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TOXICOLOGIA E CONTAMINAÇÕES AMBIENTAIS

# Isolation and identification of toxin-producing fungi associated with macroalgae

Tiago Nuno Matos Vale Pereira Guimarães

M

2021

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FACULDADE DE CIÊNCIAS

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

M.ICBAS 2021

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Dissertation for the master's degree in Toxicology and Environmental Contamination submitted to the Abel Salazar Biomedical Sciences Institute from the Porto University

Supervisor: Vitor Vasconcelos

Category: Full Professor

Affiliation: CIIMAR, Interdisciplinary Centre of Marine and Environmental Researcher

## Acknowledgments

I'd like to show my appreciation to all the people that helped me directly or indirectly during the development of this work.

To my supervisor, Dr. Vitor Vasconcelos, for accepting my application to work on this fascinating project and for accompanying and helping me in the collection of samples.

To Mariana Oliveira, for guiding me and teaching me the procedures needed to do this work.

To Jesus Gonzalez, from University of Santiago de Compostela, for helping me by analyzing of mycotoxins.

To the LEGE team, for their good mood, advice and for the help provided when needed, creating a good work environment.

To my close and dear friend, Mariana Pires, for the good times during lunch break, our cappuccino times in "–1", and for accompanying me while we write our own dissertations.

To my family, for motivating me to continue pursuing my objectives and helping me in every step of the way.

To the Agritox Project for supporting this work.

## Abstract

Marine macroalgae have been utilized for a variety of applications, however, there is still a lot of unknown knowledge regarding the fungi found in association with them.

Fungi can produce toxins, called mycotoxins, that can be responsible for health hazards in plants, animals, and humans that can range from mild to severe.

In the present work, we carried out research aiming to isolate and identify, fungi from different species of macroalgae collected in two beaches in North Portugal, Praia do Castelo do Queijo and Praia da Memória, and then analyze these fungi to detect the presence of mycotoxins.

The fungi were cultivated in Petri dishes with Potato Dextrose Agar, incubated and submitted to PCR analysis. A variety of genus were identified however the genus *Aspergillus* showed dominance displaying various species *A. carbonarius, A. fumigatus, A. giganteus, A. niger, A. piperis and A. versicolor* throughout all the collected macroalgae.

To determine the presence of mycotoxins, the samples were subjected to Ion-Trap -Time- of-flight Mass Spectrometry and each retention peak of the compounds detected were compared with existing literature to be able to confirm the existence of these mycotoxins.

A diversity of mycotoxins was found, for example, the detection of Gliotoxin that is responsible for neuroinflammatory reactions in humans and Acetophthalidin which is believed to be an inhibitor of mammalian cell cycle that could be used for anticancer treatments.

There is still a great amount of research in need to be conducted regarding fungi found in marine macroalgae however this knowledge could lead to interesting findings in medicine and pharmaceutical applications.

## Resumo

Macroalgas marinhas têm sido utilizadas para uma variedade de aplicações, no entanto, ainda existem muito desconhecimento em relação aos fungos encontrados em associação com elas.

Os fungos podem produzir toxinas, chamadas micotoxinas, que podem ser responsáveis por problemas de saúde em plantas, animais e humanos que podem variar de leves a graves.

No presente trabalho, realizámos uma investigação para isolar e identificar, a partir de diferentes espécies de macroalgas, fungos de duas praias em Portugal, Praia do Castelo do Queijo e Praia da Memória, e posteriormente analisamos estes fungos para detetar a presença de micotoxinas.

Os fungos foram cultivados em placas de Petri com Potato Dextrose Agar, incubados e submetidos a tratamento de PCR e eletroforese. Uma variedade de gêneros foram identificadas, no entanto, o género *Aspergillus* apresentou dominância exibindo várias espécies *A. carbonarius*, *A. fumigatus*, *A. giganteus*, *A. niger*, *A. piperis* e *A. versicolor* em todas as macroalgas recolhidas.

Para determinar a presença de micotoxinas, as amostras foram submetidas a Ion-Trap
-Time-of-flight Mass Spectrometry e cada pico de retenção foi comparado com a literatura existente de forma a comprovar a existência destas micotoxinas.

Uma diversidade de micotoxinas foi encontrada como, por exemplo, a deteção da Gliotoxina, que é responsável pelas reações neuroinflamatórias em humanos, e da Acetoftalidina, que se acredita ser um inibidor do ciclo celular dos mamíferos e que poderá ser usada em tratamento contra o cancro.

Ainda há uma grande quantidade de investigação a ser realizada sobre fungos encontrados em macroalgas marinhas, no entanto, esse conhecimento pode levar a descobertas interessantes na medicina e em aplicações farmacêuticas.

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# Abbreviations

$AFB_1$ - Aflatoxin B1

C.Q. - Praia do Castelo do Queijo

CIIMAR - Centro Interdisciplinar de Investigação Marinha e Ambiental

$C_9H_8O_4$ - Dehydrocarolic acid

$C_{13}H_{12}N_2O_2$ – Aspernigrin

$C_{13}H_{22}N_2O$ – Nigragillin

$C_{14}H_{16}O_5$ – Infectopyrone

$C_{14}H_{18}O_3$ - Phomapyrone A

$C_{15}H_{12}O_5$ - Rubrofusarin or TMC-256C1

$C_{15}H_{12}O_6$ – Funalenone

$C_{15}H_{14}O_6$ – Fonsecin

$C_{16}H_{14}O_5$ - Flavasperone

$C_{16}H_{16}O_6$ - Fonsecin monomethyl ether

$C_{16}H_{20}N_2$ – Costaclavine

$C_{16}H_{22}N_2O$ - Nigerazine A or Nigerazine B

$C_{18}H_{12}O_6$ – Sterigmatocystin

$C_{18}H_{22}N_2O_2$ - Roquefortine A

$C_{19}H_{23}N_3O_2$ – Preechinulin

$C_{22}H_{23}N_5O_2$ - Roquefortine C

$C_{23}H_{23}N_5O_4$ – Meleagrin

$C_{23}H_{30}N_2O_2$ - Fumigaclavine C

$C_{23}H_{39}N_5O_5S_2$ - Malformin A1

$C_{24}H_{25}N_5O_4$ – Oxaline

$C_{26}H_{34}O_7$ – Fumagillin

$C_{29}H_{30}N_4O_7$ – Tryptoquivaline

$C_{32}H_{26}O_{10}$ - Aurasperone A or Nigerone

$C_{32}H_{28}O_{11}$ - Aurasperone E =Fonsecinone D

$C_{32}H_{30}O_{12}$ - Aurasperone B

$C_{32}H_{36}N_4O_2$ - Communesin B

DNA - Deoxyribonucleic acid

DON – Deoxynivalenol

MS-IT-TOF - Ion-Trap -Time-of-flight Mass Spectrometry

PDA – Potato Dextrose Agar

P.M. - Praia da Memória

UPLC - Ultra-Performance Liquid Chromatography

# 1. Introduction

## 1.1. Fungi

Fungi are considered eukaryotes that feed by absorption of organic compounds found in the environment, are composed of hyphae and cell walls, and produce spores as a way of reproduction. The basic structural unit of most fungi is the hypha that is filamentous, branched cellular compartments with rigid cell walls composed of chitin and glucans. A mass of hyphae emerging from the same source is called mycelium, that functions as a dynamic system that responds to local conditions by changing boundary properties (Worral, 1999). Fungi are ubiquitous in almost all habitats on Earth. They have been documented in mesophotic coral ecosystems, deep-sea hydrothermal vents, arid, high-altitude deserts and the cold Arctic and Antarctic (Wainwright *et al*., 2019).

Marine fungi have been retrieved from almost every kind of abiotic and biotic substrates, such as sediments, sponges, corals, echinoderms, vertebrates, algae, in a huge diversity of habitats ranging from coastal waters to the deep biosphere. Marine fungi associated with algae are largely unexplored, despite their ecological role and potential industrial applications (Suryanarayanan, 2012). There is a gap in knowledge on marine fungi with almost 90% of the diversity to be described, mostly from uncharted waters and the basic understanding of their distribution and ecological roles is still in its early stages (Gnavi *et al*., 2017). Marine-derived fungi such as *Aspergillus* spp., apart from dominating the endosymbiont assembly of seaweeds, also dominate the fungal assembly of marine invertebrates of different geographical locations such as the North Sea, the Mediterranean, the Caribbean, and the great Barrier Reef proving their adaptation to occupy such a niche as the inner tissues of seaweeds or marine animals (Suryanarayanan, 2012).

In the last few years, mycological studies in Antarctica have focused on the fungi present in the marine ecosystem. However, the Southern Ocean remains practically unexplored with respect to mycological diversity and its possible applications in biotechnological processes (Furbino *et al*., 2018).

## 1.2. The diverse uses for macroalgae

Marine macroalgae are plant-like organisms with simple internal structures that generally live-in coastal areas and have been researched for potential uses on our lives.

These algae are considered a very useful resource for agriculture due to their high number of mineral substances, amino acids, vitamins, and plant growth regulators including auxins, cytokinin and gibberellins leading to the improvement of plants, such as potatoes, grasses, citrus plants, tomatoes, beets, and legumes (Hamed *et al*., 2018).

They are also used as livestock feed, such as in the case of poultry, with the goal of enhancing the productiveness of feed for the cost-effective production of commercially important meat and eggs, whilst also maintaining and/or improving poultry health (Kulshreshtha *et al*., 2020), they can also be used for the well-being of humans due to its antimicrobial properties, such is the case of *Kappaphycus alvarezii* that can act as an antibacterial agent towards human pathogens or the red alga *Plocamium* and *Chondrococcus* that secrete polyhalogenated monoterpenes showing antimicrobial and antitumor properties (Ismail *et al*., 2020). Research regarding the possibility of anticancer activities from macroalgae is being done through several means such as cytotoxicity activity (Martins *et al*.,2018) or the study of sulfated polysaccharides, although further research is needed it could lead to interesting medical applications (Kim *et al*., 2016).

## 1.3. Fungal growth in macroalgae worldwide

Algae represent an important isolation source of marine fungi with almost one-third of all known marine fungal species associated with these organisms. Algae-inhabiting fungi represent a taxonomically diverse group of mutualists, endosymbionts, parasites, pathogens, and saprobes, which are of evolutionary, ecological, and economical interest (Gnavi *et al*., 2017).

