Synergistic effects of extreme temperature and low salinity on foundational macroalga *Fucus vesiculosus* in the northern Baltic Sea

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Abstract

Climate change has been identified as one of the biggest current drivers of environmental change. Climate model projections for the Baltic Sea forecast increased frequency and duration of extreme temperatures, together with declines in salinity, which are expected to have impacts on the biota. In this experimental study, the interacting effects of low salinity and short-term (8 days) extreme seawater temperatures, followed by an 11-day recovery period, on the foundational macroalga, *Fucus vesiculosus*, were investigated. To account for potential variation in the responses at local scale, individuals originating from two different local populations, a warm and a cold site were included.

In experiments manipulating temperature (20 °C to 28 °C) and salinity (4 or 6), it was found that even an 8-day exposure to 26 °C or higher was detrimental to *F. vesiculosus*, causing extensive tissue necrosis. Tissue necrosis was enhanced by low salinity. Photosynthesis, measured as the steady-state electron transport rate (ETR) and maximum ETR, declined at 26 °C, and this effect was also enhanced by low salinity. Temperatures above 26 ˚C caused declines in light-limited photosynthetic efficiency (α), indicating direct physiological damage to PS II reaction centers.

After 11 days of recovery, some photosynthetic parameters recovered in the 26 °C, but not in the 28 °C treatment. It is concluded that Baltic *F. vesiculosus* populations may be severely affected even by short-term (8 days) exposure to high seawater temperatures when combined with the synergistic effects of low salinity predicted for the future Baltic Sea.

Introduction

Climate change has been identified as one of the biggest ongoing and predicted environmental changes in both terrestrial and aquatic ecosystems (Bellard et al., 2012; Doney et al., 2012; Porter et al., 2012). In sheltered, shallow sea areas, abiotic changes may be exceptionally severe. In the Baltic Sea, climate change has been identified as one of the most prominent environmental changes in the ecosystem during this century (BACC Author Team, 2008; BACC II Author Team, 2015; HELCOM, 2013). Declining sea water salinity

(Neumann, 2010) together with an increased frequency of high temperatures (Neumann et al., 2012) has been predicted. As the Baltic Sea is relatively species poor, and many species exist at the edge of their environmental tolerance (Hällfors et al., 1981), extreme events such as heat waves may have profound implications for the persistence and distribution of the biota mediated through responses of foundation species.

Temperature is one of the most important factors determining the rates of growth and photosynthesis in algae (Eggert, 2012; Raven and Geider, 1988), with resulting impacts on geographic ranges mediated by species-specific temperature tolerances (Eggert, 2012; Lüning, 1984). In addition to temperature, salinity is another major determinant of macroalgal photosynthesis, respiration, and growth (Karsten, 2012). Effects of fluctuating salinity are often dependent on temperature, with some species being tolerant of low salinities under low but not high temperatures (Karsten, 2012; Thomas et al., 1988). As different temperature and salinity combinations are experienced by algae in their natural habitat, it has been argued that temperature and salinity should not be viewed as separate environmental factors, but rather as acting in unison (Druehl and Foottit, 1985; Lobban and Harrison, 1994). This type of interaction is, however, rarely accounted for in experimental studies, which often manipulate only a single variable of interest (Forsman et al., 2016; Wernberg et al., 2012b), although interactive effects have been identified as important (Harley et al., 2012; Parmesan et al., 2013). Moreover, experimental studies simulating climate change are frequently conducted by manipulating the mean temperature (Forsman et al., 2016), while the majority of studies have failed to address the effects of extremes (Thompson et al., 2013). Extreme temperature events have been identified as a key component determining the structure and distribution of marine biodiversity under climate change, mediated through impacts on habitat-forming species such as seaweeds (Smale and Wernberg, 2013; Wernberg et al., 2012a). In the Baltic Sea, habitat-forming species such as *Fucus vesiculosus* will be exposed to both increased frequency of extreme temperatures and declining salinity. Interacting drivers may have synergistic effects exceeding those of either driver alone (Wahl et al., 2011; Wernberg et al., 2012b).

F. vesiculosus is the main habitat-forming macroalga on the hard bottoms of the northern Baltic Sea, providing shelter and increasing habitat complexity, and thus harboring a rich diversity of associated floral and faunal species (Kautsky et al., 1992; Pärnoja et al., 2014; Schagerström et al., 2014). Consequently, *F. vesiculosus* has been considered as a foundation species, crucial for the preservation of biodiversity features (Dijkstra et al., 2012; Kautsky et al., 1992; Schagerström et al., 2014). In the Baltic Sea *F. vesiculosus* lives in the lower limit of its salinity tolerance and it has been predicted to suffer from the forecasted decline in salinity (Takolander et al., 2017; Vuorinen et al., 2015). This is supported by the fact that the distributional limit of *F. vesiculosus* in the northern Baltic is at salinities of 3 or 4 (Bäck and Ruuskanen, 2000; Kautsky et al., 1992). Such a salinity limitation has been attributed to the osmotic tolerances of the gametes (Serrão et al., 1996). However, low salinity may also cause additional stress in full-grown individuals, as they will need to expend more energy in osmoregulation. Low salinity has been shown to cause increased respiration (Munda and Kremer, 1977) and reduced growth (Bäck et al., 1992). Low salinity also lowers the mannitol content in *F. vesiculosus* (Munda and Kremer, 1977), but this may be an adaptive physiological response to low salinity, as mannitol, in addition to being the main storage carbohydrate (Lehvo et al., 2001), also has important osmoregulatory functions in *F. vesiculosus* (Groisillier et al., 2014; Munda and Kremer, 1977).

