

Diversity of Fungal Endophytes from *Zostera marina* Eelgrass in False Bay Biological Preserve

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Abstract

Endophytes are organisms such as fungi and bacteria that live at least part of their life cycle inside a photosynthetic host. Fungal endophytes have been found in nearly every plant we have looked at, including seagrasses like *Zostera marina* eelgrass. As part of the host plant's microbiome, fungi can affect how its host responds to stressors in its environment, but these relationships have not been well studied in the marine environment. The community of endophytic fungi can vary per host species and from site to site. For this study, the aim was to establish the diversity of fungal endophytes in two patches of *Zostera marina* in False Bay Biological Preserve on San Juan Island, WA, USA. Surface sterilized eelgrass leaves were plated onto Potato Dextrose Agar. Cultured growths were isolated and then analyzed based on macroscopic and microscopic morphology to identify the lowest possible taxonomic rank. Two fungi were successfully cultured: a *Cladosporium* species and a *Mucor* species. There were also five macroscopically distinct bacterial colonies cultured. *Cladosporium* was only found in the subtidal patch and *Mucor* was only found in the intertidal patch, indicating site-to-site difference in the microbial communities. While both of these genera of fungal endophytes have been studied for their benefits to terrestrial crop plants, it is still unknown what costs or benefits *Z. marina* in False Bay might incur from hosting these endophytes. Further study is needed to characterize these host-fungi interactions in seagrasses.

Keywords

Fungi - Endophytes - False Bay - San Juan Island - *Zostera marina* - *Cladosporium* - *Mucor*

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Background

Endophytes are organisms that live within the tissues of a photosynthetic organism, such as plants or algae, for at least part of their life cycle without causing symptoms of disease (Shoemaker 2010). They have been found across a wide range of host plant species, latitudes, and habitats. Endophytes can be plants, algae, fungi, or bacteria (Shoemaker 2010).

Fungi are osmotrophic organisms that absorb organic matter (OM) from their environment through their cell walls (Raghukumar 2017). Generally they function either as saprobes, colonizing and decomposing dead organic matter, or as symbionts that source their food from a photosynthetic host (Raghukumar 2017, Arnold 2007). Bacteria share a similar ecological role by also living as saprobes or symbionts. However, fungi have an advantage over bacteria in that they can penetrate solid substrates and continue decomposition from the interior of particles

(Raghukumar 2017). Fungi can also be pathogenic to plants, animals, or even other fungi and thus can control populations of organisms (Raghukumar 2017). In marine habitats, fungi have been found living symbiotically in mangrove trees, sediments, driftwood, sponges, seagrasses, diatoms, and coral (Gladfelter 2019). In some habitats, the biomass of fungi is thought to be greater than that of bacteria (Gladfelter 2019).

Fungal endophytes may provide services that support their hosts' response to biotic and abiotic stressors (Hamayun 2009). In terrestrial habitats, some plants derive benefits from their fungal symbionts. For instance, arbuscular mycorrhizal fungi take minerals like phosphorus from the soil and transfer it to their host plant's roots. In turn, fungi gain carbon from the plant (Lendenmann 2011). Some fungi make toxins that repel herbivores and reduce herbivory on their hosts (Czarndeski 2012). Fungal endophytes have been found to assist in plant growth even under stressors such as drought and salt toxicity (Yan 2019). There is evidence of certain fungal endophytes providing disease resistance to their hosts, possibly by stimulating the host's immune system (Arnold 2007). Fungi also plays an important role in carbon sequestration in the soil (Clemmensen 2013). However, these host-fungi relationships are complex and in some cases they can provide no discernible benefit to the host or even become pathogenic (Shoemaker 2010, Arnold 2007). Host-endophyte interactions can vary greatly depending on host species and geographic location (Arnold 2007).

The seagrass species *Zostera marina*, commonly called eelgrass, occupies coastal temperate habitats throughout the Northern Hemisphere (Ettinger 2021). It provides important ecosystem services such as nursery habitat for fishes and invertebrates and reduction of coastal erosion (Groner 2016, Sullivan 2018). Seagrasses have been on the decline globally. *Zostera marina* in particular has been vulnerable to eelgrass wasting disease outbreaks (Sullivan 2018). The

eelgrass beds in False Bay Biological Preserve on San Juan Island in Washington are considered to be declining (Groner 2016).

