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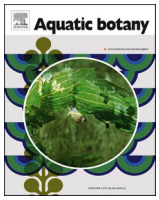

Low pH enhances germination of eelgrass (*Zostera marina* L.) seeds despite ubiquitous presence of *Phytophthora gemini*

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ABSTRACT

Seagrasses are foundation species in coastal ecosystems promoting biodiversity and community structure. Future marine carbonate chemistry under ocean acidification may enhance seagrass physiology, but little is known about how reproductive ecology and disease will integrate into future ocean conditions. A novel pathogen, *Phytophthora gemini*, infects >90 % of eelgrass, *Zostera marina,* surveyed in Northern Atlantic and Mediterranean populations reducing annual germination 6-fold. Our study investigated the combined effects of ocean acidification and *P. gemini* infection on germination of eelgrass seeds. We conducted a two-level factorial experiment crossing four pH levels (Δ0, - Δ0.3, - Δ0.6, -Δ0.9; relative to the average pH at the sampling site) with three infection levels (infected, non-infected, exposed) to determine germination rate and infection response. Prior to experimentation, flowering shoots were collected and held in flow-through seawater tanks where seeds ripened naturally. Once collected, seeds were held in copper sulfate solution (27.37 ± 1.57 ppt) and stored in darkness to mimic winter dormancy (4 °C). Before the start of the experiment, viable seeds were cultured on oomycete selective growth media to determine infection status. By the end of the experiment, 100 % of tested seeds, regardless of treatment, contained *P. gemini*. Germination rate significantly increased with decreased pH. Our findings indicate that *P. gemini* is not inhibited by ecologically relevant changes to carbonate chemistry and standard handling practices can result in effective and highly virulent disease transmission. These results confirm perennial populations of eelgrass are susceptible to infection and alerts conservationists to additional considerations necessary for successful eelgrass restoration.

1. Introduction

Ocean acidification generated from anthropogenic CO_2 emissions has resulted in disruptions to ocean carbonate chemistry (Rudd, 2014, Mason et al., 2017). The ocean acts as a carbon sink, absorbing approximately one third of total anthropogenic CO_2 (Sabine et al., 2004). Increased [$CO_{2\ (aq)}$] disrupts the balance of inorganic carbon by increasing the concentrations of carbonic acid (H_2CO_3), bicarbonate (HCO_3^-), and hydrogen ions (H^+) (Elderfield et al., 2005). The efflux of [H^+] logarithmically decreases the pH of seawater (Gattuso and Hansson, 2011). Thus, as a result of increased CO_2 emissions, the pH of the upper ocean has decreased by 0.1 units since the Industrial Revolution equating to a 30 % increase in [H^+] (Guinotte and Fabry, 2008, Gattuso and Hansson, 2011).

Seagrasses are foundational to coastal ecosystems by promoting biodiversity, stabilizing sediment, cycling nutrients, and sequestering carbon (Heck and Orth, 1980, Hemminga and Duarte, 2000, Hernan et al., 2016, Infantes et al., 2022). Species like *Zostera marina* or *Posidonia oceanica* have shown increased rates of photosynthesis, reproduction, below ground biomass, and carbohydrate production in response to increased availability of CO_2 typically associated with lower pH (Koch et al., 2012, Garrard and Beaumont, 2014, Takahashi et al.,

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2015, Zimmerman et al., 2017). *In situ* carbon enrichment can influence the elemental ratios of C:N making plant tissue more palatable for herbivores, while simultaneously decreasing phenolic compounds responsible for anti-microbial responses (Arnold et al., 2012, Hernan et al., 2016). However, there is almost no data predicting how ocean acidification will integrate into seagrass disease ecology. As an emerging field, there is an increasing interest in seagrass diseases (Nordlund et al., 2024) and how emerging pathogens will respond to changing climates and ocean chemistry.

Phytophthora gemini and its conspecifics *P. inundata, P. chesapeakensis* and *Halophytophthora* spp. are marine oomycetes recently found to infect marine angiosperms (Man in 't Veld et al., 2011). Previously, *Phytophthora* species were known as terrestrial plant pathogens famously causing the "Irish Potato Famine" (Khan et al., 2004). Through leaf blight and concomitant root and stem rot, *Phytophthora infestans* is collectively responsible for over $1 billion dollars' worth of crop loss per year (Haas et al., 2009). Recent efforts have characterized the near ubiquitous nature of *P. gemini* across eelgrass beds in the Northern Atlantic and the Mediterranean. To identify the presence of *Phytophthora,* seeds are collected and placed on an oomycete selective media (ParpH), where if positive, are sequenced to confirm identity (Man In't Veld et al., 2011, Govers et al., 2016). Over 99 % of tested eelgrass meadows are naturally infected by *Phytophthora*, yet avenues for infection are largely unknown (Govers et al., 2016). Experimental work has revealed that infection of *Phytophthora* spp. significantly reduces seed quality and its virulence (i.e. the severity of infection) results in a 6-fold reduction of germination for contaminated seeds (Govers et al., 2016). Copper sulfate ($CuCO_4$) has been shown to significantly reduce infection without inhibiting germination providing a potential solution for restoration specialists (Govers et al., 2017).

