Francielli Vilela Peres

Diversidade e Estrutura de Comunidades Microbianas em Áreas de Pockmarks e Diápiros de Sal na Margem Continental Brasileira (Sudoeste do Oceano Atlântico)

Tese submetida ao Programa de Pós-Graduação em Microbiologia do Instituto de Ciências Biomédicas da Universidade de São Paulo, para obtenção do Título de Doutor em Ciências.

São Paulo
2022

Francielli Vilela Peres

Diversity and Structure of Microbial Communities in Areas of Pockmarks and Salt Diapirs on the Brazilian Continental Margin (Southwest Atlantic Ocean)

A thesis submitted to the Graduate Program in Microbiology from the Institute of Biomedical Sciences, University of São Paulo, to obtain the title of Doctor in Sciences.

Area of Concentration: Microbiology

Advisor: Prof ª Dra. Vivian Helena Pellizari

Original version

São Paulo
2022

CATALOGAÇÃO NA PUBLICAÇÃO (CIP)
Serviço de Biblioteca e informação Biomédica
do Instituto de Ciências Biomédicas da Universidade de São Paulo

Ficha Catalográfica elaborada pelo(a) autor(a)

Peres, Francielli Vilela
Diversidade e Estrutura de Comunidades Microbianas em Áreas de Pockmarks e Diápiros de Sal na Margem Continental Brasileira (Sudoeste do Oceano Atlântico) / Francielli Vilela Peres; orientador Vivian Helena Pellizari. -- São Paulo, 2022.
123 p.

Tese (Doutorado)) -- Universidade de São Paulo, Instituto de Ciências Biomédicas.

1. Diversidade Microbiana. 2. Pockmark. 3. Sedimento Marinho. 4. Metilotrofia. 5. Halofílicos. I. Helena Pellizari, Vivian, orientador. II. Título.

UNIVERSIDADE DE SÃO PAULO

INSTITUTO DE CIÊNCIAS BIOMÉDICAS

Candidato(a): Francielli Vilela Peres

Título da Dissertação/Tese: Diversidade e Estrutura de Comunidades Microbianas em Áreas de Pockmarks e Diápiros de Sal na Margem Continental Brasileira (Sudoeste do Oceano Atlântico)

Orientadora: Vivian Helena Pellizari.

A Comissão Julgadora dos trabalhos de Defesa da Dissertação de Mestrado/Tese de Doutorado, em sessão pública realizada a **25/08/2022**, considerou o(a) candidato(a):

( ) Aprovado(a) ( ) Reprovado(a)

Examinador(a): Assinatura: .....
Nome: .....
Instituição: .....

Examinador(a): Assinatura: .....
Nome: .....
Instituição: .....

Examinador(a): Assinatura: .....
Nome: .....
Instituição: .....

Presidente: Assinatura: .....
Nome: .....
Instituição: .....

ICB USP

Cidade Universitária "Armando de Salles Oliveira", Butantã, São Paulo, SP - Av. Professor Lineu Prestes, 2415 - ICB III - 05508 000
Comissão de Ética em Pesquisa - Telefone (11) 3091-7733 - e-mail: cep@icb.usp.br

CERTIFICADO DE ISENÇÃO

Certificamos que o Protocolo CEP-ICB nº **895/2017** referente ao projeto intitulado: "***Diversidade e estrutura de comunidades microbianas em áreas de pockmarks e diápiros de sal na margem continental brasileira (Oceano Atlântico Sudoeste)***" sob a responsabilidade de ***Francielli Vilela Peres*** e orientação do(a) Prof.(a) Dr.(a) ***Vivian Helena Pellizari,*** do Departamento de Microbiologia, foi analisado pela **CEUA** - Comissão de Ética no Uso de Animais e pelo **CEPSH** – Comitê de Ética em Pesquisa com Seres Humanos, tendo sido deliberado que o referido projeto não utilizará animais que estejam sob a égide da Lei nº 11.794, de 8 de outubro de 2008, nem envolverá procedimentos regulados pela Resolução CONEP nº 466 de 2012.

São Paulo, 10 de agosto de 2017.

Profa. Dra. **Luciane Valéria Sita**
Coordenadora CEUA ICB/USP

Profa. Dra. **Camila Squarzoni Dale**
Coordenadora CEPSH ICB/USP

Eu dedico...

Ao meu pai Evaristo e à minha mãe Valdirene.
Ao meu irmão Alessandro e à minha irmã Daniele.

AGRADECIMENTOS

À Dra. Vivian Helena Pellizari pela orientação, pelos ensinamentos, confiança, pelas oportunidades incríveis e por proporcionar a realização deste sonho.

À minha querida Rosa Gamba por sempre acreditar em mim, me incentivar a ser melhor e por estar ao meu lado em todos os momentos. Você é minha inspiração, Rosinha.

A todo o time LECOM: Amanda Bendia, Alice Moura, Ana Burtarelli, Augusto Amendola, Camila Signori, Célio Jonck, Diana Duque, Fabiana Paula, Júlio Moreira, Lucas Salomão, Maria Carolina, Nailah Ahmed, Natascha Bergo, Raquel Cristina, Rebeca Lizarraga, Renato Gamba e William Gattaz pelo apoio, ensinamento, paciência e pelos momentos de descontração.

Às Dras. Amanda Bendia, Natascha Bergo e Fabiana Paula por me auxiliarem diretamente, por toda a paciência e ensinamento.

Ao Augusto Amendola, João Regis e Raissa Ramos pela confecção dos mapas e pela amizade.

Ao Dr. Michel Michaelovitch de Mahiques por todo o suporte ao projeto e a todos do Laboratório de Geologia de Margens Continentais, em especial à Samara Goya.

Ao Augusto Amendola, Amanda Bendia, Natascha Bergo, Rebeca Lizarraga e Renato Gamba por acreditarem em mim, por sempre me apoiarem e principalmente pela amizade que foi essencial nesse percurso.

Ao Instituto de Ciências Biomédicas e ao Instituto Oceanográfico e todos os professores e funcionários que sempre estiveram de prontidão para me auxiliar.

Aos meus queridos amigos: Alexandre Ferraro, Célia Gonçalves, Luana Agostini, Naiara Machado, Pamela Bernal e Patricia Shibuya pela amizade, incentivo e por tornarem meus dias melhores. Ao Isaias Garcia por todo o apoio, paciência, incentivo e por alegrar os meus dias.

Aos meus pais e irmãos pelo amor incondicional, incentivo e total apoio para que eu realizasse mais este sonho. Serei eternamente grata!

E a todos que direta ou indiretamente me ajudaram a concluir esta etapa.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

Muito obrigada!

“A pesquisa básica é como atirar uma flecha para o ar e,
onde ela cair, pintar um alvo”
(Homer Adkins Burton)

RESUMO

Peres, F. V. **Diversidade e Estrutura de Comunidades Microbianas em Áreas de Pockmarks e Diápiros de Sal na Margem Continental Brasileira (Sudoeste do Oceano Atlântico)**. 2022. 123 f. Tese (Doutorado) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2022.

Os microrganismos são as formas de vida mais diversas e abundantes do planeta, com aptidões metabólicas capazes garantir sua sobrevivência nos mais variados ambientes. Os sedimentos marinhos, por exemplo, abrigam elevada abundancia e diversidade de comunidades microbianas. Esses microrganismos desempenham papeis fundamentais nos ciclos biogeoquímicos e na sustentação de processos essenciais à vida, sendo os maiores responsáveis por dirigir os ciclos biogeoquímicos globais. Apesar da grande influência que essas comunidades exercem sobre o planeta, os sedimentos marinhos estão entre os ambientes menos compreendidos da Terra. O presente trabalho teve como objetivo geral revelar a diversidade taxonômica espacial e verticalmente distribuída em sedimentos associados a pockmarks e diápiros de sal. Além disso, nosso objetivo também foi reconstruir genomas microbianos usando os dados metagenômicos de amostras superficiais e realizar o cultivo e isolamento de microrganismos halofílicos desta região. Na Bacia de Santos, em uma área chamada de campo de pockmark, oito estações foram coletadas em locais com batimetria variando de 400 a 800 metros de profundidade compreendendo três estações em diápiros de sal, três estações em pockmarks e duas estações de sedimento marinho denominado controle. Testemunhos foram utilizados para retirar o sedimento do interior do *box corer*, e o sedimento foi segmentado em camadas de 2 cm em que apenas o sedimento mais superficial e mais profundo foram utilizados. A partir do sequenciamento na plataforma Illumina Miseq utilizando *primers* universais, observamos que os sedimentos da superfície apresentaram alta semelhança taxonômica independentemente do local amostrado, dominados principalmente por Nitrososphaeria, enquanto as comunidades do sedimento da subsuperfície apresentaram maior diversidade taxonômica. Através do cultivo de microrganismos hipersalinos foram obtidos 22 isolados em que 21 foram classificados como *Chromohalobacter* e apenas um isolado foi associado ao gênero *Salinisphaera*. A biblioteca metagenômica construída a partir de três amostras de sedimentos superficiais rendeu 77.922.888 leituras brutas que, após o processamento, foram posteriormente agrupados em 34 MAGS, em que a MAG SB_MAG_00001 exibiu a maior qualidade (completude = 94,2%, contaminação = 2,1%), e de acordo com a análise filogenômica do GTDB-Tk, foi atribuído à ordem Methylomirabiles, família CSP1-5. Apresentamos neste trabalho a primeira descrição de comunidades microbianas espacial e verticalmente distribuída nos sedimentos em áreas de pockmarks e diápiros de sal na Bacia de Santos, além da obtenção de isolados halofílicos e reconstrução do genoma de um membro de Methylomirabiles, que pertence a um grupo metilotrófico mal descrito, especialmente para ecossistemas de alto mar.

Palavras chave: Diversidade Microbiana. Pockmark. Sedimento Marinho. Oceano Atlântico. Metilotrofia. Halofílicos.

ABSTRACT

Peres, F. V. **Diversity and Structure of Microbial Communities in Areas of Pockmarks and Salt Diapirs on the Brazilian Continental Margin (Southwest Atlantic Ocean).** 2022. 123 f. Tese (Doutorado) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2022.

Microorganisms are the most diverse and abundant forms of life on the planet, with metabolic abilities capable of guaranteeing their survival in the most varied environments. Marine sediments, for example, harbor a high abundance and diversity of microbial communities. These microorganisms play fundamental roles in biogeochemical cycles and in sustaining essential life processes, being the main ones responsible for directing global biogeochemical cycles. Despite the great influence these communities have on the planet, marine sediments are among the least understood environments on Earth. The present work aimed to reveal the spatial and vertically distributed taxonomic diversity in sediments associated with pockmarks and salt diapirs. In addition, our objective was also to reconstruct microbial genomes using metagenomic data from surface samples and to carry out the cultivation and isolation of halophilic microorganisms from this region. In the Santos Basin, in an area called pockmark field, eight stations were collected in places with bathymetry ranging from 400 to 800 meters deep, comprising three stations in salt diapirs, three stations in pockmarks, and two stations of marine sediment called control. Testimonies were used to remove the sediment from inside the box corer, and the sediment was segmented into 2 cm layers in which only the most superficial and deepest sediment were used. From the sequencing on the Illumina Miseq platform using universal primers, we observed that the surface sediments showed high taxonomic similarity regardless of the sampled location, dominated mainly by Nitrososphaeria, while the subsurface sediment communities showed greater taxonomic diversity. Through the cultivation of hypersaline microorganisms, 22 isolates were obtained, 21 of which were classified as *Chromohalobacter*, and only one isolate was associated with the genus *Salinisphaera*. The metagenomic library constructed from three surface sediment samples yielded 77.922.888 raw reads which, after processing, were later grouped into 34 MAGS, in which the SB_MAG_00001 MAG exhibited the highest quality (completeness = 94.2%, contamination = 2.1%), and according to the phylogenomic analysis of GTDB-Tk, it was assigned to the order Methylomirabiles, family CSP1-5. We present in this work the first description of microbial communities spatially and vertically distributed in sediments in areas of pockmarks and salt diapirs in the Santos Basin, in addition to obtaining halophilic isolates and genome reconstruction of a member of Methylomirabiles, which belongs to a methylotrophic group. poorly described, especially for offshore ecosystems.

Keywords: Microbial Diversity. Pockmark. Marine Sediment. Atlantic Ocean. Methylotrophy. Halophilic.

LISTA DE FIGURAS

Chapter I

Chapter II

Chapter III

Chapter IV

LISTA DE TABELAS

Chapter III

Chapter IV

CONTENT

1 INTRODUCTION

Marine sediments occupy more than two-thirds of the Earth's surface and are densely populated by diverse communities of bacteria and archaea. It is estimated that the total microbial cells in marine sediments represent 0.18 to 3.6% of the living biomass on Earth (PARKES et al., 2014; HOSHINO et al., 2020). These microorganisms play key roles in biogeochemical cycles and in sustaining essential life processes. In the sediment, these organisms process organic and inorganic carbon and are largely responsible for cycling nutrients such as sulfur, iron, and nitrogen (HOSHINO et al., 2020; PELIKAN et al., 2021).

Autotrophic production at the ocean surface is responsible for supplying much of the organic carbon to deep regions (KAISER; BENNER, 2008; WOHLERS et al., 2009; MORAN et al., 2022). As this production overcomes degradation, part of this photosynthetically produced organic carbon that was not consumed by the bacterioplankton sinks and reaches the marine sediments (KAISER; BENNER, 2008; MORAN et al., 2022). Upon reaching the surface sediment, a portion of this material is consumed by microorganisms at the sediment-water interface, and what is not degraded ends up being buried (BLAIR et al., 2003). Due to this dynamic, marine sediment is the largest reservoir of organic carbon on the planet (SMITH; HOLLIBAUGH, 1993; FAKHRAEE et al., 2021).

Despite the great influence that these communities have on the planet, marine sediments are among the least understood environments on Earth (HOEHLER; JØRGENSEN, 2013; BAKER; APPLER; GONG, 2021). Factors such as the difficulty of sampling, especially in regions of great depth, the high cost demanded by research vessels, and the complexity of the communities that inhabit the sediments contribute to the fact that most of these microorganisms remain unknown (WILKINS et al., 2014; BAKER; APPLER; GONG, 2021). Characteristic factors of the ocean itself can influence the presence and complexity of these microbial communities. Salinity, temperature, light, nutrient availability, among others, are factors that interact and produce different habitats in the marine environment (ORCUTT et al., 2011; ORSI, 2018). Knowing characteristic communities of each habitat is essential to understand the adaptations and dynamics of factors associated with these ecosystems.

In recent decades, there has been greater access to genomic techniques, which has led to an increase in studies aimed at understanding microbial communities in marine sediments (FRASER et al., 2018; HOSHINO et al., 2020; BENDIA et al., 2021; BRUCE et al., 2022). With the advancement of these techniques, it was possible to perceive the dimension of the

diversity of taxa not yet cultivated in the laboratory. It is estimated that more than 75% of microbial genera in marine sediments are composed of uncultivated strains (PARKES et al., 2014; LLOYD et al., 2018; BAKER; APPLER; GONG, 2021). Techniques using genome assembly into metagenomes (MAGs), for example, have successfully revealed numerous microbial genomes, especially in the marine environment, which have even proved to be a source of new evolutionary lineages (NATHANI et al., 2021; BRUCE et al., 2022).

This genomic exploration allows new insights into the real role of microorganisms in marine sediments, bringing insights into trophic interactions, metabolic versatility, and ecological adaptations (ZHOU et al., 2019; WASMUND et al., 2021). In addition to the connection between the metabolic functions and the taxonomic identity of these communities.

In this context, the present work aimed to use next-generation sequencing techniques, genome assembly, and cultivation-dependent techniques to perform the taxonomic and metabolic description of microbial communities in deep sediments in a pockmark field and salt diapirs in the Santos Basin, in addition to cultivating and isolating halophilic microorganisms from this region. The present work was developed within the scope of the project "Morphological Features of the Continental Slope of the Southeastern Margin of Brazil: Active Tectonics Versus Modern Oceanographic Conditions" supported by FAPESP (Process 2014/08266-2) under the coordination of Professor Michel Michaelovitch de Mahiques of the Oceanographic Institute of USP (IOUSP) and carried out at the Laboratory of Microorganism Ecology at IOUSP.

2 OBJECTIVES

General objectives

The present work will aim to reveal the taxonomic diversity of bacteria and archaea distributed spatially and vertically in sediments associated with pockmarks and salt diapirs in the Santos Basin, in the deep Southwest Atlantic Ocean. In addition, our objective will also be to reconstruct microbial genomes using metagenomic data from surface samples, providing a general description of the potential microbial metabolisms of the sampled points. We will focus on the potential roles of MAGs more fully, especially those belonging to groups poorly described for open ocean ecosystems. The cultivation and isolation of halophilic microorganisms from this region will also be targeted.

Specific objectives

1. The taxonomic and functional diversity of bacteria and archaea in surface and subsurface sediments of samples collected from pockmarks, salt diapirs and control sediments will be evaluated through 16S gene sequencing (Chapter II).

2. We will describe the main microbial metabolisms found in surface sediment samples and we will reconstruct microbial genomes focusing on the potential role of microorganisms with the most complete genomes through metagenomic data obtained by sequencing on the Illumina Hiseq platform (Chapters II and IV).

3. We will perform a hypersaline enrichment culture of pockmark sediments, salt diapirs and control sediment, and we will perform a description of the taxonomic diversity of enriched cultures compared to environmental sediment through 16S gene sequencing (Chapter III).

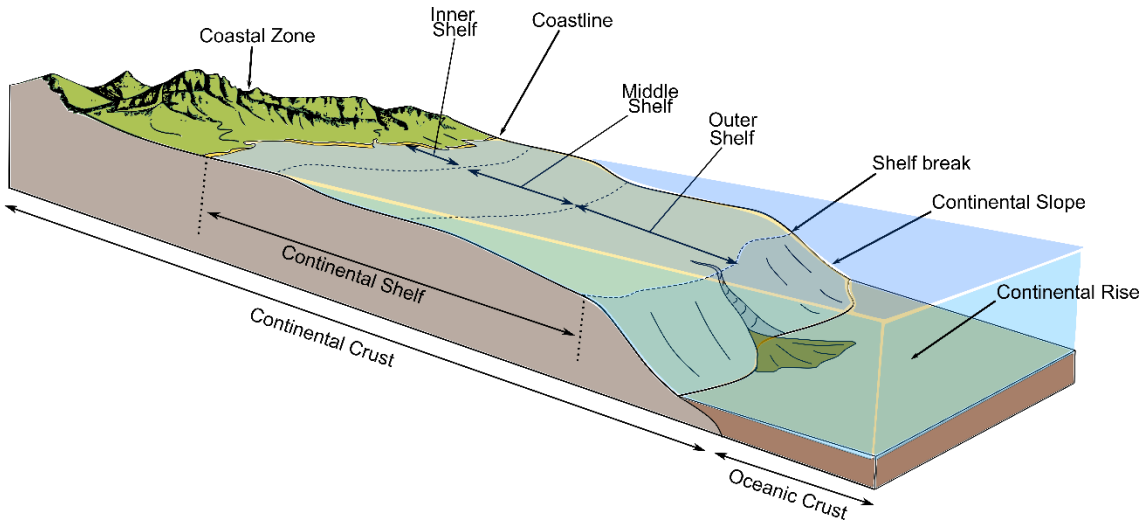
4. We will isolate and describe the taxonomic diversity of halophilic isolates from the sediment of pockmarks, salt diapirs, and control sediments using Sanger sequencing (Chapter III).

CHAPTER - I REVIEW

I.1 Continental Margins

Continental margins are unique ecosystems with specific and complex characteristics that connect terrestrial and marine processes, playing a critical role in global biogeochemical cycles (ROMANS; GRAHAM, 2013; LAMB, 2014). The morphology of these areas is the result of interactions between tectonics, oceanographic processes, and climate change (TORSVIK et al., 2009). In a simplified way, the continental margins can be formed by the continental shelf, shelf break, and slope (**Figure 1**).

Figure 1. The basic composition of the continental margins shows the coastal zone, inner shelf, coast, middle shelf, outer shelf, shelf break, continental slope, and continental elevation.



Source: João Regis dos Santos Filho, 2022.

The continental shelf is often considered a distinct environment that is heavily influenced by its internal hydrographic factors. This region is washed by relatively shallow marginal waters, reaching less than 200 m in depth (TYSON; PEARSON, 1991; LAMB, 2014). The continental slope is referred to as the steepest portion between the continent and the ocean basin, delimiting the end of the continental shelf (CACCHIONE; PRATSON; OGSTON, 2002; HERNÁNDEZ-MOLINA; LLAVE; STOW, 2008). The slope is highly variable, changing from gentle slopes to steep slopes (CACCHIONE; PRATSON; OGSTON, 2002; LAMB, 2014).

Although the continental margin represents only 20% of the world's ocean area, these environments are responsible for up to 50% of all marine productivity (SMITH, S V, HOLLIBAUGH, 1993; BAUER; DRUFFEL, 1998). Much of the material produced from primary production is sedimented and reaches the bottom sediments where it is capable of

sustaining great benthic diversity (SOETAERT et al., 1998; CORLISS et al., 2009; PINCKNEY, 2018). Consequently, the rates of settlement, accumulation of organic matter, and carbon burial are higher in shelf sediments when compared to open sea sediments (MULLER-KARGER et al., 2005; BARCELLOS et al., 2020).

Continental platforms and slope regions can harbor numerous geological features with unique characteristics, such as pockmarks, diapirs, and submarine canyons (SUMIDA et al., 2004; HARRIS; WHITEWAY, 2011; DE MAHIQUES et al., 2017). These regions constitute the largest erosive features of the continental margin, harboring a high diversity of fauna and microbial communities (LEVIN; SIBUET, 2012; SPERLING; FRIEDER; LEVIN, 2016).

Different geomorphological structures on the ocean floor can affect the bottom currents, which can influence the distribution of nutrients and the dispersion and establishment of microbial and faunal communities (SNELGROVE et al., 2018; SERRA et al., 2020). The different microbial metabolic processes in marine sediments are essential for ocean biogeochemistry and the mineralization of organic matter on the seafloor (SNELGROVE et al., 2018; HOSHINO et al., 2020). Understanding the different metabolic processes and carbon utilization pathways in these regions is extremely important, especially in heterogeneous areas with different geomorphological characteristics (ORSI, 2018; HOSHINO et al., 2020; BAKER; APPLER; GONG, 2021).

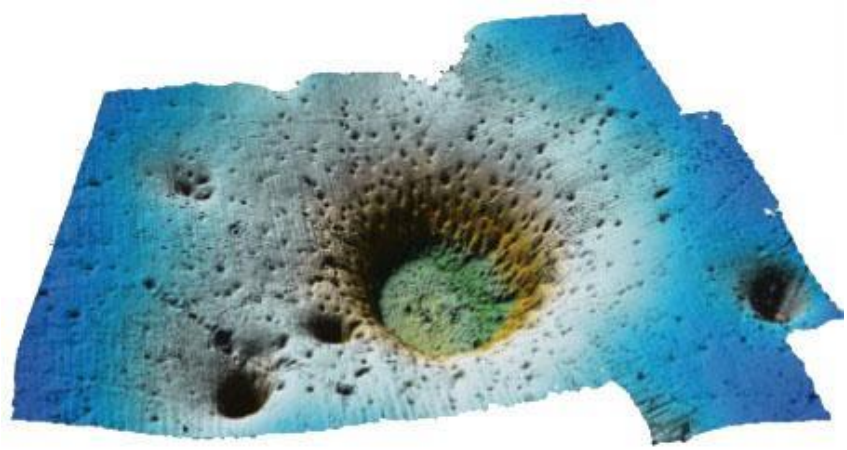
Due to the complexity of the communities of microorganisms that inhabit sediments in shelf and slope regions, for a long time, the metabolic and structural functions of these communities were unknown (HOSHINO et al., 2020; BAKER; APPLER; GONG, 2021). The advancement of sequencing and bioinformatics techniques allowed the discovery of new taxonomic and metabolic groups, opening more space for discussions about the real role of these microorganisms in the continental shelf and slope sediments (IVERSON et al., 2012; SHARON; BANFIELD, 2013; DINASQUET; TIIROLA; AZAM, 2018).

I.1.1 Pockmarks

Pockmarks were discovered in the 1960s with the advent of acoustic mapping systems. These structures are craters that can be found in the deep ocean, continental platforms, slopes, fjords, estuaries, and lake environments (HOVLAND; GARDNER; JUDD, 2002; PILCHER; ARGENT, 2007; ZHANG et al., 2020). Pockmarks can be found with variations in shape, however, circular and elliptical are the most common (DUARTE et al., 2017; ZHANG et al., 2020; TANG et al., 2021).

They do not have high edges and can have different diameters, from less than 1 meter to more than 1500 meters, and can be up to 150 meters deep. The most common pockmarks vary between 10 and 250 meters in diameter with 1 to 25 meters in depth (PILCHER; ARGENT, 2007; TASIANAS et al., 2018; BATCHELOR et al., 2022). These structures can be found as chains of circular or aligned pockmarks and smaller craters can merge, forming regions with large pockmarks surrounded by smaller craters (**Figure 2**) (HOVLAND; GARDNER; JUDD, 2002; DUARTE et al., 2017; BATCHELOR et al., 2022).

Figure 2. Bathymetric image in the North Sea showing pockmarks with different diameters. Data was collected by ROV multibeam with 0.2m resolution.



Source: Adriano Mazzini, Center for Evolution and Earth Dynamics (CEED) at the University of Oslo).

They are often associated with gas reservoir areas and can indicate potential sources of hydrocarbons. For that reason, these areas have special attention from the oil exploration industry (STROZYK et al., 2018; TASIANAS et al., 2018; TANG et al., 2021). In most cases reported worldwide, the fluids involved in the formation of pockmarks are mainly methane gas and other fluids with low permeability (HOVLAND; GARDNER; JUDD, 2002; JUDD; HOVLAND, 2007; LI et al., 2020; O'REILLY et al., 2021).

The gas and/or fluid existing in the subsurface can migrate to more superficial layers through tectonic faults or porous lithology until it finds more resistant layers that block its passage or until they are emitted to the surface (GEHRMANN et al., 2021). The tension of the sedimentary layers controls the flow of these gases from their reservoirs to the surface. Pockmarks are thought to form quickly when a pressurised subsurface containing gases or fluid is suddenly released to the sediment surface (STROZYK et al., 2018; GEHRMANN et al., 2021; MICALLEF et al., 2022).

Pockmarks with active flows are rarely observed, making it difficult to understand the mechanisms of gas expulsion between one interval and another, in addition to the time between these occurrences being unknown (JUDD; HOVLAND, 2007; WEBB, 2009). These craters can remain emitting gas or fluid from the subsurface to the surface at slower rates, for indefinite periods, until they become inactive (JUDD; HOVLAND, 2007). Inactive pockmarks can be activated by new pulses of gas or fluids as these tend to overflow through existing cracks instead of new ones being formed (HOVLAND; SOMMERVILLE, 1985; HOVLAND; GARDNER; JUDD, 2002; DUARTE et al., 2017).

Pockmarks are interesting not only from a geological point of view but also biologically (PIMENOV et al., 2008; ZEPPILLI; CANALS; DANOVARO, 2012; PIMENOV et al., 2008; GIOVANNELLI et al., 2016). These gaseous emissions can occur over long geological periods, causing the development of topographies with unique characteristics (JUDD; HOVLAND, 2007). Chemical reactions between the emanating methane and seawater (rich in sulfate ions) produce carbonate precipitation as a result of anaerobic methane oxidation and sulfate reduction (SHUBENKOVA et al., 2010; HONG et al., 2016).

These chemosynthetic microbial communities can supply energy and nutrients to the local fauna from chemical energy, thus constituting the basis of the food chain in these regions (ZEPPILLI; CANALS; DANOVARO, 2012). hese areas of exudation of hydrocarbons on the seabed have specific microbial communities and aggregations of epifauna with these microorganisms, which may evidence the continuous supply of carbon sources (PIMENOV et al., 2008; CAMBON-BONAVITA et al., 2009; O'REILLY et al., 2021). These microorganisms have high population densities and are highly relevant for controlling the emission of methane and related compounds into the hydrosphere from the seabed (NICKEL et al., 2012; IDCZAK et al., 2020; O'REILLY et al., 2021).

In inactive pockmarks and the adjacent sediment, the sedimentation of carbon compounds, for example, sea snow, terrestrial runoff, and fecal pellets of zooplankton, through the water column is the main factor responsible for microbial diversity (ORCUTT et al., 2011;

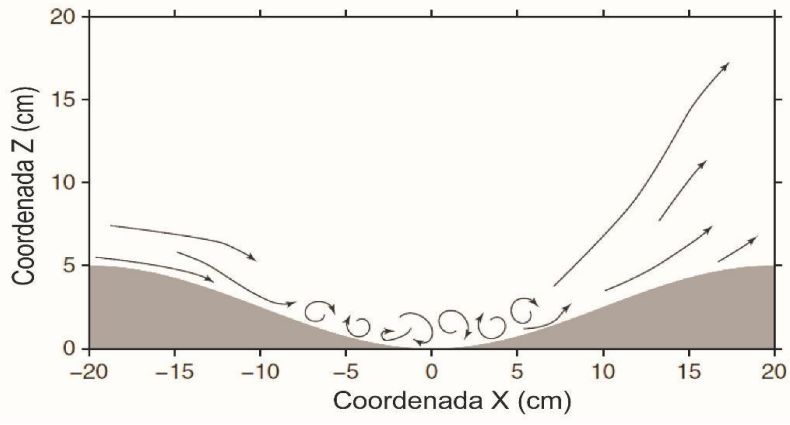
HAVERKAMP; HAMMER; JAKOBSEN, 2014; HONG et al., 2016). However, some studies report that the sedimentation rate is different in inactive pockmarks and surrounding sediments (BARTLETT et al., 2010; PAU; HAMMER, 2013). The total of organic carbon detected in sediments can consist of simple and complex compounds such as lipids, humic acids, sugars, and proteins in addition to inorganic carbon compounds as such bicarbonate, carbonate, and dissolved carbon dioxide (ORCUTT et al., 2011).

The microorganisms in the sediment decompose organic matter and provide dissolved organic carbon, which can be used by other groups in the microbial Community (ORCUTT et al., 2011; BOEUF et al., 2019; NAWAZ et al., 2022). In addition to the carbon cycle, microorganisms in the sediment participate in the nitrogen cycle, which is obtained from compounds of nitrate, nitrite, ammonia, and amino acids, or the decomposition of organic matter (ORCUTT et al., 2011; YU et al., 2018; BOEUF et al., 2019; NAWAZ et al., 2022).

In addition to sedimentation, another difference found between inactive pockmarks and the surrounding sediment is sediment granulometry. Inside the pockmarks, finer granulometry sediments are described, which may be related to the action of bottom currents that act differently when going through a depression, becoming slower due to the depth of the cavity (**Figure 3**) (PAU; HAMMER, 2013; HAVERKAMP; HAMMER; JAKOBSEN, 2014).

The accumulation of fine sediments inside the inactive crater is a consequence of its inactivity (HAVERKAMP; HAMMER; JAKOBSEN, 2014a). In contrast, in active pockmarks, during the expulsion of gases or fluids, the fine sediment is resuspended in the water column and is deposited around the cavity, making the interior of active pockmarks composed mainly of coarser-grained sediments (CATHLES; SU; CHEN, 2010; HAVERKAMP; HAMMER; JAKOBSEN, 2014b).

Figure 3. A schematic illustrating the flow pattern of the sea current as it passes through a crater.



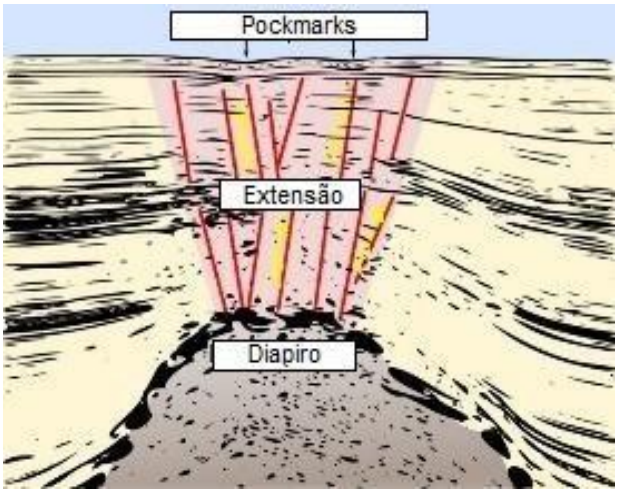
Source: Pau et al., 2013.

I.1.2 Salt of Diapirs

Salt diapirs are large masses composed of different salts with distinct properties and characteristics and with locomotion capacity (KEKEN, 1993; STEWART, 1995; JACKSON, M. PA., VENDEVILLE, B., SCHULTZ-ELA, 2003). Pure saline rocks are basically composed of halite (sodium and chlorite), gypsum, and anhydride, which together are called salt (STEWART, 2006). The movement of this saline mass is conceptually known as halokinesis (HUDEC; JACKSON, 2007). An important characteristic of this salt is its viscosity, which, when subjected to high pressures, contributes to its behaviour as a fluid, and depending on the mineralogy and conditions of pressure and temperature, it is considered an unstable fluid (KEKEN, 1993; STEWART, 1995; HUDEC; JACKSON, 2007).

The upward migration of the salt diapir causes fractures and ruptures in the rock layers in a penetrative way, and its ascent on the ocean floor will depend on the resistance of the layers superimposed on its top (HUDEC; JACKSON, 2007; DE MAHIQUES et al., 2017). This movement of salt may be responsible for the presence of pockmarks in the same area since the pre-formed cracks by diapirs facilitate the escape of gas and fluids (JUDD; HOVLAND, 2009; DE MAHIQUES et al., 2017b) (**Figure 4**).

Figure 4. Schematic illustration of a salt diapir migrating to the sediment surface. The movement of salt causes flaws in the upper sedimentary layers. That facilitates the displacement of gases and fluids and contributes to the formation of pockmarks.



Source: Adapted from DE MAHIQUES et al., 2017.

Upon reaching the surface of the marine sediment, diapirs can cause changes in the bathymetric relief and interact with regional bottom currents (NELSON; BARAZA; MALDONADO, 1993; LLAVE, et al., 2008; PALOMINO et al., 2016). Diapirs begin as anticlines or dunes and evolve into domes, columns, walls, and more complex features such as

the shape of a mushroom (DAVISON et al., 2000; ALSOP, 2014). The size of these structures can vary from 3 to 15 km in diameter, and they can unite and form long chains of up to 50 km in length (PAUTOT; PICHON, 1970; STEWART, 2006).

These saline structures began to gain attention in 1901 with the discovery of Spindletop in Beaumont (Texas), which revealed the important role of salt in the formation of hydrocarbon accumulations in saline sedimentary basins (STEWART, 1995; DRACHEV, 2015). This ability to accumulate pockets of hydrocarbons is due to another important property of salt: the absence of porosity and permeability (DAVISON et al., 2000; COLEMAN et al., 2018). This allows the salt to seal off fluids and gases, thus preventing the free migration of this material. The saline masses together with the hydrocarbons trapped constitute the pre-salt reservoirs.

In Brazil, in 2007, a large area of pre-salt was discovered on the continental margin, and it is estimated that the formation of salt layers in this region was formed 100 million years ago, when the continents America and Africa separated (BEASLEY; DRIBUS, 2010; DE SOUZA; SGARBI, 2019). This area extends between the Campos, Espírito Santo, and Santos Basins, covering an area of 149 thousand square kilometers (BEASLEY; DRIBUS, 2010; BEASLEY; DRIBUS, 2010). This is the largest oil reservoir discovered in the southern hemisphere in recent decades (BARRA et al., 2021).

Another very relevant characteristic of these saline diapirs, when reaching the surface of the sediment, is the ability to modify the sedimentary environment of the area, making the surrounding sediment excessively rich in salt (DRACHEV, 2015). Natural environments with large amounts of salt are called hypersaline environments. These sites act as selection favours for communities of organisms since high salt concentrations cause a cellular osmotic imbalance in organisms that are not adapted to these conditions (KUSHNER, 1968; OREN, 2002; OREN, 2008). Biological membranes are naturally permeable to water, causing the organism to be unable to maintain levels of water in its interior higher than the surrounding environment (GALINSKI, 1995; ROESSLER; MÜLLER, 2001; OREN, 2008).

These hypersaline environments are dominated by halophilic organisms, mainly bacteria, and archaea (OREN, 2008; SANTOS et al., 2012). These microorganisms participate in the oxidation of organic matter in these regions by anaerobic, facultative, and aerobic metabolism (DASSARMA; ARORA, 2001; BUTTURINI et al., 2022). It is estimated that the metabolic diversity of these microorganisms is as wide as their phylogenetic diversity (OREN, 2008; OREN, 2015).

The Santos Basin is known to be influenced by salt tectonics and with evidence of the presence of numerous saline diapirs in the area, together with the presence of dozens of

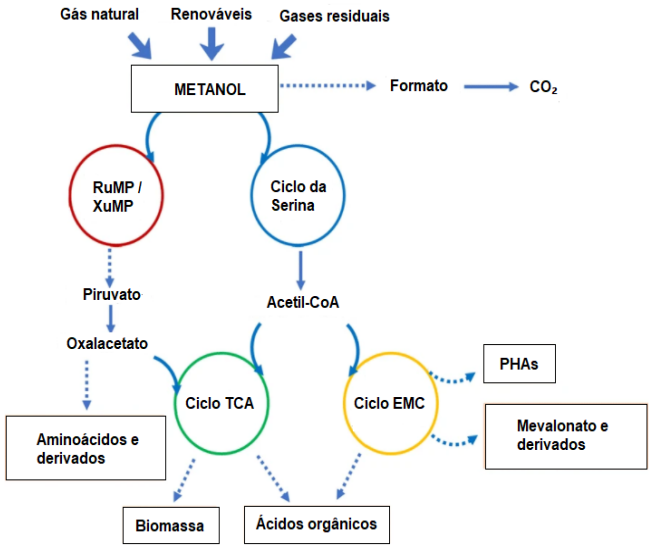
pockmarks (SUMIDA et al., 2004; SUMIDA et al., 2004). However, there are no microbiological studies on saline diapirs in the South Atlantic or elsewhere in the world where salt diapirs have been found.

I.2 Methylotrophic Microorganisms

Methylotrophy, as a metabolic process, has been known since the beginning of the 20th century. It is defined as the ability of some organisms to use reduced carbon compounds, without carbon-carbon (C1) bonds, as their only source of carbon and energy (CHISTOSERDOVA; KALYUZHNAYA; LIDSTROM, 2009); (CHISTOSERDOVA, 2015); (CHISTOSERDOVA; KALYUZHNAYA, 2018). The microorganisms responsible for this metabolism play an important role in the global cycle of carbon, nitrogen, and sulfur. The growth of methylotrophs is supported by methane, methanol, as well as methylated amines, halogenated methane, and methylated sulfur species (KELLY; MURRELL, 1999; CHISTOSERDOVA, 2011; HUG et al., 2016).

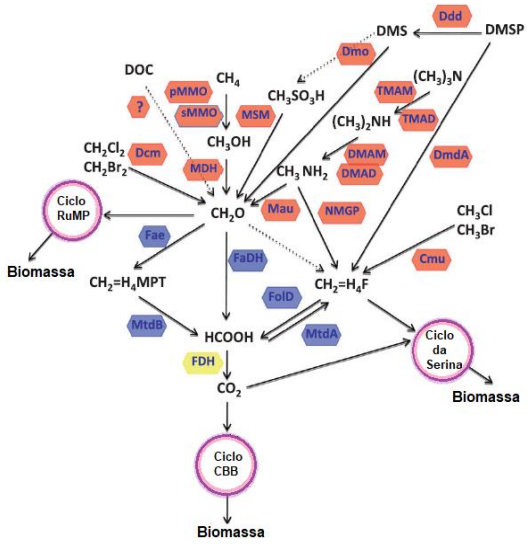
Methylotrophs act as the main biological sink for several methylated greenhouse gases, in addition to being targets for bioremediation processes, production of fuels, and various chemicals for industrial use (**Figure 5**) (HANSON, 1980; CHISTOSERDOVA, 2018; SAKARIKA; GANIGUÉ; RABAEY, 2022). The use of methanol can oxidise methanol to formaldehyde and formaldehyde to CO_2, and the assimilation of carbon compounds such as formaldehyde or CO_2, or a combination of both (HEUX et al., 2018) (**Figure 6**).