It is known that fungal diversity in and around seagrass meadows varies between oceans and from site to site (Ettinger 2021, Shoemaker 2010). It is not well known what controls distributions of fungal symbionts in the marine environment or how they are functioning ecologically. Stressors specific to the marine environment such as noise pollution have been shown to degrade fungal symbionts of a seagrass species (Solé 2021). There is still much to be learned about marine host-fungi interactions. However, given research on fungi-plant interactions in terrestrial habitats, it seems possible that fungal endophytes in *Z. marina* could play key roles in nutrient availability, pathogen resistance, and carbon sequestration in eelgrass meadows.

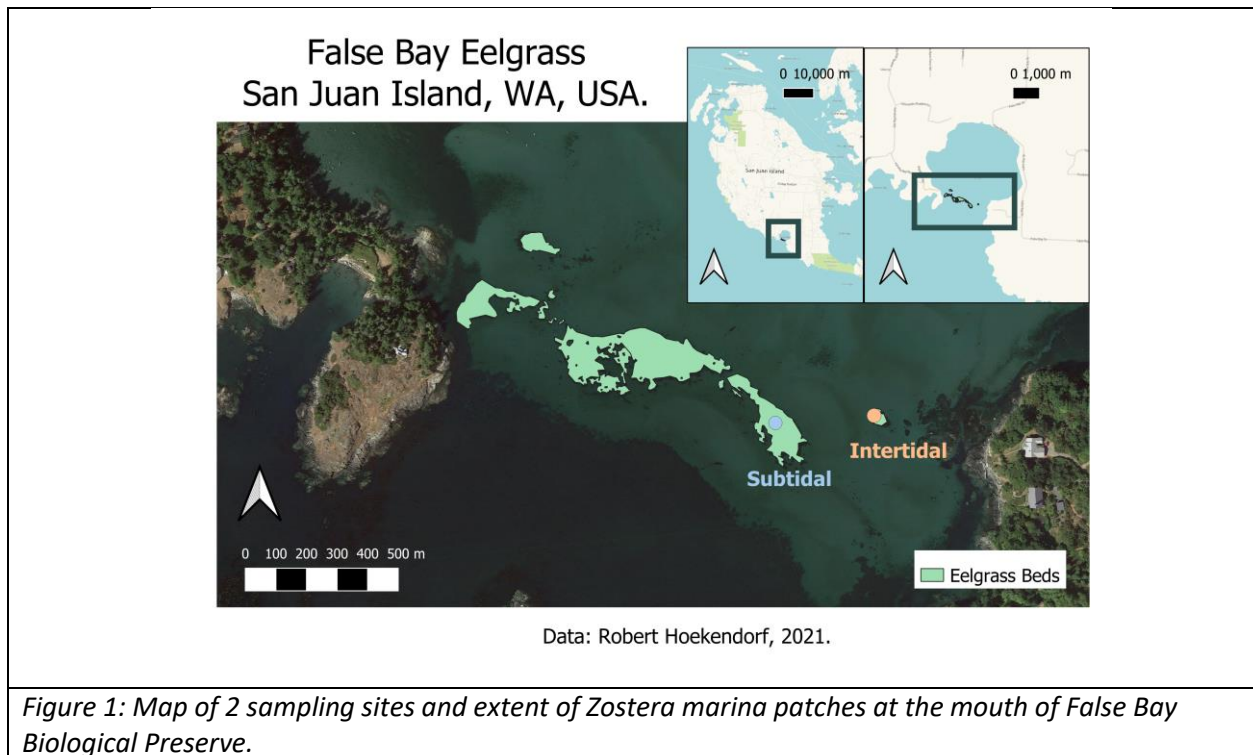
Given the declines in *Z. marina* and prevalence of eelgrass wasting disease, further understanding of the eelgrass microbiome is critical in predicting eelgrass meadow resilience to stressors. The objective for this study is to establish the diversity of fungal endophytes in False Bay eelgrass.

Methods

Sampling

Fungal symbionts have been found in all tissues of *Z. marina*, with the highest diversity found in rhizomes (Shoemaker 2010, Ettinger 2021). However, since False Bay eelgrass is considered to be in a state of decline (Groner 2016), I chose to sample leaf tissues only as this sampling is not destructive to the rooting structures. Samples were collected from False Bay Biological Preserve on San Juan Island, Washington, USA.