Algae derived fungi can be associated with a variety of algae, including brown (e.g., *Agarum clathratum*, *Fucus* sp., *Laminaria* sp., *Sargassum* sp.), green (e.g., *Ulva* sp., *Enteromorpha* sp., *Flabellia* sp.), or red (e.g., *Chondrus* sp., *Dilsea* sp., *Ceramium* sp.) algae. The most described fungi associated with algae belong to the Ascomycota phyla and are represented by a wide diversity of genera such as *Acremonium*, *Alternaria*, *Aspergillus*, *Cladosporium, Phoma, Penicillium, Trichoderma, Emericellopsis, Retrosium, Spathulospora, Pontogenia,* and *Sigmoidea (*Patyshakuliyeva *et al*., 2020).

Interest in the study, of macroalgae and the identification and detection of algae derived fungi, in *Agarum clathratum*, a brown macroalgal species, and other seaweed species has risen recently due to its immunomodulatory and antioxidant activities, however, a large amount of seaweed wastes are deposited on the shores of Korea peninsula, and a mass of *A. clathratum* has accumulated on the northeast coast of Korea causing serious environmental problems so a study was conducted to detect the presence of fungi in these macroalgae. 89 species were identified where it was detected that *Acremonium, Corollospora,* and *Penicillium* were the dominant genera, and *Acremonium fuci* and *Corollospora gracilis* were the dominant species (Seobihn *et al*., 2019).

*Sargassum* (Phaeophyceae), a large canopy-forming brown macroalga, is found throughout temperate and tropical seas. In the Caribbean, *Sargassum* beaching is becoming a frequent and increasingly problematic issue; several massive *Sargassum* beaching events since 2011 have negatively impacted local environments, fisheries, and tourism. *Sargassum ilicifolium* were collected from eight island locations, in Singapore, to determine fungal biodiversity. The results obtained showed that all locations were dominated by unclassified fungi, proving that there's still a lot of research needed to be done regarding marine fungal species (Wainwright *et al*., 2019).

The sugar kelp *Saccharina latissima* dominates many temperate coastal ecosystems, plays key ecological roles, and presents important economic potential. However, its microbiota remains poorly investigated, although it could play an important role in algal
fitness. Research done to this species of macroalgae, on the coast of Scotland, detected fungal communities dominated by *Ascomycota* and *Basidiomycota*, in particular, Mycosphaerellaceae, Psathyrellaceae, and Bulleribasidiaceae. One hundred fungal Amplicon Sequence Variants were identified per algal individual, showing a diverse fungal community associated with S. *latissimi.* These communities were spatially organized as highly diverse patches within the sampled algal tissues and whilst good sequencing coverage allowed to detect the diversity present in the samples, couldn't retrieve all the fungal community present within each alga. The unexpected diversity made the definition of a core fungal microbiota difficult (Tourneroche *et al*., 2020).

## 1.4. The properties of Macroalgae derived fungi

Fungi might show a systemic growth within its host, which is, for example, the case of *Mycophycias ascophylli*. It remains associated with the algal host, *Ascophyllum nodosum* and *Pelvetia canaliculata* throughout its life cycle, it also protects the photobiont from desiccation and has a nutritional dependence on its host (Zuccaro *et al*., 2008).

Saprobic fungi, that can be protected by Antarctic macroalgae, can produce enzymes with the potential to degrade the algal biomass and may be involved in a complex network of biogeochemical cycles and cycling of organic matter, which may be available to other marine organisms in the food cycle (Furbino *et al*., 2018).

When compared with other fungi, endophytic fungi isolated from marine macroalgae are one of the main sources of natural active substances. Many kinds of pure compounds with high bioactivities, such as alkaloids, anthraquinones, and steroids, have been separated from the secondary metabolites of *Aspergillus.* This fungi genus displays strong algicidal activity against red tide algae and might be one of the important sources of active compounds against red tide algae, so more attention should be shown towards *Aspergillus* and anthraquinones (Fengping *et al*., 2019).

*Penicillium* species are amongst the most common fungi found in a diverse range of habitats including extreme environments with high salinity. The *Penicillium* species isolated as endosymbionts from different seaweeds revealed cytotoxicity and inhibitory activities against fungal pathogens. It is noteworthy that marine-derived *Penicillium* species are important producers of relevant secondary metabolites such as antimicrobial and anticancer compounds (Gonçalves *et al*., 2019).

Fungi could show a potential preference for tissue and host where the taxonomic diversity and abundance of isolates may differ between algal organs tested in brown algae. This pattern of fungal colonization may be explained by the differences in chemical composition and defense in algal species and organs (Vallet *et al*. 2018)

Marine fungi associated with macroalgae are an ecologically important group that have a strong potential for industrial applications. Several macroalgae derived fungi obtained from *Fucus sp.* can degrade algal polymers and can grow on terrestrial biomass and produce enzymes involved in degradation of plant cell wall polysaccharides (Patyshakuliyeva *et al*., 2020).

There has been an effort to find the bioremediation potential of fungi, such as the elimination of decaying seaweeds from populated areas where some species, *A. fuci, Alfaria terrestris, Hypoxylon perforatum, P. madriti,* and *Pleosporales sp*., showed high enzyme activity (Seobihn *et al*., 2019).

## 1.5. Mycotoxins

Mycotoxins can be considered as natural products produced by fungi that evoke a toxic response when introduced in low concentrations to higher vertebrates and other animals by a natural route (Bennett, 1987). They are also secondary metabolites defined as metabolic intermediates or products, found as a differentiation product in restricted taxonomic groups, not essential to growth and life of the producing organism, and biosynthesized from one or more general metabolites by a wider variety of pathways than is available in general metabolism (Bennett, 1989).

Mycotoxigenic fungi are frequently found as contaminants of food and feed, infesting crop plants in the field or agricultural commodities during storage. The most common mycotoxins are aflatoxin (e.g., aflatoxin B1), fumonisins, zearalenone (ZEN), type B trichothecenes (e.g., deoxynivalenol; DON), type A trichothecenes (e.g., T-2 toxin) and ochratoxin A (Gruber-Dorninger *et al*., 2019).

Aflatoxins are potent carcinogens and, in association with hepatitis B virus, are responsible for many thousands of human deaths per year (Adeyeye, 2016).

The contamination by mycotoxins may lead to the deterioration of the ingredients and may also cause severe health problems for animals and humans. When the ingestion of these toxins by fish takes place, it might not only cause distress to their health but also be passed through the food chain to its consumers and lead to serious health risks. Mycotoxicosis is the name given to the intoxication that happens in animals and humans as a consequence of the consumption of one or more mycotoxin that can result in disease or death (Oliveira *et al*., 2020).

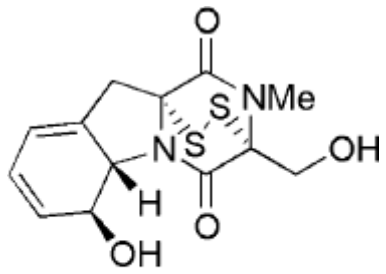
Among the mycotoxins, Aflatoxin $B_1$ ($AFB_1$), are one of the most studied mycotoxins, especially in fish, duo to the fact that its natural occurrence being found in tropical countries and for being known as a human carcinogen and a potent hepatotoxin. It has been reported to cause disruption on the reproductive system in both sexes in animals, however, few studies have been conducted in aquatic animals

Another problematic mycotoxins are the ones produced by Fusarium, Deoxynivalenol (DON), that might cause acute and chronic toxic effects (Marijani *et al*., 2019).

The members of the genus *Fusarium* are responsible for the production of a variety of chemically different phytotoxic compounds, such as fusaric acid, fumonisins (fumonisin B1), beauvericin (BEA), enniatin (ENN), moniliformin (MON) and trichothecenes that cause morphological, physiological, and metabolic effects in crops such as necrosis, chlorosis, growth inhibition, wilting, inhibition of seed germination and effects on calli (Ismaiel *et al*., 2015).

Gliotoxin is a pyrazinoindole with a disulfide bridge spanning a dioxo-substituted pyrazine ring (figure 1) and it is another mycotoxin that is produced by several fungal species e.g., *Aspergillus fumigatus, Eurotium chevalieri, Gliocladium fimbriatu*m and some *Trichoderma* and *Penicillium* species. This toxin may cause apoptosis, prevent NF- kB activation by inhibition of the proteasome and inhibits angiogenesis (Sharf *et al*., 2011).

It has deleterious effects due to its neurotoxic properties, aggravating clinical symptoms of diseases, such as multiple sclerosis, causing an increased neuroinflammation (Fraga-Silva *et al*., 2019).




Gliotoxin

Figure 1. Molecular structure of Gliotoxin (adapted from Scharf et al, 2012)

Fungi can develop a considerable amount of toxic chemicals, most of which are not found in routine analysis and since some of the toxin's effects can be subtle, they may go undetected, so the true scale of mycotoxins in the environment is still unknown. (Ciegler, n.d.)

## 1.6. Objectives

The main objective of this research is to unravel de potential mycotoxins production by fungi associated with macroalgae. The specific objectives are:

1- To isolate and identify fungi associated with marine macroalgae with potential use in human or animal food, using microscopy and molecular biology techniques.

2- To detect potential toxins produced by fungi with analytical chemistry techniques.

3- To evaluate the potential of biotechnological applications of the isolated fungi.

# 2. Materials and methods

## 2.1. Sampling

Macroalgae were collected in two beaches in Northern Portugal in the vicinity of Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR) headquarters. These beaches, despite being close to each other (9 km apart) have different characteristics, being that Castelo do Queijo has less exposure to wave activity but more impacted by anthropogenic activities while Praia da Memória has more exposure to wave activity and less impacted from an anthropogenic point of view (figure 2). A total of three samplings of different macroalgae species were made along the coast of Praia do Castelo do Queijo (41°10'6.28"N, 8°41'27.36"W) and Praia da Memória (41°13'42.63"N, 8°43'18.98"W).

The first sampling was conducted on the 7$^{th}$ of September 2020 at Praia do Castelo do Queijo, the second was on the 19$^{th}$ of April 2021 at Praia da Memória and the third was on the 11$^{th}$ of June 2021 at both beaches. Pieces of algae were cut, photographed on site, and placed in sterile plastic containers, transported to the laboratory and maintained at 4 °C until isolation. All sampling process was made during low tide to be able to collect a larger diversity of macroalgae.

