Interspecific transmission of Seagrass Wasting Disease from Pacific oysters, *Crassostrea gigas,* to eelgrass, *Zostera marina*

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Abstract

In order to feed the growing population of the world in more sustainable ways, more governments and other actors are turning to the aquaculture of marine species to provide for the increasing need for sustenance. Oysters, specifically *Crassostrea gigas,* are an important aquaculture species, and have been introduced into many areas for this purpose, however welack understanding of their direct interactions with cohabiting species. One such species that commonly coccurs in areas with *C. gigas* is *Zostera marina,* eelgrass, which is affected by Seagrass Wasting Disease (SWD). The transmission dynamics of SWD from oysters to eelgrass needs more study. A laboratory experiment was conducted where Pacific oysters, *C. gigas*, were exposed to *Labyrinthula zosterae* and transferred over to tanks containing naive eelgrass to test if oysters were a vector for *Labyrinthula zosterae*. The resulting data shows that disease prevalence of directly inoculated treatments and oyster vectoring of disase were similar. Disease severity was also highest in the inoculated treatments, with direct inoculation being highest. Overall, the findings show that transfer of oysters from infected waters to non-infected waters can introduce disease and subsequent infection. These findings should be used to inform general aquaculture practices and the management and land use practices of oyster beds and possibly other farmed shellfish species**.**

Background

Eelgrass (*Zostera marina*) performs many ecosystem services which are vital to the coastal health and function of marine ecosystems, such as oxygen production, carbon sequestration, nutrient cycling, sediment stabilization, enhanced biodiversity, and acting as nursery sites for fishes and other aquaculture and fished species that provide sustenance. (Orth et al. 2006, Lilley and Unsworth, 2014). However, eelgrasses face many stressors that have

decreased the overall coverage and effectiveness of eelgrass, with rates of decline being up to 7% per year (Waycott et al, 2009). Seagrass wasting disease, (SWD), caused by the protist *Labyrinthula zosterae* (Muehlstein et al, 1991), is a large stressor on eelgrass, with multiple outbreaks being recorded and ongoing outbreaks affecting both coasts of the United States that are hypothesized to be contributing to eelgrass population declines. (Muehlstein et al, 1988).

Pacific Oysters (*Crassotrea gigas*) are an introduced aquaculture species, and they have accounted for over $250 million in landing profits in the United States (NOAA 2019), providing both economic importance and nutritional sustenance. Bivalve aquaculture has shown positive and negative impacts for seagrass communities, with eelgrass density declining with increasing oyster density (Tallis et al. 2009). Oysters in turn benefit from seagrass co-culturing, with densities of bivalve species being 1.6 times higher in seagrass beds than out in a meta-analysis (Fales et al. 2020). Further laboratory experiments show that eelgrass both naturally infected and exposed to *L. zosterae* have reduced disease and pathogen intensity when grown with oysters. (Groner et al. 2018, Agne et al. in review). Field experiments in the Northern Gulf of Mexico have shown restoration of oyster beds can benefit eelgrass restoration efforts as well, but more studies are needed on determining the extent of the synergies and their mechanisms (Sharma et al, 2016) The possibility of disease transmission from oysters to eelgrass needs to be studied more, as method of transmission and other factors are currently unknown and can affect the effectiveness of a possible synergistic relationship. From this background information and gap in knowledge, an experiment was conducted with oysters infected with *L. zosterae* that were placed in tanks with naive *Zosterae marina* to quantify disease transmission. By quantifying SWD prevalence, severity, and the ability of oyster to transmit the disease and act as vectors in lab experiments, the results of this could help better inform efforts for eelgrass restoration and

conservation, as well as land use planning in aquaculture with oysters and other species, especially when moving or transferring oysters to create new bed sites near eelgrasses

Hypothesis

H_{O1} : *C. gigas* oysters will be not transmit SWD to naive *Z.marina*

H_{A1}: *C. gigas* oysters exposed to *L. zosterae* will transmit SWD to naive *Z. marina*

