Centro de Investigación Científica y de Educación Superior de Ensenada, Baja California

Doctorado en Ciencias en Ciencias de la Vida con orientación en Microbiología

Metagenomic analysis of fungal diversity of marine sediments from the Gulf of Mexico

Tesis
para cubrir parcialmente los requisitos necesarios para obtener el grado de Doctor en Ciencias

Presenta:

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Resumen de la tesis que presenta **Lluvia Beatriz Vargas Gastélum** como requisito parcial para la obtención del grado de Doctor en Ciencias en Ciencias de la Vida con orientación en Microbiología.

Análisis metagenómico de la diversidad fúngica en sedimentos marinos del Golfo de México

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Los hongos han sido descritos como los participantes principales en la degradación de compuestos orgánicos. A pesar de su importancia, la presencia, la abundancia y la distribución de la comunidad fúngica ha sido poco estudiada y comprendida, sobre todo en ambientes marinos. El presente estudio describe los patrones de distribución y las características del hábitat asociadas a la micobiota de los sedimentos marinos profundos (1000 m hasta >3500 m) colectados de la Zona Económica Exclusiva (ZEE) de México en el golfo de México (GM). Para expandir nuestro conocimiento sobre la comunidad fúngica y su distribución en los sedimentos, se obtuvieron muestras procedentes de 39 estaciones colectadas en cuatro campañas en años diferentes (2013, 2015, 2016 y 2017), se extrajo su ADN, se amplificó el espaciador interno transcrito 1 (ITS 1, por sus siglas en inglés), y se secuenció en la plataforma MiSeq de Illumina. Durante el análisis de las secuencias de la campaña del 2013, se identificaron errores en el set de datos, los cuales no permitieron su correcto análisis, por lo que se decidió descartar esos resultados. En las campañas realizadas en el 2015, 2016 y 2017, durante el procesamiento de las muestras de sedimento, se enfrentaron diferentes retos metodológicos que ayudaron a determinar el correcto procesamiento de las muestras y el análisis de las secuencias obtenidas. El uso de una comunidad control de referencia y añadir diferentes controles negativos fue la mejor opción utilizada y probada para superar estos retos. Mientras que la comunidad de referencia ayudó en el procesamiento de datos y la selección de la base de datos taxonómica, los controles negativos ayudaron a disminuir la señal de contaminación en las muestras. Se obtuvo un total de 4,421 Unidades Taxonómicas Operacionales (OTU, por sus siglas en inglés), de las cuales la mayoría de las asignaciones taxonómicas pertenecían a miembros del filo Ascomycota, hongos no identificados y al filo Basidiomycota. Se observó que las dos capas del sedimento muestreadas (0-5 cm y 5-10 cm) por estación compartían los mismos grupos fúngicos, pero la capa profunda contiene una menor abundancia de estos grupos; este resultado indicó que estas dos capas continuas, podrían presentar las mismas características, por lo que, si se desean observar cambios en la comunidad fúngica, se debería muestrear una capa más profunda. Se encontraron diferencias entre estaciones en relación con la abundancia de ciertos órdenes, tales como Eurotiales, Saccharomycetales, Capnodiales y hongos no identificados, que también estuvieron presentes en todas las estaciones. La mayoría de las estaciones compartieron 31 OTUs, los cuales incluyen géneros de hongos reportados de forma global, tales como *Penicillium*, *Rhodotorula* y *Cladosporium*. Se identificó una comunidad conservada y una transitoria, la cual sugiere una dependencia o adaptación a la dinámica del hábitat, respectivamente. Estos resultados permitieron identificar diferencias de la micobiota a través de un amplio rango geográfico con diferentes características fisicoquímicas de los sedimentos. Las diferencias encontradas en la riqueza y composición taxonómica de los hongos se correlacionaron principalmente con contenido de carbono, carbonatos y material terrígeno, que pueden ser los factores importantes que delimitan la distribución fúngica. Así mismo, la localización geográfica de las estaciones muestreadas sugiere un efecto latitudinal en la composición de la comunidad fúngica. Este estudio representa un esfuerzo para comprender la diversidad fúngica en un hábitat complejo, donde las características geográficas, físicas y químicas delimitan su composición y distribución.

Palabras clave: Golfo de México, sedimentos marinos profundos, comunidad fúngica, región ITS 1.

Abstract of the thesis presented by **Lluvia Beatriz Vargas Gastélum** as a partial requirement to obtain the Doctor of Science degree in Life Sciences with orientation in Microbiology

Metagenomic analysis of fungal diversity of marine sediments from the Gulf of Mexico

Abstract approved by:

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PhD. Meritxell Riquelme Pérez

Thesis Director

Fungi are the major participants of the microbiota in the degradation of organic materials. Despite their importance, the fungal community occurrence, abundance, and distribution remain largely understudied and misunderstood, especially in marine environments. This study describes the distribution patterns and associated habitat characteristics of the mycobiota of deep-sea sediments (1000 m and >3500 m depth) collected from the Mexican Exclusive Economic Zone (EEZ) of the Gulf of Mexico (GoM). To extend our knowledge on the fungal community and its distribution in deep-sea sediments, Internal Transcribed Spacer 1 (ITS 1) amplicons were sequenced by Illumina MiSeq from 39 stations sampled across four campaigns in different years (2013, 2015, 2016 and 2017). During the analyses of samples from the 2013 campaign, the results indicated inconsistencies and errors, so it was decided to discard those datasets. In the rest of the campaigns analyzed (2015, 2016 and 2017), during the processing of the sediment samples a mock community control and different negative controls were included, which helped to determine the correct processing of the samples and the sequences. While the mock community helped to process the data and select the fungal databases, the negative controls helped to identify contamination. A total of 4,421 Operational Taxonomic Units (OTUs) were obtained, from which the majority of the assignments corresponded to members of the Ascomycota, unidentified fungi and Basidiomycota. When analyzing the taxonomic composition at different depths of the corer (0-5 cm and 5-10 cm), the same fungal groups found in the top layer of the corer, were found in the layer below, although in less abundance in the latter; this indicated that the sediments in these two layers may present similar characteristics, and to compare fungal communities, a deeper layer should be sampled. Differences across stations were found in the abundance of certain fungal orders including Eurotiales, Saccharomycetales, Capnodiales and unidentified fungi, which were also present in all stations. The majority of the stations shared a mere 31 OTUs, including the worldwide reported genera *Penicillium*, *Rhodotorula* and *Cladosporium*. Both a transient and a conserved community were identified, suggesting their dependence or adaptation to the habitat dynamics, respectively. These results allowed identifying differences of the mycobiota across a wide range of geographic locations with different sediment physicochemical properties and depths. The differences found in fungal richness and taxonomic composition were correlated principally with carbon, carbonates and terrigenous content, which could be the potential drivers that delimit fungal distribution. Also, the correlation among fungal community and the geographic location of the sampling stations suggests a latitudinal effect on the fungal community composition. This study represents an effort to understand the fungal diversity in a complex habitat, where the geographic, physical and chemical properties delimit their composition and distribution.

Keywords: Gulf of Mexico, deep-sea sediments, fungal community, ITS 1 region.

Dedication

Este trabajo se lo quiero dedicar a mi familia:

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Table of content

Figure list

Table list

Chapter 1. Introduction

Fungal communities play multiple key roles in the ecosystem. They represent the majority of biomass on soils, decompose organic material, providing nutrients to plants, and act as biomarkers of the ecosystem health (Borneman and Hartin, 2000). Despite the current knowledge on the existing fungal species, most of them remain uncharacterized; in the last two decades, a conservative number of ~1.5 million fungal species had been estimated (Hawksworth and Rossman, 1997). The estimation of the Earth's fungal richness depends mostly in the different extrapolation techniques. While some recent studies estimate at least 6 million species, considering the fungus to plant ratio (17:1) (Taylor et al., 2014), others remains more cautious and estimate between 2.2 to 3.8 million fungal species by taking into account fungus to plant ratios, and also species discovery and molecular sequence data (Hawksworth and Lucking, 2017). These studies highlight the lack of knowledge and disparity between estimations on the real fungal diversity on Earth, a problem that arises from the lack of an ideal method that allows identifying the undescribed fungal species and from the vast amount of environments that remain to be sampled. In addition, there are no realistic estimates that include fungi associated with animals or fungi from freshwater and marine environments (Richards et al., 2012).

Fungi comprise a wide variety of organisms capable of inhabiting nearly all ecosystems, including forests (Buée et al., 2009), meadows (Brodie et al., 2003; Hunt et al., 2004), arid and semi-arid ecosystems (Aguilera et al., 1999; Cregger et al., 2012; Durrell and Shields, 1960; Oliveira et al., 2013; Porras-Alfaro et al., 2011; Rillig et al., 2003; Romero-Olivares et al., 2013; Vargas-Gastélum et al., 2015), tundra (Lentendu et al., 2011; Schadt et al., 2003; Wallenstein et al., 2007; Zinger et al., 2009), fresh water environments (Comeau et al., 2016; Raja et al., 2008) and marine deep-sea sediments (Edgcomb et al., 2011; Lai et al., 2007; Nagahama and Nagano, 2012; Xu et al., 2018b; Zhang et al., 2015; Zhang et al., 2016). Within an ecosystem, fungi contribute to nutrient cycles by degrading or assimilating complex organic compounds into simpler molecules that can be easily assimilated by themselves or other organisms; furthermore, fungi participate in pathogenic or mutualistic interactions with a variety of eukaryotic hosts (Mohopatra, 2008).

The distribution of fungi in an environment is influenced by suitable substrate availability, hydrostatic pressure, temperature, pH, humidity and potential hosts (Arnolds, 2007). Varying values of these factors can induce changes in fungal species abundance and richness, and in turn trigger mechanisms of adaptation, such as thermo and osmotic tolerance (Magan, 2007). Fungal communities can also be affected by anthropogenic activities. For example, the exposure to organic and inorganic contaminants

can disturb the fungal activity, and impact different biogeochemical cycles such as those of carbon, nitrogen, and phosphorus (Gadd, 2007), therefore, altering ecosystem functioning.

In the Gulf of Mexico (GoM) two important anthropogenic disasters have occurred: the spill of the Ixtoc platform in Bahía de Campeche in March 6th, 1979, in which a total of 3.3 million barrels of oil were spilled in 10 months (Jernelöv and Lindén, 1981); and more recently, in April 20th, 2010, 60 km southeast of the Louisiana coast, the Deepwater Horizon (DH) platform collapsed due to an explosion in the tower. For about 3 months, until July 15th, 2010, approximately 4.9 million barrels were spilled into the ocean (Lu et al., 2012; Mendelssohn et al., 2012).

The exposure to a high concentration of hydrocarbons significantly affected the biological communities of the GoM. After the DH spill subsequent studies reported damage on marine corals (Macondo, 61 km from Louisiana, USA) reflected as stress signals and tissue loss of the corals (White et al., 2012), as well as on fisheries, which suffered a greater impact, with environmental and economic losses of millions of dollars (Mancera-Pineda et al., 2013).

The oil, besides persisting in the water column, adheres to organic particles of higher density that tend to sediment (Payne et al., 2003). Once in the sediments, the oil composition and concentration could affect the microbial communities (Al-Nasrawi, 2012). It had been suggested that in these environments communities of microorganisms that may play an important role in the hydrocarbons degradation/assimilation could be present (Kimes et al., 2013). Therefore, after an oil spill there could be an increase of microorganisms capable of degrading those compounds, including a few fungal genera (*Aspergillus*, *Candida*, *Cladosporium*, *Fusarium*) with a high tolerance to oil due to their adaptability and physiology (Al-Nasrawi, 2012).

It is not easy to assess the impact of human-caused disasters on the fungal communities from marine sediments, mainly because (1) we lack information about the fungal diversity pre-oil spills, and (2) the techniques that evaluate them are inadequate due to the complexity of microbial communities. The continuous development of improved high throughput sequencing methods (e.g., Roche 454, Illumina, Ion Torrent) allow studying microbial communities, including fungi, and deepens our knowledge on their diversity and function at a specific time.

The advances in high throughput sequencing (HTS) platforms have helped the development of methods in metagenomics and metatranscriptomics, obtaining larger amounts of data at lower cost and

shorter time (Jones, 2009), and that provide information about the diversity and putative function of different organisms in environmental samples. A combination of both methods has helped us to understand the ecological role of the different microbial communities (Kirchman, 2012). Metabarcoding and shotgun sequencing have helped to disentangle communities' compositions; metabarcoding is a PCR-based technique, which enriches the microbial fraction of certain groups (*i.e*. bacteria, archaea, fungi), improving their detection; and shotgun sequencing is a PCR-free technique, which could facilitate calculating the real abundance of the members of the community (Tedersoo et al., 2015a). For surveys of fungal communities of marine sediments of the GoM, those methodologies have the potential to provide a novel perspective about the community structure and functioning.

In marine ecosystems about 500 species, which include yeast/filamentous forms and lineages, have been reported as obligate marine fungi (Mohopatra, 2008; Richards et al., 2012). The use of high throughput sequencing platforms will allow us to acquire a better knowledge on fungal diversity and activity, by providing a greater amount of data, which helps in the detection of low-abundance members of the community and in obtaining a more detailed data of gene expression profiles.

Oil spills result in high hydrocarbons concentrations but the effects on the fungal communities in the marine sediments of the GoM remain unknown. Previous examination of microbial diversity pre- and post- DH oil spill in coastal communities in the USA demonstrated dramatic changes on the community structure, including fungi (Bik et al., 2012). Therefore, it is necessary to know the baseline of the mycobiota diversity from marine sediments to detect changes after disturbances due to ecological disasters. Fungal species such as *Candida albicans*, *C. dubliniensis* and *Penicillium citrinum* have been reported as capable of assimilating hydrocarbons as substratum (Kaczorek et al., 2008; Polman et al., 1994). These species have been found abundantly in the USA coast (Bik et al., 2012), as well as in deep-sea sediments of the Mexican Exclusive Economic Zone (EEZ) from the GoM (Vargas-Gastélum et al., in prev). However, detecting fungi that can degrade/assimilate hydrocarbons does not necessarily imply that these fungi are associated to oil spills in the area as they could be part of the characteristic microbiota in sediments of the GoM.

1.1. Background

1.1.1. Gulf of Mexico.

The GoM is a semi-closed basin that represents a large and productive ecosystem located between Mexico, the United States and Cuba (Lara-Lara et al., 2008; Love et al., 2013). This basin is connected with the Atlantic Ocean via the strait of Florida and with the Caribbean Sea via the Yucatan channel (Lara-Lara et al., 2008).

The GoM is divided by countries into different EEZ, where the corresponding country has rights over the exploitation of resources and exploration (NOAA, 2013). The EEZ of Mexico, represents the 55% (0.9×10^6 km^2) of the total area of the GoM (Figure 1).

There are seasonal changes, which can be observed in this semi-enclosed basin, such as the dry season in spring (February-May), rainy season in summer and autumn (June-October), and "north" season or cold anticyclonic fronts in winter (October-February) (de Lanza Espino and Gómez Rojas, 2004; Lara-Lara et al., 2008). The bathymetry varies depending on the area: the depth in the intertidal zone, is <20 m, in the continental shelf from 20 to <180 m (Figure 1), in the continental slope from 180 to 3,000 m, and in the abyssal areas (Sigsbee deep: deepest part of the GoM) greater than 3,000 m; these zones represent the 38%, 22%, 20% and 20% respectively of the total area of the GoM (Love et al., 2013). The deep-sea comprises those areas with more than 200 m of depth, low biological productivity and currents of no more than 0.25 knots (0.1286 meters per second) (Lara-Lara et al., 2008).

The bottom sediments from the Mexican EEZ are mud dominated with some sand dominant areas in the Yucatan Bank (Jenkins, 2011). Most of the sediments in the continental shelf and slope are terrigenous type arising from continental land erosion and receiving permanent input of nutrients from four important rivers: Pánuco, Coatzacoalcos, Papaloapan and Grijalva-Usumacinta (Figure 1) (de Lanza Espino and Gómez Rojas, 2004).

The biological processes in the sediments depend almost entirely on the organic matter that travel across the water column as product of the primary production (PP) (García-Villalobos and Escobar-Briones, 2007). From the total PP, only 1.5% of the organic particles are deposited on the sediments in the open ocean, and 17% on the slopes: most of this is oxidized, while the 0.5-3% in the continental shelf and slopes and the 0.014% in the deep-sea, are buried (Seiter et al., 2004; Singh et al., 2010). During cold anticyclonic

fronts on winter, the deep-sea sediments received a larger amount of particles due to the mixing of the water column and the contribution of the rivers; while in summer, the smallest amount of particles is received due to the water column thermohaline stratification (differences between temperature and salinity from different water masses) (Escobar Briones, 2004).

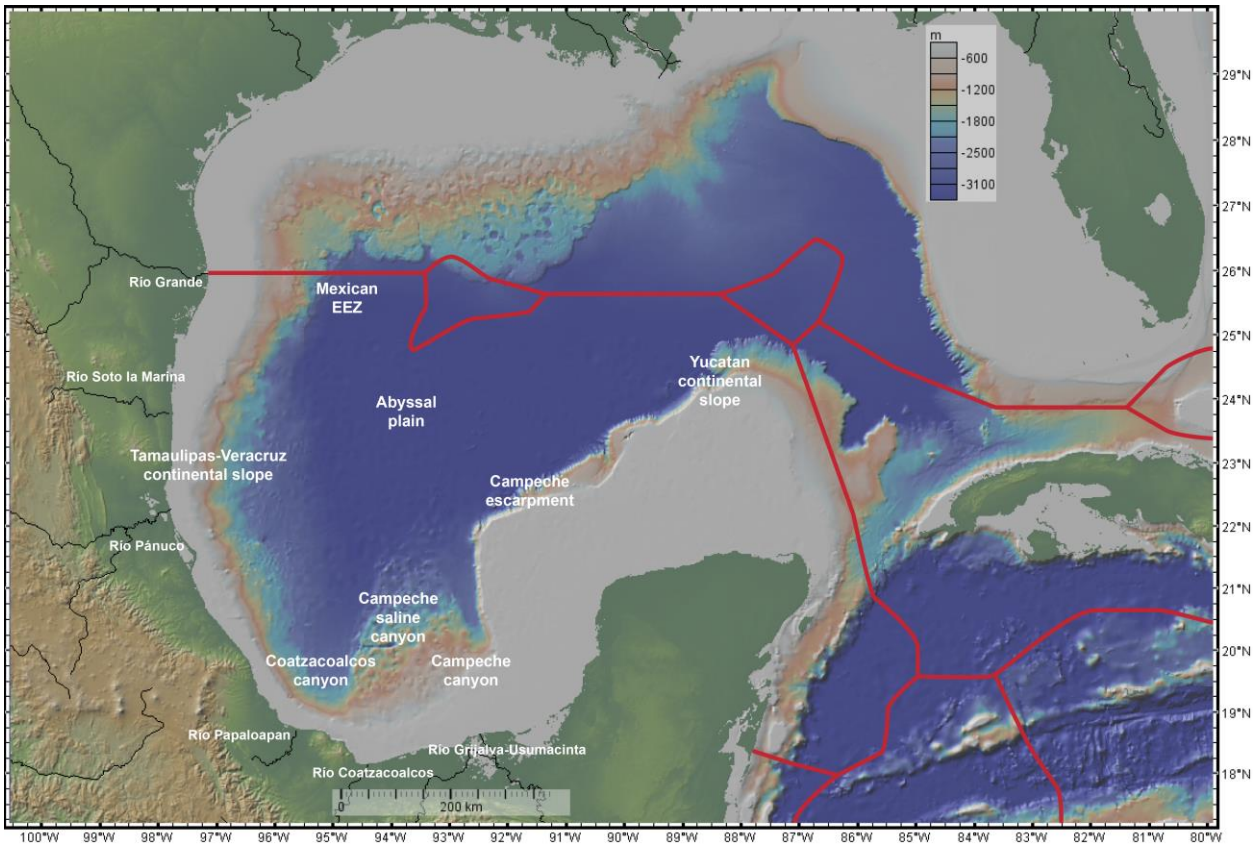

Figure 1. Map of the Gulf of Mexico: Mexican Exclusive Economic Zone, regions and bathymetry and important rivers. Black lines represent the rivers. Red lines represent the delimitation of EEZ.

Our understanding about deep-sea processes of the GoM is based on the knowledge of these different areas, including primarily their physical and chemical characteristics. However, there is a lack of knowledge about their biological components, despite the different threats that could possibly affect the biological diversity of these environments. Among these threats one should consider industrial waste (urban and from ships), mineral, oil and gas extraction (Lara-Lara et al., 2008). In order to improve prevention and restoration plans, we need to know and understand the ecosystem structure and functioning (Love et al., 2013), in all their aspects.

1.1.2. Marine fungi: their role in marine ecosystems

The ocean includes an extensive number of habitats, from coastal waters (characterized as highly photosynthetic environments) to deep-sea waters and sediments (Redou et al., 2015). The participation of bacteria, which act as decomposers and parasites in each marine trophic level, has been extensively studied (Xu et al., 2014). In contrast, the role of fungi in these environments has only recently started to be studied (Raghukumar, 2017b).

In marine environments, fungi are found from the photic zone in the water column to sediments over 3 km deep (Raghukumar, 2006). In terms of surface extension, the sediments represent the largest environment in the ocean in terms of surface extension (Bongiorni, 2012), characterized by high hydrostatic pressure (0.1 MPa/10 m), low temperatures (2-4°C) (Nagano et al., 2010; Xu et al., 2014), and absence of photosynthetic processes due to the lack of light. Their growth and activity depend almost entirely on the material that is transported to the sediments as a product of the biological pump (Raghukumar, 2017b). From this material, the labile components are quickly degraded first, while the most complex material such as humic components, accumulate in the sediments because they are slowly degraded (Raghukumar, 2017b). Fungi inhabiting marine sediments are responsible for degrading organic matter and are able to endure extreme conditions such as low temperatures, high pressure and lack of oxygen (Damare and Raghukumar, 2008).

The most accepted definition of marine fungi was proposed by Kohlmeyer and Kohlmeyer (1979), who described obligate fungi as those that can grow exclusively on marine environments, and facultative fungi as those that can grow both on marine and terrestrial environments. This definition of marine fungi suggests that the fungi can be seen as an ecological and physiological defined group, rather than as a taxonomical group (Hyde et al., 2000). Among the 1,500 fungal species estimated in marine environments (Hyde et al., 1998; Kohlmeyer and Kohlmeyer, 1979), approximately 530 species are considered obligate marine fungi (Damare et al., 2012; Jones et al., 2009). The marine fungi *Sphaeria scirpicola var typharum* (Desmaziéres, 1849) and *Sphaeria posidoniae* (Durien and Montagne, 1869) were the first facultative and obligate marine fungi described, respectively. The first evidence of fungal existence in deep sea sediments came from wood panels submerged at 1,000 m depth (Barghoorn and Linder, 1944), and years later on the Atlantic abyssal plane at 4,450 m (Roth et al., 1964). Subsequent studies reported cultivable species including *Cladosporium* sp., *Alternaria* sp., *Aspergillus sydowi* and *Penicillium* sp. at a depth of more than 4,450 m. Those species have been described as terrestrial organisms, indicating that can adapt to high hydrostatic pressure (Mohopatra, 2008).

One model proposes that as plants and other life forms, fungi evolved from sea to land, and this invasion of terrestrial ecosystems was possible for the appearance of mycorrhizal-like organisms, which belonged to Glomeromycota fungi and their association to plants roots and other phototrophs organisms (Heckman et al., 2001). This theory was supported by fossil evidence from near 400 Mya ago, but studies based on 18S molecular clocks indicates the origin of fungi around 600 Mya ago (Raghukumar, 2017c), rising new arguments on the real origin and divergence of fungi. Some authors favor the idea that fungi came from freshwater habitats, based on the recovery of a high abundance of sequences corresponded to basal fungi in these environments (Richards et al., 2012). It has been suggested that some terrestrial fungal species were adapted to marine environments, as is the case of the Halosphaeriales and the Lulworthiales (Spatafora et al., 1998). Another example is found in the Dothideomycetes, which have been reported as merely terrestrial species, but there are many observations from this clade documented in marine environments as well (Suetrong et al., 2009). Ascomycota and Basidiomycota phyla have been reported as the most commonly found on marine ecosystems, especially the yeast forms (Blackwell, 2011), which are abundant on the first few centimeters of the sediment and their density can reach about 2000 viable cells/g (Kutty and Philip, 2008). Fungi in marine environments can behave as parasites, as symbionts or as saprobes: *Chytridium polysiphoniae* and many marine ascomycetes are parasites of algae (Richards et al., 2012), *Pharcidia balani* is a symbiont of microalgae (Kohlmeyer and Kohlmeyer, 1979), and *Amylocarpus encephaloides* is a saprobe that grows in wood substrates (Rämä et al., 2014).

The dynamics of fungal communities on this type of ecosystems is still highly unknown. A study on the Peru Margin sampled sediments from different depths (5, 30, 50, 70, 91 and 159 meters below sea surface), and discovered that fungi are present and active in all sampled depths (Orsi et al., 2013b). Transcripts related to carbohydrate, amino acid and lipid metabolism, as well hydrolases were identified, suggesting an active participation of fungi on different substrate degradation processes.

In the coastline along the GoM, through cultivation methods, some studies reported as the most abundant species the obligate marine fungus *Corollospora maritima* as well as the facultative marine fungus *Cladosporium cladosporioides* (González et al., 1998; Velez et al., 2015; Velez et al., 2013). *Arenariomyces majusculus*, *A. parvulus*, *Leptosphaerella* sp. (Velez et al., 2013), *Alternaria longipes*, *Emericella violacea*, *Chaetomium globosum* and *Lasiodiplodia theobromae* (González et al., 1998) were reported as the less abundant fungi in the samples. In addition, these studies reported changes in species richness and diversity in different sampled sites near important rivers affected by anthropogenic activities such as discharges of wastewaters and waste from petroleum developments in the area (Velez et al., 2015; Velez et al., 2013).

Metagenomic studies using Next Generation Sequencing (NGS) techniques, have reported that the hydrocarbons derived from the oil spill of the DH platform have strongly influenced the dynamics of microbial populations in the GoM (Bik et al., 2012). The proportion of fungi, specifically, *Candida dubliniensis, Lodderomyces elongisporus* and *Penicillium chrysogenum* increased in sand and water from affected areas in the U.S. (Widger et al., 2011). On the other hand, it has also been reported the presence of yeast of the genera *Rhodotorula*, *Cryptococcus* and *Candida*, in oil agglomerations found in the coast of Florida in summer 2010, and it is believed to be a result of the oil spill from the DH platform (Albu et al., 2011).

In deep-sea environments from the GoM, the fungal diversity of methane seeps (from U.S. waters) has been recovered through culture-independent methods (Thaler et al., 2012). From these methane seeps, only 39 fungal sequences were recovered, with Ascomycota and Basidiomycota being the most abundant, and the most abundant recovered phylotype could be related to the novel DSF-group1 (from Ascomycota), a group that has been previously described in deep-sea sediments from the Pacific Ocean (Nagano et al., 2010). Methane seeps are known to be formed when there is an increase in pressure, forcing the hydrocarbon-enriched water to ascend through the sediment; it is known that the emerging methane is produced by the microbial decomposition of the organic matter in anoxic conditions, so the presence of fungi could suggest an active participation in these processes, but more studies are needed to demonstrate the ecological role of fungi in this extreme environment.

1.1.3. Environmental factors influencing the marine fungal distribution

There are only a handful of studies reporting fungi from deep-sea environments. Similarly, only a few reports exist describing the physicochemical properties for these environments, making it difficult to correlate the presence of these organisms to the environment characteristics. The Peru Margin study explained in the above section, correlated the fungal community and activity with dissolved and total organic carbon, and sulfides (Orsi et al., 2013a). In subsurface sediments (at more than 1900 meters below seafloor), some fungal genera such as *Cryptococcus*, *Rhodotorula*, *Penicillium* and *Meyerozyma* were mostly correlated with depth (Redou et al., 2014). In another study in sediments from the high Artic, the fungal diversity was mostly influenced by salinity, organic carbon, silicates and phosphates content (Zhang et al., 2015). And, in sediments from subtropical Chinese seas, water depth, temperature, salinity, total nitrogen and C/N ratio influenced the distribution of the fungal communities (Li et al., 2016).

At a global scale, the distance from the equator and the mean annual precipitation has an effect in the distribution and richness of terrestrial fungi (Tedersoo et al., 2014), but these factors do not seem to be the same for marine fungi, whose distribution in the water column has been correlated with temperature and salinity (Booth and Kenkel, 1986), and in sediments it has been correlated with geographic (particularly depth) and environmental physicochemical characteristics (i.e., oxygen and nitrate content) (Tisthammer et al., 2016). Nevertheless, there is no consensus yet on which drivers shape the fungal community composition and diversity from regional to global spatial scales.

While these studies represent an important effort to understand the dynamics of fungal communities in the deep-sea environments, more extensive effort in the characterization of the mycobiota at regional scales is needed to decrease uncertainties in distribution models associated with these organisms.

1.1.4. Fungal diversity in marine sediments from the GoM: the XIXIMI campaigns effort

The XIXIMI (*xiximi*: traditional Nahuatl for "*spill*") campaigns began in 2010 as part of the multi-institutional project entitled "Defining the baseline of deep waters from the GoM in response to the Deepwater Horizon oil spill". This project has as one of the principal objectives to establish a baseline of the oceanographic, geochemical and biological characteristics of the Mexican territorial waters of the GoM, in order to detect the influence of the Deepwater Horizon spill on this environment, and subsequently monitor the impact of oil spills in the short, intermediate and long terms. As part of the subproject aimed the characterization of the baseline biological diversity, fungi were included as important undescribed organisms on the GoM.

To accomplish the main objective of the project, a first effort was undertaken during XIXIMI 1, 2 and 3 campaigns in 2010, 2011, 2013, respectively. A total of 10 stations per campaign were sampled on the Mexican EEZ (Figure 2) using a Soutar core, and on each station, 5 replicate samples were obtained from each core.

The fungal diversity of the samples from the XIXIMI 1 and 2 campaigns was obtained by construction of a library and subsequent Sanger sequencing, and the samples from the XIXIMI 3 campaign were processed by amplification of the ITS region of the rDNA and subsequent Illumina MiSeq sequencing

(the methodology and results of XIXIMI 3 campaign are shown on subsequent chapters as part of this work). The results of the XIXIMI 1 and 2 campaigns allowed detecting some phylotypes such as *Candida* spp., *Penicillium citrinum*, *Alternaria* spp., *Phoma exigua*, *Chytriomyces angularis*, and *Aureobasidium* spp., among others. The results of these campaigns represent the first overview of the fungal community on deep-sea sediments of the GoM belonging to the Mexican EEZ. Some of the phylotypes found have been described as capable to assimilate hydrocarbons, i.e. *Candida albicans* (Kaczorek et al., 2008) and *Penicillium citrinum* (Polman et al., 1994), but more research is needed to detect the fungal phylotypes that are actively degrading these kind of compounds.

