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Marine *Phytophthora* species can hamper conservation and restoration of vegetated coastal ecosystems

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Phytophthora species are potent pathogens that can devastate terrestrial plants, causing billions of dollars of damage yearly to agricultural crops and harming fragile ecosystems worldwide. Yet, virtually nothing is known about the distribution and pathogenicity of their marine relatives. This is surprising, as marine plants form vital habitats in coastal zones worldwide (i.e. mangrove forests, salt marshes, seagrass beds), and disease may be an important bottleneck for the conservation and restoration of these rapidly declining ecosystems. We are the first to report on widespread infection of *Phytophthora* and *Halophytophthora* species on a common seagrass species, *Zostera marina* (eelgrass), across the northern Atlantic and Mediterranean. In addition, we tested the effects of *Halophytophthora* sp. Zostera and *Phytophthora gemini* on *Z. marina* seed germination in a full-factorial laboratory experiment under various environmental conditions. Results suggest that *Phytophthora* species are widespread as we found these oomycetes in eelgrass beds in six countries across the North Atlantic and Mediterranean. Infection by *Halophytophthora* sp. Zostera, *P. gemini*, or both, strongly affected sexual reproduction by reducing seed germination sixfold. Our findings have important implications for seagrass ecology, because these putative pathogens probably negatively affect ecosystem functioning, as well as current restoration and conservation efforts.

1. Background

Phytophthora species, fungi-like oomycetes, are known to be pathogenic to many terrestrial plants species, causing a range of symptoms including root rot, stem rot and leaf blight. *Phytophthora* diseases yearly cause high economical damage to agriculture and loss of fragile ecosystems [1]. *Phytophthora infestans*, or potato blight, the best-known *Phytophthora* species, caused a massive die-off of potato plants in the nineteenth century, resulting in the death of millions during the great Irish famine [2–4]. Even today, potato loss due to this pathogen is estimated at more than 1 billion euros yearly in the European Union only [5]. Another member of the *Phytophthora* genus, *Phytophthora ramorum*, is currently harming forest ecosystems in California by causing mass mortality in oaks [6,7]. *Phytophthora ramorum* is a very successful invasive pathogen that can infect over

109 recorded host species. Its use of more resistant hosts to maintain infectious stages, makes it very difficult to combat [8,9]. In addition, invasive *Phytophthora* species may also cause great harm to fragile systems and communities, such as in southwest Australia, where *Phytophthora cinnamomi* has infected many endemic tree species, turning eucalyptus forest into grass-dominated savannahs [10].

In contrast to the extensive knowledge of terrestrial infections, not much is known about marine *Phytophthora* species [1]. Only recently, two species of *Phytophthora*, *Phytophthora gemini* and *Phytophthora inundata*, were discovered in plant material and seeds of eelgrass, *Zostera marina*, from The Netherlands [11]. Similarly, *Halophytophthora* species are commonly found in salt water systems. However, *Halophytophthora* species, inhabiting brackish and salt water habitats, were recently positioned in a genus separate from *Phytophthora* and seem only distantly related to *Phytophthora* species based on rDNA-ITS sequences [12]. *Halophytopthora* species have, in contrast to pathogenic *Phytophthora* species, only been described as saprophytes—organisms living on organic matter—that play an important role as decomposers in primarily mangrove ecosystems [13–15]. Although some saprophytes can become pathogenic under favourable conditions [16,17], virtually nothing is currently known about possible pathogenicity of marine *Phytophthora* and *Halophytophthora* spp. species on marine plants [11].

The ambiguity about the potential pathogenicity of marine *Phytophthora* and *Halophytophthora* species is disconcerting because recent findings show that at least two *Phytophthora* species can contaminate marine plants [11] (electronic supplementary material, table S1). Massive disease-driven die-offs of seagrasses [18,19] and mangroves [20,21] illustrate the potential harm to a population as a consequence of an outbreak. For instance, the pathogen *Labyrinthula zosterae* was the purported pathogen responsible for the loss of up to 90% of the *Z. marina* beds across the North Atlantic region in the 1930s [19,22], which eventually resulted in the loss of ecological properties such as the rich faunal or waterfowl communities often associated with *Z. marina* [23,24]. Thus, the ecological ramifications of such large die-offs are not limited to the infected species alone.

Marine plants such as salt marsh plants, mangroves and seagrasses are typically habitat-forming species that are vital to ecosystem functioning and provide important ecosystem services (e.g. flood protection, carbon and nutrient storage, biodiversity enhancement) [25–28]. In addition, these vegetated coastal ecosystems are globally disappearing [29,30], and costly restoration efforts, with various success rates, are being undertaken to halt and revert these losses. Given the vital functions of marine plants, their worldwide declines, and the restoration efforts being undertaken to reverse these losses, it is important to identify agents of infection that may contribute to declines or may prevent successful restoration.

