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MSc

Green Roofs as Biodiversity Promoters in Urban Coastal Areas

Ana Francisca Silva Carvalho

Dissertação de Mestrado apresentada à
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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

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Abstract

The rapid expansion of city areas causes alterations in a variety of ecological processes and functions. It impacts biodiversity by altering the environmental conditions and destroying the natural habitat. Green roof technology is a nature-based solution that consist of vegetation installed on a constructed structure. Depending on the typology of green roof considered, they may provide a wide range of ecosystem services, such as management of the stormwater runoff, energy savings through internal building's temperature regulation, mitigate the urban heat island effect and increase biodiversity.

Plants in natural habitats benefit from a variety of interactions with soil microorganisms, which are particularly relevant around plant roots. Microbial diversity has a key role in improving plants survival and productivity. However, in green roofs, plants are subjected to different conditions than in natural environment and the knowledge about this associated biodiversity is still limited. Although this technology is already supported by research, the knowledge is still scarce, when considering green roof application to coastal areas and their role as biodiversity promoters.

The overall aim of this work was to study the biotic community of an extensive green roof, located in Porto city (Portugal), by following its dynamics along time. Two sampling campaigns were carried out, where samples of substrate and the rhizosphere of the most abundant plant species (*Helichrysum italicum*, *Festuca scoparia* and *Delosperma cooperi*) were collected. The microorganisms' biocenosis associated to the rhizosphere of the select plants species and to the substrate, was undertaken by optical microscopy and molecular biology tools.

The microscopic analysis revealed the constant presence of ciliates, flagellates, testate amoeba and diatoms in the different samples. The taxonomic characterization of the microbial communities of the substrate and plants' rhizosphere, obtained by 16S Rrna gene sequencing, showed that the most dominant phyla were Proteobacteria, Acidobacteriota, Bacteroidota, Planctomycetota and Actinobacteriota. Regarding the sequencing of the 18S Rrna gene, the three most abundant phyla were subsequently Amorphea, SAR (Stremenopiles, Alveolata and Rhizaria) and Archeoplastida.

The microscopic analysis and the molecular biology approach showed that there were no substantial differences, concerning the microbial composition, between the different plants' rhizosphere and between them and the substrate, although future research should deepen seasons dynamics. Further studies on the role of these biocenosis' organisms may confirm their usefulness as bioindicators of green roofs performance.

Keywords: Green roofs; Biodiversity; Urbanization; Rhizosphere’s Biocenosis

Resumo

A rápida expansão das áreas urbanas causa alterações numa variedade de processos e funções ecológicas – interfere na biodiversidade, alterando as condições ambientais e destruindo os habitats naturais. As coberturas verdes são uma solução baseada na natureza que consiste em vegetação instalada numa estrutura edificada. Dependendo da sua tipologia, as coberturas verdes podem fornecer uma ampla gama de serviços de ecossistema como, gestão de águas pluviais, poupança de energia por meio da regulação da temperatura interna do edifício, mitigação do efeito da ilha de calor urbana e promoção da biodiversidade.

As plantas, em habitats naturais, beneficiam de uma variedade de interações com os microrganismos do solo, que são particularmente relevantes em torno das suas raízes. A diversidade microbiana tem um papel fundamental na melhoria da produtividade e sobrevivência das plantas. No entanto, em coberturas verdes, as plantas estão sujeitas a condições diferentes das do ambiente natural e o conhecimento sobre a biodiversidade associada a estas ainda é limitado. Embora esta tecnologia seja suportada por vários estudos científicos, o conhecimento é ainda escasso quando se consideram coberturas verdes em áreas costeiras e o seu papel como promotores da biodiversidade.

O objetivo geral deste trabalho é estudar a comunidade biótica do substrato de uma cobertura verde extensiva, localizado na cidade do Porto (Portugal), seguindo a sua dinâmica ao longo do tempo. Foram realizadas duas campanhas de amostragem, para recolher amostras do substrato e da rizosfera de espécies vegetais selecionadas (*Helichrysum italicum, Festuca scoparia* e *Delosperma cooperi*). A biocenose dos microrganismos associados à rizosfera das espécies de plantas selecionadas e ao substrato, foi realizada por microscopia ótica e ferramentas de biologia molecular.

A análise microscópica revelou a constante presença de ciliados, flagelados, amebas com teca e diatomáceas nas diferentes amostras. A caracterização taxonómica das comunidades microbianas do substrato e da rizosfera das plantas foi obtida por sequenciação do gene 16S Rrna, e mostrou que os filos mais representados foram Proteobacteria, Acidobacteriota, Bacteroidota, Planctomycetota e Actinobacteriota. Em relação à sequenciação do gene 18S Rrna, os microrganismos eucariotas mais abundantes foram identificados como Amorphea, SAR (Stremenopiles, Alveolata e Rhizaria) e Archeoplastida.

As análises microscópica e molecular não evidenciaram diferenças consideráveis, no que diz respeito à composição microbiana da rizosfera das diferentes plantas e entre elas e o substrato. Estudos adicionais sobre o papel desses microrganismos eucariotas poderão confirmar sua utilidade como bioindicadores do desempenho das coberturas verdes.

Palavras-chave: Coberturas verdes; Biodiversidade; Urbanização; Biocenose de Rizosfera

Table of Contents:

List of Tables

Chapter 2 Material and Methods

Chapter 3 Results and Discussion

Annexes

List of Figures

Chapter 1 Introduction

Chapter 2 Material and Methods

Chapter 3 Results and Discussion

Annexes

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Chapter 1
Introduction

1.1 Urbanization Challenges

The demand for available area is increasing in order to accommodate the actual growing population needs and its concentration in areas of high economic activity. Consequently, the concept of urbanization appeared as urban areas expanded (Weng, 2007). The Department of Economic and Social Affairs of the United Nations reported that 55% of the population was residing in urban areas in 2018, and it's expected that the urbanization rate will increase to 68% by 2050 (United Nations, 2019).

Urbanization consists of a process by which the natural landscapes are replaced by manufactured elements, mostly impervious surfaces such as roads and buildings (Schneider and Woodcock, 2008). These constructions affect ecosystems by changing the flow of matter and energy (Acho-Chi, 1998), that often causes alterations in a variety of ecological processes and functions like water and land quality (Ren et al., 2003), greenhouse gases emission (Ala-Mantila et al., 2014) and impacts on biodiversity by altering the conditions and destroying the natural habitat (Guetté et al., 2017).

In developed cities, roofs occupy approximately 40-50% of the total impermeable surface (Elvidge et al., 2004). The alteration of properties of the roof space to a green area can help lessen the negative effects of urbanization and improve the local ecosystems.

Urban development strategies such as green roofs are considered a nature-based solution that can be applied in new constructions but also for buildings retrofit (Bianchini and Hewage, 2012; Gagliano et al., 2014; Cascone et al., 2018; Calheiros and Stefanakis, 2021). Nature-based solutions can support the cities shift from linear to circular resource management addressing the urban circularity challenges through (i) 'restoring and maintaining the water cycle';(ii) 'water and waste treatment, recovery and reuse'; (iii) 'nutrient recovery and reuse'; (iv) "material recovery and reuse'; (v) 'food and biomass production'; (vi) 'energy efficiency and recovery'; and (vii) 'building system recovery' (Atanasova et al., 2021). Nature-based solutions are defined as "solutions that are inspired and supported by nature, which are cost-effective, simultaneously provide environmental, social and economic benefits and help build resilience" (EC, 2019).

1.2 Green Roofs Ecosystems Services

Green roof technology briefly consists of an engineered system (several layers) with vegetation placed on a substrate, implemented on a structure (Vijayaraghavan, 2016; Xing et al., 2017). They present numerous environmental, economic and social benefits, in addition, to provide ecosystem services such as increase rainwater retention capacity (mitigating floods and problems in precipitation peaks), improve microclimate and consequent reduction of the heat island effect, energy costs reduction (due to thermal insulation of buildings), promotion of air quality, noise reduction and increase the lifespan of the roof building (Oberndorfer et al., 2007; Shafique et al., 2018; Calheiros and Stefanakis, 2021). Besides that, they can act as important stepping stone structures for plants and animals, increasing urban biodiversity (Brenneisen, 2006).

One of the most important layers of a green roof is the plant coverage. Besides being the visible part of the system, it influences the biological and hydrological dynamics of the green roof. Plants are also key contributors to ecosystem services delivered by these infrastructures including air and water runoff quality (Dvorak and Volder, 2010; Speak et al., 2012; Vijayaraghavan and Joshi, 2014), thermal performance (Cook-Patton and Bauerle, 2012) and wildlife habitat (Brenneisen, 2006). However, green roofs are exposed to a variety of harsh abiotic factors that are not favorable for plant growth. Factors such as wind, water availability, intense solar radiation and isolation from ground-level habitats make the survival of the vegetation layer difficult (Aloisio et al., 2017).

When designing a green roof, it is also important to consider the climate conditions of the region where it will be implemented when choosing the type of substrate and vegetation (Sutton, 2015). Most of the research in the subject is focused on green roofs located in regions with temperate, dry and cool continental climates (Kazemi and Mohorko, 2017). There is clearly a gap in the research on green roofs under the extreme variations and climate events associated with urban coastal areas (Silva et al., 2019) such the one where the green roof of this study is located.

The diversity of this type of research taking into account the different climates and respective challenges can assist in the development of climate-specific guidelines to better design and select the different components of the green roof (Kazemi and Mohorko, 2017).

1.3 Types of Green Roofs

Green Roofs also known as eco-roofs, living roofs or vegetated roofs are a modern modification of the concept of a roof garden serving as an ecological roof that offers social and environmental benefits. They are classified taking into account their structural components such as the substrate thickness and their maintenance requirements (Allnut et al., 2014). In Figure 1 it is shown the classification of green roofs and the typical layers that they comprised, based on technical guidelines (ANCV, 2019). Mainly they can be considered as: intensive, semi-intensive and extensive green roofs.

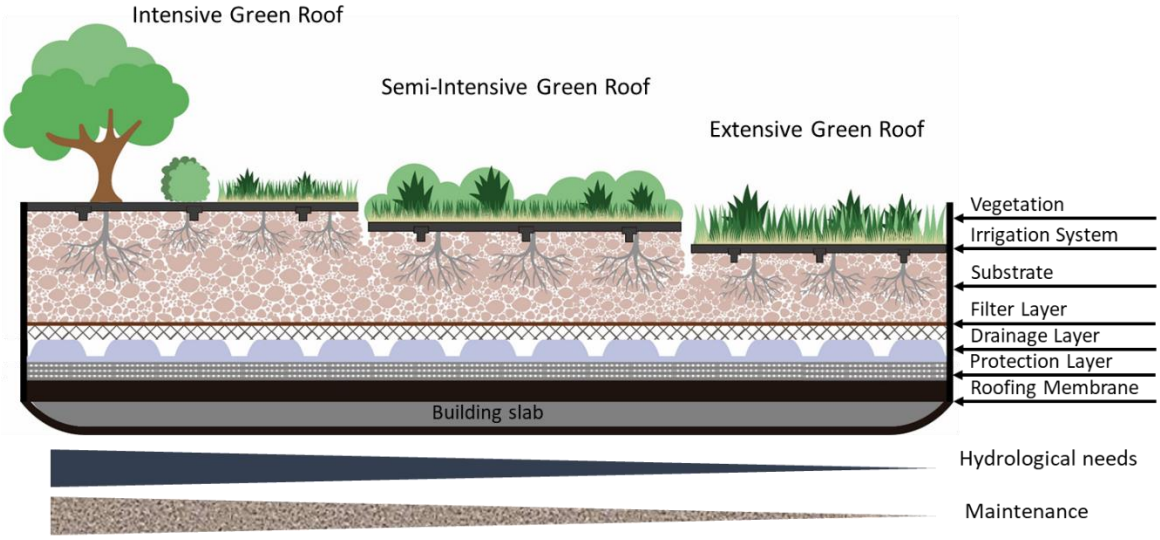


Figure 1: Typical green roof cross-sectional layers and classification (Source: Calheiros and Stefanakis, 2021)

Extensive green roofs are usually light, allowing their installation in existing buildings, without renewing the structural support of the building. They use shallow substrates, usually between 8 and 15 cm, the maintenance is kept to minimum and irrigation is reduced. The vegetation layer is normally composed of grasses, mosses and sedums species that are able to survive on shallower substrate depths and require lower nutrient and water levels (ANCV, 2019; Calheiros and Stefanakis, 2021). On the other hand, intensive green roofs have a deep substrate, usually more than 25 cm, capable of supporting a wide variety of plants species with more robust and longer roots, such as trees. This type of green roofs requires a substantial investment since the implementation and maintenance costs are higher when compared to an extensive green roof. Their level of maintenance, regarding the irrigation and fertilization specifications,

are similar to a normal garden (Oberndorfer and Reid, 2007). They are generally accessible and designed to create recreational spaces and leisure areas for social interactions.

In addition to the intensive and extensive coverage, there are also semi-extensive coverage, which includes characteristics of the previous two. They usually require a substrate depth of between 15 and 25 cm, which can be planted with a wider range of plants than extensive green cover, namely shrubs and woody plants. The irrigation and maintenance requirements depend on the plant species installed (Dunnett and Nolan, 2004).

1.4 The Rhizosphere: The Plant-Root Interface

Plants, in natural habitats, benefit from a variety of interactions with soil microorganisms. There are more interactions close to the roots in a zone called, the rhizosphere. The rhizosphere is a hot spot of soil biodiversity driven primarily by plant roots, whose exudates provide nutrients for microbes, mostly bacteria, that increase their activity (Freitas et al., 2007). The growth of bacterial biomass attracts the subsequent trophic levels, protozoa and nematodes, improving nutrient recycling (Chen et al., 2007).

