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Irene Marianna Fugalli **Characterization of fungal diversity in dolphins from the Portuguese coast**

Universidade do Minho
Escola de Ciências

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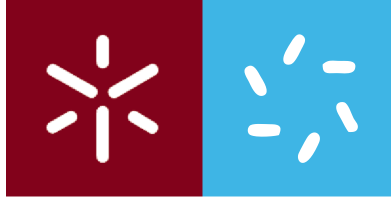
Dezembro de 2017



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Characterization of fungal diversity in dolphins from the Portuguese coast

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Professora Célia do Sacramento Santos Pais

e do
Doutor Ricardo Franco-Duarte

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“L'uomo che è cieco alle bellezze della natura ha perduto metà del piacere di vivere"

Sir Robert Baden Powell

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Caracterização da diversidade fúngica em golfinhos da costa portuguesa

RESUMO

A composição da comunidade microbiana presente em mamíferos marinhos foi recentemente indicada como uma ferramenta para avaliar o estado de saúde das suas populações e, indiretamente, do ecossistema marinho. Esta comunidade pode ainda avaliar o risco de doenças infecciosas emergentes e eventos zoonóticos. Atualmente encontra-se bastante informação sobre o microbioma bacteriano. Contudo, há um reduzido conhecimento sobre o microbioma fúngico. O objetivo desta tese é caracterizar o microbioma fúngico cultivável de populações de golfinhos da zona costeira do norte de Portugal para determinar se este pode ser usado como ferramenta de triagem de saúde de comunidades e ecossistemas, bem como avaliar o papel dos golfinhos como potenciais vetores de fungos patogénicos. Para tal, as estirpes de fungos foram isoladas de amostras de golfinhos coletados pela Sociedade Portuguesa de Vida Selvagem nomeadamente da cavidade oral e do espiráculo de indivíduos das espécies *Phocoena phocoena, Delphinus delphis e Stenella coeruleoalba,* encontrados nas praias portuguesas em 2014 e 2015. Os isolados foram identificados por sequenciação das regiões ITS dos genes de DNA ribossomal e, em alguns casos, dos genes da ß-tubulina e do factor de elongação 1α (EF-1α), comparando as sequências obtidas com bases de dados online.

Nos 122 isolados obtidos foram identificadas 25 espécies de fungos diferentes, indicando que uma comunidade fúngica rica está presente em golfinhos selvagens. Várias das espécies identificadas são de interesse clínico, especialmente como agentes patogénicos oportunistas, e a sua presença em golfinhos pode ser resultado da existência de um elevado número de fatores de *stress* no ecossistema. Análises de similaridade entre os golfinhos indicam que o microbioma fúngico pode ser específico da comunidade, sugerindo um potencial em estudos de ecologia. Este trabalho representa a primeira caraterização da comunidade microbiana fúngica de golfinhos de Portugal.

Characterization of fungal diversity in dolphins from the Portuguese coast

ABSTRACT

The study of the composition of the microbial community harbored by marine mammals has been recently indicated as a tool to assess the health status of these populations and, indirectly, of marine ecosystem. Moreover, it serves to assess the risk of new emerging infectious diseases and zoonotic events. Nowadays, there is much information about the bacterial microbiome, however knowledge about the fungal microbiome is very reduced.

The aim of this thesis is to characterize the cultivable fungal microbiome of free-ranging dolphin populations of northern Portugal shores, to determine if it may be used as a health screening tool of communities and ecosystem, as well as to assess the role of dolphins as vectors of fungal pathogens.

To do this, fungal strains were isolated from cultured tissue samples collected by the Sociedade Portuguesa de Vida Selvagem from blowhole and oral cavity of stranded individuals of *Phocoena phocoena, Delphinus delphis* and *Stenella coeruleoalba*, found along Portuguese beaches in 2014. The isolates were identified by sequencing the ITS regions of rDNA genes and, in some cases, the ß-tubulin and elongation factor 1α (EF-1α) genes. Identification was achieved by comparing the obtained sequences with online databases.

25 different fungal species were identified from 122 isolates, indicating that a rich and diverse fungal community is hosted by free-ranging dolphins. Several of the identified species were of clinical interest, especially as opportunistic pathogens, and their presence in dolphins may result from high stress factors in the ecosystem. The analysis of similarity among dolphins indicates that the fungal microbiome may be community specific, suggesting a potential in ecology studies.

This work represents the first characterization of the fungal microbial community of free-ranging dolphins of Portugal.

TABLE OF CONTENTS

AGRADECIMENTOS v

RESUMO vii

ABSTRACT ix

TABLE OF CONTENTS xi

LIST OF ABBREVIATIONS xiv

LIST OF FIGURES xv

Chapter I - Introduction 1

1.1 Marine mammals and marine ecosystem degradation 3

1.2 Marine mammals in Portugal 4

1.2.1 Species studied in this work 4

1.2.2 Studies on Portuguese dolphins 5

1.3 The Kingdom of Fungi 6

1.3.1 Characteristics and ecology 6

1.4 Emerging Infectious Diseases in dolphins 8

1.5 Relevant fungal species in dolphins 9

1.6 Studying the microbiota of marine mammals 13

1.6.1 Microbiome and mycobiome 13

1.6.2 Molecular identification of fungi 14

1.6.3 Objectives 16

Chapter II - Materials and Methods 19

2.1 Samples collection 21
2.2 Growth and isolation of fungi 22
2.2.1 Culture Media 22
2.2.2 Sample inoculation 22
2.3. DNA extraction 23
2.3.1 DNA extraction from filamentous fungi 23
2.3.2 DNA extraction from yeasts 24
2.4 Species Identification 24
2.4.1.PCR amplification 24
2.4.2 Sequence analysis 28
2.5 Data analysis 28
Chapter III - Results 31
3.1 Analysis of fungal isolates 33
3.2 Species diversity analysis 36
Chapter IV - Discussion 47
4.1 Analysis of fungal growth 49
4.1.1 Fungal diversity according to body sites 49
4.1.2 Fungal diversity according to different temperatures and culture media 50
4.2. Fungal identification 50
4.2.1 List of identified species. 50

4.2.3 Final remarks 56

4.3 Richness and diversity analysis. 57

Chapter V - Conclusions 59

Chapter VI - Bibliography 63

Appendix 73

Appendix 1 - Tables 75

Appendix 2 - Reagents for buffer solution preparation 85

LIST OF ABBREVIATIONS

EID	Emerging Infectious Diseases
PCB	Poly Chlorinated Biphenyl
DNA	Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
rDNA	ribosomal DNA
RNA	Ribonucleic Acid
LSU	Large Subunit
SSU	Small Subunit
ITS	Internal Transcribed Spacer
EF	Elongation Factor
SPVS	Sociedade Portuguesa da Vida Selvagem
BTAM	Banco dos Tecidos dos Animais Marinhos
PDA	Potato Dextrose Agar
SAB	Sabouraud
TES	N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid
TAE	Tris-acetate-EDTA
QC	Query Coverage
ID	Identity score

LIST OF FIGURES

Figure 1.1: (A): Qualitative diagram showing the concept of 'rare biosphere', that is the ensemble of microbiotas that represents less than 1% of the total microbial community. In (B) is shown the ration between fungi and bacteria in a human fecal sample. Fungi are part of the 'rare biosphere'. Figure adapted from (Huffnagle & and Noverr, 2013). 14

Figure 1.2: Schematic representation of the ITS region of rDNA. Light blue rectangles represent the coding regions, black lines the ITS region. Red triangles show the anchor point of the its1-4 primer pair. Primer names are in lower case to distinguish them from region names (upper case). The sequence is in the 5'-3' order....................15

Figure 1.3: Schematic representation of the β-tubulin gene, in which the conserved coding parts are fully filled and the high variability introns are filled with crossing lines. The Bt1 and Bt2 primer pairs are also shown. In this study, the Bt2a/b pair was used. Adapted from (Glass & Donaldson, 1995). 16

Figure 2.1: left: the localities where the dolphins were found. Right: illustration representing the body sampling sites in analyzed dolphins....................21

Figure 3.1: Number of yeasts (grey) and filamentous fungi (green) isolated from each dolphin tested. Blue bars represent the total of isolates. 33

Figure 3.2: Yeasts (grey) and filamentous fungi (green) isolates obtained from blowhole and oral cavity samples. 34

Figure 3.3: Yeasts (grey) and filamentous fungi (green) isolates obtained from the different culture media and temperatures considered in this work. 35

Figure 3.4: Isolated species for each dolphin at 17 °C using PDA (top) and Sabouraud (bottom). Each color corresponds to a different identification, as reported in the legend. 41

Figure 3.5: Isolated species for each dolphin at 30 °C using PDA (top) and Sabouraud (bottom). Each color corresponds to a different identification, as reported in the legend. 42

Figure 3.6: Shannon index and evenness value for each sample that was incubated. The evenness was calculated by using a value of H'_{max} = 3.22. 44

Figure 4.1: Strain of *Debaryomyces hansenii* isolated from oral cavity of dolphin 7, using PDA medium at 17 °C. 51

Figure 4.2: Strain of *Rhodotorula mucilaginosa* isolated from oral cavity of dolphin 1, using Sabouraud agar at 17 °C. 51

Figure 4.3: Strain of *Candida zeylanoides* isolated from oral cavity of dolphin 4, using Sabouraud agar at 30 °C. 52

Figure 4.4: Left: Strain of *Penicillium* sect. *Chrysogena* isolated from oral cavity of dolphin 2, using YPD agar at 17 °C. Center: Strain of *Penicillium restrictum* isolated from oral cavity of dolphin 5, using PDA at 30 °C. Right: Strain of *Penicillium waksmanii* isolated from blowhole of dolphin 8, using PDA at 17 °C. 53

Figure 4.5: Strain of *Cladosporium cladosporioides* isolated from oral cavity of dolphin 11, using PDA at 17 °C. 54

Figure 4.6: Strain of *Fusarium oxysporum* isolated from blowhole of dolphin 7, using Sabouraud agar at 30 °C. 54

Figure 4.7: Strain of *Beauveria bassiana* isolated from the blowhole of dolphins 6, using Sabouraud at 30 °C. 55

Figure 4.8 Left: Strain of *Phlebiopsis sp.* isolated from oral cavity of dolphin 11, using PDA at 17 °C. Right: Strain of *Trametes sp.* isolated from blowhole of dolphin 9, using Sabouraud agar at 17 °C. 56

LIST OF TABLES

Table 2.1: Primer pairs used for DNA amplification and fungal identification. ……24

Table 2.2: PCR conditions used for the amplification of the selected regions……… 25

Table 3.1: Fungal species and genera isolated from dolphin sample cultures. For each species, the frequency and the colonization percentage is reported. ……39

Table 3.2: Sorensen similarity index calculated for the different incubation conditions (temperature and culture medium) considered in this study. Genus-level identifications and unidentified species were not considered……43

Table 3.3: Sorensen similarity index for each sample pair. Nonzero values were highlighted in bold. ……45

Table A.1: List of the dolphins from which the cultured samples were isolated. The data was collected by SPVS researchers that also performed the necropsy. Most of the dolphins were found stranded after an accidental capture with a xavega, a kind of fishing net used by Portuguese traditional fisheries……75

Table A.2: Isolates obtained from the cultured samples of dolphin 1 and their identification. ……77

Table A.3: Isolates obtained from the cultured samples of dolphin 2 and their identification. ……77

Table A.4: Isolates obtained from the cultured samples of dolphin 4 and their identification. ……77

Table A.5: Isolates obtained from the cultured samples of dolphin 5 and their identification. ……78

Table A.6: Isolates obtained from the cultured samples of dolphin 6 and their identification. ……79

Table A.7: Isolates obtained from the cultured samples of dolphin 7 and their identification. 80

Table A.8: Isolates obtained from the cultured samples of dolphin 8 and their identification. 81

Table A.9: Isolates obtained from the cultured samples of dolphin 9 and their identification. 82

Table A.10: Isolates obtained from the cultured samples of dolphin 11 and their identification. 83

Table A.11: Isolates obtained from the cultured samples of dolphin 12 and their identification. 83

Table A.12: Isolates obtained from the cultured samples of dolphin 13 and their identification. 83

Chapter I - Introduction

1.1 Marine mammals and marine ecosystem degradation

Marine ecosystems are nowadays subject to a great degradation, the causes of which are of anthropic origin, namely excessive resource exploitation, pollution, intensive fishing, acidification of water, invasive species introduction, and global climate changes (Jackson, 2008). Because of this, many animal species which dwell in seawater and oceans are -or will be before long- on the brink of extinction.

Amongst the threatened species, marine mammals have a very important role inside the marine ecosystem, because they can be regarded as sentinel species of the ecosystem health status (Bossard, 2011). These animals are on top of the food pyramid, are long lived and possess plenty of fatty tissue that can store chemical substances for a long period. These factors make the marine mammals very sensitive to pollutants and toxic compounds. Moreover, they are the closest sea-dweller organisms to the *Homo sapiens*, and many of them, such as harbor porpoises and dolphins, live close to the coast-lines, thus share the environment with human populations and are more subject to the anthropic degradation factors (Van Bressem, et al., 2009).

The appearance of emerging infectious diseases (EID) is another consequence caused by the previously mentioned anthropogenic factors. In particular, these are epidemic events caused by agents that were not known to cause diseases in normal, unstressed conditions. EIDs represent a main concern regarding the human health, because some of them can be transmitted from marine mammals to humans, given their similarity, thus causing zoonotic events (Waltzek, et al., 2011). Of special concern are the EIDs caused by fungal microorganisms due to their high morbidity and zoonosis risk, and the larger efforts required to treat them.

1.2 Marine mammals in Portugal

1.2.1 Species studied in this work

A wide diversity of marine mammals can be found along the Portuguese coast-line including whales, dolphins, seals and otters. Regarding cetaceans, the highest stranding rates are registered in Spain and Portugal, mostly because of their coast extension (Bento, et al., 2016). In this thesis, samples from *Phocoena phocoena,* commonly named harbor porpoise, and *Delphinus delphis,* or short-beaked common dolphin, were analyzed.

The common dolphin inhabits cool to tropical seawaters of Atlantic and Pacific oceans, spreading from coastal to offshore waters (Hammond, et al., 2008). The species is not threatened, even if several risk factors exist. Dolphins are often killed in accidental captures, generally during tuna fishing activities, because they get caught in fishing nets. Other factors recognized to cause a decline in *D. delphis* populations are reduction of resources caused by overfishing of anchovy and sprat, the chemical contamination of the environment, and environmental changes such as water temperature and habitat degradation (Bearzi, et al., 2003).