Figure 5. Scheme demonstrating the production of several chemical compounds from the oxidation of methanol derived from different sources and the use of different metabolic pathways. Solid arrows represent one-step reactions, while dotted arrows show multi-step



Source: Adapted from Zhang et al., 2018.

Figure 6. Simplified diagram showing the main metabolic modules of methylotrophy with the main substrates and intermediates.



The primary assimilation modules (demethylation and dehalogenation) are represented in red. In blue are the modules for handling formaldehyde (methyl-H4F). The yellow dehydrogenase module is shown in yellow and the assimilation modules in purple. Dashed lines represent non-enzymatic reactions or lack of biochemical knowledge. Acronyms: RuMP (Ribulose Monophosphate), CBB (Calvin-Benson-Bassham). Adapted from Chistoserdova, 2011.

Methylotrophic microorganisms can be anaerobic, aerobic, or optional and occur in various aquatic and terrestrial environments (KELLY; MURRELL, 1999; CHISTOSERDOVA, 2015; CHISTOSERDOVA, 2018). Aerobic methylotrophic have already been reported in diverse environments, such as aerated and flooded soils in swamps, deserts, trundles, pastures, rhizosphere, and phyllosphere of various plants and in open and coastal ocean waters (KOLB, 2009; CHISTOSERDOVA, 2015; FISCHER et al., 2021).

Methanol is considered one of the most abundant non-methane hydrocarbons in the troposphere. It is considered a volatile oxygenated organic compound (OVOCs) involved in the main atmospheric processes on the planet (SINGH et al., 2001; HEIKES et al., 2002; LEGREID et al., 2007). Methanol triggers the formation of tropospheric ozone and indirectly has a global warming potential three times greater on a hundred-year basis than carbon dioxide (BEALE et al., 2013). Methanol source studies for a long time concentrated on terrestrial systems being a residual product of plants (with about 50–280 teragrams (Tg) per year) (HEIKES et al., 2002; BEALE et al., 2013; BATES et al., 2021).

In 2002, Heikes et al (2002) suggested that the world's oceans could also be an important source of atmospheric methanol. However, the computational model efforts estimated that oceanic sources of methanol are comparable to emissions from terrestrial plants, especially in

oligotrophic regions (MILLET et al., 2008; BATES et al., 2021). Beale et al (2013) described that only the Atlantic Ocean could contribute about 3 Tg per year of atmospheric methanol.

In addition to the sources of methanol in situ in the oceans, the concentration of this gas in marine environments is also attributed to external deposits of terrestrial origin (BATES et al., 2021). Due to the volatility of methanol produced in the terrestrial environment (estimated annual emission of 70–350 Tg) (HEIKES et al., 2002; BATES et al., 2021), a large part of this production is deposited in the oceans through air-water exchange, diffusion, and precipitation (BEALE et al., 2013). The major source of methanol production in the oceans by phytoplankton.

Half of the global methanol production by photosynthesis as a by-product of exudate may exceed emissions from land plants (DIXON et al., 2013; BATES et al., 2021). Estimates made for the water column showed methanol concentrations ranging from 27 nM to 429 nM (DIXON; BEALE; NIGHTINGALE, 2011a; DIXON; BEALE; NIGHTINGALE, 2011b; READ et al., 2012; BATES et al., 2021) and estimates for marine sediment ranged from 0.3 μM to 100 μM (FISCHER et al., 2021; XU et al., 2021), with the concentration of methanol in the sediments significantly higher than in the water column.

Relatively high turnover rates of this gas in the water column (<1 day) suggest rapid metabolization, preventing methanol from reaching anoxic marine sediments (ZHUANG et al., 2018). In marine sediments, the turnover of methanol is slower than in the water column, estimated between 22 and 100 days (ZHUANG et al., 2018; FISCHER et al., 2021)(ZHUANG et al., 2018). These explain the lower concentrations of methanol in the water column, however, it is not clear how methanol is produced on the surface by phytoplankton or exchanged between air-water, reaches deep sediment (DIXON; BEALE; NIGHTINGALE, 2011b; ZHUANG et al., 2018).

It is known that phytoplankton converts up to 35% of the assimilated carbon into pectin, lignin, and galactans (methoxylated polysaccharides) and the demethoxylation of these compounds by aerobic microorganisms from the water column and anaerobes in the sediment also release methanol (DIXON et al., 2013; ZHUANG et al., 2018). The availability of this compound in marine environments allows methylotrophic bacteria to use it as a carbon and energy source (CHISTOSERDOVA, 2015; CHISTOSERDOVA; KALYUZHNAYA, 2018).

The enzyme methanol dehydrogenase (MDH) is responsible for catalyzing the first step of the oxidation of methanol to formaldehyde. For a long time, it was thought that this first stage of oxidation was possible only by the calcium-dependent MDH (Ca 2+) action known as MxaFI (CHISTOSERDOVA, 2011). MxaFI is a quinone pyrroloquinoline (PQQ) located in the

periplasm and basically constituted of genes encoding large (MxaF) and small (MxaI) subunits of MDH, electron acceptors (MxaG), proteins for binding Ca^{2+} (MxaACKL) and other genes whose functions are not yet understood (CHISTOSERDOVA, 2011; CHU; LIDSTROM, 2016).

However, a gene similar to the MxaFl coder was discovered in 1995, called xoxF (TUBERT et al., 2015; CHU; LIDSTROM, 2016). This gene is more widespread than MxaFI and was described in several methylotrophic groups (CHISTOSERDOVA, 2011; GOOD et al., 2019). Through phylogenetic analysis it was shown that xoxF can be divided into five different clades, called xoxF1 to xoxF5 (CHISTOSERDOVA, 2011; KELTJENS et al., 2014).

Some methylotrophs may have only xoxF and no other genes encoding MDH, while other microorganisms such as *Methylotenera mobilis* may have up to two xoxF4 and no mxaF (TAUBERT et al., 2015; CHISTOSERDOVA; KALYUZHNAYA, 2018). It was demonstrated that xoxF is present in all known methylotrophic and present in the most diverse environments, especially coastal marine and other aquatic environments, suggesting its strong ecological role (TAUBERT et al., 2015; GOOD et al., 2019). It is currently known that the oxidation of methanol to formaldehyde in both methanotrophic and methylotrophic microorganisms can be catalysed by a Ca^{2+} dependent MxaFI or a XoxF-type methanol dehydrogenase, with some methylotrophic genomes encoding only XoxF (CHISTOSERDOVA, 2011; POL et al., 2014; CHISTOSERDOVA; KALYUZHNAYA, 2018).

In both methanol dehydrogenases, there is a relationship with the presence of rare earth elements (REEs), composed of lanthanides (Ln^{3+}) plus scandium and yttrium. These phenomenon is known as lanthanide exchange, in which it was shown that nanomolar amounts of these elements were sufficient to activate MDH xoxF suggested as an active transport of REEs into the cell (POL et al., 2014; WANG et al., 2020; CHISTOSERDOVA, 2019).

Regulatory mechanisms were also observed that intercede the exchange between mxaF and xoxF in response to the presence or absence of REEs. XoxF can be found inside cells even in the absence of these elements. However, its activation is only carried out when REEs are present (POL et al., 2014; WANG et al., 2020; SKOVRAN; RAGHURAMAN; MARTINEZ-GOMEZ, 2019). It has been shown that REEs also transcriptionally regulates the expression of the two MDH enzymes (maxFI and xoxF) in several methylotrophic microorganisms (RAMACHANDRAN; WALSH, 2015; FISCHER et al., 2021).

If maxFI were removed, it would not cause negative effects on the growth of microorganisms as long as lanthanides were present in the medium (POL et al., 2014; SKOVRAN; RAGHURAMAN; MARTINEZ-GOMEZ, 2019). Ln^{3+} are stronger than Ca^{2+},

due to their much higher Lewis acidity, acting on the xoxF enzyme and allowing the removal of electrons from methanol (POL et al., 2014).

Pieces of evidence of REEs in the oxidation of methanol have caused exponential growth in studies on the role of REEs in bacterial metabolism (POL et al., 2014; CHISTOSERDOVA, 2016; WANG et al., 2020; FISCHER et al., 2021). XoxF was able to use several REEs elements as cofactors, innovating the field of inorganic biochemistry and revealing the biological importance of these elements, which were considered biologically inactive (SKOVRAN; MARTINEZ-GOMEZ, 2015; GOOD et al., 2019; WANG et al., 2020; GOOD et al., 2019; YANPIRAT et al., 2020). So far, xoxF is the only known REE-dependent enzyme (KELTJENS et al., 2014; POL et al., 2014; CARL-ERIC et al., 2020; YANPIRAT et al., 2020; GOVINDARAJU et al., 2022).

REFERENCES

ALSOP, G. I. Physical modelling of fold and fracture geometries associated with salt diapirism. **Salt Tectonics, Geological Society Special Publiation**, v. 100, n. 100, p. 227–241, 2014.

BAKER, B. J.; APPLER, K. E.; GONG, X. New Microbial Biodiversity in Marine Sediments. **Annual Review of Marine Science**, v. 13, p. 161–175, 2021.

BAKER, B. J.; APPLER, K. E.; GONG, X. New Microbial Biodiversity in Marine Sediments. **Annual Review of Marine Science**, v. 13, p. 161–175, 2021.

BARCELLOS, R. L. et al. Sedimentary Organic Matter Characterization on a Tropical Continental Shelf in Northeastern Brazil. **International Journal of Geosciences**, v. 11, n. 06, p. 393–419, 2020.

BARRA, T. A. et al. Deconvolution of post- and pre-salt petroleum sources in southeastern offshore Brazilian basins. **Organic Geochemistry**, v. 153, p. 104146, 2021.

BARTLETT, R. et al. Lithological controls on biological activity and groundwater chemistry in Quaternary sediments. **Hydrological Processes**, v. 24, n. 6, p. 726–735, 2010.

BATCHELOR, C. L. et al. The morphology of pockmarks on the north-east Antarctic Peninsula continental shelf. **Antarctic Science**, v. 12, p. 1–12, 2022.

BATES, K. H. et al. The Global Budget of Atmospheric Methanol: New Constraints on Secondary, Oceanic, and Terrestrial Sources. **Journal of Geophysical Research: Atmospheres**, v. 126, n. 4, p. 1–23, 2021.

BAUER, J. E.; DRUFFEL, E. R. M. Ocean margins as a significant source of organic matter to the deep open ocean. **Nature**, v. 392, n. 6675, p. 482–485, 1998.

BEALE, R. et al. Methanol, acetaldehyde, and acetone in the surface waters of the Atlantic Ocean. **Journal of Geophysical Research: Oceans**, v. 118, n. 10, p. 5412–5425, 2013.

BEASLEY, C. J.; DRIBUS, J. R. Brazil ' s Presalt Play. **Oilfield Review Autumn**, v. 22, p. 28–37, 2010.

BENDIA, A. G. et al. Microbial perspective on the giant carbonate ridge Alpha Crucis (Southwestern Atlantic upper slope). **FEMS Microbiology Ecology**, v. 97, n. 8, p. 1–14, 2021.

BLAIR, N. E. et al. The persistence of memory: The fate of ancient sedimentary organic carbon in a modern sedimentary system. **Geochimica et Cosmochimica Acta**, v. 67, n. 1, p. 63–73, 2003.

BOEUF, D. et al. Biological composition and microbial dynamics of sinking particulate organic matter at abyssal depths in the oligotrophic open ocean. **Proceedings of the National Academy of Sciences of the United States of America**, v. 116, n. 24, p. 11824–11832, 2019.

BUTTURINI, A. et al. Oigin, accumulation and fate of dissolved organic matter in an extreme hypersaline shallow lake. **Water Research**, p. 118727, 2022.

CACCHIONE, D. A.; PRATSON, L. F.; OGSTON, A. S. The shaping of continental slopes by internal tides. **Science**, v. 296, n. 5568, p. 724–727, 2002.

CAMBON-BONAVITA, M. A. et al. Deep-Sea Research II Diversity, and distribution of methane-oxidizing microbial communities associated with different faunal assemblages in a giant pockmark of the Gabon continental margin. **Deep-Sea Research Part II**, v. 56, n. 23, p. 2248–2258, 2009.

CARL-ERIC, W. et al. Lanthanide-Dependent Methylotrophs of the Family. **Applied and Environmental Microbiology**, v. 86, n. 1, p. 1–18, 2020.

CATHLES, L. M.; SU, Z.; CHEN, D. The physics of gas chimney and pockmark formation, with implications for assessment of seafloor hazards and gas sequestration. **Marine and Petroleum Geology**, v. 27, n. 1, p. 82–91, 2010.

CHISTOSERDOVA, L. Modularity of methylotrophy, revisited. **Environmental Microbiology**, v. 13, n. 10, p. 2603–2622, 2011.

CHISTOSERDOVA, L. Methylotrophs in natural habitats: current insights through

metagenomics. **Applied Microbiology and Biotechnology**, v. 99, n. 14, p. 5763–5779, 2015.
CHISTOSERDOVA, L. Lanthanides: New life metals? **World Journal of Microbiology and Biotechnology**, v. 32, n. 8, p. 1–7, 2016.
CHISTOSERDOVA, L. Applications of methylotrophs: can single carbon be harnessed for biotechnology? **Current Opinion in Biotechnology**, v. 50, p. 189–194, 2018.
CHISTOSERDOVA, L. New pieces to the lanthanide puzzle. **Molecular Microbiology**, v. 111, n. 5, p. 1127–1131, 2019.
CHISTOSERDOVA, L.; KALYUZHNAYA, M. G. Current Trends in Methylotrophy. **Trends in Microbiology**, v. 26, n. 8, p. 703–714, 2018.
CHISTOSERDOVA, L.; KALYUZHNAYA, M. G.; LIDSTROM, M. E. The expanding world of methylotrophic metabolism. **Annual Review of Microbiology**, v. 63, p. 477–499, 2009.
CHU, F.; LIDSTROM, M. E. XoxF acts as the predominant methanol dehydrogenase in the type I methanotroph Methylomicrobium buryatense. **Journal of Bacteriology**, v. 198, n. 8, p. 1317–1325, 2016.
COLEMAN, A. J. et al. How, where, and when do radial faults grow near salt diapirs ? **The Geological Society of America**, v. 46, n. 7, p. 655–658, 2018.
CORLISS, B. H. et al. Deep-sea benthic diversity linked to seasonality of pelagic productivity. **Deep-Sea Research Part I: Oceanographic Research Papers**, v. 56, n. 5, p. 835–841, 2009.
DASSARMA, S.; ARORA, P. Halophiles. **Encyclopedia of life sciences**, v. 1, p. 1–9, 2001.
DAVISON, I. et al. Geometry and late-stage structural evolution of Central Graben salt diapirs, North Sea. **Marine and Petroleum Geology**, v. 17, p. 499–522, 2000.
DE MAHIQUES, M. M. et al. An extensive pockmark field on the upper Atlantic margin of Southeast Brazil : spatial analysis and its relationship with salt diapirism. **Heliyon**, n. August 2016, p. e00257, 2017a.
DE MAHIQUES, M. M. et al. An extensive pockmark field on the upper Atlantic margin of Southeast Brazil: spatial analysis and its relationship with salt diapirism. **Heliyon**, v. 3, n. 2, p. e00257, 2017b.
DE SOUZA, L. S.; SGARBI, G. N. C. The Santos Basin in Brazil: Geology, exploration, and production of oil and natural gas. **Boletin de Geologia**, v. 41, n. 1, p. 175–195, 2019.
DINASQUET, J.; TIIROLA, M.; AZAM, F. Enrichment of bacterioplankton able to utilize one-carbon and methylated compounds in the coastal Pacific ocean. **Frontiers in Marine Science**, v. 5, n. SEP, p. 1–13, 2018.
DIXON, J. L. et al. Gradients in microbial methanol uptake: Productive coastal upwelling waters to oligotrophic gyres in the Atlantic Ocean. **ISME Journal**, v. 7, n. 3, p. 568–580, 2013.
DIXON, J. L.; BEALE, R.; NIGHTINGALE, P. D. Microbial methanol uptake in northeast Atlantic waters. **ISME Journal**, v. 5, n. 4, p. 704–716, 2011a.
DIXON, J. L.; BEALE, R.; NIGHTINGALE, P. D. Rapid biological oxidation of methanol in the tropical Atlantic: Significance as a microbial carbon source. **Biogeosciences**, v. 8, n. 9, p. 2707–2716, 2011b.
DRACHEV, S. S. Salt Diapirism in the Oceans and Continental Margins Classification of Salt Diapirs. **Encyclopedia of Marine Geosciences**, p. 1–8, 2015.
DUARTE, D. et al. Identification and characterization of fluid escape structures (pockmarks) in the Estremadura Spur, West Iberian Margin. **Marine and Petroleum Geology**, v. 82, p. 414–423, 2017.
FAKHRAEE, M. et al. A largely invariant marine dissolved organic carbon reservoir across Earth's history. **Proceedings of the National Academy of Sciences of the United States of America**, v. 118, n. 40, 2021.
FISCHER, P. Q. et al. Anaerobic microbial methanol conversion in marine sediments. **Environmental Microbiology**, v. 23, n. 3, p. 1348–1362, 2021.
FRASER, M. W. et al. Metagenomic Evidence of Microbial Community Responsiveness to

Phosphorus and Salinity Gradients in Seagrass Sediments. **Frontiers in Microbiology**, v. 9, n. July, p. 1–11, 2018.
GALINSKI, E. A. **Osmoadaptation in Bacteria**. [s.l: s.n.]. v. 37
GEHRMANN, R. A. S. et al. Porosity and free gas estimates from controlled source electromagnetic data at the Scanner Pockmark in the North Sea. **International Journal of Greenhouse Gas Control**, v. 109, n. April, 2021.
GIOVANNELLI, D. et al. Diversity and distribution of prokaryotes within a shallow-water pockmark field. **Frontiers in Microbiology**, v. 7, n. JUN, p. 1–21, 2016.
GOOD, N. M. et al. Contrasting in vitro and in vivo methanol oxidation activities of lanthanide-dependent alcohol dehydrogenases XoxF1 and ExaF from Methylobacterium extorquens AM1. **Scientific Reports**, v. 9, n. 1, p. 1–12, 2019.
GOVINDARAJU, A. et al. Employing methylotrophs for a green economy: one-carbon to fuel them all and through metabolism redesign them. **Current Opinion in Microbiology**, v. 67, p. 102145, 2022.
HANSON, R. S. Ecology, and Diversity of Methylotrophic Organisms. **Advances in Applied Microbiology**, v. 26, n. C, p. 3–39, 1980.
HARRIS, P. T.; WHITEWAY, T. Global distribution of large submarine canyons: Geomorphic differences between active and passive continental margins. **Marine Geology**, v. 285, n. 1–4, p. 69–86, 2011.
HAVERKAMP, T. H. A.; HAMMER, Ø.; JAKOBSEN, K. S. Linking geology, and microbiology: Inactive pockmarks affect sediment microbial community structure. **PLoS ONE**, v. 9, n. 1, 2014a.
HAVERKAMP, T. H. A.; HAMMER, Ø.; JAKOBSEN, K. S. Linking Geology, and Microbiology : Inactive Pockmarks Affect Sediment Microbial Community Structure. **PLoS ONE**, v. 9, n. 1, p. e85990, 2014b.
HEIKES, B. G. et al. Atmospheric methanol budget, and ocean implication. **Global Biogeochemical Cycles**, v. 16, n. 4, p. 80-1-80–13, 2002.
HERNÁNDEZ-MOLINA, F. J.; LLAVE, E.; STOW, D. A. V. Chapter 19 Continental Slope Contourites. **Developments in Sedimentology**, v. 60, n. 08, p. 379–408, 2008.
HOEHLER, T. M.; JØRGENSEN, B. B. Microbial life under extreme energy limitation. **Nature Reviews Microbiology**, v. 11, n. 2, p. 83–94, 2013.
HONG, W. L. et al. Removal of methane through hydrological, microbial, and geochemical processes in the shallow sediments of pockmarks along eastern Vestnesa Ridge (Svalbard). **Limnology and Oceanography**, v. 61, p. S324–S343, 2016.
HOSHINO, T. et al. Global diversity of microbial communities in marine sediment. **Proceedings of the National Academy of Sciences of the United States of America**, v. 117, n. 44, p. 27587–27597, 2020.
HOVLAND, M.; GARDNER, J. V.; JUDD, A. G. The significance of pockmarks to understanding fluid flow processes and geohazards. **Geofluids**, v. 2, n. 2, p. 127–136, 2002.
HOVLAND, M.; SOMMERVILLE, J. H. Characteristics of two natural gas seepages in the North Sea. **Marine and Petroleum Geology**, v. 2, n. 4, p. 319–326, 1985.
HUDEC, M. R.; JACKSON, M. P. A. Terra infirma : Understanding salt tectonics. **Earth Science Reviews**, v. 82, p. 1–28, 2007.
HUG, L. A. et al. Critical biogeochemical functions in the subsurface are associated with bacteria from new phyla and little studied lineages. **Environmental Microbiology**, v. 18, n. 1, p. 159–173, 2016.
IDCZAK, J. et al. A geophysical, geochemical, and microbiological study of a newly discovered pockmark with active gas seepage and submarine groundwater discharge (MET1-BH, central Gulf of Gdańsk, southern Baltic Sea). **Science of the Total Environment**, v. 742, 2020.

IVERSON, V. et al. Untangling genomes from metagenomes: Revealing an uncultured class of marine euryarchaeota. **Science**, v. 335, n. 6068, p. 587–590, 2012.
JACKSON, M. PA., VENDEVILLE, B., SCHULTZ-ELA, C. D. Structural Dynamics of Salt Systems. **Annual Review of Earth and Planetary Sciences**, n. November, 2003.
JØRGENSEN, B. B. et al. Sediment oxygen consumption: Role in the global marine carbon cycle. **Earth-Science Reviews**, v. 228, n. March, 2022.
JUDD, A. G. et al. The geological methane budget at continental margins and its influence on climate change. **Geofluids**, v. 2, n. 2, p. 109–126, 2002.
JUDD, A.; HOVLAND, M. Seabed fluid flow: the impact on geology, biology, and the marine environment. **Choice Reviews Online**, v. 45, n. 01, p. 45- 0294-45–0294, 2007.
JUDD, A.; HOVLAND, M. **Seabed fluid flow: the impact on geology, biology and the marine environment**. [s.l.] Cambridge University Press, 2009.
KAISER, K.; BENNER, R. Erratum: Major bacterial contribution to the ocean reservoir of detrital organic carbon and nitrogen (Limnology and Oceanography 53, 1, (99-112)). **Limnology and Oceanography**, v. 53, n. 3, p. 1192, 2008.
KEKEN, P. E. VAN. The effective viscosity of rocksalt : implementation of steady-state creep laws in numerical models of salt diapirism. **Tectoniphysics**, v. 225, p. 457–476, 1993.
KELLY, D. P.; MURRELL, J. C. Microbial metabolism of methanesulfonic acid. **Archives of Microbiology**, v. 172, n. 6, p. 341–348, 1999.
KELTJENS, J. T. et al. PQQ-dependent methanol dehydrogenases: Rare-earth elements make a difference. **Applied Microbiology and Biotechnology**, v. 98, n. 14, p. 6163–6183, 2014.
KOLB, S. Aerobic methanol-oxidizing Bacteria in soil. 2009.
KUSHNER, D. J. Halophilic Bacteria. **Advances in Applied Microbiology**, v. 10, p. 73–99, 1968.
LAMB, K. G. Internal wave breaking and dissipation mechanisms on the continental slope/shelf. **Annual Review of Fluid Mechanics**, v. 46, p. 231–254, 2014.
LEGREID, G. et al. Oxygenated volatile organic compounds (OVOCs) at an urban background site in Zürich (Europe): Seasonal variation and source allocation. **Atmospheric Environment**, v. 41, n. 38, p. 8409–8423, 2007.
LEVIN, L. A.; SIBUET, M. Understanding continental margin biodiversity: A new imperative. **Annual Review of Marine Science**, v. 4, p. 79–112, 2012.
LI, J. et al. Broadband Acoustic Inversion for Gas Flux Quantification—Application to a Methane Plume at Scanner Pockmark, Central North Sea. **Journal of Geophysical Research: Oceans**, v. 125, n. 9, 2020.
LLAVE, E.; HERNANDEZ-MOLINA, F.J.; STOW, D.A.V.; FERNANDEZ-PUGA, M.C.; GARCIIA, M.; VAZQUEZ, J.T.; MAESTRO, A.; SOMOZA, L.; RIO, V. D. Reconstructions of the Mediterranean Outflow Water during the quaternary based on the study of changes in buried mounded drift stacking pattern in the Gulf of Cadiz. **Marine Geophysical Researches**, v. 28, n. 2007, p. 379–394, 2008.
LLOYD, K. G. et al. Phylogenetically Novel Uncultured Microbial Cells Dominate Earth Microbiomes. **mSystems**, v. 3, n. 5, 2018.
MICALLEF, A. et al. Multiple drivers and controls of pockmark formation across the Canterbury Margin, New Zealand. **Basin Research**, n. February, p. 1–26, 2022.
MILLET, D. B. et al. New constraints on terrestrial and oceanic sources of atmospheric methanol. **Atmospheric Chemistry and Physics**, v. 8, n. 23, p. 6887–6905, 2008.
MORAN, M. A. et al. The Ocean's labile DOC supply chain. **Limnology and Oceanography**, v. 67, n. 5, p. 1007–1021, 2022.
MULLER-KARGER, F. E. et al. The importance of continental margins in the global carbon cycle. **Geophysical Research Letters**, v. 32, n. 1, p. 1–4, 2005.
NATHANI, N. M. et al. 309 metagenome assembled microbial genomes from deep sediment

samples in the Gulfs of Kathiawar Peninsula. **Scientific Data**, v. 8, n. 1, p. 1–8, 2021.
NAWAZ, M. Z. et al. Understanding Interaction Patterns within Deep-Sea Microbial Communities and Their Potential Applications. **Marine Drugs**, v. 20, n. 2, p. 1–18, 2022.
NELSON; BARAZA; MALDONADO. Mediterranean undercurrent sandy contourites, Gulf of Cadiz, Spain. **Sedimentary Geology**, v. 82, p. 103–131, 1993.
NICKEL, J. C. et al. Characterization of microbial activity in pockmark fi elds of the SW-Barents Sea. **Marine Geology**, v. 332–334, p. 152–162, 2012.
O'REILLY, S. S. et al. Production of methane and gaseous compounds by surface microbial activity in a small pockmark field, Dunmanus Bay, Ireland. **Estuarine, Coastal and Shelf Science**, v. 255, n. May 2020, 2021.
ORCUTT, B. N. et al. Microbial Ecology of the Dark Ocean above, at, and below the Seafloor. **Microbiology and Molecular Biology Reviews**, v. 75, n. 2, p. 361–422, 2011.
OREN, A. Diversity of halophilic microorganisms: Environments, phylogeny, physiology, and applications. **Journal of Industrial Microbiology and Biotechnology**, v. 28, n. 1, p. 56–63, 2002.
OREN, A. Microbial life at high salt concentrations: phylogenetic and metabolic diversity. **Saline Systems**, v. 4, n. 1, p. 1–13, 2008.
OREN, A. Halophilic microbial communities, and their environments. **Current Opinion in Biotechnology**, v. 33, n. Figure 1, p. 119–124, 2015.
ORSI, W. D. Ecology, and evolution of seafloor and subseafloor microbial communities. **Nature Reviews Microbiology**, v. 16, n. 11, p. 671–683, 2018.
PALOMINO, D. et al. Multidisciplinary study of mud volcanoes and diapirs and their relationship to seepages and bottom currents in the Gulf of Cádiz continental slope (northeastern sector). **Marine Geology**, v. 378, p. 196–212, 2016.
PARKES, R. J. et al. A review of prokaryotic populations and processes in sub-seafloor sediments, including biosphere: Geosphere interactions. **Marine Geology**, v. 352, p. 409–425, 2014.
PAU, M.; HAMMER, T. Sediment mapping and long-term monitoring of currents and sediment fluxes in pockmarks in the Oslofjord, Norway. **Marine Geology**, v. 346, p. 262–273, 2013.
PAUTOT, G. U. Y.; PICHON, X. L. E. Continuous Deep Sea Salt Layer along North Atlantic Margins related to Early Phase of Rifting. **Nature**, v. 227, p. 351–354, 1970.
PELIKAN, C. et al. Anaerobic bacterial degradation of protein and lipid macromolecules in subarctic marine sediment. **ISME Journal**, v. 15, n. 3, p. 833–847, 2021.
PILCHER, R.; ARGENT, J. Mega-pockmarks and linear pockmark trains on the West African continental margin. **Marine Geology**, v. 244, p. 15–32, 2007.
PIMENOV, N. V. et al. Microbiological and biogeochemical processes in a pockmark of the Gdansk depression, Baltic Sea. **Microbiology**, v. 77, n. 5, p. 579–586, 2008.
PINCKNEY, J. L. A Mini-Review of the Contribution of Benthic Microalgae to the Ecology of the Continental Shelf in the South Atlantic Bight. **Estuaries and Coasts**, v. 41, n. 7, p. 2070–2078, 2018.
POL, A. et al. Rare earth metals are essential for methanotrophic life in volcanic mud pots. **Environmental Microbiology**, v. 16, n. 1, p. 255–264, 2014.
RAMACHANDRAN, A.; WALSH, D. A. Investigation of XoxF methanol dehydrogenases reveals new methylotrophic bacteria in pelagic marine and freshwater ecosystems. **FEMS Microbiology Ecology**, v. 91, n. 10, p. 1–10, 2015.
READ, K. A. et al. Multiannual observations of acetone, methanol, and acetaldehyde in remote tropical Atlantic air: Implications for atmospheric OVOC budgets and oxidative capacity. **Environmental Science and Technology**, v. 46, n. 20, p. 11028–11039, 2012.
ROESSLER, M.; MÜLLER, V. Osmoadaptation in bacteria and archaea: common principles and differences. **Environmental Microbiology**, v. 3, n. 12, p. 743–754, 2001.

ROMANS, B. W.; GRAHAM, S. A. A Deep-Time Perspective of Land-Ocean Linkages in the Sedimentary Record. p. 69–94, 2013.
S. SERRA, C. et al. Tectonic evolution, geomorphology and influence of bottom currents along a large submarine canyon system: The São Vicente Canyon (SW Iberian margin). **Marine Geology**, v. 426, n. May, p. 106219, 2020.
SAKARIKA, M.; GANIGUÉ, R.; RABAEY, K. Methylotrophs: from C1 compounds to food. **Current Opinion in Biotechnology**, v. 75, p. 1–8, 2022.
SANTOS, F.; YARZA, P.; PARRO, V.; MESUGUER, I.; RSSELLÓ-MORA, R.; ANTÓN, J. **Culture-Independent Approaches for Studying Viruses from Hypersaline EnvironmentsApplied and Environmental Microbiology**, 2012.
SHARON, I.; BANFIELD, J. F. Genomes from metagenomics. **Science**, v. 342, n. 6162, p. 1057–1058, 2013.
SHUBENKOVA, O. V. et al. Microbial community of reduced pockmark sediments (Gdansk Deep, Baltic Sea). **Microbiology**, v. 79, n. 6, p. 799–808, 2010.
SINGH, H. et al. Evidence from the pacific troposphere for large global sources of oxygenated organic compounds. **Nature**, v. 410, n. 6832, p. 1078–1081, 2001.
SKOVRAN, E.; MARTINEZ-GOMEZ, N. C. Just add lanthanides. **Science**, v. 348, n. 6237, p. 862–863, 2015.
SKOVRAN, E.; RAGHURAMAN, C.; MARTINEZ-GOMEZ, N. C. Lanthanides in methylotrophy. **Current Issues in Molecular Biology**, v. 33, p. 101–115, 2019.
SMITH, S V, HOLLIBAUGH, J. T. Coastal metabolism, and the ocean organic carbon balance. **Reviews of Geophysics**, v. 31, n. 92, p. 75–89, 1993.
SNELGROVE, P. V. R. et al. Global Carbon Cycling on a Heterogeneous Sea fl oor. v. 33, n. 2, 2018.
SOETAERT, K. et al. Assessing organic matter mineralization, degradability and mixing rate in an ocean margin sediment (Northeast Atlantic) by diagenetic modeling. p. 519–534, 1998.
SPERLING, E. A.; FRIEDER, C. A.; LEVIN, L. A. Biodiversity response to natural gradients of multiple stressors on continental margins. **Proceedings of the Royal Society B: Biological Sciences**, v. 283, n. 1829, 2016.
STEWART, S. A. Synthesis of salt tectonics in the southern. **Marine and Petroleum Geology**, v. 12, n. 5, p. 457–475, 1995.
STEWART, S. A. Implications of passive salt diapir kinematics for reservoir segmentation by radial and concentric faults. **Marine and Petroleum Geology**, v. 23, p. 843–853, 2006.
STROZYK, F. et al. Giant pockmark formation from Cretaceous hydrocarbon expulsion in the western Lower Saxony Basin, The Netherlands. **Geological Society Special Publication**, v. 469, n. 1, p. 519–536, 2018.
SUMIDA, P. Y. G. et al. Seabed pockmarks associated with deepwater corals off SE Brazilian continental slope, Santos Basin. **Marine Geology**, v. 207, n. 1–4, p. 159–167, 2004.
TANG, Q. et al. Using seabed acoustic imagery to characterize and classify seabed sediment types in the pockmark area of the North Yellow Sea, China. **Applied Acoustics**, v. 174, 2021.
TASIANAS, A. et al. High-resolution 3D seismic study of pockmarks and shallow fluid flow systems at the Snøhvit hydrocarbon field in the SW Barents Sea. **Marine Geology**, v. 403, n. 9037, p. 247–261, 2018.
TAUBERT, M. et al. XoxF encoding an alternative methanol dehydrogenase is widespread in coastal marine environments. **Environmental Microbiology**, v. 17, n. 10, p. 3937–3948, 2015.
TORRES-BELTRÁN, M. et al. The metabolic core of the prokaryotic community from deep-sea sediments of the southern Gulf of Mexico shows different functional signatures between the continental slope and abyssal plain. **PeerJ**, v. 9, 2021.
TORSVIK, T. H. et al. A new scheme for the opening of the South Atlantic Ocean and the dissection of an Aptian salt basin. p. 1315–1333, 2009.

TYSON, R. V.; PEARSON, T. H. Modern and ancient continental shelf anoxia. **Modern and ancient continental shelf anoxia**, n. 58, p. 1–24, 1991.

WANG, L. et al. Preference for particular lanthanide species and thermal stability of XoxFs in Methylorubrum extorquens strain AM1. **Enzyme and Microbial Technology**, v. 136, n. January, p. 109518, 2020.

WASMUND, K. et al. Publisher Correction: Genomic insights into diverse bacterial taxa that degrade extracellular DNA in marine sediments (Nature Microbiology, (2021), 6, 7, (885-898), 10.1038/s41564-021-00917-9). **Nature Microbiology**, v. 6, n. 8, p. 1102, 2021.

WEBB, K. E. Ecology, and geology of pockmarks. **Physics of Geological Processes Department of Biology Faculty of Mathematics and Natural Sciences University of Oslo**, 2009.

WILKINS, M. J. et al. Trends and future challenges in sampling the deep terrestrial biosphere. **Frontiers in Microbiology**, v. 5, n. SEP, p. 1–8, 2014.

WOHLERS, J. et al. Changes in biogenic carbon flow in response to sea surface warming. **Proceedings of the National Academy of Sciences of the United States of America**, v. 106, n. 17, p. 7067–7072, 2009.

XU, L. et al. Methyl-compounds driven benthic carbon cycling in the sulfate-reducing sediments of South China Sea. **Environmental Microbiology**, v. 23, n. 2, p. 641–651, 2021.

YANPIRAT, P. et al. Lanthanide-dependent methanol and formaldehyde oxidation in methylobacterium aquaticum strain 22A. **Microorganisms**, v. 8, n. 6, p. 1–17, 2020.

YU, T. et al. Difference of nitrogen-cycling microbes between shallow bay and deep-sea sediments in the South China Sea. **Applied Microbiology and Biotechnology**, v. 102, n. 1, p. 447–459, 2018.

ZEPPILLI, D.; CANALS, M.; DANOVARO, R. Pockmarks enhance deep-sea benthic biodiversity: a case study in the western Mediterranean Sea. p. 832–846, 2012.

ZHANG, K. et al. ORIGINAL RESEARCH PAPER A preliminary study on morphology and genesis of giant and mega pockmarks near Andu Seamount, Nansha Region (South China Sea). **Marine Geophysical Research**, v. 41, n. 1, p. 1–12, 2020.

ZHUANG, G. C. et al. Microbial metabolism of methanol and methylamine in the Gulf of Mexico: insight into marine carbon and nitrogen cycling. **Environmental Microbiology**, v. 20, n. 12, p. 4543–4554, 2018.

ZHOU, Z. et al. Genomic and transcriptomic insights into the ecology and metabolism of benthic archaeal cosmopolitan, Thermoprofundales (MBG-D archaea). **ISME Journal**, v. 13, n. 4, p. 885–901, 2019.

CHAPTER II - WIDESPREAD METHYLOMIRABILALES ALONG THE SOUTHWESTERN ATLANTIC UPPER SLOPE: AN OVERLOOKED METHANOL-OXIDISING METABOLISM FOR THE DEEP SEA

This chapter was submitted to Scientific Reports, Nature

Abstract

Continental slopes can play a significant contribution to marine productivity and carbon cycling. These regions can harbour distinct geological features, such as salt diapirs and pockmarks, in which their depressions may serve as natural sediment traps where different compounds can accumulate. We investigated the prokaryotic communities in surface (0-2cm) and subsurface (18-20 or 22-24cm) sediments from a salt diapir and pockmark field in Santos Basin, Southwest Atlantic Ocean. Metabarcoding of 16 samples revealed that surface sediments were dominated by the archaeal class Nitrososphaeria, while the bacterial class Dehalococcoidia was the most prevalent in subsurface samples. Sediment strata were found to be a significant factor explaining 27% of the variability in community composition. However, no significant difference was observed among geomorphological features. We also performed a metagenomic analysis of three surface samples and analysed the highest quality metagenome-assembled genome retrieved. The genome was assigned to the family CSP1-5, phylum Methylomirabilota. This non-methanotrophic methylotroph contains genes encoding for methanol oxidation and Calvin Cycle pathways, along with diverse functions that may contribute to its adaptation to deep-sea habitats and oscillating environmental conditions. By integrating metabarcoding and metagenomic approaches using phylogenetic tools, we reported that CSP1-5 is widespread in Santos Basin slope sediments, indicating the potential importance of methanol metabolism in this region. Finally, in a 16S rRNA gene public database investigation, we argued that CSP1-5 sequences might be misclassified as Methylomirabilaceae (the methanotrophic clade) and, therefore, the role of these organisms and the methanol cycling could also be neglected in other environments.

Keywords: CSP1-5; Pockmarks, Salt diapirs; Methylotroph; Metabarcoding; Metagenome assembled genome

II.1 INTRODUCTION

The continental margins are complex and unique environments that interconnect terrestrial and marine processes, playing a significant role in the biogeochemical cycles of carbon and nitrogen [1]. Although continental shelves and slopes represent only 20% of the ocean area, these regions can account for up to 50% of marine productivity [2, 3]. The mineralisation of organic matter in these areas occurs at a much faster rate when compared to what occurs in open sea sediments, allowing the regenerated nutrients to quickly return to their natural cycles [4, 5].

Continental slopes often comprise a wide range of physical and geological features that can affect nutrient distribution and, consequently, biodiversity [6]. This variability is particularly important for benthic microbial communities, which are known to respond to changes in nutrient availability and carbon substrate type [7]. As microbial processes are essential for ocean biogeochemistry and organic matter mineralization [5], it is crucial to understand their roles in local and regional carbon cycles.

