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BARCELONA

**Seagrass responses to climate change:
effects of warming and the interaction
with local stressors**

**Respuestas de las angiospermas marinas al cambio climático:
efectos del calentamiento y la interacción
con estresores locales**

Yaiza Ontoria Gómez



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Yaiza Ontoria Gómez
2020



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Seagrass responses to climate change



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Ontoria, Y. Seagrass responses to climate change: effects of warming and the interaction with local stressors. PhD Thesis. Universitat de Barcelona, Barcelona. Spain.

Cover design: Israel Cascón

TESIS DOCTORAL



UNIVERSITAT DE
BARCELONA

Departamento de Biología Evolutiva, Ecología y Ciencias Ambientales
Doctorado en Ecología, Ciencias Ambientales y Fisiología Vegetal

Seagrass responses to climate change: effects of warming and the interaction with local stressors

Respuestas de las angiospermas marinas al cambio climático:
efectos del calentamiento y la interacción con estresores locales

Memoria presentada por **Yaiza Ontoria Gómez** para optar al Grado de Doctora por la
Universidad de Barcelona

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Departamento de Biología Evolutiva, Ecología y Ciencias Ambientales
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A mi familia

A mi abuela, mi balcón al mar

*Parli'm d'aquells móns llunyans,
de les espècies per catalogar
que ningú mai ha vist abans.
Porti'm a aquells mars remots,
on els indicadors de profunditat
diuen que és de valents baixar.
Un viatge fragmentat. Un fascicle setmanal.
Sóc l'home que busca.
Perquè sempre he volgut ser part d'una tripulació.
Perquè no hi ha color si em fa dir què vull ser de gran:
Jo, Jacques Cousteau.
Mil balenes a tocar.
Sentir l'electrostàtica i el mar.
Calypso ve, Calypso va.
Ser-hi sense haver-hi estat.
Veure els colors dels esculls de corall.
Vostè escafandre, jo xandall
confiï amb mi, anem més avall!
Que amb aquest comandament puc eternitzar el moment
o puc tornar enrere.
Perquè sempre he volgut ser part d'una tripulació.
Que jo amb vostè vull anar tan lluny, tan lluny que no hi arribi la ficció.
Perquè sempre he volgut ser part d'una tripulació.
Perquè hi ha coses noves sota el sol que esperen un explorador.
Monsieur Cousteau, per què a vostè els taurons no li fan por?
Monsieur Cousteau, a mi no em cal l'Alta Definició.
Monsieur Cousteau, com més avall te'n vas, hi ha més pressió.
Monsieur Cousteau, creuant l'oceà des d'una habitació.
Monsieur Cousteau, la mare em crida des del menjador.*

“Monsieur Cousteau” (Els Amics de les Arts)

AGRADECIMIENTOS

Es el momento de echar la vista atrás. Más de 4 años han pasado desde ese 1 de Mayo de 2015, día en el que comencé el doctorado. Ecología marina, ¡qué bonito! Plantas marinas ¡ala, vas a bucear! Cambio climático ¡es la moda! Sí. El doctorado era todo eso, sí. Y mucho más. Ni por asomo podía imaginarme lo que me deparaban esos próximos años: amigos, risas, curiosidad, motivación, búsqueda bibliográfica, inmersiones, experimentos, datos, R, más datos, hoja en blanco, miedo, revisiones, congresos, viajes, estancias, submission, sueño, deadlines, paper rejected, frustración, dudas, superación, paper accepted, lágrimas, satisfacción... En definitiva, un largo camino lleno de subidas, bajadas, obstáculos superados y la culminante llegada a la cima. Una cima que ha sido alcanzada gracias al apoyo de cada uno de vosotros.

En primer lugar, gracias a los directores y tutor (¡tercer director realmentel!) de esta tesis, Marta Pérez, Juanma Ruiz y Javier Romero. Por abrirme las puertas y darme la oportunidad de realizar el tan perseguido doctorado. Por vuestra confianza, acompañamiento y paciencia infinita. Por cada una de las inolvidables campañas, con sus momentos dentro y fuera del agua, los “encierros” en Fonolleres y los paseos en barca a las Medas buscando inspiración. Gracias también por las innumerables versiones (de ida y vuelta) de cada uno de los trabajos, los tropecientos colores en cada word.doc, y todas las cosas “*a comentar*” resaltadas en amarillo chillón...todo eso que me ha ayudado a crecer. Juanma, gracias por llevarme a tierra “sana” y salva después de perder el ancla y el equilibrio en Cabo Tiñoso. Marta, por tu cercanía, comprensión y por poner orden. Y, por supuesto, por involucrar (véase también *enredar*, *liar*) a quien no podía no formar parte de todo esto. Javier, gracias por aceptar tan tentadora invitación y no arrepentirte nunca (...) (¡que lo sé yo!). Por cada una de tus palabras que he tenido que buscar en Google (y que seguramente ahora tendría que volver a buscar...), por atender las llamadas de auxilio los viernes a las 15h y por tu empujón para hacerme ir siempre hacia delante y jamás pa’trá.

Gracias a los miembros que forman la comisión de seguimiento de esta tesis, Esperança Gacia, Teresa Alcoverro y José Luis Sánchez Lizaso, por vuestros siempre constructivos comentarios y por la buena coordinación.

A Neus. Perfecta compañera y mejor amiga. Por hacer fácil lo difícil y posible lo imposible. Por enseñarme a crecer, a enfrentarme y a superar. Por haber sido imprescindible en este camino y por traer de vuelta a Peter Pan.

A los Jordis. Gracias por llegar y llenar de motivación, alegría, fuerza y energía el despacho. Por vuestro ánimo y constante disposición a echar una mano y por aguantar y entender los momentos más complicados de la recta final. Moltès gràcies als dos! A Aurora, quien me acogió con los brazos abiertos y ha estado presente siempre, a pesar de haber saltado al otro lado del charco. Gracias por tus consejos y por demostrar que sí se puede.

A Dani, Alba y Marta. Mis compañeros de vagón de la montaña rusa a la que nos hemos subido. Mil gracias por cada uno de los momentos vividos, por las alegrías, las risas, los cafés,

las penas, penurias y las remontadas. Por no rendiros y por llegar juntos hasta aquí. Ahora, a por el “Chapter 6”. A cada uno de los compañeros del departamento que han formado parte de esta aventura: Graciela, Ignasi, Myrto, Pau, Eneko, Pol T, Pol C, María..., gracias por estar cada día y por todos los momentos juntos. A Luisa, por ser la alegría en estado puro, por tu confianza, tu locura y tu fuerza. A Quentin, por haber sufrido las agonías no de uno, sino de dos doctorandos, y sobrevivir para poder contarlo.

A mis murcianicos y murcianicas. Al los *Posidonios*, Jaime, José Miguel, Lázaro, Arantxa, Maridol, Judith, Rocío y al pequeño Unai. A Marina, Mara, Sole e Irene. Y, por supuesto, a Tania, un gran descubrimiento. A todos vosotros, gracias por recibirme con los brazos abiertos en las tantas visitas y por acogerme durante las estancias más largas. Jornadas infinitas en el IEO, idas y venidas a la una, dos, tres...de la mañana para los encuentros nocturnos con el rotavapor, semanas de siete días laborables y fuego cayendo del cielo en pleno Julio. Gracias por ponerle sonrisas, alegría y buen humor a cada uno de aquellos días y hacer que tal rutina se afrontase con mil ganas. Y, en definitiva, por hacer tan fácil la convivencia y por vuestra amistad, jacho pijo, qué buenos momentos!

I am very grateful to my Australian family. To all the people I met in Perth during my first international internship. Thank you Kathryn McMahon for giving me the chance to work with your research group and to develop research that has risen to be a part of this thesis. Thanks also for your closeness, confidence and your fostering. Thanks to the seagrass girls, Caitlyn, Nicole, Sian, Marta and Simone, for welcoming me, for always being willing to help me and for make me feeling part of your team. Endless thanks to Chanelle (Zimbabwina), my soul sister from the other side of the world. Thanks for being part of this journey, for your kindness and hospitality since the first day we met, for all your help and unconditional support. I could not imagine that such a little person would become “the biggest problem of my life”. I will never forget our reunions at the beach, looking for the meaning of life while enjoying our plastic cups of wine, neither we as being “weird”. PhD done. What do you reckon mate? I am also very grateful to Paul Lavery, Casper, Emily, Anna, Oscar, Gloria...and all the ECU people who were a part of my time there. Thanks Trish, “darling”, for being my maternal figure in Perth. Gracias a Iñigo, Joi y Elena, mi familia en Australia. Mil gracias por vuestra acogida, vuestro cariño y verdadera amistad. Me hicisteis sentir como en casa desde el primer día, como una más de la familia. Gracias de corazón por hacerlo todo tan fácil. Familia bonita allá donde las haya.

I would like to thank Catherine Collier, Sven Uthike and Andrew Negri for giving me the chance to work at AIMS and develop an experiment that, although not included in this thesis, it is expected to be published in the future. Thanks to Florita and Frances for all your help in the field and lab.

A Dorle, por estos más de 4 años de convivencia y por unos cuantos más de amistad (¡he perdido la cuenta!). Por hacerlo todo tan fácil y por ser mi ejemplo a seguir de esfuerzo y constancia. Y cómo no, por compartir esas tardes de sábado de manta, secuestros y asesinatos.

A mis *gartibles*, que, a pesar de la distancia, hacéis que os sienta cerca y habéis contribuido a que llegue hasta aquí. Gracias Jojo, Tania, Alba, Vir y Celia, por creer en mí, apoyarme y no

dejarme caer en los momentos más complicados. Por las llamadas llenas de risas, alegrías, llantos, gritos y desahogos. Por los ánimos en forma de canción, por la recarga de pilas con visitas y escapadas exprés y por los rincones que hemos descubierto juntos. Por compartir coloretos y por estar siempre.

A mis incondicionales, Tamara, Lidia, Raquel, Miguel, Elena, Isra, Carol y Sara. Por apoyarme tras la decisión un poco loca de hacer un doctorado, por intentar entender mi trabajo (aunque algunos aún creéis que trabajo con algas..., ¡os lo perdono!), y por estar en los buenos y en los malos momentos. Gracias por las visitas, siempre con chute de energía y que sabían a poco. Por comprender el enclaustramiento de los últimos meses buscando la luz al final del túnel y por ayudarme a encontrarla. Gracias por ser como sois y por el empujón para el sprint final.

A mi familia. A mis padres, por creer en mí, por animarme a perseguir mis sueños (por locos que parezcan) y por haberme enseñado que rendirse es de cobardes. Por hacerme sentir siempre cerca, por tender vuestra mano para sujetar la toalla y evitar que la tirase en los momentos más críticos. Por inculcarme que la constancia es la clave del éxito. A mi hermano, Raúl, y a Patricia, por hacerme la tía más feliz del mundo y por todo vuestro apoyo. A Lucas y Jorge, mis personitas favoritas. Por llegar y revolucionar, por vuestra inagotable energía y por ser mi constante motivación. Os quiero hasta el infinito y más allá.

ADVISORS' REPORT

Dra. Marta Pérez Vallmitjana, professor at the Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals (Universitat de Barcelona), Dr. Juan Manuel Ruiz Fernández, research professor at the Instituto Español de Oceanografía, advisors, and Javier Romero Martinengo, professor at the Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals (Universitat de Barcelona), tutor of the PhD thesis entitled “Seagrass responses to climate change: effects of warming and the interaction with local stressors”,

INFORM, that the research studies developed by Yaiza Ontoria for her Doctoral Thesis have been organized in four chapters, which correspond to four scientific papers listed below (two published, two intended to be submitted in the next months), plus a general Introduction and a general Discussion;

and CERTIFY, that the work has been carried out by Yaiza Ontoria, participating actively in all the tasks: setting the objectives, conceiving and performing the analyses, executing the experiments, handling and analyzing the results and writing the manuscripts.

Finally, we certify that the co-authors of the publications listed below that conform this doctoral thesis, will not use them in another PhD thesis.

Barcelona, 5th December 2019

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List and publication status of the chapters of this thesis

Part of the results of this PhD thesis have been published in indexed international journals:

Chapter 1: Ontoria, Y.¹, Bernardeau-Esteller, J.², Marín-Guirao, L.², Sandoval-Gil, JM.³, García-Muñoz, R.², Pérez, M.¹, Romero, J.¹, Ruiz, JM.² Seagrass species with contrasting ecological strategies reveal differential tolerance to warming. (In preparation).

Chapter 2: Ontoria, Y.¹, Gonzalez-Guedes, E.¹, Sanmartí, N.¹, Bernardeau-Esteller, J.², Ruiz, JM.², Romero, J.¹, Pérez, M.¹ (2019). Interactive effects of global warming and eutrophication on a fast-growing Mediterranean seagrass. *Marine Environmental Research*, 145, 27-38. Impact factor: 3.445

Chapter 3: Ontoria, Y.¹, Cuesta-Gracia, A.¹, Ruiz, JM.², Romero, J.¹, Pérez, M.¹ (2019). The negative effects of short-term extreme thermal events on the seagrass *Posidonia oceanica* are exacerbated by ammonium. *PLoS ONE*, 14 (9), e0222798. Impact factor: 2.776

Chapter 4: Ontoria, Y.¹, Webster, C.⁴, Said, N.⁴, Ruiz, JM.², Pérez, M.¹, Romero, J.¹, McMahon K.⁴ High salinities buffer the negative effects of warming on functional traits of the seagrass *Halophila ovalis*. (In preparation).

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In addition, the results of this thesis have been presented at international and national conferences (12th International Seagrass Biology Workshop, Gwynedd, Wales, UK, 2016; 13th International Seagrass Biology Workshop, Singapore, 2018; I Congreso de Jóvenes Investigadores del Mar, Cádiz, Spain, 2018).

Funding sources of this PhD thesis

For the completion of this PhD thesis, I have been awarded a postgraduate scholarship (FPI) of the Ministry of Economy and Competitiveness (BES-2014-069593), in the framework of the project of R+D+i “Resilience of seagrass meadows to global warming: an analysis based on ecophysiological, population and ecosystem responses” (RECCAM, CTM2013-48027-C3-1/2-R). Moreover, part of the work presented in this thesis has been funded by the project of R+D+I “Benthic marine vegetation responses to stress: critical transitions, resilience and management opportunities” (UMBRAL, CTM2017-86695-C3-1-R, Ministry of Economy and Competitiveness).

In addition, I have been awarded two grants for short-term internships in two research centers (EEBB-I-17-12572 and EEBB-I-18-13047), both in Australia. A first internship was at the Edith Cowan University and a second one at the Australian Institute of Marine Sciences in collaboration with the James Cook University.

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ABSTRACT

Coastal ecosystems are highly threatened worldwide by multiple anthropogenic stressors that act at a range of spatial scales, from local to the global, and adversely affect their ecological functions and associated biodiversity. Global warming is one of the most pervasive stressors, and the assessment of how the species (or other levels of biological organization) react to it is an urgent need in a rapidly warming world. Moreover, thermal stress rarely acts in isolation from other stressors. The potential synergies among global warming and local stressors is of particular concern in what it regards foundation species, such as the case of seagrasses, due to their crucial role in maintaining the structure and function of coastal ecosystems. The main objective of this PhD thesis is to improve the knowledge of how warming alone and in combination with different local factors can affect seagrasses. This research has been conducted based upon mesocosm experiments submitted to different temperatures (and, in some cases, to other agents), and plants responses measured from biochemical to population levels. The results obtained are considered an approach to what may occur in the real world, always acknowledging the limitations of our methodology.

Chapter 1 revealed different tolerance to warming among the two main Mediterranean seagrass species, *Cymodocea nodosa* and *Posidonia oceanica*. *C. nodosa* tolerates temperature increases much better than *P. oceanica* probably due to its life story (opportunistic), habitat (from confined waters to the open sea) and biogeographical affinity (tropical and subtropical). This will potentially cause changes in the distribution area of these two species in the Mediterranean under a future scenario of warming.

As shown in Chapter 2, an increase in nutrient concentration in water does not modify the response of *C. nodosa* to warming. However, the increase of organic matter in sediment clearly worsens, synergistically in some plant traits, the effects of warming, entailing a hazardous combination for plant survival. *P. oceanica*, in turn, is severely affected by conditions of high nutrient content and high temperatures (Chapter 3), again displaying synergistic effects, and confirming not only a thermal sensitivity in this species greater than in *C. nodosa*, but also a greater vulnerability to the exacerbation of thermal effects by other local stressors.

Finally, the interactive effects of warming and salinity (Chapter 4) in an estuarine seagrass species, *Halophila ovalis*, in southwestern Australia resulted beneficial for plant survival, as the negative effect of warming was buffered by concomitant salinity increases.

Overall, this research highlights the complexity of global warming effects in at least two aspects. Firstly, the multiplicity of biological levels at which those effects act and, secondly, the importance of studying not only isolated effects of temperature increases but also their joint effect with other stressors. Advances in these two directions will yield more realistic predictions concerning global warming and seagrass ecosystems and help to develop management policies to protect seagrass ecosystems in a changing world.

RESUMEN

Los ecosistemas costeros están altamente amenazados en todo el mundo por múltiples factores estresantes antropogénicos que actúan en un rango de escalas espaciales, desde lo local a lo global, y afectan negativamente sus funciones ecológicas y la biodiversidad asociada. El calentamiento global es uno de los factores estresantes más generalizados, y la evaluación de cómo reaccionan las especies (u otros niveles de organización biológica) es una necesidad urgente en un mundo en rápido calentamiento. Además, el estrés térmico rara vez actúa aislado de otros factores estresantes. Las posibles sinergias entre el calentamiento global y los estresores locales son motivo de especial preocupación en lo que respecta a las especies fundadoras de hábitat, como el caso de las angiospermas marinas, debido a su papel crucial en el mantenimiento de la estructura y función de los ecosistemas costeros. El objetivo principal de esta tesis doctoral es enriquecer el conocimiento de cómo el calentamiento, solo y en combinación con diferentes factores locales, puede afectar a las angiospermas marinas. Esta investigación se ha llevado a cabo en base a experimentos de mesocosmos sometidos a diferentes temperaturas (y, en algunos casos, a otros agentes), y a las respuestas de las plantas medidas desde el nivel bioquímico hasta el nivel de población. Los resultados obtenidos se consideran una aproximación de lo que puede ocurrir en el mundo real, siempre reconociendo las limitaciones de nuestra metodología.

El capítulo 1 reveló una tolerancia al calentamiento diferente entre las dos principales especies de angiospermas marinas del Mediterráneo, *Cymodocea nodosa* y *Posidonia oceanica*. *C. nodosa* tolera los aumentos de temperatura mucho mejor que *P. oceanica*, probablemente debido a su historia de vida (oportunista), hábitat (desde aguas confinadas hasta mar abierto) y afinidad biogeográfica (tropical y subtropical). Potencialmente, esto provocará cambios en el área de distribución de estas dos especies en el Mediterráneo en un escenario futuro de calentamiento.

Como se muestra en el capítulo 2, un aumento en la concentración de nutrientes en el agua no modifica la respuesta de *C. nodosa* al calentamiento. Sin embargo, el aumento de la materia orgánica en el sedimento empeora claramente, sinérgicamente en algunos rasgos de la planta, los efectos del calentamiento, lo que supone una combinación peligrosa para la supervivencia de la planta. *P. oceanica*, a su vez, se ve gravemente afectada por condiciones de alto contenido de nutrientes y altas temperaturas (capítulo 3), nuevamente mostrando efectos sinérgicos y confirmando no solo una sensibilidad térmica en esta especie mayor que en *C. nodosa*, sino también una mayor vulnerabilidad a la exacerbación de los efectos térmicos por otros estresores locales.

Finalmente, los efectos interactivos del calentamiento y la salinidad (capítulo 4) en una especie de angiosperma marina de estuarios, *Halophila ovalis*, en el suroeste de Australia resultaron beneficiosos para la supervivencia de las plantas, ya que el efecto negativo del calentamiento fue amortiguado por el aumento simultáneo de la salinidad.

En general, esta investigación destaca la complejidad de los efectos del calentamiento global en al menos dos aspectos. En primer lugar, la multitud de niveles biológicos en los que actúan

esos efectos y, en segundo lugar, la importancia de estudiar no solo los efectos aislados de la temperatura, sino también su efecto conjunto con otros factores estresantes. Los avances en estas dos direcciones generarán predicciones más realistas sobre el calentamiento global y los ecosistemas de angiospermas marinas y ayudarán a desarrollar políticas de gestión para protegerlos en un mundo cambiante.

GENERAL INTRODUCTION

A THREATENED WORLD

Paraphrasing an expression that has become popular, we would say: “Global change is coming!”. However, it would be much more realistic to state: “Global change has come!”.

We live in a world where humans have profound impacts on the global environment, and our activities have modified almost every part of the planet. Human population growth, increased resource consumption, land use changes, pollution, and energy use are drivers of global change, which impacts equally ecological systems and human societies. Almost 7 billion people live at present on Earth, and world population is expected to rise to 9 billions by 2050. The rapid population growth and the increased demand for natural resources such as crops, seafood, energy, wood, minerals and other raw materials are at the base of global environmental change. Landscapes are being modified worldwide as natural land covers (grasslands, forest, wetlands) have been converted to areas dominated by human activities, that is, agriculture, forestry, cities, infrastructures, among many others. Those land use changes cause the retraction of natural habitats, with a plethora of detrimental effects for biodiversity, ecosystem function and the abiotic environment, including the release of greenhouse gases to the atmosphere. Rising pollution, mainly attributed to the increased use of petroleum and the development of new synthetic products such as plastics, solvents, pesticides and other chemicals are other consequences of human activities. In addition, the environment has to face urban waste disposal and runoff from agriculture, causing eutrophication in rivers, lakes and coastal waters (Alcamo, 2002; Galloway, 2001) or the increasing amount of plastics washed into rivers and oceans (Ryan 2015; Schmidt et al., 2017).

Such scenario has been a matter of concern for scientists since at least the first half of the 20th century, and this concern has been ever growing since then. In the last decades, and additional threat has been incorporated to the long list outlined above. Although with historical background rooted in the beginning of the industrial revolution, the release of greenhouse gases to the atmosphere has come into the scene as one of the hot topics in environmental science. Nowadays, most of the worldwide energy production relies on the fossil fuels, accounting for 85 % of all energy used. The burning of fossil fuels is the largest source of emissions of CO₂ (and other greenhouse effect gases) into the atmosphere (about 8.5 billion tons each year), to which other human sources should be added (livestock, land use changes, rice culture...). The accumulation of such gases has reached levels that are unprecedented in at least the last 800.000 years (IPCC, 2014), causing a clear and significant increase in global temperature which, in turn, has resulted in severe drought and floods, heat waves, seawater acidification, ocean warming, reduction on the amounts of snow and ice and sea level rise, among other undesirable consequences. If the burning of fossil fuels continues at current rates, global temperatures are predicted to increase by 4 °C by the year 2100 (IPCC 2007).

THE BIG GLOBAL THREAT: GLOBAL WARMING

While it is difficult to rank in order of importance the different threats outlined above, the plethora of consequences of climate change, and among them global warming (on which we

are going to focus our attention), are probably among those causing a greatest concern, due to their pervasiveness, their harmfulness and their persistence in time.

Global warming has, in fact, two faces. On the one hand, we have a progressive thermal increase that affects, to a greater or lesser extent, all natural environments on Earth, and has been taking place, probably, since at least the second half of the 19th century. On the other hand, and maybe less known by the general public, we have the increasing frequency of discrete, extreme thermal events usually known as heat waves, understood as sporadic episodes (from days to a few weeks) in which temperature is well above the average in that time of the year. Despite confusing claims with a clear ideological bias, both aspects are not pessimistic predictions, but an increasingly measurable reality. In effect, not only air and sea surface average temperatures have risen by 0.4 - 0.8 °C in the past century (IPCC 2001) and are expected to rise between 1.1 and 6.4 °C by the end of 21st century (Figure 1), but also the intensity and frequency of heatwaves have increased (see below) and are expected to further increase (Collins et al., 2013; IPCC 2007).

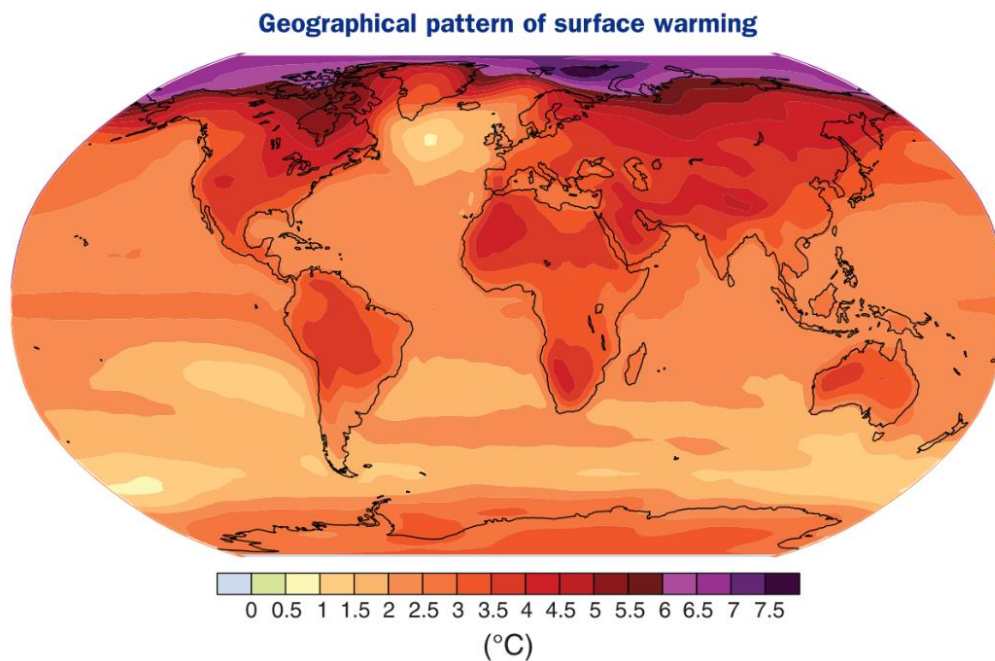


Figure 1. Projected surface temperature changes for the late 21st century (2090-2099) (IPCC 2007).

Heatwaves are important climatic extreme events in both atmospheric and oceanic systems that have potential consequences, from deleterious to devastating, for human health, economies and the environment. In oceanic systems, marine heatwaves are defined as “discrete, prolonged anomalously warm-water events at particular location”; in other words, periods when daily sea-surface temperature exceeds a local seasonal threshold for at least five consecutive days (Hobday et al., 2016). Over the last century, specifically from 1925 to 2016, marine heatwaves have increased significantly in frequency and duration (34 % and 17 %, respectively), which results in a 54 % increase in annual marine heatwave days globally (Oliver et al., 2018) (Figure 2a, 2b). This has been associated with ecological alterations such as shifts in species’ distribution or changes in biodiversity patterns (Bond et al., 2015; Cavole et al., 2016; Garrabou et al., 2009; Wernberg et al., 2016) (Figure 2c, 2e), as well as with massive

mortalities of sessile benthic invertebrates, some of them being foundation species (*sensu* Dayton 1972, see below). Overall, there is a serious risk of global warming disrupting the integrity of ocean ecosystems and curtailing the goods and services they provide.

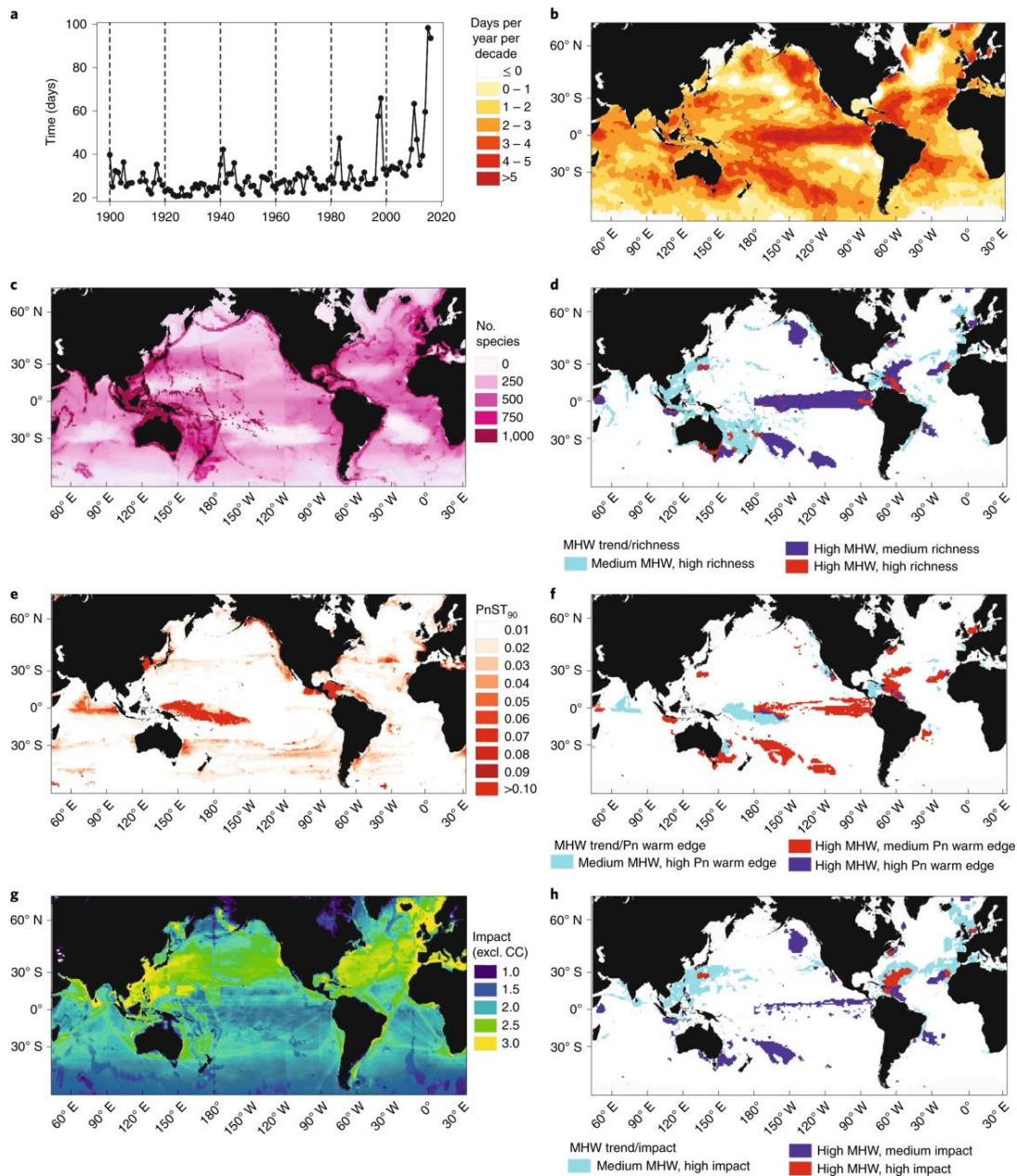


Figure 2. Global patterns of MHW intensification, marine biodiversity, proportions of species found at their warm range-edge, and concurrent human impacts. **a,b**, Globally averaged time series of the annual number of MHW days and trends in the annual number of MHW days (in the periods 1925–1954 and 1987–2016) across the global ocean. **c,e,g**, Existing data on marine biodiversity (**c**), the proportion of species within the local species pool found near their warm range edge (**e**) and non-climatic human stressors (**g**), were combined with trends in the annual number of MHW days (**b**). **d,f,h**, The resultant bivariate maps identify regions of high diversity value that may be affected by MHWs (**d**), high thermal sensitivity of species that may have been particularly vulnerable to increased MHWs (**f**) and high levels of non-climatic human stressors where MHW intensification has affected concurrently on marine ecosystems (**h**). Pn, proportion; PnST₉₀, proportion of species beyond 90% species thermal range; excl. CC, excluding climate change. (Smale et al., 2019).

CHANGES IN THE ENVIRONMENT

Stressful factors and their combined effects

The action of human activities on ecosystems is termed with different names (pressures, impacts, stressors, disturbances...), which, despite pointing to the same direction, are associated to slightly different concepts. To discuss these concepts in deep is beyond the scope of this introduction, and, for the sake of simplicity, we will consider warming as a stress. A **stressful factor** or, simply, **stressor** is “*an abiotic or biotic variable that exceeds its range of normal variation, and adversely affects individual physiology or population performance in a statistically significant way*” (Barret et al., 1976; Auerbach 1981). The stressors can be either natural or anthropogenic and can be classified as local or global, depending on the spatial extent of their causes and/or action. While the separation among local and global stressors is, at first sight, sharp and clear, its application becomes confuse when, for example, a given pollutant whose concentration has increased locally (local stressor) is transported and distributed (by currents, winds...) worldwide (global stressor). Beyond these subtleties, it should be taken into account that the wider the spatial extent of an stressor origin and effects, the harder becomes its management, as it usually requires collaboration among countries, not always with common interests or priorities.

Indeed, the concurrence of multiple stressors at the same time is a common situation and then opens the door for synergistic interactions worsening the effects of the stressors in isolation, and jeopardizing not only the biological processes and ecosystems functions but also the global biodiversity (Brook et al., 2008; Folke et al., 2004; Paine et al., 1998).

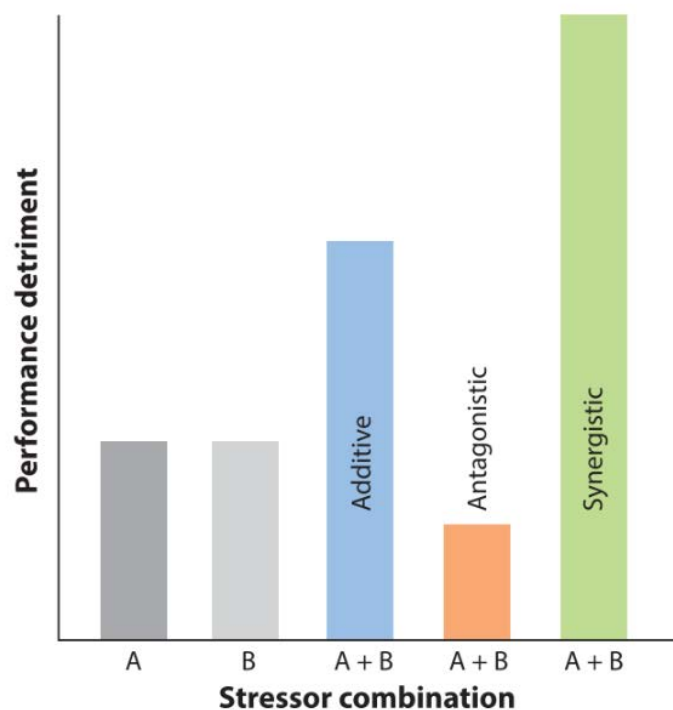


Figure 3. Conceptual diagram of possible effects of multiple stressors (Gunderson et al., 2016).

The impossibility of predicting consequences for the natural world based on evidence from a single stressor (Vinebrooke et al., 2004) or even in a simple additive model including several stressors, due to the abovementioned synergies, justifies the growing interest and the urgent need to better understand the combined effect of multiple stressors. At first glance, it is fundamental to identify and define the different ways in which different stressors can interact. Two-three possibilities are considered at this respect, following the different authors: additive effect (the joint effect is the sum of the effects of both stressors in isolation), synergy (the combined effect is greater than the additive effects) or antagonism (the combined effect is lower than the additive effects) (Todgham and Stillman, 2013) (Figure 3). For some authors, when additivity occurs there is no interaction, while the term “interactive effects” is reserved to either synergy or antagonism.

Cascading effects across different levels of biological organization

Warming affects the living world at all levels of biological organization, from subindividual (molecular, biochemical...) through individual level through populations and communities to biomes and to the biosphere as a whole (Figure 4, referred to climate change; Bellard et al., 2012). A thermal increase can alter gene expression, biochemical reactions or physiological mechanisms, as well as modify population dynamics, biotic interactions or the effects of abiotic factors. It can also influence species distributions, altering thus landscapes and biomes (Koh et al., 2004; Leadley et al., 2010; Sala et al., 2005; Walther 2010; Yang and Rudolf, 2010). All across those biological organization levels, downscaling and upscaling effects occurs.

At the species level, when a potentially detrimental environmental change occurs (e.g. warming) the species fitness decreases, due to the impairment of relevant physiological or ecological capacities (growth, food intake, reproductive output...), eventually leading to local or, in the worst case, global extinction (Chevin et al., 2010). Species extinction can be thus one of the drastic consequences of global warming, although it is important to note that only relative few species became extinct during the Quaternary period (Botkin et al., 2007). Much more often, and at least to some extent, species have mechanisms to mitigate the potential negative effects of environmental changes, through adaptive responses, which are either due to micro-evolution (that is, through selection of existing or new genotypes) or to phenotypic plasticity, that allows a very short-term response, involving the expression of physiological, morphological or behavioral traits counteracting the potential fitness loss. Overall, species can change in three different (but not mutually exclusive) ways: spatially (moving to areas with appropriate conditions), temporally (adjusting life cycle events to match the new climatic conditions) and in themselves (physiological and behavioral changes) (Bellard et al., 2012).

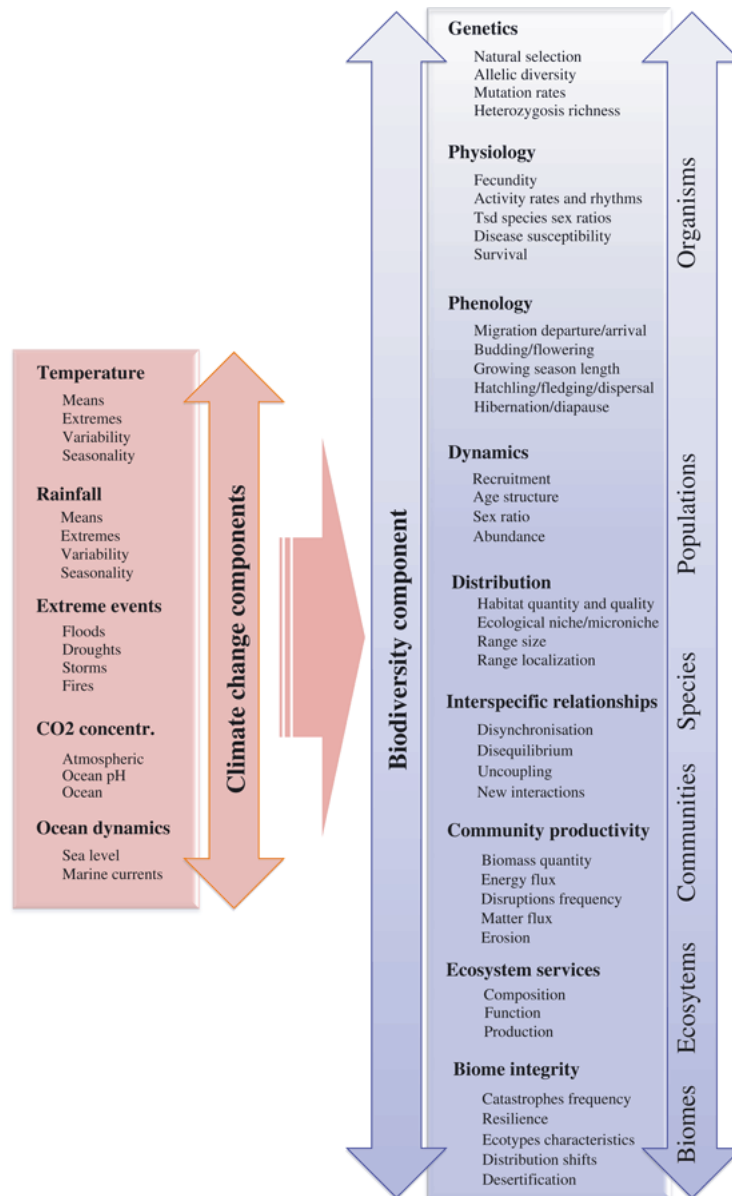


Figure 4. Summary of some of the predicted aspects of climate change and some examples of their likely effects on different levels of biodiversity (Bellard et al., 2012).

COASTAL ECOSYSTEMS

Importance, pressures and current status

Coastal marine systems are among the most ecologically and socio-economically valuable of our planet. Just as an example, and without putting too much emphasis on the approach based on the monetary value of nature, it has to be said that, while the services of ecological systems and the associated goods provision of the entire biosphere have an estimated annual value of US\$33 trillion, about 32 % of this estimated value comes from coastal ecosystems (US\$10.6 trillion) (Costanza et al., 1997).

Beyond their putative monetary value, the truth is that coastal ecosystems provide a broad array of goods and services that benefit the society and are practically necessary for the survival and well-being of a significant proportion of the world's population that depends on them. Some of those goods and services are food provision, energetic resources, natural products, wetland protection against floods and storms, climate and weather regulation, nutrients regulation, carbon sequestration, water quality maintenance and leisure and recreation opportunities (Costanza et al., 1997, UNEP 2006).

At present, 41 % of the human global population lives within 100 km of the coastline (Martínez et al., 2007; UNEP 2007), and the growing development of anthropogenic activities, many of them environmentally threatening, have caused that coastal areas are currently highly altered and endangered worldwide (Gilman et al., 2008; Polidoro et al., 2010) (Figure 5).

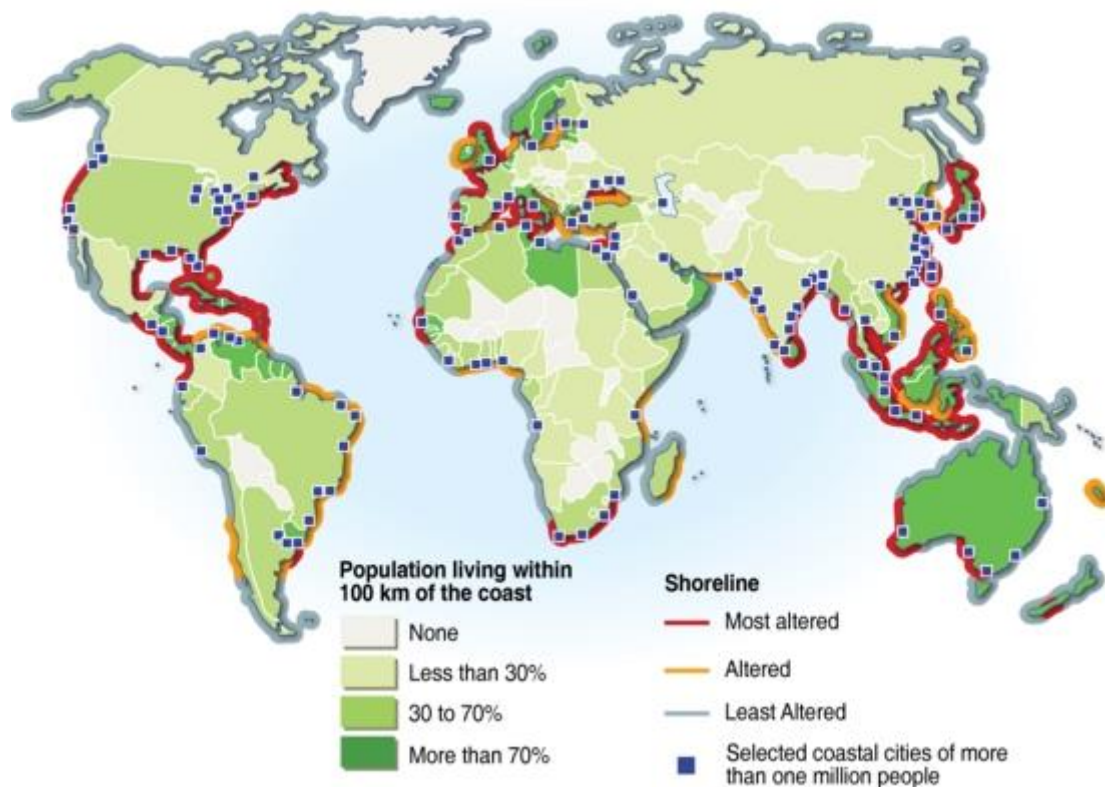


Figure 5. Coastal population and altered land cover in coastal areas (UNEP 2007).

Thus, overfishing, introduction of invasive species, waste disposal, dredging or nutrient loading are some of the numerous human activities that impact on coastal ecosystems, altering their integrity and reducing their resilience against environmental changes. In addition, increased greenhouse gases in the atmosphere causes a suite of physical and chemical changes in the marine coastal ecosystems with detrimental consequences, often still uncertain (Figure 6).

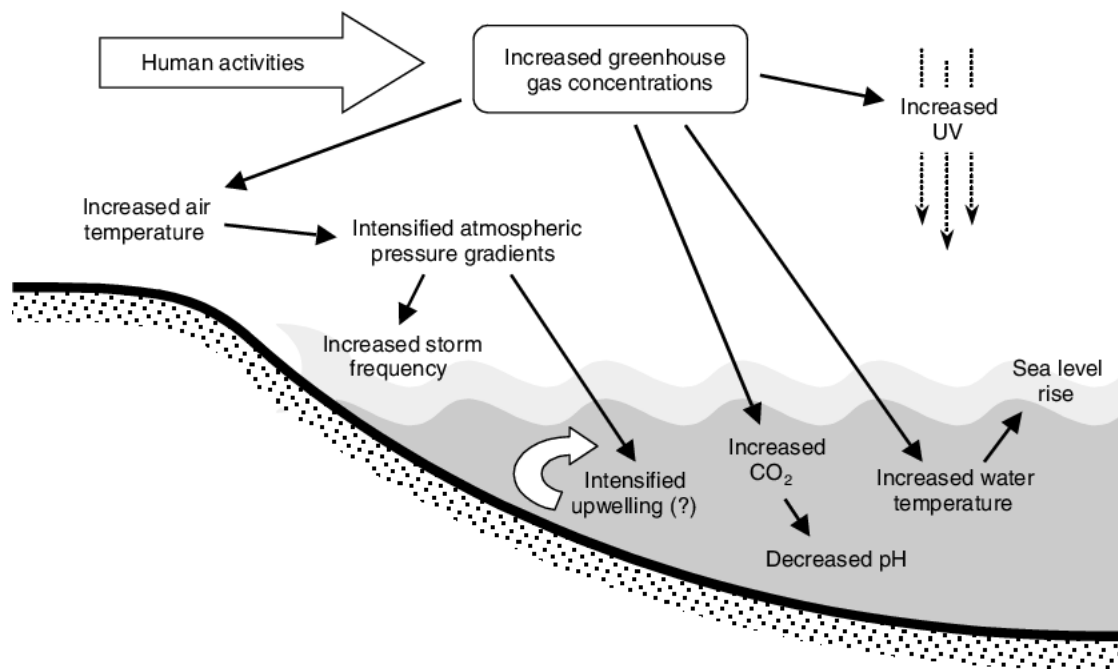


Figure 6. Abiotic changes associated with climate change (Harley et al., 2006).

Foundation species, a key piece for coastal ecosystems

Foundation species are defined as “a single species that defines much of the structure of a community by creating locally stable conditions for other species and by modulating and stabilizing fundamental ecosystem processes” (Dayton 1972). The effects of foundation species (sometimes called engineering species, Jones et al., 1994) is maybe one of the most relevant case of facilitation, with great consequences (both functional and structural) at the ecosystem level (and beyond), and many of the most important coastal communities (such as corals, mangroves, algal forests, seagrass meadows and many others) are based on the structure and performance of those foundation species. Their sensitivity to climate change is a matter of major concern, as any negative impact on a foundation species propagates to other organisms by a cascading effect with potential catastrophic consequences at the ecosystem level (e.g. Hoegh-Guldberg 1999; Hughes et al., 2013). Unfortunately, the detrimental effects of climatic (and associated) drivers on foundation species (e.g. gorgonians, corals, kelps and seagrasses), with dramatic effects on the rest of the community, have been repeatedly reported in the last times (Hoegh-Guldberg and Bruno, 2010).

Seagrass meadows are a paradigmatic example of what has been said above. In effect, they provide relevant goods and services (Beaumont et al., 2007; Costanza et al., 1997; Hemminga and Duarte, 2000). At this respect, it should be reminded that they are highly productive (1.1 % of the total marine primary production, Duarte and Chiscano, 1999), they stabilize the sediment in coastal areas thus helping to prevent coastal erosion (Koch et al., 2009), they provide habitat for a numerous fish and shellfish of high commercial value (Duarte 2000), they improve water quality through nutrient cycling, they produce oxygen and release it to the water column and sediment, (Duarte 2002), and they are considered a hotspot

of carbon sequestration in the biosphere (Fourqurean et al., 2012). Extensive seagrass beds are widespread worldwide (Figure 7) in a number of different environments (open and semi-confined waters, lagoons, estuaries...) between the coastline down to ca. 90 m depth (Duarte 1991). Different impacts of human activities are causing severe declines of these valuable habitats (Orth et al, 2006; Waycott et al., 2009), and the rate of such decline has been accelerated in the last decades. Thus, while before 1940 seagrass meadows were estimated to have experienced a rate of decline of 0.9 % total area per year, it was increased to 7 % per year from 1990 (Waycott et al., 2009; but see de los Santos et al., 2019). That results in a 29 % disappeared seagrass meadows area since 1879 (Figure 8).

Natural processes such as storms and hurricanes damages, or biological disturbances (diseases, grazing, invasive species...) have been identified and documented as causes of the seagrass loss, although the main current drivers at present are directly linked to human activities that cause, excessive nutrient loadings, waste accumulation, pollution or mechanical damage (Collins et al., 2010; Howarth et al., 2000; Orth et al., 2006), to which global warming (Collier and Waycott, 2014; Marbà and Duarte, 2010) should be added (e.g. Fraser et al., 2014). Seagrasses are, therefore, excellent ecosystem models where to address the effects of interacting stressors.

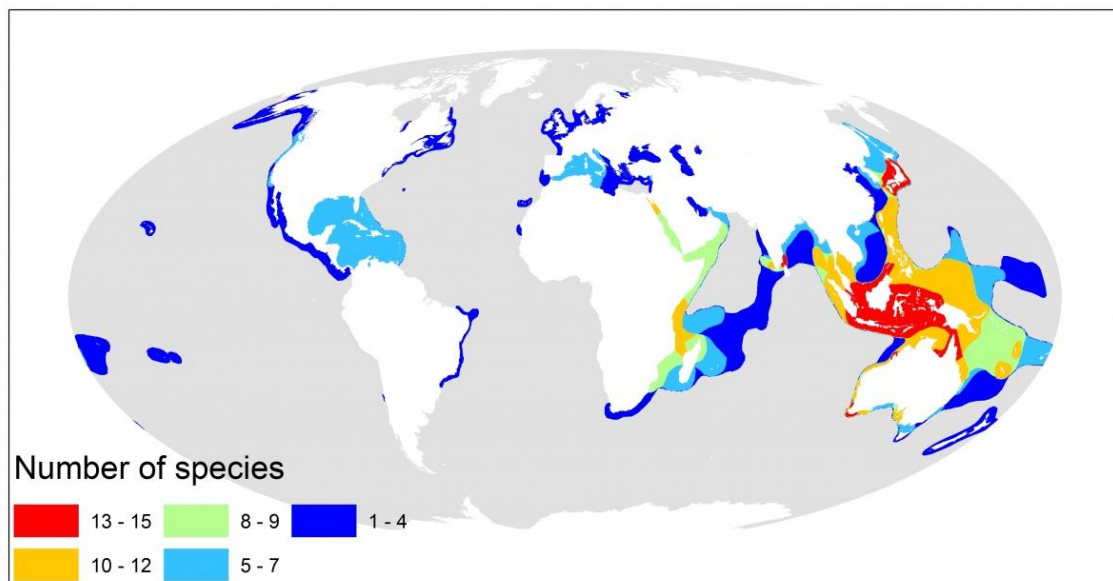


Figure 7. Global distribution of seagrass species richness (UNEP-WCMC, Green and Short FT (2003)). URL: <http://data.unep-wcmc.org/datasets/9>.

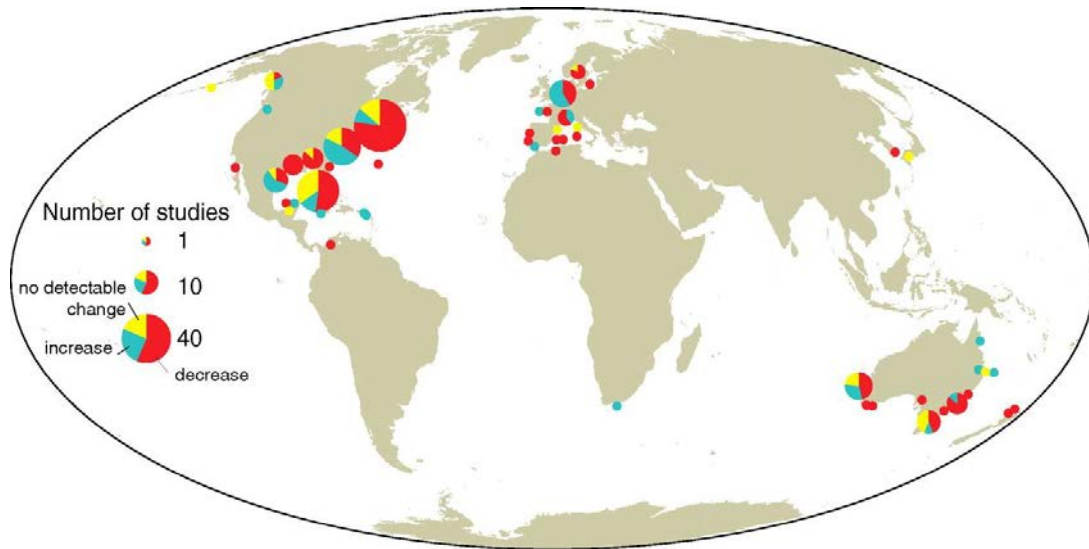


Figure 8. Global map indicating changes in seagrass area plotted by coastline regions (Waycott et al., 2009).

Under the present scenario of accelerated warming, it is necessary to understand, firstly, how and to which extent seagrasses will be affected by temperature increases, and identify temperature thresholds beyond which the effects become deleterious; secondly, attention should be paid to how different local stressors will interact with global warming, potentially worsening the effects on seagrasses. As managing climate change, at least in the short term, faces enormous difficulties, it seems more than urgent to put management efforts on other, more affordable, local stressors, so as at least to maintain seagrass resilience.

AIMS OF THE
THESIS AND
STUDY SPECIES

OBJECTIVES

The main objective of this PhD thesis is, in very general terms, to contribute to elucidate how global warming impacts seagrasses. More specifically, we attempt to understand the main seagrass responses to warming and the potential protective mechanism behind, paying attention at the interspecific variability in strategies and capacity in acclimation to warming. Furthermore, given the fact that warming rarely acts alone in nature and it often overlaps with numerous other stressors, this work also addresses the combined effects of warming with those stressors, with particular focus on eutrophication and salinity.

This general aim is undertaken through an approach defined by:

- (i) the experimental conditions, obtained through different thermal treatments at different time scales (from few days to several weeks), in isolation or in combination with other stressors, simulating heat waves in future scenarios of global warming predicted for the end of the XXI century.
- (ii) the responses determined, which consisted in the measurement of plant functional traits at different plant integration levels, including physiological (e.g. chlorophyll a fluorescence-related parameters), individual (e.g. necrosis, growth...) and population responses (e.g. demographic balance). In some cases, we attempted to get insights about the mechanisms underlying the responses.
- (iii) the species investigated (*Posidonia oceanica*, *Cymodocea nodosa* and *Halophila ovalis*).