Sampling of *Z. marina* leaves took place on November 2 and November 12 in False Bay at two sites: an intertidal patch and a subtidal patch (Fig. 1). These patches were accessed from a private residence near the mouth of False Bay by snorkeling. A total of 26 eelgrass leaf blades, 13 from each site, were removed from eelgrass beds using scissors or hands. Samples were transported to the lab and placed in zip-top plastic bags and stored in a refrigerator at $\sim 4^{\circ}\text{C}$ for up to 48 hours.



Processing

Processing methods were modeled after previous studies (Shoemaker 2010, Ettinger 2021). Petri dishes were prepared with autoclaved Potato-Dextrose Agar (PDA) mixed with unfiltered seawater from Friday Harbor Labs. These plates were sealed with parafilm and stored in the fridge prior to use.

Eelgrass leaf samples were numbered S1 through S26. Each sample leaf was surface sterilized for 5 seconds in 95% ethanol, 2 minutes in 10% bleach (sodium hypochlorite), and 2 minutes in 70% isopropyl alcohol. Sample was then immersed and rinsed in filtered reverse osmosis water for 1 minute.

Samples were plated under a fume hood. Approximately 1 cm of each end of the sterilized leaf sample was cut and removed with flame-sterilized scissors. Five approximately 1 cm pieces were cut from the remaining sample and placed on PDA using flame-sterilized tweezers. Plates were sealed with parafilm. Plates were then placed upside down on a countertop in a closed room in the dark at ~22°C.

Plates were checked every 24-48 hours for fungal growth. Morphologically distinct growths were then isolated onto new PDA plates using fume hood and flame-sterilized tools. Once isolated and allowed to form distinct growth patterns, plates were then analyzed.

Analysis

Isolated samples were analyzed using both macroscopic morphological traits and microscopic features. Macroscopic features observed included color, reverse color, texture, and growth pattern. Plates were also observed under a dissecting microscope.

A Nikon 50i Eclipse compounding microscope was utilized to identify microscopic features. Flame-sterilized needles were used to remove a small amount of the culture. Sample was placed onto a wet-mount slide. Wet-mount slides were prepared with multiple methods, including filtered reverse osmosis water, 95% ethanol, and lactophenol cotton blue stain (Leck 1999). Features such as types of spore-bearing structures, spore shape, size, presence of asci, presence of spore appendages, presence of septate or coenocytic hyphae or pseudohyphae, color of

hyphae, as well as other features. Photos were captured with a Lumenera Infinity microscope camera.

Morphological features were referenced to Kohlmeyer (1971), Kohlmeyer (1991), Sutton (2014), Kidd (2016), Raghukumar (2017), and the University of Adelaide Mycology website (2021) for identification to the lowest taxonomic level possible.