Santos Basin (SB) is a marginal basin located on the Southwestern Atlantic margin. In this region, the continental upper to middle slope presents high declivity and is marked by numerous kilometre-scale seafloor features, including pockmarks and exhumed salt diapirs [8, 9]. Pockmarks are crater-like depressions formed by the sudden release of fluid (predominantly methane) in the form of a gas or liquid to the surface [10, 11].

In Santos Basin, fluid expulsion is facilitated by moving large volumes of salt, weakening the upper sedimentary layers [9]). Due to gravitational forces, these salt masses show vertical movement (diapir), which may or may not be exposed on the marine sedimentary surface [8, 12]. The pockmark field on the SB continental slope has been reported to contain more than nine hundred depressions related to this geological feature and salt diapirs. Although the majority of pockmarks in this area are thought to be not actively seeping gas, acoustic data and metal proxies have suggested the presence of recent to sub-recent seepage activities in some of the pockmarks [9, 13–16]. In addition, the unique characteristics of the seafloor in this area may provide a myriad of habitats for diverse but yet poorly explored microbial communities [17].

Continental slopes often present high sediment accumulation and organic matter deposition [9, 18]. In addition, the depressions as pockmarks may serve as natural sediment traps [19], where the sedimentation of different compounds can occur, including methanol, a highly abundant reduced carbon in the marine environment [20, 21]. This C1 hydrocarbon has a fast turnover in this environment, on the order of a few days, indicating its significance in

biological cycles, mainly as a source of carbon and energy for methylotrophic microorganisms [22, 23]. Investigations on the methanol sources and turnover have long been focused on terrestrial systems [24], and it was only recently that the role of the oceans in the global methanol cycle began to be questioned [25–28]

Versatile metabolic capabilities provide the seafloor microbial communities with different strategies to couple with oligotrophic and variable conditions. The importance of C1 metabolism in deep-sea sediments has been recently suggested by Torres-Beltrán et al (2021) [29], including methanol oxidation to formaldehyde and methylamine degradation. The authors also detected functions related to formaldehyde oxidation on the continental slope of the southern Gulf of Mexico. Yet, the identity, metabolic capabilities, and distribution of these organisms demand further exploration. While the metabolism of some methylotrophic groups has been vastly studied, others have only recently emerged from metagenome-assembled genomes (MAGs). Hug et al (2016) [30] described a genome of a non-methanotrophic methylotroph from the phylum Methylomirabolota (former NC10), a cluster mostly known for its nitrate-dependent methanotroph members, which has raised questions regarding the phylogenetic boundaries of the different methylotrophic groups in this phylum.

This study used metabarcoding and metagenomics to investigate the prokaryotic diversity in surface and subsurface sediments collected at the pockmark and salt diapir field on the SB continental slope. Through genome reconstruction from metagenomic data, we described the MAG of a methylotroph from the order Methylomirabilales, which was widespread across SB sediments. We further explored its adaptations to the deep-sea environment and then argued its potential metabolic capabilities and roles in the carbon cycle along the SB continental slope.

II.2 MATERIAL AND METHODS

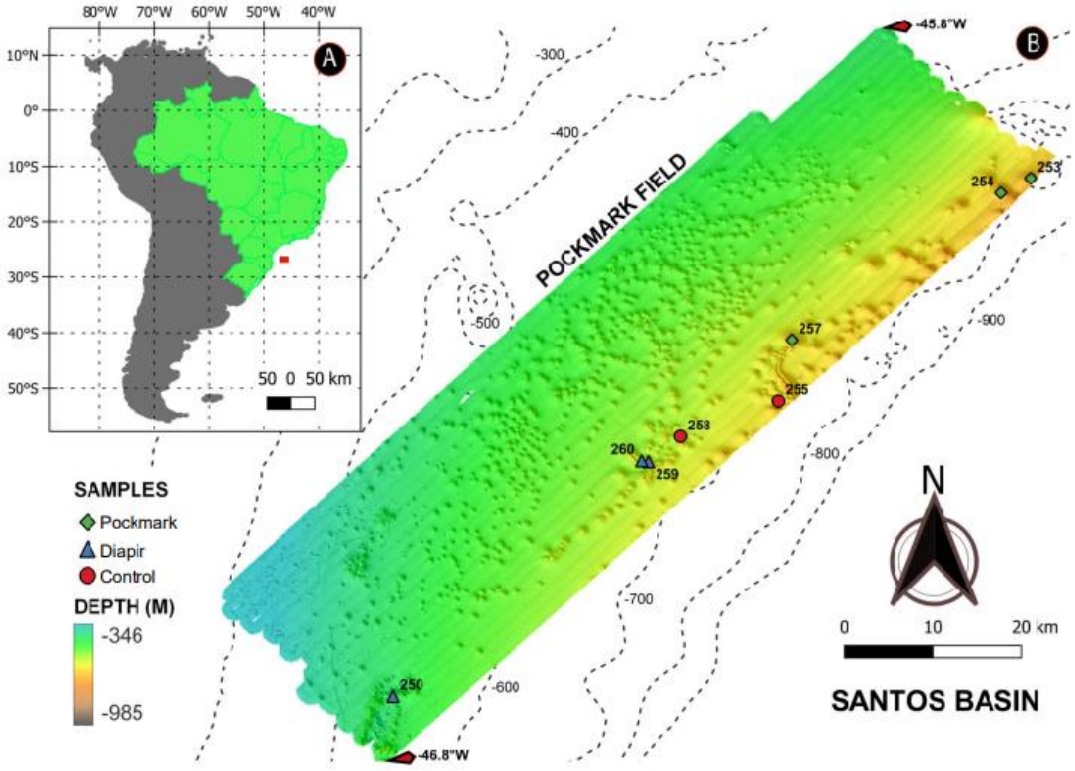
II.2.1 Study area and sediment sampling

The Santos Basin (SB) is located on the southern continental margin of Brazil between latitudes 27 °S and 26 °N, covering an area of 350 thousand km². SB is limited to the north by Alto de Cabo Frio and to the south by Cabo Santa Marta Grande in Alto de Florianópolis [31, 32]. Under the hydrographic point of view, the area of study is located in the transition between two water masses, the South Atlantic Central Water (SACW) and the Antarctic Intermediate Water (AAIW) [33]. The SACW ($T \leq 18.5^{o}C$, $S \geq 35.3$) occupies the pycnocline level [34],

while the AAIW presents temperatures between 3ºC and 6ºC and salinities between 34.2 and 34.5 [35].

We collected sediment samples using a stainless steel box corer (BX-650) (50 cm x 50 cm) with a maximum penetration of 60 cm. Cylindric corers were used to collect sediments within the box corer while maintaining the sediment stratification. The sediment cores were sliced into 2 cm layers with sterile spatulas and placed in whirl pak bags, then stored at -20 °C until processing. For this study, we selected eight stations with bathymetry ranging from 400 to 800 metres approximately, comprising three stations located in salt diapirs, three stations in pockmarks, and two stations in the adjacent marine seafloor considered as control sediments, without pockmarks and salt diapirs influence (**Figure 1**). We used the superficial (0-2 cm) and the deepest sediment layer (16-18 or 22-24 cm), from now on called surface and subsurface strata, respectively (**Table S1**).

Figure 1. Map of the study region located in the Santos Basin, highlighting the sampling sites where structures related to pockmarks and salt diapers were found on the Brazilian continental margin.



Source: Raissa Basti Ramos, 2022.

II.2.2 Taxonomic profiling of the prokaryotic community

DNA was extracted from 0.25 g of sediment using the Power Soil DNA Isolation Kit (Qiagen, Germany), following the manufacturer's specifications. DNA integrity was verified by electrophoresis in 1% (v/v) agarose gel, and concentration was assessed using the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The V4-V5 hypervariable region of the 16S rRNA gene was amplified with the universal primers 515F and 926R [36, 37]. The initial PCR reaction consisted of a denaturation step at 95 ºC for 3 minutes, followed by 35 cycles with 95 ºC for 30 seconds, annealing at 57 °C for 30 seconds, extension at 72 °C for 30 seconds, and final extension at 72 °C for 5 minutes. Library preparation and sequencing were performed by Mr DNA/Molecular Research (Shallowater, TX, USA), using Illumina Miseq platform (2 x 250 bp system). Bioinformatic and statistical tools used to analyse the 16S rRNA gene sequences are described in Supplementary Methods. 16S rRNA gene sequencing data are available in the National Center for Biotechnology Information Sequence Read Archives under BioProject ID PRJNA818533.

After sequencing, paired-end reads were initially imported and demultiplexed into the Qiime2 software version 2019.10 [38]. Graphic inspection of quality profiles was performed, low-quality reads (below Phred score 30) were trimmed, and the chimeras were removed with the aid of the Dada2 software [39]. After quality control, the amplicon sequence variants (ASVs) were determined using the Dada2 software [39] into the Qiime2 package. Taxonomy was assigned through feature-classifier classify-sklearn and SILVA database v. 138. ASV richness, Chao1, and Shannon diversity indexes were calculated using *phyloseq* [40] and *vegan* packages [41], and *ggplot2* [42] was used for graphing in R v. 4.1.0 (R Development Team, 2018).ASVs were normalised by *varianceStabilizingTransformation*, using the R package "*DESeq2*" [43]. Similarities among samples and site groups were examined using ordinated weighted Unifrac normalised distance and visualised by non-metric multidimensional scaling (MDS). Alpha diversity, statistical analysis, and data visualisation were carried out in R using the *Phyloseq* package [40]. Differences in the microbial communities among sites and depths were assessed by permutational multivariate analysis of variance (PERMANOVA) [44]. 16S rRNA gene sequencing data are available in the National Center for Biotechnology Information Sequence Read Archives under BioProject ID PRJNA818533.

II.2.3 Metagenomic analysis for metagenome-assembled genome (MAG) recovery

We performed shotgun metagenomic analysis of three surface sediment samples from the following stations: 259 (diapir area), 260 (diapir area), and 255 (control area). The metagenomic libraries were prepared using Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA), and sequencing was performed in Illumina Hiseq platform (2 x 150 pb system) at the Woods Hole Institute's Marine Biology Laboratory, as part of the "Deep Carbon Observatory's Census of project Deep Life". Raw reads were filtered through the SICKLE software with phred >30 (Joshi and Fass, 2011) and then used for genome reconstruction through the anvi'o pipeline v. 7.1 [45]. Co-assembly was performed using the Megahit software [46], and the contigs with size >4,000 bp were selected for binning through the CONCOCT software [47]. Bins were manually refined using *anvi-refine* [45] and then quality checked with CheckM v. 1.0.7 [48]. Bins were taxonomically classified based on genome phylogeny according to the *classify* workflow (classi- fy_wf) from the Genome Taxonomy Database Toolkit (GTDB- Tk v. 1.3.0) and the Genome Taxonomy Database (GTDB; release 202) [49]. Metagenome raw sequences are available in the GenBank repository under BioProject ID PRJNA818670.

For further analyses, we selected the MAG with the highest quality scores, the SB_MAG_00001 (94.2% completeness and 2.1% contamination), classified within the Methylomirabilota phylum (formerly NC10 phylum). Prediction and annotation of ORFs were performed using prokka v.14.5 [50]. Ghost-KOALA (genus_prokaryotes) (Kanehisa et al., 2016) and SEED Subsystem through RASTtk [51] were used for functional annotations of the predicted protein sequences. MetabolismHMM tool v. 1.9 (https://github.com/elizabethmcd/metabolisHMM) and DRAM (Distilled and Refined Annotation of Metabolism) tool v. 1.2.4 (https://github.com/shafferm/DRAM) were used to annotate genes related to sulphur, nitrogen, and carbon metabolisms.

For pangenomic analysis, we selected three genomes from the order Methylomirabilaliles, available in the NCBI (National Center for Biotechnology Information): *Candidatus Methylomirabilis oxyfera* (Ca. *M. oxyfera*) (NCBI:txid671143), *Candidatus Methylomirabilis limnetica* (Ca. *M. limnetica*) (NCBI: txid671143), and NC10 bacterium CSP1-5 (NCBI:txid1640516). The functional annotation was also carried out for the three reference genomes. Pangenome was performed following the anvi'o v.7.1 pipeline [45, 52] to carry out genome comparison and gene cluster identification, considering the following parameters: COG annotation, NCBI blastp for amino acid sequence similarity search, 0.5 for minbit, 1 for the gene cluster minimal occurrence and 2 for MCL inflation and euclidean

distance. In the pangenome, we selected the following gene sets for further investigation regarding the predicted proteins annotated by GhostKOALA (genus_prokaryotes) [53]: Core - genes present in all genomes analysed; Singletons - genes present only in the SB_MAG_00001; and CSP1-5 - genes shared only by the CSP1-5 representative and SB_MAG_00001. The metabolic reconstruction model of SB_MAG_00001 was performed using annotations from DRAM, MetabolismHMM and GhostKOALA. The MAG was deposited in Figshare under DOI10.6084/m9.figshare.20080031.

II.2.4 Construction of the phylogenetic tree

A phylogenetic tree was built to verify the phylogenetic relationships among members of the Methylomirabilota phylum recovered from our sediment samples using the different approaches. First, selected all sequences assigned to this phylum found in our 16S rRNA gene sequencing data (23 ASVs), and then we extracted the 16S rRNA gene sequence from the MAG SB_MAG_00001 using the barrnap software (version 0.9, https://github.com/tseemann/barrnap). In addition, 16S rRNA gene sequences were extracted from the reference genomes Ca *M. oxyfera,* Ca *M. limnetica,* and CSP1-5. Finally, we used 34 16S rRNA gene sequences retrieved from the Silva database that showed at least 95% similarity with our Methylomirabilota 16S gene sequences and the 16S sequence extracted from our MAG SB_MAG_00001. The sequences obtained from the Silva database were retrieved from marine and terrestrial environments distributed worldwide.

All sequences were aligned using the Mega X software [54] through the clustalW algorithm that uses progressive alignment methods [55]. This algorithm calculates an approximate distance matrix between pairs of sequences based on alignment scores. The phylogenetic tree was constructed by the maximum likelihood method with Bootstrap replications equal to 999.

II.3 RESULTS AND DISCUSSION

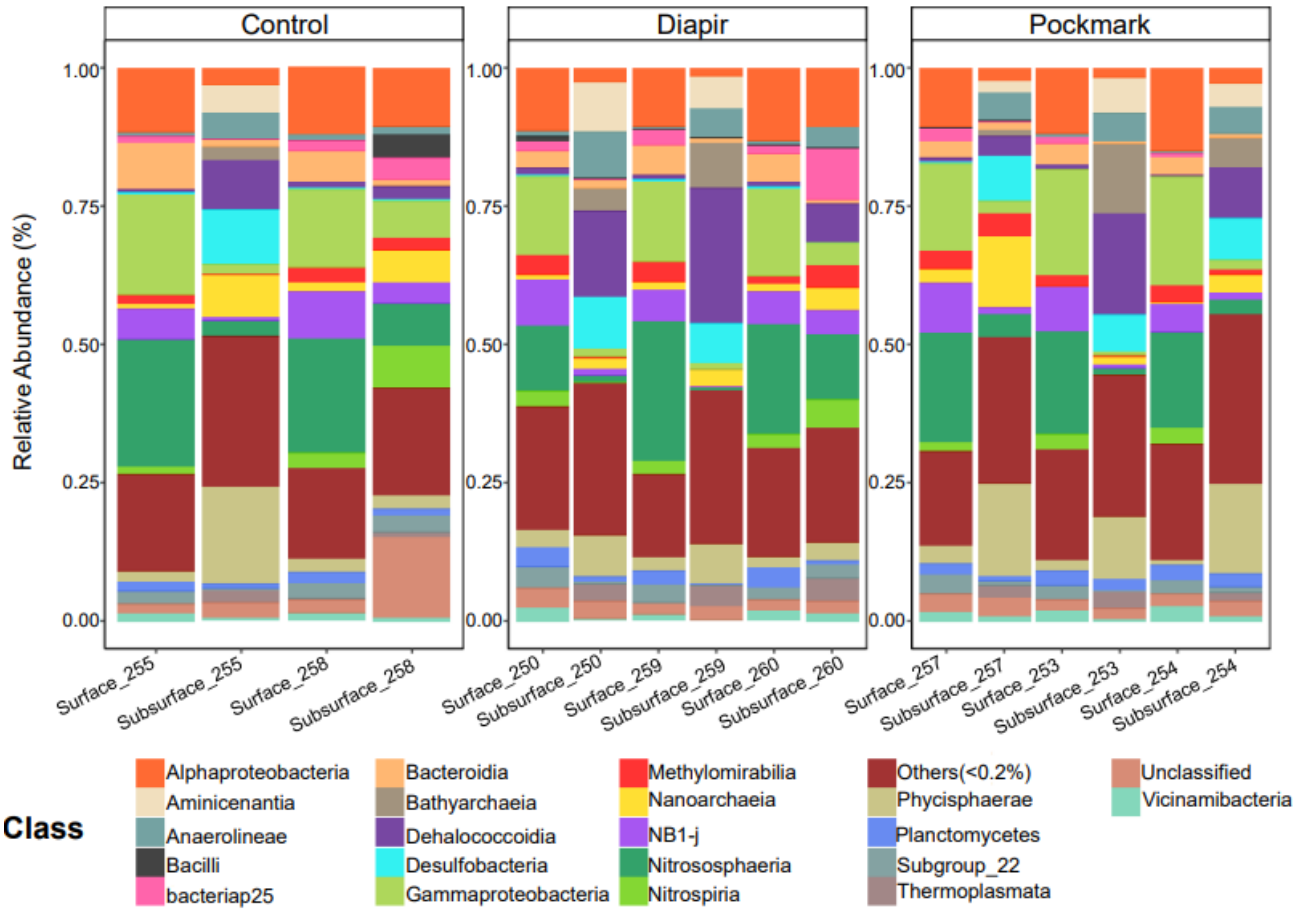
II.3.1 Microbial taxonomic diversity in sediments from the Brazilian continental slope

The 16S rRNA gene sequencing of the 16 samples yielded a total of 573,850 quality-filtered reads, divided into 11,034 amplicon sequence variants (ASVs) (0,03 cut-off). Of those, 9,244 ASVs were assigned to Bacteria and 1,788 to Archaea. Richness and diversity, assessed using Chao1 and Shannon indexes (**Table S2)**, did not change significantly across the types of

seabed samples (control, diapir, pockmark) and between the two sediment strata (surface and subsurface strata) (**Table S3**).

Surface sediments were dominated by the archaeal class Nitrososphaeria (Crenarchaeota) across all seabed types, accounting for up to 25% of the taxonomic assignments (**Fig. 2**). Nitrososphaeria is one of the most widely distributed classes of Archaea, found in a diversity of environments [56, 57], including marine water and sediment [58]. It comprises chemolithoautotrophic ammonia-oxidising taxa, which play an important role in the marine nitrogen and carbon cycles [58]. Alpha and Gammaproteobacteria comprised between 11% and 18% of the ASVs assignments. These classes have also been reported as part of the dominant groups in benthic marine environments, including pockmark sediments [57, 59–62].

Figure 2. Bar graphs showing the relative abundance of bacterial and archaeal classes in the Control, Diapir and Pockmark sediment samples collected in the Santos Basin. Surface = 0-2cm and Subsurface = 16-18cm or 22-24cm.



The class NB1-J, previously described in sediment samples from active pockmarks [61], was detected in surface sediments from the SB. The class Methylomirabilia, from the phylum Methylomirabilota (former NC10), was found in all surface samples, with an average

abundance of approximately 2%. Members of this class have been identified in the most diverse environments, from freshwater to saline, and include methanotrophic and non-methanotrophic methylotrophic taxa [30, 63, 64].

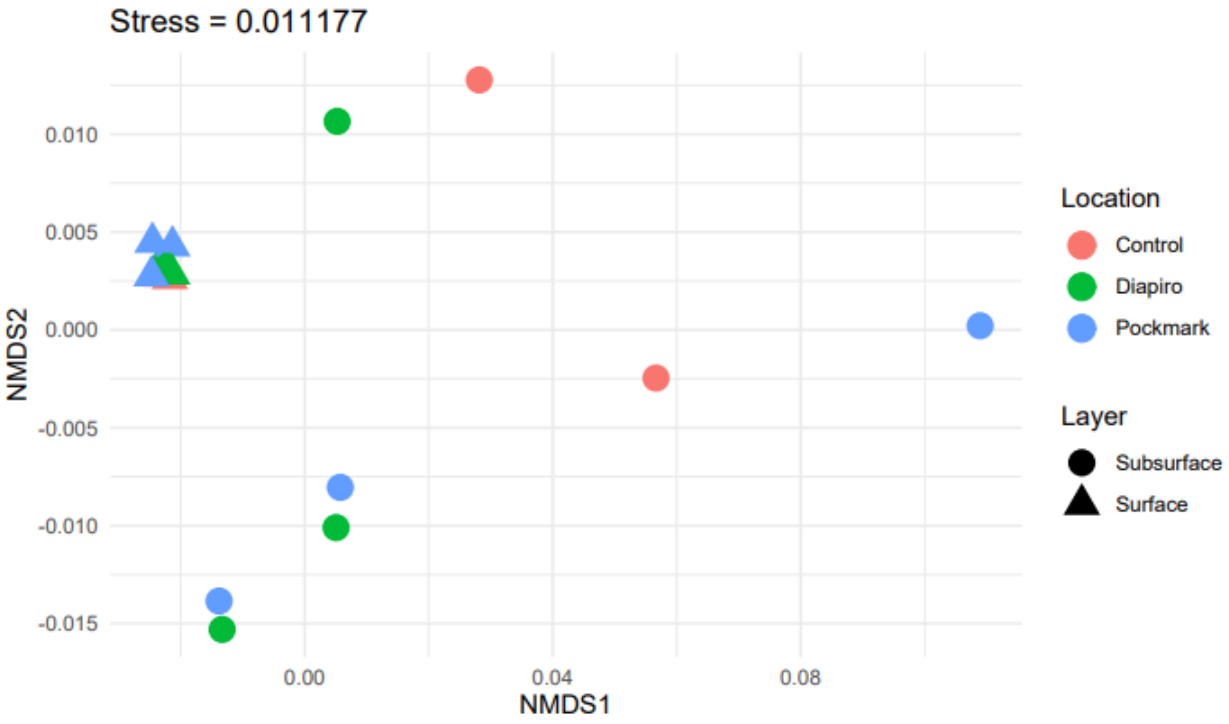
In the subsurface samples, the Dehalococcoidia represented up to 24% of the communities. Some members of this class can perform organohalide respiration, anaerobic respiration that uses halogenated organic compounds as terminal electron acceptors for hydrogen oxidation [65]. Microbial dehalogenation plays a significant role in the functioning of the halogen cycle, and many of these organisms are found in the marine environment, mainly in subsurface sediments [65, 66]. The class Phycisphaerae (phylum Planctomycetota) was also prevalent across all subsurface samples from SB, reaching up to 17% of the communities. These organisms are aerobic or facultatively anaerobic and colonise a wide variety of ecosystems, from aquatic to terrestrial and even extreme environments such as desert, saline, and thermal soils [67–70].

Desulfobacteria, known to contain sulphate-reduction bacteria, a key function in the sulphur cycling and anaerobic respiration [71], presented relative abundance between 0.2 and 9.8 % of the communities. Nitrososphaeria comprised a much smaller proportion in the most profound strata than in the surface samples. By contrast, another Crenarchaeota class, the Bathyarchaeia, was found in higher proportions in the subsurface sediments. These organisms are widely distributed and abundant in marine sediments; however, the environmental factors that control their distribution are currently unclear [72]. They are metabolically diverse and indicated by Lazar et al (2016) [73] as degraders of aromatic compounds and recalcitrant organic matter. The acetogenic ability to lithotrophically synthesise acetate from inorganic carbon has also been suggested through MAGs studies, as well as the potential ability to metabolise methane; however, these metabolisms were not yet confirmed by physiological studies since they were not yet cultivated in laboratory conditions [63, 74].

According to PERMANOVA analysis, seabed type was not a significant factor affecting the composition of the communities (**Table S4**). By contrast, 27% of the community variability ($p<0.001$) was explained by the vertical sediment strata - surface (0-2 cm) versus subsurface (16-18 cm or 22-24 cm). This finding is in agreement with previous studies showing remarkable differences in prokaryotic communities along the sediment strata, which is attributed to the sharp changes in physical and chemical conditions with depth [17, 59, 75, 76]. Another interesting pattern was revealed by NMDS ordination, where all surface sediment communities were concentrated in a tight cluster, indicating high similarity in their composition, while lower similarity was observed among subsurface samples (**Fig. 3**). As surface sediments are under

direct contact with the pelagic environment, their biotic and abiotic characteristics suffer stronger influences from the recent deposition processes and exchanges with the water column. This aspect also contributes to the higher availability of organic carbon [77, 78]. In addition, high-energy electron acceptors such as oxygen and nitrate are often available in the surface sediments [79]. By contrast, deeper sediment layers may reflect events during the deposition, such as different sedimentation rates [80], which may vary along the continental slope [81, 82].

Figure 3. Non-metric multidimensional scale (nMDS) of the surface (0-2cm) and subsurface (16-18cm or 22-24cm) sediment communities. Stress = 0,011177. Geometric shapes indicate the collection site: squares - control; circles - Diapirs; triangles – Pockmarks.



The core microbiome analysis further supported these contrasting patterns for the community similarity when comparing surface and subsurface strata. In this analysis, we asked whether there would be ASVs common to all samples within each stratum, as well as to all samples regardless of the stratum (central core microbiome). While 80 ASVs were shared by all surface sediment samples (surface core microbiome), comprising archaeal and bacterial taxa from ten different phyla, the core microbiome from the deep strata was composed of only two ASVs, which were also the central core microbiome, i.e., present in all samples (**Table S5**). These two ASVs were assigned to the Methylomirabilaceae and Hyphomicrobiaceae families, according to Silva database v.138. The Hyphomicrobiaceae family (Alphaproteobacteria) has been identified in several marine and non-marine habitats [83–85] and are morphologically and

physiologically diverse, with most of their members known to perform aerobic chemoheterotrophic metabolism. However, some representatives can grow anaerobically by denitrification or fermentation [86]. Interestingly, as the Methylomirabilales, the family Hyphomicrobiaceae also comprises methanol assimilating representatives [30, 86, 87] suggesting that C1 metabolising organisms could be widespread in the studied area.

II.3.2 SB_MAG_00001: a Methylomirabilales methylotroph retrieved from SB surface sediment samples

The metagenomic library constructed from three surface sediment samples yielded 77,922,888 raw reads. After quality filtering, the number of reads per sample was 23,406,011 (Surface_255), 19,431,982 (Surface_259) and 22,090,471 (Surface_260). The co-assembly resulted in 108,018 contigs (>1,000 bp, N50=1,492 bp), which were further binned into 34 MAGS: two high-, six medium-, and 32 low-quality drafts, according to genome quality standards suggested by Bowers et al. (2017) [88] (**Table S6**).

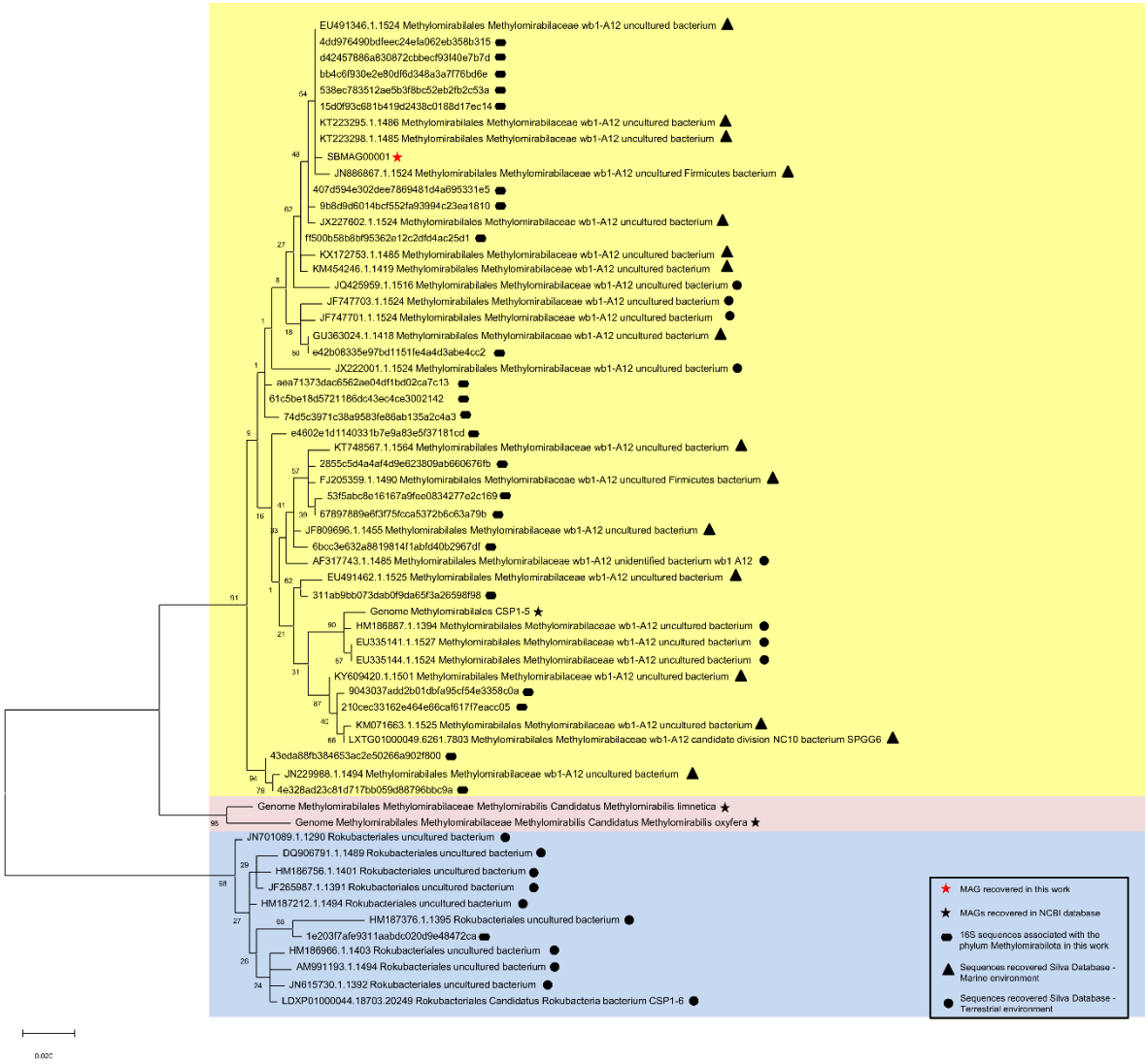
SB_MAG_00001, selected for further analyses, exhibited the highest quality parameters (completeness of 94.2% and contamination of 2.1%) and, according to the phylogenomic analysis of the GTDB-Tk, it was assigned to the order Methylomirabiles, family CSP1-5 (ANI of 97.77% with CSP1-5 sp001443495 as the closest representative genome). At this point, it is noteworthy that contrasting taxonomic assignments were observed for members of the Methylomirabilales order retrieved by the different sequencing approaches in our study. While using Silva 138 database to classify 16S rRNA gene sequences, all ASVs from the Methylomirabilales order were assigned to the family Methylomirabilaceae (including one of the two ASVs from the central core microbiome). By contrast, the genome of SB_MAG_00001 was classified within the family CSP1-5, according to GTDB-Tk. Although Methylomirabilales is still a poorly known taxon, this difference in taxonomy may have important implications for our conclusions regarding microbial function and carbon cycling, as these families have distinct key metabolic capabilities.

To date, there is no isolated culture from the order Methylomirabilales. The most studied organisms belong to the family Methylomirabilaceae (Ca. *Methylomirabilis oxyfera*, Ca. *M. limnetica*, and Ca. *M. lanthanidiphila*), which are known to carry out anaerobic oxidation of methane (AOM) coupled to nitrite reduction [64, 89, 90]. Unlike other AOM processes, they employ enzymatic machinery similar to aerobic methanotrophy, including its central enzyme, the methane monooxygenase. The oxygen required for this process is produced intracellularly by nitric oxide (NO) disproportionation [90]. However, this methane oxidising enzyme

complex is absent in CSP1-5, a genome retrieved from aquifer sediment samples [30], and characterised as a non-methanotrophic methylotroph. The CSP1-5 genome shares 89% of the 16S rRNA gene identity with Ca. *M. oxyfera.* The phylogenetic differences described between CSP1-5 and the Ca. *M. oxyfera* establish the boundaries of denitrification coupled with methane oxidation within the Methylomirabilota phylum [30].

A quick inspection revealed that the Silva database contains no family other than Methylomirabilaceae within the Methylomirabilales order. We then asked whether the CSP1-5-like microorganisms in our samples could have been misclassified by the Silva database. To answer this question, we built a phylogenetic tree using 16S rRNA gene sequences from our amplicon sequencing data classified into the Methylomirabilota phylum and the SSU fragments extracted from SB_MAG_00001 and from three reference genomes: *M. oxyfera*, *M. limnetica* and CSP1-5. In addition, we used 16S rRNA sequences downloaded from Silva (see methods for details). The phylum Methylomirabolota can be divided into four groups: *A*, *B*, C, and *D*, according to Ettwig et al. (2009) [91]. Groups *A* and *B* are considered the dominant branches and harbour the anaerobic methane-oxidising microorganisms. Members of the CSP1-5 family are housed in the group *D*. All other members of this phylum are allocated to clade *C* [91]. In the phylogenetic tree presented in **Figure 4**, the 16S rRNA gene sequences clustered in three major clades: One clade was formed only by sequences extracted from the reference genomes of the methanotrophic taxa, Ca. *M. oxyfera* and Ca. *M. limnetica* (both group A) [64, 89]. The largest cluster housed 22 ASVs obtained in this study by amplicon sequencing and the sequences extracted from the genomes of SB_MAG_00001 and CSP1-5. Therefore, the clustering pattern indicates that, similarly to the MAG SB_MAG_00001, the SB amplicon sequences are more closely related to CSP1-5 than the methanotrophic genomes. This finding suggests that those sequences may have been misclassified by the Silva database and could, in fact, belong to the same family as SB_MAG_00001, CSP1-5. In addition, this large cluster also contained 24 sequences downloaded from the Silva database (retrieved from marine and terrestrial ecosystems) and classified within the family Methylomirabilaceae. According to this analysis, previous studies may have inaccurately reported the presence of methane-oxidising microorganisms and neglected the importance of methanol metabolism in those environments (**Table S7**). The third cluster comprised 10 sequences from the Silva database and only one ASV retrieved from this study by amplicon sequencing. All members of this cluster belong to the order Rokubacteriales. Interestingly, except for the ASV obtained in this study, all sequences in the third clade were recovered from terrestrial environments.

Figure 4. Phylogenetic tree comparing the 16S sequences affiliated to the order Methylomorabilales obtained in our samples, together with the 16S sequence recovered from SB_MAG_00001.



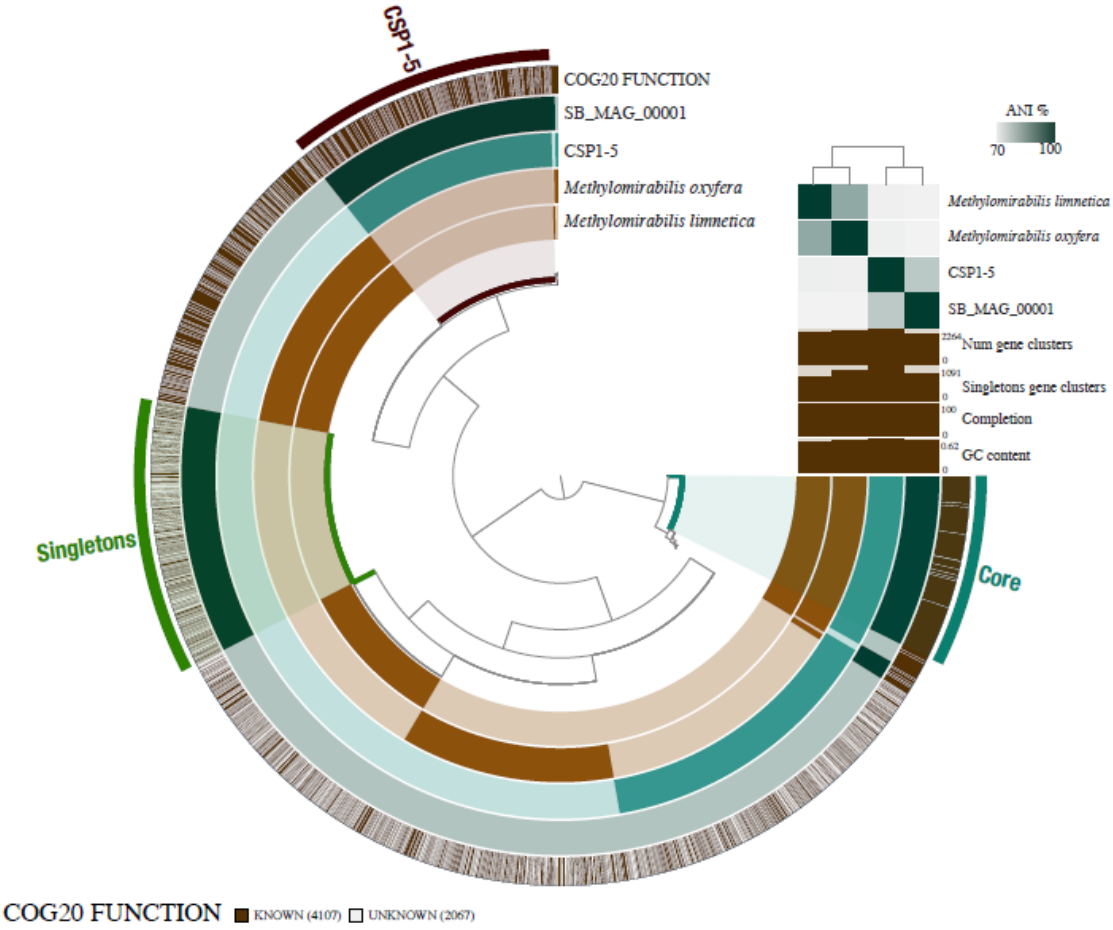
Additionally we include 16S sequences recovered from the reference genomes: Ca. Methylomirabilis oxyfera, Ca. Methylomirabilis limnetica and NC10 bacterium CSP1-5, obtained through data deposited at NCBI (National Center for Biotechnology Information). 16S sequences retrieved from the Silva 138 database, with 100% similarity with our sequences, were also used.

II.3.3 Pangenomics and potential methanol metabolism

Pangenomic analysis provided information on the similarities in gene composition between MAG SB_MAG_00001 and the three reference genomes (*M. oxyfera*, *M. limnetica* and CSP1-5). This analysis allows for the inspection of the sets of genes shared by all genomes (core) or by a particular group of genomes, as well as those unique to a single genome (singletons) [92]. A total of 6,174 gene clusters were identified across the pangenome. CSP1-5 presented the highest number of singletons among the genomes. As observed for 16S rRNA

phylogenetic analysis, SB_MAG_00001 was more similar to CSP1-5 (ANI of 76.7%) in comparison with *M. oxyfera* (ANI of 70.3%) and *M. limnetica* (ANI of 70.5%). We further analysed the gene clusters shared only between SB_MAG_00001 and CSP1-5 (844 gene clusters - denoted by the name of the family CSP1-5), the singletons (864 gene clusters), and those composing the core genes (601 gene clusters) (**Fig. 5**) and discussed their presence/absence in the metabolic reconstruction of SB_MAG_00001 (**Fig. 6)**.

Figure 5. Pan-genome analysis based on the presence/absence of 6,174 gene clusters.



Pan-genome analysis based on the presence/absence of 6,174 gene clusters, which are defined by the tree in the centre (Euclidean distance; Ward linkage). The SB_MAG_00001 is represented by the dark green bar, while the reference genomes are represented by the light green (CSP1-5) and brown (Methylomirabilis spp.) bars. The Core cluster represents the genes present in all genomes analysed, while the CSP1-5 cluster represents the genes shared by SB_MAG_00001 and the CSP1-5 representative genome. Singletons cluster indicates the genes present only in SB_MAG_00001. The heatmap represents the ANI percentage between the genomes analysed (70-100%).

