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PETRÓLEO E MEIO AMBIENTE

CAROLINA OLIVEIRA DE SANTANA

AVALIAÇÃO TAXONÔMICA E FUNCIONAL DA COMUNIDADE BACTERIANA NOS SEDIMENTOS DO RIO JULIANA- APA DO PRATIGI, BAHIA, BRASIL

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Tese apresentada ao Programa de Pós Graduação em Geoquímica: Petróleo e Meio Ambiente, da Universidade Federal da Bahia, como requisito para obtenção do grau de Doutora em Geoquímica do Petróleo e Ambiental

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Co-orientadora: Prof. Dra. Taíse Bomfim de Jesus

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Salvador, 30 de Outubro de 2020

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“A verdadeira coragem consiste em dar pequenos passos, todos os dias”

Monja Coen

RESUMO

As comunidades microbianas dos sedimentos são as responsáveis diretas pelos ciclos biogeoquímicos que sustentam os ecossistemas. Portanto, é imperativo estudar variações naturais na diversidade estrutural e funcional dessas comunidades e distingui-las dos efeitos causados pelos impactos ambientais. Esse estudo foi realizado com amostras de sedimento do Rio Juliana, Bahia, que escoa por uma área de proteção ambiental de Mata Atlântica, um bioma antropogenicamente ameaçado cuja área original já sofreu redução de 89%. Neste trabalho, amplicons do gene 16S rRNA são usados para descrever os perfis taxonômicos e prever características funcionais das comunidades procarióticas no ambiente. Sedimentos foram coletados na nascente, no vale e em duas áreas de manguezal, uma considerada natural e a outra localmente impactada por esgoto doméstico. Foram realizados experimentos de laboratório com sedimento de nascente para qualificar o impacto do glifosato (pesticida) e do óleo bruto no ciclo biogeoquímico do nitrogênio. Os resultados revelaram variações na biodiversidade procariótica induzidas por fatores naturais e por interferência antrópica. A biodiversidade natural diminui na direção do curso do rio, da cabeceira para o estuário. Observou-se a prevalência de *Proteobacteria* e *Firmicutes* nos sedimentos naturais, em todas as áreas do rio. Ao longo do curso do rio, observou-se a substituição de filos prevalentes nos ambientes de água doce, como *Bacteroidetes* e *Verrucomicrobia*, por filos prevalentes em sedimentos costeiro, como *Crenarchaeota*. Os potenciais funcionais correlacionaram-se positivamente com a biodiversidade, com exceção da fotossíntese, com tendência inversa. O impacto ambiental (esgoto, glifosato ou óleo) ocasionou mudanças estruturais e funcionais distintas nas comunidades. A presença de esgoto nos sedimentos dos manguezais levou ao aumento da biodiversidade, porém, favorecendo a colonização de táxons exógenos e associados à patogenicidade. Observou-se, também, uma expressiva diminuição de táxons nativos do manguezal natural, como *Firmicutes* e *Planctomycetes*, que contém os organismos responsáveis pelo processo anammox, impactando no ciclo do nitrogênio. A área de mangue também mostra variações estruturais que se correlacionam com as zonas de maré, devido a fatores ambientais como matéria orgânica e salinidade. Os experimentos indicaram que o consumo de nitrato em sedimentos não impactados foi de 0,90 mg/L/min. A adição de óleo e glifosato reduziu essa taxa de consumo em 15% e 50%, respectivamente. Portanto, o impacto causado pelo pesticida foi maior na atividade de transformação do nitrogênio. Após 7 dias de incubação, observou-se um aumento de biodiversidade de procariotos nos sedimentos tratados com glifosato, em comparação com o controle, indicando que a comunidade se beneficiou deste composto. O tratamento com óleo causou uma grande diminuição na biodiversidade procariótica nos sedimentos, indicando um possível grau de toxicidade para a maioria dos organismos. O filo *Firmicutes* demonstrou sensibilidade às mudanças causadas pelo processo de incubação, diminuindo expressivamente em todos os tratamentos após 7 dias, de modo que o grupo prevaleceu apenas em condições não impactadas. Filos *Proteobacteria* e *Acidobacteria*, principalmente a família *Corynebacteriaceae*, dominaram os sedimentos expostos ao óleo, sendo possivelmente os principais grupos hidrocarbonoclásticos nas amostras. Os perfis funcionais dos sedimentos impactados sugerem que a maioria dos metabolismos pode ser mantida devido à redundância funcional destas comunidades. Grande variedade de táxons contribuem para as rotas metabólicas, sugerindo que os sedimentos com maior biodiversidade apresentam também maior resiliência frente a impactos antrópicos.

Palavras-chave: Ambiente pristino, sistema fluvial, mangue, microbioma de sedimentos

ABSTRACT

Microbial communities in sediments are directly responsible for the biogeochemical cycles that sustain ecosystems. Therefore, it is imperative to study natural variations and distinguish them from the effects caused by environmental impacts on the structural and functional diversity of these communities. This study was carried out with sediment samples from the Juliana River, Bahia, which flows through an environmental protection area of the Atlantic Forest. This is an anthropogenically threatened biome whose original area has already been reduced by 89%. In this work, amplicons from the 16S rRNA gene are used to describe the taxonomic profiles and predict the functional characteristics of the prokaryotic communities. Sediment collections were carried out in the source, valley and in two mangrove areas, one natural and the other locally impacted by domestic sewage. Laboratory experiments were carried out with samples of sediments from the source, to qualify the impact of glyphosate (pesticide) and crude oil on the biogeochemical cycle of nitrogen. The results revealed variations in prokaryotic biodiversity either induced by natural and anthropic factors. In natural sediments, biodiversity decreases in the direction of the river flow, from the head to the delta. The prevalence of *Proteobacteria* and *Firmicutes* in natural sediments was observed for all areas of the river. Along the course of the river, the replacement of phyla prevalent in freshwater environments, such as *Bacteroidetes* and *Verrucomicrobia*, by phyla prevalent in coastal sediments, such as *Crenarchaeota* was observed. The functional potentials correlated positively with biodiversity, except for the for photosynthesis, with opposite trend. Environmental impacts (sewage, glyphosate or oil) result in distinct structural and functional changes. The presence of sewage in the mangrove sediments leads to an increase in biodiversity, however, it favors the colonization of exogenous taxa associated with pathogenicity. There is also a significant decrease in some native mangrove taxa, including *Firmicutes* and the *Planctomycetes* group, which are responsible for the anammox process, thus impacting the nitrogen cycle. The mangrove area also shows variations that correlate with the tidal zones and environmental variables such as organic matter and salinity. Experiments indicated that nitrate consumption in non-impacted sediments was 0.90 mg/L/min. The addition of oil and glyphosate reduced this consumption rate by 15% and 50%, respectively. Therefore, the impact caused by the pesticide was greater on the nitrogen transformation activity. After 7 days of incubation, there was an increase in the biodiversity in the sediments treated with glyphosate, compared to the control, indicating that the community benefited from this compound. The oil treatment caused a great decrease in the prokaryotic biodiversity in the sediments, indicating a possible degree of toxicity for most organisms. The *Firmicutes* phylum also showed sensitivity to changes caused by the incubation process, decreasing significantly in all treatments after 7 days, prevailing only in non-impacted conditions. *Proteobacteria* and *Acidobacteria*, mainly the family *Corynebacteriaceae*, dominated the sediments exposed to crude oil, possibly being the main hydrocarbonoclastic groups in the samples. In general, the functional profiles of sediments exposed to disturbances suggest that most metabolisms can be maintained due to the functional redundancy of these communities. A variety of taxa contributed to the different metabolic pathways, suggesting that sediments with greater biodiversity also have greater resilience against anthropic impacts.

Keywords: Pristine environment, river system, mangrove, sediment microbiome

SUMÁRIO

1 INTRODUÇÃO

A geoquímica, enquanto área de estudo, se concentra na investigação da composição química da Terra e processos envolvidos na distribuição dos elementos químicos. Nesse contexto, grande importância tem sido direcionada para os processos responsáveis pela ciclagem destes elementos nos diferentes compartimentos ambientais, pois a forma como se apresentam os ciclos interfere diretamente na forma de apresentação dos elementos de interesse. Ao investigar a ciclagem de elementos químicos na natureza, observa-se a grande relevância da biota associada aos diferentes ambientes nesses processos (KNOSSOW et al., 2015; WILD et al., 2017; WENDT-POTTHOFF et al., 2012; LAMMERS et al., 2017).

Deste modo, a microbiogeoquímica tem por objetivo investigar os processos realizados por microrganismos, que resultam em ciclagem de nutrientes no ambiente e podem ser aplicados para processos como a remediação de impactos ambientais, por exemplo (ABHISHEK et al., 2017; KILANY, 2017; NASEER; ABUALHAIL; XIWU, 2013). Cada compartimento ambiental apresenta diferentes características como consequência das interações entre os organismos e o meio abiótico, resultando em diferentes composições de comunidades microbianas e diferentes padrões de ciclagem de elementos químicos. A ciclagem destes elementos pelos microrganismos ocorre a partir da sua incorporação nos processos metabólicos que resultam em absorção e liberação de diferentes espécies químicas no meio (ASHWORTH et al., 2017; WESTON; DIXON; JOYE, 2006; CHESNOKOVA et al., 2015).

Os microrganismos nos solos e sedimentos são responsáveis por grande parte dos ciclos biogeoquímicos. No entanto, até o momento, pouco se conhece da diversidade microbiana e dos papéis funcionais exercidos pelos diferentes táxons nesse ambiente pois, apenas uma pequena parte destes organismos pode ser cultivada em laboratório (BORNEMANN et al., 2015). Assim, a partir do uso de novas ferramentas tecnológicas, a investigação dos microrganismos presentes nos sedimentos e sua importância nos processos geoquímicos locais e até globais, começa a fornecer respostas mais completas, preenchendo uma lacuna de conhecimento que permaneceu aberta por muito tempo. Nesse contexto, a genômica surge como uma alternativa às dificuldades encontradas no estudo de comunidades microbianas do solo, ao permitir a análise de DNA a partir de amostras ambientais, sem a necessidade de cultivo (APPELS et al., 2015; MARDIS, 2013).

Assim, a caracterização biogeoquímica de um dado ambiente é possível a partir de estudos genômicos em conjunto com estudos cinéticos sobre as taxas de transformação dos

elementos químicos nos diversos compartimentos ambientais (LISA et al., 2015). Dessa forma é possível obter informações acerca do perfil funcional das comunidades estudadas, identificar os grupos taxonômicos responsáveis por esses processos e as taxas a que ocorrem, permitindo uma abordagem mais ampla dos ciclos biogeoquímicos no ambiente.

O Rio Juliana está inserido inteiramente dentro da Área de Proteção Ambiental do Pratigi, uma área de remanescente de floresta Atlântica na região Baixo Sul do estado da Bahia. Apesar de sua importância para a biodiversidade, a mata Atlântica é considerada como um dos biomas mais ameaçados pelas interferências humanas no planeta, de modo que pouco ainda resta da sua conformação original, tornando urgente a necessidade de estudar as áreas ainda preservadas (SILVA e NOLASCO, 2015; DITT et al., 2013; MMA, 2004; BRASIL, 2010).

Este estudo apresenta uma caracterização em larga escala da estrutura e função das comunidades procarióticas dos sedimentos superficiais ao longo de toda a extensão do Rio Juliana (APA do Pratigi-BA). Esta área é considerada uma área modelo para caracterização de microbiomas devido ao baixo grau de antropização observado, considerando-se que quase a totalidade do bioma Mata Atlântica encontra-se fortemente impactada. Ao longo da bacia do rio Juliana, o único local em que se observa claros sinais de interferência humana é nas redondezas da cidade de Ituberá, com introdução de esgotos domésticos em uma pequena área do manguezal. Assim, a caracterização foi realizada com foco para as possíveis consequências da interferência humana nesta área protegida, de modo que estes dados poderão ser utilizados para o monitoramento ambiental da área e identificação de potenciais efeitos da degradação ambiental sobre os ciclos da natureza.

No primeiro artigo, apresenta-se os perfis estruturais e funcionais das comunidades procarióticas dos sedimentos de três regiões em bom estado de conservação (nascentes, vale e estuário) do Rio Juliana (APA do Pratigi-BA), revelando as influências das variáveis ambientais sobre a estrutura e função destas comunidades e como se diferenciam entre os três compartimentos.

O segundo artigo apresenta os impactos causados pela simulação de eventos de contaminação por pesticida (glifosato) e óleo sobre as taxas de transformação do elemento nitrogênio e a comunidade microbiana nos sedimentos da região de nascente. As variações taxonômicas e funcionais na comunidade, induzidas pela adição em laboratório de contaminantes, nos sedimentos após 7 dias também foram caracterizadas.

O terceiro artigo apresenta o estudo da ecologia de comunidades procarióticas dos sedimentos de manguezal coletados em uma área urbanizada e uma área distante em que não

se verificou a interferência antrópica. As diferenças na estrutura e potenciais funcionais bem como os principais fatores abióticos influenciando os padrões observados foram caracterizadas.

No quarto artigo, apresenta-se um estudo focado nas variações entre as comunidades procarióticas dentro do ecossistema de manguezal, de acordo com a influência das marés nos sedimentos dessa área. Para tal caracterização foi escolhida uma região de manguezal preservada e distante de possíveis interferências antrópicas.

2 OBJETIVOS

O objetivo geral de pesquisa é realizar a caracterização taxonômica e funcional da comunidade procariótica dos sedimentos de nascente, vale e estuário ao longo do Rio Juliana e determinação das taxas de transformação do nitrogênio no sistema em condições naturais e sob efeito de pressões ambientais simuladas.

São objetivos específicos:

- caracterizar as comunidades procarióticas presentes nos sedimentos do Rio Juliana coletados ao longo do seu curso (nascente, vale e estuário), bem como os potenciais de ciclagem dos elementos C, S, P e N e os fatores abióticos mais relevantes.
- identificar efeitos imediatos da adição de pesticida e óleo, dois contaminantes comuns, sobre a capacidade de remoção do nitrato nos sedimentos naturais da nascente e observar os efeitos na estrutura das comunidades procarióticas após um período de 7 dias após a exposição aos contaminantes.
- avaliar os impactos ocasionados pela constante adição de efluentes domésticos sobre as comunidades procarióticas nos sedimentos de manguezal, a partir da comparação entre uma área próxima ao centro urbano de Ituberá, e uma região distante e isolada de aglomerações humanas, considerada pristina, dentro do estuário do Serinhaém.
- descrever efeitos das variações físico-químicas influenciadas pelo regime hídrico de marés sobre as comunidades presentes nas três zonas de maré (submersa, intermaré e seca) em uma região de manguezal pristino no estuário do Serinhaém.

3 MATERIAIS E MÉTODOS

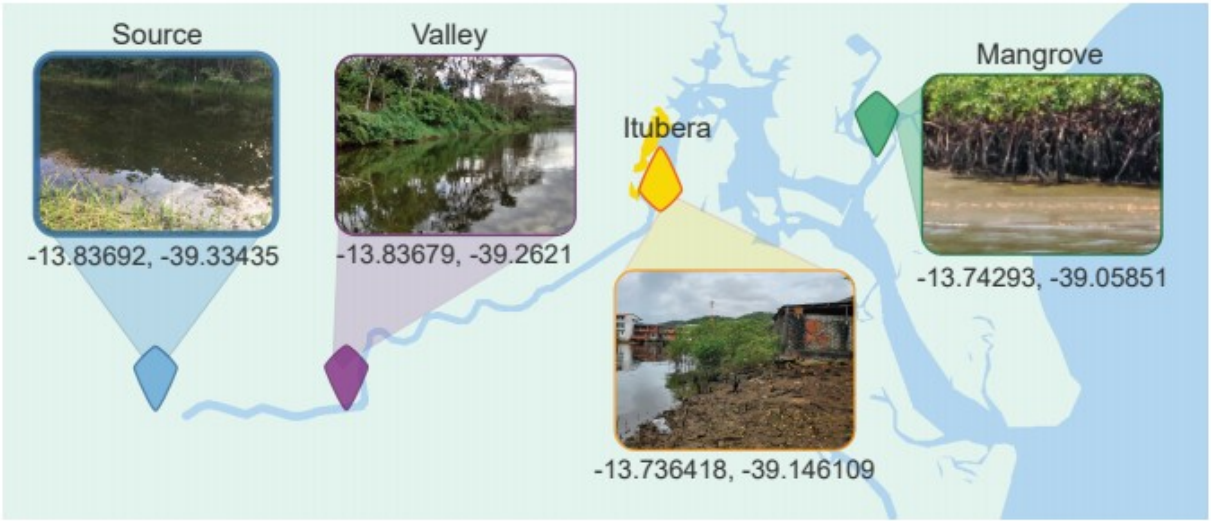
A metodologia geral do trabalho será apresentada a seguir, e as metodologias mais específicas são apresentadas em detalhe nos artigos. Figuras suplementares se encontram na seção Apêndices, ao final do documento.

3.1 ÁREA DE ESTUDO

A APA do Pratigi (Figura 3.1) está compreendida entre as coordenadas 13°35'N e 14°10'S, e 39°40'W e 38°50'E, totalizando uma área de 472.455 Km^2, que abarca cinco municípios no Baixo Sul da Bahia, limitando-se ao norte com a sub-região Recôncavo, e ao sul com a sub-região Extremo Sul (RIBEIRO et al., 2019).

A APA está dividida em Ecopólos, baseados na seguinte configuração socioespacial: Ecopólo 01: Serra do Papuã; Ecopólo 02: Vale do Juliana; Ecopólo 03: Litorâneo, onde se localiza o estuário do Serinhaém, entre os municípios de Igrapiúna e Ituberá (DA SILVA SANTOS; NOLASCO, 2017). A área de estudo apresenta como principal sub-bacia a do Rio Juliana, situado na porção centro-leste, apresentando como a maior drenagem o Rio Juliana, que nasce na Serra de Papuã e na Serra de Santa Rita e deságua no canal do Serinhaém e portanto está totalmente inserido na área protegida (BRASIL, 2004).

Figura 3.1- Localização e imagens dos pontos de coleta ao longo da Bacia do Juliana



Fonte: Santana, 2020

3.2 COLETA DE SEDIMENTOS E ÁGUA PARA SEQUENCIAMENTO E EXPERIMENTO

Para a realização das análises genômicas e experimento com microcosmos, foram coletados sedimentos e água do Rio Juliana. As amostras de sedimentos superficiais foram coletadas em 3 compartimentos (nascente, vale e manguezal) e 4 pontos de coletas no Rio Juliana em julho de 2018 e em fevereiro de 2019.

Parâmetros físico-químicos como temperatura, salinidade e oxigênio dissolvido na coluna d'água foram medidos usando um sistema de monitoramento multiparâmetros (YSI modelo 85, Columbus). Cada local de coletas apresentou diferentes características e densidades de vegetação, conforme se observa na imagem 1. As concentrações de metais não foram analisadas, considerando que estes dados foram previamente documentados por nosso grupo de estudos, e foi constatado que as concentrações encontradas refletem as características naturais da região.

Após a coleta, as amostras foram transferidas para o laboratório e os sedimentos preparados para extração de DNA e avaliação do conteúdo de matéria orgânica. Uma parcela dos sedimentos de nascente foram utilizados juntamente com a água coletada no local para a confecção dos microcosmos para experimento cinético de consumo do nitrato. O DNA genômico total foi extraído de 0,25 g de sedimento usando o kit comercial PowerSoil DNA Isolation (Qiagen, Carlsbad, CA, EUA), seguindo as orientações do fabricante. Todas as amostras de DNA foram armazenadas a -80 ºC antes da preparação da biblioteca e sequenciamento.

3.3 SEQUENCIAMENTO GENÔMICO

Após a extração do DNA, foi realizado o PCR para amplificar especificamente a região V4 do gene 16S rRNA procarioto, usando o par de primers 515F-Y (PARADA; NEEDHAM; FUHRMAN, 2016) e 806R-XT (CAPORASO et al., 2011). O sequenciamento do DNA presente nas amostras de sedimento foi realizado utilizando a plataforma Illumina MiSeq, kit V2 (300 ciclos). Após a demultiplexação, os dados foram armazenados na plataforma BaseSpace para subsequente análise bioinformática.

3.4 EXPERIMENTO CINÉTICO: TRANSFORMAÇÃO DO NITROGÊNIO EM MICROCOSMOS

Foram realizados experimentos em microcosmos contendo sedimentos e água da região de nascente do Rio Juliana para a avaliação dos efeitos de contaminantes (glifosato, uréia e óleo) na cinética de transformação do nitrogênio nestes sedimentos. Após a adição de reagentes e contaminantes, amostras de água foram coletadas para análise das concentrações de NH_4^+ e NO_3^- em 6 tempos diferentes. Para os controles positivos, microcosmos com sedimentos frescos não manipulados foram analisados em paralelo com os microcosmos de tratamento e para o controle negativo, microcosmos com sedimentos autoclavados foram analisados, a fim de certificar que as alterações observadas nos compostos nitrogenados são produtos da atividade microbiana. As concentrações das concentrações de NH4 + e NO3- nas amostras de água foram posteriormente analisadas por Cromatografia Gasosa (CG).

3.5 TRATAMENTO DE DADOS

Os dados genômicos foram analisados através de ferramentas de bioinformática. Resumidamente, as sequências foram submetidas a filtros de qualidade e eliminação de adaptadores com o uso da ferramenta Trimmomatic. Os passos posteriores foram realizados com o uso de ferramentas de análises de comunidades microbianas contidos no programa QIIME2: eliminação de ruídos e sequências quiméricas, agrupamento de sequências em unidades taxonômicas operacionais (OTUs), testes de diversidade intra e inter-locais, classificação taxonômica a partir da base de dados SILVA, a 97% de similaridade, e reconstrução filogenética das sequências.

Os arquivos com dados gerados a partir das análises pelo QIIME2 foram utilizados para posteriores análises no software R para visualização gráfica de árvores filogenética e índices de diversidade bem como realização de testes estatísticos da correlação entre as comunidades procarióticas e as variáveis ambientais observadas e criação de gráficos de distância taxonômica e filogenética das amostras.

A diversidade biológica e estrutural dos sedimentos foi calculada no QIIME2, sendo separadas em alfa e beta-diversidade. Alfa-diversidade é uma medida da biodiversidade dentro de um grupo de amostras; quanto mais elevado o índice, mais diversas são as espécies nas replicatas desse grupo. Beta-diversidade é uma medida da variação observada entre

amostras de grupos distintos, permitindo uma comparação estrutural das comunidades de diferentes áreas ou tratamentos.

A análise funcional foi realizada com o software PICRUSt2 com configurações padrão, a partir dos dados de abundâncias obtidas pelo pipeline QIIME2, de acordo com as informações presentes na plataforma KEGG. Abundâncias diferenciais significativas foram calculadas no software R. As taxas de consumo do nitrato em microcosmos foram calculadas a partir da plotagem do gráfico de dispersão e cálculo de regressão linear no EXCEL.

4 VARIATION IN PROKARYOTIC POPULATION STRUCTURES AND FUNCTIONAL PROFILES ALONG A PRISTINE RIVER SYSTEM IN THE BRAZILIAN ATLANTIC FOREST

4.1 ABSTRACT

Sediment microbiomes play important roles in the health of large-scale biomes as microbial activities drive many biogeochemical cycles. However, given the variability of biotic and abiotic environmental factors across biomes, no single microbiome can represent a biome, and understanding the diversity of microbiomes within larger biomes becomes then imperative. The Atlantic Forest is an anthropogenically threatened biome which includes tropical forest watersheds that feed into coastal mangrove forests. While areas of the Atlantic Forest are today protected by governmental conservation directives, it is estimated that 89% of the original area have been lost. Despite conservation plans such a large area is always under threat. This adds urgency to unveil the ecological variables driving the prokaryotic communities in such an environment. In this work, we use 16S rRNA metagenomic amplicons to describe the taxonomic and functional profiles of the prokaryotic communities present in the Juliana River sediments at the head or source, in the valley, and in the mangrove area, as it meets the sea. This river runs internally through an environmentally protected area. The results show that the prokaryotic communities are made of distinct populations and microbial biodiversity decreases from the head to the delta area. The mangrove shows the most distinct community between all sites. Environmental variables such as temperature, pH, salinity, organic matter, Pb and Zn correlated either positively or negatively with the variations in microbial biodiversity indexes. The metabolic potentials unveiled by metagenomics followed the same trend observed with microbial biodiversity. The highest metabolic abundance at the sediment of the river head (source) and lowest in the mangrove. A variety of bacterial families, some of which are unique to each site, make distinct contributions to the biogeochemical metabolisms. Therefore, each site's biogeochemical cycle is driven by distinct prokaryotic communities.

Keywords: Sediment microbiome, pristine river, mangrove, prokaryotic community

4.2 INTRODUCTION

Microorganisms in soils and sediments are important for most biogeochemical cycles and we have only begun to understand the scope of microbial diversity and the functional roles that they play, with the advent of Genomics (KAUR et al., 2015; MOCALI; BENEDETTI, 2010; BORNEMANN et al., 2015). This includes understanding how the microbial diversity and functional nutrient cycling may naturally vary across interconnected diversified ecosystems; particularly those that can extend through long hydrographic basins (HU et al., 2012; HUDON et al., 2017; LIU et al., 2012).

One of the most biologically diverse biomes worldwide is the Atlantic Forest, with unique local ecologies, centered around the numerous watersheds that feed into the Atlantic coast. However, it is also one of the most threatened biomes (MDDA, 2010). In Brazilian territory, the original cover of Atlantic Forest has been drastically reduced to only 11% of it's pre-Columbian size, with the majority of loss due to human activities (SILVA; NOLASCO, 2015). In the southern part of Bahia State, Brazil, one of the largest remaining fragments of the Atlantic Forest is preserved within the limits of an Environmental Protection Area (APA) named APA do Pratigi (MMA, MINISTÉRIO DO MEIO AMBIENTE, 2004). Since its creation in 1998, research on the environmental conditions of the APA have shown that the preservation efforts have been effective, maintaining at a high environmental quality (DITT et al., 2013; LOPES, 2011; MASCARENHAS et al., 2019).

Among the most important watersheds in this region is the Juliana River basin, located entirely within the protected area. This basin comprises an area of 29.975ha, with the linear course of the Juliana River running 47km long from the sources in the mountains of Papuã to the mouth of the Serinhaém estuary (MASCARENHAS et al., 2019; DITT et al., 2013). Previous studies have shown the watershed to have high environmental quality, suggesting that this basin is a great area for research in ecological, hydrological and biochemical fields, as it allows for the observation of a pristine watershed. Thus, this preserved area is a valuable and unique study object that can increase our understanding of the prokaryotic communities ecology that colonises the natural watershed in the Atlantic forest. By understanding the key factors that drive the dynamics of the distribution and environmental services played by these microbes we can acquire a better notion of how land use and occupation can impact riverine systems.

This research explores the use of 16S rRNA amplicon in order to identify the microbial diversity present in the Juliana River sediments sampled from three distinct areas (river source or head, valley and the mangrove found in the delta). In addition, the genomic analysis allowed the construction of the metabolic potentials harbored by such a microbial population, in particular regarding to autotrophic and heterotrophic use of carbon (C), along with nitrogen (N), phosphorus (P) and sulfur (S).

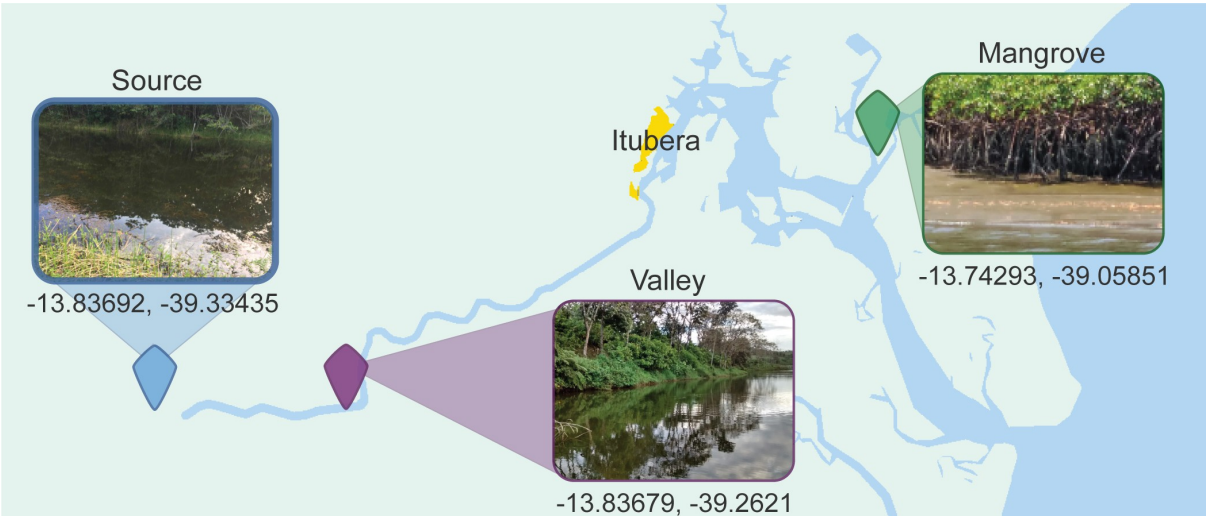
4.3 MATERIALS AND METHODS

The following topics will detail the methodology used for the present study.

4.3.1 Study area

The Juliana River Basin (Figure 4.1) within the Environmental Protection Area of Pratigi, in the southeastern part of Bahia State, Brazil. The river basin is subdivided into three administrative sections, I, II and III. Section I is the highlands of the mountains of Papuã, and are the source of the rivers in this study. Section II is the downstream hydrographic basin of the river and is largely dominated by the Atlantic Forest with some agroforestry systems. Section III is the lowest course of the hydrographic basin consisting of Quaternary sedimentary lands, hosting ecosystems ranging from tropical forest fragments to restingas, mangroves, and estuaries.

Figure 4.1- Map and schematic of sediment sampling sites.



Fonte: Santana, 2020

4.3.2 Sampling and genomics analyses

Sediment samples were collected from three different regions of the Juliana River watershed (river source, valley, and mangrove estuary). From each sample site, 3 collection points were chosen, resulting in 3 composite samples of superficial sediments (top 10 cm of the surface layer) collected with a steel cylindrical sediment core sampler, with precautions taken to avoid the disruption of rhizospheres associated with vegetation. Plant matter and other organic material were manually removed from core samples.

Physical-chemical parameters such as temperature, pH, conductivity and dissolved oxygen in the water column of each sample of the sites were measured using a multiparameter

monitoring system (YSI model 85, Columbus). Additional environmental variables such as Pb, Zn, Cd and organic matter were previously reported (PEREIRA, 2016; MASCARENHAS et al., 2019). After collection, samples were transferred to the laboratory for processing. For each sediment core an aliquot was separated and kept in the -20°C freezer for subsequent DNA extraction while the remainder of the sample was used to measure organic matter content. DNA was extracted at the Microbiology research laboratory (LAPEM) at the State University of Feira de Santana (UEFS), Bahia, Brazil. The total genomic DNA from 0.25 g of sediment using the PowerSoil DNA Isolation Kit (Qiagen, Carlsbad, CA, USA) and stored at -80 ºC before analysis.

For Mangrove samples after DNA extraction, we used PCR amplification of the V4 region of the bacterial 16S rRNA using the primer pairs 515F-Y (PARADA; NEEDHAM; FUHRMAN, 2016) and 806R-XT (CAPORASO et al., 2011). Followed by Illumina MiSeq paired-end sequencing (2x150) (GREGORY CAPORASO et al., 2012). For samples from the Source and Valley sites, after DNA was extracted samples were sent on dry ice to Novogene Bioinformatics Technology Co. Ltd. for amplification of bacterial 16S using 515F and 806R primers, followed by Illumina NovaSeq 6000 paired-end (2x250) sequencing (THOMPSON et al., 2017).

4.3.3 Data analysis

Trimmomatic (BOLGER; LOHSE; USADEL, 2014) was used to filter and trim demultiplexed sequences. For the sample from the Mangrove area, we used QIIME (CAPORASO et al., 2010) to join forward and reverse reads into single reads (join_paired_ends.py, -j 4 -p 1), which resulted in the reads of approximately 250 bp in length that were used for denoising. The denoising step was performed using DADA2 (CALLAHAN et al., 2016) in QIIME2 (BOLYEN et al., 2019), (q2cli, version 2019.4.0).Reads from Source and Valley sites were also denoised using DADA2 and then merged with the denoised sequences from the Mangrove site for further analysis.

