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Research Article

Metabarcoding of environmental samples suggest wide distribution of eelgrass (*Zostera marina*) pathogens in the north Pacific

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Abstract

Seagrass meadows provide important ecological services to the marine environment but are declining worldwide. Although eelgrass meadows in the north Pacific are thought to be relatively healthy, few studies have assessed the presence of known disease pathogens in these meadows. In a pilot study to test the efficacy of the methods and to provide foundational disease biodiversity data in the north Pacific, we leveraged metabarcoding of environmental DNA extracted from water, sediment, and eelgrass tissue samples collected from five widely distributed eelgrass meadows in Alaska and one in Japan and uncovered wide prevalence of two classes of pathogenic organisms – *Labyrinthula zosterae* and other associated strains of *Labyrinthula*, and the *Phytophthora*/*Halophytophthora* blight species complex – known to have caused decline in eelgrass (*Zostera marina*) elsewhere in the species' global distribution. Although the distribution of these disease organisms is not well understood in the north Pacific, we uncovered the presence of at least one eelgrass pathogen at every locality sampled.

Key Words

Alaska, eDNA, disease, Japan, seagrass

1. Introduction

Seagrass meadows comprise the most widespread coastal ecosystems in the world (Green and Short 2003), providing important ecological services to the marine environment and promoting ecosystem health (Costanza et al. 1997; Nordlund et al. 2016). They act as nurseries that shelter young animals, provide habitat for fish, crabs, and other invertebrates, sequester carbon (Hemminga and Duarte 2000; Duarte et al. 2005; Macreadie et al. 2014), and mitigate the impact of terrestrial pollution on the marine environment (Fernandes et al. 2009; Lamb et al. 2017).

In the north Pacific, the predominant seagrass species is eelgrass, *Zostera marina*, a widely distributed marine angiosperm adapted to the cold waters of northern high latitudes. Eelgrass meadows provide valuable habitat for diverse animal assemblages – including salmon (Salmonidae), Dungeness crab (*Metacarcinus magister*), herring (Clupeidae), and gunnels (Pholidae) to name a few – and function as an important primary producer and erosion stabilizer in coastal ecosystems (Asmala et al. 2019; Olson et al. 2019; Marin-Diaz et al. 2020). In Alaska, eelgrass meadows also provide critical foraging habitat for migrating avian species, including most of the world's population of Pacific black brant (*Branta bernicla nigricans*) (Ward et al. 2005). Eelgrass, like other seagrass species, has been declining globally (Waycott et al. 2009). Major declines of eelgrass meadows in North America have been

attributed to both localized and general human-induced events, such as the release of oil, farming induced eutrophication, residential expansion, climate change (Short and Burdick 1996; Short and Wyllie-Echeverria 1996; Orth et al. 2006; Waycott et al. 2009), and natural events, such as anoxia and disease (Short et al. 1987; Muehlstein 1992; Short and Wyllie-Echeverria 1996; Plus et al. 2003). Baseline abundance and distribution data collected from a number sites suggest eelgrass beds are stable in Alaska (Ward 2021; Ward et al. 2021), but trend data are limited to a single location (Izembek Lagoon; Ward et al. 1997; Hogrefe et al. 2014; Ward and Amundson 2019) and little is known regarding broader patterns of eelgrass across the state and elsewhere in the north Pacific.

Diseases specific to eelgrass have played roles in eelgrass meadow losses in Europe and the Atlantic and have the potential to emerge elsewhere (Govers et al. 2016; Lindholm et al. 2016). The most severe declines of eelgrass in North America occurred in the 1930s, along the Atlantic coast, where the species suffered an almost complete die-off attributed to a virulent pathogenic strain of protist, *Labyrinthula zosterae* (Short et al. 1987). The loss of eelgrass meadows promoted declines in fish and waterfowl species along the Atlantic coasts of North America and Europe (Cottam et al. 1944). Martin et al. (2016) provided evidence that both pathogenic and non-pathogenic strains of *Labyrinthula* are distributed globally, suggesting similar outbreaks could occur elsewhere. Lesions on eelgrass, attributable to the presence of *Labyrinthula* sp., were recently documented in eelgrass beds in Puget Sound, Washington (Groner et al. 2014; Groner et al. 2016), but disease lesions have yet to be reported from Alaska, the northwesternmost distribution of eelgrass in the eastern north Pacific.

