TITLE:

Role of thermal photosynthetic plasticity in the dispersal and settlement of two global Green Tide formers: *Ulva pertusa* and *U. ohnoi*.

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Abstract

The green invasive macroalgae *Ulva ohnoi* and *U. pertusa* are known for their capacity to form Green Tides across many geographic regions. Given their global ecological and economic impact, photosynthetic responses of both *Ulva* spp. were tested in laboratory experiments using short-term (3 h, 5 h) exposure to different temperatures (27-36 °C) and light regimes, simulating light deprivation during ballast water transport conditions. Heat treatment of ship' ballast water has been widely advocated as a possible treatment to prevent biological contamination. In addition, the physiological performance of *U. ohnoi* was further assessed in long-term experiments (14 d). We examined several photosynthetic parameters and physiological variables such as growth and pigment content as a measure of physiological fitness. Both species maintained stable values of F_v/F_m over several hours across temperatures, although *Ulva ohnoi* presented higher values of F_v/F_m, photosynthetic efficiency and $\mathrm{NPQ}_{\mathrm{max}}$ than *U. pertusa,* and activated dissipative protective mechanisms earlier. In the long-term experiment, *U. ohnoi* died by the 4th day at 36 °C. In surviving samples, F_v/F_m increased by day 7, regardless of temperature, and decreased by day 14; lower values pertained to 34 °C. Photosynthetic efficiency of *U. ohnoi* decreased after 1 week at 27 and 29 °C, but at 31 and 34 °C, an increase was recorded after 7 d. The highest $\mathrm{rETR}_{\mathrm{max}}$ was recorded at 29 °C, while growth optimum occurred at 27-31 °C. We postulate that short-term thermal stress affects dispersal risk, which may also explain the seasonal replacement of the two *Ulva* spp. in Japan; and that *U. ohnoi* has a higher thermal tolerance that allows its settlement in new areas, resulting in a wide distributional range. Its physiological performance under the temperatures tested suggests that *U. ohnoi* may pose a greater risk of dispersal and settlement than *U. pertusa*.

Introduction

Green Tides represent vast accumulations of unattached green macroalgae such as *Ulva* Linnaeus, *Chaetomorpha* Kützing and *Cladophora* Kützing, formed as a result of excessive growth, usually driven by eutrophication (Fletcher 1996). Lately, Green Tide events are increasing in severity, frequency and expanding geographic range, posing a common environmental problem in extensive areas of temperate coasts worldwide, with America, Europe and the Asia-Pacific regions being the most seriously affected (Ye et al. 2011). Additionally, their frequency is predicted to increase under future climate change scenarios (Gao et al. 2017). An example of the magnitude and the economical and biological impact of Green Tides was the well-documented Green Tide formed in Qingdao (China) in 2008, extending over 30 km in length; its removal required extensive labour at an estimated cost of $30 million to the province. In addition, aquaculture operations along the shore suffered losses of $100 million (Smetacek and Zingone 2013). Besides economic costs, Green Tides pose a substantial threat to the marine environment since they are associated with severe adverse effects on marine intertidal ecosystems, including modifications in ecosystem structure and a decrease in biodiversity (Raffaelli et al. 1998, Ye et al. 2011, Zhang et al. 2013).

Some green macroalgal species that cause Green Tides are relatively common in marine and estuarine habitats, and exhibit a high tolerance to variable salinity, temperature, water quality, and grow rapidly in nutrient-rich waters (Taylor et al. 2001). In Japan, Green Tides have been reported since the 1970s in many coastal areas where nutrient concentrations were higher than normal, causing serious economic and ecological problems (Hiraoka et al. 2004). Their geographical extent includes southern and western Pacific coasts around Tokyo, the Seto Inland Sea, and the islands of Shikoku and Kyushu. The conditions that lead to Green Tides in Japan include high nutrient fluxes and the lack of strong currents or wave action in relatively shallow waters (Ohno 1999). Genetic analysis revealed that Green Tides in Mikawa Bay area (Japan) were composed of up to nine species of the genus *Ulva*; *Ulva ohnoi* Hiraoka & Shimada (Kawai et al. 2007) and *Ulva pertusa* Kjellman (=*Ulva australis*, Hanyuda and Kawai 2018) being the most common ones. Their occurrence was strongly seasonal, with *U. ohnoi* dominating during autumn to early winter and *Ulva pertusa* during spring to early summer (Notoya 1999; Choi et al. 2001; Uchimura et al. 2003, Kawai et al. 2007). Optimal growth of *U. pertusa* occurs, under controlled conditions, between 20 and 25 °C (Ohno, 1977, Kakinuma et al. 2006), and that of *U. ohnoi* at 28 °C (Mata et al. 2016). The lower thermal growth limit for *U. ohnoi* was reported to be 10 °C (Notoya 1999) while *U. pertusa* can survive up to 24 h frozen at -15 ºC (Terumoto 1960). In natural environments, *U. pertusa* was reported to grow from about 0 ºC to 16-18 ºC along the Okhostsk Sea coasts, and from 27-28 ºC to about 30-31 ºC in Singapore (Tokida 1954, Teo and Wee 1983). Despite being ubiquitous, the upper thermal growth or survival limits of *U. ohnoi* are unknown. Given the extended latitudinal range occupied by Japan, seasons vary from subarctic conditions in the north to subtropical in the south. Southern Japan has very hot and humid summers, with seawater temperatures sometimes reaching 31 °C in August (Japan Meteorological Agency).

Risk assessments provide useful tools to aid managers in controlling and reducing the rate of current invasions. Additionally, they can be used to evaluate the invasion problem and choose, or develop, the most suitable operational procedures (Hewitt and Hayes 2002). Though risk assessment should ideally take into account multiple attributes of the organism and consider many abiotic factors, some physiological attributes have been reported to favour the invasion by an exotic organism. Many species are capable of modifying ecologically important features, such as growth rates and photosynthetic performance in their life cycle in response to environmental cues (Smith 2009). This phenotypical plasticity allows an invader to respond relatively quickly to its new biotic or abiotic environment and has been identified as a character supporting invasiveness (Davidson et al. 2011). Knowledge regarding the type, direction, and magnitude of induced responses is critical to decipher direct and indirect ecological effects stemming from species introductions (Smith 2009).

Summarizing, *U. ohnoi* and *U. pertusa* are significant Green Tide formers and in Japan, some blooms are believed to occur as result of rising temperatures other than eutrophication (Yabe et al. 2009). Also, considering the high summer temperatures experienced in Japan during the summer, and that heat treatment is among the most widespread on-board treatment methods to prevent biological contamination, the present study undertook a species-specific survey of the upper thermal tolerance of their photosynthetic responses. We designed short (3 and 5 h) incubations in different thermal environments (27 to 36 °C) under two conditions: a) in darkness (mimicking light environment within ballast waters), since *U. ohnoi* and *U. pertusa* are known to survive in this introduction vector (Flagella et al. 2010), and b) during exposure to light, mirroring the likely event of their transport attached to the hull of ships (or as an epibiont). Additionally, we explored the upper thermal physiological tolerance of *U. ohnoi* in extended incubations (14 days) to survey its adaptation capacity to increased temperature.

Experiments were designed to investigate the implication of thermal plasticity in the first steps of the invasive process, dispersal and settlement (Sakai at al. 2001), as well as the seasonal dominance of both species in Japan. The following working hypotheses were proposed: 1) The two *Ulva* species respond differently to thermal stress in the short-term, and responses relate to the dispersal risk to near-by regions and may further explain their seasonal replacement; 2) due to its wide distribution and year-round presence, *U. ohnoi* has a broad thermal tolerance which can facilitate its settlement in new areas. Although the capacity for dispersal and settlement is the result of many biological responses to biotic and abiotic factors, and their complex interactions, temperature is considered a major variable controlling photosynthesis and physiology in most algae, including *Ulva* spp. (Steffensen 1976; Breeman 1988; Lüning et al. 1990).

Material and Methods

Algal material

Unialgal culture strains of *Ulva ohnoi* and *Ulva pertusa* (=*U. australis*) housed in the Kobe University Macroalgal Culture Collection (KU-MACC) were used. Prior to experiments, the culture strains were grown at 20 °C (TG-100 AD Nippon Medical and Chemical Instruments, Osaka, Japan) under 120 µmol photons m^{-2} s^{-1} (LT-N10S-L LED Light, OHM Electronic Inc., Saitama, Japan), 12: 12, L: D in 1 L flasks for two months to obtain enough biomass. Light was measured placing the sensor (LICOR, LI-189, Lincoln, Nebraska, USA) at the same height as the flask. Seawater was filtered, autoclaved and enriched using Provasoli-enriched seawater (PES) (Provasoli 1968). Media were changed weekly. Continuous aeration was supplied to cultures using an air pump.

Experimental design

The photosynthetic response to short-term incubations of *U. ohnoi* and *U. pertusa* was studied by exposing two independent sets of samples for 3 h (average travel time of a short-distance ship along Japanese shores) and 5 h (average travel time of a mid-distance ship along Japanese shores) under different experimental temperatures (27, 29, 31, 34, 36 °C) in a temperature gradient chamber (NK System Nippon Medical and Chemical Instruments, Co. LTD). Both species are commonly present in the green blooms formed in Japanese winters (Kawai et al. 2007), being able to proliferate even during ice cover, which indicates a remarkable capacity to survive at low temperatures and variable salinity. The temperatures were chosen to both encompass, and further expand, the known upper thermal threshold of *U. pertusa*.

Secondly, the thermal physiological plasticity of *U. ohnoi* was assessed in a two-week experiment, maintaining the same temperatures as during the short-term exposure.

Short-term exposure experiment

Algal disks of 7 mm diameter from *U. ohnoi* and *U. pertusa* were cut (n = 50) and placed for 24 h in a culture chamber at 20 °C under 120 µmol photons $m^{-2}s^{-1}$, 12:12 L:D in Petri dishes containing culture media to allow for wound healing. Subsequently, 10 replicates were transferred into the temperature gradient chamber where the experimental temperatures were set. For each temperature treatment, half of the replicates were placed in darkness, mimicking light environment in ballast water conditions (Sutherland et al. 2001), the remaining were incubated at the same irradiance as mentioned above. Light was measured placing the sensor at the same height as the Petri dishes.

All Petri dishes were removed from the culture chamber after 3 h, and several biological variables measured (see below). A separate batch of algal disks was cut and incubated for 5 h, and treated in the same way as the first set.