Seagrass disease may be magnified by global change scenarios due to the amplification of biotic and abiotic stressors (Sullivan et al., 2018). Rapidly changing habitats (i.e., elevated seawater temperature, altered carbonate chemistry, increased nutrient loading) may push seagrasses beyond their natural physiological plasticity (Orth et al., 2006). As such, eelgrass may prove increasingly vulnerable to an otherwise uncharacterized and potentially destructive pathogen (Govers et al., 2016). Little is known about how *Phytophthora* will respond to changing oceans. Studies conducted on terrestrial *Phytophthora* demonstrate decreased virulence outside its physiological range with a pH of 3 negatively affecting the motility and germination of *Phytophthora* zoospores (Kong et al., 2012). Species survival has been reported in ranges of 3–11 units with several *Phytophthora*-species showing optimal survival and virulence around pH 7 (Kong et al., 2012). Research conducted with terrestrial *Phytophthora* has characterized its ability to navigate complex environments and its ability to "sense" plant matter in space (Kasteel et al., 2023). These studies were conducted in freshwater media (pH 7) supporting earlier work regarding optimal pH conditions. There are no data to predict how eelgrass-infecting *Phytophthora* species (*Phytophthora gemini, Phytophthora inundata, Phytophthora chesapeakensis or Halophytophthora* spp.) will respond to ocean acidification, nor how its virulence will influence eelgrass germination in changing conditions (Man in 't Veld et al., 2019).

The presence of *P. gemini* within the North Atlantic may be of increasing concern to restoration specialists as current efforts focus on using seed-based restoration techniques to increase the areal coverage of eelgrass meadows. Eelgrass restoration is contingent upon sexual reproduction and the availability of robust seedbanks to safeguard against population bottlenecks and to enhance phenotypic resiliency (Jarvis and Moore, 2010, Jarvis et al., 2014). The most successful restoration initiative utilized over 70 million seeds to restore approximately 3600 ha of eelgrass meadows (Orth et al., 2020). As sea surface temperatures are increasing, restoration specialists may begin to transplant more thermally tolerant phenotypes of eelgrass northward via the utilization of seeds originating 1000 s of km away (DuBois et al., 2022). Without proper precautions, this may unintentionally spread or infect meadows with geographically disparate phenotypes of *Phytophthora* which could display heightened virulency outside its home range (Govers et al., 2016). Restoration practices vary by latitude and phenotype, but most practices utilize large vessels stored at high salinity/low temperature to maintain seed viability through time (Marion and Orth, 2010, Infantes et al., 2016). Little is known about the mechanisms of infection for marine *Phytophthora* species, and it is possible that current practices could unintentionally concentrate *Phytophthora* within storage vessels resulting in downstream infection. Seed-based restoration is necessary for restoring eelgrass meadows and accounting for *Phytophthora* must be integrated into current strategies.

The aims of this study were twofold: 1) we sought to determine how changing carbonate chemistry modulates infection rate of *Phytophthora gemini* and 2) we aimed to explore how a range of carbonate chemistry influences eelgrass germination. Because terrestrial species of *Phytophthora* are not inhibited by low pH, we do not expect low pH to inhibit infection. In addition, we hypothesize that *P. gemini* will not inhibit the process of germination, but rather reduce survival of newly germinated embryos. This will prevent the establishment of seedlings, thereby reducing the reproductive output of eelgrass . Finally, because adult eelgrass responds favorably to increases in available CO_2, we do not expect increased CO_2 to reduce germination.

2. Methods

2.1. Seed collection, viability, and infection status

Flowering shoots of eelgrass were harvested in August 2018 from a meadow in North-West Sweden (Gullmars Fjord, Lindholmen, 57.703703°N, 11.393°E). Shoots were stored in a 1500 L seawater flow-through tank outdoors at Kristineberg Marine Station until seeds matured and were released from the flowering shoots. Following the methods of Infantes and Moksnes (2018), the tank had incoming seawater and compressed air pumped into the bottom to increase water mixing. In addition, a mesh net was placed on top of the shoots to keep them submerged and avoid desiccation. When seeds matured and fell to the bottom of the tank, they were siphoned into secondary containment. To prevent pre-mature germination and to mimic winter dormancy, seeds were then stored in sterilized seawater for 5 months in cool (4°C), dark conditions at a salinity of 27.37 ± 1.57 ppt (Infantes et al., 2016). Winter conditions followed by increased temperature and decreased salinity (mimicking early spring) can trigger germination for *Z. marina* in this region (Infantes et al., 2016). From this population, two thousand seeds were selected and treated with a 0.2 ppm copper sulfate ($CuSO_4$) solution for three months. This method has been shown to reduce the infection rate of *Phytophthora gemini* by 86 % (Govers et al., 2017). Because $CuSO_4$ readily absorbs into storage containers, the storage solutions were measured and adjusted to 2.0 ppm weekly for 12 weeks (Govers et al., 2017). Assuming robust and viable seeds, $CuSO_4$ solution at these concentrations does not negatively influence seed viability or germination (Govers et al., 2017).