The work is based exclusively in mesocosm experiments, an experimental approach that has demonstrated a great potential not only to explore the responses of seagrasses to warming but also to make robust inference about the causes of such responses. Nonetheless, all caution is exerted to acknowledge the limitations of such approach, specially when extrapolating our findings to the real world or/and at the ecosystem level.

More in detail, our general objective can be split into two specific objectives and three subobjectives:

- *Objective 1:*

To evaluate the response capacity of different seagrass species with contrasting ecological strategies to transient chronic (several weeks) warming in order to elucidate the underlying species-specific tolerance mechanisms against heat stress and their adaptive capacities to persist in future warming scenarios (Chapter 1).

Particularly, we assess the response of two seagrass species that, besides presenting evident differences in morphological attributes, as well as in their ecological strategies and evolutionary history, share a common distribution area in the Mediterranean. Such excellent case-study represents a good chance to explore the abovementioned particularities, that is, the potentially differential responses to warming and the mechanisms involved in such

responses, especially those related to energy dissipation pathways and antioxidant enzymes activities. All this is needed to determine the acclimation capacity of each species to warming.

- Objective 2:

To evaluate seagrass responses (in the short-term) to the interaction between warming and other stressful factors, that is, to the simultaneous occurrence of warming and other, more local, stressors.

The need to evaluate the joint effects of the local stressors with temperature is critical in order to capture, in a more realistic way, the effects of warming on seagrass meadows inhabiting areas already submitted to other sources of stress. Eutrophication and salinity changes are the local stressors evaluated in this work. On the one hand, the effect of eutrophication was studied by testing separately its two main components: an increase in nutrient concentrations in the water column and an increase in organic matter loading in the sediment. Firstly, we evaluate the plant response to each one of these two potential stressful factors, separately and in its combination with temperature for the Mediterranean species *Cymodocea nodosa*, (subobjective 2.1, **Chapter 2**). Secondly, we evaluate the plant response to only an increase of nutrient concentrations in the water column, separately and in combination with temperature for the Mediterranean species *Posidonia oceanica* (subobjective 2.2, **Chapter 3**). Finally, we explore the interactive effects of warming with salinity changes typically experienced by some seagrasses species that are dominant in estuarine environments, such as *Halophila ovalis* (subobjective 2.3, **Chapter 4**).

The two main hypotheses we address are:

- (i) The predicted increasing intensity and frequency of heatwaves will affect seagrass meadows. As these organisms cannot escape from such stress, they must acclimate to the coming conditions and such acclimation differs among species with different ecological strategies. Temperature increase can favor plant performance, but negative responses appear once the thermal threshold is surpassed. Both this threshold, and the protective mechanisms put in place to deal with temperature effects, are highly variable among species, and will determine their acclimation capacity.
- (ii) The ongoing global warming acts over other stressors that currently affect seagrass performance. The simultaneous action of increasing temperature and such local stressors leads to interactive effects, likely in an additive or synergistic way, thus exacerbating the negative effects of increased temperature in isolation, aggravating the effect on plant performance and hence jeopardizing plant survival and, in consequence, the persistence of the population.

STUDY SPECIES

This work is focused on three seagrass species: *Posidonia oceanica*, *Cymodocea nodosa* and *Halophila ovalis*. These species present different biological traits and life history strategies, which ranks from colonizing, opportunistic to persistent species (*sensu* Kilminster et al., 2015). (Figure 1).

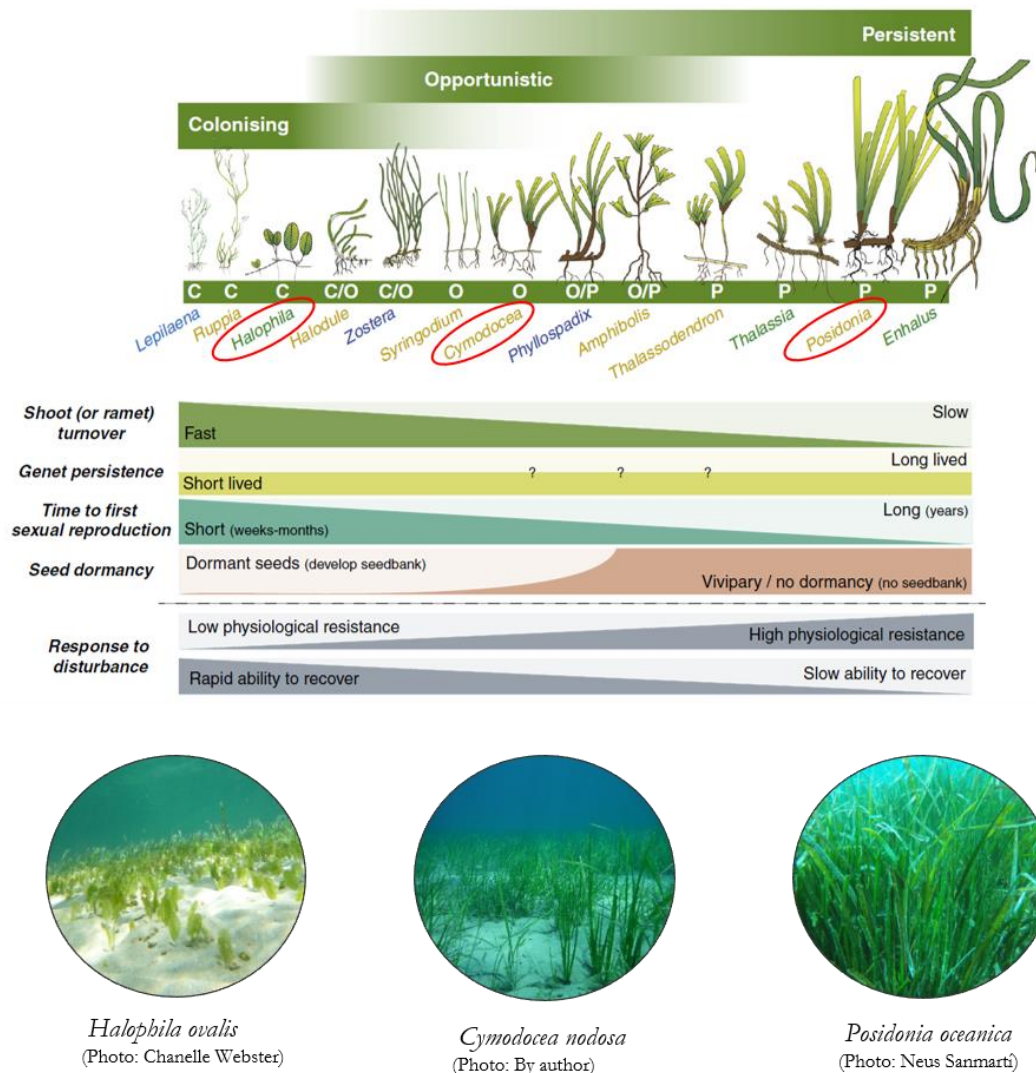


Figure 1. Diagram showing the classification of seagrass genera based on their life history strategies (colonizing, opportunistic and persistent) and the response of some of their dominant traits (extracted from Kilminster et al., 2015). Red circles in the diagram highlight the genus corresponding to the three species studied in this thesis. Above, photograph of each species.

P. oceanica and *C. nodosa* share a large distributional area along the Mediterranean coasts, forming extensive meadows dominating infralittoral environments down to depths of 30-40 m, still deeper in the Eastern basin (Olesen et al., 2002; Ruiz et al., 2009).

P. oceanica is a Mediterranean endemic, and is supposed to be one of the longest living plants on Earth. It is a large, perennial and slow growing species (Marbà and Duarte, 1998), and it is found only in open coastal waters or bays with relative stable and unaltered conditions.

C. nodosa, a medium-size, fast-growing and a high turnover species is distributed all over the Mediterranean, but also, extends to adjacent areas of the Atlantic Ocean. This species tolerates well confined waters, as well as eutrophication, and is therefore found not only in open waters (usually in habitats unsuitable for *P. oceanica*, for example due to sediment instability) but also in coastal lagoons and estuaries (Pérez and Romero, 1994; Terrados and Ros, 1991).

Halophila ovalis is a small-size and fast-growing seagrass species, distributed in temperate and tropical areas (Short et al., 2007), able to live in highly variable environments, such as estuaries or coastal lagoons, although it is also found in open coastal waters.

CHAPTER 1

**Seagrass species with contrasting
ecological strategies reveal differential
tolerance to warming**

Yaiza Ontoria*, Jaime Bernardeau-Esteller, Lázaro Marín-Guirao,
José Miguel Sandoval-Gil, Rocío García-Muñoz, Marta Pérez, Javier Romero,
Juan Manuel Ruiz

ABSTRACT

The Mediterranean Sea is highly vulnerable to global warming. The increasing frequency and intensity of extreme thermal events is threatening Mediterranean coastal ecosystems, and, specifically, seagrass meadows. Understanding the seagrass species tolerance to warming and the protective mechanisms to cope with thermal stress is imperative to predict the future of these foundation species, and, subsequently, of their associated ecosystems. We exposed the two main Mediterranean seagrass species, *Posidonia oceanica* and *Cymodocea nodosa*, to a range of temperatures (20, 24, 28 and 32 °C) for 5 weeks in a mesocosm system, after which we determined several plant functional traits (from physiological to population level) and potential thermotolerance mechanisms. In none of the two species the integrity of photosystem II (PSII) was affected by temperature but warming improved *C. nodosa* carbon balance, stimulating also leaf growth, while it impaired that of *P. oceanica*, with also negative consequences for leaf growth and shoot demographic balance. Interestingly, these contrasting responses were accompanied by a very clear differential capacity to activate thermotolerance mechanisms: *C. nodosa* was able to activate antioxidant enzymes while *P. oceanica* was not and just presented mild symptoms of activation of heat dissipation mechanism (non-photochemical quenching and xanthophyll cycle). These results suggest that, under a future scenario of global change, changes in the distribution and abundance of these two species can occur, with *C. nodosa* potentially expanding and *P. oceanica* retracting. Given the crucial roles these species play, these changes will not be without consequences for the Mediterranean coastal ecosystems.

INTRODUCTION

Warming is a global threat affecting all functional levels of life, from the molecular to the whole biosphere. The public concern about its effects has grown enormously in recent years, and the main focus of this concern seems to be the loss of the provision of goods and services to human society, and how this will affect human wellbeing (van der Geest et al., 2019). No doubt, a large part of goods and services are originated by ecosystem functions, being the response to warming of individual species, as the basic units of communities and, hence, of ecosystems, what have attracted a large part of the focus from the scientific community. In fact, how the individual species respond to warming and which mechanisms do they have to cope with temperature increase seem an unavoidable first step to predict coming changes in our living environment (Matesanz et al., 2010; Parmesan 2006), in particular in those ecosystems based on foundation species.

When a species is submitted to a shift in environmental conditions such as a thermal increase, there are three strategies tending not to loss fitness: to change the phenotype (acclimation), to change the genotype (adaptation by natural selection) or to move to another area with more favourable conditions (migration) (Nicotra et al., 2010). All three strategies are equally important, and a deep understanding of them (including their interactions) is crucial to predict the coming changes. However, acclimation seems the primary process through which species will have to deal, at least in the short term, with the ongoing rapid increases in temperature (Esmon et al., 2005; Mittler et al., 2011). From the growing evidence of a plethora of studies, it seems clear that how species react to warming is highly heterogeneous across species and is determined not only by a suite of specific functional traits, but also by their phenotypic plasticity, both aspects framed by the evolutionary and biogeographical context. Those aspects are interrelated, since the phenotypic plasticity not only refers to morphological and physiological changes of the organisms, but it is also an adaptive character in itself, thus being subject to selection and evolution (Dudley 2004; Nicotra et al., 2010).

Although not always explicitly, the assessment of species responses to global warming has been often addressed in a context of performance impairment. However, heat stress responses, and hence vulnerability to warming, can vary among species, not only in magnitude, but also, within a given range, in sign; in addition, the defence mechanisms activated to face warming impact can also differ among species. This differential response is especially relevant in species sharing a common habitat or a common biogeographical area, either if they compete (Chalanika De Silva and Asaeda, 2017) or if one predates on the other (Allan et al., 2015), and leads to the concept of “winners” (i.e. species obtaining some benefit from warming) and “losers” (i.e. species submitted to negative consequences), which in turn expands our tools to better predict changes derived from warming, in the local abundance of the species or in shifts of its distribution limits (Harley et al., 2006).

Because of global warming, oceans are currently experiencing both a progressive increase of the average seawater temperature and an increase of frequency and intensity of extreme climate events such as heat waves (Oliver et al., 2018). In coastal areas, the impact of warming is of particular concern in what it regards foundation species such as corals,

mangroves, gorgonians and seagrasses due to their crucial role in maintaining the structure and function of key ecosystems, which provide essential ecosystem services (Costanza et al., 2014; Bennett et al., 2016; Lejeune et al., 2009). The detrimental effects of warming caused by heat waves on these and other habitat-forming species (Garrabou et al., 2009; Marbà and Duarte, 2010; Thomsen et al., 2019) have been documented, and may lead potential cascading effects to the communities they support (Bennett et al., 2016; Wernberg et al., 2016).

Particularly, seagrasses are considered one of the most valuable coastal ecosystems regarding the wide range of ecosystem services they provide, including carbon sequestration, nutrient cycling and coastal protection, in addition to those referred to biodiversity (Orth et al., 2006). Yet seagrasses are highly vulnerable to warming (Collier and Waycott, 2014; Savva et al., 2018). Although moderate temperature increases can benefit seagrass performance, stimulating processes such as photosynthesis (Pérez and Romero, 1992; Zimmerman et al., 1989), growth or biomass accumulation (Bulthuis 1987; Ontoria et al., 2019a, 2019b), once a given thermal threshold is exceeded, metabolic alterations in photochemistry (Campbell et al., 2006; Ralph 1998; Repolho et al., 2017), photosynthetic and respiratory rates (Collier et al., 2011; Marín-Guirao et al., 2016, 2018), and other key metabolic process occur, leading to deleterious effects such as carbon unbalances, reduced growth or leaf structure deterioration, all these ultimately causing mortality (Collier and Waycott, 2014; Hammer et al., 2018; Lee et al., 2007; Marín-Guirao et al., 2016; Ontoria et al., 2019a). However, the available literature clearly shows that thermal thresholds and vulnerability are highly variable among species, depending for a large part on different acclimative capacities. This, on the one hand, suggests future changes in the distribution and abundances of the different species accordingly with future warming scenarios (Chefaoui et al., 2018; Collier and Waycott, 2014; Collier et al., 2011; Jordà et al., 2012). On the other hand, it points to the importance of the underlying thermotolerance mechanisms (Marín-Guirao et al., 2016, 2017, 2018; Tutar et al., 2017), which remain poorly studied (at least relative to terrestrial plants) despite the increased knowledge on the responses of seagrasses to warming.

As higher plants, it seems likely that seagrasses should have signalling pathways to sense changes in temperature as well as thermotolerance mechanisms similar to that found in other angiosperms (Mittler et al., 2011). As for instance, plants have developed protection systems against oxidative stress (Hasanuzzaman et al., 2013), caused by an unusual accumulation of toxic by-products such as reactive oxygen species (ROS) (Mittler et al., 2011; Ruelland and Zachowski, 2010). Molecular studies have shown the overexpression of some antioxidant genes in seagrasses under heat stress (Tutar et al., 2017; Purnama et al., 2019), confirming the existence of such protective mechanisms against heat stress based on those antioxidant enzymes. Another protective mechanism consisting on the dissipation of the excess energy by non- photochemical quenching (NPQ), coupled to the xanthophyll cycle (Demming-Adams and Adams, 2006), has been also described in higher plants. The operation of such a mechanism in seagrasses as a photoprotective mechanism against high irradiance stress (Ralph et al., 2002) or high salinity stress (Marín-Guirao et al., 2013a) has been evidenced, but its involvement in protection against heat stress is scarcely known to date.

In addition, other stress-protective mechanisms present in higher plants, including ion transporters, protein stabilizers (i.e. Heat Shock Proteins (HSP)) and osmoprotectants (Krasensky and Jonak, 2012; Rasheed et al., 2011), have been evidenced in some seagrass species (Massa et al., 2011; Traboni et al., 2018; Tutar et al., 2017), although their role coping with thermal stress remains unknown. At this respect, it seems urgent to investigate how these mechanisms operate in seagrasses but, specially, what is the potential they confer for acclimation in response to heat stress to the different seagrass species.

The oceans are not warming equally and the known ocean warming “hotspots” (*sensu* Pecl et al., 2014) are those areas which are warming fastest. Specifically, the Mediterranean Sea is considered an area of special vulnerability to global warming (Burrows et al., 2011; Vargas-Yáñez et al., 2008). In this region, the most abundant and extended seagrass species are *Posidonia oceanica* and *Cymodocea nodosa*, which clearly differ in their origin, ecological strategies and biogeographical affinities. Thus, while *P. oceanica*, a Mediterranean endemics, is a large long-lived and slow-growing species that occupies open coastal waters, *C. nodosa* is a medium-size fast-growing species that can be found in a wider range of environments (estuaries, coastal lagoons, confined and semi-confined waters as well as, less abundant than *P. oceanica*, in open coastal waters) with a latitudinal range from temperate to subtropical waters. Accordingly with this, a higher vulnerability to warming is usually attributed to *P. oceanica*, based on mortality events reported in relation to extreme heat waves, such as those recorded in 2003 and 2009 in the Western Mediterranean (Garrabou et al., 2009; Díaz-Almela et al., 2009; Marbà and Duarte, 2010). By contrary, *C. nodosa* is expected to be favoured by warming (Boudouresque et al., 2009; Pérez and Romero, 1992). A previous study supports this notion since experimental heat stress caused a 40 % reduction of leaf carbon balance in *P. oceanica* while *C. nodosa* remained unaffected (Marín-Guirao et al., 2016, 2018). Moreover, *C. nodosa* has been shown to display a remarkable phenotypic plasticity (Pérez and Romero, 1994; Olesen et al., 2002; Sandoval-Gil et al., 2014). This scenario (i.e. the existence of two species apparently differing in their ecological strategy and responses to warming, and sharing a large distributional area especially vulnerable to climate change) offers an excellent case-study to explore the potential differences in the response mechanisms to heat stress and the involvement in their respective, species-specific acclimation capacities.

To analyse in depth this case study, we conducted an indoor mesocosm experiment aiming at (i) to evaluate the differential responses to warming of these two seagrass species (*P. oceanica* and *C. nodosa*) and (ii) to elucidate the underlying thermotolerance mechanisms of each species, specifically those related to heat dissipation pathways and antioxidant enzymes activities. To this, and after the exposition period, we measured a suit of plant traits, including some related to plant performance (photochemistry, photosynthesis, respiration, growth...), to protective mechanisms (xanthophylls pigments, antioxidant enzyme activities...) or to plant damage (membrane peroxidation, chlorophyll pigments...). The hypothesis is that, both responses and mechanisms will largely differ between these two species, accordingly with their respective evolutive and ecological stories, and that these differences could imply profound consequences for their respective trajectories, and hence for Mediterranean ecosystems, under future scenarios of climate change.

MATERIAL AND METHODS

Plant sampling

In spring 2016 (April), healthy large fragments of rooted *P. oceanica* and *C. nodosa* were carefully collected by scuba divers in dense and healthy meadows (5m depth, over an area of ca. 300 m²) off the South Eastern coast of Spain, Isla Grosa (37° 43.7' N, 0° 42.75' W). Individual fragments were chosen so as to hold rhizomes with apical growth meristems and several connected shoots to maintain clonal plant integrity.

Collected plants were transported to the laboratory in large, temperature and oxygen-controlled coolers within two hours of their collection, and then transferred into the aquaria of the mesocosm facility at the Oceanographic Centre of Murcia (IEO, Spain; Marín-Guirao et al., 2011). Plant fragments of *P. oceanica* with a similar size and morphology were selected, attached by cable ties to a rigid plastic mesh placed into a plastic pot (on average, 50 shoots/pot) filled with cleaned coarse sediments. Furthermore, morphologically similar *C. nodosa* fragments (rhizome sections) were equally distributed and attached on wire meshes (on average, 60-70 shoot/mesh), which were immediately buried in cleaned sediment.

Mesocosm system and experimental set up

The system consisted on 12 independent tanks per species (500 L for *P. oceanica* and 100 L for *C. nodosa*) containing each four randomly selected pots (Figure 1). Each tank had its own independent seawater circulation system and control of temperature and light. In addition, nutrients, water flow, temperature and pH conditions were controlled and recorded in each tank every day (hourly for temperature and pH). Plants were maintained in the tanks under conditions close to that found *in situ* during collection (temperature: 20 °C; salinity: 37.5 psu; irradiance: 250 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$; light:dark photoperiod: 14h:10h) to acclimate for a three weeks period. For each species, after the acclimation period, temperature was maintained at 20 °C in three tanks chosen at random and increased at a fixed heating rate of 2 °C day⁻¹ in the rest of the tanks so as to reach the other three target temperatures (24 °C, 28 °C, and 32 °C). These thermal conditions were selected so as to represent present day summer months values (minimum-maximum) in the area of collection, while 32 °C approaches the maximum summer value expected to be experienced by these seagrass species at the end of the century (Marbà and Duarte, 2010; Jordà et al., 2012; IPCC, 2014). Plants were maintained for five weeks under experimental conditions, a period of time that has been demonstrated to be long enough to induce plant stress responses (Marín-Guirao et al., 2018; Traboni et al., 2018) and, at the end, plant traits assessment was conducted as explained below.

The tanks were considered the experimental units, and thus, for statistical purposes, each tank is considered an independent replicate (n=3 replicates per experimental condition). Values obtained from different shoots within a tank (number changing depending on the variable) were considered subsamples and averaged to obtain the value for the replicate.

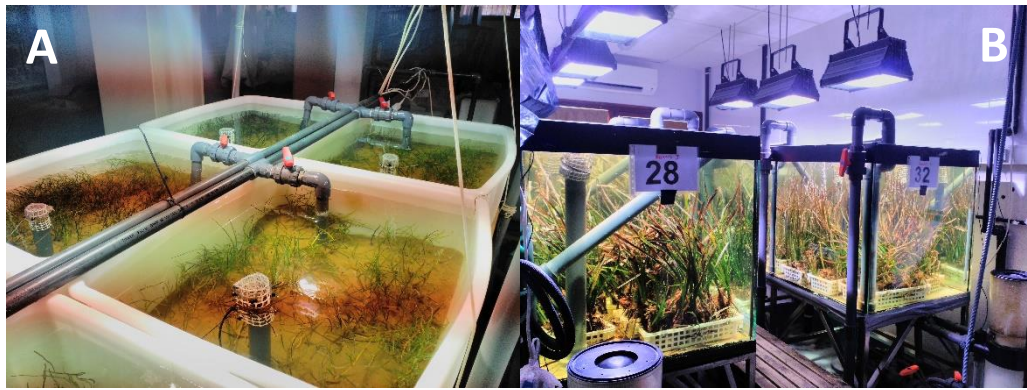


Figure 1. General view of the mesocosm systems: A) *Cymodocea nodosa* and B) *Posidonia oceanica*

Plant traits assessment

Chlorophyll a fluorescence parameters

Chlorophyll fluorescence parameters were measured in four randomly selected shoots from each tank (one shoot per pot), using a diving PAM (Pulse Amplitude fluorometer, Walz Germany) (Genty et al., 1989; Schreiber, 2004). Maximum quantum yield of PSII ($F_v/F_m = (F_m - F_o)/F_m$), where F_m and F_o are, respectively, maximum and minimum fluorescence of dark-adapted leaves, was measured in the early morning (before lights on, at 7:00 am). In order to reduce within-shoot and within-leaf variability of fluorescence parameters (Durako and Kulzelman, 2002; Gera et al., 2012), the measurements were taken on the basal portion of the second youngest leaf where values are at its optimum (Marín-Guirao et al., 2011). After three hours of illumination, effective quantum yield of PSII ($\Delta F/F_m' = (F_m' - F)/F_m'$) was measured, where F_m' is the maximum fluorescence of light-adapted leaves and F is the yield in any given state of illumination. Then, rapid light curves (RLCs) were obtained by exposition to nine increasing irradiances (from 0 to a maximum of $378 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), with a duration of 10 s each. RLCs allowed to extract other fluorescence-based parameters such as the maximum electron transport rate (ETR_{max}), as provided by the PAM WinControl program (Walz, Germany). The non-photochemical quenching (NPQ) was calculated as $\text{NPQ} = (F_m - F_m')/F_m'$ (Bilger and Björkman, 1990).

Pigment analyses

Photosynthetic pigment (chlorophyll *a* and *b*) content in leaves was analysed in one shoot per pot, from which 1 cm^2 leaf fragment was excised from the middle section of the youngest fully-developed leaf. Pigments were extracted in buffered acetone (80 %) using homogenizers. Extracted samples were maintained overnight at $4 \text{ }^\circ\text{C}$ to ensure full disaggregation of leaf material. Absorbances were measured spectrophotometrically at four specific wavelengths (470, 646, 663 and 725 nm) to calculate chlorophyll *a* and chlorophyll *b*, using the equations of Lichtenthaler and Wellburn (1983). Pigment content was expressed per leaf surface ($\mu\text{g cm}^{-2}$).

Carotenoids were obtained from chlorophyll-free extracts following the methodology described in Marín-Guirao et al. (2013b), while the HPLC analysis for xanthophyll pigment separation were conducted according to the detailed protocol in Stinco et al. (2012) and identified by chromatographic and UV/vis spectroscopic characteristics. We estimated the conversion of violaxanthin (V) into anteraxanthin (A) and zeaxanthin (Z) through the xanthophyll de-epoxidation ratio (DR), using the equation $(A + Z)/(V + A + Z)$. Xanthophyll content was expressed on mmol mol^{-1} chlorophyll *a*.

Antioxidant enzyme activities

The activities of antioxidant enzymes were measured in two randomly selected shoots of *P. oceanica* and *C. nodosa* of different pots per tank. Leaf tissues were taken from the middle section of the youngest fully-developed leaf (avoiding necrotic tissues), cleaned up of epiphytes, and kept ultrafrozen ($-80\text{ }^{\circ}\text{C}$) until analyses. Ultrafrozen leaf samples (0.5 g of fresh weight) were ground in liquid nitrogen using a mortar, and homogenized in phosphate potassium buffer 100 mM pH 7.8 (1 mM EDTA, 1 % polivinilpirrolidone), with a dilution factor of 1:6. For APX (ascorbate peroxidase) and DHAR (dehydroascorbate reductase), sodium ascorbate (10 mM) was added to the buffer. After centrifugation at 49000 g for 10 min at $4\text{ }^{\circ}\text{C}$, the supernatant was used for enzyme activity assay of SOD (superoxide dismutase), GST (glutathione-S-transferase), GPX (glutathione peroxidase), GPOX (guaiacol peroxidase), DHAR and APX, and also to determine the protein content. SOD activity was determined by colorimetric method (i.e. decrease in the absorbance at 440 nm), using the SOD Assay Kit-WST based on Dojindo's highly water-soluble tetrazolium salt. GST activity was measured spectrophotometrically (increment in the absorbance at 340 nm; extinction coefficient $9.6\text{ mM}^{-1}\text{ cm}^{-1}$) at pH 8.4 by following conjugation of the acceptor substrate (1-chloro-2, 4-dinitrobenzene, CDNB) with reduced glutathione (Habig et al., 1974; Ferrat et al., 2003). GPX activity was determined by the reduction of absorbance at 340 nm (extinction coefficient $6.2\text{ mM}^{-1}\text{ cm}^{-1}$) in a reactive solution containing phosphate buffer 100 mM pH 7.5, EDTA, sodium azide, reduced glutathione, glutathione reductase, NADPH and Hydrogen peroxide (H_2O_2), based on protocols described in Flohé and Günzler (1984) and Sureda et al. (2008). GPOX activity was determined in a reaction mixture consisted of 100 mM potassium phosphate buffer pH 6.0, 0.18 % (v/v) H_2O_2 , 1 % guaiacol and the enzyme extract; the oxidation of guaiacol by H_2O_2 was measured by the increase in absorbance at 470 nm (extinction coefficient $26.6\text{ mM}^{-1}\text{ cm}^{-1}$) according to Kato and Shimizu (1987) and Upadhyaya et al. (1985). APX and DHAR protocols were adapted to those described by Costa et al. (2015). APX activity was measured by the decrease in the absorbance at 290 nm (extinction coefficient $26.6\text{ mM}^{-1}\text{ cm}^{-1}$) in a reaction mixture of potassium phosphate buffer 100 mM pH 7.0, with a solution of ascorbate (800 μM), and H_2O_2 (20 mM) and leaf extract. For its part, DHAR activity was measured as the increase in absorbance at 265 nm ($14\text{ mM}^{-1}\text{ cm}^{-1}$) of a reaction mixture containing 100 mM potassium phosphate buffer (pH 6.1), 5 mM reduced glutathione, 800 μM dehydroascorbic acid, and leaf extract. Activities of antioxidant enzymes were expressed by soluble protein content of leaf tissues, which were determined by protein measurement with Folin phenol reagent (Lowry et al., 1951) with bovine serum albumin as standard curve.

Membrane lipid peroxidation

Lipid peroxidation is considered an indicator of cellular damage (Geffard et al., 2001) that occurs as a result of an ineffective of the antioxidant enzyme. The sampling protocol and handling of leaf tissues for the analyses of membrane lipid peroxidation was similar to that described for the activity of antioxidant enzymes. Membrane lipid peroxidation was measured following the thiobarbituric acid-reactive-substances assay described by Hodges et al. (1999) and Barrote (2005). Ultrafrozen leaf tissues were ground in liquid nitrogen using a mortar, and homogenized (dilution 1:10) in trichloroacetic acid (TCA, 20 %). Tissue homogenates were then centrifuged (3000 g, 4 °C) and supernatants were mixed with a solution of TCA (20 %) and TBA (thiobarbituric acid, 0.5 %). These solutions were heated at 90 °C during 30 min, and then centrifuged again (10000 g) during 10 min. The supernatants were then extracted and their absorbances (440, 532 and 600 nm) were measured by spectrophotometer. Equivalents of malonildialdehyde (Eq MDA; molar extinction coefficient $155 \text{ mM}^{-1} \text{ cm}^{-1}$), which is a secondary metabolite resulted from lipid peroxidation in cell's membranes, was calculated according the method described in Hodges et al. (1999) and Barrote (2005) as:

$$\text{Eq MDA (nmol ml}^{-1}\text{)} = [(A-B)/155000] \times 10^6$$

Photosynthesis and respiration rates

Photosynthesis and dark respiration rates were determined in two randomly selected shoots from each tank using a Clark type electrode (Oxygraph system, Hansatech, UK), following the methodology described in Marín-Guirao et al. (2011, 2016). From each shoot, a leaf segment of approximately 2 cm^2 was taken from the middle section of the youngest fully-developed leaf and incubated in a 20 ml chamber (DW3, Hansatech, UK) housing the electrode. Temperature of incubations was kept the same as in the corresponding experimental treatment by a controlled temperature circulating bath. Leaf fragments were exposed to $250 \text{ } \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (which is above saturating irradiance for both species, Alcoverro et al., 1998; Pérez and Romero, 1992) determining thus maximum rates of net oxygen production (net P_{max} ; $\mu\text{mol O}_2 \text{ g}^{-1} \text{ FW h}^{-1}$). After maintaining leaf segments to darkness for 10 min, dark respiration rates (R_d ; $\mu\text{mol O}_2 \text{ g}^{-1} \text{ FW h}^{-1}$) were also determined. We estimated gross photosynthesis rate (gross P_{max} ; $\mu\text{mol O}_2 \text{ g}^{-1} \text{ FW h}^{-1}$) as:

$$\text{gross } P_{\text{max}} = \text{net } P_{\text{max}} - R_d.$$

An estimated gross $P_{\text{max}}:R_d$ ratio was calculated from each incubation and used as a proxy of the leaf carbon balance (Marín-Guirao et al., 2018).

Non-structural carbohydrates content

Non-structural carbohydrates content (soluble sugars and starch) was analysed in leaves and rhizome tissues (2 cm rhizome apex fragments) samples obtained from a shoot of each pot (a total of four samples of each tissue per tank) according to the method described in Marín-Guirao et al. (2013b), based on Invers et al. (2004) and Yemm and Willis, (1954). Analyses

were performed on dried tissue at 50 °C (48 h) and finely ground. Carbohydrates were then solubilised by four sequential extractions with 95 % (v/v) ethanol at 80 °C for 15 min. The ethanol extracts were evaporated using a thermostated vacuum centrifuge (Univapo 100H, Unijet II) while the residues were dissolved in deionized water for analysis. Starch was extracted from the ethanol-insoluble residue by keeping it overnight in 0.1N NaOH. Both soluble sugars and starch were determined spectrophotometrically using an anthrone assay. The total carbohydrate content was estimated by the sum of the soluble and reserve carbohydrates and all data expressed as percentage of dry weight (% DW).

Leaf necrosis, leaf growth and net shoot change

Leaf growth was measured according to the leaf punching method proposed by Zieman (1974), adapted to the species (Alcoverro et al., 2001; Pérez and Romero, 1994). Three shoots of each pot (i.e. a total of 12 shoots per tank) were marked after the acclimation period, just before starting the temperature rise. At the end of the experiment, the marked shoots were collected, and the leaves were carefully separated to measure leaf growth, which was expressed as the surface of new tissue produced per shoot and day ($\text{cm}^2 \text{shoot}^{-1} \text{day}^{-1}$). The incidence of leaf necrosis (dark brown or black spots covering leaf tissue) was assessed in the same four shoots. Once leaves were separated from each shoot, the necrotic surface relative to the total leaf surface of each shoot was estimated visually and expressed as a percentage.

To estimate the net shoot demographic balance, all shoots were counted at the beginning and at the end of the experiment and after normalizing to initial shoot numbers, the net shoot change was computed as follows:

$$\text{net shoot change (\%)} = ((N_t - N_0) \times 100) / N_0$$

Where N_0 is the number of shoots in each tank at the beginning of the experiment and N_t is the number of shoots at the end of the experiment. Positive values indicate an increase of shoot abundance (i.e. recruitment higher than mortality) while negative values indicated a reduction (higher mortality than recruitment).

Statistical analysis

To assess overall effects of temperature and differences between species on response variables and thermotolerance defence mechanisms related variables, we applied permutational multivariate analysis of variance (PERMANOVA), using PRIMER 6 statistical package (Clarke and Gorley, 2006) and the PERMANOVA+ module (Anderson et al., 2008). PERMANOVA was based on a similarity matrix created from the Euclidean distances between samples. We considered two fixed factors: temperature (four levels: 20 °C, 24 °C, 28 °C and 32 °C) and species (two levels: *P. oceanica* and *C. nodosa*, and a total of $n=3$ replicates (tanks) for each experimental condition. We further applied univariate PERMANOVAs to assess the significance of species, temperature or their interaction (independent variables) on each plant trait determined (dependent variables).

In order to synthetically (and visually) explore our dataset, and to further evidence interspecific differences of the response variables to temperature, a principal component analysis (PCA) was applied using the software CANOCO 4.5 (Ter Braak and Smilauer, 2002).

Single-factor ANOVA tests were performed to test the effects of temperature on plant response variables within each species. Data were log and fourth root-transformed prior to the analysis if normality or homoscedasticity assumptions were violated. Temperature was treated as a fixed factor with four levels with the same three replicates ($n=3$). The significance level (α) used was 0.05. When significant effect was found, a Tukey post-hoc test was applied (Zar 1984) in order to identify significant pairwise significance. These statistical analyses were conducted using R.

RESULTS

Plant responses to temperature increase

Chlorophyll a fluorescence parameters

Changes with temperature in maximum quantum yield (F_v/F_m) and effective quantum yield ($\Delta F/F_m'$) were small, albeit significant. The only clear trend was a modest and progressive increase in F_v/F_m in *P. oceanica* with temperature, absent in *C. nodosa* (see significant interaction in Tables 1, S2 and S3; Figure 2A and 1B). F_v/F_m was always above 0.6, a critical threshold of plant health (Ritchie 2006), thus suggesting the absence of damage in the photosynthetic apparatus. Maximum electron transport rate (ETR_{max}) was significantly higher (by a factor of two) in *C. nodosa* than in *P. oceanica* at all temperature treatments. Again, no clear pattern with temperature was evident, except a mild (but significant) increasing trend in *P. oceanica*. (Figure 2C; Tables 1, S2 and S3).

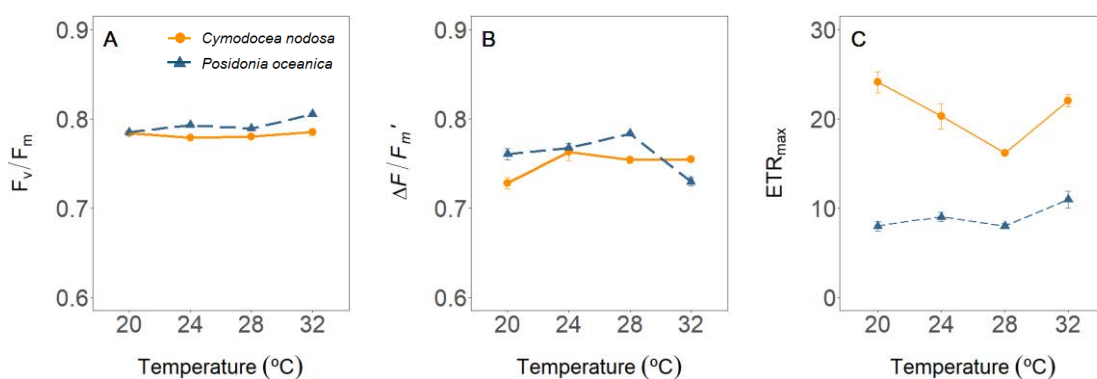


Figure 2. Photochemical responses of *P. oceanica* and *C. nodosa* plants exposed to a four temperatures range during 5 weeks. (A) Maximum quantum yield of dark-adapted leaves (F_v/F_m), (B) effective quantum yield of PSII ($\Delta F/F_m'$), and (C) maximum electron transport rate (ETR_{max}). Results are the mean and standard error of three independent replicates.

Chlorophyll pigments

Both species maintained their pigment content (chlorophyll *a* and chlorophyll *b*) at all temperatures assayed. The chlorophyll *b/a* ratio was significantly higher (up to 38 %) in *P. oceanica* than in *C. nodosa* (Figure S1; Tables 1, S2 and S3).

Photosynthesis, respiration rates and leaf carbon balance

At basal and intermediate temperatures, gross photosynthesis did not differ among species. However, at the highest temperature (32 °C) gross photosynthesis experienced a drop (ca. 30 % relative to the rest) in *P. oceanica* (Table 2A; Table 1). Respiration rates were similar between the two species, with no significant effects of the thermal treatments (Figure 3B; Table 1). Interestingly, the resulting leaf carbon balance (P/R_d) showed completely opposite responses among both species to temperature, with a clear and significant increase in *C. nodosa* and a clear decrease in *P. oceanica* (Figure 3C; Tables 1, S2 and S3).

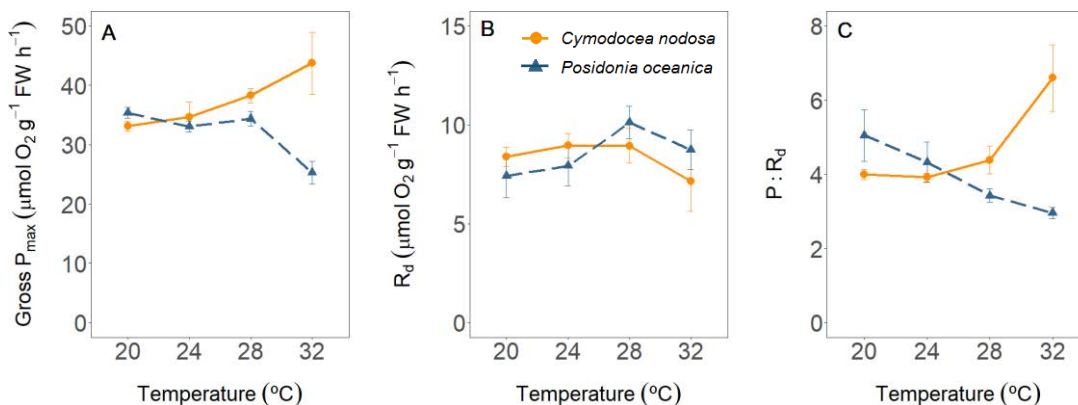


Figure 3. (A) Maximum gross photosynthetic and (B) respiration rates, and (C) leaf carbon balance (in terms of P_{max}/R_d ratio) of *P. oceanica* and *C. nodosa* plants after being exposed to a four temperatures range for five weeks (mean \pm SE, $n=3$).

Non-structural carbohydrates content

Non-structural carbohydrates (both starch and soluble fraction) content in leaf and rhizomes were significantly higher (between two and three-fold) in *C. nodosa* than in *P. oceanica*, with no relevant trends concerning the response to temperature (Figure S2; Tables 1, S2 and S3).

Leaf necrosis, leaf growth and net shoot change

While the thermal increase caused a clear decrease in leaf necrosis incidence in *C. nodosa* (80-85 % less at 28-32 °C than at 20-24 °C), it did not affect significantly its incidence in the leaves of *P. oceanica* (Figure 4A; Tables 1, S2 and S3).

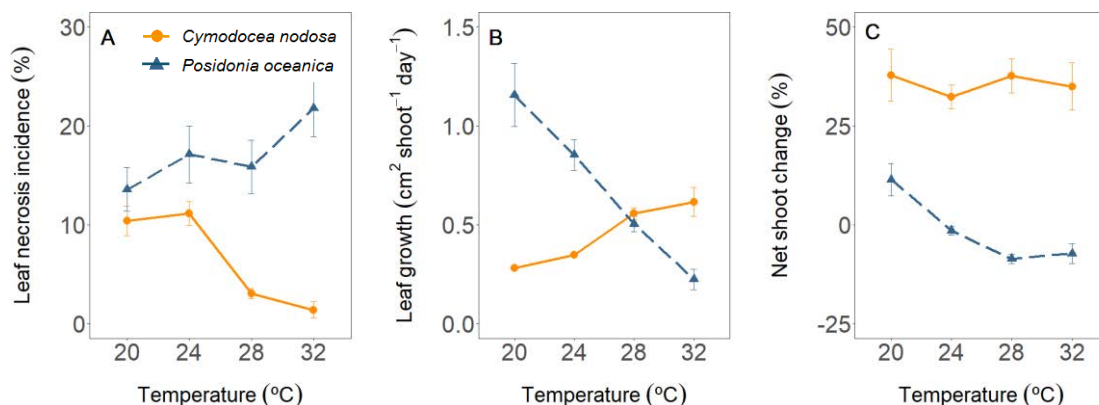


Figure 4. (A) Necrosis incidence on leaves, (B) growth rate, and (C) net shoot change of *P. oceanica* and *C. nodosa* plants after being exposed to a four temperatures range for five weeks (mean \pm SE, $n=3$).

The shoot growth rate, significantly higher in *P. oceanica* than in *C. nodosa* at low temperatures (20 and 24 °C), showed a response to warming clearly contrasting among the two species. Thus, while *C. nodosa* growth was stimulated by warming (up to 2-fold higher at 32 °C relative to control), a drastic and continuous decline across the experimental temperature range was observed in *P. oceanica* (growth values at 32 °C 81 % less than at 20 °C) (Figure 4B; Tables 1, S2 and S3).

Net shoot change was significantly higher in *C. nodosa* than in *P. oceanica* (Figure 4C; Table 1). Temperature only had a significant effect in *P. oceanica* where the balance became negative at 28 °C and 32 °C (Figure 4C; Tables 1 and S2).

Potential thermotolerance defence mechanisms

Heat dissipation pathway: Non-photochemical quenching (NPQ) and xanthophyll cycle pigments

NPQ was significantly higher (ca. two-fold) in *C. nodosa* than in *P. oceanica* and it did not show any variation with temperatures below 32 °C, temperature at which both species reacted oppositely (Figure 5A; Tables 1, S2 and S3), increasing in *P. oceanica* and decreasing (not statistically significant) in *C. nodosa* (but see significant interaction in Table 1).

Violaxanthin (V) was the most abundant xanthophyll pigment in both species, with no significant differences among them (Figure 5B). In contrast, higher concentrations of xanthophylls in a de-epoxidated state, antheraxanthin (A) and zeaxanthin (Z), were found in *C. nodosa* than in *P. oceanica* leaves (Figure 5C and 5D). Consequently, either VAZ (total xanthophyll content:total chlorophyll molar ratio) and AZ (A+Z:total chlorophyll molar ratio) were also significantly higher in *C. nodosa* than in *P. oceanica* (Figure S3). The xanthophyll de-epoxidation rate (DR) was up to 41 % higher in *C. nodosa* due to the higher content of A and Z (relative to V values), in comparison to *P. oceanica* (Figure 5E; Tables 1 and S1). The only temperature effect was found in Z content in *C. nodosa* leaves, which significantly decreased with temperature (Figure 5D; Tables 1 and S3). VAZ and AZ showed this same decreasing trend (Figure S3; Table 1), as well as DR (Figure 5E; Table 1), which was found

to be 18 % lower at 32 °C than at 20 °C. Moreover, both Z content and DR showed a significantly different response to temperature between both species (Figure 5D and 5E; Tables 1, S2 and S3, see significant interaction), with a mild increasing trend in *P. oceanica* whose statistical detection was precluded by the high variability of values at 32 °C.

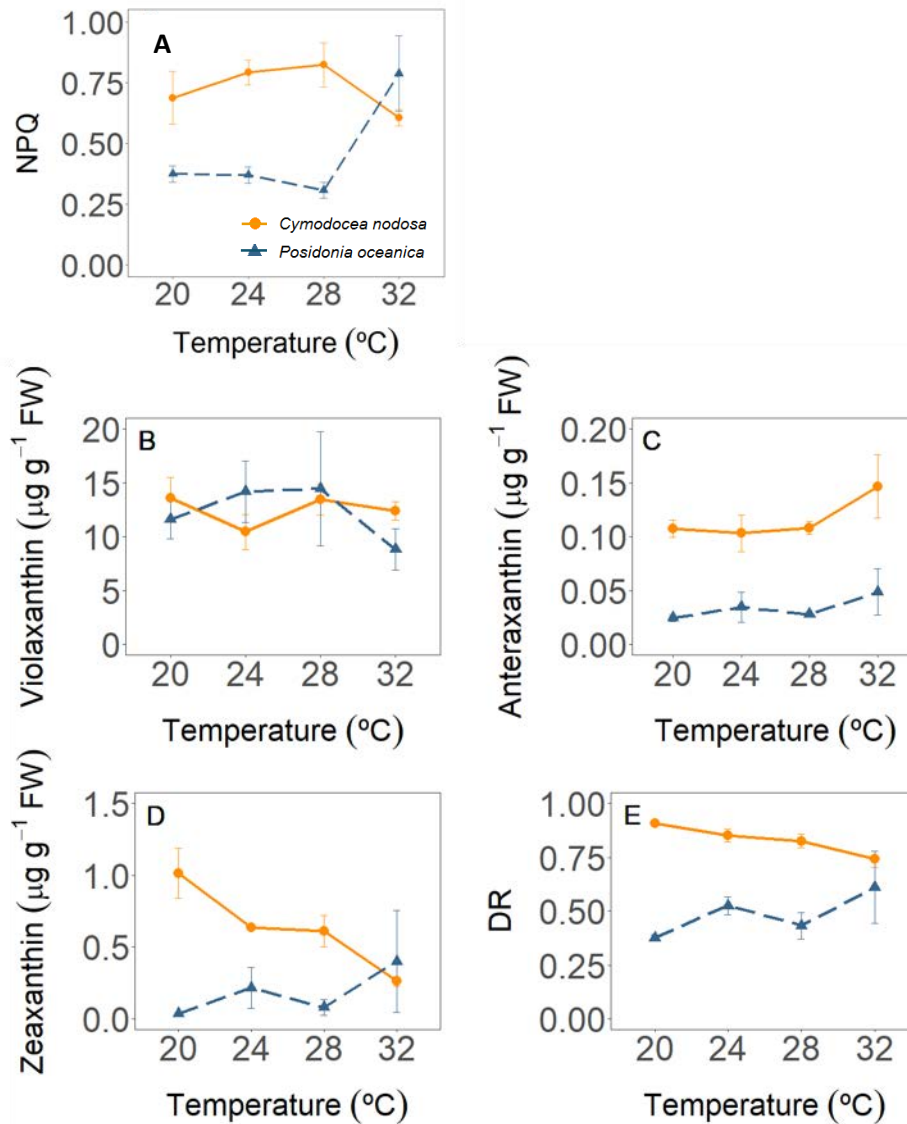


Figure 5. Energy dissipation mechanisms in *P. oceanica* and *C. nodosa* plants after being exposed to a range of four temperatures for five weeks (mean \pm SE, $n=3$). (A) non-photochemical quenching (NPQ) and (B-D) xanthophylls cycle pigments content: (B) Leaf violaxanthin, (C) antheraxanthin and (D) zeaxanthin concentration. (E) xanthophyll de-epoxidation ratio (DR).

Antioxidant enzyme activities and membrane lipid peroxidation

C. nodosa exhibited higher activity of antioxidant enzymes than *P. oceanica* at all temperatures, except a few cases (Figure 6A-F; Table 1). These differences were particularly remarkable for GPOX, with values in *C. nodosa* ca. one order of magnitude higher than those measured in *P. oceanica* (Figure 6D).

The response to temperature followed different trends in both species, the enzymatic activities tending to increase in *C. nodosa* and to decrease in *P. oceanica*, as indicated by the significant interactions between species and temperature in GPX, GST, SOD and DHAR. Specifically, in *C. nodosa*, SOD doubled and GPX was 3-fold higher at high temperatures (28 °C and 32 °C) relative to control (Figure 6A and 6E), while DHAR also increased, although in the threshold of significance ($p=0.05$, Figure 6F) (Tables 1 and S3). In the case of *P. oceanica*, the activity of GST, GPOX and SOD significantly decreased at increasing temperatures (Figure 6C, 6D and 6E).

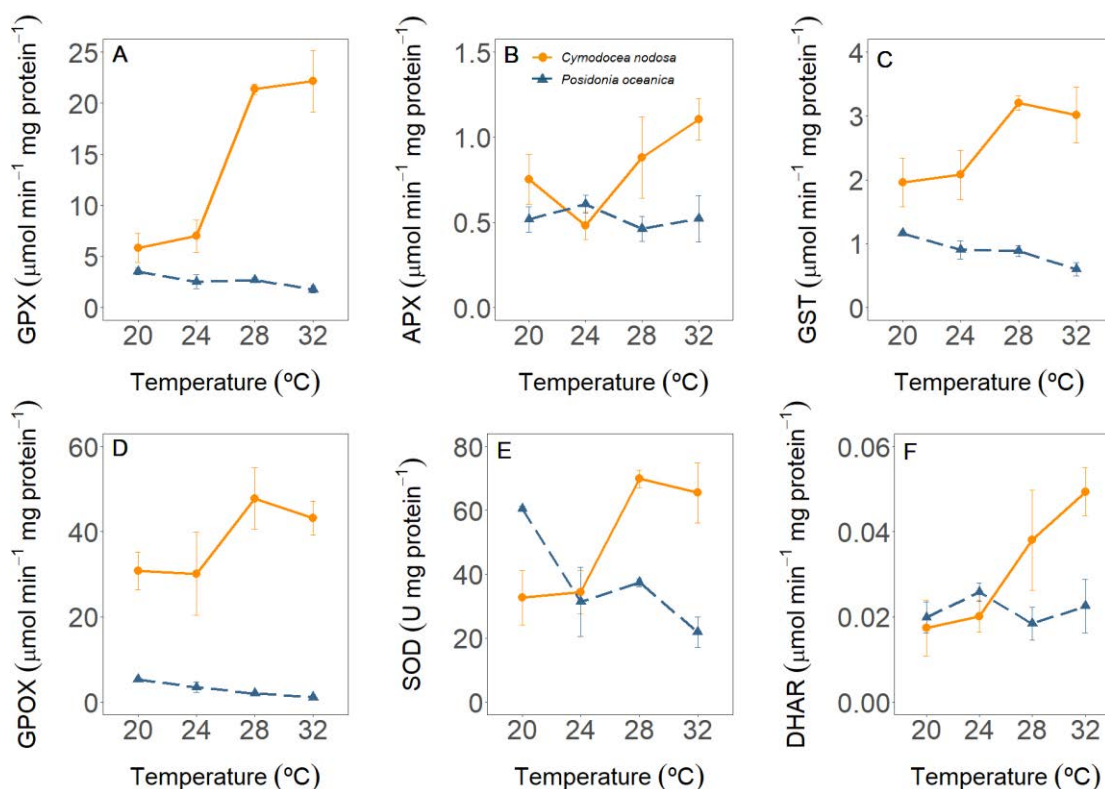


Figure 6. Activity of antioxidative enzymes (mean \pm SE, $n=3$). (A) Glutathione peroxidase (GPX), (B) ascorbate peroxidase (APX), (C) glutathione-S-transferase (GST), (D) guaiacol peroxidase (GPOX), (E) superoxide dismutase (SOD) and (F) dehydroascorbate reductase (DHAR) measured in shoots of *P. oceanica* and *C. nodosa* exposed to four temperatures for five weeks.

The concentration of MDA measured in leaves of *P. oceanica* was consistently higher (over 2-fold higher) than in those from *C. nodosa* (Figure S4; Table S1). Increasing temperature had no apparent effects in the lipid peroxidation level of leaf tissues of both species (Tables 1, S2 and S3).

Table 1. Summary of the results from univariate PERMANOVA (left) testing the significance of effects of the two independent variables, species (*P. oceanica* (P) and *C. nodosa* (C)) and temperature (20 °C, 24 °C, 28 °C and 32 °C), on the different plant responses assessment (dependent variables). ANOVA was applied to detect within- species significance on the effects of temperature on the same response variables. Numbers in bold indicate significant effects ($p < 0.05$). Post hoc indicates the significance of pairwise differences. In the species column (PERMANOVA), there is also indicated (following post-hoc analysis) the sign of the differences (P: *P. oceanica*; C: *C. nodosa*). More details of this analysis can be found on table S1).