Results

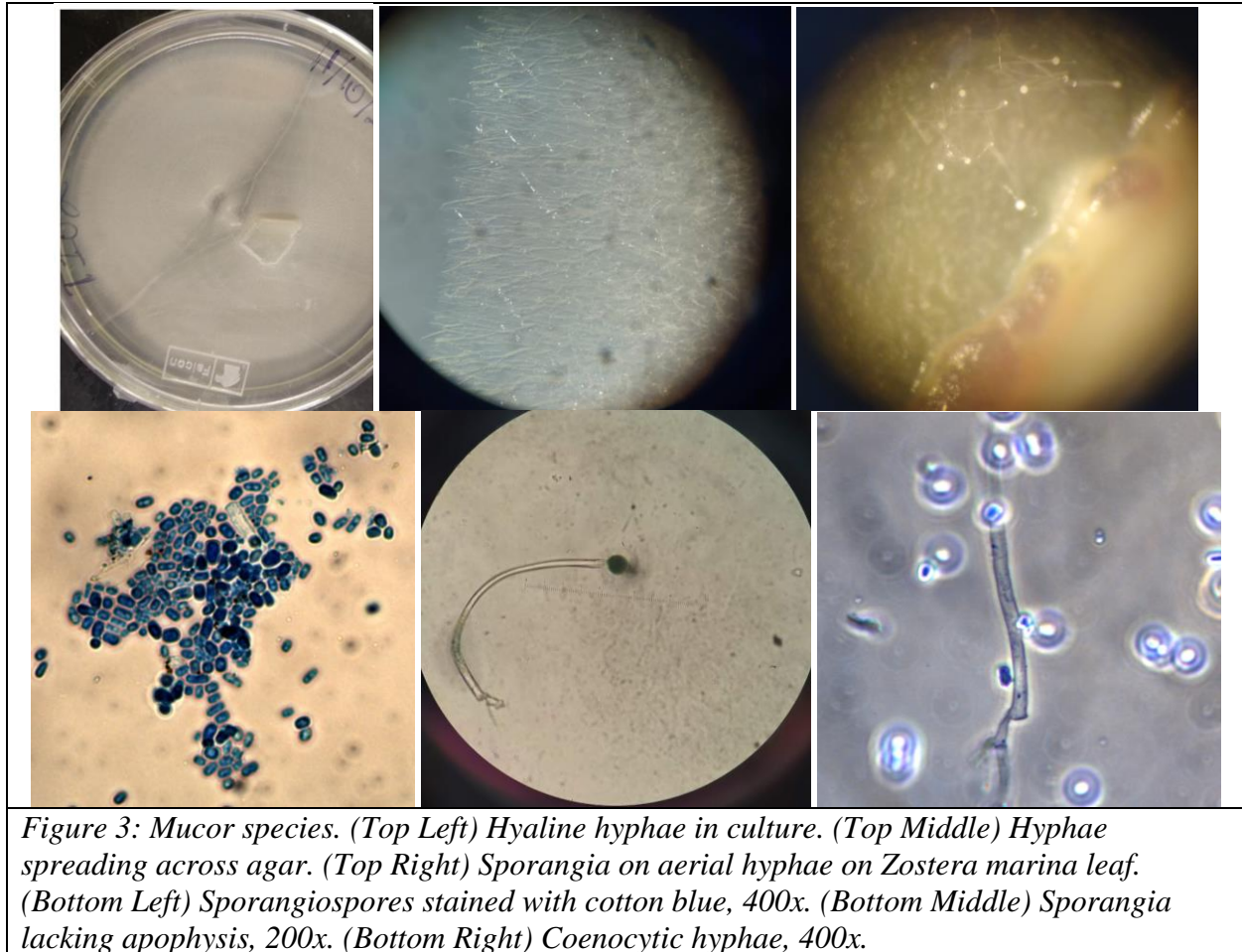
All 26 sample plates (S1-S26) were checked every 24-48 hours for growth and documented. Two plates exhibited no conspicuous growth.

Macroscopic analysis of cultures revealed fungi with two distinct growth patterns radiating out of the edge of leaf cuttings (Fig. 2). It is assumed that these growth patterns represent two distinct species.



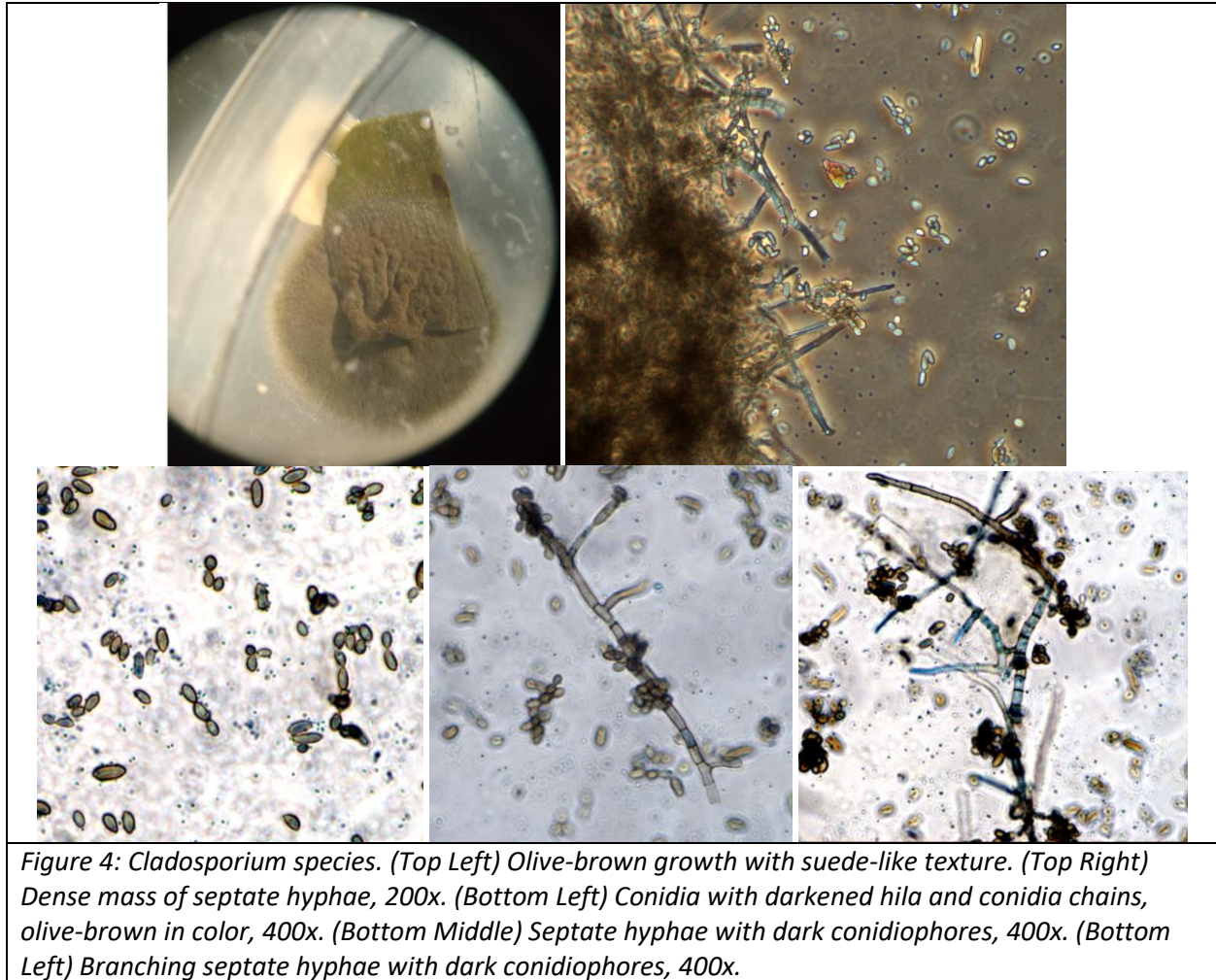
Figure 2: Isolated fungal cultures. (Top, L to R) *Mucor* sp. and *Cladosporium* sp. (Bottom) Reverse color of same cultures.