Long-term exposure experiment

Additional *U. ohnoi* disks of 7 mm were cut (n = 35) fresh weights taken and allowed for wound healing in the same conditions described above. Seven replicates were placed into experimental temperatures starting at 27ºC and increasing step-wise every 2 h by 2 degrees up to 36ºC to avoid excessive thermal shock (as already assessed in the short-term experiment) and allow some acclimation to occur. Discs were cultured for two weeks under 120 µmol photons $m^{-2}s^{-1}$, 12:12 (L:D) in plastic Petri dishes (8.5 cm diameter) containing 10 mL of PES culture media. Temperature-adjusted culture media were added once a week to avoid nutrient depletion, and thus imposing the effect of temperature alone. Disposition of the Petri dishes was randomly changed every two days within the culture chamber to assure all samples were exposed to the same light environment over the duration of the experiment.

Biological variables and parameters

Photosynthetic performance of the samples was analysed through *in vivo* chlorophyll fluorescence with a portable pulse-amplitude modulated fluorometer, Diving PAM (Walz, Effeltrich, Germany). We measured the optimal quantum yield for photosystem II (F_v/F_m) using samples kept in darkness for 30 min to ensure that all photosynthetic reaction centres were open (F_o, all QA molecules are oxidized, Schreiber et al. 1986). Dark incubations were performed at the respective experimental temperatures. When a short (800 ms) pulse of white light is applied to dark-adapted samples a quick reduction of the electron transport chain between PSII and PSI occurs. As a result, all reaction centres become closed (QA molecules are reduced) resulting in maximal fluorescence (F_m). The parameter F_v/F_m reflects the physiological fitness in photosynthetic organisms (Maxwell and Johnson 2000), with low values indicating a degree of stress (Chaloub et al. 2010, da Oliveira et al. 2013).

Additionally, Rapid Light Curves (RLC; 17, 24, 37, 53, 87, 135, 213, 373 µmol photons m^{-2} s^{-1}; 10 s each irradiance, measured using LICOR, LI-185B, Lincoln, Nebraska, USA) were measured to quantify the Relative Electron Transport Rates (rETR, calculated by the formula rETR = YIELD x PAR x 0.5; Schreiber et al. 1986), which allowed the estimation of the following photosynthetic parameters (as described in Tait et al. 2017): α

(photosynthetic efficiency at low light), $rETR_{max}$ (relative maximal electron transport rate) and I_k (irradiance of saturation of photosynthesis). Photosynthetic efficiency was calculated using the initial four points of the curves and adjusting it to a slope of the linear model (Zanolla et al. 2015). $rETR_{max}$ was calculated by fitting the rETR-Irradiance curves to the Edwards and Walker equation (1983) using Kaleidagraph (Synergy Software, Version 4.0). The light saturation parameter (I_k) was obtained from the ratio $rETR_{max}/\alpha$ (Henley 1993). We considered higher tolerance to thermal stress when these parameters remained constant (i.e. no statistical differences) across experimental temperatures (Zanolla et al. 2015).

After each light increment, a saturating pulse was given to determine the effective PSII quantum efficiency (ΔF/F'm), qP (Photochemical Quenching of Variable Chlorophyll-*a* Fluorescence) and NPQ (Non-photochemical Fluorescence Quenching). Through non-photochemical quenching the level of excitation energy in the PSII antenna can be regulated and thus prevent over-reduction of the electron transfer chain. Therefore, increasing values in this parameter imply a higher protection capacity against photo-damage (Niyogi 1999, Fracheboud and Leipner 2003).

For a quantitative NPQ as a function of E_{PAR}, NPQ versus E_{PAR} curves were fitted after Serôdio and Lavaud (2011) as all requirements were met:

$$NPQ_{fit} = NPQ_{max} * \frac{E^n}{E_{50}^n + E^n}$$

where, NPQ_{fit} is the corrected NPQ value, NPQ_{max} is the maximum NPQ value of the NPQ versus E_{PAR} curve, E_{50} is the irradiance at which NPQ attains 50 % of NPQ_{max} and *n* represents the Hill coefficient characterising the sigmoidicity of the curve. Computed data from this model allow the direct comparison of parameters from P/E and NPQ versus E_{PAR} curves (Serôdio and Lavaud 2011; Nitschke et al. 2012). NPQ_{max} presented in this study was calculated by using the former equation. The light–response curve models were fitted using a procedure based on Microsoft Excel Solver. Likewise, qP values were fitted to negative exponential curves ($qP_{fit} = a * Exp^{(-b * irradiance)}$). The slope of the curves (b) was used to compare qP curves between treatments. All model parameters were estimated iteratively by minimizing a least-squares function.

Measurements of all replicates were done at the end (3 h or 5 h) of the short-term experiments; and in the long-term experiment, once they were placed at each temperature, at the beginning (*t* = 0), after one week (*t* = 7) and at the end of the experiment (*t* = 14) at the same time of day and in the same order. For the long-term experiment, we additionally estimated the relative growth rate (RGR) using the formula: $RGR\ (\%\ d^{-1}) = (\ln (Fw_f) - \ln (Fw_i))/t) \times 100$, where *t* is time (14 d), Fw_f is the final fresh weight and Fw_i the initial fresh weight. Finally, samples at the end of the long-term experiment were stored for pigment analyses at -20 °C. Chlorophyll *a* and *b*, and total carotenoids were extracted in DMF (N, N-dimethylformamide) at 4ºC in darkness overnight and concentrations were estimated spectrophotometrically (DU730 Spectrophotometer, Beckman Coulter, Fullerton, CA, USA) according to the equations of Wellburn (1994).

Statistical data analysis

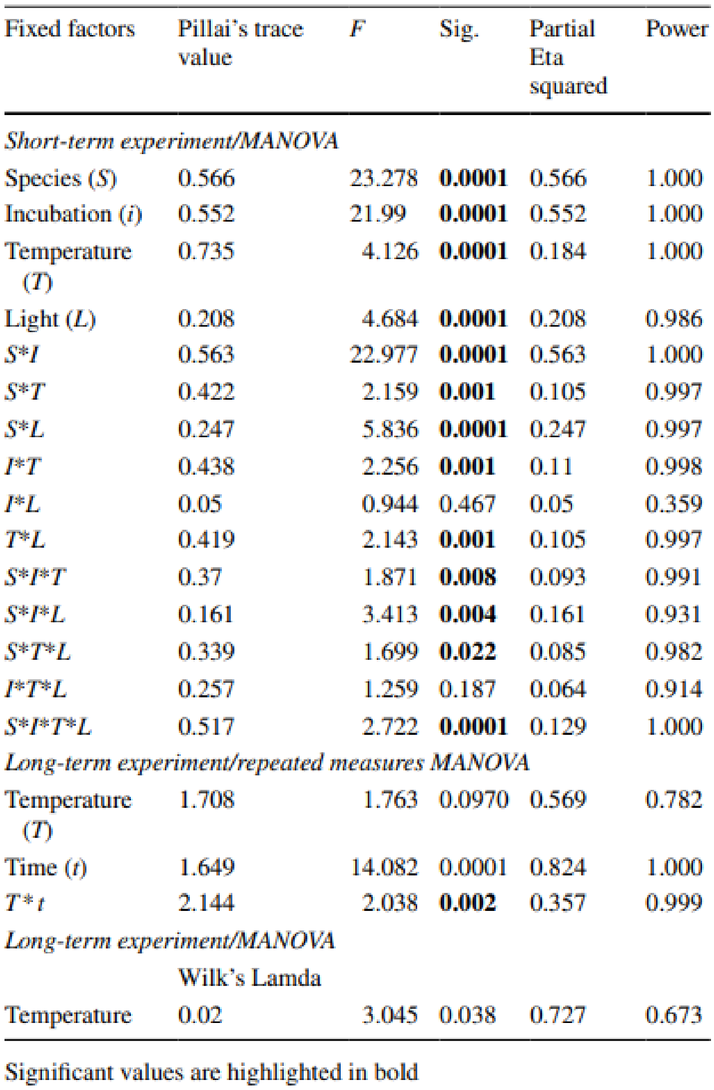
The variation between photosynthetic parameters (ETR_{max}, α and I_k), F_v/F_m and dissipative quotients (NPQ_{max}, qP_{fit}) after incubations of 3 h and 5 h to experimental temperatures (27-36 °C) and light conditions (0 and 120 μmol photons m^{-2} $s^{-}1$) for both species were tested by a multifactorial MANOVA. The requirements for MANOVA were assessed: linear regressions among variables and fixed factors, independency of observations using the Durbin Watson residual's test, multivariate homogeneity using Box M. Normality was assessed for each dependent variable as in the univariate ANOVA using Mahalanobis' distance. Multicollinearity was tested using Tolerance/VIF values. All statistical analyses were computed in SSPS version 25.0.0 (IBM SPSS statistics, IBM Corporation, Armonk, NY). For *post-hoc* comparisons between experimental temperatures we used Holm-Sidak procedure with an overall significance level of $\alpha = 0.05$.

F_v/F_m, NPQ_{max}, and qP_{fit} were measured in both species prior to short-term exposure to measure the stress caused by the temperature shock, and also to establish the time (3 h or 5 h) required for the activation of any protective response. Due to absence of normality and homogeneity of variances of the data from the short-term experiment the more conservative, Pillai's Trace multivariate test, was used. Equal cell sizes and high number of observations (n = 152) allowed the maximum power of the Pillai's test (Table 1). Following multivariate results, we examined univariate main effects with a significance of $\alpha = 0.0083$. This value was calculated because the experiment-wise α protection provided by the overall *F* test ($\alpha = 0.05$) does not extend to univariate tests. Thus, confidence levels were divided by the number of dependant variables used. Significant interactions were then identified based on this significance, the power of the test and the Partial Est Data, i.e. the amount of variance explained by that variable.

F_v/F_m, photosynthetic parameters and dissipative quotients of *U. ohnoi* from the long-term experiment (measured at t = 0, t = 7 and t = 14) were analysed using a two-way repeated measures MANOVA to detect significant differences in photosynthetic parameters over time and among temperatures. Sphericity was assessed using Mauchly's Test. Pigments and RGR of this species were analysed using a one-way MANOVA with temperature as fixed factor. In this case, all requirements were met, which permitted the application of Wilk's Lamda F test.

Differences among significant interactions were analysed using Holm-Sidak *post-hoc* with a significance of α = 0.0083 (as explained above).