Variable	PERMANOVA			ANOVA										
	Species		Temp	Sp x Temp	<i>P. oceanica</i>					<i>C. nodosa</i>				
					Post hoc					Post hoc				
	<i>p</i> -valor		<i>p</i> -valor	<i>p</i> -valor	<i>p</i> -valor	20°C	24°C	28°C	32°C	<i>p</i> -valor	20°C	24°C	28°C	32°C
F _v /F _m	0.0001	<i>P > C</i>	0.0003	0.001	0.00002	a	b	ab	c	0.2537				
ΔF/F _m '	0.0167	<i>P > C</i>	0.0002	0.0006	0.0003	a	ab	b	c	0.0181	a	b	b	b
ETR _{max}	0.0001	<i>C > P</i>	0.0007	0.0025	0.0286	a	ab	a	b	0.0030	a	a	b	a
NPQ	0.0003	<i>C > P</i>	0.2279	0.0016	0.0051	a	a	a	b	0.2495				
Chla	0.0718		0.5004	0.6558	0.6218					0.4405				
Chlb	0.8565		0.4833	0.5877	0.5646					0.3730				
Chlb/Chla	0.0001	<i>P > C</i>	0.1065	0.1503	0.103					0.554				
Gross P	0.0023	<i>C > P</i>	0.7295	0.0002	0.0025	a	a	a	b	0.1094				
R _d	0.7823		0.3392	0.4093	0.3073					0.5440				
P:R _d	0.0303	<i>C > P</i>	0.2906	0.0007	0.0489	a	ab	ab	b	0.0094	a	a	a	b
Starch Leaf	0.0001	<i>C > P</i>	0.0223	0.6748	0.4965					0.0572				
Soluble Leaf	0.0001	<i>C > P</i>	0.0148	0.3842	0.2055					0.0627				
NSCs Leaf	0.0001	<i>C > P</i>	0.013	0.6988	0.3098					0.0697				
Starch Rhizome	0.0005	<i>C > P</i>	0.0072	0.0005	0.1645					0.0001	ab	a	b	c
Soluble Rhizome	0.0001	<i>C > P</i>	0.0118	0.0022	0.2770					0.0111	ab	b	a	a
NSCs Rhizome	0.0001	<i>C > P</i>	0.011	0.0016	0.2130					0.0086	ab	b	a	a
Necrosis	0.0001	<i>P > C</i>	0.1867	0.0033	0.2501					0.0004	a	a	b	b
Leaf growth	0.0007	<i>P > C</i>	0.005	0.0001	0.0006	a	a	b	b	0.0006	a	a	b	b
Net shoot change	0.0001	<i>C > P</i>	0.0508	0.1102	0.0020	a	b	b	b	0.8597				
Violaxanthin	0.9208		0.6617	0.5126	0.6107					0.4684				
Anteraxanthin	0.0001	<i>C > P</i>	0.1663	0.8206	0.5942					0.3320				
Zeaxanthin	0.0014	<i>C > P</i>	0.5944	0.0139	0.5048					0.0030	a	a	ab	b
VAZ	0.0023	<i>C > P</i>	0.628	0.0113	0.4271					0.0056	a	ab	b	b
AZ	0.0025	<i>C > P</i>	0.627	0.0091	0.4304					0.0057	a	ab	b	b
DR	0.0001	<i>C > P</i>	0.8187	0.0414	0.3369					0.0211	a	ab	ab	b
GPX	0.0001	<i>C > P</i>	0.0001	0.0002	0.1102					0.0003	a	a	b	b
APX	0.0069	<i>C > P</i>	0.2416	0.0698	0.7210					0.1125				
GST	0.0001	<i>C > P</i>	0.1746	0.0147	0.0247	a	ab	ab	b	0.0790				
GPOX	0.0001	<i>C > P</i>	0.3344	0.1139	0.0028	a	ab	bc	c	0.2360				
SOD	0.0162	<i>C > P</i>	0.0502	0.0003	0.0099	a	b	ab	b	0.0112	a	a	b	ab
DHAR	0.0398	<i>C > P</i>	0.0659	0.0415	0.6380					0.0500				
MDA	0.0001	<i>P > C</i>	0.9684	0.6963	0.8558					0.6003				

Overview of the inter- and intraspecific different responses to warming

The multivariate analysis summarized clearly the results of our experiment. Thus, axis 1 of the PCA (Figure 7), explaining 48 % of total variance, absorbed interspecific differences, clearly separating the observations corresponding to *C. nodosa* (positive part of the axis) from

those corresponding to *P. oceanica* (negative part of the axis). The variables with the highest factor loadings on this axis (values > 0.6) were net shoot change, maximum electron transport rate, non-structural carbohydrates content (soluble + starch), maximum net photosynthetic rate, soluble carbohydrates content, anteraxanthin content, de-epoxidation ratio, some antioxidant activities (GPX, APX, GST and GPOX (positive loadings), leaf necrosis and MDA concentration (negative loading).

In turn, axis 2, explaining 18 % of the total variance, and was much related to temperature. Interestingly, observations corresponding to low temperatures appeared in the positive part of the axis for *C. nodosa*, while they appeared in the negative part for *P. oceanica*, illustrating the different response to thermal stress among the two species. The variables with the highest factor loadings on this axis (values > 0.6) were leaf growth, photosynthetic rates (both gross and net), P/R_d balance and zeaxanthin content, VAZ, AZ and effective quantum yield.

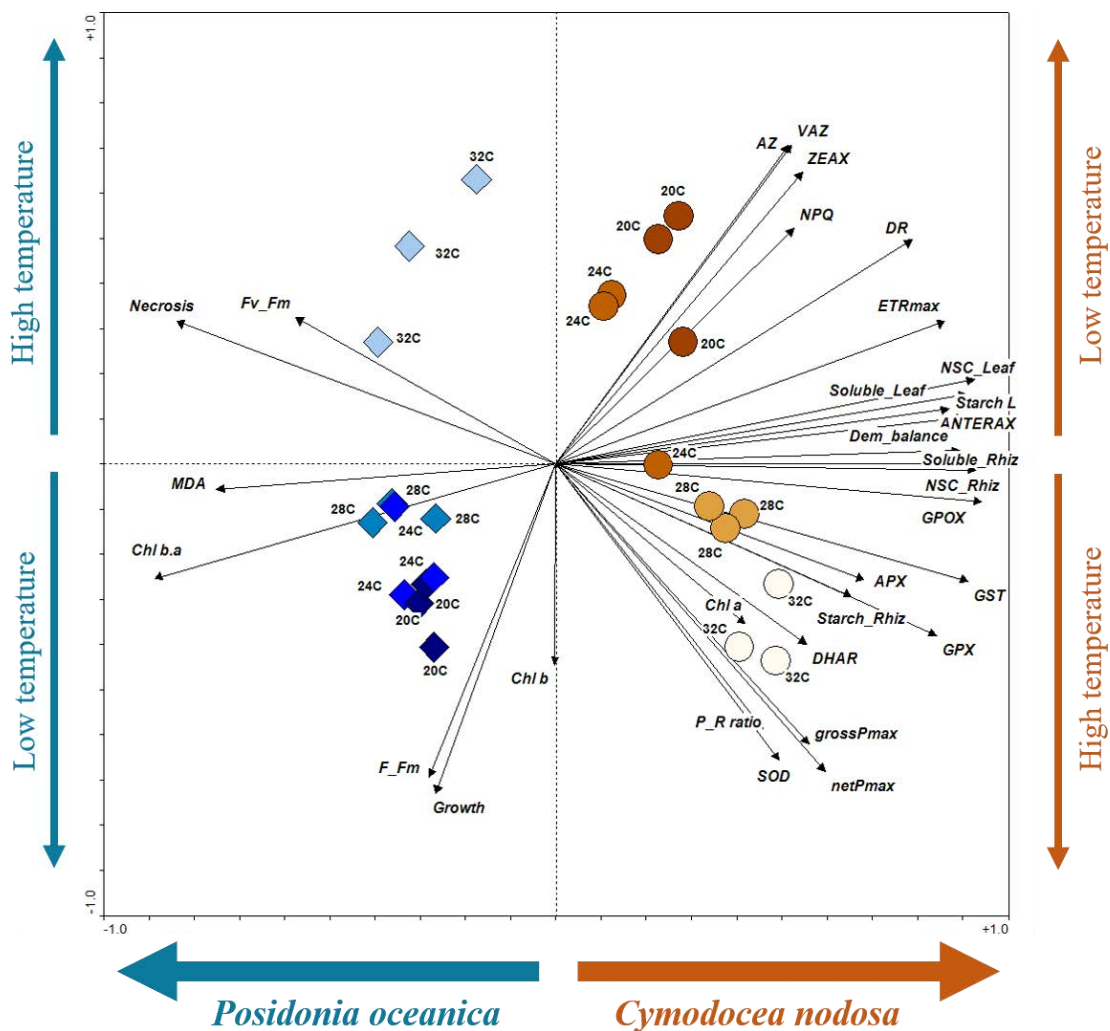


Figure 7. Plot of the principal component analyses performed including the different plant variables measured in *P. oceanica* and *C. nodosa* at the end of the five weeks exposure period to four thermal treatments. Data from *P. oceanica* are represented with blue rhombus while those from *C. nodosa* aquaria are represented with orange circles. The intensity of the tone (from clear, 32 °C, to dark, 20 °C) indicates the thermal treatment. The factor loadings of the different variables are represented as vectors (See Table S4 for the abbreviations).

DISCUSSION

This study reveals deep quantitative and qualitative differences in the responses to warming between the two dominant Mediterranean seagrass species, *P. oceanica* and *C. nodosa*, evidencing contrasting strategies to cope with thermal stress. In general, *P. oceanica* performance (i.e. leaf carbon balance, shoot growth and net shoot change) tends to be negatively affected by temperatures higher than normal (28 and 32 °C), while, oppositely, *C. nodosa* seems to perform better at those temperatures (up to 32 °C), which stimulate photosynthetic and growth rates and lowers leaf necrosis incidence. Our results suggest that this differential response is based upon the capacity of *C. nodosa* for activating protective mechanisms such as increasing the activity of antioxidant enzymes, mechanism which does not seem to operate in *P. oceanica* where, if any, the only (and with limited effects) protective mechanism appears to be related to dissipation pathways of energy excess (NPQ and xanthophyll cycle). Although no accurate predictions can be obtained from these results, it seems likely that, under a future scenario of temperature rise, changes in Mediterranean seagrass vegetation should be expected, with beneficial effects for *C. nodosa* and detrimental effects for *P. oceanica*.

Some of the negative effects of warming on *P. oceanica* reported here were already known from previous works. Specifically, the sharp decline in the gross photosynthesis rate at 32 °C, coincides with available evidences of negative effects of such high temperatures on plant photochemical efficiency (Ontoria et al., 2019a; Savva et al., 2018; Marín-Guirao et al., 2016), suggesting an upper thermal threshold likely between 28 and 32 °C. This drop in gross photosynthesis was the main driver of the observed leaf carbon balance impairment because, in contrast with results from previous works (Marín-Guirao et al., 2018), the respiration was unaffected by temperature increase. The negative effects of warming were even more evident at individual and population levels, as both shoot growth and net shoot change began to decrease before (i.e. at lower temperature) plant photosynthesis (24 °C and 28 °C for shoot growth and net shoot change, respectively; 32 °C for photosynthesis). This agrees with the idea that the optimum temperature for photosynthesis is usually higher than for growth (Lee et al., 2007) or, in other words, that growth is more sensitive to warming than photosynthesis, at least in the short term. This, in turn, has an obvious methodological implication: assessments based solely on photosynthesis can overestimate thermal threshold of the species. The “moderately warm” temperature used in the study (28 °C) can be experienced by the plants in present days (on average, 17 days yr⁻¹ in the last four years, 2015-2018, at the site from where plants were collected), suggesting that temperature-driven growth depression is already taking place in this species. In spite of this, there were no evidences of the induction of effective thermal tolerance mechanisms to cope with thermal stress. At this respect, the only finding suggesting some protective behaviour was an increase in non-photochemical quenching (NPQ) in plants experimentally exposed to 32 °C, as previously reported by Marín-Guirao et al. (2016). This, coupled to the mild (and non-significant) reduction of violaxanthin (V) content and increase in anteraxanthin (A), zeaxanthin (Z) and de-epoxidation ratio (DR) at this temperature, could be suggestive of a weak photoprotective response. In effect, it is known that xanthophyll intermediate pigments induce a photoprotective response when plants are exposed to stressful conditions, such as high

irradiance (Marín-Guirao et al., 2013a; Ralph et al., 2002). Unfortunately, this xanthophyll cycle mechanism has been poorly studied on seagrasses (Collier et al., 2008; Dawson and Dennison, 1996) and never, to our knowledge, described under thermal stress. No activation of antioxidant enzymes was detected, suggesting inability to combat increased ROS levels caused by warming, with concomitant plant damage (Huang et al., 2019; Mittler 2002).

The response to warming of *C. nodosa* greatly differed from that of *P. oceanica*, with a significant improvement of leaf carbon balance at 32 °C, again driven by the increase in gross photosynthesis rate. In addition, *C. nodosa* accumulates more carbohydrates in its rhizomes than *P. oceanica*; yet the accumulation of carbohydrates entails a major capacity of plants to tolerate heat stress and other abiotic factors, as reported for terrestrial plants (Liu and Huang, 2000; Wahid et al., 2007) and also for seagrasses (Marín-Guirao et al., 2016; Sandoval-Gil et al., 2012a). Warming also improved other plant performances, such as the leaf growth, confirming that the thermal optimum for *C. nodosa* should be slightly above 32 °C, as previous works reported negative effects at 35 °C (Marín-Guirao et al., 2016; Ontoria et al., 2019b; Pérez and Romero, 1992).

The good performances of *C. nodosa* at high temperature seems associated to the existence of effective protective mechanisms against thermal stress. This was reflected, to some extent, in values of NPQ and xanthophyll content higher than those found in *P. oceanica* but, more evidently, in the activation of antioxidant enzymes, evidencing the ability of this species to detoxify the accumulated ROS and offering an efficient protection mechanism against their toxicity. This is supported by the low levels of MDA found, which is considered a proxy of damage of the cell membrane due to lipid peroxidation by excess of ROS (Valenzuela, 1991).

Part of the response to heat stress relies on inherent physiological properties such as, in our case, the carbohydrates content, higher in *C. nodosa* than in *P. oceanica*. However, a large part of the species capacity to cope with thermal stress relies on the species phenotypic plasticity, which allows acclimation to changes and is, in turn, an adaptive trait in itself (Dudley 2004; Nicotra et al., 2010). At this respect, *C. nodosa* seems clearly to display higher plasticity, as indicated by the activation of antioxidant enzymes, resulting in a better acclimation to warming. A great plasticity relative to other environmental drivers of this species had also been reported in previous works (Pérez et al., 1994).

These contrasting behaviours facing warming in two seagrass species with overlapping geographical distribution has several possible explanations, rooted either in their phylogeography, their ecological strategy or their specific habitat requirements. On the one hand, the high thermotolerance of *C. nodosa* coincides with its biogeographical affinities and distribution not only in temperate (southern Portugal, Mediterranean) but also in subtropical (Canary islands) and tropical areas (Senegal, Cape Verde), being the rest of the species belonging to that genus of tropical affinities (Green and Short, 2003). In contrast, the distribution of *P. oceanica* is limited to the Mediterranean, with a much narrower thermal range, and the other species of the genus *Posidonia* inhabit also in temperate waters (in Australia, Green and Short, 2003). On the other hand, on the basis of their life-history traits, *C. nodosa* is classified as opportunistic, while *P. oceanica* is considered persistent (Kilminster et al., 2015). Yet it is known that opportunistic species are, in general terms, much more able

to cope with more stressful conditions, as is the case, for example, of hypersalinity stress, to which *C. nodosa* is much more resistant than *P. oceanica* (Fernández-Torquemada and Sánchez-Lizaso, 2005; Ruiz et al., 2009; Marín-Guirao et al., 2011; Sandoval-Gil et al., 2012a, 2012b) and also, as shown here, in the case of thermal stress. Finally, each species has specific habitat requirements. While *C. nodosa* is distributed from the open sea to confined or semi-confined coastal lagoons, where it grows well within a wide range of nutrient regimes, salinity and temperature (e.g. from 8 °C in winter to 32 °C in summer, personal observations and Ontoria et al., 2019b), *P. oceanica* distribution is restricted to infralittoral bottoms in open coasts or bays with a relatively high water exchange with the open sea (Procaccini et al., 2003), under environmental conditions less fluctuating and within a narrower range of variation.

Independently of these causal explanations, the experimental fact of a greater tolerance to warming in *C. nodosa* than in *P. oceanica* has, potentially, great consequences for the future of coastal Mediterranean ecosystems. However, and when analyzing such consequences, it should be reminded that our approach, based on mesocosms, has evidences, and all caution should be exerted at this respect when extrapolating our results to the possible changes at the ecosystem and landscape levels. Among those limitations, the impossibility to properly capture intraspecific variability (see Marín-Guirao et al., 2016; Winters et al., 2011), the absence in mesocosms of community level processes (i.e. biotic interactions, see Pagès et al., 2018) or the potential interaction of temperature with other stress agents (e.g. Collier et al., 2011, 2016; Egea et al., 2018; Koch et al., 2007; Ontoria et al., 2019a, 2019b) are maybe the most relevant.

Nonetheless, and despite these acknowledged limitations, the pronounced differential thermotolerance between the two main Mediterranean species, assessed within a thermal range close to what is expected to occur in the Mediterranean in a relatively near future, is beyond doubt. To a greater or to a lesser extent, warming, in the framework of realistic future scenarios, will be more favorable for one species than for the other and hence, temperature increase is reasonably expected to become a relevant driver of changes in the composition and distribution of the seagrass meadows at the Mediterranean scale. At the light of what is reported here, *C. nodosa* seems much more capable than its counterpart, *P. oceanica*, not only to cope with the warmer thermal conditions predicted to occur in the Mediterranean over the 21st century, but also to perform better than nowadays under those conditions. Thus, *P. oceanica* meadows might suffer significant alterations of their structure and functions, or even some degree of retreat of their distribution, maybe in favour to other opportunistic and more thermotolerant species, including *C. nodosa*. The extension of the meadows of the latter and the retraction of the meadows of the former will, undoubtedly, imply severe consequences for the marine coastal ecosystem as well as for the goods and services those ecosystems provide.

ACKNOWLEDGEMENTS

We thank Arantxa Ramos Segura and Neus Sanmartí for their help in the experimental set up and laboratory work, and Carla María Stinco and Antonio Meléndez-Martínez (Food Colour and Quality Laboratory, University of Sevilla) for the xanthophylls analysis. This

work was supported by the European Union and the Spanish Government through the RECCAM (Seagrass meadows resilience to global warming: an analysis based on responses at ecophysiological, population and ecosystem levels, CTM2013-48027-C3-1-R and CTM2013-48027-C3-2-R) and UMBRAL (Responses of benthic marine vegetation to stress: critical transitions, resilience, and management opportunities, CTM2017-86695-C3-1-R) projects; and by the Spanish Ministry of Economy and Competitiveness (via grant BES-2014-069593 awarded to Y. Ontoria).

CHAPTER 2

Interactive effects of global warming and eutrophication on a fast-growing Mediterranean seagrass

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ABSTRACT

Coastal ecosystems, such as seagrasses, are subjected to local (e.g. eutrophication) and global (e.g. warming) stressors. While the separate effects of warming and eutrophication on seagrasses are relatively well known, their joint effects remain largely unstudied. In order to fill this gap, and using *Cymodocea nodosa* as a model species, we assessed the joint effects of warming (three temperatures, 20 °C, 30 °C and 35 °C) with two potential outcomes of eutrophication: (i) increase in nutrients concentration in the water column (30 and 300 µM), and (ii) organic enrichment in the sediment). Our results confirm that temperature in isolation clearly affects plant performance; while plants exposed to 30 °C performed better than control plants, plants exposed to 35 °C showed clear symptoms of deterioration (e.g. decline of photosynthetic capacity, increase of incidence of necrotic tissue). Plants were unaffected by high ammonium concentrations; however, organic enrichment of sediment had deleterious effects on plant function (photosynthesis, growth, demographic balance). Interestingly, these negative effects were exacerbated by increased temperature. Our findings indicate that in addition to the possibility of the persistence of *C. nodosa* being directly jeopardized by temperature increase, the joint effects of warming and eutrophication may further curtail its survival. This should be taken into consideration in both predictions of climate change consequences and in local planning.

INTRODUCTION

Coastal ecosystems are facing multiple anthropogenic stressors that adversely affect their biodiversity and functioning (Vinebrooke et al., 2004). Such stressors are generated at a range of spatial scales, from the global to the most local. Global stressors are mostly related to climate change and include rising sea level, seawater acidification, warming and an increased frequency of heat waves (IPCC, 2013). The most prominent and pervasive stressor generated locally is probably eutrophication: increased loading of nutrients and organic matter from human activities (Nixon 2009). This has come to be considered one of the major threats confronting coastal ecosystems (Bricker et al., 2008; Hemminga and Duarte, 2000). The knowledge accumulated to date on the effects of individual stressors on key species is impressive. However, stressors rarely occur in isolation in the environment and, when acting together, they can be synergistic, additive or antagonistic (Todgham and Stillman, 2013). The interaction between stressors is now viewed as a crucial issue, to the point that it is recognized that single-factor experiments are of limited use for assessing the effects of climate change on coastal marine ecosystems subjected to other disturbances, such as eutrophication (Wernberg et al., 2012). Undoubtedly, experiments with single stressors can help us gain knowledge of the intrinsic, basic response mechanisms involved. However, the results should only be extrapolated to nature with great caution, not only due to problems associated with scaling up, but also due to potential interactions with concurrent stressors (Gunderson et al., 2016).

Seagrasses are widespread habitat-forming species of great ecological value that are exposed to multiple threats and are currently suffering declines worldwide (Waycott et al., 2009). The effects of climate change (including increased temperature and acidification) or eutrophication on the distribution, abundance and vitality of seagrasses are relatively well known (see reviews by Koch et al., 2013, for climate change; and Burkholder et al., 2007, for eutrophication), and even though their effects have, for the most part, been assessed separately (but see Campbell and Fourqurean, 2014, 2018; Ow et al., 2016). Thus, it is well known that eutrophication has two main consequences for seagrasses. On the one hand, the effects of increased nutrient concentrations are generally considered detrimental, although they strongly depend on species-specific features and on local conditions (Kilminster et al., 2015; Romero et al., 2006; Ruiz et al., 2001). Thus, while a moderate supply of nutrients to plants adapted to nutrient-poor environments can stimulate growth (Alcoverro et al., 1997; Pérez et al., 1991; Short, 1987), once a threshold is reached, it may cause negative effects on plant photosynthesis and may even curtail survival (Brun et al., 2002, 2008; Hauxwell and Valiela, 2003; van der Heide et al., 2008). These negative effects can be caused directly, mainly by ammonium toxicity (Touchette and Burkholder, 2000; van Katwijk et al., 1997); or indirectly, by stimulating phytoplanktonic, epiphytic and macroalgal overgrowth, and enhancing negative biotic interactions such as macro herbivore activity (Campbell et al., 2018; Ruiz et al., 2009; Wear et al., 1999). On the other hand, an increased supply of organic matter to the seagrass sediment, such as that caused by eutrophication, stimulates its oxygen demand, eventually leading to hypoxic or anoxic conditions (Frederiksen et al., 2008; Pérez et al., 2007). This oxygen shortage not only blocks metabolic function in seagrass roots, including respiration, growth and nutrient acquisition (Smith et al., 1988), but it also

stimulates microbial sulphate reduction, which leads to belowground seagrass organs (rhizomes and, specially, roots) being exposed to sulphide, a strong phytotoxin (Holmer and Bondagarrd, 2001). Despite seagrass having evolved a number of adaptations which increase its chances of surviving in naturally organic-rich sediments (Hasler-Sheetal and Homer, 2015), additional deposition of organic C can exceed the seagrass response capacity, and have negative effects such as reduced photosynthesis, impaired growth or, in some cases, mass mortality (Collier and Waycott, 2014; Frederiksen et al., 2008; Koch et al., 2007; Olivé et al., 2009).

Temperature affects seagrass physiology in a number of ways. It is known that increased temperature usually stimulates both photosynthesis (Campbell et al., 2006; Winters et al., 2011) and respiration (Schulze et al., 2005); but beyond some threshold, it generally increases the latter more than the former, thus leading to an impaired C balance and reduced growth (Lee et al., 2005, 2007; Marín-Guirao et al., 2016, 2018; Pérez and Romero, 1992). Temperature also affects other processes, such as for instance nutrient uptake (Borum et al., 2004; Bulthuis, 1987) or protein synthesis (Campbell et al., 2006; Marín-Guirao et al., 2017). Overall, when the temperature exceeds a given threshold, which is largely species specific, thermal stress leads to a reduction in growth (Lee et al., 2007), deterioration of shoot status and eventually shoot mortality (Marbà and Duarte 2010). The responses of seagrasses to increased temperature are relatively well documented; however, little is known of the potential distortion of these responses caused by eutrophication.

Global warming is expected to increase in the coming decades and will affect the surface waters of almost all of the world's oceans. Meanwhile, a large part of the planet's coastal areas are subjected to different degrees of eutrophication (Halpern et al., 2007), which is especially notable in industrialized countries. Consequently, many cases, thermal stress will have an impact on meadows already affected by chronic or acute eutrophication, whose responses to thermal stress will probably differ from that of unaffected plants, thus limiting our ability to make reliable and realistic predictions for future warming scenarios. To date, only a few studies have focused on the combined effects of warming and other stressors, such as anoxia (Koch et al., 2007, with *Halodule wrightii* and *Thalassia testudinum*), nutrients (Kaldy 2014, with *Zostera marina*) or light (York et al., 2013 with *Zostera muelleri*). These works seem to suggest that synergistic effects are more the rule than the exception. If this is the case, the consequences of global warming may be worse than expected based solely on studies of thermal effects. In fact, a synergistic interaction between eutrophication and seawater warming has already been suggested for the Mediterranean seagrass *Posidonia oceanica* to forecast trajectories in abundance and distribution of this seagrass species in the context of the different global climate change scenarios (Jordà et al., 2012). However, a considerable gap exists in our knowledge of the combined effects of warming and other stressors; and research is needed to confirm (or refute) the potential synergies in seagrasses, especially in species that dominate areas that are particularly sensitive to climate change.

The present study attempts to help fill this gap, by evaluating the joint effect of warming and eutrophication on a Mediterranean seagrass (*Cymodocea nodosa*). The Mediterranean is one of the regions that are expected to be most affected by warming, and

the sea surface temperature rise, already in evidence (Burrows et al., 2011; Jordà et al., 2013) may reach 3 °C by the end of the 21st century (Jordà et al., 2012); while the frequency of heat waves is also expected to increase (IPCC, 2013). Moreover, eutrophication has been identified as one of the major environmental threats to seagrass habitats in coastal areas, mainly due to loading from urban, agricultural and aquaculture wastes, particularly in the more confined environments where *C. nodosa* is dominant (Boudouresque et al., 2009). *C. nodosa* is widely distributed across a broad variety of shallow Mediterranean environments, from open coastal areas to coastal lagoons, and extends into the Atlantic, from the south of the Iberian Peninsula to the Canary Islands and Mauritania (Green and Short 2003; Mascaró et al., 2009b; Reyes et al., 1995a). Its ecological value and its capacity to survive relatively eutrophic conditions (Oliva et al., 2012), as well as its considerable phenotypic plasticity (Pérez et al., 1994; Sandoval-Gil et al., 2014), make it an interesting model species to evaluate the joint effects of increased temperatures and eutrophication.

The aim of this study is thus to explore the combined effect of a global stressor (warming) and a local stressor (eutrophication) on functional traits of *C. nodosa*. We partition the eutrophication effects into an increased nutrient concentration in the water column and an increase of organic matter loading of the sediment. We then determine the response of the plant to each one of the three stressors (elevated temperature, nutrient increase and increased organic matter loading) separately; and also to the combined effects of temperature and each of the other two. The main hypothesis we wish to evaluate is that temperature and eutrophication act synergistically, with deleterious consequences for the seagrass. To this end, we perform two fully factorial experiments on indoor mesocosms in which plants are exposed to three levels of temperature and, on the one hand, to three different nutrient concentrations and, on the other hand, to two different levels of organic matter in the sediment.

MATERIAL AND METHODS

We explored the interactive effects of eutrophication and temperature in two separate experiments. In the first experiment (INUT experiment, hereinafter), temperature increase and nutrient (ammonium) addition were applied; while in the second (TANOX experiment, hereinafter) the stressors were temperature increase and addition of labile organic C to the sediment.

Plant and sediment collection

Undamaged healthy *C. nodosa* shoots (including their rhizomes and roots) were carefully collected by hand from a shallow, undisturbed meadow (0.5 m deep) in Alfacs Bay (NW Mediterranean) in late April. Only shoots less than one year old (less than 12 scars on the vertical rhizome, Mascaró et al., 2014) were selected to reduce the effects of physiological and morphological variability between shoots of different ages (Pagès et al., 2010; Pérez and Romero, 1994). Sediment was collected from the same area, extracting the surface layer (up to 10 cm deep), and immediately sieved (1 mm pore) to exclude macroinvertebrates and

detritus. Sediment and plants were then transported separately in aerated tanks to the laboratory, where they were maintained with aeration for one night prior to the experiment being setup. Temperature was kept constant at the ambient values measured at the collection site (19.5 °C). The experiments were conducted at the Experimental Chambers Service of the University of Barcelona.

Experimental design and setup

Both experiments were conducted using cylindrical transparent aquaria (12 L capacity, 40 cm height x 20 cm diameter) placed randomly in 3 experimental chambers (2.1 m²). Each aquarium had an independent air pump providing proper aeration. The chambers allowed us to control the water temperature (20 °C, 30 °C and 35 °C) and incident light (270 μmol photons m⁻² s⁻¹), which was above the saturation irradiance for these plants (Pérez and Romero, 1992) on a 12 h:12 h light:dark photoperiod. To avoid experimental bias and minimize any uncontrolled variability, the aquaria were randomly relocated within the chambers every two days. Moreover, the aquaria were moved from one chamber to another (changing the chamber temperature) so that they spent approximately 1/3 of the experimental period in each chamber. Within 24 hours of collection, twenty shoots (with their corresponding portion of rhizome and roots) were planted in each aquarium, previously filled with 10 cm of sediment and 9 L of filtered seawater. All the aquaria were covered with plastic film to prevent water evaporation. For the TNUT experiment, a total of 27 aquaria were prepared and distributed randomly in groups of 9 in the three experimental chambers; while for the TANOX experiment, a total of 18 aquaria were distributed randomly in groups of 6 (see experimental setting in Figure. 1).

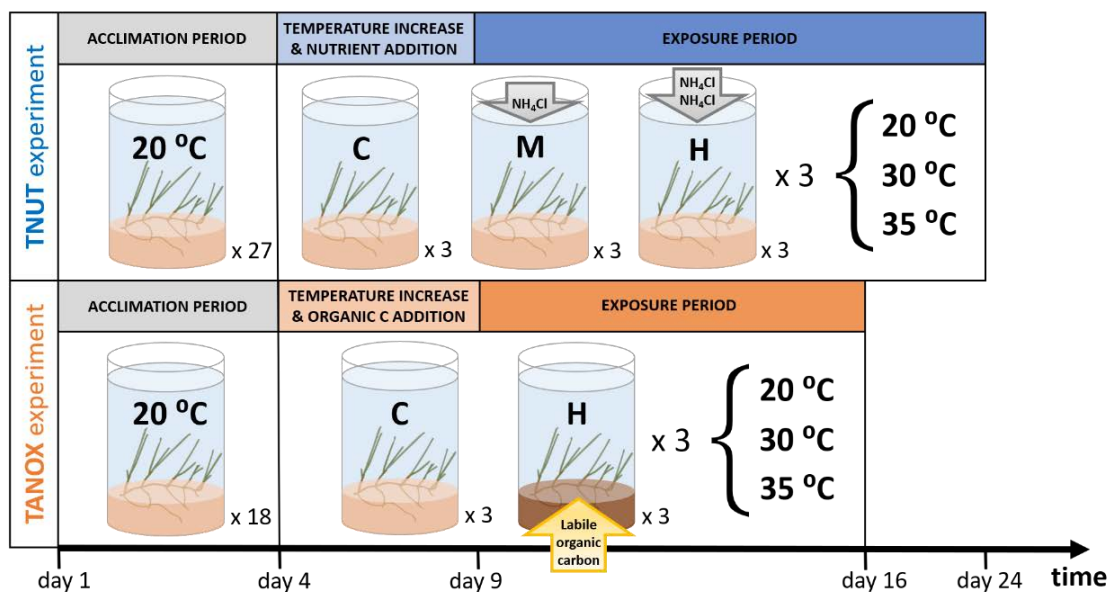


Figure 1. Experimental setting. Grey arrows indicate ammonium addition in water and yellow arrow indicates labile organic carbon addition to sediment. TREATMENTS: C, control; M, moderate and H, high (see text). Temporal axis indicates: day 1, the beginning of the experimental setup with acclimation at control temperature; day 4, end of acclimation period and progressive increase of temperature; day 9, nutrient or labile organic carbon addition and start of the exposure period; day 16, end of TANOX experiment (7 days exposition); day 24, end of TNUT experiment (15 days exposition).

The aquaria were kept at 20 °C for four days (a temperature close to that registered during sampling) to allow for plant acclimation. After the acclimation period, the temperature in two chambers was increased progressively (ca. 3 °C per day) until reaching 30 °C in one and 35 °C in the other. The third chamber was left at 20 °C as a control. The choice of the experimental temperatures was based on an unpublished 3-year temperature data series collected by the authors using continuous *in situ* recorders, indicating average summer (July to September) temperatures close to 30 °C, but peaking to 33 °C in the hottest summer. Based on this, we consider that 35 °C during heat waves is a reasonable assumption under a climate change scenario (IPCC, 2013).

For the TNUT experiment, once the experimental temperatures were reached, NH₄Cl was added to obtain the following concentrations: control (no NH₄Cl added), moderate concentration (30 µM of NH₄Cl) and high concentration (300 µM of NH₄Cl). The so-called “moderate” value (30 µM) can be observed at eutrophic sites and can trigger responses in some seagrass species (Villazán et al., 2013b). The “high” value (300 µM) represents extreme events and would only be reached during some wastewater discharges (Cabaço et al., 2008). To counterbalance plant uptake (roughly estimated from growth requirements, Pérez et al., 1994), NH₄Cl was further added to the experimental aquaria every 5 days: 3.8 mg of NH₄Cl to the moderate ammonium treatment aquaria and 7.6 mg NH₄Cl to the high ammonium treatment aquaria (with a total of two pulses after the initial addition at the beginning of the experiment). Each ammonium treatment was applied to three aquaria, chosen at random, within each temperature chamber, resulting in a complete factorial design with n=3 replicates per experimental condition. The exposure period to both factors lasted 15 days, after which time leaves in the high-temperature treatment (35 °C) began to show critical necrosis marks.

For the TANOX experiment, once the experimental temperature was reached, the organic matter treatments were applied by adding labile organic C in the form of sucrose to the sediment in the aquaria as follows: control (no sucrose addition, 0.7 % DW sediment C content in natural conditions) and high (675 g of sucrose added ≈ 15 % DW sediment C content). The labile organic C treatments were applied to three aquaria, chosen at random, within each temperature chamber, resulting in a complete factorial design with n=3 replicates per experimental condition. The experiment ended after 7 days of exposure period, when leaves in the high-temperature treatment (35 °C) began to show critical necrosis marks.

Water and sediment analysis

The concentration of ammonium in the water was analysed in each aquarium at the beginning and end of the TNUT experiment using an FP 2020 Plus fluorometer and following a standard method (Kérouel and Aminot 1997). The redox potential of the sediment was measured at the end of the TANOX experiment using a Thermo Scientific, Orion Star A211 electrode. Measurements were taken in the upper 5 cm of the sediment layer.

Plant biochemical composition

To verify that the additions of ammonium and labile organic C applied could affect plant conditions, directly (high level of nutrients) and indirectly (anoxic sediment), we determined the N content (TNUT experiment) and S content (TANOX experiment) of different plant parts. To do this, at the end of the experiments, all the remaining shoots were harvested from the aquaria and separated into leaves, rhizomes, and roots. Subsequently, all plant tissues were dried at 60 °C and then finely ground and homogenized; finally, they were weighed and packed into tin microcapsules.

For the TNUT experiment, the nitrogen content of leaves, rhizomes and roots was measured using a Carlo-Erba elemental auto-analyser. For the TANOX experiment, vanadium pentoxide was added, and the sulphur content of leaves, rhizomes and roots was determined. Samples were analysed at the Scientific and Technological Centre (CCiT) of the University of Barcelona.

Measurement of plant traits

The plant responses to the different stressors (or their combination) were assessed via measurement of a series of traits, from the physiological to population level. These included maximum quantum yield of PSII (F_v/F_m), incidence of leaf necrosis, leaf growth, rhizome elongation, and shoot demographic balance. All these variables have previously been used in the assessment of seagrass responses to stress and are related to plant health and performance (Beer et al., 1998; Frederiksen et al., 2008; Maxwell et al., 2000; Pagés et al., 2010; Romero et al., 2007). Rhizome elongation was only determined for the TANOX experiment, while all the other traits were measured in both experiments.

Maximum quantum yield of PSII (F_v/F_m) was determined using a diving PAM (pulse amplitude modulation) fluorometer (Walz, Germany) after 10 min of plant adaptation to dark conditions. Three shoots were randomly selected from each aquarium (avoiding apical shoots due to their more active growth and photosynthesis) and measurements were obtained from the basal portion of the second youngest leaves, to minimize within-shoot variability (Durako and Kunzelman, 2002; Gera et al., 2012).

The incidence of necrosis was assessed at all leaves of five shoots from each experimental condition. Leaves were carefully separated from each shoot and the percentage of necrotic surface, considered as that partially or totally covered by dark brown or black spots, was visually estimated for each leaf and averaged for each aquarium. Leaf growth was measured using a leaf punching method (Zieman, 1974) adapted to the model species (Pérez and Romero, 1994). At the beginning of the experiments, five shoots from each aquarium (avoiding apical shoots) were marked by punching a hole just above the ligule of the outermost leaf with a hypodermic needle. At the end of the experiments, the marked shoots were harvested, epiphytes were removed, and the leaves were carefully separated to measure leaf growth. Shoots were individually sorted into old and newly produced tissues, which were then dried for 48 h at 60 °C before obtaining their dry weights. Leaf growth rate was expressed as the new tissue produced per shoot and day ($\text{mg DW shoot}^{-1} \text{day}^{-1}$), averaged for

each aquarium. To measure rhizome growth, we marked two apical shoots per aquarium with a rubber band. At the end of the experiment, these shoots were harvested, the new portions of rhizome cut, and their weight determined (after drying at 60 °C until constant). Rhizome growth was then expressed as weight of new rhizome per day (mg DW rhizome day⁻¹).

To estimate the shoot demographic balance (the difference between recruitment, i.e. the number of new shoots, and mortality, i.e. the number of dead shoots), all shoots surviving at the end of the experiments were counted. We computed the instantaneous demographic balance (a) as:

$$a \text{ (days}^{-1}\text{)} = 1/t \ln (N_t/N_0)$$

where N_0 is the number of shoots planted in each aquarium at the initial time (20), N_t is the number of shoots alive in each aquarium at the end of each experiment and t is the duration of the experiment (in days). Positive values for a occur when shoot recruitment is higher than mortality, indicating a net increase in shoot abundance. Conversely, negative values would indicate a net reduction in shoot abundance, and hence a negative response to the stressor(s) considered.

Statistical analysis

For all statistical analysis, an aquarium was considered as the experimental unit, with $n=3$ replicates per experimental condition. The significance of the effects of temperature and ammonium, on the one hand, and, temperature and addition of labile organic C, on the other hand, were determined using PERMANOVA analysis based on a similarity matrix created from the Euclidean distances between samples. The analysis was run with two fixed factors: temperature (3 levels: 20 °C, 30 °C and 35 °C) and nutrients (3 levels: Control, Moderate and High, see above) for the TNUT experiment; and temperature (3 levels: 20 °C, 30 °C and 35 °C) and addition of labile organic C (2 levels: Control and High, see above) for the TANOX experiment.

For each experiment, one multivariate PERMANOVA was carried out for variables related to plant biochemical composition (N and S content of plant tissues, for the TNUT and TANOX experiment, respectively), and a second for the other variables (F_v/F_m , incidence of leaf necrosis, leaf growth, rhizome elongation, and shoot demographic balance), followed by univariate PERMANOVAs performed separately for each individual variable. As in PERMANOVA the test is produced by permutation, the usual normality assumptions of ANOVA (Anderson 2001), that were not met by most of the variables considered, is not necessary. Pairwise comparisons were performed to identify significant differences between individual treatments. In those cases, in which the number of permutations was too low (<999, Anderson et al., 2008), a Monte Carlo test was applied to establish an alternative p -value to validate the analysis. Analysis was carried out using the Primer v6 statistical package (Clarke and Gorley, 2006), in conjunction with the Windows PERMANOVA+ module (Anderson et al., 2008).

RESULTS

Culture conditions and plant biochemical composition

The different treatments (additions of nutrient and labile organic C) effectively changed the conditions under which the plants were grown. Thus, on the one hand, in the TNUT experiment, the ammonium concentrations in the water of the moderate and high treatments were increased (relative to the water in the control aquaria) to the target values at the beginning of the experiment and had decreased at the end of the experiment, despite the repeated additions of ammonium and irrespective of the thermal treatment (Table 1).

Table 1. Ammonium concentrations (in μM , mean \pm SEM, $n=3$) in the water at the beginning (just after adding 30 μM and 300 μM to the “Moderate” and “High” treatments respectively) and at the end of the TNUT experiment.

Ammonium treatment	Thermal treatment					
	20 °C		30 °C		35 °C	
	[NH ₄ ⁺] (μM)					
	Initial	Final	Initial	Final	Initial	Final
Control	1.20 \pm 0.50	1.54 \pm 0.53	0.69 \pm 0.18	1.96 \pm 1.41	8.64 \pm 29.63	17.95 \pm 8.29
Moderate	27.76 \pm 1.12	0.21 \pm 0.09	26.10 \pm 0.33	0.62 \pm 0.08	45.64 \pm 1.30	3.83 \pm 2.93
High	288.06 \pm 34.01	2.21 \pm 0.03	252.33 \pm 11.23	4.25 \pm 0.79	264.55 \pm 7.96	15.82 \pm 8.36

These results show that the plants were subjected at least to one strong initial pulse of ammonium, plus another two pulses during the experiment. On the other hand, in the TANOX experiment, the redox potential of the sediments at the end of the experiment, while maintaining positive values under control conditions, became negative in the mesocosms subjected to large additions of high labile organic C. Temperature affected the redox potential, with lower values at higher temperatures likely due to an enhancement of the bacterial activity (Table 2).

Table 2. Redox potential values (mean \pm SEM, $n=3$) of the sediment in the TANOX experiment for 7 days in three thermal treatments (20 °C, 30 °C, and 35 °C). Lower case letters indicate significant differences ($p>0.05$) between treatments.

Labile organic C treatment	Thermal treatment		
	20 °C	30 °C	35 °C
	Redox potential (mV)		
Control	180.33 \pm 8.31 ^a	137.07 \pm 16.41 ^b	78.59 \pm 9.52 ^c
High	-24.81 \pm 6.90 ^d	-230.19 \pm 11.38 ^e	-281.78 \pm 14.84 ^f

Overall, the biochemical composition of leaves (N and S content) changed in response to the treatments. In the TNUT experiment, addition of ammonium at high concentrations increased the N content of all plant tissues, up to 23 % relative to controls (Figure 2A, B and C; Table 3). In the TANOX experiment, the S content of leaves and roots was significantly

higher under conditions with an addition of labile organic C than under control conditions (Figure 2D and F; Table 3). Temperature had significant effects on biochemical composition in both experiments. The N content of leaves increased with temperature; while the N content of rhizomes decreased at the intermediate temperature. The S content of leaves and rhizomes increased with temperature; and the latter was even higher due to interactive effects between temperature and the addition of labile organic C.

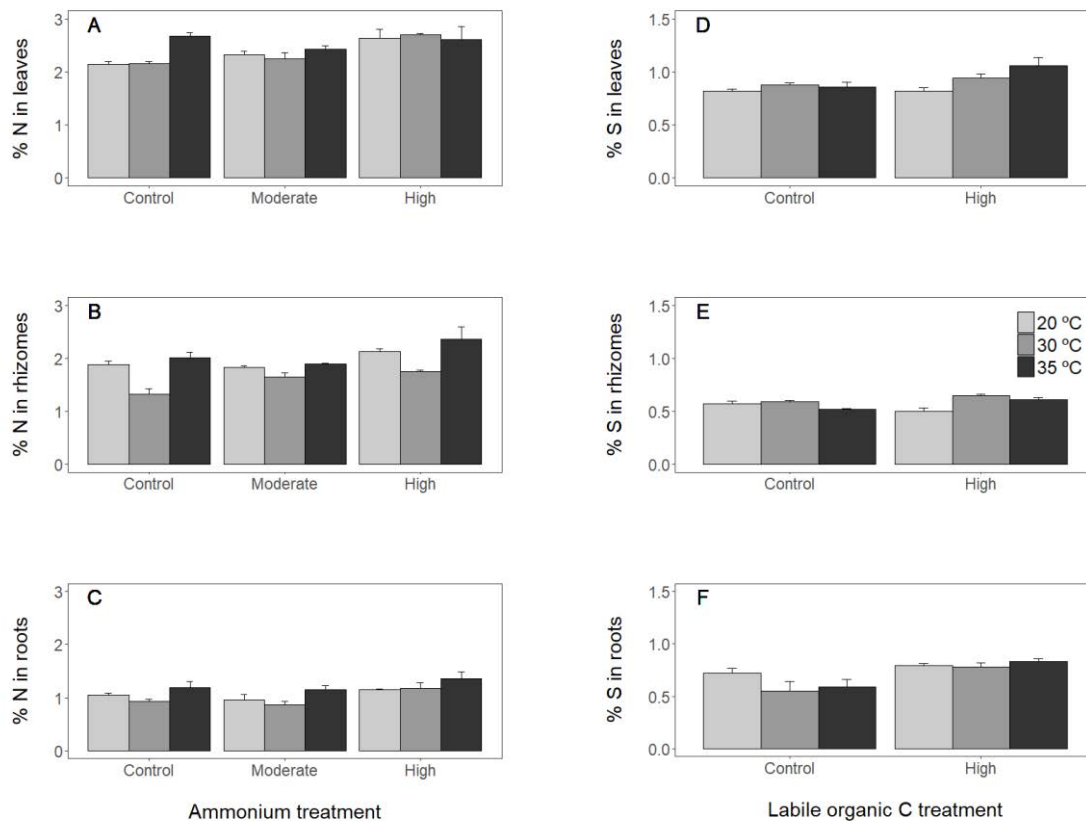


Figure 2. *Cymodocea nodosa* biochemical composition (N content (mean \pm SE, $n=3$) and sulphur content (mean \pm SE, $n=3$)) measured in (A & D) roots, (B & E) rhizomes and (C & F) leaves, at 3 thermal treatments (20 °C, 30 °C, and 35 °C, black, light grey and dark grey respectively) in the TNUT (A, B & C) and TANOX (D, E & F) experiments, expressed in percentage (%).

Table 3. Results of PERMANOVA testing for the significance of effects of temperature (20 °C, 30 °C, and 35 °C), nutrient level (Control, Moderate, and High additions) and labile organic C addition (Control and High) on plant biochemical composition. Numbers in bold indicate significant effects ($p < 0.05$). The results of the pair-wise tests are indicated in factors with significant influence.

Exp.	Variable	Source	df	SS	MS	Pseudo-F	Unique perms	P	Pair-wise	
TNUT	<i>Main test</i>									
		Temperature	2	1.802	0.901	9.839	9952	0.0001		
		Ammonium	2	1.467	0.734	8.011	9947	0.0002		
		Temp x Amm	4	0.582	0.145	1.588	9931	0.1377		
		Residual	18	1.648	0.092					
	<i>Individual test</i>									
	N leaves	Temperature	2	0.254	0.127	3.153	9952	0.0634		
		Ammonium	2	0.605	0.302	7.520	9950	0.0042	H>C=M	
		Temp x Amm	4	0.373	0.093	2.317	9958	0.0955		
		Residual	18	0.724	0.04					
	N rhizome	Temperature	2	1.257	0.629	20.145	9949	0.0001	35=20>30	
		Ammonium	2	0.594	0.297	9.519	9953	0.0012	H>C=M	
		Temp x Amm	4	0.188	0.047	1.506	9956	0.2396		
		Residual	18	0.562	0.031					
	N roots	Temperature	2	0.291	0.145	7.219	9943	0.006	35>20=30	
Ammonium		2	0.268	0.134	6.655	9945	0.0059	H>C=M		
Temp x Amm		4	0.021	0.005	0.261	9961	0.9			
Residual		18	0.363	0.02						
TANOX	<i>Main test</i>									
		Temperature	2	25.162	12.581	9.972	9961	0.001		
		Labile organic C	1	8.366	8.366	6.631	9952	0.0087		
		Temp x L. org. C	2	7.581	3.790	3.004	9940	0.0443		
		Residual	12	15.140	1.262					
	<i>Individual test</i>									
	S leaves	Temperature	2	0.06	0.03	5.686	9947	0.0228		
		Labile organic C	1	0.034	0.034	6.456	9823	0.0302	30=35>20	
		Temp x L. org. C	2	0.032	0.016	3.088	9955	0.0825	C<H	
		Residual	12	0.063	0.005					
	S rhizome	Temperature	2	0.021	0.011	10.223	9951	0.0012		
		Labile organic C	1	0.003	0.003	3.013	9851	0.1117	20=30, 20=35, 30>35	
		Temp x L. org. C	2	0.021	0.01	9.921	9952	0.0016		
		Residual	12	0.012	0.001					
	S roots	Temperature	2	0.026	0.013	1.435	9951	0.2638		
Labile organic C		1	0.146	0.146	16.286	9834	0.0029			
Temp x L. org. C		2	0.03	0.015	1.648	9954	0.2393	C<H		
Residual		12	0.108	0.009						

Effects of temperature on plant traits

Temperature had an overall significant effect on the plant traits measured in both experiments (Table 4). The maximum quantum yield of PSII (F_v/F_m) revealed that the photosynthetic apparatus maintained its integrity at 30 °C. However, F_v/F_m was significantly depressed at 35 °C (6 % and 42 % lower than under control conditions, in the TANOX and TNUT experiment, respectively), suggesting that significant damage was caused by warming (Figure 3; Table 5).

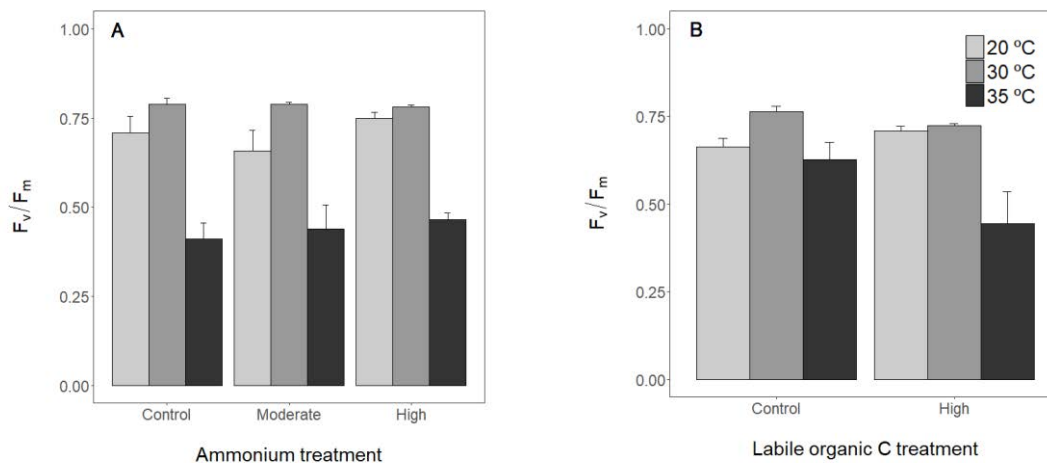


Figure 3. *Cymodocea nodosa* maximum quantum yield (mean \pm SE, $n=3$) at 3 thermal treatments (20 °C, 30 °C, and 35 °C, black, light grey and dark grey respectively) in the (A) TNUT and (B) TANOX experiments.

The incidence of necrosis on the leaves (Figure 4; Table 5) was low under control conditions (20 °C; between 7 % and 17 % in the TANOX and TNUT experiment, respectively) and at 30 °C (<8 % in both experiments), but increased significantly to 23 % - 33 % (depending on the experiment) at 35 °C. Leaf growth rates (Figure 5A and B; Table 5) showed higher values at 30 °C than under control conditions (45 %) and minimum values at 35 °C (a decrease of between 63 % and 94 % relative to control conditions, in the TNUT and TANOX experiment, respectively). Rhizome elongation (only measured in the TANOX experiment) was also significantly higher (74 %) at 30 °C than at the other two temperatures (Figure 5C; Table 5).

The shoot demographic balance (i.e. recruitment – mortality) was clearly sensitive to temperature, with a sharp increase (83 % on average, relative to the control temperature) under moderate warming (30 °C) and a clear decrease under extreme warming (35 °C), dropping to negative values in the TNUT experiment (Figure 6a; Table 5).

The effects of additions of ammonium and labile organic C on plant traits

Ammonium addition did not show any effect on any of the plant traits measured (Figures 3A, 4A, 5A and 6A; Tables 4 and 5). The addition of labile organic C did not affect the maximum quantum yield of PSII, the incidence of necrosis or the shoot demographic balance (Figures 3B, 4B and 6B; Table 5). However, it caused a significant decrease (relative to plants

grown in unaltered sediment at 30 °C) in leaf and rhizome growth rates (of 44 % and 67 % respectively; Figures 5B and c; Table 5).

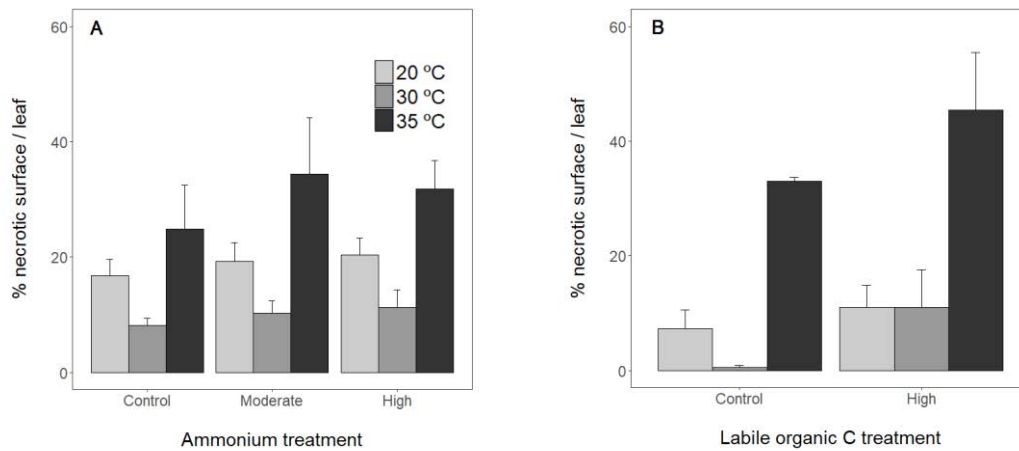


Figure 4. Leaf necrosis incidence (average necrotic surface per leaf, in %) in *Cymodocea nodosa* (mean \pm SE, $n=3$) at 3 thermal treatments (20 °C, 30 °C, and 35 °C, black, light grey and dark grey respectively) in the (A) TNUT and (B) TANOX experiments.