Figure 2. Differences between the two beaches. The first is Praia do Castelo do Queijo and the second is Praia da Memória.

## 2.2. Sample processing for fungus isolations

Macroalgae were cut into small samples (1-2 cm) and plated into Petri plates with Potato Dextrose Agar (PDA). These plates were incubated at 28 °C for 7 days. Fungal growth was observed and at the end of the incubation fungal samples were repicked to new PDA plates to fully isolate the fungi specimens (figure 3).

Samples were kept in cryopreservation tubes at -20 °C on a solution of 10% glycerol.

Figure 3. Examples of plated samples and the diversity of fungi

## 2.3. Microscopical analysis of fungal isolates

Using duct-tape over the isolated fungi, a small sample was removed and placed in a microscopic slide and then treated with lactophenol cotton blue staining. Microscopic magnification of 40x was the standard (figure 4). For the identification of the isolated strains, the fungi structure was analyzed and compared to existing information (Webster J., Weber R., 2007) and the Mycobank databases (www.mycobank.org).

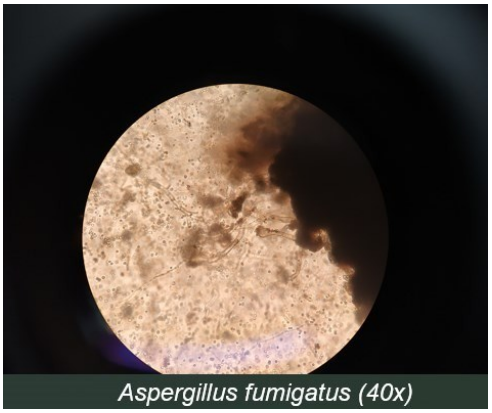



Figure 4. Fungus identified (*Aspergillus fumigatus*) using microscopical analysis

## 2.4. DNA extraction and fungus identification

The DNA extraction process was conducted following the instructions provided by the manufacturer of the commercial kit *High Pure PCR Template Preparation* (Roche Diagnostics GmbH, Mannheim, Germany) and executed in a Biological Class II flow chamber (Telstar Bio II Advance Plus).

The region ITS1-5.8S-ITS2 (table 1) was amplified using primers ITS1 (forward) and ITS4 (reverse) (White *et al*., 1999) and a thermocycler (Biometra T3000) was used to perform PCR.

Reaction mix:

- *PureTaq Ready-To-Go™ PCR Beads*
- 1,50 µl of primer ITS1 (10 µmol) (forward)
- 1,50 µl of primer ITS4 (10 µmol) (reverse)
- 18 µl of ultrapure water
- 6 µl of DNA

PCR conditions:

- Initial denaturation: 95ºC for 4,5 minutes
- Denaturation: 95ºC for 30 seconds (x40)
- Hybridization: 50ºC for 30 seconds (x40)
- Extension: 72ºC for 1 minute (x40)
- Final extension: 72ºC for 3 minutes

The negative control used was 6 µl of ultrapure water.

To determine the result of the PCR an electrophoresis was performed.

1.8g of agarose in 90 ml of Buffer solution TAE x1 were used to make the gel. 2 µl of Sybr Safe (Invitrogen) was added to the gel. A mixture was made of 3 µl loading buffer with 5 µl of DNA sample, then loaded onto the wells and run in a medium electrophoresis tray (Bio-Rad Laboratories, inc.) for 40 minutes at 120 V. The bands were analyzed using the program Image Lab.

Purification of the resulting products was made with a *High Pure PCR Product Purification Kit* (Roche Diagnostics GmbH, Mannheim, Germany). The volumes of each reagent were balanced for the amount of DNA to be purified (22 µl of DNA product) and sequencing was done at a Eurofins Genomics laboratory.

The identification of the isolates was made using molecular biology methods and to determine the species identification, the sequence of fungi was compared to other sequence species deposited in the GenBank database using BLASTn.

Table 1. Primers used (ITS1 and ITS4) during PCR analysis.

| Primers | Primers Sequence (5' -> 3') | Amplicon (size) |
|---|---|---|
| **ITS1 (forward)** | TCCGTAGGTGAACCTGCGG | ITS1-5.8S-ITS2 (400 – 600 bp) |
| **ITS4 (reverse)** | TCCTCCGCTTATTGATATGC | |

## 2.5. Toxin analysis

Agar plug samples of each fungus, 3 each, were sent to be analyzed by Ultra Performance Liquid Chromatography (UPLC), followed by Ion-Trap -Time-of-flight Mass Spectrometry (MS-IT-TOF) at University of Santiago (Pharmacology Department, Faculty of Veterinary, Lugo). The extraction was performed in a 4 ml deactivated amber glass vial. The agar plugs were mixed with 0.5 ml of the extraction solvent (acetonitrile/water/acetic acid; 79:20:1, v/v/v) and shaken for 3 mins using a vortex mixer. Before the analysis, the extract was filtered through a 0.45 µm Ultrafree-MC centrifugal filter (Milipore, USA). (González-Jartín *et al*., 2018).

### 2.5.1. UPLC- ion trap - time of flight MS detection

The separation was performed using an UPLC system (Shimadzu, Kyoto, Japan) combined with MS-IT-TOF. The UPLC is made of two LC-30AD pumps, SIL-10AC autoinjector with refrigerated rack, DGU-20A degasser, CTO-10AS column oven and a SCL-10Avp system controller. The analytical column Waters ACQUITY HSS T3 (100 mm × 2.1 mm and 1.8 µm particle size) was selected to carry out the separation, and was kept at 40 °C. The binary gradient system consisted of (A) water containing 0.1% formic acid and 5 mM ammonium formate, and (B) methanol. The gradient (14.5 min) started and was held at 0% B for 1 min. Afterwards, the proportion of eluent B was increased linearly to 50% within 3 min and then maintained for 2.5 min. Thereafter, the proportion of eluent B was increased to 100% within 3.5 min and then maintained for 2 min. Finally, the proportion of eluent B returned to 0% in 0.5 min and maintained for 2 min for column equilibration. The flow rate of the mobile phase was kept at 0.3 mL/min and the injection volume was set at 5 µL.

IT-TOF was equipped with an electrospray ionization (ESI) interface (Shimadzu, Kyoto, Japan). The operating conditions were as follows: detector voltage, 1.65 kV; nebulizing gas flow, 1.5 L/min; curved desolvation-line and heat block temperature, 200 °C; drying gas pressure,105 kPa; pressure of TOF region, 1.4 × 10−4 Pa; ion trap pressure, 1.8 × 10−2 Pa. The nitrogen generator was a NITROMAT N-075 ECO from Worthington

Creyssensac (Spain). The MS method was performed in positive full scan MS mode within the mass range 150–850.

The event time was set at 300 ms with an ion accumulation time of 20 ms and 3 repetitions. Relevant ions were isolated in MS1 scan and collision induced dissociation (CID) energy was applied over them obtaining MS2 product ion spectra (MS1–2 experiments). Collision energy parameters were set at 25% and Argon was used as collision gas at 75%.

The ion accumulation time was increased to 30 ms for MS2 stage and the precursor ion isolation was acquired within a tolerance range of 1 Dalton (Da). The mass range was calibrated prior to data acquisition employing a standard sample from Shimadzu (Kyoto, Japan) as an external reference.

Data recorded was analyzed by Shimatzu LC/MS solution software.

# 3. Results

## 3.1. Fungal identification

### 3.1.1. 1st Sampling

During the first sampling a total of eight macroalgae species were collected in Praia do Castelo do Queijo (C.Q.) (figure 5).

From seven of these macroalgae, 111 fungal isolates were. No fungal isolates were obtained from the macroalgae *Chondrus crispus*. The number of fungi isolated from each macroalgal is shown in Table 2, being *Laminaria sp*. the one with the most isolated fungi, followed by *Codium* sp.

The fungi were analyzed through the study of their morphology. Macroscopically by their growth rate and color and microscopically by the analysis of their microscopic structures with the use of lactophenol blue staining procedure.

Fungi molecular identification was determined with the use of Blast database and selected by their best hits (Query cover, Identification percentage) (Table 3), this was applied in all the three samplings executed in this study.

The distribution of fungal species in each of the macroalgal species (Table 4) shows that the genera *Aspergillus* and *Cladosporium* are the most predominant (figures 6,7).

Table 2. Number of isolated fungi from each macroalgae collected in Praia do Castelo do Queijo

| **Macroalgae** | **Nº of isolated fungi** |
|---|---|
| *Laminaria* sp. | 24 |
| *Ulva rigida* | 9 |
| *Mastocarpus stellatus* | 19 |
| *Codium* sp. | 21 |
| *Mastocarpus stellatus* | 15 |
| *Chondrus crispus* | - |
| *Fucus spiralis* | 20 |
| *Dilsea carnosa* | 3 |

Figure 5. List of macroalgae species collected in C.Q.: 1- *Fucus spiralis*, 2 and 6 – *Mastocarpus stellatus*, 3- *Dilsea carnosa*, 4- *Chondrus crispus*, 5- *Codium* sp., 7- *Ulva rigida*, 8- *Laminaria* sp.