SB_MAG_00001 contains complete or nearly complete gene sets for the following central carbon and energy metabolism pathways: glycolysis, gluconeogenesis, pyruvate oxidation, TCA cycle, pentose phosphate pathway, and Calvin Cycle. As reported for the three

reference genomes [30, 64, 89] and observed in the core cluster of the pangenome, SB_MAG_00001 also contains the lanthanide-dependent Xox-type methanol dehydrogenase (MDH), which can convert methanol to formaldehyde or directly to formate [93–95]. It has also been proposed that XoxF-MDH can oxidise formaldehyde to formate [94]. Several studies have suggested the widespread nature of Xox-MDH within methylotrophs, with its functional relevance in the environment likely comparable with the vastly studied calcium-dependent MDH (Mxa-MDH).

The metabolic reconstruction in **Figure 6** revealed complete pathway to oxide formaldehyde to formate through the 5,6,7,8-tetrahydromethanopterin-dependent route, in addition to formate dehydrogenase which can oxidise formate to CO_2 [90]. CO_2 can be assimilated through Calvin Cycle, the only C fixation pathway found in this genome and other known Methylomirabilales [90, 96]. Accordingly, the Serine Cycle for formaldehyde assimilation is absent. Using enrichment cultures and carbon isotope tracing experiments, Rasigraf et al. (2014) [96] demonstrated that the methanotrophic *M. oxyfera* assimilated exogenous $^{13}CO_2$, when provided with CH_4, indicating autotrophic CO_2 fixation. The question arises whether these non-methanotrophic methylotroph counterparts could also assimilate exogenous CO_2 (and not only from the formaldehyde degradation). Considering the wide distribution of these organisms in the deep-sea sediments of the Santos Basin slope presented in our study, this could represent an overlooked primary production process in the area. However, experimental demonstration of this capability would be required.

II.3.4 Potential metabolisms and lifestyles of the SB_MAG_00001

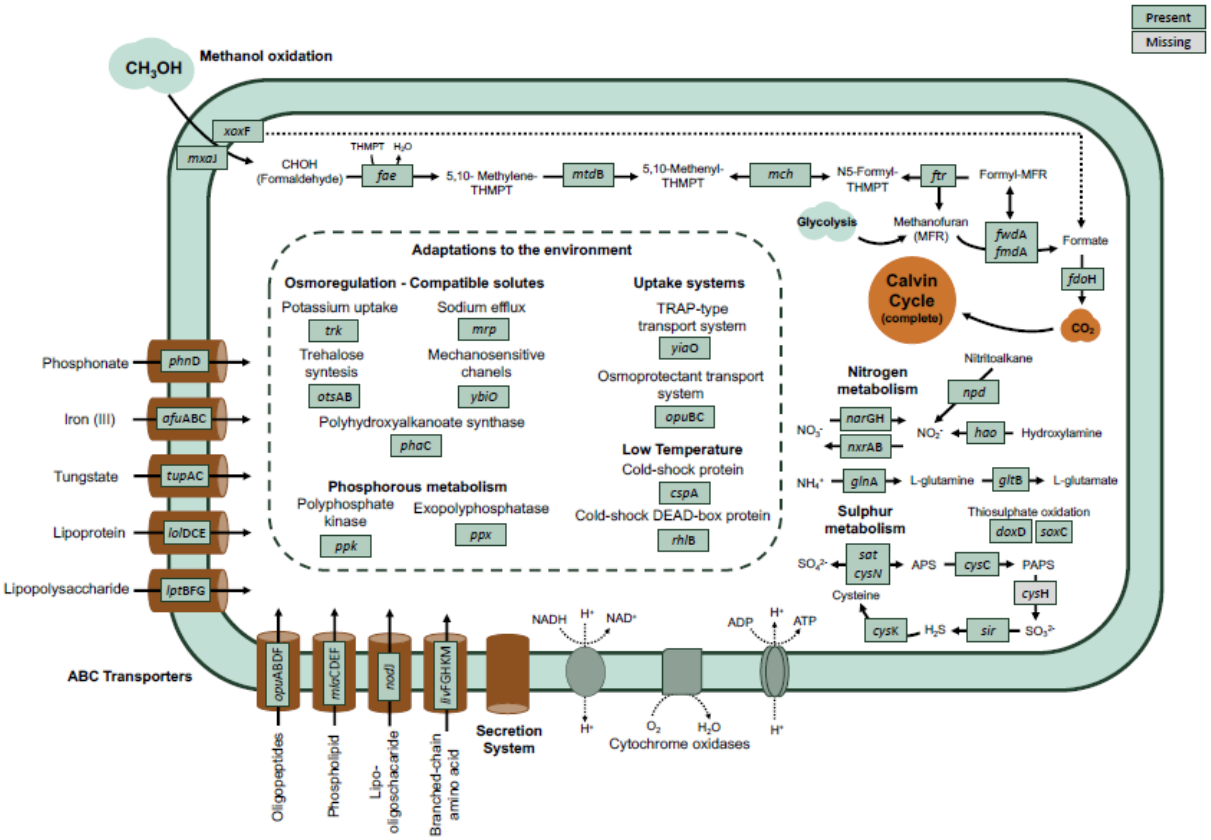
Microorganisms inhabiting continental slope sediments may endure a wide range of conditions, including oligotrophic environments, low temperature, and high hydrostatic pressure (HHP). Such conditions may select organisms with suitable niches, and by exploring the metabolic potential of SB_MAG_00001 we could shed light on the ecology and adaptation of this microorganism. Living in oligotrophic sediments demands metabolic strategies to cope with the scarce and oscillating resources. In this regard, methylotrophy might represent an advantageous trait, considering that C1 compounds seem to be present in the marine environment in high abundance [27, 97, 98].

In addition to carbon substrate, metabolic capabilities related to nutrient cycling can reveal ecological strategies to couple with the availability of reduced compounds and electron acceptors. For instance, SB_MAG_00001 contains genes for the first step of the dissimilatory nitrate reduction and denitrification (*nar*GH), pathways used as alternative respiratory

pathways under low levels of oxygen by several microorganisms [99, 100]. Furthermore, *nxr*AB, involved in nitrite oxidation, and the gene *hao*, which converts hydroxylamine to nitrite, are also present. Interestingly, *hao* was not detected in the closest relative genome, CSP1-5, but it is present in the methanotrophic genomes (*M. oxyfera* and *M. limnetica*). As for sulphur cycling, genes related to assimilatory sulphate reduction (ASR) (*sat*, *cys*N, *cys*C, *sir*, *cys*K), and thiosulphate oxidation (*sox, doxD*) were annotated. ASR is a fundamental metabolic route, as sulphur is an essential element in all organisms present in biomolecules, such as amino acids [101]. In addition, thiosulphate can be an important intermediate in the sulphur cycle in marine sediments and it might be generated from the anoxic sulphide oxidation [102].

Regarding phosphorus metabolism, genes encoding for polyphosphate kinase (PPK) and exopolyphosphatase (PPX) were detected. These enzymes are responsible for polyP accumulation and degradation, a trait that may confer an advantage in environments under high oscillation in phosphate availability and other stress types [103, 104]. In addition, the presence of the gene for phosphonate transport (*phn*D) indicates a potential use of organic phosphorus. Besides *phn*D, other transporters provided some indications of the potential ecophysiology of this methylotroph, including transporters for Iron, glutamate/ aspartate, phospholipid/ cholesterol/ gamma-HCH, amino acids, and tungstate (**Figure 6**).

Figure 6. Model prediction of SB_MAG_00001 metabolisms.



The model includes potential capabilities related to Methanol, Nitrogen (N) and Sulphur (S) metabolisms, as well the ABC transporters and the adaptations to the environment. The model was constructed with the genes annotated by DRAM, predicted proteins annotated by GhostKoala and the Hidden Markov Model (HMM) searches (Supplementary Table 8).

In deep-sea conditions, adaptations for psychrophilic and piezophilic lifestyles may confer advantages. Our sampling points ranged from 433 to 747 m deep where water temperatures can be as low as 5°C for the Antarctic Intermediate Water mass [33, 105]. Some traits, such as the expression of cold and shock proteins can have key roles in the cold seafloor [106] SB_MAG_00001 contains *csp*A and *rhl*B genes, which encode for the cold-shock protein and cold-shock dead-box protein-A, respectively, and have been suggested to have a role in the adaptation to cold conditions [107–109].

One of the major findings in cellular adaptations to high hydrostatic pressure conditions is the accumulation of solutes in the bacteria, which may play the role of a “piezolyte” acting as protein-stabilising solutes [110]. Accumulation of protein-stabilising solutes, such as β-hydroxybutyrate (PHB), is often observed in organisms living under HHP [100]. The gene related to PHB biosynthesis, polyhydroxyalkanoate synthase (*pha*C), is present in SB_MAG_00001 and CSP1-5 genomes but not in the methanotrophic genomes. Compatible solutes can confer resistance to multiple stresses, including hydrostatic and osmotic pressure

[111]. Other genes related to compatible solutes were found to be part of the SB_MAG_00001 singleton clusters: the *trk* system potassium uptake protein and the monovalent cation:H^+ antiporter, and CPA1 family *mrp,* for sodium efflux. The genes for TRAP-type transport system periplasmic protein (*yiaO*) and trehalose synthesis (*ots*AB) were present only in the CSP1-5 cluster, while the osmoprotectant transport system (*opuB*C) and the mechanosensitive channels (*ybi*O) were found in all genomes analysed. Other adaptations to high HP can only be verified at the physiological level, such as the increased proportions of unsaturated fatty acids in the membranes and higher enzyme rates under such conditions [112].

II.4 CONCLUDING REMARKS

We studied the prokaryotic communities in sediments in pockmark and salt diapir fields from the Continental Slope in Santos Basin. Using a combination of metabarcoding and metagenomic approaches associated with phylogenetic tools, we observed that a non-methanotrophic methylotroph from the order Methylomirabilales was widespread across all samples and was the highest-quality MAG retrieved. We explored the metabolic potential of this genome (classified into the family CSP1-5) and described its methanol oxidising capability, along with several genes with the potential to improve the fitness of the organism in the deep-sea environment. The widespread nature of this organism suggests a potential important role of methanol metabolism in this continental slope area. We further argued whether it could contain an overlooked autotrophic CO_2 fixation pathway.

The results also provide evidence that studies based only on metabarcoding may lead to misclassification of the members of the Methylomirabilales, which has profound relevance for the conclusions regarding the roles of the organisms in the environment, as the order contains methanotrophic (Methylomirabilaceae) and non-methanotrophic (CSP1-5) members. Ribosomal sequences misclassified as Methylomirabilaceae were retrieved from various environments, and, therefore, the relevance of methanol metabolism may be neglected in previous studies.

REFERENCES

1. Romans BW, Graham SA. A Deep-Time Perspective of Land-Ocean Linkages in the Sedimentary Record. 2013; 69–94.
2. Smith, S V, Hollibaugh JT. Coastal metabolism and the ocean organic carbon balance. *Rev Geophys* 1993; **31**: 75–89.
3. Bauer JE, Druffel ERM. Ocean margins as a significant source of organic matter to the deep open ocean. *Nature* 1998; **392**: 482–485.
4. Soetaert K, Herman PMJ, Middelburg JJ, Heip C. Assessing organic matter mineralization , degradability and mixing rate in an ocean margin sediment (Northeast Atlantic) by diagenetic modeling. 1998; 519–534.
5. Snelgrove PVR, Soetaert K, Solan M, Thrush S, Wei C, Danovaro R, et al. Global Carbon Cycling on a Heterogeneous Sea fl oor. 2018; **33**.
6. Torsvik TH, Rousse S, Labails C, Smethurst MA. A new scheme for the opening of the South Atlantic Ocean and the dissection of an Aptian salt basin. 2009; 1315–1333.
7. Goffredi SK, Orphan VJ. Bacterial community shifts in taxa and diversity in response to localized organic loading in the deep sea. *Environ Microbiol* 2010; **12**: 344–363.
8. García M, Hernández-molina FJ, Llave E, Stow DA V, León R, Fernández-puga MC. Contourite erosive features caused by the Mediterranean Out fl ow Water in the Gulf of Cadiz : Quaternary tectonic and oceanographic implications. *Mar Geol* 2009; **257**: 24–40.
9. Mahiques MM De, Jens T, Hanebuth J, Nagai RH, Bícego MC, Cesar R, et al. Inorganic and organic geochemical fingerprinting of sediment sources and ocean circulation on a complex continental margin (São Paulo Bight, Brazil). *Ocean Sci* 2017; **13**: 209–222.
10. Hovland M, Gardner J V., Judd AG. The significance of pockmarks to understanding fluid flow processes and geohazards. *Geofluids* 2002; **2**: 127–136.
11. Vaular EN, Barth T, Haflidason H. The geochemical characteristics of the hydrate-bound gases from the Nyegga pockmark field, Norwegian Sea. *Org Geochem* 2010; **41**: 437–444.
12. Nelson; Baraza; Maldonado. Mediterranean undercurrent sandy contourites , Gulf of Cadiz , Spain. *Sediment Geol* 1993; **82**: 103–131.
13. Calder BR, Fonseca L, Francolin JB. Automatic estimation of the seafloor geomorphology of the Santos Basin, Brazil. *Eos, Trans Am Geophys Union* 2002; **83**: F728.
14. Sumida PYG, Yoshinaga MY, Madureira LASP, Hovland M. Seabed pockmarks associated with deepwater corals off SE Brazilian continental slope, Santos Basin. *Mar Geol* 2004; **207**: 159–167.
15. dos Santos R, Nagaoka D, Basti R, Barbosa A, Taniguchi S, Cesar R, et al. Metal / Ca ratios in pockmarks and adjacent sediments on the SW Atlantic slope : Implications for redox potential and modern seepage. *J Geochemical Explor* 2018; **192**: 163–173.
16. Ramos RB, dos Santos RF, Schattner U, Figueira RCL, Bícego MC, Lobo FJ, et al. Deep pockmarks as natural sediment traps: a case study from southern Santos Basin (SW Atlantic upper slope). *Geo-Marine Lett* 2020; **40**: 989–999.
17. Bendia AG, Signori CN, Nakamura FM, Butarelli ACDA, Passos JG, Ramos RB, et al. Microbial perspective on the giant carbonate ridge Alpha Crucis (Southwestern Atlantic upper slope). *FEMS Microbiol Ecol* 2021; **97**: 1–14.
18. Barcellos RL, da Silva Sales de Melo MC, Sial AN, Vaz Manso V do A. Sedimentary Organic Matter Characterization on a Tropical Continental Shelf in Northeastern Brazil. *Int J Geosci* 2020; **11**: 393–419.
19. Ramos RB, Felicio R, Schattner U, Cesar R, Figueira L. Deep pockmarks as natural sediment traps : a case study from southern Santos Basin (SW Atlantic upper slope). *Geo-Marine Lett* 2020; **40**: 989–999.
20. Heikes BG, Chang W, Pilson MEQ, Swift E, Singh HB, Guenther A, et al. Atmospheric methanol budget and ocean implication. *Global Biogeochem Cycles* 2002; **16**: 80-1-80–13.

21. Bartlett R, Bottrell SH, Sinclair K, Thornton S, Fielding ID, Hatfield D. Lithological controls on biological activity and groundwater chemistry in Quaternary sediments. *Hydrol Process* 2010; **24**: 726–735.
22. Dixon JL, Beale R, Nightingale PD. Rapid biological oxidation of methanol in the tropical Atlantic: Significance as a microbial carbon source. *Biogeosciences* 2011; **8**: 2707–2716.
23. Dixon JL, Nightingale PD. Fine-scale variability in methanol uptake and oxidation: From the microlayer to 1000 m. *Biogeosciences* 2012; **9**: 2961–2972.
24. Fall R, Benson AA. Leaf methanol - The simplest natural product from plants. *Trends Plant Sci* 1996; **1**: 296–301.
25. Millet DB, Jacob DJ, Custer TG, De Gouw JA, Goldstein AH, Karl T, et al. New constraints on terrestrial and oceanic sources of atmospheric methanol. *Atmos Chem Phys* 2008; **8**: 6887–6905.
26. Beale R, Dixon JL, Arnold SR, Liss PS, Nightingale PD. Methanol, acetaldehyde, and acetone in the surface waters of the Atlantic Ocean. *J Geophys Res Ocean* 2013; **118**: 5412–5425.
27. Dixon JL, Beale R, Nightingale PD. Microbial methanol uptake in northeast Atlantic waters. *ISME J* 2011; **5**: 704–716.
28. Bates KH, Jacob DJ, Wang S, Hornbrook RS, Apel EC, Kim MJ, et al. The Global Budget of Atmospheric Methanol: New Constraints on Secondary, Oceanic, and Terrestrial Sources. *J Geophys Res Atmos* 2021; **126**: 1–23.
29. Torres-Beltrán M, Vargas-Gastélum L, Magdaleno-Moncayo D, Riquelme M, Herguera-García JC, Prieto-Davó A, et al. The metabolic core of the prokaryotic community from deep-sea sediments of the southern Gulf of Mexico shows different functional signatures between the continental slope and abyssal plain. *PeerJ* 2021; **9**.
30. Hug LA, Thomas BC, Sharon I, Brown CT, Sharma R, Hettich RL, et al. Critical biogeochemical functions in the subsurface are associated with bacteria from new phyla and little studied lineages. *Environ Microbiol* 2016; **18**: 159–173.
31. Mohriak WU. Bacias Sedimentares da Margem Continetal Brasileira. *Geol Tectônica e Recur Minerais do Bras* 2003; 151–165.
32. de Souza LS, Sgarbi GNC. The Santos Basin in Brazil: Geology, exploration and production of oil and natural gas. *Bol Geol* 2019; **41**: 175–195.
33. Dottori M, Dalsenter INB, Napolitano DC, da Silveira ICA. A dataset of temperature and salinity in the South Brazil Bight: Identifying water mass interfaces. *Data Br* 2018; **20**: 1297–1304.
34. Moller OO, Piola AR, Cristina A, Campos EJD, Mo OO. The effects of river discharge and seasonal winds on the shelf off southeastern South America. 2008; **28**: 1607–1624.
35. Sverdrup H, Johnson M, Flaming R. The Oceans Their Physics, Chemistry, and General Biology. https://publishing.cdlib.org/ucpressebooks/view?docId=kt167nb66r;chunk.id=0;doc.view=print. Accessed 23 Jun 2022.
36. Quince et al. 2011. Removing Noise From Pyrosequenced Amplicons. *BMC Bioinformatics* 2011; **12**: 834–839.
37. Parada AE, Needham DM, Fuhrman JA. Every base matters: Assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ Microbiol* 2016; **18**: 1403–1414.
38. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* 2019; **37**: 852–857.
39. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* 2016; **13**: 581–583.
40. McMurdie PJ, Holmes S. Phyloseq: An R Package for Reproducible Interactive Analysis and

Graphics of Microbiome Census Data. *PLoS One* 2013; **8**.
41. Oksanen AJ, Blanchet FG, Friendly M, Kindt R, Legendre P, Mcglinn D, et al. Package ' vegan '. 2013; 0–291.
42. Wickham H. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York. *Media* . 2009.
43. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014; **15**: 550.
44. Anderson MJ. Non-Parametric MANOVA. *Austral Ecol* 2001; 32–46.
45. Eren AM, Esen Ö, Quince C, Vineis J, Sogin M, Delmont T. Anvi'o: An advanced analysis and visualization platform for 'omics data. 2015.
46. Li D, Liu CM, Luo R, Sadakane K, Lam TW. MEGAHIT: An ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics* 2015; **31**: 1674–1676.
47. Alneberg J, Bjarnason BS, de Bruijn I, Schirmer M, Quick J, Ijaz UZ, et al. CONCOCT: Clustering cONtigs on COverage and ComposiTion. 2013; 1–28.
48. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: Assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res* 2015; **25**: 1043–1055.
49. Chaumeil PA, Mussig AJ, Hugenholtz P, Parks DH. GTDB-Tk: A toolkit to classify genomes with the genome taxonomy database. *Bioinformatics* 2020; **36**: 1925–1927.
50. Seemann T. Prokka: Rapid prokaryotic genome annotation. *Bioinformatics* 2014; **30**: 2068–2069.
51. Brettin T, Davis JJ, Disz T, Edwards RA, Gerdes S, Olsen GJ, et al. RASTtk: A modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. *Sci Rep* 2015; **5**.
52. Delmont TO, Eren EM. Linking pangenomes and metagenomes: The Prochlorococcus metapangenome. *PeerJ* 2018; **2018**: 1–23.
53. Kanehisa M, Sato Y, Morishima K. BlastKOALA and GhostKOALA: KEGG Tools for Functional Characterization of Genome and Metagenome Sequences. *J Mol Biol* 2016; **428**: 726–731.
54. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* 2018; **35**: 1547–1549.
55. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties, and weight matrix choice. *Nucleic Acids Res* 1994; **22**: 4673–4680.
56. Stahl DA, De La Torre JR. Physiology and diversity of ammonia-oxidizing archaea. *Annu Rev Microbiol* 2012; **66**: 83–101.
57. Stieglmeier M, Klingl A, Alves RJE, Rittmann SKMR, Melcher M, Leisch N, et al. Nitrososphaera viennensis gen. nov., sp. nov., an aerobic and mesophilic, ammonia-oxidizing archaeon from soil and a member of the archaeal phylum Thaumarchaeota. *Int J Syst Evol Microbiol* 2014; **64**: 2738–2752.
58. Kerou M, Schleper C. Nitrososphaera . *Bergey's Man Syst Archaea Bact* 2016; 1–10.
59. Shubenkova O V., Likhoshvai A V., Kanapatskii TA, Pimenov N V. Microbial community of reduced pockmark sediments (Gdansk Deep, Baltic Sea). *Microbiology* 2010; **79**: 799–808.
60. Haverkamp THA, Hammer Ø, Jakobsen KS. Linking Geology and Microbiology : Inactive Pockmarks Affect Sediment Microbial Community Structure. *PLoS One* 2014; **9**: e85990.
61. Giovannelli D, D'Errico G, Fiorentino F, Fattorini D, Regoli F, Angeletti L, et al. Diversity and distribution of prokaryotes within a shallow-water pockmark field. *Front Microbiol* 2016; **7**: 1–21.
62. Idczak J, Brodecka-goluch A, Katarzyna Ł, Gorska N, Klusek Z, Pezacki PD, et al. Science of

the Total Environment A geophysical, geochemical and microbiological study of a newly discovered pockmark with active gas seepage and submarine groundwater discharge (MET1-BH, central Gulf of Gda ń sk, southern Baltic Sea). *Sci Total Environ* 2020; **742**.

63. He Z, Geng S, Cai C, Liu S, Liu Y, Pan Y, et al. Anaerobic oxidation of methane coupled to nitrite reduction by halophilic marine NC10 bacteria. *Appl Environ Microbiol* 2015; **81**: 5538–5545.
64. Graf JS, Mayr MJ, Marchant HK, Tienken D, Hach PF, Brand A, et al. Bloom of a denitrifying methanotroph, 'Candidatus Methylomirabilis limnetica', in a deep stratified lake. *Environ Microbiol* 2018; **20**: 2598–2614.
65. Wasmund K, Schreiber L, Lloyd KG, Petersen DG, Schramm A, Stepanauskas R, et al. Genome sequencing of a single cell of the widely distributed marine subsurface Dehalococcoidia, phylum Chloroflexi. *ISME J* 2014; **8**: 383–397.
66. Fagervold SK, Watts JEM, May HD, Sowers KR. Sequential reductive dechlorination of meta-chlorinated polychlorinated biphenyl congeners in sediment microcosms by two different Chloroflexi phylotypes. *Appl Environ Microbiol* 2005; **71**: 8085–8090.
67. Andrew DR, Fitak RR, Munguia-Vega A, Racolta A, Martinson VG, Dontsova K. Abiotic factors shape microbial diversity in Sonoran desert soils. *Appl Environ Microbiol* 2012; **78**: 7527–7537.
68. Bohorquez LC, Delgado-Serrano L, López G, Osorio-Forero C, Klepac-Ceraj V, Kolter R, et al. In-depth Characterization via Complementing Culture-Independent Approaches of the Microbial Community in an Acidic Hot Spring of the Colombian Andes. *Microb Ecol* 2012; **63**: 103–115.
69. Schneider D, Arp G, Reimer A, Reitner J, Daniel R. Phylogenetic Analysis of a Microbialite-Forming Microbial Mat from a Hypersaline Lake of the Kiritimati Atoll, Central Pacific. *PLoS One* 2013; **8**.
70. Lage OM, Bondoso J. Planctomycetes and macroalgae, a striking association. *Front Microbiol* 2014; **5**: 1–9.
71. Jackson BE, Bhupathiraju VK, Tanner RS, Woese CR, McInerney MJ. Syntrophus aciditrophicus sp. nov., a new anaerobic bacterium that degrades fatty acids and benzoate in syntrophic association with hydrogen-using microorganisms. *Arch Microbiol* 1999; **171**: 107–114.
72. Haroon MF, Hu S, Shi Y, Imelfort M, Keller J, Hugenholtz P, et al. Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. *Nat 2013 5007464* 2013; **500**: 567–570.
73. Lazar CS, Baker BJ, Seitz K, Hyde AS, Dick GJ, Hinrichs KU, et al. Genomic evidence for distinct carbon substrate preferences and ecological niches of Bathyarchaeota in estuarine sediments. *Environ Microbiol* 2016; **18**: 1200–1211.
74. Evans PN, Parks DH, Chadwick GL, Robbins SJ, Orphan VJ, Golding SD, et al. Methane metabolism in the archaeal phylum Bathyarchaeota revealed by genome-centric metagenomics. *Science* 2015; **350**: 434–8.
75. Edlund A, Hårdeman F, Jansson JK, Sjöling S. Active bacterial community structure along vertical redox gradients in Baltic Sea sediment. *Environ Microbiol* 2008; **10**: 2051–2063.
76. Roalkvam I, Jørgensen SL, Chen Y, Stokke R, Dahle H, Hocking WP, et al. New insight into stratification of anaerobic methanotrophs in cold seep sediments. *FEMS Microbiol Ecol* 2011; **78**: 233–243.
77. Henrichs SM. Early diagenesis of organic matter in marine sediments: progress and perplexity. *Mar Chem* 1992; **39**: 119–149.
78. Arndt S, Jørgensen BB, LaRowe DE, Middelburg JJ, Pancost RD, Regnier P. Quantifying the degradation of organic matter in marine sediments: A review and synthesis. *Earth-Science Rev* 2013; **123**: 53–86.

79. Durbin AM, Teske A. Microbial diversity and stratification of South Pacific abyssal marine sediments. *Environ Microbiol* 2011; **13**: 3219–3234.
80. Jovane L. Metodologias em testemunhos marinhos. 2021.
81. Morono Y, Terada T, Nishizawa M, Ito M, Hillion F, Takahata N, et al. Carbon, and nitrogen assimilation in deep subseafloor microbial cells. *Proc Natl Acad Sci U S A* 2011; **108**: 18295–18300.
82. Jørgensen BB. Deep subseafloor microbial cells on physiological standby. *Proc Natl Acad Sci U S A* 2011; **108**: 18193–18194.
83. Hwang CY, Cho BC. Cucumibacter marinus gen. nov., sp. nov., a marine bacterium in the family Hyphomicrobiaceae. *Int J Syst Evol Microbiol* 2008; **58**: 1591–1597.
84. Xu HY, Chen LP, Fu SZ, Fan HX, Zhou YG, Liu SJ, et al. Zhangella mobilis gen. nov., sp. nov., a new member of the family Hyphomicrobiaceae isolated from coastal seawater. *Int J Syst Evol Microbiol* 2009; **59**: 2297–2301.
85. Pershina E V., Ivanova EA, Korvigo IO, Chirak EL, Sergaliev NH, Abakumov E V., et al. Investigation of the core microbiome in main soil types from the East European plain. *Sci Total Environ* 2018; **631**–**632**: 1421–1430.
86. Osaka T, Yoshie S, Tsuneda S, Hirata A, Iwami N, Inamori Y. Identification of acetate- or methanol-assimilating bacteria under nitrate-reducing conditions by stable-isotope probing. *Microb Ecol* 2006; **52**: 253–266.
87. Kim HS, Srinivasan S, Lee SS. Methyloterrigena soli gen. Nov., sp. nov., a methanol-utilizing bacterium isolated from chloroethylene-contaminated soil. *Int J Syst Evol Microbiol* 2016; **66**: 101–106.
88. Bowers RM, Kyrpides NC, Stepanauskas R, Harmon-Smith M, Doud D, Reddy TBK, et al. Minimum information about a single amplified genome (MISAG) and a metagenome-assembled genome (MIMAG) of bacteria and archaea. *Nat Biotechnol* 2017; **35**: 725–731.
89. Ettwig KF, Butler MK, Paslier D Le, Pelletier E, Mangenot S. Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* 2010; **464**: 543–548.
90. Versantvoort W, Guerrero-Cruz S, Speth DR, Frank J, Gambelli L, Cremers G, et al. Comparative genomics of Candidatus Methylomirabilis species and description of Ca. Methylomirabilis lanthanidiphila. *Front Microbiol* 2018; **9**: 1672.
91. Ettwig KF, Van Alen T, Van De Pas-Schoonen KT, Jetten MSM, Strous M. Enrichment and molecular detection of denitrifying methanotrophic bacteria of the NC10 phylum. *Appl Environ Microbiol* 2009; **75**: 3656–3662.
92. Rouli L, Merhej V, Fournier PE, Raoult D. The bacterial pangenome as a new tool for analysing pathogenic bacteria. *New microbes new Infect* 2015; **7**: 72–85.
93. Vorobev A, Beck DAC, Kalyuzhnaya MG, Lidstrom ME, Chistoserdova L. Comparative transcriptomics in three Methylophilaceae species uncover different strategies for environmental adaptation. *PeerJ* 2013; **2013**.
94. Pol A, Barends TRM, Dietl A, Khadem AF, Eygensteyn J, Jetten MSM, et al. Rare earth metals are essential for methanotrophic life in volcanic mudpots. *Environ Microbiol* 2014; **16**: 255–264.
95. Vu HN, Subuyuj GA, Vijayakumar S, Good NM, Martinez-Gomez NC, Skovran E. Lanthanide-dependent regulation of methanol oxidation systems in Methylobacterium extorquens AM1 and their contribution to methanol growth. *J Bacteriol* 2016; **198**: 1250–1259.
96. Rasigraf O, Kool DM, Jetten MSM, Sinninghe Damsté JS, Ettwig KF. Autotrophic carbon dioxide fixation via the Calvin-Benson-Bassham cycle by the denitrifying methanotroph ‘Candidatus Methylomirabilis oxyfera’. *Appl Environ Microbiol* 2014; **80**: 2451–2460.
97. Dixon JL, Sargeant S, Nightingale PD, Colin Murrell J. Gradients in microbial methanol uptake: Productive coastal upwelling waters to oligotrophic gyres in the Atlantic Ocean. *ISME J* 2013; **7**: 568–580.

98. Dinasquet J, Tiirola M, Azam F. Enrichment of bacterioplankton able to utilize one-carbon and methylated compounds in the coastal Pacific ocean. *Front Mar Sci* 2018; **5**: 1–13.
99. Song GD, Liu SM, Marchant H, Kuypers MMM, Lavik G. Anammox, denitrification and dissimilatory nitrate reduction to ammonium in the East China Sea sediment. *Biogeosciences* 2013; **10**: 6851–6864.
100. Liu P, Ding W, Lai Q, Liu R, Wei Y, Wang L, et al. Physiological and genomic features of Paraoceanicella profunda gen. nov., sp. nov., a novel piezophile isolated from deep seawater of the Mariana Trench. *Microbiologyopen* 2020; **9**: 1–16.
101. Kushkevych I, Cejnar J, Treml J, Dordević D, Kollar P, Vítězová M. Recent Advances in Metabolic Pathways of Sulfate Reduction in Intestinal Bacteria. *Cells* 2020; **9**: 1–16.
102. Wasmund K, Mußmann M, Loy A. The life sulfuric: microbial ecology of sulfur cycling in marine sediments. *Environ Microbiol Rep* 2017; **9**: 323–344.
103. Kuroda A, Ohtake H. Molecular Analysis of Polyphosphate Accumulation in Bacteria. *Biochem* 2000; **65**: 304–308.
104. Paula FS, Chin JP, Schnürer A, Müller B, Manesiotis P, Waters N, et al. The potential for polyphosphate metabolism in Archaea and anaerobic polyphosphate formation in Methanosarcina mazei. *Sci Rep* 2019; **9**.
105. Silveira, I., C., A.; Schmidt, A., C., K.; Campos, E., J., D.; Godoi, S., S.; Ikeda Y. A Corrente do Brasil ao Largo da Costa Leste Brasileira. *Rev Bras Oceanogr* 2000; **48**: 171–183.
106. Jian H, Li S, Tang X, Xiao X. A transcriptome resource for the deep-sea bacterium Shewanella piezotolerans WP3 under cold and high hydrostatic pressure shock stress. *Mar Genomics* 2016; **30**: 87–91.
107. Phadtare S, Alsina J, Inouye M. Cold-shock response and cold-shock proteins. *Curr Opin Microbiol* 1999; **2**: 175–180.
108. Iost I, Bizebard T, Dreyfus M. Functions of DEAD-box proteins in bacteria: Current knowledge and pending questions. *Biochim Biophys Acta - Gene Regul Mech* 2013; **1829**: 866–877.
109. Jiang P, Ren X, Wang W, Niu G, Li J. Arthrobacter terrae sp. nov., a psychrophilic actinobacterium with multi copies of capA gene isolated from Antarctic soil. *Antonie Van Leeuwenhoek* 2022.
110. Roumagnac M, Pradel N, Bartoli M, Garel M, Jones AA, Armougom F, et al. Responses to the Hydrostatic Pressure of Surface and Subsurface Strains of Pseudothermotoga elfii Revealing the Piezophilic Nature of the Strain Originating From an Oil-Producing Well. *Front Microbiol* 2020; **11**.
111. Kerou M, Ponce-Toledo RI, Zhao R, Abby SS, Hirai M, Nomaki H, et al. Genomes of Thaumarchaeota from deep sea sediments reveal specific adaptations of three independently evolved lineages. *ISME J* 2021; **15**: 2792–2808.
112. Roumagnac M, Pradel N, Bartoli M, Garel M, Jones AA, Armougom F, et al. Responses to the Hydrostatic Pressure of Surface and Subsurface Strains of Pseudothermotoga elfii Revealing the Piezophilic Nature of the Strain Originating From an Oil-Producing Well. *Front Microbiol* 2020; **11**.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS

Bioinformatics and statistical tools used to analyse 16S rRNA gene sequences After sequencing, paired-end reads were initially imported and demultiplexed into the Qiime2 software version 2019.10 (BOLYEN et al., 2019). Graphic inspection of quality profiles was performed, low-quality reads (below Phred score 30) were trimmed, and the chimeras were removed with the aid of the Dada2 software (CALLAHAN et al., 2016). After quality control, the amplicon sequence variants (ASVs) were determined using the Dada2 software (CALLAHAN et al., 2016) in the Qiime2 package. Taxonomy was assigned through feature-classifier classify-sklearn and SILVA database v. 138. ASV richness, Chao1, and Shannon diversity indexes were calculated using phyloseq (MCMURDIE; HOLMES, 2013) and vegan packages (OKSANEN et al., 2013), and ggplot2 (WICKHAM, 2009) was used for graphing in R v. 4.1.0 (R Development Team, 2018). ASVs were normalised by varianceStabilizingTransformation, using the R package "DESeq2" (LOVE; HUBER; ANDERS, 2014). Similarities among samples and site groups were examined using ordinated weighted Unifrac normalised distance and visualised by non-metric multidimensional scaling (MDS). Alpha diversity, statistical analysis, and data visualisation were carried out in R using the Phyloseq package (MCMURDIE; HOLMES, 2013). Differences in the microbial communities among sites and depths were assessed by permutational multivariate analysis of variance (PERMANOVA) (ANDERSON, 2001).

Supplementary Table 1. Sampling stations studied at Santos Basin continental slope and sample metadata.

Sample ID	Strata (cm) [a]	Latitude	Longitude	Depth (m) [b]	Site [c]
Surface_255	0-2	26°29.677'S	45°58.153'W	652	Control
Subsurface_255	22-24	26°29.677'S	45°58.153'W	652	Control
Surface_258	0-2	26°15.715'S	45°40.97'W	543	Control
Subsurface_258	22-24	26°15.715'S	45°40.97'W	543	Control
Surface_250	0-2	26°49.590'S	46°24.27'W	433	Diapir
Subsurface_250	16-18	26°49.590'S	46°24.27'W	433	Diapir
Surface_259	0-2	26°33.776'S	46°07.008'W	559	Diapir
Subsurface_259	22-24	26°33.776'S	46°07.008'W	559	Diapir
Surface_260	0-2	26°33.710'S	46°07.470'W	517	Diapir
Subsurface_260	22-24	26°33.710'S	46°07.470'W	517	Diapir
Surface_253	0-2	26°14.72'S	45°40.97'W	730	Pockmark
Subsurface_253	22-24	26°14.72'S	45°40.97'W	730	Pockmark
Surface_254	0-2	26°15.715'S	45°42.971'W	747	Pockmark
Subsurface_254	22-24	26°15.715'S	45°42.971'W	747	Pockmark
Surface_257	0-2	26°32.075'S	46°04.822'W	698	Pockmark
Subsurface_257	22-24	26°32.075'S	46°04.822'W	698	Pockmark

[a] Sediment stratum (depth) selected from the core of sediment collected. Surface: 0-2 cm; Subsurface: 16-18 or 22-24 cm

[b] Water column depth

[c] Sampling site geomorphological feature

Supplenentary Table 2. Richness and diversity of prokaryotes in surface and subsurface sediment samples.

Sample ID	No. of Sequences	Observed ASV	Chao1	Shannon
Control Surface				
Surface_255	25.853	744	776.354	6.136
Surface_258	49.839	1.094	1214.839	6.449
Sum	*75692*	*1.838*	*1991.193*	*12.585*
Mean ± SD	*37.846 ± 11.99*	*919 ± 175*	*995.59 ± 219.24*	*6.292 ± 0.156*
Control Subsurface				
Subsurface_255	39.456	1.176	1320.460	6.574
Subsurface _258	64.296	816	932.575	5.632
Sum	*103.752*	*1.992*	*3.277.522*	*12.206*
Mean ± SD	*51.976 ± 12.42*	*996 ± 180*	*1321.393 ± 659.76*	*6.103 ± 0.471*
Diapir Surface				
Surface_250	48.861	1.181	1288.013	6.548
Surface_259	38.970	921	971.281	6.247
Surface_260	36.265	999	1079.577	6.419
Sum	*124.096*	*3.101*	*3338.871*	*19.214*
Mean ± SD	*38.97 ± 5.41*	*999 ± 108.93*	*1079.577 ± 131.44*	*6.419 ± 0.123*
Diapir Subsurface				
Subsurface _250	42.091	1193	1348.472	6.537
Subsurface _259	32.897	917	992.607	6.224
Subsurface _260	38.272	721	790.538	5.785
Sum	*113.260*	*2831*	*3131617*	*18.546*
Mean ± SD	*38.272 ± 3.77*	*917 ± 193.64*	*992.607 ± 230.64*	*6.22 ± 0.308*
Pockmark Surface				
Surface_253	23.675	708	724.5541	6.158
Surface_254	6.972	292	293.500	5.292
Surface_257	38.816	932	1001.227	6.262
Sum	*69.463*	*1932*	*1294.727*	*11.554*
Mean ± SD	*23.67 ± 13.0*	*708 ± 265.16*	*1001.227 ± 3123.8*	*6.15 ± 0.434*
Pockmark Subsurface				
Subsurface _253	28.379	952	724.554	6.158
Subsurface _254	20.535	822	844.557	6.299
Subsurface _257	38.673	1.186	1295.572	6.556
Sum	*87.587*	*2.960*	*2864.683*	*19.013*
Mean ± SD	*28.379 ± 7.427*	*952 ± 150.61*	*844.557 ± 245.82*	*6.299 ± 0.164*

Supplementary Tables 3. Two-way ANOVA statistics evaluating the effects of strata and site on Chao1 and Shannon diversity indexes.