Materials

Oyster transmission experiment (H_{O1}): isolated *L. zosterae,* naive *Z. marina, C. gigas*, hemocytometer, OA lab setup, serological pipettes, Epsom scanner, transparency papers, oyster bags, zip ties, string, vexar plots, 30 oysters per treatment, trays, whirl packs, bleach and RO water, bucket, biohazard bags, sampling tubes and ice chest, vacuum filters, vacuum, 1 liter bottles

Methods

Oyster Transmission Experiment

Z.marina was collected from Fourth of July Beach at Friday Harbor, on July 11th (at 8.463713 N, 122.991055 W) at 8:55 am; salinity of the site at that time was taken at 27 ppt. Eelgrass individuals that had no visible signs of disease on the youngest blade were dug up and taken. 100 blades were taken as insurance for the 64 blades needed; 4 per treatment were required, with 4 treatments having blades, and 4 replications of each treatment, giving 64 blades needed. Blades were put in ice water bags to keep from decaying, put in a cooler, and then brought back to the Ocean Acidification lab. In the OA lab, blades were standardized for length, number of blades (2 blades per individual), and rhizome nodes (1 node); only one eelgrass individual per clonal rhizome was used to ensure greater variability in the replications. Blades were marked for growth with pinhole pricks, given a label, and were scaned alongside a ruler and the label; scans

were used as comparison to later disease development scans. The shoots then sat in filtered, 10ppt seawater for 24 hours, in order to mitigate any possible asymptomatic *L. zosterae* infections. The eelgrass blades were then tethered to a vexar plot with their label and put in the bottom of the tubs. *C. gigas* were bagged and tagged in preparation, with 15 oysters per bag, and 30 oyster bags total. Similar weights were used in each treatment, with the two bags per treatment being around 55 grams. A subset of oysters were photographed at the beginning and end of the experiment to analyze for oyster growth.

The following treatments were used in the experiment

1. Eelgrass without oysters or inoculation (negative control)
2. Oysters inoculated with *L. zosterae*, without eelgrass (for water sampling for disease shedding)
3. Eelgrass with non-exposed oyster
4. Eelgrass exposed to *L. zosterae*, no oyster (positive control)
5. Eelgrass with oysters inoculated with *L. zosterae*

On July 15, the similarly sized *C. gigas* oysters were inoculated with either 1000cells/mL of *L. zosterae*, or the control of sterile seawater for 24 hours. After 24 hours (on July 16th), the oysters were transferred to naive eelgrass tubs to expose the eelgrass. Oysters were strung vertically in the water column. A control treatment was also setup to have eelgrass with no oysters (to help monitor for any asymptomatic infection) and eelgrass with disease and without oyster (to compare to the oyster inoculated treatment) Setup for the tubs and treatments are shown on **Figure 1**. Ibuttons were used to continuously measure temperature across the experiment, and were placed into tubs representing each treatment; aditionally, each cooler has a temperature probe to monitor temperature in real time, with temperatures set to 14 C. The experiment then

ran for 1 week, which was maintained as such; every other day, watering pumps were to be turned off, and oysters were fed with 30% dilution of Shellfish Diet 1800; treatment 1 received 1mL and every other treatment received 2mL. Every day, the 20, 10, 5, and 1 micron filters that filter the water coming into the cooling tanks were replaced and rinsed. Every day, 25mL of germanium dioxide was added to the coolers to prevent diatom growth. Every day, water temperatures in each system and tank sand filter pressure were checked, and the drippers that pumped water into each individual treatment tub were checked to ensure they were working.

Water samples were also taken from oyster treatment (treatment 2) tubs every day to monitor for disease shedding, with 250mL being taken and placed into the 1L sterile bottles, then poured into .45 micron vacuum filters to filter out the water. The filters were then folded over and put into test tubes in an ice chest, which were then put in a -80C freezer for later quantitative PCR. On July 22, six days post inoculation, water samples were from each treatment, filtered, and cooled to run QPCR to quantify *L. zosterae* in each treatment, with the same methods used to filter water from treatment 2; these tubes were also put into the -80C freezer for later qPCR. Seven days post experiment, on July 23rd, the experiment ended. First, the oysters were removed from the tanks for sampling. The oysters were cut from their zipties and placed onto trays, with each bag being re-measured for its wet weight to see if there was any growth. A subset of oysters were measured for growth following the methods above. 4 random oysters per bag from all bags were taken, labelled, and kept in whirl packs for later qPCR analysis; the rest of the oysters were disposed according to the rules of Washington state by freezing at -20 C followed by autoclaving.