Table 1 Details of the diferent statistical multivariate analysis applied to the data



Fixed factors	Pillai's trace value	*F*	Sig.	Partial Eta squared	Power
Short-term experiment/MANOVA					
Species (*S*)	0.566	23.278	**0.0001**	0.566	1.000
Incubation (*i*)	0.552	21.99	**0.0001**	0.552	1.000
Temperature (*T*)	0.735	4.126	**0.0001**	0.184	1.000
Light (*L*)	0.208	4.684	**0.0001**	0.208	0.986
*S*I*	0.563	22.977	**0.0001**	0.563	1.000
*S*T*	0.422	2.159	**0.001**	0.105	0.997
*S*L*	0.247	5.836	**0.0001**	0.247	0.997
*I*T*	0.438	2.256	**0.001**	0.11	0.998
*I*L*	0.05	0.944	0.467	0.05	0.359
*T*L*	0.419	2.143	**0.001**	0.105	0.997
*S*I*T*	0.37	1.871	**0.008**	0.093	0.991
*S*I*L*	0.161	3.413	**0.004**	0.161	0.931
*S*T*L*	0.339	1.699	**0.022**	0.085	0.982
*I*T*L*	0.257	1.259	0.187	0.064	0.914
*S*I*T*L*	0.517	2.722	**0.0001**	0.129	1.000
Long-term experiment/repeated measures MANOVA					
Temperature (*T*)	1.708	1.763	0.0970	0.569	0.782
Time (*t*)	1.649	14.082	0.0001	0.824	1.000
T t*	2.144	2.038	**0.002**	0.357	0.999
Long-term experiment/MANOVA					
	Wilk's Lamda				
Temperature	0.02	3.045	0.038	0.727	0.673

Significant values are highlighted in bold

Results

Short-term exposure

All data obtained from the short-term experiment were analysed using MANOVA, which evaluated effects of individual factors as well as their interactions (Table 1). Interaction among factors indicates that responses to experimental conditions were species-specific. Given the significance of the applied MANOVA the univariate main effects were examined subsequently. *Ulva ohnoi* presented higher values of F_v/F_m, photosynthetic efficiency (α) and $\mathrm{NPQ}_{\mathrm{max}}$ than *U. pertusa* (Supp. Table 1a).

Optimal quantum yield

F_v/F_m of *Ulva ohnoi* was similar when exposed to 27-34 °C for 3 h or 5 h, but significantly lower values were recorded at 36 °C (Fig 1a, b; Supp. Table 1a). After 5 h (Fig. 1b), F_v/F_m of samples kept at 36 °C were 10 % lower than at 27 or 34 °C (Fig 1b). A reduction in F_v/F_m at 36 °C was also observed for *U. pertusa*. These values were 10 % lower than those recorded at 29 °C after 3 h, and 15 % than those at 34 °C after 5 h (Fig. 1c, Supp. Table 1a). *U. ohnoi* samples kept in dark presented higher values than those exposed to 120 µmol photons m^{-2} s^{-1} (Supp. Table 1a) while no such differences were recorded for *U. pertusa* (Fig 1c, d). The most important variables influencing F_v/F_m were the species (37 %), the experimental temperatures (15 %) and the interaction between incubation time and temperature (13 %, Supp. Table 1a).

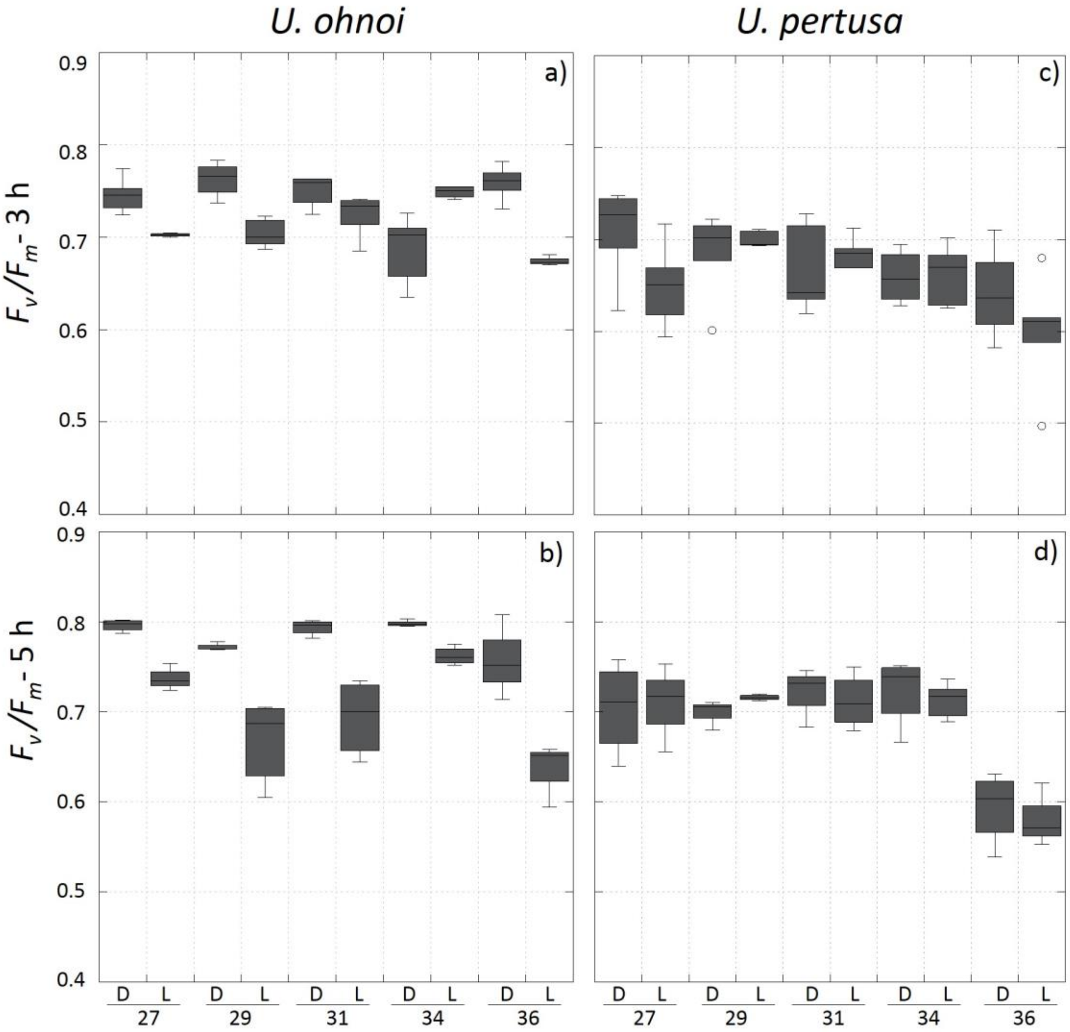


Fig. 1 Optimal quantum yield (Fv/Fm) variation in *Ulva ohnoi* (a, b) and *Ulva pertusa* (c, d) exposed to 3 h and 5 h of thermal and light treatment (D: dark; L: light). Data are presented as boxplots (n=5)

Photosynthetic parameters

Regarding $rETR_{max,}$ both species responded differently to the incubation time and temperatures, whereas light exposure was not a significant factor in shaping this parameter (Supp. Table 1a). Overall values were similar for both species (Fig 2a, b, Supp. Table 1a). Higher values of 15.11 ± 2.42 μmol $e^{-}m^{-2}$ s^{-1} (Fig 2a) were recorded at both incubation times for *U. ohnoi* at 29 °C, but after 5 h a general decay of 25 % was recorded (Fig 2a). *U. pertusa*, on the other hand, maintained similar $rETR_{max}$ over time (average values of 12.15 ± 5.81 μmol $e^{-}m^{-2}$ s^{-1}, Fig 2d); significant higher values were recorded at 29 and 31 °C (Fig 2b, Supp. Table 1a) compared to 36 °C.

Photosynthetic efficiency (α) was affected by the interaction of all fixed factors considered, but interactions between "species" and "incubation" were the most significant ones, explaining almost half of the variability presented by this parameter (Supp. Table 1a). The effect of temperature was also significant, explaining 38 % of the variation (Supp. Table 1a). The photosynthetic efficiency (α) showed a 42 % decrease at all temperatures after 5 h of incubation in *U. ohnoi*. Values calculated at 27, 29 and 31 °C were 27 % higher than those at 34 and 36 °C (Fig 2c). However, after 5 h, an average of 0.0989 ± 0.02 µmol $m^{-2} s^{-1}$ / µmol photons was maintained at all temperatures, without significant variation between individual temperatures at this time (Fig 2c, Supp. Table 1a).

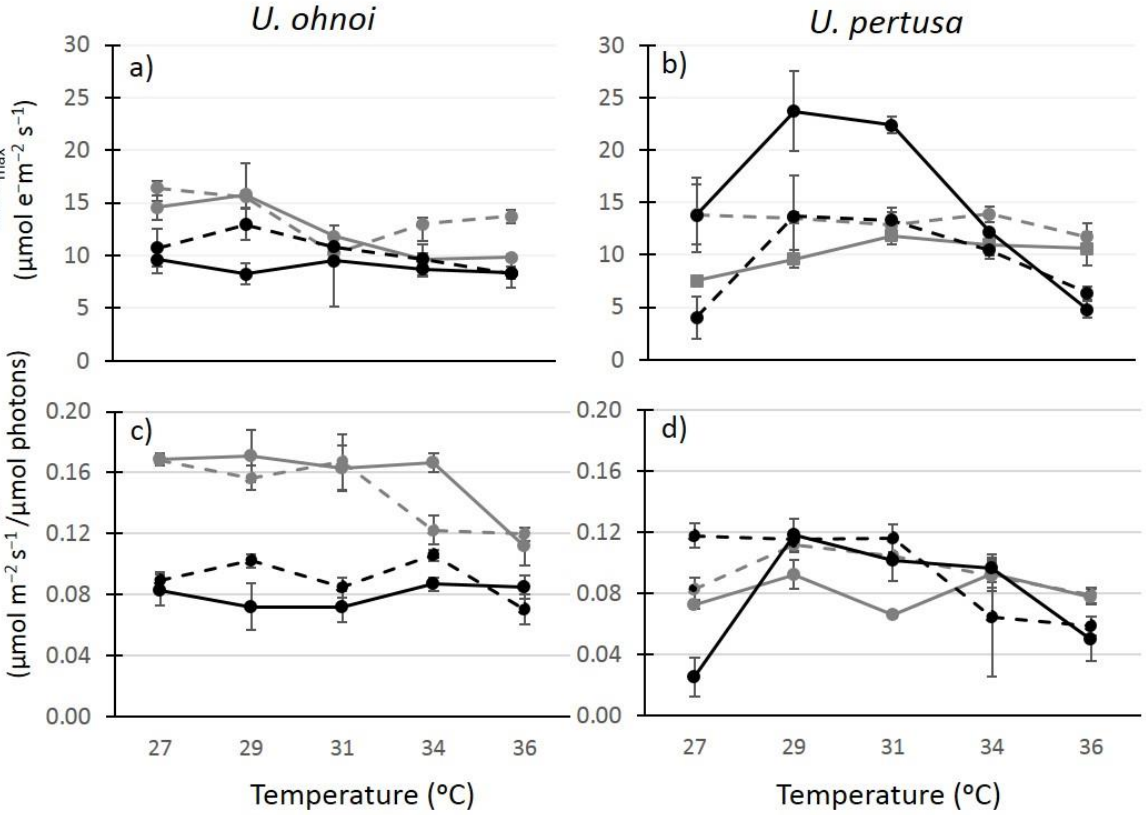


Fig 2 Photosynthetic parameters calculated for *U. ohnoi* (a, c) and *U. pertusa* (b, d) after 3 h (grey) and 5 h (black) of thermal and light (discontinuous line) /dark treatment (continuous line). Data are presented as means ± SD (n = 5).