Denoised sequences were then clustered into Operational Taxonomic Units (OTUs). Alpha-rarefaction was calculated using QIIME2. The alpha-diversity and beta-diversity tests were performed using QIIME2. The taxonomic assignment step was performed with the Vsearch (ROGNES et al., 2016) tool in QIIME2 using the Open Reference method with a

97% similarity against the reference 16S rRNA sequences in the SILVA database (Silva SSU 132) (MCDONALD et al., 2012).

Phylogenetic reconstruction was performed in QIIME2 resulting in alignment and phylogenetic tree files. Taxonomic assignment was carried out in QIIME2 using the 97% similarity representative set containing only 16S rRNA sequences. In order to remove taxa with very low frequencies from correlation analysis, all groups were required to be present within at least 2 samples with a minimum of 3 reads each. The QIIME2 zip files generated by the pipeline were exported and processed in R, with the QIIME2R package (version 0.99.12).

Correlations between taxonomic community structure and environmental variables were tested using the Vegan package (DIXON, 2003) (version 2.5-6) in R. Functional potential analysis was performed using PICRUSt2 (version 2.3.0-b) (DOUGLAS et al., 2019; BARBERA et al., 2019; CZECH; BARBERA; STAMATAKIS, 2020; LOUCA; DOEBELI, 2018; YE; DOAK, 2009) with default settings, using the observed OTU abundances generated by QIIME2. KEGG Orthologs (KOs) were subsequently analyzed for significant (p-value <= 0.05) differential abundances and were subsequently used in the construction of a heatmap of KOs.

In order to identify which species were significantly different in abundance in each sampling area we carried out a taxa enrichment analysis using the results of QIIME2. We also required the effect size to be in excess of a 5% difference in abundance between sites.

To determine which taxa were driving the differences in functional abundance between sites we also calculated taxa specific pathway enrichment using the custom sigilo.py script, relying on the relative functional abundance results of PICRUSt2. Furthermore, we require that a taxa at a single site must be significantly enriched relative to both other sites and that the taxa contributes at least 5% of the total relative functional abundance in at least one site.

The entire computational workflow is available as a repository in github: https://github.com/pspealman/project_oxun.

The source sediment and valley sediment sequencing data is available from NCBI BioProject PRJNA650560, while the mangrove sediment data is available from NCBI BioProject as accession number PRJNA608697.

4.4 RESULTS

The results are synthesized by categories as follows.

4.4.1 Taxonomy

After quality filtering of the sample set, a total of 606,159 sequences were obtained and assigned to 5,842 Operational Taxonomic Units (OTUs). From those, 5,425 OTUs and 570,389 sequences were assigned to the Bacterial kingdom, while 277 OTUs and 22,965 sequences were assigned to the Archaeal kingdom and 140 OTUs (3,805 sequences) were not assigned to prokaryotic kingdoms. In total, the sequences were assigned to 61 phyla, 149 classes, 300 orders, 464 families, 643 genera and 524 species. The unassigned and uncultured taxa are considerably more abundant at the genus and species levels. Therefore, the best resolution was obtained for the family level. Families that account for >= 1% of the total observed families and their relative abundance in each collection site are shown in Figure 4.2.

4.4.2 Prokaryotic diversity within sites

Diversity indices of the sediments from the source, valley and mangrove regions of the Juliana Basin River were analyzed and are shown in Figure 4.3. We observed that the diversity is higher in the sediments of the source area, followed by the intermediary valley area, and lower in the mangrove region, indicating a decrease in diversity along the course of the river.

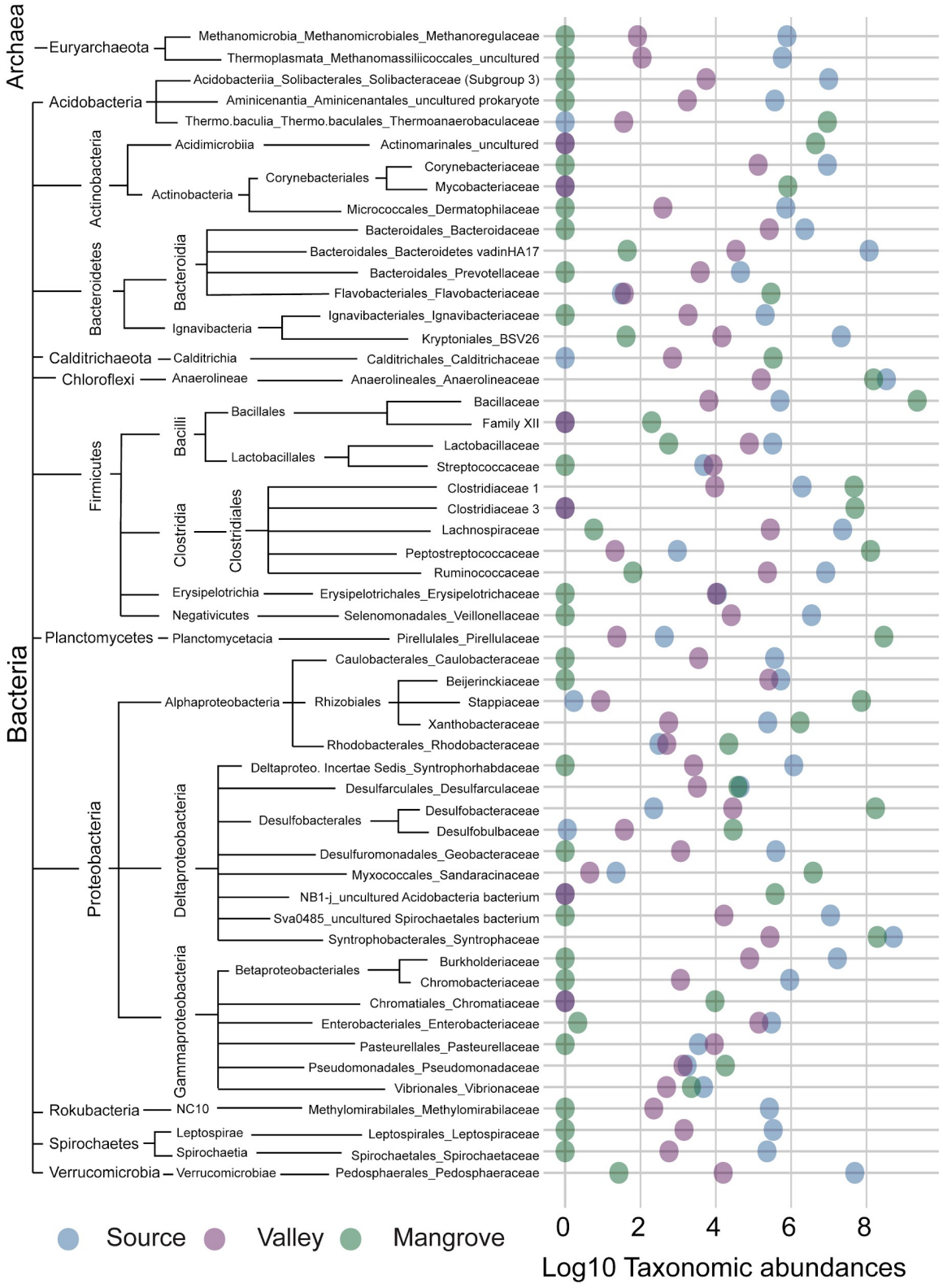
The Kruskal-Wallis statistical test (Figure 3) on Shannon's index values identified statistically significant differences between the sediments of each area (p-value <= 0.05), confirming that the regions of the Juliana River differ statistically in terms of diversity. In the paired comparison it was possible to observe that the studied areas also have significantly relevant differences between them (p-value < 0.1, α = 90% of significance).

4.4.3 Differences in community structures between sites

We compared prokaryotic communities structures from distinct areas of the Juliana river through different distance measurements and found that they present statistically significant differences (p <0.01 in all tests performed). As expected, due to the limited number of samples from each site, pairwise evaluations suggested differences between sites

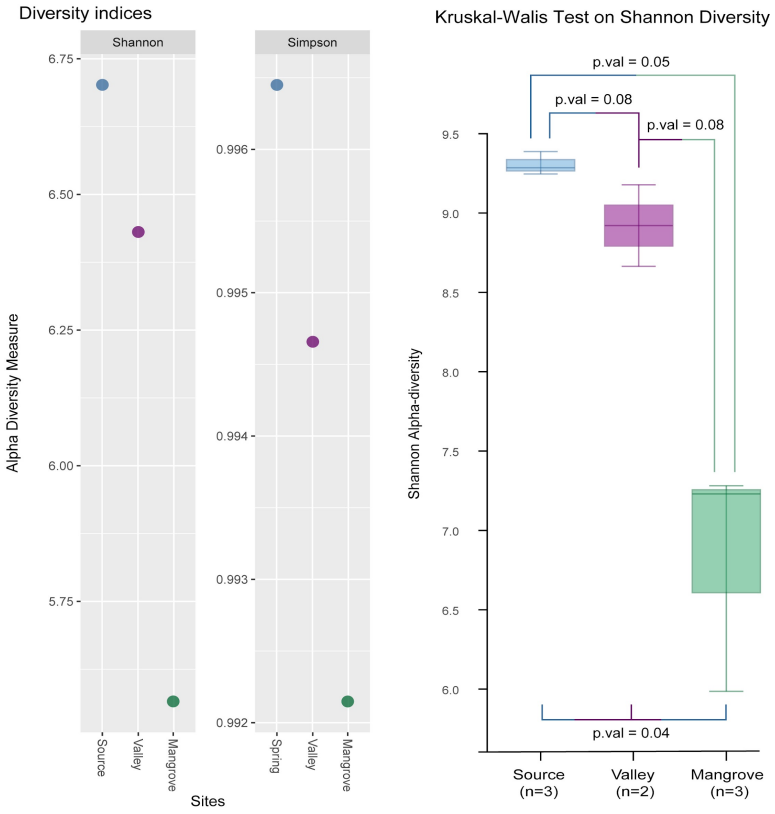
but had p-values in excess of 0.05. These differences are explicitly shown in the distance plot in which the samples for each site group closer to each other (Figure 4.4).

Figure 4.2- Taxonomic distribution of sequence depth normalized abundance between collections sites at the level of Family. All families shown contribute at least 1% of taxonomic abundances at the level of family



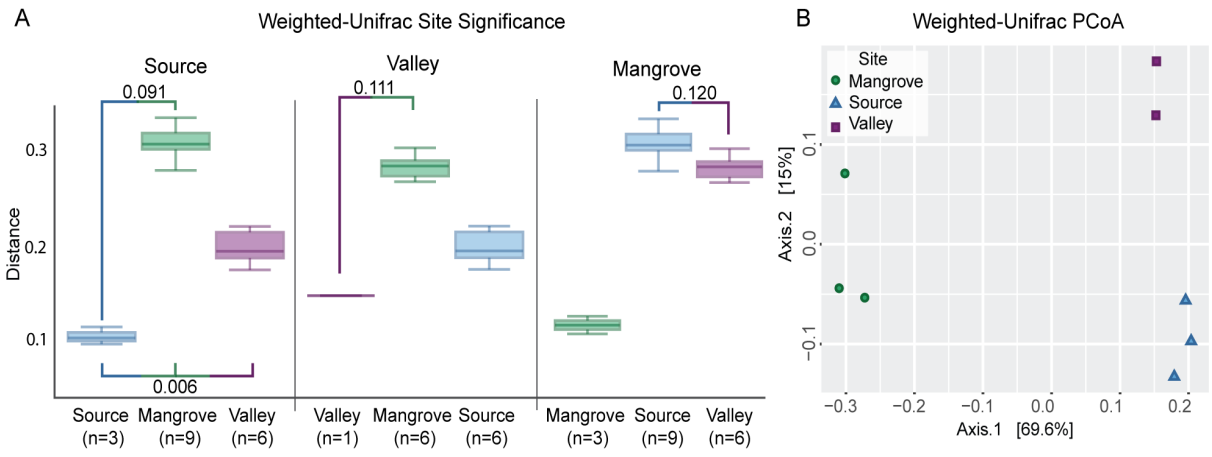
Source: Santana, 2020.

Figure 4.3- Values of richness and diversity indices (left). Boxplot of Kruskal-Wallis test of group significance with p-values (right)



Source: Santana, 2020.

Figure 4.4- A) Boxplots of PERMANOVA test for site significance based on the Weighted Unifrac distance metric. B) PCoA plot of community distances based on the Weighted Unifrac distance metric.

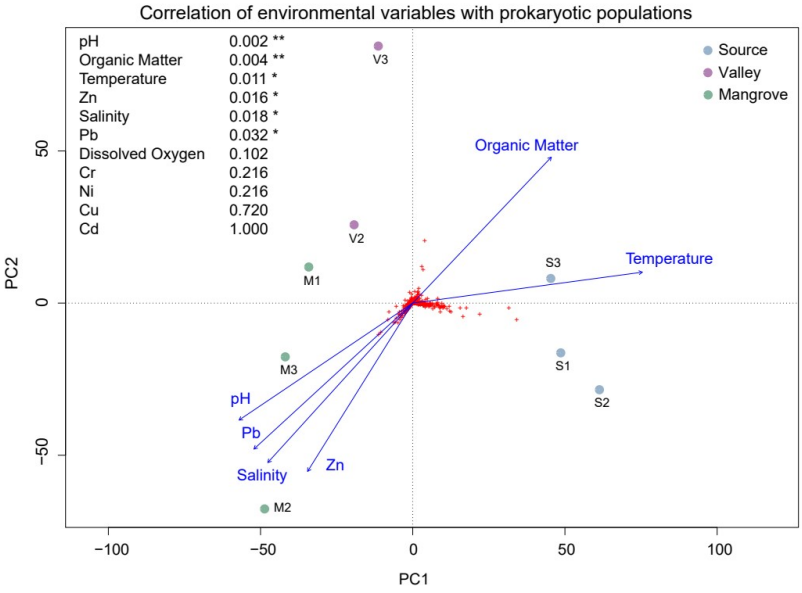


Source: Santana, 2020.

4.4.4 Influence of environmental factors to the prokaryote diversity

We investigated the correlations between the prokaryotic communities and the environmental factors such as temperature, dissolved oxygen, pH, salinity, organic matter, and heavy metals concentrations (Zn, Cu, Pb, Cr, Ni and Cd) (Figure 4.5). Significant correlations with the temperature, pH, salinity, organic matter (O.M.), Pb and Zn were found (α = 90%). In Figure 4.9, arrows lengths are scaled according to the strength of their respective correlations.

Figure 4.5- Principal Component Analysis Plot showing the effect size and directions of the environmental variables that correlated significantly with the communities at different sites



Source: Santana, 2020.

4.4.5 Functional Profiles

The analysis of functional profiles through PICRUSt2 allowed the identification of statistically significant differences in the abundances of the Carbon, Phosphate, Nitrogen and Sulfur metabolic pathways between collection sites.

Most of the carbon metabolism pathways (methane (ko00680)), carbon fixation pathways in prokaryote (ko00720), carbon fixation pathways in photosynthetic organisms (ko00710) were significantly enriched in the sediments of the source and lower in the mangrove. The photosynthesis pathway (ko00195), however showed higher abundances in the mangrove sediments (Figure 4.6).

The majority of metabolic routes for the metabolism of nitrogen (ko00910), phosphorus (ko00030) and sulfur (ko00920) showed higher abundances in the sediments of the source area, which display the higher biodiversity (Figure 4.7). These results indicate the importance of the genetic diversity in shaping the functional profiles of the communities in the river sediments.

4.4.6 Taxa contributions to functional potentials

The contributions of each microbial family to metabolic potentials regarding each nutrient cycle was investigated. Families that had significantly different functional abundances of a given metabolic pathway and contributed 5% or more for the function in at least one site are shown in Figure 4.8.

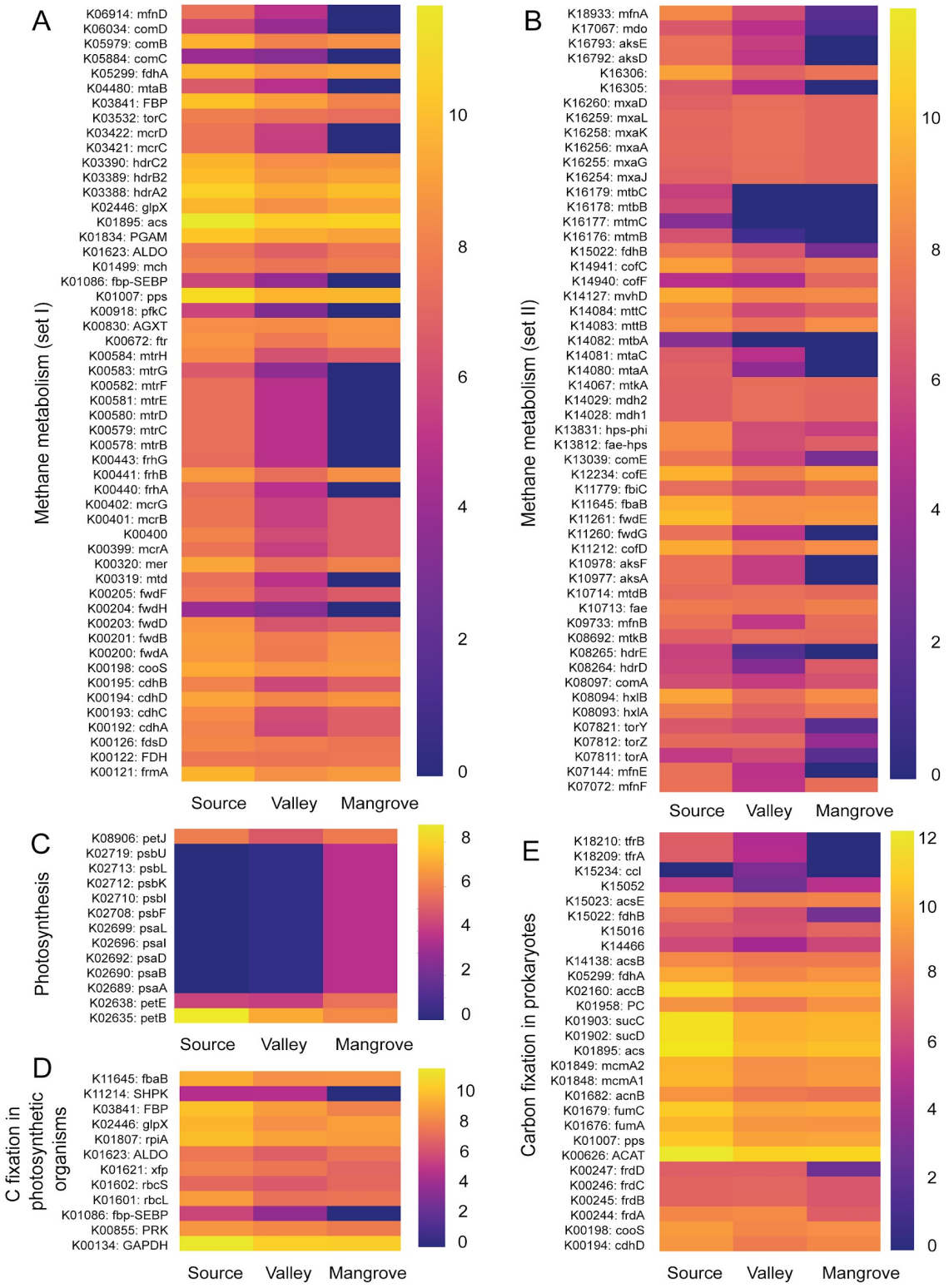
We find 11 families with large site specific potential contributions to metabolic pathways, displaying three modes of contribution: “Site specific generalist” wherein a family makes significant and distinct contributions to numerous (5 or more) pathways (*Bacterioidetes vadinHA17, Bacillaceae, Pirellulaceae, Stappiaceae,* and *Desulfobacteraceae*). “Site specific specialist” where families make significant and distinct contributions to only a small number (4 or less) of pathways (*Clostridiaceae 1, Lachnospiraceae, Ruminococcaceae,* and *Pedosphaeraceae*). And “Mixed site generalists” where a significant difference is observed in some but not all pathways between sites (*Anaerolinaceae* and *Syntrophaceae*). Potentially, these differences in modes represent different adaptation strategies for these organisms.

4.4.7 Site specific taxa enrichment

We also determined the specific taxa which significantly differed by site. Considering all taxonomic levels, the source sediments have a larger number of site specific taxa, which is consistent with the higher biodiversity observed in that area (Figure 4.9).

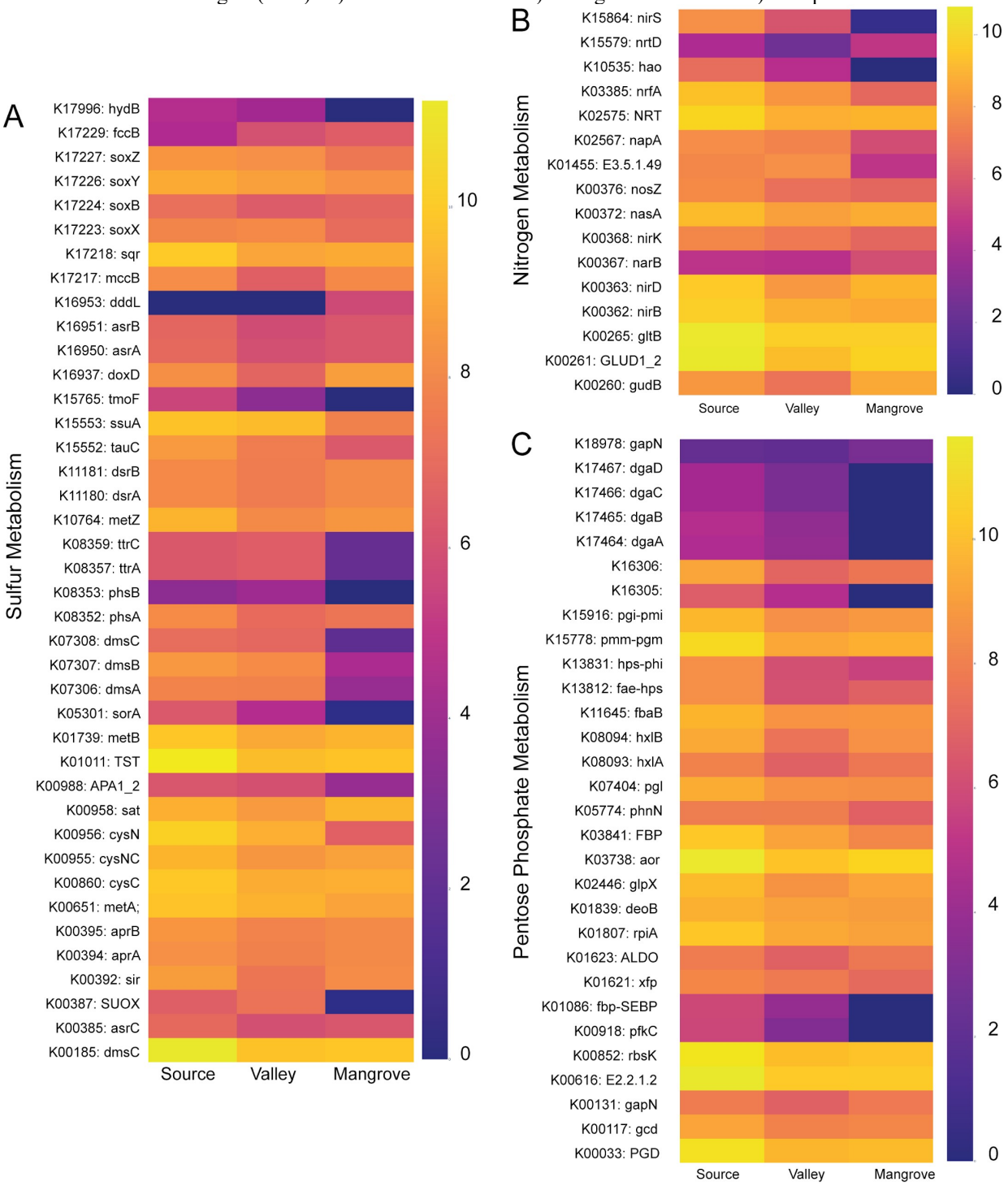
At the family level, 59 groups presented statistically different abundances in at least one of the studied sites, mostly enriched in the source sediments (n=22). Notably, despite showing the least biodiversity in the analysis, the mangrove sediments had statistical enrichment of 18 families, and many of these families were unique for this site.

Figure 4.6- Heatmaps of carbon metabolism pathways, showing relative abundances of differentially enriched KEGG Orthologies (KOs). A) Methane. B) Carbon Fixation. C) Carbon fixation in photosynthetic organisms. D) Photosynthesis



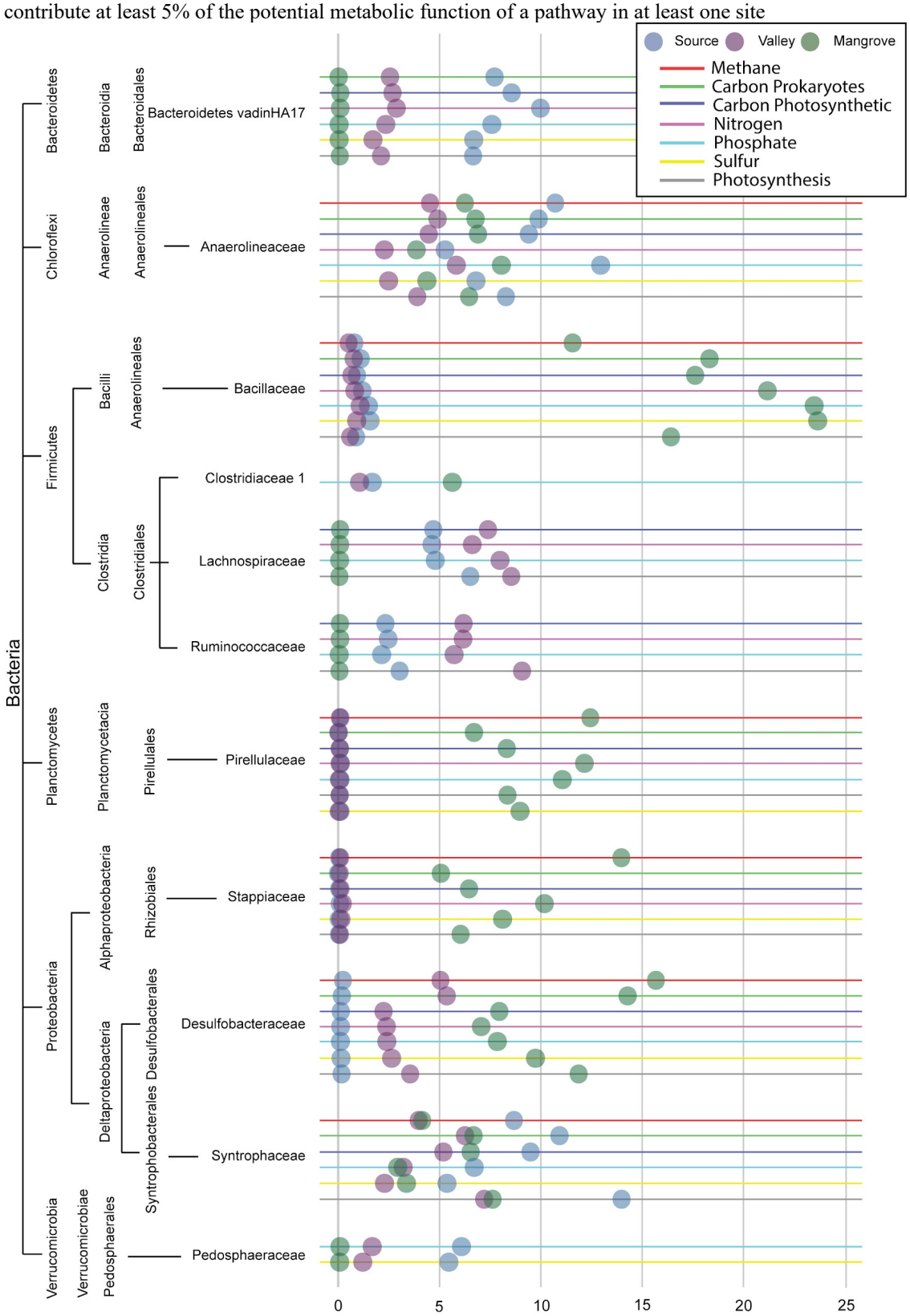
Source: Santana, 2020.

Figure 4.7- Heatmaps for metabolic pathways of nutrients cycling, showing relative abundances of differentially enriched KEGG Orthologies (KOs). A) Sulfur metabolism. B) Nitrogen metabolism. C) Phosphorus metabolism



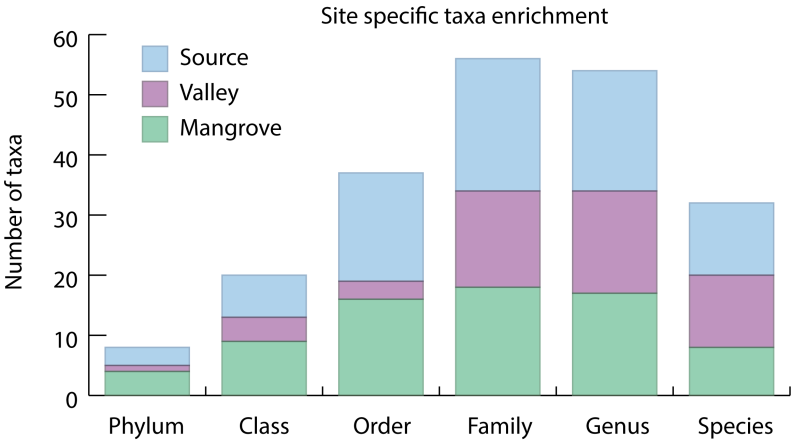
Source: Santana, 2020.

Figure 4.8- Families that have significantly different contributions to potential function between sites and that contribute at least 5% of the potential metabolic function of a pathway in at least one site



Source: Santana, 2020.

Figure 4.9- Relative abundances of groups with significantly different enrichment between collection sites at each taxonomic level



Source: Santana, 2020.

4.5 DISCUSSION

This work shows that the three distinct sampled sediments have statistically distinct prokaryotic communities. Microbial diversity reduces from the river head to its delta, which is almost 5 times lower. A potential explanation for such a decrease could be the increasing levels of anthropic interference and contamination (BERG et al., 2012; CHEN et al., 2018). Given the preserved status of the Julian River, it seems unlikely that anthropic activities are the main factors influencing the community structures. Alternatively, we consider that this decrease in biodiversity is a result of the natural variations observed in the environmental factors influencing these communities.

Previous studies that have investigated changes in microbiomes through the river-estuary-ocean interface found the same trend of decreasing diversity in the direction of the river flow, either in taxonomic or functional terms (WANG et al., 2012b; BEHERA et al., 2019; ZHANG et al., 2020). In these studies, variables such as temperature, salinity and the trophic status of each studied area greatly influenced the taxonomic and functional distribution of microorganisms, similar to what was observed in our results.

We observed that the temperature, pH, salinity, organic matter, Pb and Zn values are significantly correlated with the prokaryotic communities. In the ecology field, a well accepted concept is that biodiversity will decrease with increasing habitat harshness (STATZNER; MOSS, 2004). In this sense, it can be inferred that the mangrove sediments are a more extreme environment in comparison to the freshwater sites, selecting fewer more resistant species. Similarly, Tolkkinen et al. (2020) argue that the narrow river source areas are less environmentally extreme, usually having more canopy cover, which affects stream

diversity by maintaining water temperature and controlling litter input and primary production. In this sense, the influence of the external environment in headwater streams would provide the requirements of a hotspot for greater diversity, such as a great variety of C and nutrient sources, while extreme conditions of salinity and tidal effects (DE SANTANA et al., 2020) would be the potential cause of the decrease in prokaryotic diversity observed in the mangrove area.

The transition in population structure is significantly correlated with several environmental variables in addition to salinity and pH, such as organic matter content and temperature. The presence of the phylum *Bacteroidetes* in sediments has been previously correlated to environmental variants such as the trophic state of water and temperature (HUANG et al., 2017; DAI et al., 2016), which suggests that the availability of nutrients is higher in the source sediments than in the sediments of the valley and mangrove, possibly due to the differences in the sources of organic matter at each site. Similarly, the prevalence of *Verrucomicrobia* has also been correlated with temperature, suggesting a preference for milder and more stable temperatures and with the levels of environmental disturbance, such it is found in greater abundance in the most preserved environments (BERG et al., 2012).