The vexar plots of eelgrass were then removed from the tanks and placed onto sterile trays; the plots were removed based on treatment, starting with treatment 1, 3, 5, then 4 to mitigate contamination of the controls by the inoculated treatments. The oldest blade was cut off at its growth ridge, then gently scraped to remove any diatoms or epiphytes. The blade was then cut at the bottom of their two pin pricks, after which the top part of the growth was placed on the left most side of a transparency paper, with the bottom part being placed to the right besides it. This process was repeated with all other blades, then the tethered label was placed onto the transparency along with a ruler for rescans. An example of the scan setup is shown in **Figure 2**. Trays and rulers were disinfected between each blade plot with bleach. and put on trasparancy papers and rescanned for lesion severity, with the pinpricks from before the experiment being used to check for growth. The top 15cm (or any length above the pin pricks if less than 15cm) for the oldest blade were taken and put into test tubes for later qPCR analysis. The scans were analyzed on ImageJ for growth, the distance between pin pricks and grass sheathing, and lesion size to check for disease severity and prevalence to inform the hypothesis. Oysters from the second treatment were taken for later tissue sampling to see pathogen concentration in their gills.

Scans from ImageJ were taken of each blade to measure lesion area compared to leaf area to measure disease severity, with proportion of the population of blades being used to determine prevalence in each treatment. Tank means were taken, of which treatment means were then taken.

Results

Figure 3 shows the prevalence of Seagrass Wasting Disease in each treatment. Both direct inoculation of the water column and oyster inoculation then transferring showed the same prevalence of disease, at .875 ± .054 SE. This was followed by eelgrass with naive oysters, which had a prevalence of .375 ± .117 SE, then the control treatment of eelgrass alone at .3125 ± .107 SE.

Figure 4 shows the severity of the disease in each treatment, as measured as the mean percent area of blade that was lesioned. Directly inoculated eelgrass had the highest disease severity, with a mean of 7.065% ± .905 SE. This was followed by oyster inoculation then transferring, which was at 4.993% ± 1.466 SE. After this was eelgrass with naive oysters, which had a severity of 2.835% ± 2.597 SE, then the control at 1.305% ± .905 SE.

Discussion

Movement of oysters from one area to another can introduce pathogens that are not already present. This study was able to show disease severity and prevaalence were simliar in eelgrass directly inoculated with *Labyrinthula zosterae* and those indirectly inoculated through oyster vectors, in relation to previous work that shows other dynamics seen in the eelgrass-oyster system such as reduced pathogen loads when oysters are grown alongside eelgrass (Groner et. al 2018). As seen in Figure 4, severity was indeed lower in the oyster inoculated treatment, a result that is possibly due to the filtering component of the oyster-eelgrass dynamic. However, as shown in Figure 4, prevalence of disease was the same in both oyster inoculation and direct inoculation. Because of this, the synergy of growing oyster and eelgrass together should be looked at with more nuance, as transferring oysters from infected waters to non-infected waters can introduce more pathogens, which defeats the purpose of oyster filtration in the first place.

