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Dissertation for Master's degree in Marine Biology

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Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Luís António de Matos Barreto

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All the ones that should be here, you know.

And to Mafalda for being that special reason why we grow together, our new adventure is already here!!

**Abstract**

Compelling evidence on environmental and species distribution changes due to global climate change have greatly increased over the last decades and extreme events like marine heatwaves (MHWs) (concurrent high temperature and low nutrient) are increasing in frequency, duration and intensity. The giant kelp, *Macrocystis pyrifera* (L.) C.A. Agardh forms dense marine forests serving as a foundation species in ecosystems that are of great ecological and economic importance. Thus, the objective of this study was to test for evidence of local adaptation to conditions representative of MHWs stress in the field on a regional level, encompassing areas with different oceanographic regimes and genetic groups. Adult blades from two depths and four regions of the Northeast Pacific were exposed to simulated nutrient and thermal stress conditions. The results clearly showed that *M. pyrifera* populations at the southernmost limit displayed greater thermal tolerance in their photosynthetic physiology. Compared to the other regions, surface blades from the South suffered reduced impact on the photosynthetic apparatus, with $F_v/F_m$ values at 24ºC 15.5% higher than the regional mean. Thermally-sensitive parameters governed mainly by enzymatic processes ($rETR_{max}$ and *Ek*) were unaffected over the 15 - 24ºC range in the southernmost region, suggesting adaptation to local environmental conditions. Amongst the bottom blades collected at depth, photosynthetic responses to stress were rather similar among different regions. The F1 juvenile generation grown *in vitro* from parental source material did not exhibit a significant reductions in $F_v/F_m$ under the moderate stress temperature used (21ºC), but $rETR_{max}$ and *Ek* remained significantly higher in the Southern regions. Consistently poor photosynthetic responses to thermal stress were observed in the North-Center region (Santa Barbara) in all experiments, suggesting genetically-based fitness reduction. Our results also highlighted the need for further research on the synergistic effects of low nutrients availability and high thermal stress considering different life stages of *M. pyrifera*.

*Key-words*: Giant kelp, foundation species, climate change, thermal stress, local adaptation

**Resumo**

Evidências científicas de alterações no ambiente e distribuição de espécies devido a mudanças climáticas de caracter global, tem aumentado largamente nas últimas décadas. Eventos como as ondas de calor marinhas, têm aumentado a sua frequência (em cerca de 38%), duração e intensidade, ameaçando vários ecossistemas marinhos como recifes de coral, pradarias marinhas e florestas marinhas, assim com espécies de grande relevância económica. Espécies como as lagostas e as amêijoas foram afetadas através da redução do habitat e por *stress* térmico, o que resultou num aumento da mortalidade, na redução do tamanho dos indivíduos e da sua taxa de reprodução.

O Kelp gigante, *Macrocystis pyrifera* (L.) C.A. Agardh é o kelp com maior distribuição nos oceanos que forma extensas florestas marinhas, com uma canópia densa na superfície resultando numa grande produção primária a qual alimenta diretamente organismos herbívoros mas maioritariamente detritívoros. Devido a sua densidade e dimensão, as florestas de *M. pyrifera* são capazes de alterar as condições ambientas locais, nomeadamente reduzindo correntes e luz, criando um habitat heterogéneo, abrigando um grande número de espécies. Assim, funciona como uma espécie fundadora do ecossistema apresentando grande relevância ecológica e económica.

A fotossíntese é influenciada pela temperatura que regula as taxas de reações e movimentos de solutos dentro das células das algas tendo sido utilizada com sucesso noutros estudos, para quantificar o efeito de aumento de temperatura em kelps. Um dos métodos mais comuns para medir respostas fotossintéticas em eco-fisiologia é o fluorómetro (PAM), pois é capaz de determinar eficientemente as taxas fotossintéticas em laboratório e também no campo.

A utilização de técnicas de deteção remota como boias e satélites em estudos oceanográficos, permite obter dados a uma grande escala e com grande regularidade possibilitando a análise de padrões e fatores relevantes como por exemplo oscilações da temperatura da superfície do mar (SST) ou movimentos da camada de gelo polar. Esta técnica pode também ser utilizada para gravar eventos a uma escala temporal mais curta, como *blooms* de fitoplâncton e processos de afloramento costeiro. Dado que a macroalga *M. pyrifera* forma uma canópia densa à superfície da água do mar, a utilização de dados de satélite (capazes de detetar a fluorescência da Clorofila *a*). Esta ferramenta tornou-se

essencial na análise da distribuição, padrões sazonais de crescimento e correlação das florestas de kelp gigante com fenómenos ambientais como furacões ou eventos como o El Niño. A análise de climatologias, resumos de uma ou mais variáveis (SST ou Clorofila *a*) em escalas temporais grandes, utiliza modelos de fluxos atmosféricos e oceanográficos com enormes bases de dados e capacidade de processamento dos mesmos (como por exemplo o sistema de reanalise de dados oceanográficos ORAS4). Estes aumentam a quantidade e qualidade dos dados utilizáveis para estudar os fatores ambientais que afetam a espécie em estudo e permitem a criação de cenários de hipotéticos de alterações na distribuição destas espécies.

O objetivo deste estudo foi procurar evidências de adaptação local a condições representativas de stress relacionado com ondas de calor marinhas (temperaturas altas e baixa concentração de nutrientes) a um nível regional numa área que engloba regimes oceanografias diferentes, assim como diferentes grupos genéticos.

Em 2015, lâminas de *M. pyrifera* adultas amostradas a duas profundidades e provenientes de quatro regiões do Pacifico Noroeste foram expostas a um gradiente de água do mar a 3 temperaturas (15, 20 e 24ºC) e nutrientes (meio não enriquecido e enriquecido com Provasoli (PES)) replicados (x3), simulando condições similares fenómenos com o El Niño. Tanques de 30L contendo água do mar filtrada foram equipados com termostatos para regular a temperatura, bombas de circulação de água e aparelhos de registo de temperatura para registar o seu perfil. As condições de cultura foram: salinidade a 35ppm, ciclos de 14:10 luz:escuro a 80 $\mu E \cdot m^{-2} \cdot s^{-1}$ e os tratamentos com adição de nutrientes foram enriquecidos com 20 $mL \cdot L^{-1}$ PES.

Os resultados das experiências revelaram, com grande expressividade, que as populações de kelp gigante do limite sul da distribuição geográfica no hemisfério norte, expressavam na sua resposta fotossintética uma maior tolerância ao *stress*. Comparada com as outras regiões, as lâminas da superfície da região Sul, demonstraram uma menor perturbação no aparelho fotossintético com os valores do parâmetro $F_v/F_m$ 15.5% superiores à média regional, nos tratamentos a 24ºC. Também na região Sul, os parâmetros relacionados com os processos fotossintéticos de enzimas termo sensíveis ($rETR_{max}$ e *Ek*) foram mais elevados a 24ºC comparativamente com os tratamentos a 20ºC, o que não sucedeu nas outras regiões. Nas lâminas amostradas junto ao fundo, as repostas fotossintéticas ao *stress* apresentaram maior semelhança entre regiões. Esporófitos juvenis crescidos em

laboratório, a partir do tecido reprodutivo recolhido nas mesmas populações amostradas para as lâminas adultas, não exibiram uma redução significativa do parâmetro $F_v/F_m$, provavelmente devido à temperatura máxima utilizada (21ºC) a qual não foi suficientemente alta para causar *str*ess térmico. No entanto, os parâmetros $rETR_{max}$ e *Ek* foram significativamente mais altos na região Sul. A região Norte-Centro (Santa Barbara, Califórnia) revelou os níveis mais baixos de resposta fotossintética ao *stress* em todas as experiências, incluindo nos esporófitos F1. Este facto aponta para uma possível redução de *fitness* devido a fatores genéticos. Os nossos resultados realçam também a necessidade de futuras investigações nos efeitos sinérgicos de baixa disponibilidade de nutrientes, associada a elevadas temperaturas considerando diferentes etapas no ciclo de vida de *M. pyrifera*.

*Palavras-chave:* Kelp, espécies, espécies estruturais, alterações climáticas, *stress* térmico, adaptação local

**Contents**

**Abbreviations list**

PAM fluorometer - Pulse Amplified Modulated fluorometer

RLC - rapid light curve

PSII - photosystem II

$F_v/F_m$ - maximum quantum yield of PSII

$ETR_{max}$ - maximum electron transport rate

$rETR_{max}$ - relative maximum electron transport rate

α - photosynthetic rate in light-limited region of RLC

*Ek* - minimum saturating irradiance

N – North

NC - North-Center

SC - South-Center

S - South

SW - seawater

RuBisCO - Ribulose-1,5-biphosphate carboxylase/oxygenase

ANOVA - Analysis of variance

GLM - Generalized Linear Models

ATP - adenosine triphosphate

$V_{max}$ - maximum rate of enzymatic reaction

$K_m$ - substrate affinity

$\phi_{PSII}$ - effective quantum yield of PSII

SST - sea surface temperature

Chl *a* - chlorophyll *a*

MHWs - Marine Heat Waves

PES - Provasoli Enriched Seawater

## Introduction

Temperature is a major physical factor that defines the biogeographic distribution of species in the marine environment (Adey & Steneck, 2001; Poloczanska *et al*., 2007; Wernberg *et al*., 2016). Species range distributions are usually associated with a center where conditions are optimal and the edges where those conditions are no longer met (Lomolino *et al*., 2006). Thermal limits occur where organisms can no longer tolerate continuous temperature conditions in the environment or their fluctuations (Garrabou *et al*., 2009; Mohring *et al*., 2014). While temperature change across the latitudinal range can be broadly contiguous, environmental factors such movement of air masses, ocean currents, hurricanes, upwelling and internal waves provide extreme complexity to the marine environment resulting in great spatial (from meters to hundreds of kilometers) and temporal (from hours to decades) thermal variability (Adey & Steneck, 2001).

Compelling evidence on environmental and species distribution changes due to global climate change have greatly increased over the last decades (Selig *et al*., 2010; Wernberg *et al*., 2012a; Rosenzweig & Neofotis, 2013; Caputi *et al*., 2015). In a decadal review of scientific experiments related to climate change on marine organisms, Wernberg *et al*., (2012b) found that virtually all experiments showed significant effects of stress related to increased temperatures. The frequency of high seawater temperature anomalies on nearshore ecosystems has increased 38% (Lima & Wethey, 2012) resulting in a mean temperature increase of 0.5-1ºC (Hawkins *et al*., 2003; Ridgway, 2007). Thus, extreme events like marine heatwaves (MHWs) characterized by a sudden significant increase of seawater temperature (but see Hobday *et al*., 2016 for a recent detailed and hierarchical definition) are increasing in frequency, duration and intensity (Perkins, 2011; Hobday *et al*., 2016). Examples of such events are the northern Mediterranean Sea marine heat wave (MHW) of 2003 (Garrabou *et al*., 2009) or the 2014 positive temperature anomalies on the NE and equatorial Pacific that led to the extreme magnitude of the 2015/16 El Niño (Bond *et al*., 2015; Levine & Mcphaden, 2016). MHWs are caused by the interaction of atmospheric and oceanographic processes that can encompasses different geographical (local, regional and global) and temporal (seasonal, yearly, decadal) scales (Trenberth, 2012; Hobday *et al*., 2016).

