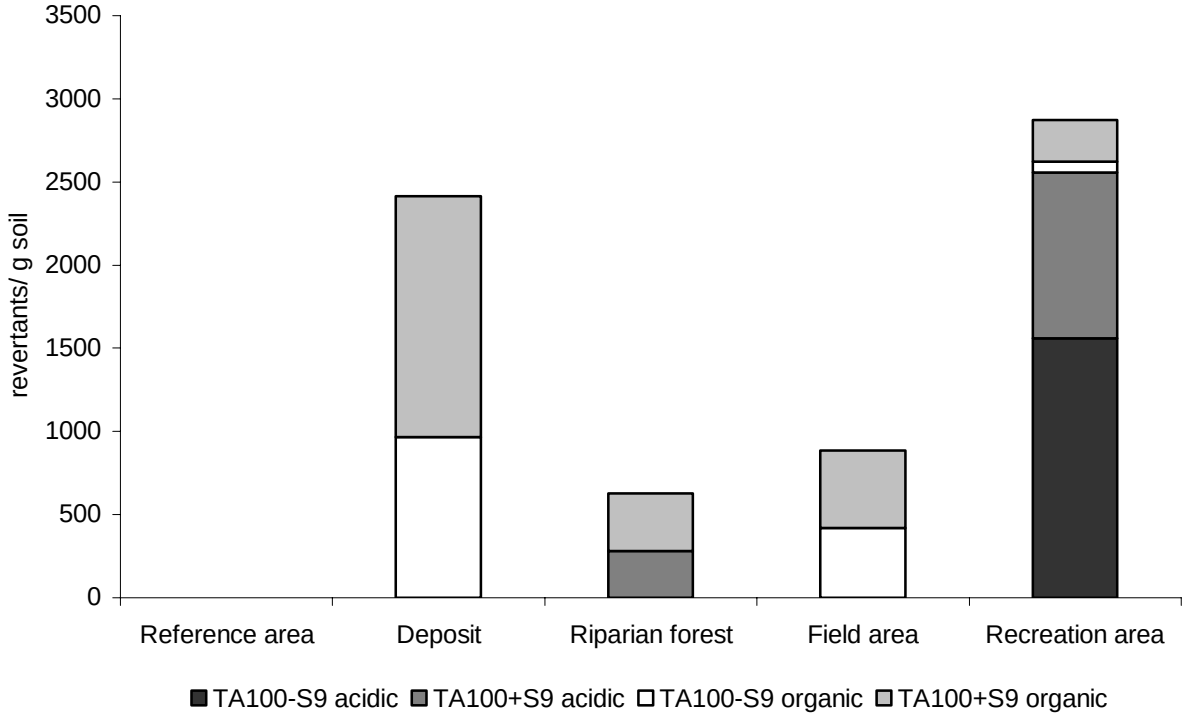

Figure 5. Mutagenic potency in revertants per gram of soil equivalent to total sum of the strains that detect base-pair substitution mutagens (TA100) in absence and presence of S9 mix in both types of extraction (acidic and organic)

