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# Standard ecological and molecular research methods and techniques for *Labyrinthula* spp.

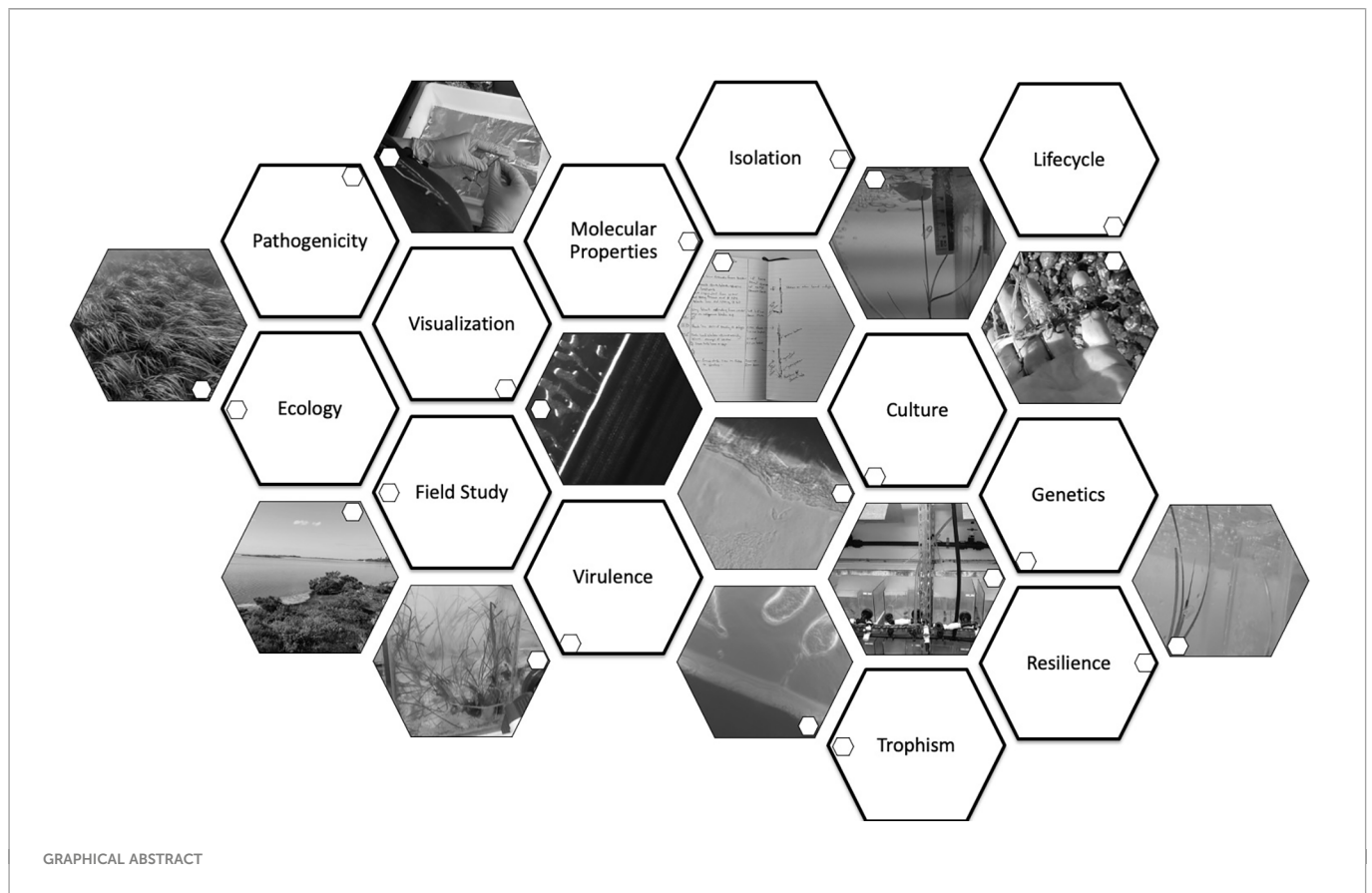
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*Labyrinthula* are unicellular protists occupying diverse spatial and functional niches, including various roles in host and ecological function, fatty acid production, pandemic marine disease and saprobic decomposition. *Labyrinthula* species span tropical and temperate climates and have been isolated from each marine coastal ecosystem tested. Our understanding of primary cellular and molecular functions of *Labyrinthula* has substantially progressed through a combination of increased global investments, research interest and technological advances. Recent advances in molecular techniques provide a toolkit for advancing ecological questions in marine infectious disease in seagrass meadows around the world. Here we provide a comprehensive review of relevant ecological and molecular techniques used in long-term research and the progression of *Labyrinthula* scholarship. Our aims in preparing this review are to: 1) share, compare and advance global *Labyrinthula* protocols, 2) increase accessibility to robust methodology to encourage the uptake of *Labyrinthula*-based questions into marine studies of molecular and ecological qualities of *Labyrinthula* and 3) encourage uptake of robust *Labyrinthula*-based questions into coastal marine studies, while also encouraging international collaborative networks across multiple fields. Lastly, we discuss gaps in the over 100 years of *Labyrinthula* research and opportunities for expanding research on this model marine organism.

## KEYWORDS

molecular protocols, bioassay, pathogenicity, *Labyrinthula* sp, seagrass, disease ecology, pathosystems, sequencing



## 1 Introduction to *Labyrinthula* sp.

Labyrinthulea are a class of heterotrophic protists containing two orders of Bigyra (Labyrinthulida and Thraustochytrida) known for their unique ability to produce exogenous sagenetosomal networks (also known as ‘slime nets’ or extracellular network) and biflagellated zoospores. Labyrinthulida are classified as Chromista and are common saprobic decomposers, parasites and pathogens of other organisms (Cavalier-Smith and Chao, 2006). They can be found in terrestrial and marine habitats, where they have been sampled from every ocean on the world, including temperate, tropical and polar regions (Raghukumar and Damare, 2011). Labyrinthulida comprises two families, Labyrinthulaceae and Aplanochytridiaceae, with the former containing the sole genus *Labyrinthula* (Anderson and Cavalier-Smith, 2012; Beakes et al., 2014; Pan et al., 2017).

*Labyrinthula* is a genus in Labyrinthulaceae described by unique ‘spindle-shaped’, ‘fungal-like’ qualities and production of distinctive colonies of transparent net-plasmodium (Pokorny, 1967; Porter, 1969). *Labyrinthula* spp. demonstrate considerable tolerance across a wide range of ecological conditions, such as variable temperature, salinity, light and nutritional resources (Vishniac, 1955; Sykes and Porter, 1973; Vergeer et al., 1995). *Labyrinthula* are also able to swiftly and efficiently transfer between a variety of biological hosts and sediment substrates (Muehlstein, 1992) and their potential roles in global disease phenomena are well documented, including observations of epidemic events in seagrass and turfgrass systems (Sullivan et al., 2013; Sullivan et al., 2018).

*Labyrinthula* was originally described from a marine alga (Cienkowski, 1867), and subsequently has been found in association with all three major algal groups, including one freshwater alga (Zopf, 1892), and even blue-green algae. In many of these observations *Labyrinthula* was considered to be pathogenic (Raghukumar 1987; Raghukumar, 1986) yet, to our knowledge *Labyrinthula* are not etiological agents of mass die-offs in marine algae. Rapid Blight (RB) however afflicts a variety of turfgrasses and is a lesser-known and reviewed pathosystem than *Labyrinthula*-driven Seagrass Wasting Disease (SWD). It was first noted in 1995, in the U.S. (Entwistle et al., 2014), with *Labyrinthula terrestris* later confirmed as the disease agent (Olsen et al., 2003; Bigelow et al., 2005). It is predominately associated with elevated salinity attributed to irrigation water (Camberato et al., 2006; Kerrigan et al., 2012) and considered an emergent disease driven by human activity (Douhan et al., 2009; Entwistle et al., 2014). Given the genetic distinctness observed between European and U.S. *Labyrinthula terrestris*, and the genetic diversity likely still hidden within the genus (Douhan et al., 2009; Chitrampalam et al., 2015; Martin et al., 2016; Lohan et al., 2020), exploring the wild host-origin(s) of the species affecting turfgrasses would be valuable (Douhan et al., 2009).

Tolerance to wide ranging ecological conditions and discoveries in advanced pathology support recognition of *Labyrinthula* as a model organism for studying infectious disease in halophytes (broadly defined here as salt-tolerant plants) across marine and terrestrial systems (Cienkowski, 1867; Watson and Raper, 1957; Pokorny, 1967; Dick, 2001; Olsen et al., 2003; Martin et al., 2016;

Popova et al., 2020). *Labyrinthula* also plays an important and lesser-known role as a saprobic decomposer and remineralizer in coastal marine ecosystems, where researchers have discovered potential ecological value derived from its position in the food web, where it decomposes organic carbon and produces the nutritious fatty acid, DHA (Kumon et al., 2003; Armenta and Valentine, 2013; Yoshioka et al., 2019).

Difficulties in detecting and culturing *Labyrinthula* and a paucity of global monitoring protocols for SWD creates a steep learning curve for examination of emerging seagrass disease events when they do occur, and the lack of local skills may hinder timely detection of seagrass die-offs following regional and localized outbreaks of SWD. Increasing anthropogenic pressure on marine coastal ecosystems, including global warming, has created an increased risk of introduction or migration by non-native biota, such as pathogenic microbes (Harvell et al., 1999; Lafferty et al., 2004; Lafferty, 2009). It is prudent to inventory, review and share current methodologies in *Labyrinthula* and SWD research to highlight key research gaps that may bottleneck progress of evaluations and timely response to increasing instance of localized epidemics and loss events. Our review and syntheses provide a globally relevant summary of methodological research in support of advancement in examinations of *Labyrinthula* impacting conservation biology and ecology.

