Labyrinthula spp. Association with Eelgrass (*Zostera marina*) Population in Morro Bay Estuary, Morro Bay, California

Tiffany X. Ren, Jessica Ormiston, Silvio Favoreto, and Laurie McConnico

Cuesta College Biological Sciences Division, San Luis Obispo, CA, USA

Abstract

Labyrinthula is linked to wasting disease and was associated with pandemic eelgrass declines in the 1930's and 1980's. Necrotic leaf tissue indicative of wasting disease is present throughout the Morro Bay Estuary, CA and the local eelgrass population (*Zostera marina*) has declined by 97%. Field and lab work (June 2018/2019) were done to determine the slime mold's presence in Morro Bay and to evaluate if it acts as a primary pathogen or opportunist. We collected "healthy" (green) and "necrotic" (green with black/ brown lesions) blades from the fore, mid and back bay (n=10 each site/blade type). In the lab, half of each blade was cultured on SSW media to detect *Labyrinthula* spp., and the other dried for DNA extraction and qPCR to quantify *Labyrinthula* spp. Excess Green Index (EGI) image analysis was used as a proxy for plant health. Both "healthy" and "necrotic" blades were positive for *Labyrinthula* spp. in culture at all sites. qPCR results (2018 samples) also confirmed *Labyrinthula* spp. on both blade types at all sites, with the greatest concentration observed in the back bay. EGI analysis showed no consistent correlation between number of parasites and severity of lesions. The ubiquitous distribution and EGI results suggest that the *Labyrinthula* spp. in Morro Bay Estuary may not be a primary pathogen, but instead is an opportunist. Ongoing investigation will allow for an assessment of the slime mold's persistence and infection patterns in the Estuary. This work pioneers the research of *Labyrinthula* spp. in Morro Bay Estuary.

Introduction

Estuaries are among the world's most productive and delicate ecosystems, susceptible to wide range of environmental changes. In California, Morro Bay is one of the state's major estuaries, and the system contributes significantly to the local economy through aquaculture, fisheries, tourism, and other services. Morro Bay has historically been dominated by eelgrass (*Zostera marina*). Eelgrass meadows provide essential fish habitat, serve as nursery grounds and offer refugia for migratory species. However, between 2007-2015, eelgrass declined 97% in Morro Bay, from 344 acres to 13 acres [Fig. 1; Morro Bay National Estuary Program (MBNEP) 2015]. For the past five years, eelgrass acreage has remained at ~10 acres with little evidence of recovery. Causes of the eelgrass decline in Morro Bay are unknown.

Eelgrass wasting disease is linked to a protist slime mold, *Labyrinthula* spp. (Fig. 2). *Labyrinthula* caused a pandemic decline (>90% loss) in eelgrass in Europe and North America in the 1930's. *Labyrinthula* mainly occur within leaf parenchyma cells, where they damage chloroplasts leading to reduced photosynthetic activity, discoloration and development of expanding brown or black necrotic spots finally resulting in leaf loss (see Fig. 3)

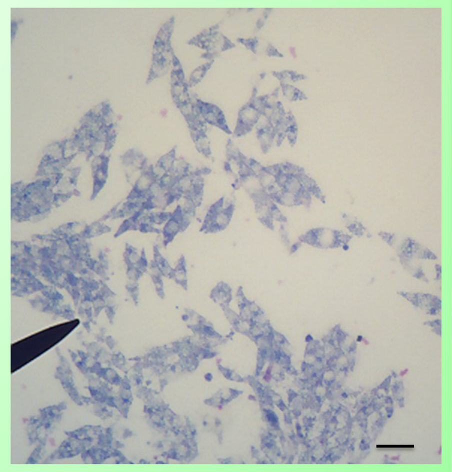

Figure 2: *Labyrinthula* spp. from eelgrass blades, cultured on SSW agar and stained with Giemsa.

Research Goal

In Morro Bay, necrotic leaf tissue is present throughout the bay, and appears more prevalent in the back bay where eelgrass is now extremely sparse. Thus, it is critical to determine if the pathogen *Labyrinthula* is present in Morro Bay, whether the necrotic tissue is associated with the presence of *Labyrinthula* and whether there is a higher prevalence of the pathogen in areas with less favorable physical conditions (the back bay with higher salinity, higher temperature, and lower light).

