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BIODEGRADAÇÃO DE HIDROCARBONETOS AROMÁTICOS POLICÍCLICOS UTILIZANDO CONSÓRCIOS MICROBIANOS VISANDO A BIORREMEDIAÇÃO DE SOLOS CONTAMINADOS

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Resumo

A biodegradação de poluentes de petróleo em um solo contaminado foi acompanhada neste estudo, avaliando o comportamento da microbiota do solo durante a utilização de hidrocarbonetos policíclicos aromáticos (HPAs) como fontes de carbono. Através da biomassa microbiana, da taxa de respiração no solo, bem como do quociente metabólico (eficiência em degradar os compostos recalcitrantes em questão), foi possível uma avaliação do impacto na microbiota nativa do solo contaminado e nos diferentes microcosmos bioaumentados com os consórcios de bactérias, fungos, e uma mistura destes consórcios por 12 semanas. Tanto a microbiota nativa quanto os solos bioaumentados demonstraram uma rápida resposta à adaptação neste ambiente contaminado pelo aumento da biomassa e das taxas metabólicas.

Durante o período de biodegradação dos HPAs, valores de evolução de CO_2 foram diminuindo e a biomassa se manteve em crescimento estável, indicando menos gasto de energia para os microrganismos sobreviverem neste solo impactado, como resposta à boa competitividade e eficiência da microbiota nativa e bioaumentada.

A biodegradação dos HPAs e a presença de metabólitos intermediários foram também avaliados, apresentando uma rápida redução das concentrações dos HPAs de baixo peso molecular (menores que 4 anéis aromáticos) em comparação com os de alto peso molecular, devido à sua biodisponibilidade e alta atividade microbiana de degradação. Todavia, a bioaumentação não demonstrou ser melhor que a microbiota nativa na degradação dos HPAs. É provável que o mecanismo de cooperação metabólica por co-metabolismo tenha sido realizado pela comunidade do solo, uma vez que vários HPAs complexos foram degradados. Diversos estudos apontam a presença de HPAs de menor peso molecular e seus respectivos produtos como indutores da degradação dos HPAs de maior peso molecular. Os metabólitos intermediários produzidos foram assimilados pelos microrganismos em processo cooperativo no solo, sendo responsáveis pela indução de enzimas e suas respectivas vias degradativas.

Os distúrbios causados no solo pela poluição com HPAs normalmente estimulam o crescimento de microrganismos capazes de sobreviver nestes compostos, causando mudanças na estrutura microbiana do solo devido às suas adaptações nos processos co-metabólicos para a manutenção da comunidade. Neste trabalho, um solo impactado com HPAs, estocado por vários anos sob refrigeração foi analisado quanto à habilidade da microbiota nativa em crescer em alguns HPAs, individualmente, ou ainda em mistura complexa. Perfis moleculares de microbiota foram observados pela técnica de PCR-DGGE utilizando fragmentos do gene RNA ribossômico 16S durante 4 semanas. O número de bandas observadas foi interpretado como os membros dominantes na comunidade, e os diferentes perfis mostraram diferentes dinâmicas de degradação dos HPAs em meio contendo ou não fatores essenciais de crescimento (micronutrientes e vitaminas). Espécies ativas metabolicamente mostraram-se predominantes na interação com a comunidade e na cooperação catabólica. Após enriquecimento do solo original por 6 meses, apenas duas bandas foram visulizadas em gel, correspondendo à duas colônias morfologicamente diferentes isoladas em meio ágar, sendo identificadas como sendo do gênero *Pseudomona,* mais provavelmente *Pseudomonas stutzeri* (98% de similaridade). Esta espécie possui alta capacidade de transformação natural no solo, gerando mutantes. Diferenças genéticas entre as colônias de *P. stutzeri* foram confirmadas por PFGE, as quais apresentaram bandas para os genes catabólicos *nah*A-dioxigenase, catecol-1,2 e 2,3-dioxigenases, responsáveis por codificar as respectivas enzimas atuantes nas principais vias metabólicas de degradação dos HPAs.

Abstract

The biodegradation of petroleum derivatives in a contaminated soil was evaluated in this study monitoring the behavior of the soil microbiota when using polycyclic aromatic hydrocarbons (PAHs) as carbon sources. Analyzing C-biomass, respiration rates (CO_2 evolution) and metabolic quotient (which means the efficiency in degrading PAHs), it was possible to evaluate the impact of the contaminated soil in the native microbiota and soil bioaugmented with bacterial and fungal consortia or a mixture of these consortia for 12 weeks in microcosms. Even the native microbiota or the bioaugmented soils performed a rapid response regarding to their adaptation into contaminated environment due to the increase of biomass and respiration rates. During the PAH biodegradation period, CO_2 evolution values remained steady, indicating less loss of energy to the survival microorganism into the impacted soil, a good competitiveness, and also an efficiency of both native and bioaugmented populations.

The biodegradation of PAHs and the production of metabolic intermediates were assessed, and low-molecular-weight (LMW-) PAHs (less than 4 rings) had fast reduction in their concentrations comparing with the high-molecular-weight (HMW-) PAHs, probably due to their bioavailability and the high microbial activity. Bioaugmentations were not better than native microbiota in the PAHs degradation performances, and it is believed that the cooperative mechanism under co-metabolism could be responsible for the degradation of HMW-PAHs. Metabolic intermediates were assimilated by microorganisms in a cooperative process, inducing some key-catabolic pathways enzymes.

The pollution of soil by PAHs could stimulate the growth of organisms capable of surviving in the presence of these compounds, changing the soil microbial structure due a catabolic adaptation process. In this study, another PAH-impacted soil, collected from a manufacturing gas plant and stored for several years was analyzed regarding to the remained ability of the native microbiota to grow on individual PAHs or on its mixtures. Molecular profiles of the microbial community were observed using PCR-DGGE of the 16S rDNA fragment for 4 weeks. The number of bands was interpretated as dominant members into the community, and differences in profiles showed different PAH degradation dynamics in mineral medium with or without micronutrients and vitamins. Catabolically activated species were predominant in the community, and after several enrichment steps for 6 months, only two bands were observed in DGGE, corresponding to two colonies showing morphological differences, identified as *Pseudomonas* genus, very close to *Pseudomonas stutzeri* (98% of similarity). This specie has high capacity of natural transformation in soil, generating some mutants. Genetical differences were found between colonies using PFGE, and the presence of catabolic genes as *nahA*-dioxygenase, cathecol-1,2- and 2,3- dioxygenases were confirmed by PCR products in agarose gel electrophoreses.

1. Introdução

A contaminação ambiental é causada pelas inúmeras atividades industriais, agro-industriais e demais atividades ligadas à vida moderna, como transportes e resíduos urbanos, as quais afetam a qualidade de vida do planeta, causando um desequilíbrio no ecossistema e ameaçando diretamente a saúde do homem. O aumento crescente de locais impactados por compostos xenobióticos lançados no ambiente pela ação antropogênica - muitos dos quais são tóxicos mesmo em baixas concentrações - é atualmente uma grande preocupação ecológica. Assim, na tentativa de resolver este problema mundial, a biorremediação é uma forma de auxílio da natureza, uma 'tecnologia limpa' que se baseia na atividade natural de microrganismos de metabolizarem compostos orgânicos, utilizando-se de diferentes estratégias para acelerar esse processo natural, minimizando os níveis de contaminação pela diminuição das concentrações destes compostos no ambiente ou ainda pelas suas mineralizações (transformação dos contaminantes à CO_2 e H_2O).

A adaptação da microbiota nos próprios locais contaminados é de grande importância neste processo, uma vez que, otimizando uma bioestimulação destes microrganismos com adição de nutrientes, O_2, temperature, umidade, entre outros fatores, a biorremediação pode perdurar a longo-prazo. Os níveis de poluição podem ainda ser amenizados através da atenuação natural neste ambientes, sem bioestimulação ou bioaumentação com microrganismos exógenos ou isolados dos próprios locais contaminados que se deseja biorremediar. No entanto, a introdução de microrganismos com grande capacidade degradativa dos compostos poluentes pode aumentar a eficiência da biorremediação pela rápida ação metabólica desses microrganismos, e o sucesso para a inoculação de organismos exógenos ao local contaminado depende da boa competitividade destes com a microbiota natural.

Uma microbiota eficiente para degradar HPAs significa microrganismos aptos a aumentarem sua respiração basal, indicando o consumo de tais compostos como fonte de carbono; seguindo um decréscimo do CO_2 liberado, o que indica a incorporação do carbono na biomassa e grande habilidade em usar os HPAs disponíveis no solo. As determinações da quantidade de carbono existente na biomassa microbiana, das taxas de atividade respiratória e também do quociente de respiração por unidade de biomassa podem indicar a performance da microbiota durante o processo de detoxificação do solo pela degradação dos compostos poluentes, diminuindo suas concentrações no ambiente. Os resultados da verificação da eficiência microbiana nos processos de biorremediação podem ser complementados ainda com a determinação da biodegradação dos compostos e a análise dos metabólitos intermediários produzidos pela microbiota no solo impactado, bem como a verificação de toxicidade destes produtos de degradação.

A comunidade microbiana, ao interagir nas condições impostas nos processos de biorremediação, produz intermediários de metabolismo, na maioria das vezes, usados como substratos de crescimento por outros microrganismos, iniciando o processo de co-metabolismo cooperativo. Os produtos de uns são imediatamente absorvidos por outros,

e os produtos decorrentes desse processo vão se tornando cada vez menos complexos, mais polares e capazes de serem usados para manutenção do metabolismo de todos.

Além dos métodos já descritos anteriormente para verificação do comportamento da microbiota quando as suas atividades metabólicas (crescimento, respiração, eficiência metabólica, etc.), ferramentas moleculares são atualmente empregadas para analisar a cooperação microbiana em solo contaminado, monitorando a diversidade microbiana total através da análise de regiões conservadas do gene RNA ribossômico 16S. Os chamados microrganismos catabolicamente ativos para a degradação dos compostos de interesse e responsáveis, conseqüentemente, pela ocorrência de cooperações metabólicas na comunidade microbiana são quantificados por métodos bioquímicos e moleculares. A presença de genes específicos que codificam determinadas enzimas-chave na degradação de compostos tóxicos e recalcitrantes podem ser também verificada e sua expressão quantificada em tempo real na microbiota.

As mudanças no perfil da microbiota e a presença de espécies dominantes e altamente adaptadas ao processo de degradação podem ser analisados rapidamente pela extração total de material genético e amplificação dos fragmentos de interesse com iniciadores (*primers*) universais ou específicos. A utilização de várias técnicas moleculares possibilita identificar mudanças do perfil eletroforético, determinando diferenças temporais na dinâmica da comunidade envolvida na remediação dos poluentes ambientais (predominância de algumas espécies, inibição de outras, etc.). Uma etapa posterior é a identificação dos microrganismos relevantes no processo e estudo de otimização das tividade metabólica da comunidade.

Ainda não há evidências de trabalhos na literatura que apresentem resultados de atividade metabólica e análise em nível molecular de comunidades microbianas em solos contaminados com HPAs, complementando os valores de degradação destes compostos. Este trabalho teve como principal objetivo analisar a atividade microbiana tanto por processos bioquímicos (C-biomassa, evolução de CO_2, qCO_2) quanto por técnicas moleculares, as quais permitem uma análise mais refinada do processo. Tais métodos foram de grande importância para a avaliação do potencial microbiano e para indicar possíveis otimizações das condições de biorremediação usando a bioaumentação para o sucesso da microbiota total em ambientes impactados com HPAs.

2. Revisão de Literatura

2.1. Hidrocarbonetos Poliaromáticos (HPAs)

Os HPAs são compostos formados pela fusão de anéis benzênicos de forma linear, angular ou agrupados. Os vários HPAs se diferenciam em números de anéis aromáticos e a posição em que estes se encontram fundidos uns aos outros. Suas características físico-químicas variam conforme seu peso molecular, sendo os HPAs de maior peso molecular mais resistentes à oxidação e vaporização, enquanto que os de menor peso molecular se encontram mais disponíveis para a degradação no ambiente (Cerniglia, 1992).

Os HPAs são considerados compostos potencialmente perigosos, devido suas atividades cancerígena e mutagênica, segundo a Agência de Proteção Ambiental (EUA), que classificou 16 deles como poluentes prioritários: naftaleno, acenaftaleno, acenaftileno, fenantreno, fluoreno, fluoranteno, antraceno, benzo[a]antraceno, criseno, pireno, benzo[a]pireno, benzo[b]fluoranteno, benzo[k]fluoranteno, dibenz[a,h]antraceno, benzo[g,h,i]perileno e indeno[1,2,3-cd]pireno, apresentados na Figura 1.

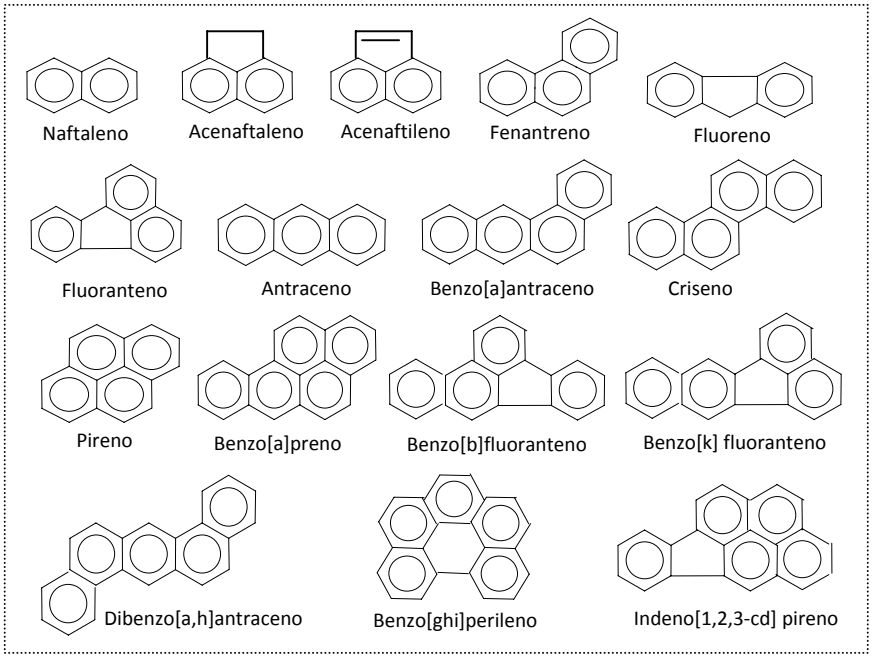

Figura 1. Estrutura molecular dos 16 HPAs considerados poluentes prioritários de acordo com a US EPA (Pothuluri e Cerniglia, 1998; Peters *et al.*, 1999).

Para os organismos vivos, os HPAs possuem propriedades tóxicas aguda (HPAs de menor peso molecular), carcinogênica, mutagênica e teratogênica (HPAs de maior pesos molecular), quando ativados por enzimas de mamíferos (por exemplo, a monooxigenase citocromo P-450) dando origem a epóxidos e reativos intermediários diol-epóxidos, que

após sucessivas oxidações e/ou hidrólises combinam com o DNA em ligação covalente, resultando em carcinogenia (Harvey, 1996).

Os HPAs têm sido acumulados no ambiente como resultado de atividades antropogênicas (combustão de carvão, petróleo, óleo, madeira, processos de defumação, queima de cigarros e combustíveis automotivos, derramamentos e vazamentos acidentais de petróleo e derivados) e de atividades biogênicas/geoquímicas, como queima natural de florestas e erupções vulcânicas (Freeman e Cotell, 1990; Lim *et al.*, 1999; Semple *et al.*, 2001 e 2003). A exposição aos HPAs pode ocorrer por inalação (poluição atmosférica), ingestão (alimentos e água contaminados) e contato dérmico direto, uma vez que são altamente lipofílicos e são absorvidos rapidamente pelo trato intestinal de mamíferos, podendo ainda causar vários tipos de cânceres como o de pulmão, intestino, pâncreas, fígado, vesícula e de pele (IARC, International Agency for Reserach on Cancer; http://www.iarc.fr; Mastrangelo *et al.*, 1996; Boffeta *et al.*, 1997).

A persistência dos HPAs no ambiente depende de vários fatores como sua estrutura química, concentração e dispersão, bem como sua biodisponibilidade nos solos, água, sedimentos e no ar. Nos solos, outros fatores também contribuem para o tempo de permanência dos HPAs, como por exemplo, o tipo de solo, pH, temperatura, níveis de oxigênio, umidade, presença de nutrientes, conteúdo de matéria orgânica e a idade do HPA na matriz do solo/sedimento (Connaughton *et al.*, 1993; Ratzinger e Alexander, 1995; Sutherland *et al.*, 1995).

2.2. HPAs na cadeia alimentar

Os alimentos podem ser contaminados com HPAs presentes no ar, uma vez que estes são volatilizados e aderidos às micropartículas de poeira que podem ser aspiradas pelo homem e animais. Além disso, podem penetrar na camada de cera das folhas dos vegetais e frutos, ou ainda penetrar na epiderme, ocorrendo bioacumulação nos tecidos adiposos.

Quando presentes no solo, os PAHs estão intimamente ligados à matéria orgânica e sedimentos, podendo ser absorvidos pelas raízes das plantas, contaminando diretamente tubérculos, hortaliças e animais que consomem a pastagem contaminada.

Na água, estes compostos podem se adsorver em partículas na superfície ou em sedimentos de rios, lagos e oceanos, devido a sua natureza hidrofóbica. Os organismos aquáticos, os animais e o homem são capazes de biotransformar HPAs pela ação da enzima monooxigenase citocromo P-450, diminuindo o acúmulo destes compostos na cadeia alimentar, porém, dentre os organismos aquáticos, os moluscos bivalves acumulam HPAs pela ausência de enzimas detoxificantes.

Nos processos de secagem e defumação, diferentes tipos de HPAs são depositados nos alimentos, bem como na tostagem, grelhagem, fritura e cozimento em altas temperaturas (Guillen *et al*., 1997; Phillips, 1999), contribuindo para a contaminação de carnes, queijos, grãos, café, óleos vegetais, etc. (Howard e Fazio, 1980; Dennis e Massey, 1983; Lawrence e Weber, 1984; De Vos *et al.,* 1990; Speer *et al.,* 1990; Lodovici *et al.,* 1995). Pesquisas realizadas no Brasil foram direcionadas para a determinação de

benzo[a]pireno em algumas categorias de alimentos, como os produtos cárneos, óleos, gorduras e derivados (Yabiku *et al.*, 1993; Pupin e Toledo, 1996a e 1996b; Camargo e Toledo, 1998 e 2000). As gorduras e óleos vegetais são fontes significativas de HPAs na dieta humana, sendo contaminados pela secagem dos grãos através dos gases de combustão e pela extração do óleo com solventes a base de petróleo. Os óleos são fontes também de contaminação indireta em alimentos à base de cereais (biscoitos, bolachas, tortas, pães e massas), por fazerem parte da composição destes alimentos (Dennis *et al.*, 1991).

Os HPAs podem ser também encontrados em bebidas como chás, café, uísque e também aguardente (Bettin e Franco, 2005). A colheita da cana-de-açúcar no Brasil (que ocorre todo ano entre maio e novembro) e em outros países é conduzida geralmente após a queima dos canaviais (Zamperlinni *et al.*, 2000). Este procedimento primitivo resulta, primeiramente, em contaminação ambiental, pois partículas de palha queimada (fuligem), bem como inúmeros compostos tóxicos dentre os quais destacamos os HPAs são introduzidos na atmosfera. Estudos realizados em amostras de fuligem da cana-de-açúcar queimada indicam a presença dos seguintes HPAs: naftaleno, acenaftaleno, acenaftileno, fluoreno, fenantreno, antraceno, fluoranteno, pireno, 1,2-benzo[e]pireno, benzo[a]antraceno, criseno, benzo[e]pireno, 1,2-benzo[b]fluoranteno, benzo[k]fluoranteno, benzo[a]pireno e benzo[ghi]perileno. A presença destes compostos em bebidas pode ser proveniente de contaminações nas etapas do processo de produção, tais como: o emprego de lubrificantes nos equipamentos; a utilização de recipientes não-adequados para o armazenamento da bebida; tanques revestidos com resinas asfálticas ricas em HPAs (Akhlaq, 1994; Hawthorne *et al.*, 2000). Apesar de bebidas contaminadas por HPAs serem potencialmente prejudiciais à saúde, pois benzo(a)pireno e criseno são compostos considerados carcinogênicos (Dennis e Massey, 1983; Fálcon, 1994; Camargo e Toledo, 2000), no Brasil até o momento não existe legislação específica sobre o assunto.

Camargo e Toledo (2002) analisaram a presença de HPAs em diversos outros alimentos como produtos lácteos, cereais, panificados, leguminosas, tubérculos, açúcares, produtos cárneos, defumados e pizza. Os resultados desta pesquisa revelaram vários alimentos que fazem parte da dieta dos brasileiros, contaminados por HPAs em níveis dentro da faixa encontrada na literatura internacional para alimentos similares. Estas informações, juntamente com dados nacionais de consumo dos alimentos analisados foram importantes para estimar a ingestão total de HPAs e avaliar os alimentos que representam maior fonte de exposição por meio da dieta. Dessa forma, caso necessário, será possível recomendar aos órgãos responsáveis pela saúde pública o monitoramento da contaminação dos alimentos identificados como importantes fontes desses carcinógenos, ajudando nas futuras resoluções relativas a limites de contaminantes em alimentos.

2.3. Degradação de HPAs por microrganismos

De maneira geral, o metabolismo de HPAs por microrganismos ocorre de maneira aeróbia, via oxidação do anel aromático pela monooxigenase citocromo P-450 de bactéria e fungos não-ligninolíticos, formando óxido de areno, que por ação de outra enzima epóxido-hidrolase forma *trans*-diidrodiol (Figura 2). Rearranjos não-enzimáticos podem ocorrer com o óxido de areno, transformando-o primeiramente em fenol, e conseqüentemente, em o-glicosídeos, o-glucuronídeos, o-sulfatos, o-xilosídeos e o-metil (Cerniglia, 1992). Bactérias também podem hidroxilar os anéis aromáticos dos HPAs pela ação das dioxigenases, originando *cis*-diidrodióis, os quais se transformam em catecol através das enzimas desidrogenases. A abertura do anel aromático se dá através de orto- e meta-fissões realizadas por enzimas catecol-dioxigenases, originando *cis, cis*- ácido mucônico e semialdeído 2-hidroximucônico, respectivamente. Já, os fungos ligninolíticos produzem enzimas ligninolíticas (lignina-peroxidase, manganês-peroxidases e lacase) que agem no HPA transformando-o em quinonas, ocorrendo posterior abertura do anel (Bamforth e Singleton, 2005).

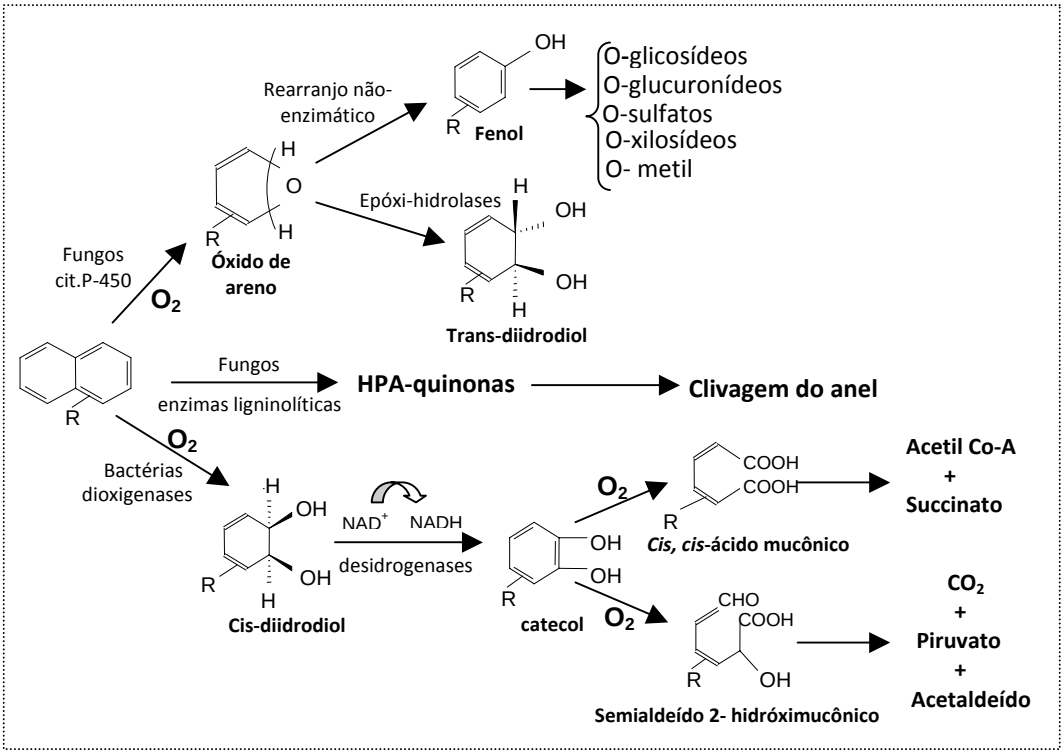

Figura 2. Rotas metabólicas da degradação de HPAs por bactérias e fungos em sistema aeróbio (Müncnerová and Augustin, 1994 - adaptação de Galli, 1994).

Bactérias, fungos, actinomicetos e algas possuem capacidade de degradar vários tipos de HPAs, e alguns como única fonte de carbono e energia. Trabalhos focados nos HPAs e microrganismos, individualmente, contemplam o estudo do comportamento de crescimento e os passos do metabolismo microbiano, a degradação do HPA com produção de metabólitos, toxicidade e o potencial de detoxificação. Quando estudados em mistura

complexa, verifica-se a utilização preferencial de certos compostos pelo microrganismo. Quando em consórcios ou em mistura de consórcios, a análise da co-metabolização dos HPAs é interessante, onde se desenvolve um mecanismo de degradação cooperativa entre os microrganismos. Quando adicionados em solos com mistura de PAHs, os resultados desta relação podem ser observados de maneira mais complexa, uma vez que vários fatores podem interferir na cooperação catabólica dos microrganismos. Consórcios microbianos podem ter maior potencial degradativo nos processos de biorremediação pela interdependência de organismos, agindo em cooperação e promovendo uma maior eficiência e versatilidade na limpeza de locais contaminados (Kanaly *et al*., 2002).

Muitos microrganismos são produtores de biosurfactantes. Estes compostos aumentar a solubilidade dos HPAs (moléculas lipofílicas) às células do microrganismo, promovendo rapidez no uso destes compostos como fonte de carbono pelo contato mais direto com enzimas catalíticas envolvidas nas suas degradações.

Geralmente, os fungos são responsáveis pela oxidação inicial dos anéis aromáticos dos HPAs na presença de oxigênio, transformando-os em metabólitos polares mais disponíveis à microbiota indígena não-adaptada aos HPAs (Kotterman *et al.*, 1998). Os fungos são capazes de metabolizar os HPAs pela ação de enzimas ligninolíticas inespecíficas (Clemente *et al.,* 2001; Silva, 2002), as quais atuam numa ampla gama de compostos poluentes, e da enzima intracelular monooxigenase citocromo P-450 (Verdin *et al.,* 2004), como já citado anteriormente. No mecanismo de degradação dos HPAs por fungos sugerido por Bezalel *et al.* (1996), a monooxigenase age primeiramente no anel aromático, seguido da atividade das enzimas ligninolíticas. O crescimento dos fungos quando em contato com HPAs pode se dar através de hifas em filamentos ou agregadas em forma de *pellet*, os quais variam suas características de acordo com a concentração disponível de tais compostos (Saraswathy and Hallberg, 2005). Alguns fungos filamentosos retiram os HPAs do meio através da adsorção micelial (da Silva *et al.*, 2003; Capotorti *et al.,* 2004) sem, necessariamente, oxidá-los. Tal adsorção pode se dar devido à baixa solubilidade destes em água. A indução das monooxigenases intracelulares e enzimas ligadas à membrana pode ocorrer pelo contato direto do HPA que, quando adsorvido, pode ser armazenado em vesículas lipídicas no interior da célula fúngica, as quais podem ter sítios da oxidação inicial dos HPAs (Verdin *et al.,* 2005).

O uso de fungos filamentosos nos processos de descontaminação dos ambientes oferece grandes vantagens e, uma vez adaptados ao local contaminado, permitem a sobrevivência do inóculo e habilidade de alcance dos poluentes através da elongação de suas hifas (Bennett e Faison, 1997), aumentando a colonização e maximizando o contato entre os compostos recalcitrantes e a parede do micélio, sugerindo potencial para uso em processos de biorremediação a longo prazo nos solos contaminados com HPAs (Potin *et al.*, 2004).

Na ausência de oxigênio, aceptores finais alternativos como sulfatos, íons ferro ou nitratos são necessários para oxidar os anéis aromáticos. Mecanismos de degradação anaeróbia de HPAs são ainda pouco elucidados: para naftaleno, a carboxilação por CO_2/HCO_3 (formando ácido 2-naftólico) seguida de sucessivas hidrogenações a ácido 2-decaidronaftóico, inicia a degradação anaeróbia do HPAs (Meckenstock *et al.*, 2000; Zhang

et al, 2000). Hidroxilações do anel aromático também podem ocorrer nesta condição anóxica, formando intermediários naftólicos (Bedessem *et al.*, 1997).

Deste modo, um melhor entendimento na diversidade da microbiota que habita os solos contaminados com poluentes xenobióticos, em especial os HPAs pelo seu potencial bioacumulativo e carcinogênico, no que diz respeito à bioaumentação e bioestimulação, pode ajudar na seleção de organismos adaptados a explorar tais ambientes.

2.4. A biorremediação de solos e a descontaminação ambiental

As técnicas convencionais aplicadas na biorremediação dos solos compreendem a escavação do material e posterior remoção do poluente para aterros adequados, ou ainda o confinamento das áreas contaminadas. Tais procedimentos possuem algumas desvantagens, como por exemplo, o risco de manuseio e transporte dos materiais contaminados para uma outra área, bem como a tomada de medida paliativa para os locais contaminados confinados, requerendo constante monitoramento e manutenção das barreiras físicas (Vidali, 2001). Os métodos físicos de remediação podem ser efetivos na redução dos níveis contaminantes a valores aceitáveis pelos órgãos de fiscalização local ou estadual, porém, sua complexidade tecnológica encarece o tratamento.