The harbor porpoise lives in cold to sub-polar waters of Northern hemisphere. Being one of the smaller marine mammals, it can be found in near-shore and never outside the continental shelf waters (Hammond, et al., 2008). Like the common dolphins, it is very prone to accidental captures by fishing nets, and suffers from the same risk factors, with some remarks. Its habitat makes the porpoise more subject to risk connected to human activity, such as coastal pollution, vessel traffic, noise and overfishing.

In the present work, samples from one individual of striped dolphin *Stenella coeruleoalba* were analyzed. This small cetacean species is widely distributed in warm and tropical waters of Indian, Pacific and Atlantic Oceans, as well as in the adjacent seas (Hammond, et al., 2008). *S. coeruleoalba* is subject to the same threats than *P. phocoena,* with the addition that in some areas, including Spain, they are

intentionally captured for human consumption or to use their meat as bait for other species.

All those species are also threatened by emerging infectious diseases, the impact of which becomes important in stressed populations, as will be explained next.

1.2.2 Studies on Portuguese dolphins

Many studies investigated the characteristics of dolphin populations of Portugal, with attention to the impact of environmental factors on them. High concentrations of heavy metals, especially mercury, were found in stranded exemplars of *Tursiops truncatus* (Monteiro, et al., 2016), *Delphinus delphis* (Zhou, et al., 2001; Monteiro, et al., 2016) and *Phocoena phocoena* (Ferreira, et al., 2016). These studies evidenced the presence of bioaccumulations of heavy metals in dolphin tissues. Monteiro et al. (2016) observed also a possible influence between the metal concentrations and the proximity to anthropogenic sources. Organochlorine compounds and a polychlorinated biphenyl (PCB) are also easily accumulated by the dolphin blubber. In fact, high levels were reported for *T. truncatus* individuals along the Portuguese shores (Borrell, et al., 2006).

The impact of noise caused by naval traffic was studied for *T. truncatus* in the Sado estuary area (Luís, et al., 2014), showing that the dolphins reduce significantly their communication in presence of noise sources and avoid completely the noisiest areas. Similarly, a study revealed a high incidence of skin disorders in bottlenose dolphins (Harzen & Brunnick, 1997), and advanced the hypothesis that such bloom could be related with stress factors arising from high pollutant concentrations and poor water quality (low oxygen levels), a typical condition of many estuaries.

Information on the fungal microbiome of wild-ranging dolphins of Portugal is, until now, lacking. On the other hand, there are studies targeting specific fungi on captive dolphins that will be discussed in section 1.5. However, captive individuals are an extreme case because they live in a much more stressing environment with respect to wildlife populations. Recently, the microbiome of a *S. coeruleoalba* found

stranded in Portugal (Godoy-Vitorino, et al., 2017) was characterized, on different body sites, with the aid of high-throughput sequencing techniques. This study showed a difference in microbiome composition from site to site and a relevant presence of bacteria with clinical interest.

1.3 The Kingdom of Fungi

In this section, the distinctive features of Fungi were reviewed, and some taxonomic notions were introduced. If not otherwise specified, the present exposition is based on (Dismukes, et al., 2003) and (Deacon, 2006).

1.3.1 Characteristics and ecology

Fungi are eukaryotic organisms that possess a series of unique features that make them different from animals and plants. Unlike animals, their cells have walls made up by chitin and glucans and not, as plants, of cellulose. Moreover, they are heterotrophic organisms, lacking chlorophyll. They can secrete enzymes that digest the nutrients present in the medium where they grow. Nutrients are then absorbed across the cell wall and membrane. Their structure is simpler with respect to those of plants and animals. They present themselves as chains of tubular cells, separated or not by septa, which arrange themselves to make filaments called hyphae, that exhibit an apical growth, or as single independent cells, arranged in colonies, that replicate by budding. The fungi of the former type are commonly called *filamentous fungi,* while the latter are called *yeasts*. Filamentous fungi are often found as a mass of branched hyphae that is called mycelium.

Some species may be dimorphic, as *Candida albicans*, once they can grow either as yeasts or filamentous fungi, depending upon the growing conditions. Fungi can reproduce by means of sexual or asexual reproduction, and some can reproduce in both modes during different stages of their life cycle. The sexual-reproducing form

is called *teleomorph*, while the other, *anamorph.* Fungi can also reproduce by means of spores that may be produced in great quantity and spread from structures called fruiting bodies that, in some cases, can reach considerable sizes.

Fungi are ubiquitous organisms and can be found in a wide class of environments, including soil, water, dead organisms, both animal and vegetal, and living tissues. According to their feeding habit, fungi can be grouped in three distinct categories: parasites, symbionts and saprophytes. Parasite fungi obtain their nutrients from a living organism and may kill the host tissues as a part of the feeding process. Symbiont fungi establish a collaborative relation with the host organisms, providing them with nutrients they can't produce. Saprophytes grow on dead organic matter, such as wood rot, and play a major role in decomposing organic compounds and recycle nutrients.

Humans can also be affected by mycoses, but the number of fungal species that attack humans and other mammals is very low: it was reported that only 100 out of 150000 known fungal species are clinically relevant (Kohler, et al., 2015). In normal conditions, humans have high immune defenses against mycotic infections, but when the immune system is compromised, many otherwise harmless fungal species may opportunistically invade an organism, and cause serious clinical situations. Some cases will be discussed in section 1.5.

Under the light of this, it is possible to understand how the composition of the fungal microfauna hosted by wild-ranging dolphins is strictly related to the ecosystem status. The presence of relevant stress factors can cause a decrease of immune defenses within the population, and then opportunistic fungi infections can occur. If such fungi are identified from cultured tissue samples of dolphins, then this is a symptom of ecosystem degradation.

1.4 Emerging Infectious Diseases in dolphins

The EIDs can be caused by bacterial, viral or fungal agents. Due to their high infectivity and pathogenicity, they are often responsible for outbreaks that can spread quickly in different regions and populations (Van Bressem, et al., 2009). The appearance of EIDs and the status of the ecosystems are closely related. In fact, many of the pathogenic agents may inhabit the host organism without any harm. However, environmental stress factors, as food shortage, pollution, interaction with human activities and habitat degradation could result in a depression of the immune system of the dolphin. This would result in some microorganisms being able to grow uncontrolled and eventually cause a serious disease. EID are also associated with zoonotic and epizootic risk, because infected populations could act as vectors and reservoirs of pathogenic microorganisms. Regarding marine mammals, not only they inhabit densely populated areas, but are also widely used in sea parks and various attractions, where direct contact between humans and dolphins is often encouraged. This makes the study of microbial strains in dolphins even more important.

EIDs have various consequences. They could cause a high rate of mortality in affected populations and change the population reproductive capacity (Bossard, 2011). Due to this, they could change the balance among the different members of an ecosystem and produce a negative impact on already stressed habitats (Epstein, et al., 2003).

One of the most renowned EIDs in dolphins, briefly discussed here, is the Cetacean Morbillivirus (CeMV) (Van Bressem, et al., 2014). This endemic virus in cetacean populations can cause pneumonia, meningoencephalitis, lymphatic cell disruption and eventually death on those individuals with a compromised immune system (Domingo, et al., 1990; Duignan, et al., 1992). This EID was identified for the first time during the years 1987-1988, when a CeMV outbreak hit the populations of *T. truncatus* dwelling along the Atlantic coast of North America. It has been estimated that that event killed 50% of the individuals (Lipscomb, et al., 1994). During the

years 1987-1990, the CeMV decimated the coast populations of *P. phocoena* of Netherlands and UK (Kennedy, 1998). Later, outbreaks have been recorded in the population of *Stenella coeruleoalba* and *Megas globocephalus* (Raga, et al., 2008). Recently, it has been shown that the CeMV is endemic in Portuguese and Galician populations of *S. coeruleoalba* (Bento, et al., 2016).

1.5 Relevant fungal species in dolphins

Fungal microorganisms may constitute one of the main vectors of zoonosis. As a matter of fact, it has been shown that 18 out of 23 fungal species identified in an infected skin sample coming from a *Pseudorca crassidens* individual, were already identified in human mycoses (Mouton, et al., 2015). Moreover, even commonly non-pathogenic fungi may become a serious threat for health, especially for immunosuppressed or stressed individuals. For this reason, fungi should be regarded as potential causes for not yet discovered EIDs in dolphin populations. Thus, a screening of fungal diversity in dolphins is of utmost importance. However, unlike what was done regarding bacteria, information about microbial community in free-ranging dolphins is scarce.

The principal fungal species found in dolphins were reviewed, highlighting the zoonotic risk when available.

Lacazia loboi

Lobomycosis, caused by the yeast-like fungus *Lacazia loboi*, has been found in many dolphin species (Van Bressem, et al., 2009). The fungus can be found in water, soil or vegetation and can easily penetrate a host, by infecting a wound after a traumatic event. In dolphins, it can develop granulomatous skin lesions, which can be larger than 30 cm (Reif, et al., 2008). The *L. loboi* mainly affects the American populations of *T. Truncatus* and *Sotalia guianensis*. Epidemic episodes have been recently

observed in *T. truncatus* populations in North Carolina (Rotstein, et al., 2009) and in the Indian River Lagoon, Florida (Reif, et al., 2006).

Concerning humans, *L. loboi* is responsible for the Jorge Lobo disease, or lacaziosis. It is a subcutaneous chronical mycosis, in which the fungus penetrates the skin after a traumatic event or an insect bite and causes verrucous lesions. It is mainly diffused in central and South America (Carvalho, et al., 2015).

Regarding the dolphin-to-human transmission, direct inoculation could happen when handling infectious tissue samples, because of poor hygiene conditions or laboratory accidents (Waltzek, et al., 2011; Rosa, et al., 2009). However, excluding these sporadic events, no evidence has been found for direct zoonosis (Reif, et al., 2013). However, it has been suggested that the marine habitat could be a reservoir of infection (Bermudez, et al., 2009). *L. loboi* is uncultivable, meaning it does not grow in standard culture media and conditions.

Candida sp.

Candidiasis is the clinical condition caused by an infection by yeasts belonging to the genus *Candida*, causing a wide range of superficial and soft tissue infections, as well as deep systemic infections, especially in nosocomial environments (Dismukes, et al., 2003). It is widely found in captive dolphins (Higgins, 2000), and it was also cultured from samples coming from free-ranging dolphins (Morris, et al., 2011). In general, *Candida* species are commonly associated with soft tissue regions of blowhole, esophagus, vagina and anal area of cetaceans, and causes severe local skin infections in captive, immunosuppressed individuals (Mouton & Botha, 2012).

In a study of air samples blown from captive dolphins (Takahashi, et al., 2010) in an aquarium, it was shown by molecular identification and sequence comparison that two *Candida* isolates from the breath of a dolphin and from the breath of one volunteer working at the aquarium, were the same strain, indicating an airborne zoonotic potential for this genus.

Rhodotorula sp.

Rhodotorula is a genus of basidiomycetous yeasts with a typical red coloration. They are ubiquitous in nature, and can be found in air, soils, lakes, ocean water, milk, and fruit juice (Wirth & and Goldani, 2012). Concerning human health, *Rhodotorula* is responsible for a wide spectrum of infections including fungemia, endocarditis, peritonitis, meningitis and disseminated disease (Dismukes, et al., 2003). The infection is often associated with intravascular catheters utilization in patients receiving antibiotic therapy or chemotherapy, hence immunocompromised (Dismukes, et al., 2003). Recently, the concern about this genus increased, since mycotic infections caused by *Rhodotorula sp.* were encountered in various animals, including the sea lion (Wirth & and Goldani, 2012). It was also found in the pool waters of captive bottlenose dolphins (Buck, 1980), along with many *Candida* strains.

Cryptococcus sp.

Cryptococcus fungi are a family of encapsulated yeasts, responsible for cryptococcosis, an opportunistic disease that targets immunocompromised subjects and can cause pneumonia and meningitis (Dismukes, et al., 2003). It is commonly found in avian guano, especially in those of pigeons. The *Cryptococcus* may cause zoonoses: it was reported that the occurrence of an infection caused by *Cryptococcus laurentii* in captive dolphins in Portugal, could be related with an improper depuration of water and contamination from avian guano (Martins, et al., 2002). Cryptococcoses caused by *C. neoformans var. gattii* were recorded in *T. truncatus* (Miller, et al., 2001) and *Stenella longirostris* (Rotstein, et al., 2010).

Debaryomyces sp.

Debaryomyces hansenii is a yeast common in various substrates and in some cheeses, and it has the distinctive features of a high salt tolerance and the capability to produce toxins that kill other yeasts (Banjara, et al., 2016). This species is considered pathogenic in some rare cases, and recent investigation showed that its pathogenicity is rarer than previously thought (Desnos-Ollivier, et al., 2008).

Fusarium sp.

Fusarium are filamentous fungi known as plant pathogens. Recently, they have been recognized as the main cause of fusariosis, a serious mycotic opportunistic infection (Dismukes, et al., 2003). *Fusarium* is highly invasive at the level of veins, causing hemorrhages and tissue necrosis. In addition, it can produce a wide range of mycotoxins. It has been also proved that a specific commitment of the immune system, called neutropenia, is the critical factor for fusariosis outbreak.

Interestingly, a fatal, non-cutaneous infection caused by *Fusarium* was reported for a *T. truncatus* in 2010, for the first time (Staggs, et al., 2010). Before that, it was found as a cause of mycotic dermatitis in several marine mammals, cetaceans and pinnipedia (Fresca, et al., 1996), but no deadly fusariosis cases were reported.

Aspergillus sp.

The filamentous genus *Aspergillus,* member of the *Ascomycota*, is at the basis of several diseases in humans. It is a ubiquitous saprophytic mould and it may trigger allergic responses (allergic pulmonary aspergillosis), colonize the host generating a fungus ball (aspergilloma) or develop an invasive colonization of external ear or lungs (Dismukes, et al., 2003), as well as skin and nails. *Aspergillus* is likely to infect immunosuppressed individuals that enter in contact with its airborne spores. Aspergillosis has been reported several times in dolphins, causing pulmonary infections (Reidarson, et al., 1998) that are often fatal if untreated (Delaney, et al., 2013). A molecular analysis (Lança, et al., 2010) carried on 6 common dolphins[1] , killed by pulmonary aspergillosis, revealed a very high sequence homology with the isolated *Aspergillus fumigatus* strains. *Aspergillus fumigatus* was also identified from free-ranging *T. truncatus* individuals from the Southeastern United States (Morris, et al., 2011), even if its identification was done on a morphological basis. It was proposed that the emergence of an ear aspergillosis in a *Phocoena phocoena,* a very

[1] The referenced work does not report whether the individuals were captive or wild-ranging.

serious condition for dolphins, since they rely on echolocation, may be related with high concentration of bio-toxins in the porpoise tissues (Prahl, et al., 2009).