These events are also affecting larger areas of the globe, threatening several marine ecosystems and key species (Lima & Wethey, 2012; Wernberg *et al*., 2012a). Coral reefs are among the most sensitive ecosystems to anthropogenic related environmental changes (Halpern *et al.,* 2007) and recent extreme MHWs caused extensive bleaching and disease (Selig *et al.*, 2010; Smale & Wernberg, 2012b). Seagrass meadows (*e.g Posidonia oceanica)* also responded negatively after the two extreme MHWs events (2003 and 2006) in the Mediterranean when shoot mortality rates exceeded recruitment (Marbà & Duarte, 2010). Similar results were obtained in a laboratory experiment mimicking an ocean warming scenario on the seagrass *Zostera noltii*, with reduced photosynthetic performance and shoot density (Repolho *et al*., 2017). Species with great economic relevance such as lobsters and scallops were also affected by the loss of habitat and direct thermal stress resulting in increased mortality, individual size reduction and reproductive rates (Mills *et al*., 2013; Caputi *et al*., 2015). Kelp forests and fucoid beds have also registered negative effects caused by rising seawater temperature events on fundamental processes such as growth, reproduction, recruitment and resilience to disturbances (Ladah & Zertuche-González, 2007; Pearson *et al.*, 2009 ; Wernberg *et al*., 2012a; Nicastro *et al.*, 2013; Jueterbock *et al*., 2014; Mota *et al*., 2015)

Photosynthesis is greatly influenced by temperature (Raven & Geider, 1988; Kirk, 2011) which regulates the rate of enzymatic reactions and solute movements within algal cells (Raven & Geider, 1988; Davison, 1991; Tamburic *et al*., 2014) and has been successfully used to quantify the effects of temperature increase on kelps (Henkel & Hofmann, 2008; Wernberg *et al*., 2010). While low temperatures reduce photosystem 2 (PSII) activity (Baker, 1991), high temperatures can result in protein denaturation and damage to the membrane systems of the photosynthetic apparatus (Daniel *et al.,* 1996). Thus, photosynthesis is often used as an indicator for algal stress response to temperature (Hurd *et al.*, 2014). As photosynthesis depends on the electron transport rate (ETR) in order to maintain cellular redox balance, metabolic homeostasis, and energy production (ATP) for the organism, this system is highly conservative in response to environmental *(e.g* temperature oscillations) and physiological changes (McDonald *et al*., 2011). The other facet of photosynthesis, i.e., the Calvin cycle biochemical pathway through which inorganic carbon is fixed, is temperature dependent via effects on enzyme activity. Moderate temperature increase may enhance

photosynthesis by increasing maximum rates of enzymatic activity, beyond which further temperature increase may reduce the maximum rate ($V_{max}$) and/or substrate affinity ($K_m$) of key enzymes such as RuBisCO. Limitation of photosynthetic process by inorganic carbon availability, carbonic anhydrase activity, diffusion and active transport of $CO_2$ across the chloroplast membrane may also occur at higher non-acclimated temperatures, contributing to declining photosynthetic rates (Davison, 1991).

One of the most commonly used methods to measure photosynthetic responses in ecophysiological research is the Pulse Amplified Modulated (PAM) fluorometer, as it can efficiently determine photosynthetic rates in laboratory and field conditions (Beer *et al*., 1998; Ralph & Gademann, 2005; Roleda *et al*., 2009; Terada *et al*., 2016). These instruments generally have built-in software routines allowing efficient measurement of the maximum quantum yield of PSII ($F_v/F_m$), or the effective quantum yield of PSII under illumination ($\phi_{PSII}$). $\phi_{PS}$II together with information on illumination can be used to construct photosynthesis-irradiance curves, often as "rapid light curves" (RLCs) when illumination steps are given briefly (over several seconds). Declining $F_v/F_m$ values imply a reduction in the maximum quantum yield of PSII photochemistry and therefore disturbance (short term photoacclimation) or damage to the photosynthetic apparatus (Lichtenthaler *et al*., 2005). RLCs are a powerful tool that provide more detailed information on the maximum electron transport rate ($ETR_{max}$), while the initial slope (α) of the RLC provides information about the relative efficiency of (PSII) photochemistry and the minimum saturating irradiance ($E_k$) reveal the dominance of photochemical quenching (Ralph & Gademann, 2005) and can be used as a photoacclimation indicator (Sakshaug *et al*., 1997).

The giant kelp, *Macrocystis pyrifera* (L.) C.A. Agardh is the most widely distributed kelp taxon in the oceans, distributed through the northeast and southeast Pacific Ocean, in the southwest Atlantic Ocean, South Africa, Australia, New Zealand and also around most sub-Antarctic Islands (Graham *et al.*, 2007)). It has an heteromorphic life cycle in which the microscopic haploid gametophyte (male and female) alternates with a micro and macroscopic diploid sporophyte (Ladah & Zertuche-González, 2007; Graham & Halpern, 2008). *M. pyrifera* forms dense forests with thick canopy at the water surface where the majority of the sporophyte biomass is located (Nyman *et al*., 1993; Graham *et al*., 2007), resulting in high

net primary production (NPP) between 0.42 and 2.38 kg dry mass m-2 $y^{-1}$ (Graham & Halpern, 2008). While this productivity feeds secondary production via direct grazing, most of this carbon follows the detrital pathway or is exported (Graham, 2004; Dean *et al*., 1984). Due to their size, density and primary production, *M. pyrifera* forests are able to modify the local environmental conditions providing a heterogeneous habitat which can harbour a high number of species (Santelices & Ojeda, 1984; Graham, 2004). Therefore, this species serves as a foundation species in ecosystems that are of great ecological and economic importance (Lee *et al*., 2009; Graham *et al*., 2007; Byrnes *et al*., 2011).

Due to the great importance of *M. pyrifera* as a structuring species in the Northeast Pacific, several studies have been conducted in recent years to understand the oceanographic processes affecting geographical range and genetic diversity of this species (Alberto *et al*., 2010, 2011; Bell *et al*., 2015; Johansson *et al*., 2015). Across the broad latitudinal distribution of *M. pyrifera* in the Northeast Pacific, regionally heterogeneous environmental conditions raise the hypothesis that populations may be locally adapted. In general, adaptive genetic responses to the environment can occur on a time scale of years to hundreds of years (Crispo *et al*., 2010). Examples of local adaptation to warmer and nutrient limited sea-water were already described for example in *Fucus serratus* (Jueterbock *et al.*, 2014); but see Pearson *et al* (2009) for potential mal-adaptation in the same species) and coral reef endosymbionts (Howells *et al*., 2012).

The use of remote sensing techniques such as oceanographic buoys and satellite data has greatly increased our knowledge of the marine environment as continuous observations allow for pattern analysis of relevant factors (*e.g.* sea surface temperature (SST) oscillations, ice shelf movements) and can record short-lived events (*e.g.* phytoplankton blooms, upwelling event) (Joint & Groom, 2000; Paolo *et al.*, 2015). Because *M. pyrifera* forms dense canopies at the water surface where most of the adult sporophyte biomass is located (Nyman *et al*., 1993; Graham *et al*., 2007) remote sensing by satellite data analysis has become a fundamental tool to assess kelp forest distribution, seasonal growth and correlation with environmental phenomena like hurricanes or El Niño events (North *et al.*, 1993; Graham *et al*., 2007; Cavanaugh *et al*., 2013). The analysis of climatologies (long-term summary of a single or multiple variables (*e.g.* SST, Chl *a*), ocean models, atmospheric forcing fluxes and

oceanographic observations by data integration in reanalysis systems (*e.g.* ocean reanalysis system ORAS4) provide improved quality controlled data sets that can be used to assess how environmental conditions affect target species and provide plausible scenarios concerning species range distribution shifts (Lee *et al*., 2009 ; Balmaseda *et al*., 2013)

Understanding the physiological responses of *M. pyrifera* to stress conditions will help to predict and possibly minimize reductions in range distribution. Although several studies have focused on the response of *M. pyrifera* to thermal stress (North & Zimmerman, 1984; Deyseher & Dean, 1986; Rothausler *et al*., 2009; Xu *et al*., 2013) a regional comparison between population responses that encompass areas with different oceanographic regimes and genetic groups has yet to be performed.

*Objectives:*

This study aims to test for evidence of local adaptation to conditions representative of heat waves in the field (i.e., concurrent high temperature and low nutrient [inorganic N] stress) in populations of the giant kelp *Macrocystis pyrifera* at its southern limit in the northern hemisphere, in a region that encompasses distinct genetic groups and biogeographic thermal barriers, by comparing southern limit populations with those near the center of the range. This aim is approached by comparing ecophysiological responses of adult blades and F1 juvenile sporophytes from eight populations representing four distinct genetic regions.

## Materials and Methods

In all tasks, the model used was the giant kelp *Macrocystis pyrifera* in the northeast Pacific between Baja California, Mexico and central California, U.S.A., an area covering around 1300 km of coastline that includes major biogeographic and thermal transition zones (Fig 1).

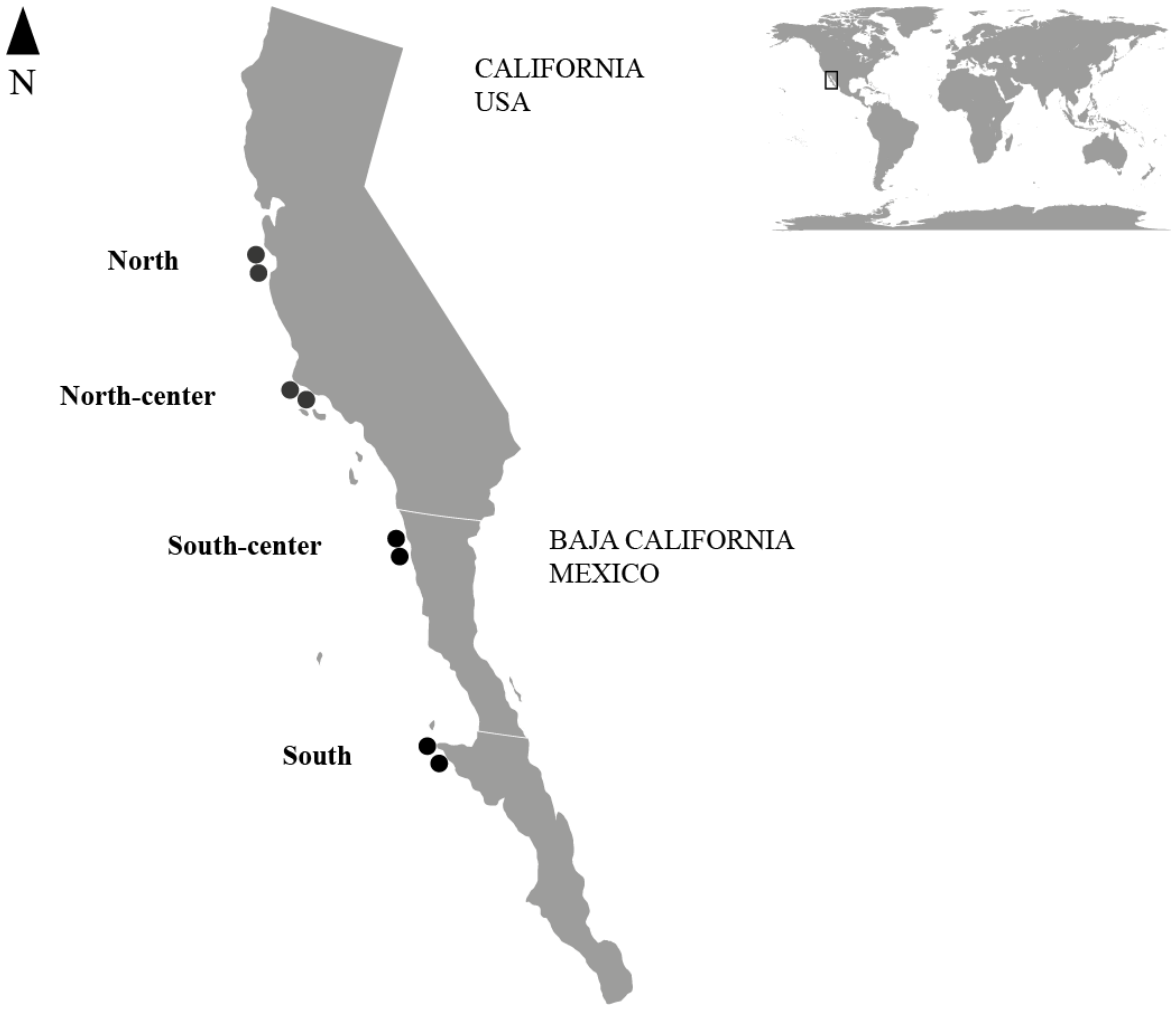



**Figure1**: Locations of the 8 populations (2 populations per region) sampled along the coast of California, USA (regions North, North-Center) and Baja California, Mexico (regions South-Center and South) (see Annex I for coordinates).