A biorremediação é um processo de utilização de organismos vivos ou de seus sistemas enzimáticos para o tratamento do ambiente contaminado, com o objetivo de diminuir os níveis de contaminantes até à detoxificação total do ambiente pela mineralização dos compostos poluentes. Tal processo é considerado mais barato, pelo emprego de tecnologia de mais baixo custo, tendo geralmente uma boa aceitação pública, uma vez que usa microrganismos ou plantas, podendo ser realizado *in situ* (no próprio local contaminado), não havendo necessidade de maiores contaminações com transporte e possíveis acidentes de manuseio no transporte do material contaminado para fins de remediação. Na sua aplicação prática, a biorremediação deve demonstrar que a remoção de contaminantes é atribuída à biodegradação dos poluentes a níveis inferiores aos existentes e os produtos de degradação devem ser menos tóxicos que os compostos anteriormente existentes (Pritchard *et al*, 1992).

Diferentes técnicas de biorremediação podem ser empregadas, dependendo do grau de saturação dos poluentes no solo, levando em conta suas propriedades físico-químicas, umidade, aeração, temperatura ambiente, sendo definidas como técnicas *in situ* – realizadas no local contaminado (solo e águas subterrâneas) com o mínimo de perturbação – ou *ex situ* – material é escavado ou bombeado para ser tratado no próprio local contaminado (*on-site*) ou transportando para longe do local contaminado para destinação e tratamento adequados.

A biorremediação *in situ* pode ter algumas limitações como a profundidade do solo e difusão suficiente de oxigênio, onde pode ser empregada técnicas de *bioventilação* (fluxo baixo de ar para estimular a atividade da microbiota natural do solo), "*biosparging*" (injeção de ar sob pressão na água subterrânea, aumentando a mistura de ar na zona saturada e, conseqüentemente, o contato entre solo e água substerrânea), *bioestimulação* (adição de oxigênio e nutrientes – fontes de N, P, S) e ainda a

bioaumentação (adição de microrganismos exógenos ou do próprio local contaminado, após algum enriquecimento). Já a biorremediação *ex situ* compreende tratamentos como: "*landfarming",* onde o solo contaminado é escavado e espalhado em um leito preparado, facilitando a degradação aeróbia da comunidade natural do solo; *biopilhas*, pilhas de solo colocadas em células e cobertas para estimular tanto a degradação aeróbia quanto a anaeróbia e controlar temperatura e umidade; *compostagem,* combinação de solo contaminado com compostos orgânicos para o desenvolvimento e estimulação da microbiota, como os resíduos agroindustriais e esterco; e *biorreatores*, usado para pluma contaminada bombeada, lodo de esgoto, sedimentos e solo em um sistema fechado, tendo possibilidade de melhor controle das condições de degradação.

Nos Estados Unidos, o número de locais tratados através da biorremediação vem crescendo nos últimos 20 anos (dados disponíveis no site da EPA - National Priority List; http://www.epa.com). No Brasil, das 1.822 áreas contaminadas no Estado de São Paulo, os HPAs estão presentes como os principais poluentes de contaminação, juntamente com solventes aromáticos e combustíveis líquidos. De acordo com dados da Companhia de Tecnologia e Saneamento Ambiental (site da CETESB; http://www.cetesb.sp.org.br), os processos de biorremediação ainda estão sendo pouco empregados, provavelmente devido à necessidade de maiores adequações do processo para ações *in situ* a curto-prazo. Algumas áreas com uma mistura complexa de contaminantes, onde a remediação rápida se faz necessária pelo perigo à saúde pública, várias técnicas conjugadas de biorremediação podem ser empregadas no intuito de acelerar o processo que será extendido ainda a longo-prazo, uma vez que a microbiota estará adaptada ao local, onde os compostos que já alcançaram os limites aceitáveis pelos órgãos fiscalizadores poderão ser mineralizados pela ação intermitente dos microrganismos.

A biorremediação no nosso país deve ser olhada com especial atenção, uma vez que dispomos de uma microbiota rica, um clima tropical e solos com características que propiciam a vida microbiana.

2.5. Uso da bioaumentação na biorremediação de solos

O solo é considerado o maior depositório de contaminantes orgânicos, os quais penetram através das práticas agrícolas, aterros de resíduos e atividades industriais (Semple *et al.* 2001). Os poluentes orgânicos possuem grande capacidade de interação com a matriz do solo e, por isso, tal interação é tema de importantes e recentes estudos sobre sua desorção e biodisponibilidade destes compostos para serem biodegradados (Alexander, 2000; Macleod *et al.*, 2001; Semple *et al.*, 2004). A introdução de microrganismos em ambientes a serem descontaminados já provou sua eficácia na degradação de vários compostos poluentes e recalcitrantes no solo (Sarkar *et al.*, 2005).

O isolamento e caracterização de microrganismos capazes de degradarem HPAs de alto peso molecular torna-se particularmente importante para aumentar o sucesso de processos de biorremediação *in situ*.

A bioaumentação deve ser feita utilizando microrganismos com conhecida habilidade metabólica para os solos contaminados, capazes ainda de competirem com a

microbiota natural dos locais de tratamento e manterem sua competência na degradação dos compostos alvos. A taxa de biodegradação natural em solos contaminados é, na maioria das vezes baixa, devido à falta de microrganismos na microbiota natural com capacidade de metabolizar os contaminantes (Juhasz *et al.* 2000).

Nos solos contaminados com HPAs onde foi realizada bioaumentação com consórcio bacteriano metabolicamente ativo para estes compostos, o aumento das taxas de degradação foi diretamente proporcional à diminuição de níveis de toxicidade e mutagenicidade no solo (Juhasz *et al.*, 2000), gerando produtos menos tóxicos. No entanto, April e colaboradores (1990) observaram um aumento de 4,5 vezes na toxicidade enquanto as taxas de degradação dos HPAs aumentavam em solo contaminado com resíduos de refinação de petróleo, devido ao acúmulo de metabólitos polares como os compostos fenólicos e diidrodióis gerados da degradação incompleta destes compostos aromáticos.

Para uma rápida remoção de HPAs é necessária uma grande população de indivíduos metabolicamente ativos, capazes de competir por nutrientes com a microbiota local, o que é desejável em qualquer experimento de bioaumentação em biorremediação. A bioestimulação por nutrientes com adição de substratos e compostos inorgânicos, bem como aeração, pode proporcionar uma melhor degradação de poluentes pela microbiota natural e maior sobrevivência das espécies introduzidas. Vários nutrientes, tais como sais inorgânicos usados como fontes de N e P, uréia, biossólidos, esterco, palha, material de compostagem, podem ser adicionados para melhorar a eficiência dos processos de biorremediação, sustentando diversas populações de microrganismos com potencial para a degradação de contaminantes orgânicos, incluindo HPAs (Rosenberg *et al.*, 1992; Walworth e Reynolds, 1995; Kästner e Mahro, 1996; Cho *et al.*, 1997; Williams *et al.,* 1999; Namkoong *et al*., 2002). Os biosurfactantes, substâncias produzidas pelos microrganismos e já discutidos anteriormente, auxiliam na solubilização de moléculas hidrofílicas pelo aumento da superfície de contato entre os compostos e as células microbianas, sendo largamente empregados nos processos de biorremediação de óleos e poluentes orgânicos do petróleo. Os biosurfactantes podem ser produzidos por conseqüência de bioestimulação, otimizando o metabolismo da microbiota para sucesso do processo. Além disso, a fração de substâncias húmicas do solo desempenha importante papel como surfactantes naturais, estimulando a atividade microbiana e promovendo a atenuação natural nas áreas impactadas (Conte *et al*., 2005).

Em experimentos com fitorremediação, o aumento da dissipação de PAHs na rizosfera ocorreu devido à estimulação da comunidade microbiana dentro dessa área, tendo estes organismos um papel importante na degradação de HPAs de alto peso molecular. Os microrganismos degradadores desses compostos aumentaram dentro da rizosfera pela inoculação de *Rhizobium leguminosarum* bv. *trifolii* (Johnson *et al.*, 2005).

Estudos sobre a degradação de HPAs pela microbiota natural de solos contaminados, com ou sem estimulação orgânica/inorgânica, têm demonstrado que os HPAs com menor peso molecular são degradados primeiramente, uma vez que estes se encontram mais biodisponíveis e possuem menor complexidade de estrutura que os HPAs de maior peso molecular (Stringfellow *et al.,* 1995). Os autores sugerem ainda uma possível competitividade de HPAs pela enzima envolvida na degradação inicial desses compostos,

ocorrendo uma inibição inicial da degradação dos HPAs de maior peso molecular. Portanto, faz-se necessário a inoculação no solo de microrganismos capazes de degradarem de maneira eficiente HPAs de maior peso molecular (Wilson e Jones, 1993).

Juhasz e colaboradores (1996 e 1997) isolaram alguns microrganismos de um solo contaminado com HPAs, e quando em consórcio foram capazes de crescer em uma mistura de HPAs contendo de 4 a 6 anéis aromáticos em meio líquido, tanto quando inoculados individualmente, como também em consórcio. Em outro trabalho, Juhasz *et al.* (2000), utilizou o mesmo consórcio microbiano em um solo contaminado com HPAs coletado de um pólo petroquímico, verificando uma diminuição significativa dos HPAs, incluindo os de maior peso molecular como benzo(a)pireno e dibenzo(a,h)antraceno. Ainda neste estudo realizados pelos mesmos autores, a microbiota natural teve apenas uma pequena capacidade de degradação dos HPAs de menor peso molecular, e nem mesmo com a bioestimulação usando extrato de levedura, a comunidade nativa pôde aumentar seu potencial degradativo.

Boonchan e colaboradores (2000) observaram que a degradação de HPAs com 5 anéis aromáticos (pireno, benzo[a]pireno, benzo[a]antraceno, criseno, dibenzo[a,h]-antraceno) como únicas fontes de carbono pelo consórcio de bactérias em meio líquido de sais na presença também do fungo *Penicillium janthinellum*, foi superior quando comparando o consórcio e o mesmo fungo inoculados separadamente. Algumas enzimas que estão envolvidas na degradação de HPAs são induzíveis, ou seja, são sintetizadas apenas quando o substrato está presente, e é possível que HPAs de menor peso molecular ou produtos de degradação destes possam induzir a síntese de enzimas necessárias para o catabolismo dos demais PAHs e moléculas aromáticas mais complexas (Heitkamp and Cerniglia, 1988, Hamzah e Al-Baharna, 1994, Juhasz *et al.*, 1997).

Analisando a dinâmica da comunidade microbiana nos processos de biorremediação de solo contaminado com creosoto, Viñas e colaboradores (2005) observaram que os experimentos bioestimulados com C:N:P (300:10:1), relação de nutrientes considerada ótima para descontaminação de solos contendo hidrocarbonetos totais de petróleo (TPHs) e HPAs (Atagana, 2004), obtiveram menores níveis de degradação destes compostos, quando comparados com o solo apenas aerado.

Assim sendo, para a biorremediação de locais contaminados, a bioaumentação com microrganismos é de grande importância para o aumento das taxas de degradação dos poluentes xenobióticos (Atlas, 1991; Brodkorb e Legge, 1992; Banerjee *et al.*, 1995; Trzesickia-Mlynarz e Ward, 1996) e os protocolos de inoculação de microrganismos no solo possuem papel fundamental no sucesso da atividade de degradação (Kästner *et al.*, 1998). Para o sucesso do processo deve-se ajustar ainda as condições do solo para otimizar o potencial de degradação da microbiota natural e dos microrganismos inoculados.

Os avanços da genética na área da bioengenharia molecular possibilitaram a criação de microrganismos geneticamente modificados que, de acordo com testes preliminares em laboratório, podem crescer rapidamente em áreas contaminadas com poluentes orgânicos persistentes, como os HPAs, uma vez que a capacidade de degradação dos microrganismos nativos é melhorada apenas por mutações ao acaso ou pela transferência horizontal de genes catabólicos para outros indivíduos da microbiota. Mesmo com a

possibilidade de se bioaumentar locais contaminados com organismos geneticamente modificados (OGMs), poucos microrganismos alçaram ainda um estágio para aplicação *in situ* devido à necessidade de energia extra para impor a presença de genes exógenos ao seu material genético (Ang *et al.*, 2005), isso sem mencionar a questão da aceitação de OGMs para aplicação direta no ambiente em grande escala, podendo se restringir apenas a processos de bioremediação *ex situ* devidamente controlado, uma vez que ainda estão sendo estudadas maneiras de expressão celular para o controle dos microrganismos no ambiente. Outros trabalhos discutem ainda a viabilidade dos OGMs nos processos de biorremediação (Giddings, 1998; Sayler e Ripp, 2000; Watanabe, 2001).

Mesmo diante do crescente desenvolvimento tecnológico voltado para a área da genética, o planeta abriga milhares de espécies de microrganismos capazes de serem potenciais biorremediadores de áreas impactadas. Os estudos de otimização das condições-chaves importantes para o seu crescimento e sobrevivência nessas áreas (tais como temperatura, pH, umidade do solo, concentração de oxigênio), fazendo o uso da bioestimulação e bioaumentação com a própria microbiota local enriquecida ou microrganismos degradadores previamente selecionados têm alcançado resultados promissores para o sucesso nos processos de biorremediação em campo.

2.6. A biomassa de carbono e a respiração microbiana avaliando a eficiência da microbiota do solo

A biomassa de carbono no solo é constituída de resíduos vegetais e animais nos vários estágios de decomposição, humus estabilizado, carbono inerte em forma de carvão mineral e vegetal, e microrganismos, os quais correspondem de 1-5% do carbono orgânico total do solo. O carbono também é encontrado em 47% do peso seco da célula microbiana e, avaliando a biomassa de carbono, pode-se obter informações de mudanças rápidas nas propriedades orgânicas do solo que podem ser causadas por rotação de cultura, manejo de solo, aeração ou devastação de florestas, verificação da regeneração do solo após a retirada da camada superficial, bem como avaliação dos efeitos de compostos tóxicos no solo, muitas vezes antropogênicos e recalcitrantes, advindos da poluição ambiental (hidrocarbonetos derivados de petróleo, PCBs, herbicidas, metais pesados, etc.) (Sparling, 1997; Gregorich *et al.*, 1997; Andréa e Hollweg, 2004).

A biomassa microbiana é uma estimativa da massa microbiana viva total e varia em termos temporais e espaciais, sendo determinada por fatores bióticos (tipo de vegetação, relações ecológicas nos micro-ambientes do solo) e abióticos (mudança nas propriedades físico-químicas do solo, sazonalidade de umidade e temperatura). Uma vez que a comunidade microbiana é sensível a tais variações e, por apresentar relação direta na ciclagem de nutrientes e fluxo de energia, a biomassa de carbono é utilizada como um bioindicador dos níveis de matéria orgânica no solo e também da qualidade do solo (De-Polli e Guerra, 1999; Gama-Rodrigues, 1999; Nielsen e Winding, 2002).

O crescimento microbiano pode ser avaliado também com base na concentração de algumas substâncias intracelulares ou ligadas à membrana (proteínas, ergosterol, LPFAs) e paredes celulares (ácido teicóico) ou ainda na atividade de algumas enzimas

(desidrogenase, urease, fosfatase, etc), sendo estas auxiliares na interpretação das condições funcionais da biomassa microbiana (De-Polli e Guerra, 1999).

Uma outra medida indireta da biomassa microbiana no solo é a respiração basal (evolução de CO_2), que indica diretamente a atividade de microrganismos heterótrofos (Kennedy e Smith, 1995; Paul e Clark, 1996; Dilly, 2001; Nielsen e Winding, 2002). A taxa de respiração basal por unidade de biomassa microbiana é denominada quociente metabólico, qCO_2 (Anderson e Domsch, 1985 e 1993). O qCO_2 está embasado na Teoria de Odum (1969 e 1985) e é considerado um índice de mudança da qualidade da atividade microbiana em resposta a fatores de estresse no solo e também indica a eficiência da biomassa em utilizar as fontes de carbono disponíveis no solo (Wardle e Ghani, 1995). Em solos não estressados, a ocorrência de uma perturbação - alteração ambiental rápida - pode aumentar o qCO_2 reduzindo a eficiência microbiana; enquanto que em solos estressados - em equilíbrio, porém com falta de nutriente, oxigênio ou em pH desfavorável - a ocorrência de uma perturbação pode diminuir o qCO_2, aumentando a eficiência microbiana de incorporar uma fração significativa de carbono na biomassa, perdendo menos carbono em forma de CO_2 pela respiração (Wardle, 1994; Gama-Rodrigues, 1999).

Portanto, tanto a biomassa microbiana quanto à respiração basal e o quociente metabólico podem ser utilizados como indicadores de mudanças na qualidade dos solos (Brookes, 1995; Giller *et al.*, 1998).

2.7. O uso de técnicas moleculares nos processos de biorremediação

Estudos moleculares *in situ* são de extrema relevância, pois caracterizam a estrutura da comunidade microbiana e indicam as mudanças da diversidade desta comunidade no local contaminado, bem como a dinâmica da degradação dos compostos poluentes, uma vez que 90-99% dos microrganismos potencialmente degradadores em solos contaminados não são isolados e cultivados com sucesso em condições de laboratório (Rozsak e Colwell, 1987; White, 1995).

O perfil genético de fragmentos amplificados de DNA, através da técnica de PCR (*polymerase chain reaction*), tem sido atualmente analisado em estudos de comunidades microbianas em solos, estuários, lagos e rios (Muyzer *et al.,* 1995; Ferris *et al.,* 1996; Murray *et al.,* 1996; Heuer *et al.,* 1997; Ovreås e Torsvik, 1998; Torsvik *et al.,* 1998; Zwart *et al.,* 1998).

O método de amplificação de fragmentos de genes utilizando a polimerização em cadeia pela Taq DNA-polimerase, enzima produzida pelo microrganismo termofílico *Thermus aquaticus* e comercializada para reações de PCR, permite a multiplicação em escala exponencial dos fragmentos de DNA previamente extraídos, para diversas análises moleculares, como visualização de bandas em gel de agarose após eletroforese, indicando a presença de genes de metabolização de compostos de interesse; ou ainda para uso em géis de poliacrilamida com gradiente desnaturante no intuito de monitorar a dinâmica microbiana em um processo de biodegradação em solo contaminado.

A técnica de PCR consiste na adição em um microtubo do DNA total extraído da matriz a ser analisada ou do próprio crescimento individual de microrganismos (Figura 3a); um tampão contendo $MgCl_2$ que atua como co-fator para a Taq-DNA polimerase, o par de iniciadores específicos (fragmentos curtos de fita simples de DNA no sentido 5'-3' e 3'-5') para o gene de interesse usados para iniciar a amplificação de algum ponto específico no DNA; os nucleotídeos A, T, G e C; e a enzima Taq polimerase que atua na síntese de novos fragmentos a partir do molde de DNA. Ciclos sucessivos de desnaturação do DNA em fita simples, anelamento dos iniciadores e extensão dos fragmentos pela Taq-polimerase vão realizando a amplificação exponencial dos fragmentos do DNA de interesse (Figura 3b).

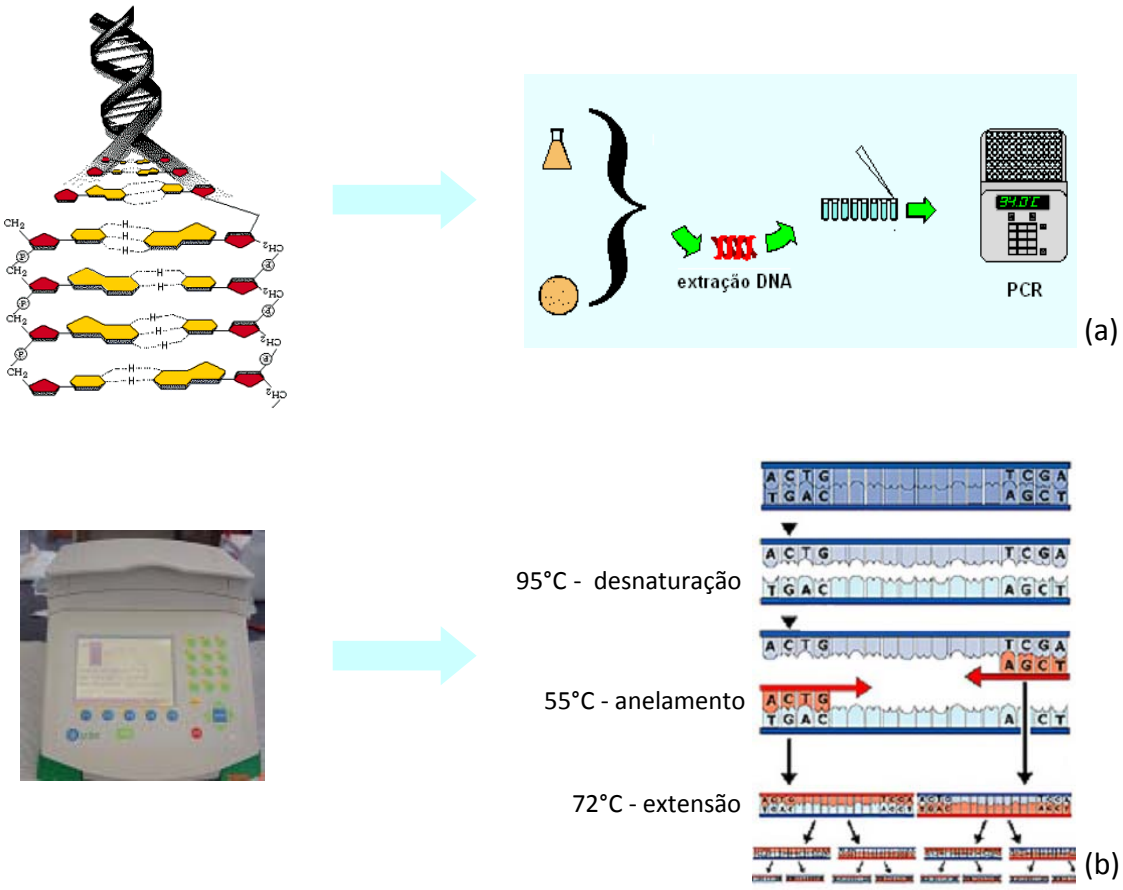

Figura 3. Molécula de DNA (genoma total) extraída de um microrganismo ou de uma microbiota em matriz de solo ou água (a), e esquema de uma reação de amplificação em cadeia de polimerase (PCR) em termociclador (b).

O perfil das populações de bactérias em diferentes matrizes ambientais tornou-se mais apurado com a utilização das técnicas moleculares, tais como eletroforeses em gel desnaturante por gradiente químico ou de temperatura (DGGE, TGGE), em gel de agarose com campo pulsado (PFGE), e ainda análise do comprimento de fragmentos ribossomais polimórficos terminais (T-RFLP) e da região intergênica entre as unidades ribossomais (RISA), hibridização fluorescente *in situ* (FISH), sondas de oligonucleotídeos de fragmentos

de RNAr marcados, amplificação de DNA polimórfico ao acaso (RAPD), análise de restrição de DNAr amplificado (ARDRA), entre outras.

Neste trabalho será tratado, em especial, sobre a técnica de eletroforese em gel de poliacrilamida com gradiente de desnaturação (DGGE) para monitoramento da dinâmica microbiana durante o processo de degradação dos HPAs. Uma avaliação preliminar da heterogeneidade microbiana do solo e da atividade metabólica da comunidade pode ser promovida pelo DGGE em tempo real (RT-DGGE), de acordo com Zocca e colaboradores (2004). Análises da expressão de genes do RNA mensageiro em tempo real têm sido usadas para verificar o metabolismo de HPAs de baixo peso molecular, onde o PCR com transcrição reversa pode identificar a expressão de genes catabólicos para a degradação de naftaleno, por exemplo (Wilson *et al,* 1999) .

A técnica de DGGE foi desenvolvida por Fisher e Lerman (1979), complementada pelos mesmos autores em 1983, como sendo uma técnica sensível para diferenciar fragmentos de DNA de dupla hélice que atingiam seu *melting point* em determinada concentração de gel desnaturante (promovido por formamida e uréia), enquanto migrava por eletroforese. Essa técnica separa produtos de PCR de mesmo comprimento e com diferentes seqüências de bases nitrogenadas, de acordo com o comportamento de abertura das fitas quando os fragmentos atingem seus respectivos *melting points*.

Os produtos são gerados por PCR através da amplificação do fragmento do gene RNA ribossomal 16S, utilizando iniciadores (*primers*) específicos contendo um grampo-GC ligado a um dos iniciadores. O grampo é rico em guanina e citosina, variando de 30 a 50 nucleotídeos. Tais bases, possuindo ligações triplas por ponte de hidrogênio formando dupla hélice, previnem a dissociação completa das fitas em fita única (Muyzer and Smalla, 1998). As comparações de seqüências de nucleotídeos mostram que há algumas regiões no gene do RNAr 16S que são altamente conservadas entre os organismos, e outras regiões variam em diferentes graus. A variabilidade nestas regiões aumenta quando se aumenta a distância filogenética das espécies (Woese, 1992).

Por serem os produtos amplificados geralmente de tamanhos iguais, quando migrados em gel contendo concentrações crescentes de cima para baixo de gradiente desnaturante, cada amplicon vai cessando sua migração no gel na medida em que 50% da dupla hélice se desnatura, siginificando que atingiram o *melting point*, ocorrendo então a separação das diferentes espécies microbianas (Muyzer *et al.*, 1993 e Muyzer, 1999). As espécies que apresentam *melting points* mais baixos, alcançam posições superiores no gel, enquanto que aquelas que apresentam *melting points* mais altos, possuem maior migração, verificando a presença de bandas na base do gel (Muyzer e Smalla, 1998) – Figura 4.

A técnica de DGGE, originalmente desenvolvida para detectar espécies mutantes, tem sido considerada vantajosa no estudo de comunidades microbianas quando alguns fatores estão selecionando populações específicas de microrganismos. Este método é considerado simples e rápido para a análise qualitativa por comparação dos perfis eletroforéticos para o monitoramento de mudanças na comunidade devido a algum distúrbio ambiental. Baseado na presença ou ausência de bandas em cada amostra, o coeficiente de similaridade pode ser determinado para os perfis do DGGE, usando tanto iniciadores universais quanto específicos (Nübel *et al.,* 1997; Kowalchuk *et al*., 1997).

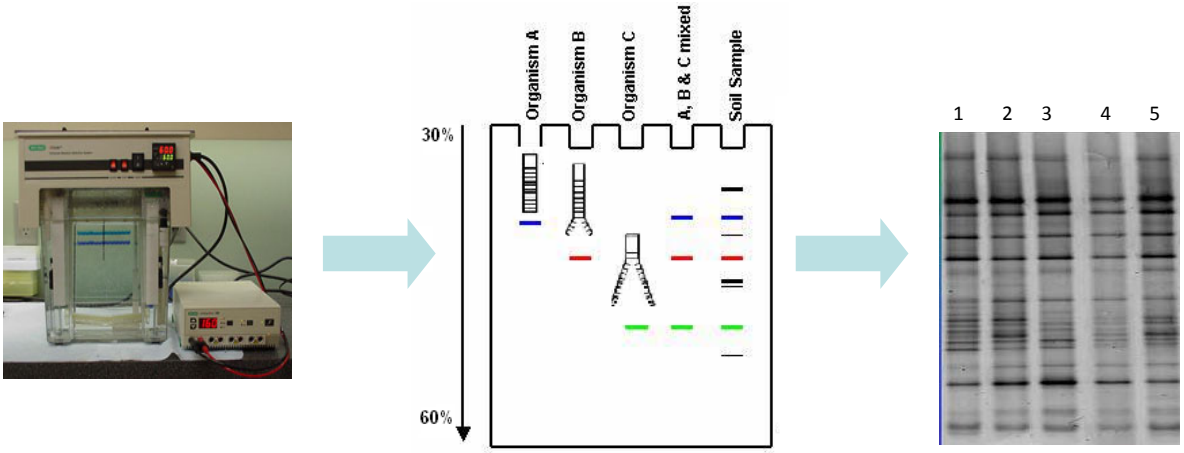

Figura 4. Técnica de DGGE utilizando produtos de PCR de uma amostra de solo, sendo separado cada organismo de acordo com seu *melting point*, originando várias bandas.

O uso de iniciadores específicos permite estudos de mudanças temporais e espaciais nas populações microbianas, avaliando o efeito de uma bioaumentação a longo prazo num processo de biorremediação, sua estabilidade e a dinâmica da microbiota (van Elsas *et al.*, 1998). No entanto, algumas dificuldades ocorrem para esta técnica, como a detecção de espécies em quantidades menores que 1% na matriz a ser avaliada e a formação de bandas *heteroduplex* (dois produtos desnaturados de PCR distintos que se ligam por re-anelamento), as quais podem contribuir para a superestimação de bandas em uma comunidade muito complexa (Ferris *et al*, 1997). No entanto, este problema pode ser resolvido utilizando tampão com maior força iônica, uma menor temperature de anelamento no PCR, uma maior concentração de *primers*, ou ainda diminuindo o número de ciclos de amplificação (Jensen e Straus, 1993). Muyzer e colaboradores (1993), analisando os fragmentos do gene RNA ribossômico 16S para identificar o perfil da comunidade microbiana em um biofilme após separação em DGGE, verificaram que o número de bandas correspondia ao número de espécies predominantes na comunidade. As bandas separadas puderam ser extraídas do gel e eluídas para sequenciamento, identificando os membros dominantes possivelmente com maiores habilidades metabólicas nestes ambientes (Muyzer e de Wall, 1994; Rölleke *et al.,* 1996; Kowalchuk *et al.,* 1997).

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3. Objetivos

Neste trabalho, foram destacados alguns objetivos importantes para o direcionamento dos estudos no período de desenvlovimento desta tese:

- Avaliar o impacto da contaminação por HPAs em um solo na microbiota nativa e também bioaumentada com consórcios de bactérias, fungos e uma mistura destes consórcios, no que diz respeito ao crescimento, à atividade respiratória microbiana (evolução de CO_2), bem como à eficiência microbiana de utilizar os HPAs como fontes de carbono (coeficiente metabólico, qCO_2);

- Determinar a remoção dos HPAs presentes nos microcosmos de solo por cromatografia gasosa com detector de ionização de chama (CG-FID) e também a produção de metabólitos intermediários da degradação dos HPAs para o solo natural impactado - microbiota local - e para os tratamentos de bioaumentação neste solo;

- Monitorar por eletroforese em gel com gradiente denaturante (DGGE) a dinâmica de uma microbiota de solo contaminado com HPAs a longo prazo, observando o comportamento de adaptação e dominância de certos organismos no decorrer da incubação com HPAs isolados ou em uma mistura com 2 a 7 anéis aromáticos;

- Identificar microrganismos predominantes e verificar a presença de genes catabolicamente ativos nos microrganismos isolados, verificando ainda possíveis diferenças genéticas entre organismos da mesma espécie.