Nevertheless, Martin et al. (2016) provided evidence that both pathogenic and non-pathogenic strains of *Labyrinthula* are distributed globally and developed a cladogenic context for both subtypes; they reported the presence of a species of *Labyrinthula* in eelgrass meadows in Kasitsna Bay on the Kenai Peninsula, Alaska, and that it belonged to a non-pathogenic clade. More recently, Menning et al. (2020) leveraged next-generation sequencing of environmental DNA (eDNA; Peters et al. 2017) in a metabarcoding approach designed to detect the presence of *Labyrinthula* sp. DNA – including strains hypothesized to be virulent (Martin et al. 2016).

Relatedly, Govers et al. (2016) reported the presence of two closely related fungi-like oomycetes species in the Atlantic: *Phytophthora gemini* and a previously undescribed species, *Halophytophthora* sp. *Zostera*. Both species are potent pathogens closely related to the potato blight (*P. infestans*), and both may hinder sexual reproductive success in eelgrass by reducing seed germination up to six-fold (Govers et al. 2016). Like *Labyrinthula*, *Phytophthora* and *Halophytophthora* disease pathogens have played a role in eelgrass meadow losses in Europe and the Atlantic (Govers et al. 2016; Lindholm et al. 2016). The first reported occurrences of members from the blight species complex *Phytophthora*/*Halophytophthora* were recently documented in Alaska waters (Menning et al. 2020), supporting the hypothesis of Govers et al. (2016) that the distribution of *Phytophthora* is likely wider than currently known.

The spatial extent of the eelgrass beds in Izembek Lagoon, the most extensively studied eelgrass bed in Alaska, is considered stable (Ward et al. 1997; Hogrefe et al. 2014). Nevertheless, its aboveground biomass has fluctuated widely in since 2012 (Ward and Amundson 2019), possibly in response to the recent increased water temperatures in the north Pacific (Bond et al. 2015) and Eastern Bering Sea (Gamito et al. 2015). Although prior work by Menning et al. (2020) found both pathogen classes in Izembek Lagoon in Alaska, the prevalence of eelgrass pathogens – which may increase in virulence with increasing water temperatures (Harvell et al. 2002) – has not been characterized across Alaska coastal regions. To better understand the presence and potential extent of these pathogens in the eastern north Pacific, we leveraged the prior eDNA approach (Menning et al. 2020) to assay environmental samples collected from five broadly distributed eelgrass beds in Alaska. For comparative purposes that would allow us to better understand the distribution of these pathogens in the broader north Pacific, we also sampled one site in Japan. In addition to Izembek Lagoon, in Alaska, we extended our sampling to the east to include two meadows in the Gulf of Alaska Large Marine Ecosystem (Kupreanof Island, in Frederick Sound, and Chignik Lagoon, on the southern coast of the central Alaska Peninsula) and three meadows in the Eastern Bering Sea Large Marine Ecosystem (Port Moller Spit and Port Moller Hot Spring, on the northern coast of the middle Alaska Peninsula, and to the north to include Safety Lagoon near Nome on the southside of the Seward Peninsula; Fig. 1). Our sampling in Japan was conducted in an eelgrass bed in Notsuke Bay, in the western north Pacific. Further, we augmented prior research (Menning et al. 2020) from Izembek Lagoon to test our initial hypothesis that there are seasonal differences in the detection of pathogenic and non-pathogenic strains of *Labyrinthula* sp.

2. Methods

2.1 Sample collection

Environmental samples were collected from the water column, sediment, and eelgrass leaves periodically between January 2016 and August 2018. Water samples were collected in 500 mL volumes and filtered through 0.22 micron filters (GTTP 04700, Millipore), which were then stored in 5 mL of Longmire Buffer (LMB) (Longmire et al. 1997) held in 15 mL Falcon tubes (Menning et al. 2018). Sediment samples were collected in 1 mL volumes and stored in 15 mL tubes containing 5 mL of LMB. Approximately eight centimeters of plant tissue (leaf) were collected and stored in 15 mL tubes containing 5 mL of LMB. Five replicates of each sample type were collected and processed individually at each location unless otherwise noted.

