DANIEL KUMAZAWA MORAIS

PETROLEUM EFFECTS ON SOIL MICROBIAL COMMUNITIES

Tese apresentada à Universidade Federal de Viçosa como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola para obtenção do título de Doctor Scientiae.

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APROVADA: 29 de Julho de 2015.

Hilário Cuquetto Mantovani	Maurício Dutra Costa (Co-orientador)
Luiz Fernando Wurdig Roesch	Moysés Nascimento

Marcos Rogério Tótola
(Orientador)

Dedicado aos meus pais. Vladimir Morais
e Maria Edina Kumazawa Morais

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BIOGRAFIA

DANIEL KUMAZAWA MORAIS, filho de Vladimir Morais e Maria Edina Kumazawa Morais, nasceu em 10 de abril de 1987 em Sinop, Mato Grosso. Em 12 de março de 2009, graduou-se no curso de Licenciatura Plena em Ciências Biológicas da Universidade Federal de Mato Grosso. Em agosto de 2009, iniciou o curso de Mestrado em Microbiologia Agrícola na Universidade Federal de Viçosa, na área de Microbiologia Ambiental, submetendo-se à defesa de dissertação em 26 de julho de 2011. Subsequentemente, ingessou no curso de Doutorado em Microbiologia Agrícola na Univerisade Federal de Viçosa, na área de Microbiologia Ambiental, submentendo-se à defesa de tese em 29 de julho de 2015.

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ABSTRACT

MORAIS, Daniel Kumazawa, D. Sc., Universidade Federal de Viçosa, July, 2015. **Petroleum effects on soil microbial communities.** Advisor: Marcos Rogério Tótola. Co-Advisors: Maurício Dutra Costa and Poliane Alfenas Zerbini.

Crude oil is still the dominant energy source in Brazil where oil consumption keeps rising since 2013, reaching nowadays 2.2% of the world's energy consumption. A recent discovery of crude oil reservoirs at the Espirito Santo, Campos and Santos basins, can represent an excellent opportunity to meet the country's economic and energetic demands. However, offshore exploration offers risks to the microbiota and the whole sea life. Microbes are responsible for nutrient cycling can degrade recalcitrant organic compounds and several species have been reported as sensitive to petroleum hydrocarbons. This work aimed to evaluate microbial community shifts in soils under crude oil contamination and assess the effects of Biodiesel co-product (BCP) as a protecting agent of soil microbiota under crude oil addition. We used soils from the Trindade Island and from the Highfield research station at Rothamsted Research, UK. We assembled microcosms of 20 grams and contaminated the soils using weathered crude oil. Soils were incubated at 26° C with moisture correction to ca. 60% water holding capacity. We used CO_2 evolution measurements to evaluate soil activity, during the incubation, and soil genomic DNA extraction, at the end of incubation period, to evaluate microbial community changes from treatments and controls. DNA was submitted to amplicon sequencing of 16S rDNA for Bacteria and Archaea and the ITS1 region for Fungi using Illumina MiSeq platform. We compared alpha and beta-diversity and taxonomic shifts. This thesis is divided in two chapters. The first describes the effects of crude oil on Trindade Island's soil microbial communities. In the second chapter we tested the protective effects of BCP on Trindade Island, Rothamsted's Bare Fallow and Grassland soils, against the amendment with crude oil. Crude oil had a major negative effect on microbial diversity for Trindade Island, but didn't change the diversity of Rothamsted agricultural soils. Taxonomy comparisons showed rise of the Actinobacteria phylum, shifts in several Proteobacteria classes and reduction of the Archaea class Nitrososphaerales. This is the first effort in acquiring knowledge concerning the effect of crude oil contamination in soils of a Brazilian oceanic island.

This information is important to guide any future bioremediation strategy that can be required.

RESUMO

MORAIS, Daniel Kumazawa, D. Sc., Universidade Federal de Viçosa, julho, 2015. **Efeito do petróleo sobre comunidades microbianas do solo.** Orientador: Marcos Rogério Tótola. Coorientadores: Maurício Dutra Costa e Poliane Alfenas Zerbini.

O petróleo é a principal fonte de energia no Brasil, onde o consumo de óleo continua subindo desde 2013, atingindo atualmente 2.2% do total de energia consumida no mundo. A descoberta recente de petróleo nas baias do Espirito Santo, Campos e Santos, pode representar uma excelente oportunidade para atender as demandas energéticas nacionais. Entretanto, a exploração de petróleo oferece riscos a microbiota e toda a vida marinha. Microrganismos são responsáveis pela ciclagem de nutrientes, podem degradar compostos orgânicos recalcitrantes e muitas espécies são reportadas como sensíveis à contaminação por hidrocarbonetos do petróleo. Esse trabalho teve o objetivo de avaliar as alterações na comunidade microbiana em solos sob a contaminação por petróleo e avaliar os efeitos do Co-produto de biodiesel (BCP) como um agente protetor da microbiota do solo perante a adição de petróleo. Foram utilizados solos da Ilha da Trindade, e da estação de pesquisa Highfield no Rothamsted Research, UK. Foram montados microcosmos com 20 gramas de solo e os tratamentos utilizaram petróleo intemperizado. Os solos foram incubados a 26° C com correção da umidade para cerca de 60% da capacidade de retenção de água dos solos. Foi utilizada a medição de evolução de CO_2 para avaliar a atividade do solo, durante o período de incubação, e a extração de DNA genômico do solo, ao final do período de incubação, para avaliar as mudanças nas comunidades microbianas dos tratamentos e controles. O DNA foi submetido para o sequenciamento de amplicons de 16S rDNA para a avaliação de Bacteria e Archaea e de amplicons da região ITS1 para a avaliação de Fungos utilizando a plataforma Illumina HiSeq. Foi feita a comparação das diversidades alpha e beta e análise das alterações taxonômicas. Essa tese está dividida em dois capítulos. O primeiro descreve os efeitos do petróleo nas comunidades microbianas do solo da Ilha da Trindade. No segundo capítulo foi testado o efeito protetor do BCP sobre a microbiota dos solos da Ilha da Trindade, do campo Bare Fallow e do campo Grassland do Rothamsted Research contra a adição de óleo. O petróleo teve um grande efeito negativo sobre a diversidade microbiana da Ilha da Trindade, mas não mudou a diversidade microbiana dos solos agrícolas do Rothamsted. A comparação taxonômica mostrou aumento do filo Actinobacteria, mudanças em várias classes de Proteobacteria e redução da classe Nitrosphaerales do filo Archaea. Esse é o primeiro esforço para aquisição de conhecimento sobre o efeito da contaminação de solos de uma ilha oceânica brasileira com petróleo. Essa informação é importante para guiar qualquer futura estratégia de biorremediação que se faça necessária.

INTRODUÇÃO GERAL

O petróleo ainda é a principal fonte de energia utilizada no mundo, suprindo 32.6% da energia consumida. Sua utilização como fonte de energia está em declínio há quinze anos, enquanto o número de reservatórios e a produção crescem (BP Statistical Review of World Energy, 2014). Apesar da sua importância econômica e alta demanda, a exploração de petróleo apresenta risco para a biodiversidade, em razão do caráter tóxico e mutagênico de alguns de seus hidrocarbonetos (Sikkema et al., 1995; Das e Chandran, 2010). Os efeitos da contaminação do solo por hidrocarbonetos do petróleo têm sido estudados desde o famoso acidente ocorrido no Alaska onde o navio tanque Exxon Valdez (1989) derramou o equivalente a 257 mil barris de petróleo no ambiente, esse foi o primeiro grande acidente de contaminação ambiental com petróleo envolvendo a ação de múltiplos países. Estudos de biorremediação feitos nesse local geraram tecnologias empregadas até os dias de hoje (Atlas, 2011). Mais atenção foi dada ao tema após o acidente envolvendo a explosão e o naufrágio da plataforma de exploração de petróleo em águas profundas, no golfo do México, resultando no vazamento de cerca de 3.2 milhões de barris. Tal vazamento se caracteriza como o maior acidente de petróleo já ocorrido (Atlas, 2011; Sammarco et al., 2013). Os dois eventos são os exemplos clássicos de contaminações com efeitos drásticos ao ambiente marinho e quase irreversíveis ao solo.

O solo abriga 60% da biomassa terrestre, essa biomassa é composta em sua maioria, por microrganismos (Singh et al., 2009; Nielsen et al., 2011). Os microrganismos são responsáveis pela ciclagem de nutrientes no ambiente, executam funções importantes na interação com organismos superiores, tem papel fundamental no desenvolvimento das plantas (Wall et al., 2008; Nielsen et al., 2011) e assim como podem causar doenças, são também muito importantes na proteção contra patógenos (Kloepper et al., 1980; Hornby, 1983, Mendes et al., 2011). Dessa forma, possuem aparato enzimático diverso e fundamental para a manutenção dos ecossistemas.

Muitos trabalhos, avaliando a contaminação de ambientes costeiros por petróleo, reportam grande alteração da microbiota (Head et al., 2006; Kostka et al., 2011 Bik et al., 2012; Lamendella et al., 2014; Rodriguez-R et al., 2015). Tais trabalhos descrevem um padrão de alterações bastante característico como a redução da diversidade microbiana, sucessão de populações microbianas atreladas às mudanças na composição dos hidrocarbonetos do petróleo e aumento da densidade de células bacterianas. A redução da diversidade e a sucessão de populações microbianas podem resultar na perda de espécies importantes relacionados aos ciclos biogeoquímicos e assim gerar graves impactos ao ambiente. Os microrganismos do solo, por serem os únicos organismos a possuírem enzimas capazes de degradar compostos orgânicos vegetais recalcitrantes (Romani et al., 2006; Masai et al., 2007), são responsáveis pela decomposição da maior parte da matéria orgânica dos solos. Embora os estudos sobre comunidades microbianas tenham se popularizado em razão do avanço das tecnologias de sequenciamento e da redução do custo por sequência (Caporaso et al., 2012), trabalhos relacionados aos efeitos do petróleo sobre a diversidade microbiana em ilhas ocânicas tropicais ainda são escassos, ficando restritos a ambientes de alta latitude (Jurelevicius et al., 2012; Wang et al., 2015).

Mais de 90% da exploração do petróleo brasileiro vem de plataformas de exploração no oceano (Lima, 2010). A maior concentração dessas plataformas está localizada nas bacias do Espírito Santo, de Campos e de Santos, litoral dos estados do Espírito Santo, Rio de Janeiro e São Paulo, respectivamente. No ano de 2008, houve a descoberta de jazidas de petróleo nessas mesmas bacias porém em regiões mais profundas (mais de 7000 metros) (Riccomini et al., 2012), região descrita como pré-sal, elevando a posição do Brasil entre os maiores produtores de petróleo no mundo (Barbassa, 2007; Lima, 2010). A recente descoberta e o início da exploração de petróleo na região denominada província do pré-sal, aumenta o risco da contaminação ambiental por hidrocarbonetos do petróleo.

Localizada a cerca de 1.160 km da costa brasileira, perpendicularmente à cidade de Vitória, capital do estado do Espírito Santo, está a Ilha da Trindade. A ilha possui 10.8 km^2, mais de 130 espécies vegetais endêmicas, diversas espécies de aves aquáticas, algumas delas possivelmente endêmicas, mais de 100 espécies de artrópodes e 129 espécies de peixes, incluindo espécies que são encontradas apenas naquele local (Gasparini e Floeter, 2001; Joyeux, 2009; Alves et al., 2011). Gasparini e colaboradores, em 1999 e 2001, mencionaram o interesse particular em estudos na Ilha da Trindade, em razão de sua posição isolada entre a dorsal meso-atlântica e a costa brasileira. Está posicionada a mais de 1.000 km da costa brasileira, 2.400 km da Ilha da Ascensão, 2.500 km das Rochas de São Paulo, 2.000 km de Santa Helena e 4.200 km da África. Dessa forma, a ilha da Trindade seria a ilha oceânica mais próxima ao maior ponto de exploração de petróleo brasileiro, estando exposta ao risco de contaminação em casos de acidentes durante os processos de extração ou transporte do petróleo. A contaminação do solo da ilha da Trindade pode levar a alteração da comunidade microbiana, de forma que espécies importantes para esse ambiente sejam perdidas.

OBJETIVOS

Este trabalho teve como objetivo avaliar as mudanças da comunidade microbiana de solos em resposta a contaminação com petróleo curado e testar o efeito protetor de um subproduto da indústria de biodiesel (BCP).

No capítulo 1 fez-se uma avaliação de médio prazo das alterações causadas pelo petróleo sobre a comunidade microbiana do solo da Ilha da Trindade, com o objetivo de conhecer seus efeitos sobre a diversidade de micro-organismos e identificar os taxa resistentes e sensíveis ao petróleo.

No capítulo 2, compara-se o efeito da adição de petróleo sobre a comunidade microbiana do solo da Ilha da Trindade ao efeito da adição de petróleo sobre a comunidade microbiana de solos agrícolas, utilizando-se uma avaliação de curto prazo. O capítulo 2 tem ainda o objetivo de avaliar o potencial do composto coproduto de biodiesel (BCP) quanto à bioestimulação e proteção das populações microbianas contra os efeitos da contaminação com petróleo.

REVISÃO BIBLIOGRÁFICA

Ilha da Trinadade

A Ilha da Trindade é uma formação vulcânica localizada no Oceano Atlântico Sul, a cerca de 1.160 km da costa brasileira, perpendicularmente à cidade de Vitória, capital do estado do Espírito Santo. A ilha possui 10.8 km^2, mais de 130 espécies vegetais endêmicas, diversas espécies de aves aquáticas, algumas delas possivelmente endêmicas, mais de 100 espécies de artrópodes e 129 espécies de peixes, incluindo espécies que são encontradas apenas naquele local (Gasparini e Floeter, 2001; Joyeux, 2009; Alves et al., 2011). A isolada localização da Ilha da Trindade, além de sua formação geológica diferenciada, justificam as pesquisas e a conservação desse ambiente (Alves et al., 2011). A ilha oceânica da Trindade faz parte de um complexo grupo de cadeias montanhosas submersas, com exemplares espalhados por todo o globo (Gad e Schminke, 2004). Podem ocorrer como picos isolados, unidos em grupos ou alinhados em cadeias. Exemplos como o de Trindade, que surge desde 5000 metros de profundidade até se expor na superfície da água, frequentemente constituem áreas de grande produtividade biológica, que sustentam grande diversidade de plâncton e organismos bentônicos. Existem mais de 50.000 montanhas submersas entre os oceanos Atlântico e Pacífico, e até o ano de 2002, apenas 5 haviam sido profundamente estudadas quanto à diversidade biológica (Gad e Schminke, 2004).

Gasparini e colaboradores, em 1999 e 2001, mencionaram o interesse particular em estudos na Ilha da Trindade, em razão de sua posição isolada entre a dorsal meso-atlântica e a costa brasileira. Está posicionada a mais de 1.000 km da costa brasileira, 2.400 km da Ilha da Ascensão, 2.500 km das Rochas de São Paulo, 2.000 km de Santa Helena e 4.200 km da África. Dessa forma, a ilha da Trindade seria a ilha oceânica mais próxima ao maior ponto de exploração de petróleo brasileiro, estando exposta ao risco de contaminação em casos de acidentes durante os processos de extração ou transporte do petróleo. Esse fato valoriza a avaliação dos efeitos da contaminação com hidrocarbonetos sobre a microbiota dos solos da Ilha da Trindade. Além disso, os estudos dos efeitos da contaminação por hidrocarbonetos de petróleo em regiões insulares tropicais são escassos, sendo grande parte dos trabalhos desenvolvidos em ambientes de clima temperado (Ruberto et al., 2003; Yergeau et al., 2007; 2012; Jurelevicius et al., 2012).

Degradação de hidrocarbonetos do petróleo

A remoção de hidrocarbonetos do solo está predominantemente relacionada à atividade microbiana e à sua capacidade de ciclagem de nutrientes. Esses processos são executados pelos microrganismos para a obtenção de carbono e energia (Sinha et al, 2011; Singh et al, 2012). Embora a radiação solar, temperatura e outros fatores físicos sejam capazes de eliminar parte dos hidrocarbonetos, a atividade dos micro-organismos é a principal forma de remoção dessa classe de contaminantes do solo (Björklöf et al., 2008; Raymond et al., 1976).

Os micro-organismos representam cerca de 60% da biomassa presente no mundo e a abundância de células no solo é estimada em 4 x 10^{30} células microbianas (Singh et al., 2009). A compreensão da diversidade, estrutura e funções das comunidades microbianas é essencial para podermos explorar o vasto potencial biotecnológico desses organismos, a maioria ainda desconhecida (Singh et al., 2009). O conhecimento sobre comunidades microbianas tem crescido, em razão de novas ferramentas desenvolvidas para a triagem de micro-organismos em larga escala, acompanhamento dos produtos de seu metabolismo, quantificação e determinação dos produtos da transcrição dos genomas, de forma a dissecar as características da biomassa presente em solos e em outros ecossistemas microbianos. Essas ferramentas nos aproximam do momento em que seremos capazes de conhecer, com tal profundidade, as interações e comunidades microbianas, que poderemos predizer o impacto de mudanças e controlar as funções presentes no solo (Singh et al., 2009; Hirsch et al., 2010; Chan et al., 2013).

Coproduto de biodiesel (BCP)

Embora seja a principal matriz energética mundial, o petróleo oferece riscos em todas as etapas de seu processamento além de contribuir com o aumento da emissão de gases do efeito estufa (Gleick et al., 2010). Uma das estratégias para reduzir a emissão de gases nocivos ao ambiente é a utilização de biocombustíveis. Após o protocolo de Kyoto, vários países adotaram a prática de utilizar o biodiesel como estratégia para reduzir as emissões de carbono para a atmosfera (Senatore et al., 2000). O biodiesel é composto por ésteres metílicos obtidos pela reação de transesterificação do óleo vegetal (Ma e Hanna, 1999). Esse processo gera um resíduo rico em glicerol e outros subprodutos como oleato de potássio e compostos orgânicos voláteis, esse resíduo é chamado de coproduto de biodiesel (BCP). O descarte desse resíduo pode se tornar um custo a mais para a produção de biodiesel. Dessa forma, estratégias de reaproveitamento do BCP têm sido criadas. Redmile-Gordon et al. (2015) observaram o aumento da matriz de exopolissacarídeos do solo em resposta a aplicação de BCP a solos agrícolas. Além disso, foi observado redução da quantidade de nitrogênio lixiviado para as águas subterrâneas em solos tratados com BCP. O BCP é composto por hidrocarbonetos solúveis em água, o oleato de potássio é um surfactante e o BCP apresenta efeito positivo sobre a biomassa microbiana do solo (Redmile-Gordon et al., 2014), dessa forma, apresenta potencial para sua utilização em processos de biorremediação de petróleo.

Sequenciamento de nova geração para descrição de comunidades microbianas

Desde antes da revolução causada pelo sequenciamento Sanger por eletroforese capilar, a revelação da informação genética era fundamental em todas as áreas da biologia. Embora as técnicas de sequenciamento de nova geração mantenham a linha conceitual de Sanger, detectando bases sequencialmente em um pequeno fragmento de DNA, onde cada fragmento é novamente sintetizado a partir de uma fita molde, as novas tecnologias de sequenciamento ampliam esse processo por meio de milhões de reações que ocorrem simultaneamente em um sistema de paralelização massivo, ou seja, mais sequencias a um menor custo (Niedringhaus et al., 2011).

Na mais recente revisão sobre o sequenciamento de genomas bacterianos, Loman e Pallen (2015) ilustram as descobertas alcançadas após o surgimento das tecnologias de sequenciamento. Os autores separam a história em 3 fases. A primeira fase descreve as descobertas alcançadas após a descoberta de Sanger et al. (1977) desenvolvendo a tecnologia de sequenciamento utilizando dideoxinucleosídeo trifosfatado para terminação da síntese. Com essa fase, surge a estratégia de fragmentação do genoma de interesse seguido da clonagem e mapeamento de fragmentos grandes e fragmentos pequenos utilizando plasmídeos, resultando na montagem do genoma completo de organismos modelos como Escherichia coli e Bacillus subtilis (Burland et al., 1993; Glaser et al., 1993). Posteriormente, novos consórcios se formaram utilizando a estratégia de fragmentação aleatória, clonagem e sequenciamento que dependia de softwares de bioinformática para a montagem dos genomas, o primeiro artigo descrevendo detalhadamente o método de montagem e anotação de um genoma foi publicado por Fleishman et al. (1995), essa estratégia estimulou o desenvolvimento de ferramentas de bioinformática para montagem, predição e anotação gênica (Phred, Phrap, Glimmer e Artemis) (Rieder et al., 1998;

Delcher et al., 1999; Rutherford et al., 2000). A partir desses avanços vários patógenos foram sequenciados, incluindo Yersinia pestis, Mycobacterium tuberculosis, Treponema pallium, Mycobacterium leprae, Tropheryma whipplei (Cole et al., 1998; Fraser et al., 1998; Eiglmeier et al., 2001; Parkhill et al., 2001; Bentley et al., 2003). Além disso, as informações metabólicas desses genomas permitiram a criação de novos meios de cultura capazes de cultivar microrganismos previamente classificados como não cultiváveis (Renesto et al., 2003).

Na segunda fase, próximo ao final do ano 2000, as tecnologias de sequenciamento de nova geração (NGS) passam a ser aplicadas na bacteriologia. Por volta de 2012 surgem aparelhos de NGS de pequeno porte e menor custo. Com essa tecnologia, o sequenciamento de genomas completos deixou os grandes conglomerados de sequenciamento e chegou as universidades e centros de pesquisa. Na mesma época surgem métodos de genômica comparativa e avanços na análise filogenética, permitindo a avaliação de amostras em escala global traçando links entre a diversidade genômica e a posição geográfica de importantes patógenos como a Salmonella tiphy (Holt et al., 2008). Foi possível também, descrever a origem da epidemia de cólera no Haiti (Chin et al., 2011) e a transferência de lepra entre humanos e tatus no sul dos Estados Unidos (Truman et al., 2011). Atualmente, qualquer microrganismo isolado em laboratório pode ter seu genoma sequenciado em questão de horas (Long et al., 2013). O serviço de saúde pública da Inglaterra, já utiliza a tecnologia de sequenciamento de nova geração (NGS) para caracterizar rapidamente surtos de Salmonella (Ashton et al., 2014), o mesmo grupo já depositou mais de 8000 genomas em bancos de dados públicos. Com os avanços na tecnologia de sequenciamento e o surgimento das ferramentas de bioinformática para NGS, começaram a surgir trabalhos investigando o ambiente de forma direta, sem as etapas de cultivo ou clonagem dos fragmentos de DNA obtidos (Edwards et al., 2006; Roesch et al., 2007). Mendes et al. (2015) demonstraram os efeitos da mudança no uso do solo sobre a comunidade microbiana, em ambiente de floresta amazônica. Li et al. (2015) descreveu a mudança no perfil de microrganismos em regiões desérticas revertidas para Oasis para a agricultura. Essas técnicas possibilitam a identificação de um imenso número de espécies microbianas que não seriam detectadas utilizando métodos tradicionais ou dependentes de clonagem (Caporaso et al., 2012).