*Common-garden experiment comparing adult blades (Experiment 1)*

On June 18, 2015, adult *M. pyrifera* blades were simultaneously sampled by SCUBA diving from two populations within each of 4 regions in the northeast Pacific: 1) north (N) (Santa Cruz), 2) north-center (NC) (Santa Barbara) - both in the U.S.A, and 3) south-center (SC) (Ensenada) and 4) south (S) (Punta Eugenia) in Baja California, Mexico (Fig. 1, see Annex I for coordinates). Populations within a region were not very distant, but direct gene flow is expected to be low based on gaps in habitat continuity between populations (Alberto *et al*., 2010). From each population, 54 blades were sampled from the surface and 54 blades near the bottom (at depth 15-20 m). In addition, 10 reproductive blades (sporophylls) were sampled for subsequent spore release. Blades were cleaned of epiphytes using paper towels, then packed in moistened paper towels and wrapped in aluminum foil. All blades were transported to Ensenada in cool-boxes packed with ice for the laboratory experiment and arrived within 24 h of collection. Samples from the Ensenada area were collected, prepared and stored in a similar way, to avoid transportation effects. Immediately on arrival at the laboratory, adult sporophyte blades were acclimated for 24h at 15 ºC in 20 $mL \cdot L^{-1}$ Provasoli seawater (SW) medium (PES; Provasoli, 1968) (Anderson, 2005).

The experimental design consisted of a replicated (×3) gradient of SW temperatures (15, 20, 24 ºC) and nutrients (non-enriched or enriched SW with PES SW medium), simulating a range of environmental conditions including El Niño (Fig. 2).

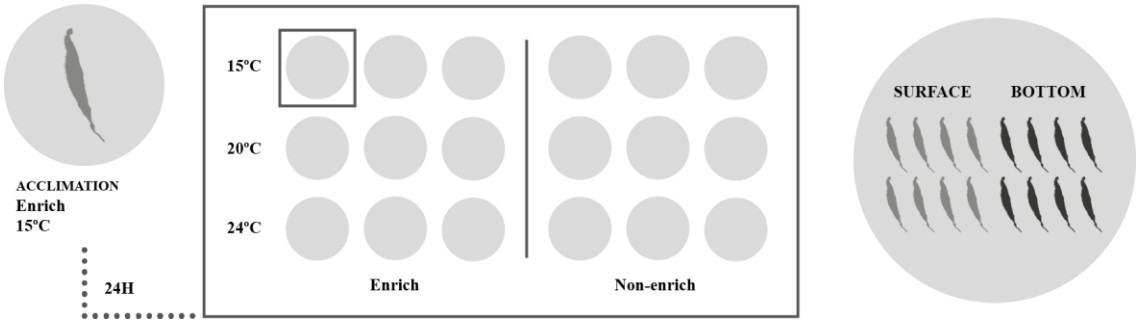



**Figure 2**: Experimental design. Acclimation for 24 h (15 ºC, with nutrients) followed by transfer of blades to 3 replicates of each of 3 temperature treatment (15, 20, 24ºC) with enriched and non-enriched seawater with PES. For each treatment condition (temperature and nutrients) a total of 3 blades per population (8 populations) x 2 depths (surface and bottom) were used.

Tanks containing 30 L filtered SW were equipped with thermostats to regulate temperature, pumps for water circulation and temperature loggers to record thermal profiles. Culture conditions were 14:10h light:dark cycle, 80 $\mu E \cdot m^{-2} \cdot s^{-1}$ and treatments with nutrients were enriched with 20 $mL \cdot L^{-1}$ PES (Anderson, 2005) at 35 ppm salinity. Blades were placed inside large open-mesh (1 cm) bags to allow individual identification throughout the experiment while not significantly reducing light and water circulation reaching the blades. Three blades per population (and from each depth within that population) were placed in each treatment tank. The maximum temperature (24ºC) was selected based on climatology analysis of the sites and thermal limits reported on literature (Hernandez-Carmona *et al*., 2001; Ladah & Zertuche-González, 2007; Rothausler *et al*., 2009)

The following physiological measurements were taken using a pulse amplified modulated (PAM) fluorometer (Diving PAM, Waltz, Germany) during the course of the experiment to allow a continuous assessment of the condition and stress responses of the algae: $F_v/F_m$ (the maximum yield of photosystem 2 (PSII) photochemistry), and rapid light curves (photosynthesis versus irradiance curves from relative electron transport rates [rETR], calculated from PSII yield [$\Delta F/F_m$] at each irradiance level). $F_v/F_m$ measurements and observation of blade condition were performed every 24h (starting at 12h) to determine if sampling was required due to tissue degradation (at higher temperatures). If so, rapid light curves were performed and tissue from each individual blade was sampled for gene expression. Tissue samples were flash-frozen in liquid nitrogen and later lyophilized in order to preserve tissue for RNA extraction. Diving PAM settings for the RLCs were the following: light curve intensity (LC-INT = 2), duration of each step (LC-WIDTH = 10seconds) and actinity light list for each step (1, 19, 45, 70, 94, 138, 219, 278 and 444). These settings allowed a plateau in the rETR curve to be reached (*i.e.*, saturated photosynthesis and in some cases photoinhibitory decline at the highest irradiance (Ralph & Gademann, 2005). RLC calibration was performed prior to the experiment using separate *M. pyrifera* blade samples.

*Oceanographic data:*

We analyzed local oceanographic features of each region prior to and during the sampling event for evaluation in light of the physiological results. Ocean temperature data were compiled on a daily basis at two depths (surface and 20 m) for each region. Data were derived from the Global Ocean Physics Reanalysis ECMWF ORAP5.0 (1979-2013), which uses a 3D model (NEMOVAR) with a bias correction scheme that combines satellite data and *in situ* measurements, for a spatial resolution of 0.25 degree x 75 depth levels (Balmaseda *et al*., 2015; Zuo & Balmaseda, 2015; Zuo *et al*., 2015). Bilinear interpolation was used to estimate the temperatures of each sampling site (e.g., Assis *et al*., 2016). An anomalously warm event (heat wave) was defined similarly to Hobday *et al*. (2016) as five or more days of temperatures warmer than the 90th percentile of a historical baseline climatology, which in our study was considered for the period 1993-2015. The climatology was defined relative to the time of year, using all data within an 11-day window centered on the time of year from which the climatological threshold was calculated. The high percentile threshold (*e.g*. 90%) was used rather than an absolute value above the climatological value as the magnitude of variability across a range of timescales varies considerably by region (Hobday *et al*., 2016).

*Effect of blade removal on physiological responses: Comparison of entire individuals vs excised blades (Experiment 2)*

In adult *Macrocystis pyrifera* sporophytes, photosynthesis is mostly performed at the canopy surface and the resulting products (mannitol and aminoacids) are translocated downwards to the youngest blades, meristems, holdfasts and sporophylls (Schmitz & Grant, 1979). As translocation in *M. pyrifera* is not fully understood, an experiment was designed in order to understand if individual responses to thermal stress (i.e. entire individuals, in this case small juveniles due to logistic constrains) differ from those of juvenile and adult excised blades. A total of 24 juveniles (smaller than 40 cm), 40 juvenile blades and 40 adult bottom blades from *M. pyrifera* sporophytes were collected from the Campo Kennedy population, in the South-center region. Two temperatures (16 and 24 ºC) and nutrients concentration (non-enriched and enriched PES-enriched SW) were used with 2 × replicates per treatment. We used a lower

number of juveniles to minimize any population impact caused by the removal of young sporophytes. Culture conditions were replicated from the first experiment (see above) and $F_v/F_m$ measurements were taken every 12h using the diving PAM fluorometer using the same settings as in the first experiment.

*Control of environmental carry-over effects: Comparison of F1 generation juvenile sporophytes (Experiment 3)*

A third experiment was designed in order to assess the ecophysiological responses of first filial (F1) generation sporophytes to thermal stress given a common growth history *in vitro*. Gametophytes developed from spores released from 10 fertile sporophylls per population used in Experiment 1were grown vegetatively in 500 mL Erlenmeyer flasks with 4 $mL \cdot L^{-1}$ of PES (Anderson, 2005) without iron, 35 ppm salinity, 20 $\mu E \cdot m^{-2} \cdot s^{-1}$ red light (LEE filters, #026 bright red) and aeration at 15ºC.

On February 2017 a gametophyte mass from each population was manually separated into the smallest pieces possible using forceps. The fragments were seeded onto a spool of cotton filament and left for 2h in air under cool (15ºC) and humid conditions to allow attachment. Seeded spools for each population were then placed separately into 20 L tanks with aerated seawater (SW) and weekly increasing concentrations of PES (6, 10, 15, 20 $mL \cdot L^{-1}$) containing iron. Incubations proceeded under white light (20, 40 60 80 $\mu E \cdot m^{-2} \cdot s^{-1}$) and 14:10 h light:dark cycle. Seawater and tanks were changed/cleaned weekly to maintain optimal culture conditions. When sporophytes were ca. 1 cm length they were gently removed from the line using forceps and grown detached with aeration (see Westermeier *et al*., 2006) in the same 20 L tanks. When sporophytes reached 6 to 10 cm they were transferred to the experimental setup. This constituted a temperature gradient (15, 17, 19 and 21ºC) in PES-enriched SW, with 3 replicates of 5 sporophytes per final temperature. A 24 h ramp between each temperature increase was used to avoid excessive stress on the small juvenile sporophytes. All individuals were initially at 15ºC, at 24 h all individuals were transferred to 17ºC except the 15ºC treatment, and so on up to 21ºC.

Rapid light curves (RCLs) were performed every 24 h for 5 days using a diving PAM. Experimental conditions were: 10 L tanks with aeration, SW with 20 $mL \cdot L^{-1}$ PES with iron, 80 $\mu E \cdot m^{-2} \cdot s^{-1}$ white light and 14:10 h light:dark cycle. Diving PAM settings for the RLCs were adjusted for the lower light conditions in the laboratory: light curve intensity (LC-INT = 2), duration of each step (LC-WIDTH = 10 s) and actinity light list for each step (0, 5, 18, 30, 42, 69, 97, 133 and 195 $\mu mol.m^{-2}.s^{-1}$). All individuals were flash-frozen in liquid nitrogen after 120 h. An additional 15 individuals were also flash-frozen at the beginning and another 15 at the end of the experiment for a transcriptomic baseline. Due to sporophyte growth problems in one of the NC populations and no sporophyte recruitment in one of the S populations, these two regions only had one replicate population. The 17ºC treatment and the NC region at 24 h were removed from the data analysis as temperature fluctuations were recorded in the 17ºC culture chamber, resulting in inconsistent ecophysiological responses and potentially compromising the data analysis and interpretation.

*Statistical analyses of experimental data*

In order to assess ecophysiological responses to temperature and nutrients in *M. pyrifera* from different regions, generalized linear models were used (GLMs; McCullagh & Nelder, 1989).

*Experiment 1) - Adult blades:* Four levels of the factor "Region" (N, NC, SC, and S), three levels of the factor "Temperature" (15, 20 and 24ºC), two levels of the factor "Nutrients" (PES-enriched and non-enriched SW) and two levels of the factor "Depth" (surface and bottom) were used as fixed effects. The response variables were minimum saturating irradiance (*Ek*), maximum potential relative ETR ($rETR_{max}$) and the photosynthetic rate in light-limited region of RLC (α), and the maximum quantum yield of PSII ($F_v/F_m$). All parameters were estimated from the rapid light curves (computed with rPackage phytotools (Silsbe & Sairah, 2015, CRAN repository), by fitting the model of Platt *et al* (1980). Analysis of variance (ANOVA) and post-hoc tests (Tukey Honest Significant differences (HSD)) were used to analyze the differences amongst treatments using R software (R Development Core Team, 2008). Fv/Fm and α were square root transformed to approximate residuals to a normal distribution. Since only a short acclimation period was possible, and to minimize any effects

of transportation on physiological responses, data from the RLCs were analyzed at the end point of the respective temperature treatments.

