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Paula Soledad María Celis Plá

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Vulnerability and acclimation of Mediterranean Sea macroalgae, to environmental stress related to climate change: use of indicators physiological state

Memòria presentada per **Paula Soledad María Celis Plá** per optar al títol de doctor per la Universitat de Barcelona

Dr. Félix Diego López Figueroa
Director de la tesi

Dr. Nathalie Korbee Peinado
Directora de la tesi

Paula S. M. Celis Plá
Doctorand

Dra. Amélia Gómez Garreta
Tutora de la tesi

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A Andrés…

A BILL OF RIGHTS FOR FUTURE GENERATIONS

"Why should we preserve a livable planet if not for our children and grandchildren?"

Jacques-Yves Cousteau

THE GENERAL ASSEMBLY,

MINDFUL of the determination proclaimed by the peoples of the world in the Charter of the United Nations to reaffirm faith in the dignity and worth of the human person and to promote social progress and better standards of life in larger freedom,

ACKNOWLEDGING that it is among the purposes of the United Nations to achieve international cooperation in solving international problems and to be a center for harmonizing the actions of nations in the attainment of these common ends,

RECOGNIZING that for the first time in history the rights of future generations to exercise options with respect to the nurture and continuity of life and the enrichment and diversity of their mental and physical environment are seriously threatened,

BELIEVING that the preservation and promotion of these rights has a claim on the conscience of all peoples and all nations,

CONVINCED that each generation has the inherent right to determine its own destiny and the corresponding responsibility to accord a similar right to future generations as an extension of the right of the living,

SOLEMNLY PROCLAIMS the necessity of securing the universal recognition of this right and this responsibility; and to this end.

DECLARES THAT:

Article 1. Future generations have a right to an uncontaminated and undamaged Earth and to its enjoyment as the ground of human history, of culture, and of the social bonds that make each generation and individual a member of one human family.

Article 2. Each generation, sharing in the estate and heritage of the Earth, has a duty as trustee for future generations to prevent irreversible and irreparable harm to life on Earth and to human freedom and dignity.

Article 3. It is, therefore, the paramount responsibility of each generation to maintain a constantly vigilant and prudential assessment of technological disturbances and modifications adversely affecting life on Earth, the balance of nature, and the evolution of mankind in order to protect the rights of future generations.

Article 4. All appropriate measures, including education, research, and legislation, shall be taken to guarantee these rights and to ensure that they not be sacrificed for present expediencies and conveniences.

Article 5. Governments, non-governmental organizations, and the individuals are urged, therefore, imaginatively to implement these principles, as if in the very presence of those future generations whose rights we seek to establish and perpetuate.

Jacques-Yves Cousteau (June, 1979)

CONTENTS

List of abbreviations

A absorptance

C internal carbon

Chl*a* chlorophyll *a*

C:N ratio between internal C and N

Chl*c* chlorophyll *c*

Cu copper concentration

Cu_T total copper concentration

DMF dimethylformamide

DPPH 2,2-diphenil-1-picrylhydrazyl

DW dry weight

E Irradiance

EC_{50} concentration required to obtain a 50% antioxidant effect

EDTA Ethylene diamine tetra-acetic acid

Ek_{ETR} saturation irradiance for ETR

Ek_{NPQ} saturation irradiance for NPQ

ETR Electron Transport Rate (through PSII)

ETR_{max} maximum electron transport rate (maximal photosynthetic capacity)

F steady-state fluorescence in light

F'_m maximum fluorescence yield (light adapted)

F'_m-F/F'm ($\Delta F/ F'_m$) effective quantum yield

F_m maximum fluorescence yield (dark adapted)

F_o minimum fluorescence yield

Fx Fucoxanthin

F_v maximum variable fluorescence yield

F_v/F_m maximum quantum yield

FW fresh weight

GES Good Environmental Status

GHG greenhouse gas

HPLC high-performance liquid chromatography

K_d attenuation coefficient

MAAs mycosporine-like amino acids

MSFD Marine Strategy Framework Directive

N internal nitrogen

NPQ non-photochemical quenching

NPQ_{max} maximum non-photochemical quenching

PAM Pulse Amplitude Modulated

PAR Photosynthetic Active Radiation (400-700 nm)

PC phenolic compounds

PC_W phenolic compound in the water

PCO Principal Component Analysis

PSII photosystem II

RLC rapid light curve

SD standard deviation

SE standard error

SST Sea Surface Temperature

UVA ultraviolet A (320-400 nm)

UVB ultraviolet B (280-320 nm)

UVR ultraviolet radiation (280-400 nm)

WFD Water Framework Directive

Y(NO) quantum yield of non-regulated non-photochemical energy loss in PSII

Y(NPQ) quantum yield of regulated non-photochemical energy loss in PSII

α_{ETR} initial slope of the ETR *vs*. Irradiance curve (photosynthetic efficiency)

α_{NPQ} initial slope of the NPQ *vs*. Irradiance curve

Resumen

Cabo de Gata-Níjar, Natural Park. September 2013. Photograph by Paula S. M. Celis Plá

En esta tesis, se evaluó la vulnerabilidad y la capacidad de aclimatación al estrés ambiental, relacionada al cambio climático, especialmente en *Cystoseira tamariscifolia (Phaeophyceae)*, y en otras especies de algas pardas *Cystoseira compressa* y *Padina pavonica* y algas rojas *Ellisolandia elongata*. Se estudiaron los efectos de los factores de estrés físico, como la irradiancia elevada de PAR (λ = 400-700 nm) y UVR (λ = 280-400 nm), la temperatura y factores de estrés químico, como nutrientes, metales pesados y CO_2. Se realizaron seis estudios experimentales variando irradiancia, temperatura, condiciones de nutrientes, acidificación y metales pesados. El enfoque común en estos estudios fue el uso bioindicadores funcionales para evaluar el estado fisiológico de estas especies de macroalgas en el Mar Mediterráneo (Mar de Alborán). Estos estudios se realizaron *in situ* en aguas ultra-oligotróficas (Parque Natural de Cabo de Gata-Níjar, Almería), aguas oligotróficas (Playa de La Araña, Málaga) y con algas transportadas a los sistemas de mesocosmos en condiciones controladas al aire libre. Además, se realizó un estudio experimental en el laboratorio con la macroalga *C. tamariscifolia*, recolectada en el Océano Atlántico Norte, en el límite norte de distribución de esta especie.

En un estudio temporal realizado en durante dos años seguidos, en las aguas costeras oligotróficas de Málaga hemos observado que *C. tamariscifolia* presentó la mayor producción fotosintética y menor vulnerabilidad en primavera respecto al resto de las estaciones del año debido a las condiciones más favorables en temperatura y nutrientes en primavera. Sin embargo, también se observó que las algas en verano pueden aclimatarse eficazmente al aumento de las condiciones de estrés, dado que se registró un aumento en actividad antioxidante. Por otra parte, la vulnerabilidad y la capacidad de aclimatación de *C. tamariscifolia* se evaluó en condiciones experimentales al aire libre, en algas recolectadas en La Playa de La Araña (Málaga), durante dos periodos del año (verano e invierno) y en dos lugares de recolección diferentes; costas rocosas (algas con fases de emersión durante el ciclo diario) y pozas intermareales (algas siempre sumergidas durante el ciclo diario). La actividad fotosintética en el invierno, estimada mediante la transporte de electrones (ETR_{max}), en el momento inicial (antes de la incubación) fue mayor en las algas que creen rocas costeras que en las pozas intermareales El ETR_{max} en algas recolectadas en pozas intermareales fue mayor en verano que en invierno. Por el contrario, el rendimiento máximo cuántico (F_v/F_m), indicador de la fotoinhibición fue menor en el verano, respecto a la época de invierno en las algas recolectada de rocas o pozas intermareales. Estas respuestas, indican la existencia de fotoinhibición en *C. tamariscifolia* en verano y una alta vulnerabilidad respecto a las algas

en el período de invierno, como se observó en el estudio de temporal realizado en el campo. La producción fotosintética estimada como ETR_{max}, fue más alta en las algas recolectadas del intermareal rocoso que la de las pozas intermareales. Esto puede estar relacionado con el uso del CO_2 del aire durante los periodos de emersión y una mayor incorporación de nitrato durante la rehidratación en las algas de intermareal rocoso comparado con algas que crece en micro hábitats de pozas (siempre sumergido). Además, del ETR_{max} obtenido a partir de la función ETR-irradiancia de curvas rápidas de luz, se determinó el ETR durante el ciclo diario bajo radiación solar (ETR *in situ*). El ETR *in situ* fue mayor en verano que en el invierno, tanto en las algas del intermareal rocoso como de las recolectadas de pozas intermareales. En general, el ETR *in situ* bajo radiación solar fue mayor que el ETR_{max} obtenido de las curvas rápidas de luz. Los datos *in situ* explicaron mejor el estado fisiológico de ETR_{max} ya que, el ETR *in situ* representa una producción real mientras que ETR_{max} se relaciona con una producción potencial. El contenido de nitrógeno interno y compuestos fenólicos fueron mayores en las algas recogidas en invierno que en verano, sin embargo, la actividad antioxidante fue mayor en la estación verano. La disminución de compuestos fenólicos en la estación de verano, puede estar relacionada con una mayor liberación al agua, un proceso que tiene una función fotoprotectora, así como también para esta estación del año no se descarta la acumulación de otros compuestos fotoprotectores como los carotenoides.

La capacidad de fotoaclimatación en el corto plazo a la radiación natural, fue evaluada a través del trasplante en la estación de verano en el Parque Natural del Cabo de Gata-Níjar (aguas ultraoligotróficas) mediante algas recogidas desde 0,5 y 2,0 m de profundidad y luego incubadas en aguas superficiales, al 100% y al 70% de la irradiancia en superficie mediante el uso de filtros neutros. En este estudio se utilizaron las macroalgas; *Cystoseira tamariscifolia* y el alga roja calcárea, *Ellisolandia elongata*. Con el objetivo de probar los efectos interactivos entre los factores de estrés radiación, nutrientes y las dos profundidades de origen de ambas algas. La actividad fotosintética (es decir ETR_{max}), en *C. tamariscifolia* proveniente de algas profundidades (0,5 y 2,0 m), fue mayor en condiciones de sombra y sin enriquecimiento de nutrientes. Estas diferencias podrían explicarse por la alta penetración de la radiación UV (alta transparencia del agua, provocando fotoinhibición en las algas de superficie). En cambio en *E. elongata*, la actividad fotosintética (ETR_{max}), fue también mayor en condiciones de sombra, pero con enriquecimiento de nutrientes. También se observó, que las algas provenientes de la profundidad mayor (2,0 m) en ambas condiciones de radiación, fueron

menos vulnerables que las algas recogidas en aguas superficiales (0,5 m) y condiciones de radiación completa (100%). El F_v/F_m como se esperaba, fue mayor en las algas sombreadas (70% de la radiación competa), que en las algas de radiación completa, en condiciones de no enriquecimiento de nutrientes. El elevado amortiguamiento no fotoquímico (NPQ_{max}) indica una alta capacidad de fotoprotección. El patrón de algas tipo-Sol (fotófilas) se mostró en las algas trasplantadas desde 2,0 a 0,5 m: aumento de la ETR_{max} y la Ek (intensidad de saturación) y disminución de la eficiencia (α_{ETR}). El contenido interno de nitrógeno, como indicador del estado nutricional en *C. tamariscifolia*, fue mayor en algas recogidas en aguas superficiales con enriquecimiento de nutrientes. Se observó una rápida respuesta al enriquecimiento de nutrientes, y la fotoprotección en *C. tamariscifolia* ya que el contenido de compuestos fenólicos incrementó rápidamente en algas recolectadas a 0,5 m bajo condiciones de enriquecimiento de nutrientes pero independientemente de las condiciones lumínicas de incubación. En contraste, en algas recolectadas en 2,0 m de profundidad, el contenido de fotoprotectores fue mayor en condiciones de no enriquecimiento de nutrientes y también independientes de las condiciones lumínicas durante la incubación. Esto podría explicarse por la alta radiación en aguas poco profundas (0,5 m), ya que, los compuestos fenólicos se pueden liberar como una prevención ante el daño solar, como una estrategia de fotoprotección. El contenido de carotenoides fue menos influenciado por la radiación o los nutrientes a excepción, del carotenoide; violaxantina, el cual fue mayor, después del enriquecimiento de nutrientes. En *C. tamariscifolia*, el contenido de violaxantina fue mayor en las condiciones de radiación completa y en aguas más profundas (2,0 m). Sin embargo, anteraxantina y β-caroteno fueron significativamente afectados por la interacción de factores, radiación y nutrientes.

En este estudio, se demuestra que los indicadores funcionales, (1) fisiológicos; ETR_{max}, F_v/F_m y NPQ_{max}, (2) el indicador nutricional (C: N) y (3) el indicador bioquímico del estrés en *C. tamariscifolia* recolectada en el límite norte de distribución geográfica (sur de Inglaterra, Reino Unido) fueron sensibles a las variaciones de cobre y nitrato en el agua, respondiendo con efectos interactivos. *C. tamariscifolia* presentó una alta resistencia al cobre, sin efectos aparentes de daño después de 14 días de cultivo, incluso a 2,0 μM CU_T de concentración de cobre. Encontramos una mayor concentración de cobre interno, en los tratamientos de exposición a altas concentraciones de cobre, independiente de las condiciones de nitrato. Los fenoles fueron liberados principalmente en los altos niveles de cobre y en condiciones de enriquecimiento de nitrato, mostrando una

correlación positiva con la actividad antioxidante. Por lo tanto, la mayor actividad antioxidante se produce en el tratamiento de estrés más alto es decir, alta concentración de Cu combinado con altos niveles de nitrato. La Clorofila *a* y *c*, así como también la fucoxantina, fueron mayores en altos niveles cobre, independiente de las condiciones de nutrientes. En general, al final del período experimental, los niveles de cobre medidos parecen ser más favorables fisiológicamente en *C. tamariscifolia* a bajos niveles de nitrato. En altos niveles de cobre con altos niveles de nitratos, el estado fisiológico en *C. tamariscifolia* también fue favorable. Estos resultados sugieren que la interacción entre el cobre y nitrato puede dar una alta resistencia al cobre en el alga *C. tamariscifolia* sin efectos de daño aparente después de 14 días de cultivo.

En la gradiente natural de pH en el Mar Mediterráneo (Isla de Vulcano, Italia), se estudiaron los efectos interactivos entre la radiación solar, el CO_2 y los niveles de nutrientes en el alga parda no calcárea; *Cystoseira compressa* y el alga parda calcárea; *Padina pavonica*. Tanto *C. compressa* y *P. pavonica* fueron fisiológicamente beneficiadas por el aumento de DIC en el agua (carbono inorgánico disuelto). Se observó que la magnitud de la respuesta de estas macroalgas depende de la disponibilidad de nutrientes y de la radiación. En *C. compressa* y *P. pavonica*, la actividad fotosintética (ETR_{max}) fue mayor en condiciones de CO_2 ambiental, expuestas a radiación total y en con las condiciones naturales de nutrientes (niveles bajos). El rendimiento cuántico máximo (F_v/F_m), en *C. compressa* fue mayor en alto CO_2, sombreado de la radiación solar y agua enriquecida con nutrientes. En contraste, en *P. pavonica* el rendimiento máximo fue mayor en niveles naturales de nutrientes. En *C. compressa*, en los tratamientos de alto CO_2, el contenido interno de carbono y la actividad antioxidante fueron mayores en tratamientos de menor radiación, con y sin enriquecimiento de nutrientes. Las concentraciones de Clorofila *a*, fenoles y fucoxantina fueron mayores en condiciones de enriquecimiento de nutrientes. Sin embargo, el rendimiento máximo (F_v/F_m) y la eficiencia fotosintética (α_{ETR}) fueron mayores, sin enriquecimiento de nutrientes. En *P. pavonica*, observamos que en los tratamientos de concentraciones altas de CO_2, se registró un mayor contenido de carbono interno, F_v/F_m, α_{ETR} y Clorofila *a* independientemente de los niveles de nutrientes, así como también se observaron mayores concentraciones de compuestos fenólicos en condiciones de nutrientes enriquecidos y con radiación solar completa. Por otra parte, la actividad antioxidante fue mayor en condiciones de sombra y en tratamientos de enriquecimiento de nutrientes. El contenido interno de nitrógeno, en *C. compressa* fue mayor en incubaciones a altas concentraciones

de CO_2, con baja radiación solar y con nutrientes. En contraste, en *P. pavonica* el contenido interno de nitrógeno fue mayor en condiciones ambientales de CO_2, con menor radiación solar y condiciones de enriquecimiento de nutrientes.

El contenido de nitrógeno interno, aumentó significativamente en los tratamientos de incremento de nutrientes, lo que confirma que estas algas necesitan de nutrientes, ya que viven en aguas oligotróficas del Mar Mediterráneo. En *C. compressa* se registró un aumento de los compuestos fenólicos en condiciones de incremento de los niveles de CO_2 en aguas enriquecida con nutrientes, independiente de las condiciones de luz.

El incremento de temperatura (+4°C) provocó una disminución de los compuestos fenólicos sin embargo, sólo en las algas que crecen bajo condiciones de CO_2 ambiental. Por lo tanto, el nivel interno de fenoles se mantuvo constante con el aumento de la temperatura bajo condiciones de acidificación, lo que permitió mantener la capacidad antioxidante. En *C. compressa* y *P. pavonica*, la actividad antioxidante (es decir EC_{50}) se vieron afectados por las interacciones entre los niveles de luz y CO_2. EC_{50} tendió a ser mayor en tratamientos de menor radiación y altas concentraciones de CO_2, con y sin adición de nutrientes, lo que sugiere una correlación positiva con compuestos fenólicos y su uso como antioxidantes para prevenir el fotodaño. NPQ_{max}, la producción de fenoles y bajos EC_{50} indican que en condiciones elevadas de CO_2, las algas tendrán una alta capacidad de fotoprotección. Nuestros hallazgos refuerzan la evidencia que las algas pardas podrían proliferar en las condiciones ambientales previstas en el futuro de acurdo a los modelos de cambio climático, acidificación del océano pero si las condiciones físico-químicas lo permiten es especialmente en lo relativo a los niveles de nutrientes y la radiación solar.

También se estudiaron los efectos de la temperatura y el CO_2 en dos poblaciones de *Cystoseira tamariscifolia* recogidas de aguas ultraoligotróficas y oligotróficas, en sistemas de experimentación al aire libre bajo condiciones controladas, con el objetivo de evaluar los efectos interactivos de la temperatura y los niveles de CO_2 en condiciones que simulan los escenarios futuros asicados al cambio climático. La actividad fotosintética (ETR_{max}) fue mayor en los niveles elevados de CO_2 en algas recogidas de ambos lugares. Después del período experimental (28d), el rendimiento cuántico máximo (F_v/F_m) fue mayor en *C. tamariscifolia* de aguas ultraoligotróficas con tratamientos de incremento de temperatura (+4ºC) y niveles de CO_2 ambiental. Por el contrario, en algas recolectadas en aguas oligotróficas, el F_v/F_m fue mayor en los tratamientos de altos niveles de CO_2 y temperatura ambiental. Por lo tanto, el efecto interactivo de la temperatura y el CO_2 fue

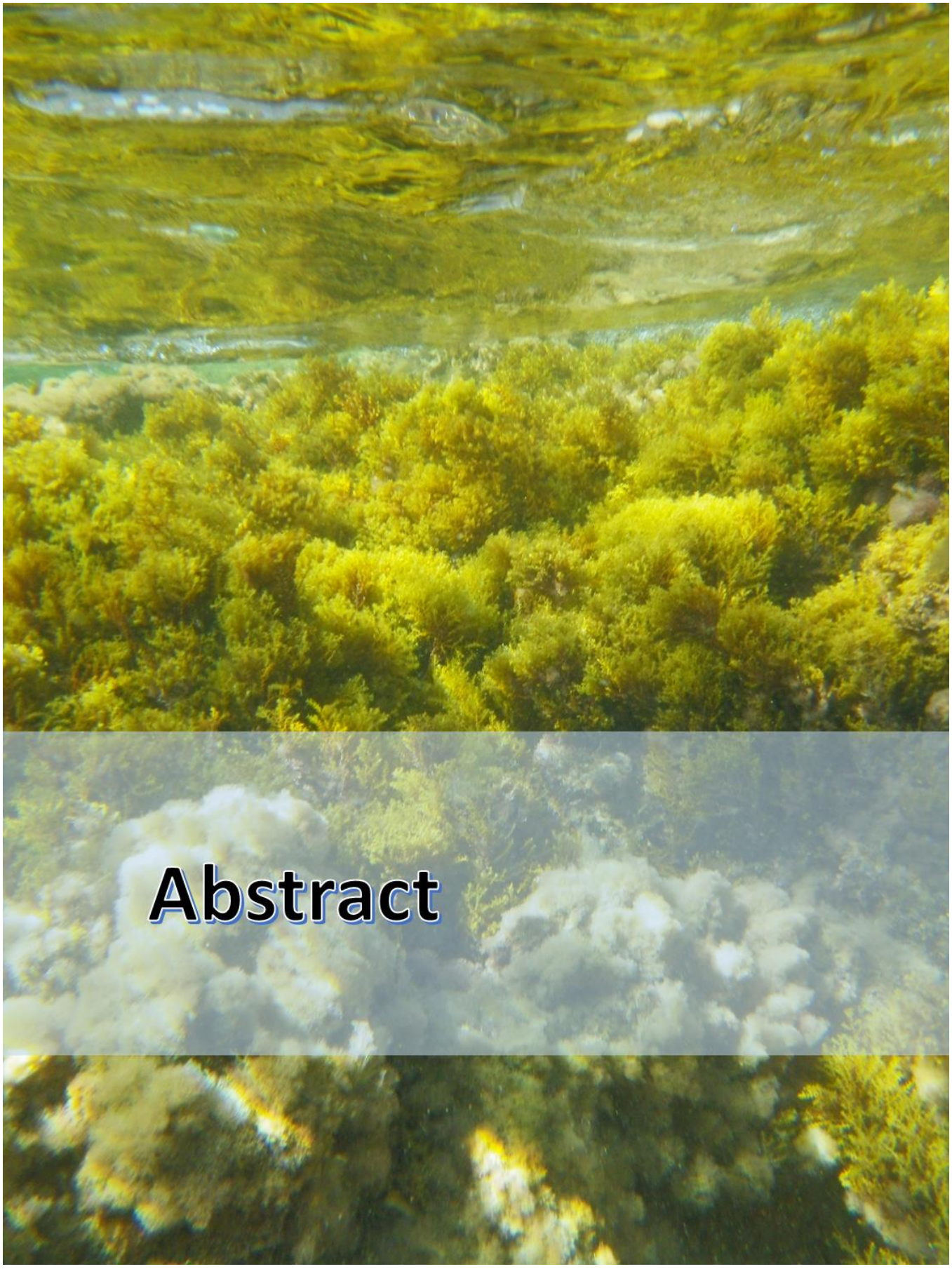
diferente para algas recogidas bajo diferentes tratamientos de radiación y condiciones de nutrientes. No sabemos si *C. tamariscifolia* de ambos sitios corresponden a diferentes ecotipos, pero al menos los resultados indican la importancia de la historia lumínica y nutritiva de las macroalgas en respuestas a los factores del cambio climático. Por otra parte, el contenido interno de nitrógeno y carbono fue mayor en condiciones de altos niveles de CO_2 y la temperatura ambiente independiente del lugar de recolección de las algas; esto indica una menor vulnerabilidad y un buen estado fisiológico en esas condiciones.

Se encontraron beneficios del aumento del DIC (carbono inorgánico disuelto) en el incremento del crecimiento de *C. tamariscifolia*, siendo las respuestas fisiológicas más aceleradas en algas provenientes de ambientes ultraoligotróficos que de algas provenientes de ambientes oligotróficos. El aumento de la temperatura, tiene un efecto negativo sobre la tasa de crecimiento de las algas de aguas oligotróficas. Aunque la biomasa en *C. tamariscifolia* aumentó en condiciones elevadas de CO_2; la biomasa de algas recogida de aguas ultraoligotróficas fue mayor que las algas provenientes de aguas oligotróficas. Esto sugiere que hay efectos interactivos entre el origen de las poblaciones y las condiciones de CO_2. Se propone que las algas recolectadas en aguas ultraoligotróficas puedan aprovechar el carbono del sistema acuático de forma más eficiente que las algas de aguas oligotróficas cuando los nutrientes no se limitan en extremo. Los compuestos fenólicos fueron mayores en las algas provenientes de aguas ultraoligotróficas con altos niveles de CO_2 que en las algas provenientes de aguas oligotróficas con altos niveles de CO_2 y temperatura ambiental. La mayor concentración de compuestos fenólicos en las algas de las aguas ultraoligotróficas, puede estar relacionado con la fotoaclimatación a altos niveles de radiación solar en aguas costeras con alta transparencia. Una correlación positiva entre los compuestos fenólicos y actividad antioxidante expresada como EC_{50} indica que los compuestos fenólicos pueden prevenir el fotodaño. En las algas provenientes de aguas oligotróficas los niveles de fenoles después de 28 d de incubación, fueron menores, pero la concentración de carotenoides fue mayor en las algas provenientes de ambientes oligotróficos, lo que sugiere una mayor importancia de los carotenoides en la fotoprotección de las algas crecidas en ambientes oligotróficos. Como se sugirió anteriormente, las algas que crecen bajo condiciones de mayor estrés, es decir en aguas ultraoligotróficas parecen tener respuestas fisiológicas más amplificadas, respecto a las variaciones de las variables físico-químicas que las que crecen en aguas oligotróficas.

En este estudio, la disminución del rendimiento cuántico máximo y la tasa máxima de transporte de electrones, el aumento de compuestos fenólicos y de la actividad antioxidante o el aumento de la relación C: N se produce en condiciones de estrés y por lo tanto, se validan como indicadores de estrés. Además, es posible evaluar la dirección de la respuesta fisiológica es decir, positiva o negativa ante los cambios esperados, como factores de cambio climático u otros impactos antropogénicos; como la eutrofización (aumento de los niveles de nitrato en la columna de agua) o la contaminación por metales pesados. Sin embargo, por otro lado, el aumento de los compuestos fenólicos se produce también en condiciones favorables (expresada con altas tasas fotosintéticas) y que muestra una relación con la actividad antioxidante y la producción algal. Este no es un resultado extraño ya que una alta actividad fotosintética se relaciona con una producción alta de oxígeno lo que puede producir estrés oxidativo. La disipación no fotoquímica, el consumo de oxígeno a través de la reacción Mehler y el incremento de la actividad antioxidante, son mecanismos de fotoprotección que permite sobrevivir a *C. tamariscifolia* en condiciones de alta producción de oxígeno. La acumulación de compuestos fenólicos en condiciones de aumento de los niveles de nitrato y de CO_2 o la liberación de fenoles bajo mayor radiación solar en *C. tamariscifolia*, nos muestra que esta especie tiene mecanismos bioquímicos eficaces para aclimatarse a las variaciones esperadas en los factores del cambio climático aunque esté limitado por la temperatura.

Los compuestos fenólicos están relacionados con el metabolismo secundario, pero en *C. tamariscifolia*, la relación directa y positiva que se encuentran con la actividad fotosintética y nitrógeno interna en todos los experimentos parece vincularlos con el metabolismo primario. En resumen, el aumento de los niveles de CO_2 y la alta radiación solar, pero sin llegar a condiciones de fotoinhibición, serán condiciones favorables para el crecimiento y fisiología de esta especie de alga. El enriquecimiento de nitrato, reduce el estrés provocado por el exceso de radiación solar o contaminación por cobre, debido a que los mecanismos de fotoprotección resultan favorecidos por el aumento de nitrato. Sin embargo, el efecto positivo de CO_2 y nitrato depende de la temperatura, la temperatura de verano en el campo o 4°C de aumento de la temperatura en los experimentos en mesocosmos al aire libre provocaron un estrés fisiológico. En consecuencia, la acidificación del océano será más favorable para *C. tamariscifolia*, sólo en condiciones que no impliquen un aumento alto de temperatura (4ºC) y sin limitación de nutrientes. La oligotroficación que se observa en determinadas zonas del mar Mediterráneo sería desfavorable para las comunidades *Cystoseira tamariscifolia* en un escenario de cambio

climático. Los datos sobre la vulnerabilidad y la aclimatación a factores de cambio climático de *Cystoseira tamariscifolia*, *Ellisolandia elongata, Cystoseira compressa* y *Padina pavonica* presentes en este estudio, pueden ayudar a la gestión de las comunidades de macroalgas, principalmente en las áreas protegidas. Además, los datos fisiológicos y bioquímicos ayudarán a predecir los efectos del cambio climático sobre los compuestos bioactivos con capacidad antioxidante y su potencial biotecnológico; como los compuestos fenólicos, aminoácidos tipo micosporinas y carotenoides.



Abstract

Cabo de Gata-Níjar, Natural Park. September 2013. Photograph by Paula S. M. Celis Plá

In this thesis, the vulnerability and capacity of acclimation to environmental stress related to Climate change mainly in *Cystoseira tamariscifolia* and other species as *Ellisolandia elongata, Cystoseira compressa* and *Padina pavonic*a are evaluated. The effects of physical stressors as elevated irradiance of PAR (λ=400-700 nm) and UVR (λ=280-400 nm) and temperature and chemical stressors as nutrient, heavy metals and CO_2, separately and in interaction with different levels were evaluated. Six experimental studies were conducted under varying irradiance, temperature, nutrient conditions, acidification and heavy metals. The common approach in these studies was the use functional bioindicators to evaluate the physiological state macroalgal species of Mediterranean Sea (Alboran Sea) in studies conducted both *in situ* in ultra-oligotrophic (Cabo de Gata-Níjar Natural Park, Almeria) and oligotrophic waters (La Araña beach, Malaga) and with algae transported to controlled experimental systems under *out-door* conditions. In addition, an indoor experiment study was performed in *Cystoseira tamariscifolia* collected in the North Atlantic Ocean, the northern limit of distribution of this species.

We observed in a seasonal study conducted in two subsequent years in oligotrophic coastal waters of Malaga that *C. tamariscifolia* presented the highest production and it was less vulnerable in spring than those in the rest seasons through the year due to irradiance that is more favorable, temperature and nutrient environmental conditions. However, we also observed that the algae in summer could efficiently acclimate to increased stress conditions by the increase its antioxidant activity. Then, the vulnerability and capacity of acclimation of *Cystoseira tamariscifolia* were evaluated in outdoor experimental conditions in algae collected in La Araña (Málaga) in two periods of the year (summer and winter) and from two sites , rocky shores (algae with emersion phases during the daily cycle) and rockpools (algae always immersed during the daily cycle). The photosynthetic activity estimated as electron transport rate (ETR_{max}) at initial time (before the incubation) was higher in rocky shore than in rockpool collected algae but only wintertime. In rockpool-collected algae, ETR_{max} was higher in summer than that in wintertime. In contrast, the maximal quantum yield as indicator of photoinhibition (F_v/F_m) was lower in summer time respect to the winter time in algae collected from both sites. These responses indicated photoinhibition for *C. tamariscifolia* in summer time and high vulnerability respect to the winter periods as it was suggested in the seasonal study in the field. The highest production (ETR_{max}) in rocky shore algae is related to the use of CO_2 from the air during emersion periods and higher nitrate incorporation during

rehydration after drying periods compared alga growing in rockpools (always immersed). In addition to ETR_{max} obtained from the fitting of ETR-irradiance function from rapid light curves, ETR was determined during the daily cycle under solar radiation (*in situ* ETR). In summer time, *in situ* ETR was higher in algae collected than that winter in both rocky shore and rockpools collected algae. *In situ* ETR under solar radiation was higher than ETR_{max} fitted form rapid light curves. The *in situ* data explained better the physiological state than ETR_{max} since *in situ* ETR is an actual production whereas ETR_{max} is a potential production. Internal nitrogen content and phenolic compounds were higher in algae collected in winter than in summer, however the highest antioxidant activity was reached in summer time. The decrease of phenols in summer time is related to its higher release to the water, a process with photoprotective role, but in addition, it is not discarded the accumulation of other photoprotector compounds as carotenoids.

The photoacclimation capacity in the short term to natural solar irradiance was evaluated by transplant approach in summer time in Cabo de Gata-Nijar (ultraoligotrophic waters) by using algae collected form 0.5 and 2.0 m and incubated in surface waters to 100% and 70% of surface irradiance that were covered with neutral mesh. In addition to *C. tamariscifolia*, the red calcareous macroalga, *Ellisolandia elongata,* was studied. In this experiment, we tested interactive effects between light, nutrient and two different depths for both algae. The photosynthetic activity (i.e. ETR_{max}) in *C. tamariscifolia* collected from both 0.5 and 2.5 depth waters was higher in shade conditions without nutrient enrichment. These differences could be explained by the high penetration of UV radiation (high water transparency provoking photoinhibition in algae of the surface waters). In contrast in *E. elongata,* the photosynthetic activity (i.e. ETR_{max}), was also higher in shade conditions but with nutrient enrichment. The vulnerability was lower in algae collected from 2.0 depth in different light conditions, respect to the algae collected from shallow waters and full light conditions. F_v/F_m as expected was higher shaded that in full light conditions but under non-enriched nutrient condition. The elevated NPQ_{max} indicates high photoprotection capacity. Sun type pattern is showed in transplanted algae from 2.0 to 0.5 m such as increase of ETR_{max} and Ek and decrease of $\alpha_{ETR.}$ The nitrogen internal content, as indicator of the nutritional status in *C. tamariscifolia* was highest in algae collected from shallow waters with nutrient enrichment. The response to nutrient enrichment was very rapid. The photoprotection in *C. tamariscifolia* by phenolic compounds was higher in shallow water (0.5 m) under nutrient enrichment conditions independent of the light. In contrast, in depth waters (2.0)

the photoprotectors content was higher in non-enrichment conditions independent of the light conditions. This could be explained because of the high irradiance in shallow waters, phenolic compounds can be released preventing the photodamage as a photoprotection strategy. The carotenoids were less influenced by irradiance or nutrients with the exception of violaxanthin that had higher content after nutrient enrichment. In *C. tamariscifolia,* the content of violaxanthin was higher in the simulated deeper irradiance. However, antheraxanthin and β-carotene were significantly affected by the interaction of factors, irradiance and nutrients.

In this study, it is shown that the functional indicators, (1) physiological ETR_{max}, F_v/F_m and NPQ_{max}, (2) nutritional indicator (C:N) and (3) biochemical indicator of stress in *C. tamariscifolia* collected in the northern geographical distribution (southern England, UK) were sensible to Copper and nitrate variations and they respond as interactive effect. The data show complex interaction effects between copper and nitrate on the ecophysiological variables. *C. tamariscifolia* presented a high resistance to copper with no apparent damage effects after 14 d culture even at 2.0 μM Cu_T. We found higher internal copper and in the water under exposed to high copper concentration, independent of the nutrient conditions. Phenols were released mainly in high copper levels and with nitrate enrichment conditions and positive correlation with antioxidant, activity was found. Thus, the highest antioxidant activity was produced in the highest stress treatment i.e. high Cu but also at high N levels. Chl*a*, *c* and fucoxanthin contents were higher in high coper levels independent of the nitrate conditions. In general, at the end of the experimental period, middle copper levels seems to be more favorable in low nitrate conditions, respect to the physiological status of *C. tamariscifolia*. In contrast, in high copper levels with high nitrate levels, the physiological status in *C. tamariscifolia* was also favorable. These results suggest that interaction between copper and nitrate can give a high resistance to copper of *C. tamariscifolia* with no apparent damage effects after 14 d culture.

In the natural pH gradient in the Mediterranean Sea (Vulcano Island, Italy), interactive effects between light, CO_2 and nutrient levels were studied *in Cystoseira compressa* and in the calcareous alga *Padina pavonica*. Both *C. compressa* and *P. pavonica* were benefited as physiological level by the increases of DIC (dissolved inorganic carbon), but the extent of the algal response, depends upon nutrient and light availability. The photosynthetic activity responses (i.e. ETR_{max}), in *C. compressa* and *P. pavonica* were higher in ambient CO_2 with full light and ambient nutrient conditions.

Maximal quantum yield (F_v/F_m), in *C. compressa* was higher in high CO_2, shaded with enriched nutrient conditions; in contrast, in *P. pavonica* was higher in non-enrichment levels. In *C. compressa*, elevated CO_2 treatments resulted in higher carbon content and antioxidant activity in shaded conditions both with and without nutrient enrichment they had more Chl*a*, phenols and fucoxanthin with nutrient enrichment and higher quantum yield (F_V/F_m) and photosynthetic efficiency (α_{ETR}) without nutrient enrichment. In *P. pavonica*, elevated CO_2 treatments had higher carbon content, F_V/F_m, α_{ETR}, and Chl*a* regardless of nutrient levels they had higher concentrations of phenolic compounds in nutrient enriched, fully lit conditions and more antioxidants in shaded, nutrient enriched conditions. The nitrogen internal content, in *C. compressa* was higher in high CO_2 in shaded and enrichment nutrient conditions; in contrast, in *P. pavonica* was higher in ambient CO_2 in shaded with nutrient levels. Nitrogen content increased significantly in fertilized treatments, confirming that these algae were nutrient limited in this oligotrophic part of the Mediterranean. The phenolic compounds increase in *C. compressa* in high CO_2 levels with nutrient enrichment independent of the light conditions.

The increased temperature (4^oC) provoked a decrease of polyphenols however only in algae grown under ambient CO_2 conditions. Thus, the internal level of phenols remained constant with the increasing temperature under acidification conditions allowing maintaining the antioxidant capacity. In *C. compressa* and *P. pavonica*, antioxidant activity and EC_{50} were affected by the interactions between light levels and CO_2. EC_{50} tended to be higher in shaded, high CO_2 treatments with and without nutrient addition, suggesting a positive correlation with phenolic compounds and their use as antioxidants to prevent photodamage. Together, NPQ_{max}, phenol production and EC_{50} indicate that in elevated CO_2 conditions some species will have a higher capacity for photoprotection. Our findings strengthen evidence that brown algae can be expected to proliferate as the oceans acidify where physicochemical conditions, such as nutrient levels and light, permit.

Temperature and CO_2-dependent processes in two populations of the *Cystoseira tamariscifolia* collected from ultraoligotrophic and oligotrophic waters were studied in out-door experimental system to assess the interactive effects of temperature and CO_2 expected levels in future scenarios. The photosynthetic activity (ETR_{max}) was higher in high CO_2 levels in algae collected from both sites. After experimental period (28d), the maximal quantum yield (F_v/F_m) was higher in *C. tamariscifolia* from ultraoligotrophic

waters under increased temperature (+4ºC) and ambient CO_2 levels. In contrast, for oligotrophic waters, F_v/F_m was higher in high CO_2 levels and ambient temperature. Thus, the interactive effect of temperature and CO_2 was different in algae collected under different light and nutrient history grown conditions. We do not know if *C. tamariscifolia* from the two sites correspond to different ecotypes but at least the results indicate the importance of light and nutrient history of the macroalgae in the responses to climate change factors. The nitrogen and carbon internal content for both sites was higher in high CO_2 levels and ambient temperature, this indicate less vulnerability for this treatments and good state for this macroalgae. We found benefits of DIC (dissolved inorganic carbon) increase on growth rate in *C. tamariscifolia* being the physiological responses more accelerated in ultraoligotrophic than in oligotrophic harvested algae. Temperature increased has negative effect on growth rate only in algae from oligotrophic waters. Although the biomass in both *C. tamariscifolia* increased in elevated CO_2 conditions, biomass of algae collected from ultraoligotrophic waters was higher than algae collected from oligotrophic waters. This suggest *C. tamariscifolia* had interactive effects between origin of populations and CO_2 conditions, probability that algae collected in ultraoligotrophic waters maybe can capitalize carbon of the aquatic system when the nutrient is not limited in extreme.

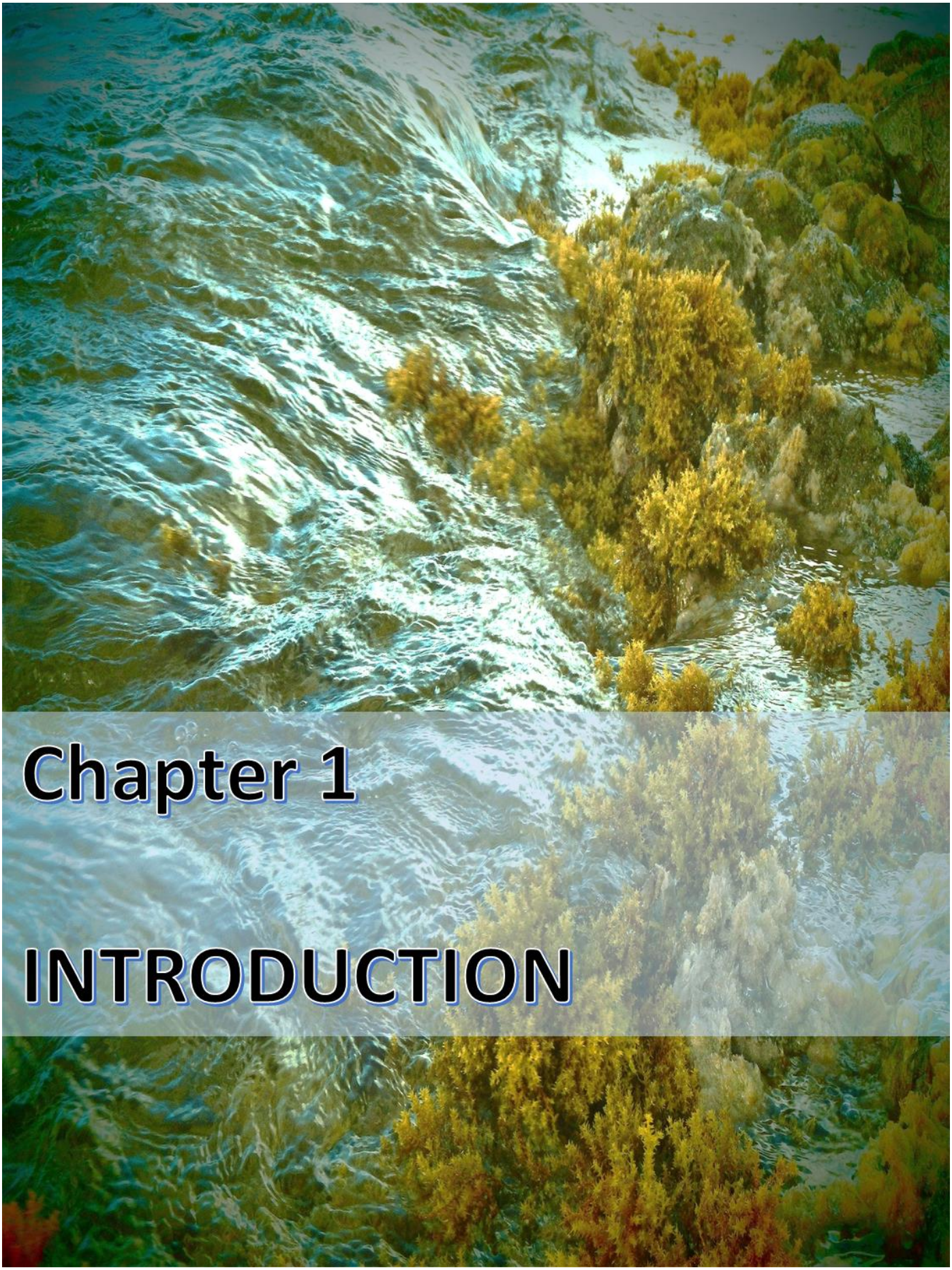
The phenolic compounds were higher in algae from ultraoligotrophic waters in high CO_2 waters than that form oligotrophic waters in high CO_2 waters in ambient temperature. The higher concentration of phenolic compounds in algae from ultraoligotrophic waters can be related to the photoacclimation to high irradiance levels in coastal waters with high transparency. A positive correlation between phenolic compounds and antioxidant activity expressed as EC_{50} indicates that phenolic compounds can prevent photodamage. In algae from oligotrophic waters the levels of phenols after 28 d incubation was lower, but carotenoids higher than that from oligotrophic grown algae suggesting more importance of carotenoid for photoprotection in oligotrophic grown algae. As it was suggested, the algae growing under more stress conditions i.e., ultraoligotrophic compared to oligotrophic waters seem to have more amplified physiological responses to the variations of physical chemical variables.