CHAO1	**SS**	**df**	**MS**	**F**	**P**
(S) Surface:Subsurface	52896.8	1	52896.8	0.7	0.4206
(L) Control:Diapir:Pockmark	250570.95	2	125285.48	1.67	0.2326
S x L	84649.68	2	42324.84	0.56	0.5867
Error	825423.66	11	75038.51		
Total	1213541.09	16			
SHANNON	**SS**	**df**	**MS**	**F**	**P**
(S) Surface:Subsurface	0.01	1	0.01	0.07	0.7962
(L) Control:Diapir:Pockmark	0.15	2	0.08	0.5	0.6197
S x L	0.5	2	0.25	1.67	0.2326
Error	1.65	11	0.15		
Total	2.31	1			

Site= Control, Diapir and Pockmark. Strata= Surface (0-2cm) and Subsurface (16-18cm or 22-24cm).

Supplementary Table 4. Permutational analysis of variance (PERMANOVA) evaluating the effects of site and strata on community composition.

Factor	Df	SumsOfSqs	MeanSqs	F.Model	R^2	P value
Site	2	0.6665	0.33324	1.1750	0.11858	0.231
Strata	1	1.5204	1.52044	5.3611	0.27052	0.001
Site:Strata	2	0.5975	0.29875	1.0534	0.10631	0.360
Residuals	10	2.8361	0.28361	0.50459	0.35164	
Total	15	5.6205			1.00000	

Site= Control, Diapir and Pockmark. Strata= Surface (0-2cm) and Subsurface (16-18cm or 22-24cm).

Supplementary Table 5. Core Microbiome analysis – list of ASVs shared by all samples (central), only between surface samples, and only between subsurface samples.

ASV_ID	Domain	Phyla	Class	Order	Family
Central Core Microbiome					
88ece184badcd461a1c452b9168238dd	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae
d42457886a830872cbbecf93f40e7b7d	Bacteria	Methylomirabilota	Methylomirabilia	Methylomirabilales	Methylomirabilaceae
Surface Core Microbiome					
04013e82bfa1a8727a8d9230ad85f757	Bacteria	Proteobacteria	Alphaproteobacteria	Kiloniellales	Kiloniellaceae
0498ad663b7f79af564bbd917becf6a4	Archaea	Crenarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae
07f4ec67a67b6e689a8b1f3bc7043e83	Bacteria	Proteobacteria	Alphaproteobacteria	uncultured	uncultured
0b678d1f0f13f61e6383bb6e0948c798	Bacteria	Dadabacteria	Dadabacteriia	Dadabacteriales	Dadabacteriales
10b767aa94756f5887aeb2cb7fc9f2f4	Bacteria	Gemmatimonadota	PAUC43f	PAUC43f	PAUC43f
140a6b70a72fcedca42dad6d25f5fd98	Bacteria	Proteobacteria	Gammaproteobacteria	AT-s2-59	AT-s2-59
1c1edb73165695a5bd9ca622dc2b711f	Archaea	Crenarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae
1d1d67547136a33fcdfcd306f5702f6e	Bacteria	Proteobacteria	Alphaproteobacteria	Kiloniellales	Kiloniellaceae
20c3f85fb8a8652ec8b1330d433aed32	Bacteria	Proteobacteria	Alphaproteobacteria	Kiloniellales	Kiloniellaceae
21ace7371bd6993fc047f6187e925dbf	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methyloligellaceae
25733a39b1dfa679b2d0712dff87f258	Bacteria	Proteobacteria	Gammaproteobacteria	MBMPE27	MBMPE27
2b78b7292871cab7dd6e4ab1518ef6a3	Bacteria	NB1-j	NB1-j	NB1-j	NB1-j
2d2d4c698a867a06755afc4dce823452	Bacteria	Gemmatimonadota	BD2-11	BD2-11	BD2-11
2d6449d9ef1747714ad9b8242947215b	Bacteria	Proteobacteria	Gammaproteobacteria	MBMPE27	MBMPE27
2e48c01cef7f4daf8c36285a101c0197	Bacteria	Proteobacteria	Alphaproteobacteria	-	-
2f898363289df017eb456cd4434e976e	Bacteria	Dadabacteria	Dadabacteriia	Dadabacteriales	Dadabacteriales
30d230e4e00057dfdf4adde04627529d	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Nitrincolaceae
33885eacf84739fdf59109d9a30c4143	Bacteria	Proteobacteria	Alphaproteobacteria	-	-
33df8b69f75f0e4b605028dbfc54ea96	Archaea	Crenarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae
34bb86368caea06b45a8f23fea1dbc5c	Archaea	Crenarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae
3f1bc0782561cd249c71637d97a7738f	Archaea	Crenarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae
41a20373352d33616e961818dbb179e5	Bacteria	Proteobacteria	Gammaproteobacteria	Nitrosococcales	Nitrosococcaceae
4436051b8caa9b2f2ea3bdacd1afdeb9	Bacteria	Proteobacteria	Gammaproteobacteria	Steroidobacterales	Woeseiaceae
45ff6775638a7dc8be1a5966055555d2	Archaea	Crenarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae
471f8559df7c30dbc2b2d0a0aeed68ed	Bacteria	Nitrospirota	Nitrospiria	Nitrospirales	Nitrospiraceae
4c16b55ededa3ba039f6662cc72e63f6	Bacteria	Proteobacteria	Alphaproteobacteria	-	-

Supplementary Table 5 – continuation

ASV_ID	Domain	Phyla	Class	Order	Family
502c02860f623e5ff8795422903cbfde	Bacteria	Acidobacteriota	Vicinamibacteria	Vicinamibacterales	uncultured
56bbc504d74551cd72635d16d0bac851	Bacteria	Proteobacteria	Alphaproteobacteria	Kiloniellales	Kiloniellaceae
57c59f2ccbca5b03a1e04670dd8f0010	Archaea	Crenarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae
5a304aed857826a705f0a14cefb2db9f	Bacteria	Proteobacteria	Gammaproteobacteria	AT-s2-59	AT-s2-59
63e33a7d7b5a21a9df845a4a05847bc9	Bacteria	Gemmatimonadota	BD2	BD2	BD2
65e42fc434451815d377d184a77fe1c7	Bacteria	Proteobacteria	Gammaproteobacteria	Steroidobacterales	Woeseiaceae
68c9a3dacdbfd3b63b79cb29e6673b4b	Bacteria	Proteobacteria	Gammaproteobacteria	Steroidobacterales	Woeseiaceae
6c8fe3f5b0b240007f0ffea2faeaae73	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	KF-JG30-B3
6fd0f2ed0e237e81dcadcbab72284bde	Bacteria	Proteobacteria	Alphaproteobacteria	Kiloniellales	Kiloniellaceae
702e8b2107edfe1dbe47dba8bc5d185c	Bacteria	Proteobacteria	Gammaproteobacteria	Gammaproteobacteria	Unknown
7079b0bac05957134fe21be3636f405c	Bacteria	-	-	-	-
739d21ef1089dd8495682f60f7727ed9	Bacteria	Bacteroidota	Bacteroidia	Cytophagales	Cyclobacteriaceae
73a76d201ec82c666a150f6cfb7473c4	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Shewanellaceae
7485b488409ea7697e23e79766acdd86	Bacteria	Proteobacteria	Gammaproteobacteria	EPR3968-O8a-Bc78	EPR3968-O8a-Bc78
77701520d52299d7f15e2871502f1664	Bacteria	Planctomycetota	Pla3_lineage	Pla3_lineage	Pla3_lineage
7cac67fe6bbff3aeb2194c826f91f565	Bacteria	Proteobacteria	Alphaproteobacteria	-	-
7dcf93ffa46f9927ef6df7ab89a09613	Bacteria	Nitrospirota	Nitrospiria	Nitrospirales	Nitrospiraceae
7e7d25eb23ad32f12e195b377e4b37ba	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Nitrosomonadaceae
83365de7a4bd74406238426c3e5d34d2	Bacteria	Proteobacteria	Gammaproteobacteria	Nitrosococcales	Nitrosococcaceae
850e720604e35b0e152d821aa5391dc0	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methyloligellaceae
8816d7893041e98f9cad4704883a48d8	Bacteria	Planctomycetota	Planctomycetes	Pirellulales	Pirellulaceae
8c504df61b282e1fa88491325c5e00d8	Bacteria	-	-	-	-
8cfb8c64de2d14262e2b7ba2da2b683b	Bacteria	Proteobacteria	Alphaproteobacteria	Kiloniellales	Kiloniellaceae
8f4c1632a796a6609c6368164542e423	Bacteria	Proteobacteria	Alphaproteobacteria	Kiloniellales	Kiloniellaceae
8f6ed589c9351e9b5f6b0ffe9e7c100e	Bacteria	Proteobacteria	Alphaproteobacteria	Defluviicoccales	uncultured
9843e7cd10cfbdd58b222aa2b44c6e37	Bacteria	Nitrospirota	Nitrospiria	Nitrospirales	Nitrospiraceae
9a1c885c36a0ece622a7f8f92c07cd7d	Bacteria	Acidobacteriota	AT-s3-28	AT-s3-28	AT-s3-28
9cb32e4ce15398ecb5d3c94f335eb296	Bacteria	NB1-j	NB1-j	NB1-j	NB1-j
9e5dcea274a19a7008d2265efff2923a	Bacteria	Proteobacteria	Gammaproteobacteria	EPR3968-O8a-Bc78	EPR3968-O8a-Bc78
a8c2c0f8a2812df378266e804e702aa9	Bacteria	Proteobacteria	Gammaproteobacteria	Gammaproteobacteria	Unknown
aa834f0722681701e21903fa1b9329bb	Bacteria	Proteobacteria	Alphaproteobacteria	Kiloniellales	Kiloniellaceae

Supplementary Table 5 – continuation

ASV_ID	Domain	Phyla	Class	Order	Family
ab418e07af33521ba183bb4b836b5168	Archaea	Crenarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae
ab8701fe9bc69d2545f1d1841be43ca0	Bacteria	Proteobacteria	Gammaproteobacteria	Nitrosococcales	Nitrosococcaceae
add9c0f8a87fa265a195397a8d0d58b6	Archaea	Crenarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae
af562fbdd67ae04b1ae167190232b522	Bacteria	Proteobacteria	Alphaproteobacteria	-	-
b1cf9092b41e5db7ba632d91fd070bd8	Bacteria	Proteobacteria	Gammaproteobacteria	Gammaproteobacteria	Unknown
b713c9afb89b316e229a19441dff4d1b	Bacteria	Gemmatimonadota	PAUC43f	PAUC43f	PAUC43f
b7f91f8b2b617fadf4d321ca6eaf0313	Bacteria	NB1-j	NB1-j	NB1-j	NB1-j
b8748189b9c61925bb5f0eddb7b3e734	Bacteria	Gemmatimonadota	PAUC43f	PAUC43f	PAUC43f
bdc959f8622c41e5649e80f6989a239a	Archaea	Crenarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae
c1dd3fba8751fc4558951c6225bf78a9	Bacteria	Gemmatimonadota	BD2-11	BD2-11	BD2-11
c3e3dbe5247b1352b53856ff86aac1c1	Bacteria	Proteobacteria	Gammaproteobacteria	MBMPE27	MBMPE27
c3eefd9de45c9afc27757e0931c955b1	Archaea	Crenarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae
c67a44e7c182d82abd4d193f45a00d5c	Bacteria	Acidobacteriota	Subgroup_22	Subgroup_22	Subgroup_22
c8d97f24b030206f07d1c54b2a631393	Bacteria	NB1-j	NB1-j	NB1-j	NB1-j
d85f37f01b54e59aaa9b5194385dd433	Bacteria	Myxococcota	bacteriap25	bacteriap25	bacteriap25
dbefeb8a83c0e0cc2b9f8490f851e228	Bacteria	Proteobacteria	Gammaproteobacteria	MBMPE27	MBMPE27
e01197bd4113e43decca0c3663e67f43	Bacteria	Bacteroidota	Bacteroidia	Cytophagales	Cyclobacteriaceae
e5f6e798a12918409bf6fc6ae3bc509e	Archaea	Crenarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae
e6a468f5c87528890754b9440ce9c39b	Bacteria	Bacteroidota	Bacteroidia	Cytophagales	Cyclobacteriaceae
e7b305d64864e6b6342640ef7cd475c4	Bacteria	NB1-j	NB1-j	NB1-j	NB1-j
ed3e7227f2817d733a9c26b6a87af048	Bacteria	Proteobacteria	Alphaproteobacteria	Kiloniellales	Kiloniellaceae
ef63eb3f3f5a1749e2025c173dd78b1c	Bacteria	Planctomycetota	Planctomycetes	Pirellulales	Pirellulaceae
f33200e3502fdd17e126d1333fb55140	Bacteria	Bacteroidota	Bacteroidia	Flavobacteriales	Crocinitomicaceae
Subsurface Core Microbiome					
88ece184badcd461a1c452b9168238dd	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae
d42457886a830872cbbecf93f40e7b7d	Bacteria	Methylomirabilota	Methylomirabilia	Methylomirabilales	Methylomirabilaceae

Supplementary Table 6. List of MAGs recovered from the sediment samples, their taxonomic classification and quality parameters.

MAGs	Completeness (%)	Contamination (%)	Contig no.	CG %	N50	Total length	GTDB-TK
SB_MAG_00001	94.244	2.158	403	58.583	7968	2397160	Bacteria; Methylomirabilota; Methylomirabilis, CPS1-5; CPS1-5
SB_MAG_00002	90.647	2.877	323	57.953	9268	2379090	Bacteria; Nitrospirota; Nitrospiria; Nitrospirales; Nitrospiraceae
SB_MAG_00003	83.453	1.438	520	44.422	3070	1518180	Bacteria; Proteobacteria; Gammaproteobacteria; Burkholderiales; Nitrosomonadaceae; GCA2721545
SB_MAG_00004	82.014	2.158	928	57.198	3165	2823452	Bacteria; Proteobacteria; Gammaproteobacteria; Burkholderiales; Nitrosomonadaceae; GCA2721545
SB_MAG_00005	76.978	2.158	2.328	54.233	2573	5918495	Bacteria; Proteobacteria; Gammaproteobacteria; GCA-2729495; GCA-2729495; GCA2729495
SB_MAG_00006	72.661	0.719	1049	63.004	3646	3537226	Bacteria; Proteobacteria; Gammaproteobacteria; HK1
SB_MAG_00007	71.942	2.877	515	40.507	2826	3537226	Bacteria; Proteobacteria; Gammaproteobacteria; UBA4486; UBA4486
SB_MAG_00008	71.942	2.877	2024	53.166	2000	4170056	Bacteria; Binatota; Binatia; UBA9968
SB_MAG_00009	66.906	2.158	1984	62.711	2381	4744342	Bacteria; Proteobacteria; Gammaproteobacteria; HK1
SB_MAG_00010	63309	2.158	841	66363	1957	1698391	Bacteria; Proteobacteria; Alphaproteobacteria;Kiloniellales
SB_MAG_00011	60.431	2.158	2299	57.615	1864	4436461	Bacteria; Proteobacteria; Gammaproteobacteria;Woeseiales; Woeseiaceae;
SB_MAG_00012	58.992	1.438	963	66.436	2056	2034755	Bacteria; Proteobacteria; Alphaproteobacteria; Kiloniellales;Kiloniellaceae;
SB_MAG_00013	57.554	0.000	455	34.752	2205	1040817	Bacteria; Dadabacteria; UBA1144
SB_MAG_00014	53.956	1.438	3617	59.202	1889	7172570	Bacteria; Tectomicrobia; Entotheonellia; Entotheonellales
SB_MAG_00015	50.617	2.469	235	34.209	1951	475213	Archaea; Crenarchaeota;Nitrososphaeria;Nitrososphaerales; Nitrosopumilaceae;
SB_MAG_00016	51.234	3.703	336	33.915	2171	751328	Archaea; Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae
SB_MAG_00017	50.000	2.469	172	33.744	3972	608712	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae;CSP1-1
SB_MAG_00018	50.000	4.321	370	33.771	2163	819900	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae
SB_MAG_00019	46.762	2.158	2042	66.128	1817	3842992	Bacteria;Myxococcota;UBA9160
SB_MAG_00020	46.043	2.877	638	66.476	1975	1299393	Bacteria;Proteobacteria;Alphaproteobacteria;Kiloniellales
SB_MAG_00021	43.884	1.438	691	48.608	1851	1322597	Bacteria; Nitrospirota;Nitrospiria;Nitrospirales;UBA8639;UBA8639
SB_MAG_00022	43.827	3.086	246	34.212	2190	557612	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae
SB_MAG_00023	40.287	1.438	1466	65.685	1804	2742135	Bacteria;Proteobacteria;Alphaproteobacteria;UBA6615
SB_MAG_00024	40.123	1.851	128	34.001	3182	379099	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae;CSP1-1
SB_MAG_00025	40.123	1.851	261	33.521	2319	615250	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae;CSP1-1
SB_MAG_00026	38.848	0.719	608	56.672	2073	1308546	Bacteria;Proteobacteria;Gammaproteobacteria;Woeseiales;Woeseiaceae;SZUA-117
SB_MAG_00027	40.123	3.703	411	33.581	2092	892541	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae
SB_MAG_00028	40.123	4.938	377	34.542	1974	767898	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae
SB_MAG_00029	34.532	0.000	569	53.863	2121	1243523	Bacteria; Proteobacteria; Gammaproteobacteria; Acidiferrobacterales; SPGG2; SPGG2;

SB_MAG_00030	37.654	3.703	341	33.619	1973	704382	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae
SB_MAG_00031	35.802	3.703	214	33.417	2826	579426	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae;CSP1-1

Supplementary Table 6 – continuation

MAGs	Completeness (%)	Contamination (%)	Contig no.	CG %	N50	Total length	GTDB-TK
SB_MAG_00032	30.246	1.234	184	33.889	2364	478985	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae;CSP1-1
SB_MAG_00033	30.246	1.851	120	34.555	3043	346885	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae
SB_MAG_00034	30.864	6.172	382	33.449	2081	814736	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae

Supplementary Table 7. Information about sequences was taken from the Silva v.138 database and used to build the phylogenetic tree.

Access number	Isolation source	Location information	Alignment Identity	DOI
AF317743	Cave-freshwater samples	Nullarbor region of Australia	100%	DOI: 10.1046/j.1462-2920.2001.00187.x
AM991193	Karst spring water - groundwater	Switzerland:Yverdon-les-Bains	87.82%	Unpublished
DQ906791	Subsurface soil	Oman	87.91%	Unpublished
EU335141	Saturated C horizon soil aggregate - Soil	Melton Branch Watershed, Oak Ridge, TN.	94.83%	DOI: 10.1128/AEM.01787-07
EU335144	Saturated C horizon soil aggregate	Melton Branch Watershed, Oak Ridge, TN.	87.66%	DOI: 10.1128/AEM.01787-07
EU491346	seafloor lavas from Hawai'i South Point X3	East Pacific Rise (EPR)	95.14%	DOI: 10.1038/nature06899
EU491462	Seafloor lavas from Hawai'i South Point X3	East Pacific Rise (EPR)	94.04%	DOI: 10.1038/nature06899
FJ205359	Deep marine sediments, depth:2725m	Pacific Ocean: Lau Basin	94.04%	Unpublished
GU363024	marine sediment from the South China Sea	South China Sea	97.11%	Unpublished
HM186756	Saturated zone of the Hanford Site 300 Area subsurface - River sediment	Hanford Site 300 Area near Richland, Washington state (USA)	93.79%	DOI: 10.1111 / j.1462-2920.2011.02659.x
HM186887	Saturated zone of the Hanford Site 300 Area subsurface - River sediment	Washington - EUA	97.13%	DOI: 10.1111 / j.1462-2920.2011.02659.x
HM186966	Saturated zone of the Hanford Site 300 Area subsurface - River sediment	Washington - EUA	94.29%	DOI: 10.1111 / j.1462-2920.2011.02659.x
HM187212	Saturated zone of the Hanford Site 300 Area subsurface - River sediment	Washington - EUA	87.88%	DOI: 10.1111 / j.1462-2920.2011.02659.x
HM187376	Saturated zone of the Hanford Site 300 Area subsurface - River sediment	Washington - EUA	91.11%	DOI: 10.1111 / j.1462-2920.2011.02659.x
JF265987	White microbial mat from lava tube wall - Cave	Portugal: Gruta da Malha, Terceira, Azores	94.90 %	DOI: 10.1089 / ast.2010.0562
JF747701	Underwater Cave - hypersaline groundwater	Dominican Republic - Manantial del Toro	95.14 %	Unpublished
JF747703	Underwater Cave - hypersaline groundwater	Dominican Republic - Manantial del Toro	94.82 %	Unpublished
JF809696	Hypersaline basin - Deep-Sea Hypersaline Sediments	Medea - Mediterranean Sea	94.23 %	DOI: 10.1264 / jsme2.ME12045
JN229988	Subseafloor sediment deeply buried coral carbonates and sediments	Porcupine Seabight – Atlantic Ocean	95.52 %	Unpublished
JN615730	Tan microbial mat from lava tube wall - Cave	Gruta da Malha, Terceira, Azores - Portugal	93.97 %	Unpublished
JN701089	Yellow microbial mat from lava tube wall- Cave	Gruta Madre de Deus Terceira, Azores - Portugal	97.83 %	Unpublished
JN886867	Carbonate sediments	South West Indian Ridge	94.16 %	Unpublished
JQ425959	saline Soil layer (0-10 cm)	Old Texcoco lake - Mexico	93.88 %	Unpublished

Supplementary Table 7 – continuation

Access number	Isolation source	Location information	Alignment Identity	DOI
JX222001	Subsurface aquifer sediment	USA:Rifle - Colorado	93.31 %	DOI: 10.1111/j.1574-6941.2012.01363.x
JX227602	Sediment collected from station WS0902 deep-sea polymetallic nodules and the surrounding sediments	Clarion-Clipperton Fracture Zone – Pacific Ocean	95.34%	DOI: 10.1016 / j.dsr.2013.05.004
KM071663	Deep-sea hydrothermal vent sediments	East Pacific Ocean	93.38 %	Unpublished
KM454246	Marine sediment	Maluku Strait - Indonesian	96.83 %	Unpublished
KT223295	Slope sediment - a depth of 1250 m	Orca Basin - Gulf of Mexico	89.91 %	DOI: 10.30564 / jasr.v2i2.930
KT223298	Slope sediment	Orca Basin - Gulf of Mexico	91.25%	Unpublished
KT748567	Seafloor basalt (3 km depth)	Dorado Outcrop, East Pacific Rise	93.19 %	DOI: 10.3389/fmicb.2015.01470
KX172753	marine Sediment	Gulf of Mexico	97.71 %	DOI: 10.3389/fmicb.2016.01384
KY609420	Fe-Si-rich low-temperature hydrothermal precipitates	Lau Basin - Australia-Pacific	91.07 %	DOI: 10.1111 / j.1574-6941.2012.01367.x
LDXP01000044	Sediment at 5m depth aquifer adjacent to the Colorado River	Rifle, Colorado - USA	84.87 %	DOI: 10.1111 / 1462-2920.12930
LXTG01000049	Sediment sample at the water-sediment interface Oxic Deep-Sea Sediments	Central gyre - Pacific Ocean	92.81 %	DOI: 10.1128/AEM.01023-16.

CHAPTER III - MICROBIAL DIVERSITY AND HALOPHILIC ENRICHMENTS OF A POCKMARKS FIELD'S SEDIMENT FROM SANTOS BASIN CONTINENTAL SLOPE

Abstract

The Santos Basin, in the Southwest Atlantic, comprises a region of shelf break, marked by the presence of numerous geomorphological features and irregularities in the ocean floor, caused mainly by the movement of large pockets of salt in the subsurface. Large volumes of salt can be exhumed from the ocean floor, forming large salt mounds called salt diapirs. This movement of salt towards the surface can weaken and cause cracks in the rocky layers of the oceanic subsurface, favoring the formation of other features such as pockmarks. In the Santos Basin we found the co-occurrence of these two geomorphological features (salt diapirs and pockmarks), offering us a unique opportunity to explore the microbial communities of this region. For this study, surface sediment samples were collected at five previously established stations: two stations in saline diapirs, two stations for seafloor sampling in the region (control) and one station in a pockmark. The depths of the stations ranged from approximately 300 to 800 meters. Part of the collected sediment was used to carry out the enrichment cultures with HM medium (25% NaCl) and later we performed the sequencing of the 16S rRNA gene of the enriched cultures together with the environmental sediment using universal primers 515F and 926R on the Illumina Miseq platform. We obtained 400,765 quality-filtered reads that were identified in 3,199 amplicon sequence variants (ASVs), of which 2,850 were assigned to the Bacteria Domain and only 348 to the Archaea Domain. We observed high taxonomic diversity in environmental sediment samples when compared to enriched culture samples. The sediment samples were similar in taxonomic composition, all dominated by archaea of the Nitrosopumilaceae family, with relative abundances ranging from 19% to 25%. In the enriched samples, we observed a decrease in the abundance and diversity of taxonomic groups, in which the especially moderate halophilic groups stood out and obtained high relative abundance. Families such as Idiomarinaceae represented 10% to 85% of the identified communities, followed by families such as Halomonadaceae and Marinobacteriaceae. After enrichment of the sediment samples, the cultures underwent the process of isolation of microorganisms, in which 22 isolates belonging to two distinct families of bacteria were obtained: Halomonadaceae and Salinisphaeraceae, both belonging to the Gammaproteobacteria class. The present work is a pioneer in the collection of information on halophilic microorganisms in areas of salt diapirs and pockmarks in the Brazilian continental margin. Here we describe the great diversity of microorganisms in the marine surface sediment, as well as the isolation and identification of little described halophiles on the seafloor.

Keywords: Halophilic; Microbial ecology; Deep Sea; Salt Diapirs; Pockmark; Southwest Atlantic

III.1 INTRODUCTION

Marine sediments cover up to 70% of the planet's surface and microbial life in this region contributes substantially to global biogeochemical cycles and is a key component of the entire terrestrial system (DELONG, 1997; SCHRENK; HUBER; EDWARDS, 2010; BOWLES et al., 2014). Sediment microbial communities can occupy aerobic and anaerobic niches with a low concentration of organic matter and with the most diverse conditions of temperature, pressure, and salinity (SEYMOUR, 2014; SALAZAR; SUNAGAWA, 2017; MAPELLI et al., 2022). However, the taxonomic diversity in the marine sedimentary environment and the spatial distribution of these communities are not fully known, especially at great depths where access to these samples becomes a challenge (PEDRÓS-ALIÓ, 2006; SALAZAR; SUNAGAWA, 2017).

The continental slope presents the steepest portion between the continent and the ocean basin, being the main means of transporting sediment from the shelf to the abyssal plain. This region is strongly influenced by physical and biological processes, and the dominant source of organic matter comes from the sedimentation of particulate organic carbon from the photic zone (CACCHIONE; PRATSON; OGSTON, 2002; DE ALMEIDA; KOWSMANN, 2015).

In the Santos Basin (SB), in the Southwest Atlantic, this shelf-break region is marked by the presence of numerous geological features and irregularities in the ocean floor, mainly caused by the movement of large pockets of salt in the subsurface (HUDEC; JACKSON, 2007; PALOMINO et al., 2016). The movement of salt is called halocygenesis, and on continental margins of the Atlantic type, salt tends to move vertically, favouring the formation of saline diapirs (MOHRIAK, 2003; MOHRIAK, 2003). Diapirs are large salt reservoirs, that when exposed to the sea surface cause changes in bathymetric relief and interact with the regional bottom current, in addition to making the surrounding sediment excessively saline (DAVISON et al., 2000; STEWART, 2006; DE MAHIQUES et al., 2017; COLEMAN et al., 2018).

The extensional faults generated by the movement of salt are also related to the formation of crater-shaped features called pockmarks. These features are generated by the sudden expulsion of large volumes of gas and/or fluids (most often methane gas) that is facilitated by the weakening of the plaques caused by halocygenesis (DE MAHIQUES et al., 2017; STROZYK et al., 2018).

In SB we found the co-occurrence of saline diapirs and pockmarks. However, without reports of active gas escape in the craters (CALDER; FONSECA; FRANCOLIN, 2002; SUMIDA et al., 2004). Despite being an economically very important area for the country and

being under the great influence of salt, there are no studies that show the presence of microorganisms adapted to these conditions in the study area.

Halophilic (salt-loving) organisms can be found in the three Domains of life: Eukarya, Bacteria, and Archaea. However, most are composed of prokaryotic microbial life, mainly bacteria (OREN, 1999; QUILLAGUAMÁN et al., 2010; SANTOS et al., 2012; DASSARMA; DASSARMA, 2015). Halophilic microorganisms constitute the natural communities of saline ecosystems. These environments are globally distributed and show wide variations in salt concentration (OREN, 2008; SETATI, 2010; ANDREI; BANCIU; OREN, 2012). Saline environments are selection factors for microbial communities since high salt concentrations cause a cellular osmotic imbalance in organisms that are not adapted to this condition (OREN, 1999; OREN, 2015; YADAV; SINGH; MATHUR, 2015).

Halophilic microorganisms are taxonomically and physiologically diverse and are considered important sources of different enzymes that are more stable in saline, thermal and alkaline environments than the same enzymes of their non-halophilic counterparts (SETATI, 2010; MORENO et al., 2013; ZHOU et al., 2016). Despite actual or potential applications in numerous fields of science, the taxonomic description of halophilic microbial communities and their spatial distribution in deep marine environments is almost nonexistent.

The present work is a pioneer in the collection of information on halophilic microorganisms in areas of salt diapirs and pockmarks in the Brazilian continental margin. Using enrichment techniques and cultivation of halophilic microorganisms from marine sediment, associated with 16S rRNA gene sequencing techniques on the Illumina Miseq platform, we reported high microbial diversity in all sampled points, as well as the isolation and sequencing of two halophilic bacteria, little sampled in deep-sea ecosystems.

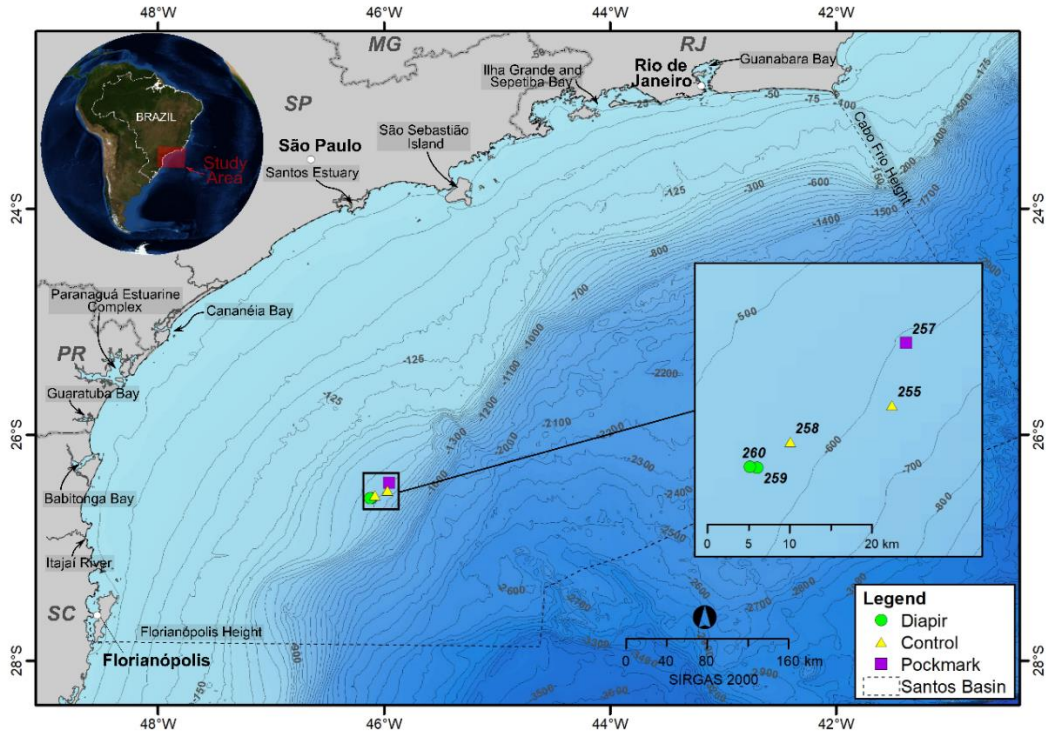
III.2 MATERIALS AND METHODS

III.2.1 Study area and sample collection

The Santos Basin, located on Brazil's southern continental slope between latitudes 27°S and 26°N, has an area of 350 thousand km² (SILVEIRA et al., 2000). The slope regions are influenced by the Brazil Current (CB) that forms along the Brazilian continental margin and is part of the current system that makes up the western edge of the South Atlantic Subtropical Gyre (PETERSON; STRAMMA, 1991). The vertical structure of the water bodies of the Brazil Current is characteristic of the South Atlantic. It is constituted in the first 1.500 meters by Tropical Water (TA), Central Water of the South Atlantic (ACAS), Antarctic Intermediate Water (AIA), Deep Water of the North (APAN) and Antarctic Bottom Waters (AAF) (SILVEIRA et al., 2000).

In the Pockmark field, in SB, sediment samples were collected from five previously established stations (**Figure 1**), based on previous works carried out in the area (CALDER; FONSECA; FRANCOLIN, 2002; SUMIDA et al., 2004; DE MAHIQUES et al., 2017b). The depths of the stations ranged from approximately 300 to 800 meters, comprising samples of pockmarks, salt diapirs, and samples of the seabed in the region (here called control) (**Table S1**).

Figure 1. Map of the study region in the pockmark field located in the Santos Basin, highlighting the sampling stations.



The green circles indicate stations with salt diapirs, yellow triangles indicate control stations, and the purple square indicates the pockmark station. Source: João Regis dos Santos Filho, 2022.

The collection was carried out on board the Oceanographic Ship Alpha-Crucis of the Oceanographic Institute of the University of São Paulo in July 2016, using a stainless steel Box Corer (BX-650) (50 cm x 50 cm) with a maximum penetration of 60 cm. With the aid of cylindrical cutters, the cores were collected from inside the box and stratified every 2 cm with sterile spatulas. The surface sediment (0-2 cm) from all stations was divided into two parts: one was used to enrich the culture, while the other was placed in whirl pak bags and then stored at -20 °C for further sequencing of the 16S gene.

III.2.2 Culture enrichment, DNA extraction, and 16S rRNA gene sequencing

The enrichment of the culture of all station was carried out by adding the sediment (approximately 2 grams) directly into glass flasks (50 ml) containing hypersaline culture medium (25% NaCl) (HM - Halobacterium medium) (BOHACEK, 1968). All cultures were incubated aerobically and without agitation at 20 °C. The basal culture medium consisted of 250 grams of sodium chloride (NaCl), 5 grams of magnesium chloride hexahydrate (($MgCl_2$) $6H_2O$), 5 grams of potassium chloride (KCl), 5 grams of ammonium chloride (NH_4Cl), and 1 gram of yeast extract to 1 liter of water and adjusted to pH 7.0. After two months of incubation,

all samples showed turbidity and growth of microorganisms, confirmed by electron microscopy.

In order to eliminate possible dead and non-halophilic microbial cells from the sediment, a repeat of the original enrichment was performed: 100 µl of the original enriched culture in 30 ml of HM medium (25% NaCl) and kept under the same conditions. The cultures showed growth after approximately two months of incubation and were then subjected to DNA extraction with a PureLink Genomic DNA kit (Thermo Fisher Scientific, USA) according to the manufacturer's specifications.

DNA from environmental sediment samples from the five collection stations was also extracted from 0.2 grams of sediment using the Power Soil DNA Isolation Kit (MoBio Laboratories, USA), also according to the manufacturer's specifications.

DNA from enriched culture samples and environmental sediment samples was quantified by the Qubit 1.0 fluorescence detector (Life Technologies, USA) with the Qubit® dsDNA HS Assay kit (Life Technologies, USA). Library preparation and sequencing were performed by Mr. DNA/Molecular Research (Shallowater, TX, USA), using the Illumina Miseq platform (2x250 bp system). The V4-V5 hypervariable region of the 16S gene was sequenced using universal primers 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 926R (5'-CCGYCAATTYMTTTRAGTTT-3') (QUINCE ET AL., 2011; PARADA; NEEDHAM; FUHRMAN, 2016).

The initial PCR reaction consisted of denaturation at 95 °C for 3 minutes, followed by 35 cycles at 95 °C for 30 seconds, annealing at 57 °C for 30 seconds, extension at 72 °C for 30 seconds and final extension at 72 °C for 5 minutes. A second PCR reaction was performed to add indices to each 16S rRNA gene amplicon to assemble the libraries. Libraries were purified by magnetic beads using the AMPure XP Bead Kit (Beckman Coulter), quantified and normalized. The final pooling step was performed by grouping all libraries and sequencing on the aforementioned platform.

III.2.3 Analysis of 16S rRNA gene sequencing data

The sequences received from Mr. DNA/Molecular Research were imported and demultiplexed in Quantitative Insights Into Microbial Ecology 2 (Qiime2) version 2019.10 (BOLYEN et al., 2019). Subsequently, the sequences were demultiplexed and the quality profiles were inspected; the readings that showed low quality (below Phred score 30) were cut and the chimaeras were removed with the aid of Dada2 software (CALLAHAN et al., 2016). Using the same software, in the Qiime2 package, good quality sequences were used to

determine the amplicon sequence variants (ASVs) for each sample through a SILVA v.138 database classification learning plugin (KLINDWORTH et al., 2013).

The ASVs were normalised by varianceStabilizingTransformation using the R package "DESeq2" (LOVE; HUBER; ANDERS, 2014). The taxonomic, heatmap, and veendiagram plots were performed using the phyloseq (MCMURDIE; HOLMES, 2013), ggplot2 (WICKHAM, 2009), vegan (OKSANEN et al., 2013), heatmap (ZHAO et al., 2014), and venndiagram (CHEN; BOUTROS, 2011) in the R software (R Core Team, 2018). ASV richness, Chao1, and Shannon diversity indexes were calculated using phyloseq (MCMURDIE; HOLMES, 2013). The availability of datasets from the enrichment culture sequencing datasets is available in the GenBank repository under BioProject ID PRJNA824322, while the 16S rRNA gene data from the sediment samples is in the same repository under BioProject ID PRJNA818533.

III.2.4 Cultivation and isolation of halophilic microorganisms

The enriched cultures were then subcultured in triplicates using 100 µl of the enrichment culture in 30 ml of HM 25% NaCl medium. To maximize the chances of recovering halophilic microorganisms, the same transfer was performed in HM medium with a concentration of 10% NaCl, also in triplicate. All cultures were maintained aerobically at 20 °C. After approximately two months, all HM 25% NaCl cultures showed cell growth, verified by electron microscopy, while cultures maintained in HM 10% NaCl showed growth after two weeks of incubation.

After this step, the transfer to the solid medium was carried out, respecting the NaCl concentrations of the respective liquid medium. The solid medium was composed of the same medium (HM) with the addition of 2% marine agar.

The plates were filled with 50 ml of culture medium, sealed with plastic film, and kept in a BOD oven to avoid dissection of the medium due to the high concentration of salt and the long incubation period. All plates were kept at 20 °C. Colony growth was first observed in a 25% NaCl medium after four months of incubation. In HM medium at 10% NaCl, the first colonies appeared after one month of incubation.

To obtain pure cultures, single colonies were collected from all plates and transferred to new solid media. From the HM 25 % NaCl cultures we obtained five pure cultures, while from the HM 10% NaCl cultures we obtained 17 pure cultures. All pure cultures were stored at -80 °C in an isolation medium supplemented with 30% glycerol.

