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Microbial Diversity in Coastal Ecosystems and Assessment of Elevated Water Temperature and Acidification in Microbial Mats

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ABSTRACT

Microorganisms are key players in the biogeochemistry of coastal environments; however, little is known about the microbial diversity and the influence of physicochemical variables on microbes living in the coast. In this work, the microbial community structure and composition in different coastal ecosystems (a coastal lagoon, hypersaline sediments, microbial mats and endoevaporites) from Yucatán and Baja California Sur, Mexico, was explored and characterized. Moreover, the effect of elevated water temperature and acidification in microbial mats was studied, in order to explore the response on nitrogen fixation and oxygen production/consumption. High-throughput sequencing of 16S rRNA gene showed differences in the structure of microbial communities for each analyzed ecosystem. In addition, salinity, temperature, and redox potential were the principal environmental variables that explained the variance of microbial populations. Most of the microorganisms detected were related to the biogeochemical cycling of carbon, nitrogen, and sulfur. Sediments along the coastal lagoon in Yucatán (Celestún) displayed members from Methanosaetaceae, ANME 1-b, Sandaracinaceae, Aminicenantes, Thaumarchaeota, Thermoplasmatales, Bathyarchaeota and Lokiarchaeota. The microbial component of hypersaline sediments, microbial mats and endoevaporites from Yucatán and Baja California Sur, presented representatives from Proteobacteria, Bacteroidetes, Chloroflexi Crenarchaeota, Euryarchaeota, Asgardeota, Diapherotrites and Nanoarchaeaeota. In addition, increased water temperature and acidification in mats was positively related to nitrogen fixation, oxygen production and respiration, suggesting that those variables (associated with climate change) would promote a higher activity of coastal microbial mats. This study highlights the unexplored microbial diversity living in coastal ecosystems and their environmental characteristics, providing information on uncultured microorganisms not previously reported for those sites. This work represents an effort to increase the knowledge of microbial diversity in coastal ecosystems; however, future studies are required to understand the specific ecological role of certain detected groups.

Keywords: Coastal ecosystems, hypersaline environments, microbial mats, 16S rRNA amplicon sequencing, climate change.

RESUMEN

Los microorganismos son actores clave en la biogeoquímica de los ambientes costeros; sin embargo, poco se sabe sobre la diversidad microbiana y la influencia de las variables fisicoquímicas en los microbios que viven en la costa. En este trabajo se exploró y caracterizó la estructura y composición de la comunidad microbiana en diferentes ecosistemas costeros (una laguna costera, sedimentos hipersalinos, tapetes microbianos y endoevaporitas) de Yucatán y Baja California Sur, México. Además, se estudió el efecto del incremento de temperatura del agua y acidificación en tapetes microbianos, con el fin de explorar la respuesta sobre la fijación de nitrógeno y la producción/consumo de oxígeno. La secuenciación de alto rendimiento del gen 16S ARNr mostró diferencias en la estructura de las comunidades microbianas para cada ecosistema analizado. Además, la salinidad, la temperatura y el potencial redox fueron las principales variables ambientales que explicaron la varianza de las poblaciones microbianas. La mayoría de los microorganismos detectados fueron relacionados con el ciclo biogeoquímico del carbono, nitrógeno y azufre. Los sedimentos a lo largo de la laguna costera de Yucatán (Celestún) mostraron miembros de Methanosaetaceae, ANME 1-b, Sandaracinaceae, Aminicenantes, Thaumarchaeota, Thermoplasmatales, Bathyarchaeota y Lokiarchaeota. El componente microbiano de sedimentos hipersalinos, esteras microbianas y endoevaporitas de Yucatán y Baja California Sur, presentó representantes de Proteobacteria, Bacteroidetes, Chloroflexi Crenarchaeota, Euryarchaeota, Asgardeota, Diapherotrites y Nanoarchaeaeota. Además, el aumento de la temperatura del agua y la acidificación de las esteras se relacionaron positivamente con la fijación de nitrógeno, la producción de oxígeno y la respiración, lo que sugiere que esas variables (asociadas con el cambio climático) promoverían una mayor actividad de las esteras microbianas costeras. Este estudio destaca la diversidad microbiana inexplorada que vive en los ecosistemas costeros y sus características ambientales, proporcionando información sobre microorganismos no cultivados no reportados previamente para esos sitios. Este trabajo representa un esfuerzo por incrementar el conocimiento de la diversidad microbiana en los ecosistemas costeros; sin embargo, se requieren estudios futuros para comprender el papel ecológico específico de ciertos grupos detectados.

Palabras clave: Ecosistemas costeros, ambientes hipersalinos, tapetes microbianos, secuenciación de amplicones del gen 16S ARNr, cambio climático.

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Thanks to my mother and father, for their guidance in life.

I find it elevating and exhilarating

to discover that we live in a universe

which permits the evolution of molecular machines

as intricate and subtle, as we.

Carl Sagan

https://youtu.be/XGK84Poeynk

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INTRODUCTION

Microbial biodiversity

Tiny organisms, known as microbes, are distributed around the world, in all kinds of ecosystems. They exist in our body, in water, air, and soils. They can be found in cities, forests, and oceans as well as in extreme environments, like deserts, polar regions, and volcanoes. Microbes are invisible to the human eye and they were not discovered until the XVII century, with the invention of the microscope. Anton Van Leeuwenhoek, a Dutch dedicated to the sale of fabrics, manufactured a microscope for the first time in order to observe the quality of seams. Later, he observed a diverse type of samples (rainwater, blood and others) under the microscope and discovered the microbial world. Thenceforth, human vision and understanding about nature, diseases, and technology, completely changed (Opal et al., 2009).

The first studies on microorganisms were performed to investigate their morphology, describing sizes, shapes (cocci, rod, spiral) and arrangement of microbial cells (diplo, strepto, sarcinae). The second big goal has been to cultivate microbes in the laboratory. Microbial cultivation is performed creating different growth media that mimic natural conditions. Nevertheless, artificial systems are far away to simulate the chemistry of the microbe's natural habitat. It is estimated that more than 99% of microbial species cannot be cultured by traditional techniques, while the 1% of microbes that can be cultured are not representative of the total diversity in nature (Pham and Kim, 2012). In recent years, the development of molecular biology and bioinformatics have permitted access to the uncultured microbial world of almost any environment. Culture-independent methods based either on sequencing of targeted genes (e.g. 16S rRNA gene as taxonomic standard marker and functional genes) or by sequencing all the genomic content of a sample, has allowed a gene-based exploration of microbial communities (Escalante and Pajares, 2014; Knight et al., 2012). These technologies have revolutionized our understanding of the microbial diversity, evolution, and ecological interactions from small to large scales in our planet.

Nowadays, we know that microbes are the most abundant form of life on Earth. Microorganisms constitute about 60% of the Earth's live biomass (Whitman et al., 1998). It has been estimated that Earth probably hosts $>10^{30}$ microbial cells, exceeding the number of stars in the universe by nine orders of magnitude (Knight et al., 2012). At the present age, more than 99 bacterial phyla are recognized (Parks et al., 2018) and 14 archaeal lineages have been described (Adam et al., 2017). Massive collaborative efforts to characterize microbial life in our planet are carried out by different organizations, such as The Earth Microbiome Project (Gilbert et al., 2014) and the Tara Oceans foundation (Sunagawa et al., 2020). The unseen microbial diversity is being intensely studied all around the world.

Microbes living in coastal ecosystems

Coastal habitats are areas both along and close to marine shorelines, where the land meets the sea. It is estimated that the world coastline is about 312, 000 km and because of its large area, shorelines profoundly influence the world ocean, Earth's climate, and human activities (Das and Khan, 2005).

Microbes play a fundamental role in driving coastal ecosystem dynamics. (Danovaro and Pusceddu, 2007). Bacteria and Archaea are key players in the production of organic matter since many of them are able to fix carbon from inorganic sources. Degradation of complex organic substrates to their basic compounds is also mediated by microorganisms. Because microbes have evolved a huge variety of energy metabolisms, they can use organic and/or inorganic electron donors and acceptors, allowing them to participate in the biogeochemical cycling of the six major building elements of life (carbon, hydrogen, nitrogen, oxygen phosphorus and sulfur) and that shape the biogeochemistry of coastal and terrestrial ecosystems of our planet (Flemming et al., 2019; Offre et al., 2013).

While their impact is at a global scale, microbial processes occur at the level of single cells and are intimately dependent on interactions between microorganisms and the surrounding physical and chemical environment (Braga et al., 2016; Pham and Kim, 2012). Environmental features, such as salinity, temperature, alkalinity, and nutrients form a framework of physico-chemical factors that influence prokaryotic diversity, because those variables demand metabolic

specialization of microbes to prosper in a given environment (Graham et al., 2019; Lozupone and Knight, 2007). In this sense, ecosystems along the coastline have strong physical and chemical gradients due to the continental-ocean interaction. The understanding of biodiversity and the influence of physicochemical variables on microbes is necessary to figure the main drivers of the prokaryotic community dynamics and function on coastal environments (Liu et al., 2018; Lindström and Langenheder, 2012).

Coastal microbial mats

Coastal habitats include sandy beaches, estuaries, mangroves, coastal lagoons, salt marshes, among others. In these ecosystems, marine flooding and intense solar radiation often support the development of photosynthetic microbial mats (Stal, 2001).

Coastal microbial mats are sophisticated biofilms that flourish on the top of the sediments (Gerdes, 2010). As its name describes, mats look like a carpet or rug, and can be easily mistaken with simple mud, but are living structures made by microbial life. Microbes produce a matrix of extracellular polymeric substances embedded with nutrients, grains of sediments, and organic matter, reaching a thickness of a few centimeters (Stal, 2001). Frequently, new live mats grow on top of older mats that have died, creating very thick sequences. However, the most living and active mat is just at the first centimeter. Here, microbes have a vertical stratification at a fine millimeter scale, distributed in green, orange, and purple colorful layers, where each layer represents a predominance of a different kind of microbes, with a special lifestyle (Gerdes, 2010; Des Marais, 2003). This stratification occurs as a result of diverse environmental factors, such as oxygen availability and sulfide. Mats are based on autotrophy, the fixation of inorganic carbon into biomass, which occurs either photosynthetically or chemosynthetically (Stal, 2001). Then, organic materials are decomposed by aerobic/anaerobic degradation by aerobes/fermenters. Some heterotrophic activity generates toxic sulfide and sulfate, which are used by green/purple sulfur bacteria and by sulfate reducers, respectively (Seckbach and Oren, 2010; Stolz, 2000).

Microbial mats are ecologically relevant in coastal environments. They contribute to the stabilization of soils and sediments, producing organic materials that enrich the sediment with nutrients (Stolz, 2000). Mats participate in the recycling of some chemical elements like carbon,

nitrogen, and sulfur (Des Marais, 2003). They can clean the water and release some gases to the atmosphere such as oxygen, hydrogen, carbon dioxide and methane (Coban et al., 2018; Hoehler et al., 2001). Mats are also a food source for animals. Some flies, worms and birds eat small pieces of mats, supporting the local food web (Stal, 2001).

Studies on mats have investigated their ecological contribution on modern and past Earth and explored their relevance in astrobiology. Evidence from fossilized microbial mats situates their occurrence to 3.5 Ga in the geological record, indicating that mats constitute the oldest reliable form of life organization known on Earth (Seckbach and Oren, 2010). Geological data and laboratory studies have revealed the importance of microbial mats in the history of Earth. It is believed that, in the past, the abundance and high activity of mats was responsible for creating the oxygen-rich atmosphere that we breathe, during the Great Oxidation Event approximately 2.5 billion years ago (Gutiérrez-Preciado et al., 2018). Furthermore, as they release carbon dioxide and methane, identified as greenhouse gases, they have also contributed to the regulation of Earth's climate, helping to create a warmer atmosphere that has made the Earth a more habitable planet (Hoehler et al., 2001). For these reasons, scientists believe that mats can prosper on other similar rocky planets or moons, and are studying mats to recognize their characteristics, if they occur on other worlds.

Climate change and microorganisms

Climate change is defined as an alteration in the average weather conditions such as temperature, rainfall, wind, and hurricanes, in a region over a long period of time (Parry, 1996). Currently, the evidence of climate change in several regions of the world is strong, observed as the melting of glaciers (Dyurgerov and Meier, 2000), atmospheric concentrations of greenhouse gases (Bodelier and Steenbergh, 2014), precipitation patterns (Dore et al., 2005) and ocean acidification (Boyd, 2011). Climate change may be due to natural processes within the climate system, or to anthropogenic external forcing (Matthews et al., 2004). International efforts to understand causes and effects of climate change are necessary to protect biodiversity and human life.

Given their global importance, coastal environments are a major focus of concern regarding the potential impacts of climate change (Sánchez-Arcilla et al., 2016). Macroscopic organisms such

as fishes, corals, mangroves, and humans, have been relatively well studied. In contrast, the contribution and susceptibility of microorganisms and microbial mats to a changing climate have received little attention (Reinold et al., 2019).

HYPOTHESES

1) Microbial biodiversity between different types of coastal ecosystems (i.e. coastal lagoon sediments, microbial mats from natural and human-made hypersaline environments) is different.

2) Physicochemical environmental conditions drive microbial communities across studied ecosystem. Then, it is expected to find a relationship between the taxonomic composition and environmental variables across the different ecosystems studied.

3) Since the increase in water temperature and acidification influences the biology of marine organisms, then, it is expected that they will also affect the functioning of microbial mats, particularly, on nitrogen fixation, oxygen production and microbial respiration.

MAIN OBJECTIVE

To better understand microbial biodiversity patterns in coastal environments, the main objective of this PhD dissertation was to characterize microbial communities from diverse coastal ecosystems (sediments from a coastal lagoon, hypersaline sediments, microbial mats and endoevaporites) and investigate some environmental features that potentially influence their distribution, using gene amplicon sequencing and bioinformatic analysis. In addition, through culture-dependent methods, I studied the response of microbial mats to elevated water temperature and acidification, simulating a climate change scenario, in order to explore the effect on nitrogen fixation and oxygen fluxes in those structures.

SPECIFIC GOALS

To characterize the benthic microbial community structure and composition in three differentiated zones (oligohaline, marine and the mixing) of a transitional coastal lagoon in the Southern Gulf of Mexico through 16S rRNA gene Illumina-sequencing.

To investigate the microbial and micro-eukaryotic community structure and composition of hypersaline sediments and microbial mats developing in Exportadora de Sal, Guerrero Negro, Mexico, using independent Illumina amplicon sequencing of 16S rRNA and 18S rRNA genes.

To characterize the microbial structure and composition of coastal microbial mats from four localities in the Yucatán Peninsula, by 16S rRNA gene sequencing on the Illumina platform.

To evaluate the response of microbial mats to an increase of water temperature and acidification in greenhouse incubations as an assessment under a climate change scenario.

CHAPTER 1

Community structure and distribution of benthic Bacteria and Archaea in a stratified coastal lagoon in the Southern Gulf of Mexico

In Chapter 1, the benthic microbial community structure from Celestún Lagoon, a tropical coastal lagoon located at Yucatán, Mexico was studied. High-throughput sequencing of 16S rRNA gene to unravel the microbial assemblages in sediments along the lagoon was performed. Differentially distributed taxa were found, being zonation and salinity the principal variables that explained the variance of microbial communities.

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Abstract

Coastal lagoons are important aquatic systems with strong physicochemical gradients, where the participation of microorganisms in biogeochemical cycles has been well recognized; however, to date, the microbial diversity and distribution in these environments remains under-investigated. Here, three distinguished regions (oligohaline, marine and the mixing) of a transitional coastal lagoon were explored, to characterize the structure and composition of benthic microbial communities through 16S rRNA gene Illumina-sequencing, for both Bacteria and Archaea domains. Principal coordinate analysis showed differences in the community structure according to the analyzed zones. PERMANOVA analysis evidenced that, of the measured variables, sample zonation and salinity were the main environmental factors explaining the variance of the prokaryotic assemblages. Differentially abundant microbial taxa were detected for each region of the lagoon by LEfSe analysis. Representative members of anaerobic methanogens/methanotrophs (Methanosaetaceae, ANME 1-b and WSA2) were enriched in the oligohaline sediments, while the coastal marine zone had a community represented mainly by Sandaracinaceae, Aminicenantes and Thaumarchaeota (Group-C3). The sediments in the mixing zone had higher abundance of Flavobacteriaceae, Syntrophobacteraceae and uncultured Thermoplasmatales, Bathyarchaeota and Lokiarchaeota. This study expands the available information of the composition and distribution of uncultured Bacteria and Archaea in transitional coastal lagoons, contributing to a systematic understanding of the functioning of these ecosystems.

Keywords: Transitional coastal lagoon microbial diversity, 16S rRNA gene illumina sequencing, LEfSe analysis

Introduction

Coastal lagoons are transitional zones located in the continental ocean-interphase that provide important supporting ecosystem services, such as nutrient retention, flood control and sediment stabilization (Pérez-Ruzafa et al., 2011). These ecosystems are important biodiversity reservoirs, characterized by high primary production, which are ecologically viable for fisheries and ecotourism (Pérez-Ruzafa et al., 2011; Säwström et al., 2016). Prokaryotes thriving in coastal lagoons represent the basis of ecosystem functioning (Danovaro and Pusceddu, 2007). Bacteria and Archaea are key players in greenhouse gas emission, in the production and decomposition of organic matter and in biogeochemical cycling of primary elements, such as carbon, nitrogen and sulfur (Azam and Malfatti, 2007; Danovaro and Pusceddu, 2007). Due to their geographical location, coastal lagoons usually have strong physical and chemical gradients of salinity, nutrients concentrations, turbidity, and organic matter content. These environmental features demand significant physiological versatility that enable the survival of microorganisms under these transitional freshwater-seawater environments (Säwström et al., 2016). In consequence, some studies assessing the diversity of microorganisms in these ecosystems have demonstrated that microbial communities can be shaped by a variety of environmental factors, such as salinity, temperature, and pH, or even by hydrological and latitudinal aspects (Lozupone and Knight, 2007; Lindström and Langenheder, 2012; Liu et al., 2018).

Previous studies focused on the microbial diversity inhabiting transitional freshwater-marine ecosystems and their potential role in ecosystem functioning has recently been addressed with the development of molecular biology techniques (Danovaro and Pusceddu, 2007; Armougom and Raoult, 2009; Liu et al., 2018; Matcher et al., 2018), revealing higher bacterial and archaeal diversity for freshwater systems than in marine environments (Lozupone and Knight, 2007; Lindtröm and Langenheder, 2012). In addition, concomitant differences in the microbial community composition have been reported. For example, fresh-water sediments usually are composed by members of Acidobacteria, Actinobacteria, Chlorobi and Crenarchaeota (Xie et al., 2014), while marine sediments are generally dominated by Deltaproteobacteria, Gammaproteobacteria and uncultured Thaumarchaeota and Euryarchaeota (Wang et al., 2012; Lloyd et al., 2013). At estuarine conditions, few studies have been conducted in the Northern

Hemisphere (Kirchman et al., 2005) and in the Baltic Sea (Klier et al., 2018). However, the composition and diversity of the prokaryotic communities in tropical estuaries are poorly understood (Lindstöm and Langenheder, 2012; Liu et al., 2018; Matcher et al., 2018).

In the northwestern coast of the Yucatán Peninsula (Southern Gulf of Mexico) is located Celestún Lagoon, a shallow coastal lagoon with approximately 22 km in length and 2 km wide, bordered by mangrove forest. The southern part is permanently connected to the sea, while several springs lead to the discharge of groundwater to the northern and middle parts, giving to the lagoon an estuarine salinity gradient throughout (Herrera-Silvera, 1994, 1995; Vega-Cendejas and Arreguí-Sánchez, 2001; Stalker et al., 2014). Based on water quality and hydrologic characteristics, previous works have recognized three principal regions in the lagoon: the inner oligohaline zone, the coastal marine area, and the mixing zone between these two areas (Herrera-Silveira, 1995; Tapia-González et al., 2008). In this study, we used high-throughput sequencing of 16S rRNA gene to characterize the benthic microbial community structure and composition of Bacteria and Archaea along three differentiated zones in a coastal lagoon in the Southern Gulf of Mexico. Moreover, the potential ecological role of these microorganisms is discussed.

Materials and methods

Sample collection and physical and chemical characterization

Surficial sediments (cores 8 cm width × 8 cm length, 3–5 cm depth) were collected, in triplicate, in September 2017 in three zones of the Celestún Lagoon, inner mixing and marine (Table 1), which corresponded to the eastern, middle, and western areas of the lagoon (Fig. 1). All stations had an average water depth of 1.5 m. Sediment samples were hand-collected during high tide and immediately homogenized into 50 ml sterile plastic containers and transported to the laboratory on ice. Samples were stored at -80 °C for further physical, chemical and molecular analyses. Salinity, pH, temperature, and dissolved oxygen were measured in situ from surface water using a portable multi-parameter analyzer (YSI-85, YSI Incorporated Inc., USA). Salinity was measured using the Practical Salinity Scale. The content of carbon and nitrogen in the sediments was obtained with an elemental analyzer in the laboratory (Flash-Smart™, Thermo Fisher Scientific, Waltham, NJ). All *in situ* and laboratory measurements were performed in triplicate.

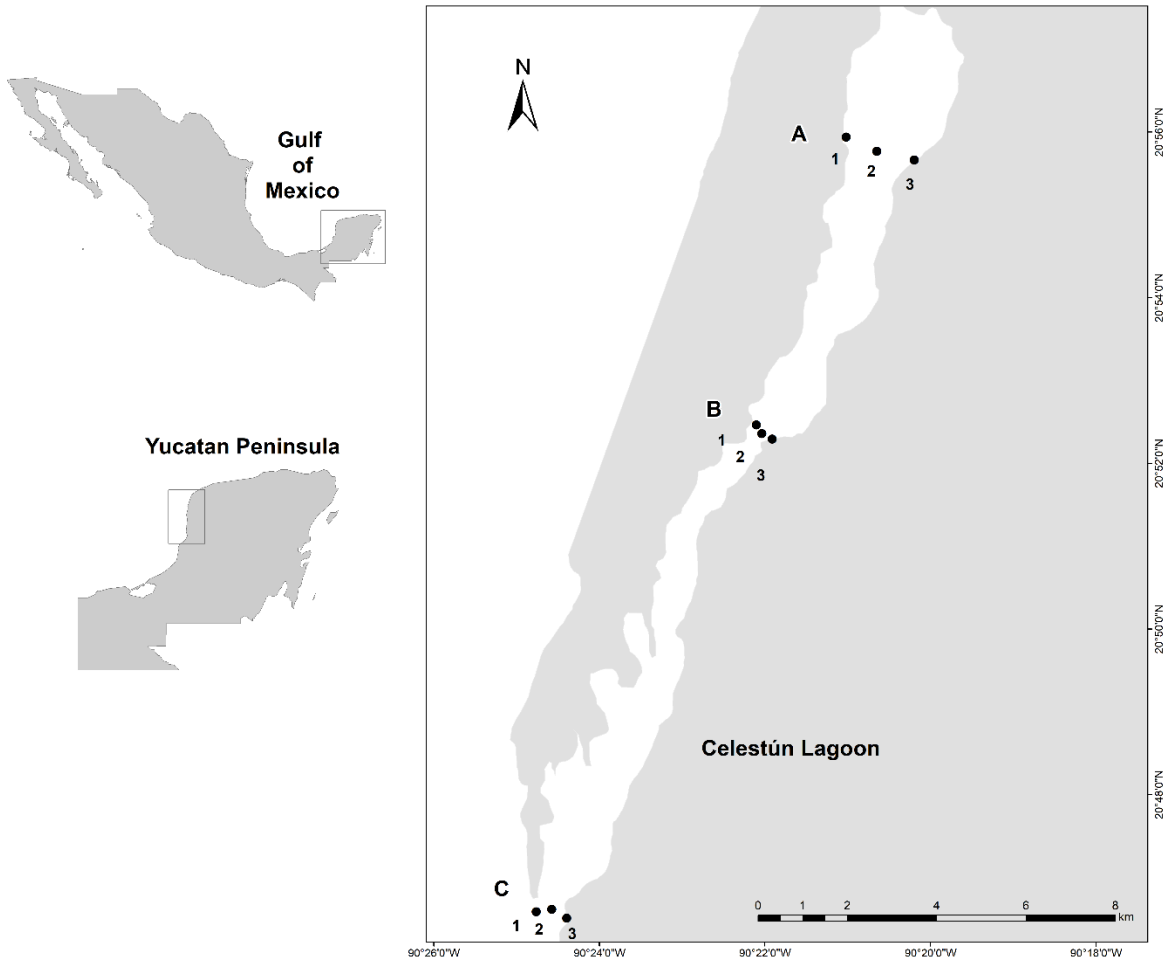

Fig. 1. Location of Celestún Lagoon and the sampling stations along the recognized inner, mixing and marine zone from the lagoon.

DNA extraction, PCR amplification and sequencing

Environmental DNA was extracted, in duplicate, from 0.25 g of each homogenized sediment. Samples were lysed with a TissueLyser LT (Qiagen, Hilden, Germany), and DNA was obtained using the DNeasy PowerSoil Kit (Qiagen, Germantown, MD) according to the manufacturer's protocol. An extraction blank (spin column with no sample added) was processed alongside the

samples. DNA quality was evaluated by 1% agarose gel. DNA extracts were pooled per sampling site. 16S rRNA gene fragments were amplified using the universal bacterial primers sets 16SF/16SR, covering the V3 and V4 regions and the archaeal suits of primers Arch0519/1041, spanning the V4–V6 regions and following the thermocycling conditions described by Klindworth et al. (2013). Each PCR reaction (20 µl) included 2 µl of DNA (10–20 ng/µl), 0.5 µl of each primer (10 µM) and 10 µl of 2 × Phusion High-Fidelity MasterMix (Thermo Scientific, Waltham, MA, USA). PCR products were obtained in duplicate from pooled DNA and then mixed in equal amounts for further Illumina sequencing.