In this study, the decrease of maximal quantum yield and electron transport rate , the increase of phenolic compounds and antioxidant activity or the increase of C:N ratio are produced in stress conditions and thus they are validated as stress indicator. In addition, it is possible to evaluate the direction of the physiological response i.e. positive or

negative to expected changes under climate change factors or other anthropogenic impacts, as eutrophication (increased nitrate levels in the water column) or pollution by heavy metals. However, on the other hand, the increase of phenolic compounds is also produced under increased photosynthetic activity showing a link between antioxidant and algal production. This not a strange result since a high photosynthetic activity is related to a high oxygen production which can be produced oxidative stress. Non-photochemical quenching, oxygen consumption through Mehler reaction and increased antioxidant activities are down regulation mechanisms to survive under promoted oxygenic scenario.

Phenolic accumulation under increased nitrate and CO_2 levels or the release of phenols under increased irradiance in *C. tamariscifolia* shows us that this species has effective biochemical mechanisms to acclimate for the expected variations in climate change factors although this is limited by temperature. Phenolic compounds are related to secondary metabolism but in *C. tamariscifolia* but the direct positive relation found with photosynthetic activity and internal nitrogen in all experiments seem to link the phenols to primary metabolism. In summary, increased CO_2 under high irradiance, but not photoinhibitory, conditions will be favorable growth and physiological responses. The nitrate enrichment reduced stress provoked by irradiance or pollution by Cooper due to the photoprotection mechanisms are favored by nitrate increase. However, the positive effect of CO_2 and nitrate is dependent on temperature, summer temperature in the field or 4^oC increased temperature in outdoor experiments provoked physiological stress. Consequently, ocean acidification will be favorable for *C. tamariscifolia* only under no very high increase of temperature, less 2-3^oC and without nutrient limitation.

The oligotrophication produced in certain areas of Mediterranean Sea will be unfavorable for *Cystoseira tamariscifolia* communities in a climate change scenario. The data on vulnerability and acclimation to climate change factors of *Cystoseira tamariscifolia, Ellisolandia elongata* and *Padina pavonica* presented in this study can help the management of macroalgal communities, mainly in protected areas. In addition, the physiological and biochemical data will help to predict the effects of climate change on bioactive compounds with antioxidant capacity and their potential biotechnological uses as phenolic compounds, mycosporine like aminoacids and carotenoids.



Chapter 1

INTRODUCTION

Cabo de Gata-Níjar, Natural Park. September 2012. Photograph by Paula S. M. Celis Plá

1.1 Global scenarios

The Intergovernmental Panel on Climate Change (IPCC 2014) concluded as key findings in the synthesis the report as follows: (1) Human influence on the climate system is clear, (2) we are disrupting our climate, the most risks are severe and pervasive and they are producing irreversible impacts, and (3) we have to limit climate change and build a more prosperous environment.

Anthropogenic greenhouse gas (GHG) emissions since the pre-industrial era have driven large increases in the atmospheric concentrations of carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) (Figure 1.1). Since 1750, atmospheric CO_2 concentrations have increased from approximately 280 to 400 ppm in 2014. By the end of 2100, atmospheric CO_2 concentrations are expected to be between 800-1000 ppm (IPCC 2014), according to the 'business-as-usual' CO_2 emission scenario (Brewer 1997, Caldeira and Wickett 2003). The ocean has absorbed about 30% of the emitted anthropogenic CO_2, causing ocean acidification. About half of the anthropogenic CO_2 emissions between 1750 and 2011 have occurred in the last 40 years (Figure 1.1) (IPCC 2014).

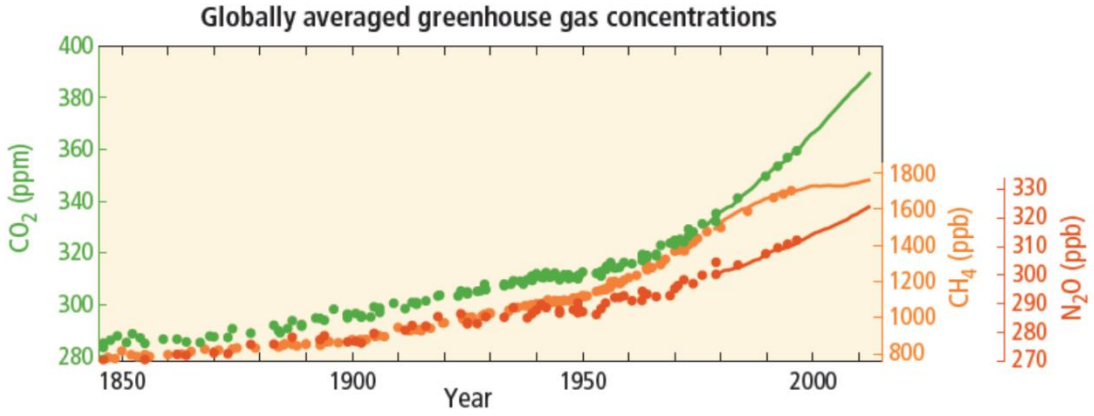


Figure 1.1 Figure of the document, Intergovernmental Panel on Climate Change (2014). Atmospheric concentrations of the greenhouse gases carbon dioxide (CO_2, green), methane (CH_4, orange) and nitrous oxide (N_2O, red) determined from ice core data (dots) and from direct atmospheric measurements (lines).

The global-scale in future scenarios such as ocean warming and acidification can also influence local scale as coastal water habitats (Harley et al. 2006, Helmuth et al. 2010, Russell and Connell 2012) (Figure 1.1).

The temperature in the seawater has been increasing by 0.13°C per decade over the last 50 years, mainly due to the increase of CO_2 levels in the atmosphere (Figure 1.2a and b). On a global scale, the ocean warming is largest near the surface, and the upper 75 m warmed by 0.11 [0.09 to 0.13] °C per decade over the period 1971 to 2010 (IPCC 2014) (Figure 1.2a and b).

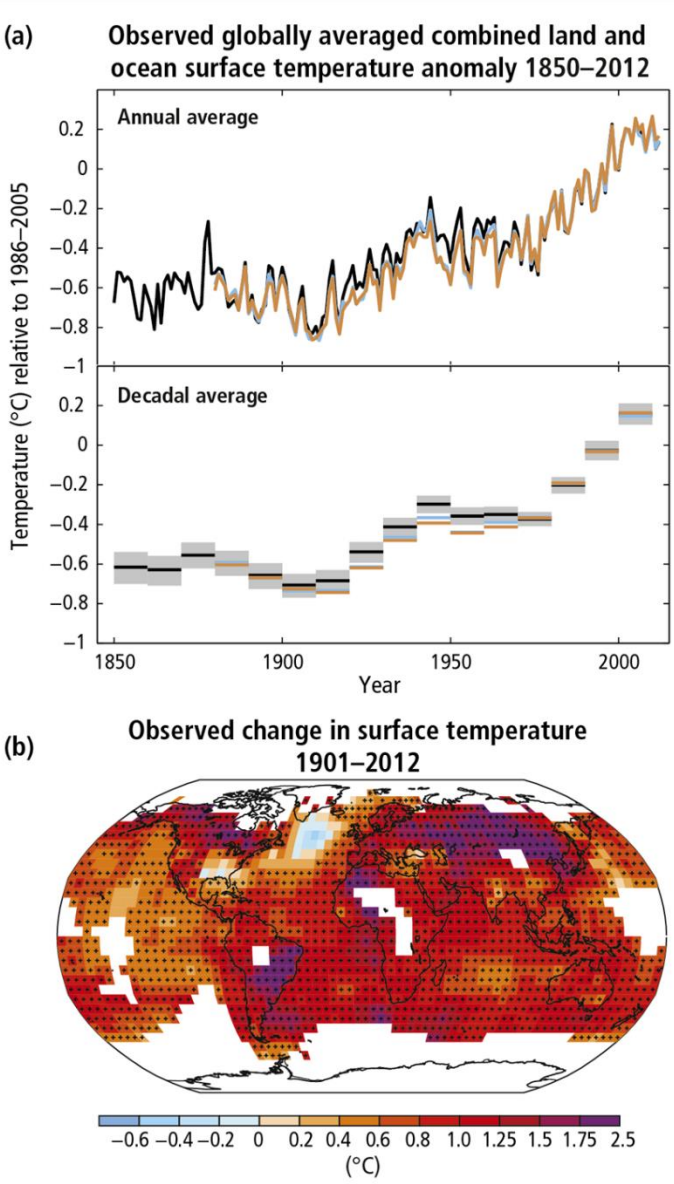


Figure 1.2 Figure of the document Intergovernmental Panel on Climate Change (2014). (a) Annually and globally averaged combined land and ocean surface temperature anomalies relative to the average over the period 1986 to 2005. Colors indicate different data sets. (b) Map of the observed surface temperature change, from 1901 to 2012, derived from temperature trends determined by linear regression from one data set (orange line in Figure 1.2). Trends have been calculated where data availability permitted a robust estimate (i.e. only for grid boxes with greater than 70% complete records and more than 20% data availability in the first and last 10% of the time period), other areas are white. Grid boxes where the trend is significant, at the 10% level, are indicated by a + sign.

Moreover, the ocean chemistry indicates that the rise in atmospheric CO_2 levels will affect carbonate chemistry with an increasing pCO_2 in seawater. Values lowered of the mean ocean surface pH by 0.1 units from preindustrial values with a predicted future with decrease to 0.3-0.4 units by 2100 are expected (Johnson et al. 2012) (see Figure 1.3). Recently, the National Aeronautics and Space Administration (NASA) has released data showing how temperature and rainfall patterns worldwide may change through the year 2100 because of growing concentrations of greenhouse gases in Earth's atmosphere (Figure 1.3).

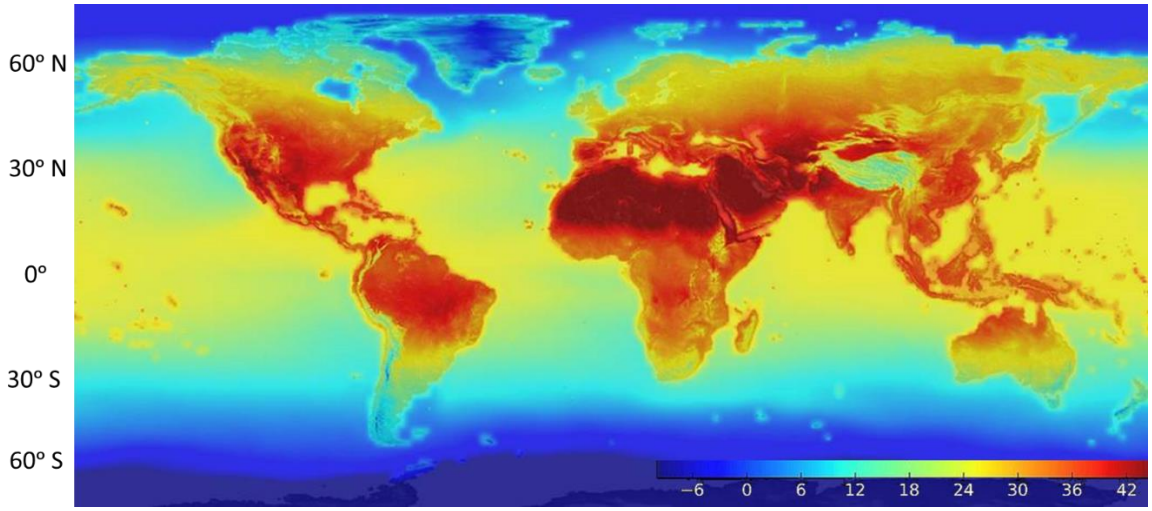


Figure 1.3 Figure of the National Aeronautics and Space Administration (NASA) global data set combines historical measurements of surface temperature with data from climate simulations using the best available computer models to provide forecasts of how global temperature (shown here) and precipitation might change up to 2100 under different greenhouse gas emissions scenarios. Data obtained from NASA, published 9 June 2015. (http://www.nasa.gov/press-release/nasa-releases-detailed-global-climate-change-projections)

Each of the last three decades has been successively warmer at the Earth's surface than any preceding decade since 1850 (IPCC 2014). Several studies show increase of the sunlight that reaches the Earth's surface. Surface solar global radiation showed a widespread reduction/increase from the 1950s to the 1980s not only in the infra-red region of the spectra but also in the photosynthetic active radiation waveband (400-700 nm) and short wavelengths (UVB, λ=280-315 nm and UVA, λ = 315-400 nm).

Since the 1980s a phenomenon that has been named as global dimming/brightening has been observed (Stanhill and Cohen 2001, Wild et al. 2005). This phenomenon is produced for changes in the transparency of the atmosphere due to variations in cloud

characteristics and changes in anthropogenic aerosol emissions (Stanhill and Cohen 2001, Wild et al. 2005). As result, various studies show an increased in global solar irradiance in Spain and a tendency to increase during the 1985 - 2010 period (+ 3.9 Wm^{-2} per decade). Solar irradiance has increased about + 5.1 Wm^{-2} per decade in Southeastern of the Mediterranean Sea, province of Málaga (Sánchez-Lorenzo et al. 2013). Moreover, changes in the irradiance and light quality can promote photosynthetic activity, but it also can inhibit many biological processes if radiation becomes excessive (Barber and Andersson 1992).

On the other hand, UVB integrated daily irradiances are affected by the ozone levels. The changes in stratospheric ozone depletion and the resulting increase of UVB radiation at the surface of the earth (see Figure 1.4) represent the main threats to the terrestrial and aquatic ecosystems.

Simulations of UVB change 2090-2000 according to two models (RCP45 and RCP85) which considers key processes that change the surface UV radiation: atmospheric dynamic and chemistry affecting ozone in the stratosphere and troposphere, aerosols and clouds in the troposphere and changes in surface albedo (Watanabe et al., 2012). The prediction models on the evolution of ozone layer indicate that in spite of the expected reduction at the end of XXI century of the CFCs levels to levels of 1980. UVB radiation will decrease or increase on 2095 compared to 1980 levels in the Northern Hemisphere (30-60ºN) depending on the used models (Hegglin and Sheperd 2009, Watanabe et al., 2011).

According to Hegglin and Sheperd (2009), UVR radiation reaching the earth surface will decrease in the Northern hemisphere (30ºN to 60ºN) and in the Southern hemisphere (30ºN to 60ºN). But not in the tropics or in the Southern hemisphere at latitudes over 60ºS which will exhibit increase in UVR at rates of +3.8% for the former and +10% for the latter mainly due to large climate-induced changes in ultraviolet index and stratosphere-troposphere ozone flux. Nevertheless, at some sites of Northern Hemisphere, UVB irradiance may continue to increase because of continuing reduction in aerosol extinction since 1990 (McKenzie et al. 2011).

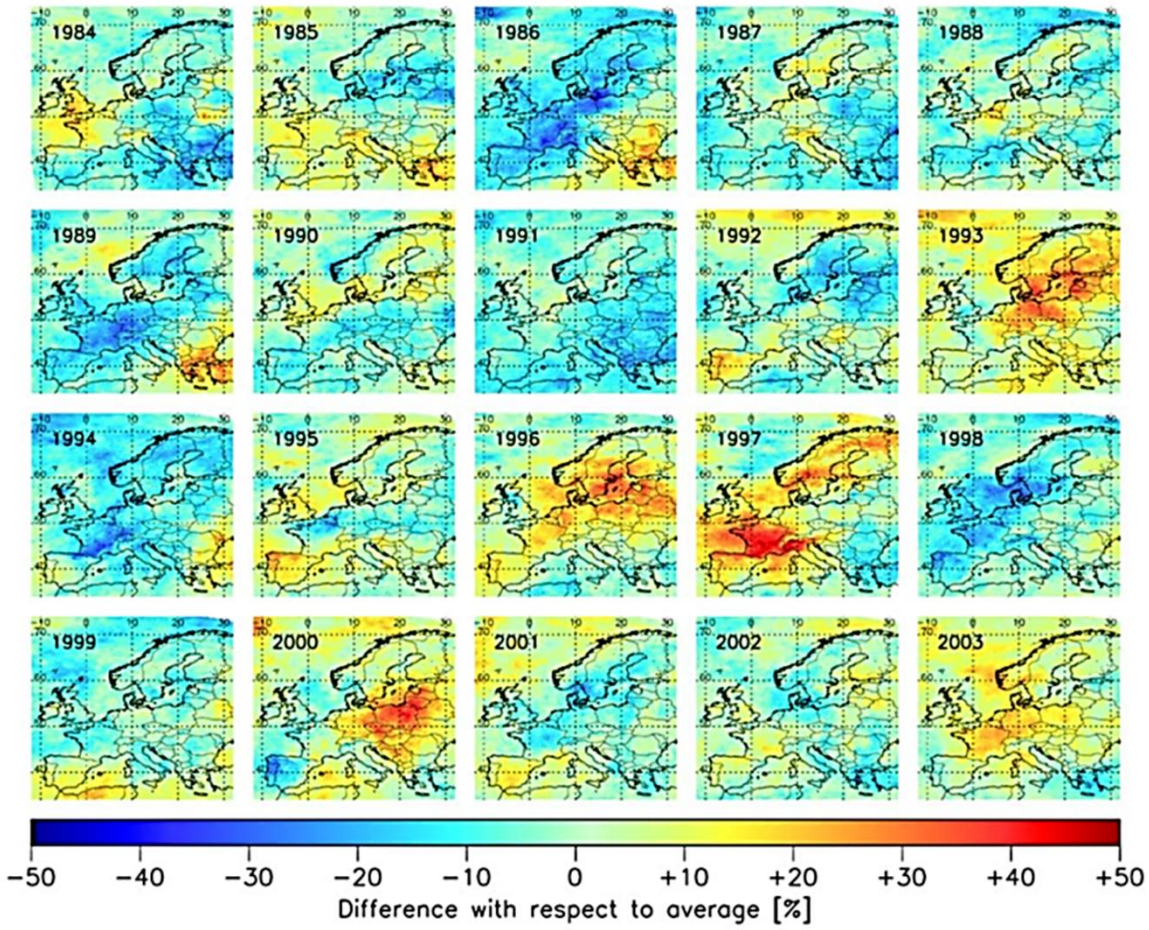


Figure 1.4 Daily dose maps from January 1 (1984) to August 31 (2003). Shows an example the year-to-year variability of the surface UV radiation over Europe of the METEOSAT satellite (*fide* J. Verdebout and J. Gröbner of the European Commission - Joint Research Centre - Institute for Health and Consumer Protection, Italy).

A new earth system model, MIROC-ESM-CHEM, has been used for the simulation, which considers key processes that change the surface UV radiation: atmospheric dynamics and chemistry affecting ozone in the stratosphere and troposphere, aerosols and clouds in the troposphere, and changes in surface albedo with sea ice and snow cover (Watanabe et al. 2011).

In contrast to previous assessments considering only the effect of long-term change in stratospheric ozone, the simulated long-term behavior of UV radiation is strongly affected by other processes. In RCP8.5 model, UV-B radiation decreases in the NH mid latitudes after 2000 following increasing total column ozone. In contrast, UV-B radiation in RCP4.5 is projected to stay at the 2000 level until 2040, and increase afterward to reach +6% in 2100. This increase in UV-B radiation is unexpected from the nearly constant total column ozone in this period, implying the importance of reductions in aerosols and

clouds. According to Watanabe et al. (2011), future all-sky UV-B, radiation in the NH mid latitudes depends most strongly on the choice of future socioeconomic scenario. Reductions in aerosols and clouds are expected to overcompensate for the effect of ozone recovery.

In the last 20 years, a good number of studies have been conducted on the effects of UVB radiation on macroalgae showing negative effects at molecular, physiological and ecological levels (Häder and Figueroa et al. 1997, Figueroa and Gómez 2001, Wiencke et al. 2004, Bischoff et al. 2006, Häder et al. 2007, Gao and Zheng 2010). The algae present different sensitivity to UVB according to species, morphology and life cycles (Dring et al. 1996, Franklin and Forster 1997, Altamirano et al. 2004). The vulnerability of plants to UV radiation is related to the balance between photodamage and photoprotection and photorepair mechanisms of DNA mediated by PAR and UVR (Mitchell and Karentz 1993, Murata et al. 2007). Accumulation of lipids and water-soluble antioxidants and the activation of antioxidant enzymes (Cockell and Kowland 1999) and the accumulation of UV screen photoprotectors as mycosporine-like aminoacids (MAAs) in red macroalgae (Karsten et al. 1998, Korbee-Peinado et al. 2004) and phenolic compounds in brown algae (Pavia et al. 1997, Connan et al. 2004, Abdala et al. 2006). These responses are partly offset by various protection strategies such as morphological adaptations (Ma and Gao 2009), avoidance, screening, photochemical quenching, repair (Roy 2000) and depth distribution (zonation). Consequently driving the structure of the coastal system as a correlation with the sensitivity of the algae to UV radiation i.e. the supralittoral species present less DNA damage and higher repair rate than the algae grown in the subtidal area (Bischof et al. 1998, Gómez et al. 2004, Wiencke et al. 2006). Growth and primary production can also be reduced by UV radiation (Grobe and Murphy 1998, Aguilera et al. 1999, Pang et al. 2001, Bischof et al. 2006).

The photoinhibition of photosynthesis under conditions of solar radiation depends on the daily changes in irradiance, vertical light attenuation, or the combination of both (Häder and Figueroa 1997). In addition, in the site in the intertidal system, macroalgae presented different sensitivity according to its morpho-functional pattern (Gómez et al. 2004, Bischof et al. 2006, Gómez and Huovinen 2011).

1.2 Climate change and Global change

Climate change is referred to change in the state of the climate that can be identified by changes in the mean climate and/or the variability of its properties and that persists for an extended period, typically decades or longer. Climate change may be due to natural internal processes or external forcing such as modulations of the solar cycles, volcanic eruptions and persistent anthropogenic changes in the composition of the atmosphere or in land use (IPCC 2014). The Framework Convention on Climate Change (UNFCCC), in the Article 1, defines climate change as 'a change of climate which is attributed directly or indirectly to human activity that alters the composition of the global atmosphere and which is in addition to natural climate variability observed over comparable time periods'. The UNFCCC thus makes a distinction between climate change attributable to human activities altering the atmospheric composition and climate variability attributable to natural causes.

Historically, habitat loss and over-exploitation of key species have been the main human impacts on landscapes (Jackson et al. 2001). In the last century, this list has grown to include pollution (in particular excess nitrogen), invasive species, and more recently climate change (Kappel 2005, Dudgeon et al. 2006, Venter et al. 2006), among many other stressors (see Figure 1.5). The predicted increase human population to about 10.500 million at the end of this century will produce elevation of the intensity of anthropogenic stressors, i.e. environmental and biotic factors that exceed their natural ranges of variation due to human activities (Sanderson et al. 2002, Halpern et al. 2007).
Human influences at regional and local scales such as eutrophication (Smith and Schindler 2015), will interact with other ambient physicochemical conditions and potential stressors such as temperature (Roleda and Hurd 2012) and solar irradiance, including UV radiation (Bischof et al. 2006, Gao and Zheng 2010, Russell et al. 2011, Yildiz et al. 2013). In addition, the increase of temperature and CO_2 concentration may affect synergistically the physiology and performance of organisms, marine biodiversity and affect biological interactions among species (Harley et al. 2006, Kroeker et al. 2010, Johnson et al. 2012) (See Figure 1.5).

Thus, in addition to the impact of climate change on algal communities, it is necessary to evaluate the impact of anthrophonic activities no directly related to climate change. These

anthropogenic impacts occur at different spatial-temporal scales being the most important global stressors: eutrophication, pollution with waste compounds of chemical industry or intensive agriculture and acid rain or ocean acidification. The whole change derived from climate change and due to other anthropogenic activities is denominated global change.

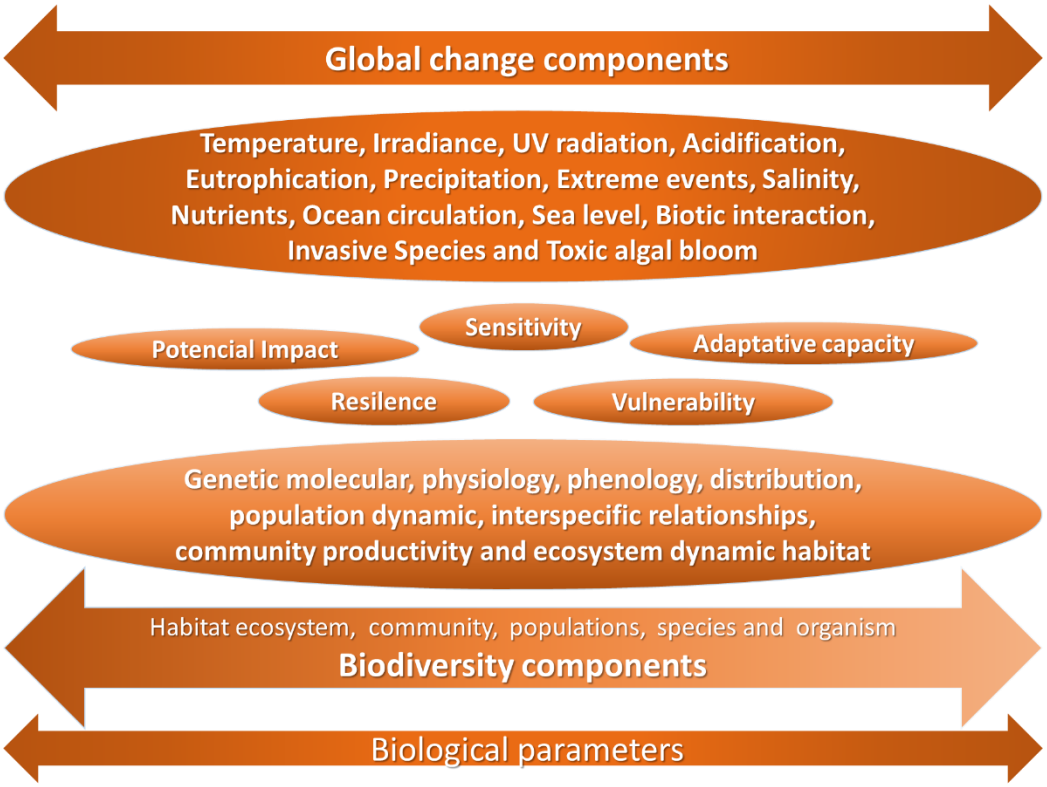


Figure 1.5. Principal stressors or components of the Global change and effects that cause in the Ecosystem, community, population, species and organism, and different mechanisms that the ecosystem use for counteract this impact.

1.3 Climate and global change in the Oceans

The ocean absorbs atmospheric carbon dioxide into solution at the sea surface. Like land plants, the phytoplankton and marine macrophytes, use carbon dioxide to produce their organic biomass via photosynthesis.

Historically, the oceans have absorbed about 30% of the emitted anthropogenic CO_2 since 1750, causing ocean acidification. About 40% of the anthropogenic CO_2 emissions have remained in the atmosphere. The rest is removed from the atmosphere by sinks, and stored

in natural carbon cycle reservoirs. Sinks from ocean uptake and vegetation with soils account, in roughly equal measures, for the remainder of the cumulative CO_2 emissions. In open ocean, the phytoplankton vary greatly in their size, function, and response to environmental and ecosystem changes or stresses such as ocean acidification. Thus are especially important, because oligotrophic and mesotrophic regions (<0.1 and 0.1-1mg Chl*a* m^{-3} near the surface, respectively) (Figure 1.6) account for 80%of the total net primary production of the oceans (Field et al. 1998).

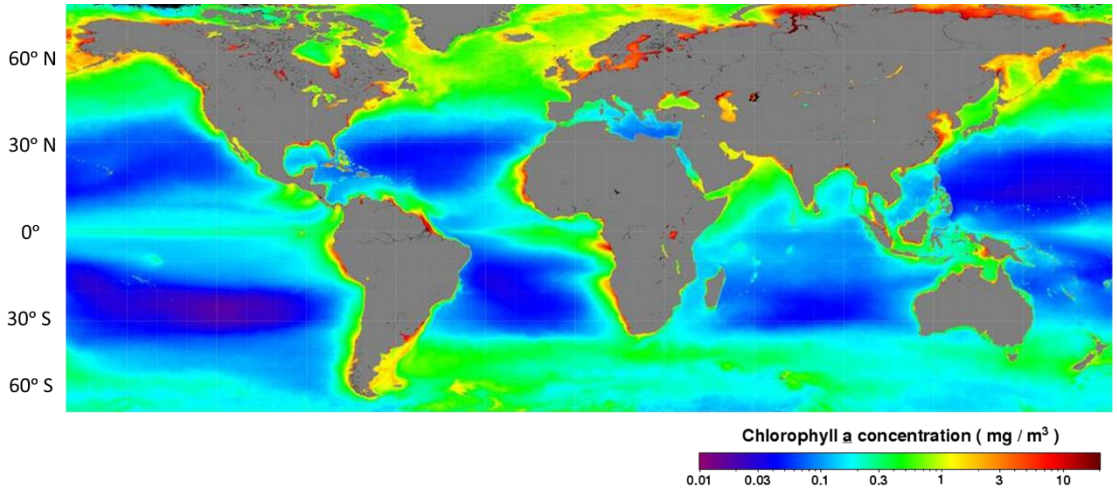


Figure 1.6 Figure of the National Aeronautics and Space Administration (NASA) global data set by NASA's PACE. Global distribution phytoplankton biomass, presented as the mean annual chlorophyll *a* concentration derived from remotely sensed fluorescence signals, was obtained 13 March 2015. Data was obtained from NASA (http://www.nasa.gov/press/2015/march/new-nasa-mission-to-study-ocean-color-airborne-particles-and-clouds)

The increase of CO_2 in the oceans due to increased atmospheric CO_2 levels is altering the concentrations of dissolved inorganic carbon (DIC) in surface waters and the consequence is the decrease of pH (acidification) phenomena know as Ocean acidification (OA). The CO_3^{2-} levels are falling, which is expected to corrode marine carbonates, whilst CO_2 and HCO_3^- levels are rising which can stimulate photosynthesis (Cornwall et al. 2012, Connell et al. 2013, Celis-Plá et al. 2015). Ocean acidification (OA) acts together with other global changes (e.g., warming, progressively lower oxygen levels) and with local changes (e.g., pollution, eutrophication), and act directly in the marine ecosystems affecting numerous environmental services especially in coastal areas (Barbier et al. 2011) leading to interactive, complex and amplified impacts for species and ecosystems. They are currently threatened by anthropogenic global climate change

(Hoegh-Guldberg and Bruno 2010), that is affecting biodiversity and ecosystem services at multiple scales (Schröter et al. 2005). The ways in which Ocean Acidification affects communities of primary producers is likely to vary regionally, depending on the species present and abiotic factors such as temperature, light and nutrient availability (Giordano et al. 2005, Brodie et al. 2014, Hofmann et al. 2014). As some primary producers are better able to capitalize on increasing carbon availability than others, this is expected to alter marine communities (Hepburn et al. 2011, Connell et al. 2013, Koch et al. 2013, Gaylord et al. 2015).

1.4 Climate and global change in coastal habitats

The coastal and marine environments are a major provider of goods and services. It also hosts an invaluable biodiversity that forms complex ecosystems that are threatened by human activities such as agriculture, fisheries, aquaculture, shipping, urbanization and tourism.

The Joint Research Centre (JRC) provides scientific and technical support for the implementation of the Marine Strategy Framework Directive, which aims to protect marine waters. The Marine Strategy Framework Directive's aim to achieve a Good Environmental Status (GES) of all European Seas by 2020 by assessing European marine waters, determining their environmental status and establishing monitoring programs. This center also disseminates datasets and information from satellite observations and numerical modelling as required for the analysis of coastal and marine ecosystem status and trends, including climate change, on the scale of European and regional seas. An Integrated Maritime Policy for the EU is essential to achieving EU commitments with respect to biodiversity targets, climate change mitigation and efficient use of natural resources. In order to achieve these targets and objectives, we need a better understanding of the inner workings of the many diverse seas and coastal systems. Following the Marine Strategy Framework Directive (MSFD) adopted by the European Council in May 2008, EU Member States must achieve or maintain Good Environmental Status (GES) of their marine and coastal waters by 2020. The Marine Strategy Framework Directive (MSFD) define the good environmental status is necessary to provide for the preparation at national level of an appropriate framework, including marine research operations and monitoring, for informed policymaking (2008/56/EC) and the action in areas of application of MSFD (Spanish jurisdictional waters).

The main stressors associated to climate and global change in coastal waters in addition to ocean acidification are the eutrophication and pollution and the rise of sea level

1.4.1 Eutrophication and pollution of coastal waters

Over-enrichment of water by nutrients such as nitrogen and phosphorus. It is one of the leading causes of water quality impairment. The two most acute symptoms of eutrophication are hypoxia (or oxygen depletion) and harmful algal blooms. The Marine systems are currently threatened by anthropogenic global climate change (Hoegh-Guldberg and Bruno 2010) that is affecting biodiversity and ecosystem services at multiple scales (Schröter et al. 2005). Ferreira et al. (2011) defining eutrophication as a process driven by enrichment of water nutrients, especially nitrogen and/or phosphorus compounds, leading to: increased growth, primary production and biomass of algae; changes in the balance of organisms; and water quality degradation.

In addition to urban sewage, pollution by different effluents from industrial activity are contaminating the coastal waters. Among the contaminants, heavy metals are producing risks in human health due to the bioaccumulation in the marine food chain. Metals occur naturally in seawater at low concentrations and some, such as copper, act as essential micronutrients for marine biota (Lobban and Harrison, 1994).The domestic sewage and urban storm-water, as well as industrial and agricultural effluents represent an important input of metals to marine ecosystems (Perez et al. 2005, Li et al. 2009, Sarkar et al. 2011). Elevated concentrations that may occur in coastal waters and sediments due to contamination near mines, harbours, and industrial activity (Nriagu, 1990) can pose a threat to marine life and can be toxic when absorbed by macroalgae. Among metals, copper is an essential element for metabolic processes in marine algae, required for electron transport in photosynthesis (plastocyanin) and by various enzyme systems (Burkhead et al. 2009), but it is also one the most toxic to marine algae after mercury (Gledhill et al. 1997). In this study, the effect of cooper in combination with different nutrient supply on algal physiology is studied.

1.4.2 Sea level

The increased surface temperature in the ocean or warming ocean has prompted decreased to the snow cover, the global mean sea level and therefore increased of the sea level in

the marine ecosystem in general. According to IPCC (2014), global mean sea level rose by 0.19 [0.17 to 0.21] m shown in the period 1901-2010, (see Figure 1.7). The rate of sea level rise since the mid-19th century has been larger than the mean rate during the previous two millennia. It is very likely that the mean rate of global averaged sea level rise was 1.7 [1.5 to 1.9] mm/yr between 1901 and 2010 and 3.2 [2.8 to 3.6] mm/yr between 1993 and 2010.

In Northern Hemisphere was observed that ice cover has decreased since the mid-20th century by 1.6 [0.8 to 2.4] percentage per decade for March and April, and 11.7% per decade for June, over the 1967 to 2012 period. In consequence, the temperatures have increased in most regions of the Northern Hemisphere since the early 1980s, with reductions in thickness and areal extent of ice cover in some regions.

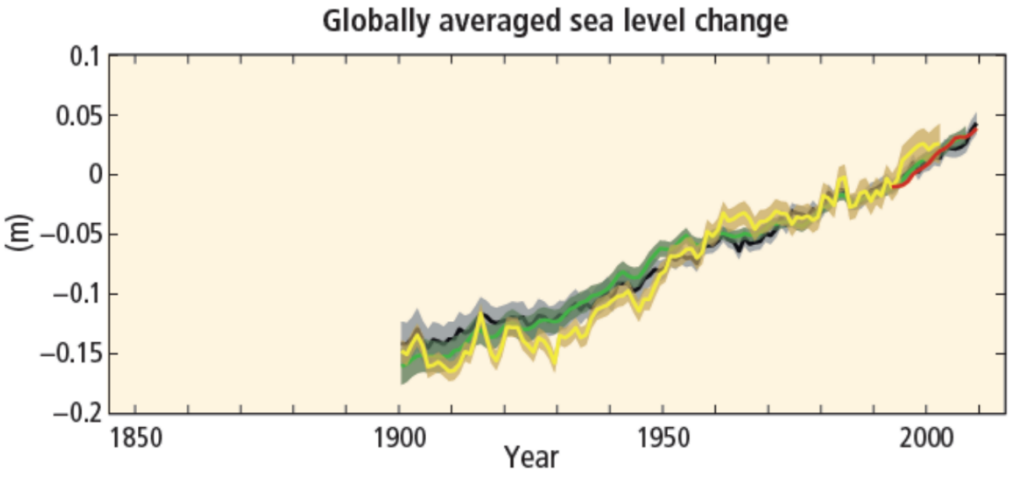


Figure 1.7 Figure of the document, Intergovernmental Panel on Climate Change (2014). Annually and globally averaged sea level change relative to the average over the period 1986 to 2005 in the longest-running dataset. Colors indicate different data sets. All datasets are aligned to have the same value in 1993, the first year of satellite altimetry data (red). Where assessed, uncertainties are indicated by colored shading.

These changes in the coastal ecosystems related to the global sea level are subject to considerable and increasing anthropogenic pressure because of human demographic (Halpern et al. 2008) with over-enrichment of water by nutrients such as nitrogen and phosphorus. It is one of the leading causes of water quality impairment, and two consequences or symptoms of eutrophication in the ocean are hypoxia (or oxygen depletion) and harmful algal blooms. Human effects as land-based activities affect the

runoff of pollutants and nutrients into coastal waters (Nelson and Nusse 2004, Gumbiner 2005) and can remove, alter, or destroy natural habitat (Halpern et al. 2008).

1.5 Mediterranean and Alboran Sea

The Mediterranean Sea is the largest of the semi enclosed European seas, bordering the continents of Europe, Africa and Asia. It consists of two major basins, the western and the eastern (Western Mediterranean, Eastern Mediterranean), separated by the Strait of Sicily and further subdivided into smaller regional seas such as the Alboran Sea (Figure 1.8). The Mediterranean Sea is linked to the Atlantic Ocean through the Strait of Gibraltar, to the Black Sea through the Dardanelles and to the Red Sea through the Suez Canal. Water exchange with these exterior seas is small; nevertheless, the exchange with the Atlantic Ocean, saline water that exits through the Strait of Gibraltar influences the thermohaline circulation in the northern Atlantic Ocean (Candela 2001) (Figure 1.8). A complete revision on hydrographic, chemistry, biological and human impact in the Mediterranean has been published recently "The Mediterranean Sea: its history and present challenges" edited by Steffano Goffredo and Zvi Dubinsky (2015).

The gradient temperature is about 10ºC from 24ºC to about in the western and eastern part respectively. The sea surface temperature 2-3ºC in the eastern Mediterranean whereas the increase in the western part has been only 0.8-1ºC according to the data of Joint Research Center.

The Mediterranean Sea (Figure 1.8) is an oligotrophic basin (with the exception of areas affected by sewage, river flows and upwelling) where the nutrient balance is maintained by water exchange mainly through the Straits of Gibraltar and minor contribution through Bosphorus and Suez Channel (Finkl 2015). The water exchange through the Strait of Gibraltar occurs in a two-layer flow (Parrilla and Kinder 1987). The input influence of the nutrient-poor Atlantic Water (AW) in the Alboran Sea is combined with a layer an outflow of saltier and nutrient-rich Mediterranean waters progresses towards the Atlantic Ocean (Minas et al. 1991, Béthoux et al. 1998, Gómez et al. 2001) (see Figures 1.9 and 1.10).

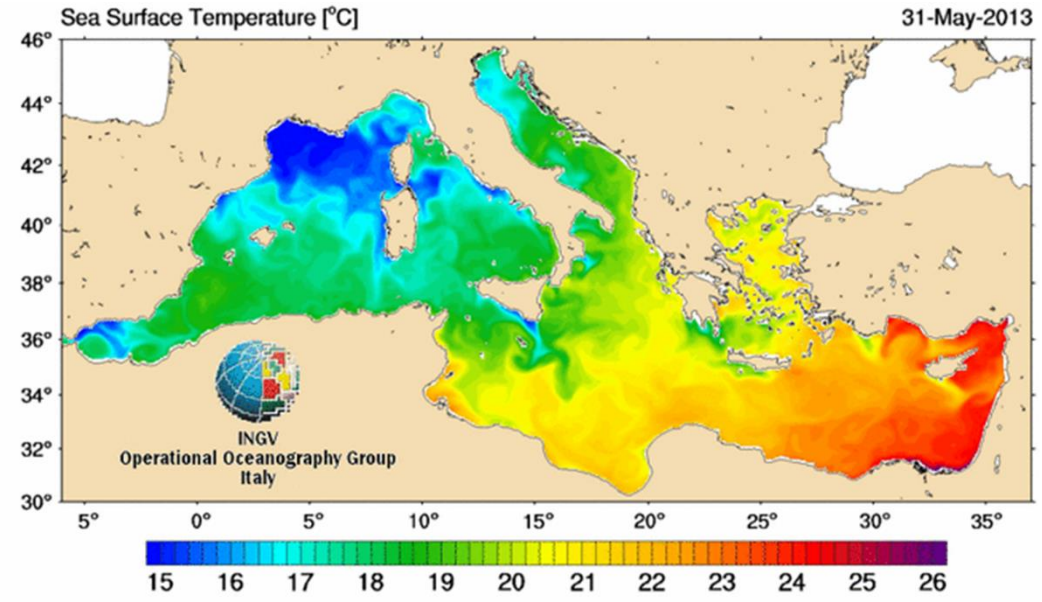


Figure 1.8 Figure of the Operation Oceanography Group (Italy) global data set by INGV. Mediterranean Sea, the mean temperature 31 May 2013. Data was obtained from INGV (http://www.ingv.it/en/).