In this study, the responses of *F. vesiculosus* from two local northern Baltic populations to the combined effects of short-duration heat wave and low salinity were investigated. More specifically, the acclimatory responses to short-term (8 days) extreme temperatures, followed by an 11-day recovery period, were examined in order to unravel whether high temperature and low salinity have synergistic effects. Importantly, local populations may have divergent responses to environmental factors. Individuals may have adapted or acclimated to prevailing local conditions, such as temperature, or may be exposed to varying levels of other local factors or stressors, such as herbivory, which may affect how they respond to environmental stimuli. However, when assessing the effects of global climate change on species, site-scale effects, local adaptation or plasticity is rarely taken into account, although including these factors may greatly alter the expected impacts (Benito Garzón et al., 2011; Valladares et al., 2014). The Baltic *F.*

vesiculosus population is adapted to relatively low temperatures, with lower temperature tolerances and optima for growth and photosynthesis than the Atlantic populations (Nygård and Dring, 2008). In the Atlantic, the southern range of *F. vesiculosus* has shifted 1.250 km northwards in the last 30 years as a consequence of elevated seawater temperatures in southern areas (Nicastro et al., 2013), and substantial range shifts for the future have been projected by ecological niche models (Jueterbock et al., 2013). For Baltic *F. vesiculosus*, laboratory experiments have demonstrated that high temperatures (28 °C) can cause severe mortality in full-grown individuals, even after a short exposure (Graiff et al., 2015). It has also been suggested that the temperature tolerance of *F. vesiculosus* depends on co-occurring fluctuations in salinity (Russell, 1987). Thus, the aim of this study was to investigate the combined effects of low salinity and high temperature. To account for possibility of variability in the responses of local populations, specimens were collected from two local lagoons, one site having predominantly warmer summer temperatures due to limited water exchange and the other predominantly lower temperatures due to its proximity to the open sea.

2. Material & Methods

2.1. Sampling and experimental design

The experiment was carried out in August 2015 at Tvärminne Zoological Station (TVZ) in SW Finland. *F. vesiculosus* specimens were collected from two nearby sites off the islands of Brännskär and Granbusken, located approximately 4 km apart. The sampling sites were labeled “warm” (Brännskär) and “cold” (Granbusken), respectively. The “warm” site at Brännskär is a sheltered, shallow (maximum depth 1.5 m) lagoon that warms up rapidly during the summer. The “cold” site at Granbusken is a relatively sheltered bay in the outer archipelago with colder water due to direct contact with the open sea. Both sites have similar salinity conditions, following regional salinity fluctuations (monitoring data from Tvärminne Zoological Station 2016). The water temperature at both sites was monitored in July/August 2015 using underwater loggers (Hobo Pendant, Onset Corporation) floating freely approximately 30 cm above the

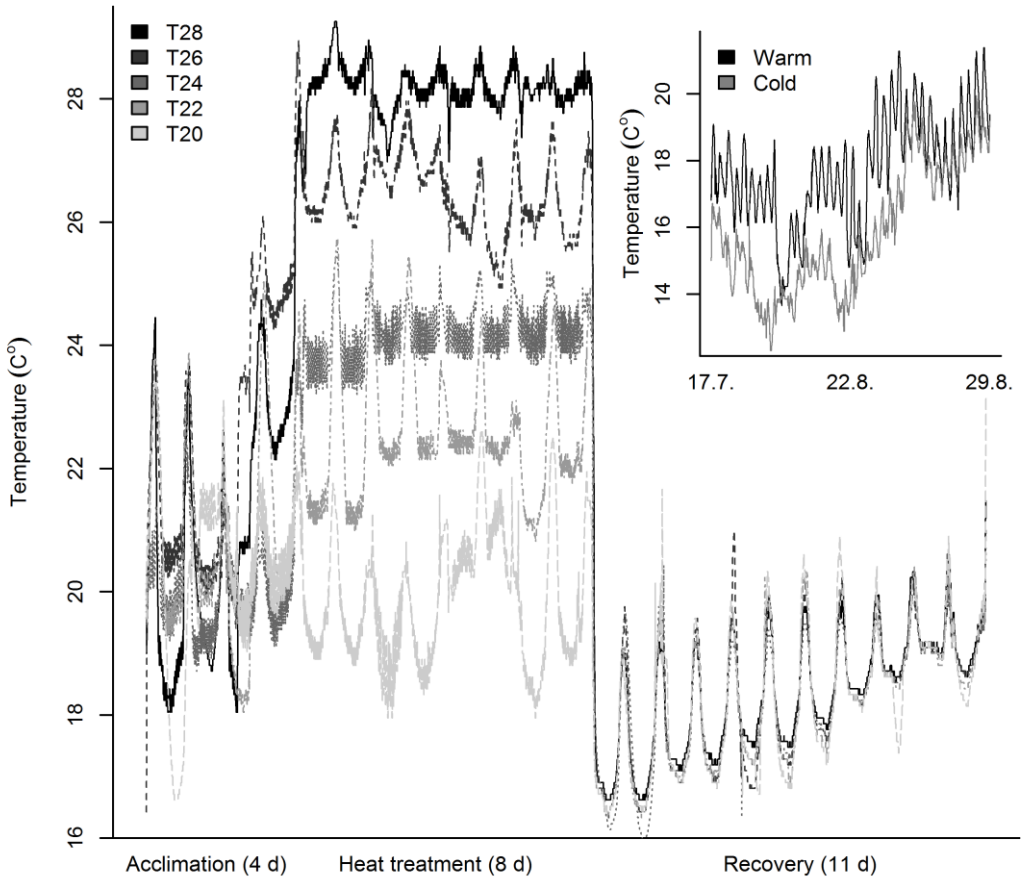
bottom at a depth of 1.5 meters, and logging with 15 minute intervals. July/August temperatures were recorded, since the annual seawater temperatures are typically highest during these months (FMI, 2016; Haapala, 1994). The temperatures at the two sites ranged from 13.7 to 21.4 °C at the warm site and from 12.3 to 20.0 °C at the cold site, the warm site having a larger diurnal range of fluctuations (Fig. 1 inset).