## 2 Ecological investigations

Despite their ecological value and ubiquity in the environment, co-infections and phenotypic variability can make *Labyrinthula* species challenging to identify and evaluate in the field. As genetic signatures and ecological datasets grow (Collado-Mercado et al., 2010; Martin et al., 2016; Lohan et al., 2020) combined molecular and ecological approaches will enhance our understanding of both the biology and ecology of cultured, as-yet uncultured, or unculturable, genotypes of *Labyrinthula*, whose hidden diversity has been predicted to be significant (Adl et al., 2012). Much remains to be discovered regarding the broader ecological characteristics of ‘elusive’ protists like *Labyrinthula*, including their role in the food web, connections to marine disease ecology, pathogen transmission, distribution patterns and diversity in seagrass and other marine ecosystems. Identification of pathways for maintaining resilient seagrass and macroalgal ecosystems are critically needed to protect and restore global habitats, especially those recognized and conserved as high-value resources in marine areas.

### 2.1 Microbiome

The role of *Labyrinthula* in the seagrass and macroalgae microbiome and biofilm production is gaining attention (Hurtado-McCormick et al., 2020; Popova et al., 2020; Trevathan-Tackett et al., 2020; Wang et al., 2020; Beatty et al., 2022). The larger role of *Labyrinthula* in microbial ecology, including pathology of other organisms (O’Kelly, 2005) and trophic food webs is largely unknown. However, microbial drivers of seagrass health have been discovered to increase host resilience (Martin et al., 2019), suggesting

that, similar to marine corals (Rosado et al., 2019), development of a pro-biotic treatment may provide some support for seagrass or other populations facing exacerbating disease. Relatedly, host microbiome changes were associated with prevalence of SWD in eelgrass, with the potential of some enriched taxa (e.g. Cellvibrionaceae, Colwelliaceae, and Granulosicoccaceae) to exacerbate disease (Beatty et al., 2022).

### 2.2 Signs of disease

SWD appears in infected seagrass plants as small dark patches of black or brown spots or streaks, typically in leaf tissues. Within days, the streaks expand and coalesce, causing rapid loss of photosynthetic ability and buoyancy in leaves. The ‘stains’ symptomatic of SWD may eventually take over the entire leaf, and in the process, cells may be transferred in the water column or by leaf-to-leaf contact. RB in turfgrasses progresses in a very similar fashion to SWD, with water-logged grass tissues becoming discolored (often chlorotic to near transparent) and eventually dying (Kopeck et al., 2004). These patches may eventually join to form larger areas of collapsed turf (Olsen et al., 2003). Extended periods of time between large-scale disease events has hindered long-term data collection.

### 2.3 Pathosystems

Interactions among hosts, pathogens and their environment are known to regulate disease in plants, including seagrasses (Sullivan et al., 2013). SWD is sometimes described as a syndrome, rather than a specific ‘disease’ as there are often co-factors implicated in disease progression (Porter, 1986; Brakel et al., 2019; Duffin et al., 2020). The pathosystems model recognizes the complex and intersectional roles of multiple environmental parameters and thresholds in determining the health of both pathogen and host, including their co-interactions. The core relationships between age, salinity, temperature, light and associated host-pathogen (*Seagrass species–Labyrinthula species*) relationships have well-demonstrated impacts on occurrences and severity of disease (Groner et al., 2014; Groner et al., 2016; Dawkins et al., 2018).

### 2.4 Transmission

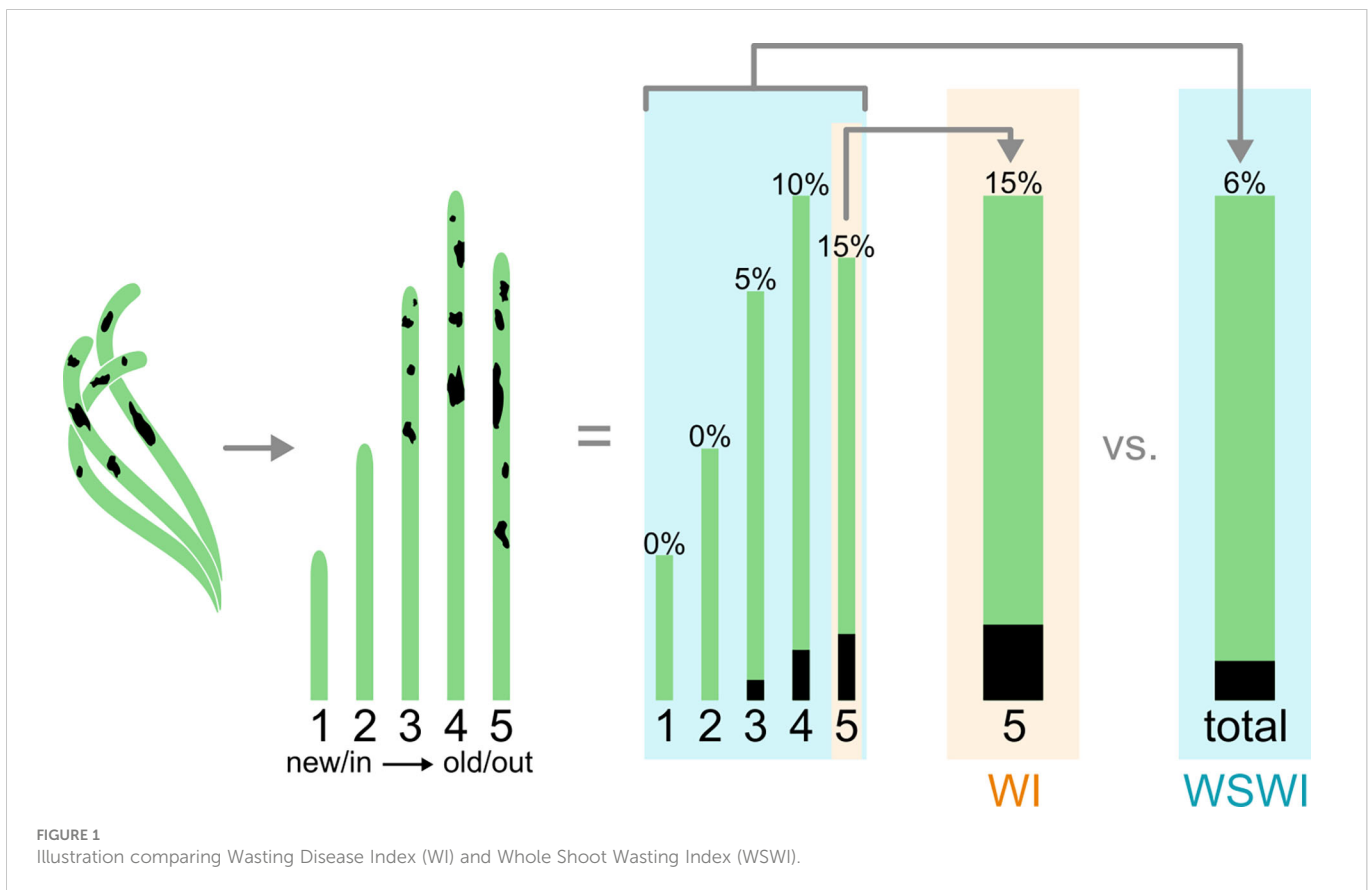
*Labyrinthula* are ubiquitous in plant, water and sediment samples from a variety of saline terrestrial and marine environments, including halophytes in salt lakes (Amon, 1978), golf courses (Olsen et al., 2003), diatoms, algae (Jepps, 1931) and seagrass around the world (Vergeer and den Hartog, 1994). Inhabiting a range of halophytes, and even amoeba (Dyková et al., 2008), *Labyrinthula* have been identified from all nearshore marine environments of the world it has been sampled (Vergeer and den Hartog, 1994; Raghukumar and Damare, 2011). Evidence of both leaf-to-leaf and water borne disease transmission have been demonstrated (Muehlstein et al., 1988; Martin et al., 2016). Research initially focused on leaf-to-leaf transfer as a primary mode of transmission, thus pathogenicity studies were initiated using replicate inoculated leaf tissue vectors aimed at mimicking natural direct contact conditions

(Muehlstein et al., 1988). Newly developed metabarcoding and molecular investigation techniques targeting *Labyrinthula* spp. indicate alternate transmission pathways in life-cycle and various microhabitats, including sediment and water columns (Lohan et al., 2020), and ballast water samples (Galil and Hülsmann, 1997; Lohan et al., 2016). Variability in transmission mode and potential vectors indicate unknown modes of transmission might affect *Labyrinthula* pathosystems, especially in the wild. Pathways for transmissions are an active area of research in *Labyrinthula*.