Although few studies have reported on *Phytophthora* presence on marine host plants (electronic supplementary material, table S1), it remains unclear (i) how widespread *Phytophthora* and related *Halophytophthora* species are in vegetated marine ecosystems, (ii) if these marine *Phytophthora* and *Halophytophthora* species are harmful to foundation species such as seagrasses and (iii) which environmental conditions promote infection. We therefore used the widespread marine foundation species *Z. marina* as a model species and collected *Z. marina* plant material from across six countries to determine *Phytophthora* spp. and *Halophytophthora* spp. presence across the North Atlantic and Mediterranean region. Secondly, we conducted a laboratory experiment to determine potential harmful effects of *P. gemini* and *Halophytophthora* sp. Zostera under various environmental conditions.

2. Material and methods

(a) Collection of seed material and pre-treatment

To investigate the potential occurrence of *Phytophthora* and *Halophytophthora* species in *Z. marina* across the Atlantic, we obtained representative samples of *Z. marina* leaves and seeds from Denmark (Limfjord, 56.913° N, 9.993° E), Sweden (Lindholmen, 57.703° N, 11.939° E), The Netherlands (Oosterschelde 51.672° N, 4.131° E), France (Thau Lagoon, 43.446° N, 3.663° W) and the USA (Chesapeake Bay, VI, 37.567° N, 76.101° W). These sites were chosen based on the availability of seed material from restoration projects on these sites. These seeds were not used for the experiment, but analysed for only the presence of *Phytophthora* and *Halophytophthora* species, as described below. For the experiment, we collected 6 kg of *Z. marina* aboveground biomass, including seed material, on the island of Sylt, Germany (54.799° N, 8.296° E) in early September 2014. The material was transported to the laboratory where seeds were removed from seed-bearing shoots. For the experiment, we carefully selected seeds under the microscope with ethanol-disinfected equipment in order to select only fully developed, mature seeds with a hardened seed coat, to mimic natural seed development [31]. Selection was needed, as half of the harvested seeds had not yet matured on the shoot and using these would have biased the outcome of our experiment. A subset of these seeds (160 seeds) was individually tested for *Phytophthora* spp. or *Halophytophthora* spp. infection before the start of the experiment, using both visual and molecular techniques as explained below.

(b) Experimental set-up

We tested the effects of winter temperature and sediment type—two main factors controlling winter survival of organisms (and thus both seeds and *Phytophthora*) buried in the sediment [32]—on *Phytophthora* infection and seed germination in a full-factorial laboratory experiment with four treatments and 16 replicates per treatment. Both sediment types and winter temperatures reflected field conditions encountered in restoration experiments in the Dutch Wadden Sea, yielding variable results with regard to restoration success. We applied two temperatures (5.4°C and 12.4°C) mimicking cold and warm winters in the Dutch Wadden Sea [33]. These treatments were crossed with two sediment types: sandy and muddy sediment (electronic supplementary material, table S2) from two contrasting sites where eelgrass restoration experiments have been conducted since 2011. The sediment was sieved (5 mm) and homogenated prior to the start of the experiment. The sediment was tested for *Phytophthora* and *Halophytophthora* presence, but no *Phytophthora* or *Halophytophthora* was found in the sediment.

The experiment was conducted in 0.5-m high round glass tubes with a diameter of 60 mm. To each experimental unit, we added a 0.2 m sediment layer and a 0.25 m layer of synthetic seawater (27‰). Every experimental unit was equipped with a separate pump for aeration to prevent cross-infection among experimental units. This system was sufficient to aerate the entire water column, which is representative of the Wadden Sea winter conditions [34]. Treatments were randomly assigned to the experimental units. After addition of sediment and water, tubes were left for 2 days to ensure sediment stabilization, after which 10 seeds were added to each experimental unit with

ethanol-disinfected forceps to prevent cross-infection of seeds. Seeds were subsequently covered by an approximately 5 mm layer of sediment to mimic natural conditions in the field. Seeds were incubated for 110 days in the experimental units and salinity and temperature were checked twice weekly. After 110 days, the experiment was terminated and the sediment from each unit was carefully sieved (800 µm) to retrieve seeds. On average, 8.3 out of 10 seeds were retrieved from each tube, 13% of the seeds had already germinated before retrieval (69 of the 531 retrieved seeds), with no effects of treatments. Seeds that had already germinated during the experiment were treated similar to the other seeds for further analysis. Retrieved seeds were individually stored in 1.5 ml Eppendorf tubes filled with 200 µl, 23‰ seawater at 4°C for 2 days. Subsequently, tubes containing seeds were moved to the specialized *Phytophthora* laboratory for further analysis.