Microbial communities have a key role in improving plant tolerance to drought (Xi et al., 2018), protection from pathogens (Wehner et al., 2011), access to limiting nutrients (Jacoby et al., 2017), salt tolerance (Numan et al., 2018), productivity (Welbaum et al., 2010), and stabilization of the substrate (Nabnera et al., 2006). They are also involved in some soil processes, such as decomposition, mineralization of organic matter, and biogeochemical cycles that have been recognized as key components of ecosystems (Huang et al., 2005). Besides the important interaction between plants and soil microorganisms, they also contribute to benefits awarded to green roofs, as they can regulate a variety of ecosystem services, such as the removal of organic pollutants (Abdu et al., 2017).

The choice of plant species for a green cover will affect the communities of microorganisms present as well as the functions that can be performed by the cover. The chemical constituents of plant tissues, root exudates and plant residues can affect microbial biomass, composition in species of microorganisms, and rates of microbial activity (Steinauer et al., 2016). The choice of the growth substrate will also have a significant effect on microbial communities of green roofs, since microbial diversity depends on environmental conditions (biochemical and physical factors). Growth substrate pH, that depends on the amount of organic matter, strongly influences the

incorporation of organic carbon (C) in the soil and nitrogen (N) in the microbial biomass. The variation in the amount of organic matter in the substrate can promote the survival of certain microorganisms and hinder the persistence of others (Cho et al., 2016).

There are several functional groups of microorganisms that are important for plant maintenance and play different roles in their interactions. One of the most essential group of microorganism for the survival of certain plants are the N_2-fixing bacteria that convert atmospheric N to an accessible form for plants (Igiehon and Babalola, 2018). Herbaceous plants in green roofs form many of these associations with nitrogen-fixing bacteria convenient to the wide range of benefits these bacteria provide to plants.

Protozoa are also part of the edaphic micro-fauna; they primarily control bacterial growth being an essential component of soil ecosystems. Therefore, they play a fundamental role in decomposition, as they are consumers of bacteria, fungi, other protists and even small invertebrates. As predators, they transfer nutrients to higher trophic levels and can protect plants from parasites, as they control these populations (Bonkowski, 2004).

In the presence of protozoa, plants develop an extensive and highly branched root system. This increase in roots is related to the production of auxins by some protozoa such as amoebae and production of nitrate by nitrifying bacteria whose development is stimulated by the presence of amoeboid protozoa (Krome et al., 2010). The presence of these molecules leads to the growth of lateral roots, which consequently allow to absorb more nutrients, also increasing the release of exudates, which thus stimulate even more bacterial growth, and naturally the presence of more protozoa (Krome et al., 2010).

As protozoa are on the base of the heterotrophic eukaryotic food web, they are essential components in soil ecosystems. Their dynamics and community structures provide a powerful means for assessing and monitoring changes in the biotic and abiotic environment making them valuable bioindicators (Foissner, 1999). Protozoa, such as ciliates, are used as bioindicators of the variation of CO_2 fluxes in the soil and certain ciliates can be used as bioindicators for the soil's oxygen regime (Foissner, 1999; Gabilondo et al., 2018).

1.5 Soil Protists

Protists include all eukaryotes except animals, plants and fungi and although essentials they are often a forgotten component of soil microbiome. They have key roles in regulating and shaping soil communities, as they are present in the microbial food webs as consumers of, bacteria (bacterivorous), fungi and other small eukaryotes, releasing

nutrients that enhance plant's growth. They may also have potential to be used as bioindicators of soil and environment quality (Foissner, 1999).

1.5.1 Diversity and Role of Soil Protists in Ecosystems

Protists are present in all biomes on Earth and their diversity and communities' structure depends on habitats. Their morphology and lifestyle are diverse: they can be either heterotrophs ("protozoa"), or photoautotrophs ("algae") or even, mixotrophic (Geisen and Bonkowski, 2018).

Photosynthetic protists, such as diatoms, are more abundant in the sunlit soil layers where they contribute to the formation of biological crusts (Bamforth, 2008). Even though they represent a small part of all soil protists, they provide an important C input in soils (Schmidt et al., 2016).

Heterotrophic soil protists are essential to nutrient cycling by releasing nutrients via microbial predation, making them available to plants, thus stimulating growth (Bonkowski and Clarholm, 2012). Most of them are bacterivorous meaning that they obtain energy and nutrients primarily or entirely from bacteria consumption (Clarholm, 2005). As the C:N ratio of protist is higher than that of their bacterial preys, they excrete nitrogen, mostly as ammonia (NH_3), to the soil making it available for all organisms (Sherr et al., 1983). Some heterotrophic protists also prey on small eukaryotes, for example, large testate amoebae can consume nematodes and rotifers (Yeates and Foissner, 1995). They are traditionally classified based on locomotion organelles morphology (Figure 2) in three categories: ciliates, flagellates and, naked and testate amoeba.

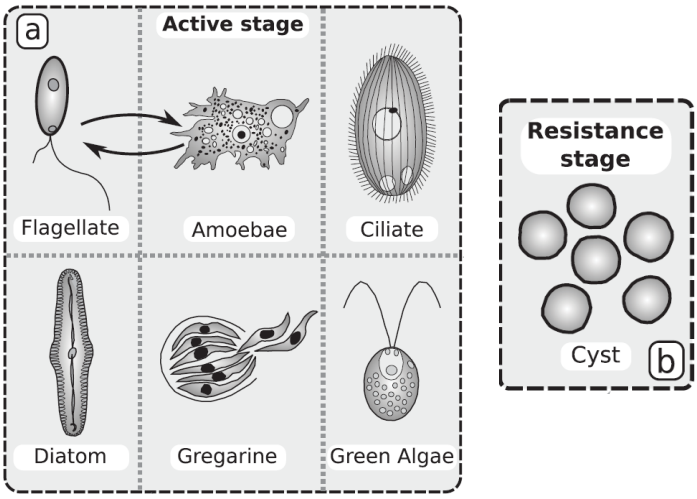


Figure 2: Typical life stages of soil protists: a) active stages; b) cysts – a shelter against harsh environmental conditions (adapted from: (Geisen et al., 2018)

Ciliates are the only group of protozoa that are monophyletic, that is, a group of organisms that are classified in the same *taxon* sharing a common recent ancestor. They commonly have hundreds of “short flagella” (cilia) on their cell bodies maintaining a recognizable shape. In soil habitats, Colpodea and Haptoria are the classes of the most found ciliates (Foissner and Oertel, 2009).

Flagellates have flagella (normally one, two or few) as locomotion organelles that can help to direct food particles to ingestion and, may function as sensory organelles. They tend to maintain a fairly constant body shape and are a paraphyletic group, which means that, they form a taxonomic group that does not include all descendants of a common ancestor. Amoebae are organisms with flexible cell shapes that form cell extensions called pseudopodia. Certain amoeboids are testate, that is, they have a test or shell generally produced by them, that it’s used for protection according environmental conditions and predation. The tests are normally composed of proteins, thickened by, most often, silica or calcium carbonate. Other use mineral scales from their prey or material collected in their surroundings (Lahr et al., 2015).

1.5.2 Soil Challenges to Protists

Soil presents a number of highly variable conditions subjecting protists to desiccation, and to freeze-thaw cycles making protists survival very challenging (Geisen et al., 2018). For autotrophic protist there are also the limiting factor of the sunlight only reaching the upper part of the soil. As well, the pore space between the soil particles, limit the distribution of the microorganisms in soil creating niches for the protist and their prey which can hamper the predation process (Geisen et al., 2018). Considering these challenges protists survival depends on its abilities. Most of them have the ability of form resistant structures, cysts, that are very efficient in preserving and protecting protist from environmental stress such as drought (Lewis and Trainor, 2012). Cysts are coccoid structures that are regulated by different mechanisms and the cyst wall composition is diverse (Samuelson and Robbins, 2011).

Many photosynthetic protist, like diatoms, can survive in low light conditions in soil because they have the ability of becoming temporarily heterotrophic and consume the dissolved organic nutrients in the soil by osmotrophy (Lewin, 1953).

1.5.3 Soil Protists as Bioindicators

Soil protists exhibit a series of characteristics that can make them useful bioindicators either as monitoring tools for soil parameters or for the performance of green roofs. They respond to environmental changes more quickly than macroscopic organisms due to, their short generations times. Another advantage is that, as they are abundant and diverse, small samples are sufficient to obtain valuable data (Adl and Vadakattu, 2006; Payne, 2013). Despite their potential as bioindicators, soil protists are not yet very used with this purpose. Studies about protists has been neglected and so there are little information, many species are still not described and there are fewer protist specialists, than specialists on prokaryotes, soil invertebrates and fungi (Adl et al., 2007; Pawlowski et al., 2012).

Soil protist communities are affected by key environmental factors, such as soil moisture, temperature, pH and light intensity. Soil moisture is a limiting factor for the development of soil protists as they need water to be active and for all of their functions. The water availability regulates soil protists diversity (community composition) and density (Geisen et al., 2014). Soil moisture also influences the oxygen availability, as the excess of water leads to anoxia in soils. In these conditions, the growth rates of protists tend to decrease, being different protist have specific tolerances for anoxia (Fenchel and Finaly, 1990; Fenchel, 2014). Another factor that affects soil protists is temperature that directly affects the soil moisture through drought and freezing (Bamforth, 1973). Some protists are able to survive in frost and/or desiccation (Müller et al., 2010; Anderson, 2016). Local soil pH and conductivity are additional selective parameters that affect the diversity, activity and density of protists (Mitchell et al., 2013). The diversity of photosynthetic protists often decreases under low pH (Antonelli et al., 2017).

The abundance of photosynthetic protist are affected by light intensity as well as abundance of theirs protist predators (Seppey et al., 2017). The dispersal of heterotrophic protists is directly affected by UV and red light (Miura and Siegert, 2000).

Protist's diversity is also shaped by the availability of nutrients such as C and N. The density and diversity of soil protists, such as algae, testate amoebae and ciliates varies tightly along the soil gradient of N (Bernasconi, 2014). On the other hand, testate amoebae's diversity and density were reduced by the addition of experimental C and phosphorus (P) (Krashevska et al., 2014).

Different rhizospheres provided by the plant species considered affect the associated soil protist communities. Root exudates and differences in the quality of litter will form

the ideal conditions for a certain group of protists and attract specifics bacterial and fungi communities (Acosta-Mercado' and Lynn, 2004).

A combination of biotic and abiotic factors affects the protist communities but are still far from being completely understood. Soil protists also respond to anthropogenic perturbations such as pollution and high carbon dioxide (CO_2) concentrations (Geisen et al., 2018)

1.6 Soil Prokaryotes

Prokaryotes (BACTERIA and ARCHAEA) are the most dominant and diverse form of life in soil and are indispensable to the Earth systems processes (Whitman et al., 1998). A single gram of soil may harbour between 10^8 (bulk soil) to 10^{10} (rhizosphere) prokaryotic cells and with an estimated diversity of 4×10^3 to 8×10^6 species (Torsvik et al., 1990; Portillo et al., 2013). Bacteria can live and proliferate in a much broader range of environmental conditions than most eukaryotes can. They have a broader metabolic diversity, besides being phototrophic or heterorganotrophic they can be chemolithotrophic. Several bacteria can use H_2, H_2S or Fe^{2+} as an energy source which is a characteristic exclusive of bacteria (Vidyalakshmi et al., 2009). Regarding the need or tolerance of oxygen, bacteria also cover the full spectrum from obligate anaerobic to obligate aerobic. Prokaryotes also have an important role in the nutrient cycling of soils, especially in C, N, and P cycles, that are essential elements for all living beings (Zhang et al., 2019).

Bacteria are involved in the process of demineralization of organic carbon into single carbon molecules that then can be return to the atmosphere as CO_2. This carbon dioxide then again is fixed into organic carbon via photosynthesis by plants and other bacteria such as cyanobacteria (Gougoulias et al., 2014). As for the N cycle, some bacteria are capable of fixing atmospheric N_2 and convert to ammonium (NH_4^+). Plants need N for growth. However, they are incapable of uptake N_2 so they associate themselves with root-colonizing rhizobacteria that have this ability to convert to NH_4^+(González-López et al., 2005).

The role of bacteria in the P cycle is to convert, directly or indirectly, P into a bioavailable form (inorganic soluble orthophosphate). Only a minority of soil P is bioavailable for plants because the majority is either in an inorganic insoluble form or in an organic form like incorporated in biomass (Tapia-Torres et al., 2016).

1.6.1 Soil Bacteria in the Rhizosphere

The rhizosphere is an area where the plants get into direct contact with soil bacteria via their roots. It's a "hot spot" of a large variety of different carbon sources originating from rhizodeposits and root exudates that attract the bacteria from the bulk soil into the rhizosphere (Dennis et al., 2010). Different components of root exudates, characteristic of the plant genome, attract and repel certain microorganisms' species (Olanrewaju et al., 2019).

For instance, *Rhizobium* is a bacteria genus that is attract to specific flavonoids released from legume roots (Thies et al., 2001). After infecting the plant, they form nodules in the roots where they fix N_2 from the atmosphere into ammonia, a more useful form of nitrogen to the plant (Figure 3). In return, they receive carbon compounds as energy source from the plant (Lindström and Mousavi, 2020). However, some plant species have the oppositive effect, they repel and reduce the microbial community diversity and richness, just as presented by Maul and Drinkwater (2010). Understanding linkages between plant species influence on microbial community structure and the subsequent direct and indirect effects on microbial community functionality will be key in predicting how ecosystem processes change (Eviner et al., 2006).