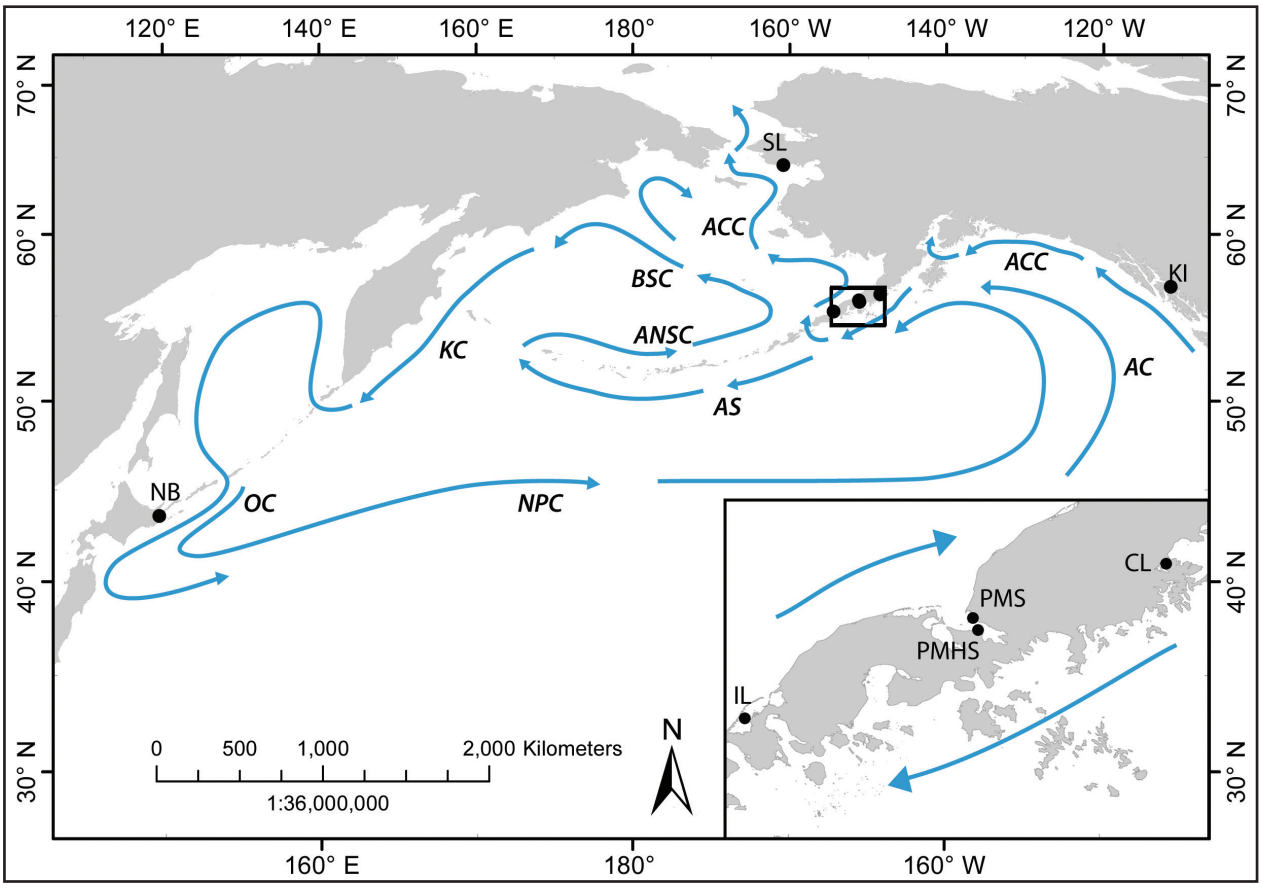

Figure 1. Locations of sampling sites and major oceanic currents in the north Pacific. Eastern Bering Large Marine Ecosystem sites: SL = Safety Lagoon, PMS = Port Moller Spit, PMHS = Port Moller Hot Spring, IL = Izembek Lagoon. Gulf of Alaska Large Marine Ecosystem sites: CL = Chignik Lagoon and KI = Kupreanof Island. NB = Notsuke Bay, Japan. Arrows indicate general direction of major oceanic currents: AC = Alaska Current; ACC = Alaska Coastal Current; ANSC = Aleutian North Slope Current; AS = Alaska Stream; BSC = Bering Sea Current; KC = Kamchatka Current; NPC = North Pacific Current; OC = Oyashio Current.