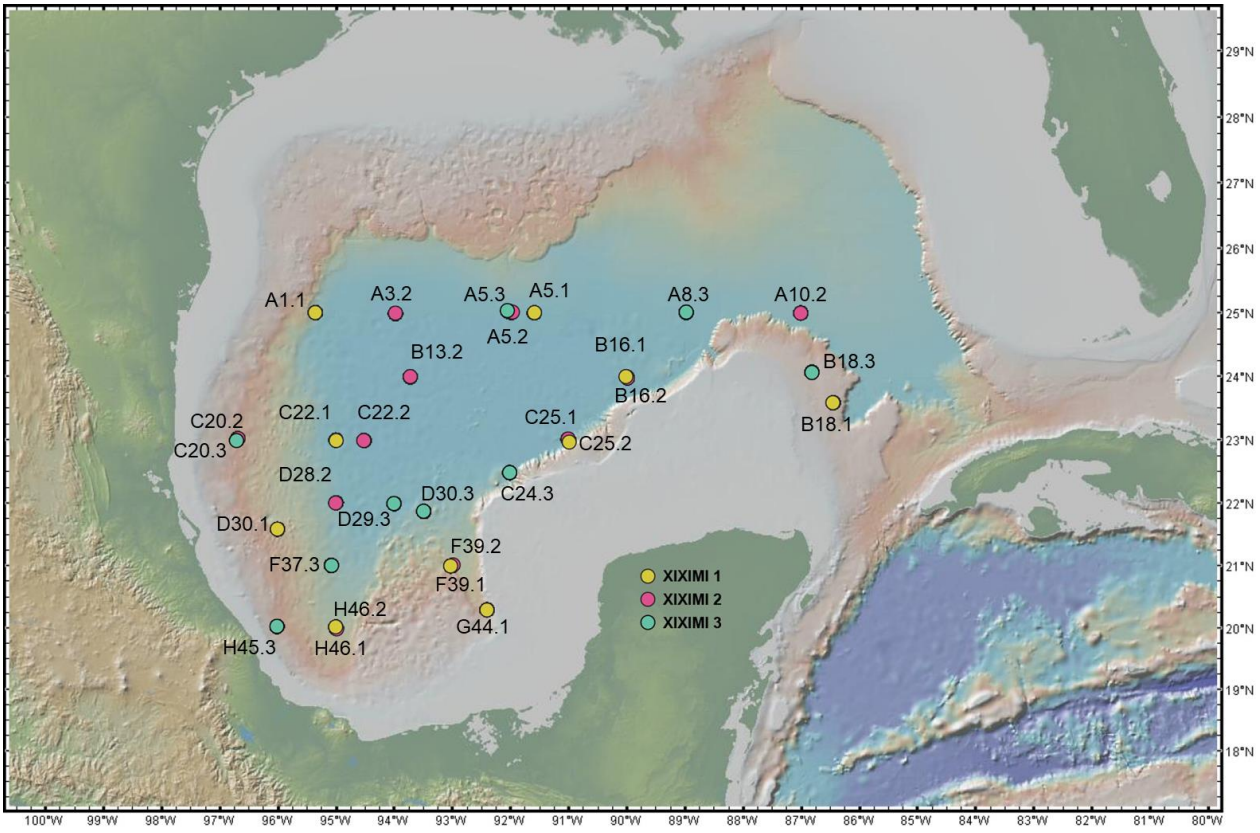

Figure 2. XIXIMI 1, 2 and 3 campaigns. Sampling sediment stations belonging to each campaign.

The XIXIMI campaigns are now part of the Gulf of Mexico Research Consortium (CIGoM) with the project "Implementation of oceanographic observation networks for the evaluation of potential contingencies related to the exploration and production of hydrocarbons in the deep-water region of the Gulf of Mexico". The CIGoM project is divided into different “action lines”, focused on different aspects of the GoM; the XIXIMI campaigns belong to Line 2 (baseline and environmental monitoring).

1.1.5. ITS region as barcode tool for fungal identification

To evaluate the status of fungal communities in different environments it is necessary the precise identification of the species that they comprise. Obtaining a composition profile is rather complicated; moreover, the majority of studies on fungal communities have been directed to identify the cultivable species (Brunner et al., 2007), making it difficult to obtain a community profile due to the fact that there are many cryptic species that cannot be distinguished from others that grow slowly and are overtaken or masked by species with rapid growth.

Molecular tools have eliminated the biases from culture-dependent methods, and have allowed the identification of microorganisms of interest with the use of different "barcoding" regions.

The genes that codify for ribosomal RNA are found arranged in arrays that include repeated transcriptional units (18S - 5.8S – 28S), two variable regions (internal transcribed spacer; ITS1 and ITS2), as well as two external sequences (external transcribed spacer, ETS) (Brunner et al., 2007; Buchan et al., 2002; Jeewon and Hyde, 2007; Korabecna, 2007).

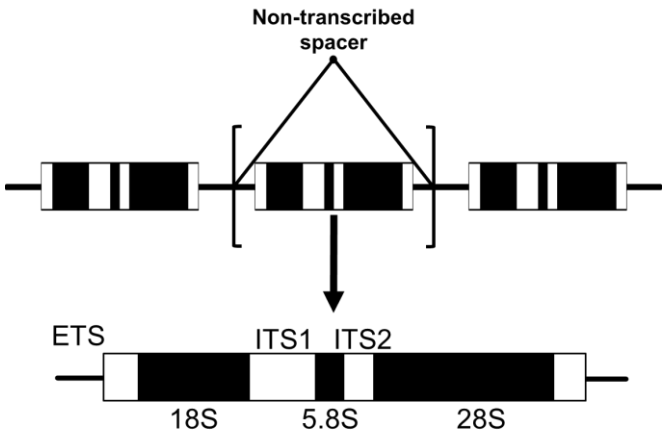

Figure 3. Organization of eukaryotic rRNA genes. Modified from Deacon (2006).

The most popular barcoding sequences for fungal identification are the transcribed internal spacers (ITS1 and ITS2) (Buée et al., 2009; Wang et al., 2011). Those regions show variability in size and

sequence, allowing identification to genus and species levels (Korabecna, 2007; Lai et al., 2007; Nilsson et al., 2008; Schoch et al., 2012; Tedersoo et al., 2010). The number of ITS copies per fungal cell be typically more than 250, making this region and ideal target gene for fungal identification, especially in studies where a low concentration of genomic DNA is recovered from the environment to be studied (Nilsson et al., 2009b).

The use of these two variable regions (ITS1 and ITS2) has allowed the advancement of fungal diversity studies. The design of fungal specific oligonucleotides enabled the amplification of the region of interest, including all fungal taxa (Bidartondo and Gardes, 2005). Currently there is a wide range of universal and specific oligonucleotides for each taxa. White et al. (1990) designed the first pair of universal oligonucleotides (ITS1/ITS2 and ITS3/ITS4) for fungi. Gardes and Bruns (1993) designed specific oligonucleotides for the Basidiomycota (ITS1F-ITS4). One of the encountered problems was the co-amplification of plants rDNA. For this reason Martin and Rygiewicz (2005) designed more specific oligonucleotides for Ascomycota and Basidiomycota (NSA3 NSI1, 58A1F, 58A2F, 58A2R, NLB4, NLC2) to try to eliminate the co-amplification of other eukaryotic organisms (Figure 4).

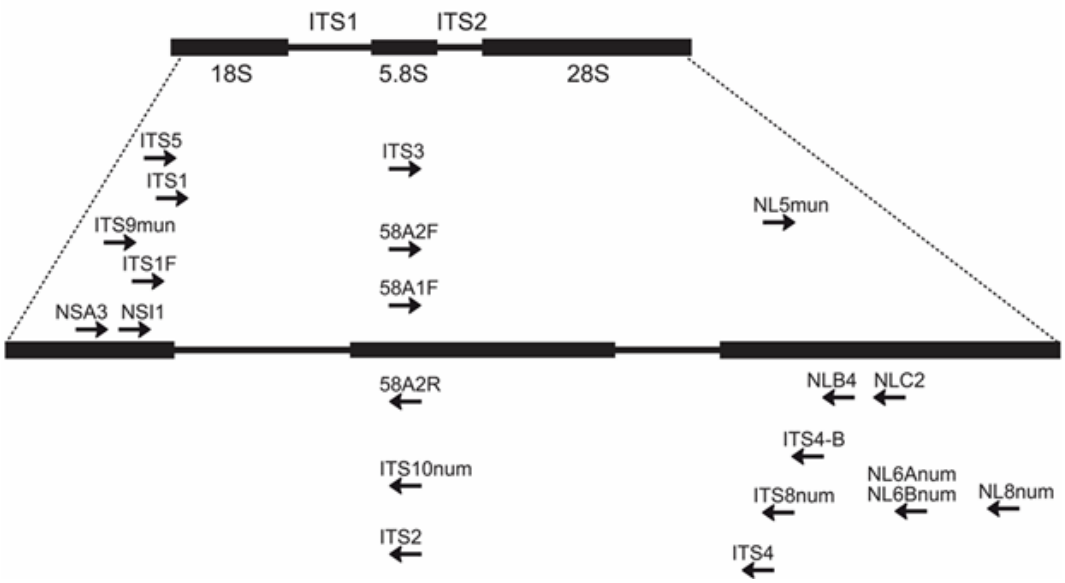

Figure 4. Location and orientation of oligonucleotides for the amplification of different regions of rDNA. Modified from Martin and Rygiewicz (2005).

It has been suggested that the ITS1 region has a higher variability among species than the ITS2 region (Monard et al., 2013; Nilsson et al., 2008); thus using the ITS1 region for fungal community studies could offer a better resolution to species level. Nilsson et al. (2008) demonstrated that this is not entirely correct, since not all fungal groups have an ITS region equally variable. The decision to use the ITS1 or ITS2 regions in diversity studies depends on the specific aim of the study, although the available databases for the ITS1 region contain many more entries than for databases of the ITS2 region.

1.1.6. The study of fungal communities using High Throughput Sequencing

In the last decade, the growing interest to know the fungal diversity on the ecosystems has led to test new technologies that allow the sequencing of entire fungal communities (Nilsson et al., 2009a). This interest is associated with the prosperous development of HTS, which allowed big advances on the study of fungal communities, giving a broad overview of the richness and abundance of these organisms in different environments.

The emergence of NGS has allowed the development of efficient HTS techniques (Xu, 2006), besides offering a faster and cheaper alternative to conventional techniques (Quince et al., 2011). The most widely used sequencing platforms are Illumina HiSeq and MiSeq, which are based on sequencing by synthesis. MiSeq platform produces up to a total of 15 Gb of data, up to 25 million reads (*c*. 600 bp) and has a capacity to process 96 samples; HiSeq produces a total of 120 Gb of data, up to 400 million sequences (150 bp), and has a capacity to process 36 samples (Illumina, 2019). Other platforms like Ion Torrent, based also on sequencing by synthesis, has the capacity to produce reads ranging from 100 to 400 bp length, although it produces less reads than Illumina, up to 5.5 million reads (Thermo-Fisher-Scientific, 2019). After the discontinuation of the 454 Pyrosequencing platform from Roche, these platforms mentioned above are the most widely used, specially Illumina.

There are another sequencing platforms such as Nanoballs (Complete Genomics) and MinION (Oxford Nanopore Technologies). These two technologies consist in sequencing by ligation and changes in electrical conductivity, respectively. Nanoballs gives an output of only 35 bp reads, while MinION can produce longer reads (more than 10 kb), representing a good choice for metagenomics studies, but the analysis of the data associated to long reads provided by this technology is still a work in progress

(Krehenwinkel et al., 2019); various algorithms testing error profiles provide non-comparable and very different results (Magi et al., 2018), making it difficult to replicate the analyses.

The most common methods to assess fungal communities in a given environment are by either target-gene sequencing or shotgun sequencing. Both methods apply HTS. The target-gene sequencing approach, is based in the amplification of a barcode gene (i.e. ITS region for fungi), whereas in the shotgun method, the data obtained corresponds to the entire microbial community (Bacteria, Archaea, Fungi, Nematoda, among others). The target-gene sequencing generates an enrichment of the fungal fraction of the community, is sensitive to PCR and entails primer bias (Lindahl et al., 2013). The shotgun sequencing does not amplify the entire fungal fraction of the community. This hinders the reconstruction the eukaryotic genomes among the recovered metagenome, because they are several orders of magnitude larger than the bacterial genomes, making difficult to obtain a real overview of the fungal portion of the microbial community (Tedersoo et al., 2015a). One of the most important disadvantages of studying fungi (and eukaryotes in general) through shotgun sequencing is that fungal genomes databases are not enriched in comparison with bacteria genome databases, and this results in a poor fungal identification, even in samples where fungi are the most abundant organisms (Nilsson et al., 2019).

1.2. Justification

Marine sediments are environments that are still highly unexplored. The diversity of organisms in sediments is huge, with an estimated number of $10^6 – 10^9$ total species (98,100 described species). Current studies of marine sediments have mostly focused on bacterial diversity, while fungi, despite their highly developed metabolism, have been largely ignored. In studies that evaluate microbial communities in oil-impacted marine ecosystems, changes in the microbial community structure have been observed, with a higher prevalence of certain fungal groups than prokaryotes. This leads to question ourselves, which fungi are developing in those environments? And, are they actively participating on the assimilation of hydrocarbons?

In our working group, preliminary studies to evaluate the fungal diversity of marine sediments from the Mexican EEZ of the GoM have suggested that the Ascomycota is the predominant phylum, which was enriched for species potentially capable of degrading hydrocarbons. In those studies, three sampling campaigns (XIXIMI 1, 2 and 3) were carried out. In XIXIMI 1 and 2 campaigns the replicates from each

station were pooled and the fungal diversity was detected by the construction of clone libraries and subsequent Sanger sequencing. Comparative analyses were performed to determine whether fungal diversity varied within different areas of the same core could not be conducted. Previously, the construction of a library was biased and did not allow the correct detection of all phylotypes present in a sample. In the XIXIMI 3 and subsequent campaigns it was intended to sample a major number of stations to cover a larger area of the GoM, and this would allow us to expand the knowledge of the fungal diversity and their activity on deep-sea sediments from the GoM. This would be achieved by the sequencing of different replicates per station and using next generation sequencing approaches. The results of these analyses will provide information to establish the baseline mycobiota of the GoM and detect the differences within the same station, across stations and across zones.

1.3. Hypothesis

- There will be differences in the fungal community composition found among sampling stations, and these differences will correlate with the geographical location and physicochemical properties of each station.

- There will be differences in fungal community composition of stations sampled on the same geographic location in different years.

- There will be differences in richness and abundance of fungal phylotypes corresponding to the different depths (0-5 cm and 5-10 cm) of the same core.

1.4. Objectives

1.4.1. General objectives

Determine the structure of the fungal community of deep-sea sediments from the GoM, to establish the baseline mycobiota, their temporal community dynamics and the potential of geographical location and physicochemical properties affecting their distribution.

1.4.2. Specific objectives

- Determine the fungal community composition of the deep-sea sediments from the GoM.
- Determine the differences across the sampled stations.
- Determine the temporal variations in abundance and taxonomic composition of the fungal community.
- Determine the influence of geographical location and physicochemical properties in the fungal community distribution of the sampled stations.

Chapter 2. Methodology

2.1. Sampling procedure during XIXIMI campaigns

Four sampling campaigns XIXIMI 3, XIXIMI 4, XIXIMI 5 and XIXIMI 6 were conducted in the Mexican EEZ of the GoM from February 19th to March 10th, 2013, August 27th to September 16th, 2015, June 10th to June 21th, 2016, and August 15th to September 8th, 2017, respectively, on board of the Research vessel *Justo Sierra (http://www.buques.unam.mx/especificacionesJS.htm)*.

A total of ten stations were sampled in XIXIMI 3, 4 and 5 campaigns (XIXIMI 3: A5, A8, B18, C20, C23, C24, D29, D30, F37 and H45; XIXIMI 4: A1, A5, A7, B14, B18, C22, E31, G44, H45 and H47; XIXIMI 5: TS1, A3, A5, B11, B15, B18, C22, D28, and G44; Figure 5). In the XIXIMI 6[1] campaign only 8 stations were sampled (B12, B18, C22, D26, D27, E33, G44 and H48), and an extra sample from G44 station was obtained as replicate (G44R).

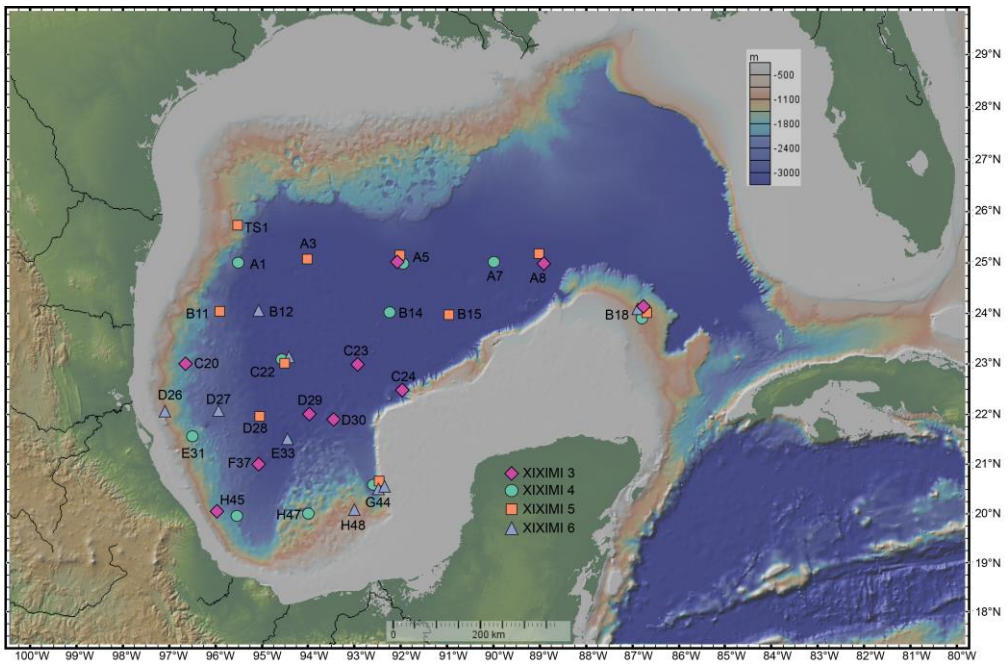

Figure 5. Map of the Gulf of Mexico and stations sampled during XIXIMI 3, 4, 5 and 6 campaigns.

[1] XIXIMI 6 campaign. A total of ten stations were planned to collect sediments, but during the campaign, three hurricanes (Harvey, Irma and María) interfered with the original sampling plan.

To collect sediment samples, a multicorer or a box corer was used (Table 1; Figure 6). Some of the stations were sampled in all campaigns (A5, A8, B18, C22, H45, H47 and G44) in order to obtain information about temporal variation, and the other stations were sampled trying to cover much area as possible.

Table 1. Sampling stations location and characteristics.

Campaign	Station	Longitude	Latitude	Depth	Region*	Corer
XIXIMI 3	A5	267.9534	25.0281	3,520	AP	Multicorer
	A8	271.0169	25.00999	3,488	AP	Multicorer
	B18	273.1793	24.07572	1,208	YCS	Multicorer
	C20	263.3061	23.02876	1,749	TVCS	Multicorer
	C23	267.0147	22.99776	3,741	AP	Multicorer
	C24	267.9821	22.48894	3,551	CE	Multicorer
	D29	265.9958	21.99697	3,559	AP	Multicorer
	D30	266.5086	21.87153	2,960	AP	Multicorer
	F37	264.9138	21.01251	3,087	AP	Multicorer
	H45	263.9819	20.02935	1,739	CZC	Multicorer
XIXIMI 4	A1	264.4609	25.00892	2,429	TVCS	Box corer
	A5	267.9954	25.00432	3,528	AP	Box corer
	A7	269.9854	24.96048	3,534	AP	Box corer
	B14	267.6834	24.00448	3,734	AP	Box corer
	B18	273.2087	23.916	1,183	YCS	Box corer
	C22	265.4357	23.00753	3,721	AP	Multicorer
	E31	263.4727	21.50767	1,549	TVCS	Multicorer
	G44	267.3688	20.5246	2,470	CC	Multicorer
	H45	264.391	19.98707	2,159	CZC	Multicorer
	H47	265.9822	20.0004	1,342	CSD	Multicorer
XIXIMI 5	TS1	264.459	25.741	1,355	TVCS	Box corer
	A3	265.987	25.05	3,685	AP	Box corer
	A5	267.99	25.124	3,513	AP	Box corer
	A8	270.947	25.124	3,477	AP	Box corer
	B11	264.054	24.058	2,298	TVCS	Box corer
	B15	268.976	23.977	3,708	AP	Box corer
	B18	273.273	23.977	1,242	YCS	Box corer
	C22	265.443	22.988	3,717	AP	Box corer
	D28	264.94	21.984	3,721	AP	Box corer
	G44	267.483	20.536	2,353	CC	Multicorer
XIXIMI 6	B12	264.8832	24.05016	3,508	AP	Multicorer
	B18	273.1165	24.05007	1,150	YCS	Box corer
	C22	265.4833	23.00024	3,727	AP	Multicorer
	D26	262.8832	22.0168	966	TVCS	Multicorer
	D27	263.9999	22.00001	2,722	TVCS	Multicorer
	E33	265.4998	21.48334	3,431	AP	Multicorer
	G44	267.5	20.50019	2,384	CC	Multicorer
	G44R	267.5	20.50004	2,374	CC	Multicorer
	H48	266.9832	20.01673	1,201	CC	Multicorer

*Regions: AP, Abyssal Plain; CC, Campeche canyon; CE, Campeche escarpment; CSD, Campeche saline domo; CZC, Coatzacoalcos canyon; TVCS, Tamaulipas-Veracruz cont. Slope; YCS, Yucatan continental slope.

Previous to XIXIMI 3 campaign, 1.7 mL micro-centrifuge tubes were filled with 5 mL of sucrose buffer 25% w/v, in order to preserve the DNA of the organisms present in the collected sediment. In each station, 1 cc sterile syringes (needleless) were introduced into one of the cores (Figure 6-A) to collect sediment from the top 5 cm (depth A: 0-5 cm). Five samples were obtained (subsamples) from each station. For XIXIMI 4, 5 and 6 campaigns, five samples (sub-samples) were taken from one of the halves of a core by using a 10 mL syringe. Each sub-sample was divided in two (depth A: 0-5 cm and depth B: 5-10 cm) and preserved in 15 mL tubes containing 5 mL of sucrose buffer 25% w/v, obtaining a total of 10 samples per station. After sampling, the tubes were stored at -20°C (Figure 6-B).

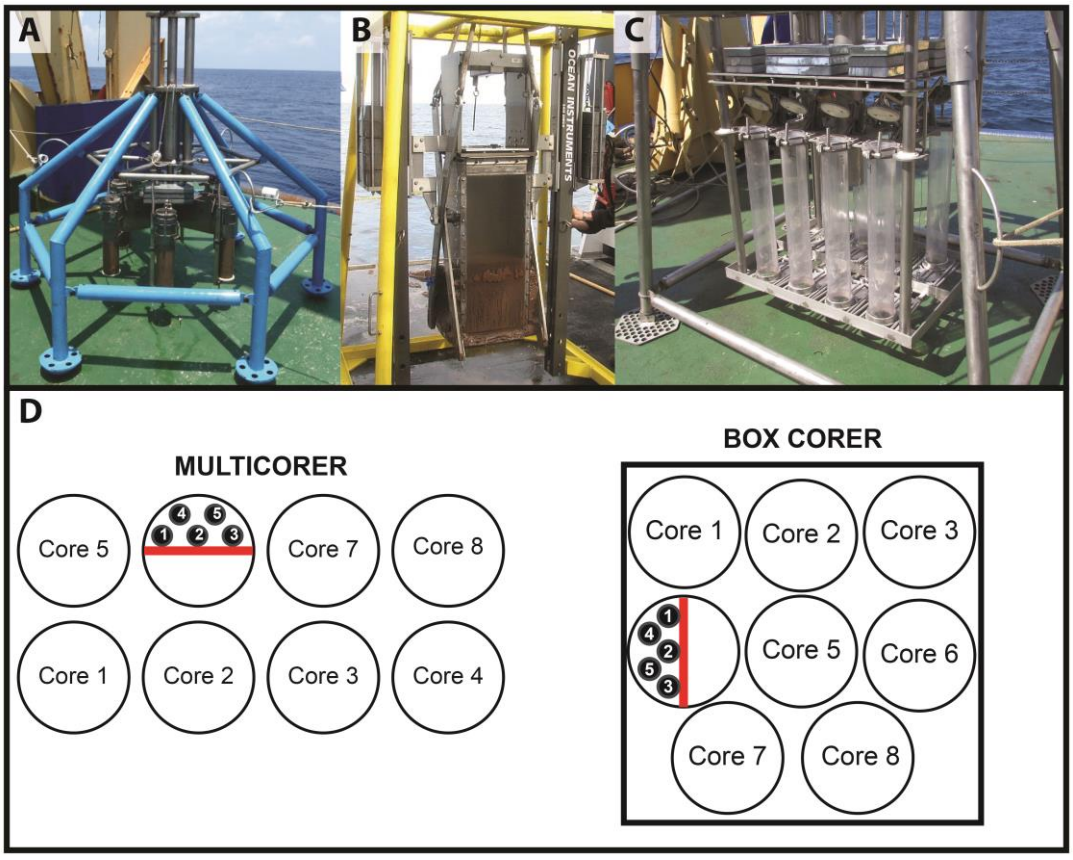

Figure 6. Sampling strategy. (A) Multicorer used during the XIXIMI 3 campaign to sample the sediments. (B) Box corer used in sample collection in XIXIMI 4, 5 and 6 campaigns. (C) Multicorer used to collect sediment samples from XIXIMI 4, 5 and 6. (D) Scheme showing subsampling of half of the core used in multicorer and box corer.

2.2. Laboratory processing

Samples from all campaigns were centrifuged (Eppendorf 5415D centrifuge, Hamburg, Germany) at 13,000 rpm for 1 min in order to eliminate the sucrose buffer and water.

The DNA from all the samples processed was extracted by using a Power Soil DNA Extraction Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA), but with certain modifications, which are explained in the sections below.

2.2.1. STRATEGY 1. Molecular processing of sediment samples from XIXIMI 3, 4 and 5 campaigns

From XIXIMI 4 campaign, three sub-samples were selected from depth A (0-5 cm) and depth B (5-10 cm), and for XIXIMI 3 and 5 campaigns, three sub-samples were selected from depth A.

DNA extraction of samples from XIXIMI 4 was performed following the supplier instructions, and using 0.25 gr of sediment, while DNA extraction from XIXIMI 3 and 5 campaigns were performed using a modified protocol of the kit which included, as first step, the addition of 200 µl of phenol:chloroform:isoamyl alcohol (Tips and FAQ portion of MoBio website), in order to improve the extraction. The concentration and purity of DNA was estimated by using a NanoDrop (Thermo Fisher Scientific Inc., Model: Lite NanoDrop, Waltham, MA, USA).

Samples were sent to Mr DNA (Shallowater, Texas) for the amplification and sequencing of the ITS region. The ITS region was amplified using primers ITS1-F (5´-CTTGGTCATTTAGAGGAAGTAA-3´; Gardes & Bruns, 1993) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´; White *et al*., 1990) and the sequencing was conducted by employing a 300-bp paired-end on an Illumina MiSeq (Illumina, Inc.). A total of 60 samples from XIXIMI 4 were sent out for sequencing. For XIXIMI 3 and 5, 30 samples for each campaign were sent out for sequencing together.

2.2.2. STRATEGY 2. Molecular processing of sediment samples from XIXIMI 4, 5 and 6 campaigns

This strategy was implemented when the output from the strategy 1 did not give reliable results (See chapter 3).

During the preparation of sampling material, different negative controls were included, which consisted in sterile microcentrifuge tubes containing 1 mL of sterile H_2O HPLC, that were opened during different preparation steps: during syringe preparation; during buffer sucrose filtration and tubes preparation. An extra control was included during DNA extraction.

In order to have a qualitative and quantitative control, a mock community was included. This positive control comprised genomic DNA from 21 different fungal strains obtained from the Microbiology Department of CICESE (Table 1). Fungal cultures were grown in plates on different media and temperatures (Table 1), during 1 to 2 weeks.

Table 2. Mock Community composition.

Strain	Media	Growth temperature (°C)
Aspergillus nidulans	PDA	30
Botrytis cinerea	PDA	25
Candida albicans	YPD	37
Candida orthosilopsis	YPD	37
Coprinopsis cinerea	YMG + Tryptophan	30
Fusarium oxysporum	PDA	25
Lasiodiplodia theobromae	PDA	25
Mucor rouxii	SDA	25
Neurospora crassa	MMV	30
Penicillium	PDA	30
Phomopsis	PDA	25
Saccharomyces cerevisiae	YPD	30
Schizosacharomyces pombe	YPD	30
Setophoma	PDA	25
Sordaria macrospora	PDA	30
Trichoderma asperellum	PDA	25
Trichoderma atroviridae	PDA	25
Trichoderma harzianum	PDA	25
Unknown fungi - 1	PDA	30
Unknown fungi - 2	PDA	30
Ustilago maydis	YPD	25

YPD: Yeast Extract-Peptone-Dextrose. YMG: Yeast Extract-Malt Extract-Glucose. MMV: Vogel minimal medium. SDA: Sabouraud-Dextrose-Agar. PDA: Potato-Dextrose-Agar. Fungal strains were obtained from the Microbiology Department in CICESE.

The mycelium was recovered from the plate using a sterile spatula, and then it was grinded in a mortar and pestle with liquid nitrogen. The genomic DNA was extracted using 0.25 gr of mycelium powder and the Power Soil DNA Extraction Kit, following supplier instructions. The recovered DNA was measured using a NanoDrop (Thermo Fisher Scientific Inc., Model: Lite NanoDrop, Waltham, MA, USA).

In order to obtain the complete sequence of the ITS1 region from each fungus present in the mock community control, a PCR was performed from 10 ng/µl of extracted genomic DNA and using the universal primers ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3'). *Neurospora crassa* and HPLC water were used as positive and negative control, respectively. Thermal cycling consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 40 sec, 58°C for 45 sec and 72°C for 30 sec, and a final denaturation step at 72°C for 10 min. PCR products were confirmed by 1% agarose gel electrophoresis and purified using the QIAquick Gel Extraction Kit, following the supplier instructions. The purified PCR products were sent out for sequencing to Eton Biosciences Inc. (San Diego, CA, USA). Once the sequences were received, these were processed with the BioEdit program (Hall, 1999). The sequences were submitted to a BLAST analysis in the NCBI database to confirm the species identification. Only the species whose molecular identification matched the Laboratory fungal record were used to construct the mock community control. *Sordaria macrospora*, *Aspergillus nidulans*, *Trichoderma harzianum* and *Setophoma* were excluded because no clear identification resulted from BLASTN analysis.

From each campaign (XIXIMI 4, 5 and 6), three sub-samples were selected by station, only taking into account the depth A (0-5 cm). The XIXIMI 4 sub-samples analyzed in the Strategy 2, were the same as the analyzed in the Strategy 1.

For two days, the selected sub-samples samples were lyophilized using a FreeZone 2.5 Liter Benchtop Freeze Dryer (Labconco Corporation, Kansas City, MO, USA). The genomic DNA was extracted using only 0.15 g of sediment, via a modified protocol of the Power Soil DNA Extraction Kit which included, as first step, the addition of 100 µL of phenol:chloroform:isoamyl alcohol and a 2 minutes incubation, followed by 30 sec vortex.

The concentration of DNA was estimated in a Qubit 4 Fluorometer using a high sensitivity dsDNA Qubit Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA).

For the amplification of the entire ITS1 region from environmental DNA extracted from the sediment samples, a touchdown PCR (Korbie and Mattick, 2008) was carried out in 50 µL PCR reactions

(Table 3). To the primer set used in these PCR reactions, adapters for Illumina sequencing were added in the 5' end of each primer (Figure 7). The PCR consisted in an initial denaturation step at 95°C for 10 min, followed by 10 cycles of 94°C for 45 sec, 65°C-55°C for 45 sec (the annealing temperature decrease 1°C each cycle) and 72°C for 75 sec, followed by 27 cycles of 95°C for 45 sec, 58°C for 45 sec and 72°C for 30 sec, and a final denaturation step at 72°C for 10 min. PCR products were confirmed by running 5 µL of each reaction on a 1% agarose gel electrophoresis (Figure 8).

Table 3. Components for PCR1 reactions.

PCR reagent	Final concentration in the reaction
Genomic DNA	10 ng
Colorless GoTaq Reaction Buffer	1X
$MgCl_2$	3 mM
Nucleotide mix	0.8 mM
ITS1F (Forward primer)	0.3 µM
ITS2 (Reverse primer)	0.3 µM
GoTaq® DNA Polymerase*	0.03 u
BSA	0.8 mg μL^{-1}
HPLC water	-----

*Promega, Radnor, PA, USA

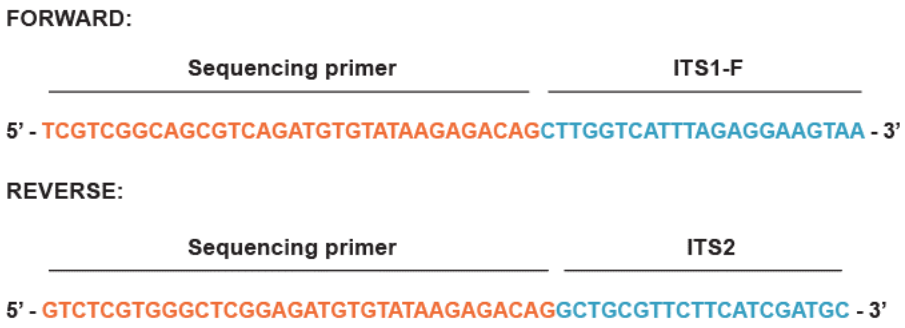

Figure 7. Set of primers modified for the amplification of the ITS1 region. ITS1-F and ITS2 represent the fungal universal primers.