Amplicons were purified using AMPure XP magnetic beads, according to the supplied protocol (Beckman Coulter Genomics, Brea, CA, USA). PCR products were indexed using Nextera XT Index kit version 2 (Illumina, San Diego, CA, USA), following the Illumina's 16S Metagenomic Sequencing Library Preparation protocol. PCR barcoded amplicons were again purified as previously described, and then were quantified with a Qubit 3.0 fluorometer (Life Technologies, Malaysia). The correct size of the amplicons was verified on an Advanced QIAxcel (QIAGEN, USA). The individual barcoded amplicons were diluted on 10 mM Tris (pH 8.5) and pooled in equimolar concentrations (9 pM). Paired-end sequencing (2 × 300 bp) was performed with the MiSeq platform (Illumina, San Diego, CA, USA) using a MiSeq Reagent Kit V3 (600 cycles). Sequencing was performed in the Aquatic Pathology laboratory at CINVESTAV-Mérida. All sequences obtained in this study were deposited in the NCBI Sequence Read Archive (BioProject PRJNA429278).

Bioinformatic analyses

Clean reads per sample were obtained with a minimum length of 250 bp. The demultiplexed fastq were processed with the QIIME2 (2017.11) pipeline (Caporaso et al., 2010). The error correction and denoising to resolve the amplicon sequence variants (ASV) of Illumina reads were performed with the DADA2 plugin, removing chimeras with the "consensus" method (Callahan et al., 2016, 2017). Representative sequences of ASVs were taxonomically assigned with the V-SEARCH consensus taxonomy classifier plugin (Rognes et al., 2016), using the SILVA database (v. 128) as a reference. The representative sequences were aligned with the MAFFT algorithm (Katoh and Standley, 2013), and the alignment was filtered for nonconserved and gapped positions to build a

phylogenetic tree with the fasttree algorithm (Price et al., 2010). Data were normalized among samples by sub-sampling to the lowest sequence count (73,000 reads for Bacteria and 75,000 for Archaea). The abundance tables were exported to the R environment, and the statistical analysis and visualization were performed with the phyloseq (McMurdie and Holmes, 2013), vegan (Oksanen, 2011) and ggplot2 (Wickham, 2009) libraries. A Principal Coordinate Analysis (PCoA) was calculated with the unweighted UniFrac distance (Lozupone and Knight, 2005) to evaluate differences among samples, based on presence/absence of the microbial taxa. The alpha diversity indexes, as observed by ASVs, Shannon and Simpson, were calculated. Then, a Kruskal-Wallis test was performed in order to detect significant differences among the sampled sites. A PERMANOVA (Permutational Multivariate Analysis of Variance) test was calculated with the adonis function (Anderson, 2001; Oksanen, 2011), to assess the effect of the environmental variables and sample zonation (variables used as independent factors) on the structure of the microbial communities, for each independent bacterial/archaeal data set (Anderson, 2001). The differential abundances of the ASVs among the zones were determined with a Linear Discriminant Analysis (LDA) Effect Size (LEfSe) (Segata et al., 2011) at ASV level, using a cutoff of LDA >2, and a p-value <0.05 for the internal Krustal Wallis and Wilcoxon tests.

Results

Physical and chemical characterization of stratified zones of Celestún Lagoon

The physical and chemical properties of Celestún Lagoon are summarized in Table 1. Results are means of triplicate measurements in situ or from laboratory analysis of pooled sediments. Salinity ranged between 20’ and 25.90’ along sampled sites from Celestún. The lowest salinity measurements were registered in the inner and mixing zones and, as expected, salinity increased in the marine area, except for C3 station. The amount of dissolved oxygen was higher in the marine zone (4.74 ± 0.58 mg L^{-1}) and in the inner zone (2.98 ± 0.86 mg L^{-1}) as compared to the level observed in the mixing area (2.06 ± 0.32 mg L^{-1}). The average temperature of the sites ranged from 29 to 33 °C. The pH showed similar values for all stations monitored (8.1 ± 0.3). The carbon, nitrogen, and hydrogen fraction (in %, wt/wt) of the sediments varied at 13.3 ± 0.9, 0.4 ± 0.1 and 0.5 ± 0.2, respectively.

Table 1: Geographic coordinates and physicochemical characteristics of the sampling sites along Celestún Lagoon. Salinity was measured using the Practical Salinity Scale.

Zone	Station	Geoposition	Salinity	Temp (°C)	pH	Dissolved oxygen (mg L^{-1})	Carbon (%)	Nitrogen (%)
Inner Zone	A1	20.932343° -90.350288°	6.4(±1.9)	30.0(±0.84)	8.0(±0.25)	3.7(±0.24)	15.2(±1.62)	0.35(±0.07)
	A2	20.929485° -90.344135°	4.1(±0.2)	31.3(±0.98)	8.3(±0.02)	3.4(±0.20)	12.5(±0.05)	0.45(±0.05)
	A3	20.927705° -90.336604°	2.0(±0.1)	29.8(±0.20)	8.1(±0.02)	1.8(±0.30)	13.7(±0.08)	0.56(±0.01)
Mixing Zone	B1	20.874400° -90.368370°	7.7(±0.1)	31.8(±0.28)	8.1(±0.01)	2.0(±0.09)	12.8(±1.69)	0.64(±0.04)
	B2	20.872666° -90.367290°	5.5(±0.1)	31.9(±0.77)	8.1(±0.01)	2.4(±0.02)	12.9(±0.04)	0.54(±0.03)
	B3	20.871549° -90.365180°	4.8(±0.2)	29.9(±0.14)	7.8(±0.02)	1.6(±0.12)	13.1(±0.27)	0.55(±0.01)
Marine Zone	C1	20.776452° -90.412721°	25.9(±0.3)	33.6(±0.21)	8.3(±0.01)	3.9(±0.19)	11.6(±0.08)	0.40(±0.06)
	C2	20.776939° -90.409605°	23.7(±0.7)	33.1(±0.20)	8.3(±0.01)	5.3(±0.01)	12.5(±0.01)	0.22(±0.07)
	C3	20.775128° -90.406584°	4.4(±0.01)	33.3(±0.10)	8.1(±0.01)	4.9(±0.11)	14.6(±0.51)	0.71(±0.06)

Bacterial and archaeal community structure analysis

PCoA calculated on an unweighted UniFrac distance matrix showed a differentiated distribution of microbial taxa according to Celestún zones for both Bacteria and Archaea domains (Fig. 2). The PERMANOVA test analysis suggested that among the environmental data, sample zonation and salinity were of statistical significance, explaining 31% and 16–17% of the total variance of both bacterial/archaeal communities, respectively. Moreover, dissolved oxygen was of statistical significance explaining 15% for Archaea (Table 2).

Fig. 2. PCoA based on the unweighted UniFrac metric of Bacteria (a) and Archaea (b) diversity using 16S rRNA gene sequences. The zonation explained 31% of the variance of the microbial communities.

Table 2: Adonis test performed on the UniFrac distance matrix, based on 16S rRNA gene sequencing data and the environmental parameters. Asterisks denote data that are statistically significant.

Variable	Bacteria		Archaea	
	R^2	P	R^2	P
Zone	0.31	0.007*	0.31	0.006*
Salinity	0.16	0.004*	0.17	0.001*
Carbon	0.13	0.32	0.12	0.23
Nitrogen	0.13	0.66	0.11	0.25
pH	0.13	0.17	0.13	0.10
Dissolved oxygen	0.15	0.07	0.15	0.031*

In this study a total of 1,382,171 bacterial raw reads were obtained, while archaeal reads accounted for 2,341,355 sequences. After denoising and chimera remotion, we obtained 760,032 and 932,347 tags for Bacteria and Archaea, respectively (Online Appendix A1). Observed bacterial ASVs in analyzed samples from Celestún ranged from 658 to 954, with the highest ASVs number in the mixing zone. Shannon and Simpson diversity indexes varied from 6.07 to 6.57 (mean 6.36 ± 0.13) and 0.995–0.998 (0.996 ± 0.0008), respectively, and did not significantly differ among the three stratified zones. In turn, observed archaeal ASVs reached between 513 and 1458; Shannon and Simpson indexes of the samples varied from 4.7 to 5.9 (5.55 ± 0.39) and 0.941–0.995 (0.974 ± 0.015), respectively (Appendix A1). Interestingly, the bacterial alpha diversity indexes did not change among Celestún zones, and only the archaeal alpha diversity was significantly higher in the coastal marine zone ($p < 0.05$).

Bacterial and archaeal community composition

The bacterial community composition in the studied sediments from Celestún was composed of 35 phyla, but only 22 lineages were represented in an abundance >1% (Fig. 3). Proteobacteria, Chloroflexi, Bacteroidetes and Planctomycetes were the dominant taxa, followed by Spirochaetae Acidobacteria, Actinobacteria, Cyanobacteria and Verrucomicrobia. Proteobacteria accounted for 26–34% of the relative abundance in all analyzed sediment samples, mainly integrated by Desulfobacteraceae (4–8%), Desulfarculaceae (1–5%), Rhodobacteraceae (1–5%) and Chromatiaceae (1–4%) families (Online Appendix A2). Abundances of Chloroflexi ranged between 7 and 28%, being represented by Anaerolineaceae (3–10%), and uncultured members of Dehalococcoidia (1–4%). The Bacteroidetes (9–17% relative abundance) included Marinilabiaceae (1–5%), Flavobacteriaceae (1–4%) and Saprospiraceae. The main component of observed Planctomycetes (3–7%) belonged to the Planctomycetaceae family (1–5%). Furthermore, identified genus-level diversity of benthic bacterial communities from Celestún were mainly composed of Spirochaeta 2, Caldithrix, Desulfatiglans, Sva0081 and SEEP-SRB1 (Online Appendix A3).

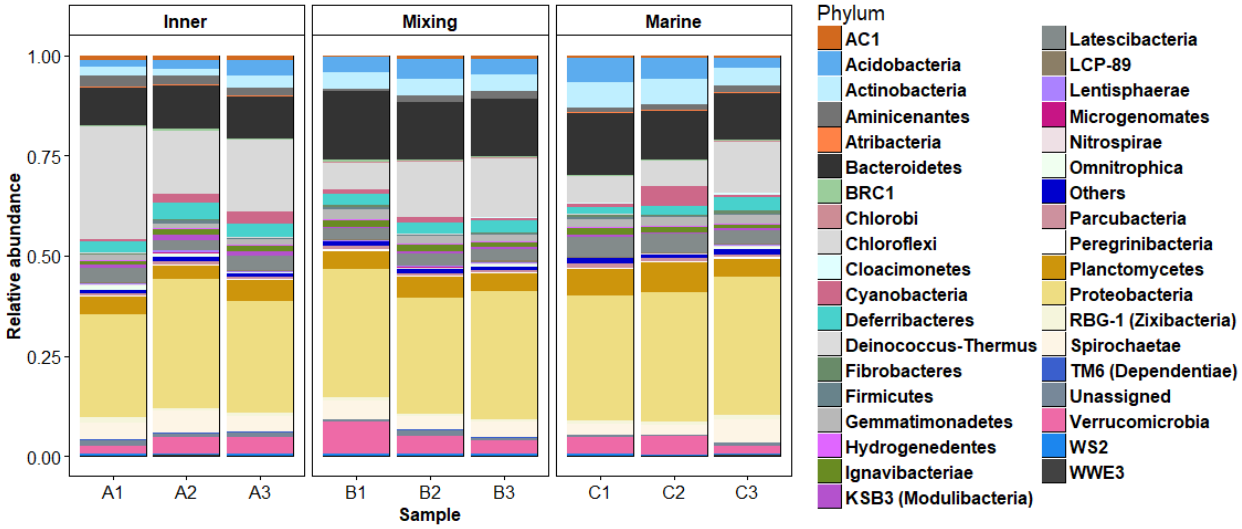

Fig. 3. Stacked bar charts showing relative abundance of the bacterial community composition at phylum level, detected in sediment samples from Celestún Lagoon. Phyla <1% were grouped in "Others".

16S rRNA gene sequences retrieved from Celestún sediments were affiliated to 12 archaeal phyla (Fig. 4). The best represented groups in all analyzed samples were Euryarchaeota, Thaumarchaeota, Woesearchaeota (DHVEG-6), Bathyarchaeota and Lokiarchaeota, with a range in their relative abundances between 15 and 54%, 8–36%, 7–30%, 12–25% and 7–21%, respectively. In addition, low abundances (~2%) of other lineages, such as Aenigmarchaeota, Ancient Archaeal Group (AAG), Diapherotrites and WSA2 environmental group were also detected (Fig. 5). Detailed analysis at family level allowed us to identify representatives within Euryarchaeota, belonging to Marine Benthic Group D (MBGD) and DHVEG-1 (11–25%) and Marine Group III (4–22%). Furthermore, low abundances (1–3%) of ANME-1b clade, Methanosaetaceae, CCA47, 20c-4, AMOS1A-4113-D04, ANT06-05 and VC2.1 Arc6 were also found (Online Appendix A4).

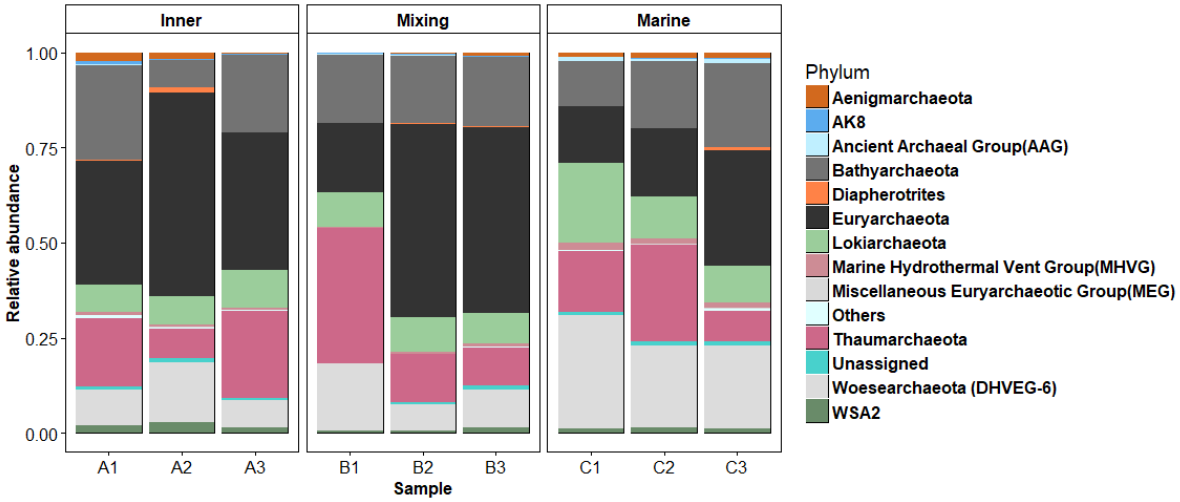

Fig. 4. Stacked bars showing relative abundance of the archaeal community composition at phylum level, detected in sediment samples from Celestún Lagoon. Phyla <1% were grouped in "Others".

Differential distribution of bacterial and archaeal communities in Celestún Lagoon

The analysis performed with LEfSe at ASV-level revealed a clear distinction in the microbial communities among the three stratified zones of Celestún Lagoon. A total of 71 bacterial and 163 archaeal ASVs showed LDA values higher than 2 (Online Appendix A5 and Online Appendix A6). The differentially abundant taxa detected in the inner zone from Celestún were composed of Bacteroidetes (the family Marinilabiaceae and ASVs from the order Sphingobacteriales), Spirochaetae, Proteobacteria (Desulfobacterales, Syntrophobacterales and SAR324 clade), Chromatiales, Deferribacteres, Modulibacteria, Cyanobacteria (Pleurocapsa), Chloroflexi (Anaerolineales) and Gemmatimonadetes. Interestingly, the LEfSe analysis identified some archaeal ASVs related to uncultured Bathyarchaeota, Euryarchaeota (MBGD, DHVEG-1, ANME-1b), Thaumarchaeota (Group C3 and environmental groups AMOS1A-4113-D04 and CCA47), Lokiarchaeota and Aenigmarchaeota. Moreover, ASVs from the pMC2A209, AK8 and WSA2 (20a-9) clades were also represented in the inner zone from the lagoon.

The distinct bacterial ASVs detected in the mixing zone were Bacteroidetes (the orders BD2-2, Sphingobacteriales, Cytophagales and Flavobacteriales), Proteobacteria (Desulfobacterales, Chromatiales, Desulfuromonadales, Syntrophobacterales, Myxococcales and KI89A clade), Chloroflexi (Anaerolineales), Ignavibacteriae and Acidobacteria (Holophagae-Subgroup 23).

Meanwhile, the archaeal diversity observed corresponded to uncultured Euryarchaeota (MMBGD and DHVEG-1), Thaumarchaeota (Group C3) and unassigned Bathyarchaeota, Lokiarchaeota and Woesearchaeota (DHVEG-6).

Bacterial ASVs in the coastal marine zone from Celestún Lagoon belonged to Proteobacteria (Sandaracinaceae and Hyphomicrobiaceae families and the orders Rhodobacterales, Xanthomonadales, Desulfarculales, Desulfobacterales, Rhodospirillales and Thiotrichales), Bacteroidetes (Flavobacteriales, Desulfarculales, Cytophagales and the family Marinilabiaceae), Gemmatimonadetes (BD2-11 terrestrial group), Spirochaetae, Aminicenantes and Acidobacteria (Holophagae-Subgroup 10). On the other hand, unassigned archaeal ASVs belonging to the Lokiarchaeota, Thaumarchaeota and Bathyarchaeota phyla obtained the highest LDA values (~3.5) in the analyzed coastal marine zone (Appendix A6). The differentiated abundances of the ASVs among Celestún zones, and their relative abundance are shown in Figs. 5 and 6.

Fig. 5. Differentiated abundances of the bacterial ASVs among Celestún zones and their relative abundance of the whole bacterial community in percentage.

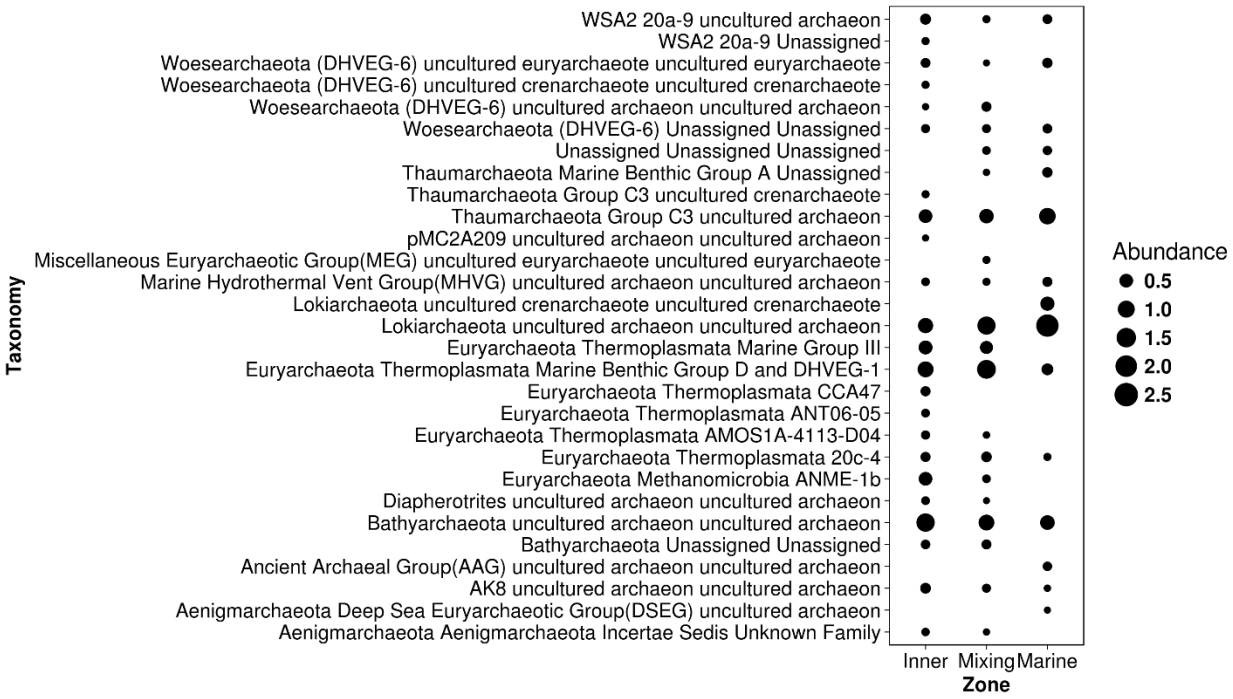

Fig. 6. Differentiated abundances of the archaeal ASVs among Celestún zones and their relative abundance of the whole archaeal community in percentage.

Discussion

Previous studies conducted on Celestún Lagoon have been focused on the hydrology (Herrera-Silvera, 1994, 1995), energy fluxes (Vega-Cendejas and Arreguí-Sánchez, 2001), parasites communities (Vidal-Martínez et al., 2002) and benthic macroorganisms (Hernández-Guevara et al., 2008). In this study, we employed a high-throughput sequencing approach to perform, for the first time, a characterization of the differentiated distribution of the prokaryotic community composition from Celestún Lagoon. Samples were obtained at sites representing the stable inner, mixing and marine zones of the lagoon, where salinity was the principal driver controlling the distribution and abundance of microbial assemblages in the lagoon.

Benthic microbial diversity

In the following section, we will discuss on the dominant prokaryotic communities from Celestún (Fig. 3). According to our results, Proteobacteria were mostly represented by Desulfobacteraceae, Desulfarculaceae and Rhodobacteraceae (Online Appendix A2), which include strictly anaerobic sulphate-reducing bacteria, aerobic and anaerobic

photo/chemoheterotrophs and heterotrophs growing on methylated compounds (Kuever et al., 2005a, 2005b; Lidbury et al., 2015). Members of Anaerolineaceae are chemolitho-organo-heterotrophs, degraders of carbohydrates and amino acids, and some of them are also hydrogenogens (Yamada et al., 2005; Yamada and Sekiguchi, 2009). Moreover, Bacteroidetes detected in this study (Marinilabiaceae, Flavobacteriaceae and Saprospiraceae) (Online Appendix A2) include facultative aerobic and strictly anaerobes, chemoorganotrophs and chemolithotrophs, as well as non-photosynthetic microorganisms widely distributed in aquatic environments (Rosenberg, 2014; McIlroy and Nielsen, 2014). Finally, members of Planctomycetaceae have been recognized as chemoorganoheterotrophs; nevertheless, this family also embraces the anammox bacteria. Frequently, this group only constitutes a few percent of the microbial community in coastal waters and marine sediments, but its ecological importance in the nitrogen cycle has been extensively demonstrated (Fuerst and Sagulenko, 2011).

Based on previous studies, Euryarchaeota and Thaumarchaeota are the phyla which include the best-known archaeal groups. Similar to our results, Euryarchaeota has been also observed as the predominant archaeal taxon in ecosystems (Offre et al., 2013). Members of this phylum are metabolically diverse (autotrophs, heterotrophs, chemolithotrophs), being abundant in lakes, marine waters, soils, and sediments (Teske and Sørensen, 2008). Thaumarchaeota has been identified as a clade of microorganisms ecologically relevant in ammonium oxidation (Offre et al., 2013), and some members appear to be versatile chemoorganotrophs, potentially growing on carbohydrates and amino acids (Beam et al., 2014; Adam et al., 2017). The Woesearchaeota is a novel phylum, of which the genomic features have suggested a possibly heterotrophic, symbiotic, or parasitic lifestyle (Castelle et al., 2015; Castelle and Banfield, 2018). Single-cell genomics, as well as metagenomic analysis of Bathyarchaeota, have suggested their capacity for methane production from methylated compounds (Evans et al., 2015), CO2 fixation via acetogenesis (He et al., 2016) and dissimilatory nitrite reduction to ammonium (Lazar et al., 2016). Notably, based on Bathyarchaeota abundance in methanogenic anoxic environments, some reports have also proposed their potential role in novel models of anaerobic methane oxidation (Saxton et al., 2016; Valenzuela et al., 2017). The phylum Lokiarchaeota is an emerging lineage commonly found in anaerobic marine and estuarine sediments (Adam et al., 2017). Metagenomic studies have suggest that some of them might be anaerobes, autotrophs, and hydrogen-dependents (Sousa et al., 2016; Castelle and Banfield, 2018).

Spatial distribution of bacterial and archaeal communities

In this section, we will discuss on the taxa with higher LDA values. Displayed bacterial ASVs in the inner zone were related to sulphate reducers (Desulfobacteraceae, Desulfobulbaceae) and different Archaea affiliated to Methanosaetaceae, WSA2(20a-9) clade, MBGD and ANME-1b. All the microorganisms detected by this approach have been previously reported as key players in the methane cycle, suggesting that methanogenesis/methanotrophy are predominantly performed under oligohaline conditions on this type of stratified ecosystems. Members of the family Methanosaetaceae are recognized as acetoclastic methanogens. WSA2 clade was originally proposed as a class within Euryarchaeota (Nobu et al., 2016), but further phylogenetic analyses of the mcrA and 16S rRNA genes, distinguished this group as a new archaeal phylum, the Verstraetearchaeota. Metabolic reconstruction evidenced that members of Verstraetearchaeota encode the genes required for methylotrophic methanogenesis (Vanwonterghem et al., 2016). This taxon usually appears in low abundances, widespread across diverse environments, such as marine sediments, lakes, and hot springs (Nobu et al., 2016, McKay et al., 2017), and there are no reports about its contribution to methane emissions in tropical coastal lagoons. On the other hand, MBGD and ANME-1b are well known taxa involved in anaerobic methane oxidation. Previous studies have identified the presence of ANME-1b in shallow marine sediments (Lee et al., 2016) and wetlands (Valenzuela et al., 2017). Since traditional studies have reported that members of ANME-1b are involved in anaerobic methane oxidation coupled with sulphate reduction (Cui et al., 2015), recently, Valenzuela et al. (2017) reported, in a similar wetland, a novel anaerobic methanotrophic process, coupled with the reduction of the humic fraction of natural organic matter.