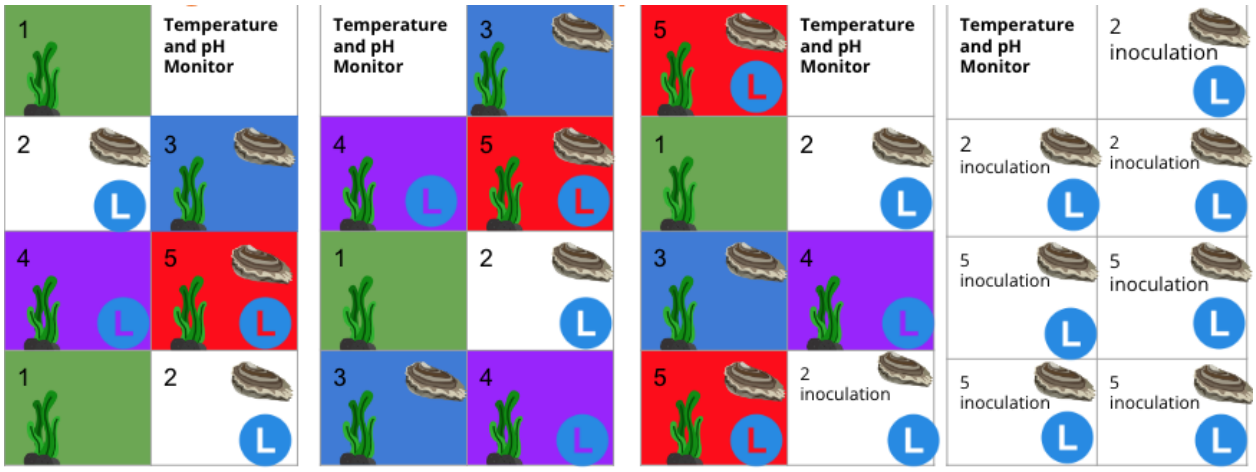
Further research should look into the mechanisms vectoring, as this is still unknown. Our findings show vectoring from oysters to eelgrass occurred when infected oysters were transferred to non-infected waters, but the mechanism through which the *L. zosterae* moved from the oyster (i.e. in tissues or on the shell) to the water needs more study. Some avenues to look at are the depositing of the pathogen through the pseudofeces, or the pathogen sticking to the host oyster shell, and whether or not the pathogen is being amplified in the oyster (Burge et al 2016). Other data collected during the experiment will later be analyzed. The water samples taken from the oyster inoculation (treatment 2) will inform the pathogen loads present in the water when an inoculated oyster is transferred; this will help to see if the pathogen is replicated in or on the oyster when being vectored and thus increasing its prevalence in the water column. The eelgrass blades taken for qPCR will be used to more accurately quantify the severity of the disease by seeing pathogen load in the eelgrass tissues. Lastly, the subsets of the oysters from bags will be taken for qPCR on the gills and other areas of the oyster, to see where the pathogen is on the oyster vector and how prevalent they are in possible vectoring organs.

Conclusion

Overall, our study showed that SWD prevalence resulting from exposure to inoculated oysters and direct inoculation were the same, the SWD severities of both these treatments were the highest of the four treatments. This leads to evidence to reject the null hypothesis, and shows that oysters had an ability to infect naive *Z. marina* with *L. zosterae*. Further avenues of research should try to test the mechanism of transmission that oysters can vector the pathogen; it is unknown whether the pathogen is sticking to the shells of the oyster and are introduced into the water column upon transferring, or the oysters filter the disease in their gills and then deposit through pseudofeces, or some other possible transfer mechanism. These findings should be used

to inform land use and aquaculture planning, as the transfer of oysters from one bed to another for the creation of oyster farms could lead to the introduction of new diseases to an area.

Figure 1:



Legend:

Number indicates treatments (1-5)

1. Eelgrass with no oysters or innoculation
2. Oysters inoculated with laby, no eelgrass (for water sampling)
3. Eelgrass with non-exposed oyster
4. Eelgrass exposed to *L. zosterae*, with no oysters
5. Eelgrass with oysters inoculated with *L. zosterae*

The 2s and 5s on the rightmost side (labeled with inoculation) will be the oyster inoculation for treatment 2 and 5

Through this system, each treatment will be in different coolers to account for any slight variation of temperatures or lighting that could occur