Vegetative thallus apices (48 ± 10 mm) were collected at the beginning of August 2015 at the two sites from a depth of 0.5 meters by snorkeling. The apices were kept constantly submerged and immediately transported to an outdoor greenhouse at TVZ, where the experiment was conducted. A greenhouse setting was chosen instead of a climate chamber to maintain the specimens in natural light conditions. The apices were stored in a 100-liter container with constant seawater flow-through for 2 days.

After this, the apices were placed in glass jars (volume 1 L), with 2 marked individuals in each, one from the warm and the other from the cold site, resulting in an average algal biomass of 440 ± 120 mg/jar. The jars were placed in temperature-controlled water baths (Hailey 300 W aquarium heaters, with cooling provided with seawater flow-through). The temperature of each water bath was raised from the ambient 19 °C to the target temperature during the course of one day, except for the two highest temperature treatments, where the temperature was raised in increments of 5 °C over 2 days to avoid rapid temperature shocks.

The samples were exposed to five temperature treatments, 20 °C (control), 22 °C, 24 °C, 26 °C, and 28 °C, which are referred to with the codes T20, T22, T24, T26, and T28, respectively. The temperature in the 1-l jars was monitored every 10 minutes using temperature loggers (Hobo Pendant, Onset Corporation) placed in each bath. The mean logged temperatures during the heat treatment were 19.8, 22.5, 24.1, 26.2, and 27.9 °C, and the standard deviation across all treatments was 1.4 °C, caused by diurnal fluctuations in ambient temperatures (Figure 1). In this way, the treatments reflected the natural daily variation in temperature that the algae are also exposed to in the sea. The duration of the temperature treatments was 8 days, followed by an 11-day recovery period. At the start of the recovery period, all heaters were switched off, and the temperature followed the ambient seawater temperature, which rose slightly towards the end of the experiment (Figure 1).

138 At present, high seawater temperatures (> 23 °C), with duration of 8 days, have been recorded in the study
139 area in July (FMI, 2016), caused by thermal stratification, which may increase surface water temperature as
140 much as 10 °C over the course of few days (Haapala, 1994). Breakdown of thermal stratification by
141 upwelling may similarly cause abrupt decreases in surface water temperatures, which have been recorded
142 to decline as much as 10 °C (Haapala, 1994). Rapid warming has been observed in the Northern Baltic, with
143 rates of 1 °C per decade since 1990s (HELCOM, 2013), with summer temperatures increasing three times
144 faster than the global warming rate (MacKenzie and Schiedek, 2007). The lowest annual salinity values in
145 the area are observed in May/June, which is somewhat before the highest temperatures, which typically
146 occur in July/August. However, the mean annual salinity levels in the Baltic, especially in the Northern
147 areas, have been predicted to decline in the future because of increased freshwater input, and the *F.*
148 *vesiculosus* populations in the Northern Baltic are thus expected to be experiencing salinity levels around 4
149 by the end of the ongoing Century (Vuorinen et al., 2015). If the mean salinity levels decline as predicted,
150 the low salinity (~4) will be a prevalent salinity level also during the summer seasons with the highest
151 seawater temperatures in the future. Thus our experimental set-up reflects the effect of expected future
152 summertime extreme temperature events (Neumann et al., 2012), coupled with predicted declines in mean
153 salinity levels (Meier, 2006; Vuorinen et al., 2015).



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155 Figure 1. Logged water temperatures during the experiment in the temperature treatments (T20- T28) and
156 at the two sites from where specimens were collected (inset).

157

158 Twelve jars were included in each temperature treatment, which were allocated to two salinity treatments,
159 "ambient" (5.9 units) and "low" (4.2 units). The low salinity was achieved by diluting filtered (25 µm)
160 seawater pumped from the adjacent bay with ion-exchanged water (Miele E 318). The "ambient" salinity
161 treatment consisted of filtered seawater. Both treatments were enriched by adding $NaNO_3$ and $H_2KO_4P_4$
162 from a stock solution to obtain mean nutrient concentrations of 150 µg/l NO_2 + NO_3 and 30 µg/l PO_4,
163 respectively, to avoid nutrient limitation. The water in the jars was vigorously aerated and changed every
164 second day. Salinity and temperature were measured daily with a VWR EC-300 conductivity meter. The
165 salinities measured during the experiment in these treatments were 4.18 ± 0.06 and 5.89 ± 0.09. These are
166 referred to as salinities 4 and 6.

167 Irradiance in the experiment followed the ambient light environment. The PAR (400–750 nm) photon flux
168 density measured at the top of the jars at midday was 136 ± 3.5 µmol photons m^{-2} s^{-1} (OceanOptics
169 USB2000+), which corresponds to in situ irradiance at the depth of 2-3 meters in August (Lindström, 2000).

170 After the experiment, all algal specimens were frozen (-20 °C) for mannitol analysis.

171 *2.2. Parameters measured*

172 In order to study the integrative physiological effects of temperature and salinity, growth as increase in
173 fresh weight (FW) was measured after the heat treatment and after the recovery period. Growth in length
174 was also measured, and provided identical information to FW measurements (data not shown); hence, FW
175 values were used to determine growth rates. The mannitol content in the algae was analyzed, since it is the
176 key storage compound in *F. vesiculosus* (Lehvo et al., 2001) and plays an important role in osmoregulation
177 (Munda and Kremer, 1977).