2.2 DNA extraction

Stored environmental samples were vortexed and eDNA extracted from a 400 µL subsample of the LMB-preserved sample using a Qiagen DNeasy Blood and Tissue kit (Qiagen) following manufacturers suggested protocols, with the exception that volumes were doubled. To avoid contamination, all extractions were conducted in a laboratory in which Polymerase Chain Reactions (PCRs) have never been conducted and which is separated physically from laboratories in which PCRs are conducted.

2.3 DNA library preparation and sequencing

Environmental DNA libraries were prepared using custom primers, a two-step PCR protocol, and sequenced using an Illumina MiSeq following Menning et al. (2020). Briefly, a first PCR was conducted using each eDNA sample with each locus specific primer separately to develop amplicons. After the first PCR excess primers and dNTPs were removed using ExoSap (Affymetrix), all loci were quantified by fluorometry using a Quant-IT Broad Range kit (Thermo Fisher Scientific), normalized to equal concentrations, and pooled by sample. To produce barcoded amplicons, a second PCR was performed on each locus pooled sample using I5 and I7 Nextera XT index primers. All second step PCR products were quantified by fluorometry using a High Sensitivity Quant-IT kit (Thermo Fisher Scientific), normalized to equal concentrations, and pooled. Quantified pooled PCR products were electrophoresed on a 1% polyacrylamide gel to estimate PCR fragment sizes, gel purified using Qiagen Gel purification kit (Qiagen), quantified by fluorometry using a Quant-IT High Sensitivity kit (Thermo Fisher Scientific), diluted to 2 nM concentrations following Illumina guidelines (Illumina Document # 15039740 v01), re-quantified by fluorometry using a Quant-IT High Sensitivity kit, and further diluted to 20 pM following the Illumina NextSeq Protocol A (Illumina Document #15048776 v02) for library dilution. All remaining steps for library preparation followed Illumina MiSeq protocols (Illumina Part #15034097 Rev. B). Both the eDNA library and PhiX were subsequently diluted to 15 pM. Sequencing was performed using an Illumina MiSeq 300 cycle v2 reagent kit (2 X 151 paired-end cycle runs) (Illumina Part #MS-102-2002) on an Illumina MiSeq with a 30% PhiX spike.