*Experiment 2) - Entire individual vs removed blade:* ANOVA and post-hoc tests (Tukey HSD) were used to analyze the difference between treatments using $F_v/F_m$ as the variable for two levels of factor "Temperature" (16 and 24ºC), two levels of the factor "Nutrients" (PES-enriched and non-enriched seawater) and three levels of factor "Tissue" (juvenile, juvenile blade and adult blade). $F_v/F_m$ was square rooted transformed and the GLMs fitted using the Gaussian family (highest AIC) and resulting residuals were approximate to the normal distribution.

*Experiment 3) - F1 generation juvenile sporophytes:* Four levels of the factor "Region" (N, NC, SC and S), three levels of the factor "Temperature" (15, 19 and 21ºC) and two levels of the factor "Nutrients" (PES-enriched and non-enriched SW) were used as fixed effects with the response variables α, *Ek*, $ETR_{max}$ (estimated by the Diving PAM software Win-Control 3, Walz GmbH, Effeltrich, Germany) and $F_v/F_m$. GLMs were fitted using the Gaussian family distribution for all variables (selected by AIC comparison with the available distributions for R). The variable $F_v/F_m$ was square root transformed and residuals were approximate to the normal distribution. Analysis of variance (ANOVA) and Tukey (HSD) were used to analyze the differences amongst treatments using R software (R Development Core Team, 2008). For the first and third experiments, we used populations within the same region as a replicate for that region.

## Results

*Common-garden experiment comparing adult blades (Experiment 1)*

Our results showed that *Macrocystis pyrifera* blades from all regions performed well at 15ºC and 20ºC but were rapidly stressed at 24ºC. At this temperature, more than 88 % of the blades had lower mean $F_v/F_m$ values compared with the other treatments. At 24ºC there was tissue degradation (*e.g.* necrotic tissue and loss of pigmentation) and mucilage production. Therefore, the experimental treatments at this temperature had to be terminated earlier while at the other temperatures the blades remained in culture in good condition. Rapid light curves (RLCs) parameters (α, *Ek* and $rETR_{max}$) and $F_v/F_m$ measured at the end point of each temperature treatment confirmed that 24ºC treatment rapidly stressed *M. pyrifera* blades (Fig. 3). Since no main effect or interactions involving nutrients were detected, non-enriched and PES-enriched treatments were combined for the plots in Figure 3.

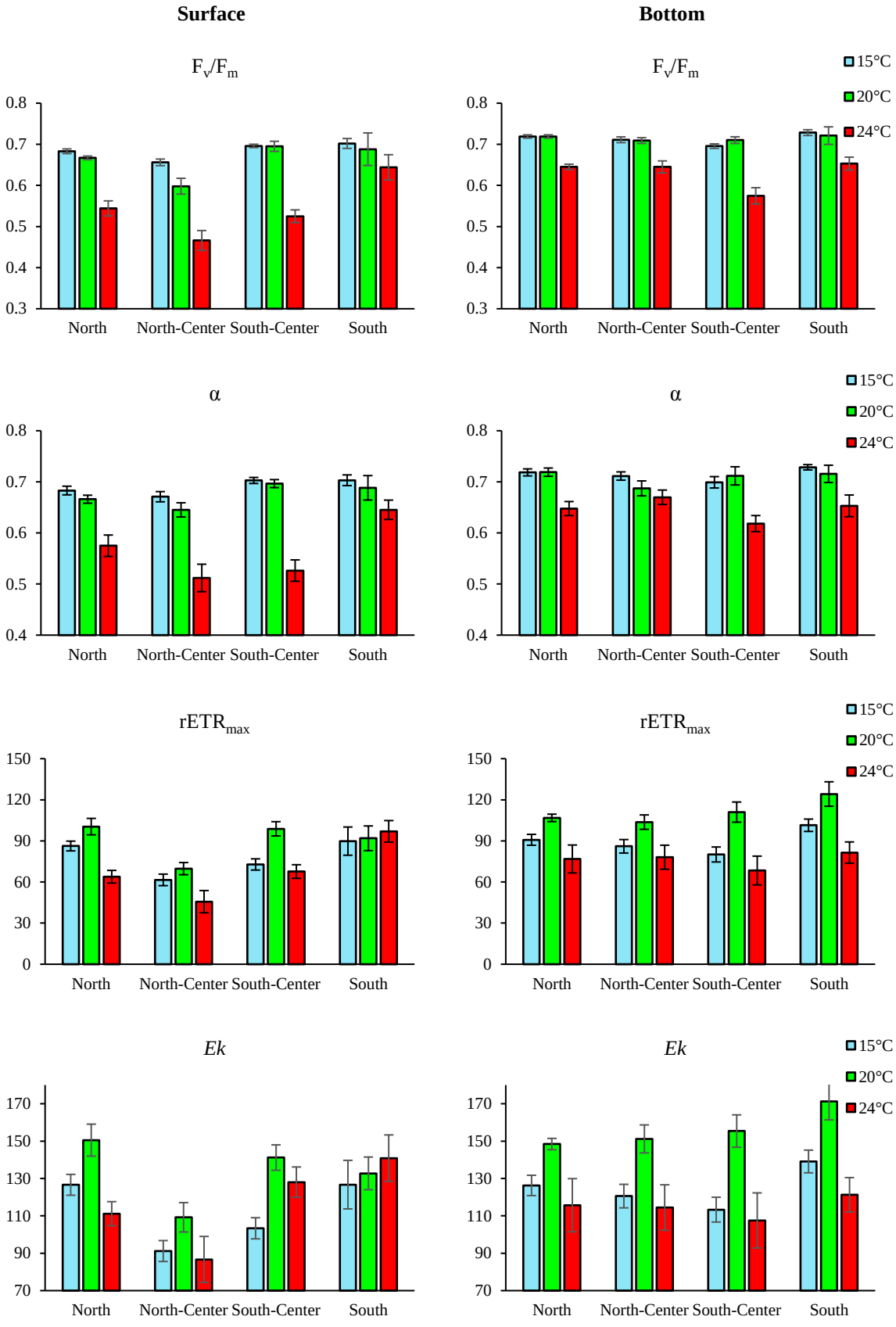

Surface
Bottom
$F_v/F_m$
α
$rETR_{max}$
*Ek*
15°C
20°C
24°C
North
North-Center
South-Center
South

**Figure 3:** Maximum quantum yield of PSII ($F_v/F_m$), photosynthetic rate in light-limited region of RLC (α), relative maximum electron transport rate ($rETR_{max}$) and minimum saturating irradiance (*Ek*) mean values (± mean standard error) for 3 temperatures (15, 20, 24ºC) and 4 regions (North, North-Center, South-Center and South) of adult blades over time. Different letters indicate significant differences among temperatures within each region and variable using Tukey HSD.

*Maximum quantum efficiency of PSII ($F_v/F_m$)*

Decreasing values of $F_v/F_m$ can provide a rapid indication of stress affecting PS II in the dark-adapted state. With $F_v/F_m$ as response variable, ANOVA indicated signification interactions due to Region x Temperature, Temperature x Depth and Region x Depth (Table 1).

The interaction between Region x Temperature revealed significant differences between regions in the response to thermal stress: In the N, NC, and SC regions, $F_v/F_m$ was lower at 24ºC than at either 20ºC or 15ºC (and attributable mainly to stress in surface blades), but in the S region significantly lower $F_v/F_m$ was only detected between 24ºC and 15ºC ($p = 0.040$). The region differences were clear when responses at 24ºC between regions at 24ºC were considered; $F_v/F_m$ was higher at 24ºC in the S versus either SC or NC regions ($p = 9.5 \times 10^{-5}$ and $2.3 \times 10^{-6}$, respectively), but was marginally insignificant between S and N regions ($p = 0.066$).

Post-hoc tests (Tukey HSD) indicated that the interaction between Temperature x Depth (*i.e.*, surface and bottom blades) was due to greater resilience of $F_v/F_m$ in bottom blades at 20ºC and 24ºC ($p = 0.001929$ and $p < 0.0001$, respectively), while in 15ºC control conditions no differences in blade sampling depth were detected ($p > 0.25$).

Finally, the interaction between Region x Depth was due to significant contrasts involving surface blades between regions; more specifically $F_v/F_m$ in NC surface blades was lower overall than in all other regions (NC < N, $p = 0.00136$; NC < SC, $p = 0.00027$; NC < S, $p = 8 \times 10^{-7}$). In addition, bottom blades had significantly greater $F_v/F_m$ than surface blades in the N and NC regions, but not in the SC or S (Fig. 3).

*Light-limited photosynthetic rates* (α)

Analysis of $\alpha$ values revealed a significant interaction between Region x Temperature x Depth and post-hoc tests (Tukey HSD) highlighted that regional differences regarding the response to thermal stress (24ºC) were due to significantly higher $\alpha$ values on the S region surface blades compared to NC and SC regions ($p$ = 7.19 x $10^{-5}$ and $p$ = 2.82 x $10^{-5}$, respectively) as other interactions were non-significant ($p > 0.275$). The response to thermal stress of the S region surface blades showed surprising stress tolerance, as all post-hoc tests between surface blades at 24ºC and either 15 or 20ºC temperatures were non-significant ($p > 0.742$), indicating that α was little affected over this temperature range. The same contrasts were significant for all other regions ($p \leq 0.00023$ in all cases). Also all regional comparisons between the 24ºC treatments for the bottom blades were non-significant ($p \geq 0.698$). The effect of the 24ºC surface blades on the interaction was also attested by the analysis of the $\alpha$ values between and within the 15ºC control and 20ºC that showed no significant differences between or within depths considering all possible regional combinations (total 120 combinations) with only one residual significant value ($p = 0.046$) . Because both α and $F_v/F_m$ represent aspects of photosynthetic efficiency (Kirk, 2011), the values reported were quite similar among temperatures within each region (Fig. 3).

*Maximum electron transport rate ($rETR_{max}$)*

Blades from all regions (exception for the S region surface blades) followed the same pattern in both depths as $rETR_{max}$ values were higher at 20ºC, indicating increased photosynthetic performance when exposed to 20ºC and photosynthetic inhibition by thermal stress when exposed to 24ºC (Fig. 3).The post-hoc analysis of the interaction Region x Depth indicated that the significant regional differences were cause by significantly lower values in NC surface blades compared to other regions from the same depth (NC < N, $p < 0.001$; NC < SC, $p = 0.002$; NC < S, $p < 0.001$); no significant contrasts were found between regions when analyzing the blades collected from the bottom ($p > 0.257$). Treatment temperatures (*e.g.* 15, 20 and 24ºC) differed significantly, with 18.5% and 29.5% higher $rETR_{max}$ values in blades at 20ºC, when compared to 15ºC and 24ºC, respectively ($p < 0.001$) and 11% higher at 15ºC

than at 24ºC ($p < 0.001$). Again, the S region surface blades showed tolerance to thermal stress (*i.e.* 24ºC) with no significant differences in $rETR_{max}$ compared at 24ºC compared with 20ºC and 15ºC (Fig. 3) ($p = 1.000$).