In the Alboran Sea, there are oceanographic differences between the western (W) and Eastern (E) sector as it is illustrated in Table 1 taking reported data by Ramírez et al. (2005), Mercado et al. (2005, 2007, 2012) and Cortés et al. (2012). The eastern sector presented warmer waters and lower level of nutrients than that in the western sector (Table 1, Figure 1.9). The salinity of surface waters is slightly higher in E compared W sector (Table 1). The nutrient levels are submitted to seasonal regime, i.e. nitrate and silicates levels are generally higher in spring and winter in W sector associated to coastal upwelling (Figure 1.9) whereas in E sector the spring maximal is not observed and only at winter the nitrate level clearly is increased (Table 1). Ammonium in contrast presented different pattern with higher levels in spring and summer in W sector probably associated to urban sewage of the increased population during the summer period. Phosphate concentration did not present a high marked seasonal pattern as the case of nitrate being higher in W than E sector.

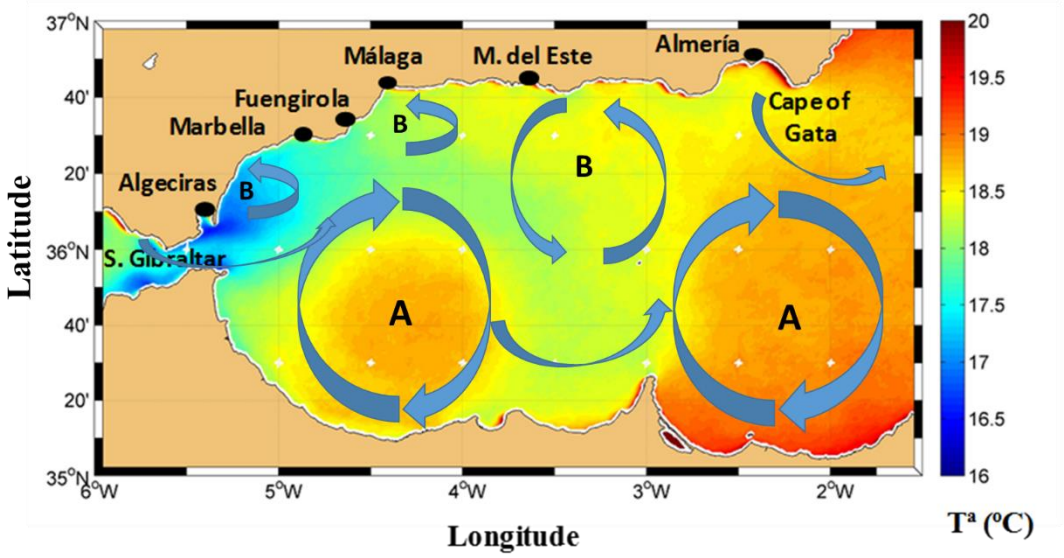


Figure 1.9 Map of the climatological surface temperature (ST) of the Alboran Sea. The map has been composed from the average of daily images captured by the satellite MODIS-Aqua from 2002 to 2012 (http://oceancolor.gsfc.nasa.gov/cms/data/aqua). Mediterranean Sea with a water circulation (blue lines) the mean summer temperature in Celsius degree with (A) with anticyclonic gyres with low algal productivity and warmer waters and (B) zones upwelling and increased primary productivity.

Nitrate and phosphate concentrations at the eastern sector coast are lower by 30-40%, on average. Nitrate: phosphate molar ratio is lower than Redfield ratio (16:1) in the coastal areas of both sub-basins, although deviation from that theoretical ratio is higher in the eastern that that in the western sector. Overall, these data would indicate that nitrate limits the algal growth in the Alboran Sea in contrast to those occurred in most of Mediterranean Sea where primary productivity is mainly limited by phosphate (Siokou-Frangou et al. 2010).

Beyond these climatologic values, nutrient concentrations vary strongly throughout the seasonal cycle, at least in the western sector where nitrate concentrations in spring are higher than in the other seasonal periods (Table 1). In addition to the differences observed in nutrient concentrations, the N:Si:P molar ratio is slightly higher than the Redfield ratio (16:16:1) in the out flowing Mediterranean waters than that in the Atlantic Water (Béthoux et al. 2002, Dafner et al. 2003). The seasonal changes in the water column stratification during the entire annual cycle and intensity of the Atlantic water inflow, affecting the strength of upwelling off the Malaga coast (Figure 1.10) (Ramírez et al. 2005).

Table 1. Seasonal changes in physical and chemical features of the western (W) and eastern (E) waters of the Alboran Sea. Salinity, nitrate, ammonium, phosphate and N: P ratio (mean values ± SE, n=180) according to Ramírez et al. (2005) and Mercado et al. (2007 and 2012). *nd*: no data.

Parameters	***Sector***	***Spring***	***Summer***	***Autumn***	***Winter***
T^a (^{o}C)	W	15.91±1.14	18.91±2.09	17.81±1.66	14.88±0.45
	E	13.91±0.16	22.16±2.21	18.30±1.30	14.89±0.36
Salinity	W	37.14±0.45	36.87±0.29	36.72±0.34	36.93±0.28
	E	38.22±0.01	36.99±0.22	37.72±0.27	37.42±0.21
Nitrate (µmol L^{-1})	W	1.59±1.44	0.58±1.07	0.62±0.77	1.52±1.07
	E	0.49±0.55	0.25±0.99	0.32±0.80	1.04±0.73
Ammonium (µmol L^{-1})	W	0.35±0.20	0.53±0.75	0.19±0.27	0.18±0.10
	E	nd	nd	nd	nd
Phosphate (µmol L^{-1})	W	0.15±0.09	0.12±0.08	0.14±0.01	0.14±0.05
	E	0.11±0.05	0.11±0.17	0.10±0.10	0.10±0.05
Silicate (µmol L^{-1})	W	1.87±0.78	1.0±0.81	1.15±0.49	1.70±0.92
	E	0.86±0.63	0.62±0.85	0.87±0.63	1.28±0.47
N:P molar ratio	W	16.0±21.3	4.3±6.6	7.4±10.9	13.4±12.3
	E	5.8±4.97	3.8±7.3	4.7±5.7	7.7±6.1
Chlorophyll a	W	1.45±0.99	0.92±0.69	1.21±0.94	1.22±1.14
(µg L^{-1})	E	0.63±0.57	0.38±0.78	0.92±1.03	1.01±0.61

The horizontal distribution pattern of *in situ* Chl*a* matches the one described for nutrients. In fact, strong decreasing gradients of Chl*a* from coast to offshore and from western to eastern sector are typical features of the northern Alboran Sea (Rodríguez et al. 1998 and 2001, Ramírez et al. 2005, Macías et al. 2007). The intensity of the coast-offshore gradients varies seasonally and normally is higher in the western sector coinciding with its higher concentration of nutrients (Ramírez et al. 2005, Mercado et al. 2007). According to the data gathered by Cortés et al. (2012), the highest Chl*a* concentration registered in the most superficial layer of the western sector is 16.8 µg L^{-1}, although occurrence of Chl*a* concentrations higher than 4 µg L^{-1} is relatively scarce (less than 3% out of the registered data).

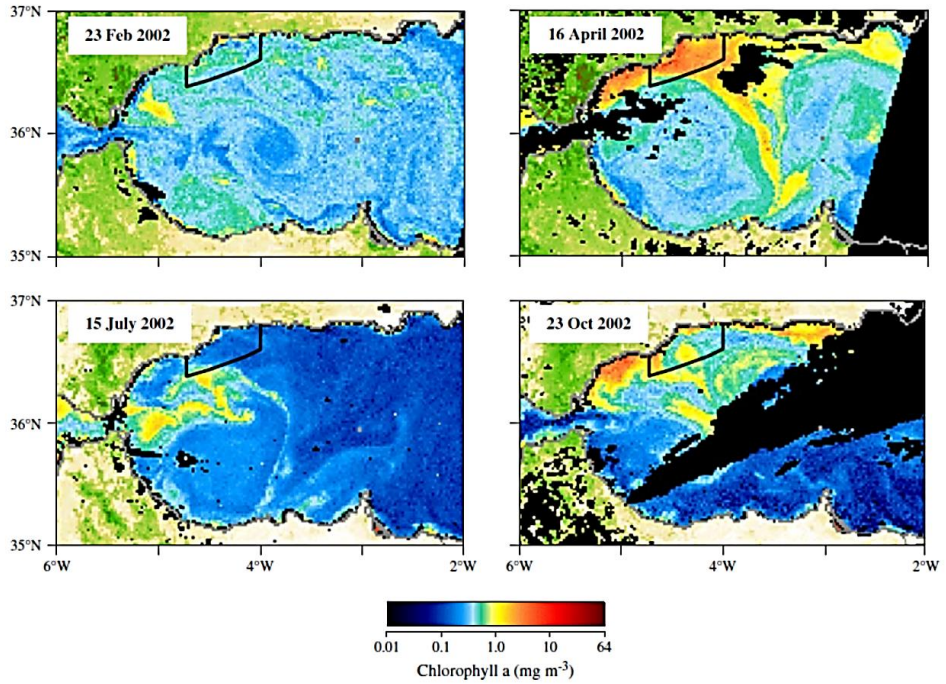


Figure 1.10 Figure of the SeaWiFS project images (NASA) (processed level 3) (*fide* Ramírez et al 2005). Data of selected for the different seasonal. The area under study is shown in the map.

Most commonly, Chl*a* concentration is within the range of 0.5 to 2 µg L^{-1}. In contrast, the highest concentration of Chl*a* registered in the W sector is 5 µg L^{-1} and occurrence of Chl*a* peaks higher than 2 µg L^{-1} is very scarce (less than 3%). The most frequent range of concentrations in the E sector is 0.1-0.5 µg L^{-1}.

Concentrations of Chl*a* recorded during the 2002 year for SeaWiFS images (Ramirez et al. 2005), are similar to that reported by other studies during transient upwelling events in the Alboran Sea (Minas et al. 1991, Arin et al. 2002). As indicated by the high nutrients and Chl*a* concentrations found in spring (Ramírez et al. 2005, Mercado 2007). Thereby, the annual peaks of nutrient concentration and chlorophyll *a* tend to be detected in spring (Mercado et al. 2005 and 2006, Ramírez et al. 2005 and 2006). Studies made for Mercado et al. (2007) indicates different concentrations of the nutrients in the Mediterranean Sea waters, specifically in the Alboran Sea. The temporal series of the nutrient and Chl*a* in Alboran Sea can be also observed by using remote images (Figure 1.10) composed from 1992 to 2002. The increase of surface chlorophyll *a* concentration in April and October was clear observed in a study conducted in 2002 by Ramírez et al. (2005). On the other, the low levels of chlorophyll *a* in the anticyclone gyres are observed.

The coastal waters of the Alboran Sea are exposed to very high daily irradiance as demonstrated by the high values of monthly climatological incident irradiance registered for different coastal stations located at the northern coast (Sancho et al. 2012). For instance, minimum and maximum of daily irradiance averaged monthly in Málaga city (western sector) ranges from 3 to 8 Kwh m^{-2} d^{-1} obtained in January and July, respectively (Sancho et al. 2012). These values are the highest described for the whole Mediterranean Sea. Furthermore, the coastal waters of the northern Alboran Sea are little affected by discharges from rivers and/or streams due to the geo-morphologic features of the basin and its climate regime (semi-arid in the eastern extreme). Consequently, both factors high isolation degree and relatively low concentration of allocthonous material would imply that the phototrophic organisms growing at the surface layer of the Alboran Sea are often exposed to very high daily solar irradiance. The means annual global radiation series over Spain, including coastal areas, show a tendency to increase during the 1985–2010 period, with a significant linear trend of +3.9 Wm^{-2} per decade (Sánchez-Lorenzo et al. 2013). Similar significant increases were observed in the mean seasonal series, with the highest rate of change during summer (+6.5 Wm^{-2} per decade) and secondly in autumn (+4.1 W m^{-2} per decade) and spring (+3.2 Wm^{-2} per decade).

Cortés et al. (2012) analyze transparency of the water column in the northern Alboran Sea, from values of Secchi disc depth (ZSD, Table 2) obtained in serial research surveys performed from 1994 to 2010. As expected from the Chl*a* concentrations, transparency is lower in western than that in eastern sector. Thus, the euphotic zone depth (i.e. depth at which 1% surface irradiance reaches, $ZD_{1\%}$) varies normally between 15 and 50 m in western sector where occurrence of $ZD_{1\%}$ values lower than 10 m is infrequent (they represent less than 10% of registered items). The variation range of $ZD_{1\%}$ in the eastern is narrower (40-55 m), than in the western sector and only 1% of $ZD_{1\%}$ values registered are lower than 20 m.

Apart of these transparency data, reports describing the underwater light field in the Alboran Sea are scarce and only a few values of PAR and UVR diffusive attenuation coefficient (K_d) can be found in the literature (Table 2). Pérez-Rodríguez (2000) reported K_d values lower in the eastern than western coast although in both sectors K_d was low in comparison with the values reported for coastal areas of other regions (Kirk 1994, Dring and Lüning 1994, Wiencke et al. 2000, Figueroa et al. 2002, Figueroa and Gómez 2001a, Bermejo et al. 2013).

Table 2. Optical properties of the coastal waters of the western and eastern sectors of the Alboran Sea. Secchi disc depth represents the means calculated for spring-summer (lowest value) and autumn-winter (highest value) in different stations sampled from 1992 to 2005. K_d and D1% values are the means of different samplings performed in stations located out of Fuengirola (western sector), Marina del Este (eastern sector) and Cape of Gata (eastern limit). Kd: attenuation coefficient of the downward radiation (m^{-1}) for different spectral bands (PAR: 400-700 nm, UVA: 315-400 nm, UVB, 280-315 nm) and at 305 and 340 nm. $D_{1\%}$, depth at which 1% of the surface irradiance reaches estimated for different spectral bands. The irradiance was determined by using a Multifilter radiometer PUV 500. The UVA and UVB irradiance was calculated according to Orce and Helbling (1997). Sources of data: Secchi discs by Cortés et al. (2012) and K_d values by Pérez-Rodríguez (2000) and Figueroa and Gómez (2001a).

Parameters	***Western sector***	***Eastern sector***	***Eastern limit***
Secchi disc depth (m)	9 - 11	15 - 20	-
$K_d(PAR)(m^{-1})$	0.102 ± 0.009	0.110 ± 0.023	0.070 ± 0.002
$K_d(UVA)(m^{-1})$	0.275 ± 0.023	0.215 ± 0.021	0.105 ± 0.013
$K_d(UVB)(m^{-1})$	0.387 ± 0.043	0.390 ± 0.032	0.220 ± 0.024
Kd(305) (m-1)	0.415 ± 0.005	0.430 ± 0.068	0.225 ± 0.025
Kd(340) (m-1)	0.305 ± 0.005	0.240 ± 0.068	0.120 ± 0.022
$D_{1\%}(PAR)(m)$	45.12 ± 4.28	41.86 ± 4.68	65.78 ± 6.48
$D_{1\%}(UVA)(m)$	16.74 ± 1.76	21.42 ± 2.31	43.85 ± 4.53
$D_{1\%}(UVB)(m)$	11.89 ± 1.21	11.80 ± 1.23	20.93 ± 2.21
D1%(305) (m)	11.10 ± 0.10	10.71 ± 2.41	20.46 ± 2.42
D1%(340) (m)	15.10 ± 0.10	19.18 ± 2.41	38.37 ± 2.42

According to the oceanographic characteristics of the W and E sector, the waters can be classified as oligotrophic and ultra-oligotrophic, respectively according to the classification proposed by OCDE (1982). In this study, the physiological responses to climate change factor have been evaluated in macroalgae collected from both E and W sectors of Alboran Sea.

1.6 Marine macroalgae in coastal areas affected by climate changes and species analyzed in this study

These futures scenarios can affect species more vulnerable because they live closer to their absolute tolerance limits (Harley et al. 2006). The species with higher temperature tolerance will be better able to cope with global warming (Calosi et al. 2008). Macroalgal communities of the brown algae and some seagrasses will be affected by high CO_2 concentrations, either maintaining or accelerating their physiological processes (Figueroa and Gómez 2001a, Hall-Spencer et al. 2008, Porzio et al. 2011, Roleda et al. 2012). The change the structure of the community, can be caused by the ocean acidification because can to lower growth rates in marine calcifies (Guinotte and Fabry 2008, Martin and Gattuso 2009), and higher growth of the no calcifying organisms (Kuffner et al. 2008, Connell and Russell 2010). The negative impact algal communities with structural role in the ecosystem can produce a subsequent loss of habitat for many other species (Olabarria et al. 2012). In addition, the increasing CO_2 in the oceans might increase photosynthetic activity and affecting nutrient uptake systems.

The effects of environmental degradation, it have observed in littoral ecosystems of the Mediterranean Sea and Atlantic North Ocean, such as in seaweeds of the orders Laminariales and Fucales, involving the disappearance of sensitive ecosystem "habitat-forming" or "engineer" species (Lüning 1990, Giaccone et al. 1994, Arévalo et al. 2007, Figueroa et al. 2014, Pérez-Lloréns et al. 2014). Different seaweeds of the order Fucales (Feldmann 1937, Giaccone 1973) are suffering a general decline (Thibaut et al. 2005, Serio et al. 2006, Díaz-Almela et al. 2007, Fernández 2011, Pérez-Lloréns et al. 2014) and habitat destruction or degradation. Considered as threat to the diversity, structure, functioning and services they provide of marine coastal ecosystems in the Mediterranean Sea (Claudet and Fraschetti 2010, Coll et al. 2010, Lotze et al. 2006).

In this study, we have investigated the effect of climate factors on a habitat and key forming species in the intertidal and subtidal (Gómez-Garreta et al. 2001) of Alboran Sea in macroalgae collected from Western and Eastern sectors. Corresponding to oligotrophic and ultra-oligotrophic waters, respectively, as the non-calcareous species *Cystoseira tamariscifolia* (Hudson) Papenfuss (Phaeophyceae, Fucales) (Figure 1.11A), and other calcareous species growing in the same coastal habitat as *Padina pavonica* (Phaeophyta) and *Ellisolandia elongata* (Rhodophyta). *C. tamariscifolia* is distributed from Scotland

to Mauritania and it can be found in the Mediterranean area with Atlantic water influence (Gómez-Garreta ed. 2001, Bunker et al. 2010) (Figure 1.11A, B and 1.12A) *C. tamariscifolia* is a habitat-forming species that dominates intertidal and shallow-subtidal W Mediterranean communities in pristine sites and oligotrophic waters (Figure 1.12B). Thalli present a single axis, with green-bluish iridescence, to almost 1 m in height, attached to the substratum by disc or by thick, branched haptera that may be fused. Axis cylindrical has several decimeters in length and 3-10 mm in diameter, often branched. Apex of the axis is not very prominent and covered by small spines. In *C. tamariscifolia*, the receptacles are not very compact and they are located in the apices on the top of branches. Plants are fertile throughout the year, although receptacles are most developed in spring and summer. In this study we have investigated on *C. tamariscifolia* collected from western sector of Alboran Sea (La Araña Beach, Malaga) and eastern sector (Cabo de Gata-Níjar Natural Park, Almeria) and from Atlantic waters (Plymouth, UK).

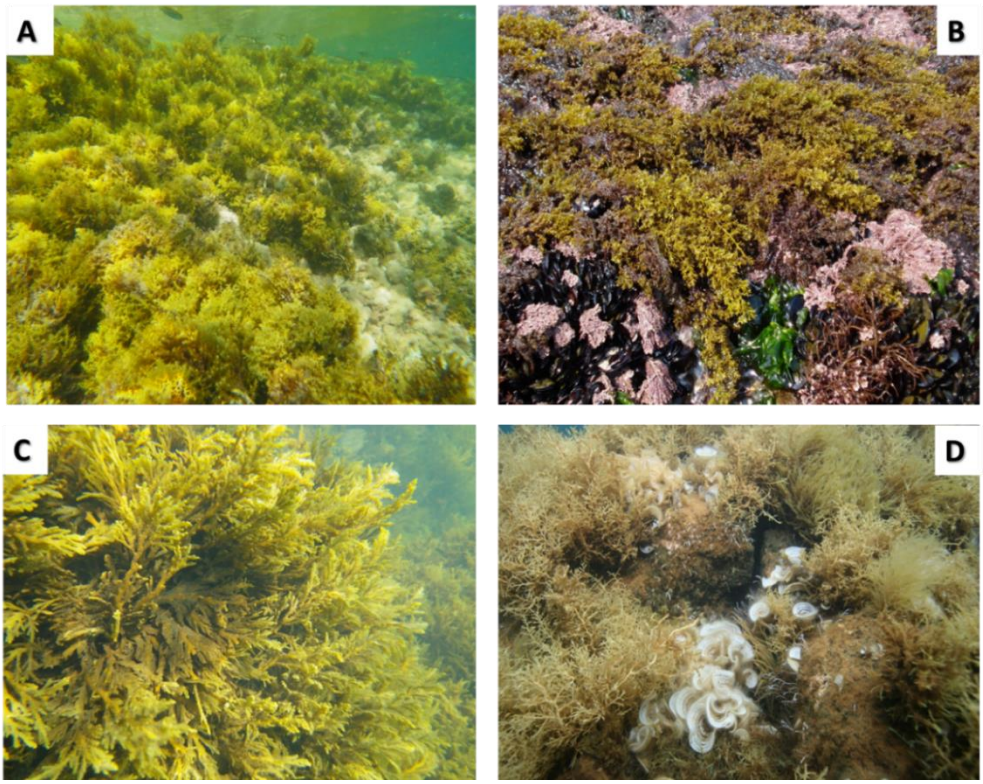


Figure 1.11 Different intertidal marine ecosystems dominated by structural complex macroalgae communities (See Figure 1.12A). (A) Intertidal forest of *Cystoseira tamariscifolia* in Isleta del Moro (Cabo de Gata-Níjar Natural Park at 1.0 m of depth; Photography by P. Celis-Plá, See Figure 1.12B). (B) Macroalgal in an ecosystem dominated by *Cystoseira tamariscifolia* and *Ellisolandia elongata* in "La Araña" beach, Málaga (Photography by F.L. Figueroa, See Figure 1.12B). (C) Shallow *Cystoseira compressa* in pH gradient, Vulcano, Italy (0.5 m of depth; Photography by P. Celis-Plá, See Figure 1.12C) and (D) Intertidal of *Padina pavonica* in pH gradient, Vulcano, Italy (0.5 m of depth; Photography by J.M. Hall- Spencer, See Figure 1.12C).

Ellisolandia elongata (Ellis and Solander) Hind and Saunders (Florideophyceae, Corallinales) (Figure 1.11B and 1.12B). *E. elongata* is an articulated calcareous species that dominates benthic intertidal communities replaced by ulvacean algae at intermediate levels of nutrient enrichment (Arévalo et al. 2007). Resembling a small bush, up to 20 cm in height (Braga et al. 2009), it is a perennial species and can occupy both sun and shaded habitats (Häder et al. 1997, Figueroa and Gómez 2001). *E. elongata* specimens have been recorded as fertile tetrasporophyte phase throughout the year (Rodríguez and Polo 1986) (Figure 1.11B and 1.12B).

Padina pavonica (Phaeophyceae, Dictyotales) is a calcified macroalgae (Figure 1.11D). *P. pavonica* is a widely distributed warm-temperate species (Figure 1.12C). Despite its wide distribution, reports of fertile gametophytes are rare and descriptions of their sexual reproductive structures are scarce and almost exclusively from the Mediterranean Sea (Gómez-Garreta et al. 2007).

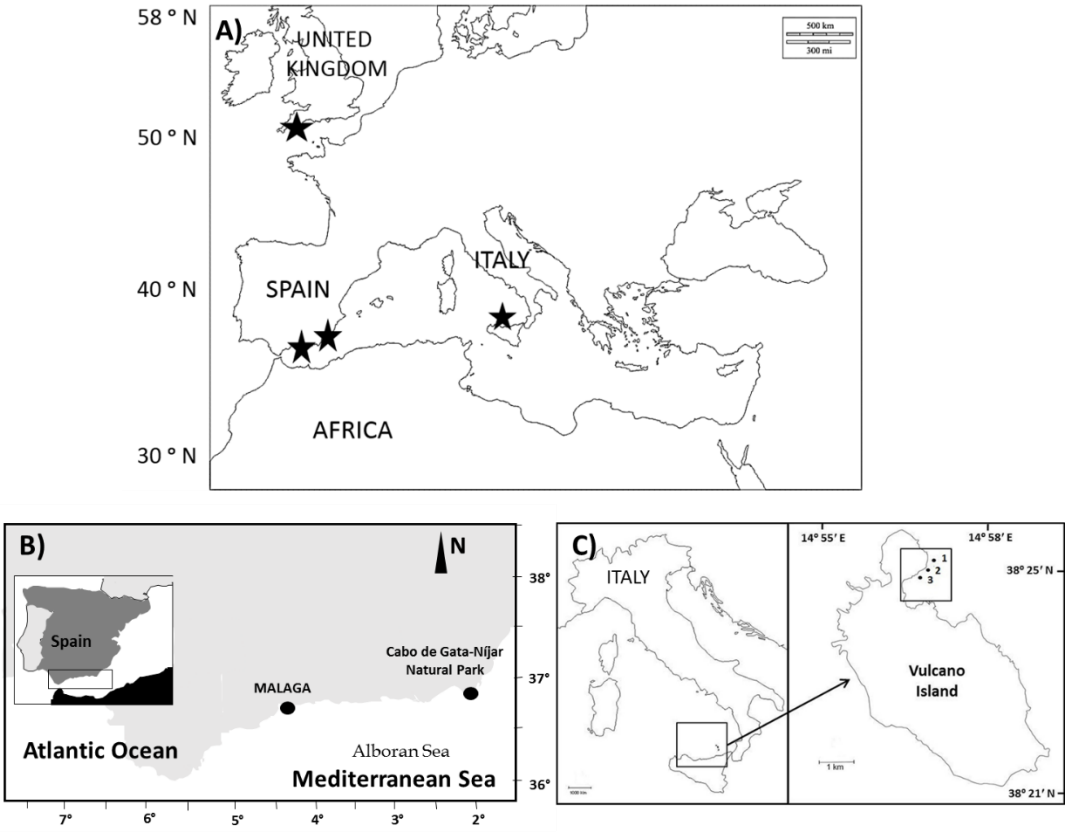


Figure 1.12 A) Map of the Europe, star symbol localizes sites of collected algae in this study. Three areas of the collected macroalgae are shown in the map, A) United Kingdom, B) Spain and C) Italy.

In addition, the effects of climate change factors have been studied *in situ* on non-calcareous and calcareous brown macroalgae in Vulcano Island (Italy), *Cystoseira*

compressa and *Padina pavonica* respectively (Figure 1.11C and 1.12C). *Cystoseira compressa* (Phaeophyceae, Fucales) (Figure 1.12C) is distributed from Sicily (Figure 1.12C), Adriatic Sea and recently the Columbretes Island. Plant caespitose, to 10-20 cm in height. Axes are very short, 1 cm high. Apices of the axes are smooth. All branches flattened and arranged in a single plane. Receptacles are in the apices of the terminal branchlets. Plants are fertile in September (Gómez-Garreta et al. 2001).

1.7 Interactive/additive effect of variable of climate and global change: synergistic or antagonistic interaction responses in macroalgae

The main investigations on climate or global change have been conducted at species level and there is still scarce information on the interactive effect of climate change variables on the structure, diversity and primary production of the algal and aquatic macrophytes communities (Lüning 1990, Häder and Figueroa 1997, Bischof et al. 2006). It has observed that the sensitivity of macroalgae to UV radiation maybe influenced by global changes such as, ocean acidification, eutrophication or increasing CO_2 and temperature as synergistic or antagonistic interaction effects on the photosynthetic activity occurring (Bischof et al. 2006). It is still more scarce the number of studies on the interaction between factors of global climate change (increased CO_2, temperature and UVR) at regional scale and the relation of the pattern of biodiversity and function of ecosystems (Naeem et al. 1999). There are a great number of studies on the individual effects of UVR and nutrients on organisms (Häder et al. 2007) but it is scarce the number of studies on the interaction between UV radiation and nutrient availability (Villafañe et al. 2003). Because, most studies are reduce to analyze the effect of the increase of CO_2, acidification, solar UV radiation or temperature separately (Franklin and Forster 1997, Bischof et al. 2006, Häder et al. 2007).

Research carried out both in the field (*in situ*) and in out-door experimental systems, to study of the interactive effects, includes variables as solar radiation, nutrients, temperature, acidification, etc. These studies can help to understand and assess the mechanisms of acclimatization to global climate change (Villafañe et al. 2003, Wiencke et al. 2004, Figueroa et al. 2009, Figueroa and Korbee 2010, Martínez et al. 2012). Previous studies on the physiological state of macroalgae to stress, have been evaluated by photoinhibition, photoprotection, systems integration and assimilation of nutrients,

growth patterns, reproduction and morphogenesis (Häder and Figueroa 1997, Villafañe et al. 2003, Stengel et al. 2014, Figueroa et al. 2014).

Thus, a novel aspect is to analyze the interaction of solar radiation with other climate variables such as temperature change and other anthropogenic disturbances such as the availability of nutrients in coastal waters. The interplay of factors can change the sign of the effect of a single factor (antagonistic effect) or, conversely, accentuate (synergistic effect). Therefore, the analysis of multiple factors acting at different rates and scales has recently become a "hot-point" of research (Xenopoulos et al. 2002, Doyle et al. 2005).

1.8 Structural bioindicators to evaluate the ecological status of coastal waters: can also be used as indicators of climate change?

The analysis of the impact of climate or global change on macroalgae can have consequences in the ecological status of the coastal waters. The Water Framework Directive (WFD, 2000/60/EC) of the European Parliament and Council establishing a framework for Community action in the field of water policy (WFD). Directive 2000/60/EC of the European Parliament and of the Council (23 October 2000) establishing a framework for Community action in the field of water policy, through of the definition of the Coastal waters, including their seabed and subsoil, are an integral part of the marine environment, and as such should also be covered by this Directive.
The ecological status is an expression of the quality of the structure and functioning of aquatic ecosystems associated with surface waters. The ecological status is directly related to human activities: urban, industrial and agricultural effluents, urban pressure on the line coast among others, therefore related to climate change impacts and consequences. The implementation of the action would increase the ecosystem capacity resilience and can reduce the vulnerability of these waters to climate change stresses. In the context the WFD, one of the four biological quality elements (BQEs) proposed for coastal waters are macroalgae. They have been used as good indicators of the water quality because their sedentary condition, integrate the effects of long term exposure of nutrient or other pollutants resulting in a decrease or even disappearance of the most (Díez et al. 1999), sensitive species and its replacement by highly resistant, nitrophilous or opportunistic species (Murray and Littler 1978). The use of macroalgae as bioindicators

to assess pollution in the marine environment has been proved successful in many ecological studies (Orfanidis et al. 2001, Arévalo et al. 2007).

Macroalgae has been used as biological indicators in different European geographical areas as region Atlantic Ocean by research groups of United Kingdom and Ireland (Wells and Wilkinson 2002, Wells et al, 2007), region Mediterranean by groups of Greece (Orfanidis et al, 2001), France (Thibaut et al. 2005). For example, the specific richness, Wilkinson and Tilley (1979) reported that the richness remains broadly constant in absence of environmental alterations, over days, month, season and years. Other example is the ratio of ecological-functional status group. Wells et al. (2007) proposed to functional groups according to the classification of Littler et al. (1983). In Spain, in the case of the waters of the coast of Catalonia, with use the Littoral and sublittoral Cartography (CARLIT) index (Ballesteros et al. 2007, Arévalo et al. 2007) and in Andalusia with use the Reduced Species List (RSL) index (Hernández et al. 2008, Bermejo et al. 2012) and CARLIT (Bermejo et al. 2013). Arévalo et al (2007) applied methods based in functional form group of macroalgae.

They reported changes in the species composition and structure of Mediterranean macroalgal dominated communities form upper sublittoral zone described along a gradient of nutrient enrichment form urban sewage outfall. *Ulva*-dominated communities only appear close to sewage outfall, *Corallina*-dominated communities replace ulvacean at intermediate levels of nutrient enrichment and *Cystoseira*-dominated communities thrive in the reference site. The functional group approach is the adequate since it is linked to the concept of bioindicator species and to the progressive increase in the structural complexity of aquatic ecosystems (Gorostiaga et al. 2008). *Cystoseira* species are considered to grow mainly in high-quality waters according to the criteria of Water Framework Directive of the European Union (WFD, 2000/60/EC) and they are indicator of waters with high quality ecological status including Andalusia Coast (according to Ballesteros et al. 2007, Arévalo et al. 2007, Bermejo et al. 2013).

1.9 Functional indicators to evaluate the impact of climate change factors on macroalgae

This study has been conducted in two regions according to WFD, Mediterranean and Atlantic. The interactive effects of photosynthetic irradiance, UV radiation, temperature,

nutrient availability and ocean acidification are studied with different approaches and geographical areas. Figueroa and Korbee (2010) suggested different functional indicators as complementary approach to indicators based in the structure communities, biodiversity and richness as it was commented above.

The functional indicators suggested and tested in the frame of the research in the Project entitled, *Ecological status and vulnerability of aquatic ecosystems to climate changes: biological, ecological and functional indicators monitoring the adaptation responses of macrophytes to the environmental stress* (ECOLIFE), and also in the research project: *Interactive effects of acidification and climate change variables (UV radiation and temperature) on the ecophysiology of marine macrophytes of Andalusian Mediterranean* (INTERACID) are as follows:

(1) Photosynthesis estimated as *in vivo* chlorophyll *a* fluorescence: optimal quantum yield and maximal electron transport rate as *in vivo* chlorophyll fluorescence associated to Photosystem II (PAM fluorometer)
(2) Functional indicators of nutrient status: stoichiometry i.e. C:N:P ratios.
(3) Functional indicator of Stress: heat shock proteins, proteases and reactive oxidative. Species (ROS) and other hand, antioxidants and photoprotectors.

In summary, the functional indicators can be useful for both the definition of the ecological status of the coastal waters in a more dynamic approach than that of structural based indicators (Figure 1.13). The combination and integration of morpho-function approach as it has have been conducted by different authors (Markager and Sand-Jensen 1994, Gómez et al. 2005, Gómez and Huovinen 2011, Figueroa et al. 2014), can help to improve predictions of the impact of global change on algal communities. In addition, functional indicators can be used as key variables to analyze the physiological responses to environmental changes both measured in the field or under controlled condition simulated the expected variation according to the models of climate change.

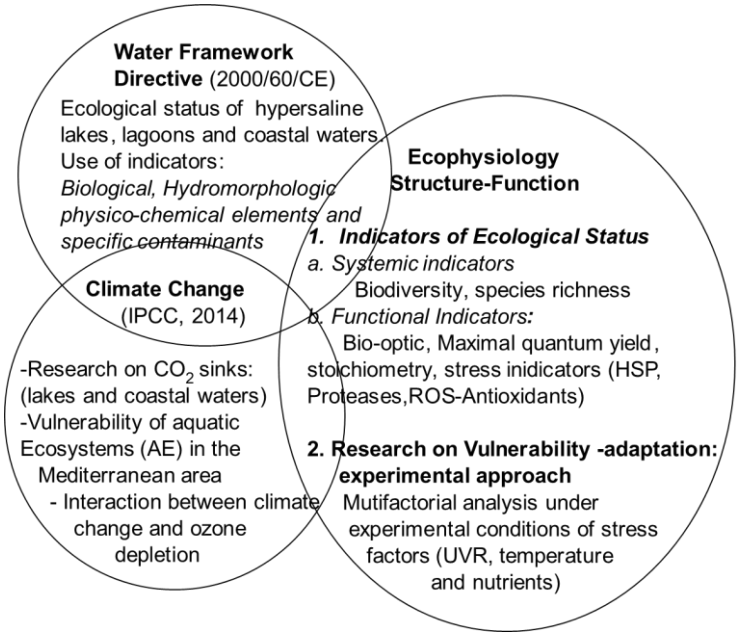


Figure 1.13 several criteria for integrate ecology and ecophysiological studies with the enriched interaction on methodology and design i.e. multifactorial approaches. Integrate trophic (energetic) and community approach. This is one of the most important threat of aquatic ecology. Integrate the designed investigation according to Water Framework Directive of UE and the research on the vulnerability and adaptation of organisms and impact on ecosystems of climate change.

1.9.1 Functional indicators: Photosynthesis estimated as *in vivo* chlorophyll *a* fluorescence

Historically, the gas measurements of either O_2 evolution or CO_2 fixation have been the most accepted methods to estimate photosynthetic activity. O_2 production has been measured by estimating the concentration of dissolved O_2 in water samples using colorimetric methods such as the Winkler method or electrochemically by using Clark-type electrodes. Carbon dioxide fixation has been widely measured by infrared gas analyzers (IRGA) and more recently by following the incorporation of inorganic radiolabeled $^{14}CO_2$ or $NaH_{13}CO_3$. The later commercialization of oxygen optodes sensors allowed for the optical estimation of oxygen evolution based on a luminescence reaction that it is quenched in the presence of oxygen. However, photosynthetic activity can be estimated by measuring the fluorescence emission or heat dissipation of PSII, which are directly related to photochemistry. By using *in vivo* fluorescence of chlorophyll *a* photosystem II (PSII) associated (Figure 1.14).

In a summarized way, fluorescence emission of chlorophyll *a* of PSII is a complementary

pathway to others two de-excitation processes, which also took place during the photosynthesis. The fluorescence emission competes with a photochemical energy conversion, which takes place in the reaction centres of PS II and nonradioactive energy dissipation, which occurs at the antenna and the reaction centres levels. Therefore, two basic types of fluorescence quenching, photochemical (qP) and non-photochemical (qN), can be distinguished. Thus, for a correct interpretation of fluorescence emission, it is necessary to know the relative contribution of each quenching mechanisms to the global de-excitation process (see Figure 1.14).

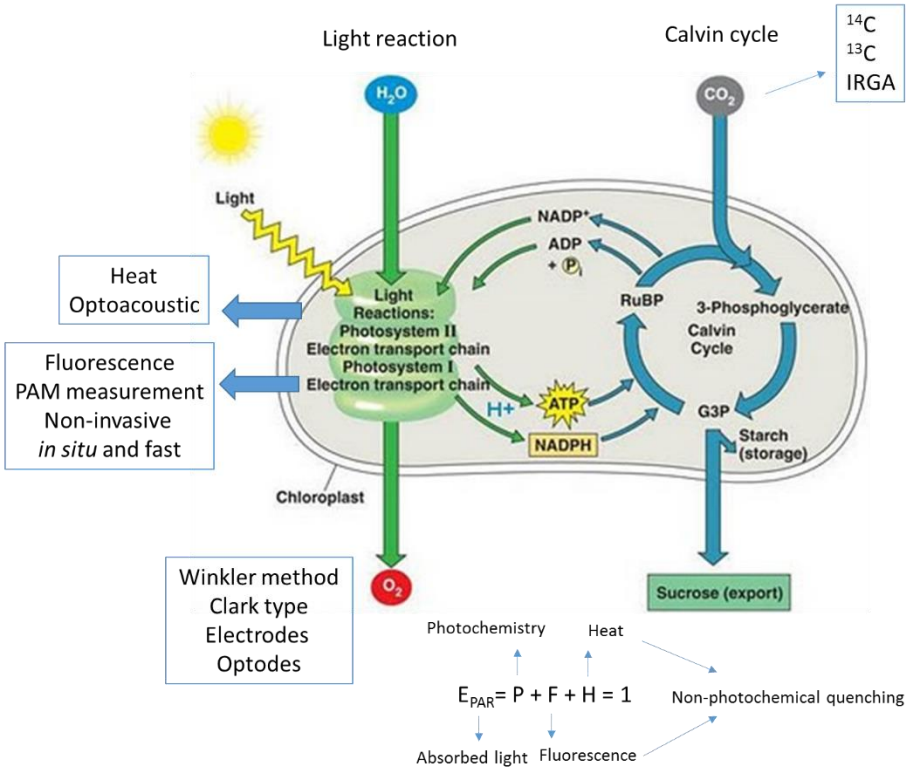


Figure 1.14 Global view of the photosynthetic processes and different techniques for measurements photosynthetic activity; Gas measurements of either O_2 evolution or CO_2 fixation, O_2 production has been measured by estimating the concentration of dissolved O_2 in water samples using colorimetric methods such as the Winkler method or electrochemically by using Clark-type electrodes. Oxygen optodes sensors allowed for the optical estimation of oxygen evolution based on a luminescence reaction that is quenched in the presence of oxygen. Carbon dioxide fixation measured by infrared gas analyzers (IRGA). In addition, photosynthetic activity can be estimated by measuring the fluorescence emission or heat dissipation of PSII, which are directly related to photochemistry through of the Pulse-amplitude modulated fluorometers (PAM).

To maintain high primary production and growth rates, the macroalgae must balance the light and dark reactions of photosynthesis. In the light reactions of photosynthesis, energy is captured and electrons are passed along different carriers to form the energy storage molecules ATP and NADPH (see Figure 1.15 for details).