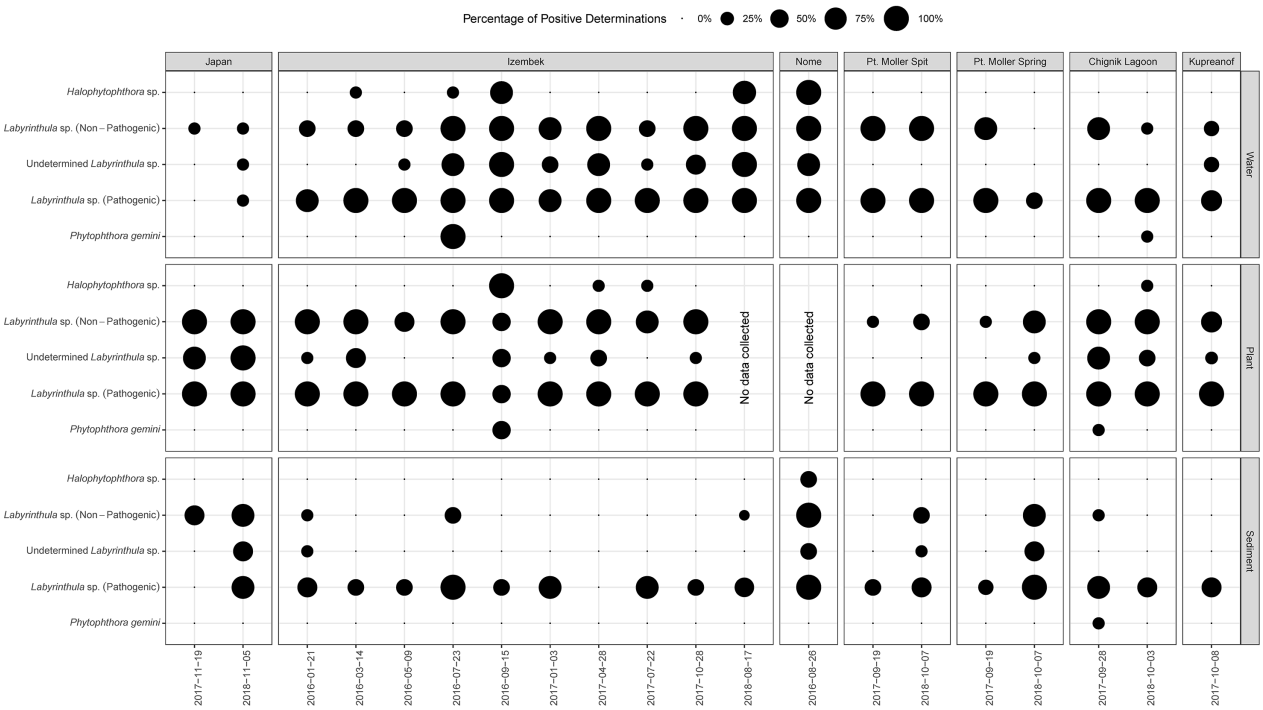

Figure 2. Percentage of genomic reads of potential pathogen lineages: *Labyrinthula* sp. (Pathogenic, Non-pathogenic, Undetermined), *Halophytophthora* sp. Zostera, and *Phytophthora gemini* in *Z. marina* leaves, sediment, and water column from Notsuke Bay, Japan during fall sampling in 2017 and 2018; Izembek Lagoon, Alaska, during seasonal collections in 2016–2017, and in summer 2018; middle Alaska Peninsula eelgrass beds in Port Moller (two sites) and Chignik Lagoon, Alaska, during fall sampling in 2017 and 2018; Safety Lagoon, Nome, Alaska in Fall 2016; and Scow Bay, Kupreanof Island in the Alexander Archipelago, southeastern Alaska in Fall 2017.

2.4 Bioinformatic analyses

All de-multiplexed data were retrieved from the Illumina MiSeq and analyzed in the same manner as Menning et al. (2018) using the reference database developed by Menning et al. (2020) with the exception that the default BLAST+ parameters reward/penalty were changed to 1/-3 respectively and the gapopen/gapextend parameters were set at 1/1 to ensure at least a 99% match to the reference database. Quality-filtering to remove sequencing errors was conducted by only including match count information that exceeded 0.01% of the total number of reads passing filter per sample in the MiSeq run (Bokulich et al. 2012). Identifications including matches to both pathogenic and non-pathogenic strains were identified as "Undetermined *Labyrinthula* sp." Bioinformatic analyses were conducted using the USGS Yeti Supercomputer (USGS Advanced Research Computing).

3. Results

We observed annual and seasonal variation in the presence of blight species in Izembek Lagoon. We detected *Halophytophthora* sp. from only one water sample and one plant sample in Izembek Lagoon during the summer of 2016, whereas we detected reads from *P. gemini* in plant samples collected in summer 2017 and in water samples during the summers of 2016 and 2018. In contrast, we found indicators of both non-pathogenic and pathogenic *Labyrinthula* strains in Izembek Lagoon during nearly every sampling period (Fig. 2).

On the Alaska Peninsula, in samples collected from Chignik Lagoon, *Halophytophthora* sp. was only found in one plant sample and *Phytophthora gemini* was found only once in each of the sample types. However, we found pathogenic and non-pathogenic strains of *Labyrinthula* sp. at all sites sampled along the Alaska Peninsula (Port Moller Spit, Port Moller Hot Spring, and Chignik Lagoon) (Fig. 2).

We detected reads from *P. gemini* in samples collected in Safety Lagoon, the northwesternmost Alaska lagoon sampled, in the summer of 2016; we note that reads from *P. gemini* were also found in Izembek Lagoon, another Eastern Bering Sea Large Marine Ecosystem (LME) locale, during the same period. In contrast, we did not detect reads from *P. gemini* in samples collected in 2017 from Scow Bay (Kupreanof Island), the southeasternmost site sampled. Reads from *Halophytophthora* sp. were not detected in samples collected from Safety Lagoon or Scow Bay; however, reads from *Labyrinthula* spp., including pathogenic lineages, were detected at these locations (Fig. 2).