Na terceira fase, descrita por Loman e Pallen (2015) como a fase das sequencias longas, os autores explicam que o alto rendimento no número de sequencias obtido pelas técnicas de NGS foi alcançado em detrimento do tamanho das sequencias. Dessa forma, a era de alto rendimento foi marcada pela criação de métodos voltados para a análise de fragmentos menores que 500 pares de bases. Já na nova fase surgem tecnologias como a “Single Molecule Real-Time” (SMRT) da PacBio (English et al., 2012) e o MinION da Oxford Nanopore (Clarke et al., 2009). As duas tecnologias oferecem milhares de sequencias com tamanho médio entre 10.000 e 30.000 pares de bases. As sequencias longas permitem maior facilidade na montagem dos genomas, na resolução de regiões repetitivas e no fechamento de gaps. Embora bastante promissoras, ambas as tecnologias ainda apresentam alta taxa de erro e ainda estão limitadas ao sequenciamento de genomas isolados. Poucos trabalhos são voltados para o sequenciamento de material genético ambiental e não apresentam vantagens em relação ao uso de tecnologias de fragmentos curtos (Franzén et al., 2015; Freedman e Zak, 2015).

REFERÊNCIAS

Alves, R. J., Silva, N. G. D., Aguirre-Muñoz, A. 2011. Return of endemic plant populations on Trindade Island, Brazil, with comments on the fauna. In C. R. Veitch, M. N. Clout, & D. R. Towns (Eds.), Island invasives: eradication and management. Proceedings of the International Conference on Island Invasives. Gland, IUCN, Auckland (pp. 259-263).

Ashton, P. M., Peters, T., Ameh, L., McAleer, R., Petrie, S., Nair, S., Muscat, I., Pinna, E., Dallman, T. 2014. Whole Genome Sequencing for the Retrospective Investigation of an Outbreak of Salmonella Typhimurium DT 8. PLoS currents, 7.

Atlas, R. M., Hazen, T. C. 2011. Oil biodegradation and bioremediation: a tale of the two worst spills in US history. Environmental science & technology, 45(16), 6709-6715.

Barbassa, A. G. Fato Relevante, Análise da Área de Tupi. Petrobras, 2007. Disponível em: http://siteempresas.bovespa.com.br/consbov/ArquivosEibe.asp?site=&protocolo=140478. Acessado em 10 de junho de 2015.

Bentley, S. D., Maiwald, M., Murphy, L. D., Pallen, M. J., Yeats, C. A., Dover, L. G., Relman, D. A. (2003). Sequencing and analysis of the genome of the Whipple's disease bacterium Tropheryma whipplei. The Lancet, 361(9358), 637-644.

Bik, H. M., Halanych, K. M., Sharma, J., Thomas, W. K. 2012. Dramatic shifts in benthic microbial eukaryote communities following the Deepwater Horizon oil spill.

Björklöf, K., Salminen, J., Sainio, P., & Jørgensen, K. 2008. Degradation rates of aged petroleum hydrocarbons are likely to be mass transfer dependent in the field. Environmental geochemistry and health, 30(2), 101-107.

British Petroleum; BP statistical review of world energy. BP, London, UK. 2014. Accessible at: bp.com/statisticalreview

Burland, V., Plunkett, G., Daniels, D. L. Blattner, F. R. 1993. DNA sequence and analysis of 136 kilobases of the Escherichia coli genome: organizational symmetry around the origin of replication. Genomics 16 (3), 551–561

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S. M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J. A., Smith, G., Knight, R. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME journal, 6(8), 1621-1624.

Chan, Y., Van Nostrand, J. D., Zhou, J., Pointing, S. B., Farrell, R. L. 2013. Functional ecology of an Antarctic dry valley. Proceedings of the National Academy of Sciences, 110(22), 8990-8995.

Chin, C. S., Sorenson, J., Harris, J. B., Robins, W. P., Charles, R. C., Jean-Charles, R. R., Bullard, J., Webster, D. R., Kasarskis, A., Peluso, P., Paxinos,E. E., Yamaichi, Y., Calderwood, S. B., Mekalanos, J. J., Schadt, E. E., Waldor, M. K. 2011. New England Journal of Medicine, 364(1), 33-42.

Clarke, J., Wu, H. C., Jayasinghe, L., Patel, A., Reid, S., Bayley, H. 2009. Continuous base identification for single-molecule nanopore DNA sequencing. Nature nanotechnology, 4(4), 265-270.

Cole, S., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E. 3rd., Tekaia, F., Badcock, K., Barrell, B. G. 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature, 393(6685), 537-544.

Das, N., Chandran, P. 2010. Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnology research international, vol 2011. 13 p.

Delcher, A. L., Harmon, D., Kasif, S., White, O. & Salzberg, S. L. 1999. Improved microbial gene identification with GLIMMER. Nucleic Acids Res. 27, 4636–4641.

Edwards, R. A., Rodriguez-Brito, B., Wegley, L., Haynes, M., Breitbart, M., Peterson, D. M., Alexander, S., Alexander, E. C., Rohwer, F. 2006. Using pyrosequencing to shed light on deep mine microbial ecology. BMC genomics, 7(1), 57.

Eiglmeier, K., Parkhill, J., Honore, N., Garnier, T., Tekaia, F., Telenti, A., Cole, S. T. 2001. The decaying genome of Mycobacterium leprae.Leprosy review, 72(4), 387-398.

English, A. C., Richards, S., Han, Y., Wang, M., Vee, V., Qu, J., Qin, X., Muzny, D. M., Reid, J. G., Worley, K. C., Gibbs, R. A. 2012. Mind the gap: upgrading genomes with Pacific Biosciences RS long-read sequencing technology. PLoS ONE 7, e47768.

Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J. F., Dougherty, B. A., Merrick, J. M. McKenney, K., Sutton, G., FitzHugh, W., Venter, J. C. (1995). Whole-genome random sequencing and assembly of Haemophilus influenzae Rd. Science, 269(5223), 496-512.

Franzén, O., Hu, J., Bao, X., Itzkowitz, S. H., Peter, I., & Bashir, A. 2015. Improved OTU-picking using long-read 16S rRNA gene amplicon sequencing and generic hierarchical clustering.Microbiome, 3(1), 1.

Fraser, C. M., Norris, S. J., Weinstock, G. M., White, O., Sutton, G. G., Dodson, R., Venter, J. C. 1998. Complete genome sequence of Treponema pallidum, the syphilis spirochete.Science, 281(5375), 375-388.

Freedman, Z., e Zak, D. R. 2015. Soil bacterial communities are shaped by temporal and environmental filtering: evidence from a long-term chronosequence. Environmental microbiology.

Gad, G., Schminke, H. K. 2004. How important are seamounts for the dispersal of meiofauna. Archive of Fishery and Marine Research, 51(Suppl 1), 3.

Gasparini, J. L., e Floeter, S. R. 2001. The shore fishes of Trindade Island, western South Atlantic.Journal of Natural History, 35(11), 1639-1656.

Gasparini, J. L., Moura, R. L., & Sazima, I. 1999. Stegastes trindadensis n. sp.(Pisces: Pomacentridae), a new damselfish from Trindade Island, off Brazil. Boletim Do Museu Mello Leitão, 10, 3-11.

Glaser, P., Kunst, F., Arnaud, M., Coudart, M. P., Gonzales, W., Hullo, M. F., Ionescu, M., Lubochinsicy, B., Marcelino, L., Moszer, I., Presecan, E., Santan, M., Scwelzer, J., Vertes, A., Rapoport, G., Danchin, A. 1993. Bacillus subtilis genome project: cloning and sequencing of the 97 kb region from 325° to 333deg. Molecular microbiology, 10(2), 371-384.

Gleick, P.H., Adams, R.M., Amasino, R.M., Anders, E., Anderson, D.J., Anderson, W.W., Anselin, L.E., Arroyo, M.K., Asfaw, B., Ayala, F.J., Bax, A., Bebbington, A.J., Bell, G., Bennett, M.V.L., Bennetzen, J.L., Berenbaum, M.R., Berlin, O.B., Bjorkman, P.J.,

Blackburn, E., Blamont, J.E., Botchan, M.R., Boyer, J.S., Boyle, E.A., Branton, D., Briggs, S.P., Briggs, W.R., Brill, W.J., Britten, R.J., Broecker, W.S., Brown, J.H., Brown, P.O., Brunger, A.T., Cairns, J., Jr., Canfield, D.E., Carpenter, S.R., Carrington, J.C., Cashmore, A.R., Castilla, J.C., Cazenave, A., Chapin, F.S., III, Ciechanover, A.J., Clapham, D.E., Clark, W.C., Clayton, R.N., Coe, M.D., Conwell, E.M., Cowling, E.B., Cowling, R.M., Cox, C.S., Croteau, R.B., Crothers, D.M., Crutzen, P.J., Daily, G.C., Dalrymple, G.B., Dangl, J.L., Darst, S.A., Davies, D.R., Davis, M.B., de Camilli, P.V., Dean, C., Defries, R.S., Deisenhofer, J., Delmer, D.P., Delong, E.F., Derosier, D.J., Diener, T.O., Dirzo, R., Dixon, J.E., Donoghue, M.J., Doolittle, R.F., Dunne, T., Ehrlich, P.R., Eisenstadt, S.N., Eisner, T., Emanuel, K.A., Englander, S.W., Ernst, W.G., Falkowski, P.G., Feher, G., Ferejohn, J.A., Fersht, A., Fischer, E.H., Fischer, R., Flannery, K.V., Frank, J., Frey, P.A., Fridovich, I., Frieden, C., Futuyma, D.J., Gardner, W.R., Garrett, C.J.R., Gilbert, W., Goldberg, R.B., Goodenough, W.H., Goodman, C.S., Goodman, M., Greengard, P., Hake, S., Hammel, G., Hanson, S., Harrison, S.C., Hart, S.R., Hartl, D.L., Haselkorn, R., Hawkes, K., Hayes, J.M., Hille, B., Hoekfelt, T., House, J.S., Hout, M., Hunten, D.M., Izquierdo, I.A., Jagendorf, A.T., Janzen, D.H., Jeanloz, R., Jencks, C.S., Jury, W.A., Kaback, H.R., Kailath, T., Kay, P., Kay, S.A., Kennedy, D., Kerr, A., Kessler, R.C., Khush, G.S., Kieffer, S.W., Kirch, P.V., Kirk, K., Kivelson, M.G., Klinman, J.P., Klug, A., Knopoff, L., Kornberg, H., Kutzbach, J.E., Lagarias, J.C., Lambeck, K., Landy, A., Langmuir, C.H., Larkins, B.A., Le Pichon, X.T., Lenski, R.E., Leopold, E.B., Levin, S.A., Levitt, M., Likens, G.E., Lippincott-Schwartz, J., Lorand, L., Lovejoy, C.O., Lynch, M., Mabogunje, A.L., Malone, T.F., Manabe, S., Marcus, J., Massey, D.S., McWilliams, J.C., Medina, E., Melosh, H.J., Meltzer, D.J., Michener, C.D., Miles, E.L., Mooney, H.A., Moore, P.B., Morel, F.M.M., Mosley-Thompson, E.S., Moss, B., Munk, W.H., Myers, N., Nair, G.B., Nathans, J., Nester, E.W., Nicoll, R.A., Novick, R.P., O'Connell, J.F., Olsen, P.E., Opdyke, N.D., Oster, G.F., Ostrom, E., Pace, N.R., Paine, R.T., Palmiter, R.D., Pedlosky, J., Petsko, G.A., Pettengill, G.H., Philander, S.G., Piperno, D.R., Pollard, T.D., Price, P.B., Jr., Reichard, P.A., Reskin, B.F., Ricklefs, R.E., Rivest, R.L., Roberts, J.D., Romney, A.K., Rossmann, M.G., Russell, D.W., Rutter, W.J., Sabloff, J.A., Sagdeev, R.Z., Sahlins, M.D., Salmond, A., Sanes, J.R., Schekman, R., Schellnhuber, J., Schindler, D.W., Schmitt, J., Schneider, S.H., Schramm, V.L., Sederoff, R.R., Shatz, C.J., Sherman, F., Sidman, R.L., Sieh, K., Simons, E.L., Singer, B.H., Singer, M.F., Skyrms, B., Sleep, N.H., Smith, B.D., Snyder, S.H., Sokal, R.R., Spencer, C.S.,

Steitz, T.A., Strier, K.B., Suedhof, T.C., Taylor, S.S., Terborgh, J., Thomas, D.H., Thompson, L.G., Jian, R.T.T., Turner, M.G., Uyeda, S., Valentine, J.W., Valentine, J.S., Van Etten, J.L., Van Holde, K.E., Vaughan, M., Verba, S., Von Hippel, P.H., Wake, D.B., Walker, A., Walker, J.E., Watson, E.B., Watson, P.J., Weigel, D., Wessler, S.R., West-Eberhard, M.J., White, T.D., Wilson, W.J., Wolfenden, R.V., Wood, J.A., Woodwell, G.M., Wright, H.E., Jr., Wu, C., Wunsch, C., Zoback, M.L., 2010. Climate Change and the Integrity of Science. Science 328, 689-690.

Head, I. M., Jones, D. M., & Röling, W. F. 2006. Marine microorganisms make a meal of oil. Nature Reviews Microbiology, 4(3), 173-182.

Hirsch, P. R., Mauchline, T. H., Clark, I. M. 2010. Culture-independent molecular techniques for soil microbial ecology. Soil Biology and Biochemistry, 42(6), 878-887.

Holt, K. E., Parkhill, J., Mazzoni, C. J., Roumagnac, P., Weill, F. X., Goodhead, I., Dougan, G. 2008. High-throughput sequencing provides insights into genome variation and evolution in Salmonella Typhi. Nature genetics,40(8), 987-993.

Hornby, D. 1983. Suppressive soils. Annual review of phytopathology, 21(1), 65-85.

Joyeux, J. C. 2009. New records of fishes for Trindade-Martin Vaz oceanic insular complex, Brazil. Zootaxa, 2298, 45-54.

Jurelevicius, D., Alvarez, V. M., Peixoto, R., Rosado, A. S., Seldin, L. 2012. Bacterial polycyclic aromatic hydrocarbon ring-hydroxylating dioxygenases (PAH-RHD) encoding genes in different soils from King George Bay, Antarctic Peninsula. Applied Soil Ecology, 55, 1-9.

Kloepper, J. W., Leong, J., Teintze, M., & Schroth, M. N. 1980. Pseudomonas siderophores: a mechanism explaining disease-suppressive soils. Current microbiology, 4(5), 317-320.

Kostka, J. E., Prakash, O., Overholt, W. A., Green, S. J., Freyer, G., Canion, A., Delgardio, J., Norton, N., Hazen, T. C., Huettel, M. 2011. Hydrocarbon-degrading bacteria and the bacterial community response in Gulf of Mexico beach sands impacted by the Deepwater Horizon oil spill. Applied and environmental microbiology, 77(22), 7962-7974.

Lamendella, R., Strutt, S., Borglin, S., Chakraborty, R., Tas, N., Mason, O. U., Hultman, J., Prestat, E., Hazen, T. C., Jansson, J. K. 2014. Assessment of the Deepwater Horizon oil spill impact on Gulf coast microbial communities. Frontiers in microbiology, 5.

Li, C. H., Tang, L. S., Jia, Z. J., Li, Y. 2015. Profile Changes in the Soil Microbial

Community When Desert Becomes Oasis. PloS one, 10(10).

Lima, J. A. M. A energia que vem do mar: a herança energética do mar brasileiro. Cienc. Cult. [online], v.62, n.3, p.25-8, 2010.

Loman, N. J. e Pallen, M. J. 2015. Twenty years of bacterial genome sequencing. Nature Reviews Microbiology, 6(6), 477-487.

Long, S. W., Williams, D., Valson, C., Cantu, C. C., Cernoch, P., Musser, J. M., Olsen, R. J. 2013. A genomic day in the life of a clinical microbiology laboratory. Journal of clinical microbiology, JCM-03237.

Ma, F., e Hanna, M. A. 1999. Biodiesel production: a review. Bioresource technology, 70(1), 1-15.

Masai, E., Katayama, Y. Fukuda, M. 2007. Genetic and biochemical investigations on bacterial catabolic pathways for lignin-derived aromatic compounds. Bioscience Biotechnology & Biochemistry, 71, 1–15.

Mendes, L. W., Tsai, S. M., Navarrete, A. A., de Hollander, M., van Veen, J. A., & Kuramae, E. E. (2015). Soil-borne microbiome: linking diversity to function. Microbial ecology, 1-11.

Mendes, R., Kruijt, M., de Bruijn, I., Dekkers, E., van der Voort, M., Schneider, J. H., Piceno, Y. M., DeSantis, T. Z., Andersen, G. L., Bakker, P. A. H. M., Raaijmakers, J. M. 2011. Deciphering the rhizosphere microbiome for disease-suppressive bacteria. Science, 332(6033), 1097-1100.

Niedringhaus, T. P., Milanova, D., Kerby, M. B., Snyder, M. P., Barron, A. E. 2011. Landscape of next-generation sequencing technologies. Analytical chemistry, 83(12), 4327-4341.

Nielsen, U. N., Ayres, E., Wall, D. H., & Bardgett, R. D. 2011. Soil biodiversity and carbon cycling: a review and synthesis of studies examining diversity–function relationships. European Journal of Soil Science, 62(1), 105-116.

Parkhill, J., Wren, B. W., Thomson, N. R., Titball, R. W., Holden, M. T. G., Prentice, M. B., Barrell, B. G. 2001. Genome sequence of Yersinia pestis, the causative agent of plague.Nature, 413(6855), 523-527.

Raymond, R. L., Hudson, J. O., Jamison, V. W. 1976. Oil degradation in soil. Applied and Environmental Microbiology, 31(4), 522-535.

Redmile-Gordon, M. A., Armenise, E., Hirsch, P. R., e Brookes, P. C. 2014. Biodiesel Co-Product (BCP) Decreases Soil Nitrogen (N) Losses to Groundwater. Water, Air, & Soil

Pollution, 225(2), 1-15.

Redmile-Gordon, M. A., Evershed, R. P., Kuhl, A., Armenise, E., White, R. P., Hirsch, P. R., Goulding, K. W. T., Brookes, P. C. 2015. Engineering soil organic matter quality: Biodiesel Co-Product (BCP) stimulates exudation of nitrogenous microbial biopolymers. Geoderma, 259, 205-212.

Renesto, P., Crapoulet, N., Ogata, H., La Scola, B., Vestris, G., Claverie, J. M., Raoult, D. 2003. Genome-based design of a cell-free culture medium for Tropheryma whipplei. The Lancet,362(9382), 447-449.

Riccomini, C., Sant, L. G., Tassinari, C. C. G. 2012. Pré-sal: geologia e exploração. Revista USP, (95), 33-42.

Rieder, M. J., Taylor, S. L., Tobe, V. O. Nickerson, D. A. 1998.Automating the identification of DNA variations using quality-based fluorescence re-sequencing: analysis of the human mitochondrial genome. Nucleic Acids Res. 26, 967–973.

Rodriguez-R, L. M., Overholt, W. A., Hagan, C., Huettel, M., Kostka, J. E., & Konstantinidis, K. T. (2015). Microbial community successional patterns in beach sands impacted by the Deepwater Horizon oil spill. The ISME journal.

Roesch, L. F., Fulthorpe, R. R., Riva, A., Casella, G., Hadwin, A. K., Kent, A. D., Daroub, S. H., Camargo, F. A., Farmerie, W. H., Triplett, E. W. 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. The ISME journal, 1(4), 283-290.

Romani, A. M., Fischer, H., Mille-Lindblom, C. Tranvik, L. J. 2006. Interactions of bacteria and fungi on decomposing litter: differential extracellular enzyme activities. Ecology, 10, 2559–2569.

Ruberto, L., Vazquez, S. C., e Mac Cormack, W. P. 2003. Effectiveness of the natural bacterial flora, biostimulation and bioaugmentation on the bioremediation of a hydrocarbon contaminated Antarctic soil. International Biodeterioration & Biodegradation, 52(2), 115-125.

Rutherford, K., Parkhill, J., Crook, J., Horsnell, T., Rice, P., Rajandream, M. A., Barrell, B. 2000. Artemis: sequence visualization and annotation.Bioinformatics, 16(10), 944-945.

Sammarco, P. W., Kolian, S. R., Warby, R. A., Bouldin, J. L., Subra, W. A., Porter, S. A. 2013. Distribution and concentrations of petroleum hydrocarbons associated with the BP/Deepwater Horizon Oil Spill, Gulf of Mexico. Marine pollution bulletin, 73(1), 129-143.

Sanger, F., Nicklen, S., Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors.Proceedings of the National Academy of Sciences,74(12), 5463-5467.

Senatore, A., Cardone, M., Rocco, V., Prati, M. V. 2000. A comparative analysis of combustion process in DI diesel engine fueled with biodiesel and diesel fuel (No. 2000-01-0691). SAE Technical Paper.

Sikkema, J., De Bont, J. A., Poolman, B. 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiological reviews, 59(2), 201-222.

Singh, A., Singh, B., & Ward, O. 2012. Potential applications of bioprocess technology in petroleum industry. Biodegradation, 23(6), 865-880.

Singh, B. K., Campbell, C. D., Sorenson, S. J., & Zhou, J. 2009. Soil genomics. Nature Reviews Microbiology, 7(10), 756-756.

Sinha, S., Chattopadhyay, P., Pan, I., Chatterjee, S., Chanda, P., Bandyopadhyay, D., Das, K., Sen, S. K. 2011. Microbial transformation of xenobiotics for environmental bioremediation. African Journal of Biotechnology, 8(22).

Truman, R. W., Singh, P., Sharma, R., Busso, P., Rougemont, J., Paniz-Mondolfi, A., Kapopoulou, A., Brisse, S., Scollard, D. M., Gillis, T. P., Cole, S. T. 2011. Probable zoonotic leprosy in the southern United States. New England Journal of Medicine, 364(17), 1626-1633.

Wall, D. H., Bradford, M. A., St. John, M. G., Trofymow, J. A., Behan-Pelletier, V., Bignell, D. E., Dangerfield, M. J., Parton, W. J., Rusek, J., Voigt, W., Wolters, V., Gardel, H. Z., Ayuke, F. O., Bashford, R., Beljakova, O. I., Bohlen, P. J., Brauman, A., Flemming, S., Henschel, J. R., Johnson, D. L., Jones, T. H., Kovarova, M., Kranabetter, J. M., Kutny, L., Lin, K-C., Maryati, M., Masse, D., Pokarzhevskii, A., Rahman, H., Sabará, M. G., Salamon, J-A., Swift, M. J., Varela, A., Vasconcelos, H. L., White, D., Zou, X. 2008. Global decomposition experiment shows soil animal impacts on decomposition are climate dependent. Global Change Biology, 14, 2661–2677.

Yergeau, E., Kang, S., He, Z., Zhou, J., Kowalchuk, G. A. 2007. Functional microarray analysis of nitrogen and carbon cycling genes across an Antarctic latitudinal transect. The ISME journal, 1(2), 163-179.