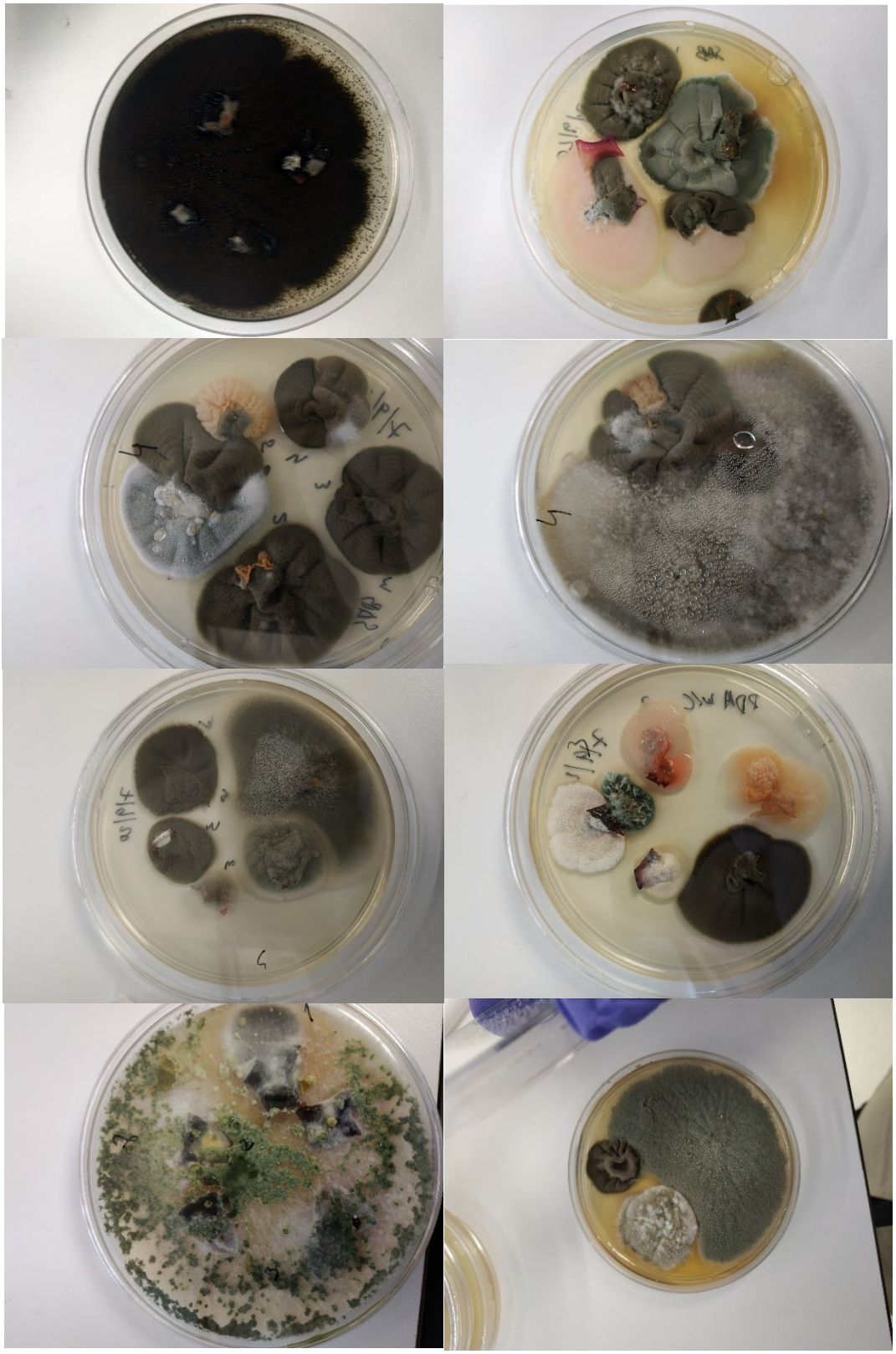


Figure 6. Macroscopic view of the diversity of fungi found as a result of the 1st sampling.

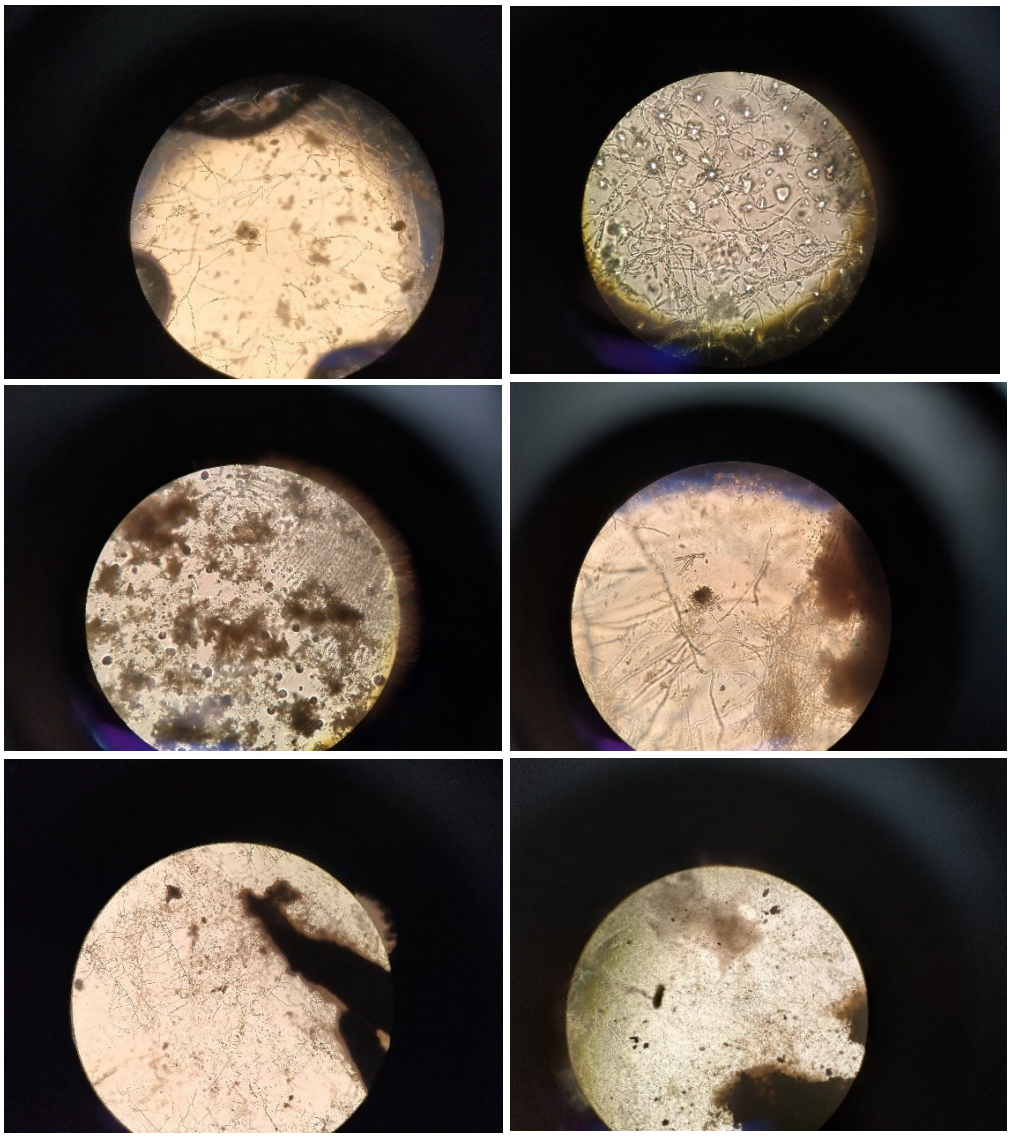


Figure 7. Microscopic view of the diversity of fungi found as a result of the 1st sampling.

Table 3. Query cover and identification percentage of each fungus species/genus from Blast analysis - 1st sampling

| Fungi | Query Cover (%) | Identification (%) |
|---|---|---|
| *Aspergillus fumigatus* | 81 | 99.64 |
| *Aspergillus giganteus* | 99 | 99.82 |
| *Aspergillus carbonarius* | 95 | 100 |
| *Aspergillus versicolor* | 98 | 99.81 |
| *Aspergillus niger* | 98 | 99.82 |
| *Acremonium fuci* | 100 | 94.95 |
| *Cladosporium* sp. | 99 | 100 |
| *Cladosporium ramotenellum* | 99 | 100 |
| *Cladosporium halotolerans* | 98 | 100 |
| *Cladosporium cladosporioides* | 99 | 100 |
| *Aureobasidium* sp. | 99 | 99.82 |

| | | |
|---|---|---|
| *Aureobasidium pullulans* | 99 | 99.45 |
| *Diaporthe* sp. | 97 | 99.81 |
| *Rhodotorula mucilaginosa* | 99 | 99.66 |
| *Botrytis cinerea* | 99 | 100 |
| *Penicillum* sp. | 100 | 97.70 |
| *Penicillium corylophilum* | 97 | 99.82 |
| *Penicillum glabrum* | 99 | 99.45 |
| *Pezicula* sp. | 43 | 88.06 |
| *Plectosphaerella cucumerina* | 96 | 98.47 |
| *Alternaria* sp. | 98 | 98 |
| *Alternaria alternata* | 99 | 99.81 |
| *Fusarium* sp. | 100 | 100 |
| *Fusarium oxysporum* | 97 | 100 |
| *Syncephalastrum* sp. | 99 | 93.96 |
| *Syncephalastrum racemosum* | 57 | 97.74 |
| *Pithomyces* sp. | 99 | 96.19 |
| *Trichoderma* sp. | 99 | 99.22 |
| *Trichoderma atroviride* | 99 | 100 |
| *Candida* sp. | 84 | 89.36 |
| *Radulidium subulatum* | 96 | 99.57 |

Table 4. Distribution of identified fungi in each macroalgae collected in Praia do Castelo do Queijo

| Fungi / Algae | *Laminaria* sp. | *Ulva rigida* | *Mastocarpus stellatus* | *Codium* sp. | *Dilsea carnosa* | *Chondrus crispus* | *Fucus spiralis* |
|---|---|---|---|---|---|---|---|
| *Aspergillus fumigatus* | X | | X | | | | |
| *Aspergillus giganteus* | | X | | | | | |
| *Aspergillus carbonarius* | | | X | | | | |
| *Aspergillus versicolor* | | | | X | | | |
| *Aspergillus niger* | | | X | | | | |
| *Acremonium fuci* | X | | | | | | |
| *Cladosporium* sp. | X | | X | X | | | |
| *Cladosporium ramotenellum* | X | | X | | | | |
| *Cladosporium halotolerans* | | | | X | | | |
| *Cladosporium cladosporioides* | X | X | X | X | | | |
| *Aureobasidium* sp. | | | X | | | | |
| *Aureobasidium pullulans* | X | | | | | | |
| *Diaporthe* sp. | X | | | X | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Rhodotorula mucilaginosa* | X | X | | | | | |
| *Botrytis cinerea* | X | | | | | | |
| *Penicillum* sp. | | | X | | | | X |
| *Penicillium corylophilum* | X | | | | | | |
| *Penicillum glabrum* | | | X | | | | |
| *Pezicula* sp. | X | | | | | | |
| *Plectosphaerella* sp. | X | | | | | | |
| *Alternaria* sp. | X | | | | | | |
| *Alternaria alternata* | | | | | | | X |
| *Fusarium* sp. | | | X | | | | |
| *Fusarium oxysporum* | | | | | | | X |
| *Syncephalastrum* sp. | | | | X | | | |
| *Syncephalastrum racemosum* | | | | X | | | |
| *Pithomyces* sp. | | | X | | | | |
| *Trichoderma* sp. | | | | X | | | X |
| *Trichoderma atroviride* | | | | | | | X |
| *Candida* sp. | | | | | X | | |
| *Radulidium subulatum* | | | | | X | | |
| Total | 13 | 3 | 11 | 8 | 2 | 0 | 5 |

### 3.1.2. 2nd Sampling

A total of eight different species of macroalgae (figure 8) were collected in Praia da Memória (P.M.) resulting in 29 fungal isolates (Table 4).