Beyond the mere abundance, the contents of organic matter and nutrients are also environmental factors that affect microbial communities. It has been observed that the bioavailability of essential nutrients, such as nitrogen and phosphorus, are lower in mangrove sediments than in freshwater sediments, possibly due to tidal variations that cause frequent nutrient washout or erosion (BEHERA et al., 2019) and the increased salinity that hinder the accessibility of the microbial community to the soil organic matter, as well as the prevalence of humic-like substances (WANG et al., 2010; CECCON et al., 2019). Because the structural characteristics of dissolved and particulate OM in aquatic environments have large effects on the contents, species and availability of nutrients such as N and P and heavy metals (DONG et al., 2020), it is expected that this will have considerable effects in aquatic microbial productivity and community composition (TOLKKINEN et al., 2020).

The prevalence of the phyla *Proteobacteria* and *Firmicutes* in the sediments of all the studied areas is in agreement with the literature for most soils and sediments (TVEIT et al., 2013; JOST, 2007; YADAV et al., 2015; ANDREOTE et al., 2012; IMCHEN et al., 2018; SU et al., 2018) and is mainly due to the high morphological and physiological diversity of these groups that allow for their colonization of various habitats. Aside from these generalists, there is a gradual decrease and replacement of phyla going down stream from source to valley to mangrove in the Juliana river. Broadly, this is the loss or replacement of phyla that prevail in

freshwater, such as *Bacteroidetes* and *Verrucomicrobia*, for that prevail in coastal environments, such as *Crenarchaeota* (THIELE et al., 2017).

The greater incidence of the phylum *Euryarchaeota* in the source sediments is notable because most organisms belonging to this phyla are considered halophilic and with preference for higher temperatures and have only recently been observed in more moderate environments (BERG et al., 2012; CHEN et al., 2018; KORZHENKOV et al., 2019).

The river source presented the majority of taxa enrichment, with the valley sediments having the least. Although the mangrove site had the least biodiversity, several taxa were observed as unique to that environment, including members of the families *Bacillaceae, Clostridiaceae 1, Pirellulaceae,* and *Stappiaceae*. As described below, these families also contribute significantly to the metabolic potentials in mangrove sediments, suggesting that the biogeological processes at this site are largely distinct from those occurring in the freshwater sites.

These results confirm that the mangrove site has a very different community compared to the two freshwater sites. As noted previously, not only can the increased salinity act as a limiting factor for freshwater taxa by selecting for more saline resistant taxa, but also change the patterns of nutrient bioavailability in the sediments. The findings of taxa such as *Xanthobacteraceae* and *Clostridiaceae* to be enriched in these sediments can reinforce this hypothesis since such groups are recognized for being resistant to adverse conditions (OREN, 2014; WIEGEL; TANNER; RAINEY, 2006), while the enrichment of taxa involved in sulfur cycling such as *Desulfobacteraceae*, *Desulfobulbaceae* and *Chromatiaceae* in marine environments has also been previously observed (WANG et al., 2012b; IMHOFF, 2014).

From the functional analysis we observe the tendency for metabolism associated KO abundances to be statistically higher in the sediments of the source, relative to those at the Juliana River valley and its delta or mangrove. We also find higher prokaryotic diversity in the sediments of this area, suggesting that there might be more groups acting on the diverse stages of nutrients metabolism in sediments. This agrees with previous observations that many functions in microbial ecosystems are redundant (BARNES; CARTER; LEWIS, 2020; TOLKKINEN et al., 2020). Because redundant functionality can grant a biome greater resilience to environmental impact, the headwaters may be more resilient than the mangrove to anthropogenic impact.

Focusing in the carbon metabolic pathways, the microbial diversity observed in the mangrove sediment showed higher diversity or KOs for the photosynthesis pathways . In our study we found the photosynthetic families *Chromatiaceae* and *Rhodobacteraceae*,

representatives of the phototrophic purple sulfur bacteria (IMHOFF, 2014; PUJALTE et al., 2014), to be enriched in the mangrove sediments. These results suggest that this is a more important metabolism in estuaries than in upstream areas of the river. This trend has also been observed previously (FORTUNATO; CRUMP, 2015), and is possibly correlated with the fact that oceanic waters have relatively higher abundances of aerobic photosynthetic bacteria and anoxygenic phototrophic bacteria (KOLBER et al., 2000; WAIDNER; KIRCHMAN, 2007). These organisms would have advantages over non-photosynthetic groups in situations, such as mangrove sediments, where nutrient availability is constrained.

Nitrogen metabolism often involves multiple groups participating in particular stages of the transformation of this element (HU et al., 2012; OUYANG; NORTON; STARK, 2017). We find higher abundances of KOs associated with nitrogen metabolism in the source sediments, which could be associated with the high biodiversity in this area. Another factor that could affect the nitrogen cycling in the studied areas is the diversity and bioavailability of carbon in the different sediments, which can impact the nitrification and denitrification capacities of the environment (TRIMMER et al., 2012; REN et al., 2019). In such situations, were the diversity and availability of nutrients is low, microorganisms such as members of the family *Beijerinckiaceae*, which was statistically enriched in the mangrove sediments, would show some metabolic advantages as the group is capable of fixing nitrogen, enabling their survival in habitats in which such element is scarce (MARÍN; ARAHAL, 2014).

The abundances of metabolic routes of phosphorus indicate that there are more groups acting in the different stages of the P cycle in the source area. The structure of the organic matter in the sediments is also an important factor for the cycling of this element. The prevalence of humic-like organic matter in the marine sediments could be a limiting factor for the activity of alkaline phosphatase, which would hinder the transformation of organic P into inorganic P (DONG et al., 2020). In this sense, it could be hypothesized that the higher content of humic-like substances in the mangrove sediments, which makes them a “sink” for many contaminants (DOBBSS et al. 2016), also leads to the lower availability of phosphorus for microbial transformations in this area.

Likewise, the abundances of the sulfur metabolism pathway routes can be correlated to the higher microbial diversity observed in the sediment samples at the river source, in comparison to the valley and mangrove. According to the literature this metabolism is, however, quite widespread in coastal and marine sediments; mainly due to the presence of sulfate-reducing bacteria (BAKER et al., 2015). The dissimilatory sulfate reduction (DSR) to sulfide is a major pathway for sulfur transformation by anaerobic microorganisms in marine

environments and this process is also highly depending on the organic carbon and S availability (JØRGENSEN et al. 2019).

Soil microbiomes present high functional versatility, which is the potential for several taxa to present metabolic activities of a given pathway (JENKINS et al., 2017; LAKSHMANAN; SELVARAJ; BAIS, 2014; JIAO; CHEN; WEI, 2019). Additionally, it is common that the metabolism of different elements occurs coordinately in the organism as is the case of the regulation of sulfur oxidation and nitrate reduction in many organisms within the *Gammaproteobacteria* class (BAKER et al., 2015). This is consistent with our findings as we observed the contributions of the diverse taxa to the studied metabolic pathways. Furthermore, taxonomic abundance also appears to correlate with the potential contribution to metabolism, such as in the mangrove, where the lower diversity seems to lead to fewer families making larger contributions to nutrient cycling. Our analysis showed that the most abundant families also encode genes for many different metabolisms.

Thus, given the higher prokaryotic diversity and subsequent higher versatility observed, it could be hypothesized that the sediments of the source area of the Juliana river are the more eco-physiologically resilient compartiment of this watershed. Nevertheless, other characteristics intrinsic to the different collection sites can be associated with the risks posed by anthropic and natural disturbances for the ecosystem functions. The high affinity of the OM in mangrove sediments for the contaminants like metals and other elements would hinder their bioavailability for the prokaryotes, while the salinity highly affects this organic matter availability. The presence of plants could help with the consumption of excessive nutrients and metallic species, while the temperature and pH could also be important for these interactions and speciation of elements in the interface sediment-water and plant assimilation (MOREIRA et al., 2016; LEONCIO et al., 2020).

4.6 CONCLUSION

In this study we conducted an investigation of the structural and functional profiles of the prokaryotic communities in the sediments of different areas along a preserved riverine system, from the head or source to the delta of the Juliana River. This river runs through the Pratigi Environmental Protection Area, Brazil. The results provide us information on the composition of the communities along the river and how it correlates with the spatial variability of the environmental factors, since the entire watershed is in good preservation

status, a lacking condition to most riverine systems worldwide. The phyla *Euryarchaeota*, *Bacteroidetes* and *Verrucomicrobia* exhibited the tendency for lower abundance in the estuarine sediments in comparison to the freshwater samples, while *Crenarchaeota* and *Planctomycetes* prevailed in the saline water environment. The differences could be observed in lower taxonomic levels as well, indicating that the collected sites at the Juliana river basin show distinct taxonomic profiles. These taxa can be further studied as possible indicators for the monitoring of ecosystem health for each riverine region, either in the Juliana basin or other watersheds in the Atlantic Forest. Diversity measures showed that communities differ statistically, such as sediments of the source area were the most diverse, while the mangrove sediments were the least diverse but most distinct microbiome. This indicates that, considering the microbiology, river sediments should not be considered as an unique environment, but diverse environments. Despite the lower diversity, a considerable number of families were enriched in the mangrove sediments, suggesting that the increased salinity and harsh conditions could be selecting fewer and unique taxa in this area. Salinity, as well as the organic matter content, pH, temperature, Pb and Zn levels were confirmed as important environmental factors that influence the communities structures. Organic matter content and structure also appears to have large influences in the patterns of metabolic potentials in the sediments. Results showed that some metabolic pathways for C, S, P and N cycles are significantly different between the river areas, with the source sediments showing a higher diversity for KOs. Higher functional and structural diversity observed in the river head suggests higher functional redundancy. Such higher functional redundancy may affect the ecosystem resilience to environmental impact. Finally, other environmental factors that were not evaluated in this study could be also important for the observed patterns, such as the seasonality which has relevant effects on the other variables addressed in the research.

5 THE IMPACT OF LAB SCALE CONTAMINATION EVENTS ON PROKARYOTIC COMMUNITIES OF THE JULIANA RIVER SEDIMENTS IN THE NORTHEAST OF BRAZIL

5.1 ABSTRACT

Given their proximity to agricultural areas and road sides, rivers are often exposed to environmental pollution by pesticides, fertilizers, crude oil. The cycling of mineral nutrients is the central component that defines ecosystem health. This research discusses the effects of crude oil and glyphosate pollution events on prokaryotic populations present in the sediment sampled at the river head or source. This is a pristine freshwater river site located within an environmental protection area at Brazillian Atlantic Forest without any known previous exposure to such pollutants. We found that immediately after exposure to glyphosate the nitrate consumption rate decreases to 50% of the untreated control, while the equivalent treatment with oil results in a 15% decrease of consumption rate. This suggests that glyphosate has a large and acute effect on the population's capacity for nitrate consumption. We also find large-scale changes in the prokaryotic population structure after a 7 day incubation period for both glyphosate and crude oil. We find that these altered populations have significant changes in metabolic pathways according to the contaminant they were exposed. Taken together, this suggests that contamination by glyphosate and crude oil can cause deleterious changes to the microbiome of pristine riverine systems.

Keywords: River contamination, sediment microbiome, nitrogen kinetics, Petroleum, Glyphosate

5.2 INTRODUCTION

Riverine systems often receive, transport and process different kinds of natural and human-derived materials or pollutants. These systems also spread these substances to downstream waters bodies such as lakes and oceans. Thus, they may cause significant perturbation on biogeochemical cycling of large scales (HELTON et al. 2011; KUPILAS et al., 2017). Perturbations such as those caused by agricultural and urban use are recognized to cause significant changes in natural characteristics of rivers, when compared to the undisturbed areas (YOUNG; MATTHAEI; TOWNSEND, 2008; LAMBERT et al., 2017). Nevertheless, rivers are not simple inert conduits that merely transport nutrients from landscape to coastal environments, instead numerous and important transformations of nutrients, pollutants, and trace elements occur in riverine systems (TRIMMER et al., 2012).

In Brazil, aquatic ecosystems are threatened by intensive agriculture's use of pesticides such as glyphosate, which have already led to the contamination and degradation of

rivers in numerous different regions (DELLAMATRICE; MONTEIRO, 2014). Aquatic ecosystems are also threatened by coastal oil spills that can affect the lower river areas and estuarine regions as pipeline leakage and storage that can occur further upstream (BEGALISHVILI et al. 2012).

The communities and, consequently, the functional roles of microbes involved in N cycling, such as the removal of NO_3^- in river sediments can be greatly affected by the presence of contaminants or perturbations in the environment, and the ecologies of many river systems have already been overwhelmed in this capacity (BEAULIEU et al., 2011).

In recent years, glyphosate, a systemic herbicide with a broad spectrum of action, has become a major participation in the world herbicide market especially with the introduction of genetically modified glyphosate resistant crops (MORAES and ROSSI, 2010). Glyphosate is of particular concern in regards to water resources because as an agricultural herbicide it is often used in close proximity to agricultural water supplies (CABRERA; COSTA; PRIMEL, 2008). Additionally, the existence of glyphosate resistant crops allows for overuse without concern for crop damage and because specific surfactant formulations can have increased toxicity in aqueous environments (VAN BRUGGEN et al., 2018). Moreover, glyphosate use is not limited to agricultural activities, being often applied for weed control along highways and railroads and even for private uses such as in gardening and weed control in residential areas or commercial sites (POIGER et al., 2017).

Pesticides in the aquatic environment can be degraded by chemical routes, photolysis, or by microorganisms. Glyphosate can be present in aqueous environments as either dissolved or associated with suspended sediment and has a medium-span half-life between 7 to 14 days (GIESY; DOBSON; SOLOMON, 2000; CABRERA; COSTA; PRIMEL, 2008). This herbicide in soil environments has been shown to alter the microbial balance to favor resistant microbiota while suppressing sensitive microorganisms (MORAES and ROSSI, 2010).

Currently, over 30% of the global energy use comes from petroleum hydrocarbons derived from crude oil (IEA, 2020). Crude oil is composed of different hydrocarbon molecules consisting, primarily, of straight chained hydrocarbons (alkanes), cycloalkanes and various aromatic hydrocarbons (BABALOLA et al. 2016). Since human activity is reliant on oil to meet its energy demands, environmental deterioration caused by petroleum pollution is an important issue (XU et al., 2018). While petroleum and petroleum-product toxicity depends on chemical composition and hydrocarbons structure, research has proven that it can be harmful to plants, animals, and can even accumulate in food chains (EDET; ANIKA; UMOREN, 2019). Increasing demand and changing methods of extraction, storage, and

processing have increased the distances petroleum travels from source to market with concomitant increases in accidents, leaks, and both freshwater and saltwater spills (ROBIDOUX et al., 2018).

In the environment, some indigenous microorganisms can utilize the total petroleum hydrocarbons (TPH) of crude oil as source of carbon and energy and break them down to simpler non-toxic compounds such as CO_2 and H_2O, in a process called demineralization (YUDONO et al., 2010). However, the presence of oil is still highly toxic to microorganisms, and can, in turn, lead to disruptions in local nutrient cycles, as is the case of the nitrogen cycling with species and functional genes involved in nitrification being reduced in the presence of petroleum sub-products (VAN DORST et al., 2014).

Considering the importance of microorganisms in biogeochemical cycles in aquatic ecosystems and the constant threat of pollution, it is important to know the extent of the disruptions that can be caused by the addition of contaminants. As previously described, the introduction of contaminants such as glyphosate and oil can impact microbial communities, in some cases changing their whole structures. This altered microbiome in turn, has an altered metabolic function and capacity which can have severe consequences for higher order organisms and the biome as a whole. Thus, it is important to understand the impact that environmental contaminants exert on microbial populations in aquatic ecosystems.

We aim to study the effects the common contaminants glyphosate and crude oil have on pristine river system microbiomes and their nitrate removal capacity. To achieve this we use river sediments and water from a preserved tropical riverine system, namely the Juliana river, that is entirely situated inside a protected area of the Atlantic Forest to create microcosms simulating the natural conditions. The samples were treated with either glyphosate or crude oil, since these are very common contaminants in aquatic systems. To determine the immediate effect these contaminants had on nitrate removal we measured the nitrate kinetics over the course of an hour and half after exposure. To examine changes in prokaryotic community structures we maintained treated and non-treated microcosms incubated over a seven-days exposure period before performing DNA extraction and 16S rRNA amplicon sequencing for genomic analysis.

5.3 MATERIAL AND METHODS

The following topics will detail the methodology used for the present study.

5.3.1 Study area

The Juliana River watershed (Figure 5.1), is contained within the Pratigi Environmental Protection Area (APA), one of the largest remaining Atlantic forest regions located in the Southern Lowlands of Bahia State, Brazil. It is located between coordinates 13°35' N and 14°10' S, and 39°40' W and 38°50' E, with a total area of 472,455 Km^2. The Juliana River basin rises and flows within the boundaries of the APA and is formed by ten sub-basins, sheltering extensive fragments of dense ombrophilous forest. The site is located in an area with a tropical rainy climate, due to the proximity to the sea, with average monthly rainfall over 600 mm and annual rainfall 1500 mm.

5.3.2 Sampling of water and sediments

Sediment samples were collected in triplicates at the source area of the Juliana River watershed, from 3 collection points, resulting in 3 composite samples of superficial sediments (top 10 cm of the surface layer) collected with a steel cylindrical sediment core sampler and stored in plastic bags inside termic boxes during transportation to the laboratory. Before the assemblage of microcosms, plant matter and other organic material were manually removed.

After measuring the physical-chemical parameters (temperature, pH, conductivity and dissolved oxygen) of the source water in the sample sites with a multiparameter monitoring system (YSI model 85, Columbus), water samples were collected and stored in plastic bottles kept in termic boxes during the transport to the laboratory. At the Laboratory of Environmental Geochemistry and Catalysis (LGCA) at the State University of Feira de Santana, Bahia, the water and sediments were prepared for the microcosms. For each sediment core an aliquot was separated and kept in the -20°C freezer for subsequent DNA extraction while the remainder of the sample was used for the experiments. The experiments were performed less than 24 hours after the collections.

5.3.3 N Transformations in microcosm

For each treatment three microcosms containing river sediments and water were prepared (Figure 5.1). For the composition of the microcosms we used 500 mL erlenmeyers containing 200g of fresh sediment and 400 mL of river water filtered through A20 paper filter. KNO_3 was added to the filtered river water in order to reach the concentration of 350 mg/L of nitrate for subsequent kinetics analysis. The aeration of the microcosms was provided

with the use of aquarium hoses and air pumps which guaranteed the air flux in the surface water.

For the positive controls, three microcosms with fresh untreated sediments were analyzed in parallel with the treatment microcosms. For the negative control, three microcosms with autoclaved sediments were analyzed, in order to certify that the observed changes in nitrogen compounds were products of microbial activity.

For evaluating the influence of agrarian pesticides on the transformations of nitrate in the sediments, we added the glyphosate pesticide to the concentration of 15 mg/L, as this concentration has been observed as the LC_{50} in previous work with freshwater fish (ALBINATI et al., 2009). For this experiment we followed the order: addition of fresh sediments, introduction of filtered water added with nitrate (350 mg/L) followed by the addition of glyphosate to the concentration of 15 mg/L of water.

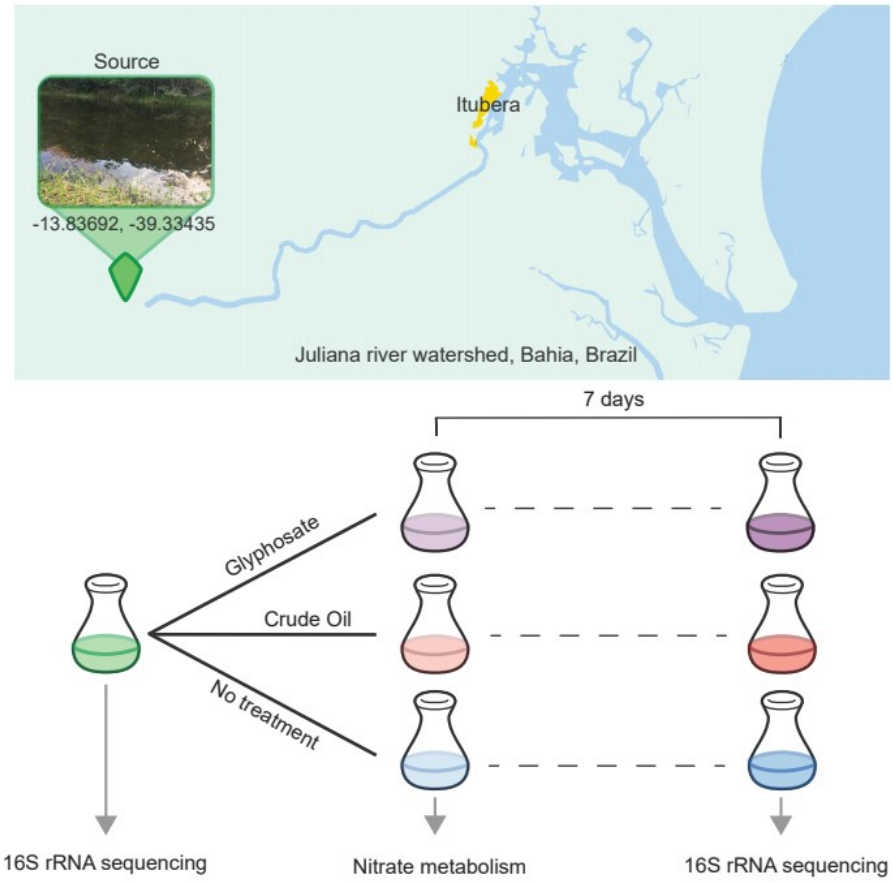
In order to determine the effects of crude oil on the kinetics of nitrate consumption, 2 mL was added to each of the 3 microcosms to the concentration of 1% v/v of sediment (EDET; ANIKA; UMOREN, 2019). The order followed for assembling the microcosms was: introduction of fresh sediments to the Erlenmeyers, addition of crude oil and mechanic mixing with the sediments followed by the addition of river filtered water enriched in nitrate (350 mg/ L)

Following addition of reagents and contaminants, water samples were collected in order to analyze concentrations of NO_3^- immediately after (t0) and then every 10 minutes until t3 (30 min). The samplings at the t4 and t5 were performed 30 minutes after the previous ones, totalizing 1h 30min. Nitrate concentrations in the water samples were subsequently analyzed through Ion Chromatography (IC) at the Laboratory of Petroleum Studies (LEPETRO) at the Federal University of Bahia (UFBA), Brazil.

5.3.4 Microbial communities before and after seven- days exposure

After N transformation experiments, the microcosms for each contaminant treatment were incubated at room temperature (varying between 23 - 30 celsius degrees) for 7 days, as this is the minimal average life span of glyphosate in an aqueous environment (GIESY; DOBSON; SOLOMON, 2000). No addition of contaminants was performed in order to observe how the prokaryotic community would respond to punctual perturbations in the first 7 days, thus helping understand how contamination with pesticide and crude oil could affect the microorganisms immediately after the exposure.

Figure 5.1- Juliana River Basin showing the collection site in the river source area (top). The experimental workflow for kinetic and genomic analysis (bottom).



Source: Santana, 2020.

5.3.5 DNA extraction and sequencing

The sediment DNA was extracted from field sediments stored at -20 and from experiment sediments after 7 days of exposure to contaminants. Separate DNA extractions were made from each Erlenmeyer that composed the treatment replicates. Finally, the DNA was analyzed for comparing the prokaryotic communities from the natural environment with the post incubation communities for each treatment and control.

The total genomic DNA from all the microcosms was extracted from 0.25 g of sediment using the Power Soil DNA Isolation Kit (Qiagen, Carlsbad, CA, USA) at the Environmental Geochemistry and Catalysis Laboratory (LGCA) at the State University of Feira de Santana (UEFS). All DNA samples were stored at -80º C before analysis. After DNA extraction, we used PCR to specifically target the V4 region of the bacterial 16S rRNA using the primer pair 515F-Y(PARADA; NEEDHAM; FUHRMAN, 2016) and 806R-XT (CAPORASO et al., 2011). DNA sequencing was performed using Illumina MiSeq platform,

V2 kit (300 cycles) at the Laboratory of Microbial Ecology and Biotechnology (Lembiotech) at the Federal University of Ceará, Brazil.

5.3.6 Data analysis

The kinetics of nitrate consumption in the microcosms was analyzed through excel software using the linear regression model. The results were plotted in a unique graph to allow better comparison between treatments and control.

After sequencing, we used the Trimmomatic (BOLGER; LOHSE; USADEL, 2014) to filter and trim demultiplexed sequences. QIIME pipeline (CAPORASO et al., 2011) was used to join forward and reverse reads into single reads. Reads were then denoised using DADA2 (CALLAHAN et al., 2016) in QIIME2 (BOLYEN et al., 2019), (q2cli, version 2019.4.0) and clustered into Operational Taxonomic Units (OTUs). Alpha-rarefaction was calculated using QIIME2 and a variety of alpha-diversity and beta-diversity tests were performed through QIIME2.

Taxonomic assignment was performed with Vsearch (ROGNES et al., 2016) in QIIME2 using Open Reference with 97% similarity against the reference 16S rRNA sequences in SILVA database (Silva SSU 132), (MCDONALD et al., 2012). Phylogenetic reconstruction was carried out in QIIME2 using the representative sequences for each OTU and a QIIME2 feature classifier trained using the 97% similarity representative set containing only 16S rRNA sequences (e.g. silva_132_97_16S.fna). All groups were required to be present within at least 2 samples with a minimum of 3 reads each.

QIIME2 tree files were accessed in R (version 3.4.4) using QIIME2R and Metacoder (FOSTER; SHARPTON; GRÜNWALD, [s.d.]). Posterior analysis was performed using Phyloseq (MCMURDIE; HOLMES, 2013). Analyses in R were plotted using ggplot2 (MCMURDIE; HOLMES, 2013; VILLANUEVA; CHEN, 2019).

Functional analysis was performed using PICRUSt2 (version 2.3.0-b) (DOUGLAS et al., 2019; BARBERA et al., 2019; CZECH; BARBERA; STAMATAKIS, 2020; LOUCA; DOEBELI, 2018; YE; DOAK, 2009) with default settings. KEGG Orthologs (KOs) were analyzed for significant (p-value <= 0.05) differential abundances and a heatmap of KOs with differential abundance between treatments was then generated.

In order to identify which species were significantly different in abundance in each treatment we carried out a taxa enrichment analysis. OTUs were combined into assigned taxa using the results of QIIME2. To be defined as significantly different, there must be at least

100 counts of the taxa in at least one treatment. The sets of observations between sites must be statistically significant (p-val <= 0.05) as calculated by the one-way non-parametric test Kruskal-Wallis H. And finally, we also required the effect size to be in excess of a 20% difference in abundance between treatments. In order to determine taxa exclusive to a single treatment with a high degree of certainty we required a taxa to have been observed 0 times (using unnormalized abundances) at one site and at least 100 counts at the other, with a minimum of 10[10 | 20] counts per replicate.

We also calculated metabolic pathway enrichment specific to a given taxa at a given taxonomic level. For this we relied on the relative functional abundance results of PICRUSt2. We then applied Kruskal-Wallis H test to identify taxa that had significantly different taxa-specific pathway abundances. Furthermore, we require that a taxa at a single treatment must be significantly enriched relative to the other sets and that the taxa contributes at least 5% of the total relative functional abundance in at least one treatment.

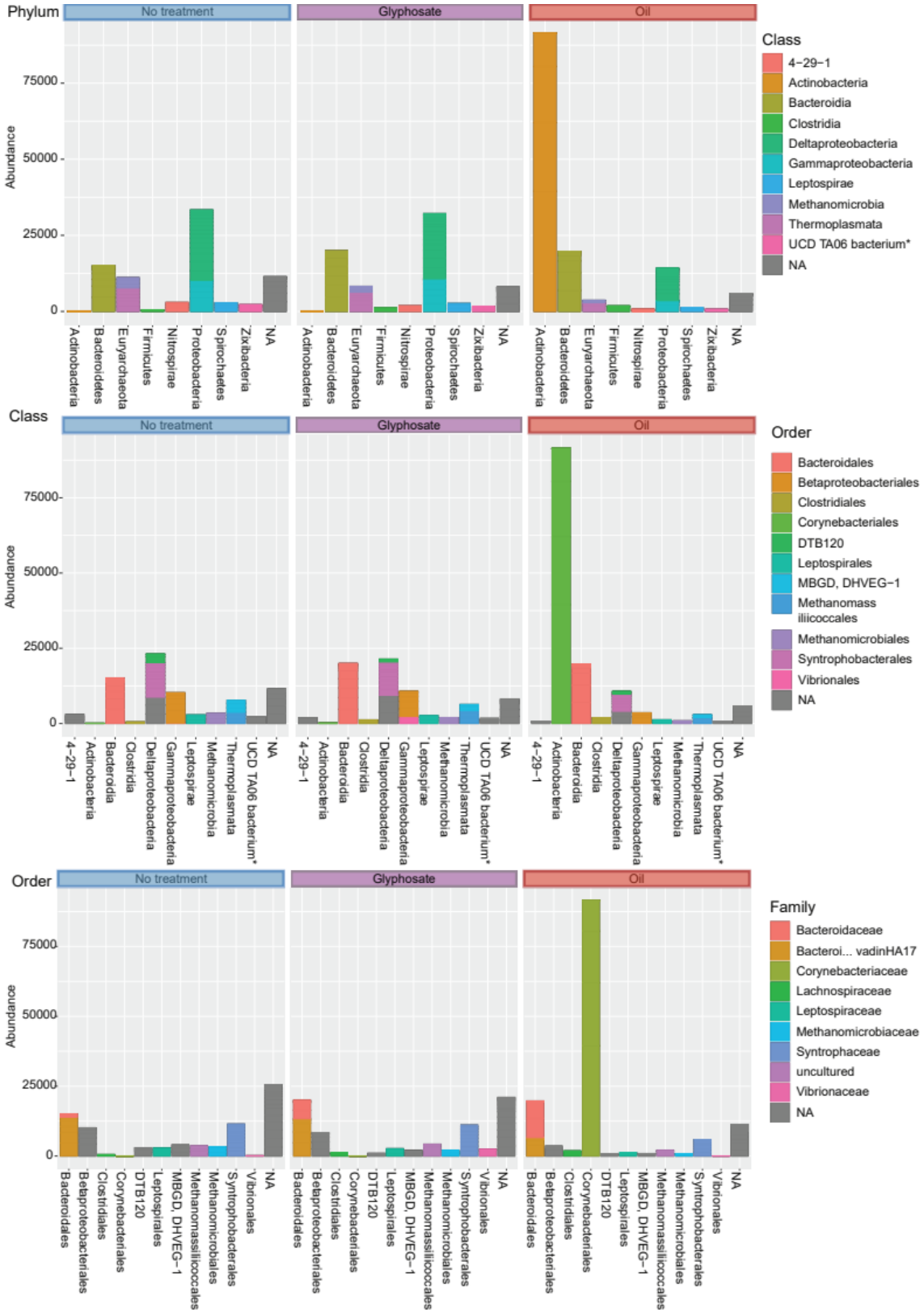
5.4 RESULTS

The results are synthesized by categories as follows.

5.4.1 Taxonomy

Sediments taken from the river source and analyzed right after collection had the highest contributions of amplicons belonging to the phyla *Proteobacteria*, *Bacteroidetes*, *Firmicutes* and *Euryarchaeota*, in this order. After 7 days of incubation with no treatment we observed a decrease in the abundance of *Firmicutes*, but also a relative stability of abundance of the other 3 most abundant phyla (Figure 5.2.) (Supplemental Figure 1). Similar decreases were observed in the microcosms treated with glyphosate after 7 days of exposure. For the microcosms treated with oil we observed a decrease in *Proteobacteria* and *Euryarchaeota* and a large increase of abundance for the phylum *Actinobacteria* after 7 days of exposure. Thus, the treatment with pesticide did not seem to produce large changes in the community, in comparison to the control samples, but the oil treatment seemed to favor the *Actinobacteria* group to outperform the *Proteobacteria*, creating a larger difference.

Figure 5.2- Barplots of taxa abundances for each treatment and control after 7 days of incubation



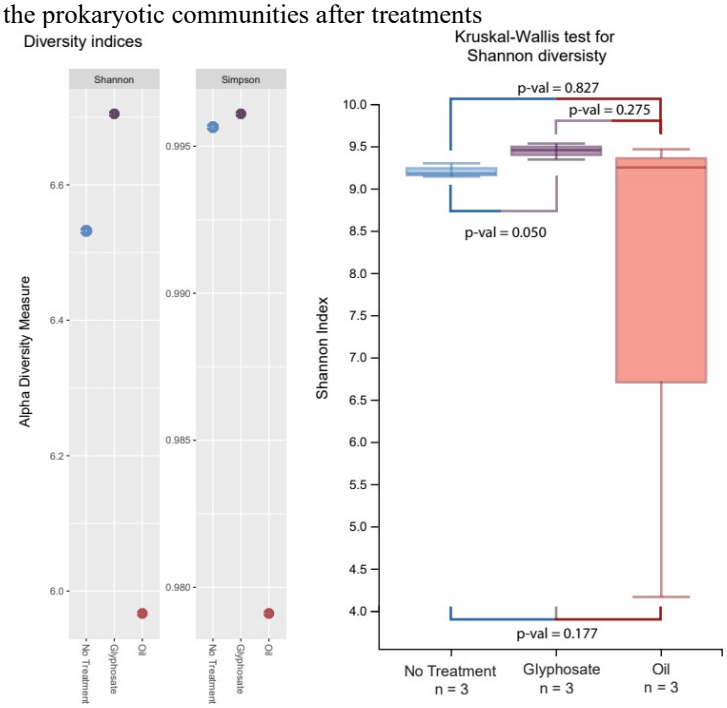
Source: Santana, 2020.

