Luan Renato dos Passos Aires

AVALIAÇÃO ESPAÇO-TEMPORAL DA MICROBIOTA SEDIMENTAR DA LAGOA DA CONCEIÇÃO (FLORIANÓPOLIS, SANTA CATARINA) E PROSPECÇÃO DE CIANOBACTÉRIAS E PROTEOBACTÉRIAS POTENCIALMENTE PRODUTORAS DE BIOHIDROGÊNIO

Dissertação submetida ao Programa de Pós-graduação em Biotecnologia e Biociências da Universidade Federal de Santa Catarina para a obtenção do Grau de Mestre em Biotecnologia e Biociências

Orientador: Prof. Dr. Leonardo Rorig
Coorientadora: Profª Dra. Maria Luiza Schmitz Fontes

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Esta Dissertação foi julgada adequada para obtenção do Título de "Mestre em Biotecnologia e Biociências", e aprovada em sua forma final pelo Programa de Pós-Graduação em Biotecnologia e Biociências

Florianópolis, 18 de Abril de 2017.

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“Não podemos resolver nossos problemas utilizando o mesmo raciocínio que os criaram.”

(Albert Einstein)

RESUMO

Neste trabalho avaliou-se a estrutura da comunidade microbiana em sedimentos da Lagoa da Conceição (Florianópolis-SC). Dois sítios foram escolhidos: um representando a “zona morta” – região sujeita a eventos sazonais de anoxia e outro em local de prevalente normoxia. Água de fundo e sedimento superficial dos dois pontos foram coletados para a obtenção das variáveis abióticas e bióticas, respectivamente. A caracterização da diversidade procariótica foi obtida pelo sequenciamento de um fragmento da região V3-V4 do gene 16s rRNA de sedimentos coletados entre o final da primavera e início do verão austral dos anos de 2015 e 2016. Sedimentos da “zona morta” apresentaram abundância relativa procariótica significativamente maior de Archaea ($p<0.001$) do que no ponto sujeito à normoxia. Os filos Cyanobacteria e Proteobacteria foram também mais abundantes no sítio sujeito à normoxia ($p<0.01$ e $p<0.001$), além de apresentarem correlações positivas com os nutrientes nitrito + nitrato em águas de fundo, o que indica possíveis efeitos de eutrofização no local. Os sedimentos coletados em ambos os sítios foram utilizados para a preparação de colunas

de Winogradsky as quais foram incubadas sob temperatura e luminosidade constantes por um período de até dois meses. A combinação da técnica de Sergei Winogradsky com a de sequenciamento de nova geração mostrou-se eficaz no enriquecimento e identificação dos grupos de micro-organismos alvos deste trabalho, dobrando o número de OTUs para gêneros de Cianobactérias e quase triplicando o de Proteobactérias. Biofilmes verde-azulados e de coloração púrpura foram mais frequentes em colunas feitas com sedimentos sujeitos à normoxia e consórcios de três espécies de cianobactérias (*Synechococcus* sp., *Leptolyngbya* sp. e *Oscillatoria* sp.) bem como de bactérias púrpuras não-sulfurosas (*Rhodobium* sp.) foram obtidos a partir da inoculação em meio BG-11 e RCV, respectivamente. Esta foi a primeira caracterização da microbiota de sedimentos da Lagoa da Conceição e servirá para elucidar os processos biogeoquímicos que ocorrem neste ecossistema, bem como nortear processos de produção de biohidrogênio em escalas industriais.

Palavras-chave: Biohidrogênio, Colunas de Winogradsky, Cyanobacteria, Proteobacteria.

ABSTRACT

This work evaluated the structure of the microbial community in sediments of Lagoa da Conceição (Florianópolis-SC). Two sites were chosen: one at the "dead zone" area, subjected to seasonal anoxia events and the other representing an area of prevalent normoxia. Bottom water and top sediments were collected to obtain abiotic and biotic variables, respectively. The characterization of prokaryotic diversity was obtained by sequencing a fragment of the V3-V4 region of the 16s rRNA gene from sediments collected between late spring and early austral summer of 2015 and 2016. Sediments collected in the "dead zone" presented higher relative abundances for the Archaea Domain ($p<0.001$), whereas Bacteria was higher in the site subjected to constant normoxia. Cyanobacteria and Proteobacteria phyla were also more abundant in the site under frequent normoxia ($p<0.01$ and $p<0.001$), besides presenting strong positive correlations with the nutrients nitrite + nitrate in the bottom waters, which indicates possible effects of eutrophication at the site. Sediments collected at both sites were used for the preparation of Winogradsky columns, which were incubated under constant temperature

and luminosity for a period of up to two months. The combination of the Sergei Winogradsky technique with new generation sequencing techniques proved to be effective in enriching and identifying the target groups of microorganisms in this work, doubling the number of OTUs for Cyanobacteria genera and nearly tripling that of Proteobacteria. Blue-green and purple biofilms were more frequent in columns made with normoxia-bearing sediments and consortia of three species of cyanobacteria (*Synechococcus* sp., *Leptolyngbya* sp. and *Oscillatoria* sp.), as well as purple non- sulphurous bacteria (*Rhodobium* sp.) were obtained from inoculation in BG-11 and RCV medium, respectively. This was the first sediment microbiome of the Conceição Lagoon and will serve to elucidate biogeochemical processes that occur in this ecosystem, as well as guiding processes of biohydrogen production in industrial scales.

Keywords: Biohydrogen, Cyanobacteria, Proteobacteria, Winogradsky column.

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LISTA DE ABREVIATURAS E SIGLAS

BIOM – do inglês, *Biological observation matrix*

CS – do inglês, *Central sector*

DGGE – do inglês, *Denaturing Gradient Gel Electrophoresis*

H_2 – Hidrogênio

LC – Lagoa da Conceição

$M^{-2}\,s^{-1}$ – Metros quadrados por segundo

NGS – do inglês, *next generation sequencing*

nm – Nanômetros

NPK – Nitrogênio, fósforo e potássio

NS – do inglês, *North sector*

OTU – do inglês, *operational taxonomic unit*

PCR – do inglês, *polymerase chain reaction*

PNS – do inglês, *purple non-sulphur*

$T_{(0)}$, $T_{(1),}$
$T_{(2)}$ e $T_{(3)}$ Tempos de incubação

WGS – do inglês, *whole genome sequencing*

WCs – Colunas de Winogradsky

LISTA DE SÍMBOLOS

®	Marca Registrada
µ	Unidade métrica (micrón)
E	Einsteins
°C	Graus Celsius

SUMÁRIO

INTRODUÇÃO

Os ecossistemas marinhos costeiros estão entre os mais produtivos e diversos da Terra, fornecendo mais de US$ 14 trilhões anualmente em bens alimentícios e matéria bruta (HARLEY et al., 2006). Mudanças climáticas globais e impactos antropogênicos sob esses ecossistemas têm sido considerados, em consenso pela comunidade científica, como ameaças ao equilíbrio da biodiversidade do planeta (IPCC, 2014). Práticas agrícolas como o excesso na utilização de fertilizantes resultam em grande aporte de nutrientes nos solos os quais, quando não consumidos localmente, são drenados pela chuva para corpos de água e estuários (TILMAN, 1999; DI & CAMERON, 2002). A maior disponibilidade de nutrientes, principalmente fósforo e nitrogênio, ambos presentes na composição de fertilizantes (NPK) e essenciais para a síntese de biomoléculas como ácidos nucléicos e aminoácidos, favorecem florações sazonais fitoplanctônicas (HIGHTON et al., 2016). A elevada produção primária por cianobactérias, outras micro e macroalgas resulta em um acúmulo de matéria orgânica nos sedimentos, favorecendo o crescimento de organismos procarióticos heterotróficos

aeróbicos e consequente agravação dos processos de eutrofização e hipoxia (DIAZ & ROSENBERG, 2008; SINKKO et al., 2013).

A Lagoa da Conceição (LC) é um ecossistema lagunar de constante influência antrópica. Estudos de projeções demográficas indicam que a população urbana às margens da laguna cresceu aproximadamente 6,3% ao ano, entre 2001 e 2015, sendo que o crescimento populacional encontrado no Estado de Santa Catarina para o mesmo período foi de 1,5% ao ano (CAMPANARIO, 2007). Na LC são observados efeitos de eutrofização artificial causados pelo aumento da forma reduzida de nitrogênio, a proliferação de micro e macroalgas oportunistas e a falta de oxigênio nas águas de fundo da região central favorecida pela acentuada estratificação da coluna d'água e pela baixa troca de água com o mar adjacente (FONSECA & BRAGA, 2006; FONTES et al., 2011). Estudos indicam que quando a LC ainda não era ligada ao mar adjacente a haloclina ainda não era estruturada (ASSUMPÇÃO et al., 1981) e uma vez que a abertura permanente do canal que liga a LC ao oceano Atlântico foi concretizada, a estratificação física da coluna d'água da região central da Lagoa tornou-se permanente, com frequente anoxia na água de fundo, descrita primeiramente por Odebrecht & Caruso (1987) e confirmada

por posteriores trabalhos (SIERRA DE LEDO & SORIANO SIERRA, 1994; FONSECA, 2004; FONTES, 2004; FONSECA & BRAGA; 2006; FONTES et al., 2011; FONTES & ABREU, 2012).

A principal fonte de carbono e de nutrientes nos sedimentos é a matéria orgânica (SCHIMEL, 1995) e o tempo de remineralização dessa matéria orgânica é governado pela velocidade em que micro-organismos a consomem, sendo a taxa de degradação da matéria influenciada pela estrutura microbiana presente nesse, bem como pelas condições ambientais (temperatura, pH, porosidade, etc.), (GRIGGS et al., 2013). O isolamento e cultivo em laboratório de micro-organismos que participam desses processos é um dos maiores desafios para o avanço e a compreensão dos processos biogeoquímicos (ROUSK & BÅÅTH, 2011; STEIN & NICOL, 2011). Para isso, é notória a necessidade de se identificar os fatores ambientais que regem a atividade e os mecanismos de interação entre micro-organismos autotróficos e heterotróficos (TRESEDER et al., 2012). Além do mais, solos e sedimentos são conhecidos por conterem uma diversidade muito elevada de micro-organismos. Sendo assim, compreender a estrutura da comunidade microbiana presente

em solos de diferentes ecossistemas tem se tornado foco de diversos grupos de pesquisa (LOZUPONE & KNIGHT, 2007).

