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Effects of environmental parameters on chytrid infection prevalence of four marine diatoms: a laboratory case study

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Abstract: The influence of environmental factors on the infection susceptibility of four different marine diatom host species to chytrid infection was tested under laboratory conditions, using host and parasite isolates obtained from diverse coastal areas in north-west Iceland in 2015. Specifically, a total of 120 monoclonal marine diatom host cultures of *Navicula*, *Nitzschia*, *Rhizosolenia* and *Chaetoceros* were exposed to their chytrid parasites *Chytridium* type I and *Rhizophydium* type I and II in Hellendahl glass staining jars which were subdivided in two compartments by nylon filters (mesh size 5 µm). Infection densities were assessed at different temperatures (5, 15, 20°C), salinities (0, 5, 10, 20, 40), photon fluence rates (PFR; 10, 50, 100, 200 µmol photons m^{-2} s^{-1}) and photoperiods (24 h dark, 8:16 h, 16:8 h light:dark and 24 h light) after 168 h exposure, using the one-factor-at-a-time method. In addition, growth rates and proline concentrations of the non-infected monoclonal host cultures were determined. In most cases, decreasing growth rates during the acclimatisation process to abiotic stressors were directly related to increases of proline in the host cells. Significant positive associations of infection densities to cell based proline concentrations were predominantly observed in the high-PFR assays and 24-h daylight treatments. At least for half of the tested host-parasite pairs, positive correlations of proline and parasite prevalence were found. In addition, chytrid abundance was also positively correlated with host densities of *Navicula* sp., *Rhizosolenia* sp. and *Chaetoceros* sp. Only in *Nitzschia* sp., was parasite density negatively associated with proline and showed no significant relationship to host densities, suggesting that other physiological/biochemical factors related to stress might have an impact on the susceptibility of this peculiar host diatom species.

Keywords: chytrids; marine diatoms; parasite-host dual cultures; proline concentrations; stress physiology.

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Introduction

Diatoms (Bacillariophyta) are a highly diverse and abundant group of microalgae which are key primary producers at the bottom of most autotrophic food webs in benthic and planktonic habitats. Particularly in freshwater ecosystems, diatoms have been shown to be common hosts for parasites, such as representatives of the phylum Chytridiomycota (chytrids). Chytrids are true fungi and are characterised by cell walls composed of chitin (James et al. 2006). When conditions are favourable for growth, the asexual life cycle is completed relatively rapidly, resulting in the release of a large number of zoospores into the aqueous environment, which are often host-specific, highly infective and extremely virulent (e.g. Gleason et al. 2011). These parasites cause frequent epidemics in lakes which have been studied periodically during the past century (e.g. Canter 1951, Sparrow 1960, Ibelings et al. 2004, Gsell et al. 2012, Sime-Ngando 2012, Voigt et al. 2013, Carney and Lane 2014). Thereby chytrids have been reported to play significant roles in controlling population sizes, in the transfer of carbon from relatively inedible substrates at the producer trophic level to higher trophic levels (Kagami et al. 2007), and in the biodiversity and succession of diatoms (Van Donk and Ringelberg 1983, Van Donk 1989).

Although marine true fungi have been reported as low in abundance and species richness in some studies (De Vargas et al. 2015, Richards et al. 2015), rDNA sequences of marine true fungi retrieved in metagenomic surveys revealed possible new species (Massana and Pedrós-Alió 2008). The Chytridiomycota alone accounted for more than

60% of the rDNA sequences sampled in six near-shore sites around Europe (Massana et al. 2015, Richards et al. 2015) and, in Arctic and sub-Arctic coastal habitats, they have been described as the most abundant fungal group (Hassett and Gradinger 2016, Hassett et al. 2016). Particularly planktonic diatoms such as *Pseudo-nitzschia pungens* (Grunow *ex* Cleve) Hasle (Hanic et al. 2009), *Chaetoceros* Ehrenberg, *Rhizosolenia* Brightwell (Scholz 2015), *Bellerochea* Van Heurck and *Cylindrotheca closterium* (Ehrenberg) Lewin *et* Reimann [=*Ceratoneis closterium* (Elbrächter and Schnepf 1998, Scholz et al. 2014a, 2016a)] were identified as host species for such zoosporic pathogens. Particularly in infections of the microphytobenthos, mainly epipelic taxa of the orders Naviculales (e.g. *Navicula digitoradiata*) (Gregory) Ralfs and Achnanthales (e.g. *Achnanthes brevipes* Agardh) were recorded in the temperate Solthörn tidal flat (Scholz et al. 2014a, 2016b), using sporangium morphology of the chytrids to distinguish different morphotypes. Similarly, the morphology of zoosporangia was used to distinguish chytrids in sediment samples from the north-west Icelandic coast (Scholz 2015). The diatom taxa infected by chytrids comprised representatives of the Thalassiophysales (e.g. *Amphora ovalis*) (Kützing) Kützing, Fragilariales (*Fragilaria striatula* Lyngbye) and Naviculales (e.g. *Diploneis bombus*) (Ehrenberg) amongst others (Scholz et al. 2016b).

When conditions are favourable for growth, the asexual life cycle in many chytrids is completed relatively rapidly resulting in the release of a large number of zoospores into the water column. According to Sparrow (1960), population densities can increase or decrease suddenly with changing environmental conditions. In several cases, pathogen periodicity was primarily related to host cell density (e.g. Anderson and May 1979, Ibelings et al. 2011), whereas no single physico-chemical factor has been found that fully explains the dynamics of chytrid epidemics in the field (e.g. Van Donk and Bruning 1992, Scholz et al. 2016b). This in part may be because several physico-chemical parameters commonly change simultaneously in aquatic systems. Abiotic factors can affect the growth rate of an alga as well as that of its parasite, making it difficult to discern environmental effects from field observations (e.g. Van Donk and Ringelberg 1983). For example, Bruning (1991a) showed that, under laboratory conditions, increase in temperature consistently decreased the number of zoospores produced per sporangium of the freshwater chytrid *Rhizophydium planktonicum* Canter, under both limiting and saturating light conditions for the host (*Asterionella*). In contrast, development time of the sporangia sharply decreased with an increase in temperature (Bruning 1991b).

Compatible solutes are highly soluble, low-molecular-weight organic molecules without net charge at physiological pH (Kirst and Wiencke 1995, Karsten 2012). Particularly in marine diatoms, the relationships between environmental stress factors, such as fluctuating salinity or irradiance, and the increased physiological acclimation potential of diatoms in relation to such compounds have been studied in recent years (e.g. Scholz and Liebezeit 2012). In algae, compatible solutes are restricted to four major classes of solutes: sugars and polyols, free amino acids and derivatives, quaternary ammonium compounds, and tertiary sulphonium compounds (Bisson and Kirst 1995, Erdmann and Hagemann 2001). Among the compatible solutes, proline appears to be the most widely distributed osmolyte accumulated by marine diatoms under osmotic (Plettner 2002, Krell 2006, Krell et al. 2007, Scholz and Liebezeit 2012), temperature and UV stress (Scholz and Liebezeit 2013, Scholz et al. 2014b). Proline has been shown to function as a stabilizer of macromolecules such as proteins and membranes during dehydration, as well as a nitrogen-storage compound and energy source after stress release (Aspinall and Paleg 1981, Delauney and Verma 1993).