*Minimum saturating irradiance (Ek)*

The ANOVA analysis of *Ek* for adult blades showed a significant main effect for Temperature, and a significant interaction between Region x Depth (i.e., blade type). The patterns of the response were similar to $ETR_{max}$ with a clear peak at 20ºC for the majority of the regions (Fig. 3). The Region x Depth interaction (Table 1) resulted from significantly lower *Ek* values in NC surface blades (96.3) compared to the N (130.4407), SC (124.069) and S (134.25) regions (all $p \leq 0.001$) and no significant differences were found between surface blades for the other 3 regions ($p > 0.824$). Regional comparisons for *Ek* in bottom blades were non-significant ($p > 0.438$). At 20ºC, *Ek* values were 19.6% and 20.8% higher overall compared to 15ºC and 24ºC, respectively ($p < 0.001$ for both comparisons), while no significant differences were found between 15ºC and 24ºC ($p = 0.783$). This result clearly demonstrates the thermal dependence of *Ek* with a peak near 20ºC in N, NC, and SC regions. In contrast, *Ek* for S region surface blades was temperature independent across the thermal range tested, with no significant differences detected (all $p = 1.000$; Fig. 3). The thermal dependence of both *Ek* and $ETR_{max}$ is expected, since they are governed by the rate of enzymatic processes, and our results suggest that S region blades were better able to adjust photosynthetic rates across a broad temperature range between 15-24ºC, in contrast to surface blades from more northerly regions.

The overall picture that emerged was that the NC region (Santa Barbara, Southern CA) was particularly sensitive to thermal effects on $F_v/F_m$, particular for surface blades. Overall, bottom blades showed greater resilience than surface blades. There was also a clear tendency in the S region towards thermal stress resilience, followed, surprisingly, by N regional populations (Santa Cruz, Central CA).

**Table 1:** Four-way ANOVA for the effects of Region, Temperature, Nutrients and Depth on the maximum quantum yield of PSII ($F_v/F_m$), photosynthetic rate in light-limited region of RLC (α), maximum electron transport rate ($ETR_{max}$) and minimum saturating irradiance (*Ek*) mean values of adult blades. Significant p-values are marked in bolt.

**$F_v/F_m$**

| Factor | df | SS | MS | F | p-value |
|---|---|---|---|---|---|
| Region | 3 | 0.063 | 0.021 | 8.268 | < **0.001** |
| Temperature | 2 | 0.488 | 0.244 | 96.204 | < **0.001** |
| Nutrients | 1 | 0.002 | 0.002 | 0.646 | 0.422 |
| Depth | 1 | 0.138 | 0.138 | 54.239 | < **0.001** |
| Region:Temperature | 6 | 0.044 | 0.007 | 2.867 | **0.010** |
| Region:Nutrients | 3 | 0.018 | 0.006 | 2.368 | 0.070 |
| Region:Depth | 3 | 0.072 | 0.024 | 9.524 | < **0.001** |
| Temperature:Nutrients | 2 | 0.006 | 0.003 | 1.158 | 0.315 |
| Temperature:Depth | 2 | 0.031 | 0.015 | 6.058 | **0.003** |
| Nutrients:Depth | 1 | 0.001 | 0.001 | 0.366 | 0.545 |
| Region:Temperature:Nutrients | 6 | 0.022 | 0.004 | 1.459 | 0.191 |
| Region:Temperature:Depth | 6 | 0.020 | 0.003 | 1.331 | 0.242 |
| Region:Nutrients:Depth | 3 | 0.008 | 0.003 | 1.038 | 0.376 |
| Temperature:Nutrients:Depth | 2 | 0.006 | 0.003 | 1.095 | 0.336 |
| Region:Temperature:Depth:Nutrients | 6 | 0.018 | 0.003 | 1.174 | 0.319 |

**α**

| Factor | df | SS | MS | F | p-value |
|---|---|---|---|---|---|
| Region | 3 | 0.016 | 0.005 | 4.265 | **0.010** |
| Temperature | 2 | 0.277 | 0.138 | 110.182 | < **0.001** |
| Nutrients | 1 | 0.000 | 0.000 | 0.006 | 0.939 |
| Depth | 1 | 0.074 | 0.074 | 58.612 | < **0.001** |
| Region:Temperature | 6 | 0.030 | 0.005 | 3.918 | **0.001** |
| Region:Nutrients | 3 | 0.002 | 0.001 | 0.545 | 0.652 |
| Region:Depth | 3 | 0.015 | 0.005 | 3.985 | **0.008** |
| Temperature:Nutrients | 2 | 0.005 | 0.003 | 2.056 | 0.130 |
| Temperature:Depth | 2 | 0.029 | 0.015 | 11.570 | < **0.001** |
| Nutrients:Depth | 1 | 0.000 | 0.000 | 0.005 | 0.943 |
| Region:Temperature:Nutrients | 6 | 0.012 | 0.002 | 1.572 | 0.155 |
| Region:Temperature:Depth | 6 | 0.018 | 0.003 | 2.413 | **0.027** |
| Region:Nutrients:Depth | 3 | 0.002 | 0.001 | 0.504 | 0.680 |
| Temperature:Nutrients:Depth | 2 | 0.001 | 0.001 | 0.415 | 0.661 |
| Region:Temperature:Depth:Nutrients | 6 | 0.011 | 0.002 | 1.520 | 0.171 |

**rETRmax**

| Factor | df | SS | MS | F | p-value |
|---|---|---|---|---|---|
| Region | 3 | 17909 | 5970 | 9.516 | < 0.001 |
| Temperature | 2 | 60023 | 30012 | 47.839 | < 0.001 |
| Nutrients | 1 | 364 | 364 | 0.58 | 0.447 |
| Depth | 1 | 13760 | 13760 | 21.934 | < 0.001 |
| Region:Temperature | 6 | 3278 | 546 | 0.871 | 0.516 |
| Region:Nutrients | 3 | 228 | 76 | 0.121 | 0.948 |
| Region:Depth | 3 | 8223 | 2741 | 4.369 | 0.005 |
| Temperature:Nutrients | 2 | 592 | 296 | 0.472 | 0.624 |
| Temperature:Depth | 2 | 1148 | 574 | 0.915 | 0.402 |
| Nutrients:Depth | 1 | 94 | 94 | 0.15 | 0.699 |
| Region:Temperature:Nutrients | 6 | 3712 | 619 | 0.986 | 0.435 |
| Region:Temperature:Depth | 6 | 3916 | 653 | 1.04 | 0.399 |
| Region:Nutrients:Depth | 3 | 3495 | 1165 | 1.857 | 0.137 |
| Temperature:Nutrients:Depth | 2 | 286 | 143 | 0.228 | 0.796 |
| Region:Temperature:Depth:Nutrients | 6 | 7123 | 1187 | 1.892 | 0.082 |

**Ek**

| Factor | df | SS | MS | F | p-value |
|---|---|---|---|---|---|
| Region | 3 | 26036 | 8679 | 7.816 | < 0.001 |
| Temperature | 2 | 74820 | 37410 | 33.693 | < 0.001 |
| Nutrients | 1 | 807 | 807 | 0.727 | 0.395 |
| Depth | 1 | 7284 | 7284 | 6.56 | 0.011 |
| Region:Temperature | 6 | 6562 | 1094 | 0.985 | 0.435 |
| Region:Nutrients | 3 | 551 | 184 | 0.165 | 0.920 |
| Region:Depth | 3 | 15432 | 5144 | 4.633 | 0.003 |
| Temperature:Nutrients | 2 | 264 | 132 | 0.119 | 0.888 |
| Temperature:Depth | 2 | 6577 | 3288 | 2.962 | 0.053 |
| Nutrients:Depth | 1 | 227 | 227 | 0.204 | 0.652 |
| Region:Temperature:Nutrients | 6 | 7125 | 1188 | 1.07 | 0.381 |
| Region:Temperature:Depth | 6 | 7887 | 1315 | 1.184 | 0.314 |
| Region:Nutrients:Depth | 3 | 7744 | 2581 | 2.325 | 0.075 |
| Temperature:Nutrients:Depth | 2 | 242 | 121 | 0.109 | 0.897 |
| Region:Temperature:Depth:Nutrients | 6 | 13871 | 2312 | 2.082 | 0.055 |

*Oceanographic data*

Short term climatology (2.5 months centered on the sampling day) showed a latitudinal gradient of temperature increase towards the south but with only small differences between the SC and NC regions, particularly during the days before the sampling day (vertical lines; Fig.4).

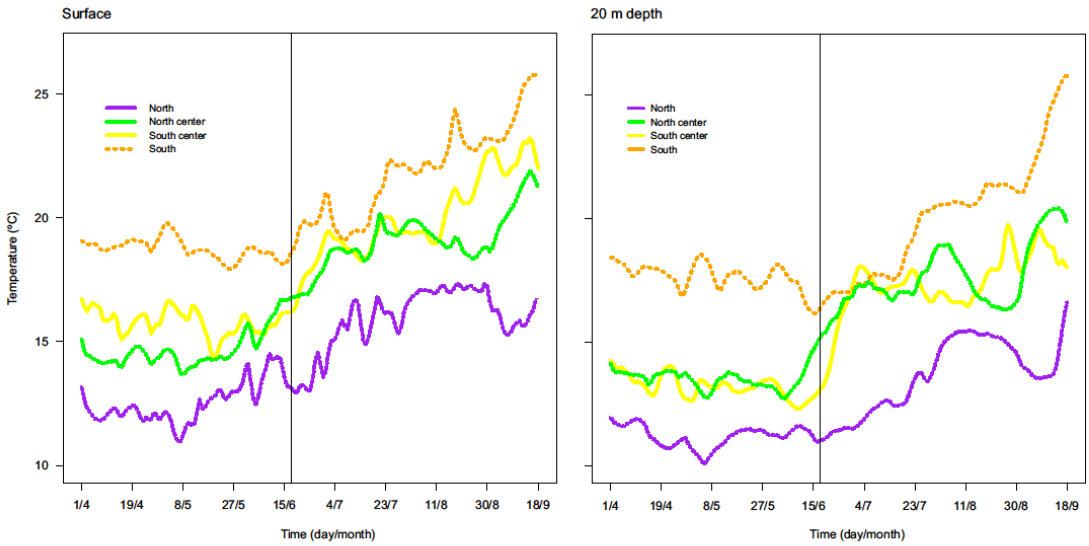
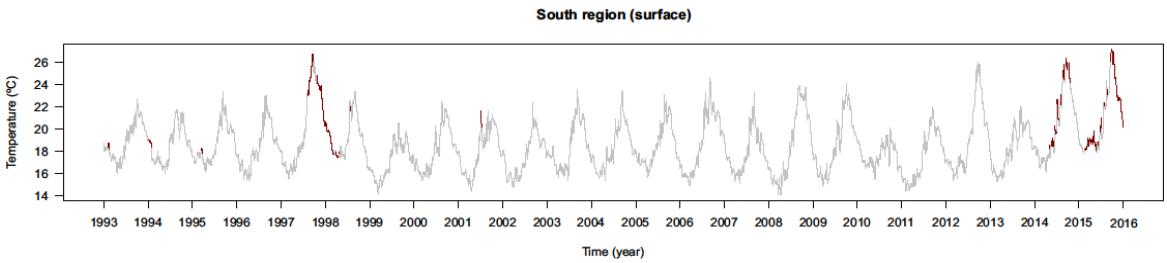



**Figure 4:** Daily mean seawater temperature (ºC) on the 4 regions (North, North-Center, South-Center, South), at the surface (left) and 20m depth (right) centered on our sampling day (17/06/2017; vertical lines) using data collected from the Global Ocean Physics Reanalysis ECMWF ORAP5.0.

Previously to our sampling, the S region registered a continuous high temperature while the remaining regions presented a pattern of increasing temperatures. The NC region actually had higher temperatures than the SC region over several days prior to sampling, especially at 20m depth. Seawater (SW) temperatures at 20 m depth showed lower values when compared to the surface. On the sampling day, cooler 20 m SW had a mean difference to the warmer surface of between 2 and 3ºC. During the months following the sampling, a persistent pattern of water temperature increase was predominant in which 25ºC was reached in the S region by mid-September.