From the intertidal site, two samples grew what is likely a *Mucor* species. There was no *Mucor* growth from the subtidal samples. These growths had hyaline (colorless) hyphae and had a simple radial growth pattern (Fig. 2). Analysis of the sample plates through a dissecting microscope revealed distinctive hyaline “pinhead” sporangia (asexual spore-bearing structure) on simple, unbranched aerial hyphae (Fig. 3). With the compound microscope, one non-apophysate (lacking basal swelling) sporangia with a globose head was visualized. Spores were simple and pill-shaped with no appendages. Several coenocytic (multinucleate, lacking septa or “walls”) hyphae were observed but some septate (having clear septa or “walls”) hyphae were present. Large, spherical, non-apophysate sporangia and hyaline hyphae that are coenocytic when young are key features of the *Mucor* genus (University of Adelaide 2021, Raghukumar 2017, Sutton 2014).



A *Cladosporium* species was cultured in 8 sample plates from the subtidal site. There was no *Cladosporium* growth from intertidal samples. In culture, this fungus was dark olive green at the center with a wrinkled surface and white edge (Fig. 4). The reverse color is a greenish-black. Microscopy revealed septate hyphae with darkened conidiophores (asexual spore-bearing structures) (Fig. 4). Conidiophores grew directly off the hyphae. Conidia (asexual spores) were obovoid (egg-shaped) to ellipsoid with raised, darkened scars (hila) where they broke from the conidiophore. Conidia appeared green and often formed chains that readily disarticulate. In these chains, the “youngest” conidia, or the one that was directly attached to the conidiophore, was