A preparação de colunas Winogradsky (WCs) para o estudo de sedimentos é uma técnica barata e de alta reprodutibilidade, na qual culturas de enriquecimento são feitas com solo ou sediment e incubadas sob luz. Ao longo do tempo, há o desenvolvimento de gradiente químico que favorece a separação de consórcios microbianos com grande diversidade metabólica, tanto vertical quanto horizontalmente (da superfície para o interior das colunas), resultando em nichos diversos para o crescimento microbiano. A luz serve como fonte de energia para os organismos fotoautotróficos e assim, desenvolve-se um microcosmo semi-fechado dependente de luz. As colunas de Winogradsky são frequentemente aplicadas na investigação da diversidade metabólica microbiana, mas também têm sido usadas em outras aplicações, como no enriquecimento ou o isolamento de novas bactérias, biorremediação (DE SOUSA et al., 2012), e para a geração de biohidrogênio (LOSS et al., 2013). A coluna de Winogradsky pode também ser um modelo de ecossistema microbiano útil para estudar as influências ambientais na estrutura e dinâmica

da comunidade procariótica e viral (RUNDELL et al., 2014; ESTEBAN et al., 2015).

Como a busca pela produção, armazenamento e distribuição energética são assuntos de elevada importância sócio-econômica e ambiental, a aplicação dessa técnica como meio de investigação de consórcios produtores de biocombustíveis é altamente viável. Encontrar formas que substituam a queima de combustíveis fósseis por tecnologias sustentáveis está entre os dezessete “objetivos para transformar nosso mundo” propostos na agenda 2030, sendo sustentabilidade abordada em quatorze destes itens (ONU, 2015). Mudanças climáticas têm sido o motor do desenvolvimento de tecnologia de energias renováveis, como no acordo sobre mudanças climáticas de Copenhague de 2009, que prevê a redução da emissão de CO_2 de 25-40% até 2020 e de 80-90% até 2050, para que o acréscimo da temperatura do planeta se mantenha dentro do limite de 2 °C (IPCC, 2014).

Dentre as alternativas sugeridas para a substituição da queima de combustíveis fósseis, ato de participação importante no aumento de gases de efeito estufa na atmosfera e, consequentemente, aquecimento global, o gás hidrogênio (H_2) é cotado como o combustível do futuro. Como carregador energético de alta flexibilidade e baixa “pegada” de carbono,

H_2 pode ser utilizado como uso final nos setores energéticos dos transportes, indústrias e residências. Atualmente, 40% de hidrogênio é produzido a partir de gases naturais, 30% de óleo pesado e nafta, 18% a partir do carvão, 4% a partir da eletrólise da água e apenas 1% a partir de biomassa. O hidrogênio é usado na combustão direta em motores de combustão interna ou como combustível para células combustíveis (CARDOSO et al., 2014).

LOSS *et al.* descreveram a produção biológica de hidrogênio a partir de consórcios de bactérias púrpuras não-sulfurosas (PNS) isoladas de sedimentos da Lagoa da Conceição quando cultivados em meio RCV modificado na presença de ácidos orgânicos como o acetato e butirato. O consórcio de PNS aplicado nesse estudo havia sido isolado de WCs preparadas com sedimentos da Lagoa da Conceição coletados no sítio sujeito às recorrentes eventos de anoxia (LOSS et al., 2013). A icaracterização do consórcio limitou-se a utilização da técnica de DGGE (*Denaturing Gradient Gel Electrophoresis*), revelando quatro prováveis espécies pertencentes ao filo Proteobacteria. Com isso, observou-se que com uma simples amostra de sedimento coletada de um sítio na Lagoa da Conceição e incubada em coluna de Winogradky foi possível produzir hidrogênio em escala de bancada. No

entanto, as espécies de procariotos que produziram o H_2 não puderam ser identificadas naquele momento devido à limitação da técnica utilizada.

Essa foi a maior motivação para o desenvolvimento desta dissertação – busca de micro-organismos com potencial para a produção de bio-hidrogênio por meio da aplicação de colunas de Winogradsky em combinação com o sequenciamento de um fragmento do gene 16s para a identificação das espécies presentes, assim como descrição do microbiota da Lagoa da Conceição.

A dissertação será apresentada em dois capítulos de acordo com os objetivos gerais.

1. OBJETIVOS

2.1 Objetivos Gerais

1) Descrição do microbioma de sedimentos superficiais da Lagoa da Conceição, Florianópolis – SC (CAPÍTULO 1).

2) Caracterização da estrutura microbiana com potencial para produção de hidrogênio de sedimentos da Lagoa da Conceição (CAPÍTULO 2).

2.2 Objetivos Específicos

1. Avaliação da variação temporal da comunidade microbiana em sedimentos superficiais de sítios sujeitos a recorrentes eventos de anoxia e sítios sob constante normoxia (CAPÍTULO 1);

2. Descrição da comunidade microbiana de sedimentos enriquecidos e não enriquecidos em coluna de Winogradsky (CAPÍTULO 2);

3. Avaliação das amostras para a prospecção de cepas de Cianobactérias e Proteobactérias potencialmente produtoras de hidrogênio (CAPÍTULO 2).

CAPÍTULO I: MICROBIAL COMMUNTY STRUCTURE FROM SEDIMENTS OF A SUBTROPICAL, COASTAL LAGOON SUBJECTED TO ANOXIC EVENTS

Abstract

This study aimed to evaluate the spatiotemporal variability within the microbial community of sediments collected from two distinct locations in a subtropical, coastal lagoon with a seasonal dead zone. Samples were collected between late spring and early summer of 2015/2016. DNA was extracted from the sediments, and16s rRNA genes were paired-end sequenced through the Illumina MiSeq platform. A total of 219,709 high quality sequences were obtained and 1,546 operational taxonomic units were clustered at 97% identity using the Silva 119 database as reference. Taxonomic diversity, estimated at a domain, phylum, class and family levels significantly differed with space and time. Sediments of site where dead zones are frequent presented higher abundance of Archaea sequences compared to site 82, under constant bottom oxic waters. Proteobacteria, Chloroflexi and Euryarcheota were dominant prokaryotic phyla, where

Proteobacteria predominated in site 82, whereas Chloroflexi and Euryarcheota at site 33. Cyanobacteria were the twelfth most abundant phylum, and was significant higher at site 82 in November, reaching 14% of all prokaryotic phyla, while other time periods of the same site it remained <5%. This rise in Cyanobacteria at site 82 was strongly and positively correlated to nitrate+nitrite and dissolved oxygen levels, and inversely to salinity. Prokaryotic community structure of sediments collected at site 33 was more stable with time than those of site 82. This is the first description of the Conceição Lagoon microbiota and the first of the south Brazilian coastal lagoons. Some of the most abundant taxa within the Archaea domain detected in the sediments of the lagoon are found in seepage of other marine systems throughout the world, indicating the presence of active bottom sediments.

Keywords: 16s rRNA gene, Coastal eutrophication, Dead zones, Suboxia, Bacteria

Introduction

The intense urbanization of coastlines and the exponential increase in the nutrients runoffs are among the main factors impacting the biodiversity and biogeochemical cycles of coastal systems worldwide (KOWARIK, 2011). Microorganisms play an important role in aquatic ecosystems functioning, both in the water column and in the sediments. As prokaryotes respond quickly to anthropogenic environmental changes, such as to warming and pollution, they are considered as sentinels of ecosystem health. Human and natural extreme interventions can, thus, disrupt the microbial community composition commonly observed in an aquatic system (BLASER et al., 2016).

Bacterioplankton community composition can be regulated by either bottom up or top down pressures, or by both. The availability of resources such as: nutrients, dissolved oxygen, dissolved organic carbon and light shape the community structure since they select those taxa more adapted to one or more conditions over others (FALKOWSKI et al., 2008). Top down pressures, such as grazing and viral lyses also may alternate the community structure by cell-to-cell encounter rates (TSAI et al., 2015). In the past few decades' depth-related changes of microbial communities from sediments of lenthic

freshwater and marine systems have been investigated through different methodologies, e.g. comparison between oxic and anoxic conditions (HOLLIBAUGH et al., 2001; HUMAYOUN et al., 2003; URAKAWA et al., 2001; ZHAO et al., 2012), and sub-saline shallow lakes (FERRER et al., 2011).

It is well known that traditional microbiological techniques, such as culture-dependent methods, are not adequate for microbial ecological studies, since much of them do not grow in general culture media. Next generation sequencing (NGS) of the whole DNA present in an environmental samples has been widely used as an alternative in microbial studies; however, the analyzes are still expensive for large datasets when the main goal is to understand beta diversity of prokaryotic communities (SHOKRALLA et al., 2012). The sequencing of the universal gene present in all prokaryotic cells, 16S rRNA, has been extensively applied in the investigation of taxonomic microbial studies in marine ecosystems (WELCH & HUSE, 2011), and in sediments of coastal lagoons (FERNANDEZ et al., 2016a).

Conceição Lagoon is a shallow, subtropical, coastal lagoon in Southern Brazil that hosts seasonal “dead zones” where intense, seasonal suboxia is found below the halocline (> 3m deep) in the central-southern (CS) sector (FONSECA &

BRAGA, 2006; BARROS et al., 2017), represented by site 33 and normoxia is constantly observed in the bottom waters of the northern (N) sector, represented by site 82. Previous studies have reported higher microbial biomass in the hypoxic-suboxic bottom waters of CS sector when compared to the Northern sector [15,16] and the presence of purple sulfur bacteria, genus *Chromatium*, in the bottom waters of site 33 was observed in the interior of the large *Chromatium* cells through light microscopy [17].

Here, we aimed (1) to evaluate the variability of prokaryotic community structure in the top sediments from two distinct locations in Conceição Lagoon and (2) to detect the main drivers of such variability.

Materials and methods

Study site, sampling and DNA extraction

A total of four sampling campaigns were carried out between late austral spring and summer of 2015/2016 (November and December of 2015, January and March of 2016). Sediments and water were sampled in sites 33 and 82 (-27°36’S - 48°27’W and 27°52’S - 48°45’W, respectively) (figure 1), representing Central-Southern (CS) and Northern

(N) sectors, respectively; described in previous studies (FONTES & ABREU, 2009; FONTES et al., 2011).

Temperature and dissolved oxygen of the water column were measured *in situ* using an oximeter (YSI, mod. 550A), salinity was measured using a thermo-salinometer model EC 300 YSI and PAR (photosynthetically active radiation), a radiometer Biosciences LI-Cor (model 2501). Water samples were collected with a van Dorn bottle for nutrient analyses (nitrate + nitrite, phosphate, ammonium), filtered in the laboratory in 0.45 μm glass fiber filters and the filtrate frozen at -20°C until analyses in the laboratory. Nutrients of the bottom waters were quantified according to Grasshoff et al (1999).

Five replicates of 300 g of top 5-cm sediments from each site (33 and 82) were collected with a sediment sampler and taken to laboratory where replicates were pooled together and subsequent subsamples were kept frozen at -80 ^{o}C until analysis. DNA extraction was performed from 0.25 g of sediment for each sample, in duplicates, using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., USA), adding a pre-heated step (65°C in water bath for 10 minutes) prior to cell lysis processes by bead beating. DNA was quantified by fluorometry using a Qubit® 2.0 (Thermo-Fisher).