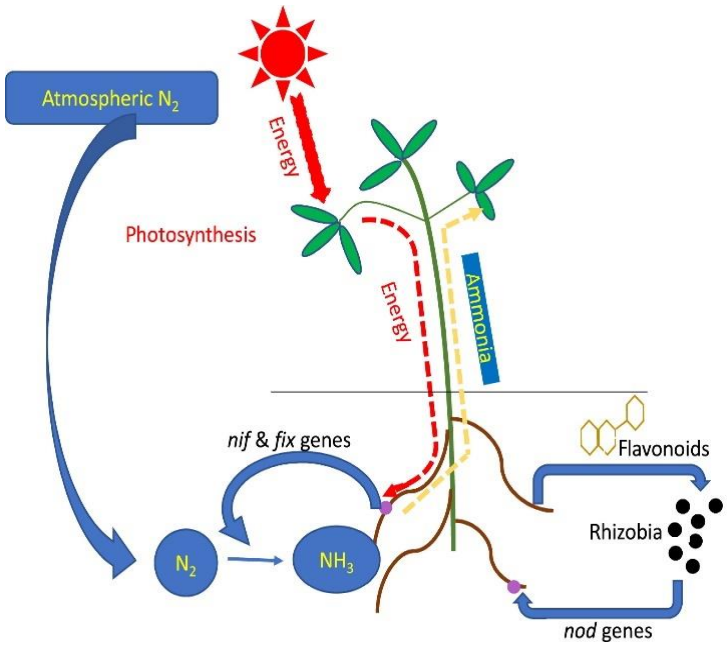


Figure 3: General interaction between N_2-fixing bacteria and plant roots (Source: (Lindström and Mousavi, 2020)

Many herbaceous plants found on green roofs, such as plants from the Fabaceae family, are able to form many of these associations with the N_2-fixing bacteria due to the wide range of benefits that the N_2-fixing bacteria provide to plants (Saikia et al., 2014; Sánchez-Pardo and Zornoza, 2014). By inoculating green roof substrates with N-fixing bacteria, it is likely that green roof vegetation will exhibit increased survival (McGuire et al., 2015).

1.7 Thesis Aim and Outline

The overall goal of this thesis is to assess the microorganisms' community of an extensive green roof, located in Porto city (Portugal), an urban coastal area, by following its dynamics along time. By characterizing the green roof substrate microbial biocenosis, we expect to gather knowledge to support the understanding of their role in the green roofs' performance.

It is well known the importance of microbial communities in the performance of plants in their survival and productivity. However, in green roofs, plants are subjected to different conditions than in natural environments and the knowledge about the biotic community associated is still limited. Most studies to date were done in prototypes at laboratory scale. Thus, there is a need to carry out studies on green roofs in a real context and that are already established. As considering local conditions, such coastal areas.

The present thesis was focused on the microbial biocenosis associated to the substrate and the rhizosphere of the three most abundant plant species present in the green roof (*Helichrysum italicum*, *Festuca scoparia* and *Delosperma cooperi)*.

The specific aims of this thesis were the following:

(1) Evaluate the microorganisms' biocenosis associated to the rhizosphere of the selected plant species and to the substrate, using optical microscopy and molecular biology tools
(2) Assess the existence of specific microbial diversity associated with each plant species and the substrate

This thesis is divided in four chapters. The first chapter corresponds to a general introduction to the work, approaching the urbanization challenges, the green roofs framework, namely its definition as a nature-based solution, importance, types, and its impact and functioning as a system. As well the diversity and role of soil protist and prokaryotes in the plants' rhizosphere. The second part corresponds to the section of

materials and methods, where are described the methodologies and materials used to accomplish each work objective. It is detailed the procedure carried out on the samples campaign, the microscopic analysis of the eukaryotic biocenoses and the 16S rRNA and 18S rRNA gene sequencing and metabarcoding analysis targeting prokaryotic and eukaryotic communities, respectively. The third chapter corresponds to the results description and its respective discussion. Lastly, the chapter four corresponds to the main conclusions and future work directions. In Annex I are listed the communications in Scientific Symposiums.

Chapter 2
Materials and Methods

2.1 Study Area

It was selected for the study area an extensive green roof on a private property (Figure 4) located on the western Iberian in an urban coastal area in Porto city, Portugal (Matosinhos; 41°09'56.9"N; 8°41'09.1"W). It is located at, approximately, 216.12 meters from the Atlantic coast (Figure 5).

According to Köppen-Geiger climate classification system, the climate of the coastal area of Matosinhos, Portugal is considered temperate - Type C (Peel et al., 2007). It is integrated in one of the two Cs climate varieties, classified as Csb (Annex II). Csb is characterized by a temperate climate with dry and mild summers and rainy winters (IPMA, n.a.). The coastal area is characterized by daily and seasonal temperature variations, humidity, wind erosion, precipitation fluctuations and heavy storm events.

Based on the information provided by the contractor, the green roof was constructed in 2016, with an area of 60 m^2, with 10 cm depth of technical substrate Landlab sedum. The mineral part of the commercial substrate (Annex III) consisted of light expanded clay (2 - 4 mm of diameter) and special volcanic rock (3 – 9 mm diameter) and the organic part included pine bark humus (0 – 15 mm diameter) and blonde peat (0 - 40 mm diameter), with addition of river gravel to protect from the wind action. The vegetation area displays a diverse selection of plant species such as *Allium schoenoprasum*, *Armeria maritima*, *Delosperma congestum*, *Delosperma cooperi*, *Festuca scoparia*, *Helichrysum italicum*, *Origanum vulgare*, *Sedum kamtschaticum* and *Thymus vulgaris.* There is not direct access to the green roof being only visited for maintenance or to collect samples.

Figure 4: Extensive green roof – Study Area (Porto, Portugal)

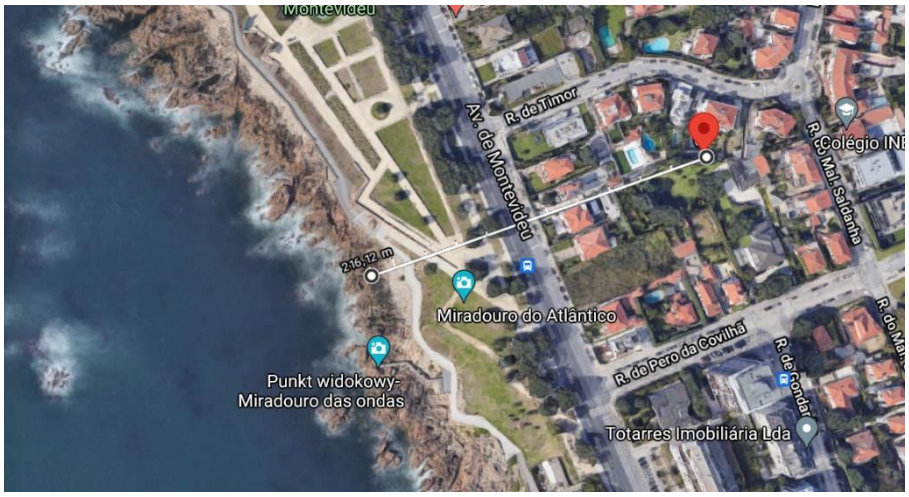


Figure 5: Study area represented as a red icon and representation of the distance between the study area and the Atlantic coast (Image obtained through google maps).

This study was focused on the rhizosphere of three most abundant plant species present in the extensive green roof – *D. cooperi* (Dc), *H. italicum* (Hi), *F. scoparia* (Fs) - and on the substrate (SUB) (Figure 6).

H. italicum (Fam. Asteracecea) is a xerophytic shrub, 10 to 30 cm high, branched at the base with small, linear, hairy leaves that give the plant an overall grey hue until the appearance of the yellow flowerheads in June or July. It grows in dry, stony areas at an impressive range of altitudes from sea level to more than 2,000 m, widespread mainly in the Western European Mediterranean region (Polunin, 1981). Their secretions are endowed with various biological activities with pharmaceutical and aromatic properties (Viegas et al., 2014). Papafotiou et al. (2013) reported that *H. italicum* were found suitable for use in Mediterranean extensive or semi-intensive green roofs.

D. cooperi (Fam. Aizoaceae) is a perennial plant that can reach a height of 20 to 40 cm, with fleshy leaves and trailing stems. *D. cooperi* grows in the cold semi-arid climate of Eastern Cape (South Africa) with the ability to grow at a wide range of altitudes (http://pza.sanbi.org/delosperma). Plants from this family are storage succulents that possess the facultative crassulacean acid metabolism (CAM); depending on soil moisture conditions, many of these plants shift between C3 metabolism and CAM (Cushman and Borland, 2002).

Provenzano et al. (2010) tested succulent and non-succulent species for their tolerance to drought in extensive green roofs located in the Mediterranean area. It was reported that, *D. cooperi* was more tolerant to drought stress compare to the other succulent species.

F. scoparia (Fam. Poaceae) are widespread in the holarctic region but also inhabit cool and temperate areas in the southern hemisphere. They grow in a large variety of different

habitats, from wetlands to xeric ecosystems, and are especially well adapted to extreme conditions in mountains and in arctic and subantarctic areas (Inda et al., 2008). This species is also on of recommend for intensive vegetated roofs in Virginia, USA (Fairfax County Public Works and Environmental Services, 2007).

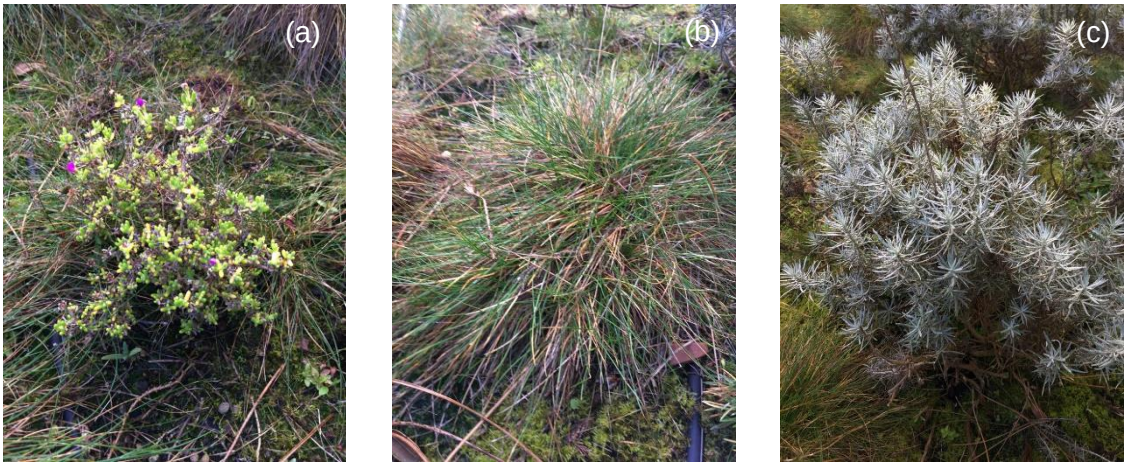


Figure 6: Selected plant species from the extensive green roof: (a) *Delosperma cooperi* (b) *Festuca scoparia* and (c) *Helichrysum italicum*

2.2 Substrate Analysis

Samples from the substrate of the extensive green roof were collected in the first campaign at 9thof November 2020, for physicochemical characterization. A total volume of 500 mL of substrate retrieved from four areas of the green roof, distant from plant rhizosphere influence, were pulled to form a composite sample, and placed on a sterile bag with a spatula. The samples were then sent to EOR Chemical Analysis Laboratory for a summary analysis (Table 1).

Table 1: Summary of commercial substrate analysis considering the parameter and method of analysis.

Parameter	Method
Texture	Manual/ Gomes & Silva (1962) – PT.SO.10
pH (H_2O)	Potentiometry/ ISO 10390:2005 - PT.SO.02
pH (KCl)	Potentiometry/ ISO 10390:2005 - PT.SO.02
Organic matter	EAM/ Walkley-Black - PT.SO.12
Extractable phosphorus	EAM/ Egner-Riehm - PT.SO.13
Extractable potassium	EAA / NF X 31-108:1992 - PT.SO.08
Need of limestone	Potentiometry/ Taboadela e Ojea - PT.SO.11
Electric conductivity	Conductimetry/ ISO 11265:1994 - PT.SO.03
Extractable calcium	EAA / NF X 31-108:1992 - PT.SO.08
Extractable magnesium	EAA / NF X 31-108:1992 - PT.SO.08
Total nitrogen	Kjeldahl modified/ ISO 11261:1995 - PT.SO.06

2.3 Sampling Procedure and Microscopic Analysis of Eukaryotic Biocenoses

The rhizosphere microorganisms' biocenosis of the select plants species (*H. italicum*, *F. scoparia* and *D. cooperi*) and also associated to the substrate, were observed by optical microscopy. A volume of 50 mL of substrate from the rhizosphere (approximately 8 cm deep) of four plants aleatory selected from each selected specie (*H. italicum* - Hi*, F. scoparia* - Fs and *D. cooperi* - Dc) and substrate samples (SUB) collected from an area without plants, was pulled to form a composite sample, and placed on a sterile bag with a spatula.

At the laboratory, to each composite sample, 8 mL of distilled water were added, for microscopy analysis of substrate suspensions. They were kept at room temperature throughout the microscopic observation process, with the occasional addition of water as it evaporated.