(c) Visual identification of *Phytophthora* and *Halophytophthora* species

All seeds, those from the survey and the experiment, including germinated seeds, were individually placed on sterile 12 wells tissue culture plates with a growth area of 3.8 cm^2 and a selective growth medium (ParpH) [35]. ParpH is an oomycete-selective agar growth medium to which selected antibiotics are added to promote growth of *Phytophthora*, *Halophytophthora* and *Pythium* species and to suppress growth of non-pythiaceous fungi [35]. Seeds were incubated on ParpH for four weeks in total with a natural daylight cycle at room temperature (18–20°C) with 2–3 ml of artificial seawater (20‰) added to every well/seed. After 3 and 7 days, the presence or the absence of *P. gemini* and *Halophytophthora* sp. Zostera was scored for each individual seed (531 in total) by visual identification, based on colony morphology on ParpH (figure 1*a*) [35]. To distinguish between the two species, branching and roughness of the mycelium was checked by microscope (100×). Visual identification of the oomycetes grown on ParpH was checked on 22% of the infected seeds [36] by growing the isolated oomycete on cherry decoction agar (CHA) and potato dextrose agar (PDA), where colony morphology of *P. gemini* and *Halophytophthora* sp. Zostera can clearly be distinguished (figure 1*b–e*). *Phytophthora gemini* can also be identified by the incidental double sporangia growth on a sporangiophore (by microscope) when cultured on the plate (electronic supplementary material, figure S2). By contrast, *Halophytophthora* sp. Zostera does very rarely grow sporangia when cultured and does not have double sporangia. More detailed information about this method and microscopy on *P. gemini* can be found in Man in 't Veld *et al*. [11]. In addition, seed germination was scored up to four weeks of seed incubation. A selection of four samples of colonies grown on CHA was additionally identified by molecular analysis (see below).

Additionally, to test whether *P. gemini* and *Halophytophthora* sp. Zostera were not only present on the seed coat, but also within the seed itself, we determined *P. gemini* and *Halophytophthora* sp. Zostera growth on ParpH of a separate selection of smashed seeds (not used in the experiment) of which seed coats were first thoroughly disinfected by ethanol and hypochlorite.

(d) Molecular identification of *Phytophthora* and *Halophytophthora* species

Isolations of *P. gemini* and *Halophytophthora* sp. Zostera were made from *Z. marina* seeds on ParpH agar containing pentachloronitrobenzene (Sigma/P7626) 25 mg l^{-1}, pimaricin (Sigma/P-440) 0.0005%, ampicillin (Sigma/A-9393) 250 mg l^{-1}, rifampicin (Sigma/R-8626) 10 mg l^{-1} and hymexazol (Sigma/T-4014) 50 mg l^{-1}. Outgrowing colonies were transferred to CHA [37].

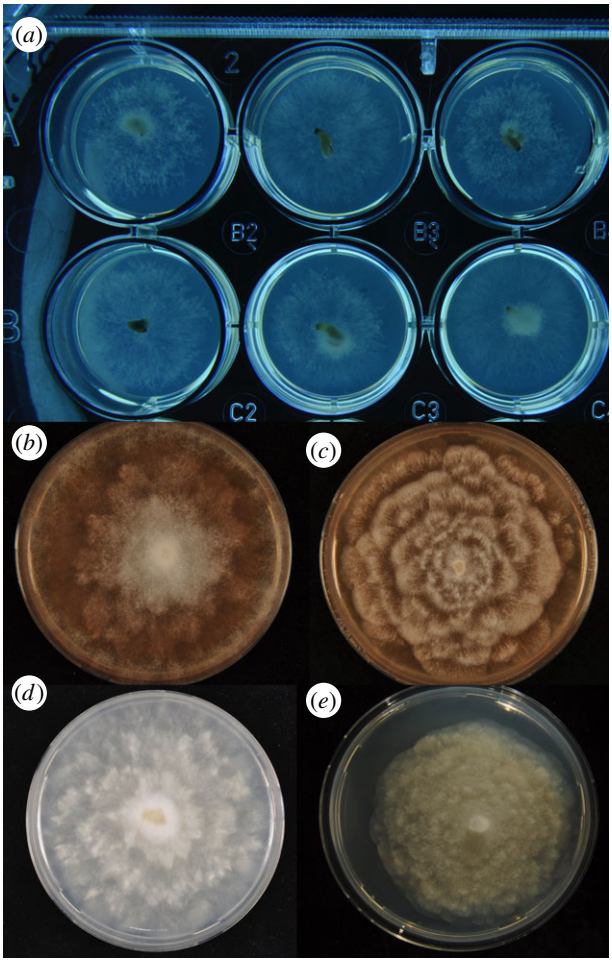

Figure 1. Colony morphology of *Phytophthora gemini* and *Halophytophthora* sp. Zostera on ParpH medium. (*a*) *Zostera marina* seeds are individually cultured on ParpH medium to determine infection. All seeds in this picture are infected by *P. gemini* and the seed in the right corner (well A3) is infected by both *P. gemini* and *Halophytophthora* sp. Zostera. (*b*) Colony morphology of *P. gemini* on CHA, (*c*) colony morphology of *Halophytophthora* sp. Zostera on CHA, (*d*) colony morphology of *P. gemini* on PDA, and (*e*) colony morphology of *Halophytophthora* sp. Zostera on PDA. (Online version in colour.)