Figure 2

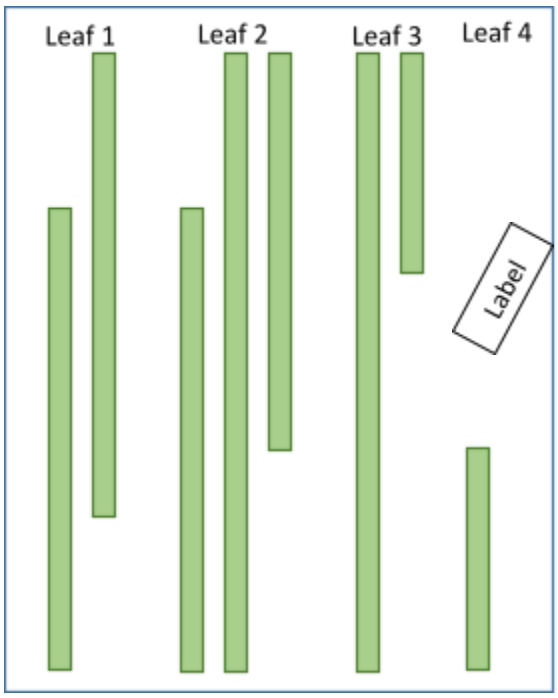


Figure and breakdown methods adapted from MVA 2019

Figure 3

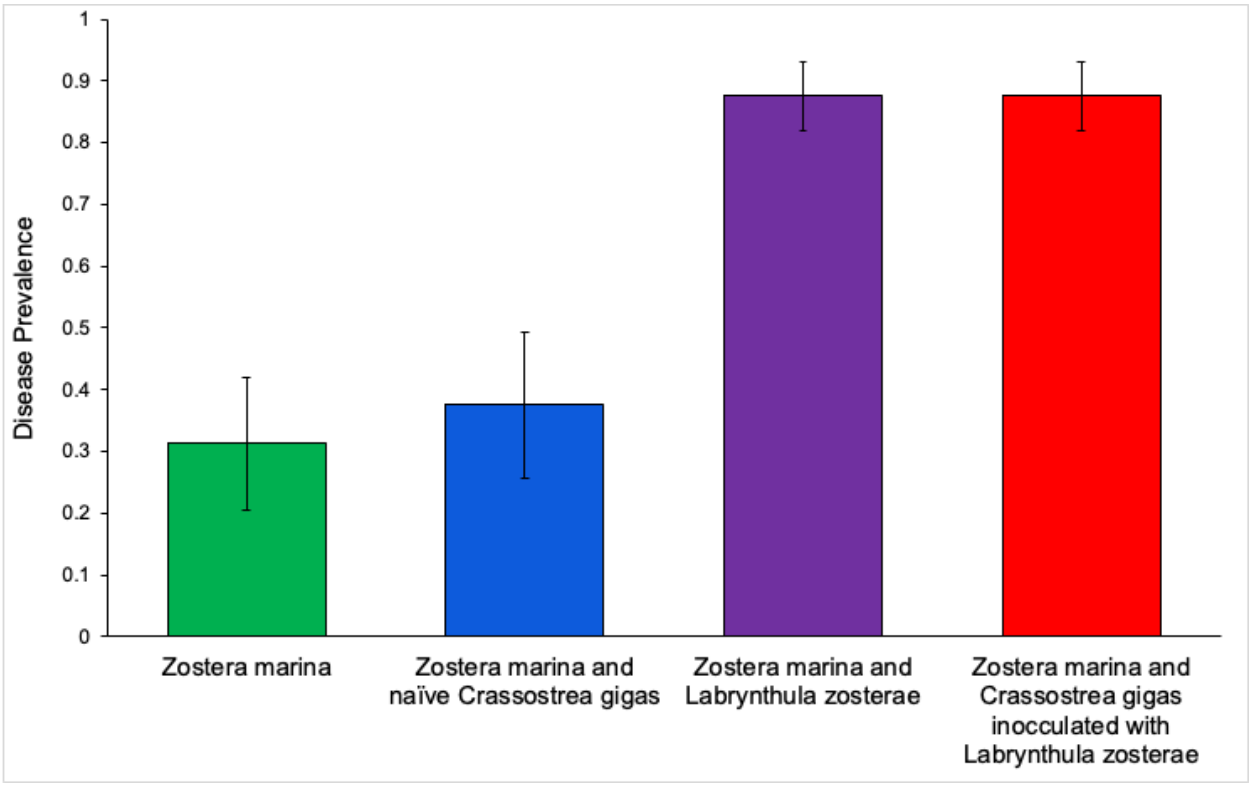
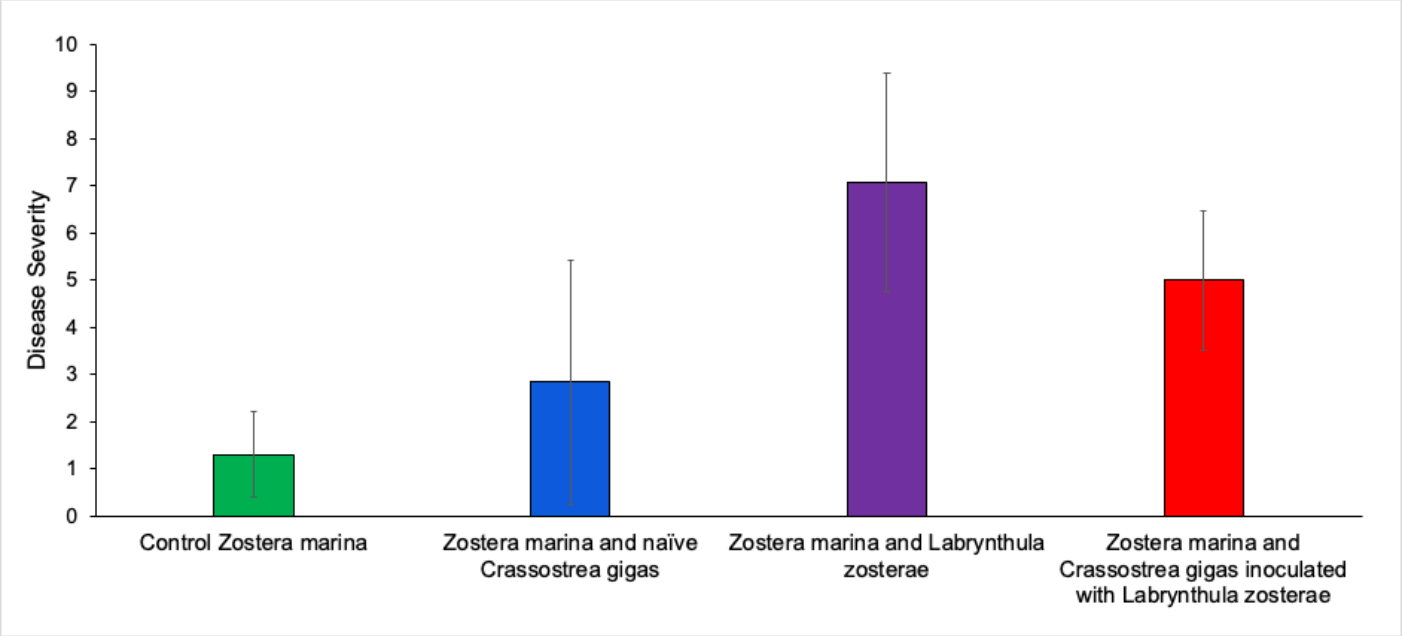


Figure 4


Disease Severity
10
9
8
7
6
5
4
3
2
1
0
Control Zostera marina
Zostera marina and naïve Crassostrea gigas
Zostera marina and Labrynthula zosterae
Zostera marina and Crassostrea gigas inoculated with Labrynthula zosterae

Expected Conclusions:

Hypothesis 1: I expect that the null hypothesis will be rejected, as oysters should be able to transmit *L. zosterae* based on previous studies (Agnew, *in press*) and infect eelgrass, despite the method of transmission from oysters to eelgrass being unknown

These findings will help inform the conservation and restoration efforts of eelgrass in relation to their declines from SWD. Furthermore, this could provide insight to possible synergies between eelgrass conservation and oyster aquaculture, as seagrasses can remove carbon that acidifies the ocean and prevents shellfish from creating shells, and oysters can filter out *L. zosterae* (Groner et al, 2018). leading to better land use planning for putting oyster bed aquaculture nearer to eelgrass beds