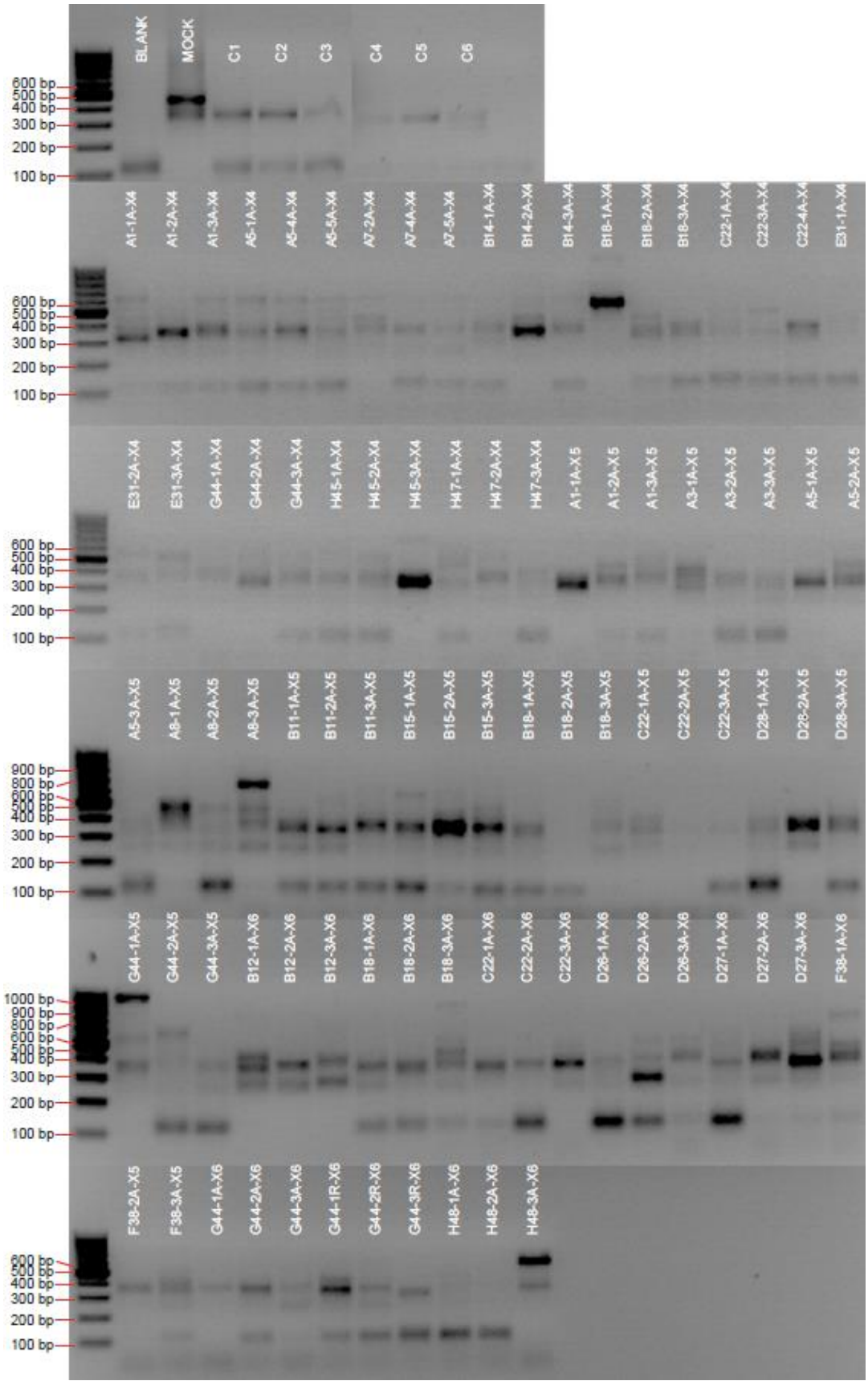

Figure 8. PCR products from ITS 1 region of rDNA from genomic DNA of sediment samples.

From the remaining 45 µL PCR reaction, the PCR products were purified using the Just-a-plate™ 96 PCR purification kit (Charm Biotech San Diego, CA, USA) following supplier instructions. The objective of this cleaning step was to remove any artificial PCR product observed in the PCR 1 as well as the remaining PCR components in the reaction. PCR products were confirmed by running 5 µL of the reaction on a 1% agarose gel electrophoresis (Figure 10).

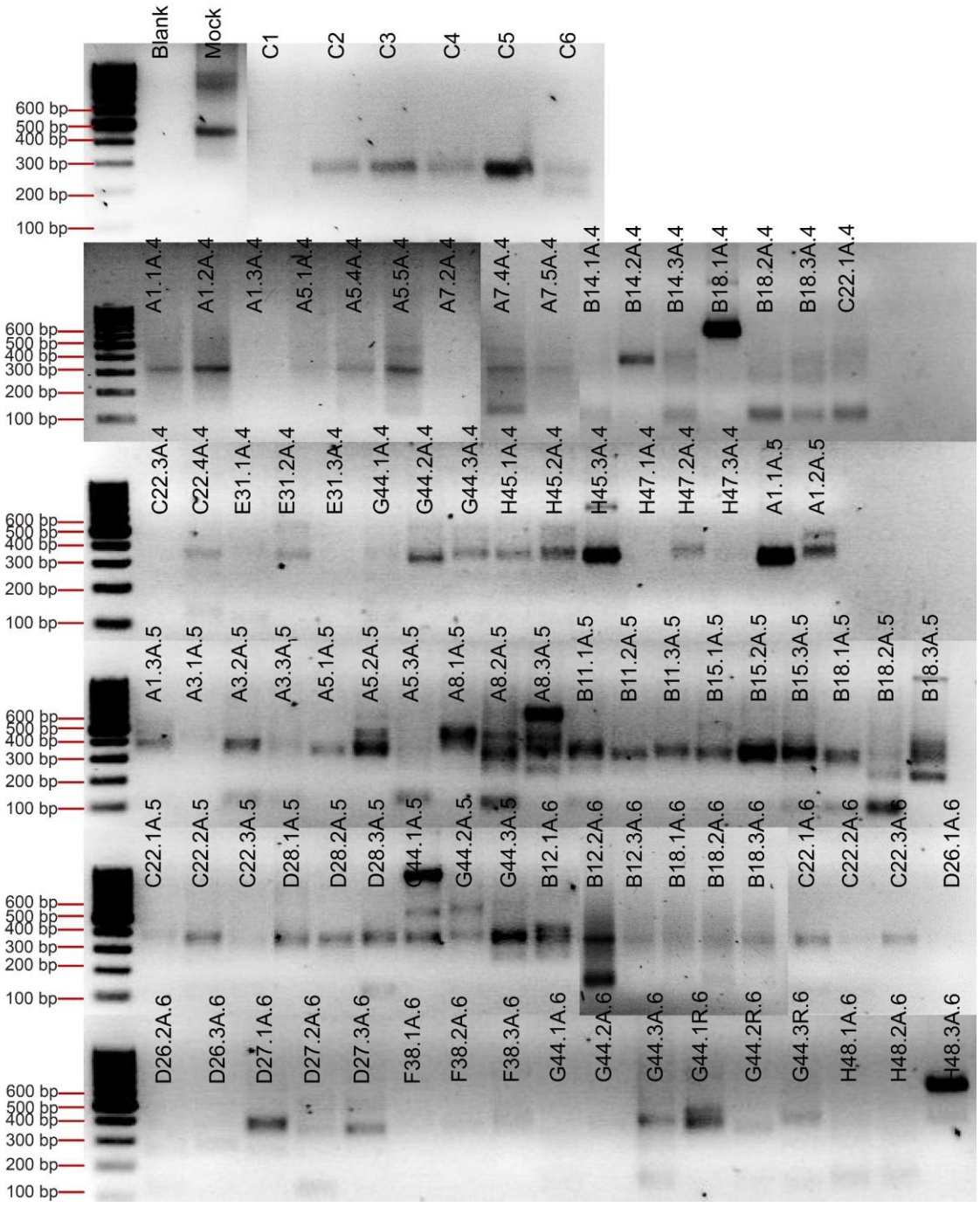

Figure 9. Purified PCR products from PCR 1.

To process all the samples in the same sequencing run, a second PCR was needed to perform the indexing of all the PCR products. Before this step, an assignation of a specific pair of indexes for each sample was made (Annex 4). These indexes were previously synthetized including the Illumina adapters and the sequencing primer (Figure 10).

Figure 10. Design of the set of Illumina adaptors and indexes – PCR 2. The sequencing primer represents the sequence from the PCR 1.

Once all the samples had a pair of indexes assigned, the PCR reactions were prepared. This PCR was carried out on 25 µL (Table 4). The PCR 2 reaction consisted in an initial denaturation step of 95°C for 3 min, followed by 8 cycles of 95°C for 30 sec, 61°C for 30 sec and 72°C for 30 sec, and a final extension step at 72°C for 5 min. The PCR products were visualized by loading 5 µL of the reaction in a 1% agarose gel (Figure 11).

Table 4. Components for PCR 2 reactions.

PCR component	Concentration	Volume
Genomic DNA	-----	1 µL
Colorless GoTaq Reaction Buffer	5X	6 µL
$MgCl_2$	25 mM	3.6 µL
Nucleotide mix	10 mM	2.4 µL
ITS1F (Forward primer)	10 µM	0.6 µL
ITS2 (Reverse primer)	10 µM	0.6 µL
GoTaq® DNA Polymerase*	5 u	0.25 µL
BSA	10 mg μL^{-1}	2.4 µL
HPLC water	-----	8.15 µL

*Promega, Radnor, PA, USA

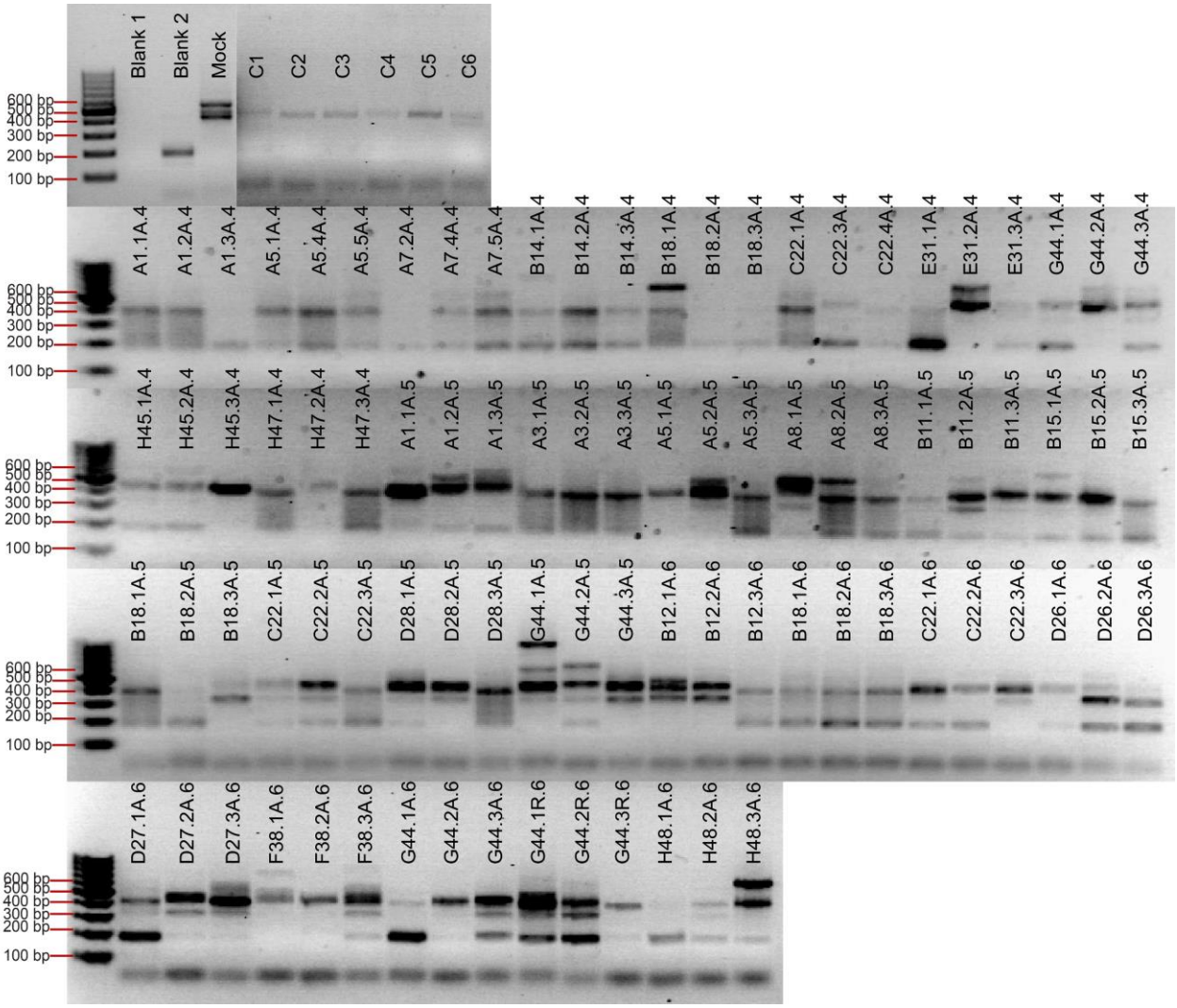

Figure 11. Indexed PCR products from PCR 2.

In order to obtain equal amounts of PCR products and a final purification of the PCR reactions, the remaining volume of each sample was processed through SequalPrep™ Normalization Plate Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). All the PCR products were quantified using a Qubit 4 Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

During the clustering process in the Illumina platform, the shorter fragments (in a mixture of fragments) are preferentially amplified over the larger fragments, so the samples with similar fragment sizes were pooled together. The concentrations of the pooled samples were measured using a Qubit 4 Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The DNA concentration of the pooled samples was measured and a final pool was constructed with a concentration of 1,300 pM.

Following the instructions of the MiSeq® Reagent Kit v3 of 600 cycles for a 300 bp paired-end sequencing, (Illumina, San Diego, CA, USA), the pool denaturalization step was performed as follows:

a) From the pool, 10 µL at 1300 pM were taken and mixed with 10 µL of NaOH (0.2 N) and then incubated for 5 minutes. The final concentration of the reaction was 650 pM.

b) Twenty µL of Tris-HCL were added to the 20 µL of the reaction and incubated for 5 min. The final concentration of the reaction was 325 pM.

c) To obtain a pool of 700 µL final volume at 5.5 pM, from the pool in b), 11.85 µL were taken and 688.15 µL of HT1 (Illumina kit provided) were added.

Also, the dilution and denaturation of PhiX[2] was carried out as follows:

a) To 1 µL de PhiX (10,000 pM), 1.5 µL of H_2O were added, to obtain a concentration of 4000 pM in 2.5 µL of PhiX.

b) To those 2.5 µL from step a), 2.5 µL of NaOH (0.2 N) were added, to obtain a final volume of 5 µL to 2000 pM.

c) A total of 495 µL of HT1 were added to the 5 µL from step b), obtaining a final volume of 500 µL of PhiX at 20 pM.

d) Lastly, the PhiX library was diluted again adding 507.5 µL of HT1, to obtain a final concentration of 5.5 pM in 700 µL final volume.

The sequencing was performed on Illumina MiSeq platform from the Division of Experimental and Applied Biology the Centro de Investigación y de Educación Superior de Ensenada (CICESE).

[2] PhiX is used as control during sequencing to reduce library variations. PhiX represents a library from a well-characterized genome.

2.3. Bioinformatic and statistical analyses

2.3.1. STRATEGY 1. Operational Taxonomic Units (OTUs) and Amplicon Sequence Variants (ASVs)

For each sequencing run, forward (*R1.fastq*) and reverse (and *R2.fastq*) files corresponding to the paired-end data, were provided by the sequencing service.

For the bioinformatic analysis, only *R1.fastq* files were used, this was due to the absence of complementarity of the forward and reverse reads.

2.3.1.1. Operational Taxonomic Units (OTUs) assignment and troubleshooting

The quality of the reads in the *R1.fastq* was analyzed using FastQC program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (for details see Annex 1).

The reads were filtered and processed using QIIME 1 (Caporaso et al., 2010) as follows:

- The XIXIMI 3, 4 and 5 reads were analyzed separately up to the quality filtering step, because the barcode used from some samples of XIXIMI 4 was the same than the one used for some samples from XIXIMI 3 and 5.
- The reads where merged into a single file in order to compare across campaigns and stations.
- The *.fastq* file were demultiplexed by barcode.
- Only sequences with a high quality were chosen (quality score =/>26) and the reads orientations were checked. Other quality scores cut offs such as 28 and 30 were tested[3].

[3] Some changes in the quality control and clustering of the reads were tested, in order to mend the number of operational taxonomic units obtained using this pipeline.

- UCLUST (Edgar, 2010), USEARCH (Edgar, 2010) and RDP (Wang et al., 2007) algorithms were tested[2] at a 97% identity threshold to cluster sequences into Operational Taxonomic Units (OTUs).

- The taxonomic assignment (to species level) was carried out using the BLAST algorithm (Altschul et al., 1990) against the UNITE fungal database (Abarenkov et al., 2010). The OTUs that resulted on a "No Blast Hit" assignation, were queried using a nucleotide BLAST and those that were not identified as fungi, were eliminated.

- To establish differences between samples, a frequency OTU table was obtained and singletons were discarded.

An additional pipeline called UPARSE was tested in order to compare across the results obtained by the different pipelines used (Edgar, 2013).

2.3.1.2. Amplicon Sequence Variants (alternative pipeline)

The *R1.fastq* and *R2.fastq* files from each sequencing run[4] were divided by sample ID (according to barcode sequence file) using the Fastq Processor (Mr DNA, Shallowater, Texas).

The quality of the reads from each sample was analyzed using the FastQC program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (for details see Annex 2).

The reads were filtered and processed using the DADA2 pipeline (Callahan et al., 2016) in R program v.4.0 (R Development Core Team, 2008) based on the open-source Bioconductor project (Huber et al., 2015):

- Due to the amplicon size (>600 bp), it was not possible to pair-end the forward and reverse files. Only the *R1.fastq* (forward) files were used for the analysis.

[4] Two sequencing runs from XIXIMI 3 and 5 were received, which means that some samples from the first sequencing were re-sequenced. To address this issue, equal samples in both runs were compared with FastQC and the sample with the best quality profile was selected to continue with the analysis.

- The files from all sequencing runs were processed together. To analyze differences across stations and campaigns, only the files from XIXIMI 4 campaign corresponding to 0-5 cm were used to compare with XIXIMI 3 and 5 files. An extra analysis with XIXIMI 4 files was made to compare between depths 0-5 cm and 5-10 cm.

- The quality profile of each file was examined in order to establish the length of the reads. The DADA2 pipeline requires all reads to be the same length. The established length was 240 bp. The quality filtering of the reads was established by an Expected Error of 1.

- A dereplication function was applied to the reads, where identical sequences were combined into unique sequences and a corresponding abundance.

- The dada function was applied to the sequences in order to remove all the errors and identify the real biological unique sequences. This function is based in the assumption that biological sequences are more common and more observed than error sequences. After this step, the unique sequences are called Amplicon Sequence Variants (ASV).

- A removal chimeras step was applied. The chimera detection was based on the comparison of each Sequence Variant against others in the same dataset, removing those than can be reproduced by the joint of two sequences.

- The taxonomic assignment of each ASV was carried out using the RDP algorithm (Wang et al., 2007) against the UNITE fungal database (Abarenkov et al., 2010).

- To establish differences between samples, a frequency ASV table was obtained.

2.3.1.3. STRATEGY 1. Statistical analysis

Differences on the fungal community based on Shannon Wiener diversity index and richness (Chao 1) were assessed using the Phyloseq package (McMurdie and Holmes, 2013) in R program v 3.4.0. ANOVA tests were applied in order to establish statistical differences by sub-samples and stations. A rarefaction analysis was carried out using Past v 2.17 (Hammer et al., 2001).

Profiles of relative abundances of the ITS sequences per sample were obtained using the BiodiversityR package (Kindt and Coe, 2005) in R program v 3.4.0.

To analyze the similarity of the fungal composition among stations and campaigns a Non-metric Multidimensional Scaling (NMDS) analysis was performed using Vegan package (Oksanen et al., 2013) and an analysis of variance (ANOVA) was conducted using the ANOSIM function with the Vegan package (Oksanen et al., 2013). Both analyses were carried out in R v.3.01 (R Development Core Team, 2008).

2.3.2. STRATEGY 2. Operational Taxonomic Units (OTUs)

From the sequencing run, *R1.fastq* and *R2.fastq* files were obtained for each sample (demultiplexed files). The demultiplexed files were processed as follows:

- To remove portions of DNA sequences that did not originate from ITS1 (18S and 5.8S), the resulting demultiplexed fastq files were processed using ITSx 1.0.11 (Bengtsson-Palme et al., 2013). This step was carried out using the open source script fastq-from-ITSx (Darcy, 2018; https://github.com/darcyj/fastq-from-ITSx) because the original ITSx program do not accept the fastq files as input format.

- The fastq files containing only ITS1 sequences were subjected to quality filtering, using an expected number of errors of 0.5 and an ambiguous bases threshold of 0.

- A chimera analysis was performed using the open source VSEARCH tool (Rognes et al., 2016) and as reference sequences, the UCHIME reference dataset v28.06.2017 (Nilsson et al., 2015) was used.

- The sequences were clustered into Operational Taxonomic Units (OTUs) with a 97% of identity threshold, using the UCLUST algorithm (Edgar, 2010). OTUs represented by one sequence (singletons) were removed.

- From each OTU, the longest sequence was selected as representative, and used to assign the taxonomy. The taxonomic assignment was carried out using the BLAST algorithm (Altschul et al.,

1990), against the NCBI nucleotide database. Entrez limits were applied to our database in order to minimize the number of unculturable gut fungus (Neocallimastigomycota) and to remove all bacteria.

- The OTU table containing the frequency of each OTU and the taxonomic identification was constructed: to analyze the sub-samples, the OTU was kept as is, while to analyze differences among stations, the sequences from each sub-sample were pooled by station. From OTU formation until OTU frequency table, was conducted using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010).

- The OTUs corresponding to unidentified Fungi were submitted to a BLASTN search in the NCBI database, in order to improve the taxonomy assignment; this assignment was based on the best Blastn match. For the final frequency table, only OTUs identified as fungi were kept.

To diminish the presence of contaminant sequences, the data was treated as follow:

- For OTUs present both in controls and samples, the proportion of the number of sequences in the controls and in the samples was calculated (Nguyen et al., 2015), and those contaminants representing more than 1% of the total sequences in the samples were removed. The OTUs that were first removed were those on which the sequence counts in the controls was higher than in the samples, since the proportion would tend to zero.

- The OTUs identified as *Neurospora crassa* (known as laboratory contaminant) were removed.

At the end of this processing, we obtained an OTU table containing the frequencies of each OTU. This table was used to perform statistical analyses.

2.3.2.1. STRATEGY 2. Statistical analyses

The alpha diversity was estimated by calculating the number of observed OTUs and the Shannon-Wiener diversity index for each sub-sample and station. The index was calculated using the Phyloseq package v.1.22.3 (McMurdie and Holmes, 2013).

To visualize how much the stations are connected in terms of OTUs, the number of shared and unique (OTUs present only in one station) OTUs among stations was calculated using the *make_otu_network.py* function in QIIME and then visualized as OTU network with Cytoscape v3.7.0 (Shannon et al., 2003).

To identify patterns of community structure among sub-samples and stations, a bar plot based on the abundance of fungal classes was constructed using the *summarize_taxa.py* function in QIIME. The stations bar plot was coupled with a hierarchical dendrogram computed using Ward's clustering algorithm to matrices of Euclidean distances. The dendrogram was computed using the *hclust* function from Vegan package (Oksanen et al., 2013).

Multiple linear regressions were performed to identify the relationship between richness/ diversity indices, and geographic location and sediment physicochemical properties.

The OTU frequency table was transformed into a presence/absence table and an OTU table normalized by proportion (Weiss et al., 2017). To test which table should be used for the statistical analyses, both were submitted first to *rankindex* function of Vegan package to select the best dissimilarity index that fits better to the data. Then, the *vegdist* function was used to compute the distances. To analyze the similarity of the fungal composition among stations using both tables, a Non-metric Multidimensional Scaling (NMDS) was performed using the *metaMDS* function, establishing 3-dimension ordination; the NMDS results were visualized in a 2-dimension plot and stressplots with NMDS values were constructed in order to visualize the correlation based on the stress and the goodness of fit. Finally, a Procrustes based test using the *protest* was used in order to select the most appropriate dataset for our analysis.

Once the table with the best fit was selected, the stations were categorized by geographic location (latitude and longitude), depth, region, and type of core (*i.e.* box corer, multicorer; Table 1), to detect differences or patterns among groups. The categorical variables were managed as follows: for variables such as latitude and longitude, the geographic location was grouped to the nearest degree (e.g. 25.00892 degree-north to 25; 265.98224 degree-east to 265); similarly, for depth, the samples were grouped every 500 m. To test statistical meaning of categorical variables, a permutational multivariate analysis of variance (PERMANOVA) was conducted using the *adonis* function, followed by Pairwise comparisons and p-adjusted values computed by Bonferroni correction using *pairwise.adonis* function from pairwiseAdonis package (Martinez Arbizu, 2019).

In order to compare beta diversity of the fungal communities taking into account the categorical variables explained above, a beta dispersion analysis using the *betadisper* function from Betapart package was conducted (Baselga and L. Orme, 2012). The Betadisper analysis was based on the distance given by the *vegdist* function, and significance was computed by ANOVA test using the *anova* function and p-adjusted values were computed by Bonferroni correction using *p.adjust* function. Pairwise comparisons were made among groups using *pairwise.t.test* function.

To test whether the type or corer (box corer or multicorer) had an effect on the OTU composition, the stations were arranged by type of corer, and NMDS and beta dispersion analyses were carried out as explained above. Moreover, to test whether the corer type could be masking a correlation between the fungal community and sediment physicochemical properties, a Mantel test was carried out using the Euclidean distance matrices of the numerical variables and community data (presence/absence OTU table).

A distance decay analysis was performed in order to determine the significance of the relationship among community dissimilarity and geographic distance, followed by a Mantel test performed to corroborate the significance of the relationship. For this analysis, Euclidean distance matrixes were calculated for the OTU table, the geographic location (latitude and longitude) and a distance decay analysis.

To detect the potential interactions between OTU fungal dataset (presence/absence OTU table) and the geographic location and sediment physicochemical properties as variables, a Canonical Correspondence Analysis (CCA) was undertaken using the *cca* function. To select the better model for the CCA analysis, the *ordiestep* function was used, which employ using a stepwise linear regression, and variable selection using the Akaike Information Criterion (Ripley et al., 2015). The significance of the model was tested by an ANOVA with the *anova* function, followed by the calculation of p-adjusted values, which were computed by Bonferroni correction using *p.adjust* function. The OTU table used in the analysis did not include station G44R.6, because there is not sediment characteristic data available for this replicate station. B18 stations were not included either, because previous research had demonstrated that this sampling site in the Yucatan slope is an outlier in comparison with other areas across the GoM (Díaz-Asencio et al., 2019).

The *hclust*, *rankindex*, *vegdist*, *protest*, *metaMDS*, *adonis*, *mantel*, *cca*, *ordistep* functions were performed with the Vegan package (Oksanen et al., 2013). All the statistical analyses were carried out in RStudio v. 1.1.456 (R Development Core Team, 2008).

Chapter 3. Results

3.1. STRATEGY 1. Results

3.1.1. DNA concentration

Values of genomic DNA concentration for all sub-samples from each station were averaged by depth (A or B) for XIXIMI 4 campaign, and by station for XIXIMI 3 and 5 campaigns.

For samples from the XIXIMI 4 campaign, the genomic DNA concentrations at different depths (A or B) were almost equal on each station; concentrations from depth A ranged from 8.74 ng/µl (A7.A. 4) to 29.5 ng/µl (G44.A.4), and from depth B ranged from 6.7 ng/µl (A7.B.4) to 20.8 ng/µl (G44.B.4) (Annex 3). A variance analysis (ANOVA) indicated that no differences were found between depths from the same station ($P>0.05$), but across stations, the G44.4 station presented significant differences ($P<0.05$) with almost all the stations, except with A1.4, while A1.4 only displayed significant differences ($P<0.05$) with C22.4, B14.4 and A7.4 samples (Figure 12-A). For XIXIMI 3 samples, the values ranged from 8.5 ng/µl (B18.3) to 21.4 ng/µl (H45.3), and no significant differences were found across stations ($P>0.05$; Figure 9-B; Annex 3). For XIXIMI 5 campaign, the values of DNA concentration ranged from 4.16 ng/µl (B14.5) to 15.3 ng/µl (G44.5); the G44.5 station displayed significant differences with TS1.5, A5.5, B15.5, C22.5 and D28.5 stations ($P<0.05$; Figure 12-C; Annex 3).

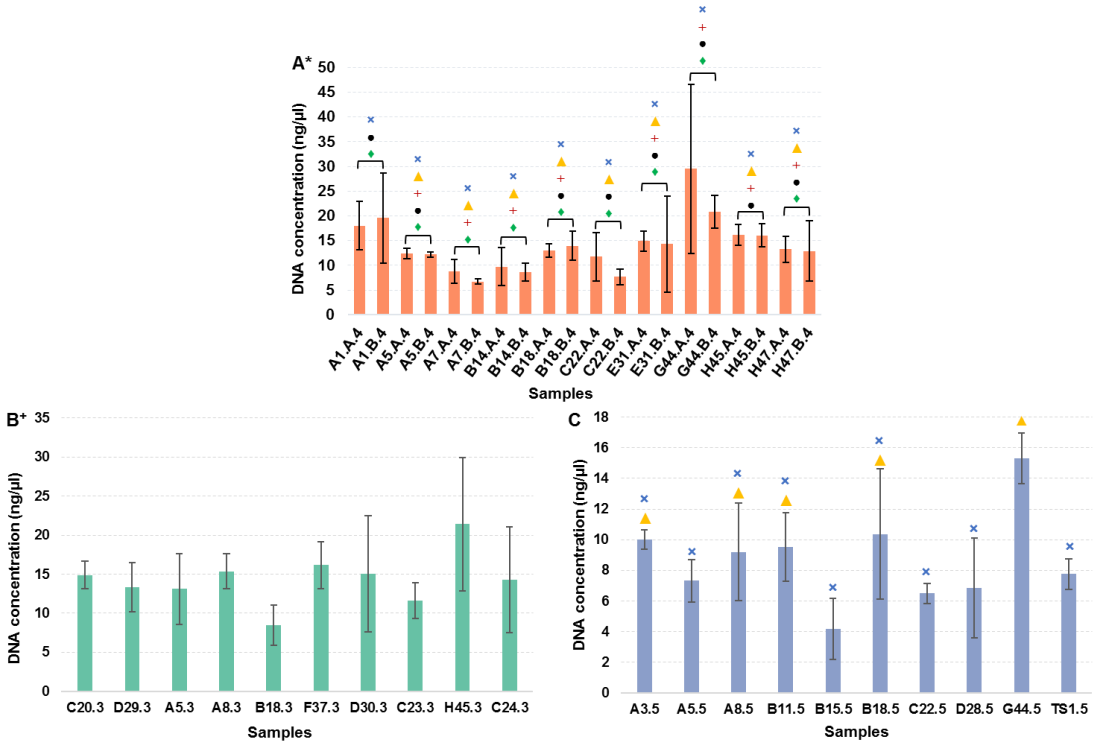

Figure 12. Average concentration of genomic DNA of each station from the different campaigns. Bars correspond to standard deviation bars. (A) Depth 0-5 cm. (B) Depth 5-10 cm. Statistical differences are represented by symbols between stations. *****No differences were found between depths on the same station. Symbols correspond to differences across stations. **+**No differences were found across stations.

