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

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12

13   **Abstract**

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

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

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

31   **Introduction**

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

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

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

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

78 In this study, the responses of *F. vesiculosus* from two local northern Baltic populations to the combined
 79 effects of short-duration heat wave and low salinity were investigated. More specifically, the acclimatory
 80 responses to short-term (8 days) extreme temperatures, followed by an 11-day recovery period, were
 81 examined in order to unravel whether high temperature and low salinity have synergistic effects.
 82 Importantly, local populations may have divergent responses to environmental factors. Individuals may
 83 have adapted or acclimated to prevailing local conditions, such as temperature, or may be exposed to
 84 varying levels of other local factors or stressors, such as herbivory, which may affect how they respond to
 85 environmental stimuli. However, when assessing the effects of global climate change on species, site-scale
 86 effects, local adaptation or plasticity is rarely taken into account, although including these factors may
 87 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

113 bottom at a depth of 1.5 meters, and logging with 15 minute intervals. July/August temperatures were
 114 recorded, since the annual seawater temperatures are typically highest during these months (FMI, 2016;
 115 Haapala, 1994). The temperatures at the two sites ranged from 13.7 to 21.4 °C at the warm site and from
 116 12.3 to 20.0 °C at the cold site, the warm site having a larger diurnal range of fluctuations (Fig. 1 inset).

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

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

128 The samples were exposed to five temperature treatments, 20 °C (control), 22 °C, 24 °C, 26 °C, and 28 °C,
 129 which are referred to with the codes T20, T22, T24, T26, and T28, respectively. The temperature in the 1-l
 130 jars was monitored every 10 minutes using temperature loggers (Hobo Pendant, Onset Corporation) placed
 131 in each bath. The mean logged temperatures during the heat treatment were 19.8, 22.5, 24.1, 26.2, and
 132 27.9 °C, and the standard deviation across all treatments was 1.4 °C, caused by diurnal fluctuations in
 133 ambient temperatures (Figure 1). In this way, the treatments reflected the natural daily variation in
 134 temperature that the algae are also exposed to in the sea. The duration of the temperature treatments was
 135 8 days, followed by an 11-day recovery period. At the start of the recovery period, all heaters were
 136 switched off, and the temperature followed the ambient seawater temperature, which rose slightly
 137 towards the end of the experiment (Figure 1).

138 At present, high seawater temperatures ($> 23^{\circ}\text{C}$), 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).

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

Twelve jars were included in each temperature treatment, which were allocated to two salinity treatments, “ambient” (5.9 units) and “low” (4.2 units). The low salinity was achieved by diluting filtered (25 μm) seawater pumped from the adjacent bay with ion-exchanged water (Miele E 318). The “ambient” salinity treatment consisted of filtered seawater. Both treatments were enriched by adding NaNO_3 and $\text{H}_2\text{KO}_4\text{P}_4$ from a stock solution to obtain mean nutrient concentrations of 150 $\mu\text{g/l}$ $\text{NO}_2 + \text{NO}_3$ and 30 $\mu\text{g/l}$ PO_4 , respectively, to avoid nutrient limitation. The water in the jars was vigorously aerated and changed every second day. Salinity and temperature were measured daily with a VWR EC-300 conductivity meter. The salinities measured during the experiment in these treatments were 4.18 ± 0.06 and 5.89 ± 0.09 . These are 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 $136 \pm 3.5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (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,

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

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

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

203

2.2.1 Growth measurements

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

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

$$RGR = \frac{100 \cdot \ln(N_t \div N_0)}{t} \quad (\text{Eq. 1})$$

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 in days.

2.2.2. Fluorescence measurements

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

$$217 \quad \text{ssrETR} = \frac{\Delta F}{F_m'} \times \text{PAR} \times 0.5 \text{ (Eq. 3)}$$

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

222 α and rETRmax were measured by attaching a dark leaf clip to each thallus apex, after which the rapid light
 223 curve protocol of the Diving-PAM was immediately (<10 s) initiated without allowing any dark adaption, as
 224 recommended by Ralph and Gademann (2005). Eight levels of increasing light intensities from 0 to 734
 225 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ were applied using the internal halogen lamp of the Diving-PAM. The duration of each
 226 light increment was 10 s (Edwards and Kim, 2010; Ralph and Short, 2002; Ralph and Gademann, 2005). rETR
 227 versus PAR curves were fitted to the model of Platt et al. (1981) with the R package “phytotools” (Silsbe
 228 and Malkin, 2015), and the parameters for α and rETRmax were solved from the equation. RLC
 229 measurements were conducted at the end of the recovery period. The light sensor of the Diving-PAM was
 230 used in determining the photon flux density in RLC and ssrETR measurements, when the halogen lamp was
 231 used as an external light source. The Diving-PAM light sensor was calibrated against a factory-calibrated
 232 light sensor (LI-COR LI-1000).

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

235

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 H₂O 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
<u>Heat treatment</u>						
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***			
<u>Recovery period</u>						
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			

275

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).