The microbial groups detected as enriched for the mixing zone (Fig. 5; Online Appendix A5) have been previously recognized as predominantly aerobic or facultative heterotrophs, chemoorganotrophs and chemolithoautotrophs, commonly recognized as involved in the degradation of organic matter (Lino et al., 2010; Kuever, 2014; Spring et al., 2015; Kielak et al., 2016). Because the mixing zone receives water inputs from both the inner and the marine zones, previous reports have shown high ammonium, nitrite, and nitrate concentrations over the other two zones (Herrera-Silvera, 1994, 1995; Vega-Cendejas and Arreguí-Sanchez, 2001). In fact, based on the water characteristics, this is a unique zone considered as mesotrophic (Tapia-Gonzalez et al., 2008). We hypothesized that those high nutrient concentrations

previously reported would support the development of these metabolically diverse microbial communities.

From the microbial groups detected by LefSe for the marine zone (Fig. 5; Online Appendix A5 and A6), members of Sandaracinaceae and Hyphomicrobiaceae have been shown to be facultative anaerobes, thriving mainly in soils and marine environments (Mohr et al., 2012; Oren and Xu, 2014). The Rhodospirillales order integrates a phylogenetic cluster of anoxygenic phototrophic purple bacteria, abundant in the water column (Imhoff et al., 2005). Aminicenantes was firstly identified in sediments from Yellowstone Park, but then it has subsequently been identified in terrestrial and marine habitats. The metabolic capabilities of this clade are currently unknown, as well as its ecological role on coastal ecosystems (Farag et al., 2014). The environmental Thaumarchaeota Group-C3 has been reported as an acetate-consuming archaeon in similar marine sediments (Na et al., 2015). These differentiated bacterial/archaeal taxa could be related to some water characteristics in the marine zone from Celestún, since here, the lowest ammonium, silicate, nitrite, and nitrate concentrations have been reported (Herrera-Silvera, 1994, 1995; Tapia-Gonzalez et al., 2008). Several Archaeal ASVs differently enriched for the mixing and marine zone corresponded to uncultured members of Thaumarchaeota, Bathyarchaeota, Lokiarchaeota and Woesearchaeota (DHVEG-6) (Appendix A6). Unfortunately, due to the lack of information associated to database sequences, it was impossible an assignment at lower taxonomic levels. Thus, further efforts for the isolation of archaeal representatives from coastal ecosystems are needed (Auguet et al., 2010; Offre et al., 2013; Lazar et al., 2017).

Microbial community structure and environmental variables

In this study, the water measurements were determined from surficial water, although all the stations had an average water depth of 1.5 m. Even though there are not previous reports about stratification in the water column for the Celestún Lagoon, we hypothesized that surface conditions may do not exactly reflect the same conditions at the bottom, near the sediments. Previous works have only reported horizontal stratification of the lagoon among analyzed zones (inner, mixing and marine) (Herrera-Silvera, 1994; Tapia-Gonzalez et al., 2008; Stalker et al., 2014), but further studies are required to understand if there is a vertical stratification in the water column on this coastal lagoon.

As evidenced by PCoA and PERMANOVA analysis, sample zonation was the main factor linked to the presence of specific microbial taxa, followed by salinity (Table 2). Several studies developed in lakes (Wu et al., 2006), wetlands (Wang et al., 2012) and lagoons (Pavloudi et al., 2016) have shown that salinity is the major driver determining the microbial communities prevailing, due to the strong selective pressure promoted by the osmotic stress (Lozupone and Knight, 2007). A clear example of this was found in the coastal marine zone, where the microbial communities of site C3 separated from the other samples in the PCoA (Fig. 2), probably due to the lower salinity condition of the station, as compared to the other two sites from the zone (Table 1). Since salinity could be used as a measure of the origin of the water, it can be hypothesized that freshwater discharge would occur in the eastern part of the mouth of the lagoon. Stalker and colleges (2014) reported that the freshwaters springs appeared to be an eastern boundary phenomenon in Celestún, mainly observed in the inner and mixing zone from the lagoon. Here, we found that freshwater influence would occur in the mouth of the lagoon, but it is unclear if it is seasonally consistent.

The estimated bacterial diversity and richness did not significantly change along the lagoon, despite the differentiated distribution of the bacterial ASVs that was evident among the three stratified zones (Appendix A1, A5 and A6). Celestún Lagoon has been described as a highly productive ecosystem, with high organic matter content in sediments, derived from several nutrient inputs, which can support the development of diverse microbial communities (Herrera-Silvera, 1994; Tapia-Gonzalez et al., 2008). Moreover, the archaeal alpha diversity was significantly higher only in the marine zone. The coastal marine zone, in fact, was characterized by an estuarine salinity (Table 1). In agreement, some studies examining the change in archaeal communities in a salinity gradient have found that the archaeal diversity increases at estuarine salinities, as compared to freshwater and marine ecosystems (Webster et al., 2015; Xie et al., 2014). A recent survey analyzing the diversity and distribution of Archaea in global estuarine ecosystems has suggested that salinity, as well as the latitude, are the two major factors driving the distribution of archaeal communities in these ecosystems (Liu et al., 2018).

Celestún represents an important coastal lagoon in the Yucatán Peninsula, of which the hydrology has been well characterized (Herrera-Silveira, 1995; Stalker et al., 2014). This study contributes to a better understanding of the benthic microbial diversity in Celestún and associates the detected taxa to some environmental features that influence their distribution.

However, we are aware that part of the unexplained variance in the community structure could be associated to other environmental variables related to the zonation not measured here. Thus, further studies are required to link specific taxa to changes on particular nutrients gradients on this ecosystem.

Conclusions

The results obtained in this work highlight a wide variety of both bacterial and archaeal communities thriving on coastal lagoons. Alpha diversity (Shannon and Simpson indexes) did not change significantly along the three stratified zones in the lagoon (oligohaline, marine and the mixing) for Bacteria, and only the archaeal diversity was significantly higher in the marine zone. Moreover, differentially abundant microbial taxa were identified for each region, as supported by statistical analyses. The detailed comparison of the benthic microbial community composition presented in this work will contribute to further understanding of the ecosystem functions and biogeochemical cycling within the coastal lagoon and in similar estuarine and coastal environments.

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CHAPTER 2

Microbial community profiling of Bacteria, Archaea and Eukarya in hypersaline microbial mats and endoevaporites from Guerrero Negro, Baja California Sur, Mexico.

In Chapter 2, the prokaryotic and micro-eukaryote community structure of sediments, microbial mats and endoevaporites, developing in the solar saltern “Exportadora de Sal”, Guerrero Negro, Mexico was studied. Novel microbial lineages not previously reported for these human-made hypersaline environments were found.

This chapter is in the final stages of preparation for a journal submission, as a short communication paper:

Microbial community profiling of Bacteria, Archaea and Eukarya in hypersaline microbial mats and endoevaporites from Guerrero Negro, Baja California Sur, Mexico.

Abstract

In this work, the prokaryotic and micro-eukaryote community structure and composition of hypersaline sediments (4% salinity), microbial mats (at 6% and 8%) and endoevaporites (16%), developing in multiple ponds from the solar salterns operated by Exportadora de Sal, in Baja California Sur, Mexico was studied, using independent Illumina amplicon sequencing of 16S rRNA and 18S rRNA genes. Different microbial assemblages were found among analyzed samples. Bacterial community composition was mostly related to Proteobacteria, Bacteroidetes, Chloroflexi and Cyanobacteria. Archaeal groups belonged to Crenarchaeota, Euryarchaeota, Asgardeota, Diapherotrites and Nanoarchaeaeota. Micro-eukaryote community was dominated by Opisthokonta, Archaeplastida and the SAR Supergroup. These results highlight the unexplored microbial miscellany thriving in hypersaline ponds, providing for the first time a full overview of the microbiota inhabiting these sites and increasing the available information about the uncultured halophile microbial communities.

Keywords: Illumina sequencing, microbial diversity, coastal solar saltern, marine halophiles, extreme environments.

Introduction

Hypersaline environments, defined as those with a higher salinity than seawater (3.5% of salinity), are commonly found in coastal intertidal zones where evaporation exceeds sea/freshwater input (Reitner and Thiel, 2011). Coastal hypersaline ecosystems include natural saline lakes, saline soils, coastal lagoons, salt flats (sabkha), salt marshes, and human-made solar salterns (Rich and Maier, 2015). Solar salterns are based on a large-scale multi-pond system, where seawater is evaporated until sea salt can be harvested (Javor, 2002). It is well known that at salinities from approximately 6 to 16%, laminated microbial mat ecosystems flourish in evaporation ponds (Wong et al., 2016). At higher salinities (17-36%), massive precipitation of gypsum occurs and within the gypsum crust, stratified communities of pigmented microorganisms develop (Tazaz et al., 2013; Oren et al., 1995; Rothschild et al., 1994). Both microbial mats and gypsum crust endoevaporites are functional complex ecosystems, that harbor a wide metabolic and phylogenetic diversity of the tree domains of life (Bacteria, Archaea and Eukarya). These microbial structures have been model of study for several purposes, including biochemical cycling research, biotechnology potential, and their implications in astrobiology (Javor, 2002; Tazaz et al., 2013).

Exportadora de Sal, S.A. de C.V. (ESSA) is a system of solar salterns located at Guerrero Negro, Baja California Sur (BCS), northwestern Mexico, comprising 13 interconnected concentration areas. These extensive seawater evaporation ponds have been the subject of microbial ecology research for more than 50 years (Des Marais, 2010). Studies using clone libraries and 454 sequencing have reported members of Bacteroidetes, Proteobacteria, Cyanobacteria and Choroflexi in mats from sites ESSA-A1 (salinity 6%) and ESSA-A4 (P4n5) (salinity 8%) (García-Maldonado et al., 2018; Harris et al., 2013; Ley et al., 2006), while Bacteroidetes, Alpha Proteobacteria and Cyanobacteria have been reported in brine and endoevaporites at higher salinities (18-38%) (Dillon et al., 2013; Sahl et al., 2008). Archaeal diversity has been less studied. Using lipid biomarker and molecular methods, Thermoplasmatales and Halobacteria has been detected as dominant archaeal taxa in microbial mats from site ESSA-A4 (P4n1) (salinity 8%) (Jahnke et al., 2014; Orphan et al., 2008). Robertson and colleagues (2009) studied mats from site ESSA-A4 (P4n1) and observed Euryarchaeota and Crenarchaeota as the best phyla represented. In the case of Eukaryotic communities, Feazel and colleagues (2008) characterized some nematode (Monhysteridae and Rhabdolaimidae), arthropods (Besorus ludirus and Bryocamptus pygmaeus) and fungi

(Ascomycota) from site ESSA-A4 (P4n5). More recently, using qPCR and metagenomics, the abundance and diversity of fungi were characterized in different layers of a microbial mat from site P4n5, revealing *Thermothelomyces*, *Pyricularia*, *Fusarium*, *Colletotrichum*, *Aspergillus*, *Botrytis*, *Candida* and *Neurospora* as the predominant taxa.

Even though several molecular studies have been performed on ESSA, the systematic composition of Bacteria, Archaea and Eukarya among different ponds has not been fully explored. In this work, based on 16S rRNA gene and 18 rRNA gene sequencing, the prokaryotic and micro-eucaryotic community structure developing in sediments, soft microbial mats and gypsum-encrusted endoevaporites is characterized, for a better understanding about the halophile microbiota in this system from ESSA. In addition, the potential ecological role of these microorganisms is discussed.

Materials and methods

Sampling

Sampling was performed in four sites of ESSA in November 2016: (1) sediments from the pumps station zone near ESSA-A1 (A1-Pumps); (2) microbial mats from ESSA-A1 (A1n4) and (3) ESSA-A4 (A4n1); (4) endoevaporites from ESSA-A8 (A8) (Fig. 7) Coordinates of sampling sites are shown in Table 3. Triplicate samples were taken from the first centimeter of the surface, homogenized, and placed in 50-ml sterile conical Falcon™ tubes. Salinity was measured from surface water using a portable American Optical refractometer. Temperature was measured *in situ* using a YSI multiparameter sonde. Samples were immediately transferred in ice to the laboratory for DNA isolation.

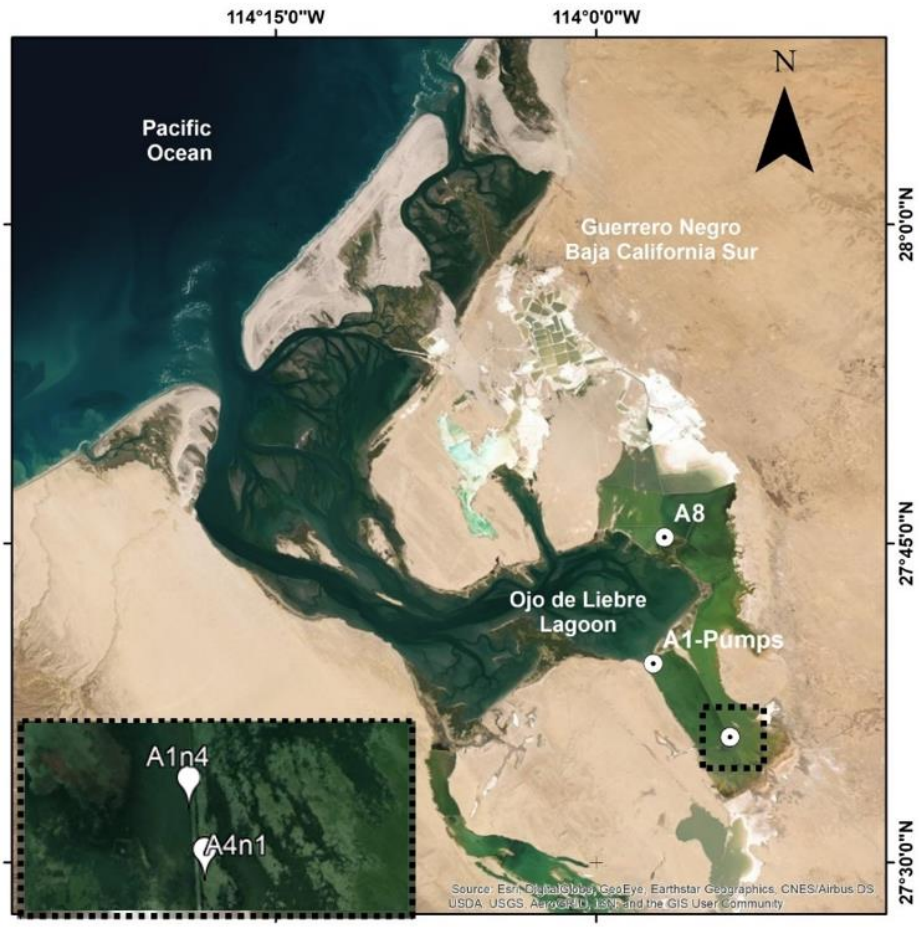

Fig. 7: Sampling sites at Baja California Sur, Mexico, in several ponds from ESSA.

Total environmental DNA from each locality was extracted by triplicate from 0.25 g of sample. Cell lysis was performed with a TissueLyser LT (Qiagen, Hilden, Germany) and DNA was extracted using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA). A sample blank (spin column with no sample supplied) was processed alongside the extractions. DNA quality was analyzed by 1% agarose gel. DNA extracts were homogenized per sampling site. 16S rRNA gene fragments from Bacteria were amplified using the universal primers sets 16SF/16SR, spanning the V3 and V4 regions. For Archaea, the primers Arch0519/1041, covering the V4-V6 regions were used. Both primers characteristics and thermocycling conditions are described by Klindworth and colleagues (2013). Universal eukaryotic primers F566 and R1200, that covers the V4 and V5 regions, were used for amplification of 18S rRNA gene, as described by Hadziavdic and colleagues (2014). Each PCR reaction (20 µl) was performed with 2 µl of DNA (5 ng/µl), 0.5 µl of each primer (10 µM) and 10 µl of 2× Phusion High-Fidelity MasterMix (Thermo Scientific, Waltham, MA, USA).

Library preparation of amplicons was performed according to Cadena and colleagues (Cadena et al., 2019). Paired-end sequencing (2 × 300 bp) was carried out on the MiSeq platform (Illumina, San Diego, CA, USA), with a MiSeq Reagent Kit V3 (600 cycles). Sequencing was performed in the Aquatic Pathology Laboratory at CINVESTAV-Mérida. It is important to highlight that only one sample was sequenced per site, this limitation should be considered when interpreting molecular data.

Bioinformatic work

The demultiplexed data were analyzed with the QIIME2 (2017.11) pipeline (Caporaso et al., 2010). Amplicon sequence variants (ASV) were obtained using DADA2 plugin (Callahan et al., 2016). Representative ASVs were taxonomically assigned using V-SEARCH (Rognes et al., 2016) with the SILVA small subunit ribosomal RNAs (16S/18S) v132 databases as references. Then, the representative sequences were aligned with the MAFFT algorithm (Katoh, 2002) and filtered for unconserved and gapped positions to build a phylogenetic tree with fasttree (Price et al., 2010). Data were normalized among samples by sub-sampling to lowest reads count (100 000 reads for Bacteria, 25 000 for Archaea and 29 000 Eukarya). The resulted data were exported to the R environment with the phyloseq (McMurdie and Holmes, 2014) and ggplot2 (Gómez-Rubio, 2017) libraries. The alpha diversity indexes as observed ASVs and Shannon Index were calculated for all studied sites. For the bacterial, archaeal, and eukaryotic data sets, the taxonomic classification was used for a hypothesized functional role in the ecosystem, found on main metabolic capabilities described in literature for those taxonomic groups. This, based on niche conservatism, which states that closely related linages share similar niches (Wiens and Graham, 2005).

Results

Physicochemical characteristics of sampling sites

The water salinity increased across the analyzed ponds from 4, 6, 8 and 16% corresponding to sites A1-Pumps, A1n4, A4n1 and A8, respectively. *In situ* water temperature ranged from 18 to 20.1 ºC (Table 3).

Table 3: Physicochemical characteristics and type of sample collected from the studied sites.

Sampling site	Geographic coordinates	Salinity (%)	Temperature (ºC)	Macrostructure
A1-Pumps	N: 27º 39.363, W: 113º 57.324	4	18.0	Sediment
A1n4	N: 27º 35.909, W: 113º 53.744	6	25.0	Microbial mat
A4n1	N: 27º 35.899, W: 113º 53.730	8	19.0	Microbial mat
A8	N: 27º 45.300, W: 113º 56.803	16	20.1	Endoevaporite

Microbial community structure and alpha diversity

Sequencing stats from samples indicating sequence reads before and after denoising were incorporated in Table 4. Bacterial ASVs detected in all analyzed samples ranged 628-2803, observing the highest number in the A1-Pumps. Bacterial diversity estimated with the Shannon Index varied from 5.4 to 7.9 H'. In turn, observed archaeal ASVs ranged from 238 to 516, finding a higher diversity in the endoevaporites from site A8. Unfortunately, eukaryotic sequences from sediments of A1-Pumps were discarded to further bioinformatic analysis due to low-quality reads obtained (data no shown). Nonetheless, eukaryotic observed ASVs of the other samples reached 37-105, retrieving more ASVs with increasing salinity. Shannon index changed between 1.0 and 2.9 H'.

Table 4: Stats from samples indicating sequence reads before and after denoising and chimera remotion and alpha diversity indexes obtained. N. A. = Not applicable.

Sample	Bacteria				Archaea				Eukarya			
	Input reads	Clean reads	Obser-ved ASVs	Shannon (H´)	Input reads	Clean reads	Obser-ved ASVs	Shannon (H´)	Input reads	Clean reads	Obser-ved ASVs	Shannon (H´)
A1-Pumps	633176	220422	2803	7.4	42240	39976	516	5.2	N.A.	N.A.	N.A.	N.A.
A1n4	284223	115984	1612	6.8	58289	50693	238	4.3	31934	31225	37	1.0
A4n1	560279	184141	2000	6.9	119600	112489	473	4.8	32115	30813	49	1.2
A8	549870	127463	628	5.4	102675	89399	274	2.2	31828	30227	105	2.9

Microbial community composition

Bacteria

All the classified sequences were correctly affiliated to 29 bacterial phyla, being Proteobacteria, Bacteroidetes, Cyanobacteria and Chloroflexi the best represented clades. Analyzed sediments from A1-Pumps were dominated by Proteobacteria (Alphaproteobacteria, 23.6%; Gammaproteobacteria 12.3%) and Bacteroidetes (Bacteroidia, 27.4%). For site A1n4, the most abundant bacterial groups corresponded to Bacteroidia (26.1 %), Deltaproteobacteria (10.7%) and Gammaproteobacteria (9.0%). Samples from A4n1 were represented by Bacteroidia (19.6 %), Deltaproteobacteria (11.9 %) and Anaerolineae (9.5 %). Interestingly, in the highest salinity pound A8, the favored taxa belonged to Oxyphotobacteria (26.8 %), Rhodothermia (26.8 %) and Alphaproteobacteria (8.8 %) (Fig. 8).

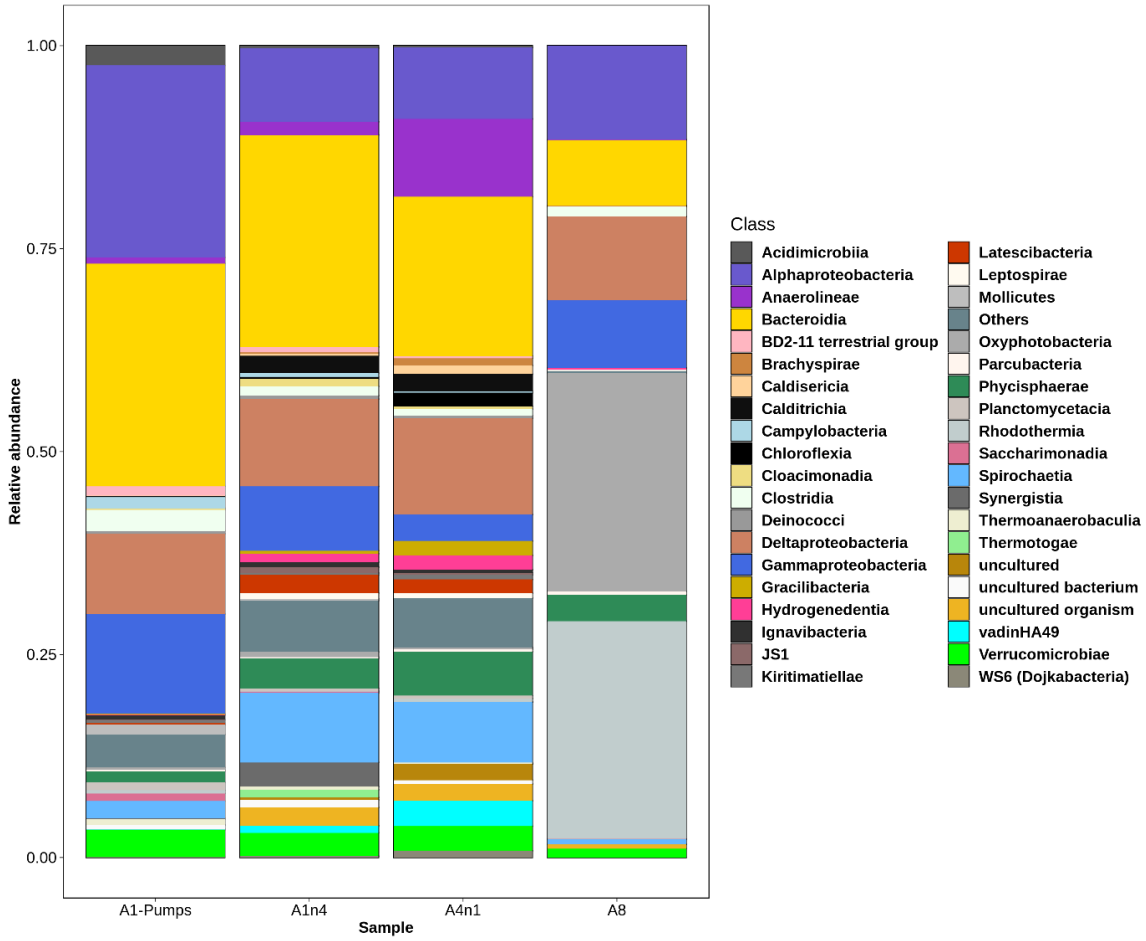

Fig. 8: Stacked bar charts showing relative abundance of the bacterial community composition at Class level, obtained from analyzed hypersaline sediments, microbial mat and endoevaporites. Phyla < 1% are collapsed in “Others”.

Archaea

The archaeal community from studied samples was composed of 6 Phyla: Euryarchaeota, Crenarchaeota, Diapherotrites, Asgardaeota, Nanoarchaeaeota and Thaumarchaeota. Sediments from A1-Pumps were composed of Crenarchaeota (Bathyarchaeia, 33.5%), Euryarchaeota (Halobacteria, 20.6%) and Asgardaeota (Lokiarchaeia, 20.8%). Microbial mats from A1n4 were integrated by Nanoarchaeaeota (Woesearchaeia, 44.3%), Diapherotrites (Micrarchaeia, 18.8%) and Asgardaeota (Odinarchaeia, 11.1%). Mats from A4n1 contained Diapherotrites (Micrarchaeia, 65.7%) and Asgardaeota (Lokiarchaeia, 20.9%). Finally, endoevaporites from A8 displayed members of Euryarchaeota (Halobacteria, 59.9%) and Nanoarchaeaeota (Woesearchaeia, 22%; Nanohaloarchaeia, 10.8%) (Fig. 9).