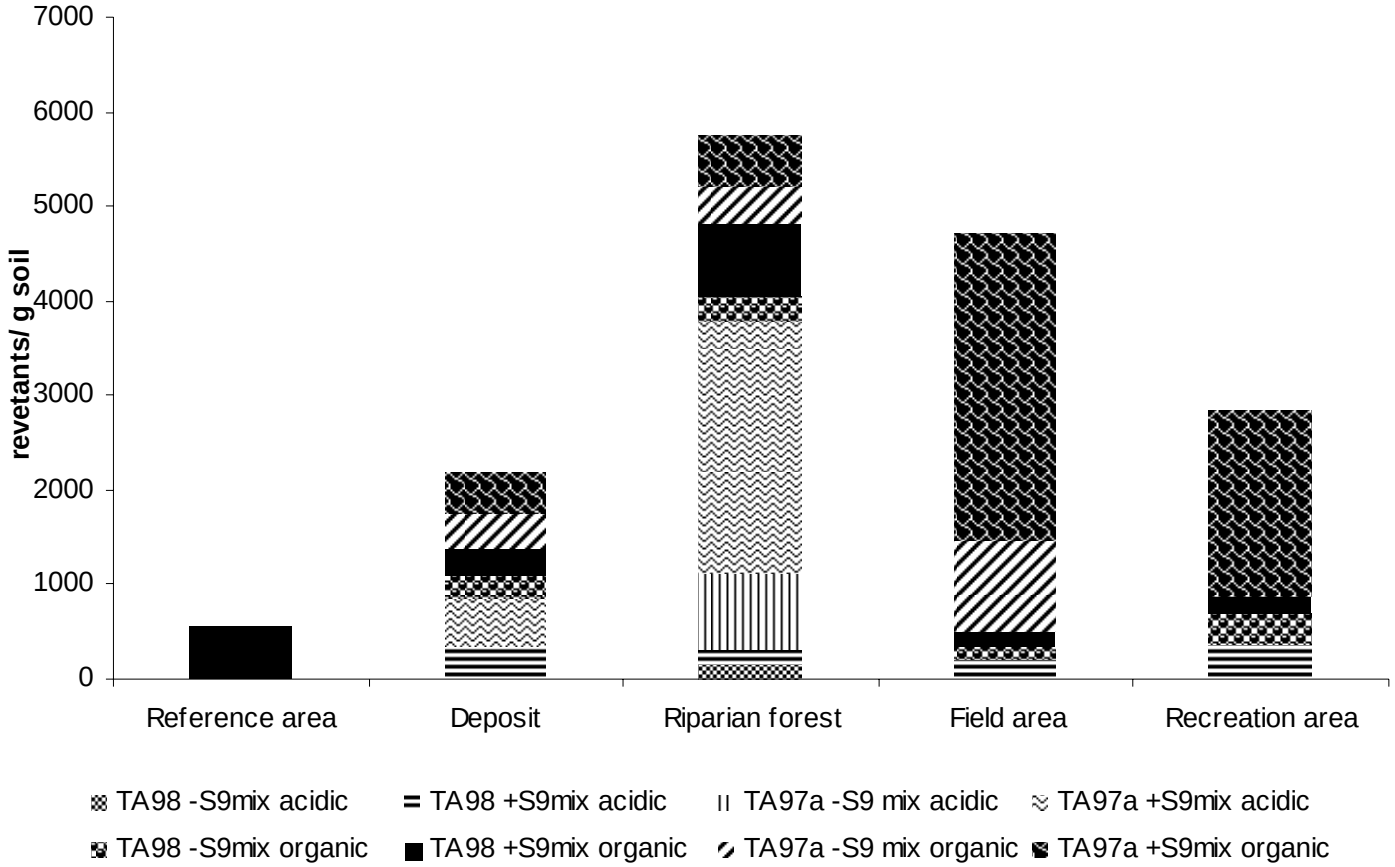

Figure 6. Mutagenic potency in revertants per gram of soil equivalent to total sum of the strains that detect frameshift mutagens (TA98 and TA97a) in absence and presence of S9 mix in both types of extraction (acidic and organic).

4. Considerações finais

Este trabalho foi realizado com uma parceria entre o Programa de Pós-Graduação em Ecologia da Universidade Federal do Rio Grande do Sul e a Fundação Estadual de Proteção Ambiental Henrique Luís Roessler (FEPAM-RS). Está inserido em duas problemáticas de grande importância em estudos ecológicos: o efeito biológico dos contaminantes presentes em depósitos de carvão associado à identificação de substâncias com potencial genotóxico em amostras de solo. Estes dois aspectos foram, até hoje, pouco explorados nas pesquisas científicas, tanto em nível de abrangência mundial quanto no Brasil.

A utilização do carvão mineral fóssil como fonte de energia no Sul do Brasil em grande escala somado ao alto potencial poluidor dessa matriz energética ressaltam a importância de estudos visando o diagnóstico, o monitoramento e a fiscalização de sua utilização, além da disposição final de seus resíduos, por meio de agências de controle ambiental. Algumas dessas agências, como é o caso da FEPAM-RS e da CETESB-SP (Companhia de Tecnologia de Saneamento Ambiental de São Paulo), já utilizam ensaios para detectar a ação de substâncias mutagênicas em amostras ambientais.