In the present study, four parasite-host tandem cultures (*Chytridium* sp./*Navicula* sp., *Rhizophydium* type I/*Nitzschia* sp., *Rhizophydium* type IIa/*Rhizosolenia* sp., *Rhizophydium* type IIb/*Chaetoceros* sp.; Figure 1, Table 1) were used to test the hypothesis that pathogen infection rates rise with increasing physiological stress of the diatom hosts, driven by variations in abiotic environmental conditions. Staining jars divided in two compartments by nylon filters (mesh opening 5 μm) were utilised in combination with variations in abiotic environmental parameters (four different temperatures, five salinities as well as four photon fluence rates and photoperiods) to investigate changes in infection prevalence of monoclonal diatom-host cultures.

Materials and methods

Chemicals and organisms

If not otherwise mentioned, all chemicals used in this study were of the highest purity from Sigma/Aldrich Chemical Co (St. Louis, MO, USA). The isolation and taxa identification was conducted according to the methods described in Scholz et al. (2017). Figure 2 gives a short overview about the methods used.

Experimental design

All experiments were conducted for 168 h, employing in each case the "one-factor-at-a-time" method (Figure 2).

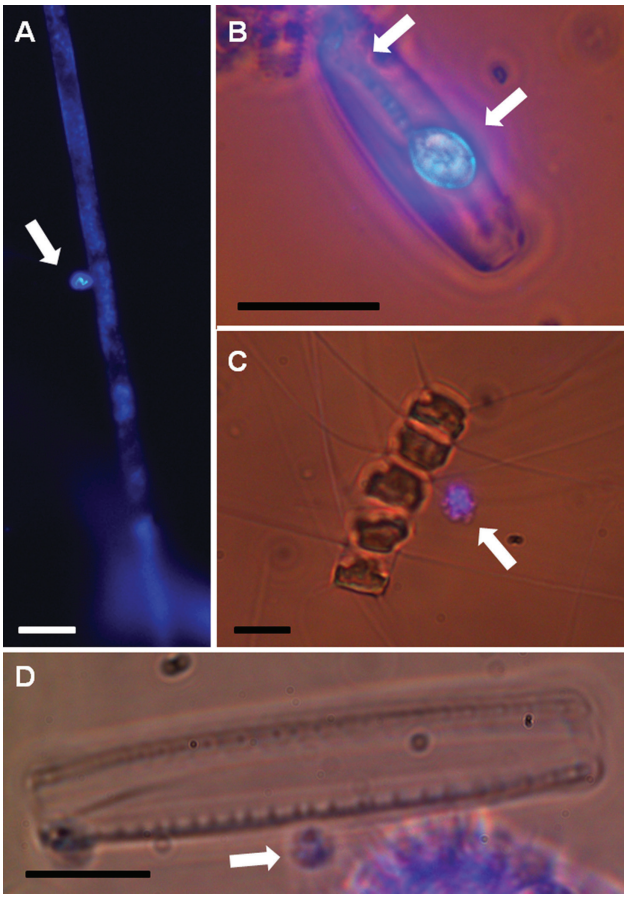

Figure 1: Diatoms infected by different chytrids isolated from sediment and phytoplankton samples collected in different areas in north-west Iceland (for description of the area, see Scholz and Einarsson 2015). Black and white scale bars indicate 10 µm. Pictures were taken under fluorescence light using Calcofluor White. (A) *Rhizosolenia/ Rhizophydium* type II; (B) *Navicula/Chytridium* type I; (C) *Chaetoceros/ Rhizophydium* type II; (D) *Nitzschia/Rhizophydium* type I.

Table 1 gives an overview of the control and experimental conditions employed during this study.

For the infection experiments, autoclaved Hellendahl glass staining jars (rectangular, 90.2 mm high, 44.5 mm wide, 58.8 mm long, containing eight slide holders; Isolab, Wertheim, Germany) were utilised, using nylon filters (Spectrum™ Spectra Mesh™; Fisher Scientific, Schwerte, Germany; 47 mm in diameter; mesh opening 5 µm) as barriers between infected diatom hosts (host-parasite dual cultures) and monoclonal test cultures. To prevent anoxic conditions, autoclaved cotton wool was used to close the jars. In parallel, monoclonal hosts were cultured in 250-ml Erlenmeyer flasks under the same conditions as those in the infection experiments (control assays; cf. Table 1). All cultures were adapted to the standard culturing conditions given in Table 1 at a saturating nitrogen concentration of 800 µM, for 2 weeks, using f/2 medium. The nitrogen to phosphorus ratio of the medium during all experiments was 17:1. Furthermore, sodium nitrate ($NaNO_3$) was used as the sole nitrogen source. The experiments were started by adding 30 ml of infected and monoclonal (uninfected) test cultures into the different chamber compartments and flasks (cell density: $6.8–9.2\times10^6$ cells ml^{-1}; infection prevalence of the infected cultures: 70%±5%), containing already 30 ml fresh culture medium. The different salinities (0, 5, 10, 20, 30, 40) were obtained by dissolving defined quantities of artificial seawater salt (Tropic Marin®) in de-ionized water. The experiments were conducted in an incubator (Innova 42R, Eppendorf New Brunswick Co, Inc., Hamburg, Germany). For the temperature experiments, 5, 15 and 20°C (all±0.2) were used, whereas for the different photon fluence rates 10, 50, 100, 200 µmol photons m^{-2} s^{-1} (all±5; Master TL-D 18W/840 light, Phillips, Germany) were applied in addition to the photoperiods 24 h dark, 16:8 h and 8:16 h light:dark and 24 h light. Salinity, temperature, pH and conductivity were measured using handheld probes (YK-31SA, YK-2001PH SI Model 33, Engineered Systems and Designs-Model 600, Philips W9424). Photon fluence rates (PFR, 400–700 nm) were measured with an underwater spherical quantum sensor LI-193SA connected to a Licor Data Logger LI-250A (LI-COR Lincoln, NE, USA). Every 2 days, 5 ml of the medium were replaced by fresh medium in all test assays to prevent

Table 1: Overview of the abiotic conditions employed in the different experiments.