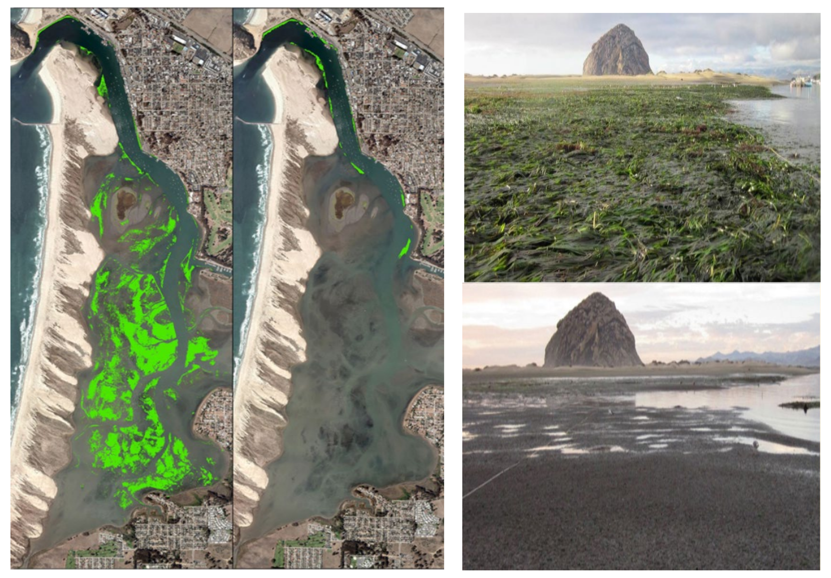

Figure 1: (a) Aerial maps highlighting the large extent of eelgrass declines in Morro Bay from 2007 (left) to 2015 (right). (b) Field photographs illustrating the change in eelgrass abundance at one location in Morro Bay between 2010 (top) and 2012 (bottom).

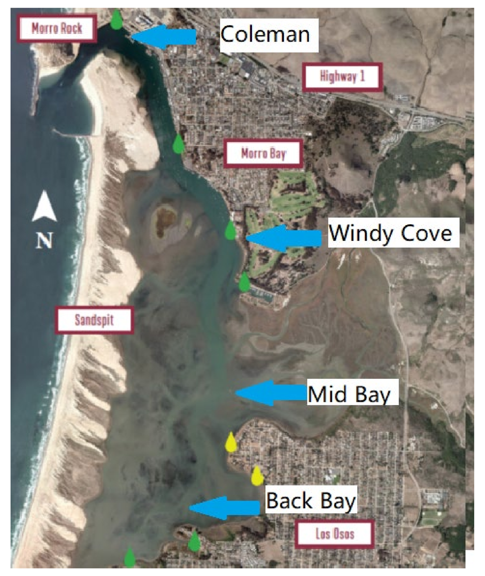

Figure 3: Map of Morro Bay Estuary, Morro Bay, CA indicating four sampling sites used for eelgrass collection (Coleman, Windy Cove, Mid bay and Back Bay).

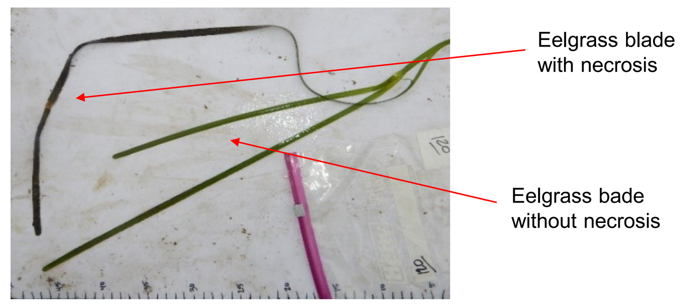

Figure 4: Green and necrotic eelgrass blades observed against white board for visual inspection at time of collection.

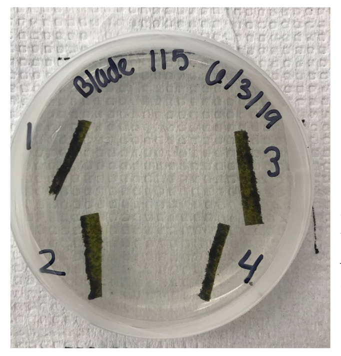

Figure 5: SSW media plate prepared for *Labyrinthula* culture with bisected eelgrass fragments.