Yergeau, E., Sanschagrin, S., Beaumier, D., Greer, C. W. 2012. Metagenomic analysis of the bioremediation of diesel-contaminated Canadian high arctic soils. PloS one, 7(1), 1-10.

CHAPTER 1

RESPONSES OF MICROBIAL COMMUNITY FROM TROPICAL PRISTINE COASTAL SOIL TO CRUDE OIL CONTAMINATION

Responses of microbial community from tropical pristine coastal soil to crude oil contamination

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ABSTRACT

Brazilian offshore crude oil exploration has increased after the discovery of new reservoirs in the region known as pré-sal, in a depth of 7.000 m under the water surface. Oceanic Islands near these areas represent sensitive environments, where changes in microbial communities due to oil contamination could cause the loss of metabolic functions, with catastrophic effects to the soil services provided from these locations. This work aimed to evaluate the effect of petroleum contamination on microbial community shifts (Archaea, Bacteria and Fungi) from Trindade Island coastal soils. Microcosms were assembled and divided into two treatments, control and contaminated (weathered crude oil at the concentration of 30 g kg^{-1}), in triplicate. Soils were incubated for 38 days, with CO_2 measurements every four hours. After incubation, the total DNA was extracted, purified and submitted for high-throughput target sequencing of 16S rDNA, for Bacteria and Archaea domains and Fungal ITS1 region, using the Illumina MiSeq platform. Three days after contamination, the CO_2 emission rate peaked at more than 20x the control and the emissions remained higher during the whole incubation period. Microbial alpha-diversity was reduced for contaminated-samples. Fungal relative abundance of contaminated samples was reduced to almost 40% of the total observed species. Taxonomy comparisons showed a rise of the Actinobacteria phylum, shifts in several Proteobacteria classes and reduction of the Archaea class Nitrososphaerales in oil contaminated microcosms. This is the first effort in acquiring knowledge concerning the effect of crude oil contamination in soils of a Brazilian

oceanic island. This information is important to guide any future bioremediation strategy that may be required.

INTRODUCTION

The offshore petroleum exploration offers risks to the whole seaecosystems, as their hydrocarbons are toxic, mutagenic, teratogenic and carcinogenic (Hentati et al, 2013; Mckee et al, 2013). These toxic compounds tend to accumulate in the environment after spillage events, but factors such as temperature, sun light, high exchange of gases and biological activity can remove the lighter portions of the crude oil in the first weeks after leakage. However, the recalcitrant portion of the oil stays in the environment for years (Huesemann et al., 2002; Trindade et al., 2005). The British Petroleum review of 2015, states that crude oil is still the dominant energy source in Brazil and that the consumption has kept rising since 2013. The recent discovery of crude oil reservoirs in the so-called pré-sal (pre-salt) reservoir is considered an excellent opportunity to supply the country’s economic and energety demands (Lima, 2010), but possible oil spills events should be a major concern.

Trindade Island is located at the South Atlantic Ocean, 1,160 km from the city of Vitória, capital of Espirito Santo State, Brazil, and is the closest oceanic island to these new Brazilian petroleum offshore exploration areas. It hosts a peculiar and endangered biodiversity (Alves & Castro, 2006; Mohr et al., 2009), so the development of conservation approaches to maintain these unique ecosystems is required. It is well known that microbes are fundamental to several soil processes, including changes on physicochemical properties and degrading recalcitrant and toxic compounds (Elliot et al., 1996; Haritash & Kaushik, 2009). The expected scientific benefits from increasing knowledge on Trindade Island soil microbial diversity and functionality are extensive, including a better understanding of the roles played by these communities to empowering bioremediation actions.

The input of a mixture of hydrocarbons, for example crude oil, directly influences the structure of microbial populations in soils (Hamamura et al., 2006). In contamination events, changes in soil properties, such as crude oil viscosity increasing, ageing, sorption of nutrients and toxicity cause the microbial community to shift towards profiting oil resistant populations. Some microorganisms are capable of degrading crude oil hydrocarbons through a number of aerobic and anaerobic metabolic pathways, using these compounds as sources of carbon and energy (Zobell, 1946; Atlas, 1981; Haritash & Kaushik, 2009) comprising an appropriate target for studies focused on alleviating any possible impacts of soil contamination.

In the last 10 years, after the development of the Next Generation Sequencing (NGS) technology, microbial community studies have undergone a major boost (Caporaso et al., 2012; Loman and Pallen, 2015; Markowitz et al., 2015). Nevertheless, research related to crude oil contamination is primarily focused on the water column, without applying NGS (Huetel et al., 2014; Rodrigues et al., 2015), or only performed after an accidental contamination event (Lamendella et al., 2014; Rodriguez et al., 2015), lacking any proper control.

Crude oil hydrocarbons are expected to impact soil microbial communities through toxic effects of the oil components, enriching the environment with hydrocarbon degrading microorganisms. Here, we aimed to evaluate the microbial community shifts (Archaea, Bacteria and Fungi) from Trindade Island coastal soil under crude oil contamination, using state of the art NGS approach on a controlled microcosm experiment, in order to access the whole soil microbiota, including the nonculturable and low abundance ones.

MATERIAL AND METHODS

Sampling site and soil analysis

Trindade Island soil was randomly sampled, 10 soil cores with 6 cm of diameter to the depth of 0-10 cm, from the northeast shoreline of Trindade (coordinates: 20°30' S and 29° 19' W), under influence of native vegetation (Cyperus atlanticus). Soil cores were bulked, sieved (< 2mm) and stored at 4 °C, for 20 days, until microcosm assembly (Figure 1). The sampling expedition took place through April 2013. A total of 10 chemical variables (pH, P-rem, P, K, S, Ca^{2+}, Mg^{2+}, OM, N and C), plus soil texture, were assessed in the soil analysis. The protocol references and results are shown in Table 1, in the results section.

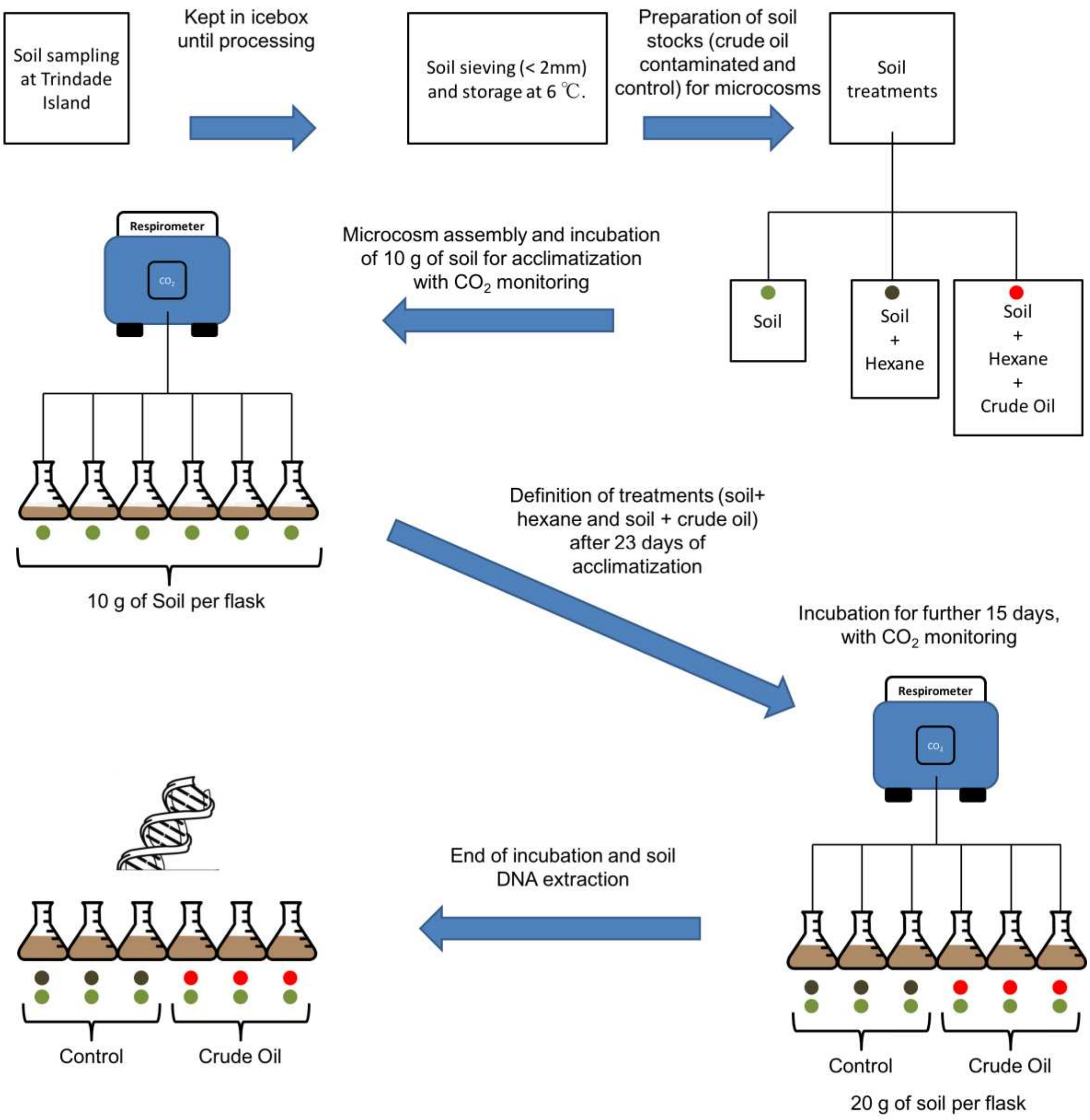

Figure 1 – Experimental design representation

Table 1 – Summary of physicochemical data for surface soil cores (0 - 10 cm) sampled at the northeast coast of Trindade Island - Brazil.

Characteristic	Unit	Value
pH – H_2O		5.6
Soil texture		Sandy Loam
P-rem (1)	mg L^{-1}	26.5
P(2)	mg kg^{-1}	1290.8
K(2)		180.33
S(3)		5.63
Ca^{+2}(4)	$cmol_c$ kg^{-1}	9.84
Mg^{+2}(4)		2.78
OM	%	0.64
N		0.19
C(5)		0.37

(1) Remaining phosphorus (Alvarez et al., 2000). (2) Extracted with Mehlich – 1. (3) Extracted with monocalcium phosphate in acetic acid (HOEFT et al., 1973). (4) Extracted with KCl 1 mol L^{-1}. (5) Walkley and Black method/OM = C.org * 1.724.

Soil treatment with crude oil

Firstly, to simulate the ageing of crude oil exposed to environmental conditions during spillage events, we heated 500 mL of crude oil to 90 °C and incubated for two hours in a fume hood. The resulting aged crude oil was a material highly viscous and difficult to work with. To obtain homogenous mixing of oil with soil, we dissolved the aged crude oil in hexane and applied it to a subsample of each experimental soil (Figure 1). Studies regarding the degradation or extraction of hydrocarbons from soil systems routinely use organic solvents for spiking of soil with these hydrocarbons, and it is well known that organic solvents are harmful for native microbial communities within soils (Maliszewska-Kordybach, 1993; Brinch et al., 2002). Therefore, hexane was also added to soils without crude oil to create a hexane-only contaminated control stock. These hexane (and crude oil + hexane) exposed soil stocks were kept in a fume hood until all hexane had evaporated. We then added 10 g of the control stock soil (hexane evaporated) to the flasks corresponding to 'Control', and made up to 20 g with the corresponding soil that had not been exposed to hexane. The same procedure was repeated for the stock soils contaminated with crude oil, corresponding to the treatment 'Crude Oil'. This combination method was required to repopulate the native soil microbial community injured by hexane. The final concentration of crude oil was 30 g kg^{-1}. The flasks were incubated at 26° C and the soil moisture was kept at 60% of its water holding capacity (remoistened periodically with deionised water upon reaching c. 50% water holding capacity).

Experimental design

To evaluate the effects of crude oil on the soil microbial community, we assembled six soil microcosms in borosilicate Wheaton® (USA) respirometer flasks of 250 mL, containing 10 g of soil (dry weight). The microcosms were incubated at 26° C for 23 days, and the microbial activity was monitored by quantifying CO_2 emissions every 4 hours, using a continuous-flow respirometer coupled to an infrared CO_2 detector (TR-RM8 Respirometer Multiplex – Sable System) (Heinemeyer et al., 1989). After this 23 days, acclimatizing period, three flasks (3 replicates) received further 10 g of stock soil treated with hexane for 'Control' and three flasks (3 replicates) received further 10 g of stock soil treated with crude oil and hexane, 'Crude Oil' treatment. The final concentration of the Crude Oil in this treatment was 30 g kg^{-1}. After the settlement of the treatment replicates, the incubation continued for a further 15 days (Figure 1). After this period the samples were frozen using liquid nitrogen and stored at -80 °C until total community DNA extraction.

Molecular analyses

DNA extraction and quality check

Genomic DNA was extracted and purified from each soil sample (0.5 g) using the PowerMax® Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) following manufacturer's instructions. Purity of the extracted DNA was checked using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) (260/280 nm ratio) and DNA concentration was determined using Qubit® 2.0 fluorometer and dsDNA BR Assay kit (Invitrogen™). Integrity of the DNA was confirmed by electrophoresis in a 0.8 % agarose gel with 1 X TAE buffer.

High-throughput sequencing

Sequencing was done on the Illumina MiSeq® platform (Caporaso et al. 2012) at the High-throughput Genome Analysis Core (HGAC), Argonne National Laboratory (Illinoi, USA). Bacterial and archaeal 16S rRNA genes were amplified using primers

515F (5’-GTGCCAGCMGCCGCGGTAA-3’) and 806R (5’-GGACTACHVGGGTWTCTAAT-3’) for paired-end microbial community analysis (Caporaso et al., 2011). Fungal ITS1 region was amplified using primers ITS1F (5’-CTTGGCCATTTAGAGGAAGTAA-3’) and ITS2 (5’-GCTGCGTTCTTCATCGATGC-3’) using the method described by Smith & Peay (2014).

Data analysis

We applied the 16S and ITS bioinformatics pipeline recommended by the Brazilian Microbiome Project, available at http://brmicrobiome.org (Pylro et al, 2014). Briefly, this pipeline uses QIIME (Caporaso et al., 2010) and Usearch 7.0 (Edgar, 2010) for filtering low quality sequences, clustering sequences of high similarity, diversity analysis, diversity comparisons and graphical plotting. For fungal ITS analysis we also used the software ITSx (Bengtsson-Palme, 2013) for taxonomic assignment improvement. The sequencing depth can affect alpha and beta diversity analysis, therefore, we used the strategy of rarefaction (randomly sub-sampling of sequences from each sample) to equalize the number of sequences per sample and to evaluate the sufficiency of the sequencing effort. We also used the Good’s coverage (Good, 1953) index to assess the coverage reached using the rarefaction level chosen. The microbial diversity changes were measured using the alpha diversity metrics: PD whole tree (for 16S rRNA gene only), Chao (Chao, 1984) and observed species. For beta-diversity estimations, we generated distance matrixes using the phylogenetic method weighted unifrac (Lozupone et al, 2005) for 16S rRNA gene sequences and the Bray-Curtis (Bray & Curtis, 1957) method for ITS sequences. We plotted the beta-diversity distance matrixes using a bi-dimensional Principal Coordinates Analysis (PCoA) and the clusters were evaluated using the cluster quality analysis (cluster_quality.py script on QIIME) (Caporaso et al., 2010), calculating the ratio of mean “distances between samples from different clusters” to mean “distances between samples from the same cluster”. The hypothesis testing method used to compare taxonomic differences between treatments was made using the bioconductor EdgeR package (Robinson, et al., 2010). The count matrix was normalized through the relative log expression (RLE) proposed by Anders and Huber (2010), where the median count is calculated from the geometric mean of all columns and the median ratio of each sample to the median library is used as the scale factor. The p-values were corrected using the Benjaming-Hochberg false discovery rate method (FDR). The R script used in this analysis is described and available at http://github.com/kdanielmorais.

RESULTS

Soil respiration and physicochemical characteristics

The Trindade Island soil physicochemical properties are listed at Table 1. The first 23 days of incubation didn't show any difference in CO_2 emissions between the 6 microcosms. Differences were detected only on the 24th day, after the implementation of the treatments (Figure 2). CO_2 emission rate of oil-contaminated samples increased 8 x compared to the control in the first 4 hours (Figure 2). Three days after contamination, emission rate peaked at more than 20 times the control. After the addition of crude oil CO_2 emissions of the oil treated samples continued to be higher than the control treatment until the sampling of the DNA on the 38th day. At the last day of incubation (38th), CO_2 emission rate of the contaminated treatment was still almost 4 times higher than the control (Figure 2).

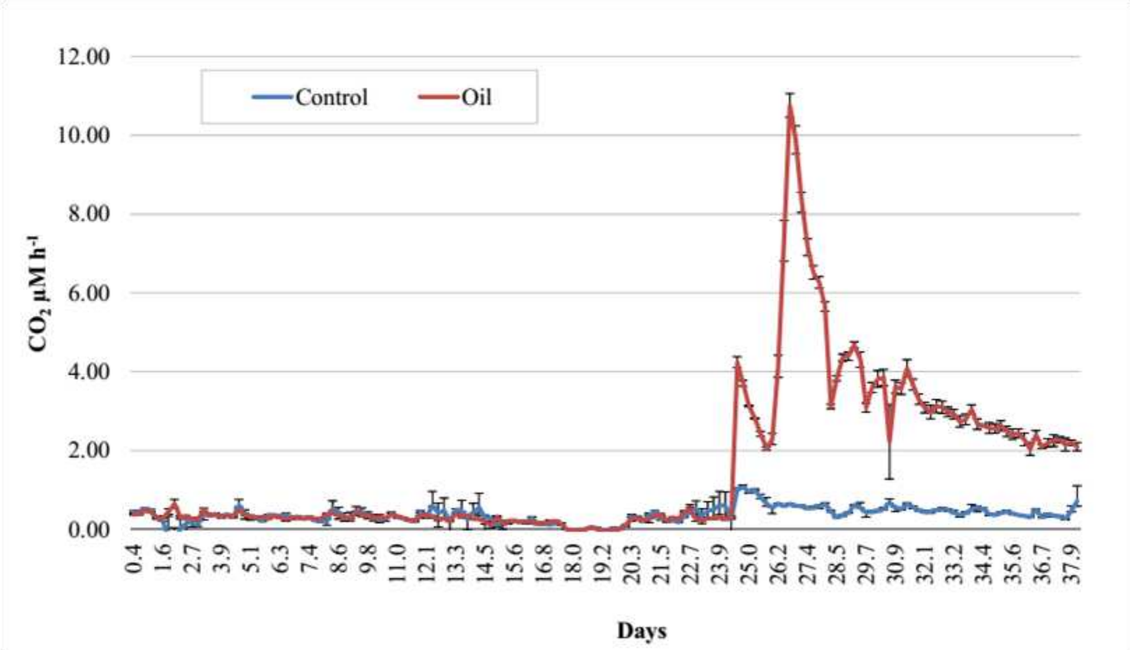

Figure 2 - Respirometry analysis of Trindade Island coastal soil microcosms. Average CO_2 emission rates evaluated during 38 days of incubation. Emissions until 24 days represent the acclimatizing period without oil addition. Readings after 24 days show the differences in CO_2 emissions after establishing the two treatments (Control and Oil). The microcosms were incubated at 26 °C and CO_2 emission was monitored by an automated respirometer coupled to an infrared CO_2 detector.

Sequencing output

A total of 314,748 joined and quality filtered 16S rRNA gene Illumina® barcoded reads, and 424,269 single end quality filtered fungal ITS Illumina® barcoded reads were obtained from the soil samples (Table S1). The oil-contaminated treatment yielded a smaller number of sequences. To minimize the effects of sequencing depth variation on diversity analysis and taxa comparison, we applied the rarefaction method (random subsampling of sequences). Estimates of alpha and beta-diversity were based on evenly rarefied OTU matrices (45,695 sequences per sample for Bacteria and Archaea and 25,315 sequences per sample for Fungi).

Diversity comparisons

The alpha diversity indexes used in this experiment represent species richness (Tables 2 and 3). We compared treatment's effects over Bacteria/Archaea community using the estimators Faith's PD (phylogenetic measure of diversity based on total branch length of phylogeny captured by a sample, proposed by Faith, 1992), the Chao-1 (estimator of total species richness proposed by Chao, 1984), and observed species (number of species detected) (Table 2). The effects on Fungal community was measured using only the Chao-1 and Observed species estimators, as there was not an ITS1 phylogenetic tree available to use the Faith's PD estimator. All metrics yielded similar results for Bacteria/Archaea and Fungi. The comparison between the two treatments shows a significant reduction of diversity upon the addition of oil for Bacteria, Archaea and Fungi. The fungal community was the most sensitive group to the oil addition, showing a reduction of ~40% for the indexes Chao1 and Observed species (table 3).

The rarefaction analysis (Figures 3 A and B), which plots the operational taxonomic unit (OTU) richness as a function of sequencing depth, and the Good´s coverage shows that sequencing effort was sufficient to capture the Bacterial, Archaeal and Fungal diversity of samples. The analysis also confirms that crude oil had a reductive effect on microbial diversity.

The beta diversity analysis (Figure 4) was performed using Weighted Unifrac for 16s rRNA gene and Bray-Curtis for fungal intergenic spacer ITS1 due to the lack of a phylogenetic tree for ITS1 marker. Both methods showed two very distinct clusters separating the treatments Control and Crude Oil (Cluster quality. 16S = 2.36 and ITS = 2.14).

Table 2 –Average (n = 3) alpha diversity comparison between the treatments control and crude oil for bacteria and archaea groups.

Metrics	**Control**	Std. Err.	**Crude Oil**	Std. Err.	p-value*
Rarefaction level	45690	-	45690	-	-
Good's coverage	0.987	0.001	0.989	0.002	-
PD whole tree	177.51	1.69	164.87	3.72	0.012546
Chao 1	3107.12	39.7	2796.51	149.7	0.047083
Observed species	2679.23	36.9	2443.20	78.5	0.018392

*Two-sample parametric t-test.

Table 3 – Average (n = 3) alpha diversity comparison between the treatments control and crude oil for fungi.

Metrics	**Control**	Std. Err.	**Crude Oil**	Std. Err.	p-value*
Rarefaction level	25315	-	25315	-	-
Good's coverage	0.99	0.001	0.99	0.001	-
Chao 1	100.58	12.1	69.96	12.9	0.0548
Observed species	96.46	11.8	67.8	11.7	0.0681

*Two-sample parametric t-test.