Pure mycelium with a surface area of 1 cm^2 was placed in a 1.5-ml micro centrifuge tube with a secure flattop cap (Superlock tubes; BIOzymTC) containing a stainless steel bead (4 mm diameter) and 300 µl of extraction buffer (0.02 M phosphate-buffered saline, 0.05% Tween T25, 2% polyvinylpyrrolidone and 0.2% bovine serum albumin). The tube was placed in a bead mill (Mixer Mill MM300; Retsch) for 80 s at 1800 beats min^{-1}. The mixture was centrifuged for 5 s at maximum speed in a micro centrifuge at 16 100*g* and 75 µl of the resulting supernatant was used for DNA isolation.

Automated DNA isolation was performed with the KingFisher 96 magnetic particle processor (Thermo Electron Corporation, Breda, The Netherlands) using the QuickPick Plant DNA kit from Bio-Nobile (Isogen Life Science, IJsselstein, The Netherlands), according to a protocol developed by the manufacturer. Briefly, 5 µl of proteinase K and 50 µl of lysis buffer were added to 75 µl of the supernatant described above. After 30 min of incubation at 65°C, 5 µl of MagaZorb Magnetic Particles and 125 µl of binding buffer were added. Particle-bound DNA was washed twice with 200 µl of washing buffer and DNA was eluted in 50 µl of elution buffer and further purified using polyvinylpolypyrrolidone (PVPP) (Sigma, Zwijndrecht, The Netherlands) columns. The columns were prepared by filling Axygen Multi-Spin columns (Dispolab, Asten, The Netherlands) with 0.5 cm of PVPP, placing