4. Capítulo 1

Metabolic efficiency of microorganisms in a soil impacted with polycyclic aromatic hydrocarbons using bioaugmentation*

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Abstract

A PAH-contaminated soil was assessed with regard to the microbial behavior to degrade a mixture of these complex hazardous compounds. Microbial biomass, respiratory activity and metabolic efficiency to use such compounds as carbon sources were determined in PAH-impacted soil microcosms. Bioaugmentation experiments were performed using a bacterial consortium, previously isolated from a diesel oil-contaminated site; a soil fungal consortium, selected as good degraders of PAHs; each fungal strain, inoculated individually; and a mixture of both consortia. The soil microcosms were conducted through 12 weeks, and results of the microbiota performance into soil without PAHs, impacted with PAHs, and bioaugmented with microorganisms were then evaluated. The non-bioaugmented native microbiota had some contribution for the PAHs degradation in the impacted soil. Both native microbiota and bioaugmented microorganisms performed a rapid response of growth and respiratory activity, showing great adaptation and competitiveness. The carbon biomass and respiratory activity into PAH-contaminated microcosms increased - indicating the use of PAHs as carbon source, followed by a drop when microorganisms showed effectiveness in spending less energy to survive in the soil with disturbance. Recovery in the microbial efficiency was observed by decreasing the CO_2 evolution level, and then a steady microbial growth showed that even native or inoculated microorganisms were able to grow and survive into PAH-contaminated soil. They showed a rapid adaptation and played some metabolic cooperation into soil.

Key-words: polycyclic aromatic hydrocarbons (PAHs); biodegradation; bioaugmentation; C-biomass; microbial respiration; metabolic quotient.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are formed by fused benzene rings in linear, angular or group arrangements and are considered priority pollutants due to their toxic, mutagenic and carcinogenic potential (Cerniglia, 1992). Sources of pollution with PAHs include natural emissions (forest burning, volcanoes), and anthropogenic activities (incomplete combustion of fuel, crude oil and wood, cigarettes, and industrial activities such as processing of petroleum products and coal, incineration, etc.) (Reid *et al.*, 2000; Semple *et al.,* 2001 and 2003). Because of their hydrophobicity, PAHs are naturally associated with soil and sediment particles, becoming less available to degradation.

It is still difficult to explain clearly the mechanism of biodegradation of a complex mixture of contaminants by heterogeneous microbial communities (Toledo *et al,* 2006). Such degradations depend on the bacterial choice and type of PAH mixture (Bouchez *et al.*, 1995). Furthermore, the impact of some PAHs on the rates of other PAHs disappearance revealed some interaction between them, when enzyme induction occurs by the pre-exposure of microbiota to some other PAH (Bauer and Capone, 1988). The metabolic cooperation by several microorganisms may result in an enhanced PAH utilization, since metabolic intermediates produced by some organisms may serve as substrates to the growth of some others (Keck *et al.*, 1989; Bhatnagar and Fathepure, 1991).

The use of native microbiota from contaminated soils in bioremediation is of great interest as it may be more adapted than some commercial microbial inocula which could be out-competed by indigenous microorganisms (Grosser *et al.*, 1995). However, when the natural microbiota does not show any ability to degrade the pollutant compounds, the bioaugmentation using microorganisms with desired degradation capability is a possible means to enhance the success of the process (Vogel, 1996).

Microbial activities into soil can be evaluated by some factors such as biomass content, microbial respiration and the relation between respiration and growth (qCO_2), considered as good indicators of pollution degree in contaminated soils (Anderson, 1982; Anderson and Domsch, 1990; Wardle and Ghani, 1995; Kuperman and Margaret, 1997; Marin *et al.*, 2005).

In this work, we focused on the behavior of soil microorganisms, when the soil was impacted with PAHs and bioaugmented with bacterial and fungal consortia, soil fungi individually, as well as a mixture of these consortia, assessing the biomass growth, respiratory activity and the microbial efficiency of PAH biodegradation.

2. Materials and methods

2.1. Chemicals

Naphthalene, phenanthrene, anthracene, pyrene, dibenzo[a]anthracene, benzo[a] pyrene (all PAHs >98% of purity) were purchased from Sigma-Aldrich, Brazil. Bacterial and fungal growth media were acquired from Oxoid, Brazil. Reagents for protein assay were obtained from BioRad Laboratories, USA, and all other chemical were high-purity analytical grade.

2.2. Screening assays

Bacteria isolated from a diesel oil-contaminated site (Paulínia, SP, Brazil) and belonging to the culture collection of The Microbial Systematics and Physiology Laboratory (FEA-UNICAMP, Brazil) were screened for their capability of degrading pyrene. Forty two strains were inoculated onto minimal media plates containing per liter: K_2HPO_4 0.4 g, KH_2PO_4 0.4 g, $(NH_4)_2PO_4$ 0.4 g, NaCl 0.3 g, 1 ml of micronutrients solution (Bogardt and Hemmingsen, 1992), 1 ml of vitamins solution (Wolin *et al.*, 1963), plus agar 1.5% and pyrene 400 $mg.l^{-1}$ in dimethylformamide. Both solutions were sterilized by filtration (0.22 μm, Millipore) and added to medium after sterilization.

Another rapid screening was performed at minimal medium and pyrene 1% in ethereal solution, sprayed over the surface of agar plates, followed by bacterial inoculation and incubation at 30°C for 7-14 days for both screenings. The pyrene was utilized in the screening assays since this PAH is relative persistent (4-rings) into the environment and often used as a model substrate in degradation studies of high-molecular-weigh (HMW-) PAHs (Gaskin and Bentham, 2005). Bacteria that reached the fastest growth in both screening during incubation period were selected to perform the microcosm experiments.

The soil fungi used in this study were also chosen according to previous research conducted in our laboratory. They are the best degraders of PAHs in liquid media under aerobic (Clemente *et al.*, 2001) and microaerobic conditions (Silva and Durrant, 2007 – writing manuscript).

2.3. Microbial consortia

The bacterial consortium was composed of 5 strains of bacteria [*Chromobacterium* sp. (1), *Enterobacter aglomerans* (2), B1F and B5A (3 and 4) – Gram-positive rods, and B3G (5) – Gram-positive cocci]. The sequencing procedure is on the way to these unidentified strains, as part of this work. Bacteria were grown in NA (nutrient agar) for 7 days and transferred to flasks containing nutrient broth plus pyrene 200 $mg.l^{-1}$ for 18 hours at 30°C/150 $rev.min^{-1}$, followed by centrifugation at 4°C for 10 minutes. Cells were washed twice with phosphate buffer 0.1 M pH 7.0, and suspended in the same buffer volume as

used for cultivation. The inoculum of each strain was standarded as 10^5cells.g soil^{-1}, and added directly into the respective microcosms.

The three soil filamentous fungi strains used in the microcosms: *Achremonium* sp., *Aspergillus* sp and *Verticillium* sp, were previously grown for 72 hours in liquid medium containing glucose 2%, yeast extract 0.5%, peptone 1%, malt extract 1% and pyrene 200 mg.l^{-1} at 30°C/100 rev.min^{-1}. Fungal hyphae were centrifuged and washed twice with phosphate buffer as described previously, and 12.5 mg.g soil^{-1} (wet weight) of mycelia were added as inoculum of each fungal strain in the respective microcosms. The mixture of both consortia was prepared in flasks adding sterile distilled water to help in the mixture of microorganisms by shaking, followed by inoculation into the respective microcosm.

2.4. Soil sample

A non-contaminated soil was collected from an experimental site of the Campinas Agronomic Institute (IAC-Pindorama, SP, Brazil), and analyzed for its physic-chemical characteristics before the artificial contamination with a defined concentration of PAHs. The soil sample had 9.9% clay, 9.8% silt, 80.3% total sand, 50 g.dm^{-3} organic matter, 16 mg.dm^{-3} P, 3 mmol$_c$.dm^{-3} K; high levels of Ca (65 mmolc.dm^{-3}), Mg (13 mmol$_c$.dm^{-3}) and micronutrients (0.23 mg.dm^{-3} B, 0.9 mg.dm^{-3} Cu, 47 mg.dm^{-3} Fe, 48.4 mg.dm^{-3} Mn, 5.8 mg.dm^{-3} Zn), according to IAC-Laboratory of Soil and Plant Analysis (Campinas, SP, Brazil).

The soil collected was dried at constant weight at room temperature for some days and sieved through a 2-mm mesh. Initial pH (6.16) was measured in case of any necessary adjustment to optimize conditions to the PAH degradation by the native microbiota or by the augmented microbial communities. The water content of soil was adjusted to 50% of the water-holding capacity, and after 24 hours the initial number of bacteria and fungi was determined before microcosm studies.

2.5. Preparing soil microcosms

PAHs were added previously to 400 g of soil in 1.5 l jar-microcosms, and PAH final concentrations in each microcosm were defined as follows (mg.kg soil^{-1}): naphthalene and phenanthrene, 150 each; anthracene and pyrene, 100 each; benzo[a]anthracene and benzo[a]pyrene, 50 each. After thorough mixing with a sterile glass rod, bioaugmentations were performed with the bacterial and fungal consortia, each fungi separately, and the mixture of these consortia. The period of incubation was 90 days at 24-25°C in the dark, and all microcosms treatments were run in triplicate: non-contaminated soil (soil-PAHs), and contaminated soils containing: only the native microbiota (soil+PAHs), the bacterial consortium 5BAC (five bacterial strains), each fungus separately (*Achremonium* sp*., Verticillium* sp., *Aspergillus* sp.), the fungal consortium 3F (three fungal strains), and the mixture of these consortia.

The soil aeration into microcosms was carried out weekly by mixing the soil with a sterile glass rod, and soil moisture was amended to 50% of water hold capacity by adding sterile distilled water when needed.

2.6. Determining microbial growth

2.6.1. Counting plates method

Soil samples (1 g) were extracted from microcosms weekly, after mixing the soil with a sterile glass rod, and placed into a flask containing 10 ml of peptone solution 0.1% (w/v). A 100 µl aliquot was transferred to microplate wells containing 900 µl of the same peptone solution. A 10-fold serial dilution was carried out into the microplate, and 100 µl of each well content were spread onto PCA (plate counting agar) followed by incubation at 30°C for 48 hours to the bacterial cells count per gram of soil, and onto PDA (potato dextrose agar) for 7 days at the same temperature to the fungal cells count per gram of soil.

2.6.2. Analysis of microbial C-Biomass using the fumigation-extraction method

The microbial C-biomass was analyzed by the fumigation–extraction method (Vance *et al.*, 1987, modified) using 5 g soil samples in duplicate, collected weekly during a 90-day period to determine C-biomass in different treatments of bioaugmentation into PAHs-impacted soil. One of the duplicates was fumigated with ethanol-free chloroform (25 ml) in a desiccator lined with wet filter paper to maintain humidity. Chloroform was placed in a beaker with glass beads, the desiccator was closed and vacuum was applied for a few minutes to fumigate the samples - which remained in the close desiccator for 24 hours – to break the microbial cells and release all cytoplasm contents of microorganisms in the soil (Powlson and Jenkinson, 1976; Frighetto, 2000). The excess chloroform steam was removed from samples applying vacuum to the desiccator 3-5 times using distilled water instead of chloroform. According to this methodology, microbial biomass is proportional to the increase of extractable organic carbon after fumigation. Non-fumigated extracts contain only extracellular organic matter, whereas fumigated extracts contain the intracellular organic matter (biomass) and also extracellular (Hofman and Dusek, 2003). The released cellular material was extracted with K_2SO_4 0.5 M (40 ml) for 30 minutes under rotatory agitation (150 rev.min^{-1}), followed by filtration of the extracts using filter paper Watman n°42.

C-biomass of fumigated and non-fumigated extracts was determined by oxidation of the organic matter (8 ml extract) with $K_2Cr_2O_7$ 66.7 mM (2 ml) and H_2SO_4:H_3PO_4 (2:1; 15 ml), using a heater block at 100°C for 30 minutes. Afterwards, tubes containing oxidized extracts were cooled down and transferred to flasks, where 25 ml of distilled water and 5 drops of indicator solution 1,10-phenanthroline-ferrous sulphate 25 mM were added to a back titration of the dichromate excess, using $[(NH_4)_2Fe(SO_4)_2 .6H_2O]$ 0.0333 mol.l^{-1} in H_2SO_4 0.4 mol.l^{-1}. The color of the initial solution was green and; after turning-point

titration, raddish-red. The extractable carbon was calculated assuming that 1 ml de $K_2Cr_2O_7$ 66.7 mM or 0.4 N is equivalent to 1,200 μg C, and in accordance with the equation as follows (Alef and Nannipieri, 1998):

C (μg.l^{-1}) = [(H-S)/C] x [(N x V/A) x E x 1,000], where: H = ml of consumed solution by the hot blank titration (refluxed); S = ml of consumed solution by the sample titration; C = ml of consumed solution by cold blank titration (unrefluxed); N = normality of $K_2Cr_2O_7$ solution; V= volume of $K_2Cr_2O_7$ added to the reaction mixture; A = aliquot of extract; E = conversion of Cr^{+6} to Cr^{+3}.

Transforming C (μg.l^{-1}) to C (μg.g soil^{-1}):

C (μg.g soil^{-1}) = C (μg.l^{-1}) x [k/(DW+W)], where: K = volume of extractant; DW = dry weight of soil (in gram); W = soil water (%DW:100).

Calculating the microbial biomass:

C-Biomass (μgC.g dry soil^{-1})= 2.64 x E_c, where: 2.64 is k_{ce} factor to conversion of extractable carbon to microbial biomass, and $E_c = C_{fumigated} - C_{non\text{-}fumigated}$.

2.7. Determination of microbial basal respiration and metabolic efficiency

Microbial respiratory activities in PAHs-impacted soil microcosms were evaluated based on a cumulative CO_2 evolution, according to Ferreira *et al.* (1999) - modified. A CO_2-trapping system was set for each microcosm, where NaOH 0.5 M (20 ml) was added in a small plastic glass in an aluminum tripe. The jars were closed for some period of hours, and opened to determine the microbial respiration, repeating this procedure during 90 days. After incubation for the respective time periods, microcosms were opened and $BaCl_2$ 1 M (5 ml) was added into the CO_2-trapping NaOH solution. A $BaCO_3$ precipitation appeared as indicative of CO_2 recovery, and the volume was transferred to flasks, adding 3 drops of phenolphthalein alcoholic solution 1M. The NaOH excess in the solution was then titred with HCl 1.196 M and the amount of CO_2 released in each microcosm during microbial respiration was inversely proportional to the volume of HCl used in the titration. The CO_2 production during microbial basal respiration was calculated using the equation as follows:

mg C-CO_2 g.soil^{-1}= (V_b-V_s) x M x Eq , where: V_b = ml of acid solution necessary to titrate the blank (jars with trapping-CO_2 system with no soil); V_s = ml of acid solution necessary to titrate the NaOH excedent in the sample; M = HCl molarity; Eq = equivalent weight or carbon.

The microbial metabolic quotient rate (qCO_2) was also determined as the microbial activity (respiration in μg.h^{-1} C-CO_2) per unit of microbial biomass (μgC. g dry soil $^{-1}$), as described by Anderson (1994).

2.8. Statistical analysis

Statistical significances of data from microcosm experiments were conducted by analysis of variance and the averages were compared by the Tukey test at $p \leq 0.05$ to verify differences among treatment to the same week, and also among weeks to each treatment.

3. Results and discussion

3.1. Microbial growth

The initial soil microbiota before PAH microcosm settings was measured related to bacteria and fungi populations: 3.5 x 10^6 and 6.5 x 10^4 CFU.g $soil^{-1}$, respectively. Some changes in the microbial community could occur due to the addition of pollutants compounds that may serve as carbon source. In the initial 1-week period, all treatments increased three orders of magnitude in cell numbers, except for the non-impacted soil containing the native microbiota (soil+PAHs). Up to week-4, the number of bacteria per gram of soil remained between 10^8-10^9 CFU .g $soil^{-1}$, followed by a slight decrease of cell numbers, that was sustained about 10^8 CFU.g $soil^{-1}$ through week-12 (Figure 1).

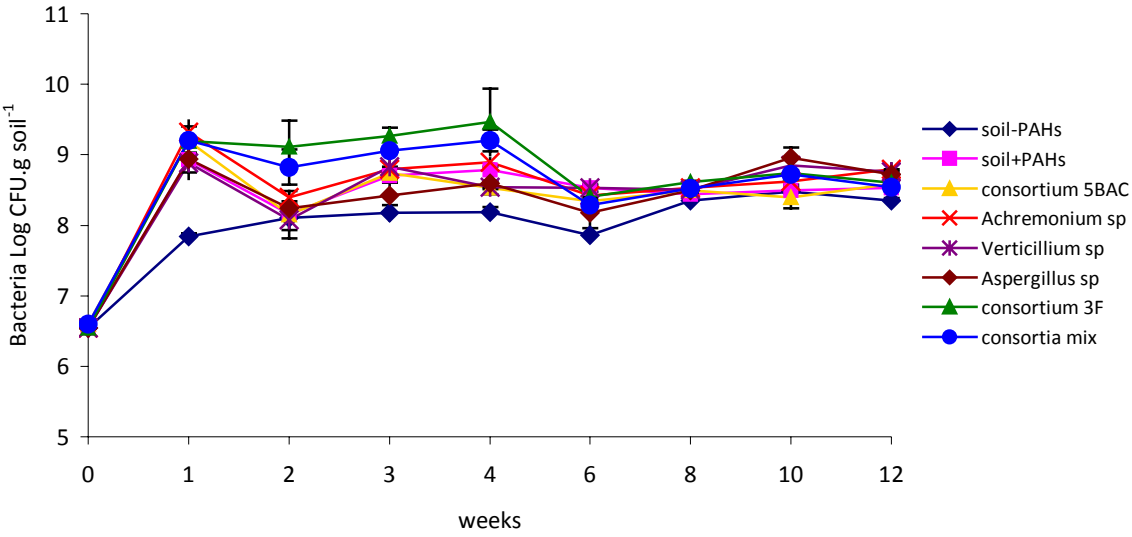

Figure 1. Bacterial growth in soil microcosms: non-impacted soil (soil-PAHs), PAH-impacted soil containing only the native microbiota (soil+PAHs), soil bioaugmented with bacterial consortium (consortium 5BAC), fungal consortium (consortium 3F), each fungus separately (*Achremonium* sp, *Aspergillus* sp, *Verticillium* sp), and the mixture of both consortia (consortia mix). All data are mean of triplicate determination and error bars represent SD.

Some authors have shown that after bioaugmentation a total soil microbiota doubled in 7 days, reaching a 10-fold-increased CFU, followed by a decrease after 7 days (Kästner *et al.*, 1998), and declining factors of ≈100 CFU.g $soil^{-1}$ in 30 days, as also

observed by Ogunseitan *et al.* (1991) and Wagner-Döbler *et al*. (1992). On the other hand, in experiments conducted by Bento *et al.* (2005), the number of diesel-oil degrading microorganisms decreased after week-1, and was not affected by the degradation process until the end of week-12. According to Scelza *et al.* (2006), the indigenous microbiota of a PAH-contaminated soil was inhibited by the addition of bacterial cells. Moreover, microbial population in a phenanthrene-contaminated soil bioaugmented with bacteria dropped 10^2 CFU.g soil^{-1} after a 9-week incubation period. These authors attributed this fact to a limitation of nutrients, suggesting high inoculation or reseeding the soil after a period of time to ensure adequate degradation of phenanthrene levels (Trzesickia-Mlynarz and Ward, 1996).

The present study showed that, neither the bioaugmentation nor the presence of PAHs seem to be an inhibitory factor for the growth of the native microbiota and the bioaugmented soil microorganisms. Statistical differences among each treatment to a same week were analyzed by Tukey test in 95% of confidence. The treatment containing the non-impacted soil (soil-PAHs) was statistically lower than the other treatments at week-1 (Table 1). From this period on, the bacterial growth into the non-bioaugmented microcosm containing only the native microbiota (soil+PAHs) did not show statistically significant difference among treatments using bioaugmentation, indicating a fast adaptation of the soil microbiota after the disturbance with PAHs. Analyzing each treatment in the whole period of the microcosms incubation: soil+PAHs, the both consortia, *Verticillium* sp., and *Aspergillus* sp. did not show statistical differences from week-1 to week-12.

Table 1. Bacterial growth over time (12 weeks) in soil microcosms receiving different treatments

Microcosm treatments	Bacteria log UFC . g soil $^{-1}$							
	Week 1	Week 2	Week 3	Week 4	Week 6	Week 8	Week 10	Week 12
Soil – PAHs	7.84± 0.06 aA	6.00± 0.00 aB	8.15± 0.15 aE	8.18± 0.10 abcB	7.84± 0.14 aA	8.33± 0.15 aBC	8.48± 0.00 abCD	8.34± 0.05 aBD
Soil + PAHs	8.77± 0.38 bA	7.25± 0.08 bA	8.70± 0.00 acB	8.78± 0.06 abcA	8.52± 0.10 aA	8.44± 0.04 abA	8.48± 0.12 abA	8.53± 0.03 abA
Consortium 5BAC	9.04± 0.43 bA	7.24± 0.68 bA	8.71± 0.18 acB	8.53± 0.03 abcA	8.33± 0.12 aA	8.49± 0.07 abA	8.33± 0.30 bA	8.58± 0.10 abA
Achremonium sp	9.23± 0.33 bA	7.07± 0.10 abBC	8.77± 0.18 acD	8.88± 0.11 abcAB	8.42± 0.12 aC	8.53± 0.02 abBC	8.62± 0.09 abBC	8.77± 0.17 bBC
Verticillium sp	8.88± 0.43 bA	7.10± 0.30 abA	8.74± 0.39 acB	8.51± 0.18 abcA	8.52± 0.11 aA	8.5± 0.09 abA	8.88± 0.21 aA	8.76± 0.07 bA
Aspergillus sp	8.93± 0.11 bA	7.14± 0.12 abAC	8.33± 0.35 aB	8.59± 0.06 abcAD	7.89± 0.77 aBCDE	8.49± 0.07 abAE	8.90± 0.30 aAE	8.71± 0.11 bAE
Consortium 3F	9.19± 0.04 bA	7.45± 0.74 bA	9.22± 0.24 bcB	8.06± 0.94 bAB	8.38± 0.15 aAB	8.61± 0.04 bAB	8.74± 0.07 abA	8.60± 0.05 abAB
Consortia mix	9.20± 0.06 bA	8.21± 0.50 bA	9.06± 0.02 bcB	9.13± 0.31 cAC	8.28± 0.00 aB	8.52± 0.06 abBC	8.72± 0.07 abAB	8.54± 0.06 abBC

The same lower case letters are not statistically different among treatments, and the same capital letters are not statistically different among weeks to each treatment by the Tukey test (p<0.05), ±SD (n=3).

Analyzing the fungal growth into microcosms (Figure 2), each fungus added to the soil, and also the native microbiota had a drop in cell numbers at week-1, differing from results of bacterial growth (Figure 1). Both microbial consortia and its mixture did not show significant changes in the number of fungal cells in this period. The oxygen was supplied by mixing microcosms to stimulate mainly the fungal population and to avoid the inefficient metabolism due to lack of oxygen.

In this case, fungi could probably be inhibited by bacterial growth in the beginning of their adaptation into impacted soil. PAHs could be a reason for decreasing number of fungal cells in this period. Fungi have increased the growth after week-1 and it is likely to be an adaptative phase to these fungi. Both bacterial and fungal growth reached some

stability in the number of cells from week-4 on, showing the capability of adaptation into contaminated soil and possible cooperative metabolism between these two groups of microorganisms. Nannipieri *et al.* (2002) reported that level of enzymes responsible for degradation can increase without any change in the microbial biomass.

The initial differences in the fungal cells into the microcosms might have a response effect when comparing the bioaugmented treatments related to the initial adaptation of the cells and their performance through 12 weeks. The microcosms that received more fungal inoculum (3F and consortia mix) did not suffer high depletion of fungal cells in the initial period than the other treatments.

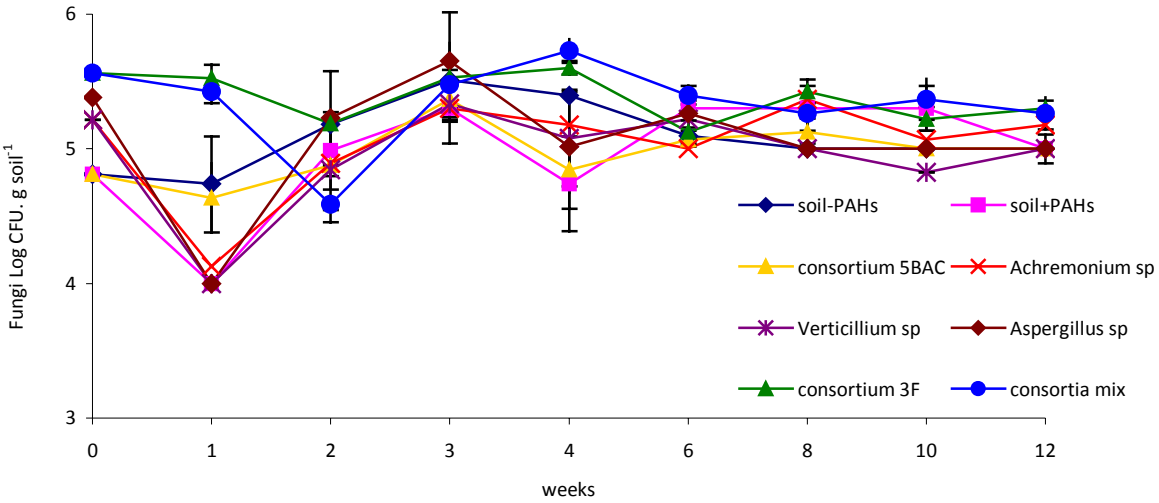

Figure 2. Fungal growth in soil microcosms: non-impacted soil (soil-PAHs), PAH-impacted soil containing only the native microbiota (soil+PAHs), soil bioaugmented with bacterial consortium (consortium 5BAC), fungal consortium (consortium 3F), each fungus separately (*Achremonium* sp, *Aspergillus* sp, *Verticillium* sp), and the mixture of both consortia (consortia mix). All data are mean of triplicate determination and error bars represent SD.

At week-1, the fungal consortium and the mixture of consortia had statistically higher values of fungal growth (Table 2), and fungi inoculated separately did not differ statistically from the non-contaminated soil and the impacted soil containing only the native microbiota. From the week-2 on, soil containing only the native microbiota did not show statistical differences compared with other bioaugmentation treatments, except to the fungal consortium at week-4 and consortia mix at weeks-2 and -4. All microcosms showed fungal growth remaining statistically similar during the whole period, indicating steady growth.

Table 2. Fungal growth over time (12 weeks) in soil microcosms receiving different treatments

Microcosm treatments	Fungi log UFC . g soil $^{-1}$							
	Week 1	Week 2	Week 3	Week 4	Week 6	Week 8	Week 10	Week 12
Soil – PAHs	4.00± 1.00 aA	6.00± 0.00 acB	5.50± 0.10 aB	5.39± 0.03 abB	5.09± 0.09 aB	5.00± 0.00 aAB	5.00± 0.00 aBA	5.00± 0.00 aAB
Soil + PAHs	4.00± 0.00 aA	7.40± 0.41 bC	5.30± 0.00 aB	4.00± 1.00 aA	5.25± 0.24 aB	5.25± 0.24 aB	5.25± 0.24 aB	5.15± 0.15 aAB
Consortium 5BAC	4.43± 0.51 acA	7.00± 0.00 abC	5.30± 0.30 aB	4.67± 0.58 abAB	5.06± 0.10 aAB	5.10± 0.17 aAB	5.00± 0.00 aAB	5.00± 0.00 aAB
Achremonium sp	4.10± 0.17 aB	7.00± 0.00 abC	5.26± 0.24 aA	5.13± 0.23 abA	5.00± 0.00 aA	5.32± 0.28 aA	5.06± 0.10 aA	5.18± 0.00 aA
Verticillium sp	4.00± 0.00 aA	6.55± 0.78 abdB	5.28± 0.27 aC	4.80± 0.72 abAC	5.16± 0.28 aC	5.00± 0.00 aAC	5.00± 0.00 aAC	5.00± 0.00 aAC
Aspergillus sp	4.00± 0.00 aA	7.00± 0.00 abD	5.60± 0.27 aB	4.78± 0.68 abAC	5.18± 0.31 aBC	5.00± 0.00 aBC	5.00± 0.00 aBC	5.00± 0.00 aBC
Consortium 3F	5.49± 0.20 bcAB	6.55± 0.78 abeB	4.98± 0.98 aAC	5.59± 0.10 bAB	5.10± 0.17 aAC	5.40± 0.17 aAB	5.20± 0.17 aAC	5.29± 0.11 aAB
Consortia mix	5.40± 0.17 bcA	5.58± 0.08 cdeA	5.28± 0.49 aA	5.70± 0.17 bA	5.38± 0.14 aA	5.30± 0.07 aA	5.30± 0.30 aA	5.22± 0.24 aA

The same lower case letters are not statistically different among treatments, and the same capital letters are not statistically different among weeks to each treatment by the Tukey test (p<0.05), ±SD (n=3).

Microorganisms responsible for the decomposition and mineralization of the organic fraction use part of the compounds contained in the residues as sources of nutrients and energy to their biomass formation (Brookes, 1995; Pontes, 2002).

In general, according to the C-biomass contents in the soil samples (Figure 3), the bioaugmented treatments showed higher values of biomass than non-contaminated soil and contaminated soil containing only the native microbiota, which was expected by adding more biomass into the system. Furthermore, the biomass production in these microcosms indicated that such microorganisms from soil did not have an expressive impact by the PAHs addition. When the soils were bioaugmented, some oscillation in the biomass were verified, suggesting that different microorganisms present into the microbiota were adjusting their mechanisms to survive in the PAH-contaminated soil, creating probably a dependent cooperation. All treatments has shown an increase of biomass up to week-4, as also observed in counting plates method, however, fungal consortium and the consortia mix had more differences in the biomass growth.