3.1.2. Sequencing results, OTUs-ASVs and alpha diversity

From XIXIMI 3 samples, a total of 3,345,351 sequences were obtained and a total of 1,494,352 sequences remained after applying quality control. Station D30.3 had the highest number of sequences (102,962 sequences), while H45.3 station had the lowest number of sequences (19,381 sequences) (Table 5). From XIXIMI 4 samples, a total of 3,489,154 sequences were obtained, and after quality control processing, a total of 2,798,204 sequences remained. Overall, depth A had a higher number of sequences than depth B, with 1,783,513 and 1,705,641 sequences, respectively. Station H45.A.4 had the highest number of sequences (280,452 sequences), while A1.A.4 station had the lowest number of sequences (58,270sequences) (Table 5). And, from XIXIMI 5 campaign, a total of 2,988,904 sequences were obtained, and after quality control, 1,148,363 sequences remained. Station C22.5 had the highest number of sequences (192,372) and station A3.5 had the lowest number of sequences (52,518) (Table 5).

When number of OTUs were obtained using the UCLUST algorithm explained in methodology, a total of 649,803 OTUs were identified, the majority of them represented by only one (542,510 OTUs) or

two (36,003 OTUs) sequences, which means that the sequences were not clustering together, even at a 97% similarity threshold. To address this problem, different quality control settings were changed (quality cutoff from 28 to 25) and different clustering algorithms were tested (UCLUST, USEARCH and RDP), although still a higher number of OTUs were obtained (more than 300,000 OTUs). As a final test, the UPARSE pipeline was tested (Edgar, 2013), but a high number of OTUs represented by one sequence was still obtained (more than 300,000 OTUs). To overcome this problem, a pipeline called DADA2 was tested (Callahan et al., 2016), in which all the identical sequences are combined into a unique group: Amplicon Sequence Variant (ASV). The results presented below represent the analysis by ASVs.

A total of 5,869 ASVs were obtained from all stations belonging to depth A. A total of 2,421 ASVs were obtained for XIXIMI 3, with stations C20.3 and A5.3 presenting on average the highest number of ASVs (194.66 and 173.66, respectively), and C24.3 and C23.3 presenting on average the lowest number of ASVs (88.66 and 51.33, respectively) (Table 5). For XIXIMI 4, a total of 2,455 ASVs were obtained (depth A and B). Depth A, presented an average number of 132 ASVs, and depth B an average number of 114 ASVs; depths A and B shared a 99.2% of the ASVs. Stations H45.A.4 and G44.A.4 had in average the highest number of ASVs (191 and 170, respectively), while A1.A.4 and A7.B.4 had in average the lowest number of ASVs (76 and 68, respectively) (Table 5). The average number of ASVs varied between depths A and B: some stations, such as H45.A.4 had more ASVs than H45.B.4 station, and the A1.A.4 station had a lower number of OTUs than A1.B.4 station. For XIXIMI 5, a total of 2,362 ASVs were obtained. A8.5 and B11.5 presented in average the highest number of ASVs with 154 and 138 respectively, while A5.5 and TS1.5 had the lowest average value of ASVs (103 and 62, respectively) (Table 5).

Table 5. Richness and diversity indexes for the studied stations.

	Stations	Sequences (Before)•	Sequences (After)•	Obs. ASV▲	Chao 1▲	Shannon Wiener▲
X3	A5.3	363,844	147,810	173.66 ± 39.95	173.66 ± 39.95	2.81 ± 0.34
	A8.3	307,822	108,386	124.33 ± 20.42	124.33 ± 20.42	2.36 ± 0.39
	B18.3	550,162	210,324	152.33 ± 11.59	152.33 ± 11.59	2.27 ± 0.24
	C20.3	317,276	153,315	194.66 ± 34.93	194.66 ± 34.93	2.3 ± 0.23
	C23.3	299,032	160,888	51.33 ± 6.42	51.33 ± 6.42	2.56 ± 0.19
	C24.3	336,351	177,038	88.66 ± 8.38	88.66 ± 8.38	2.98 ± 0.57
	D29.3	428,487	226,618	104.66 ± 25.5	104.66 ± 25.5	2.68 ± 0.43
	D30.3	279,037	183,105	146.33 ± 24	146.33 ± 24	2.53 ± 0.18
	F37.3	156,144	59,412	118.66 ± 35.1	118.66 ± 35.1	2.65 ± 0.45
	H45.3	307,196	67,456	129.66 ± 92.39	129.66 ± 92.39	2.61 ± 0.04
X4	A1-A	70,958	58,270	76.33 ± 45	76.33 ± 45	2.65 ± 0.89
	A1-B	196,458	157,826	139 ± 37.58	139 ± 37.58	2.96 ± 0.21
	A5-A	187,739	150,231	122.66 ± 14.46	122.66 ± 14.46	2.9 ± 0.27
	A5-B	121,361	97,156	104.66 ± 25	104.66 ± 25	2.53 ± 0.46
	A7-A	124,698	101,549	86.33 ± 20.13	86.33 ± 20.13	2.27 ± 0.79
	A7-B	96,711	77,548	68 ± 16.7	68 ± 16.7	2.15 ± 0.43
	B14-A	221,294	182,733	112.66 ± 9.45	112.66 ± 9.45	1.71 ± 0.36
	B14-B	249,552	210,372	108.33 ± 86.11	108.33 ± 86.11	1.8 ± 0.47
	B18-A	156,887	105,327	167.33 ± 17.03	167.33 ± 17.03	2.88 ± 0.67
	B18-B	183,399	145,469	163.33 ± 29.5	163.33 ± 29.5	3.3 ± 0.28
	C22-A	177,006	140,606	97 ± 38.74	97 ± 38.74	2.47 ± 0.45
	C22-B	167,806	132,785	90.33 ± 7.02	90.33 ± 7.02	1.88 ± 0.74
	E31-A	238,747	195,312	150 ± 4.35	150 ± 4.35	2.92 ± 0.46
	E31-B	151,520	126,431	79.33 ± 34.55	79.33 ± 34.55	2.03 ± 0.11
	G44-A	148,037	120,590	170 ± 18.73	170 ± 18.73	3.58 ± 0.14
	G44-B	209,403	170,135	143.66 ± 76.46	143.66 ± 76.46	3.15 ± 0.28
	H45-A	334,390	280,452	191.33 ± 38.21	191.33 ± 38.21	2.75 ± 1.02
	H45-B	179,817	143,969	122 ± 19.15	122 ± 19.15	2.78 ± 0.72
	H47-A	123,757	84,182	152 ± 17.34	152 ± 17.34	3.46 ± 0.4
	H47-B	149,614	117,261	122 ± 39.68	122 ± 39.68	3.2 ± 0.41
X5	A3.5	219,550	52,518	107 ± 62.35	107 ± 62.35	2.14 ± 0.48
	A5.5	305,957	103,469	103.33 ± 40.52	103.33 ± 40.52	1.72 ± 0.95
	A8.5	373,285	112,958	154.66 ± 55.94	154.66 ± 55.94	2.62 ± 0.27
	B11.5	351,134	98,710	138 ± 24.06	138 ± 24.06	1.67 ± 0.41
	B15.5	400,702	186,810	109.66 ± 44.06	109.66 ± 44.06	2.04 ± 0.25
	B18.5	245,132	76,189	128 ± 29.86	128 ± 29.86	2.62 ± 0.3
	C22.5	336,821	192,372	113.66 ± 50.93	113.66 ± 50.93	2.09 ± 0.78
	D28.5	417,349	172,692	133.66 ± 12.66	133.66 ± 12.66	2.09 ± 0.67
	G44.5	199,278	63,788	134.66 ± 94.11	134.66 ± 94.11	2.36 ± 0.81
	TS1.5	139,696	88,857	62 ± 29.05	62 ± 29.05	2.03 ± 0.18

• Values represent the sum of sub-samples by depth A (0-5 cm) and B (5-10 cm) of the XIXIMI 4 campaign and the sum of sub-samples by station of the XIXIMI 3 and 5 campaigns. ▲Values represent the average of sub-samples by depth A (0-5 cm) and B (5-10 cm) of the XIXIMI 4 campaign and the average of sub-samples by station of the XIXIMI 3 and 5 campaigns.

All the stations presented the same number of observed richness and Chao 1 index values (Table 5). The rarefaction curves showed that all XIXIMI 3 stations reach the plateau (Figure 13-A). The same was observed for rarefaction curves of samples from depth A and B of XIXIMI 4 campaign, which all reached the plateau (Figure 13-B).

Among all the campaigns, the highest values of Shannon-Wiener diversity index were observed for G44.4 and H47.4 stations (Table 5). For XIXIMI 3, Shannon-Wiener index indicated that sample C24.3 presented on average the highest diversity value of 2.98, while samples C20.3 and B18.3 presented on average the lowest diversity values (2.3 and 2.27, respectively) (Table 5). For XIXIMI 5, this index indicated that station A8.5 presented the highest diversity value (2.62) and A5.5 and B11.5 had the lowest diversity values (1.72 and 1.67, respectively) (Table 5).

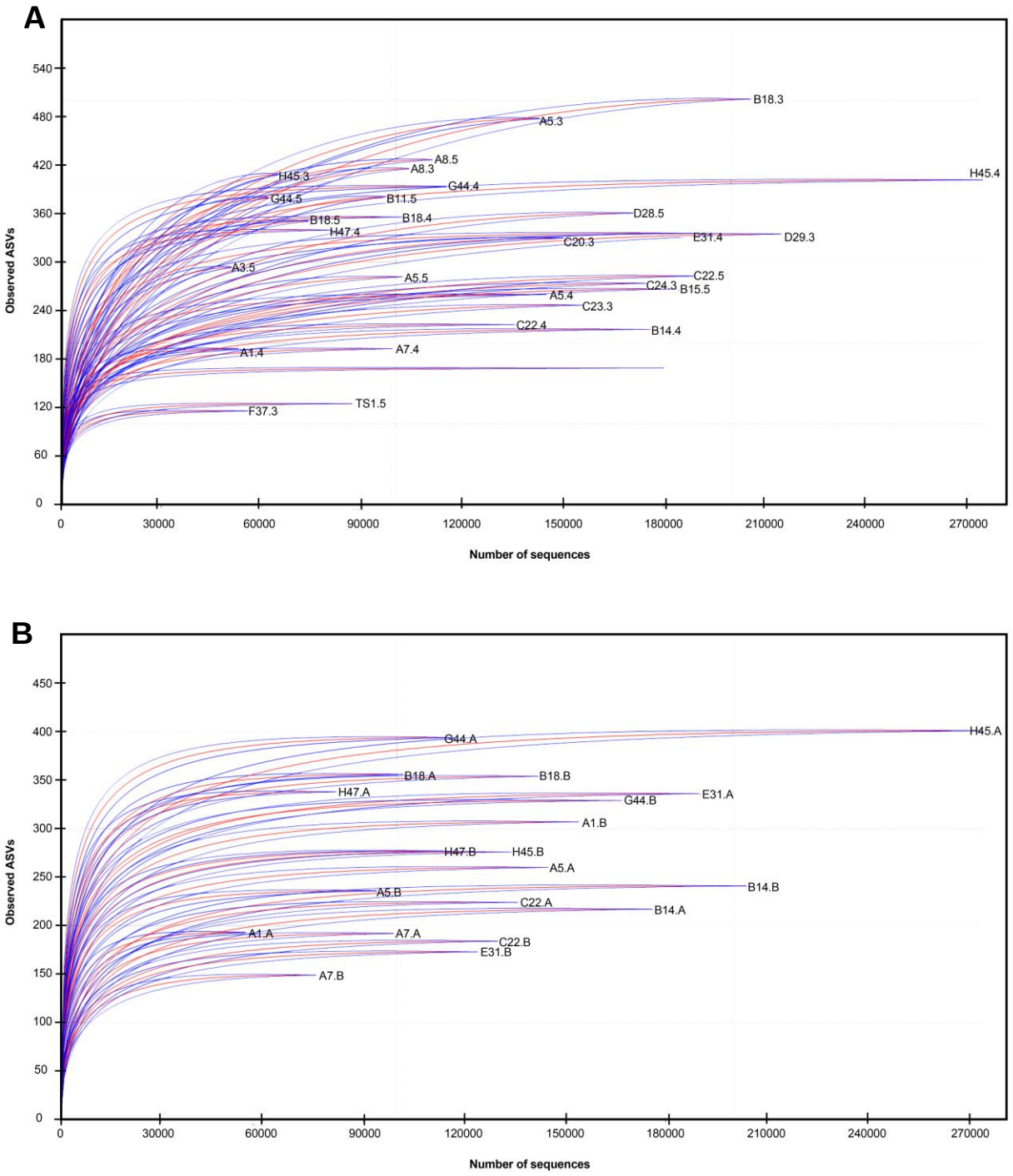

Figure 13. Rarefaction curves describing the observed fungal richness across stations. (A) The graph displays the number of Amplicon Sequence Variants (ASVs) as a function of the number of sequences obtained across all stations in all XIXIMI campaigns; for the comparison across campaigns, only depth A from XIXIMI 4 campaign was taken into account. (B) The graph displays the number of Amplicon Sequence Variants (ASVs) as a function of the number of sequences obtained in depth A and B from XIXIMI 4 campaign. Blue lines represent the Standard error.

3.1.3. Taxonomic composition

From the 5,869 ASVs, a total of 5,664 ASVs belonged to Fungi, 59 to Animalia, Chromist and Protista, and 146 were not assigned. Four phyla were identified: Ascomycota, Basidiomycota, Chytridiomycota and Mucoromycota. From a total of 122 genera identified, 83 belonged to Ascomycota, 36 to Basidiomycota, one to Chytridiomycota and two to Mucoromycota. Among sub-samples within the same station, differences on abundance were observed; an example of this was the Basidiomycota group from sub-samples of D30.A.3, E31.A.4 and A5.A.5 (Figure 14). Between depth A and B, differences were found at the phylum level. An example of this is station E31, where the Basidiomycota in depth B had a higher abundance than in depth A (Figure 15-A). Differences were found in the presence-absence and abundance of classes between different depths within the same station; for example, in B14 the Microbotriomycetes had a higher abundance in depth B than in depth A (Figure 15-B).

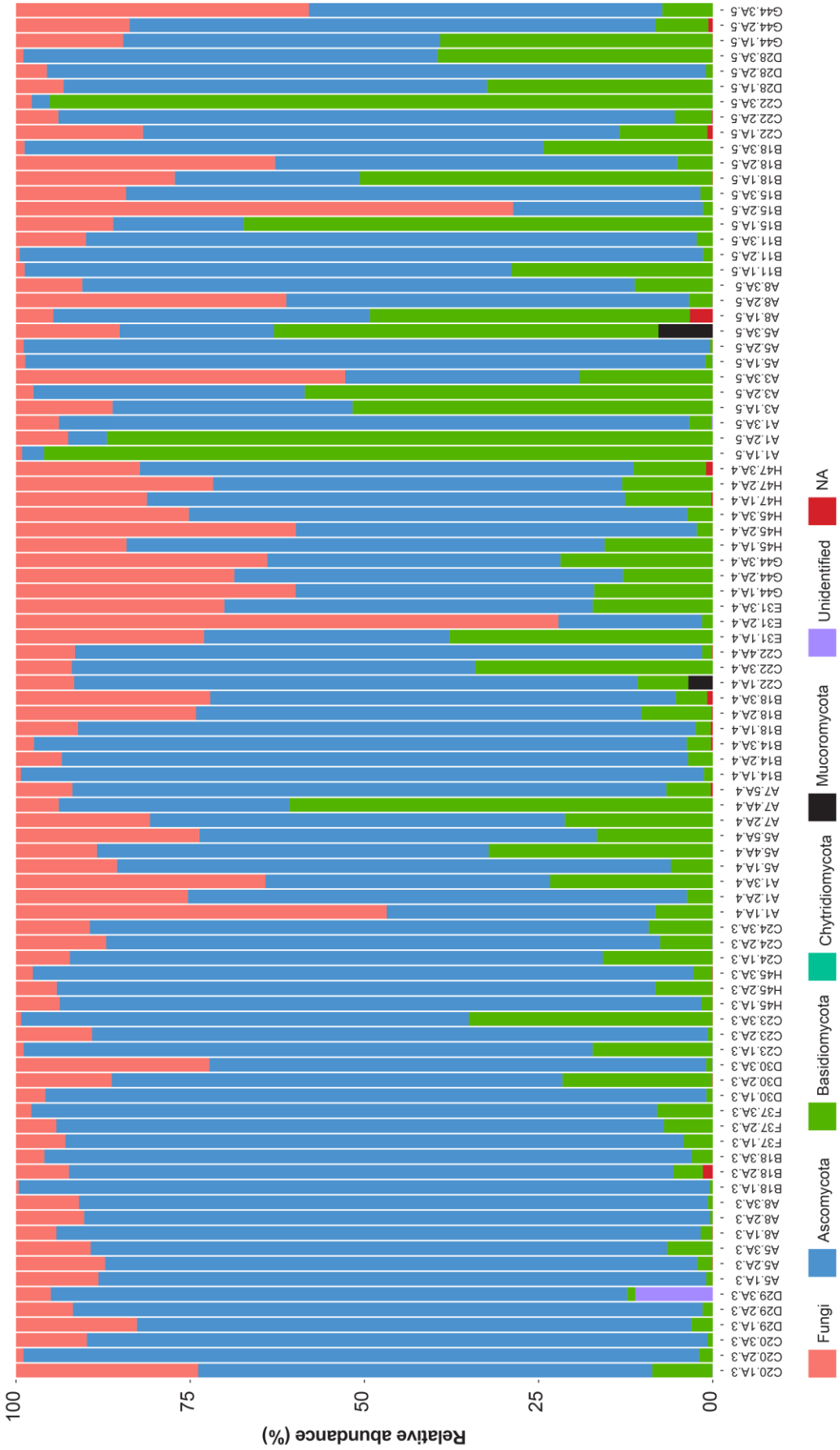

Figure 14. Relative abundance of fungal phyla among sub-samples.

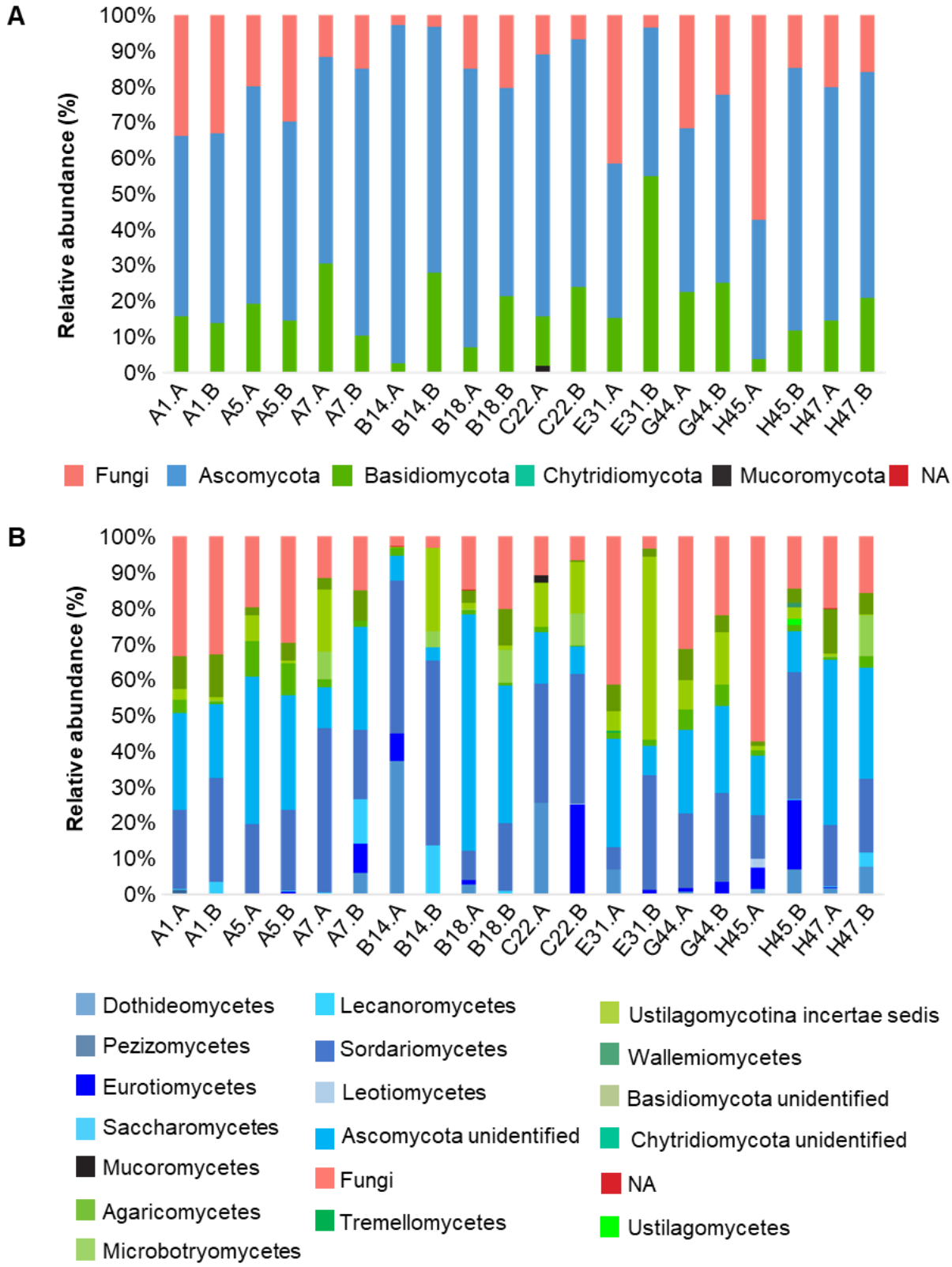

Figure 15. Relative abundance of fungal groups corresponding to depths A and B from the XIXIMI 4 campaign. (A) Displays phyla abundances. (B) Displays classes abundances.

From XIXIMI 3 a total of four phyla were identified (Ascomycota, Basidiomycota, Chytridiomycota, and Mucoromycota). The most abundant phylum was the Ascomycota with 85% of the identified ASVs, followed by unidentified Fungi with 8%; the least abundant phyla were the Chytridiomycota and the Mucoromycota with less than 1% (Figure 16). In this campaign, differences across stations were observed, specifically with regards to the abundance of Basidiomycota; while in some stations such as C23.3 this group represents the 10%, in others it represents only a 0.36% (A8.3) and a 0.58% (D29.3). In this campaign 65 genera were identified, where the most abundant were Neurospora and Meyerozyma with 35% and 24%, respectively, and the least abundant were an unidentified genus of Schizoporaceae and Uromyces, with less than 0.001% of the identified ASVs (Figure 17). In this campaign, differences were found across stations, notably on the abundance of Neurospora, and unidentified genera belonging to Ascomycota and unidentified fungi (Figure 17).

From XIXIMI 4, a total of four phyla were identified (Ascomycota, Basidiomycota, Chytridiomycota, and Mucoromycota), as well as unidentified fungi, and unidentified sequences classified as not assigned (considering depths A and B). From the identified groups, the Ascomycota was the most abundant phylum with 59.6% of the ASVs, followed by unidentified fungi with 22% of the ASVs and the Basidiomycota with 17.90% of the ASVs; while the other groups including the Mucoromycota and the not assigned represented less than 1% of the identified ASVs (Figure 16). A total of 18 fungal classes were identified; with the Sordariomycetes (present in all samples), unidentified Ascomycota and unidentified fungi being the ones with the highest abundance values. The fungal classes with the least abundance values were an unidentified class of Chytridiomycota and the Lecanoromycetes (Figure 17).

Four groups were identified from the XIXIMI 5 campaign, where the most abundant were the Ascomycota and the Basidiomycota with 56% and 28% respectively, and the least abundance values were for Mucoromycota with less than 1% of the identified ASVs (Figure 16). In this campaign 65 genera were identified, and the most abundant were *Neurospora* (17%) and *Rhodotorula* (16%), the least abundant genera were unidentified Lecanoromycetes with values >0.01% (Figure 17). Across stations, the abundances varied mostly on *Neurospora*, *Hortaea*, *Rhodotorula* and unidentified Ascomycota (Figure 17).

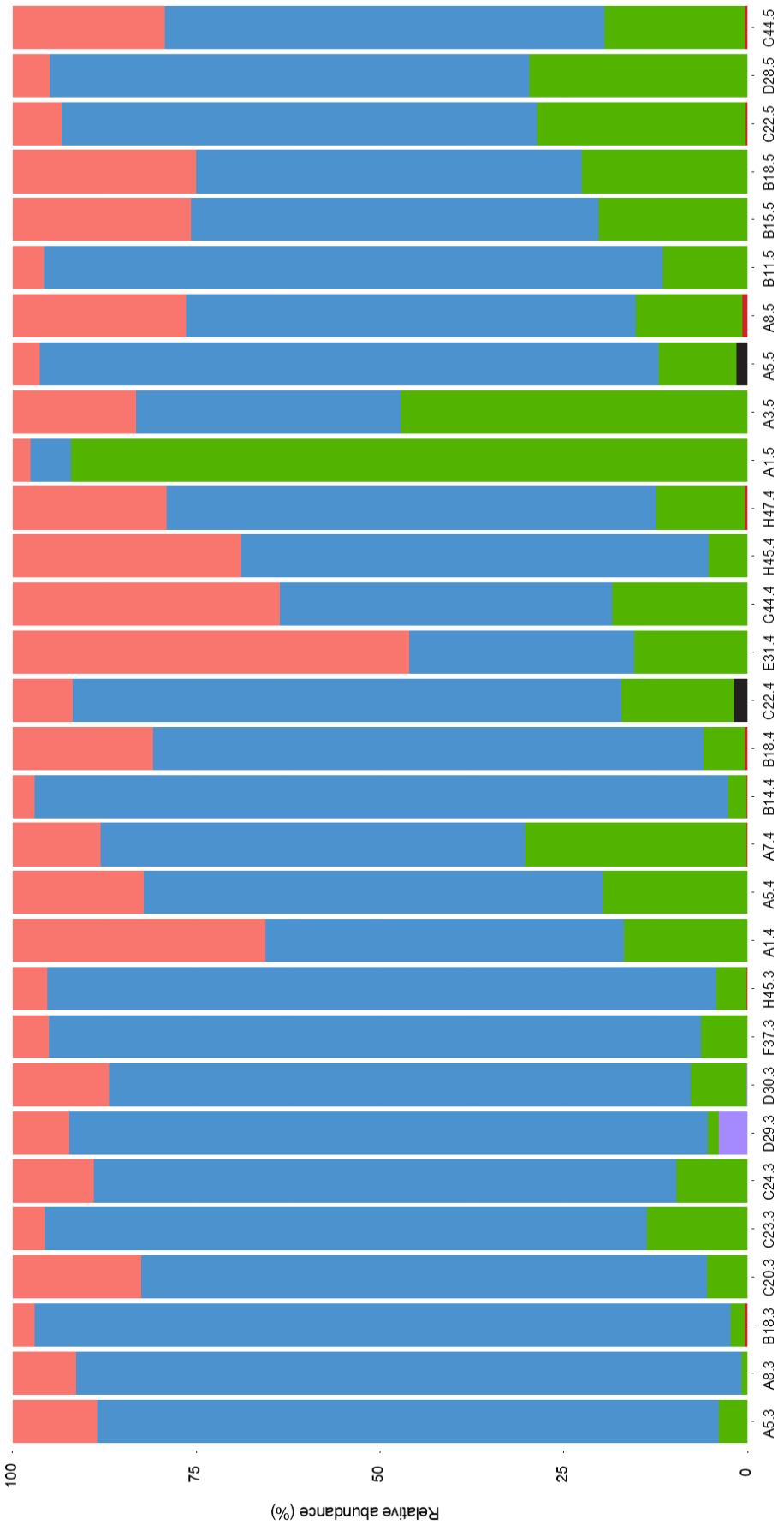

Figure 16. Relative abundance of fungal phyla among stations on the different XIXIMI campaign.

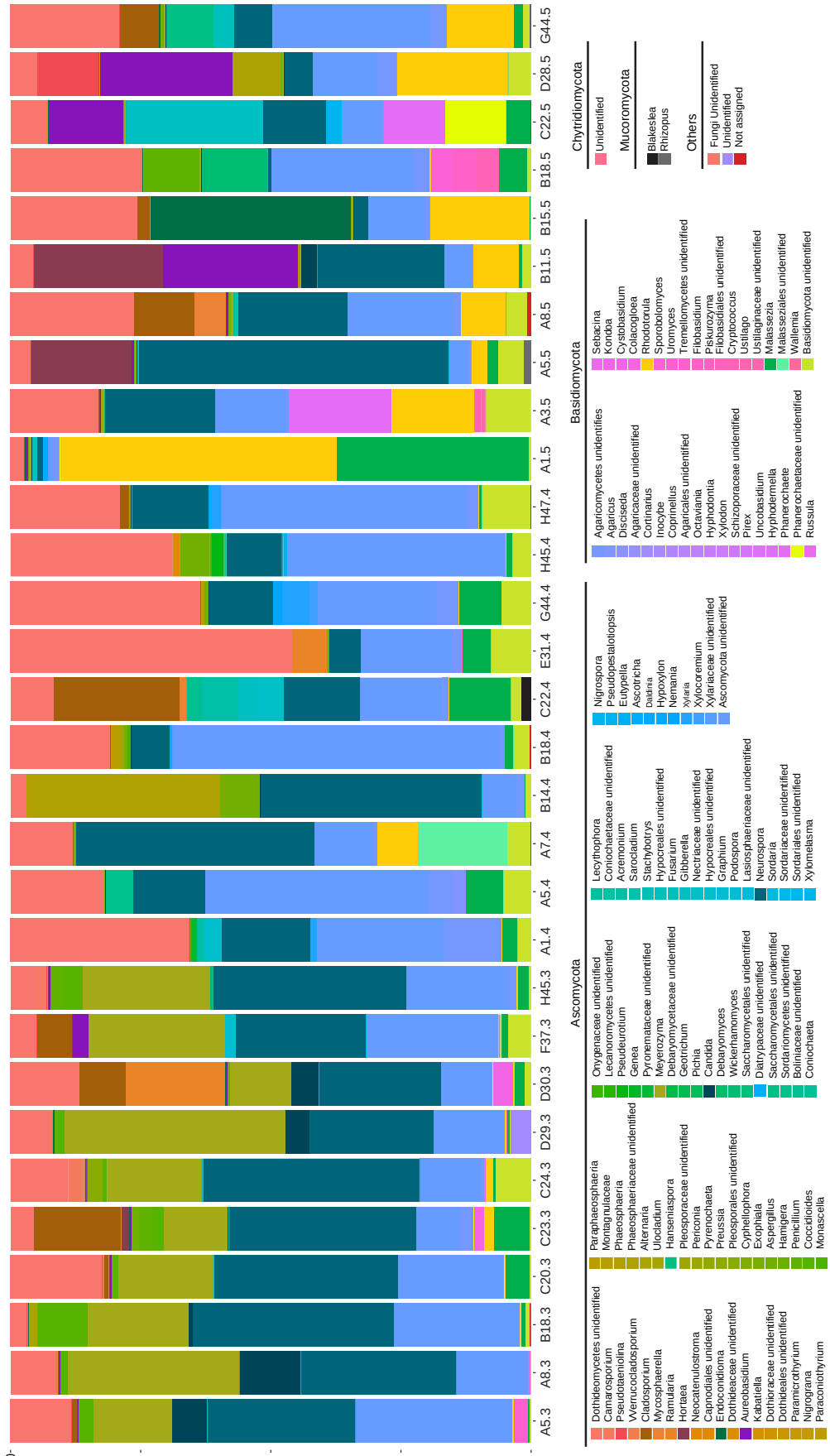

Figure 17. Relative abundance of fungal genera among stations on the different XIXIMI campaigns.

3.1.4. Beta diversity

The Non-metric Multidimensional Scaling (NMDS) analysis revealed clustering of stations by campaign (Figure 18). These NMDS results coincided with the ANOSIM test (P<0.05), which indicated statistical differences in fungal community composition across campaigns. While the stations from XIXIMI 3 and 5 were closer on the ordination plot, the stations from XIXIMI 4 campaigns were grouped together (Figure 18).