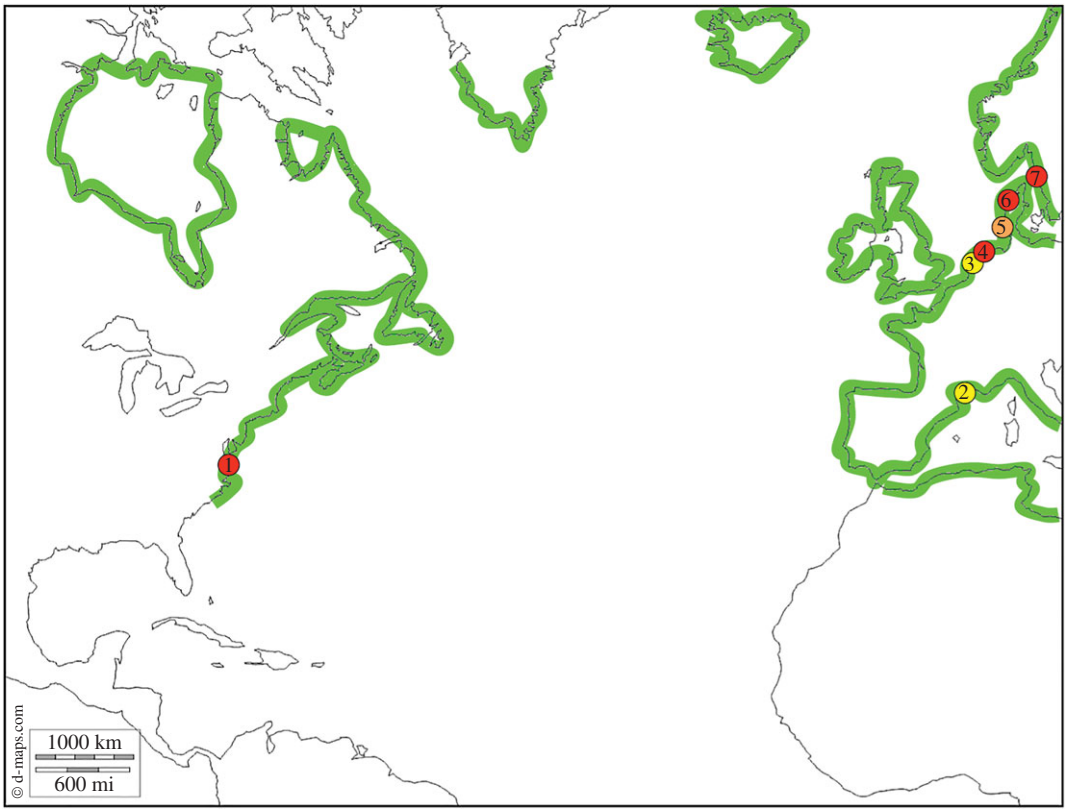

Figure 2. Map of the North Atlantic region with the distribution of *Z. marina* (green), with indicated locations (circles) where *Halophytophthora* spp. (yellow dots), *Phytophthora* spp. (red dots), or both *Halophytophthora* spp. and *Phytophthora* spp. were found in *Z. marina* plant material. Numbers indicate sites: (1) Cheseapeake Bay, USA, (2) Thau Lagoon, France, (3) Oosterschelde, The Netherlands, (4) Grevelingen, The Netherlands (as described in Man in 't Veld *et al*. [11]), (5) Sylt, Germany, (6) Limfjord, Denmark, (7) Lindholmen, Sweden. In total, three species of *Phytophthora* were found: *Phytophthora gemini* (sites 4, 5, 7), *Phytophthora inundata* (sites 4 and 6), *Phytophthora* sp. Chesapeake (site 1). In addition, several *Halophytophthora* species were found (sites 2, 3, 5). (Online version in colour.)

them on empty reaction tubes, and washing twice with 250 µl of DNase- and RNase-free water by centrifuging the columns for 5 min at 4000*g*. The DNA suspension was applied to a PVPP column and centrifuged for 5 min at 4000*g*. The flow-through fraction was used as the template for sequence analysis.

The complete nuclear rDNA ITS1–5.8S-ITS2 region was amplified with primers ITS1 and ITS4 for *Phytophthora* [38] or ITS4 and ITS5 for *Halophytophthora* using the PCR profile described by Goodwin *et al*. [39]. Sequences were edited using Geneious v. 6.1.6 (Biomatters, New Zealand). Sequences were aligned with selected sequences from GenBank and alignments of sequences were made by Muscle using MEGA 5.05. Phylogenetic analysis was performed by neighbour joining using MEGA 5.05. Bootstrapping was done with 1000 replicates.

(e) Chemical sample analysis

In addition, we conducted analyses of sediment characteristics and sediment biogeochemistry, because winter sediment conditions may affect seed viability and infection. Before the start of the experiment, four sediment samples per sediment type were dried (48 h, 60°C). Sediment grain size was analysed on sieved (1 mm) samples by laser diffraction on a Malvern (Master 2000, UK) particle size analyser. Sediment organic matter content was determined by weight loss on ignition at 550°C. Porewater for sulfide measurements was sampled 2 days prior to the end of the experiment using Rhizon soil moisture samplers (Eijkelkamp Agrisearch Equipment, Giesbeek, The Netherlands). Sulfide was measured according to the method described in Govers *et al*. [36]. We measured porewater sulfide concentrations since this indicates sediment anoxia (sulfide is only produced in anoxic conditions), and sulfide is toxic to most eukaryotic life, including marine plants [40] and perhaps also oomycetes, potentially affecting seed survival and infection by *Phytophthora* and *Halophytophthora* species.

(f) Statistical analysis

Infection and germination were analysed using generalized linear mixed models (GLMM, lme4-package in R v. 3.01) with binomial distribution. Treatments were included as fixed factors (sediment and winter temperature), and experimental unit was included as random factor. First, we tested the complete model with all treatments and interactions and stepwise reduced the model by excluding non-significant interactions, starting with the most complex interactions. By this method, we reduced the statistical model until only significant factors were left [41]. GLMM results are reported as B = model estimate, s.e. B = standard error of B, *z* value, and *p*-value. *P*-values of less than 0.05 were considered statistically significant. Total effects of infection (infected or not, regardless by which species) and differences between *P. gemini* and *Halophytophthora* spp. were additionally analysed by χ^2-tests. Results from the sulfide measurements were tested by a two-way ANOVA on log-transformed data. Normality of the data was checked on the residuals.

3. Results

Phytophthora spp. and *Halophytophthora* spp. infection on *Z. marina* appears to be widespread across the Atlantic (figure 2), as we discovered four different *Phytophthora* and *Halophytophthora* species within *Z. marina* seeds and on plant material from six different countries. We found *P. gemini* in *Z. marina* seeds from Sweden (Lindholmen), *P. inundata* in seed material from Denmark (Limfjord), *P. gemini* (GenBank