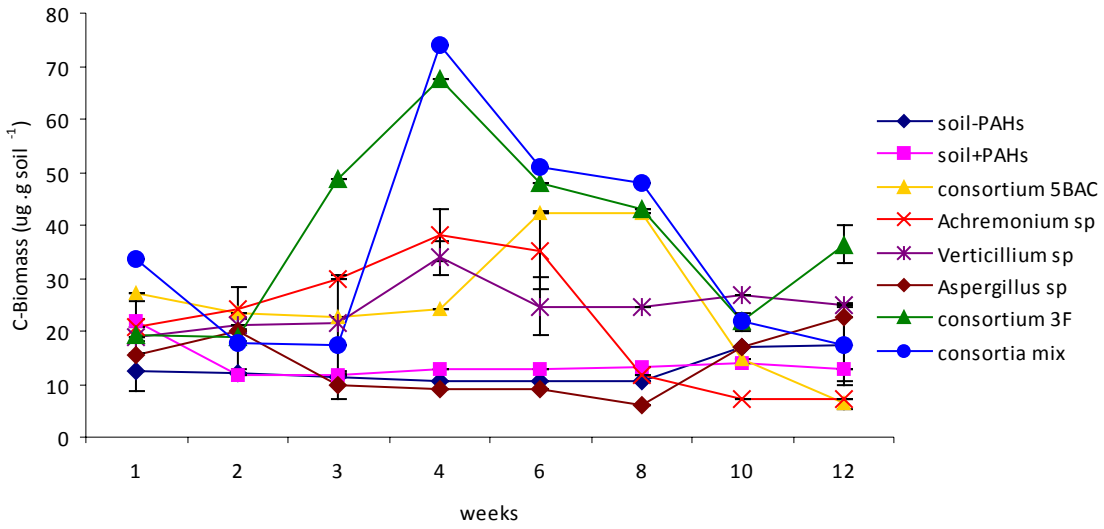

Figure 3. Microbial C-biomass in soil microcosms: non-impacted soil (soil-PAHs), PAH-impacted soil containing only the native microbiota (soil+PAHs), soil bioaugmented with bacterial consortium (consortium 5BAC), fungal consortium (consortium 3F), each fungus separately (*Achremonium* sp, *Aspergillus* sp, *Verticillium* sp), and the mixture of both consortia (consortia mix). All data are mean of triplicate determination and error bars represent SD.

Since fungi were inoculated in a mycelial form might favor the establishment of fungi into soil and contribute to higher levels of PAHs degradation (specially LMW-PAHs as observed in chapter 2) by maximizing mechanical contact and enzymatic stimulation, whereas spores inoculation is less successful due to a competitive interaction with indigenous soil microorganisms, expressing a poor catabolic potential as related previously by Potin *et al.* (2004).

A decrease in biomass values from week-6 to week-10 in soils containing *Achremonium* sp., the fungal consortium and the consortia mix, differs from the microbial growth by counting cell number. The reason for a loss in C-biomass was possibly due to the lack of homogeneous sampling, even when the soil was extensive mixed, once fungal mycelia contribute to a great amount of C-biomass content.

Table 3. C-biomass over time (12 weeks) in soil microcosms receiving different treatments

Microcosm treatments	C biomass (μgC . g dry soil^{-1})							
	Week 1	Week 2	Week 3	Week 4	Week 6	Week 8	Week 10	Week 12
Soil – PAHs	12.52± 0.13 aA	11.94± 0.12 aA	11.36± 0.11 aA	10.58± 0.11 aA	10.54± 0.11 aA	10.59± 0.11 aA	16.69± 0.17 aA	17.53± 10,02 aA
Soil + PAHs	21.84± 16.38 aA	11.52± 0.12 aA	11.70± 0.12 aA	12.69± 0.13 aA	12.81± 0.13 aA	13.24± 0.13 aA	13.90± 0.14 bA	13.00± 0.13 aA
Consortium 5BAC	27.29± 0.29 aC	23.23± 0.24 bA	23.01± 0.23 abA	24.23± 0.25 bA	43.22± 0.42 bcB	42.33± 0.42 bB	14.54± 0.15 abD	6.26± 1.25 aE
Achremonium sp	20.62± 0.21 aAB	24.27± 7.96 bAB	30.00± 0.30 bA	334,76± 6.10 cdA	35.27± 14.87 bcA	11.74± 0.12 aB	7.34± 0.07 cB	7.27± 0.08 aB
Verticillium sp	19.00± 0.19 aA	21.00± 0.21 abA	21.45± 13.65 aA	33.86± 0.34 dA	24.69± 10.66 abA	27.19± 0.25 cA	26.65± 0.27 dA	25.05± 0.25 aA
Aspergillus sp	15.47± 10.00 aABCD	19.86± 0.20 acAC	9.75± 3.90 aA	9.02± 0.12 aB	9.00± 0.29 aB	9.07± 0.46 aB	16.96± 0.17 aCD	22.54± 0.23 aD
Consortium 3F	19,10± 4.55 aA	18.76± 1.11 abA	48.73± 0.49 cB	67.72± 0.68 eC	48.00± 0.00 cB	43.05± 0.43 bB	21.89± 0.22 eA	22.55± 5.02 aA
Consortia mix	33.66± 26.38 aAB	17.65± 6.62 abA	17.54± 0.18 aA	74.07± 0.74 fC	51.00± 0.00 cBCD	47.37± 0.48 bAC	21.81± 2.43 eAD	17.53± 15.64 aA

The same lower case letters are not statistically different among treatments, and the same capital letters are not statistically different among weeks to each treatment by the Tukey test (p<0.05), ±SD (n=3).

Analyzing week-1, no statistically significant differences were found among treatments. From the week-2 on, the values indicated the great C-biomass production to the bacterial consortium microcosm and also bioaugmented with *Achremonium* sp, with no statistical difference between these treatments. At week-4, the fungal consortium and the mixture of consortia produced the highest value of biomass, differing statistically from the other treatments. In general, soil containing only the native microbiota did not differ statistically from the bioaugmented soils, excepting the both consortia and the consortia mix at weeks 4, 6 and 8. According to the growth performances of each microcosm, these same microcosms showed statistically significant differences during the experimental period.

The techniques to determine microbial growths - number of cells and C-biomass content - were important to complement data of the bacterial and fungal performances into each microcosm, since the microbial number of cells was determined separately. These both techniques could confirm growth peaks at week-4, what is probably associated with high depletion on LMH-PAHs and production of metabolic intermediates (Chapter 2).

3.2. Respiratory activity and metabolic efficiency

The potential of microbial activity was evaluated by a rapid increase of microbial respiration rates and biomass. Furthermore, the basal respiration of heterotrophic microorganisms can be used to measure indirectly the microbial biomass into soil (Kennedy and Smith, 1995; Paul and Clark, 1996; Dilly, 2001; Nielsen and Winding, 2002).

During the week-1 (≅168 hours), both non-contaminated soil and the bioaugmented microcosms did show increased levels of CO_2 evolution due to the high basal respiratory activity (Figure 4). The bacterial consortium and the consortia mix performed higher respiratory levels during the 12-week period, followed by slow drops in the respiration rates from week-1 to week-3 (≅168-504 hours). Slight oscillations tending to a steady performance to the microbial respiration level was then observed after week-4 (from the 720-hour on). At week-1, the CO_2 evolution to *Aspergillus* sp., the fungal consortium and the consortia mix were statistically higher than other treatments, and these consortia together showed statistical similarity only to the fungal consortium (Table 4). The contaminated soil containing only the native microbiota did not show statistically significant differences comparing with bioaugmentation treatments from the week-2 on, except to the fungal consortium (weeks 3 and 10), the non-impacted soil (week-4), and to the mixture of consortia (week-10).

Analyzing the behavior of each treatment during 12 weeks (Table 4), only the bacterial consortium had statistical similarity among weeks. This treatment also showed one of the slighter respiration rates (Figure 4).

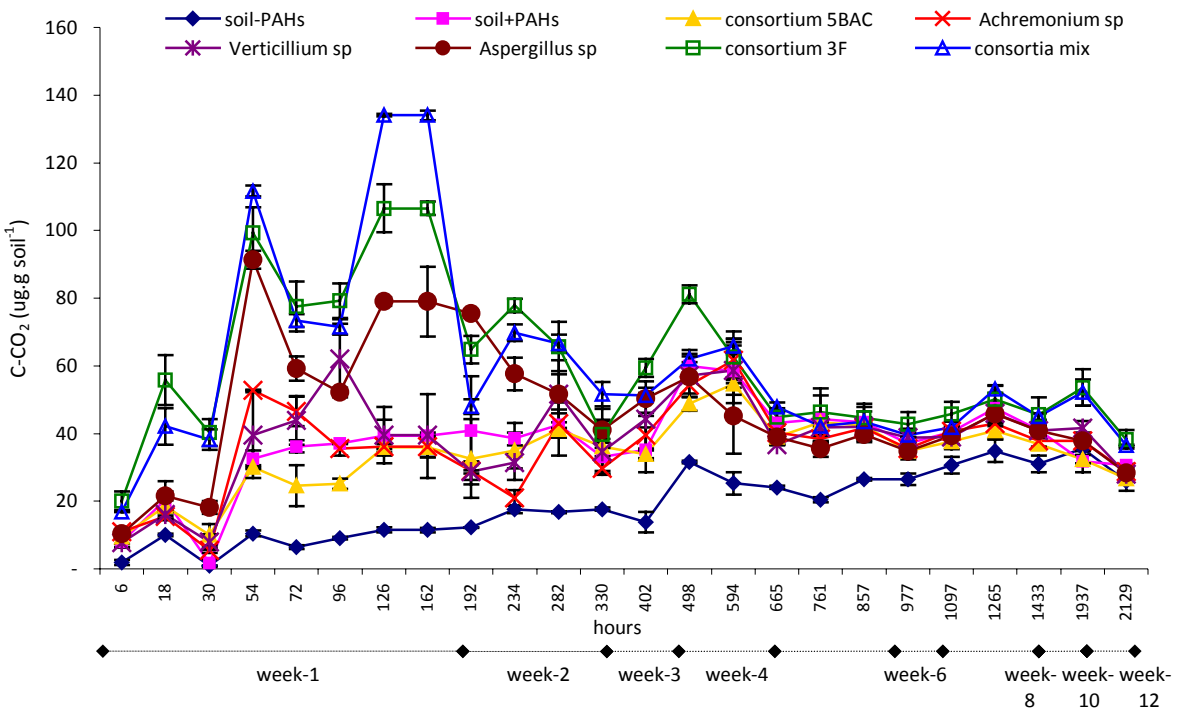

Figure 4. CO_2 evolution rates in soil microcosms: non-impacted soil (soil-PAHs), PAH-impacted soil containing only the native microbiota (soil+PAHs), soil bioaugmented with bacterial consortium (consortium 5BAC), fungal consortium (consortium 3F), each fungus separately (*Achremonium* sp, *Aspergillus* sp, *Verticillium* sp), and the mixture of both consortia (consortia mix). All data are mean of triplicate determination and error bars represent SD.

Table 4. Microbial respiration over time (12 weeks) in soil microcosms receiving different treatments

Microcosm treatments	CO_2 evolution (μg C-CO^2 . g soil $^{-1}$)							
	Week 1	Week 2	Week 3	Week 4	Week 6	Week 8	Week 10	Week 12
Soil – PAHs	12,26± 0.17 aA	17,64± 0.74 aA	31,61± 0.59 aB	24,11± 0.70 aC	30,67± 3.48 aDE	31,08± 3.48 aDE	35,50± 0.13 aD	25,99± 4.02 aCE
Soil + PAHs	40,90± 22.59 acAB	33,08± 5.88 abA	59,98± 6.62 bB	43,13± 5.22 bcAB	40,45± 1.74 acAB	41,12± 0.67 acAB	31,75± 4.42 aA	30,68± 0.40 abA
Consortium 5BAC	32,59± 23.32 acA	36,21± 15.90 abA	48,76± 3.99 bA	38,98± 4.65 bcA	37,55± 4.27 acA	36,97± 3.04 acA	32,42± 2.56 aA	26,70± 1.72 aA
Achremonium sp	29,00± 5.39 adA	29,65± 3.78 abAB	54,24± 3.53 bC	39,16± 1.90 bcAD	39,52± 2.64 acBDE	39,82± 3.64 acBD	40,27± 4.69 acD	29,56± 2.40 acAE
Verticillium sp	28,72± 0.94 aeA	34,55± 2.41 abA	57,18± 12.67 bB	36,66± 0.15 bA	38,98± 4.18 acA	40,81± 3.40 acA	41,53± 4.18 acA	27,95± 2.70 aA
Aspergillus sp	75,54± 1.80 befA	41,11± 5.26 abB	56,69± 2.06 bC	38,98± 4.21 bcB	39,25± 1.42 acB	40,90± 2.55 acB	37,86± 1.78 adB	28,31± 1.57 aD
Consortium 3F	64,78± 8.13 cfAB	39,64± 14.67 abAC	81,10± 5.20 cB	44,87± 5.17 bcAC	45,77± 7.10 bcAC	45,54± 10.18 bcAC	53,58± 10.72 bcAD	38,22± 5.50 bCD
Consortia mix	47,99± 4.28 cdfA	51,60± 5.30 bAB	61,99± 1.62 bB	47,82± 2.84 cAC	41,84± 3.17 bcAC	45,00± 1.88 bcAC	52,15± 7.75 bcdAB	36,43± 0.54 bcC

The same lower case letters are not statistically different among treatments, and the same capital letters are not statistically different among weeks to each treatment by the Tukey test ($p<0.05$), ±SD ($n=3$).

In a non-stressed soil (e.g. non-impacted soil used in this study), the basal respiration of microbiota can fastly increase with the addition and rightly consumption of PAHs, considering a rapid environmental disturbance. Microbial activity efficiency when using PAHs as carbon source was verified to all treatments. A comparison was done between the contaminated soil containing the native microbiota and the non-impacted soil, and there was an evidence of a very poor microbial activity by respiration rates in this latter treatment (Figure 4). Such decrease in the CO_2 evolution after week-1 is related to the period of exhaustion of available organic pollutant (e.g. LMW-PAH), since the microorganisms opted by some less complex PAHs as carbon source into a mixture of several PAHs.

According to Marin *et al.* (2005), the CO_2 released gradually decreased when easy fraction of hydrocarbons disappeared, remaining the most recalcitrant fraction of pollutants. Moreover, high respiration rates indicate either impact into soil and high level of productivity (Islan and Weil, 2000). An increase of microbial activity can be accompanied with unaffected cells growth indicating that specialized microorganisms are adjusting to substrate and other conditions, increasing their basal respiration and limiting the microbial growth (Devinny and Chang, 2000). Considering the metabolic quotient (qCO_2) an index of change in the microbial activity as response to stress factors may be also interpretated as a microbial efficiency since it measures the energy necessary to maintain the metabolic activity in relation to the energy necessary to increase biomass (Bardgett and Saggar, 1994).

In the literature was found that qCO_2 reflects the efficiency in using the carbon sources, and discriminates the mature level of the microbiota (Marin *et al.*, 2005). According to these authors, the disturbance in the natural soil could increase qCO_2 by increasing respiration per unit of biomass to degrade the PAHs in a landfarming of oil refinery sludge. Such levels did not have a significant decrease probably due to the soil system do not reached a balance after removal of the most part of PAHs. However, this present study showed increased rates of qCO_2 up to week-3, period of intense respiratory activity (Figure 5), followed by some drop on qCO_2 values. In this period, CO_2 evolution has also decreased levels (Figure 4). Soil microcosm microorganisms were able to survive in PAHs, and maintain their metabolic potential. A certain stability on metabolic quotient values showed that both microbial growth and respiration rate remained with very few

variations up to week-12, except in the *Achremonium* sp. and *Aspergillus* sp. microcosms. This fact indicates likely slow PAH mass transfer to the microbial biomass and a better adaptation microbial populations into soil microcosms.

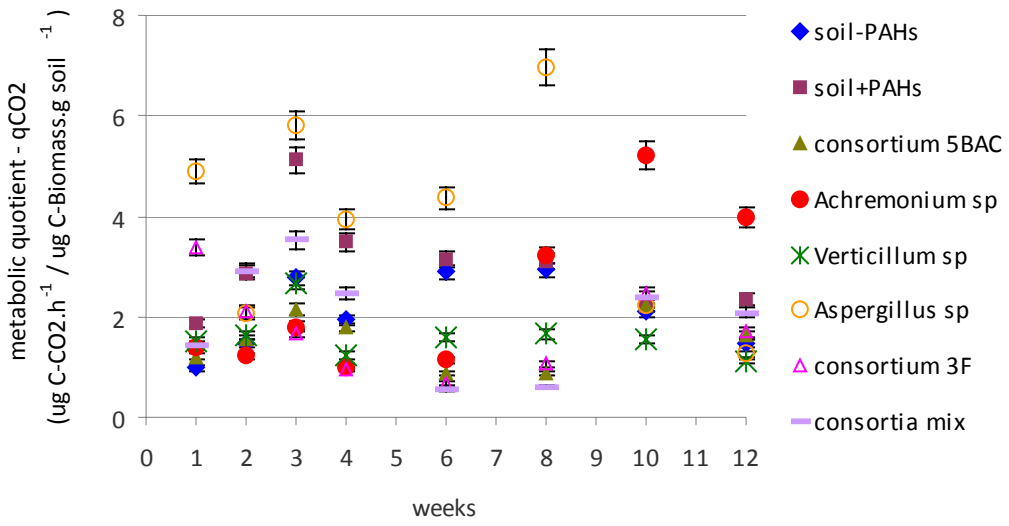

Figure 5. Microbial metabolic quotient n soil microcosms: non-impacted soil (soil-PAHs), PAH-impacted soil containing only the native microbiota (soil+PAHs), soil bioaugmented with bacterial consortium (consortium 5BAC), fungal consortium (consortium 3F), each fungus separately (*Achremonium* sp, *Aspergillus* sp, *Verticillium* sp), and the mixture of both consortia (consortia mix). All data are mean of triplicate determination and error bars represent SD.

Table 5. Metabolic quotient over time (12 weeks) in soil microcosms receiving different treatments

Microcosm treatments	Metabolic quotient, qCO_2 [($\mu g.\ h^{-1} C\text{-}CO^2$) ($\mu gC\ .\ dry\ soil^{-1})^{-1}$]							
	Week 1	Week 2	Week 3	Week 4	Week 6	Week 8	Week 10	Week 12
Soil – PAHs	0.98± 0.05 aD	1.48± 0.07 adA	2.78± 0.14 aB	1.94± 0.10 aC	2.90± 0.15 aB	2.94±0.15 aB	2.09±0.11 aC	1.48±0.07 acA
Soil + PAHs	1.87± 0.09 Ba	2.87± 0.14 bB	5.13± 0.26 bE	3.48± 0.17 bC	3.16± 0.16 aBC	3.11± 0.16 aBC	2.28± 0.11 aAD	2.36± 0.12 bD
Consortium 5BAC	1.19± 0.06 acE	1.56± 0.08 dA	2.15± 0.11 cB	1.79± 0.09 aC	0.89± 0.04 bcD	0.87± 0.04 bD	2.23± 0.11 aB	1.64± 0.08 cAC
Achremonium sp	1.41± 0.07 cA	1.22± 0.06 aAB	1.81± 0.09 cC	1.01± 0.05 cB	1.15± 0.06 bAB	3.23± 0.16 aD	5.21± 0.26 bE	3.96± 0.20 dF
Verticillium sp	1.51± 0.08 cA	1.65± 0.08 dA	2.67± 0.13 aC	1.23± 0.06 cB	1.58± 0.08 dA	1.65± 0.08 cA	1.56±0.08 cA	1.12± 0.06 eB
Aspergillus sp	4.88± 0.24 dA	2.07± 0.10 cfB	5.81± 0.29 dD	3.94± 0.20 dC	4.36± 0.22 eAC	6.97± 0.35 dE	2.23± 0.11 aB	1.26± 0.06 aeF
Consortium 3F	3.39± 0.17 eC	2.11± 0.11 cfD	1.66± 0.08 eA	0.97± 0.05 cB	0.68± 0.03 cE	1.06± 0.05 bB	2.46± 0.12 aF	1.70± 0.09 acA
Consortia mix	1.43± 0.07 cD	2.92± 0.15 bE	3.53± 0.18 eF	2.48± 0.12 eA	0.56± 0.03 cB	0.61± 0.30 bB	2.39± 0.12 aAC	2.08± 0.10 bC

The same lower case letters are not statistically different among treatments, and the same capital letters are not statistically different among weeks to each treatment by the Tukey test (p<0.05), ±SD (n=3).

At week-1, the non-impacted soil did not have statistically significant difference with the bacterial consortium treatment, which showed the lowest values of qCO_2, verifying more efficiency to use PAH to transform them in biomass (Table 5). At week-2, even the impacted soil containing only the native microbiota, or the mixture of consortia showed the highest values of qCO_2 with no statistical differences between them, indicating a better use of PAHs as carbon source to the microbial respiration, more than to the microbial growth. Analyzing week-3 through 6, the qCO_2 in the native microbiota microcosm was statistically higher and different from the other bioaugmented

treatments, what confirmed less efficiency to use PAHs as carbon source to increase biomass. Furthermore, statistical similar values of qCO_2 were found between non-impacted soil and *Achremonium* sp at week-3, the bacterial consortium at week-4, and the native microbiota at week-6. At week-8, both consortia and the mixture of them had the qCO_2 statistically lower than the other treatments. At week-12, the consortia mix did not show statistical difference with the native microbiota.

When treatments were analyzed separately along the weeks, the Tukey test showed that the lowest statistically different values of qCO_2 were found at week-1 for the non-impacted soil and the impacted soil containing only the native microbiota; at week-4 for *Achremonium* and *Verticillium* ssp.; at weeks 6 and 7 for bacterial consortium and the mixture of consortia; and at week-12, for *Verticillium* and *Aspergillus* ssp. microcosms. The non-impacted soil and the soil containing only the native microbiota increased qCO_2 values up to the week-3, and the bioaugmented soil could decrease such values along weeks, showing some efficiency during these period to use PAHs as part of biomass instead of a high activity respiration.

Generally, consortia with different species of bacteria and bacteria-fungi have been used to improve degradation rates of pollutants (Trzesickia-Mlynarz and Ward, 1995; Park *et al.*, 1999; Boonchan *et al.* 2000). Nonetheless, pure culture of bacteria when introduced into soil may not persist by limitation of competitiveness, and degradation can be observed just to sterilized soils (Kästner *et al.*, 1998). In bioaugmentation practices, when a fungus-bacteria was used the clean-up PAHs, a initial ring oxidation could be realized by fungi, where as bacteria might use its metabolic products to grow and degrade PAH by enzymatic activation, creating a mutually dependent relationship between them, in a cooperative catabolism (Sack *et al.*, 1997; Kotterman *et al.*, 1998; Boonchan *et al.,* 2000).

In this study, bacterial growth was higher than fungal growth in the beginning of the degradation period, suggesting that fungi need some additional adaptation period than bacterial population. Nevertheless, even not increasing biomass, extracellular non-specific ligninolytic enzymes or intracellular monooxigenases can be produced and activated by the presence of PAHs to act in the initial degradation of such compounds.

According to Jacobsen and Petersen, (1992a and 1992b), bioaugmentation inoculation levels of 10^7-10^8 are considered sufficient to establish degradation activity. In other studies, Boonchan *et al.* (2000) reported approximately 10^6 cells.g $soil^{-1}$ as a good inoculum in PAH-impacted soil. In the present study, even at lower cell densities (10^5 cells. g $soil^{-1}$), microorganisms inoculated into microcosms were able to compete with the native microbiota and help in the PAH degradation.

It is possible that a higher inoculum reaches faster adaptation by decreasing the lag phase populations and also the PAH depletion time, differing in somehow from natural microbiota performance. The density of introduced populations is an important ecological consideration in determining establishment success (Ramadan *et al.,* 1990; Warren *et al.*, 1992; Wagner *et al.*, 1994), however, a high cell density does not guarantee such establishment, since the native microbiota is adapted to the soil conditions (Massol-Deiá *et al.*, 2005). The persistence and growth of an inoculated strain in any soil depend on its ability to utilize local resources, and sometimes microorganisms used in bioaugmentation survive well only when a particular contaminant is present (Cunliffe and Kertesz, 2006).

4. Conclusions

The microbial adaptation was shown through the catabolic activity of PAH-degrading microorganisms. In the microcosm treatments, nutrients were not amended in the soil and microbial activities using PAHs was supported by the soil organic matter, minerals as sources of nitrogen and phosphorus, and the co-metabolism process generated by the microbial community. Both native microbiota and bioaugmented microorganisms performed a rapid use of the PAHs as carbon source to respiratory activities, showing an adaptative response and effectiveness when spending less energy to survive in the soil receiving PAHs disturbance. A recovery in the microbial efficiency observed by a decrease in the CO_2 evolution level following a stable microbial growth indicated that the native and inoculated microorganisms are able to grow and survive into PAH-contaminated soil. They reached a rapid adaptation and played some metabolic cooperation into soil.

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5. Capítulo 2

Bioremediation of a PAH-contaminated soil bioaugmented with microbial consortia

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Abstract

The biodegradation of several PAHs into contaminated soil and the presence of metabolic intermediates were evaluated into contaminated soil microcosms with native microbiota and/or bioaugmented with bacterial and fungal consortia, soil fungi individually, and the mixture of these consortia. The total concentration of PAHs in microcosms (600 mg.kg soil $^{-1}$) did not show inhibitory effect in the natural microbiota or into the bioaugmented soils. Low-molecular-weigh PAHs depletions were marked by rapid and faster initial reduction, due to their bioavailability and high microbial degradation activity. Bioaugmentation did not affect the biodegradation efficiency in PAH-contaminated soils. It is possible that metabolic intermediate are used as co-substrate for the degradation of other PAHs, and the presence of LMW-PAHs may induce catabolic enzymes activated by the presence of these compounds or key-intermediates from PAHs metabolism.

Key-words: *polycyclic aromatic hydrocarbons (PAHs); biodegradation; bacteria; fungi; metabolic intermediates.*

1. Introduction

The petroleum-derivative compounds, polycyclic aromatic hydrocarbons (PAHs), are formed by incomplete combustion of fuel, oil, coal, petroleum, and wood (Wild and Jones, 1995), composed of linear, angular and group benzenic rings arrangements. They occur into soil, water, groundwater, air, sediments, animals and plants as a result of both natural and anthropogenic activities. Because of their mutagenic and carcinogenic properties, PAHs are the most extensively investigated of the chemical pollutants (Cerniglia, 1992).

PAHs are transformed throughout several metabolic reactions by cooperation among microorganisms in real environment and the bioremediation has been shown to be effective in remediating low-molecular-weight (LMW-) PAH contaminated soil (Mueller *et al.*, 1991; Banerjee *et al.*, 1995; Kästner and Mahro, 1996), however, high-molecular-weight (HMW-) PAHs are more recalcitrant and need more efficient microorganisms in optimal conditions to perform such degradation. The mineralization of HMW-PAHs decreases when increasing the residence time in soil (Erickson *et al.*, 1993), where PAHs is strongly associated with organic matter by several mechanisms (Khan and Ivarson, 1982). An effective bioremediation strategy depends on the ability of microorganism to degrade the target compounds towards mineralization or to a very low level with production of non-toxic metabolites. The degradative performance of any inoculum into soil depends on soil type and other environmental conditions that may be not easy to control *in situ* (Juhasz *et al*., 2000).

Fungi and bacteria are capable of degrading partially or completely HMW-PAHs by co-metabolism when LMW-PAHs or the products of their degradation help degradation of other HMW-PAHs, even in individual experiments or in consortia (Boonchan *et al.*, 2000; Juhasz and Naidu, 2000; Marcoux *et al.*, 2000; Gauthier *et al.*, 2003).

Bioaugmentation is quite important to remediate contaminated sites containing high levels of HMW-PAHs, and amended microorganisms may overcome the indigenous catabolic limitation (Juhasz and Naidu, 2000). Some consortia and co-cultures added to PAH-contaminated soil were able to degrade several PAHs into the soil matrix (Juhasz, 1998; Kotterman *et al.*, 1998; Stanley *et al.,* 1999; Boonchan *et al.*, 2000). Some information on metabolic products from consortia degradation experiments may elucidate mechanisms of interaction between indigenous and bioaugmented exogenous organisms, contributing to improve the bioremediation efficiency of PAHs (Luan *et al.*, 2006).

The aim of this work was to investigate the levels of PAH removal from contaminated soil and the production of metabolic intermediates in native microbiota and soil bioaugmented with bacterial or fungal consortia, some individual soil fungi and a mixed consortia.

2. Materials and methods

2.1. Reagents

All PAHs (>98% of purity) and chemicals were purchased from Sigma-Aldrich, Brazil. Dichloromethane at chromatographical grade was obtained from Mallinckrodt, USA, and dimethylformamide from Merck, Brazil. Culture media were acquired from Oxoid, Brazil.

2.2. Microorganisms

Forty two bacteria belonging to the culture collection from The Systematic and Microbial Physiology Laboratory, Faculty of Food Engineering, UNICAMP, Brazil, were isolated from a diesel oil-contaminated site in the Paulínia city, SP, Brazil, in a previous work (Jacobucci-Carvalho, 2000), and screened in this present work, for their ability to degrade pyrene added to agar minimal medium at final concentration of 400 $mg.l^{-1}$, and using 1% pyrene etheral solution sprayed on the surface of agar minimal medium plates. The minimal medium contained per liter: K_2HPO_4 0.4 g, KH_2PO_4 0.4 g, $(NH_4)_2PO_4$ 0.4 g, NaCl 0.3 g, 1 ml of microelements solution (Bogardt and Hemmingsen, 1992) and 1 ml of vitamins solution (Wolin *et al.*, 1963). Both solutions were sterilized by filtration (0.22 μm, Millipore) and added to the autoclaved medium. Plates were inoculated and incubated at 30°C for 7-14 days for both screenings.

The strains with the fastest growths were selected to run the experiments in soil microcosms, and soil fungi were chosen due to the best degradations of PAHs in liquid media under aerobic (Clemente, 2001) and microaerobic conditions (Silva, 2007 – manuscript under corrections).