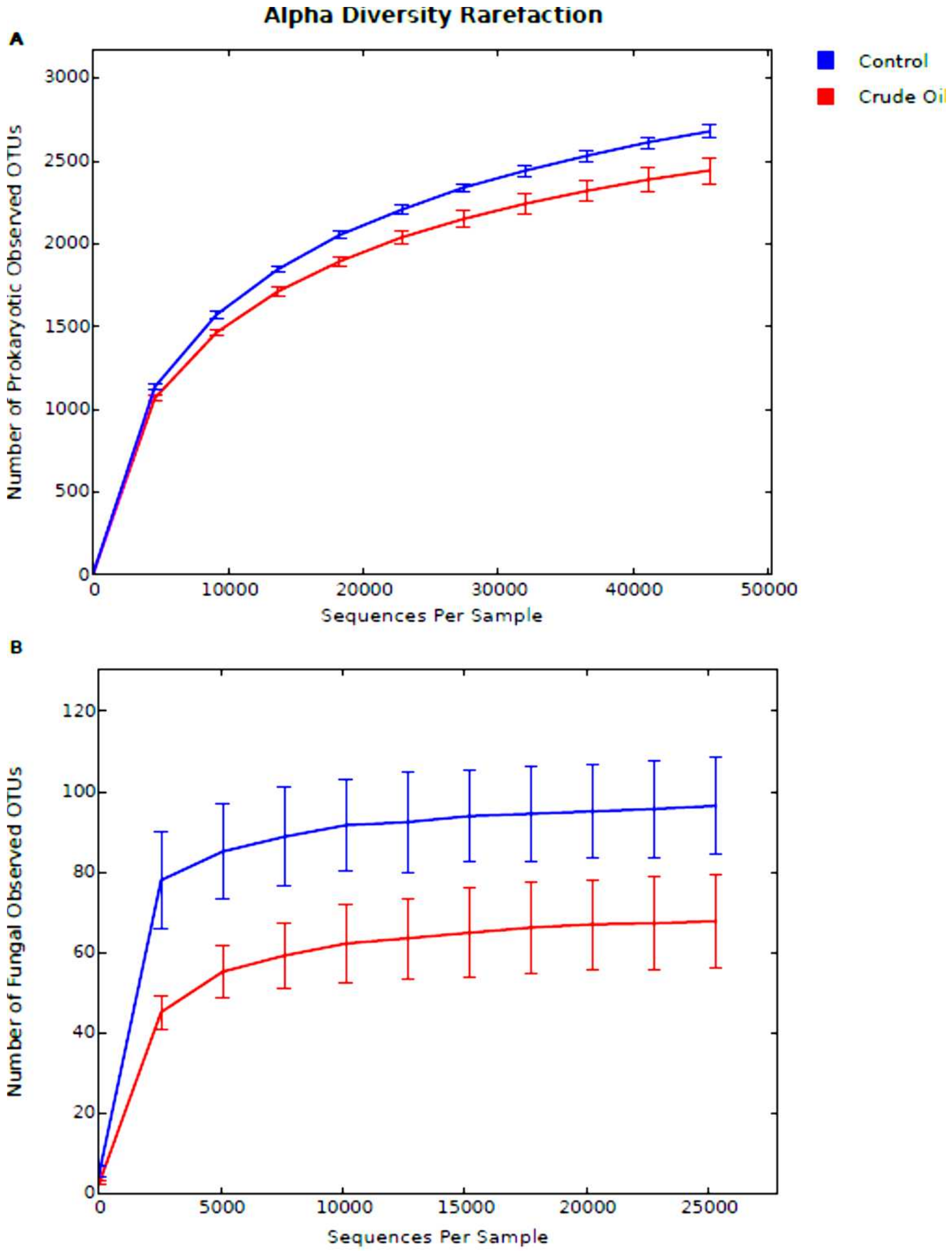

Figure 3 - Average alpha diversity rarefaction plot for (A) Bacteria/Archaea and (B) Fungi. It shows the number of observed species at a random pool of sequences in different depths.

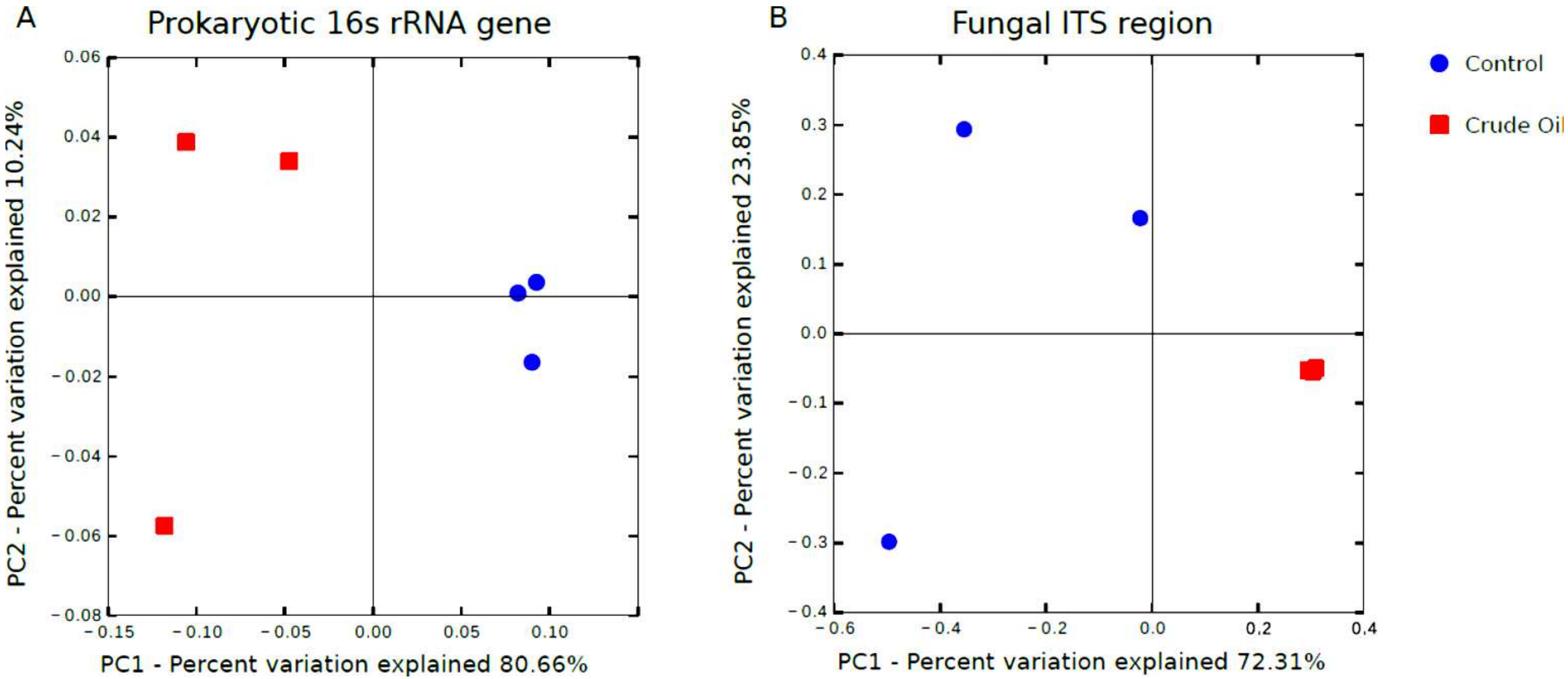

Figure 4 - Principal coordinate analysis (PCoA). A total of 6 soil samples were analysed by amplicon sequencing. Sequences were rarefied at the same sequencing depth and abundance matrixes were generated using taxa tables summarized at the lowest possible taxonomic level, ranging from phylum to specie. (A) 16S rDNA amplicon sequences coordinates analysis, generated with Weighted Unifrac distance matrix, explaining 90.90% of variation. (B) Fungal ITS1 region amplicon sequences coordinate analysis, generated with Bray-Curtis distance matrix, explaining 96.16% of variation.

Taxonomic comparison

The taxonomic distributions of Bacteria/Archaea are shown in Figure 5 at phylum level. The control treatment show 6% of sequences to be from the Archaea domain, 93.4% from Bacteria domain and 0.5% were not assigned to any taxa from the GreenGenes database of May 2013 (DeSantis et al., 2006). For Archaea, we found only three representatives: the genus Nitrosphaerales belonging to the phylum Crenarchaeota, the order E2 belonging to the phylum Euryarchaeota and the order YLA114, belonging to the phylum Parvarchaeota. The addition of oil reduced the relative abundance of Archaea to 2.7%.

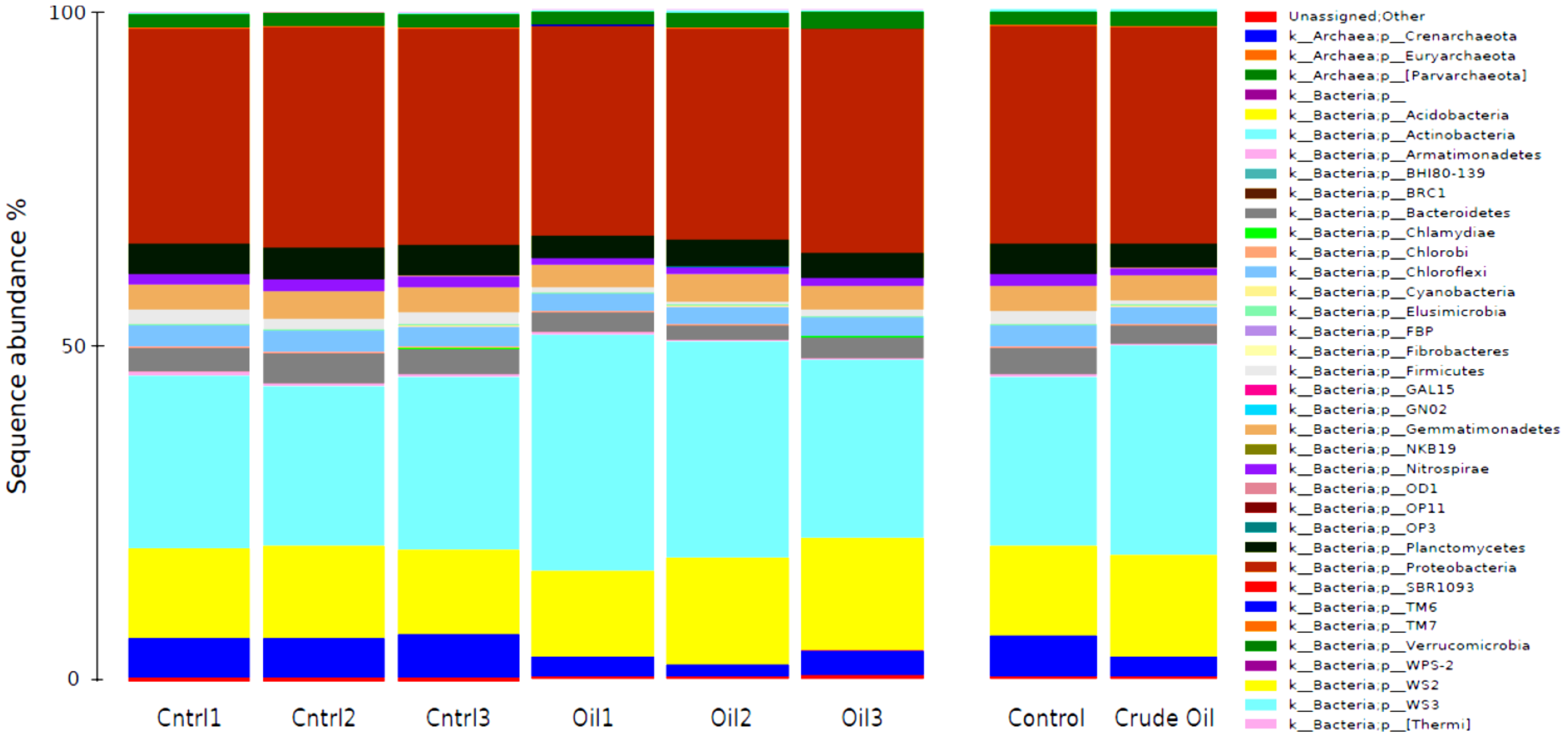

Figure 5 - Relative abundance of Bacteria and Archaea phyla using 16S rDNA sequences. Samples are disclosed isolated and as an average of each treatment.

We identified 225 orders in the bacterial group of the control samples. The most abundant bacterial orders in the control were Acidobacteria order iii1-15 (7%), Rhizobiales (6.5%), Rubrobacterales (6.3%), Nitrospirales (6.1%), Xanthomonadales (4.8%), Syntrophobacterales (4.2%), Gaiellales (4%) and Myxococcales (4%). Oil-contaminated samples presented 224 orders, and the most abundant orders were Actinomycetales (17%), Acidobacteria order iii1-15 (8.5%), Rhizobiales (6.4%), Burkholderiales (4%), Xanthomonadales (3.9%), Chloroacidobacteria order RB41 (3.4%), Sphingomonadales (3%), Acidimicrobiales (2.9%). The abundance of 57 taxa was significantly different between Control and Crude Oil (Table 4).

Fungal taxonomy analysis (Figure 6) was assessed using the UNITE database version 7 (Kõljalg et al., 2005). 5% of the reads from non-contaminated soil were not assigned to any taxonomic group. For the crude oil treatment, only 0.7% of the sequences did not match to a taxon. We found 29 orders in the fungal group of the control samples. The most abundant orders in the control were Hypocreales (41%), Mortierellales (27%) and Sordariales (7.5%). Oil-contaminated samples presented 29 orders, and the most abundant orders were Mortierellales (70%), Hypocreales (24%) and Botryosphaeriales (1.1%). Abundance of 6 taxa was significantly different between control and oil contaminated soils (Table 5).

Table 4 – Bacterial/Archaeal OTUs presenting an average absolute abundance significantly different between the treatments “Oil” and “Control”, under the EdgeR Fisher’s exact test and α = 0.05.

Taxa	Control	Crude Oil	q-value*
g__Candidatus Nitrososphaera	10275.0	4098.0	3.77E-03
p__Acidobacteria;o__DS-18	546.0	740.0	9.05E-03
p__Acidobacteria;o__Sva0725	370.0	704.0	9.20E-05
p__Actinobacteria;g__Iamia	93.0	206.0	1.41E-02
p__Actinobacteria;f__Actinosynnemataceae	17.0	71.0	6.66E-07
p__Actinobacteria;g__Gordonia	1.0	51.0	2.69E-02
p__Actinobacteria;f__Intrasporangiaceae	42.0	186.0	1.03E-07
p__Actinobacteria;f__Micrococcaceae	264.0	467.0	1.02E-03
p__Actinobacteria;g__Nocardia	6.0	13766.0	2.76E-79
p__Actinobacteria;f__Nocardiaceae	18.0	91.0	2.20E-06
p__Actinobacteria;f__Nocardioidaceae	392.0	1158.0	4.43E-12
p__Actinobacteria;g__Aeromicrobium	179.0	506.0	3.11E-07
p__Actinobacteria;g__Nocardioides	57.0	105.0	1.13E-02
p__Actinobacteria;g__Pimelobacter	6.0	799.0	1.82E-19
p__Actinobacteria;g__Amycolatopsis	2.0	88.0	5.19E-10
p__Actinobacteria;f__Streptomycetaceae	166.0	3743.0	2.19E-48
p__Actinobacteria;g__Streptomyces	380.0	613.0	6.03E-04
p__Actinobacteria;g__Actinomadura	32.0	71.0	3.75E-03
p__Actinobacteria;c__MB-A2-108	80.0	130.0	2.16E-02
p__Actinobacteria;f__Rubrobacteraceae	4085.0	1590.0	1.72E-04
p__Actinobacteria;g__Rubrobacter	6674.0	1710.0	4.18E-08
p__Bacteroidetes;g__Crocinitomix	62.0	0.0	5.30E-11
p__Bacteroidetes;g__Fluviicola	568.0	53.0	9.23E-05
p__Firmicutes;o__Bacillales	109.0	33.0	3.84E-02
p__Firmicutes;g__Alicyclobacillus	102.0	30.0	1.45E-02
p__Firmicutes;g__Bacillus	1680.0	504.0	5.32E-05
p__Firmicutes;g__Virgibacillus	162.0	65.0	1.61E-02
p__Firmicutes;g__Cohnella	54.0	14.0	1.71E-02
p__Firmicutes;f__Thermoactinomycetaceae	52.0	7.0	1.08E-04
p__Nitrospirae;g__Nitrospira	1356.0	591.0	8.71E-04
p__Planctomycetes;c__Pla3	169.0	65.0	4.17E-02
p__Planctomycetes;o__B97	127.0	52.0	4.41E-02
p__Proteobacteria;c__Alphaproteobacteria	95.0	214.0	1.33E-05
p__Proteobacteria;f__Caulobacteraceae	62.0	105.0	1.02E-02
p__Proteobacteria;g__Phenylobacterium	52.0	159.0	2.03E-06
p__Proteobacteria;o__Ellin329	579.0	754.0	2.80E-02
p__Proteobacteria;f__Rhizobiaceae	142.0	223.0	1.29E-02
p__Proteobacteria;o__Rhodospirillales	1862.0	1003.0	2.52E-02
p__Proteobacteria;g__Phaeospirillum	33.0	83.0	6.83E-04
p__Proteobacteria;o__Rickettsiales	55.0	13.0	3.75E-03
p__Proteobacteria;f__Alcaligenaceae	88.0	152.0	1.25E-02
p__Proteobacteria;f__Burkholderiaceae	1.0	110.0	9.60E-14
p__Proteobacteria;g__Burkholderia	6.0	108.0	8.14E-16

p__Proteobacteria;f__Comamonadaceae	611.0	4498.0	1.14E-14
p__Proteobacteria;g__Delftia	15.0	289.0	1.34E-09
p__Proteobacteria;g__Cupriavidus	25.0	258.0	1.09E-17
p__Proteobacteria;f__Entotheonellaceae	1030.0	472.0	1.11E-03
p__Proteobacteria;f__Bacteriovoracaceae	185.0	35.0	2.80E-02
p__Proteobacteria;f__Syntrophobacteraceae	7112.0	3975.0	3.01E-02
p__Proteobacteria;f__Alteromonadaceae	969.0	54.0	5.43E-05
p__Proteobacteria;g__Cellvibrio	101.0	22.0	1.37E-04
p__Proteobacteria;f__Moraxellaceae	3.0	271.0	4.08E-02
p__Proteobacteria;g__Acinetobacter	6.0	436.0	3.44E-06
p__Proteobacteria;g__Perlucidibaca	9.0	1496.0	3.11E-07
p__Proteobacteria;g__Arenimonas	60.0	9.0	2.93E-04
p__TM7;c__SC3	127.0	17.0	2.41E-08
p__TM7;c__TM7-1	50.0	7.0	1.77E-04

*p-values corrected by the FDR method.

Table 5 – Fungal OTUs presenting an average absolute abundance significantly different between the treatments “Crude Oil” and “Control”, under the EdgeR Fisher’s exact test and α = 0.05.

Taxa	Control	Crude Oil	q-value*
p__Ascomycota;f__Clavicipitaceae	96.3	2442.6	1.96E-16
p__Ascomycota;g__Fusarium	834.0	19800.6	7.25E-16
p__Zygomycota;g__Mortierella	11430.0	69846.3	2.55E-08
p__Ascomycota;o__Hypocreales;	378.0	1148.3	1.01E-05
p__Ascomycota;g__Lecanicillium	0.0	135.0	3.02E-05
p__Ascomycota;f__Bionectriaceae	14704.3	202.3	8.01E-04

*p-values corrected by the FDR method.

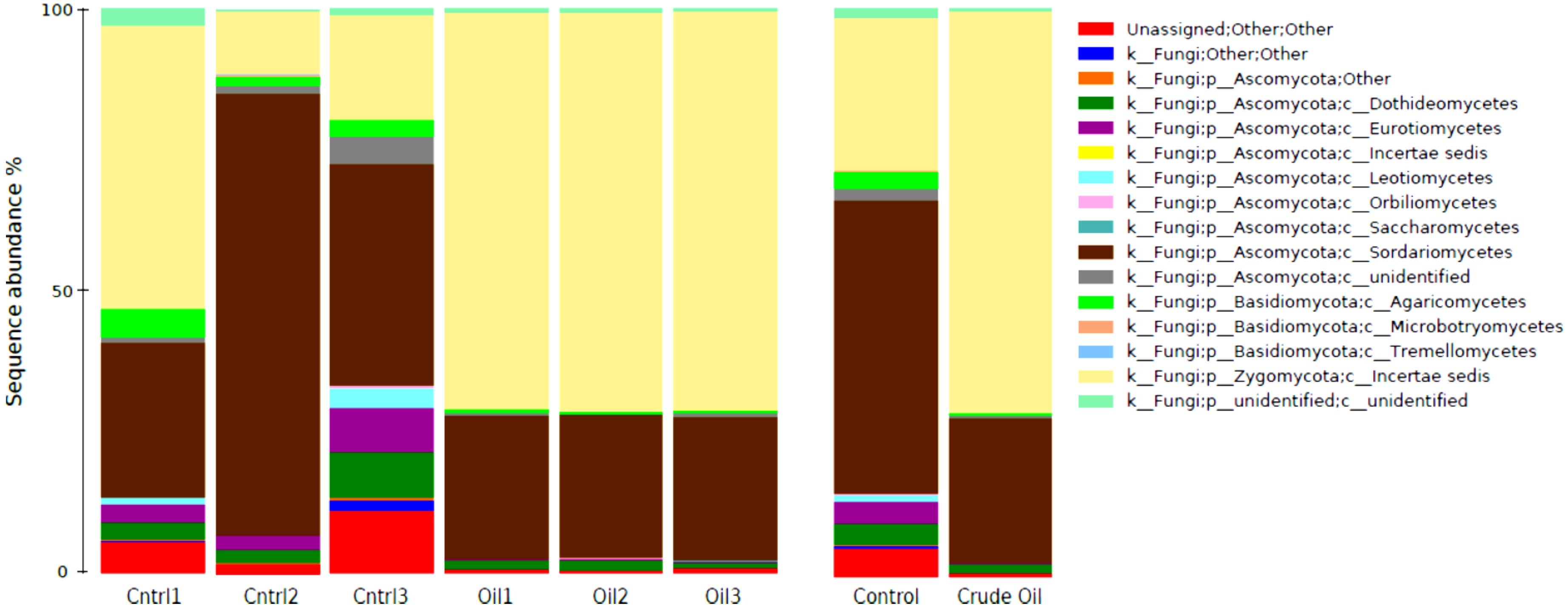

Figure 6 - Relative abundance of Fungi phyla using ITS1 region sequences. Samples are disclosed isolated and as an average of each treatment.

DISCUSSION

In this study, we have applied high throughput sequencing to evaluate the effect of crude oil contamination on Trindade Island soil microbiota. We found that crude oil had a deleterious effect on microbial alpha-diversity (Tables 2 and 3). This result is similar to that obtained by Yang (2014), as crude oil was thought to have an eco-toxicological effect. The higher amount of CO_2 evolved in the crude oil treated-soil (Figure 2) is related to the oil stressing effect (Franco et al., 2004), and the further peaks observed in the Figure 2, might be related to different fractions of oil being degraded according to its bioavailability.