Chemical energy produced during the light reactions is used to convert CO_2 into carbohydrates in the Calvin-Benson cycle (Figure 1.14). The dark reactions occur in the stroma and different enzymes, including ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), are involved in the reactions of the Calvin- Benson cycle. The energy necessary to fix one molecule of carbon dioxide during the dark phase is produced during the light reaction in the form of NADPH (two molecules) and ATP (three molecules). In addition, the reduction of a molecule of nitrate to glutamate requires one molecule of ATP per eight electrons provided by NADPH and reduced ferredoxin. Since the reaction centers of both photosystems must work in series for continuous oxygen evolution, meaning that it is necessary equal number of charge separations, and since four electrons (four photons in PSII) are required to oxidize two water molecules to evolve one molecule of oxygen, a minimum of eight photons are required for the production of each O_2. For each carbon fixed, two molecules of NADPH (four electrons) are required (Figure 1.15). Thus, the minimum quantum requirement for the fixation of one carbon atom is also eight photons (Kirk 1994, Raven and Johnson 2002) but 10 photons per oxygen produced or C assimilated is the more accepted ratio.

Photosynthetic components, that are involved in the light reactions are embedded in the thylakoid membrane and include photosystem II (PSII) (Figure 1.15), the cytochrome b6f complex, photosystem I (PSI), and the ATP-synthase complex. The electron transport chain between photosystem II and I leads to the formation of a protein (H+) gradient along the thylakoid membrane that provides energy for the synthesis of ATP by the ATP-synthase complex. In photosystem I, harvested light energy excites the reaction center protein P700, which loses electrons to the electron acceptor. The energy is then transferred to membrane ferredoxin (Fd) to NADP reductase (FNR) and consequently NADPH is formed. The production of NADPH leaves the photosystems with a deficit of electrons.

Therefore, the oxidized reaction center of photosystem I is restored to its original state by an electron from plastocyanin, whereas photosystem II is reduced by the oxidation of water, simultaneously releasing O_2.

The carbon and nitrogen assimilation by plants requires reducing power and ATP. Reducing power and ATP can be supplied by the light-dependent reactions of photosynthesis, or by glycolysis and respiration. The carbon skeletons are supplied by carbohydrate oxidation that ultimately are formed by photosynthetic carbon reduction.

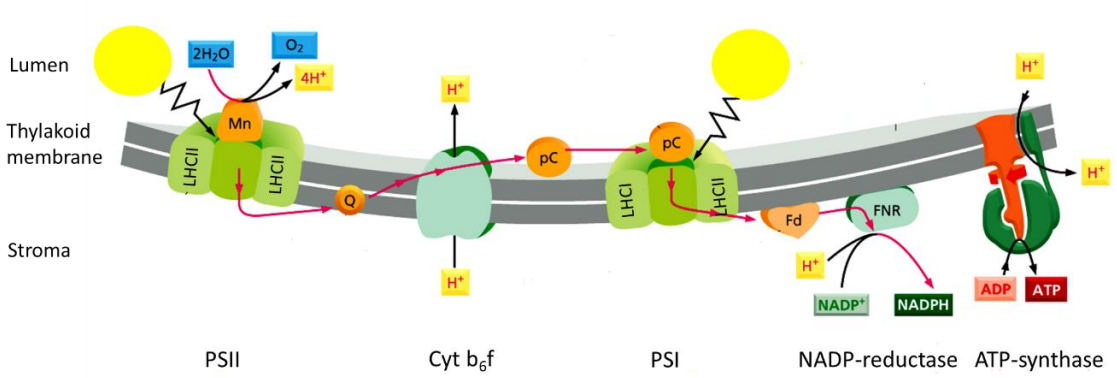


Figure 1.15 The light reactions of photosynthesis. During linear electron transport; light energy is captured by pigment-protein complexes (LHCII) in photosystem II (PSII) and transferred to the reaction center protein P680. When P680 acquires sufficient excitation, energy it loses electrons to a phaeophytin molecule and the two electron acceptors plastoquinone QA and QB are reduced (Mn). Electrons are transferred from the plastoquinone pool (Q) to photosystem I (PSI) by the subsequent reduction of the cytochrome b6f complex (Cyt b6f) and plastocyanin (PC). The electron transport chain between photosystem II and I leads to the formation of a protein (H+) gradient along the thylakoid membrane that provides energy for the synthesis of ATP by the ATP-synthase complex. In photosystem I, harvested light energy excites the reaction center protein P700, which loses electrons to the electron acceptor. The energy is then transferred to membrane ferredoxin (Fd) to NADP reductase (FNR) and consequently NADPH is formed. The production of NADPH leaves the photosystems with a deficit of electrons. Therefore, the oxidized reaction center of photosystem I is restored to its original state by an electron from plastocyanin, whereas photosystem II is reduced by the oxidation of water, simultaneously releasing O_2. In addition to alternative electron transport, excess light energy maybe dissipated as heat by non-photochemical (NPQ) processes in the light harvesting complexes, including xanthophyll pigment cycle.

Nitrogen assimilation is therefore a process intimately connected to carbon metabolism. Nitrogen assimilation pathway is a two-step process, first with NO_3^- reduction to NO_2^- through the Nitrate reductase (NR); (1) and then to ammonium throgh Nitrite reductase (NiR) (NH_4^+):

$$NO_3^- + NAD(P)H + 2e^- + H+ \rightarrow NO_2^- + NAD(P)+ + H_2O \quad (1)$$

Nitrate (NO_3^-) is taken up by the cells and translocated across the plasmalemma by energy-dependent processes. Once inside the cells, any excess can be stored within vacuoles, while a fraction is being metabolized in the cytoplasm by reduction to nitrite, via the enzyme nitrate reductase (NR), and using NAD(P)H as electron donor. In turn nitrite (NO_2^-) is transported to chloroplasts and reduced to ammonium, prior to assimilation into organic compounds by enzyme nitrite reductase (NiR), by means of reduced ferredoxin (Fdred) as electron source.

$$NO_2^- + 6Fd\ (red) + 6e^- + 8H+ \rightarrow NH_4^+ + 6Fd\ (ox) + 2H_2O \quad (2)$$

Nitrite and ammonium ions cannot be accumulating inside cells, as they are cytotoxic through producing pH change and inducing reactive nitrogen species (RNS) and oxidative damage. Consequently, their incorporation into organic compounds must be relatively fast, in order to prevent accumulation and toxicity. In the case of photosynthetic organisms, present a variety of mechanisms to regulate and control the expression of those enzymatic activities involved in nitrogen assimilatory pathways.

In vivo chlorophyll a fluorescence measurements

In order to conduct rapid light curves (RLCs) (according to Schreiber et al. 1995). *Fo* (basal fluorescence from fully oxidized reaction centers of PSII) and *Fm* (maximal fluorescence from fully reduced PSII reaction center), were determined in darkness to obtain the maximal quantum yield (F_v/F_m) being F_v the difference between *Fm* and *Fo* (see Figure 1.16) (Schreiber et al. 1995).

(1) Maximum quantum yield of PSII (F_v/F_m) as an indicator of physiological status of macroalgae and photoinhibition (Schreiber et al. 1986).
(2) Electron transport rate (ETR) to estimate of photosynthetic capacity (Figueroa et al. 2003).
(3) Non-photochemical quenching (NPQ) as an indicator of photoprotection.
(4) Photosynthetic pigments and thallus absorptance as indicators bio-optical characteristics.

The effective quantum yield ($\Delta F/Fm'$) was calculated according to Schreiber et al. (1995) (Fig. 1.16):

$$\Delta F/Fm' = (Fm' - F)/Fm' \quad (3)$$

Where *Fm´* is the maximal fluorescence induced with a saturating white light (halogen lamp) pulse and *F* the current steady-state fluorescence in light-adapted algae.

The Electron transport rate (ETR) was calculated according to Schreiber et al. (1995) as follows:

$$ETR\ (\mu mol\ electrons\ m^{-2}\ s^{-1}) = \Delta F/Fm' \times E \times A \times F_{II} \quad (4)$$

Where *E* is the incident PAR irradiance, *A*, is the thalli absorptance as the fraction of incident irradiance that is absorbed by the algae estimated by using a PAR sensor (see Figueroa et al. 2009) and F_{II} is the fraction of chlorophyll associated to PSII (400-700nm) being 0.8 in brown and 0.15 in red algae macroalgae (Figueroa et al. 2014).

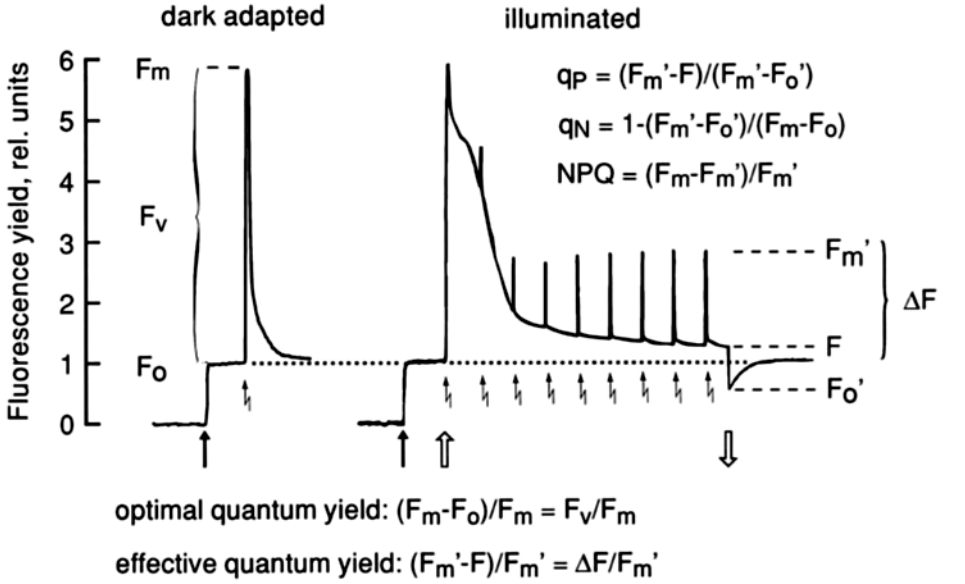


Figure 1.16 Kinetics of fluorescence emission determined by PAM fluorometer. Black arrows indicate the turning-on the measuring light; Break-arrow denoted saturation pulse; Red arrows mean turning-on and turning-off actinic light. Pink arrow indicates far-red pulse. The main fluorescence parameters are: Optimal quantum yield: F_v/F_m = $(F_m-F_o)/F_m$, Effective quantum yield: $\Delta F/F'_m$ = $(F'_m – Ft)/ F'_m$, Photochemical quenching: qP =$(F'_m-F_t)/(F'_m-F'_o)$, Non-photochemical quenching: qN =1-$[(F'_m-F'_o)/(F'_m-F_t)]$ and NPQ = (Fm-Fm')/Fm'.

ETR parameters as maximum ETR (ETR_{max}) and the initial slope of ETR versus irradiance curves (α_{ETR}) as estimator of photosynthetic efficiency, was obtained from the tangential function reported by Eilers and Peeters (1988). Thus, the electron transport rate (ETR) as rapid light curves (RLC) was determined after 20s of the exposure, to the twelve incremental irradiances (E1=9.3, E2=33.8, E3=76, E4=145, E5=217, E6=301, E7=452, E8=629, E9=947, E10=1403, E11=2084 and E12=3444 µmol m^2 s^{-1}) of white light by using the Diving-PAM equipment.

Quantum yield of non-light induced non-photochemical fluorescence quenching. The Y(NO) is fraction of energy passively dissipated in form of heat and fluorescence, mainly due to closed PSII reaction centers (Kramer et al 2004).

$$Y(NO) = F/F_m \quad (5)$$

Where *F* the current steady-state fluorescence in light-adapted algae and *Fm* the maximal fluorescence from fully reduced PSII reaction center.

Quantum yield of non-light induced (ΔpH and zeaxanthin-dependent) non-photochemical fluorescence quenching. The Y(NPQ) is fraction of energy dissipated in form of heat via regulated photoprotective NPQ mechanisms (Kramer et al. 2004).

$$Y(NPQ) = 1 - Y(II) - Y(NO) \quad (6)$$

Non-photochemical quenching (NPQ) was calculated according to Schreiber et al. (1995) as:

$$NPQ = (Fm-Fm')/Fm' \quad (7)$$

Maximal NPQ (NPQ_{max}) and the initial slope of NPQ *versus* irradiance curves (α_{NPQ}) were obtained from the tangential function of NPQ *versus* irradiance function according to Eilers and Peeters (1988).

1.9.2 Functional indicators: of nutrient status: stoichiometry i.e. C:N:P ratios

Stoichiometric ratios to evaluate the physiological status of the seaweeds (according to Figueroa and Korbee 2010). Sterner et al. (1997) proposed the hypothesis Light Nutrients (LNH**)** which predictive models. However, the investigation have treated partial aspects as structure and succession of nano-plankton (Xenopoulos and Frost 2003) or functional aspects of algal community (Xenopoulos et al. 2002, Lichtman et al. 2002). The nitrogen availability i.e. limitation affects a lot of process in macrophytes not only the photosynthetic capacity (Pérez-LLoréns et al. 1996) but also the content of proteins (Henly et al. 1991, Vergara et al. 1995), and photoprotection mechanisms (Korbee et al. 2005, Huovinen et al. 2006). The limitation of N reduces the cell size (García-Pichel 1994). The increase of the relation cell surface/volume not only favoured nutrient incorporation but also decrease the attenuation of UVR (antagonist effect). The accumulation of MAAs (photoprotectors) with the increasing of both nitrate and ammonium availability suggest that these substances can have several functions, UV-screen and antioxidant capacities but also reservoir of N (Korbee-Peinado et al. 2004,

Korbee et al. 2005, De la Coba 2009). The nitrogen of MAAs can be used when the N sources is reduced (anticipating strategy) as it has been suggested for phycobiliproteins (Algarra and Rüdiger 1993, Tandeu de Marsac and Houmard 1993, Talarico and Maranzana 2000, Singh et al 2008). The limitation of P also reduced the photosynthetic rate in macroalgae (Flores-Moya et al. 1997). The interaction UVR-P is very complex with both synergic or antagonist effect depending of the temporal scale (Medina-Sánchez et al. 2006).

1.9.3 Functional indicator of stress: biochemical indicators

Korbee et al (2010) suggested as functional indicator of environmental stress as follows: heat shock proteins, proteases and reactive oxidative species (ROS) and other hand antioxidants and photoprotectors. In this study, we have analysed as biochemical indicators, photoprotectors with antioxidant activity:

(1) Phenolic compounds, photoprotective compounds in brown algae (Pavia et al. 1997). In addition, antioxidant substances in brown algae measurement through DPPH assay (Connan et al. 2006).
(2) Mycosporine like aminoacids, as photoprotectors in red algae with antioxidant capacity (De la Coba et al. 2009).
(3) Carotenoids due to their antioxidant capacities (Takaichi 2011).

In high irradiance conditions such as Mediterranean waters, the concentrations of photoprotectors (i.e. polyphenols, mycosporine and xanthophyll cycle) (according to Connan et al. 2004, Korbee et al. 2004, Abdala-Díaz et al. 2006, Demmig-Adams and Adams 2006, Celis-Plá et al. 2014b) show phenotypic plasticity in response to changes in environmental parameters. Such as salinity, nutrients, light quality and irradiance availability, and herbivory (Pavia and Toth 2000, Honkanen and Jormalainen 2002, Swanson and Druehl 2002, Abdala-Díaz et al. 2006). The intertidal macroalgae can be under to high solar irradiance, survive and grow, under this stressful condition of the intertidal system, thanks to active photoprotection mechanisms, such as accumulation of UV screen substances and increase of antioxidant capacity, among others (Häder and Figueroa 1997, Hanelt and Figueroa 2012, Figueroa et al. 2014, Celis-Plá et al. 2014a).

(1) Phenolic compounds

Phlorotannins are a group of phenolic compounds. Phlorotannins constitute an extremely heterogeneous group of molecules (structure and polymerization degree heterogeneity) providing a wide range of potential biological activity (Table 3) (Burtin 2003). Phlorotannins are localized in physodes, which are membrane-bound cytoplasmic vesicles, and the fusion of physodes with cell membranes results in a secretion of phlorotannins (Bartsch et al. 2008, Li et al. 2009, Lüder and Clayton 2004).

Table 3. Figures are given in percentage of dry weight Gallic acid (*) equivalents and phloroglucinol (**).

Seaweed	***Concentration***	***Author***
Laminaria/Sacharina spp.	0.2-2.6% *	Connan et al. (2006)
	~0.2% *	Connan et al. (2004)
	<0.4% *	Repérez and Sausa- Calixto (2001)
	1.3-3.1% *	Hammerstrom et al. (1998)
	Up to 5.3% *	References in Horn (2000)
Fucus spp.	<0.4% *	Repérez and Sausa- Calixto (2001)
	0.7-8.5% *	Haug and Larsen (1958)
	1.0-12.2% *	Haug and Larsen (1958)
	>2% (2-6%) *	Connan et al. (2004)
Ascophyllum spp.	0.5-14% *	References in Horn (2000)
	4-13% *	Haug and Larsen (1958)
	4-13% *	Pavia and Åberg (1996)
	5% *	Connan et al. (2006) and Pavia et al. (1997)
Undaria spp.	<0.4% *	Repérez and Sausa- Calixto (2001)
Sargassum spp.	1.1-2.3% *	Wong and Cheung (2001a)
	2-3% *	Zubia et al. (2008)
	6% *	Connan et al. (2006)
	12.7% *	Cho et al. (2007)
Cystoseira tamariscifolia	3.1-3.6% **	Celis-Plá et al. (2014a)
	2.5-3.4% **	Celis-Plá et al. (2014b)
	2-8% **	Abdala-Díaz et al. (2006)
Cystoseira compressa	2.5% **	Celis-Plá et al. (2015)
Padina pavonica	1.2% **	Celis-Plá et al. (2015)
	0.5-1.0% **	Betancor et al. (2014)
Cystoseira humilis	2-12% **	Betancor et al. (2015)
Lessonia nigrescens	1-2.5% **	Gómez and Huovinen (2010)

In brown macroalgae, the UV screen compounds (Table 3), with antioxidant capacity are the polyphenols; the most common are the phlorotannins, halogenated polymers of phloroglucinol (1,3,5-trihydroxybenzene) accounting for 2-6% of the dry weight of the algae (Ragan and Glombitza 1986) (Table 3). Phlorotannins content, spatial and temporarily varies due to environmental factors such as the availability of nitrogen

(Ragan and Glombitza 1986, Arnold et al. 1995), UV radiation, solar and artificial (Pavia et al. 1997, Pavia and Brock 2000).

These compounds can be photoprotection mechanisms; absorb UV radiation (280-400 nm), chelate metal ions (Bischof et al. 2006, Hanelt and Figueroa 2012), and effective chemical defenses against some marine vertebrate and invertebrate herbivores (Steinberg 1992, Targett and Arnold 1998).

(2) Mycosporine like Amino acids

In red algae, the tolerance to inhibit the excessive light, including UV, is driven by the accumulation of mycosporine-like amino acids (MAAs) (De la Coba et al. 2009, Carreto and Carignan 2011). Mycosporine-like amino acids (MAAs) are water-soluble compounds that have in common a cyclohexenone or cyclohexenimine chromophore conjugated with the nitrogen substituent of an amino acid. MAAs are widely distributed among freshwater and marine organisms (Shick and Dunlap 2002). These compounds have been broadly studied because its photoprotection capacity as UV-screen substances that has been inferred from their efficient UV absorption and from their light-dependent synthesis induction (Karsten et al. 1998).

Nutrient enrichment increases the photoprotection capacity of seaweeds due to the increase in the protein content, MAAs (Korbee-Peinado et al. 2004, Huovinen et al. 2006, Figueroa et al. 2012). The main function of MAAs is photoprotection, especially for its ability to absorb short wavelengths of UVA and UVB regions of the spectra. This coupled with high photo-stability make them effective agents protective against UV radiation. Various types of aquatic organisms have been used to study the properties and functions of the MAAs (Korbee et al. 2006).

(3) Carotenoids

Other essential part of this set of photoprotective mechanisms relies on carotenoids, play a role in preventing damage in the photosynthetic apparatus. These act as light harvesting pigments and are important by quenching triplet chlorophyll (chl) molecules, by scavenging singlet oxygen and by regulation the rate of thermal energy dissipation (Demmig - Adams and Adams 2006, Esteban et al. 2008). Carotenoids are produced in plastids by successive action of enzymes on linear lycopene, which initially leads to the formation of α, and β-carotene. Zeaxanthin is produced through the β-branch of the carotenoid biosynthetic pathway by hydroxylation of β-carotene. The final steps

catalyzed by an epoxidase that forms anteraxanthin and violaxanthin (Esteban et al. 2008, Gross and Jakob 2010). One of the most important photoprotective mechanisms available to algae is an ability to dissipate excess thermal energy (Adams et al. 2006). The thermal dissipation measured, as non-photochemical PSII fluorescence quenching (NPQ) triggered by the trans-thylakoidal proton gradient (ΔpH) and zeaxanthin (ZEA) synthesis through the xanthophyll cycle (Gilmore 1997). In addition, recognized as the most important photoprotective mechanisms in higher plants and several algal divisions (Demmig-Adams and Adams 1996, Niyogi et al. 2001, Rodrigues et al. 2002).

Natural pH gradient, Vulcano, Italy. March 2013. Photograph by Paula S. M. Celis Plá

Thesis outline

Most studies are reduced to analyze the effects of the increase of CO_2, acidification, solar UV radiation or temperature separately and there is a lack of knowledge on the interactive effects of climate change variables on the structure, diversity, primary production of the macroalgae communities. It is important to analyze the interaction of climate change variables such as temperature and CO_2 increase and other anthropogenic changes under different light climate and nutrient availability. The present study aimed to fill gaps in this knowledge on the synergistic or antagonistic interaction of variables of the global change, vulnerability and capacity of acclimation of the macroalgae, under the solar radiation, nutrients, temperature, ocean acidification and heavy metals (copper). We carried out experiments both in the field (*in situ*) and in out-door experimental systems, using of the functional bioindicators for evaluating the ecophysiological responses in macroalgae.

The functional indicators not only will give us information on vulnerability and acclimation capacity to climate change factors. Moreover, they can help us to integrate morphofunctional responses providing for the ecophysiological responses and the knowledge can be transferred to improve the management of the aquatic environment in order to reach high/good ecological status according to Water Framework Directive (WFD) and Good Environmental Status (GES) according to The Marine Strategy Framework Directive (MSFD).

The following questions were addressed in this thesis and presented in six subchapters:

Subchapter 2.1 What is the vulnerability and capacity of acclimation of *Cystoseira tamariscifolia* through the seasons related to ecophysiological responses? Are there relations between photosynthetic activity, stoichiometry (C:N) polyphenols and antioxidant activity of this species and the changes through the year of physicochemical variables the coastal waters?

Expected results: *C. tamariscifolia* presents the highest production in spring since this is the season with favorable daily-integrated irradiance (high but not photoinhibitory), optimal temperature for growth (18-21^{o}C) and nutrient conditions. In summer time, acclimation mechanisms are activated as increased of phenolic compounds, antioxidant activity and energy dissipation (high non-photochemical quenching).

Subchapter 2.2 What is the vulnerability and capacity of acclimation of *Cystoseira tamariscifolia*, in algae collected in summer and winter, submitted to emersion/immersion regime (algae collected from rocky shores versus rocky pool collected algae, respectively), and incubated in an experimental controlled system under out-door conditions?

Expected results: algae from rocky shores present higher production than that collected from rockpools because the increased CO_2 supply during the emersion periods will favor photosynthetic activity and nitrate incorporation (during rehydration after drying periods). Since nitrogen was not limited during the experimental period, the physiological responses will be mainly affected by the natural solar irradiance and temperature conditions.

Subchapter 2.3 Are there interactive effects of irradiance and nutrient availability on photosynthesis and biochemical composition of *Cystoseira tamariscifolia* and *Ellisolandia elongata*, collected from two different depths, on the physiological and biochemical responses in oligotrophic waters?

Expected results: Nitrate enrichment will increase photosynthetic activity and photoprotection mechanisms based on the accumulation of photoprotectors with antioxidant capacity (Polyphenols and carotenoids) and the increased of non-photochemical quenching. Algae growing in shallow waters present higher resistance to increased irradiance than that form 2.0 m depth. Light and shade patterns in the photosynthetic equipment are expected according to the growth depth and short-term after the transplant of algae to increased or decreased irradiance. The ratio of C:N will be increased under light stress conditions.

Subchapter 2.4 How does the effects of copper on photophysiology and biochemistry in *Cystoseira tamariscifolia* can be modulated by nitrate enrichment?

Expected results: Nitrate enrichment reduced the potential toxic effect of Copper due to the increase of photosynthetic activity with interactive effects of nitrate-copper, synergic or antagonist depending on the concentration of copper. The increase of antioxidant substances and C:N are expected to be produced under stress conditions provoked by cooper at high level.

Subchapter 2.5 Are there interactive effects of ocean acidification on functional responses of *Cystoseira compressa* and *Padina pavonica* with irradiance and nutrient levels in a natural gradient of pH in an *in situ* coastal water study?

Expected results: Ocean acidification will induce carbon assimilation and photosynthesis in the non-calcareous alga *Cystoseira compressa* whereas the physiological state of the calcareous macroalga *Padina pavonica* will be negative affected by decalcification. Nitrate enrichment can reduce photoinhibition in algae exposed to high solar irradiance rate due to increase photosynthetic activity. Antioxidant activity and C:N ratio increased in the unfavorable environmental condition for each species.

Subchapter 2.6 How is changed the physiological pattern and biochemical composition in an experimental mesocosms study according to two levels of CO_2 and temperature simulating expected future levels of both variables of *Cystoseira tamariscifolia* collected in two sites, one from ultra-oligotrophic and other from oligotrophic coastal waters?. Are there interactive effects of CO_2 and temperature?

Expected results: functional indicators related to photosynthesis and internal nitrogen will be increased by CO_2 and nutrient enrichments under ambient temperature. High temperature and low nitrate availability produce an increase of biochemical responses against the stress (increase of polyphenols and antioxidant activity) with an increase of C:N ratio. Ultraoligotrophic grown algae are expected to have more accelerated responses than that in oligotrophic waters for acclimation to stress conditions.

To answer these questions, six experimental studies were performed under varying irradiance, temperature, nutrient conditions, acidification and metals. The common approach in these studies was the evaluation of the functional bioindicators and comparison between macroalgae species of Mediterranean Sea (Alboran Sea) in studies conducted both in situ in ultra-oligotrophic (Cabo de Gata-Níjar Natural Park, Almeria) and oligotrophic waters (La Araña, Malaga) and with algae transported to controlled experimental systems under out-door conditions. In addition, an in-door experiment study was performed in *Cystoseira tamariscifolia* collected in the North Atlantic Ocean, the northern limit of distribution of this species.

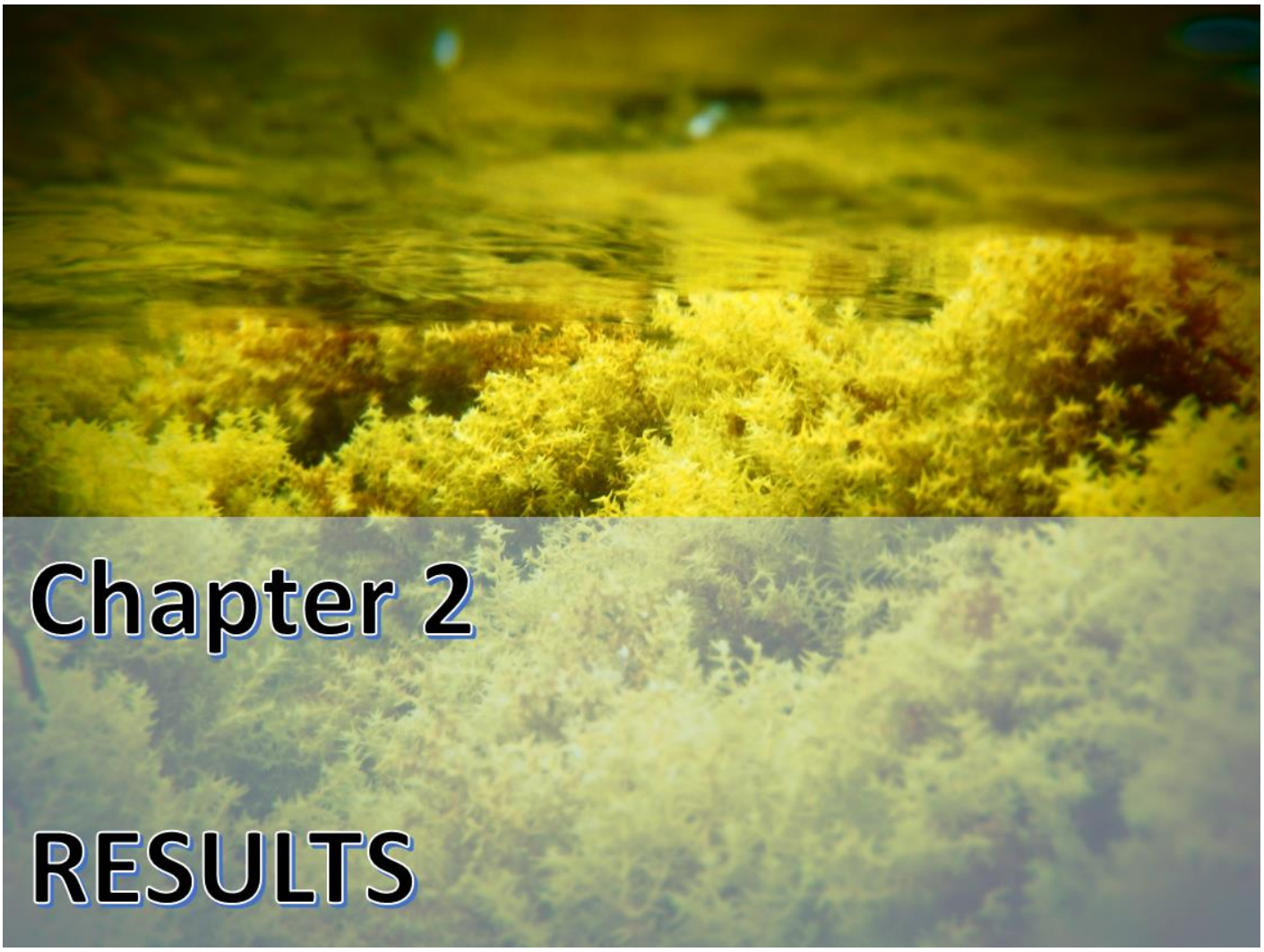
In ***Subchapter 2.1***, address of the first of the main objectives proposed. In chapter 1, are assessed the ecophysiological responses throughout the season for two years in the intertidal macroalga *Cystoseira tamariscifolia* (Ochrophyta) in oligotrophic coastal

waters affected by urban activity (La Araña, Malaga). In ***Subchapter 2.2***, are assessed in out-door experimental system, the vulnerability and capacity of acclimation of *Cystoseira tamariscifolia* according to physiological and biochemical pattern Subchapter 1). The experiments were conducted with macroalgae collected from La Araña (Málaga) in two periods of the year (summer and winter) and from two sites , rocky shores (algae with emersion phases during the daily cycle) and rocky pools (algae always immersed during the daily cycle).

In ***Subchapter 2.3***, Irradiance effects in short-term time were studied in interaction with nutrient availability. Ecophysiological and biochemical responses were assessed in *Cystoseira tamariscifolia* and *Ellisolandia elongata* collected from two different growth depths (0.5 and 2.0 m) in Cabo de Gata-Nijar Natural Park (ultraoligotrophic waters) and incubated to 100% and 70% as a transplant experiment approach. The principal aim of this chapter was investigate on if there are independent and/or interactive effects of ambient radiation and nutrient availability. In ***Subchapter 2.4***, interactive effects of nutrient availability and copper on photophysiology and biochemistry in *Cystoseira tamariscifolia* collected in the North of Atlantic Ocean (in the limit north of the distribution for this species) was assessed in door under laboratory conditions.

In ***Subchapter 2.5***, the effects of two irradiance and nutrient levels *in situ* in a natural coastal water pH gradient on photosynthesis and biochemical composition in a non-calcifying and calcify brown macroalgae *Cystoseira compressa* and *Padina pavonica*, respectively, were analyzed in the context of the interactive responses to nutrient, light and CO_2.

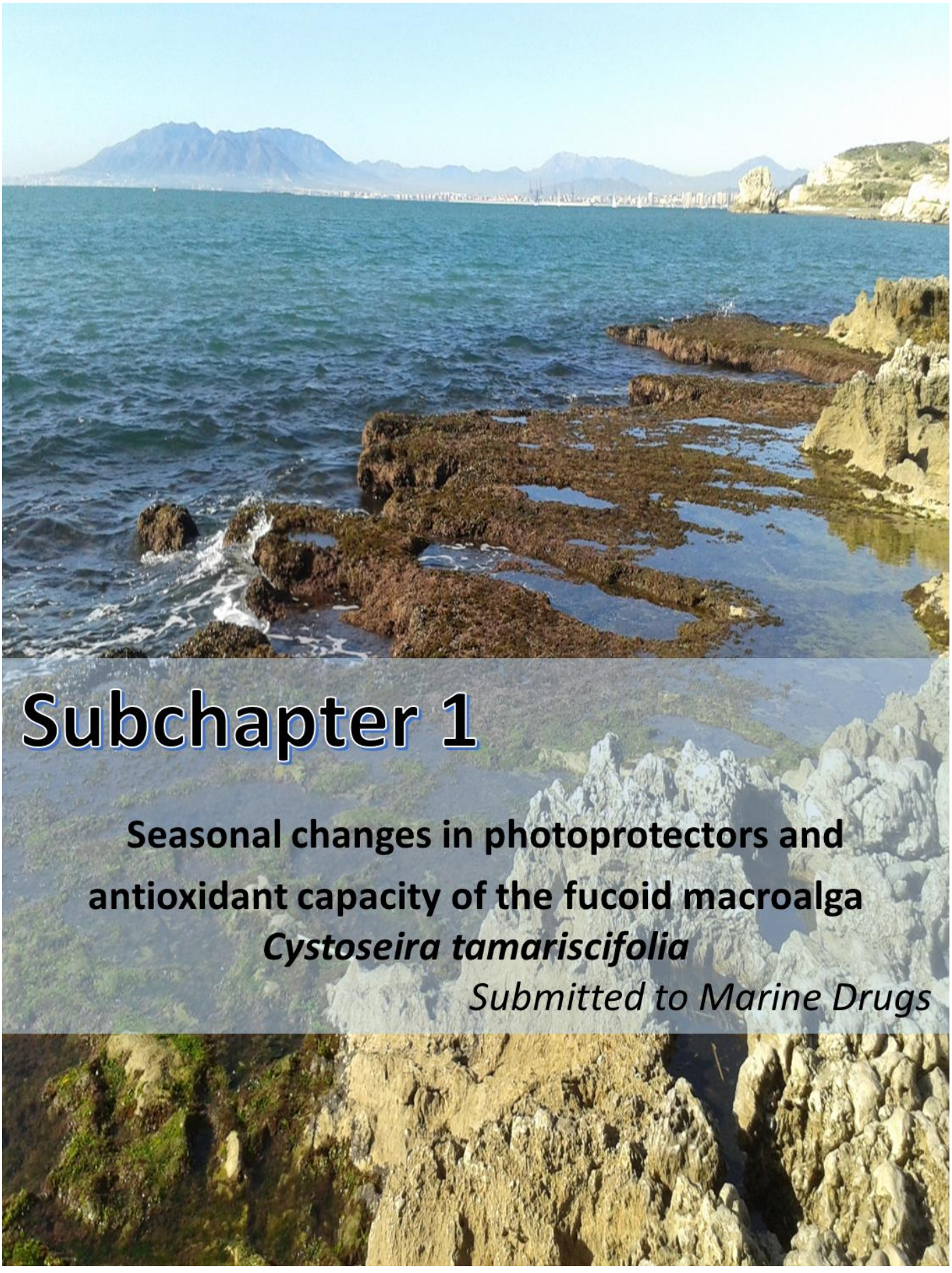
Finally, in ***Subchapter 2.6***, temperature and CO_2-dependent processes in two populations of the *Cystoseira tamariscifolia* collected from ultraoligotrophic and oligotrophic waters, were studied in out-door experimental system to assess the interactive effects of temperature and CO_2 expected levels in future scenarios. Growth, photo-physiology and biochemical responses were assessed to analyze the possible effects of global warming on macroalgal communities and species distribution.



Chapter 2

RESULTS

Cabo de Gata-Níjar, Natural Park. September 2012. Photograph by Paula S. M. Celis Plá



La Araña Beach. Winter 2013. Photograph by Paula S. M. Celis Plá

Seasonal changes in photoprotectors and antioxidant capacity of the fucoid macroalga *Cystoseira tamariscifolia*

Paula S.M. Celis-Plá[1,2*], Zenilda L. Bouzon[3], Jason M. Hall-Spencer[4], Eder C. Schmidt[3], Nathalie Korbee[1] and Félix L. Figueroa[1]

[1]Departamento de Ecología, Universidad de Málaga, Campus de Teatinos s/n, 29071 Málaga, España.
[2]Laboratorio de Botánica, Facultad de Farmacia, Universidad de Barcelona, Joan XXIII s/n, Barcelona 08028, España.
[3]Plant Cell Biology Laboratory, Department of Cell Biology, Embryology and Genetics, Federal University of Santa Catarina, 88049-900, CP 476, Florianópolis, SC, Brazil.
[4]Marine Biology and Ecology Research Centre, Plymouth University, Plymouth PL4 8AA, UK.

*Corresponding author at: Tel: +34952131672; fax: +34952137386, e-mail address: paulacelispla@uma.es

1.1 Abstract

Seaweeds produce chemicals that have widespread commercial applications; here we assesses seasonal changes in the biochemistry and photophysiology of the fucoid *Cystoseira tamariscifolia* in southern Spain. Total carbon and nitrogen contents, phenolic compounds, antioxidant activity and photosynthetic activity were determined seasonally over two years. Carbon, nitrogen and photoprotective phenolic contents were higher in winter and spring than in summer and autumn. Antioxidant activity was highest in spring and there was a positive correlation between phenolic compounds and antioxidant activity (EC_{50}). Photosynthetic capacity (ETR_{max}) and photosynthetic efficiency (α_{ETR}) were highest in spring and there was a positive correlation between ETR_{max} with phenolic content. Increased irradiance in spring enhanced algal productivity, antioxidant activity and the production of photoprotective compounds but in summer, nutrient depletion due to thermal stratification of the coastal waters reduced the photoprotective capacity of *C. tamariscifolia*. In conclusion, spring would be the best period to harvest *C. tamariscifolia* to extract photoprotectors and antioxidants for commercial products, although the environmental impacts would need to be carefully assessed.

Keywords: Algal productivity, Antioxidants, *Cystoseira tamariscifolia*, Chlorophyll fluorescence, Phenols, UV protection, Mediterranean Sea, Phycology.

1.2 INTRODUCTION

Macroalgae in temperate regions, such as the southern coast of Iberian Peninsula in Spain , are exposed to dramatic daily and seasonal changes in photosynthetically active (PAR) and ultraviolet (UV) light (Häder and Figueroa 1997, Gómez et al. 2001). They have evolved photoprotective mechanisms to help them cope with high light levels; intertidal algae can prevent UV damage using polyphenols that help dissipate light energy (Bischof et al. 2006, Goss and Jakob 2010, Hanelt and Figueroa 2012). They also use antioxidants as carotenoids i.e. xanthophyll cycle in photoprotection although they can be also accessory light harvesting pigments. Carotenoids are highly efficient natural scavengers of O_2 and play an essential role in the protection of plants against excess light and photooxidative stress (Demmig-Adams and Adams, 2002, Cantrell et al. 2003, Stahl and Sies 2007).

In brown algae, phenolic compounds are acetate-malonate-derived polymers of phloroglucinol (Sattler 1974) which can be accumulated under high irradiance and act as photoprotectors against UV radiation and they are powerful antioxidants, as scavenging harmful reactive oxygen species (Cruces et al. 2012). In addition, phenolics help reduce DNA damage (Gómez and Huovinen 2010), they can chelate metal ions, reducing metal toxicity (Connan et al. 2004, Stengel et al 2005), and they are effective chemical defences against a wide range of herbivores (Steinberg 1992, Arnold et al. 2012).

In stressful conditions, phenolics can be released from thalli that react rapidly with proteins and carbohydrates to form a UV-absorbing exudate (Swanson and Druehl 2002, Koivikko et al. 2005, Celis-Plá et al. 2014a). Phlorotannin contents show phenotypic plasticity in response to environmental changes, factors as salinity, nutrients, light, and herbivory (Swanson and Druehl 2002, Abdala-Díaz et al. 2006, Celis-Plá et al. 2014b). Phlorotannins derived from marine brown algae have been investigated for their medical benefits, including anti-inflammatory and hyaluronidase inhibitory activities (Vinay and Kim 2012). Ranges of compounds produced by brown algae are incorporated into nutraceutical (a portmanteau of the words nutrition and pharmaceutical) products as antioxidants (Ahn et al. 2007, Heo et al. 2009). And for purported benefits as photoprotectors, as antiplasmin inhibitors, to reduce allergies, for skin whitening,

anti‑HIV-1, antibacterial, and anticancer activities (Sugiura et al. 2007, Artan et al. 2008, Le et al. 2009, Heo et al. 2010).

In this study, we investigated on *Cystoseira tamariscifolia* (Hudson) Papenfuss, (Phaeophyceae, Fucales) which is can be abundant in waters of high ecological quality in the Mediterranean (Bermejo et al. 2013). We use standard biochemical indicators (C, N, photoprotectors and antioxidant concentrations) and photosynthetic physiology (maximum quantum yield of photosystem II and electron transport rate) to evaluate environmental stress in this macroalga, adopting methods used by Figueroa and Korbee (2010) and Celis-Plá et al. (2014a). In addition, we examined the cell morphology of *C. tamariscifolia* in summer, to explore the location the phenolic compounds.

Our aim was to assess seasonal variability in the abundance of commercially valuable UV screen and antioxidant compounds in *C. tamariscifolia* to inform potential exploitation of these resources. In addition, we assessed the effects of seasonal changes in solar radiation on photosynthetic activity (as *in vivo* chlorophyll fluorescence), polyphenol content and antioxidant activity in this habitat-forming macroalga and we provide detailed cell morphology of this alga.

1.3 MATERIALS AND METHODS

Sampling

Nine *Cystoseira tamariscifolia* thalli were collected at least 2 m apart at 0.1-0.4 m above Chart Datum at 10 am monthly from July 2012 to June 2014 from rocky substrata on La Araña beach, Málaga, Spain (36º 42'N, 4º 19'W). Live material was transported in cooled containers and samples were frozen *in situ* using liquid nitrogen for biochemical analyses. Photographs of the habitat were taken in spring, summer, autumn and winter (Figure 1).