5.4.2 Diversity analyses

By comparing the sediments from the natural environment with the sediments of the control microcosms we found a statistically significant drop in the prokaryotic diversity after 7 days of incubation with no contaminants (Supplemental Figure 2), suggesting that the incubation alters the prokaryotic populations independently from the exposure to contaminants.

Next, we compared the prokaryotic diversity between the treated and untreated microcosms (Figure 5.3). We find significantly higher diversity (KW, p-value=0.05) indices in the samples exposed to the pesticide glyphosate relative to the control. Although the average diversity in the oil treated samples is very low because of the variability between replicates, this is not a statistically significant finding. Taken together, these data suggest that treatment with either glyphosate or oil affects the amount of diversity in the sediment.

Figure 5.3- Diversity indices for each treatment and boxplots displaying the significance of differences between the prokaryotic communities after treatments



Source: Santana, 2020.

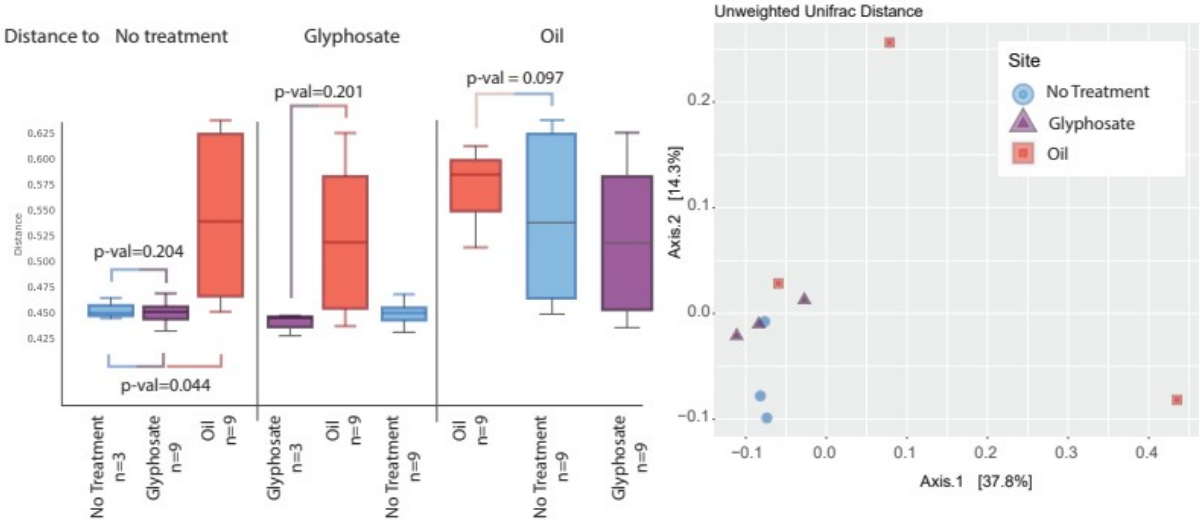
5.4.3 Differentiation of prokaryotic communities after treatments

Beta-diversity is a distance measure of the amount of biodiversity between two groups, the higher the distance between two groups the greater the difference in both the abundances

of taxa and the specific taxa that are present at each site. First, we compared the sediments from the natural environment with the sediments of the control microcosms and although there is separation between the two samples it is not statistically significant (PERMANOVA, p-value = 0.1) (Supplemental Figure 3).

When comparing the samples of treatment and pre-treatment sets we observed that changes in the prokaryotic communities caused by the addition of contaminants were statistically significant (p-value <0.05) although no paired comparison was significant (Figure 5.4). As shown in the PCoA plot for Unweighted Unifrac distance metric, the replicates of the treated samples fell at various distances from the control, suggesting that the divergence in populations within the microcosms can vary greatly between replicates.

Figure 5.4- Boxplots of the PERMANOVA comparison of the differences between communities under different treatments (left). PCoA plot displaying the community distances based on the Unweighted Unifrac metric



Source: Santana, 2020

5.4.4 Functional analysis

After observing that the exposure to the treatments led to statistically significant changes in the prokaryotic communities, we evaluated the functional profiles for the cycling of the elements C, N, P and S. The metabolic pathways that presented significant differences in abundance for at least one treatment are shown in Figure 5.5.

The main metabolisms for carbon transformations are subdivided into carbon fixation (photosynthetic or non-photosynthetic) and methane metabolism. Overall, there is a decrease in abundance for the majority of methane metabolism routes in the sediments treated with oil,

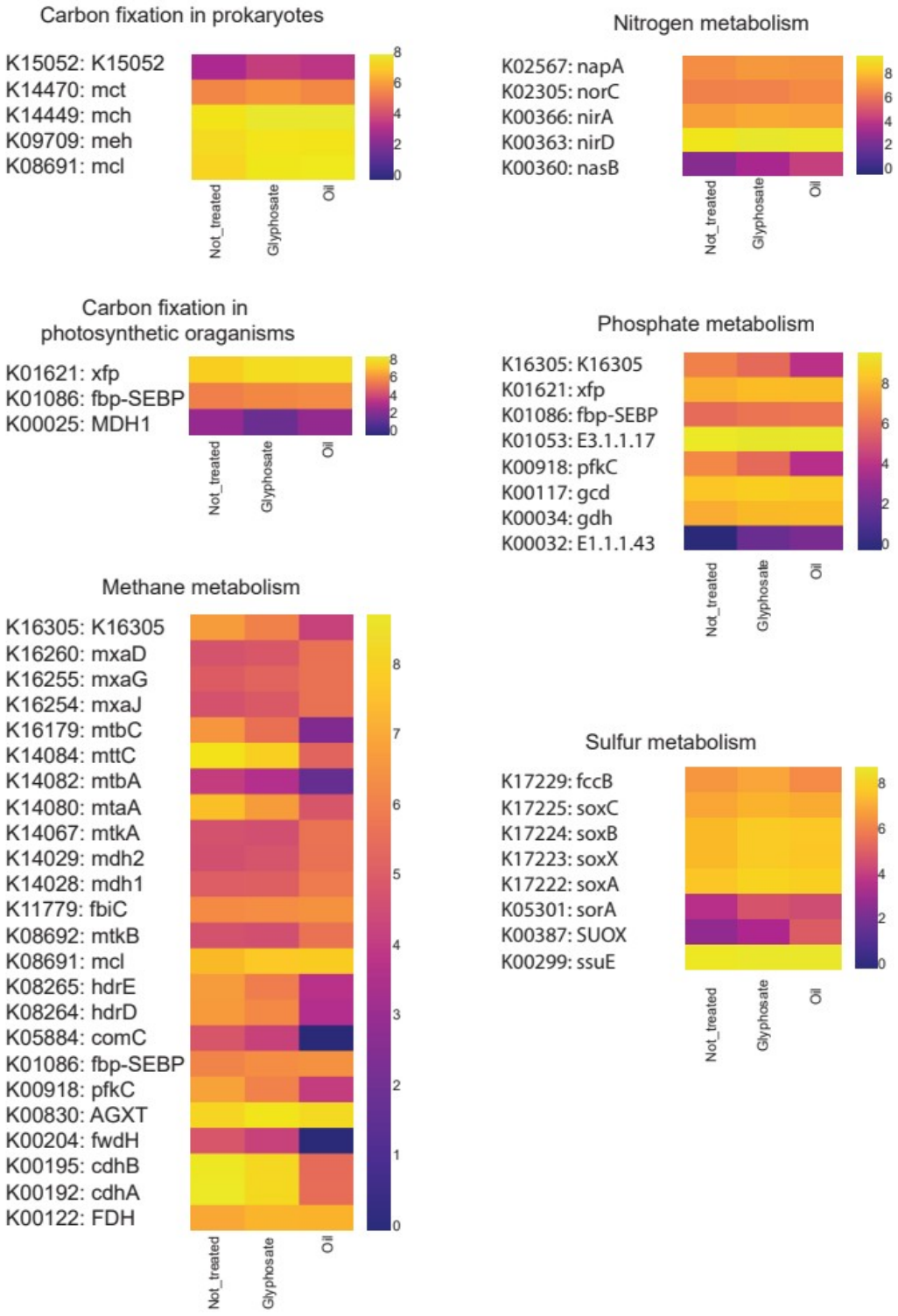
while most of the carbon fixation pathways increased in abundance for the sediments tread with glyphosate and oil in comparison to the not treated ones. The not treated sediments presented slightly lower abundances for the majority of the metabolic pathways of the nitrogen, phosphate and sulfur cycles.

A separate analysis of functional enrichment across all KOs with 10% greater ("High") or lower ("Low") abundance relative to the control was performed (Figure 5.6). There was a large percentage (23%) of overlap between glyphosate and oil treatments and two complete modules (modules with each component present) were found in this overlap: the anoxygenic photosystem II (M00597) and the benzene to catechol degradation module (M00548). These enrichments may represent the relative increase of taxa well suited to survival in disrupted environments. Notably, these modules are much more abundant in the oil treatment. A large percentage (30%) of KOs were de-enriched ("low") in the treatments, mostly due to the widespread loss of the Archeal taxa, such as the de-enrichment of the Archeal RNA exosome (K03679, K07573, K11600, K12589).

5.4.5 Taxa contributions to functional potentials

We also sought to determine which families were the main drivers of the functional potentials in each treated condition. All the families that presented large contributions (>5%) to the metabolic pathways for the element transformations in at least one treatment are represented in Figure 5.7. While many of the taxa with large contributions are also taxa that present large abundances in the treatment samples, this trend does not extend across all pathways. For instance, the three Archeal families *Methanomicrobiaceae*, *Methanoregulaceae*, and *Methanosaetaceae* contribute greatly to the methane metabolism pathway in the control samples but do not contribute significantly to other metabolic pathways. Likewise, other families also appear to be metabolic specialists: *BSV26*, *Lachnospiraceae*, *Chromobacteriaceae*, *Burkholderiaceae*, and *Pedosphaeraceae*. Conversely, several families appear to function as metabolic generalists, capable of making significant contributions to many (>5) metabolic pathways. Notably, 7 out of 9 of the metabolic specialists families dominate in the no treatment control, suggesting the treatments may be deleterious to specialists. A similar pattern is not observed for the generalists however, as these families are found to dominate in near equal numbers under all conditions.

Figure 5.5- Heatmaps showing the abundance of metabolic pathways (KOs) for C, N, S and P metabolisms

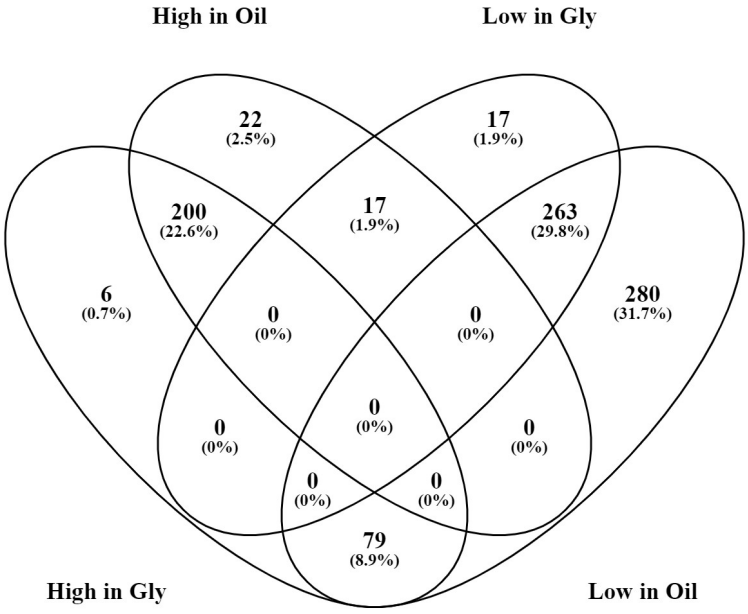


Source: Santana, 2020.

5.4.6 Taxa extinction driven by treatments

We next investigated families that could no longer be identified in at least one of the treatments (Figure 5.8). From the 20 families that were extinct after the experiment period, 9 had gone extinct in the control microcosms, 8 in glyphosate, and 7 extinct in oil, with some amount of overlap. Taxa abundance in oil is generally higher than the other conditions suggesting that the crude oil conditions provided a generally more suitable environment for such groups, in comparison to the control and glyphosate microcosms.

Figure 5.6- Venn diagram displaying the abundances of KOs with 10% enrichment or de-enrichment relative to the control samples

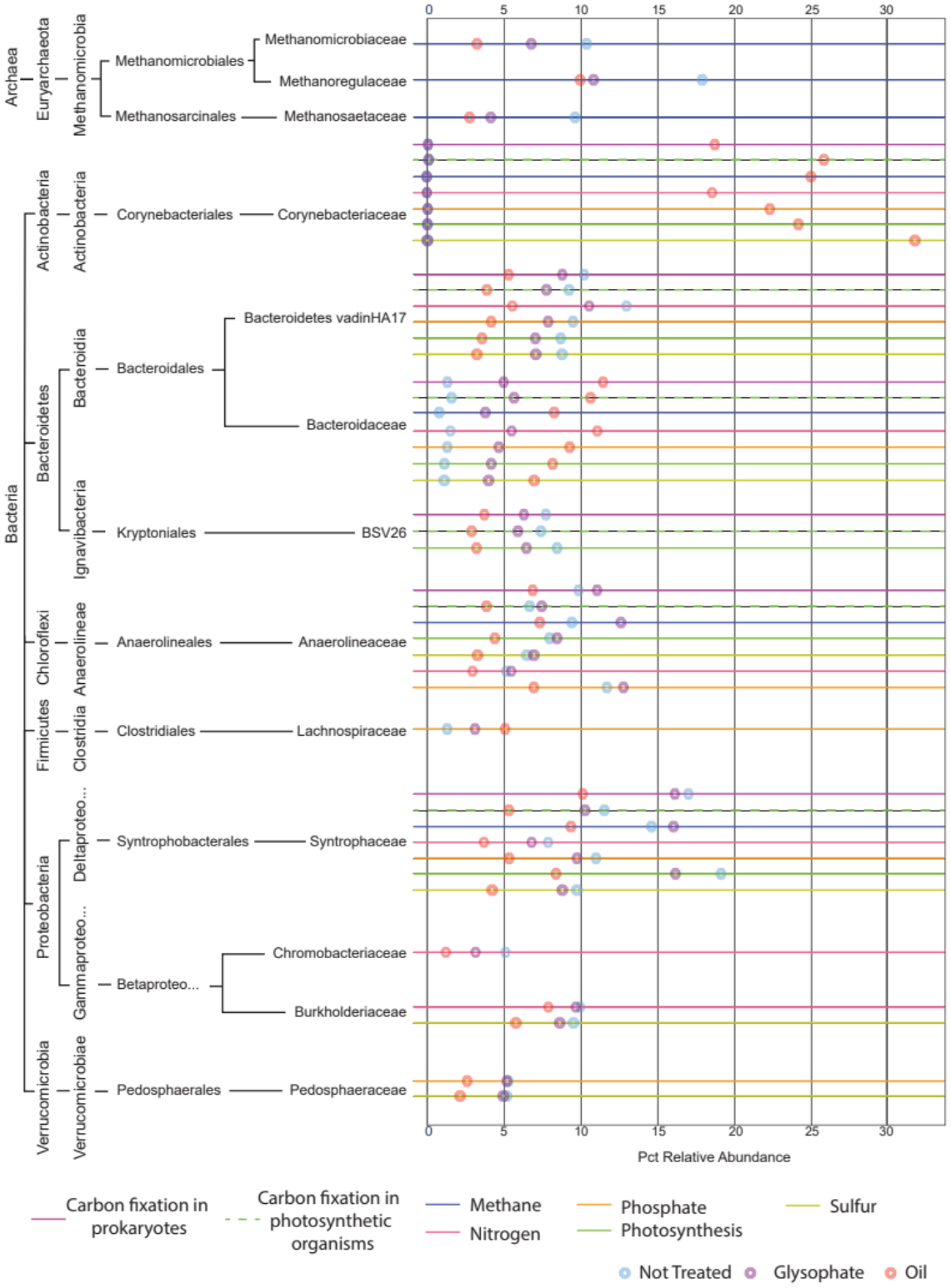


Source: Santana, 2020.

5.4.7 Effects of glyphosate and crude oil on nitrate consumption kinetics

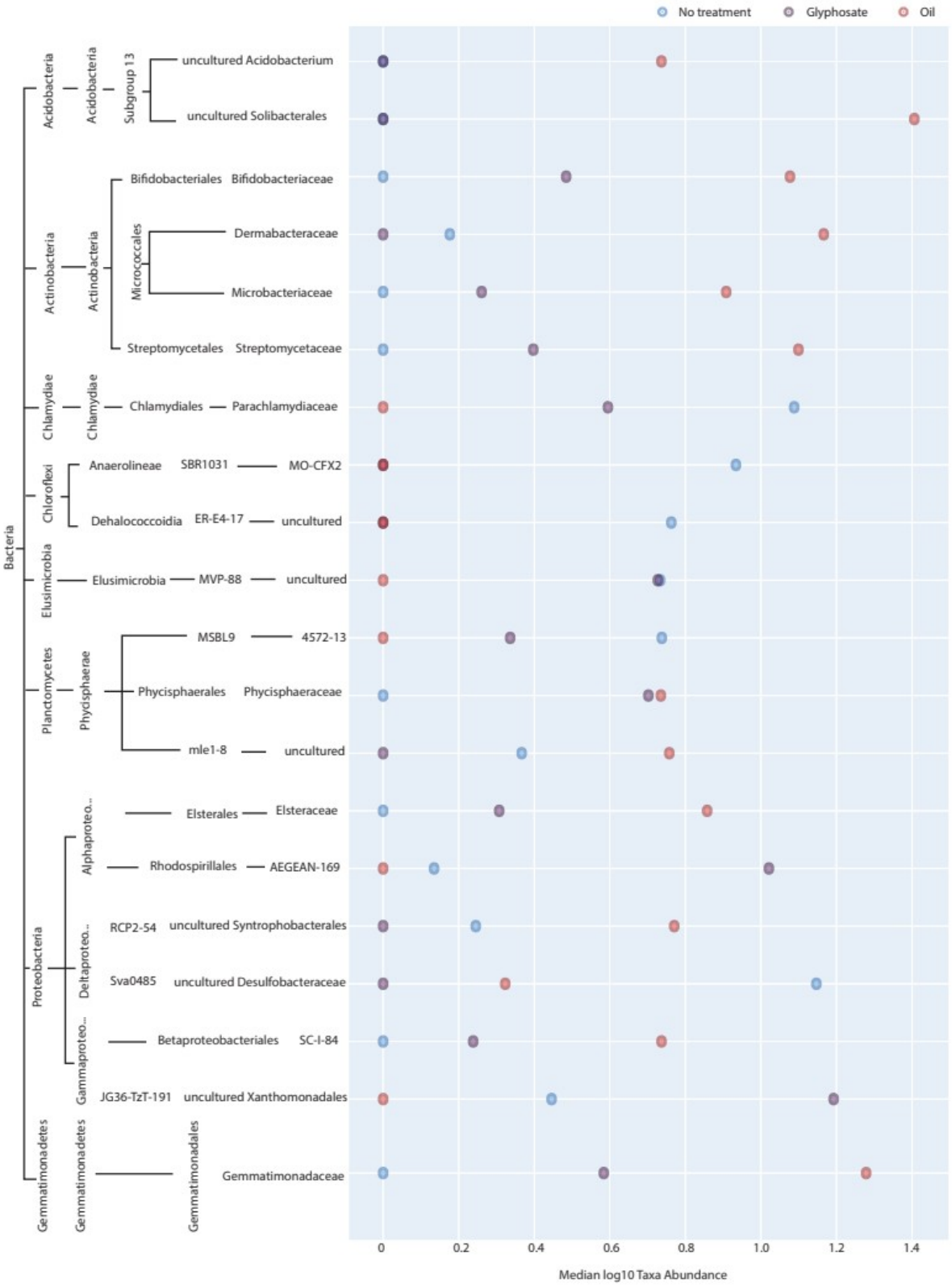
We characterized the rates of nitrate consumption in the sediments in natural conditions and under the influence of crude oil and glyphosate immediately after addition (Figure 5.9). The natural nitrate consumption rate was 0.90 mg/L/min and the crude oil introduction event decreased nitrate consumption rate in 15% (0.76 mg/L/min). A stronger effect was caused with the experiment using glyphosate. Nitrate consumption rate was 0.45 mg/L/min, a reduction of 50% in the transformation kinetics. These results indicate that the addition of glyphosate has a stronger disturbing effect on the nitrogen biogeochemical cycle immediately after the event.

Figure 5.7- Relative contributions of prokaryotic families to the metabolic pathways in each treatment



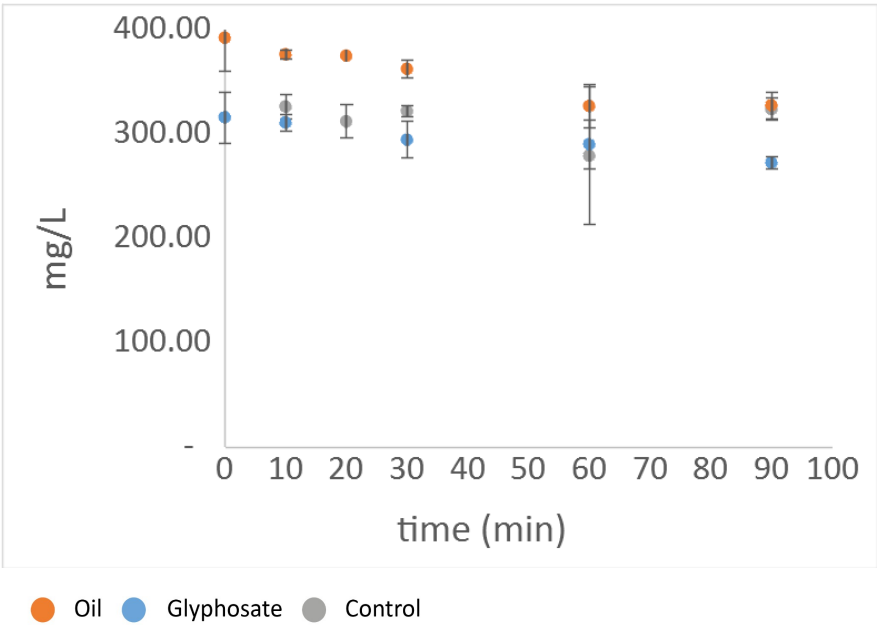
Source: Santana, 2020

Figure 5.8- Families that went extinct in one or two treatments after 7 days of exposure to contaminants or control incubation



Source: Santana, 2020.

Figure 5.9- Linear model of the kinetics of nitrate consumption on the sediments of the Juliana river source under different treatments



Source: Santana, 2020.

5.5 DISCUSSION

Pristine river sediments were exposed to two kinds of common pollutants, glyphosate and crude oil, in order to evaluate the immediate effect these pollutants had on the capacity of the organisms to metabolise nitrate and the effects of the exposure on the prokaryotic population structure over seven days. An important change in the patterns of prokaryotic diversity and community structures after the 7 day period was observed and the incubation process itself led to a large decrease in median biodiversity. The biodiversity in the glyphosate samples was higher than the control, and in the experiments with oil it was lower. While surprising, the higher biodiversity in the glyphosate samples could be associated with new resource niches which were open with the caused metabolic impact. Conversely, the lower biodiversity in the oil treatment may be due to the success of *Corynebacteriaceae* to rise to dominance in one of the replicates.

The most important usage of glyphosate by microorganisms is as a source of C and N for the synthesis of microbial biomass (WANG et al., 2016). However, the effects glyphosate has on communities are largely dependent on environmental conditions and, thus, the sediment characteristics are also important for determining the fate of the pesticide and effects on the microbiota (BUSSE et al., 2001). While in the experiment all plant material was

removed before addition of the pesticide, we nonetheless observed altered community structures.

Distinct groups of microorganisms are important for bioremediation of impacted areas, being either tolerant to petroleum or capable of degrading these compounds, (NKANANG et al., 2018; YUDONO et al., 2010; TALAIEKHOZANI et al., 2015). However, oil spills can negatively affect key microbial groups that are associated with the biogeochemical cycles (BABALOLA et al. 2016; EDET; ANIKA; UMOREN, 2019). We observed a decreased biodiversity in the sediments exposed to oil and a large increase in a subgroup of *Actinobacteria*, the family *Corynebacteriaceae*, suggesting that the oil had some level of toxicity to the majority of taxa. This family has previously been found in sediments affected by oil spill and in oil degrading communities, with representatives of *Proteobacteria* (JIMÉNEZ et al., 2007; THAVASI; JAYALAKSHMI; BANAT, 2011; NDIBE; EUGENE; USMAN, 2019), the last being long known as important groups in oil impacted sediments (REUNAMO et al., 2016; GAO et al., 2014; BRAKSTAD; LØDENG, 2005). The findings suggest that the main carriers of bioremediation of oil in these sediments would be the members of *Corynebacteriaceae*.

Most taxonomic groups showed small differences between the samples of pre-treatment and not treated microcosms after 7 days, for the most abundant phyla, except for *Firmicutes*, on which the incubation process caused a substantial decrease in abundance. However, despite the intense drop in abundance of *Firmicutes*, the *Lachnospiraceae* family was relevant to the P cycle (> 5%) in the oil treatment. This result could be a reflection of the low biodiversity caused by the oil, which would make families with low abundance have important contributions to this pathway or it could indicate the relevance of this element for the group. While *Lachnospiraceae* is not very explored in the literature, other clostridial members have shown great dependency of phosphate concentrations for some important processes, as well as high-affinity phosphate uptake systems (FISCHER et al., 2006), suggesting that they may be metabolic specialists.

Many taxa contributed to several metabolisms and this could be mainly correlated with the abundance of the groups, as exemplified by the large contributions of *Corynebacteriaceae* to all metabolic pathways in the oil treated microcosms. This is possibly due to the functional redundancy, when many organisms in an environment can provide all or part of the function of a pathway, that is usually observed in the soil communities (BARNES; CARTER; LEWIS, 2020; TOLKKINEN et al., 2020). It is notable that while these metabolic generalists are most

likely to play a role in the event of taxa replacement and community structure changes, they are not only observed as dominant taxa in the polluted sites but in pristine ones too.

The numerous functional pathways that were increased by the addition of crude oil and glyphosate, suggest that the changes in the community structures under stressful conditions favoured groups with higher efficiency in these metabolisms. This is especially notable for the oil treatment, where the increase in functional abundance is coupled with a great loss of biodiversity. A potential reason for the decrease in function and biodiversity in the control sediments after 7 days is that the added contaminants possibly served as nutrient and carbon sources for some taxa, while the control samples may have become nutrient depleted faster during the incubation. In this sense, glyphosate can provide some C and N for the community, while the processes of hydrocarbon degradation, such as benzene metabolism, usually depends on anaerobic electron accepting processes such as iron, nitrate and sulfate reduction (BERDUGO-CLAVIJO; GIEG, 2014; WANG et al., 2016), which in turn would increase the metabolism potentials for the different elements.

The enriched KOs observed in the glyphosate treatment included pathways associated with glyphosate resistance. One of such metabolisms is driven by the enzyme N-Acetyltransferase that is involved in the aminoglycoside resistance process, being previously selected under glyphosate exposure (BOTE et al., 2019). The enrichment of CAMP resistance modules (M00744) is also notable, since, despite not being directly known to grant resistance to glyphosate, they are frequently present in organisms with resistance (KURENBACH et al., 2015). The enrichment of a class of enzymes (glycosyltransferase) GALT29A (K00786) associated with glyphosate resistance in plants (PIASECKI et al., 2019) is also an indicative of the capacity of the microbial community to this metabolism. Finally, the enrichment in the cytochrome complex III respiratory module (M00151) is interesting, as one of the potential side targets of glyphosate is the cytochrome complex II (PEIXOTO, 2005), suggesting that taxa with higher abundances of the downstream complex may have some additional resistance to the effects of glyphosate.

While the pathways for benzene degradation and the anoxygenic photosystem II are also enriched in the glyphosate treated samples, they are both in greater abundance in the oil treated samples (22% and 152%, respectively). Additionally, the enrichment of both the catechol meta-cleavage pathway module (M00569), a major metabolism for the degradation of aromatic compounds, and the methane oxidation to formaldehyde module (M00174) was observed. This suggests that taxa able to metabolise hydrocarbons such as methane and benzene were more fit in the oil treated samples.

However, the majority of the methane metabolic potentials decreased in treated samples when compared to the control, especially for oil trial. The main methanogenic families in the sediments, *Methanomicrobiaceae*, *Methanosaetaceae* and *Methanoregulaceae* in fact showed a lower contribution of carbon metabolism genetic information, although many studies show the importance of methanogenic organisms for oil degradation processes (JI et al., 2019; VILCÁEZ et al., 2018; JONES et al., 2008). We hypothesize that environmental conditions in the microcosms were not adequate for the proper biodegradation of oil by the methanogens possibly due to the lack of a required low redox potential condition for sustaining the anaerobic consortium (DOLFING; LARTER; HEAD, 2008). Thus, the presence of organisms with the potential to degrade the contaminant are not a guarantee of a success in the maintenance of their ecological attributes and functional activities.

The decrease in nitrate consumption kinetics caused by addition of glyphosate could correlate with previous observation that microbial groups involved in nitrogen cycle are sensitive to pesticide contamination (ZABALOY et al., 2016). Glyphosate can pose toxicity to some microorganisms by the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPPS), while the presence of the C-Plyase enzymatic complex can allow for it to be metabolized (SVIRIDOV et al., 2015). This indicates that the fraction of microorganisms that are negatively affected by glyphosate in these sediments is big enough that the interference on the nitrogen cycle is immediately observed after contamination.

Although the crude oil led to a larger decrease in biodiversity after 7 days, its immediate effect on the nitrate transformation kinetics seemed to be less disruptive than the effects of glyphosate. The consumption of nitrate is an important route for oil fractions metabolism, since nitrogen is often a limiting factor to biological hydrocarbon degradation (WALWORTH et al., 2007), as some polyaromatic hydrocarbons have high degradability under denitrifying conditions (BOLLIGER et al., 1999; SCHÜRMANN et al., 2003). Thus, we hypothesize that despite being affected by the introduction of crude oil in the system, the microbial community readily started to use the nitrate in the microcosm for its biodegradation, which resulted in a smaller difference in transformation rates.

The families that succeeded in the oil-contaminated microcosms while being extinct in another treatment mostly belonged to the phyla *Proteobacteria*, *Actinobacteria* and *Acidobacteria*, which could be expected considering the literature (NDIBE; EUGENE; USMAN, 2019; YU et al., 2020; BIDJA ABENA et al., 2020; OBI; ATAGANA; ADELEKE, 2016). These taxa are likely to be the main drivers of the initial oil degradation process using the available nitrate in the system, as it has been shown to be an important nutrient for

biostimulation of hydrocarbonoclastic bacteria (LEONCIO et al., 2020; LIMA et al., 2012). Although some of these families are still uncultured, the results indicate that the source sediments of the Juliana river potentially have an important prokaryotic oil degrading community, beyond the *Corynebacteriaceae* family.