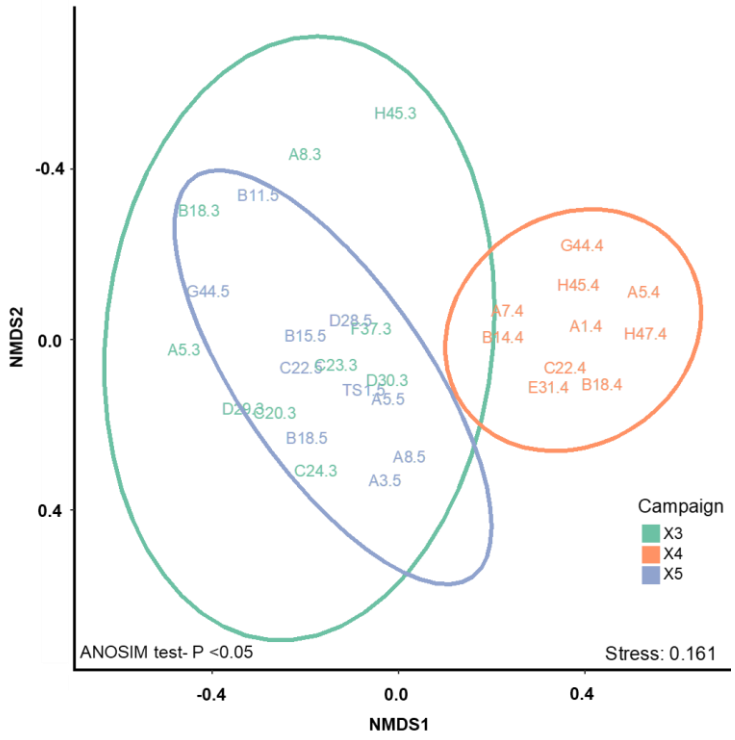

Figure 18. Non-metric Multidimensional Scaling (NMDS) ordination of the similarities in fungal composition across campaigns.

In general, the results from the analysis by ASVs, even when this was the best option to process the sequences from all campaigns, indicate a high prevalence of the genus *Neurospora* in the samples. Interpretation of the results has to be conducted with lots of caution. *Neurospora* could be a contaminant fungus if we consider that the samples were processed in a laboratory where *Neurospora crassa* is routinely grown and propagated; the non-inclusion of negative and positive controls during the processing did not allow interpreting whether this fungus is a real contaminant. The results obtained from the NMDS analysis raised some additional concerns. Stations from XIXIMI 3 and XIXIMI 5 displayed clustering; these

samples from the two campaigns were sequenced in the same Illumina run, and in a different run from samples from XIXIMI 4, which could be creating a bias in the observed community. Due to these concerns, this entire dataset (XIXIMI 3, 4 and 5) was discarded for subsequent analyses. Therefore STRATEGY 2 was implemented. This strategy included controls (negative and positive) and the samples from all the campaigns were sequenced together.

3.2. STRATEGY 2. Results

3.2.1. Positive and negative controls results

Sequences belonging to the mock community (positive control), were submitted to two different databases to obtain taxonomic assignments; the first one was performed using the UNITE database, and the second one was performed using the NCBI database. The UNITE database failed to identify four mock community species, and failed in the assignment of *Fusarium* species, *Penicillium*, *Candida*, and *Neurospora crassa*, which was identified as *Neurospora terricola* (Table 6). The NCBI database, did not identify correctly (Table 6).

Table 6. Comparison among databases in the identification of Mock Community control.

Mock community composition	NCBI	UNITE
Aspergillus ochraceus		
Candida albicans		
Candida orthopsilopsis		Genus *Candida*
Coprinopsis cinerea		
Fusarium oxysporum		
Fusarium sp.		
Lasiodiplodia sp.		
Macrophomina phaseolina		
Neurospora crassa		*Neurospora terricola*
Penicillium chrysogenum		Genus *Penicillium*
Penicillium roqueforti		Genus *Penicillium*
Rhizomucor variabilis var regularior		
Saccharomyces cerevisiae		
Schizossacharomyces pombe		
Botrytis cinerea		
Trichoderma atroviridae		
Tricoderma asperellum		
Ustilago maydis		

*Green color means identified species. Red color means non identified species.

A total of 562,261 raw sequences were obtained from the controls, and after quality control, 366,979 sequences remained for subsequent processing. The control with the largest number of sequences was C6, and the control with the largest number of OTUs was C1 (Table 7).

Table 7. Number of sequences and OTUs of controls.

Control	Number of sequences*	Number of OTUs
C1. Sucrose buffer (sterile)	47,845	96
C2. Sucrose buffer filtration	41,440	69
C3. During syringe preparation	63,727	87
C4. H_2O research vessel	27,160	26
C5. Sucrose buffer research vessel	92,503	39
C6. H_2O during DNA extraction	77,526	40
Blank	16,778	42

*Number of sequences after quality control.

The taxonomic composition of the controls was dominated by the genera *Rhodotorula Cladosporium*, *Penicillium*, unidentified fungi, *Candida*, *Aspergillus*, *Trichoderma* and *Neurospora* (Annex 5).

3.2.2. Sequencing results

A total of 8,563,339 raw sequences were obtained from Illumina sequencing. From those, 562,261 sequences corresponded to the controls included during the sampling campaigns and the experimental procedures and the remaining 8,001,078 sequences corresponded to the samples from the three campaigns. The XIXIMI 4 campaign contained the fewest raw sequences (2,435,016), followed by XIXIMI 5 with 2,717,128 sequences and XIXIMI 6 with 2,848,934 sequences. After processing the reads in the ITSx program to obtain the ITS 1 region and after applying quality control steps, a total of 2,584,642 sequences remained for subsequent analyses. The number of sequences per station ranged from a minimum of 5,077 (A5.4) to a maximum of 229,518 (D26) (Table 8).

In total, 8,949 OTUs were obtained at 97% similarity threshold. Singletons, OTUs more abundant in the controls than in the samples, and OTUs identified as *Neurospora crassa*, amounting to a total of

1,293 OTUs, were deleted. A total of 7,656 OTUs remained after all the above quality filters were applied. The stations A5.4 and B14 contained some of the lowest numbers of OTUs, while the stations C22.6, D26 and G44.6 contained the highest numbers of OTUs (Table 8).

Table 8. Number of sequences and OTUs through the quality control and alpha diversity.

	Station	Number of sequences after quality control and contamination removal	Observed number of OTUs after singletons and contamination removal	Number of sequences*	Observed number of OTUs**	Shannon Wiener
X4	**A1**	40392	542	32630	286	2.41
	A5	5077	303	2851	93	2.77
	A7	54380	515	50309	322	2.12
	B14	5363	272	3802	87	2.95
	B18	9970	392	8969	142	1.54
	C22	41854	499	36667	231	1.84
	E31	97684	739	69178	515	2.12
	G44	84922	723	51644	332	2.25
	H45	83507	624	64235	370	1.27
	H47	43493	543	23784	242	1.83
X5	**TS1**	145015	871	119249	719	2.56
	A3	22044	396	21118	183	0.87
	A5	99631	800	89396	482	1.61
	A8	18264	443	10087	164	2.28
	B11	175746	899	171726	714	2.34
	B15	64037	609	62699	414	2.49
	B18	39740	622	35724	290	2.2
	C22	67226	665	55329	451	1.92
	D28	130513	845	125952	613	2.31
	G44	90113	785	78528	484	2.21
X6	**B12**	130136	796	120400	555	1.99
	B18	10412	449	7203	141	2.56
	C22	212347	1146	181168	717	1.71
	D26	229518	1299	218599	861	2.19
	D27	123003	918	100808	520	1.85
	E33	135789	903	72014	491	1.93
	G44	185347	1213	114582	784	2.34
	G44R	134194	877	117525	606	2.2
	H48	104925	914	98850	556	1.85

* Number of sequences after quality control and identified as fungi.

** Number of Operational Taxonomic Units (OTUs) identified as fungi.

3.2.3. OTUs taxonomic assignments

From the resulting 8,949 OTUs, 4,421 were identified as fungi and the remaining 3,235 OTUs as Metazoa, Viridiplantae and No Blast Hit assignments. Overall, the fungal taxonomically assigned OTUs from all the sediment samples belonged to six phyla, 25 classes, 51 orders, 98 families, and 148 genera. From the 4,421 fungal OTUs analyzed, 2,872 (64.96%) correspond to Ascomycota, 638 (14.43%) to Basidiomycota, 17 (0.38%) to Chytridiomycota, 37 (0.84%) to Glomeromycota, three (0.07%) to Mucoromycota, one (0.02%) to Neocallimastigomycota and 853 (19.29%) to unidentified fungi (Annex 6). Approximately 50% of the BLAST results from the unidentified fungi assignments revealed that the highest hit similarity corresponds to sequences belonging to studies from marine sediments. The Ascomycota phylum and unidentified fungi were the most abundant in the majority of the stations, and the Basidiomycota was the most abundant phylum in some sub-samples of a few stations such as A5.5, C22.5, and D28 from the abyssal plain (AP), and B11 from the Tamaulipas-Veracruz continental slope (TVCS), with abundances between ~50% and 90% (Figure 19-A and B; Annex 6).

Some fungal groups were identified in all stations. The Eurotiales, Saccharomycetales and Capnodiales were the most abundant orders and were present in all stations (Figure 19-A). The unidentified fungi presented a greater abundance in stations from the TVCS (A1 and D26), the Yucatan continental slope (YCS; B18.A) and the AP (A8, A5.4, B14); the Eurotiales order presented a high abundance in stations from AP (C22.6 and E33), the TVCS (D27), Campeche canyon (CC; G44.6 and H48) and Campeche saline canyon (CSC; H47); the Saccharomycetales abundance was higher in the AP and TVCS stations (B12 and E31, respectively); and the Capnodiales were the dominant order in AP (A3) and Coatzacoalcos canyon (CZC; H45) (Figure 19-A).

Stations from the AP were found clustering with stations from other regions (dendrogram in Figure 19-B), while TVCS stations clustered only with stations from AP and CC. This organization seems to be influenced by the most abundant groups, including unidentified fungi, Dothideomycetes, Eurotiomycetes, Saccharomycetes and Microbotryomycetes (Figure 19-B).

Differences in abundance and composition were found among the same stations sampled in different campaigns. A5.4 harbored primarily members of Ascomycota as well as unidentified fungi, while members of the Basidiomycota predominated in the following year (A5.5). For B18, unidentified fungi dominated in all three campaigns. However, B18.6 presented a greater abundance of Eurotiomycetes and Dothideomycetes than B18.4 and B18.5, while B18.5 had a greater abundance of Saccharomycetes, than

B18.4 and B18.6 (Figure 19-A and B; Annex 7). Samples C22.4, C22.5 and C22.6 differed mostly in the abundance of the order Eurotiales. Among samples G44.4, G44.5, G44.6 small differences were found in terms of unidentified fungi, but the composition of other fungal groups largely differed; for instance, the Sordariomycetes was the most abundant class in G44.4, while the Tremellomycetes was the most abundant in G44.5. Replicate samples G44.6 and G44R.6 differed in terms of composition; while G44.6 was dominated by Eurotiomycetes, Sordariomycetes and Cystobasidiomycetes, G44R.6 was dominated by Dothideomycetes and Eurotiomycetes (Figure 19-A and B).

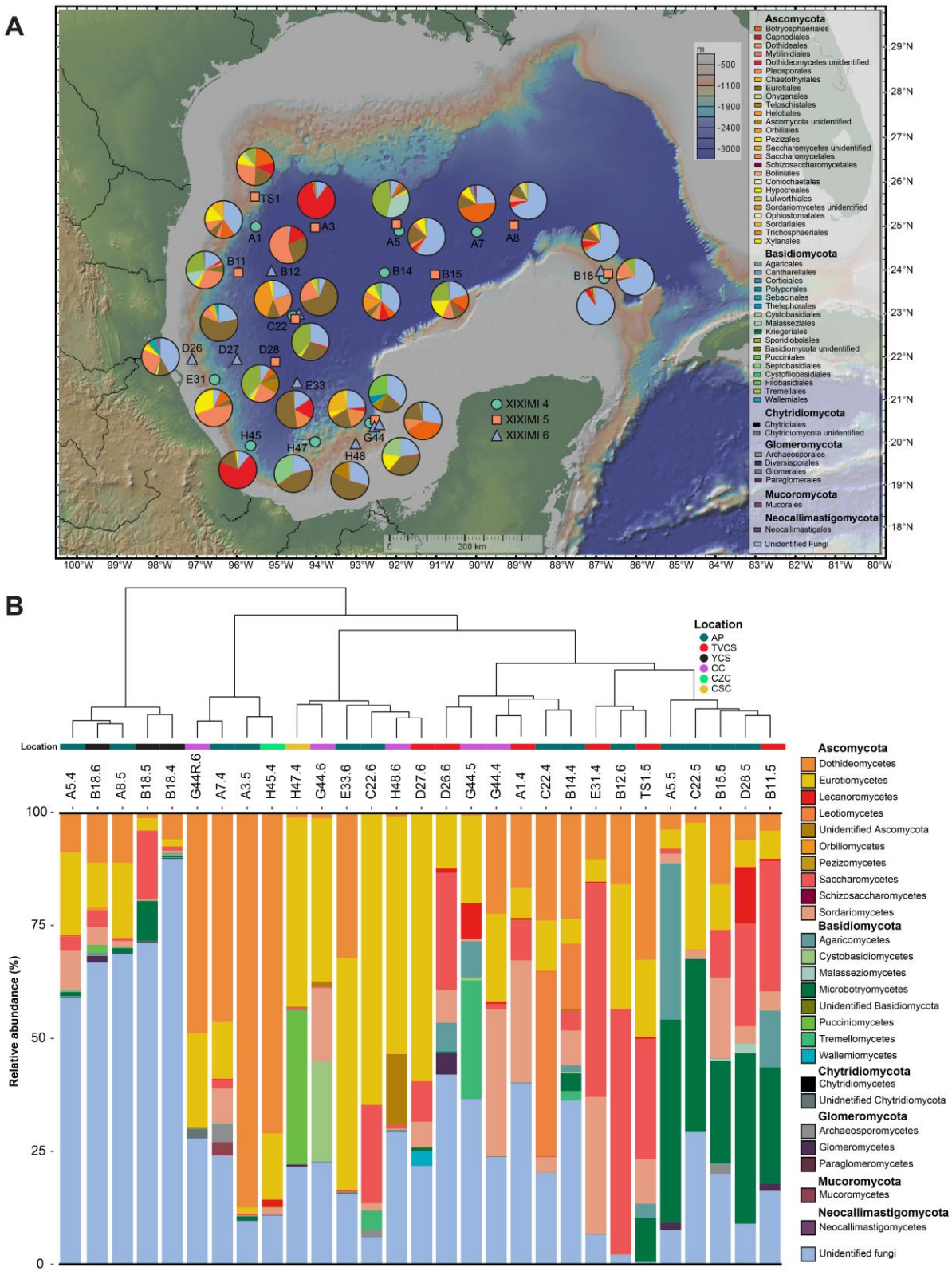

Figure 19. Taxonomic profiles of fungi among sampling stations in the Gulf of Mexico. (A) Distribution map of the relative abundance at order level among the sampling stations. (B) Hierarchical clustering represented as a dendrogram coupled to a bar plot of relative abundance at the class level among the sampling stations. The dendrogram was calculated using Euclidean distance and Ward clustering.

3.2.4. Alpha diversity

The highest Shannon Wiener diversity index was found for B14 and A5.4 stations, while the lowest values were obtained for A3 and H45 stations (Table 8).

Significant correlations were found among the number of observed OTUs with longitude, water content, carbonate, nitrogen and terrigenous content (Table 9; $P<0.05$). It is worth noting that despite the significant relationships, the explained variance ranged between R^2 = 0.13 and 0.30. No significant correlations were found with Shannon diversity index (Table 9).

Table 9. Summary of parameter of linear relationships of the Number of OTUs and Shannon index with environmental variables.

Dependent variable	Independent variable	B0	m	Adj. R^2	P-value
Observed number of OTUs	**Latitude**	1298.37	-38.08		0.08
	Longitude	11782.15	-42.54	0.29	0.002*
	Depth	516.85	-0.03		0.41
	Water content	-1285.32	26.58	0.29	0.002*
	Carbonates	661.52	-7.31	0.24	0.005*
	Carbon	246.6	252.6		0.26
	Terrigenous	-113.72	8.03	0.30	0.001*
	Nitrogen	103.8	3698.4	0.13	0.03*
	C/N ratio	924.31	-62.16		0.051
Shannon Wiener	**Latitude**	1.10	0.04		0.34
	Longitude	-3.84	0.02		0.45
	Depth	2.13	1.68 x10-5		0.84
	Water content	2.73	-0.01		0.59
	Carbonates	2.00	0.002		0.67
	Carbon	1.99	0.14		0.76
	Terrigenous	2.24	-0.002		0.66
	Nitrogen	2.09	-0.09		0.97
	C/N ratio	1.68	0.04		0.46

B0: Intercept; m = slope; *Significant values

3.2.5. Shared and unique OTUs among stations

Network analysis displayed how the OTUs are partitioned across stations (Figure 20); this analysis revealed that just nine OTUs were common in all the 29 stations, while there are some specific OTUs (i.e. from 1 to 5) shared among less than 28 stations.

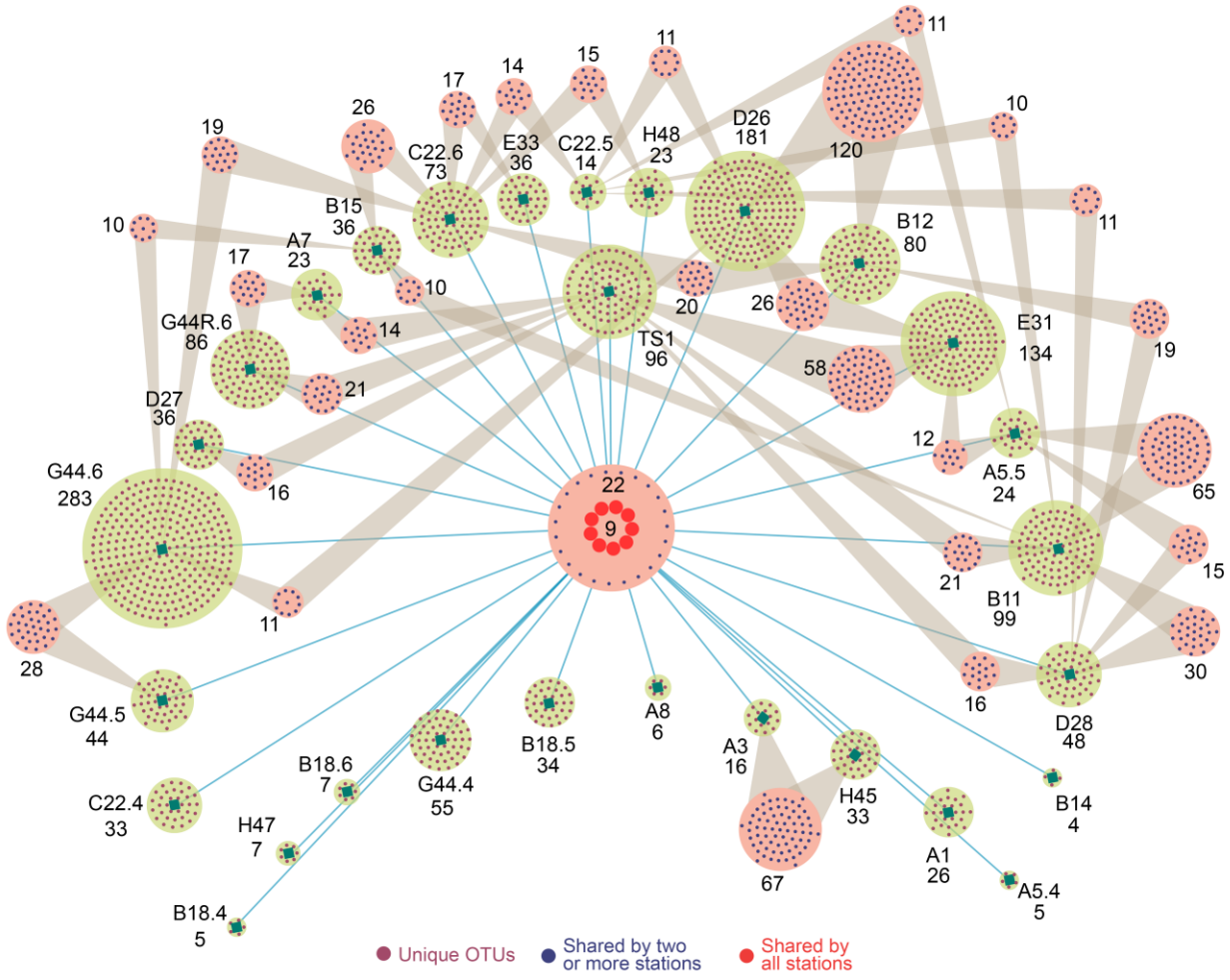

Figure 20. Cytoscape OTUs network illustrating the 4,421 fungal OTUs.

Most stations were compositionally distinct. The stations that shared the largest number of OTUs were B12 and D26 (120 OTUs), followed by A3 and H45 (68 OTUs), A5.5 and B11 (65 OTUs), and TS1 and E31 (59 OTUs) (Figure 20). Stations D26 and G44.6, and E31 showed the largest number of unique OTUs (181, 283 and 134 OTUs, respectively), while A5.4, B14 and B18.4 showed the lowest number of unique OTUs (5, 4 and 5, respectively; Figure 20).

Differences in the observed richness were found among the same stations sampled in different campaigns (Table 8). In some cases (A5.A, C22.A, and G44.A) more than four-fold differences in the number of OTUs were obtained in different years: 93 OTUs in A5.4 and 482 OTUs in A5.5; 231 OTUs in C22.4 , 451 OTUs in C22.5 , and 717 OTUs in C22.6 ; 332 OTUs in G44.4 , 484 OTUs in G44.5, 784 OTUs in G44.6, and 606 OTUs in the replica station G44R.6 (Table 8). Across the different campaigns, the A5.A stations shared 45 OTUs, B18.A stations shared 36 OTUs, C22.A stations shared 37 OTUs and G44.A stations shared 55 OTUs (Figure 21-A to E). G44.6 and G44R.6 shared a total of 44 OTUs (Figure 21-E).

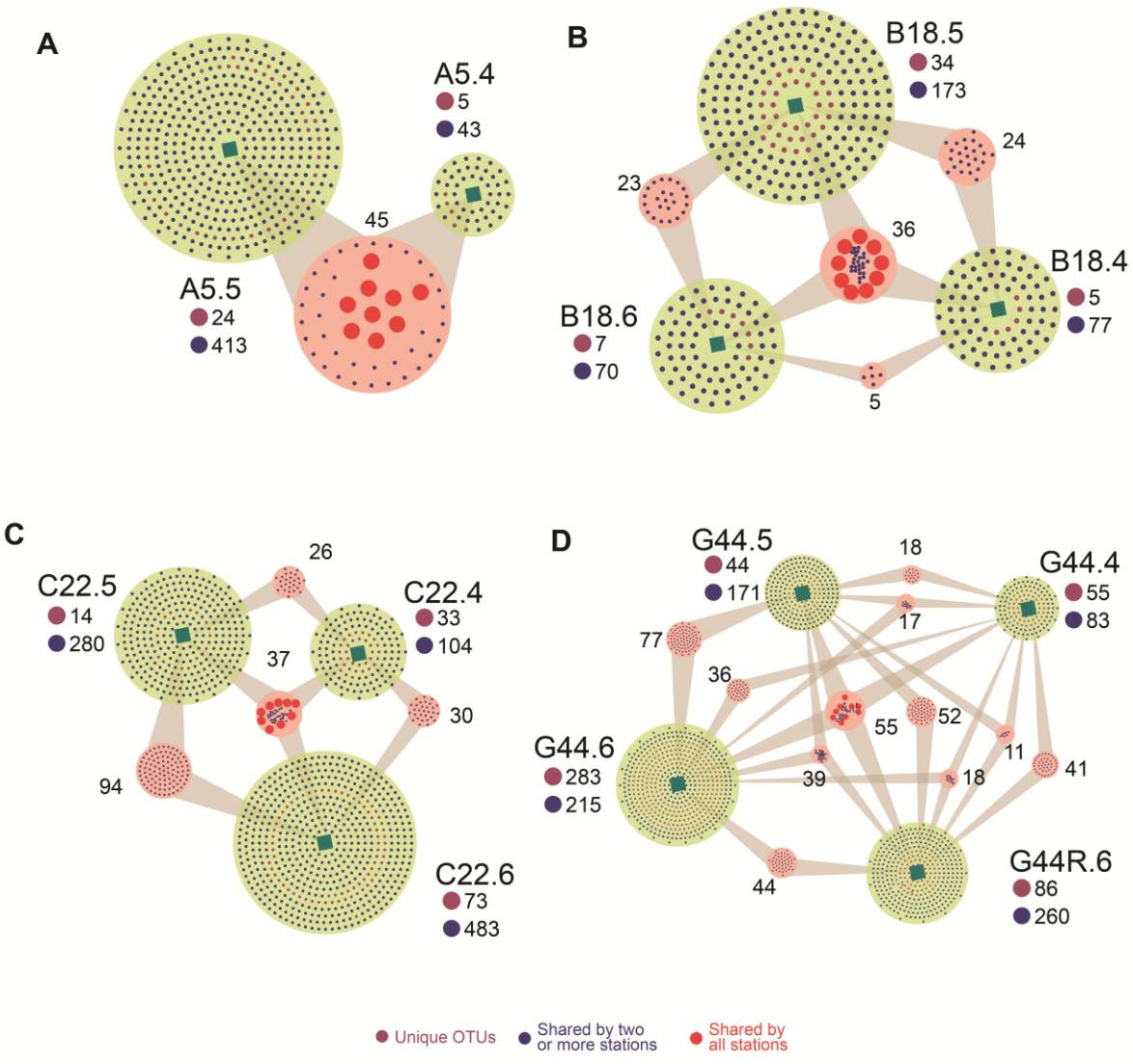

Figure 21. Cytoscape OTUs network illustrating sharing and unique OTUs among common stations. (A) Shared OTUs between the A5 station sampled in XIXIMI 4 and XIXIMI 5. (B) Shared OTUs in the station B18 sampled in XIXIMI 4, 5 and 6. (C) Shared OTUs in the station C22 sampled in XIXIMI 4, 5 and 6. (D) Shared OTUs in the station G44 sampled in XIXIMI 4, 5 and 6; and shared OTUs between G44 and replica G44R.

3.2.6. Selection of the better OTU table and beta diversity analyses

When the different OTU tables (presence-absence and normalized table) were submitted to *rankindex* function to select the best dissimilarity index that fits better to each table, Euclidean distance was the best option for the presence-absence OTU table, and Bray-Curtis distance for the normalized OTU table. The stress values from the NMDS analysis of each table were different: for the presence-absence table, the stress was 0.1358 and for the normalized table was 0.1454 (Figure 22-A). The stressplots obtained in the analysis indicated a better fit for the presence-absence table (Figure 22-B). Also, the Procrustes analysis showed a Procrustes sum of squares of 0.6097, a correlation in a symmetric Procrustes rotation of 0.6248 and a significance of 0.001, indicating a significant correlation between both datasets, suggesting that both, normalized and presence-absence table gave similar results. However, it is important to notice that the correlation coefficient is lower than expected (expected to be near 0.9). We decided to take into account the results from the presence-absence table, because a better fit and stress value is obtained with these results.

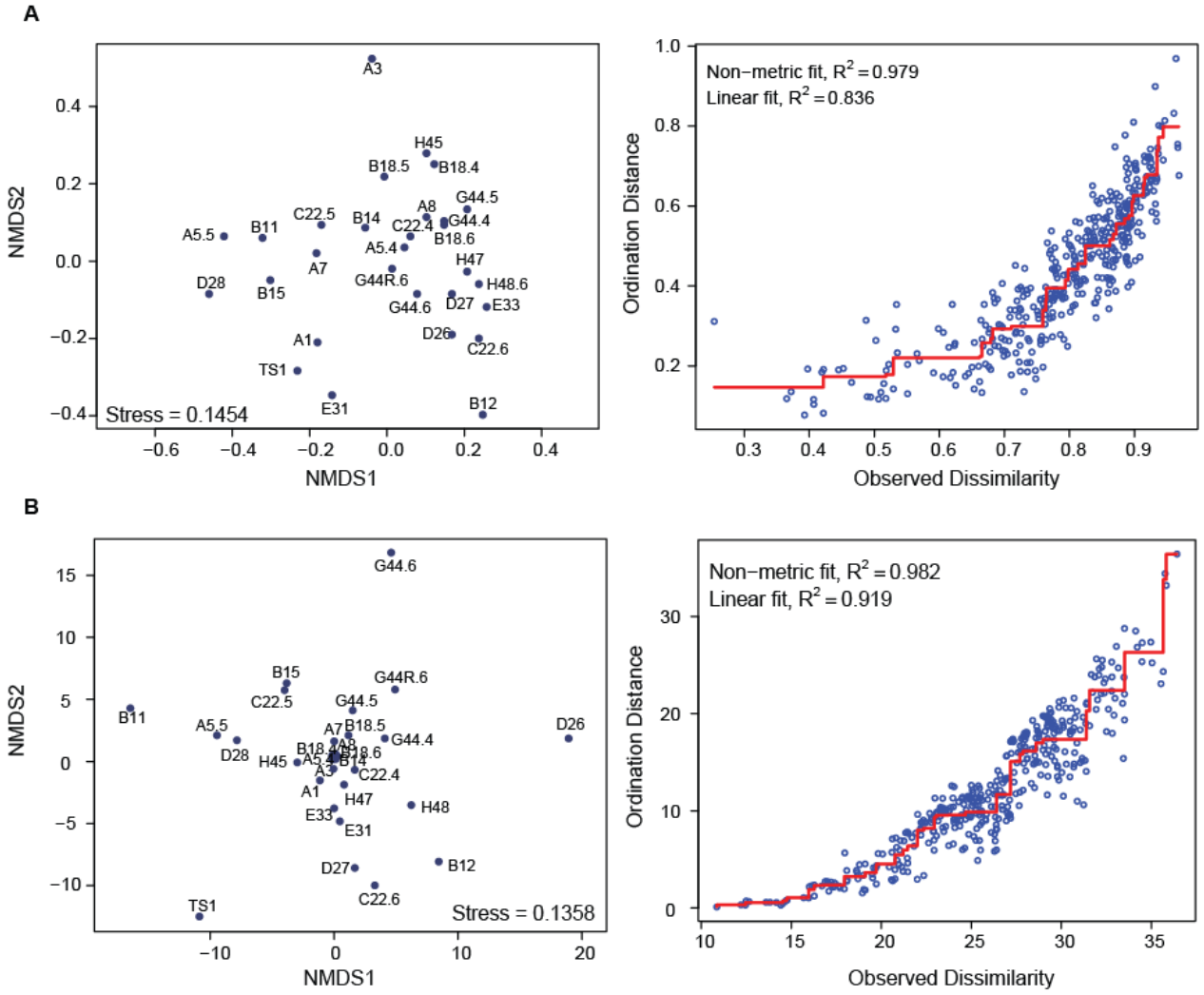

Figure 22. NMDS analysis and stressplots from normalized and presence-absence OTU tables. (A) NMDS and stressplot results using normalized OTU table. (B) NMDS and stressplot results using presence-absence OTU table.

The NMDS analysis (stress=0.1358) based on presence-absence OTU table, did not reveal an obvious clustering of stations (Figure 22-A). Similarly, additional NMDS analyses arranging stations by categorical groups indicated no significant correlations between fungal community composition and regions, latitude, longitude or depth (Figure 23-B, C, D and E; Table 10; Annex 8).