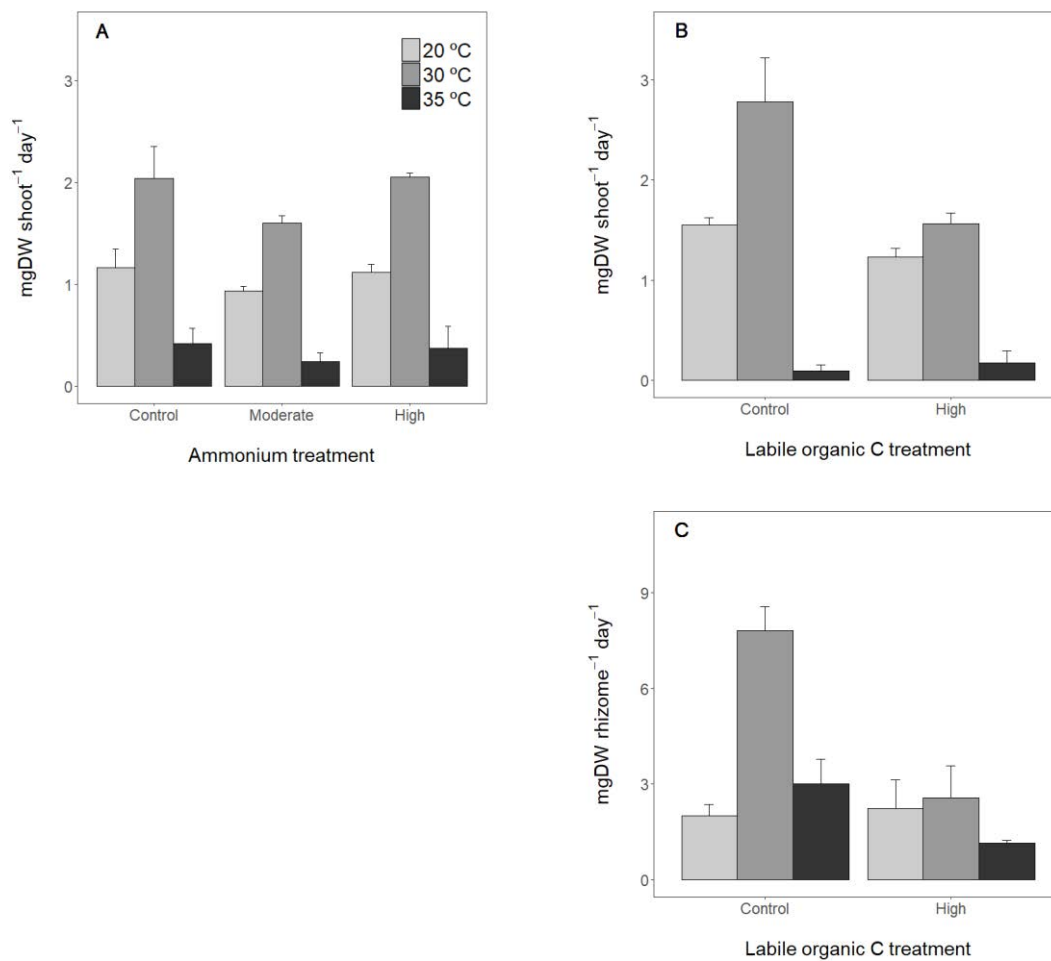


Figure 5. *Cymodocea nodosa* growth rate (mean \pm SE, $n=3$) at 3 thermal treatments (20 °C, 30 °C, and 35 °C, black, light grey and dark grey respectively) in two experiments: (A) Leaf growth rate in the TNUT experiment; (B) Leaf growth rate in the TANOX experiment; (C) Rhizome growth rate in TANOX experiment.

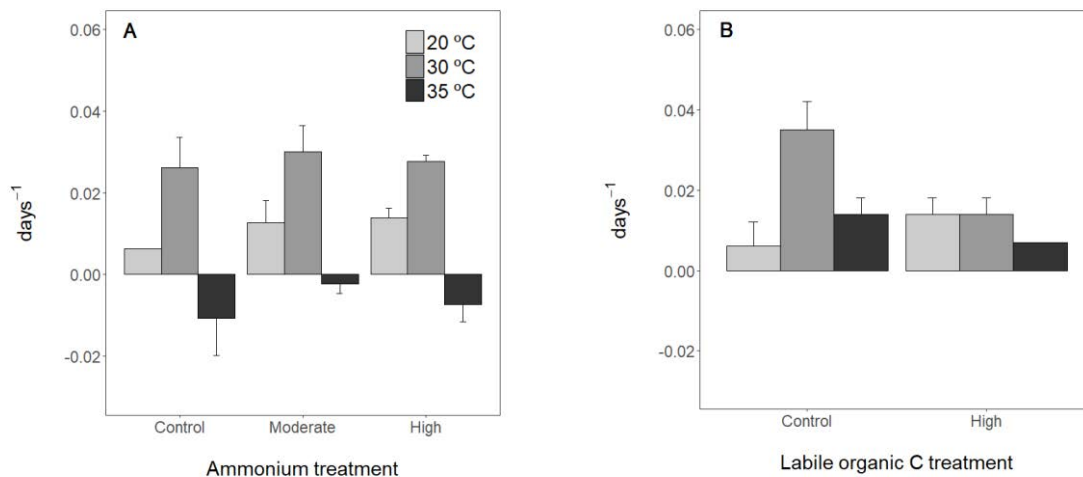


Figure 6. *Cymodocea nodosa* shoot demographic balance (mean \pm SE, $n=3$) at 3 thermal treatments (20 °C, 30 °C, and 35 °C, black, light grey and dark grey respectively) in the (A) TNUT and (B) TANOX experiments.

Interactive effects

Our results did not show any significant interaction between warming and ammonium addition (TNUT experiment) in terms of their effects on plant traits. In contrast, we found interactive effects of warming and the addition of labile organic C to the sediment (TANOX experiment), both overall (Table 4) and in individual traits. Thus, the stimulation of leaf and rhizome growth at intermediate temperatures and the improvement of the shoot demographic balance were cancelled by labile organic C. In addition to this, with the normal organic C content of the sediment, high temperature (35 °C) did not alter rhizome growth or the demographic balance, but it did in the sediment with labile organic C added to it.

Table 4. Multivariate PERMANOVA testing for the significance of the general effect of temperature (20 °C, 30 °C, and 35 °C), nutrient level (Control, Moderate, and High additions) and labile organic C addition (Control and High) on plant traits. Numbers in bold indicate significant effects ($p < 0.05$).

Experiment	Source	df	SS	MS	Pseudo-F	Unique perms	P
Main test							
TNUT	Temperature	2	1725.100	862.540	8.612	9950	0.0030
	Ammonium	2	50.622	25.311	0.253	9949	0.7830
	Temp x Amm	4	144.960	36.241	0.362	9958	0.8326
	Residual	18	1802.700	100.150			
TANOX	Temperature	2	105420	52709	105.350	9948	0.0001
	Labile organic C	1	435410	435410	870.260	9882	0.0001
	Temp x L. org. C	2	25298	12649	25.282	9948	0.0001
	Residual	12	6003.800	500.320			

Table 5. Results of PERMANOVA testing for the significance of effects of temperature (20 °C, 30 °C, and 35 °C), nutrient level (Control, Moderate, and High additions) and labile organic C addition (Control and High) on each plant trait. Numbers in bold indicate significant effects ($p < 0.05$). The results of the pair-wise tests are indicated in factors with significant influence.

Var.	Exp.	Source	df	SS	MS	Pseudo-F	Unique perms	P	Pair-wise
Fv/Fm	TNUT	Temperature	2	0.543	0.272	52.937	9952	0.0001	20=30>35
		Ammonium	2	0.012	0.006	1.206	9947	0.3277	
		Temp x Amm	4	0.012	0.003	0.597	9943	0.6722	
		Residual	18	0.092	0.005				
	TANOX	Temperature	2	0.139	0.069	11.488	9956	0.001	30>20>35
		Labile organic C	1	0.016	0.016	2.595	9851	0.1297	
		Temp x L. org. C	2	0.04	0.02	3.283	9951	0.0555	
		Residual	12	0.072	0.006				
Necrosis	TNUT	Temperature	2	1714.000	856.990	8.566	9957	0.0031	30<20=35
		Ammonium	2	50.242	25.121	0.251	9954	0.7805	
		Temp x Amm	4	144.860	36.215	0.362	9962	0.8436	
		Residual	18	1800.900	100.050				
	TANOX	Temperature	2	4060.000	2030.000	23.683	9948	0.0006	20=30<35
		Labile organic C	1	354.090	354.090	4.131	9859	0.0655	
		Temp x L. org. C	2	62.753	31.376	0.366	9956	0.7057	
		Residual	12	1028.600	85.714				
Leaf growth rate	TNUT	Temperature	2	10.538	5.269	53.080	9950	0.0001	30>20>35
		Ammonium	2	0.367	0.183	1.848	9950	0.186	
		Temp x Amm	4	0.091	0.023	0.228	9951	0.9224	
		Residual	18	1.787	0.099				
	TANOX	Temperature	2	12.713	6.357	54.210	9955	0.0001	30>20>35
		Labile organic C	1	1.070	1.070	9.125	9851	0.0058	C>H
		Temp x L. org. C	2	1.327	0.663	5.657	9951	0.0075	
		Residual	12	1.407	0.117				
Rhizome growth	TANOX	Temperature	2	38.329	19.164	15.536	9954	0.0011	30>20=35
		Labile organic C	1	23.510	23.510	19.059	9739	0.0012	C>H
		Temp x L. org. C	2	22.905	11.452	9.284	9948	0.0035	
		Residual	12	14.803	1.234				
Shoot demographic balance	TNUT	Temperature	2	0.005	0.003	33.785	9964	0.0001	30>20>35
		Ammonium	2	0.001	0.001	1.097	9957	0.3624	
		Temp x Amm	4	0.001	0.001	0.152	9962	0.9594	
		Residual	18	0.001	0.001				
	TANOX	Temperature	2	0.001	0.001	6.767	9952	0.0119	30>20=35
		Labile organic C	1	0.001	0.001	3.571	9758	0.0887	
		Temp x L. org. C	2	0.001	0.001	5.231	9959	0.0219	
		Residual	12	0.001	0.001				

DISCUSSION

Climate change is having an impact on a world that has already been altered by a panoply of local stressors. Our results show how cumulative stress, in this case derived from the joint action of warming and eutrophication, can worsen, either through additive or interactive effects, the negative consequences of each stressor acting in isolation.

As already known, temperature increases can negatively affect different functional mechanisms of seagrasses (Koch et al., 2013). The response patterns and the thresholds involved are largely species specific (Campbell et al., 2006; Collier et al., 2011). In the model species used here (*C. nodosa*), moderate warming seems to be beneficial for the plant, whose performance (photosynthesis, growth and shoot demographic balance) increase at 30 °C, relative to those found at the basal spring temperature (control) of 20 °C. This pattern is fully consistent in the two experiments we conducted. This is in accordance with previous reports for this species, suggesting an optimum temperature close to 30 °C (Olsen et al., 2012; Pérez and Romero 1992; Savva et al., 2018; Terrados and Ros 1995; Tutar et al., 2017). In contrast, plant performance was severely depressed at 35 °C; not only relative to the 30 °C optimum, but also relative to control conditions. This suggests there is a thermal threshold of clear negative effects on plant activity between 30 °C and 35 °C. This thermal threshold is relatively high (see Lee et al., 2007 for comparisons), and it is in accordance with the subtropical distribution of this species (Green and Short 2003; Reyes et al., 1995) and its facultative habitat in confined environments, where summer temperatures can easily be 5 °C above open sea temperatures.

Exposure to extreme thermal values damaged the integrity of the photosynthetic apparatus, as shown by a clear drop in F_m/F_v to values below those considered acceptable for healthy plants (0.7-0.8, Campbell et al., 2006; Ralph et al., 1998), as previously found for other seagrass species (e.g. *T. testudinum*; Koch et al., 2007; e.g. *Z. noltii*; Massa et al., 2009). While photosynthesis is depressed, respiration is probably stimulated by thermal stress (not measured in this study; but see Pérez and Romero, 1992), leading to impairment of the C budget (Collier and Waycott, 2014), which could be the cause of the reduced growth and the low to negative shoot demographic balance observed in our experiments. Plants exposed to high temperatures may have to use their energy reserves (stored non-structural carbohydrates) to cope with this stress and the consequent energy requirement (Collier et al., 2011; Massa et al., 2009), probably leading to exhaustion of the internal C reserves (Marín-Guirao et al., 2018). Indeed, thermal stress also affects other metabolic processes, causing, for instance, oxidative stress (Tutar et al., 2017), and ultimately affecting plant health, which deteriorated in our experiments as shown by the increase in the incidence of necrosis.

Reducing the shoot demographic balance can be critical for *C. nodosa*. This species has a very high shoot turnover, with a yearly shoot mortality reaching 1/2 to 2/3 of the total number of shoots in unaltered meadows. This mortality takes place in late summer to autumn and is balanced by massive recruitment in late spring (Mascaró et al., 2014). Any event altering the shoot demographic balance, such as a heat wave, will cause a drop in seagrass density, eventually leading to meadow extinction. This is relevant for projections of

distribution and abundance of this species in future warming scenarios since the frequency and intensity of heat waves are predicted to increase (IPCC, 2013). Those predictions suggest that the threshold temperature (thermal tolerance limit) could be reached during these extreme climate events, mainly in confined areas such as shallow bays or lagoons. However, the threshold is quite unlikely to be reached in the open sea, where warming will be much more moderate and could have beneficial effect on the species which could extend its distribution, maybe at the expenses of the Mediterranean species *P. oceanica*, which is much more sensitive to warming (Marín-Guirao et al., 2016; Olsen et al., 2012).

Regarding eutrophication, *C. nodosa* is affected by an increase in organic matter in the sediment but not by pulses in nutrient concentrations. None of the traits studied were modified by addition of ammonium, despite the high concentrations attained (up to 300 μM) and the fact that ammonium was depleted from the aquaria. A coarse N mass balance, estimating N incorporation in the plant through leaf growth, new shoots and N increase in tissues, suggests that most of this depletion was caused by plant activity, being microbial activity in the sediment the most likely explanation for the rest. Seagrasses seem to be unable to downregulate N uptake, probably due to a lack of inhibitory feedback mechanisms (Touchette and Burkholder, 2000). This failure in regulation could generate ammonium accumulation in cells, which in turn may have toxic effects (Invers et al., 2004). However, while some species seem to be more vulnerable to this toxicity (e.g. *Z. marina*, Burkholder et al., 1992; van Katwijk et al., 1997; Villazán et al., 2013b; and *Z. noltei*, Moreno-Marín et al., 2016) others show great resistance (*C. nodosa*, Egea et al., 2018 and *Z. marina*, Kaldy et al., 2014). It has been suggested that the key mechanism to endure large ammonium pulses may be an efficient mechanism that is capable of rapidly converting the excess of ammonium into organic forms (Brun et al., 2002; Invers et al., 2004). Second-order (indirect) effects of ammonium pulses, such as an increase in epiphytic load or a decrease in water transparency, were not studied here and cannot be ruled out. In contrast, the addition of labile organic C had a detrimental effect on plants. The organic additions to the sediment seemed to enhance bacterial respiration and thus oxygen demand, leading to oxygen exhaustion and anoxic conditions (up to -290 mV of redox potential). Under these conditions, sulphate reduction is stimulated, resulting in sulphide accumulation. Consistently with this, we found higher sulphur contents in our exposed plants than in controls. The oxidation of sulphide to sulphur compounds that are further stored in tissues has been shown to be a mechanism that can help cope with sulphide intrusion. However, once the capacity of detoxification of this mechanism is surpassed, the detrimental effects appear (Hasler-Sheetal and Holmer, 2015). Although *C. nodosa* is highly resistant to eutrophication (Oliva et al., 2012), highly negative values (such as those created in our experiment, close to -250 mV) clearly seem to be harmful for plant production and fitness.

Beyond the effects of warming and eutrophication highlighted above, and given that both stressors will act jointly in most real-world conditions, the assessment of their potential interactions is of great interest. Our results show that there were no interactive effects between warming and ammonium; but in contrast the effects of warming on key processes (leaf and rhizome growth and the demographic balance) were strongly mediated by the amount of labile organic C in the sediment. The interaction between temperature and organic

matter was detected at the individual (leaf and rhizome growth) and population (shoot demographic balance) level, but not at the physiological one (F_v/F_m). Clearly, the processes affected are critical for meadow persistence, which underlines the relevance of such interactions for the prediction of future seagrass meadow dynamics. However, the mechanisms through which these interactions function have not been elucidated by our work. A possible explanation would be a synergistic effect on environmental conditions. This is supported by the fact that the addition of labile organic C and temperature decreased sediment redox potential synergistically, probably through the stimulation of oxygen demand and cascading effects on sulphide production and plant performances. Other mechanisms, including the amplification of sulphide effects by temperature, should not be ruled out.

Despite multiple stressors studies have increased in the last decades, our results add evidence to the need to further assess the interactive effects of different stressors, and understanding how the organisms or communities will respond to the impact of multiple co-occurrent stressors is still a matter of concern (Côté et al., 2016). Seemingly, synergistic effects are quite frequent, as revealed by Crain et al. (2008), which found in a review focused on coastal ecosystems that 36 % of the cases examined showed synergy. A thorough literature search on interactive effects on seagrass ecosystems (Table 6) confirms that synergy is more the rule (50 %) than the exception (36 % additive; only a small part of the studies found antagonistic interaction). There is an urgent need to incorporate those interactive effects to improve predictions of the consequences of climate change in marine ecosystems, which can be seriously underestimated when assessing thermal effects in isolation. In addition, results such as those presented here can support strategies to increase ecosystem resilience to climate change by managing other stressors at a local or regional scale. In this respect, shallow bays and coastal lagoons, which are more vulnerable to both extreme thermal events and eutrophication, may represent a critical scenario for the survival of seagrass species growing close to their upper thermal limit (York et al., 2013, Koch et al., 2007), but also an opportunity to test the above mentioned strategies.

Even though our findings, it is important to keep in mind that the results of this work were obtained from a mesocosm experiment focusing only on two factors (warming and eutrophication) without considering any other disturbance that may be found in the environment. In this sense, they should only be extrapolated to natural conditions cautiously. In spite of these limitations, this research highlights the importance of evaluating the impact of global and local stressors jointly; not only to generate more realistic predictions of the impacts that climate change might have, but also to design and implement strategies to improve (or at least not to impair) seagrass resilience to global warming.

ACKNOWLEDGEMENTS

This study was funded by the European Union and the Spanish Government through the RECCAM project (seagrass meadows resilience to global warming: an analysis based on responses at ecophysiological, population and ecosystem levels, CTM2013-48027-C3-1-R and CTM2013-48027-C3-2-R); and by the Spanish Ministry of Economy and Competitiveness (via grant BES-2014-069593 awarded to Y. Ontoria).

Table 6. Synthesis of multiple stressors studies on seagrasses and the interaction type effects.

Stressor 1	Stressor 2	Stressor 3	Overall interaction type	Species	Source
Temperature	Nitrate and ammonium		Additive	<i>Zostera marina</i>	Kalry 2014
Temperature	Ammonium		Additive	<i>Cymodocea nodosa</i>	This study
Temperature	Labile Organic C		Synergy	<i>Cymodocea nodosa</i>	This study
Temperature	Acidification		Additive	<i>Zostera nolii</i>	Repolho et al., 2017
Temperature	Herbicide		Antagonism	<i>Halophila ovalis</i>	Wilkinson et al., 2017
Temperature	Light		Additive	<i>Zostera muelleri</i>	York et al., 2013
Temperature	Light		Synergy	<i>Halodule uninervis</i> and <i>Zostera muelleri</i>	Collier et al., 2011
Temperature	Floods		Synergy	<i>Amphibolis antarctica</i>	Fraser et al., 2014
Temperature	Macroalgae		Synergy	<i>Zostera marina</i>	Höfhe et al., 2011
Temperature	Salinity		Synergy	<i>Zostera marina</i>	Salo and Pedersen, 2014
Temperature	Sulfide		Synergy	<i>Halodule wrightii</i> and <i>Thalassia testudinum</i>	Koch et al., 2007
Nitrate	Acidification		Additive	<i>Thalassia testudinum</i>	Campbell and Fourqurean, 2014
Nitrate	Acidification		Additive	<i>Thalassia hemprichii</i> and <i>Halodule uninervis</i>	Ow et al., 2016
Nitrate and phosphate	Acidification		Additive	<i>Thalassia testudinum</i>	Campbell and Fourqurean, 2018
Nitrate, ammonium and phosphate	Waves		Synergy	<i>Zostera nolii</i>	La Nabe et al., 2012
Ammonium	Light		Synergy	<i>Zostera marina</i>	Villazán et al., 2013b
Ammonium	Macroalgae		Antagonism	<i>Zostera nolii</i>	Moreno-Maín et al., 2016
Ammonium	Salinity		Synergy	<i>Zostera marina</i>	Villazán et al., 2015
Organic matter	Burial		Synergy	<i>Posidonia oceanica</i>	Ceccherelli et al., 2018
Temperature	Ammonium		Synergy	<i>Zostera marina</i>	Moreno-Maín et al., 2018
Temperature	Ammonium		Additive/Synergy	<i>Cymodocea nodosa</i>	Egea et al., 2018
Ammonium	Phosphate		Antagonism	<i>Zostera nolii</i>	Brun et al., 2008



CHAPTER 3

The negative effects of short-term extreme thermal events on the seagrass *Posidonia oceanica* are exacerbated by ammonium additions

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ABSTRACT

Global warming is increasingly affecting our biosphere. However, in addition to global warming, a panoply of local stressors caused by human activities is having a profound impact on our environment. The risk that these local stressors could modify the response of organisms to global warming has attracted interest and fostered research on their combined effect, especially with a view to identifying potential synergies. In coastal areas, where human activities are heavily concentrated, this scenario is particularly worrying, especially for foundation species such as seagrasses. In this study we explore these potential interactions in the seagrass *Posidonia oceanica*. This species is endemic to the Mediterranean Sea. It is well known that the Mediterranean is already experiencing the effects of global warming, especially in the form of heat waves, whose frequency and intensity are expected to increase in the coming decades. Moreover, this species is especially sensitive to stress and plays a key role as a foundation species. The aim of this work is thus to evaluate plant responses (in terms of photosynthetic efficiency and growth) to the combined effects of short-term temperature increases and ammonium additions. To achieve this, we conducted a mesocosm experiment in which plants were exposed to three thermal treatments (20 °C, 30 °C and 35 °C) and three ammonium concentrations (ambient, 30 μM and 120 μM) in a full factorial experiment. We assessed plant performance by measuring chlorophyll fluorescence variables (maximum quantum yield (F_v/F_m), effective quantum yield of photosystem II ($\Delta F/F_m'$), maximum electron transport rate (ETR_{max}) and non-photochemical quenching (NPQ)), shoot growth rate and leaf necrosis incidence. At ambient ammonium concentrations, *P. oceanica* tolerates short-term temperature increases up to 30 °C. However, at 35 °C, the plant loses functionality as indicated by a decrease in photosynthetic performance, an inhibition of plant growth and an increase of the necrosis incidence in leaves. On the other hand, ammonium additions at control temperatures showed only a minor effect on seagrass performance. However, the combined effects of warming and ammonium were much worse than those of each stressor in isolation, given that photosynthetic parameters and, above all, leaf growth were affected. This serves as a warning that the impact of global warming could be even worse than expected (based on temperature-only approaches) in environments that are already subject to eutrophication, especially in persistent seagrass species living in oligotrophic environments.

INTRODUCTION

Climate change represents a major threat to coastal ecosystems worldwide. The urgent need to gain a better understanding of its impact on the performance of organisms and the subsequent cascading effects that cause changes in ecological functions and ecosystem services is a widespread concern (Hallett et al., 2018; Harley et al., 2006; Parmesan and Yohe, 2003). Warming is probably the most pervasive effect of global change and is expected to cause ocean surface temperatures to rise by between 2.6 °C and 4.8 °C by 2100 (IPCC 2014). Aside from this progressive warming, most climatic models predict that temperature extremes will increase in frequency and intensity in the coming decades (Collins et al., 2013; IPCC 2007; Meehl and Tebaldi, 2004; Oliver et al., 2018; Schär and Jendritzky, 2003). These so-called heat waves increase temperature by several degrees above the historical mean, usually last for days or a few weeks and seem to be especially deleterious for the biota, thereby increasing concern and attracting a great deal of attention in recent years as key drivers of change (Meehl and Tabaldi, 2004; Oliver et al., 2018; Wernberg et al., 2016). In addition to global warming, a panoply of stressors caused by human activity (Halpern et al., 2015) is already affecting our environment. Thus, warming will impact ecosystems that are heterogeneously affected, to varying degrees, by a range of other stressors, most of them local in origin. The risk that these local stressors could profoundly modify the response of organisms to warming, thereby altering predictions based solely on thermal responses, is gaining attention and in recent years has fostered a growing interest in assessing the combined effects of warming and other stressors (Brown et al., 2013; Côté et al., 2016; Gunderson et al., 2016), especially with a view to identifying possible synergies (Dunne 2010; Darling and Côté, 2008).

Such a scenario is a particular threat to coastal areas, where human activities are concentrated, thereby generating a wide array of stressors that could potentially interact with warming (continuous or pulsed) and decrease the resilience of the biota. This is especially worrying in the case of foundation species such as corals, gorgonians and seagrasses due to the propagation of the effects, which may extend to other organisms and have ecosystem-wide implications (Díaz-Almela et al., 2007; Coma et al., 2009; Marbà and Duarte, 2010; Hoegh-Guldberg 1999; Hughes et al., 2013). Seagrasses in particular have demonstrated great sensitivity not only to warming (Marbà and Duarte, 2010; Collier and Waycott, 2014), but also to other stressors of local origin, including eutrophication (Waycott et al., 2009). Seagrass habitats are considered some of the most valuable coastal ecosystems in terms of the provision of goods and ecological services (Orth et al., 2006), thus making the assessment of the combined effects of warming and other stressors a major challenge for the scientific community.

Indeed, on the one hand, temperature is widely known to be one of the main ecological factors that determines seagrass performance, survival and distribution limits (see reviews by Koch et al., (2013) and Lee et al., (2007)), and the potential effects of temperature rises are subject to an increasing number of studies. It is well known that a moderate temperature rise can be favourable for plant physiology, since it stimulates photosynthesis. However, it also stimulates the respiration rate and, since the latter increases at a faster rate

than the former, this can generate a carbon imbalance in plants if it exceeds a certain threshold (Bulthuis 1987; Collier and Waycott, 2014; Greve et al., 2003; Lee et al., 2007; Marín-Guirao et al., 2018; Moore and Short, 2006; Pérez and Romero, 1992). Similarly, it has been demonstrated that photochemical reactions are highly sensitive to thermal stress, which causes damage to the photosystem II (PSII) reaction centres (Repolho et al., 2017; York et al., 2013) that is irreversible beyond a certain threshold (e.g. 37.5 °C, *Halophila ovalis* (Ralph 1998); 40-45 °C, *Zostera capricorni*, *Syringodium isoetifolium*, *Cymodocea rotundata*, *Cymodocea serrulata*, *Halodule uninervis*, *Thalassia hemprichii* and *H. ovalis* (Campbell et al., 2006)). Negative responses of seagrasses to warming have also been reported at individual and population level, including shoot growth impairment (Hendriks et al., 2017; Repolho et al., 2017), an increase in leaf shedding and a reduction in above-ground biomass (York et al., 2013). In some cases, elevated temperatures have been shown to cause plant mortality (Collier and Waycott, 2014; Kaldy and Shafer, 2013; Marbà and Duarte, 2010) and even alter the geographic limits of seagrass distribution (Collier et al., 2011; Massa et al., 2009).

On the other hand, the continuous rise in local nutrient enrichment sources as a consequence of the increasing human population growth and rapid development in coastal areas means that eutrophication is considered a major threat to coastal ecosystems (Díaz and Rosenberg, 2008; Green and Short, 2003; Nixon and Fulweiler, 2009; Orth et al., 2006). Eutrophication can negatively affect seagrasses in particular, either directly or indirectly (Burkholder et al., 2007). The direct effects of nutrient loading, despite the fact that an adequate nutrient supply is fundamental for plant performance (Kaldy 2014), include damage caused to seagrasses by excessive inorganic nitrogen (e.g. *Zostera marina* (Burkholder et al., 1992, 1994; van Katwijk et al., 1997); *Zostera noltii* (Brun et al., 2002)). In this sense, the toxicity of high ammonium concentrations has been reported in several studies (Brun et al., 2002, 2008; Moreno-Marín et al., 2016; van der Heide et al., 2008; Villazán et al., 2013a, 2013b), which observed the negative effects of ammonium on several physiological and morphological response variables, including a reduction in primary production and significantly decreased shoot, rhizome and root elongation rates, thus affecting plant survival.

Further research on the isolated effects of each of these two stressors (nutrient loading and warming) on seagrasses is required, but efforts should also be made to assess their combined action, not only to increase knowledge of the expected responses in a realistic multi-stressor scenario, but also to improve the reliability of our predictions about seagrass ecosystem changes in the coming years. In this regard, temperature is already known to exacerbate the negative effects of other stressors such as organic matter-enriched sediments (*Halodule wrightii* and *Thalassia testudinum* (Koch et al., 2007); *Cymodocea nodosa* (Ontoria et al., 2019b)) and changes in salinity (*Z. marina* (Salo and Pedersen, 2014)), which act synergistically with thermal stress. Some other works have reported additive effects of temperature and other stressors (e.g. light availability, *Zostera muelleri* (York et al., 2013); acidification, *Z. noltii* (Repolho et al., 2017); and nutrients, *Z. marina* (Kaldy 2014)), and, much less commonly, an antagonistic interaction of temperature and a second stressor (e.g. herbicide, *Halophila ovalis* (Wilkinson et al., 2017)). All these studies suggest that plant response to the combined impact of temperature and other stressors is largely species-specific and probably depends on the

functional traits of the specific plant, but knowledge of this topic with respect to seagrass communities remains scarce and incomplete.

Given the global nature of warming, and the pervasive presence of eutrophication, studying the combined effects of warming and nitrogen loading is crucial to understanding the future of coastal communities dominated by seagrasses, especially in light of the specific plant traits of seagrass foundation species (Marbà and Duarte, 1998). Although some progress has been made in this area (Egea et al., 2018; Kaldy 2014; Moreno-Marín et al., 2018; Ontoria et al., 2019b), studies that explore this interaction, especially in persistent seagrass species (*sensu* Kilminster et al., 2015) such as those belonging to the *Posidonia* genus, remain surprisingly scarce.

The Mediterranean endemic species *P. oceanica* is an excellent model for exploring the issues described above. On the one hand, *P. oceanica* is a paradigm of a persistent species (Arnaud-Haond et al., 2012; Marbà et al., 2002) and a key foundation species in Mediterranean oligotrophic waters, where it provides critical habitats and other ecosystem services. Due to its high sensitivity to stress and vulnerability to coastal deterioration, *P. oceanica* meadows have undergone a substantial decline over the last 50 years (Marbà et al., 2011). Consequently, it has been one of the main targets of efforts to protect and manage the Mediterranean marine environment in the last 20 years (Boudouresque et al., 2012). On the other hand, sea surface temperature in the Mediterranean is increasing at a much faster rate than in the global oceans (IPCC 2007; Vargas-Yañez et al., 2007) and, at the same time, temperature extremes and heat waves are becoming more common in this region. Moreover, eutrophication is considered a major threat to and stressor for this seagrass, especially near highly populated areas along the Mediterranean coastline, where the first problems of eutrophication were detected as far back as the 1960s (Karydis and Kitsiou, 2012).

While the effects of eutrophication on this species are relatively well known (Holmer et al., 2003; Invers et al., 2004; Pérez et al., 2007; Ruiz et al., 2001), the effects of warming have only recently started being documented (Beca-Carretero et al., 2018; Guerrero-Meseguer et al., 2017; Marbà and Duarte, 2010; Marín-Guirao et al., 2016, 2017, 2018; Olsen et al., 2012; Savva et al., 2018) and, to the best of our knowledge, there is no information on the potential effects of the interaction between these two stressors.

The aim of this study is thus to explore both the individual and combined effects of warming, by simulating the effects of a short-term extreme temperature event, and eutrophication, through nutrient loading in the form of ammonium, in the persistent seagrass species *P. oceanica*. In order to achieve this, we evaluated physiological and individual plant responses to a short-term temperature increase (lasting days) and the interactive effects of ammonium additions. To do so, we conducted an indoor mesocosm experiment in which plants were exposed to three thermal treatments and three levels of ammonium concentration in a full factorial experiment.

MATERIAL AND METHODS

Plant collection

Divers hand-picked healthy plant fragments of *P. oceanica* with at least four interconnected vertical shoots (apical shoots were avoided) in late September 2016 from an eight-metre deep meadow in Cala Montgó (42° 06' 23" N / 3° 10' 16" E, NE coast of Spain), where allowances to collect plants fragments for scientific purposes are not required.

Plants were transported in aerated tanks to the laboratory and aerated overnight until the experimental setup the following day. The experiment was performed in the University of Barcelona's Experimental Fields Service.

Experimental design and setup

For the experiment, we chose three thermal treatments (20 °C, 30 °C and 35 °C) and three ammonium concentrations: ambient seawater (control), 30 µM (moderate) and 120 µM (high).

The temperatures were chosen to represent the following scenarios: 20 °C, close to the temperature of the study site at the collection time, according to a temperature data series recorded by continuous *in situ* temperature data loggers (Figure 1), obtained by the authors in Medas Islands (at a depth of 5 m), an area close to the collection site (< 5 km); 30 °C, an anomalously high temperature, likely to be reached in the coming years during heat waves (as a reference, > 28 °C recorded during recent heat waves by (Coma et al., 2009; Marbà and Duarte, 2010), and relatively common in the Eastern Mediterranean basin (Galli et al., 2017); and 35 °C, a temperature during an extreme heat wave that could be reached in the mid-term future (the temperature is predicted to increase by 4-5 °C in the western Mediterranean by the end of the 21st century, as per IPCC (2014), Marbà and Duarte (2010), and Sánchez et al., (2004). With respect to nutrients, the “moderate” value (30 µM) and the “high” value (120 µM) are the lowest and highest values, respectively, observed in sites affected by sewage discharge (Arévalo et al., 2007; Mozetič et al., 2008) in the Mediterranean Sea, and similar values have been used in previous experimental approaches (Kaldy 2014; Ontoria et al., 2019b).

The plants were incubated in cylindrical and transparent aquaria (12 L capacity, 40 cm height x 20 cm diameter), each with its own independent air pump and filled with 10 L filtered seawater (Figure 2). The plants were incubated in water to avoid possible confounding effects from sediment. Within 24 hours of collection, a single rhizome fragment bearing four interconnected vertical shoots (apical shoots were avoided) was put in each of the 27 aquaria and covered with plastic film to prevent water evaporation. The aquaria were then distributed randomly in three experimental chambers (2 x 1 x 1.5 m, 9 aquaria per chamber), under controlled temperature and light conditions. The chambers were kept at 210-223 µmoles photons m⁻² s⁻¹, above the saturation irradiance of these plants (Alcoverro et al., 1998; Pirc 1986; Ruiz and Romero, 2001; Ruiz et al., 2001), under a 12h/12h light/dark photoperiod. Light was provided by daylight fluorescent tubes. The three chambers were maintained at 20 °C for four days to allow for plant acclimation. After the acclimation period,

the temperature was progressively increased (at a maximum rate of 3 °C/day) until it reached 30 °C in one chamber and 35 °C in the other after 5 days, while the third was kept at 20 °C as a control. After the experimental temperatures were reached, appropriate amounts of NH_4Cl were added to obtain the ammonium concentration treatments mentioned above. Ammonium was added just once at the beginning of the experiment, to simulate an ammonium pulse. While thermal treatments were differentiated in three chambers, ammonium treatments were applied to three randomly chosen aquaria in each chamber, which resulted in a complete factorial design with three replicates per experimental condition. The experiment ended after seven days of exposure to both stressors (temperature and ammonium), when necrosis marks in plants exposed to the highest temperature (35 °C) indicated critical damage to the plant. In order to minimize uncontrolled variability due to small heterogeneities in light and/or temperature, all aquaria were randomly relocated within the chamber every two days. Moreover, each set of nine aquaria was moved from one chamber to another (changing the chamber temperature to maintain the thermal treatments) to ensure that each aquarium spend the same time in each chamber. This was done to discard a potential “chamber effect” and avoid pseudoreplication (Ontoria et al., 2019b).

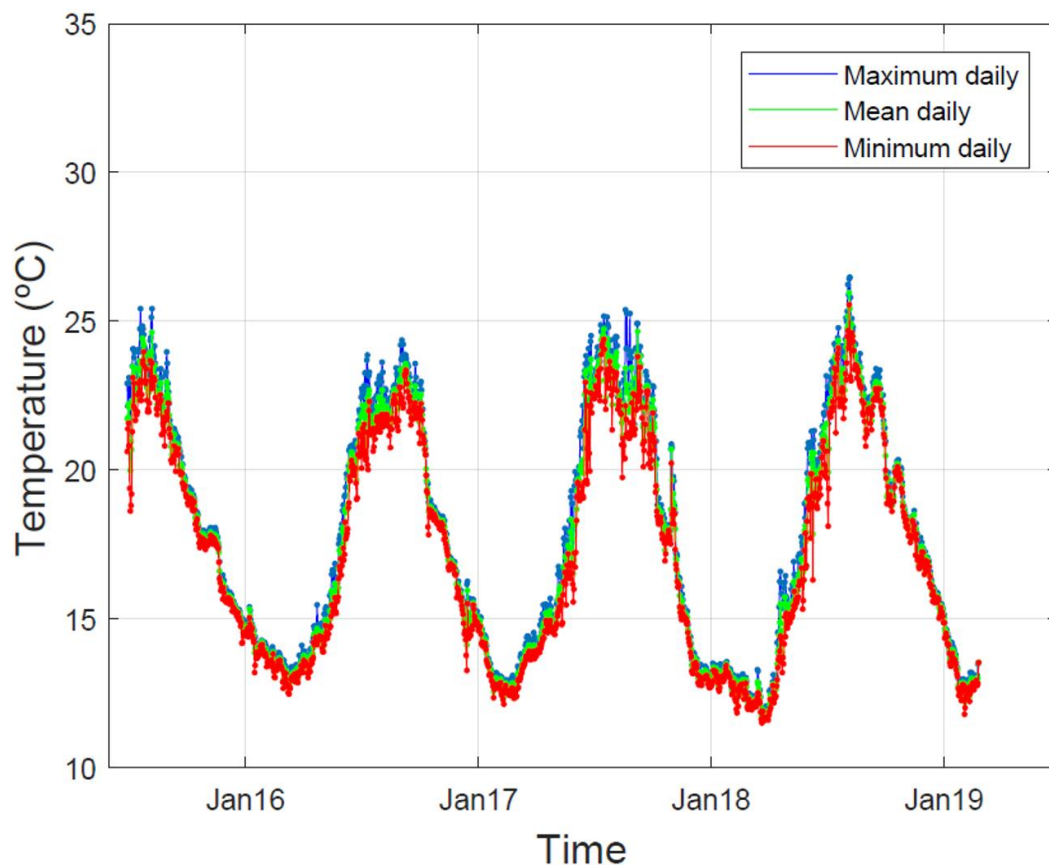


Figure 1. Three-years temperature data series recorded at 5 m deep in Medas Islands (NW Mediterranean Sea).



Figure 2. Overview of the mesocosm system within the experimental chamber.

Water analyses

Nutrients concentration in the water (ammonium, nitrite, nitrate and phosphate) in each aquarium was analysed at the beginning (just after the experimental ammonium additions) and at the end of the experiment, using an FP-2020 Plus Fluorescence Detector, in accordance with standard methodology (K erouel and Aminot, 1997).

Plant trait response

A number of physiological and individual plant traits were measured at the end of the experiment to determine plant responses. These were maximum quantum yield (F_v/F_m), effective quantum yield of PSII ($\Delta F/F_m'$), maximum electron transport rate (ETR_{max}), non-photochemical quenching (NPQ), incidence of necrosis on the leaves and shoot growth rate.

Chlorophyll fluorescence parameters were determined in three randomly selected shoots from each aquarium using a diving PAM (pulse-amplitude Modulated fluorometer, Walz, Germany). The measurements were obtained from the basal portion of the second youngest leaf to avoid within-shoot variability (Durako and Kunzelman, 2002; Gera et al., 2012). F_v/F_m was measured by the saturation pulse method after a 10-minute period of dark adaptation. After three hours of illumination, leaves were exposed to increasing photosynthetic photon flux density values (0, 5, 19, 17, 129, 235, 277, 503 and 676 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) at intervals of 10 s to perform rapid light curves (RLCs), which made it possible to obtain $\Delta F/F_m'$, ETR and NPQ measurements. $\Delta F/F_m'$ and NPQ values extracted from RLCs were those obtained at a similar irradiance to plants that were maintained (210-223 $\mu\text{moles photons m}^{-2} \text{s}^{-1}$), while ETR_{max} corresponded to the maximum ETR value obtained in each curve.

The necrosis incidence was assessed in leaves from three shoots in each experimental condition. Leaves were carefully separated from each shoot and the percentage of necrotic surface (dark brown or black spots covering leaf tissue) relative to the total leaf surface was visually estimated in each leaf and averaged for each aquarium. Shoot growth was measured using a leaf marking technique (Zieman 1974) adapted to our species (Alcoverro et al., 2001; Short and Duarte, 2001). On the first day of the experiment, all shoots in each aquarium were marked by punching a hole just above the ligule with a hypodermic needle. At the end of the experiment, the shoots were harvested, the epiphytes carefully removed, and three shoots separated to measure shoot growth. Each shoot was sorted into old and new tissue. Plant material was dried for 48 hours at 60 °C and weighed to obtain the dry weight. Shoot growth rate was expressed as the new tissue produced per shoot and day (mg DW shoot⁻¹ day⁻¹), and then averaged for each aquarium.

Statistical procedures

The statistical significance of the effects of temperature and ammonium found between treatments was tested using PERMANOVA analyses based on a similarity matrix created from the Euclidean distances between samples. The aquarium was considered as the experimental unit, with a total of $n=3$ replicates for each experimental condition. The value for each variable in each replicate is the averaged value for this variable obtained from the three shoots (subsamples) used from each aquarium. Two fixed factors were used to run the analyses: temperature (three levels: 20 °C, 30 °C and 35 °C) and ammonium (ambient water, 30 μM and 120 μM).

Multivariate PERMANOVA was performed for plant response variables and univariate PERMANOVA analyses were subsequently carried out individually for each plant trait. As the PERMANOVA statistical test is produced by permutation, the usual ANOVA normality assumptions (Anderson 2001) were not necessary. Differences between treatments were evaluated using pairwise comparisons, and a Monte Carlo test was carried out to obtain an alternative p-value in order to validate the analysis when the number of permutations was too low (<999 , Anderson et al., (2008)). All analyses were performed using the Primer v6 statistical package Clarke and Gorley (2006) in conjunction with the Windows PERMANOVA+ module (Anderson et al., 2008).

RESULTS

Nutrient experimental conditions

The initial ammonium concentrations obtained in water ranged from 0.25-0.7 μM , 32-60 μM and 121-132 μM in samples from the control, moderate and high treatments, respectively. At the end of the experiment, ammonium concentrations were very low (less than 1 μM in most treatments, except in two cases: the control (no ammonium added) at high temperature, where some ammonium production took place, and in the high concentration treatment at

35 °C, where the final concentration was ca. 70 μM , 60 % of the initially supplied (Table 1). Concentrations of other nutrients were in the normal range for the NW Mediterranean waters and did not change significantly during the experiment.

Table 1. Ammonium concentrations (in μM , mean \pm SEM, $n=3$) in the water at the beginning (just after ammonium additions) and at the end of the experiment.

Ammonium treatment	Thermal treatment					
	20 °C		30 °C		35 °C	
	NH ₄ ⁺ (μM)					
	Initial	Final	Initial	Final	Initial	Final
Control	0.25 \pm 0.08	0.43 \pm 0.18	0.33 \pm 0.14	0.27 \pm 0.16	0.73 \pm 0.27	3.01 \pm 1.97
Moderate	40.24 \pm 2.18	0.41 \pm 0.12	59.87 \pm 16.87	0.28 \pm 0.23	32.15 \pm 1.40	0.85 \pm 0.43
High	131.83 \pm 2.27	0.15 \pm 0.09	123.77 \pm 4.47	0.63 \pm 0.26	121.27 \pm 4.63	72.49 \pm 18.83

Chlorophyll fluorescence parameters

Temperature had a significant effect on all chlorophyll fluorescence parameters measured (Table 2). Maximum and effective quantum yields (F_v/F_m and $\Delta F/F_m'$, respectively) and maximum electron transport rate (ETR_{max}) showed a similar response pattern, with values at 30 °C unaltered and a substantial decrease (38 %, 81 % and 73 %, respectively) at 35 °C (in both cases relative to controls at 20 °C) (Figure 3A, B & C). Non-photochemical quenching (NPQ) (Figure 3D) showed slightly higher values at 30 °C (up to 17 % more) and lower values at 35 °C (58 %, in both cases relative to controls).

Overall, ammonium additions had negative effects in all but one chlorophyll fluorescence parameter ($\Delta F/F_m'$, ETR_{max} , and NPQ), which decreased by 19 %, 19 % and 41 %, respectively, irrespective of the amount added. Interestingly, NPQ increased at 30 °C in plants submitted to no ammonium addition and moderate ammonium addition but did not at high ammonium concentrations. This is suggestive of a synergistic effect but, given the significance level of the interaction ($p=0.0582$), by no means conclusive.

In contrast, the combined effect of temperature and ammonium on decreasing F_v/F_m was clearly synergistic. As mentioned above, warming alone (35 °C) depressed F_v/F_m in the absence of ammonium additions, while ammonium additions at the control temperature did not cause any effects (Table 2). However, when ammonium was added and plants were warmed (35 °C), F_v/F_m was depressed to 54-87 %, relative to controls. At 35 °C and under high ammonium concentrations, F_v/F_m was below 0.1, thus indicating critical damage to the photosynthetic apparatus (Figure 3A).

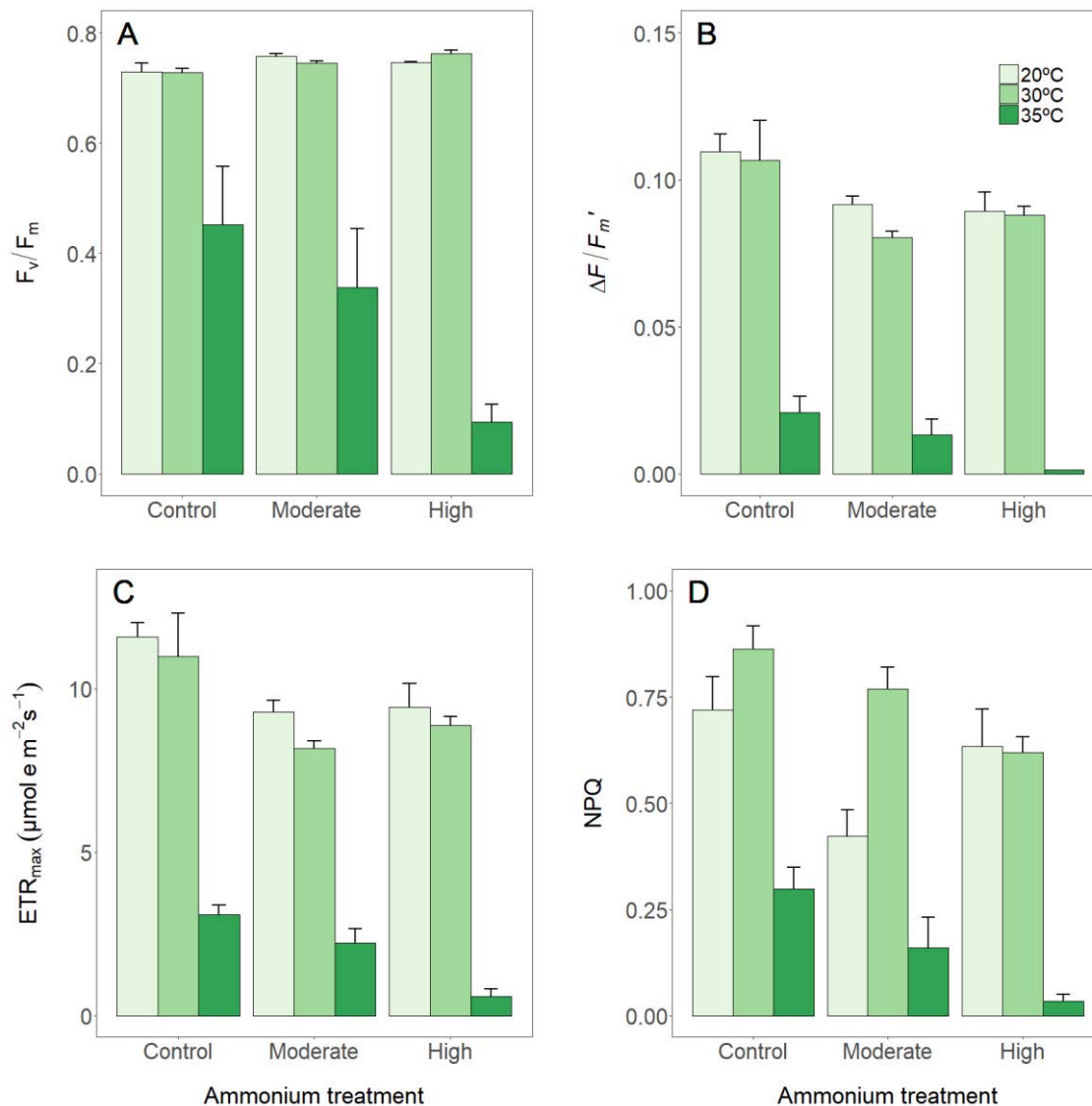


Figure 3. Photochemical responses of *P. oceanica* plants to temperature increase and ammonium addition: (A) Maximum quantum yield of dark-adapted leaves (F_v/F_m), (B) effective quantum yield of PSII ($\Delta F/F_m'$), (C) maximum electron transport rate (ETR_{max}), and (D) non-photochemical quenching (NPQ). Each variable was measured (mean \pm SE, $n=3$) at three thermal treatments and at three ammonium concentrations, after 7 days of exposure.

Leaf necrosis incidence

Temperature had a significant effect on leaf necrosis, with an incidence of up to 25 % higher at 35 °C than at 20 °C and 30 °C (Figure 4, Table 2). Ammonium addition also appeared to increase necrosis incidence, although the effect was only marginally significant ($p=0.0692$), likely due to the high variability of this variable.

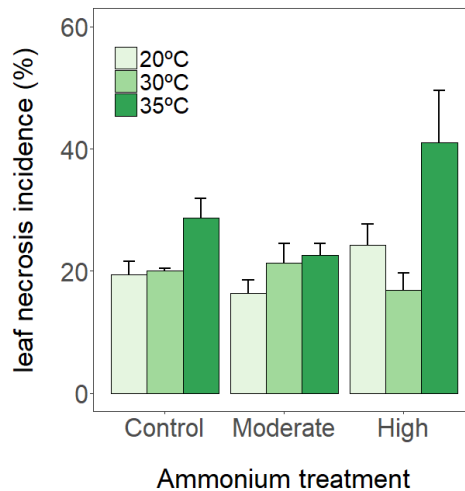


Figure 4. *P. oceanica* leaf necrosis incidence (mean \pm SE, $n=3$) at three thermal treatments and at three ammonium concentrations, after 7 days of exposure.

Shoot growth rate

Both temperature and ammonium had a significant overall effect on plant growth, with a negative effect of temperature and a positive effect (at the moderate concentration only) of ammonium (Figure 5, Table 2). However, these overall effects are misleading, since both stressors showed a clear synergistic interaction ($p=0.0399$) that made their combined effect relatively complex. Thus, the positive effect of moderate ammonium concentrations on growth occurred only at the control temperature, while it disappeared at 30 °C and became negative at 35 °C. Interestingly, the negative effects of extreme temperature (35 °C) were considerably higher at the high ammonium concentration (65 % growth rate reduction) than at the control ammonium concentration (40 %).

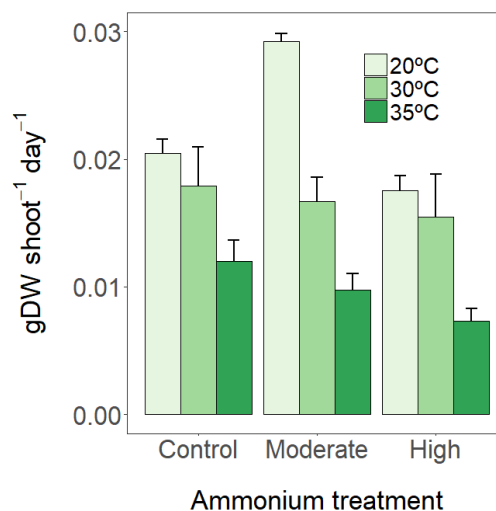


Figure 5. Shoot growth. *P. oceanica* shoot growth rate (mean \pm SE, $n=3$) at three thermal treatments and at three ammonium concentrations, after 7 days of exposure.

Table 2. Results of PERMANOVA (multivariate and univariate analysis) testing for the significance of temperature (20 °C, 30 °C, and 35 °C) and nutrient concentration (C: ambient; M: moderate, 30 µM and H: high, 120 µM) effects on plant traits. Bold values indicate significant effects ($p < 0.05$). The results of the pairwise tests are indicated in factors with significant influence.

Variable	Source	df	SS	MS	Pseudo-F	P	Unique perms	Pairwise
<i>Main test</i>								
	Temperature (T)	2	1098.4	549.2	12.857	0.0004	9953	
	Ammonium (A)	2	276.8	138.4	3.24	0.0531	9960	
	T X A	4	416.16	104.04	2.4356	0.0741	9950	
	Residual	18	768.9	42.717				
<i>Individual test</i>								
F_v/F_m	Temperature (T)	2	1.21	0.61	76.43	0.0001	9935	20=30>35
	Ammonium (A)	2	0.05	0.03	3.25	0.0588	9955	
	T X A	4	0.15	0.04	4.75	0.0073	9957	35C > 35H
	Residual	18	0.14	0.01	0.00			
$\Delta F/F_m'$	Temperature (T)	2	0.04	0.02	180.52	0.0001	9941	20=30>35
	Ammonium (A)	2	0.002	0.001	8.99	0.0019	9957	C>M=H
	T X A	4	0.0004	0.0001	0.81	0.5396	9946	
	Residual	18	0.0020	0.0001				
ETR_{max}	Temperature (T)	2	364.50	182.25	183.78	0.0001	9953	20=30>35
	Ammonium (A)	2	27.33	13.66	13.78	0.0005	9946	C>M=H
	T X A	4	5.06	1.26	1.28	0.3177	9965	
	Residual	18	17.85	0.99				
NPQ	Temperature (T)	2	1.65	0.83	76.83	0.0001	9948	30>20>35
	Ammonium (A)	2	0.21	0.11	9.87	0.0017	9952	C>M=H
	T X A	4	0.12	0.03	2.85	0.0582	9952	30C ≥ 30M ≥ 30H
	Residual	18	0.19	0.01				
Necrosis	Temperature (T)	2	731.00	365.50	8.76	0.0019	9958	20=30<35
	Ammonium (A)	2	249.21	124.61	2.99	0.0692	9940	
	T X A	4	410.83	102.71	2.46	0.0725	9960	
	Residual	18	750.71	41.71				
Growth	Temperature (T)	2	0.0007	0.0004	33.34	0.0001	9936	20>30>35
	Ammonium (A)	2	0.0001	0.0001	5.55	0.0123	9943	C=M, C=H, M>H
	T X A	4	0.0001	0.00004	3.25	0.0399	9956	35C > 35H
	Residual	18	0.0002	0.00001				

DISCUSSION

While warming has a clear negative effect on most of the variables measured, ammonium additions seem to exert only a moderate impact on plant performance when acting in isolation. However, we detected synergy between both factors in the response of two-three important plant traits, one related to the integrity of the photosynthetic system (maximum quantum yield), the second related to the capacity of the plant to activate photoprotective mechanisms (NPQ, only suggestive, as indicated based on p-value) and the third related to plant production (shoot growth rate), all of which are critical to plant survival. This serves as a warning that the impact of global warming on seagrass meadows already subject to eutrophication could be worse than expected.