Before seeding the experiment, all seeds were screened for viability using sinking velocity as a proxy (Marion and Orth, 2010, Infantes and Moksnes, 2018). All seeds were dropped individually into a graduated cylinder filled with ultra-pure seawater (32 ppm) with forceps sterilized with 90 % molecular grade ethanol (Govers et al., 2016). Sinking velocity was calculated by timing each seed's descent through 30 cm of seawater using a stopwatch (+/- 0.25 s estimated error). Seeds were considered viable if they had a sinking velocity greater than 5 cm s^{-1} and were considered non-viable if they had a sinking velocity less than 4 cm s^{-1} (Marion and Orth, 2010, Infantes and Moksnes, 2018). Non-viable seeds were excluded from the experiment.

Three infection levels of *P. gemini* were used in this experiment: *i)* non-infected seeds, *ii)* infected seeds and *iii)* seeds exposed infection. To assign viable seeds to the relevant infection treatment, infection status was determined prior to the start of the experiment. We used an

oomycete-selective growth medium, known as ParpH, to visualize the presence or absence of *P. gemini* (Jeffers and Martin, 1986). Tweezers sterilized with 90 % ethanol were used to transfer viable seeds onto plates with sterilization occurring after each seed (Govers et al., 2016). Seeds remained on the media with 2–3 mL of autoclaved seawater for 7 days at 18–20 °C. After incubation, seeds were individually examined for presence of *P. gemini* verified by thin branching colonies using a 100x light microscope (Man in't Veld et al., 2011, Govers et al., 2016). Seeds were assigned to infected or non-infected treatments depending on the presence of branching colonies (Fig. 1C, D). Infected seeds were assigned to the 'Infected' treatment. Non-infected seeds were divided between 'Non-Infected' and 'Exposed' treatments. Seeds in the 'Exposed' treatment were placed in experimental units that had an additional filter bag with a sample of ParpH-positive media added to the mesocosm. The inclusion of an 'Exposed' treatment aimed to determine if seeds needed to be in direct contact with *P. gemini* for disease transmission. Once screening was complete, forty seeds were subsampled from the population to molecularly confirm the presence of *P. gemini* by isolating *Phytophthora* DNA using PCR and Sanger sequencing.

2.2. Experimental setup

A seawater flow-through mesocosm system was designed and constructed at Kristineberg Marine Research Station (Fig. 1A, B). Prior to experimental setup, all materials were sterilized (autoclaved or 10 % bleach solution) to eradicate any additional source of disease. This included header tanks, tubing, valve connectors, beakers, and nylon bags used for seeds. The experiment ran on a 14/10 hr light/dark cycle with PAR measurements averaging 200 μmol m^2 s^{-1} and an average ambient salinity of 33.10 +/- 0.45 ppt. Because *Phytophthora* has an average size class of 40.2 – 57.2 microns, including water filtration was necessary to the experimental design (Man in't Veld et al., 2011). As such, source water from the surface of Gullsmars Fjord passed through a 50-micron and then a 20-micron filter to ensure that no additional oomycetes were introduced into the experiment.

The experiment utilized a Latin square design (Hurlbert, 1984) with two header tanks corresponding to each target pH level (n = 2; Δ 0, $-\Delta$ 0.3, $-\Delta$ 0.6, $-\Delta$ 0.9). The pH of the source water averaged 8.09 ± 0.15 for the duration of the experiment and is referred to as Δ 0. These values were used to generate each subsequent pH treatment and are referenced here: $-\Delta$ 0.3 ≈ 7.8, $-\Delta$ 0.6 ≈ 7.5, $-\Delta$ 0.9 ≈ 7.2. Headers were continuously filled with filtered surface seawater via the previously described flow-through system at the Kristineberg Marine Research Station. Air was bubbled into each header tank to prevent hypoxia. The pH was controlled by bubbling pure-CO_2 in the header basins using continuous pH-stat systems (Aqua Medic, Germany). Experimental acidification treatments were continuously maintained at each target pH and adjusted from average ambient conditions through routine, twice weekly pH measurements and subsequent adjustment to the pH-stat systems. Each header tank fed six individual experimental units (800 mL containers). Water overfilled each experimental unit (250 mL min^{-1} or 15x $hour^{-1}$) and exited the flow-through system ensuring that water was never exchanged between treatments.