The list above shows that many clinically relevant fungi were found in dolphins. This means that the study of the fungal community of free-ranging individuals is not only useful as a tool, to check the population health and the ecosystem status, but also to assess the potential health risk, that marine mammals may represent, especially for those people with lower immune defenses. This makes the characterization of dolphin-hosted fungi even more important.

1.6 Studying the microbiota of marine mammals

1.6.1 Microbiome and mycobiome

Recently, the study and characterization of the microbiome, that is the ensemble of microorganisms hosted by an individual, led to promising results in this purpose (Bik, et al., 2016). A very important part of the microbiome is represented by the mycobiome (Huffnagle & and Noverr, 2013), its fungal fraction. In fact, despite being but a fraction of the total microbiome, as illustrated in Figure 1.1, fungi are more likely associated to greater zoonotic risks. Despite this, there is much less information about the cetacean mycobiome with respect to the bacterial part, because the growth and identification of fungal microorganisms is much more difficult.

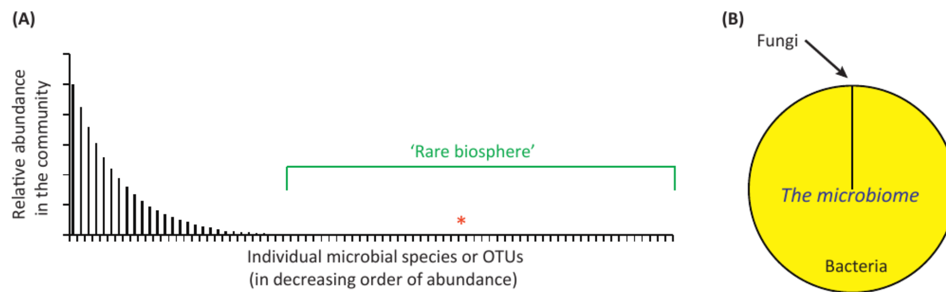


Figure 1.1: (A): Qualitative diagram showing the concept of 'rare biosphere', that is the ensemble of microbiotas that represents less than 1% of the total microbial community. In (B) the ratio between fungi and bacteria in a human fecal sample is shown. Fungi are part of the 'rare biosphere'. Figure adapted from (Huffnagle & and Noverr, 2013).

1.6.2 Molecular identification of fungi

In the past, the identification of fungal species was mainly done by morphological identification, studying, for instance, the shape and features of the reproductive apparatus. Morphological identification often fails to provide a species-level identification because of the appearance of cryptic speciation, hybridization and convergent evolution. Moreover, many species that were named differently, turned out to be the anamorph and teleomorph stage of the same species (Raja, et al., 2017). However, this technique may still be important in some cases, e.g. when there is no sequence to compare with or the sequences are reported without binomial name (Hyde, et al., 2010).

Molecular identification is based on the study and comparison with databases of specific DNA sequences of the organism to identify, and it was made possible by the advent of PCR amplification DNA sequencing techniques. Regarding the identification of fungi, the most important region is the ribosomal DNA (rDNA). Ribosomes are capable to read the genetic code (using RNA) and to translate it, by assembling proteins from it. Since their function is fundamental for life, the sequences that codify the Large Subunit (LSU) and the Small Subunit (SSU), the two building blocks of a ribosome, are highly conserved; in other words, they evolve very slowly. These sequences are suitable for phylogenetic studies, in which comparison of distant species is needed, but are not suitable for species-level identifications.

In rDNA small, non-coding regions are also present, called Internal Transcribed Spacer (ITS), whose role is not yet clearly known. These regions are poorly conserved, that is they evolve faster therefore they can be used for species-level identification. According to these considerations, and to the fact that the rDNA is present repeatedly inside the fungal DNA, making this region particularly suitable for amplification, primer pairs its1 and its4 were developed to target the ITS1 and ITS2 regions and the coding 5.8S region that is in between (White, et al., 1990), as shown in Figure 1.2.

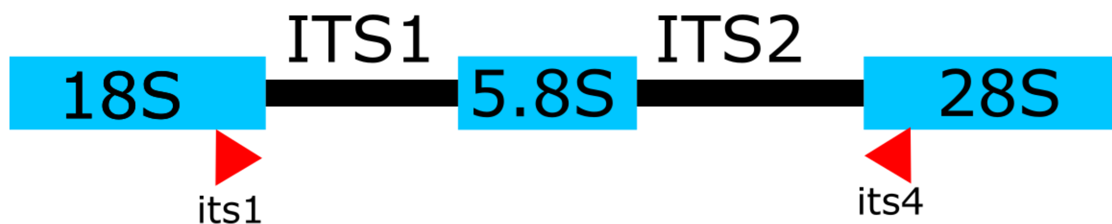


Figure 1.2: Schematic representation of the ITS region of rDNA. Light blue rectangles represent the coding regions, black lines the ITS region. Red triangles show the anchor point of the its 1-4 primer pair. Primer names are in lower case to distinguish them from region names (upper case). The sequence is in the 5'-3' order.

The primers were designed to attach to the ends of 18S gene of SSU and 28S gene of LSU. This choice makes the primers universal, that is, they may be used with every fungal species, because the 18S and 28S genes will be the same, due to their high conservation. The resulting amplified sequence will be 600-800 base pairs long, depending upon the species. The success of molecular identification based upon ITS regions is such that it was recognized as the universal barcode marker for fungi (Schoch, et al., 2012). However, there are some cases in which the ITS region does not report a good species level identification, because the interspecific variation may be too low for some genera, such as *Fusarium, Aspergillus* and *Penicillium* (Raja, et al., 2017; Balajee, et al., 2009). In such cases, amplifying other high variable part of the rDNA may be helpful. One widely used choice are the D1 and D2 regions that are located inside the 28S gene, near its 5'end, and can be targeted using the NL1/NL4 primer pair (O'Donnel, 1993). Another region of interest is the gene that codes for

the elongation factor (EF1), which display interspecific polymorphism for *Fusarium* genus (O'Donnell, et al., 1998).

Apart from rDNA, other conserved parts of the genome may be used for species level identification. The gene that codifies the β-tubulin, a building block of the protein tubulin structure, represented in Figure 1.3, is also well conserved (Glass & Donaldson, 1995) and provided species-level identification for various filamentous fungi genera.

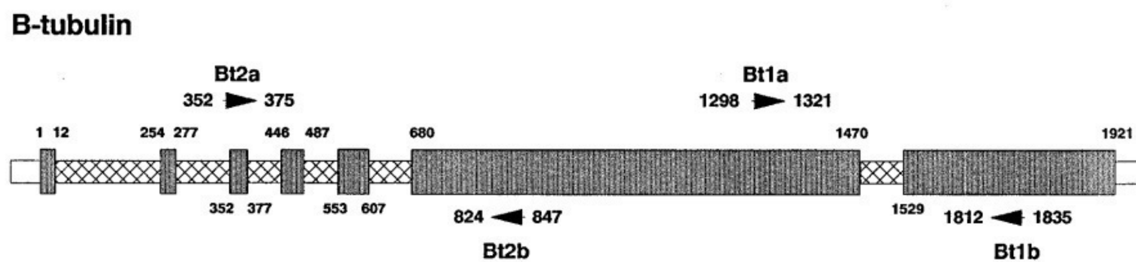


Figure 1.3: Schematic representation of the β-tubulin gene, in which the conserved coding parts are fully filled and the high variability introns are filled with crossing lines. The Bt1 and Bt2 primer pairs are also shown. In this study, the Bt2a/b pair was used. Adapted from (Glass & Donaldson, 1995).

1.6.3 Objectives

The main aim of this thesis is to give a first characterization of the mycobiome of Portuguese free-ranging dolphins that will be achieved by analyzing samples taken from the blowhole and the oral cavity of stranded specimens. The fungal isolates will be identified to species-level by using molecular methods, namely by sequencing the ITS regions of rDNA genes and, for some fungi, the ß-tubulin and elongation factor 1α (EF-1α) genes.

By this, we expect to determine whether wild-ranging dolphins could act as a reservoir of fungal pathogen agents that could trigger disease outbreaks, and could represent a risk to human health. It will be possible to give a first description of the mycobiome hosted by healthy dolphins, which knowledge is extremely important, since changes in its composition could be related with the dolphin health status and environmental stress factors. Moreover, this study will investigate the effect of culture media and temperature on the species that grow in culture. Finally, we will

check if the mycobiome characterization could potentially be used to study the interactions between groups.

Chapter II - Materials and Methods

2.1 Samples collection

A total of 11 stranded or accidentally captured dolphins in Portuguese Coast were sampled (Figure 2.1, left). The species studied, the capture and death dates, and eventual evidence of diseases are described in Table A.1 of appendix 1.

The sampling was performed within 12 hours after death (decomposition code 1 or 2) by the researchers of Portuguese Society of Wild Life (SPVS). All samples are conserved at the marine animal tissue bank (BTAM), located in the University of Minho facilities. Fungal diversity was evaluated using swab samples of blowhole and oral cavity, stored in saline solution (Figure 2.1, right).

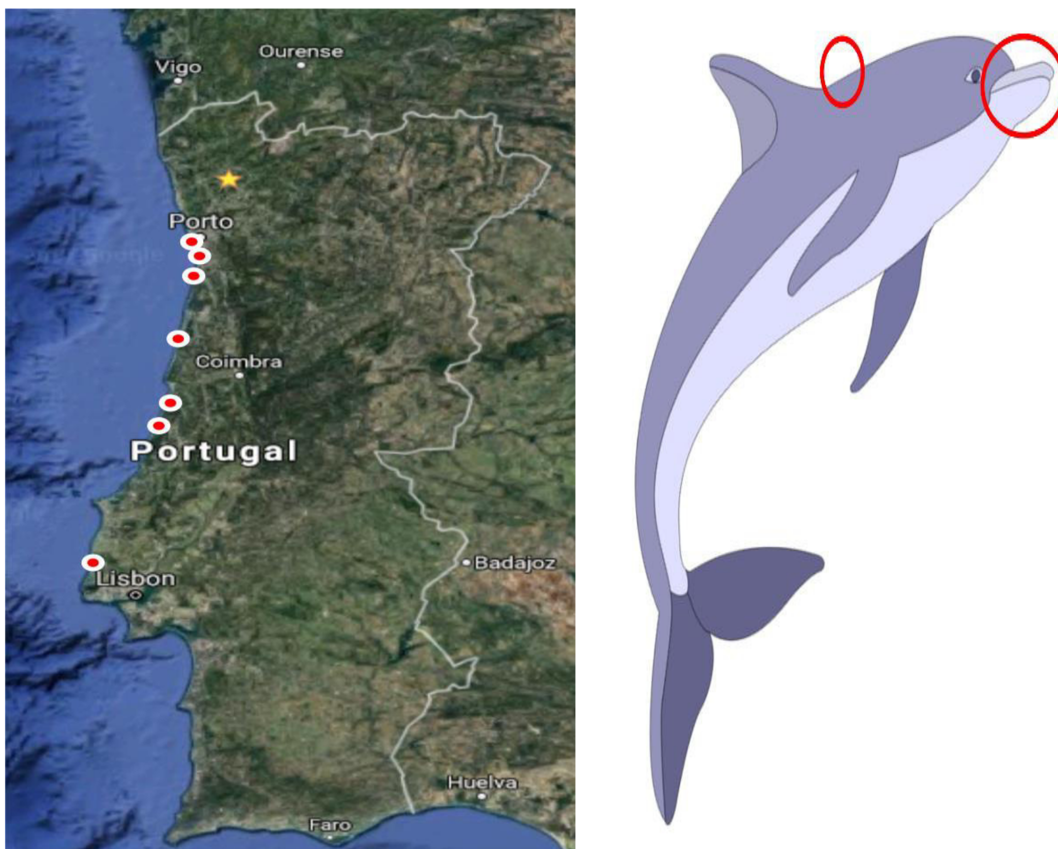


Figure 2.1: left: the localities where the dolphins were found. Right: illustration representing the body sampling sites in analyzed dolphins.

2.2 Growth and isolation of fungi

2.2.1 Culture Media

All samples collected were cultured using potato-dextrose-agar (PDA) and Sabouraud-agar (SAB) supplemented with 0.05 g/L chloramphenicol in order to prevent the growth of bacteria and obtain a higher diversity of fungal species. To search for lipophilic yeasts, Sabouraud agar supplemented with olive oil was used.

2.2.2 Sample inoculation

All samples collected from the oral cavity and blowhole were directly stored at -80 °C, in glycerol 30% (v/v), into swab tubes. For the inoculation step, all samples were placed at room temperature, well agitated in the Vortex and cultured in the different medium plates using the respective swab.

The plates were incubated at 17 °C, the average water temperature in which dolphins live, and 30 °C, the optimal temperature for the growth of pathogenic fungi.

After two days, all plates were checked to evaluate growth, count the colonies and record their aspect. The plates were checked again after 9 and 16 days. The number and appearance of the grown fungi were registered and the plates were photographed.

Yeast colonies were observed by microscopy and isolated using the same initial conditions. The replica process was repeated 2-3 times to ensure the isolation. The isolates were numbered with a specific code. The first number refers to the dolphin number and the second indicates the provenience of the sample, namely “1” for blowhole and “6” for oral cavity. Following, temperature (17 °C or 30 °C) and culture medium, “P” for PDA and “S” or “Y” for Sabouraud, were also annotated.

2.3. DNA extraction

2.3.1 DNA extraction from filamentous fungi

Filamentous fungi present cell walls resistant to standard DNA extraction techniques (van Burik, et al., 1998). Thus, a process to disrupt the cell wall and release the DNA is necessary. To extract the DNA from filamentous fungi, we followed a protocol in which the cellular lysis was performed by cold maceration, followed by a chemical treatment to remove contaminants (do Vale, 2012).

Under sterile conditions, 500 µL of fungal biomass were added to a 2mL tube. The tube was submerged in liquid nitrogen and biomass was macerated using a pestle. Maceration was repeated three times, with the addition of 3x200 µL of TES buffer (see appendix 2). After maceration, the tube was placed in a thermo-block at 95-100 °C for 5 minutes, agitated for a few seconds and left to cool down at room temperature. Two centrifugations were performed, first at 3000 rpm, 4 °C for 10 minutes and next at 13000 rpm, 4 °C for 3 minutes, to improve the DNA extraction process.

A total of 600 µL of Chloroform: Isoamyl Alcohol (24:1) solution was added and subsequently the mixture was agitated and centrifuged at 14000 rpm for 10 minutes, at 4 °C. This step separated the mixture in three layers. The top layer contained the DNA and the second and third layers contained proteins and contaminants, respectively.