A certain amount of interest lies in characterizing the thermal response of foundation species to warming. In the case of *P. oceanica*, such studies are relatively scarce (see below). In our case, based on the chlorophyll fluorescence responses and other plant traits, it would seem that *P. oceanica* tolerates short-term (i.e. one-week) temperature increases up to 30 °C. This tolerance might be partially attributed to the plant's capacity to activate photoprotective mechanisms (e.g. associated with xanthophyll cycle pigments (Marín-Guirao et al., 2012, 2016; Ralph and Gademann, 2005)) at this temperature, as suggested by the increasing, albeit not statistically significant, NPQ trend (at 30 °C). In addition, neither the necrosis incidence of leaves nor shoot growth were affected by 30 °C, in line with the findings of previous studies (Olsen et al., 2012), which would support its thermal tolerance to temperature increases up to 30 °C. By contrast, we observed negative changes in all variables measured at 35 °C. Thus, the decrease in F_v/F_m and $\Delta F/F_m'$, at 35 °C, indicates a severe reduction in the functionality of the photosynthetic apparatus (Campbell et al., 2003). At the same time, the electron transport chain and, therefore, the electron transport capacity (ETR_{max}) were severely affected by this high temperature, which could be attributed to a negative effect on the PSII donor side (Duarte et al., 2016), as reported in previous studies (*Z. noltii*, Repolho et al., (2017)). This suggests that the heat dissipation pathway likely linked to the xanthophyll cycle found at 30 °C seems to be inhibited when temperature reaches 35 °C, as demonstrated by the drastic reduction in NPQ. This loss of capacity to dissipate the excess thermal energy could have induced damage to the PSII and consequently reduced the photosynthetic capacity of the plants (Ashraf and Harris, 2013). Impairment of photosynthesis or a likely increase on respiration rates, are probably some of the causes behind the clear reduction in leaf growth that was observed, and certainly triggered other negative effects on plant fitness (reserve accumulation, rhizome growth and probably many others). Finally, the higher leaf necrosis incidence, which is a common plant response to several stressors, including salinity (Pagès et al., 2010; Salo et al., 2014) and eutrophication (Ceccherelli et al., 2018; Roca et al., 2016), in plants exposed to 35 °C indicates not only a loss of functionality of the photosynthetic systems, but also tissue damage and cell death.

In this regard, based on the thermal sensitivity of this species to high temperatures, as described above (Marbà and Duarte, 2010; Pagès et al., 2018; Savva et al., 2018), our results and the findings of other studies (Marín-Guirao et al., 2018; Olsen et al., 2012; Traboni et

al., 2018; Tutar et al., 2017), we suggest a thermal threshold for *P. oceanica* of between 30 °C and 35 °C.

Ammonium additions negatively and moderately affect most of the chlorophyll fluorescence-related variables measured ($\Delta F/F_m'$, ETR_{max} and NPQ), independently of temperature (see non-significant interactions in Table 2). No effect of ammonium was detected on F_v/F_m at control or moderately high temperatures (20 °C and 30 °C). In addition, we observed a positive effect of moderate ammonium addition on shoot growth at the control temperature, consistent with the nutrient-limited condition of this species (Alcoverro et al., 1997; Invers et al., 2004). Therefore, it would seem that the toxicity of ammonium in *P. oceanica* at basal temperatures is much lower than in other seagrass species, which are mostly colonizing and opportunistic (*sensu* Kilminster et al., (2015)) species (*Z. noltii*, Moreno-Marín et al., (2016); *Z. marina*, van Katwijk et al., (1997)). However, the most relevant finding of our experiment was that the negative effects of ammonium additions appear when temperature increases, thus leading to interactive effects between both stressors. Thus, maximum quantum yield (F_v/F_m) was clearly affected by ammonium, but only at extreme temperatures (35 °C), thereby indicating temperature-dependent ammonium toxicity. This toxicity is likely related to the damage of the photosynthetic machinery which, due to its inability to fix C, hindered the assimilation of ammonium in non-toxic forms (Invers et al., 2004; Leoni et al., 2008). In addition, our results suggest that the interaction between both stressors affected the plant's capacity to activate photoprotective mechanisms, as indicated by a lack of activation of NPQ mechanism at 30 °C under high ammonium concentration. Our findings indicate that moderate ammonium additions stimulated shoot growth at control temperature while this stimulation was lost at 30 °C and 35 °C. Moreover, the thermal effects of extreme temperatures (35 °C) were clearly worse at high ammonium concentrations, as growth rates in this treatments combination were 42 % lower than those found at 35 °C without ammonium addition.

Even though several studies in opportunistic species have revealed that the combined effects of temperature increase and ammonium are not detrimental (*Z. marina*, Kaldy (2014); Moreno-Marín et al., (2018); *C. nodosa*, Ontoria et al., (2019b)), or may even favour plant primary production (*C. nodosa*, Egea et al., (2018)), our results indicated a negative synergistic effect between both stressors in *P. oceanica*, a species considered to be persistent, thus leading to the conclusion that the future impact of warming could be much worse for plants subject to high ammonium loading than for plants living in relatively pristine environments. These findings are consistent with a large number of studies, which have also reported synergistic effects between two simultaneous stressors on seagrasses (Collier et al., 2011; Ontoria et al., 2019b; Villazán et al., 2013b, 2015). However, most of these studies have focused on colonizing and opportunistic seagrass species; further studies are therefore required to shed light on the response of this, and other, persistent seagrass species to simultaneous exposure to two or more stressors.

As highlighted in the introduction, exploring the effects of climate change on coastal ecosystems already threatened by local factors is critical to determining and understanding the future of such ecosystems. Performing factorial experiments, which allow two or more

stressors to be combined simultaneously with a view to exploring plant response, could help predict future scenarios. Although some caution should be exercised when scaling our results up to real-world ecosystems, mainly due to our limited spatial and temporal scales, it is clear that our findings serve as a warning not only about the effects of global warming, but also about the synergies between warming and other local stressors. The predicted rise in the frequency and intensity of heat waves in the Mediterranean Sea (IPCC 2007; Jordà et al., 2012; Vargas-Yañez et al., 2007) may be tolerated by the plant in the short term, but as duration (Marín-Guirao et al., 2018) and/or intensity increase, plant photosynthesis and growth will be curtailed and persistence will likely be compromised. Moreover, other stressors such as eutrophication, especially in persistent seagrass species such as *P. oceanica* living in oligotrophic environments, can worsen the negative effects of warming. Consequently, these heightened effects might threaten the survival of these important seagrass meadows (Jordà et al., 2012).

Although this research is not fully conclusive, and more extensive experiments, in the field whenever possible, are needed for a proper upscaling to the real world, our results clearly indicate a need to broaden the focus to include the potential interaction with other stressors when attempting to assess the effects of global warming. This is required not only to obtain more accurate, reliable and realistic predictions and therefore aid adaptive management, but also to act against global stressors at local level. In effect, attenuating local stressors may represent one way to alleviate the effects of global warming, or at least ensure they do not worsen.

ACKNOWLEDGEMENTS

We thank Neus Sanmartí for her help in the plant sampling necessary for conducting this experiment, and Rocío García Muñoz for her help in the temperature data analysis and graphics. This work was supported by the European Union and the Spanish Government through the RECCAM (Seagrass meadows resilience to global warming: an analysis based on responses at ecophysiological, population and ecosystem levels, CTM2013-48027-C3-1-R and CTM2013-48027-C3-2-R) and UMBRAL (Responses of benthic marine vegetation to stress: critical transitions, resilience, and management opportunities, CTM2017-86695-C3-1-R) projects; and by the Spanish Ministry of Economy and Competitiveness (via grant BES-2014-069593 awarded to Y. Ontoria).



CHAPTER 4

High salinities buffer the negative effects
of warming on functional traits of the
seagrass *Halophila ovalis*

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ABSTRACT

Coastal ecosystems, especially estuaries are subject to environmental fluctuations. If two of the main environmental factors that can affect plant performance, temperature and salinity, are altered under a future scenario of climate change, this may be detrimental for ecosystem persistence. This study examined the response of the seagrass *Halophila ovalis* to a short-term salinity and temperature (simulating a heatwave) increase, in isolation and in combination, through two indoor mesocosm experiments. Warming caused detrimental effects, a decline in photosynthetic efficiency (F_v/F_m) and an increase in maximum photosynthetic rates (Gross P_{max}), revealing symptoms of thermal stress, a decrease in leaf TNCs content and severe reduction in plant growth. Salinity increase in isolation did not alter plant performance, however, interestingly, in combination with high temperature the Gross P_{max} were higher and photosynthetic efficiency (α) was not impacted. Overall, higher salinities might ameliorate the negative effects of high temperatures, buffering the impact of climate change.

INTRODUCTION

Global change is threatening ecosystems worldwide (Bellard et al., 2012; IPCC 2014) and is considered a major driver of the erosion of marine biodiversity (Poloczanska et al., 2013). Coastal ecosystems are particularly vulnerable to global change because they are exposed to a range of cumulative impacts, including eutrophication and physical alterations due to engineering works such as marinas or walls. These and many others are strongly linked to human population pressures on the coast (Halpern et al., 2015; Orth et al., 2006). Increased temperatures also impact coastal ecosystems, often in an additive or synergistic way with other stressors (Humanes et al., 2016; Koch et al., 2007; Ontoria et al., 2019a, 2019b; York et al., 2013). The current gradual increase of sea surface temperature, which is predicted to rise, on average, between 0.6 and 1.5 °C in the next 40 years (IPCC, 2014) is causing deleterious or even lethal impacts on some aquatic organisms (Garrabou et al., 2009; Smale and Wernberg, 2013). In addition, this gradual thermal rise is accompanied by extreme events such as heat waves where temperatures increase well above background levels (up to ca. 5 °C) and can persist for more than five days (Hobday et al., 2016). These are especially detrimental for ecosystems integrity and persistence (IPCC 2014; Karl and Trenberth, 2003; Oliver et al., 2018; Smale et al., 2019).

Specially, estuaries and other transitional waters are particularly subject to large environmental fluctuations which pose significant physical forcing and influence ecological relationships (Day et al., 2012). Furthermore, estuaries are influenced by a wide range of physical drivers, which make them particularly vulnerable to climate change (Hallett et al., 2018), which is expected to change the “rules of play” or the regime under which fluctuations occur.

Fluctuations in salinity are a feature of estuarine environments, where both gradual and abrupt changes in salinity can occur. Organisms inhabiting estuaries are generally euryhaline, being able to thrive under a wide range of salinities. This ability is attributed to features that confer tolerance to salinity changes, including adjustments through a series of metabolic pathways, physiological traits and molecular or gene networks (Gupta and Huang, 2014). With progressive warming and the increased frequency and intensity of heat waves, evaporation rates are likely to increase resulting in increases in salinity, especially where flushing with fresh or marine water is limited. On the other hand, the predicted increasing intensity of rainfall at particular times of year could temporally and abruptly reduce salinity in these systems (Hallett et al., 2018). How these climate change drivers manifest will depend on the type of estuary, including the patterns of freshwater inputs and exchange with ocean waters, physical features such as flushing times, and morphological features such as water depth and volume.

The impact of global warming is of particular concern for species considered as ecosystem engineers, such as the habitat forming corals, mangroves, gorgonians, kelps or seagrasses, as it will have cascading effects on ecosystem functions and biodiversity (Hoegh-Guldberg et al., 2007; Smale et al., 2019; Wernberg et al., 2012). Vegetated communities provide structure and other key ecosystem functions as primary production, fueling coastal

trophic networks and contributing to other ecosystem services (e.g. acting as carbon sinks, buffering acidification) (Beaumont et al., 2007; Duarte et al., 2005; Twilley and Day, 1999). Specifically, seagrasses are one of the most productive ecosystems on Earth (Hemminga and Duarte, 2000), providing multiple ecosystem services and are highly valued economically and ecologically (Orth et al., 2006). The sensitivity and vulnerability of seagrasses to warming is becoming increasingly evident (Nowicki et al., 2017, 2019; Pearce and Feng, 2013; Thomson et al., 2015). Negative effects of warming on plant performance (Collier et al., 2011; Marín-Guirao et al., 2018; Ontoria et al., 2019a, 2019b) as well on shoot survival (Díaz-Almela et al., 2009; Marbà and Duarte, 2010) have resulted in detrimental effects in these ecosystems (Kendrick et al., 2019; Waycott et al., 2009).

However, warming rarely acts in isolation from other factors. Temperature increases might affect plant performance not only directly but also through its interaction with plant tolerance mechanisms to other factors such as changes in salinity (Sandoval-Gil et al., 2012a, 2012b, 2014) or grazing pressure (Hernán et al., 2017; Eklöf et al., 2008). In this regard, although it is largely accepted that global warming represents a real threat for coastal ecosystems worldwide (Harley et al., 2012; IPCC 2014; Poloczanska et al., 2013), it is difficult to predict how warming will affect the resilience of those ecosystems that are currently found in fluctuating environments, such as the estuaries, and whose survival largely depends upon robust regulating mechanisms (Hallett et al., 2018).

Temperature affects seagrass performance from the molecular to population level (Beca-Carretero et al., 2018; Campbell et al., 2006; Collier and Waycott, 2014; Collier et al., 2011; Marín-Guirao et al., 2016, 2017, 2018; Ontoria et al., 2019a, 2019b; Ruiz et al., 2018; Savva et al., 2018; Traboni et al., 2018). Changes in salinity, in turn, can also alter physiological functioning and, consequently, influence plant growth and survival (Garrote-Moreno et al., 2015; Marín-Guirao et al., 2013a, 2013b, 2017; Piro et al., 2015; Ruiz et al., 2009; Sandoval-Gil et al., 2012a, 2012b, 2014; Touchette and Burkholder, 2000). However, and despite the recent growing knowledge (restricted to some seagrass species and geographical areas), thermal and salinity tolerance thresholds and acclimation mechanisms are still poorly understood for seagrasses overall, compared to terrestrial halophytes and marine algae. In this regard, there is still a gap in knowledge about the combined effect or interactions between temperature and salinity on seagrasses, particularly for those species living in estuarine environments where both factors fluctuate and interact. This is highly relevant when trying to predict the ecological consequences of global change on seagrasses.

It is predicted that estuaries in Mediterranean climate regions, as southwestern Australia, will experience high temperatures and marine salinities in summer that favor seagrass growth (Forbes and Kilminster, 2014). *Halophila ovalis* is one of the most common seagrass species found in estuaries of southwestern Australia. It is a fast growing, colonizing species (*sensu* Kilminster et al., 2015) with a rapid ability to recover. *H. ovalis* has a wide tolerance range, occurring in waters between 10 °C and 40 °C (Ralph 1998) and from 5-45 psu (Hillman 1995; Tyerman 1982), which coincides with its broad distribution and abundance in estuarine environments. Previous short-term experiments (five days, Ralph 1998) with laboratory-cultured plants revealed that while *H. ovalis* has its optimum

photosynthetic range between 25 and 30 °C, its tolerance to salinity can range from 9-52 psu. However, the lack of knowledge about the responses to periods longer than five days (see Ralph 1998) to each one of these factors, as well as the interaction implies that there are opportunities to improve our understanding of tolerance limits.

The present study aims to explore the response of an estuarine seagrass, *H. ovalis*, to climate change pressures of warming and salinity changes and, specifically, to assess whether temperature increases affect plant tolerance to salinity fluctuations. To do this, two indoor mesocosm experiments were performed to evaluate plant responses to changing salinity under thermal increase, at the physiological, individual and population levels.

MATERIAL AND METHODS

Two independent short-term experiments were conducted in order to assess the response of *H. ovalis* to increases in temperature under different salinity conditions. The two experiments were required to enable measurements across a range of plant scales: physiological, individual and population levels and also with a range of treatments that were not possible in a single experiment due to technical and logistical constraints. In the first experiment (experiment A, hereinafter), photosynthesis-irradiance curves were performed under five levels of temperature and two salinity conditions, after 1 day of exposure period. In the second experiment, (experiment B, hereinafter), photochemical responses, carbon reserves, plant growth and survival were measured in plants subjected to two temperature levels and two salinity conditions for 13 days. Both experiments were conducted at the School of Science aquarium facilities at Edith Cowan University (Western Australia).

Experiment A: Photosynthesis-Irradiance curves

Plant collection, experimental set up and incubation equipment

Plants for the experiment were collected in late spring-early summer 2017 in the Swan-Canning Estuary (southwestern Australia). The Swan-Canning estuary is a shallow estuary permanently open to the ocean. It is characterized by two distinct ‘summer’ and ‘winter’ phases due to the region’s Mediterranean climate. During summer (December – March), air temperatures are high, and rainfall is typically low. Climatological (1961 – 1990) averages for the Perth metropolitan area, where the estuary is located, range from 29–31 °C and 10–19 mm (Bureau of Meteorology, <http://www.bom.gov.au>). Water temperature and salinity are greatly influenced by these climatic conditions and fall between 22–24 °C and 33–38 psu respectively (Hillman 1995; <http://wir.water.wa.gov.au/Pages/Water-Information-Reporting.aspx>). The majority of *H. ovalis* meadows occur in shallow, subtidal areas (< 2 m) of the estuary. Peak growth and reproduction are generally observed during summer months as a consequence of the favorable conditions (Forbes and Kilminster, 2014). Contrastingly, during winter (June – August) air temperatures are cooler ~19–19.4 °C whilst the majority (~80 %) of rainfall occurs and averages between 125–126 mm (Bureau of Meteorology, <http://www.bom.gov.au>).

Fragments of *H. ovalis* (including three or four pair of leaves, rhizomes, roots and a growing tip), termed “ramets”, were carefully collected and transported in a cooler box filled with *in situ* seawater to the laboratory. Dark adapted yields were measured from three randomly chosen plants in the field using a diving PAM (pulse-amplitude modulated) fluorometer (Walz, Germany). These measurements were used afterwards as a dark-adapted yield reference to confirm that plants in the experimental tanks had acclimated to the laboratory conditions after planting. Temperature and salinity at collection time were on average: 23 °C and 34 psu and were used to set the acclimation conditions in the laboratory. Plants were standardized to four leaf pairs with an apical meristem (the growing tip) and were carefully cleaned by hand to remove epiphytes before planting. Ramets were then planted into aquarium tanks (54 L capacity, 600 x 300 x 300 mm), previously filled with 10 cm depth of unsorted and washed quartz river sand and 52 L of seawater at 20 °C with a salinity of 34 psu. Each tank had its own independent sump tank containing a pump, filter (300 µm foam block) and aquaria heaters which controlled water temperature. Incident light on plants ($180 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, based on saturating irradiance for *H. ovalis* according to Strydom et al., 2017) was provided by a marine aquarium Light Emitting Diode (LED) modules with a full spectrum light (MarinTech™ Pty Ltd) on a 12 h: 12 h light: dark photoperiod. After two days of acclimation, temperature was progressively increased or decreased (1 °C day^{-1}) in the tanks until reaching the experimental temperatures (20 °C, 24 °C, 28 °C, 31 °C and 34 °C). This range of temperatures was established in order to cover the natural temperature range this species experiences during spring and summer in the area where they were collected. The highest temperature treatment was above what is experienced on average in the estuary and was chosen to simulate a heat wave event. Simultaneously, salinity was progressively increased (2 psu day^{-1}) from 34 psu (salinity during acclimation days) until reaching 40-42 psu (high salinity) in half of the tanks, keeping the other half at 34 psu (low salinity). This high salinity is above what is generally experienced in this estuary and might be potentially reached in some parts of the estuary as a result of changes in the climatic conditions. Thus, ten different conditions were set up: five different temperatures with each temperature treatment having two salinity treatments. Once the experimental conditions were reached in the tanks, plants were left for 24 hours before measuring maximum quantum yield on five randomly chosen plants to confirm their acclimation. Yields indicated were equal or higher to those measured in the field at the time of collection: 0.65-0.71, hence the plants were considered acclimated.

P-I determinations, curve fitting and extraction of photosynthetic parameters

Seagrass respiration and photosynthesis were measured via the consumption or production of O₂ after 24 hours incubation period to each corresponding experimental condition. Plants were incubated in sealed transparent acrylic chambers, with a diameter of 52 mm and a length of 150 mm (volume = 318 ml). Water within the chamber was circulated using a small submersible pump with a flow rate of 7000 ml hr⁻¹. Dissolved oxygen concentrations within the chambers were measured using FireSting™ 3 mm robust REDFLASH technology sensors (Pyrosience) inserted through the chamber wall and connected through a 4-channel meter to a computer recording O₂ concentrations (mg L⁻¹) To maintain a stable temperature ($\pm 0.25\text{°C}$) chambers were submerged in a 300 L tank containing 150 L of seawater, which

was circulated through a chiller-heater unit set to the appropriate experimental temperature. For temperature treatments above 30 °C saltwater was made using aquarium salt and milli-Q water to remove the additive effect of microbial respiration. The internal temperature of the chamber was also measured using a submersible temperature sensor connected to the FireSting O₂ machine. Light was provided by full spectrum LED light units (GrowPro 320; MarinTech™ Pty Ltd) suspended above the chambers, providing light intensities from 30 to 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Prior to each incubation, the oxygen electrodes were calibrated using a 2-point method (0 % and 100 % air saturated water) calibration as per the manufacturer's instructions. For P-I determinations, four-five *H. ovalis* ramets (apical meristem with four preceding leaf pairs connected to rhizome) were placed into the chambers (Figure 1). Five replicate plant chambers and a blank (no plant material) were established and placed into the temperature-controlled tank. The chambers were then covered in aluminium foil to exclude light and the inlet of each chamber was connected to its individual pump, to allow the chamber to be flushed whilst the plant material was left to dark adapt for 30 minutes. When dark adapted, the chamber outlet was connected to the pump to create a sealed system. Once sealed, the dissolved oxygen concentration in each chamber was monitored every second. Monitoring continued in the dark for at least 20 minutes after the slope of dissolved oxygen vs time stabilised. The foil was then removed, and photosynthetic rates were measured for 10 minutes (once the slope had stabilised) at each of the 7 light intensities (30, 60, 90, 120, 180, 240, and 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). At the end of the experiment whole plants were removed from the chambers and were separated into above (leaves) and below ground (roots and rhizomes) tissue. Fresh weight was recorded before drying the plants (48 hours at 60 °C) and reweighing for dry weight determination.

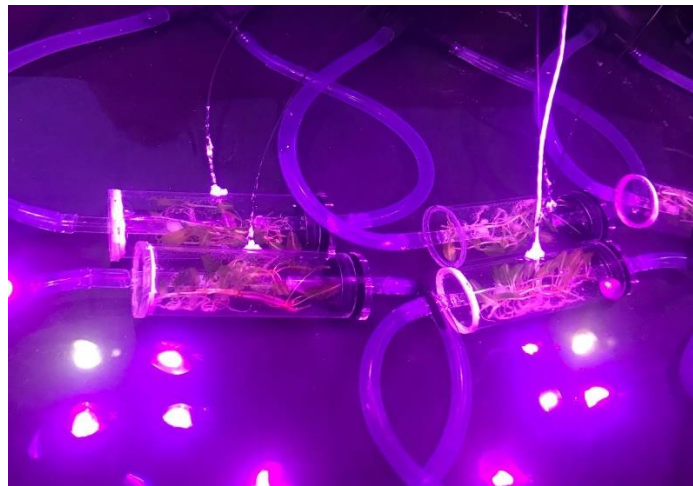


Figure 1. Incubation of *Halophila ovalis* plants in acrylic chamber for P-I curve determination (experiment B).

For each replicate incubation (and control) at all unique temperature-light intensity combinations, oxygen concentration was plotted against time after discarding the first 2 minutes of data, which was considered a stabilisation period. The portion of the remaining data used to determine the rate, was confined to that where the R² value was greater than 0.9.

At the two lowest light intensities, due to noise, a lower R^2 value of 0.5 was used. Rates of oxygen exchange were normalised to g DW of seagrass hr^{-1} . Oxygen concentrations within the control chamber (containing no seagrass) were measured throughout the experiment as a procedural control, data was accepted if there was a stable line with little variation, neither decreasing nor increasing in oxygen concentration. This was achieved in all trials.

Photosynthetic parameters were extracted from the photosynthetic-irradiance (P-I) curves using the least-squares method. For each incubation, PI curves were fitted to the data using the hyperbolic tangent model equation of Chalker (1981):

$$P = \text{Gross } P_{\max} \times \tanh\left(\frac{\alpha \times I}{GP_{\max}}\right) - R,$$

where P is the net rate of photosynthesis ($\text{mg O}_2 \text{ g DW}^{-1} \text{ h}^{-1}$), Gross P_{\max} is the maximum gross photosynthesis ($\text{mg O}_2 \text{ g DW}^{-1} \text{ hr}^{-1}$), α is the photosynthetic efficiency estimated as the slope for the linear portion (light-limited portion) of the PI curve, I is irradiance ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$), and R is the rate of oxygen consumption in the dark ($\text{mg O}_2 \text{ g DW}^{-1} \text{ h}^{-1}$).

NP_{\max} was defined as the maximal net rate of photosynthesis ($\text{mg O}_2 \text{ g DW}^{-1} \text{ h}^{-1}$) and was calculated as:

$$NP_{\max} = \text{Gross } P_{\max} - R$$

The half-saturating irradiance (I_k ; $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) was calculated as:

$$I_k = (NP_{\max} + R) / \alpha$$

Experiment B: Plant functional traits responses

Plant collection, mesocosm system and experimental set up

Undamaged healthy *H. ovalis* ramets were carefully collected by hand from a shallow, undisturbed meadow (< 0.5 m deep) in spring-early summer 2017 in Peel Harvey Estuary (southwestern Australia). Similar to the Swan-Canning estuary, the Peel-Harvey Estuary is a shallow, permanently open estuary and the salinity and temperature regimes reflect the hot, dry conditions during summer. Between December to March, rainfall and temperature range between 12.5–19.6 mm and 28–31 °C, respectively (Bureau of Meteorology, <http://www.bom.gov.au>). In the estuary, water temperatures average between 21–23 °C which also becomes increasingly hypersaline over the summer from 35 psu in December to 43 psu by March (<http://wir.water.wa.gov.au/Pages/Water-Information-Reporting.aspx>). The estuary was opened to the ocean via the Dawesville channel causing salinity to become more stable and similar to marine values (~ 35 psu) except during winter when most of the rainfall occurs (Water and Rivers Commission, 1998, link: https://www.water.wa.gov.au/__data/assets/pdf_file/0019/5356/10564.pdf). This change has favoured the establishment of *H. ovalis* meadows, the dominant seagrass species in the estuary.

At the time of collection, the average field temperature was 20 °C and salinity 34 psu. Plants were transported to the laboratory and kept under constant temperature (20 °C) overnight before planting. Nine ramets of *H. ovalis* plants (standardized to two pairs of leaves, rhizome, roots and a growing tip) were planted in aquarium tanks as described above. The ramets were shorter than in the experiment A as the plants were naturally more fragmented in the field.

In order to determine plant response to warming when growing under different levels of salinity, *H. ovalis* plants were exposed to two levels of temperature (control and high) and two levels of salinity (low and high) simultaneously in a short-term experiment lasting for 13 days. Control temperature condition was set as the average field temperature (24 °C) in late spring while 30 °C was set up as the high thermal treatment. The latter temperature is above the average summer temperatures and was selected to simulate the temperature that might be reached in these estuarine environments during extreme warming events such as heat waves. The salinity measured in the field during plant collection was 34 psu and it was considered the low salinity treatment. The high salinity tested (40-42 psu), in turn, is within the range experienced in some parts of the estuary, but not where the seagrass was collected. Water temperature and salinity were monitored every two days by a WTW™ conductivity meter throughout the experiment. Plants were acclimated at 24 °C (progressive increasing of 1 °C day⁻¹) and 34 psu for seven days, after which temperature and salinity were increased progressively (1 °C day⁻¹ and 2 psu day⁻¹, respectively) in the appropriate treatment tanks until reaching the temperature and salinity experimental treatments. It took 13 days to reach treatment conditions, then the plants were exposed for another 13 days to different temperature and salinity treatments as follows: control temperature and low salinity (24 °C, 34 psu), high temperature and low salinity (30 °C, 34 psu), control temperature and high salinity (24 °C, 40-42 psu) and high temperature and high salinity (30 °C, 40-42 psu). For each treatment, four replicate tanks were established (total n=16 independent tanks) and randomly allocated. Incident light (200-220 μmol photons m⁻² s⁻¹, above the saturation irradiance for these plants) was measured using an underwater quantum sensor (MicroPAR) and maintained on a 12 h: 12 h light: dark photoperiod.



Figure 2. Overview of the mesocosm system of the experiment B.

Plant traits assessment

Chlorophyll a fluorescence parameters

In each tank, chlorophyll a fluorescence parameters were measured in three randomly selected shoots from three independent ramets using a diving PAM fluorometer. Maximum quantum yield (F_v/F_m) measurements were obtained in the middle portion of the leaf by the saturation pulse method after an overnight dark adaptation. After being exposed to illumination conditions for three hours, an increasing photosynthetic photon flux density was applied directly to the leaf at intervals of 10 s to perform rapid light curves (RLCs), which were fitted to the equation described by Jassby and Platt (1976) using SigmaPlot (version 11) in order to estimate the maximum electron transport rate (ETR_{max}).

Carbohydrates content

Total non-structural carbohydrates (TNCs) analyses was performed separately in leaves and rhizome material. All ramets from each tank were pooled thus having a total of four replicate samples of each tissue per treatment. Samples were analyzed for soluble sugars and starch content following the anthrone assay described in Marín-Guirao et al., (2013b), based on Invers et al., (2004) and Yemm and Willis, (1954).

Plant growth and survival

At the beginning of the experiment, all the ramets were tagged to be able to identify the new tissue produced in the experiment. All newly produced material (leaves, petioles, rhizomes and roots) from each ramet was dried at 60 °C for 48 h and weighed all together. Plant growth was expressed as mg DW ramet⁻¹ day⁻¹. At the end of the experiment, the number of surviving ramets in each tank was estimated and expressed in percentage relative to the initial number of ramets planted.

Statistical analyses (Experiments A and B)

PERMANOVA analyses based on a similarity matrix created from the Euclidean distances between samples was performed to test the statistical significance of the effects of temperature and salinity on seagrass response parameters (fixed factors). For the experiment A, we had 10 experimental conditions (with five replicates for each), resulting from the combination of five temperatures (20 °C, 24 °C, 28 °C, 31 °C and 34 °C) and two salinities (low and high, i.e. 34 psu and 40-42 psu). For experiment B, the two factors had two levels (temperature: 24 °C and 30 °C, salinity: 34 psu and 40-42 psu), resulting in four experimental conditions, with four replicate tanks for each.

Firstly, multivariate PERMANOVA analysis was run (separately for each experiment) for all response variables and then univariate PERMANOVA analyses were individually performed for each variable (experiment A: photosynthetic and respiration rates, half-saturating irradiance and photosynthetic efficiency; experiment B: F_v/F_m , ETR_{max} , TNC content in leaves and rhizomes, plant growth rate and ramets survival). As the

PERMANOVA statistical test is produced by permutation, it was not necessary to meet the usual ANOVA normality assumptions (Anderson, 2001). When main factors had a significant effect on the variable, pair wise comparisons were performed to identify significant differences between treatments. When the number of permutations was too low (< 999, Anderson et al., 2008), a Monte Carlo test was conducted in order to establish an alternative p-value for analysis validation. The significance level (α) used was $p=0.05$ in all tests performed. All analyses were performed using the Primer v6 statistical package (Clarke and Gorley, 2006) in conjunction with the Windows PERMANOVA+ module (Anderson et al., 2008).

RESULTS

Experiment A: Photosynthesis-Irradiance curves

Typical P-I curves with no photoinhibition was observed in each experimental condition in *H. ovalis* plants (Figure 3).

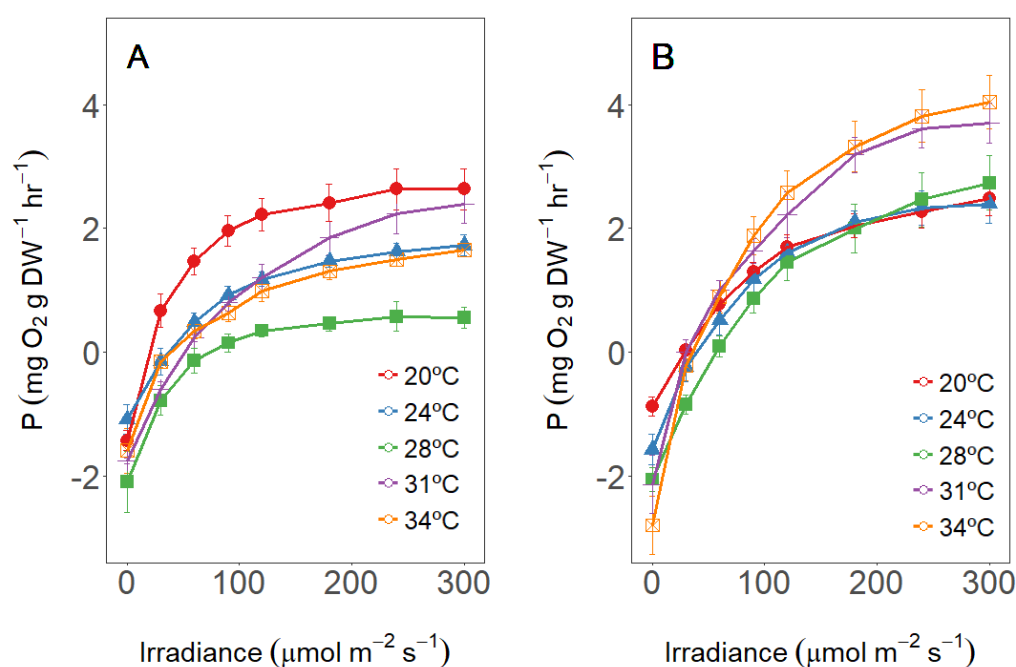


Figure 3. Photosynthesis-Irradiance curves of *Halophila ovalis* plants at five temperatures (20 °C, 24 °C, 28 °C, 31 °C and 34 °C) and two salinities: A) low (34 psu) and B) high (40-42 psu).

All of the photosynthetic parameters extracted from the photosynthesis-irradiance (P-I) curves were affected by the treatments, either temperature only, salinity only, both and/or an interaction between the two (Figure 4). Maximum gross photosynthesis values ranged from 2.6 to 6.6 mg O₂ g DW⁻¹ hr⁻¹, and there was a significant interaction between the two factors (Table 1). At low salinity, the effect of temperature was unclear, with highest values at 20 °C and 31 °C (3.9 and 4.1 mg O₂ g DW⁻¹ hr⁻¹, respectively) and lowest at 24 °C and 28 °C (2.6 mg O₂ g DW⁻¹ hr⁻¹) (Figure 4A). In contrast, at high salinity, temperature had

a clear and positive effect on maximum gross photosynthesis, reaching $6.6 \text{ mg O}_2 \text{ g DW}^{-1} \text{ hr}^{-1}$ at $34 \text{ }^\circ\text{C}$. Respiration rates ranged from 0.84 to $2.61 \text{ mg O}_2 \text{ g DW}^{-1} \text{ hr}^{-1}$ (Figure 4B). Temperature had a positive and significant effect on respiration, resulting in an increase of 81% at the highest temperature ($34 \text{ }^\circ\text{C}$) relative to the lowest ($20 \text{ }^\circ\text{C}$). Salinity did not significantly affect the respiration rate (Table 1). Half-saturating irradiance (I_k) ranged from 62 to $126 \text{ } \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Figure 4C). Salinity affected I_k significantly (Table 1) with higher I_k at higher salinities. Temperature, in turn, did not affect I_k (Table 1). Photosynthetic efficiency (α) ranged from 0.03 to $0.07 \text{ mg O}_2 \text{ g DW}^{-1} \text{ hr}^{-1} / \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ (Figure 4D). No individual effects of temperature nor salinity were found, but there was a significant interaction (Table 1). This is due to the opposite response of α with temperature, decreasing at low salinity (from 0.07 to $0.04 \text{ } \mu\text{mol O}_2 \text{ g DW}^{-1} \text{ hr}^{-1}$) and increasing at high salinity (from 0.03 to $0.07 \text{ mg O}_2 \text{ g DW}^{-1} \text{ hr}^{-1} / \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$).

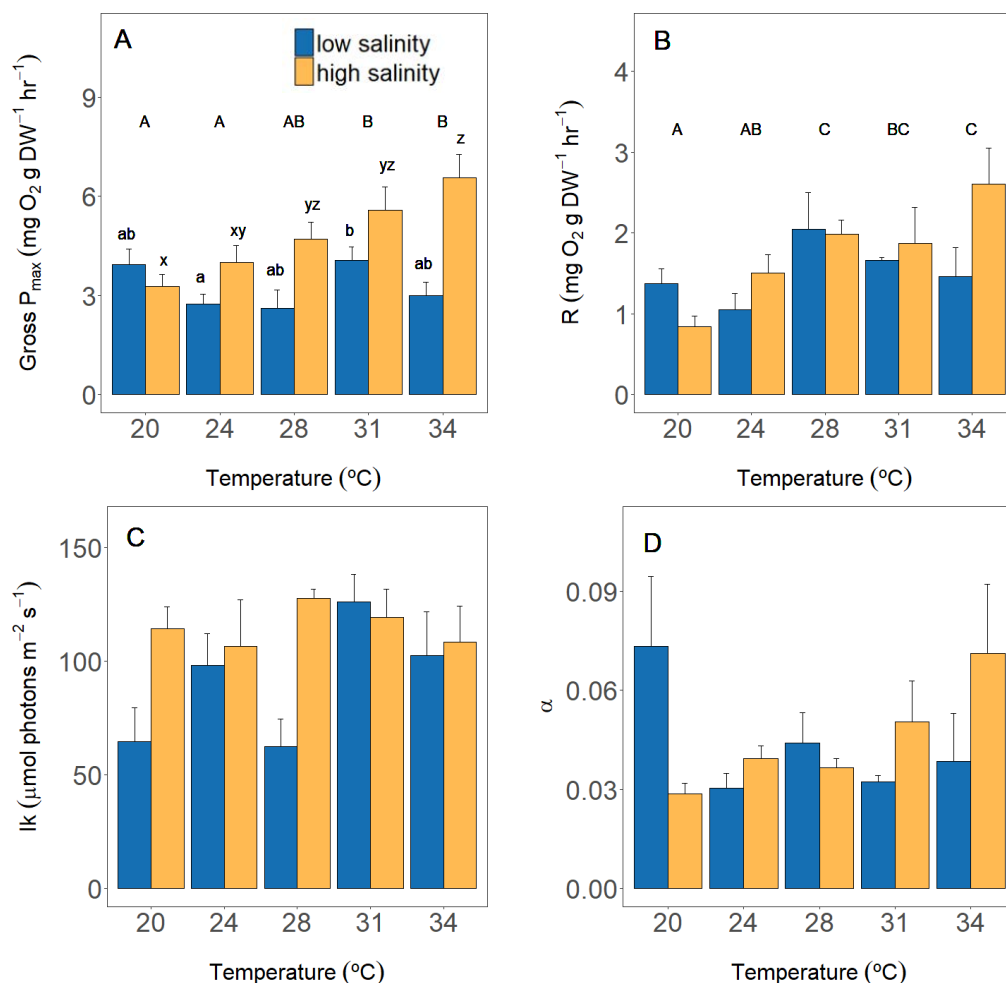


Figure 4. Photosynthetic parameters obtained from P-I curves for *Halophila ovalis* plants: A) maximum gross photosynthesis (Gross P_{max}), B) respiration rate (R), C) half-saturating irradiance (I_k) and D) photosynthetic efficiency (α) at five temperatures (20 °C, 24 °C, 28 °C, 31 °C and 34 °C) and two salinities (low (34 psu), and high (40-42 psu)) in the experiment A. Capital letters over the bars represent significant differences between thermal treatments (independent from salinity); lower case letters represent significant differences between thermal treatments at each salinity separately (a and b for low salinity treatment and x, y and z for high salinity treatment).

Table 1. Results of PERMANOVA (multivariate and univariate analysis) testing for the significance of temperature (20 °C, 24 °C, 28 °C, 31 °C and 34 °C) and salinity (low, 34 psu and high, 40-42 psu) effects on photosynthetic parameters extracted from P-I curves obtained from experiment A. Bold values indicate significant effects ($p < 0.05$).

Source	df	MS	Pseudo-F	P	Unique perms	
Main test						
Temperature (T)	4	2131.6	1.8384	0.1243	9955	
Salinity (S)	1	6785.9	5.8525	0.0176	9945	
T x S	4	2341.2	2.0192	0.1062	9948	
Residual	36	1159.5				
Individual test						
Gross P_{max}						
Temperature (T)	4	4.334	3.472	0.0151	9949	
Salinity (S)	1	26.919	21.565	0.0001	9830	High > Low
T x S	4	5.440	4.358	0.0068	9965	
Residual	36	1.248				
Respiration						
Temperature (T)	4	1.744	3.856	0.0098	9952	
Salinity (S)	1	0.659	1.457	0.2333	9865	
T x S	4	0.921	2.037	0.1018	9947	
Residual	36	0.452				
I_k						
Temperature (T)	4	1235.200	1.260	0.3055	9961	
Salinity (S)	1	6738.500	6.876	0.0131	9840	High > Low
T x S	4	2167.100	2.211	0.0876	9955	
Residual	36	979.980				
alpha						
Temperature (T)	4	0.001	1.027	0.4018	9946	
Salinity (S)	1	0.000	0.043	0.8375	9817	
T x S	4	0.002	3.111	0.0223	9961	
Residual	36	0.001				

Experiment B: Plant functional traits responses

The potential photosynthetic capacity of plants had a negative effect with temperature increase. High temperature (30 °C) significantly depressed maximum quantum yield (F_v/F_m) by 40 % (Figure 5A; Table 2). Maximum electron transport rate (ETR_{max}) was significantly affected by temperature with a 28 % increase at the high temperature, relative to the control (Figure 5B; Table 4).

High temperature significantly reduced TNCs in leaves by 28 % compared to plants at the control temperature (Figure 5C; Table 2), while TNCs in rhizome were not affected by temperature nor by salinity (Figure 5D; Table 2). Plant growth was significantly affected by high temperature, as indicated by a reduction of 55 % on growth rate compared to the

controls (Figure 5E; Table 2). Temperature did not show any effect on plant survival. Interestingly, survival was favored under high salinity conditions as indicated by the increase of 21 % of surviving ramets (Figure 5F; Table 2).

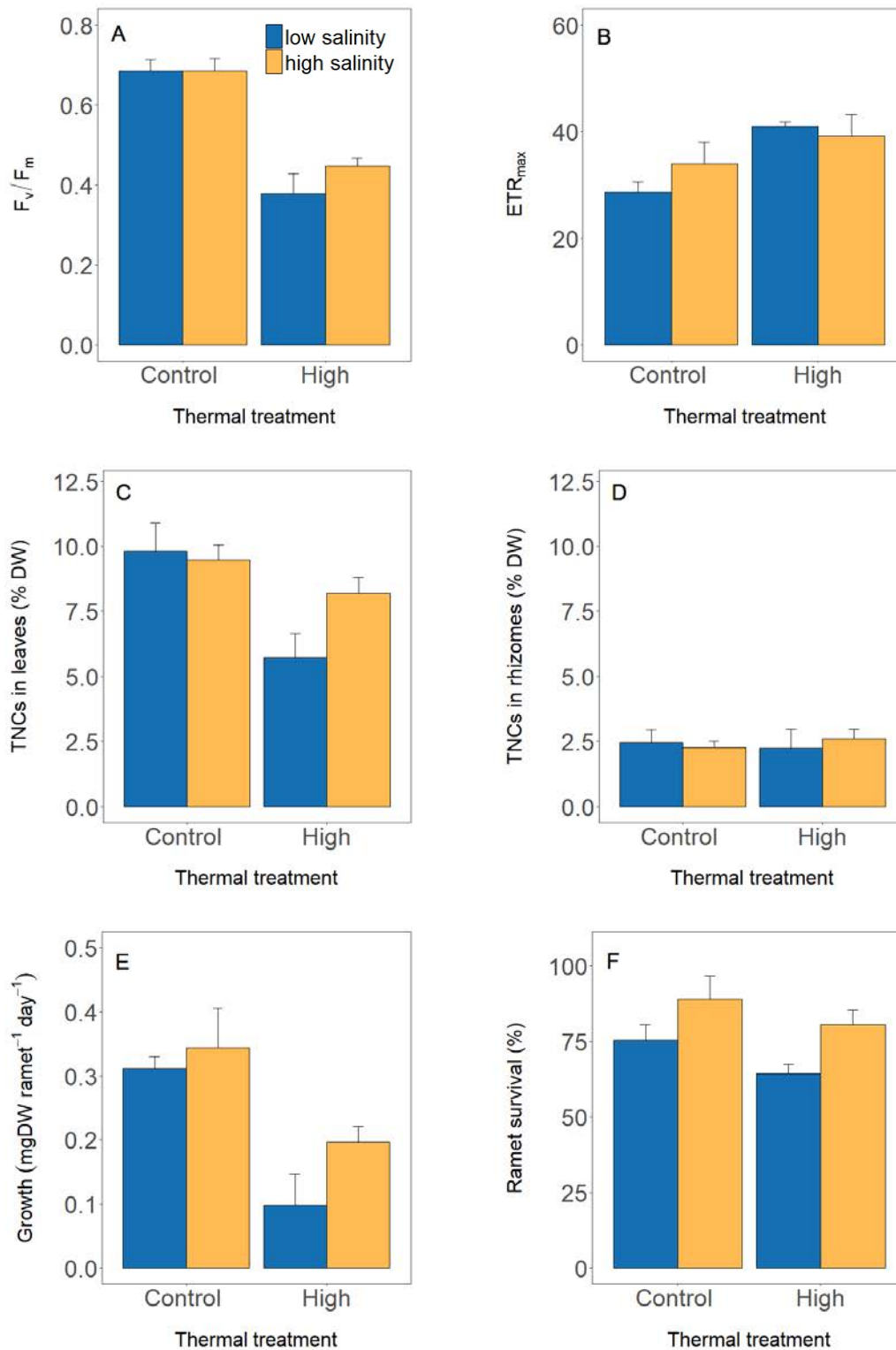


Figure 5. *Halophila ovalis* plant traits: A) maximum quantum yield, B) maximum electron transport rate, C) total non-structural carbohydrates (TNCs) content in leaves and D) TNCs content in rhizomes, E) growth rate and F) ramet survival, measured in plants (mean \pm SE, n=4) exposed to two thermal treatments (control (24 °C) and high (30 °C)) and two levels of salinity (low (34 psu), and high (40-42 psu)) for 13 days.

Table 2. Results of PERMANOVA (multivariate and univariate analysis) testing for the effect of temperature and salinity on plant functional traits of *Halophila ovalis* after growing for 13 days under experimental conditions in the experiment B. Bold values indicate significant effects ($p < 0.05$).

Source	df	MS	Pseudo-F	P	Unique perms	
Main test						
Temperature (T)	1	702.91	4.26	0.046	9936	
Salinity (S)	1	895.98	5.43	0.026	9937	
T x S	1	63.99	0.39	0.673	9936	
Residual	12	165.01				
Individual test						
F_v/F_m						
Temperature (T)	1	0.30	62.56	<0.001	9854	Control > High
Salinity (S)	1	0.00	1.01	0.333	9814	
T x S	1	0.00	1.01	0.322	9816	
Residual	12	0.00				
ETR_{max}						
Temperature (T)	1	303.10	8.06	0.014	9843	High > Control
Salinity (S)	1	11.49	0.31	0.589	9824	
T x S	1	49.04	1.30	0.274	9830	
Residual	12	37.63				
TNCs leaves						
Temperature (T)	1	28.96	12.66	0.003	9823	Control > High
Salinity (S)	1	4.56	1.99	0.185	9816	
T x S	1	7.98	3.49	0.085	9849	
Residual	12	2.29				
TNCs rhizome						
Temperature (T)	1	0.01	0.01	0.905	9834	
Salinity (S)	1	0.03	0.03	0.862	9839	
T x S	1	0.31	0.33	0.578	9848	
Residual	12	0.95				
Growth						
Temperature (T)	1	0.13	18.16	0.002	9851	Control > High
Salinity (S)	1	0.02	2.39	0.145	9835	
T x S	1	0.00	0.61	0.448	9811	
Residual	12	0.01				
Survival						
Temperature (T)	1	370.41	2.98	0.111	9741	
Salinity (S)	1	879.88	7.09	0.026	9663	High > Control
T x S	1	6.65	0.05	0.823	9757	
Residual	12	124.13				

DISCUSSION

Estuaries are influenced by a wide range of climate drivers, which make them particularly vulnerable to global change. Heatwaves combined with increasing salinity are two expected outcomes of climate change that can impact estuarine ecosystems (Hallet et al., 2018), including seagrass communities. In this work, the response of *H. ovalis* to temperature and salinity, both individually and in combination, over a duration that represents a heatwave event (Hobday et al., 2016), was assayed in two indoor mesocosm experiments. Overall, our results evidence negative effects of the heatwave but, interestingly, high salinities seem to buffer the impacts of transient warming.

Symptoms of thermal stress were detected at both physiological and plant levels. The sensitivity of the photosynthetic apparatus to warming was reflected by the decline of F_v/F_m to values below those commonly accepted for healthy plants (0.7-0.8, Campbell et al., 2006; Ralph, 1998), which indicates a damage of the photosynthetic apparatus of the plant after 13 days of exposure to high temperature (30 °C). However, this negative effect of temperature seemed to have no negative consequences in maximum gross photosynthesis and maximum electron transport rate. This lack of decline in maximum gross photosynthesis might be attributed to the short duration of the experiment A (1 day). However, it is consistent with previous studies on other fast-growing seagrass species inhabiting estuarine environments (e.g. *Cymodocea nodosa*, Marín-Guirao et al., 2018; *Halodule uninervis*, Collier et al., 2011; *Halophila johnsonii*, Fernández-Torquemada et al., 2005), where photosynthesis was either unaffected or affected positively by increasing temperatures. As our plants were photosynthetically active throughout the whole range of temperatures tested (20-34 °C), the critical temperature threshold for photosynthesis was not reached in this experiment, and a photosynthetic thermal optimum for this species cannot be drawn from here. The observed increase of ETR_{max} at high temperature is consistent with the enhancement of photosynthetic rates suggesting high rates of inorganic carbon assimilation. Moreover, ETR_{max} enhancement could also respond to the activation of alternative electron sinks that can be activated under stressful conditions to alleviate PSII damage (Sharkey 2005, Marín-Guirao et al., 2016), as indicated by the reported decline in F_v/F_m in our experiment.

As expected, the respiration rate did increase with temperature, as has been observed in other species (e.g. *Cymodocea serrulata*, *Halodule uninervis* and *Zostera muelleri*, Collier et al., 2017; *Posidonia oceanica* and *C. nodosa*, Marín-Guirao et al., 2018; *Thalassia hemprichii* and *Enhalus acoroides*, Pedersen et al., 2016). This increase in respiration, together with a decrease in photosynthetic efficiency (α), seemed to have an overall negative effect at the individual plant scale, as indicated by a decrease in growth rate and in the carbohydrate content in the leaves at high temperature. This can be attributed, most likely, to the use of internal resources to cope with the energetic demands increased respiration. In fact, reductions in growth have been reported for many species of seagrasses worldwide when the optimal temperature is exceeded (Collier and Waycott, 2014; Collier et al., 2011; Olsen et al., 2012; Ontoria et al., 2019a, 2019b; Traboni et al., 2018). Our results support the well known observation that the optimum temperature for photosynthesis is usually higher than for growth (Lee et al., 2007). A reliable explanation is that, under thermal stress, despite the photosynthetic rate being

maintained, a greater amount of carbon fixed by photosynthesis is used by respiration at the expense of the carbon allocation to growth and storage. Despite reduced growth, warming did not affect plant survival over the 13 days period of exposure. This has not been the case in other studies where increased mortality of *H. ovalis* was observed following 6 days of thermal stress (Collier et al., 2014). These differences can be attributed not only to the different temperatures used (30 °C in this study, 40-43 °C in Collier et al., 2014) but also to the different environments the plants live in (temperate areas here, tropical ones in Collier et al., 2014). Different tolerance responses of a single species to environment factors based on the location they grow in have also been found in Mediterranean seagrass species (e.g. Marín-Guirao et al., 2018) highlighting the need for local understanding when predicting impacts of changing environmental conditions. Furthermore, our study has shown that the eurythermal species *H. ovalis* (den Hartog 1970; Hillman 1985) tolerates thermal shock up to 30 °C for 13 days, at least in terms of mortality, widening the previous 4 days tested by Ralph (1998).

Salinity on its own (that is, at control temperatures, 20-24 °C in experiment A and “low” -24 °C- in experiment B) had no negative effects on plant performance. This was relatively unexpected, as other studies have documented enhanced respiration with increased salinity, as for instance *P. oceanica*, *C. nodosa* or *Zostera japonica* (Marín-Guirao et al., 2011; Sandoval-Gil et al., 2012a; Shafer et al., 2011). Notwithstanding, respiration reduction at high salinities of 40-60 psu was described in a congeneric species (*H. johnsonii*, Fernández-Torquemada et al., 2005). Photosynthetic efficiency (α) in our experiment decreased with salinity at control temperatures, indicating a decline in plant functioning at high salinity conditions. Photosynthesis was stimulated by high salinities, similar to the study of Fernández-Torquemada et al., (2005) on *H. johnsonii* where photosynthesis was favored by acute salinity exposure up to 40 psu, but this was opposite to the response of a larger more persistent species, *P. oceanica*, which reduced photosynthetic rate with high salinity (Sandoval-Gil et al., 2014).