Methods

We collected 80 blades of eelgrass from four sites in Morro Bay Estuary: Coleman, Windy Cove, Mid Bay and Back Bay (Fig. 3; MBNEP 2017). The criteria for the collection were: 1. Exterior (oldest) blades 2. Blade tip preserved (rounded at apex) 3. Under visual inspection, ten blades/site had no signs of necrosis and ten had clear signs of necrotic tissue (Fig. 4). We cut the blades longitudinally in 2 halves, one half for culture and another for DNA analysis. Blade sections used for culture were cut transversally in 2cm fragments and seeded in SSW media (Fig. 5). Plates were inspected daily for ten days, for signs of *Labyrinthula* growth. The blade section used for DNA isolation was dehydrated and processed using the QIAamp DNA Mini Kit (Qiagen) following the manufacturer's instructions. We used Nanovue (G&E) for DNA quantification. The pre-amplification reaction started with 100ng of total DNA, 500 nmol of flanking primers (R/F) for 16 cycles using GE HealthCare Illustra PCR tubes. The amplified samples were diluted (1:60) and used for quantification of *Labyrinthula* ribosomal DNA gene copies using qPCR. Reactions were carried out with 500 nmol of primers (R/F) and 250 nmol of FAM labeled probe. The number of parasites was calculated by comparison with a standard curve created with a known number of cultured *Labyrinthula.*

We used the Woebbecke's Excess Green Index (EGI) as a surrogate measure of photosynthetic potential and overall blade health. We photographed all culture plates under controlled light conditions. Areas of interest (blade fragments) were selected from each image and RGB channel analysis was carried out using ImageJ software (NIH). Excess Green Index was calculated using the formula $ExG = 2g - r - b$. We also used the area measurement function of ImageJ to calculate percentage of abnormal tissue in each sample.

Conclusion

Preliminary data analysis suggests that *Labyrinthula* has an opportunistic behavior pattern in association with eelgrass in the Morro Bay Estuary. Our data shows the ubiquitous distribution of the slime mold in the estuary. We found *Labyrinthula* in healthy blades, as well as blades with necrotic tissue at all study sites. The Excess Green Index (EGI) showed no correlation with the number of parasites in sites where the eelgrass population was considered healthy. However, in sites where eelgrass is subjected to desiccation stress and increased tidal variation, we observed a trend of increased necrosis (low EGI) and higher number of parasites. The ongoing investigation of abiotic stressors for eelgrass in Morro Bay Estuary will help to clarify the complex ecological association between these two species.

Acknowledgements

We thank the Cuesta College Applied and Environmental Microbiology 210M students from 2018/2019 for their assistance in the field and in the laboratory. We also thank Cuesta College for the use of facilities and our funding sources: Morro Bay National Estuary Program, National Science Foundation and Cuesta College Foundation.