Works Cited

Colleen A. Burge, Collin J. Closek, Carolyn S. Friedman, Maya L. Groner, Cody M. Jenkins, Amanda Shore-Maggio, Jennifer E. Welsh, The Use of Filter-feeders to Manage Disease in a Changing World, Integrative and Comparative Biology, Volume 56, Issue 4, October 2016, Pages 573–587, https://doi.org/10.1093/icb/icw048

Groner ML, Burge CA, Kim CJS, Rees E and others (2016) Plant characteristics associated with widespread variation in eelgrass wasting disease. Dis Aquat Org 118:159-168. https://doi.org/10.3354/dao02962

Heather M. Tallis, Jennifer L. Ruesink, Brett Dumbauld, Sally Hacker, Lorena M. Wisehart "Oysters and Aquaculture Practices Affect Eelgrass Density and Productivity in a Pacific Northwest Estuary," Journal of Shellfish Research, 28(2), 251-261, (1 April 2009)

Lisa K. Muehlstein, David Porter, Frederick T. Short, LABYRINTHULA ZOSTERAE SP. NOV., THE CAUSATIVE AGENT OF WASTING DISEASE OF EELGRASS, ZOSTERA MARINA, Mycologia, Volume 83, Issue 2, 1991, Pages 180-191, http://www.jstor.org/stable/3759933

Michelle Waycott, Carlos M. Duarte, Tim J. B. Carruthers, Robert J. Orth, William C. Dennison, Suzanne Olyarnik, Ainsley Calladine, James W. Fourqurean, Kenneth L. Heck, Jr., A. Randall Hughes, Gary A. Kendrick, W. Judson Kenworthy, Frederick T. Short, and Susan L. Williams,

Accelerating loss of seagrasses across the globe threatens coastal ecosystems, PNAS, Volume 106, Issue 30, July 28, 2009, Pages 12377-1238, https://www.pnas.org/doi/abs/10.1073/pnas.0905620106

Muehlstein, Lisa & Porter, David & Short, Frederick. (1988). Labyrinthula sp., a marine slime mold producing the symptoms of wasting disease in eelgrass, Zostera marina. Marine Biology. 99. 465-472. 10.1007/BF00392553.

NOAA 2019
https://media.fisheries.noaa.gov/2021-05/FUS2019-FINAL-webready-2.3.pdf?null=

Richard J. Lilley, Richard K.F. Unsworth,
Atlantic Cod (Gadus morhua) benefits from the availability of seagrass (Zostera marina) nursery habitat, Global Ecology and Conservation,Volume 2, 2014, Pages 367-377, ISSN 2351-9894, https://doi.org/10.1016/j.gecco.2014.10.002.
(https://www.sciencedirect.com/science/article/pii/S235198941400050X)

Robert J. Orth, Tim J. B. Carruthers, William C. Dennison, Carlos M. Duarte, James W. Fourqurean, Kenneth L. Heck, A. Randall Hughes, Gary A. Kendrick, W. Judson Kenworthy, Suzanne Olyarnik, Frederick T. Short, Michelle Waycott, Susan L. Williams, A Global Crisis for Seagrass Ecosystems, BioScience, Volume 56, Issue 12, December 2006, Pages 987–996, https://doi.org/10.1641/0006-3568(2006)56[987:AGCFSE]2.0.CO;2

Robin J. Fales, Fiona C. Boardman, Jennifer L. Ruesink "Reciprocal Interactions between Bivalve Molluscs and Seagrass: A Review and Meta-Analysis," Journal of Shellfish Research, 39(3), 547-562, (28 December 2020)

Sharma, S., Goff, J., Moody, R.M., Byron, D., Heck, K.L., Jr., Powers, S.P., Ferraro, C. and Cebrian, J. (2016), Do restored oyster reefs benefit seagrasses? An experimental study in the Northern Gulf of Mexico. Restor Ecol, 24: 306-313. https://doi.org/10.1111/rec.12329