The first samples were collected from the green roof on 9th of November of 2020 and the second on 26th of April of 2021. In each sampling campaign, the relative humidity and temperature of the air, at the location, were measured with an OH503 logger (Greutor).

Temporary preparations in glass slides with 50 µL of each composite sample's suspension (Hi, Fs, Dc and SUB), covered with 24 x 24 mm lamellae, were observed under the optical microscope (Olympus BX41). For both campaigns, 15 observations (lamellas) were carried out; observations were done after 8, 16, 99, 100 and 101 days of the first campaign date and after 1, 2, 3, 22, 25 and 88 days of the second campaign date.

Whenever possible, a photographic record was made with the camera installed on the microscope and subsequently the registration and identification of the observed microorganisms was carried out using appropriate bibliography (Siemensma, 2021).

2.4 Molecular Biology Analyses

The rhizosphere and substrate sample collection procedure for molecular biology analyses was the same than used for microscopy. In the laboratory, samples were kept frozen at -80ºC until further processing.

2.4.1 DNA Extraction, Quantification and Sequencing

Environmental DNA (eDNA) was extracted from 500 mg of each sample using DNeasy PowerSoil Kit® (Qiagen) following the manufacturer's instructions. Extracted eDNA was eluted in a final volume of 100 µL of Elution Buffer of 10 mM of Tris ((hydroxymethyl)aminomethane).

The quantification of the eDNA extracted was carried out in Qubit™ 3 Fluorometer (ThermoFisher Scientific) using Qubit dsDNA HS (High Sensitivity) Assay Kit (Invitrogen). Samples were frozen at -20ºC until further processing.

The eDNA obtained was then used for 16S rRNA and 18S rRNA gene sequencing and metabarcoding analysis targeting prokaryotic and eukaryotic communities, respectively. The sequencing method includes the amplification of the hypervariable V4-V5 region (≈412 bp) of the 16S rRNA gene fragment using the forward primer 515YF (5'-GTGYCAGCMGCCGCGGTAA-3') and reverse primer Y926R (5'-CCGYCAATTYMTTTRAGTTT-3') (Parada et al., 2016) and the amplification of the V4 region of the 18S SSU rRNA gene with the primer pair TAReuk454FWD1 (5′-CCAGCASCYGCGGTAATTCC-3′) and TAReukREV3_modified (5′-ACTTTCGTTCTTGATYRATGA-3′) (Maritz et al., 2017). The paired-end sequencing was carried out by Illumina MiSeq® platform at Genoiseq (Cantanhede, Portugal).

Quality processing of the sequenced data was conducted at Genoinseq company: raw reads were extracted from Illumina MiSeq® System in fastq format and quality-filtered using PRINSEQ version 0.20.4(Schmieder and Edwards, 2011) to remove sequencing adapters, trim bases with an average quality lower than Q25 in a window of 5 bases and reads with less than 100 bases. The forward and reverse reads present in the fastq files were merged by overlapping paired-ends reads with AdapterRemoval version 2.1.5 (Schubert et al., 2016) using default parameters.

2.4.2 Bioinformatic Analysis

In the post sequence processing, merged reads in fastq format were converted into fasta format using Mothur software (version 1.35.1; https://www.mothur.org/).

All sequences were submitted (aligned, quality checked and classified) by the Next Generation Sequencing (NGS) analysis pipeline of the SILVA rRNA gene database project (SILVAngs 1.3) (Quast et al., 2013), and processed using SILVAngs default parameters.

Quality steps were automatically performed by SILVAngs: each read was aligned using the SILVA Incremental Aligner (SINA v1.2.10 for ARB SVN (revision 21008)) (Pruesse et al., 2012) against the SILVA SSU rRNA SEED and quality controlled (Quast et al., 2013). In the step of quality control, reads shorter than 50 aligned nucleotides and reads with more than 2% of ambiguities, or 2% of homopolymers, respectively, were excluded from further processing. Putative contaminations and artefacts, reads with a low alignment quality (50 alignment identity, 40 alignment score reported by SINA), were identified and excluded from downstream analysis.

After these initial steps of quality control, identical reads were identified in the dereplication process. Unique reads were clustered (OTUs) and the reference read of each OTU was classified. Dereplication and clustering were done using cd-hit-est (version 3.1.2; http://www.bioinformatics.org/cd-hit) (Li and Godzik, 2006) running in accurate mode, ignoring overhangs, and applying identity criteria of 1.00 and 0.98, respectively. The classification was performed by a local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (release 132; http://www.arb-silva.de) using blastn (version 2.2.30+; http://blast.ncbi.nlm.nih.gov/Blast.cgi) with standard settings (Camacho et al., 2009). The classification of each OTU reference read was mapped onto all reads that were assigned

to the respective OTU. Reads without any BLAST hits or reads with weak BLAST hits, where the function "(% sequence identity + % alignment coverage)/2" did not exceed the value of 93, remain unclassified and assigned to the metagroup "No Relative" in the SILVAngs fingerprint.

2.4.3 Downstream Analysis

Taxonomic abundance tables at different levels, phylum (higher) and genus (lower) have been produced to show the relative abundance of each taxonomic group (*taxa*) within each sample, the total number of sequences assigned to each taxonomic path.

A summary of sequence processing, rarefaction curves of alpha diversity created in SILVAngs analysis platform and graphs of relative abundance of 16S and 18S rRNA genes per sample were presented.

Considering the overall microbial communities' relative abundance, a distance matrix-based method, namely, non-metric multidimensional scaling (nMDS) according to the Bray Curtis similarity was accomplished to look for patterns of biodiversity. A hierarchical cluster analysis was also performed with PRIMER 6 (version 6.1.13) & PERMANOVA + (version 1.0.3) in order to complete information of nMDS considering the dissimilarity between samples. Species diversity was calculated using the Shannon Index using the same program to assess changes in microbial biodiversity between the samples from the rhizosphere of the different plant species and the substrate.

Chapter 3
Results and Discussion

3.1 Substrate Characterization

The physicochemical characterization of the commercial substrate performed by EOR Chemical Analysis Laboratory are summarized in Table 2.

Soil pH is an important indicator of soil quality as it plays an important role in many soil processes such as the solubility and availability of nutrients, microbial activity and a variety of physicochemical processes involved in biogeochemical cycling and ion exchange control. Also provides information regarding the liming and fertilization requirements (Hue, 2011). A pH range of 6 to 7 is generally most favorable for plant growth as most plant nutrients are available in this range. This pH range is also the most favorable for microbial activities that contribute to the availability of N, sulfur (S) and P in soils (Soil Science Division Staff, 2017). In the present study the pH was within this range.

According to the Manual de Fertilização das culturas of LQARS/INIA (2005), the values of organic matter, extractable phosphorus, extractable potassium and extractable magnesium are classified as very high.

Table 2: Summary of commercial substrate physicochemical characteristics sampled from the extensive green roof.

Parameter	**Units**	**Substrate**
Texture	-------	Substrate
pH (H_2O)	-------	7.2
pH (KCl)	-------	6.4
Organic matter	%	>7.0 (65.2)
Extractable phosphorus	ppm P_2O_5	572
Extractable potassium	ppm K_2O	347
Need of limestone	$CaCO_3$ ton/ha	0.0
Electric conductivity	mS/cm (25ºC)	0.28
Extractable calcium	ppm CaO	12535
Extractable magnesium	ppm MgO	1727
Total nitrogen	mg/g N	10.62

The commercial substrate used in the studied extensive green roof is an exclusive mixture of the Landlab company (Annex II) with the following characteristics: pH corrected to 5.5 –6.5, dry weight 750 –850 kg/m^{-3} saturated weight 900 – 1000 kg/m 3

;natural humidity 50 –60%; air capacity 37.3% v/ v; organic matter 13.8% and water easily used taxa 6.8% v/v (Monteiro et al., 2017). The same commercial substrate was used in other studies. According to Silva et al. (2019) the physicochemical analysis of the substrate showed a high content of organic matter but an acidic pH (pH <7). This shows that the implementation and all the connections with the soil microorganisms can alter the substrate properties making it more favorable to plant survival and productivity. A previous study by Monteiro et al. (2017) about the substrate influence on aromatic plant growth in extensive green roofs in a Mediterranean climate, report that this substrate was effective for establishment and growth of aromatic species, being one of them *H. italicum*, allowing 100% survival of all plants.

3.2 Biotic community characterization

3.2.1 Microscopic analysis of eukaryotic biocenosis

Substrate samples were collected from the rhizosphere area of the three selected plant species, *D. cooperi* (Dc), *F. scoparia* (Fs), *H. italicum* (Hi), and substrate with no plants (SUB). Two sampling campaigns were carried out: November 9th of 2020 and April 26th of 2021.

At the first campaign, fifteen optical microscopy observations (lamellae) were performed for each sample. The first observations were done seven days after the campaign date, followed by observations after 8, 16, 99, 100 and 101 days (Annex IV). Different *taxa* were observed on the 15 observations (lamellae) of each sample (Table 3).

The microscopic analysis revealed the constant presence of ciliates, flagellates, testate amoebas and diatoms in all samples. Various cysts and nematodes were also very common in most samples (Figure 7).

Table 3: Optical microscopy observations to identification of organisms' *taxa,* present (x) in the samples of the first campaign (9th November 2020).

	SAMPLES			
TAXA	SUB	Dc	Fs	Hi
Micro Ciliates	**X**		**X**	**X**
Ciliates	**X**	**X**	**X**	**X**
Halteria			**X**	**X**
Colpoda				**X**
Stylonichia				**X**
Flagellate	**X**	**X**	**X**	**X**
Testate amoeba	**X**	**X**	**X**	**X**
Euglypha	**X**	**X**	**X**	**X**
Trinema	**X**	**X**	**X**	**X**
Difflugia	**X**	**X**	**x**	**X**
Cysts	**X**	**X**		**X**
Diatoms	**X**	**X**	**X**	**X**
Heliozoa				
NEMATODA	**X**	**X**		**X**
ROTIFERA		**X**		
PLATHELMINTHES		**X**	**X**	

Note: Substrate sample (without plants) - SUB; Rhizosphere sample from *Delosperma cooperi* (Dc), *Festuca scoparia* (Fs) and *Helichrysum italicum* (Hi).

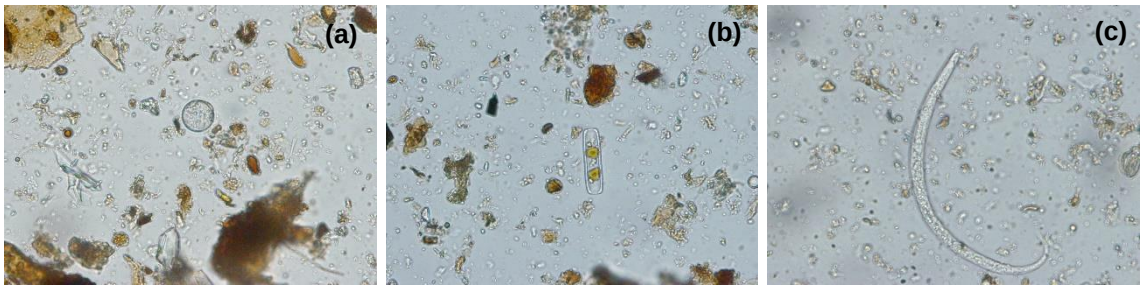


Figure 7: Observed microorganisms in brightfield optical microscopy of the samples from the first campaign. Cyst, 400x (a); Diatom, 400x (b); Nematode, 400x (c).

Relatively to the second sampling campaign, fifteen observations (lamellae) were also performed for each sample (Table 4). The first observation was performed 1 day after the campaign date, followed by observations after 2, 3, 22, 25 and 88 days (Annex V).

Table 4: Optical microscopy observations to identification of organisms' *taxa,* present (x) in the samples of the second campaign (26th April 2021).

	SAMPLES			
TAXA	Sub	Dc	Fs	Hi
Micro Ciliates			**X**	**X**
Ciliates	**X**	**X**	**X**	**X**
Aspidisca	**X**	**X**		**X**
Halteria	**X**	**X**	**X**	**X**
Colpoda	**X**	**X**		**X**
Vorticella			**X**	
Stylonichia	**X**	**X**	**X**	**X**
Flagellate	**X**	**X**	**X**	**X**
Testate amoeba	**X**	**X**	**X**	**X**
Euglypha	**X**	**X**	**X**	**X**
Trinema	**X**	**X**	**X**	**X**
Difflugia	**X**	**X**	**X**	**X**
Cysts	**X**	**X**	**X**	**X**
Diatoms	**X**	**X**	**X**	**X**
Heliozoa	**X**	**X**		
NEMATODA		**X**	**X**	**X**
ROTIFERA	**X**			**X**

Note: Substrate sample (without plants) - SUB; Rhizosphere sample from *Delosperma cooperi* (Dc), *Festuca scoparia* (Fs) and *Helichrysum italicum* (Hi).

The results from the microscopic observations of the samples from the second campaign show the constant presence of ciliates, such as *Halteria and Stylonichia,* flagellates, testate amoebas, diatoms and various cysts in all samples.

Among the testate amoebas, forms of the genus *Difflugia*, *Trinema* and *Euglypha* have been identified quite frequently in all samples and in both of the campaigns (Figure 8).