Temperature climatologies were compiled for the S region on the surface and bottom (20 m depth) over the last 22 years (Fig. 5)

a)




b)

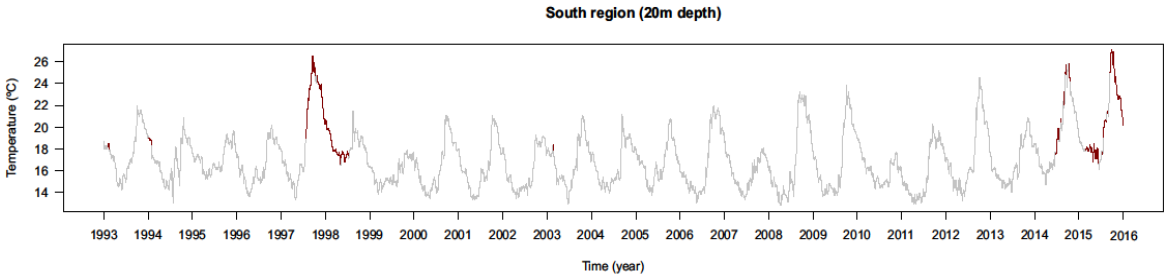



**Figure 5:** Climatologies of the South region surface (a) and 20m depth (b) over the past 22 years. Marine heat waves (MHWs) are marked in red (according to Hobday *et al*., 2016).

Marine heat waves (an anomalously warm event lasting five or more days, with temperatures warmer than the 90th percentile based on a 30-year historical baseline period [Hobday *et al*., 2016] greatly increased during the El Niño event the 1997-98, but also in 2014 and 2015 including during our sampling campaign (Table 4).

**Table 2:** Climatologies resume of the sampling sites (region) over the last 22 years for the seawater surface and 20m depth for the years with marine heat waves (MHW) and mean temperature (ºC) during MHWs.

| Region | Depth | Years with heat waves | Mean temperature during Heat wave (ºC) |
|---|---|---|---|
| **North** | Surface | 1993 1995 1997 1998 2005 2009 2014 2015 | 15.447±0.958 |
| | 20m depth | 1993 1994 1995 1996 1997 1998 2003 2005 2006 2014 2015 | 14.329±1.984 |
| **North-center** | Surface | 1993 1994 1995 1996 1997 1998 2001 2005 2006 2009 2013 2014 2015 | 15.869±1.048 |
| | 20m depth | 1993 1995 1996 1997 1998 2000 2005 2006 2009 2013 2014 2015 | 15.902±1.019 |
| **South-center** | Surface | 1995 1996 1997 1998 2005 2006 2010 2013 2014 2015 | 17.990±1.130 |
| | 20m depth | 1993 1995 1996 1997 1998 2001 2003 2005 2006 2010 2014 2015 | 17.070±1.820 |
| **South** | Surface | 1993 1994 1995 1997 1998 2000 2001 2006 2014 2015 | 21.243±2.173 |
| | 20m depth | 1993 1994 1997 1998 2003 2014 2015 | 20.185±2.463 |

All regions registered MHW during 2014-15 in both depths which suggests recent increase on the frequency of these extreme events.

*Comparison of entire individuals vs excised blades (Experiment 2)*

The photosynthetic response ($F_v/F_m$) over time for three different types of blades (entire juveniles, excised adults and juvenile blades) to thermal and nutrient stress is shown in (Fig. 6).

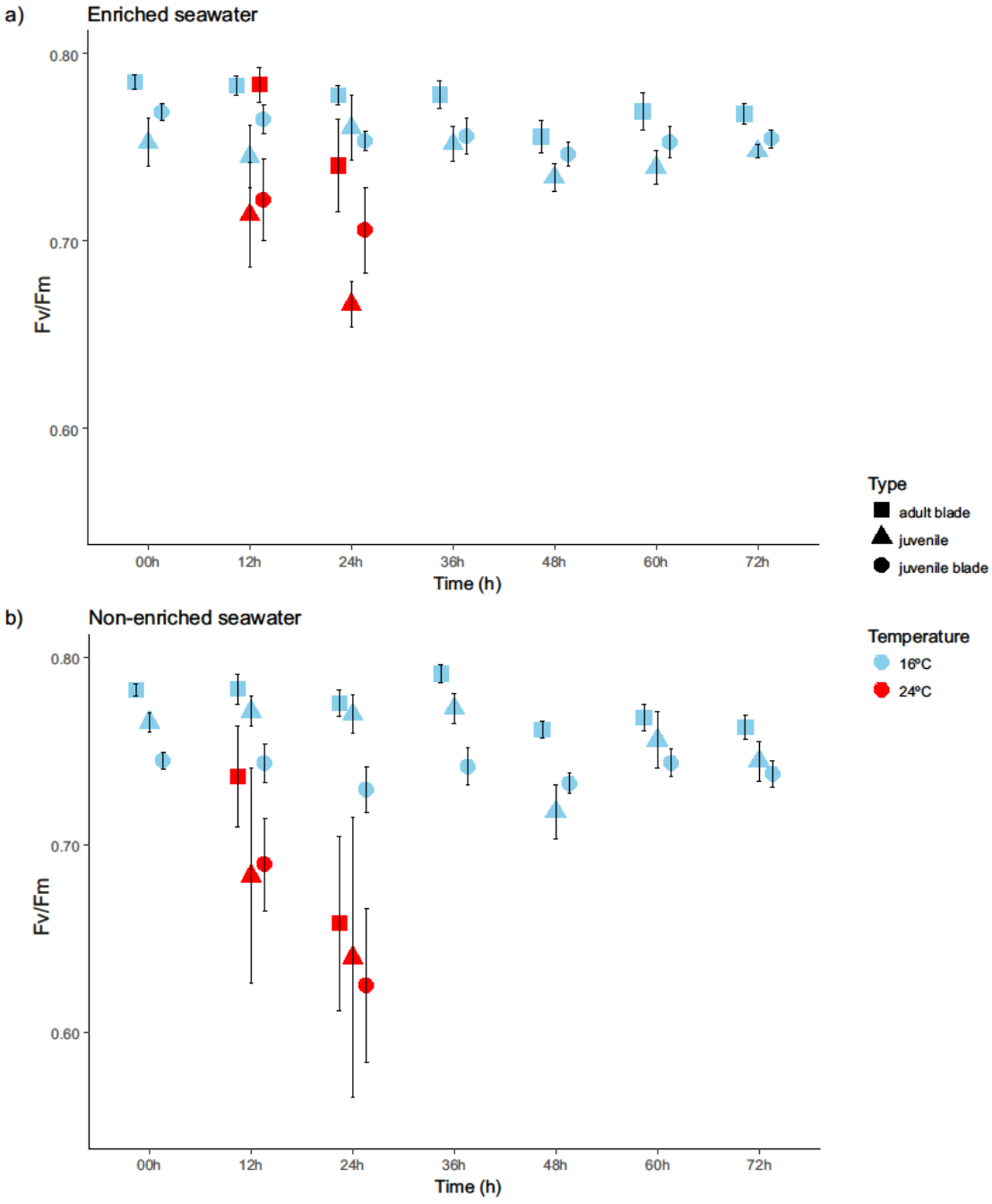



**Figure 6**: Maximum quantum yield of PSII ($F_v/F_m$) mean values (± mean standard error) for 2 temperatures (16 and 24ºC) and 3 types (adult blade, juvenile blade and juvenile) on a) enriched seawater with PES (20 $mL.L^{-1}$) and b) non-enriched seawater, over time.

As in the previous experiment, blades and entire individuals at 16ºC showed rather constant $F_v/F_m$ over time while exposure to 24ºC resulted in a rapid decrease, indicating a severe decline in PSII energy conversion efficiency. The ANOVA showed an interaction between Temperature x Nutrients and a significant main effect for the factor Tissue (Table 3). The post-hoc analysis (Tukey HSD) on the factor Tissue (*i.e.* juvenile, excised juvenile and adult blades) indicated no significant differences between the entire juvenile and the juvenile blade ($p = 0.995$), supporting the experiment design using excised blades in Experiment 1. Also in this analysis, significantly higher $F_v/F_m$ values were found for adult blades (0.766) when compared with entire juvenile (0.737) and juvenile blade (0.736) ($p = 0.0000065$ and $p = 0.0000001$, respectively). Post-hoc tests (Tukey HSD) indicated that the interaction between Temperature x Nutrients was due to higher temperature as tissue at 24ºC in enriched SW had significant lower $F_v/F_m$ values than 16ºC non-enriched and by nutrients when temperatures were at 24ºC (see Annex II for mean values and *p-values*). The only exception was between 16ºC enriched and 16ºC non-enriched where differences in $F_v/F_m$ were non-significant ($p = 0.848$). This experiment supports the use of excised blades as surrogate for entire individuals for *M. pyrifera* and that high temperature (24ºC) in conjugation with low nutrients availability affects this species ecophysiological response, while having no effect at lower (non-stressful) temperatures (16ºC), at least for 72h.

**Table 3:** Three-way ANOVA for the effects of Temperature, Nutrients and Type on the maximum quantum yield of PSII ($F_v/F_m$) mean values. Significant p-values are marked in bolt.

| Variable | Factor | df | SS | MS | F | p-value |
|---|---|---|---|---|---|---|
| $F_v/F_m$ | Temperature | 1 | 0.117 | 0.117 | 122.682 | < **0.001** |
| | Nutrients | 1 | 0.010 | 0.010 | 10.795 | **0.001** |
| | Tissue | 2 | 0.037 | 0.018 | 19.293 | < **0.001** |
| | Temperature:Nutrients | 1 | 0.022 | 0.022 | 22.603 | < **0.001** |
| | Temperature:Tissue | 2 | 0.005 | 0.003 | 2.640 | 0.072 |
| | Nutrients:Tissue | 2 | 0.005 | 0.002 | 2.526 | 0.081 |
| | Temperature:Nutrients: Tissue | 2 | 0.001 | 0.001 | 0.532 | 0.588 |

*Comparison of F1 generation juvenile sporophytes (Experiment 3)*

F1 generation juvenile sporophytes of *M. pyrifera* grown *in vitro* and exposed to a thermal gradient over a 5-day simulated heat wave (15, 19 and 21ºC) showed few region-specific patterns of stress induced by temperature increase, based on photosynthetic response parameters ($F_v/F_m$ and the RLCs parameters α, $ETR_{max}$ and *Ek*; Fig. 7).