	Temperature (°C)	Salinity	PFR (µmol photons m^{-2} s^{-1})	Daylength (h, light:dark)
Control	10±0.2	30	30±5	12:12
Experiments[a]				
Temperatures	**5, 15, 20±0.2**	30	30±5	12:12
Salinities	10±0.2	**0, 5, 10, 20, 40±0.1**	30±5	12:12
PFR	10±0.2	30	**10, 50, 100, 200±5**	12:12
Daylength	10±0.2	30	30±5	**24:0, 8:16, 16:8, 0:24**

PFR, photon flounce rate.
[a]In each case the experiments were conducted as one-factor-at-a-time (e.g. 5°C, salinity of 30, PFR 30, 12:12 light:dark cycle).
Values in bold are the varied factor in the experiment. e.g. for temperature experiment it was 5, 15 or 20°C.

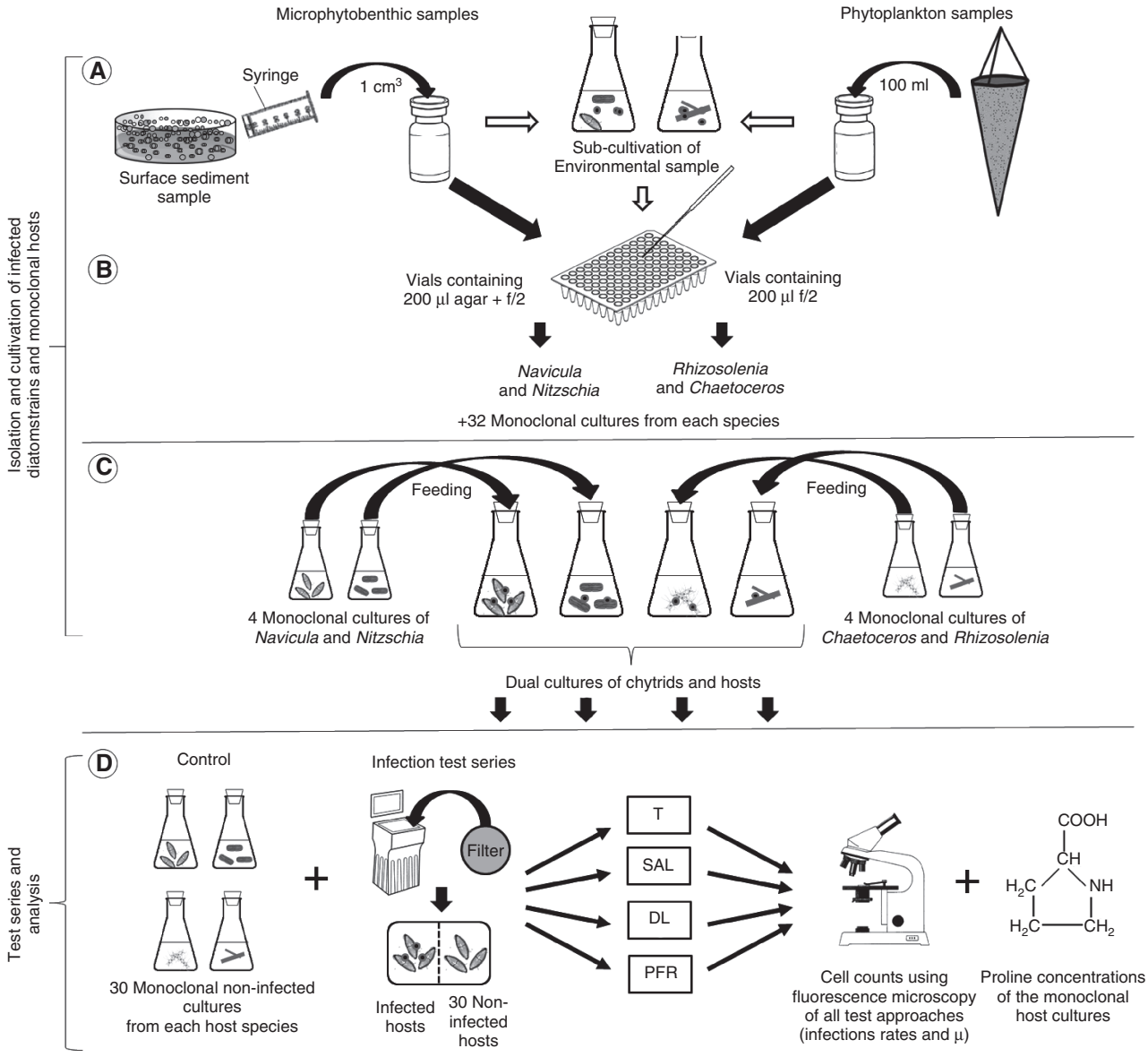

Figure 2: Methods used for the isolation (A–B) and cultivation (C) of the four chytrid-host pairs and 32 monoclonal host cultures, as well as the test series and analysis (D).
In each case, individual cells were isolated with a micropipette and transferred into microwells containing 0.5 ml of the culture medium f/2-Si (Guillard and Ryther 1962, with and without agar) and sterile filtered natural seawater (50% v/v). Each isolate was maintained in 250-ml batch cultures under sterile conditions at 10°C, 12:12 h light:dark regime and at a photon fluence rate of 30 µmol photons m^{-2} s^{-1} (Scholz et al. 2017).

nutrient depletion. The position of the chambers and flasks was randomly changed every other day to eliminate any location effect due to minor changes in external conditions.

Diatom cell counts, visualisation of infections and calculation of the infection prevalence

Diatoms were fixed in Lugol's solution (2% v/v) and counted under a light microscope (Olympus BX51, equipped with a BX-RFA reflected fluorescence system, Hamburg, Germany), using a Neubauer improved counting chamber with 0.1 mm depth (LO Laboroptik GmbH, Germany). Cells were counted that exhibited red fluorescence under ultraviolet light. At least 500 cells were counted in each sample at 400 × magnification. The specific growth rate (μ) was calculated with the following equation:

$$\mu = \frac{\ln_{c168} - \ln_{c0}}{t_{168} - t_0}$$

where c_{168} and c_0 are the number of cells at time t_{168} (h) and t_0.

The infection prevalence was determined by counting 100 µl of the formaldehyde-fixed-(2% v/v) test cultures in triplicate, considering sporangia attached to host cells, under 1000 × magnification using Fluka Analytical Calcofluor White staining (18909-100ML-F) in combination with UV-light. The percentage of infected cells was calculated by dividing the number of infected cells by the total number of host cells. The mean number of chytrids per cell (host) was also calculated, by dividing the total number of parasites attached to algal cells by the total number of host cells, to normalise the cell density among treatments. This value is referred to as the mean intensity of infection (Holfeld 2000), reflecting the number of pathogens that succeed in attaching to their host.