Despite the toxic effect, some taxa are able to utilise oil hydrocarbons as a source of carbon and energy being favoured by oil amendment, and gradually overcoming the populations lacking those abilities. The effects on fungal diversity were more marked than that observed on prokaryotic diversity, corroborating Embar (2006), who reported a rapid increase in abundance and shift in diversity in the fungal community in response to oil contamination. The strong effect of oil on the fungal diversity may also be explained by metabolic differences between eukaryotes and prokaryotes. This effect relates to the increased toxicity of polycyclic aromatic hydrocarbons, present in crude oil, after metabolic activation mediated by the enzyme cytochrome P450 (CYP) of eukaryotes. The majority of carcinogens in the environment are inert by themselves and require the metabolic activation by CYP, in order to exhibit carcinogenicity (Shimada and Fuji-Kuriama, 2003). The CYP genes belong to the superfamily of dioxygenases, present in all domains of life. Genes that code for dioxygenases in prokaryotes are related to toxin and xenobiotic degradation, while in eukaryotes CYP genes may be related to a plethora of functions, ranging from biosynthesis of hormones to chemical defence in plants (Werck-Reichhard et al., 2000).

We observed the formation of two distinct clusters representing the control samples and the crude oil contaminated samples during the analysis of beta-diversity (Figure 4). We found that bacterial/archaeal oil-contaminated replicates showed a broader spread in the PCoA, while oil-contaminated replicates in fungal communities are clustered more tightly (figure 4B). Because of selective pressure, the taxa resistant to the contamination event and the populations able to degrade hydrocarbons will gradually outnumber the rest of the community in the curse of succession (Yang et al., 2014). Therefore, as oil presented a toxic effect, we would expect that the bacterial community of contaminated samples would show a more compact clustering, as happened with the fungal community. However, as the bacterial community comprises c. 30x more OTU than the fungal, the shifts in the bacterial relative abundance might be more related to soil microhabitats present in each replicate, than with the oil toxic effects. This phenomenon was previously observed (Juck et al., 2000; Liang et al., 2011; Yang et al., 2014), and could be explained by the appearance of new niches in the contaminated soil with further fulfilment of these niches by previously not detected (low abundance) taxa.

Soil is the most diverse environment on earth (Vogel et al., 2009), and many of the native microorganisms possess the ability to resist and degrade crude oil hydrocarbons (Franco et al., 2004; Head et al., 2006). In this study, we detected the relative abundance community shifts in Actinobacteria, Proteobacteria, Firmicutes and Planctomycetes. The phylum Actinobacteria had its abundance increased in response to crude oil addition. We detected shifts in one unidentified OTU from the family Streptomycetaceae, one OTU from the genus Streptomyces and one OTU from the order Solirubrobacterales. Interestingly, the genera Nocardia represented less than 0.01% of the total sequences in the control samples and shifted to 9.4% of the sequences in the crude oil samples (Table 4). Several studies have reported Actinobacteria as a good option for removing recalcitrant hydrocarbon, since they are known for the production of extracellular enzymes that degrade a wide range of complex hydrocarbons. Also, many species of Actinobacteria are able to produce biosurfactants that enhance hydrocarbons solubility and bioavailability (Pizzul et al., 2007; Kim & Crowley, 2007; Balachandram et al., 2012; da Silva et al., 2015). The Actinobacteria phylum is recognized as the main alkane degrader in polar soils (Aislabie et al., 2006), besides producing multiple types of antifungals, antivirals, antibiotics, immunosuppressives, anti-hypertensives and antitumorals (Benedict, 1953; Omura et al., 2001; Khan et al., 2011; de Lima Procópio et al., 2012). Rodriguez et al. (2015) reported a significant rise in Gamma and Alphaproteobacteria relative abundance from beach sand of Florida coast, in response to the crude oil plume from the Deepwater Horizon Drilling rig accident in the Gulf of Mexico. Although some research has reported the prevalence of Gram-negative bacteria upon soils contaminated with heavily weathered petroleum (Kaplan & Kitts, 2004), our work shows a big shift on Gram-positive Actinobacteria. Our results also corroborates with Chikere et al. (2009) who reported the prevalence of Actinobacteria after oil addition using cultivation dependent techniques. Grosshard et al. (2004) detected the inhibition of several proteobacterias by actinomycete strains isolated from the German Wadden sea, while Burgess et al. (1999) report that, antibiotic production may be triggered by several factors as presence of chemical substances, substrate availability, population density and many others.

We did not detect a shift in the general relative abundance of the Proteobacteria phylum (Figure 4) but, the relative abundance of the classes inside this phylum showed a significant change (Table 5). Alpha and Deltaproteobacteria classes had a major relative abundance reduction in the contaminated samples. The reduction of these two classes might even be connected, considering that, the Alphaproteobacteria with the biggest reduction was a member of the Rhodospirillales order, which is composed mainly by purple non sulphur photosynthetic microorganisms. This group fix carbon using hydrogen as an electron donor, and the member of the Deltaproteobacteria phylum that suffered the biggest reduction belongs to the Syntrophobacteraceae family, a family known for releasing H_2 as a product of organic acids fermentation. This ecological interaction is called syntrophy (McInerey et al., 1981), and its presence could be happening as both groups were reduced by c. 50%. The Beta and Gammaproteobacteria classes rose in their relative abundance in response to crude oil treatment. The member of the Betaproteobacteria class with the biggest increase belonged to the family Comamonadaceae, this family is known by its heterotrophic denitrification capability (Khan et al., 2002) using organic compounds as electron donors. The only Archaea with significant differences between treatments, Nitrososphaera, is an autotrophic ammonia-oxidizer (Mußmann et al., 2011) and represented 6.1% of the total sequences in the control. In the crude oil contaminated treatment this relative abundance was reduced to 2.8%. Urakawa et al. (2012) evaluating the responses of ammonia-oxidizing Archaea and Bacteria to crude oil hydrocarbons, showed that Archaea are several times more sensitive than Bacteria. The reduction of this Archaea and the increase in the relative abundance of the Comamonadaceae family individual (Table 5), mentioned above, reinforces the hypothesis raised to explain the broader cluster observed in Bacteria beta-diversity (Figure 4A). This phenomenon was not observed for Fungi, as in the control samples we were able to detect 12 well distributed classes and in the contaminated samples, more than 95% of the sequences belonged to the classes Sordariomycetes and

Incertae. The Incertae class was represented by only one genera, Mortierella, and its relative abundance in the contaminated samples reached 70.3%. Mortierella is a Zygomycota and is known as an oleaginous microorganism, it accumulates lipids and has even been used as a strategy for biodiesel production (Ratledge, 2002; Kumar et al., 2011).

This is the first study reporting the effect of crude oil contamination in soils of the Trindade Island, a Brazilian oceanic island threatened by possible oil spills due petroleum exploration. Our results reinforces the importance of microbial diversity analysis in insulated environments, pointing out the impact of crude oil on microbial communities shifts from unexplored environments. Moreover, these findings indicate the biotechnological potential of degrading hydrocarbons soil microorganisms, fostering further studies aiming to relieve any oil contamination occurrence on Trindade Island.

REFERENCES

Aislabie, J., Saul, D. J., Foght, J. M. (2006) Bioremediation of hydrocarbon contaminated polar soils. Extremophiles 10: 171–179.

Alves, R. J. V., & Castro, J. W. (2006) Ilhas oceânicas brasileiras: da pesquisa ao manejo. MMA Secretaria de Biodiversidade e Florestas. Brasília, DF, Brasil. 298 p.

Anders, S., Huber, W. (2010). Differential expression analysis for sequence count data Genome Biology 11, R106.

Atlas, R. M. (1981). Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiological reviews, 45(1), 180.

Balachandran, C., Duraipandiyan, V., Balakrishna, K., & Ignacimuthu, S. (2012). Petroleum and polycyclic aromatic hydrocarbons (PAHs) degradation and naphthalene metabolism in Streptomyces sp.(ERI-CPDA-1) isolated from oil contaminated soil. Bioresource Technology, 112, 83-90.

Benedict, R. G. (1953). Antibiotics produced by actinomycetes. The Botanical Review, 19(5), 229-320.

Bengtsson-Palme, J., Ryberg, M., Hartmann, M., Branco, S., Wang, Z., Godhe, A., Nilsson, R. H. (2013). Improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and other eukaryotes for analysis of environmental sequencing data. Methods in Ecology and Evolution, 4(10), 914-919.

Brinch, U. C., Ekelund, F., & Jacobsen, C. S. (2002). Method for spiking soil samples with organic compounds. Applied and Environmental Microbiology, 68(4), 1808-1816.

British Petroleum; BP statistical review of world energy. BP, London, UK. 2014. Accessible at: bp.com/statisticalreview

Burgess, J. G., Jordan, E. M., Bregu, M., Mearns-Spragg, A., & Boyd, K. G. (1999). Microbial antagonism: a neglected avenue of natural products research. Journal of Biotechnology, 70(1), 27-32

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., Reeder, J., Sevinsky, J. R., Turnbaugh, P. J., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. Nature methods, 7(5), 335-336.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S. M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J. A., Smith, G., Knight, R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME journal, 6(8), 1621-1624.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N., Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the National Academy of Sciences, 108(Supplement 1), 4516-4522.

Chikere, C. B., Okpokwasili, G. C., & Chikere, B. O. (2009). Bacterial diversity in a tropical crude oil-polluted soil undergoing bioremediation. African Journal of Biotechnology, 8(11).

da Silva, F. S. P., Pylro, V. S., Fernandes, P. L., Barcelos, G. S., Kalks, K. H. M., Schaefer, C. E. G. R., Tótola, M. R. (2015). Unexplored Brazilian oceanic island host high salt tolerant biosurfactant-producing bacterial strains. Extremophiles, 19(3), 561-572.

de Lima Procópio, R. E., da Silva, I. R., Martins, M. K., de Azevedo, J. L., & de Araújo, J. M. (2012). Antibiotics produced by Streptomyces. The Brazilian Journal of infectious diseases, 16(5), 466-471.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D. Hu, F. Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and environmental microbiology, 72(7), 5069-5072.

Edgar,RC (2010) Search and clustering orders of magnitude faster than BLAST, Bioinformatics 26(19), 2460-2461.

Edwards, R. A., Rodriguez-Brito, B. Wegley, L. Haynes, M., Breitbart, M., Peterson, D. M., Saar, M. O., Alexander, S. Alexander, E. C., Rohwer, F. (2006). "Using pyrosequencing to shed light on deep mine microbial ecology". BMC Genomics 7: 57.

Elliott, L. F., Lynch, J. M., Papendick, R. I., Stotzky, G., Bollag, J. M. (1996). The microbial component of soil quality. Soil biochemistry Volume 9, 1-21.

Embar, K., Forgacs, C., & Sivan, A. (2006). The role of indigenous bacterial and fungal soil populations in the biodegradation of crude oil in a desert soil. Biodegradation, 17(4), 369-377.

Faith, D. P. (1992). Conservation evaluation and phylogenetic diversity. Biological conservation,61(1), 1-10.

Franco, I., Contin, M., Bragato, G., & De Nobili, M. (2004). Microbiological resilience of soils contaminated with crude oil. Geoderma, 121(1), 17-30.

Grossart, H. P., Schlingloff, A., Bernhard, M., Simon, M., & Brinkhoff, T. (2004). Antagonistic activity of bacteria isolated from organic aggregates of the German Wadden Sea. FEMS microbiology ecology, 47(3), 387-396.

Hamamura, N., Olson, S. H., Ward, D. M., & Inskeep, W. P. (2006). Microbial population dynamics associated with crude-oil biodegradation in diverse soils. Applied and Environmental Microbiology, 72(9), 6316-6324.

Haritash, A. K., & Kaushik, C. P. (2009). Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. Journal of hazardous materials, 169(1), 1-15.

Head, I. M., Jones, D. M., & Röling, W. F. (2006). Marine microorganisms make a meal of oil. Nature Reviews Microbiology, 4(3), 173-182.

Heinemeyer, O., Insam, H., Kaiser, E. A., & Walenzik, G. (1989). Soil microbial biomass and respiration measurements: an automated technique based on infra-red gas analysis. Plant and soil,116(2), 191-195.

Hentati, O., Lachhab, R., Ayadi, M., & Ksibi, M. (2013). Toxicity assessment for petroleum-contaminated soil using terrestrial invertebrates and plant bioassays. Environmental monitoring and assessment, 185(4), 2989-2998.

Hoeft, R. G., Walsh, L. M., and D. R. Keeney. 1973. Evaluation of various extractants for available soil sulfur. Soil Sci. Soc. Am. Proc. 37: 401-404.

Huettel, M., Berg, P., Kostka, J. E. (2014). Benthic exchange and biogeochemical cycling in permeable sediments. Marine Science, 6.

Juck, D., Charles, T., Whyte, L., Greer, C. (2000) Polyphasic microbial community analysis of petroleum hydrocarbon-contaminated soils from two northern Canadian communities. FEMS Microbiology Ecology 33: 241–249

Kaplan, C. W., & Kitts, C. L. (2004). Bacterial succession in a petroleum land treatment unit. Applied and Environmental Microbiology, 70(3), 1777-1786.

Khan, S. T., Horiba, Y., Yamamoto, M., & Hiraishi, A. (2002). Members of the family Comamonadaceae as primary poly (3-hydroxybutyrate-co-3-hydroxyvalerate)-degrading denitrifiers in activated sludge as revealed by a polyphasic approach. Applied and environmental microbiology, 68(7), 3206-3214.

Khan, S. T., Komaki, H., Motohashi, K., Kozone, I., Mukai, A., Takagi, M., & Shinya, K. (2011). Streptomyces associated with a marine sponge Haliclona sp.; biosynthetic genes for secondary metabolites and products. Environmental microbiology, 13(2), 391-403.

Kim, J. S., & Crowley, D. E. (2007). Microbial diversity in natural asphalts of the Rancho La Brea Tar Pits. Applied and environmental microbiology, 73(14), 4579-4591.

Kõljalg, U., Larsson, K. H., Abarenkov, K., Nilsson, R. H., Alexander, I. J., Eberhardt, U., Erland, S., Hoiland, K., Kjoller, R., Larsson, E., Pennanen, T., Sen, R., Taylor, A. F., Tedersoo, L., Vrålstad, T., Ursing, B. M. (2005). UNITE: a database providing web based methods for the molecular identification of ectomycorrhizal fungi. New Phytologist, 166(3), 1063-1068.

Kostka, J. E., Prakash, O., Overholt, W. A., Green, S. J., Freyer, G., Canion, A., Delgardio, J., Norton, N., Hazen, T. C., Huettel, M. (2011). Hydrocarbon-degrading bacteria and the bacterial community response in Gulf of Mexico beach sands impacted by the Deepwater Horizon oil spill. Applied and environmental microbiology,77(22), 7962-7974.

Kumar, I., Ramalakshmi, M. A., Sivakumar, U., Santhanakrishnan, P., & Zhan, X. (2011). Production of microbial oils from Mortierella sp for generation of biodiesel livestock. African Journal of Microbiology Research, 5(24), 4105-4111.

Liang, Y., Van Nostrand, J. D., Deng, Y., He, Z., Wu, L., Zhang, X., Li, G., Zhou, J. (2011) Functional gene diversity of soil microbial communities from five oil-contaminated fields in China. The ISME Journal 5: 403–413.

Lima, J. A. M. A energia que vem do mar: a herança energética do mar brasileiro. Cienc. Cult. [online], v.62, n.3, p.25-8, 2010.

Loman, N. J., & Pallen, M. J. (2015). Twenty years of bacterial genome sequencing. Nature Reviews Microbiology.

Maliszewska-Kordybach, B. (1993). The effect of temperature on the rate of disappearance of polycyclic aromatic hydrocarbons from soils.Environmental pollution, 79(1), 15-20.

Markowitz, V. M., Chen, I. M. A., Chu, K., Pati, A., Ivanova, N. N., & Kyrpides, N. C. (2015). Ten Years of Maintaining and Expanding a Microbial Genome and Metagenome Analysis System.Trends in microbiology, 23(11), 730-741.

McInerney, M. J., Bryant, M. P., Hespell, R. B., & Costerton, J. W. (1981). Syntrophomonas wolfei gen. nov. sp. nov., an anaerobic, syntrophic, fatty acid-oxidizing bacterium. Applied and Environmental Microbiology, 41(4), 1029-1039.

McKee, R. H., Schreiner, C. A., Nicolich, M. J., & Gray, T. M. (2013). Genetic toxicity of high-boiling petroleum substances. Regulatory Toxicology and Pharmacology, 67(2), S75-S85.

Mohr, L. V., Castro, J. W. A., Costa, P. M. S., & Alves, R. J. V. (2009). Ilhas oceânicas brasileiras: da pesquisa ao manejo.

Mueller, M., & Wallace, R. (2008). Enabling science and technology for marine renewable energy. Energy Policy, 36(12), 4376-4382.

Mussmann, M., Brito, I., Pitcher, A., Damsté, J. S. S., Hatzenpichler, R., Richter, A., Nielsen, J. L., Müller, A., Damis, H., Wagner, M., Head, I. M. (2011). Thaumarchaeotes abundant in refinery nitrifying sludges express amoA but are not obligate autotrophic ammonia oxidizers.Proceedings of the National Academy of Sciences,108(40), 16771-16776.

Ōmura, S., Ikeda, H., Ishikawa, J., Hanamoto, A., Takahashi, C., Shinose, M., Takahashi, Y., Horikawa, H., Nakazawa, H., Osonoe, T., Kikuxhi, H., Shiba, T., Sakaki, Y, Hattori, M. (2001). Genome sequence of an industrial microorganism Streptomyces avermitilis: deducing the ability of producing secondary metabolites. Proceedings of the National Academy of Sciences,98(21), 12215-12220.

Pizzul, L., del Pilar Castillo, M., & Stenström, J. (2007). Effect of rapeseed oil on the degradation of polycyclic aromatic hydrocarbons in soil by Rhodococcus wratislaviensis. International biodeterioration & biodegradation, 59(2), 111-118.

Rahman, K. S. M., Thahira-Rahman, J., Lakshmanaperumalsamy, P., & Banat, I. M. (2002). Towards efficient crude oil degradation by a mixed bacterial consortium. Bioresource technology, 85(3), 257-261.

Ratledge, C. (2002). Regulation of lipid accumulation in oleaginous micro-organisms.Biochemical Society Transactions, 30(6), 1047-1049.

Robinson, M. D., McCarthy, D. J. & Smyth. J. K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics, 26(1): 139–140.

Rodriguez-R, L. M., Overholt, W. A., Hagan, C., Huettel, M., Kostka, J. E., & Konstantinidis, K. T. (2015). Microbial community successional patterns in beach sands impacted by the Deepwater Horizon oil spill. The ISME journal, 9(9):1928-40.

Roesch, L. F., Fulthorpe, R. R., Riva, A., Casella, G., Hadwin, A. K., Kent, A. D., Daroub, S. H., Camargo, F. A., Farmerie, W. G., Triplett, E. W. (2007). Pyrosequencing enumerates and contrasts soil microbial diversity. The ISME journal, 1(4), 283-290.

Seabra, A. A. D., Freitas, G. P. D., Polette, M., & Casillas, T. (2011). The promising oil province of the pre-salt. Revista Direito GV, 7(1), 57-74.

Shimada, T., & Fujii Kuriyama, Y. (2004). Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1. Cancer science, 95(1), 1-6.

Smith, D. P., & Peay, K. G. (2014). Sequence depth, not PCR replication, improves ecological inference from next generation DNA sequencing. PLoS One, 9(2), e90234.

Urakawa, H., Garcia, J. C., Barreto, P. D., Molina, G. A., & Barreto, J. C. (2012). A sensitive crude oil bioassay indicates that oil spills potentially induce a change of major nitrifying prokaryotes from the Archaea to the Bacteria. Environmental pollution,164, 42-45.

Venkateswaran, K., Iwabuchi, T., Matsui, Y., Toki, H., Hamada, E., & Tanaka, H. (1991). Distribution and biodegradation potential of oil-degrading bacteria in North Eastern Japanese coastal waters.FEMS Microbiology Letters, 86(2), 113-121.

Vogel, T. M., Hirsch, P., Simonet, P., Jansson, J. K., Tiedje, J., van Elsas, J. D., Bailey, M. J. Nalin, R., Philipot, L.(2009). TerraGenome: a consortium for the sequencing of a soil metagenome. Nature Reviews Microbiology, 7, 252.

Werck-Reichhart, D., & Feyereisen, R. (2000). Cytochromes P450: a success story. Genome Biol, 1(6), 3003-1.

Yang, S., Wen, X., Zhao, L., Shi, Y., & Jin, H. (2014). Crude oil treatment leads to shift of bacterial communities in soils from the deep active layer and upper permafrost along the China-Russia Crude Oil Pipeline route. PloS one, 9(5).

Zobell, C. E. (1946). Action of microorganisms on hydrocarbons. Bacteriological reviews, 10(1-2), 1.

ADDITIONAL INFORMATION AND DECLARATIONS

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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Supplementary table

Table S1 – Number of quality filtered Illumina® barcoded sequences

Treatments	16S rRNA gene	Fungal Intergenic Spacer (ITS1)
Crude Oil	45690	25315
Crude Oil	47917	46921
Crude Oil	52889	55579
Control	54458	91866
Control	55487	100736
Control	58337	103852
Total	314748	424269

CHAPTER 2

EMPOWERING THE SOIL MICROBIAL COMMUNITY TO WITHSTAND TOXIC HYDROCARBON CONTAMINATION

Empowering the soil microbial community to withstand toxic hydrocarbon contamination

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ABSTRACT

Petroleum hydrocarbons may cause problems in all domains of life, from shifts in microbial communities (bacteria, phytoplankton and zooplankton) to mutagenic effects, alteration of embryonic development, life spam and behavior of vertebrates. Biodiesel Co-Product (BCP) is now a highly available and cost-effective material for many pourposes, including soil amendment. BCP was shown to promote increased extracellular investment of N into amino acids present in the extracellular matrix (or 'EPS') relative to the simple carbon source glycerol. Soil is considered the most diverse environment on Earth. We aimed to compare the effects of crude oil contamination on three soils not previously exposed to oil contamination. Furthermore, we hypothesized that a microbial community given BCP would maintain greater diversity when exposed to contamination with crude oil than a community not given BCP. An early flush of CO_2 was observed when BCP was applied to the soils with and without oil. BCP did not affect ATP or alpha diversity in Grassland soils, nor alpha diversity of Bare Fallow. The data distribution obtained with Principal Coordinates Analysis (PCoA) shows a very tight clustering of the samples by type of soil. Grassland and Bare Fallow soils had high differences of OTU abundances between the treatments Control and Oil and Trindade Island soil had very low differentially abundant OTUs between these two tratmetns. It is necessary to evaluate long-term effects of crude oil addition on different types of soil. This study was part of a larger project, which intends to describe immediate changes in microbial functions under the same conditions of this experiment. This experiment was the first comparison between agricultural soils and a tropical pristine soil under crude oil contamination and a bioremediation strategy using a by-product from the biodiesel industry.

Keywords: Next Generation Sequencing; microbial community analysis; alpha diversity; beta diversity; biodiesel co-product (BCP), differential abundance.