Table 5 shows the distribution of fungi species between the macroalgae, demonstrating the dominance of *Aspergillus fumigatus* amongst the samples (figures 9.10).


Figure 8. List of macroalgae species collected in P.M.: 1- *Chondrus crispus*, 2- *Mastocarpus stellatus*, 3- *Fucus spiralis*, 4- *Caulacanthus ustulatus*, 5,6- *Ulva rigida*, 7- *Gelidium* sp., 8- *Grateloupia turuturu*, 9- *Osmundea pinnatifida*

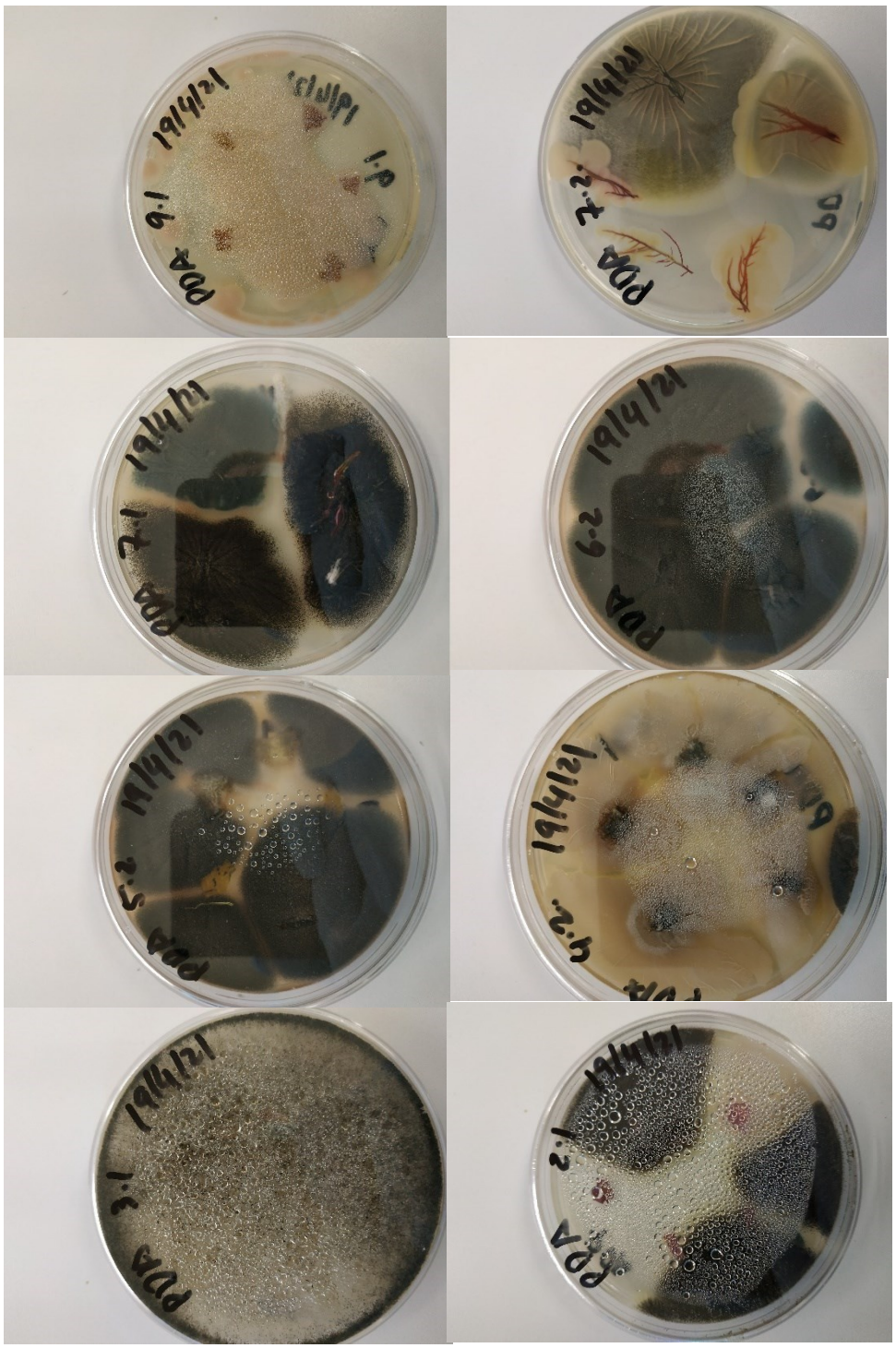


Figure 9. Macroscopic view of the diversity of fungi found as a result of the 2nd sampling.

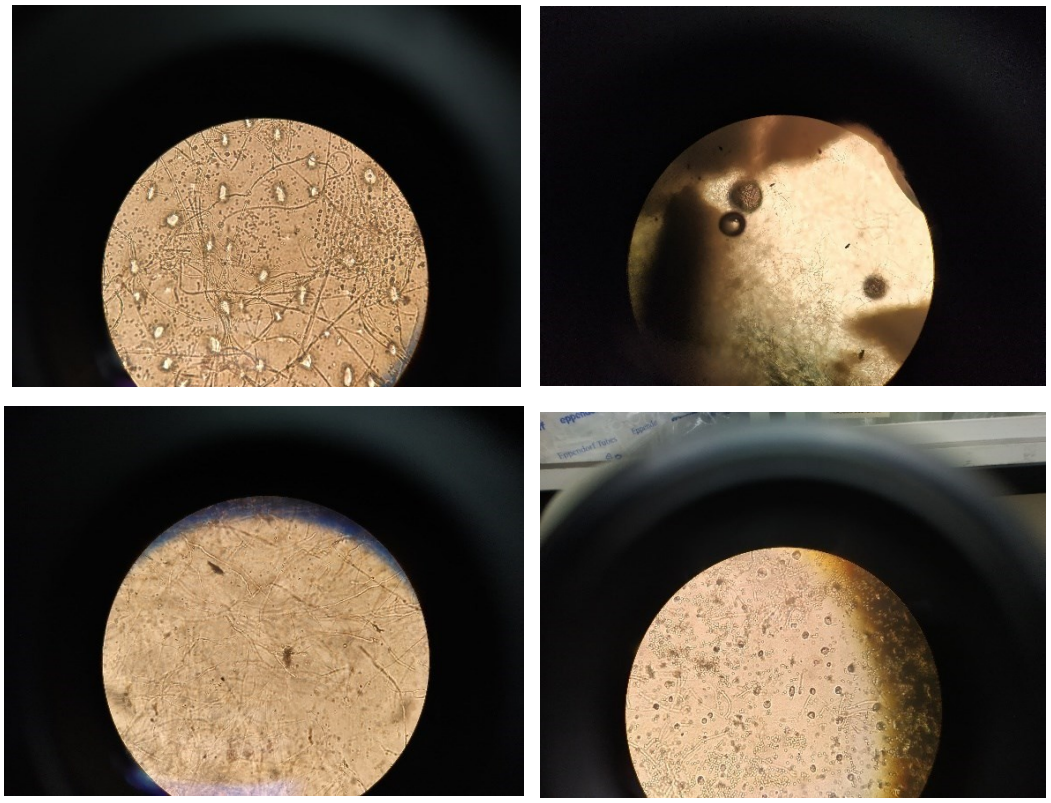


Figure 10. Microscopic view of the diversity of fungi found as a result of the 2nd sampling.

Table 5. Number of isolated fungi from macroalgae collected in Praia da Memória

| **Macroalgae** | **Nº of isolated fungi** |
|---|---|
| *Chondrus crispus* | 1 |
| *Mastocarpus stellatus* | 3 |
| *Fucus spiralis* | 3 |
| *Caulacanthus ustulatus* | 9 |
| *Ulva rigida* | 6 |
| *Gelidium* spp | 6 |
| *Grateloupia turuturu* | - |
| *Osmundea pinnatifida* | 1 |
| Total | 29 |

Table 6. Query cover and identification percentage of each fungus species/genus from Blast - 2nd sampling

| Fungi | Query Cover (%) | Identification (%) |
|---|---|---|
| *Aspergillus fumigatus* | 99 | 97.10 |
| *Aspergillus piperis* | 98 | 99.82 |
| *Aspergillus niger* | 98 | 99.54 |
| *Cryptococcus diffluens* | 97 | 100 |
| *Galactomyces sp.* | 99 | 100 |
| *Penicillium stecki* | 92 | 100 |
| *Rhizopus stolonifer* | 100 | 100 |
| *Rhizopus oryzae* | 99 | 99.32 |

Table 7. Distribution of identified fungi in each macroalgae collected in Praia da Memória

| Fungi \ Macroalgae | *Caulacanthus ustulatus* | *Chondrus crispus* | *Fucus spiralis* | *Gelidium sp.* | *Grateloupia turuturu* | *Mastocarpus stellatus* | *Osmundea pinnatifida* | *Ulva rigida* |
|---|---|---|---|---|---|---|---|---|
| *Aspergillus fumigatus* | | | | x | | | x | x |
| *Aspergillus piperis* | | | | | | x | | |
| *Aspergillus niger* | | | x | | | | | |
| *Cryptococcus diffluens* | | x | | | | | | |
| *Galactomyces sp.* | x | | | | | | | |
| *Penicillium stecki* | | | | x | | | | |
| *Rhizopus stolonifer* | | | x | | | | | |
| *Rhizopus oryzae* | x | | | | | | | |
| Total | 2 | 1 | 2 | 2 | | 1 | 1 | 1 |

### 3.1.3. 3rd Sampling

This sampling was conducted to compare similar macroalgae between both beaches (P.M. and C.Q.). The selected macroalgae were *Fucus spiralis*, *Mastocarpus stellatus* and *Ulva rigida* (figure 11).