The top layer was transferred to a new 2 mL tube and the previous step was repeated twice. 1/10 of the total volume of sodium acetate 3M and 2.5X of the total volume of absolute ethanol were added. The solution was homogenized by reverting 50/60 times and centrifuged at 14000 rpm for 30 minutes. Then, the supernatant was discarded and the pellet was washed twice, with 500 µL

of 70 %(v/v) ethanol. Between and after washes, the sample was centrifuged as 14000 rpm for 10 minutes and the supernatant was discarded. Afterwards, the

pellet was dried at room temperature, suspended in ultrapure H_2O and placed at 4 °C overnight.

The following day, the sample was heated using a thermo-block at 65 °C for 45 minutes to dissolve the DNA pellet. Finally, the DNA concentration was measured using a Nanodrop spectrophotometer, and stored at –20 °C.

2.3.2 DNA extraction from yeasts

DNA extraction from yeasts was performed either by colony PCR according to (Vaz, et al., 2011), or by using the JetQuick purification spin kit according to the manufacturer's instructions.

2.4 Species Identification

2.4.1.PCR amplification

For species identification, different regions or genes were amplified and sequenced, namely ITS region, D1/D2 region, β-tubulin gene and elongation factor 1α gene (EF-1α). This selection was performed according to the databases available.

The primers used in this work were ITS1 and ITS4 (White, et al., 1990), NL1 and NL4 (O'Donnel, 1993), Bt2a and Bt2b (Glass & Donaldson, 1995), and EF1 and EF2 (O'Donnell, et al., 1998) (Table 2.1).

Table 2.1: Primer pairs used for DNA amplification and fungal identification.

Primers	**Sequence (5'-3')**
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC

Table 2.1 (continued).

NL1	GCATATCAATAAGCGGAGGAAAAG
NL4	GGTCCGTGTTTCAAGACGG
Bt2a	TGGGCYAARGGYCACTACACYGA
Bt2b	TCAGTGAACTCCATCTCRTCCAT
EF1	ATGGGTAAGGA(A/G) GACAAGAC
EF2	GGA(G/A) GTACCAGT(G/C) ATCATGTT

The PCR conditions used for the amplification of each region are described in Table 2.2. The DNA concentration used was optimized using serial dilutions. Negative and positive controls were used for each PCR run. PCR products were stored at 4 °C until purification.

Table 2.2: PCR conditions used for the amplification of the selected regions.

ITS1/4 and NL1/4	
PCR MIX	**Concentration**
Ultrapure H_2O	-
Taq Buffer 10X	1 X
$MgCl_2$ 25mM	2 mM
ITS1/NL1 10 μM	0.4 μM
ITS4/NL4 10 μM	0.4 μM
dNTPs 10mM	0.2 mM
Taq-polymerase (5U/ μL)	1.5 U
DNA template	100 ng

Table 2.2 (continued).

PCR Conditions ITS1/4		
Initial denaturation		95 °C for 6 minutes
35 cycles:	Denaturation	95 °C for 20 seconds
	Annealing	53 °C for 20 seconds
	Extension	72 °C for 1 minute
Final extension		72 °C for 5 minutes
PCR Conditions NL1/4		
Initial denaturation		94 °C for 3 minutes
33 cycles:	Denaturation	95 °C for 1 minute
	Annealing	52 °C for 30 seconds
	Extension	72 °C for 1 minutes
Final extension		72 °C for 6 minutes

Bt2a/b	
PCR MIX	**Concentration**
Ultrapure H_2O	-
Taq Buffer 10X	1X
MgCl2 25 mM	2 mM
Bt2a 10 µM	0.4 µM
Bt2b 10µM	0.4 µM
dNTPs 10 mM	0.2 mM
Taq-polymerase (5U/ µL)	1 U
DNA template	250 ng

PCR Conditions		
Initial denaturation		95 °C for 10 minutes
38 cycles:	Denaturation	94 °C for 1 minute
	Annealing	64.5 °C for 50 seconds
	Extension	72 °C for 1 minutes
Final extension		72 °C for 10 minutes

Table 2.2 (continued).

EF1/EF2	
PCR MIX	**Concentration**
Ultrapure H_2O	-
Taq Buffer 10mM	1X
$MgCl_2$ 50 mM	1.5 mM
EF1 10 µM	0.2µM
EF2 10 µM	0.2 µM
dNTPs 10 mM	0.2 mM
Taq-polymerase (5U/ µL)	5 U
DNA template	100 ng
PCR Conditions	
Initial denaturation	94 °C for 5 minutes
35 cycles: Denaturation	95 °C for 30seconds
35 cycles: Annealing	47 °C for 45 seconds
35 cycles: Extension	72 °C for 2 minutes
Final extension	72 °C for 5 minutes

To confirm PCR amplification, agarose gel electrophoresis was performed using 1.5 % agarose gel TAE 1X. A ladder marker (BenchTop 1kb, Promega Corporation) was used to confirm PCR product size. Samples were run at 400 mA, 100 V for 30-40 minutes. Gel was stained using Midori Green (Nippon Genetics) and DNA bands were observed using a trans-illuminator.

After confirmation, PCR products were purified using a NZYgelpure (NZYTech) kit according to manufacturer's instructions, and eventually stored at -20 ° C.

2.4.2 Sequence analysis

PCR fragments were sequenced in STAB VIDA laboratory (www.stabvida.com). Obtained sequences (Forward and Reverse) were aligned and analyzed using CodonCode Aligner software, version 7.0 (www.codoncode.com).

The contigs blast was performed using NCBI (https://blast.ncbi.nlm.nih.gov) and MycoBank (www.mycobank.org) databases.

The identification was considered successful at the species level if both the query coverage (QC) and sequence pairwise identity (ID) were equal or higher than 97 %. These thresholds are in line with the average intraspecific sequence divergence in the kingdom of fungi, 3.33 % (Raja, et al., 2017).

2.5 Data analysis

To characterize quantitatively the species richness within a sample and the population difference between samples, several indices were calculated following a commonly used approach (Figueira & Barata, 2007). The Shannon diversity index was calculated for the set of fungal isolates of each sample. It is defined as $H = -\sum_i \ p_i \cdot \ln(p_i)$, that is the negative of the sum of the proportion p_i of each species times its natural logarithm. H is related to the species richness and their abundances: it is 0 if there is only one species in a population and increases with the number of species, but decreases if one the species becomes dominant. The evenness value is obtained straightforwardly from H by dividing it with the value H'_{max} of the maximal possible diversity, defined as $H'_{max} = \ln S$, S being the total number of unique species in all sets.

To compare the similarity between two samples, some value to measure it is required. In this regard, we considered the Sorensen similarity index *s.* Given two sets of individuals containing species a and species b, with species c that appear in

both sets, then $s = \frac{2c}{a+b}$. The Sorensen index equals 0 if no species are in common, then there is complete dissimilarity, and equals 1 if $c=a=b$, that is when two sets have the same number of species and all are in common.

Chapter III - Results

3.1 Analysis of fungal isolates

To isolate fungi from Portuguese wild ranging marine mammals, samples from the blowhole and the oral cavity of 11 dolphins were grown. The samples were collected by the researchers of the SPVS society from dolphins found stranded or accidentally captured along the Portuguese coasts. Information about the recovery date and site, sex and results of a post-mortem analysis can be found in Table A.1 of Appendix 1. The obtained isolates were grown in different media and at different temperatures, 17 °C and 30 °C. A total of 122 fungal strains were isolated, with an average of 11.09 isolates per dolphin. The morphological analysis of the isolates resulted in 83 yeasts (68.03 %) and 39 filamentous fungi (31.97 %). Bacterial strains were not considered.

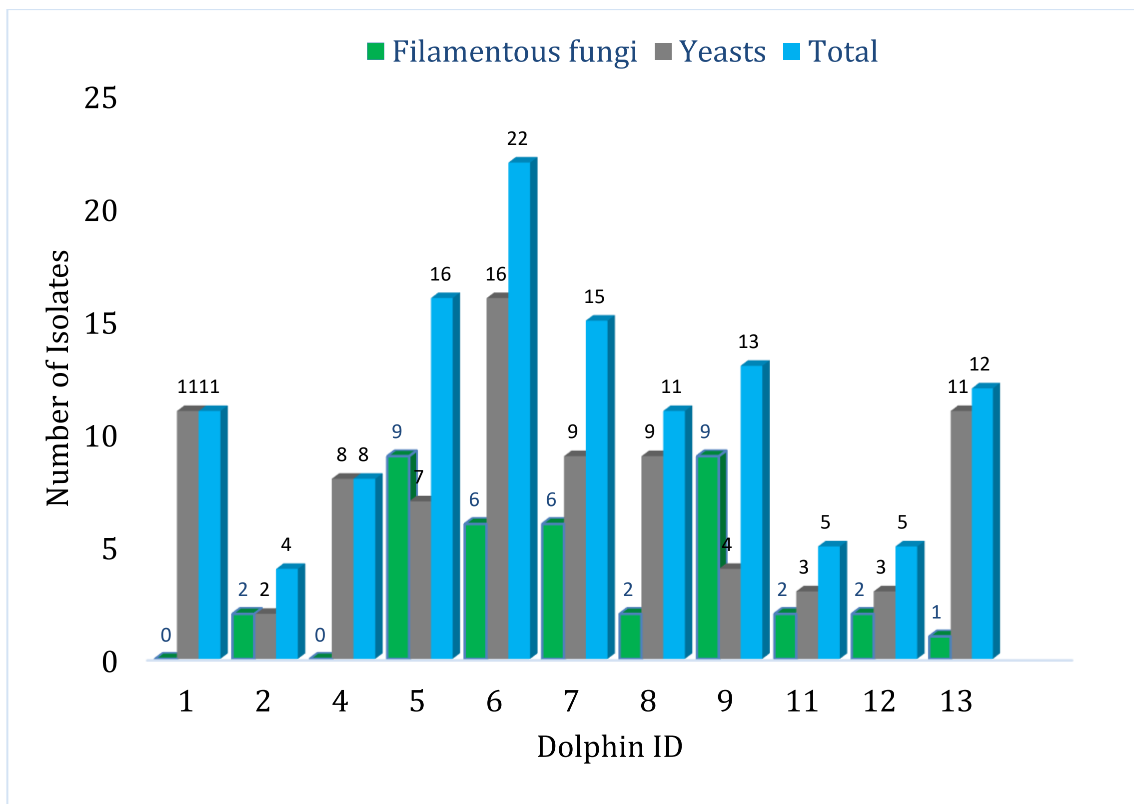


Figure 3.1: Number of yeasts (grey) and filamentous fungi (green) isolated from each dolphin tested. Blue bars represent the total of isolates.

The number of yeasts and filamentous fungi isolated from each dolphin is shown in Figure 3.1. The number of isolates fluctuates between 22 isolates (dolphin 6) to 4 isolates (dolphin 2) according to the dolphin studied. The data represented in Figure 3.1 also shows that in the majority of dolphins (with exception of dolphins 5 and 9), yeast strains were more frequent. Moreover, dolphin 2 presented the same number of yeasts and filamentous fungi isolates.

Comparing the different body sites, it is possible to observe that samples from oral cavity presented a higher number of isolates than samples isolated from blowhole (Figure 3.2). Interestingly, the ratio between yeasts and filamentous fungi isolates also differed when sampling sites were compared. Blowhole presented a higher percentage of filamentous fungi (43.14%, 22 isolates), while the oral cavity presented only 23.94% (17 isolates).

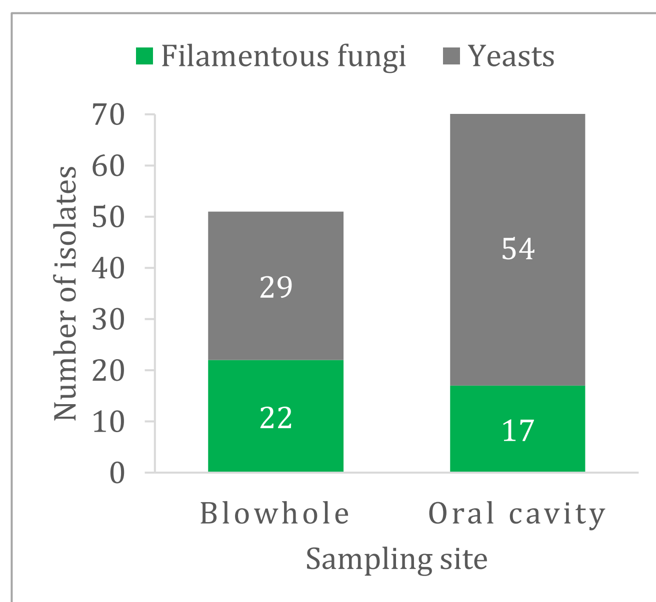


Figure 3.2: Yeasts (grey) and filamentous fungi (green) isolates obtained from blowhole and oral cavity samples.

To observe if the use of different fungal growth conditions influenced the number and morphology of isolates, all samples were cultured using PDA and Sabouraud medium at 17 °C and 30 °C (Figure 3.3). Comparing the different media, a similar number of isolates were obtained (60 isolates for PDA and 62 isolates for

Sabouraud). Moreover, the same tendency was observed when comparing different temperatures (63 isolates at 17 °C and 59 isolates at 30 °C).

Comparing the different combinations, PDA cultures at 17 °C presented the highest number of isolates (n=35). In contrast, PDA cultures at 30 °C presented only 25 isolates. Figure 3.3 also showed that yeast strains were predominant in all four conditions.

Regarding filamentous fungi, Sabouraud cultures at 30 °C presented the higher number of isolates (12 isolates). However, the highest ratio of filamentous fungi was obtained in Sabouraud cultures at 17 °C (37.03 % of filamentous fungi).