INTRODUCTION

Crude oil exploration, transport, storage, refining and consumption are activities that bring risk to the environment. As shown in many articles, the leakage of crude oil or its derivatives cause damage to soil, water and air, through the release of recalcitrant and toxic compounds (D. M. Di Toro et al, 2007). Petroleum hydrocarbons may cause problems in all domains of life, from shifts in microbial communities (bacteria, phytoplankton and zooplankton) to mutagenic effects, alteration of embryonic development, life spam and behavior of vertebrates (G. Perhar and G. B. Arhonditsis, 2014, J. P. Incardona et al, 2014, D. Kochhann et al, 2015). In reason of its disastrous effects, petroleum spillage is now considered the main threat to marine ecosystems (G. Perhar and G. B. Arhonditsis, 2014).

The diversification of the energy industry into the renewable sector and favorable combustion dynamics of biodiesel (Coniglio et al., 2013) mean that Biodiesel Co-Product (BCP) is now a highly available and cost-effective material for many pourposes, including soil amendment. BCP contains approximately 49% glycerol and 12% of fatty acid methyl esters from (Table 1). It was previously demonstrated that BCP rapidly increased the microbial biomass in an arable soil (Redmile-Gordon et al., 2014). In the aforementioned study, BCP enabled microbial transformations of nitrate-N to organic forms and thus prevented losses of nitrogen from the soil. Furthermore, BCP was shown to promote increased extracellular investment of N into amino acids present in the extracellular matrix (or ‘EPS’) relative to the simple carbon source glycerol (Redmile-Gordon et al., under review 1). EPS is thought to support improved enzyme efficiency by increasing the contact between enzyme and substrate in aquatic systems (Romani et al., 2009) and soils (Redmile-Gordon et al., under review2), and embody tolerance against toxic hydrocarbon (Kang & Park, 2010).

Microorganisms are key components of the carbon cycle, therefore, possessing the enzymatic apparatus to produce and degrade a plethora of organic carbon molecules. It is well known that prokaryotes are efficient on utilizing recalcitrant petroleum hydrocarbons as source of carbon and energy. In 1946, Claude E. Zobel published the classical review about the microorganisms’ ability of oil degradation. Ronald Atlas’ review (1981) presents an environmental perspective on the role of bacteria, algae and fungi, comparing taxonomy, environmental conditions and chemical characteristics of crude oil. That review highlights the increasing importance of this field of research in line with the global trend for spillages of greater magnitude. At that time, the use of molecular approaches to prospect biodegradation functions in the environment was expensive and the absence of high throughput methods prevented the deepening (or expansion) of our knowledge about the existence, organization and regulation of genes and metabolic pathways involved in hydrocarbon biodegradation.

Although the term "metagenomics" have been used for the first time in 1998 by Handelsman and his collaborators, results from studies using "next generation sequencing" technology to describe environmental microbial samples were published only in 2005 by Poinar and colleagues, describing ancient DNA samples from Mammouth, followed by those of Robert Edwards (2006) about the microbial ecology of deep mines.

Soil is considered the most diverse environment on Earth. Torsvik and colleagues (1996) estimated that one gram of soil could hold up to 6000 species of bacteria, based on E. coli genome size. Results from Roesch et al. 2007, using next generation sequencing, show that community richness and diversity are closely related to the use of land; they also obtained richness results that agreed with the estimations made by Torvisk et al. (1996).

The recent achievements in the field of nucleotide sequencing have improved our capabilities to study complex microbial communities. Such advances made it possible to describe the microbial community profile of highly diverse and complex matrices like soil (Roesch et al., 2007; Caporaso et al., 2012).

Here we aimed to compare the effects of crude oil contamination on three soils not previously exposed to oil contamination and with highly contrasting physicochemical properties (Table 2) and management. Furthermore, we hypothesized that a microbial community given BCP would maintain greater diversity when exposed to contamination with crude oil than a community not given BCP.

MATERIAL AND METHODS

Experimental design

Three soils were used to examine the effect of crude oil contamination and of BCP treatment. Two soils were sampled from the Highfield long-term experiment at Rothamsted Research, Hertfordshire, UK and one soil was sampled at Trindade Island, Brazil, harboring the species Cyperus atlanticus. We defined 4 treatments: Control samples (Cntrl), Crude oil addition (Oil) (50 g kg^{-1}), Biodiesel Co-product (BCP) (3 g kg^{-1}) and a Combination of Crude oil and Biodiesel Co-product (Comb). The microcosms were assembled with 20 grams of soil in bijou bottles of 50 mL. The microbial biodegradation activity was measured by CO_2 emission quantified using the MicroResp^{tm} method (Campbel et al., 2003).

The Biological Chemistry department of Rothamsted Research Institute supplied Biodiesel co-product characteristics (Table 1).

Table 1 - Composition (%) of biodiesel co-product (BCP).

Potassium hydroxide (KOH)	Potassium soap (oleate equivalent)	Biodiesel (FAME)	Volatile organics*	Glycerol	H_2O
0.1	29.3	12.1	7.3	49.3	1.9

*Volatile organics at 105 °C.
FAME stands for fatty acid methyl ester.

Sampling site and soil analysis

The two soils from Rothamsted Research's Highfield site were under different cultivation regimes. One soil was under a bare fallow regime since 1959 and the other soil was under a grassland regime since 1949 (P. Hirsch et al., 2009). Soils were randomly sampled at a depth of 0 – 10 cm. Trindade Island soil was sampled to the same depth from the northeast shoreline area (coordinates: 20°30' S and 29° 19' W), under influence of native vegetation (Cyperus atlanticus). The soils were sampled randomly along the shoreline, following the distribution of C. atlanticus. Cores were bulked, sieved (<2mm) and stored at 4°C until the microcosms were assembled. The sampling expedition occurred through April 2013 and was supported by the Brazilian Navy and PROTRINDADE Research Program. The National Council for Scientific and Technological Development (CNPq) provided all approvals and permits (project grant number 405544/2012-0 and authorization access to genetic resources process number 010645/2013-6) to conduct the study within this protected area. The field study did not involve endangered or protected species.

Soil treatment with crude oil and BCP

In order to achieve the homogenous mixing of oil with soil, hexane was used as a carrier. To eliminate the effect of hexane from the experimental design, the following procedure was adopted. Firstly, to simulate the ageing of crude oil exposed to environmental conditions upon spillage, we heated 500 mL of crude oil to 90^0 C and incubated for two hours in a fume hood. The resulting aged crude oil was a material highly viscous and difficult to work with. To facilitate application to soil, we dissolved the aged crude oil in hexane and applied to a subsample of each experimental soil. Hexane was also added to soils without crude oil to create a hexane-only contaminated control stock. These hexane (and crude oil + hexane) exposed soil stocks were kept in the fume hood until all hexane had evaporated. We then added 10 g of the control stock soil (hexane evaporated) to the bottles corresponding to treatments ‘Control’ and ‘BCP’, and made up to 20 grams with the corresponding soil that had not been exposed to hexane. The same procedure was repeated for the stock soils that received the amendment of oil, corresponding to the treatments ‘Crude Oil’ and ‘Comb’ (crude oil + BCP). This procedure was adopted to repopulate the native soil microbial community decimated by hexane. The BCP was added to soils (treatments ‘BCP and Comb’) as an aqueous solution of pH 7 at the beginning of the incubation period. The final concentration of crude oil and BCP was 3 g kg^{-1} and 3 g kg^{-1} respectively. After microcosm setup, the bottles were incubated at 26^0 C and the soil moisture was kept at 60% of its water holding capacity (remoistened periodically with deionized water upon reaching c. 50% of the water holding capacity). In order to stimulate the microbial activity, at the 10th and 15th day of incubation, the microcosms were amended with 5 g of stock soil (hexane evaporated) to the treatments ‘Control’ and ‘BCP’ and 5 g of oil + hexane-contaminated soil stock to the treatments ‘Crude Oil’ and ‘Comb’ (crude oil + BCP).

ATP quantification analysis

ATP is a useful substitute for the 'biomass C' method when C is made highly available (Joergensen and Raubuch, 2002) or when non-biomass C might be suspected to be solubilized through fumigation with chloroform. Considering that these soils were contaminated with crude oil, we favoured the ATP approach as a measure of microbial biomass. The methodology was the described by Redmile-Gordon et al., (2011).

Molecular analyses

DNA extraction and quality check

Genomic DNA was extracted and purified from each soil sample (0.5 g) using the PowerMax® Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) following the manufacturer's instructions. The purity of the extracted DNA was verified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) (260/280 nm ratio), DNA concentration was determined using Qubit® 2.0 fluorometer and dsDNA BR Assay kit (Invitrogen™). The integrity of the DNA was confirmed by electrophoresis in a 0.8 % agarose gel with 1 X TAE buffer.

High-throughput sequencing

Sequencing was done on the Illumina MiSeq® platform (Caporaso et al. 2012) at the High-throughput Genome Analysis Core (HGAC), Argonne National Laboratory (Illinoi, USA). Bacterial and archaeal 16S rRNA genes were amplified using primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') for paired-end microbial community.

Data analysis

Respiratory data was compared using the R package and the ExpDes (Cite ExpDes) library to perform the ANOVA and Tukey test between treatments and environments. The graphs were generated using the SigmaPlot software (Systat Software, San Jose, CA).

We applied the 16S bioinformatics pipeline recommended by the Brazilian Microbiome Project available at http://brmicrobiome.org (Pylro et al., 2014) that apply Qiime and Usearch 7.0 (Cite Usearch) for filtering, clustering and diversity analysis. The microbial community data was evaluated using the balance weighted phylogenetic diversity (BWPD) (McCoy & Matsen, 2013) to compare the alpha diversity between soils and treatments and Principal Coordinates Analysis (PCoA) to compare the beta diversity between treatments and environments. The phylogenetic method used to produce the distance matrix was the weighted unifrac (Lozupone et al, 2005) and the distances between treatments were compared using the two-sided Student's two-sample t test. The full tables of statistical tests are in the supplementary material.

In order to evaluate the differential abundance of species among samples, we used the method recommended by McMurdie and Holmes, (2014). The mentioned authors suggest the normalization of OTU counts using the relative log expression (RLE) scaling factor (Anders & Huber, 2010) between two groups of samples. After the normalization step, McMurdie and Holmes (2014) suggest the exact test (Robinson & Smith, 2008) for differences in the means between the two groups, assuming a negative-binomial distribution. These analysis were performed using the edgeR package (Robinson & Smith, 2010). The complete RScript developed for this study is available at https://github.com/kdanielmorais/diff_abundance.

RESULTS AND DISCUSSION

Soil respiration and physicochemical characteristics

An early flush of CO_2 was observed when BCP was applied to the soils with and without oil (figure 1, A, B and C). After this initial flush had passed (day 3), the cumulative CO_2 increase was greater in Grassland soil given BCP + Oil than with oil alone (figure 1-A), indicating either that the BCP was providing a sustained source of carbon or that its co-addition was supporting the respiration of crude oil-associated carbon. Opposite to Grassland, the increase of CO_2 release after treating the soils was only noticed after the 10th day of incubation for Bare Fallow and Trindade soils. These two soils showed very low respiration rates in comparison to Grassland soil, what can be explained by their low biomass content (figure 3).

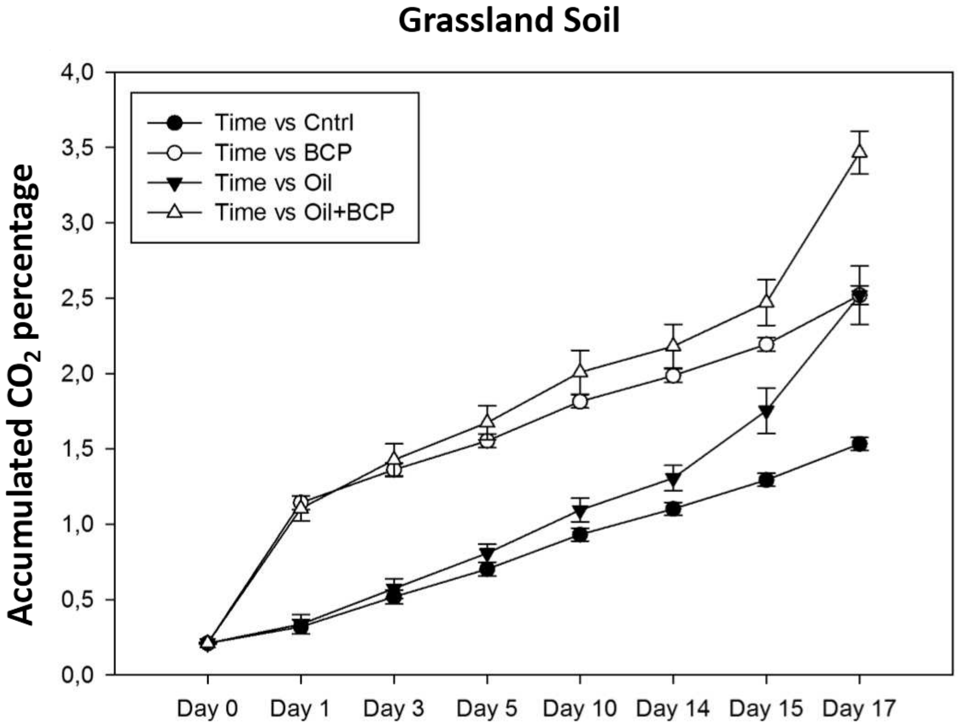

Figure 1 (A) Accumulated CO_2 evolution of the treatments Control, Oil, BCP and Comb for Grassland soil, during the 17 days of incubation. The measurement was made using the MicroResp^{TM} method.

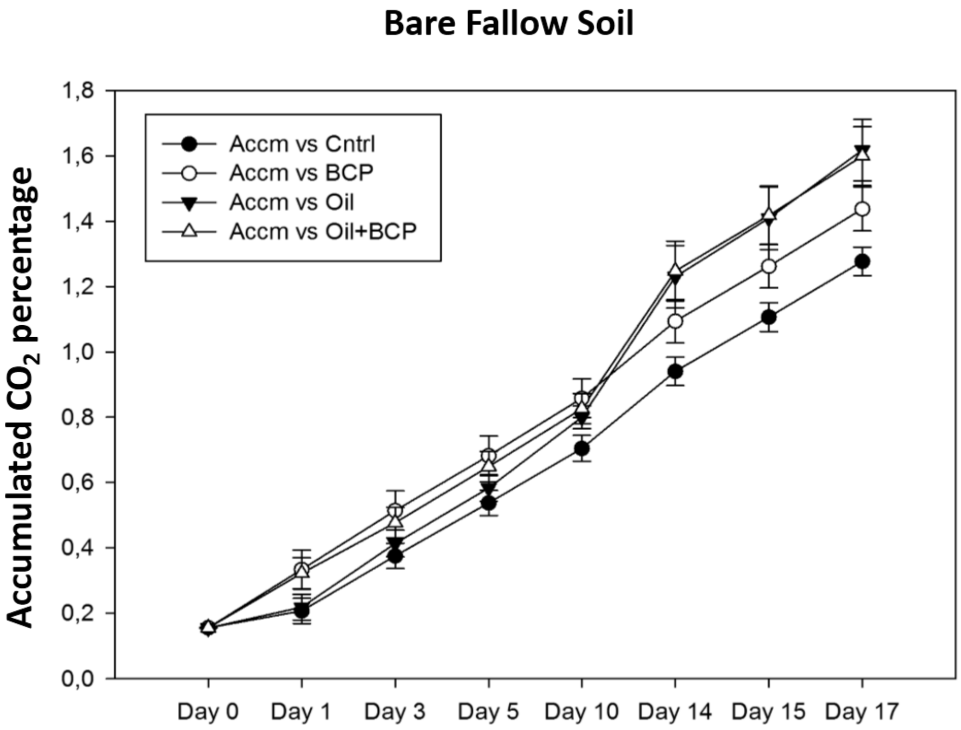

Figure 1 (B) Accumulated CO_2 evolution of the treatments Control, Oil, BCP and Comb for Bare Fallow soil, during the 17 days of incubation. The measurement was made using the MicroResp^{TM} method.

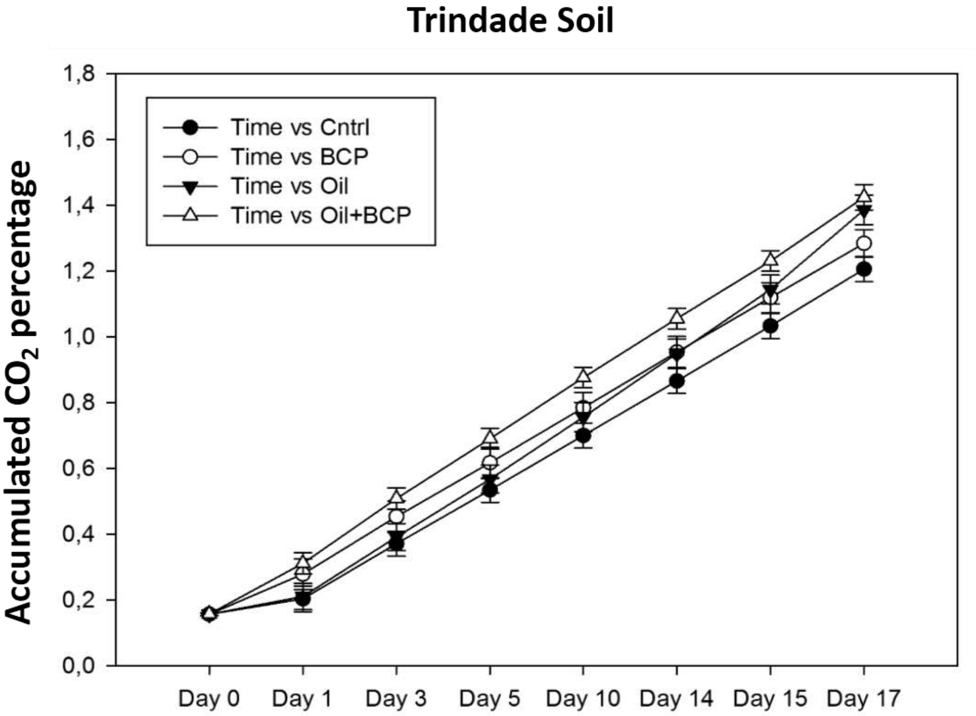

Figure 1 (C) Accumulated CO_2 evolution of the treatments Control, Oil, BCP and Comb for Trindade Island soil, during the 17 days of incubation. The measurement was made using the MicroResp^{TM} method.

The three soils have contrasting physicochemical properties (Table 2). The parental material, resident biota, local topography and time of formation, explain these contrasting characteristics (Brady & Weil, 2002). Trindade Island soil showed low organic matter in comparison with Grassland, but high content of basic nutrients and available phosphorus (Table 2). These characteristics relate to the nature of the soils used in the study. High fertility is common in soils from shore regions, like Trindade Island. High carbon and nitrogen contents are expected in soils receiving root exudates as Rothamsted’s Highfield Grassland. Bare Fallow soil has the characteristics of a soil without vegetation coverage for a long period. The leaching process occurring more easily without temporary immobilization in plant biomass may explain its low nutrient concentration. Another factor rely on the lack of plants to stimulate the microbial community and, therefore, nutrient cycling.

Table 2 - Summary physicochemical information for surface soil cores (0 - 10 cm) sampled at Rothamsted Research's High Field site (Bare Fallow and Grassland) and Trindade Island - Brazil.

Sample	pH	Soil texture	P-avail(1)	P(3)	K(4)	Ca^{+2}(3)	Mg^{+2}(3)	OM	N
			mg kg^{-1}	mg kg^{-1}		$cmol_c$ kg^{-1}		%	
Bare Fallow	6.83	Silty Loam	2.32	49.75	31.98	1.03	0.42	0.85	0.09
Grassland	6.43	Silty Loam	11.30	128.09	163.77	6.18	0.60	3.98	0.34
Trindade	5.60	Sandy Loam	/	1290.80	180.33	9.84	2.78	0.64	0.19

(1) Pipet method. (1) Available Phosphorus (Olsen et al., 1954). (3) Extracted with Mehlich – 1. (4) Extracted with KCl 1 mol L^{-1}.

Soil microbial biomass and diversity

In order to evaluate the effects of oil and the protective effect of BCP against hydrocarbon toxicity, we decided to merge the graphs containing the informations of biomass (ATP quantification) and the alpha diversity metric, (balance weighted phylogenetic diversity) (McCoy & Matsen, 2013) (figure 3).

Biodiesel Co-product (BCP) amendment increased the microbial biomass of Bare Fallow soil and Trindade Island soil and alpha diversity (BWPD) of Trindade Island. BCP did not affect ATP or alpha diversity in Grassland soils, nor alpha diversity of Bare Fallow. The Liebig's law, also known as "the law of the minimum", states that the scarcest resource (limiting factor) controls growth (Ploeg et al, 1999). Bare Fallow and Trindade Island soils have low carbon content, while Grassland soils have very high organic carbon concentration. The addition of BCP added the nutrient that was limiting the microbial growth in Bare Fallow and Trindade, but for Grassland, another factor seems to be limiting the microbial growth.

Crude oil had a deleterious effect on microbial biomass of Grassland and Bare Fallow soils, but not in the soil of Trindade (figure 3). It is well known that polycyclic hydrocarbons present in oil have a toxic effect on non-resistant cells (Hentati et al, 2013). Oil contamination also increased alpha diversity (BWPD metric) in Bare Fallow. Although the result of a toxic compound like hydrocarbons present in oil causing the increase of Bare Fallow's microbial diversity sounds controversial, it is known that soils hold a high degree of functional redundancy, especially among bacterial taxa, with very far phylogenetic groups performing similar processes (Allison & Martiny, 2008). If the groups of higher abundance struggle against crude oil toxicity, we would expect that less abundant groups, resistant to crude oil, would carry on the functions recently lost. The abundance of these taxa, that just got space in the competition, will now increase, causing the effect of higher diversity seen in figure 3 A. This effect may be caused by the reduction of populations density of dominant species (figure 3 A), so that the less abundant species start to increase and occupy available niches, so they can better compete. This shifts result in increased diversity.

The combination of oil and BCP to Bare Fallow increased alpha diversity and ATP concentration. We speculate that this was possible thanks to the positive effect of BCP on the microbiota of this soil. BCP contains approximately 29 % of potassium soap (Table 1) and may have enhanced the availability of oil hydrocarbons to Bacterial groups able to degrade these compounds. The protective effect observed for Bare Fallow was not seen for Grassland and did not alter the microbial biomass of Trindade Island.