III.2.5 Extraction, sequencing, and analysis of isolates

Genomic DNA from 22 isolates was extracted using the Purelink Genomic DNA kit (Invitrogen from Thermo Fisher Scientific, Carlsbad, USA), and the total DNA concentration was quantified using the Qubit dsDNA HS kit (Thermo Fisher Scientific, São Paulo, Brazil), both following the manufacturer's instructions. Amplification of the 16S rRNA gene was performed using primers 21F (5' -TTC CGG TTG ATG CYG CGG A-3') and 1492R (5' -GGT TAC CTT GTT ACG ACT-3') for the Archaea Domain and 28F (5'-AG AGT TTG ATC CTG GCT CAG-3') 1492R (5'-GGT TAC CTT GTT ACG ACT-3') for the Bacteria Domain (DELONG, 1992).

The polymerase chain reaction (25 µL reaction) was performed using Gotaq Mix Hot Start, 0.25 µL of each primer, and 2 µL of DNA template extracted from isolated colonies. For primers 21F and 1492R, the PCR conditions were as follows: an initial denaturation temperature of 95 °C for 3 minutes, followed by 30 cycles of 94 °C for 1 minute, 53 °C for 30 seconds, 72 °C for 1 minute, and a final extension at 72 °C for 10 minutes.

For primers 28F and 1492R, the PCR conditions were as follows: an initial denaturation temperature of 95 °C for 3 minutes, followed by 30 cycles of 94 °C for 1 minute, 53 °C for 30 seconds, 72 °C for 1 minute, and a final extension at 72 °C for 10 minutes. After amplification, the PCR product was purified with the DNA Clean & ConcentratorTM kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's specifications. Sanger sequencing of the forward and reverse sequences was performed by the company Sustainable Development and Environmental Monitoring (DSMA) (Mogi das Cruzes, São Paulo, Brazil).

After obtaining the raw data, the forward and reverse sequences were analyzed for quality, and the contigs of each sample were assembled in the BioEdit Sequence Alignment Editor v.7.2.5 program (HALL, 1999). Using the SILVA v.138 database (High-Quality Ribosomal RNA Databases), the sequences of the isolates were identified.

To phylogenetically correlate the isolates in this work, a phylogenetic tree was constructed using also reference genomes taken from the Silva v.138 database. We used two isolates of the genus *Chromohalobacter*, an isolate of the genus *Salinisphaera* together with two reference genomes belonging to *Chromohalobacter salexigens*, one referring to the genus *Salinisphaera shabanensis* and finally a genome belonging to *Cobetia marina*, a moderately halophilic bacterium used here as an external member.

The sequences of the isolates together with the reference genomes were submitted to multiple alignments between the sequences using the MAFFT v.7.4 program (KATOH; TOH, 2008). The Mega X v.11.0.11 software (KUMAR et al., 2018) was used to cut the sequences and perform the phylogenetic tree by the maximum likelihood method with Bootstrap replication number equal to 999.

III.3 RESULTS AND DISCUSSION

III.3.1 Microbial diversity in marine sediment and enrichment cultures

Ten samples were used for the sequencing of the 16S rRNA gene: five samples from environmental sediment and five samples submitted to aerobic enrichment culture. We obtained 400.765 quality-filtered reads identified as 3.199 amplicon sequence variants (ASVs) (0.03 cut-off), of which 2.850 were assigned to the Bacteria Domain and only 348 to the Archaea Domain. Microbial diversity was evaluated using the Chao1, and Shannon indexes, and when we performed the culture enrichment through a selective medium for halophilic microorganisms, we observed higher values of richness and diversity in the sediment samples when compared to the culture enriched samples (**Table 1**).

Table 1. The number of sequences, number of amplicon sequence variants (ASV), richness (Chao1) and diversity (Shannon) of microbial communities in environmental sediment samples as well as enrichment cultures.

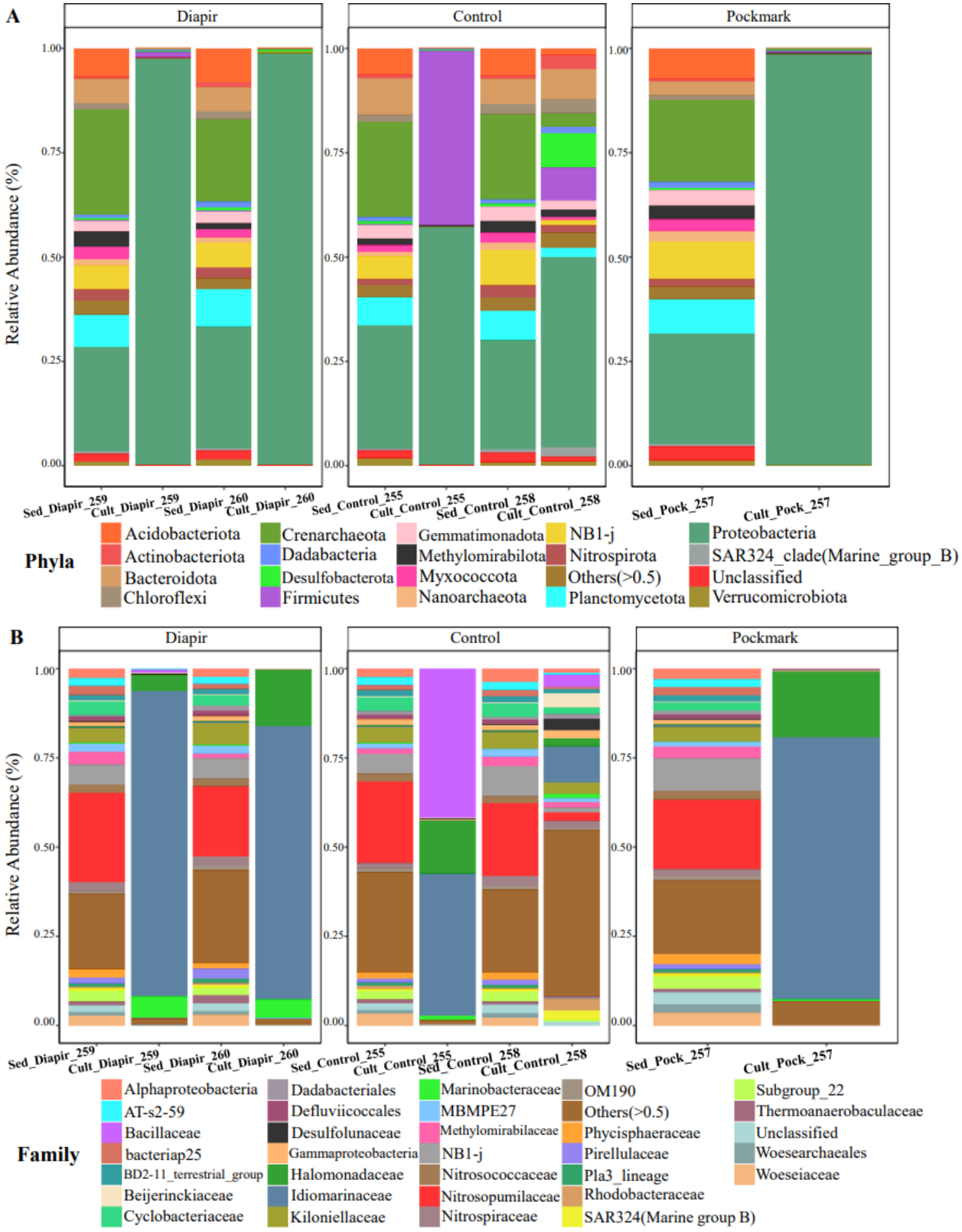
Sample ID	No. of Sequences	Observed ASV	Chao1	Shannon
Control Sediment				
Sed_Control_ 255	25.823	787	788.3636	6.204
Sed_Control_ 258	49.800	1.272	1278.0000	6.555
Control Culture				
Cult_Control_255	30.750	55	55.0000	1.484
Cult_Control_258	1.931	91	91.3750	1.218
Diapir Sediment				
Sed_Diapir_ 259	38.947	1.026	1026.7692	6.327
Sed_Diapir_ 260	36.225	1.107	1108.8000	6.510
Diapir Culture				
Cult_Diapir_259	70.529	141	141.0667	0.981
Cult_Diapir_260	57.994	106	106.4286	1.115
Pockmark Sediment				
Sed_Pock_ 257	38.777	1.035	1038.4375	6.336
Pockmark Culture				
Cult_Pock_257	49.989	91	91.3750	1.218

The composition of prokaryotic communities varied between sediment samples and samples from enriched cultures. The environmental sediment samples showed very similar microbial composition, with a predominance of the phyla Proteobacteria, with relative abundance varying among 25% and 30%, Crenarchaeota (19% to 25%), and Planctomycetota

(6% to 9%). The samples enriched with salt diapir sediment and pockmark sediment showed almost total dominance of the phylum Proteobacteria (above 97%). The control sediment sample from station 255 showed a similar abundance between the phyla Proteobacteria and Firmicutes, while the control sediment sample from station 255 showed a dominance of Proteobacteria, however, it also showed a high diversity of other phyla (**Figure 2A**).

When analysing at the family level (**Figure 2B**), we observed that the environmental sediment samples were again similar to each other regardless of the collection site, with a predominance of the Nitrosopumilaceae group (phylum - Crenarchaeota). Nitrosopumilaceae are widely distributed in the ocean and can be deposited on the seafloor through the water column (KEROU; SCHLEPER, 2016). These organisms can survive in marine sediment using ammonia produced by aerobic mineralization of organic matter (STIEGLMEIER et al., 2014; KEROU; ELOY ALVES; SCHLEPER, 2016). Despite the high relative abundance of these ammonia-oxidizing archaea in the sampled sediments, after enrichment culture, Nitrosopumilaceae ranged from 0% in the pockmark sample to 2.5% in the Cult_Control_258 sample, especially because the HM medium is not compatible with the physiology of this group.

Figure 2. The relative abundances of bacterial and archaeal taxonomic composition for (A) phyla and (B) Family**.**



Only phyla and families with more than 0.5% relative abundance are represented. Other groups are in "Others".

Kiloniellaceae, an Alphaproteobacteria abundant in our environmental sediment samples, is involved in the nitrogen cycle. This organism was initially isolated from marine macroalgae, being aerobic with denitrification potential (WIESE et al., 2009; WIESE et al., 2020). This family represented 4% to 6% of the families sampled in the sediment, while in the enriched samples this group was found only in the Cult_Control_258 sample, representing 3% of the community. Despite being a group with moderately halotolerant members, its ideal growth is around 3% NaCl (WIESE et al., 2009), justifying the low presence of this family in cultures enriched with 25% NaCl.

NB1-J, was also an important group in the environmental sediment samples, varying from 5.4% in the Sed_Control_255 sample to 9% in the Sed_Pock_257 sample. There is little information on the functions of NB1-j in marine environments. In a study carried out on the metagenome of marine sponges, the participation of this group in nitrogen metabolism was also suggested (DE VOOGD et al., 2015). NB1-j has also been reported in active pockmark sediments, but it is not yet possible to say the role of this organism in these environments (GIOVANNELLI et al., 2016). In the enriched culture samples, this group was found only in two samples, ranging from 0.01% to 1.2%.

In general, the microbial communities from enrichment cultures showed a high prevalence of a few bacterial groups, especially halophiles belonging to the order Alteromonadales and Oceanospirillales. Idiomarinaceae was the dominant family in all enriched ones except for the Cult_Control_255 sample, in which the dominant family was Bacillaceae with 41.8% followed by Idiomarinaceae with 40% relative abundance. The Idiomarinacea family is composed of halophilic organisms and all its members have been isolated from saline environments, mainly marine habitats (IVANOVA et al., 2000; IVANOVA; FLAVIER; CHRISTEN, 2004; FRANCO et al., 2021).

They are aerobic and differ from other marine bacteria, especially by their ability to grow in wide ranges of pH, temperature, and NaCl concentration (IVANOVA et al., 2000; DONACHIE et al., 2003; IVANOVA; FLAVIER; CHRISTEN, 2004; FLORES-FERNÁNDEZ et al., 2019). This great physiological versatility could justify the abundant presence of this family in all analysed crops. In sediment samples that did not undergo the enrichment process, Idiomarinacea presented an abundance of less than 0.6% in all samples.

ASVs attributed to Halomonadaceae were also abundant in the enriched samples, reaching up to 18.3% of the community. This family contains almost exclusively halophilic or halotolerant microorganisms (OREN, 2008). They were primarily isolated from marine and/or saline environments and, like Idiomarinaceae, can grow across a broad salinity gradient (VENTOSA et al.,

1989; GARCÍA; VENTOSA; MELLADO, 2005; OREN, 2008). The high abundance of this family is very interesting, since they are organisms of great biotechnological interest, especially for their ability to synthesise osmoprotectants, biopolymers, biosurfactants, and mediate the degradation of aromatic compounds (GARCÍA; VENTOSA; MELLADO, 2005; CORTI MONZÓN et al., 2018; FLORES-FERNÁNDEZ et al., 2019). When comparing the presence of this family in the environmental sediment samples, we observed that the maximum abundance found was 0.12%.

Another important group in the enriched samples was the Marinobacteriaceae, which reached up to 6% of the identified families. This group is composed of several halophilic representatives that are widely found in marine and salt lake sediments (ZHANG; LIN; CHEN, 2018; ZHANG et al., 2022). In our environmental sediment samples, this family did not reach a relative abundance of above 0.05%.

Interestingly, of all the enriched samples, the Cult_Control_258 sample presented the greatest diversity of taxonomic groups when compared to the other enrichments, in which the other groups (relative abundance below 0.5%) represented 46.6% of the identified groups (**Table S2**).

III.3.2 Differential favoring of microbial communities from sediment to enrichment culture

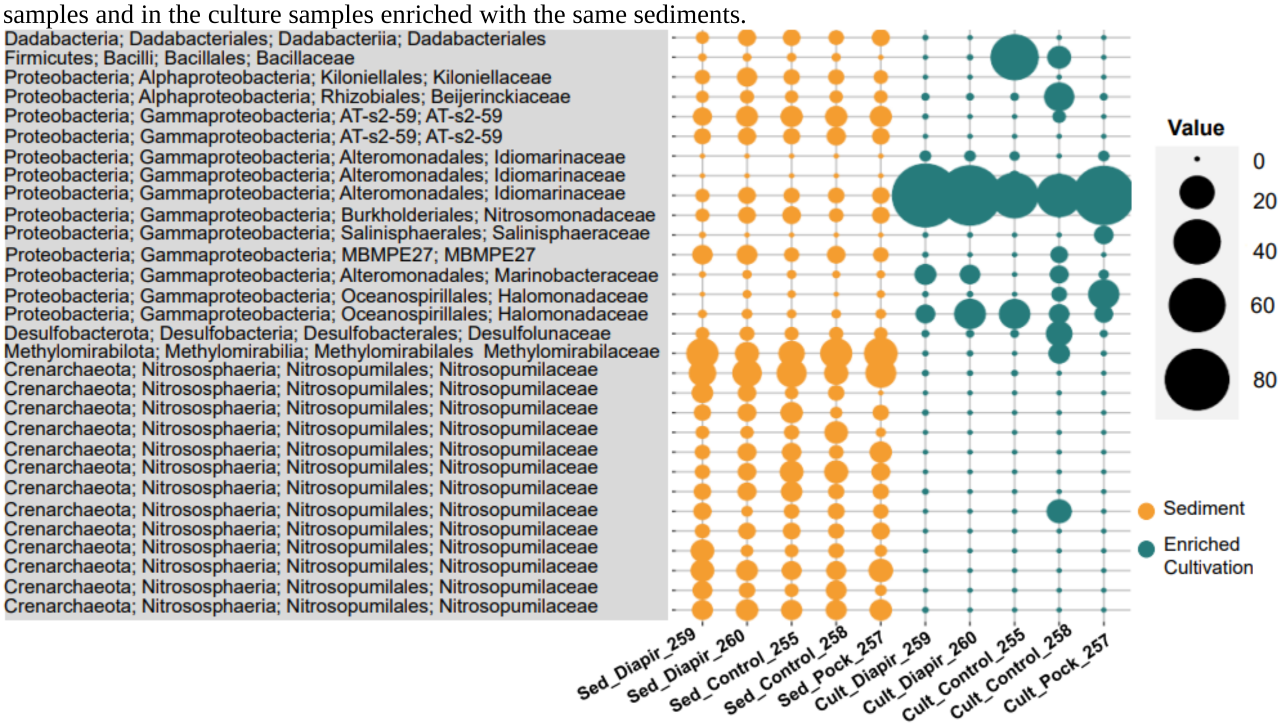
When analysing the 30 most abundant ASVs of all the 16S ribosomal gene sequenced samples, it is possible to notice a pattern in the abundance and distribution of these ASVs among the environmental sediment samples and the enriched culture samples (**Figure 3**). As previously demonstrated, the Nitrosopumilaceae family stands out among the environmental sediment samples, showing here the presence of 13 different ASVs belonging to this group, and only one of these ASVs was also found in one of the enriched culture samples.

Another family that stood out in the environmental sediment samples was Methylomirabilaceae. This ASV was also present in all sediment samples and was found in only one enriched culture sample. Methylomirabilaceas encompass methanotrophic methylotrophs and non-methanetrophic methylotrophs (ETTWIG et al., 2009; HUG et al., 2016). It is worth mentioning that in the study area it was not possible to find evidence of methane escape from the various pockmarks recorded in the region, however, the oxidation of methanol is a remarkable process in the oceans where it is estimated that this C1 compound can reach concentrations of up to 420 nM (DIXON et al., 2011; READ et al., 2012).

When we focus on the enriched culture samples, it is possible to observe the decrease in taxonomic diversity and the favoring of halophilic groups, mainly in terms of their abundance, when compared to environmental sediment samples. Families such as Marinobacteriacea, Halomonadaeace, Salinisphaeraceae, and mainly Idiomarinaceae stood out among the 30 most abundant ASVs. These halophilic groups were also present in some samples of environmental sediment, however, in low relative abundance.

Despite harboring mainly moderate halophiles, these families have members with great metabolic versatility and high tolerance to NaCl variations in the environment (TANG et al., 2018; FLORES-FERNÁNDEZ et al., 2019). HM medium with 25% NaCl is a medium used for extreme halophiles, however, these moderate halophilic groups were able to grow and dominate samples from enriched cultures.

Figure 3. Classification of the 30 most abundant ASVs of Archaea and Bacteria in the environmental sediment samples and in the culture samples enriched with the same sediments.



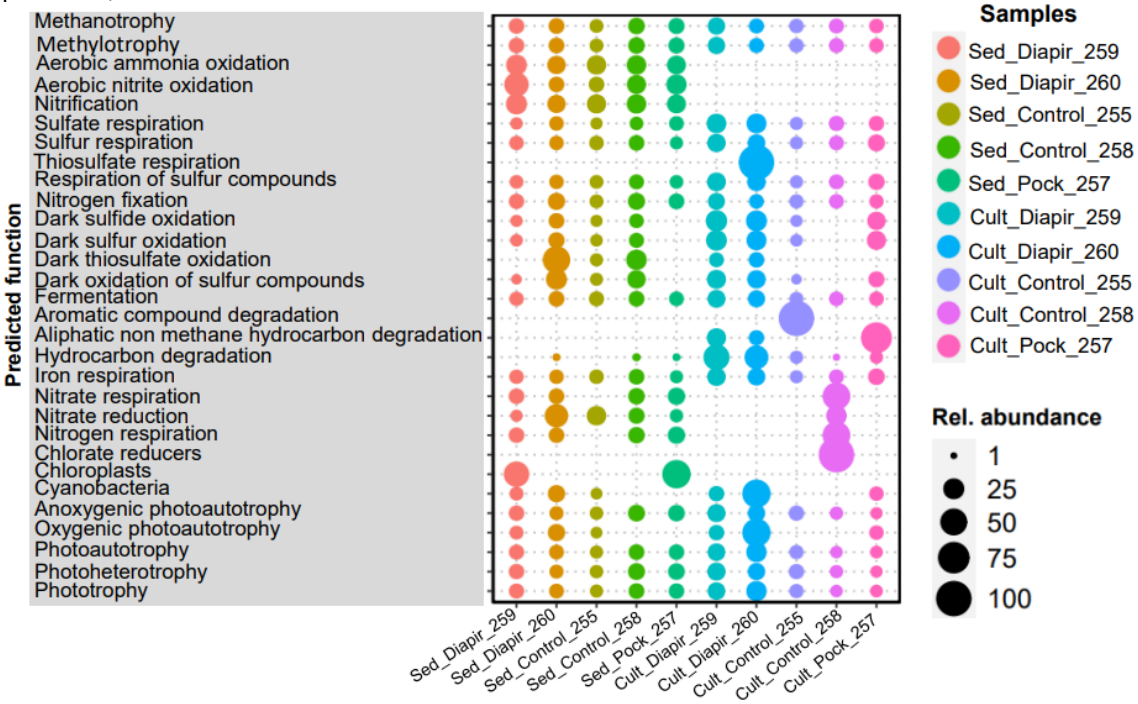
The size of the circles is related to the relative abundance of each ASV. ASVs were organised by families.

III.3.3 Predicted functions in environmental sediment and enriched cultures samples

The 3.199 ASVs detected in the samples were searched in the FAPROTAX database to explore and infer their functional potential based on phylogenetic relationships with cultured representatives. Predicted metabolisms varied between samples, especially when comparing environmental sediment samples with enriched culture samples. Methanotrophy, methylotrophy, sulphate and sulphur respiration, nitrogen fixation, fermentation, iron

respiration, anoxygenic and oxygenic photoautotrophy, photoheterotrophy were predicted functions in all samples with differences in relative abundance between samples (**Figure 4**).

Figure 4. Predictions of the potential metabolism of microbial communities in the sediment of salt diapir, control, pockmark, and enrichment cultures.



The size of the circles represents the relative abundance of each function. Different colours indicate different samples.

The main functions associated with the microbial communities of the environmental sediment were related to the nitrogen cycle: aerobic ammonia oxidation; aerobic nitrite oxidation; nitrate reduction; nitrate respiration; nitrogen respiration; and nitrification, which we can correlate with the high relative abundance of communities participating in the nitrogen cycle found in the analysis of the ribosomal 16S gene.

The predicted functions of enriched cultures vary between samples. The Cult_Control_255 sample was the only one to present metabolism related to aromatic compound degradation, while the other control sample was the only one to present functions related to chlorate reducers.

Salt diapir enriched culture samples showed similar metabolic predictions, highlighting mainly hydrocarbon degradation, thiosulfate respiration, non-methane aliphatic hydrocarbon degradation, dark sulfide oxidation, and dark sulfur oxidation.

Through this profile of predictions and metabolic inferences, we highlight again the importance of the nitrogen cycle in the marine sediments of this region. The marine nitrogen cycle is quite complex and plays a central role in ocean biogeochemistry, with the ability to

influence the cycle of other compounds, such as carbon and phosphorus (ZEHR; WARD, 2002). The entry of nitrogen into this environment can occur through riverine inflows, atmospheric precipitation, N_2 fixation, the burial of organic matter in the sediment from the water column, and sedimentary denitrification (ZEHR; WARD, 2002; HOSHINO et al., 2020).

The sampled points are located on the slope, a region with a high continental influence. This is the main form of transport of sediments from the shelf to the abyssal plain, which may contribute to the entry of nitrogen compounds into the region. Although each form of nitrogen (nitrate, nitrite, ammonium, and organic nitrogen, for example) has a different level of reactivity, this complex cycle directly influences marine biological processes (STROZYK et al., 2018; TASIANAS et al., 2018; HOSHINO et al., 2020).

III.3.4 Identification of Isolates from Sediment Enrichment Cultures

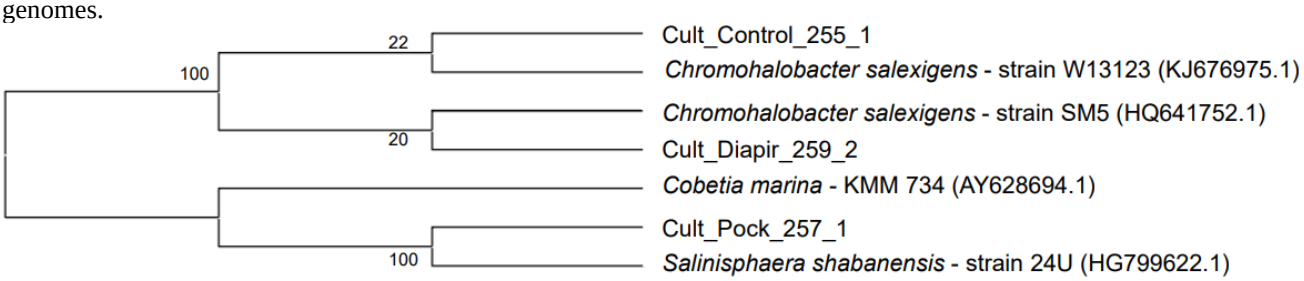
From the enriched culture samples, 22 isolated microorganisms were obtained, of which 17 were obtained from the HM medium with 10% NaCl and only 5 were isolated from the medium that remained with 25% NaCl. All isolates belonged to two distinct families of bacteria: Halomonadaceae and Salinisphaeraceae, both belonging to the class Gammaproteobacteria (**Table S2**).

All isolates from the Halomonadaceae family were identified as belonging to the genus *Chromohalobacter*. These bacteria are gram-negative bacilli, strictly aerobic and chemoorganotrophic. They are moderately halophilic, but have great versatility in terms of salt tolerance and can grow on a wide range of single carbon compounds (CÁNOVAS et al., 1996; ARAHAL et al., 2001). The osmoadaptation found in *Chromohalobacter* is obtained through the de novo synthesis of ectoine, hydroxylated and hydroxytoin, being able to rapidly regulate the concentration of these solutes in response to osmotic variations in the medium (ARAHAL et al., 2001; OREN et al., 2005).

This great versatility and tolerance to salt concentration possibly allowed this organism to grow even in the medium enriched with 25% NaCl, however, the incubation time was considerably longer. The second family isolated (Salinisphaeraceae), for example, could not be isolated in the medium enriched with 25% NaCl, being isolated only after migration to the medium containing 10% NaCl. Interestingly, when comparing the 16S gene sequencing carried out in the environmental sediment samples, we noticed that the relative abundance of the Halomonadaceae family ranged from 0.09% to 0.12%, while in the enriched cultures there was an increase in the abundance of this family, which varied from 2.2% to 18.3% (**Table S3**).

Although the genus *Chromohalobacter* has already been found in different studies in marine environments (FRANCO et al., 2021; BLANDÓN et al., 2022; BLANDÓN et al., 2022), the work carried out on microbiology in pockmark fields did not report the presence of the Halomonadaceae family and, consequently, there is no record of the *Chromohalobacter* genus in similar regions, even in inactive pockmark fields (CAMBON-BONAVITA et al., 2009; OMOREGIE et al., 2009; HAVERKAMP; HAMMER; JAKOBSEN, 2014; GIOVANNELLI et al., 2016; IDCZAK et al., 2020; O'REILLY et al., 2021). According to the Silva database v.138, all sequences of our isolates belonging to the genus *Chromohalobacter* correspond to 100% similarity with the sequences of *Chromohalobacter salexigens* (formerly *H. elongata* DSM 3043), a highly remarkable species for its tolerance to salt (ARAHAL et al., 2001) (**Figure 5**).

Figure 5. Phylogenetic tree constructed with bacterial isolates from enrichment cultures and different reference genomes.



Two isolates of the genus *Chromohalobacter* and one isolate of the genus *Salinisphaera* were used together with two reference genomes belonging to *Chromohalobacter salexigens*, one referring to the genus *Salinisphaera shabanensis* and finally a genome belonging to *Cobetia marina* as an external member.

In this work, we obtained only one isolate belonging to the Salinisphaeraceae family. This family comprises a single genus (S*alinisphaera*) and all members have been isolated from marine or oceanic and with high salinity environments (ANTUNES et al., 2003; PARKES et al., 2007; CRESPO-MEDINA et al., 2009; SHIMANE et al., 2013).

They have coccoid or short rod morphologies and are composed of moderate or halotolerant halophilic, aerobic and heterotrophic (except for the species *S. hydrothermalis*, which is a facultative chemolithoautotroph) (ANTUNES et al., 2003). The osmoregulation process is aided by the accumulation of ectoine and betaine in its interior to avoid damage caused by salt (ANTUNES et al., 2003). The growth range of this organism is quite wide for oxygen, temperature, and NaCl concentration. Growth can occur between 1% and 28% of NaCl.

However, its optimal growth is 10% and the temperature range for growth can occur between 5 °C and 42 °C, showing the great versatility of this group.

When analyzing the relative abundance of this family in the environmental sediment samples, we observed that this group was absent in all samples collected in the control sediment and had low abundance in the pockmark and salt diapir samples. In the enriched culture samples, we observed a very low relative abundance (less than 0.009%) in the salt diapir samples, and a higher abundance was observed only in the pockmark sample (4.3%), the sample from which this isolate was obtained. Salinisphaeraceae was the third most abundant family in the culture sample enriched with pockmark sediment.

According to the Silva database (v.138), the sequence of this isolate has 100% similarity with the species *Salinisphaera shabanensis*, described by Antunes et al. (2003). This organism was first isolated from the brine-seawater interface at Shaban Deep in the Red Sea. Like members of *Chromohalobacteria*, *Salinisphaera* was also not previously reported in pockmark fields and salt diapirs, constituting the first isolates in such environments, especially in the South Atlantic.

III.4 CONCLUSION

In the present work, high taxonomic diversity was observed in environmental sediment samples and the prevalence of halophilic groups in samples enriched in HM medium. Sediment samples were similar in taxonomic composition, all dominated by archaea of the Nitrosopumilaceae family, while the sediment samples enriched in HM medium families such as Idiomarinaceae, Halomonadaceae, and Marinobacteriaceae were dominant. From cultures enriched with saline diapir sediment, control, and pockmark sediment, 22 isolates belonging to two distinct genera of bacteria were obtained: *Chromohalobacter* and *Salinisphaera*, both belonging to the class Gammaproteobacteria and little described in the deep sea. The present work is a pioneer in the cultivation and isolation of halophilic microorganisms from pockmark fields and salt diapirs on the Brazilian continental margin, in the Southeast Atlantic Ocean. We also emphasize the importance of combining dependent and independent cultivation techniques and the need for more studies focused on this region.

REFERENCES

ANDREI, A. Ş.; BANCIU, H. L.; OREN, A. Living with salt: Metabolic and phylogenetic diversity of archaea inhabiting saline ecosystems. **FEMS Microbiology Letters**, v. 330, n. 1, p. 1–9, 2012.

ANTUNES, A. et al. Salinisphaera shabanensis gen. nov., sp. nov., a novel, moderately halophilic bacterium from the brine-seawater interface of the Shaban Deep, Red Sea. **Extremophiles**, v. 7, n. 1, p. 29–34, 2003.

ARAHAL, D. R. et al. Chromohalobacter salexigens sp. nov., a moderately halophilic species that includes Halomonas elongata DSM 3043 and ATCC 33174. **International Journal of Systematic and Evolutionary Microbiology**, v. 51, n. 4, p. 1457–1462, 2001.

BLANDÓN, L. M. et al. Diversity of cultivable bacteria from deep-sea sediments of the Colombian Caribbean and their potential in bioremediation. **Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology**, v. 115, n. 3, p. 421–431, 2022.

BOHACEK, J. Deoxyribonucleic Acid Base Composition of some Marine and Halophilic Micrococci. **Journal of Applied Bacterology**, v. 31, n. 2, p. 215–219, 1968.

BOLYEN, E. et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. **Nature Biotechnology**, v. 37, n. 8, p. 852–857, 2019.

BOWLES, M. W. et al. Global rates of marine sulfate reduction and implications for sub-sea-floor metabolic activities. **Science**, v. 344, n. 6186, p. 889–891, 2014.

CACCHIONE, D. A.; PRATSON, L. F.; OGSTON, A. S. The shaping of continental slopes by internal tides. **Science**, v. 296, n. 5568, p. 724–727, 2002.

CALDER, B. R.; FONSECA, L.; FRANCOLIN, J. B. Automatic estimation of the seafloor geomorphology of the Santos Basin, Brazil. **Eos, Transactions, American Geophysical Union**, v. 83, n. 47, Suppl., p. F728, 2002.

CALLAHAN, B. J. et al. DADA2: High-resolution sample inference from Illumina amplicon data. **Nature Methods**, v. 13, n. 7, p. 581–583, 2016.

CAMBON-BONAVITA, M. A. et al. Deep-Sea Research II Diversity, and distribution of methane-oxidizing microbial communities associated with different faunal assemblages in a giant pockmark of the Gabon continental margin. **Deep-Sea Research Part II**, v. 56, n. 23, p. 2248–2258, 2009.

CÁNOVAS, D. et al. Osmoprotectants in Halomonas elongata: High-affinity betaine transport system and choline-betaine pathway. **Journal of Bacteriology**, v. 178, n. 24, p. 7221–7226, 1996.

CHEN, H.; BOUTROS, P. C. Venn Diagram: a package for the generation of highly-customizable Venn and Euler diagrams in R. **Mathematics Magazine**, v. 57, n. 5, p. 297, 2011.

COLEMAN, A. J. et al. How, where, and when do radial faults grow near salt diapirs ? **The Geological Society of America**, v. 46, n. 7, p. 655–658, 2018.

CORTI MONZÓN, G. et al. New Findings on Aromatic Compounds' Degradation and Their Metabolic Pathways, the Biosurfactant Production, and Motility of the Halophilic Bacterium Halomonas sp. KHS3. **Current Microbiology**, v. 75, n. 8, p. 1108–1118, 2018.

CRESPO-MEDINA, M. et al. Salinisphaera hydrothermalis sp. nov., a mesophilic, halotolerant, facultatively autotrophic, thiosulfate-oxidizing gammaproteobacterium from deep-sea hydrothermal vents, and emended description of the genus Salinisphaera. **International Journal of Systematic and Evolutionary Microbiology**, v. 59, n. 6, p. 1497–1503, 2009.

DASSARMA, S.; DASSARMA, P. Halophiles, and their enzymes: Negativity put to good use. **Current Opinion in Microbiology**, v. 25, n. Table 1, p. 120–126, 2015.

DAVISON, I. et al. Geometry and late-stage structural evolution of Central Graben salt diapirs, North Sea. **Marine and Petroleum Geology**, v. 17, p. 499–522, 2000.