5.6 CONCLUSION

The effects of contaminating glyphosate and crude oil on the structure and functional potentials of the prokaryotic community in the sediments of a preserved riverine system were investigated. We measure both the immediate effects of these contaminants to the processes of nitrate transformation and the population scale changes over the course of a 7 day exposure. The immediate impacts of these contaminants on the nitrate transformation rates, show a higher interference of the glyphosate in this function, which led to a larger decrease (50%) in nitrate removal in the system. The genomic findings reveal that the biodiversity is decreased in the control microcosms after 7 days, suggesting a rapid depletion of nutrients and the importance of environmental factors that could be tested in the laboratory. The increase in diversity on the glyphosate treatment microcosms suggests the use of this compound as a nutrient source in the system, while the lower diversity in the oil treated microcosms suggests some level of toxicity. The functional analysis showed that the addition of contaminants led to an increase of many metabolic pathways correlated with the required routes of biodegradation of the contaminants. It is assumed that the metabolic functions were mainly carried out by the most abundant taxa in each treatment. Nonetheless, it was observed that the information regarding metabolic potentials was relatively stable throughout the experiments. This indicates a significant potential that the microbial community shows for promoting ecological resilience under stress. Finally, we observed that the community structure will change according to the type of impact, resulting in significantly different communities, but also found that these sediments are capable of keeping the main metabolisms of the studied elements at least for the first 7 days of testing. Some taxa in the sediments resisted the oil contamination, while going extinct at the control and glyphosate microcosms, and showed the metabolic potential to use the crude oil as substrate. This indicates that some indigenous prokaryotes in these source sediments could be further studied for oil bioremediation. It is also important to consider that the crude oil fractions can take longer times to be bioavailable and that this could also influence the dynamics in the microcosms. Similarly, populations

within the glyphosate samples may harbor additional mechanisms of glyphosate resistance. Importantly, there was intrinsic variance in populations in replicates of all samples, most notably a replicate of the Oil sample where *Corynebacteriaceae* became the dominant taxa. This variance in populations represents competing experimental shifts of prokaryotic populations exposed to pollution and suggests that additional replicates may have given rise to alternative evolutions. More research should also be carried out in order to observe the effects of longer term exposure and compare it between the other compartments of the river basin, given its importance as a preservation area.

6 DOMESTIC SEWAGE DISCHARGES INDUCE LARGE-SCALE CHANGES IN DIVERSITY AND PROKARYOTIC COMMUNITIES FUNCTIONAL ADAPTATIONS IN MANGROVE SEDIMENTS

6.1 ABSTRACT

Mangroves are tropical ecosystems with strategic importance for local and global scales. They are also under considerable environmental stress due to fragmentation, degradation, and urbanization. However, the complete understanding of how anthropogenic actions can affect biodiversity and microbial functional adaptations is still lacking. In this study, we carried out 16S rRNA amplicon analysis using sediment samples from two distinct mangrove areas located within the same estuarine system. The first sampling area was located around an urban area, being impacted by domestic sewage discharges and the second was a pristine site. The results show significant changes in the structure of the communities. Biodiversity along with microbial functional potential increased in the impacted area. The environmental factors of organic matter and pH, salinity, dissolved oxygen, Ni and Zn were significantly correlated with the observed shifts in the communities. The results also suggest that the contamination by domestic sewage caused the extinction of taxa that were abundant at the pristine site, including taxa associated with the anammox process, thus altering the N cycle. Conversely, the impacted site was enriched in prokaryotic families that are known as human pathogens.

Keywords: Mangrove; eutrophication; urban sewage; sediment microbiome

6.2 INTRODUCTION

Mangrove forests are coastal ecosystems mostly present in tropical and subtropical areas, representing more than 70% of the world's coastline (YAO et al., 2020; SINGH; RAMANATHAN; K., 2005; IMCHEN et al., 2018). Brazil has the largest area of mangrove forests in the world (CABRAL et al., 2016; HUERGO et al., 2018; MOITINHO et al., 2018). These ecosystems are recognized to be of strategic importance for climate mitigation, due to the capacity of sequestration and storage of large amounts of carbon (HOWARD et al., 2017; MOITINHO et al., 2018) as well as means of protecting the coastal area from erosion and rising sea levels. Many aspects of mangrove ecological health and function are tied to microbial metabolic activities that play essential roles in large-scale biogeochemical nutrient cycling (IMCHEN et al., 2017). Thus, there is a significant scientific interest with the area and several metagenomic studies have been reported in the past few years (FERNANDES et al., 2014; MARCOS et al., 2018; YUN; DENG; ZHANG, 2017; ZHOU et al., 2017).

Globally, mangrove ecosystems are threatened by habitat degradation and loss due to anthropogenic disturbances such as urbanization, development, and increasing population densities in coastal areas (GONG et al., 2019; NESME et al., 2016; FERNANDES et al., 2014; IMCHEN et al., 2018). In Brazil, where a large number of mangrove studies have been performed, numerous studies have been conducted in disturbed mangroves (ANDREOTE et al., 2012; SANDERS et al., 2014; CABRAL et al., 2016; MOITINHO et al., 2018), mostly without direct comparison to undisturbed sites. Undisturbed mangrove sites have been conserved within Brazil's Environmental Protection Areas (APA), where human activities are limited, in order to maintain the most natural conditions. The Serinhaém estuary and mangrove forest in the Pratigi APA, encompasses a 32 km long area. The Pratigi APA is characterized by the presence of small urban areas interspersed within dense Atlantic Rainforest vegetation (CORRÊA-GOMES et al., 2005). It is important to consider that, before the definition of the Pratigi Protection Area in 1998, the urban locations were already present, and were integrated in the efforts for a sustainable use of the natural resources (MMA, MINISTÉRIO DO MEIO AMBIENTE, 2004). Despite the presence of these urban areas, the APA has received highly satisfactory quality indices (LOPES, 2011; DITT et al., 2013; MASCARENHAS et al., 2019). Even with this high level of environmental quality globally, some local anthropogenic disturbance is still present, as is the case of the existence of constructions in a deforested spot of the mangrove around the city of Ituberá (Figure 6.1). In this location, it is possible to observe clear signs of anthropogenic impacts, such as raw sewage runoff and the withdrawal of the native vegetation within the immediate vicinity.

The existence of a small locus of disturbance within a largely conserved estuarine system provides for an opportunity to study how human activities impact microbial populations. We hypothesize that the input of domestic runoff and sewage leads to a more eutrophic environment close to the urban area, and that the higher bioavailability of nutrients favors the colonization of exogenous opportunistic prokaryotes, while leading to the extinction of the more sensitive endogenous taxa. This would, ultimately, create significantly distinct communities with distinct metabolic activities. To accomplish this we conducted 16S rRNA amplicon analysis in both preserved and impacted mangrove areas within the Atlantic Rainforest region of the Pratigi APA, as well as measured environmental variables for each site.

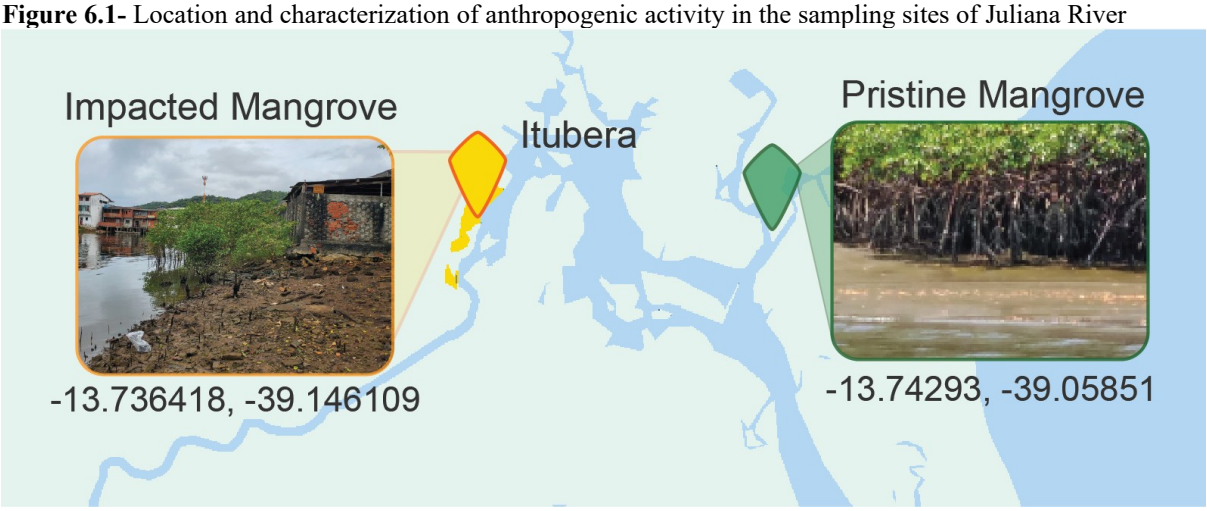
6.3 MATERIAL AND METHODS

The following topics will detail the methodology used for the present study.

6.3.1 Study area

The Serinhaém estuary (Figure 6.1) is located within the limits of the Environmental Protection Area of Pratigi (APA Pratigi) in the state of Bahia, Brazil between the coordinates 13º55'48"S and 38º57'13''W. The APA is defined as an area of sustainable use aiming for protection of remnants of the Brazilian Atlantic Forest and associated ecosystems, such as restingas and mangroves in which extractive and agricultural activities are controlled but still allowed (RIBEIRO et al., 2019). This estuarine region covers approximately 32 km of the Juliana River basin, a riverine system that is completely inside the limits of the protected area, and flows into Camamu Bay, where it meets the Atlantic Ocean (DA SILVA SANTOS; NOLASCO, 2017). Ituberá is a relatively small urban area of approximately 28,000 people within the Pratigi APA with a strong economic focus on tourism.

Figure 6.1- Location and characterization of anthropogenic activity in the sampling sites of Juliana River



Source: Santana, 2020.

6.3.2 Sampling and genomic analysis

The superficial sediment samples from both the impacted and the pristine mangrove areas of the Serinhaém Estuary were collected in triplicate (top 10 cm of the surface layer and 3 samples in each site) using a cylindrical sediment core sampler. The impacted collection site

was located in the boundaries of Ituberá and exhibited several signs of disturbance such as deforestation, presence of human habitations, and runoff of domestic sewage. The pristine collection site exhibited no visible signs of anthropogenic disturbance or pollution and was located several kilometers away from the impacted mangrove area. Samples from both sites had plants and other macroscopic organic materials removed, with care taken to avoid the collection of rhizosphere associated sediments.

This research assessed physical-chemical parameters such as dissolved oxygen, conductivity, pH and temperature in the water column using a multiparameter monitoring system (YSI model 85, Columbus). For each sediment core an aliquot was separated for the measurement of organic matter content and the other part was kept in the -20°C freezer for DNA extraction.

Total genomic DNA was extracted with the use of the PowerSoil DNA Isolation Kit (Qiagen, Carlsbad, CA, USA) from 0.25g of each sediment cores and stored at -80º C before amplification. For amplification of the V4 region of the bacterial 16S rRNA we performed PCR using the primer pairs 515F-Y (PARADA; NEEDHAM; FUHRMAN, 2016) and 806R-XT (CAPORASO et al., 2011) with a thermal cycler using the following program: for 2.5 µl of each sample, 5 µl the forward and reverse primers and 12.5 µl of the 2x KAPA HiFi HotStart ReadyMix were added, to the total volume of 25µl, and the samples were then subjected to following cycles: 1 x 95°C for 3 minutes, 25 x 95°C for 30 seconds, 1 x 55°C for 30 seconds and 1 x 72°C for 30 seconds and 1 x 72°C for 5 minutes.

Amplicons of the V4 region of the 16S rRNA gene were added with Illumina sequencing adapters and dual-index barcodes using the Nextera XT indices Kit according to manufacturer's directions (Illumina, San Diego, CA, USA). DNA paired-end sequencing (2x150) (GREGORY CAPORASO et al., 2012) was performed using Illumina MiSeq platform, V2 kit (300 cycles).

6.3.3 Data analysis

Demultiplexed sequences were filtered and trimmed with Trimmomatic (BOLGER; LOHSE; USADEL, 2014). For the samples from the Pristine Mangrove site, QIIME (CAPORASO et al., 2010) was used to join forward and reverse reads into single reads, resulting in the reads of approximately 250 bp in length that were used for subsequent steps. We used DADA2 (CALLAHAN et al., 2016) in QIIME2 (BOLYEN et al., 2019) to perform

the denoising step. The reads from the Impacted Mangrove were also denoised using DADA2. These were then merged with the denoised sequences from the Pristine Mangrove site for further analysis.

Sequences resulting from the denoising step were then clustered into Operational Taxonomic Units (OTUs). The alpha-diversity and beta-diversity tests were performed using QIIME2.

For taxonomic assignment we used the Vsearch (ROGNES et al., 2016) tool in QIIME2 with the Open Reference method with a 97% similarity against the reference 16S rRNA sequences in SILVA database (Silva SSU 132) (MCDONALD et al., 2012). Phylogenetic reconstruction was performed in QIIME2 resulting in alignment and phylogenetic tree files. Taxonomy assignment was carried out in QIIME2 using the representative sequences for each OTU and a QIIME2 feature classifier trained using the 97% similarity representative set containing only 16S rRNA sequences. In order to remove taxa with very low frequencies from correlation analysis, all groups were required to be present within at least 2 samples with a minimum of 3 reads each. The QIIME2 zip files generated by the pipeline were exported and processed in R, with the QIIME2R package (version 0.99.12).

Correlations between taxonomic community structure and environmental variables found in the samples sites were tested using the Vegan package (DIXON, 2003) in R.

We used PICRUSt2 (version 2.3.0-b) (DOUGLAS et al., 2019; BARBERA et al., 2019; CZECH; BARBERA; STAMATAKIS, 2020; LOUCA; DOEBELI, 2018; YE; DOAK, 2009) with default settings for functional analysis using the observed OTU abundances generated by QIIME2. KEGG Orthologs (KOs) were analyzed for significant (p-value <= 0.05) differences and were then used for the construction of the heatmaps of KOs with differential abundance between pristine and impacted mangrove. Pathway analysis of these KOs was performed using KEGG Mapper (KANEHISA; SATO, 2020). For a pathway to be considered we required that the entire pathway needed to be significantly enriched at a single site (ie. ‘complete’).

In order to identify which species were significantly different in abundance in each sampling area we carried out a taxa enrichment analysis. OTUs were combined into assigned taxa using the results of QIIME2. To be defined as significantly different, there must be at least 100 counts of the taxa in at least one site. The sets of observations between sites must be statistically significant (p-val <= 0.05) as calculated by the Kruskal-Wallis H test, a one-way non-parametric test. And finally, we also required the effect size to be in excess of a 20% difference in abundance between sites. In order to determine taxa exclusive to a single site

with a high degree of certainty we required a taxa to have been observed 0 times (using unnormalized abundances) at one site and at least 100 counts at the other, with a minimum of 10 counts per replicate.

We also calculated metabolic pathway enrichment specific to a given taxa at a given taxonomic level. For this we relied on the relative functional abundance results of PICRUSt2 and applied Kruskal-Wallis H test to identify taxa that had significantly different taxa-specific pathway abundances. Furthermore, we require that a taxa at a single site must be significantly enriched relative to the other site and that the taxa contributes at least 5% of the total relative functional abundance in the site.

The entire computational workflow is available as a repository in github: https://github.com/pspealman/Project_Impact.

The impacted mangrove sediment sequencing data is available from NCBI BioProject PRJNA650560, while the pristine mangrove sediment data is available from NCBI BioProject as accession number PRJNA608697.

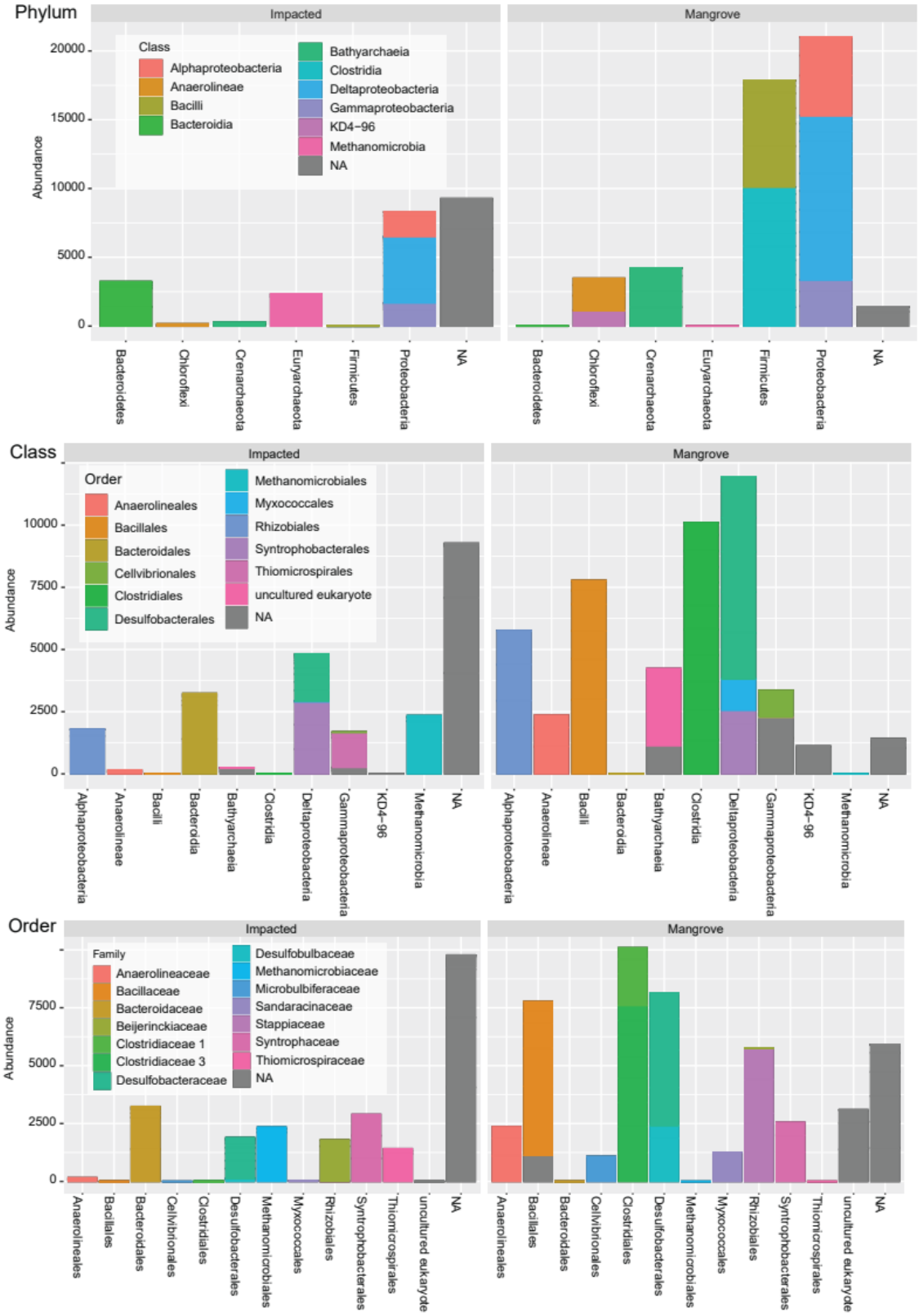
6.4 RESULTS

The results are synthesized by categories as follows.

6.4.1 Taxonomic profiles of the mangrove sediment communities

The prokaryotic communities in mangrove sediments of the two sites in the Serinhaém estuary are mainly composed of Bacteria with 90.5% of all sequences (469,754 sequences). Archaean groups accounted for 7.9% (40,837 sequences) and unassigned groups were 1.6% (8,313 sequences). The two mangrove areas presented considerable differences at the phylum level for taxa with more than 1% abundance in the dataset with *Crenarchaeota*, *Firmicutes* and *Chloroflexi* only appearing in the unimpacted mangrove area and *Euryarchaeota* and *Bacteroidetes* only found in the polluted sediments. Thus, 99% of all prokaryotic taxa belong to 6 phyla. The only phylum with more than 1% of the sequences that was present in both mangrove areas was *Proteobacteria*, with higher abundances in comparison to the other phyla and with classes *Alphaproteobacteria*, *Deltaproteobacteria* and *Gammaproteobacteria* also present in impacted and pristine sediments (Figure 6.2). Below the family level we observed a high dominance of unassigned and uncultured groups.

Figure 6.2- Relative abundances of prokaryotic groups for each detected taxonomic level

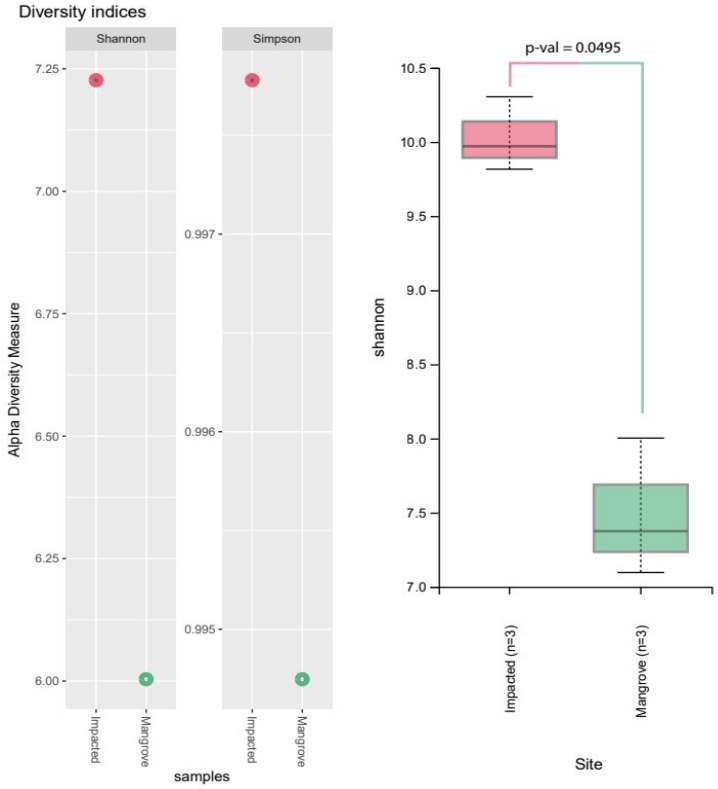


Source: Santana, 2020.

6.4.2 Prokaryotic diversity in mangrove sediments

The comparisons between the two collection sites show that the eutrophication caused by sewage discharge led to a significantly higher diversity (Kruskal-Wallis test on Shannon diversity index, p-val < 0.05) in the impacted mangrove sediments relative to the pristine sediments (Figure 6.3). This indicates that the domestic sewage increased the diversity and bioavailability of nutrients in the impacted mangrove sediments.

Figure 6.3- Diversity indices of each sampling site and statistical analysis of the differences observed in diversity between sites



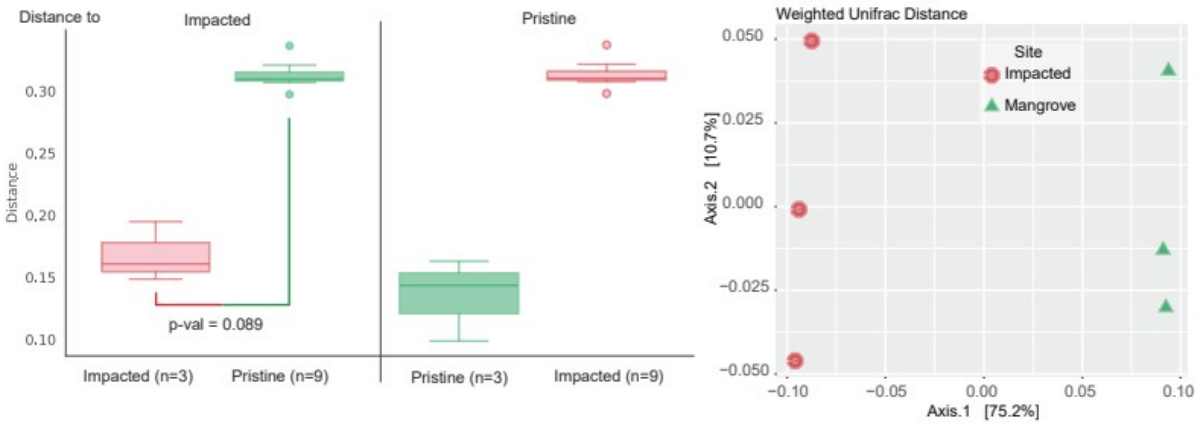
Source: Santana, 2020.

6.4.3 Structural differences between communities

The prokaryotic communities of the sediments from the two mangrove areas were compared for structural differences (Figure 6.4). It is possible to observe that the samples from each site group closer to each other in the Principal Coordinate Analysis (PCoA) plot, suggesting the existence of two separate communities. This is confirmed by the PERMANOVA analysis that found the differences to be statistically significant ($p < 0.1$). This suggests that the anthropogenic impact in these mangrove sediments resulted in a distinct

prokaryotic community structure, which could also be a result of the interactions with the other environmental variables.

Figure 6.4- Boxplots of PERMANOVA statistical test on prokaryotic communities between sites (left). PCoA plot of Weighted Unifrac distance metrics (right)



Source: Santana, 2020.

6.4.4 Functional profiles

Functional profiles of the two mangrove sites were calculated in order to identify statistically different abundances in the potential metabolisms of Nitrogen, Carbon, Phosphate and Sulfur between the pristine and impacted mangrove sites (Figures 6.5 and 6.6).

The carbon metabolism heatmaps are subdivided in carbon fixation for photosynthetic organisms (ko00710), prokaryotes (ko00720) and methane (ko00680) metabolism, to allow a better visualization (Figure 5). Generally, the carbon fixation pathways present higher functional abundance in the impacted site than in the pristine mangrove.

The results show that the impacted site is richer in the formaldehyde assimilation pathway (M00345), 2-Oxocarboxylic acid chain extension (M00608), and Serine biosynthesis metabolisms (M00020). The impacted site is also richer in the reductive pentose phosphate cycle associated with photosynthetic organisms (M00166, Calvin Cycle). The same was observed with the upstream crassulacean acid metabolism pathways (CAM), either affected by light (M00169) or dark (M00168) (CRAWFORD et al., 1984); (LÜTTGE, 2015). Additionally, for carbon fixation in prokaryotes, the impacted site is enriched in the second carbon step of the citrate cycle (M00011).

Interestingly, the results show no complete pathway enrichment in the pristine site but, it is consistent with the hypothesis that the impacted site is highly productive at its eutrophicated state.

The heatmap (Figure 6.6) shows enrichment for the nitrogen metabolism in the anthropized site of the mangrove, with a significant increase in the assimilatory nitrate reduction (nitrate to ammonia) pathway (M00531). The functional profiling of the phosphorus metabolism has also shown a tendency for an enrichment of KOs in the impacted mangrove area. This enrichment includes the pentose phosphate pathway (M00004), both oxidative (M00006) and non-oxidative phases (M00007), as well as the archeal pathway (M00580).

For the sulfur metabolism, some KOs are absent or nearly absent in the pristine sediments while generally present in the impacted area. A significant enrichment in the assimilatory sulfate reduction (M00176) and Cysteine biosynthesis (M00021) pathways was observed in the impacted sediments. Many metabolic KOs for photosynthesis were absent in the impacted sediments while having low abundance in the pristine area, but generally, the sediments near the urban area presented the majority of enrichment for photosynthetic pathways, and in fact are significantly enriched in F-type ATPase ATP synthesis (M00157). Taken together, these findings suggest that the high abundance and diversity in the impacted area leads to more enrichment in the majority of metabolic pathways for the studied elements.

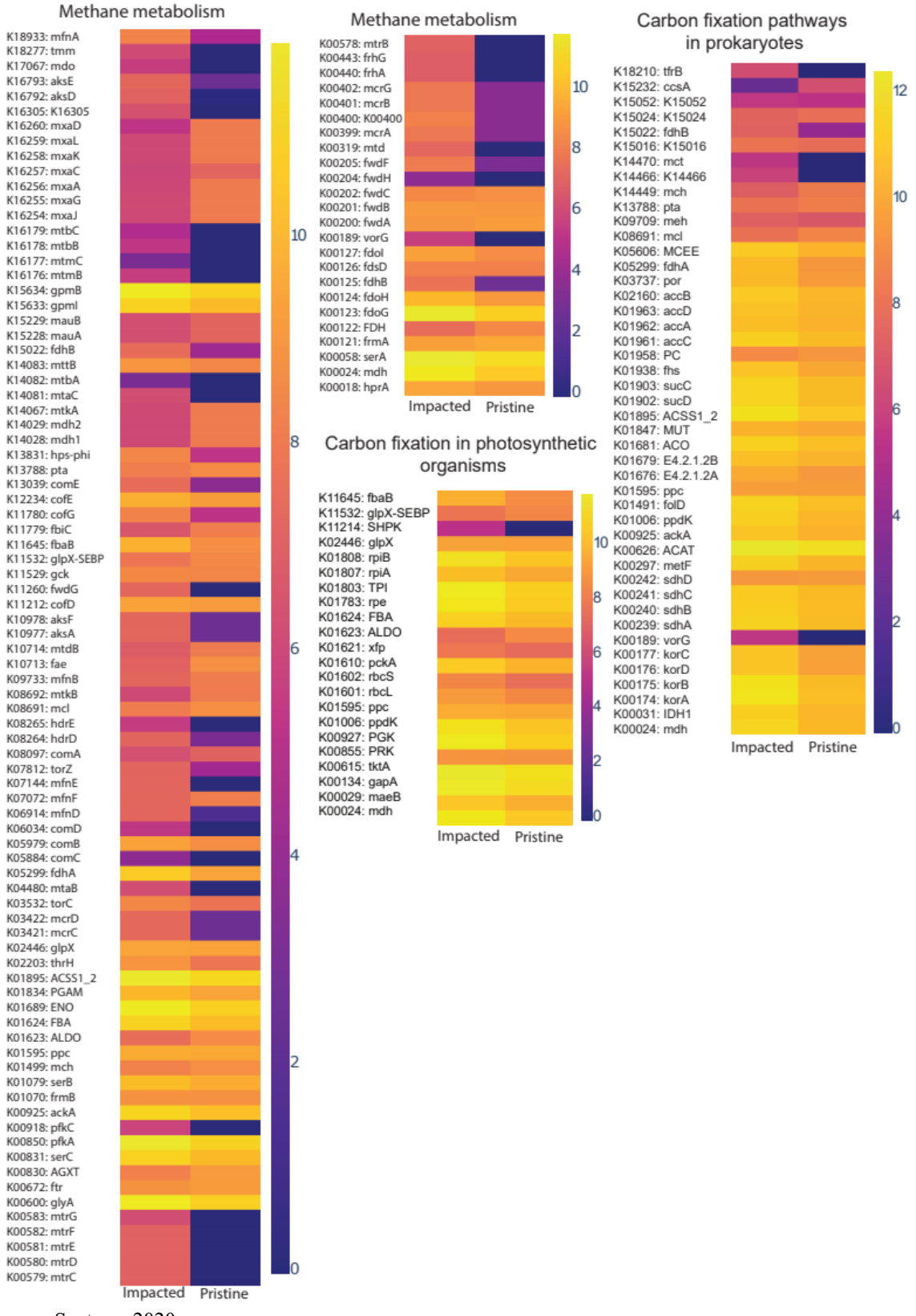
6.4.5 Taxa enrichment and exclusivity

Some taxa were found to be present in both mangrove areas, but with statistically significant differences ($p<0.05$) in abundance. The figure 6.7 shows all the families that showed large effect size differences between the sites (>20%). The two studied areas show near parity between the number of enriched taxa (14 enriched in the pristine, 13 enriched in the impacted). This suggests that the introduction of urban waste at the impacted site may lead to decreases in abundance of sensitive taxa from the pristine sediments but also, that some other taxa adapt well to the more eutrophic environment.