Harvesting and proline analysis

Non-infected diatom cells from the control assays were harvested by centrifugation at 680 *g* for 10 min (Heraeus Biofuge Primo R, Thermo Fisher Scientific GmbH, Dreieich Germany). Proline concentrations were measured spectrophotometrically with ninhydrin according to the method of Bates et al. (1973) as modified by Nothnagel (1995). In brief, biomass was re-suspended in 400 µl H_2O, and 2 ml of ninhydrin-reagent [1.25 g ninhydrin in 50 ml 6 M phosphoric and acetic acid (3:2 v/v)] and 10 µl acetic acid (75%) were added. The mixture was shaken (15 s) and heated at 100°C (WNB 7-45, Memmert, Schwabach, Germany) for 1 h. After this, 3 ml toluene was added and centrifuged for 5 min at 1631*g*. Absorbance of the toluene phase was measured at 520 nm (BioTek Epoch; BioTek Instruments, Winooski, VT, USA). Proline concentrations in the control and treatments were estimated from a calibration curve of L-proline (CAS 147-85-3, Merck), and are expressed as fmole $cell^{-1}$ and pmol μl^{-1}.

Statistical analysis

To assess variability of the diatom taxa (specific growth rate μ) and the infection prevalence, one-way analysis of variance (ANOVA) with Tukey's honest significant difference (HSD) post hoc test with Bonferroni corrections was performed on log-transformed data, testing each species separately. Data are expressed as relative difference (%). A p-value of <0.05 was considered as significant. The relationships between the growth rates, infection prevalence and concentrations of proline were evaluated using non-parametric Spearman's rank correlation (r_s) to avoid problems associated with non-normal data distribution. These tests were performed with the program XLSTAT 2011, Version 2011.2.08 (Addinsoft, New York, NY, USA).

Lack of infection in an individual can be due to different aspects as discussed by Scholz et al. (2017). The inability to distinguish between different scenarios may confound relationships between the predictor variables and parasite density. Therefore, the analysis of host susceptibility was restricted to infected individuals. In order to obtain independent variables, the data for infection prevalence and growth rates were used as abundances only (=host and parasite or chytrid density), whereas the proline concentrations were utilised as pmol · μl^{-1}. If not otherwise mentioned, all data in the analyses were log transformed to reduce heteroscedasticity in the data. Differences between treatments were tested using a one-way ANOVA, testing each approach separately, and pair-wise comparisons used Tukey's HSD. Data analyses used the base and lattice packages in R (Sarkar 2008, R Core Team 2013).

Logistic regression models for each host-parasite pair were used to determine significant abiotic and biotic contributors to the infection prevalence or density, utilizing JMP® 8.0.2 (SAS Institute Inc., Cary, NC, USA) and R (R Core Team 2010). Proline data for each species were square root transformed ($n = 360$). To preserve predictive power, the model was run initially using all abundances of one host diatom and its parasite as predictor variables. In the present study, the duration of the experiments ($t = 168$ h) may be an important predictor of parasite density. Therefore, duration was tested as a random intercept in the model. This initial test was used to decide whether generalized linear mixed models (GLMMs) or generalised linear models (GLMs) were most appropriate for the present data (Bolker et al. 2009). To do so, a saturated model was fitted with the same fixed-effect structure, using the lme4 package for GLMM. Subsequently, a likelihood ratio test (LRT) was used to investigate whether the inclusion of a random intercept was warranted. The LRT suggested that the inclusion of the random intercept did not significantly improve the model ($\chi^2 = 3.1$, d.f. $= 1$, $p = 0.07$), which prompted the use of a GLM for the remainder of the analysis.

After removing non-significant predictor variables, a second iteration was run with the addition of cell-free proline concentrations to determine whether infection status varies with stress physiology. Significant effect variables were evaluated post hoc using contingency

analysis (chi-square) and the relationships between categorical groups were further assessed using Mann-Whitney U-tests. Alpha levels were adjusted using Holm-Bonferroni corrections for multiple comparisons (Holm 1979).

Results

Growth responses of diatom-hosts to varying abiotic factors

Growth of the non-infected diatom hosts, measured as the mean increase in cell numbers (specific growth rate μ) after 168 h, was highly variable in relation to the abiotic parameters tested in the experiments (Figure 3A–D). Significant differences were found between mean growth rates of the benthic species and the planktonic ones independent of the tested parameter. Particularly in the 10°C control assay, the mean growth rates of the planktonic species were higher than those of the benthic species, ranging from 0.71 and 0.87 μ day^{-1} for *Navicula* sp. and *Nitzschia* sp. to 1.08 and 1.26 μ day^{-1} for *Chaetoceros* sp. and *Rhizosolenia* sp. (ANOVA: $F_{2,66} = 31.8$, $p < 0.0001$; Figure 3A). Increased mean growth rates were recorded for *Rhizosolenia* sp. and *Chaetoceros* sp. in the test using different photon fluence rates (PFR), being the highest at 100 μmol photons m^{-2} s^{-1} with a photoperiod of 12:12 h light:dark, a temperature of 10°C and a salinity of 30 (up to 52% difference to the control assay grown at 30 μmol photons m^{-2} s^{-1}, ANOVA: $F_{1,67} = 34.5$, $p < 0.0001$; Figure 3A, C). In contrast, both planktonic species showed significantly lower mean growth rates in the high temperature assays (up to 46% lower in comparison to the control assay at 10°C, ANOVA: $F_{1,33} = 28.5$, $p < 0.0001$; Figure 3A). Furthermore, the mean growth rates of all tested species in relation to low salinity and 24 h dark cycle treatments were significantly reduced in comparison to the control assay (salinity of 30; 12:12 light:dark cycle; up to 93.6% lower; ANOVA: $F_{1,67} = 33.2$, $p < 0.0001$; Figure 3B, D).