DE ALMEIDA, A. G.; KOWSMANN, R. O. **Geomorfologia Do Talude Continental E Do Platô De São Paulo**. [s.l.] Elsevier Editora Ltda., 2015.
DE MAHIQUES, M. M. et al. An extensive pockmark field on the upper Atlantic margin of Southeast Brazil: spatial analysis and its relationship with salt diapirism. **Heliyon**, v. 3, n. 2, p. e00257, 2017a.
DE MAHIQUES, M. M. et al. An extensive pockmark field on the upper Atlantic margin of Southeast Brazil : spatial analysis and its relationship with salt diapirism. **Heliyon**, n. August 2016, p. e00257, 2017b.
DE VOOGD, N. J. et al. Bacterial community composition and predicted functional ecology of sponges, sediment and seawater from the thousand islands reef complex, West Java, Indonesia. **FEMS Microbiology Ecology**, v. 91, n. 4, p. 1–12, 2015.
DELONG, E. F. Archaea in coastal marine environments. **Proceedings of the National Academy of Sciences of the United States of America**, v. 89, n. 12, p. 5685–5689, 1992.
DELONG, E. F. Marine microbial diversity: The tip of the iceberg. **Trends in Biotechnology**, v. 15, n. 6, p. 203–207, 1997.
DIXON JL, BEALE R, NIGHTINGALE PD. Rapid biological oxidation of methanol in the tropical Atlantic: Significance as a microbial carbon source. *Biogeosciences* 2011; **8**: 2707–2716.
DONACHIE, S. P. et al. Idiomarina loihiensis sp. nov., a halophilic γ-proteobacterium from the Lō'ihi submarine volcano, Hawai'i. **International Journal of Systematic and Evolutionary Microbiology**, v. 53, n. 6, p. 1873–1879, 2003.
ETTWIG, K. F. et al. Enrichment and molecular detection of denitrifying methanotrophic bacteria of the NC10 phylum. **Applied and Environmental Microbiology**, v. 75, n. 11, p. 3656–3662, 2009.
FLORES-FERNÁNDEZ, C. N. et al. Molecular characterization of protease producing Idiomarina species isolated from Peruvian saline environments. **Microbiology and Biotechnology Letters**, v. 47, n. 3, p. 401–411, 2019.
FRANCO, N. R. et al. Bacterial composition and diversity in deep-sea sediments from the southern Colombian Caribbean sea. **Diversity**, v. 13, n. 1, p. 1–13, 2021.
GARCÍA, M. T.; VENTOSA, A.; MELLADO, E. Catabolic versatility of aromatic compound-degrading halophilic bacteria. **FEMS Microbiology Ecology**, v. 54, n. 1, p. 97–109, 2005.
GIOVANNELLI, D. et al. Diversity and distribution of prokaryotes within a shallow-water pockmark field. **Frontiers in Microbiology**, v. 7, n. JUN, p. 1–21, 2016.
HAVERKAMP, T. H. A.; HAMMER, Ø.; JAKOBSEN, K. S. Linking geology, and microbiology: Inactive pockmarks affect sediment microbial community structure. **PLoS ONE**, v. 9, n. 1, 2014.
HOSHINO, T. et al. Global diversity of microbial communities in marine sediment. **Proceedings of the National Academy of Sciences of the United States of America**, v. 117, n. 44, p. 27587–27597, 2020.
HUDEC, M. R.; JACKSON, M. P. A. Terra infirma: Understanding salt tectonics. **Earth Science Reviews**, v. 82, p. 1–28, 2007.
HUG, L. A. et al. Critical biogeochemical functions in the subsurface are associated with bacteria from new phyla and little studied lineages. **Environmental Microbiology**, v. 18, n. 1, p. 159–173, 2016.
IDCZAK, J. et al. Science of the Total Environment A geophysical, geochemical and microbiological study of a newly discovered pockmark with active gas seepage and submarine groundwater discharge (MET1-BH, central Gulf, southern Baltic Sea). **Science of the Total Environment**, v. 742, 2020.
IVANOVA, E. P. et al. Idiomarina gen. nov., comprising novel indigenous deep-sea bacteria from the Pacific Ocean, including descriptions of two species, Idiomarina abyssalis sp. nov.

and Idiomarina zobellii sp. nov. **International Journal of Systematic and Evolutionary Microbiology**, v. 50, n. 2, p. 901–907, 2000.
IVANOVA, E. P.; FLAVIER, S.; CHRISTEN, R. Phylogenetic relationships among marine Alteromonas-like proteobacteria: Emended description of the family Alteromonadaceae and proposal of Pseudoalteromonadaceae fam. nov., Colwelliaceae fam. nov., Shewanellaceae fam. nov., Moritellaceae fam. nov., Ferri. **International Journal of Systematic and Evolutionary Microbiology**, v. 54, n. 5, p. 1773–1788, 2004.
KATOH, K.; TOH, H. Recent developments in the MAFFT multiple sequence alignment program. **Briefings in Bioinformatics**, v. 9, n. 4, p. 286–298, 2008.
KEROU, M.; ELOY ALVES, R. J.; SCHLEPER, C. Nitrososphaeria . **Bergey's Manual of Systematics of Archaea and Bacteria**, p. 1–8, 2016.
KEROU, M.; SCHLEPER, C. Nitrososphaera . **Bergey's Manual of Systematics of Archaea and Bacteria**, p. 1–10, 2016.
KLINDWORTH, A. et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. **Nucleic Acids Research**, v. 41, n. 1, p. 1–11, 2013.
KUMAR, S. et al. MEGA X: Molecular evolutionary genetics analysis across computing platforms. **Molecular Biology and Evolution**, v. 35, n. 6, p. 1547–1549, 2018.
LOVE, M. I.; HUBER, W.; ANDERS, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. **Genome Biology**, v. 15, n. 12, p. 1–21, 2014.
MAPELLI, F. et al. Bacterial community structure, and diversity along the halocline of Tyro deep-sea hypersaline anoxic basin. **Annals of Microbiology**, v. 72, n. 1, 2022.
MCMURDIE, P. J.; HOLMES, S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. **PLoS ONE**, v. 8, n. 4, 2013.
MOHRIAK, W. U. Bacias Sedimentares da Margem Continetal Brasileira. **Geologia, Tectônica e Recursos Minerais do Brasil**, p. 151–165, 2003.
MORENO, M. DE L. et al. Halophilic bacteria as a source of novel hydrolytic enzymes. **Life**, v. 3, n. 1, p. 38–51, 2013.
O'REILLY, S. S. et al. Production of methane and gaseous compounds by surface microbial activity in a small pockmark field, Dunmanus Bay, Ireland. **Estuarine, Coastal and Shelf Science**, v. 255, n. May 2020, 2021.
OKSANEN, A. J. et al. Package ' vegan '. n. January, p. 0–291, 2013.
OMOREGIE, E. O. et al. Microbial methane oxidation and sulfate reduction at cold seeps of the deep Eastern Mediterranean Sea. **Marine Geology**, v. 261, n. 1–4, p. 114–127, 2009.
OREN, A. Bioenergetic Aspects of Halophilism WHY CERTAIN PHYSIOLOGICAL GROUPS OF MICROORGANISMS ARE ABSENT IN. **Society**, v. 63, n. 2, p. 334–348, 1999.
OREN, A. et al. How to be moderately halophilic with broad salt tolerance: Clues from the genome of *Chromohalobacter salexigens*. **Extremophiles**, v. 9, n. 4, p. 275–279, 2005.
OREN, A. Microbial life at high salt concentrations: Phylogenetic and metabolic diversity. **Saline Systems**, v. 4, n. 1, p. 1–13, 2008.
OREN, A. Halophilic microbial communities, and their environments. **Current Opinion in Biotechnology**, v. 33, n. Figure 1, p. 119–124, 2015.
PALOMINO, D. et al. Multidisciplinary study of mud volcanoes and diapirs and their relationship to seepages and bottom currents in the Gulf of Cádiz continental slope (northeastern sector). **Marine Geology**, v. 378, p. 196–212, 2016.
PARADA, A. E.; NEEDHAM, D. M.; FUHRMAN, J. A. Every base matters: Assessing small subunit rRNA primers for marine microbiomes with mock communities, time series, and global field samples. **Environmental Microbiology**, v. 18, n. 5, p. 1403–1414, 2016.
PARKES, R. J. et al. Biogeochemistry and biodiversity of methane cycling in subsurface marine sediments (Skagerrak, Denmark). **Environmental Microbiology**, v. 9, n. 5, p. 1146–

1161, 2007.
PEDRÓS-ALIÓ, C. Marine microbial diversity: can it be determined? **Trends in Microbiology**, v. 14, n. 6, p. 257–263, 2006.
PETERSON, R. G.; STRAMMA, L. Upper-level circulation in the South Atlantic Ocean. **Progress in Oceanography**, v. 26, n. 1, p. 1–73, 1991.
QUILLAGUAMÁN, J. et al. Synthesis and production of polyhydroxyalkanoates by halophiles: Current potential and future prospects. **Applied Microbiology and Biotechnology**, v. 85, n. 6, p. 1687–1696, 2010.
QUINCE ET AL., 2011. Removing Noise From Pyrosequenced Amplicons. **BMC bioinformatics**, v. 12, n. 9, p. 834–839, 2011.
READ, K. A. et al. Multiannual observations of acetone, methanol, and acetaldehyde in remote tropical Atlantic air: Implications for atmospheric OVOC budgets and oxidative capacity. **Environmental Science and Technology**, v. 46, n. 20, p. 11028–11039, 2012.
SALAZAR, G.; SUNAGAWA, S. Marine microbial diversity. **Current Biology**, v. 27, n. 11, p. R489–R494, 2017.
SANTOS, F.; YARZA, P.; PARRO, V.; MESUGUER, I.; RSSELLÓ-MORA, R.; ANTÓN, J. **Culture-Independent Approaches for Studying Viruses from Hypersaline EnvironmentsApplied and Environmental Microbiology**, 2012.
SCHRENK, M. O.; HUBER, J. A.; EDWARDS, K. J. Microbial provinces in the subseafloor. **Annual Review of Marine Science**, v. 2, n. 1, p. 279–304, 2010.
SETATI, M. E. Diversity and industrial potential of hydrolaseproducing halophilic/halotolerant eubacteria. **African Journal of Biotechnology**, v. 9, n. 11, p. 1555–1560, 2010.
SEYMOUR, J. R. A sea of microbes: the diversity and activity of marine microorganisms. **Microbiology Australia**, v. 35, n. 4, p. 183, 2014.
SHIMANE, Y. et al. Salinisphaera japonica sp. nov., a moderately halophilic bacterium isolated from the surface of a deep-sea fish, malacocottus gibber, and emended description of the genus *Salinisphaera*. **International Journal of Systematic and Evolutionary Microbiology**, v. 63, n. PART6, p. 2180–2185, 2013.
SILVEIRA, I., C., A.; SCHMIDT, A., C., K.; CAMPOS, E., J., D.; GODOI, S., S.; IKEDA, Y. A Corrente do Brasil ao Largo da Costa Leste Brasileira. **Revista Brasileira de Oceanografia**, v. 48, n. 2, p. 171–183, 2000.
STEWART, S. A. Implications of passive salt diapir kinematics for reservoir segmentation by radial and concentric faults. **Marine and Petroleum Geology**, v. 23, p. 843–853, 2006.
STIEGLMEIER, M. et al. Nitrososphaera viennensis gen. nov., sp. nov., an aerobic and mesophilic, ammonia-oxidizing archaeon from soil and a member of the archaeal phylum Thaumarchaeota. **International Journal of Systematic and Evolutionary Microbiology**, v. 64, n. PART 8, p. 2738–2752, 2014.
STROZYK, F. et al. Giant pockmark formation from Cretaceous hydrocarbon expulsion in the western Lower Saxony Basin, The Netherlands. **Geological Society Special Publication**, v. 469, n. 1, p. 519–536, 2018.
SUMIDA, P. Y. G. et al. Seabed pockmarks associated with deepwater corals off SE Brazilian continental slope, Santos Basin. **Marine Geology**, v. 207, n. 1–4, p. 159–167, 2004.
TANG, X. et al. Complete genome of Cobetia marina JCM 21022T and phylogenomic analysis of the family Halomonadaceae. **Journal of Oceanology and Limnology**, v. 36, n. 2, p. 528–536, 2018.
TASIANAS, A. et al. High-resolution 3D seismic study of pockmarks and shallow fluid flow systems at the Snøhvit hydrocarbon field in the SW Barents Sea. **Marine Geology**, v. 403, n. 9037, p. 247–261, 2018.
VENTOSA, A. et al. Classification of "*Chromobacterium marismortui*" in a new genus, *Chromohalobacter* gen. nov., as *Chromohalobacter marismortui* comb. nov., nom. rev.

International Journal of Systematic Bacteriology, v. 39, n. 4, p. 382–386, 1989.
WICKHAM, H. **ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York**. [s.l: s.n.]. v. 35
WIESE, J. et al. *Kiloniella laminariae* gen. nov., sp. nov., an Alphaproteobacterium from the marine macroalga *Laminaria saccharina*. **International Journal of Systematic and Evolutionary Microbiology**, v. 59, n. 2, p. 350–356, 2009.
WIESE, J. et al. Genome analysis of the marine bacterium *Kiloniella laminariae* and first insights into comparative genomics with related Kiloniella species. **Archives of Microbiology**, v. 202, n. 4, p. 815–824, 2020.
YADAV, D.; SINGH, A.; MATHUR, N. Review Article Halophiles-A Review. **International Journal of Current Microbiology and Applied Sciences**, v. 4, n. 12, p. 616–629, 2015.
ZEHR, J. P.; WARD, B. B. Nitrogen cycling in the ocean: New perspectives on processes and paradigms. **Applied and Environmental Microbiology**, v. 68, n. 3, p. 1015–1024, 2002.
ZHANG, M. et al. The effect of salinity on ammonium-assimilating biosystems in hypersaline wastewater treatment. **Science of the Total Environment**, v. 829, p. 154622, 2022.
ZHANG, X.; LIN, Y.; CHEN, G. Q. Halophiles as Chassis for Bioproduction. **Advanced Biosystems**, v. 2, n. 11, p. 1–12, 2018.
ZHAO, S. et al. Heatmap3: An improved heatmap package with more powerful and convenient features. **BMC Bioinformatics**, v. 15, n. 10, p. 15–16, 2014.
ZHOU, H. et al. Characterization of pyrene degradation by halophilic *Thalassospira* sp. strain TSL5-1 isolated from the coastal soil of Yellow Sea, China. **International Biodeterioration and Biodegradation**, v. 107, p. 62–69, 2016.

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Samples list, a collection station, sample identification, latitude, longitude, depth, and geomorphological feature from SB.

Station	Sample ID	LAT	LONG	Depth (m)	Geomorphological feature
259	Sed_Diapir_259	26°33.776'S	46°07.008'W	559	Salt of diapir
260	Sed_Diapir_260	26°33.710'S	46°07.470'W	517	Salt of diapir
255	Sed_Control_255	26°29.677'S	45°58.153'W	652	Control
258	Sed_Control_258	26°15.715'S	45°40.97'W	730	Control
257	Sed_Pock_257	26°32.075'S	46°04.822'W	543	Pockmark

Supplementary Table 2. Taxonomic identity of bacterial strains isolated from culture enriched with salt diapir sediment, control, and pockmark in SB. All isolated strains were identified using the Silva v.138 database.

Isolated ID	Geomorphological feature	Station	Depth (m)	Isolated from HM		Taxonomy
				10%	25%	
Diapir_260_1	Salt of diapir	259	559		x	Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*
Control_258_2	Control	258	730		x	Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*
Pock_257_1	Pockmark	257	543		x	Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*
Pock_257_2	Pockmark	257	543		x	Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*
Pock_257_3	Pockmark	257	543		x	Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*
Diapir_259_1	Salt of diapir	259	559	x		Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*
Diapir_259_2	Salt of diapir	259	559	x		Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*
Diapir_259_3	Salt of diapir	259	559	x		Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*
Diapir_260_1	Salt of diapir	260	517	x		Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*
Diapir_260_2	Salt of diapir	260	517	x		Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*
Diapir_260_3	Salt of diapir	260	517	x		Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*
Control_255_1	Control	255	652	x		Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*
Control _255_1.1	Control	255	652	x		Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*
Control _255_2	Control	255	652	x		Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*
Control _255_3	Control	255	652	x		Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*
Control _258_1	Control	258	730	x		Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*
Control _258_2	Control	258	730	x		Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*
Control _258_2.2	Control	258	730	x		Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*
Control _258_3	Control	258	730	x		Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*
Pock_257_1	Pockmark	257	543	x		Proteobacteria;Gammaproteobacteria;Salinisphaerales;Salinisphaeraceae; *Salinisphaera shabanensis*
Pock_257_2	Pockmark	257	543	x		Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*
Pock_257_3	Pockmark	257	543	x		Proteobacteria;Gammaproteobacteria;Pseudomonadales;Halomonadaceae;*Chromohalobacter salexigens*

Supplementary Table 3. Relative abundance of the families Halomonadaceae and Salinisphaeraceae in the sediment samples of salt diapirs, control, and pockmark station, and the samples of culture enriched with the same sediments. Analysis based on sequencing of the 16S ribosomal gene.

Family	**Relative abundance (%)**									
	Control 255	Pockmark 257	Control 258	Diapir 259	Diapir 260	Culture 255	Culture 257	Culture 258	Culture 259	Culture 260
Halomonadaceae	0.0929	0.1289	0.0602	0.0513	0.1297	14.8943	18.3280	2.1750	4.5172	15.6361
Salinisphaeraceae	0	0	0	0.0102	0	0	4.3869	0	0.0085	0.0068

CHAPTER IV - METABOLIC POTENTIALS AND GENOME-RESOLVED METAGENOMICS OF SALT DIAPIRS' SEDIMENTS IN POCKMARKS FIELDS FROM THE DEEP SOUTHWEST ATLANTIC OCEAN

This chapter is under review by the authors for journal submission

Abstract

Marine sediment composes a vast and dynamic ecosystem with physical and chemical gradients shaped by biotic and abiotic processes and potentially inhabited by bacteria and archaea. Microbial communities in marine sediment are responsible for the largest share of ocean biomass, mediating global biogeochemical cycles. Despite the importance of microorganisms in marine sediment, they are poorly understood, and obtaining a pure culture of microorganisms, especially from complex environments, is quite difficult, limiting our knowledge about the real local diversity. Improvements in computing infrastructure and greater accessibility of high-throughput DNA sequencing technologies have provided new insights into microbial diversity and interactions. The present work aimed to describe potential microbial metabolisms and reconstruct microbial genomes using metagenomic data. The pockmark field, in the Santos Basin, occupies a polygonal area of 130 × 30 km and is located on the shelf break. Sediment samples from this area were collected in July 2016 aboard the Research Vessel Alpha Crucis using a stainless-steel box. Samples Diapir_259 and Diapir_260 were collected from salt diapir stations and sample Control_255 was collected from the station without a defined geomorphological feature (here called control). Only the surface layer (0 – 2 cm) was collected with sterile spatulas and stored in sterile bags. DNA was extracted and paired-end shotgun metagenomic sequencing (2 x 150 bp) was performed on an Illumina HiSeq platform. Metagenomic sequencing was performed at Woods Hole Institute's Marine Biology Laboratory, as part of the "Deep Carbon Observatory's Census of Project Deep Life". The metagenomic library constructed from three surface sediment samples of salt diapirs and control sediment yielded 77.922.888 raw readings which, after processing, were later grouped into 34 MAGS. Only 8 MAGs showed completeness above 70% and were selected for taxonomic and metabolic detailing using the DRAM software. From the recovered MAGs, our results provide the first information about the microbial communities of the sediment and the possible metabolisms predominant in the sampled areas. We emphasize the importance of these communities in the carbon, sulfur and nitrogen cycles in the pockmark fields of the deep Southwest Atlantic.

Keywords: Metagenomic; Deep Sea; Salt Diapirs; Pockmark; Southwest Atlantic

IV.1 INTRODUCTION

The marine seafloor comprises vast and dynamic ecosystems with physical and chemical gradients shaped by biotic and abiotic processes (PARKES et al., 1994; PARKES et al., 2014). Microbial communities in marine sediments account for the largest fraction of ocean biomass, mediating global biogeochemical cycles (ORCUTT et al., 2011; ARNDT et al., 2013). Despite the importance of microorganisms in marine sediments, their diversity remains poorly understood, and a considerable portion is not yet cultivable in the laboratory (DURBIN; TESKE, 2011; HOSHINO et al., 2020; BLANDÓN et al., 2022). Particularly in the deep sea, these uncultivated strains make up a large fraction of the seafloor microbiome (PARKES et al., 2014; HOSHINO et al., 2020).

Obtaining pure cultures of microorganisms, especially from complex environments, is very challenging, limiting our knowledge about the real local diversity. However, advances in high-throughput DNA sequencing technologies have provided new insights into microbial diversity and their potential interactions (IVERSON et al., 2012; ABRAM, 2015; CARDENAS et al., 2015). Metagenomic sequencing comprises an independent-cultivation technique that allows the recovery of complete or partial microbial genomes, which can give clues regarding their potential metabolic roles within an ecosystem (TRINGE et al., 2005; ABRAM, 2015). This sequencing enables the assembly of genomes of microorganisms that make up only 1% of the population in ocean waters and marine sediments, providing numerous advances in the area (SHARON; BANFIELD, 2013).

The extension of the continental margin of Brazil is one of the largest on Earth, harbouring several sedimentary basins with numerous geomorphological characteristics. The Santos Basin (SB), located in the southeastern portion of the Brazilian continental margin, houses an area of pockmark fields because more than 900 depressions associated with this formation have already been recorded in this region (SUMIDA et al., 2004; DE MAHIQUES et al., 2017). In addition to the pockmarks, this area is characterised by being influenced by salt tectonics and contains numerous salt diapirs (GARCIA et al., 2012; SCHATTNER et al., 2018).

Several geological and chemical characterisation studies have been carried out in these pockmark and salt diapir fields (SUMIDA et al., 2004; DE MAHIQUES et al., 2017b; DOS SANTOS et al., 2018; SCHATTNER et al., 2018; RAMOS et al., 2020). However, there is still no description of microorganisms inhabiting the area. Here, we aimed to study sediment-associated microorganisms collected in salt diapirs in a pockmark field using genome-resolved metagenomics and metagenomics approaches, in which we were able to describe the metabolic potentials of the microorganisms found.

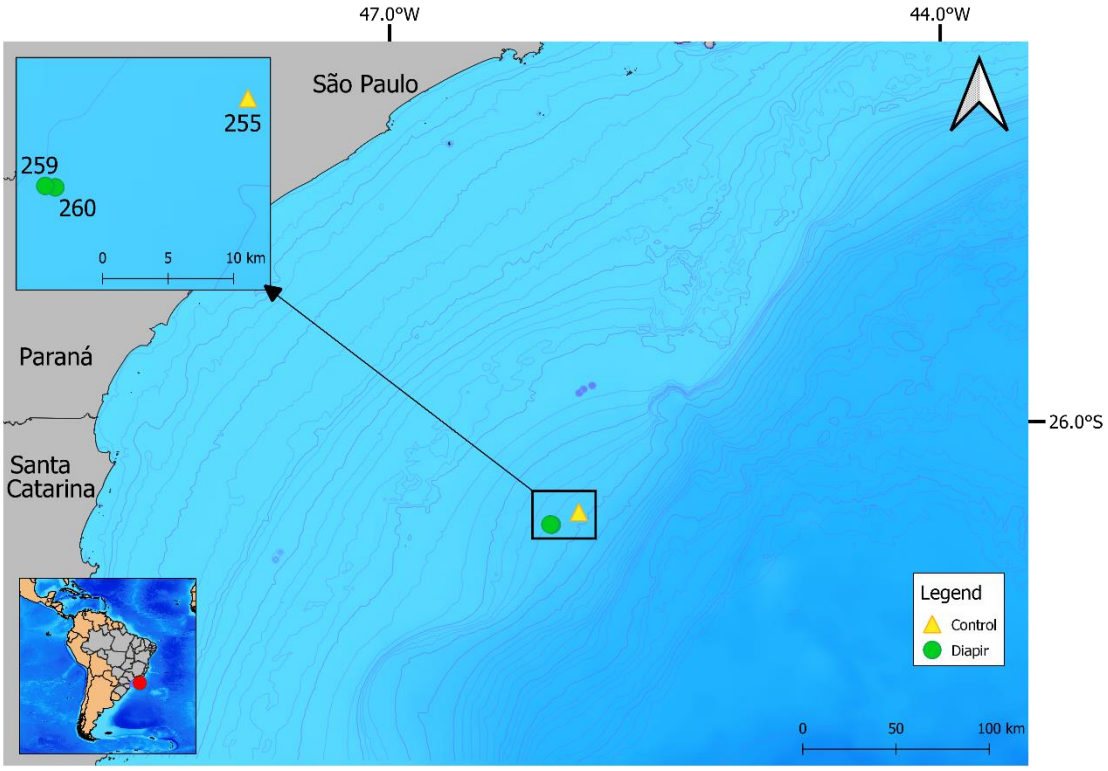
IV.2 MATERIALS AND METHODS

IV.2.1 Study area and sediment sampling

The Santos Basin (SB) spans the coast of the states of Rio de Janeiro, São Paulo, Paraná, and Santa Catarina, occupying approximately 350 thousand km² (SILVEIRA et al., 2000). In SB the water mass is formed by the Brazil Current (BC), which is formed by the stacking of water masses that are characteristic of the South Atlantic, composed in the first 1,500 meters by Tropical Water (AT), Central Water of the South Atlantic (ACAS), Intermediate Water Antarctica (AIA), Northern Deep Water (APAN) and Antarctic Bottom Water (AAF) (SILVEIRA et al., 2000).

This pockmark field, in SB, occupies a polygonal area of 130 × 30 km along with the continental and was initially described by Calder et al (2002). Sediment samples were collected in July 2016 aboard the Research Vessel Alpha Crucis (Oceanographic Institute of the University of São Paulo) at three stations (**Figure 1**) using a stainless-steel box (BX-650) (50 cm x 50 cm) with a maximum penetration of 60 cm. Samples Diapir_259 and Diapir_260 were collected from salt diapir stations and sample Control_255 was collected from the station without a defined geomorphological feature (here called control) (**Table S1**). Cylindrical corers were used to remove the sediment from the box corer. The surface layer (0 – 2 cm) was collected with sterile spatulas and stored in swirl bags and then stored at -20 °C until processed.

Figure 1. A map of the study region in the pockmark field located in the Santos Basin, highlighting the sampling stations. The green squares indicate stations in salt diapirs and the yellow triangle indicates control sampling.



Source: Augusto Miliorini Amendola, 2022.

IV.2.2 DNA extraction and shotgun metagenomics

DNA was extracted from 0.25 g of surface sediment (0-2 cm) using the Power Soil DNA Isolation Kit (MoBio Laboratories, USA), according to the manufacturer's specifications. The sediment from each station was extracted 10 times to obtain sufficient DNA concentration for metagenomic sequencing, totaling 2.5 g of extracted sediment per station. DNA concentration was assessed with the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, São Paulo, Brazil), and a Qubit Fluorometer 1.0, both following the manufacturer's instructions. DNA integrity was verified on a 1% agarose gel.

Paired-end shotgun metagenomic sequencing (2 x 150 bp) was performed on an Illumina HiSeq platform following standard methods as provided by the manufacturer, and library preparation was performed using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA), with a magnetic bead clean-up step before library preparation. Metagenomic sequencing was performed at Woods Hole Institute's Marine Biology Laboratory, as part of the "Deep Carbon Observatory's Census of Project Deep Life". Raw metagenome sequences are available in the GenBank repository under BioProject ID PRJNA818670.

IV.2.3 Taxonomic and functional annotation of metagenomic reads

Initially, reads were trimmed, and then low-quality reads (Phred score <30) were discarded through the SICKLE software. Then, filtered reads were analysed using the open-source online server Metagenomics Rapid Annotation Subsystem (MG-RAST) (MEYER et al., 2008). After uploading filtered reads in MG-RAST, a normalisation step was performed using the default parameters, in which ambiguous base and duplicated sequences are removed and a new quality check is performed. Subsequently, the sequences were screened for potential protein-coding genes, and annotation was carried out through the SEED Subsystems database with a minimum e-value of 1e-5 and minimum alignment of 50 bp and 60% identity. The National Center for Biotechnology Information (NCBI) Reference Sequence Database (Refseq) was selected for taxonomic classification. The MG-RAST data were analysed in R (R Development Core Team), using the vegan (OKSANEN et al., 2013) end ggplot2 (WICKHAM, 2009) packages.

IV.2.4 Assembly and reconstruction of metagenome-assembled genomes (MAGs)

We performed a co-assembly approach to optimise the quantity and quality of MAGs, as suggested in a previous study (EREN et al., 2015). Filtered reads were used for co-assembly and genomic reconstruction was performed using the anvi'o v. 7 pipeline (EREN et al., 2015). Co-assembly was performed using MEGAHIT v1.2.9 software (LI et al., 2015), and contigs with a size >4.000 bp were selected for binning using CONCOCT v1.1 software (ALNEBERG et al., 2013). The resulting bins were manually refined using anvi-refine (EREN et al., 2015) and then their qualities were checked with CheckM v. 1.0.7 (PARKS et al., 2015). Bins were taxonomically classified using the Genome Taxonomy Database Toolkit classification workflow (GTDB-Tk v. 1.3.0, database release 202).

We used the DRAM software (Distilled and Refined Annotation of Metabolism, v. 1.2.4) (SHAFFER et al., 2020) for functional annotation of the high and medium-quality MAGs (completeness >70% and contamination <3%). This tool uses hidden marker models of KEGG proteins (ARAMAKI et al., 2020) to identify and annotate protein-coding genes, and thus it was possible to annotate genes related to nitrogen (N_2), sulphur, methane, and carbon metabolisms. For more details about DRAM see: https://github.com/WrightonLabCSU/DRAM/wiki/1.-How-DRAM-Works. The data analysis was carried out with R software (R Development Core Team), using the packages vegan (OKSANEN et al., 2013) and ggplot2 (WICKHAM, 2009).

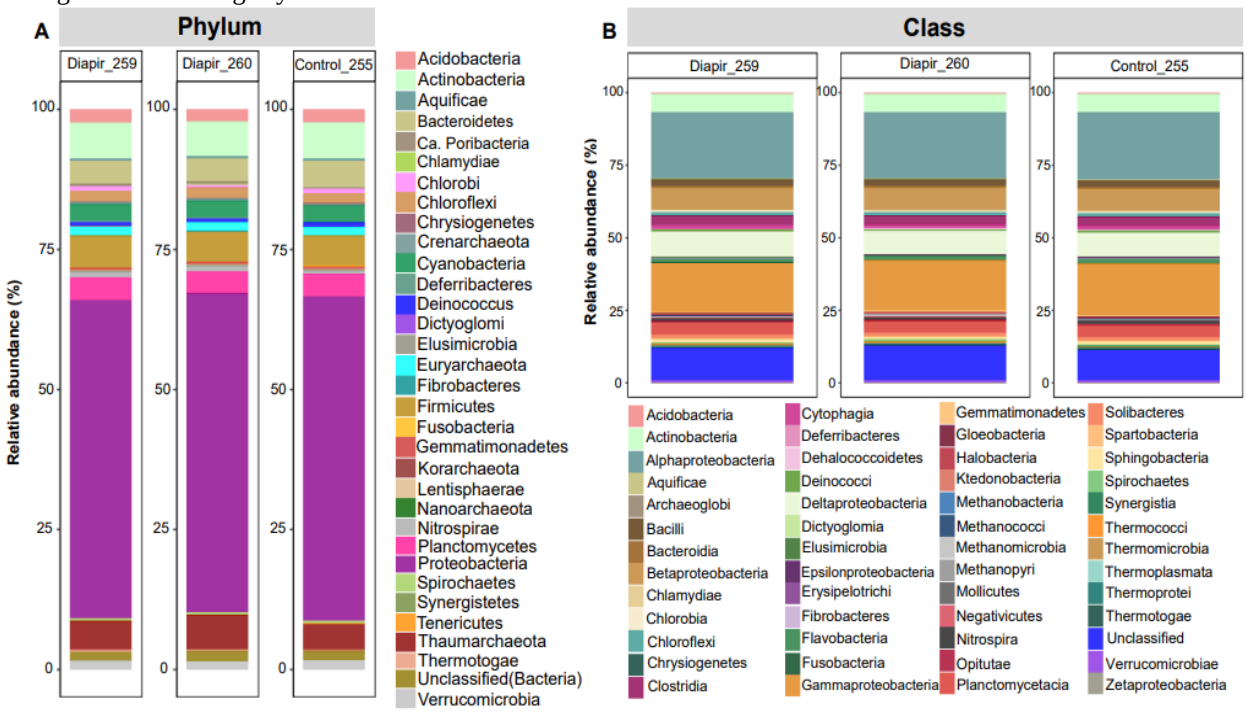
IV.3 RESULTS AND DISCUSS

IV.3.1 Taxonomic profile of microbial communities

Shotgun metagenomic sequencing of the three surface sediment samples from the salt diapir and control stations yielded 77.922.888 raw reads. After quality filtering, the number of reads per sample was 23.406.011 (Control_255), 19.431.982 (Diapir_259) and 22.090.471 (Diapir_260).

Through taxonomic analysis, it was possible to identify 32 phyla, of which five belonged to the Archaea and the others to Bacteria. Despite the sampling stations being geographically separated, the taxonomic composition of the microbial communities was very similar among them, with the most abundant phylum being Proteobacteria, representing more than 50% of the relative abundance, followed by Actinobacteria (6%, 6%, and 7%, respectively), Firmicutes (6%, 6%, and 5%, respectively), Bacteroidetes (4%, 4%, and 5%, respectively), Thaumarchaeota (6%, 5%, and 5%, respectively) and Planctomycetes (4% in all samples). All other phyla represented less than 3% relative abundance in each sample (**Figure 2A**). The classes observed as dominant were: Alphaproteobacteria, with more than 22% relative abundance in all samples, followed by Gammaproteobacteria (up to 15%), Deltaproteobacteria (up to 8%), Betaproteobacteria (up to 7%), Actinobacteria (up to 6%), Planctomycetacia and Clostridia (both up to 3%) (**Figure 2B**).

Figure 2. Relative abundances of microbial community taxonomy based on the annotation of shotgun metagenomics readings by MG-rast.



Representation at phylum (A) and class (B) levels.

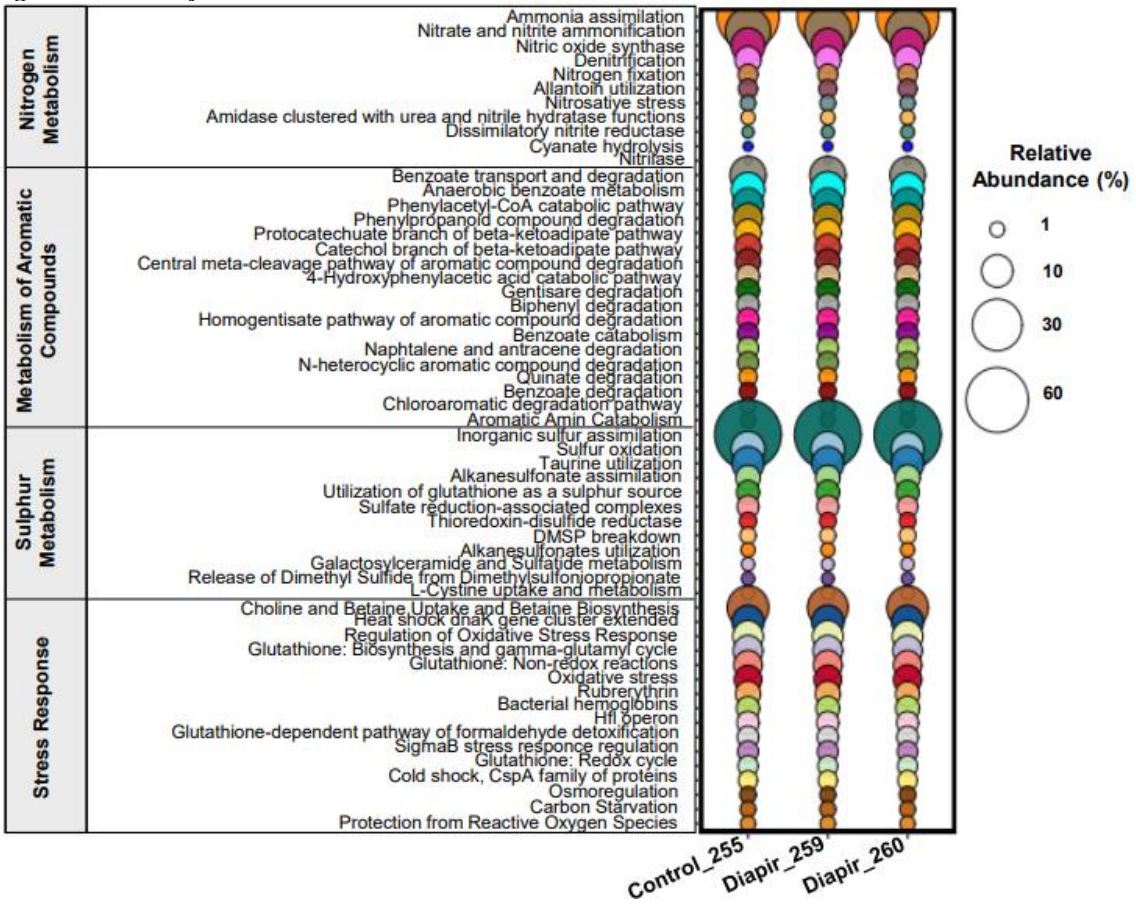
Although two samples were taken in salt diapir areas, they were not different in composition when compared to the control, even considering the microbial groups related to saline environments, such as Halobacteria. One explanation for the taxonomic similarity among the samples could be the presence of similar physical and oceanographic conditions along with the sediment-water interface, such as temperature and salinity, which could favour certain taxonomic groups in addition to the current flowing in the same direction, which could aid in dispersion and mixing of the microbial groups in the sediments of the area.

In the area, there is an influence of the Brazil Current, which flows toward the south, bordering the southern continent and, thus, bathing the SB (SIGNORINI et al., 1989; SILVEIRA et al., 2000). The points sampled here range from 517 to 652 meters in depth, which fits the entire area in the same water mass: Antarctic Intermediate Water (AAIW). This water mass is found at depths ranging from 500 to 1.200 meters, with temperatures ranging from 3 to 6 °C and salinities ranging from 34.2 to 34.6 (SILVEIRA et al., 2000).

IV.3.2 Metabolic profile of microbial communities

The study of the relationship between microorganisms and biogeochemical cycles is of great relevance to understanding the marine ecosystem (MADSEN, 2011; PARKES et al., 2014; BAKER; APPLER; GONG, 2021). This information provides basic clues about different pathways of transformation, production, and cycling of organic matter (GRIGGS et al., 2013; BAKER; APPLER; GONG, 2021). We were able to have an overview of the main potential microbial metabolisms present in our samples. We focus on the following categories: nitrogen metabolism; degradation of aromatic compounds; sulphur metabolism; and stress response (**Figure 3**).

Figure 3. Relative abundance of functional profiles of microbial communities generated by annotation of metagenomic reads by MG-rast.



Nitrogen is a limiting factor for oceanic biological production and influences other equally relevant biogeochemical cycles, such as the carbon and phosphorus cycle (HUTCHINS; FU, 2017; KUYPERS; MARCHANT; KARTAL, 2018; PAJARES; RAMOS, 2019). Nitrogen is naturally present in the environment, either as a product of the degradation of organic and

inorganic compounds, fixation of gaseous nitrogen, or gas exchange with the atmosphere (CHO; AZAM, 1963; PAJARES; RAMOS, 2019).

This element is found in several chemical forms and numerous microorganisms participate in its cycling. However, certain groups collaborate with different stages of nitrogen transformation (KUYPERS; MARCHANT; KARTAL, 2018).

In our samples, the most abundant nitrogen cycle metabolism was ammonia assimilation (an average value of 49.5%). The conversion of N_2 to ammonium is a biologically very important process since many microorganisms rely on this reaction to obtain nitrogen for growth (for amino acids) while transforming an inert form into a form that is biologically available to other microorganisms (ZEHR; WARD, 2002; JETTEN, 2008; SMITH et al., 2016).

The degradation of aromatic compounds is of great importance for the biogeochemical carbon cycle since these compounds can constitute a quarter of plant biomass and represent up to about 20% of terrestrial biomass (DURAN; CRAVO-LAUREAU, 2016; GONZÁLEZ-GAYA et al., 2019). These compounds are abundant and structurally diverse, and their degradation is mainly controlled by aerobic and anaerobic bacteria (BUGG et al., 2011). The aromatic structure makes this substrate inert to oxidation or simple reduction, which requires elaborate strategies for the degradation of these compounds, such as the cleavage of the central ring, which eliminates the aromatic character of this molecule.

In our samples, the metabolism of aromatic compounds was mainly related to the degradation of benzoate (an average value of 14%), an intermediate product formed in the fermentation of organic matter (FUCHS; BOLL; HEIDER, 2011). The organic matter that reaches the marine sediment can be of autochthonous origin (primary production, for example) and be more easily degraded or be allochthonous (terrestrial organic matter), whose degradation is slower (COLE, 1999; ZONNEVELD et al., 2010). Dissolved organic matter is responsible for more than 97% of all organic matter found in marine waters, being a major carbon reservoir on the planet (CALVERT, 1987; PARKES et al., 2014). The microbial communities in the sediment, especially those in the surface sediment, process organic and inorganic carbon, contributing to the cycling of this material.

Sulphur is an essential element in the terrestrial biosphere, and microorganisms play important roles in this element's biogeochemical cycle. Different metabolisms related to the sulphur cycle and its intermediates were highlighted in our results, especially inorganic sulphur assimilation (average value of 58.6%), a pathway used by almost all microbial groups to reduce sulphate or sulphite in organic sulphur compounds (L-cysteine, for example) (KAWANO; SUZUKI; OHTSU, 2018). In natural environments, the metabolic processes involved in the

sulfur cycle are spatially segregated according to the supply of reduced and oxidized compounds (FIKE; BRADLEY; ROSE, 2015; MORAN; DURHAM, 2019).

IV.3.3 Description and taxonomic assignment of MAGs

In this approach, the reads of the three samples were co-assembled, which makes it possible to obtain high-quality MAGs, thus taking advantage of the differential abundances of the contigs of each sample (**Table S2**). This approach also allows the recovery of low-abundance microbial genomes (NARASINGARAO et al., 2012; URITSKIY; DI RUGGIERO, 2019). Co-assembly of paired readings resulted in 108,018 contigs greater than 1.000 bp and with N50 equal to 1.492 bp, which were binned and resulted in a total of 34 MAGs. Two MAGs were classified as high-quality drafts (>90% complete, <5% contamination), 16 as medium-quality draft (>50% complete, <10% contamination), and 16 as low-quality draft (<50% complete, <10% contamination), according to genome quality standards suggested by Bowers et al (2017) (**Table S3**).

Regarding the taxonomic classification (GTDB, release 202), 20 MAGs were classified as Bacteria and 14 as Archaea. The bacterial phyla of MAGs were: Methylomirabilota, Nitrospirota, Myxococcota, Proteobacteria, Tectomicrobia, Binatota, and Dadabacteria, while all MAGs belonging to archaea were classified in a single phylum: Crenarchaeota. A total of 25 MAGs could be classified at the family level, namely: CPS1-5, Nitrospiraceae, Nitrosomonadaceae, GCA-2729495, UBA4486, UBA8639, SPGG2, Woeseiaceae, Kiloniellaceae, and Nitrosopumilaceae.

A total of eight MAGs were selected for DRAM analysis according to their completeness (>70%) and contamination score (<3%). These 8 MAGs belong to the Bacteria Domain and were assigned to the classes: Methylomirabilis (MAG_00001), Nitrospiria (MAG_00002), Gammaproteobacteria (MAG_00003, MAG_00004, MAG_00005, MAG_00006, and MAG_00007) and Binatia (MAG_00008) (**Table 1**).

Table 1. List of the 8 selected MAGs and their taxonomic classification based on GTDB-Tk.