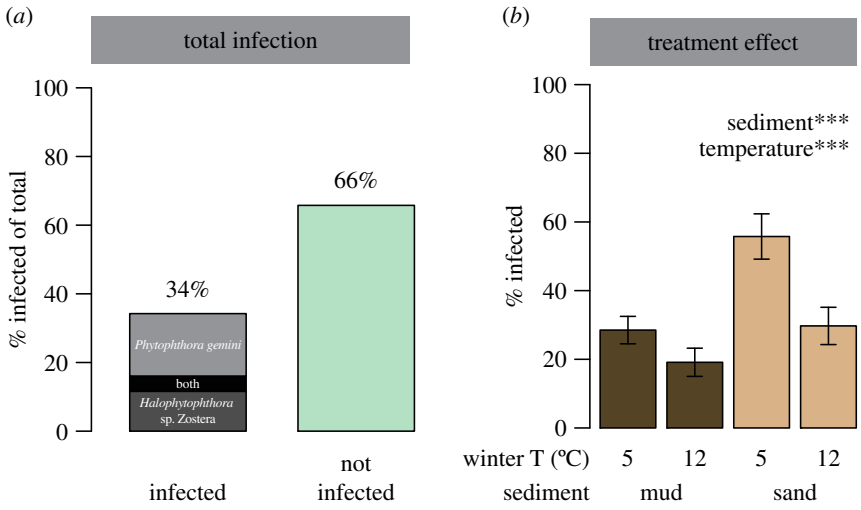

Figure 3. Infection of all retrieved *Z. marina* seeds by *P. gemini* or *Halophytophthora* sp. Zostera after the 110 days incubation experiment. (*a*) Percentage of infected seeds versus non-infected seeds and (*b*) treatment effects (winter temperature, sediment type) on seed infection. Stars indicate significant differences between treatments (***$p < 0.001$), error bars represent s.e.m. (Online version in colour.)

ID. KT986006) and *Halophytophthora* sp. Zostera (GenBank ID. KT986007) in both seed and plant material from Germany (Sylt) (electronic supplementary material, figure S1), and an unknown species of *Phytophthora* (GenBank ID: Man in 't Veld *et al*. [42]) in seed material from Chesapeake Bay, United States, and unknown *Halophytophthora* species in seed and plant material from France (Thau lagoon) and The Netherlands (Oosterschelde).

Ninety-nine per cent of all collected seeds, including those used in the experiment, were infected by either *Halophytophthora* sp. Zostera, *P. gemini* or both species as tested after collection, prior to the incubation experiment. Forty-nine per cent of these seeds were infected by *Halophytophthora* sp. Zostera, 18% by *P. gemini*, and 33% were infected by both oomycete species. On average, 38% of these infected seeds germinated, which indicates that infection is not only associated with dead seeds. Surprisingly, percentages of infection were reduced after 110 days of incubation in the sediment. Only 34% of all seeds remained infected, whereas 66% of the seeds were no longer infected (figure 3*a*). As we retrieved 83% of all seeds, this shift could not be explained by seed loss in the experiment. Of all seeds after incubation, 12% were infected by *Halophytophthora* sp. Zostera, 18% by *P. gemini* and 4% by both species.

Environmental conditions during incubation strongly affected (the reduction of) infection of *Z. marina* seeds by *P. gemini* and *Halophytophthora* sp. Zostera (figure 3*b*). In sand, 1.8× more seeds were infected than in mud (43 versus 24% respectively, GLMM: $B = 0.9809$, s.e. $B = 0.2684$, $z = 3.655$, $p < 0.001$), and in the lower winter temperature, 1.7× more seeds were infected compared with the higher winter temperature (42 versus 24%, respectively, GLMM: $B = -0.9627$, s.e. $B = 0.2671$, $z = -3.604$, $p < 0.001$). The effects of these environmental factors on *Z. marina* seed infection were very similar for both oomycete species (GLMM: *P. gemini* winter temperature, $B = -0.9968$, s.e. $B = 0.3592$, $z = -2.775$, $p = 0.005$, sediment type, $B = 0.9284$, s.e. $B = 0.3586$, $p = 0.009$, *Halophytophthora* sp. Zostera winter temperature $B = -1.1099$, s.e. $B = 0.4062$, $z = -2.733$, $p = 0.006$, sediment type $B = 1.2768$, s.e. $B = 0.4152$, $z = 3.075$, $p = 0.002$).

Infection had strong, negative effects on *Z. marina* seed germination (figure 4, χ^2, $p < 0.001$). Infected seeds had 6× lower germination than non-infected seeds, as only 4% of the infected versus 23% of the non-infected seeds germinated. Both *Halophytophthora* sp. Zostera and *P. gemini* had similar negative effects and did not differ in putative pathogenicity (χ^2, $p = 0.55$).

Environmental conditions (winter temperature, sediment type) did not directly affect seed germination of *Z. marina*. Only infection by either or both *P. gemini* and *Halophytophthora* sp. Zostera (negatively) affected seed germination (GLMM: $B = -2.1576$, s.e. $B = 0.4395$, $z = -4.910$, $p < 0.001$), reducing germination by six times. This trend was similar for both *Halophytophthora* sp. Zostera and *P. gemini* (GLMM: *P. gemini*, $B = -1.6769$, s.e. $B = 0.4824$, $z = -3.476$, $p < 0.001$, *Halophytophthora* sp. Zostera, $B = -2.9289$, s.e. $B = 1.0211$, $z = -2.868$, $p = 0.004$).