178 Photosystem II (together with enzymes of the Calvin-Benson cycle) has been identified as the most
179 sensitive component of the photosynthetic apparatus to thermal stress (Allakhverdiev et al., 2008; Eggert,
180 2012; Ralph, 1998). To quantify the responses of PS II photochemistry to thermal and salinity treatments,
181 several fluorescence parameters were measured. These were the steady-state relative electron transport
182 rate in saturating light (ssrETR) and two rapid light curve parameters: the linear slope of the light-limited
183 region of the curve (α), which indicates the efficiency of photochemistry in the light-limited state, and the
184 maximum relative electron transport rate (rETRmax). ssrETR and rETRmax are proxies for the performance
185 of photosynthesis under saturating irradiance, and thus indicate the status of the electron transport chain.

186 α and rETRmax were determined using the rapid light curve (RLC) protocol (Ralph and Gademann, 2005). In
187 RLC measurement, photosynthetic tissue is subjected to relatively short increments of increasing
188 irradiances, each followed by determination of the effective quantum yield ($\Delta F/F_m'$) (Ralph and Gademann,
189 2005). In the light-limited region of the curve, photobiology is limited by light availability and the efficiency
190 of photosynthetic energy capture (α) (Enríquez and Borowitzka, 2011). After the onset of light saturation,

191 the curve reaches a plateau, and a maximum electron transport rate (rETRmax) is reached. In this state,
192 photosynthesis is limited by the reaction rates of the electron transport chain components (Ralph and
193 Gademann, 2005). As illumination times are quite short (often around 10s) in the RLC methodology, a
194 photosynthetic steady state is not attained (Ralph and Gademann, 2005). To determine whether the
195 treatments affected the enzymatic machinery required to sustain steady-state photosynthesis in saturating
196 irradiances, e.g. enzymes related to carbon fixation, ssrETR was also measured under saturating light.

197 In addition to these parameters, the maximum potential quantum efficiency of PS II photochemistry (F_v/F_m)
198 was measured every 2 days throughout the experiment. Detailed description of the measurements are
199 given in Supplementary Information.

200 As absorption factors were not measured for the apices, ETR values are given as the relative electron
201 transport rate, rETR (Ralph and Gademann, 2005). All fluorescence measurements were conducted using a
202 Diving-PAM underwater pulse amplitude modulated fluorometer (Walz GmbH, Germany).

203

204 *2.2.1 Growth measurements*

205 The fresh weight (1 mg precision, Sartorius CP 3202S) of each apex was measured before and after the
206 temperature treatment, and after the recovery period.

207 Relative growth rate (RGR) was calculated from FW after Wiencke and Fischer (1990) as

208 $$RGR = \frac{100 * \ln(N_t \div N_0)}{t}$$ (Eq. 1)

209 where RGR is the daily growth in percent FW, N_t is the FW at day t, N_0 is the initial FW, and t is time interval
210 in days.

211 *2.2.2. Fluorescence measurements*

ssrETR was measured by attaching the thallus apex to a dark leaf clip to ensure a standard distance between the fiber optics and the sample, and attaching the clip to the fiber optics of the Diving-PAM. Irradiance of 194 µmol photons m^{-2} s^{-1} was supplied by the internal halogen lamp of the Diving-PAM for four minutes, after which the effective quantum yield, $\Delta F/F_m'$ (Genty et al., 1989) was measured. ssrETR was calculated using the formula

$$ssrETR = \frac{\Delta F}{F_m'} \times PAR \times 0.5 \quad \text{(Eq. 3)}$$

where $\Delta F/F_m'$ is the effective quantum yield under actinic light, PAR is the photon flux density of photosynthetically active radiation (provided by the halogen lamp), and 0.5 is the factor accounting for the assumed equal partitioning of photons between photosystems I and II (Genty et al., 1989). ssrETR was measured twice, after the heat exposure and the recovery period.

α and rETRmax were measured by attaching a dark leaf clip to each thallus apex, after which the rapid light curve protocol of the Diving-PAM was immediately (<10 s) initiated without allowing any dark adaption, as recommended by Ralph and Gademann (2005). Eight levels of increasing light intensities from 0 to 734 µmol photons m^{-2} s^{-1} were applied using the internal halogen lamp of the Diving-PAM. The duration of each light increment was 10 s (Edwards and Kim, 2010; Ralph and Short, 2002; Ralph and Gademann, 2005). rETR versus PAR curves were fitted to the model of Platt et al. (1981) with the R package “phytotools” (Silsbe and Malkin, 2015), and the parameters for α and rETRmax were solved from the equation. RLC measurements were conducted at the end of the recovery period. The light sensor of the Diving-PAM was used in determining the photon flux density in RLC and ssrETR measurements, when the halogen lamp was used as an external light source. The Diving-PAM light sensor was calibrated against a factory-calibrated light sensor (LI-COR LI-1000).

To assess the function of fluorescence measurements as fitness proxies, all fluorescence parameters were correlated with growth rate and mannitol (see Supplementary information, Fig S2-S4).

236 *2.2.3. Mannitol analyses*

237 Mannitol was extracted and analyzed following the HPLC protocol of Karsten et al. (1991). Mannitol was
238 extracted into MQ H_2O in a water bath at +80 °C for 4 h with ultra-sonication. The mannitol concentration
239 was analyzed with a Waters Acquity® UPLC system (Waters, Milford MA, USA) attached to a Waters Synapt
240 G2 QTOF HDMS mass spectrometer (Waters, Milford MA, USA) via an ESI ion source.