α of *U pertusa*, on the other hand, was insensitive to temperature, incubation time and light regime (Fig 2d), yet presented lower overall values than α of *U. ohnoi* (0.115 ± 0.039 *vs* 0.100 ± 0.034 µmol $m^{-2} s^{-1}$ / µmol photons for *U. ohnoi* and *U. pertusa*, respectively)

I_k (light saturation of photosynthesis) remained similar across species, incubation times, temperatures and light regimes (Supp Fig 1, Supp. Table 1a).

Dissipative quotients

The effect of thermal treatment and/or light regime applied to the algae did not translate into changes of NPQ_{max}, as no significant differences were observed between treatments (Supp. Table 1a and 2a, b) at any incubation time. However, *U. ohnoi* presented higher values of NPQ_{max} than *U. pertusa* (0.658 ± 0.193 *vs* 0.577 ± 0.192, respectively, Supp. Table 1a, b). Compared to initial values (0.45 ± 0.06) NPQ_{max} of *U. ohnoi* was higher even after 3 h of thermal treatment and remained high after 5 h. By contrast, *U. pertusa* was able to active this mechanism only after 5 h (Supp. Table 1a, 2a, b).

Light intensity and incubation time affected qP_{fit} values, and this influence depended on the species considered (Supp. Table 1a, 2c, d). Interestingly, this effect was independent of temperature. *Ulva ohnoi* presented significantly higher values of qP_{fit} after 5 h compared to 3 h (Supp. Table 1a, 2c, d) and initial values (0.007 ± 0.001). Samples kept in darkness scored higher values at all incubation times and temperatures. *U. pertusa* remained invariable to changes in light intensities and incubation times. qP_{fit} of *U. pertusa* exposed to experimental conditions did not differ significantly from initial values.

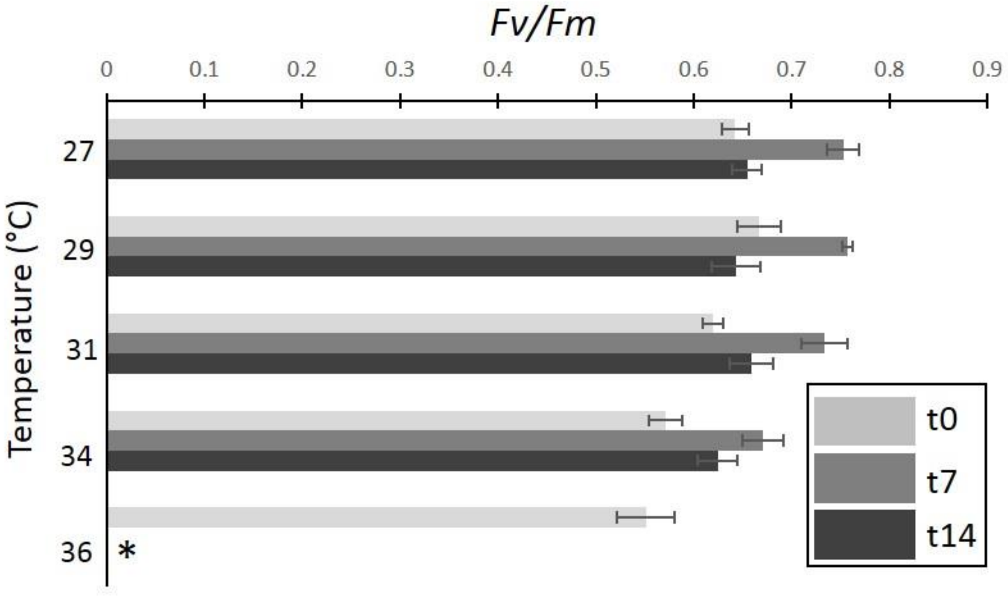


Fig 3. Optimal quantum yield (F_v/F_m) of *Ulva ohnoi* samples exposed to varying temperatures at the beginning (t = 0, light grey), after one week (t = 7, medium grey) and at the end (t = 14, dark grey) of the long-term (14 d) experiment. Data are presented as means ± SD (n = 7). * indicates no data available.

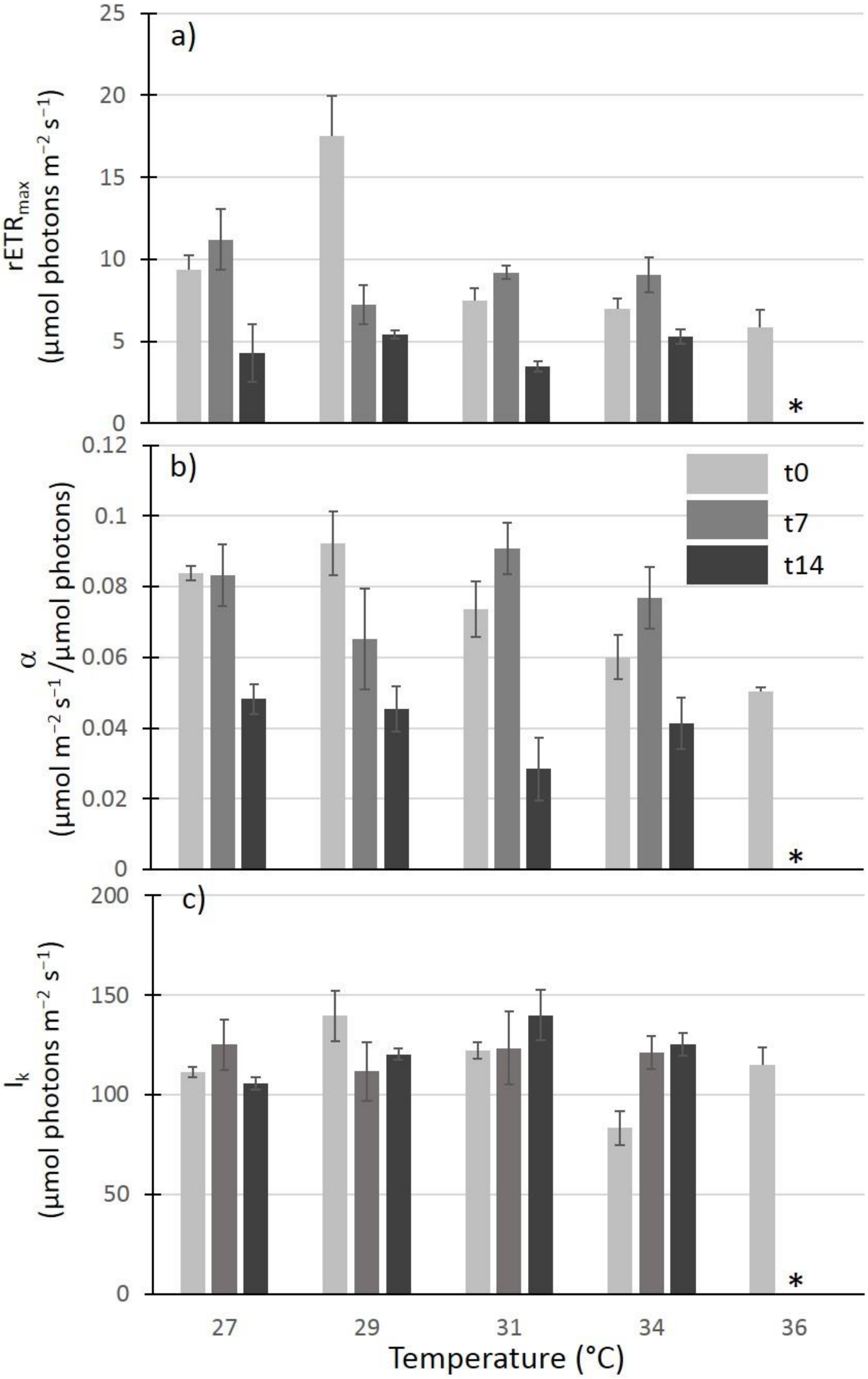


Fig 4 Photosynthetic parameters from the rapid light curves (RLC) of *Ulva ohnoi* at different temperatures at the beginning (t = 0, light grey), after one week (t = 7, medium grey) and at the end (t = 14, dark grey) of the long-term (14 d) exposure experiment. Data are presented as means ± SD (n = 7). * indicates no data available.

Long-term exposure of *Ulva ohnoi*

Samples of *U. ohnoi* kept at 36 °C had decoloured completely and died by day 4 of the experiment. No sign of sporulation or recovery was detected. Thus, results presented in Figs. 3, 4 and 5 only refer to isolates held at 27, 29, 31 and 34 °C. Two-way repeated measures MANOVA depicted interactions among time and temperature (Supp. Table 1b). Given these significances, the univariate main effects were examined.

Optimal quantum yield

Both time and temperature impacted on F_v/F_m independently, but their interaction was insignificant (Supp. Table 1b). F_v/F_m values of all algal samples exhibited a similar trend, with an initial increase after the first week of the experiment, followed by a decrease to initial values by the end of the experimental period (Fig. 3, Supp. Table 1b). Values of samples kept at 34°C were significantly lower than all other treatments (Fig 3).