There was very little diversity of fungi species found in both locations (figure 12,13), *Aspergillus fumigatus* showed a clear dominance by appearing in all the different macroalgae species, followed by *Talaromyces sp.* (Table 7).

Table 8. Number of isolated fungi from each macroalgae collected in both beaches

| **Nº of isolated fungi** | **Castelo do Queijo** | **Praia da Memória** |
|---|---|---|
| *Fucus spiralis* | 7 | 8 |
| *Mastocarpus stellatus* | 7 | 8 |
| *Ulva rigida* | 6 | 6 |
| Total | 20 | 22 |

Figure 11. List of macroalgae species collected in both beaches: 1- *Chondrus crispus*, 2- *Ulva rigida*, 3- *Mastocarpus stellatus*, 4- *Fucus spiralis*

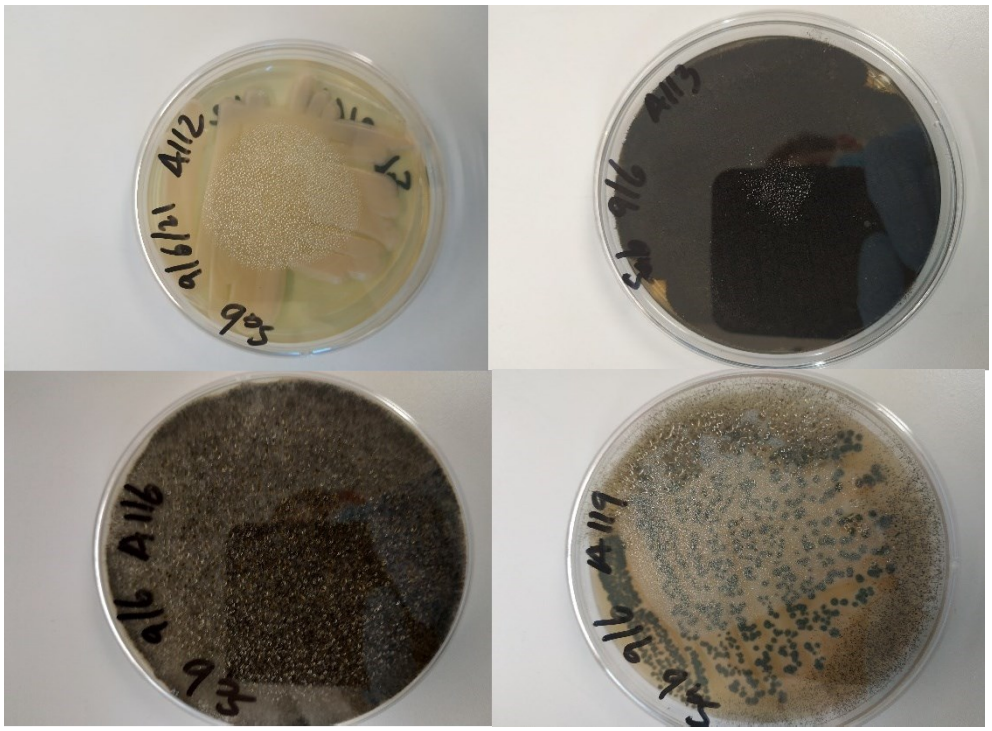


Figure 12. Macroscopic view of the diversity of fungi found as a result of the 3rd sampling.

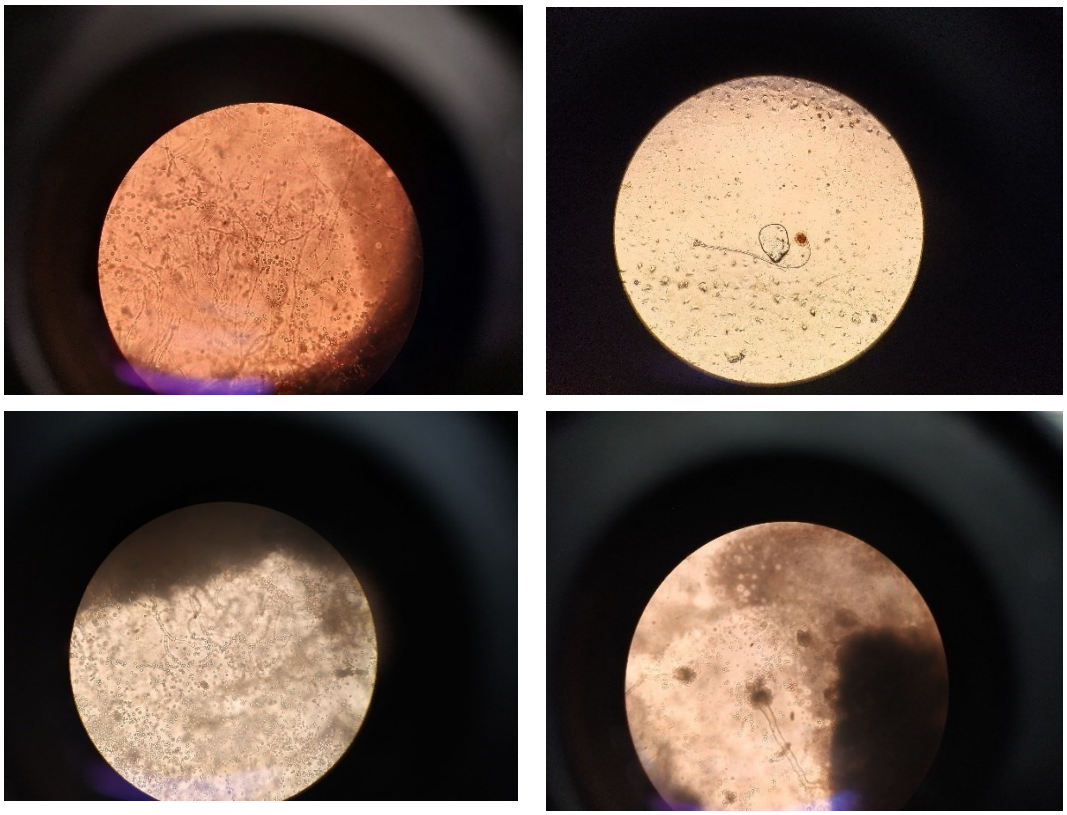


Figure 13. Microscopic view of the diversity of fungi found as a result of the 3rd sampling.

Table 9. Query cover and identification percentage of each fungus species/genus from Blast – 3rd sampling

| Fungi | Query Cover (%) | Identification (%) |
|---|---|---|
| *Aspergillus fumigatus* | 99 | 99.82 |
| *Fusarium oxysporum* | 89 | 99.01 |
| *Talaromyces* sp. | 99 | 98.13 |
| *Talaromyces verruculosus* | 95 | 99.44 |

Table 10. Distribution of identified fungi in each macroalgae from both beaches

| Fungi \ Algae | *Fucus spiralis* (P.M.) | *Fucus spiralis (C.Q.)* | *Mastocarpus stellatus (P.M.)* | *Mastocarpus stellatus (C.Q.)* | *Ulva rigida (P.M.)* | *Ulva rigida (C.Q.)* |
|---|---|---|---|---|---|---|
| *Aspergillus fumigatus* | x | x | x | x | x | x |
| *Fusarium oxysporum* | | | | | x | |
| *Talaromyces* sp. | x | | | x | | |
| *Talaromyces verruculosus* | | | | | x | |
| Total | 2 | 1 | 1 | 2 | 3 | 1 |

## 3.2. Mycotoxin analysis

Some of the fungi samples were selected, due to previously their species have been identified, to be submitted to Ultra Performance Liquid Chromatography (UPLC) and MS-

IT-TOF analysis resulting in the detection of certain compounds (Table 8).

These compounds were compared to existing literature to determine the compatibility with the molecules (figures 14 – 23).

Table 11. List of compounds found in fungi species through the use of UPLC-MS

| **Fungi species** | **Compounds** |
|---|---|
| *Alternaria sp.* | Phomapyrone A<br>Infectopyrone<br>Deoxyradicinol |
| *Aspergillus carbonarius* | Rubrofusarin<br>Flavasperone<br>Fonsecin<br>Fonsecin monomethyl ether |
| *Aspergillus fumigatus* | Acetophthalidin<br>Agroclavine<br>Asperfumoid<br>Bisdethiobis(methylthio)gliotoxin<br>Bisdechlorogeodin<br>Cyclotryprostatin A<br>Demethoxyfumitremorgin C<br>Fumiquinazoline C/D<br>Fumiquinazoline F<br>Festuclavin<br>Fumigaclavine A/B/C<br>Fumiquinazoline F/G<br>Fumitremorgin C<br>Gliotoxin<br>Monomethylsulochrin<br>Pyripyropene A<br>Terezine D<br>Tryptoquivaline<br>(F/J)/Chaetominine/Isochaetominine<br>Trypacidin<br>6-Methoxyspirotryprostatin B |
| *Aspergillus giganteus* | Tryptoquivaline |
| *Aspergillus niger* | Nigragillin<br>Aspernigrin<br>Nigerazine A or Nigerazine B<br>Flavasperone<br>Funalenone<br>Aurasperone E =Fonsecinone D<br>Aurasperone A or Nigerone<br>Aurasperone B<br>Malformin A1 |
| *Aspergillus versicolor* | sterigmatocystin |
| *Penicillium sp.* | Roquefortine C<br>Oxaline<br>Meleagrin |
| *Pithomyces sp.* | Nigragillin<br>Fonsecin<br>Fonsecin monomethyl ether |
| *Syncephalestrum racemosum* | Dehydrocarolic acid |

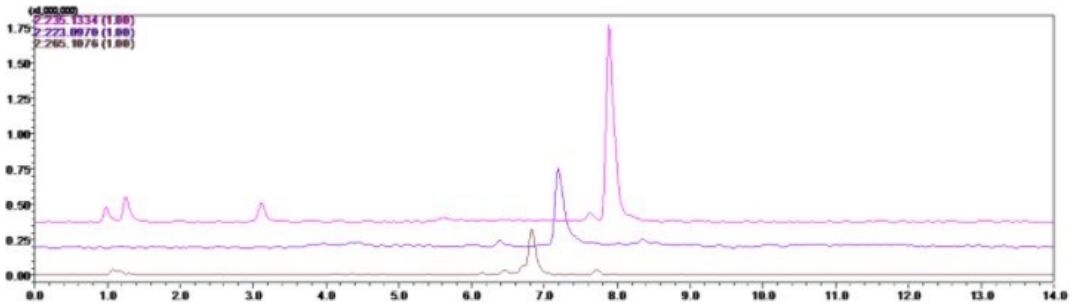


Figure 14. Results of UPLC-MS analysis of the fungi sample *Alternaria sp*.