### 2.5 Wasting disease index

Leaf-based analyses of lesion size and disease severity metrics can be visually estimated using the Wasting Disease Index (WI) method (Burdick et al., 1993). The WI reveals characteristics about the most severe infections of host tissue and may be valuable at a molecular or physiological level. This metric may be used to model a worst-case epidemic infection. Still, a high WI does not say anything about how the infection is affecting the rest of the shoot, since most other leaves can be healthy even if the WI is high on a given leaf. On the other hand, the Whole Shoot Wasting Index (WSWI) determines to what extent the infection affects the whole plant, which can be averaged over a sample population to understand how the infection is affecting population dynamics and to understand resilience (Figure 1). Burdick et al. (1993) argue that WI is a better measurement of infection than WSWI since the range of disease values is larger, making it easier to monitor variation in disease over time. A major limitation with the

WI more broadly is that it only measures the visual outcome of the infection. It does not reveal anything about the abundance of *Labyrinthula* cells or physiological responses to infection on the leaf, shoots, or below-ground energy reserves. More recently, the WI has been coupled with histology (Groner et al., 2014) and quantitative PCR methods (Bergmann et al., 2011; Duffin et al., 2020) to provide greater resolution for measures of density and abundance of *Labyrinthula* associated with a specific leaf or plant. For example, researchers found *Labyrinthula* load and areal lesion extent in turtlegrass exhibit a logistic relationship, suggesting pathogen load does not scale linearly across lesion sizes (Duffin et al., 2020). Investigations of cell counts reveal threshold levels of infection may exist before a leaf shows any symptoms and that cell counts may experience a dramatic decline in abundance when they are associated with larger lesion areas (> ~1/3 of leaf area). The relative presence of *Labyrinthula* may be further moderated through host immunity, which can be spatially and temporally variable (Bockelmann et al., 2013; Duffin et al., 2020). An additional constraint with WI is that black lesions can be a result of necrosis by other reasons besides *Labyrinthula* infection. Depending on the plant species and health of the plant it can be difficult to distinguish between lesions, heat stress and hydrogen sulfide toxicity. It is therefore difficult to be 100% sure lesion measurements give a fair picture of the infection severity. By including a second measuring technique, e.g., qPCR, plants with necrosis can be examined for the presence and abundance of the pathogen, hence corroborating the WI findings. Thus, it is important for researchers to distinguish between the different indexing methods and in either case use a quantitative





cell measurement when investigating *Labyrinthula* infection. Improved measurement techniques have included taking photographs of each leaf used in WI and WSWI and assessing visual characteristics with image software capable of analyses, such as ImageJ (Trevathan-Tackett et al., 2018a) and emerging ‘artificial intelligence’ tools, such as EeLISA (Rappazzo et al., 2021; Aoki et al., 2022).

## 2.6 Collection guidance

Place-based, or ‘field’ sampling has often been the first step in initiating *Labyrinthula* collections and investigations worldwide. Responsible scientific investigations (Minteer and Collins, 2008) in *Labyrinthula*, wasting disease and other related research may include obtaining legal permits and conducting sampling practices that encourage the recognition and conservation of natural processes, and local communities. This is especially true on long-term monitoring sites; while minimizing harm to vulnerable plant roots and sediments, we can honor and respect local traditional ecological knowledge, people and society (Pierotti and Wildcat, 2000). Practically, when sampling eelgrass for *Labyrinthula*, take care to avoid sampling the outer-most leaves as they are older and could be senescing or turning colors for any number of reasons. Older leaves are further likely to have higher densities of fungi and other microbial contaminants as they naturally acquire epiphytes and fouling organisms with age (Opsahl and Benner, 1993). Specific recommendations for collecting methods are provided in the Supplemental Manual.

## 2.7 Considerations for sampling design

Field collections targeting *Labyrinthula* in monocultural halophytes (such as seagrass and turf grass) generally involves cluster sampling or modified cluster sampling (Madden and Hughes, 1999; Garcias-Bonet et al., 2011). Sampling individuals and populations across variable spatial and temporal conditions can be performed through stratified or randomized grid, transect or other data point locations. The total number of samples required to test hypotheses may be quite variable based on the type of samples being collected, *Labyrinthula* lifecycle, or nuances of the specific questions being asked.

## 3 Laboratory protocols

Many techniques have been utilized to study the biology of *Labyrinthula* in the laboratory, including culture-based isolation, molecular and morphological characterization, pathogenicity assays, transmission dynamics, host immunity responses, fatty acid analyses, microscopy, and virulence. More recently, emerging research on host immunity (Duffin et al., 2020), discovery of *Labyrinthula*’s role in primary DHA production (Kumon et al., 2002; Yoshioka et al., 2019) and the recent sequencing of the first *Labyrinthula* genome (Tan

et al., 2021) have highlighted the importance and potential for advancement of discoveries related to this unique protist.

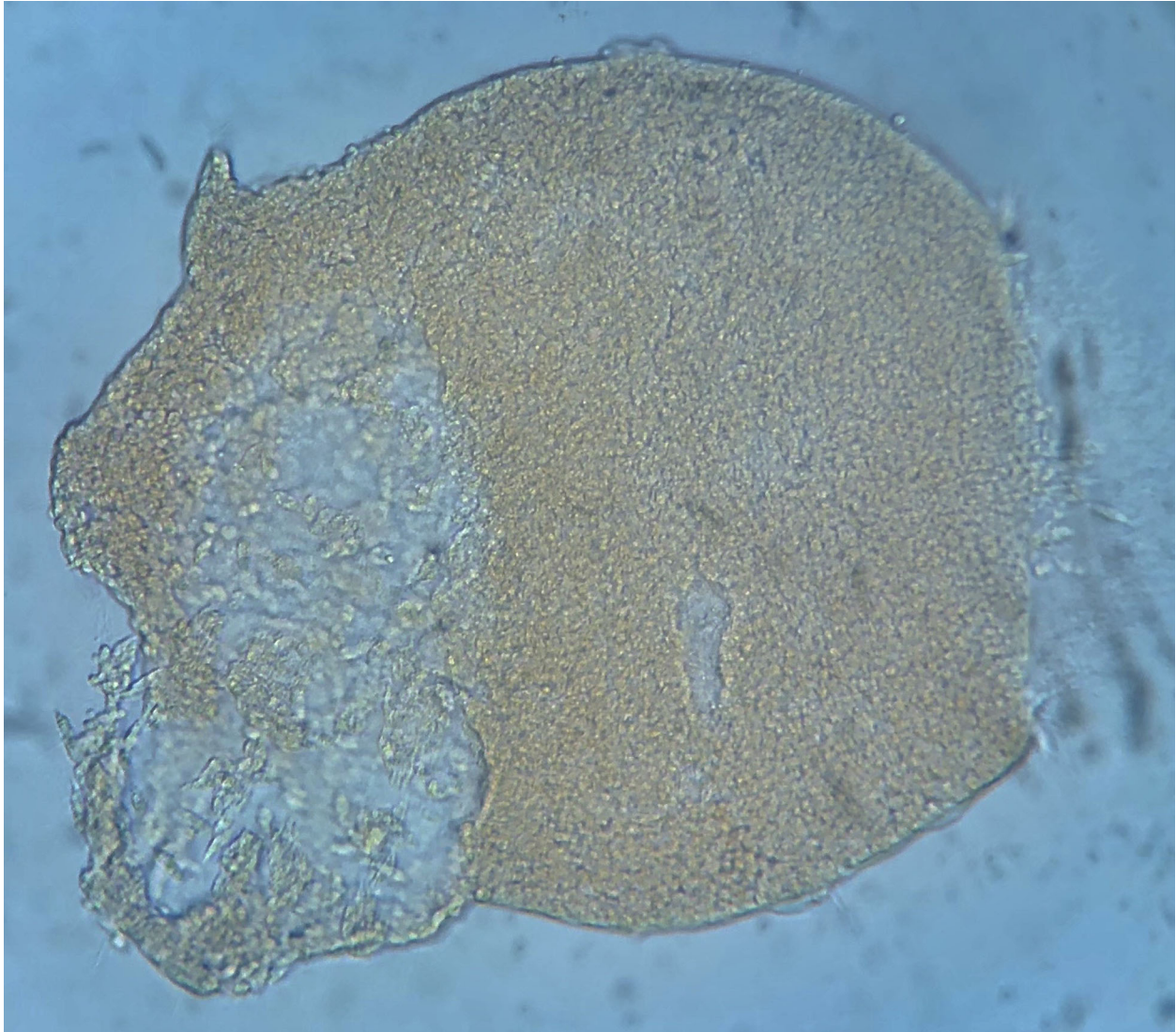
## 3.1 Species identification and classification

*Labyrinthula* spp. (Cienkowski) were discovered through early field sampling and laboratory culture of marine microbes, and are described in the literature as early as 1867 (Cienkowski, 1867). The family only consists of one genus and has been variably classified between fungi and slime molds since that time, though more recent evaluations have established their position among the Stramenopiles, adjacent to the oomycetes (Porter, 1990; Leander and Porter, 2001; Tsui et al., 2009; Leano and Damare, 2012; Beakes et al., 2014).

### 3.1.1 Morphology

*Labyrinthula* can be identified in culture through observation of distinctive fusiform cells embedded within hyaline ectoplasmic networks, or ‘slimeways’ (Porter, 1969). Individual *Labyrinthula* cells are colorless, though dense aggregations may have some yellowish/orangish coloring (Figure 2), measuring between 5  $\mu\text{m}$  – 30  $\mu\text{m}$  long by 3–8  $\mu\text{m}$  wide (Pokorny, 1967; Muehlstein et al., 1988; Leano and Damare, 2012). Each *Labyrinthula* cell contains a single large central nucleus, flanked by 2 large vacuoles where stores of lipid bodies are easily visible (Jepps, 1931; Young, 1943; Porter, 1969). *Labyrinthula* cells also contain specialized posteriorly oriented organelles, including dictyosomes and an endoplasmic reticulum (Porter, 1969).

Early in isolation, *Labyrinthula* cells appear enigmatic, as actin-myosin reactions in the ectoplasm generate distinctive gliding motion of cells within the colony. Active cells may travel up to 50  $\mu\text{m}$  per minute (Dietz and Schnetter, 1999; Preston and King, 2005). The plasmodium generating organelle allows the cells to develop sometimes extensive networks, which are used by the cells for both motility and osmotic feeding (Porter, 1969) by secreting exoenzymes for digestion during nutrient uptake (Tsui et al., 2009). The unique ectoplasmic networks generated by *Labyrinthula* grow in root-like, or ‘lacey’ patterns (Vergeer and den Hartog, 1994). They are most often observed following a period of intensive axenic culturing and growth in a laboratory setting. Cell colonies are readily identifiable through microscopy where they can be observed forming distinctive and robust fractal-like vegetative cell communities in culture (Figure 3) (Jepps, 1931; Young, 1943; Trevathan-Tackett et al., 2018a). In the literature, *Labyrinthula* colonies have been variably described by morphological qualities, however there is agreement in their tendency to branch out from single-point infections. Growth characteristics of the colony may be characterized by cellular alignments, density, agar penetration and cellular sizes (Sullivan et al., 2013; Sullivan et al., 2017; Trevathan-Tackett et al., 2018a). Finally, care should be taken to avoid confusing *Labyrinthula* with a closely-related and commonly co-occurring taxa, such as aplanochytrids, especially for smaller-bodied *Labyrinthula* spp. (Figure 2, Leander et al., 2004). With experience and under similar culture conditions, aplanochytrids can appear to have a more distinct nucleus, be more rounded, and have a more ‘dotted line’ like



**FIGURE 2**  
Image of large aggregate/sorus-like mounding of *Labyrinthula* sp. cells from liquid culture, isolated from diseased tissue of *Thalassia testudinum*. Yellowish or orangish color is common in such aggregates.

presentation when observing continuity among cells in the leading edge/arm of a colony on agar (Leander et al., 2004).