Sulfide concentrations of the sediments were generally low (less than 5 $\mu mol\, l^{-1}$), although warmer winter temperatures resulted in significantly higher sulfide production in the muddy sediment (30 $\mu mol\, l^{-1}$, $F_{1,53} = 7.22$, $p = 0.009$) due to higher decomposition rates.

4. Discussion

Until now, nothing was known about the presence and potential harmful effects of *Phytophthora* and *Halophytophthora* species on marine vascular plants [11,43]. Here, we showed for the first time that occurrence of *Halophytophthora* sp. Zostera and *Phytophthora* spp. is widespread in plant and seed material of the marine foundation species *Z. marina*, with natural infection numbers as high as 99%. Moreover, we provide the first account of putative pathogenicity of *P. gemini* and *Halophytophthora* sp. Zostera on *Z. marina*, as germination of seeds infected by either or both species was nearly six times lower. Overall, these findings suggest that both *Halophytophthora* sp. Zostera and *Phytophthora* spp. are common in *Z. marina* beds, and that, depending on prevailing environmental conditions, these oomycetes can hamper sexual reproduction in *Z. marina* populations by decreasing seed germination.

Clearly, our analyses do not yet provide definitive proof for pathogenicity of *P. gemini* and *Halophytophthora* sp.

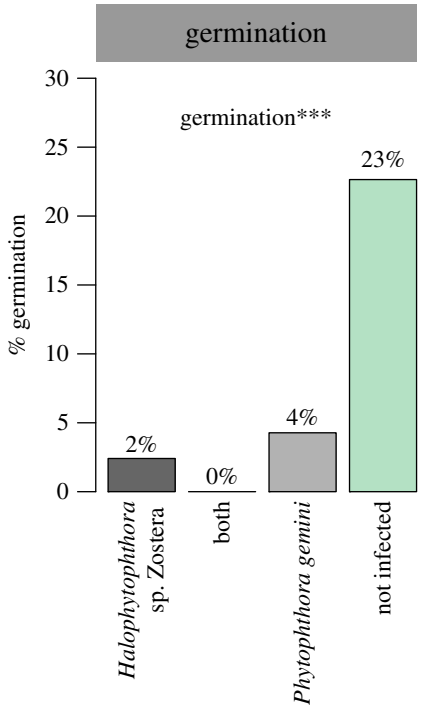

Figure 4. Seed germination of *Z. marina* in relation to *Phytophthora* and *Halophytophthora* infection after the incubation experiment. Stars indicate a significant effect of infection on *Z. marina* seed germination (***$p < 0.001$), error bars represent s.e.m. (Online version in colour.)

Zostera according to Koch's postulates. Yet, we suggest it is unlikely that both species merely acted as saprophytes in our experiment. First, because both were already present on healthy, living seed material (over 38% germinated at the start), which is highly unusual for saprophytes, but general behaviour for pathogens that need to colonize living plants [44]. Second, all known *Phytophthora* species display pathogenic behaviour to some degree [1] and have very limited ability to compete as saprophytes [45,46].

Although previously considered as tropical to subtropical saprophytes [15,47], more recently, Nigrelli & Thines [48] isolated two *Halophytophthora* species (sp. 1 and sp. 2) from leaf litter from a temperate coastal area (German Bight). Low temperature preference indicated that these strains had probably been overlooked, and not recently introduced. This may also be the case for the strain reported here, which is only the second account of *Halophytophthora* spp. in a temperate area. Previously overlooked contact between seagrasses and *Halophytophthora* spp. is also suggested from multiple tropical seagrass species that were found to produce secondary metabolites that inhibit growth of *Halophytophthora spinosa* [49,50]. As marine algae are known to produce many defensive secondary metabolites [51], it is possible that antibacterial and anti-fungal defences are also widespread among seagrasses, as anti-fouling mechanisms or to prevent infection by pathogens [50]. The decline from 99 to 34% infection over the course of our experiment may also indicate the presence of such defences. Nevertheless, as virtually all *Z. marina* seeds became infected and many remained infected with lack of germination as an apparent consequence, any potential chemical defences against the *Phytophthora* and *Halophytophthora* species reported here seem insufficient.

We found that environmental conditions in winter strongly affected the infection. Surprisingly, we observed a drop in infection by both *Halophytophthora* sp. Zostera and *P. gemini* during the simulated winter period in all treatments. Apart from potential chemical defences, this may be due to low winter temperatures. Winter is generally considered the major period of pathogen mortality, with higher winter temperatures generally lessening this bottleneck [52]. Contrastingly, however, we found less infected seeds in the high-temperature treatment (figure 3*b*). Thus, although winter appears to be a bottleneck for *Halophytophthora* sp. Zostera and *P. gemini* both species seem to prefer colder (5°C) over warmer winter temperatures (12°C).