Com relação aos estudos de mutagenicidade em amostras de solo, existe um número bastante reduzido de pesquisas quando se comparam aos números de trabalhos envolvendo os demais compartimentos ambientais (águas superficiais, atmosfera, sedimentos). Na FEPAM, até o momento, não tinham sido propostos estudos utilizando solos contaminados com alvo na investigação de substâncias mutagênicas. Essa nova abordagem permitiu, então, a elaboração de um protocolo básico para análise de extratos de compostos orgânicos e inorgânicos de amostras de solos.

A comparação de alguns parâmetros dos processos de extração de compostos para análise de amostras de solo permitiu identificar as melhores condições dentre as

testadas. No caso de extratos orgânicos, utilizando o solvente diclorometano, que extrai a fração moderadamente polar dos compostos, a extração acelerada com solvente (ASE) foi mais eficiente que o processo de sonicação. O processo ASE permitiu a separação de um maior número de compostos de diferentes grupos funcionais, incluindo ésteres (ftalatos), aminas aromáticas, ácidos carboxílicos e hidrocarbonetos alifáticos. Cabe ressaltar que compostos desse último grupo são marcadores da presença de resíduos de combustíveis fósseis.

Para extratos inorgânicos, em que os estudos são mais raros, foram avaliados além de processos de extração (sonicação ou agitação mecânica), o solvente mais eficiente (água ou solução ácida) e o procedimento do ensaio de mutagênese mais adequado (pré-incubação ou microssuspensão). A análise dos resultados evidenciou que a agitação mecânica utilizando solução ácida para o preparo do extrato, associada ao método de microssuspensão se constituiu em um protocolo eficiente para detecção de atividade mutagênica nas amostras de solo testadas. Essas condições foram sugeridas para estudos de solos potencialmente contaminados por substâncias genotóxicas através do ensaio *Salmonella*/microssoma.

A área selecionada para o presente estudo está localizada em uma unidade termelétrica de baixo potencial energético com solo potencialmente contaminado por resíduos gerados pela queima do carvão e disposição de suas cinzas. Os locais de amostragem buscaram avaliar os potenciais impactos residuais no solo, partindo de depósito de cinzas pesadas em processo de recuperação. Foram investigadas as rotas de dispersão potencial dos poluentes, por lixiviação e escoamento superficial ou por dispersão atmosférica. Com estes objetivos, as amostras foram retiradas de áreas localizadas em nível inferior (mata ciliar do rio Jacuí e campo úmido pastejado) e superior em relação ao depósito (área de recreação infantil), respectivamente.

A avaliação da mutagenicidade em associação com análises químicas nessas amostras de solo revelou a contribuição de resíduos de carvão na contaminação de solos próximos. O depósito de cinzas em recuperação apresentou um grande número de compostos orgânicos oriundos da queima incompleta do carvão. Muitas dessas substâncias foram também encontradas nas áreas imediatamente abaixo, confirmando o transporte desses contaminantes até áreas adjacentes por lixiviação e escoamento superficial. A área de recreação localizada acima do nível do depósito teve, por sua vez, a presença de orgânicos típicos de resíduos de carvão, sendo esses provenientes da dispersão atmosférica.

As respostas para mutagenicidade dos extratos inorgânicos parecem estar relacionadas com os resultados da quantificação dos metais, onde áreas adjacentes como mata ciliar e área de recreação apresentam maior potencial mutagênico e maior concentração de metais que o próprio depósito. A elevada concentração desses elementos pode estar associada à deposição dos resíduos de forma constante e por longo período de tempo, somada à concentração natural dos metais nesses locais. Os valores de pH encontrados nas amostras de solo foram similares ao pH dos extratos testados. A realização do procedimento de extração ácida aumenta a relevância ecológica das análises, uma vez que a extração inorgânica mimetizou condições normais de lixiviação do solo.

A utilização de linhagens específicas para estudar a contribuição de nitrocompostos (YG1041 e YG1042) na atividade mutagênica comprovou os resultados das análises químicas. Em locais como o depósito e o campo, onde foram identificados nitrocompostos, como a bis-(octil-fenil)amina, a mutagenicidade observada nessas linhagens foi acentuada, quando comparada aos resultados de suas respectivas linhagens parentais.