### 3.1.2 Reproduction

*Labyrinthula* cells reproduce both sexually and asexually (Figure 4). Vegetative cells multiply through binary fission or mitotic division of vegetative cells. This is considered to be the most commonly used strategy for replication and propagation of *Labyrinthula* cells in naturally occurring colonies (Pokorny, 1967). A cyst-like resting phase, possibly asexual, is also reported (Pokorny, 1967; Amon, 1978). Sexual modes of reproduction have been described through accounts of aggregating plasmodia (Pokorny, 1967), sporulating cells, and zoospore recovery (Amon and Perkins, 1968; Perkins and Amon, 1969). Several accounts of cells restructuring in culture to form dense aggregations of cells with nucleated walls are described as 'sori' (Pokorny, 1967). The 'spores' aggregating inside the 'sori' are extremely difficult to visualize and

often usual staining methods are not effective (Jepps, 1931). However, bi-flagellated zoospores were isolated from a sorus and photographed using a scanning electron microscope (Amon and Perkins, 1968). Imaging and assessment of chemical signaling and quorum-sensing behaviors has been described for other organisms, such as bacteria (De Kievit and Iglewski, 2000), but is generally lacking for stramenopiles (Hassani et al., 2018). The role of chemotaxis in *Labyrinthula* life cycle and behavior are unknown.

### 3.1.3 Systematics

The first attempt to morphologically and taxonomically describe separate *Labyrinthula* (Cienkowski) species evaluated and summarized cell structure, colony morphology, physiology, ecology and pathogenic considerations, for 10 species (*L. macrocystis*, *L. minuta*, *L. vitellina* var. *vitellina*, *L. vitellina* var. *pacifica*, *L. cienkowski*, *L. zopfii*, *L. valkonovii*, *L. chattonii*, *L. algeriensis*, *L. roscoffensis*, *L. coenosystis*) of known isolates (Pokorny, 1967). A review of taxonomy reveals early nomenclature was



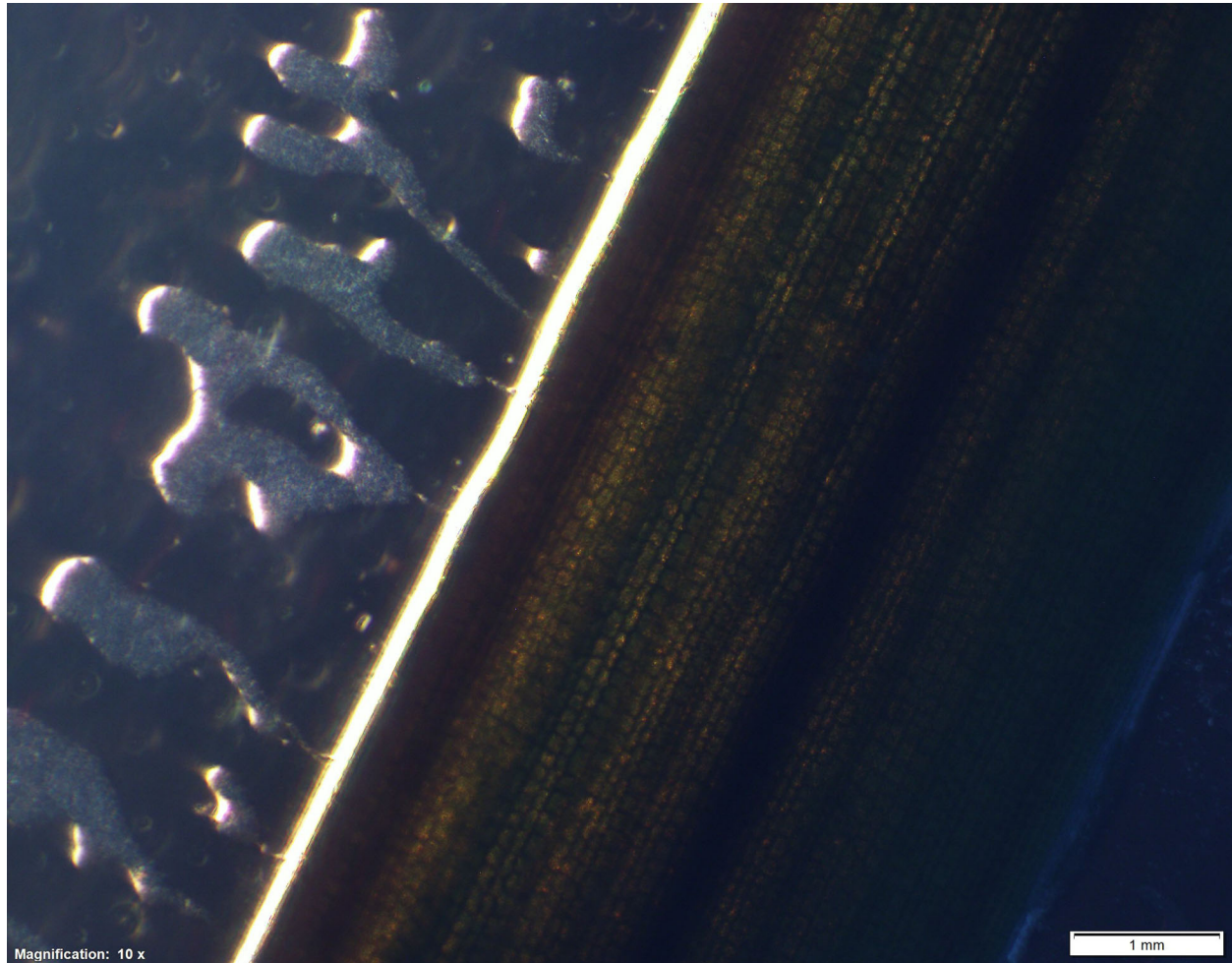


FIGURE 3  
Labyrinthula colonies exuding from *Zostera* leaf margin.

abandoned when two epithets emerged in the literature as pathogenic phenomenon were discovered in *Labyrinthula*, including *L. zosterae* (Porter, 1990) affecting seagrasses and *L. terrestris* (Bigelow et al., 2005) affecting turf grasses. A third species, *Labyrinthula diatomea*, is more recently described and is associated with marine sediments where it primarily, if not entirely, consumes diatoms (Popova et al., 2020). There may be redundancy in the descriptions of cultured specimens based on morphological features, however, molecular sequence-based approaches help clarify, replicate and underpin resolution efforts in this arena (e.g. Martin et al., 2016).

Metabarcoding studies commonly target the V9 section of the 18S rDNA gene to describe protist communities (Amaral-Zettler et al., 2009). Huge data collection surveys like the Tara Ocean Expedition or the Earth Microbiome Project accumulate an immense database of 18S rDNA sequences and associated environmental data. These efforts drive advances in protistan phylogeny and accelerate our understanding of ecological needs for otherwise cryptic taxa, including Labyrinthulomycetes (Pan et al., 2017). Global *Labyrinthula* surveys, including two nuclear DNA sequencing methods (18S and ITS amplicons) report there are at least 16-21 *Labyrinthula* species covering both terrestrial and marine habitats (Bigelow et al., 2005;

Martin et al., 2016). ITS sequences are more commonly used as a barcode for fungi phylogeny (Schoch et al., 2012). Therefore, both currently used molecular markers have high quality reference data and are a good tool for discriminating taxonomic units and to describe diversity within the genus *Labyrinthula*. Newer whole genome approaches will eventually resolve phylogenetic uncertainties.

Given the plastic and somewhat featureless nature of *Labyrinthula* cells at the level of gross morphology, genetic signatures are a critical pathway for resolution. In the last decade, molecular techniques have been more commonly used to identify *Labyrinthula* spp. cultured from field samples (see Section 3 for details on molecular markers). Progress has been made in classifying *Labyrinthula* in the context of other genera and families in this order (Pan et al., 2016; Pan et al., 2017). The most recent key developed to describe distinguishable morphological characteristics of species in this genus was prepared over 20 years ago (Dick, 2001). Genetic evidence for Labyrinthulomycetes species diversity has recently leaned more on barcoding-type approaches, but also ecological context, to establish speciation and develop a stable taxonomic key (Martin et al., 2016). Given the robust record of publishing species descriptions, recent advances in genome



FIGURE 4  
Cell myosis in *Labyrinthula*. (A) mature cell and (B) freshly split cells are present.

sequencing (Tan et al., 2021) and success in developing prototypic genetic-level species delineations (Martin et al., 2016), there are new opportunities to resolve historic and ongoing discoveries into speciation and evolution of *Labyrinthula*.

## 3.2 From leaf to culture

To isolate and identify *Labyrinthula* from field collections, targeted sections of leaf tissue may be extricated from leaf samples and transferred to sterile agar media for growth and isolation (Young, 1943; Muehlstein, 1992). The use of high-quality clear agar results in the best colony growth visualizations. Isolated cultures can be used for cellular and molecular investigations, including diagnostics, growth assays, fatty acid analyses, visualizations and long-term sample storage.