All pH treatments were crossed with 3 *P. gemini* infection treatments: *i)* non-infected seeds, *ii)* infected seeds and *iii)* seeds exposed infection. A total of 960 eelgrass seeds were divided into 12 treatment combinations (n = 4); resulting in 48 mesocosms each containing 20 seeds tested for *Phytophthora* infection. Before initiating the experiment, seeds experienced a sequential freshwater shock to help lyse the seed coat. The salinity of autoclaved seawater was lowered to 5 ppt for 12 hours and then gradually increased to 30 ppt over a two-day period (De Cock, 1980). Air and water temperature started at 12°C and were increased to 18°C in week 3 of the experiment (incrementally increased by 2°C every 2 days) to help stimulate germination (Infantes et al., 2016). Low salinity in combination with increasing temperatures are thought to simulate springtime cues for germination (Infantes et al., 2016). Seeds within each mesocosm were secured within a nylon mesh bag to prevent seeds from exiting the flow-through system. Seeds were allowed to incubate in the treatments for 90 days. Germination and mortality were assessed weekly with mortality defined as the disintegration of the

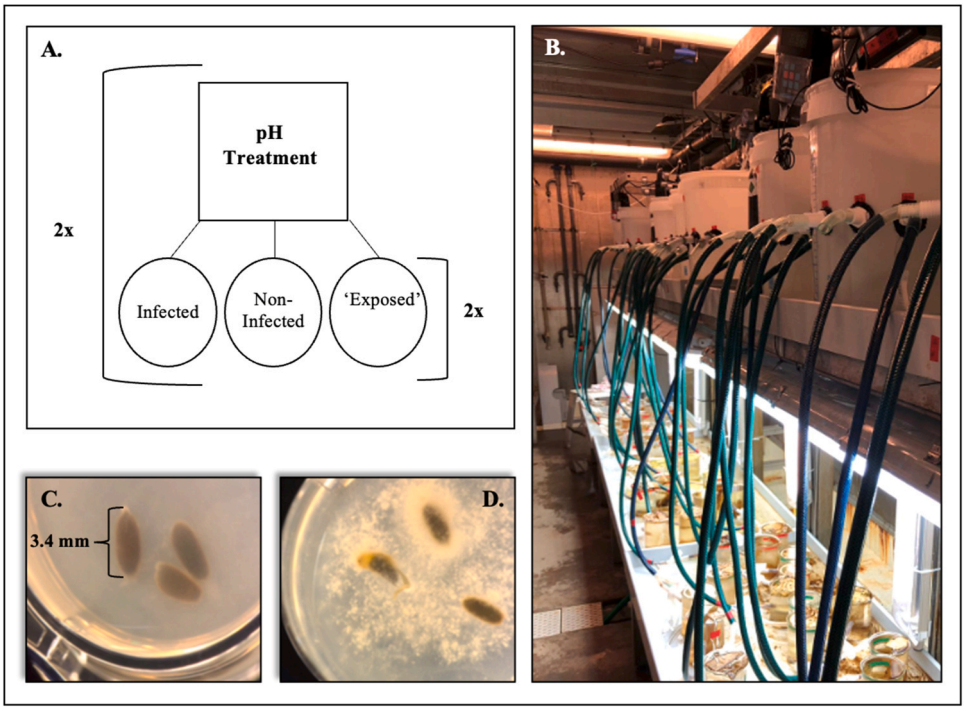

Fig. 1. A) provides a brief experimental schematic. Each pH treatment was replicated twice (n = 2). Each header tank (represented here as "pH treatment") fed 6 mesocosms, each designated with a distinct infection treatment. Each infection treatment at each pH was replicated 4 times. **B)** shows how the experimental design was executed. **C)** shows three eelgrass seeds with no presence of infection by *Phytophthora gemini*. **D)** represents seeds with confirmed infection of *Phytophthora gemini*.

embryo confirmed by "squish test" (Marion and Orth, 2010). Seedling developmental stages were recorded weekly using Xu et al., (2016) (but see Churchill, 1983) as a reference for development.

After the experiment was terminated, mesocosms and seeds were screened for infection using a pear baiting method (Method described in Erwin and Ribeiro, 1996). Pears were suspended in each mesocosm for 72 hours. Presence of infection was noted as deep discoloration of the peel. The wound was excised and incubated on ParpH media for 7 days. In addition, a subsample of 5 seeds per experimental unit were also incubated on ParpH-plates. At the end of the incubation period, both pears and seeds were stored in 100 % ethanol and kept in −80°C freezer until molecular analysis.

2.3. Carbonate chemistry

The pH electrodes of the pH-stat system were calibrated using AquaMedic pH buffers before the initiation of the experiment (AquaMedic). The pH-stat system was adjusted twice weekly to maintain target pH treatments. pH_T was measured across header tanks and replicates twice weekly using a Metrohm 827 pH (Metrohm, Switzerland) glass electrode. The electrode was calibrated on the total scale using TRIS and AMP buffers (CRM from Universite de Liege). Water samples were taken twice weekly for Total Alkalinity (A_T, 60 mL) from each header basin and from a random subsample of 28 mesocosms to ensure that each mesocosm was measured once a week. Each water sample was also measured for salinity (psu) and temperature (°C) using a handheld thermometer and a refractometer. Samples for A_T were filtered on GF/F membranes and analyzed at 22°C using an SI Analytics TitroLine alpha plus automatic titrator. Carbonate chemistry was calculated using A_T and pH_T using CO2SYS v2.1 with dissociation constants set to Mehrbach et al. (1973) refit by Dickson and Millero (1987) (Lewis and Wallace, 1998).