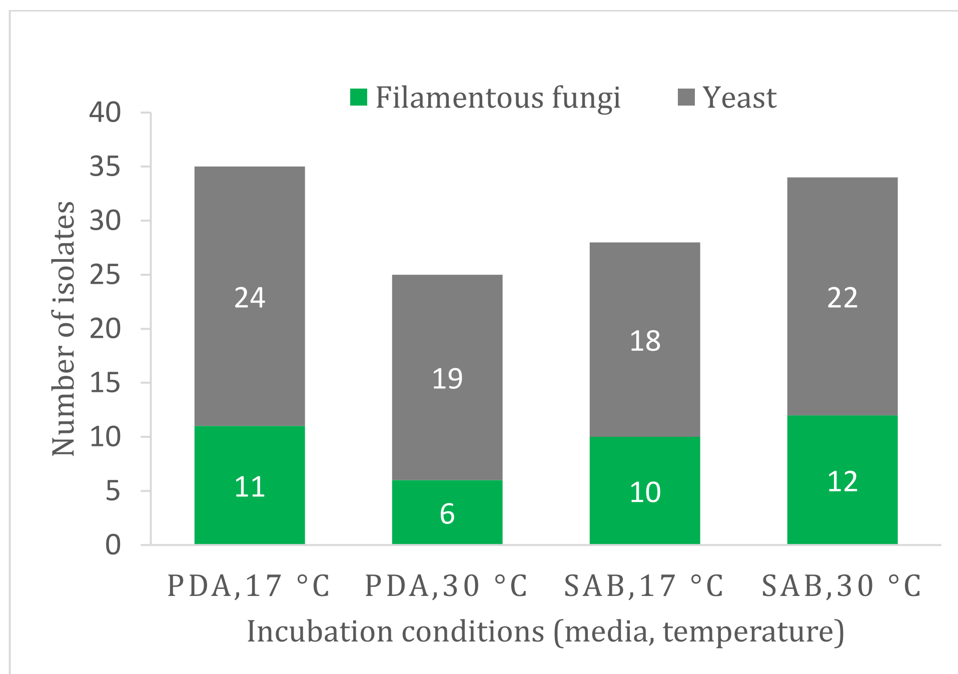


Figure 3.3: Yeasts (grey) and filamentous fungi (green) isolates obtained from Potato dextrose agar (PDA) and Sabouraud (SAB) media at 17 °C and 30 °C.

3.2 Species diversity analysis

A total of 108 isolates (88.52 %) were successfully identified at the species level; 10 isolates were identified at the genus/group level (8.20 %); and 4 (3.28 %) were not successfully identified.

For all 108 isolates identified a total of 25 species were found. The list containing all identified species and genera is reported in Table 3.1. This table also presents the frequency of each species, calculated according to the number of identified isolates, and the percentage of colonization, calculated according to the number of dolphins colonized with each species.

The most frequently isolated yeast species were *Rhodotorula mucilaginosa* (30 isolates, 25.42 %), *Candida zeylanoides* (21 isolates, 17.80 %) and *Debaryomyces hansenii* (14 isolates, 11.86 %), isolated from six, six and seven dolphins, respectively.

Regarding filamentous fungi, *Fusarium oxysporum* (8 isolates, 6.78 %), *Cladosporium cladosporioides* (4 isolates, 3.39 %) *and Penicillium* sect. *chrysogena* (3 isolates, 2.54 %) were the most frequently found, isolated from two, four and two dolphins, respectively.

Comparing the isolates obtained using different culture medium and incubation temperatures it was possible to observe differences in fungal richness and diversity (Figure 3.4 and 3.5). Cultures of PDA at 17 °C and Sabouraud at 30 °C presented the highest fungal diversity, with 13 and 14 different species respectively. Using the other two conditions only 9 different species were obtained.

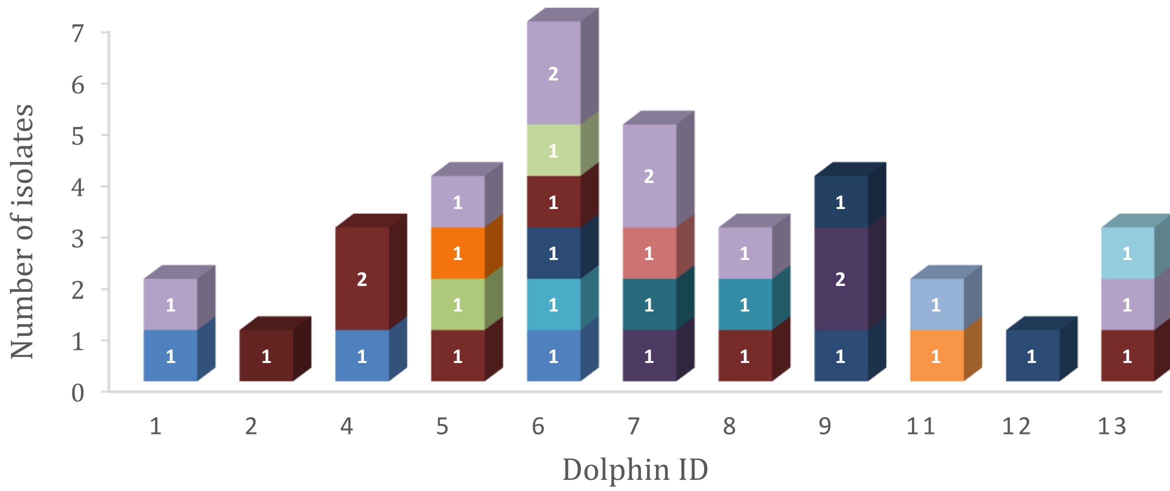
The use of different media and temperatures allow the observation of the ability of species to grow at different conditions. In fact, the results showed that *Aureobasidium pullulans, D. hansenii, R. mucilaginosa, C. zeylanoides* and *F. oxysporum* were the only species isolated from all growth conditions. All the other

species just grew using one media/temperature combination, except *Rhodotorula diobovata*, which grew using PDA at 17 °C and Sabouraud at 30 °C.

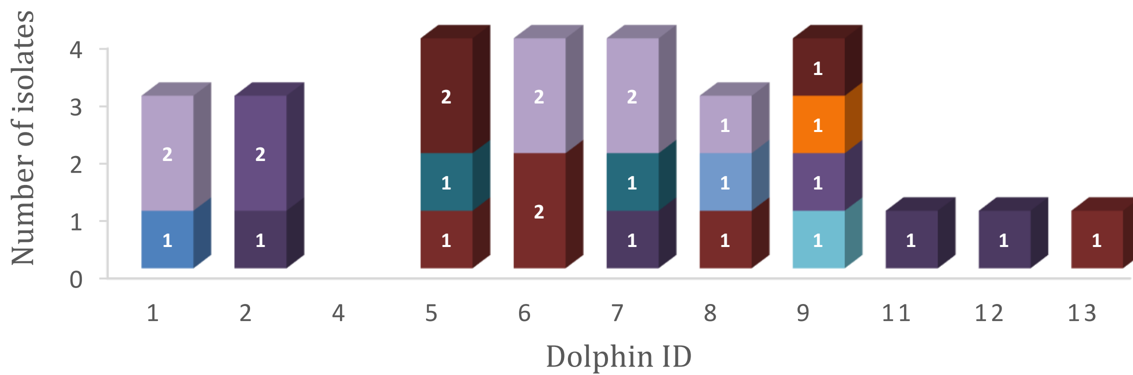
Table 3.1: Fungal species and genera isolated from dolphin sample cultures. For each species, the frequency and the colonization percentage is reported.

Dolphin	1	2	4	5	6	7	8	9	11	12	13	Total	Frequency (%)	Colonization (%)
Aspergillus sect. *Versicolores*					1							1	0.85	9.09
Aureobasidium pullulans	4		4		1							9	7.63	27.27
Beauveria bassiana					1							1	0.85	9.09
Candida atmosphaerica						1						1	0.85	9.09
Candida zeylanoides			4	2	5		4	1			5	21	17.80	54.55
Cladosporium sp.					2							2	1.69	9.09
Cladosporium cladosporioides					1			1		1	1	4	3.39	36.36
Cladosporium ramotenellum									1			1	0.85	9.09
All Cladosporium												7	5.93	45.45
Cryptococcus oeirensis					1							1	0.85	9.09
Debaryomyces hansenii		1		1		3		2	3	3	1	14	11.86	63.64
Filobasidium uniguttulatum					1							1	0.85	18.18
Fusarium oxysporum				4		4						8	6.78	18.18
Penicillium sp.				1				1				2	1.69	18.18
Penicillium adametzii							1					1	0.85	9.09
Penicillium antarcticum						1						1	0.85	9.09
Penicillium atramentosum				1						1		2	1.69	18.18
Penicillium cecidicola								2				2	1.69	9.09
Penicillium sect. *chrysogena*		2						1				3	2.54	18.18
Penicillium pancosmium								1				1	0.85	9.09
Penicillium restrictum				1								1	0.85	9.09
Penicillium rubens								1				1	0.85	9.09
Penicillium waksmanii							1					1	0.85	9.09
All Penicillium												15	12.71	54.55
Phialemonium dimorphosporum					1	1						2	1.69	18.18
Phlebiopsis sp.									1			1	0.85	9.09
Phlebiopsis crassa								1				1	0.85	9.09
Rhodotorula sp.					1							1	0.85	9.09
Rhodotorula sloofiae											1	1	0.85	9.09
Rhodotorula diobovata					1						1	2	1.69	18.18
Rhodotorula mucilaginosa	7			4	6	5	5				3	30	25.42	54.55
All Rhodotorula												34	28.81	54.55
Trametes sp.								1				1	0.85	9.09

ISOLATED SPECIES IN PDA AT 17 °C



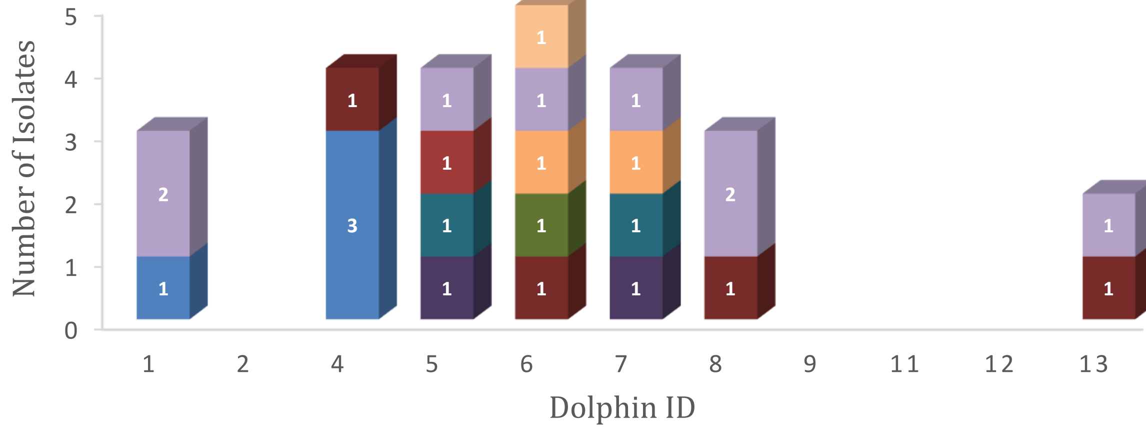
ISOLATED SPECIES IN SAB AT 17 °C



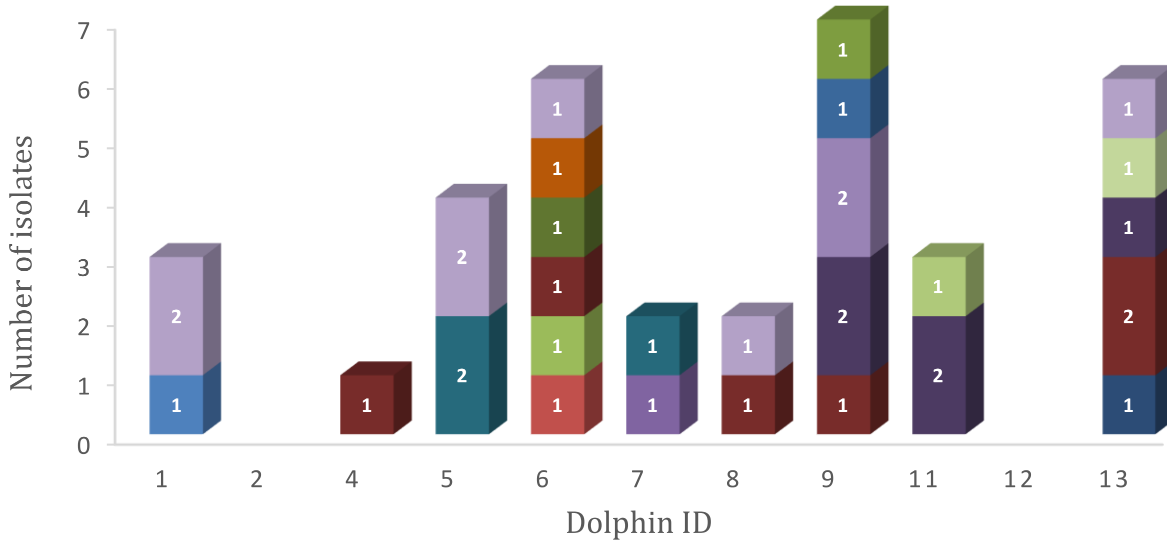
- *A. pullulans*
- *Aspergillus sect. Versicolores*
- *B. bassiana*
- *C. atmosphaerica*
- *C. oeirensis*
- *C. ramotenellum*
- *C.cladospoiroides*
- *C.zeylanoides*
- *Cladosporium sp*
- *D. hansenii*
- *F. oxysporum*
- *F. uniguttulatum*
- *P. adametzii*
- *P. antarcticum*
- *P. atramentosum*
- *P. cecidicola*
- *P. crassa*
- *P. dimorphosporum*
- *P. pancosmium*
- *P. restrictum*
- *P. rubens*
- *P. sect. chrysogena*
- *P. waksmanii*
- *Penicillium sp.*
- *Phlebiopsis sp.*
- *R. sloofiae*
- *R. diobovata*
- *R. mucilaginosa*
- *R. sloofiae*
- *Rhodotorula sp.*
- *Trametes sp.*
- *Unidentified*

Figure 3.4: Isolated species for each dolphin at 17 °C using PDA (top) and Sabouraud (bottom). Each color corresponds to a different identification, as reported in the legend.

ISOLATED SPECIES IN PDA AT 30 °C



ISOLATED SPECIES IN SAB AT 30 °C



A. pullulans	*Aspergillus sect. Versicolores*	*B. bassiana*	*C. atmosphaerica*
C. oeirensis	*C. ramotenellum*	*C.cladospoiroides*	*C.zeylanoides*
Cladosporium sp	*D. hansenii*	*F. oxysporum*	*F. uniguttulatum*
P. adametzii	*P. antarcticum*	*P. atramentosum*	*P. cecidicola*
P. crassa	*P. dimorphosporum*	*P. pancosmium*	*P. restrictum*
P. rubens	*P. sect. chrysogena*	*P. waksmanii*	*Penicillium sp.*
Phlebiopsis sp.	*R. sloofiae*	*R. diobovata*	*R. mucilaginosa*
R. sloofiae	*Rhodotorula sp.*	*Trametes sp.*	*Unidentified*

Figure 3.5: Isolated species for each dolphin at 30 °C using PDA (top) and Sabouraud (bottom). Each color corresponds to a different identification, as reported in the legend.