16s rRNA gene sequencing and data analysis

The DNA region amplified, prior to sequencing by paired-end Illumina Miseq platform, was a V3-V4 multivariable fragment of the prokaryotic 16s RNA gene, using a set of primers (U341F and 806R) with high coverage for both Archaea and Bacteria groups that results in amplicons of approximately 400 bp (TAKAHASHI et al., 2014). Briefly, the computational analyses applied were: forward and reverse reads were merged using the software PEAR 0.9.10 (ZHANG et al., 2014), and then followed by a workflow modified from the BMPOS (Brazilian Microbiome Project) (PYLRO et al., 2016), using the softwares QIIME (Quantitative Insights Into Microbial Ecology) 1.9.1 (CAPORASO et al., 2010) and USEARCH7 (EDGAR, 2013). The complete modified workflow used in this study is uploaded to GitHub repository under the name "Bioenergia-Lagoa" (https://github.com/vinisalazar/BioEnergia-Lagoa). The sequence parameter for clustering by similarity into operational taxonomic units (OTUs) was 97%, and then classified taxonomically using SILVA 119 database (QUAST et al., 2013). Finally, the BIOM table generated was normalized to the sample with lower counts.

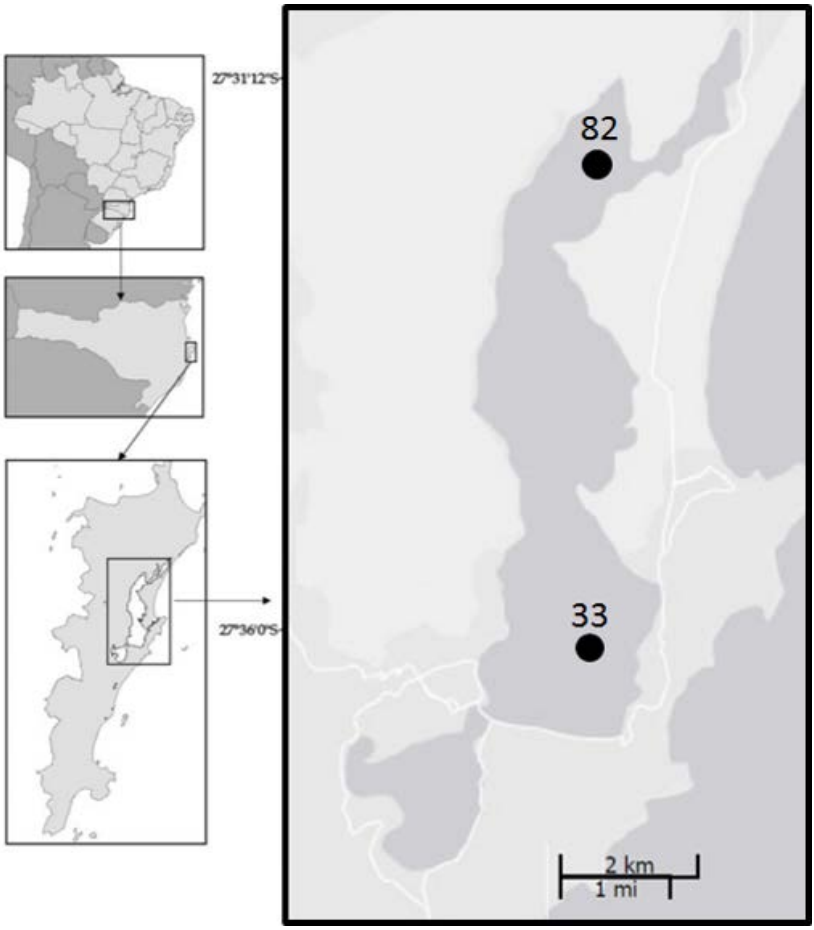

Figure 1: **Sampling sites in Conceição Lagoon.** Site 82 at Northern sector (N), and site 33 at central-southern sector (CS).

Statistical analysis

Analysis of variance (Two-way ANOVA) and redundancy analysis were carried out using the software R 3.3.1 for each of the most abundant domains, phyla, classes, orders and families of OTUs, which were obtained from the BIOM table against the abiotic variables. The multivariate

analyses such as cluster and heat-maps for phyla and families were done in Visualization and Analysis of Microbial Population Structures (VAMPS) using the Bray-Curtis index (HUSE et al., 2014).

Results

Overlaying bottom water metadata

Average salinity was higher (mean= 29.9 ppt) at site 33 (frequent hypoxic/suboxic site) than at site 82 (mean= 16.9 ppt), where minimum and maximum values were 28.8 and 31.4 at site 33, and 15.6 and 18.2 at site 82, respectively. Temperatures were similar in both sites, with an average of 24.9°C, and minimum and maximum values of 23.3°C and 26°C, respectively. Site 33 presented lower dissolved oxygen (DO) (mean = 5.39 mg/L) when compared to site 82 (mean = 8.76 mg/L). Maximum OD concentration was detected at site 82 in November, 9.7 mg/L. Ammonium and phosphate were higher at site 33 (mean= 18.57 and 0.45 μM, respectively) than site 82 (mean= 4.64 and 0.24 μM, respectively). Nitrite + nitrate, on the other hand, presented higher concentrations at site 82 (mean= 0.40 μM) than at site 33 (mean= 0.19 μM).

Dynamics of prokaryotes in the sediments

A total count of 219,709 high quality sequences reads was obtained in this study, which was assigned to a total of 1,546 operational taxonomic units (OTUs) at 97% identity in the sediments of Conceição lagoon.

Abundance of Archaea was significantly higher in site 33 when compared to site 82, where they contributed of 20% to 23% of total prokaryotes, against only 8% to 19% of total prokaryotes at site 82 (figure 2). Consequently, the highest contribution of Bacteria in all samples was registered at site 82, especially in November; when the bottom waters of that site had the lowest salinity and highest nitrite + nitrate levels (Table 1).

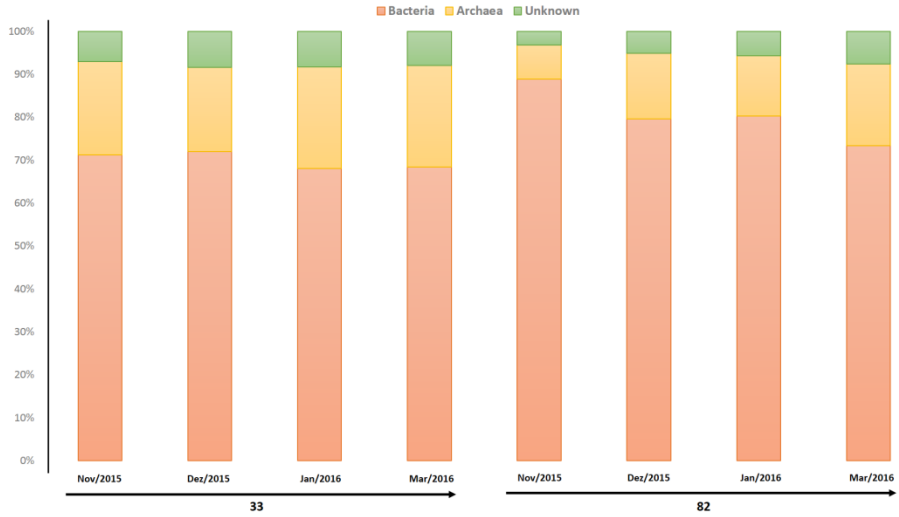

Figure 2: **Relative abundance of Archaea and Bacteria domains in sediments of Conceição Lagoon.**

Proteobacteria and Chloroflexi were the most abundant phyla among Bacteria domain in all samples, followed by Euryarchaeota, the most abundant phylum among Archaea. Their relative abundances varied from 20-24.2%, 18.6-24.8% and 15.2-18.6%, respectively, at site 33 and from 29.3-40.1%, 13.2-23.7% and 7.4-16.3%, respectively, at site 82. Cyanobacteria has showed to be the twelfth most abundant phylum, with an average of 2% of the community throughout the samples, except for site 82 in November when a significant increase reaching 14% of total prokaryotes was observed (Fig. 3). The same sample also showed the highest relative

abundances of Proteobacteria (40%) and the lowest of values of Chloroflexi (13.2%) and Euryarchaeota (7.4%) (Table 2). Overall, the analysis of variance showed that the differences in these most abundant taxa are statistically significant, and that "sites" explained these differences better than "months" (Table 2).

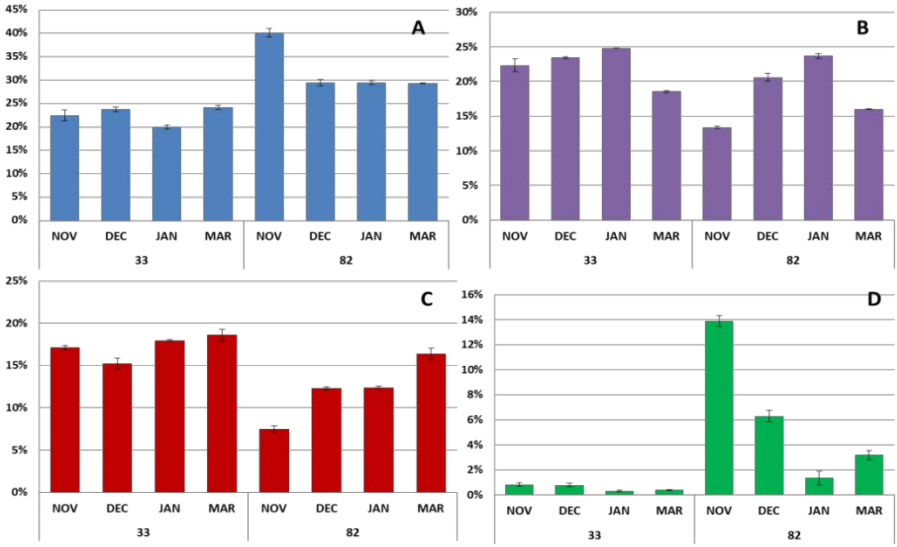

Figure 3: **Phyla diversity of the microbial community.** Proteobacteria (A), Chloroflexi (B), Euryarchaeota (C) e Cyanobacteria (D).