241

242 *2.2.4. Statistical analyses*

243 The effects of temperature, salinity, site, and their interactions on growth rates, the mannitol content,
244 ssrETR, α, and rETRmax were analyzed with generalized least squares (GLS) regression models using
245 restricted maximum likelihood (REML) estimation for the weights, allowing for heterogeneity, and
246 maximum likelihood (ML) estimation for the fixed components (Zuur et al., 2009). GLS was used because,
247 when plotting fitted values against covariates, residual patterns indicated heterogeneity, especially at the
248 highest temperatures.

249 In all analyses, salinity and site were treated as factorial covariates, and the effect of temperature was
250 modeled with cubic regression splines with three or four degrees of freedom (James et al., 2013). The
251 model selection protocol outlined by (Zuur et al., 2009) was followed. Model assumptions were inspected
252 by plotting normalized residuals against fitted values and all covariates. If heterogeneity was encountered,
253 different variance structures per treatment were specified (Zuur et al., 2009). The appropriate variance
254 structure was selected as the one with the lowest Akaike Information Criterion. For the fixed components
255 of the model, p-values of < 0.05 were used as a threshold for accepting a variable in the final model. As
256 individuals from the two sites were kept in the same jars, the jar identification (id) was included as a
257 random factor in the starting model, but as the likelihood ratio tests did not support including jar id in the
258 final model, it was subsequently dropped from all analysis.

259 All statistical analyses were performed in the R software environment, version 3.2.0 (R Core Team, 2015).

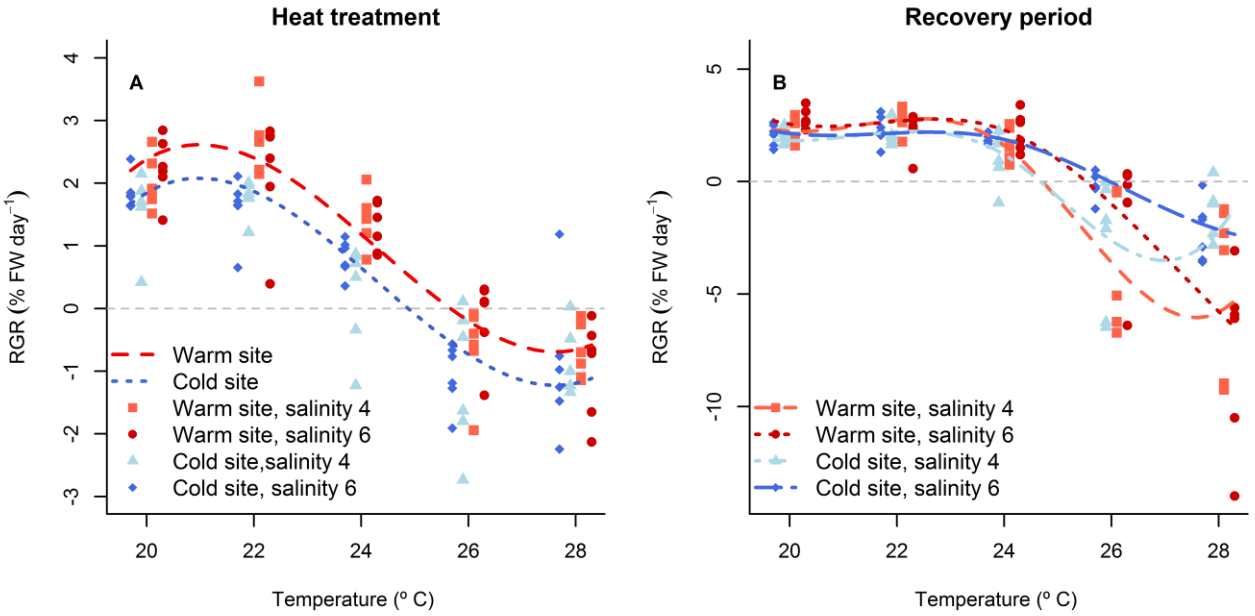
3. Results

3.1. Growth rates and mannitol content

During the heat treatment, temperature and site affected the growth rates, whereas the interactive effects of salinity were only observable during the recovery period (Table 1, Fig. 2). Low salinity already caused declines in growth rates at 24 °C, and above this temperature it caused more extensive tissue necrosis (Table 1, Fig. 2b). In T26, the growth rate of individuals exposed to low salinity was 2.6% FW day^{-1} lower than for individuals growing in ambient salinity (Fig. 2b). In T28, RGR was consistently negative, because of excessive tissue necrosis in all treatments. However, individuals from the "warm" site suffered from more severe tissue necrosis, having 3.63% FW day^{-1} lower growth rates than individuals from the "cool" site (Fig. 2b). During the heat treatment, individuals from the warm site had consistently higher growth rates (Table 1, Fig. 2a). However, they were ultimately most affected by the highest temperatures, as displayed during the recovery period (Fig. 2b).

Table 1. Regression analysis results for relative growth rate and mannitol. Significance levels: *<0.05, **<0.01, ***<0.001.