Interactive effects of temperature and salinity in maximum gross photosynthesis and in (α) (photosynthetic efficiency at low irradiances) were identified. Interestingly, the interactions were positive. Thus, while maximum gross photosynthesis remained more or less constant with rising temperature, it was stimulated at high salinity when temperature reached 28 °C and above. Moreover, photosynthetic efficiency (α) decreased with temperature, suggesting increased vulnerability to water turbidity events under a warming scenario. However, in plants submitted to high salinity, the photosynthetic efficiency presented levels similar to control temperature and salinity conditions values. Plants exposed to high salinity can activate different acclimation mechanisms including changes in the cellular ion and solute concentrations to the modification of the cell wall (Sandoval-Gil et al., 2012b; Touchette 2007). These were not assessed in this study, but these types of plant responses combined with others induced by high temperatures (e.g. antioxidant enzymes, Tutar et al., 2017), may have led to the observed increase in photosynthesis. If this was the case, then the TNCs content in leaves of plants grown under high salinity and high temperature, would not have as big a decline. Our results did show a trend of dampening the reduction in carbohydrates caused by high temperature with high salinity, but the interaction

was not significant (p -value of 0.085). However, at the population level, plant survival was higher at high salinity. These observations suggest that this species has developed mechanisms to acclimate to increased salinity, as has been reported for other seagrass species (Marín-Guirao et al., 2017). This is not surprising as *H. ovalis* is a euryhaline species with a distribution across a wide range of environments, in terms of the salinity regime.

The notion that some consequences of climate change (e.g. increased salinity) can dampen those of others (e.g. warming) has great interest and warrants pursuing further. This finding is based on the two complementary experiments presented here which were designed to broadly explore responses and performance at different plant scales (from physiology to population, *sensu* O'Brien et al., 2018) to increased temperature and salinity. Although slightly different exposure levels and durations were used, and the populations of *H. ovalis* were collected in different estuaries, there was consistency in the responses giving confidence in the general value of our results for this species, at least for those populations inhabiting the temperate area where we worked. On the other hand, it is largely accepted that mesocosms have the advantage of working under controlled conditions, allowing to assess individual and combined effects of different stressors, but they do not fully replicate the complexity of the natural environment. So, field observations or natural experiments examining the responses of seagrass meadows to heatwave events across a salinity gradient would be very valuable to compliment this work for the future.

This work contributes to an improved understanding of how multiple factors interact to influence aquatic plants in coastal ecosystems. This is timely and relevant considering the range of possible factors involved in the decline of coastal ecosystems worldwide. We have found that *H. ovalis* populations living in variable salinity environments, such as in the estuaries of southwestern Australia, may be negatively impacted by more frequent and extreme warming events. However, if this warming, in turn, results in high salinity conditions through increased evaporation and reduced rainfall, these high salinities will buffer plant survival and reduce the negative effect of temperature on plant performance. These findings suggest that the effects of short-term warming (i.e. heatwaves) might not be as negative as predicted while, in a future scenario of global change, longer term warming could be more detrimental for populations, due to the expected reductions in growth rate. There are opportunities for further research to improve predictions of the effects of global change in fluctuating environments such as considering other possible drivers and stressors, how other species will respond and investigating responses in the more complex field setting.

ACKNOWLEDGEMENTS

We thank Caitlyn O'Dea, Sian McNamara and Elena Álvarez for their help in the field and laboratory. Laboratory facilities were provided by School of Science and Centre for Marine Ecosystems Research at ECU. We thank Department of Water and Environmental Regulation staff Dr Kieryn Kilminster, Marta Sánchez Alarcón and Katherine Bennett for logistical and in-kind support of this project. Carbohydrates analysis were performed at the Oceanographic Center of Murcia (Spanish Institute of Oceanography), and we thank Neus

Sanmartí and Rocío García-Muñoz for their assistance. The Spanish Government supported this work through the RECCAM (CTM2013-48027-C3-1/2-R) and UMBRAL (CTM2017-86695-C3-1-R) projects, and the fellowships BES-2014-09593 and EEBB-I-17-12572, awarded to Y. Ontoria.

GENERAL DISCUSSION

This thesis has shed light on some of the responses of seagrasses to global change with a special focus on temperature increase, but also assessing the effects of eutrophication and salinity, and not only the effects of these stressors in isolation, but also their potential interactions. Based on mesocosm experiments, the response to warming, is, in general terms, the same for the three temperate seagrass species studied in this work (*Posidonia oceanica*, *Cymodocea nodosa* (Chapter 1) and *Halophila ovalis* (Chapter 4)): negative effects on plant performance appear when a critical thermal threshold is exceeded. The extent of those negative effects, as well as the thermal thresholds, largely differ among species, mostly based on their habitat, ecological strategies and biogeographical affinities. Nevertheless, and albeit interesting, this kind of results must be extrapolated with caution to real ecosystems, on the one hand because of the shortcomings of the approach and the difficulties of the scale-up, that will be addressed further. On the other hand, we must also take into consideration the already altered status of most ecosystems submitted to the global warming stress. It is a matter of fact that the action of a given stressor erodes the resilience of the ecosystem, making it more vulnerable to others (Chapin III et al., 2009). To support this notion, in this thesis, we have demonstrated, for two Mediterranean species, how the combination of eutrophication and warming can be much worse than the isolated effect of each one of them in isolation (Chapter 2 and Chapter 3). Surprisingly, the simultaneous occurrence of high salinity and warming appears not only to not be detrimental for an estuarine species but it appears that high salinity conditions buffer the negative effects of warming (Chapter 4). All this confirms the need for making efforts to better understand the effects of increasing temperatures attending to the variability of other environmental changes, especially those potentially causing detrimental effects on organisms.

The results obtained throughout this research have been individually discussed in depth in each one of the four chapters encompassed in this thesis. To avoid reiterations, this general discussion aims to go further and, not losing sight of our findings, make a more synthetic analysis of the possible effects of global change, at the light of what has been indicated in the previous paragraphs. In this sense, a first section explores seagrass responses through different and interacting plant integration levels and suggests a generalized framework to address the effects of global warming on seagrasses. A second section highlights the importance of interactive effects of global warming, while a third section analyzes limitations of mesocosm experiments. Finally, a fourth section focuses on the most promising directions for future research.

SCALING SEAGRASS RESPONSES

The worldwide extended concern about the future of seagrasses relies on the possible loss of the ecosystem functions and services they provide. Given that world's seagrass distribution seems to have been reduced by 30 % after industrial revolution (Waycott et al., 2009), mostly due to anthropogenic pressures, increasing efforts are now focused on developing more effective science based mainly on the improvement of monitoring, conservation, restoration and management outcomes (Kilminster et al., 2015; Orth et al., 2006; Unsworth et al., 2015). Although these efforts seem to commence to render some

results (de los Santos et al. 2019), much better knowledge is still needed to deepen our understanding of the effects of stressors on seagrasses and, specially, a framework into which to integrate the abundant information already available.

In this sense, it has to be reminded that, in most cases, seagrass deterioration in response to a given stress only becomes evident when signs of degradation are visibly appreciable, i.e. when meadows lose integrity, decline ostensibly or retract. However, those are generally the ultimate consequences of initially much more subtle changes in response to stressors taking place at low (e.g. biochemical, physiological) biological organization levels. This hierarchy of responses through multiple biological organization levels is to some extent a tenet not only in seagrass biology, but in different approaches related to ecology. In the specific case of seagrasses, it has been proposed that their response to a given stressor can be “small and fast”, “intermediate” or “large and slow”, following combined increasing spatial and temporal scales (O’Brien et al., 2018; Figure 1). Obviously, those responses and scales are not independent, and upscaling and downscaling effects occur, leading thus to potentially complex effects.

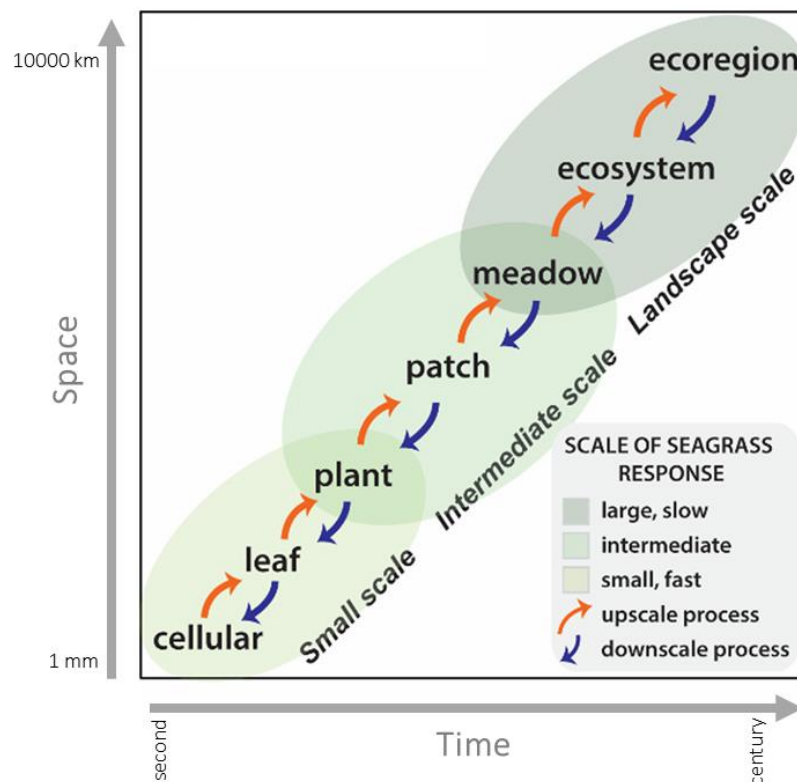


Figure 1. Scale of seagrass responses to environmental changes. Responses can be “small and fast”, “intermediate” or “large and slow”. Responses at one scale can influence those at higher or lower scales (adapted from O’Brien et al., 2018).

This framework fits particularly well in the intellectual challenge of addressing the effects of temperature as a stressful factor. However, a close examination of those effects and the multiscale nature of the response show that the diagram in figure 1 is too simplistic. At this

respect, it has to be acknowledged that the first response to (almost) any environmental change occurs at the **molecular level** (see Figure 2, scaling effects of a stressor). It is known that heat stress alters the expression of thousands of genes involved in a myriad of biological processes. As for instance, temperature increase can, on the one hand, stimulate the transcript of genes encoding for Heat Shock Proteins (HSP) with chaperone-like functions, for antioxidant enzymes, for COX proteins related to electron transport in respiratory chains or for enzymes related to programmed cellular death (Tutar et al., 2017). On the other hand, it can also decrease expression of other genes, as some related to carbon assimilation (Marín-Guirao et al., 2016; Purnama et al., 2019). These changes at the molecular level have evident influences in the next, **biochemical level**, impairing essential functions (e.g. decrease in the expression of genes encoding for enzymes related to carbon assimilation which leads to a lesser activity in the Calvin cycle). However, not all the effects at the biochemical level derive from effects at the molecular one, as thermal stress can affect biochemical processes directly, that is, independently of changes in gene expression. In effect, photosynthesis is specially sensitive to temperature, either in terms of integrity of the photosynthetic apparatus (and, specifically, of PSII: Campbell et al., 2006; Chapter 2 and Chapter 3 of this thesis) or in terms of photosynthetic electron transport chains (George et al., 2007). Moreover, respiratory rates usually increase with temperature because respiratory electron transport chains are also highly sensitive to thermal stress (Collier et al., 2011; Marín-Guirao et al., 2018; Pedersen et al., 2016), decreasing photosynthesis/respiration balance. In addition, heat stress can uncouple enzymes and metabolic pathways, causing the accumulation of harmful reactive oxygen species (ROS). To add further complexity, some of the effects at the molecular level do not propagate as negative effects at the biochemical level but, in contrast, dampen the consequences of malfunctions caused by temperature. This is the case, for example, of overexpression of genes encoding for antioxidant enzymes, scavenging ROS, or for the COX proteins, counteracting to some extent the negative changes in respiratory chains (Das and Roychoudhury, 2014; Tutar et al., 2017).

Changes at the biochemical level, in turn, scale-up to the next, **physiological level**. There is a great availability of evidences of scaling effects of temperature to this level, including a decline in carbohydrates storage, leaf growth impairment (Olsen et al., 2012; Chapter 2 and Chapter 3 of this thesis) or depressed content of deterrent substances, whose production is very much linked to the primary carbon metabolism (Vergès et al., 2008). Most, if not all, of these effects are direct consequences of the impairment of the carbon balance (Alcoverro et al., 2001), although direct effects of temperature should not be discarded (e.g. effect on cell mitosis, Koutalianou et al., 2016). At this level, flowering induction by thermal stress in one ecotype of *Posidonia oceanica* has been documented (Ruiz et al., 2018; see also Díaz-Almela et al., 2007). It has to be remarked that, in this case, the mechanism is rooted in the molecular level (expression of floral genes, Marín-Guirao et al., 2019) but the effects are at the population level. Moreover, and although as a mere speculation, flowering induction by thermal stress can be viewed as a response of the plant (production of new genotypes, possibility of colonization of more favorable areas) counteracting the negative effects of temperature rise.

The detrimental effects of temperature at the **population level**, also rooted in previous levels, and mostly in terms of increased shoot mortality (Collier and Waycott, 2014; Marbà and Duarte, 2010; Chapter 1 and Chapter 2 of this thesis), or in the reduction of seed germination capacity (Guerrero-Meseguer et al., 2017; Pereda-Briones et al., 2019), are well documented. Moreover, temperature causes lower elongation rates in rhizomes (Chapter 2), resulting in a decrease in the rate of production of new shoots, which in turn implies a decrease in “birth” (recruitment) rates from the population perspective. Finally, changes in the demographic balance (births vs. deaths) at population level would obviously result in changes in the density, cover and areal extension of seagrass meadows.

At the **community level**, the consideration of biological interactions, including at least competition, predation, amensalism and mutualism, adds further complexity to the whole picture. These aspects, in comparison with those described in previous levels, have been less investigated. However, it is known that warming can alter either herbivory (Jiménez-Ramos et al., 2017; Pagès et al., 2018), competitive interactions (between seagrass species, Chapter 1, or maybe with macroalgae) or amensalism (temperature effects on epiphytes, Lepoint et al., 1999). Some of these effects, again, are at least in part a consequence of upscaling from lower levels (e.g. at biochemical level, a decrease in production of chemical defenses), while others appear as direct effects of temperature (e.g. thermal stimulation of herbivores activity). Along with those upscaling effects, downscaling influences from the community level to those below appear also here, including changes in carbon balance due to reduction in photosynthetic biomass or the induction of chemical defenses (Sanmartí et al., 2014; Vergés et al., 2007).

Finally, when we go beyond the community level (**ecosystem, landscape, biosphere levels**), new actors, new mechanisms and new processes emerge. On the one hand, we found other stressors, either natural or anthropogenic that can exacerbate (Chapter 2 and Chapter 3) or, in some cases, dampen (Chapter 4) the effects of temperature rising. As demonstrated in this thesis, this issue is of particular concern, and will deserve further attention in this discussion. On the other hand, effects of temperature in large scale processes (such as species range distribution shifts, Hyndes et al., 2016; Lonhart et al., 2019), in other environmental factors (for example, oxygen consumption in the sediment, see for example Chapter 2) or in ecosystems other than seagrass meadows but functionally linked to them (Hyndes et al., 2012; Ricart et al., 2017) should be taken into consideration. As these larger levels have been not directly investigated in this thesis, we will not go further on in discussing them.

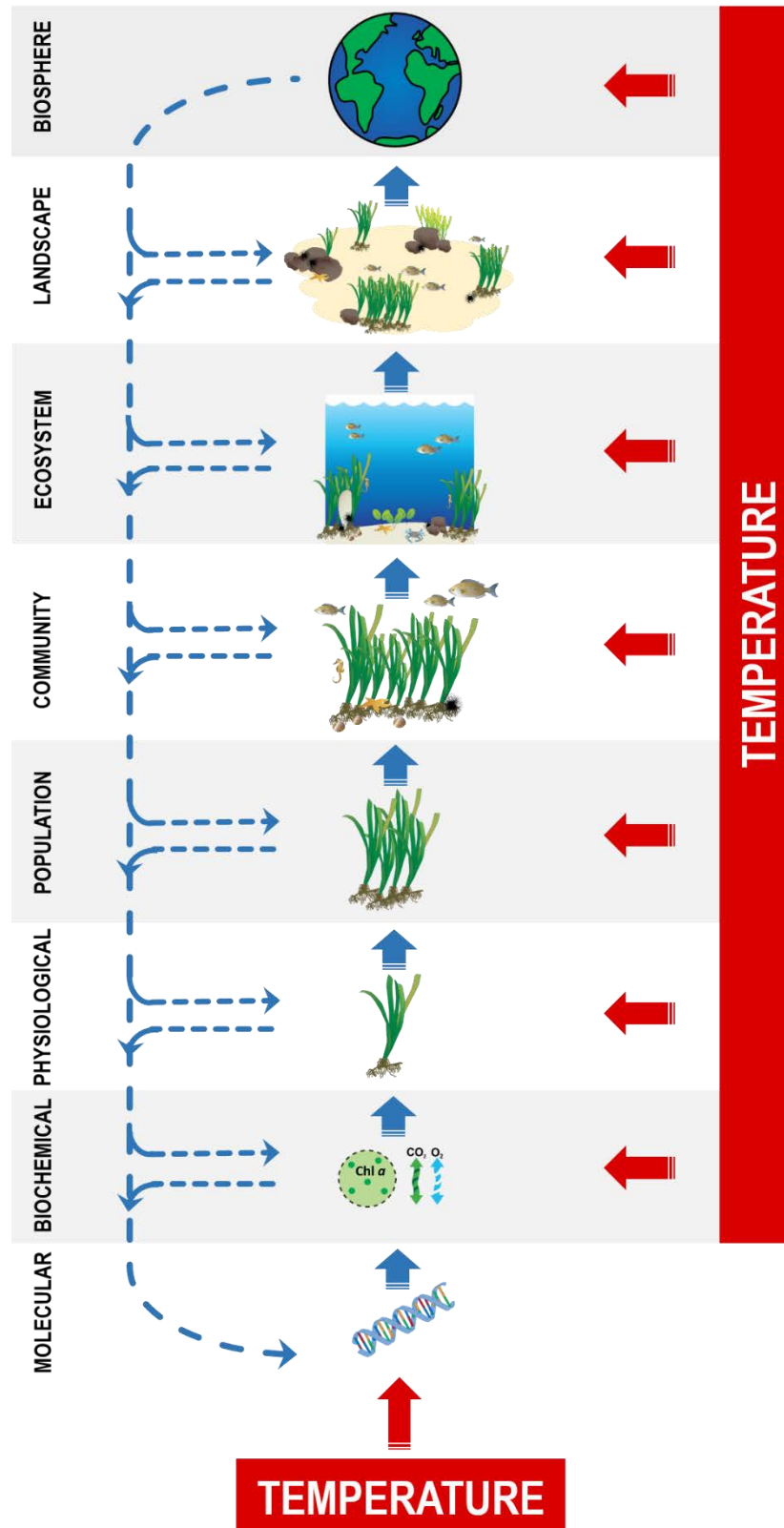


Figure 2. Scaling-up effects of temperature (blue arrows) and down scaling effects (dashed blue arrows) through the different levels of biological organization. Direct effects of temperature at each level (red arrows).

The previous paragraphs provide, in our opinion, a reliable framework to understand the effects of climate change on seagrass ecosystems. However, at least two additional remarks are needed to not overlook important aspects. First, and although this framework intends to be of general validity for seagrasses, when going into the detail of the responses and the mechanisms implied, the differences among species seems to be the rule (Campbell et al., 2006; Collier et al., 2011; Marín-Guirao et al., 2016). We have explored these differences in Chapter 1, and the results evidence that thermal effects and thermal responses, assessed in *Posidonia oceanica* and *Cymodocea nodosa*, were not only very different among them but, in some instances, opposite. Therefore, from the available literature and our own results, it seems clear that the *universe* of seagrasses will by no means respond to warming as a whole. Instead, some species will be more affected ('losers') than others ('neutrals'), and even others will be favored, at least within a given thermal range ('winners'; see also Pagès et al., 2018). This notion is essential to accept that the coming change in underwater landscapes will be not only a seagrass meadow regression but, in some instances, a species replacement or a species redistribution. Secondly, our framework can wrongly transmit the perception that increasing temperature has only negative effects on seagrasses at multiple levels, eventually propagating upscale and downscale. We have, however, already suggested the existence of mechanisms dampening negative effects, such as ROS scavenging enzymes or induction of flowering, among many others. These mechanisms are adaptive and have originated along the evolutionary history of each species. To a greater or lesser degree, all seagrass species have some plasticity to acclimate to thermal stress (Chapter 1). This, in turn, brings to the scene evolution (Grant et al., 2017; Parmesan 2006), and reminds us that thermal increase is probably already a selective pressure. With the results of our thesis, we cannot go deeper into this aspect, but a warning against making predictions based solely upon present biological traits of the species is probably needed.

INTERACTION AMONG STRESSORS

A large part of this thesis (specifically Chapters 2, 3 and 4) delves into the combined effects of a global stressor (warming) with a local one (eutrophication, salinity) on seagrass performance. Eutrophication is considered a serious threat for coastal ecosystems, and it is caused mainly by local to regional sources such as aquaculture, some industrial waste disposal, agriculture runoffs or urban effluents, among other causes. Eutrophication is a major concern worldwide (Diaz and Rosenberg, 2008; Nixon and Fulweiler, 2009), and, specifically, the Mediterranean Sea has experienced eutrophication problems at least since the 60s, mainly in coastal areas (Kitisiou and Karydis, 2011; Krom et al., 2010; UNEP/FAO/WHO 1996). The effects of eutrophication on seagrasses have been repeatedly evaluated (Burkholder et al., 1992; Ruiz et al., 2001; Short et al., 1995; see review of Burkholder et al., 2007) and will not be further discussed here. The concern, and the question, is if and to which extent the reaction of seagrasses to a new stressor (warming) will be worsened by to the existence of a previous stressor (eutrophication). This question has been assessed in this thesis, as far as we have been able, for two species, *P. oceanica* and *C. nodosa*, which again differed in the response. *P. oceanica* seemed greatly sensitive to the combined effects of eutrophication and warming, and high nutrient concentrations clearly

worsened its already weak tolerance to temperature. Although we were not able to evaluate the joint effects of increased organic matter in sediment and warming in this species due to technical constraints, given its sensitivity to organic enrichment (Holmer et al., 2003; Pérez et al., 2007), it is reasonable to assume also a rather negative response. In contrast, *C. nodosa* seemed greatly tolerant to high nutrient concentrations, which did not aggravate the negative warming effects found, but not to additions of organic matter to sediment, which negatively and synergistically affected growth rate and demographic balance. At the light of these results, it seems urgent to make all efforts to decrease eutrophication in coastal Mediterranean waters (and elsewhere) in order to not impair the resilience of seagrasses to warming. To say it with big words, fighting against eutrophication is fighting against climate change consequences.

However and, to some extent, surprisingly, not all potentially concurring stressors have negative effects when combined with warming. The effects of salinity, which is also predicted to increase in confined or semi-confined coastal waters (Hallet et al., 2017) and assessed in southwestern Australia with the species *Halophila ovalis*, effects revealed unexpected results (Chapter 4). While high temperatures cause severe damage on plant performance, increased salinity seems to buffer those negative effects. Within the limitations of our study (mesocosm approach, see below; limited salinity range, see Chapter 4), this suggests not only that the combination of high temperature and high salinity conditions does not account for the mortality events reported in the area (Kieryn Kilminster, pers. comm.) but also that the effects of some drivers resulting from climate change (warming) can be alleviated by others (salinity increase), at least in some estuarine seagrasses.

LIMITATIONS OF MESOCOSM EXPERIMENTS

The results of this thesis have been obtained from experimental works conducted in indoor mesocosm systems. Although the validity of the information obtained from mesocosm experiments has been questioned (Benton et al., 2007), such information is highly valuable as long as it is cautiously interpreted and extrapolated. To extract general conclusions from the results of this thesis, we consider important to acknowledge and address critically the limitations of the approach.

The experimental approach based on mesocosms is commonly used to overcome the logistic constraints the marine researcher faces when attempting to control and manipulate the environmental conditions underwater. Despite their technical complexity, mesocosms are relatively affordable, and then are commonly used as alternatives to correlational approaches, not only in seagrasses (e.g. this work; Egea et al., 2018; Sandoval et al., 2014) but also in a number of other organisms (Pagès-Escolà et al., 2018; Rich et al., 2018; Russell et al., 2009). It has to be reminded that robust inference can only be made based on experimental, reliable and accurate designs, and those designs are much more easily reachable using mesocosms.

However, mesocosm experiments are not a panacea, and the inferences obtained, albeit robust, should be treated with great caution when attempting to translate them to

predictions and generalizations. Concerning design, the selection of the treatments levels, the number of experimental units and the replicates among treatments usually determine the size of the experimental system, and, consequently, represent a first shortcoming due to limitations imposed by the capacity of the facility used. An optimization of designs is then required, but, in those designs, it is fundamental to not skimp on the independent replicates, that must properly integrate the variability of the phenomena assessed. Equally critical is that the treatment levels established capture the range and magnitude of variation relevant to the context of the experiment. Finally, physicochemical conditions are extremely controlled and do not reflect the natural variation of the real world, moving away the experiment from how nature works (Carpenter, 1996). Moreover, the role of factors others than those specifically addressed in the experiment and their variability is occluded.

Beyond this, probably the main restrictions of the approach are related to time and space scales. The time span of this kind of experiments is necessarily short (from days to weeks, more rarely months) relative to the time scale of some processes at the individual/physiological scale (growth, reproduction, reserves accumulation and mobilization, among others) and, not to say, above (see Figure 1). Usually, the most technically sound installations allow for longer times, but care should be taken to not create artifacts due to plant health deterioration under culture conditions. For example, in our simple mesocosms used in Chapters 2 and 3, the experiment could not last for more than 14 days, while in the more sophisticated facility used in Chapter 1 we were able to expose the plants for 5 weeks. In any case, a mesocosm experiment is a kind of clip of video only, reflecting a given time lapse belonging to a much more extended dynamics. Determining the optimum time for conducting the experiment, according to the objective to reach and the seasonality (if any) of the processes examined are key aspects to take into consideration.

The limitation of the spatial scale should also be taken into account. Firstly, individuals (ramets, shoots, clones) are collected from a single population, and even if attention is paid so as to perform a good spatial sampling, hardly the most optimal sampling will capture *in toto* the phenotypic and genotypic variability of that population (and distribute it randomly among microcosms), not to say when considering variability at large geographical areas or across all the species distribution range. Secondly, relevant processes occurring at community level and above (see the second section of this discussion) cannot be, for the most, reproduced within mesocosms. In fact, therefore, the mesocosm approach only addresses a part of the whole diagram in figure 2.

Taking into account all these limitations, it is clear that this thesis does not aim to be sententious about the future of seagrasses. Considering the whole ecosystem as a puzzle, the union of the small pieces (in this case, increasing the knowledge about the responses at the lower integration levels of the plant) will help to complete the whole puzzle.

DIRECTIONS FOR FUTURE RESEARCH

We have brought light about a few specific issues after more than four years of efforts, but we have also extended the list of the questions that remain open. We attempt here a short summary about those open questions.

Where are the (thermal) limits?

In Chapter 1 four temperatures were assessed to evaluate *Posidonia oceanica* and *Cymodocea nodosa* responses to long-term (5 weeks) exposure to increased temperatures. These results, in combination with those of previous works (Savva et al., 2018; Olsen et al., 2012) allow to propose a thermal threshold for these two seagrass species, as has been proposed for other seagrass species worldwide (e.g. Collier et al., 2017). This represents a basic information that can be of some interest in combination with projections of temperature rise (IPCC, 2007; Vargas-Yañez et al., 2007). It should not be forgotten, however, that the concept of thermal optimum depends on the plant function assessed (Chapter 1), and such thermal optimum and limits must be explored for different plant functions. Moreover, further efforts are needed to explore, within each species, the geographic variability of their tolerance, their plasticity and the functional (and evolutionary) consequences of their genetic variability. Such advances will make possible to produce more accurate predictions about the future of seagrass ecosystems.

Delayed effects and recovery

Global warming can be viewed as a progressive and constant temperature increase, and its effects understood as a kind of linear response to the stressor. This view is by no means prevalent in the scientific literature, but is present more than desirable in the collective *imaginarium* of the human society. Yet a relevant part of reported effects of global warming are linked to discrete events, such as heat waves, which seems specially harmful not only for seagrasses but also for a panoply of other organisms (Garrabou et al., 2009; Wernberg et al., 2013). Indeed, such heat waves are predicted to increase in frequency and intensity (IPCC 2013; Meehl and Tebaldi, 2004), and thus the assessment of short-term warming events (from days to weeks) have been increasingly present in the literature, being the present thesis an example. There is a large consensus about the results of these short-term effects, which are, for the most, detrimental (but see, for example, Chapter 2, or Pagès et al., 2017). However, what happens once the extreme event has passed? While some negative effects can appear after cessation of the heat wave, it is also possible that the plant recovers some of its functions (e.g. Marín-Guirao et al., 2016; Ralph 1998). We would like to highlight the importance of incorporating an experimental period after removing (or cessation) the stressor in this type of experiments, thus considering the chance of recovery (or for expressing delayed effects). More realistic, although also more complex, would be the same kind of approach but considering the interaction among stressors, that is, considering which stressors other than warming modifies either the delayed response or the recovery capacity of the plant.

The risk of tipping points

Cumulating experimental evidences, supported by a solid theoretical background, indicates that the response of ecosystems to stress is rarely linear. Instead, ecosystems show nonlinear behaviour, often aside with critical transitions and tipping points (Carpenter et al., 2011; May 1977; Scheffer and Carpenter, 2003; Scheffer et al., 2001). While this theoretical framework has been widely applied to a wide array of case studies (macroalgae and herbivores: Boada et

al., 2017; seagrass and lucinid bivalves with endosymbiotic sulfide-oxidizing bacteria: Fouw et al., 2018; predatory fishes in mountain lakes, Koel et al., 2019, among many others), there is a surprising lack of works exploring the possibility of warming triggering critical transitions, or weakening ecosystem resilience to other stressors and then indirectly facilitating those critical transitions, in marine ecosystems. A more explicit approach of the science of global warming to this conceptual framework could prevent undesirable surprises in the near future.

Would increasing biodiversity confer resilience to climate change?

The contention of species diversity as an element conferring resilience to ecosystem against disturbances or stressful conditions has been assessed repeatedly (Downing et al 2010; Isbell et al., 2015). Given the near complete monospecific nature of the foundation species in seagrass meadows, the concept of specific biodiversity can, to some extent, be replaced by that of genetic (or genotypic) diversity. In effect, it has been shown that genotypic diversity confers high resistance to heat waves in *Zostera marina* (Ehlers et al., 2008; Reusch et al., 2005). The matter deserves further exploration for at least two reasons: (i) if genetic diversity increases resistance to warming in seagrasses, all efforts should be done not only to conserve seagrasses themselves, but also their genetic diversity; and (ii) the heterogeneity in resistance to warming among genotypes to certain extent parallels the heterogeneity in the response to warming of different species, already mentioned elsewhere in this discussion. Consequently, in the same manner we talk about species being “losers” and “winners”, there will be also winner and loser genotypes, open thus an stimulating evolutionary perspective.

Where do we expect to go through such experiments?

It is almost impossible to address all the subjects outlined above (as well as others equally relevant) based solely on mesocosm experiments. It is therefore necessary to combine those small-scale experimental approaches with more extended field work that would reduce the artificiality of the experimental context and would broaden the space scale, thus fixing some of the limitations mentioned previously. Although, in our context, field manipulative experiments are technically very complex, efforts should be done, maybe using natural temperature gradients or applying opportunistic strategies (e.g. after a heat wave). Other tools, such as modeling, can help to transcend the useful but narrow universe of mesocosms.

Seagrass ecosystems, as complex systems, are much more than the sum of their parts, and each part is much more than the sum of their sub-parts; and so on. To understand how big parts work, it is necessary to understand how the smallest parts do, in its individuality and in its ensemble to function as a whole. Just, as if they were the pieces that make up a puzzle, one of the most valuable puzzles hidden under the ocean waters. Understanding the pieces of the puzzle and how they fit are unavoidable steps to protect it from the threats of global change.

CONCLUSIONS

1. The performances of the three seagrass species studied in this thesis were clearly influenced by experimental increases in temperature, although there was a great interspecific variability in the responses and in the thermal thresholds above which the effects became detrimental. Such variability is most likely the result of their different life stories, ecological strategies and biogeographical affinities.
2. A very different thermotolerance and response to warming was found between the two main Mediterranean species (*Posidonia oceanica* and *Cymodocea nodosa*), based on diverging strategies to cope with thermal stress. In effect, while in *C. nodosa* antioxidant enzymes were activated in response to warming, seemingly an effective thermal protective mechanism, *P. oceanica* did not and, in turn, seemed only to lightly activate dissipation pathways (NPQ and xanthophyll cycle) although with limited effects.
3. These differences might potentially cause changes on the distribution and abundance of these two species in the Mediterranean, with a potential extension of *C. nodosa* meadows and a contraction of those of *P. oceanica*, which might be replaced by other opportunistic and more thermotolerance species, overall entailing relevant consequences not only for the goods and services those ecosystems provide but also for the whole Mediterranean ecosystem.
4. The action of warming in combination with other widespread stressors such as eutrophication can worsen, either in an additive or synergistic manner, the predicted effects of warming alone. Both Mediterranean species studied showed synergistic effects, in *P. oceanica* when combining high nutrient concentration in water and increased temperature, and in *C. nodosa* when combining the organic enrichment of sediment and increased temperature. As those synergies seem to be more the rule than the exception, efforts should be done to properly assess combinations of stressors, more than stressors in isolation.
5. Surprisingly, we have found one stressor (increased salinity) that, instead of causing additional deterioration in combination with thermal increase for the estuarine seagrass species *Halophila ovalis*, buffered the detrimental thermal effects. Although this result should be viewed with caution due to experimental limitations, it underlines the complexity of nature and the difficulties faced when attempting predictions on global warming consequences.
6. Thermal stress acts at different levels of biological organization, from the molecular to the ecosystem and beyond, and upscale and downscale through these levels, resulting in a network of interactions within the organisms, between the organisms (of the same or different species), between the organisms and the environment and even within and between larger systems. The complexity of this network is overwhelming, but unpacking, even partially, some of its components is probably one of the main challenges science is facing today.

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**SUPPLEMENTARY
MATERIAL**

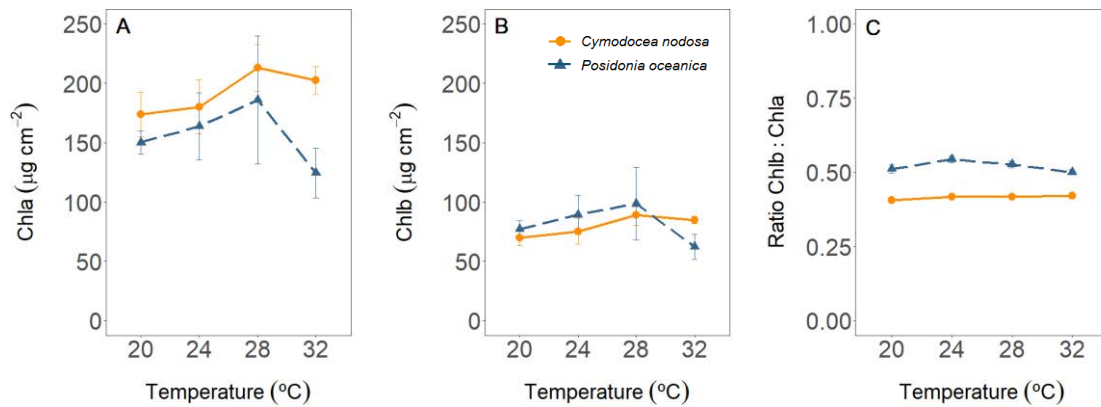


Figure S1. Chlorophyll *a* content (A), chlorophyll *b* content (B) and molar ratio chlorophyll *b*: chlorophyll *a* (C) in leaves of *P. oceanica* and *C. nodosa* exposed to four experimental temperatures for five weeks. Values are reported as mean \pm SE, $n=3$.

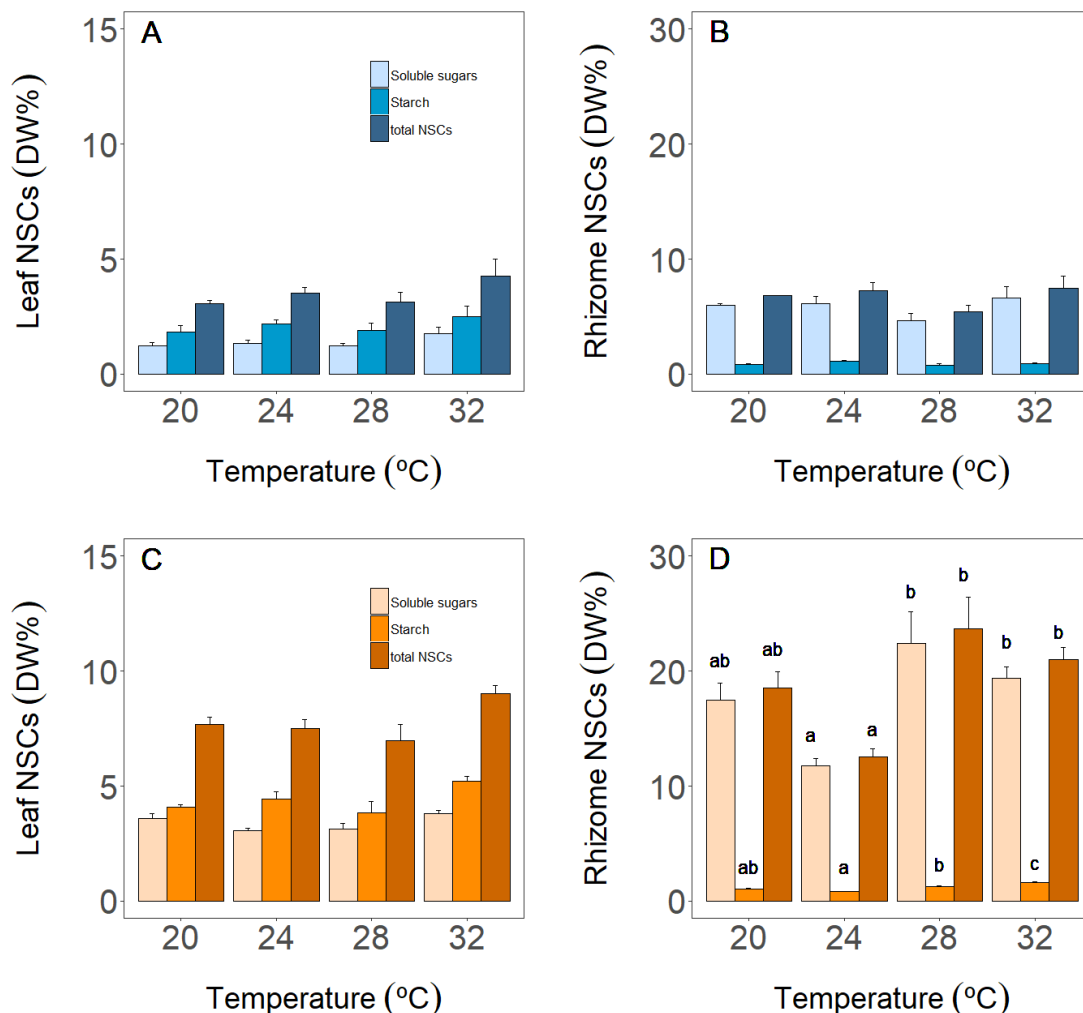


Figure S2. Carbohydrates (NSCs, either in the form of starch or soluble sugars) content in leaves (left panel) and rhizomes (right panel) of *P. oceanica* (A and B) and *C. nodosa* (C and D) plants exposed to four experimental temperatures. Statistical differences among temperatures are indicated by different letters. Values are reported as mean \pm SE, $n=3$.

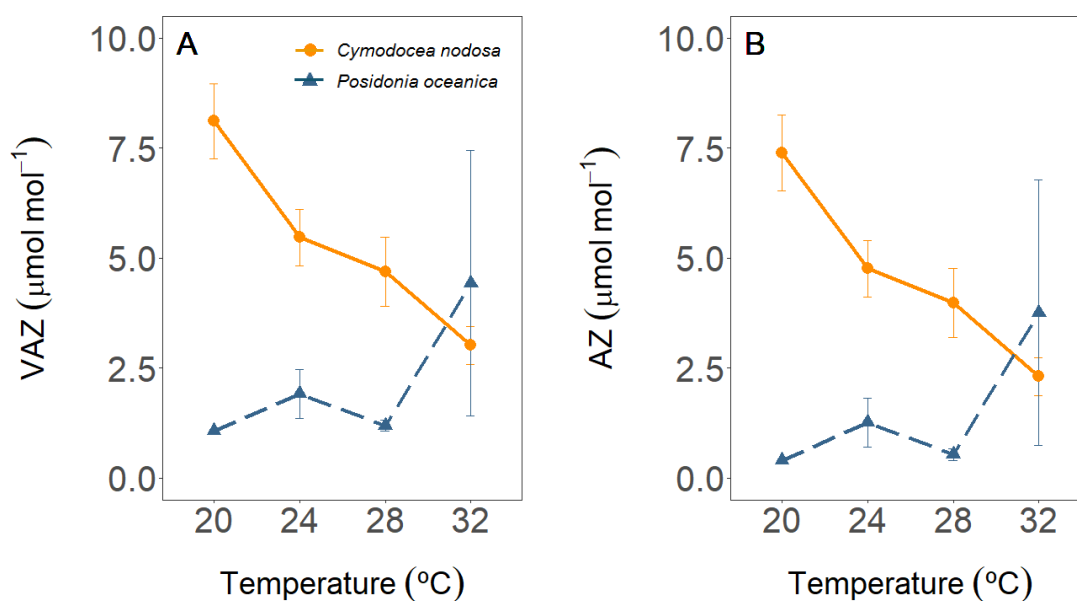


Figure S3. Xanthophylls cycle pigments (mean \pm SE, $n=3$) in *P. oceanica* and *C. nodosa* plants after being exposed to a range of four temperatures for five weeks. (A) Total xanthophyll concentration relative to chlorophyll content (VAZ) and (B) the degree of conversion of the xanthophyll cycle to AZ (A+Z:total chlorophyll molar ratio).

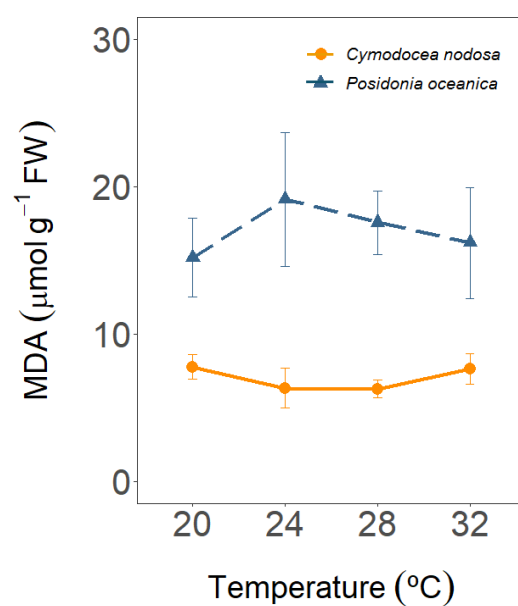


Figure S4. Membrane lipid peroxidation (expressed as MDA equivalents, see text), measured in shoots of *P. oceanica* and *C. nodosa* exposed to a range of four temperatures. Values are reported as mean \pm SE, $n=3$.

Table S1. Results of PERMANOVA (multivariate and univariate analysis) testing for the significance of effects of temperature (20 °C, 24 °C, 28 °C and 32 °C) and species (*P. oceanica* and *C. nodosa*) on plant response variables after plants were exposed to increased temperatures for five weeks. Numbers in bold indicate significant effects ($p < 0.05$).

Variable	Source	df	MS	Pseudo-F	P	Unique perms
<i>Main test</i>						
	Species (Sp)	1	2.31E+05	63.2100	0.0001	9946
	Temperature (Te)	3	3876.4	1.0610	0.3961	9942
	Sp \times Te	3	5194.6	1.4218	0.2271	9933
	Residual	16	3653.6			
	Total	23				
<i>Individual test</i>						
F _v /F _m	Species (Sp)	1	7.26E-04	70.757	0.0001	9834
	Temperature (Te)	3	1.55E-04	15.133	0.0003	9955
	Sp \times Te	3	1.01E-04	9.8565	0.001	9964
	Residual	16	1.03E-05			
	Total	23				
$\Delta F/F_m'$	Species (Sp)	1	6.48E-04	7.1131	0.0167	9850
	Temperature (Te)	3	1.15E-03	12.601	0.0002	9944
	Sp \times Te	3	1.09E-03	11.91	0.0006	9953
	Residual	16	9.12E-05			
	Total	23				
ETR _{max}	Species (Sp)	1	817.36	394.02	0.0001	9733
	Temperature (Te)	3	23.686	11.418	0.0007	9950
	Sp \times Te	3	16.275	7.8453	0.0025	9952
	Residual	16	2.0744			
	Total	23				
NPQ	Species (Sp)	1	0.42817	22.16	0.0003	9856
	Temperature (Te)	3	3.08E-02	1.5923	0.2279	9955
	Sp \times Te	3	0.14523	7.5163	0.0016	9952
	Residual	16	1.93E-02			
	Total	23				
Chl _a	Species (Sp)	1	7893.8	3.7428	0.0718	9840
	Temperature (Te)	3	1813.2	0.85973	0.5004	9956
	Sp \times Te	3	1191.9	0.56514	0.6558	9943
	Residual	16	2109.1			
	Total	23				

<i>Chlb</i>						
	Species (Sp)	1	22.488	3.71E-02	0.8565	9842
	Temperature (Te)	3	549.44	0.90722	0.4833	9961
	Sp \times Te	3	418.19	0.69051	0.5877	9950
	Residual	16	605.63			
	Total	23				
<i>Chlb/Chla</i>						
	Species (Sp)	1	6.63E-02	238.48	0.0001	9808
	Temperature (Te)	3	6.52E-04	2.3459	0.1065	9952
	Sp \times Te	3	5.79E-04	2.084	0.1503	9955
	Residual	16	2.78E-04			
	Total	23				
<i>Gross P_{max}</i>						
	Species (Sp)	1	176.7	11.124	0.0023	9833
	Temperature (Te)	3	7.0352	0.44291	0.7295	9956
	Sp \times Te	3	122.43	7.7079	0.0002	9968
	Residual	16	15.884			
	Total	23				
<i>R_d</i>						
	Species (Sp)	1	0.21794	7.64E-02	0.7823	9833
	Temperature (Te)	3	3.4108	1.1958	0.3392	9949
	Sp \times Te	3	2.8691	1.0059	0.4093	9949
	Residual	16	2.8523			
	Total	23				
<i>P:R_d</i>						
	Species (Sp)	1	3.6811	5.378	0.0303	9835
	Temperature (Te)	3	0.92053	1.3449	0.2906	9960
	Sp \times Te	3	6.4848	9.4741	0.0007	9949
	Residual	16	0.68448			
	Total	23				
<i>Starch Leaf</i>						
	Species (Sp)	1	31.906	102.71	0.0001	9823
	Temperature (Te)	3	1.2298	3.9589	0.0223	9953
	Sp \times Te	3	0.15513	0.49939	0.6748	9952
	Residual	16	0.31064			
	Total	23				
<i>Soluble Leaf</i>						
	Species (Sp)	1	24.039	239.98	0.0001	9779
	Temperature (Te)	3	0.46152	4.6072	0.0148	9946
	Sp \times Te	3	0.10803	1.0784	0.3842	9948
	Residual	16	0.10017			
	Total	23				
<i>NSCs Leaf</i>						
	Species (Sp)	1	111.33	172.39	0.0001	9818
	Temperature (Te)	3	2.8814	4.4614	0.013	9968
	Sp \times Te	3	0.3178	0.49208	0.6988	9960
	Residual	16	0.64584			
	Total	23				

Starch Rhiz						
Species (Sp)	1	0.51761	21.561	0.0005	9848	
Temperature (Te)	3	0.13659	5.6896	0.0072	9952	
Sp \times Te	3	0.30267	12.608	0.0005	9947	
Residual	16	2.40E-02				
Total	23					
Soluble Rhiz						
Species (Sp)	1	852.09	177.07	0.0001	9819	
Temperature (Te)	3	25.126	5.2213	0.0118	9953	
Sp \times Te	3	37.581	7.8097	0.0022	9967	
Residual	16	4.8122				
Total	23					
NSCs Rhiz						
Species (Sp)	1	894.61	180.96	0.0001	9821	
Temperature (Te)	3	26.893	5.4398	0.011	9966	
Sp \times Te	3	43.465	8.7919	0.0016	9950	
Residual	16	4.9437				
Total	23					
Necrosis						
Species (Sp)	1	671.60	53.79	0.0001	9822	
Temperature (Te)	3	22.17	1.78	0.1867	9953	
Sp \times Te	3	88.67	7.10	0.0033	9952	
Residual	16	12.49				
Total	23					
Growth						
Species (Sp)	1	0.32725	20.592	0.0007	9813	
Temperature (Te)	3	9.42E-02	5.9298	0.005	9955	
Sp \times Te	3	0.47746	30.044	0.0001	9950	
Residual	16	1.59E-02				
Total	23					
Net shoot change						
Species (Sp)	1	8286.9	166.94	0.0001	9833	
Temperature (Te)	3	153.6	3.0943	0.0508	9957	
Sp \times Te	3	117.07	2.3584	0.1102	9954	
Residual	16	49.64				
Total	23					
Violaxanthin						
Species (Sp)	1	0.22932	1.17E-02	0.9208	9865	
Temperature (Te)	3	11.271	0.57338	0.6617	9954	
Sp \times Te	3	15.855	0.80657	0.5126	9961	
Residual	16	19.658				
Total	23					
Antheraxanthin						
Species (Sp)	1	4.09E-02	56.56	0.0001	9826	
Temperature (Te)	3	1.36E-03	1.8841	0.1663	9960	
Sp \times Te	3	2.22E-04	0.30667	0.8206	9952	
Residual	16	7.23E-04				
Total	23					

Zeaxanthin						
	Species (Sp)	1	1.2058	16.811	0.0014	9833
	Temperature (Te)	3	4.81E-02	0.67069	0.5944	9943
	Sp \times Te	3	0.3182	4.436	0.0139	9945
	Residual	16	7.17E-02			
	Total	23				
VAZ						
	Species (Sp)	1	60.3	14.158	0.0023	9852
	Temperature (Te)	3	2.7168	0.6379	0.628	9958
	Sp \times Te	3	18.102	4.2503	0.0113	9955
	Residual	16	4.259			
	Total	23				
AZ						
	Species (Sp)	1	58.473	13.696	0.0025	9807
	Temperature (Te)	3	2.6951	0.63128	0.627	9957
	Sp \times Te	3	18.042	4.226	0.0091	9954
	Residual	16	4.2692			
	Total	23				
DR						
	Species (Sp)	1	0.71523	51.227	0.0001	9838
	Temperature (Te)	3	4.59E-03	0.32892	0.8187	9946
	Sp \times Te	3	4.18E-02	2.9962	0.0414	9958
	Residual	16	1.40E-02			
	Total	23				
GPX						
	Species (Sp)	1	787.01	142.25	0.0001	9865
	Temperature (Te)	3	106.03	19.165	0.0001	9960
	Sp \times Te	3	131.9	23.841	0.0002	9962
	Residual	16	5.5325			
	Total	23				
APX						
	Species (Sp)	1	0.46218	9.4211	0.0069	9832
	Temperature (Te)	3	7.53E-02	1.5341	0.2416	9959
	Sp \times Te	3	0.14014	2.8566	0.0698	9956
	Residual	16	4.91E-02			
	Total	23				
GST						
	Species (Sp)	1	16.855	84.558	0.0001	9825
	Temperature (Te)	3	0.38033	1.9081	0.1746	9957
	Sp \times Te	3	0.99495	4.9915	0.0147	9952
	Residual	16	0.19933			
	Total	23				
GPOX						
	Species (Sp)	1	7354.9	107.23	0.0001	9827
	Temperature (Te)	3	83.122	1.2119	0.3344	9956
	Sp \times Te	3	163.97	2.3907	0.1139	9962
	Residual	16	68.589			
	Total	23				

SOD						
	Species (Sp)	1	984.36	7.3474	0.0162	9832
	Temperature (Te)	3	444.6	3.3185	0.0502	9956
	Sp \times Te	3	1544.8	11.531	0.0003	9954
	Residual	16	133.97			
	Total	23				
DHAR						
	Species (Sp)	1	5.50E-04	4.9131	0.0398	9843
	Temperature (Te)	3	3.34E-04	2.9834	0.0659	9955
	Sp \times Te	3	3.89E-04	3.4678	0.0415	9956
	Residual	16	1.12E-04			
	Total	23				
MDA						
	Species (Sp)	1	602	31.786	0.0001	9820
	Temperature (Te)	3	16.835	8.89E-02	0.9684	9963
	Sp \times Te	3	91.645	0.4839	0.6963	9951
	Residual	16	18.939			
	Total	23				

Table S2. Results of one-way ANOVA assessing the significance of temperature on *Posidonia oceanica* plant response variables after plants were exposed to four temperatures for five weeks. Numbers in bold indicate significant effects ($p < 0.05$).

Variable	Factor	Df	SS	MS	F	p-value
F_v/F_m	Temperature	3	0.0007	0.0002	50.537	0.00002
	Residual	8	0.0000	0.0000		
$\Delta F/F_m'$	Temperature	3	0.0046	0.0015	22.639	0.0003
	Residual	8	0.0005	0.0001		
ETR _{max}	Temperature	3	17.5526	5.8509	5.138	0.0286
	Residual	8	9.1094	1.1387		
NPQ	Temperature	3	0.0663	0.0221	9.525	0.0051
	Residual	8	0.0186	0.0023		
Chl _a	Temperature	3	5929.4288	1976.4763	0.619	0.6218
	Residual	8	25527.7565	3190.9696		
Chl _b	Temperature	3	2221.9559	740.6520	0.726	0.5646
	Residual	8	8165.0875	1020.6359		
Chl _b /Chl _a	Temperature	3	0.0033	0.0011	2877.000	0.103
	Residual	8	0.0031	0.0004		
Gross P _{max}	Temperature	3	188.3666	62.7889	11.941	0.0025
	Residual	8	42.0655	5.2582		
R _d	Temperature	3	12.4516	4.1505	1.418	0.3073
	Residual	8	23.4210	2.9276		
P:R _d	Temperature	3	7.8100	2.6033	4.107	0.0489
	Residual	8	5.0714	0.6339		
Starch Leaf	Temperature	3	0.8740	0.2913	0.868	0.4965
	Residual	8	2.6861	0.3358		
Soluble Leaf	Temperature	3	0.0832	0.0277	1.916	0.2055
	Residual	8	0.1158	0.0145		
NSCs Leaf	Temperature	3	0.1144	0.0381	1.408	0.3098
	Residual	8	0.2167	0.0271		
Starch Rhizome	Temperature	3	0.2489	0.0830	2.210	0.1645
	Residual	8	0.3003	0.0375		

Soluble Rhizome	Temperature	3	6.2173	2.0724	1.543	0.2770
	Residual	8	10.7478	1.3435		
NSCs Rhizome	Temperature	3	7.9549	2.6516	1.870	0.2130
	Residual	8	11.3453	1.4182		
Necrosis	Temperature	3	107.3309	35.7770	1.668	0.2501
	Residual	8	171.6369	21.4546		
Leaf growth	Temperature	3	1.4831	0.4944	18.298	0.0006
	Residual	8	0.2162	0.0270		
Net shoot change	Temperature	3	752.3321	250.7774	12.863	0.0020
	Residual	8	155.9639	19.4955		
Violaxanthin	Temperature	3	62.3984	20.7995	0.639	0.6107
	Residual	8	260.2575	32.5322		
Antheraxanthin	Temperature	3	0.0010	0.0003	0.669	0.5942
	Residual	8	0.0041	0.0005		
Zeaxanthin	Temperature	3	0.1050	0.0350	0.850	0.5048
	Residual	8	0.3296	0.0412		
VAZ	Temperature	3	21.9386	7.3129	1.036	0.4271
	Residual	8	56.4571	7.0571		
AZ	Temperature	3	21.8133	7.2711	1.028	0.4304
	Residual	8	56.5998	7.0750		
DR	Temperature	3	0.0144	0.0048	1.309	0.3369
	Residual	8	0.0292	0.0037		
GPX	Temperature	3	4.6513	1.5504	2.779	0.1102
	Residual	8	4.4640	0.5580		
APX	Temperature	3	0.0323	0.0108	0.455	0.7210
	Residual	8	0.1892	0.0237		
GST	Temperature	3	0.4805	0.1602	5.438	0.0247
	Residual	8	0.2356	0.0294		
GPOX	Temperature	3	2.0301	0.6767	11.622	0.0028
	Residual	8	0.4658	0.0582		
SOD	Temperature	3	2434.6184	811.5395	7.629	0.0099
	Residual	8	850.9865	106.3733		

DHAR	Temperature	3	0.0001	0.0000	0.591	0.6380
	Residual	8	0.0004	0.0001		
MDA	Temperature	3	26.7002	8.9001	0.255	0.8558
	Residual	8	279.3483	34.9185		

Table S3. Results of one-way ANOVA assessing the significance of the effects of temperature on *Cymodocea nodosa* plant response variables after plants were exposed to four temperatures for five weeks. Numbers in bold indicate significant effects ($p < 0.05$).