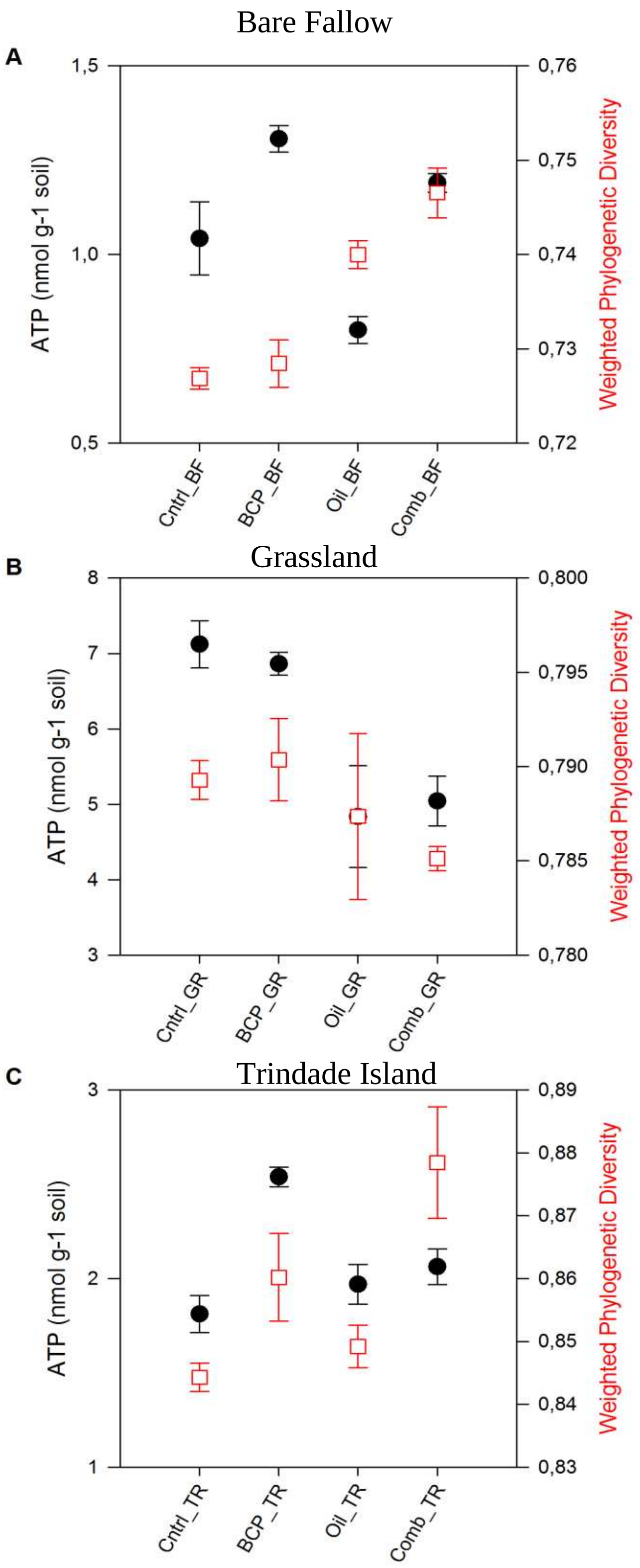

Figure 2 Soil biomass measured through ATP and alpha diversity index microbial balance weighted phylogenetic diversity (BWPD). The graphs represent the biomass (ATP) and alpha diversity (BWPD) responses of the soils (A) Bare Fallow, (B) Grassland and (C) Trindade Island to the treatments Control (Cntrl), Biodiesel Co-product (BCP), Crude oil (Oil) and Combination of Crude (Comb).

Beta Diversity comparison

We used the weighted UniFrac distance metrics (method that calculates phylogenetic distances between samples considering higher weight to more abundant taxa/branch tips) to estimate the beta diversity and the results were presented as a three-axis principal coordinates analysis (PCoA) (figure 4).

The data distribution obtained with Principal Coordinates Analysis (PCoA) (Figure 4) shows a very tight clustering of the samples by type of soil (p-value Benjaming-Hochberg corrected = 1.89 e^{-300}). It can be concluded that soil microbial communities are very divergent between the three areas. The differences between microbial communities of the three areas is significantly higher than the differences of microbial communities at same area exposed to the treatments with oil and BCP. For this reason, the beta diversity analysis was also performed separately within each soil type (Bare Fallow, Grassland and Trindade Island, Fig 5,6 and 7 respectivelly), this approach allowed us to see the separation caused by treatments. Bare Fallow soil showed two clusters separated by the presence of oil (p-value Benjaming-Hochberg corrected = 0.00398) and separated the treatments oil and comb (p-value Benjaming-Hochberg corrected = 5.65^{-6}), but did not show differences between the treatment cntrl and bcp (figure 6). Grassland soils formed two clusters separated by the presence and the absence of crude oil, differences were significative under t-test at 0.05 and p-value Benjaming-Hochberg corrected. The Trindade Island soil showed 4 well resolved clusters (p-value Benjaming-Hochberg corrected = 0.05) representing the 4 treatments (cntrl, BCP, crude oil and comb).

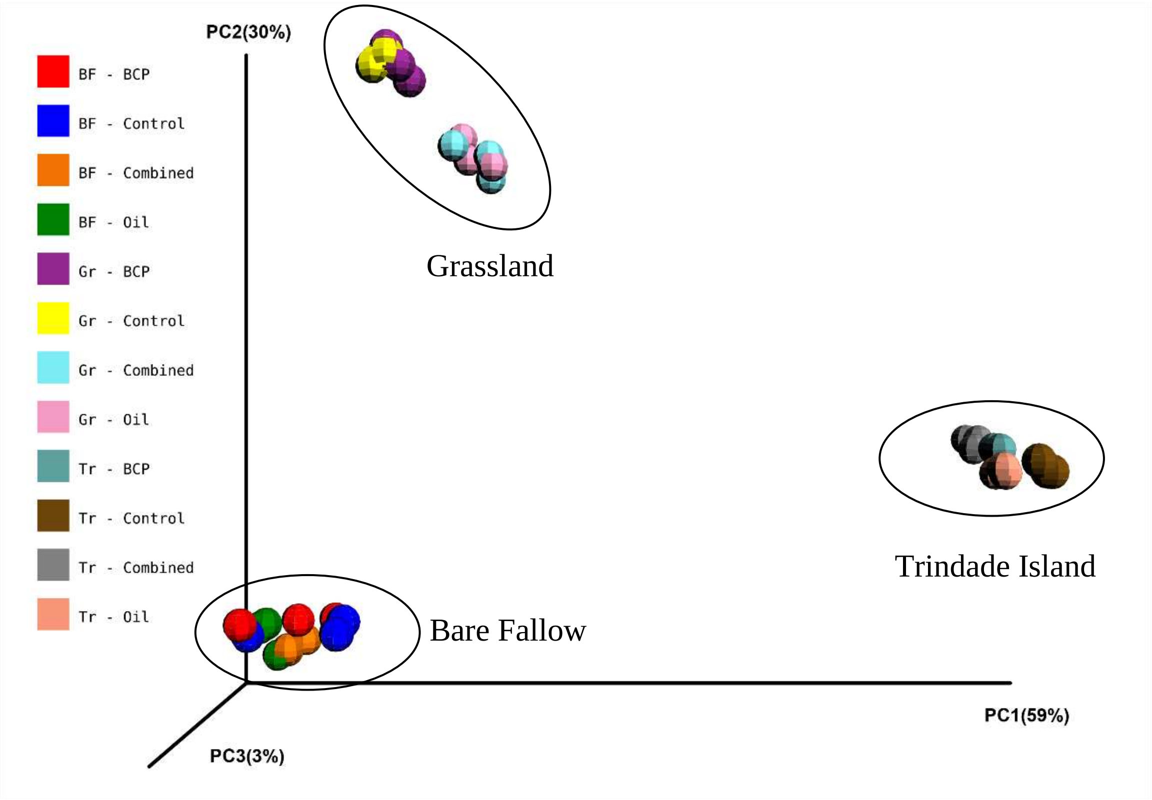

Figure 3 Principal coordinate analysis (PCoA) of weighted unifrac distances generated from taxa tables, summarized at the genus level, exposed to treatments with biodiesel co-product (BCP) and oil.

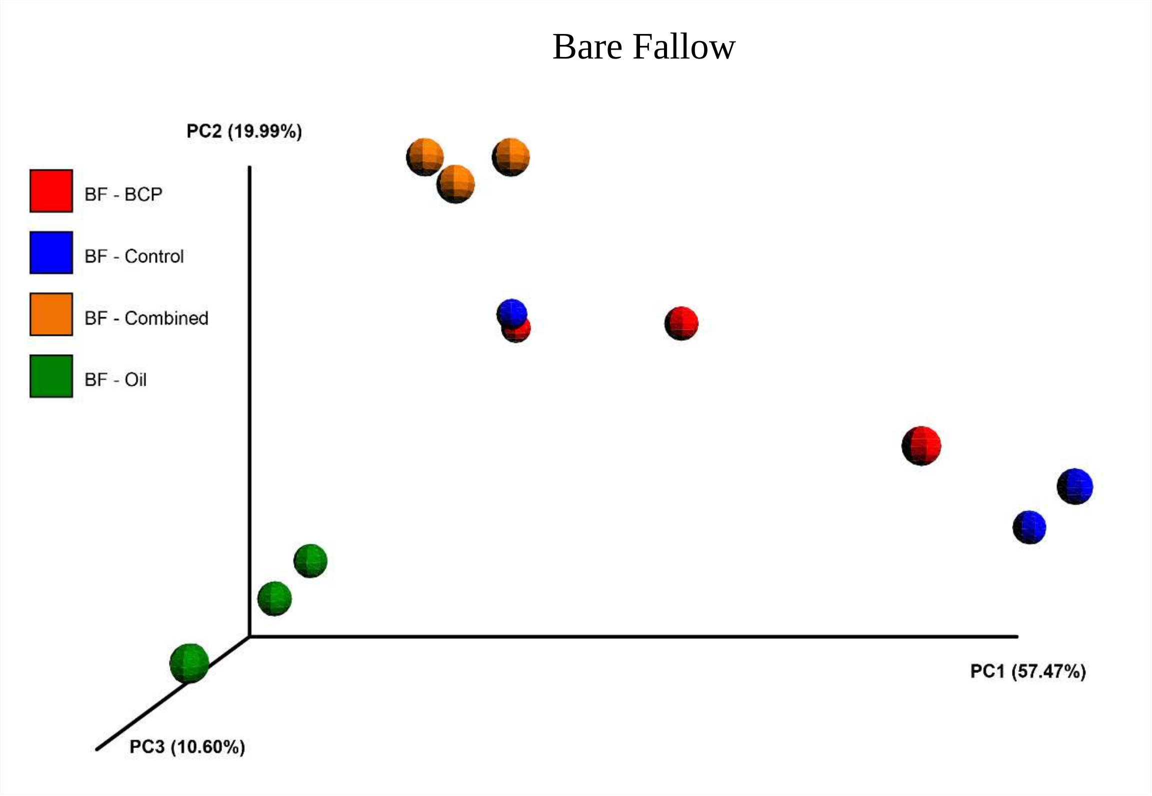

Figure 4 Principal coordinate analysis (PCoA) of weighted unifrac distances generated from Bare Fallow soil’s taxa tables, summarized at the genus level, exposed to treatments with biodiesel co-product (BCP) and oil.

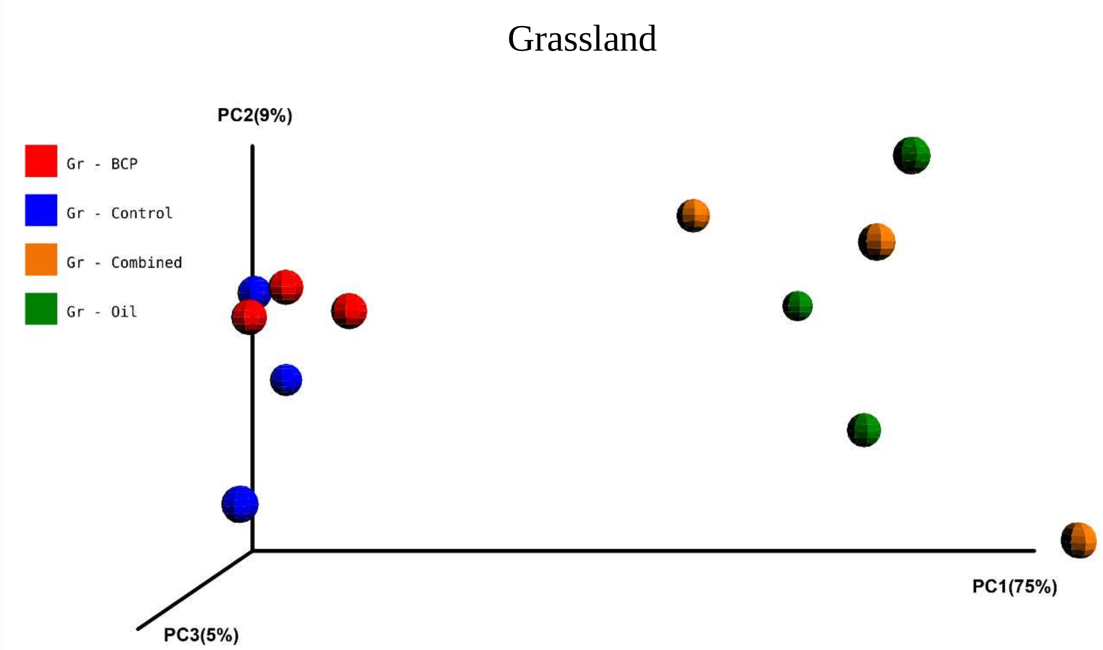

Figure 5 Principal coordinate analysis (PCoA) of weighted unifrac distances generated from Grassland soil’s taxa tables, summarized at the genus level, exposed to treatments with biodiesel co-product (BCP) and oil.

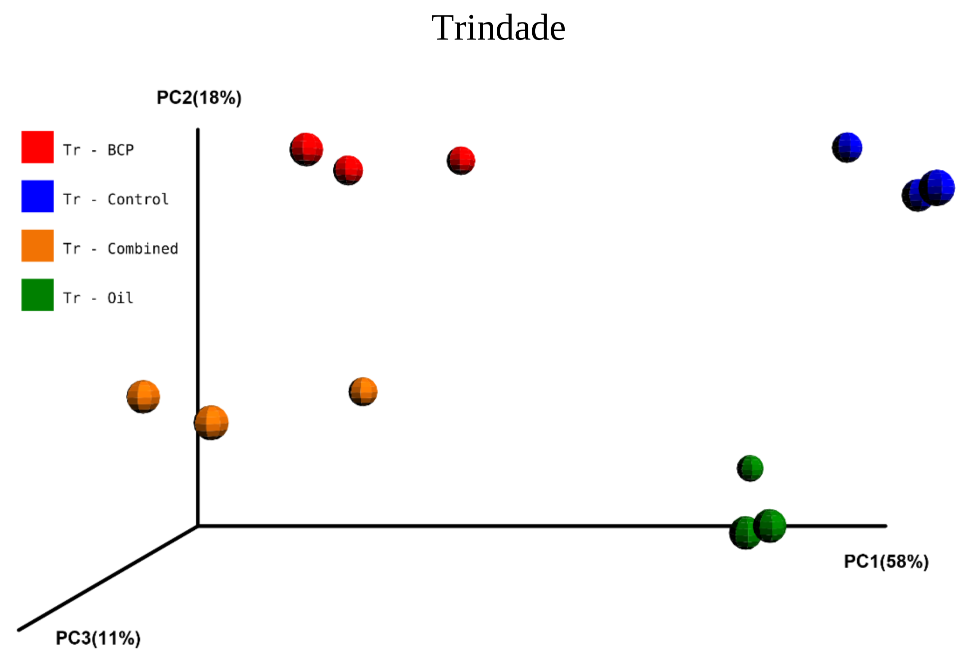

Figure 6 Principal coordinate analysis (PCoA) of weighted unifrac distances generated from Trindade Island soil's taxa tables, summarized at the genus level, exposed to treatments with biodiesel co-product (BCP) and oil.

Taxonomic comparison

The relative abundances of the phyla detected on the work reported here, are presented in table S2. The Bare Fallow control soil show ca. 0.1% of the sequences to be from the Archaea domain, 99.3% from Bacteria domain and 0.2% were not assingned to any taxa from the GreenGenes database (DeSantis et al., 2006). The Grassland control soil show ca. 0.1% of the sequences to be from the Archaea domain, 99.5% from Bacteria domain and 0.4% were not assingned to any taxa from the GreenGenes database. The Trindade Island control soil show ca. 3.1% of the sequences to be from the Archaea domain, 96.7% from Bacteria domain and 0.5% were not assingned to any taxa from the GreenGenes database. We detected 6 Archaeal orders and 288 Bacterial orders in the present study. The most abundant Archaea found in the three soils was the Candidatus nitrophaera. This specie belongs to the phylum Crenarchaetoa and correspond to ca. 90% of the Archaea community detected in this experiment. The Candidatus nitrosphaera presence is related to the ammonia oxidation activity (Hatzenpichler et al., 2008) and the high nitrogen content found at Trindade Island suggest the presence of a niche for this specie. Much attention must be addressed to this organisms considering its high abundance in Trindade soil and the knowledge of Archaea domain being the most abundant ammonia oxidizing prokaryotes in soil (Leninger et al., 2006).

The most abundant phyla in the Control treatment of Bare Fallow were Proteobacteria (31.4%), Acidobacteria (21.5%), Actinobacteria (12.1%), Firmicutes (10.6%), Gemmationadetes (9.9%), Verrucomicrobia (6.4%), Bacteroidetes (2.1%), Chloroflexi (1.6%) and Planctomyces (1.6%). The most abundant phyla in the Control treatment of Grassland were Verrucomicrobia (30.1%), Proteobacteria (26.6%), Acidobacteria (16.2%), Actinobacteria (9.5%), Bacteroidetes (5.2%), Firmicutes (4.6%), Planctomyces (2.7%), Gemmationadetes (1.8%), Chloroflexi (0.9%). The most abundant phyla in the Control treatment of Trindade Island were Actinobacteria (33.7%), Proteobacteria (29.3%), Acidobacteria (13.2%), Gemmationadetes (5.7%), Bacterioidetes (3.1%), Crenarchaeota (3.1%), Chloroflexi (3.1%), Planctomycetes (3.0%), Verrucomicrobia (1.6%) and Firmicutes (1.2%).

To compare the taxonomical differences between treatments we applied the Student-t test with p-values FDR-corrected on absolute abundance tables at the lowest taxonomic level (genera) (Suplementary data), but this method did not detect OTUs with significative abundance differences. Therefore, we assessed the variation of OTU abundances between treatments using the Edge R pipeline (Robinson & Smith, 2010), recommended by McMurdie and Holmes (2013). This approach takes into account that during samples sequencing, using NGS technologies, the total number of reads obtained for each sample is not the same. The R script for running this analysis on R software is available at https://github.com/kdanielmorais/diff_abundance and in table S3.

As we are interested on differences between the treatments in each soil and not in differences between soils, the discussion will be focused on comparisons in pairs of samples, between treatments within each soil (figure 8). Bare Fallow and Grassland soils did not show any OTU differentially abundant significant ($\alpha = 0.05$), when comparing the treatments cntrl and BCP (figure 8A and 8B). This result agrees with the results of beta diversity (dissimilarities between samples), by which it is not possible to separate the samples control and BCP in the phylogenetic distance comparison (figure 5 and figure 6). However, there was a drastic change of OTU abundances in Trindade Island soil when BCP was applied (figure 9C). The changes in OTU abundances observed in the Trindade Island soil means that, although the alpha diversity have rised (figure 3 C) the overall microbial community ended up being higly divergent from its origin (Control treatment), in the same manner, the increase of microbial biomass for Bare Fallow and the absense of differentially abundant OTUs, suggest that BCP did not alter the microbial community of this environment.

Grassland and Bare Fallow soils had high differences of OTU abundances between the treatments Control and Oil and Trindade Island soil had very low differentially abundant OTUs between these two tratmetns. This result is very similar to what was discussed for ATP and alpha diversity analyses (figure 3). Bare Fallow and Grassland showed a strong change of ATP and diversity upon addition of oil, both soils had a reduction of microbial biomass and Bare Fallow increased its microbia diversity, while Trindade Island did not have a significant difference of ATP nor alpha diversity. These observations linked to the effects of BCP on Bare Fallow, reinforce that the addition of BCP may have helped to protect soils contaminated with crude oil, when having characteristics like Bare Fallow soil, low biomass, low nutrient availability and low fertility, but did not show any protective effect on Grassland and Trindade Island soils.

The comparison between the treatments Control and Comb, for Bare Fallow and Grassland, yielded (13 and 63, respectivelly) lower differentially abundant OTUs than the comparison between the treatments Control and Oil for these two soils (23 and 73, respectivelly). Trindade Island showed 282 OTUs differentially abundant between the treatments Control and Comb (figure 8 C), being 169 OTUs more abundant in Control and 113 OTUs more abundant in Comb. The treatments Control and Comb, of Trindade Island, showed the highest number of OTUs differentially abundant, this might be an accumulative effect of BCP and Oil addition. Within the 169 OTUs more abundant in the Control samples, when comparing to Comb samples, we detected the 4 species belonging to the genus Streptomyces, 1 specie belonging to the genus Pimelobacter, 4 species belonging to the genus Bacillus, 4 subspecies beloging to the Archaeal specie Candidatus nitrososphaera. This two aforementined groups (Bacillus and Candidatus nitrososphaera) were detected in higher abundance also in the Comb treatment when compared to the control. This could be explained by the presence of different species or subspecies or could be individuals that lack the molecular apparatus to produce the enzymes necessary to degrade oil hydrocarbons. Molecular experiments have described C. nitrosophaera as presenting a higly dynamic genome, presenting multiple IS elements/transposases and genomic islands (Bose & Grossman, 2011; Spang et al., 2012).

The biggest difference between the treatments of Grassland soil was in the Verrucomicrobia phylum. The genus DA_101 belonging Verrucomicrobia occurred only in soils of Rothamsted (Bare Fallow and Grassland) and is the most abundant taxum in Grassland soil. The treatment Oil caused the biggest impact (73 OTUs) on OTU abundances, comparing to the Control treatment. The phylum Verrucomicrobia had 10 species more abundant in Control treatment, when comparing to Oil treatment (Suplementary Tables). This group is highly selected by roots exudates and its functions are not completely known (da Rocha et al., 2013).