The most abundant classes that presented significant spatiotemporal variability within the lagoon were Deltaproteobacteria, Thermoplasmata, Dehalococcoidia and

Anaerolineae, which oscillated from 18.0-20.5%, 9.5-13.5%, 10.5-14.0% and 6.5-10.0%, respectively, at site 33 and from 17.0-22.0%, 6.0-12.5%, 2.0-9.0% and 7.5-14.0%, respectively, at site 82 (Fig. 4, table 2). Deltaproteobacteria (p. Proteobacteria), Dehalococcoidia (p. Chloroflexi), and Thermoplasmata (p. Euryarchaeota) were significantly lower at site 82 in November compared to other samples, which is concurrent with the lowest salinity and highest nitrate + nitrite levels observed. Anaerolineae (p. Chloroflexi) did not show the same trend, where abundance at site 82 in November was high. As Proteobacteria were the predominant phylum in the sediments of the lagoon, we chose to show the predominant Classes of Proteobacteria. Deltaproteobacteria oscillated from 18.86% in November to 20.35% in March at site 33 and from 16.88% in November to 21.77% in March at site 82. Gammaproteobacteria oscillated from 2.71% in November to 3.06% in March at site 33 and from 17.72% in November to 6.01% in March at site 82. Alphaproteobacteria and Proteobacteria Incertae Sedis contributed to <1% of Proteobacteria (Fig. 5, table 2). In regards to the most abundant families with identified taxonomy detected in the sediments are Desulfarculaceae (10-12% at site 33 and 7-14% at site 82), Anaerolineaceae (6-10% at site 33 and 5-12% at site 82),

Marine benthic group D and DHVEG-1 (8-10% at site 33 and 5-12% at site 82), and Spirochaetaceae, Desulfobacteraceae, Thiotrichaceae, with respective relative abundances means of 4%, 3% and 2%.

Multivariate analyses (spatial and temporal)

The dissimilarity index calculated for the prokaryotic community structure based on taxonomic level of phylum and family showed that the community composition was more dissimilar with space than with time (Fig. 6 A and B), and that site 82 in November had the most dissimilar community in this study (Fig. 6B).

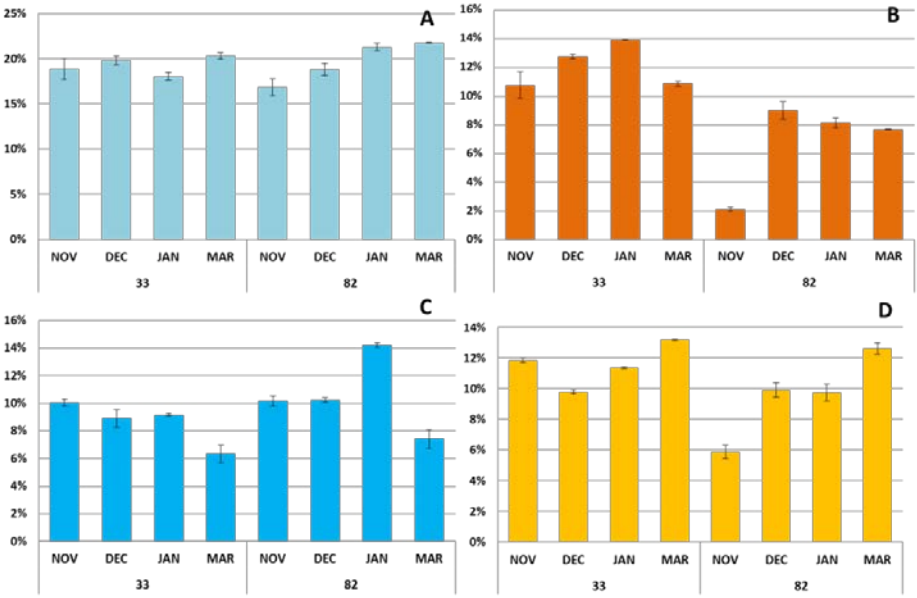

Figure 4: **Diversity of the four most abundant classes in the lagoon's sediments.** Deltaproteobacteria (A), Thermoplasmata (B), Anaerolineae (C) amd Dehalococcoidia (D). Relative abundance showed significant temporal differences for A, B (*) and D (**); spatial significant differences for B (*) e D (***). $p<0,05$(*); $p<0,01$(**) and $p<0.001$(***).

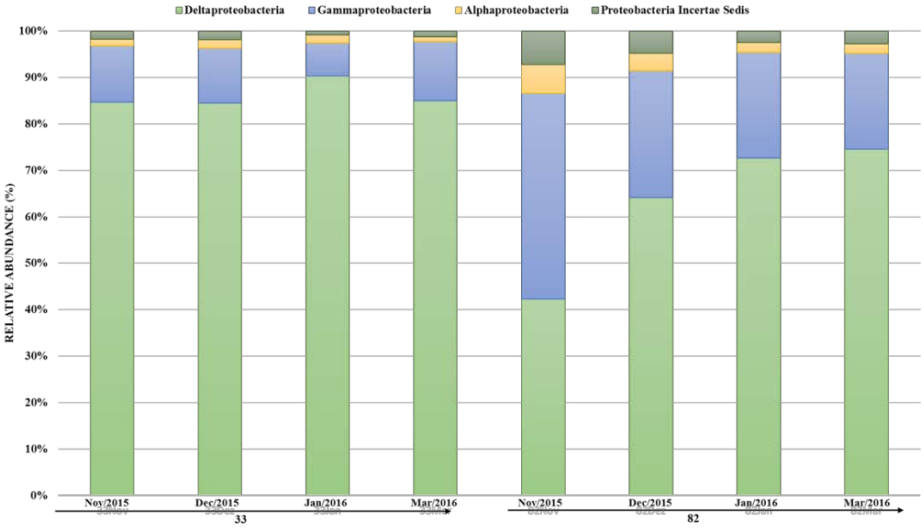

Figure 5: **Relative abundance of major Proteobacteria classes in the sediments of Conceicao Lagoon.** Significant differences are observed with time for classes Alphaproteobacteria (***), Deltaproteobacteria (*), Gammaproteobactéria e Proteobactéria Incertae Sedies (**). p<0,05(*); p<0,01(**) e p<0.001(***).

The RDA (Fig. 7) showed that nitrate + nitrite and DO were positively correlated with Cyanobacteria and Proteobacteria at site 82, and more strongly in November. SIMPER results reinforce that by showing them as the main phyla responsible for 14.35% of total dissimilarity between sites 33 and 82, followed by Thaumarcheota, responsible for 4.86%, and Euryarchaeota for 4.21% of the dissimilarity between sites (data not shown).

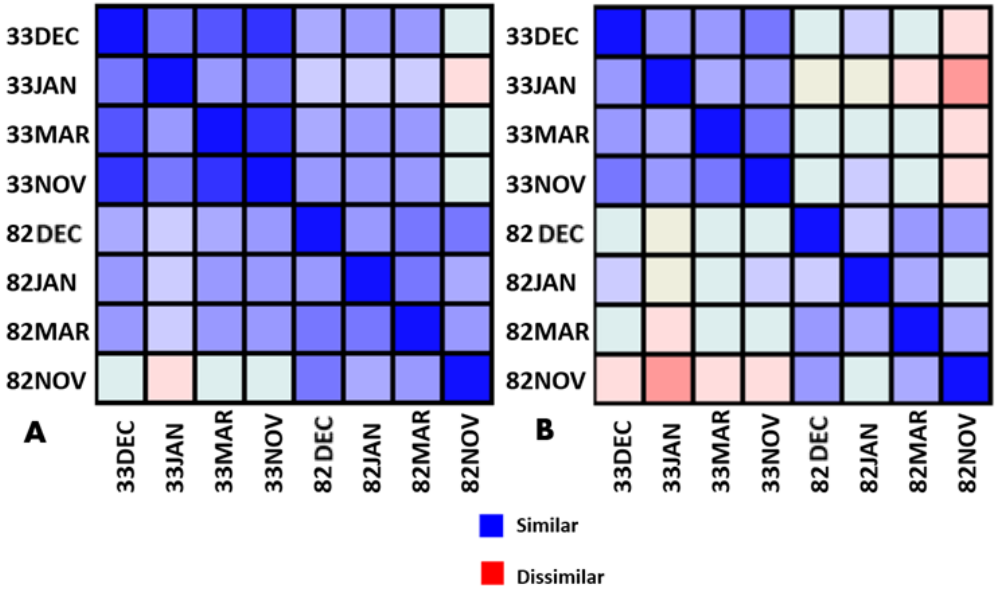

Figure 6: **Dispersion analysis for taxonomic composition: microbial phyla (A) and microbial families (B).** Data was standardized and normalized for the sample with lowest number of reads (7,488) and dissimilarity calculated using Bray-Curtis index in a heat-map color scale.

For family-level, the predominant families were similar as shown in sup. mat. figure 1; however, the rare families such as Deep Sea Euryarchaeotic Group (DSEG) (d. Archaea, p. Euryarchaeota, c. Halobacteria, o. Halobacteriales, g. Aenigmarchaeota), Thiotrichaceae (d. Bacteria, p. Proteobacteria, c. Gammaproteobacteria, o. Thiotrichales), uncultured archaeon (d. Archaea, p. Euryarchaeota, c.

Halobacteria, o. Halobacteriales), Granulosicoccaceae (d. Bacteria, p. Proteobacteria, c. Gammaproteobacteria, o. Chromatiales), Chromatiaceae (d. Bacteria, p. Proteobacteria, c. Gammaproteobacteria, o. Chromatiales), and Chlorobiaceae (d. Bacteria, p. Chlorobi, c. Chlorobea, o. Rhodospirillales) were responsible for 17.60% of the dissimilarity (data not shown).

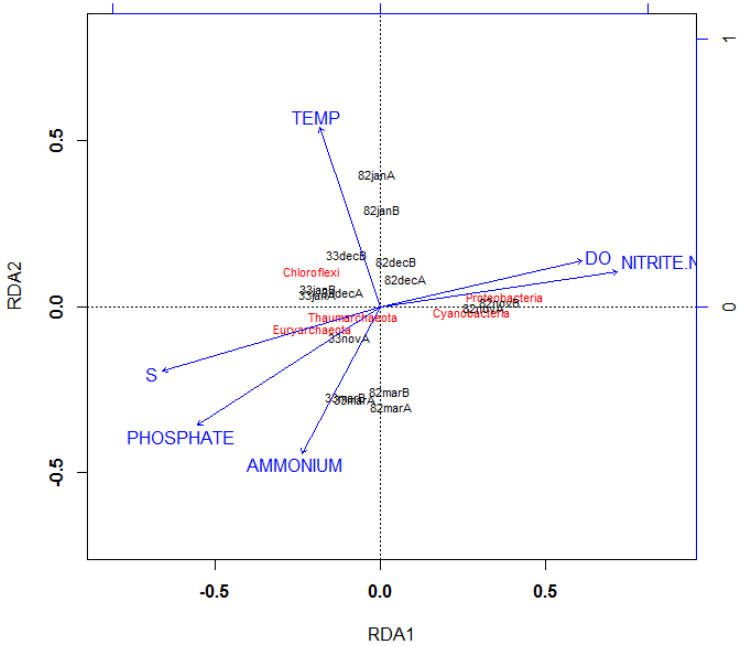

Figure 7: **Redundancy analysis (RDA) of the top 7 prokaryotic groups at phylum level using transformed (log+1) abiotic data.**

Discussion

In the present study, we examined the structure of microbial community of sediments collected from two sites of Conceição Lagoon subjected to different water stratification and bottom dissolved oxygen (DO) concentrations, according to previous studies of Fontes and Abreu (FONTES & ABREU, 2009; FONTES & ABREU, 2012). It is known that the longer an ecosystem suffers from regular recurrent hypoxia the more difficult it is to restore oxygen conditions of the dead zone back to life (RABALAIS, 2015), indicating that even though site 33 presents elevated salinity, high urbanization and water stratification, its microbial community may still be resilient to regenerate local oxygen concentrations seasonally. According to Diaz and Rosenberg (2008), coastal hypoxia follows predictable patterns of eutrophication, in which a third phase of progression is set when low DO levels are found recurrently and hypoxia becomes seasonal (DIAZ & ROSENBERG, 2008), similar to what is found at site 33 of Conceição Lagoon.