No samples from Notsuke Bay, Japan yielded reads from *Halophytophthora* sp. or *Phytophthora gemini*. Interestingly, pathogenic strains of *Labyrinthula* sp.

were found in all samples in both 2017 and 2018 but no non-pathogenic strains of *Labyrinthula* sp. were found in the sediment or water column in 2017 in Japan (Fig. 2).

All Illumina MiSeq data can be found at NCBI BioProject PRJNA548352, and sample information can be found in Menning et al. (2021).

4. Discussion

Our prior research found two classes of known or suspected eelgrass pathogens – *Labyrinthula* and *Halophytophthora* spp./*Phytophthora* spp. – in the largest eelgrass bed in Alaska, Izembek Lagoon (Menning et al. 2020), but that study did not address the pathogens' wider distribution in north Pacific waters, where Alaska represents the northwesternmost extent of eelgrass range in North America. The results of this pilot study suggest the two classes of known or suspected pathogens on *Z. marina* are more widely distributed in the north Pacific than previously demonstrated; although *Halophytophthora* spp. and *Phytophthora* spp. were not detected in eDNA samples collected from Scow Bay (Kupreanof Island) in southeast Alaska's Alexander Archipelago, Safety Lagoon (Nome, Alaska), or Notsuke Bay (Japan), they were detected in Chignik Lagoon on the Alaska Peninsula. These results indicate that the distribution of disease pathogens is not well understood and that continued sampling is required.

Sequences from *Labyrinthula* strains, including from pathogenic and non-pathogenic clades (Martin et al. 2016), were detected at all sites. We did not test the pathogenicity of *Phytophthora* and *Halophytophthora* strains in north Pacific waters, but Govers et al. (Govers et al. 2016) suggest that pathogenic strains of both blight species cause widespread infection of eelgrass across the northern Atlantic and Mediterranean by reducing seed germination in eelgrass. Notably, both classes of pathogens – virulent strains of *Labyrinthula*, and blight species – were detected in eelgrass meadows at Izembek Lagoon.

Izembek Lagoon. A prior eDNA study (Menning et al. 2020) uncovered annual and seasonal variation in the presence of blight species in Izembek Lagoon (one of the largest and most productive eelgrass meadows in North America), and we extended those data with collections made in August 2018. The basis for the ephemeral occurrence of *Halophytophthora* sp. at Izembek remains unclear, but its introduction to the lagoon may be linked to migratory birds and/or to water transport via the Alaska Coastal Current (Fig. 1) from other locales along the Alaska Peninsula (Menning et al. 2020). There also appeared to be a shift in the presence of *Halophytophthora* from the water column to plants, beginning in July and extending into September (Fig. 2). Thus, blight species are not present on plant tissue during winter months, but rather attach to plants during the summer months, with unknown effect. In contrast, *Labyrinthula* species, including virulent lineages, are found year-round in Izembek Lagoon. Environmental factors such as temperature changes may be responsible for seasonal differences in pathogen occurrence of blight species and the observed variability of positive determinations of *Labyrinthula* sp., although further research is required to test this.

Middle Alaska Peninsula. We only detected *Phytophthora gemini* and *Halophytophthora* sp. during different sampling events in Chignik Lagoon. However, *Labyrinthula* sp. were found in almost all eDNA samples collected at all three locations on the Alaska Peninsula, which were sampled during the months of September and October (Fig. 2). This suggests that the virulent lineages of *Labyrinthula* are resident in lagoons along the middle portion of the Alaska Peninsula. This differs from Izembek Lagoon, where *Halophytophthora* sp. was detected only during summer months. It is unclear whether these differences were due to sampling bias or some environmental factors such as water temperature or differences of water conditions between the Gulf of Alaska LME and those found in the Bering Sea LME. Additional sampling across seasons in these and additional lagoons is needed to address this disparity.