The biotic and abiotic selective pressures can lead certain native taxa of the mangrove to be locally extinct near the impacted site. On the other hand, some taxa that exist in the impacted site may not be adapted to the environment found at the pristine site. For this reason, the taxa that are exclusive to a given area were also determined. All the families that were unique to one of the mangrove sites are represented in the figure 6.8, which shows that the

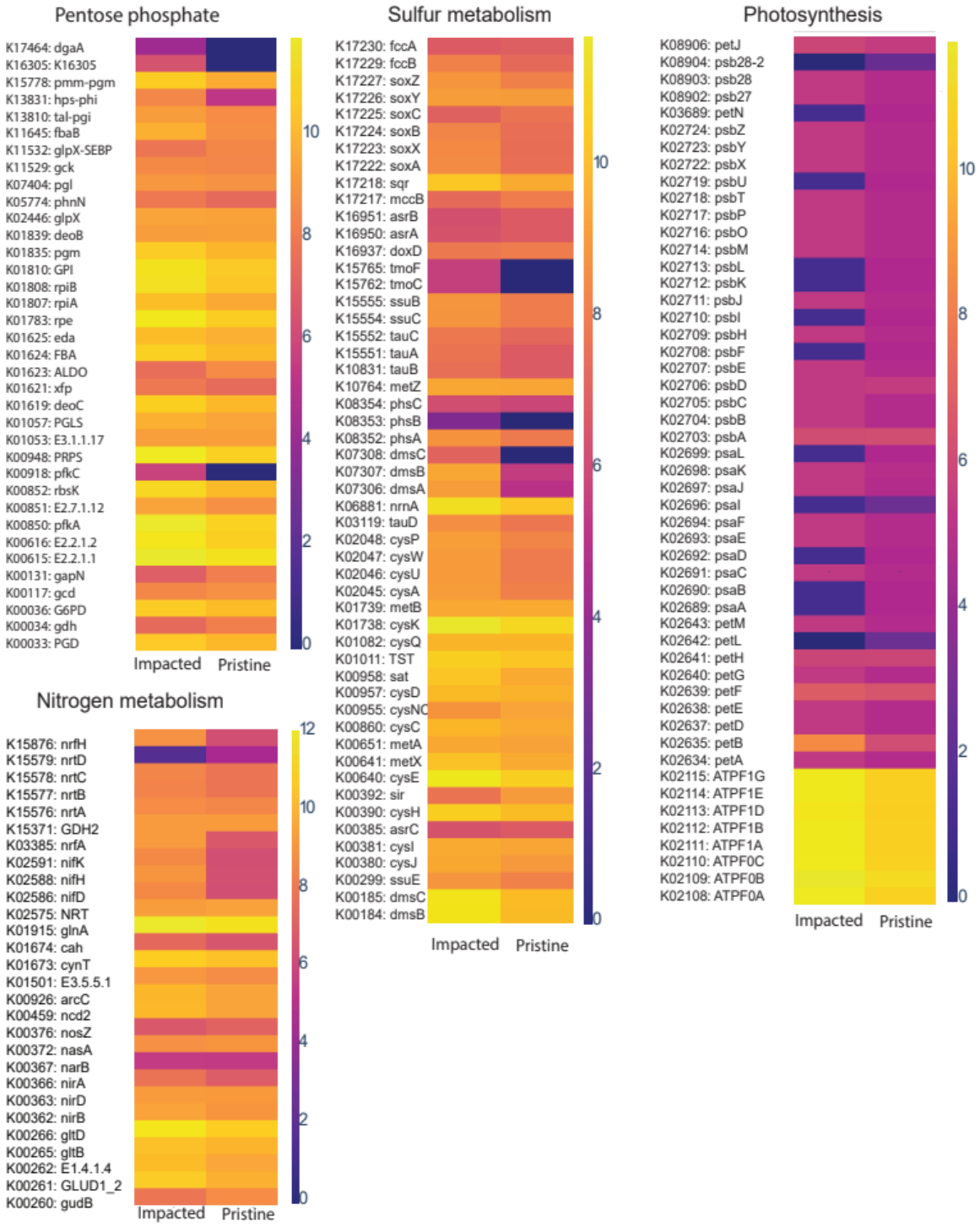
majority of these families were found in the sediments of the impacted mangrove, in accordance with the findings that the impacted site supports high microbial biodiversity.

Figure 6.5- Heatmaps for abundances of carbon metabolic pathways at each studied site



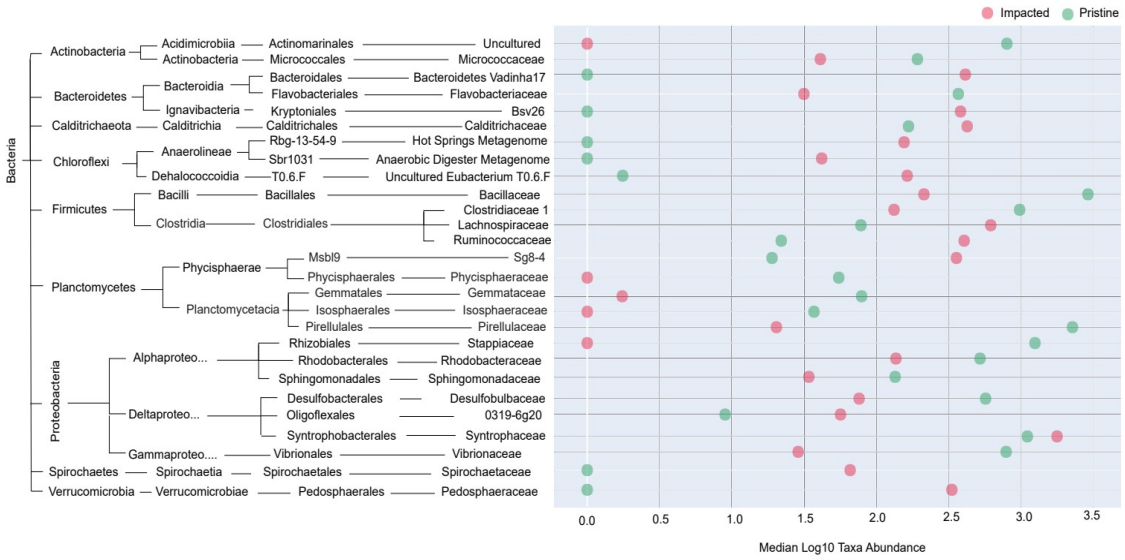
Source: Santana, 2020.

Figure 6.6- Heatmaps of metabolic pathways abundances for nitrogen, phosphorus, photosynthesis and sulfur, comparing the two mangrove collection sites



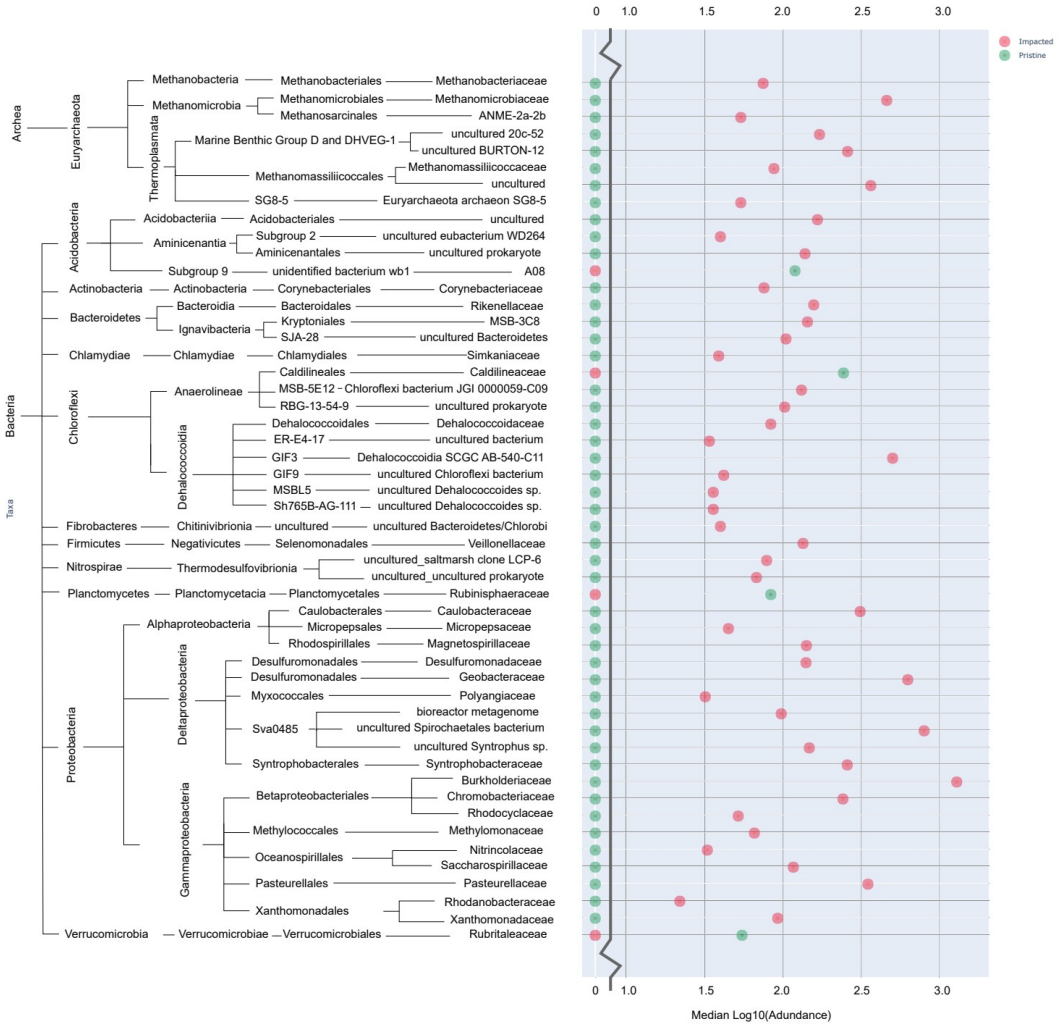
Source: Santana, 2020.

Figure 6.7- Microbial families observed in both sites, but with significant differences in abundance values



Source: Santana, 2020.

Figure 6.8- Microbial families that were exclusively observed in one of the mangrove sites



Source: Santana, 2020.

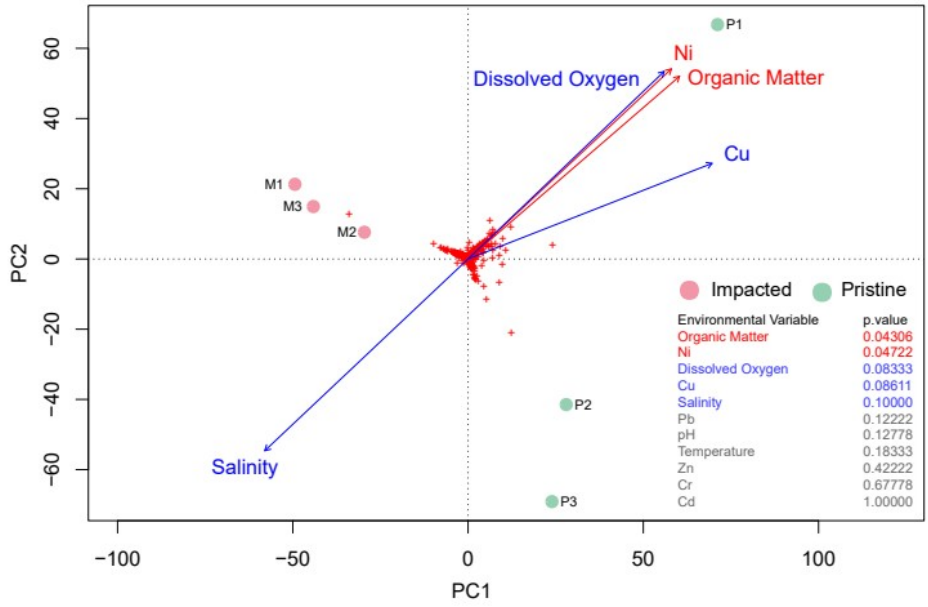
6.4.6 Influences of environmental factors

The analysis was carried out in order to test the correlation between each site's community structure with the assessed environmental factors. The results show a significant correlation between the prokaryotic communities and the variables: organic matter, dissolved oxygen (D.O.), salinity, Ni and Cu (Figure 6.9). These results confirm the importance of external environmental factors to the microbiomes of each site.

6.4.7 Contributions of distinct taxa to the metabolic potentials

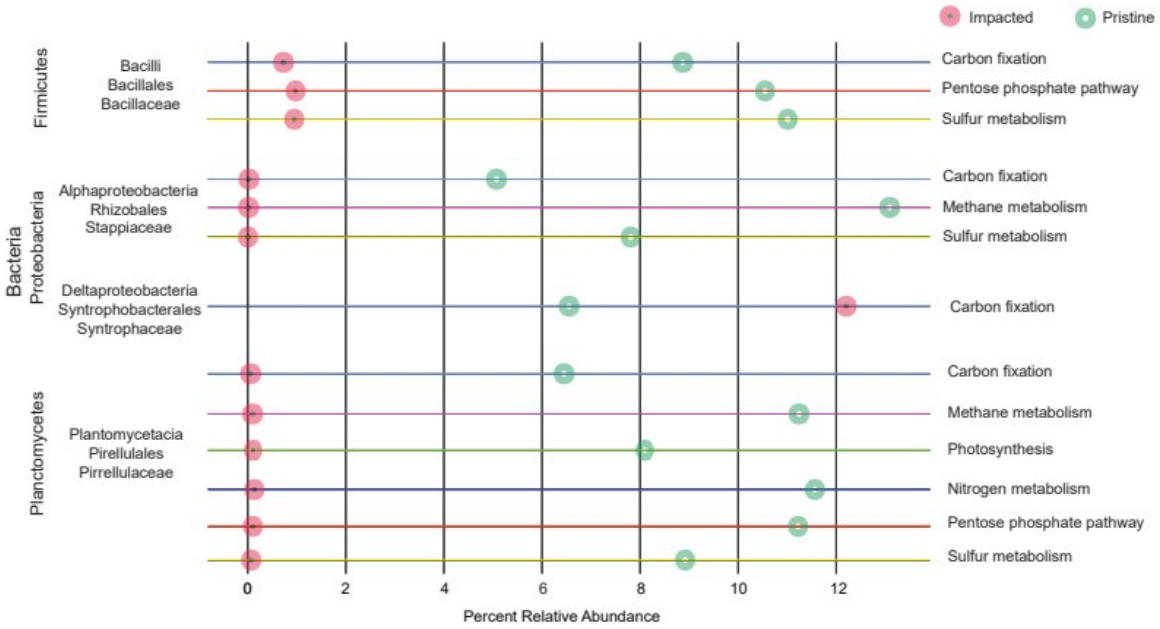
This research analyzed the contributions that each family makes for the functional metabolism of C, N, P and S. Prokaryotic families that contributed above 5% relative abundance to some of these metabolisms are displayed in Figure 6.10. The results show that the majority of the families with large metabolism contributions are in the pristine mangrove sediments, which display the lower biodiversity. Only the family Syntrophaceae presents large contributions in the two sediments, specifically for the carbon fixation metabolism, being higher for the impacted area. This indicates that a lower biodiversity leads to more taxa with large contributions to the functional potentials.

Figure 6.9- Principal component analysis describing the correlations between the environmental variables and communities structures



Source: Santana, 2020.

Figure 6.10- Relative contributions of the microbial families that contribute for > 5% of the metabolic potentials in at least one site



Source: Santana, 2020.

6.5 DISCUSSION

The results show that microbial biodiversity is higher in the impacted mangrove sediments than in the pristine site. Similar observations of the anthropic effects on diversity have been made previously in the comparison between mangroves in a preserved area and in an anthropized area (FERNANDES et al., 2014). Indeed, the urbanization process is known for its capacity to change the natural ecological spaces with high impacts to ecosystem services as well (CHEN et al., 2020).

The prokaryotic communities of the studied mangrove sediments were greatly influenced by environmental factors, such as the abundance of organic matter, nickel, copper, dissolved oxygen, and salinity. Previous study has shown that copper and other metal contents are directly associated with the lithology of the area (DA SILVA PEREIRA, 2016).

Organic matter is one of the most significant environmental variables affecting microbial diversity. Since mangrove sediments do not naturally have large amounts of available nutrients (Behera et al. 2019), these environments rapidly become eutrophic when exposed to the constant presence of urban tailings, leading to greater biomass production and biodiversity (GLIBERT, 2017; FERNANDES et al., 2014). Thus, the introduction of

domestic sewage in this specific area of the estuary has possibly caused eutrophication, altering the structure of the local prokaryotic community.

The findings of different taxa contributing to the metabolisms of the studied nutrient cycles are in accordance with the premise that sediment microbial communities present elevated versatility (BARNES; CARTER; LEWIS, 2020). In the case of the two mangrove sites, the potentials for element metabolisms were spread as a function of biodiversity, suggesting that the lower the biodiversity, the higher the importance of particular groups to the nutrient cycles.

Considering the high dependency of the ecosystem functions on fewer taxa observed in the pristine sediments, it could be expected that the disturbance caused by the introduction of urban waste would imbalance the elements cycles, even impairing some important metabolisms. The analyses of functional profiles shows, however, an opposite trend where the potentials for these metabolisms are higher in the impacted mangrove site. Urbanization is one of the main causes for environmental eutrophication worldwide, consisting of a source of different structures and higher amounts of organic matter and other nutrients (DONG et al., 2020), which can stimulate the increase of diversity as the competition for nutrients is lower (GLIBERT, 2017). This suggests that depending on the type of anthropogenic impact, the microbiome is capable of adapting to maintain the nutrient cycles.

Changes in organic matter and nutrient bioavailability caused by domestic sewage contamination have previously been associated with shifts in the diversity of sulfate-reducing bacteria (SRB) and sulfur-oxidizing bacteria (SOB) (MEYER et al., 2016). Thus, such an environmental impact can interfere with the sulfur cycle, as well as, with the carbon, nitrogen and phosphorus cycles (TOLKKINEN et al., 2020). The presence of genetic material associated to the biogeochemical cycles is not, however, a guarantee of a healthy ecosystem since several eukaryotic and even some prokaryotic species could be negatively affected by the eutrophication or other impacts caused by domestic sewage contamination (HOUSER; RICHARDSON, 2010). These interferences could, thus, have a cascading effect in the system.

The enrichment in the formaldehyde assimilation pathway in the impacted site could also be associated with human interference, as this compound is commonly found in animal waste, building materials, pesticides, and fertilizers. While formaldehyde assimilation is a common intermediary in the oxidation of methane by methanotrophs, it is also known that the influx can alter microbiomes (DIXON, 2003), suggesting that the prokaryotes at this site may have been selected to take advantage of this additional carbon source present in the urban

runoff. Although less prevalent than the methane metabolism pathway, the impacted site showed enrichment in other general carbon fixation pathways.

The results show a widespread variance in the abundance of taxa between sites and that some native taxa were extinguished in the impacted area. Although the majority of taxa persisted in the impacted site, some families were a characteristic of the pristine sediment, suggesting that human interference with sewage discharge is the main variable associated with disappearance of taxa. Evidence in support of this includes the absence of the subgroup 9 of *Acidobacteria* in the impacted sediments, since this group shows a strong negative correlation with the organic matter concentration in the environment (KIELAK et al., 2016).

It is also important to notice the absence of the entire phylum *Planctomycetes* in the impacted area. As the totality of *anammox* bacteria belong to this phylum, this implies that N cycling has fundamentally changed between the two areas (MARDANOV et al., 2019; WANG et al., 2012a). This suggests that the removal of the N from the impacted system is then totally carried by denitrifying organisms. While the ecological aspects of the families *Caldilineaceae* and *Rubritaleaceae* are still not well documented, their extinction in the impacted mangrove suggests that these groups could be oligotrophic or sensitive to some of the important components of domestic sewage.

Similarly, some of the taxa that were enriched or unique for the impacted mangrove area could be directly correlated with the discharge of domestic sewage. The prevalence of the family *Pasteurellaceae* is an example, since most of its subgroups are known pathogens of vertebrates and are not usually found outside hosts (CHRISTENSEN et al., 2014). Additionally, many genera belonging to the family *Spirochaetaceae* are known for causing a variety of human diseases such as syphilis, Lyme disease, leptospirosis, periodontal disease, among others (KARAMI et al., 2014). Furthermore, members of the *Ruminococcaceae* are among the most abundant groups found in the mammalian gut environment (BIDDLE et al., 2013). Finally, the enrichment in the uncultured group of *Bacteroidales* could be further evidence of fecal contamination, and this group has been proposed as a predictor of waterborne pathogens (SCHRIEWER et al., 2010).

These results are likely to be a consequence of the removal of native vegetation for the construction and occupation of houses with low sanitation infrastructure.

6.6 CONCLUSION

This study describes the effects of urban effluent on the prokaryotic communities of mangrove sediments, by means of comparing samples taken from both impacted and pristine areas of the same estuarine system. The analysis confirmed a significant change in the structure of the communities and an elevation of prokaryotic biodiversity in the sediments of the urbanized mangrove area. The functional analysis showed that the metabolism of the studied elements could be maintained and that the metabolic potentials in the impacted mangrove were higher than in pristine mangroves, probably due to the higher biodiversity in the area. The findings of diverse groups contributing to metabolic pathways suggests higher versatility in the impacted mangrove sediments, where the functions are carried out by a greater number of groups, while fewer groups are responsible for large functional contributions in the pristine site. Based on these results, it is possible to suggest that the microbiome of the pristine mangrove area presents a lower resilience to impacts that could result in a decrease in biodiversity. These results were found to be correlated with environmental factors that greatly impact the availability of organic carbon and nutrients such as organic matter and salinity. Despite increasing biodiversity, the effects of urban waste on the mangrove ecosystem as a result of domestic sewage introduction showed some clear negative effects, such as the extinction of some prokaryotic groups, as well as the colonization by human pathogens in the microbiome of the impacted area. In this sense, the relative stability observed in functional terms does not imply that the ecosystem is not negatively impacted. Finally, the results confirm the hypothesis that the two different areas of the serinhaém estuary present distinct microbiomes both in structural and functional terms. Further research would be valuable in order to address alternatives for the mitigation of impacts in this area and investigation of the recovery processes that could possibly take place in a recovering environment.

7 DIFFERENTIAL ANALYSIS OF PROKARYOTIC COMMUNITIES AND FUNCTIONAL PROFILES OF MANGROVE TIDAL ZONE SEDIMENTS FROM THE PRISTINE SERINHAÉM ESTUARY, BRAZIL

7.1 ABSTRACT

Mangrove forests are intertidal ecosystems that constitute a large portion of the world's coastline. Mangroves are composed of, and reliant upon, microhabitats defined by the tides. We are only beginning to understand microhabitat biodiversity and their role in nutrient cycling. Unfortunately, the majority of mangrove genomic studies have been conducted on anthropogenically impacted areas. Recent work has shown that even mildly disrupted mangroves can be severely affected leading to decreases in biodiversity and local extinctions. Here, we seek to characterize prokaryotic populations and their involvement in nutrient cycling across the tidal zones of a pristine mangrove forest. We collected mangrove sediments from an Environmental Protection Area within the Brazilian Atlantic Forest. Samples were collected in triplicate from zones below, between, and above the tidal waterline. Using 16S amplicon sequencing we found that these zones have different prokaryotic communities. A functional analysis found significant differences in nutrient cycling related functions between zones. Finally, we identified taxa with significant differences in populations between zones and their contribution to functional abundances. Our findings contrast those observed in anthropogenically impacted mangroves and suggest that some aspects of mangrove zonation may be compromised by human activity. We urge caution in generalizing from anthropogenically impacted habitats.

Keywords: Mangrove tidal zones, sediment microbiomes, prokaryotic diversity

7.2 INTRODUCTION

Mangrove ecosystems constitute a large portion of tropical and subtropical coastlines (YUNUS et al., 2011; DOS SANTOS et al., 2011). These ecosystems are characterized by periodic tidal flooding that leads to varying environmental conditions across small spatiotemporal scales, with levels of nutrients, oxygen, and salinity periodically fluctuating, resulting in frequent anaerobic conditions and a wide range of redox potentials in the sediments (ANDREOTE et al., 2012; LIN et al., 2019). These dynamic conditions, in turn, lead to high microbial diversity, and these microbes play essential roles in the functioning and maintenance of the greater ecosystem (IMCHEN et al., 2017; LIN et al., 2019; HUERGO et al., 2018).

Although mangroves have previously been shown to have a diverse prokaryotic population, distinct from the regions they border (ie. mountain forest and restinga), only recently there has been work to understand the differences between mangrove microhabitats,

that are mainly a result of tidal variations. The previous research that sought to characterize the prokaryotic microbiome across the tidal zones of the mangrove ecosystem, were mostly conducted in anthropogenically impacted areas (ROCHA et al., 2016; ZHANG et al., 2018); LV et al., 2016). In a pristine mangrove area, an investigation based in fingerprint analysis (T-RFLP) of the tidal influences on microbial communities did not identify large differences between tidal zones (MENDES; TSAI, 2018).

The mangrove forests in Brazil are connected with the Atlantic Forest, which is one of the most biodiverse biomes on the planet. In Brazilian coast, mangrove trees are primarily composed of the genera *Rhizophora*, *Avicennia*, *Laguncularia* and *Conocarpus* (PUPIN; NAHAS, 2014). Unfortunately, the Atlantic Forest and associated ecosystems are highly threatened by anthropogenic disturbances such as logging and farming, as well as habitat loss and fragmentation due to human encroachment, resulting in a severe decline in its original area (DITT et al., 2013; PUPIN; NAHAS, 2014; NOGUEIRA et al., 2015). However, in the southern part of Bahia State, Brazil, a significant fragment of the Atlantic Forest remains preserved within the Environmental Protection Area (APA) of Pratigi (MMA, MINISTÉRIO DO MEIO AMBIENTE, 2004). Recent studies on the environmental conditions of the area show that preservation efforts initiated in 1998 have been generally effective, resulting in high environmental quality relative to most mangroves, both in Brazil and globally (DITT et al., 2013; LOPES, 2011; MASCARENHAS et al., 2019). This preserved area constitutes an convenient site for the understanding of the ecology of unimpacted mangrove forests. This is relevant because, due to the above described variety of anthropogenic influences, conserved mangrove areas are much less prevalent than impacted ones, which can be confirmed by the increased number of studies made in anthropogenically impacted mangroves.

Thus, in order to improve our understanding of mangrove ecology, in this study we characterize the prokaryotic microbiota from sediments of three tidal zones in the pristine mangrove of the Serinhaém estuary, within the Pratigi APA, using 16S rRNA amplicon. This approach allows us to identify diverse taxa without the laborious task of culturing them (KAUR et al., 2015; BORNEMANN et al., 2015; NESME et al., 2016) and is a more powerful tool in comparison to classic techniques such as T-RFLP (DE VRIEZE et al., 2018). Furthermore, we assess the community structure and functional aspects of these prokaryotes to achieve a deeper understanding of the terrestrial processes at work in different environments.

Considering mangrove zonation as driven, primarily, by tide variation, we hypothesized that sediments of different mangrove regions would differ significantly in

richness and composition of prokaryotic communities as a result of differences in the physicochemical properties. We also hypothesize that the intertidal zone has the highest diversity as a result of the highly dynamic conditions. We assessed the prokaryotic communities, the influence of environmental variables and the functional profiles of these sediments. We also identified the possible taxa driving the different nutrient cycles between zones. Our study provides insight into the role of microbes in the functioning of mangrove forests and establishes a baseline for monitoring the health of this important ecosystem, since this information is still scarce for pristine mangrove sites.

7.3 MATERIALS AND METHODS

The following topics will detail the methodology used for the present study.

7.3.1 Study area

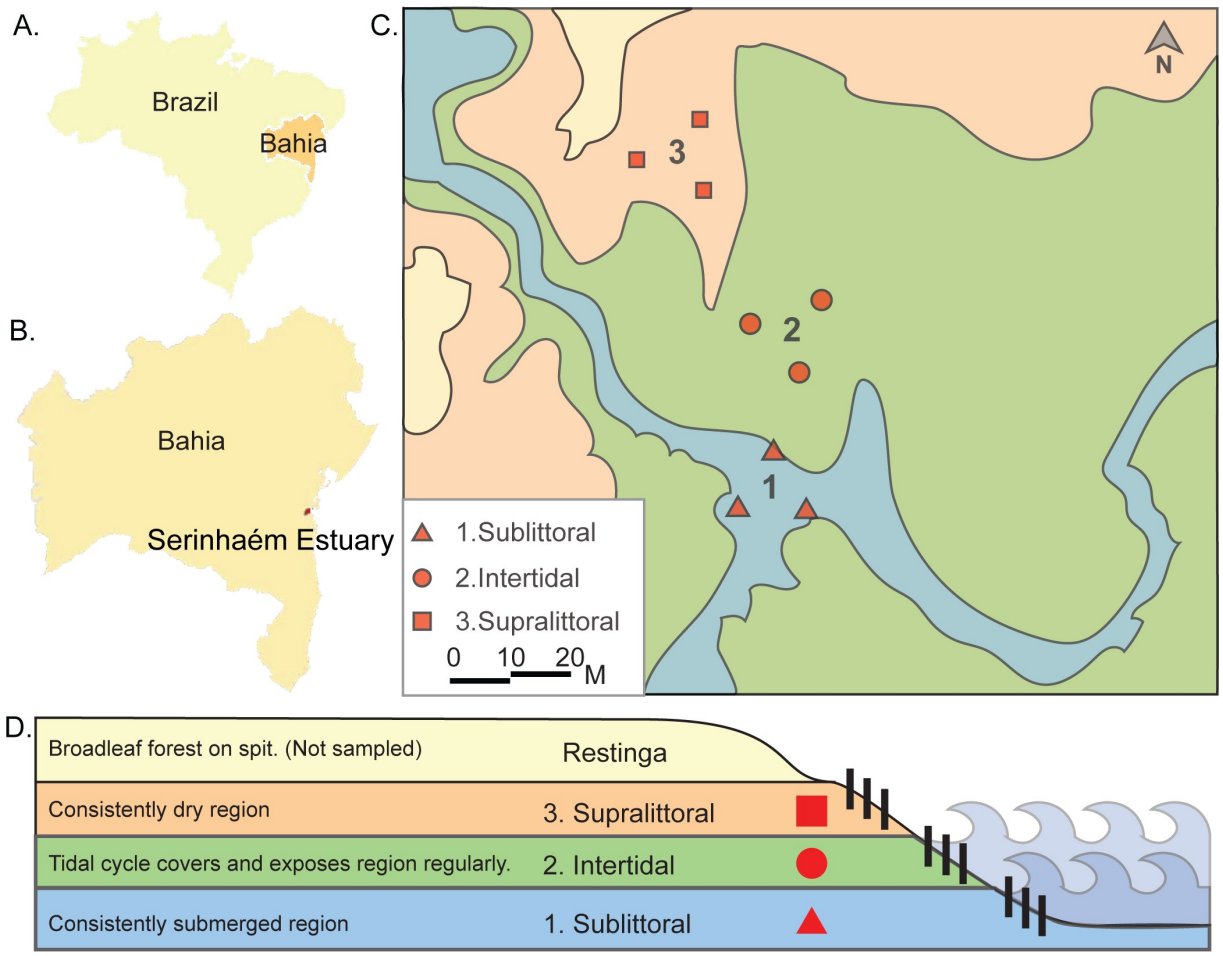
The Serinhaém Estuary is located in the Low South Region of Bahia State, Brazil (Figure 7.1), between the coordinates 13°35'S and 14°10'S and 39°40’W and 38°50’W. The estuary is within the Pratigi Environmental Protection Area (APA), one of the few remaining Atlantic forest regions with a total area of 85 686 ha. The estuary enclose a 32 km long portion of the lower Juliana River Basin and empties directly into Camamu Bay (CORRÊA-GOMES et al., 2005).

7.3.2 Sampling and DNA extraction

Samples were collected from 3 tidal zones (centered around 13°42'59.0"S, 39°01'35.9"W) in the Serinhaém estuary in July 2018 during the morning low tide period. No sites exhibited signs of anthropogenic disturbance or pollution. From each tidal zone, 3 samples of superficial sediments (top 10 cm of the surface layer) were collected with a cylindrical sediment core sampler with precautions taken to avoid the disruption of rhizospheres associated with vegetation. To ensure a broad representation of each zone, sample sites were located a minimum of 15m from each other in a triangle and for each site, 3 sediment cores randomly collected. Plant and other organic material was manually removed from core samples.

Physical-chemical parameters such as temperature, salinity and dissolved oxygen in the water column were measured using a multiparameter monitoring system (YSI model 85, Columbus). Each zone had different vegetation densities, with the sublittoral zone having the greatest plant density, and the supralittoral the least, with almost no vegetation. Metal concentrations were not collected as previous analysis performed by our lab (Jesus, T.B.) found no significant difference in metal concentrations relative to background within the Serinhaém estuary. After collection, samples were transferred to the laboratory and an aliquot was separated and kept in the -20°C freezer for subsequent DNA extraction while the remainder of the sample was used for measuring organic organic matter content using 'loss-on-ignition' method (NELSON and SOMMERS, 1996). The total genomic DNA was extracted from 0.25 g of sediment using the PowerSoil DNA Isolation Kit (Qiagen, Carlsbad, CA, USA). All DNA samples were stored at -20º C before analysis.

Figure 7.1- Map and schematic of sediment sampling sites. Brazil (A) and Bahia (B). Relation of the three sampling sites within each zone (1. sublittoral, 2. intertidal, 3. supralittoral, (C). Schematic showing the topographic and tidal relation of each sampling site (D)



Source: Santana, 2020.

7.3.3 Library preparation and sequencing

After DNA extraction, we used PCR to specifically target the V4 region of the bacterial 16S rRNA using the primer pair 515F-Y (PARADA; NEEDHAM; FUHRMAN, 2016) and 806R-XT (CAPORASO et al., 2011). DNA sequencing was performed using Illumina MiSeq platform, V2 kit (300 cycles).