Variable	Factor	Df	SS	MS	F	p-value
F_v/F_m	Temperature	3	0.0001	0.0000	1.650	0.2537
	Residual	8	0.0001	0.0000		
$\Delta F/F_m'$	Temperature	3	0.0021	0.0007	6.125	0.0181
	Residual	8	0.0009	0.0001		
ETR _{max}	Temperature	3	102.3292	34.1097	11.331	0.0030
	Residual	8	24.0818	3.0102		
NPQ	Temperature	3	0.0898	0.0299	1.671	0.2495
	Residual	8	0.1433	0.0179		
Chl _a	Temperature	3	3085.9756	1028.6585	1.001	0.4405
	Residual	8	8217.2779	1027.1597		
Chl _b	Temperature	3	680.9257	226.9752	1.191	0.3730
	Residual	8	1524.9827	190.6228		
Chl _b :Chl _a	Temperature	3	0.0004	0.0001	0.748	0.554
	Residual	8	0.0014	0.0002		
Gross P _{max}	Temperature	3	0.1198	0.0399	2.789	0.1094
	Residual	8	0.1145	0.0143		
R _d	Temperature	3	6.3882	2.1294	0.767	0.5440
	Residual	8	22.2162	2.7770		
P:R _d	Temperature	3	0.3515	0.1172	7.761	0.0094
	Residual	8	0.1208	0.0151		
Starch Leaf	Temperature	3	3.2807	1.0936	3.830	0.0572
	Residual	8	2.2842	0.2855		
Soluble Leaf	Temperature	3	1.1520	0.3840	3.673	0.0627
	Residual	8	0.8363	0.1045		
NSCs Leaf	Temperature	3	6.8523	2.2841	3.495	0.0697
	Residual	8	5.2279	0.6535		
Starch Rhizome	Temperature	3	1.0689	0.3563	34.012	0.0001
	Residual	8	0.0838	0.0105		

Supplementary material

Soluble Rhizome	Temperature	3	181.9046	60.6349	7.322	0.0111
	Residual	8	66.2467	8.2808		
NSCs Rhizome	Temperature	3	203.1184	67.7061	7.994	0.0086
	Residual	8	67.7543	8.4693		
Necrosis	Temperature	3	225.1873	75.0624	21.332	0.0004
	Residual	8	28.1501	3.5188		
Leaf growth	Temperature	3	0.1098	0.0366	18.781	0.0006
	Residual	8	0.0156	0.0019		
Net shoot change	Temperature	3	59.6761	19.8920	0.249	0.8597
	Residual	8	638.2726	79.7841		
Violaxanthin	Temperature	3	18.9807	6.3269	0.933	0.4684
	Residual	8	54.2626	6.7828		
Antheraxanthin	Temperature	3	0.0037	0.0012	1.326	0.3320
	Residual	8	0.0075	0.0009		
Zeaxanthin	Temperature	3	0.3225	0.1075	11.314	0.0030
	Residual	8	0.0760	0.0095		
VAZ	Temperature	3	40.5170	13.5057	9.245	0.0056
	Residual	8	11.6866	1.4608		
AZ	Temperature	3	40.3969	13.4656	9.201	0.0057
	Residual	8	11.7079	1.4635		
DR	Temperature	3	0.0437	0.0146	5.785	0.0211
	Residual	8	0.0202	0.0025		
GPX	Temperature	3	709.1309	236.3770	22.497	0.0003
	Residual	8	84.0565	10.5071		
APX	Temperature	3	0.6139	0.2046	2.748	0.1125
	Residual	8	0.5957	0.0745		
GST	Temperature	3	3.6454	1.2151	3.291	0.0790
	Residual	8	2.9537	0.3692		
GPOX	Temperature	3	710.3291	236.7764	1.740	0.2360
	Residual	8	1088.4110	136.0514		
SOD	Temperature	3	3533.6065	1177.8688	7.290	0.0112
	Residual	8	1292.6074	161.5759		

DHAR	Temperature	3	0.0021	0.0007	4.066	0.0500
	Residual	8	0.0014	0.0002		
MDA	Temperature	3	5.8440	1.9480	0.658	0.6003
	Residual	8	23.6747	2.9593		

Table S4. Variables represented in the principal component analyses (PCA) and their abbreviations as they appear in the plot.

Variable	Abbreviation (in plot)
Maximum quantum yield	Fv_Fm
Effective quantum yield	F_Fm
Maximum electron transport rate	E'TRmax
Non-photochemical quenching	NPQ
Chlorophyll a in leaves	Chl <i>a</i>
Chlorophyll b in leaves	Chl <i>b</i>
Chlb/Chla ratio	Chl <i>b.a</i>
Maximum net photosynthesis	netPmax
Maximum gross photosynthesis	grossPmax
Photosynthesis:Respiration ratio	P_Rd ratio
Starch content in leaves	Starch L
Soluble content in leaves	Soluble Leaf
Total non-structural carbohydrates in leaves	NSC Leaf
Starch content in rhizome	Starch Rhiz
Soluble content in rhizome	Soluble Rhiz
Total non-structural carbohydrates in rhizome	NSC Rhiz
Leaf necrosis	Necrosis
Leaf growth	Growth
Net shoot change	Dem_balance
Anteraxanthin	ANTERAX
Zeoxanthin	ZEAX
Total xanthophyll concentration relative to chlorophyll content	VAZ
A+Z:total chlorophyll molar ratio	AZ
Xanthophyll de-epoxidation ratio	DR
Glutathione peroxidase	GPX
Ascorbate peroxidase	APX
Glutathione-S-transferase	GST
Guaiacol peroxidase	GPOX
Superoxide dismutase	SOD
Dehydroascorbate reductase	DHAR
Membrane lipid peroxidation	MDA

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Interactive effects of global warming and eutrophication on a fast-growing Mediterranean seagrass

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ARTICLE INFO

Keywords:

Global warming
Eutrophication
Cymodocea nodosa
Nutrients
Anoxia
Ammonium
Interactive effect

ABSTRACT

Coastal ecosystems, such as seagrasses, are subjected to local (e.g. eutrophication) and global (e.g. warming) stressors. While the separate effects of warming and eutrophication on seagrasses are relatively well known, their joint effects remain largely unstudied. In order to fill this gap, and using *Cymodocea nodosa* as a model species, we assessed the joint effects of warming (three temperatures, 20 °C, 30 °C and 35 °C) with two potential outcomes of eutrophication: (i) increase in nutrients concentration in the water column (30 and 300 μM), and (ii) organic enrichment in the sediment). Our results confirm that temperature in isolation clearly affects plant performance; while plants exposed to 30 °C performed better than control plants, plants exposed to 35 °C showed clear symptoms of deterioration (e.g. decline of photosynthetic capacity, increase of incidence of necrotic tissue). Plants were unaffected by high ammonium concentrations; however, organic enrichment of sediment had deleterious effects on plant function (photosynthesis, growth, demographic balance). Interestingly, these negative effects were exacerbated by increased temperature.

Our findings indicate that in addition to the possibility of the persistence of *C. nodosa* being directly jeopardized by temperature increase, the joint effects of warming and eutrophication may further curtail its survival. This should be taken into consideration in both predictions of climate change consequences and in local planning.

1. Introduction

Coastal ecosystems are facing multiple anthropogenic stressors that adversely affect their biodiversity and functioning (Vinebrooke et al., 2004). Such stressors are generated at a range of spatial scales, from the global to the most local. Global stressors are mostly related to climate change and include rising sea level, seawater acidification, warming and an increased frequency of heat waves (IPCC, 2013). The most prominent and pervasive stressor generated locally is probably eutrophication: increased loading of nutrients and organic matter from human activities (Nixon, 2009). This has come to be considered one of the major threats confronting coastal ecosystems (Bricker et al., 2008; Hemminga and Duarte, 2002). The knowledge accumulated to date on the effects of individual stressors on key species is impressive. However, stressors rarely occur in isolation in the environment and, when acting together, they can be synergistic, additive or antagonistic (Todgham and Stillman, 2013). The interaction between stressors is now viewed as

a crucial issue, to the point that it is recognized that single-factor experiments are of limited use for assessing the effects of climate change on coastal marine ecosystems subjected to other disturbances, such as eutrophication (Wernberg et al., 2012). Undoubtedly, experiments with single stressors can help us gain knowledge of the intrinsic, basic response mechanisms involved. However, the results should only be extrapolated to nature with great caution, not only due to problems associated with scaling up, but also due to potential interactions with concurrent stressors (Gunderson et al., 2016).

Seagrasses are widespread habitat-forming species of great ecological value that are exposed to multiple threats and are currently suffering declines worldwide (Waycott et al., 2009). The effects of climate change (including increased temperature and acidification) or eutrophication on the distribution, abundance and vitality of seagrasses are relatively well known (see reviews by Koch et al., 2013, for climate change; and Burkholder et al., 2007, for eutrophication), and even though their effects have, for the most part, been assessed separately

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<https://doi.org/10.1016/j.marenvres.2019.02.002>

Received 11 December 2018; Received in revised form 30 January 2019; Accepted 1 February 2019

Available online 05 February 2019

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(but see Campbell and Fourqurean, 2014, 2018; Ow et al., 2016). Thus, it is well known that eutrophication has two main consequences for seagrasses. On the one hand, the effects of increased nutrient concentrations are generally considered detrimental, although they strongly depend on species-specific features and on local conditions (Kilminster et al., 2015; Romero et al., 2006; Ruiz et al., 2001). Thus, while a moderate supply of nutrients to plants adapted to nutrient-poor environments can stimulate growth (Alcoverro et al., 1997; Pérez et al., 1991; Short, 1987), once a threshold is reached, it may cause negative effects on plant photosynthesis and may even curtail survival (Brun et al., 2002, 2008; Hauxwell et al., 2003; van Der Heide et al., 2008). These negative effects can be caused directly, mainly by ammonium toxicity (Touchette and Burkholder, 2000; Van Katwijk et al., 1997); or indirectly, by stimulating phytoplanktonic, epiphytic and macroalgal overgrowth, and enhancing negative biotic interactions such as macro herbivore activity (Campbell et al., 2018; Ruiz et al., 2009; Wear et al., 1999). On the other hand, an increased supply of organic matter to the seagrass sediment, such as that caused by eutrophication, stimulates its oxygen demand, eventually leading to hypoxic or anoxic conditions (Frederiksen et al., 2008; Pérez et al., 2007). This oxygen shortage not only blocks metabolic function in seagrass roots, including respiration, growth and nutrient acquisition (Smith and Piedrahita, 1988), but it also stimulates microbial sulphate reduction, which leads to below-ground seagrass organs (rhizomes and, specially, roots) being exposed to sulphide, a strong phytotoxin (Holmer and Bondgaard, 2001). Despite seagrass having evolved a number of adaptations which increase its chances of surviving in naturally organic-rich sediments (Hasler-Sheetal and Holmer, 2015), additional deposition of organic C can exceed the seagrass response capacity, and have negative effects such as reduced photosynthesis, impaired growth or, in some cases, mass mortality (Collier and Waycott, 2014; Frederiksen et al., 2008; Koch et al., 2007; Olivé et al., 2009).

Temperature affects seagrass physiology in a number of ways. It is known that increased temperature usually stimulates both photosynthesis (Campbell et al., 2006; Winters et al., 2011) and respiration (Schulze et al., 2005); but beyond some threshold, it generally increases the latter more than the former, thus leading to an impaired C balance and reduced growth (Lee et al., 2005, 2007; Marín-Guirao et al., 2016, 2018; Pérez and Romero, 1992). Temperature also affects other processes, such as for instance nutrient uptake (Borum et al., 2004; Bulthuis, 1987) or protein synthesis (Campbell et al., 2006; Marín-Guirao et al., 2017). Overall, when the temperature exceeds a given threshold, which is largely species specific, thermal stress leads to a reduction in growth (Lee et al., 2007), deterioration of shoot status and eventually shoot mortality (Marbà and Duarte, 2010). The responses of seagrasses to increased temperature are relatively well documented; however, little is known of the potential distortion of these responses caused by eutrophication.

Global warming is expected to increase in the coming decades and will affect the surface waters of almost all of the world's oceans. Meanwhile, a large part of the planet's coastal areas are subjected to different degrees of eutrophication (Halpern et al., 2007), which is especially notable in industrialized countries. Consequently, many cases, thermal stress will have an impact on meadows already affected by chronic or acute eutrophication, whose responses to thermal stress will probably differ from that of unaffected plants, thus limiting our ability to make reliable and realistic predictions for future warming scenarios. To date, only a few studies have focused on the combined effects of warming and other stressors, such as anoxia (Koch et al., 2007, with *Halodule wrightii* and *Thalassia testudinum*), nutrients (Kaldy, 2014, with *Zostera marina*) or light (York et al., 2013 with *Zostera muelleri*). These works seem to suggest that synergistic effects are more the rule than the exception. If this is the case, the consequences of global warming may be worse than expected based solely on studies of thermal effects. In fact, a synergistic interaction between eutrophication and seawater warming has already been suggested for the

Mediterranean seagrass *Posidonia oceanica* to forecast trajectories in abundance and distribution of this seagrass species in the context of the different global climate change scenarios (Jordà et al., 2012). However, a considerable gap exists in our knowledge of the combined effects of warming and other stressors; and research is needed to confirm (or refute) the potential synergies in seagrasses, especially in species that dominate areas that are particularly sensitive to climate change. The present study attempts to help fill this gap, by evaluating the joint effect of warming and eutrophication on a Mediterranean seagrass (*Cymodocea nodosa*). The Mediterranean is one of the regions that are expected to be most affected by warming, and the sea surface temperature rise, already in evidence (Burrows et al., 2011; Jordà et al., 2013) may reach 3 °C by the end of the 21st century (Jordà et al., 2012); while the frequency of heat waves is also expected to increase (IPCC, 2013). Moreover, eutrophication has been identified as one of the major environmental threats to seagrass habitats in coastal areas, mainly due to loading from urban, agricultural and aquaculture wastes, particularly in the more confined environments where *C. nodosa* is dominant (Boudouresque et al., 2009).

C. nodosa is widely distributed across a broad variety of shallow Mediterranean environments, from open coastal areas to coastal lagoons, and extends into the Atlantic, from the south of the Iberian Peninsula to the Canary Islands and Mauritania (Green and Short, 2003; Mascaró et al., 2009; Reyes et al., 1995). Its ecological value and its capacity to survive relatively eutrophic conditions (Oliva et al., 2012), as well as its considerable phenotypic plasticity (Pérez and Romero, 1994; Sandoval-Gil et al., 2014), make it an interesting model species to evaluate the joint effects of increased temperatures and eutrophication.

The aim of this study is thus to explore the combined effect of a global stressor (warming) and a local stressor (eutrophication) on functional traits of *C. nodosa*. We partition the eutrophication effects into an increased nutrient concentration in the water column and an increase of organic matter loading of the sediment. We then determine the response of the plant to each one of the three stressors (elevated temperature, nutrient increase and increased organic matter loading) separately; and also to the combined effects of temperature and each of the other two. The main hypothesis we wish to evaluate is that temperature and eutrophication act synergistically, with deleterious consequences for the seagrass. To this end, we perform two fully factorial experiments on indoor mesocosms in which plants are exposed to three levels of temperature and, on the one hand, to three different nutrient concentrations and, on the other hand, to two different levels of organic matter in the sediment.

2. Material and methods

We explored the interactive effects of eutrophication and temperature in two separate experiments. In the first experiment (TNUT experiment, hereinafter), temperature increase and nutrient (ammonium) addition were applied; while in the second (TANOX experiment, hereinafter) the stressors were temperature increase and addition of labile organic C to the sediment.

2.1. Plant and sediment collection

Undamaged healthy *C. nodosa* shoots (including their rhizomes and roots) were carefully collected by hand from a shallow, undisturbed meadow (0.5 m deep) in Alfacs Bay (NW Mediterranean) in late April. Only shoots less than one year old (less than 12 scars on the vertical rhizome, Mascaró et al., 2014) were selected to reduce the effects of physiological and morphological variability between shoots of different ages (Pagès et al., 2010; Pérez and Romero, 1994). Sediment was collected from the same area, extracting the surface layer (up to 10 cm deep), and immediately sieved (1 mm pore) to exclude macro-invertebrates and detritus. Sediment and plants were then transported separately in aerated tanks to the laboratory, where they were

maintained with aeration for one night prior to the experiment being setup. Temperature was kept constant at the ambient values measured at the collection site (19.5 °C). The experiments were conducted at the Experimental Chambers Service of the University of Barcelona.

2.2. Experimental design and setup

Both experiments were conducted using cylindrical transparent aquaria (12 L capacity, 40 cm height x 20 cm diameter) placed randomly in 3 experimental chambers (2.1 m²). Each aquarium had an independent air pump providing proper aeration. The chambers allowed us to control the water temperature (20 °C, 30 °C and 35 °C) and incident light (270 μmol photons m⁻² s⁻¹), which was above the saturation irradiance for these plants (Pérez and Romero, 1992) on a 12h:12h light:dark photoperiod. To avoid experimental bias and minimize any uncontrolled variability, the aquaria were randomly relocated within the chambers every two days. Moreover, the aquaria were moved from one chamber to another (changing the chamber temperature) so that they spent approximately 1/3 of the experimental period in each chamber. Within 24 h of collection, twenty shoots (with their corresponding portion of rhizome and roots) were planted in each aquarium, previously filled with 10 cm of sediment and 9 L of filtered seawater. All the aquaria were covered with plastic film to prevent water evaporation. For the TNUT experiment, a total of 27 aquaria were prepared and distributed randomly in groups of 9 in the three experimental chambers; while for the TANOX experiment, a total of 18 aquaria were distributed randomly in groups of 6 (see experimental setting in Fig. 1).

The aquaria were kept at 20 °C for four days (a temperature close to that registered during sampling) to allow for plant acclimation. After the acclimation period, the temperature in two chambers was increased progressively (ca. 3 °C per day) until reaching 30 °C in one and 35 °C in the other. The third chamber was left at 20 °C as a control. The choice of the experimental temperatures was based on an unpublished 3-year temperature data series collected by the authors using continuous *in situ* recorders, indicating average summer (July to September) temperatures close to 30 °C, but peaking to 33 °C in the hottest summer. Based on this, we consider that 35 °C during heat waves is a reasonable assumption under a climate change scenario (IPCC, 2013).

For the TNUT experiment, once the experimental temperatures were reached, NH₄Cl was added to obtain the following concentrations: control (no NH₄Cl added), moderate concentration (30 μM of NH₄Cl)

and high concentration (300 μM of NH₄Cl). The so-called “moderate” value (30 μM) can be observed at eutrophic sites and can trigger responses in some seagrass species (Villazán et al., 2013). The “high” value (300 μM) represents extreme events and would only be reached during some wastewater discharges (Cabaço et al., 2013). To counter-balance plant uptake (roughly estimated from growth requirements, Pérez and Romero, 1994), NH₄Cl was further added to the experimental aquaria every 5 days: 3.8 mg of NH₄Cl to the moderate ammonium treatment aquaria and 7.6 mg NH₄Cl to the high ammonium treatment aquaria (with a total of two pulses after the initial addition at the beginning of the experiment). Each ammonium treatment was applied to three aquaria, chosen at random, within each temperature chamber, resulting in a complete factorial design with n = 3 replicates per experimental condition. The exposure period to both factors lasted 15 days, after which time leaves in the high-temperature treatment (35 °C) began to show critical necrosis marks.

For the TANOX experiment, once the experimental temperature was reached, the organic matter treatments were applied by adding labile organic C in the form of sucrose to the sediment in the aquaria as follows: control (no sucrose addition, 0.7% DW sediment C content in natural conditions) and high (675 g of sucrose added ≈ 15% DW sediment C content). The labile organic C treatments were applied to three aquaria, chosen at random, within each temperature chamber, resulting in a complete factorial design with n = 3 replicates per experimental condition. The experiment ended after 7 days of exposure period, when leaves in the high-temperature treatment (35 °C) began to show critical necrosis marks.

2.3. Water and sediment analysis

The concentration of ammonium in the water was analysed in each aquarium at the beginning and end of the TNUT experiment using an FP 2020 Plus fluorometer and following a standard method (Kérouel and Aminot, 1997). The redox potential of the sediment was measured at the end of the TANOX experiment using a Thermo Scientific, Orion Star A211 electrode. Measurements were taken in the upper 5 cm of the sediment layer.

2.4. Plant biochemical composition

To verify that the additions of ammonium and labile organic C applied could affect plant conditions, directly (high level of nutrients) and indirectly (anoxic sediment), we determined the N content (TNUT experiment) and S content (TANOX experiment) of different plant parts. To do this, at the end of the experiments, all the remaining shoots were harvested from the aquaria and separated into leaves, rhizomes, and roots. Subsequently, all plant tissues were dried at 60 °C and then finely ground and homogenized; finally, they were weighed and packed into tin microcapsules.

For the TNUT experiment, the nitrogen content of leaves, rhizomes and roots was measured using a Carlo-Erba elemental auto-analyser. For the TANOX experiment, vanadium pentoxide was added, and the sulphur content of leaves, rhizomes and roots was determined. Samples were analysed at the Scientific and Technological Centre (CCiT) of the University of Barcelona.

2.5. Measurement of plant traits

The plant responses to the different stressors (or their combination) were assessed via measurement of a series of traits, from the physiological to population level.

These included maximum quantum yield of PSII (F_v/F_m), incidence of leaf necrosis, leaf growth, rhizome elongation, and shoot demographic balance. All these variables have previously been used in the assessment of seagrass responses to stress and are related to plant health and performance (Beer et al., 1998; Frederiksen et al., 2008; Maxwell

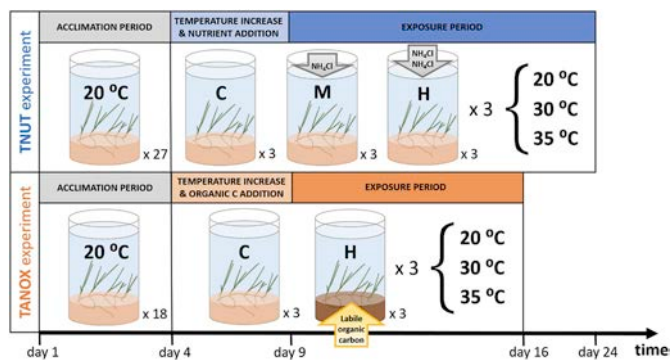


Fig. 1. Experimental setting. Grey arrows indicate ammonium addition in water and yellow arrow indicates labile organic carbon addition to sediment. TREATMENTS: C, control; M, moderate and H, high (see text). Temporal axis indicates: day 1, the beginning of the experimental setup with acclimation at control temperature; day 4, end of acclimation period and progressive increase of temperature; day 9, nutrient or labile organic carbon addition and start of the exposure period; day 16, end of TANOX experiment (7 days exposition); day 24, end of TNUT experiment (15 days exposition). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and Johnson, 2000; Pagès et al., 2010; Romero et al., 2007). Rhizome elongation was only determined for the TANOX experiment, while all the other traits were measured in both experiments.

Maximum quantum yield of PSII (F_v/F_m) was determined using a diving PAM (pulse amplitude modulation) fluorometer (Walz, Germany) after 10 min of plant adaptation to dark conditions. Three shoots were randomly selected from each aquarium (avoiding apical shoots due to their more active growth and photosynthesis) and measurements were obtained from the basal portion of the second youngest leaves, to minimize within-shoot variability (Durako and Kunzelman, 2002; Gera et al., 2012).

The incidence of necrosis was assessed at all leaves of five shoots from each experimental condition. Leaves were carefully separated from each shoot and the percentage of necrotic surface, considered as that partially or totally covered by dark brown or black spots, was visually estimated for each leaf and averaged for each aquarium.

Leaf growth was measured using a leaf punching method (Zieman, 1974) adapted to the model species (Pérez and Romero, 1994). At the beginning of the experiments, five shoots from each aquarium (avoiding apical shoots) were marked by punching a hole just above the ligule of the outermost leaf with a hypodermic needle. At the end of the experiments, the marked shoots were harvested, epiphytes were removed, and the leaves were carefully separated to measure leaf growth. Shoots were individually sorted into old and newly produced tissues, which were then dried for 48 h at 60 °C before obtaining their dry weights. Leaf growth rate was expressed as the new tissue produced per shoot and day ($\text{mg DW shoot}^{-1} \text{ day}^{-1}$), averaged for each aquarium.

To measure rhizome growth, we marked two apical shoots per aquarium with a rubber band. At the end of the experiment, these shoots were harvested, the new portions of rhizome cut, and their weight determined (after drying at 60 °C until constant). Rhizome growth was then expressed as weight of new rhizome per day ($\text{mg DW rhizome day}^{-1}$).

To estimate the shoot demographic balance (the difference between recruitment, i.e. the number of new shoots, and mortality, i.e. the number of dead shoots), all shoots surviving at the end of the experiments were counted. We computed the instantaneous demographic balance (a) as:

$$a (\text{days}^{-1}) = 1/t \ln (N_t/N_0)$$

Where N_0 is the number of shoots planted in each aquarium at the initial time (20), N_t is the number of shoots alive in each aquarium at the end of each experiment and t is the duration of the experiment (in days).

Positive values for a occur when shoot recruitment is higher than mortality, indicating a net increase in shoot abundance. Conversely, negative values would indicate a net reduction in shoot abundance, and hence a negative response to the stressor(s) considered.

Table 1

Ammonium concentrations (in μM , mean \pm SEM, $n = 3$) in the water at the beginning (just after adding 30 μM and 300 μM to the “Moderate” and “High” treatments respectively) and at the end of the TNUT experiment.

Ammonium treatment	Thermal treatment					
	20 °C		30 °C		35 °C	
	[NH ₄ ⁺] (μM)					
	Initial	Final	Initial	Final	Initial	Final
Control	1.20 \pm 0.50	1.54 \pm 0.53	0.69 \pm 0.18	1.96 \pm 1.41	8.64 \pm 29.63	17.95 \pm 8.29
Moderate	27.76 \pm 1.12	0.21 \pm 0.09	26.10 \pm 0.33	0.62 \pm 0.08	45.64 \pm 1.30	3.83 \pm 2.93
High	288.06 \pm 34.01	2.21 \pm 0.03	252.33 \pm 11.23	4.25 \pm 0.79	264.55 \pm 7.96	15.82 \pm 8.36

2.6. Statistical analysis

For all statistical analysis, an aquarium was considered as the experimental unit, with $n = 3$ replicates per experimental condition. The significance of the effects of temperature and ammonium, on the one hand, and, temperature and addition of labile organic C, on the other hand, were determined using PERMANOVA analysis based on a similarity matrix created from the Euclidean distances between samples. The analysis was run with two fixed factors: temperature (3 levels: 20 °C, 30 °C and 35 °C) and nutrients (3 levels: Control, Moderate and High, see above) for the TNUT experiment; and temperature (3 levels: 20 °C, 30 °C and 35 °C) and addition of labile organic C (2 levels: Control and High, see above) for the TANOX experiment.

For each experiment, one multivariate PERMANOVA was carried out for variables related to plant biochemical composition (N and S content of plant tissues, for the TNUT and TANOX experiment, respectively), and a second for the other variables (F_v/F_m , incidence of leaf necrosis, leaf growth, rhizome elongation, and shoot demographic balance), followed by univariate PERMANOVAs performed separately for each individual variable. As in PERMANOVA the test is produced by permutation, the usual normality assumptions of ANOVA (Anderson, 2001), that were not met by most of the variables considered, is not necessary. Pairwise comparisons were performed to identify significant differences between individual treatments. In those cases, in which the number of permutations was too low (< 999 , Anderson et al., 2008), a Monte Carlo test was applied to establish an alternative p-value to validate the analysis. Analysis was carried out using the Primer v6 statistical package (Clarke and Gorley, 2006), in conjunction with the Windows PERMANOVA + module (Anderson et al., 2008).

3. Results

3.1. Culture conditions and plant biochemical composition

The different treatments (additions of nutrient and labile organic C) effectively changed the conditions under which the plants were grown. Thus, on the one hand, in the TNUT experiment, the ammonium concentrations in the water of the moderate and high treatments were increased (relative to the water in the control aquaria) to the target values at the beginning of the experiment and had decreased at the end of the experiment, despite the repeated additions of ammonium and irrespective of the thermal treatment (Table 1).

These results show that the plants were subjected to at least to one strong initial pulse of ammonium, plus another two pulses during the experiment. On the other hand, in the TANOX experiment, the redox potential of the sediments at the end of the experiment, while maintaining positive values under control conditions, became negative in the mesocosms subjected to large additions of high labile organic C. Temperature affected the redox potential, with lower values at higher

Table 2

Redox potential values (mean \pm SEM, $n = 3$) of the sediment in the TANOX experiment for 7 days in three thermal treatments (20 °C, 30 °C, and 35 °C). Lower case letters indicate significant differences ($p > 0.05$) between treatments.

Labile organic C treatment	Thermal treatment		
	20 °C	30 °C	35 °C
	Redox potential (mV)		
Control	180.33 \pm 8.31 ^a	137.07 \pm 16.41 ^b	78.59 \pm 9.52 ^c
High	-24.81 \pm 6.90 ^d	-230.19 \pm 11.38 ^e	-281.78 \pm 14.84 ^f

temperatures likely due to an enhancement of the bacterial activity (Table 2).

Overall, the biochemical composition of leaves (N and S content) changed in response to the treatments. In the TNUT experiment, addition of ammonium at high concentrations increased the N content of all plant tissues, up to 23% relative to controls (Fig. 2a, b and c; Table 3). In the TANOX experiment, the S content of leaves and roots was significantly higher under conditions with an addition of labile organic C than under control conditions (Fig. 2d and f; Table 3). Temperature had significant effects on biochemical composition in both experiments. The N content of leaves increased with temperature; while the N content of rhizomes decreased at the intermediate temperature. The S content of leaves and rhizomes increased with temperature; and the latter was even higher due to interactive effects between temperature and the addition of labile organic C.

3.2. Effects of temperature on plant traits

Temperature had an overall significant effect on the plant traits measured in both experiments (Table 4). The maximum quantum yield

of PSII (F_v/F_m) revealed that the photosynthetic apparatus maintained its integrity at 30 °C. However, F_v/F_m was significantly depressed at 35 °C (6% and 42% lower than under control conditions, in the TANOX and TNUT experiment, respectively), suggesting that significant damage was caused by warming (Fig. 3; Table 5).

The incidence of necrosis on the leaves (Fig. 4; Table 5) was low under control conditions (20 °C; between 7% and 17% in the TANOX and TNUT experiment, respectively) and at 30 °C (< 8% in both experiments), but increased significantly to 23%–33% (depending on the experiment) at 35 °C.

Leaf growth rates (Fig. 5a and b; Table 5) showed higher values at 30 °C than under control conditions (45%) and minimum values at 35 °C (a decrease of between 63% and 94% relative to control conditions, in the TNUT and TANOX experiment, respectively). Rhizome elongation (only measured in the TANOX experiment) was also significantly higher (74%) at 30 °C than at the other two temperatures (Fig. 5c; Table 5).

The shoot demographic balance (i.e. recruitment – mortality) was clearly sensitive to temperature, with a sharp increase (83% on average, relative to the control temperature) under moderate warming (30 °C) and a clear decrease under extreme warming (35 °C), dropping to negative values in the TNUT experiment (Fig. 6a; Table 5).

3.3. The effects of additions of ammonium and labile organic C on plant traits

Ammonium addition did not show any effect on any of the plant traits measured (Figs. 3a, 4a and 5a and 6a; Tables 4 and 5). The addition of labile organic C did not affect the maximum quantum yield of PSII, the incidence of necrosis or the shoot demographic balance (Figs. 3b, 4b and 6b; Table 5). However, it caused a significant decrease (relative to plants grown in unaltered sediment at 30 °C) in leaf and rhizome growth rates (of 44% and 67% respectively; Fig. 5b and c; Table 5).

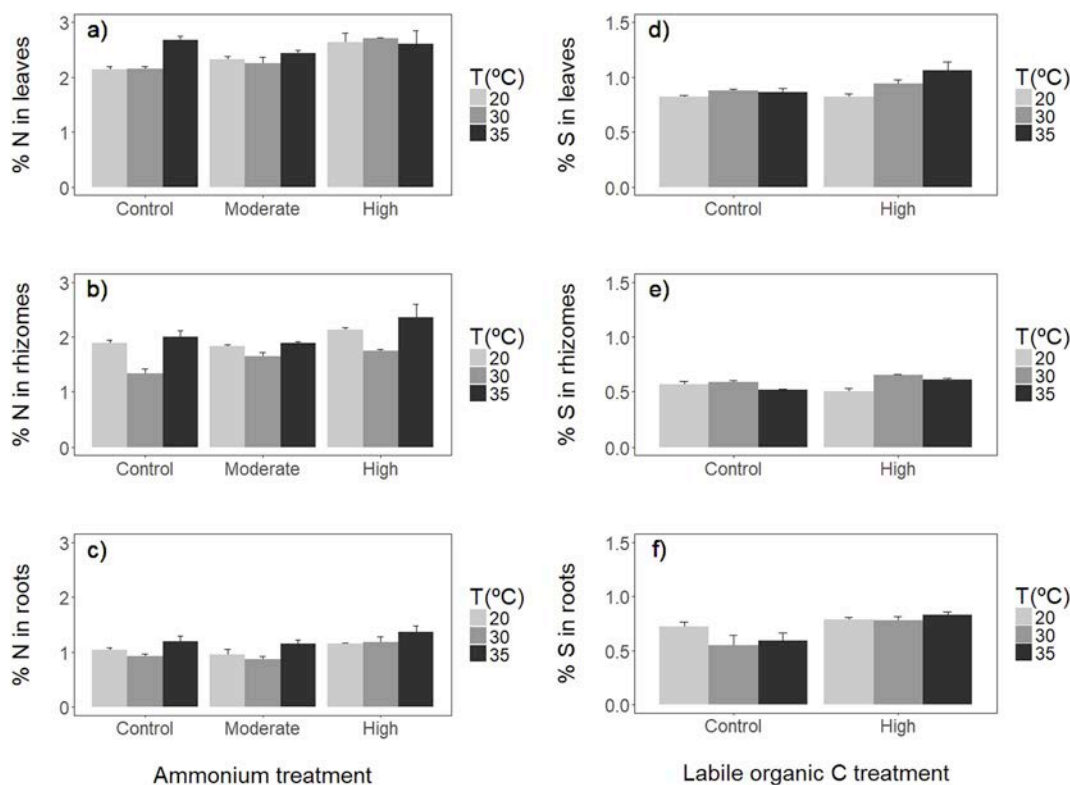


Fig. 2. *Cymodocea nodosa* biochemical composition (N content (mean \pm SE, $n = 3$) and sulphur content (mean \pm SE, $n = 3$)) measured in (a & d) roots, (b & e) rhizomes and (c & f) leaves, at 3 thermal treatments (20 °C, 30 °C, and 35 °C, light grey, dark grey and black respectively) in the TNUT (a, b & c) and TANOX (d, e & f) experiments, expressed in percentage (%).

Table 3

Results of PERMANOVA testing for the significance of effects of temperature (20 °C, 30 °C, and 35 °C), nutrient level (Control, Moderate, and High additions) and labile organic C addition (Control and High) on plant biochemical composition. Numbers in bold indicate significant effects ($p < 0.05$). The results of the pair-wise tests are indicated in factors with significant influence.

Experiment	Variable	Source	df	SS	MS	Pseudo-F	Unique perms	P	Pair-wise
TNUT	Main test								
		Temperature	2	1.802	0.901	9.839	9952	0.0001	
		Ammonium	2	1.467	0.734	8.011	9947	0.0002	
		Temp x Ammonium	4	0.582	0.145	1.588	9931	0.1377	
		Res	18	1.648	0.092				
	N leaves	Temperature	2	0.254	0.127	3.153	9952	0.0634	
		Ammonium	2	0.605	0.302	7.520	9950	0.0042	H > C = M
		Temp x Ammonium	4	0.373	0.093	2.317	9958	0.0955	
		Res	18	0.724	0.04				
	N rhizome	Temperature	2	1.257	0.629	20.145	9949	0.0001	35 = 20 > 30
		Ammonium	2	0.594	0.297	9.519	9953	0.0012	H > C = M
		Temp x Ammonium	4	0.188	0.047	1.506	9956	0.2396	
		Res	18	0.562	0.031				
	N roots	Temperature	2	0.291	0.145	7.219	9943	0.006	35 > 20 = 30
		Ammonium	2	0.268	0.134	6.655	9945	0.0059	H > C = M
		Temp x Ammonium	4	0.021	0.005	0.261	9961	0.9	
Res		18	0.363	0.02					
TANOX	Main test								
		Temperature	2	25.162	12.581	9.972	9961	0.001	
		Labile organic C	1	8.366	8.366	6.631	9952	0.0087	
		Temp x Lab. org. C	2	7.581	3.790	3.004	9940	0.0443	
		Res	12	15.140	1.262				
	S leaves	Temperature	2	0.06	0.03	5.686	9947	0.0228	
		Labile organic C	1	0.034	0.034	6.456	9823	0.0302	30 = 35 > 20
		Temp x Lab. org. C	2	0.032	0.016	3.088	9955	0.0825	C < H
		Res	12	0.063	0.005				
	S rhizome	Temperature	2	0.021	0.011	10.223	9951	0.0012	
		Labile organic C	1	0.003	0.003	3.013	9851	0.1117	20 = 30, 20 = 35, 30 > 35
		Temp x Lab. org. C	2	0.021	0.01	9.921	9952	0.0016	
		Res	12	0.012	0.001				
	S roots	Temperature	2	0.026	0.013	1.435	9951	0.2638	
		Labile organic C	1	0.146	0.146	16.286	9834	0.0029	
		Temp x Lab. org. C	2	0.03	0.015	1.648	9954	0.2393	C < H
Res		12	0.108	0.009					

3.4. Interactive effects

Our results did not show any significant interaction between warming and ammonium addition (TNUT experiment) in terms of their effects on plant traits. In contrast, we found interactive effects of warming and the addition of labile organic C to the sediment (TANOX experiment), both overall (Table 4) and in individual traits. Thus, the

stimulation of leaf and rhizome growth at intermediate temperatures and the improvement of the shoot demographic balance were cancelled by labile organic C. In addition to this, with the normal organic C content of the sediment, high temperature (35 °C) did not alter rhizome growth or the demographic balance, but it did in the sediment with labile organic C added to it.

Table 4

Multivariate PERMANOVA testing for the significance of the general effect of temperature (20 °C, 30 °C, and 35 °C), nutrient level (Control, Moderate, and High additions) and labile organic C addition (Control and High) on plant traits. Numbers in bold indicate significant effects ($p < 0.05$).

Experiment	Source	df	SS	MS	Pseudo-F	Unique perms	P
TNUT	Main test						
	Temperature	2	1725.100	862.540	8.612	9950	0.0030
	Ammonium	2	50.622	25.311	0.253	9949	0.7830
	Temp x Ammonium	4	144.960	36.241	0.362	9958	0.8326
	Res	18	1802.700	100.150			
TANOX	Temperature	2	105420	52709	105.350	9948	0.0001
	Labile organic C	1	435410	435410	870.260	9882	0.0001
	Temp x Labile organic C	2	25298	12649	25.282	9948	0.0001
	Res	12	6003.800	500.320			

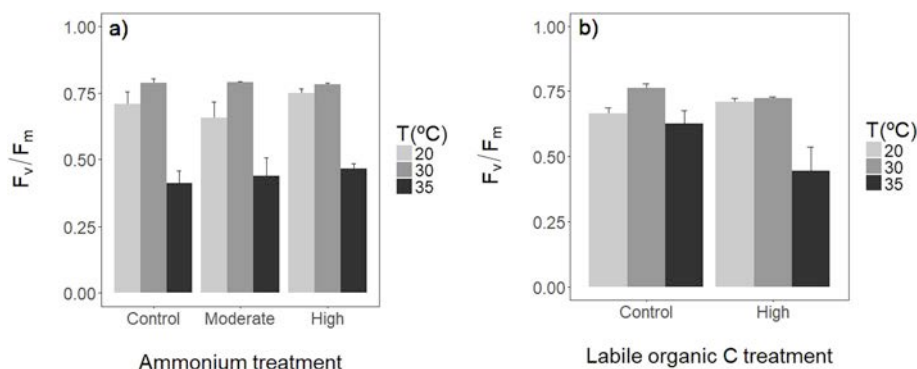


Fig. 3. *Cymodocea nodosa* maximum quantum yield (mean \pm SE, $n = 3$) at 3 thermal treatments (20 °C, 30 °C, and 35 °C, light grey, dark grey and black respectively) in the (a) TNUT and (b) TANOX experiments.

Table 5

Results of PERMANOVA testing for the significance of effects of temperature (20 °C, 30 °C, and 35 °C), nutrient level (Control, Moderate, and High additions) and labile organic C addition (Control and High) on each plant trait. Numbers in bold indicate significant effects ($p < 0.05$). The results of the pair-wise tests are indicated in factors with significant influence.

Variable	Experiment	Source	df	SS	MS	Pseudo-F	Unique perms	P	Pair-wise	
Fv/Fm	TNUT	Temperature	2	0.543	0.272	52.937	9952	0.0001	20 = 30 > 35	
		Ammonium	2	0.012	0.006	1.206	9947	0.3277		
		Temp x Ammo.	4	0.012	0.003	0.597	9943	0.6722		
		Res	18	0.092	0.005					
	TANOX	Temperature	2	0.139	0.069	11.488	9956	0.001	30 > 20 > 35	
		Labile organic C	1	0.016	0.016	2.595	9851	0.1297		
		Temp x Lab. org. C	2	0.04	0.02	3.283	9951	0.0555		
		Res	12	0.072	0.006					
	Necrosis	TNUT	Temperature	2	1714.000	856.990	8.566	9957	0.0031	30 < 20 = 35
			Ammonium	2	50.242	25.121	0.251	9954	0.7805	
Temp x Ammonium			4	144.860	36.215	0.362	9962	0.8436		
Res			18	1800.900	100.050					
TANOX		Temperature	2	4060.000	2030.000	23.683	9948	0.0006	20 = 30 < 35	
		Labile organic C	1	354.090	354.090	4.131	9859	0.0655		
		Temp x Lab. org. C	2	62.753	31.376	0.366	9956	0.7057		
		Res	12	1028.600	85.714					
Leaf growth rate		TNUT	Temperature	2	10.538	5.269	53.080	9950	0.0001	30 > 20 > 35
			Ammonium	2	0.367	0.183	1.848	9950	0.186	
	Temp x Ammonium		4	0.091	0.023	0.228	9951	0.9224		
	Res		18	1.787	0.099					
	TANOX	Temperature	2	12.713	6.357	54.210	9955	0.0001	30 > 20 > 35	
		Labile organic C	1	1.070	1.070	9.125	9851	0.0058	C > H	
		Temp x Lab. org. C	2	1.327	0.663	5.657	9951	0.0075		
		Res	12	1.407	0.117					
	Rhizome growth rate	TANOX	Temperature	2	38.329	19.164	15.536	9954	0.0011	30 > 20 = 35
			Labile organic C	1	23.510	23.510	19.059	9739	0.0012	C > H
Temp x Lab. org. C			2	22.905	11.452	9.284	9948	0.0035		
Res			12	14.803	1.234					
Shoot demographic balance	TNUT	Temperature	2	0.005	0.003	33.785	9964	0.0001	30 > 20 > 35	
		Ammonium	2	0.001	0.001	1.097	9957	0.3624		
		Temp x Ammonium	4	0.001	0.001	0.152	9962	0.9594		
		Res	18	0.001	0.001					
	TANOX	Temperature	2	0.001	0.001	6.767	9952	0.0119	30 > 20 = 35	
		Labile organic C	1	0.001	0.001	3.571	9758	0.0887		
		Temp x Lab. org. C	2	0.001	0.001	5.231	9959	0.0219		
		Res	12	0.001	0.001					

4. Discussion

Climate change is having an impact on a world that has already been altered by a panoply of local stressors. Our results show how cumulative stress, in this case derived from the joint action of warming

and eutrophication, can worsen, either through additive or interactive effects, the negative consequences of each stressor acting in isolation.

As already known, temperature increases can negatively affect different functional mechanisms of seagrasses (Koch et al., 2013). The response patterns and the thresholds involved are largely species

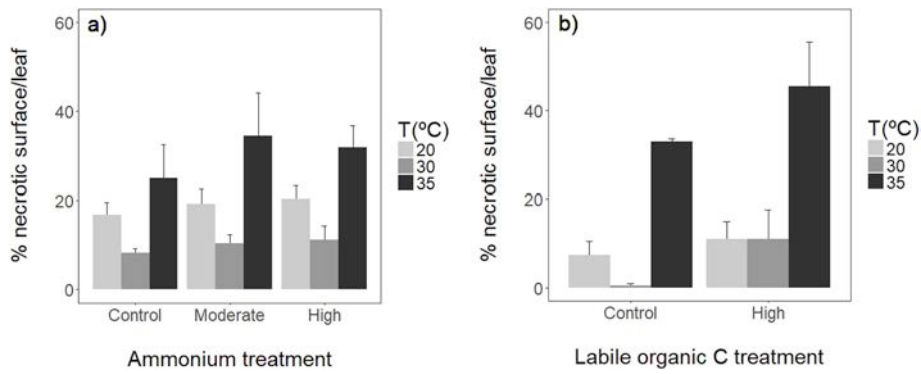


Fig. 4. Leaf necrosis incidence (average necrotic surface per leaf, in %) in *Cymodocea nodosa* (mean \pm SE, $n = 3$) at 3 thermal treatments (20 °C, 30 °C, and 35 °C, light grey, dark grey and black respectively) in the (a) TNUT and (b) TANOX experiments.

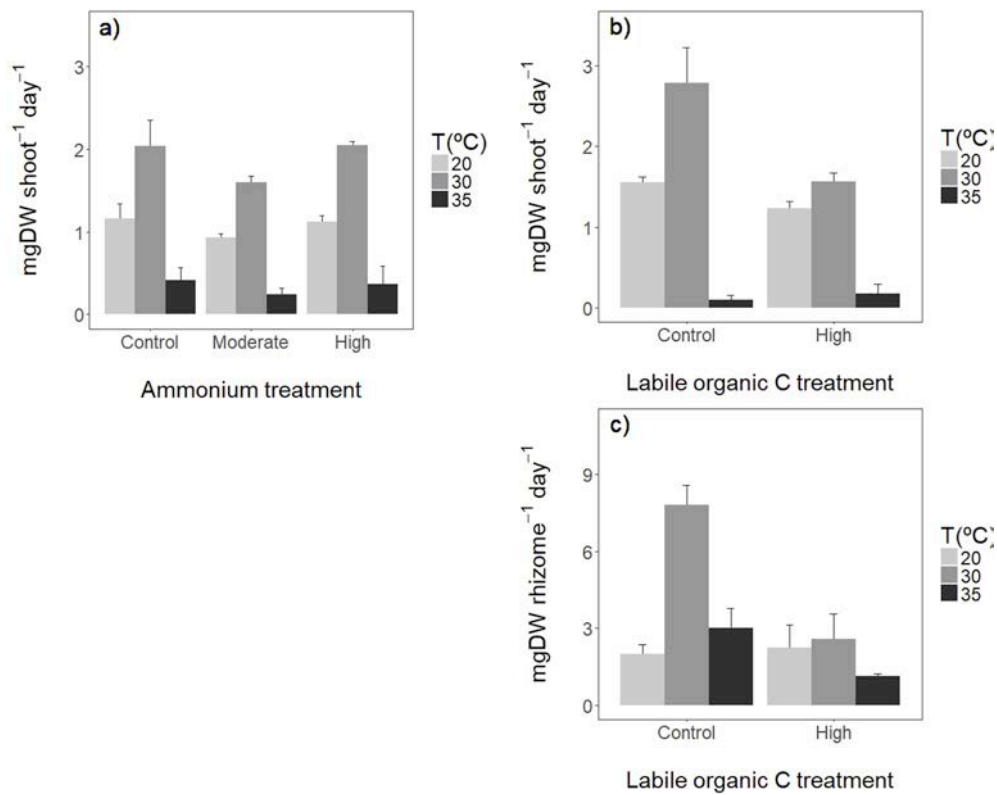


Fig. 5. *Cymodocea nodosa* growth rate (mean \pm SE, $n = 3$) at 3 thermal treatments (20 °C, 30 °C, and 35 °C, light grey, dark grey and black respectively) in two experiments: (a) Leaf growth rate in the TNUT experiment; (b) Leaf growth rate in the TANOX experiment; (c) Rhizome growth rate in TANOX experiment.

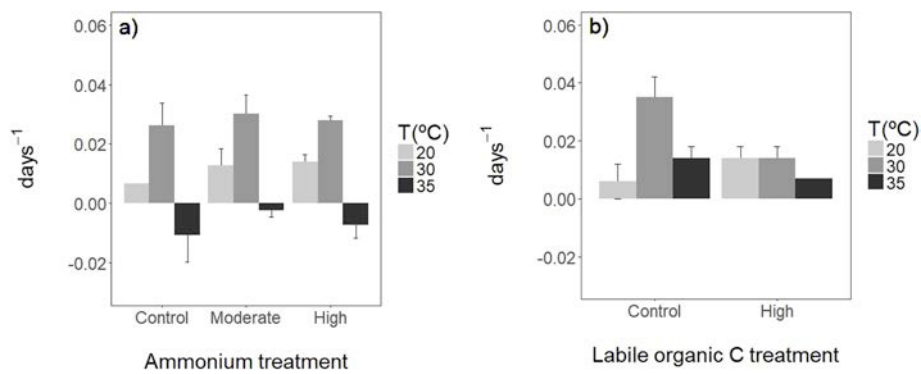


Fig. 6. *Cymodocea nodosa* shoot demographic balance (mean \pm SE, $n = 3$) at 3 thermal treatments (20 °C, 30 °C, and 35 °C, light grey, dark grey and black respectively) in the (a) TNUT and (b) TANOX experiments.

specific (Campbell et al., 2006; Collier et al., 2011). In the model species used here (*C. nodosa*), moderate warming seems to be beneficial for the plant, whose performance (photosynthesis, growth and shoot demographic balance) increase at 30 °C, relative to those found at the basal spring temperature (control) of 20 °C. This pattern is fully consistent in the two experiments we conducted. This is in accordance with previous reports for this species, suggesting an optimum temperature close to 30 °C (Olsen et al., 2012; Pérez and Romero, 1992; Savva et al., 2018; Terrados and Ros, 1995; Tutar et al., 2017). In contrast, plant performance was severely depressed at 35 °C; not only relative to the 30 °C optimum, but also relative to control conditions. This suggests there is a thermal threshold of clear negative effects on plant activity between 30 °C and 35 °C. This thermal threshold is relatively high (see Lee et al., 2007 for comparisons), and it is in accordance with the subtropical distribution of this species (Green and Short, 2003; Reyes et al., 1995) and its facultative habitat in confined environments, where summer temperatures can easily be 5 °C above open sea temperatures.

Exposure to extreme thermal values damaged the integrity of the photosynthetic apparatus, as shown by a clear drop in F_m/F_v to values below those considered acceptable for healthy plants (0.7–0.8, Campbell et al., 2006; Ralph, 1998), as previously found for other seagrass species (e.g. *T. testudinum*; Koch et al., 2007; e.g. *Z. noltii*; Massa et al., 2009). While photosynthesis is depressed, respiration is probably stimulated by thermal stress (not measured in this study; but see Pérez and Romero, 1992), leading to impairment of the C budget (Collier and Waycott, 2014), which could be the cause of the reduced growth and the low to negative shoot demographic balance observed in our experiments. Plants exposed to high temperatures may have to use their energy reserves (stored non-structural carbohydrates) to cope with this stress and the consequent energy requirement (Collier et al., 2011; Massa et al., 2009), probably leading to exhaustion of the internal C reserves (Marín-Guirao et al., 2018). Indeed, thermal stress also affects other metabolic processes, causing, for instance, oxidative stress (Tutar et al., 2017), and ultimately affecting plant health, which deteriorated in our experiments as shown by the increase in the incidence of necrosis.

Reducing the shoot demographic balance can be critical for *C. nodosa*. This species has a very high shoot turnover, with a yearly shoot mortality reaching 1/2 to 2/3 of the total number of shoots in unaltered meadows. This mortality takes place in late summer to autumn and is balanced by massive recruitment in late spring (Mascaró et al., 2014). Any event altering the shoot demographic balance, such as a heat wave, will cause a drop in seagrass density, eventually leading to meadow extinction. This is relevant for projections of distribution and abundance of this species in future warming scenarios since the frequency and intensity of heat waves are predicted to increase (IPCC, 2013). Those predictions suggest that the threshold temperature (thermal tolerance limit) could be reached during these extreme climate events, mainly in confined areas such as shallow bays or lagoons. However, the threshold is quite unlikely to be reached in the open sea, where warming will be much more moderate and could have beneficial effect on the species which could extend its distribution, maybe at the expenses of the Mediterranean species *P. oceanica*, which is much more sensitive to warming (Marín-Guirao et al., 2016; Olsen et al., 2012).