Abiotic parameters

Photosynthetic active radiation (PAR, λ=400-700 nm), Ultraviolet A radiation (λ=320-400 nm) and Ultraviolet B radiation (λ=280-320 nm) (Figure 2) was measured continuously in air using an UV-PAR Multifilter radiometer NILU-6 (Geminali AS, Oslo, Norway) located on the roof Central Services for Research building (University of Málaga).

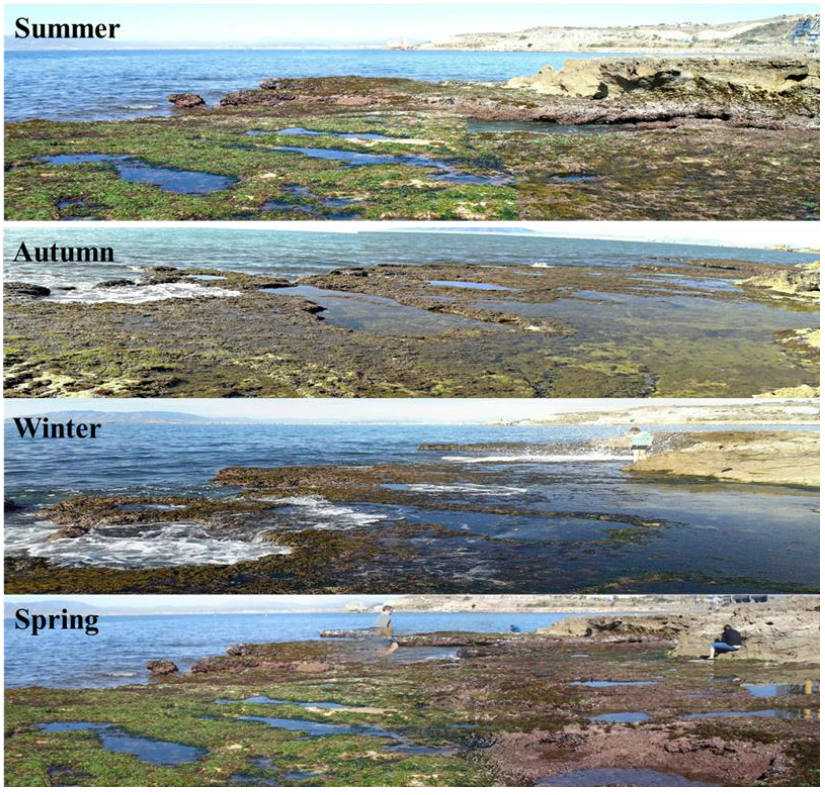


Figure 1. La Araña rocky shore in southern Spain showing high perennial coverage of the brown alga C*ystoseira tamariscifolia* and spring/summer blooms of *Ulva* spp. in 2013.

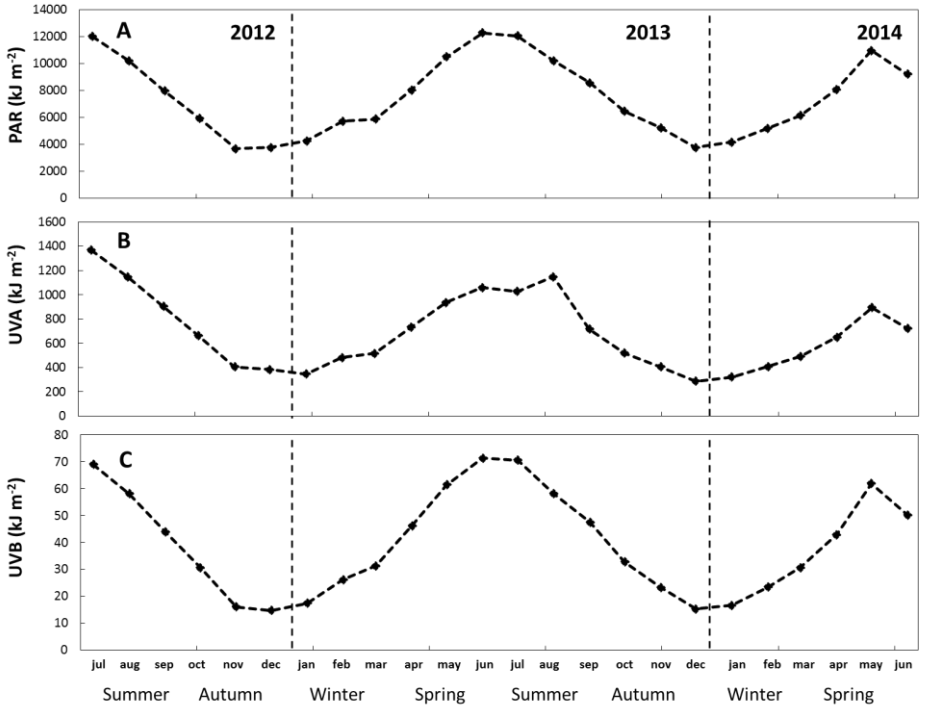


Figure 2. Daily integrated irradiance per month of A) PAR (400-700 m), B) UVA (320-400 nm) and C) UVB (280-320 nm) at La Araña rocky shore in 2012- 2014.

Seawater temperature was logged every minute of the day at Coast Network of wave monitoring buoys of state harbours of Spain, number 1514 (REDCOS) located at (36º 42'N, 4º 19'W). Seawater nitrate (µmol L^{-1}), ammonium (µmol L^{-1}), phosphate (µmol L^{-1}) and N: P ratio and Chlorophyll *a* (µmol L^{-1}) (Table 1), data were obtained from Ramírez et al. (2005) and Mercado et al. (2007, 2012) from 36º 60'N 4º 10'W.

Cell morphology

Macroalgal tissue samples ca 5 mm in length were collected in summer 2013; for light microscopy these were and fixed in 2.5% paraformaldehyde in 0.1 M (pH 7.2) phosphate buffer overnight then dehydrated in aqueous ethanol solutions before infiltration with Historesin (Leica Historesin, Heidelberg, Germany). Then, 5 µm length sections were stained with 0.5% Toluidine Blue (TB-O), pH 3.0 (Merck Darmstadt, Germany) and investigated with an epifluorescence microscope (Olympus BX 41) equipped with Image Q Capture Pro 5.1 Software (Qimaging Corporation, Austin, TX, USA). Samples for Transmission Electron Microscopy were fixed with 2.5% glutaraldehyde, 2.0% paraformaldehyde, and 5 mM $CaCl_2$ in 0.075 M sodium cacodylate buffer (pH 7.2) plus 0.2 M sucrose and caffeine 1% overnight. The material was then fixed with 1% osmium tetroxide for 4 hours, dehydrated in a graded acetone series and embedded in Spurr's resin. Thin sections were stained with aqueous uranyl acetate followed by lead citrate. Four replicates were made for each experimental group; two samples per replicate were then examined under TEM (JEM 1011 JEOL Ltd., Tokyo, Japan, at 80 kV).

Biochemical variables

The dry weight of algal carbon and nitrogen contents was determined using an element analyzer (model CNHS-932, LECO Corporation, Michigan, USA). Polyphenol concentrations were measured using 0.25 g fresh weight samples pulverized in a mortar and pestle with sea-sand using 2.5 mL of 80% methanol. This mixture was stored overnight at 4ºC then centrifuged at 4000 rpm for 15 min at 4ºC and the supernatant was collected to measure the phenolic compound content colorimetrically using Folin-Ciocalteu reagent. Phloroglucinol (1,3,5-trihydroxybenzene, Sigma P-3502) was used as standard. Finally, the absorbance was determined at 760 nm using a Shimadzu UVMini-1240 spectrophotometer. Phenolic concentration was expressed as mg g^{-1} dry weight after

determining the fresh to dry weight ratio in the tissue (the ratio was 5.6). The results are expressed as average ± Standard Error from 9 replicates.

The antioxidant activity DPPH (2,2-diphenyl-1-picrylhydrazyil) assay (i.e. EC_{50}), of seaweed extracts was estimated by reducing the stable free radical DPPH. The supernatant used for phenolic compound measurements was used for DPPH analysis; 150 µL of DPPH prepared in 90% methanol (90MeOH: $10H_2O$) were added to each extract. The reaction was complete after 30 min in a dark dark room at ~20° C and the absorbance was read at 517 nm in a spectrophotometer (UVMini-1240 model, Shimadzu, Columbia, USA). A calibration curve made with DPPH was used to calculate the remaining concentration of DPPH in the reaction mixture after incubation. Values of DPPH concentration (mM) were plotted against plant extract concentration (mg DW mL^{-1}) to obtain the oxidation index EC_{50}, which represents the concentration of the extract, expressed as mg DW mL^{-1}, required to scavenge 50% of the DPPH in the reaction mixture. Ascorbic acid was used as positive control (Celis-Plá et al. 2014a).

Photosynthetic physiology

In vivo chlorophyll *a* fluorescence associated with Photosystem II was determined using a portable pulse amplitude modulated fluorometer (Diving-PAM, Walz GmbH, Germany). Apical pieces of macroalgal thalli were introduced in 10 mL incubation chambers to obtain rapid light curves for each treatment. F_o and F_m were measured after 15 minutes in darkness to obtain the maximum quantum yield (F_v/F_m) being $F_v=F_m-F_o$, F_o the basal fluorescence of 15 min dark adapted thalli and F_m maximal fluorescence after a saturation light pulse of >4000 µmol m^{-2} s^{-1} (Schreiber *et al*., 1995). The electron transport rate (ETR) was determined after 20 s exposure in twelve increasing irradiances of actinic white light (halogen lamp provided by the Diving-PAM) according to Celis-Plá et al. (2014a). The ETR was calculated according to Schreiber et al. (1995) as follows:

$$ETR\ (\mu mol\ electrons\ m^{-2}\ s^{-1}) = \Delta F/F'_m \times E \times A \times F_{II} \quad (1)$$

Where $\Delta F/F'm$ is the effective quantum yield, being $\Delta F = Fm'-Ft$ (Ft is the intrinsic fluorescence of alga incubated in light and Fm' is the maximal fluorescence reached after a saturation pulse of algae incubated in light). E is the incident PAR irradiance expressed

in µmol photons m^{-2} s^{-1}, A is the thallus absorptance as the fraction of incident irradiance that is absorbed by the algae (see Figueroa et al. 2003) and F_{II} is the fraction of chlorophyll related to PSII (400-700 nm) being 0.8 in brown macroalgae (Figueroa et al. 2014). ETR parameters as maximum electron transport rate (ETR_{max}) and the initial slope of ETR versus irradiance function (α_{ETR}) as estimator of photosynthetic efficiency were obtained from the tangential function reported by Eilers and Peeters (1988). Finally, the saturation irradiance for ETR (Ek_{ETR}) was calculated from the intercept between ETR_{max} and α_{ETR}. Non-photochemical quenching (NPQ) was calculated according to Schreiber et al. (1995) as:

$$NPQ= (Fm-Fm')/Fm' \quad (2)$$

Maximal NPQ (NPQ_{max}) and the initial slope of NPQ *versus* irradiance function (α_{NPQ}) were obtained from the tangential function of NPQ *versus* irradiance function according to Eilers and Peeters (1988).

Statistical analyses

Pearson correlation coefficients were calculated and tested between all measured dependent variables. Interactive effects between physiological variables were analyzed using ANOVA. This test was performed for *C. tamariscifolia* including year and season (two-way) as fixed factors for biochemical variables and season (one-way) with four levels, for the photosynthetic variables. Homogeneity of variance was tested using Cochran tests and by visual inspection of the residuals. Student Newman-Keuls tests (SNK) were performed after significant ANOVA interactions. All data conformed to homogeneity of variance. Analyses were carried out using SPSS v.21 (IBM, USA).

1.4 RESULTS

Environmental conditions

Cystoseira tamariscifolia was abundant in all seasons, along with abundant *Ulva* spp. in spring and summer (Figure 1). The seawater temperature ranged from 14-23 ^{o}C (Table 1) with a peak summer average daily irradiance of *ca*. 10165 kJ m^{-2} for PAR, 1051 kJ m^{-2}

for UVA and 57.5 kJ m^{-2} UVB (Figure 2A-C). Seasonal nitrate (NO_3^-) concentrations ranged from 0.6-1.5 mg L^{-1} (Ramírez et al. 2005, Mercado et al. 2007, 2012).

Table 1. Seasonal changes in mean (± SE) surface seawater temperature (mean values ± SE, n=2144) (according to REDCOS) and salinity, nitrate, ammonium, phosphate and N: P ratio (mean values ± SE, n=180) off La Araña near Málaga (Southern Spain) according to Ramírez et al. (2005) and Mercado et al. (2007, 2012).

	Units	Summer	Autumn	Winter	Spring
Tº	ºC	18.91±2.09	17.81±1.66	14.88±0.45	15.91±1.14
Salinity		36.87±0.29	36.72±0.34	36.93±0.28	37.14±0.45
Nitrate	µmol L^{-1}	0.58±1.07	0.62±0.77	1.52±1.07	1.59±1.44
Ammonium	µmol L^{-1}	0.53±0.75	0.19±0.27	0.18±0.10	0.35±0.20
Phosphate	µmol L^{-1}	0.12±0.08	0.14±0.01	0.14±0.05	0.15±0.09
N:P molar ratio		4.3±6.6	7.4±10.9	13.4±12.3	16.0±21.3
Chlorophyll *a*	µmol L^{-1}	0.92±0.69	1.21±0.94	1.22±1.14	1.45±0.99

Seawater nitrate concentrations were approximately 2.5 times higher in winter and spring than in summer and autumn. Ammonium (NH_4^+) varied through the year from 0.1 to 0.5 mg L^{-1} and was 2.7 times higher in summer than in autumn and winter, 1.4 times higher than in spring. The phosphate (PO_4^{3-}) concentration varied little (0.12 to 0.15 mg L^{-1}) in all seasons. Chlorophyll *a* concentrations were highest in spring with 1.45 mg L^{-1} and lowest in summer with 0.92 mg L^{-1}, respectively (Table 1).

Morphological observations

Cystoseira tamariscifolia samples have olive green in colour, with a cylindrical frond and branches irregularly (Figure 3A).

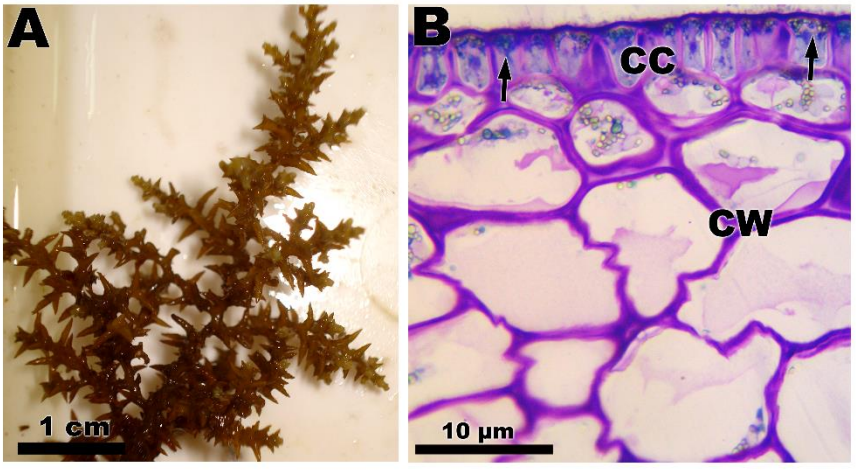


Figure 3. A) *Cystoseira tamariscifolia* from La Araña in Summer 2013, B) detail of cortical cells (CC) showing metachromatic reaction in the cell walls (CW), indicating the presence of acidic sulfated polysaccharides. Note the presence of physodes (arrows).

C. tamariscifolia was stained with toluidine Blue had a metachromatic reaction in the cell wall, indicating the presence of acidic sulphated polysaccharides.

In the cytoplasm of cortical and subcortical cells, a large quantity of dark blue and yellow physodes was observed (Figure 3B, arrows). In the cortical cells the physodes are located near in the surface of thallus (Figure 3B, arrows). When observed by transmission electron microscopy, the cortical cells had numerous chloroplasts (Figure 4A) and physodes (Figures 4A and C) with a thick cell wall (Figures 4A and C) that was embedded with phenolic compounds (Figure 4B arrows). Mitochondria were associated with the chloroplasts (Figure 4D) which had the typical internal organization of brown algae with thylakoids aggregated in bands (Figure 4D). Lipid droplets (plastoglobuli) were situated between the thylakoids (Figure 4D) and plasmodesmata cell connections were seen (Figure 4E).

Biochemical responses

The carbon and nitrogen content of *C. tamariscifolia* was significantly higher in winter and spring; the C: N ratio was significantly lower in winter (Figure 5, S1). The amount of phenols was also significantly affected by season, being highest in spring when whereas antioxidant activity (EC_{50}) was significantly reduced (Figure 5, S1).

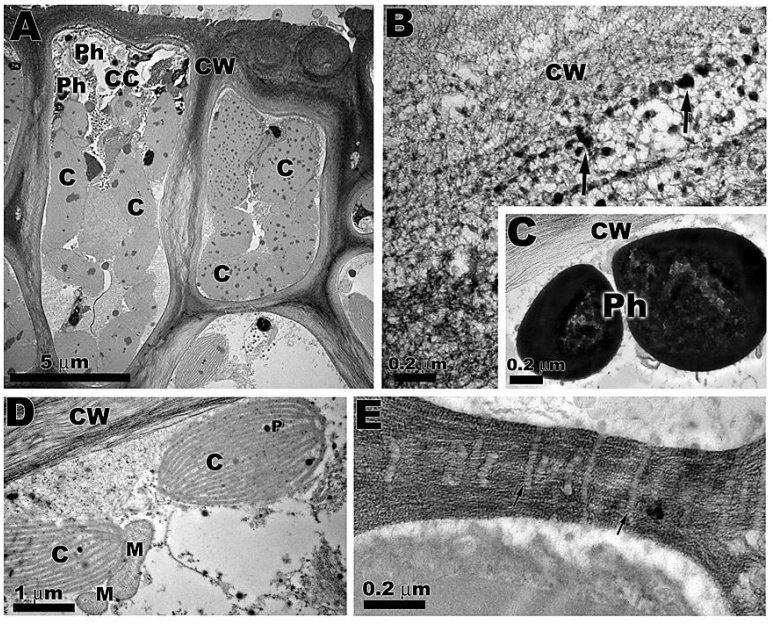


Figure 4. Transmission electron microscopy images of *Cystoseira tamariscifolia* from La Araña in summer 2013. A) Cortical cell showing a many chloroplasts (C), physodes (Ph) and thick cell wall (CW). B) Arrows indicate present of phenolic compounds in cell wall. C) Detail of physodes in cortical cell. D) Chloroplast with plastoglobuli (P) and associated mitochondria (M). E) Detail of plasmodesmata (arrows).

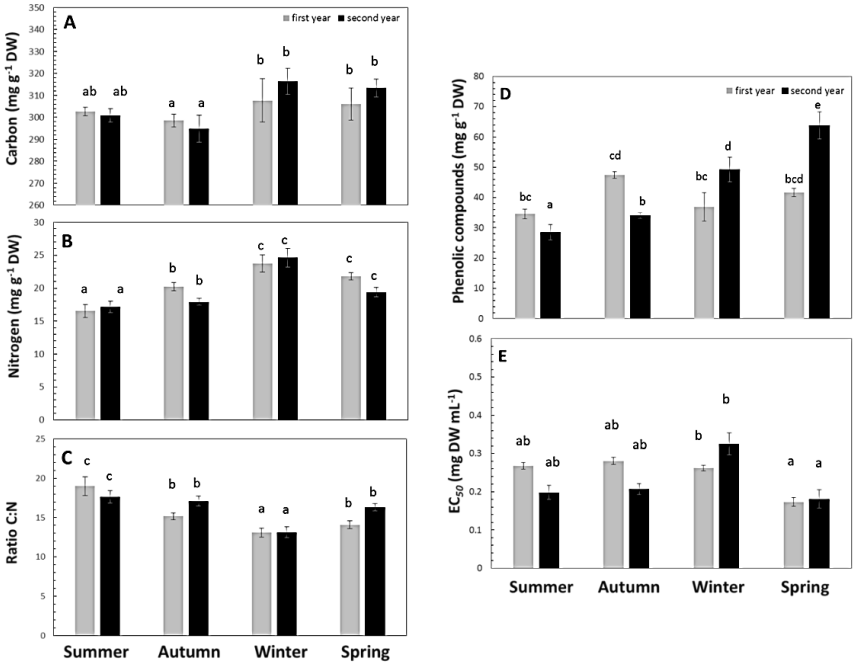


Figure 5 A) Carbon internal content (expressed as mg g^{-1} DW), B) Nitrogen internal content (expressed as mg g^{-1} DW) and C) Ratio C:N to *Cystoseira tamariscifolia* in season (summer, autumn, winter and spring). D) Phenolic compounds (expressed as mg g^{-1} DW) and E) Antioxidant activity (EC50; expressed as mg DW mL^{-1}) to *Cystoseira tamariscifolia* in season (summer, autumn, winter and spring).

Physiological responses

F_v/F_m was not significantly affected by season although it tended to be higher in winter; maximal electron transport rate (ETR_{max}) was the highest in spring and photosynthetic efficiency (α_{ETR}) was significantly affected by season, being lowest in winter (Table 2, S2). The irradiance of saturation of curve (Ek_{ETR}) was not significantly affected by season, but tended to be higher in winter and spring.

Table 2. Photosynthetic physiology of *Cystoseira tamariscifolia* on a rocky shore at La Araña beach, Málaga (Southern Spain) in summer, autumn, winter and spring 2013-14. Maximal quantum yield (F_v/F_m), maximal electron transport rate (ETR_{max}, expressed in µmol m^{-2} s^{-1}), photosynthetic efficiency (α_{ETR}), irradiance of saturation of ETR (Ek_{ETR}), maximal non-photochemical quenching (NPQ_{max}), irradiance of saturation of NPQ (Ek_{NPQ}) and ETR_{max}/NPQ_{max} (mean ± SE, n=9). Lower-case letters denote significant differences after SNK test.

	Summer	Autumn	Winter	Spring
F_v/F_m	0.71 ± 0.01	0.71 ± 0.02	0.69 ± 0.02	0.71 ± 0.01
ETR_{max}	52.18 ± 3.39^{a}	53.01 ± 2.23^{a}	55.14 ± 4.29^{a}	70.65 ± 6.58^{b}
α_{ETR}	0.41 ± 0.02^{b}	0.39 ± 0.01^{b}	0.27 ± 0.01^{a}	0.36 ± 0.03^{b}
Ek_{ETR}	137.95 ± 0.06	136.52 ± 17.81	235.64 ± 49.84	272.38 ± 84.51
NPQ_{max}	1.39 ± 0.11	1.27 ± 0.16	1.61 ± 0.18	1.38 ± 0.14
Ek_{NPQ}	301.28 ± 33.22^{ab}	395.56 ± 48.98^{b}	190.99 ± 27.83^{a}	295.61 ± 66.12^{ab}
ETR_{max}/NPQ_{max}	42.32 ± 4.91	52.14 ± 15.68	38.77 ± 3.93	61.96 ± 7.43

The highest non-photochemical quenching (NPQ_{max}) occurred winter, although no statistically significant seasonal differences were found (Table 2, S2). The irradiance of saturation (Ek_{NPQ}) was significantly higher in autumn and the relationship between ETR_{max} (production) and NPQ_{max} (photoprotection) was highest in spring (Table 2, S2). A positive correlation was found between phenolic compounds and antioxidant activity, between antioxidant activity and nitrogen internal content, through all seasons, and positive correlation between EC_{50} and ETR_{max} and between photosynthetic efficiency (Table S3).

1.5 DISCUSSION

In brow algae, antioxidant, photoprotective, antiplasmin, antiallergic, antiviral antibacterial and anticancer properties have been reported (Sugiura et al. 2007, Artan et al. 2008, Heo et al. 2010). For example, he phloroglucinol from *Ecklonia cava* are widely used in Asian medicines, foods and cosmetics (Le et al. 2009). In our study, we found that as the short days of winter lengthened into spring in southern Spain this stimulated an upsurge in photoprotectors, antioxidants, and productivity in *Cystoseira tamariscifolia* as they laid down stores of nitrogen and carbon. The phenol and antioxidant capacity of this seaweed fell in summer, which we suggest was due to nutrient depletion as sea surface waters became gradually more oligotrophic due to thermal stratification in the heat of the Mediterranean summer.

Where nutrient levels permit, brown algal phenols are stimulated by high light levels (Pavia and Brock 2000) but peak phenol content is often not found in summer since nitrate concentrations can become limiting (Pavia and Toth 2000, Cabello-Pasini et al. 2011). This certainly seems true for *C. tamariscifolia*, which has higher phenolic contents when nitrates are most abundant (Celis-Plá et al. 2014b). In addition, the photoprotection in brown macroalgae, is regulated through the released of the phenols, thus, the produce the phenols during periods of UVR stress, and phenolics can be released into seawater, both are considerate such as other mechanism of the photoprotection (Swanson and Druehl 2002, Koivikko et al. 2005, Polo et al. 2014, Celis-Plá et al. 2014a).

In this study, the highest content of phenolic compounds in *C. tamariscifolia*, reached in springtime. PC was about 5-7.0% which is within the range of the highest levels found in

brown algae from northwest Europe (Pavia and Åberg 1996, Connan et al., 2004), southern Chile (Gómez and Huovinen 2010, Cruces et al. 2012) and in *Cystoseira* spp. from other areas of Mediterranean sea (Abdala-Díaz et al. 2006, Celis-Plá et al. 2014b, Celis-Plá et al., 2015). The level of phlorotannins of brown algae of Southern Chile were lower than that found in *C. tamariscifolia* in this study in spite of the light and nutrient levels are high in Southern Chile. In addition to the possible differences due to they are different species, other explanation is that the samples collected in the field in Gómez and Huovinen (2010) and Cruces et al. (2012) were not immediately preserved in liquid nitrogen as in this study. As in other reports, phenolic compounds were clearly located by using light and electron microscopy in phenolic contain vesicles known as physodes which are presented more in cortical compared to medullary cells (Schoenwaelder 2008, Gómez and Huovinen 2010).

Cystoseira tamariscifolia is able to acclimate to high UVB by upregulating UV screen substances that also act as antioxidants (Figueroa et al. 2014). Connan et al. (2004) showed that mid-shore brown algae (such as *Fucus spiralis*, *F. vesiculosus*, *Ascophyllum nodosum*) tend to have higher phenol content and antioxidant activity, than those found in the low intertidal or sublittoral zone (such as *F. serratus*, *Bifurcaria bifurcata*, *Himathalia elongata* and *Laminaria digitata*). This suggest that the PC is to protect them against exposure to higher UV irradiance levels.

The results of the internal N and C, show a nutritional state seems to be more favorable in winter and spring than in summer and autumn grown algae as a lower C:N in algae collected in winter and spring were found. This suggest close relationship with production of phenols in this season time. Additionally, algae collected in summer and autumn seemed not to accumulate N compounds after nitrate enrichment due to a low uptake rate, this suggest high acclimation in high irradiance, because seemed that algae accumulate N during winter and spring as reservoir for periods of the high irradiance. This result could be related with the high amount of energy that this algae demands during summer, the period in which the activation of photoprotection and acclimation mechanisms may occur (Figueroa et al. 2014). This could also be an indicator of a good physiological status, i.e., accumulation of secondary metabolic compounds in nutrient replete conditions to be used

under stress conditions as high irradiance and low nitrate conditions (Celis-Plá et al. 2014a).

C. tamariscifolia collected in summer, spring and winter had higher values of NPQ than those collected in autumn. Demmig-Adams and Adams (2006) described a high values of NPQ indicate active photoprotective mechanisms, which are highly related to the xanthophyll cycle. Maximal photosynthetic capacity (i.e. ETR_{max}) and photosynthetic efficiency (i.e. α_{ETR}) were higher in *C. tamariscifolia* collected in springtime. In spring, high daily integrate irradiance of PAR (*ca* 102.72 MJm^{-2} and 18.1ºC) favored photosynthetic activity in *C. tamariscifolia* compared with the winter and autumn period (*ca* 51.5 MJm^{-2} and 15.5ºC). These results suggest more productivity in *C. tamariscifolia* in this season time.

These seasonal and environmental variations on the biochemical composition and bioactivity of compounds in *C. tamariscifolia* given important information for the correct management in this species. Due to the harvesting of macroalgae without a correct planification, can drastically reduce the natural populations, it is important to have knowledge of the reproductive phases and vegetative biomass for proper management from natural populations for correct management. In *C. tamariscifolia,* the thalli are fertile throughout the year, although receptacles are most developed in spring and summer (Gómez-Garrta et al. 2001).

Some examples of the excessive harvesting it has been shown in 2011. *Fucus serratus*, *Palmaria palmata* and *Porphyra* spp. were harvested to 328 tons, 322 tons and 25 tons, respectively in Brittany (unpublished data). The establishment of a canopy of Ulva spp., a seasonal opportunistic green alga (Stagnol et al. 2013), amplified harvesting impact on the community. Other example, is in the coasts of Brittany (France), these seaweeds have been collected during the middle of the 16th century for the iodine industry and are now harvested for their alginates (Bixler and Porse 2010). Some studies have shown that commercial harvesting has different effects according to the target species and is necessary make emphasizes the long-term monitoring of the natural populations of seaweeds.

1.6 CONCLUSIONS

Higher acclimation capacity and less vulnerability of *C. tamariscifolia* was higher in algae collected in spring than the rest of seasonal periods. Antioxidant activity increase in spring as the carbon and nitrogen internal and phenolic compounds. This suggest their uses as antioxidants to prevent photodamage. In addition, phenol production and antioxidant activity indicate that under the high irradiance, *C. tamariscifolia* presents a high capacity for photoprotection as it has been reported in experiments conducted in mesocosms exposing the algae to variation of light, temperature and CO_2 and nutrient levels. In contrast, in summer time the algae presented high dynamic photoinhibition of photosynthesis, with a decrease in electron transport rate, and increase of high non-photochemical quenching, active photoprotective mechanisms, which are highly related to the xanthophyll cycle.

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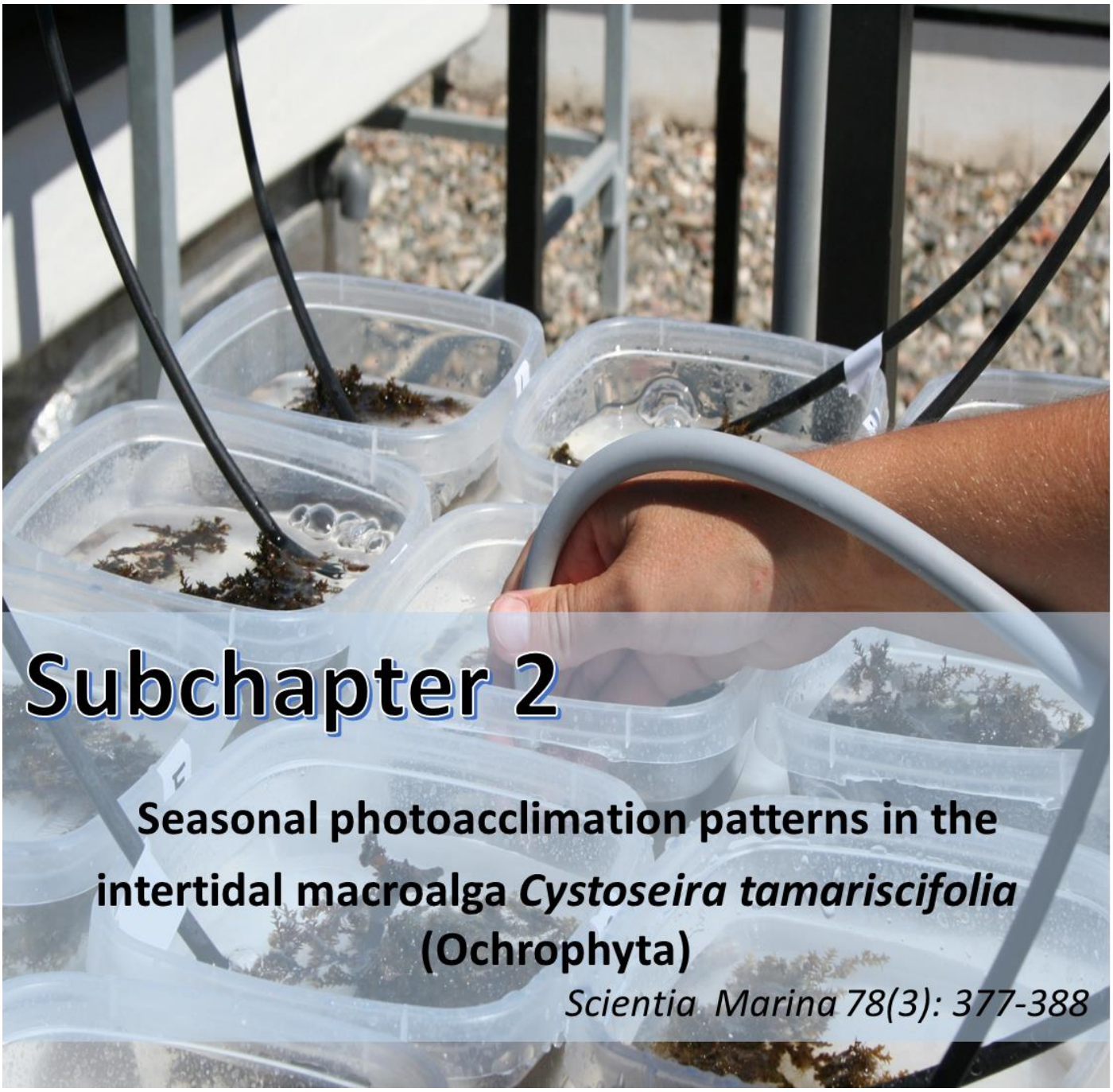

Subchapter 2

Seasonal photoacclimation patterns in the intertidal macroalga *Cystoseira tamariscifolia* (Ochrophyta)

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Málaga. Summer 2011. Photograph by Paula S. M. Celis Plá

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Seasonal photoacclimation patterns in the intertidal macroalga *Cystoseira tamariscifolia* (Ochrophyta)

Paula S. M. Celis-Plá [1,2], Nathalie Korbee [1], Amelia Gómez-Garreta [2], Félix L. Figueroa [1]

[1] Departamento de Ecología, Universidad de Málaga, Campus de Teatinos s/n, 29071 Málaga, España.
E-mail:paulacelispla@uma.es
[2] Laboratorio de Botánica, Facultad de Farmacia, Universidad de Barcelona, Joan XXIII s/n, 08028 Barcelona, España

2.1 Summary: *Cystoseira tamariscifolia* thalli collected from rocky shores and rockpools in winter and summer in Southern Spain were incubated for 7 days in UV transparent cylindrical vessels under outdoor conditions. Photosynthetic activity estimated as in vivo chlorophyll *a* fluorescence of photosystem II, photosynthetic pigments, antioxidant activity (DPPH assay), phenolic compounds and total internal C and N contents were determined after short-term (3 d) and mid-term (7 d) periods. Maximum quantum yield of PSII (F_v/F_m) was significantly higher in field-collected algae and after 7 d incubation in winter than in summer. In rocky shores and rockpools thalli, maximum electron transport rate (ETR_{max}) and photosynthetic efficiency (α_{ETR}) were much higher in summer than in winter. ETR of outdoor-grown thalli (in situ ETR) showed a daily pattern, with a decrease at noon in both winter and summer (3rd and 7th days). We found much higher antioxidant activity in thalli collected in summer than in winter. However, the concentration of internal UV screen substances (polyphenols) was higher in winter than in summer, whereas the release of phenolic compounds was lower. The highest capacity of acclimation in *C. tamariscifolia* found in summer and RS with emersion periods was explained by the highest dynamic photoinhibition, energy dissipation (non-photochemical quenching) and antioxidant activity (EC_{50}).

Keywords: antioxidant activity; *Cystoseira tamariscifolia*; phenolic compounds; photoinhibition; photoprotection.

Patrones estacionales de fotoaclimatación en el alga intermareal, *Cystoseira tamariscifolia* (Ochrophyta)

Resumen: Talos de *Cystoseira tamariscifolia* recolectados en pozas y plataformas rocosas intermareales (Sur de España) en invierno y en verano se incubaron bajo radiación solar durante 7 días en recipientes cilíndricos de metacrilato transparentes a la radiación UV. Se estimó la actividad fotosintética a través de la fluorescencia de la clorofila *a* asociada al fotosistema II, el contenido de pigmentos fotosintéticos y compuestos fenólicos, actividad antioxidante y el contenido total en C y N internos tras 3 y 7 días de incubación. Los valores iniciales del rendimiento cuántico máximo (F_v/F_m) fueron significativamente mayores en algas recolectadas en invierno que en verano mientras que la tasa de transporte electrónico máximo (ETR_{max}) y la eficiencia fotosintética fueron mayores en verano que en invierno en ambas zonas. Por otra parte, la tasa de transporte electrónico determinada bajo radiación solar presentó un patrón diario, con una disminución a mediodía, tanto en invierno como en los períodos de verano. La actividad antioxidante fue mayor en algas recogidas en verano; sin embargo, la concentración interna de compuestos fenólicos fue mayor en invierno que en verano, mientras que en la tasa de excreción se observó lo contrario. La alta capacidad de aclimatación en *C. tamariscifolia* en algas sometidas a emersión en las plataformas rocosas en verano se explica por su alta fotoinhibición dinámica, su capacidad de disipación de energía (amortiguamiento no fotoquímico, NPQ) y su actividad antioxidante (EC_{50}).

Palabras clave: actividad antioxidante; *Cystoseira tamariscifolia*; compuestos fenólicos; fotoinhibición; fotoprotección.

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2.2 INTRODUCTION

Macroalgae in temperate regions, such as the Mediterranean coast of Spain, are exposed to high daily integrated solar irradiance, both ultraviolet (UV) and photosynthetically active (PAR) (Häder and Figueroa 1997). The high irradiance and transparency of shallow water in this region suggest that macroalgae have de-

veloped efficient photoprotection mechanisms to tolerate light stress (Figueroa and Gómez 2001). In fact, intertidal macroalgae subject to high solar irradiance and desiccation can survive and grow under the stressful conditions of the intertidal system due to active photoprotection mechanisms such as dynamic photoinhibition, accumulation of UV screen substances and increase in antioxidant capacity (Häder and Figueroa 1997, Korbee et al. 2006, Bischof et al. 2006, Hanelt and Figueroa 2012).

Brown algae accumulate UV screen compounds (polyphenols) under high PAR and UVR (Pavia et al. 1997). In addition to the UV screen capacity, phenolic compounds also have strong antioxidant activity (Connan et al. 2006, Cruces et al. 2012), thus reducing DNA damage (Gómez and Huovinen 2010). Phlorotannins are phenolic compounds identified in brown algae constituting up to 25% dry weight (Targett et al. 1992). Concentrations of phlorotannins show phenotypic plasticity in response to changes in environmental parameters, such as salinity, nutrients, light quality and irradiance availability, and herbivory (Peckol et al. 1996, Pavia et al. 1997, Pavia and Toth 2000, Honkanen and Jormalainen 2002, Swanson and Druehl 2002, Abdala-Díaz et al. 2006). Phenolic compounds can also be released from the thalli into alkaline medium of seawater under stressful conditions, reacting rapidly with both proteinaceous and carbohydrate substances to form UV-absorbing complexes (Dujmov et al. 1996, Swanson and Druehl 2002, Koivikko et al. 2005). Hence, it is very important to determine both internal and released polyphenols in order to evaluate the photoprotective capacity of these compounds (Koivikko et al. 2005, Gómez and Huovinen 2010, Cruces et al. 2012).

Cystoseira species are considered to grow mainly in high-quality waters according to the Water Framework Directive of the European Union (WFD, 2000/60/EC) and they are indicator of waters with high-quality ecological status including the Andalusia Coast (Ballesteros et al. 2007, Arévalo et al. 2007). In our study, *Cystoseira tamariscifolia* (Hudson) Papenfuss, was selected as a model species because it is an abundant and is a key species on the southern shores of the Mediterranean Sea. The evaluation of photosynthetic and antioxidant activities in thalli collected at different places in short spatial scales such as rocky shores (RS, thalli exposed to the air during some time during the daily cycle) and rockpools (RP, thalli always immersed in the seawater and with a high rate of water renewal) can give information on the vulnerability and acclimation capacity of this species to environmental changes. In addition, it is important to analyse the relation between photosynthetic activity and energy dissipation by using *in vivo* chlorophyll *a* fluorescence and polyphenol content and antioxidant activity in thalli collected in summer and winter and submitted to an emersion/immersion regime (RS- versus RP-collected thalli) and incubated under outdoor conditions.

Two physiological indicators have been used to evaluate the physiological status of seaweeds (Figueroa and Korbee 2010): (1) maximum quantum yield of PSII (F_v/F_m) as an indicator of physiological status of macroalgae and photoinhibition (Schreiber et al. 1986) and (2) electron transport rate (ETR) as an indicator of photosynthetic capacity (Figueroa et al. 2003). Two biochemical indicators of stress conditions have also been used: (1) stoichiometric ratios (C:N) as an indicator of nutritional status and (2) the content of phenolic compounds, such as photoprotective and antioxidant substances in brown algae (Pavia et al. 1997, Connan et al. 2004, Abdala-Díaz et al. 2006).

The hypothesis is that thalli submitted to high stress conditions in the natural environment have a greater acclimation capacity and less vulnerability to increased solar irradiance in the short to medium term (3 and 7 days).

2.3 MATERIALS AND METHODS

Species and sampling

C. tamariscifolia (Hudson) Papenfuss, (Phaeophyceae: Fucales) was randomly collected in winter (February, 2011) and summer (June and July, 2011) at La Araña beach, Málaga, southern Spain (36°42'N, 4°19'W) in the morning (before 11:00 am local time). The samples were collected in RS (areas in high zones of the platform) and in RP (with a high rate of water renewal). RS-collected thalli are exposed to air during low tide, when they are subjected to temperature and desiccation stress, while RP-collected thalli are always submerged but may be exposed to temperature stress when the pool is isolated from the sea during low tide. Thalli were transported under cold conditions to the laboratory in order to avoid damage to the biological material. Rocky shores and rockpools are very close to each other (a distance of less than 1.0-1.5 m). Samples for biochemical analysis were frozen in situ using liquid nitrogen.