2.4. Molecular analysis

To confirm the presence of *Phytophthora* in the experiment, a subset of samples was analyzed using PCR and DNA sequencing. Samples were collected by scraping ParpH-medium for colonies. DNA was extracted from colonies using an alkaline DNA extraction protocol (Birnboim, 1983; Rudbeck and Dissing, 1998). DNA was added to a PCR master mix containing fungal-specific primers (ITS1 and ITS4) to amplify PCR products (Martin and Rygiewicz, 2005). Amplicons were excised from gel and sent to University of Leiden's Genome Technology Center to confirm species identification. Sanger sequencing confirmed infection to be from fungal pathogen *Phytophthora gemini*.

2.5. Statistics

For parametric testing, data was first plotted to assess the normality of the residuals. Homogeneity of variance was tested using Levene's test. The α value was assigned to 0.05. All data is reported as means ±standard deviation.

Significance between pH treatments was assessed using a One-Way ANOVA. A Two-Way ANOVA was used to assess the relationship between pH and Infection. Infection (in %) was calculated as the number of infected seeds or pear skins divided by the number of samples. To determine if infection significantly increased by the conclusion of the experiment, seed and pear infection rates were analyzed using a χ^2 test. Mortality (in %) was calculated as the total number of dead seeds per mesocosm (i.e. seeds with disintegrated embryos) divided by the number of weeks of the experiment (13 weeks). Analysis of covariance (ANCOVA) was used to determine how infection level and pH determined mortality through time. Germination rate was first calculated as the total number of seeds germinated per mesocosm divided by the number of weeks of the experiment. Each rate was binned to the average pH of its corresponding treatment (Table 1). Germination rate was plotted as a function of pH and fit with a linear model. Of the 48 datapoints, three were identified as outliers (as determined by Q3 + 1.5xIQR or Q1 − 1.5xIQR; IQR = interquartile range) and removed from the analysis. In doing so, we significantly improved both QQ plot and model fit. Statistical analyses and data visualization were performed using R studio ver. 1.4.1106 using the R package Tidyverse (Wickham et al. 2019).

3. Results

3.1. Carbonate chemistry

Acidification treatments (Δ 0, $-\Delta$ 0.3, $-\Delta$ 0.6, $-\Delta$ 0.9) were successfully maintained over the course of the experiment within the header tanks (Table 1; S1), and within the experimental mesocosms (Table S2; S3). Header tanks were significantly different with respect to pH_T ($F_{1,3}$= 50.4, $p < 0.0001$), pCO_2 ($F_{1,3}$= 20.72, $p < 0.0001$), and Total Carbon (C_T) ($F_{1,3}$ = 30.37, $p < 0.0001$). Mesocosms were significantly different with respect to pH_T ($F_{1,3}$ = 356.737, $p < 0.0001$), pCO_2 ($F_{1,3}$= 117.084, $p < 0.0001$), and Total Carbon (C_T) ($F_{1,3}$ = 124.89, $p < 0.0001$). However, when testing between differences of pH and infection level, there were no significant differences between pH_T ($F_{1,2}$ = 0.303, p = 0.739), pCO_2 ($F_{1,2}$= 0.384, p = 0.681), and Total Carbon (C_T) ($F_{1,2}$ = 1.694, p = 0.186). Therefore, carbonate chemistry was significantly different for each pH treatment, but did not significantly vary between infection levels.

3.2. Infection

Post-experiment seed tests revealed that *P. gemini* was present in 100 % of experimental replicates (Fig. 2) demonstrating a significant increase in infection at the seed level throughout the course of the experiment (χ^2-test, $p < 0.001$). As such, this effectively removed the "non-infected" and "exposed" treatments. Similarly, post-experiment water tested using pear-baits revealed that *P. gemini* zoospores were present in 58 % of experimental units (Fig S3). This demonstrated a significant increase in infection throughout the course of the experiment (χ^2-test, $p = 0.024$). Since infection was only tested at the beginning and end of the experiment, we cannot determine when infection occurred over the course of the 90-day experiment.

3.3. Mortality and germination

Average seed mortality was 21.25 ± 1.61 % after 90 days with no significant effect at the level of infection or pH (ANCOVA, model: $F_{3,48}$ = 0.81, p = 0.50). Germination rate was significantly affected by pH (linear model: 13.8 – 1.4 * pH_T; R^2 = 0.25, $F_{(1,43)}$ = 14.4, $p < 0.001$). The germination rate decreased linearly with increasing pH (Fig. 3) resulting in a 140 % higher germination rate at low pH compared to the control. Our experiment resulted in 398 of the 960 seeds successfully passing to Stage 1 (lysis of seed coat and elongation of the cotyledon (Churchill, 1983, Xu et al., 2016)). This corresponds to ~ 42 % of the overall population germinating with only 3 seedlings establishing over the course of the experiment (Table S4).