Regarding eutrophication, *C. nodosa* is affected by an increase in organic matter in the sediment but not by pulses in nutrient concentrations. None of the traits studied were modified by addition of ammonium, despite the high concentrations attained (up to 300 µM) and the fact that ammonium was depleted from the aquaria. A coarse N mass balance, estimating N incorporation in the plant through leaf growth, new shoots and N increase in tissues, suggests that most of this depletion was caused by plant activity, being microbial activity in the sediment the most likely explanation for the rest. Seagrasses seem to be unable to downregulate N uptake, probably due to a lack of inhibitory feedback mechanisms (Touchette and Burkholder, 2000). This failure in regulation could generate ammonium accumulation in cells, which in

turn may have toxic effects (Invers et al., 2004). However, while some species seem to be more vulnerable to this toxicity (e.g. *Z. marina*, Burkholder et al., 1992; Van Katwijk et al., 1997; Villazán et al., 2013; and *Z. noltei*, Moreno-Marín et al., 2016) others show great resistance (*C. nodosa*, Egea et al., 2018 and *Z. marina*, Kaldy, 2014). It has been suggested that the key mechanism to endure large ammonium pulses may be an efficient mechanism that is capable of rapidly converting the excess of ammonium into organic forms (Brun et al., 2002; Invers et al., 2004). Second-order (indirect) effects of ammonium pulses, such as an increase in epiphytic load or a decrease in water transparency, were not studied here and cannot be ruled out.

In contrast, the addition of labile organic C had a detrimental effect on plants. The organic additions to the sediment seemed to enhance bacterial respiration and thus oxygen demand, leading to oxygen exhaustion and anoxic conditions (up to –290 mV of redox potential). Under these conditions, sulphate reduction is stimulated, resulting in sulphide accumulation. Consistently with this, we found higher sulphur contents in our exposed plants than in controls. The oxidation of sulphide to sulphur compounds that are further stored in tissues has been shown to be a mechanism that can help cope with sulphide intrusion. However, once the capacity of detoxification of this mechanism is surpassed, the detrimental effects appear (Hasler-Sheetal and Holmer, 2015). Although *C. nodosa* is highly resistant to eutrophication (Oliva et al., 2012), highly negative values (such as those created in our experiment, close to –250 mV) clearly seem to be harmful for plant production and fitness.

Beyond the effects of warming and eutrophication highlighted above, and given that both stressors will act jointly in most real-world conditions, the assessment of their potential interactions is of great interest. Our results show that there were no interactive effects between warming and ammonium; but in contrast the effects of warming on key processes (leaf and rhizome growth and the demographic balance) were strongly mediated by the amount of labile organic C in the sediment. The interaction between temperature and organic matter was detected at the individual (leaf and rhizome growth) and population (shoot demographic balance) level, but not at the physiological one (F_v/F_m). Clearly, the processes affected are critical for meadow persistence, which underlines the relevance of such interactions for the prediction of future seagrass meadow dynamics. However, the mechanisms through which these interactions function have not been elucidated by our work. A possible explanation would be a synergistic effect on environmental conditions. This is supported by the fact that the addition of labile organic C and temperature decreased sediment redox potential synergistically, probably through the stimulation of oxygen demand and cascading effects on sulphide production and plant performances. Other mechanisms, including the amplification of sulphide effects by temperature, should not be ruled out.

Despite multiple stressors studies have increased in the last decades, our results add evidence to the need to further assess the interactive effects of different stressors, and understanding how the organisms or communities will respond to the impact of multiple co-occurring stressors is still a matter of concern (Côté et al., 2016). Seemingly, synergistic effects are quite frequent, as revealed by Crain et al. (2008), which found in a review focused on coastal ecosystems that 36% of the cases examined showed synergy. A thorough literature search on interactive effects on seagrass ecosystems (Table 6) confirms that synergy is more the rule (50%) than the exception (36% additive; only a small part of the studies found antagonistic interaction). There is an urgent need to incorporate those interactive effects to improve predictions of the consequences of climate change in marine ecosystems, which can be seriously underestimated when assessing thermal effects in isolation. In addition, results such as those presented here can support strategies to increase ecosystem resilience to climate change by managing other stressors at a local or regional scale. In this respect, shallow bays and coastal lagoons, which are more vulnerable to both extreme thermal events and eutrophication, may represent a critical scenario for the

Table 6
Synthesis of multiple stressors studies on seagrasses and the interaction type effects.

Stressor 1	Stressor 2	Stressor 3	Overall interaction type	Species	Source
Temperature	Nitrate		Additive	<i>Zostera marina</i>	Kaldy (2014)
Temperature	Ammonium		Additive	<i>Cymodocea nodosa</i>	This study
Temperature	Labile Organic C		Synergy	<i>Cymodocea nodosa</i>	This study
Temperature	Acidification		Additive	<i>Zostera noltii</i>	Repolho et al. (2017)
Temperature	Herbicide		Antagonism	<i>Halophila ovalis</i>	Wilkinson et al. (2017)
Temperature	Light		Additive	<i>Zostera muelleri</i>	York et al. (2013)
Temperature	Light		Synergy	<i>Halodule uninervis</i> and <i>Zostera muelleri</i>	Collier et al. (2011)
Temperature	Floods		Synergy	<i>Amphibolis antarctica</i>	Fraser et al. (2014)
Temperature	Macroalgae		Synergy	<i>Zostera marina</i>	Höffle et al. (2011)
Temperature	Salinity		Synergy	<i>Zostera marina</i>	Salo and Pedersen (2014)
Temperature	Sulfide		Synergy	<i>Halodule wrightii</i> and <i>Thalassia testudinum</i>	Koch et al. (2007)
Nitrate	Acidification		Additive	<i>Thalassia testudinum</i>	Campbell and Fourqurean (2014)
Nitrate	Acidification		Additive	<i>Thalassia hemprichii</i> and <i>Halodule uninervis</i>	Ow et al. (2016)
Nitrate and phosphate	Acidification		Additive	<i>Thalassia testudinum</i>	Campbell and Fourqurean (2018)
Nitrate, ammonium and phosphate	Waves		Synergy	<i>Zostera noltii</i>	La Nafie et al. (2012)
Ammonium	Light		Synergy	<i>Zostera marina</i>	Villazán et al. (2013)
Ammonium	Macroalgae		Antagonism	<i>Zostera noltii</i>	Moreno-Marín et al. (2016)
Ammonium	Salinity		Synergy	<i>Zostera marina</i>	Villazán et al. (2015)
Organic matter	Burial		Synergy	<i>Posidonia oceanica</i>	Ceccherelli et al. (2018)
Temperature	Ammonium	Light	Synergy	<i>Zostera marina</i>	Moreno-Marín et al. (2018)
Temperature	Ammonium	Acidification	Additive/Synergy	<i>Cymodocea nodosa</i>	Egea et al. (2018)
Ammonium	Phosphate	Light	Antagonism	<i>Zostera noltii</i>	Brun et al. (2008)

survival of seagrass species growing close to their upper thermal limit (York et al., 2013; Koch et al., 2007), but also an opportunity to test the above mentioned strategies.

Even though our findings, it is important to keep in mind that the results of this work were obtained from a mesocosm experiment focusing only on two factors (warming and eutrophication) without considering any other disturbance that may be found in the environment. In this sense, they should only be extrapolated to natural conditions cautiously. In spite of these limitations, this research highlights the importance of evaluating the impact of global and local stressors jointly; not only to generate more realistic predictions of the impacts that climate change might have, but also to design and implement strategies to improve (or at least not to impair) seagrass resilience to global warming.

Author contributions

YO, EG, NS, JMR, JR and MP planned and designed the study. YO, EG and NS conducted fieldwork, performed the experiments, samples processing and lab work. YO and JB analyzed the data. YO, JR and MP led the writing of the paper with contributions from the rest of authors. All Authors reviewed the manuscript.

Acknowledgements

This study was funded by the European Union and the Spanish Government through the RECCAM project (seagrass meadows resilience to global warming: an analysis based on responses at ecophysiological, population and ecosystem levels, CTM2013-48027-C3-1-R and CTM2013-48027-C3-2-R); and by the Spanish Ministry of Economy and Competitiveness (via grant BES-2014-069593 awarded to Y. Ontoria).

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RESEARCH ARTICLE

The negative effects of short-term extreme thermal events on the seagrass *Posidonia oceanica* are exacerbated by ammonium additions

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OPEN ACCESS

Citation: Ontoria Y, Cuesta-Gracia A, Ruiz JM, Romero J, Pérez M (2019) The negative effects of short-term extreme thermal events on the seagrass *Posidonia oceanica* are exacerbated by ammonium additions. PLoS ONE 14(9): e0222798. <https://doi.org/10.1371/journal.pone.0222798>

Editor: Silvia Mazzuca, Università della Calabria, ITALY

Received: April 3, 2019

Accepted: September 6, 2019

Published: September 19, 2019

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Data Availability Statement: The data are available in the Figshare repository <https://doi.org/10.6084/m9.figshare.8949410.v1>.

Funding: This work was supported by the European Union and the Spanish Government through the RECCAM (Seagrass meadows resilience to global warming: an analysis based on responses at ecophysiological, population and ecosystem levels, CTM2013-48027-C3-1-R and CTM2013-48027-C3-2-R) and UMBRAL (Responses of benthic marine vegetation to stress:

Abstract

Global warming is increasingly affecting our biosphere. However, in addition to global warming, a panoply of local stressors caused by human activities is having a profound impact on our environment. The risk that these local stressors could modify the response of organisms to global warming has attracted interest and fostered research on their combined effect, especially with a view to identifying potential synergies. In coastal areas, where human activities are heavily concentrated, this scenario is particularly worrying, especially for foundation species such as seagrasses. In this study we explore these potential interactions in the seagrass *Posidonia oceanica*. This species is endemic to the Mediterranean Sea. It is well known that the Mediterranean is already experiencing the effects of global warming, especially in the form of heat waves, whose frequency and intensity are expected to increase in the coming decades. Moreover, this species is especially sensitive to stress and plays a key role as a foundation species. The aim of this work is thus to evaluate plant responses (in terms of photosynthetic efficiency and growth) to the combined effects of short-term temperature increases and ammonium additions. To achieve this, we conducted a mesocosm experiment in which plants were exposed to three thermal treatments (20°C, 30°C and 35°C) and three ammonium concentrations (ambient, 30 μM and 120 μM) in a full factorial experiment. We assessed plant performance by measuring chlorophyll fluorescence variables (maximum quantum yield (F_v/F_m), effective quantum yield of photosystem II ($\Delta F/F_m'$), maximum electron transport rate (ETR_{max}) and non-photochemical quenching (NPQ)), shoot growth rate and leaf necrosis incidence. At ambient ammonium concentrations, *P. oceanica* tolerates short-term temperature increases up to 30°C. However, at 35°C, the plant loses functionality as indicated by a decrease in photosynthetic performance, an inhibition of plant growth and an increase of the necrosis incidence in leaves. On the other hand, ammonium additions at control temperatures showed only a minor effect on seagrass performance. However, the combined effects of warming and ammonium were much worse than those of each stressor in isolation, given that photosynthetic parameters and, above all, leaf growth were affected. This serves as a warning that the impact of global warming

critical transitions, resilience, and management opportunities, CTM2017-86695-C3-1-R) projects; and by the Spanish Ministry of Economy and Competitiveness (via grant BES-2014-069593 awarded to Y. Ontoria). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

could be even worse than expected (based on temperature-only approaches) in environments that are already subject to eutrophication, especially in persistent seagrass species living in oligotrophic environments.

Introduction

Climate change represents a major threat to coastal ecosystems worldwide. The urgent need to gain a better understanding of its impact on the performance of organisms and the subsequent cascading effects that cause changes in ecological functions and ecosystem services is a widespread concern [1–3]. Warming is probably the most pervasive effect of global change, and is expected to cause ocean surface temperatures to rise by between 2.6°C and 4.8°C by 2100 [4]. Aside from this progressive warming, most climatic models predict that temperature extremes will increase in frequency and intensity in the coming decades [5–9]. These so-called heat waves increase temperature by several degrees above the historical mean, usually last for days or a few weeks and seem to be especially deleterious for the biota, thereby increasing concern and attracting a great deal of attention in recent years as key drivers of change [7,8,10]. In addition to global warming, a panoply of stressors caused by human activity [11] is already affecting our environment. Thus, warming will impact ecosystems that are heterogeneously affected, to varying degrees, by a range of other stressors, most of them local in origin. The risk that these local stressors could profoundly modify the response of organisms to warming, thereby altering predictions based solely on thermal responses, is gaining attention and in recent years has fostered a growing interest in assessing the combined effects of warming and other stressors [12–14], especially with a view to identifying possible synergies [15,16].

Such a scenario is a particular threat to coastal areas, where human activities are concentrated, thereby generating a wide array of stressors that could potentially interact with warming (continuous or pulsed) and decrease the resilience of the biota. This is especially worrying in the case of foundation species such as corals, gorgonians and seagrasses due to the propagation of the effects, which may extend to other organisms and have ecosystem-wide implications [17–21]. Seagrasses in particular have demonstrated great sensitivity not only to warming [19,22], but also to other stressors of local origin, including eutrophication [23]. Seagrass habitats are considered some of the most valuable coastal ecosystems in terms of the provision of goods and ecological services [24], thus making the assessment of the combined effects of warming and other stressors a major challenge for the scientific community.

Indeed, on the one hand, temperature is widely known to be one of the main ecological factors that determines seagrass performance, survival and distribution limits (see reviews by [25,26]), and the potential effects of temperature rises are subject to an increasing number of studies. It is well known that a moderate temperature rise can be favourable for plant physiology, since it stimulates photosynthesis. However, it also stimulates the respiration rate and, since the latter increases at a faster rate than the former, this can generate a carbon imbalance in plants if it exceeds a certain threshold [22,26–31]. Similarly, it has been demonstrated that photochemical reactions are highly sensitive to thermal stress, which causes damage to the photosystem II (PSII) reaction centres [32,33] that is irreversible beyond a certain threshold (e.g. 37.5°C, *Halophila ovalis* [34]; 40–45°C, *Zostera capricorni*, *Syringodium isoetifolium*, *Cymodocea rotundata*, *Cymodocea serrulata*, *Halodule uninervis*, *Thalassia hemprichii* and *H. ovalis* [35]). Negative responses of seagrasses to warming have also been reported at individual and population level, including shoot growth impairment [32,36], an increase in leaf shedding

and a reduction in above-ground biomass [33]. In some cases, elevated temperatures have been shown to cause plant mortality [19,22,37] and even alter the geographic limits of seagrass distribution [38,39].

On the other hand, the continuous rise in local nutrient enrichment sources as a consequence of the increasing human population growth and rapid development in coastal areas means that eutrophication is considered a major threat to coastal ecosystems [24,40–42]. Eutrophication can negatively affect seagrasses in particular, either directly or indirectly [43]. The direct effects of nutrient loading, despite the fact that an adequate nutrient supply is fundamental for plant performance [44], include damage caused to seagrasses by excessive inorganic nitrogen (e.g. *Zostera marina*; [45–47]; *Zostera noltii*; [48]). In this sense, the toxicity of high ammonium concentrations has been reported in several studies [48–53], which observed the negative effects of ammonium on several physiological and morphological response variables, including a reduction in primary production and significantly decreased shoot, rhizome and root elongation rates, thus affecting plant survival.

Further research on the isolated effects of each of these two stressors (nutrient loading and warming) on seagrasses is required, but efforts should also be made to assess their combined action, not only to increase knowledge of the expected responses in a realistic multi-stressor scenario, but also to improve the reliability of our predictions about seagrass ecosystem changes in the coming years. In this regard, temperature is already known to exacerbate the negative effects of other stressors such as organic matter-enriched sediments (*Halodule wrightii* and *Thalassia testudinum*, [54]; *Cymodocea nodosa*, [55]) and changes in salinity (*Z. marina*; [56]), which act synergistically with thermal stress. Some other works have reported additive effects of temperature and other stressors (e.g. light availability, *Zostera muelleri*; [33]; acidification, *Z. noltii*; [32]; and nutrients, *Z. marina*; [44]), and, much less commonly, an antagonistic interaction of temperature and a second stressor (e.g. herbicide, *Halophila ovalis*; [57]). All these studies suggest that plant response to the combined impact of temperature and other stressors is largely species-specific and probably depends on the functional traits of the specific plant, but knowledge of this topic with respect to seagrass communities remains scarce and incomplete.

Given the global nature of warming, and the pervasive presence of eutrophication, studying the combined effects of warming and nitrogen loading is crucial to understanding the future of coastal communities dominated by seagrasses, especially in light of the specific plant traits of seagrass foundation species [58]. Although some progress has been made in this area [44,55,59,60], studies that explore this interaction, especially in persistent seagrass species (*sensu* [61]) such as those belonging to the *Posidonia* genus, remain surprisingly scarce.

The Mediterranean endemic species *P. oceanica* is an excellent model for exploring the issues described above. On the one hand, *P. oceanica* is a paradigm of a persistent species [62,63] and a key foundation species in Mediterranean oligotrophic waters, where it provides critical habitats and other ecosystem services. Due to its high sensitivity to stress and vulnerability to coastal deterioration, *P. oceanica* meadows have undergone a substantial decline over the last 50 years [64]. Consequently, it has been one of the main targets of efforts to protect and manage the Mediterranean marine environment in the last 20 years [65]. On the other hand, sea surface temperature in the Mediterranean is increasing at a much faster rate than in the global oceans [6,66] and, at the same time, temperature extremes and heat waves are becoming more common in this region. Moreover, eutrophication is considered a major threat to and stressor for this seagrass, especially near highly populated areas along the Mediterranean coastline, where the first problems of eutrophication were detected as far back as the 1960s [67].

While the effects of eutrophication on this species are relatively well known [68–71], the effects of warming have only recently started being documented [19,27,72–77] and, to the best

of our knowledge, there is no information on the potential effects of the interaction between these two stressors.

The aim of this study is thus to explore both the individual and combined effects of warming, by simulating the effects of a short-term extreme temperature event, and eutrophication, through nutrient loading in the form of ammonium, in the persistent seagrass species *P. oceanica*. In order to achieve this, we evaluated physiological and individual plant responses to a short-term temperature increase (lasting days) and the interactive effects of ammonium additions. To do so, we conducted an indoor mesocosm experiment in which plants were exposed to three thermal treatments and three levels of ammonium concentration in a full factorial experiment.

Material and methods

Plant collection

Divers hand-picked healthy plant fragments of *P. oceanica* with at least four interconnected vertical shoots (apical shoots were avoided) in late September 2016 from an eight-metre deep meadow in Cala Montgó (42° 06' 23" N / 3° 10' 16" E, NE coast of Spain), where allowances to collect plants fragments for scientific purposes are not required.

Plants were transported in aerated tanks to the laboratory and aerated overnight until the experimental setup the following day. The experiment was performed in the University of Barcelona's Experimental Fields Service.

Experimental design and setup

For the experiment, we chose three thermal treatments (20°C, 30°C and 35°C) and three ammonium concentrations: ambient seawater (control), 30 µM (moderate) and 120 µM (high).

The temperatures were chosen to represent the following scenarios: 20°C, close to the temperature of the study site at the collection time, according to a temperature data series recorded by continuous *in situ* temperature data loggers (Fig 1), obtained by the authors in Medas Islands (at a depth of 5 m), an area close to the collection site (< 5 km); 30°C, an anomalously high temperature, likely to be reached in the coming years during heat waves (as a reference, > 28°C recorded during recent heat waves by [18,19]), and relatively common in the Eastern Mediterranean basin (Galli et al., 2017) [78]; and 35°C, a temperature during an extreme heat wave that could be reached in the mid-term future (the temperature is predicted to increase by 4–5°C in the western Mediterranean by the end of the 21st century, as per [4,19,79]). With respect to nutrients, the “moderate” value (30 µM) and the “high” value (120 µM) are the lowest and highest values, respectively, observed in sites affected by sewage discharge [80,81] in the Mediterranean Sea, and similar values have been used in previous experimental approaches [44,55].

The plants were incubated in cylindrical and transparent aquaria (12 L capacity, 40 cm height x 20 cm diameter), each with its own independent air pump and filled with 10 L filtered seawater. The plants were incubated in water to avoid possible confounding effects from sediment. Within 24 hours of collection, a single rhizome fragment bearing four interconnected vertical shoots (apical shoots were avoided) was put in each of the 27 aquaria and covered with plastic film to prevent water evaporation. The aquaria were then distributed randomly in three experimental chambers (2 x 1 x 1.5 m, 9 aquaria per chamber), under controlled temperature and light conditions. The chambers were kept at 210–223 µmoles photons m⁻² s⁻¹, above the saturation irradiance of these plants [71,82–84], under a 12h/12h light/dark photoperiod. Light was provided by daylight fluorescent tubes.

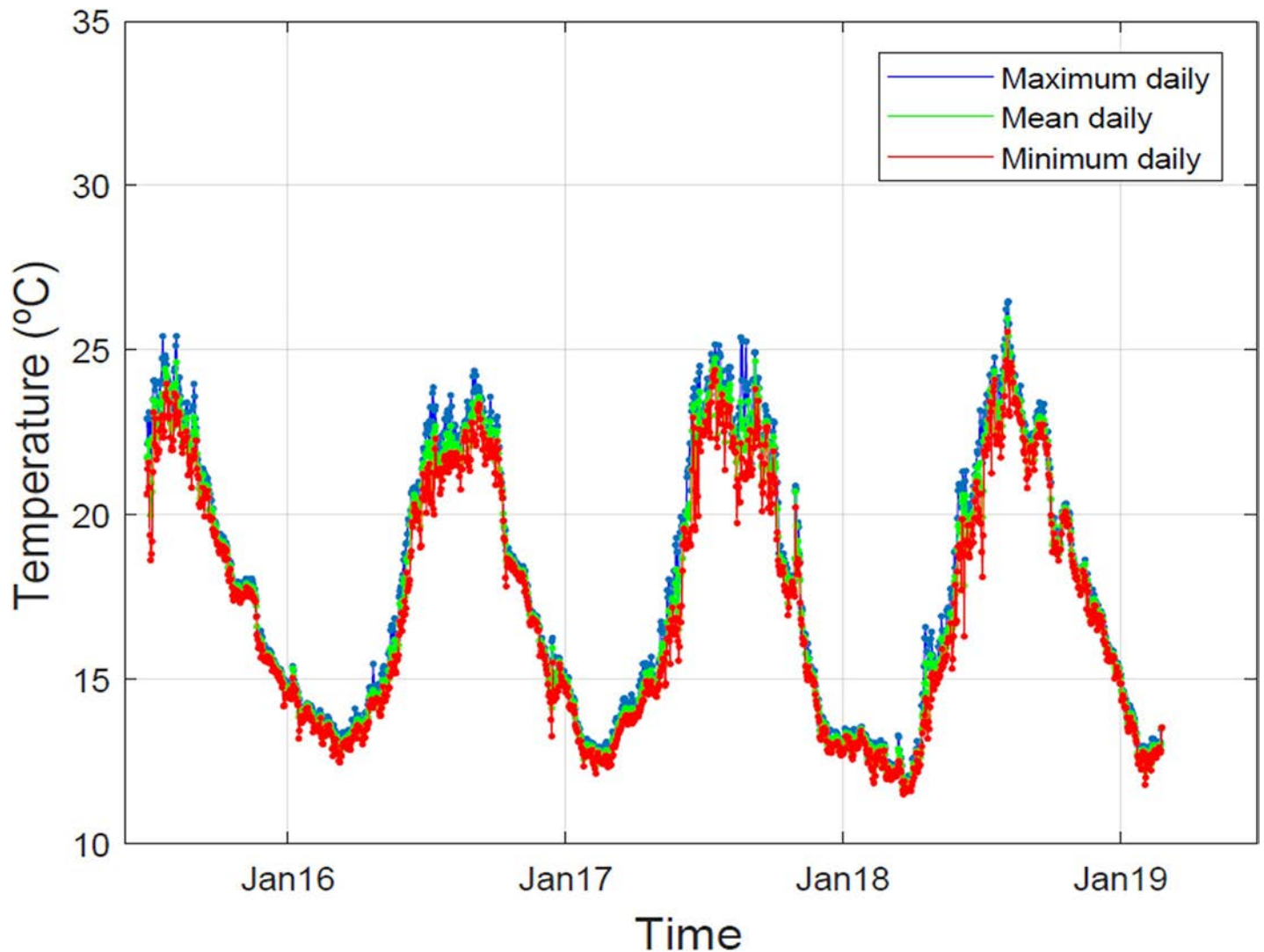


Fig 1. Three-years temperature data series recorded at 5 m deep in Medas Islands (NW Mediterranean Sea).

<https://doi.org/10.1371/journal.pone.0222798.g001>

The three chambers were maintained at 20°C for four days to allow for plant acclimation. After the acclimation period, the temperature was progressively increased (at a maximum rate of 3°C/day) until it reached 30°C in one chamber and 35°C in the other after 5 days, while the third was kept at 20°C as a control.

After the experimental temperatures were reached, appropriate amounts of NH_4Cl were added to obtain the ammonium concentration treatments mentioned above. Ammonium was added just once at the beginning of the experiment, to simulate an ammonium pulse.

While thermal treatments were differentiated in three chambers, ammonium treatments were applied to three randomly chosen aquaria in each chamber, which resulted in a complete factorial design with three replicates per experimental condition.

The experiment ended after seven days of exposure to both stressors (temperature and ammonium), when necrosis marks in plants exposed to the highest temperature (35°C) indicated critical damage to the plant.

In order to minimize uncontrolled variability due to small heterogeneities in light and/or temperature, all aquaria were randomly relocated within the chamber every two days.

Moreover, each set of nine aquaria was moved from one chamber to another (changing the chamber temperature to maintain the thermal treatments) to ensure that each aquarium spend the same time in each chamber. This was done to discard a potential “chamber effect” and avoid pseudoreplication (Ontoria et al., 2019).

Water analyses

Nutrients concentration in the water (ammonium, nitrite, nitrate and phosphate) in each aquarium was analysed at the beginning (just after the experimental ammonium additions) and at the end of the experiment, using an FP-2020 Plus Fluorescence Detector, in accordance with standard methodology [85].

Plant trait response

A number of physiological and individual plant traits were measured at the end of the experiment to determine plant responses. These were maximum quantum yield (F_v/F_m), effective quantum yield of PSII ($\Delta F/F_m'$), maximum electron transport rate (ETR_{max}), non-photochemical quenching (NPQ), incidence of necrosis on the leaves and shoot growth rate.

Chlorophyll fluorescence parameters were determined in three randomly selected shoots from each aquarium using a diving PAM (pulse-amplitude Modulated fluorometer, Walz, Germany). The measurements were obtained from the basal portion of the second youngest leaf to avoid within-shoot variability [86,87]. F_v/F_m was measured by the saturation pulse method after a 10-minute period of dark adaptation. After three hours of illumination, leaves were exposed to increasing photosynthetic photon flux density values (0, 5, 19, 17, 129, 235, 277, 503 and 676 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) at intervals of 10 s to perform rapid light curves (RLCs), which made it possible to obtain $\Delta F/F_m'$, ETR and NPQ measurements. $\Delta F/F_m'$ and NPQ values extracted from RLCs were those obtained at a similar irradiance to plants that were maintained (210–223 $\mu\text{moles photons m}^{-2} \text{s}^{-1}$), while ETR_{max} corresponded to the maximum ETR value obtained in each curve.

The necrosis incidence was assessed in leaves from three shoots in each experimental condition. Leaves were carefully separated from each shoot and the percentage of necrotic surface (dark brown or black spots covering leaf tissue) relative to the total leaf surface was visually estimated in each leaf and averaged for each aquarium.

Shoot growth was measured using a leaf marking technique [88] adapted to our species [89,90]. On the first day of the experiment, all shoots in each aquarium were marked by punching a hole just above the ligule with a hypodermic needle. At the end of the experiment, the shoots were harvested, the epiphytes carefully removed, and three shoots separated to measure shoot growth. Each shoot was sorted into old and new tissue. Plant material was dried for 48 hours at 60°C and weighed to obtain the dry weight. Shoot growth rate was expressed as the new tissue produced per shoot and day ($\text{mg DW shoot}^{-1} \text{day}^{-1}$), and then averaged for each aquarium.

Statistical procedures

The statistical significance of the effects of temperature and ammonium found between treatments was tested using PERMANOVA analyses based on a similarity matrix created from the Euclidean distances between samples. The aquarium was considered as the experimental unit, with a total of $n = 3$ replicates for each experimental condition. The value for each variable in each replicate is the averaged value for this variable obtained from the three shoots (subsamples) used from each aquarium. Two fixed factors were used to run the analyses: temperature (three levels: 20°C, 30°C and 35°C) and ammonium (ambient water, 30 μM and 120 μM).

Multivariate PERMANOVA was performed for plant response variables and univariate PERMANOVA analyses were subsequently carried out individually for each plant trait. As the PERMANOVA statistical test is produced by permutation, the usual ANOVA normality assumptions [91] were not necessary. Differences between treatments were evaluated using pairwise comparisons, and a Monte Carlo test was carried out to obtain an alternative p-value in order to validate the analysis when the number of permutations was too low (<999, [92]). All analyses were performed using the Primer v6 statistical package [93] in conjunction with the Windows PERMANOVA+ module [92].

Results

Nutrient experimental conditions

The initial ammonium concentrations obtained in water ranged from 0.25–0.7 μM, 32–60 μM and 121–132 μM in samples from the control, moderate and high treatments, respectively. At the end of the experiment, ammonium concentrations were very low (less than 1 μM in most treatments, except in two cases: the control (no ammonium added) at high temperature, where some ammonium production took place, and in the high concentration treatment at 35°C, where the final concentration was ca. 70 μM, 60% of the initially supplied (Table 1). Concentrations of other nutrients were in the normal range for the NW Mediterranean waters and did not change significantly during the experiment.

Chlorophyll fluorescence parameters

Temperature had a significant effect on all chlorophyll fluorescence parameters measured (Table 2). Maximum and effective quantum yields (F_v/F_m and $\Delta F/F_m'$, respectively) and maximum electron transport rate (ETR_{max}) showed a similar response pattern, with values at 30°C unaltered and a substantial decrease (38%, 81% and 73%, respectively) at 35°C (in both cases relative to controls at 20°C) (Fig 2A, 2B & 2C). Non-photochemical quenching (NPQ) (Fig 2D) showed slightly higher values at 30°C (up to 17% more) and lower values at 35°C (58%, in both cases relative to controls).

Overall, ammonium additions had negative effects in all but one chlorophyll fluorescence parameter ($\Delta F/F_m'$, ETR_{max} , and NPQ), which decreased by 19%, 19% and 41%, respectively, irrespective of the amount added.

Interestingly, NPQ increased at 30°C in plants submitted to no ammonium addition and moderate ammonium addition but did not at high ammonium concentrations. This is suggestive of a synergistic effect but, given the significance level of the interaction ($p = 0.0582$), by no means conclusive.

In contrast, the combined effect of temperature and ammonium on decreasing F_v/F_m was clearly synergistic. As mentioned above, warming alone (35°C) depressed F_v/F_m in the absence of ammonium additions, while ammonium additions at the control temperature did not cause any effects (Table 2). However, when ammonium was added and plants were warmed (35°C),

Table 1. Ammonium concentrations (in μM, mean ± SEM, n = 3) in the water at the beginning (just after ammonium additions) and at the end of the experiment.

Ammonium treatment	Thermal treatment					
	20°C		30°C		35°C	
	NH ₄ ⁺ (μM)					
	Initial	Final	Initial	Final	Initial	Final
Control	0.25 ± 0.08	0.43 ± 0.18	0.33 ± 0.14	0.27 ± 0.16	0.73 ± 0.27	3.01 ± 1.97
Moderate	40.24 ± 2.18	0.41 ± 0.12	59.87 ± 16.87	0.28 ± 0.23	32.15 ± 1.40	0.85 ± 0.43
High	131.83 ± 2.27	0.15 ± 0.09	123.77 ± 4.47	0.63 ± 0.26	121.27 ± 4.63	72.49 ± 18.83

<https://doi.org/10.1371/journal.pone.0222798.t001>

Table 2. Results of PERMANOVA (multivariate and univariate analysis) testing for the significance of temperature (20°C, 30°C, and 35°C) and nutrient concentration (C: Ambient; M: Moderate, 30 µM and H: high, 120 µM) effects on plant traits. Bold values indicate significant effects (p<0.05). The results of the pairwise tests are indicated in factors with significant influence.

Variable	Source	df	SS	MS	Pseudo-F	P	Unique perms	Pairwise
	<i>Main test</i>							
	Temperature (T)	2	1098.4	549.2	12.857	0.0004	9953	
	Ammonium (A)	2	276.8	138.4	3.24	0.0531	9960	
	T X A	4	416.16	104.04	2.4356	0.0741	9950	
	Residual	18	768.9	42.717				
	<i>Individual test</i>							
F_v/F_m								
	Temperature (T)	2	1.21	0.61	76.43	0.0001	9935	20 = 30>35
	Ammonium (A)	2	0.05	0.03	3.25	0.0588	9955	
	T X A	4	0.15	0.04	4.75	0.0073	9957	35C > 35H
	Residual	18	0.14	0.01	0.00			
$\Delta F/F_m'$								
	Temperature (T)	2	0.04	0.02	180.52	0.0001	9941	20 = 30>35
	Ammonium (A)	2	0.002	0.001	8.99	0.0019	9957	C>M = H
	T X A	4	0.0004	0.0001	0.81	0.5396	9946	
	Residual	18	0.0020	0.0001				
ETR_{max}								
	Temperature (T)	2	364.50	182.25	183.78	0.0001	9953	20 = 30>35
	Ammonium (A)	2	27.33	13.66	13.78	0.0005	9946	C>M = H
	T X A	4	5.06	1.26	1.28	0.3177	9965	
	Residual	18	17.85	0.99				
NPQ								
	Temperature (T)	2	1.65	0.83	76.83	0.0001	9948	30>20>35
	Ammonium (A)	2	0.21	0.11	9.87	0.0017	9952	C>M = H
	T X A	4	0.12	0.03	2.85	0.0582	9952	30C ≥ 30M ≥ 30H
	Residual	18	0.19	0.01				
Necrosis								
	Temperature (T)	2	731.00	365.50	8.76	0.0019	9958	20 = 30<35
	Ammonium (A)	2	249.21	124.61	2.99	0.0692	9940	
	T X A	4	410.83	102.71	2.46	0.0725	9960	
	Residual	18	750.71	41.71				
Growth								
	Temperature (T)	2	0.0007	0.0004	33.34	0.0001	9936	20>30>35
	Ammonium (A)	2	0.0001	0.0001	5.55	0.0123	9943	C = M, C = H, M>H
	T X A	4	0.0001	0.00004	3.25	0.0399	9956	35C > 35H
	Residual	18	0.0002	0.00001				

<https://doi.org/10.1371/journal.pone.0222798.t002>

F_v/F_m was depressed to 54–87%, relative to controls. At 35°C and under high ammonium concentrations, F_v/F_m was below 0.1, thus indicating critical damage to the photosynthetic apparatus (Fig 2A).

Leaf necrosis incidence

Temperature had a significant effect on leaf necrosis, with an incidence of up to 25% higher at 35°C than at 20°C and 30°C (Fig 3, Table 2). Ammonium addition also appeared to increase necrosis incidence, although the effect was only marginally significant (p = 0.0692), likely due to the high variability of this variable.

Shoot growth rate

Both temperature and ammonium had a significant overall effect on plant growth, with a negative effect of temperature and a positive effect (at the moderate concentration only) of

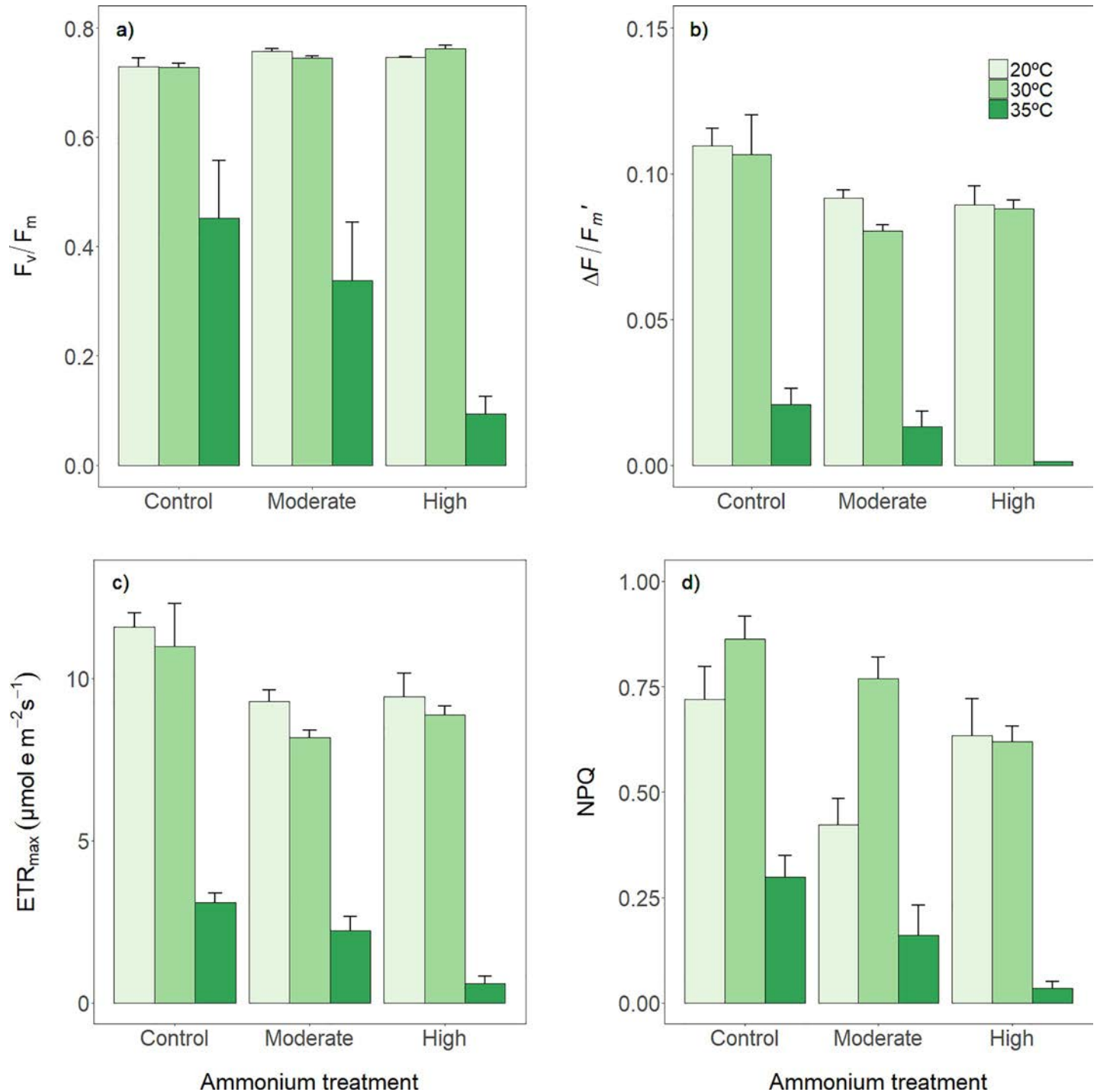


Fig 2. Photochemical responses of *P. oceanica* plants to temperature increase and ammonium addition. (a) Maximum quantum yield of dark-adapted leaves (F_v/F_m), (b) effective quantum yield of PSII ($\Delta F/F_m'$), (c) maximum electron transport rate (ETR_{max}), and (d) non-photochemical quenching (NPQ). Each variable was measured (mean \pm SE, $n = 3$) at three thermal treatments and at three ammonium concentrations, after 7 days of exposure.

<https://doi.org/10.1371/journal.pone.0222798.g002>

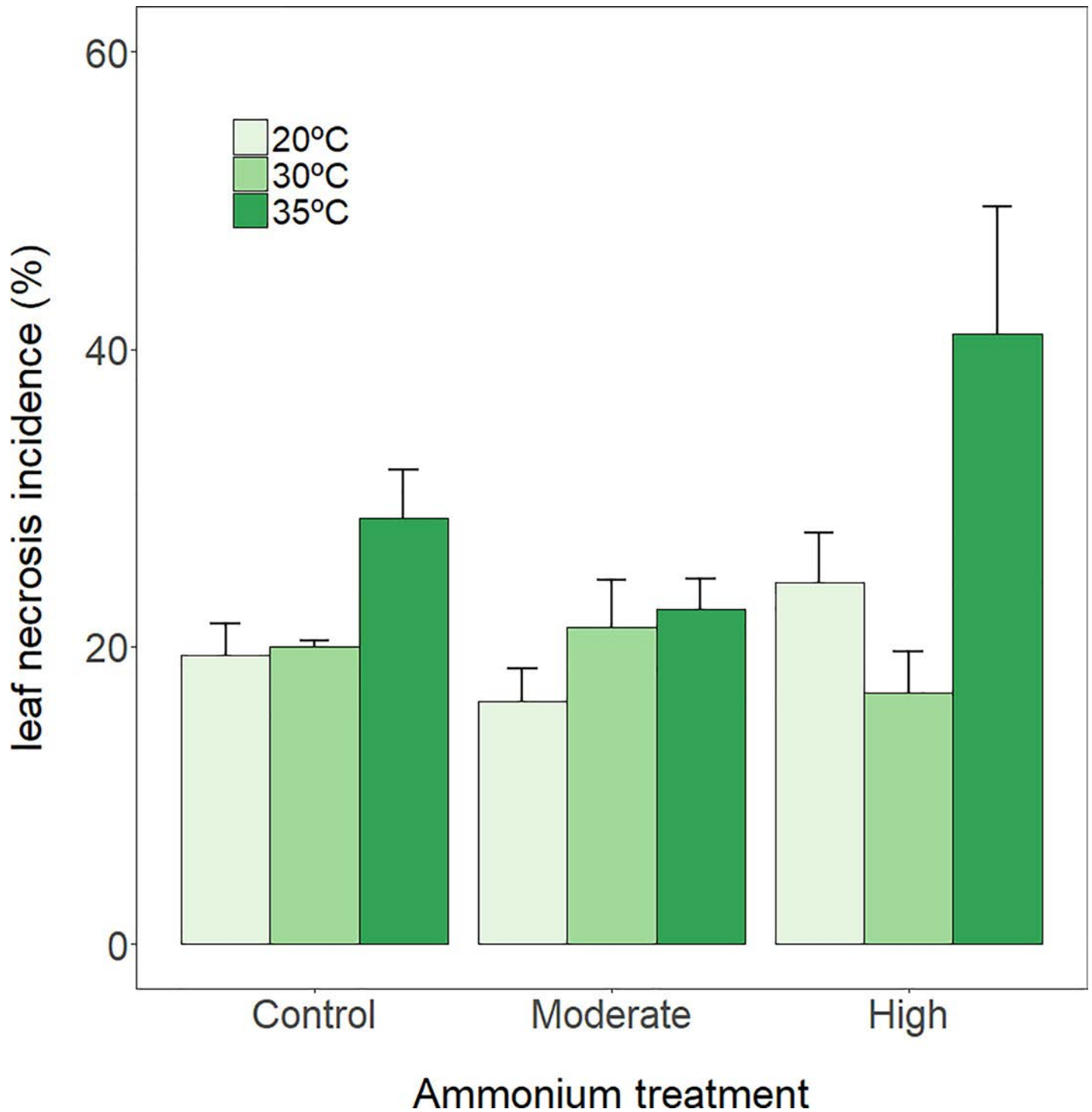


Fig 3. Leaf necrosis incidence. *P. oceanica* leaf necrosis incidence (mean \pm SE, $n = 3$) at three thermal treatments and at three ammonium concentrations, after 7 days of exposure.

<https://doi.org/10.1371/journal.pone.0222798.g003>

ammonium (Fig 4, Table 2). However, these overall effects are misleading, since both stressors showed a clear synergistic interaction ($p = 0.0399$) that made their combined effect relatively complex. Thus, the positive effect of moderate ammonium concentrations on growth occurred

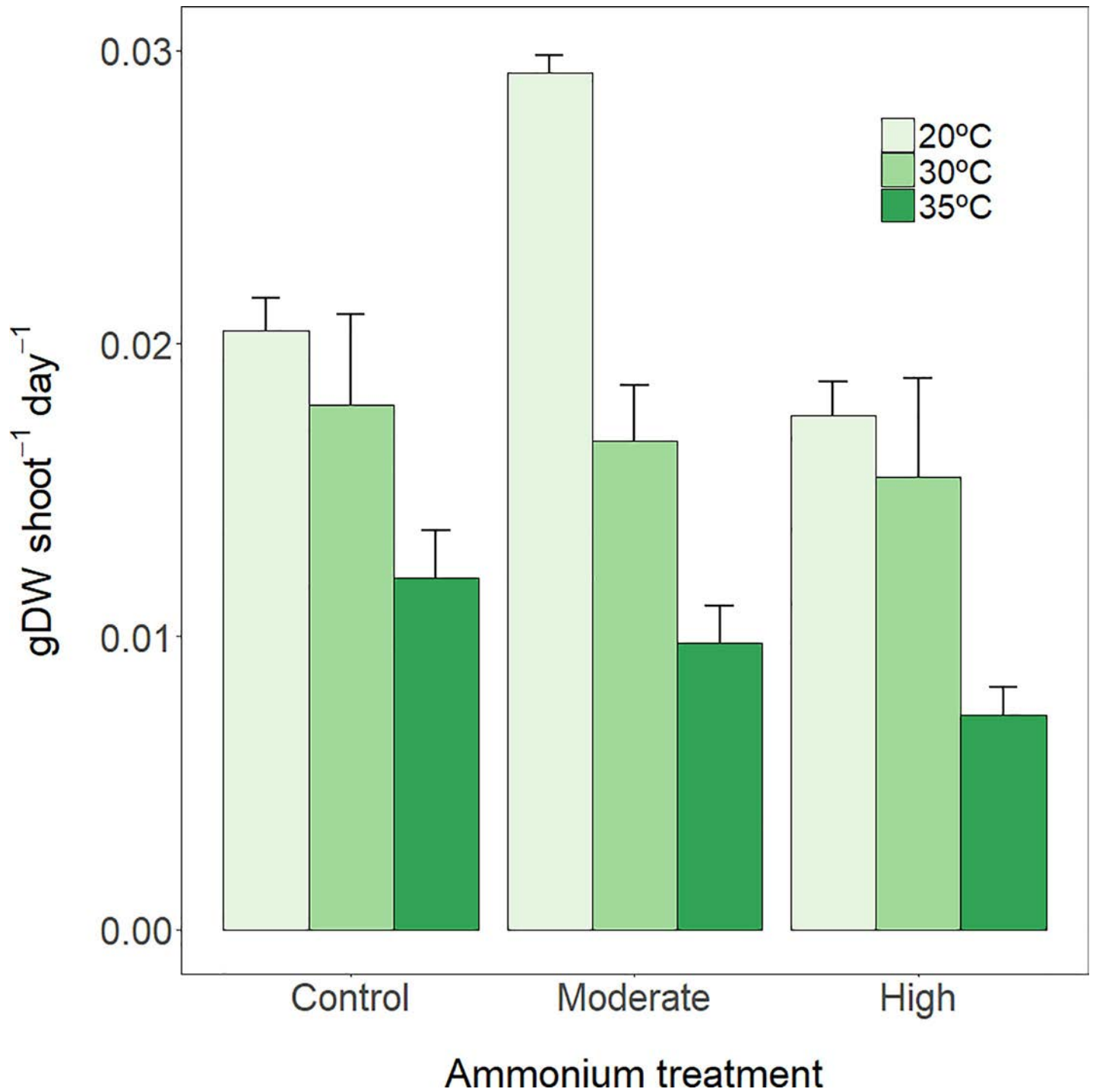


Fig 4. Shoot growth. *P. oceanica* shoot growth rate (mean \pm SE, $n = 3$) at three thermal treatments and at three ammonium concentrations, after 7 days of exposure.

<https://doi.org/10.1371/journal.pone.0222798.g004>

only at the control temperature, while it disappeared at 30°C and became negative at 35°C. Interestingly, the negative effects of extreme temperature (35°C) were considerably higher at the high ammonium concentration (65% growth rate reduction) than at the control ammonium concentration (40%).

Discussion

While warming has a clear negative effect on most of the variables measured, ammonium additions seem to exert only a moderate impact on plant performance when acting in isolation. However, we detected synergy between both factors in the response of two-three important plant traits, one related to the integrity of the photosynthetic system (maximum quantum yield), the second related to the capacity of the plant to activate photoprotective mechanisms (NPQ, only suggestive, as indicated based on p-value) and the third related to plant production (shoot growth rate), all of which are critical to plant survival. This serves as a warning that the impact of global warming on seagrass meadows already subject to eutrophication could be worse than expected.

A certain amount of interest lies in characterizing the thermal response of foundation species to warming. In the case of *P. oceanica*, such studies are relatively scarce (see below). In our case, based on the chlorophyll fluorescence responses and other plant traits, it would seem that *P. oceanica* tolerates short-term (i.e. one-week) temperature increases up to 30°C. This tolerance might be partially attributed to the plant's capacity to activate photoprotective mechanisms (e.g. associated with xanthophyll cycle pigments; [74,94,95]) at this temperature, as suggested by the increasing, albeit not statistically significant, NPQ trend (at 30°C). In addition, neither the necrosis incidence of leaves nor shoot growth were affected by 30°C, in line with the findings of previous studies [76], which would support its thermal tolerance to temperature increases up to 30°C.

By contrast, we observed negative changes in all variables measured at 35°C. Thus, the decrease in F_v/F_m and $\Delta F/F_m'$, at 35°C, indicates a severe reduction in the functionality of the photosynthetic apparatus [96]. At the same time, the electron transport chain and, therefore, the electron transport capacity (ETR_{max}) were severely affected by this high temperature, which could be attributed to a negative effect on the PSII donor side [97], as reported in previous studies (*Z. noltii*, [32]). This suggests that the heat dissipation pathway likely linked to the xanthophyll cycle found at 30°C seems to be inhibited when temperature reaches 35°C, as demonstrated by the drastic reduction in NPQ. This loss of capacity to dissipate the excess thermal energy could have induced damage to the PSII and consequently reduced the photosynthetic capacity of the plants [98]. Impairment of photosynthesis or a likely increase on respiration rates, are probably some of the causes behind the clear reduction in leaf growth that was observed, and certainly triggered other negative effects on plant fitness (reserve accumulation, rhizome growth and probably many others). Finally, the higher leaf necrosis incidence, which is a common plant response to several stressors, including salinity [56,99] and eutrophication [100,101], in plants exposed to 35°C indicates not only a loss of functionality of the photosynthetic systems, but also tissue damage and cell death.

In this regard, based on the thermal sensitivity of this species to high temperatures, as described above [19,77,102], our results and the findings of other studies [27,76,103,104], we suggest a thermal threshold for *P. oceanica* of between 30°C and 35°C.

Ammonium additions negatively and moderately affect most of the chlorophyll fluorescence-related variables measured ($\Delta F/F_m'$, ETR_{max} and NPQ), independently of temperature (see non-significant interactions in Table 2). No effect of ammonium was detected on F_v/F_m at control or moderately high temperatures (20°C and 30°C). In addition, we observed a positive effect of moderate ammonium addition on shoot growth at the control temperature, consistent with the nutrient-limited condition of this species [69,105]. Therefore, it would seem that the toxicity of ammonium in *P. oceanica* at basal temperatures is much lower than in other seagrass species, which are mostly colonizing and opportunistic (*sensu* [61]) species (*Z. noltii*; [53]; *Z. marina*; [47]). However, the most relevant finding of our experiment was that the negative effects of ammonium additions appear when temperature increases, thus leading to

interactive effects between both stressors. Thus, maximum quantum yield (F_v/F_m) was clearly affected by ammonium, but only at extreme temperatures (35°C), thereby indicating temperature-dependent ammonium toxicity. This toxicity is likely related to the damage of the photosynthetic machinery which, due to its inability to fix C, hindered the assimilation of ammonium in non-toxic forms [69,106]. In addition, our results suggest that the interaction between both stressors affected the plant's capacity to activate photoprotective mechanisms, as indicated by a lack of activation of NPQ mechanism at 30°C under high ammonium concentration. Our findings indicate that moderate ammonium additions stimulated shoot growth at control temperature while this stimulation was lost at 30°C and 35°C. Moreover, the thermal effects of extreme temperatures (35°C) were clearly worse at high ammonium concentrations, as growth rates in this treatments combination were 42% lower than those found at 35°C without ammonium addition.

Even though several studies in opportunistic species have revealed that the combined effects of temperature increase and ammonium are not detrimental (*Z. marina*, [44,60]; *C. nodosa*, [55]), or may even favour plant primary production (*C. nodosa*, [59]), our results indicated a negative synergistic effect between both stressors in *P. oceanica*, a species considered to be persistent, thus leading to the conclusion that the future impact of warming could be much worse for plants subject to high ammonium loading than for plants living in relatively pristine environments. These findings are consistent with a large number of studies, which have also reported synergistic effects between two simultaneous stressors on seagrasses [39,52,55,107]. However, most of these studies have focused on colonizing and opportunistic seagrass species; further studies are therefore required to shed light on the response of this, and other, persistent seagrass species to simultaneous exposure to two or more stressors.

As highlighted in the introduction, exploring the effects of climate change on coastal ecosystems already threatened by local factors is critical to determining and understanding the future of such ecosystems. Performing factorial experiments, which allow two or more stressors to be combined simultaneously with a view to exploring plant response, could help predict future scenarios.

Although some caution should be exercised when scaling our results up to real-world ecosystems, mainly due to our limited spatial and temporal scales, it is clear that our findings serve as a warning not only about the effects of global warming, but also about the synergies between warming and other local stressors. The predicted rise in the frequency and intensity of heat waves in the Mediterranean Sea [6,66,108] may be tolerated by the plant in the short term, but as duration [27] and/or intensity increase, plant photosynthesis and growth will be curtailed and persistence will likely be compromised. Moreover, other stressors such as eutrophication, especially in persistent seagrass species such as *P. oceanica* living in oligotrophic environments, can worsen the negative effects of warming. Consequently, these heightened effects might threaten the survival of these important seagrass meadows [108].

Although this research is not fully conclusive, and more extensive experiments, in the field whenever possible, are needed for a proper upscaling to the real world, our results clearly indicate a need to broaden the focus to include the potential interaction with other stressors when attempting to assess the effects of global warming. This is required not only to obtain more accurate, reliable and realistic predictions and therefore aid adaptive management, but also to act against global stressors at local level. In effect, attenuating local stressors may represent one way to alleviate the effects of global warming, or at least ensure they do not worsen.

Acknowledgments

We thank Neus Sanmartí for her help in the plant sampling necessary for conducting this experiment, and Rocío García Muñoz for her help in the temperature data analysis and

graphics. This work was supported by the European Union and the Spanish Government through the RECCAM (Seagrass meadows resilience to global warming: an analysis based on responses at ecophysiological, population and ecosystem levels, CTM2013-48027-C3-1-R and CTM2013-48027-C3-2-R) and UMBRAL (Responses of benthic marine vegetation to stress: critical transitions, resilience, and management opportunities, CTM2017-86695-C3-1-R) projects; and by the Spanish Ministry of Economy and Competitiveness (via grant BES-2014-069593 awarded to Y. Ontoria).

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Everyone is a genius.

*But if you judge a fish by its ability to climb a tree,
it will live its whole life believing that it is stupid.*

Albert Einstein