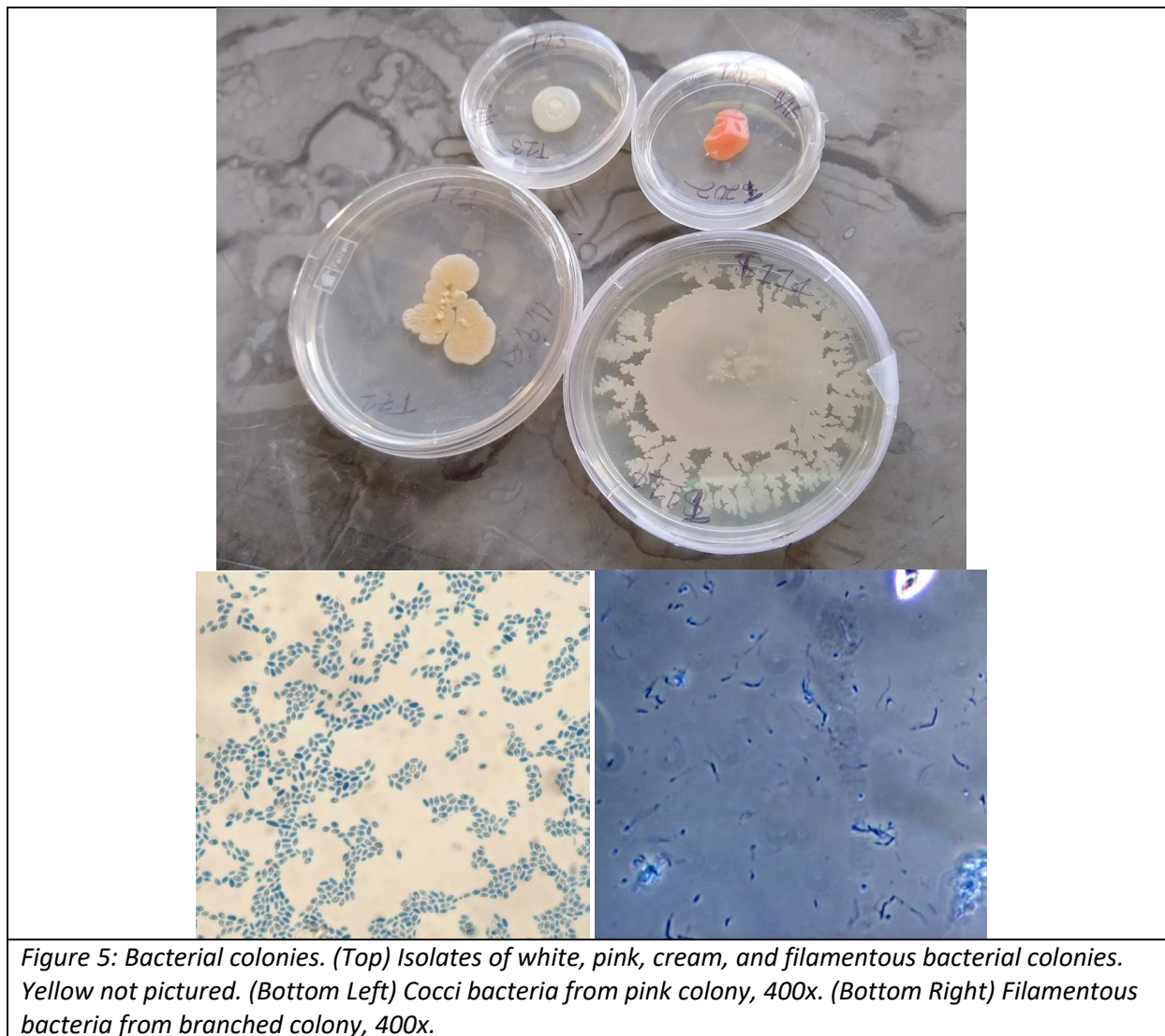
often shield-shaped. Conidia chains, darkened hila, and the shield-shaped conidia are distinctive of this genus (University of Adelaide 2021, Bensch 2012).



Mucoid growths were common in most sample plates, indicating presence of bacteria (Shi 2019) (Fig. 5). These mucoid growths are assumed to be endophytic bacteria, as they appeared to be growing out of cut leaf tissue. Some of these bacterial growths co-occurred with fungi in the same leaf cutting. Bacterial growths in culture were distinguished by color, including white, cream, yellow, and pink. Microscopy of pink and cream-colored colonies revealed ellipsoid cells suggestive of cocci bacteria (Kaiser 2021)(Fig. 5). When isolated in new cultures,