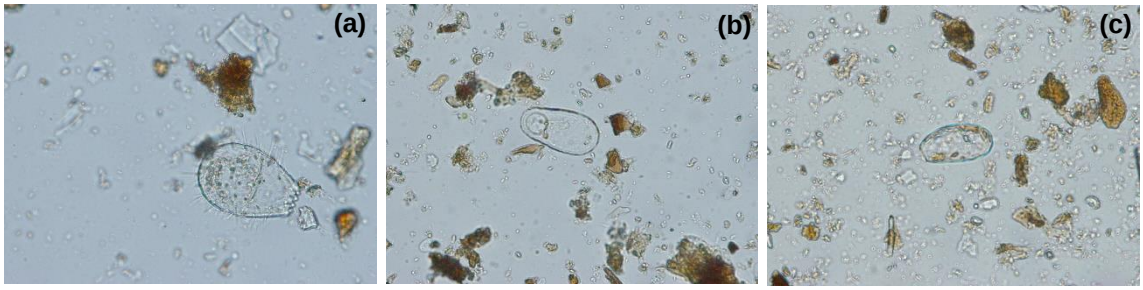


Figure 8: Most abundant genus of testate amoebas found in the suspensions of the samples from the extensive green roof, in brightfield optical microscopy. *Euglypha,* 400x (a), *Trinema,* 400x (b); *Difflugia,* 400X (c).

Moreover, the most abundant genus of ciliates identified in both campaigns were *Colpoda, Stylonichia* and *Halteria* (Figure 9). Ciliates are generally very abundant in the soil with a diversity similar to that found in aquatic environments (Madoni, 2011).

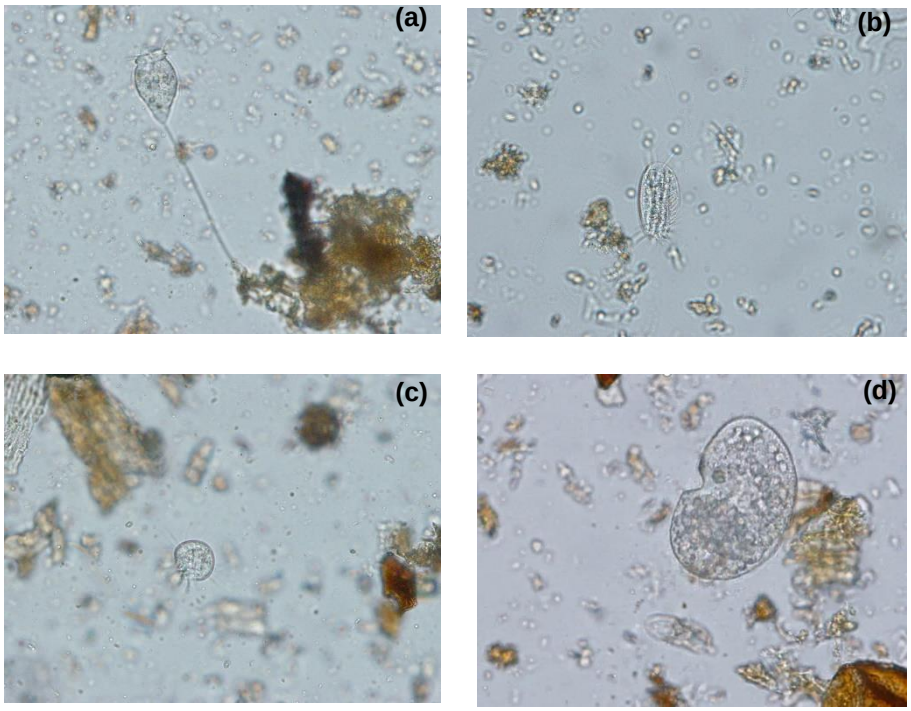


Figure 9: Most abundant genus of ciliates found in the suspensions of the samples from the extensive green roof, in brightfield optical microscopy. *Vorticella,* 400x (a); *Aspidisca*, 400x (b); *Halteria*, 400x (c); *Colpoda,* 400X (d).

Colpoda is a genus of free-living ciliates that are, normally, dominant in wastewater treatment systems by activated sludge (Madoni, 1994).

Protozoa, such as ciliates, flagellates and naked amoebae, was been reported to significantly increase plant growth by improving nutrient availability (mainly nitrogen) due to grazing on bacterial populations in the rhizosphere but also by non-nutritional effects, such the promoting microorganisms or suppressed pathogenic bacteria (Alphei et al., 1996)

Other microorganisms found with less abundance were testate amoeba genus, *Arcella*, phagotrophic amoeboid protists Heliozoa, genus of single-celled flagellated algae *Euglena* and also some metazoans as rotifers (Figure 10).

A higher abundance of testate amoeba and diatoms compared with other microorganisms were notable in all samples from the different sampling campaigns.

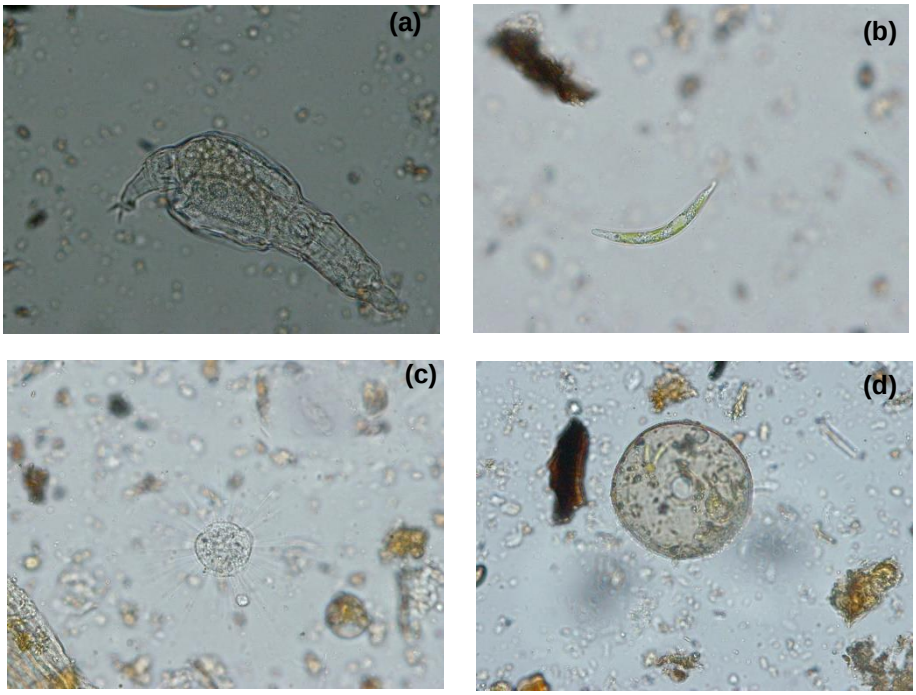


Figure 10: Less abundant microorganisms found in the suspensions of the samples from the extensive green roof, in brightfield optical microscopy. Rotifera, 400x (a); *Euglena*, 400x (b); Heliozoa, 400x (c); *Arcella* (testate amoebae), 400X (d).

Similarly, a study conduct in green roofs' pilot systems with different substrates show the presence of microfauna mainly represented by ciliates, testate amoeba and nematodes (Pinto et al., 2017)

Diatoms can produce an extracellular matrix of mucopolysaccharides which it will aggregate the soil and thus leads to a subsequently reduce in water loss by evaporation, limit soil erosion and improve water infiltration (Hoffmann, 1989; Jewson et al., 2006).

3.2.2 Taxonomy of microbial communities at the substrate and rhizosphere level of the selected plant species

A total of 374,642 of 16S rRNA gene sequences were generated through SILVAngs pipeline. After quality filtering, were removed 28768 low quality sequences (7.68%) and the number of sequences decreased to a total of 345,874 sequences (92.32%) generated from 8 samples. From those, 336,662 (89.86%) were classified and 9,212 (2.46%) were considered as unknown phyla ("No Relative").

The taxonomic characterization of the substrate and plant rhizosphere microbial communities obtained by 16S rRNA gene sequencing, detected a total of 63 phyla belonging to the three domains: Archaea, Bacteria and Eukarya, being Bacteria the most represented domain. Graphics from the SILVAngs pipeline (Figure 11) showed the most dominant phyla with their respective relative abundances: Proteobacteria was the most abundant phylum detected (25-31%-), considering all samples. Besides Proteobacteria, the five most dominant phyla were Acidobacteriota (11-14%), Bacteroidota (10-12%), Planctomycetota (9-15%) and Actinobacteriota (6-14%). There were not substantial differences observed between the different plants' rhizospheres and between them and the substrate.

A previous study by Mitchell et al. (2018) on the microbiome of 6 green roofs located in the U.S.A reported that the majority of bacterial 16S rRNA gene sequences were affiliated with the Proteobacteria and Actinobacteria phyla. The dominant phyla in our results are also similar with the one observed in 4 different soil types across a large transect of the western hemisphere which found that 40% of total bacterial sequences were Proteobacteria, followed by Bacteriodetes, Acidobacteria and Actinobacteria (Roesch et al., 2007). Furthermore, bacteria detected in the present study, were identified as Proteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia, Bacteroidetes, Chloroflexi and Planctomycetes. These phyla are also dominant in soil libraries showing that commercial substrates have a microbial biodiversity similar to that of natural soils (Janssen, 2006).

Silva et al.(2019) studied green roof systems under coastal conditions using the same commercial substrate as the extensive green roof in our study and reported that the substrate composition was the main factor influencing microbial phyla abundance, being the dominant phyla similar with our results.

Regarding the most abundant archaeal 16S rRNA gene sequences reported in green roofs microbiome, *Thaumarchaeota* followed by *Nanoarchaeota* and *Euryarchaeota* were the most abundant phyla (Mitchell et al., 2018). Just as presented in our results, these three taxa were between the most abundant phyla but being *Nanoarchaeota* the most dominant one.

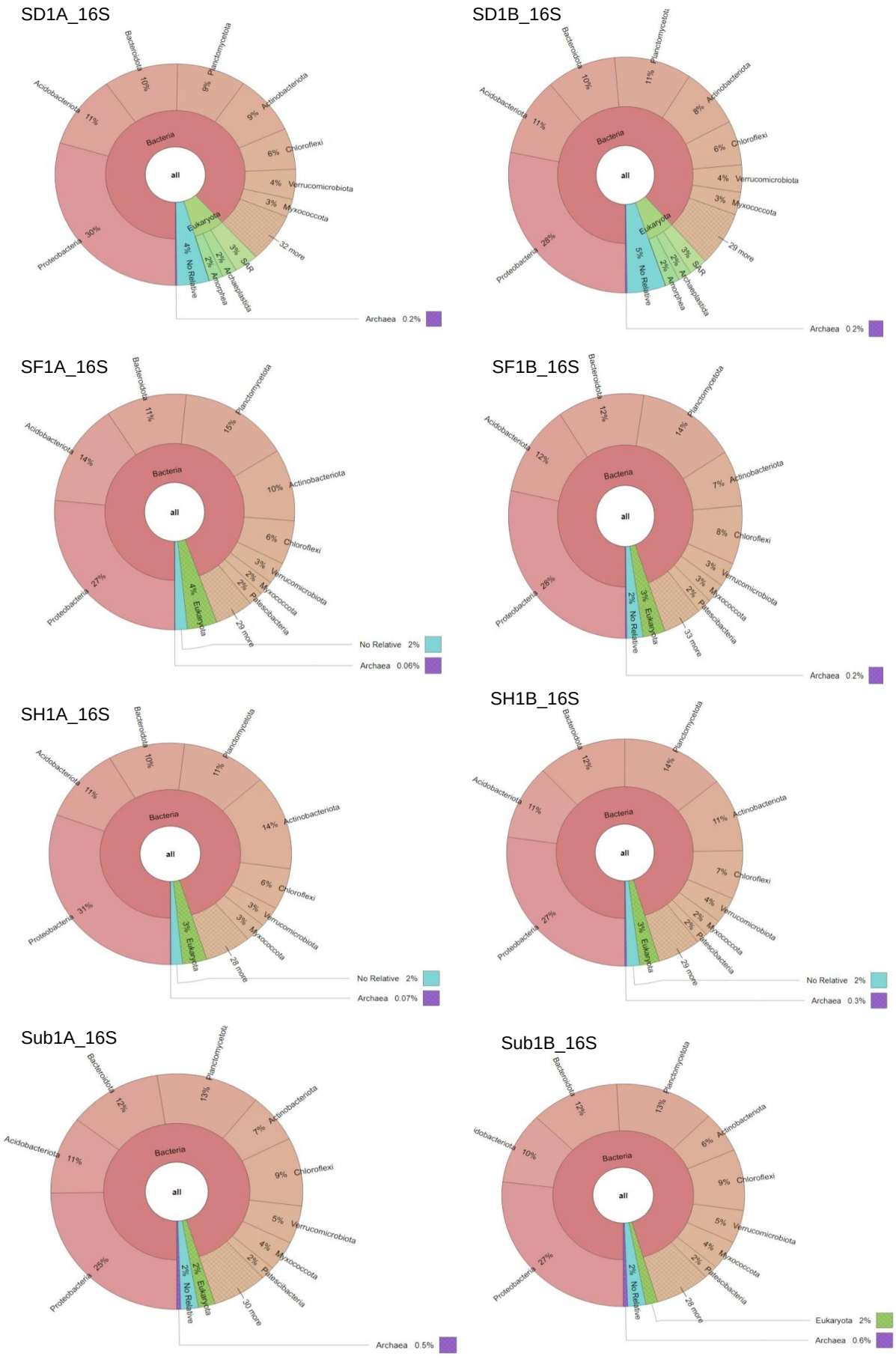


Figure 11. Taxonomic profile of the prokaryotic community at phylum level of substrate and rizosphere. Graphics were generated through SILVAngs pipeline (from the processing of 16S gene sequences): SD1A/B: Duplicated Samples from *Delosperma cooperi* rhizosphere, SF1A/B: Duplicated Samples from *Festuca scoparia* rhizosphere, SH1A/B: Duplicated Samples from *Helichrysum italicum* rhizosphere, Sub1A/B: Duplicated Samples from the substrate.