Results

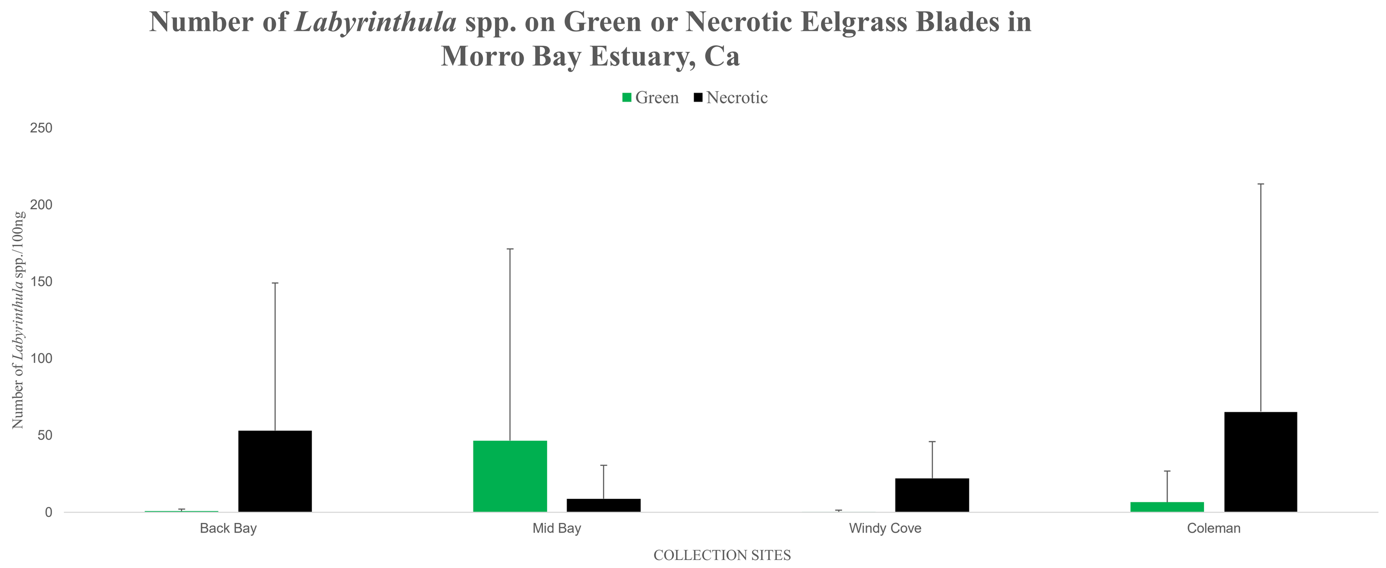

Figure 6: Average number (+SD) of *Labyrinthula* spp. per 100 ng of total DNA from green and necrotic eelgrass samples collected from Morro Bay Estuary. Samples were pre-amplified by PCR and detection of *Labyrinthula* performed by qPCR. Two outlier samples (1285 and 3777 *Labyrinthula*/100ng of DNA) were removed from the Coleman Green group; these samples hosted a greater number of *Labyrinthula* spp.. While higher average parasite concentration was observed on necrotic samples from Back Bay, Windy Cove and Coleman, the distribution of *Labyrinthula* spp. was ubiquitous in the estuary.

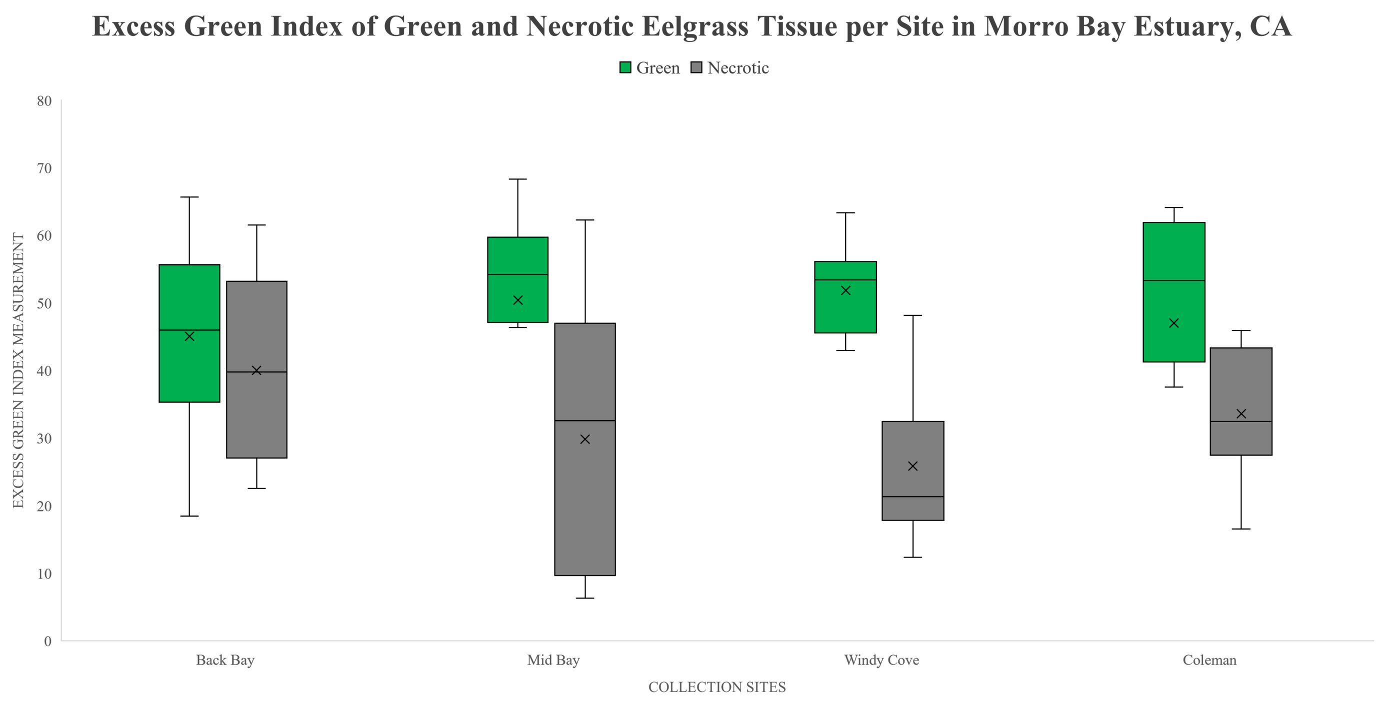

Figure 7: Excess Green Index (EGI) for healthy (green) and necrotic (gray) blades at four collection sites. Box plots show Median (line) and Mean (X) values, quartiles and maximum/minimum values (whiskers). Healthy blades (green) from the Back Bay had a slightly lower average EGI than healthy blades (green) from other sites. The overlap in average EGI for healthy (green) and necrotic blades from Back Bay also suggests that both groups of plants sampled from this site were less healthy compared to other sites sampled.