The bacterial consortium (5BAC) was composed by 5 bacterial strains: *Chromobacterium* sp (1), *Enterobacter aglomerans* (2), B1F (3) and B5A (4) – gram-positive rods, and B1H (5) – gram-positive cocci. The latter three strains have their 16S rDNA sequencings on the way. Bacteria were grown in 20 ml of nutrient broth (NB) plus pyrene 200 $mg.l^{-1}$ for 18 hours at 30°C/150 $rev.min^{-1}$, followed by centrifugation at 4°C/10,000 g for 10 minutes. Cells were washed twice with phosphate buffer 0.1 M pH 7.0, and suspended with the same volume of buffer as used to the growth liquid medium. Flasks were kept under refrigeration until the inoculum standardization to 10^5 $cells.g\ soil^{-1}$ in each microcosm by counting viable cells per ml of medium.

Achremonium sp., *Aspergillus* sp. and *Verticillium* sp. were used in the individual bioaugmentation by growing them for 72 hours at 30°C/100 $rev.min^{-1}$ in liquid medium containing glucose 2%, yeast extract 0.5%, peptone 1%, malt extract 1% and pyrene 200 $mg.l^{-1}$. Mycelia were washed twice with phosphate buffer, and 12.5$mg.g\ soil^{-1}$ (wet weight) of each strain biomass was added as inoculum into microcosms.

The mixture of both consortia was prepared in flasks using the respective quantity of inocula as described for each strains, and adding sterile distilled water to help in the mixture of microorganisms by shaking, before inoculation into 'consortia mix' microcosm.

2.3. Soil microcosms

A forest soil belonging to an experimental site in Pindorama, SP, Brazil was kindly collected by The Campinas Agronomic Institute, Campinas-SP, Brazil, and analyzed related to its physic-chemical and biological characteristics (Table 1).

Table 1.Physic-chemical and microbial content of soil, according to the Laboratory of Soil and Plant Analysis (IAC, Campinas, SP, Brazil)

Soil characteristics	
Clay (%)	9.9
Silt (%)	9.8
Total sand (%)	80.3
Organic matter (g dm^{-3})	50
P (mg dm^{-3})	16
K ($mmol_c$ dm^{-3})	3
Ca ($mmol_c$ dm^{-3})	65
Mg ($mmol_c$ dm^{-3})	13
Micronutrients:	
B (mg dm^{-3})	0.23
Cu (mg dm^{-3})	0.9
Fe (mg dm^{-3})	47
Mn (mg dm^{-3})	48.4
Zn (mg dm^{-3})	5.8
PH	6.16
Bacteria (CFU g $soil^{-1}$)	3.5×10^6
Fungi (CFU g $soil^{-1}$)	6.5×10^4

The soil collected was dried at room temperature for some days, sieved through a 2-mm mesh and the water content in the soil was adjusted to 50% of the water-holding capacity. The soil was artificially contaminated with a defined concentration of PAHs: naphthalene and phenanthrene, 150 mg.kg $soil^{-1}$ each; anthracene and pyrene, 100 mg.kg $soil^{-1}$ each; benzo[a]anthracene and benzo[a]pyrene, 50 mg.kg $soil^{-1}$ each. PAHs were previously added into 1.5 L glass-jar microcosms, and after solvent evaporation, 400 g of soil were vigorously homogenized with a sterile glass rod, followed by bioaugmentation procedures. Microcosms were then incubated for a 12-week period at 24-25°C, in the dark.

All the microcosm treatments were run in triplicate: non-impacted soil (soil-PAHs), impacted soil containing only the native microbiota (soil+PAHs), soils bioaugmented with the bacterial consortium 5BAC (five bacteria strains), each fungus separately (*Achremonium* sp., *Verticillium* sp., *Aspergillus* sp.), the fungal consortium 3F (three fungi strains), and the mixture of both consortia. Soil oxygenation was performed weekly and 50% of water hold capacity was adjusted when necessary.

2.4. Extraction of PAHs from contaminated soil

PAHs extraction from the soil matrix was modified from US EPA (1992). Briefly, 1 g of soil sample was collected from each microcosm and placed into flasks containing dichloromethane (DCM, 10 ml), Na_2SO_4 (1 g), and then sonicated for 3 minutes (30-second interval each 1 minute) under ice bath. The extraction procedure (DCM/sonication) was repeated twice more, and then the extract was centrifuged for 5 minutes at 10,000 x g / 4°C. Afterward, supernatant was rota-vaporated, and PAHs were suspended in 1ml of DCM. Samples were transferred to 2 ml brown vials (Agilent) and stored at -20°C for degradation analysis.

2.5. Degradation of PAHs

An aliquot of 1 μl was taken from each vial using a 10 μl syringe (Agilent) and injected in splitless mode into a gas chromatographer (Shimadzu GC-14A) equipped with a flame ionization detection (GC-FID), using a BPX-5 column - fused silica in 5% phenyl polysilphenylene-siloxane as bonded phase (25 m x 0.22 mm ID x 0.25 μm film thickness, SGE-Australia) under helium flow at 0.7 ml min^{-1}. The temperature program was performed as follows: 65°C for 1 minute, and 25°C/minute up to 140°C. The second temperature ramp was 10°C/minute up to 290°C, remaining in this final temperature for 8 minutes. Injector and detector temperatures were set at 240°C and 300°C, respectively.

2.6. Production of metabolic intermediates

Phenolic compounds, such a hydroxilated aromatic sub-products generated from PAHs degradation were determined according to Guerin and Jones (1988) with modifications, using the Folin-Ciocalteau reagent (Box, 1983). Briefly, a 1 g-soil was placed into a flask containing 10 ml of sterile distilled water and shaked for 2 hours at 200 rev. min^{-1} (room temperature). The soil extract was then sonicated for 5 minutes in ice bath, settled and the volume was added to a tube containing 1.5 ml of Na_2CO_3 200 g l^{-1}. The Folin-Ciocalteau reagent was added (0.5 ml), and the tubes were incubated for 30 minutes at 20°C. A volume of 1 ml was placed in a cuvette and read in a spectrophotometer (Shimadzu UV-1201) at 750 nm. Different concentrations of resorcinol (from a 100 $\mu g.ml^{-1}$ stock solution) were used to the standard curve. The blank was run using the sterile contaminated soil, and the quantity of metabolic intermediates produced was expressed as mg of resorcinol equivalents (RE) per kilogram of soil.

2.7. Statistical analysis

Statistical significances of data from microcosm experiments were conducted by analysis of variance and the averages were compared by the Tukey test at $p \leq 0.05$ to verify differences among treatment to the same week, and also among weeks to each treatment.

3. Results and discussion

3.1. Determining the PAHs removal

The initial amount of PAHs into the soil influences the PAH degradation level, and the PAH mass transfer rates of these compounds to microorganisms (Tiehm *et al.*, 1997). In this study, the total concentration of PAHs in microcosms (600 mg.kg soil^{-1}) did not show inhibitory effect to the native microbiota, or to the bioaugmented treatments (data to be published elsewhere). LMW-PAHs depletions were marked by an initial rapid reduction, followed by a slower decrease overtime up to a steady residual concentration in soil, probably representing the non-available fraction or the biodegradation-resistant fraction (Figure 1). Such performances were also verified by Sabaté *et al.* (2006), when soil microbiota used only the portion of available PAHs as carbon source, transforming them into C-biomass, water and CO_2.

During week-1, LMW-PAHs (naphthalene, phenanthrene and anthracene) were degraded fastly both in the native microbiota and in the bioaugmented microcosms, except to the soil bioaugmented with *Verticillium* sp. and with the fungal consortium, which showed some resistance to the naphthalene degradation. This fact was verified possibly due to the naphthalene production associated with some efficiency in the other PAHs depletion, since naphthalene is an intermediate in the HMW-PAH degradation.

Previous studies have demonstrated that degradation of naphthalene was inhibited, where as rates of phenanthrene and pyrene decreased in a mixture of PAHs using bacteria isolated from PAH-contaminated soil (Guha *et al.*, 1999). These authors defend that the low degradation rate of the more degradable PAH was due to the competitive inhibition, and the enhance of degradation rates to the more recalcitrant PAHs was because of the simultaneous biomass growth on multiple substrates.

In this study, naphthalene degradation seems not to be inhibited up to week-3; however, from this period on the removal values did not change, remaining 24.69 - 28.49 mg.kg soil^{-1}, as showed in Figure 1, suggesting that only available naphthalene portion was used by microorganisms in all treatments. This fact was also verifyed by Viñas *et al.* (2005), when phenanthrene and anthracene were not completely degraded due to the low availability of residual quantities of these compounds after 45 days.

Semple *et al.* (2006) characterized the behavior of some bacteria in the soil PAHs degradation, and after a *plateau* of phenanthrene mineralization, the number of cells of microorganisms remained steady. A rapid use of fresh phenanthrene by bacteria was still observed by these authors, and neither inoculation of active catabolic bacteria nor

nutrients increased the mineralization period, once there was no longer available substrate which was incorporated in a small portion into the biomass. The limited bioavailability of pollutants results from their adsorption to soil particles or a covalent linkage with organic matter (Kästner *et al.*, 1999; Friedrich *et al.*, 2000).

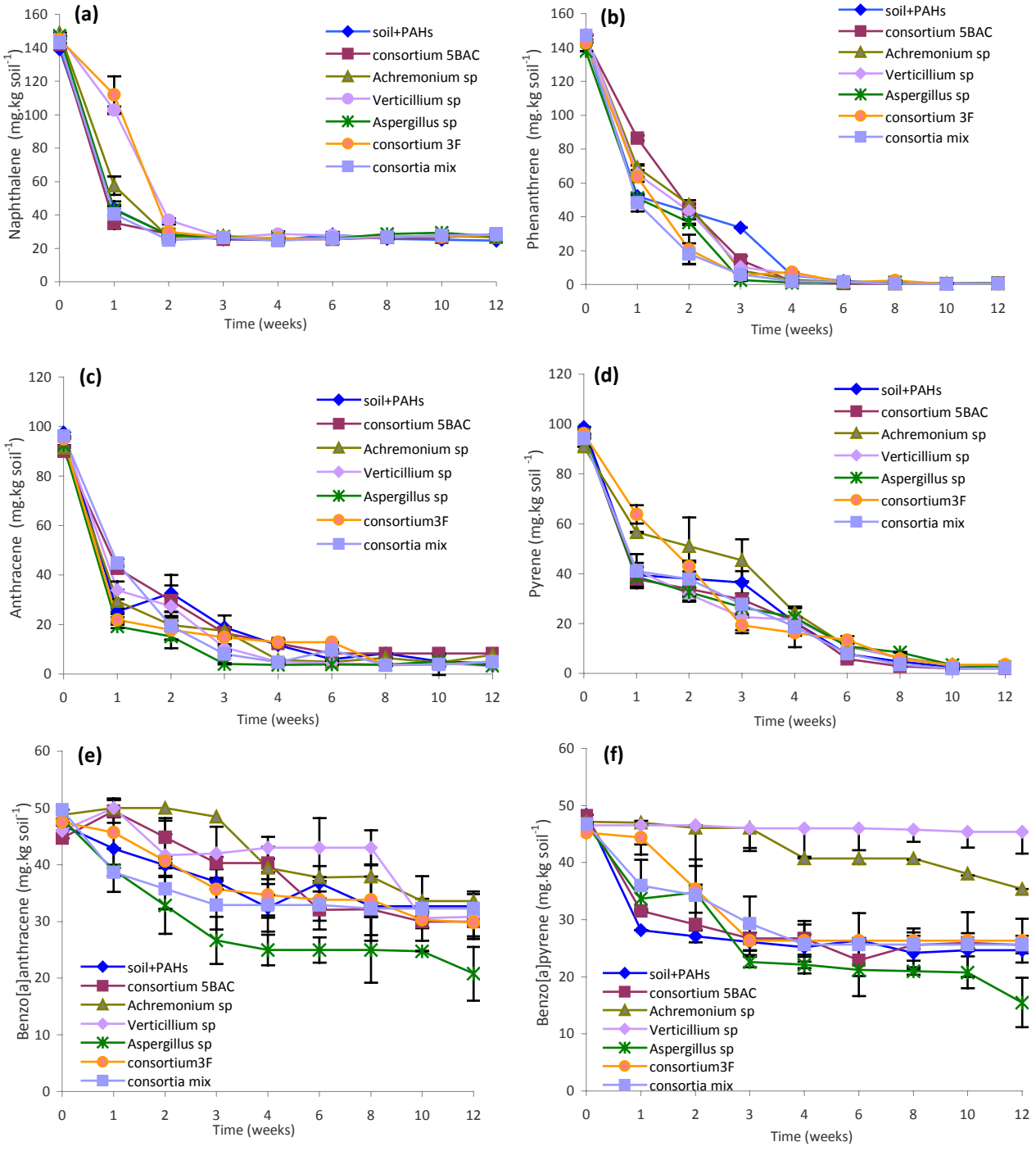

Figure 1. PAH degradation in soil microcosms during 12 weeks: (a) naphthalene, (b) phenanthrene, (c) anthracene, (d) pyrene, (e) benzo[a]anthracene, (f) benzo[a]pyrene. All data are mean of triplicate determinations and error bars represent SD.

There are some reports on the bioaugmentation of PAH-contaminated soil, where four and five- ring PAHs are more recalcitrant, and LMW-PAHs are effectively removed (Aprill *et al.*, 1990; Park *et al.*, 1990; Mueller *et al.*,1991). When analyzing other LMW-PAHs in the present work, phenanthrene levels decreased to less than 1 mg.kg soil^{-1} during the 12-week period, and anthracene reached less than 10 mg.kg soil^{-1} in all microcosms. A rapid loss of LMW-PAHs up to week-4 was due to their bioavailability and high microbial activity in all treatments.

According to Eggen and Majcherczyk (1998) and Eggen (1999), the potential of PAH degradation declines when the number of aromatic rings increases. This fact was confirmed in the present study, when HMW-PAHs showed lower and less degradation than LMW-PAHs, since these last compounds are generally less recalcitrants and more bioavailable to microorganisms.

Heitkamp and Cerniglia (1989) stated that soil is a complex matrix where degradation of PAHs occurs by co-metabolism mechanisms with other undefined organic material. Therefore, it is believed that PAHs degradation was a result from a co-metabolic process in this present study, when degradation of HMW-PAHs by consortia in a bioaugmentation process occurred in co-metabolism after utilization of LMW-PAHs or other soil component as carbon source and energy. This fact could be also observed by Juhasz and colleagues (1997), and Boonchan *et al. (*2000), whose studied the degradation of PAHs with more than 4 rings. The addition of a small quantity of LMW-PAHs can stimulate degradation of HMW-PAHs (Dean-Ross *et al.*, 2002). These authors have also observed evidences of interactive effects by simultaneous utilization of PAHs in a complex mixture.

In this study, the rates of PAHs detoxification by the both native microbiota and bioaugmented soils were dependent on the number of aromatic rings of the PAH compounds, likewise in experiments carried out by Pott and Henrysson (1995) and Juhasz *et al.* (2000).

Kästner *et al.* (1998) verified that a native microbiota could degrade phenanthrene and anthracene: 1.5 and 0.07 mg.kg soil^{-1}, respectively, in 98 days. Nevertheless, when introducing the consortium BP9 into soil, degradation of PAHs by microbiota was inhibited. In this present study, it was reached approached removal values even in a mixture with more complex PAHs. Onother study have demonstrated that over the 91-day period, degradation of naphthalene, phenanthrene, pyrene and benzo[a]pyrene in bioaugmented soil with bacterial consortium reached 1.96, 1.50, 0.65 and 0.04 mg.kg soil^{-1}, respectively (Juhasz *et al.*, 2000). According to our results, only phenanthrene and pyrene showed similar rates of degradation comparing with the previous authors.

In a creosote-contaminated soil, indigenous microbiota readly degraded LMW-PAHs, however, HMW-PAHs rates were unaffected (Park *et al,*1990; Mueller *et al.*, 1991). In other soil with the same contaminating compound, three and four aromatic rings among PAHs were the most abundant, whereas LMW-PAHs reached 80-100% of degradation during a 45-day period (Viñas *et al.*, 2005).

A sterile soil spicked with a PAH mixture was bioaugmented with a bacterial consortium and *Stenotrophomonas maltophilia*: pyrene (250 mg.kg soil^{-1}) was completely mineralized in 30 days; chrysene, benzo[a]anthracene, benzo[a]pyrene and dibenzo[a,h]

anthracene degradation reached 24 to 48% after 100 days into soil inoculated with *Penicillium janthinellum*; and when in co-culture, the range of degradation of these PAHs increased to 40-80% (Boonchan *e al*, 2000). *Stenotrophomonas maltophilia* and the bacterial consortium could cometabolically mineralize benzo[a]pyrene in the presence of pyrene, indicating some relation with PAH-catabolic enzymes activity , when pyrene or its degradation products may compensate the lack of enzyme activity, inducing them to degrade benzo[a]pyrene. In this case, pyrene and benzo[a]pyrene may also share a common lower metabolic pathway which is regulated by pyrene (Boonchan *et al.*, 2000).

Chávez-Gómez and co-workers (2003) reported the use of several bacterial-fungal co-cultures inhibiting the microbial synergism in the phenanthrene removal. The authors have pregrown fungi on sugarcane bagasse pith to confer better growth and survival into soil. When in fungal consortia, some fungi have the ligninolytic enzymes significantly stimulated by their interspecific interaction, as described by Chi *et al.* (2007), when the oxidation of PAHs to polar water-soluble products with more bioavailability can increase the rates of mineralization of these metabolites by bacteria.

In the present study, the soil fungi used to bioaugmentation were isolated from a forest soil rich in humic compounds that stimulate the production of ligninolytic enzymes responsible for acting in complex aromatic molecules, including PAHs. In general, PAH degradation values did not show statistically significant differences among treatments during the experimental period, except to naphthalene (week-10), phenanthrene (weeks 2 and 3), anthracene and benzo[a]anthracene (week-4), and benzo[a]pyrene (weeks 10 and 12), as shown in Table 2. Such differences are explained by some possible different interactions among microorganisms that are degrading PAHs in the soil.

The impacted soil containing only the native microbiota showed lower statistical significant value of phenanthrene degradation than in bioaugmented treatments at week-2, as well as in the last three last treatments at week-3 (Table 2). However, the native microbiota had higher value of naphthalene degradation (week-10) in comparison with *Aspergillus* sp. microcosm, which showed an opposed more degradative behavior to benzo[a]anthracene degradation (week-4), probably caused by an efficient microbial cooperation in this microcosm.

Table 2. PAH degradation over time (12 weeks) in soil microcosms receiving different treatments

Microcosm treatments	PAH degradation (mg .kg soil^{-1})						
	Naphthalene	Phenanthrene		Anthracene	Benzo[a]pyrene		Benzo[a]anthracene
	Week 10*	Week 2	Week 3	Week 4	Week 10	Week 12	Week 4
Soil + PAHs	25.22± 0.34 a	116.00± 9.84 a	33.31± 21.29 a	11.70± 4.78 ab	24.65± 0.27 ab	27.59± 8.36 ab	32.39± 5.93 ab
Consortium 5BAC	26.46± 0.89 ab	44.80± 39.40 b	14.47± 9.93 ab	14.15± 5.88 b	25.92± 8.19 ab	30.56± 13.88 ab	42.66± 5.77 b
Achremonium sp	26.33± 1.30 ab	47.20± 5.19 b	8.26± 3.04 ab	5.63± 1.17 ac	45.77± 16.14 a	53.33± 27.28 a	39.41± 3.18 ab
Verticillium sp	27.65± 1.58 ab	43.44± 15.76 b	10.21± 7.37 ab	5.00± 0.73 ac	32.97± 3.91 ab	37.45± 5.43 ab	42.98± 3.81 b
Aspergillus sp	29.27± 2.74 b	53.95± 3.44 b	2.65± 1.26 b	3.78± 0.37 ac	20.77± 5.60 b	15.49± 2.99 b	24.93± 5.40 c
Consortium 3F	26.81± 0.74 ab	20.75± 13.04 b	5.52± 6.53 b	7.28± 1.70 bc	31.14± 2.94 ab	33.71± 3.36 ab	34.68± 8.03 ab
Consortia mix	27.68± 1.26 ab	24.63± 4.18 b	5.82± 2.55 b	4.69± 1.03 a	33.10± 11.27 ab	35.01± 14.29 ab	33.16± 3.58 ab

* all weeks showed in this tablehad statistical differences among treatments

The same lower case letters are not statistically different among treatments by the Tukey test (p<0.05), ±SD (n=3).

None of microcosms showed statistically significant differences to pyrene degradation during the 12-week incubation period. This PAH was not shown in table 2 due to statistic similar values among degradation performances for both native microbiota and bioaugmented soils. There are some previous reports that also demonstrated success in the PAH biodegradation achieved by a soil native microbiota (Horinouchi *et al.*, 2000; Roper and Pfaender, 2001). To Guerin (1999), an indigenous population of PAH-degrading microorganisms could remove 82-97% of LMW-PAHs and up to 35% HMW-PAHs in a creosote-contaminated site. Furthermore, some humic substances into the soil may have an important role to the natural attenuation as natural surfactants (Conte *et al.*, 2005), promoting some microbial activity. In the present study, we verified that the bioaugmentation did not affect the efficiency in the degradation of PAH-contaminated soil by the native microbiota, as also suggested in some other studies (Launen *et al.*, 2002; Saponaro *et al.,* 2002).

Stanley *et al.* (1999) analyzed a bacterial community incubated with *Penicillium janthinellum* in a basal salt medium, showing increased values of benzo[a]pyrene degradation when comparing only bacteria community or the fungus inoculated separately. Such fungus was not able to mineralize benzo[a]pyrene as sole carbon source, however, 16-64% of degradation was visualized in form of polar metabolites when some co-substrates was added into the medium (e.g. pyrene, peptone, glucose). According to these authors, *Stenotrophomonas maltophilia* and a bacterial consortium reached 13% and 32% of benzo[a]pyrene degradation after 56 days, in the presence of pyrene. The co-culture *S. maltophilia*/*P. janthinellum* also resulted in a significant degradation of benzo[a]pyrene, 58%, after the same period and using the same co-substrate. The results achieved in the present study corroborate the previous information from literature, when fungi were inoculated separately in PAH-soil microcosms, and showed capability of several PAHs degradation when in a cooperative metabolism with the native microbiota and the bacterial consortium.

3.2. Analyzing metabolic intermediates in PAH degradation

Metabolic intermediates may increase or conserve their toxicity related to the parental PAH, and produce an inhibitory effect on microbial population (Sabaté *et al.,* 2006). The accumulation of metabolic products may reduce the viability of degraders to use PAHs. Toxic metabolic intermediates may be a reason for the lack of complete degradation of PAHs in the soil matrix (Kazunga and Aitken, 2000; Kazunga *et al.*, 2001), and the accumulation of PAH metabolites after fungi transformation is of some concern since fungal monooxigenases oxidize PAHs to epoxides and dihydrodiols (Sutherland, 1992). However, in a soil mixed community, when several metabolic intermediates are generated and used at the same time, might balance in some manner such toxicity. In a bioaugmentation treatment with a bacterial consortium, intermediate metabolites less toxic than PAHs have been produced during degradation or mineralization of three- to five- aromatic rings (Juhasz *et al.*, 1998). Some bacteria survived in soil without developing their PAH degradative capacities. It means that such microorganisms may rely on

metabolites produced by fungal catabolism for survival. Kotterman *et al*. (1998) and Stanley *et al.* (1999) reported that PAH degradation resulted in a significant decrease in mutagenicity of soil extract, and when inoculating a co-culture into soil, a decrease in PAH mutagenicity effect was observed. Fungal pre-oxidation of PAHs can increase the bacterial potential to degrade these compounds. Moreover, fungi have been described as potential degraders of PAHs in soil (Cortes-Espinosa *et al,* 2006) and the metabolic intermediates of such degradation could be easily degraded by a microbial consortium from a polluted soil (Capotorti *et al.*, 2005).

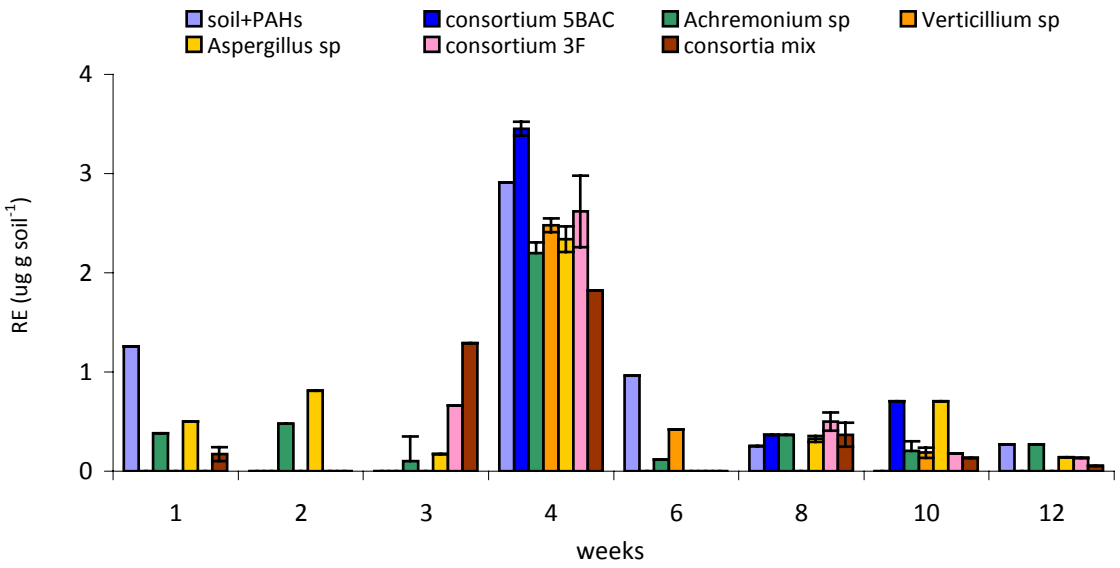

Figure 2. Values of Resorcinol Equivalent (RE) to the metabolic intermediates produced during degradation of PAHs into soil microcosms. All data are mean of triplicate determinations and error bars represent SD.

In the present study, the native microbiota, *Achremonium* sp., *Aspergillus* sp. and the consortia mix could accumulate some RE metabolites on week-1, followed by its consumption on week-2 by the native microbiota and the consortia mix (Figure 2). The bacterial and fungal consortia did not generate significant RE values at weeks-1 and 2, probably due to a high assimilation of intermediate products by co-metabolism among microorganisms in these microcosms. RE metabolites reappeared in the fungal consortium and the consortia mix microcosms at week-3, increasing their values at week-4. According to PAH-removal performances in this period (Figure 1), a great amount of LMW-PAHs was biodegraded up to week-4, showing the highest RE production in all microcosms. After this period, the rates of PAHs degradation decreased slightly, suggesting the preference of both native microbiota and bioaugmented community to use less complex substrates in their metabolisms, since its levels had a considerable drop after this period.

The native microbiota was also able to produce and use metabolic intermediates, showing efficiency in using them to the microbial maintenance and survival in PAH-impacted soil. Analyzing RE production at weeks 1 and 4 to this microcosm, it was verified concomitant peaks of CO_2-evolution (Chapter 1, Figure 4), what indicates PAH consumption followed by RE production. The absence of such products at weeks 2 and 3 is probably explained by some steady CO_2-evolution, however with RE consumption in an adaptation period up to week-4. In this same week, high RE values found to all treatments was precedent of CO_2-evolution peaks. Furthermore, at week-4, microbial community in all treatments could produce RE, where bioaugmented microcosms showed less RE values as a result of more ability in the prompt RE consumption by some microorganisms.

Juhasz *et al.* (1997) studied phenanthrene degradation by a consortium 'community 5' was accompanied by a RE increase (maximum of 4.5 $mg.l^{-1}$) after 7 days in liquid medium, followed by a decrease to 2 $mg.l^{-1}$ after three weeks. In another consortium 'community 4' no expressive amount of RE was generated during growth in phenanthrene. According to the same authors, RE increased values to 12.5 and 8.2 $mg.l^{-1}$, analyzing two different *Burkholderia cepacia* strains, respectively, followed by a drop in these values to 6 and 5 mg. l^{-1} after 3 weeks, respectively.

In the present study, peak of RE was produced by bacterial consortium in a maximum value of 3.45 $\mu g.g\ soil^{-1}$ (week-4); while all other microcosms reached RE values lower than 2 $\mu g.g\ soil^{-1}$ (Figure 2), the minimal value reached in the literature, as discussed previously. This production may be different depending on the occurrence of interactions between microorganisms in each microcosm, when RE peaks can appear and disappear depending on the metabolic cooperation in the soil community.

Luan *et al.* (2006) have reported several products from PAH degradation, originated from an enriched bacterial consortium grown on fluorene, phenanthrene, and pyrene, showing production and consumption peaks of metabolic intermediates by a cooperative degradation process. In a mixture of PAHs, Chen and Aitken (1999) analyzed the importance of the co-substrate supplied on benzo[a]pyrene mineralization medium: *Pseudomonas saccharophilia* only grew when another LMH-PAH (e.g. phenanthrene) or salicylate (a PAH degradation production) were added as inducers of enzymatic syntheses to the benzo[a]pyrene mineralization. Some other studies addressed that consortia and pure cultures are able to degrade benzo[a]pyrene as a part of the PAH mixture (Juhasz and Naidu, 2000).

In the present study, from a 4-week period on, metabolites were still being produced together with a slow HMW-PAHs degradation, and it is believed that metabolic intermediates are used as co-substrate for the degradation of other PAHs. Furthermore, the presence of LMW-PAHs may induce catabolic enzymes activated by the presence of these compounds or still by the key-intermediates from metabolism of PAHs.

4. Conclusions

PAHs into contaminated soil were degraded by both native and exogenous microorganisms added to soil. The native microbiota had a rapid adaptation in the impacted soil most likely by the rich amount of organic matter. Such bioaugmentation possibly will be potentially more efficient in soils with less or different organic matter content, when the native microbiota has less capability of PAHs degradation. LMW-PAHs were removed faster than HMW-PAHs, and the cooperative metabolization of several PAHs into soil by microorganisms was possible, since HMW-PAHs had their degradation extended after depletion of great amount of LMW-PAHs. The production of key-metabolic intermediates could have a possible role in the activation of these PAHs. In all microcosms, it is possible the use of metabolism intermediates produced, immediately by some microorganisms into the microbiota. The PAHs degradation rates, and the ability of production and consumption of such metabolites, which are possibly less toxic than PAHs, sign the consortia and the native microbiota as potential organisms to be added into bioremediation processes due to their fast adaptation in a site with a short-term contamination considered as a period of stress to the soil microbiota.