3.2.3 Alpha-diversity Analysis

The alpha diversity rarefaction curves provide information about the sequencing coverage in each sample based on the number of OTUs registered. The results indicated that microbial diversity was not fully covered from the sequencing depth in the samples, meaning that sequence coverage was not sufficient to represent all *taxa* present in and on concrete, since a plateau phase was not reached (Figure 12).

The Shannon diversity index reflects the diversity within each sample, considering the observed number of *taxa* and their abundance. The results allowed to infer that (Table 5), the samples from the substrate (Sub_1A/B) correspond to the variables with lower diversity values (Shannon index between 5.108 – 5.127). The highest diversity results obtained correspond to the samples from the *D. cooperi* rhizosphere (5.221-5.316) followed by *H. italicum* (5.223 – 5.261) and *F. scoparia* (5.121 – 5.155).

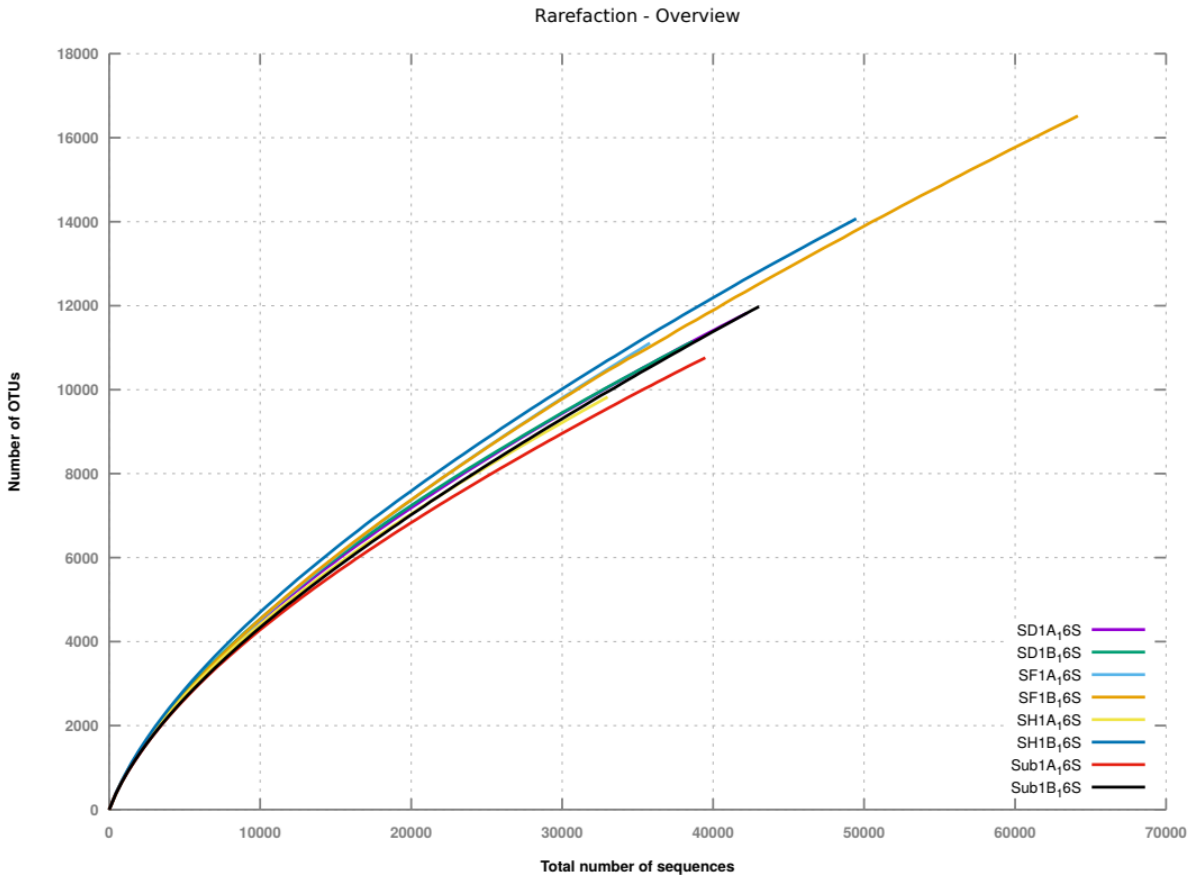


Figure 12: Rarefaction curves of the prokaryotic community from the 8 samples. (SD1A/B: Duplicated Samples from *Delosperma cooperi* rhizosphere, SF1A/B: Duplicated Samples from *Festuca scoparia* rhizosphere, SH1A/B: Duplicated Samples from *Helichrysum italicum* rhizosphere, Sub1A/B: Duplicated Samples from the substrate)

Table 5: Alpha-diversity metrics (Shannon diversity index and Richness – Observed number of genera) (SD1A/B: Samples from *Delosperma cooperi* rhizosphere, SF1A/B: Samples from *Festuca scoparia* rhizosphere, SH1A/B: Samples from *Helichrysum italicum* rhizosphere, Sub1A/B: Samples from the substrate)

Samples	Shannon Diversity Index	Richness
SD1A_16S	5.316	859
SD1B_16S	5.221	821
SF1A_16S	5.121	766
SF1B_16S	5.155	776
SH1A_16S	5.261	767
SH1B_16S	5.223	786
Sub1A_16S	5.127	730
Sub1B_16S	5.108	739

A higher diversity in the rhizosphere of the selected plants compared with a lower diversity in the substrate, it would be expected as the plant exudates would attract bacteria increasing the microbial biodiversity (Alawiye and Babalola, 2019).
Similar results were reported in the master thesis Silva et al. (2019) where a lower diversity was detected in substrate before the implementation of vegetation that increased when the plants were implemented.

3.2.4 Beta-diversity analysis

Non-metric Multi-Dimensional Scaling (nMDS) reflects a representation of community patterns based on similarity. The results reported in Figure 13, showed that there are two clearly distinct groups: one composed by all samples from the rhizosphere of the selected plant species and the second group composed by the duplicated substrate samples, highlighting the high level of similarity within the two clusters
A dendrogram (Figure 14) was also accomplished to underline the above results. The hierarchical clustering analysis of the 16S rRNA gene sequence allowed to observe a clear pattern considering the similarity between the duplicate samples of the same plant species and the duplicate samples from the substrate.

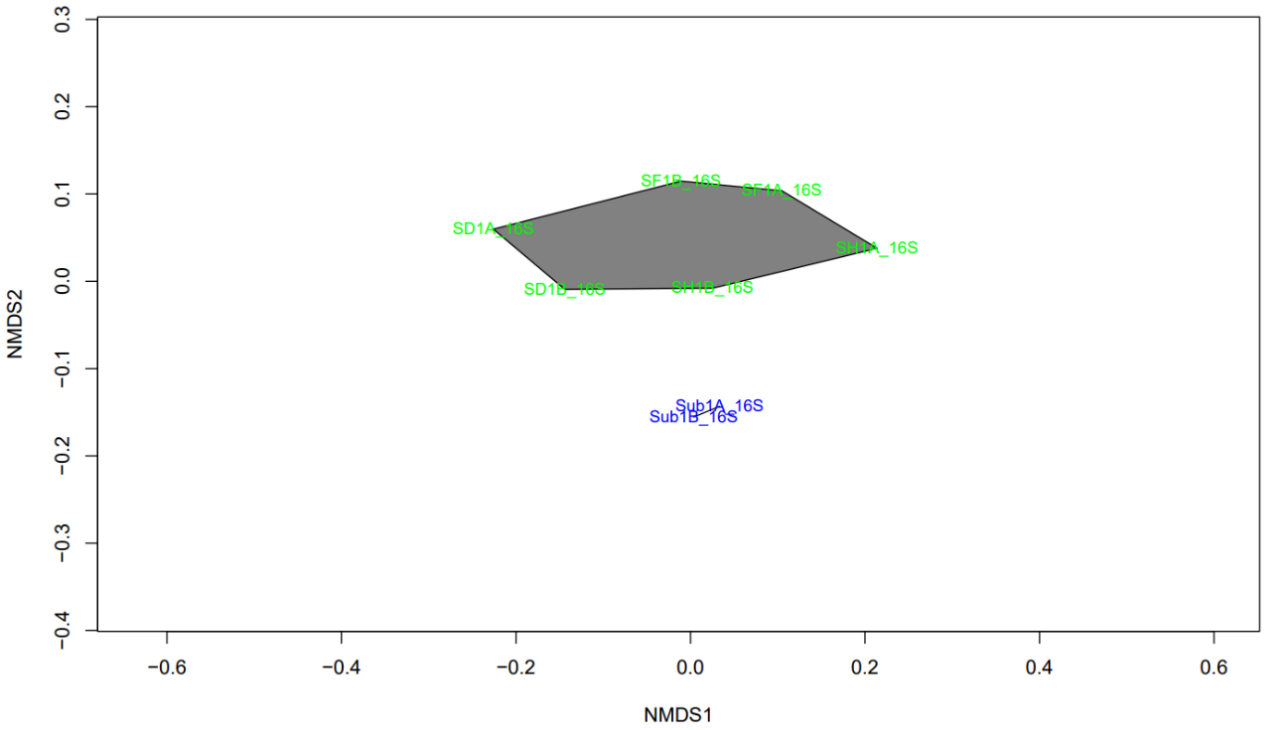


Figure 13: Non-metric multi-dimensional scaling (nMDS) based on Bray Curtis similarity considering all the samples in analysis (SD1A/B: Duplicated Samples from Delosperma cooperi rhizosphere, SF1A/B: Duplicated Samples from Festuca scoparia rhizosphere, SH1A/B: Duplicated Samples from Helichrysum italicum rhizosphere, Sub1A/B: Duplicated Samples from the substrate)

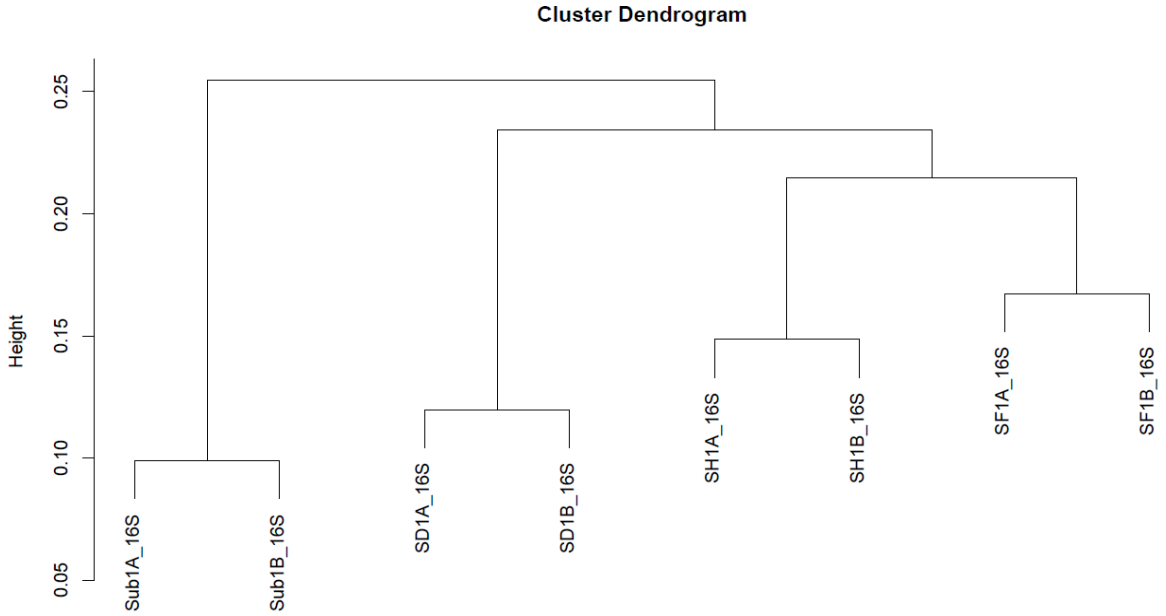


Figure 14: Cluster analysis based on Bray Curtis similarity considering all samples in analysis. (SD1A/B: Duplicated Samples from *Delosperma cooperi* rhizosphere, SF1A/B: Duplicated Samples from *Festuca scoparia* rhizosphere, SH1A/B: Duplicated Samples from *Helichrysum italicum* rhizosphere, Sub1A/B: Duplicated Samples from the substrate)

We also can highlight a closer similarity of samples from the rhizosphere of the selected plant species between each other, and a lower similarity between the substrate samples and the samples from the plants' rhizospheres. The closest similarity was between the rhizosphere samples from *F. scoparia* and *H. italicum*.

The results of prokaryotic community characterization, overall, showed distinct patterns between the samples from the substrate and the samples from the rhizosphere of the selected plants. Moreover, it was possible to access that the plants implemented in the same substrate-type, differ in their rhizosphere microbiome. Schmid et al. (2018) also reported that, by using 16S rRNA gene sequencing, microbial community structure in the plant rhizosphere was primarily determined by soil legacy and by plant species identity, meaning that plant diversity can strongly affect belowground community composition and diversity.