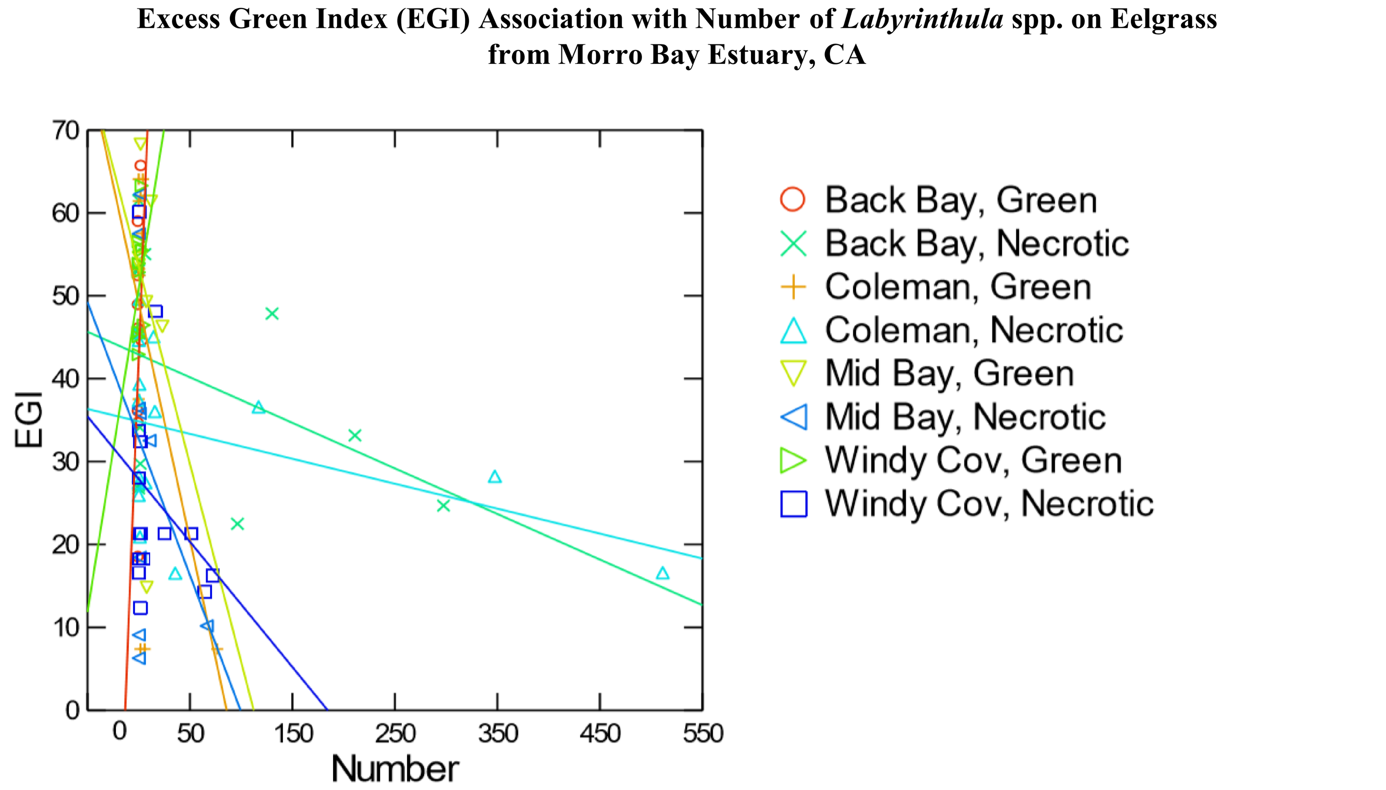

Figure 8: EGI (Excess Green Index) vs. number of *Labyrinthula* spp.. Linear regressions are displayed based on the collection site and blade type. A clear negative correlation between EGI and # of *Labyrinthula* was observed on necrotic samples from Back Bay and Coleman. The other sites/groups showed no distinct positive or negative correlation. We did not perform statistical analysis due to the limited data set.