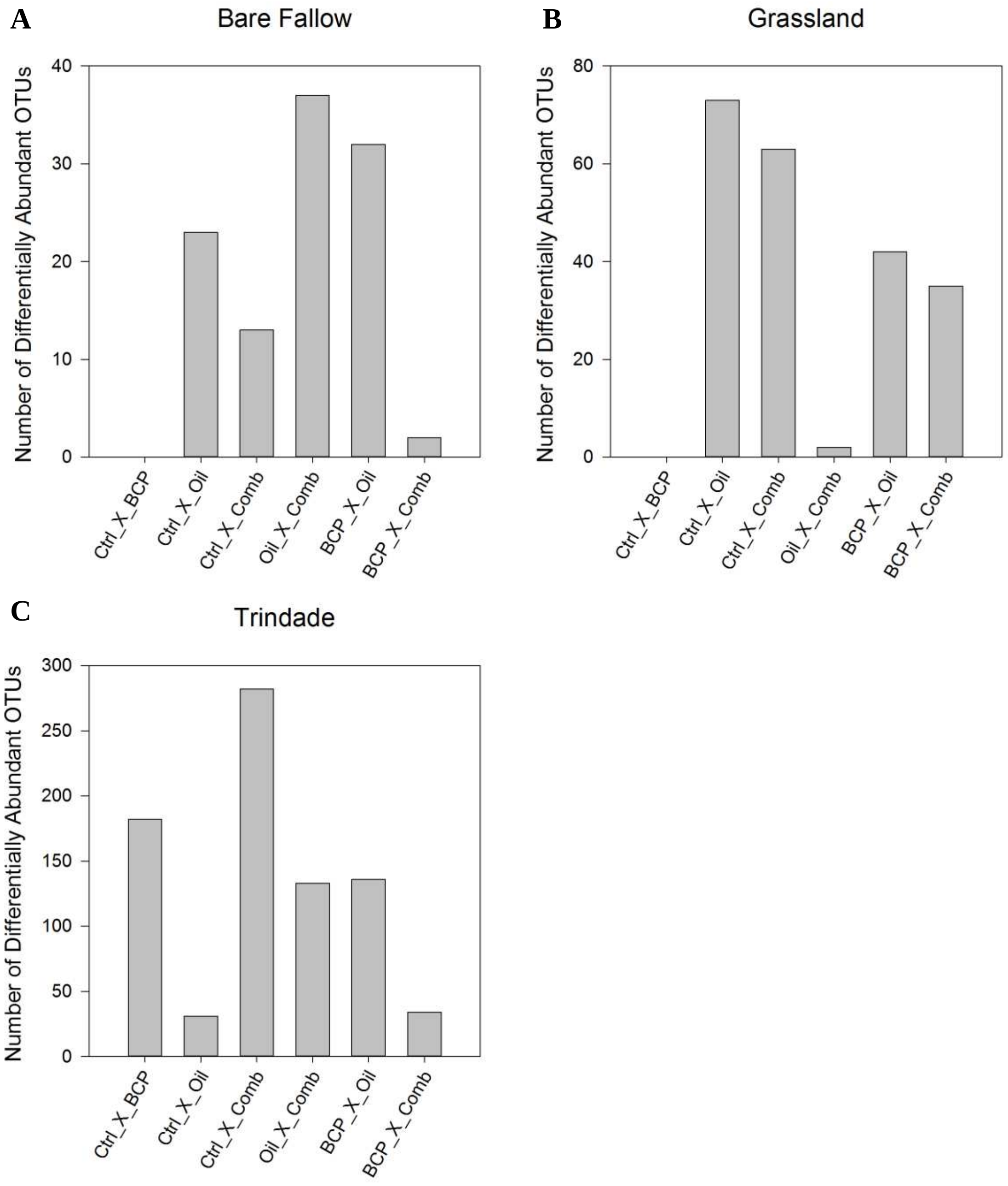

Figure 7 Number of differentially abundant (DA) OTUs between treatments (Cntrl, BCP, Oil and Comb). This bar plot shows the treatments compared two by two, using Edge R “exact test”, after normalization of the OTUs counting table using the RLE method.

CONCLUSIONS AND FUTURE PERSPECTIVES

BCP improved soil biodiversity in a short time scale (17 days) and using a very low dosage, when carbon availability was a limiting factor (Trindade Island). It helped to protect soil biomass from deleterious crude oil effects (Bare Fallow) and improved biomass of Trindade Island soil. Crude oil had no immediate negative effect on Trindade Island biomass or biodiversity and caused the reduction of agricultural soils biomass (Grassland and Bare Fallow). Beta diversity analysis was able to detect changes in the phylogenetic distances between contaminated samples and non-contaminated samples.

It is necessary to evaluate long-term effects of crude oil addition on different types of soil. This study was part of a larger project, which intends to describe immediate changes in microbial functions under the same conditions of this experiment. This experiment was the first comparison between agricultural soils and a tropical pristine soil under crude oil contamination and a bioremediation strategy using a by-product from the biodiesel industry.

The analysis of taxonomic differences using the DLE transformation and the exact test based on the negative-binomial distribution was capable to detect changes in short time soil experiments.

REFERENCES

Allison, S. D., Martiny, J. B. H., 2008. Resistance, resilience, and redundancy in microbial communities. Proceedings of the National Academy of Sciences of the United States of America 105, 11512e11519

Alvarez, V. H. V. R. F. Novais, L. E. Dias and J. A. Oliveira. 2000. Determinação e uso do fósforo remanescente. B. Inf. SBCS 25: 24-32.

Anders, S., Huber, W. 2010. Differential expression analysis for sequence count data Genome Biology 11, R106.

Atlas, R. M. 1981. Microbial Degradation of Petroleum Hydrocarbons: an Environmental Perspective. Microbiological Reviews, p. 180-209

Bokulich, N. A., Subramanian, S., Fait, J. J., Gevers, D., Gordon, J. I., Knight, R., Mills, D. A., Caporaso, J. G., 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat. Methods. 10: 57-59.

Bose, B., Grossman, A. D. 2011. Regulation of horizontal gene transfer in Bacillus subtilis by activation of a conserved site-specific protease.Journal of bacteriology, 193(1), 22-29.

Brady, N. C., Weil, R. R. 2002. The nature and properties of soils.

Campbell, C. D., Chapman, S. J., Cameron, C. M., Davidson, M. S., Potts, J. M. 2003. A rapid microtiter plate method to measure carbon dioxide evolved from carbon substrate amendments so as to determine the physiological profiles of soil microbial communities by using whole soilAppl. Environ. Microbiol., 69 (6), pp. 3593–3599.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S. M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J. A., Smith, G., Knight, R. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME journal, 6(8), 1621-1624.

da Rocha, U. N., Plugge, C. M., George, I., van Elsas, J. D., van Overbeek, L. S. 2013. The rhizosphere selects for particular groups of acidobacteria and verrucomicrobia. PloS one,8(12), e82443.

Di Toro, D. M., McGrath, J. A., Stubblefields, W. A. 2007. Predicting the toxicity of neat and weathered crude oil: toxic potential and the toxicity of saturated mixtures. Environmental Toxicology and Chemistry, Vol. 26, No. 1, pp. 24–36.

Edwards, R. A., Rodriguez-Brito, B., Wegley, L., Haynes, M., Breitbart, M., Peterson, D. M., Saar, M. O., Alexander, S., Alexander, E. C., Rohwer, F. 2006. Using pyrosequencing to shed light on deep mine microbial ecology. BMC Genomics 7: 57.

Hatzenpichler, R., Lebecleva, E. V., Spieck, E., Stoecker, K., Richter, A., Daims, H. 2008. A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring. Proc Natl Acad Sci USA 105: 2134–2139.

Hentati, O., Lachhab, R., Ayadi, M., & Ksibi, M. 2013. Toxicity assessment for petroleum-contaminated soil using terrestrial invertebrates and plant bioassays. Environmental monitoring and assessment. 185(4), 2989-2998.

Hirsch, P. R., Gilliam, L. M., Sohi, S. P., Williams, J. K., Clark, I. M., & Murray, P. J. 2009. Starving the soil of plant inputs for 50 years reduces abundance but not diversity of soil bacterial communities. Soil Biology and Biochemistry, 41(9), 2021-2024.

Incardona, J. P., Gardner, L. D., Linbo, T. L., Brown, T. L., Esbaugh, A. J., Mager, E. M., Tagal, M. (2014). Deepwater Horizon crude oil impacts the developing hearts of large predatory pelagic fish. Proceedings of the National Academy of Sciences, 111(15), E1510-E1518.

Johnston, A. E., Poulton, P. R., & Coleman, K. 2009. Soil organic matter: its importance in sustainable agriculture and carbon dioxide fluxes. In D. L. Sparks (Ed.), Advances in Agronomy, Vol 101, pp. 1–57.

Jorgensen, R. G., Raubuch, M. 2002. Adenylate energy charge of a glucose-treated soil without adding a nitrogen source. Soil Biology & Biochemistry 34, 1317-1324.

Kang, Y. S., Park, W. 2010 Protection against diesel oil toxicity by sodium chloride-induced exopolysaccharides in Acinetobacter sp. strain DR1. Journal of bioscience and bioengineering,109(2), 118-123.

Kochhann, D., Jardim, M. M., Domingos, F. X. V., Val, A. L. 2015. Biochemical and behavioral responses of the Amazonian fish Colossoma macropomum to crude oil: The effect of oil layer on water surface. Ecotoxicology and environmental safety, 111, 32-41.

Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G. W., Schleper, C. 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature, 442(7104), 806-809.

Lozupone C. Knight R. 2005 UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol 71: 8228–8235.

Matsen, F. A. 2015. Phylogenetics and the Human Microbiome. Syst Biol. 64 (1): e26-e41.

McCoy, C. O., & Matsen IV, F. A. 2013. Abundance-weighted phylogenetic diversity measures distinguish microbial community states and are robust to sampling depth. PeerJ, 1, e157.

Mcmurdie, P. J., Holmes, S. 2014 Waste not, want not: why rarefying microbiome data is inadmissible. PLoS computational biology, v. 10, n. 4, p. e1003531.

Olsen, S. R., Cole C. V., Watanabe, F. S., Dean, L. A. 1954. Estimation of available phosphorus in soils by extraction with sodium bicarbonate. U.S. Department of Agriculture Circular 939, USDA, Washington, DC.

Perhar G. Arhonditsis, G. B. 2014. Aquatic ecosystem dynamics following petroleum hydrocarbon perturbations: A review of the current state of knowledge. Journal of Great Lakes Research. 40 Supplement 3 56–72

Poinar, HN; Schwarz, C, Qi, J, Shapiro, B, Macphee, RD, Buigues, B, Tikhonov, A, Huson, D, Tomsho, LP, Auch, A, Rampp, M, Miller, W, and Schuster, SC 2006. Metagenomics to Paleogenomics: Large-Scale Sequencing of Mammoth DNA. Science311 (5759): 392–394.

Pylro, V. S., Roesch, L. F. W., Morais, D. K., Clark, I. M., Hirsch, P. R., & Tótola, M. R. 2014. Data analysis for 16S microbial profiling from different benchtop sequencing platforms. Journal of microbiological methods, 107, 30-37.

Pylro, V. S., Roesch, L. F., Ortega, J. M., Amaral, A. M., Tótola, M. R., Hirsch, P. R. 2014. Brazilian Microbiome Project: Revealing the unexplored microbial diversity - Challenges and prospects. MicrobEcol 67: 237-241.

van der Ploeg, R. R., B¨ohm, W., Kirkham, M. B. 1999. On the origin of the theory of mineral nutrition of plants and the law of the minimum, Soil Science Society of America Journal 63, 1055–1062.

Redmile-Gordon, M., White, R. P., Brookes, P. C., 2011. Evaluation of substitutes for paraquat in soil microbial ATP determinations using the trichloroacetic acid based reagent of Jenkinson and Oades (1979). Soil Biology and Biochemistry 43, 1098-1100.

Redmille-Gordon M., Armenise, E., Hirsch, P. R., Brookes, P. C. 2014. Biodiesel Co-Product (BCP) Decreases Soil Nitrogen (N) Losses to Groundwater. Water Air Soil Pollut. 225:1831.

Robinson, M. D, and Smyth, G. K. 2008. Small-sample estimation of negative binomial dispersion, with applications to SAGE data. Biostatistics, 9, 321-332.

Robinson, M. D., McCarthy, D. J., Smyth, G. K. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics, 26(1), 139-140.

Roesch, L. F., Fulthorpe, R. R., Riva, A., Casella, G., Hadwin, A. K., Kent, A. D., Triplett, E. W. 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. The ISME journal, 1(4), 283-290.

Spang, A., Poehlein, A., Offre, P., Zumbrägel, S., Haider, S., Rychlik, N., Wagner, M. 2012. The genome of the ammonia-oxidizing Candidatus Nitrososphaera gargensis: insights into metabolic versatility and environmental adaptations. Environmental microbiology, 14(12), 3122-3145.

Spitzer, M. W., Rappsilber, J. T. M. 2014. BoxPlotR: a web tool for generation of box plots. Nature Methods. V. 11. P. 121-122.

Torsvik, V., Sorheim, R., Goksoyr, J. 1996. Total bacterial diversity in soil and sediment communities a review. Journal of Industrial Microbiology. 17, 170-17.

Torsvik, V., Øvreås, L., & Thingstad, T. F. 2002. Prokaryotic diversity--magnitude, dynamics, and controlling factors. Science, 296(5570), 1064-1066.

Zobel, C. Action of microorganisms on hydrocarbons. Bacteriol. Rev. 10:1-49. 1946

ADDITIONAL INFORMATION AND DECLARATIONS

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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Supporting data

Supporting data can be accessed through the following link: https://drive.google.com/open?id=0B8Ms461m3C-Bfkt1SXVuTTQtVjhKSkcxWDhqTEVsWGRTcWgwMWNDOF9td2lCUnBqaEVMTmM

The material linked above contains the results of the differential abundance Exact test based on the negative-binomial distribution. The results are organized in 18 tables representing the treatments compared two by two within each soil, as explained in figure 8. The material contains also the raw table of absolute OTU counts from all samples. These files were too big to attach to this document.

Suplementary Table 1
Taxonomic community compositions.

Taxa	**Bare Fallow**				**Grassland**				**Trindade Island**			
	Cntrl	**BCP**	**Oil**	**Comb**	**Cntrl**	**BCP**	**Oil**	**Comb**	**Cntrl**	**BCP**	**Oil**	**Comb**
Unassigned;Other	0.002106	0.00208	0.00252	0.0028149	0.004236	0.00451	0.00357	0.0039242	0.00521	0.00516	0.00474	0.005614
k__Archaea;p__Crenarchaeota	0.001389	0.00172	0.00188	0.0026836	0.00101	0.00081	0.00114	0.0008564	0.031198	0.04722	0.03596	0.055056
k__Archaea;p__Euryarchaeota	6.11E-05	5.05E-06	2.03E-05	0	0	0	0	0	5.48E-05	3.89E-05	2.77E-05	5.90E-05
k__Archaea;p__[Parvarchaeota]	2.22E-05	1.51E-05	2.03E-05	5.14E-05	1.51E-05	3.37E-05	4.30E-05	2.81E-05	0.000349	0.00016	0.00011	0.000246
k__Bacteria;p__	3.33E-05	2.52E-05	3.55E-05	4.57E-05	5.05E-06	1.45E-05	0	2.01E-05	0.00013	3.34E-05	8.30E-05	2.95E-05
k__Bacteria;p__AD3	0.000239	0.00031	0.00013	0.0001542	1.51E-05	0	4.77E-06	2.81E-05	0	0	0	0
k__Bacteria;p__Acidobacteria	0.215306	0.23944	0.21244	0.2015222	0.162939	0.15472	0.13447	0.1347371	0.132641	0.09682	0.13038	0.105913
k__Bacteria;p__Actinobacteria	0.121308	0.11432	0.11316	0.1447895	0.095483	0.10804	0.18783	0.1963138	0.337305	0.32746	0.34786	0.340019
k__Bacteria;p__Armatimonadetes	0.007514	0.00648	0.00709	0.0070973	0.000237	0.00042	0.00036	0.0003377	0.002243	0.00161	0.00198	0.001563
k__Bacteria;p__BHI80-139	0	0	0	0	0	0	0	0	0	0	1.38E-05	0
k__Bacteria;p__BRC1	9.45E-05	3.53E-05	3.05E-05	5.14E-05	2.02E-05	6.75E-05	6.21E-05	6.84E-05	0.000259	0.00017	0.00018	8.36E-05
k__Bacteria;p__Bacteroidetes	0.020668	0.03035	0.0319	0.0348527	0.052946	0.04921	0.04532	0.0421448	0.031313	0.02696	0.02836	0.029266
k__Bacteria;p__Chlamydiae	0.000178	0.00027	0.00022	0.0002627	0.000793	0.0008	0.00084	0.0008001	0.000414	0.00072	0.0003	0.001111
k__Bacteria;p__Chlorobi	7.22E-05	0.00014	0.00012	0.000177	0.000374	0.00033	0.0002	0.000197	0.001346	0.00097	0.0012	0.000649
k__Bacteria;p__Chloroflexi	0.016383	0.01403	0.01103	0.011688	0.009657	0.00921	0.01079	0.0109282	0.030326	0.02363	0.02462	0.02401
k__Bacteria;p__Cyanobacteria	0.002484	0.00319	0.00522	0.0061095	0.000727	0.00056	0.00063	0.0008805	0.000688	0.00109	0.00082	0.00148
k__Bacteria;p__Elusimicrobia	0.00123	0.00152	0.0017	0.002101	0.00219	0.00207	0.00206	0.002251	0.00096	0.00099	0.00119	0.00121

k__Bacteria;p__FBP	0.00018	0.00014	0.00015	0.000194	1.01E-05	2.41E-05	9.55E-06	2.81E-05	0.00017	0.00017	0.00021	0.00018
k__Bacteria;p__FCPU426	5.00E-05	0.0001	1.02E-05	2.28E-05	1.51E-05	0	4.77E-06	5.63E-05	0	0	0	0
k__Bacteria;p__Fibrobacteres	0.000167	0.00013	6.60E-05	4.00E-05	0.000247	0.00025	0.0002	0.000197	0.00016	0.00015	0.0001	0.000147
k__Bacteria;p__Firmicutes	0.106686	0.11416	0.16246	0.1637061	0.04603	0.0353	0.04707	0.0486824	0.012628	0.03091	0.01338	0.030092
k__Bacteria;p__GAL15	0	0	0	0	0	0	0	0	2.49E-05	3.89E-05	0.0001	5.90E-05
k__Bacteria;p__Gemmatimonadetes	0.09945	0.07547	0.05814	0.060038	0.01804	0.0192	0.01968	0.020272	0.05799	0.03975	0.05001	0.03678
k__Bacteria;p__MVP-21	0	0	0	0	5.05E-06	2.41E-05	0	0	2.99E-05	1.67E-05	0	0
k__Bacteria;p__NKB19	1.67E-05	0	0	0	0	0	0	0	4.99E-06	1.11E-05	2.77E-05	9.83E-06
k__Bacteria;p__Nitrospirae	0.003874	0.00421	0.00227	0.0024609	0.003726	0.00331	0.0034	0.0034055	0.011322	0.01019	0.01237	0.009468
k__Bacteria;p__OD1	6.11E-05	6.56E-05	0.0001	0.0001313	5.05E-06	3.86E-05	1.91E-05	4.02E-06	0.000184	0.00014	0.00021	9.34E-05
k__Bacteria;p__OP11	0	1.51E-05	5.08E-06	1.71E-05	1.51E-05	0	4.77E-06	0	9.97E-06	3.34E-05	4.61E-06	1.97E-05
k__Bacteria;p__OP3	3.33E-05	4.54E-05	5.08E-06	4.00E-05	1.51E-05	2.41E-05	9.55E-06	4.02E-05	0.00012	0.00013	0.0001	0.000167
k__Bacteria;p__Planctomycetes	0.015967	0.01284	0.0128	0.0108429	0.026963	0.02663	0.02338	0.0257123	0.030072	0.02584	0.02553	0.026355
k__Bacteria;p__Proteobacteria	0.314935	0.30282	0.30679	0.2843888	0.266141	0.27586	0.2931	0.2789228	0.293952	0.34272	0.30083	0.311555
k__Bacteria;p__SBR1093	0	0	0	0	0	0	0	0	3.49E-05	1.67E-05	1.38E-05	0
k__Bacteria;p__Spirochaetes	5.56E-06	8.08E-05	0	5.71E-06	1.51E-05	2.41E-05	4.77E-06	0	0	0	0	0
k__Bacteria;p__TM6	0.0002	0.00032	0.00044	0.0003597	0.001464	0.00171	0.00137	0.0015037	0.000319	0.00057	0.0004	0.000806
k__Bacteria;p__TM7	0.000217	0.0003	0.00038	0.0004968	0.000136	0.00018	0.00016	0.0002292	0.000479	0.00043	0.00033	0.000369
k__Bacteria;p__Tenericutes	0.000111	4.54E-05	5.08E-06	2.85E-05	0.000202	0.00019	0.00016	0.0001528	0	0	0	0

k__Bacteria;p__Verrucomicrobia	0.064049	0.07099	0.06606	0.0600673	0.301509	0.30187	0.21949	0.2217286	0.016606	0.01478	0.01717	0.01639
k__Bacteria;p__WPS-2	0.003473	0.00338	0.00227	0.0020498	5.05E-06	3.37E-05	2.39E-05	4.02E-06	3.49E-05	5.56E-06	2.77E-05	2.95E-05
k__Bacteria;p__WS2	0	0	0	0	0	0	0	0	4.49E-05	2.78E-05	2.30E-05	1.97E-05
k__Bacteria;p__WS3	0.001412	0.00096	0.00053	0.000708	0.004806	0.00453	0.0046	0.0055445	0.001266	0.00097	0.00118	0.001086
k__Bacteria;p__[Thermi]	1.11E-05	5.05E-06	0	0	0	0	0	0	0.00011	6.67E-05	0.00016	4.92E-05

Average relative abundance of dominant phyla and subphyla as calculated for each of the 4 replicates for each treatments

CONCLUSÕES GERAIS

O petróleo aumentou a atividade microbiana no solo litorâneo da Ilha da Trindade e reduziu a diversidade de arqueias, bactérias e fungos, em uma avaliação de médio prazo, 38 dias. A comunidade fúngica foi mais sensível a contaminação com petróleo em comparação a comunidade bacteriana e após a contaminação com petróleo, os fungos tiveram 2 grupos representando 90% da população total.

O experimento de contaminação de curto prazo, 17 dias, avaliou a comunidade bacteriana do solo litorâneo da Ilha da Trindade e de solos agrícolas (solo nu e solo sob crescimento de gramíneas). Os três solos possuem comunidades microbianas bastante distintas, assim como as características físicas e químicas desses ambientes. Esse experimento não detectou alteração da diversidade bacteriana para o solo da Ilha da Trindade, detectou aumento da diversidade para o solo nu e uma tendência a redução da diversidade para o solo sob o crescimento de gramíneas como resultado da adição de petróleo. Os solos agrícolas tiveram redução na biomassa quando petróleo foi aplicado. No experimento de curto prazo, houve também a avaliação do efeito da adição de BCP. O composto BCP aumentou a diversidade de bactérias no solo litorânea da Ilha da Trindade e apesar de não ter efeito sobre a diversidade dos solos agrícolas (solo nu e sob gramíneas), teve um efeito positivo para a biomassa microbiana do solo de Trindade e do solo agrícola nu. O BCP teve ainda efeito protetor sobre a biomassa do solo nu quando adicionado juntamente com o petróleo e apesar do pequeno efeito sobre a biomassa do solo de Trindade, houve um grande aumento da diversidade.

Os resultados deste trabalho são importantes por salientarem os efeitos nocivos do petróleo sobre a comunidade microbiana dos solos, aumentar a compreensão sobre as mudanças na comunidade microbiana em diferentes tipos de solos e por demonstrar a aplicação de métodos estatísticos mais eficientes para a comparação taxonômica entre ambientes ultra diversos como o solo.