Apart from temperature, sediment conditions also affected infection: in sand 1.8× more seeds remained infected compared with mud. This may be attributed to the local sediment characteristics, as the organic, fine-grained muddy sediment promoted decomposition rates of organic matter, resulting in anoxic conditions with enhanced sulfide levels (see Material and methods—Experimental set-up). Similar to other *Phytophthora* species [53,54], both *P. gemini* and *Halophytophthora* sp. Zostera survival were suppressed by anoxic conditions. This may be due to lack of oxygen or the production of sulfide (up to 180 $\mu mol\, l^{-1}$ in our experiment), which is toxic to many organisms, including seagrasses [40,55–57].

Wasting disease was previously the only well-known disease in *Z. marina*, causing large-scale declines in *Z. marina* beds across the Atlantic in the 1930s [18,19]. The Labyrinthulomycete *Labyrinthula zosterae*, a genus related to *Phytophthora*, has been identified as the pathogen causing wasting disease [58,59]. Although *L. zosterae* has been shown to be able to cause wasting disease symptoms [58], not all species from the *Labyrinthula* genus are pathogenic [59]. Indeed, recent work has shown that current isolates from European *Z. marina* populations display varying virulence [60,61], and *Labyrinthula* spp. seem to be very common in northern *Z. marina* beds [62]. This implies that *L. zosterae* may not be pathogenic under non- or low-stress conditions [60,63,64], possibly as a result of low pathogenicity, a strong defence reaction of the host, or both. Given our finding of additional putative pathogens in *Z. marina* beds, we may have to revisit the pathology of *Z. marina*, and perhaps also of seagrasses in general. It is highly conceivable that *Phytophthora* spp. and *Halophytophthora* spp. have been infecting *Z. marina* populations across the Atlantic for some time, and perhaps even in concert with *Labyrinthula zosterae*. Wasting disease-infected *Z. marina* plants may have been more susceptible to infection by *Phytophthora* and *Halophytophthora* species and vice versa. Hence, it is even possible that these oomycetes contributed to the epidemic of wasting disease in the 1930s.

5. Implications for conservation

Our results demonstrate that *Phytophthora* spp. and *Halophytophthora* spp. are likely common in *Z. marina* beds across the North Atlantic and Mediterranean, and suggest that both are pathogenic on seeds, with potentially important implications for *Z. marina* sexual reproduction and population fitness. This in turn may affect restoration and conservation efforts, especially in intertidal *Z. marina* populations that depend almost entirely on sexual reproduction. Of all seagrass restoration trials initiated in the last decades (less than 1700), over 50% aimed to restore *Z. marina* beds [65]. This means that pathogens affecting seagrass restoration efforts can be very

costly. For instance, based on our experimental results, we estimate that *Phytophthora* or *Halophytophthora* infection have reduced seed germination by at least 44% in current *Z. marina* restoration projects in the Dutch Wadden Sea.

Our novel insights into these pathogens allow us to optimize sediment conditions. For example, our study shows that anoxia during incubation may reduce infection—which appears to be unknowingly applied in restorations with seed from Chesapeake Bay, where seed germination is highest following storage in low oxygen conditions [31]. Our findings emphasize the need for a mechanistic understanding of such results and call for the development of potentially more efficient mechanism-based methods to reduce infection such as seed treatment with copper-based compounds or phosphonates [66–68].

As this is the first study showing putative pathogenicity of *P. gemini* and *Halophytophthora* sp. Zostera, we are only scratching the surface of the consequences of oomycete infections for *Z. marina* and marine plants in general. Considering potent pathogenicity of species of the *Phytophthora* genus, some urgent questions arise: can we definitively identify these putative pathogens as causal agents of the observed symptoms (decreased germination) according to Koch's postulates? Are these oomycetes only putatively pathogenic to *Z. marina*, or also to other seagrasses or marine plant species? What plant life stages (seed, seedling, adult, seed production) do *Phytophthora* and *Halophytophthora* species affect? Does *Z. marina* produce anti-fungal secondary metabolites to inhibit *Phytophthora* and *Halophytophthora* species as observed in tropical species? What conditions promote or reduce infection? We thus stress the need for further research on *Phytophthora* and *Halophytophthora* infections of marine plant species that often form the ecological foundation of coastal ecosystems, and are increasingly targeted for conservation and restoration [69].

Data accessibility. Experimental data from this manuscript are made publicly available in the Dryad Digital Repository http://dx.doi.org/10.5061/dryad.k72dj. Molecular species data have been submitted to GenBank, GenBank numbers are provided in the main text and electronic supplementary material, figure S1.

Authors' contributions. L.G., T.v.d.H., T.B., M.v.K. and J.H. generated hypotheses and designed research; L.G., J.H. and T.H. conducted the experiment and laboratory analysis; J.M., W.Mih.V. and P.v.R. performed *Phytophthora* analysis and molecular analysis; L.G. and T.v.d.H. analysed data; and L.G., W.Mih.V., J.M., B.O., P.R., T.B., J.H., M.v.K. and T.v.d.H. wrote the paper.

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