Phomapyrone A → $C_{14}H_{18}O_3$ m/z 235,1334

Exact mass and theoretical fragmentation pattern compatible with the molecule.

Deoxyradicinol → $C_{12}H_{14}O_4$ m/z 223,0970

Exact mass and theoretical fragmentation pattern compatible with the molecule.

Infectopyrone → $C_{14}H_{16}O_5$ m/z 265,1076

Exact mass and fragmentation pattern compatible with the molecule. Fragmentation showed a m/z 139 fragment, which is likely due to the pyrone-moiety (Ivanova *et al*. 2010).

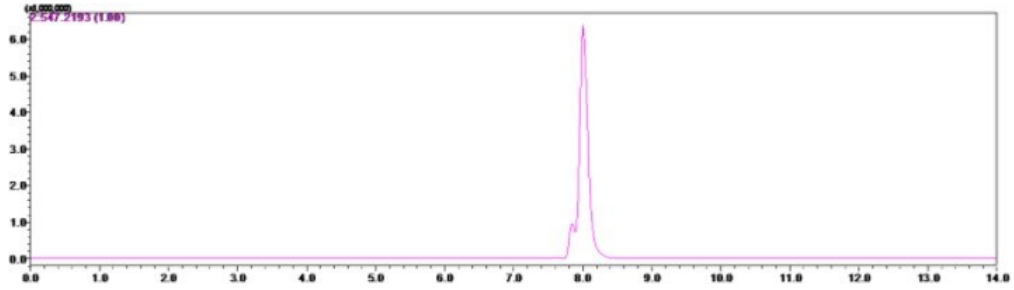


Figure 15. Results of UPLC-MS analysis of the fungi sample *Aspergillus giganteus*

Tryptoquivaline → $C_{29}H_{30}N_4O_7$ m/z 547,2193

Exact mass and theoretical fragmentation pattern compatible with the molecule.

Roquefortine C → $C_{22}H_{23}N_5O_2$ m/z 390,1930

Exact mass and fragmentation pattern compatible with the molecule. It was verified by analysing a standard

Oxaline → $C_{24}H_{25}N_5O_4$ m/z 448,1985

Exact mass and fragmentation pattern compatible with the molecule (Kim *et al*., 2012).

Meleagrin → $C_{23}H_{23}N_5O_4$ m/z 434,18228

Exact mass and one fragment compatible with the molecule reported (Kim *et al*., 2012).

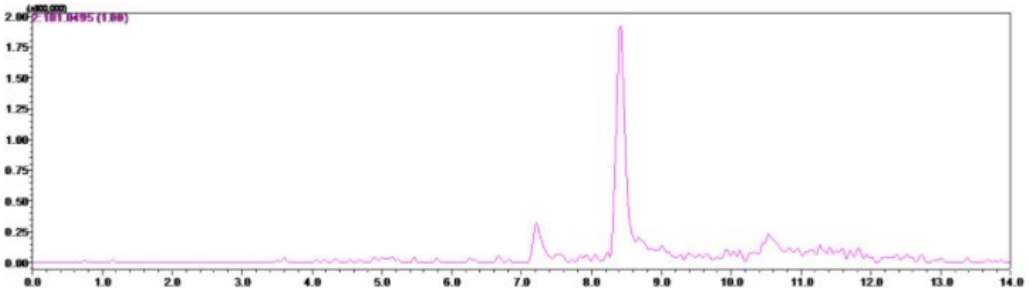


Figure 18. Results of UPLC-MS analysis of the fungi sample *Syncephalestrum racemosum*

Dehydrocarolic acid → $C_9H_8O_4$ m/z 181,04953

Exact mass and theoretical fragmentation pattern compatible with the molecule.

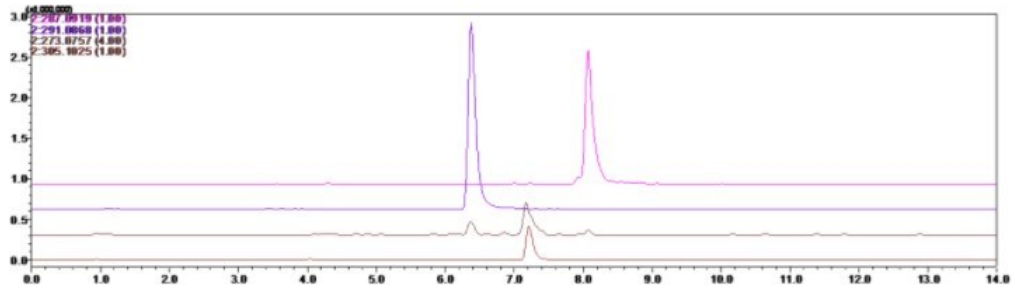


Figure 19. Results of UPLC-MS analysis of the fungi sample *Aspergillus carbonarius*

The profile of metabolites is compatible with the literature, so they were identified based on the exact mass (Nielsen *et al*., 2009).

Flavasperone → $C_{16}H_{14}O_5$ m/z 287,0919

Fonsecin → $C_{15}H_{14}O_6$ m/z 291,0868

Fonsecin monomethyl ether → $C_{16}H_{16}O_6$ m/z 305,1025

Rubrofusarin or TMC-256C1 → $C_{15}H_{12}O_5$ m/z 273,0757

These compounds are isomers

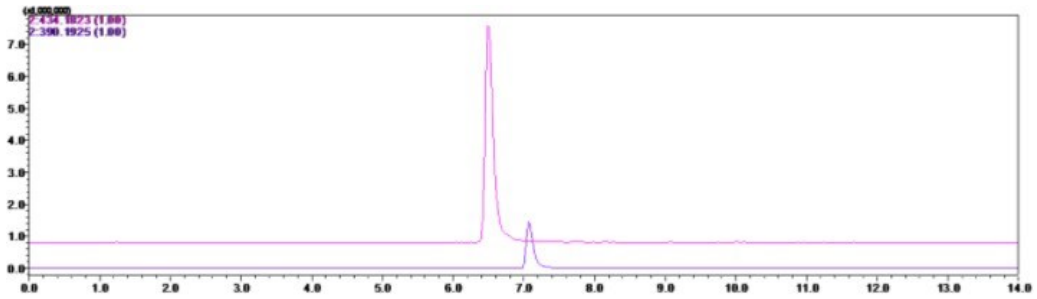


Figure 20. Results of UPLC-MS analysis of the fungi sample *Cladosporium sp.*

Meleagrin → $C_{23}H_{23}N_5O_4$ m/z 434,18228

Exact mass and one fragment compatible with the molecule (Kim *et al.*, 2012).

Roquefortine C → $C_{22}H_{23}N_5O_2$ m/z 390,1930

Exact mass and fragmentation pattern compatible with the molecule. It was verified by analyzing a standard

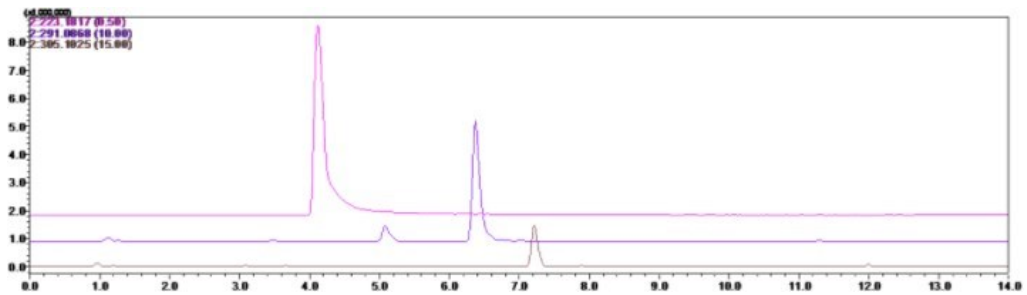


Figure 21. Results of UPLC-MS analysis of the fungi sample *Pithomyces sp.*

Nigragillin → $C_{13}H_{22}N_2O$ m/z 223,1817

Exact mass and theoretical fragmentation pattern compatible with the molecule.

Fonsecin → $C_{15}H_{14}O_6$ m/z 291,0868

Exact mass and theoretical fragmentation pattern compatible with the molecule.

Fonsecin monomethyl ether → $C_{16}H_{16}O_6$ m/z 305,1025

Exact mass and theoretical fragmentation pattern compatible with the molecule.

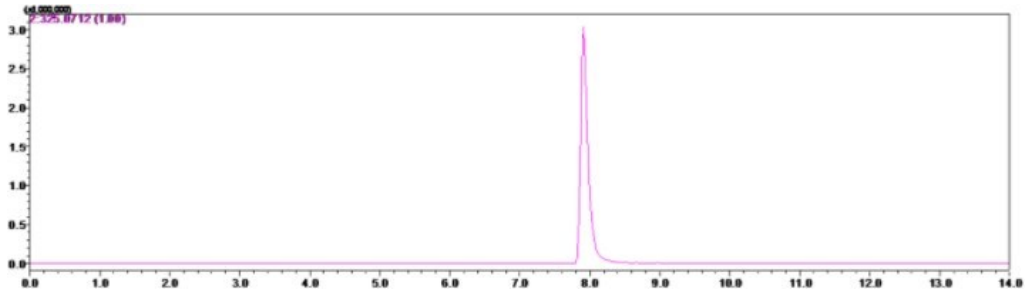


Figure 22. Results of UPLC-MS analysis of the fungi sample *Aspergillus versicolor*

Sterigmatocystin → $C_{18}H_{12}O_6$ m/z 325,0712

Exact mass and fragmentation pattern compatible with the molecule (González-Jartín *et al*., 2021).