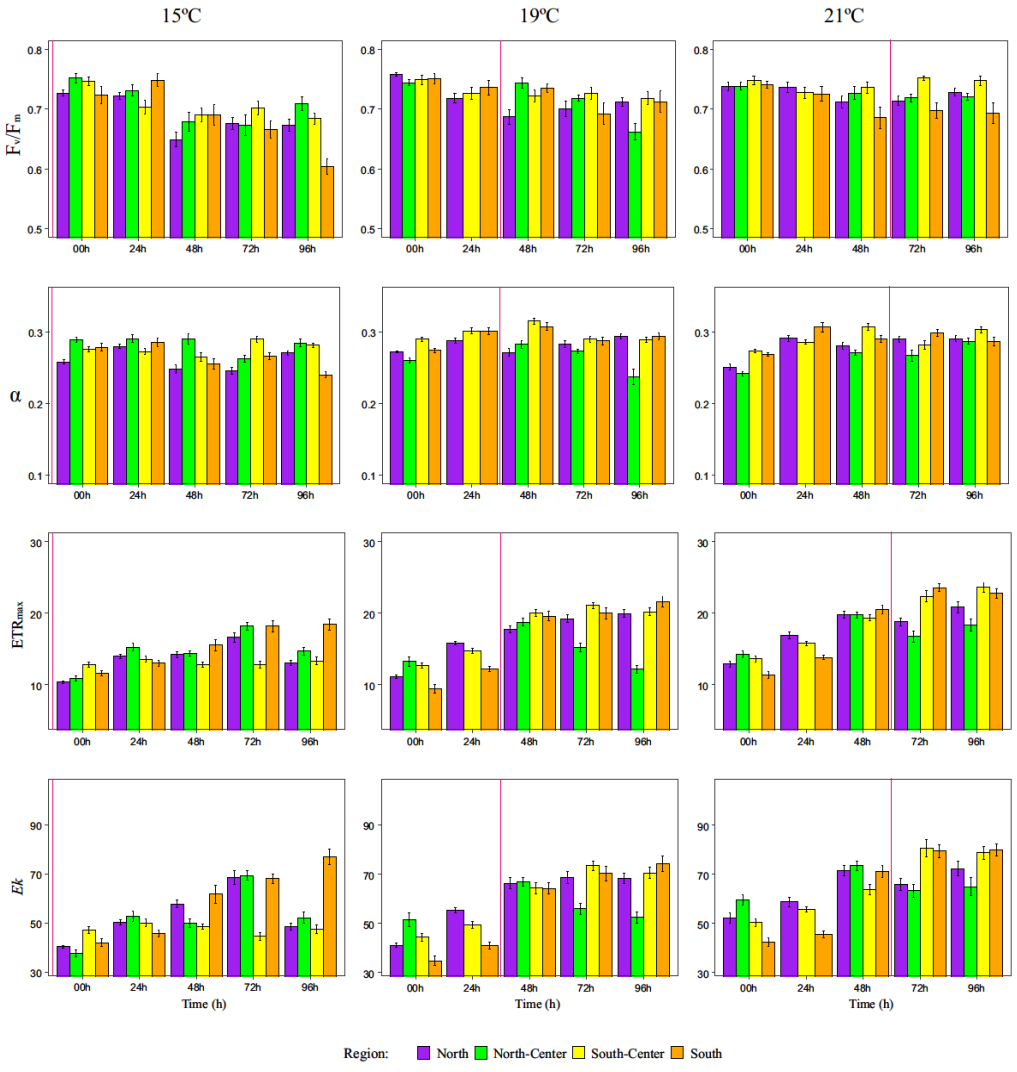



**Figure 7**: Maximum quantum yield of PSII ($F_v/F_m$), photosynthetic rate in light-limited region of RLC (α), maximum electron transport rate ($ETR_{max}$) and minimum saturating irradiance (*Ek*) mean values (± mean standard error) for 3 temperatures (15, 19, 21ºC) and 4 regions (North, North-Center, South-Center and South) of F1 juvenile sporophytes over time. Vertical red line indicates the starting point for each temperature (time point when sporophytes were at that specific temperature for 24h). Different letters indicate significant differences among regions within each sampling time (00, 24, 48, 72 and 96h) for each temperature using Tukey HSD. North-Center region data at 24h on 19 and 21ºC was removed due to values inconsistences.

*Maximum quantum efficiency of PSII ($F_v/F_m$)*

Values for $F_v/F_m$ in F1 juvenile sporophytes increased with increasing temperature as by the last sampling time (96 h) sporophytes at 21ºC had higher $F_v/F_m$ (0.725) when compared with the control (15ºC) (0.671) although this increase was only significant for the SC region ($p$ = 0.006) (Fig. 7). The interaction Region x Temperature (Table 4) was analyzed with post-hoc tests (Tukey HSD), which showed that within the same temperature, significant regional differences were only found at 21ºC, in which the S region had significant lower $F_v/F_m$ values (0.670) compared to N (0.721) and SC (0.749) regions ($p$ = 0.021 and $p$ = 0.0000042, respectively), but was not significantly different to the NC region (0.719; $p$ = 0.087).

*Light-limited photosynthetic rates (α)*

The photosynthetic efficiency of juvenile F1 sporophytes as assessed from the initial slope of ETR vs. irradiance curves ($\alpha$). With α as response variable, post-hoc tests on the interaction Region x Temperature reveled significant differences within temperature treatments, but in contrast to $F_v/F_m$ no differences were found at 21ºC (all $p > 0.252$), but only at 19ºC and 15ºC. At 15ºC α values from the N region sporophytes (0.260) were significantly lower than either the NC (0.273) or SC (0.277) regions ($p$ = 0.00043; $p$ = 0, respectively), while SC had significantly greater $\alpha$ than the S region (0.266, $p$ = 0.0097456). All regions showed a significant increase in α values at 21ºC compared with controls at 15ºC (S > 9%, $p$ = 0.001; SC > 4.9%, $p$ = 0.011; N 11.2%, $p$ = 0) except for NC region (> 1.1%, $p$ = 0.999).

*Maximum electron transport rate ($rETR_{max}$)*

An increase of $ETR_{max}$ values with temperature and also time was observed during the experiment (Fig. 7). The analysis of the interaction Region x Temperature indicated a significant increase of regional differences with increasing temperature within the same region as $ETR_{max}$ values were significantly higher at 21ºC compared to the 15ºC control (N > 33.4%; NC > 17.9%; SC 43%; S > 42%) ($p < 0.0000322$). All regional contrasts at 21ºC

differ significantly ($p < 0.044$) except for S-SC regions ($p = 0.972$) but at 15ºC significant differences were only found between NC region and the N and SC regions ($p = 0.034$ and 0.014, respectively). At 21ºC the sporophytes from S and SC regions showed significant higher $ETR_{max}$ (24.01 and 22.96, respectively) compared with NC and S regions (17.55 and 19.79, respectively) ($p < 0.004$).

*Minimum saturating irradiance (Ek)*

F1 sporophyte *Ek* values followed a similar pattern to $ETR_{max}$ as the Region x Temperature interaction pointed again to significant contrasts at higher temperatures, with tissue within each region at 21ºC showing *Ek* mean values 31% greater overall than 15ºC control (all $p \leq 0.002$). Regional differences were also higher at 21ºC as northern regions showed significantly lower *Ek* values (N = 68.8 and NC = 63.88) compared with the southern regions (SC = 79.49 and S = 82.40; all $p \leq 0.001$). No significant differences were found within the groups (N-NC, $p = 0.836$ and SC-S, $p = 0.999$). At 15ºC, a strong reduction in *Ek* was observed at 96 h, but interestingly S region sporophytes *Ek* values continued to increase, being at least 32.33% higher than the other regions.

**Table 4**: Two-way ANOVA for the effects of Region and Temperature on the maximum quantum yield of PSII ($F_v/F_m$), photosynthetic rate in light-limited region of RLC (α), maximum electron transport rate ($ETR_{max}$) and minimum saturating irradiance (*Ek*) mean values of F1 juvenile sporophytes. Significant p-values are marked in bolt.

| Variable | Factor | df | SS | MS | F | p-value |
|---|---|---|---|---|---|---|
| $F_v/F_m$ | Region | 3 | 0.033 | 0.011 | 9.577 | < **0.001** |
| | Temperature | 2 | 0.009 | 0.004 | 3.771 | **0.023** |
| | Region:Temperature | 6 | 0.024 | 0.004 | 3.502 | **0.002** |
| *α* | Region | 3 | 0.049 | 0.016 | 28.820 | < **0.001** |
| | Temperature | 2 | 0.087 | 0.043 | 76.160 | < **0.001** |
| | Region:Temperature | 6 | 0.037 | 0.006 | 10.730 | < **0.001** |
| $ETR_{max}$ | Region | 3 | 426 | 142 | 15.650 | < **0.001** |
| | Temperature | 2 | 12133 | 6066 | 668.050 | < **0.001** |
| | Region:Temperature | 6 | 1232 | 205 | 22.610 | < **0.001** |
| *Ek* | Region | 3 | 2460 | 820 | 5.216 | **0.0014** |
| | Temperature | 2 | 107682 | 53841 | 342.405 | < **0.001** |
| | Region:Temperature | 6 | 11060 | 1843 | 11.722 | < **0.001** |

## Discussion

### *Regional differences and potential for local adaptation*

The results of our common garden experiment clearly showed that populations of *M. pyrifera* at the southernmost limit of the species distribution in the northern hemisphere displayed greater thermal tolerance in their photosynthetic physiology. This suggests an important effect of the highly selective thermal conditions of the southernmost kelp habitat. This better performance of South (S) region populations was more clearly seen in the blades collected at the water surface, which were able to maintain similar maximum electron transport rate ($ETR_{max}$) values at temperatures between 15-24ºC, and in contrast to more northerly populations. The rather clear patterns of thermal tolerance in the S region surface blades was much less evident in blades from deeper waters (bottom blades), the responses of which were rather similar between regions.

Given the constraints of the experiment, in particular the short pre-acclimation period, these differences can either be attributed to intrinsic (*i.e*., genetically-determined) variation, or to plasticity induced by an environmental history of higher temperatures in southern surface waters (up to 20ºC; Fig. 4). Wave height increase might also have impacted the kelp forests of the south region as the hurricane Blanca, the second of the season, moved near the populations two weeks before our sampling campaign. In the field, the southern population (Bahia Tortugas) had a lower canopy cover (sparse distribution of individuals) and showed signs of greater stress (lower pigmentation, damaged tissue) compared with populations in more northern regions. The differences observed between the S and SC regions are all the more intriguing given that genetic analysis with microsatellites (neutral genetic markers) indicated that these two regions form a single genetic cluster (Johansson *et al*., 2015). We conclude that, while the results are consistent with local genetic adaptation, this hypothesis will require further analysis and confirmation via the transcriptomes extracted from the individuals across the experiments, particularly those derived from the F1 generation cultured *in vitro* from parental reproductive material.

Individuals from the North-Center (NC) region showed a consistently poor photosynthetic response to thermal and nutrients stressors compared with other regions using both acclimated field-collected blades (Experiment 1) and in vitro F1 sporophytes (Experiment 3). As F1 juveniles were grown in common-garden laboratory conditions, the effects of environmental history can safely be excluded (assuming no or limited carry-over across generations and life-history phases). It can therefore be concluded that the NC populations are intrinsically less able to cope with stress conditions due to genetic factors. A possible reason might be elevated levels of self-fertilization and inbreeding in NC populations. Previous studies in the Santa Barbara bay area showed that self-fertilization greatly reduced *M. pyrifera* fitness (survival to adulthood, zygote production, development of reproductive structures and fecundity) (Raimondi *et al*., 2004) . The study of Johansson *et al*., (2013) reported a high self-fertilization rate (32-44%) and strong correlation between inbreeding depression and mortality. Although this hypothesis requires an objective study and analysis at the regional level, our results suggest that reproductive strategies and constraints such as inbreeding and self-fertilization may be important factors regulating regional differences underlying local population dynamics.

The North (N) and South-Center (SC) regions adult blades showed similar intermediate photosynthetic responses to stress conditions with no significant differences between them (Table 1, Fig. 3). N region blades from both depths were in very good conditions, with almost no tissue damage or mucilage production and good pigmentation which might reflect local field conditions prior to our experiment as SW temperatures were below 15ºC in the area. The good ecophysiological conditions might have contributed to the increase of photosynthetic efficiency of surface blades (α) compared to the other regions but $F_v/F_m$ and $ETR_{max}$ were lower than the S region, suggesting greater stress on the photosynthetic apparatus. The N region kelp forests are located ~ 300 km north of the phylogeographical break of Point Sal (Johansson *et al*., 2015), 50Km north of the biogeographic break of Point Conception, a boundary characterized by a 5ºC north-south temperature gradient caused by the interaction of the cold California current with the California Eddy (Burton, 1998; Eberl *et al*., 2013). The mean surface temperature during a MHW over the last 22 years is 15.447ºC ± 0.958 in this region. This suggests that these populations are not exposed to the same level of thermal/nutrient stress experienced by the southern regions, and may putatively be less

able to cope with such stresses. Our regional analysis did not, however, show a clear latitudinal gradient of ecophysiological response of the giant kelp to thermal and nutrient stresses, which could be due to several factors: heterogeneity of oceanographic/atmospheric processes (Pacific decadal oscillation (PDO), El Niño and La Niña, California current, California Eddy, upwelling areas, internal waves) (Burton, 1998; Fram *et al*., 2008; Ladah *et al*., 2012 ; Newman *et al*., 2016; Jacox *et al*., 2016) , or, as suggested by other authors, or by more local processes that interact at a smaller (population) scales such as competition, dispersal and reproduction, wave exposure, dispersal, forest depth and density (Dayton, 1999; Edwards, 2004; Edwards & Hernández-Carmona, 2005; Cavanaugh *et al*., 2011; Cavanaugh *et al*., 2013; Johansson *et al*., 2015). Thus, in order to understand and predict possible implications of climate change on *M. pyrifera* forests on a regional level, further studies should focus on understanding the fundamental processes that act locally and how they interact with larger scale processes.