3.2.5 Taxonomy of eukaryotic communities at the substrate and rhizosphere level of the selected plant species

Regarding the sequencing of the 18S rRNA gene, a total of 631,491 sequences were generated through SILVAngs pipeline. The number of sequences decreased to 513,917 (81.38%) after the removal of 117,574 (18.62%) sequences with low-quality. From those, 445,827 (70.60%) were classified and 68,090 (10.78%) were not classified.

A total of 31 phyla were identified in all 8 samples. Among them, the most dominant phyla were Eukaryota (Figure 15). Considering all the samples, the three, clearly, most abundant phyla were Amorphea (relative abundance 37-44%), SAR (Stramenopiles, Alveolates and Rhizaria) (22-31%) and Archeoplastida (16-24%).

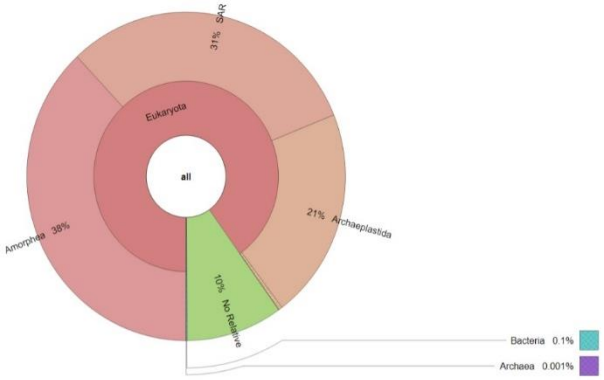
At present, the great majority of living eukaryotes can be assigned to one of four "supergroups": Archaeplastida, SAR, Excavata and Amorphea (Simpson and Eglit, 2016). The most abundant phylum detected in all samples was Amorphea (previous known as "unikonts"), a supergroup that unites two major taxa, Amoebozoa and Obazoa. Amoebozoa includes naked lobose amoebae and testate ones. The taxon SAR, acronym for Stremenopiles, Alveolata and Rhizaria, is the second most abundant in the samples that include different organisms such as diatoms and ciliates. Archeoplastida is the group containing essentially all of the primary algae, being that the most majority of species in this assemblage are photosynthetic.

Microorganisms such as ciliates, diatoms, naked and testate amoebae that are part of the most abundant phyla, were also observed by optical microscopy in the samples analyzed.

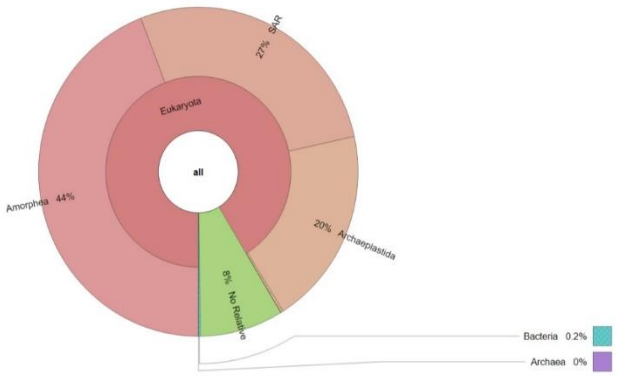
Numerous studies have examined microbial community composition and associated ecosystem services in natural environments being that the identities and functions of the urban microbiota are only now begin to be uncovered (McGuire et al., 2015). However, the same biotic and abiotic factors that operate in unconstructed environments will also likely be operating in green roof communities.

Singer and collaborators (2021) conducted a comparison of protist diversity based on standardized high throughput 18S rRNA gene sequencing of soil, freshwater and marine environmental DNA. Our results are in accordance with the ones they reported that the most relative abundant phyla of the overall protist *taxa* in soil environments were Stramenopiles, Alveolates and Rhizaria (SAR), Archeaplastida, Amoebozoa and Excavata. Relatively to the analysis of the functional diversity ciliates (phylum SAR) were overall the second richest group of consumers in soil ecosystems. In relation to the phototrophic groups, Archeaplastida were widespread in soils (Singer et al., 2021).

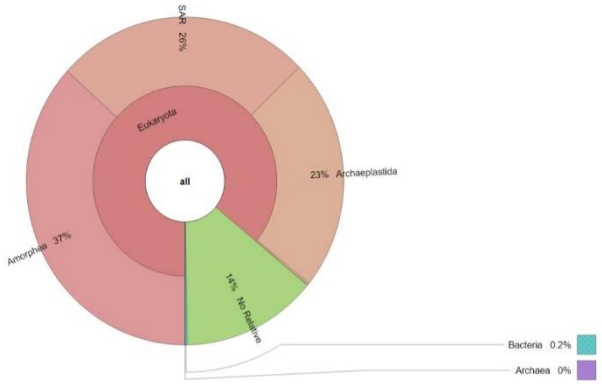
SD1A_18S



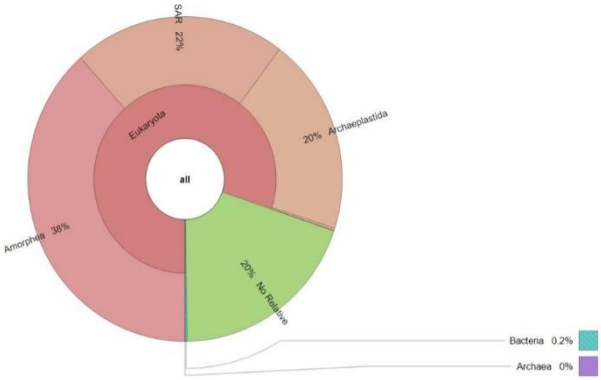
SD1B_18S



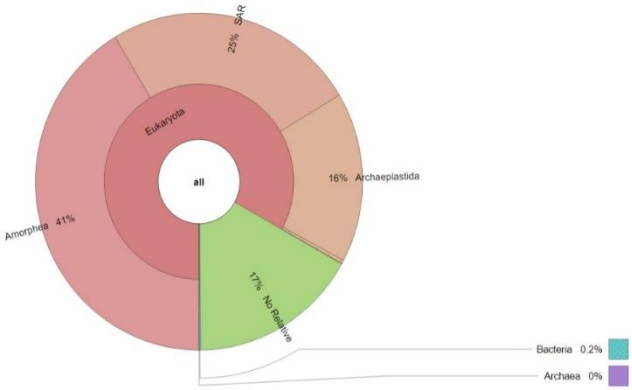
SF1A_18S



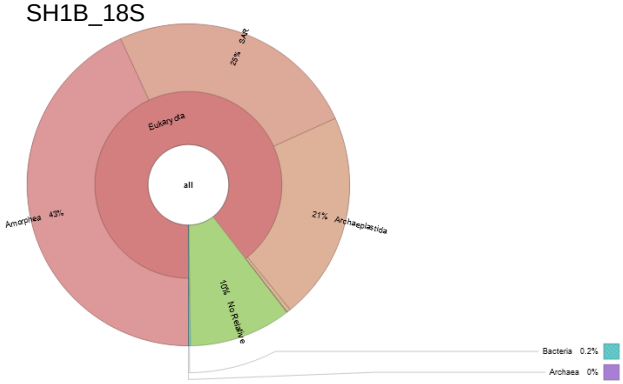
SF1B_18S



SH1A_18S



SH1B_18S



Sub1A_18S

Sub1B_18S

Figure 15: Taxonomic profile of the eukaryotic community at phylum level of substrate and rizosphere. Graphics were generated through SILVAngs pipeline (from the processing of 18S gene sequences: SD1A/B: Duplicated Samples from *Delosperma cooperi* rhizosphere, SF1A/B: Duplicated Samples from *Festuca scoparia* rhizosphere, SH1A/B: Duplicated Samples from *Helichrysum italicum* rhizosphere, Sub1A/B: Duplicated Samples from the substrate)

The alpha diversity rarefaction curves provide information about the sequencing coverage in each sample based on the number of OTUs registered. The results indicated that eukaryotic diversity was not fully covered from the sequencing depth in the samples, meaning that sequence coverage was not sufficient to represent all *taxa* present in and on concrete, since a plateau phase was not reached (Figure 16).

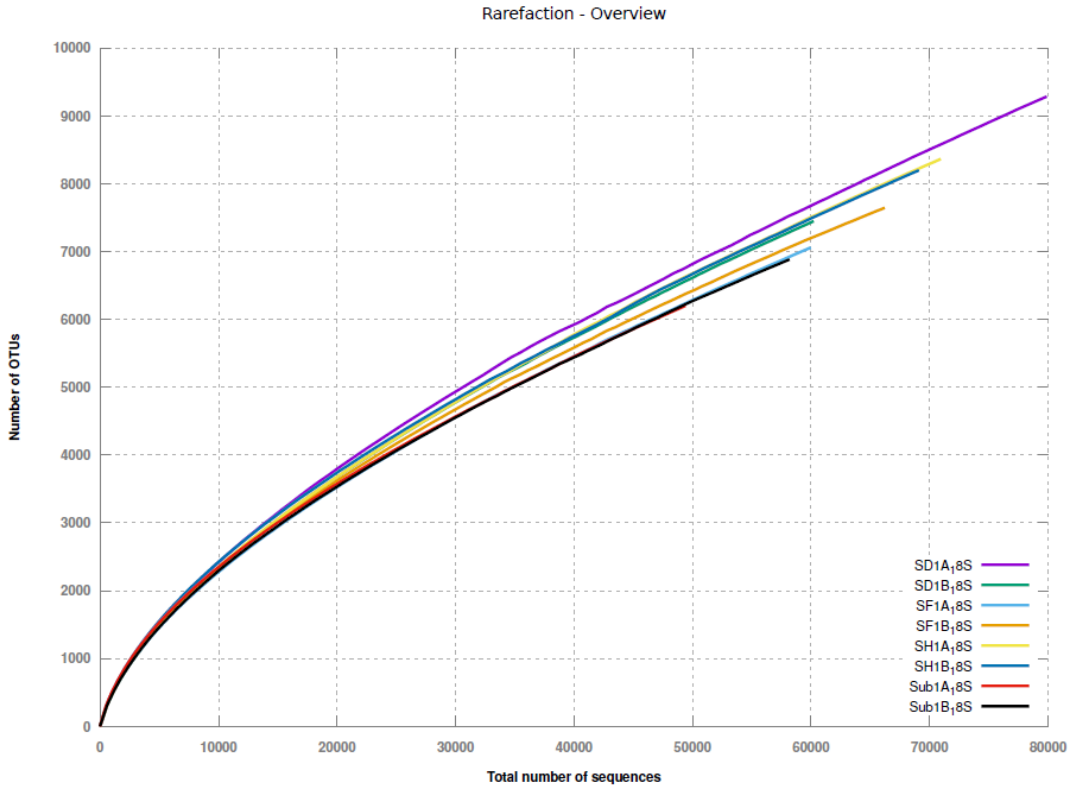


Figure 16: Rarefaction curves of the eukaryotic community from the 8 samples. (SD1A/B: Duplicated Samples from *Delosperma cooperi* rhizosphere, SF1A/B: Duplicated Samples from *Festuca scoparia* rhizosphere, SH1A/B: Duplicated Samples from *Helichrysum italicum* rhizosphere, Sub1A/B: Duplicated Samples from the substrate)

Chapter 4
Conclusion and Future Perspectives

Although the importance of soil microorganisms is well-known in natural terrestrial ecosystems, there is a lack of information regarding their taxonomic diversity or functional role in green roofs ecosystems (McGuire et al., 2015).

This study provides important information about the microbial communities present in a green roof under urban coastal conditions. Characterizing the microorganisms associated to the rhizosphere of specific plants species and understanding the dynamics of the microbial communities along time are topics of great interest on the research of green roofs' technology.

The vegetation layer together with the substrate selected have a role in shaping the microbial communities as well as the climate conditions where the green roof is installed. In this research it was used as study case, a mature extensive green roof located in an urban coastal area in Porto city (Portugal).

The taxonomic characterization of the prokaryotic communities of the substrate and plants' rhizosphere, obtained by 16S rRNA gene sequencing, showed that the most dominant bacteria were identify belonging to the phyla Proteobacteria, Acidobacteriota, Bacteroidota, Planctomycetota and Actinobacteriota.

Regarding the eukaryotic communities, obtained by the sequencing of the 18S rRNA gene, the three most abundant phyla were subsequently Amorphea, SAR (Stremenopiles, Alveolata and Rhizaria) and Archeoplastida.

For the microscopic analysis, the most observed microorganism were protists such as ciliates, flagellates and testate amoeba, as it happens in aquatic and natural soils environments (Varma and Buscot, 2005; Geisen et al., 2017). There were no substantial differences between the forms of organisms found in the rhizosphere of the selected plants species and between them and the samples from the substrate.

Most studies on this subject are conducted in green roofs pilot systems or under laboratory conditions. There is a gap in the research of studies of longer duration and higher plant density that would help understand the temporal changes in microbial communities between seasons and explore their potential as the green roof system mature and plants reproduce. This study intended to contribute to fill that gap being, in the future, further studies necessary, having in consideration growing seasons, substrate properties, different plant species and/or climate conditions, in order to elucidate about the potential of using these microorganisms as bioindicators of the green roof performance.

Regarding the optical microscopy analysis, the most observed microorganism were ciliates, flagellates, testate amoeba and diatoms. There were no substantial differences between the forms of organisms found in the rhizosphere of the selected plants species and between them and the samples from the substrate.

As green roofs became an important structure in cities in order to mitigate the impacts of the urbanization on the environment and even so the impacts of the climate change, is of great importance to study all the elements that can affect its performance. A better understanding of the connection of microbial communities and the survival and productivity of the vegetation lays can hence increase the durability of the system with the minimum maintenance.