4. Discussion

Our results confirm the presence of *Phytophthora gemini* in perennial eelgrass seeds supporting earlier work conducted by Govers et al. (2016). Most importantly, this study finds active infection in the presence of *P. gemini* zoospores, confirming that non-infected seeds can become infected when exposed to *P. gemini*. Our results also demonstrate that while low pH facilitates germination rate, it also does not inhibit infection of *P. gemini*. Additionally, our results highlight the extremely infectious nature of *P. gemini* given that all experimental units regardless

Table 1
Carbonate chemistry for the header tanks over the course of the experiment. Values are represented as mean ± standard deviation for each treatment to include replicates (n=3) and averaged over 90 days. The carbonate system speciation (pCO_2, C_T, Ω_{Ca}, Ω_{Ar}) was calculated using pH_T and A_T using CO_2SYS (Lewis and Wallace, 1998), with dissociation constants from Mehrbach et al., (1973), refitted by Dickson and Millero (1987).

Treatment	Temp(∘C)	Salinity (ppt)	A_T (μmol kg^{-1})	pH_T	pCO_2 (μatm)	C_T (μmol kg^{-1})	Ω_{Ca}	Ω_{Ar}
Δ 0	19.06 ± 1.20	33.10 ± 0.45	2352.78 ± 50.00	8.09 ± 0.15	405.04 ± 214.15	2084.65 ± 87.47	4.74 ± 1.07	3.06 ± 0.69
Δ 0.3	19.05 ± 1.23	33.10 ± 0.45	2335.32 ± 29.99	7.63 ± 0.21	1503.82 ± 969.13	2262.07 ± 83.18	2.08 ± 0.74	1.34 ± 0.48
Δ 0.6	18.20 ± 2.13	33.10 ± 0.45	2330.27 ± 40.85	7.43 ± 0.17	2174.12 ± 1042.19	2331.01 ± 64.81	2.05 ± 2.51	0.81 ± 0.28
Δ 0.9	18.75 ± 1.52	33.10 ± 0.45	2309.98 ± 45.11	7.19 ± 0.14	3679.33 ± 1244.53	2389.92 ± 65.33	0.74 ± 0.21	0.48 ± 0.14

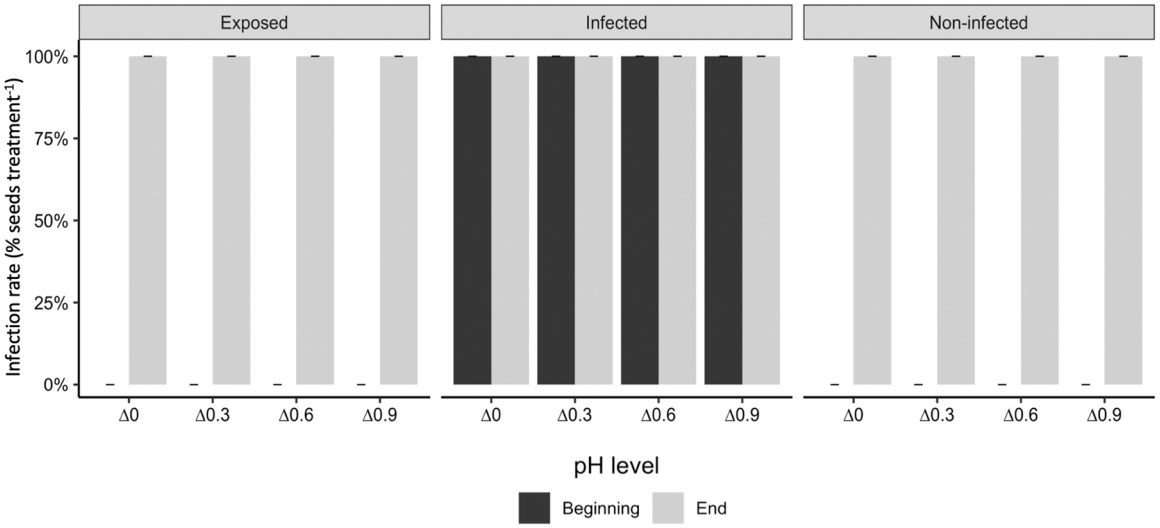

Fig. 2. The presence of *P. gemini* at the beginning of the experiment (black = week 0) versus the end (grey = week 13) using ParpH plating and PCR confirmation. Each bar is a subsample of 5 eelgrass seeds randomly selected from each experimental unit with error bars representing the standard deviation from the mean.