The strong positive correlation between DO and nitrite + nitrate and Proteobacteria and Cyanobacteria at site 82 in November suggests a more photoautotrophy-dominated community in this sector. Previous studies have reported that complex secondary metabolites produced by freshwater cyanobacteria strains may favour the following growth of

heterotrophic bacteria (HARADA, 2004). Another study on strain isolation showed that waters in which the abundance of *Anabaena* sp. was the highest were the ones with the biggest abundance of Alphaproteobacteria (*g. Sphignomonas*), and that lakes, rivers and brackish waters with frequent cyanobacterial predominance presented high diversity of cultivable heterotrophic bacteria, some previously unknown or uncultured (BERG et al., 2009). This may be an indicative that the resiliency found in site 82 might be related to seasonal blooms of cyanobacteria and their intense primary production, especially during late austral spring. The variations in climatology, such as the intense and atypical El Nino that persisted through the late austral spring in 2015, might have contributed to the high precipitation levels during this season (HUGHES et al., 2015), causing nutrients runoff to estuaries and coastal ecosystems (RABALAIS, 2015), including Conceição Lagoon.

On the other hand, the elevated salinity has shown to explain the higher abundance of the phyla Euryarchaeota at site 33. Two of the most abundant archaeal classes found in our samples, Halobacteria and Thermoplasmata, have been previously described at sediments from photic-oxic and oxygen-depleted layers, respectively, from the hypersaline lake

of the Kiritimati atoll (SCHNEIDER et al., 2013). This last cited taxon have been previously identified at Helgoland mud area through pyrosequencing, presenting positive correlations with the total organic carbon in sediments (ONI et al., 2015). Most of the sequences that were found in this work are relative to the uncultured Thermoplasmatales groups (Marine Benthic Group D and DHVEG-1), known for its high abundance in other anoxic sediments (LLOYD et al., 2013), in water column and sediment of anoxic deep-sea hydrothermal vents (TAKAI & HORIKOSHI, 1999) and in sediments of the hypersaline laguna Tebenquiche (FERNANDEZ et al., 2016b). Studies carried out in Tibetan lakes have shown that salinity is the most important factor shaping microbial diversity and structure, and that the least present groups are the most sensitive to this abiotic variable (YANG et al., 2016). Site 33 also presented higher relative abundance ($p<0.05$) of the phylum Aminicenantes (mean= 3.8%), indicating possible hydrocarbon or ruminal-associated contamination (FARAG et al., 2014) of the central-south area of the lagoon, subject to more intense urbanization than the Northern sector (FONSECA & BRAGA, 2006).

The most abundant class of Proteobacteria, Deltaproteobacteria, Orders Desulfarculales and

Desulfobacterales (sulfate-reducing bacteria) were predominant in all samples but in site 82 in November, which indicates lower sulphate-reducing metabolism in this area. High DO in bottom waters may have limited the growth of these anaerobic bacteria (MUYZER & STAMS, 2008) in the top sediments in November. These taxa are often in elevated abundance in deep sediments of the Gulf of Mexico, the second human-caused hypoxic area in the world (RABOTYAGOV et al., 2014), and are shown to play an important role on polyaromatic hydrocarbons, alkanes, and alkenes degradation during oil spill at this coastal ecosystem (GOLDING et al., 2013). This fact reinforces our hypothesis of possible hydrocarbon contamination at site 33, but further functional diversity studies and experiments are essential to track any contamination source.

The third and fourth predominant classes of *Chloroflexi* (green non-sulphur bacteria), Anaerolineae and Dehalococcoidia, significantly higher at site 33, were also found dominating other hypoxic-anoxic sediments throughout the world. For example: deep sedimentary community of the pelagic pacific ocean subseafloor (WALSH et al., 2015), freshwater sediments from Qinghai-Tibetan Lakes (YANG et al., 2016), ponds of two poultry feeding constructed wetlands

(Louisiana, USA) (JEONG & HAM, 2016) and at the Gulf of Finland, one of the most severely eutrophic areas of the Baltic Sea (SINKKO et al., 2013).

Conclusions

This study will help to elucidate the impact of the permanent opening, in 1982, of the channel that connects the studied “dead zone” to the Atlantic Ocean. The high salinity at the central sector, site 33, seems to be an important factor for the seasonal anoxic events in this region of Conceição Lagoon, since the constant entrance of marine waters has led to a permanent stratification of the water column. This stability of the water column at site 33 along with the predominant authochothonous organic material in the area are responsible for the maintaining a more stable microbial community structure, when compared to site 82, a less anthropogenic impacted region.

Overall, this study provided the first detailed spatio-temporal study of the distribution of prokaryotes in the top 5-cm of the sediments of Conceição Lagoon. We conclude that the major differences are observed with space and that as the water column, there are differences in the microbial community of the sediments of two sectors of the lagoon. A

more detailed study in the functional diversity needs to be conducted to explain the role of these shifts in the biogeochemical cycles.

Acknowledgements

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SUPPLEMENTARY MATERIALS

Sample	S.D. (m)	T (°C)	S (ppt)	DO (mg L^{-1})	NH_4^+ (µM)	NO_3^-+NO_2^-(µM)	PO_4^{3-} (µM)
33NOV	5.5	23.3	28.80	9.25	20.98	0.73	0.47
33DEC	5.5	25.8	29.60	3.99	3.80	0.28	0.47
33JAN	5.5	26.0	31.40	4.41	24.23	0.32	0.37
33MAR	5.5	24.5	29.93	3.91	25.28	0.29	0.47
82NOV	6.0	23.6	15.60	9.70	13.77	1.47	0.22
82DEC	5.5	26.6	17.00	8.74	1.25	0.41	0.28
82JAN	5.5	29.2	18.20	8.03	1.63	0.64	0.21
82MAR	5.5	27.1	16.93	8.57	1.93	0.52	0.25

Table 1: Physical-chemical parameters of the bottom waters of Conceição Lagoon concomitant to sediment sampling dates. S.D. = sampling depth, T = temperature, DO = dissolved oxygen, NH_4^+ = ammonium, NO_3^- + NO_2^- = nitrate + nitrite, PO_4^{3-} = phosphate.

TAXA		Months[1]	Sites[1]	Min (%) #33	Max (%) #33	Mean (%) #33	Min (%) #82	Max (%) #82	Mean (%) #82
Domains	Archaea	*	***	0.19	0.23	**0.22**	0.08	0.19	**0.14**
	Bacteria	*	***	0.68	0.73	**0.70**	0.73	0.89	**0.80**
Phyla	Proteobacteria	n.s.	***	0.20	0.25	**0.23**	0.29	0.41	**0.32**
	Chloroflexi	**	**	0.18	0.25	**0.22**	0.13	0.24	**0.18**
	Cyanobacteria	*	**	0.00	0.01	**0.01**	0.01	0.14	**0.06**
	Euryarchaeota	**	***	0.15	0.19	**0.17**	0.07	0.17	**0.12**
Classes	Deltaproteobacteria	n.s.	*	0.18	0.21	**0.19**	0.18	0.22	**0.20**
	Thermoplasmata	*	*	0.09	0.14	**0.12**	0.06	0.13	**0.10**
	Anaerolineae	**	*	0.06	0.10	**0.09**	0.07	0.19	**0.11**
	Dehalococcoidia	**	***	0.10	0.14	**0.12**	0.02	0.09	**0.07**
	Gammaproteobacteria	*	***	0.01	0.03	**0.02**	0.06	0.19	**0.10**
	Proteobacteria Incertae Sedis	*	***	0.00	0.01	**0.00**	0.01	0.03	**0.01**
	Alphaproteobacteria	n.s.	**	0.00	0.00	**0.00**	0.01	0.03	**0.01**

Table 2: Minimum, maximum, average values and variance analysis of the most representative taxa and reliability levels.

[1] n.s. = p>0.05, * = p<0.05, ** = p<0.01 and *** = p<0.001

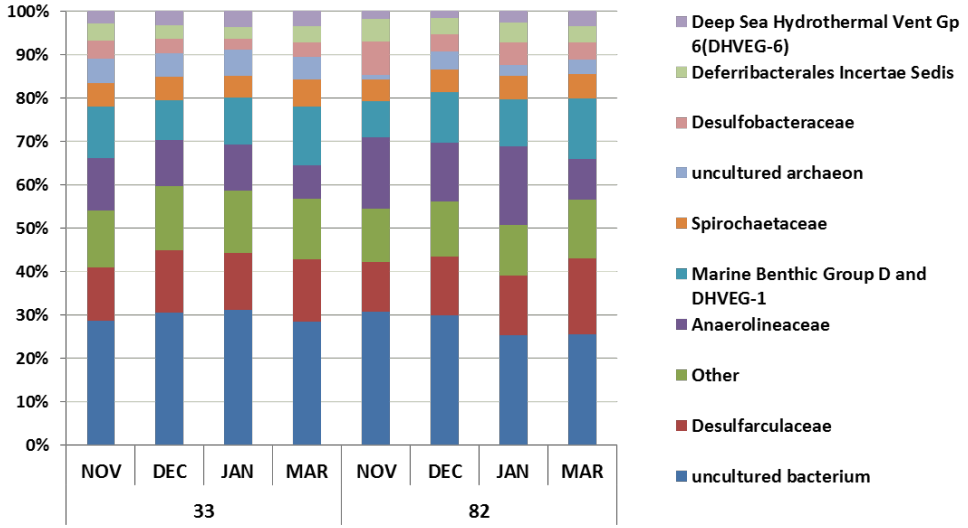

Figure S1: **Top 10 most abundant families in Conceição Lagoon's sediments.**

CAPÍTULO II

BIOPROSPECTION OF HYDROGEN PRODUCING CYANOBACTERIA AND PROTEOBACTERIA USING WINOGRADSKY COLUMNS

Introduction

Although fossil fuel-based generation of energy has promoted exponential industrial development in the past few decades, the current global energy scenario requires diversification on acquisition of energy and fuel options. Hydrogen, the most abundant element on Earth, is considered to be the fuel of the future, since its combustion produces water as the only by-product, a 100% clean energy (GAZEY et al., 2006). This sustainable energy alternative have great chances to take place in the global scenario upon the unprecedented carbon dioxide levels in the atmosphere and global warming (CHANDRASEKHAR et al. 2015; JIANG et al. 2016). Additionally, this potentially versatile energy yields high-energy of 122 kJ/g per unit mass, which is 2.75-fold higher than that of hydrocarbon fuels, and can be produced through methane reforming, electrolysis of water or by microorganisms (CHRISTOPHER & DIMITRIOS, 2012). The biological hydrogen production is the process with the least energy loss of