### 3.2.1 Tissue preparation and growth media

Prior to sampling collected plant material for transfer onto agar media, it is advisable to pre-treat tissues to reduce contaminants (See Supplemental Manual). Labyrinthulids are able to utilize a range of microbial or plant substrates (See Supplemental Manual) for survival (Sykes and Porter, 1973). This is consistent with findings

about their diverse biogeographic range, hosts, and trophism. Variabilities in nutrients and other additives in media may have a substantial impact on the cell and colony characteristics of isolates. Accordingly, care should be taken with the intent of using any culture medium, as the consequences of different media on growth results remains an open question, especially with regards to affecting strain virulence. A summary of standard agar and liquid culture methods successfully used to culture *Labyrinthula* can be found in the Supplementary Manual.

## 3.3 Axenic culture

After *Labyrinthula* is identified growing away from the host tissues and onto selected sterile culture media, it can be transferred repeatedly to new petri-dishes. Active and relatively rapid identification and transfer of *Labyrinthula* is required in initial phases of axenic cell and colony isolation. A common source of contamination in cultures are fungi, bacteria and other protists from the original seagrass segments. The use of antibiotics and germanium dioxide in the sterile seawater agar media helps to avoid and minimize bacterial and diatom contaminations (See Supplementary Manual).



### 3.4 Quantifying cell and colony growth

When examining one or more factors suspected of impacting *Labyrinthula* cell and colony growth, assays may be required. Assays provide a tool for experimentation of environmental and laboratory conditions that may favor or hinder the growth or pathogenesis of *Labyrinthula*.

#### 3.4.1 Growth assay

Since *Labyrinthula* does not grow fully homogeneous in liquid culture or on agar plates, it is difficult to control *Labyrinthula* cell densities at the start of any assay. For this reason, it is important to advance protocol to standardize precise conditions and controls to account for the overall variance in *Labyrinthula* growth between replicates. The length of the assay will depend on how fast isolates are growing. All assays should be stopped before the colony reaches the edge of the tank, petri dish or well-plate. Those types exhibiting more agar-surface growth tend to grow faster and often reach the edge within days, while predominantly in-agar types are usually slower and may never reach the edge. Given the phenotypic plasticity observed in *Labyrinthula*, care should be taken to make sure the *Labyrinthula* isolate used for all replicates of the assay exhibit similar cell size and shape, and that they are growing at roughly the same rate. Thus, colonies used in assays should be the same age, and maintained by transferring to new agar plates at the same time (ideally, not more than 1 week or so old). Finally, as noted by [Martin et al. \(2009\)](#), abiotic conditions (and likely biotic, as well) can alter both colony extent and cell densities within colonies/ectoplasmic networks, with experimental designs and questions dictating whether both measures are necessary. Three key parameters (dish size, borer size and incubation time) can be altered to fit the need of the assay. For example, if the assay is used to investigate *Labyrinthula* growth on seagrass extracts it can be beneficial to use smaller petri dishes or well-plates to minimize the amount of extracts used for each replicate ([Trevathan-Tackett et al., 2015](#); [Jakobsson-Thor et al., 2018](#)). If long term data are required, a larger dish and longer incubation time may be needed.

### 3.5 Cell quantification by hemocytometer

Cell counts using hemocytometers is a common, easy and inexpensive (though sometimes time-consuming) method for quantifying cell numbers. Due to the adhesive nature of *Labyrinthula* cells and colonies to surfaces and each other, accurate quantification of *Labyrinthula* in suspension interferes with both direct counting and absorbance-based cell quantifications. Through informal tests of counting cells *via* hemocytometer in suspension, 25-second vortex durations of *Labyrinthula* with 100- $\mu$ m glass or zirconia/silica beads emerged as a useful method of separating cells to facilitate counting ([Dawkins et al., 2018](#)).

### 3.6 Visualization

Given *Labyrinthula*'s generally hyaline appearance, histological and microscopic investigations have been critical to species

discoveries, including the creation of detailed drawings of *Labyrinthula* cellular and colony morphology ([Jepps, 1931](#); [Young, 1943](#)). It can be very difficult to detect *Labyrinthula* colonies with the naked eye, though it is possible with experience. Methods to track live *Labyrinthula* cells and visualize their distinct qualities and behaviors will improve our knowledge of cellular and colony functional traits.

#### 3.6.1 Histology

Staining of plant tissues has been effective in revealing important cellular characteristics of *Labyrinthula*, including descriptions of key structures and functions ([Jepps, 1931](#); [Young, 1938](#); [Watson and Raper, 1957](#); [Armstrong et al., 2000](#); [Preston and King, 2005](#)). Early visualizations were drawn by [Jepps \(1931\)](#) observed from stained and fixed *Labyrinthula* cells, as viewed through a microscope. More recently, color images of histological investigations of seagrass demonstrate the presence of *Labyrinthula* cells within the leaf tissue of its seagrass host ([Groner et al., 2014](#); [Groner et al., 2016](#)). For example, in our experience and that of [Harrington and Hageage \(2003\)](#) when utilizing a fluorescing stain, it is strongly advised to carefully evaluate stain performance under particular conditions, as it may vary by organism, pH, production batch, and many other factors. Notably for seagrasses, background fluorescence can vary significantly by species (e.g., [Figure 5A, B](#)), leaf age (possibly related to cuticle thickness and/or composition), degree of fouling and damage to the epidermis ([Figures 5A–C](#)), and microscope filters used.

#### 3.6.2 Microscopy

An inverted microscope with phase contrast allows for the best visualization of cell and colony formations and measurements needed to verify the presence of *Labyrinthula* organisms. Scanning electron microscopy (SEM) has been successfully used to view zoospores ([Amon and Perkins, 1968](#); [Perkins and Amon, 1969](#)) and ultrastructure ([Perkins, 1972](#)) in *Labyrinthulomycota*. To improve microscopic images of cultured colonies, staining and light manipulations may be required. Exploration and discovery of these and other visualization tools may aid in further discoveries of temporal and spatial qualities of host-pathogen relationships and trophic transfers.

### 3.7 Culture maintenance and storage

As isolates are acquired, it may be desirable to slow *Labyrinthula* colony growth while maintaining genotypes or healthy populations in the laboratory. There are storage options for researchers who may no longer require rapid and intensive growth in their collection. Short-term (day-to-day) storage of sealed isolates is possible for rapid growth of cultured colonies. Temperature thresholds can be strain specific and thus, the optimum temperature for culturing isolates will be different depending on climatic adaptation. Generally, cultures can be grown at approximately 20 °C. When longer time periods between transfers are needed, this can be achieved by storing growth plates under chilled conditions (~4 °C). Samples may also be stored for longer periods, (month-month) in controlled light and temperature chambers. Cryopreservation allows for samples to be stored at -80 °C for up to six months ([Trevathan-Tackett et al., 2018b](#)). However,

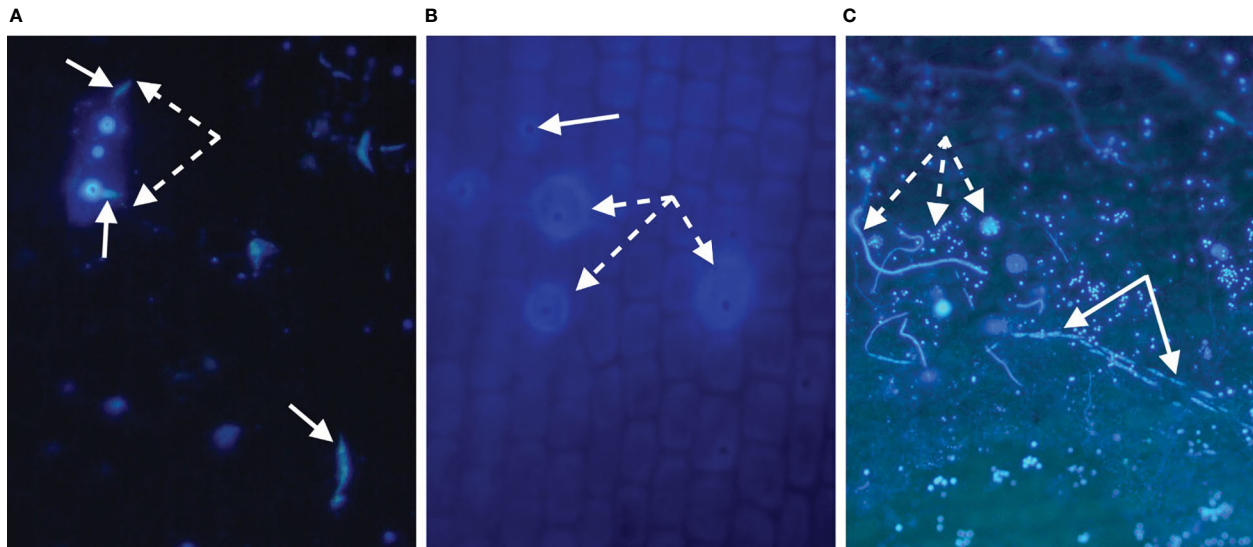


FIGURE 5

(A-C). Evidence of *Labyrinthula* sp. infection using u/v light microscopy with the fluorescent stain, Calcofluor White (CW). CW binds cellulose of host cell walls (less so for waxy cuticle regions), and the chitin within *Labyrinthula* sp. and fungal cell wall structures. (A) *Thalassia testudinum* surface showing rectangular epidermal cell (dashed arrows), infected with *Labyrinthula* sp. *in vitro*, fluorescing from the inside where CW has gained access to the inner walls, and thus is brighter than neighboring cells. Individual *Labyrinthula* sp. cells (solid arrows), some of which appear to be entering (or exiting) epidermal holes (bright spots, for which fluorescing edges have bound stain, and dark center/hole with no stain). (B) Field-collected *Zostera marina* epidermal surface from diseased (bleached/lesioned) tissue, where apparent *Labyrinthula* sp. hole-like damage (solid arrow) has allowed stain to bind inner structures of some cells (dashed arrows); focused slightly below the surface wall, hence rounder appearance. (C) Field-collected *Thalassia testudinum* epidermal surface of green tissue located 3 mm from diseased (brown/lesioned) tissue edge; stained with CW and showing trail of *Labyrinthula* sp. cells (solid arrows) co-occurring with apparent fungal structures (dashed arrows: hyphae, spores [white spots without dark centers], appressoria), emphasizing the potential for interactions among the host and its microbiome.

regular thawing, culturing and re-cryopreservation may keep viable isolates vital for longer timescales. For important isolates, duplicating efforts to maintain genotypes, especially through collaborative storage agreements with other institutions and facilities has been important in long-term studies of *Labyrinthula*, as cultures can die off for no obvious reasons and historical knowledge and testing of the same isolates has a powerful effect on background needed for effective experimental design and interpretation of statistical results.