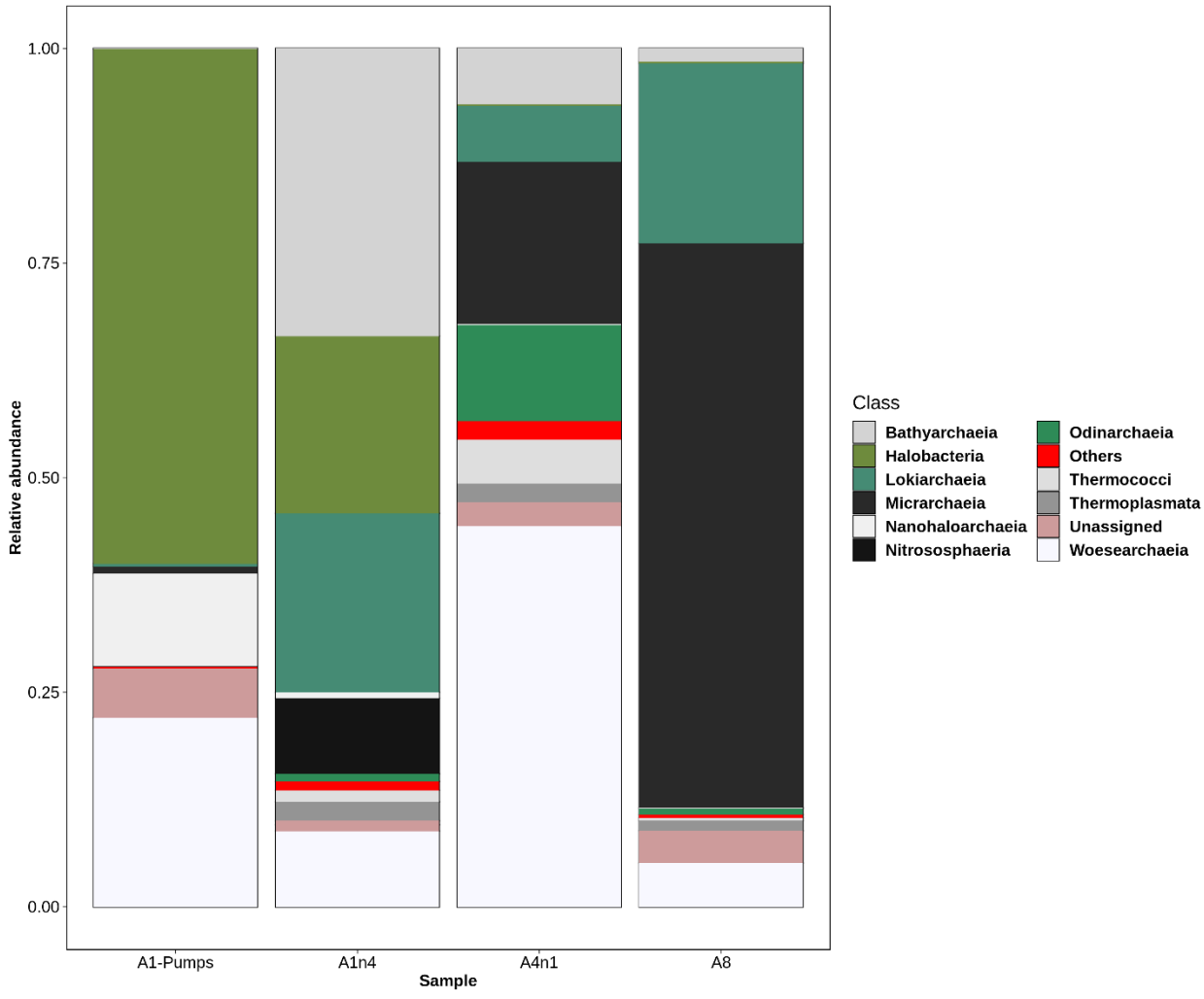

Fig. 9: Stacked bar charts showing relative abundance of the archaeal community composition at Class level, obtained from analyzed hypersaline sediments, microbial mat and endoevaporites. Phyla < 1% are collapsed in “Others”.

Eukarya

Sequencing of 18S rRNA gene of microbial mats and endoevaporites allowed to identify 3 micro-eukaryotic phyla major represented: Opisthokonta, Archaeplastida, and the SAR Supergroup. A1n4 was mainly dominated by Opisthokonta (Holozoa, 83.8%) and Archaeplastida (Chloroplastida, 13.4%). Microbial mats from A4n1 had a complete dominance of Opisthokonta (Holozoa, 75.9%; Nucletmycea, 13.8%). Gypsum crust endoevaporites from A8 showed high abundances of Opisthokonta (Nucletmycea, 36%) and SAR (Stramenopiles, 12.1%) (Fig. 10).

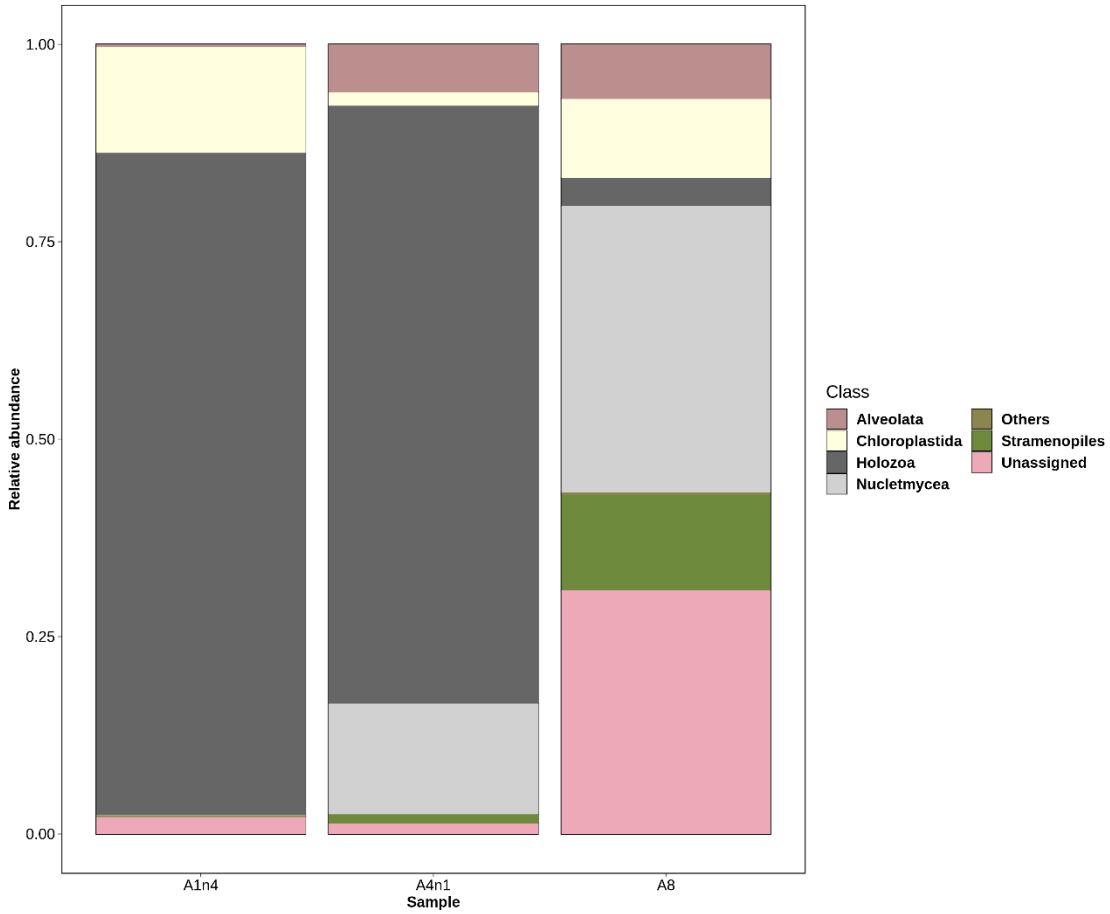

Fig. 10: Stacked bar charts showing relative abundance of the micro-eukaryotic community composition at Class level, obtained from analyzed hypersaline sediments, microbial mat and endoevaporites. Phyla < 1% are collapsed in “Others”.

Discussion

Solar salterns are one of the best examples of ecosystems with natural and strong chemical gradient. These hypersaline habitats are based on flow-through pond systems, with

environmental conditions relatively stable over time where microbial macrostructures, such as microbial mats and endoevaporites, often develop (Des Marais, 2010; Javor, 2002). Therefore, these ecosystems represent unique model systems to investigate microbial mats and biochemical cycling and, there are many studies using traditional microbiological techniques on the ecology of multi-pond salterns (Dillon et al., 2013; Rothrock and Garcia-Pichel, 2005). Here, based on 16S rRNA and 18S rRNA genes Illumina massive sequencing, the composition and structure of Bacteria, Archaea and Eukarya developing in sediments, soft microbial mats, and gypsum-encrusted endoevaporites from the Exportadora de Sal saltern (ESSA) in Guerrero Negro, BCS, Mexico, were characterized. ESSA saltern has been intensively studied (Des Marais, 2010; Feazel et al., 2008; García-Maldonado et al., 2018; Harris et al., 2013; Ley et al., 2006). However, this study represents the first use of next generation sequencing to examine the phylogenetic miscellany across sites in a wide salinity range (6-16%), characterizing locations not previously reported, which increase the known microbial diversity for hypersaline environments.

Bacteria

Bacteria domain is the best-known microbial group in this hypersaline system. The high relative abundances of Proteobacteria, Bacteroidetes and Chloroflexi observed in analyzed samples (Fig. 8) have been also reported in previous studies characterizing the bacterial diversity of microbial mats and endoevaporites from ESSA (García-Maldonado et al., 2018; Harris et al., 2013; Ley et al., 2006). Representatives of Proteobacteria in microbial mats are recognized for hydrogen metabolism, sulfate reduction, chemoorganotrophy and/or chemolithoautotrophy, with potential ecological roles as fermenters (Harris et al., 2013; Sahl et al., 2008). Bacteroidetes usually are conspicuous in the photic zone of the mats. Thus, it has been suggested that this group would be important phototrophs in the site, although it also includes heterotrophic aerobic and facultatively anaerobic organisms (Harris et al., 2013). Chloroflexi was suggested in Elkhorn Slough mats to be related to photoheterotrophy (Burow et al., 2012). However, dark filamentous clades of the Anaerolineae responsible for uptake of acetate have also been observed in microbial mats from ESSA-A4 (Lee et al., 2014). In this study, we found a dominance of Cyanobacteria (Oxyphotobacteria) in the highest saline site (16%). These results agree with previous works on evaporite crust from Area 9, where they appeared as colorful green strata due to organisms with photosynthetic pigment (Sahl et al., 2008). It is well known that Cyanobacteria are primary producers at the basis of the microbial

foodweb in hypersaline systems, involved in the production of organic matter, and in nitrogen fixation (Stal, 1995).

Archaea

Archaeal community composition was clearly different in each analyzed pound (Fig. 9). Dominance of Bathyarchaeia and Halobacteria was observed in the site with lower salinity (4%). It has been proposed that Bathyarchaeia are degraders of organic matter and some of them are methane producers (Zhou et al., 2018). Halobacteria in saline system constitute a physiologically diverse group, including anaerobic fermenters, chemoorganotrophs and sulfur reducers (Oren, 2008). Microbial mats from A1n4 were composed of Woesearchaeia, Micrarchaeia, and Odinarchaeia. Woesearchaeia has been observed in coastal zones, where they may exhibit fermentative and symbiotic lifestyles (Wang et al., 2019). Low abundances of Micrarchaeia have been observed in other extreme environments, such as radioactive sites (Vázquez-Campos et al., 2019) and in acid mine drainage (Pei et al., 2019), although its ecological role remains unresolved. Mats from A4n1 contained Micrarchaeia and Lokiarchaeia. Similarly, Lokiarchaeia was detected in analogous microbial mats incubated under methanogenic conditions (García-Maldonado et al., 2018). These microorganisms are believed to be autotrophic or with hydrogen-dependent metabolism (Sousa et al., 2016). Finally, not surprisingly, endoevaporites from A8 were dominated for Halobacteria, which agrees with previous studies (Sahl et al., 2008). However, representatives of Nanohaloarchaeia were also well represented. Nanohaloarchaea has been observed in hypersaline lakes and in similar endoevaporites (García-Maldonado et al., 2018; Narasingarao et al., 2012). Recent studies suggest that Nanoarchaeota live as parasites, or possibly as symbionts of hydrogenotrophs (Jarett et al., 2018).

Eukarya

Holozoa was the major micro-eukaryote linage represented in microbial mats from A1n4 and A4n1 (Fig. 10). Similar results have been reported in mats from a magnesium sulfate hypersaline lake from Washington (Bernstein et al., 2017). However, according to our results, A1n4 was composed of Rhabdocoela, while A4n1 was constituted by Enoplia. Rhabdocoela is a group of important grazers flatworms, commonly found in salt marshes (Armonies, 1986). Enoplia is a phylogenetic clade of nematode, abundant in coastal environments, such as beaches and estuaries (Venekey, 2010). Gypsum crust endoevaporites from A8 showed high

abundances of Opisthokonta (Aspergillaceae) and SAR (Stramenopiles). Aspergillaceae are related to saprophytic fungi that has been also reported by culture-independent approach as dominant taxa in halites from the Atacama Desert (Dong et al., 2019). Recently, the genus *Aspergillus* has been proposed to participate in denitrification in similar microbial mats from Guerrero Negro (Maza-Márquez et al., 2021). Thraustochytriaceae are heterotrophic fungus-like protist, abundant in coastal waters (Liu et al., 2017), also detected in benthic mats from Hot Lake (Bernstein et al., 2017). This group of protists is becoming important for biotechnological purposes, such as food additive in aquaculture industries (Leyland et al., 2017).

Alpha diversity

As a general pattern, we observed that diversity and richness for Bacteria and Archaea decreased with increasing salinity (Table 4). It is well known that salinity is the major factor shaping microbial communities because salinity stress requires a sophisticated metabolic specialization (Lozupone and Knight, 2007). In addition, a decreasing trend of microbial diversity along gradients of increasing salinity has previously been observed through different habitat types (Cadena et al., 2019; Nemergut et al., 2010; Wang et al., 2011). On the other hand, only few studies have emphasized the high eukaryotic diversity in hypersaline environments (Feazel et al., 2008; Tyrrell et al., 2013) and halites (Dong et al., 2019). Therefore, the present results contribute to the knowledge of the eukaryotic assemblages and its alpha diversity of hypersaline sites.

Conclusions

This study highlights the bacterial, archaeal, and micro-eukaryotic microbiome and its putative functional capabilities in poorly investigated hypersaline sediments, microbial mats, and gypsum crust endoevaporites. A wide biodiversity was found, with representative members from more than 40 different microbial phyla, involved in the recycling of principal nutrients and in the organic matter degradation. The information of these microbial groups will allow to create novel techniques for the cultivation and research for further metabolic characterization of these taxa.

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CHAPTER 3

First characterization of coastal microbial mats in the Yucatán Peninsula, Mexico

In Chapter 3, four different localities at Yucatán were explored looking for microbial mats. This work report for the first time naturally developed coastal microbial mats in Sisal, Progreso, Dzilam and Ría Lagartos, Yucatán, Mexico.

This chapter corresponds to a study done during the dry season. However, a second sampling during the rainy season was performed and I am processing that information. When the results are final, all data will be gathered to make a paper that includes the seasonal changes of mats.

Abstract

In this study, we report for the first time an exploration of the physicochemical characteristics and the prokaryotic diversity of three different type of microbial mats from the Yucatán Peninsula, Mexico, a karstic ecosystem. Our results indicated that floating microbial mats occurred at lower salinity (2.2%), while flat and pustular mats were detected in hypersaline sites (6-9%). Different mineral composition of mats was revealed by XRD analysis; however, aragonite, calcite and halite were common minerals for all the studied samples. Based on the high throughput sequencing of the16S rRNA gene, differences in the microbial communities were observed and statistical analyses evidenced that salinity, redox potential, and temperature, explained the variance of the prokaryotic assemblages. Microbial biodiversity was associated to the biochemical cycling of key elements, such as carbon, nitrogen, and sulfur. Floating mats were dominated by members of Bacteroidetes (Saprospiraceae, 6.5%; Lentimicrobiaceae, 4.7%) and Proteobacteria (Chromatiaceae, 4.5%), while flat and pustular mats were more similar between them, containing Bacteroidetes (Saprospiraceae, 6.2-8.3%), Spirochaetes (Spirochaetaceae, 5.8-8.3%), Chloroflexi (uncultured Anaerolineae, 4.8-5.0%) and Planctomycetes (Phycisphaeraceae, 4.3-4.1%). This work contributes to the understanding of the distribution, physicochemical characteristics, and microbial diversity of coastal microbial mats, increasing the available information about microbial mats developing in karstic ecosystems.

Keywords: microbial mats, karstic ecosystem, 16S rRNA gene amplicon sequencing

Introduction

Microbial mats are complex associations of several functional groups of microbes that grow on a solid substrate (Stal, 2001). Mats can occur in a wide variety of aquatic ecosystems, such as hot springs, hypersaline ponds, dry and hot deserts, alkaline lakes, and coastal intertidal sediments (Stal, 1994). On these sites, mats flourish where there is little substrate competition from plants or protection from grazing organisms (Gerdes, 2010). The development of a microbial mat on sediments is usually initiated by cyanobacteria. Excess of fixed carbon by phototrophs is exudated as extracellular polymeric substances, that form a matrix where microorganisms are embedded with grains of sediments (Stolz, 2000). Mats colonize inter-tidal flats that are low in nutrients and periodic inundation causes desiccation and strong variations in salinity and temperature (Stal, 2001).

At present, the most-studied marine microbial mats are located at Guerrero Negro (Baja California, Mexico) (Des Marais, 2010), the Ebro Delta (Iberian Peninsula) (Guerrero et al., 1993), Elkhorn Slough (California, USA) (D'haeseleer et al., 2017) and at Shark Bay (Australia) (Reinold et al., 2019). Different types of coastal microbial mats have been reported, but karstic environments have been relatively less studied compared to other ecosystems around the world.

Karst is defined as a special type of landscape containing caves and underground water that developed on soluble rocks, such as limestone, marble, dolomite, and gypsum (Ford and Williams, 2007). The Yucatán Peninsula (YP) is one of the most extensive karst systems known on the planet (Bauer-Gottwein et al., 2011). The YP is a big limestone platform with a surface of 165, 000 km^2, comprising the Mexican states of Campeche, Yucatán, Quintana Roo, and parts of Tabasco, as well as northern Belize and Guatemala (Bauer-Gottwein et al., 2011). The climatic regime has three defined seasons: dry (March-June), rainy (July-October) and north-winds season (November-February) (Herrera-Silveira and Ramírez-Ramírez, 1998). The coastal characteristics of the YP permits the formation of shallow, swampy, brackish-to-saline estuaries along the shoreline (Perry et al., 2003; Herrera-Silveira and Ramírez-Ramírez, 1998). Coastal hydrological features of YP have shown that the evaporation is much greater to the water contributions, allowing the development of several solar salterns in the coastal zone (Ortíz-Milán, 2009). Even though all those environmental conditions are promising for the development of coastal microbial mats, there is not current information on these ecosystems.

Thus, the objective of this work is to explore and characterize the prokaryotic community structure of microbial mats from the Yucatán Peninsula, by 16S rRNA gene sequencing.

Materials and methods

Sampling and physicochemical characterization

Microbial mats were collected in May 2019 from coastal zones located at Sisal, Progreso, Dzilam y Ría Lagartos (Fig. 11). Surficial mat cores (8 cm width × 8 cm length, 3–5 cm depth) were sampled in triplicate. Subsequently, three sub-cores (1 cm × 1 cm) per core were taken, to obtain 9 representative samples per locality. Sub-cores were immediately stored in liquid nitrogen and transported to the laboratory for further molecular analysis. Some physicochemical variables, such as salinity, temperature, pH and redox potential were measured *in situ* from interstitial water with a portable multi-parameter analyzer.

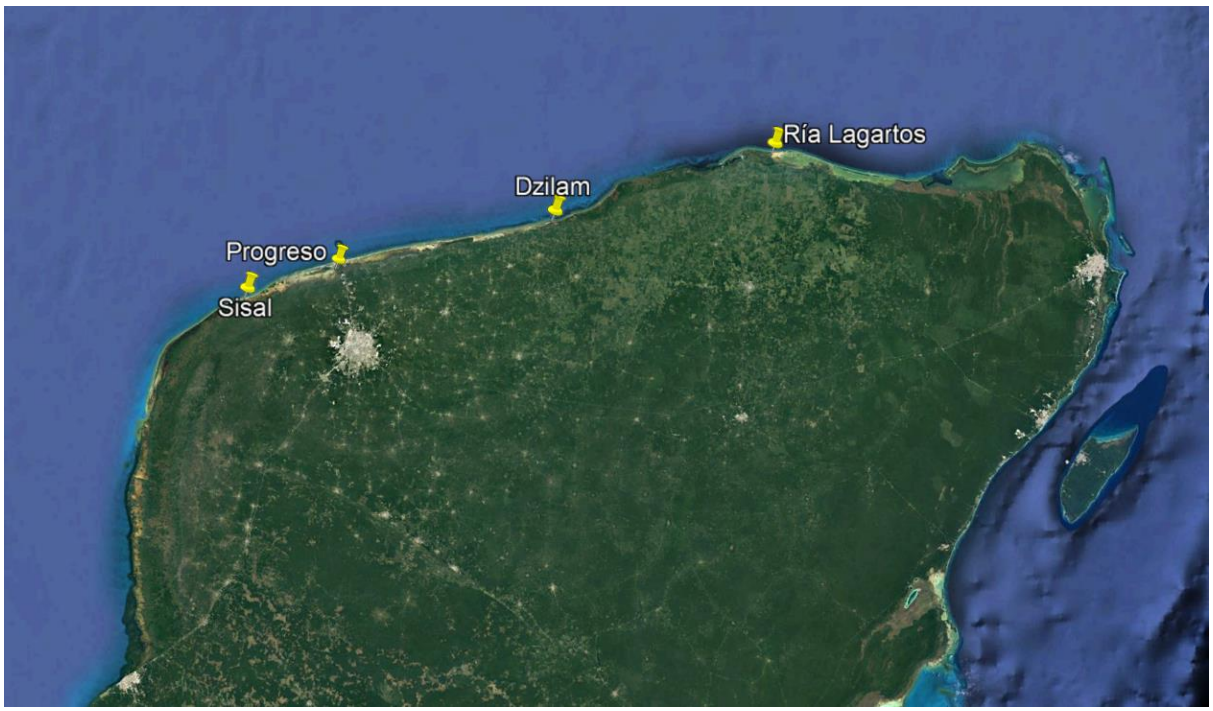

Fig. 11: Sampling sites of microbial mats from the Yucatán Peninsula.

Mineralogical analysis by XRD

The mineral composition of the mats was achieved by X-ray diffraction (XRD). Mats were finely macerated (<20 μm) and analyzed with a Bruker D-8 Advance (IUC, Indore) diffractometer, with Cu tube (kα: 1.5406 Å) operated at 30 mÅ and 40 kV at CINVESTAV-Mérida.

DNA extraction and amplicon library construction for sequencing

Environmental DNA from each locality (n=9) was extracted from 0.25 g of microbial mat sample. A TissueLyser LT (Qiagen, Hilden, Germany) was used for cell lysis and DNA was extracted with the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) using the conventional instructions. A blank (spin column with no sample supplied) was processed alongside the extractions. DNA quality was corroborated with a 1% agarose gel.

16S rRNA gene fragments from Bacteria and Archaea were amplified using the universal primers sets 515F-Y and 926R (Parada et al., 2008), covering the V4-V6 regions. Primer's characteristics and thermocycling conditions are reported by Parada et al. (2008). PCR reactions (20 μl) were done with 2 μl of DNA (5 ng/μl), 0.5 μl of each primer (10 μM) and 10 μl of 2× Phusion High-Fidelity MasterMix (Thermo Scientific, Waltham, MA, USA).

PCR fragments were purified with the magnetic beads (AMPure XP) (Beckman Coulter Genomics, Brea, CA, USA). Then, amplicons were indexed with The Nextera XT Index kit version 2 (Illumina, San Diego, CA, USA), following Illumina's 16S Metagenomic Sequencing Library Preparation protocol. Barcoded fragments were secondly purified and quantified with the Qubit 3.0 fluorometer (Life Technologies, Malaysia). Correct size of PCR products was confirmed on Advanced QIAxcel (QIAGEN, USA). Barcoded PCR-amplicons were diluted on 10 mM Tris (pH 8.5) and pooled in equimolar concentrations (9 pM). Paired-end sequencing (2 × 300 bp) was performed on the MiSeq platform (Illumina, San Diego, CA, USA), with a MiSeq Reagent Kit V3 (600 cycles), in the Aquatic Pathology Laboratory at CINVESTAV-Mérida.

Demultiplexed reads were analyzed with the QIIME2 (2017.11) pipeline (Caporaso et al., 2010). For denoising and to resolve amplicon sequence variants (ASV), we used the DADA2 plugin, eliminating chimeras with the "consensus" method (Callahan et al., 2016).

Representative ASVs were assigned with V-SEARCH plugging (Rognes et al., 2016) using the SILVA small subunit ribosomal RNAs (16S) v132 database as reference. Representative reads were aligned with the MAFFT algorithm (Katoh, 2002) and filtered to build a phylogenetic tree with fasttree (Price et al., 2010). Data were normalized by sub-sampling to lowest reads count per sample (12 900). Graphic visualization and statistical analysis were done on the R environment using phyloseq (McMurdie and Holmes, 2014) and ggplot2 (Gómez-Rubio, 2017) libraries. In addition, the alpha diversity from samples (observed ASVs, Chao1 and Shannon indexes) was calculated.

Results

Physicochemical characteristics

Different types of microbial mats were found, denominated as floating, pustular, and flat mats. Salinity from studied sites ranged between 2.2% and 9.8 %. The lowest salinity measurements corresponded to Sisal, while the higher salinity occurred at Ría Lagartos. The average interstitial water temperature of the sites varied from 31 to 37° C. The pH showed similar values for all monitored sites (7.3 ± 0.2). Redox potential ranged from −177 to -308 mV. The physicochemical properties of the sampled microbial mats are summarized in Table 5.