O uso de ensaios sensíveis como o *Salmonella*/microssoma permitiu avaliar a presença, persistência e dispersão de substâncias que podem causar danos à saúde humana e ao meio ambiente ainda de maneira precoce. O diagnóstico e monitoramento da área em andamento como solicitado pela FEPAM (FEPAM, 2005), visa acompanhar o processo de recuperação desse sítio contaminado a partir do depósito de cinzas. Os estudos já relatados nesse processo institucional evidenciaram alterações em alguns parâmetros de qualidade das águas subterrâneas, incluindo a presença de efeitos tóxicos agudos e crônicos durante algumas amostragens. Ainda relata alterações na qualidade das águas superficiais e dos sedimentos no rio Jacuí a partir de dados químicos. As informações disponíveis desse sítio em recuperação justificam a busca de um biomarcador sensível para amostras de solo e que permita definir a presença de substâncias biodisponíveis em dosagens precoces.

O ensaio *Salmonella*/microssoma permitiu detectar efeitos mutagênicos nessa matriz complexa. As estratégias empregadas de partição da amostra, caracterização química associada e linhagens específicas para investigar presença de efeitos moleculares diversos permitiram definir rotas de dispersão dos contaminantes e o acompanhamento preventivo de possíveis impactos ao ambiente e à saúde humana. Sob essas perspectivas, esses biomarcadores despontam como ferramentas úteis em estudos ambientais para verificar efeitos dos poluentes em sistemas biológicos, possibilitando o avanço da Ecologia como ciência a partir de abordagens moleculares.

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6. Apêndice

6.1 Avaliação de áreas de influência de uma termelétrica a carvão através de ensaio de genotoxicidade

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Resumo

Cinzas provenientes da queima de carvão podem apresentar em sua constituição diversas substâncias com potencial genotóxico, incluindo compostos orgânicos e metais pesados. Estas cinzas são lançadas no ambiente por usinas termelétricas e podem contaminar suas áreas adjacentes. No Brasil, não existem trabalhos que avaliem os impactos ambientais em área sob influência de usinas termelétricas a carvão utilizando o ensaio *Salmonella*/microssoma. Desta forma, o objetivo desta pesquisa é avaliar o potencial genotóxico de amostras de solo adjacentes a uma usina termelétrica a carvão e de um depósito de cinzas de carvão em processo de recuperação através deste bioensaio. Amostras de solos superficiais serão coletadas em áreas sob influência de uma usina termelétrica a carvão no estado do Rio Grande do Sul e então serão feitos extratos orgânicos e solubilizados aquosos para o emprego do ensaio *Salmonella*/microssoma em presença e ausência de ativação metabólica com as linhagens TA98, TA100, TA97a e possivelmente YG1021 e YG1024. Os resultados obtidos neste trabalho poderão contribuir para implementação deste bioensaio no biomonitoramento de solos.

Palavras-chave: amostras de solo, cinzas de carvão, ensaio Salmonella/microssoma, extratos aquosos, extratos orgânicos.

Abstract

Evaluation of areas under the influence of a coal-fired power plant through genotoxicity assay

Ashes originated from coal burning may contain in its constitution several substances with genotoxic potential, including organic compounds and heavy metals. These ashes are launched in the environment by power plants and may contaminate its sorrounding areas. In Brazil, there are no works evaluating the environmental impacts in the area under influence of coal-fired power plants using the *Salmonella*/microsome assay. Therefore, the aim of this research is to evaluate the genotoxic potential of soil samples adjacent to a coal-fired power plant and of a coal bottom ash deposit in process of recuperation through this bioassay. Superficial soils samples will be collected in areas under influence of a coal-fired power plant in Rio Grande do Sul state, and then organic extracts and aqueous solubilized for the *Salmonella*/microsome assay will be performed in presence and absence of metabolic activation with the strain TA98, TA100, TA97a and possible YG1021 and YG1024. The results of this work may contribute for the implementation of this bioassay in the biomonitoring of soils.

Key words: soil samples, coal bottom ash, Salmonella/microsome assay, aqueous extracts, organic extracts.

Introdução

O solo, do ponto de vista de retenção de poluentes, difere dos demais compartimentos ambientais (água e ar) na medida que inexiste um deslocamento contínuo, como no caso da circulação atmosférica e dos cursos d'água, podendo acarretar no aumento do tempo de permanência dos contaminantes em nível local. Além disso, o solo funciona como um filtro para águas contaminadas percolantes e como um grande reservatório de gases, a partir de intensos processos físicos, químicos e biológicos, necessitando assim, de cuidados especiais em seu uso (Sisinno & Moreira, 1996; Stenberg, 1999; Costa & Costa, 2004).