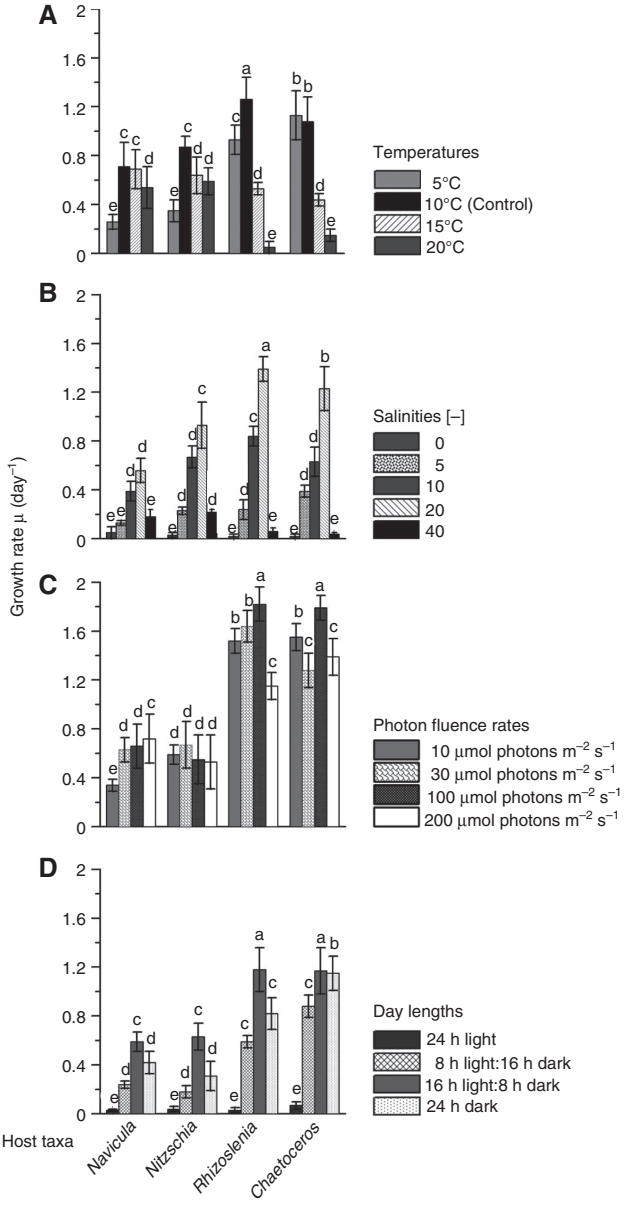

Figure 3: Diatom growth rates (μ day^{-1}) in relation to different environmental parameters (A) temperatures: 5, 10 (=control assay), 15, 20°C; (B) salinities: 0, 5, 10, 20, 40; (C) photon fluence rates: 10, 30, 100, 200 μmol photons m^{-2} s^{-1}, and (D) daylengths: 24 h dark, 8:16 h, 16:8 h light:dark and 24 h light).
The control assays of each species were maintained at a temperature of 10°C, salinity of 30, 30 μmol photons m^{-2} s^{-1} and 12:12 h light:dark cycle (cf. Table 1). Due to redundancy of the results the control is only once depicted in A. Results are mean values of 30 clones per species (n = 90). Bars labelled with different letters are significantly different at p = 0.01 (repeated measures ANOVA with Tukey's HSD post hoc test with Bonferroni corrections).

Variations in infection prevalence

The monoclonal cultures of the diatom hosts showed high variability in their susceptibility to infection under different values of the abiotic variables (Figure 4), ranging from a mean prevalence of 0.16% ± 0.9% (e.g. *Navicula* sp. at salinities of 0 and 40, Figure 4B) to 89.9% ± 1.5% (*Chaetoceros* sp. at a salinity of 20, Figure 4B). Particularly the monoclonal cultures of the benthic species *Navicula* sp. and *Nitzschia* sp. showed differences in their mean prevalence of infections by *Chytridium* and *Rhizophydium*,

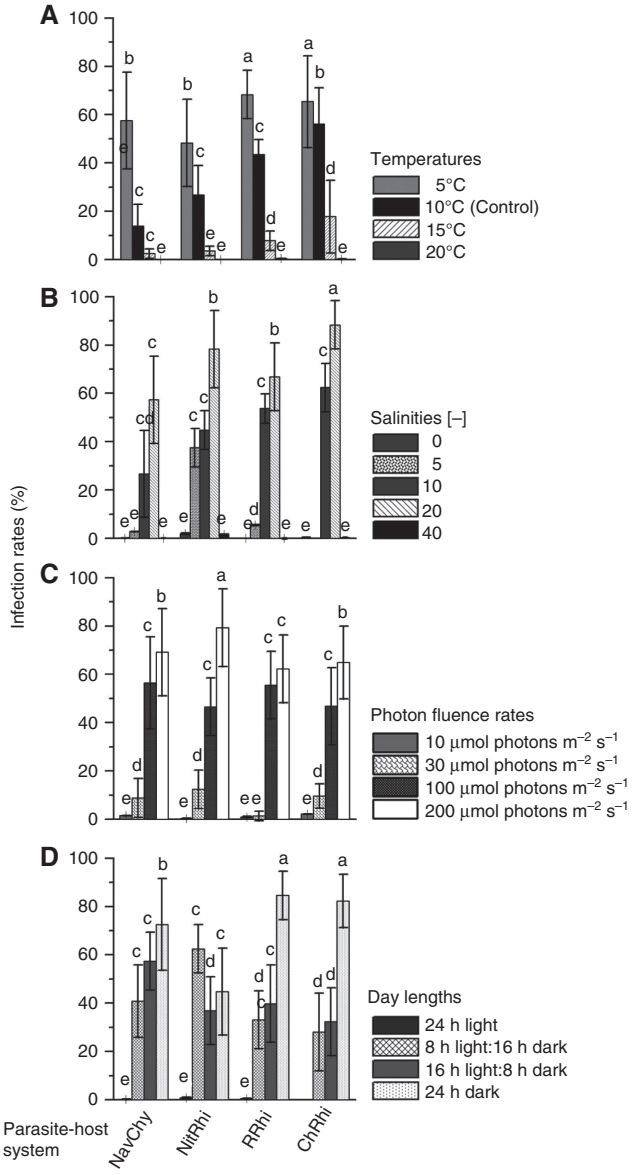

Figure 4: Diatom host susceptibility to infection by chytrids in relation to different environmental parameters (A) temperatures: 5, 10 (=control assay), 15, 20°C; (B) salinities: 0, 5, 10, 20, 40; (C) photon fluence rates: 10, 30, 100, 200 µmol photons m^{-2} s^{-1}, and (D) daylengths: 24 h dark, 8:16 h, 16:8 h light:dark and 24 h light). Infection rates obtained after 168 h incubation time of the hosts [*Navicula* sp. (NavChy 101-130), *Nitzschia* sp. (NitRhi 101-130), *Rhizosolenia* sp. (RRhi 101- 130) and *Chaetoceros* sp. (ChRhi 101-130)]. The control assays of each species were maintained at a temperature of 10°C, salinity of 30, 30 µmol photons m^{-2} s^{-1} and 12:12 h light:dark cycle (cf. Table 1). Due to redundancy of the results the control is only once depicted in A. Results are mean values of 30 clones per species (n = 90). Bars labelled with different letters are significantly different at p = 0.01 (repeated measures ANOVA with Tukey's HSD post hoc test with Bonferroni corrections).

respectively, in relation to the tested variables, being the highest for temperature and daylength (up to 16.8% differences in the mean infection rates of *Navicula* sp. and *Nitzschia* sp. in the daylength and temperature assays; Figure 4A, D).