Table 5: Physicochemical characterization of sampled sites and type of macrostructure.

Locality	Macrostructure	Salinity (%)	Temperature (°C)	pH	Redox potential (mV)
Sisal	Floating mats	2.2 (±0.22)	31.4 (±0.72)	7.4 (±0.07)	-299.6 (±20.08)
Progreso	Pustular mat	6.0 (±0.36)	34.6 (±1.29)	7.2 (±0.06)	-271.1 (±21.07)
Progreso	Flat mat	8.8 (±0.54)	32.4 (±1.63)	7.4 (±0.11)	-177.7 (±60.33)
Dzilam	Flat mat	6.8 (±1.51)	31.9 (±0.34)	6.9 (±0.09)	-308.2 (±23.74)
Ría Lagartos	Flat mat	9.8 (±1.9)	37.3 (±1.9)	7.2 (±1.9)	-207.0 (±1.9)

Nine different minerals were detected by XRD analysis of microbial mats (Fig. 12). Remarkably, calcite and halite were present in all the studied microbial mats (floating, flat, and pustular). Beidellite and quartz were only found in flat mats from Progreso. The comparative mineral composition of microbial mats is shown in Table 6.

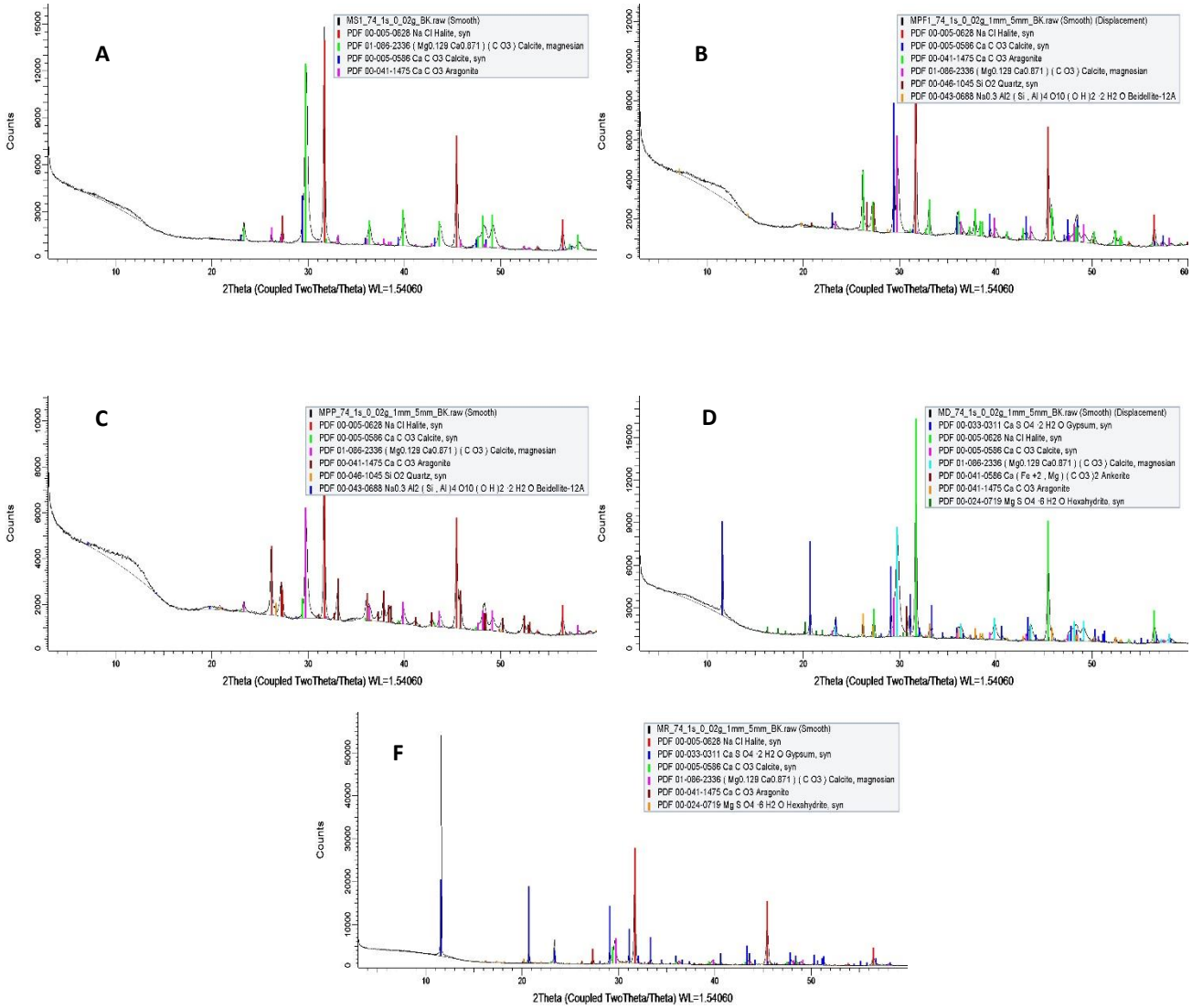

Fig. 12: X-ray diffraction of microbial mat samples. Floating microbial mats from Sisal (A); flat (B) and pustular (C) mats from Progreso; flat microbial mats from Dzilam (D) and Ría Lagartos (F).

Table 6: Mineral composition of studied microbial mats from the Yucatán Peninsula.

Microbial mats /minerals	Ankerite	Aragonite	Beidellite	Calcite	Calcite-magnesium	Gypsum	Halite	Hexahedrite	Quartz
Floating - Sisal	-	x	-	x	x	-	x	-	-
Flat - Progreso	-	x	x	x	x		x	-	x
Pustular - Progreso	x	x	-	x	x	x	x	-	-
Flat - Dzilam	x	x	-	x	x	x	x	x	-
Flat - Ría Lagartos	-	x	-	x	-	x	x	x	-

Microbial community structure analysis

Three types of microbial mats were used for further molecular analysis: floating (from Sisal), pustular (Progreso) and flat (Progreso) (Fig. 13).

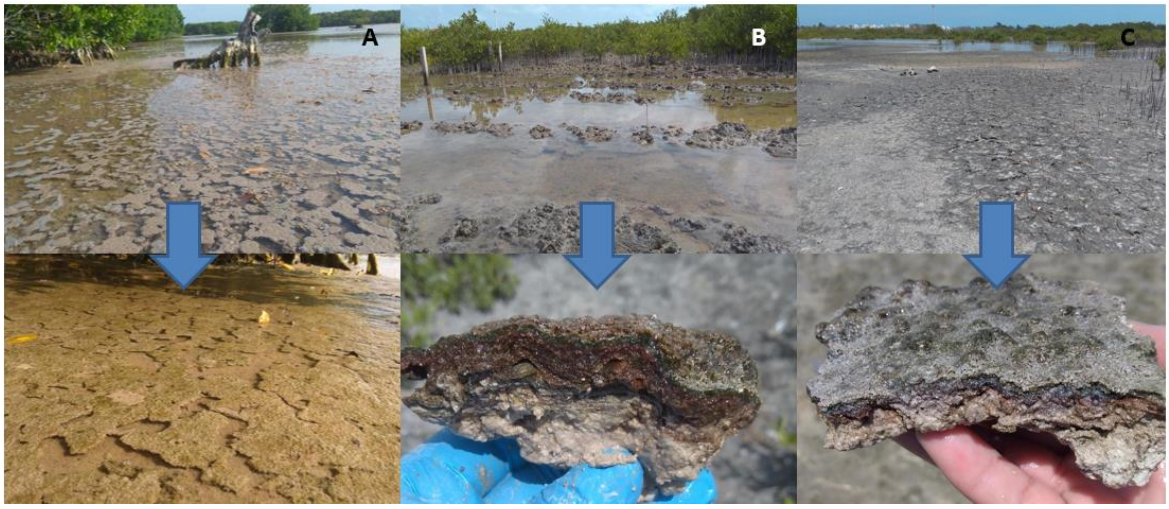

Fig. 13: Different types of coastal microbial mats. Floating microbial mats from Sisal (A). Pustular (B) and flat (C) microbial mats from Progreso, Yucatán, Mexico.

PCoA estimated on a weighted and unweighted UniFrac distance showed that microbial community structure from mats were different among the different sampled sites (Fig. 14).

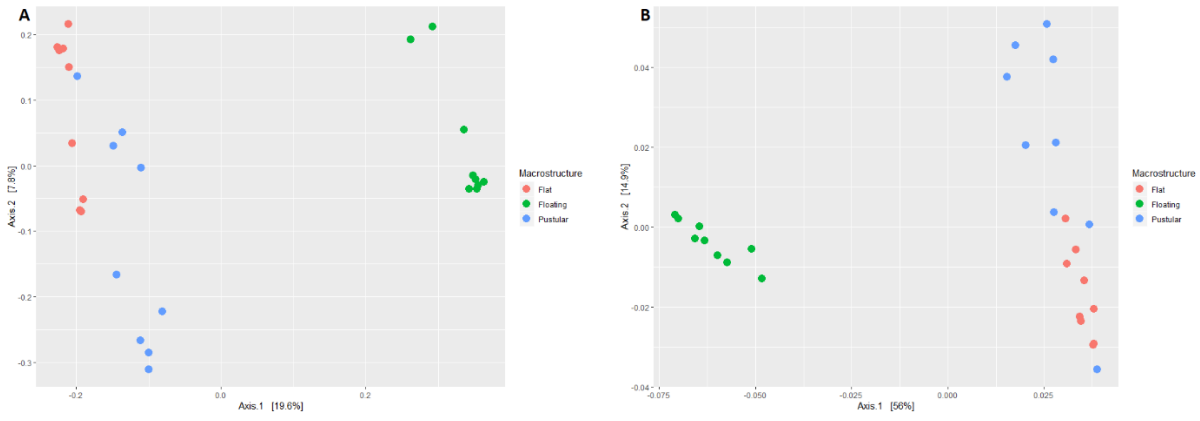

Fig. 14: PCoA calculated on the unweighted (A) and weighted (B) Unifrac metric, based on 16S rRNA gene amplicon sequences.

Observed bacterial ASVs retrieved from microbial mat samples varied from 1155 to 606. Shannon diversity index ranged from 6.55 to 5.90. Alpha diversity measurements were not significantly different among samples (Table 8). The PERMANOVA analysis suggested that salinity, temperature, and redox potential, were of statistical significance, explaining 18%, 12% and 14% of the variance of microbial communities, respectively (Table 7).

Table 7: PERMANOVA calculated on the UniFrac distance matrix, using 16SrRNA gene sequences from microbial mats and environmental data. Asterisks denote statistically significant data.

Variable	P	R^2
Salinity	0.005*	0.18
Temperature	0.005*	0.12
pH	0.339	0.06
Redox potential	0.005*	0.14

Table 8: Alpha diversity calculated from 16S rRNA gene amplicons from microbial mats.

Type of microbial mat	Observed	Chao1	Shannon
Flat	808 (±110)	831 (±127)	6.24 (±0.14)
Floating	842 (±126)	868 (±143)	6.16 (±0.15)
Pustular	853 (±184)	879 (±208)	6.22 (±0.21)

Microbial community composition

Bacterial biodiversity was related to 29 phyla, but the bacterial community composition was dominated by four taxa: Bacteroidetes, Proteobacteria, Chloroflexi and Planctomycetes (Fig. 15). Bacteroidetes (36-13%) contained members from Bacteroidia (33-12%), Rhodothermia (5-1%) and Ignavibacteria (2-0.2%). Furthermore, Proteobacteria (27-16%) included representatives from

Gammaproteobacteria (15-3%), Alphaproteobacteria (11-4%) and Deltaproteobacteria (12-9%) classes. Moreover, Chloroflexi (27-3%) consisted of Anaerolineae (19-5%) and Chloroflexia (4-1%). Meanwhile, Planctomycetes (15-3%) was represented by Phycisphaerae (9-2%) (Fig. 16).

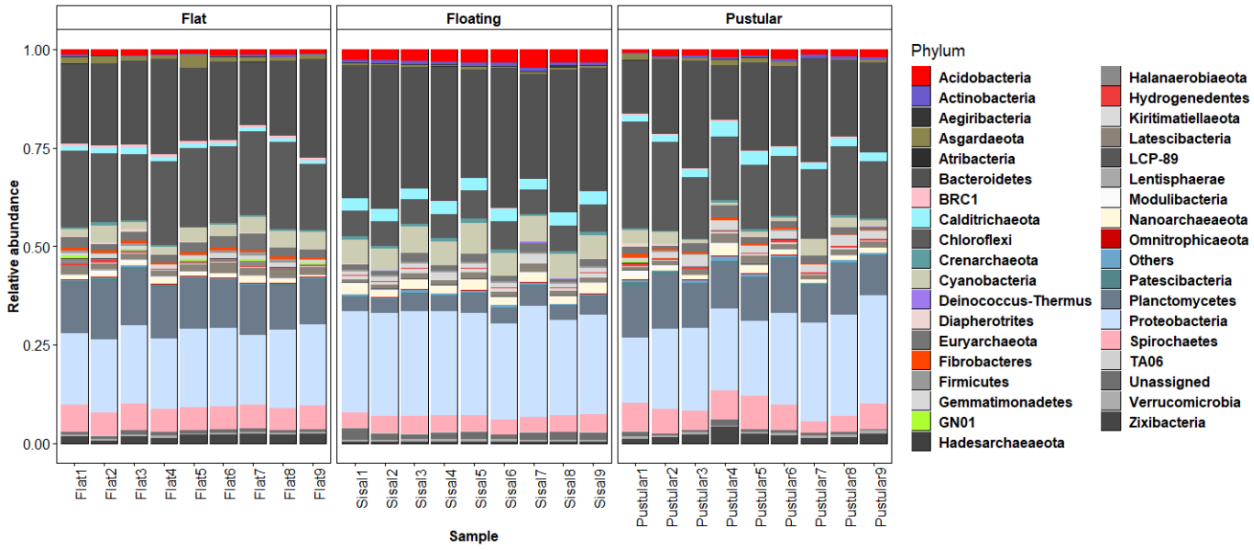

Fig. 15: Relative abundance of the microbial biodiversity at phylum level, from studied microbial mats. Phyla represented <1% were grouped in “Others”.

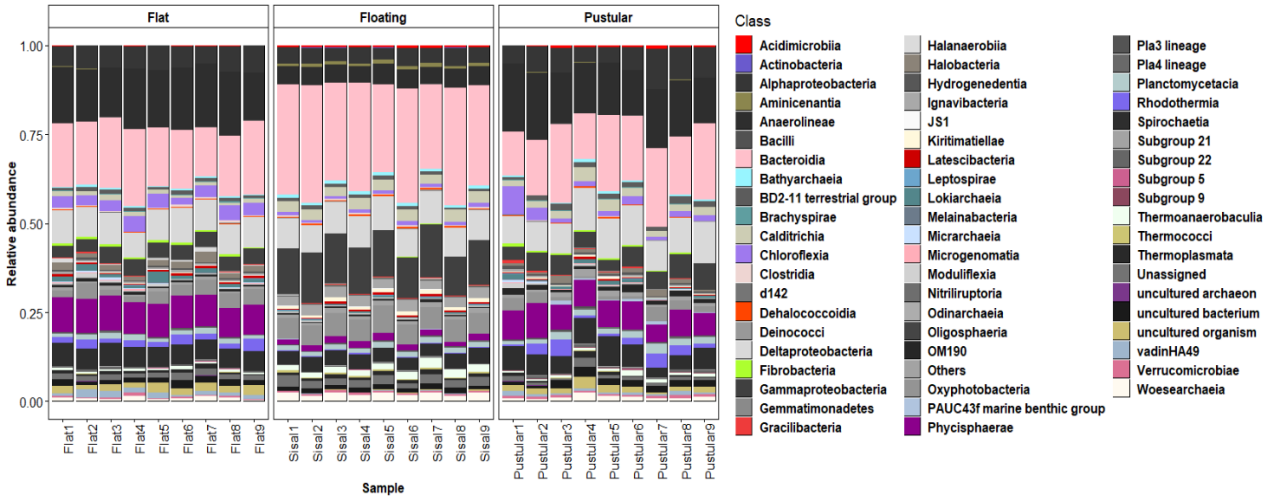

Fig. 16: Relative abundance of the microbial biodiversity at class level, from studied microbial mats. Classes represented <1% were grouped in “Others”.

Discussion

Previous studies in the YP have reported the presence of microbial mats and microbialites in Laguna Bacalar (Yanez-Montalvo et al., 2020a), in freshwater Cenotes (Yanez-Montalvo et al., 2020b; Schmitter-Soto et al., 2002) and in the Chicxulub crater (Schaefer et al., 2020). Here, we report for the first time the existence of microbial mats alongside the coast for four different localities, Sisal, Progreso, Dzilam and Ría Lagartos, where different types of microbial mats were found with distinctive physicochemical characteristics.

Prokaryotic community structure and physicochemical characteristics of mats

Benthic microbial mats are laminated systems that usually grow in flat formations, also known as smooth mats (Franks and Stolz, 2009; Allen et al., 2009). This type of macrostructure was common in Progreso, Dzilam and Ría Lagartos. In contrast with flat mats, based on their surface morphology, pustular mats have an amorphous, gelatinous, tufted structure (Allen et al., 2009). Pustular microbial mats were only found in Progreso, associated to mangroves in restoration. A wide diversity of minerals associated to the carbon, aluminum, iron, and sulfur cycles were found in flat and pustular mats (Table 6). In addition, microbial diversity from pustular and flat mats was similar between them (Fig. 12 and Fig 13). Microbial assemblages were related to Saprospiraceae and Spirochaetaceae, being these microorganisms facultative aerobes, chemoorganotrophs and chemolithotrophs, also distributed in aquatic environments (McIlroy and Nielsen, 2014). Members of Chloroflexi (uncultured Anaerolineae) were also represented, with potential chemolitho-organo-heterotrophs, growing on carbohydrates and amino acids and hydrogenogens (Yamada et al., 2005; Yamada and Sekiguchi, 2009). Phycisphaeraceae was found in less abundance as compared to previous reports on anaerobic ammonium oxidation and aerobic oxidation of methane (Jasmin et al., 2017).

Microbial mats are frequently found over sedimentary surfaces; however, physical disruption originated by temperature, gas production or flooding can promote the release of the mat from the sediment, turning it into a floating mass in water. Floating microbial mats have been found in salt lakes (John and Paton, 2009), caves (Reboul et al., 2019) and hot springs (Lacap et al., 2007). Coastal floating microbial mats occurring in Sisal (salinity 2.2%), presented the least amount of

minerals (Aragonite, Calcite and Halite), suggesting the relevance of carbon precipitation in those structures. Microbial biodiversity of mats corresponded to Saprospiraceae, Lentimicrobiaceae and Chromatiaceae (Fig. 12 and Fig 13). Saprospiraceae and Lentimicrobiaceae are degraders of the organic matter, common in organic-rich anoxic environments (Sun et al., 2016). In turn, Chromatiaceae are commonly referred to as phototrophic purple sulfur bacteria that grow in anaerobic environments that use sulfide for photosynthesis (Imhoff, 2014).

On the other hand, salinity, temperature, and redox potential were the environmental variables that explained the variance in the microbial communities. It is well known that salinity and temperature are key drivers of microbial populations. Salinity originates an osmotic stress that requires a complex metabolic specialization (Lozupone and Knight, 2007). Temperature impacts on the metabolisms of microorganisms, affecting microbial biodiversity and biogeochemical cycling (Hicks et al., 2018; Alser et al., 2016; Cole et al., 2013). Remarkably, prokaryotic alpha diversity did not change among studied floating, flat and pustular microbial mats (Table 8) which highlight the huge microbial diversity of these microbial structures. Future work to examine the seasonal variations in the microbial diversity and environmental characteristics of mats will allow to understand the ecological role of these structures on coastal ecosystems from the Yucatán Peninsula.

Conclusions

In this study, we report for the first time the prevalence of coastal microbial mats in four locations of the Yucatán Peninsula. Different types of microbial macrostructures were found, formally named as flat, floating, and pustular microbial mats. The main minerals that built those mats were aragonite, calcite, and halite, showing the importance of carbon precipitation in the construction of mats. Microbial biodiversity was unraveled using massive sequencing of the16S rRNA gene, showing some differences in the microbial community composition of mats. Floating mats were dominated by Saprospiraceae, Lentimicrobiaceae and Chromatiaceae, while flat and pustular mats were more similar, displaying members from Spirochaetaceae, uncultured Anaerolineae and Phycisphaeraceae. The principal variables related to the community structure were salinity, redox potential, and temperature, suggesting the relevance of physicochemical micro-gradients that

influence microbial diversity. This work reports on the distribution, environmental characteristics, and microbial biodiversity of coastal microbial mats in karstic ecosystems, which contributes to the knowledge on microbial ecosystems in coastal habitats.

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CHAPTER 4

Assessing the effect of elevated water temperature and acidification on photosynthetic microbial mats

In Chapter 4, the response of coastal microbial mats under a simulated climate change scenario was evaluated. After one month of incubation, significant changes in the microbial activity of mats were found.

In this work, biogeochemical evidence of changes in the microbial activity of the mats was found. At the end of the experiment, samples were frozen for further molecular analysis. With all the data, an article will be written to report these findings.

Abstract

Coastal microbial mats have been present on Earth for billions of years and several studies have been performed to understand their ecological relevance in present and ancient planetary life. Projections of future Earth estimates an upcoming global warming with several effects, such as increasing ocean temperature and acidification, among others. The aim of this Chapter was to evaluate the response of photosynthetic microbial mats to a climate change scenario, at the temperatures projected for the year of 2100. Microbial mats from Elkhorn Slough, California, were collected, transported, and incubated under greenhouse conditions for one month and then samples were taken for a diel cycle analysis. Interestingly, nitrogen fixation, oxygen production and respiration, were significantly higher in amended warmer-acidified mats as compares to controls. This study highlights the ecological changes of coastal microbial mats in a climate change scenario, this information is useful to understand the future of microbial mats ecosystems on coastal environments.

Introduction

Microorganisms are recognized as having an essential role in the functioning of ecosystems. They are important players in nutrient cycling in the global food web. In marine environments, microbial primary production substantially contributes to carbon dioxide sequestration. In terrestrial biomes, microorganisms and their activities regulate the organic carbon content in soils and the provision of macronutrients (such as nitrogen and phosphorus) to bigger organisms. Additionally, microbes participate in shaping the atmosphere, by the release of greenhouse gases including carbon dioxide, methane, and nitrous oxide. The microbial world has a great significance driving Earth climate and recent studies have indicated the central role and global importance of microorganisms in climate change biology (Cavicchioli et al., 2019; Reinold et al., 2019).

Climate change is defined as a change in the usual weather conditions of a specific region. This phenomenon has been widely documented at a global scale, occurring due to natural and anthropogenic sources (Parry, 1996). In this sense, coastal environments are major focus of concern due to their global relevance (Spalding et al., 2014).

Photosynthetic microbial mats are organo-sedimentary coastal ecosystems vulnerable to climate change like any other coastal environment (Pearl et al., 2003; Ahrendt et al., 2009). Field studies on hypersaline microbial mats from San Salvador Island, The Bahamas, have pointed out the effect of hurricane activity on the cyanobacterial diversity and functioning of mats (nitrogen and carbon dioxide fixation), concluding that microbial mats ecosystems are well indicators of climate change (Pearl et al., 2003). Laboratory experiments of photosynthetic microbial mats from the island of Highborne Cay, The Bahamas, examined the impact of increased CO_2 in the growing of mats. The authors concluded that CO_2 did not alter the microbial diversity and carbon precipitation; however, some taxa related to sulfate-reducing bacteria were enriched (Ahrendt et al., 2014). The potential impact of climate change in microbial mats and stromatolites from Shark Bay, Australia, has also been discussed by Reinold and colleagues (2009); however, the effects on the microbial diversity and functioning of mats and stromatolites are still under-investigated.

In coastal ecosystems, the psychochemical variables associated to climate change that may drive ecological responses in organisms include wave energy, rise of sea level, upwelling events,

freshwater inputs, temperature change, and ocean acidification (Hewitt et al., 2016; Spalding et al., 2014). Among those variables, increment in temperature and acidification in water, strongly influence the physiology of organisms, such as seagrasses, marine macroalgae and coral reefs (Hoegh-Gulberg et al., 2017; Koch et al., 2013).

In the present work, we evaluated the effect of temperature increase and acidification in water, on the oxygen production and nitrogen fixation of coastal microbial mats, as an assessment of a climate change scenario.

Materials and methods

Sample collection and experimentation

Microbial mats were collected from a marine tidal zone located in the Elkhorn Slough estuary at Monterey Bay, California, USA, in January 2020. Twelve cores (12 cm width × 20 cm length, 1 cm depth) were carefully dissected and transported to a greenhouse located at the NASA Ames Research Center. Mats were placed in six flow boxes (flumes), each of which contained two mats in black acrylic boxes, covered with 5 cm of seawater from site that was circulating through each flow box. The water circulation in the flumes was disconnected during weekends as a simulation of natural desiccation. The salinity of water was 3.5%, the temperature was 19°C and had a pH of 8.0. Microbial mats were placed under natural solar irradiance for three weeks prior to experimentation.

Experiments were amended to the projected year 2100, increasing 5°C the water surface temperature (Sakalli, 2017) and decreasing pH from 8 to 7.6. Three control flumes (19 ˚C; pH 8) and three warmer flumes (25 ˚C; pH 7.6) were maintained for one month and then diel cycle experiments were carried out.