To further study the impact of the growing conditions on the identified species, the Sorensen similarity index was calculated between the sets of species grouped by culture medium and incubation temperature. Results reported in Table 3.2 showed similarity between all the different conditions. This fact is due to the ability of some species to grow in all different conditions. In fact, comparing the species isolated from PDA at 17 °C and from Sabouraud at 30 °C, it is possible to observe 8 common species.

Table 3.2: Sorensen similarity index calculated for the different incubation conditions (temperature and culture medium) considered in this study. Genus-level identifications and unidentified species were not considered.

Sorensen index	PDA, 17 °C	PDA, 30 °C	SAB, 17 °C	SAB, 30 °C
PDA, 17 °C	--	0.38	0.38	0.48
PDA, 30 °C		--	0.56	0.40
SAB, 17 °C			--	0.40
SAB, 30 °C				--

Shannon diversity index and the closely related Evenness provide a quantitative description of the species diversity. These indexes were calculated for the species identified in each incubated sample, distinguishing between those obtained in blowhole or in oral cavity cultures. These results allowed the detection of differences in the fungal communities from the two sampling sites (Figure 3.6). Some variations were also observed with samples from the same dolphin but from different body sites.

The sample with the highest diversity rate is the blowhole sample from dolphin 6, that presented an H'=2.04. Considering the 25 different identified species, the maximum possible diversity is H'_{max}=3.22. This value was used to calculate the Evenness. Low evenness values indicate that the species are generally not evenly distributed across samples, with samples 9.6 and 6.1 only showing values slightly above 0.5.

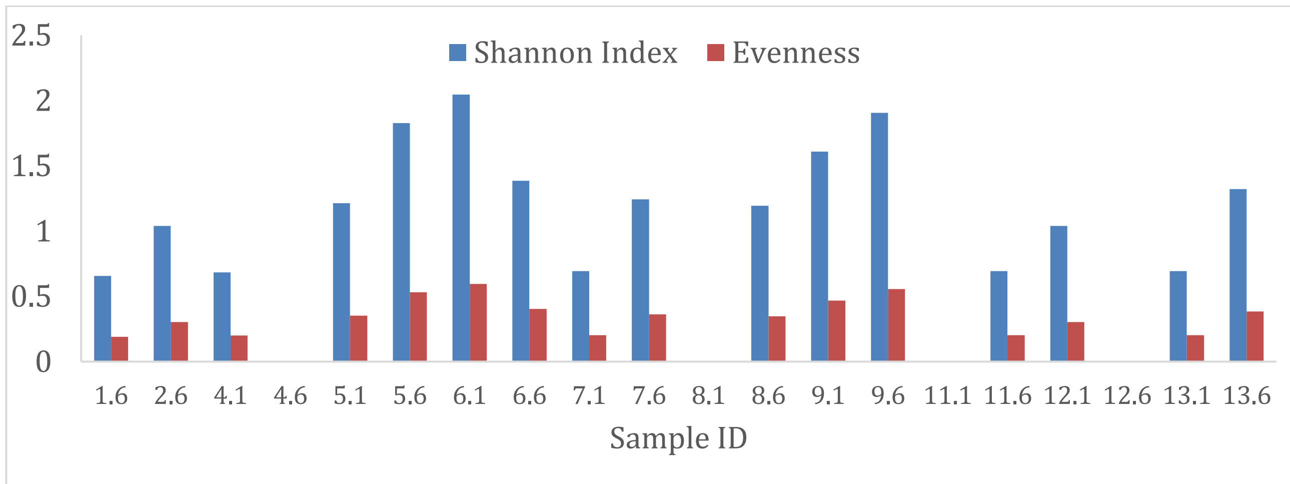


Figure 3.6: Shannon index and evenness value for each sample that was incubated. The evenness was calculated by using a value of H'_{max} = 3.22.

Finally, the Sorensen similarity index was calculated by comparing each sample separately (Table 3.3). Results demonstrated that species isolated from each body site are different from sample to sample. However, samples from dolphins 5 to 8 seemed to be more related with respect to the remaining ones: common species can always be found by comparing any two samples. The species similarity between blowhole and oral cavity of the same dolphin is rather low, being equal or less to 0.5 for all dolphins, except for dolphin 4. While sample 11.6 contained species that were identified only once and, thus, had no common species with any other samples, sample 13.6 contained the most frequent species and because of this shared species with all the other samples.

Table 3.3: Sorensen similarity index for each sample pair. Nonzero values were highlighted in bold font.

Sample	1.6	2.6	4.1	4.6	5.1	5.6	6.1	6.6	7.1	7.6	8.1	8.6	9.1	9.6	11.1	11.6	12.1	12.6	13.1	13.6
1.6	-	0.00	**0.50**	0.00	**0.33**	**0.25**	**0.36**	**0.29**	**0.50**	**0.29**	**0.66**	**0.33**	0.00	0.00	0.00	0.00	0.00	0.00	**0.50**	**0.29**
2.6		-	0.00	0.00	0.00	**0.22**	0.00	0.00	0.00	**0.25**	0.00	0.00	0.00	**0.40**	**0.50**	0.00	**0.67**	**0.50**	0.00	**0.20**
4.1			-	**0.67**	0.00	**0.25**	**0.40**	**0.33**	0.00	0.00	0.00	**0.40**	**0.29**	0.00	0.00	0.00	0.00	0.00	0.00	**0.29**
4.6				-	0.00	**0.29**	**0.20**	**0.33**	0.00	0.00	0.00	**0.40**	**0.33**	0.00	0.00	0.00	0.00	0.00	0.00	**0.33**
5.1					-	**0.20**	**0.15**	**0.22**	**0.67**	**0.22**	**0.40**	**0.25**	0.00	0.00	0.00	0.00	**0.29**	0.00	**0.33**	**0.22**
5.6						-	**0.27**	**0.36**	**0.25**	**0.40**	**0.29**	**0.40**	**0.18**	**0.15**	**0.29**	0.00	**0.22**	**0.29**	**0.29**	**0.55**
6.1							-	**0.29**	**0.18**	**0.14**	**0.20**	**0.31**	**0.14**	**0.25**	0.00	0.00	**0.17**	0.00	**0.36**	**0.43**
6.6								-	**0.29**	**0.40**	**0.33**	**0.44**	**0.20**	0.00	0.00	0.00	0.00	0.00	**0.29**	**0.40**
7.1									-	**0.29**	**0.33**	**0.33**	0.00	0.00	0.00	0.00	0.00	0.00	**0.50**	**0.29**
7.6										-	**0.33**	**0.22**	0.00	**0.17**	**0.33**	0.00	**0.22**	**0.33**	**0.29**	**0.40**
8.1											-	**0.40**	0.00	0.00	0.00	0.00	0.00	0.00	**0.67**	**0.33**
8.6												-	**0.22**	0.00	0.00	0.00	0.00	0.00	**0.33**	**0.44**
9.1													-	**0.17**	0.00	0.00	0.00	0.00	0.00	**0.20**
9.6														-	**0.25**	0.00	**0.40**	**0.25**	**0.22**	**0.17**
11.1															-	0.00	**0.50**	**1.00**	0.00	**0.33**
11.6																-	0.00	0.00	0.00	0.00
12.1																	-	**0.50**	**0.40**	**0.25**
12.6																		-	0.00	**0.33**
13.1																			-	**0.29**
13.6																				-

Chapter IV - Discussion

4.1 Analysis of fungal growth

A total of 122 fungal isolates were obtained from oral and blowhole samples of 11 dolphins. As reported in Figure 3.1, the number of isolates obtained from each dolphin was variable, ranging from 4 (dolphin 4) to 22 (dolphin 6) isolates. These results indicate a high fungal diversity, different across the analyzed dolphins.

The isolation rates and the ratio between filamentous fungi and yeasts is dependent on the sampling site and could be affected by different temperatures and culture media.

4.1.1 Fungal diversity according to body sites

In Figure 3.2 it was possible to compare the number of isolates obtained from oral cavity and blowhole. These results show that oral cavity present a higher number of isolates, however a lower ratio of filamentous fungi.

These differences could be related with several factors. For example, blowhole is part of the cetacean respiratory tract, however oral cavity is not connected with this system. In this way, blowhole is in contact with airborne organisms, while oral cavity is more exposed to microorganisms from water or food.

Clinical studies performed in humans (Underhill & Iliev, 2014) revealed that oral cavity presents a remarkable fungal diversity, with at least 75 different species isolated from healthy individuals. In contrast, the remaining respiratory tract presents low fungal diversity due to the action of the alveolar macrophage cells. However, in immunocompromised individuals some inhaled filamentous fungi spores proliferate in lower respiratory tract, causing fungal infections (Chowdhary, et al., 2016).

Our results suggest some similarity between humans and dolphins, revealing a higher fungal diversity in oral cavity and, at the same time, a higher number of filamentous fungi isolates in blowhole.

4.1.2 Fungal diversity according to different temperatures and culture media

As observed in Figure 3.3, yeast strains were more frequent than filamentous fungi in all the conditions studied. Pashley et al. (Pashley, et al., 2012) demonstrated the preference of yeasts to grow in PDA medium, even in the presence of antibiotic substances. However, in our study only small differences were observed comparing PDA and Sabouraud media, both as 17 °C and 30 °C. In what regards the different temperatures, 17 °C and 30 °C, differences were not evident. However, in order to maximize the fungal diversity, both media and temperatures should be considered.

4.2. Fungal identification

4.2.1 List of identified species.

Debaryomyces hansenii was isolated from 7 dolphins (percentage of colonization of 63,64%), resulting in the most common species. This species was isolated using all conditions considered for this study, as well as in both body sites, blowhole and oral cavity.

D. hansenii is known to produce mycocin, a toxin able to kill other competing yeasts such as *Candida* species (Banjara, et al., 2016). This fact could explain the dominant growth of *D. hansenii* in different environments.

Due to his high tolerance to saline environments sea water is a common habitat for *D. hansenii* (Breuer & Harms, 2006), supporting this species as one of the most common in dolphins oral cavity and blowhole.

Figure 4.1: Strain of *Debaryomyces hansenii* isolated from oral cavity of dolphin 7, using PDA medium at 17 °C.

Rhodotorula sp. was the most frequently isolated genus (34 isolates, 28.8 %), being observed in six dolphins, with a percentage of colonization of 54.55 %. The most frequent species (30 isolates, 25.4%), found in all 6 dolphins, was *Rhodotorula mucilaginosa,* which was isolated in all conditions studied*. R. diobovata* was found in two dolphins (2 isolates, 1.69%) and *R. sloofiae* in one dolphin (1 isolate, 0.85%). Other *Rhodotorula* strain was also isolated, however the species identification was not successful.

Rhodotorula species are easily found in sea water, both near-shore and deep-sea (Kutty & Philip, 2008), therefore it is not surprising the high frequency of this genus in this study.

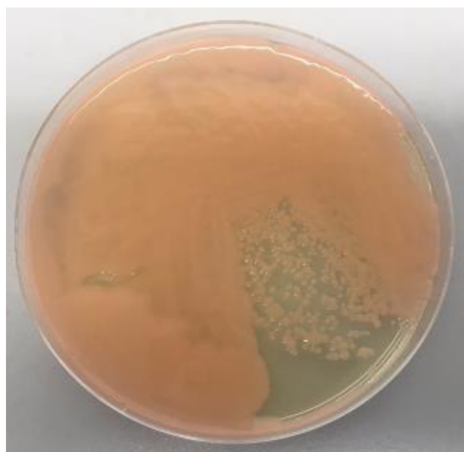


Figure 4.2: Strain of *Rhodotorula mucilaginosa* isolated from oral cavity of dolphin 1, using Sabouraud agar at 17 °C.

Candida zeylanoides was isolated from six dolphins (percentage of colonization of 54.45 %), with a total frequency of 17.8% (considering all conditions studied). Previous studies also reported *Candida zeylanoides* as frequent in sea environment (Kutty & Philip, 2008).

Regarding *Candida* species, *Candida atmosphaerica* was also isolated, however from only one dolphin.

Figure 4.3: Strain of *Candida zeylanoides* isolated from oral cavity of dolphin 4, using Sabouraud agar at 30 °C.

The species discussed above were also frequently isolated from salmonids in Chile (Raggi, et al., 2014). In the same study, it was suggested that *D. hansenii* and *R. mucilaginosa* are part of the microbial flora of the gut, and that its presence was linked with the carnivorous diet of salmons. In our study, it is possible to suggest similar conclusions due to the high frequency of these species in the dolphin samples studied.

Other frequently isolated species was *Aureobasidium pullulans*, isolated from 3 dolphins (percentage of colonization of 27.27%). *A. pullulans* is a ubiquitous black yeast frequently isolated from sea water (Zalar, et al., 2008). It could also be part of the mycobiome of wild-ranging dolphins, even if it was not so frequent as the other species.

Regarding filamentous fungi, the most frequently isolated genus was *Penicillium,* presenting the highest intragenic diversity. It is known that several *Penicillium* species can be isolated from high salinity environments and could produce mycotoxins (Butinar, et al., 2011).

It should be kept in mind that in the majority of times ITS sequences are not able to distinguish between closely related species. However, they could distinguish between species sections, that is a group of similar species defined upon phylogenetical data (Visagie, et al., 2014). That is why some strains, namely *Penicillium* sect. *Chrysogena*, could not be further identified at the species level.

Studies about isolation of *Penicillium* species from marine mammals are very scarce. Our study demonstrates the presence of unreported *Penicillium* species, such as *P. waksmanii, P. adametzii* and *P. antarcticum*, in Portuguese wild-ranging dolphins.

Figure 4.4: Left: Strain of *Penicillium sect. Chrysogena* isolated from oral cavity of dolphin 2, using YPD agar at 17 °C. Center: Strain of *Penicillium restrictum* isolated from oral cavity of dolphin 5, using PDA at 30 °C. Right: Strain of *Penicillium waksmanii* isolated from blowhole of dolphin 8, using PDA at 17 °C.

Cladosporium sp. strains were isolated from 5 dolphins (percentage of colonization of 45.45%), being *Cladosporium cladosporioides* the most frequently isolated species (4 isolates, with a frequency of 3.39%). *C. ramotenellum* was also identified once. *Cladosporium sp.* showed preference for PDA medium, since six of the isolates grew in this medium. Remarkably, 5 *Cladosporium sp.* strains were isolated from blowhole samples, showing a high affinity with the respiratory tract.