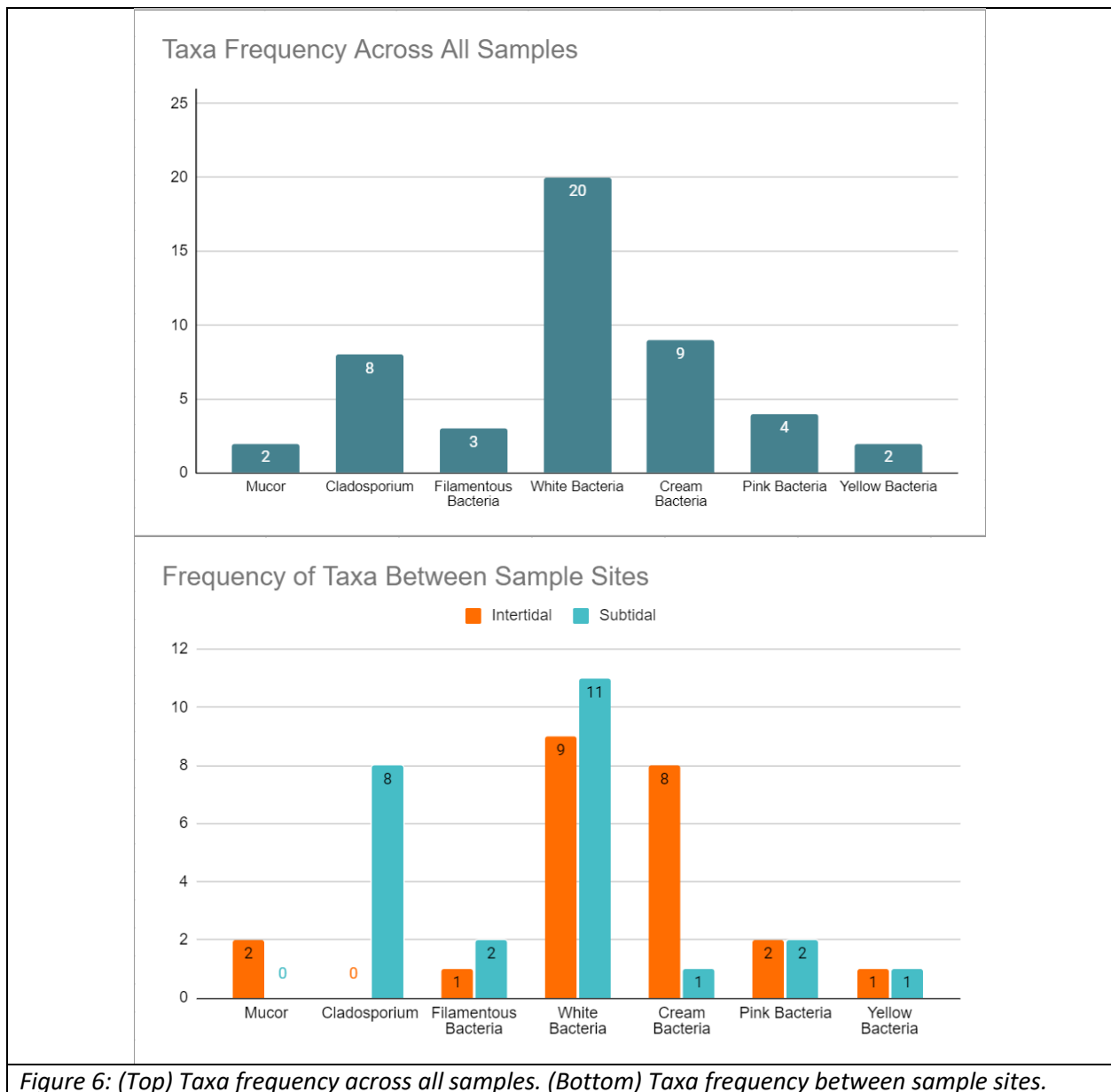
bacteria exhibited limited spread across the media surface compared to fungi and rarely covered the whole plate. Many colonies developed a dry, wrinkled surface film after several days. White mucoid growths were the most common growth across all 26 leaf samples and the most common growth in both sample sites. There were 8 cream mucoid growths in the intertidal site but only 1 in the subtidal site.

An unidentified culture grew in 3 sample plates, with one from the intertidal site and 2 from the subtidal site. This growth was colorless and had a distinctive branching macroscopic growth pattern. Under the dissecting microscope, the cultures did appear fungus-like when young. However, structures were not well visualized under the compounding microscope even at 400x magnification. The fungal cultures were easily visualized at 200x. Structures present appeared filamentous, but were too small to determine more detail (Fig. 5). Given the size of the structures, it is likely this was filamentous bacteria (Kaiser 2021).



Overall, white bacterial colonies were the most abundant isolate from all samples (Fig. 6). Cream bacterial colonies were the second most abundant isolate. The third most abundant isolate as well as the most abundant fungus was the *Cladosporium* species. *Mucor* fungus and yellow bacterial colonies were tied as the least abundant isolates from all samples.

There were some notable findings between intertidal and subtidal sample sites (Fig. 6). *Mucor* was only isolated from the intertidal patch while *Cladosporium* was only isolated from the subtidal patch. Cream bacterial colonies were far more abundant in the intertidal patch than subtidal. White bacterial colonies were highly abundant in both intertidal and subtidal *Z. marina* samples.