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Apêndices

1. Curvas de calibração em solo para determinação da degradação de HPAs

O mesmo solo natural coletado para os experimentos em microcosmo foi artificialmente contaminado em triplicata, utilizando em cada repetição uma determinada concentração dos respectivos HPAs, totalizando 5 concentrações diferentes para cada um. Os HPAs foram extraídos dos solos e injetados em CG-FID utilizando a mesma metodologia empregada para todas as amostras e os gráficos a seguir são resultados dos valores em triplicata para cada HPA extraído [Ribani, M., Bottoli, C.B.G., Collins, C.H., Jardim, I.C.S.F., Melo, L.F.C. 2004. Validação em métodos cromatográficos e eletroforéticos. **Química Nova**, 27(5), 771-780].

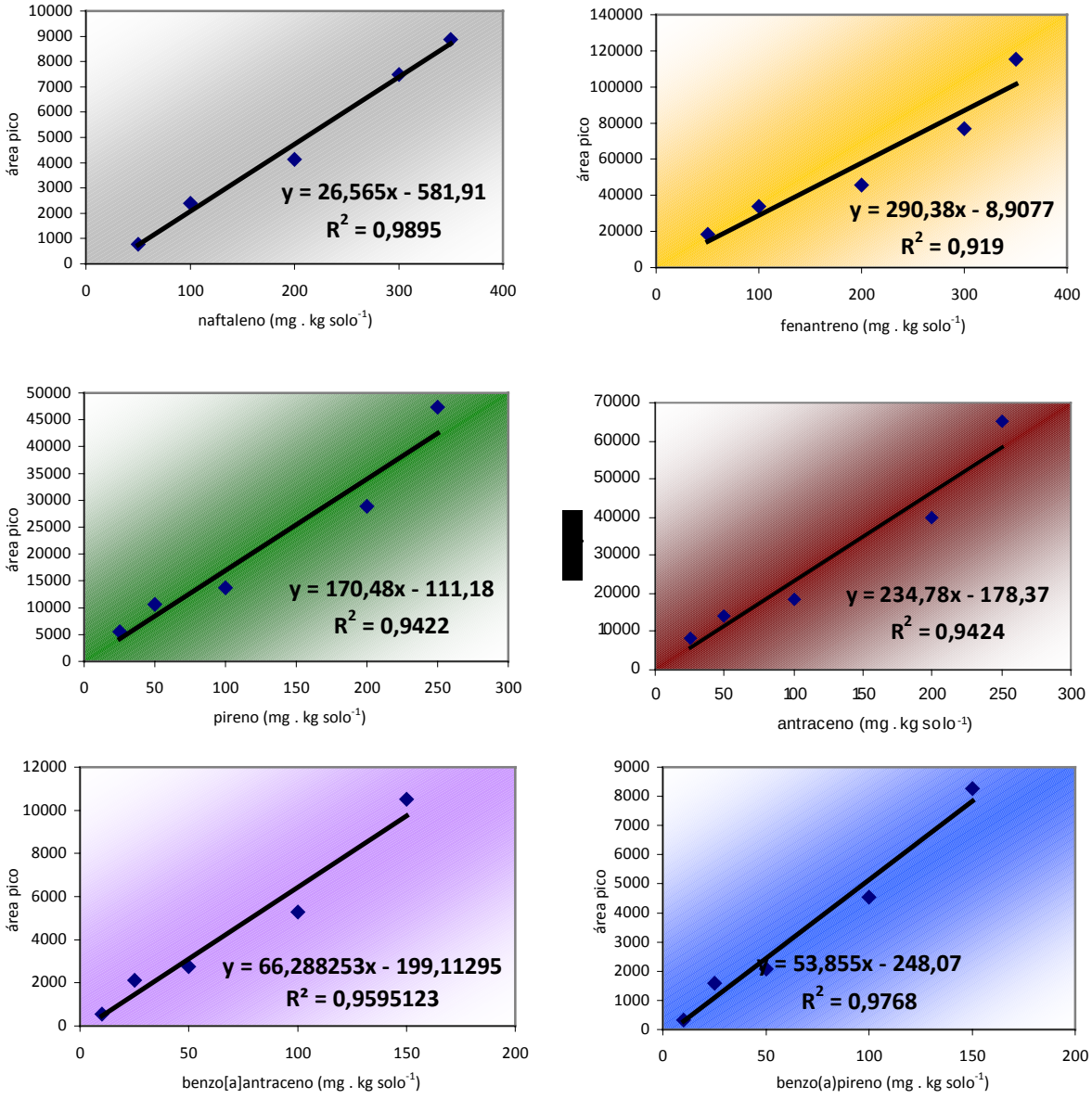

Figura 3. Curvas de calibração em matriz de solo para determinação da biodegradação de HPAs nos microcosmos

2. Curva Padrão de Resorcinol para determinação da produção de metabólitos intermediários

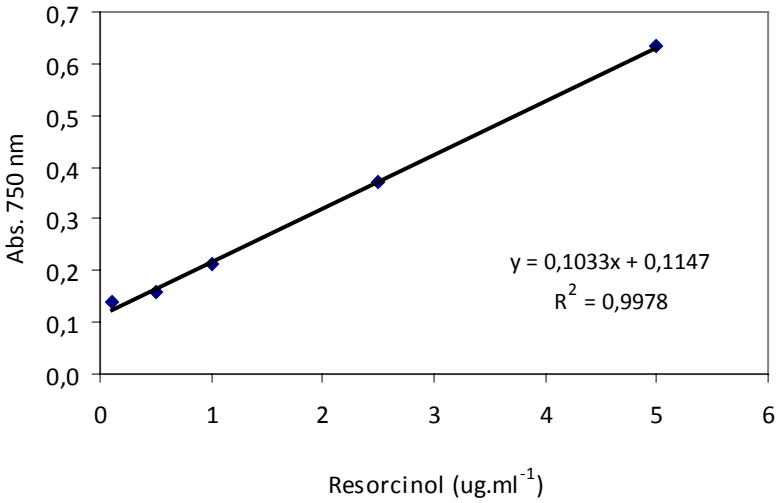

Figura 4. Curva Padrão do resorcinol para determinação de metabólitos intermediários da degradação dos HPAs em microcosmos de solo.

6. Capítulo 3

Application of molecular fingerprinting for analysis of a PAH-contaminated soil microbiota growing in the presence complex PAHs*

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) constitute a group of priority pollutants which are present at high concentrations in the soils of many industrial contaminated sites. Pollution by these compounds may stimulate growth of organisms able to live in these environments causing changes in the structure of the microbial community due to some cooperative process of metabolization of toxic compounds. A long-term PAH-contaminated soil was stored for several years and used to analyze the native microbiota regarding their ability to grow on pyrene, benzo[a]pyrene, as well as in mixtures of LMW- and HMW-PAHs. Molecular profiles of the microbial community was assessed by PCR-DGGE of 16S rRNA gene, and the number of bands observed in DGGE analyses was interpreted as dominant microbial members into the bacterial community. Results of PAH-contaminated soil microorganisms showed different profiles in the degradative dynamics when some nutrients were added. Predominant species may play a significative role while growing and surviving on PAHs, and some other metabolically active species have emerged to interact themselves in a cooperative catabolism of PAHs.

Key-words: *polycyclic aromatic hydrocarbons (PAHs); biodegradation; PCR; DGGE; microbial community.*

1. Introduction

PAH-contaminated soil is a complex and dynamic biological system where native microbial communities are capable of degrading some low-molecular-weight polycyclic aromatic hydrocarbons (LMW-PAHs), such as naphthalene, phenanthrene, fluorene; however, the ability for high-molecular-weight (HMW-) PAHs degradation is still limited (Juhasz and Naidu, 2000). Bacteria act on the degradation of the PAHs through the production of dioxygenases, which incorporate atoms of oxygen at benzenic ring, resulting in the formation of cis-dihydrodiol (Kanaly and Harayama, 2000), followed by some dehydroxylated intermediates that enter in the TCA-cycle (Abate et al., 1999). The native soil microbiota has different phases of adaptation to PAHs and by-products of PAHs degradation may influence the biodegradability rates of others PAHs (Beckles et al., 1998; Bouchez et al., 1995). The presence of LMW-PAHs may increase the biodegradability of HMW-PAHs and metabolic activity of the microorganisms by providing alternative carbon and energy sources for growth (Juhasz and Naidu, 2000). The advantages of using native microorganisms for bioremediation include their better and faster adaptation and the avoidance of other impacts on the environment (Canet et al., 2001). Researches on *in situ* bioremediation have been pursued with selected bacterial strains isolated from the contaminated sites (Mishra et al. 2001), where bioaugmentation is optimized with potentially degrader organisms, or biostimulated with ratios of oxygen and C:N:P, which provide greater efficiency of microbiota to degrade PAHs.

Studies regarding ecology and diversity of the microbial community were limited in the past because only a minimal proportion of the microbial population is cultivable. Fingerprinting profiles of rDNA fragments amplified by PCR have been used to study microbial communities (Muyzer *et al.,* 1995; Ferris *et al.,* 1996; Torsvik *et al.,* 1998), in freshwater lakes (Zwart *et al.,* 1998), estuaries (Murray *et al.,* 1996), and soils (Heuer *et al.,* 1997; Ovreas and Torsvik, 1998). Over this last decade, the profile of bacterial populations in different matrices became more accurate with the application of molecular methods to directly detect DNA and RNA in microbial ecosystems, and this development in molecular biology has found extensive applications in the field of microbial ecology.

Denaturing Gradient Gel Electrophoresis (DGGE) is a useful tool to analyze the initial step of microbial communities composition when some factors are selecting specific populations (e.g. environmental disturbance by pollutants). This technique compares the fingerprinting profiles of samples to identify temporal and spatial differences in community structure or to monitor changes into the community due to the soil impact by contaminants. Based on the presence or absence of bands in each sample, similarity coefficient can be determined for DGGE profile when using general and/or specific primers (Nübel *et al.,* 1997; Kowalchuk *et al*., 1997). The use of group-specific primers allows the study of temporal and spatial changes of microbial populations, evaluating the long-term effect of bacterial inoculants on the stability and dynamic of microbiota in ecosystems (van Elsas *et al.,*1998). In this present study our aim was to verify changes on the microbial community dynamics of a long-term contamination of soil from a manufacturing gas plant (MGP) site when growing in PAHs, identifying some predominant species involved in the biodegradation process.

2. Materials and methods

2.1. Chemicals

PAHs (>98% of purity) were purchased from Sigma. Reagents for the denaturing gradient gel electrophoresys preparation were acquired from BioRad Laboratories (USA). DNA extraction and purification kits were obtained from MoBio Laboratories Inc. (USA) and Promega (Australia), respectively. Sequencing kit was purchase from Beckman Coulter Australia Pty. Ltd.

2.2. Soil samples and PAH experiments

The soil collected previously (Juhasz *et al.*, 2000) from an abandoned MGP-site located near Port Melbourne, Victoria, Australia, and containing high concentration of PAHs, were stored at 4°C for several years. Aliquots of this contaminated-soil "Bin#4" has been taken to be used in some growth experiments in the presence of PAHs.

PAH degradation experiments were performed with the original contaminated soil inoculated into 50 ml serum bottle flasks tightly closed with polypropylene lid and aluminum cap. Different treatments have been applied to 2 g-soil community in 20 ml of salt minimal medium (Juhasz, 1998) containing or not vitamins and micronutrients (N) (Bogardt and Hemmingsen, 1992) added in flasks containing: (A) LMW-PAHs phenanthrene and fluorene, 100 $mg.l^{-1}$ each; (B) pyrene, 100 $mg.l^{-1}$; (C) benzo[a]pyrene, 50 $mg.l^{-1}$; (D) HMW-PAHs benzo[a]anthracene and dibenzo[a,h]anthracene, 50 $mg.l^{-1}$ each; (E) coronene, 20 $mg.l^{-1}$; and finally (F) a mixture of PAHs using the previous concentrations. Treatments A-F were then carried out with salt medium and all the PAH combinations described above, where as treatments AN-FN were performed with the salt medium amended with micronutrients and vitamins plus the same PAH combinations. Flasks were incubated at 30°C/150 $rev.min^{-1}$ in the dark for 30 days. Sampling was done each 7 days for genomic DNA extraction.

2.4. Extraction and purification of genomic DNA

Cells were removed from culture media by centrifugation at 1,500 x g/4°C for 5 minutes, and pellet containing also soil particles was suspended in a proper solution according to the UltraClean™ microbial isolation kit protocol provided by the manufacturer (MoBio Laboratories Inc, USA), using chemical/mechanical cells breakdown to release the genetic materials. Organic and inorganic substances as cells debris and proteins were then precipitated, followed by filtration of DNA in a silica membrane. Genomic DNA was extracted, and a 1% agarose solution containing 0.5 µl ethidium bromide (5 mg ml^{-1}) was prepared in 50 ml 1 x TBE buffer pH 8 (from a 10 x TBE stock solution containing per liter: 108 g trizma-base, 55 g boric acid, 7.4 g EDTA). The samples

(5 µl) added to 6 x loading buffer (1 µl, Genework) were loaded into the gel and run for one hour at 100 V with 1 x TBE as running buffer. Lambda DNA/*Eco* RI + *Hind* III marker (Fermentas) was run in parallel with DNA samples to estimate the amount and quality of DNA extracts, comparing the intensity of bands with the bands in the marker. The gel was photographed on a UV translumination using Quantity One computer program (BioRad).

The purification of DNA extracts was performed due to the presence of humic acid and some other substances that may interfere in the PCR amplification, using a Wizard® DNA Clean-up system protocol (Promega). Purified DNA templates were stored at -20°C to be used in PCR-DGGE experiments.

2.5. Polymerase Chain Reactions

The amplification reactions were prepared in a total volume of 25 µl, containing 2.5 µl of a 10 x PCR buffer (Roche; 100 mM Tris, 500 mM KCl, 15 mM $MgCl_2$), 0.5 µl of dNTP 40 mM (0.2 mM dATP, dCTP, dGTP and dTTP), 1 U Taq DNA polymerase enzyme (Roche), 10 µM of forward and reserve primers (Sigma-Genosys), and 10 ng of DNA template. A primer set from the V3 region into ribosome: PRBA338f - GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG-3') and PRUN518r (5'-ATTACCGCGGCTGCTGG–3') was used to generate a 236bp product. According to Ovreås *et al.* (1997), the forward primer complements a conserved region among members of the Bacteria domain, and the reverse primer is based on a universally conserved region of this domain. The PCR cycle program was set with an initial denaturing step at 95°C for 4 minutes, followed by 30 cycles of 92°C for 1 minute (denaturation), 55°C for 1 minute (annealing), and then 72°C for 1 minute (extension). At the end of cycling, 10 minutes of a final extension was performed. Volumes of 1µl of amplified fragments were run in a 1.5% agarose gel in 1 X TBE buffer. GeneRuler™ DNA Ladder Mix (Geneworks) was used as marker to analyze concentration and quality of the PCR products. To better estimate the concentration of amplified product to be loaded into DGGE wells, some dilutions from samples were made when necessary, and to check the reproducibility of the PCR products, replicate of amplifications and DGGE gels were run.

2.6. Denaturing Gradient Gel Electrophoresis

DNA from the microbial community was analyzed to assess the total diversity and generate a profile of the community. Changes in profiles could indicate some changes into the community. The DGGE technique was performed in a DCode™ Universal Mutation Detection System (BioRad). PCR products (100-200 ng) added in 2 x loading buffer into a microtube were loaded onto 8% (w/v) polyacrylamide (bisacrylamide stock 37.5:1 – BioRad) gels in 0.5 x TAE buffer (20 mM Tris, 10 mM sodium acetate, 0.5 mM Na_2-EDTA, pH 8.0). The gels were made with denaturing gradient from 30 to 60%, where 100% of denaturant solution contains 7 M urea and 40% formamide. DGGE was run at 60°C for 3 hours and 200 V. After electrophoresis, the gel was soaked into glacial acetic acid 10% for

15 minutes, washed twice with ultra pure water (Milli Q system), followed by 15 minutes in methanol 50%, washed twice more, and then the final staining process with SYBR Green I nucleic acid gel dye (1:10,000 in 0.5 x TAE buffer, pH 8.0). The stained gels were photographed under UV translumination using the Quantity One computer program (BioRad).

2.7. Sequencing of predominant bands and isolated colonies

Genomic DNA from predominant bands and isolated colonies were extracted and amplified using a set of primers 27-forward (5'-AGAGTTTGATCMTGGCTCAG-3´) and 518-reverse (5'-ATTACCGCGGCTGCTGG–3'). Preparation of templates for sequencing was performed using CEQ Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter) according to the manufacturer protocol. Samples were sequenced in the Laboratory of Genetics and Molecular Microbiology in Australian Commonwealth Scientific and Research Organization (CSIRO). The sequence identification was performed using BLASTN from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/).

3. Results and discussion

The most probable number of microorganisms into the original PAH-contaminated soil stored for several years was performed (7 x 10^5 cells g of $soil^{-1}$), and the soil "Bin#4" was inoculated into the PAH flasks. Turbidity was an indicative of microbial growth, and color changes on the liquid media might be due to the presence of intermediate metabolites, or still possible reaction between co-metabolites and the medium components.

A soil contaminated with PAHs indicated a reduction of bacterial diversity in experiments carried out by Nakatsu *et al.* (2000) when comparing with some agricultural soils. Analyzing PCR-amplified 16S rDNA fragments by Muyzer *et al.* (1993) to profile community of a bacterial biofilm after separation of the product using DGGE, the number of bands corresponded to the number of predominant members in the microbial community.

At day-zero, when the original PAH-impacted soil was inoculated in liquid medium and/or nutrients, genomic DNA were also extracted, purified, amplified and loaded in the DGGE (Figures 1-4, lines 1, 6 and 11), showing two prominent bands as a initial soil profile. The presence of two dominant bacteria were then verified prior to the incubation experiments in PAHs. In a long-term contaminated environment, some species are liable to be dominated by those microorganisms able to survive in toxic contaminants, showing less diverse but catabolically versatile bacterial community than those in non-impacted environment (Atlas *et al.*,1991; Lindstrom *et al.,* 1999). Comparison of DGGE profiles of the microbiota 16S rDNA PCR amplification products has indicated absence of a high diversity of species in the PAH-contaminated soil; however, intensity of bands has shown that these species were abundant enough to be detected. In a study demonstrated by Venosa *et al.* (1999), the microbial population monitored in a field experiment to evaluate bioremediation of crude oil, showed evidence of environmental stress by all gram-negative bacteria, even having strong bands at day-zero. Microorganisms have a wide range of responses to the environmental stress, and changes of the population level include selection for more resistant species with a concomitant change in overall diversity (Ford, 2000).

In the present work, samples corresponding to soil microbiota in pyrene, benzo[a]pyrene and a mix of PAHs were loaded in the same gel to follow profiles comparison of microbiota dynamics when using only pyrene - the most 4-ring degradable PAH by microorganisms served as a model to HMW-PAH studies, benzo[a]pyrene - the most toxic and mutagenic PAH with high resistance of biodegradation, and a mixture of several PAH – with probable metabolic cooperation and co-metabolic growth in the microbial community. Analyzing soil microbiota in pyrene (Figures 1-2, lines 1-5). New bands were detected during the 4-week period, starting in the early week-1, suggesting adaptation of other species into soil able to grow using pyrene as carbon source. Band-2 showed less intensity during this period, probably due to the action of new competitive microorganisms (bands 4-9), which bands were not detected before exposing soil with fresh PAHs.

It is believed that a cometabolic relationship has been expressed in the gel in this period, due to the raise of some organisms using products of pyrene degradation generated by predominant bacteria in the first weeks. Results from soil microbiota in benzo[a]pyrene (lines 6-10) indicated that the presence of this PAH caused a considerable impact in the community, since a very weak intensity of bands 2, 3 and 4 was visualized at weeks 1 and 2, showing a re-adaptation of these species from the week-3 on when previous predominant bands from day-zero were present again; however, species corresponding to band 3 showed some predominance into the degradation process.

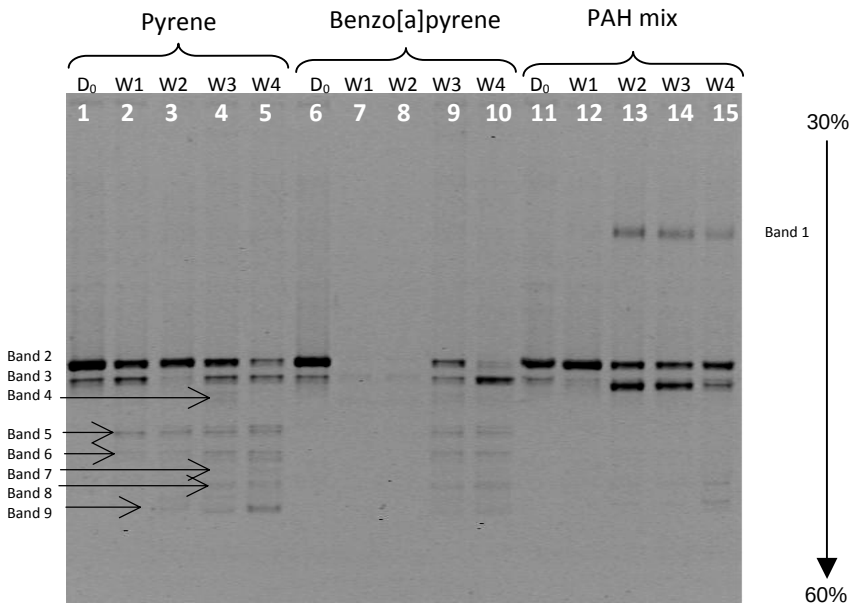

Figure 1. DGGE profiles of 16S rDNA amplicons of *Bacteria* domain obtained from PAH-contaminated soil incubated in minimal medium plus pyrene (lines 1-5); benzo[a]pyrene (lines 6-10); and mix of PAHs (lines 11-15) for 4 weeks (W1-W4)."Day-zero" (D_0) are shown in lines 1, 6 and 11.

When a mixture of PAHs was used as carbon source by the soil microbiota, bands from "day-zero" in this treatment were still predominant through the 4-week period. Nevertheless, the bands present in previous treatments were no longer showing as defined bands (lines 11-15). One additional species was detected at week-2 (band-1), remaining on all period and suggesting ability to survive in several complex PAHs under co metabolism, probably growing by using intermediate metabolites as carbon source produced by other microorganisms when degrading PAHs. Ramirez *et al.* (2001) verified that a microbial community isolated from contaminated soil could achieve more complete degradation of contaminants due to its ability to use byproducts of degradation as carbon source.

Regarding the treatments performed with pyrene, benzo[a]pyrene and a mixture of PAHs plus trace elements and vitamins (Figure 2), some differences have also been shown on DGGE profiles. Microbiota incubated for a 2-week period in pyrene (line 3), showed the presence of band-2 and more predominance of band 4, suggesting facility to grow and survive into the medium when supplied with these nutrients, showing a certain

dependence to the production and activation of catabolic enzymes responsible for degradation of pyrene. Bands 5, 6 and a new species (band 12) remained in the pyrene profile up to week-4 (lines 1-5). Results from soil microorganisms in benzo[a]pyrene plus nutrients (lines 6-10) showed that the microbiota suffered less growth inhibition in the beginning of incubation than in medium with no nutrient addition (Fig. 1, lines 6-10), however, less bands were visualized in the profile in week-4 in medium with nutrients. During the 4-week period, the behavior of microbiota was similar to day-zero. Band-2 was no longer predominant, as also observed in experiments with the same PAH without nutrients, where as band-3 remained predominant during the whole period. It may be possible that vitamins and microelements help a faster growth of microbiota and its adaptation in the presence of benzo[a]pyrene.

In the soil incubated in a mixture of PAHs and nutrients, further new bands (9-11 in lines 11-15) have indicated enough growth of some other species when vitamins and microelements were present in the medium. Band-1 was present after week-2, as observed in previous results with no addition of nutrients. Some species may not be affected by nutrients addition in pyrene, benzo[a]pyrene and a mixture of PAHs, however, some other bacteria showed growth enough to be detected in the presence of nutrients. The appearance of new bands on the bottom of the gel have been considered as better performance of cometabolism in a cooperative metabolization of PAHs.

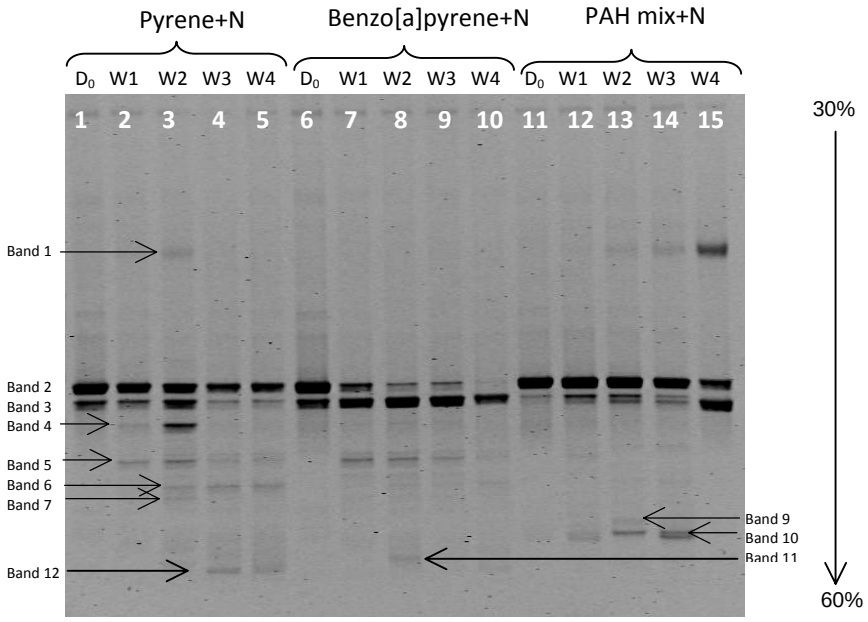

Figure 2. DGGE profiles of 16S rDNA amplification products from PAH-contaminated soil bacterial communities growing in minimal medium plus nutrients (vitamins and microelements) in pyrene (lines 1-5); benzo[a]pyrene (lines 6-10); and mix of PAHs (lines 11-15) for 4 weeks (W1-W4). "Day-zero" (D_0) are shown in lines 1, 6 and 11.

The microbial community profiles in a mixture of LMW-, HMW-PAH and coronene, individually (Figure 3), have indicated the same bands 2 and 3 verified in pyrene, benzo[a]pyrene and the mix of PAHs as predominant in the soil microbiota. In experiments with LMW-PAHs, an additional band 13 has appeared in the profile,

remaining up to week-4 (lines 1-5). The dominant bands became weakly detected, specially band 2, indicating that such specie may receive some inhibition when phenanthrene plus fluorine were used as carbon sources, however, some other bacteria developed some growth adaptation in the presence of these LMW-PAHs.

When the soil microbiota was growing in only HMW-PAHs, the dominance of bands 2 and 3 decreased, and any other band showed good resolution during the incubation period. Is likely that the presence of only HMW-PAHs in the medium did not induce these PAH degradation. Some LMW-PAHs can be added to the medium containing HMW-PAHs and induce their metabolism by activating metabolic common pathways, or stimulating co-metabolism by the use of some metabolic intermediate products from LMW-PAHs as carbon source to some other bacteria that will degrade HMW-PAHs, however, not using them to grow (Boonchan *et al.*, 2000; Juhasz and Naidu, 2000; Marcoux *et al.*, 2000; Gauthier *et al.*, 2003). It is necessary to understand the whole microbial ecology, including microorganisms that are not responsible for the degradation, since they might influence the behavior of the degrading bacteria in a microbial interaction (Sei *et al.*, 2004).

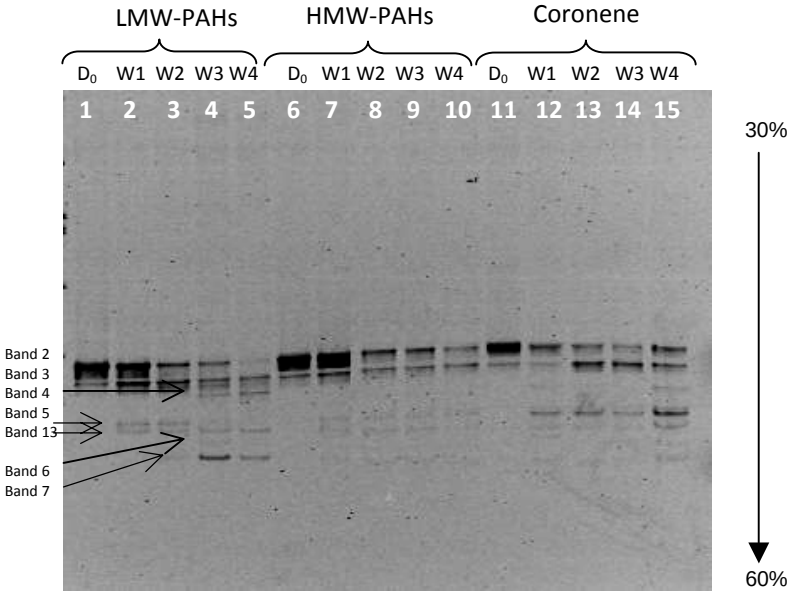

Figure 3. DGGE gel of 16S rDNA fragments of *Bacteria* domain obtained from PAH-contaminated soil incubated in minimal medium plus LMW-PAHs (lines 1-5); HMW-PAHs (lines 6-10); and coronene (lines 11-15) for 4 weeks (W1-W4). "Day-zero" (D_0) are shown in lines 1, 6 and 11.

Profiles of microbiota in coronene (lines 11-15) have shown the gradual presence of bands during the period of cultivation as shown in lines 12 to 15, when a total of 7 bands were present in this last period of incubation (week-4), indicating a better performance of microbiota when using coronene as sole carbon source, comparing with other treatments. It is possible that sub-products of coronene degradation have been used by predominant bacteria at day-zero, confirming the metabolic cooperation in the degradation of coronene. In this case, the high number of aromatic rings of coronene did not show negative effect in the growth of soil microbiota, comparing with the others less complex PAHs.