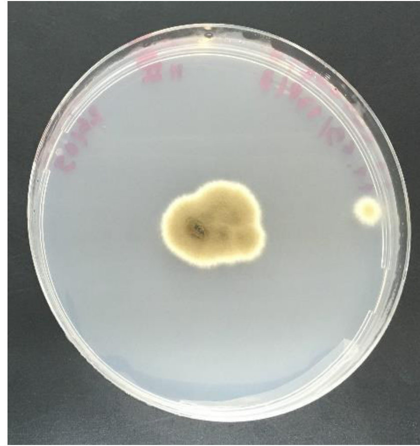


Figure 4.5: Strain of *Cladosporium cladosporioides* isolated from oral cavity of dolphin 11, using PDA at 17 °C.

The filamentous fungi *Fusarium oxysporum* was isolated exclusively from the blowhole of two dolphins (colonization percentage 18.18 %) using all conditions considered. The presence of *F. oxysporum* is interesting because it is a renowned plant parasite also responsible for fungal infections in humans and marine mammals. Moreover, this species is not part of the dolphins normal mycobiome, being its prevalence in respiratory tract probably related with pulmonary infection. *F. oxysporum* pathogenicity is confirmed by the preference to grow at 30 °C, which is the dolphins body temperature. *F. oxysporum* could be waterborne, since it was found in seawater samples (Palmero & Iglesias, 2009).

Figure 4.6: Strain of *Fusarium oxysporum* isolated from blowhole of dolphin 7, using Sabouraud agar at 30 °C.

Several other fungal species were isolated, but, unlike the previous ones, occurred much less frequently. *Beauveria bassiana* was isolated from the blowhole of dolphin 6 and grew at 30 °C. Although *B. bassiana* can be isolated from marine sediments (Suresh & Chandrasekaran, 1999), it was not possible to find case reports of its presence in any marine mammal. The activity of *B. bassiana* is well established against insects, however no studies have demonstrated pathogenicity against marine mammals.

Figure 4.7: Strain of *Beauveria bassiana* isolated from the blowhole of dolphins 6, using Sabouraud at 30 °C.

Aspergillus sect. *Versicolores* strain was also isolated from the blowhole sample of dolphin 6. Regarding oral cavity of dolphin 6, *Cryptococcus oeirensis* and *Filobasidium uniguttulatum* strains were isolated at 30 °C and 17 °C respectively.

It is important to note that *F. uniguttulatum* is a teleomorph form of *Cryptococcus uniguttulatum*, therefore two *Cryptococcus*–related strains were isolated from dolphin 6. It is also interesting to note that the two forms grew at different temperatures.

The pathogenic fungi *Phialemonium dimorphosporum* (also known as *P. curvatum)* was isolated from oral cavity samples of dolphins 6 and 7 at 30 °C. Finally, a *Trametes sp.* strain and two *Phlebiopsis sp.* strains were isolated. Those species are referred as corticioid fungi because, in their natural habitat, they usually grow on

dead trunks and branches. The presence of those species in dolphin samples could be through the debris carried by rivers, especially after floods or violent weather.

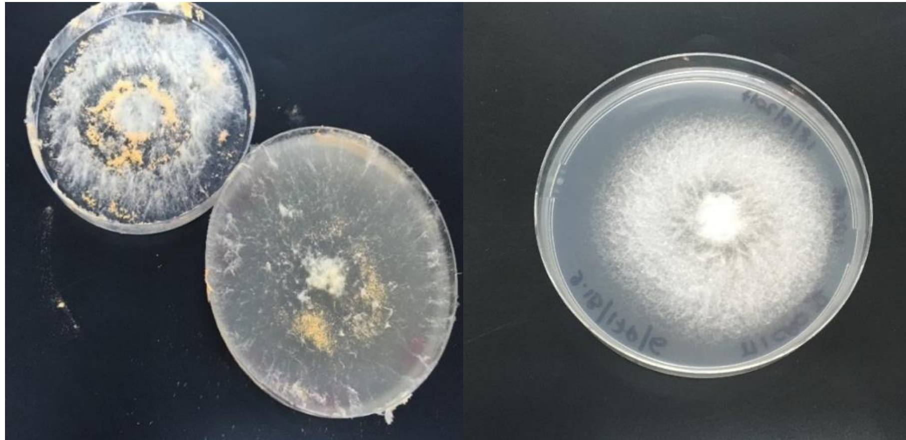


Figure 4.8 Left: Strain of *Phlebiopsis sp.* isolated from oral cavity of dolphin 11, using PDA at 17 °C. Right: Strain of *Trametes sp.* isolated from blowhole of dolphin 9, using Sabouraud agar at 17 °C.

4.2.3 Final remarks

The results obtained suggested that *D. hansenii, C. zeylanoides, R. mucilaginosa,* and *A. pullulans* could be part of the dolphins mycobiome. This conclusion is based on their widespread colonization, since they are common in seawater and have low pathogenicity. *Cladosporium* and *Penicillium* genera could also be part of the dolphins mycobiome, however it is necessary to study other dolphins to confirm this hypothesis.

Regarding the uncommon species, it is possible to conclude that the majority of pathogenic strains presented affinity to grow at 30 °C, which is the dolphins body temperature. Moreover, the majority of pathogenic species were isolated from blowhole, suggesting respiratory infections.

Several pathogenic species were isolated from dolphin 6, such as *Aspergillus* sect. *Versicolores*, *Cryptococcus oeirensis*, *Phialemonium dimorphosporum* and *Beauveria bassiana*. This dolphin also presented the highest fungal diversity. These data could

mean that the immune system of this dolphin was compromised, and allowed the opportunistic growth of fungal species.

4.3 Richness and diversity analysis.

It was already discussed how the choice of culture medium and incubation temperature could influence the isolation rates of filamentous fungi and yeast. Using the molecular identification results, it is possible to compare the species encountered in the different cultures.

Table 3.1 reported the Sorensen similarity index calculated between all the growing conditions considered. Results showed low values, below 0.5, indicating that each culture contained unique species, that were not isolated elsewhere.

5 species grew in all the conditions considered, that is *R. mucilaginosa, D. hansenii, C. zeylanoides, F. oxysporum* and *A. pullulans,* were capable to grow in every medium and temperature. Only 3 species could grow in two different conditions, that is *C. cladosporioides, R. diobovata* and *P. atramentosum*, while all the others appeared only in one medium and temperature.

This reflects the fact that the fungal microbial community hosted by dolphin soft tissues is diverse, and that its screening, at least regarding the culturable part, requires the use of different culture media and temperatures, to avoid the risk of missing some species, or underestimating them.

A quantitative assessment of the richness in species of each sample is represented by the Shannon index, that was calculated and shown for each sample in Figure 3.4. As for the isolation rates, the Shannon index was non-uniform across the dolphins. The richest one was dolphin 6, with a value of 2.04 for *H´*. Practically, no set of isolates taken from one dolphin could be representative of the total fungal diversity.

The Sorensen value measures the similarity in species composition between two groups, and could be used to check the similarities between the isolate populations from single samples, as reported in Table 3.3. Analyzing these results, it emerged that the mycobiome encountered in the blowhole and oral cavity had different composition, even within the same dolphin. This was expected, because a difference in the yeast/filamentous fungi ratio between the two sites was already found.

By comparing any two samples obtained from dolphin 5 to 8, it is always possible to find at least one common species. This could indicate a certain degree of similarity in the fungal population amongst these dolphins. By looking at the information regarding their accidental capture site, it resulted that all of them were recovered at Praia de Mira. It could be guessed that the similarity in the fungal population is related to the fact that those individuals belonged to the same pod. Because of this, they had more contacts between themselves, with a potential sharing of fungal strains.

Chapter V - Conclusions

This work represents the first attempt to characterize the fungal microbial community of oral cavity and respiratory tract of free-ranging dolphins (*Delphinus delphis, Phocoena phocoena* and *Stenella coeruleoalba)* of Portugal coast. The use of molecular identification techniques allowed the identification of 25 different fungal species, using samples of 11 dolphins.

It was shown that the use of different culture media and incubation temperatures greatly enhanced the species diversity of the isolated fungi. From this, it is possible to conclude that a combination of different culture media and incubation temperatures is needed to avoid missing some fungal species. In particular, the use of high incubation temperatures (30 °C) enhances the growth of pathogenic filamentous fungi.

Regarding the body sites, it was possible to observe that the mycobiome of oral cavity and respiratory tract was different: the isolation of filamentous fungi from blowhole was considerably higher. This could suggest that the conditions of the two body sites are different. Moreover, in cetaceans, the oral cavity is not part of respiratory tract, which means that they are not exposed to the same fungal vectors, such as food.

Regarding the mycobiome composition, several yeast species were identified, namely *Debaryomyces hansenii, Rhodotorula mucilaginosa* and *Candida zeylanoides.* Those species were frequently isolated in the majority of dolphins studied and are commonly isolated from seawater. Concerning filamentous fungi, *Cladosporium sp.* and *Penicillium sp.* were very frequent, suggesting that could be part of the mycobiome of healthy dolphins as well. However, it should be clarified whether their presence could be related to an infection.

Several well-known pathogenic fungi were isolated, such as *Cryptococcus oeirensis, Filobasidium uniguttulatum*, *Fusarium oxysporum*, *Phialemonium dimorphosporum* and *Aspergillus* Sect. *Versicolores*. The high prevalence of pathogenic strains could indicate the presence of severe stress factors that altered dolphin immune response, allowing the development of fungal infections.

This shows that the mycobiome composition could be related with the dolphins stress level and environment. Concerning the dolphins health implications of these results it is possible to observe that they could host high diversity of pathogenic fungi, causing outbreaks. Human health is also concerned, because the identified species are known human pathogenic agents, especially for immunocompromised individuals. For these reason, the study of dolphin fungal mycobiome is very important to identify the epizootic and zoonotic risks.

Our results also suggested that the fungal community composition might change between different groups. For example, a high similarity of fungal diversity of a group of *P. phocoena* found in the same beach was observed. Low similarity was found between those and dolphins found in different regions. This suggests that dolphins are vectors of fungal species and are able to diffuse them. Moreover, mycobiome characterization may be used as a tool both to assess the health status of groups and to study the interactions between populations.

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Appendix

Appendix 1 - Tables

Table A.1: List of the dolphins from which the cultured samples were isolated. The data was collected by SPVS researchers that also performed the necropsy. Most of the dolphins were found stranded after an accidental capture with a xavega, a kind of fishing net used by Portuguese traditional fisheries.

ID	*Species*	*Sex*	*Stranding site*	*Death cause*	*Necropsy results*
1	*Stenella coeruleoalba*	F	Algarve	Stranding	Lesions on the skin and in the gastric tract, specifically in the tongue and first and second stomach.
2	*Delphinus delphis*	F	Espinho	Capture	Poxovirus infections and tattoo skin lesions edema and petechiae in the lungs, an edema in the pericardium, petechiae in ventricles and in the mesenteric lymph node, stained pancreas.
3	*Delphinus delphis*	F	Ericeira	Capture	High parasite content in lungs. Several internal organs swollen.
4	*Delphinus delphis*	F	Praia de Mira	Capture	Marble-looking lungs with presence of fibrin, swollen pulmonary lymph node and pus inside left lung. Petechiae on left ventricle and swollen adrenal glands, with hemorrhagic cortex.
5	*Delphinus delphis*	F	Praia de Mira	Capture	Poxovirus infection. Petechiae in lungs and pulmonary lymph nodes of heterogeneous aspect. Hemorrhagic spleen. Swollen lymph node in colon.
6	*Delphinus delphis*	F	Praia de Mira	Capture	Poxovirus infection. Bleeding prescapular lymph node with black dots, petechiae on ventricles with the right one hypertrophic. Black dots on the lung lympho-nodes. hemorrhagic adrenals.
7	*Delphinus delphis*	M	Praia de Mira	Capture	Hemorrhagic Spleen. Swollen lymph node in Colon.
8	*Delphinus delphis*	F	Praia de Mira	Capture	Poxovirus infection and small round lesions on skin. Blood-stained and slightly swollen prescapular lymph node, with black stains. Very swollen colon and mesentheric lymph nodes. Black spots on Pancreas. Petechiae in lungs. Hemorrhagic adrenal glands medulla.
9	*Delphinus delphis*	F	Foz do Douro	Capture	Swollen, blood-stained and white-spotted prescapular lymph node. Ammonia smell from thoracic cavity, blowhole and trachea. Swollen and blood-stained pulmonary lymph nodes. swollen mesentheric lymph node. Fibrosis in liver. Adrenal glands with hermorrhagic cortex.
11	*Phocoena phocoena*	F	São Pedro de Moel	Capture	Poxovirus infection. Swollen and hemorrhagic prescapular lymph node. Hemorrhagic lymph nodes in lungs and mesentherium. Hemorrhagic adrenal glands.
12	*Delphinus delphis*	F	Valadares	Capture	Blood-stained prescapular lymph node. Small-sized heart. Liver with thickened ducts. Hemorrhagic adrenal glands. Yellow pus inside mammal glands ducts. Vomit inside oral cavity.
13	*Phocoena phocoena*	F	Osso da Baleia	Capture	Lesion on tongue. Blood-stained prescapular, pulmonary and mesentheric lymph nodes. Swollen thymus. Scars in liver. Spotted pancreas. Blood-stained and congested kidneys.

Table A.2: Isolates obtained from the cultured samples of dolphin 1 and their identification.

Isolate ID	*Num. of colonies*	*Macroscopic aspect*	*Identification*
1.6B/17S/1	2	Yeast colonies. Pink, bright and viscous.	*Rhodotorula mucilaginosa*
1.6B/17S/2	2	Yeast colonies. Pink and opaque.	*Rhodotorula mucilaginosa*
1.6B/17S/3	5	Yeast colonies. White and opaque.	*Aureobasidium pullulans*
1.6B/17P/1	11	Yeast colonies. Pink, bright and very viscous.	*Rhodotorula mucilaginosa*
1.6B/17P/2	3	Yeast colonies. White, bright and viscous.	*Aureobasidium pullulans*
1.6B/30S/1	5	Yeast colonies. Pink, bright and viscous.	*Rhodotorula mucilaginosa*
1.6B/30S/2	3	Yeast colonies. Pink and opaque.	*Rhodotorula mucilaginosa*
1.6B/30S/3	4	Yeast colonies. White, small and opaque	*Aureobasidium pullulans*
1.6B/30P/1	3	Yeast colonies. Pink, bright and viscous.	*Rhodotorula mucilaginosa*
1.6B/30P/2	2	Yeast colonies. Pink and opaque.	*Rhodotorula mucilaginosa*
1.6B/30P/3	4	Yeast colonies. White, small and viscous.	*Aureobasidium pullulans*

Table A.3: Isolates obtained from the cultured samples of dolphin 2 and their identification.