Discussion

In this study of False Bay eelgrass, two fungal taxa were isolated and five distinct bacterial colonies were isolated. It was assumed for this study that cultured taxa were endophytes of the eelgrass leaves. However, since most sample leaves also had epiphytic algae, it is possible that some taxa may be symbionts of the algae.

Identification of fungi by morphology alone proved very difficult, even with multiple visual resources. The body of evidence of endophytes specific to *Z. marina* is still small. Locating asexual spore-bearing structures on slides amongst the spores and hyphae was difficult. In using wet mount slides, it was also difficult to get clear focus on structures as structures were located on different planes within the stain on the slide. Furthermore, there are a number of limitations to identifying fungi morphologically. Some groups of fungi may appear morphologically identical but differ genetically (Senanayake 2020). An individual fungus species can also alter its morphology in response to environmental conditions, including media (Senanayake 2020). Some fungi will grow in culture without sporulating. Spores are a key feature of identification. Also, some fungi simply cannot be cultured (Raghukumar 2017). Genetic analysis would be ideal for future studies to confirm identifications.

The Mucoromycota phylum, commonly called pin molds, are identified by presence of their asexual reproductive structures called sporangia (Sutton 2014, Raghukumar 2017, University of Adelaide 2021). *Mucor* spp. are common food spoilage fungi (Sutton 2014). They are commonly present in soil, on dung, and in cut hay (Sutton 2014). While it is unclear how hosting *Mucor* may affect *Z. marina*, there have been several studies on terrestrial *Mucor* endophytes. Various *Mucor* endophyte species have been found to tolerate heavy metal-contaminated soils (Deng 2011, Domka 2019), to remove metals such as cadmium, lead, copper, and zinc from growth media (Deng 2011, Zahoor 2017), and to accelerate the growth of host plants under toxic metal

stress conditions (Domka 2019). It can also make phosphate soluble, potentially fertilizing plants in toxic conditions (Zahoor 2017). Further study is needed to assess whether *Mucor* endophytes provide resilience to *Z. marina* in heavy metal toxic conditions.

Ascomycota is a large phylum of fungi distinguished by sexual spores contained within a sac-like ascus (ascospores) (Raghukumar 2017). However, there are also several Ascomycota that have lost their ability to reproduce sexually called anamorphs (Kohlmeyer 1971). *Cladosporium* are anamorphs (Bensch 2012). Ascomycota are the most common plant foliar endophytes found (Arnold 2007). The *Cladosporium* genus are habitat generalists and are common air molds (University of Adelaide 2021). *Cladosporium* spp. are the most commonly cultured fungal endophytes from *Zostera marina* (Ettinger 2021, Shoemaker 2010). Some species of *Cladosporium* endophytes produce metabolites that inhibit seed germination of non-host plants. For instance, one species extracted from multiple common crop plants such as *Helianthus annuus* sunflower and *Cucumis sativus* cucumber were found to inhibit the germination of lettuce seeds (Waqas 2013). *Cladosporium sphaerospermum*, a species isolated from air as well as humans and plants, has been found to make products that promote growth of plant shoot length (Hamayun 2009).

Bacterial endophytes were more common than fungi in the leaf samples. As previously mentioned, bacteria in marine environments act similarly to fungi as saprobes and symbionts (Raghukumar 2017). Bacterial endophytes in *Z. marina* are thought to facilitate nitrogen and sulfur cycling for their hosts (Ettinger 2021).

Future Directions

In subtidal site samples, all *Cladosporium* growth co-occurred with white mucoid growths. Larger sample sizes would be needed to test if there is a correlation between these. White bacterial colonies were ubiquitous throughout both sample sites so this is possibly incidental.

Only one media, PDA, was used for this study. It is possible that other media may exhibit different fungal endophytes in culture. Future studies may find different taxa by using multiple media types or experimenting with salinity levels in the agar mix, as different species of fungi can require different environmental conditions for culture growth (Keral 2019). Sampling from roots or rhizomes will likely yield a wider diversity of species (Shoemaker 2010, Ettinger 2021). A larger sample size in general may also find more species in the leaves, as previous studies found more taxa as sample size increased (Ettinger 2021).

Future studies could also explore possible benefits or fitness costs of fungal infection to *Zostera marina*. As these interactions are generally species and location specific, multiple studies would be needed to assess how *Z. marina* interacts with *Mucor* and *Cladosporium* specifically in False Bay Biological Preserve.

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