Photosynthetic parameters

The pattern of rapid light curves (Supp. Fig 1) was similar to that of F_v/F_m values: rETRs at most irradiances were higher at day 7 than during initial measurements, and then decreased by day 14. This trend was even maintained at 34°C, but here values of rETR were generally lower than at other temperatures. Saturating irradiances were achieved between 100 and 140 µmol photons m^{-2} s^{-1} (except at 29 °C at t = 7), above which rETR declined at all temperatures.

Maximum electronic transport rates (ETR_{max}) displayed different trends depending on temperature (Fig 4a). Values measured at t = 0 were highest at 29 °C, but similar at the other temperatures (Supp. Table 1b). At 27 and 31 °C, a significant increase by t = 7 was followed by a decrease (to values lower than initials) at t = 14 Supp. Table 1b). At 29 °C, ETR_{max} decreased steadily from t = 0. At 34 °C, final values recovered to initial values, going through a temporal, but significant, increase.

Photosynthetic efficiency (α) at 27, 29 and 31 °C declined with time (Fig 4b) but at 34 °C, a slight increase was observed after 7 days, followed by a decrease by day 14. At the beginning of the experiment, α was highest in samples cultured at 27 and 29 °C, followed by samples at 31 and 34 °C. However, after one week, α measured in 27 and 29 °C-grown samples was significantly lower than those kept at 31 or 34 °C. This trend was maintained until the end of the experiment.

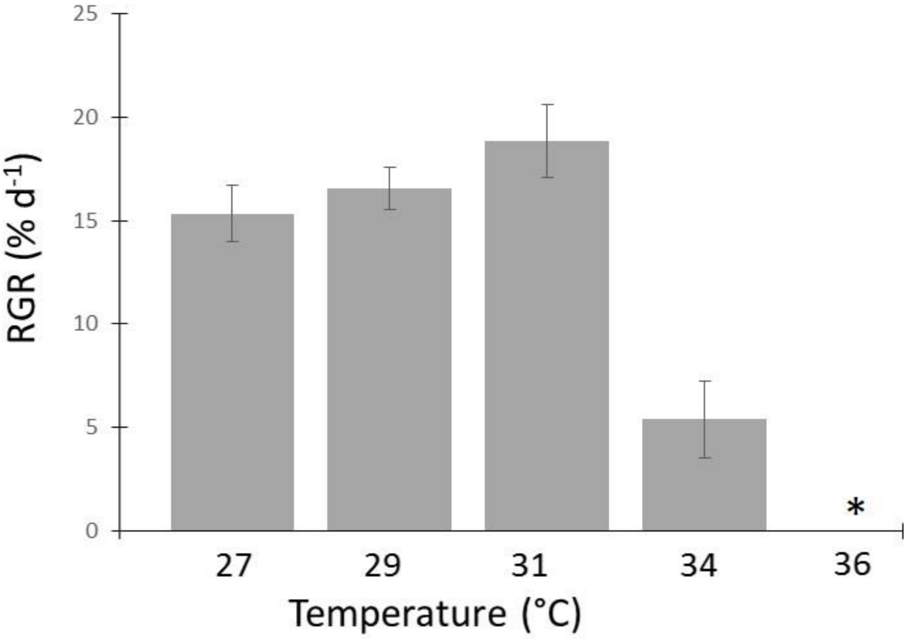


Fig 5 Relative growth rate (% d^{-1}) of *Ulva ohnoi* at experimental temperatures. Data are presented as means ± SD (n = 7). * indicates no data available.

The effect of temperature on the light saturation of photosynthesis (I_k) changed over time at all temperatures but not among temperatures (Fig 4c). Trends were temperature-specific; for example, at 29 °C I_k decreased with time, whereas at 31 and 34 °C, values significantly increased (Fig 4c; Supp. Table 1b).

Dissipative quotients

NPQ_{max} remained constant across all temperatures but values obtained at the beginning of the experiment were higher than those recorded after 14 days of treatment (Supp. Table 1b, 2a, b). Values of qP_{fit} were similar among temperatures, but this parameter decreased after 7 days and remained constant until the end of the experiment (Supp. Table 1b, 2a, b).

Growth and pigment content

One-way MANOVA provided significant results to the questioned fixed factor (Supp. Table 1c). A maximum growth rate of 18.8 ± 1.8 % d^{-1} was observed at 31 °C; although this was18 % higher than at 27 and 29 °C, it was not statistically different. Significantly lower rates were recorded at 34 °C (Fig 5, Supp. Table 1c), and no samples maintained at 36 °C survived more than 4 days.

After 14 d of culture, samples grown at 34 °C contained less chlorophyll *a* (43 % less) and chlorophyll *b* (36 % less) than those at 27 and 29 °C (Fig 6, Supp. Table 1c). Carotenoid concentrations remained similar (average

0.54 ± 0.19 mg/g FW, Supp. Table 1c) across experimental temperatures (27-34 °C), as did total pigment content (2.61 ± 1.19 mg/g FW). Despite the considerable changes in chlorophyll contents, effects were not found to be significant (Supp. Table 1c).

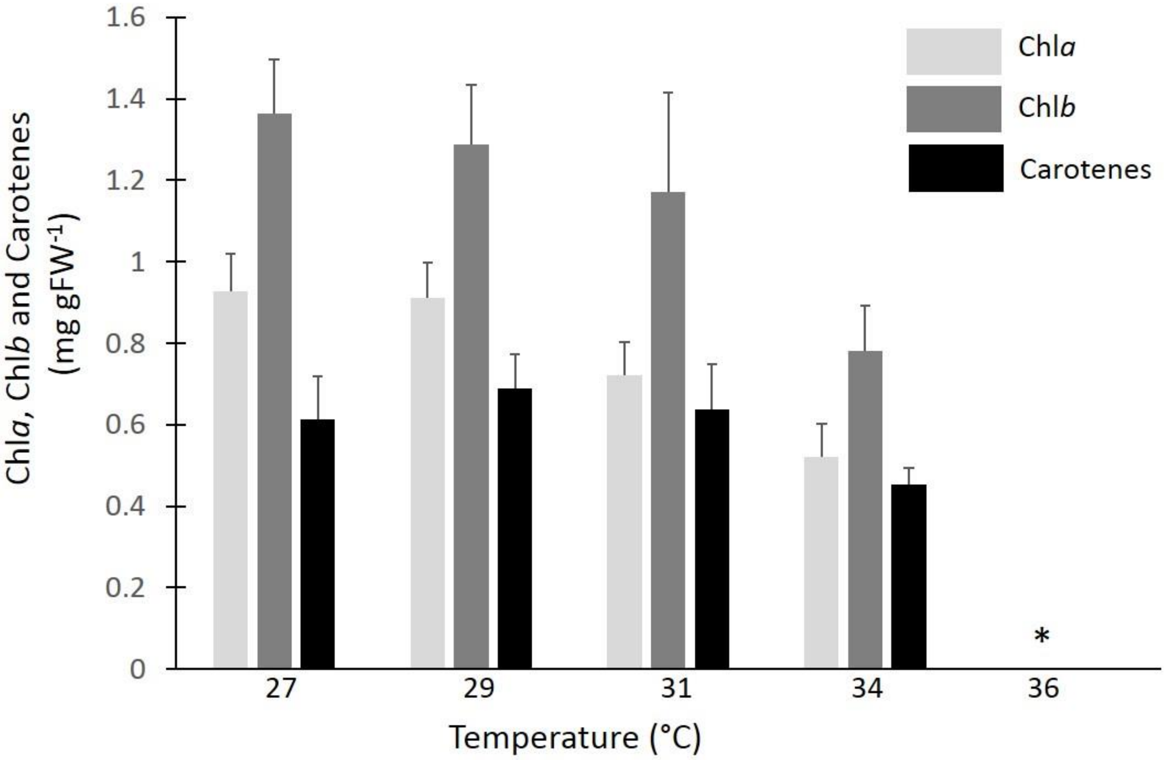


Fig 6 Pigment content (chl*a*, chl*b* and carotenes) of *U. ohnoi* samples exposed to experimental temperatures at the end of the long-term (14 d) experiment. Data are presented as means ±SD (n = 7). *Pigments of samples kept at 36 °C were not measured due to inability to survive at this temperature

Discussion

We studied the importance of vegetative traits, such as photosynthetic plasticity related to successful dispersal and settlement of *Ulva pertusa* and *U. ohnoi* using controlled exposure to different light and temperature treatments. Both species are invasive and are noticeable Green Tide formers. The laboratory experiments revealed a remarkable tolerance and adaptive capacity to high temperature exposure of *U. ohnoi*. Additionally, the significant interactions between experimental conditions and species, identified by MANOVA tests, indicate that both species differ in their response to increasing temperature, confirming the initial hypothesis of differential tolerance to thermal stress. The implications of these findings on dispersal risk are further addressed below. Long-term exposure highlighted a reduced fitness *U. ohnoi* at 34 °C, but the consistency of the values estimated for most parameters at 27- 31 °C indicates that this species has an exceptional resistance to temperature, which supports our second hypothesis that *U. ohnoi* may pose high risk of dispersal and settlement.

In general, at first, both species were negatively affected by the thermal exposure as indicated by the general decay in F_v/F_m, which was statistically lower, but remained within c. 27 % of the initial values (0.78 ± 0.008). The lower values of $\mathrm{F_v/F_m}$ displayed by *U. pertusa* than by *U. ohnoi* at all temperatures, independently of time exposure, indicate a higher sensitivity to temperature changes of the former species. Likewise, $\mathrm{rETR_{max}}$ and α measured after 3 h were higher in *U. ohnoi* than in *U. pertusa* at all temperatures, but there was a similar reduction after 5 h of treatment in both species (though *U. pertusa* presented higher values of $\mathrm{rETR_{max}}$ after 5 h at 27, 29 and 31 °C). Maximum thermal survival in *U. pertusa* was reported to be 7 days at 33 °C, after which the cuticle layer breaks down and marginal cells from the periphery are released (Kakinuma et al. 2006). Therefore, better performance in short- and long-term incubations thus indicates that *U. ohnoi* copes better with rising temperatures.