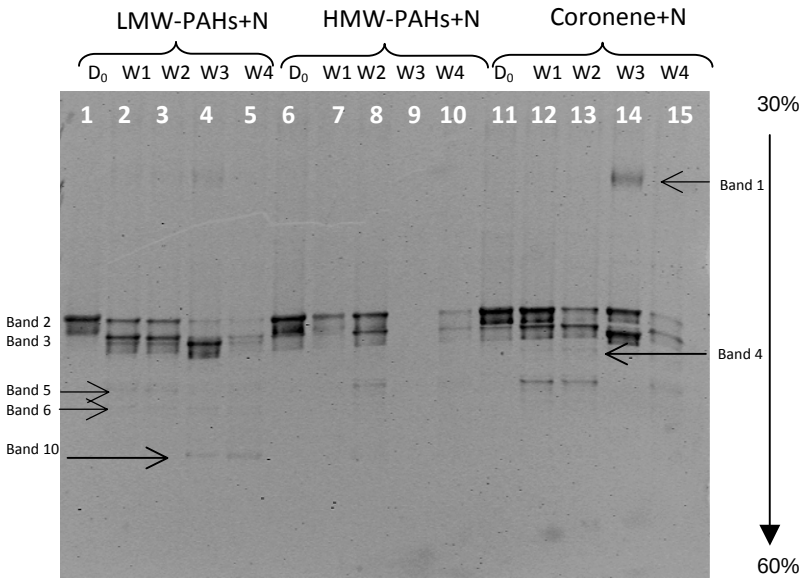

Figure 4. DGGE profiles of 16S rDNA fragments from PAH-contaminated soil bacterial communities incubated in minimal medium plus nutrients (vitamins and microelements) in LMW-PAHs (lines 1-5); HMW-PAHs (lines 6-10); and coronene (lines 11-15) for 4 weeks (W1-W4). "Day zero" (D_0) are shown in lines 1, 6 and 11.

The use of nutrients in media containing soil microbiota in LMW-, HMW- PAHs and coronene (Figure 4) did not show any other new bands, and predominant bands from day-zero had a notable decrease in their intensities in the end of the 4-week period (lines 5, 10 and 15). The diversity of bands also decreased and profiles showed inhibition of some organisms indicated by a very few intensity of bands in the presence of nutrients. The presence of some essential growth factors such as microelements and vitamins (e.g. used as co-factor of several enzymes involved in the degradation of pollutants) is associated with such inhibition of the microbiota for unknown reasons. In some results verified by Viñas *et al.* (2005), mainly HMW-PAHs were significantly degraded when nutrients were not added. These authors also suggest that there are some complex interactions between bacteria and nutrient conditions in the medium, influencing the biodegradation ability of microbiota. According to Cerniglia (1984), there are three types of microbial PAH degradation: complete mineralization, non-specific oxidation and co-metabolic transformation. This last mechanism seems to be possible in this present work to explain some new bands appearing in the course period after predominance of some other species responsible for the initial PAH degradation.

The genus *Pseudomonas* has been the subject of much research as regards its ability to degrade PAHs, being identified in the present study as predominant bands in the original Bin#4 when growing on PAH. The presence of some other species indicated likely existence of different mechanisms for assimilating PAHs in liquid culture, since bacterial community growing under nutrient limitation adapt their catabolic enzyme activities in relation to the type of compounds found in feed media (LaPara *et al.,*2006). Piskonen *et al.* (2005) have shown that dominance of *P. putida* G7 decreased according to DGGE

profiles, and other dominant microbial species have appeared. In a characterization of a microbial community of a PAH-contaminated site, most of the isolates were gram-negative and the members of the genus *Pseudomonas* accounted for 66.6% of the microbial population (Zocca *et al.,*2004). The strong dominance of the genus *Pseudomonas* was confirmed in our study, during the 4-week period, showing two predominant bands. Sequencing of these bands confirmed high similarity with the two colonies on agar plate, identified as very close to *Pseudomonas stutzeri* (Silva *et al.* 2007).

In this study, Bin#4 soil was inoculated in PAH media as sole carbon source and it is believed that in treatments without nutrients, bacteria could obtain mineral-N from salts in the minimal medium for production of proteins, enzymes, DNA, RNA and other N-molecules degrading PAHs or utilizing metabolites from their degradation. In the media containing nutrients, a slight increase of some new bands have been noticed, however, in general the profiles did not increase the diversity by supplement of nutrients, causing also a depletion of bands intensity when soil microbiota was growing in phenanthrene + fluorene, benzo[a]anthracene + dibenzo[a,h]anthracene, and coronene.

In situ molecular studies are of extreme relevance because it is possible to characterize the structure of the microbial community and changes on the diversity of this community, as well as the dynamics of the degradation of pollutants, since 90-99% of the potentially degrading microorganisms cannot be isolated and cultivated successfully under laboratory conditions (Rozsak and Colwell, 1987; White, 1995). Depending on the distribution and the bioavailability of contaminants, the abundance and distribution of microbial degraders are controlled. Moreover, the existence of different niches in soil permits the coexistence of different degrader species (Friedrich *et al.,* 2000). The long exposure period of contaminants in the soil (e.g. the MGP-soil impacted with high levels of PAHs and stored for several years) may indicate great levels of catabolic activity (Reid *et al.*, 2002; Lee *et al.,* 2003). Further research is necessary to better understand the microbial interaction within PAH-degrading microbiota, regulatory mechanisms of PAH degradation, and also the co-metabolization through several catabolic activities to degrade such compounds.

4. Conclusions

The soil microbiota was capable of growing in PAHs, and when analyzing DGGE of PCR-amplified 16S rDNA fragments, predominant organisms related to PAH degradation have been detected. DGGE proved to be a very important first step tool for obtaining information related to the dynamic of all treatments, in the original soil and when growing in PAHs. The addition of nutrients could both increase the intensity of more dominant bands and also decrease some bands intensity and inhibit some other bands expression, depending on the presence of PAHs in different mixtures. Results have shown that contaminated soil microorganisms had different profiles in the degradative dynamic of PAHs when some essential factors of growth were added. Predominant species may play a significative role while growing and surviving on PAHs, and some other metabolically active species have emerged to interact themselves in a cooperative catabolism of PAHs.

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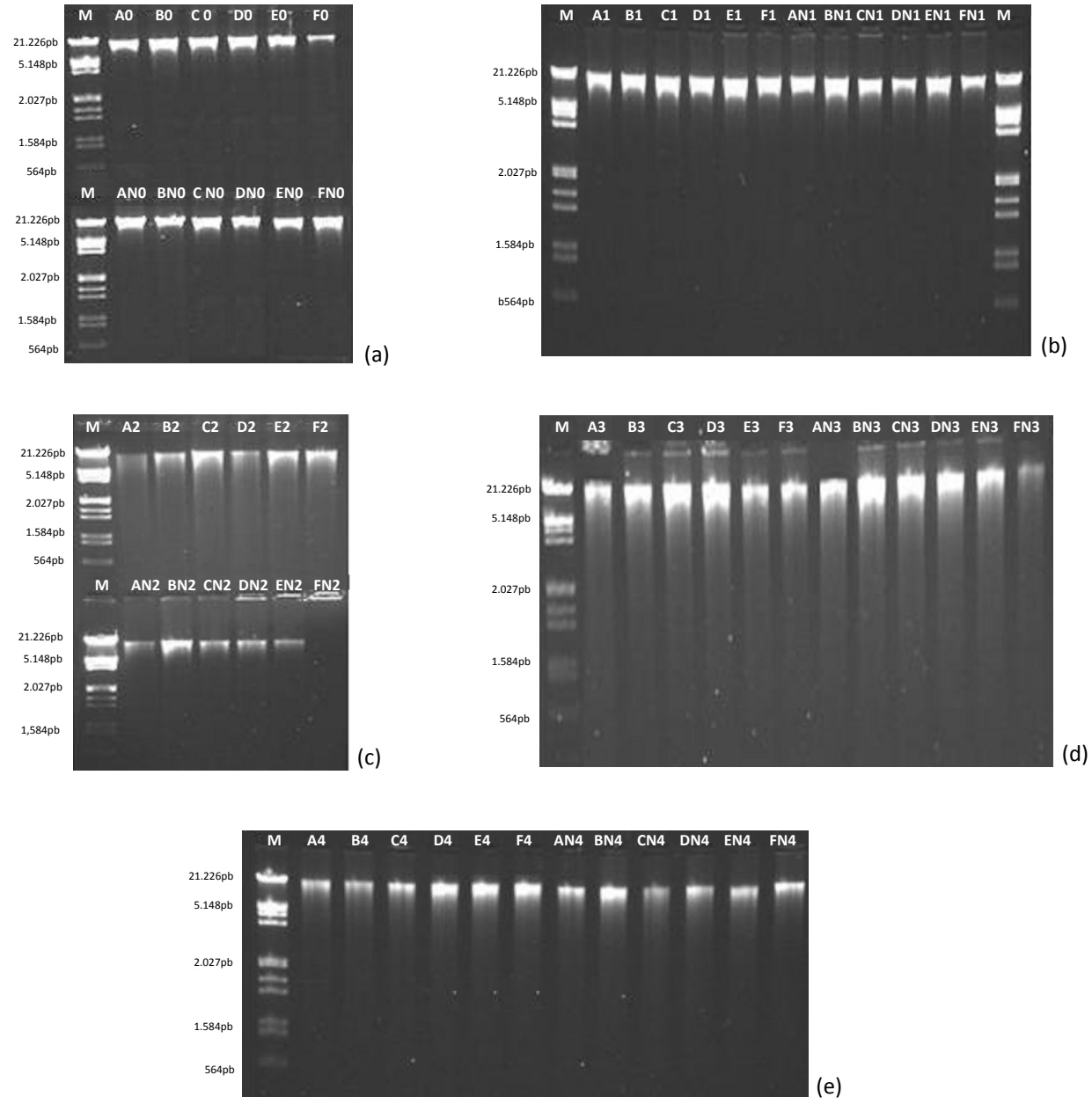
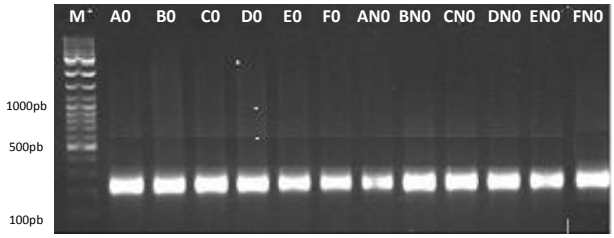
Apêndices

1. Perfil eletroforético do DNA total em gel de agarose, extraído dos experimentos de solo contaminado com HPAs

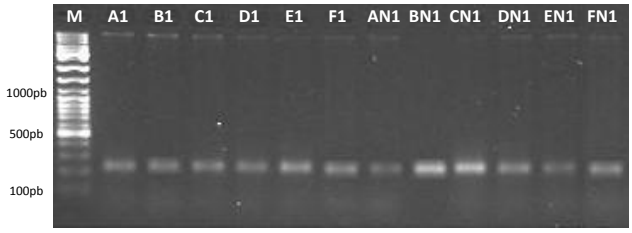


Figuras 5a-5e. Perfis eletroforéticos de DNA total em gel de agarose: (M) marcador de DNA Lambda DNA/*Eco RI* + *Hind III*; (A0-F0 e AN0-FN0) tratamentos no "dia zero" sem adição de nutrientes e com nutrientes, respectivamente. Os números indicam as semanas de coleta do material genético para análise. Exemplo: A1-F1 e AN1-FN1 indicam tratamentos sem e com nutrientes na semana-1, respectivamente; A2-F2 e AN2-FN2 para a semana-2, e assim por diante.

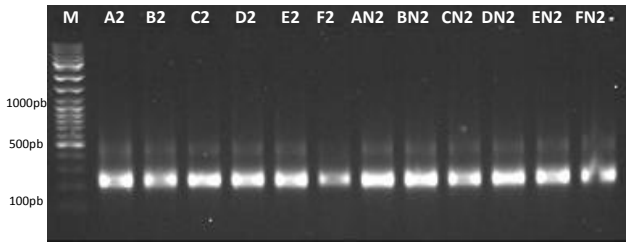
2. Eletroforese em gel de agarose do fragmento do gene RNAr 16S, amplificados em PCR para experimentos em DGGE



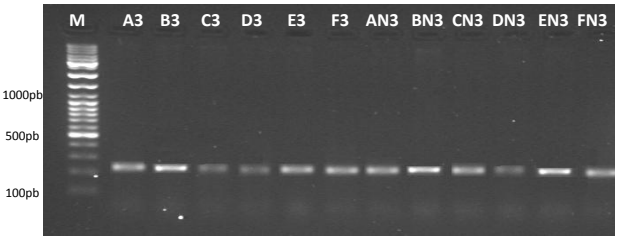
(a)



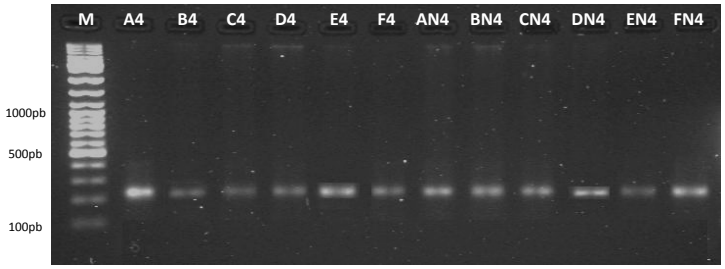
(b)



(c)



(d)



(e)

Figuras 6a-6e. Perfis eletroforéticos do fragmento do gene RNAr 16S amplificado por PCR: (M) marcador de DNA GeneRuler™ Ladder Mix (Geneworks) com diferentes pesos moleculares; (A0-F0 e AN0-FN0) tratamentos no "dia zero" sem adição de nutrientes e com nutrientes, respectivamente. On números indicam as semanas de coleta do material genético para análises - exemplo: A1-F1 e AN1-FN1 correspondem aos tratamentos sem e com nutrientes na semana-1, respectivamente; A2-F2 e AN2-FN2 para a semana-2, e assim por diante.

7. Capítulo 4

Molecular analyses of *Pseudomonas stutzeri* identified in a MGP-contaminated soil enriched for a long period with complex PAHs*

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Abstract

A soil collected from a manufactured-gas-plant contaminated with petroleum hydrocarbons and PAHs was enriched over a period of six months in liquid salt minimum medium and a mixture of several PAHs. Two morphologically different colonies were predominant on agar plates in the end of the enrichment period. A PCR amplification and subsequent sequencing of 16S rRNA genes showed that the colonies were genetically identified as very close to *Pseudomonas stutzeri* (98% of similarity). It is hypothesized that these isolates represent genetic variants of the same species. The presence of other non-cultivable species from this soil was verified with DGGE, and after six months of successive enrichments only *P. stutzeri* bands were seen in the gel. Furthermore, when using pulsed field gel electrophoresis (PFGE) to compare these strains, it was clear that more than one biotype of *P. stutzeri* was present. The presence of catabolic genes responsible for PAH degradation, the alpha subunit gene of initial PAH-dioxygenases (*nah*A) and cathecol-1,2 (C12O) and 2,3- (C23O) dioxygenases were detected by PCR in *P. stutzeri* colonies isolated from agar plates. The enrichment of soil microbiota has an important role in obtaining microorganisms that may be catabolically active in the bioaugmentation of polluted soil. *P. stutzeri*, which was the only species to survive after several months of enrichment in PAHs is possibly an organism with great potential for bioaugmentation in bioremediation processes.

Key-words: *polycyclic aromatic hydrocarbons (PAHs); DGGE; PFGE; C12O; C23O; nahA; Pseudomonas.*

1. Introduction

Recent advances on the molecular techniques to study microbial diversity, including extraction of DNA/RNA from environmental samples, PCR amplification using general and specific genes/primers, analysis of the amplified fragments in electrophoresis to verify population dynamics or the presence of catabolic genes, as well as cloning and sequencing of these DNA fragments to identify predominant microorganisms that have key-functional roles related to the degradation of polycyclic aromatic hydrocarbons (PAHs) are important for microbial cooperation studies in bioremediation (Ranjard et al. 2000; Watanabe and Hamamura, 2003). The small sub-unit of the 16S rRNA gene reveals that if some micro-niches exist independently, they change and interact among themselves (Macrae, 2000). Selective enrichments is the basic method to isolate individual or consortium of microorganisms with good capability in degrading specific compounds (Walter, 1997). Culture enrichment has been chosen as the method to isolate microorganisms able to degrade PAHs (Dagher *et al.*, 1997; Bastiaens *et al.,* 2000). PAH-degrading bacteria can also be isolated from geographically diverse sites using the 16S rRNA sequence technique (Widada *et al.,* 2002*).* Molecular biology approaches have been used to evaluate bioremediation process to detect some catabolic genes into PAH-degrading microbiota and to characterize degradative plasmids and expressions of catabolic genes (Sayler and Ripp, 2000). Specific inducers of genetic operons can also be used to increase the degradation of pollutants. A bioaugmentation with enriched microorganisms is a useful strategy to an inexpensive bioremediation process (Seklemova *et al.*, 2001; Barathi and Vasudevan, 2001), and the analysis of the total microbial genome results in a better understanding of the selection of microbial groups under influence of such enrichment process.

In this present work, the microbial profile from a PAH-contaminated soil stored for several years and after a long period of enrichment was evaluated, verifying genetical differences between predominant species, and detecting the presence of some catabolic genes responsible for PAH degradation.

2. Materials and methods

2.1. Chemicals

All reagents to DGG- and PFG- electrophoresis were purchased from BioRad Laboratories (USA) and Sigma (USA). PAHs (>98% of purity) were obtained from Sigma. DNA extraction and purification kits were acquired from MoBio Laboratories Inc. (USA) and Promega (Australia), respectively. The sequencing kit was purchased from Beckman Coulter Australia Pty. Ltd. Primer sets to catabolic genes investigations were designed by Sigma-Genosys (USA).

2.2. Soil enrichments

Soil samples were taken from "Bin#4", collected previously from an abandoned MGP-plant located in Melbourne, Australia (Juhasz *et al.*, 2000) and stored at 4°C for several years. Successive enrichment cycles were performed for six months in 50 ml of salt minimal medium containing per liter: 3 g KH_2PO_4, 6 g Na_2HPO_4, 2 g NH_4Cl, 5 g NaCl, 0.1 g $MgSO_4.7H_2O$ (Juahsz, 1998), adding 2.5% of both vitamins and microelements solutions sterilized in a 0.22 µm Millipore membrane. All PAH stock solutions were prepared in dimethylformamide at the following concentration: phenanthrene, flourene, pyrene, benzo[a]pyrene, benzo[a]anthracene 10 mg ml^{-1}; and coronene 5 $mg.ml^{-1}$. A mixture of PAHs were added to the medium in the following respective final concentration to each PAH: phenanthrene and pyrene - 100 $mg.l^{-1}$; chrysene, benzo[a]pyrene, benzo[a] anthracene and dizenzo[a,h]anthracene - 50 $mg.l^{-1}$; and coronene - 10 $mg.l^{-1}$. Twenty-five grams of PAHs-contaminated soil was added to 250 ml of minimal medium, followed by agitation at 200 $rev.min^{-1}$/room temperature for 1 hour to dissolve the soil particles, and then 10% of the mixture was used as inoculum. Each seven days, up to month-2, and then each 15 days, up to the month-6, the same amount of inoculum was transferred to a fresh salt minimum medium plus PAHs described previously. An aliquot of each enrichment was taken to a serial 10-fold dilution in 0.1% peptone to isolate colonies. Each dilution of enriched microbiota (100 µl) was transferred to nutrient agar plates to identify the diversity of cultivable microorganisms during the enrichment period.

2.3. Genomic and purification of DNA extracts

At the end of six months, DNA extraction was performed by centrifugation for 10 minutes at 1,500 x g, and then suspension of cells and soil particles in a UltraClean™ microbial isolation kit solution, according to the protocol of manufacturer (MO BIO Laboratories Inc, USA). Genomic DNA was extracted and run in 1% agarose gel containing 0.5 µl ethidium bromide (5 mg ml^{-1}), and prepared in 50 ml 1 x TBE buffer pH 8 (from a 10 x TBE stock solution containing 108 g trizma-base, 55 g boric acid, 7.4 g EDTA). The samples (5 µl) plus 6 x loading buffer (1 µl, Genework) were loaded into the gel and run for one hour at 100 V with 1 x TBE used as running buffer. A lambda DNA/EcoRI+HindIII marker was run in parallel with DNA samples to estimate the amount and quality of DNA extracts, comparing the intensity of DNA bands with the DNA markers. The gel was photographed on a UV translumination using Quantity One program (BioRad). Purification of DNA extracts was performed using a Wizard® DNA Clean-up system, using a DNA purification resin, washing with 80% of isopropanol and suspending DNA extracts with TE buffer (50 µl; 10 mM Tris-HCl, 1 mM EDTA - pH 8.0) to elute the bound DNA fragments, according to the manufacturer's protocol (Promega). Samples of DNA templates were stored at -20°C until use.

2.4. PCR-DGGE and sequencing of colonies

Amplification reactions were prepared in a total volume of 25 µl, containing 2.5 µl of 10 x PCR buffer (Roche; 100 mM Tris, 500 mM KCl, 15 mM $MgCl_2$), 0.5 µl 40 mM dNTP (0.2 mM dATP, dCTP, dGTP and dTTP), 1 U Taq DNA polymerase (Roche), 10 µM of each primer (Sigma-Genosys; forward and reverse), and 10 ng of DNA template. A primer set belonging to the V3 region: PRBA338f - GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGG CGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG-3') and PRUN518r (5'-ATTACCGCGGC TGCTGG–3') was used to generate a 236 bp product. The PCR cycle program performed an initial denaturing step at 95°C for 4 minutes, followed by 30 cycles of 92°C for 1 minute (denaturation), 55°C for 1 minute (annealing), 72°C for 1 minute (extension). At the end of the cycling, a final extension time (10 minutes) was done, and the quality and concentration of DNA were analyzed by running 1 µl of PCR samples in 1.5% agarose gel as described previously for the genomic DNA extraction. GeneRuler™ DNA Ladder Mix (Geneworks) was used as marker.

DNA from the PAH- contaminated soil stored for several years and after six months of enrichment was analyzed to assess the total diversity and to generate a profile of the communities, and changes in the profiles could indicate some disturbance into the community structure. The DGGE technique was performed with DCode™ Universal Mutation Detection System (BioRad). PCR products (100-200 ng) in 2 x loading buffer were loaded onto 8% (w/v) polyacrylamide (bisacrylamide stock 37.5:1 – BioRad) gels in 0.5 x TAE buffer (20 mM Tris, 10 mM sodium acetate, 0.5 mM Na_2-EDTA, pH 8.0). The gels were made with denaturing gradient from 30 to 60%, where 100% of denaturant solution contains 7 M urea and 40% formamide. DGGE was run at 60°C for 3 hours at 200 V. After electrophoresis, the gel was soaked into glacial acetic acid 10% for 15 minutes, washed twice with ultra-pure water (Milli Q system), followed by 15 minutes in methanol 50%, washed twice more, and finally stained with a SYBR Green I nucleic acid solution (1:10,000 in 0.5 x TAE buffer pH 8.0). The gels were photographed on a UV transluminator using the Quantity One computer program (BioRad).

Genomic DNA from two predominant colonies showing visual differences in their morphologies on agar plates were extracted and amplified as described previously for posterior sequencing. Preparation of templates for automated sequencing was performed using CEQ Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter) according to the manufacturer's protocol. Samples were then analyzed in a sequencer machine at The Laboratory of Genetic and Molecular Microbiology belonging to the Australian Commonwealth Scientific and Research Organization (CSIRO). The identification was performed by comparing similarity of nucleotides in the BLASTN from the National Center for Biotechnology Information (NCBI).

2.5. Pulse Field Gel Electrophoresis (PFGE) of *Pseudomonas* colonies

This technique was performed according to Cantor *et al.* (1988) - modified. Briefly, two different colonies of *P. stutzeri* were grown overnight in 25 ml of nutrient broth, and cells were then collected by centrifugation at 1,500 x g / room temperature for 5 minutes. The pellets were resuspended in PIV solution (5 ml; 10 mM Tris-HCl, 1 M NaCl, pH 7.6), centrifuged for 5 minutes as above and washed four more times. Afterwards, pellets were suspended in PIV (10 ml) and incubated on ice for 10 minutes. Suspensions were transferred to a 37°C shaking water bath and incubated for 10 minutes, and 500 µl aliquots were separated in minitubes, adding the same volume of warm 1% agarose in PIV in each. After mixing, suspensions were placed in agarose block molds (Pharmacia), and incubated on an ice bath for 2 hours. Agarose blocks were removed from the molds and transferred to schott bottles containing fresh EC lyses solution (5 ml; 6 mM Tris-HCl, 100 mM EDTA, 1 M NaCl, 20 µg ml^{-1} DNA free-RNAse, 10 mg ml^{-1} lysozyme, 0.5% sarkosyl, 0.2% deoxycholate; pH 7.6). Blocks were incubated at 37°C and transferred into the ESP solution (5ml; 0.5M EDTA, 1% sarkozyl, 1 mg ml^{-1} proteinase K; pH 9.0), following incubation at 45°C overnight. Thereafter, the blocks were washed in TE buffer for 30 minutes at 45°C and this procedure was repeated three more times. After washing, the blocks were stored in TE buffer at 4°C until use.

Half an agarose block was used for each restriction endonuclease digest, washing twice in TE buffer at 45°C for 15 minutes, then digested overnight at 37°C in sterile ultrapure water (115 µl) containing 40 U of *SpeI* enzyme (5' ACT$^{\downarrow}$AGT 3'; Biolabs), NEB2 restriction buffer (15 µl, Biolabs) and BSA (1 mg ml^{-1}, Biolabs). Digested blocks were washed four times in TE buffer at 45°C before being transferred into the loading wells of a 1% agarose gel melted in 0.1 x TBE buffer. Fragments sizes were compared with lambda marker (48.5 – 727.5 kbp, Biolabs), and yeast chromosome marker (225-1,900 kbp; Biolabs). The PFGE was performed using a Clamped Homogenous Electric Field (CHEF) Apparatus (Pharmacia) with HEX electrode, using the following parameters: 170V, 12°C, 10 V cm^{-1} of field strength, 0.1 x TBE running buffer, and pulse program of 5 seconds for 10 hours, 25 seconds for 10 hours and 30 seconds for 15 hours. After running, gel was stained in ethidium bromide (0.5 µg ml^{-1}) for 30 minutes, visualized under UV light, and photographed in a UV transluminator using the Quantity One computer program (BioRad).

2.6. Determination of PAHs catabolic genes

The presence of specific genes in *P. stutzeri* involved in the PAHs degradation was verified using primers from a gene encoding the alpha subunits of initial PAH-dioxygenase - PSEf/PSEr (Moser and Stahl, 2001); catechol-1,2- and -2,3-dioxygenase - C12Of/C12Or and C23Of/C23Or, respectively (Sei *et al.,* 1999). Amplification reactions with the set of primers PSEf (5`-AAA AGA GCT GTA TGG CGA GT -3') and PSE1r (5`-CCG ATA GAA GCC ACG ATA ACT -3') was performed following the same protocol described for the previous PCR

reactions. When using the primers C12Of (5' 5`- GCC AAC GTC GAC GTC TGG CA -3) and C12Or (5`- CGC CTT CAA AGT TGA TCT GCG TGG T -3'); C23Of (5`- AAG AGG CAT GGG GGC GCA CCG GTT CGA TCA – 3`) and C23Or (5`- CCA GCA AAC ACC TCG TTG CGG TTG CC - 3`), the annealing temperature has been changed to 59°C and 57°C, respectively, after some attempts to avoid artifact bands caused by progressive raise of annealing temperature when amplifying catechol-dioxygenases genes fragments.

3. Results and discussion

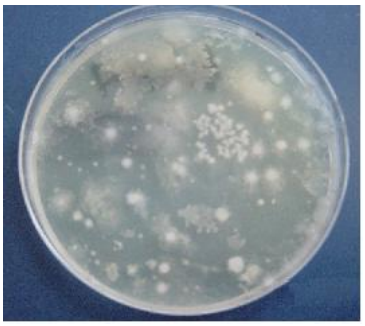
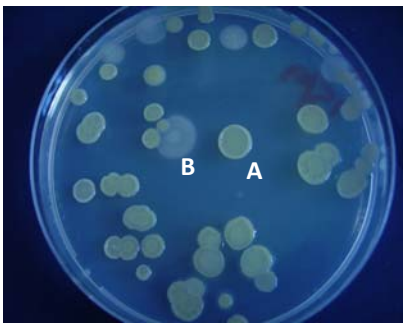
3.1. DGGE and sequencing

Pollution by petroleum hydrocarbons stimulates some changes in the structure of microbial communities in contaminated areas, and the identification of the key organisms that play roles in pollutant biodegradation is important for understanding, evaluating and developing bioremediation strategies (Harayama *et al.*, 2004). Many efforts have been made to characterize bacterial communities, to identify responsible degraders, and to elucidate catabolic potential of these degraders.

Microbial diversity is characterized by several different numbers of species and their relative abundance into community, and microorganisms potentially degraders of PAHs may be predominant due to their rapid adaptation. In this work, few bands could be observed in DGGE profile from the original contaminated soil (Figure 2) with a initial population of 7×10^5 cells.g $soil^{-1}$ (Figure 1a), where only two predominant bands were detected as the best survivers in the MGP-soil under a long-term contamination process. DGGE profile of the microbiota 16S rDNA PCR amplification products has indicated absence of a high diversity of species in the PAH-contaminated soil, however, the intensity of bands showed that these species were abundant enough to be detected.

The presence of some other species indicated a likely existence of different mechanisms to use PAHs (Andreoni *et al.*, 2004), and since bacterial community grows under nutrient limitations, they adapt their catabolic enzyme activities in accordance with the type of compounds found in the feed media (LaPara *et al.,*2006).

In the present study, the soil microbiota showed only two bands after six months of enrichment in several PAHs (line 3), corresponding to the two different colonies isolated on agar plates (lines 4 and 5). In a long-term contaminated environment some species are liable to be dominated by those microorganisms able to survive in toxic contaminants, showing then less diversity but catabolically versatile bacterial community than those in non-impacted environment (Atlas *et al.*,1991; Lindstrom *et al.,* 1999). Enrichment cultures were also performed to better evaluate the PAH-transforming potential of the soil bacterial community (Zocca *et al.*, 2004).


(a)


(b)

Figure 1. (a) Microbiota from PAH-impacted soil (Bin#4) stored for several years; (b) the same soil enriched for 6 months in salt minimal medium plus a mix of PAHs. The two different colonies where identified as *P. stutzeri*.