Proline accumulation under different abiotic parameters

Mean proline concentrations per cell, obtained from the assays after 168 h exposure to different abiotic growth conditions, varied considerably (Figure 5), ranging from 11.5 ± 2.3 fmole $cell^{-1}$ in the 16:8 light:dark assay up to 195.8 fmole $cell^{-1}$ in the 24 h daylight treatment (both Figure 5D). In addition, significantly increased mean cell based proline concentrations were also found in the high temperature assays (up to 87% above the control assay; ANOVA: $F_{1,68} = 30.7$, $p < 0.0001$; Figure 5A) as well as in the highest and lowest salinity treatments (up to 91%; ANOVA$_{\text{salinity of 40 vs. control}}$: $F_{1,67} = 31.1$, and ANOVA$_{\text{salinity of 0 vs. control}}$: $F_{1,69} = 32.3$, $p < 0.0001$; Figure 5B) in all tested species.

Discussion

Relationship between growth of the diatom hosts and the physiological variable proline

In most of the cases in the present study, decreases in growth rates during the acclimatisation process to abiotic stressors were directly related to increases of proline in the host cells (Table 2). Similar responses were also recorded in recent studies on diatoms in the Wadden Sea area during the acclimatisation process to high and low salinities, temperature and ultraviolet radiation (UVR; Scholz and Liebezeit 2012, 2013, Scholz et al. 2014b). These studies indicated that the physiological acclimatisation processes in benthic diatoms were still ongoing while in parallel suppressing the ability to invest in growth, i.e. increase of cell numbers. In these studies, the species-specific growth rates did not vary significantly over the tested stress parameter ranges during short-term exposures (1–12 h). Only during the long-term experiments (30 days) were distinct variations found, occurring in the lowest as well the highest salinities, temperatures and UVR-treatments (Scholz and Liebezeit 2012, 2013, Scholz et al. 2014b). In this context, the 168-h experimental period of the present study was identified as the most suitable interval. Benthic diatom species adapted to northern Icelandic coastal habitats were stable in their growth rates over a longer time during exposure to extreme environmental parameters as compared to related taxa originating from temperate areas

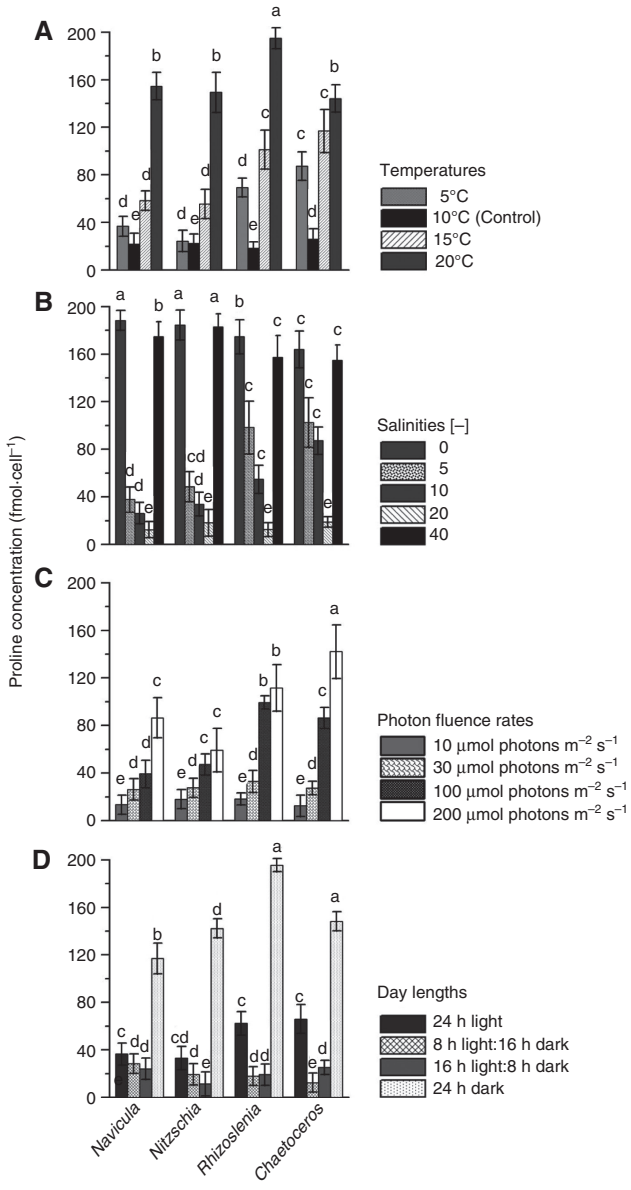

Figure 5: Proline concentrations of the diatom hosts *Navicula* sp., *Nitzschia* sp., *Rhizosolenia* sp. and *Chaetoceros* sp after 168 h exposure to different environmental parameters (A) temperatures: 5, 10 (=control assay), 15, 20°C; (B) salinities: 0, 5, 10, 20, 40; (C) photon fluence rates: 10, 30, 100, 200 µmol photons m^{-2} s^{-1}, and (D) daylengths: 24 h dark, 8:16 h, 16:8 h light:dark and 24 h light). The control assays of each species were maintained at a temperature of 10°C, salinity of 30, 30 µmol photons m^{-2} s^{-1} and 12:12 h light:dark cycle (cf. Table 1). Due to redundancy of the results the control is only once depicted in A. Results are mean values of 30 clones per species (n = 90). Bars labelled with different letters are significantly different at p = 0.01 (repeated measures ANOVA with Tukey's HSD post hoc test with Bonferroni corrections).

such as the Wadden Sea (data unpublished). This conspicuous growth stability might be attributed to their continuous exposure to fluctuating environmental conditions during low-tide. Particularly during the northern Icelandic summer, durations of 24 h sun and high-light conditions coupled with UV exposure, strong temperature fluctuations and desiccation make these adaptations explainable (cf. Scholz and Einarsson 2015). In strong contrast, phytoplankton species experience much less severe and fluctuating environmental conditions compared to microalgae on tidal flats, which is reflected in a much higher sensitivity to physical stressors. Particularly in the treatments using a PFR of 200 µmol photons m^{-2} s^{-1} ($p < 0.05$, $r_s > 0.7$) and photoperiods of 8:16 h and 24:0 h light:dark ($p < 0.05$, $r_s > 0.63$ for both light treatments; Table 2) significant positive associations of cell based proline concentrations to growth rates were observed.