	Growth rate			**Mannitol**		
	L	df	p	L	df	p
Heat treatment						
Temperature*Salinity*Site	1.976	3	0.577			
Temperature*Salinity	2.974	3	0.395			
Temperature*Site	4.626	3	0.201			
Salinity*Site	1.262	1	0.261			
Salinity	1.726	1	0.188			
Site	**26.174**	**1**	**< 0.001*** **			
Temperature	**169.15**	**3**	**< 0.001*** **			
Recovery period						
Temperature*Salinity*Site	6.343	4	0.174	20.725	**4**	**< 0.001*** **
Temperature*Salinity	**13.185**	**4**	**0.010***			
Temperature*Site	**13.852**	**4**	**0.007****			
Salinity*Site	0.005	1	0.938			



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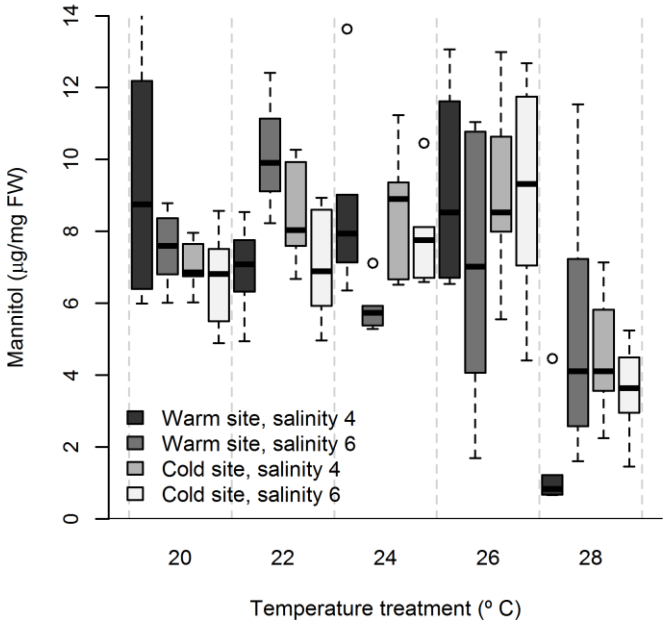
276 Figure 2. Relative growth rates during a) heat treatment, b) the recovery period. Lines represent the
277 regression model fit. In panel a), only 2 lines are shown, one for each site, as salinity had no significant
278 effect. Negative RGR values indicate tissue necrosis. Note the different scale of the two plots.

279 The mannitol content varied according to the site, salinity, and temperature (3-way interaction, Table 1). In
280 both periods, a general increasing trend was observed until T26, after which the mannitol content
281 substantially declined (Fig. 3). In T28, the warm site individuals exposed to low salinity had the lowest
282 mannitol content of all treatments (Fig. 3). Regression analysis revealed that the interaction between the
283 three variables (temperature, salinity, and site) was significant in explaining variation in the mannitol
284 concentration during the recovery period (Table 1).



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286 Figure 3. Mannitol content at the end of the recovery period.

287

288 Table 2. Statistical analysis results for chlorophyll fluorescence measurements. Significance levels: *<0.05,

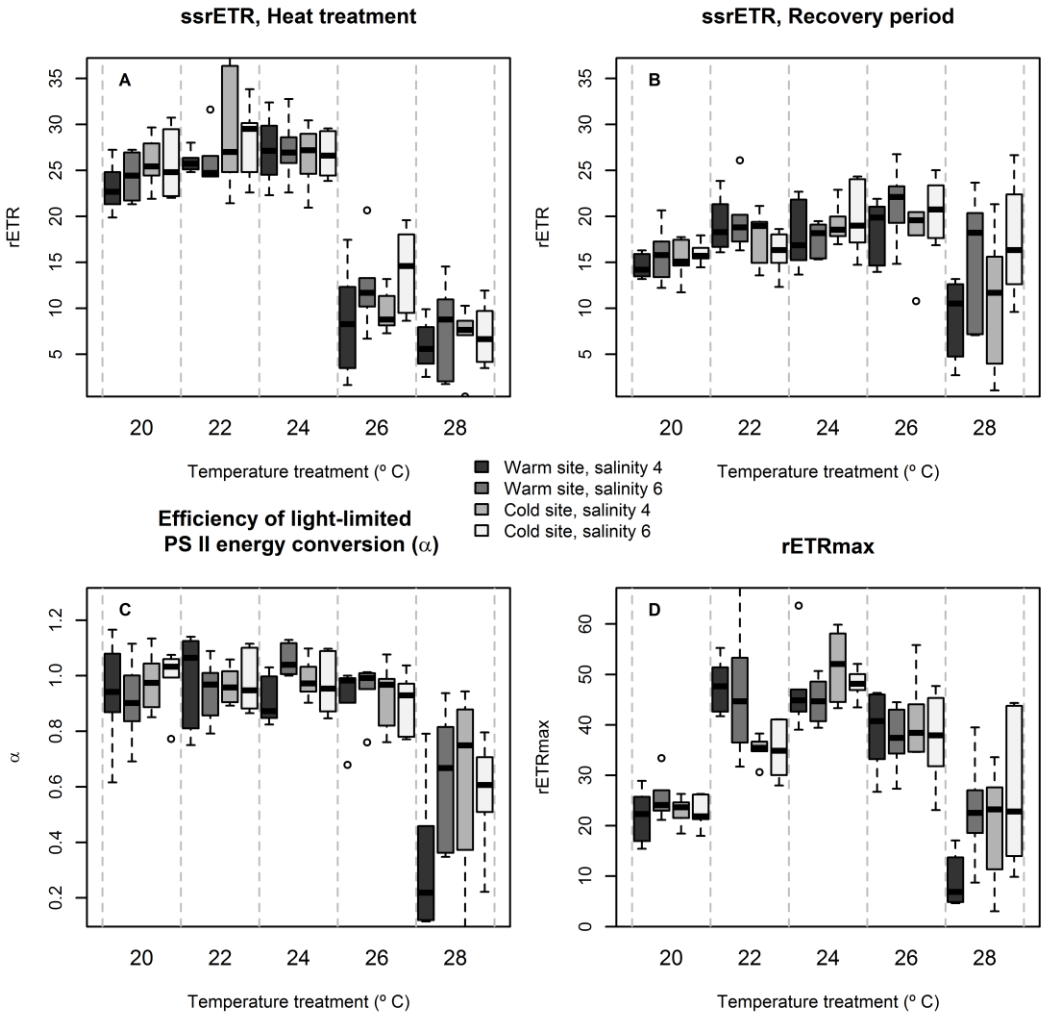
289 **<0.01, ***<0.001.