Despite *U. ohnoi* being a relatively newly-described species (Hiraoka et al. 2004), its presence has been reported from most continents (see algaebase.org for current distribution) and it is considered highly invasive (Zenetos et al. 2005, Kawai et al. 2007). However, the thermal limits of survival and growth for this species have not previously been addressed. The results provided in this study thus constitute valuable baseline data of this noticeable Green Tide former. Overall analysis of all studied parameters in short- and long-term incubations provide an insight of how metabolism changes during acclimating to stress Of particular interest in this context is the variability of the photosynthetic efficiency and $\mathrm{rETR_{max}}$ of *U. ohnoi* observed here. While α was higher at the beginning of the experiment at 27 and 29 °C - which is in line with an inversely proportional relation with temperature (Davison 1991) - an unexpected increase of this parameter was recorded after 7 d at both 31 and 34

°C. Similarly, $rETR_{max}$ was highest at 29 °C throughout of the experiment. Such an uncoupling of the maximum photosynthetic rate (at 29 °C) and growth optima (at 27- 31 °C) has been extensively reported, as growth integrates the effect of temperature on the total metabolism (Davison 1991; Eggert 2012). The higher tolerance at increasing temperatures exhibited by *U. ohnoi* could be due to a faster activation of heat dissipation mechanisms, which would prevent photosynthetic/tissue damage from occurring. In fact, NPQ_{max} and qP_{fit} were higher in samples exposed to the treatments after only 3 h (compared to initial values), and remained high after 5 h; by contrast, NPQ_{max} in *U. pertusa* increased after 5 h of treatment, and qP_{fit} remained invariable. Regarding I_k, changes in this parameter in response to temperature might be masked by using chlorophyll fluorescence methods, as only P-E curves measured by gas exchange measurements can undoubtedly reveal light saturation of photosynthesis.

The differences observed between short- and long-term performances can be attributed to a high plasticity in response to thermal variation (Zanolla et al. 2015) and ultimately, a high acclimation potential for *U. ohnoi*. The long-term experiment revealed an optimum for growth at 27-31 °C, and a remarkable survival temperature of 34 °C. Interestingly, *U. ohnoi* used for this study exhibited a higher upper thermal limit for growth than specimens studied by Mata et al. (2016); these authors recorded fast growth up to 28 °C which then declined at higher temperatures. Optimum growth occurred across a broader range of temperatures in our experiment, but maximum temperature for survival was similar in both studies (34-34.5 °C). It is thus possible that the Japanese isolate used in this study and the Australian isolate investigated by Mata et al. (2016) could, in fact, represent different taxa, or at least strains. Such adaptations of local populations of the same species (ecotypes) with different temperature tolerances have previously been reported for *Asparagopsis taxiformis* lineage 2, across invasive and native populations (Zanolla et al. 2015). Considering the distribution of *U. ohnoi*, cryptic diversity can be suspected, though genetic analysis of the available sequences is needed to clarify this point. In several cases, widely distributed (and invasive) genera, such as *Asparagopsis* Montagne (Andreakis et al. 2007; Dijoux 2014), *Codium* Stackhouse (Provan 2005) and *Caulerpa* J. V. Lamouroux (Meusnier et al. 2004) have proven to be species complexes with different thermal photosynthetic features (Raniello et al. 2004; Zanolla et al. 2015), levels of polyploidy (Varela-Alvarez et al. 2012), morphologies (Trowbridge 1996; Zanolla et al. 2014) and invasive potentials (Zanolla et al. 2018). Another factor to be considered is that genetic (and, as a result, physiological plasticity) diversity was reduced in the present laboratory cultures used, compared to wild natural populations.

Implications for increased dispersal

Some traits that increase probabilities of successful transit, such as tolerance to harsh and variable environmental conditions, might also better equip invaders to thrive and reproduce in the place of arrival after their association with the vector (Crooks and Rilov 2009). The most significant pathways for the transport of invasive macroalgae are associated with shipping vectors (ballast water and fouling of hulls), aquaculture and the aquarium trade (Ruiz et al. 2000; Carlton 2001; Ribera Siguan 2003; Hewitt et al. 2004). While technologies applying varying treatment methods are being developed to manage ballast waters (e.g., Taylor et al. 2002; Werschkun et al. 2012), many of such treatments are time consuming, and costly, and therefore can only be implemented during long voyages, but not on short-distance routes. Heat treatment of ballast water over extended periods is not always practicable, particularly where many vessels operate on short coastal journeys with short times between ballasting and deballasting (Macdonald and Davidson 1997; McCollin et al. 1999). There are concerns over the physical impacts of dealing with large volumes of heated water, the energy costs of heating the water and the possible environmental impacts of hot water discharge. Thus, the risk of biological contamination in adjacent areas increases.

Our results indicate that the physiological plasticity of *U. ohnoi* and *U. pertusa* enables them to bear the transport conditions in Japan, both in ballast waters, attached to the hull of vessels as epibionts and also in a wide thermal range. A similar photosynthetic plasticity to varying temperatures was observed for other invasive seaweeds, including the tetrasporophytes of *Asparagopsis taxiformis* Trevisian de Saint Leon (Zanolla et al. 2015). Even considering the constraints imposed, the short-term experiments represent a useful tool to measure the stress tolerance of a species, especially since experiments aimed to assess how these species deal with drastic changes that likely occur during transport.

In spite of being present in several locations around the Japanese archipelago, studying the short dispersal capacity of these species remains important; not only because of the expansion of the species *per se*, but propagule pressure also refers to the incorporation to the population of new genetic variants that might be more virulent than others (Colautti et al. 2006) which could disrupt the frequency and intensity of the Green Tides.

Differences between samples kept in the dark and light during the short-term experiment should be interpreted cautiously, given that the methodology employed to measure the photosynthetic parameters (PAM fluorometry) is highly dependent on light conditions experienced immediately before measurement. On the other hand, the clear trends in initial stimulation, followed by reduction in parameters such as F_v/F_m and rETR suggest that the chosen parameters were indicative of physiological adjustments to different temperatures, in particular in the long-term experiment.

Implications for settlement- Ulva ohnoi

Photosynthetic performance of *Ulva ohnoi* during short-term exposure was higher than that of *U. pertusa* at high temperatures, and growth rates remained high when exposed over 2 weeks to 31 °C. Results therefore suggest that the seasonal pattern of the occurrence of *Ulva pertusa* and *U. ohnoi* described from Japan (*U. ohnoi* dominates during autumn to early winter, and *U. pertusa* during spring to early summer; Kawai et al. 2007) might thus be due to a range of factors. These include competition, grazer preferences, salinity, photoacclimation, nutrient requirements and responses to nutrient enrichment (Nelson et al. 2008)- rather than physiological performance alone- controlling changes in relative abundance of the two species at different times of the year.

Although the thermal tolerance is not the only factor influencing the settlement success of an invasive organism, it is considered among the most critical factors influencing the survival of seaweeds in new environments (Sakai et al. 2001). Thus, our data suggest that the broad photosynthetic plasticity during exposure to high temperatures evidenced by *U. ohnoi* can contribute to the high settlement risk for Japan and near-by regions even in summer, given the upper thermal for growth of 31 °C (summer maximum temperature in southern Japan, Japan Meteorological Agency) and the high thermal limit of survival for the species of 34 °C.

Conclusions

Our results indicate that Green Tides formed by *U. ohnoi* may increase in frequency and will be favoured by rising of global temperatures as recently suggested by Gao et al. (2017). Regarding frequency, the arrival of new genetic variants to an established population implies increased propagule pressure, which is a significant predictor of both invasiveness and invasibility (Colautti et al. 2006). Both species showed a remarkable tolerance to high temperatures (*U. ohnoi* was capable to survive at 36 °C for 4 days), which implies that high temperature alone would not constrain their dispersal or settlement. It is thus likely that their photosynthetic plasticity, conferring tolerance to harsh and variable environmental conditions, increases the probability of a successful transit and also allows them to survive and reproduce in the hosting ecosystem (Crooks and Rilov 2009). Additionally, in-depth studies of the physiological performance of targeted species are of pivotal importance for providing essential input data for species distribution models to underpin the forecasting of anthropogenic effects on patterns of biodiversity at different spatial scales (Guisan and Thuiller 2005).

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Figure and table captions

Fig 1 Optimal quantum yield (F_v/F_m) variation in *Ulva ohnoi* (a, b) and *Ulva pertusa* (c, d) exposed to 3 h and 5 h of thermal and light treatment (D: dark; L: light). Data are presented as boxplots (n = 5).

Fig 2 Photosynthetic parameters calculated for *U. ohnoi* (a, c) and *U. pertusa* (b, d) after 3 h (grey) and 5 h (black) of thermal and light (discontinuous line) /dark treatment (continuous line). Data are presented as means ± SD (n = 5).

Fig 3. Optimal quantum yield (F_v/F_m) of *Ulva ohnoi* samples exposed to varying temperatures at the beginning (t = 0, light grey), after one week (t = 7, medium grey) and at the end (t = 14, dark grey) of the long-term (14 d) experiment. Data are presented as means ± SD (n = 7). * indicates no data available.

Fig 4 Photosynthetic parameters from the rapid light curves (RLC) of *Ulva ohnoi* at different temperatures at the beginning (t = 0, light grey), after one week (t = 7, medium grey) and at the end (t = 14, dark grey) of the long-term (14 d) exposure experiment. Data are presented as means ± SD (n = 7). * indicates no data available.

Fig 5 Relative growth rate (% d^{-1}) of *Ulva ohnoi* at experimental temperatures. Data are presented as means ± SD (n = 7). * indicates no data available.

Fig 6 Pigment content (chl*a*, chl*b* and carotenes) of *U. ohnoi* samples exposed to experimental temperatures at the end of the long-term (14 d) experiment. Data are presented as means ±SD (n = 7). *Pigments of samples kept at 36 °C were not measured due to inability to survive at this temperature.

Supplementary material

Supp Fig 1 Relative electron transport rate (rETR)-irradiance curves in *U. ohnoi* at experimental temperatures at the beginning (t = 0), after one week (t = 7) and at the end (t = 14) of the long-term (14 d) exposure experiment. Data are presented as means ± SD (n = 7).

Supp Fig 2 Saturation parameter of photosynthesis (I_k) calculated for *U. ohnoi* and *U. pertusa* after 3 and 5 h of thermal and light/dark treatment. Data are presented as means ± SD (n = 5).

Supp Table 1 Univariate ANOVA tests derived from the different MANOVA tests shown in Table 1. Significant values ($p < 0.0083$) are highlighted in bold (see M & M).

Supp Table 2 Effect of temperature (27, 29, 31, 34 and 36ºC) on the maximum non-photochemical quenching NPQ_{max} (relative units) calculated from NPQ versus E_{PAR} curves from short-term (3h, 5h, light and dark conditions) and long-term (14 d) experiments for *Ulva ohnoi* (a) and *U. pertusa* (b). qP model fit data (relative units) derived from the model fit of qP versus E_{PAR} curves (see Material and Methods section) are displayed for all experiments conducted in *U. ohnoi* (c) and *U. pertusa* (d). Data are means (± SD) (n = 5-7).