Experimental design

C. tamariscifolia were acclimated for 48 h in a polyvinyl methacrylate UV transparent vessel (Plexiglass XT- 29080) with a final volume of 1.5 L seawater covered with two layers of neutral density filters to remove 65% of full solar radiation (PAR+UVA+UVB, mesh with pore size 1 mm^2) in order to reduce the risk of photoinhibition during the acclimation period in the experimental vessels. Twelve cylindrical vessels with 250 g of thalli were placed in tanks of 250 L with circulating fresh water to control the temperature of the system. After the acclimation time, thalli were incubated to full solar radiation in the same outdoor systems located on the roof of the building of the Central Services for Research (University of Malaga) for 7 days. The experiment was performed in both winter (from 8 to 16 February 2011) and summer (from 27 June to 5 July 2011). Six replicates for thalli collected from RS and another six for those collected from RP were used in each period. Seawater was N-enriched at the beginning of the experiment with $NaNO_3$ reaching a maximum final concentration of 50 µM NO_3^-. Seawater was renewed and N-enriched in the experimental vessels after

3 days. The incubation temperature reached maximum temperature values of 18°C in winter and 22°C in summer, with temperature oscillations during the day and night of 2-3°C in winter and 1-2°C in summer. The temperature was maintained by using a cooling unit Titan-500 (Aqua Medic, Bissendorf, Germany) with a submersible pump for water circulation (Ocean runner OR Aqua Medic, Bissendorf, Germany). The tempera- ture was measured using a HOBO U22 Water Temp Pro v2 logger (Onset Computer Corporation, Mas- sachusetts, USA). Algae were continuously aerated inside the cylinders using a 3010-1 HPEMODEL air pump (HPE Technology, Barcelona, Spain). Measure- ments of photosynthetic parameters and biochemical analysis were done in field-collected algae and after
3 and 7 days of incubation. Samples for biochemical analysis were stored at –80°C until analysis.

Incident solar irradiance of PAR (400-700 nm), UVA (320-400 nm) and UVB (280-320 nm) was meas- ured continuously in air using an NILU-6 UV-PAR Multifilter radiometer (Geminali AS, Oslo, Norway). The irradiances of UVA and UVB were calculated from the data of the different UV filters according to Høiskar et al. (2003).

Photosynthesis and energy dissipation by using in vivo chlorophyll *a* fluorescence

In order to conduct rapid light curves (RLCs) according to Schreiber et al. (1995), apical algal pieces were collected from each treatment in field-collected algae and after 3 and 7 days of incubation (in the morning) and introduced in incubation chambers with
10 mL seawater at the 12 incremental irradiances (E1=9.3, E2=33.8, E3=76, E4=145, E5=217, E6=301, E7=452, E8=629, E9=947, E10=1403, E11=2084 and E12=3444 µmol photons m^{-2} s^{-1}) of white light (halo- gen lamp provided by the Diving-PAM).

F_0 (basal fluorescence from fully oxidized reaction centers of PSII) and F_m (maximum fluorescence from fully reduced PSII reaction centre) were determined after 15 minutes in darkness to obtain the maximum quantum yield (F_v/F_m), F_v being the difference between F_m and F_0 (Schreiber et al. 1995).

The effective quantum yield ($\Delta F/F_{m'}$) was calcu- lated according to Schreiber et al. (1995):

$$\Delta F/F_{m'} = (F_{m'} - F)/F_{m'} \quad (1)$$

where $F_{m'}$ is the maximum fluorescence induced with a saturating white light (halogen lamp) and F is the cur- rent steady-state fluorescence in light-adapted thalli.

The ETR was calculated according to Schreiber et al. (1995) as follows:

$$\text{ETR (µmol electrons } m^{-2}\ s^{-1}) = \Delta F/F_{m'} \times E \times A \times F_{II} \quad (2)$$

where E is the incident PAR irradiance, A, is the ab- sorptance of the thalli of the fraction of incident ir- radiance estimated using a PAR sensor with a cosine response (Licor 192 SB) according to Figueroa et al. (2009), and F_{II} is the fraction of chlorophyll associated with PSII (400-700 nm) being 0.8 in brown macroalgae (Figueroa et al. 2014). Both maximum ETR (ETR_{max}) and the initial slope of ETR versus irradiance curves (α_{ETR}) as an estimator of photosynthetic efficiency were obtained from the tangential function reported by Platt and Gallegos (1980).

In addition, to test and characterize the effect of the light quality changes by decreasing the irradiance of the Diving-PAM halogen lamp, i.e. to decrease the propor- tion of blue light (Hanelt et al. 2003), effective quan- tum yields were also measured using red light (light- emitted diodes) provided by PAM-2000 or Water PAM fluorometer. No significant differences were found in the effective quantum yield data in RLCs conducted by halogen lamp (Diving-PAM) and red light of PAM-
2000 and Water PAM in a wide range of irradiances from 18 to 2200 µmol m^{-2} s^{-1} (data not shown). Thus, the ETR as RLCs was determined after 20 seconds of exposure. In addition, ETR was calculated from the measurements of effective quantum yield using For- mula (2) of algae apical parts in the vessels during daily cycles; this ETR to distinguish from the ETR of RLCs is called in situ ETR. Measurements were conducted twice in winter (6:00,8:00, 10:00, 12:00, 14:00, 16:00 and 18:00 GMT), and twice in summer (08:00, 10:00; 12:00, 14:00, 16:00, 18:00 and 20:00 GMT).

Non-photochemical quenching (NPQ) was calculated according to Schreiber et al. (1995) as:

$$NPQ = (F_m - F_{m'})/F_{m'} \quad (3)$$

Maximum NPQ (NPQ_{max}) and the initial slope of NPQ (α_{NPQ}) versus irradiance curves were obtained from the tangential function of NPQ according to Jassby and Platt (1976).

Biochemical variables

Total internal carbon and nitrogen contents on a dry weight (DW) basis were determined using a CNHS-
932 model element analyser (LECO Corporation, Michigan, USA).

Chlorophyll *a* (Chl *a*) and carotenoids pigments were determined in samples (0.025 g fresh weight) taken in six replicates from field-collected algae and after 3 and 7 days of exposure. Chl *a* and Chl c_1+c_2 were extracted in
1 mL of 90% acetone neutralized by magnesium carbon- ate hydroxide and measured in a spectrophotometer (UV Mini-1240 model, Shimazdu, Columbia, USA) using the formula reported by Ritchie (2008).

Total phenolic compounds (polyphenols) were determined using 0.25 g fresh weight (FW). Samples were pulverized in a mortar and pestle with sea-sand using 2.5 mL of 80% methanol. The mixture was kept overnight at 4°C and then centrifuged at 4000 rpm for
15 min and the supernatant was collected. Total phe- nolic compounds were determined colorimetrically using Folin-Ciocalteu reagent (Folin and Ciocalteu
1927). Phloroglucinol (1,3,5-trihydroxybenzene, Sigma P-3502) was used as a standard. Finally, the ab-orbance was determined at 760 nm using a Shimadzu UVMini-1240 spectrophotometer (Celis-Plá et al.

2014). Phenolic concentration was expressed as mg g^{-1} DW after determining the fresh to dry weight ratio in the tissue (the ratio was 5.6). The results are expressed as average±standard deviation from six replicates of each treatment.

The release of polyphenols (PR) in the seawater was determined by measuring the optical density in a spectrophotometer (UVMini-1240 model, Shimadzu, Columbia, USA) at the maximum absorption of polyphenols in the seawater, i.e. 270 nm (Ragan and Craigie 1980). The water samples were taken from waters in which *C. tamariscifolia* were growing. The concentration, expressed as mg g^{-1} DW day^{-1}, was obtained using phloroglucinol dissolved in seawater as standard. PR was determined after 3 and 7 days of incubation.

The antioxidant activity of seaweed extracts was estimated indirectly using the method based on reducing the stable free radical DPPH (2,2-diphenyl-1-picrylhydrazyl), according to Blois (1958). The same supernatant used for phenolic compounds was used for DPPH analysis. 150 µL of DPPH prepared in 90% methanol (90MeOH: 10H_2O) were added to each extract. The reaction was complete after 30 min in the dark at room temperature (~20°), and the absorbance was read at 517 nm in a spectrophotometer (UVMini-1240 model, Shimadzu, Columbia, USA). The calibration curve made with DPPH was used to calculate the remaining concentration of DPPH in the reaction mixture after incubation. Values of DPPH concentration (mM) were plotted against plant extract concentration (mg DW mL^{-1}) in order to obtain the oxidation index, EC_{50}, which represents the concentration of the extract, expressed as mg DW mL^{-1}, required to scavenge 50% of the DPPH in the reaction mixture. Ascorbic acid was used as positive control (Celis-Plá et al. 2014).

Statistical analysis

The effects of the treatments on the ecophysiological variables were analysed by a three-way ANOVA (Underwood 1997). This test was performed for *C. tamariscifolia* including season, day and thalli origin (RS and RP) as fixed factors. The design allows testing for interactive and additive effects. Homogeneity of variance was tested using the Cochran test and by visual inspection of the residuals. Student Newman-Keuls tests (SNK) were performed after significant ANOVA interactions (Underwood 1997). Analyses were done with SPSS v.21 (IBM, USA).

2.4 RESULTS

Solar radiation and temperature

The daily integrated irradiance in the air during the experimental period of PAR, UVA and UVB is represented in Figure 1. The daily integrated irradiance in the period from 8 to 16 February 2011 was 0.21 MJ m^{-2} of UVB, 4.53 MJ m^{-2} of UVA and 54.3MJ m^{-2} of PAR, whereas from 27 June to 5 July it was 0.64 MJ m^{-2} of

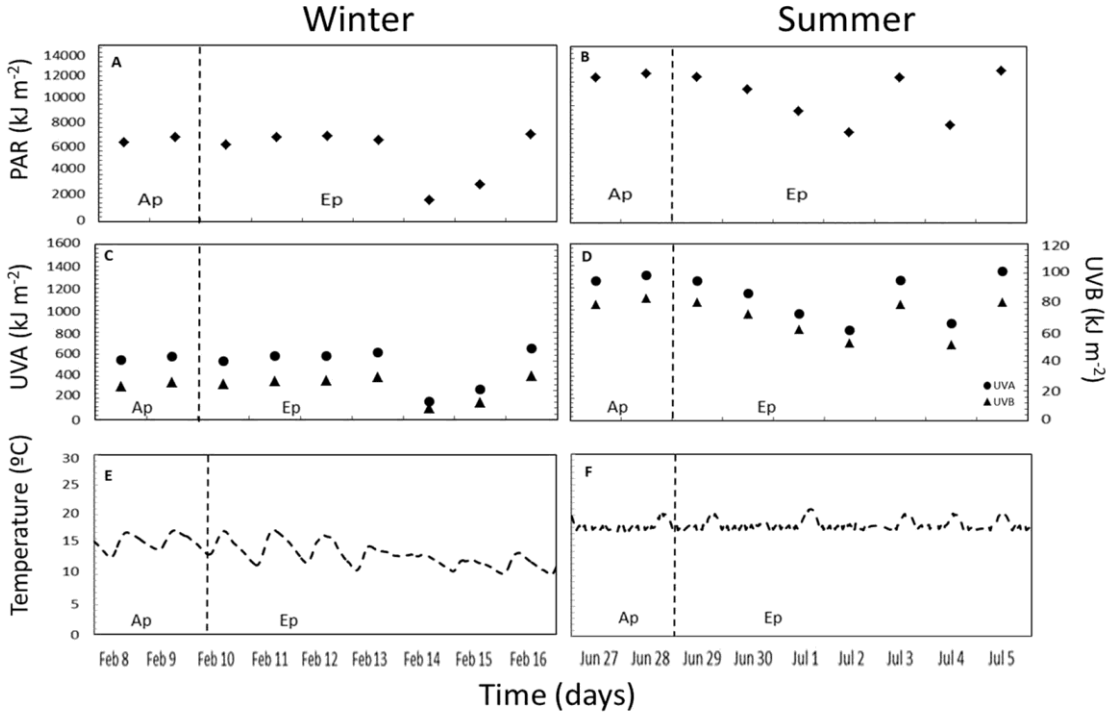

Fig. 1. – Daily integrated irradiance (DIE) in the air expressed as kJ m^{-2} of PAR (400-700 m) (A, B), UVA (320-400 nm) and UVB (280-320 nm) (C, D) in winter (February) (A, C) and summer (July) (B, D) (days). The harvesting of *C. tamariscifolia* was conducted at time 0, then the algae were incubated in 1.5-L cylindrical vessels for 2 days under decreased solar irradiance conditions (acclimation period, AP). After this period, the algae were incubated for 7 d under full solar irradiance (experimental period, EP). Average daily integrated irradiance was calculated from 8 to 16 February in winter and from 27 of June to 5 July in summer. Underwater temperature in the experiment in winter (E) and summer (F)

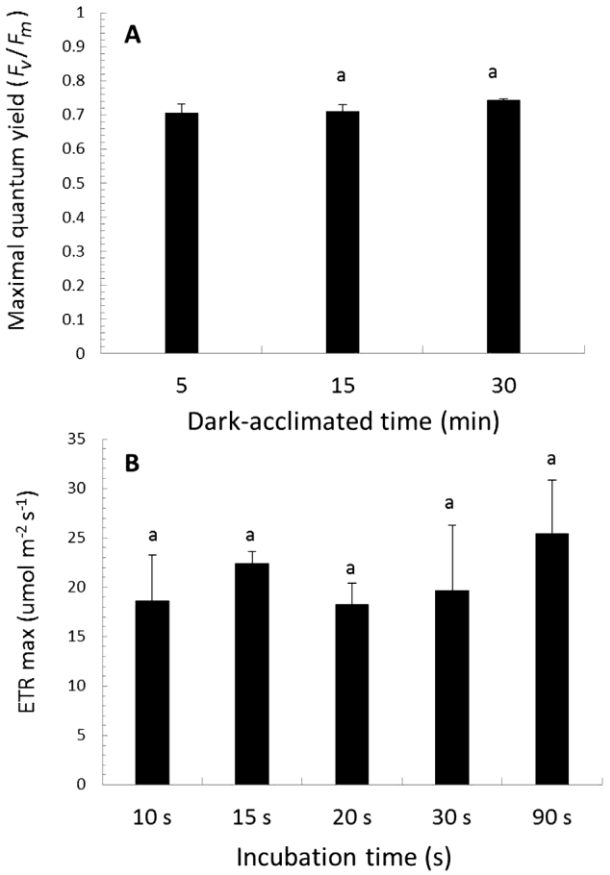

Fig. 2. – Measurement of maximum quantum yield (F_v/F_m) after incubation of *C. tamariscifolia* thalli in different time periods in darkness (5, 15 and 30 min) (A). Different incubation time (10, 15, 20, 30 and 90 s) under increased irradiances of actinic light (PAR) to estimate the ETR_{max} (B).

UVB, 10.3 MJ m^{-2} of UVA and 99.8 MJ m^{-2} of PAR;

about 3.1 times (UVB), 2.3 times (UVA) and 1.8 times (PAR) higher in summer than in winter (Fig. 1). Most of the time the sky was cloudless in winter experiments except for days 4 and 5 (Fig. 1A, C) whereas, in the summer experiment thin clouds were also observed on days 3 to 5 (Fig. 1B, D). The average underwater temperature in the incubation vessels was maintained at 18°C in winter and 22°C in summer during the day and at 12°C in winter and 17°C in summer during the night (Fig. 1E, F).

Physiological and biochemical responses

In order to determine the optimal incubation time in darkness to estimate F_v/F_m, thalli were incubated in darkness for 5, 15 and 30 min (Fig. 2A). No significant differences were found among the tested times and 15 min was selected as it is the most common dark exposure time found in the literature. In order to determine the optimal incubation time in RLCs to reach steady-state conditions of effective quantum yield and ETR, thalli were incubated under different increased intensities for 10, 15, 20, 30 and 90 seconds of incubation time in each actinic light. No significant differences were found between 20 and 90 seconds (Fig. 2B) and

Table 2. – ANOVA results testing for the effect of Seasons, Time and Origin of algae (RP; RS) on the photosynthetic parameters; maximum quantum yield (F_v/F_m), electron transport rate (ETR_{max}) exp ressed as µmol electrons m^{-2} s^{-1}, photosynthetic efficiency (α_{ETR}) as the initial slope of ETR versus irradiance rapid light curves (RLCs), saturated irradiance of ETR (Ek_{ETR}, expressed in µmol m^{-2} s^{-1}) and maximum non-photochemical quenching (NPQ_{max}) of *Cystoseira tamariscifolia*; significant differences at $\alpha<0.05$ are shown in bold.

		df	MS	F	p
F_v/F_m	Season (S)	1	0.054	57.58	**0.00**
	Time (T)	1	0.019	20.28	**0.00**
	Origen algae (O)	1	0.006	6.58	**0.01**
	S×T	1	0.005	5.67	**0.02**
	S×O	1	0.002	2.51	0.12
	T×O	1	0.001	0.80	0.37
	S×T×O	1	0.001	0.62	0.43
	Residual	40	0.001		
ETR_{max}	Season (S)	1	35794.31	463.63	**0.00**
	Time (T)	1	5.21	0.07	0.80
	Origen algae (O)	1	1843.20	23.87	**0.00**
	S×T	1	3.77	0.05	0.83
	S×O	1	1.81	0.02	0.88
	T×O	1	166.41	2.16	0.15
	S×T×O	1	67.98	0.88	0.35
	Residual	40	77.20		
α_{ETR}	Season (S)	1	0.243	214.64	**0.00**
	Time (T)	1	0.000	0.40	0.53
	Origen algae (O)	1	0.005	4.00	**0.05**
	S×T	1	0.001	0.64	0.43
	S×O	1	0.018	15.65	**0.00**
	T×O	1	0.001	0.45	0.50
	S×T×O	1	0.001	0.70	0.41
	Residual	40	0.001		
Ek	Season (S)	1	89766.27	93.58	**0.00**
	Time (T)	1	697.71	0.73	0.40
	Origen algae (O)	1	9229.51	9.62	**0.00**
	S×T	1	345.94	0.36	0.55
	S×O	1	4915.13	5.12	**0.03**
	T×O	1	603.87	0.63	0.43
	S×T×O	1	284.56	0.30	0.59
	Residual	40	959.26		
NPQ_{max}	Season (S)	1	7.71	16.48	**0.00**
	Time (T)	1	2.66	5.68	**0.02**
	Origen algae (O)	1	1.78	3.82	0.06
	S×T	1	0.37	0.79	0.38
	S×O	1	0.58	1.23	0.27
	T×O	1	2.28	4.88	**0.03**
	S×T×O	1	0.80	1.70	0.20
	Residual	40	0.47		

Table 1. – Maximum quantum yield (F_v/F_m), electron transport rate (ETR_{max}) expressed as µmol electrons m^{-2} s^{-1}, photosynthetic efficiency (α_{ETR}) as the initial slope of ETR versus irradiance rapid light curves (RLCs) and maximum non-photochemical quenching (NPQ_{max}) in winter (February) and summer (July) *C. tamariscifolia* collected from rockpools and rocky shores (field algae). Data are expressed as mean±standard deviation of n=6. Different letters indicate significant differences between period of times or collection microhabitat for each variable.

	Winter		Summer	
	Rockpools	Rocky shores	Rockpools	Rocky shores
F_v/F_m	$0.75±0.01^{b}$	$0.72±0.01^{a}$	$0.70±0.01^{a}$	$0.70±0.01^{a}$
ETR_{max}	$58.9±4.4^{a}$	$88.8±7.9^{b}$	$89.4±1.1^{b}$	$98.37±4.6^{b}$
α_{ETR}	$0.17±0.02^{a}$	$0.26±0.01^{b}$	$0.44±0.03^{c}$	$0.43±0.01^{c}$
NPQ_{max}	$1.06±0.21^{a}$	$1.41±0.41^{a}$	$3.41±1.11^{b}$	$1.99±0.31^{ab}$

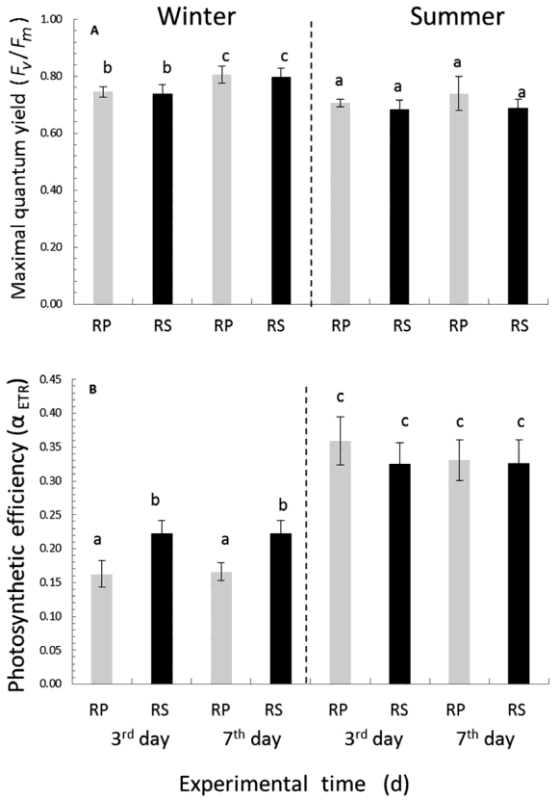

Fig. 3. – Maximum quantum yield (F_v/F_m) (A) and photosynthetic efficiency (α_{ETR}) (B) in *C. tamariscifolia* during the experimental period (3rd and 7th day) in algae incubated in 1.5-L cylindrical vessels. The algae were collected in winter (February) and summer (July) and from rockpools (RP) and rocky shores (RS). Data are expressed as mean±standard deviation of n=6 and lower-case letters denote significant differences after SNK test.

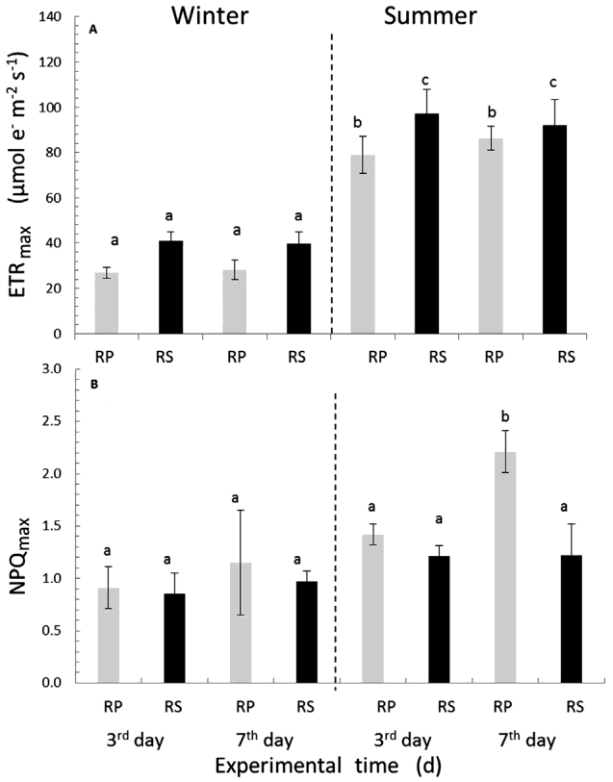

Fig. 4. – Maximum electron transport rate (ETR_{max}) (A) expressed as µmol electrons m^{-2} s^{-1} and maximum non-photochemical quenching (NPQ_{max}) (B) in *C. tamariscifolia* during the experimental period (3rd and 7th days) in algae incubated in 1.5-L cylindrical vessels. The algae were collected in winter (February) and summer (July) from rockpools (RP) and rocky shores (RS). Data are expressed as mean±standard deviation of n=6 and lower-case letters denote significant differences after SNK test.

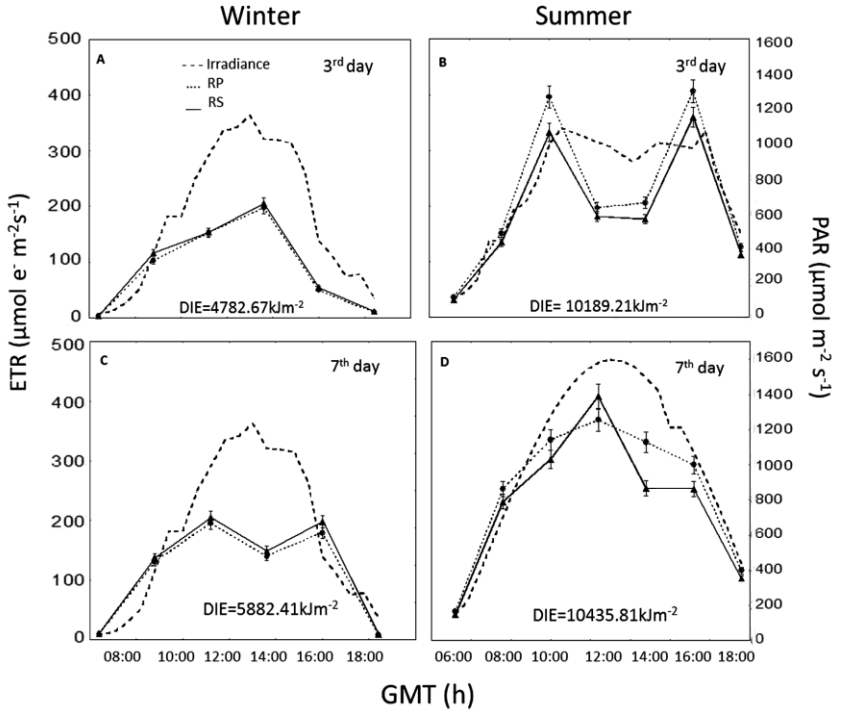

Fig. 5. – Daily cycle of in situ ETR expressed as µmol electrons m^{-2} s^{-1} in *C. tamariscifolia* during the experimental period (3rd and 7th days) in algae incubated in 1.5-L cylindrical vessels. The algae were collected in winter (February) (A, C) and summer (July) (B, D) and from rockpools (RP) (dotted lines) and rocky shores (solid lines) (RS). The daily irradiance of PAR expressed as µmol photons m^{-2} s^{-1} (straight lines). The average daily integrated irradiance (DIE) expressed as kJ m^{-2} during the experimental period is indicated.

therefore 20 seconds was selected as most common time used in the literature.

F_v/F_m was significantly ($p<0.05$) higher in winter than in summer both just after alga harvesting (Table 1) and after incubation for 3 and 7 days (Table 2 and Fig. 3A). F_v/F_m in *C. tamariscifolia* showed a significant interaction between season and time. F_v/F_m increased during the experimental time only in winter whereas, in summer a significant decrease was observed (Table 2 and Fig. 3A). The collection microhabitat (RS or RP) did not affect the values of F_v/F_m.

ETR_{max} ($F_{1,8}$= 15.6, $p<0.05$) and α_{ETR} ($F_{1,8}$=11.7, $p<0.05$) was higher in thalli collected in summer than in winter (Table 1). ETR_{max} and α_{ETR} showed a significant interaction between season and time. In winter, α_{ETR} (Table 2 and Fig. 3B) increased during the experimental time, whereas in summer a_{ETR} remained constant. ETR_{max} in the experimental period was higher in thalli collected from RS than from RP, in thalli collected in summer (Table 2 and Fig. 4A); whereas, α_{ETR} showed the same pattern only in winter (Table 2 and Fig. 3B).

NPQ_{max} was higher in field algae collected in summer than in winter and no significant differences between thalli from RP and RS were found (Table 1). NPQ_{max} showed a significant interaction between time and origin of the algae. However, during the incubation period it was higher only in thalli collected from RP in summer, at the end of the experimental period (Table 2 and Fig 4B).

In situ ETR in outdoor experiments showed a daily pattern in both winter (Fig. 5A, C) and summer (Fig. 5B, D). In winter, the irradiance around noon was about 900 µmol m^{-2} s^{-1}, whereas in summer it was 1600 µmol m^{-2} s^{-1} (Fig. 5). Though the daily integrated irradiance was about two times lower in winter than in summer, the ETR decreased on the 7th day in the first season. However, in summer the decrease in ETR occurred at the 3rd but not at the 7th day. The decrease in ETR in the experimental period in winter was higher in thalli collected from RP than from RS (Fig. 5A, C); whereas in summer (Fig. 5B, D) it was higher in thalli collected from RS. The period of decrease of ETR was 4 hours in winter, whereas in summer it was 6 hours. However, in both seasons, the ETR reached similar values.

Total internal N content was higher and C:N ratio lower in thalli collected in winter than in summer. ANOVA results showed a significant interaction between season and origin of the algae (Table 3 and 4). After 3 and 7 days of incubation, winter-grown thalli maintained higher levels of N and consequently lower levels of C:N than summer-grown ones (Table 3 and 4).

In summer Chl *a* and Chl c_1+c_2 concentrations were 1.6±0.4 mg g^{-1} DW and 0.28±0.04 mg g^{-1} DW, in field-collected algae, respectively, whereas, in winter they were 1.2±0.2 mg g^{-1} DW and 0.17±0.04 mg g^{-1} DW. After 3 and 7 days of incubation, Chl *a* showed a significant interaction between season and origin of the algae, and between time and origin of the algae (Table 3 and 5). Chl *c*1+*c*2 showed a significant interaction between season and origin of the algae (table 3 and 5). A higher content of Chl *c*1+*c*2 was found in thalli in winter.

Table 3. – ANOVA results, testing for the effect of Season, Time and Origin of algae (RP; RS) on the total internal nitrogen, C:N ratio, photosynthetic pigments (Chl *a* and Chl c_1+c_2), phenolic compounds, phenolic compounds released and EC_{50} of *Cystoseira tamariscifolia*. Significant differences at $\alpha<0.05$ are shown in bold.

		df	MS	F	p
Nitrogen	Season (S)	1	408.80	110.98	**0.00**
	Time (T)	1	3.49	0.95	0.34
	Origen algae (O)	1	8.09	2.19	0.15
	S×T	1	2.72	0.74	0.40
	S×O	1	29.61	8.04	**0.01**
	T×O	1	2.80	0.76	0.39
	S×T×O	1	0.34	0.09	0.76
	Residual	40	3.68		
Ratio C:N	Season (S)	1	205.10	79.14	**0.00**
	Time (T)	1	0.98	0.38	0.54
	Origen algae (O)	1	11.84	4.57	**0.04**
	S×T	1	0.21	0.08	0.78
	S×O	1	15.32	5.91	**0.02**
	T×O	1	0.05	0.02	0.89
	S×T×O	1	0.47	0.18	0.67
	Residual	40	2.59		
Chl *a*	Season (S)	1	0.88	7.37	**0.01**
	Time (T)	1	0.02	0.19	0.67
	Origen algae (O)	1	0.15	1.31	0.26
	S×T	1	0.01	0.01	0.93
	S×O	1	0.64	5.38	**0.03**
	T×O	1	0.66	5.55	**0.02**
	S×T×O	1	0.01	0.11	0.74
	Residual	40	0.12		
Chl c_1+c_2	Season (S)	1	0.065	35.67	**0.00**
	Time (T)	1	0.000	0.00	0.97
	Origen algae (O)	1	0.005	2.73	0.11
	S×T	1	0.000	0.26	0.62
	S×O	1	0.017	9.02	**0.00**
	T×O	1	0.004	2.31	0.14
	S×T×O	1	0.001	0.60	0.44
	Residual	40	0.002		
Phenolic compounds	Season (S)	1	1226.40	63.22	**0.00**
	Time (T)	1	23.49	1.21	0.28
	Origen algae (O)	1	220.94	11.39	**0.00**
	S×T	1	129.15	6.66	**0.01**
	S×O	1	637.88	32.88	**0.00**
	T×O	1	175.16	9.03	**0.00**
	S×T×O	1	8.04	0.41	0.52
	Residual	40	19.40		
Release of phenolic compounds	Season (S)	1	0.07	1.55	0.22
	Time (T)	1	0.67	14.61	**0.00**
	Origen algae (O)	1	0.63	13.70	**0.00**
	S×T	1	0.25	5.53	**0.02**
	S×O	1	0.01	0.09	0.76
	T×O	1	0.09	2.00	0.16
	S×T×O	1	0.10	2.08	0.16
	Residual	40	0.05		
EC_{50}	Season (S)	1	3.48	202.76	**0.00**
	Time (T)	1	0.35	20.46	**0.00**
	S×T	1	0.03	1.94	0.17
	S×O	1	0.48	28.26	**0.00**
	T×O	1	0.11	6.50	**0.01**
	S×T×O	1	0.01	0.06	0.81
	Residual	40	0.02		

The phenolic compound content was higher ($F_{1,8}$=5.8, $p<0.05$) in field-collected algae in winter than in summer. After the experimental periods, ANOVA results showed significant interactions between season and time, between season and origin of the algae and between time and origin of the algae (Table 3 and 6). In winter, phenol concentration increased from 25 to 41 mg g^{-1} DW in RP algae from the 3rd to 7th d incubation but decreased from 41 to 27 mg g^{-1} DW in RS algae. In summer, phenolic compounds did not change in RP

Table 4. – Concentration of internal N expressed as mg g^{-1} DW and C:N ratio in *C. tamariscifolia* collected in winter (February) and summer (July) from rocky shores (RS) and rockpools (RP) from field material (field algae) and after 3 and 7 d incubation in 1.5-L UV-transparent cylindrical vessels under solar radiation (incubated algae). Data are expressed as mean±standard deviation of n=6 and lower-case letters denote significant differences after SNK test.

		Winter		Summer	
		N	C:N	N	C:N
Field algae	RP	21.0±0.5 [b]	13.9±0.4 [a]	18.2±1.3 [a]	15.7±1.0 [b]
	RS	22.2±0.5 [b]	13.3±0.7 [a]	18.6±1.2 [a]	14.8±1.1 [b]
Incubated algae 3rd day	RP	22.1±1.6 [c]	12.5±1.2	15.4±2.6 [a]	17.8±2.3
	RS	22.0±1.7 [c]	12.8±0.8	18.2±1.9 [b]	15.5±1.7
Incubated algae 7th day	RP	23.8±2.3 [c]	12.2±1.2	15.8±1.6 [a]	17.4±2.1
	RS	22.4±1.2 [c]	12.8±1.5	17.8±2.0 [b]	15.6±1.9

Table 5. – Concentration of internal Chl *a* and Chl c_1+c_2 expressed as mg g^{-1} DW in *C. tamariscifolia* collected in winter (February) and summer (July) from rocky shores (RS) and rockpools (RP) from field material (field algae) and after 3 and 7 d incubation in 1.5-L UV-transparent cylindrical vessels under solar radiation (incubated algae). Data are expressed as mean±standard deviation of n=6 and lower-case letters denote significant differences after SNK test.

		Winter		Summer	
		Chl *a*	Chl c_1+c_2	Chl *a*	Chl c_1+c_2
Field algae	RP	1.31±0.28 [ab]	0.19±0.05 [ab]	2.01±0.55 [b]	0.23±0.09 [b]
	RS	1.09±0.11 [a]	0.13±0.07 [a]	1.70±0.26 [ab]	0.19±0.02 [ab]
Incubated algae 3rd day	RP	1.53±0.31	0.22±0.04 [c]	1.04±0.30	0.13±0.03 [a]
	RS	1.68±0.20	0.23±0.02 [c]	1.60±0.42	0.19±0.04 [b]
Incubated algae 7th day	RP	1.74±0.26	0.26±0.05 [c]	1.21±0.41	0.13±0.05 [a]
	RS	1.36±0.37	0.21±0.02 [c]	1.36±0.44	0.18±0.07 [b]

Table 6. – Content of phenolic compounds (PC) expressed as mg g^{-1} DW and antioxidant activity as EC_{50} (mg DW mL^{-1}, DPPH method) in *C. tamariscifolia* collected in winter (February) and summer (July) from rocky shores (RS) and rockpools (RP) from field material (field algae) and after 3 and 7 d incubation in 1.5-L cylindrical vessels under solar radiation (incubated algae). Data are expressed as mean±standard deviation of n=6 and lower-case letters denote significant differences after SNK test.

		Winter		Summer	
		PC	EC_{50}	PC	EC_{50}
Field algae	RP	36.94±5.09 [b]	1.10±0.14[c]	31.72±3.60 [a]	0.81±0.32 [b]
	RS	47.12±6.26 [b]	0.50±0.07 [a]	32.17±2.46 [a]	0.40±0.05 [a]
Incubated algae 3rd day	RP	25.73±2.10 [a]	1.30±0.20 [c]	27.01±4.78 [a]	0.60±0.11 [a]
	RS	41.95±5.16 [b]	0.82±0.11 [b]	27.01±5.21 [a]	0.54±0.07 [a]
Incubated algae 7th day	RP	35.04±2.03 [b]	1.42±0.14[d]	28.13±2.73 [a]	0.64±0.05 [a]
	RS	41.99±3.02 [b]	1.15±0.09 [c]	22.12±2.75 [a]	0.75±0.08 [a]

(27-28 mg g^{-1} DW) and RS algae (27 to 22 mg g^{-1} DW) (Table 3 and 6).

The release of polyphenols expressed as mg g^{-1} DW d^{-1} after the experimental period showed a significant interaction between season and time. PR, after 3 days of incubation was similar in thalli collected from RP in both seasonal periods (Table 3 and 7), whereas after 7 days the release was higher in summer than in winter, particularly in thalli collected from RS. The release expressed as percentage of the internal content was clearly higher after 7 days of incubation in summer- than in winter-collected *C. tamariscifolia*. After 7 days, the percentage of release was 3 and 5 times higher in summer-collected RS and RP algae, respectively, than in winter-collected ones (Table 3 and 7). Antioxidant activity estimated as the oxidation index, EC_{50}, in *C. tamariscifolia* was higher in field- collected algae in summer than in winter. EC_{50} showed significant interactions between season and origin of the algae and between time and origin of the algae. It remained higher in summer-collected thalli than in winter-collected thalli in the experimental period (Table 3 and 6). Meanwhile, only in winter was the anti- oxidant activity higher in RS-grown than in RP-grown algae during the experimental period. In winter, the antioxidant activity decreased during the experimental time, whereas in summer no significant differences during the experimental period were found.

Table 7. – Release of phenolic compounds in seawater using phloroglucinol as standard in *C. tamariscifolia* collected in winter (February) and summer (July) from rocky shores (RS) and rockpools (RP). Data are expressed as mean (SD) percentages of phenol released to total internal content (% release) after 3 and 7 d culture in 1.5-L cylindrical vessels under solar radiation, n=6 and lower-case letters denote significant differences after SNK test. PR, phenol released (mg g^{-1} DW d^{-1}).

		Winter PR	Summer PR	Winter % release	Summer % release
3rd day	RP	0.31±0.05[a]	0.35±0.08[a]	3.58	3.85
	RS	0.73±0.20[b]	0.55±0.23[b]	5.22	6.15
7th day	RP	0.11±0.03a	0.23±0.05a	1.17	3.63
	RS	0.17±0.04[a]	0.47±0.13[b]	1.64	8.41

2.5 DISCUSSION

Photosynthetic capacity (ETR_{max}) and photosynthetic efficiency (α_{ETR}) were higher in *C. tamariscifolia* collected in summer than in winter and these differences were maintained after 7 days of incubation in cylindrical vessels under full solar radiation and the temperature of each season. In summer, during the experimental period, high daily integrated irradiance of PAR (99.77 MJ m^{-2}) and temperature (22°C

day/17°C night) favoured photosynthetic activity in *C. tamariscifolia* compared with the winter period (54.26 MJ m^{-2} and 18°C day/12°C night). These results are in agreement with its latitudinal and zonal distribution in the coastal areas (Lüning 1990, Thibaut et al. 2005). A positive correlation between the ETR_{max} calculated from the RLC and the in situ ETR_{max} from the daily cycles of ETRs ($r=0.89$, $p<0.001$, $n=60$) was found: the latter were always 4.5 times higher. Parameters derived from RLCs as ETR_{max} or *Ek* are sensitive to diurnal fluctuations as the effective and maximum quantum yields of PSIIs (Belshe et al. 2007).

ETRs calculated in daily cycle (in situ ETR) tend to be higher than in RLCs, as was described by Longstaff et al. (2002). The ETR was also higher under solar radiation (in situ measurements) than under the halogen lamp provided by the Diving-PAM (RLC determination). This result can be explained by the different light qualities of the radiation sources, i.e., the solar radiation has a much higher blue:red light ratio than the halogen lamp of the Diving-PAM, contributing to a higher electron flow by accessory pigments (carotenoids) to chlorophyll. Brown algae showed a high photosynthetic quantum yield in blue light according to the action spectra for photosynthesis reported by Lüning and Dring (1985).

The microhabitat of collection (RS or RP), in addition to the season, affected the photosynthetic pattern in field collected *C. tamariscifolia*. Higher ETR_{max} and α_{ETR} in RS than in RP field-collected algae was observed, but only in winter. One possible explanation for this pattern is that the emersion periods in RS algae favoured photosynthetic activity by direct incorporation of CO_2 from the air. *C. tamariscifolia* can grow in RP, where it is always submerged during the daily period. On the other hand, *C. tamariscifolia* growing in RS may be subjected to different cycles of desiccation and rewetting, increasing atmospheric CO_2 uptake and nutrient incorporation; the inorganic carbon uptake in *C. tamariscifolia* growing in rockpools depend mainly on the amount of dissolved HCO_3^- and carbon concentration mechanisms (CCMs) through carbonic anhydrase (CA), with the consequent energy cost (Falkowski 1997). It has been reported that intertidal algae under moderate desiccation conditions have higher nitrogen and phosphate uptakes (Lobban and Harrison 1994, Nygard and Dring 2008), photosynthetic rates (Dring and Brown 1982, Mercado et al. 1998). The carbon incorporation under emersion is higher than that under submerged conditions due to the direct uptake of CO_2 (Flores-Moya et al. 1998).

Internal N content was higher in winter- than in summer-collected algae, as is expected according to nitrate content in the water (Ramírez et al. 2005). Interestingly, these differences were maintained after 7 days of incubation in cylindrical vessels in spite of the nitrate enrichment (maximum level of 50 mM). Therefore, the nutritional state seems to be more favourable in winter- than in summer-grown algae as a lower C:N in thalli collected in winter was found. Additionally, thalli collected in summer seemed not to accumulate N compounds after nitrate enrichment due to a low uptake rate. It seemed that thalli accumulate N during winter as a reservoir. This result could be related to the high amount of energy that these macroalgae demand in summer, the period in which the activation of photoprotection and acclimation mechanisms may occur (Hanelt and Figueroa 2012).