Host and chytrid densities as infection prevalence determining factor in relation to abiotic parameters

In three out of the four tested marine host-parasite pairs, a direct relationship between host and chytrid densities was revealed by the GLM analysis (Figure 6A–D), supporting the observations of several studies in the freshwater environment that the primary factor determining the presence of a particular parasite is the availability of suitable hosts (e.g. Canter and Jaworski 1981, Bruning and Ringelberg 1987, Bruning 1991b, Ibelings et al. 2004, Kagami et al. 2004). Besides host availability, diverse environmental conditions, including temperature, turbulence, light, nutrient concentrations, and biotic factors, such as predation, have been shown to influence the growth rate of fungal parasites in microalgae (Canter and Jaworski 1981, Bruning and Ringelberg 1987, Bruning 1991b, Kagami et al. 2004). In the present study, broad patterns regarding the influence of environmental abiotic factors on the infection susceptibility of the tested marine host-parasite systems were detected, including the highest mean values of infection prevalence at (i) a temperature of 5°C, (ii) a salinity of 20 and (iii) a photon fluence rate of 200 µmol photons m^{-2} s^{-1}. In addition, the fact that infections occurred at 20°C is noteworthy, suggesting that fungi may be able to become epidemic under both winter and summer conditions, namely under the combination of low temperature and low irradiance in winter, and of high temperature and high irradiance in summer. Even though in the present study only one factor at a time was tested, the increase of infection rates at a standard temperature of 10°C in combination with high photon fluence rates such as 100 and 200 µmol photons m^{-2} s^{-1}, indicated the potential of the tested parasites to become epidemic also during the Icelandic summer conditions. Furthermore,

Table 2: Non-parametric Spearman's rank correlations (r^2-values) for proline as physiological variable vs. growth rates and infection prevalences of four marine diatom hosts exposed to different abiotic treatments.

Species variables	Growth rate (μ)				Infection prevalence			
	Navicula	*Nitzschia*	*Rhizosolenia*	*Chaetoceros*	*Navicula*	*Nitzschia*	*Rhizosolenia*	*Chaetoceros*
Temperature treatments (°C)								
5	−0.102[a]	0.092[a]	−0.182[a]	0.243[a]	−0.328[b]	−0.519[b]	**0.099**[a]	**0.109**[a]
15	n.s.	n.s.	−0.432[c]	−0.634[b]	−0.184[a]	−0.318[c]	−0.686[b]	−0.407[c]
20	−0.847[b]	−0.603[b]	−0.867[b]	−0.827[b]	–	–	−0.833[b]	−0.742[b]
Salinity treatments								
0	−0.894[b]	−0.803[b]	−0.903[b]	−0.794[b]	−0.911[b]	−0.843[b]	–	−0.861[b]
5	0.089[a]	n.s.	−0.441[c]	−0.429[c]	n.s.	**0.114**[a]	−0.524[b]	−0.792[b]
10	−0.326[c]	−0.106[a]	n.s.	0.098[a]	−0.232[a]	−0.197[a]	**0.366**[c]	**0.542**[b]
20	−0.418[c]	−0.510[c]	−0.617[b]	−0.573[b]	−0.536[b]	−0.619[b]	−0.673[b]	−0.626[b]
40	−0.786[b]	−0.793[b]	−0.846[b]	−0.841[b]	−0.918[b]	−0.791[b]	−0.931[b]	−0.705[b]
Photon fluence rate treatments (μmol photons m^{-2} s^{-1})								
10	−0.114[a]	−0.236[a]	−0.693[b]	−0.702[b]	n.s.	−0.156[a]	−0.213[a]	n.s.
50	−0.198[a]	−0.183[a]	−0.582[b]	−0.671[b]	**0.398**[c]	**0.243**[a]	−0.214[a]	**0.417**[c]
100	−0.482[c]	n.s.	−0.301[c]	−0.247[a]	−0.246[a]	−0.313[c]	**0.683**[b]	**0.712**[b]
200	−0.536[b]	n.s.	**0.792**[a]	**0.728**[b]	**0.207**[a]	−0.457[c]	**0.715**[b]	**0.622**[b]
Daylengths (h, light:dark)								
0:24	−0.205[a]	−0.201[a]	−0.432[c]	−0.143[a]	n.s.	n.s.	−0.372[a]	–
8:16	**0.109**[b]	**0.094**[a]	−0.247[a]	−0.391[b]	−0.596[b]	−0.423[c]	−0.142[a]	−0.229[a]
16:8	−0.273[a]	−0.483[c]	−0.509[b]	−0.457[c]	−0.616[b]	−0.486[c]	−0.394[c]	−0.193[a]
24:0	−0.434[c]	−0.691[b]	**0.637**[b]	**0.635**[b]	**0.253**[b]	−0.267[c]	**0.696**[c]	**0.686**[b]
Control	−0.133[a]	−0.427[c]	−0.748[b]	−0.559[b]	n.s.	−0.142[a]	−0.413[c]	−0.349[c]

[a] > 0.001, [b] < 0.0001, [c]0.001, n.s., not significant.
Values in bold are the varied factor in the experiment. e.g. for temperature experiment it was 5, 15 or 20°C.

the present results also showed that, under some combinations of temperature and light, no epidemic development seems to be possible. Regarding the low infection rates recorded during the 24 h dark cycle treatment in the present study, it was shown that the infectivity of the zoospores and the number of zoospores per sporangium decreased when the host became light limited, and the development time of the sporangia was also slightly reduced (Ibelings et al. 2004). In the case of infections of *Asterionella* Hassall by *Rhizophydium* Schenk, which were kept completely in the dark, it was found that zoospores were not able to attach to the host in the absence of light (Bruning 1991a,b). Reduced infectivity of chytrid zoospores at low irradiances has also been reported by others (e.g. Canter and Jaworski 1986). According to Ibelings et al. (2004), chytrid zoospores may use excretion products of the host (related to photosynthesis) to find or recognize host cells, so it is possible that light limitation interferes with chemotaxis.

Stress factors, proline accumulation and their relationship to host susceptibility

Regarding the influence of stress factors, reflected by increased proline concentrations, on the susceptibility of hosts, the results of the conducted experiments showed a relatively uneven pattern with only minor differences between benthic and planktonic diatoms. Only the minority of the host-parasite pairs showed positive associations regarding the relationship of infection densities to cell based proline concentrations accumulated during exposure to different abiotic parameters (25%, Table 2). Strong positive associations were predominantly observed in the high-PFR assays as well as 24 h daylight treatments ($p < 0.05$, $r_s > 0.6$, Table 2). In contrast, increased proline concentrations in relation to abiotic factors were in the majority of the cases negatively correlated (65%) to infections or the relation was not significant (9%, Table 2), suggesting that the accumulation of proline may not be involved in each infection process. Furthermore, the GLM analysis showed for at least two out of the four tested marine host-parasite pairs a strong positive correlation between proline concentrations and chytrid densities. Particularly, in the benthic host-diatom *Navicula* sp. and the planktonic species *Rhizosolenia* sp. (Figure 6E, G) direct relationships of the tested variables low-temperature, high-PFR and 24 h daylight to the infection susceptibility and the biochemical stress indicator proline were observed. In contrast, a strong negative correlation between proline concentrations and chytrid densities was