all, and is favourable in ambient temperature and atmospheric pressure (WANG & WAN, 2009). The biological hydrogen or biohydrogen production is carried out by fermenting microbes (dark anaerobic process, and most studied mechanism) and by phototrophic organisms (such as eukaryotic algae, cyanobacteria and purple sulphur and non-sulfur phototrophic bacteria) (MAO et al., 2005). The phototrophic production can occur through direct photolysis (oxygenic photosynthesis) or indirect, where the CO_2 is transformed during the Calvin cycle into simple sugars and stored as polysaccharides, which later may be consumed through dark fermentation (Redwood et al. 2009). The biohydrogen production by direct photolysis is limited by oxygen; however, in the indirect photolysis, H_2 and O_2 are separated by their generation in different compartments or time. The photolysis is catalyzed by nitrogenases and hydrogenases, which are both found in purple sulphur and purple non-sulfur bacteria, and in Cyanobacteria. Only hydrogenases are found in eukaryotic microalgae (REDWOOD et al., 2009). Cyanobacteria are potential and versatile H_2 producing microorganisms for carrying a variety of genes involved in the uptake hydrogenase, bidirectional hydrogenase and nitrogenase pathways (TAMAGNINI et al., 2002; LEINO et al., 2014).

Several studies have been showing that the maximum efficiency in production of biohydrogen is reached when multiple organisms and processes are associated (Masset et al 2012; Cai et al., 2012). Sediments and soils host some of the most diverse microbial communities on planet (LOZUPONE & KNIGHT, 2007). It is estimated that 200 million tonnes of hydrogen are produced and consumed annually in anoxic sediments (NANDI & SEGUPTA, 1998).

The Winogradsky column (WC), a method created by Sergei Winogradsky in the late 1800's, is a simple way to study metabolic dynamics of photolithotrophic microorganisms from soil and sediments, by the formation of a vertical chemical gradient in oxygen and sulphur formed adding sulphate and a carbon source in the bottom of the column exposed to light (DWORKIN & GUTNICK, 2012).

Considering that less than 1% of the Earth's microorganisms are able to grow in traditional culture media in the laboratory, the application of WC in ecological and prospection of microorganisms for biotechnology studies is promising. Therefore, when this method is combined to next generation sequencing (NGS) techniques it allows the growth and the taxonomic characterization of microbes involved in synthrophic processes that would be energetically unfavourable

if separated (HOLLIBAUGH et al., 2001; ESTEBAN et al., 2015).

The application WCs in conjunction with molecular techniques is recent, either in microbial ecology studies (RUNDELL et al., 2014; ABBASIAN et al., 2015; ESTEBAN et al., 2015) or in biotechnological applications (LOSS et al. 2013). These previous studies have shown an increase in Proteobacteria and Cyanobacteria with time in the Winogradsky column; the two groups that have largely been reported as hosting biohydrogen producers.

The sediments of Conceição Lagoon store large amounts of the gas (Klein pers. communication), making it an excellent system for bioprospection of hydrogen producers. Consequently, simultaneous WC and molecular techniques were applied for the quantification of the biohydrogen production by microorganisms isolated from sediments of Conceição Lagoon. On the other hand, the authors were not able to identify the species within the isolated photoheretotrophic consortium of purple non-sulfur bacteria (PNS - a group of Proteobacteria) due to limitations of the method used (LOSS et al 2013).

Thus, this study aimed to identify the spatio-temporal variability of Cyanobacteria and Proteobacteria of sediments of

the Conceição Lagoon using a combination of WCs and NGS techniques to optimize the prospection of biohydrogen-producers among these two groups.

Materials and methods

Sediment and water sampling

Bottom water and sediments were used as inocula for the WCs were sampled at two distinct sites at Conceição Lagoon; **S1** (-27°36'S - 48°27'W), previously described as a vertical stratified water column subjected to seasonal suboxia/anoxia or "dead zones"; and **S2** (27°52'S - 48°45'W), where bottom water normoxia is constant. Five replicates of 300 g of top 5-cm of sediments from these two sites were collected with a sediment sampler in November 2015, brought to the laboratory where they were pooled together. Part of the sediment was frozen at -80°C for posterior analysis (to – initial time), and the other part used for the preparation of the WCs.

Winogradsky columns, incubation and DNA extraction

Transparent polyethylene terephthalate bottles (250 mL) of 15 cm high and 5 cm of diameter (with the bottle necks cut-off)

were used as Winogradsky columns. The bottom of the WCs were inoculated with 100mL of sediments enriched with $CaCO_3$ 0,15M; $CaSO_4$ 0,1M and 2g of chopped cellulose (= *enriched* sediments), followed by addition of 30mL of sediments (= *unenriched* sediments) and completed with 60mL of bottom waters of the respective sampling sites. A total of eighteen columns were prepared: from two sites, three times ($t_{(1)}$= 1 week, $t_{(2)}$ 1 month and $t_{(3)}$= 2 months) and triplicates for site and time. The columns were covered with parafilm and placed into a BOD incubator (SL-224/364) under constant photoperiod (±85 μmol/m^2/s) (adaptation according to previous studies of Fontes et al 2011; Loss et al. 2013; Esteban et al. 2015) and temperature at 21 °C (field temperature) (Fig. 1). All columns were carefully place-alternated 2x/day in order to guarantee similar light exposure to all columns. After each pre-defined time, six WCs were removed and frozen at -80 °C for posterior sawing. Each column was sawed at the enriched/unenriched+water interface, then thawed and well homogenized for immediate DNA extraction.

Figure 1: **Initial incubation time of Winogradsky Columns (18 columns shown in the photo), $T_{(0)}$.**

DNA extraction, high-throughput sequencing and OUT picking

For DNA extraction, 0.25g of WC samples (raw sediments $t_{(0)}$, *enriched* $t_{(1)}$, $t_{(2)}$, $t_{(3)}$ and *unenriched* $t_{(1)}$, $t_{(2)}$, $t_{(3)}$ – in triplicate = 40 samples) were done using a PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., USA), adding a pre-heated step (65°C in water bath for 10 minutes) prior to cell lysis processes by beads beating. DNA was quantified in a Qubit® 2.0 fluorometer before 16S amplification and consequently paired-end sequenced in the Neoprospecta Facility through Illumina Miseq platform. The region chose for

amplification was a V3-V4 multivariable fragment of the 16S rRNA gene, using a set of primers (U341F and 806R) with high coverage for both Archaea and Bacteria groups that results in amplicons of approximately 400bp (TAKAHASHI et al., 2014). Briefly, forward and reverse reads were merged using the software PEAR 0.9.10 and only high-quality merged sequences were used (ZHANG et al., 2014). Sequence analyses were conducted with the BMPOS (Brazilian Microbiome Project Operational System) for Illumina data (PYLRO et al., 2016) using QIIME (Quantitative Insights Into Microbial Ecology) 1.9.1 (CAPORASO et al., 2010) and USEARCH7 (EDGAR, 2013). The sequence parameter for similarity of operational taxonomic units (OTUs) was 97%. Sequences were then classified taxonomically using SILVA 119 database (QUAST et al., 2013), and the BIOM table generated was normalized to the sample with lower counts.

qPCR

In order to guarantee the viability of RNA after extraction to determine the hydrogen-producing genes expressions in Cyanobacteria, the experiment was repeated without freezing the columns and RNA extracted immediately after end of incubation. Sediments and bottom water were

sampled from the same sites S1 and S2 in March of 2016, and all WCs preparation and incubation were done exactly as described previously, in triplicate. RNA was then immediately extracted at to, after 1 month using RNA PowerSoil® Total RNA Isolation kit (MoBIO, Laboratories Inc.) and quantified in NanoDrop 2000 (Thermo Scientific).

Four reference genes were chosen to target cyanobacteria based on Pinto et al. (2012) and three functional genes described as expression synthesis of enzymes involved in hydrogen production in Cyanobacteria used were *nifH* - conventional nitrogenase, *hupL – uptake hydrogenase* and *hoxY* – bidirectional hydrogenase (LEINO et al., 2014). As nifH was one of the more stable genes with time, it was not used in this study.

cDNA synthesis and gene expression analyses

cDNA synthesis was performed with specific primer amplification by reverse transcription with M-MLV reverse transcriptase (Invitrogen, Waltham, MA, EUA), according to manufacturer instructions.

The RT-qPCR amplifications were performed on a ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). PCR reactions were carried out in a final

volume of 10µL, containing 5µL of diluted cDNA (1:100), 1X SYBR Green (Invitrogen, Waltham, MA, EUA), 0.025mM dNTP, 1X PCR buffer, 3mM MgCl2, 0.25U Platinum Taq DNA Polymerase (Invitrogen, Waltham, MA, EUA) and 200nM of each reverse and forward primer. The RT-qPCR conditions were set as follows: 94°C for 5 minutes, 40 cycles of 94°C for 15 seconds, 60°C for 10 seconds and 15 seconds at 72°C. Samples were analyzed in technical triplicates, and a template negative control was included. Also, an RNA template was included to confirm the absence of contaminant genomic DNA.

Statistical Analyses

Analysis of variance (Two-way ANOVA) and redundancy analysis for the most abundant phyla against the incubation conditions were carried out using the software R 3.3.1. For qPCR, the most stable pair of genes was used to normalize the gene expression levels of the functional genes in Microsoft Excel (2007) using the Geomean function. Statistical analyses were conducted using Kruskal– Wallis test (non-parametric one way analysis of variance by ranks) in STATISTICA version 10.0 (StatSoft, Tulsa, OK) and the percentages of increasing functional gene expression compared

to control genes were calculated according to Siboni et al. (2014).

Results and Discussion

Proteobacteria and Cyanobacteria distribution in Winogradsky columns

A total of 2,222 operational taxonomic units (OTUs) were assigned at 97% identity with a total count of 480,792 high-quality sequence reads. Thirty eight phyla were identified in the initial time (t_0) and forty-six phyla after the incubation in WCs.