	ssrETR			α			rETRmax		
	L	df	p	L	df	p	L	df	p
Heat treatment									
Temperature*Salinity*Site	0.551	4	0.968						
Temperature*Salinity	6.189	4	0.185						
Temperature*Site	3.414	4	0.491						
Salinity*Site	0.190	1	0.662						
Salinity	2.904	1	0.083						
Site	2.867	1	0.090						
Temperature	**224.67**		**< 0.001*** **						
Recovery period									
Temperature*Salinity*Site	0.547	3	0.908	3.497	3	0.321	1.431	4	0.838
Temperature*Salinity	**9.415**	**3**	**0.024***	1.015	3	0.797	8.474	4	0.075
Temperature*Site	3.519	3	0.318	2.304	3	0.511	**26.386**	**4**	**< 0.001*** **
Salinity*Site	0.478	1	0.502	2.796	1	0.094	**25.297**	**4**	**< 0.001 *** **
Salinity				1.080	1	0.298			
Site	0.049	1	0.823	0.135	1	0.713			
Temperature				**30.755**	**3**	**< 0.001 *** **			

290

291 *3.2. Fluorescence measurements*

292 The steady-state electron transport rate rapidly declined in T26 and T28 during the heat treatment, but the
293 overall decline was not associated with either salinity or site (Fig. 4a, Table 2). However, in T26 and T28,
294 individuals exposed to low salinity had a lower ssrETR (Fig. 4a). During the recovery period, ETR rates
295 recovered in T26 but not in T28 (Fig. 4b). The synergistic effects of high temperature and low salinity
296 caused declines in ssrETR (Table 2, Fig. 4b), with the ssrETR in T28 being approximately 40% lower at a
297 salinity of 4 (Fig. 4b).



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299 Figure 4. Steady-state relative electron transport rate (ssrETR) after heat treatment (a) and after recovery
300 (b), the quantum efficiency of light-limited PS II photochemistry (α, c), and maximum relative electron
301 transport rate (rETRmax, d).

The efficiency of light-limited photochemistry (α) was relatively unaffected by temperatures below T28, showing only a minor decline in T26 (Fig. 4c). α was not affected by site or salinity; however, in T28, individuals from the warm site at a salinity of 4 expressed the steepest declines (Fig. 4c).

After the recovery period, the two populations differed in photosynthetic rates, expressed as rETRmax, in the different temperature treatments (Fig. 4d, Table 2). The individuals from the warm site expressed the highest rETRmax rates in T22, whereas the cold site individuals had peak values at T24. The rETRmax of both populations declined at T26, followed by a steeper decline at T28 (Fig. 4d).

F_v/F_m declined rapidly declined during the experiment in the two highest temperature treatments, while low salinity caused lower F_v/F_m during the recovery period (Fig. S1). F_v/F_m showed relationship between growth rate and mannitol only in individuals exposed to highest temperature treatments, with extensive tissue necrosis (Fig. S2). However, as F_v/F_m was measured every 2 or 3 days throughout the experiment, the temporal patterns of stress development are observable in the F_v/F_m data (Fig S1).

Discussion

The present results demonstrate the synergistic negative effects of high temperature and low salinity on *F. vesiculosus*, with the combined effect exceeding in magnitude that of either variable alone. The effects were especially apparent in the recovery period. Exposing individuals to low salinity slowed the recovery of growth and photosynthesis following heat exposure and increased tissue necrosis caused by high temperature.

The growth rate during the heat treatment already declined at 24 °C, and at 26 °C and 28 °C, substantial tissue necrosis, starting from the apical meristem at the thallus tip, was observed in almost all individuals. As the growth rate integrates over a range of physiological processes, it provides a good approximation of the overall impact on the fitness of individuals.

The warm site individuals had consistently higher growth rates during heat treatment (Fig. 2a), which indicates that they were more tolerant of the relatively high temperatures used in the study. The synergistic effect of low salinity and high temperature was observed during the recovery period, when significantly lower growth rates were observed in individuals subjected to low salinity at 26 °C. On the other hand, the effect of low salinity at 20–24 °C was negligible, which suggests that mature *F. vesiculosus* individuals tolerate lowered salinity reasonably well (Larsen and Sand-Jensen, 2006). However, when combined with an interacting stressor such as high temperature, abrupt adverse effects may emerge.