Isolate ID	*Num. of colonies*	*Macroscopic aspect*	*Identification*
2.6C/17Y/1	13	Yeast colonies. White and opaque.	*Debaryomyces hansenii*
2.6C/17Y/2	1	Filamentous fungus. White and greenish.	*Penicillium* sect. *Chrysogena*
2.6C/17Y/3	1	Filamentous fungus. White and brown.	*Penicillium* sect. *Chrysogena*
2.6B/17P/1	>100	Yeast colonies. Pink and opaque with regular contours.	*Unidentified*

Table A.4: Isolates obtained from the cultured samples of dolphin 4 and their identification.

Isolate ID	*Num. of colonies*	*Macroscopic aspect*	*Identification*
4.1B/17P/1	2	Yeast colonies. White, big and viscous and filament forming.	*Aureobasidium pullulans*

Table A.4 (continued).

4.1B/17P/2	3	Yeast colonies. White, opaque and regular contours.	*Candida zeylanoides*
4.1B/30P/1	*1*	*Yeast colonies. White, big and viscous and filament forming.*	*Aureobasidium pullulans*
4.1B/30P/3	1	Yeast colony. White, big and viscous and filament forming.	*Aureobasidium pullulans*
4.1B/30P/4	1	Yeast colony. White, big and viscous and filament forming.	*Aureobasidium pullulans*
4.1B/30P/5	1	Yeast colony. White, big, opaque and regular contours.	*Candida zeylanoides*
4.1B/30S/1	3	Yeast colony. White, small opaque and regular contours.	*Candida zeylanoides*
4.6B/17P/1	3	Yeast colony. White, big and viscous and filament forming	*Candida zeylanoides*

Table A.5: Isolates obtained from the cultured samples of dolphin 5 and their identification.

Isolate ID	***Num. of colonies***	***Macroscopic aspect***	***Identification***
5.1B/17P/1	3	Filamentous fungi. White and green.	*Penicillium atramentosum*
5.1B/17S/1	1	Filamentous fungi. White and green.	*Unidentified*
5.1B/17S/2	3	Filamentous fungi. White and pink.	*Fusarium oxysporum*
5.1B/30P/1	3	Yeast colonies. Pink, opaque and with regular contours.	*Rhodotorula mucilaginosa*
5.1B/30P/2	1	Filamentous fungus. White and pink .	*Fusarium oxysporum*
5.1B/30S/1	3	Filamentous fungi. White and pink.	*Fusarium oxysporum*
5.1B/30S/2	1	Yeast colony. Pink and bright.	*Rhodotorula mucilaginosa*
5 .1B/30S/3	1	Filamentous fungus. White.	*Fusarium oxysporum*
5.6B/17P/1	3	Yeast colonies. White, opaque, small and with regular contours.	*Candida zeylanoides*
5.6B/17P/2	1	Yeast colony Pink, bright.	*Rhodotorula mucilaginosa*
5.6B/17P/3	2	Filamentous fungi. White and green.	*Penicillium sp.*
5.6B/17S/1	2	Yeast colonies. White, opaque with regular contours.	*Candida zeylanoides*
5.6B/17S/2	2	Filamentous fungi. White and green.	*Unidentified*

Table A.5 (continued).

5.6B/30P/1	1	*Yeast colony. White, opaque with regular contours.*	*Debaryomyces hansenii*
5.6B/30P/2	2	Filamentous fungi. White and with yellow contours.	*Penicillium restrictum*
5.6B/30S/1	2	Yeast colonies. Pink, opaque with regular contours.	*Rhodotorula mucilaginosa*

Table A.6: Isolates obtained from the cultured samples of dolphin 6 and their identification.

Isolate ID	*Num. of colonies*	*Macroscopic aspect*	*Identification*
6.1B/17P/1	>100	Yeast colonies. White, small, opaque and with regular contours.	*Candida zeylanoides*
6.1B/17P/2	>50	Yeast colonies. Light pink, shiny and viscous.	*Rhodotorula diobovata*
6.1B/17P/3	1	Yeast colony. White, big and viscous. Formation of dark pigments starting from the interior of the colony.	*Aureobasidium pullulans*
6.1B/17P/4	26	Filamentous fungi. Green.	*Cladosporium cladosporioides*
6.1B/17P/5	20	Yeast colonies. Dark pink, opaque and regular contours .	*Rhodotorula mucilaginosa*
6.1B/17S/2	28	Yeast colonies. White, small, opaque and with regular contours.	*Candida zeylanoides*
6.1B/17S/3	20	Yeast colonies. Pink, small and opaque with regular contours.	*Rhodotorula mucilaginosa*
6.1B/30P/1	>60	Yeast colonies. Light pink, bright and viscous.	*Rhodotorula sp.*
6.1B/30P/2	>50	Yeast colonies. White, small, opaque and with regular contours.	*Candida zeylanoides*
6.1B/30P/3	37	Filamentous fungi. Green.	*Cladosporium sp.*
6.1B/30S/1	3	Filamentous fungi. Dark green.	*Aspergillus* sect. *Versicolores*
6.1B/30S/2	>100	Filamentous fungi. Green.	*Cladosporium sp.*
6.1B/30S/3	1	Filamentous fungus. White.	*Beauveria bassiana*

Table A.6 (continued).

6.1B/30S/4	60	Yeast colonies. White, small, opaque and with regular contours.	*Candida zeylanoides*
6.6A/17S/2	3	Yeast colony. White, small and opaque with regular contours.	*Candida zeylanoides*
6.6B/17P/1	26	Yeast colonies. Pink, bright with regular contours.	*Rhodotorula mucilaginosa*
6.6B/17P/2	7	Yeast colonies. Beige, medium, opaque and with regular contours.	*Cryptococcus oeirensis*
6.6B/17S/1	1	Yeast colony. Pink, bright and with regular contours.	*Rhodotorula mucilaginosa*
6.6B/30P/1	8	Yeast colonies. Pink, bright and with regular contours.	*Rhodotorula mucilaginosa*
6.6B/30P/3	1	White, big and opaque colony with regular contours.	*Phialemonium dimorphosporum*
6.6B/30S/1	1	Yeast colony. White, big and with regular contours.	*Filobasidium uniguttulatum*
6.6B/30S/2	3	Yeast colonies. Pink, bright and with regular contours.	*Rhodotorula mucilaginosa*

Table A.7: Isolates obtained from the cultured samples of dolphin 7 and their identification.

Isolate ID	***Num. of colonies***	***Macroscopic aspect***	***Identification***
7.1B/17P/1	2	Yeast colonies. Pink, bright and with regular contours.	*Rhodotorula mucilaginosa*
7.1B/17P/2	4	Filamentous fungi. White and pink.	*Fusarium oxysporum*
7.1B/17S/1	2	Filamentous fungi. White and pink.	*Fusarium oxysporum*
7.1B/17S/2	2	Yeast colonies. Pink, opaque and with regular contours.	*Rhodotorula mucilaginosa*
7.1B/17S/3	1	Yeast colony. Pink, very bright and with regular contours.	*Rhodotorula mucilaginosa*
7.1B/30P/1	3	Yeast colonies. Pink, bright and with regular contours.	*Rhodotorula mucilaginosa*
7.1B/30P/2	2	Filamentous fungi. White and pink.	*Fusarium oxysporum*

Table A.7 (continued).

7.1B/30S/1	1	Filamentous fungus. White and pink.	*Fusarium oxysporum*
7.6B/17P/1	1	Yeast colony. White, opaque, and with regular contours.	*Debaryomyces hansenii*
7.6B/17P/2	1	Yeast colony. Pink, bright and with regular contours.	*Rhodotorula mucilaginosa*
7.6B/17P/3	1	Filamentous fugus. White and green	*Penicillium antarcticum*
7.6B/17S/1	1	Yeast colony. White, opaque with regular contours.	*Debaryomyces hansenii*
7.6B/30P/1	1	White, bright and filaments producing colony.	*Phialemonium dimorphosporum*
7.6B/30P/2	1	Yeast colonies. White, opaque, and with regular contours.	*Debaryomyces hansenii*
7.6B/30S/1	1	Yeast colony. White, opaque with regular contours.	*Candida atmosphaerica*

Table A.8: Isolates obtained from the cultured samples of dolphin 8 and their identification.

Isolate ID	*Num. of colonies*	*Macroscopic aspect*	*Identification*
8.1B/30P/1	1	Yeast colonies. Pink, bright and with regular contours.	*Rhodotorula mucilaginosa*
8.6B/17P/1	1	Filamentous fungus. Dark green with white contours.	*Penicillium waksmanii*
8.6B/17P/2	10	Yeast colonies. Pink, some shine with regular contours.	*Rhodotorula mucilaginosa*
8.6B/17P/3	4	Yeast colonies. White and small, opaque, and with regular contours.	*Candida zeylanoides*
8.6B/17S/1	>10	Yeast colonies. White, opaque, and small with regular contours.	*Candida zeylanoides*
8.6B/17S/2	10	Yeast colonies. Pink, some with regular contours. .	*Rhodotorula mucilaginosa*
8.6B/17S/3	1	Filamentous fungus. White with a green halo.	*Penicillium adametzii*
8.6B/30P/1	10	Yeast colonies. White, opaque with regular contours.	*Candida zeylanoides*
8.6B/30P/3	>100	Yeast colonies. Pink, some shine and with regular contours.	*Rhodotorula mucilaginosa*

Table A.8 (continued).

8.6B/30S/1	12	Yeast colonies. Pink, some shine with regular contours.	*Rhodotorula mucilaginosa*
8.6B/30S/2	4	Yeast colonies. White, small, opaque, and with regular contours.	*Candida zeylanoides*

Table A.9: Isolates obtained from the cultured samples of dolphin 9 and their identification.

Isolate ID	***Num. of colonies***	***Macroscopic aspect***	***Identification***
9.1B/17P/6	1	Filamentous fungus. White.	*Trametes sp.*
9.1B/17S/6	1	Filamentous fungus. Large, circular and white.	*Phlebiopsis crassa*
9.1B/30S/2	>100	Filamentous fungi. White with green center.	*Penicillium pancosmium*
9.1B/30S/3	3	Filamentous fungi. Brown with dark brown center.	*Penicillium cecidicola*
9.1B/30S/5	>20	Yeast colonies. Beige, opaque with regular contours.	*Candida zeylanoides*
9.6B/17P/1	1	Yeast colony. White, opaque with regular contours.	*Debaryomyces hansenii*
9.6B/17P/2	>100	Yeast colonies. White, opaque with regular contours.	*Debaryomyces hansenii*
9.6B/17P/3	1	Filamentous fungus. Black.	*Cladosporium cladosporioides*
9.6B/17S/1	5	Yeast colonies. Beige, opaque with regular contours.	*Unidentified*
9.6B/17S/5	1	Filamentous fungus. White with green center.	*Penicillium sp.*
9.6B/17S/6	1	Filamentous fungus. White with dark green center.	*Penicillium* sect. *Chrysogena*
9.6B/30S/1	>20	Filamentous fungi. White with green center.	*Penicillium rubens*
9.6B/30S/3	1	Filamentous fungus. White with green center and red pigmentation.	*Penicillium cecidicola*

Table A.10: Isolates obtained from the cultured samples of dolphin 11 and their identification.

Isolate ID	*Num. of colonies*	*Macroscopic aspect*	*Identification*
11.1B/17S/2	>100	Yeast colonies. White, opaque with regular contours.	*Debaryomyces hansenii*
11.1B/30S/3	>100	Yeast colonies. White, opaque with regular contours.	*Debaryomyces hansenii*
11.1B/30SA/5	1	Yeast colony. White, opaque with regular contours.	*Debaryomyces hansenii*
11.6B/17P/3	>50	Filamentous fungi. Olive-green to brown.	*Cladosporium ramotenellum*
11.6B/17P/4	1	Filamentous fungus. White and pink.	*Phlebiopsis sp.*

Table A.11: Isolates obtained from the cultured samples of dolphin 12 and their identification.

Isolate ID	*Num. of colonies*	*Macroscopic aspect*	*Identification*
12.1B/17P/5	1	Filamentous fungus. Black	*Cladosporium cladosporioides*
12.1B/30SA/1	1	Filamentous fungus. White.	*Penicillium atramentosum.*
12.1B/30SA/2	10	Yeast colonies, white, small and opaque, with regular contours.	*Debaryomyces hansenii*
12.6B/17S/5	1	Yeast colonies, white, small and opaque, with regular contours.	*Debaryomyces hansenii*
12.6B/30SA/1	>30	Yeast colonies, white, small and opaque, with regular contours.	*Debaryomyces hansenii*

Table A.12: Isolates obtained from the cultured samples of dolphin 13 and their identification.

Isolate ID	*Num. of colonies*	*Macroscopic aspect*	*Identification*
13.1B/17P/1	>30	Yeast colonies. Pink, bright and viscous with regular contours.	*Rhodotorula mucilaginosa*
13.1B/30S/1	1	Filamentous fungus. Olive-green.	*Cladosporium cladosporioides*
13.6B/17P/2	2	Yeast colonies. Light pink with regular contours.	*Rhodotorula sloofiae*

Table A.12 (continued).

13.6B/17P/3	4	Yeast colonies. White, opaque with regular contours.	*Candida zeylanoides*
13.6B/17S/10	4	Yeast colonies. White, opaque with regular contours.	*Candida zeylanoides*
13.6B/30P/1	2	Yeast colonies. Pink, bright and viscous with regular contours.	*Rhodotorula mucilaginosa*
13.6B/30P/8	4	Yeast colonies. White, opaque with regular contours.	*Candida zeylanoides*
13.6B/30S/1	2	Yeast colonies. Pink, bright and viscous.	*Rhodotorula mucilaginosa*
13.6B/30S/5	8	Yeast colonies. White, opaque with regular contours.	*Candida zeylanoides*
13.6B/30SA/2	1	Yeast colony. White, opaque with regular contours.	*Candida zeylanoides*
13.6B/30SA/3	>20	Yeast colonies. White, opaque with regular contours.	*Debaryomyces hansenii*
13.6B/30SA/1	1	Yeast colony. Dark pink, translucid with regular contours.	*Rhodotorula diobovata*

Appendix 2 - Reagents for buffer solution preparation

TAE buffer

- Tris base in water
- Glacial acetic acid
- 0.5 M EDTA (pH 8.0) solution

TES buffer

- 0.05 M EDTA
- 20% Sucrose
- 1M Tris base or 0.01M HCl (pH 8.0)

Loading buffer

- Glycerol 100%
- Tris HCl 1M (pH 8.0)
- EDTA 0.5 M (pH 8.0)
- Bromophenol blue
- Distilled water