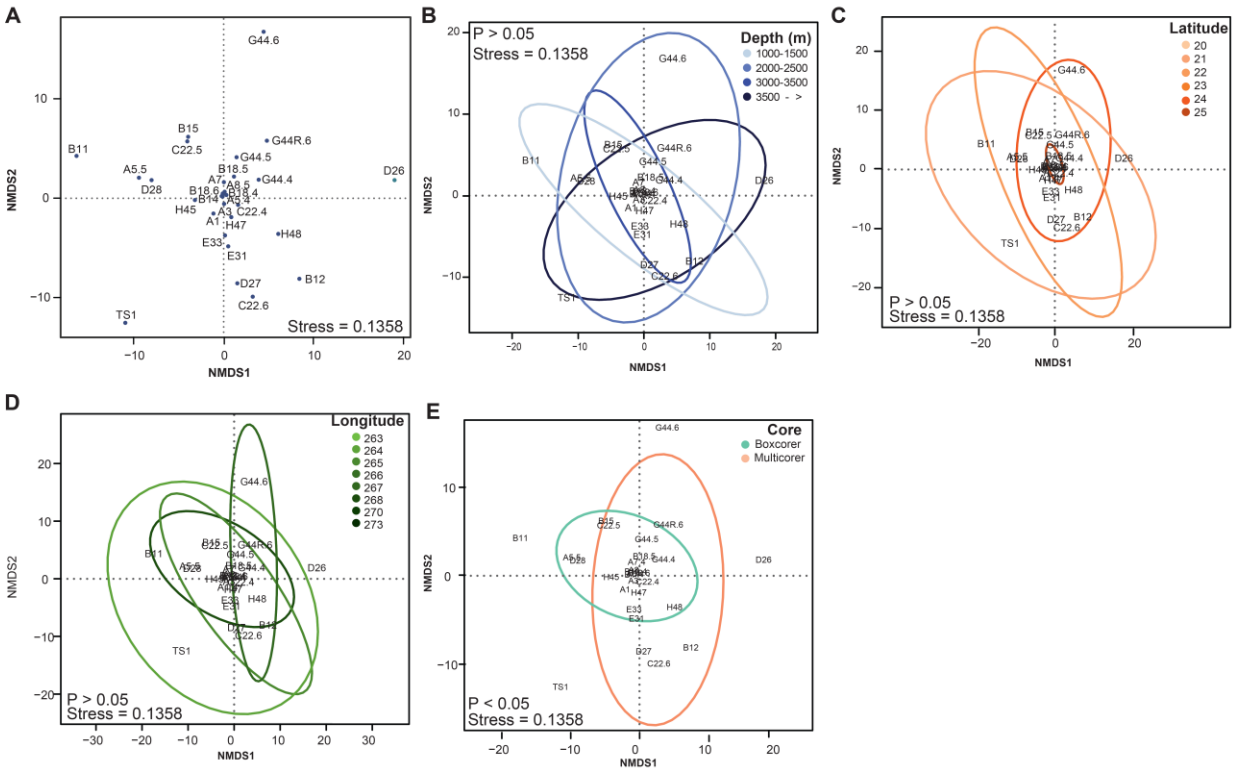

Figure 23. Non-metric Multidimensional Scaling (NMDS) ordination of the similarities in fungal composition. (A) NMDS, based on Euclidean distance matrix. NMDS based on Euclidean distance matrix of stations similarities grouped by (B) Depth, (C) Latitude, (D) Longitude and (E) Corer.

Table 10. PERMANOVA analysis on NMDS ordination.

PERMANOVA	Df	Sums of Sqs	Mean Sqs	F. Model	R^2	P (>F)	P adjusted
Latitude	6	5	1864.6	372.93	1.0985	0.19277	P>0.05
Residuals	22	23	7808.1	339.48	0.80723		
Total	28	28	9672.7		1		
Longitude	9	7	2513	358.99	1.053	0.2598	P>0.05
Residuals	19	21	7159.7	340.94	0.7402		
Total	28	28	9672.7		1		
Depth	3	1002.3	334.11	0.96338	0.10363	0.664	P>0.05
Residuals	25	8670.4	346.81		0.89637		
Total	28	9672.7			1		
Region	5	1682.1	336.42	0.96835	0.1739	0.546	P>0.05
Residuals	23	7990.6	347.42		0.8261		
Total	28	9672.7			1		
Core	1	633.6	633.58	1.8925	0.0655	0.001	0.024*
Residuals	27	9039.1	334.78		0.9345		
Total	28	9672.7			1		

*Significant values after Bonferroni correction.

However, when analyzing the variance of OTU composition of stations arranged by categorical groups, the beta dispersion analysis indicated significant differences by region, latitude and longitude (P<0.05; Figure 24-A, C and D; Table 11). Additionally, a pairwise analysis indicated significant differences within each categorical group: stations from the TVCS differed from those of the YCS (P<0.05; Figure 23-A; Annex 8), latitude 21 stations differed from latitude 25 stations (P<0.05; Figure 23-C, Annex 8), and longitude 273 stations differed from those of longitude 264, 265 and 267 (P<0.05; Figure 23-D, Annex 8). The beta dispersion analysis did not show significant differences in the variance of OTU composition of stations categorized by depth (P>0.05, Figure 23-B; Table 11).

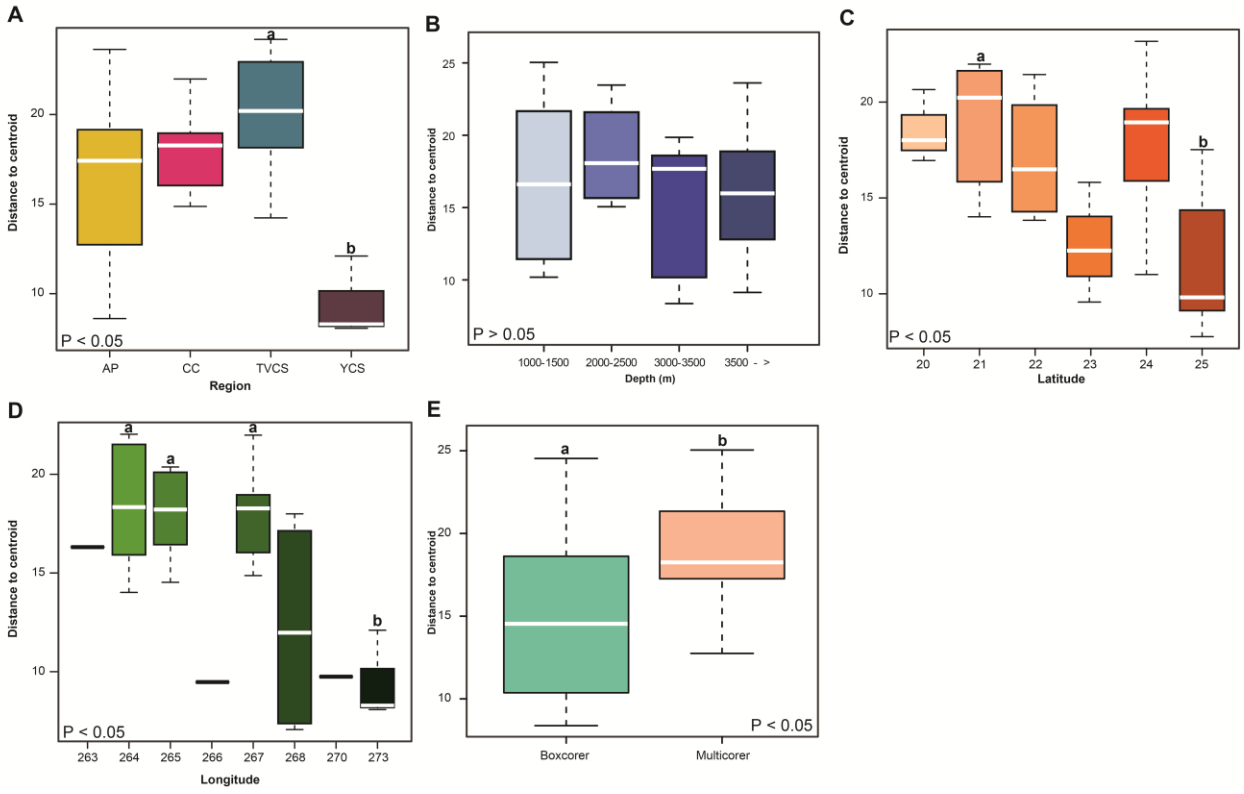

Figure 24. Beta dispersion across stations. Box plot of multivariate dispersions (beta dispersion) of stations similarities grouped by (A) region, (B) depth, (C) Latitude, (D) Longitude and (E) Depth. Beta dispersion significant values refer to pairwise analysis after P-adjusted values by Bonferroni correction.

Table 11. ANOVA analysis on Beta Dispersion.

ANOVA	Df	Sums of Sqs	Mean Sqs	F. Model	P (>F)	P adjusted
Latitude	5	246.15	49.23	3.9103	0.01035	0.0206*
Residuals	23	289.56	12.59			
Longitude	7	396.02	56.574	5.5781	0.000977	0.0003*
Residuals	21	212.98	10.142			
Depth	3	49.71	16.571	0.717	0.5511	1
Residuals	25	577.75	23.11			
Region	3	233.52	77.841	4.5741	0.01182	0.0236*
Residuals	23	391.41	17.018			
Core	1	102.71	102.707	4.9414	0.03479	0.0695
Residuals	27	561.2	20.785			

*Significant values after Bonferroni correction.

NMDS and beta dispersion analyses indicated an effect of the type of corer (box corer or multicorer) in the OTU composition (Figure 23-E, Figure 24-E). However, when the stations were analyzed separately by corer type, the results from PERMANOVA analysis on the NMDS and ANOVA on the beta dispersion analysis, did not show any significant values when comparing with the different categorical variables. The same was observed when the Mantel correlation test was performed using the numerical variables (sediment physicochemical properties), no significant correlations were found among the different stations grouped by corer type and the variables (Annex 9).

To test the effect of the geographical location (latitude and longitude) on the OTU composition among stations, a distance decay analysis was performed. A significance distance decay relationship was found for the fungal community composition along the geographical distance (Mantel $r = 0.1308$, $P<0.05$; Figure 25).

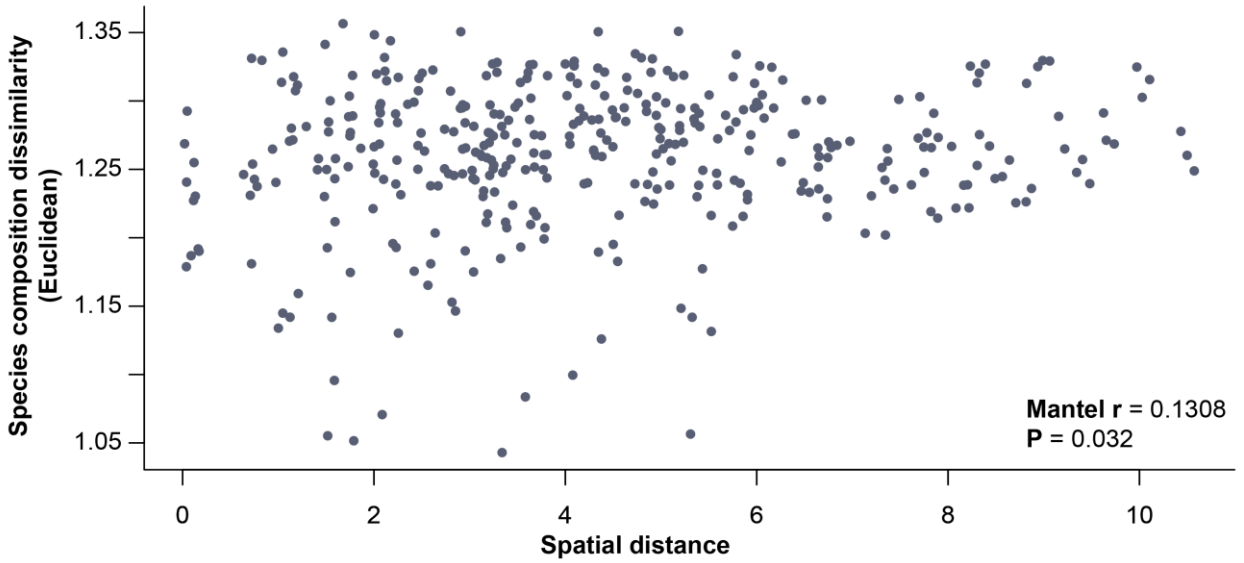

Figure 25. Distance-decay for fungal community along environmental distance. Relationship between geographic distance and fungal community distance based on Euclidean dissimilarity.

3.2.7. Constrained analysis

Before performing the Canonical Correspondence Analysis (CCA), a variable selection was made by the Akaike Information Criterion (AIC). In this analysis the lowest AIC values represented the best model that fits with the community data. All the sediment physicochemical properties were tested and only carbon, carbonates and terrigenous content (Table 11) were selected to construct the model. The model with the selected variables was statistically significant ($P<0.05$; Table 12).

The final CCA model, constructed with the model selection of the AIC was constructed, and accounted for a total inertia equaling 7.92 in community and environment associations. The environmental variables explain a total of 1.21 of the constrained inertia, representing 15.3% of the total variability. Carbon content was the first most important variable ($P<0.01$), followed by terrigenous and carbonate content (Figure 26).

Table 12. Model selection by Akaike Information Criteria and Monte Carlo permutation test of the selected model.

	Variable	Df	AIC	F	P (<0.05)	
Akaike Information Criteria	**Carbonates**	1	207.64	1.2628	0.015	
	Terrigenous	1	207.62	1.2462	0.01	
	Carbon	1	207.78	1.3918	0.005	
Monte Carlo permutation test		**Df**	**ChiSquare**	**F**	**Pr (>F)**	**Bonferroni correction**
	Model	3	1.2124	1.2648	0.001*	0.002*
	Residual	21	6.7099			

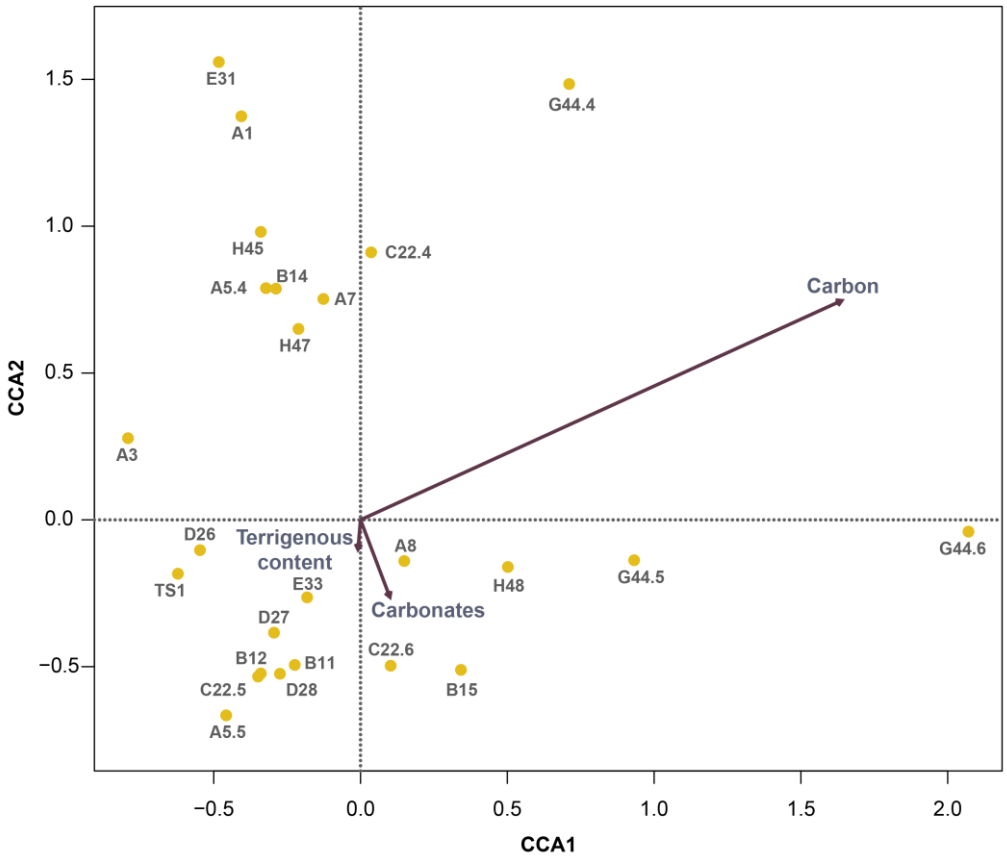

Figure 26. Canonical Correspondence Analysis (CCA) showing the relationship between sediment characteristics and fungal community composition. Ordination diagram of the fungal community of stations together with sediment physicochemical characteristics: Carbon, Carbonate and Terrigenous content. Direction and length of arrows show the degree of correlation between fungal community composition and the variables.

Chapter 4. Discussion

The present study describes the fungal community of deep-sea sediments from the GoM identified through the amplification of the ITS region of the fungal rDNA followed by HTS. The taxonomic assignment of the diverse OTUs detected (4,420 OTUs), indicated that fungi belonging to Ascomycota and unidentified fungi are the major components of the fungal community in sediments from the GoM. A significant correlation was found between the geographic location of the stations and the fungal community, suggesting that the further away the stations are from each other the greater the differences of the fungal community structure. Additionally, some sediment physicochemical properties such as carbon, carbonates and terrigenous content were found as the most important factors influencing the fungal community of the GoM.

4.1. Positive and negative controls are necessary for a good quality control of the sequences processing

The development of NGS has allowed obtaining large datasets at a low cost, in comparison with traditional sequencing methods. This massive sequencing technology has allowed the study of model and non-model organisms, supporting the advancement of disciplines such as microbial ecology and evolution (Escalante et al., 2014). NGS has enabled the analysis of microbial communities and provided an overview of the entire community, including fungi associated with plant systems (mycorrhizae, endophytes and pathogens), free-living saprotrophs, and fungi from extreme environments (Lindahl et al., 2013).

The study of fungal communities (identification and quantification of species) requires different technical considerations (from sampling to laboratory manipulation and sequencing) to obtain reliable data and subsequent conclusions. The results obtained from the processing of sediment samples explained in Strategy 1, did not give reliable results due to the difficulty to process the sequences through regular OTU analysis, the presence and abundance of fungi belonging to the *Neurospora* genus, and the similarity in the taxonomic composition of the stations from XIXIMI 3 and 5, which were sequenced together. The total genomic DNA extracted from these sediment samples was sent to a company, who obtained the ITS region by PCR and sequenced it by Illumina; through all the processing in the laboratory, no controls were included to identify possible contamination sources (Nguyen et al., 2015) and no positive controls (*i. e.* mock community) were included either, which could have been of great help for the correct analysis of the sequences and to evaluate the quality of the sequencing (Bakker, 2018; Lindahl et al., 2013).

It was observed that the majority of the sequences received by the sequencing service did not have a good quality; only 44% of the total raw sequences were kept for subsequent analysis, and not all the sequences were in a 5' – 3' orientation. It is important to consider that different technical problems could have occurred during the processing of the samples, especially in the PCR process. One of the principal problems was the lack of knowledge on how the ITS-PCR was performed by the sequencing company; during the ITS-PCR from Strategy 2, some problems were identified during the amplification of the ITS1 region from the genomic DNA step: a) products smaller than 100-120 bp were present, which was resolved with the purification (size selection) of the PCR products having the correct size; and b) difficulty to amplify the ITS 1 region, which was resolved by carrying out a touchdown PCR. In the Strategy 1, no information was provided about this type of problems. The lack of knowledge on how the samples were processed limits the capacity to detect problems in the raw sequences. As mentioned above, a questionable result was the detection of *Neurospora* in the sequences; the presence of this fungus in the dataset was dubious because the model organism *Neurospora crassa* is routinely studied in the laboratory, where the samples were processed. To our knowledge, it is not common to find this fungus in these types of environments. *N. crassa* has been only reported in an study of a hydrothermal vent in Guaymas, Sonora, Mexico (Edgcomb et al., 2002).

In the Strategy 2 the mock community control helped to choose the right taxonomic database for the identification of OTUs, and the negative controls allowed the detection of contaminant fungi. One aspect that should be highlighted in the taxonomic assignment of the OTUs belonging to the mock community, was the identification of *Neurospora crassa*, which by the UNITE database was erroneously assigned to *Neurospora terricola*; this could by due to the misrepresentation of *N. crassa* in the database in comparison with *N. terricola*, four *vs.* 555 sequences, respectively. From the total raw sequences, 6.56% of those sequences belonged to the negative controls. Currently, there is not a specific criterion to handle the sequences found in controls, because some of the fungi identified could be present in the studied environment, making it difficult to differentiate whether they are a real contamination or are in fact present in the samples (Nguyen et al., 2015). It was found that in the sediment samples there was presence of fungal OTUs, which were also identified in the controls. Therefore, rather than discarding all those OTUs, the proportion of each OTU (number of sequences in the control in relation with the number of sequences in the sample) was removed from the sediment samples.

4.2. Operational Taxonomic Units versus Amplicon Sequence Variants

An Operational Taxonomic Unit (OTU) is commonly defined as a cluster of sequences, which could belong to an organism or a taxonomic rank as family, genus or even at species level (Edgar, 2013). A percentage of similarity (commonly 97%) among sequences being studied is necessary to cluster those sequences into OTUs. There are two commonly used approaches to cluster the sequences into OTUs: closed and open reference OTUs and *de novo* clustering. During the closed reference process, the OTUs are formed by using a curated taxonomic database (as UNITE database), and the sequences that do not match any sequence in the database are discarded. During the open reference database, instead of discarding those sequences, they are clustered by a *de novo* process; finally, the *de novo* process consisted in clustering all the sequences among them to construct the OTUs (Edgar, 2019). The problem with the use of a reference database for OTU clustering is that for environments (as or marine), the community is not well studied, so, there is an underrepresentation of species and their genetic variability, and it is difficult to capture novel diversity; the advantage of *de novo* OTU clustering is that this approach can capture genetic variability, but the major disadvantage is that the OTUs cannot be compared across studies (Callahan et al., 2017); this is because if certain OTUs are obtained in one dataset, when merging with another dataset from other sequencing run, those OTUs can form other OTUs with the new sequences, and in consequence can differ in their taxonomic identification.

To solve this problem, when comparing different sequencing runs, the Amplicon Sequence Variants can be applied. The ASVs are inferred by a *de novo* process, capturing all the genetic variability present in the dataset. The reason on why the ASVs worked better for the dataset used in Strategy 1 was that the DADA2 pipeline used for this analysis, took the assumption that biological sequences are more common (or abundant) than errors (Callahan et al., 2017; Callahan et al., 2016). When in Strategy 1 the *de novo* OTU formation was applied, the majority of the OTUs were formed by only one sequence, suspecting that those OTUs could be a representation of error sequences, which the DADA2 pipeline could identify correctly. It is important to notice that even when ASVs (for Strategy 1) or OTUs (for Strategy 2) were used, differences in the taxonomic composition and abundance were observed among sub-samples from the same station, so overall results are preserved over different type of approaches.

Even when the ASVs represent a very good approach for the study of microorganism diversity, it is still not a commonly used method in ecology in comparison with the OTUs. Therefore, in the Strategy 2 the ASVs was not selected as a method to evaluate the fungal communities.

4.3. The use of sub-samples enhances obtaining higher fungal richness and detecting spatial heterogeneity

During the analyses of the sequences from Strategy 2, a total of 4,421 fungal OTUs were obtained from the 29 samples of the deep-sea sediments from the GoM, which represented a high recovery rate of OTUs and suggests that the deep-sea sediments from the GoM harbor an extraordinarily high fungal richness. In studies, where the same primers and sequencing platform were used, the number of OTUs identified as fungi were relatively low: 420 OTUs from four samples in Okinawua in Japan (Zhang et al., 2016). In other studies using a different set of universal primers and sequencing platforms, the number of recovered fungal OTUs was also low; 113 OTUs from samples in the High Artic (Zhang et al., 2015) and 1,752 OTUs in 130 samples from six European sites (Richards et al., 2015). Although it is difficult to make comparisons with the results obtained in those studies, given the differences in sequencing platforms, primers, and data treatments, the results presented here provide a new dataset of the fungal community from the GoM at a large spatial scale. In addition, most metagenomic studies do not report whether sub-samples are used, and if so whether the genomic DNA from the sub-samples is pooled and then sequenced; this practice could affect the number of reads obtained since the sequencing effort will be greater for non-pooled sub-samples sequenced separately, than those pooled and sequenced. Furthermore, when sequencing sub-samples, there could be more possibilities to identify environmental/genetic variability (Prosser, 2010).

The majority of the studies that asses the diversity of marine fungi in deep-sea sediments use culture-dependent methods (Burgaud et al., 2009; Damare et al., 2006; Jebaraj et al., 2010; Raghukumar et al., 2004; Redou et al., 2015; Takami, 1999), clone libraries (Nagano et al., 2010; Singh et al., 2011; Singh et al., 2012b; Xu et al., 2014), or a combination of both techniques (Singh et al., 2012a; Singh et al., 2010; Zhang et al., 2016). Moreover, just a few of them use HTS, previously via 454 pyrosequencing and currently with Illumina platforms (Redou et al., 2014; Richards et al., 2015; Zhang et al., 2015; Zhang et al., 2016). The increased use of HTS to analyze marine fungal communities from deep-sea sediments is notably extending our current knowledge on the marine mycobiota.

4.4. Deep-sea sediments harbor both, a transient and a conserved fungal community

The OTU network analysis showed the shared and the unique OTUs present in each station. The unique OTUs may represent the community with the highest turnover rate, that is, a rapidly changing community fully dependent on the physicochemical properties unique to that location. In contrast, the shared OTUs represent a more stable fungal community well-adapted to the habitat dynamics. Also, these OTUs might reflect the divergence among all the stations, and the genetic variability in the ITS1 region sequences for the same taxonomic assignment. Overall, the majority of the stations shared a total of 31 OTUs, and only 9 of those OTUs were shared among all the stations. The taxonomic assignment of the 31 OTUs corresponded to the genera *Penicillium*, *Rhodotorula*, *Cladosporium*, *Aspergillus*, *Meyerozyma*, *Schizophyllum*, *Trichoderma*, *Alternaria*, *Clavispora* and *Candida*. The first five genera are considered ubiquitous fungi, which have been found distributed in other deep-sea sediments worldwide and at different depths (Nagahama et al., 2011; Nagano et al., 2010; Redou et al., 2015; Roth et al., 1964; Singh et al., 2011; Takami, 1999; Xu et al., 2018a; Xu et al., 2018c; Zhang et al., 2014). Also, some genera as *Penicillium* and *Rhodotorula*, have been found in the Mariana Trench, classified as the deepest zone of the ocean (Nagano et al., 2010; Takami et al., 1997). Genera identified as unique from each station, were found in a very low abundance (>0.01%). Only *Lulwoana* genera was identified as obligate marine in the A8 station (abundance of >0.05%); this group has been identified as endophyte in roots of *Posidonia oceanica* (Torta et al., 2015).

4.5. Ascomycota and unidentified fungi dominate the seafloor of the GoM

The vast majority of the identified OTUs were classified as Ascomycota, which is commonly reported as the most abundant phylum in deep-sea sediments (Barone et al., 2018; Zhang et al., 2016), independently of the methodology used for the fungal identification (Amend et al., 2019). Also, a large abundance of unidentified fungi was obtained. Similar results were also obtained by other study in the Mediterranean sea, which reported as the most abundant group the unidentified fungi (Barone et al., 2018). The detection of these unknown or unclassified fungi suggests that deep-sea sediments harbor a largely unknown fungal community, which could include indigenous fungi and species of potential biotechnological importance (Barone et al., 2018; Zhang et al., 2015).

Taxonomic groups including Chytridiomycota, Glomeromycota, Mucoromycota and Neocallimastigomycota were also detected, but in low abundance. It has been suggested that the low abundance of these groups could be the result of the bias from the primers used for the amplification of the ITS1 region (Tedersoo et al., 2015b). The universal primers ITS1F and ITS2 are more likely to amplify ITS1 regions from Ascomycota and Basidiomycota, rather than other fungal groups (Amend et al., 2019; Op De Beeck et al., 2014). The use of multiple or group-specific primers might overcome this artifact (Singh et al., 2012b). Also, there is a bias introduced by the databases since Dikarya fungi have been, through years, the major targets of sequencing initiatives (Richards et al., 2012), enriching the databases with sequences belonging to this fungal subkingdom. Currently, there is not an ITS database that comprises sufficient marine fungal data to improve the taxonomic assignment. A larger effort is needed to enrich the databases of fungi from marine environments.

Commonly detected fungi in deep-sea sediments were also identified in the present study. The taxonomic analysis allowed the detection of *Malassezia*, which is considered ubiquitous in deep-sea sediments (Raghukumar, 2017a). In the present study *Malassezia* was present in two campaigns (XIXIMI 5 and 6) only in 10 stations and in low abundance (1% per station). Among all the genera found, the OTUs related to the novel fungal DFS Group 1 was detected only in 5 stations (B14, C22.5, C22.6, D26, and G44.4), representing an abundance of ~0.7% among all the stations. This group, generally detected in oxygen-depleted deep-sea sediments, was first described by Nagano et al. (2010) from deep-sea sediments from Japanese islands, and is closely related to *Metschnikowia bicuspidate*, which is parasitic on planktonic organisms in deep-sea environments. The DSF Group 1 has been reported in deep-sea sediments collected from methane cold-seeps of Japan Sagami-Bay (Nagahama et al., 2011), methane seeps of the GoM (USA waters) (Thaler et al., 2012), the Mariana Trench of the Pacific Ocean (Xu et al., 2014) and the Chinese Seas (Li et al., 2016). The Neocallimastigomycota phylum, a group classified as obligate anaerobic (Raghukumar, 2017a), was found also in low abundance (~0.0002%) in A7 and B11 stations. The first 10 cm of the sediments from the studied area are thought to be part of the oxygenic layer. Thus, the Neocallimastigomycota, typical of anoxic habitats, would be expected to be more abundant in deeper sediment layers. The low abundance encountered of these groups in top layers of the sediments may result from mixing layers during sediment sampling.

4.6. The high recovery rate of terrestrial fungi could be associated to the influence of GoM rivers

The input of sediments from diverse terrestrial origins may contribute to enlarge the diversity of fungi in marine sediments (Takishita et al., 2006). For instance, rivers are some of the largest sources of organic matter and terrestrial organisms (Li et al., 2016). The Mexican EEZ from the GoM receives sediments from at least six rivers, which could help to increase the input of terrestrial organisms, including fungi. The above could explain the high abundance of fungi common in terrestrial environments in all the stations, which in turn demonstrates the capacity of these organisms to adapt to this habitat and to extreme conditions (Lai et al., 2007). The CCA analysis indicated that the terrigenous content has an effect in the fungal community from the analyzed sediments. The terrigenous content (also known as terrigenous sediments) has a land origin. In the GoM the terrigenous content from river discharge represents an important input of organic material (Díaz-Asencio et al., 2019). The CCA analysis also indicated a significant correlation between the fungal community composition, carbon and carbonate contents. These correlations suggest that the carbon component of the sediments is a limiting resource for the fungal community. It has been suggested that fungi can contribute to the transport of carbon by macroaggregates (Amend et al., 2019), this process can occur by the mixing of humic material with sediment particles (microaggregates), where filamentous fungi trap those particles into macroaggregates (Raghukumar et al., 2010). The carbonate content is important since fungi participate in the degradation of calcareous material (Gleason et al., 2017). In addition, it is important to consider that some of the terrestrial fungi identified in the marine sediment samples could come partially from other sources (Nagano et al., 2010). It is rather difficult to detect and confirm potential contaminations in this type of sampling.

Surprisingly, the canonical correspondence analysis revealed no significant correlation between fungal composition and depth, contradicting other studies where the fungal community composition and abundance change with depth (Gong et al., 2015; Roth et al., 1964; Zhang et al., 2015).

4.7. The geographic location may have an influence on the fungal community

The distance decay analysis indicated a major dissimilarity on the fungal composition as the distance between stations becomes larger. This is easily observed when comparing the taxonomic composition of stations from latitude 25 (belonging to AP) with stations from latitude 21 (belonging to CSC

and TVCS); i.e. the abundance of fungal orders such as the Eurotiales is larger in latitude 21 than in latitude 25, and the abundance of unidentified fungi is larger in latitude 25 than in latitude 21. These latitudinal differences could be associated to the distance of the stations from the GoM shores and the influence of the terrestrial material in the community found: while the stations in the lower latitudes of the GoM are closer to the shore, the sampled stations in higher latitudes are more broadly dispersed across the GoM. On the other hand, in terrestrial ecosystems it has been shown that there is a great variability among samples separated by short distances (Vargas-Gastélum et al., 2015), and the same can occur in marine sediments, due to the vertical and horizontal heterogeneity across the sediments. This short distance variability was observed in G44.6 and the replicate G44R.6 (sampled at the same geographic location during the same campaign). At large spatial scales, abiotic characteristics of the environment and dispersal limitation might be influencing the distribution of fungal communities in marine sediments from GoM. The composition of fungal communities can fluctuate depending on the variability across the landscape (Tian et al., 2018). It has been suggested that the biogeography of microorganisms in deep-sea sediments can be influenced by physical processes such as deep-ocean circulation or biotic processes such as bioturbation by macrofauna (Orcutt et al., 2011), thereby regulating the fungal dispersion in this environment.