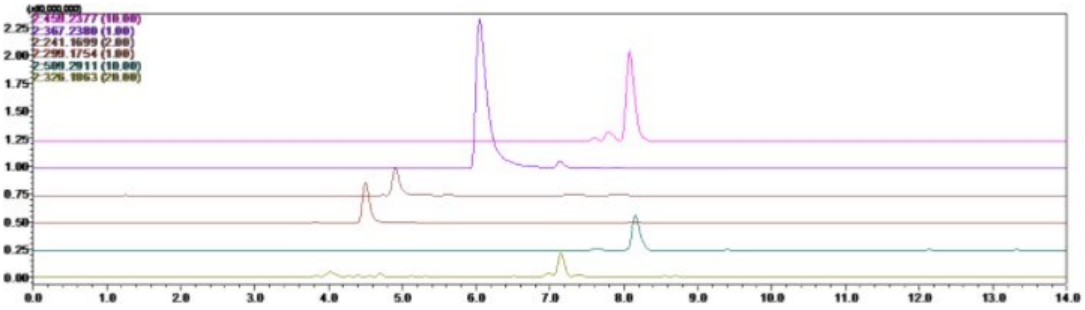


Figure 23. Results of UPLC-MS analysis of the fungi sample *Fusarium oxysporum*

Fumagillin → $C_{26}H_{34}O_7$ m/z 459,23773

Exact mass and fragmentation pattern compatible with the molecule (Grijseels *et al*., 2016).

Fumigaclavine C → $C_{23}H_{30}N_2O_2$ m/z 367,23800

Exact mass and fragmentation pattern compatible with the molecule (Castillo *et al*., 2016).

Costaclavine → $C_{16}H_{20}N_2$ m/z 241,16992

Exact mass and fragmentation pattern compatible with the molecule, fragment ions are typical of ergoline derivative (Kozlovskii *et al*., 2003).

Roquefortine A → $C_{18}H_{22}N_2O_2$ m/z 299,17540

Exact mass and fragmentation pattern compatible with the molecule (Leite *et al.,* 2020).

Communesin B → $C_{32}H_{36}N_4O_2$ m/z 509,29110

Exact mass and theoretical pattern compatible with the molecule.

Preechinulin → $C_{19}H_{23}N_3O_2$ m/z 326,18630

Exact mass and theoretical pattern compatible with the molecule (Visagie *et al*., 2017)

# 4. General discussion

## 4.1. Isolation and identification

With the results obtained from the samplings we can determine that certain species of fungi are more prolific in Portugal seashore than others, for instance, the case of *Aspergillus fumigatus* that grew in several different species of macroalgae.

During the process of identification using the Blast tool, we found some of the fungi difficult to determine the species due to getting several top hits of different species of the same genus, so in those cases they were identified only by their genus, for example, *Galactomyces* sp., so using other kinds of primers that are more precise, for example β-tubulin, could help determine these species.

Comparing the three samplings and locations, we can determine that the location and season could influence the fungal growth on macroalgae. The 1st sampling was the one that showed a larger diversity and number of fungi and was collected in Praia do Castelo do Queijo at the end of the summer. This beach is exposed to anthropogenic influences duo to the fact that it's a very popular beach and is also exposed to sewer disposal. These influences, and the fact that the samples were collected at the end of the summer where the beach experiences greater activity which in turn causes much more pollution than in the other seasons, could be

responsible for the larger number of fungi collected. In comparison with the 2nd sampling, that was conducted in Praia da Memória, the number of fungi found were fewer, since this beach is less populated therefore with fewer exposure to anthropogenic effects, and the collection occurred during spring resulting in a less polluted water.

The 3rd sampling was made to compare both beaches with the same species of macroalgae during the same season. It was found that there was a balance between both beaches regarding the number of fungi, however, it's in this sample that we found the larger number of *Aspergillus fumigatus* in both locations. This could be caused by contamination during isolation but could also be caused by the water activity (Medina *et al.*, 2015) that made *Aspergillus fumigatus* reproduce in greater numbers, further research is needed to determine this factor.

Overall, the genus *Aspergillus* was the most preponderant amongst all the fungi identified in the analysis being able to identify *A. carbonarius, A. fumigatus, A. giganteus, A. niger, A. piperis and A. versicolor.*

One of the species of macroalgae analyzed, *Chondrus crispus*, showed barely any fungi growth, from the 3 samplings, only one fungus was found which was the one collected from Praia da Memória. This could mean that these species of macroalgae have antifungal properties and further research is needed.

## 4.2. Mycotoxin detection

After the analysis of the results obtained from the mass spectrometry of the fungi samples, we were able to detect that some fungi species showed the presence of mycotoxin activity, as seen in table 11.

Some of the resulting mycotoxins displayed potential for biotechnological applications while others have been identified has pathogenic to plants, animals and/or humans.

In the case of the fungi species from the genus *Alternaria*, three mycotoxins were detected. two of these, Phomapyrone A (Soledade M., Pedras C.,2015) and Deoxyradicinol (Beni Tal *et al*., 1985), were also found in a fungal pathogen called *Leptosphaeria maculans* that's responsible for the blackleg disease in plants. Macroalgae are an excellent natural bio source in different aspects of agricultural fields (Hamed *et al*., 2018), so the presence of these kinds of metabolites could lead to potential crop diseases if the macroalgae aren't properly treated before their use as fertilizer, while Infectopyrone has been studied for antibacterial properties (Zhou Xue-Ming *et al*., 2014). The fungi species from the genus *Penicillium* also resulted in the detection of three mycotoxins. Roquefortine C is

considered a neurotoxin that causes convulsions in mice (Arnold D. L. *et al*., 1978) but it's also found in blue cheese (Scott P. M. and Kennedy P. C., 1976) demonstrating that the concentrations are responsible for the toxicity, in the blue cheese the concentration of Roquefortine C is low, so the neurotoxic effect isn't detected, while Oxaline and Meleagrin are both cellular inhibitors. Oxaline inhibits the cellular proliferation and induces cell cycle arrest in G2/M phase in Jurkat cells (Koizumi Y. *et al*, 2004) and Meleagrin also has cellular inhibitor properties, and both are being considered has a possible application to arrest the proliferation of cancer cells (Du L. *et al*., 2010).

In the fungi *Syncephalestrum racemosum*, only one mycotoxin, Dehydrocarolic acid, was detected however there's few research regarding this mycotoxin, also found in species of the *Penicillium* genus as a metabolic product. (Bracken A., Raistrick H.,1947).

The fungus from the genus *Pithomyces*, had the presence of three mycotoxins. Nigragilin has displayed insecticidal properties (Isogai A. *et al*., 1975), Fonsecin was identified as a stabilizing ligand of c-*myc*G-quadruplex DNA, this is another case of a mycotoxin being research due to its antitumoral properties (Lee H. *et al*., 2010) and there's few research regarding the possible applications of Fonsecin monomethyl ether as it's considered a synthetic compound of the methylation of Fonsecin.

From the sample of *Fusarium oxysporum*, six mycotoxins were present, however, only 2 have been researched about their possibility for biotechnological applications. Fumigaclavine C induces apoptosis in MCF-7 breast cancer cells (Li, Y.-X. *et al.,* 2013) and Fumagillin inhibits angiogenesis and suppresses tumour growth (Ingber, D., Fujita, T., Kishimoto, S. *et al.*, 1990).

Roquefortine, as previously mentioned, could cause convulsions in mice while there's few research about Costaclavine, Communesin B and Preechinulin.

There's a substantial presence of *Aspergillus* species in the data obtained. In the case of *Aspergillus giganteus*, a single mycotoxin was detected, Tryptoquivaline that is considered a tremorgenic metabolite, causing tremors in animals (Clardy J. *et al*., 1975). In *Aspergillus versicolor,* also only one was found, Sterigmatocystin, that causes necrosis of the liver and kidneys in animals (Purchase I.F.H. *et al.*, 1969) while *Aspergillus carbonarius* showed the presence of 4 mycotoxins that are being studied for potential biotechnological application, two of these already previously mentioned, Fonsecin and Fonsecin monomethyl ether, Rubrofusarin inhibits enzymes responsible for the development of diabetes (Paudel P. *et al.*, 2019) and Flavasperone causes selective inhibition of Acyl-CoA:cholesterol

acyltransferase and could be a possible use to help fight the development of atherosclerosis (Sakai K. *et al*., 2008).

*Aspergillus fumigatus,* was the species presenting the greatest number of mycotoxins, being detected 21 mycotoxins in total across several samples leading us to believe that, not only is this species very dominant, but it also appears to be one of the most pathogenic to humans and animals for demonstrating toxins such is, for example, the case of Gliotoxin, that exerts a variety of immunosuppressive effects by modulating the immune response and prompting the apoptosis in different cells (Scharf *et al*., 2012), Tryptoquivaline, previously mentioned as a tremorgenic metabolite to animals and even Trypacidin, that's considered to be cytotoxic to lung cells (Gauthier T. *et al.*, 2012). However, we also find other mycotoxins that could be used as anticancer treatment such is the case of Acetophthalidin which is believed to be an inhibitor of mammalian cell cycle (Cui *et al.*, 1996), and as previously suggested with Oxaline and Meleagrin, could inhibit the development of cancer cells or Pyripyropene A, a mycotoxin that, such as Flavasperone, inhibits Acyl-CoA:cholesterol acyltransferase, so it's another possible mycotoxin to help with the treatment for atherosclerosis (Ohshiro T. *et al.,* 2011).

*Aspergillus fumigatus* is a good example of a fungi species that displays a variety of mycotoxins that can either be useful as tools for pharmaceutical and medical applications but can also cause serious health problems in humans and animals. Amongst the fungi genus analyzed in this study, *Aspergillus* can be considered the most dangerous and *Ulva rigida*, *Mastocarpus stellatus* and *Fucus spiralis*, could be potentially considered the most toxic macroalgae for containing a larger presence of this fungi genus.

## 5. Conclusions and future perspectives

We can conclude that macroalgae carry a diverse biome of fungi species. However, exposure to pollution, such as the anthropogenic type, can influence their diversity and growth.

From the macroalgae collected, the genus *Aspergillus* was the one most prolific growing in most of the macroalgae species.

Mycotoxins can be found in fungi associated with algae and some can be pathogenic to plants, animals, and humans.

Further research is needed to be performed on different beaches so we can have a larger data for comparison and to better analyze these impacts.

There's still a lack of information regarding fungi found in macroalgae which could

lead to possible development of pharmaceuticals derived from the knowledge of these fungi, or even antifungal treatments from macroalgae that could have antifungal properties, such is possibly the case of *Chondrus crispus*, however, the mycotoxins detected in this study show great promise as biotechnological applications in the pharmaceutical and medical areas.

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