The taxa Proteobacteria and Cyanobacteria accounted for 50% and 6% of phyla in t_o, with an increase of 2.4 and 2-fold in the number of OTUs, respectively. After a week of incubation, the columns started to present an evident biofilm formation and initial stratification, especially in WCs prepared with S2 sediments. After one month, evident green and purple biofilms could be noticed in the columns S2, while S1 columns only displayed a green biofilm even after a month of incubation (Fig. 2).

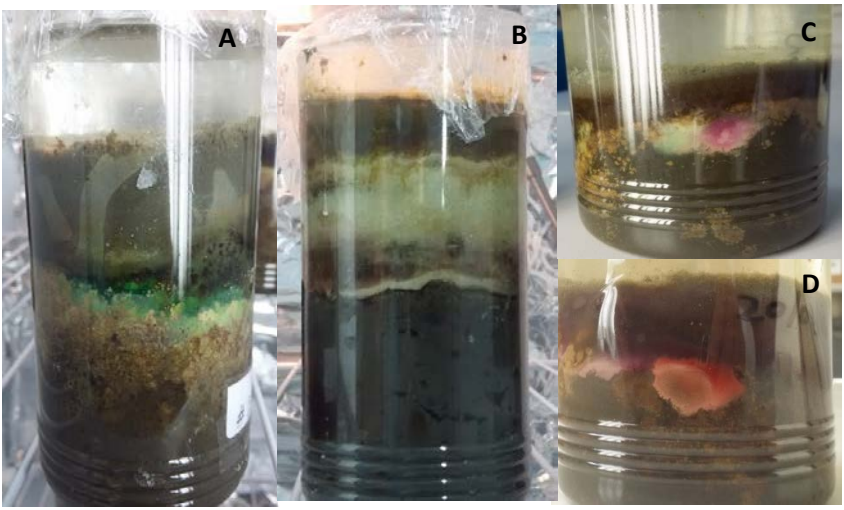

Figure 2: **Biofilm formation in Winogradsky Columns. Typical blue-green mat in a S1(T_1) column (A), a very stratified S1(T_2) column (B), a green nearby a purple biofilm formation in a S2(T_1 – experiment 2) column (C) and a large purple-red biofilm in a S1(T_2** – experiment 2)**.**

Proteobacteria was significantly more abundant in S2 column ($p<0.05$) (Fig.3). The majority of Proteobacteria identified in the enriched layers of both S1 and S2 columns were Deltaproteobacteria in all times. However, the unenriched layers showed a higher equitability of these five classes of Proteobacteria, especially after one month of incubation (Fig. 4). The most pronounced temporal increase in the abundance was in Alphaproteobacteria, which initially represented < 5%

of Proteobacteria in S1 and S2, and reaching 27% and 29% after two months of incubation ($t_{(3)}$), respectively (Fig. 4).

As previously described by Loss *et al.* (2013), the PNS bacteria consortium isolated from Conceição Lagoon sediments produced biohydrogen levels in RCV medium under the presence of two organic acids, acetate and butyrate, at maximum concentrations of 143.56 mL/L and 135.41 mL/L, respectively. This consortium was described as consisting of four different species of purple non-sulfur bacteria, a group of Proteobacteria isolated from the same site, S1.

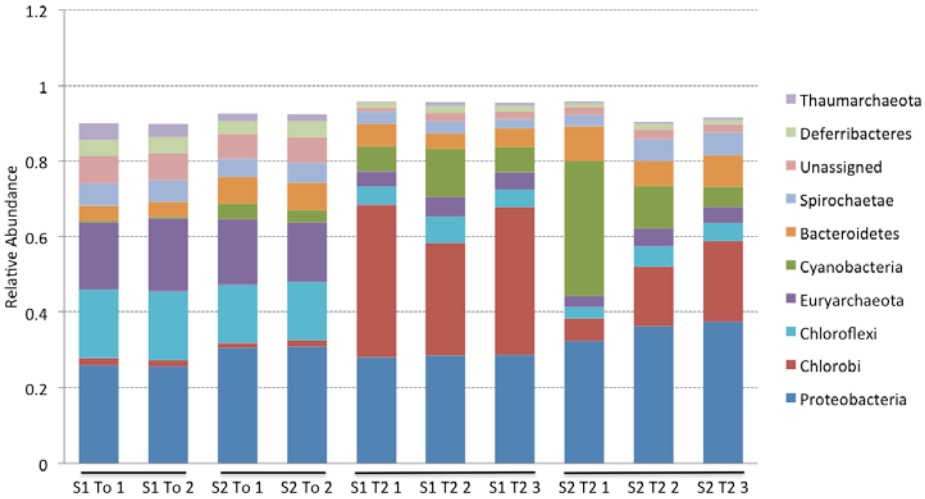

Figure 3: **Temporal microbial diversity variation of the main phyla in WCs (second experiment, prior to *qPCR*).**

Furthermore, the alpha-diversity of Alphaproteobacteria increased 4 times (from 9 to 38 genera) after two months of incubation when compared to t_o (sup. Material table 1), which some have been described as sulfate-reducers. Rare genera of alphaproteobacteria, such as *Rhodovulum* sp. (p. Proteobacteria, c. Alphaproteobacteria, o. Rhodobacterales, f. Rhodobacteraceae), were not found in t_o. However, they became abundant in both S1 and S2 columns with time (sup. Material, table 2). Some strains of *Rhodovulum* sp have been described as photoheterotrophs and have been reported to produce hydrogen in RCV medium, when supplemented with different organic acids prior to incubation in photobioreactors (CAI & WANG, 2012; CAI & WANG, 2013). This finding suggests that this taxon could have potentially been one of the strains in the consortium described by Loss et al. (2013).

Regarding Cyanobacteria, *Prochlorococcus* sp., the smallest (aprox. 0.6µm of diameter) and most abundant Cyanobacteria in most marine ecosystems (FLOMBAUM et al., 2013) was predominant in the S2 columns (Fig. 5).

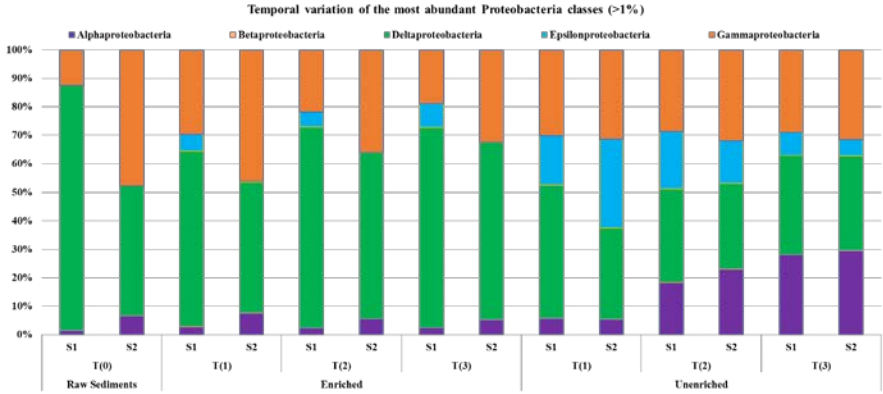

Figure 4: Development of the most abundant classes of Proteobacteria (>1%) in the enriched and unenriched sediments of the Winogradsky columns: from sites S1 (#33) and S2 (#82) incubated for 1 week T(1), 1 month T(2), and 2 months T(3). 100% represents the sum of these most relative abundant classes according to SILVA database.

These coccoid microalgae are mostly, but not exclusive, abundant in oligotrophic waters (PARTENSKY et al., 1999), and previous studies carried out by our group showed seasonal variation of this taxon in the same site of the lagoon, and positively correlated to Nitrite+Nitrate and dissolved oxygen in bottom waters (Chapter 1). The main differences separating these microorganisms from *Synechococcus* sp., are the lack of phycobilisomes, and photosynthetic pigments such as phycoerythrin and phycocyanin (BILLER et al., 2014). An initial characterization of a strain of small coccoid (aprox. 1µm in diameter) cyanobacteria isolated from our WCs have shown to grow in BG-11 medium and express phycoerythrin (data not shown), indicating the successful possible isolation of

Synechococcus sp. (Fig. 7), even being rare in the WCs (representing an average of 0.03% of total microbial community) and not present at t_o. The *unenriched* sediments after 2 months of incubation, $t_{(3)}$, presented the most alpha-diversity, and other two microbial mat forming strains of possibly *Leptolyngbya* sp. and *Oscillatoria* sp. were isolated by series dilution of these sediments and inoculated in BG-11 medium. However, under cultivation in Erlenmeyers, the microbial mats were disrupted which affected negatively their growth even though their pigmentation was maintained.

It was notable that *unenriched* halves of the columns presented higher abundance and diversity of Cyanobacteria compared to the *enriched* halves. The enrichment of cyanobacteria taxa was observed in both studied sediment samples, from raw sediments T(0) to T(3) (Fig. 5). The graphic presents all the found genera for Cyanobacteria in SILVA database, including “other” and “uncultured” taxa with the time. Columns made of sediments from S1 also presented higher abundance ($p<0.05$) of the blue-green biofilm in both *unenriched* and *enriched* halves (Fig. 2 A), possibly corresponding to the group of cyanobacteria *Lyngbya* spp., the same incubated samples that presented the lowest values for

Procholorococcus sp. (Fig. 5), while *Synechococcus* sp. presented less than 1% of all cyanobacterial community.

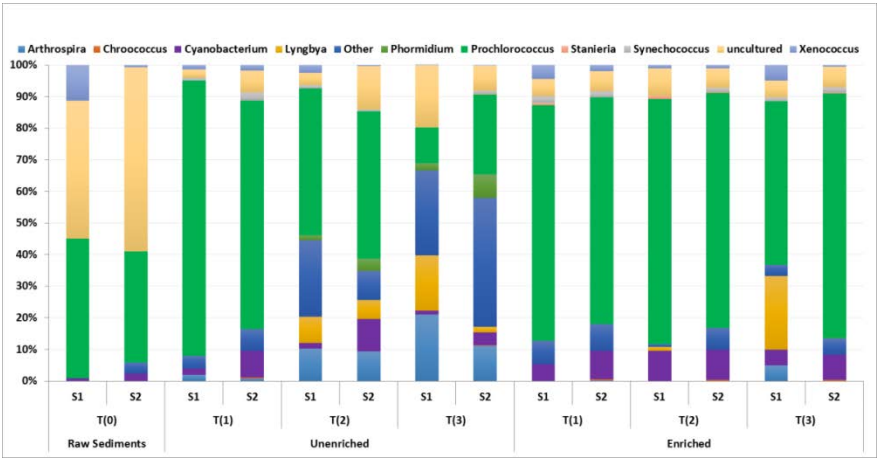

Figure 5: **Temporal changes of Cyanobacteria genera in the *enriched* and *unenriched* sediments of the Winogradsky columns.**

Esteban *et al*. (2015) found high relative abundances of *Anabaena* sp. in WCs, a group of heterocyst-forming cyanobacteria capable of fixing atmospheric N_2 and, as a by-product, potentially produce biohydrogen. Other studies identified that waters in which the abundance of *Anabaena* sp. was the highest also presented significantly higher abundance of Alphaproteobacteria (g. Sphignomonas), and that lakes, rivers and brackish waters with frequent cyanobacterial predominance presented high diversity of cultivable

heterotrophic bacteria, some previously unknown or uncultured (BERG et al., 2009). These findings indicate that WCs are strong tools for the isolation of Cyanobacteria and Proteobacteria, regardless their initial abundance.