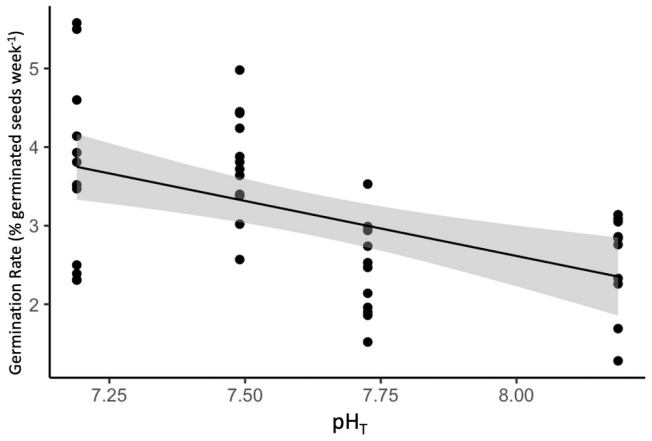

Fig. 3. Significant linear relationship between germination rate (% $week^{-1}$) and pH_T (Germination rate = 13.8 – 1.4 * pH_T (R^2 = 0.25, $F_{(1,43)}$ = 14.4, p < 0.001)). The shaded region represents 95 % confidence intervals of the linear model.

of treatment status were contaminated by the end of the experiment.

Efforts to maintain sterile conditions and eliminate cross contamination were exhaustive. Control mesocosms were only populated with seeds treated with a 12-week course of $CuSO_4$ and a confirmed negative parpH screen (Govers et al., 2017). Experimental materials were bleached or autoclaved when appropriate. We also maintained a water filtration system to exclude external contamination by particles larger than 20 microns (*Phytophthora* size class of 40.2 – 57.2 microns) (Man in't Veld et al., 2011). The experiment was designed with all available best practices for marine *Phytophthora* research, which given its novelty, is still limited (Man in't Veld et al., 2011, Govers et al., 2016, Govers et al., 2017, Man in't Veld et al., 2019). As such, we were not able to quantify inoculation levels and were limited to presence/absence assessments. Despite these limitations, we provide support to the hypothesis that infection must be present inside the seed coat with a possible mode of transmission occurring during flowering and fertilization of the ova (Man in't Veld et al., 2019). We also support the idea that once seeds lyse, the oomycete is re-released back into the water column, where once motile, can colonize, infect, and therefore inhibit newly germinated seeds from developing. Because the experiment was designed to exclude confounding variables like sediment structure or microbial community, this study speaks to the infectious nature of *Phytophthora* when uninhibited by an intact community.

As marine *Phytophthora* research is still in its nascency, no published literature addresses the specific modality of *Phytophthora* colonization, transmission, or infection. The most characterized member of its genus, the terrestrial *Phytophthora infestans* (most notably responsible for the Irish Potato famine), colonizes the surface of the seed coat as well as the seed interior, the funiculus, and the seed hairs (Vartanian and Endo, 1985, Rubin et al., 2007). Our data demonstrates the motility of *Phytophthora gemini* through the presence of *Phytophthora* cultures held in tea bags adjacent to, but not in direct contact of, uninfected seeds. In addition, because multiple points of filtration were utilized, we can comfortably assert the absence of small invertebrates serving as disease vectors (but see van Tussenbroek et al., 2016 for a description of marine "micro pollinators"). Marine *Phytophthora* research is best served by the more advanced field of terrestrial *Phytophthora* research conducted in agricultural systems. Various baiting methods (D'anjou pear, *Rhododendron* spp. whole-leaf) have been used with success to characterize the presence of *Phytophthora* spp. in stream and sediment. Terrestrial systems have repeatedly demonstrated that infested water is a known source for *Phytophthora* and other pathogenic oomycetes (Ghimire et al.,

2009, Rollins et al., 2016, Rodríguez-Padrón et al., 2019, Burgess et al., 2021, Rodríguez et al., 2021, Bourret et al., 2023). Preliminary studies using baiting methods in marine systems have been conducted, but their results are not readily available. Further studies must be conducted to elucidate the sources and sinks of infection in marine systems.

Seagrasses generate phenolic compounds in response to pathogen presence and their characterization is integral to understanding immunology and resilience (Venkataraman et al., 2023). Integrating disease response within ocean acidification and seagrass ecology is difficult as available studies are limited. Generally, adult seagrasses grown in high levels of CO_2 invest in growth, not defense (Zimmerman et al., 2017, Scartazza et al., 2017). Arnold et al. (2012) reported significant decreases in phenolic production of adult *Ruppia maritima* when exposed to elevated CO_2. In addition, a field study conducted along a naturally occurring CO_2 gradient found significant decreases in phenolic production of the Mediterranean *Cymodocea nodosa* in response to decreasing pH (Arnold et al., 2012). In contrast, an additional study designed to capture the transcriptomic response of *C. nodosa* to increased CO_2 found that elevated CO_2 upregulated enzymes involved in the production of polyphenols and isoprenoid compounds (Ruocco et al., 2017). Immunological response in the context of ocean acidification must be further investigated to determine how eelgrass will mechanistically respond to the dual variables of low pH and *P. gemini*.