Para Gilmore (2001), a contaminação dos solos constitui a maior barreira para o desenvolvimento sustentável, uma vez que este problema afeta a saúde do ecossistema e age de forma direta na perda da diversidade biológica. Entre os principais resíduos contaminantes no solo estão os metais pesados e os compostos orgânicos. A contaminação pelos metais está geralmente associada com o aumento exagerado das atividades industriais e de mineração, enquanto as altas concentrações de compostos orgânicos estão relacionadas com o uso intensivo de produtos agrícolas e de combustíveis fósseis (Wesp *et al*, 2000; Gilmore, 2001; Esteve-Nunez *et al*, 2001).

Esses dois grupos de contaminantes têm sido amplamente relatados na literatura como importantes agentes genotóxicos (mutagênicos) em amostras de solo (Knasmüller *et al*, 1998; Tsukatani *et al*, 2002; Plaza *et al*, 2005; Watanabe *et al*, 2005), colocando em risco a saúde humana e do meio ambiente. White & Claxton (2004) ressaltam que avaliar a contaminação por substâncias genotóxicas no solo não é uma tarefa fácil. O grande número de substâncias agressivas, potencialmente presentes num sítio contaminado, pode dificultar uma caracterização química, uma vez que este tipo de

análise apresenta respostas limitadas para prever a toxicidade de misturas complexas. A utilização de bioensaios, incluindo o *Salmonella*/microssoma, se apresenta como uma alternativa útil para medir a mutagênese potencial de misturas ambientais. Este ensaio vem sendo utilizado como um indicador precoce de contaminação por agentes genotóxicos (Umbuzeiro & Vargas, 2003) e mais recentemente, como indicador de ambientes restaurados por biorremediação (Plaza *et al*, 2005). Sobre o mesmo tema, pesquisas recentes têm apontado que a associação entre bioensaios, procedimentos de fracionamento de amostras e análises químicas pode ser uma estratégia eficaz para verificar a atuação de misturas complexas no ambiente (Brack,2003). No entanto, Hewitt & Marvin (2005) comentam que a utilização dessa ferramenta pode ser melhor definida durante o desenvolvimento da pesquisa, em função de incertezas geradas ao longo do trabalho.

Entre essas amostras ambientais complexas que necessitam de maiores estudos, estão os solos contaminados com resíduos oriundos da queima de carvão em usinas termelétricas, os quais apresentam entre outros constituintes, compostos orgânicos provenientes da queima incompleta do carvão e altas concentrações de metais pesados (Borm, 1997; Karuppiah & Gupta, 1997; Pires, 2002). Embora este tema seja de fundamental importância, não existem no Brasil estudos avaliando o potencial genotóxico em áreas sob influência de termelétrica a carvão, através do ensaio *Salmonella*/microssoma e, além disso, estudos de avaliação de atividade mutagênica em amostras de solo são escassos.

Desta forma, o objetivo deste trabalho será avaliar o potencial genotóxico de amostras de solo adjacentes a uma usina termelétrica a carvão e de um depósito de cinzas de carvão em processo de recuperação através do ensaio *Salmonella*/microssoma, visando

sua utilização em avaliações de solos contaminados por substâncias genotóxicas, como estimativa de impacto ecológico com reflexos em saúde humana.

Material e Métodos

O estudo será realizado na área de influência de uma usina termelétrica, localizada no estado do Rio Grande do Sul. Essa está inserida na Microrregião Carbonífera do Baixo Jacuí. Serão coletadas amostras de solo em locais de amostragem, definidos como críticos para o meio ambiente com reflexos em saúde humana. O trabalho priorizará áreas tanto em nível de altitude superior ao depósito quanto inferior, para verificar as possíveis rotas das substâncias com atividade mutagênica como assoreamento do solo e efeito das chuvas ou poluição atmosférica advinda das chaminés da usina.