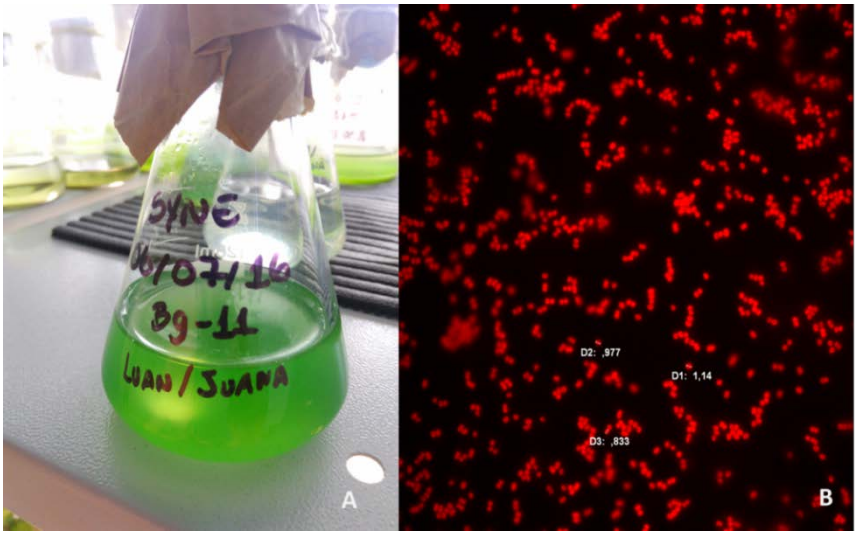

Figure 6**: Cyanobacterial strain isolated from WCs.** On the left (A), the strain while cultivated in BG-11 medium and on the right (B), epifluorescence of the strain under aprox. 550nm.

Although sediments from S2 initially presented higher abundance of Cyanobacteria, WCs with S1 sediments seemed to follow similar patterns of diversification after two months of incubation, under low abundance rate. Overall, the *enriched* sediments presented constant low abundances for S1 and a

gradual decrease for S2 of cyanobacterial individuals with time.

The RDA showed that optimization o wide range of Proteobacteria and Cyanobacteria isolation were more influenced by the origin of sediment than incubation time (Fig. 6). Definitely, unenriched sediments (top layers) of S2 column presented the highest abundance and diversity of these two phyla.

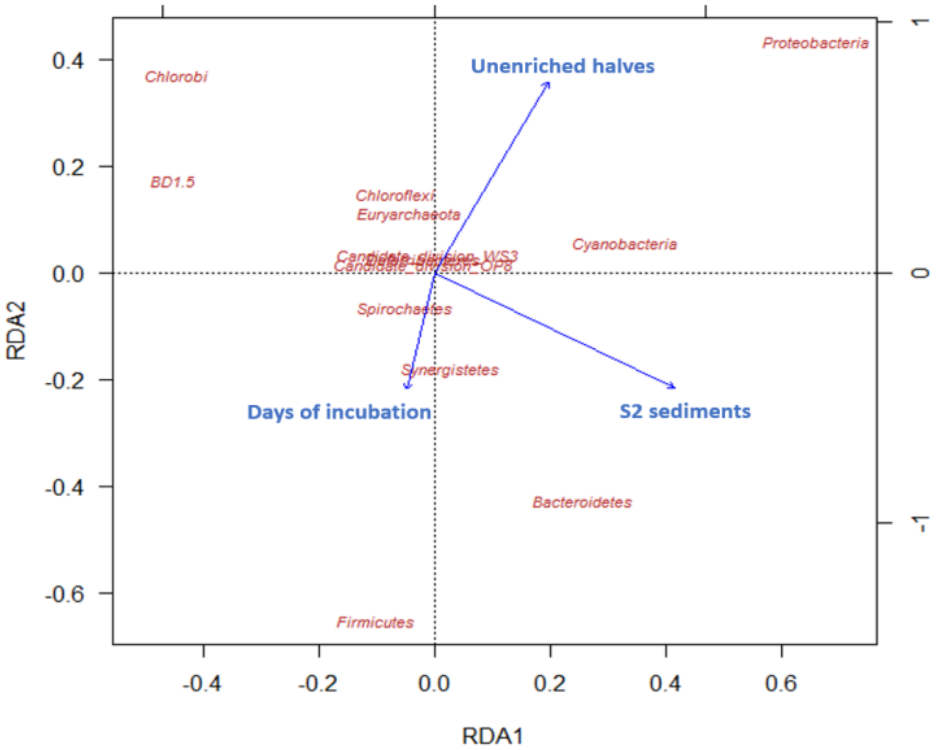

Figure 7: **Redundancy Analysis of phyla level and sediment incubation conditions (enriched and unenriched sediments = enriched halves and unenriched halves; S1 and S2 columns and days of incubation).**

Although statistical analyses showed that the abundance of Cyanobacterial groups was mainly influenced by the origin of sediment and vertical layer of sediment in the WCs, cyanobacterial alpha-diversity increased in all columns after two months of incubation (from 6 to 12 OTUs).

Previous studies have shown that Cyanobacteria produces biohydrogen in RCV medium, when supplemented with different organic acids prior to incubation in photobioreactors (CAI & WANG, 2012; CAI & WANG, 2013). Once again, there is a possibility that Cyanobacteria were present in the consortium isolated by Loss *et al*. (2013).

As observed for the phylogeny studies and statistical analyses, the expression of the two functional genes involved in the translation of enzymes related to hydrogen production in Cyanobacteria (hupL and hoxY) increased significantly ($p < 0.01$) in unenriched sediments of S2 columns (Fig. 7). Both genes were upregulated in S2 columns, showing an increment in expression levels of +17.2% for hupL and +33.3% for hoxY. S1 columns showed a small up-regulation of hupL gene expression after one month of incubation (+4.6%), but it was down-regulated after 2 months (-2.3%). On the other hand, HoxY gene was down-regulated throughout the experiment in the S1 columns (-2.66 and -2.00% respectively).

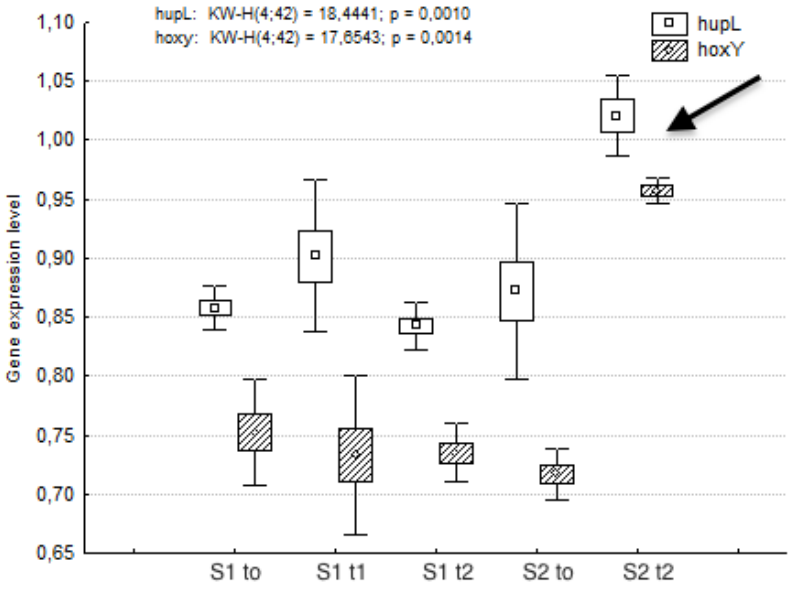

Fig. 8. Expression levels of hupL and hoxY genes involved in the synthesis of bi-directional hydrogenases in unenriched sediments of S1 and S2 WCs (to = initial time, t1 = 1 month after, t2 = 2 months after).

These findings indicate an effective increment of potentially H2-producing Cyanobacteria in S2 columns after 2 months of incubation if described by hupL and hoxY. Cyanobacteria have several other pathways involved in the H2 production: e.g. dark fermentation, direct and indirect biophotolysis (BUROW et al., 2013; NIELSEN et al., 2015).

This was the initial study of such diverse metabolism using a technique that simulates the functionality of microbial mats. With further application of different functional genes related to H2-production pathways in Cyanobacteria and Proteobacteria, and the use NGS techniques simultaneously with the implementation of multi probes within the columns, a better comprehension of temporal variability in H_2 production would be achieved.

Conclusions

The integration between Winogradsky columns and NGS techniques has shown to be effective for bioprospecting studies of phototrophic Proteobacteria and Cyanobacteria as their diversity and abundance increased with time. The Northern sector, represented here by S2, has shown to be the best area to prospect hydrogen-producing phototrophs, and that a minimum of 30 days of incubation is recommended, with optimum of 2 months.

This is the first time that the bioprospection of hydrogen-producing prokaryotes is explored in such spatio-temporal detail, and it was done in just two sites of one ecosystem. We believe that if the prospection of microorganisms and enzymes are expanded and globally

regulated, commercially hydrogen photobioreactors will soon become a reality.

4. PERSPECTIVAS

A partir dos resultados obtidos no presente estudo, novos experimentos visando à aplicação das cepas obtidas na produção de biohidrogênio em escala industrial poderão ser realizados, bem como uma exploração de diferentes sítios dentro da Lagoa da Conceição e em outros ecossistemas. As cepas descritas neste trabalho estão serão aplicadas em projetos de pesquisa de alunos de pós-graduação do curso de Engenharia e Sustentabilidade, Campus UFSC – Araranguá. Novas buscas por cepas poderão ser realizadas aplicando-se a técnica de Sergei Winogradsky, agora com o conhecimento de que sítio da Lagoa da Conceição ter como alvo, bem como o tempo de incubação para um melhor desenvolvimento de Alfaproteobactérias e Cianobactérias. Por fim, este trabalho reforça a ideia de como a ação antrópica pode afetar a biodivesidade de ecossistemas, consequentemente, diminuindo a diversidade genética e reduzindo as possibilidades de prospecção de cepas de interesse biotecnológico.

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