*Thermal limits*

This study showed that at 24ºC adult blades from almost all regions of *Macrocystis pyrifera* studied showed a strong decline in ecophysiological responses. However, the exception was surface blades from the southernmost region, which did not follow general trends with respect to temperature-sensitive photosynthetic parameters Ek and $rETR_{max}$. While $F_v/F_m$ and the slope of electron transport rate with increasing irradiance (α) showed a similar pattern of photosynthetic rate decrease with increasing temperature, $rETR_{max}$ and *Ek* increased from 15 to 20 ºC followed by a decrease at 24 ºC. This temperature trend is typical of rate-limited enzymatic processes in, e.g., Calvin cycle enzymes such as RuBisCO (Kirk, 2011). When temperature was increased to 24ºC the thermal optimum for photosynthesis was exceeded and the entire network was affected. The exception to this pattern was the S region surface blades in which $ETR_{max}$ an *Ek* did not differ between 15 and 24ºC, suggesting either pre-acclimation or adaptation to local conditions with warmer SW temperatures.

It appears that the thermal limits of photosynthetic performance were not exceeded in Experiment 3 using F1 juvenile sporophytes, in which 21ºC was the maximum exposure temperature. It is also possible that the use of gradual thermal ramping may have alleviated

the effects of thermal stress in this experiment (2ºC increments per 24 h up to the target temperature), compared with the direct shock used in the 1st and 2nd experiments. As a result, F1 sporophytes showed increasing $ETR_{max}$ and *Ek* with increasing temperature, particularly in the SC and S regions. We decided on this approach based on the results and observations of the last two experiments, as well as published experiments showing that juveniles under stress conditions survive less well than adults (Dean & Jacobsen, 1984), the small size and thickness of the F1 sporophytes and the necessity to avoid sampling necrotic tissue that would jeopardize further transcriptomic analysis. Although F1 sporophytes were not subjected to substantial disturbance to the photosynthetic apparatus, e.g., decline in $F_v/F_m$ at higher temperatures, it was nevertheless significant that differences in thermally-sensitive enzymatic processes (*Ek* and $rETR_{max}$) could be distinguished between S/SC (higher performance) and N/NC (lower performance) regions. However, further experiments will be required to explore the effects of higher temperatures on the upper thermal limits for photosynthetic performance and other (*e.g.*, growth) responses.

Climatology data for the months prior to our sampling showed that the central regions (NC and SC) shared similar thermal regimes, while the N region dominated by the cold California current had lower temperatures and S region was already experiencing high SW temperatures (Fig. 4). The low canopy cover of the southernmost populations may have reflected the prevailing thermal conditions, in addition to which local populations composed of juveniles and small adult sporophytes were still regrowing after they disappeared during the summer of 2014 (personal communication by the local fisherman). This information is supported by the 22 y climatology data of the S region (Fig. 5) which showed a great increase in MHW frequency in the summer of 2014 and spring of 2015. Selection pressure for acclimation/survival of individuals more adapted to higher temperatures prior to sampling is therefore clearly possible. Our data also showed an unprecedented increase frequency, duration and intensity of MHWs that for the first time in 22 years occurred for two consecutive years (2014-15) (see also Table 2); phenomena reported on the study area by several other authors (Bond *et al*., 2015 ; Jacox *et al*., 2016; Levine & Mcphaden, 2016; Reed *et al*., 2016; Cohen *et al*., 2017). While temperature itself can directly impact the vital processes of the giant kelp, including photosynthesis, SW temperature increase is inversely related to nutrients concentration and indeed has been widely used as a proxy for nutrient

concentration (Kamykowsk, 1977; Dugdale *et al*., 1989; Zimmerman & Kremer, 1984; Switzer *et al.*, 2003; Reed *et al*., 2016).

*Nutrient effects*

The nutrient treatments tested in Experiment 1 (enriched and non-enriched SW with PES) did not showed clear effects on the photosynthesis rates of the adult blades. This lack of effect was evident as the RLCs parameters and $F_v/F_m$ showed no significance on the Nutrients and no interaction was found with the other factors (Table 1). A likely reason is that even though *M. pyrifera* adult sporophyte nutrient and photosynthate storage capabilities are considered to be small (only 2-3 weeks) (Gerard, 1982) the short duration of the experiment (max. 72 h) did not result in significant depletion of reserves. Another reason might be that the nutrient concentration on the SW used on our experiment was already above non-limiting concentrations, even though it was collected from stationary reservoirs where the SW is stored for several days at relatively high temperatures. The analysis of the water sampled during the experiment 1 from each treatment was not available by the writing of this thesis but will certainly be used to clarify our results in future publications. On the other hand, significant nutrients effects and interaction with temperature were observed in experiment 2 (Table 3). Our results showed that at higher temperatures (*i.e.* 24ºC) photosynthetic rates ($F_v/F_m$) were significantly lower in the non-enriched treatments compared to the PES-enriched treatments, although no significant differences were found at 16ºC control. This illustrates a positive relationship between nutrient availability and thermal stress tolerance to warmer temperatures. Our results are in agreement with a recent study on another Kelp, *Saccharina japonica*, where nutrient availability significantly increased growth rates and survival of juveniles and adults (blade discs) at warmer temperatures (Gao *et al*., 2017). In two separate field experiments using nutrient fertilization, *M. pyrifera* responded positively to thermal stress (warm seawater periods) when nutrient availability was increased, resulting in reduction of adult canopy loss (North, 1983) and increase of juvenile survival (Hernandez-Carmona *et al*., 2001). Thus our further research will also include a non-enriched treatment (using controlled artificial SW) in order to understand the possible synergistic effect of nutrients at warm SW temperature on the F1 juvenile sporophytes.

*Depth effects*

Consistent differences in the photosynthetic rates between surface and bottom blades were found, with surface blades showing a lower mean $F_v/F_m$ and RLC parameters ($\alpha$, $rETR_{max}$ and *Ek*) but also higher variance within these values, especially at 24 ºC. Previous studies have found that the maximum photosynthetic and ETR rates of *M. pyrifera* blades decreased with depth and were mainly controlled by the vertical distribution of PAR in the water column (Colombo-Pallotta *et al*., 2006). In that study, surface blades were found to be adapted for high photosynthetic production with high values of $F_v/F_m$ and rETR and protected from the high light intensity by UV-absorbing compounds and protective carotenoids while bottom blades had lower photosynthetic rates and were responsible for the nutrient uptake. This makes sense as an adaptation to the local environmental conditions in the Baja California warmer months: a stratified water column with an oligotrophic warm surface layer that is breached by deep cold, nutrient rich waters pulsed by upwelling and internal waves events (Ladah, 2003; Ladah *et al*., 2012; Rodriguez *et al.*, 2016). In another study, old surface blades were found to have lower $F_v/F_m$ values and higher variability compared to bottom blades but also strong diurnal variability photosynthetic performance (Edwards & Kim, 2010). During our experiment, surface blades from all regions also showed lower $F_v/F_m$ values, lower pigmentation and were visibly more damaged than bottom blades. It is then safe to assume that the continuous exposure to UV, wave motion, higher temperatures and photosynthesis workload on surface blades resulted in higher cellular and external damage. The exposure to the experimental temperatures (24 ºC) above the algae thermal optimum resulted in more rapid degradation of the surface blades. On the other hand bottom blades were in much better physiological condition and showed higher stability between regions for all RLCs parameters compared to the surface blades, a pattern also reported previously for *M. pyrifera* (Edwards & Kim, 2010).

## Conclusions

Our findings suggests that populations of *M. pyrifera* from the southernmost region of the species distribution in the northern hemisphere, exhibit resilience to warmer ocean conditions compared to cooler, more northern regions. At the southern limit, higher photosynthetic rates under thermal stress were observed in field sampled adult blades acclimated and tested under common garden conditions. Importantly, with respect to mainly enzyme-limited photosynthetic processes ($rETR_{max}$, $Ek$), greater tolerance was seen *in vitro* F1 juveniles from the S and SC regions, suggesting that populations in these regions are locally adapted to the prevailing warmer ocean conditions. Despite the greater resilience to thermal stress, observed increasing frequency and intensity of MHWs and other disturbances such as hurricanes might result in a cumulative effect on the *M. pyrifera* forests, reducing the overall resilience of the ecosystem (Wernberg *et al*., 2010).

We also observed a generally poor photosynthetic response to thermal stress in the NC region, which also carried over to the F1 generation. Climatological data showed a thermal regime in the NC region around Santa Barbara to be at least as warm as the SC area of Baja California. Therefore our experimental observations suggest some underlying genetic basis leading to reduced fitness (e.g., possible inbreeding; Raimondi *et al.,* 2004) in this region, which forms a distinct genetic group (Johansson *et al.,* 2015).

Our experiments also demonstrated that thermal stress tolerance of *M. pyrifera* might be enhanced by nutrient availability and highlights the need for future studies on this subject. Finally, the use of excised blades as a valid surrogate for entire individuals was verified for *M. pyrifera* juveniles, at least for short experimental periods (24-72h). Our further research will focus the analysis of the transcriptomes extracted from the adult blades and F1 juveniles in order to evaluate putative regional local adaptation and assess the synergy of nutrients and temperature stress on F1 sporophytes.

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## Annexes

Annex I: Sampling sites (populations) and coordinates per region

| Region | Population name | Coordinates |
|---|---|---|
| North | Mitchell's Cove | 36° 56.896' N 122°02.066' W |
| | Pleasure Point | 36° 57.094' N 121°58.723' W |
| North-Center | Santa Barbara 1 | 34° 24.345' N 119° 52.301' W |
| | Santa Barbara 2 | 34° 23.469' N 119° 32.515' W |
| South-Center | Campo Kennedy | 31°42'00.5"N 116°40'52.7"W |
| | Arbolitos | 31°42'46.8"N 116°42'25.6"W |
| South | Punta Eugenia | 27°50'58.3"N 115°04'57.6"W |
| | Bahia Tortugas | 27°39'51.7"N 114°54'14.4"W |

**Annex II:** Tukey Honest Significant Differences (HSD) of generalized linear models (GLMs) comparison between the interaction Temperature x Nutrients reported on Table 3 on the variables maximum quantum yield of PSII ($F_v/F_m$) on the 2nd experiment and mean $F_v/F_m$ values of contrasts. Significant p-values are marked in bolt.

| Contrasts | | | | | | | |
|---|---|---|---|---|---|---|---|
| 1 | 2 | diff | lower | upper | *p-value* | Mean $F_v/F_m$ 1 | Mean $F_v/F_m$ 2 |
| 24ºC Enriched | x 16ºC Enriched | -0.02142 | -0.03377 | -0.00907 | **5.69E-05** | 0.727 | 0.762 |
| 16ºC Non-enriched | x 16ºC Enriched | -0.00246 | -0.01027 | 0.00534 9 | 0.848562 | 0.758 | 0.762 |
| 24ºC Non-enriched | x 16ºC Enriched | -0.0561 | -0.06845 | -0.04375 | **0** | 0.674 | 0.762 |
| 16ºC Non-enriched | x 24ºC Enriched | 0.018959 | 0.00660 7 | 0.03131 | **0.000503** | 0.758 | 0.727 |
| 24ºC Non-enriched | x 24ºC Enriched | -0.03468 | -0.0503 | -0.01906 | **1E-07** | 0.674 | 0.727 |
| 24ºC Non-enriched | x 16ºC Non-enriched | -0.05364 | -0.06599 | -0.04129 | **0** | 0.674 | 0.758 |