4.8. Limitations to study the temporal variability of fungal communities in deep-sea sediments in the GoM

Common stations sampled in different years were compared to test succession of fungal communities in deep-sea sediments. The results showed by the OTU network analysis indicated that G44.A shared a larger number of OTUs than A5.A, C22.A or B18.A. Stations A5.5 and B11 shared more OTUs with other stations (outside the common group) than with the corresponding stations sampled in different years (65 OTUs), suggesting that those stations are poorly connected. Variability among common stations was also observed in terms of the taxonomic composition and abundance of classes and orders for G44.A, A5.A and C22.A, while B18.A stations had a similar composition and abundance of unidentified fungi. These results might suggest variability among the stations across years. However, the complexity of the sampling makes it difficult to analyze these temporal variations, and results should be interpreted carefully. Due to ocean currents it is practically impossible to sample the same exact geographic location twice, therefore some spatial variance was unavoidable within and across campaigns. Therefore, it is hard to interpret whether the differences observed in common stations across years are due to a real temporal variation of the fungal community, small-scale spatial variability, or both.

Chapter 5. Conclusions

We explored the fungal diversity of the EEZ from the GoM and found an unexpectedly high fungal diversity, in comparison with other studies in deep-sea sediments worldwide. This suggests that the GoM may serve as a big reservoir for marine fungi, as well as terrestrial fungi with adaptative mechanisms, which remain poorly studied.

In this study it was demonstrated the importance of including both, positive and negative controls during the samples processing, which helped to improve the analysis of the sequences and to obtain reliable results.

This study describes the previously unknown fungal community of the GoM at a large-scale, allowing the identification of differences of the mycobiota across a wide range of geographic locations with different sediment characteristics and depths. Differences were found in fungal communities' composition among the sampled stations, and those differences were mainly associated to geographical location of the stations and some sediment physicochemical properties such as terrigenous, carbon and carbonates content.

The obtained results also suggested the influence of terrestrial material in the fungal diversity of the GoM, but further investigation of changes in the fungal community from the shore to deeper zones of the GoM are needed to test this hypothesis.

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Annexes

Annex 1
Raw sequences processing.

Programs:
FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)
Mothur (Schloss et al., 2009)
QIIME (Caporaso et al., 2010)
Fungal ITS Extractor (Nilsson et al., 2009a)

Databases:
UNITE - (Abarenkov et al., 2010)

Files:
R1.fastq. – Forward reads. Only one file containing the reads from all samples..
R2.fastq. – Reverse reads. Only one file containing the reads from all samples.
mapping_file.txt. – File containing samples ID, barcode sequences, primer sequences.

Scripts:
1. Prepare the data.

Checking mapping file (QIIME):

validate_mapping_file.py -m mapping_file.txt -o mapping_file_corrected.txt

Explanation: *-m* name of mapping file (input file), *-o* output directory for corrected mapping file.
Output: *mapping_file_corrected.txt* file.

Making the files compatible for QIIME processing (QIIME):

extract_barcodes.py -f seq.fastq -c barcode_single_end --bc1_len 8 -o processed_seqs/barcodes.fastq

Explanation: *-f* name of *.fastq* file (output of *make.fastq*), *-c* input is a single fastq file (*barcode_single_end*), *–bc1_len* length in base pairs of barcode, *-o* output directory.
Output: *barcodes. fastq*, file containing the extracted barcodes from *seq.fastq* file.

2. Quality filtering.

Remove low-quality reads and artifacts (QIIME).

split_libraries_fastq.py -i seq.fastq -m mapping_file_corrected.txt -b barcodes.fastq --barcode_type 8 --rev_comp_mapping_barcodes --phred_offset 33 --rev_comp_barcode -n 0 -q 26 --store_demultiplexed_fastq -o seqs.fna

Explanation: *-i* name of .fastq file (output of *make.fastq*), *-m* name of mapping file (output of *validate_mapping_file.py*), *-b* extracted barcodes .fastq file (output of *extract_barcodes.py*), --barcode_type length of barcode, *--rev_comp_mapping_barcodes* reverse complement barcode, *--phred_offset* Illumina phred score format (either 33 or 64), *--rev_comp_barcode* reverse complement

barcode reads, *-n* maximum number of ambiguous bases allowed in a sequence to retain it, *-q* maximum unacceptable quality score (26, 28, 30), *--store_demultiplexed_fastq* demultiplexed fastq file, *-o* output directory.
Output: *seqs.fna*, *seqs.fastq*, *log.txt* (quality filter results and sequences counts distribution).
Recommended checks: use of FastQC program to observe the quality of the reads after quality filtering.

Extract fungal ITS1 sequences (Fungal ITS Extractor):

sed 's/<directory>//seqs.fna
perl FungalITSExtractor.pl

Explanation: *seqs.fna* (output of *split_libraries_fastq.py*)
Output: *extracted_ITS1_seqs.fna* (file containing only ITS1 sequences)

3. Sequences analysis.

Sequences clustering into Operational Taxonomic Units (OTUs) (QIIME):

pick_otus.py -i extracted_ITS1_seqs.fna -m uclust -s 0.97 -o seqs_otus.txt

Explanation: *-i* extracted ITS sequences file (output of Fungal ITS Extractor), *-m* clustering method (uclust, usearch, rdp, *-s* similarity threshold (0.97 and 0.99), *-o* output directory.
Output: *seqs_otus.txt* file containing the formed OTUs and counting of the sequences in each group.

Extract representative sequences from each OTU (QIIME):

pick_rep_set.py -i seqs_otus.txt -f extracted_ITS1_seqs.fna -m longest -o rep_set_97.fna

Explanation: *-i* otus count file (output of *pick_otus.py*), *-f* ITS1 sequences file (output of Fungal ITS Extractor), *-m* method for picking representative sequences, *-o* output directory.
Output: File containing representative sequences of each OTU.

Identify fungal OTUs (QIIME):

assign_taxonomy.py -i rep_set_97.fna -r sh_refs_qiime_ver7_97.fasta -t sh_taxonomy_qiime_ver7.txt -m blast -o assign_taxonomy_97/

Explanation: *-i* file containing representative sequences (output of *pick_rep_set.py*), *-r* reference sequences (file from UNITE database), *-t* file mapping sequences to assigned taxonomy (file from UNITE database), *-m* taxon assignment method, *-o* output directory.
Output: file containing the ID of the representative sequence and the taxon identification (*rep_set_97_tax_assignments.txt*).

Make OTU table (QIIME):

make_otu_table.py -i seqs_otus.txt -t rep_set_97_tax_assignments.txt -o otu_table.biom

Explanation: *-i* file containing the formed OTUs (output of *pick_otus.py*), *-t* representative sequences and their identification (output of *assign_taxonomy.py*), *-o* output directory.
Output: file containing the number of times an OTU is found in each sample (format *.biom*).

Filtering singletons (QIIME):

filter_otus_from_otu_table.py -i otu_table.biom -n 2 -s 1 -o otu_table_no_singletons.biom

Explanation: *-i* file containing the number of times an OTU is found in each sample (output of *make_otu_table.py*), *-n* the minimum total observation count of an OTU, *-s* minimum number of samples an OTU must be observed, *-o* output directory.
Output: file discarding all OTUs that are observed fewer than 2 times (*otu_table_no_singletons.biom*)

Normalizing data (QIIME):

normalize_table.py -i otu_table_no_singletons.biom -a CSS -o normalized_otu_table.biom

Explanation: *-i* file discarding all OTUs that are observed fewer than 2 times (output of *filter_otus_from_otu_table.py*), *-a* normalization algorithm, *-o* output directory
Output: file containing normalized data (*normalized_otu_table.biom*).

Summary information of the representation of taxonomic groups within each sample (QIIME):

summarize_taxa.py -i otu_table_no_singletons.biom -L 2 -a -o tax_mapping.txt

Explanation: *-i* file discarding all OTUs that are observed fewer than 2 times (output of *filter_otus_from_otu_table.py*), *-L* taxonomic level to summarize (*2*-phylum, *3*-class, *4*-order, *5*-family, *6*-genus, *7*-species), *-a* if present, the absolute abundance, if missing, relative abundance, *-o* output directory.
Output: file containing the summary information of the representation of taxonomic group previously selected.

Annex 2

DADA2 pipeline.

R program
CRAN repository

Necessary packages:

Devtools v 1.13.4 (Wickham and Chang, 2015)
dada2 (Callahan et al., 2016)
ShortRead v 1.30.0 (Lawrence et al., 2016)
Vegan v 2.4-4 (Oksanen et al., 2013)
Phyloseq v 1.22.3 (McMurdie and Holmes, 2013)
ggplot2 v n 2.2.1 (Wickhan and Winston, 2016)

Load packages:

```
library(devtools)
library(dada2)
library(ShortRead)
library(vegan)
library(phyloseq)
library(ggplot2)
```

Set the working directory (example):

```
setwd("D:/Secuencias/Analisis_seqs_all_Xs_depth_A_5_NOV_2017/")
```

File parsing (only the forward Illumina reads were used):
*Move forward reads into their own directory for simplest processing

```
path <- "D:/Secuencias/Analisis_seqs_all_Xs_depth_A_5_NOV_2017/"
```
*Changing the directory at which contained the demultiplexed fastq files

Check the files in the working directory:

```
list.files(path)
```

Place filtered files in filtered/ subdirectory:
filtpath <- file.path(path, "filtered")

Make directory for filtered fqs if not already present:

```
if(!file_test("-d", filtpath)) dir.create(filtpath)
fns <- list.files(path)
fastqs <- fns[grepl(".fastq$", fns)]
```
*Changing if in the working directory contain different file extensions or to target only certain sequences

Examine quality profiles of forward reads by sample (Images in threes):
plotQualityProfile(fns[1:3])
plotQualityProfile(fns[4:6])
plotQualityProfile(fns[7:9])

Perform filtering and trimming of the reads:
*trimLeft=21 set to trim the forward primer. ITS1F-21 bp

```
for(i in seq_along(fns)) {
  fastq <- fastqs[[i]]
  fastqFilter(fn = file.path(path,fastq), fout = file.path(filtpath, fastq), truncLen=c(240),
              minLen = 180, trimLeft=21, maxN=0, maxEE=1, truncQ=2, rm.phix=TRUE,
              compress=FALSE, verbose=TRUE)
}
```

File parsing 1. indicating the path of the filtered reads

```
fns = list.files(filtpath)
fns = file.path(filtpath, fns)
```

Learn the Error Rates in filtered reads:
*On Windows, set multithread=FALSE

```
errF <- learnErrors(fns, multithread=FALSE)
plotErrors(errF, nominalQ=TRUE)
```

File parsing 2:

```
filts <- fns[grepl("fastq$", fns)] # CHANGE if different file extensions
sample.names <- sapply(strsplit(basename(filts), "_"), `[`, 1)
*Assumes filename = samplename_XXX.fastq.gz
names(filts) <- sample.names
```

Dereplication:
*Dereplication combines all identical sequencing reads into into “unique sequences” with a corresponding “abundance”: the number of reads with that unique sequence. Dereplication substantially reduces computation time by eliminating redundant comparisons.

```
drp.learn <- derepFastq(filts, verbose = TRUE)
dd.learn <- dada(drp.learn, err=NULL, selfConsist=TRUE, multithread=TRUE)
err <- dd.learn[[1]]$err_out
plot(err)
rm(drp.learn);rm(dd.learn)
```

Sample inference:

```
derep <- derepFastq(filts)
dds <- dada(derep, err=err, multithread=TRUE)
seqtab <- makeSequenceTable(dds)
uniquesToFasta(seqtab, "D:/Secuencias/Seleccion_seqs_5_NOV_2017/Results/rep_set.fna")
```

seqtab <- t(seqtab) *transpose the table.

Export data to qiime:
*Only if there are interest in analyze the data in QIIME.

seqtab <- cbind('#OTUID' = rownames(seqtab), seqtab)
*Add '#OTUID' to the header (required by biom). Export data to Qiime

write.table(seqtab, "D:/Secuencias/Seleccion_seqs_5_NOV_2017/Results/dada2_seq_table.txt", sep='\t', row.names=FALSE, quote=FALSE)

Construct sequence table and remove chimeras:

seqtab.fwd <- makeSequenceTable(dds)
seqtab.nochimera.fwd <- removeBimeraDenovo(seqtab.fwd, multithread=TRUE) # This is the RSV table used for community analyses
dim(seqtab.nochimera.fwd)
write.table(seqtab.nochimera.fwd,
"D:/Secuencias/Seleccion_seqs_5_NOV_2017/Results/ASV_table_X3_4_5.txt", sep="\t")

Fraction of chimeric sequences detected:

chimeras.fwd = 1-(sum(seqtab.nochimera.fwd)/sum(seqtab.fwd))

Constructing the data frame and exporting the data:

df.fwd = as.data.frame(seqtab.nochimera.fwd)
dim(df.fwd)
write.table(df.fwd, "D:/Secuencias/Seleccion_seqs_5_NOV_2017/Results/ASV_table_X3_4_5_df.txt", sep="\t")
table(nchar(getSequences(seqtab.nochimera.fwd)))
seqtab.nonchimera.fwd.uniques<-getUniques(seqtab.nochimera.fwd, collapse = TRUE, silence = FALSE)
write.table(seqtab.nonchimera.fwd.uniques,
"D:/Secuencias/Seleccion_seqs_5_NOV_2017/Results/ASV_sequences.txt", sep="\t")

Assign taxonomy:
*UNITE database v 7.2

fastaRef <- "sh_general_release_dynamic_28.06.2017.fasta.gz"
taxa2 <- assignTaxonomy(seqtab.nochimera.fwd, refFasta = fastaRef, multithread=FALSE, verbose=TRUE, tryRC=TRUE)
unname(head(taxa2))
write.table(taxa2, "D:/Secuencias/Seleccion_seqs_5_NOV_2017/Results/taxa_X3_4_5.txt", sep="\t")

Annex 3

Genomic DNA concentration Strategy 1.

XIXIMI 4 CAMPAIGN							
Sample	**Concentration (ng/µl)**	**Sample**	**Concentration (ng/µl)**	**Sample**	**Concentration (ng/µl)**	**Sample**	**Concentration (ng/µl)**
A1.1A.4	14.5	**A7.1B.4**	7.6	**C22.1A.4**	10.5	**G44.1B.4**	21.3
A1.2A.4	16	**A7.2B.4**	6.4	**C22.2A.4**	20.3	**G44.2B.4**	16.6
A1.3A.4	18.7	**A7.3B.4**	6.3	**C22.3A.4**	10.6	**G44.3B.4**	25.3
A1.4A.4	14.6	**A7.4B.4**	6.5	**C22.4A.4**	9.4	**G44.4B.4**	21.9
A1.5A.4	26.2	**A7.5B.4**	6.7	**C22.5A.4**	7.9	**G44.5B.4**	18.9
A1.1B.4	16.3	**B14.1A.4**	16.5	**C22.1B.4**	6.8	**H45.1A.4**	13.7
A1.2B.4	16.7	**B14.2A.4**	7.4	**C22.2B.4**	8.1	**H45.2A.4**	14.3
A1.3B.4	35.7	**B14.3A.4**	7.2	**C22.3B.4**	10.2	**H45.3A.4**	17.1
A1.4B.4	15.2	**B14.4A.4**	8.5	**C22.4B.4**	6.9	**H45.4A.4**	16.9
A1.5B.4	14	**B14.5A.4**	9	**C22.5B.4**	6.3	**H45.5A.4**	18.8
A5.1A.4	13.7	**B14.1B.4**	10.3	**E31.1A.4**	13.6	**H45.1B.4**	14.7
A5.2A.4	12	**B14.2B.4**	7.3	**E31.2A.4**	18	**H45.2B.4**	16.7
A5.3A.4	11.9	**B14.3B.4**	6.5	**E31.3A.4**	16	**H45.3B.4**	19.5
A5.4A.4	11.1	**B14.4B.4**	8.2	**E31.4A.4**	13.1	**H45.4B.4**	16.3
A5.5A.4	13.1	**B14.5B.4**	10.8	**E31.5A.4**	13.7	**H45.5B.4**	13.2
A5.1B.4	12.5	**B18.1A.4**	10.9	**E31.1B.4**	10.4	**H47.1A.4**	11.2
A5.2B.4	11.6	**B18.2A.4**	13.9	**E31.2B.4**	10	**H47.2A.4**	13.1
A5.3B.4	11.7	**B18.3A.4**	14.1	**E31.3B.4**	7	**H47.3A.4**	11.7
A5.4B.4	12.6	**B18.4A.4**	13.9	**E31.4B.4**	31.3	**H47.4A.4**	17.8
A5.5B.4	12.6	**B18.5A.4**	12.3	**E31.5B.4**	12.9	**H47.5A.4**	12.6
A7.1A.4	7.1	**B18.1B.4**	13.8	**G44.1A.4**	22.4	**H47.1B.4**	10.3
A7.2A.4	13	**B18.2B.4**	17.3	**G44.2A.4**	22.5	**H47.2B.4**	10.2
A7.3A.4	7.5	**B18.3B.4**	16.4	**G44.3A.4**	23.1	**H47.3B.4**	10.6
A7.4A.4	8	**B18.4B.4**	12.2	**G44.4A.4**	19.6	**H47.4B.4**	9.7
A7.5A.4	8.1	**B18.5B.4**	10.1	**G44.5A.4**	59.9	**H47.5B.4**	23.7

XIXIMI 3 CAMPAIGN				XIXIMI 5 CAMPAIGN			
A5.1A.3	10.4	**C24.1A.3**	11.3	**A3.1A.5**	10.7	**B18.1A.5**	8.7
A5.2A.3	18.3	**C24.2A.3**	9.5	**A3.2A.5**	9.5	**B18.2A.5**	15.2
A5.3A.3	10.6	**C24.3A.3**	22	**A3.3A.5**	9.8	**B18.3A.5**	7.2
A8.1A.3	15.9	**D29.1A.3**	12.3	**A5.1A.5**	6.3	**C22.1A.5**	5.9
A8.2A.3	12.9	**D29.2A.3**	10.9	**A5.2A.5**	8.9	**C22.2A.5**	6.4
A8.3A.3	17.3	**D29.3A.3**	16.9	**A5.3A.5**	6.8	**C22.3A.5**	7.2
B18.1A.3	6.5	**D30.1A.3**	10.2	**A8.1A.5**	8.1	**D28.1A.5**	5.9
B18.2A.3	11.4	**D30.2A.3**	11.4	**A8.2A.5**	12.8	**D28.2A.5**	4.2
B18.3A.3	7.6	**D30.3A.3**	23.6	**A8.3A.5**	6.7	**D28.3A.5**	10.5
C20.1A.3	16.9	**F37.1A.3**	15	**B11.1A.5**	12	**G44.1A.5**	16.2
C20.2A.3	14.2	**F37.2A.3**	19.6	**B11.2A.5**	7.6	**G44.2A.5**	16.3
C20.3A.3	13.6	**F37.3A.3**	13.9	**B11.3A.5**	9	**G44.3A.5**	13.4
C23.1A.3	10.5	**H45.1A.3**	13	**B15.1A.5**	2	**TS1.1A.5**	8.9
C23.2A.3	10.1	**H45.2A.3**	30	**B15.2A.5**	5.9	**TS1.1A.6**	7.4
C23.3A.3	14.3	**H45.3A.3**	21.2	**B15.3A.5**	4.6	**TS1.1A.7**	7

Annex 4

Assignation of indexes to the sequencing samples.

ID	I7_Index_ID	I5_Index_ID
A1_1A_X4	N701	S502
A1_2A_X4	N701	S503
A1_3A_X4	N701	S505
A5_1A_X4	N701	S506
A5_4A_X4	N701	S507
A5_5A_X4	N701	S508
A7_2A_X4	N701	S510
A7_4A_X4	N701	S511
A7_5A_X4	N702	S502
B14_1A_X4	N702	S503
B14_2A_X4	N702	S505
B14_3A_X4	N702	S506
B18_1A_X4	N702	S507
B18_2A_X4	N702	S508
B18_3A_X4	N702	S510
C22_1A_X4	N702	S511
C22_3A_X4	N703	S502
C22_4A_X4	N703	S503
E31_1A_X4	N703	S505
E31_2A_X4	N703	S506
E31_3A_X4	N703	S507
G44_1A_X4	N703	S508
G44_2A_X4	N703	S510
G44_3A_X4	N703	S511
H45_1A_X4	N704	S502
H45_2A_X4	N704	S503
H45_3A_X4	N704	S505
H47_1A_X4	N704	S506
H47_2A_X4	N704	S507
H47_3A_X4	N704	S508
A1_1A_X5	N704	S510
A1_2A_X5	N704	S511
A1_3A_X5	N705	S502
A3_1A_X5	N705	S503
A3_2A_X5	N705	S505
A3_3A_X5	N705	S506
A5_1A_X5	N705	S507
A5_2A_X5	N705	S508
A5_3A_X5	N705	S510
A8_1A_X5	N705	S511
A8_2A_X5	N706	S502
A8_3A_X5	N706	S503
B11_1A_X5	N706	S505
B11_2A_X5	N706	S506
B11_3A_X5	N706	S507
B15_1A_X5	N706	S508
B15_2A_X5	N706	S510
B15_3A_X5	N706	S511

ID	I7_Index_ID	I5_Index_ID
B18_1A_X5	N707	S502
B18_2A_X5	N707	S503
B18_3A_X5	N707	S505
C22_1A_X5	N707	S506
C22_2A_X5	N707	S507
C22_3A_X5	N707	S508
D28_1A_X5	N707	S510
D28_2A_X5	N707	S511
D28_3A_X5	N710	S502
G44_1A_X5	N710	S503
G44_2A_X5	N710	S505
G44_3A_X5	N710	S506
B12_1A_X6	N710	S507
B12_2A_X6	N710	S508
B12_3A_X6	N710	S510
B18_1A_X6	N710	S511
B18_2A_X6	N711	S502
B18_3A_X6	N711	S503
C22_1A_X6	N711	S505
C22_2A_X6	N711	S506
C22_3A_X6	N711	S507
D26_1A_X6	N711	S508
D26_2A_X6	N711	S510
D26_3A_X6	N711	S511
D27_1A_X6	N712	S502
D27_2A_X6	N712	S503
D27_3A_X6	N712	S505
F38_1A_X6	N712	S506
F38_2A_X6	N712	S507
F38_3A_X6	N712	S508
G44_1A_X6	N712	S510
G44_2A_X6	N712	S511
G44_3A_X6	N714	S502
G44_1_R_X6	N714	S503
G44_2A _R_X6	N714	S505
G44_3A _R_X6	N714	S506
H48_1A_X6	N714	S507
H48_2A_X6	N714	S508
H48_3A_X6	N714	S510
Blanco	N714	S511
Mock	N715	S502
C1	N715	S503
C2	N715	S505
C3	N715	S506
C4	N715	S507
C5	N715	S508
C6	N715	S510

Annex 5

Taxonomic composition at genera level from the controls.

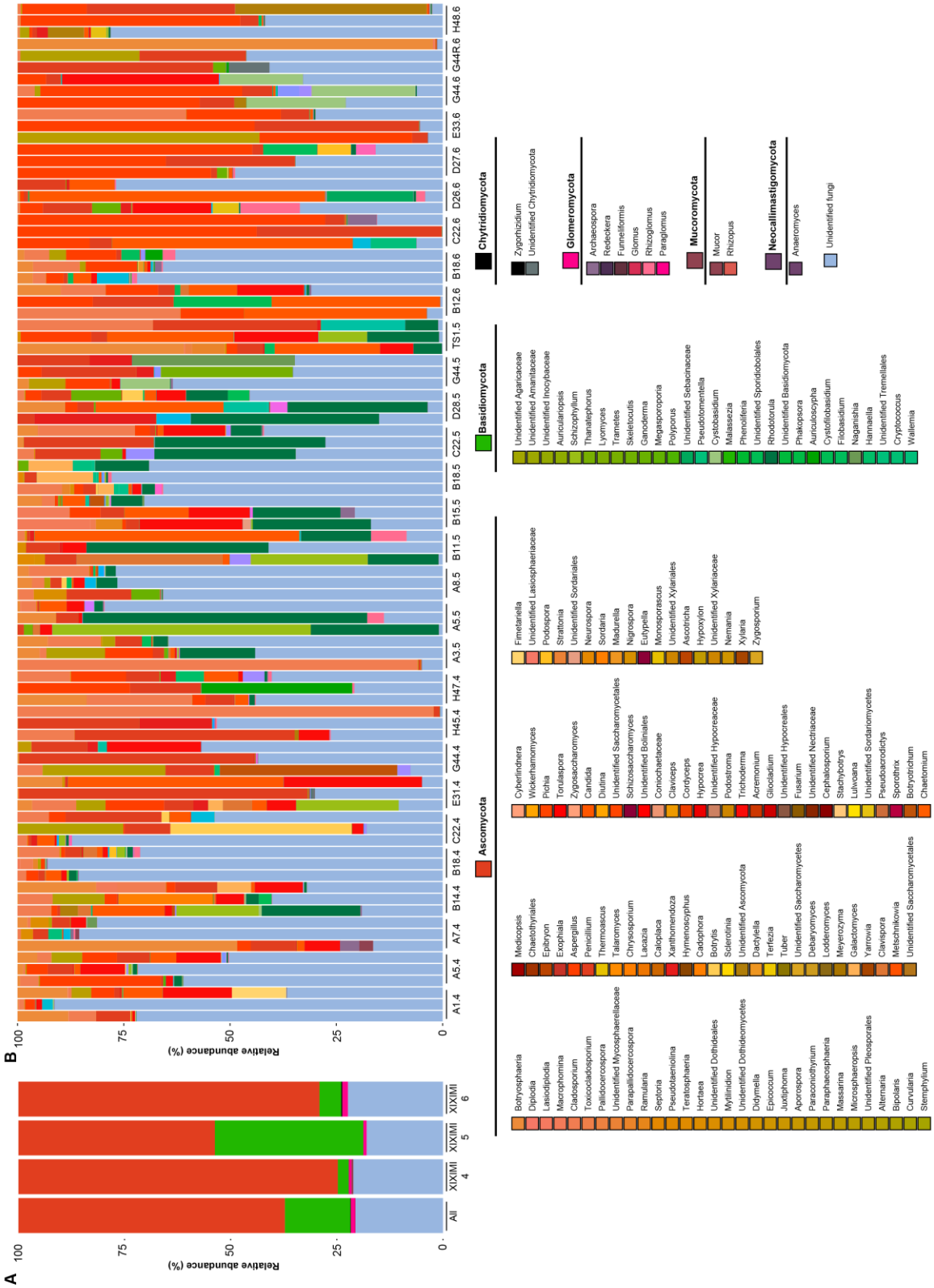
		Relative abundance (%)						
	Genera	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**Blank**
ASCOMYCOTA	Botryosphaeria	0.5721	0.0970	0.0395	0.0037	0.0011	0.0052	0.0422
	Lasiodiplodia	--	--	--	0.0037	--	--	--
	Macrophomina	0.8647	0.0073	0.0269	--	--	--	--
	Cladosporium	0.4922	0.8244	0.6400	--	0.0573	--	98.1749
	Lecanosticta	--	--	0.0016	--	--	--	--
	Pallidocercospora	0.0100	--	--	--	--	--	--
	Parapallidocercospora	--	--	0.0032	--	0.0173	0.0013	--
	Hortaea	0.0366	--	0.0016	--	--	--	--
	Teratosphaeria	0.0033	--	0.0016	--	--	0.0013	--
	unidentified Dothideales	0.5887	--	0.0016	--	--	--	--
	Coniothyrium	--	--	--	--	0.0216	--	--
	Epicoccum	--	--	--	0.0074	--	--	--
	Paraphaeosphaeria	6.1263	--	--	--	--	--	0.0060
	unidentified Montagnulaceae	0.0033	--	--	--	--	--	--
	Microsphaeropsis	0.0366	0.0339	0.1738	--	--	--	--
	unidentified Pleosporales	0.0166	0.0145	0.0126	--	--	--	0.0120
	Alternaria	0.0067	0.0194	0.0111	--	--	--	0.0060
	Pleospora	--	--	--	--	--	0.0026	--
	Stemphylium	--	--	0.0016	--	--	--	--
	unidentified Dothideomycetes	--	--	--	--	--	--	0.0060
	Aspergillus	5.3214	0.6255	1.1441	0.0148	10.1190	0.0142	0.0723
	Penicillium	5.2716	3.8260	0.3951	12.5152	0.0033	29.9522	0.0964
	Talaromyces	--	--	--	--	0.0011	0.0013	--
	Lacazia	0.0100	0.0170	0.0126	--	--	0.0026	--
	Cladia	--	--	--	--	--	0.0013	--
	Caloplaca	0.0067	0.0049	0.0047	--	--	0.0078	--
	Xanthomendoza	0.1929	0.0024	0.0221	--	0.0033	0.0013	--
	Botrytis	--	0.0097	0.0032	--	--	--	0.0482
	unidentified ascomycete	--	--	0.0016	--	--	--	--
	unidentified ascomycete	0.0166	--	--	--	--	--	0.0060
	Candida	1.0477	0.0291	0.1043	0.2989	0.0011	0.0982	0.5060
	Kurtzmaniella	0.1962	0.0145	0.0095	--	--	--	--
	Lodderomyces	0.0432	--	0.0032	--	0.0011	--	--
	Meyerozyma	0.0033	0.0145	4.8483	0.0111	--	--	--
	Galactomyces	0.0067	0.0145	0.0174	--	--	--	--

	Clavispora	0.4490	0.0315	0.0348	--	0.0011	--	--
	Metschnikowia	--	--	--	--	--	0.0026	--
	Candida	0.4390	0.1673	0.0221	0.0111	0.0206	29.3358	0.0602
	Diutina	0.7084	--	--	--	--	--	0.0060
	unidentified Candida	0.0033	0.0024	0.0016	--	--	0.0065	--
	Cyberlindnera	--	--	0.0063	--	--	--	--
	Pichia	0.7051	0.0024	--	--	--	--	--
	unidentified Saccharomycetales	--	--	--	--	--	0.0013	--
	Trichoderma	11.7937	0.0412	0.0948	0.0148	0.0033	0.0026	0.0663
	unidentified Hypocreaceae	0.0067	--	--	--	--	--	--
	Cephalosporium	--	--	--	--	--	--	0.0060
	unidentified Hypocreales	--	0.0509	--	--	--	--	--
	Fusarium	--	--	0.0016	--	--	--	--
	Volutella	--	--	--	--	--	0.0168	--
	Stachybotrys	0.0100	--	--	--	--	--	--
	Botryotrichum	0.0200	0.0097	0.0348	--	--	--	--
	Fimetariella	--	0.0024	--	--	--	--	--
	Podospora	--	0.0073	0.0126	--	--	--	--
	Chrysosporium	--	--	--	--	--	--	0.0060
	Neurospora	10.6595	0.1964	0.3587	0.1771	0.0022	0.0039	0.0843
	Sordaria	0.0499	--	0.0016	--	--	--	0.0060
	Hypoxylon	0.1164	0.0049	0.0032	--	0.0216	--	--
	Xylaria	0.0233	--	0.0016	--	--	--	--
BASIDIOMYCOTA	Schizophyllum	0.1829	0.0145	0.0442	0.0037	--	--	0.0181
	Thanatephorus	0.0466	--	0.0016	--	--	--	--
	Trametes	0.0233	0.0121	0.0174	--	--	--	--
	unidentified Sebacinaceae	0.0033	--	--	--	--	--	--
	Cystobasidium	0.1131	0.0461	0.0458	0.0037	--	--	0.0060
	Malassezia	0.0499	--	0.0032	--	--	0.0103	--
	Phenoliferia	0.0898	0.7177	0.7064	0.7564	0.8503	0.3295	0.0241
	unidentified Kriegeriaceae	--	0.0024	--	--	0.0011	--	--
	unidentified Sporidiobolales	0.0399	--	0.0016	--	--	--	--
	Rhodotorula	12.7016	92.5322	90.3334	86.1528	88.8198	40.1706	0.0723
	Phakopsora	0.0033	--	--	--	--	--	--
	Auriculoscypha	0.0831	--	0.0063	0.0074	0.0022	--	--
	Mrakia	--	--	--	--	0.0011	0.0013	--
	Filobasidium	--	0.0024	0.0032	--	--	--	--
	Naganishia	0.0333	0.0049	0.0221	--	--	--	--
	Wallemia	--	0.0024	0.0126	--	--	--	--

GLOMEROMYCOTA	Archaeospora	0.5521	--	0.0111	--	--	0.0026	--
	Funneliformis	0.0067	--	--	--	--	--	--
	Glomus	--	--	--	--	--	0.0013	--
MUCOROMYCOTA	Mucor	0.0532	--	--	--	--	--	--
	Unidentified fungi	40.1603	0.5940	0.7364	0.0184	0.0508	0.0258	0.6686

Annex 6

Taxonomic profiles of fungi among campaigns and sub-samples from each station. (A) Bar plot of relative abundance at phylum level among campaigns. (B) Bar plot of relative abundance at genus level exhibiting the differences among sub-samples belonging to the same station.