## 3.8 Laboratory experiments

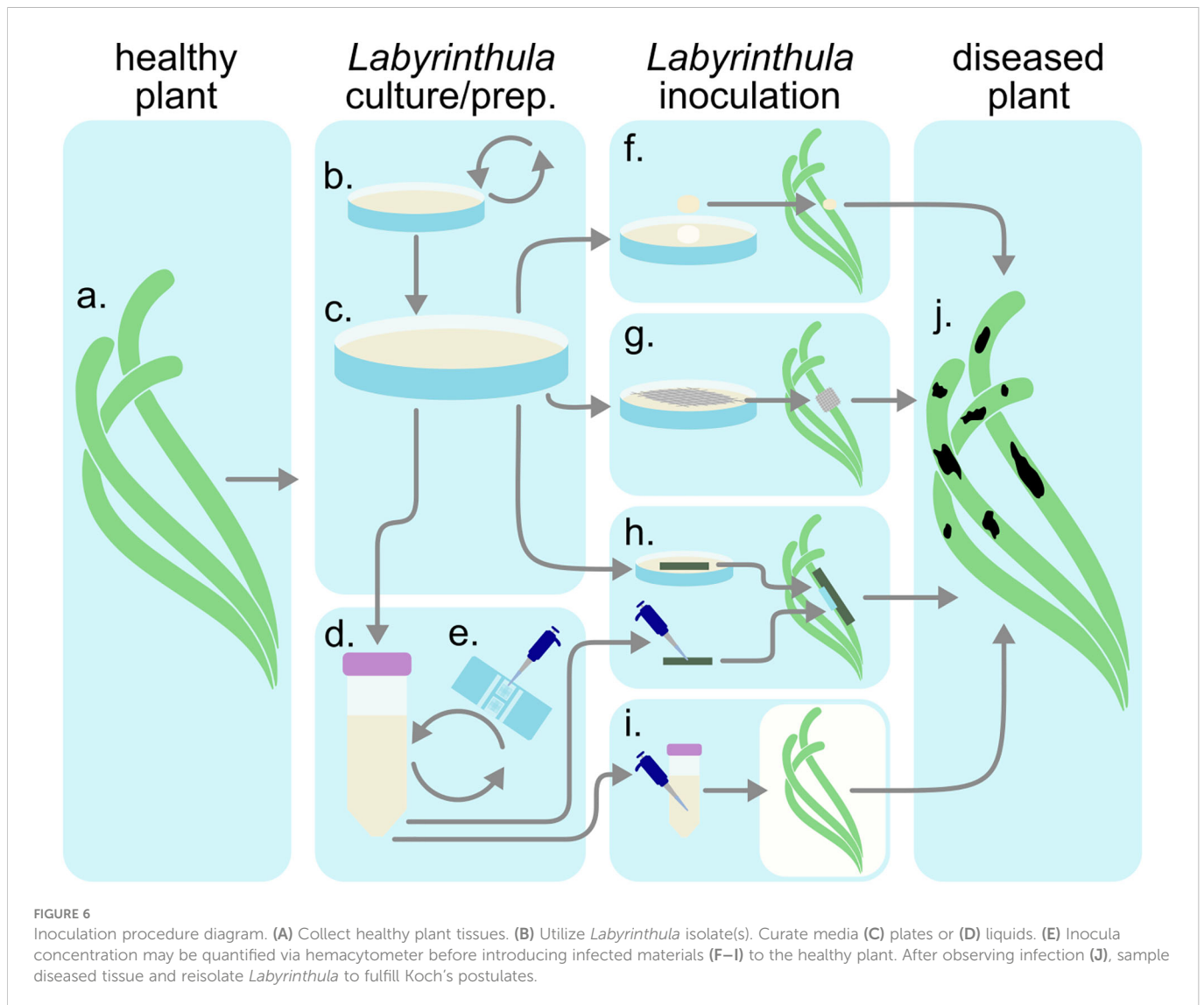
### 3.8.1 Pathogenicity

Research on *Labyrinthula* disease and ecology have primarily focused on laboratory pathogenicity assays. Investigations into seagrass pathogenicity testing of *Labyrinthula* spp. was pioneered by Charles Renn (Renn, 1936, Watson and Ordal 1957, Muehlstein, 1992; Martin et al., 2009; Sullivan et al., 2013) using *Labyrinthula* to test Koch's postulate. Direct observation of infection and pathogenicity are most common (Figure 6, See Supplemental Manual). Laboratory and field assays are both used to investigate a) pathogenicity and virulence of newly isolated cultures (Vergeer and den Hartog, 1994; Martin et al., 2016; Trevathan-Tackett et al., 2018a), b) how infection develops naturally on infected plants under various environmental conditions (Buchsbaum et al., 1990; Vergeer et al., 1995; Beets et al., 2014; Dawkins et al., 2018), and c) variability in infectiousness of healthy or asymptomatic plants by inoculating under different environmental conditions (McKone and Tanner, 2009; Trevathan et al., 2011).

Pathogenicity in seagrass has also been researched indirectly through correlation of environmental parameters, and spatial distribution and coverage (Cottam and Munro, 1954; Vergeer and den Hartog, 1994; Lathrop et al., 2001).

### 3.8.2 Experimental vectors

Different kinds of *L. zosterae* vectors, including inoculated leaf tissues, agar plugs (Jakobsson-Thor et al., 2019), gauze and water-baths (Groner et al., 2016) have been used to perform infection experiments on healthy seagrass shoots. It is important to standardize experimental vector infection protocol for pathogenicity assays. While several techniques have been used to infect plants with *Labyrinthula* (Martin et al., 2016), the majority of pathogenicity assay techniques use surface-to-surface transfer. A limitation with the method above is that it is difficult to control for *L. zosterae* quantities added during inoculation. It is important to be able to control pathogen loads when studying disease severity and other density-dependent effects. Only the *in vitro* water-borne method by Groner et al. (2016) makes it possible to evaluate and control the concentration of the *Labyrinthula* cells when performing infection experiments prior to treatment. In this method, a hemacytometer is used to determine the *Labyrinthula* cell dosage in either a concentrated inoculum applied directly to the plant or as an inoculum bath in which the ramet is immersed. Similar assays have been performed on fresh leaf sections thereby providing a relative measure of potential virulence or severity (Groner et al., 2014; Dawkins et al., 2018). This method can be used for transferring cells as mentioned above, but concentrations can also be sampled and



assessed with this method at any time to understand pathogen loading and variability due to concentration.

### 3.8.3 Mesocosms

Infection experiments have so far mainly been used to investigate direct infection effects on a given seagrass host (Short et al., 1993; Steele et al., 2005; McKone and Tanner, 2009; Garcias-Bonet et al., 2011; Trevathan et al., 2011). Recent studies on the effect of physical and chemical environmental stressors, e.g. temperature, salinity, light, pH and interactions of these factors, have shed some light on how a changing environment will affect *L. zosterae* infection (Bishop et al., 2017; Dawkins et al., 2018; Brakel et al., 2019; Jakobsson-Thor et al., 2020).

## 4 Emerging molecular and -omic toolbox

To better understand ecological and evolutionary characteristics of an organism, knowledge about multi-omics is essential. Molecular approaches are rapidly advancing research into *Labyrinthula*

genomic, transcriptomic, and metabolomic analyses. Advances in capabilities and reduced costs for molecular tools are leading to rapid advancement of discoveries related to speciation, classification, quantification, and phylogeny of *Labyrinthula* (Lohan et al., 2020). In addition, there is increasing recognition for the importance of *Labyrinthula* in host-pathogen relationships, trophic food webs, DHA production, and microbial symbioses in peer reviewed literature. Further advancement and refinement of key genetic markers and regions will continue to help researchers better understand *Labyrinthula* biology and allow for better assessments of the roles *Labyrinthula* play in marine and terrestrial ecology.

### 4.1 Extraction

High quality RNA and DNA sequences are important for successful genetic investigations. This can prove challenging because *Labyrinthula* are regularly found growing in close association with other microbes, such as other stramenopiles, bacteria, yeasts, and other contaminants (See Supplementary Manual). As such, *Labyrinthula* DNA needs to be lysed from



axenic, liquid cultures (See Section 2.3.2 Growth Media) (Martin et al., 2009; Martin et al., 2016) or from over-growth agar cultures, taking care to scrape the surface to avoid unnecessary agar collection (Trevathan-Tackett personal observation). *Labyrinthula* DNA has been extracted with repeated success using standard or modified cetyl trimethylammonium bromide (CTAB) in sufficient amounts and required quality for analyses (Honda et al., 1999; Leander and Porter, 2001). DNA extraction kits have also been repeatedly successful for isolating *Labyrinthula* genetic profiles (Bockelmann et al., 2013; Lohan et al., 2020).