Our experiment demonstrates that *Phytophthora gemini* is not inhibited by extreme pH conditions ($-\Delta$ 0.9; relative to average ambient pH). Given earlier studies on pH variability in Gullmars Fjord (Sweden), $-\Delta$ 0.9 represents the extreme range of natural variability for projected carbonate conditions by the end of the century (Dorey et al. 2013). Because low pH corresponds to an increase in available bicarbonate (HCO_3^{2-}), low pH increases available substrate for photosynthesis helping to stimulate flowering frequency in eelgrass (Palacios and Zimmerman, 2007, Zimmerman et al. 2017). It is plausible that more flowers (ergo, more seeds) will either keep pace or outpace *Phytophthora* transmission, thereby stabilizing the reproductive output. However, it is also possible that more seeds will result in more robust reservoirs for *P. gemini*. Our data supports increased germination with decreased pH, but to fully quantify that advantage is difficult in the context of disease. In fact, our data emphasizes that any added benefit of low pH to germination is uncertain when there is an active *Phytophthora* infestation.

Interpreting germination within the context of this study and previous research is important. Previous studies have promoted a freshwater treatment to stimulate germination within eelgrass seeds (De Cock, 1980, Xu et al., 2016, Yue et al., 2019). Our study utilized a freshwater treatment (5 ppt) at the start of the experiment; however, we observed no added benefit. At most one seed per replicate (15 of 48 mesocosms) germinated seven days post treatment. Our experiment resulted in 398 of 960 seeds germinating representing ~42 % of the experimental population. Of the remaining population, ~33 % of the seeds remained ungerminated and viable indicating that the freshwater treatment was neither beneficial nor detrimental to the overall health of seed population. In addition, this study continued off work conducted by Lowell et al. (2021), where the authors reported no direct impact of low pH on germination. Lowell et al. (2021) utilized two levels of pH (high $pH_T = 7.84 \pm 0.05$ vs. low $pH_T = 7.55 \pm 0.05$) compared to the range of pH reported in this paper. We believe that the significance of our results is due to the inclusion of additional pH levels. These results emphasize the importance of repeating and refining studies to more thoroughly understand ecological phenomena. Finally, because infection was present in all replicates by the end of the experiment, it is not surprising that mortality in respect to treatment and pH was not significant. Once *P. gemini* proliferated within an experimental unit, any treatment effect was superseded by infection.

Germination and infection rate must be considered through the lens of environmental realism. Little is known about *in situ* germination rates in Scandinavian waters, but seed loss is high with less than 1 % of germinated seeds developing into adult shoots (Infantes et al., 2016). This experiment reflects that estimate with 0.3 % of seeds developing into established seedlings. Our results may be skewed by the absence of sediment, nutrients, and microbial community. Emerging evidence emphasizes the importance of vegetated sediment (i.e. the associated microbial community) in facilitating seed germination (Hanley, *unpublished data*). These observations are supported by recent data indicating a 6 % increase in germination when seeds are colonized with sulfate reducing bacteria (Nakashima and Kusube, 2024). The importance of an intact community cannot be discounted when interpreting these results, however, this experiment was conducted in small, one-liter beakers. Maintaining pH for this experiment required continued vigilance and introducing sediment-microbe interactions would increase difficulty. It is also possible that the experimental design may have eliminated competition and predation among sediment-based microorganisms swinging the pendulum in favor of a *P. gemini* infestation within the mesocosms. This, in combination with high seed densities, may have influenced our end point infection results. We designed this study to optimize statistical power within experimental replicates and did not anticipate seed density to influence disease presence. Additional studies must be conducted to determine how microbial community dynamics and *in situ* seed density influence the potency of *P. gemini* and how this translates to seedling establishment.

Our findings demonstrate the potent virulency of *Phytophthora gemini* in the context of both present day and future ocean pH. Seagrasses are integral to healthy coastal ecosystems serving multiple benefits from critical nursey habitats to carbon sinks. This study presents important information for restoration and emphasizes the need to expand upon *Phytophthora* research in seagrass systems. Restoration practices must be adapted to include preventative measures against *Phytophthora*. Our results emphasize that storing seeds at high densities may increase *Phytophthora* transmission. We recommend storing seeds at low density in 0.2 ppm - 2.0 ppm $CuSO_4$ seawater to reduce *Phytophthora* infection. Not only does $CuSO_4$ significantly reduce *Phytophthora*, but emerging evidence indicates that it may stimulate germination and improve seedling establishment (Sullivan et al., 2022). Perennial meadows rely on seed banks to repopulate after disturbance events, thus the presence of *P. gemini* in these meadows may not have immediate, but long-term effects. Understanding infection rates as well as sources and sinks for disease are critical to furthering our understanding of *Phytophthora* and *Halophytophthora*. We recommend researchers to continue researching this pathogen as pathways of treatment and mitigation are necessary to ensure the long-term conservation of eelgrass meadows.

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CRediT authorship contribution statement

Sam Dupont: Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Methodology, Formal analysis, Conceptualization. **Claudia E.L. Hill:** Writing – review & editing, Visualization, Investigation, Formal analysis, Data curation. **Eduardo Infantes:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Conceptualization. **T. Erin Cox:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Laura Govers:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Bradley Peterson:** Writing – review & editing, Resources, Funding acquisition. **Kirti Ramesh:** Writing – review & editing, Supervision, Project

administration, Investigation. **Alyson Lowell:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Data curation.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use Generative AI or AI-assisted technologies in the writing process.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aquabot.2024.103805.

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