7.3.4 Data analysis

Trimmomatic (BOLGER; LOHSE; USADEL, 2014) was used to filter and trim demultiplexed sequences. QIIME (CAPORASO et al., 2010) was used to join forward and reverse reads into single reads. Reads were denoised using DADA2 (CALLAHAN et al., 2016) in QIIME2 (BOLYEN et al., 2019), and clustered into Operational Taxonomic Units (OTUs). We performed a variety of alpha-diversity and beta-diversity tests using QIIME2. Taxonomic assignment used Vsearch (ROGNES et al., 2016) in QIIME2 using Open Reference with 97% similarity against the reference SILVA database (Silva SSU 132) (MCDONALD et al., 2012). Phylogenetic reconstruction was carried out in QIIME2 using the representative sequences for each OTU and a QIIME2 feature classifier trained using the 97% similarity (e.g. silva_132_97_16S.fna). All groups were required to be present within at least 2 samples with a minimum of 3 reads each.

QIIME2 files were accessed in R using QIIME2R (version 0.99.12). Tree visualization was performed with R (version 3.4.4) using Metacoder (FOSTER; SHARPTON; GRÜNWALD, [s.d.]) (version 0.3.2). Posterior analysis was performed using Phyloseq (MCMURDIE; HOLMES, 2013) (version 1.22.3). Analyses in R were plotted using ggplot2 (MCMURDIE; HOLMES, 2013; VILLANUEVA; CHEN, 2019). Vegan (DIXON, 2003) (version 2.5-6) was used to test correlations between community structure and environmental variables. Distances were calculated using metaMDS, (engine=monoMDS, try=1000, k=3), and then fit the environmental variables using envfit (default settings, permutations=333).

Functional analysis was performed using PICRUSt2 (version 2.3.0-b) (DOUGLAS et al., 2019; BARBERA et al., 2019; CZECH; BARBERA; STAMATAKIS, 2020; LOUCA; DOEBELI, 2018; YE; DOAK, 2009) with default settings. Both the KEGG Orthologs (KOs) and MetaCyc pathways were analyzed for significant (p-value <= 0.05) differential

abundances after centered log-ratio transformation (aldex.clr) using the general-linear model method (aldex.kw) of the ALDEx2 package (ver 1.18.0).

For taxa enrichment analysis, OTU abundances were normalized by downsampling to match the least abundant zone (Intertidal). For each taxa, we required that a significant difference be found between sites using a Chi-squared, 2x3 test, with correction (scipy.stats.chi2_contingency) using the mean normalized abundance. To correct for false positives we required the distributions of unnormalized OTU abundances between sites to also be significantly different (Mann-Whitney U test, scipy.stats.mannwhitneyu, p-val <= 0.05). Finally, the effect size was required to represent at least 5% difference in log-fold abundance between sites.

For calculating taxa specific KO enrichment, we required the taxa to have at least 10% of all KO functional abundance at the given level; and the functional abundance should be significantly enriched using a Binomial exact test (Bonferroni corrected p-value <= 0.05). Additionally, the taxa must have at least three distinct KOs that meet these criteria, all within the same metabolic pathway.

The entire computational workflow is available on github: https://github.com/pspealman/COSantana_2020.

The data has been deposited as PRJNA608697 in the NCBI BioProject database: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA608697

7.4 RESULTS

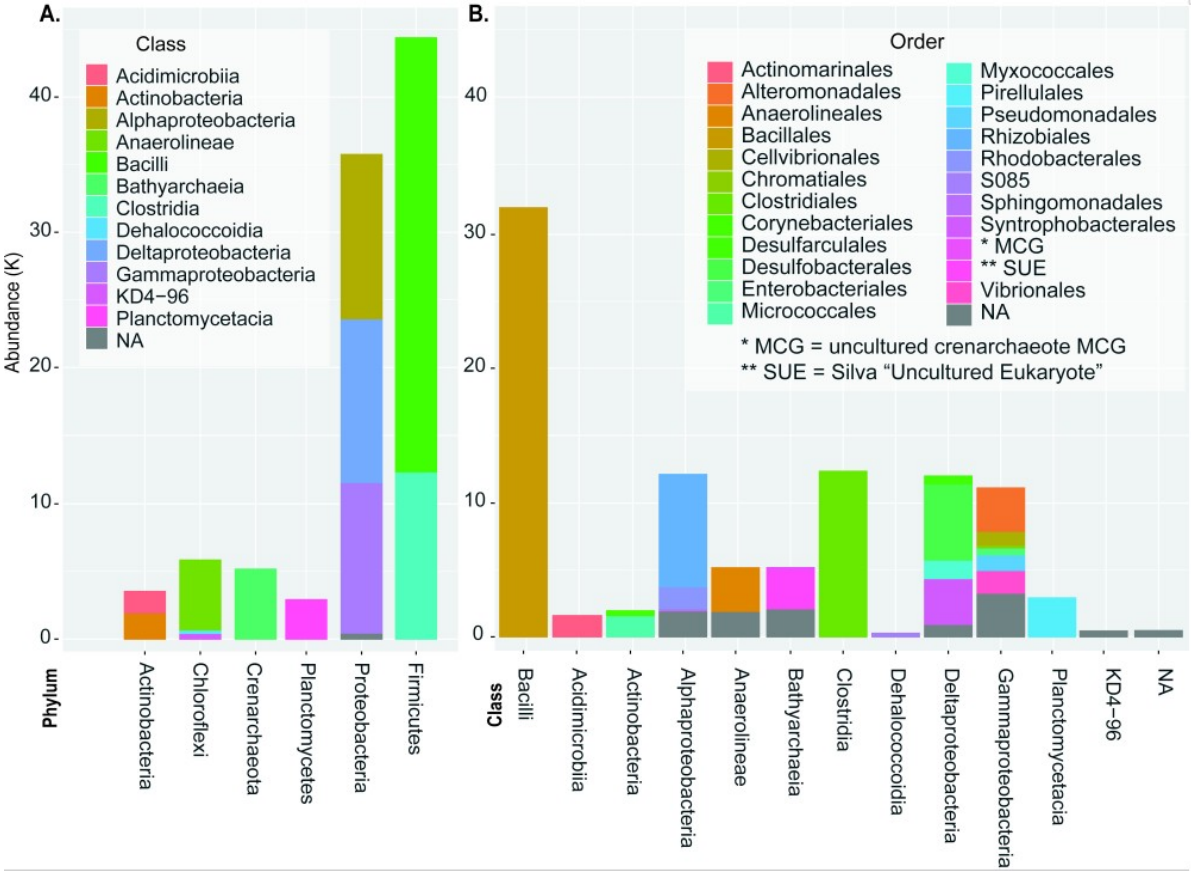
The results are synthesized by categories as follows.

7.4.1 Taxonomic composition of prokaryotic communities

Sequence clustering yielded a total of 1,709 OTUs. Of these, 94.4% were assigned to Bacteria, 5.2% were assigned to Archaea and 0.4% were not assigned to any prokaryotic kingdom. Additionally, one mis-annotated Archea taxa originally named “uncultured eukaryote” has been manually changed to “SUE” for SILVA uncultured eukaryote. All sites combined resulted in 37 phyla, 142 classes, 165 families, 142 genera and 97 species. Approximately 88% of all the sequences 6 phyla: *Proteobacteria* (30.3%), *Firmicutes* (29.4%), *Chloroflexi* (6.4%), *Planctomycetes* (5.3%), *Actinobacteria* (4.6%) and

Crenarchaeota (3.8%). Figure 7.2 shows all the classes and orders of the 6 dominant phyla in the data set. Families and genus are shown in Supplemental Figure 4.

Figure 7.2- Taxonomic distribution of prokaryotes in the sediments of the mangrove forest in the Serinhaém estuary. A) Phylum and class levels. B) Class and order levels. (K) is the abundance in thousands of sequences



Source: Santana, 2020.

7.4.2 Microbial diversity of mangrove tidal zones

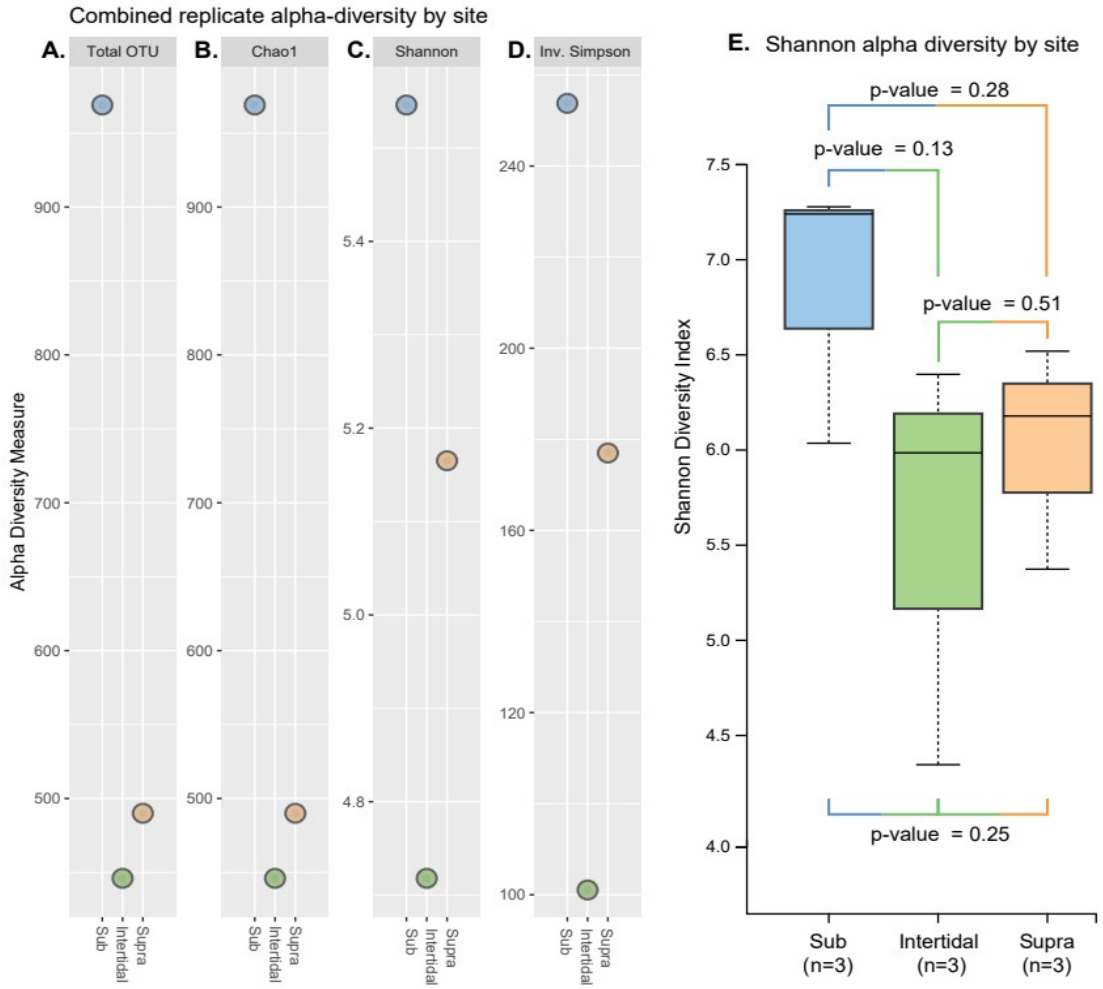
Diversity indices for each zone were calculated using QIIME2 (Figure 7.3). Overall, the sediments from the sublittoral zone were the most diverse, while the intertidal zone exhibited the lowest diversity. In terms of diversity, the differences between the sites were not statistically significant.

7.4.3 Structural differences between prokaryotic communities

We assessed differences in prokaryotic populations between zones (Figure 7.4). Through the boxplots and PCoA based on the Jaccard distance metric, it is possible to observe

significant differences in population structures between tidal zones of the mangrove (α= 0.05, 95% confidence).

Figure 7.3- Diversity measures for the sediments of each tidal zone and boxplots of the statistical analysis on the Shannon diversities

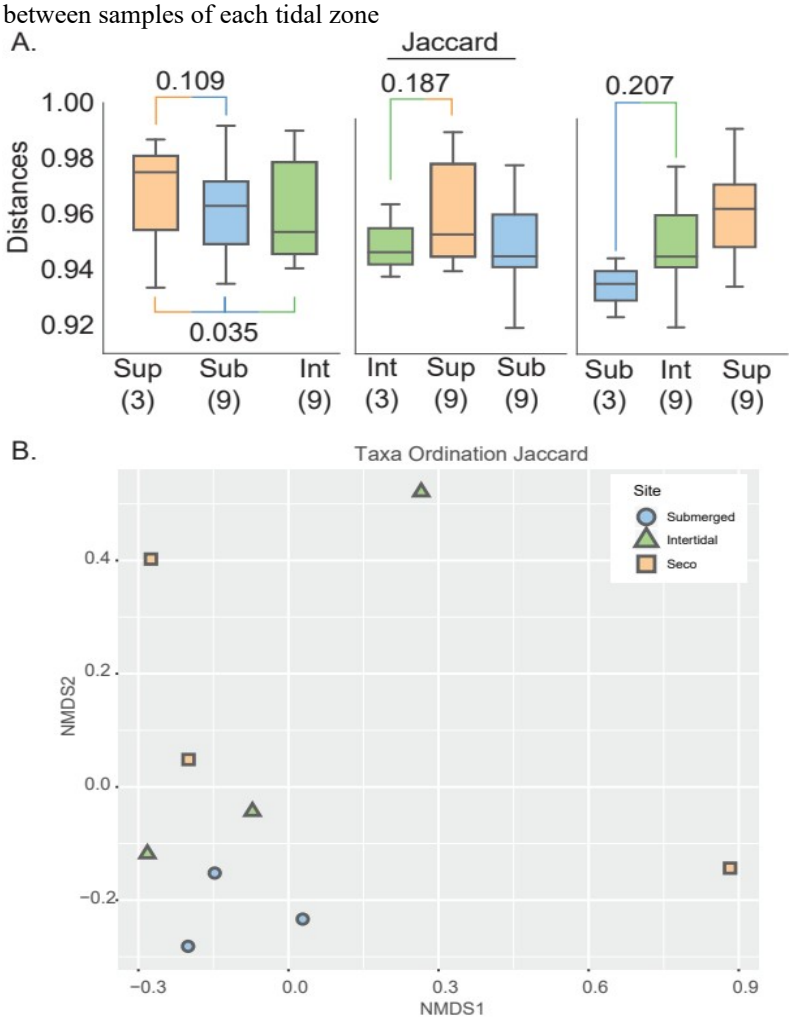


Source: Santana, 2020.

7.4.4 The influence of environmental variables

To determine if differences in population structures between zones correlated with abiotic environmental variables we measured salinity, water content, organic matter and temperature from each tidal zone. The Principal Component Analysis revealed a significant correlation between the prokaryotic populations within each zone and salinity and organic matter (Figure 7.5). Neither water content or temperature measures reflected a significant difference in community structure between zones.

Figure 7.4- Differentiation between sampling sites based on the Jaccard distance metric (α= 0.05). A) Boxplots of the PERMANOVA statistical test. B) Non-metric multidimensional scaling (NMDS) plot displaying distances between samples of each tidal zone



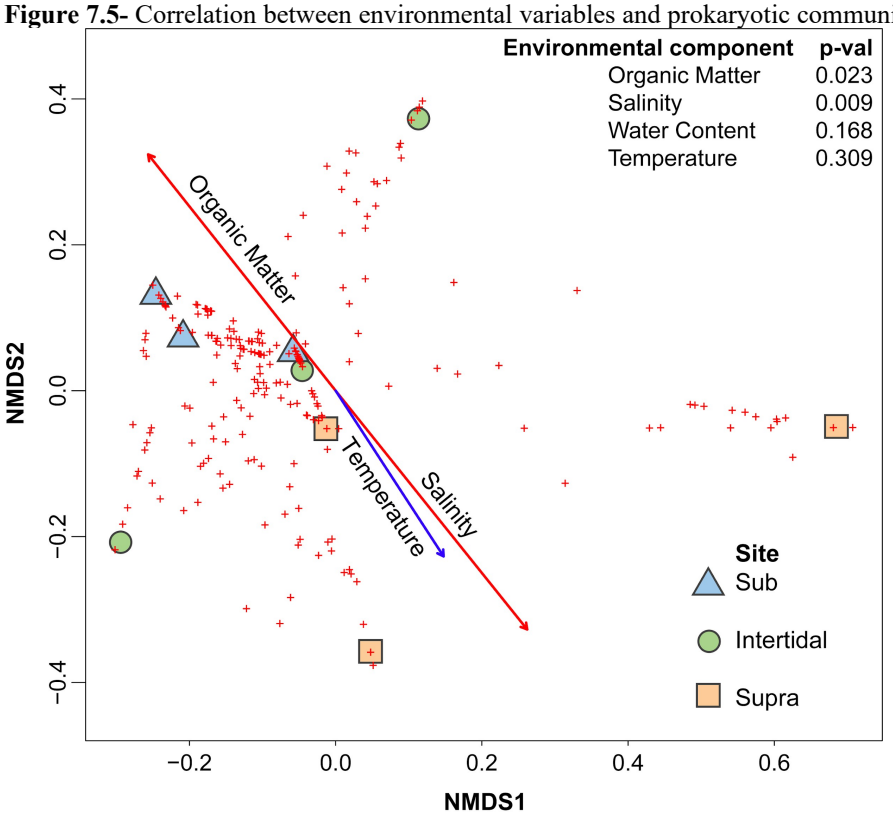
Source: Santana, 2020.

7.4.5 Taxa enrichment by tidal zones of the mangrove

A zone-specific enrichment test for each taxa was performed in order to determine significant differences in abundance between zones (Figure 7.5). Nearly every taxa (96%, 25/26) identified had greatest abundance in the sublittoral zone, with 38% (10/26) showing an inverse relationship between elevation and abundance, such that the abundance increases from the supralittoral to intertidal to sublittoral zone. From these taxa, we identified families with enrichment of metabolism associated KOs, (accounting for more than 10% of the total of a given KO) for at least 3 KOs of a metabolic pathway. The families that contribute substantially to the given metabolisms were labelled with an icon for that pathway (Figure 7.6).

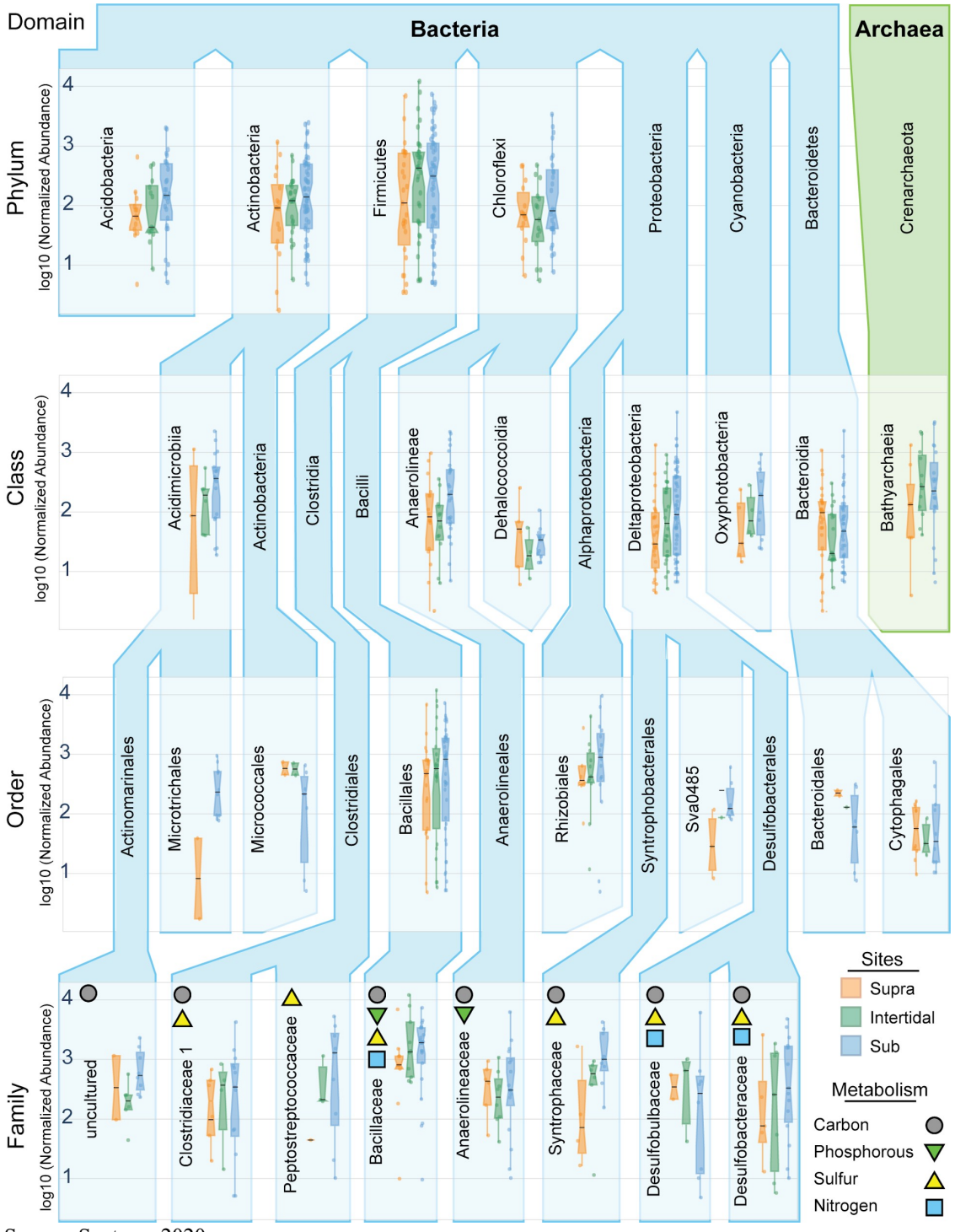
We found that 7 of the 8 enriched families make substantial (>10%) contributions to carbon metabolism associated KOs, contributing to methane metabolism associated KOs, with the exception of *Anaerolineaceae*. The contributions to sulfur metabolism associated KOs were mostly correlated with assimilatory sulfate reduction (mainly *Bacillaceae*), and dissimilatory sulfate metabolism (mainly *Syntrophaceae, Desulfobulbaceae,* and *Desulfobacteraceae*). The families *Bacillaceae, Desulfobulbaceae,* and *Desulfobacteraceae*, produce substantial amounts of nitrogen metabolism associated KOs, with *Bacillaceae* contributing to dissimilatory nitrate reduction to ammonium (DNRA) and *Desulfobulbaceae* contributing to nitrogen fixation associated KOs. Only *Anaerolineaceae* and *Bacillaceae* contribute to the general phosphorus metabolism associated KOs. Taken together, this suggests that zone-specific taxa enrichment may also contribute to differential metabolic activities at these zones.

Figure 7.5- Correlation between environmental variables and prokaryotic communities (p-value <= 0.05)



Source: Santana, 2020.

Figure 7.6- Site specific measures of taxa with significant enrichment in sediments of different tidal zones, whose mean effect size for the metabolisms exceeded 10%



Source: Santana, 2020

7.4.6 Site specific differences in metabolism associated KEGG Orthologs

To determine if there were significant differences in metabolic activity between tidal zones we calculated the functional abundance of metabolic KOs for each zone. KOs with significantly different functional abundances between zones are shown Figure 7.7. 8 KOs

show higher abundances in the sublittoral sediments, above both intertidal and supralittoral, including both members of the phosphorus D-galacturonate degradation pathway. Conversely, only one KO is enriched in the supralittoral above both sublittoral and intertidal zones and no KO is enriched in the intertidal zone. All four of the nitrogen metabolism associated KOs showed significantly lower abundance in the intertidal sediments. Taken together, the results reinforce the previously observed trend of reduced abundance in the Intertidal site, and greatest abundance at the Sublittoral zone, suggesting a positive correlation between biodiversity and functional potentials.

7.4.7 Functional potentials harbored by prokaryotes within the sediments

The contributions made by the different taxa to the genetic potentials associated with the nutrient cycles were assessed and are shown in Figure 7.8.

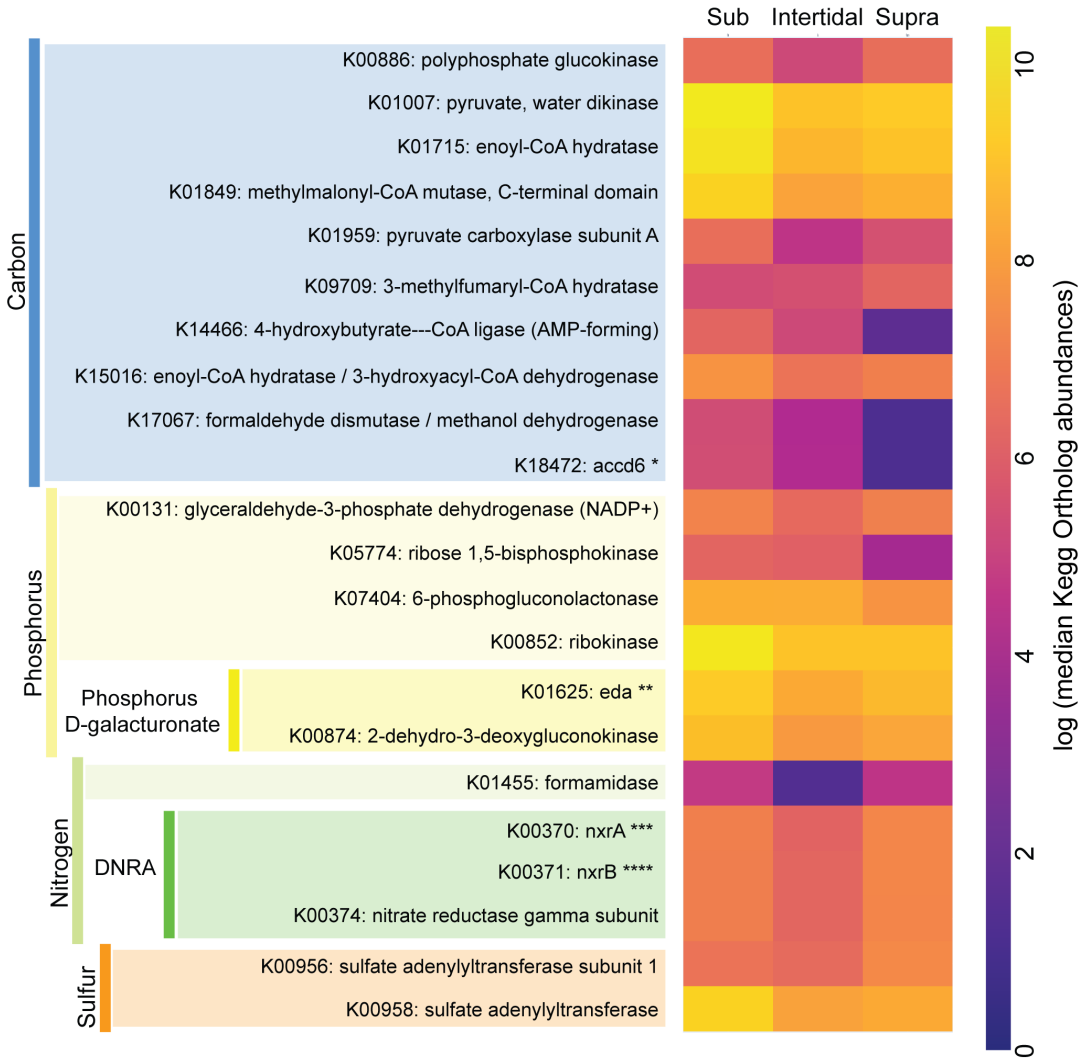
Carbon cycle pathways such as methane oxidation and methanogenesis showed enrichment in the sublittoral zone. *Syntrophaceae* family contributes significantly with different methanogenesis associated KOs, including pyruvate ferredoxin oxidoreductase subunits alpha, beta, delta, and gamma (15%, 24%, 43%, and 14%, respectively) and heterodisulfide reductase subunits A2, B2, and C2 (30%, 27% and 36%, respectively). Many *Archaeal* families contributed to the majority (>50%) of methanogenesis KOs: namely the family *Nitrosopumilaceae* and uncultured families of *Lokiarchaeia*, and *Bathyarchaeia*.

Significantly higher abundances of sulfur transformation KOs were found in the sublittoral zone. The family *Rhodobacteraceae* contributes substantially (>10%) with 12 different sulfur metabolism KOs. The families *Syntrophaceae*, *Desulfobacteraceae*, *Desulfobulbaceae* contribute to almost 90% of the KOs associated with dissimilatory sulfate reduction. Members of the order *Rhizobiales* were major drivers of the sulfur oxidizing process, contributing largely with the abundances of sulfur-oxidizing proteins (85% of SoxY and 65% of SoxZ). The family *Chromatiaceae* also contributes substantially to sulfur-oxidizing proteins SoxA (18%), SoxB (24%), SoxX (18%), and SoxZ (17%).

Similarly, P cycling KOs are, overall, more abundant in the sublittoral sediments. The family *Pseudomonadaceae*, contributes to this metabolism with a substantial amount (>40%) of associated KOs and nearly 99% of three others. In our analysis we found that *Bacillus* contributed to phosphorus metabolism associated KOs at the genus level, with 18 KOs greater than 20%, 5 of which are greater than 50%.

Significantly lower abundances for nitrogen transformation pathways were found in the intertidal zone. In the analysis, the ammonia-oxidizing archaea represented by the families *Nitrososphaeraceae* and *Nitrosopumilaceae*, make up nearly the entirety of the nitrification associates methane/ammonia monooxygenase KOs subunits A, B, C (72%, 72%, 56% and 28%, 28%, 44%, respectively). Members of the family *Clostridiaceae* in the sediments significantly contribute to nitrogen metabolism KOs. The genus *Vibrio* exhibited contributions greater than 20% to 3 nitrogen metabolism associated KOs, the genus *Marinobacter* with 5 KOs, and the genus *Bacillus* with 10 KOs. The family *Rhodobacteraceae* also has significant contributions, with 4 nitrogen metabolism associated KOs.

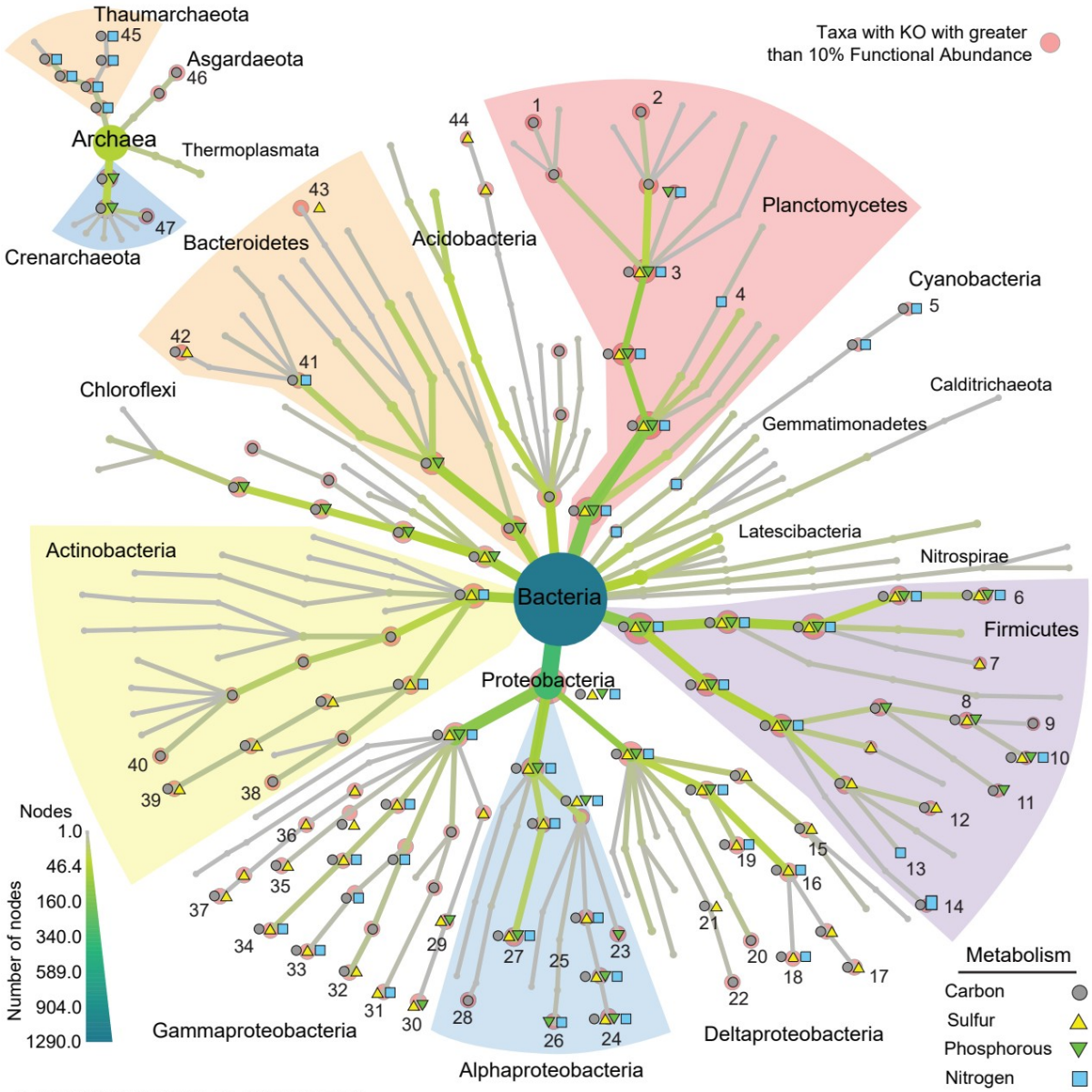
Figure 7.7- Zone specific measures of metabolism associated KEGG Orthologs with significant functional abundance differences between zones



* accd6 = acetyl-CoA/propionyl-CoA carboxylase carboxyl transferase subunit
** eda = 2-dehydro-3-deoxyphosphogluconate aldolase / (4S)-4-hydroxy-2-oxoglutarate aldolase
*** nxrA = nitrate reductase / nitrite oxidoreductase, alpha subunit
**** nxrB = nitrate reductase / nitrite oxidoreductase, beta subunit

Source: Santana, 2020.