285

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

3.2. Fluorescence measurements

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

298

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

301

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

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

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

314

315 Discussion

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

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

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

332 The mannitol content increased as a function of temperature up to 26 °C, but substantially declined at 28 °C
 333 in all treatments, and especially in the warm site individuals at low salinity (Fig. 3). Mannitol is the main
 334 storage compound of photosynthesis in brown algae (Groisillier et al., 2014; Karsten, 2012; Kirst, 1990), and
 335 it plays an important role in osmoregulation in *F. vesiculosus* (Munda and Kremer, 1977). Mannitol also
 336 correlates with the growth rate and is used as a seasonal energy storage to be utilized for growth in low
 337 light seasons (Bidwell and Ghosh, 1962; Lehvo et al., 2001). Mannitol may additionally compensate for
 338 osmotic pressure changes caused by intracellular nitrate reduction during periods of rapid growth (Karsten,
 339 2012). In addition to osmoregulatory functions, mannitol may serve as an antioxidant in algae (Groisillier et
 340 al., 2014; Iwamoto and Shiraiwa, 2005), potentially capturing free oxygen radicals accumulated under e.g.
 341 heat stress (Eggert, 2012; Takahashi and Murata, 2008). Thus, the accumulation of mannitol with increasing
 342 temperature may be caused by two different processes: temperature stimulation of photosynthesis or a
 343 stress response to harmful oxygen species. The mannitol content closely follows the maximum
 344 photosynthetic rates (Fig. 4d), which is expected, as mannitol is the main molecular sink of photosynthates
 345 in *F. vesiculosus*, and thus the slight increase in mannitol at 20–26 °C is caused by an elevated
 346 photosynthetic rate. The warm site individuals exposed to low salinity had very low mannitol contents at 28
 347 °C (Fig. 3), a pattern that was also observable in fluorescence parameters (Fig. 4), indicating that these low
 348 mannitol levels were caused by a decline in the photosynthetic capacity.

349 In the present study, photobiological parameters and the growth rate already substantially declined at 26
350 °C. Graiff et al. (2015) observed *F. vesiculosus* to grow well in the temperature range of 5–26 °C in an
351 experiment conducted in Kiel Fjord in the southwestern Baltic Sea. The difference may be explained by the
352 northern populations investigated in this study being more sensitive to high temperatures. On the other
353 hand, although differences related to thermal tolerance limits and thermal growth optima have usually
354 been attributed to regionally distinct populations (Bolton, 1983; Nygård and Dring, 2008; Pereira et al.,
355 2015), the present study shows, that even local populations separated by only a few kilometers may have
356 somewhat differing responses to heat stress.

357 In this study, exposure to extreme temperatures caused different responses in photosynthesis rates
358 (rETR_{max}, Fig. 4b) between the two local populations. Because the light curves were measured at the end
359 of the recovery period, the readings can be interpreted to reflect the stimulating effects of temperature
360 exposure on photosynthesis rates. In contrast to what was expected, the population from the cold site had
361 the highest photosynthesis rates at T24, whereas the warm site individuals had the highest rates at T22
362 (Fig. 4d). The photosynthesis rates of algae can acclimate in relation to changes in the ambient temperature
363 (Eggert, 2012), and this regulatory capability is generally higher in algae inhabiting sites with fluctuating
364 ambient temperatures (Eggert, 2012). Being a eurythermal species, *F. vesiculosus* in the Baltic Sea is
365 subjected to wide annual temperature fluctuations, and is thus expected to possess substantial phenotypic
366 plasticity in response to the changing ambient temperature. When parental algae experience high
367 temperatures, increased survival under high temperature is observed in the offspring (Li and Brawley,
368 2004) due to the accumulation of heat shock proteins such as chaperons in the embryos. Thus the observed
369 differences may arise from phenotypic plasticity caused by different temperature fluctuations experienced
370 by the individuals in the local habitats, or from genetic adaptation. As individuals from only two sites were
371 sampled, it is not possible to attribute the responses to thermal adaptation with certainty, and the
372 differences may arise also from other locally affecting factors, such as biotic interactions. The warm site
373 population subjected to extreme temperatures at a salinity of 4 displayed dramatic declines in all measured
374 parameters, while the cold site individuals did not show such steep declines. This implies that when

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

378 High temperatures increase the susceptibility to photoinhibition by impairing the mechanisms for repairing
 379 photodamage through suppression of the synthesis of PSII proteins, in particular D1, which is damaged by
 380 the accumulation of reactive oxygen species (ROS, Takahashi and Murata 2008, Allakhverdiev et al. 2008).
 381 High temperature stress also reduces CO₂ assimilation through the inhibition of Rubisco activase
 382 (Allakhverdiev et al., 2008), thus affecting the light-saturated parameters of the RLC curve, such as
 383 rETR_{max}. This, in turn, causes the accumulation of ROS, which inhibit the repair mechanisms of PSII.
 384 Photochemical reactions of photosynthesis (i.e. the light-limited region of the RLC curve) are less sensitive
 385 to ambient temperature changes compared to reactions of the Calvin cycle (Peschek and Zoder, 2001),
 386 which are regulated by enzyme kinetics (Falkowski and Raven, 2007). This was also seen in the present
 387 results, as α was relatively insensitive to temperature treatments, with the exception of 28 °C, which
 388 caused substantial declines due to direct damage to the PSII reaction centers. Such damage always leads to
 389 a decline in the slope of the PE curve (Falkowski and Raven, 2007).

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

395 **Conclusions**

396 The present results indicate that *F. vesiculosus* populations in the northern Baltic Sea are vulnerable to
 397 even relatively short periods of high temperatures (~26 °C), especially under the influence of low salinity, as
 398 predicted for the future Baltic Sea (Meier et al., 2014; Vuorinen et al., 2015). Although the distributional
 399 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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