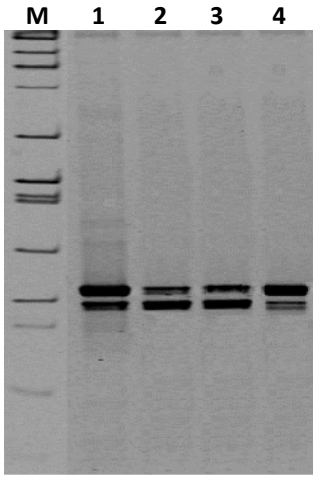


Figure 2. DGGE patterns from PAH-contaminated soil (line 1), contaminated soil enriched for 6 months (line 2), colony A (line 3), and colony B (line 4).

Sequencing from the two morphologically different colonies (Figure 1b) was performed, and *P. stutzeri* was closely identified (98% of similarity) as the only organism existing after several enrichment cycles with a high concentration of complex PAHs. It is likely that this microorganism has a mutation form and consequently different morphological colonies. The genus *Pseudomonas* has been the subject of much research as regards its ability to degrade PAHs. This strain is usually found into contaminated soils and highly grows in petroleum hydrocarbons (Dan and Mukherjee, 2007), and also mineralizes benzo[a]pyrene (Aitken *et al*., 1998). *Pseudomonas* species usually grow in a large number of hydrocarbon compounds as a source of carbon and energy, which demonstrate their efficiency to be used in bioremediation of petroleum derivatives-contaminated sites. Bioaugmentation of total petroleum hydrocarbons-contaminated soil by *P. aeruginosa* species isolated from petroleum-contaminated soil, when inoculating as a consortia, showed reduction of degradation rates and production of biosurfactants (Dan and Mukherjee, 2007). In a recent study, *P. aeruginosa* isolated from a 14-years-old petrochemical sludge of a landfarming site has still performed great growth in anthracene (up to 2g.l^{-1}) and degradation of 71% (in a concentration of 250 mg.l^{-1}) in a 48-day period (Jacques *et al.*, 2005). Other species of *Pseudomonas* in this same study were also able to grow in medium plus phenanthrene, pyrene, gasoline and diesel oil with production of biosurfactants.

Cells of *P. stutzeri* are naturally transformed by homologous chromosomal DNA and do not require treatment to become competent (Carlson *et al.*, 1983). Highly different levels of natural transformation of *P. stutzeri* have been identified into soil through the 16S rRNA gene analysis, indicating several genomovars in this species capable of transforming DNA (Sikorski *et al.*, 2002). This strong variability of the transformation phenotype is explained by many proteins involved in DNA uptake and essential to this process. DNA is released from the cells, adsorbed to the soil material and then taken up by recipient *P. stutzeri* cells, which are able to find access to and take up DNA bound on soil particles in the presence of DNAse indigenous to the soil (Sikorski *et al.*, 1998).

3.2. PFGE of *P. stutzeri* colonies

The number of pulses and the time period were set to separate small fragments (50-100 kb, using 5-second pulse time), medium-sized fragments (100-350 kb, using 25-second pulse time), and the largest fragments (250-600 kb, using 30-second pulse time). The reason for using the enzyme *SpeI* was due to the high GC-content (57-71 mol%) in *Pseudomonas*. Such enzyme cuts at the nucleotides CTAG, demonstrated to be rare in bacteria with high GC-content. Juhasz (1998) had considerable pattern results when analyzing this genus. PFDE-*SpeI* profiles from both colonies isolated from enriched Bin#4 are shown in Figure 3. A total of 12 from 18 bands were similar between lines corresponding to each strain and profiles of colonies were compared regarding the presence of common bands generating a similarity coefficient of 80% (Dice, 1945). According to the sequencing of 16S rRNA gene, the two colonies on agar plate are the same strain *P. stutzeri,* however, they have some differences in size fragments of DNA, suggesting the presence of some different sequences in the total genomic DNA. It could possibly explain the morphological differences between colonies when cultivating in agar plates.

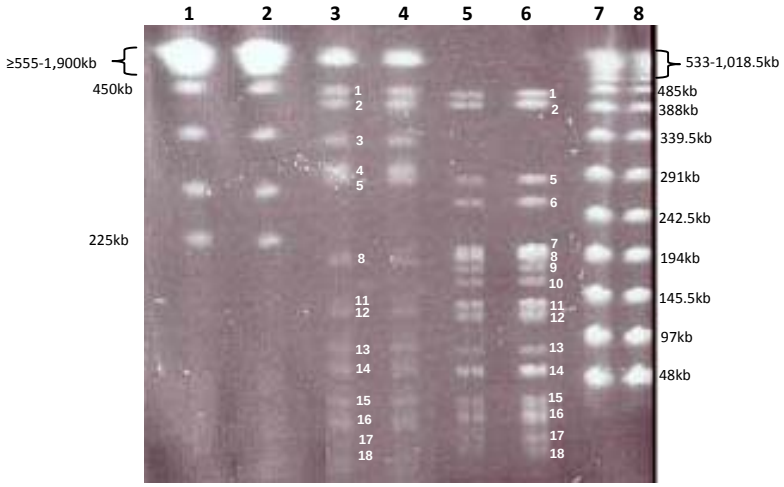


Figure 3. Pulse Field Gel Electrophoresis pattern of *SpeI* digestion of genomic DNA from colony A (lines 3 and 4) and colony B (lines 5 and 6). Yeast chromosome is shown in lines 1 and 2) as high marker, and Lambda ladder (lines 7 and 8) was used as low marker.

3.3. *P. stutzeri* and catabolic genes of PAH degradation

There is a variety of microorganism that are capable of degrading PAHs and most of these belong to the genus *Pseudomonas* having genes involved with PAH-degradative pathway. The ability of microbiota to biodegrade PAHs can be estimated by analyzing catabolic genes present in soil and the enrichment of these gene-carrying bacteria is common during the biodegradation process (Sanseverino *et al.*, 1993). The initial PAH-dioxygenase gene codes a multicomponent enzyme system that catalyses the hydroxylation of PAH to the correspondent *cis*-dihydrodiol, followed by a removal of aromatic rings leading to catechol (end of the upper pathway). In this present work, the set of primers PSE1f/PSE1r was used as target to detect the presence of PAH-dioxygenase gene in *P. stutzeri* colonies by PCR (Table 1). Such gene is commonly detected in *Pseudomonas* species (Ferrero *et al.*, 2002). PAH-degradative gene clusters in this genus are highly homologous to the naphthalene gene cluster from NAH7 plasmid in *P. putida* G7 (Cerniglia, 1993), and soil suspension on mineral medium plus PAH may have promoted the selection of more degrader-species (Piskonen *et al.,* 2005). Widada *et al.* (2002) could also characterize the molecular diversity of catabolic genes in PAH degradation by bacteria isolated from geographically diverse PAH-contaminated sites using the large subunit of terminal PAH-dioxygenase gene.

Piskonen *et al.* (2005) showed a rapid mineralization of naphthalene in soil slurry as effect of inoculation of *P. putida* G7 carrying the naphthalene dioxygenase gene (*nahA*). Furthermore, a previous study has demonstrated that *nah* genes of plasmid NAH7 also mediate the degradation of phenanthrene and anthracene (Sanseverino *et al.*, 1993). Catabolic genes decreased as a result of a gradual drop in degrader organisms. Inoculation of hydrocarbon-contaminated soil with hydrocarbon-degrading bacteria resulted in a boost of degradation rates (Whyte *et al.,* 1999). In this present work, the PSE1 fragment of *nahA* that codes the alpha-subunit of initial PAH dioxygenase was detected in both colonies of *P. stutzeri.* A product of approximately 900 bp was verified after running DNA electrophoresis in 1.5% agarose gel (Figure 4).

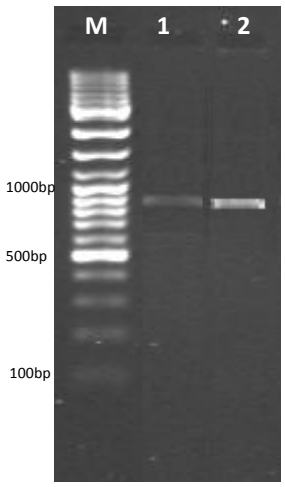


Figure 4. PCR product (≈900bp) run in 1.5% agarose gel electrophoresis using primers for the alpha subunit of initial PAH-dioxygenase in *Pseudomonas* genus to the colonies A (1) and B (2).

Table 1. PCR primers used for amplification and detection of alpha subunit of initial PAH dioxygenases and specific fragments of catechol-1,2-dioxygenase (C12O) and catechol-2,3-dioxygenase (C23O)

Primer	Sequence	PCR product (bp)	Annealing Temp. °C
PSE1f [a]	5`-AAA AGA GCT GTA TGG CGA GT -3`	894	55
PSE1r [a]	5`-CCG ATA GAA GCC ACG ATA ACT -3`		55
C12Of[b]	5`- GCC AAC GTC GAC GTC TGG CA -3`	282	59 [c]
C12Or[b]	5`- CGC CTT CAA AGT TGA TCT GCG TGG T -3		59 [c]
C23Of[b]	5`- AAG AGG CAT GGG GGC GCA CCG GTT CGA TCA – 3`	380	57 [c]
C23Or[b]	5`- CCA GCA AAC ACC TCG TTG CGG TTG CC - 3`		57 [c]

[a] *Pseudomonas* PAH dioxygenase (Moser and Stal, 2001)
[b] catechol-dioxygenase primers designed by Sei *et al.* (1999)
[c] Annealing temperatures defined in this present study according to optimization in PCR reactions

The action of C12O and C23O genes belong to the lower pathway, the most common pathway in the subsequent steps of PAH degradation (Houghton and Shanley, 1994). Most aromatic compounds are metabolized to a common intermediate catechol, which is oxidized through orto- and meta- cleavage pathways catalyzed by C12O and C23O enzymes, respectively; and these respective genes are probably good markers for detection of a whole range of aromatic compounds-degrading bacteria via catechol cleavage pathway (Sei *et al.,* 1999). Bacterial populations that have the C12O gene are primary degraders of phenol and benzoate compounds, whereas organisms containing the C23O gene increase degradation of these substrates when salicylate is present into the medium (Sei *et al.*, 2004). Changes in population of PAHs-degrading bacteria were monitored by these authors using DGGE with a set of general primers for detection of catechol-dioxygenases. These genes seem to be suitable targets to detect the presence of PAH degradation potential at the DNA level (Meyer *et al.*, 1999; Sei *et al.,* 1999). In this present work, DNA template were amplified and bands were visualized indicating the presence C12O and C23O catabolic genes in *P. stutzeri* strains isolated from enriched contaminated soil.

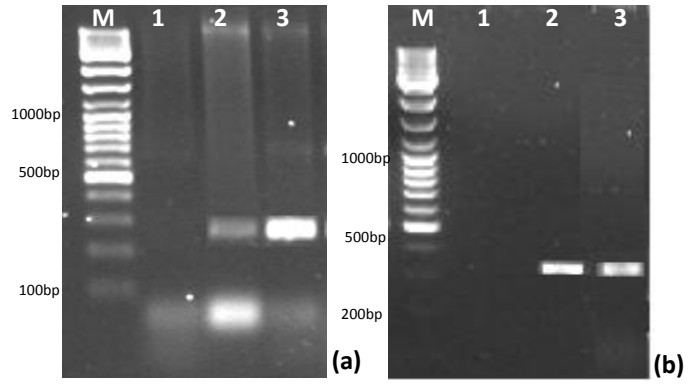


Figure 5. Products from PCR reaction using primers (a) C12O (≈282bp) and (b) C23O (≈380bp). (M) marker Generuler 100bp, (line 1) negative control, and colonies A (line 2) and B (line 3) of *Pseudomonas.*

Studies carried out by Andreoni *et al.* (2004) demonstrated a poorest biodiversity in a soil with long-term exposition to alkanes and PAHs, as well as low levels or absence of enzyme activities. Nevertheless, the enrichment of soil microbiota has an important role to obtain microorganisms catabolically actives in the bioaugmentation of polluted areas. In our results, PAH-dioxygenase and catechol-dioxygenases were detected in both *P. stutzeri* colonies isolated from the same soil after enrichment in PAHs.

Bacterial community when growing in a nutrient limitation condition (e.g. enrichment medium used in this study) can adapt their enzyme activities to the type of substrate used (e.g. PAHs), according to La Para *et al.* (2006), however, it is believed that several cycles of fresh PAH exposition to the soil microbiota could decrease some bacteria that were not able to compete with *P. stutzeri* or still survive in co-metabolism, promoting a better adaptation of this bacterium related to other organisms into soil.

4. Conclusions

A long-period of enrichment in this work has selected a microorganism able to survive in a high concentration of PAHs in the contaminated soil. Two morphologically different colonies of *P. stutzeri* were observed on agar plates in the end of the 6-month enrichment period and confirmed in the DGGE profiles of enriched-soil sample. Comparison of the total genomes using PFGE showed that more than one biotype of *P. stutzeri* were present into the enriched-soil. The presence of catabolic genes may be associated with bacteria adaptation in complex PAHs. The enrichment of soil microbiota has an important role to select microorganisms that may be catabolically active to be used in bioremediation of polluted soil. *P. stutzeri*, which was the only species to survive after several months of enrichment in PAHs is possibly an organism with great potential for bioaugmentation processes, since it was confirmed the presence of key genes responsible for the potential use of PAHs through their degradative pathways.

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Apêndices

1. DNA extraído das colônias crescidas e isoladas em ágar nutriente e do solo enriquecido para experimentos de DGGE

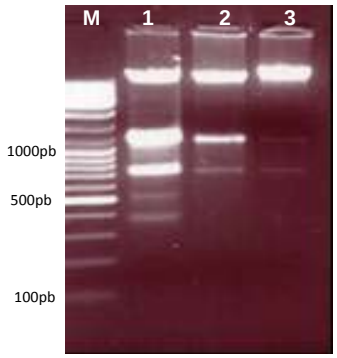
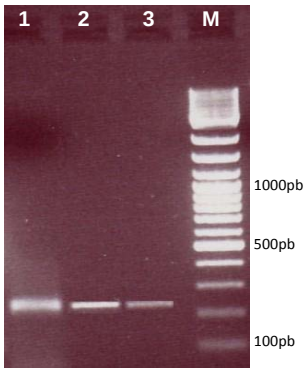


Figura 6. (M) marcador molecular Generuler 100 pb; DNA genômico das colônias A (1) e B (2); e do solo enriquecido por 6 meses (3).

2. PCR com iniciadores gerais para bactérias (334f-grampo GC e 518r) para experimentos em DGGE

Figura 7. (M) marcador molecular Generuler 100pb; produtos de PCR (236pb) das colônias A (1) e B (2), e do solo enriquecido (3).



3. PCR para sequenciamento das colônias isoladas

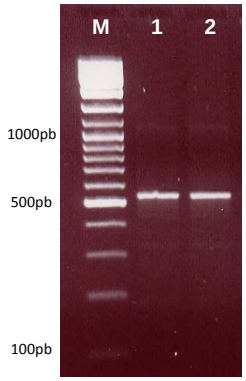


Figura 8. (M) marcador molecular Generuler 100pb; produtos de PCR gerados com iniciadores 27f e 518r (≈500pb) para serem usados em sequenciamento das colônias A (1) e B (2).

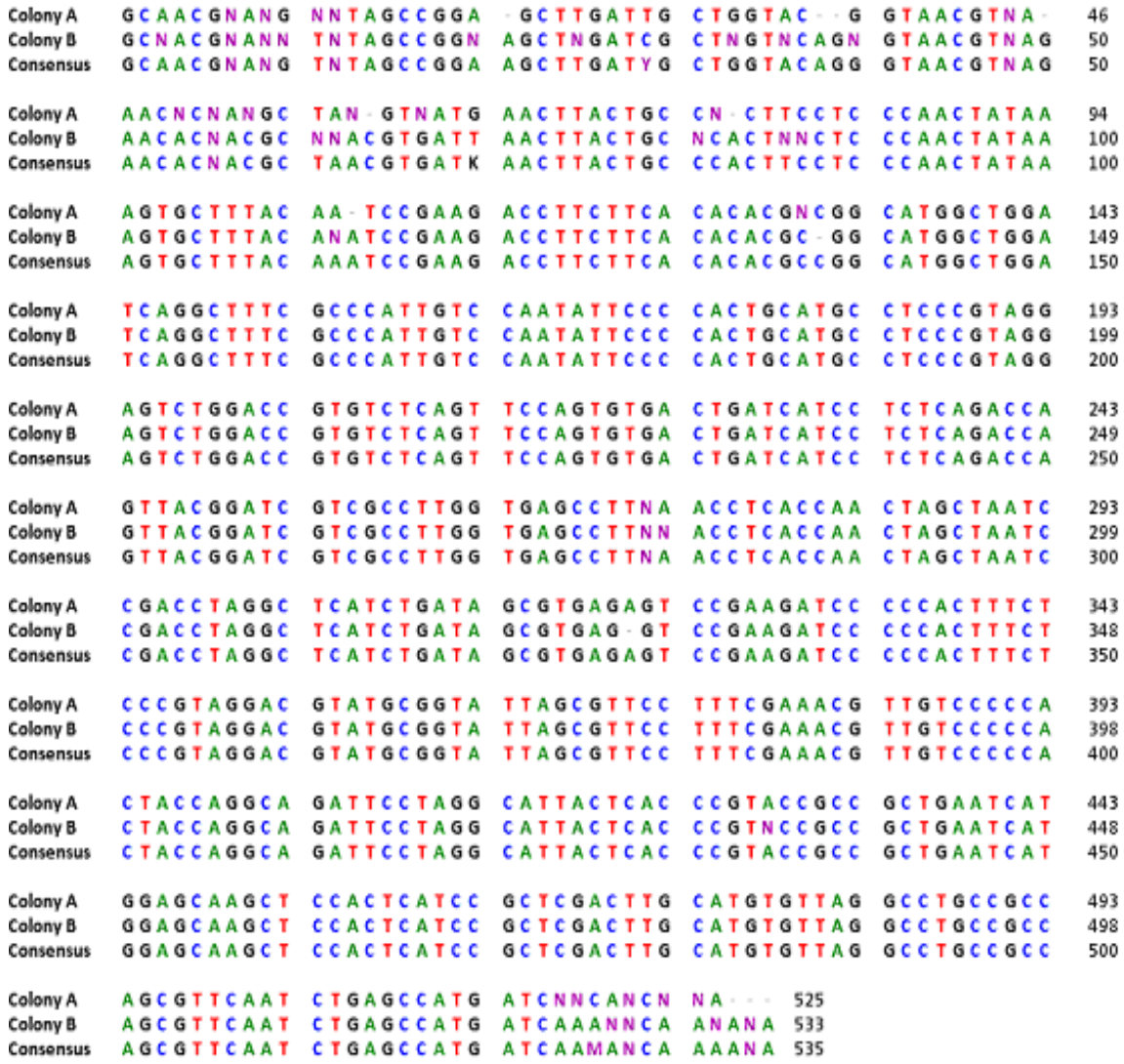


Figura 9. Alinhamento das sequências de bases do gene RNA ribossomal 16S presente das colônias isoladas do solo contaminado e no solo enriquecido. Um consenso de similaridade entre ambas as colônias foi gerado usando BioEdit Sequence Alignment Editor Version 7.0 (N =A/T/G/C/ , M =A/C , Y=T/C)

Tabela 2. Similaridade da sequência de nucleotídeos do fragmento DNAr 16S rRNA da colônia A com as demais sequências conhecidas pesquisadas no GenBank através do programa BLASTn acessado na National Center for Biotechnology Information (NCBI).

Microorganism	Accession number	16S rRNA region	% similarity to Colony A
Pseudomonas stutzeri DSM 5190T	AJ288151	5-445	98%
Pseudomonas stutzeri	AF094748	1-439	98%
Pseudomonas stutzeri ATCC 17598	AJ006104	1-431	98%
Pseudomonas sp HPC26	AY571889	13-456	98%
Uncultured bacterium clone W9	AY770949	9-452	98%
Pseudomonas sp. E1-4	DQ227347	6-449	98%
Pseudomonas sp. E2-4	DQ227348	6-449	98%
Pseudomonas sp. E4-1	DQ227349	6-449	98%
Pseudomonas sp. Hugh2814	AB247234	8-451	98%
Uncultured *Pseudomonas* sp. clone 91_19	AF467293	7-450	98%
Pseudomonas sp. PDA	AF323492	10-453	98%
Pseudomonas sp. PDB	AF323493	11-454	98%
Pseudomonas sp RNA-111	AJ387903	8-451	98%
Uncultured bacterium clone EV818BHEB5102702SAS47	DQ256364	8-451	98%
Pseudomonas sp. R-25343	AM084028	1-439	98%
Pseudomonas sp. A7-1	DQ227346	13-450	98%
Uncultured bacterium clone O1	AY770933	9-452	98%
Uncultured gamma proteobacterium clone 2532	AF467378	7-439	98%
Pseudomonas sp. JPL-1	AY030314	4-435	98%
Pseudomonas sp. BRW1	AF025349	1-431	98%
Pseudomonas sp. BRW3	AF025351	1-431	98%
Pseudomonas sp. 12a-1	AY561549	1-431	98%
Pseudomonas sp. 12b-1	AY561550	1-429	98%
Pseudomonas sp. 17a-3	AY561561	1-429	98%
Pseudomonas sp. 17a-4	AY561562	1-429	98%
Pseudomonas sp. 17-b2	AY561565	3-433	98%
Pseudomonas sp. 17-c2	AY561567	3-431	98%
Uncultured bacterium clone HBO47	DQ201249	8-425	98%
Pseudomonas stutzeri	AF152596	1-444	97%
Pseudomonas stutzeri	AB088754	1050-1492	97%
Pseudomonas stutzeri 24a36	AJ312169	5-443	97%
Pseudomonas stutzeri 24a50	AJ312171	2-440	97%
Pseudomonas stutzeri 24a80	AJ312170	3-441	97%
Pseudomonas stutzeri API-2-142	AJ410871	4-442	97%
Pseudomonas stutzeri YPF-41	AJ410872	4-442	97%
Pseudomonas stutzeri SA1	DQ059546	1048-1491	97%
Pseudomonas stutzeri ATCC 17685	AJ006103	6-449	97%
Pseudomonas stutzeri	U58660	5-443	97%
Pseudomonas stutzeri NA1	AB109011	8-449	97%
Bacterium K2-20	AY345431	12-455	97%
Pseudomonas sp. JQR2-5	DQ124297	12-455	97%
Uncultured *Pseudomonas* sp clone 349_11	AF467304	7-450	97%
Nitrogen-fixing bacterium MIS	AF214645	10-453	97%
Pseudomonas fragi	D84014	6-449	97%
Uncultured gamma proteobacterium clone 2534	AF467360	6-448	97%
Uncultured *Pseudomonas* sp. PsEJ	AF453306	8-440	97%
Uncultured bacterium clone FB46-42	AY527767	8-451	97%
Flavobacterium lutescens	M59156	15-458	97%
Pseudomonas sp. Gu5828	AY120881	8-451	97%

Tabela 3. Similaridade da sequência de nucleotídeos do fragmento DNAr 16S rRNA da colônia B com as demais sequências conhecidas pesquisadas no GenBank através do programa BLASTn acessado na National Center for Biotechnology Information (NCBI).

Microorganism	Accession number	16S rRNA region	% similarity to Colony B
Pseudomonas stutzeri DSM 5190T	AJ288151	5-430	98%
Pseudomonas stutzeri	AF094748	1-424	98%
Pseudomonas stutzeri ATCC 17598	AJ006104	1-416	98%
Pseudomonas stutzeri ATCC 17685	AJ006103	6-434	98%
Pseudomonas stutzeri phen8	AF284764	8-428	98%
Pseudomonas stutzeri SA1	DQ059546	1063-1491	98%
Pseudomonas stutzeri	AF152596	1-429	98%
Pseudomonas stutzeri 24a36	AJ312169	5-428	98%
Pseudomonas stutzeri 24a80	AJ312170	3-426	98%
Pseudomonas stutzeri 24a50	AJ312171	2-425	98%
Pseudomonas stutzeri YPF-41	AJ410872	4-427	98%
Pseudomonas stutzeri API-2-142	AJ410871	4-427	98%
Pseudomonas sp. 12a-1	AY561549	1-416	98%
Pseudomonas sp. 17-b2	AY561565	3-418	98%
Pseudomonas sp HPC26	AY571889	13-441	98%
Uncultured bacterium clone W9	AY770949	9-437	98%
Pseudomonas sp. E1-4	DQ227347	6-434	98%
Pseudomonas sp. E2-4	DQ227348	6-434	98%
Pseudomonas sp. E4-1	DQ227349	6-434	98%
Uncultured *Pseudomonas* sp. clone 91_19	AF467293	7-435	98%
Pseudomonas sp. PDA	AF323492	10-438	98%
Pseudomonas sp. PDB	AF323493	11-439	98%
Pseudomonas sp RNA-111	AJ387903	8-436	98%
Uncultured bacterium clone EV818BHEB5102702SAS47	DQ256364	8-436	98%
Pseudomonas sp. R-25343	AM084028	1-424	98%
Pseudomonas sp. A7-1	DQ227346	13-435	98%
Uncultured bacterium clone O1	AY770933	9-437	98%
Uncultured gamma proteobacterium clone 2532	AF467378	7-435	98%
Pseudomonas sp. JPL-1	AY030314	4-420	98%
Pseudomonas sp. BRW1	AF025349	1-416	98%
Pseudomonas sp. BRW3	AF025351	1-416	98%
Pseudomonas sp. 17a-3	AY561561	1-414	98%
Pseudomonas sp. 17a-4	AY561562	1-414	98%
Pseudomonas sp. 17-c2	AY561567	3-416	98%
Uncultured bacterium clone HBO45	DQ201248	8-413	98%
Uncultured bacterium clone HBO47	DQ201249	8-425	98%
Uncultured bacterium clone HBO54	DQ201255	8-413	98%
Uncultured bacterium clone HBO58	DQ201259	8-413	98%
Uncultured *Pseudomonas* sp. PsEJ	AF453306	8-436	98%
Bacterium K2-20	AY345431	12-440	98%
Pseudomonas sp. JQR2-5	DQ124297	12-440	98%
Uncultured gamma proteobacterium clone 2534	AF467360	6-434	98%
Uncultured *Pseudomonas* sp clone 349_11	AF467304	7-435	98%
Nitrogen-fixing bacterium MIS	AF214645	10-438	98%
Pseudomonas fragi	D84014	6-434	98%
Pseudomonas stutzeri NA1	AB109011	8-436	97%
Pseudomonas stutzeri	U58660	5-443	97%
Uncultured bacterium clone FB46-42	AY527767	8-436	97%

8. Conclusões finais

De um modo geral, a microbiota nativa do solo impactado artificialmente com HPAs teve uma grande contribuição na degradação destes poluentes, uma vez que não foram observadas grandes diferenças na biomassa, respiração e eficiência de metabolismo (qCO_2) em relação aos microcosmos bioaumentados. A adaptação dos microrganismos no solo contendo os HPAs não contou com a ajuda de bioestimulações, com exceção da correção da capacidade de campo e oxigenação periódica. Processos de co-metabolismo com cooperação mútua entre as populações de microrganismos do solo provavelmente tenham contribuído para uma rápida resposta ao distúrbio ambiental. O crescimento da biomassa e da taxa respiratória foram acompanhadas de consideráveis degradações dos HPAs de menor peso molecular, seguido dos HPAs de maior peso molecular, sugerindo ativação de caminhos degradativos dos HPAs mais complexos pela presença dos PAHs menores.

Para todos os microcosmos observou-se a degradação dos HPAs estudados neste trabalho. Acredita-se ainda que os produtos de degradação dos HPAs menos complexos (2-3 anéis) possivelmente estiveram também envolvidos na degradação dos HPAs de maior peso molecular, através de co-metabolismo - utilização de outros compostos para o metabolismo microbiano, resultando na degradação dos HPAs mais complexos.

Estes resultados demonstraram que, nos solos com ou sem bioaumentação com consórcios microbianos, microrganismos desta comunidade foram capazes de se adaptarem rapidamente no solo impactado, apresentando potencial de biorremediação destes compostos a curto-prazo, período considerado de estresse para uma microbiota natural de um solo recém-impactado.

Analisando uma outra microbiota proveniente de um solo contaminado com HPAs e outros derivados do petróleo; porém, a longo-prazo, alguns microrganismos que ainda sobreviviam no solo estocado por vários anos demonstraram grande capacidade de crescerem empregando adições combinadas de HPAs como única fonte de carbono. Monitorando o comportamento da microbiota no período de enriquecimento deste solo em HPAs por PCR-DGGE, usando fragmento do gene RNA ribossomal 16S, foram detectados alguns microrganismos predominantes neste processo. A adição de micronutrientes e vitaminas tiveram influência na mudança de intensidade de algumas bandas ou inibiram a presença de algumas outras, dependendo dos HPAs presentes. Tais espécies foram predominantes e mostraram potencial habilidade para sobreviverem em ambientes contaminados a longo-prazo, devido à permanência de genes que codificam enzimas importantes para a degradação de HPAs no seu material genético. Durante 6 meses de enriquecimento deste solo em uma mistura de HPAs, apenas dois tipos diferentes de colônias foram isoladas e identificadas por sequenciamento, correspondendo ao gênero *Pseudomonas* com similaridade muito próxima à espécie *stutzeri*, espécie esta dotada de grande variância genômica, verificada pela análise do genoma total destas duas colônias. A presença de genes catabólicos envolvidos na degradação de HPAs foi também confirmada para estas colônias.

Por conclusão, pôde-se estudar neste trabalho duas situações diferentes de contaminação dos solos, avaliando o comportamento da microbiota de um solo recém-impactado com HPAs, usando ou não bioaumentação com consórcios microbianos, e monitorando a dinâmica de uma microbiota de solo com histórico longo de contaminação por HPAs e outros derivados do petróleo, utilizando técnicas moleculares.

Apesar das condições diferentes, verificou-se em ambos os estudos a existência de uma alta potencialidade de adaptação e degradação dos HPAs pelos microrganismos no solo. No entanto, trabalhos futuros usando estes microrganismos em condições otimizadas de concentração dos inóculos, estimulação com nutrientes, umidade e oxigenação neessárias, em uma escala piloto, poderão render resultados interessantes usando consórcios microbianos em biorremediação de solos em biopilhas, bioreatores ou processos *in situ*.