Compliance with Ethical Standards

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Conflict of Interest: Author Marianela Zanolla declares that she has no conflict of interest. Author Raquel Carmona declares that she has no conflict of interest. Author Hiroshi Kawai declares that he has no conflict of interest. Author Dagmar Stengel declares that she has no conflict of interest. Author Maria Altamirano declares that she has no conflict of interest.

Ethical approval: This article does not contain any studies with animals performed by any of the authors.

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Fig 1

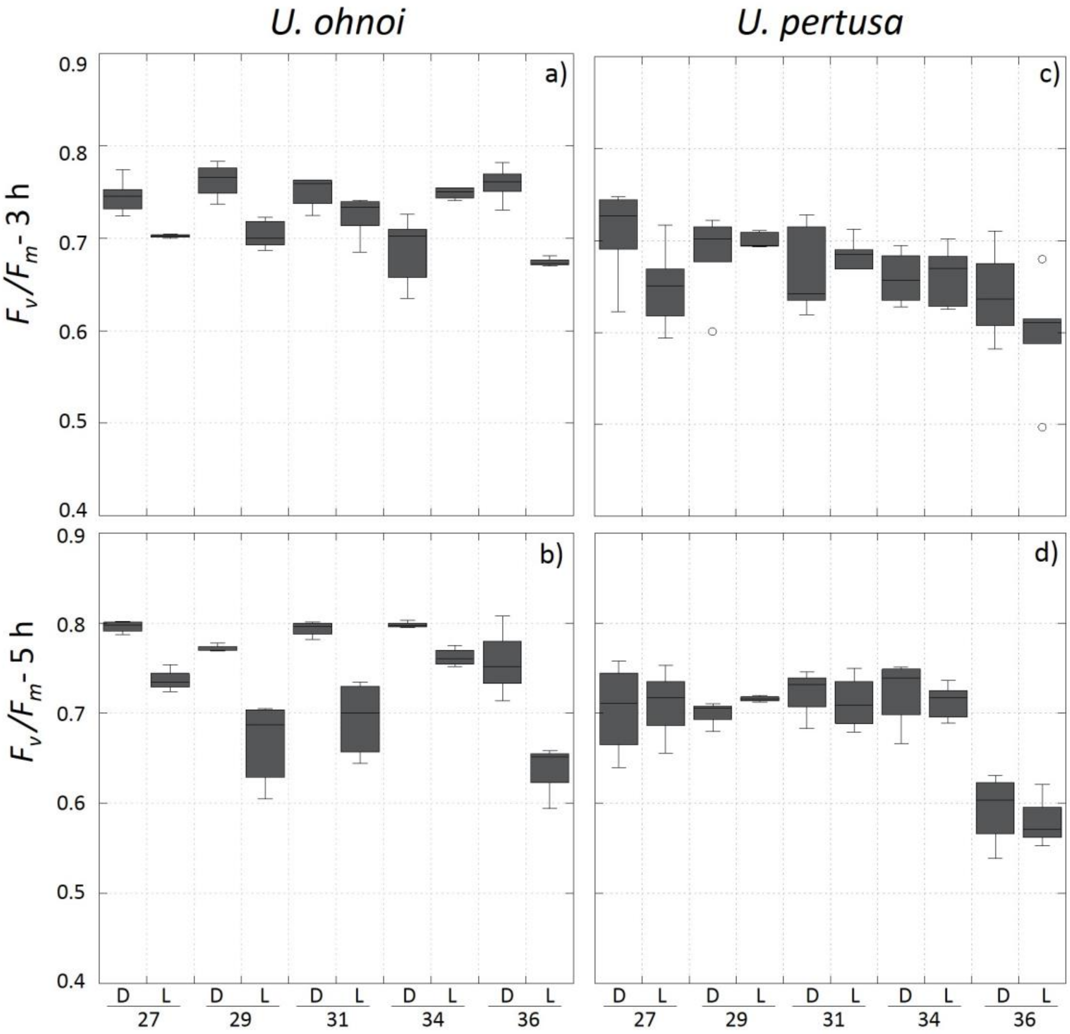

U. ohnoi
U. pertusa
a)
b)
c)
d)
F_v/F_m - 3 h
F_v/F_m - 5 h
0.9
0.8
0.7
0.6
0.5
0.4
D
L
27
29
31
34
36

Fig 2

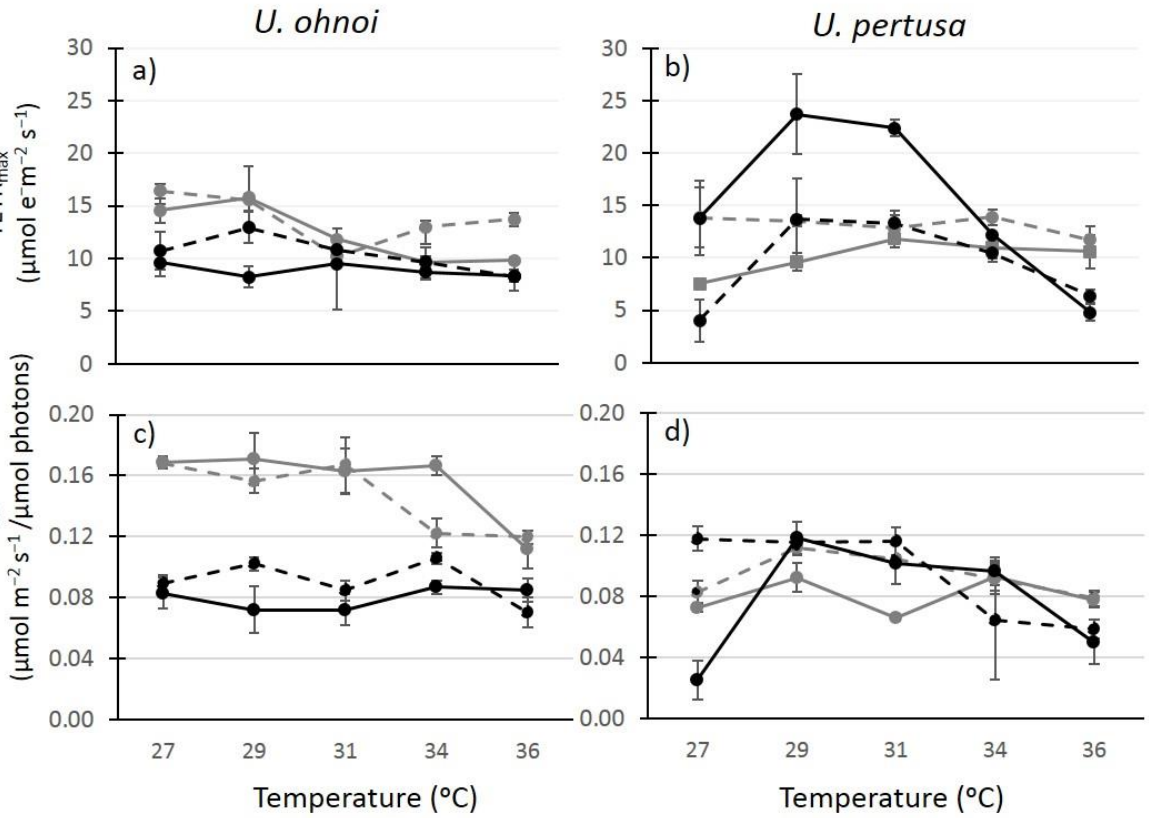
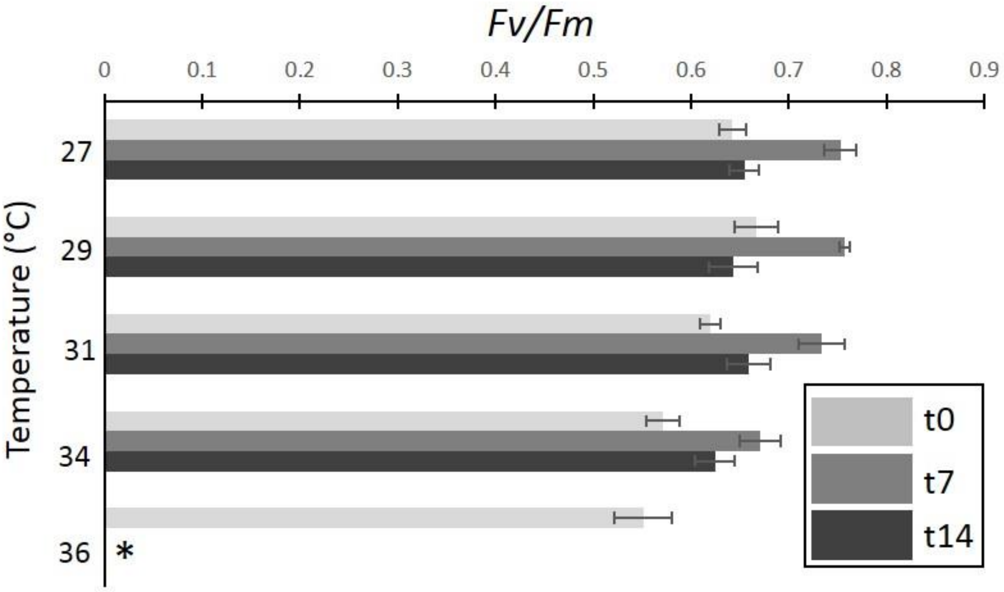

U. ohnoi
U. pertusa
a)
b)
c)
d)
rETR$_{max}$ (µmol e$^-$m^{-2} s^{-1})
α (µmol m^{-2} s^{-1}/µmol photons)
30
25
20
15
10
5
0
0.20
0.16
0.12
0.08
0.04
0.00
27
29
31
34
36
Temperature (°C)

Fig 3



Fv/Fm
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Temperature (°C)
27
29
31
34
36
*
t0
t7
t14