Distributional extremes. Safety Lagoon, Scow Bay, and Notsuke Bay. Comparison of results from the western north Pacific locale (Notsuke Bay, Japan) with the northernmost (Safety Lagoon, Alaska) and southernmost (Scow Bay) locales in the eastern north Pacific provide inference for assessing the boundaries of the distribution of both pathogen classes in the north Pacific. *Phytophthora gemini* was detected in samples collected in Safety Lagoon in the summer of 2016 but not in Notsuke Bay or Scow Bay; we note again that *P. gemini* was also found in Izembek Lagoon during same period. As hypothesized for Izembek Lagoon, *P. gemini* may be transmitted by migratory birds, and/or from ocean currents from the south passing by Safety Lagoon, such as the Alaska Coastal Current (Fig. 1) (Menning et al. 2020). Notably, *Phytophthora gemini* was not detected in samples collected in 2017 from Scow Bay, the southeasternmost site sampled, indicating the mode of transmission in more northerly sites may be something other than water currents. To date, *P. gemini* in Alaska coastal waters has only been detected during spring and summer months in eelgrass meadows located within the Eastern Bering Sea LME. Conversely, *Labyrinthula* spp., including pathogenic lineages, were detected in Safety Lagoon, Scow Bay, and Notsuke Bay indicating that *Labyrinthula* sp. may be ubiquitous in north Pacific coastal habitats; however, a more thorough survey of *Labyrinthula* occurrence is needed to validate this hypothesis. Nevertheless, our findings provide support for the hypothesis of Martin et al. (Martin et al. 2016) that *Labyrinthula* is globally ubiquitous.

Lohan et al. (2020) demonstrated a weak positive correlation between the number of seagrass species and the number of *Labyrinthula* lineages at sites along Florida's Atlantic Coast, leading to a suggestion that increased seagrass diversity might also increase diversity in *Labyrinthula.* Only two species of seagrass – *Z. marina* and *Phyllospadix serrulatus* – are known to occur in

Alaska with *Z. marina* the more common species. *P. serrulatus* is rare and highly localized and has yet to be sampled for pathogens. Given Lohan et al. (2020), we would expect that seagrass pathogen diversity to be lower in coastal waters in Alaska than Florida. We also note that Japan hosts a rich diversity of seagrass species (Miki 1934a, b; Aioi and Nakaoka 2003; Kuo et al. 2006), certainly richer than Alaska, and at least four species of *Zostera* (*Z. marina*, *Z. asiatica*, *Z. japonica*, and *Z. caespitosa*) and at least one species of *Phyllospadix* (*P. iwatensis*) are known from Notsuke Bay and other locales on Hokkaido (Nakaoka and Aioi 2001; Kumon et al. 2003). However, while pathogenic, non-pathogenic, and undetermined *Labyrinthula* strains were all detected in Notsuke Bay, no blight lineages were detected. The relationship between seagrass and pathogen diversity is a fruitful avenue for future research in the north Pacific, with Alaska representing a depauperate seagrass ecosystem.

5. Conclusion

Our research from this pilot study suggests that *Zostera marina* pathogens are found in widely dispersed eelgrass meadows in the north Pacific, and the presence of these pathogenic agents may differ annually and seasonally. It is important to note that, currently, there appears to be no detrimental impact on *Z. marina* in the northeastern Pacific by these pathogens. Continued sampling is required to determine if evolving environmental conditions will change the pathogenicity of these organisms and negatively impact *Z. marina*. Our research from this pilot study also validated our metabarcoding system and showed that it facilitates inexpensive and rapid bioassessment of the distribution of disease pathogens on eelgrass in north Pacific waters and that this approach could be used for biomonitoring and assessment of *Z. marina* throughout lagoon ecosystems in both the Gulf of Alaska and the Eastern Bering Sea LMEs. Continued sampling of eelgrass and tracking of environmental parameters related to eelgrass health and pathogen presence can provide managers with the information needed to understand the future distribution and abundance of both *Z. marina* and potential *Z. marina* pathogens.

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All demultiplexed Illumina MiSeq data can be found at NCBI BioProject PRJNA548352, and sample information will be made publicly available via data release upon publication acceptance. All other data are available in the main text.

Disclaimers

The findings and conclusions in this article are those of the author(s) and do not necessarily represent the views of the U.S. Fish and Wildlife Service.

This paper has been peer reviewed and approved for publication consistent with USGS Fundamental Science Practices (https://pubs.usgs.gov/circ/1367/).

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Supplementary material 1
Table S1. Pathogen primers
Author: Damian M. Menning
Data type: PCR primers list

Link: https://doi.org/10.3897/mbmg.5.62823.suppl1

Supplementary material 2
Reference Database accessions
Author: Damian M. Menning
Data type: NCBI accession numbers
Explanation note: The list of NCBI accsessions used in the custom reference database.

Link: https://doi.org/10.3897/mbmg.5.62823.suppl2