This result, in combination with the higher photosynthetic rate (ETR_{max}) and efficiency (α_{ETR}) in thalli collected in summer and incubated for 7 days, suggests that *C. tamariscifolia* is not limited by N in summer and it can invest the photosynthetic energy in growth. Sales and Ballesteros (2012) reported higher growth rate in *Cystoseira crinita* from the northwestern Mediterranean in summer than in winter. This is also in accordance with the higher ETR_{max} in thalli collected from RS than from RP in both seasons. Moreover, Celis-Plá et al. (2014) found higher ETR_{max} in *C. tamariscifolia* collected from 0.5 m depth waters than from 2.0 m depth waters in summer after an in situ experimental period. As in this study, ETR_{max} at initial time was also higher in algae of the intertidal zone during the emersion than the submersion period (Nitschke et al. 2012).

Most of the differences between season and growing sites observed in the thalli collected from the field (field algae) remains after 7 days of incubation under immersed conditions in cylindrical vessels. These results indicate a high resilience of this species. The higher decay observed after 3 d of exposure in summer-grown algae and after 7 d of exposure in winter-grown algae indicates a possible accumulative inhibitory effect in winter and high photoacclimation capacity in summer-grown algae. Some authors have also shown that the dynamic photoinhibitory response may be related to acclimation responses to UV radiation (Häder and Figueroa 1997, Figueroa et al. 1997, Flores-Moya et al. 1998, Figueroa et al. 2003).

In fact, studies on daily photoinhibition and full recovery in intertidal Mediterranean algae suggest that photoinhibition is a photoprotective mechanism against high solar radiation, as in higher plants, and that the pattern of photoinhibition and recovery is affected by accumulative dose (Figueroa and Viñegla 2001). An enhanced capacity for dynamic photoinhibition and subsequent recovery has been previously reported in macroalgae, including brown macroalgae from southern Spain (Häder et al. 1998, Flores-Moya et al. 1999). In summer-grown thalli collected from RP, the ETR decay was delayed, as was observed in the daily cycle or in situ measurements. Our results suggest that photoinhibi- tion can be a mechanism that protects *C. tamariscifolia* against high irradiance as observed in other intertidal seaweeds (Osmond 1994, Hanelt 1996). In addition to dynamic photoinhibition, another indicator of high photoacclimation capacity is the high energy dissipation that allows species to cope with excess excitation energy, as is the case of NPQ (Klughammer and Schreiber 2008). *C. tamariscifolia* specimens collected in summer and from RS showed higher values of NPQ than those collected in winter and from RP. High values of NPQ indicate active photoprotective mechanisms, which are highly related to the xanthophyll cycle (Demmig-Adams and Adams 2006).

Phenolic content was higher in winter than in summer. This could be an indicator of a good physiological status, i.e. accumulation of secondary metabolic compounds in nutrient-replete conditions (winter) to be used in nutrient-depleted conditions (summer) (Celis-Plá et al. 2014). Abdala-Díaz et al (2006) showed both seasonal and hourly variation in phenolic compounds depending on the daily integrated irradiance (dose) or hourly irradiances, respectively. It has been described that the variability in the phenolic content could be related to environmental factors such as herbivory, light, depth, salinity, nutrients and seasonality, as well as to intrinsic ones such as age, length and kind of tissue (see Amsler and Fairhead 2006, for review). Zubia et al. (2008) described a complexity of seasonal variations suggesting a stronger correlation between phenolic contents and local environmental factors (e.g. grazing intensity in different areas of the coral reef) than between large scale factors (i.e. months, seasons, latitude). The phenolic content and the antioxidant activity have been related to algal zonation (Connan et al. 2004). In the eulittoral and intertidal zone, some algae (*Fucus spiralis, Fucus vesiculosus, Ascophyllum nodosum*) show higher phenolic content than algae growing in the low intertidal or sublittoral zone (*Fucus serratus, Bifurcaria bifurcata, Himathalia elongata* and *Laminaria digitata*) (Connan et al. 2004). Also, contents are higher in summer when irradiance is the highest, as observed in several brown macroalgae from Brittany (Connan et al. 2004) and in *C. tamariscifolia* collected in Southern Spain (Abdala-Díaz et al. 2006). In contrast, in our study *C. tamariscifolia* showed higher phenolic content in winter than in summer, probably in relation to the high winter nitrate availability in Málaga (Ramírez et al. 2005). N can enhance the accumulation of phenolic compounds in some brown algae (Pavia and Toth 2000, Celis-Plá et al. 2014) as well as in *Ulva rigida* (Cabello-Pasini et al. 2011). In contrast to internal phenolic content, the release of phenolic compounds was similar in RP in winter and in summer, and higher in RS (Table 7), whereas the percentage of release to internal content was clearly higher in summer- than winter-collected algae, i.e. after 7 days of incubation the percentage of release was about 3 or 5 times higher in algae collected in RP and RS, respectively, in summer than in winter. Phlorotannins released to seawater from the tissues react with other substances to form UV-absorbing complexes (Craigie and McLachlan 1964, Carlson and Carlson 1984, Jennings and Steinberg 1994, Dujmov et al. 1996). However, a few data are available on quantities of released phlorotannins (Toth and Pavia 2000) or on their physiological and ecological function. Swanson and Druehl (2002) reported high excretion of phenols by increasing UV radiation. Although the effect of UV radiation on release rates was not directly examined in our study, there is a positive relationship between solar incident irradiance of PAR, UVA and UVB and rate of phenol release. The release rate of polyphenols in our study was about 3-5 times lower than that observed by Jennings and Steinberg (1994) in *Eklonia radiata* (10-24 mg g^{-1} DW d^{-1}). The release rate can be related to the light history and the species, i.e. *C. tamariscifolia* is an intertidal species subject to higher daily integrated irradiance than the subtidal species *Ecklonia radiata*. High PAR irradiances and emersion have been associated with increasing phlorotannin release rates (Ragan and Jensen 1978, Carlson and Carlson 1984). In addition, phlorotannins in macroalgae are produced and released into seawater during periods of UVA stress and they are released but under UVB i.e at concentration of >0.84 g mL^{-1}, they reduce the impact of UVB exposure in UV-sensitive kelp meiospores (Roleda et al. 2006, Huovinen et al. 2010). Taking into account that phlorotannins exhibit absorption maxima at 200 and 270 nm, the putative shielding capacity of phlorotannins would be more efficient in the case of DNA damage (caused mainly by UV-B wavelengths) than photosynthesis, for example, which is normally also affected by wavelengths in the UV-A region (Huovinen el at. 2010). Koivikko et al. (2005) also described exudation of phlorotannins to the surrounding water, and the rate of exudation was not affected by nutrient shortage. Karban and Baldwin (1997) reported an indirect defence of phlorotannins in algae, i.e. increased excretion of these compounds into the water when algae were grazed.

C. tamariscifolia showed higher antioxidant capacity in thalli collected in summer than in winter and in thalli collected from RS than from RP in spite of the lower content of internal phenols. Therefore, high anti- oxidant activity is produced in algae submitted to high solar irradiances and low internal N content. Since the internal polyphenol content is lower in summer than in winter, we suggest that the antioxidant activity in summer could be related to other internal substances such as carotenoids.

2.6 CONCLUSIONS

Photoacclimation capacity of *C. tamariscifolia* was higher in thalli collected in summer than in winter and in thalli from RS than from RP, i.e. the algae are less vulnerable to increased solar exposure when subject to more stressful conditions (e.g. high solar irradiance and low nitrate level). In thalli collected in summer from RS, photosynthetic activity was higher and photoinhibition lower after 7 days of incubation than in thalli collected in winter from rockpools. This higher acclimation capacity could be explained by: (1) high dynamic photoinhibition, as is shown during daily cycles, i.e. fast and high increase of ETR_{max} and F_v/F_m in the afternoon (high recovery); (2) high NPQ_{max}, indicating an efficient energy dissipation and high photoprotection capacity (Celis-Plá et al. 2014); and (3) high antioxidant activity (low EC_{50}), related not to internal phenolic compounds but probably to other antioxidant substances such as carotenoids. A high acclimation capacity to increased UVB radiation of *C. tamariscifolia* has recently been shown based on the accumulation of UV screen substances, high release rates of polyphenols and high antioxidant ac- tivity (Figueroa et al. 2014).

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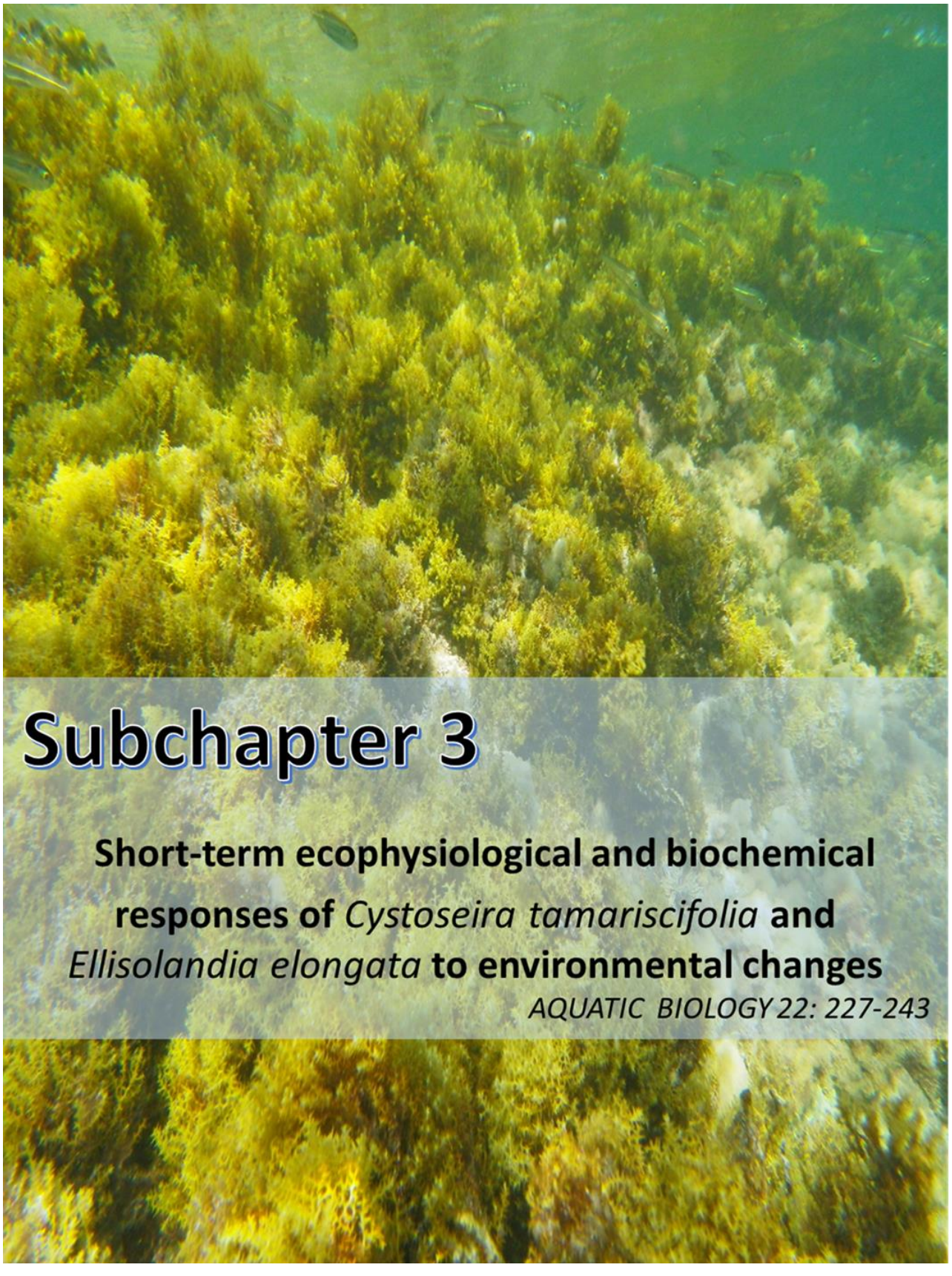

Cabo de Gata-Níjar, Natural Park. September 2013. Photograph by Paula S. M. Celis Plá

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Short-term ecophysiological and biochemical responses of *Cystoseira tamariscifolia* and *Ellisolandia elongata* to environmental changes

P. S. M. Celis-Plá[1,2,*], B. Martínez[3], E. Quintano[4], M. García-Sánchez[5], A. Pedersen[6], N. P. Navarro[7], M. S. Copertino[8], N. Mangaiyarkarasi[9], R. Mariath[10], F. L. Figueroa[1], N. Korbee[1]

[1]Department of Ecology, Faculty of Science, University of Málaga, 29071 Málaga, Spain

[2]Laboratory of Botany, Faculty of Pharmacy, University of Barcelona, 08028 Barcelona, Spain

[3]Biology and Geology Department, Rey Juan Carlos University, 28933 Móstoles, Spain

[4]Department of Plant Biology and Ecology, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), PO Box 644, 48080 Bilbao, Spain

[5]Department of Ecology and Hydrology, Faculty of Biology, Regional Campus of International Excellence 'Campus Mare Nostrum', University of Murcia, 30100 Murcia, Spain

[6] Norwegian Institute for Water Research, Department of Marine Biology, 0349 Oslo, Norway

[7]Faculty of Science, University of Magallanes, Casilla 113-D, Punta Arenas, Chile

[8]Institute of Oceanography, Federal University of Rio Grande-FURG, c.p. 474, cep 93206-900 Rio Grande (RS), Brazil

[9]Plant Biology and Biotechnology, CKN College Thiruvalluvar University, India

[10]Department of Botany, Institute of Biology, Federal University of Rio de Janeiro, Brazil

3.1 ABSTRACT: Short-term ecophysiological and biochemical responses of *Cystoseira tamariscifolia* and *Ellisolandia elongata* to changes in solar irradiance and nutrient levels were analyzed *in situ* in oligotrophic coastal waters by transferring macroalgae collected at 0.5 and 2.0 m depth and exposing them to 2 irradiance levels (100 and 70 % of surface irradiance) and nutrient conditions (nutrient-enriched and non-enriched). Both species were affected by changes in irradiance and nutrient levels. Few interactive effects between these 2 physical stressors were found, suggesting major additive effects on both species. *C. tamariscifolia* collected at 0.5 m and exposed to 70 % irradiance had the highest maximal electron transport rate (ETR_{max}), saturated irradiance (Ek_{ETR}) and chl *a* content and the lowest antioxidant activity. Under the same conditions, *E. elongata* had increased Ek_{ETR}, antheraxanthin and β-carotene content. At 100 % irradiance, *C. tamariscifolia* collected at 2.0 m had higher maximal quantum yield (F_v/F_m), photosynthetic efficiency (α_{ETR}), ETR_{max}, maximal non-photochemical quenching (NPQ_{max}), saturation irradiance for NPQ (Ek_{NPQ}), and antheraxanthin and polyphenol content increased, whereas in *E. elongata* only α_{ETR} increased. In nutrient-enriched conditions, phenolic compounds, several carotenoids and N con- tent increased in *C. tamariscifolia* at both depths. *E. elongata* from 2.0 m depth at 100 % irradiance and nutrient-enriched conditions showed increased N content and total mycosporine-like amino acids (MAAs). Our results show rapid photophysiological responses of *C. tamariscifolia* to varia- tions in *in situ* irradiance and nutrient conditions, suggesting efficient photoacclimation to envi- ronmental changes. In *E. elongata*, F_v/F_m and ETR_{max} did not change in the transplant experiment; in contrast, N content, pigment and MAAs (biochemical variables) changed. The responses of these macroalgae to nutrient enrichment indicate oligotrophic conditions at the study site and environmental stress.

KEY WORDS: *Cystoseira tamariscifolia* · *Ellisolandia elongata* · Antioxidant activity · Carotenoids · Irradiance · Nutrient · Polyphenols · Photoprotection

*Corresponding author: paulacelispla@uma.es

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3.2 INTRODUCTION

Environmental stressors can interact and have synergistic or antagonistic effects on physiological responses (Bischof et al. 2006). When multiple stressors act synergistically, there can be unpredictable effects on organisms (Xenopoulos et al. 2002). In contrast, when stressors operate in an additive way, species' responses are easier to predict (Martínez et al. 2012). It is important to understand the mechanisms of combined environmental stressors in order to predict an organism's responses to future climate scenarios. Experimental transplants can provide a better understanding of such effects (Marzinelli et al. 2009, 2011).

Benthic intertidal organisms are subjected to major changes during the tidal cycle (Davison & Pearson 1996). The responses of intertidal and benthic organisms to stressors can be very rapid, and involve adjustments in their photosynthetic and respiratory activities (Southward et al. 1995, Hoegh-Guldberg & Bruno 2010, Sorte et al. 2010). Temperate intertidal rocky communities can be dominated by habitat-forming macroalgae that drive the biodiversity and functioning of these ecosystems. The algae provide food and shelter, and also reduce environmental stress (Davison & Pearson 1996, Jones 1997, Helmuth et al. 2002, 2006). However, the increasing environmental stresses associated with climatic changes and anthropogenic impacts (e.g. coastal eutrophication, increase in UV light) can affect macroalgal communities at the biochemical, ecophysiological, morphological and population levels (Figueroa & Gómez 2001, Bischof et al. 2006).

Light availability is a key factor affecting marine environments (Huovinen & Gómez 2011). Light promotes photosynthetic activity, but can inhibit many biological processes if radiation becomes excessive (Hanelt & Figueroa 2012). Macroalgae have several photoprotective mechanisms such as energy dissipation by specific pigments (e.g. carotenoids) through the xanthophyll cycle (Goss & Jakob 2010); dynamic photoinhibition, i.e. reversible changes in photosynthetic efficiency and capacity, accumulation of ultraviolet screen compounds and increase of antioxidant activity (Gómez et al. 2011). For instance, brown algae accumulate UV screen compounds (polyphenols) with a strong antioxidant activity under high photosynthetically active radiation (PAR) and UVR (Pavia et al. 1997, Connan et al. 2004, Cruces et al. 2012), whereas the tolerance of most red algae to excessive light, including UV, is driven by the accumulation of mycosporine-like amino acids (MAAs) (de la Coba et al. 2009).

Nutrient availability is another environmental factor limiting macrophyte growth in temperate and oligotrophic habitats (Hanisak 1979, Conolly & Drew 1985). Nitrogen limitation affects many processes in macroalgae including photosynthetic capacity (Pérez-Lloréns et al. 1996), protein content (Vergara et al. 1995, Martínez & Rico 2002) and photoprotection mechanisms (Korbee-Peinado et al. 2004, Korbee et al. 2005b, Huovinen et al. 2006). Under moderate to highly desiccated conditions, some intertidal macroalgae increase their nitrogen and carbon uptake (Lobban & Harrison 1994, Flores-Moya et al. 1998, Nygard & Dring 2008). In terms of nutrient metabolism and nutrition, macroalgae vary according to their growth strategies (Lobban & Harrison 1994, Pedersen & Borum 1997). On one side, slow-growing perennial macroalgae, adapted to stable or seasonally variable N conditions, can develop large N and P storage pools (Martínez et al. 2012). At the another extreme, fast-growing opportunistic algae are unable to store large amounts, but show remarkably high N- and P-uptake rates to profit from unstable N-supply conditions (Teichberg et al. 2008). Finally, nutrient enrichment increases the photoprotection capacity of seaweeds due to the increase in protein content, MAAs (Korbee-Peinado et al. 2004, Huovinen et al. 2006, Figueroa et al. 2012) or polyphenols (Arnold & Targett 2002).

Cystoseira tamariscifolia Papenfuss (Phaeophyceae, Fucales) and *Ellisolandia elongata* (Ellis & Solander) Hind & Saunders (Florideophyceae, Corallinales) are 2 important species on Mediterranean rocky shores. *Cystoseira* spp. are indicators of high quality coastal waters (Arévalo et al. 2007, Ballesteros et al. 2007, Bermejo et al. 2013), according to the criteria of the Water Framework Directive of the European Union (WFD, 2000/60/EC). *E. elongata* is a stress-tolerant, calcareous species dominating zones subjected to disturbance.

In this study, the physiological and biochemical responses of *C. tamariscifolia* and *E. elongata*, collected from 2 different depths, were investigated in relation to the independent and/or interactive effects of ambient radiation and nutrient availability. Based on previous research on the additive effects of physical stressors on fucoid algae (Martínez et al. 2012), we hypothesized that changes in light and nitrogen will have an additive effect on *C. tamariscifolia* and *E. elongata*. Algae collected from 0.5 m depth and under nutrient enriched conditions were expected to be less vulnerable under the transplant conditions.

3.3 MATERIALS AND METHODS

Studied species

Cystoseira tamariscifolia is a habitat-forming species that dominates intertidal and shallow-subtidal Mediterranean communities in pristine sites and oligotrophic waters. Although this is a perennial species, receptacles are most developed in spring and summer (Gómez-Garreta et al. 2001). *Ellisolandia elongata* is an articulated calcareous species that dominates benthic intertidal communities replaced by ulvacean algae at intermediate levels of nutrient enrichment (Arévalo et al. 2007). Resembling a small bush and up to 20 cm in height (Braga et al. 2009), it is a perennial species and can occupy both well-lit and shaded habitats (Algarra & Niell 1987, Häder et al. 1997, Figueroa & Gómez 2001). It has been recorded to be in the fertile tetrasporophyte phase throughout the year (Rodríguez & Polo 1986).

Experimental design

The experiment was performed from September 19 to 21, 2012. *C. tamariscifolia* and *E. elongata* were randomly collected from 2 different depths (0.5 and 2.0 m) (Fig. 1a) at the 'Cabo de Gata-Níjar' Natural Park (36° 51' 0" N; 2° 6' 0" W; southwestern Mediterranean Sea, Spain). Immediately after collection, macroalgal samples (5 g fresh weight [FW]) were placed into mesh cylinders (15 cm long × 5 cm in diameter) and suspended in the water column (at a depth of 0.2 m) by a floating longline system anchored to the bottom and parallel to the coast (Fig. 1b). This system comprised 4 lines of 12 m length. Each line contained 12 cylinders (separated by 1 m). Two lines were placed at one site for the enriched nitrogen treatment and the other 2 lines were placed at another site for the non-enrichment treatment (Fig. 1b). Both sites were separated by 50 m with a small artificial breakwater between them. Each cylinder contained specimens of one unique species and collection depth (in triplicate) was fixed along each line (Fig. 1c). Two light levels were assigned within each treatment, i.e. 70 and 100 % of surface irradiance defined as PAB irradiance (PAR + UVR) under nutrient-enriched and non-enriched conditions (Fig. 1c). With regard to the irradiance treatment, a neutral screen was used which attenuates 30 % of the incident light. Half of the cylinders (containing algae from both depths) were covered with mesh (1 mm^2) to attain 70 % incoming irradiance (simulating conditions at a depth of 2.0 m, thereafter 70 $\%_{PAB}$), and the remaining cylinders were without the screen to attain 100 % incoming irradiance

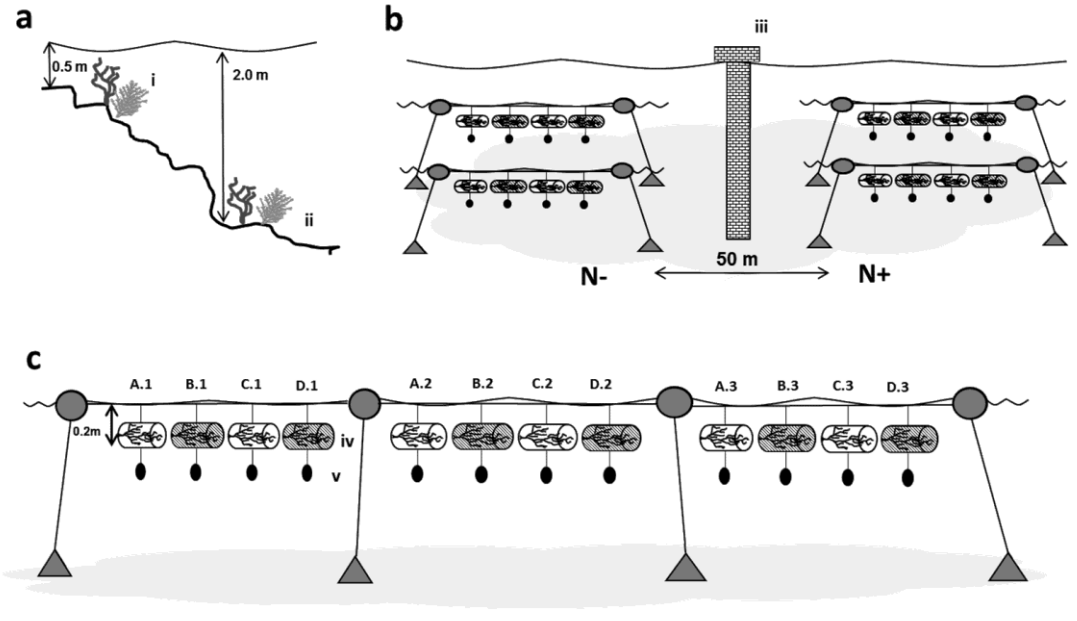

Fig. 1. (a) Depths of origin (i: 0.5 m; ii: 2.0 m) of both collected species *Cystoseira tamariscifolia* and *Ellisolandia elongata*. (b) Schematic layout of the floating lines system separated by a physical barrier (iii: breakwater) comprising 4 longline systems 50 m apart for each treatment. N+ and N− indicate nutrient-enriched and non-enriched treatments, respectively. (c) Schematic layout of one floating line system for each macroalgae with 12 cylinders (iv: cylinder; v: bag with fertilizer or sand). White cylinders (A.1, A.2, A.3, C.1, C.2 and C.3) indicate 100 $\%_{PAB}$ treatment with all replicates, and grey cylinders (B.1, B.2, B.3, D.1, D.2 and D.3) indicate 70 $\%_{PAB}$ with all replicates for both depths

(simulating a depth of 0.5 m, thereafter 100 $\%_{PAB}$). Thereby, algae collected at 0.5 m depth (shallow waters) were exposed to 70 $\%_{PAB}$ (as a transplant treatment) and 100 $\%_{PAB}$ (as a control of natural conditions at 0.5 m depth). On the other hand, those algae collected at 2.0 m depth were exposed to 100 $\%_{PAB}$ (as a transplant treatment) and 70 $\%_{PAB}$ (as a control of natural conditions at 2.0 m depth) (Fig. 1b). For the nutrient-enriched treatments, mesh bags containing 100 g of a slow-release resin-coated fertilizer (Multicote®, Haifa Chemicals) (modified from Martínez et al. 2012) and fixed below each cylinder was used to simulate nutrient enrichment. Fertilizer composition was 17 % N (NH_4^+ and NO_3^-), 17 % P (P_2O_5) and 17 % K. For non-enriched treatments, a neutral bag with 100 g of sand was used as a control of the effect of the fertilizer bag and the modifying buoyancy (Fig. 1b).

Three replicate cylinders were used for each combination of treatment level, species and depth (2 species × 2 depths × 2 irradiance levels × 2 nutrient levels), resulting in a total of 48 cylinders with macroalgal samples (Fig. 1b). Several physiological variables were obtained from the algae within each cylinder after the *in situ* experiment. These variables were also measured in *C. tamariscifolia* and *E. elongata* from natural populations (at 0.5 and 2.0 m depth) in order to know the initial values. Additionally, water nutrient concentrations, irradiance (PAR and UVR) and underwater temperature were measured during the experiment.

Environmental conditions

Nutrient enrichment (N and P) through fertilizer was assessed by taking triplicate seawater samples at both enriched and non-enriched sites. Seawater was filtered *in situ* using portable GF/F filters (Whatman), transported to the laboratory inside an isotherm bag (4°C, in darkness) and kept at −20°C (Martínez et al. 2012). Nitrate (NO_3^-), ammonium (NH_4^+) and orthophosphate (HPO_4^{3-}) were determined using an automated wet chemistry analyzer (SanPlus^{++} System, SKALAR) applying standard colorimetric procedures (Koroleff 1983).

Irradiance of solar radiation was continuously measured in the air at 3 wavelength bands (UVB = 280−315 nm, UVA = 315−400 nm and PAR = 400−700 nm) using 2 hyperspectral irradiance sensors for UV and PAR (Ramses, TrioS). Attenuation coefficients in water (Kd_{PAR} and Kd_{UVA}) were measured using PAR (QSO-SUN 2.5V) and UV-R (USB-SU 100, Onset Computer) sensors sealed within a waterproof polycarbonate box (OtterBox3000). Kd_{UVB} was not measured due to the high absorption of the polycarbonate box in the UVB spectral band (Quintano et al. 2013).

Underwater temperature was continuously measured using a HOBO U22 Water Temp Pro v2 logger (Onset Computer).

Physiological and biochemical variables

Carbon and nitrogen contents on a dry weight (DW) basis were determined using an element analyzer CNHS-932 (LECO).

In vivo chlorophyll *a* (chl *a)* fluorescence associated with Photosystem II (PSII) was determined by using a portable pulse amplitude modulated fluorometer Diving-PAM (Walz). Algal pieces were collected from natural populations (initial time) and after 60 h of incubation (for each cylinder) and were placed in 10 ml incubation chambers in order to conduct rapid light curves, one for each cylinder. F_o and F_m were determined after 15 min in darkness to obtain the maximum quantum yield (F_v/F_m), where $F_v = F_m - F_o$, F_o is the basal fluorescence of dark-adapted thalli after 15 min and F_m is the maximal fluorescence after a saturation light pulse of > 4000 µmol m^{-2} s^{-1} (Schreiber et al. 1995, Figueroa et al. 2009). The electron transport rate (ETR, µmol electrons m^{-2} s^{-1}) as rapid light curves (RLC) was determined after a 20 s exposure period in 8 increasing irradiances (E1 = 9.3, E2 = 33.8, E3 = 76, E4 = 145, E5 = 217, E6 = 301, E7 = 452, E8 = 629, E9 = 947 µmol m^{-2} s^{-1}) of white light (halogen lamp provided by the Diving-PAM). ETR was calculated according to Schreiber et al. (1995) as follows:

$$ETR = \Delta F/F_m' \times E \times A \times F_{II} \quad (1)$$

where $\Delta F/F_m'$ is the effective quantum yield, $\Delta F = F_m' - F_t$ (F_t is the intrinsic fluorescence of alga incubated in light and F_m' is the maximal fluorescence reached after a saturation pulse of algae incubated in light), E is the incident PAR irradiance expressed in µmol photons m^{-2} s^{-1}, A is the thallus absorptance as the fraction of incident irradiance that is absorbed by the algae (see Figueroa et al. 2003) and F_{II} is the fraction of chlorophyll related to PSII (400 –700 nm), being 0.8 in brown and 0.15 in red macroalgae (Grzymski et al. 1997, Figueroa et al. 2003). Maximum ETR (ETR_{max}) and the initial slope of ETR versus irradiance function (α_{ETR}), as an estimator of photosynthetic efficiency, were obtained from the tangential function reported by Eilers & Peeters (1988). Finally, the saturation irradiance for ETR (Ek_{ETR}) was calculated from the intercept between ETR_{max} and α_{ETR}.

Non-photochemical quenching (NPQ) was calculated according to Schreiber et al. (1995) as:

$$NPQ = (F_m - F_m')/F_m' \quad (2)$$

Maximal NPQ (NPQ_{max}) and the initial slope of NPQ versus irradiance function (α_{NPQ}) were obtained from the tangential function of NPQ versus irradiance function according to Eilers & Peeters (1988). Finally, the saturation irradiance for NPQ (Ek_{NPQ}) was calculated from the intercept between NPQ_{max} and α_{NPQ}.

Chl *a* and carotenoid pigments were determined in both species, whereas chlorophyll *c* (chl *c)* only in *C. tamariscifolia* and phycobiliproteins only in *E. elongata*.

Chl *a* was determined spectrophotometrically, whilst chl *c* was identified and quantified using HPLC. Both chlorophyll analyses were made by extracting pigments from thalli (25 mg FW) using 1 ml of N,N-dimethylformamide (DMF) and maintained in darkness at 4°C for 12 h. After centrifugation at 5000 × *g* for 10 min (Labofuge 400R, Heraeus, Kendro Laboratory Products), each supernatant was used to measure chlorophyll spectrophotometrically. In the case of chl *c*, the extracts were filtered (0.2 µM) before analyzing with HPLC. The chlorophyll concentrations were calculated using equations by Wellburn (1994). Carotenoid composition was determined by HPLC according to García-Sánchez et al. (2012), using commercial standards (DHI LAB Products).

Phycobiliproteins of *E. elongata* were extracted in 0.1 M phosphate buffer (pH 6.5), centrifuged at 2253 × *g* for 30 min at 4°C. Phycoerythrin (PE) and phycocyanin (PC) concentrations were calculated following Sampath-Wiley & Neefus (2007) equations.

Total phenolic compounds (polyphenols) were determined only in *C. tamariscifolia* using 0.25 g FW. Samples were pulverized in a mortar and pestle with sea sand using 2.5 ml of 80 % methanol. After keeping the samples overnight, the mixture was centrifuged at 2253 × *g* for 30 min at 4°C, and then the supernatant was collected. Total phenolic compounds were determined colorimetrically using Folin-Ciocalteu reagent (Folin & Ciocalteu 1927) and phloroglucinol (1, 3, 5-trihydroxybenzene, Sigma P-3502) as standard. Finally, the absorbance was determined at 760 nm using a Shimadzu UVMini-1240 spectrophotometer. Phenolic concentration was expressed as mg g^{-1} DW after determining the fresh to dry weight ratio in the tissue (4.3 and 1.5 for *C. tamariscifolia* and *E. elongata*, respectively). The results are expressed as mean ± SE from 3 replicates of each treatment.

Antioxidant activity, determined by the 2, 2-diphenyl-1-picrylhydrazyil (DPPH) method, was measured on the polyphenol compound extracts according to Blois (1958). Each extract had 150 µl of DPPH, prepared in 90 % methanol, added. The reaction was complete after 30 min in darkness at ambient temperature (~20°), and the absorbance was read at 517 nm in a spectrophotometer UVmini-1240 (Shimadzu). The calibration curve made from DPPH was used to calculate the remaining concentration of DPPH in the reaction mixture after incubation. Values of DPPH concentration (mM) were plotted against plant extract concentration (mg DW ml^{-1}) in order to obtain the EC_{50} value (oxidation index), which represents the concentration of the extract (mg ml^{-1}) required to scavenge 50 % of the DPPH in the reaction mixture. Ascorbic acid was used as a positive control (Connan et al. 2006).

Total MAA content was determined only in *E. elongata* using HPLC (Waters 600) as described by Korbee-Peinado et al. (2004). Results were expressed as mg g^{-1} DW after determining the fresh to dry weight ratio in the tissue (1.5 for *E. elongata).*

Statistical analysis

The effects of the *in situ* treatments on the ecophysiological response variables of *C. tamariscifolia* and *E. elongata* were assessed using ANOVA (Underwood 1997). For that purpose, 2 factors were considered: Nutrient (fixed with 2 levels) and Irradiance (fixed with 2 levels). This design allows the testing of interactive and additive effects of the variables on the ecophysiological responses. Data used in the analyses were those obtained at the end of the experimental period (after 60 h of photoacclimation). Student-Newman-Keuls tests (SNK) were performed after significant ANOVA interactions (Underwood 1997). Homogeneity of variance was tested using Cochran tests and by visual inspection of the residuals. Analyses were performed by using SPSS v.21 (IBM).

3.4 RESULTS

Environmental conditions

Nitrate (NO_3^-), ammonium (NH_4^+) and phosphate (PO_4^{3-}) concentrations at the non-enriched site were 1.34 ± 0.31 µM, 1.17 ± 0.35 µM and 0.09 ± 0.01 µM, respectively. In contrast, concentrations at the nutrient-enriched site were 107.51 ± 9.67 µM, 163.31

± 6.10 µM and 24.52 ± 1.51 µM, respectively (mean ± SE, n = 6). Hence, on average, the nutrient-enriched treatment increased nitrate, ammonium and phosphate concentrations in the water column by 80, 139 and 272 times, respectively. The average daily integrated surface irradiance for the experimental period (September 20 and 21, 2012) was 5842 KJ m^{-2} for PAR, 673.3 KJ m^{-2} for UVA and 27.3 KJ m^{-2} for UVB. The attenuation coefficients for PAR (Kd_{PAR}) and UVA (Kd_{UVA}) were 0.076 m^{-1} and 0.137 m^{-1}, respectively. The average seawater temperature at 0.2 m (mean ± SE, n = 1440) ranged between 24.42 ± 0.42°C (during the day) and 23.8 ± 0.19°C (at night).

Physiological response variables

Internal N content was higher in *Cystoseira tamariscifolia* than in *Ellisolandia elongata* (Table 1, Fig. 2). ANOVA results showed that both species from 0.5 m depth presented significantly higher N content and a lower C:N ratio under the nutrient-enriched treatment (Table 1, Figs. 2 & 3). However, the N content from 2.0 m depth samples was different for both species (Table 1, Fig. 2). *C. tamariscifolia* specimens collected from 2.0 m showed similar N content to those from 0.5 m and the C:N ratio increased under the non-enriched treatments (Figs. 2a & 3a). In contrast, *E. elongata* showed a significant interaction between nutrients and irradiance (Table 1). N content in the nutrient-enriched treatment was lower under the 100 $\%_{PAB}$ treatments and the C:N ratio was higher under the same conditions (Figs. 2b & 3b).

F_v/F_m in *C. tamariscifolia* showed a significant interaction with nutrients and irradiance in algae collected at 2.0 m depth (Table 2). Specimens of *C. tamariscifolia* transplanted to 100 $\%_{PAB}$ presented higher F_v/F_m under non-enriched treatments (Table 3). Neither of the species collected at 0.5 m

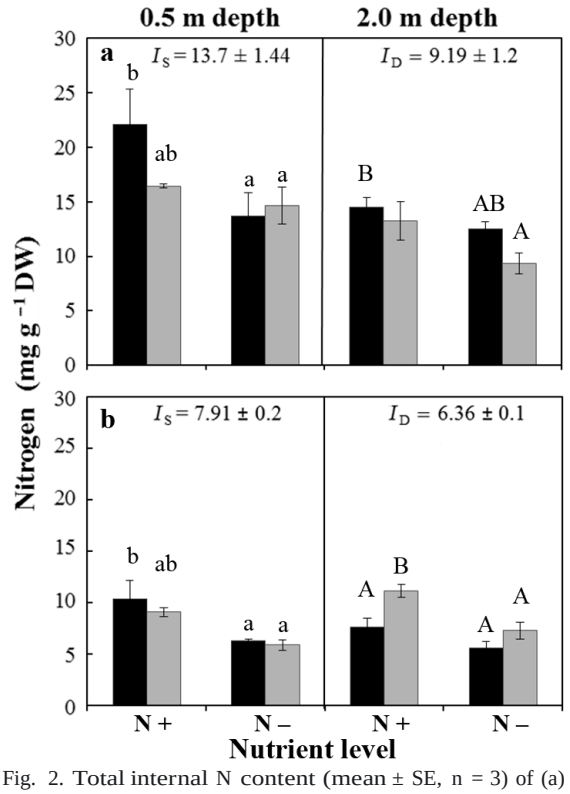

Fig. 2. Total internal N content (mean ± SE, n = 3) of (a) *Cystoseira tamariscifolia* and (b) *Ellisolandia elongata* from 0.5 and 2.0 m depth under irradiance and nutrient treatments. Black bars indicate 100 $\%_{PAB}$, and grey bars indicate 70 $\%_{PAB}$. N+ and N– indicate nutrient-enriched and non-enriched treatments, respectively. Upper values in each box indicate initial values (I_S: 0.5 m depth; I_D: 2.0 m depth). Lowercase letters denote significant differences after SNK test for 0.5 m and capital letters for 2.0 m algae

Table 1. ANOVA results after *in situ* experiment testing for the effect of irradiance and nutrients on C and N contents and C:N ratios of *Cystoseira tamariscifolia* and *Ellisolandia elongata* collected at 2 different depths. We used a significance level of α = 0.05, shown in **bold**

		df	*Cystoseira tamariscifolia*						*Ellisolandia elongata*					
			0.5 m depth			2.0 m depth			0.5 m depth			2.0 m depth		
			MS	*F*	p	MS	*F*	p	MS	*F*	p	MS	*F*	p
C	Nutrients (*N*)	1	75.5	0.276	0.613	753.7	1.366	0.276	**561.7**	**5.805**	**0.043**	149.1	1.400	0.271
	Irradiance (*E*)	1	27.9	0.102	0.758	2578.4	4.672	0.063	25.5	0.264	0.621	231.4	2.173	0.179
	N × *E*	1	144.9	0.530	0.487	108.6	0.197	0.669	2.2	0.022	0.885	**744.2**	**6.987**	**0.030**
	Residual	8	273.4			551.8			96.8			106.5		
N	Nutrients (*N*)	1	**77.6**	**5.625**	**0.045**	**25.8**	**6.639**	**0.033**	**39.9**	**14.145**	**0.006**	**25.6**	**15.540**	**0.004**
	Irradiance (*E*)	1	16.1	1.163	0.312	14.9	3.836	0.086	2.1	0.753	0.411	**20.3**	**12.321**	**0.008**
	N × *E*	1	32.9	2.382	0.161	2.7	0.695	0.429	0.5	0.189	0.675	2.5	1.543	0.249
	Residual	8	13.8			3.9			2.8			1.6		
C:N	Nutrients (*N*)	1	103.6	5.098	0.054	**154.7**	**5.962**	**0.040**	**132.0**	**23.959**	**0.001**	**182.8**	**11.883**	**0.009**
	Irradiance (*E*)	1	0.5	0.023	0.884	27.2	1.047	0.336	10.1	1.830	0.213	**149.6**	**9.723**	**0.014**
	N × *E*	1	39.9	1.963	0.199	18.5	0.712	0.423	0.0	0.006	0.941	10.3	0.668	0.437
	Residual	8	20.3			26.0			5.5			15.4		

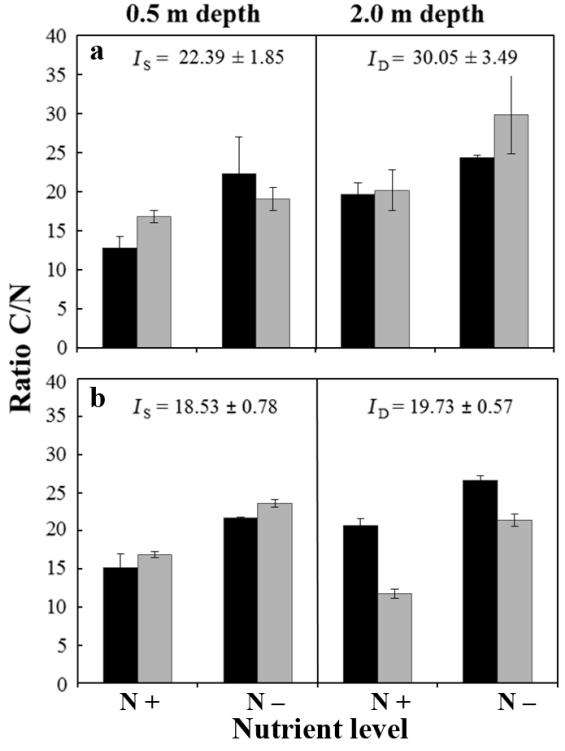

Fig. 3. C:N ratio (mean ± SE, n = 3) of (a) *Cystoseira tamariscifolia* and (b) *Ellisolandia elongata* from 0.5 and 2.0 m depth under irradiance and nutrient treatments. Black bars indicate 100 $\%_{PAB}$, and grey bars indicate 70 $\%_{PAB}$. N+ and N− indicate nutrient-enriched and non-enriched treatments, respectively. Upper values in each box indicate initial values (I_S: 0.5 m depth; I_D: 2.0 m depth)

nor *E. elongata* at 2.0 m showed significant differences (Table 2). In contrast, ETR_{max} of *C. tamariscifolia* showed significant differences among irradiance treatments (70 $\%_{PAB}$ and 100 $\%_{PAB}$) at 0.5 m depth (Table 2). This value was higher when they were transplanted to 70 $\%_{PAB}$ (Table 3). Conversely, specimens of both species collected at 2.0 m depth did not show any significant differences for either depth. α_{ETR} in *C. tamariscifolia* showed a significant interaction with nutrients and irradiances at both depths (Table 2). This value was lower at 70 $\%_{PAB}$ (transplant treatment) and non-nutrient enriched conditions. In both cases, α_{ETR} equaled initial observations from its natural habitat after incubation in the cylinders. (Table 3). To compare, *E. elongata* α_{ETR} values showed 2 different significant results depending on the depth. α_{ETR} in algae collected from 0.5 m depth showed a significant increase at the nutrient-enriched site and in the 70 $\%_{PAB}$ treatment (Tables 2 & 3). In contrast, algae collected from 2.0 m had higher α_{ETR} values under the non-enriched treatment (Tables 2 & 3).