Fig 4

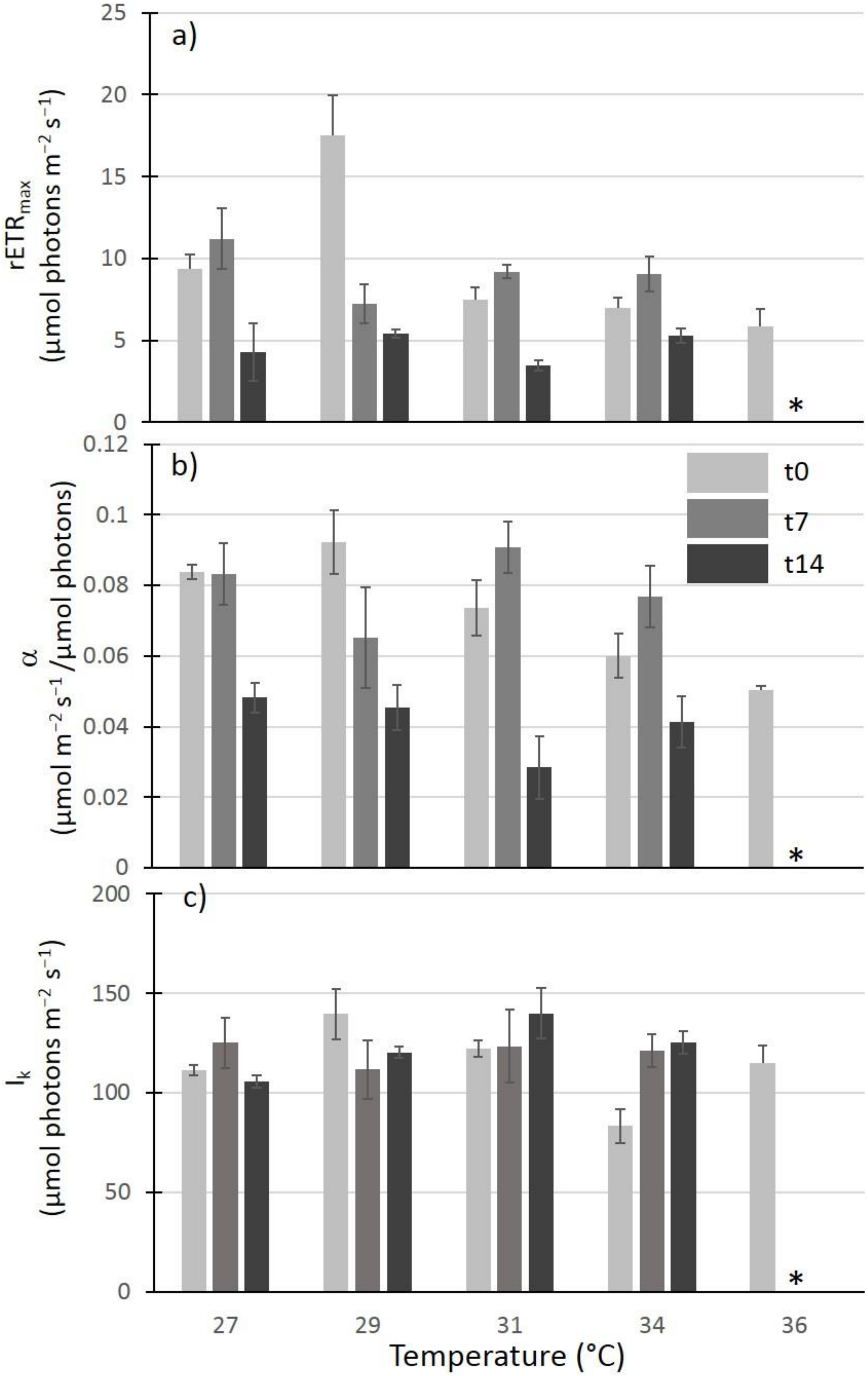

a)
rETR$_{max}$ (µmol photons m^{-2} s^{-1})
25
20
15
10
5
0
*
b)
α (µmol m^{-2} s^{-1}/µmol photons)
0.12
0.1
0.08
0.06
0.04
0.02
0
t0
t7
t14
*
c)
I$_k$ (µmol photons m^{-2} s^{-1})
200
150
100
50
0
*
27
29
31
34
36
Temperature (°C)

Fig 5

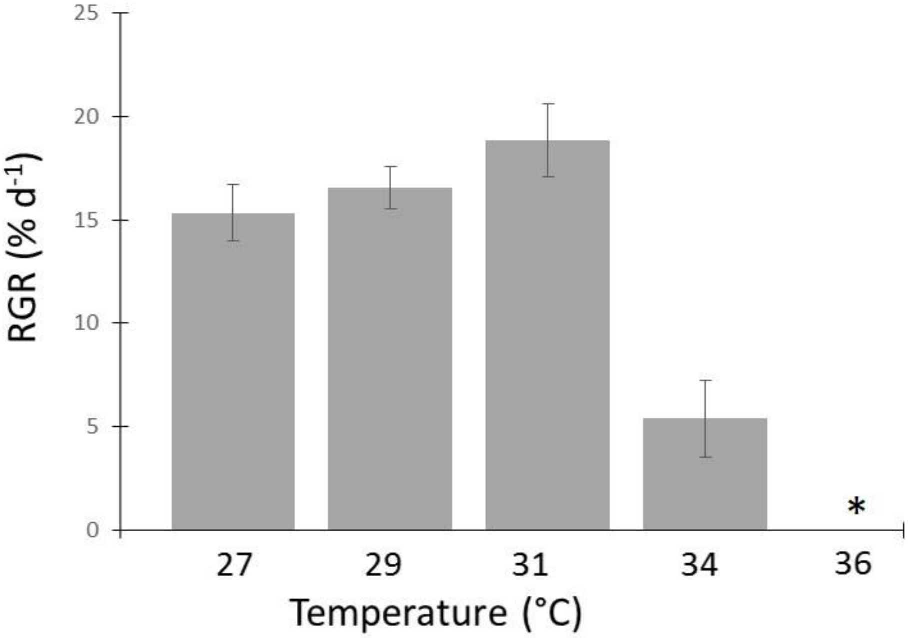


Fig 6

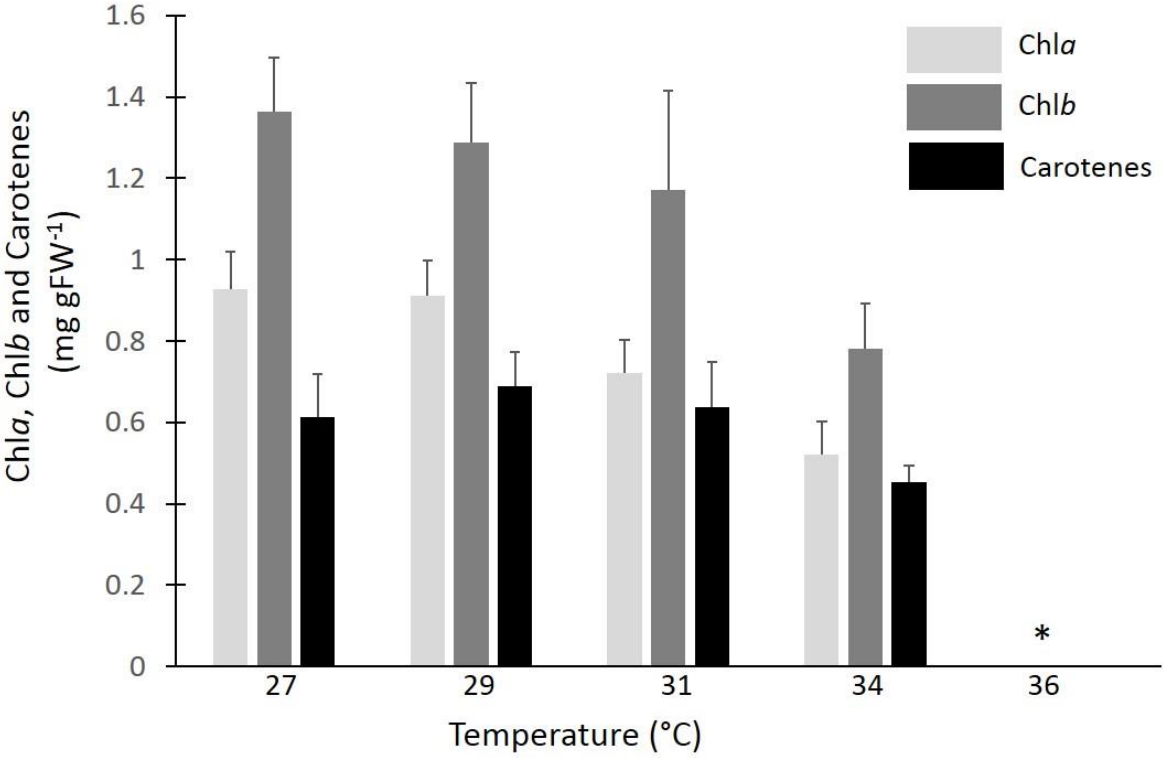

Chl*a*
Chl*b*
Carotenes
Chl*a*, Chl*b* and Carotenes (mg gFW^{-1})
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
27
29
31
34
36
*
Temperature (°C)

Table 1. Detail of the different statistical multivariate analysis applied to the data. Significant values are highlighted in bold.

	Fixed factors	Pillai's Trace value	*F*	Sig.	Partial Eta Squared	Power
Short term experiment / MANOVA						
	Species (S)	0.566	23.278	**0.0001**	0.566	1.000
	Incubation (i)	0.552	21.99	**0.0001**	0.552	1.000
	Temperature (T)	0.735	4.126	**0.0001**	0.184	1.000
	Light (L)	0.208	4.684	**0.0001**	0.208	0.986
	S*I	0.563	22.977	**0.0001**	0.563	1.000
	S*T	0.422	2.159	**0.001**	0.105	0.997
	S*L	0.247	5.836	**0.0001**	0.247	0.997
	I*T	0.438	2.256	**0.001**	0.11	0.998
	I*L	0.05	0.944	0.467	0.05	0.359
	T*L	0.419	2.143	**0.001**	0.105	0.997
	S*I*T	0.37	1.871	**0.008**	0.093	0.991
	S*I*L	0.161	3.413	**0.004**	0.161	0.931
	S*T*L	0.339	1.699	**0.022**	0.085	0.982
	I*T*L	0.257	1.259	0.187	0.064	0.914
	S*I*T*L	0.517	2.722	**0.0001**	0.129	1.000
Long term experiment / Repeated measures MANOVA						
	Temperature (T)	1.708	1.763	0.0970	0.569	0.782
	Time (t)	1.649	14.082	0.0001	0.824	1.000
	T * t	2.144	2.038	**0.002**	0.357	0.999
Long term experiment / MANOVA						
		Wilk's Lamda				
	Temperature	0.02	3.045	0.038	0.727	0.673