Annex 7

Relative abundance of fungal phyla, classes and orders in all sampled stations.

	Relative abundance (%) XIXIMI 4									
Taxon	**A1.4**	**A5.4**	**A7.4**	**B14.4**	**B18.4**	**C22.4**	**E31.4**	**G44.4**	**H45.4**	**H47.4**
Ascomycota	**59.71**	**39.32**	**68.85**	**55.94**	**9.04**	**79.67**	**93.32**	**76.13**	**89.06**	**43.71**
Dothideomycetes	16.77	8.84	46.37	23.51	5.96	23.96	10.38	22.40	71.02	1.20
Botryosphaeriales	11.46	2.60	45.82	6.60	0.61	0.03	9.77	0.06	---	0.32
Capnodiales	1.02	2.88	0.10	8.94	4.73	0.25	0.03	3.68	71.01	0.87
Dothideales	---	---	0.40	---	0.03	---	---	---	---	---
Mytilinidiales	---	---	---	---	---	---	---	---	---	---
Unidentified Dothideomycetes	---	---	---	---	---	---	---	---	---	---
Pleosporales	4.29	3.37	0.04	7.97	0.59	23.68	0.58	18.66	---	---
Eurotiomycetes	6.60	18.31	12.60	5.52	1.62	11.19	4.95	19.46	14.66	41.84
Chaetothyriales	---	---	---	---	---	---	---	0.01	---	---
Eurotiales	6.60	18.31	12.60	5.52	1.61	11.19	4.95	19.36	14.66	41.83
Onygenales	---	---	---	---	0.01	---	---	0.10	---	---
Lecanoromycetes	0.38	0.14	0.23	0.05	0.08	0.08	0.36	0.53	1.55	0.10
Teloschistales	0.38	0.14	0.23	0.05	0.08	0.08	0.36	0.53	1.55	0.10
Leotiomycetes	0.01	0.18	---	14.39	---	40.82	---	0.00	---	0.00
Helotiales	0.01	0.18	---	14.39	---	40.82	---	---	---	---
Unidentified Ascomycota	0.21	---	0.00	0.47	0.01	0.01	0.02	0.00	0.19	---
Orbiliomycetes	---	---	---	---	---	---	---	---	---	---
Orbiliales	---	---	---	---	---	---	---	---	---	---
Pezizomycetes	---	---	---	---	---	---	0.00	---	---	---
Pezizales	---	---	---	---	---	---	---	---	---	---
Saccharomycetes	8.74	3.16	1.87	4.31	0.83	0.23	47.25	1.17	0.03	0.42
Unidentified Saccharomycetes	0.01	---	---	---	---	---	---	---	---	---
Saccharomycetales	8.73	3.16	1.87	4.31	0.83	0.23	47.25	1.17	0.03	0.42
Schizosaccharomycetes	0.01	---	---	---	---	---	0.00	0.00	---	0.00
Schizosaccharomycetales	0.01	---	---	---	---	---	---	---	---	---
Sordariomycetes	27.00	8.70	7.79	7.68	0.55	3.39	30.36	32.56	1.61	0.14
Boliniales	---	---	---	---	---	---	---	---	---	---
Coniochaetales	---	---	---	---	---	---	---	---	---	---
Hypocreales	15.17	7.96	7.74	7.52	0.28	2.72	30.35	4.53	1.56	0.04
Lulworthiales	---	---	---	---	---	---	---	---	---	---
Unidentified Sordariomycetes	---	0.04	---	---	0.02	0.01	---	0.03	---	---
Ophiostomatales	---	---	---	---	---	---	---	---	---	---
Sordariales	11.82	0.14	0.04	0.08	0.23	0.11	0.01	26.07	0.05	---
Trichosphaeriales	---	---	---	---	---	---	---	---	---	---
Xylariales	---	0.56	---	0.08	0.01	0.55	---	1.92	---	0.10

Basidiomycota	**0.06**	**1.47**	**0.19**	**7.79**	**1.04**	**0.07**	**0.03**	**0.08**	**0.02**	**34.17**
Agaricomycetes	0.01	0.21	0.01	1.55	0.30	0.04	0.01	0.01	0.00	0.01
Agaricales	---	0.21	---	1.55	0.28	0.04	0.01	---	---	0.01
Cantharellales	---	---	---	---	---	---	---	---	---	---
Corticiales	---	---	---	---	---	---	---	---	---	---
Polyporales	0.01	---	---	---	---	---	---	---	---	---
Sebacinales	---	---	0.01	---	0.02	---	---	---	---	---
Thelephorales	---	---	---	---	---	---	---	---	---	---
Cystobasidiomycetes	0.02	0.18	0.02	0.26	0.17	0.01	---	0.00	---	---
Cystobasidiales	0.02	0.18	0.02	0.26	0.17	0.01	---	---	---	---
Malasseziomycetes	---	---	---	---	0.01	0.00	---	---	---	---
Malasseziales	---	---	---	---	0.01	---	---	---	---	---
Microbotryomycetes	0.01	0.95	0.03	3.89	0.41	0.01	0.02	0.08	0.02	0.06
Kriegeriales	---	0.04	---	0.03	0.02	---	0.01	0.06	0.01	---
Sporidiobolales	0.01	0.91	0.02	3.87	0.39	0.01	0.01	0.02	0.01	0.06
Unidentified Basidiomycota	---	---	0.00	---	---	---	---	---	---	---
Pucciniomycetes	---	0.04	---	---	---	---	---	---	0.00	34.10
Pucciniales	---	---	---	---	---	---	---	---	---	---
Septobasidiales	---	0.04	---	---	---	---	---	---	---	34.09
Tremellomycetes	0.02	0.11	0.13	2.03	0.14	---	---	---	---	---
Cystofilobasidiales	---	---	---	1.97	0.01	---	---	---	---	---
Filobasidiales	---	0.11	0.13	0.03	---	---	---	---	---	---
Tremellales	0.02	---	---	0.03	0.13	---	---	---	---	---
Wallemiomycetes	0.00	---	---	0.05	---	0.02	---	---	---	---
Wallemiales	---	---	---	0.05	---	0.02	---	---	---	---
Chytridiomycota	**0.11**	**---**	**---**	**---**	**0.01**	**---**	**0.05**	**0.00**	**---**	**---**
Chytridiomycetes	---	---	---	---	---	---	0.00	---	---	---
Chytridiales	---	---	---	---	---	---	---	---	---	---
Unidentified Chytridiomycota	0.11	---	---	---	0.01	---	0.05	0.00	---	---
Glomeromycota	**0.02**	**0.14**	**3.94**	**0.03**	**0.20**	**0.02**	**0.01**	**0.04**	**0.10**	**0.52**
Archaeosporomycetes	0.00	0.04	3.92	0.03	0.02	0.01	0.01	0.01	---	0.01
Archaeosporales	---	0.04	3.92	0.03	0.02	0.01	0.01	0.01	---	0.01
Glomeromycetes	0.02	0.11	0.02	---	0.18	0.01	0.01	0.02	0.10	0.51
Diversisporales	---	---	---	---	---	---	---	---	---	---
Glomerales	0.02	0.11	0.02	---	0.18	0.01	0.01	0.02	0.10	0.51
Paraglomeromycetes	---	---	---	---	---	---	---	---	---	---
Paraglomerales	---	---	---	---	---	---	---	---	---	---
Mucoromycota	**0.01**	**---**	**2.94**	**---**	**---**	**---**	**---**	**---**	**---**	**---**
Neocallimastigomycota	**---**	**---**	**0.00**	**---**	**---**	**---**	**---**	**---**	**---**	**---**
Unidentified fungi	**40.09**	**59.07**	**24.07**	**36.24**	**89.71**	**20.24**	**6.58**	**23.75**	**10.81**	**21.60**

Taxon	Relative abundance (%) XIXIMI 5									
	A3.5	A5.5	A8.5	B11.5	B15.5	B18.5	C22.5	D28.5	G44.5	TS1.5
Ascomycota	**89.29**	**11.27**	**29.85**	**43.85**	**54.42**	**19.50**	**32.42**	**51.05**	**28.54**	**86.57**
Dothideomycetes	87.41	3.84	11.15	4.05	15.95	1.27	2.34	6.22	0.73	32.63
Botryosphaeriales	0.84	3.77	1.03	---	13.65	0.07	1.15	6.14	0.17	21.39
Capnodiales	85.68	0.07	4.26	2.13	2.30	1.07	1.11	0.06	0.05	10.83
Dothideales	0.01	---	5.58	1.92	---	0.06	0.04	0.01	---	0.41
Mytilinidiales	---	---	---	---	---	---	---	---	---	---
Unidentified Dothideomycetes	---	---	---	---	---	---	---	---	---	---
Pleosporales	0.88	---	0.28	---	---	0.07	0.04	0.02	0.51	0.01
Eurotiomycetes	1.40	4.23	16.56	6.23	10.07	2.76	28.07	5.88	19.36	17.06
Chaetothyriales	---	---	---	---	---	---	0.01	---	---	---
Eurotiales	1.36	4.23	11.72	6.23	10.06	1.16	26.96	5.57	19.36	17.06
Onygenales	0.04	---	4.84	---	---	1.60	1.11	0.31	---	---
Lecanoromycetes	---	0.05	0.03	0.47	0.01	0.03	0.03	12.52	7.84	0.43
Teloschistales	---	0.05	0.03	0.47	0.01	0.03	0.03	12.52	7.84	0.43
Leotiomycetes	---	---	0.24	---	---	0.03	---	0.05	---	0.00
Helotiales	---	---	0.24	---	---	0.03	---	0.05	---	---
Unidentified Ascomycota	0.01	0.00	---	---	0.01	0.00	---	0.00	0.00	0.00
Orbiliomycetes	---	---	---	---	---	---	---	---	---	---
Orbiliales	---	---	---	---	---	---	---	---	---	---
Pezizomycetes	---	---	---	0.00	---	---	---	0.00	---	---
Pezizales	---	---	---	---	---	---	---	---	---	---
Saccharomycetes	0.39	1.02	0.53	28.85	10.52	15.05	0.07	22.65	0.04	26.63
Unidentified Saccharomycetes	---	---	---	---	---	---	---	---	---	---
Saccharomycetales	0.39	1.02	0.53	28.85	10.52	15.05	0.07	22.65	0.04	26.63
Schizosaccharomycetes	---	0.00	---	0.00	---	---	---	---	---	---
Schizosaccharomycetales	---	---	---	---	---	---	---	---	---	---
Sordariomycetes	0.07	2.12	1.35	4.24	17.87	0.36	1.91	3.73	0.56	9.82
Boliniales	0.03	---	---	---	---	---	---	---	---	---
Coniochaetales	---	---	---	---	---	---	---	---	---	---
Hypocreales	0.03	2.07	0.60	1.96	17.19	0.15	0.35	3.68	0.12	9.80
Lulworthiales	---	---	0.51	---	---	---	---	---	---	---
Unidentified Sordariomycetes	---	---	0.01	---	---	---	0.01	0.01	---	---
Ophiostomatales	---	---	---	---	---	---	---	---	---	---
Sordariales	---	0.05	0.22	0.04	0.67	0.03	0.07	0.04	0.07	0.02
Trichosphaeriales	---	---	---	---	---	---	---	---	---	---
Xylariales	---	---	0.01	2.24	---	0.19	1.48	---	0.36	---
Basidiomycota	**1.08**	**79.46**	**1.44**	**38.22**	**23.24**	**9.00**	**38.30**	**39.97**	**34.88**	**12.89**
Agaricomycetes	0.09	34.58	0.08	12.57	0.13	0.13	0.03	0.04	7.99	3.18

Agaricales	0.02	34.54	0.08	12.57	0.13	0.12	0.03	0.04	0.01	3.18
Cantharellales	---	---	---	---	---	---	---	---	---	---
Corticiales	---	0.04	---	---	---	---	---	---	---	---
Polyporales	0.06	---	---	---	---	0.01	---	---	7.98	---
Sebacinales	---	---	---	---	---	---	---	---	---	---
Thelephorales	---	---	---	---	---	---	---	---	---	---
Cystobasidiomycetes	0.01	---	0.07	---	0.01	0.01	---	0.00	0.68	0.00
Cystobasidiales	0.01	---	0.07	---	0.01	0.01	---	---	0.68	---
Malasseziomycetes	---	---	0.01	---	0.43	---	---	2.21	---	0.00
Malasseziales	---	---	0.01	---	0.43	---	---	2.21	---	---
Microbotryomycetes	0.98	44.88	1.27	25.64	22.66	8.76	38.26	37.66	0.03	9.70
Kriegeriales	0.01	---	---	---	---	---	0.01	---	---	---
Sporidiobolales	0.97	44.88	1.27	25.64	22.65	8.76	38.25	37.66	0.03	9.70
Unidentified Basidiomycota	---	---	---	---	---	---	---	---	---	---
Pucciniomycetes	---	0.00	0.01	0.00	---	---	0.00	0.00	0.00	---
Pucciniales	---	---	---	---	---	---	---	---	---	---
Septobasidiales	---	---	0.01	---	---	---	---	---	---	---
Tremellomycetes	0.01	---	---	0.01	0.01	0.10	0.01	0.06	26.17	0.01
Cystofilobasidiales	---	---	---	---	---	---	---	0.05	---	---
Filobasidiales	0.01	---	---	---	---	0.10	0.01	---	26.17	---
Tremellales	---	---	---	0.01	---	---	---	---	0.01	0.01
Wallemiomycetes	---	---	---	---	---	---	---	---	0.00	---
Wallemiales	---	---	---	---	---	---	---	---	---	---
Chytridiomycota	**---**	**0.00**	**---**	**---**	**---**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
Chytridiomycetes	---	---	---	---	---	---	---	---	---	---
Chytridiales	---	---	---	---	---	---	---	---	---	---
Unidentified Chytridiomycota	---	0.00	---	---	---	0.00	0.00	0.00	0.00	0.00
Glomeromycota	**0.05**	**1.67**	**0.05**	**1.66**	**2.32**	**0.27**	**0.01**	**0.03**	**0.03**	**0.01**
Archaeosporomycetes	0.03	0.01	---	---	2.32	0.00	---	0.01	0.03	0.00
Archaeosporales	0.03	0.01	---	---	2.32	---	---	0.01	0.03	---
Glomeromycetes	0.01	1.66	0.05	1.66	0.00	0.27	0.01	0.01	0.00	0.01
Diversisporales	---	---	---	---	---	---	---	---	---	---
Glomerales	0.01	1.66	0.05	1.66	---	0.27	0.01	0.01	---	0.01
Paraglomeromycetes	---	---	---	---	---	---	---	---	---	---
Paraglomerales	---	---	---	---	---	---	---	---	---	---
Mucoromycota	**---**	**0.01**	**---**	**0.00**	**---**	**---**	**---**	**0.00**	**---**	**---**
Neocallimastigomycota	**---**	**---**	**---**	**0.00**	**---**	**---**	**---**	**---**	**---**	**---**
Unidentified fungi	**9.58**	**7.60**	**68.66**	**16.27**	**20.02**	**71.23**	**29.27**	**8.95**	**36.55**	**0.52**

	Relative abundance (%)								
	XIXIMI 6								
Taxon	B12.6	B18.6	C22.6	D26.6	D27.6	E33.6	G44.6	G44R.6	H48.6
Ascomycota	**97.90**	**29.33**	**88.11**	**46.54**	**74.09**	**84.07**	**55.03**	**69.84**	**70.25**
Dothideomycetes	15.92	11.15	0.07	0.36	0.04	32.30	1.31	48.87	0.91
Botryosphaeriales	0.07	2.87	0.01	0.05	0.01	0.02	0.02	37.44	0.10
Capnodiales	15.84	6.41	0.05	0.26	0.01	17.47	0.97	0.31	0.64
Dothideales	---	---	---	0.03	---	0.01	---	---	0.01
Mytilinidiales	---	---	---	---	---	---	---	---	---
Unidentified Dothideomycetes	---	---	---	---	---	---	---	---	---
Pleosporales	---	1.86	0.01	0.02	0.02	14.80	0.32	11.13	0.16
Eurotiomycetes	27.59	10.07	64.64	12.01	59.43	51.24	36.13	20.87	52.61
Chaetothyriales	---	---	---	---	---	0.01	---	---	---
Eurotiales	27.59	10.01	64.63	10.08	59.36	51.23	36.13	20.22	52.60
Onygenales	---	0.06	0.01	1.93	0.08	---	0.01	0.65	0.01
Lecanoromycetes	0.02	0.06	0.05	1.01	0.03	0.08	0.04	0.01	0.10
Teloschistales	0.02	0.06	0.05	1.01	0.03	0.08	0.04	0.01	0.10
Leotiomycetes	---	0.04	---	0.00	0.01	---	0.00	---	0.01
Helotiales	---	0.04	---	---	0.01	---	---	---	0.01
Unidentified Ascomycota	0.00	0.01	0.00	0.01	0.00	0.00	1.07	0.00	15.46
Orbiliomycetes	---	0.33	---	---	---	---	---	---	---
Orbiliales	---	0.33	---	---	---	---	---	---	---
Pezizomycetes	---	---	---	---	0.00	---	---	---	---
Pezizales	---	---	---	---	---	---	---	---	---
Saccharomycetes	54.26	3.72	21.71	25.92	8.95	0.39	0.30	0.07	0.90
Unidentified Saccharomycetes	---	---	---	---	---	---	---	---	---
Saccharomycetales	54.26	3.72	21.71	25.92	8.95	0.39	0.30	0.07	0.90
Schizosaccharomycetes	---	---	---	---	0.00	---	0.00	0.00	---
Schizosaccharomycetales	---	---	---	---	---	---	---	---	---
Sordariomycetes	0.11	3.96	1.63	7.23	5.62	0.07	16.18	0.01	0.27
Boliniales	---	---	---	---	---	---	---	---	---
Coniochaetales	---	---	---	---	---	---	---	---	---
Hypocreales	0.11	3.46	1.62	5.45	0.07	0.03	14.25	0.01	0.12
Lulworthiales	---	---	---	---	---	---	---	---	---
Unidentified Sordariomycetes	---	---	---	1.76	---	---	---	---	0.09
Ophiostomatales	---	---	---	---	---	---	---	---	---
Sordariales	---	0.08	0.01	0.01	5.56	0.03	0.07	---	0.03
Trichosphaeriales	---	---	---	---	---	---	---	---	---
Xylariales	---	0.42	---	---	---	0.01	1.86	---	0.03
Basidiomycota	**0.02**	**1.96**	**4.21**	**6.66**	**4.11**	**0.26**	**22.31**	**0.19**	**0.41**

Agaricomycetes	0.01	0.07	0.03	6.36	0.00	0.05	0.03	0.01	0.09
Agaricales	0.01	0.07	0.01	0.02	---	0.03	---	---	0.03
Cantharellales	---	---	---	0.04	---	0.01	0.02	---	0.03
Corticiales	---	---	---	---	---	---	---	---	---
Polyporales	---	---	0.02	0.02	---	0.01	---	---	0.02
Sebacinales	---	---	---	6.28	---	---	---	---	0.01
Thelephorales	---	---	---	---	---	---	---	---	---
Cystobasidiomycetes	---	0.04	---	0.00	0.00	0.00	22.18	---	0.04
Cystobasidiales	---	0.04	---	---	---	---	22.18	---	0.04
Malasseziomycetes	---	---	---	0.00	---	0.00	0.00	---	0.00
Malasseziales	---	---	---	---	---	---	---	---	---
Microbotryomycetes	0.01	0.07	0.03	0.24	0.85	0.18	0.09	0.04	0.23
Kriegeriales	---	---	---	---	---	---	---	0.03	---
Sporidiobolales	0.01	0.07	0.03	0.24	0.85	0.18	0.09	0.01	0.23
Unidentified Basidiomycota	---	---	---	---	---	---	---	---	---
Pucciniomycetes	---	1.54	0.00	0.01	---	---	0.00	0.14	0.01
Pucciniales	---	---	---	---	---	---	---	---	---
Septobasidiales	---	1.54	---	0.01	---	---	---	0.14	0.01
Tremellomycetes	0.00	0.07	4.14	0.04	0.00	0.03	0.01	0.00	0.04
Cystofilobasidiales	---	---	---	---	---	---	---	---	---
Filobasidiales	---	0.06	0.04	0.04	---	0.03	0.01	---	0.04
Tremellales	---	0.01	4.10	---	---	---	---	---	---
Wallemiomycetes	---	0.17	0.00	---	3.25	---	0.00	---	---
Wallemiales	---	0.17	---	---	3.25	---	---	---	---
Chytridiomycota	**---**	**---**	**---**	**0.00**	**---**	**---**	**0.00**	**2.12**	**0.00**
Chytridiomycetes	---	---	---	---	---	---	---	---	---
Chytridiales	---	---	---	---	---	---	---	---	---
Unidentified Chytridiomycota	---	---	---	0.00	---	---	0.00	2.12	0.00
Glomeromycota	**0.04**	**1.92**	**1.65**	**4.80**	**0.02**	**0.04**	**0.01**	**0.02**	**0.06**
Archaeosporomycetes	0.03	0.49	1.62	0.01	0.00	0.02	0.00	0.02	0.01
Archaeosporales	0.03	0.49	1.62	0.01	---	0.02	---	0.02	0.01
Glomeromycetes	0.01	1.43	0.03	4.79	0.02	0.02	0.01	0.00	0.05
Diversisporales	---	---	---	---	---	---	---	---	---
Glomerales	0.01	1.43	0.03	4.79	0.02	0.02	0.01	---	0.05
Paraglomeromycetes	---	---	---	0.00	---	---	---	---	---
Paraglomerales	---	---	---	---	---	---	---	---	---
Mucoromycota	**---**	**---**	**---**	**---**	**---**	**---**	**---**	**---**	**---**
Neocallimastigomycota	**---**	**---**	**---**	**---**	**---**	**---**	**---**	**---**	**---**
Unidentified fungi	**2.03**	**66.79**	**6.03**	**42.00**	**21.78**	**15.62**	**22.65**	**27.84**	**29.27**

Annex 8

Pairwise comparisons among groups formed by categorical variables using presence/absence data.

Region

Pairs	Df	Sums Of Sqs	F. Model	R^2	P value	P adjusted
TVCS vs AP	1	0.027709139	4.0174596	0.191149	0.037	0.518
TVCS vs YCS	1	0.046107924	9.0127236	0.562848	0.013	0.182
TVCS vs CC	1	0.003396137	0.6065797	0.063142	0.592	1
TVCS vs CZC	1	0.006747459	1.0822164	0.177931	0.445	1
TVCS vs CSD	1	0.016960393	2.7202558	0.352353	0.136	1
AP vs YCS	1	0.013342536	2.0591556	0.128223	0.164	1
AP vs CC	1	0.014097089	2.1421504	0.118076	0.125	1
AP vs CZC	1	0.002043866	0.2849329	0.023194	1	1
AP vs CSD	1	0.00430564	0.6002441	0.047638	0.61	1
YCS vs CC	1	0.032701001	8.2259626	0.578236	0.016	0.224
YCS vs CZC	1	0.006799265	2.9327211	0.594544	0.25	1
YCS vs CSD	1	0.001496619	0.6455355	0.244009	1	1
CC vs CZC	1	0.003905333	0.8129669	0.168912	0.5	1
CC vs CSD	1	0.012218867	2.5435821	0.388714	0.333333	1
CZC vs CSD	1	0.003880303	NaN	1	NA	NA

Depth

Pairs	Df	Sums Of Sqs	F. Model	R^2	P value	P adjusted
2000-2500 m vs 3000-3500 m	1	0.010457026	1.554577	0.123826	0.195	1
2000-2500 m vs 3500 - > m	1	0.009758725	1.5875272	0.101846	0.196	1
2000-2500 m vs 1000-1500 m	1	0.006431161	0.8025053	0.054214	0.421	1
3000-3500 m vs 3500 - > m	1	0.002066992	0.2706427	0.024013	0.837	1
3000-3500 m vs 1000-1500 m	1	0.002750307	0.2746685	0.024362	0.743	1
3500 - > m vs 1000-1500 m	1	0.002790767	0.3196962	0.022326	0.751	1

Latitude

Pairs	Df	Sums Of Sqs	F. Model	R^2	P value	P adjusted
21 vs 25	1	0.040354196	8.2461585	0.451939	0.014	0.21
21 vs 24	1	0.003672759	0.5130138	0.048798	0.611	1
21 vs 22	1	0.002051093	0.3100312	0.042412	0.852	1
21 vs 20	1	0.003496766	0.573235	0.087207	0.573	1
21 vs 23	1	0.015132734	2.5626668	0.299284	0.156	1
25 vs 24	1	0.030016748	5.2931785	0.306085	0.021	0.315
25 vs 22	1	0.025808845	5.4310828	0.376346	0.019	0.285
25 vs 20	1	0.045636901	11.0438942	0.579918	0.008	0.12
25 vs 23	1	0.004119182	1.0333912	0.114397	0.305	1
24 vs 22	1	0.001602356	0.2204288	0.023907	0.99	1
24 vs 20	1	0.007615442	1.0935166	0.120252	0.335	1
24 vs 23	1	0.008987022	1.3181436	0.14146	0.252	1
22 vs 20	1	0.005760668	0.946484	0.159167	0.404	1
22 vs 23	1	0.008978866	1.5342175	0.234797	0.261	1
20 vs 23	1	0.020732603	4.241524	0.514653	0.1	1

Longitude

Pairs	Df	Sums Of Sqs	F. Model	R^2	P value	P adjusted
264 vs 268	1	0.018661934	2.5145247	0.264283	0.14	1
264 vs 270	1	0.016063781	2.625335	0.344291	0.144	1
264 vs 273	1	0.030255255	5.8627169	0.494214	0.03	0.84
264 vs 265	1	0.001745922	0.3042993	0.032705	0.923	1
264 vs 263	1	0.00619641	0.9765721	0.1634	0.387	1
264 vs 267	1	0.001701275	0.2988488	0.036011	0.895	1
264 vs 266	1	0.018200793	3.2843315	0.396451	0.112	1
268 vs 270	1	0.001795919	0.2403245	0.056676	0.666667	1
268 vs 273	1	0.003941102	0.6511737	0.115228	0.498	1
268 vs 265	1	0.017710933	2.7817072	0.258002	0.108	1
268 vs 263	1	0.028464971	3.6701631	0.478499	0.133333	1
268 vs 267	1	0.021065186	3.2885062	0.319629	0.1	1
268 vs 266	1	0.002987747	0.4425255	0.099611	0.733333	1
270 vs 273	1	0.00147295	0.4962928	0.141948	0.5	1
270 vs 265	1	0.015486703	3.1415715	0.343658	0.126	1
270 vs 263	1	0.025989477	5.3778917	0.72892	0.333333	1
270 vs 267	1	0.017657372	3.7597522	0.429208	0.091	1
270 vs 266	1	0.001321427	0.467885	0.189589	1	1
273 vs 265	1	0.029479924	6.8906914	0.496065	0.031	0.868
273 vs 263	1	0.039694639	11.8665622	0.798205	0.1	1
273 vs 267	1	0.032701001	8.2259626	0.578236	0.016	0.448
273 vs 266	1	0.001535359	0.7653247	0.203256	0.4	1
265 vs 263	1	0.008078809	1.5784493	0.208281	0.235	1
265 vs 267	1	0.001851222	0.3741862	0.039917	0.874	1
265 vs 266	1	0.017275255	3.8831894	0.392909	0.039	1
263 vs 267	1	0.005860215	1.190441	0.192303	0.305	1
263 vs 266	1	0.028480134	8.4011594	0.807714	0.333333	1
267 vs 266	1	0.020139754	4.8890397	0.49439	0.054	1

Core

Pairs	Df	Sums Of Sqs	F. Model	R^2	P value	P adjusted
Boxcorer vs Multicorer	1	0.03089437	4.56743	0.144688	0.024	0.024*

All values indicated with ***** represented significant Bonferroni corrected values with $P<0.05$.
AP, Abyssal Plain; CC, Campeche canyon; CSD, Campeche saline domo; CZC, Coatzacoalcos canyon; TVCS, Tamaulipas-Veracruz cont. Slope; YCS, Yucatan continental slope.

Annex 9

PERMANOVA analysis on NMDS ordination, ANOVA analysis on Beta Dispersion, and Mantel test correlations, testing the influence of Corer type on the fungal community.

	PERMANOVA	Df	SumsOfSqs	MeanSqs	F.Model	R^2	Pr(>F)	P adjusted
Multicorer	**Depth**	3	1144	381.35	0.93821	0.21964	0.721	>0.05
	Residuals	10	4064.7	406.47		0.78036		
	Total	13	5208.7			1		
	Latitude	3	1194.8	398.28	0.99227	0.22939	0.497	>0.05
	Residuals	10	4013.9	401.39		0.77061		
	Total	13	5208.7			1		
	Longitude	3	1345.5	448.5	1.161	0.25832	0.084	>0.05
	Residuals	10	3863.2	386.32		0.74168		
	Total	13	5208.7			1		
Boxcorer	**Depth**	3	803.5	267.83	0.97333	0.20977	0.467	>0.05
	Residuals	11	3026.9	275.17		0.79023		
	Total	14	3830.4			1		
	Latitude	2	663.6	331.82	1.2574	0.17326	0.138	>0.05
	Residuals	12	3166.8	263.9		0.82674		
	Total	14	3830.4			1		
	Longitude	4	1091.5	272.87	0.99627	0.28495	0.45	>0.05
	Residuals	10	2738.9	273.89		0.71505		
	Total	14	3830.4			1		

	ANOVA	Df	SumsOfSqs	MeanSqs	F.Model	Pr(>F)	P adjusted
Multicorer	**Depth**	3	107.424	35.808	3.6145	0.05323	>0.05
	Residuals	10	99.067	9.907			
	Latitude	3	44.073	14.6909	2.2722	0.1425	>0.05
	Residuals	10	64.656	6.4656			
	Longitude	3	29.889	9.9631	1.4189	0.2944	>0.05
	Residuals	10	70.218	7.0218			
Boxcorer	**Depth**	3	195.38	65.127	4.7454	0.02327	>0.05
	Residuals	11	150.97	13.724			
	Latitude	2	4.76	2.3785	0.0799	0.9237	>0.05
	Residuals	12	357.11	29.7594			
	Longitude	4	38.266	9.5665	0.3525	0.8366	>0.05
	Residuals	10	271.369	27.1369			

MANTEL CORRELATION	Multicorer		Boxcorer	
	Mantel statistic r	Significance (P)	Mantel statistic r	Significance (P)
Depth	0.05251	0.366	-0.02645	0.525
Latitude	-0.007625	0.47	0.3746	0.032
Longitude	0.3275	0.008	0.03407	0.384
Water content	0.2868	0.157	0.3024	0.05
Carbonates	0.05221	0.399	-0.09807	0.703
Carbon	0.1471	0.2	0.07427	0.344
Terrigenous	0.05817	0.392	-0.07557	0.62
Nitrogen	0.02062	0.454	0.2005	0.146
C/N ratio	-0.2055	0.893	-0.1053	0.759