The mannitol content increased as a function of temperature up to 26 °C, but substantially declined at 28 °C in all treatments, and especially in the warm site individuals at low salinity (Fig. 3). Mannitol is the main storage compound of photosynthesis in brown algae (Groisillier et al., 2014; Karsten, 2012; Kirst, 1990), and it plays an important role in osmoregulation in *F. vesiculosus* (Munda and Kremer, 1977). Mannitol also correlates with the growth rate and is used as a seasonal energy storage to be utilized for growth in low light seasons (Bidwell and Ghosh, 1962; Lehvo et al., 2001). Mannitol may additionally compensate for osmotic pressure changes caused by intracellular nitrate reduction during periods of rapid growth (Karsten, 2012). In addition to osmoregulatory functions, mannitol may serve as an antioxidant in algae (Groisillier et al., 2014; Iwamoto and Shiraiwa, 2005), potentially capturing free oxygen radicals accumulated under e.g. heat stress (Eggert, 2012; Takahashi and Murata, 2008). Thus, the accumulation of mannitol with increasing temperature may be caused by two different processes: temperature stimulation of photosynthesis or a stress response to harmful oxygen species. The mannitol content closely follows the maximum photosynthetic rates (Fig. 4d), which is expected, as mannitol is the main molecular sink of photosynthates in *F. vesiculosus*, and thus the slight increase in mannitol at 20–26 °C is caused by an elevated photosynthetic rate. The warm site individuals exposed to low salinity had very low mannitol contents at 28 °C (Fig. 3), a pattern that was also observable in fluorescence parameters (Fig. 4), indicating that these low mannitol levels were caused by a decline in the photosynthetic capacity.

In the present study, photobiological parameters and the growth rate already substantially declined at 26 °C. Graiff et al. (2015) observed *F. vesiculosus* to grow well in the temperature range of 5–26 °C in an experiment conducted in Kiel Fjord in the southwestern Baltic Sea. The difference may be explained by the northern populations investigated in this study being more sensitive to high temperatures. On the other hand, although differences related to thermal tolerance limits and thermal growth optima have usually been attributed to regionally distinct populations (Bolton, 1983; Nygård and Dring, 2008; Pereira et al., 2015), the present study shows, that even local populations separated by only a few kilometers may have somewhat differing responses to heat stress.

In this study, exposure to extreme temperatures caused different responses in photosynthesis rates (rETRmax, Fig. 4b) between the two local populations. Because the light curves were measured at the end of the recovery period, the readings can be interpreted to reflect the stimulating effects of temperature exposure on photosynthesis rates. In contrast to what was expected, the population from the cold site had the highest photosynthesis rates at T24, whereas the warm site individuals had the highest rates at T22 (Fig. 4d). The photosynthesis rates of algae can acclimate in relation to changes in the ambient temperature (Eggert, 2012), and this regulatory capability is generally higher in algae inhabiting sites with fluctuating ambient temperatures (Eggert, 2012). Being a eurythermal species, *F. vesiculosus* in the Baltic Sea is subjected to wide annual temperature fluctuations, and is thus expected to possess substantial phenotypic plasticity in response to the changing ambient temperature. When parental algae experience high temperatures, increased survival under high temperature is observed in the offspring (Li and Brawley, 2004) due to the accumulation of heat shock proteins such as chaperons in the embryos. Thus the observed differences may arise from phenotypic plasticity caused by different temperature fluctuations experienced by the individuals in the local habitats, or from genetic adaptation. As individuals from only two sites were sampled, it is not possible to attribute the responses to thermal adaptation with certainty, and the differences may arise also from other locally affecting factors, such as biotic interactions. The warm site population subjected to extreme temperatures at a salinity of 4 displayed dramatic declines in all measured parameters, while the cold site individuals did not show such steep declines. This implies that when

subjected to simultaneous multiple stressors, local populations may respond in different ways, which should be considered when making inferences of the climate-related tolerances of species based on experiments conducted with individuals sampled from a single site.

High temperatures increase the susceptibility to photoinhibition by impairing the mechanisms for repairing photodamage through suppression of the synthesis of PSII proteins, in particular D1, which is damaged by the accumulation of reactive oxygen species (ROS, Takahashi and Murata 2008, Allakhverdiev et al. 2008). High temperature stress also reduces CO_2 assimilation through the inhibition of Rubisco activase (Allakhverdiev et al., 2008), thus affecting the light-saturated parameters of the RLC curve, such as rETRmax. This, in turn, causes the accumulation of ROS, which inhibit the repair mechanisms of PSII. Photochemical reactions of photosynthesis (i.e. the light-limited region of the RLC curve) are less sensitive to ambient temperature changes compared to reactions of the Calvin cycle (Peschek and Zoder, 2001), which are regulated by enzyme kinetics (Falkowski and Raven, 2007). This was also seen in the present results, as α was relatively insensitive to temperature treatments, with the exception of 28 °C, which caused substantial declines due to direct damage to the PSII reaction centers. Such damage always leads to a decline in the slope of the PE curve (Falkowski and Raven, 2007).

An increased frequency of periods of extreme temperatures has been predicted in the Baltic as a consequence of climate change (Neumann et al., 2012). As the maximum summer seawater temperatures in the study area in Tvärminne already exceed 23 °C in the present climate (FMI, 2016; Haapala, 1994), *F.vesiculosus* populations will probably be subjected to extreme temperatures near their tolerance limit in the future.

Conclusions

The present results indicate that *F. vesiculosus* populations in the northern Baltic Sea are vulnerable to even relatively short periods of high temperatures (~26 °C), especially under the influence of low salinity, as predicted for the future Baltic Sea (Meier et al., 2014; Vuorinen et al., 2015). Although the distributional limit of *F. vesiculosus* in the northern Baltic appears to be determined by reproductive failure at low

salinities (Serrão et al., 1996), suggesting that reproductive stages would be most intolerant to environmental stressors, the results of this study indicate that mature plants may also be vulnerable to low salinity when subjected to interacting stressors such as extreme temperatures, even for relatively short periods.

The synergistic effects of low salinity and high temperature on growth rates were not observable immediately after exposure, but only after a recovery period. This highlights the importance of conducting experiments, especially with multiple interacting stressors, that include a subsequent period of recovery.

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