## 4.2 Genetic markers for phylogeny and population genetics

Phylogenetic analyses within the genus *Labyrinthula* have been performed based on several molecular markers, including 1) small subunit (SSU) ribosomal genes in 18S rDNA (Douhan et al., 2009; Bockelmann et al., 2012; Chitrampalam et al., 2015; Martin et al., 2016; Sullivan et al., 2017), 2) internal transcribed spacer (ITS) regions (Martin et al., 2016) and SL1, NS4/CITS5, actin, tubulin and Ef-alpha sequences (Tsui et al., 2009). As of November 2022, the search terminus “*Labyrinthula*” applied to NCBI GenBank nucleotide database retrieves 572 sequences of linear DNA/RNA with around 1700+ base pairs, 1 linear DNA/RNA submitted with nearly 4,758 base pairs and one complete genome containing 26,822 base pairs (<https://www.ncbi.nlm.nih.gov/nucleotide/?term=Labyrinthula>). These entries clearly reflect the dominance of ITS and 18S markers in research. Though generally regarded as a viable marker for population level genetic analyses, the ITS regions for *Labyrinthula zosterae* are surprisingly similar between isolates procured from different oceans (Bockelmann et al., 2013; Martin et al., 2016). Still, most of the published studies on *Labyrinthula* specific diversity (Douhan et al., 2009; Bockelmann, 2012; Chitrampalam et al., 2015; Martin et al., 2016; Sullivan et al., 2017) have been tested on *Labyrinthula* cultured prior to sequencing. Thus, uncultivable taxa were undiscovered prior to sequencing efforts. While ITS and 18S are the most common regions used to evaluate *Labyrinthula* thus far (Lohan et al., 2016; Lohan et al., 2020), these regions are not necessarily the “best” markers to understand speciation. Areas of the genome used to assess specific diversity in the future will depend on the questions being asked (e.g., taxonomic level), and will likely include massive multi-gene analyses from whole-genome efforts.

## 4.3 Cell quantification

Quantitative PCR (qPCR) is commonly used to detect, identify and quantify presence of oomycetes in host tissue and other environmental samples (See Supplemental Manual). Unidentified *Labyrinthula* presented in metagenomic data sets are growing rapidly in terms of both quality and quantity (Karst et al., 2018; Lohan et al., 2020), demonstrating how much remains to be discovered with regards to the biological and ecological characteristics of this elusive group of microbes. There are important applications for qPCR in plant pathology and microbial ecology (Lievens et al., 2006), including *Labyrinthula* (Bockelmann

et al., 2013; Chitrampalam et al., 2015; Martin et al., 2016; Duffin et al., 2020; Duffin et al., 2021). The great advantage of this technique is the fast and sensitive detection of *Labyrinthula* cells in host tissue without time intensive staining or culturing procedures. Further, detection of *Labyrinthula* can be achieved independent from the detection of host symptoms, which may be ambiguous. Although it would be logistically impossible to verify every strain and pathosystem, qPCR primers are likely to provide valuable first steps for various seagrass pathosystems, especially those harboring more than one pathogenic phylotype (e.g., turtlegrass; Martin et al., 2016). At least one set of primers has been designed to partly address this specific issue (Duffin et al., 2020; Duffin et al., 2021; Duffin et al. 2022). Gene expression has also been explored with qPCR in an attempt to detect immunity and stress markers in halophytes (Brakel et al., 2014).

Still, qPCR for *Labyrinthula* studies have some challenges. First, results can depend on the DNA extraction method used (Klein, 2002), making cross-study comparisons difficult. Further, false negatives can occur when the DNA sequences vary at primer binding sites (Klein, 2002). Thus, good validation with isolated *Labyrinthula* strains is necessary before developing effective qPCR assays (Duffin et al., 2020; Duffin et al., 2021; Duffin et al., 2022). Standard cell solutions need to be developed with locally occurring *Labyrinthula* isolates, as variation in copy numbers of ITS genes have been detected in other organisms (Rogers and Bendich, 1987). However, such efforts may be balanced against other assumptions, for instance, relative loading may suffice for some study questions, but provide misleading perspective for others when considering wild populations that may include more and less virulent strains (Dawkins et al., 2018; Trevathan-Tackett et al., 2018a).

## 4.4 Genome sequencing

The first whole genome for *Labyrinthula* has been assembled and published, sourced from axenic culture from southeast Australia (Tan et al., 2021) and previously identified within the pathogenic clade and confirmed *via* pathogen testing (Martin et al., 2016; Trevathan-Tackett et al., 2018a). The first genome and transcriptome provide novel insight into *Labyrinthula* phylogeny and an opportunity to explore the genes available and used by *Labyrinthula*. In the future, emerging DNA-based techniques and assessments will further improve our evolutionary, biological and ecological understanding of *Labyrinthula*, especially with regards to reproduction, growth, speciation, immunity, and virulence.

## 4.5 Biochemical analyses and metabolomics

### 4.5.1 Use

Compounds produced by *Labyrinthula* spp. and their hosts may provide valuable information on their interactions. Measurement of particular compounds or classes thereof (e.g. phenolics) may inform targeted questions, while overall metabolic signatures (usually of a certain class of compounds) of a given sample may provide broader or more exploratory information. Metabolomics has been useful in the examination of phylogeny and halophyte defense compounds. For instance, Huang et al. (2003) used fatty acid (FA) signatures to cluster

unknown isolates of thraustochytrids against a phylogeny constructed with the 18S rRNA gene. For such methods to be applicable for *Labyrinthula* phylogeny, a precise understanding of how FA are synthesized and how production may change through disease progression is required. Also, though the presence of defense compounds can be detected if there is a change in concentration between different treatments, such as between an infected and uninfected plant (Brakel et al., 2014), it is difficult to detect these defense compounds if the compounds are at the same levels and always active (constitutive defense).

#### 4.5.2 Host response mechanisms

It has been hypothesized that seagrasses defend against pathogenic *Labyrinthula* species by the production of chemical defense compounds. Overall, secondary metabolites, including chemical defenses against *Labyrinthula* are poorly understood (Zidorn, 2016). Despite research, no direct links between defense compounds and resilience have been identified thus far.

#### 4.5.3 Phenolic acids

Several studies have investigated the effect of phenolic acids on *Labyrinthula*, a well-known group of defense compounds in terrestrial plants. While some have found a positive correlation between *Labyrinthula* infection and phenolic acid production in seagrasses (Vergeer and Develi, 1997; McKone and Tanner, 2009), others suggest phenolics accumulation in diseased leaves is 'pseudo-induced' due to lesions disrupting the allocation and storage of leaf resources (Steele et al., 2005). These methods have made it possible to compare phenolic acids in seagrass shoots collected from the field, or from shoots used in different kinds of infection experiments (Groner et al., 2016; Groner et al., 2018). Still, the role of phenolics in moderating *Labyrinthula* is not certain.

#### 4.5.4 Additional defense compounds

Additional compounds with inhibitory effects on *Labyrinthula* growth have been isolated from *T. testudinum*, but the structures have not been confirmed (Trevathan-Tackett et al., 2015). The isolation was done using bioassay-guided fractionation, a common method for isolation of bioactive compounds. This method allows detection and isolation of inhibitory compounds from complex samples without targeting a specific group of compounds. However, the extraction technique used by Trevathan-Tackett et al. (2015) did not target phenolic acids. It would be beneficial for future studies to apply a broader extraction technique that also includes phenolic acids. This would better resolve whether phenolic acids are actively inhibiting *Labyrinthula* growth in seagrasses or if other compounds are responsible for this interaction. Immune-related enzymes represent another group of compounds that may act on and in response to *Labyrinthula*, at least for turtlegrass, which is thought to impact virulence. Duffin et al. (2020) developed assays for four enzymes (peroxidase, exochitinase, polyphenol oxidase, and lysozyme activity), two of which correlate with *Labyrinthula* load. Further, all are expressed constitutively in turtlegrass, and appear useful in terms of elucidating *Labyrinthula* defense strategies such as tolerance (Duffin et al., 2020), or even defense 'syndromes' (Agrawal and Fishbein,

2006; Defossez et al., 2018), in wild seagrass populations. Duffin et al. (2021) subsequently combined all four markers when evaluating host immune activity relative to *Labyrinthula* load in the field.

#### 4.5.5 Non-structural carbohydrates

Generally, the production of secondary metabolites is considered a cost to a plant, with multiple functions as a potential means to offset its toll (Erb and Kliebenstein, 2020). Still, the production of defensive compounds in plants afflicted with SWD, along with reduced host photosynthetic ability (Ralph and Short, 2002), may in turn reduce host energetic resources such as non-structural carbohydrates. As valuable measures of seagrass fitness broadly, assessments of non-structural carbohydrates (i.e. starch and/or sugar content) have also been applied to SWD systems. Brakel et al. (2019) found limited effects of *Labyrinthula* inoculation on fitness measures, including rhizome starch, leaf starch, and leaf sucrose concentrations, in eelgrass relative to other stressors (temperature, light, and salinity) in a factorial, multi-stressor experiment. In contrast, Graham et al. (2021) found that rhizome sugar content decreased with the severity of SWD in field-collected eelgrass plants.

#### 4.5.6 DHA production

Long-chain polyunsaturated FA (LCPUFA), especially the omega-3 FA docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are valuable dietary constituents for human and aquatic life (Parrish, 2009). As effective synthesizers of LCPUFA, thraustochytrids have received considerable research and industry attention (Marchan et al., 2018). Labyrinthulids are also known to produce LCPUFA, notably DHA, with much research focused on potential industrial applications of high-LCPUFA producing strains (Sakata et al., 2000; Kumon et al., 2002; Kumon et al., 2003; Kumon et al., 2005; Kumon et al., 2006; Wang et al., 2019). However, most FA studies on Labyrinthulids use single-taxon microbial (bacteria or yeasts) or derived (serum, yeast, and/or peptone-based) substrates for culture (Sakata et al., 2000; Kumon et al., 2002; Kumon et al., 2003; Kumon et al., 2005; Kumon et al., 2006; Wang et al., 2019; Yoshioka et al., 2019). Consequently, little is known about *Labyrinthula* LCPUFA production *in situ*.