Biogeochemical assays

Microbial mats were evaluated for the maximum quantum yield of the Photosystem II (Fv/Fm), with an underwater pulse-amplitude-modulated fluorometer (Diving-PAM, Walz, Effeltrich, Germany). Nitrogenase activity was measured using the acetylene reduction assay (ARA),

following the procedure of Bebout et al. (1993). Briefly, bottles (in triplicate) were prepared with a small subcore of mats (1cm diameter, 1 cm depth) and seawater from the flumes. Bottles were capped with butyl rubber stoppers and the headspace was replaced with acetylene. Vials were incubated in the flumes from which they were sampled to maintain similar light and temperature conditions. Measurements were carried out every 3 h over a diel cycle. Ethylene concentration was measured from 0.1-ml of the headspace by gas chromatography (GC), with a Shimadzu GC14A chromatograph, using 2-m Porapak N column, held at 80°C with flame ionization detector. In addition, methane gas was determined using the mentioned chromatograph, but held at 40°C (Bebout et al., 2004).

Transparent acrylic chambers were placed on the surface of the mats to measure oxygen production over a diel cycle (day and night). The produced gas in the chamber was directly sampled from the water using an optical oxygen electrode housed in a syringe (Fig. 17). The water inside the chamber was stirred prior to sampling. The water oxygen flux was analyzed for significant differences using Student's t-test between treatments.

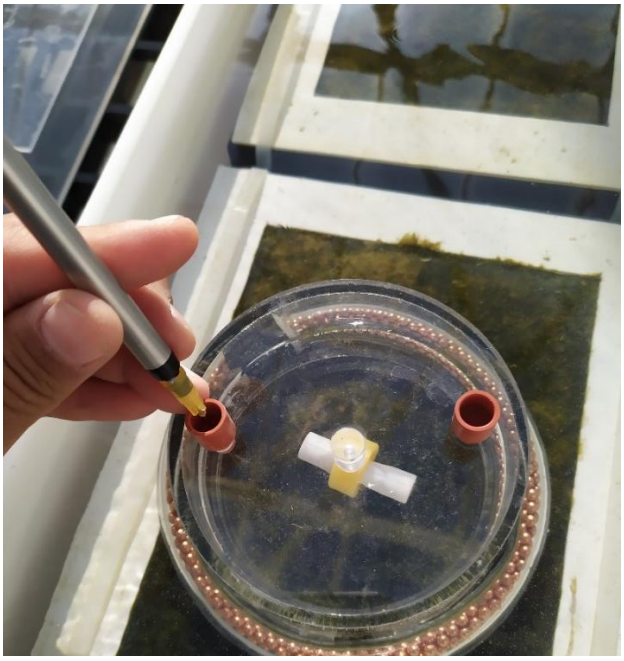

Fig. 17: Instrumentation used for oxygen flux measurements. Electrode hosed in a syringe is inserted in the chamber to analyze oxygen in water.

Results

Biogeochemical data

During the day, oxygen flux in control mats oscillate between 1127 and 694 µmol/m^2/h, while amended mats presented values ranging 1556-1376 µmol/m^2/h. During the night, a negative flux was observed, ranging from -161 to -102 µmol/m^2/h and -626 to -398 µmol/m^2/h for control and experimental mats, respectively (Fig. 18). Oxygen fluxes were significantly different between treatments at day and night (Student´s tests p-values = 0.021 and 0.001, respectively). In addition, traces of methane were detected, but not in significant amount (data not shown).

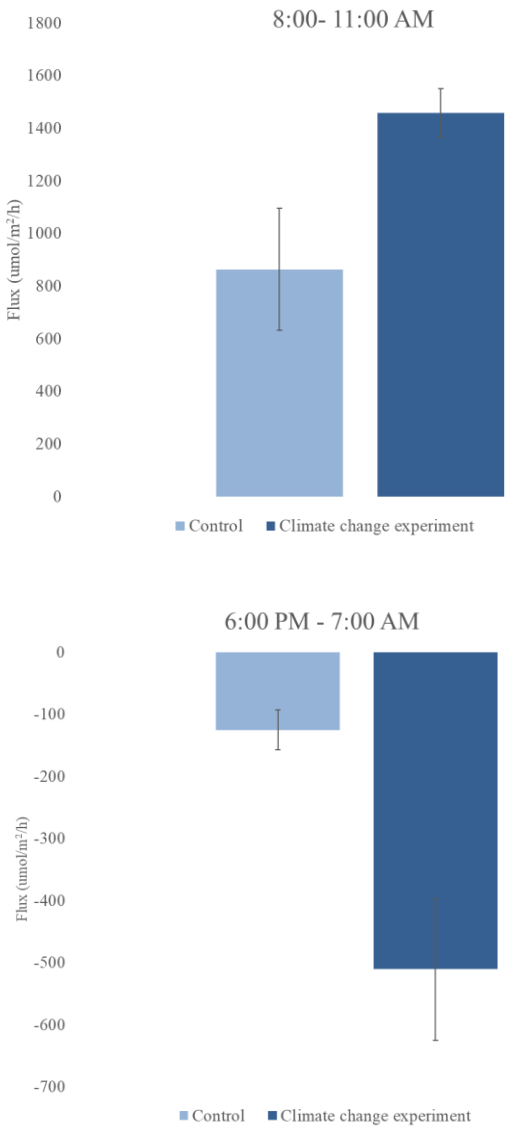

Fig. 18: Oxygen flux measurements during a diel cycle of incubated microbial mats. Data are the result of triplicate measurements per microbial mat in flumes (n=9). Positive values represent oxygen production, while negative values suggest microbial respiration.

Nitrogen fixation, measured as nitrogenase activity, mainly occurred during the night, with minimum values during the day. This means that the activity was inversely correlated to light of the day. The highest rates of acetylene reduction (nitrogen fixation) occurred between 21:00 and 00:00 h, ranging from 134 to 94 and 194 to 144 µmol/m^2/h, for control and experimental mats, respectively (Fig. 19).

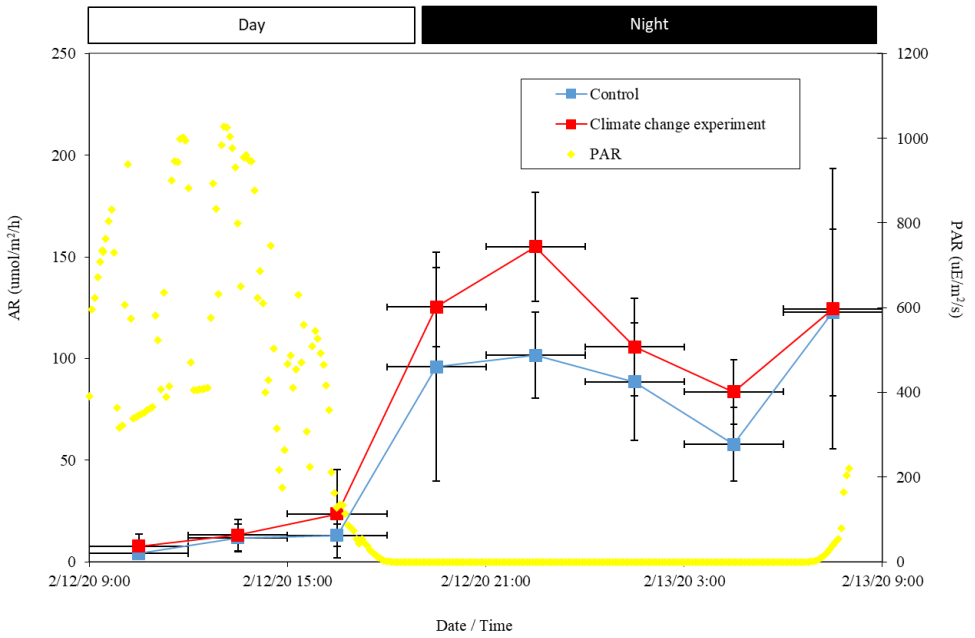

Fig. 19: Acetylene reduction (AR) rate and light measured on the diel cycle essay of coastal microbial mats. Horizontal bars represent the time between incubations. Vertical bars indicate ±1 standard deviation calculated from the average from three flumes.

The maximum quantum yield of Photosystem II was not significantly different among treatments at the two different sampling points (Fig. 20).

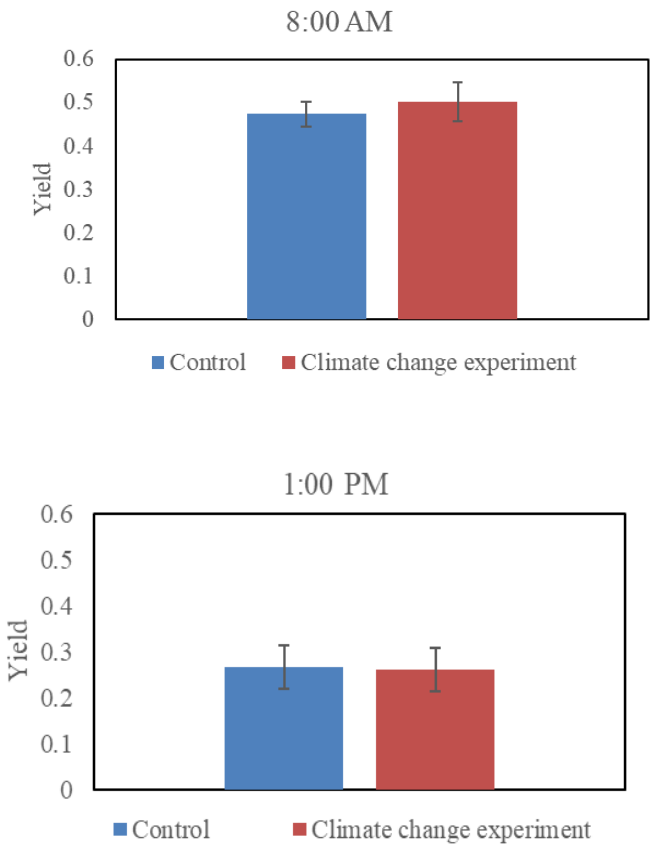

Fig. 20: PAM fluorometry measurements from control and amended warmer-acidified microbial mats. Results are means of quintuplicate measurements and error bars denote the standard deviation.

Discussion

The response of microbial mats to climate change has been relatively well documented in the Artic zone, where permafrost thawing promotes drastic changes in microbial communities and function of mats (Verleyen et al., 2010; Vincent, 2010). The relevance of heterotrophic bacteria in food webs and the increase in greenhouse gas emissions in psychrophilic mats have been observed (Valdespino-Castillo et al., 2018; Vincent, 2010). However, few studies have focused on marine, coastal microbial mats.

Marine microbial mats are frequently found in intertidal sediments that are exposed to flooding/desiccation periods, variations in salinity, drastic changes in temperature during the day/night and low nutrients input (Stal, 2001). Even though microorganisms are relatively well adapted to survive under those extreme and fluctuating conditions, it has been observed that benthic microbial communities respond to climate variations (Cavicchioli et al., 2019; Hicks et al., 2018). For example, geochemical evidence from soils, sediments, and oceans, have shown that climate change may influence microbial primary productivity, microbial decomposition, and microbial production and consumption of greenhouse gases (Cavicchioli et al., 2019; Singh et al., 2010).

After one month of incubation, we did not observe differences of Fv/Fm value between control and experimental mats, indicating that there is not photo-inhibition or damage of the photosynthetic machinery. Nitrogen fixation in control treatment was higher than previous reports on hypersaline microbial mats from Guerrero Negro (Omoregie et al., 2004), but similar to marine microbial mats from North Carolina (Bebout et al., 1993). Recently, microbial mats from the same locality (Elkhorn Slough) were evaluated for nitrogen fixation, reporting lower values than those found in the present study (Coban et al., 2021). However, those experiments occurred using natural microbial mats without a long incubation period. It is hypothesized that the incubation in the greenhouse benefited the activity of the mats, compared to their natural system, where they are more exposed to flooding and desiccation. These results highlight the potential of these microbial mats to perform nitrogen fixation when are less limited in water. In addition, we observed that nitrogen fixation increased under the climate change scenario experiment. Studies with nitrogen-fixing microorganisms have shown that climate change influences this process. Evidence from bacterial populations on coral reefs (Santos et al., 2012) and ocean waters (Wannicke et al., 2018) has shown an increase of diazotrophic communities with the increase of water and acidification. Here, we report for the first time that climate change conditions predicted to 2100 would enhance nitrogen fixation in coastal microbial mats, but further studies are needed for the understanding of the impact at a local scale.

Regarding oxygen measurements, the oxygen in water could only be measured until noon (11 AM), because after that hour, a big production of gas bubbles originated that escaped from the

water into the atmosphere. Furthermore, we could not measure oxygen in the afternoon, because the water in the flumes was so oxygenated, so that it was no longer possible to measure an increase in the oxygen concentration in the water. These results highlight the relevance of photosynthesis in microbial mats and their impact in the oxygenation of the atmosphere (Gutiérrez-Preciado et al., 2018). On the other hand, during the night, we observed a decrement in the oxygen concentration in water, suggesting a huge respiration process of mats. It is well known that oxygen is consumed by sulfide oxidation at night, where oxygen concentrations in mats can decrease to zero in the first 2 mm (Des Marais, 2003). In addition, we observed that both processes, oxygen production and respiration, significantly increased in the amended experiments simulating climate change. All the results collected in this study suggest that coastal microbial mats respond positively to increase in temperature of water and acidification, and do not release methane as greenhouse gas. Future research is needed to investigate the changes in the microbial communities, as well to calculate the potential impact at a global scale.

Conclusions

This study evaluated the response of coastal microbial mats to elevated temperature and water acidification, simulating a climate change scenario at the projected year of 2100. Greenhouse incubations and diel cycle analysis revealed some changes in the functioning of mats, where nitrogen fixation, oxygen production and respiration, significantly increased in experimental warmer-acidified mats. In addition, photo-inhibition and release of methane were not observed. These results suggest an increase in the activity of coastal microbial mats without greenhouse gas emissions (methane), but future studies need to address local and global implications for coastal habitats.

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SUMMARY AND CONCLUSIONS

Coastal ecosystems comprise a broad range of habitats, from coastal lagoons and estuaries to coral reefs, salt marshes, intertidal flats, and more. Microorganisms inhabit those ecosystems and there are three fundamental questions in microbial ecology: who they are? where are they? and what are they doing? (Boughner et al., 2016; Konopka et al., 2009). This PhD thesis is a contribution to answer those unknowns.

The microbial biodiversity from different types of coastal habitats, including samples from estuarine sediments, marine areas, hypersaline zones, microbial mats (floating, flat, and pustular) and endoevaporites, were investigated in **Chapters 1**, **2** and **3**, unraveling new phylogenetically clusters not previously reported for these ecosystems.

In **Chapter 1**, we reported the differential distribution of microbes in a stratified coastal lagoon. Microorganisms related to the methane cycle were only found in the inner zone of the lagoon (oligohaline sediments), while the mixing zone (estuarine sediments) had a predominant microbiota related to saprotrophic lifestyle. Marine sediments contained sulfate reducers and microorganisms related to the nitrogen cycle.

In **Chapter 2**, the microbial community composition from hypersaline sediments, microbial mats and endoevaporites developing in a human-made solar saltern were studied. Since bacterial communities have been relatively well documented, we investigated the less explored archaeal and micro-eukaryotic populations. In this work, we provide for the first time a complete microbial profile (Bacteria, Archaea and Eukarya) for those sites.

In **Chapter 3**, the first characterization of different type (floating, flat, and pustular) of coastal microbial mats naturally developing in the Yucatán Peninsula is reported. This is the first description of coastal microbial mats in the region.

In accordance with **hypothesis 1,** microbial biodiversity changed when analyzing different types of coastal ecosystems. This is not surprising, because each type of coastal ecosystem has a vast number of environmental variables that make it unique (salinity, temperature, hydrology, climate, etc.). This finding suggests that there is a "particular" microbiota for each type of ecosystem and

that, they are not the same microorganisms distributed in different relative abundances. This discovery highlights the immense microbial biodiversity thriving coastal environments.

There is an idea that the broad microbial biodiversity in nature occurs as a functional redundancy phenomenon, which defines the coexistence of different taxa of microorganisms that share the same set of functions and can replace each other to maintain the ecosystem stability and resilience (in case of perturbations) (Konopka et al., 2009). However, recent studies using metagenomic analysis of ocean waters found that changes in the microbial community composition are actually related to potential functional changes of microbes and ecosystems (Liu et al., 2019; Galand et al., 2018). In the present work, when analyzing different type of coastal habitats, we found differentially distributed microorganisms with different reported metabolic capabilities, providing evidence that can cast doubt on the hypothesis of functional redundancy and opens new windows for investigating the explanation of the tremendous microbial diversity in ecosystems. Therefore, the information obtained in this thesis will help to future meta studies on microbial biogeography in coastal environments and their potential ecological role.

In accordance with **hypothesis 2**, we found that regardless the type of ecosystem studied and the geographical distance, there were some physicochemical variables that influence microbial communities, being salinity, temperature, and redox potential the most relevant features. These results suggest the relevance of physicochemical micro-gradients found in sediments that strongly shape microbial biodiversity. Future research on how microorganisms deal with those variables are needed to completely understand the relationship between the micro-biotic and abiotic components in coastal ecosystems.

In **Chapter 4,** we evaluated the response of microbial mats to the increment in temperature of water and acidification, as a model of a climate change scenario. In accordance with **hypothesis 3**, we found significant changes in the activity of mats, observed in nitrogen fixation, oxygen production and respiration. Interestingly, we did not observe a decay in microbial processes and, contrarily, the mats had higher activity in the measured processes. Further research to evaluate the implications of microbial mats in a changing environment and their contribution to coastal ecosystems are still needed.

In conclusion, this PhD thesis contributes to the understanding of the microbial diversity in coastal ecosystems and highlights some environmental frameworks that influence their distribution. Moreover, the potential effect of global warming (elevated water temperature and acidification) on photosynthetic microbial mats was evaluated, indicating increased activity of mats, instead of growing limitation. This study is the basis for future studies using novel technologies in metagenomics/metatranscriptomics for a deeper comprehension on the microbial communities and their work in coastal ecosystems, as well as the potential future of mats in the coast and its repercussions at a local and global scale.

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APPENDIXES

In the following sections, I present the publications made on science communication during my doctoral studies.

APPENDIX 1

Microorganisms as an emerging technology for cleaning oil

In Appendix 1, the role of microorganisms in biotechnology is discussed, particularly, those involved in oil degradation.

This work has been published as:

Cadena S., García-Maldonado J.Q., Aguirre-Macedo M.L., 2018. Los microorganismos como tecnología emergente para la limpieza del petróleo. Avance y Perspectiva. Vol 4:2. https://avanceyperspectiva.cinvestav.mx/los-microorganismos-como-tecnologia-emergente-para-la-limpieza-del-petroleo/

¿Quiénes son los microorganismos?

Más allá de lo que apreciamos con la mirada, todos los alrededores están repletos de pequeños seres imperceptibles. No estamos hablando, por supuesto, de hadas, troles o pitufos diminutos, sino de bacterias, microalgas y protozoarios esparcidos por el ambiente.

El descubrimiento de la existencia de los microorganismos ocurrió durante el Siglo XVII y a partir de entonces cambió radicalmente la visión de la humanidad sobre el mundo natural. Anton Van Leeuwenhoek, holandés dedicado a la venta de telas, fabricó por primera vez un microscopio con el fin de observar la calidad de las costuras de sus productos. En su curiosidad expuso ante el microscopio toda clase de objetos, como agua de lluvia, agua de lagos, sangre, etcétera., y así descubrió el universo microbiano que nos rodea. Los microbios están distribuidos en prácticamente todos los rincones del planeta y desempeñan una gran variedad de funciones esenciales en los ecosistemas. Una considerable mayoría de los microorganismos ha sido estudiada debido a que algunos suelen originar enfermedades en el hombre, como tuberculosis, cólera, gripa, etcétera. Sin embargo, los microbios juegan un papel más amplio en la naturaleza, la industria y la biotecnología. Por ejemplo, muchos microorganismos son de interés comercial debido a que son necesarios para la elaboración de distintos productos como quesos, yogurts, cervezas y vinos. Novedosamente, se ha descubierto que algunos microbios tienen la capacidad de producir fuentes de energía renovables de última generación, como el metano y el hidrógeno. También existen microorganismos con el potencial de degradar o consumir sustancias tóxicas que se liberan en el ambiente como resultado de las actividades humanas, incluyendo pesticidas, metales pesados y fertilizantes. entre otros. En este texto hablaremos sobre el uso de los microorganismos con fines de bioremediación, particularmente, sobre la investigación relacionada con la búsqueda de microbios capaces de alimentarse utilizando petróleo de forma natural (Fig. 21).

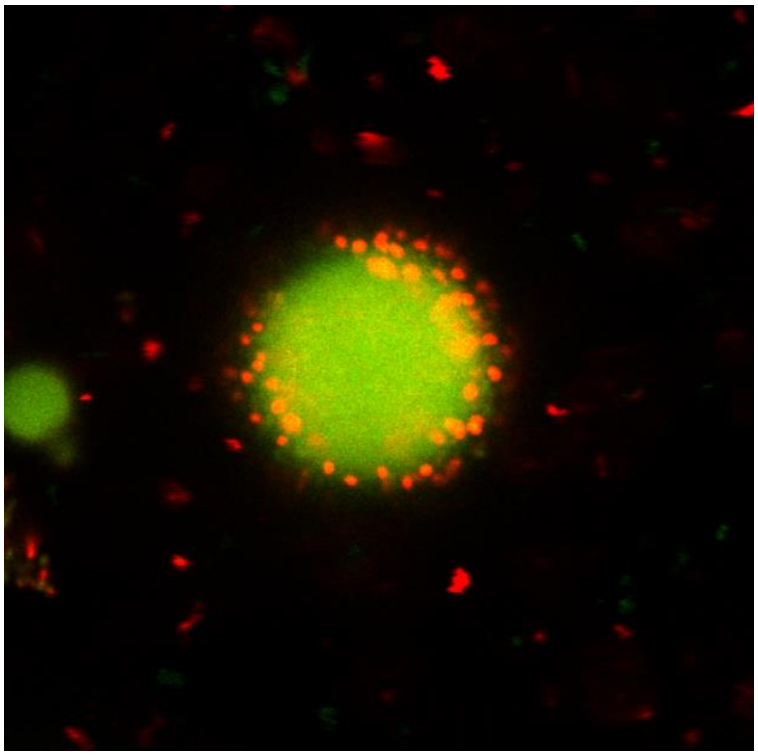

Fig. 21: Micrografía de una gota de petróleo (verde) colonizada por bacterias (rojo), utilizando microscopía CONFOCAL.

La contaminación originada por el petróleo

La contaminación por petróleo es uno de los mayores problemas ambientales en la actualidad. Las regiones con operación petrolera son vulnerables a contaminación por petróleo por actividades de exploración, extracción, transporte y refinación del crudo. Resulta entonces de vital importancia realizar investigaciones científicas que busquen la solución o mitigación de la contaminación por petróleo en los ecosistemas. En nuestro país, la principal región con actividad petrolera se encuentra dentro del Golfo de México. Esta cuenca posee más de 4 mil kilómetros de línea costera entre las penínsulas de Florida y Yucatán. El Golfo de México es una de las principales regiones del mundo donde se realizan estudios sobre la degradación de hidrocarburos debido a que históricamente se presentan derrames importantes de hidrocarburos de manera incidental. El accidente más importante y crítico en la historia reciente ocurrió en 2010 en el “Deepwater Horizon”, una plataforma petrolera compartida por Estados Unidos y México, en donde por

negligencia humana, se derramaron en el mar aproximadamente 779 mil toneladas de crudo. Debido a los daños ecológicos y económicos que este tipo de accidentes origina para nuestro país, resulta imprescindible realizar investigaciones científicas sobre el impacto de los hidrocarburos en aguas y costas, así como buscar estrategias novedosas dirigidas a la limpieza de los hidrocarburos, en caso de otro derrame petrolero.

Microorganismos degradadores de hidrocarburos

El petróleo es una mezcla compleja de diferentes compuestos llamados hidrocarburos. Existen hidrocarburos simples, denominados lineales por su estructura molecular, e hidrocarburos aromáticos, que forman moléculas cíclicas más sofisticadas. Una muestra de petróleo puede contener más de treinta diferentes tipos de hidrocarburos lineales y aromáticos. Es bien conocida la toxicidad de los compuestos del petróleo en el ecosistema y sus efectos negativos para la salud de seres vivos como plantas, peces, aves y humanos. No obstante, por increíble que parezca, se ha encontrado que algunos microorganismos son capaces de "comer petróleo", es decir, pueden degradar e integrar los hidrocarburos en su metabolismo de forma natural. Curiosamente, los microorganismos degradadores de petróleo se encuentran esparcidos por todos los océanos, sin embargo, usualmente están en bajas proporciones en el ambiente. No obstante, cuando hay un derrame de crudo, se estimula su crecimiento, provocando florecimientos de microbiota que degusta el petróleo. Diversos estudios muestran que los derrames de petróleo en agua y suelos, además de afectar la vida macroscópica (mangles, aves, peces, tortugas etcétera), también cambian la estructura de las comunidades microbianas, debido a la selección natural resultante de la presión ejercida por el petróleo.