Figure 7.8- Phylogenetic tree. Node abundances represented by color and thickness of branches and taxa significantly associated with metabolic pathways



1. Uncultured Pasteuria sp. (Pirellulaceae)
2. Uncultured marine microorganism (Pirellulaceae)
3. Pirellulaceae
4. Rubinisphaeraceae
5. Synechococcus sp. CENA143
6. Bacillus
7. Aneurinibacillus
8. Clostridiisalibacter
9. Bacterium YC−ZSS−LKJ129
10. Clostridium sp. AN−AS6E
11. uncultured Firmicutes bacterium (Brassicibacter)
12. Clostridium sensu stricto 13
13. Clostridium sensu stricto 7
14. Clostridium sp. AN−AS6C
15. Syntrophaceae
16. Desulfobacteraceae
17. Metagenome (Desulfobacteraceae)
18. Desulfatitalea
19. Desulfobulbaceae
20. Uncultured (Myxococcales)
21. Desulfarculaceae
22. Desulfatiglans
23. Xanthobacteraceae
24. Uncultured bacterium (Stappiaceae)
25. Rhizobiaceae
26. Mesorhizobium sp. KYW12
27. Rhodobacteraceae
28. Erythrobacter jejuensis
29. Pseudomonadaceae
30. Pseudomonas
31. Microbulbifer
32. Pseudoalteromonas
33. Marinobacter
34. Vibrio
35. Enterobacteriaceae
36. Chromatiaceae
37. Uncultured gamma proteobacterium (Thiohalocapsa)
38. Micrococcaceae
39. Mycobacterium
40. Uncultured Iamia sp. (Actinomarinales)
41. Flavobacteriaceae
42. Robertkochia marina
43. Uncultured Bacteroidetes bacterium (Prolixibacter)
44. Uncultured actinobacterium (Bryobacter)
45. Nitrososphaeraceae
46. uncultured Desulfurococcus sp. (Lokiarchaeia)
47. uncultured SUE (Bathyarchaeia)

Source: Santana, 2020.

7.5 DISCUSSION

Previous work has shown that mangroves forests exhibit zonation, that is site-specific variation in community structure driven by different biotic and abiotic factors, however, the vast majority of these have been conducted in anthropogenically impacted areas (PUPIN; NAHAS, 2014; MARCIAL GOMES et al., 2008; ALZUBAIDY et al., 2016; ROCHA et al., 2016; CECCON et al., 2019; EL-TARABILY, 2002; IMCHEN et al., 2017; LIN et al., 2019; ZHANG et al., 2018), confounding the makeup of the microbial populations, their abundance, and determination of environmental influences on these population structures. Importantly, the majority of this work does not consider or does not identify the mangroves under study as anthropogenically impacted, despite frequently being only a few km from dense metropolitan and industrial centers (IMCHEN et al., 2017; LIN et al., 2019; ZHANG et al., 2018; CECCON et al., 2019). Notably, studies that sought to identify differences induced by pollution and urbanization on mangroves found broad differences in prokaryotic populations in impacted areas compared to preserved mangrove areas (PUPIN; NAHAS, 2014; NOGUEIRA et al., 2015). However, this research did not study the population differences of distinct microhabitats within mangroves. Here, we extend this study of preserved mangrove areas to characterize variance in prokaryotic populations within microhabitats.

Both salinity and organic matter were significantly correlated with communities in different tidal zones, confirming the hypothesis that changes in physicochemical parameters leads to changes in the prokaryotic communities inhabiting these sediments. As the studied environmental factors were limited, we consider that these parameters are not the only pressures acting on these communities, since other variables such as granulometry, vegetation and pollutant distributions were previously correlated with mangrove sediment communities (PEIXOTO et al., 2011; COLARES; MELO, 2013; ROCHA et al., 2016). Thus, a variety of environmental factors can generate niche variations that influence the structure of communities.

The analysis confirmed that the differences observed in the sediment communities of the different tidal zones are statistically significant. While some of the differences observed between tidal zones are partially explained by environmental variables it is also possible that they are influenced by additional factors, such as fungal and eukaryotic microbes, and even plant rhizome contamination (ROCHA et al., 2016; ZHANG et al., 2017).

The higher biodiversity was found in the sublittoral mangrove sediments, while the intertidal zone had the lowest biodiversity. The higher density of vegetations observed in the

sublittoral area may, in part, explain the higher diversity of the prokaryotic populations we identified there, since rhizospheres are usually highly diverse compartments (BENNETT; KLIRONOMOS, 2019; MILLER; PERRON; COLLINS, 2019). Additionally, the microbiome of the mangrove can be heavily influenced by eukaryotic communities (SIMÕES et al., 2015), which would be invisible to our 16S rRNA amplicon sequencing method. Thus, our understanding of prokaryotic community structures will be greatly increased if complimented with rhizome, fungal, and eukaryotic populations information.

Our data suggests that the intertidal sediments of mangrove forests have lower prokaryotic diversity than those in the constant environments in the supralittoral and sublittoral regions. One possible explanation to this observation is that the constant shifts in environmental parameters creates a harsh condition that selects only the most resistant taxa, since habitat harshness is known to affect biodiversity (STATZNER; MOSS, 2004).

Functional profiling identificated higher metabolic potentials for the cycles of C, N, P and S in the sublittoral region. The higher abundances of KOs found at the sublittoral zone is possibly due to the greater taxonomic diversity that was also observed for this region. However, it remains an open question if the sublittoral zone's greater abundance of taxonomic diversity and enrichment in metabolic function correlate with a resilience to environmental perturbations.

Generally, diverse communities with organisms possessing redundant metabolic functions may be more stable against perturbations as the organisms will respond differently to stressors, thus increasing the likelihood of the survival of some taxa (GIRVAN et al., 2005). However, while the diversity of taxa at the sublittoral site may grant it certain advantages, as a more robust ecosystem, it is also in a more perilous position as the water itself is frequently the carrier of contamination from rivers, as is the case for urban waste (YUNUS et al., 2011), and from the oceans through the tides, as the case with oil spill contamination (CABRAL et al., 2016). Thus, it is important to consider that different parts of the mangrove tidal zone would be exposed in different levels of contamination and that this could affect the organisms in a site-specific manner.

The identification of a rich and divergent set of taxa associated with the diverse nutrient cycles in mangrove sediments was expected due to the previous observations of the microbial diversity in these environments (ROCHA et al., 2016; CECCON et al., 2019; CABRAL et al., 2016; MENDES; TSAI, 2014; ZHAO; BAJIC, 2015) and was confirmed in this study.

7.6 CONCLUSION

In this study the taxonomic and functional structures of sediments from three distinct tidal zones of a pristine mangrove site was assessed and correlated with the relevant environmental factors. The abundance of diverse prokaryotic taxa in mangrove sediments within distinct zones exhibited variance in species diversity, taxa enrichment and functional profiling.

These observed differences suggest that distinct communities occupy the sediments in different mangrove tidal zones. This variance is significantly associated with the abiotic environmental variables salinity and organic matter. Our functional profile analysis suggests enrichment of metabolic pathways between tidal zones. Surprisingly, we observed that the intertidal region, the most biophysically dynamic site in the mangrove forest, presents the least biodiversity. One possible explanation of this is that the dynamic, cyclic, tidal environment is itself harsher than that found in the other tidal zones. Conceptually, this property of a dynamic environment defining a selective niche, similar to a physical barrier, is worthy of further study. Further exploration of this, as well as a further exploration of whether different groups of organisms actively participate in element cycling is the subject of future studies.

8 DISCUSSÃO GERAL

Distintos padrões de diversidade microbiana (procariotos de 16S rRNA) foram observados, durante as análises. Considerando-se os sedimentos de regiões preservadas do Rio Juliana, a diversidade foi maior nas nascentes, decrescendo ao longo do curso do rio, sendo mais baixa na região estuarina. Este resultado foi similar ao reportado em literatura (WANG et al., 2012b; BEHERA et al., 2019; ZHANG et al., 2020). Este padrão se modifica em sedimento de manguezal impactado por esgoto doméstico. Neste caso foi observado um expressivo aumento na biodiversidade. Considera-se que as constantes descargas de esgotos domésticos nessa região criam um ambiente mais eutrofizado, que tem grande potencial para aumentar a biodiversidade (GLIBERT, 2017). No entanto, foram encontradas espécies consideradas exógenas nesse ambiente que ajudam a caracterizar esse tipo de impacto.

O sedimento de manguezal não impactado apresentou uma variação natural na estrutura de procariotos que se correlaciona com as as zonas de maré. A diversidade em termos de riqueza de espécies é mais elevada na região submersa e menor na região intermediária. Resultados similares foram reportados em literatura ((IMCHEN et al., 2017; LIN et al., 2019; ZHANG et al., 2018; CECCON et al., 2019). No entanto, tais estudos foram realizados em áreas impactadas ou próximas a grandes centros urbanos e, portanto, não distinguem com clareza as diferenças entre ambientes pristinos e impactados (PUPIN; NAHAS, 2014; NOGUEIRA et al., 2015). Portanto, esse estudo apresenta uma forma mais eficiente de caracterização da variação estrutural microbiana entre esses ambientes.

Nos testes realizados no microcosmos, o sedimento da nascente apresenta variações significativas após 7 dias do impacto com glifosato ou óleo. Nos sedimentos tratados com glifosato, por exemplo, a diversidade procariótica foi maior do que o sedimento controle. Por outro lado, o sedimento exposto ao óleo apresentou uma diminuição da diversidade em termos de riqueza. Os resultados sugerem que populações microbianas foram favorecidas pela adição do glifosato e permitem hipotetizar que este foi utilizado como fonte de carbono e nutrientes para biossíntese, assim como observado anteriormente por Wang et al. (2016). Por outro lado, a presença do óleo parece apresentar maior efeito tóxico para as populações microbianas presentes nesse ambiente.

Nos sedimentos das áreas preservadas ao longo da bacia do Rio Juliana foi possível observar a prevalência dos filos *Proteobacteria* e *Firmicutes*, que se deve principalmente à elevada diversidade morfofisiológica destes organismos (TVEIT et al., 2013; JOST, 2007; YADAV et al., 2015; ANDREOTE et al., 2012; IMCHEN et al., 2018; SU et al., 2018),

responsável pelo seu sucesso em diferentes habitats. No entanto, a partir do estudo da região de manguezal impactada e da simulação de contaminação dos sedimentos de nascente, foi possível observar que o filo *Firmicutes* apresenta uma grande vulnerabilidade aos impactos ambientais, apresentando grande redução em ambas as situações.

O grupo *Proteobacteria*, por outro lado, permaneceu abundante mesmo nas situações de impacto ambiental. A predominância do filo *Proteobacteria* somente foi superada pelo expressivo crescimento da família *Corynebacteriaceae* pertencente ao filo *Actinobacteria* nos sedimentos de nascente que foram impactados com o óleo. A capacidade de organismos de ambos os grupos para degradar os hidrocarbonetos de petróleo tem sido bem explorada na literatura (THAVASI; JAYALAKSHMI; BANAT, 2011; NDIBE; EUGENE; USMAN, 2019). As principais diferenças taxonômicas observadas nos sedimentos de manguezal impactado por esgoto doméstico foram a colonização por diversas famílias de microrganismos reconhecidamente patogênicos e extinção de táxons presentes nos sedimentos pristinos, demonstrando os impactos negativos da intervenção humana no ambiente.

De modo geral, variáveis ambientais tais como a temperatura, pH, salinidade, matéria orgânica e concentração de metais apresentaram grande influência para a estruturação das comunidades procarióticas nos sedimentos ao longo do curso do Rio Juliana, o que tem sido constantemente observado na literatura (WANG et al., 2012b; BEHERA et al., 2019; ZHANG et al., 2020). No entanto, o efeito dessas variáveis abióticas na estrutura microbiana é diferente do causado por impacto ambiental. A salinidade foi a principal variável influenciando a diferença na estrutura microbiana observada entre as comunidades das áreas de água doce em comparação com a região estuarina.

De modo geral, os perfis funcionais dos sedimentos do Rio Juliana indicam uma correlação positiva com a biodiversidade das amostras, de modo que os sedimentos com maior biodiversidade apresentaram maiores potenciais metabólicos. Estes resultados devem-se provavelmente à redundância funcional observada nestes ambientes (BARNES; CARTER; LEWIS, 2020; TOLKKINEN et al., 2020).

Dessa forma, observou-se uma maior diversificação do potencial para a ciclagem de nutrientes na região das nascentes, seguido pelos sedimentos do vale, mas, sendo menor nos sedimentos do manguezal. Com o aumento de diversidade procariótica ocasionado pela eutrofização do manguezal próximo à cidade de Ituberá, observou-se também um maior potencial metabólico nesta área. O mesmo pôde ser observado nos sedimentos de diferentes zonas de maré do manguezal. A exceção foram os sedimentos submetidos à contaminação por

óleo, que tiveram menor biodiversidade, porém apresentaram maior potencial metabólico para o metabolismo de hidrocarbonetos.

Quanto à cinética de consumo do nitrato nos microcosmos, observou-se que a adição de glifosato resultou em maior maior impacto imediato. Este resultado foi, possivelmente, uma consequência da sensibilidade, observada em estudos anteriores, de organismos nitrificantes à presença do glifosato (ZABALOY et al., 2016; SVIRIDOV et al., 2015). Além disso, o menor impacto imediato do óleo, em comparação com o glifosato, poderia estar relacionado com o fato de que organismos hidrocarbonoclásticos podem utilizar o nitrato dos sedimentos para processos envolvidos na degradação de hidrocarbonetos (WALWORTH et al., 2007; BOLLIGER et al., 1999; SCHÜRMANN et al., 2003)

As diferenças nos perfis metabólicos dos sedimentos submetidos a diferentes condições de pressão ambiental demonstram a capacidade que estes microbiomas têm de se adaptar para a utilização dos diferentes recursos disponíveis. Um exemplo é o enriquecimento de rotas metabólicas associadas à assimilação de formaldeído na comunidade dos sedimentos de manguezal impactado por esgotos domésticos. Comum nestes tipos de efluentes, o formaldeído é importante para a atividade de organismos metanotróficos e tem reconhecido efeito de alterar populações microbianas (DIXON, 2003). Assim, observou-se uma seleção de organismos capazes de utilizar um recurso que se tornou abundante nesta área. O mesmo tipo de processo parece ter ocorrido nos sedimentos submetidos à contaminação por óleo e pesticida, apesar do curto tempo de exposição.

9 CONCLUSÃO GERAL

O presente estudo identificou as principais características estruturais e funcionais das comunidades procarióticas nos sedimentos superficiais de todos os compartimentos que compõem a bacia do Rio Juliana, na APA do Pratigi- BA. A pesquisa permitiu estabelecer a diferenciação da mudanças estruturais causadas por eventos naturais ou de perturbação antrópica. Para tanto, foram também realizado experimentos em laboratório, além da caracterização direta da comunidade microbiana em diferentes pontos do Rio Juliana, BA.

As três áreas do rio apresentaram comunidades estatisticamente distintas em termos taxonômicos e funcionais. A diversidade de procariotos em termos de riqueza é maior nas nascentes e naturalmente decresce ao longo do curso do rio, sendo mais baixa na região estuarina. O aumento de salinidade foi o fator abiótico mais significativo para essa mudança.

A adição de petróleo e glifosato em microcosmos contendo sedimentos da região da nascente mostraram que a presença do óleo resultou na maior perda de biodiversidade. Os dados do potencial funcional, no entanto, mostraram um enriquecimento de organismos capazes de metabolizar hidrocarbonetos. A presença de glifosato resultou em um leve aumento na diversidade em comparação com o controle, sugerindo que este composto pode ter servido como uma fonte a mais de carbono e potencialmente menos tóxico que o óleo. O impacto da adição de glifosato no sedimento causou uma maior redução na taxa de consumo de nitrato do que a adição do óleo. Esse resultado sugere que pode existir uma diferença entre o potencial funcional derivado das análises taxonômicas e a medida *in situ*. Portanto, o estudo do potencial impacto na diversidade microbiana ligada aos ciclos biogeoquímicos devem seguir em paralelo a testes realizados *in situ*.

O potencial metabólico da comunidade microbiana do sedimento exposto a despejos urbanos foi maior que o da área não impactada. Esse resultado sugere que a variação da diversidade está diretamente associada à disponibilidade de recursos energéticos adicionais introduzidos no ambiente pelo esgoto doméstico. Observou-se, no entanto, a possível extinção de grupos microbianos que caracterizam o ambiente sem impacto e concomitante introdução de grupos patogênicos na região impactada.

Os sedimentos nas diferentes zonas de maré no estuário do Serinhaém apresentam comunidades procarióticas significativamente distintas, com maior diversidade na zona alagada e menor diversidade na zona intermediária, que tem períodos emersos e submersos. Os potenciais metabólicos para ciclagem de S, P, N, e C destas comunidade também foram

influenciados pela diversidade, sendo mais elevados nos sedimentos submersos. Os resultados indicam que o manguezal não pode ser considerado como um ecossistema único no que se refere aos fatores ambientais prevalentes e às comunidades microbianas associadas. Assim, os impactos ambientais poderiam ter efeitos distintos em cada zona de maré.

Finalmente, a partir dos resultados obtidos no presente estudo, foi possível estabelecer uma base de dados ecológicos que poderão ser explorados mais profundamente no futuro. Os perfis metabólicos dos microbiomas das distintas regiões estudadas, bem como a atuação dos grupos procarióticos individuais em determinadas funções ecossistêmicas poderão ser confirmados a partir de análises metatranscriptômicas destes sedimentos. Para futuras pesquisas, uma observação mais profunda dos impactos da adição de contaminantes neste sistema poderá ser obtida a partir de experimentos de longo prazo, aliados a análises de RNA e da análise dos demais compartimentos do Rio Juliana.

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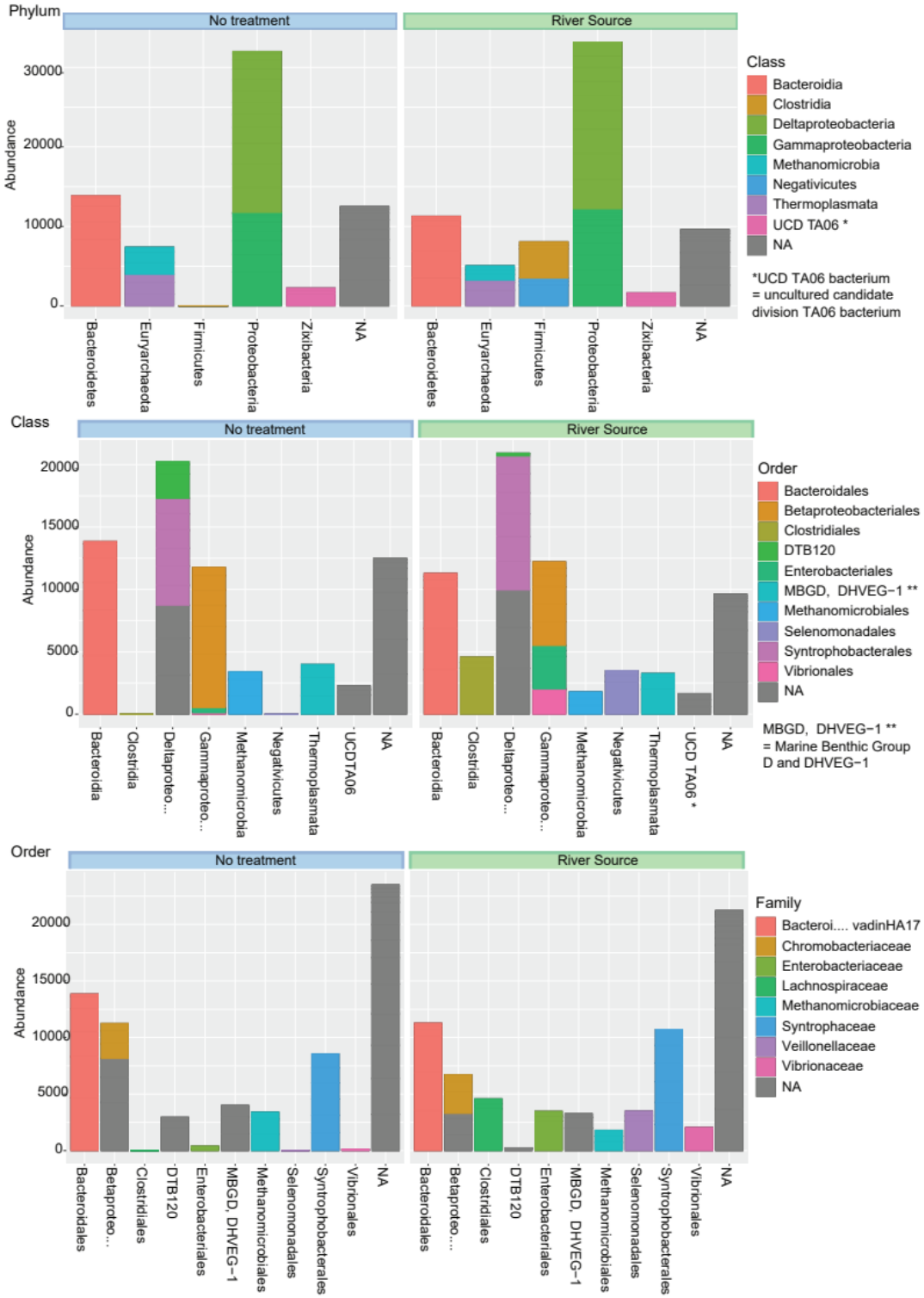
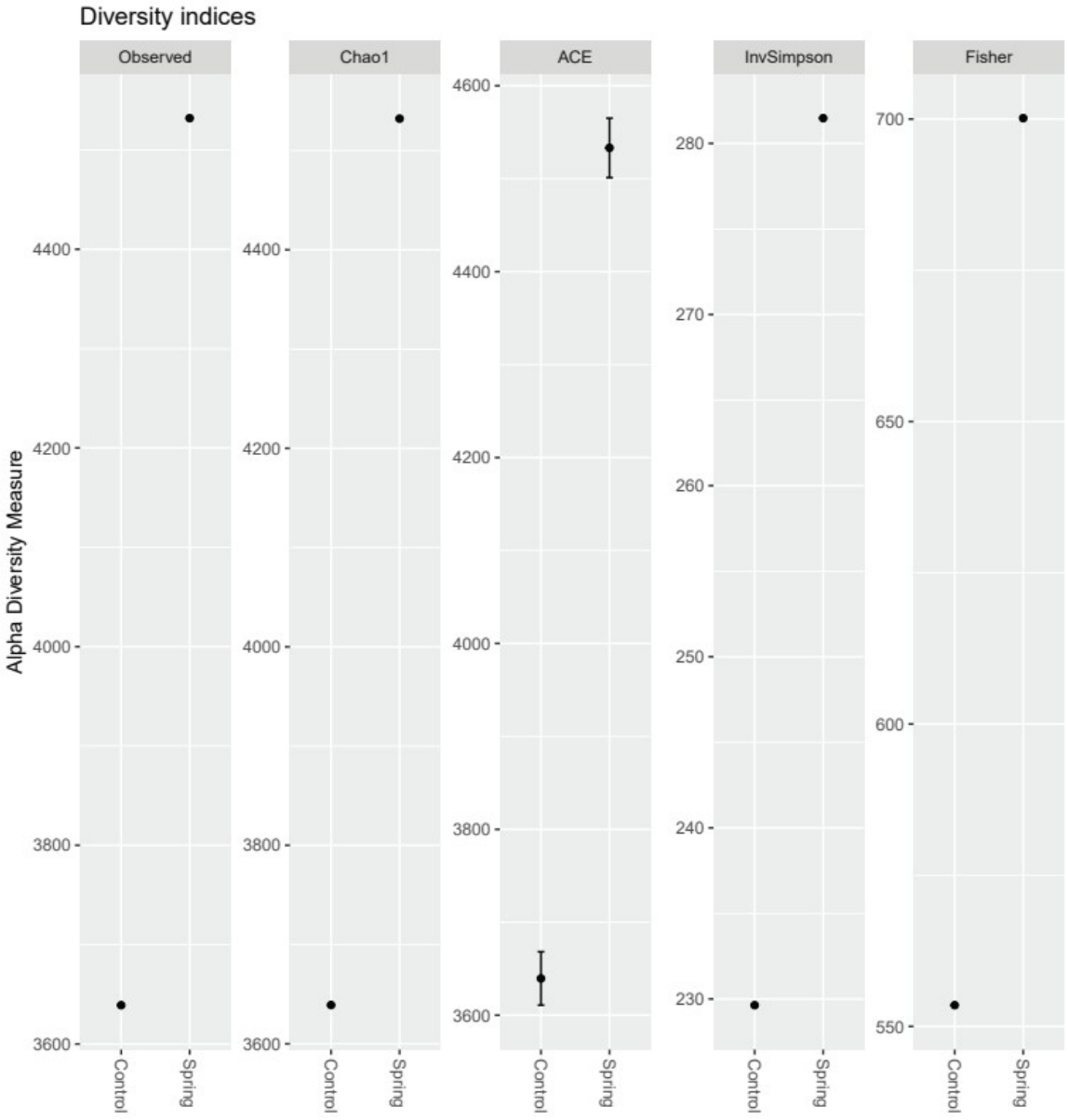
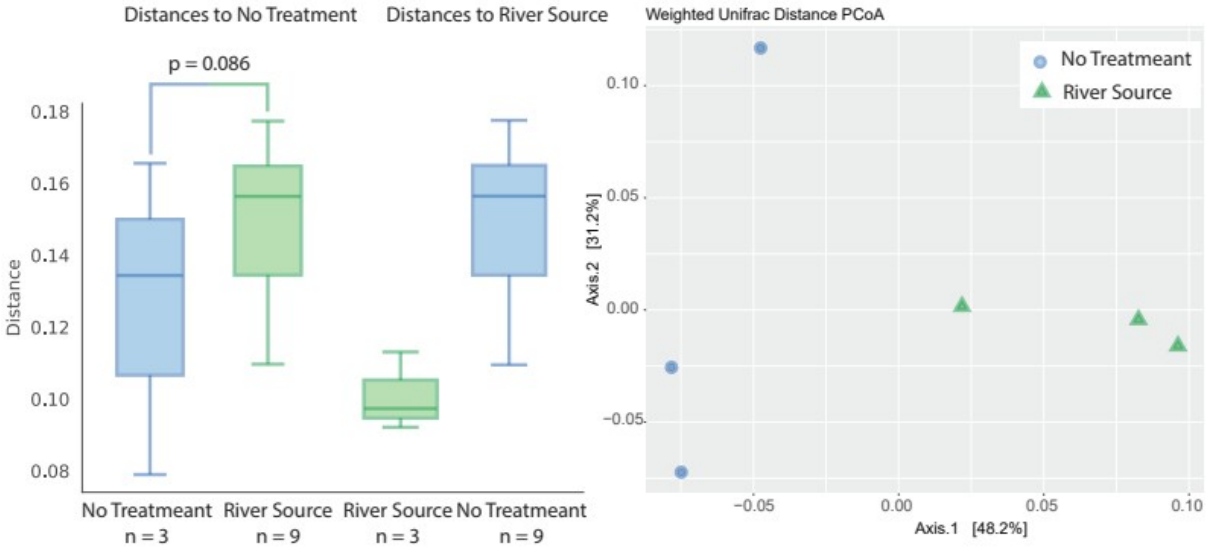
APÊNDICE A - Supplemental Figure 1. Taxonomic distributions of taxa at the levels of order and family.



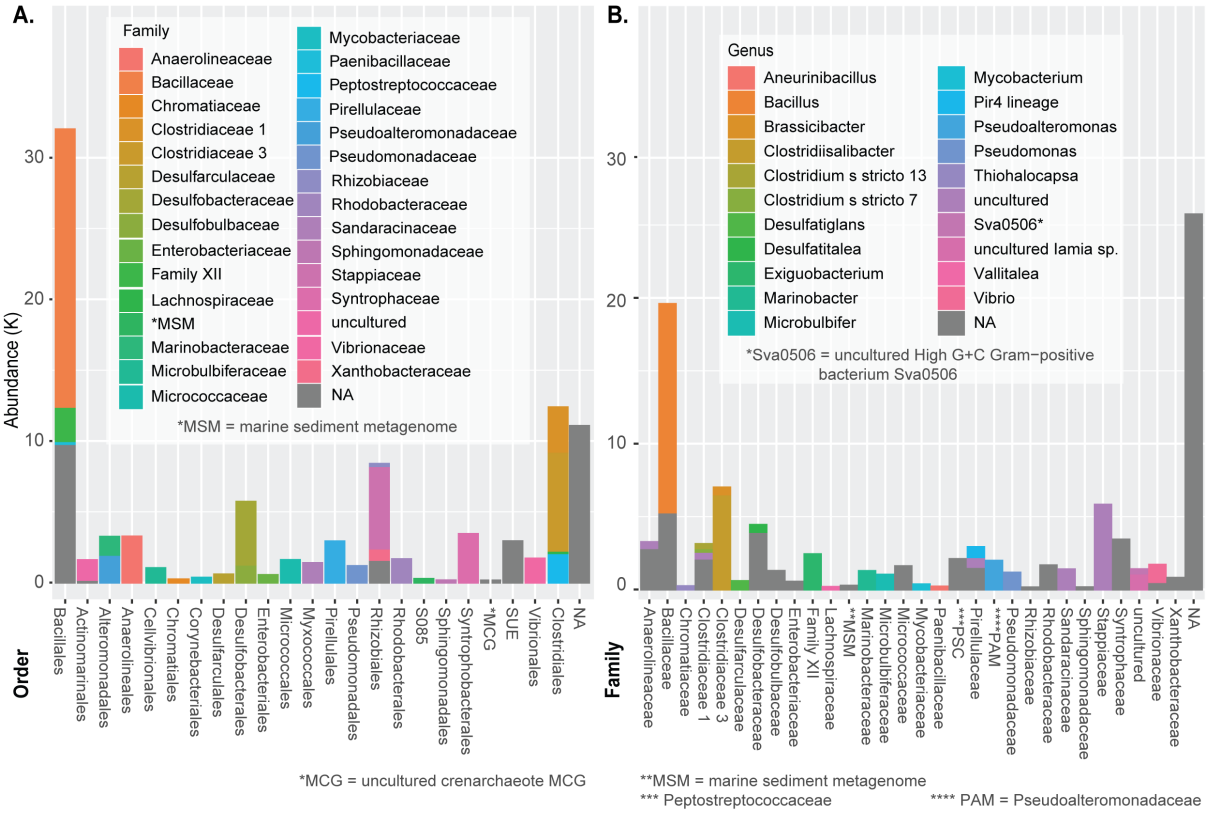
APÊNDICE B - Supplemental Figure 2. Alpha diversity indices for comparison between the natural and control (after 7 days of experiment) samples.



APÊNDICE C - Supplemental Figure 3. Beta diversity comparison between samples from the river source and samples of the experiment control after 7 days of incubation.



APÊNDICE D - Supplemental Figure 4. Taxa identified within the samples shown as stacked bar plots.



S232 Santana, Carolina Oliveira de

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