#### 4.5.7 DHA and pathogenic *Labyrinthula*

Pathogenic *Labyrinthula* can produce substantial amounts of DHA (Yoshioka et al., 2019). Grown on a modified serum seawater agar and an eelgrass-based agar (see Growth Media), *Labyrinthula* isolates from diseased *Z. marina* produced DHA as their dominant FA. In both laboratory-inoculated and field-collected eelgrass tissues, DHA content was greater in diseased tissue than healthy tissue, suggesting that pathogenic *Labyrinthula* produces DHA *in situ* (Yoshioka et al., 2019). For Yoshioka et al. (2019), plant DHA content associated with disease was detectable but modest. Fatty acid changes in diseased plants are likely subject to other variables that may affect the plant-pathogen relationships. Lastly, surface metabolites, including FA of *Z. marina*, can inhibit or modulate microbes that in turn may affect disease (Papazian et al., 2019), suggesting more work is needed to understand production and the role of microbes in modulating FA levels in plants.

## 5 Review, synthesis and future directions

Molecular work describing microbe-microbe and microbe-plant interactions in *Labyrinthula* has led to ongoing technological advances, shedding light on important topics related to *Labyrinthula*, such as speciation (Martin et al., 2016; Popova et al., 2020), gene expression (Brakel et al., 2014; Jakobsson-Thor et al., 2020), global pathogenicity (Sullivan et al., 2017; Trevathan-Tackett et al., 2018a) and host immunity (Duffin et al., 2020; Duffin et al., 2021). Thus, research attentive to Rapid Blight, Seagrass Wasting Disease, and broader ecological implications of *Labyrinthula* in critical ecosystem functions, such as disease, decomposition, host immunity and primary production and transfer of fatty acids are ongoing.

A significant gap remaining in biological research of *Labyrinthula*, requiring both cellular and molecular advancement, are life-cycle studies. Heterokont flagella are a defining feature of the stramenopiles, yet there is a distinct lack of information about zoospore production and identification in *Labyrinthula*, and it is entirely lacking in relation to those taxa thought to be pathogenic. Sporulation has been reported in *Labyrinthula*, but recovery and positive identification of motile zoospores has not been made or imaged with SEM in a laboratory since initial reports (Amon and Perkins, 1968). Subsequent attempts to observe and replicate sporulation and visualize the results have been unsuccessful. Understanding life phases of *Labyrinthula* is critical for advancing our understanding of complex marine systems. Further, testing the possible roles zoospores play in progressive phases of disease, or seagrass and marine ecology more broadly (including saprobic decay, remineralization and trophic transfer), may also include fixed factors such as seasonality and phases of growth. Thus, confirmation of a flagellated dispersive stage and a consistent laboratory method for obtaining zoospores is needed.

Additionally, more work is needed to understand the connections between *Labyrinthula* and DHA production, including potential roles for pathogenic strains in trophic transfer. If fatty acid (FA) production is increased in pathogenic *Labyrinthula*, questions remain about its biological and ecological relevance. For instance, Jain et al. (2005) suggest that lipids, including DHA, are important as energy reserves and for building the ectoplasmic network in thraustochytrids. The ectoplasmic network enzymatically breaks down plant tissues through *Labyrinthula* infection (Muehlstein, 1992), so FA production may be related to pathogenesis in seagrass, or other organisms. If FA production is indeed pathology-related, then *Labyrinthula* may have additional ecological consequences or advantages for nutritional resources it provides to its consumers. Saprotrophic *Labyrinthulomycetes* and similar microbes are known to be an important link between detrital resources and higher consumers (Raghukumar, 2002). Whether pathogenic *Labyrinthula* provide substantial FA *in situ* and how production relates to infection should be verified. Future work with FA analyses should aim to understand how tissue- and plant-level changes to FA correspond with disease, and whether such changes may have individual, community and ecosystem-level effects. Clarity at both scales of *Labyrinthula* FA production will be informative for understanding infection and developing new tools (possibly in concert with

genomic analyses) while providing a more holistic view of *Labyrinthula* ecology.

Coupled research in seagrass immunity, pathogen virulence, transmission (Dawkins et al., 2018; Duffin et al., 2020), stressor identification and environmental threshold assessments (Brakel et al., 2019) are of great importance to seagrass conservation and restoration efforts. Host susceptibility and immunity remain important areas for ongoing research. As more halophyte pathosystems are investigated, it will be interesting to assess variability in tolerance and defense mechanisms between different families of seagrass and other hosts to determine how interactive pathosystems work in favor or against *Labyrinthula* and other disease organisms. For instance, how do environmental conditions mediate gene-expression? What affect may *Labyrinthula* have on seagrass co-infections with other pathogenic organisms? Studies such as these may reveal important or different immune responses in seagrasses as well. Since seagrasses are not monophyletic, discoveries of variability in host immune defenses and vulnerabilities between individuals and populations will provide useful insight into observed variabilities in transmission, symptoms and virulence for this organism (Trevathan-Tackett et al., 2018a) and those impacts on community ecology. Further, exploration into how disease events impact ecosystem biodiversity and host coevolution long-term might be shaped by *Labyrinthula* associations (Duffin et al., 2020) and also warrants attention.

It is notable in this age of heightened awareness and risks of global pandemics that little research has looked at the role and relationships of *Labyrinthula* colonies to surrounding zoological communities, including its role in the microbiome, such as roles for moderating or exacerbating agents of human diseases in marine environments (Lamb et al., 2017), or the creation of 'biofilms' that serve as antimicrobial barriers or primary colonizers of marine environments. Thus, research is urgently needed to advance of our understanding about the role that *Labyrinthula* and other marine pathogens play in basic ecology of coastal ecosystems, especially when assessing issues of human health and threats of extinction to rarer halophytes, especially in monocultures. Though research has demonstrated that seagrass meadows reduce the presence of harmful microbes (Lamb et al., 2017), the mechanisms for this reduction, including the role of other seagrass-associated microbes in that capture, are unknown.

The potential for *Labyrinthula* to cause widespread loss of seagrasses worldwide has been established, yet even in critically valuable meadows, disease monitoring and inclusion in management objectives are often scant or missing. The presence of *Labyrinthula* has been confirmed in all non-polar major coastal systems, including mangroves, marshes, algae and seagrasses and with various pathogenicity (Martin et al., 2016). What we are still unclear about is whether severe infections with pathogenic *Labyrinthula* are a result of emerging pathogenic species described with clear genetic and morphological characteristics in the literature, or if environmental anomalies are driving virulence or mutation of these (more typically) saprobic organisms. Such a perspective may be slightly clearer for the Rapid Blight caused by *Labyrinthula*, as it is considered to be an emergent disease and an advancing threat to higher latitude cool-season turfgrasses in both the U.S. and Europe, and probably globally (Entwistle et al., 2014). A remaining knowledge gap is identifying the host(s) these terrestrial types may be derived



from. The phylogenetic work presented in Martin et al. (2016), hints at saltmarsh plants as a possible starting point for such an investigation. Finally, advances are needed to understand deeper phylogenetic relationships and evolutionary history relative to marine *Labyrinthula*, especially in regards to the freshwater origins of the only fully marine angiosperms – seagrasses.

## 6 Summary

Researchers have repeatedly found Labyrinthulomycetes are biologically and ecologically important microorganisms (Gleason et al., 2013; Sullivan et al., 2013; Gleason et al., 2014; Scholz et al., 2016). Improved techniques for sampling, isolation, culture, measurement, visualizations, and long-term storage of *Labyrinthula* and other co-occurring pathogens are necessary to advance our capacity for answering lingering questions about *Labyrinthula*'s lifecycle, trophic roles, pathogenicity and virulence, microbiome, and other ecological relationships. Advances in available methods and tools for cellular and molecular investigations of *Labyrinthula* have progressed our understanding of this microbe's importance and the need for continued research. We reviewed complex ecological roles for *Labyrinthula* in saprobic decomposition, creation of biofilms, primary trophic pathways for DHA in marine systems and incidence of pandemic diseases in halophytes, and presence of emerging diseases afflicting saline, brackish and terrestrial systems. *Labyrinthula* mediate structural ecosystems and are also mediated by conditions in the ecosystems themselves. For these reasons *Labyrinthula* has become a model marine microbe for investigating questions at a variety of functional scales with regards to halophytic environments, as it is not wholly host-specific and plays several key roles in the environment, including remineralization, trophic transfer and infectious disease.

A lack of capacity related to genome sequencing has previously stunted studies needed to advance research into *Labyrinthula* phylogeny, cellular function, virulence evolution, epidemiology, host-immunity and gaps in our understanding of *Labyrinthula* life cycle and gene expression. Now that the genome of *Labyrinthula* has been mapped (Tan et al., 2021), we expect even more break-throughs are possible. Advancements in species level discussions about the genetic basis and specific relevance of observed plasticity in phenotypic and trophic characteristics, especially related to environmental or biological stressors/factors are forthcoming as work on gene expression continues. In addition, continued advancements in ecological research of *Labyrinthula* will require effective mobilization and automation of these biological investigation protocols and techniques. Basic biological investigations are still required to describe and study *Labyrinthula* speciation, life cycles and pathosystems before critical ecological questions, such as relative importance in coastal carbon cycling, trophic pathways, and critical host-pathogen thresholds can be answered.

Through this review we have increased international capacity, collaboration and knowledge for the roles for *Labyrinthula* in complex global systems. It is critically important for researchers to collaborate at the international level and to share methodological resources needed to answer globally relevant questions about *Labyrinthula* biology and ecology as we balance an awareness of risk for catastrophic losses alongside obvious benefits from potential trophic contributions of DHA, enzyme production and important nutrient and mineral cycling.

## Author contributions

BS conceptualized, developed the outline and managed authors for this paper; BS, ST-T, RY, JB, SJ-T, ME and DM contributed to writing and editing the manuscript; RY, BS and DM created figures; BS, RY, DM and STT contributed funds for publication and ST-T wrote the Statement of Contributions. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2023.1092587/full#supplementary-material>

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