Algunos microorganismos son denominados "facultativos", debido a que pueden cambiar su alimentación habitual por petróleo. También existen los microorganismos llamados "obligados", que metabolizan estrictamente petróleo y sólo crecen utilizando los hidrocarburos como sustrato. Se reporta que existen distintos microorganismos capaces de degradar el petróleo, como las microalgas y los hongos, sin embargo, las bacterias son los microorganismos más estudiados capaces de comer el petróleo naturalmente. A través de distintos estudios realizados en zonas contaminadas con petróleo, se reporta que las bacterias poseen dos rutas metabólicas principales para la degradación de los hidrocarburos. En la primera ruta, las bacterias utilizan el oxígeno

atmosférico para degradarlos. En la segunda, las bacterias trabajan en ausencia del oxígeno del aire y ligan la degradación de los hidrocarburos a otros elementos como el nitrógeno, hierro o azufre. En la actualidad, la investigación científica se dirige a la descripción y evaluación de la actividad de estas bacterias en los ecosistemas naturales para su subsecuente recuperación o aislamiento. Este conocimiento es útil para posteriormente realizar cultivos en el laboratorio y así, montar experimentos que hagan más eficiente la degradación de los hidrocarburos de forma natural. Para la degradación de los hidrocarburos existen distintas estrategias que involucran el uso de bacterias. Una de ellas es llamada "bio-estimulación" y consiste en añadir nutrientes que ayuden a impulsar energéticamente a los microorganismos y así, lleven a cabo el proceso de la degradación del petróleo de forma más eficiente y en un menor tiempo. Otra estrategia es denominada "bioaumentación" y en ella, primero es necesario aislar un grupo de bacterias capaces de llevar a cabo la degradación del petróleo. Después se adiciona este consorcio microbiano al ambiente, con el fin de sustituir los microorganismos nativos por microorganismos especializados en la limpieza del petróleo. Ambas técnicas son ampliamente investigadas para remediar con mayor eficacia los ecosistemas dañados por derrames de petróleo

Usos tecnológicos de las bacterias que comen petróleo

Novedosamente se ha encontrado que algunos microorganismos al mismo tiempo que degradan el petróleo, también generan productos secundarios de interés tecnológico. Un reciente descubrimiento reporta un grupo de bacterias llamadas Pseudomonas, con capacidad de producir material "bio-plástico" semejante al plástico convencional utilizado en vasos y envases, simultáneamente a la degradación de hidrocarburos. Este material es producido debido a que los microorganismos almacenan dentro de sus células el carbono que obtienen de la degradación de los compuestos del petróleo. El "bio-plástico" que producen estos microorganismos tiene la suficiente maleabilidad y resistencia para potencialmente ser utilizado comercialmente y así suplir el uso del plástico convencional, conocido por generar graves daños al ecosistema debido a su poca degradabilidad (recordemos que se calcula que una simple botella de plástico persistirá en el ambiente entre 100-1000 años). Desafortunadamente, la principal limitación consiste en que las cantidades industriales de plástico que demanda el consumo humano, aún no pueden ser abastecidas por este tipo de tecnologías hasta que no se impulse su investigación y desarrollo. Otro

caso interesante ocurre con un grupo de microorganismos llamado archaeas metanogénicas. Estos microbios producen metano naturalmente y también pueden degradar hidrocarburos. El metano que generan es un subproducto de la metabolización del petróleo. Lo sorprendente es que el metano producido puede ser utilizado como fuente de energía alterna, ya sea como fuente de calor o para generar electricidad. En conclusión, los microorganismos son los seres más abundantes del planeta, debido a que en una sola gota de agua existen millones de estos seres, aunque no los podamos observar a simple vista. Sus capacidades metabólicas son muy variadas, por lo cual son excelentes modelo de estudio con potencial biotecnológico, aplicado a la industria, medicina y degradación de contaminantes. Para el caso de la contaminación por petróleo, se ha comprobado que es posible utilizar a los microorganismos como una tecnología para la limpieza del crudo. Además, debido a que el petróleo es un compuesto rico en energía, cuando los microorganismos comen petróleo, éste puede ser "re-utilizado" en otros sub-productos, como plásticos y biogas. Futuras investigaciones son necesarias para que estas tecnologías estén disponibles a nuestro alcance y se puedan aplicar en la cotidianidad (Fig. 22).

Fig. 22: Experimento de degradación de hidrocarburos a meso-escala (2500 litros) con agua marina proveniente del Golfo de México.

APPENDIX 2

The role of microorganisms on the methane cycle

In Appendix 2, the participation of microorganisms in methane production and consumption is discussed.

This work has been published as:

Cadena, S., Cervantes, F.J., Falcón, L., García-Maldonado, J.Q., 2019. The role of microorganisms on the methane cycle. Frontiers for Young Minds. 7:133. doi: 10.3389/frym.2019.00133

Abstract

Have you heard about methane gas? Maybe the word methane is not familiar to you, but in fact, this gas is widely found in our daily lives, in our atmosphere, and in the solar system. Methane is a gas that is naturally produced in all kinds of environments, and it comes from the breakdown of organic (formerly living) materials. Methane gas is effective at trapping heat and it also burns very easily. So, methane is one of the most important fuels for humans. Additionally, the methane in the atmosphere helps regulate the climate on Earth. However, the amount of methane in the atmosphere has been steadily increasing for the past 200 years, which concerns the scientific community. Surprisingly, recent studies have indicated that levels of methane are regulated by tiny microbes. In this article, we encourage you to learn about the methane cycle, the microbes that make and eat methane, and why more research is needed on this gas.

What is methane and why is it important to humans?

Methane is a simple compound, formed by one atom of carbon and four atoms of hydrogen (CH_4). Methane exists as a gas in the environment and is one of the most important fossil fuels for human society. When the methane molecule breaks down, it produces heat. Because of this property, some of our homes are fueled by methane gas, which is used to cook, heat our water, and fuel our furnaces and fireplaces. Methane can also be collected and transformed into electricity, serving as a natural energy source. Methane is also found in animal burps and farts (yes, you read correctly, farts!). Methane is one of the most abundant gases produced in the digestive tract as food is broken down. To summarize, methane is a common atmospheric gas. Remarkably, methane production and breakdown on Earth are processes driven mainly by microorganisms.

Microorganisms (microbes) are the smallest life forms known, invisible to unaided eyes. They are found in all habitats and ecosystems on Earth, in our daily surroundings as well as the most hostile and extreme habitats. Although they are extremely small, the diversity and abundance of microorganisms are enormous and remarkable. Recent estimates predict that 90–99% of the microbial species on Earth are still undiscovered (Kopf et al., 2016). Microbes are the major players in the recycling of organic matter and important nutrients on Earth. They also regulate the

production and breakdown of some atmospheric gases, including carbon dioxide, the oxygen we breathe, and of course, methane.

Methane has drawn the attention of the scientific community because its concentration in the atmosphere has almost tripled, since the Industrial Revolution began in the eighteenth century. Importantly, some studies indicate that these recent increases in atmospheric methane are happening more quickly as compared to geological time scales. Suggesting the influence of human activities associated to methane emissions. The problem with increased methane in the atmosphere is that, methane gas has the ability to trap the heat energy from the Sun and prevent this heat energy from returning to space, resulting in something known as the green-house effect. This heat-trapping capacity is very important, because it helps the Earth to stay warm enough to sustain life (Kasting, 2004). However, too much methane accumulation impacts the climate and contributes to global warming. Today, the methane cycle is a major research topic, since we need a deeper understanding of where all the methane on earth comes from and how it is transformed.

Methane production in ecosystems

There are two known forms of methane production on Earth, called non-biological and biological methane sources. Non-biological methane production occurs without the participation of living organisms. Non-biological methane can be released by volcanoes or formed underground, under high pressures and temperatures. These geological processes normally involve the transformation of rocks that are melted with heat and water (Fig. 23). Biological methane production is only done by microorganisms. The current estimates suggest that 90–95% of the methane released into the atmosphere has a biological origin and is produced exclusively as a result of microbial activity!

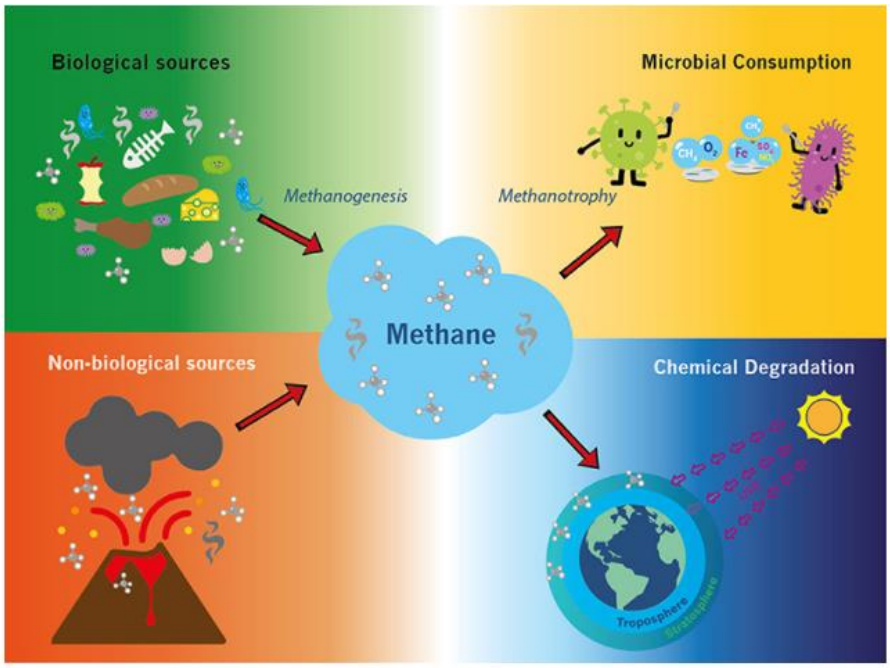

Fig. 23: Diagram of the methane cycle showing sources of methane production and methane breakdown on Earth.

The process of biological methane production is called methano-genesis. The best studied methane-producing microorganisms are named methanogenic archaea or simply methanogens. Methanogens have a complex metabolism that allows them to create methane as they produce the energy they need to survive. Interestingly, atmospheric oxygen which we need to breath and obtain energy, is toxic to some methanogens, so these microorganisms are generally found in areas where oxygen is limited or absent, such as underground, in the sediments at the bottom of lakes, lagoons, wetlands, and oceans, and even inside the intestines of all types of animals, including worms, termites, cows, and humans.

Methanogenesis is the terminal step in the food chain that occurs in the absence of atmospheric oxygen. This gas is produced as a consequence of the total degradation of organic matter, where complex molecules are degraded into their most basic compounds and then are converted to methane by methanogens. This means that in all kinds of environments, the remains of dead organisms, such as plants and animals are slowly decomposed by microbes (Fig. 23). This allows

the return of the nutrients to the food chain, and the last step involves methane production (Conrad, 2009).

Once methane is produced, how is it removed from the environment?

Removal of methane from the environment also occurs by both non-biological and biological methods. The main way that atmospheric methane is removed occurs by a non-biological method, which takes place in the zones of the atmosphere known as the troposphere and the stratosphere. These are the lowest layers of Earth's atmosphere, from 0 to 10 km and 10 to 50 km above sea level, respectively. In these zones, methane is broken down by chemical reactions driven by ultraviolet light from the sun. It is calculated that more than 90% of the methane in the atmosphere is broken down through this process (Fig. 23).

Biological removal of methane on Earth, as incredible as it seems, is exclusively performed by microbes!

There are some microorganisms that "eat" methane to get energy. This process is named methanotrophy and the microbes that carry out this process are called methanotrophs. "Trophos" means "one who is nourished from." Methanotrophs inhabit ecosystems where methane is produced, mainly under the surface of soil or sediments. Because these methanotrophs live under the soil, atmospheric methane does not come into contact with those organisms. Since the methanotrophs cannot break down the methane in the atmosphere, it accumulates. However, a very interesting phenomenon happens here. Somehow, methane produced in soils gets trapped between the soil particles and is actually there where methanotrophs take the gas for its consumption. This prevents methane from being released from the soil into the atmosphere, significantly impacting the atmospheric methane budget. As an example, it has been estimated that ~40–60% of the methane produced in wetland habitats is consumed by microbes before it can escape into the atmosphere. This means that methanotrophs are very important in soils, to prevent the release of greenhouse gases into the atmosphere where they can contribute to global warming.

Methanotrophs can eat methane both in the presence and in the absence of atmospheric oxygen. Methanotrophs that can tolerate oxygen, actually use it in the process of breaking down methane.

Regularly, these microbes are found in soils where oxygen starts to be absent because it cannot penetrate the compressed soil-particles. These oxygen minimum zones contain most of the methanotrophs and are found in all kinds of ecosystems on Earth.

Methanotrophs that do not use oxygen to break down methane, prefer to use other exotic sources of energy, accompanying the methane with some fraction of the organic matter, or with sulfur, nitrogen, and even some metals, such as iron or manganese. Here, methane is the big meal and the other elements are the complements. Interestingly, this process was firstly hypothesized by geo-chemical evidence, but remained elusive until the early 2000s, because it is extremely difficult to grow these microbes in the lab to study them.

Methane beyond earth

Here on Earth, microorganisms play a big role in the recycling of methane. So, we could say that methane is related to the presence of life on our planet. Surprisingly, recent evidence obtained by telescopes and remote artifacts has identified methane in other places in our solar system, including on Mars and on Saturn's icy moons Titan and Enceladus (Taubner et al., 20015). This is very exciting and makes us wonder if there are some kinds of microbes in those places that are producing or consuming that methane!

Methane on Mars was first identified with Earth-based telescopes in the early 2000s, and its presence was proven when the Mars rovers Spirit and Opportunity explored that planet (Fig. 24). The scientific community has been wondering if this methane originates from biological processes, but all the scientific evidence collected so far indicates that this methane comes from non-biological sources. Currently, there is no evidence of biological activity on the surface of Mars, but the research continues, because we know that methane also is a source of energy for some microbial life.

Fig. 24: If there is methane in other parts of our solar system, could microbes be there, too?

Two spacecraft missions, Cassini-Huygens and Voyager have been studying Saturn. Both spacecrafts have found evidence of organic molecules, including methane on Saturn's moons, Titan and Enceladus (Fig. 24). These moons have a lot of water and ice on their surfaces, probably similar to the polar ice caps on Earth. As strange as it seems, data suggest that both Titan and Enceladus have oceans of liquid methane, ethane, and nitrogen that form lakes and rivers, covered with rocks of water-ice.

Fig. 24 illustrates methane gas detected on other planetary bodies of our Solar System.

The exploration of extreme environments on Earth, such the Antarctic ice shelves, can help us understand the origin and evolution of extraterrestrial methane. Currently, scientists are studying how microbes can survive in permanently ice-covered ecosystems, because if we can better understand the methane cycle in extreme environments here on Earth, that would help us to also understand how methanogenesis and methanotrophy could potentially exist on the extreme environments of other planets. It is only natural to predict that methanogens and methanotrophs

could be amongst the creatures inhabiting other planetary bodies … and that we are not alone in the universe, but share it with a wide range of microbes!

Glossary

Microbes/Microorganisms: Very small forms of life including bacteria, fungi, and some diminutive algae.

Organic Matter: All cells and substances made by living organisms, including living and dead animals and plants.

Metabolism: All the chemical reactions needed to keep a cell or organism alive. Metabolism refers to how living things make and break down nutrients.

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APPENDIX 3

Exploring Mexico's hypersaline microbial mats and their biotechnological potential

In Appendix 3**,** the biotechnological potential of hypersaline microbial mats is discussed.

This work has been accepted for publication, in collaboration with the Mexican Network of Extremophiles:

Cadena, S., Ramírez-Serrano, R., Angulo, C., García-Maldonado J.Q., 2021. Explorando los tapetes microbianos hipersalinos de México y su potencial biotecnológico.

¿Sabes que son los ambientes hipersalinos? Son lugares donde la salinidad es mayor a la del agua de mar. La mayoría de estos ambientes se encuentra en sitios cercanos a la costa donde el agua marina se evapora constantemente, originando la acumulación del cloruro de sodio (sal común o sal de mesa). Estos sitios tienen un aspecto muy particular y generalmente presentan coloraciones muy llamativas con tonalidades rosa a rojizo (Fig. 25 A). Por sus propiedades, la sal es estresante para las células de todos los seres vivos, incluso plantas y animales. Por ello, en los ecosistemas hipersalinos es difícil encontrar peces o algas. No obstante, estos sitios están repletos de microorganismos extremos, que viven "felices" entre la sal.

En los ecosistemas hipersalinos generalmente se desarrollan tapetes microbianos (Fig. 25 B). ¿Sabes qué son los tapetes microbianos? Se les denomina así a las estructuras gelatinosas coloridas incrustadas con granos de arena, que se desarrollan sobre la superficie del suelo, dando un aspecto de tapete o alfombra. Los tapetes microbianos, están construidos por microorganismos y forman múltiples capas horizontales de colores verde, anaranjado, púrpura y negro, resultado de la organización de los diferentes estilos de vida de los microorganismos (Fig. 25 C). Debido a su singularidad, los científicos han estudiado a los tapetes microbianos hipersalinos y han encontrado un impresionante potencial biotecnológico.

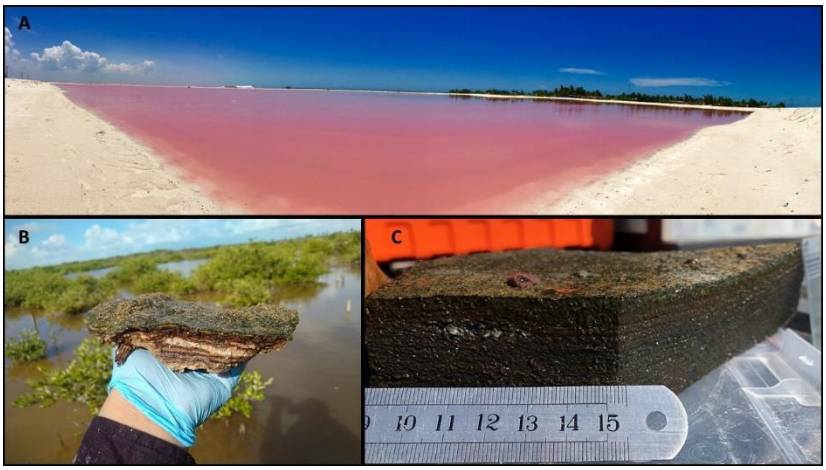

Fig. 25: Ambiente hipersalino en Las Coloradas, Yucatán, México (A); Tapetes microbianos de la Península de Yucatán (B) y Guerrero Negro, Baja California Sur, México (C).

Los tapetes microbianos tienen una contribución vital en el ecosistema. Estas estructuras son capaces de limpiar el agua de mar, reciclando nutrientes como nitrógeno, carbono y azufre. Además, producen gases atmosféricos relevantes, tales como oxígeno, hidrógeno, metano y dióxido de carbono. Algunas aves y gusanos marinos suelen alimentarse de tapetes. Aunque no sean muy populares, los tapetes microbianos son importantes en la naturaleza y en la cadena trófica (alimenticia).

Diversos estudios han mostrado que los tapetes microbianos han existido en la Tierra por millones de años. El registro fósil data estas estructuras antes de la aparición de plantas o animales, posicionándolos como uno de los ecosistemas más antiguos que se conocen y que han permanecido hasta nuestros días. Estudios previos han revelado que, durante la Tierra primitiva, los tapetes microbianos podrían haber contribuido en cambios atmosféricos a escala global, proporcionando el aire rico en oxígeno que hoy respiramos. Además, dado que los tapetes microbianos se desarrollan en condiciones extremas de salinidad e irradiación solar, los científicos creen que estas estructuras tienen el potencial de resistir las condiciones de otros planetas. Por increíble que parezca, existe todo un campo de investigación científica dedicada a la detección de rastros de actividad microbiana en otros cuerpos planetarios, como Marte.

Una cualidad importante de los tapetes microbianos es la alta diversidad de microorganismos que poseen y la complejidad de las interacciones microbianas que se llevan a cabo dentro del tapete. Por lo tanto, actualmente se busca identificar a los microorganismos que viven en los tapetes, para poder cultivarlos en el laboratorio y posteriormente evaluar su aplicación biotecnológica.

Los tapetes microbianos han sido de gran importancia en el proceso de producción de sal industrial, ya que favorecen a una mayor calidad y pureza de la sal. No obstante, el potencial biotecnológico de los tapetes microbianos es muy diverso. Algunos investigadores han buscado microbios que promuevan el crecimiento de plantas en suelos áridos. También, se han realizado investigaciones con tapetes microbianos para aislar microorganismos productores de energías alternativas, como el metano e hidrógeno; y de bioplásticos que tarden menos tiempo en degradarse que el plástico de origen petroquímico. Otras investigaciones buscan utilizar a los microorganismos de los tapetes para la limpieza del agua, probando su capacidad para remover contaminantes naturales ricos en

nitrógeno o petróleo. Las aplicaciones de los tapetes microbianos son muy diversas y aún se sigue siendo explorando su potencial biotecnológico.

Los tapetes microbianos más estudiados en México son los de Guerrero Negro, en Baja California Sur, donde las condiciones ambientales hipersalinas favorecen su desarrollo de forma impresionante, tanto, que han sido estudiados por científicos de todo el mundo y han sido un modelo de estudio para las investigaciones antes mencionadas. Sin embargo, los tapetes microbianos hipersalinos se desarrollan en diferentes lugares de México, como en la Península de Yucatán, donde recientemente se han encontrado importantes extensiones de tapetes microbianos en las lagunas costeras de Celestún, Progreso, Dzilam y Río Lagartos. A pesar del esfuerzo que se ha hecho por caracterizar la diversidad de tapetes microbianos en el país, aún existen muchas localidades con características climáticas que podrían promover el desarrollo de tapetes microbianos como Sonora, Michoacán y Oaxaca.

En conclusión, los tapetes microbianos hipersalinos son estructuras de gran relevancia ecológica que están a nuestros alrededores y generalmente pasan desapercibidos para el hombre. Su estudio permite hacer interpretaciones en distintas áreas del conocimiento, como la ecología y la biotecnología. ¿Tú, conoces o has visitado algún lugar con tapetes microbianos hipersalinos?

APPENDIX 4

Manual for the preparation of 16S rRNA gene libraries for Illumina sequencing

In Appendix 4**,** a manual for the preparation of 16S rRNA libraries (Illumina sequencing) for conventional laboratories was developed.

This work has been accepted for publication, in collaboration with the Mexican Network of Extremophiles:

Cadena S., Gamboa-Muñoz A.M., García-Maldonado J.Q., 2021. Manual para la preparación de bibliotecas del gen 16S ARNr para secuenciación Illumina.

Introducción

El gen 16S ARNr es el marcador molecular más utilizado en el estudio de comunidades microbianas (Case et al., 2007). La secuenciación masiva del gen 16S ARNr es una herramienta poderosa, que proporciona información relevante de los microorganismos procariontes hacia la comprensión del funcionamiento de los ecosistemas (Pichler et al., 2017; Sanschagrin y Yergeau, 2014). El proceso completo consiste en la extracción de ADN y PCR de la muestra; preparación de bibliotecas; la secuenciación por sí misma y el análisis bioinformático (Hess et al., 2020). Independientemente del método de secuenciación utilizado, todas las tecnologías requieren de una elaboración de bibliotecas para secuenciación, las cuales son cargadas dentro del secuenciador. Así, la correcta preparación de bibliotecas es fundamental para la obtención de resultados de calidad (Hess et al., 2020; Head et al., 2014).

En general, los pasos clave en la preparación de bibliotecas son: 1) obtención de productos de PCR de calidad; 2) limpieza de los amplicones; 3) incorporación de índices; 4) cuantificación y normalización de la biblioteca; 5) desnaturalización de la biblioteca y 6) carga en el equipo (Fig. 26) (Amplicon, Clean-Up and Index, 2013). El objetivo de este manual es proveer una guía ordenada, para preparar bibliotecas de amplicones del gen 16S ARNr, para su secuenciación a través de la plataforma MiSeq de Illumina.

Materiales y equipo

La preparación de bibliotecas de secuenciación comienza con la obtención de productos de PCR del gen 16S ARNr de muestras ambientales. A continuación, se enlistan los materiales necesarios para la preparación de bibliotecas (Tabla 9).

Tabla 9. Lista de materiales, equipos y reactivos utilizados en los distintos pasos para la preparación de bibliotecas de secuenciación del gen 16S ARNr por Illumina.

Consumibles y equipos generales	PCR	Limpieza de amplicones	Incorporación de índices	Desnaturalización de la biblioteca
Micropipetas y puntas	2× Phusion High-Fidelity MasterMix (Thermo Scientific)	Tris 10 mM pH 8.5	2× Phusion High-Fidelity MasterMix (Thermo)	Buffer de resuspension (RSB) ó10 mM Tris pH 8.5
Viales (250 y 1000µl)	Primer F con adaptador Illumina	Perlas AMPure XP (Beckman Coulter)	Kit de indices Nextera XT (Illumina)	HT1 (Buffer de hibridación)
Termociclador	Primer R con adaptador Illumina	Etanol (EtOH) 80% recién preparado	Agua grado molecular	NaOH 0.2 N fresco
Electroforesis capilar (QIAxcel, bioanalyzer, etc.)	Agua grado molecular	Soporte magnético	Gradilla TruSeq (opcional)	PhiX Control Kit v3
Fluorometro (Qubit)				Cartucho MiSeq

Descripción del método

Obtención de los productos de PCR y primera purificación

1. Realizar la amplificación del gen 16S ARNr con cualquier de los cebadores 16S Forward/16S Reverse, que incluyen los adaptadores para secuenciación de Illumina (Klindworth et al., 2013). Cada reacción de PCR (20 µL) se realiza con 2 µL de ADN (~5 ng/µL), 0.5 µL de cada cebador (10 µM) y 10 µL de 2×Phusion High-Fidelity MasterMix (Thermo Scientific). La amplificación se realiza con una desnaturalización inicial a 95° C – 3 min, con 25 ciclos de 95°C – 30 s, 55°C– 30 s, 72°C – 30 s y una extensión final a 72°C – 5 min, finalmente se verifica el tamaño del amplicon (~550pb) en el sistema QIAxcel Advanced o por geles de agarosa (1%) (Fig. 27).

2. Para la purificación, transferir el producto de PCR (~25 µL) a un tubo de 1.5mL, posteriormente agregar 20 µL de perlas AMPure XP (Beckman Coulter), y mezclar suavemente con la pipeta e incubar a temperatura ambiente por 5 minutos. Colocar el tubo en un soporte magnético durante 2 minutos, trascurrido el tiempo retirar y desechar el sobrenadante, sin tocar las perlas adheridas al magneto.