Os locais de amostragem serão demarcados com GPS e definidos de forma aleatória 15 pontos em cada, coletados em uma profundidade de 0-10cm, sendo removidos os resíduos vegetais. As 15 sub-amostras serão homogeneizadas a fim de formar uma amostra composta para cada local de amostragem. Em seguida, as amostras serão armazenadas em frascos de vidro a 4ºC, transferidas para o laboratório, onde deverão passar pelo processo de secagem à temperatura ambiente por até 48 horas, peneiradas (2mm) e novamente acondicionadas a 4ºC até o momento da extração (modificado de Watanabe *et al.*, 2000).

As amostras de solo (15 g) serão submetidas à extração orgânica com diclorometano (200 mL) em dois ciclos de dez minutos pela técnica de ultra-som, sendo seus extratos pré-filtrados, passados em coluna cromatográfica com placa filtrante contendo sulfato de sódio e celite e concentrados em rotavapor, metodologia modificada

de Tsukatani *et al.* (2002), pela substituição do metanol pelo diclorometano, uma vez que este solvente é considerado por diversos autores como eficiente na extração de contaminantes mutagênicos (Wesp *et al.*, 2000; Courty *et al.*, 2004), inclusive em misturas complexas ambientais (Nielsen, 1992).

A partir das amostras de solo será também feito um solubilizado aquoso utilizando água destilada estéril (solo: água, 1:2, g/mL), para investigar principalmente a ação genotóxica dos metais pesados. A suspensão será colocada no ultra-som durante dois ciclos de 20 minutos, sendo posteriormente centrifugada a 13.000 x g, por 15 minutos a 4°C, filtrada (0,45 micrômetros), dividida em alíquotas e estocadas a 4ºC para avaliação da genotoxicidade, adaptado de Monarca *et al.* (2002). A escolha dessa técnica se deu em função de ter um procedimento metodológico semelhante à extração orgânica, facilitando comparações posteriores.

Para avaliar a atividade genotóxica dos extratos orgânicos e do solubilizado aquoso será empregado o ensaio *Salmonella*/microssoma no procedimento de pré-incubação em presença e ausência de fração de metabolização hepática (Maron & Ames, 1983). Serão utilizadas as linhagens *Salmonella typhimurium* TA98 e TA97a**,** que detectam a ação de mutagênicos que causam erro no quadro de leitura, sendo que a TA97a é descrita na literatura como mais sensível a metais pesados (Pagano & Zeiger, 1992); a linhagem TA100**,** que caracteriza o efeito de substituição de pares de bases (Maron & Ames, 1983). Poderão ser utilizadas para melhor caracterização dos extratos orgânicos, linhagens específicas para definir a presença de nitroderivados: YG1021 e YG1024 (Umbuzeiro & Vargas, 2003).

Para a avaliação da atividade genotóxica, a amostra será considerada positiva se o número de revertentes por placa da amostra for, no mínimo, duas vezes maior que o

número de revertes observados no controle negativo, em presença de curva dose-resposta significante testada pelo *software* SALANAL (Vargas *et al.*, 1993).

Associada ao estudo de genotoxicidade, será realizada a caracterização química dos metais pesados por espectrometria de absorção atômica e a caracterização dos compostos orgânicos por cromatografia gasosa acoplada ao espectrômetro de massa. As respostas obtidas a partir da caracterização química poderão auxiliar a interpretação dos resultados do ensaio biológico, verificando a possível existência de correlações entre a genotoxicidade potencial e as concentrações dos contaminantes ambientais.

Resultados esperados

Os dados obtidos neste trabalho poderão contribuir para implementação do ensaio *Salmonella*/microssoma no biomonitoramento de solos contaminados por resíduos de carvão no país, a partir da (i) investigação da atividade genotóxica nas amostras coletadas no depósito de cinzas de carvão em recuperação; (ii) avaliação do potencial genotóxico das amostras de solo das áreas sob influência da usina, quer seja pelo assoreamento do solo, quer seja pela dispersão de poluentes na atmosfera e (iii) verificação da origem predominante da atividade genotóxica das amostras de solo, a partir da associação entre estudos de caracterização química dos principais grupos de compostos presentes nas amostras ambientais e as respostas observadas no marcador biológico para genotoxicidade.

Os resultados desta pesquisa serão divulgados na forma de dissertação no Programa de Pós Graduação em Ecologia (UFRGS), artigos científicos e da apresentação em congressos de áreas correlacionadas.

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7. Anexos (normas das revistas)

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