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Annexes

Annex I: Communications in Scientific Symposiums

Carvalho A., Mucha A.P., Tomasino M.P., Mina I., Calheiros C. S. C. The role of green roofs as biodiversity promoters in urban coastal areas. 1st EUGLOH Plant Science Meeting/ 5th Meeting in Functional Biology and Biotechnology of Plants. University of Porto and Université Paris-Saclay (Oral Presentation).

Carvalho A., Mucha A.P., Tomasino M.P., Mina I., Calheiros C. S. C. Green Roofs as biodiversity promoters in urban coastal areas. Blue Think Conference 2021. CIIMAR (Poster).

Carvalho, Ana F., Mucha, Ana P., Mina, Isabel A. P., Calheiros, Cristina S.C. 2021.The role of green roofs as biodiversity promoters in urban coastal areas. 2021. Livro de Resumos do 14.º Encontro de Jovens Investigadores da U.PORTO. ISBN 978-989-746-295-5 (Poster)

Carvalho A., Mucha A.P., Mina I., Calheiros C. S. C. 2020. The role of green roofs as biodiversity promoters in urban coastal areas. 4th Meeting – online edition – Functional Biology and Biotechnology of Plants. 02/12/2020. Biology department of the Faculty of Sciences – University of Porto, Porto, Portugal. (Poster)

Annex II: Köppen-Geiger climate classification system

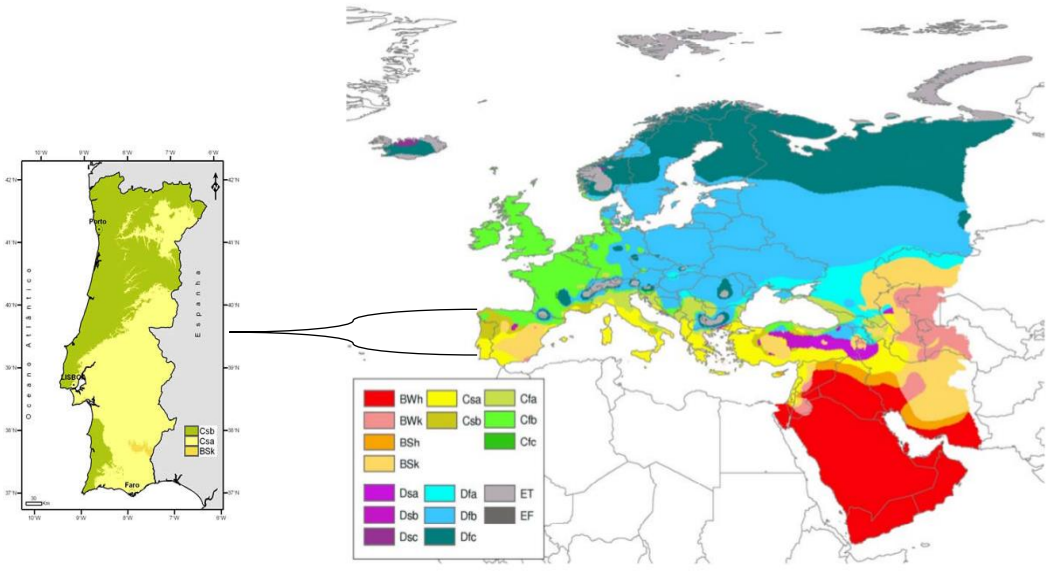
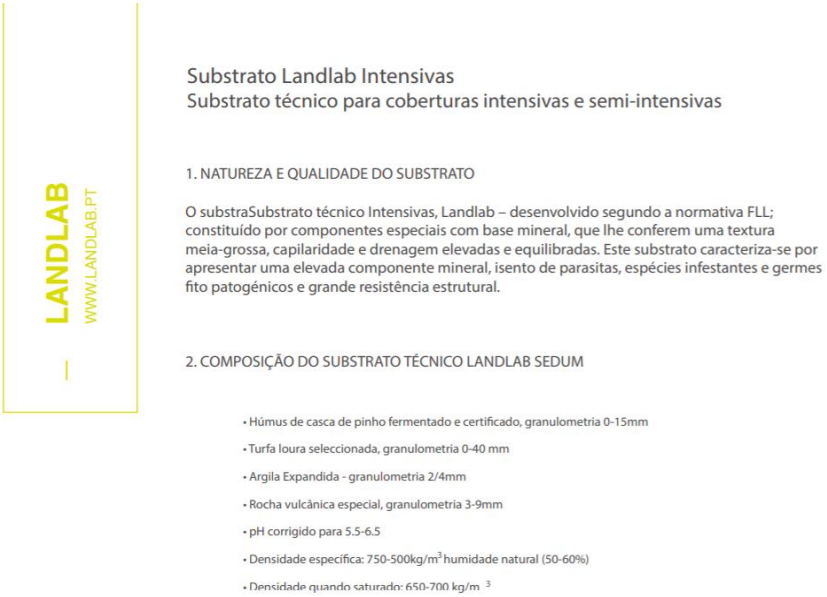


Figure 17: Site climate description by Köppen-Geiger climate classification system (adapted from Peel et al., 2007)

Annex III: Commercial substrate data sheet



LANDLAB
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Substrato Landlab Intensivas
Substrato técnico para coberturas intensivas e semi-intensivas

1. NATUREZA E QUALIDADE DO SUBSTRATO

O substraSubstrato técnico Intensivas, Landlab – desenvolvido segundo a normativa FLL; constituído por componentes especiais com base mineral, que lhe conferem uma textura meia-grossa, capilaridade e drenagem elevadas e equilibradas. Este substrato caracteriza-se por apresentar uma elevada componente mineral, isento de parasitas, espécies infestantes e germes fito patogénicos e grande resistência estrutural.

2. COMPOSIÇÃO DO SUBSTRATO TÉCNICO LANDLAB SEDUM

- Húmus de casca de pinho fermentado e certificado, granulometria 0-15mm
- Turfa loura seleccionada, granulometria 0-40 mm
- Argila Expandida - granulometria 2/4mm
- Rocha vulcânica especial, granulometria 3-9mm
- pH corrigido para 5.5-6.5
- Densidade especifica: 750-500kg/m^3 humidade natural (50-60%)
- Densidade quando saturado: 650-700 kg/m ^{3}

Figure 18: Commercial substrate data sheet

Annex IV: Registration tables of the optical microscopy observations to identification of organisms' taxa, present (x) in the samples of the first campaign (9th November 2020).

Table 6: Optical microscopy observations to identification of organisms' *taxa*, present (x) in the samples from the substrate of the first campaign (9th November 2020). Observations after 7, 8, 16, 99, 100 and 101 days of the campaign date.

	Substrate					
	Time					
TAXA	7 days	8 days	16 days	99 days	100 days	101 days
Micro ciliates	**X**					
Ciliates		**X**				
Aspidisca						
Halteria						
Colpoda						
Vorticella						
Stylonichia						
Flagellated			**X**		**X**	
Testate amoeba				**X**	**X**	**X**
Euglypha				**X**	**X**	
Trinema				**X**	**X**	**X**
Difflugia				**X**		
Cysts				**X**	**X**	**X**
Diatoms				**X**	**X**	
Heliozoa						
Nematoda						
Rotifera						
Plathelminthes						

Table 7: Optical microscopy observations to identification of organisms' *taxa*, present (x) in the samples from the rhizosphere of *Delosperma cooperi* of the first campaign (9th November 2020). Observations after 7, 8, 16, 99, 100 and 101 days of the campaign date.

Rhizosphere of Delosperma cooperi

	Time					
TAXA	7 days	8 days	16 days	99 days	100 days	101 days
Micro ciliates						
Ciliates		**X**		**X**		
Aspidisca						
Halteria						
Colpoda						
Vorticella						
Stylonichia						
Flagellated			**X**			
Testate amoeba				**X**	**X**	**X**
Euglypha				**X**		**X**
Trinema				**X**	**X**	**X**
Difflugia						
Cysts				**X**		
Diatoms				**X**	**X**	**X**
Heliozoa						
Nematoda	**X**		**X**			
Rotifera				**X**		
Plathelminthes		**X**				

Table 8: Optical microscopy observations to identification of organisms' *taxa*, present (x) in the samples from the rhizosphere of *Festuca scoparia* of the first campaign (9th November 2020)..Observations after 7, 8, 16, 99, 100 and 101 days of the campaign date.

Rhizosphere of Festuca scoparia

	Time					
TAXA	7 days	8 days	16 days	99 days	100 days	101 days
Micro ciliates		**X**				
Ciliates						**X**
Aspidisca						
Halteria					**X**	**X**
Colpoda						
Vorticella						
Stylonichia						
Flagellated	**X**		**X**			
Testate amoeba			**X**	**X**	**X**	
Euglypha				**X**	**X**	
Trinema				**X**		
Difflugia						
Cysts						
Diatoms				**X**	**X**	
Heliozoa						
Nematoda						
Rotifera						
Plathelminthes		**X**	**X**			

Table 9: Optical microscopy observations to identification of organisms' *taxa*, present (x) in the samples from the rhizosphere of *Helichrysum italicum* of the first campaign (9th November 2020).Observations after 7, 8, 16, 99, 100 and 101 days of the campaign date.

	Rhizosphere of Helichrysum italicum					
	Time					
TAXA	7 days	8 days	16 days	99 days	100 days	101 days
Micro ciliates	**X**	**X**				
Ciliates					**X**	
Aspidisca						
Halteria				**X**	**X**	**X**
Colpoda						**X**
Vorticella						
Stylonichia					**X**	
Flagellated					**X**	**X**
Testate amoeba				**X**		
Euglypha				**X**		
Trinema				**X**		
Difflugia				**X**		
Cysts				**X**		
Diatoms				**X**		**X**
Heliozoa						
Nematoda			**X**			
Rotifera						
Plathelminthes						

Annex V: Registration tables of the optical microscopy observations to identification of organisms' taxa, present (x) in the samples of the second campaign (26th April 2021).

Table 10: Optical microscopy observations to identification of organisms' *taxa*, present (x) in the samples from the substrate of the second campaign (26th April 2021). Observations after 7, 8, 16, 99, 100 and 101 days of the campaign date.

	Substrate					
	Time					
TAXA	1 day	2 days	3 days	22 days	25 days	88 days
Micro ciliates						
Ciliates	X		X	X	X	
Aspidisca					X	
Halteria			X	X	X	
Colpoda	X					
Vorticella						
Stylonichia			X			
Flagellated	X	X			X	
Testate amoeba	X	X	X	X	X	X
Euglypha	X	X	X	X	X	X
Trinema	X	X	X	X		X
Difflugia		X	X	X		
Cysts	X	X	X	X		X
Diatoms	X	X	X	X	X	X
Heliozoa				X		
Nematoda						
Rotifera	X	X		X	X	
Plathelminthes						

Table 11: Optical microscopy observations to identification of organisms' *taxa*, present (x) in the samples from the rhizosphere of *Delosperma cooperi* of the second campaign (26th April 2021). Observations after 7, 8, 16, 99, 100 and 101 days of the campaign date.

	Rhizosphere of Delosperma cooperi					
	Time					
TAXA	1 day	2 days	3 days	22 days	25 days	88 days
Micro ciliates						
Ciliates	X	X	X	X		X
Aspidisca						X
Halteria				X		
Colpoda			X			
Vorticella						
Stylonichia						X
Flagellated				X		
Testate amoeba	X	X	X	X	X	X
Euglypha	X	X	X	X	X	X
Trinema	X	X	X	X	X	X
Difflugia	X					
Cysts	X	X	X	X		X
Diatoms	X	X	X	X	X	X
Heliozoa				X	X	
Nematoda	X	X	X			
Rotifera						
Plathelminthes						

Table 12: Optical microscopy observations to identification of organisms' *taxa*, present (x) in the samples from the rhizosphere of *Festuca scoparia* of the second campaign (26th April 2021). Observations after 7, 8, 16, 99, 100 and 101 days of the campaign date.

	Rhizosphere of Festuca scoparia					
	Time					
TAXA	1 day	2 days	3 days	22 days	25 days	88 days
Micro ciliates				X		
Ciliates		X	X	X	X	
Aspidisca						
Halteria		X	X	X	X	
Colpoda						
Vorticella			X	X	X	
Stylonichia			X		X	
Flagellated	X		X		X	X
Testate amoeba	X	X	X	X	X	X
Euglypha	X	X	X	X	X	X
Trinema	X	X	X	X	X	X
Difflugia				X		
Cysts	X		X	X	X	
Diatoms	X	X		X		X
Heliozoa						
Nematoda		X	X	X		
Rotifera						
Plathelminthes						

Table13: Optical microscopy observations to identification of organisms' *taxa*, present (x) in the s samples from the rhizosphere of *Helichrysum italicum* of the second campaign (26th April 2021). Observations after 7, 8, 16, 99, 100 and 101 days of the campaign date.

	Rhizosphere of Helichrysum italicum					
	Time					
TAXA	1 day	2 days	3 days	22 days	25 days	88 days
Micro ciliates		X				
Ciliates	X	X		X	X	X
Aspidisca				X		X
Halteria		X			X	
Colpoda	X					
Vorticella						
Stylonichia		X		X		
Flagellated				X		
Testate amoeba	X	X	X	X	X	X
Euglypha	X	X	X	X	X	X
Trinema		X	X	X	X	X
Difflugia		X		X		X
Cysts	X	X	X	X		X
Diatoms	X	X	X	X	X	X
Heliozoa						
Nematoda		X	X			
Rotifera		X				
Plathelminthes						