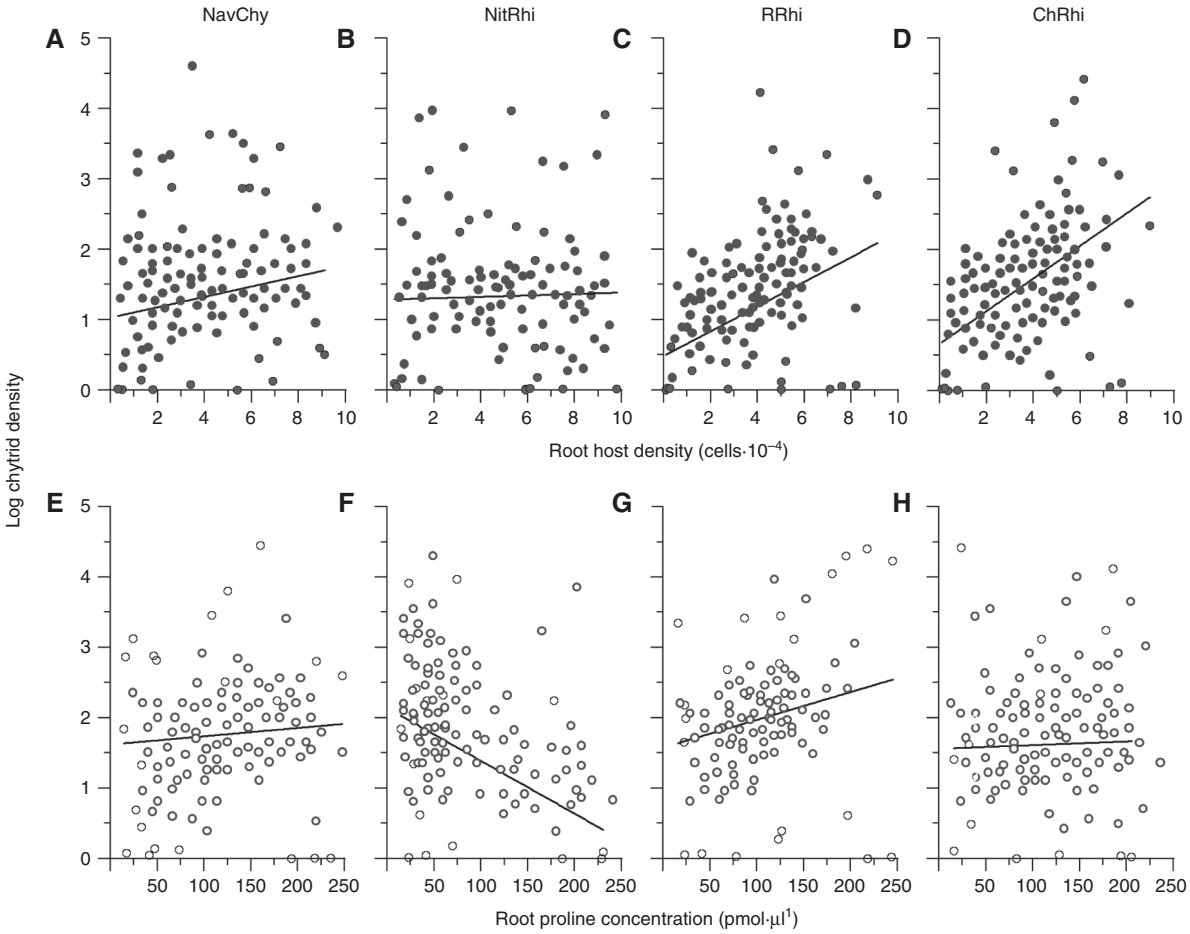

Figure 6: Relationships between infection intensity in *Navicula* sp. (NavChy), *Nitzschia* sp. (NitRhi), *Rhizosolenia* sp. (RRhi) and *Chaetoceros* sp. (ChRhi) and either host-diatom density (A–D) or proline concentration (E–H). In the logistic regression models for each host-parasite pair, parasite density correlates positively with proline concentrations in *Navicula* sp (E) and *Rhizosolenia* sp (G), whereas in *Nitzschia* sp. (F) the correlation was negative. Furthermore, parasite density correlates positively with host density in *Navicula* sp (A) and *Rhizosolenia* sp (C). Circles represent individual cells.

observed in the benthic host-diatom *Nitzschia* sp. (Figure 6F), whereas for the planktonic host-species *Chaetoceros* sp. only a weak correlation was found (Figure 6H). However, in *Nitzschia* sp. the infection prevalence was not strongly correlated to growth or to proline concentrations, pointing to other effects or variations in the biochemical composition as examined in the present investigation. In this context, the present study provides only first hints about correlations between physiological stress responses and infection susceptibility of the host cells. Besides proline, several other free amino acids have been detected in marine diatoms with possible functions as organic osmolytes, such as glutamic acid (Fujii et al. 1995, Nothnagel 1995), alanine (Nothnagel 1995) or taurine (Jackson et al. 1992). Further studies are necessary, using, for example, the 5-litre culture system developed by Paul et al. (2013) for their studies of diatom-diatom interactions in order to obtain higher biomass for fatty acid and amino acid analysis by GC-MS and HPLC. In addition, further factors, such as pesticides and pharmaceuticals (man-made anti-inflammatories, antibiotics, anti-depressants) present in coastal waters (e.g. Van den Wyngaert et al. 2014, Van Donk et al. 2016), pH values (theme complex: ocean acidification), and ultraviolet radiation may have significant effects on variations of the host physiology with secondary impacts on the host susceptibility to infections.

Conclusions

The present study shows the impacts of selected abiotic parameters on growth, infection susceptibility of the diatom hosts *Navicula, Nitzschia, Rhizosolenia* and *Chaetoceros* and the accumulation of the amino acid proline as

a biochemical stress indicator. The monoclonal cultures of the diatom hosts showed high variability in their susceptibility to infections in relation to the tested abiotic parameters. The statistical results indicate a direct relationship between environmental stressors and host susceptibility in at least half of the tested host diatom species (*Navicula*, *Rhizosolenia*). Other biochemical stress compounds such as dimethylsulphoniopropionate (DMSP) or glycine betaine might be involved in the susceptibility of the host species *Nitzschia* and *Chaetoceros* and this hypothesis needs to be further investigated.

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Bionotes

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Graphical abstract

Bettina Scholz, Wim Vyverman, Frithjof C. Küpper, Halldór G. Ólafsson and Ulf Karsten

Effects of environmental parameters on chytrid infection prevalence of four marine diatoms: a laboratory case study

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Research article: The influence of environmental factors on the infection susceptibility of four different marine diatom host species to chytrid infection was tested under laboratory conditions, using host and parasite isolates obtained from diverse coastal areas in north-west Iceland in 2015.

Keywords: chytrids; marine diatoms; parasite-host dual cultures; proline concentrations; stress physiology.