MAGs	GTDB-TK taxonomy	Total length	N. of Contig	N50	CG %	Completeness	Contamination	Draft Quality
MAG_00001	Bacteria; Methylomirabilota; Methylomirabilis, CPS1-5; CPS1-5	2397160	403	7968	58.583	94.244	2.158	High
MAG_00002	Bacteria; Nitrospirota; Nitrospiria; Nitrospirales;Nitrospiraceae	2379090	323	9268	57.953	90.647	2.877	High
MAG_00003	Bacteria; Proteobacteria; Gammaproteobacteria; Burkholderiales; Nitrosomonadaceae; GCA2721545	1518180	520	3070	44.422	83.453	1.438	Medium
MAG_00004	Bacteria; Proteobacteria; Gammaproteobacteria; Burkholderiales; Nitrosomonadaceae; GCA2721545	2823452	928	3165	57.198	82.014	2.158	Medium
MAG_00005	Bacteria; Proteobacteria; Gammaproteobacteria; GCA-2729495; GCA-2729495; GCA2729495	5918495	2.328	2573	54.233	76.978	2.158	Medium
MAG_00006	Bacteria; Proteobacteria; Gammaproteobacteria; HK1	3537226	1049	3646	63.004	72.661	0.719	Medium
MAG_00007	Bacteria; Proteobacteria; Gammaproteobacteria; UBA4486; UBA4486	3537226	515	2826	40.507	71.942	2.877	Medium
MAG_00008	Bacteria; Binatota; Binatia; UBA9968	4170056	2024	2000	53.166	71.942	2.877	Medium

IV.3.4 Potentials for carbon metabolism in MAGs recovered from SB samples

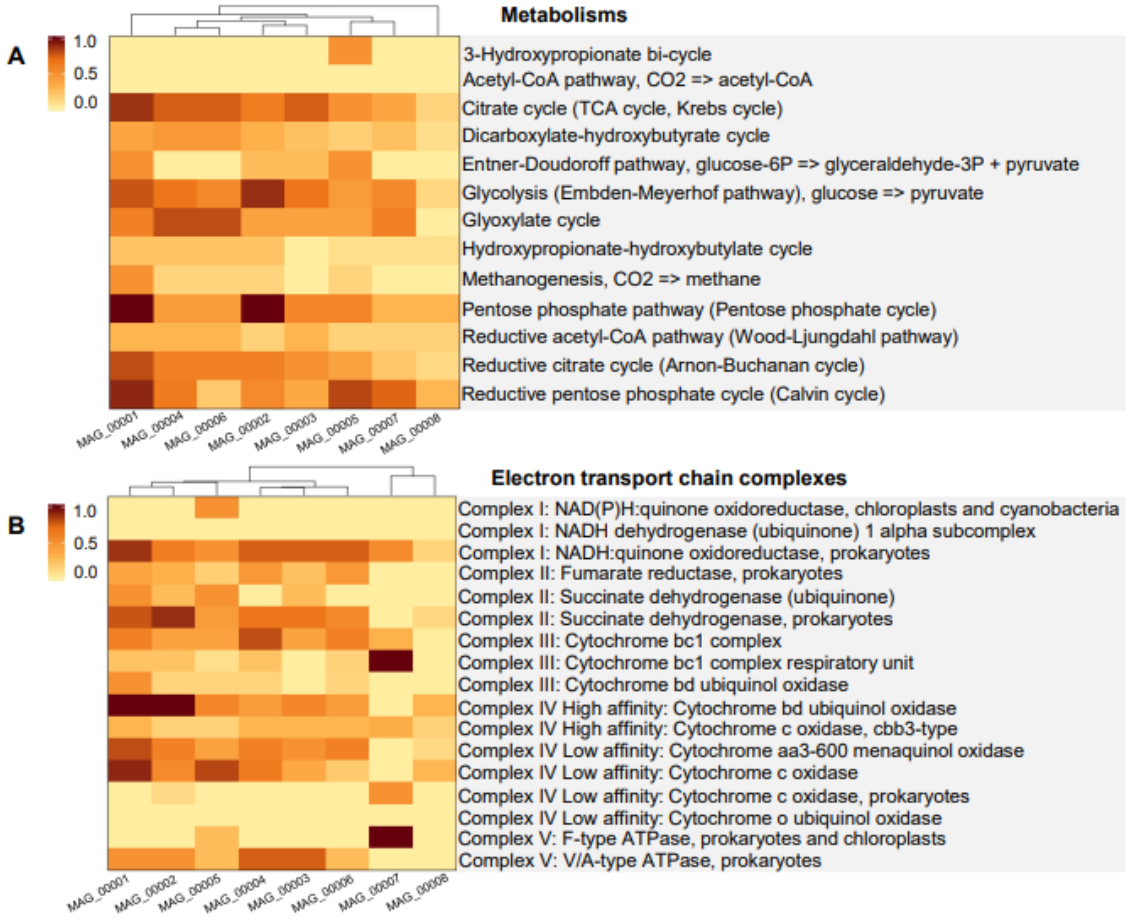
Through functional analyses of the 8 selected MAGs, it was possible to observe that genes related to Citrate cycle (Krebs cycle), Dicarboxylate-hydroxybutyrate cycle, Glycolysis (Embden-Meyerhof pathway), Pentose phosphate pathway (Pentose phosphate cycle), Reductive acetyl-CoA pathway (Wood-Ljungdahl pathway), Reductive citrate cycle (Arnon-Buchanan cycle) and Reductive pentose phosphate cycle (Calvin cycle) were present in all MAGS, however, with different pathway completions, The MAGs MAG_00001 (Methylomirabilota) and MAG_00002 (Nitrospirota) showed a complete Pentose phosphate pathway, while MAG_00007 (Proteobacteria) and MAG_00008 (Binatota) showed the lowest proportions of genes for this pathway (**Fig.4A**).

In other pathways such as Glycolyses, Citrate cycle, and Reductive Citrate cycle, we observed the same pattern, in which MAG_00001 and MAG_00002 have greater completeness of genes for these pathways. Genes related to the Calvin cycle were detected in higher completion in the MAGs of Methilomirabilis (MAG_00001) and Gammaproteobacteria (MAG_00005). This pathway is nature's most important autotrophic CO_2 fixation process (HÜGLER; SIEVERT, 2011), along with the 3-Hydroxypropionate pathway, in which genes were found only in MAG_00005.

Genes belonging to the Methanogenesis pathway were observed in low abundance in MAGs MAG_00002, MAG_00004, MAG_00005, and MAG_00006, and only MAG_00001 showed a higher proportion of these genes, which can be explained by the known methylotrophic, but not methanotrophic, metabolism of the CSP1-5 family (HUG et al., 2016).

Different genes related to electron transport chain complexes, associated with aerobic respiration, were also identified in the analysed MAGs (**Figure 4B**). Except for MAG_00007, all the other MAGs showed genes for Complex IV High affinity: Cytochrome bd ubiquinol oxidase, but only MAG_00001 and MAG_00002 showed this complete pathway.

Figure 4. DRAM annotations of metagenome-assembled genomes (MAGs) of the eight MAGs selected for analysis.

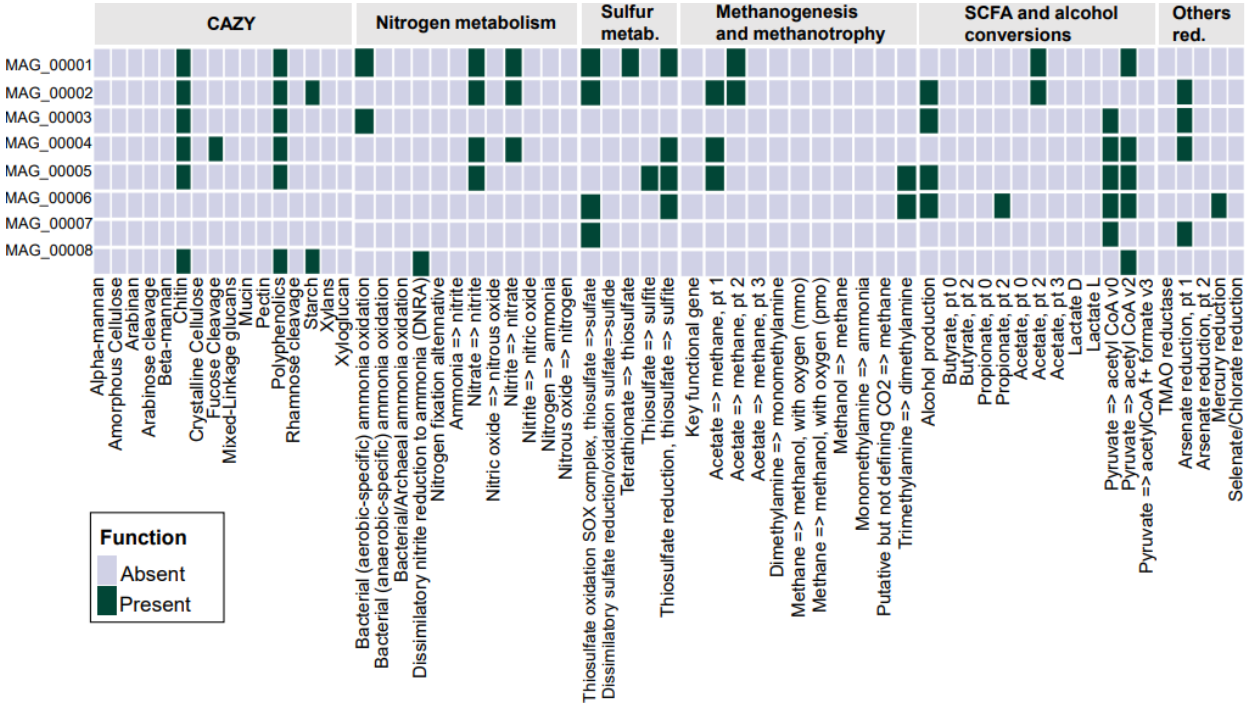


The MAGs were selected based on their completeness (> 70%) and contamination (< 3%) scores. Stronger colors indicate greater completeness of relevant metabolic pathways (A) and electron transport chain complexes (B).

IV.3.5 Metabolic potentials of MAGs: substrates degradation, sulfur and nitrogen metabolisms

The presence of carbohydrate-active enzymes (CAZymes) degradation genes in the MAGs was evaluated and we observed that only three MAGs showed genes to degrade the greatest number of substrates: MAG_00002 (chitin, polyphenolics, and starch), MAG_00004 (chitin, flucose cleavage, and polyphenolics) and MAG_00008 (chitin, polyphenolics, and starch), By contrast, in MAG_00006 and MAG_00007 (both Gammaproteobacteria) no CAZymes degradation gene was detected (**Figure 5**).

Figure 5. DRAM annotations of metagenome-assembled genomes (MAGs) from salt diapir sediments and control in SB.



MAGs were selected based on completeness (> 70%) and contamination (< 3%) scores. The colors in the heat map represent the presence or absence of relevant metabolic functions in each MAG.

Among the 8 MAGs analysed, 6 had chitin degradation genes, which can be an advantageous trait since chitin is the most abundant biodegradable polymer in the oceans and can be used as a source of carbon and energy (KURITA, 2001). The degradation of recalcitrant compounds is also an important process for the marine food web, as it releases carbon and nitrogen into the trophic chain (KURITA, 2001; BHATTACHARYA; NAGPURE; GUPTA, 2007). Carbohydrates in general are extremely important in the marine environment, making up the largest fraction of organic matter in the oceans (SMITH, S V, HOLLIBAUGH, 1993; ARNOSTI et al., 2021). They are produced mainly by phytoplankton and macroalgae, and play a special role in continental shelf regions where there is high primary production (THOMAS et al., 2004; HEHEMANN; BORASTON; CZJZEK, 2014). The polysaccharides synthetised by these algae are degraded and consumed along the water column (ACHA et al., 2004). However, a fraction can reach regions of great depths, being an important source of carbon for the region (GAO et al., 2017). Heterotrophic microorganisms degrade high molecular weight polysaccharides into low molecular weight substrates, which is critical for the carbon cycle on Earth (HEHEMANN; BORASTON; CZJZEK, 2014). For this carbohydrate degradation, marine microorganisms need different enzymes, which are fundamental for the subsequent

central sugar metabolism (KLIPPEL et al., 2014). However, our knowledge about these enzymatic capacities in the deep sea is still little explored (KLIPPEL et al., 2014; ARNOSTI et al., 2021).

Processes related to the nitrogen cycle were also evaluated, and different genes were found in our MAGs. Genes for nitrification, one of the most important steps in the nitrogen cycle, including ammonium oxidation genes, were present in MAG_00001 (Methylomirabilota) and MAG_00003 (Nitrosomonadaceae). The nitrification process involves two steps that include the oxidation of ammonium via hydroxylamine to nitrite and, later, the conversion to nitrate; it is a key process in the cycling of nitrogen in marine sediments (PURKHOLD et al., 2000; SEYMOUR, 2014). The first step of nitrification is carried out by a limited number of microorganisms, which include a few bacteria and archaea (TREUSCH et al., 2005). MAG_00003, which was classified as Nitrosomonadaceae, is related to a known chemolithoautotrophic ammonia-oxidising group, containing genes to perform this step (GARRITY; BELL; LILBURN, 2015), as found in our results. Genes for the following steps in the pathway were also identified: oxidation of nitrite to nitrate and the conversion of nitrate to nitrite in MAGs MAG_00001, MAG_00002, MAG_00004, and MAG_00005.

Genes for dissimilatory nitrate reduction to ammonia (DNRA) were found only in MAG_00008. This MAG was assigned to the Binatia class, a taxon containing only uncultured and poorly characterised bacteria. Using genome-resolved metagenomics Rodríguez-Ramos et al (2021) showed that members of this class are able to encode the dissimilatory reduction of nitrite to ammonium (DNRA).

We observed that different genes related to the sulphur cycle were found in the MAGs: oxidation of thiosulfate to sulphate (by SOX complex) (MAG_00001, MAG_00002, MAG_00006, and MAG_00007) tetrathionate to thiosulfate (MAG_00001) thiosulfate to sulphite (MAG_00005) and reduction of thiosulfate to sulphite (*rdlA* gene) (MAG_00001, MAG_00004, MAG_0000,5, and MAG_00006).

Among the genes evaluated, the only one that was not represented in any MAG was the reduction and oxidation of sulphate to sulphide. Sulphur intermediates such as thiosulfate ($S_2O^{2-}_3$), tetrathionate (S_4O^{2-6}), and sulphite (SO^{2-3}_) can be produced during the oxidation of sulphide. These intermediate compounds can be reduced back to sulphide form or further oxidised to sulphate. Possessing genes to utilise sulphur intermediates can be advantageous, since these compounds have a high redox potential, and thiosulfate, for example, is quite stable in marine sediments (JORGENSEN; BAK, 1991; JØRGENSEN; FINDLAY; PELLERIN, 2019).

Pockmarks are formed mainly by the sudden release of methane gas (JUDD; HOVLAND, 2007; JUDD; HOVLAND, 2009). These craters can remain emitting gas for indefinite periods. However, the dynamics of activation and deactivation of pockmarks are still not well understood, and these craters can return to activity at any time (CHAND et al., 2008; CATHLES; SU; CHEN, 2010; DE MAHIQUES et al., 2017a). During the collection, there was no evidence of active pockmarks in the area (SUMIDA et al., 2004; DE MAHIQUES et al., 2017a). However, the degradation of organic matter, which is deposited on the ocean floor, can produce a substantial part of methane (REEBURGH, 2007) (WEBER; WISEMAN; KOCK, 2019) (BEULIG et al., 2019), and can thus sustain methanotrophic communities.

Through the taxonomic results, we observed the presence of only two methylotrophs: CSP1-5 (MAG_00001), already described in other works as a classic methylotroph (HUG et al., 2016; VERSANTVOORT et al., 2018), and Binatia (MAG_00008), Despite Binatia being a poorly studied and not yet cultivated group, carried out a study using eleven MAGs of the Bin18 and Binatales orders, in which the presence of genes encoding copper membrane monooxygenases (CuMMOs) belonging to a family of enzymes that includes particulate methane monooxygenase (pMMO) was demonstrated, Genes encoding CuMMO subunits were observed in all MAGs evaluated with an organisation similar to that found in the pMMO operon of Verrucomicrobia, methanotrophic Proteobacteria, and Methylomirabilis (CSP1-5) (VERSANTVOORT et al., 2018).

Despite the great metabolic diversity presented, it is important to note that the recovered MAGs have completeness that ranged from 71% to 94%, so the absence or low occurrence of a gene does not mean its absence in the genome, but it may mean non-recovery of these genes in our analyses.

IV.4 CONCLUSION

In summary, the metagenomic analysis of the sediment collected in salt diapirs along the pockmark field and the control area showed great taxonomic diversity in addition to allowing the assembly of diverse draft genomes. These genomes belong to taxa still little explored and uncultured, mainly for the deep-sea environment. When considering the study area, the reported biogeochemical cycles, and the recovered MAGs, our results provide the first information about the sedimentary communities and possible biogeochemical processes by using gene and genome-level approaches through metagenomics. Finally, our study brings greater clarity to the role of these microorganisms in the carbon, sulfur, and nitrogen cycles potentially occurring in the pockmark fields of the Southwest Atlantic and reinforce the importance of further investigations to elucidate whether they are actively performing these ecological processes.

REFERENCES

ABRAM, F. Systems-based approaches to unravel multi-species microbial community functioning. **Computational and Structural Biotechnology Journal**, v. 13, p. 24–32, 2015.

ACHA, E. M. et al. Marine fronts at the continental shelves of austral South America: Physical and ecological processes. **Journal of Marine Systems**, v. 44, n. 1–2, p. 83–105, 2004.

ALNEBERG, J. et al. CONCOCT: Clustering cONtigs on COverage and ComposiTion. p. 1–28, 2013.

ARAMAKI, T. et al. KofamKOALA: KEGG Ortholog assignment based on profile HMM and adaptive score threshold. **Bioinformatics**, v. 36, n. 7, p. 2251–2252, 2020.

ARNDT, S. et al. Quantifying the degradation of organic matter in marine sediments: A review and synthesis. **Earth-Science Reviews**, v. 123, p. 53–86, 2013.

ARNOSTI, C. et al. The Biogeochemistry of Marine Polysaccharides: Sources, Inventories, and Bacterial Drivers of the Carbohydrate Cycle. **Annual Review of Marine Science**, v. 13, p. 81–108, 2021.

BAKER, B. J.; APPLER, K. E.; GONG, X. New Microbial Biodiversity in Marine Sediments. **Annual Review of Marine Science**, v. 13, p. 161–175, 2021.

BEULIG, F. et al. Cryptic CH 4 cycling in the sulfate–methane transition of marine sediments apparently mediated by ANME-1 archaea. **ISME Journal**, v. 13, n. 2, p. 250–262, 2019.

BHATTACHARYA, D.; NAGPURE, A.; GUPTA, R. K. Bacterial chitinases: Properties and potential. **Critical Reviews in Biotechnology**, v. 27, n. 1, p. 21–28, 2007.

BLANDÓN, L. M. et al. Diversity of cultivable bacteria from deep-sea sediments of the Colombian Caribbean and their potential in bioremediation. **Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology**, v. 115, n. 3, p. 421–431, 2022.

BOWERS, R. M. et al. Minimum information about a single amplified genome (MISAG) and a metagenome-assembled genome (MIMAG) of bacteria and archaea. **Nature Biotechnology**, v. 35, n. 8, p. 725–731, 2017.

BUGG, T. D. H. et al. The emerging role for bacteria in lignin degradation and bio-product formation. **Current Opinion in Biotechnology**, v. 22, n. 3, p. 394–400, 2011.

CALDER, B. R.; FONSECA, L.; FRANCOLIN, J. B. Automatic estimation of the seafloor geomorphology of the Santos Basin, Brazil. **Eos, Transactions, American Geophysical Union**, v. 83, n. 47, Suppl., p. F728, 2002.

CALVERT, S. E. Oceanographic controls on the accumulation of organic matter in marine sediments. **Geological Society Special Publication**, v. 26, n. 26, p. 137–151, 1987.

CARDENAS, E. et al. Forest harvesting reduces the soil metagenomic potential for biomass decomposition. **ISME Journal**, v. 9, n. 11, p. 2465–2476, 2015.

CATHLES, L. M.; SU, Z.; CHEN, D. The physics of gas chimney and pockmark formation, with implications for assessment of seafloor hazards and gas sequestration. **Marine and Petroleum Geology**, v. 27, n. 1, p. 82–91, 2010.

CHAND, S. et al. Gas hydrate stability zone modelling in areas of salt tectonics and pockmarks of the Barents Sea suggests an active hydrocarbon venting system. **Marine and Petroleum Geology**, v. 25, n. 7, p. 625–636, 2008.

CHO, B. C.; AZAM, F. Major role of bacteria in biogeochemical fluxes in the ocean's interior. **Deep Sea Research and Oceanographic Abstracts**, v. 10, n. 1–2, p. 129, 1963.

COLE, J. J. Aquatic microbiology for ecosystem scientists: New and recycled paradigms in ecological microbiology. **Ecosystems**, v. 2, n. 3, p. 215–225, 1999.

DE MAHIQUES, M. M. et al. An extensive pockmark field on the upper Atlantic margin of Southeast Brazil : spatial analysis and its relationship with salt diapirism. **Heliyon**, n. August 2016, p. e00257, 2017a.

DE MAHIQUES, M. M. et al. An extensive pockmark field on the upper Atlantic margin of

Southeast Brazil: spatial analysis and its relationship with salt diapirism. **Heliyon**, v. 3, n. 2, p. e00257, 2017b.
DOS SANTOS, R. et al. Metal / Ca ratios in pockmarks and adjacent sediments on the SW Atlantic slope : Implications for redox potential and modern seepage. **Journal of Geochemical Exploration**, v. 192, n. February, p. 163–173, 2018.
DURAN, R.; CRAVO-LAUREAU, C. Role of environmental factors and microorganisms in determining the fate of polycyclic aromatic hydrocarbons in the marine environment. **FEMS Microbiology Reviews**, v. 40, n. 6, p. 814–830, 2016.
DURBIN, A. M.; TESKE, A. Microbial diversity and stratification of South Pacific abyssal marine sediments. **Environmental Microbiology**, v. 13, n. 12, p. 3219–3234, 2011.
EREN, A. M. et al. Anvi'o: An advanced analysis and visualization platform for 'omics data. n. October, 2015.
FIKE, D. A.; BRADLEY, A. S.; ROSE, C. V. Rethinking the ancient sulfur cycle. **Annual Review of Earth and Planetary Sciences**, v. 43, p. 593–622, 2015.
FUCHS, G.; BOLL, M.; HEIDER, J. Microbial degradation of aromatic compounds- From one strategy to four. **Nature Reviews Microbiology**, v. 9, n. 11, p. 803–816, 2011.
GAO, B. et al. Genome sequencing reveals the complex polysaccharide-degrading ability of novel deep-sea bacterium Flammeovirga pacifica WPAGA1. **Frontiers in Microbiology**, v. 8, n. APR, p. 1–15, 2017.
GARCIA, S. F. DE M. et al. Structural modeling based on sequential restoration of gravitational salt deformation in the Santos Basin (Brazil). **Marine and Petroleum Geology**, v. 35, n. 1, p. 337–353, 2012.
GARRITY, G. M.; BELL, J. A.; LILBURN, T. Nitrosomonadaceae fam. nov. . **Bergey's Manual of Systematics of Archaea and Bacteria**, p. 1–1, 2015.
GONZÁLEZ-GAYA, B. et al. Biodegradation as an important sink of aromatic hydrocarbons in the oceans. **Nature Geoscience**, v. 12, n. 2, p. 119–125, 2019.
GRIGGS, D. et al. Microbial regulation of global biogeochemical cycles. **Nature**, v. 495, n. 7441, p. 305–307, 2013.
HEHEMANN, J. H.; BORASTON, A. B.; CZJZEK, M. A sweet new wave: Structures and mechanisms of enzymes that digest polysaccharides from marine algae. **Current Opinion in Structural Biology**, v. 28, n. 1, p. 77–86, 2014.
HOSHINO, T. et al. Global diversity of microbial communities in marine sediment. **Proceedings of the National Academy of Sciences of the United States of America**, v. 117, n. 44, p. 27587–27597, 2020.
HUG, L. A. et al. Critical biogeochemical functions in the subsurface are associated with bacteria from new phyla and little studied lineages. **Environmental Microbiology**, v. 18, n. 1, p. 159–173, 2016.
HÜGLER, M.; SIEVERT, S. M. Beyond the Calvin cycle: Autotrophic carbon fixation in the ocean. **Annual Review of Marine Science**, v. 3, p. 261–289, 2011.
HUTCHINS, D. A.; FU, F. Microorganisms and ocean global change. **Nature Microbiology**, v. 2, n. May, p. 1–11, 2017.
IVERSON, V. et al. Untangling genomes from metagenomes: Revealing an uncultured class of marine euryarchaeota. **Science**, v. 335, n. 6068, p. 587–590, 2012.
JETTEN, M. S. M. The microbial nitrogen cycle. **Environmental Microbiology**, v. 10, n. 11, p. 2903–2909, 2008.
JORGENSEN, B. B.; BAK, F. Pathways and microbiology of thiosulfate transformations and sulfate reduction in a marine sediment (Kattegat, Denmark). **Applied and Environmental Microbiology**, v. 57, n. 3, p. 847–856, 1991.
JØRGENSEN, B. B.; FINDLAY, A. J.; PELLERIN, A. The biogeochemical sulfur cycle of marine sediments. **Frontiers in Microbiology**, v. 10, n. APR, p. 1–27, 2019.

JUDD, A.; HOVLAND, M. Seabed fluid flow: the impact on geology, biology, and the marine environment. **Choice Reviews Online**, v. 45, n. 01, p. 45- 0294-45–0294, 2007.
JUDD, A.; HOVLAND, M. **Seabed fluid flow: the impact on geology, biology and the marine environment**. [s.l.] Cambridge University Press, 2009.
KAWANO, Y.; SUZUKI, K.; OHTSU, I. Current understanding of sulfur assimilation metabolism to biosynthesize l-cysteine and recent progress of its fermentative overproduction in microorganisms. **Applied Microbiology and Biotechnology**, v. 102, n. 19, p. 8203–8211, 2018.
KLIPPEL, B. et al. Carbohydrate-active enzymes identified by metagenomic analysis of deep-sea sediment bacteria. **Extremophiles**, v. 18, n. 5, p. 853–863, 2014.
KURITA, K. Controlled functionalization of the polysaccharide chitin. **Progress in Polymer Science (Oxford)**, v. 26, n. 9, p. 1921–1971, 2001.
KUYPERS, M. M. M.; MARCHANT, H. K.; KARTAL, B. The microbial nitrogen-cycling network. **Nature Reviews Microbiology**, v. 16, n. 5, p. 263–276, 2018.
LI, D. et al. MEGAHIT: An ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. **Bioinformatics**, v. 31, n. 10, p. 1674–1676, 2015.
MADSEN, E. L. Microorganisms and their roles in fundamental biogeochemical cycles. **Current Opinion in Biotechnology**, v. 22, n. 3, p. 456–464, 2011.
MEYER, F. et al. The metagenomics RAST server - A public resource for the automatic phylogenetic and functional analysis of metagenomes. **BMC Bioinformatics**, v. 9, p. 1–8, 2008.
MORAN, M. A.; DURHAM, B. P. Sulfur metabolites in the pelagic ocean. **Nature Reviews Microbiology**, v. 17, n. 11, p. 665–678, 2019.
NARASINGARAO, P. et al. De novo metagenomic assembly reveals abundant novel major lineage of Archaea in hypersaline microbial communities. **ISME Journal**, v. 6, n. 1, p. 81–93, 2012.
OKSANEN, A. J. et al. Package ‘ vegan ’. n. January, p. 0–291, 2013.
ORCUTT, B. N. et al. Microbial Ecology of the Dark Ocean above, at, and below the Seafloor. **Microbiology and Molecular Biology Reviews**, v. 75, n. 2, p. 361–422, 2011.
PAJARES, S.; RAMOS, R. Processes and Microorganisms Involved in the Marine Nitrogen Cycle: Knowledge and Gaps. **Frontiers in Marine Science**, v. 6, n. November, 2019.
PARKES, RJ, CRAGG BA, BALE SJ, GETLIFF JM, GOODMAN K, ROCHELLE PA, FRY JC, WEIGHTMAN AJ, H. S. Deep bacterial biosphere in. **Nature**, v. 371, n. September, p. 410–413, 1994.
PARKES, R. J. et al. A review of prokaryotic populations and processes in sub-seafloor sediments, including biosphere: Geosphere interactions. **Marine Geology**, v. 352, p. 409–425, 2014.
PARKS, D. H. et al. CheckM: Assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. **Genome Research**, v. 25, n. 7, p. 1043–1055, 2015.
PURKHOLD, U. et al. Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: Implications for molecular diversity surveys. **Applied and Environmental Microbiology**, v. 66, n. 12, p. 5368–5382, 2000.
RAMOS, R. B. et al. Deep pockmarks as natural sediment traps : a case study from southern Santos Basin (SW Atlantic upper slope). **Geo-Marine Letters**, v. 40, p. 989–999, 2020.
REEBURGH, W. S. Oceanic methane biogeochemistry. **Chemical Reviews**, v. 107, n. 2, p. 486–513, 2007.
RODRÍGUEZ-RAMOS, J. A. et al. Microbial Genome-Resolved Metaproteomic Analyses Frame Intertwined Carbon and Nitrogen Cycles in River Hyporheic Sediments. p. 1–33, 2021.
SCHATTNER, U. et al. A detailed look at diapir piercement onto the ocean fl oor : New evidence from Santos Basin , o ff shore Brazil. **Marine Geology**, v. 406, n. April, p. 98–108,

2018.
SEYMOUR, J. R. A sea of microbes: the diversity and activity of marine microorganisms. **Microbiology Australia**, v. 35, n. 4, p. 183, 2014.
SHAFFER, M. et al. DRAM for distilling microbial metabolism to automate the curation of microbiome function. **Nucleic Acids Research**, v. 48, n. 16, p. 8883–8900, 2020.
SHARON, I.; BANFIELD, J. F. Genomes from metagenomics. **Science**, v. 342, n. 6162, p. 1057–1058, 2013.
SIGNORINI, S. R. et al. Corrente do Brasil: estrutura térmica entre 19º e 25ºS e circulação geostrófica. **Boletim do Instituto Oceanográfico**, v. 37, n. 1, p. 33–49, 1989.
SILVEIRA, I., C., A.; SCHMIDT, A., C., K.; CAMPOS, E., J., D.; GODOI, S., S.; IKEDA, Y. A Corrente do Brasil ao Largo da Costa Leste Brasileira. **Revista Brasileira de Oceanografia**, v. 48, n. 2, p. 171–183, 2000.
SMITH, S V, HOLLIBAUGH, J. T. Coastal metabolism and the ocean organic carbon balance. **Reviews of Geophysics**, v. 31, n. 92, p. 75–89, 1993.
SMITH, J. M. et al. Factors influencing nitrification rates and the abundance and transcriptional activity of ammonia-oxidizing microorganisms in the dark northeast Pacific Ocean. **Limnology and Oceanography**, v. 61, n. 2, p. 596–609, 2016.
SUMIDA, P. Y. G. et al. Seabed pockmarks associated with deepwater corals off SE Brazilian continental slope, Santos Basin. **Marine Geology**, v. 207, n. 1–4, p. 159–167, 2004.
THOMAS, H. et al. Enhanced Open Ocean Storage of CO_2 from Shelf Sea Pumping. **Science**, v. 304, n. 5673, p. 1005–1008, 2004.
TREUSCH, A. H. et al. Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. **Environmental Microbiology**, v. 7, n. 12, p. 1985–1995, 2005.
TRINGE, S. G. et al. Comparative metagenomics of microbial communities. **Science**, v. 308, n. 5721, p. 554–557, 2005.
URITSKIY, G.; DI RUGGIERO, J. Applying genome-resolved metagenomics to deconvolute the halophilic microbiome. **Genes**, v. 10, n. 3, 2019.
VERSANTVOORT, W. et al. Comparative genomics of Candidatus Methylomirabilis species and description of Ca. Methylomirabilis lanthanidiphila. **Frontiers in Microbiology**, v. 9, n. JUL, p. 1–10, 2018.
WEBER, T.; WISEMAN, N. A.; KOCK, A. Global ocean methane emissions dominated by shallow coastal waters. **Nature Communications**, v. 10, n. 1, p. 1–10, 2019.
WICKHAM, H. **ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York**. [s.l: s.n.]. v. 35
ZEHR, J. P.; WARD, B. B. Nitrogen cycling in the ocean: New perspectives on processes and paradigms. **Applied and Environmental Microbiology**, v. 68, n. 3, p. 1015–1024, 2002.
ZONNEVELD, K. A. F. et al. Selective preservation of organic matter in marine environments; Processes and impact on the sedimentary record. **Biogeosciences**, v. 7, n. 2, p. 483–511, 2010.

SUPPLEMENTARY MATERIAL

Supplementary Table 1: General information about Sample ID, a collection station, latitude and longitude, station depth, and station geomorphological formation.

Sample ID	Station	Latitude	Longitude	Depth (m)	Geomorphological formation
Control_255	255	26°29.677'S	45°58.153'W	652	Control
Diapir_259	259	26°33.776'S	46°07.008'W	559	Salt of Diapir
Diapir_260	260	26°33.710'S	46°07.470'W	517	Salt of Diapir

Supplementary Table 2: Relative abundance of contigs supplied from each sample for mounting the eight selected MAGs.

	Relative Abundance (%)		
MAG ID	**Control_255**	**Diapir_259**	**Diapir_260**
MAG_00001	0.302377586135	0.455224572974	0.242397840891
MAG_00002	0.235432550612	0.358504373407	0.406063075981
MAG_00003	0.445856947753	0.188583301264	0.365559750982
MAG_00004	0.378338604998	0.288405761341	0.333255633661
MAG_00005	0.293056736581	0.341801210382	0.365142053036
MAG_00006	0.461590072979	0.268785847037	0.269624079984
MAG_00007	0.410969840238	0.236610551844	0.352419607918
MAG_00008	0.307615847252	0.380446529623	0.311937623125

Supplementary Table 3. List of 34 MAGs recovered and their taxonomic classification based on GTDB-Tk, total length, number of contigs, N50, CG%, percent completeness and contamination, and draft quality.

MAG ID	GTDB-TK taxonomy	Total length	Contig no.	N50	CG %	Completeness	Contamination	Draft Quality
MAG_00001	Bacteria; Methylomirabilota; Methylomirabilis, CPS1-5; CPS1-5	2397160	403	7968	58.583	94.244	2.158	High
MAG_00002	Bacteria; Nitrospirota; Nitrospiria; Nitrospirales; Nitrospiraceae	2379090	323	9268	57.953	90.647	2.877	High
MAG_00003	Bacteria; Proteobacteria; Gammaproteobacteria; Burkholderiales; Nitrosomonadaceae; GCA2721545	1518180	520	3070	44.422	83.453	1.438	Medium
MAG_00004	Bacteria; Proteobacteria; Gammaproteobacteria; Burkholderiales; Nitrosomonadaceae; GCA2721545	2823452	928	3165	57.198	82.014	2.158	Medium
MAG_00005	Bacteria; Proteobacteria; Gammaproteobacteria; GCA-2729495; GCA-2729495; GCA2729495	5918495	2.328	2573	54.233	76.978	2.158	Medium
MAG_00006	Bacteria; Proteobacteria; Gammaproteobacteria; HK1	3537226	1049	3646	63.004	72.661	0.719	Medium
MAG_00007	Bacteria; Proteobacteria; Gammaproteobacteria; UBA4486; UBA4486	3537226	515	2826	40.507	71.942	2.877	Medium
MAG_00008	Bacteria; Binatota; Binatia; UBA9968	4170056	2024	2000	53.166	71.942	2.877	Medium
MAG_00009	Bacteria; Proteobacteria; Gammaproteobacteria; HK1	4744342	1984	2381	62.711	66.906	2.158	Medium
MAG_00010	Bacteria; Proteobacteria; Alphaproteobacteria;Kiloniellales	1698391	841	1957	66363	63309	2.158	Medium
MAG_00011	Bacteria; Proteobacteria; Gammaproteobacteria;Woeseiales; Woeseiaceae;	4436461	2299	1864	57.615	60.431	2.158	Medium
MAG_00012	Bacteria; Proteobacteria; Alphaproteobacteria; Kiloniellales;Kiloniellaceae;	2034755	963	2056	66.436	58.992	1.438	Medium
MAG_00013	Bacteria; Dadabacteria; UBA1144	1040817	455	2205	34.752	57.554	0.000	Medium
MAG_00014	Bacteria; Tectomicrobia; Entotheonellia; Entotheonellales	7172570	3617	1889	59.202	53.956	1.438	Medium
MAG_00015	Archaea; Crenarchaeota;Nitrososphaeria;Nitrososphaerales; Nitrosopumilaceae	475213	235	1951	34.209	50.617	2.469	Medium
MAG_00016	Archaea; Crenarchaeota;Nitrososphaeria;Nitrososphaerales; Nitrosopumilaceae	751328	336	2171	33.915	51.234	3.703	Medium
MAG_00017	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae;CSP1-1	608712	172	3972	33.744	50.000	2.469	Medium
MAG_00018	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae	819900	370	2163	33.771	50.000	4.321	Medium
MAG_00019	Bacteria;Myxococcota;UBA9160	3842992	2042	1817	66.128	46.762	2.158	Low
MAG_00020	Bacteria;Proteobacteria;Alphaproteobacteria;Kiloniellales	1299393	638	1975	66.476	46.043	2.877	Low
MAG_00021	Bacteria; Nitrospirota;Nitrospiria;Nitrospirales;UBA8639;UBA8639	1322597	691	1851	48.608	43.884	1.438	Low
MAG_00022	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae	557612	246	2190	34.212	43.827	3.086	Low
MAG_00023	Bacteria;Proteobacteria;Alphaproteobacteria;UBA6615	2742135	1466	1804	65.685	40.287	1.438	Low
MAG_00024	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae;CSP1-1	379099	128	3182	34.001	40.123	1.851	Low
MAG_00025	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae;CSP1-1	615250	261	2319	33.521	40.123	1.851	Low
MAG_00026	Bacteria;Proteobacteria;Gammaproteobacteria;Woeseiales;Woeseiaceae;SZUA-117	1308546	608	2073	56.672	38.848	0.719	Low
MAG_00027	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae	892541	411	2092	33.581	40.123	3.703	Low
MAG_00028	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae	767898	377	1974	34.542	40.123	4.938	Low
MAG_00029	Bacteria; Proteobacteria; Gammaproteobacteria; Acidiferrobacterales; SPGG2; SPGG2;	1243523	569	2121	53.863	34.532	0.000	Low
MAG_00030	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae	704382	341	1973	33.619	37.654	3.703	Low
MAG_00031	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae;CSP1-1	579426	214	2826	33.417	35.802	3.703	Low
MAG_00032	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae;CSP1-1	478985	184	2364	33.889	30.246	1.234	Low
MAG_00033	Archaea;Crenarchaeota;Nitrososphaeria;Nitrososphaerales;Nitrosopumilaceae	346885	120	3043	34.555	30.246	1.851	Low
MAG_00034	Archaea;Crenarchaeota;Nitroosphaeria;Nitrososphaerales;Nitrosopumilaceae	814736	382	2081	33.449	30.84	6.172	Low

3. CONCLUSIONS

In this thesis, we present the results obtained from the study of prokaryotic communities in sediments in pockmark fields and salt diapirs on the continental slope of the Santos Basin. We use a combination of metabarcoding and metagenomic approaches associated with phylogenetic tools, together with cultivation-dependent techniques.

In chapter II we highlight the presence of a non-methanotrophic methylotroph of the order Methylomirabilales that was diffused in all samples and was the highest quality MAG recovered. We explored the metabolic potential of this genome (CSP1-5 family) and described its ability to oxidise methanol, along with several genes with the potential to improve the organism's fitness in the deep-sea environment. The widespread nature of this organism suggests an important potential role for methanol metabolism in this area of the continental slope. The results also provided evidence that studies based on metabarcoding alone can lead to misclassification of Methylomirabilales members, which has profound relevance to conclusions about the organisms' roles in the environment. We also highlight the taxonomic diversity found in the surface and subsurface samples, with a high prevalence of the archaea Nitrososphaeria in the surface samples.

In chapter III, dependent and independent techniques of cultivation were used, through which we observed high taxonomic diversity in samples of environmental sediments and the predominance of halophilic groups in samples enriched in HM medium. Sediment samples were similar in taxonomic composition, all dominated by archaea of the Nitrosopumilaceae family, while the sediment samples enriched in HM medium families, such as Idiomarinaceae, Halomonadaceae, and Marinobacteriaceae, were dominant. From cultures enriched with saline diapir sediment, control, and pockmark, 22 isolates belonging to two distinct families of bacteria were obtained: Halomonadaceae, and Salinisphaeraceae, both belonging to the class Gammaproteobacteria. The present work is a pioneer in the cultivation of halophilic microorganisms from salt diapirs and pockmarks on the Brazilian continental margin, in the Southeast Atlantic Ocean. Here we describe a great diversity of microorganisms in the marine surface sediment, as well as the isolation and identification of halophiles little described on the seabed.

In chapter IV, the metagenomic analysis of the sediment collected in salt diapirs along the pockmark field and the control area showed great taxonomic diversity, in addition to allowing the assembly of several draught genomes. These genomes belong to taxa still little explored and uneducated, mainly for the deep-sea environment. Our results bring greater clarity

to the role of these microorganisms in the carbon, sulphur, and nitrogen cycles that potentially occur in the Southwest Atlantic pockmark fields and reinforce the importance of further investigation to elucidate whether they are actively carrying out these ecological processes.