3. Realizar dos lavados con 195 µL de etanol al 80%, incubar en el soporte magnético durante 30 segundos, retirar el etanol y desechar el sobrenadante. Al final del segundo lavado, hay que asegurar que se retire el exceso de etanol. Dejar secar las perlas a temperatura ambiente durante 5 minutos, retirar el tubo del soporte magnético y añadir 52.5 µL de Tris 10mM a pH 8.5. Posteriormente mezclar suavemente con la pipeta e incubar a temperatura ambiente durante 2 minutos. Colocar el tubo en el soporte magnético durante otros 2 minutos y transferir a un tubo limpio 50 µL del sobrenadante sin tocar las perlas

Incorporación de índices (Segundo PCR) y segunda purificación

4. Utilizando el kit de índices NexteraXT (Illumina), se prepara una única combinación de índices 1N (i7) y 2S (i5) para cada muestra. La reacción de PCR (25 µL) se realiza con 5µL del producto purificado, 2µL de cada índice (N y S) previamente asignados a cada muestra y 12.5 µL de 2×Phusion Flash High-Fidelity MasterMix (Thermo Scientific). El programa del termociclador es el mismo que en la amplificación del gen 16S ARNr, pero con 8 ciclos

5. Realizar la segunda purificación de la biblioteca como se describió anteriormente en la primera amplificación, usando 28 µL de perlas AMpure XP (Beckman Coulter) y 22.5 µL de Tris 10 mM a pH 8.5. Finalmente, validar la biblioteca con 1 µL del producto purificado en el sistema QIAxcel Advanced (QIAGEN), se esperan fragmentos de ~630pb (Fig. 27).

Cuantificación y normalización de la biblioteca

6. Cuantificar cada biblioteca con el fluorómetro Qubit 3.0 (Life Technology) y calcular la concentración de las bibliotecas (nM) (Fig. 28), basado en el tamaño medio de los amplicones (Tabla 10) según lo determinado por el sistema QIAxcel Advanced (QIAGEN).

7. Diluir cada biblioteca a 4nM con Tris 10mM pH 8.5 y tomar alícuotas de 5µL para obtener un pool de la biblioteca final con índices únicos (Tabla 10).

Desnaturalización y dilución de la biblioteca del pool 16S

8. Mezclar 5 µL del pool 16S (4nM) y 5 µL de NaOH a 0.2N. Posteriormente, incubar por 5 minutos a temperatura ambiente, adicionar 5 µL de RSB para detener la desnaturalización y agregar 990 µL de la solución HT1 para obtener una concentración final de 20 pM y mantener en hielo.

9. Mezclar 300 µL del pool 16S desnaturalizado con 300 µL de HT1 para obtener una concentración final de 10 pM y mantener en hielo. La concentración recomendada de la biblioteca es de 8-12 pM.

Desnaturalización y dilución del control PhiX

10. Mezclar 2 µL de PhiX [10nM] con 3 µL de Tris 10mM a pH 8.5, para obtener una dilución de 4nM, adicionar 5 µL de NaOH a 0.2N, incubar por 5 minutos a temperatura ambiente y agregar 990 µL de la solución HT1 para obtener una concentración de 20 pM.

11. Diluir la solución del PhiX a la misma concentración que la biblioteca 16S [10pM], invertir varias veces el tubo, dar un pulso de centrífuga y mantener en hielo.

Combinación de la biblioteca del pool 16S y el control PhiX

12. Seleccionar el porcentaje deseado de PhiX, generalmente se utiliza 5% de PhiX. Para ello, mezclar 570 µL de la biblioteca de 16S y 30 µL PhiX ambos a 10pM y mantener la mezcla en hielo, posteriormente incubar a 96°C por 2 minutos, después invertir el tubo y colocar en hielo por 5 minutos, finalmente cargar 600 µL en cartucho Illumina.

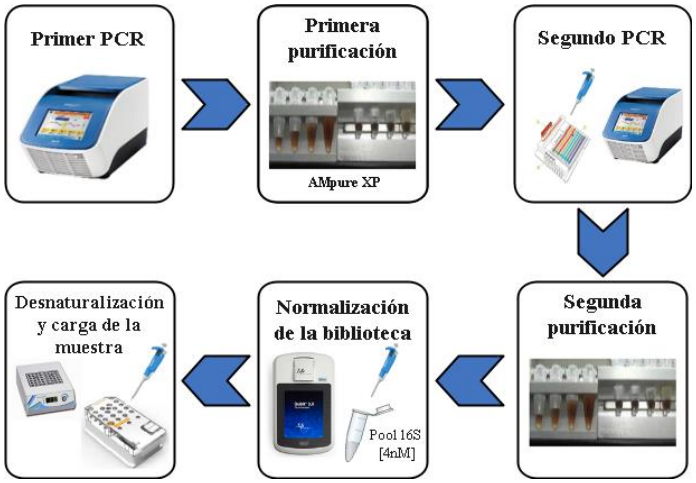

Fig. 26: Esquema del proceso seguido para la preparación de bibliotecas a partir del gen 16S ARNr

Resultados

Visualización de los productos de PCR del gen 16S ARNr y la adición de índices (tags) para secuenciación Illumina.

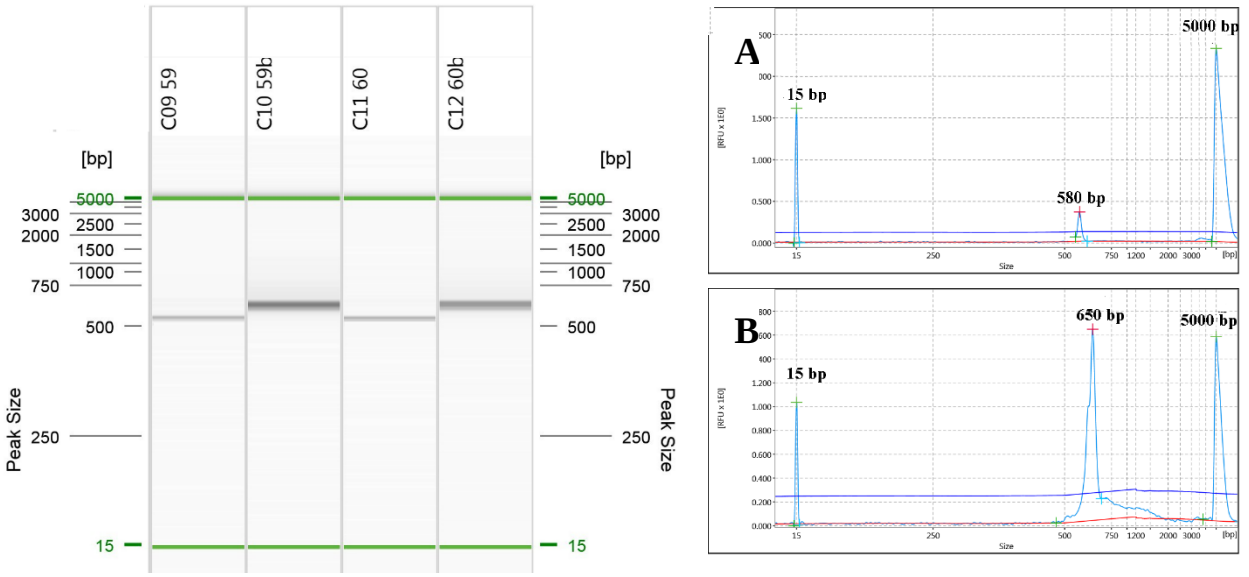

Fig. 27. En la imagen de la izquierda se muestra el primer y segundo PCR del gen 16S ARNr en dos muestras ambientales, se aprecia la diferencia de tamaños entre ambas amplificaciones. En los

electroferogramas (derecha) se distinguen bandas específicas de tamaño 580 pb en el primer PCR (A) y al incorporar los índices Nextera XT el amplicon presenta un tamaño superior de 650 pb (B).

Formula para el cálculo de la concentración (nM) de la biblioteca (*)

$$[\text{nM}] = \frac{\left[\text{ADN } \frac{\text{ng}}{\mu\text{L}}\right]}{\left(660 \frac{\text{g}}{\text{mol}}\right)(\text{tamaño medio de biblioteca})} \text{ x } 10$$

Tabla 10. Registro de datos necesarios en cada etapa para la preparación de bibliotecas.

**Cantidad de muestra requerida para diluir a 4nM. ***Cantidad de tris requerido para dilución.

	Muestra	Índice P5 (S)	Índice P7 (N)	ADN [ng/uL]	QUBIT [ng/uL] 2a Purific.	1er PCR (pb)	2o PCR Index (pb)	Molaridad (nM) *	uL/muestra (4 nM)**	uL Tris pH=8.5***
1	T0-M5	513	701	4.7	5.48	494	561	14.6	13.7	36.3
2	T0-M6	513	702	4.3	2.4	496	563	6.4	18.8	11.2
3	T0-M7	513	703	3.9	3.43	494	577	9.1	22.0	28.0
4	T0-M8	513	704	2.2	1.96	513	571	5.2	15.4	4.6
							568 promedio			

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APPENDIX 5

Microbial mats: signs of primitive life and search for life elsewhere

In Appendix 5, the main characteristics of mats and their role in nature are summarized. Additionally, the relevance of microbial mats in astrobiology is discussed.

This work has been submitted for publication to Frontiers for Young Minds and review is ongoing:

Cadena, S., Maza-Márquez, P., Ramírez, S., Grim, S., García-Maldonado, J.Q., Prufert-Bebout, L., Bebout, B., 2021. Microbial mats: signs of primitive life and search for life elsewhere.

Abstract

Some microorganisms grow together to build structures known as microbial mats. These mats form vertical colorful multilayered sheets, whose characteristics depend on environmental conditions of sunlight, humidity, sediment-substrate, and available nutrients. Microbial mats are found in aquatic ecosystems such as ocean, lakes, and coastal lagoons, as well as in extreme environments, like deserts, polar regions, and hot springs. A robust fossil record indicate that mats were a common form of life in the primitive Earth, existing in our planet for billions of years! Therefore, the study of modern mats offers clues to understand microbial life now and on primitive Earth. Moreover, evidence shows that mats have played a relevant role in the regulation of Earth's climate. For these reasons, scientist believe that it could be possible that mats can prosper on other similar rocky planets and are studying mats to recognize their characteristics, if they occur on other worlds.

Keywords: microorganisms, microbial mats, biofilms

Microbes working big!

Microorganisms are tiny living beings that you can hardly see with your naked eyes, as most of them are constituted by one single cell. They live with us, in our daily surroundings, in the soil, water and air. To look at them we need to use a microscope. However, sometimes microbes work together to get the most advantages from the environment and build big structures, observable to our eyes. For example, lichens, with their deceptive plant-like appearance, are the result of an interesting relationship between algae and fungi, forming flakes or leafless branches on trees or rocks. Yogurt, vinegar, cheese, and bread are produced by fermentation processes, performed by congregates of microbes. Some plants have root nodules, inhabited by microbes that help the plant to take nutrients from the environment. Commonly, when you let your food go to waste, you can observe a biofilm of microbes growing on it.

In nature, many microorganisms live in the soil, between water and minerals and can form big solid constructions.

Sometimes, the high density of nutrients allows microbes to reproduce by millions, growing attached to the grains of soil or sand, creating structures that look like normal rocks or mud, but are actually living structures fabricated by multitude of microscopic organisms.

There are different types of “rocky microbe structures”. For example, some of them are known as microbialites (Yañez-Montalvo et al., 2019), endoevaporites, oncolites and stromatolites (Fig. 28). Each one of those structures has special characteristics, shaped under singular environmental circumstances of evaporation, salinity, solar radiation, and humidity, that we will not further detail here. Microbial mats are, more precisely, another type of structure built by microbes (Fig. 28).

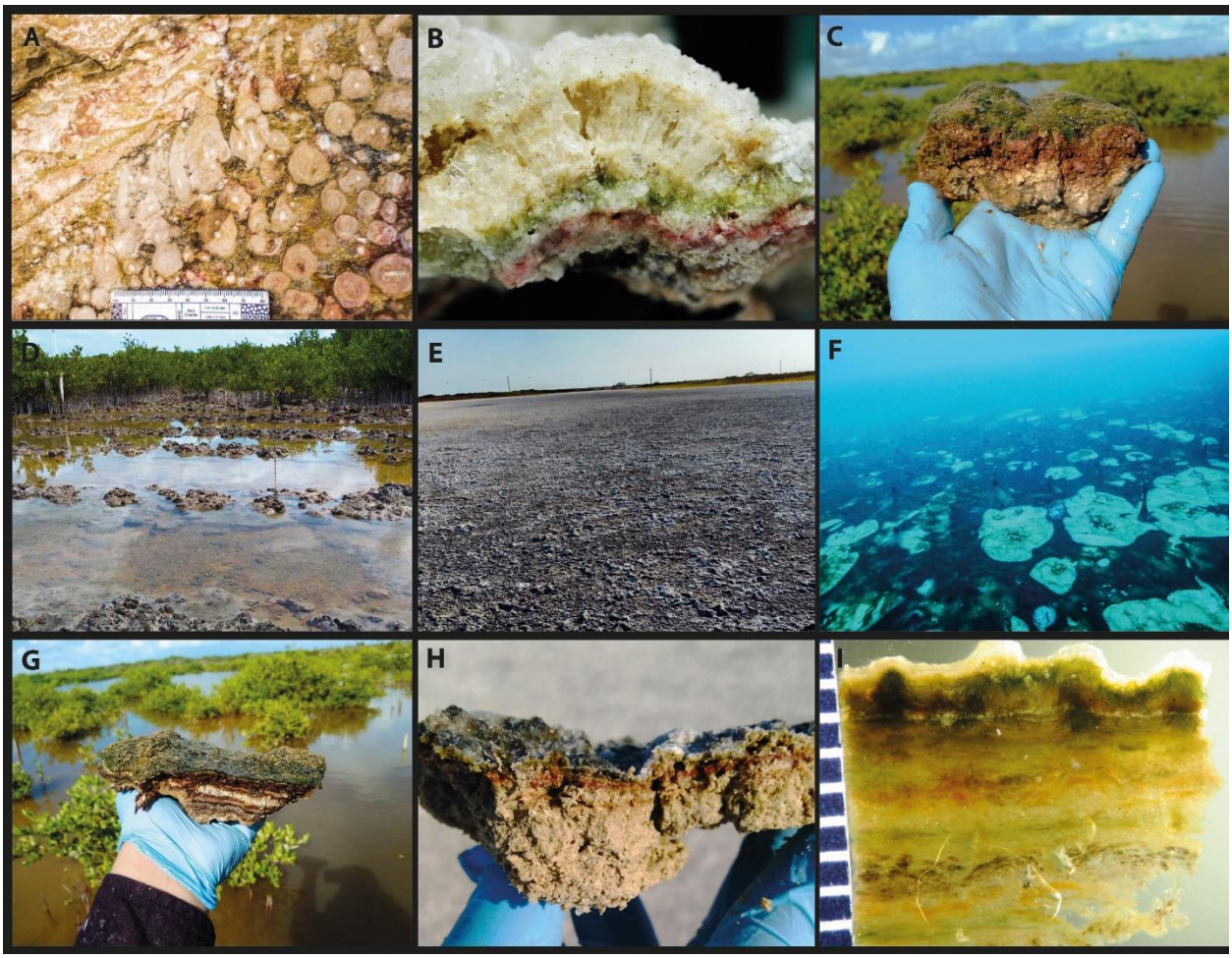

Fig. 28. Examples of structures made by microbes using soil, water, rocks and minerals: oncolites from Casey Falls locality, Canning Basin, Western Australia (photo credit: Heidi Allen) (A); endoevaporite (B); microbial mat (C). Microbial mats from the Yucatán Peninsula in Mexico (D), (E), (G), (H), and from the Middle Island Sinkhole, Lake Huron in North America (F) (photo credit: John Bright, NOAA Thunder Bay National Marine Sanctuary). A cross-section of a microbial mat from Guerrero Negro, Mexico (I).

What are microbial mats and what are they doing?

To build a microbial mat, energy and water are needed. The water can be provided by hot springs, lagoons, or a coastal shoreline, and microorganisms use the solar light as their main energy source. Then, microbes can flourish on top of the floor, embedded in a matrix with nutrients and grains of sand or soil, building mats that can reach a thickness of a few centimeters (Fig. 28 C-F). In some cases, new live mats grow on top of older mats that have died, creating very thick sequences (Fig. 28 G-H). As its name suggests, mats seem to be a carpet or rug on the floor, extended over small areas or covering large surfaces. In addition to their horizontal expansion, mats have an interesting vertical stratification at a fine millimeter scale. Microbes are generally distributed in green, orange, red and purple layers, where each layer represents a predominance of a different kind of microbes, with a special lifestyle (Fig. 28 I). This stratification occurs as a result of diverse environmental factors, such as sunlight and oxygen availability. All the microbes contained in the mat work together to sustain themselves and to interact with their surrounding environment.

Fig. 29 illustrates different places on Earth where microbial mats can be found.

Studies have shown that microbial mats are ecologically relevant. They contribute to the stabilization of soils and sediments, producing organic materials that enrich the sediment with nutrients. Mats participate in the recycling of some chemical elements like carbon, nitrogen and sulfur. They can clean the water and release some gases such as oxygen, hydrogen, carbon dioxide and methane to the atmosphere. Mats are also a food source for animals. Some flies, worms and birds “eat” small pieces of mats, supporting the local food web (Seckbach and Oren, 2010).

Fig. 29: Microbial mats across different ecosystems on Earth. Pink-orange features are meant to represent mats.

Microbial mats at a global scale

Nowadays, mats can often be found in tropical coastal lagoons, estuaries, and bays, but they may be difficult to find because they can grow to substantial size in places where there is little substrate competition from plants or protection from grazing organisms. However, mats are extensively found in the fossil record, indicating that billions of years ago in the ancient Earth, these structures were abundant around the world. Just think about it! Before dinosaurs, fishes and plants existed, it was only water and soil. There, this microbial structure proliferated on rocky-sandy surfaces. Evidence from fossilized microbial mats situates their occurrence to 3.5 Ga in the geological record, just 1 Ga after Earth was formed! This means that microbial mats are one of the oldest forms of life organization and have persisted all this time. So, study of mats helps to determine their ecological contribution on modern and past Earth.

Geological data and laboratory studies have revealed the importance of microbial mats in the history of Earth. It is believed that, in the past, the abundance and high activity of mats was responsible for creating the oxygen-rich atmosphere that we breathe. Furthermore, as they release carbon dioxide and methane, identified as greenhouse gases, they have also contributed in the regulation of Earth's climate, helping to create a warmer atmosphere that has made the Earth a more habitable planet (Hoeler et al., 2001).

Mats as models for extraterrestrial life

Microbial mats have been observed in extreme ecosystems. They have been found in marine and hyper-saline areas around coasts and in deserts soils. Also, they can be formed in polar regions, attached to the permafrost and in some glaciers. Mats have been discovered living at high temperature, close to volcanoes and hot springs. In addition, they have been identified in the deep ocean, under harsh conditions of light and pressure (Fig. 29).

Since mats can grow with extreme variations of sunlight, water, temperature or salinity, scientists believe that it would be possible that microbial mats exist beyond Earth, growing in another similar rocky planet or moons. The surface of planets and most of the moons in the Solar System are not suitable places to harbor life, due to the high incidence of solar radiation they receive, and the lack of a protective atmosphere. But evidence suggest that some of the planets and moons of the outer planets have internal water oceans protected by thick iced layers. Besides, in the event of detecting a signal of life from these remote places, it is more likely to associate it with a type of microbial life, instead of big animals or plants. Therefore, studying mats and their characteristics is a valuable tool to help scientists to recognize signatures of microbial life on other worlds if occurs. These biosignatures include both the gases that mats make as well as the larger structures that they build, constructed over rocky, sandy or mineral surfaces. The larger structures would be far easier to see and detect, with the cameras and instruments that are on board the spacecrafts than the microorganisms alone.

Currently, scientists are looking for live or fossilized mats in different places of the Solar System. The planet Mars as well as the Titan and Enceladus, two moons of the planet Saturn, have geological characteristics that are promising for microbial mats formation. Mars has a rocky dry surface, but recently, the NASA's Mars Reconnaissance Orbiter provided strong evidence of liquid

water flowing on present day (Nazari-Sharabian et al., 2020). The spacecraft missions Cassini-Huygens and Voyager were sent to study Saturn and its moons (Giuseppe et al., 2018). Both spacecrafts have found evidence of water and polar ice on Titan and Enceladus, probably like those found in the polar ice caps here on Earth. There is no evidence of microbial life or any kind of life prospering outside of our planet yet, but the study of analogous extreme ecosystems on Earth helps to define hypotheses about the conditions needed for the development and evolution of microbial life elsewhere in the universe and, to design strategies and devices that give us the possibility to find it.

How to study mats?

Mats are everywhere on Earth. They can be found in mild and extreme environments, as well as in accessible or hard to reach places. Current research is conducted based on field trips and expeditions, to investigate the ability of mats and microbes to survive across different ecosystems, under diverse environmental conditions. This information is relevant to understand the ecological role of mats, and the limits of sunlight, water, temperature etc., where these microorganisms can operate.

In addition to studying mats in the field, pieces of mats are transported to the laboratory, where it is possible to simulate a more controlled environment for long-term experiments and, use chemical and molecular tools to understand how microbes are living and what they can do. For example, we can incubate mats under greenhouse-controlled conditions and uses mall microsensors, to measure how much oxygen they produce and how much carbon dioxide they consume (Fig. 30).

In conclusion, microbial mats are complex ecosystems that serve as excellent models to study microbial diversity and evolution. Mats are found all over Earth in different ecosystems, shapes, and sizes. And just like them, scientist interested in the study of mats can be found all around the world. Do you know, or have visited any place where mats would grow?

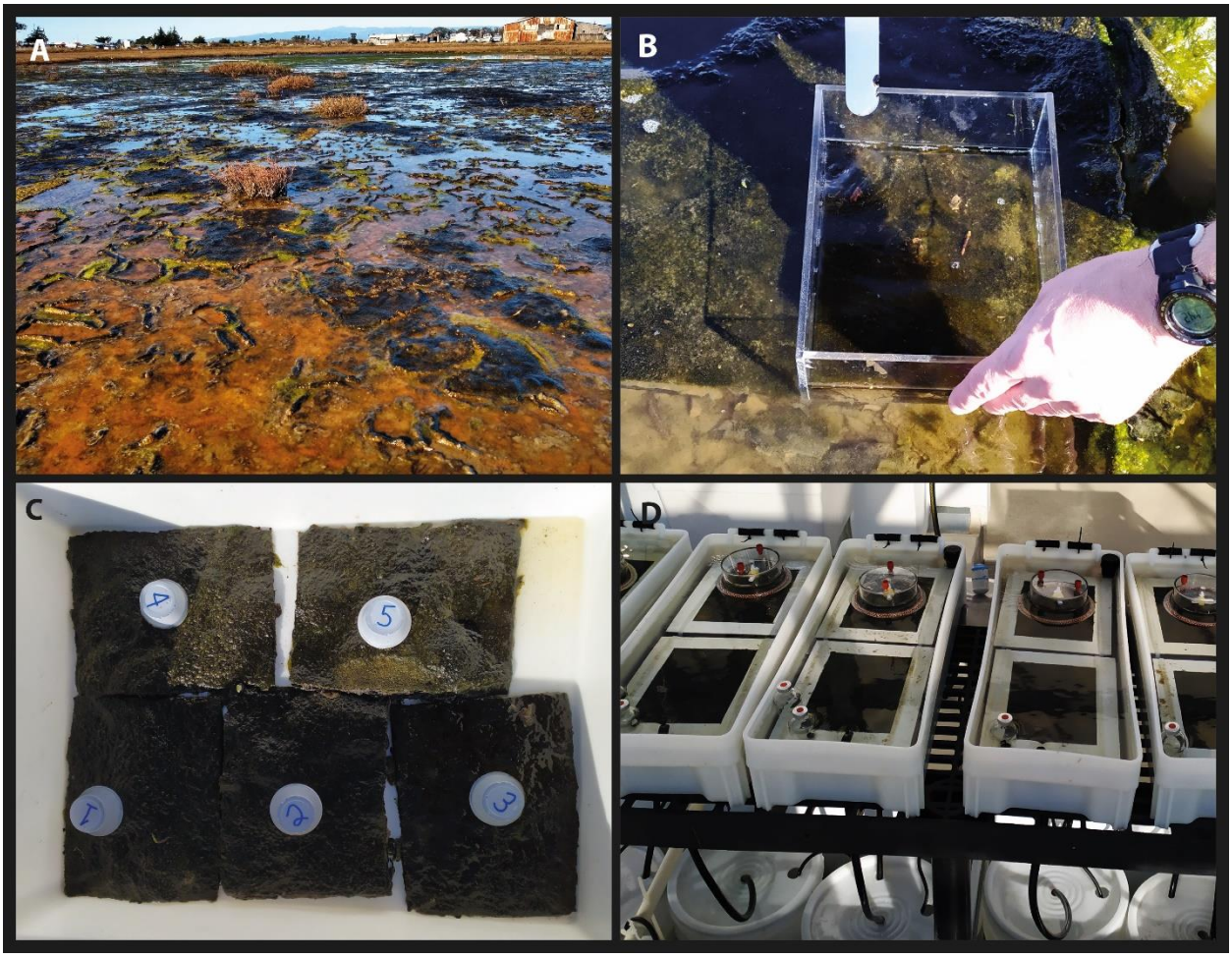

Fig. 30: Microbial mats from Elkhorn Slough, California. A view of the collecting area (A), sampling (B) and transportation (C) to incubate them under greenhouse conditions at facilities of the NASA's Ames Research Center (D).

Glossary

Biofilm: a cover of microorganisms sticks to each other, adhered to a surface

Root nodules: small aggregation of microorganisms incrusted in the roots of some plants, primary legumes

Microbialite: an amorphous sedimentary deposit made carbonate, mediated by microorganisms

Endoevaporite: crystallized gypsum-halite matrix embedded by microbes.

Oncolites: a type of spherical or ovoid microbialite

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