In *C. tamariscifolia* collected from 0.5 m depth, Ek_{ETR} showed a significant interaction with nutrients and irradiance. In algae collected at 0.5 m depth under 70 $\%_{PAB}$ in the non-enriched treatment, Ek_{ETR} was higher than in the other 3 combinations of treatments (Table 3). However, in algae collected from 2.0 m depth, Ek_{ETR} did not show any significant differences (Table 2). On the other hand, in *E. elongata*, Ek_{ETR} at both depths showed significant differences with the nutrients (Table 2). Ek_{ETR} values for algae collected from 0.5 m depth were higher in non-enriched treatments, whereas in algae from 2.0 m depth, the values were higher in nutrient-enriched treatments (Table 2).

NPQ_{max} in *C. tamariscifolia* showed significant differences due to nutrient treatments in algae collected from 0.5 m depth, and a significant interaction was observed with nutrients and irradiance in algae collected from 2.0 m depth (Table 2). In algae from both depths, NPQ_{max} was higher in non-enriched treatments, whereas the NPQ_{max} increased under 100 $\%_{PAB}$ conditions in algae collected from 2.0 m depth (Table 3). NPQ_{max} did not show any significant differences among treatments in *E. elongata* (Table 2), in contrast to *C. tamariscifolia* which showed significant differences due to nutrients at both depths (Table 2). Ek_{NPQ} values in algae collected from 0.5 m were higher in enriched treatments, whereas values were higher under non-enriched treatments in algae from 2.0 m (Table 3). Finally, Ek_{NPQ} showed no significant differences among treatments in *E. elongata* (Table 2).

Pigment content

Chl *a* in *C. tamariscifolia* increased significantly when algae from 0.5 m depth were exposed to lower irradiance levels (70 $\%_{PAB}$ treatment). Similar results were found for chl *c* in algae collected from 2.0 m (Tables 4 & 5). Chl *c* content in *C. tamariscifolia* collected from 0.5 m was significantly higher in the nutrient-enriched treatment than in the non-enriched one (Tables 4 & 5). Chl *a* and *c* contents were initially higher in algae collected from 0.5 m (Table 5). Chl *a* in *E. elongata* did not present any significant differences among treatments (Tables 4 & 5).

PC content was significantly higher in the nutrient-enriched treatment in *E. elongata* collected from 0.5 m depth. In contrast, PE content did not show any differences after the experiment (Tables 4 & 5).

The carotenoids fucoxanthin and violaxanthin in *C. tamariscifolia* showed a significant increase under nutrient-enriched treatment in algae from 0.5 m depth

Table 2. ANOVA results after *in situ* experiment testing for the effect of irradiance and nutrients on photosynthetic parameters of *Cystoseira tamariscifolia* and *Ellisolandia elongata* collected at 2 different depths. We used a significance level of $\alpha = 0.05$, shown in **bold**. F_v/F_m: maximal quantum yield, α_{ETR}: photosynthetic efficiency, ETR_{max}: maximal electron transport rate, Ek_{ETR}: saturated irradiance of ETR, NPQ_{max}: maximal non-photochemical quenching, Ek_{NPQ}: saturated irradiance of NPQ

		Cystoseira tamariscifolia						*Ellisolandia elongata*					
	df	0.5 m depth			2.0 m depth			0.5 m depth			2.0 m dep		
		MS	*F*	p	MS	*F*	p	MS	*F*	p	MS	*F*	p
F_v/F_m													
Nutrients (*N*)	1	0.001	0.214	0.656	0.000	0.047	0.834	0.001	0.202	0.665	0.012	5.293	0.050
Irradiance (*E*)	1	0.005	1.228	0.300	0.002	0.702	0.426	0.000	0.156	0.703	0.005	2.129	0.183
N × *E*	1	0.013	3.408	0.102	**0.019**	**5.925**	**0.041**	0.000	0.036	0.854	0.007	3.153	0.114
Residual	8	0.004			0.003			0.003			0.002		
α_{ETR}													
Nutrients (*N*)	1	**0.025**	**29.197**	**0.001**	0.001	0.927	0.364	**0.026**	**16.605**	**0.004**	**0.029**	**19.660**	**0.002**
Irradiance (*E*)	1	0.002	2.948	0.124	0.001	1.076	0.330	**0.009**	**5.491**	**0.047**	0.006	4.160	0.076
N × *E*	1	**0.008**	**9.009**	**0.017**	**0.007**	**6.695**	**0.032**	0.007	4.680	0.062	0.000	0.010	0.921
Residual	8	0.001			0.001			0.002			0.001		
ETR_{max}													
Nutrients (*N*)	1	2468.2	4.427	0.069	2320.8	4.728	0.061	0.122	0.294	0.602	0.009	0.019	0.895
Irradiance (*E*)	1	**3773.9**	**6.769**	**0.032**	2139.0	4.358	0.070	0.093	0.224	0.648	0.000	0.001	0.978
N × *E*	1	345.6	0.620	0.454	710.5	1.448	0.263	0.110	0.264	0.621	0.017	0.036	0.854
Residual	8	557.5			490.8			0.416			0.470		
Ek_{ETR}													
Nutrients (*N*)	1	**102164.0**	**20.450**	**0.002**	27666.1	2.289	0.169	**101.4**	**26.275**	**< 0.001**	**68.4**	**14.259**	**0.005**
Irradiance (*E*)	1	**82554.8**	**16.525**	**0.004**	47574.2	3.937	0.083	8.4	2.188	0.177	18.2	3.796	0.087
N × *E*	1	**36962.9**	**7.399**	**0.026**	288.0	0.024	0.881	3.9	1.019	0.342	8.7	1.819	0.214
Residual	8	4995.8			12085.2			3.9			4.8		
NPQ_{max}													
Nutrients (*N*)	1	**1.186**	**9.827**	**0.014**	**12.065**	**71.55**	**< 0.001**	0.002	0.060	0.813	0.000	0.001	0.979
Irradiance (*E*)	1	0.000	0.002	0.969	0.883	5.234	0.051	0.000	0.004	0.951	0.001	0.066	0.803
N × E	1	0.020	0.169	0.692	**0.946**	**5.608**	**0.045**	0.002	0.069	0.799	0.001	0.066	0.803
Residual	8	0.121			0.169			0.033			0.021		
Ek_{NPQ}													
Nutrients (*N*)	1	**558682.0**	**13.364**	**0.006**	**11110334**	**94.365**	**< 0.001**	113.6	0.578	0.469	79.1	0.154	0.705
Irradiance (*E*)	1	48629.6	1.163	0.312	609492.6	5.177	0.052	37.1	0.189	0.676	18.8	0.036	0.853
N × *E*	1	9645.9	0.231	0.644	216.7	0.002	0.967	37.5	0.191	0.674	18.8	0.036	0.853
Residual	8	41803.7			117737.9			196.5			515.2		

(Tables 4 & 5). In contrast, carotenoid content in algae collected from 2.0 m depth was significantly higher the under 70 $\%_{PAB}$ treatment (Tables 4 & 5). Additionally, antheraxanthin and β-carotene in *C. tamariscifolia* collected at the same depth had a significant interaction between nutrients and irradiance. Both compounds increased significantly at 70 $\%_{PAB}$ in the non-enriched treatment site (Tables 4 & 5). In *E. elongata*, fucoxanthin, antheraxanthin and β-carotene contents in algae collected from 0.5 m depth showed a significant increase in the 70 $\%_{PAB}$ irradiance treatment (Tables 4 & 5). Additionally, fucoxanthin content increased significantly in algae cultured under nutrient-enrichment conditions (Tables 4 & 5). Zeaxanthin content did not show any differences after the *in situ* experiment (Tables 4 & 5) for either species.

Total phenolic compounds. Total phenolic compounds in *C. tamariscifolia* were significantly different among nutrient treatments in algae from both 0.5 and 2.0 m depths (Table 6). Additionally, algae collected from 2.0 m showed significant differences in both irradiance treatments (Table 6). In algae collected from 0.5 m depth, the total phenolic compounds were higher in the nutrient-enriched treatment (Fig. 4a). In *C. tamariscifolia* from 2.0 m depth, the increase of phenolic compounds was higher under 100 $\%_{PAB}$ than under 70 $\%_{PAB}$, whereas this increase was higher under non-enrichment than that under the enrichment treatment (Fig. 4a).

Antioxidant activity (EC_{50}). EC_{50} in *C. tamariscifolia* collected at 0.5 m depth showed a significant interaction between nutrients and irradiance

Table 3. Maximal quantum yield (F_v/F_m), photosynthetic efficiency (α_{ETR}), maximal electron transport rate (ETR_{max}, µmol m^{-2} s^{-1}), saturated irradiance of ETR (Ek_{ETR}, µmol m^{-2} s^{-1}), maximal non-photochemical quenching (NPQ_{max}) and saturated irradiance of NPQ (Ek_{NPQ}, µmol m^{-2} s^{-1}) (mean ± SE, n = 3) of *Cystoseira tamariscifolia* and *Ellisolandia elongata* in relation to irradiance (70 $\%_{PAB}$ and 100 $\%_{PAB}$) and nutrient (Nutrients+ and Nutrients−) treatments. Initial values (I_S: 0.5 m depth; I_D: 2.0 m depth) are shown in the first column and in **bold** for each depth. Lowercase letters denote significant differences after SNK test for algae collected at 0.5 m and capital letters for algae collected at 2.0 m

	I_S	0.5 m depth				I_D	2.0 m depth			
		Nutrients+		Nutrients−			Nutrients+		Nutrients−	
		100 $\%_{PAB}$	70 $\%_{PAB}$	100 $\%_{PAB}$	70 $\%_{PAB}$		100 $\%_{PAB}$	70 $\%_{PAB}$	100 $\%_{PAB}$	70 $\%_{PAB}$
Cystoseira tamariscifolia										
F_v/F_m	**0.72 ± 0.01**	0.69 ± 0.03	0.71 ± 0.01	0.74 ± 0.02	0.63 ± 0.05	**0.61 ± 0.01**	0.67 ± 0.02	0.72 ± 0.01	0.74 ± 0.03	0.64 ± 0.01
α_{ETR}	**0.33 ± 0.01**	0.36 ± 0.02^{bc}	0.38 ± 0.01^{b}	0.32 ± 0.01^{b}	0.24 ± 0.01^{a}	**0.28 ± 0.01**	0.28 ± 0.01^{A}	0.31 ± 0.01^{AB}	0.34 ± 0.01^{B}	0.28 ± 0.03^{A}
ETR_{max}	**67.98 ± 8.91**	67.23 ± 11.51^{a}	91.97 ± 9.13^{ab}	85.18 ± 21.98^{ab}	131.38 ± 6.64^{b}	**63.51 ± 8.66**	57.01 ± 7.76	99.09 ± 2.26	100.21 ± 12.25	111.51 ± 20.95
Ek_{ETR}	**204.18 ± 36.74**	182.77 ± 25.51^{a}	237.66 ± 28.43^{a}	256.31 ± 59.35^{a}	533.20 ± 40.98^{b}	**228.67 ± 34.52**	201.92 ± 29.18	318.05 ± 10.38	288.16 ± 39.04	423.88 ± 116.744
NPQ_{max}	**1.11 ± 0.14**	0.34 ± 0.08^{a}	0.41 ± 0.01^{ab}	1.05 ± 0.04^{b}	0.96 ± 0.38^{ab}	**1.05 ± 0.04**	0.44 ± 0.01^{A}	0.46 ± 0.14^{A}	3.01 ± 0.28^{C}	1.91 ± 0.34^{B}
Ek_{NPQ}	**602.73 ± 39.93**	697.69 ± 39.93^{ab}	881.71 ± 219.08^{b}	322.85 ± 4.11^{a}	393.47 ± 78.28^{a}	**312.47 ± 11.16**	1302.26 ± 91.89^{A}	860.03 ± 165.71^{A}	3235.21 ± 61.18^{B}	2775.96 ± 326.32^{B}
Ellisolandia elongata										
F_v/F_m	**0.53 ± 0.06**	0.501 ± 0.037	0.519 ± 0.011	0.521 ± 0.043	0.527 ± 0.016	**0.49 ± 0.03**	0.465 ± 0.031	0.456 ± 0.023	0.480 ± 0.034	0.570 ± 0.019
α_{ETR}	**0.017 ± 0.001**	0.013 ± 0.003^{a}	0.024 ± 0.001^{b}	0.009 ± 0.002^{a}	0.009 ± 0.002^{a}	**0.011 ± 0.001**	0.016 ± 0.001^{AB}	0.012 ± 0.004^{A}	0.026 ± 0.001^{C}	0.022 ± 0.002^{BC}
ETR_{max}	**1.65 ± 0.19**	1.41 ± 0.43	1.77 ± 0.05	1.4 ± 0.42	1.38 ± 0.42	**1.36 ± 0.39**	1.42 ± 0.38	1.51 ± 0.51	1.44 ± 0.34	1.38 ± 0.30
Ek_{ETR}	**95.27 ± 5.46**	103.63 ± 10.21^{a}	75.41 ± 3.96^{a}	146.66 ± 11.87^{b}	148.63 ± 16.08^{b}	**115.71 ± 19.44**	85.35 ± 18.51^{A}	127.04 ± 11.87^{B}	54.65 ± 10.94^{A}	62.24 ± 9.35^{A}
NPQ_{max}	**0.51 ± 0.18**	0.45 ± 0.09	0.42 ± 0.14	0.45 ± 0.09	0.47 ± 0.08	**0.36 ± 0.11**	0.446 ± 0.09	0.446 ± 0.9	0.42 ± 0.07	0.46 ± 0.05
Ek_{NPQ}	**78.72 ± 28.94**	72.05 ± 3.86	64.99 ± 12.04	74.66 ± 7.78	74.78 ± 6.43	**47.41 ± 9.11**	71.57 ± 10.16	71.57 ± 10.16	68.93 ± 14.24	63.93 ± 16.65

(Table 6). In the non-enriched treatment, EC_{50} was higher (lower antioxidant activity) than in the other treatment combinations (Fig. 4b). In algae collected at 2.0 m depth, significant differences were only found in nutrient-enriched treatments (Table 6), i.e. EC_{50} was higher (lower antioxidant activity) in the nutrient-enriched treatment (Fig. 4b) than in the non-enriched treatment.

Total MAA content. Total MAA content in *E. elongata* was higher in algae collected at 0.5 m depth than in those collected at 2.0 m (Fig. 5a). MAA content in algae from 0.5 m depth showed a significant increase under 100 $\%_{PAB}$ in nutrient-enriched treatments (Table 7, Fig. 5a). In contrast, total MAA content in algae collected from 2.0 m depth was significantly higher at 100 $\%_{PAB}$ for both enriched and non-enriched nutrient treatments (Table 7, Fig. 5a). The most abundant MAAs detected in this species were shinorine (50 to 60 %) and palythine (approx. 40 %), other MAAs such as asterina-330 were present in trace amounts. After the *in situ* experiment, algae collected from 2.0 m depth showed significantly higher palythine content under nutrient-enriched treatments, and shinorine increased in non-enriched treatments (Table 7, Fig. 5b,c). In contrast, algae collected from 0.5 m did not show any differences (Table 7).

3.5 DISCUSSION

We found high photoacclimation in *Cystoseira tamariscifolia* and *Ellisolandia elongata*, with photosynthetic parameters and biochemical composition changing in response to the short-term irradiance and nutrient treatments (60 h). The algae collected from 0.5 m depth had a higher production (ETR) and efficiency (α_{ETR}) than those from 2.0 m depth. These differences can be explained by the high transparency in the coastal waters of Cabo de Gata-Níjar Natural Park, allowing high penetration of both PAR and UVR, which can produce negative biological effects such as photoinhibition or DNA damage. In our study, the attenuation coefficients for PAR (Kd_{PAR}) and UVA (Kd_{UVA}) were 0.076 m^{-1} and 0.137 m^{-1}, respectively. Figueroa & Gómez (2001) described these coefficients with similar results for PAR (Kd_{PAR}) and UVA (Kd_{UVA}), 0.070 m^{-1} and 0.100 m^{-1}, respectively, and a Kd_{UVB} value of 0.22 m^{-1} in the same coastal area.

Table 4. ANOVA results after *in situ* experiment testing for the effect of irradiance and nutrients on the photosynthetic pigment content of *Cystoseira tamariscifolia* and *Ellisolandia elongata* collected at 2 different depths. We used a significance level of $\alpha = 0.05$, shown in **bold**; nd: no data

	df	*Cystoseira tamariscifolia*						*Corallina elongata*					
		0.5 m depth			2.0 m depth			0.5 m depth			2.0 m depth		
		MS	*F*	p	MS	*F*	p	MS	*F*	p	MS	*F*	p
Chl *a*													
Nutrients (*N*)	1	0.177	1.085	0.328	0.624	3.579	0.095	0.034	3.971	0.081		nd	
Irradiance (*E*)	1	**2.152**	**13.170**	**0.007**	0.040	0.231	0.644	0.040	4.694	0.062			
N × *E*	1	0.038	0.233	0.642	0.770	4.416	0.069	0.005	0.635	0.448			
Residual	8	0.163			0.174			0.009					
Chl *c*													
Nutrients (*N*)	1	**0.015**	**7.653**	**0.024**	0.000	0.162	0.698		nd			nd	
Irradiance (*E*)	1	0.000	0.064	0.807	**0.012**	**6.201**	**0.038**						
N × *E*	1	0.000	0.002	0.963	0.001	0.318	0.588						
Residual	8	0.002			0.002								
Phycoerythrin													
Nutrients (*N*)	1		nd			nd		0.860	3.418	0.102		nd	
Irradiance (*E*)	1							0.421	1.672	0.232			
N × *E*	1							0.017	0.066	0.803			
Residual	8							0.252					
Phycocyanin													
Nutrients (*N*)	1		nd			nd		**0.067**	**5.903**	**0.041**		nd	
Irradiance (*E*)	1							0.06	5.31	0.05			
N × *E*	1							0.001	0.113	0.745			
Residual	8							0.011					
Fucoxanthin													
Nutrients (*N*)	1	**184106.8**	**9.560**	**0.015**	4812.6	0.229	0.645	**36.41**	**6.890**	**0.030**		nd	
Irradiance (*E*)	1	2085.6	0.108	0.751	**132991.1**	**6.328**	**0.036**	**78.77**	**14.904**	**0.005**			
N × *E*	1	256.3	0.013	0.911	4799.9	0.228	0.646	5.92	1.120	0.321			
Residual	8	19257.5			21016.5			5.29					
Violoxanthin													
Nutrients (*N*)	1	**3327.25**	**13.924**	**0.006**	6.55	0.032	0.863	0.102	1.326	0.283		nd	
Irradiance (*E*)	1	1.74	0.007	0.934	**2108.12**	**10.235**	**0.013**	0.200	2.590	0.146			
N × *E*	1	18.83	0.079	0.786	107.35	0.521	0.491	0.150	1.950	0.200			
Residual	8	238.96			205.98			0.077					
Anteraxanthin													
Nutrients (*N*)	1	0.08	0.004	0.953	**43.88**	**28.707**	**0.001**	89.32	4.422	0.069		nd	
Irradiance (*E*)	1	31.07	1.460	0.261	2.62	1.713	0.227	**204.13**	**10.106**	**0.013**			
N × *E*	1	7.77	0.365	0.562	**9.00**	**5.885**	**0.041**	3.59	0.178	0.684			
Residual	8	21.28			1.53			20.20					
Zeaxanthin													
Nutrients (*N*)	1	54.69	1.455	0.262	2.02	0.066	0.804	3.58	1.727	0.225		nd	
Irradiance (*E*)	1	0.06	0.002	0.969	0.55	0.018	0.896	4.67	2.257	0.171			
N × *E*	1	85.64	2.278	0.170	9.10	0.297	0.601	0.41	0.199	0.667			
Residual	8	37.59			30.63			2.07					
β-carotene													
Nutrients (*N*)	1	**623.87**	**6.230**	**0.037**	**328.83**	**7.710**	**0.024**	13.12	4.432	0.068		nd	
Irradiance (*E*)	1	97.13	0.970	0.354	58.13	1.363	0.277	**45.76**	**15.465**	**0.004**			
N × *E*	1	457.29	4.566	0.065	**1016.02**	**23.821**	**0.001**	2.32	0.785	0.401			
Residual	8	100.14			42.65			2.96					

Table 5. Pigment contents (mean values ± SE, n = 3) of *Cystoseira tamariscifolia* and *Ellisolandia elongata* collected at 2 different depths (0.5 m and 2.0 m) in relation to irradiance (70 $\%_{PAB}$ and 100 $\%_{PAB}$) and nutrient (Nutrients+ and Nutrients−) treatments. Chl *a*, chl *c*, phycoerythrin and phycocyanin contents are expressed in mg g^{-1} DW. Fuxocanthin, violaxanthin, antheraxanthin, zeaxanthin and β-carotene contents are expressed in μg g^{-1} DW. Initial values (I_S: 0.5 m depth; I_D: 2.0 m depth) are shown in the first column and in **bold** for each depth. Uppercase letters denote significant differences after SNK test in algae collected at 2.0 m depth, nd: no data

	I_S	0.5 m depth				I_D	2.0 m depth			
		Nutrients+		Nutrients−			Nutrients+		Nutrients−	
		100 $\%_{PAB}$	70 $\%_{PAB}$	100 $\%_{PAB}$	70 $\%_{PAB}$		100 $\%_{PAB}$	70 $\%_{PAB}$	100 $\%_{PAB}$	70 $\%_{PAB}$
Cystoseira tamariscifolia										
Chl *a*	**4.87 ± 0.27**	1.51 ± 0.07	2.24 ± 0.44	1.15 ± 0.11	2.11 ± 0.02	**2.11 ± 0.29**	1.73 ± 0.3	1.34 ± 0.23	0.77 ± 0.26	1.39 ± 0.1
Chl *c*	**0.17 ± 0.01**	0.18 ± 0.01	0.18 ± 0.03	0.11 ± 0.01	0.11 ± 0.02	**0.08 ± 0.01**	0.08 ± 0.02	0.16 ± 0.02	0.09 ± 0.02	0.13 ± 0.02
Fucoxanthin	**557.6 ± 1.5**	611.9 ± 28.3	629. ± 119.5	354.9 ± 38.2	390.5 ± 95.6	**265.8 ± 50.4**	292.5 ± 68.4	543.1 ± 88.5	292.5 ± 76.4	463 ± 98.3
Violaxanthin	**60.47 ± 2.56**	82.88 ± 3.23	86.15 ± 12.64	52.09 ± 6.13	50.34 ± 10.51	**29.76 ± 5.79**	38.38 ± 9.56	70.87 ± 7.9	42.88 ± 7.49	63.41 ± 8.02
Antheraxanthin	**13.89 ± 0.59**	14.27 ± 2.54	9.44 ± 2.65	12.51 ± 1.91	10.89 ± 3.34	**9.06 ± 1.51**	6.51 ± 0.37^{AB}	5.71 ± 1.04^{A}	8.61 ± 0.84^{B}	11.27 ± 0.28^{C}
Zeaxanthin	**13.58 ± 1.91**	8.03 ± 1.17	2.83 ± 0.70	6.95 ± 2.03	12.44 ± 6.52	**12.91 ± 1.81**	12.36 ± 3.47	10.19 ± 2.64	9.81 ± 2.00	11.11 ± 4.21
β-carotene	**71.15 ± 5.57**	20.50 ± 11.08	7.39 ± 2.46	22.57 ± 1.63	29.23 ± 1.38	**33.99 ± 2.29**	20.76 ± 0.93^{B}	6.76 ± 0.9^{A}	12.83 ± 2.98^{AB}	35.64 ± 6.8^{C}
Ellisolandia elongata										
Chl *a*	**0.43 ± 0.04**	0.35 ± 0.05	0.51 ± 0.01	0.29 ± 0.08	0.36 ± 0.00	**0.21 ± 0.01**	0.24 ± 0.03	0.39 ± 0.06	nd	0.38 ± 0.01
Phycoerythrin	**1.54 ± 0.05**	2.61 ± 0.28	2.16 ± 0.47	2.00 ± 0.13	1.7 ± 0.10	**0.86 ± 0.15**	0.74 ± 0.05	0.69 ± 0.12	nd	0.45 ± 0.08
Phycocyanin	**0.16 ± 0.01**	0.59 ± 0.06	0.42 ± 0.09	0.42 ± 0.03	0.29 ± 0.01	**0.27 ± 0.03**	0.18 ± 0.00	0.16 ± 0.02	nd	0.1 ± 0.02
Fucoxanthin	**7.76 ± 2.42**	4.12 ± 0.20	10.64 ± 2.42	2.04 ± 0.18	5.76 ± 1.03	**3.72 ± 0.38**	1.91 ± 0.38	3.33 ± 0.52	nd	4.25 ± 0.66
Violaxanthin	**0.49 ± 0.09**	0.67 ± 0.27	0.71 ± 0.06	0.26 ± 0.02	0.75 ± 0.15	**0.37 ± 0.01**	0.32 ± 0.02	0.43 ± 0.07	nd	0.35 ± 0.01
Antheraxanthin	**21.31 ± 1.73**	14.84 ± 4.31	22.00 ± 0.85	8.29 ± 0.91	17.63 ± 2.61	**7.34 ± 0.77**	10.00 ± 1.57	19.41 ± 1.54	nd	17.70 ± 1.06
Zeaxanthin	**3.15 ± 0.12**	3.67 ± 0.31	2.79 ± 0.06	5.13 ± 1.48	3.51 ± 0.66	**5.98 ± 1.92**	2.66 ± 0.51	6.09 ± 1.66	nd	8.01 ± 0.28
β-carotene	**11.95 ± 0.52**	6.77 ± 1.62	9.8 ± 0.45	3.81 ± 0.66	8.59 ± 0.8	**4.03 ± 0.83**	5.08 ± 1.02	10.63 ± 0.62	nd	5.05 ± 2.57

The C:N ratio was more favorable physiologically (< 23) in *C. tamariscifolia* from 0.5 m than in algae from 2.0 m (> 30). On the other hand, the elevated NPQ_{max} indicated high photoprotection capacity. The sun-type photosynthetic pattern of the species analyzed is shown by the high Ek_{ETR} values (200 to 220 μmol photons m^{-2} s^{-1}) in algae collected at both 0.5 and 2.0 m (initial conditions). These values were lower than those reported by Celis-Plá (2011) and Figueroa et al. (2014, this Theme Section) in *C. tamariscifolia* growing in a nearby coastal area of the Mediterranean Sea but subjected to emersion conditions, in contrast to the subtidal species of Cabo de Gata-Níjar, i.e. higher nutrient and irradiance levels than those found in this study.

According to the physiological status, algae grown at 0.5 m will be less vulnerable to higher irradiance conditions (100 $\%_{PAB}$) than algae grown at 2.0 m. At the initial natural conditions, the phenolic compounds (photoprotectors) in *C. tamariscifolia* are expected to be higher in algae grown at 0.5 m than at 2.0 m. However, in algae collected at 0.5 m depth, the phenolic compounds were lower than algae collected at 2.0 m, during the initial period. This can be explained as a consequence of the high irradiance found at 0.5 m, since phenolic compounds could be released under high solar irradiance, preventing the photodamage as a photoprotection strategy (Abdala-Díaz et al. 2006). Photoacclimation responses were also affected by nitrate supply in general; nitrate enrichment increased the photosynthetic rate and the accumulation of photoprotectors. This indicates that the algae are nutrient-limited in this oligotrophic system (Figueroa & Gómez 2001).

C. tamariscifolia collected from 0.5 m depth maintained ETR values 60 h after transferring to 100 $\%_{PAB}$ in both nutrient conditions, but phenolic compounds and internal N content increased only in nutrient-enriched conditions. The transplantation to 70 $\%_{PAB}$ provoked an increase in ETR_{max}, indicating that algae at 0.5 m depth were photoinhibited under initial conditions. The increase of ETR_{max} at 70 $\%_{PAB}$ is related to a decrease in NPQ_{max}, indicating less energy dissipation as a consequence of decreased

Table 6. ANOVA results after *in situ* experiment testing for the effect of irradiance and nutrients on the phenolic compounds and antioxidant activity (EC_{50}) of *Cystoseira tamariscifolia* collected at 2 different depths. We used a significance level of α = 0.05, shown in **bold**

	df	0.5 m depth			2.0 m depth		
		MS	*F*	p	MS	*F*	p
Phenolic compounds							
Nutrients (*N*)	1	**262.2**	**7.956**	**0.022**	**107.7**	**5.955**	**0.041**
Irradiance (*E*)	1	30.1	0.912	0.367	**331.7**	**18.346**	**0.003**
N × *E*	1	36.1	1.094	0.326	0.3	0.014	0.908
Residual	8	33.0			18.1		
EC_{50}							
Nutrients (*N*)	1	0.014	1.417	0.268	**0.094**	**10.86**	**0.011**
Irradiance (*E*)	1	0.032	3.273	0.108	0.001	0.078	0.787
N × *E*	1	**0.068**	**6.918**	**0.030**	0.034	3.919	0.083
Residual	8	0.010			0.009		

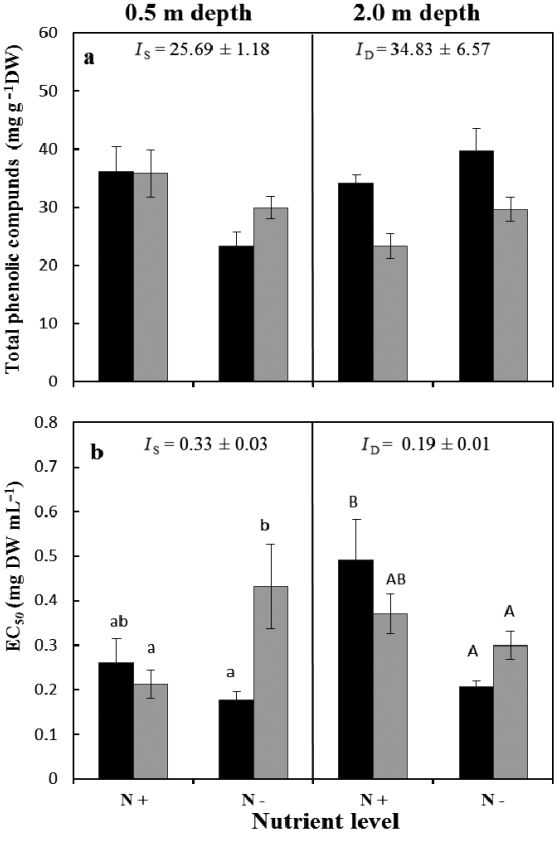

Fig. 4. (a) Total phenolic compounds and (b) antioxidant activity (EC_{50}) (mean ± SE, n = 3) of *Cystoseira tamariscifolia* from 0.5 and 2.0 m depths under irradiance and nutrient treatments. Black bars indicate 100 $\%_{PAB}$, and grey bars indicate 70 $\%_{PAB}$. N+ and N− indicate nutrient-enriched and non-enriched treatments, respectively. Upper values in each box indicate initial values (I_S: 0.5 m depth; I_D: 2.0 m depth). Lowercase letters denote significant differences after SNK test for algae collected at 0.5 m depth and capital letters for algae collected at 2.0 m

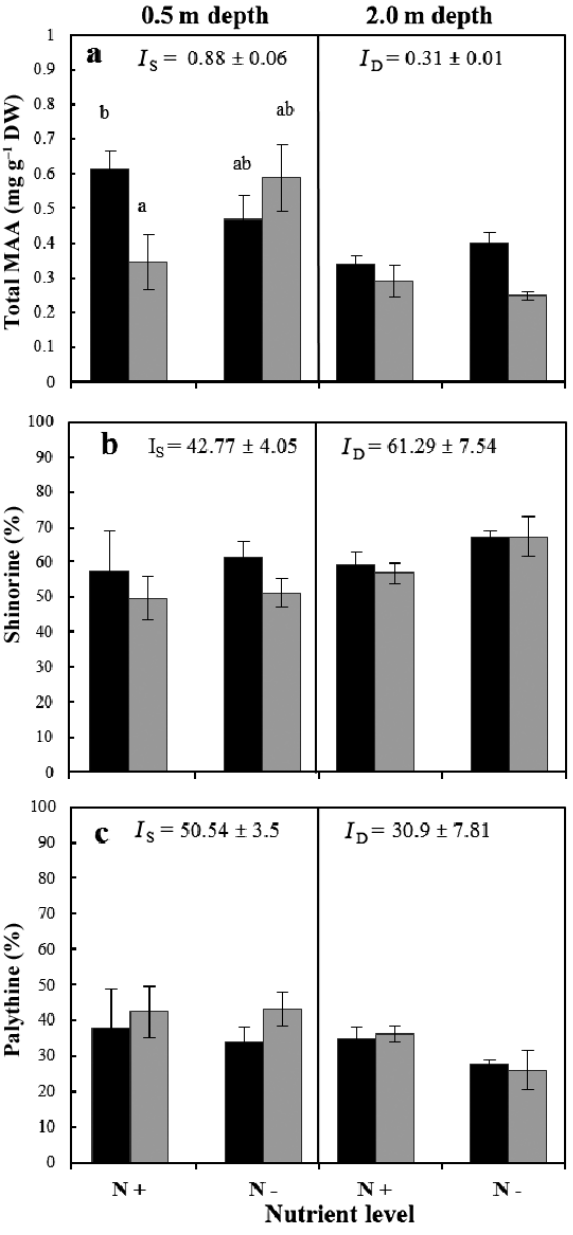

Fig. 5. (a) Total mycosporine-like amino acid (MAA) content and percentages of (b) shinorine and (c) palythine (mean values ± SE, n = 3) in *Ellisolandia elongata* from 0.5 and 2.0 m depth under irradiance and nutrient treatments. Black bars indicate 100 $\%_{PAB}$, and grey bars indicate 70 $\%_{PAB}$. N+ and N− indicate nutrient-enriched and non-enriched treatments, respectively. Upper values in each box indicate initial values (I_S: 0.5 m depth; I_D: 2.0 m depth). Lowercase letters denote significant differences after SNK test

irradiance, at least in the short-term period analyzed. In any case, prolonged time can eventually reduce the values of ETR_{max} due to less available energy at 2.0 m than that at 0.5 m in spite of photoinhibition. The ETR_{max} of algae collected from 2.0 m depth when transplanted to 100 $\%_{PAB}$ increased only in non-

Table 7. ANOVA results after *in situ* experiment testing for the effect of irradiance and nutrients on total mycosporine-like amino acid (MAA) content, and percentages of shinorine and palythine of *Ellisolandia elongata* collected at 2 different depths. We used a significance level of $\alpha = 0.05$, shown in **bold**

	df	0.5 m depth			2.0 m depth		
		MS	*F*	p	MS	*F*	p
Total MAA content							
Nutrients (*N*)	1	0.008	0.427	0.532	0.000	0.096	0.764
Irradiance (*E*)	1	0.016	0.917	0.366	**0.030**	**10.453**	**0.012**
N × *E*	1	**0.114**	**6.471**	**0.035**	0.008	2.857	0.129
Residual	8	0.018			0.003		
% Shinorine							
Nutrients (*N*)	1	24.93	0.151	0.708	**252.61**	**5.92**	**0.041**
Irradiance (*E*)	1	239.16	1.448	0.263	4.81	0.113	0.746
N × *E*	1	6.09	0.037	0.852	4.88	0.114	0.744
Residual	8	165.13			42.69		
% Palythine							
Nutrients (*N*)	1	8.21	0.051	0.827	**230.52**	**6.35**	**0.036**
Irradiance (*E*)	1	143.23	0.885	0.374	0.14	0.004	0.952
N × *E*	1	18.23	0.113	0.746	7.84	0.216	0.654
Residual	8	161.87			36.28		

enriched treatments, but both internal N and phenolic compound contents increased under nutrient enrichment.

PAR and UVR can cause photoinhibition, which can be defined as the light-dependent decline in photosynthetic capacity and maximal photosynthetic efficiency as a consequence of the dominance of photodamage versus photorepair processes (Osmond 1994, Gómez et al. 2004). It is also thought that photoinhibition is a down-regulation mechanism to quench excessive solar energy (Demmig-Adams et al. 2008). However, in *C. tamariscifolia*, no photoinhibition was observed. Intertidal macroalgae from southern Spain have low photoinhibition at noon and high recovery capacity during daily cycles due to high energy dissipation (Figueroa et al. 1997, Häder et al. 1997, 1998).

Photosynthetic efficiency α_{ETR}, ETR_{max} and MAAs in *E. elongata* collected from 0.5 m depth decreased after transfer to 100 $\%_{PAB}$ under both nutrient conditions, but internal N contents increased only under nutrient-enriched conditions. The transplant to 70 $\%_{PAB}$ provoked an increase of α_{ETR} and ETR_{max} only under nutrient-enriched conditions; however, internal N content and MAAs decreased in both nutrient treatments, indicating that algae grown at 0.5 m depth can be photoinhibited under initial conditions. The level of ETR_{max}, α_{ETR} and MAAs in algae collected from 2.0 m depth increased when they were transplanted to 100 $\%_{PAB}$ under both nutrient treatments; however, the internal N content decreased in both nutrient treatments. The transplantation of algae collected from 2.0 m depth to 70 $\%_{PAB}$ caused a higher α_{ETR} and ETR_{max} in both nutrient conditions; however, internal N content and MAAs increased in nutrient-enriched conditions.

In general, in both species collected from 0.5 m depth, the addition of nutrients increased their photosynthetic efficiency. The photosynthetic response was also affected by irradiance levels. Although the initial values of NPQ_{max} in *C. tamariscifolia* were similar, NPQ_{max} decayed at both depths under nutrient enrichment and Ek_{NPQ} only increased in the enriched treatment. Furthermore, in *C. tamariscifolia* collected at 2.0 m depth, an interaction between light and nutrients was observed, where transplanted algae (to 100 $\%_{PAB}$) under non-enriched treatment showed an increase in NPQ_{max} and Ek_{NPQ} in all treatments. At high nutrient availability, it seems that algae collected from 0.5 m depth had higher levels of photoprotective compounds (phenols) or increased size of antenna (higher content of chl *c* and fucoxanthin were observed). This could be due to high antioxidant activity and less requirement for the dissipation of energy in the form of heat (low NPQ_{max}) or due to less UV radiation that could be reaching the photosynthetic apparatus. However, in *C. tamariscifolia* collected at 2 m depth after the transplant conditions (70 $\%_{PAB}$), high levels of accessory pigments were found. These differences were independent of the nutrient treatment. The phenolic compounds and antioxidant activity were affected by irradiance and nutrients as single factors in the first case, and by the interaction of both factors in the second case. For the other carotenoids, similar results were found in *C. tamariscifolia* collected from 0.5 m depth.

Carotenoid contents were less influenced by irradiance or nutrients with the exception of violaxanthin that had higher content after nutrient enrichment. On the other hand, in *C. tamariscifolia* collected from 2.0 m depth, violaxanthin content was higher in the simulated deeper irradiance (70 $\%_{PAB}$), as was found in other accessory pigments. However, antheraxanthin and β-carotene were significantly affected by the interaction of irradiance and nutrients. In *E. elongata* collected from 0.5 m depth, an effect of irradiance was found. The responses found in this study for both species are similar to those described by Demmig-Adams & Adams (1996). The response of the xanthophyll cycle and light absorption could re-

flect a regulatory and photoprotective response that down-regulates the delivery of excitation energy into the electron-transport chain to match the rates at which products of electron transport can be consumed in these leaves. Goss & Jakob (2010) indicate that the xanthophyll cycle represents an important photoprotection mechanism in plant cells. This suggests a relationship between higher photosynthetic rates and a higher activity of the xanthophyll cycle. However, the presence of a functional xanthophyll cycle in red algae is uncertain (Andersson et al. 2006, Schubert et al. 2006). In fact, the predominant presence of red algae in intertidal zones and coral reefs suggests a highly efficient capacity to withstand elevated irradiance levels and large diurnal light fluctuations due to tides and aerial exposure (Schubert et al. 2011).

E. elongata possesses high reflectance under high solar radiation, allowing it to live in areas of high radiation and sun exposure due to a skeleton composition of calcium carbonate (Häder et al. 1997). These authors described a high reflectance under high solar radiation exposure in *E. elongata*, which can be advantageous under elevated solar irradiance reducing photoinhibition in this species.

Connan et al. (2004) found higher levels of phenols in summer in several brown macroalgae off Brittany related to higher solar irradiance. Similarly, Abdala-Díaz et al. (2006) found higher phenol content in summer than in winter in *C. tamariscifolia* collected in southern Spain in the morning. However, at noon the levels were similar in both seasons due to the high release of polyphenols in summer. In our study, the phenolic content in *C. tamariscifolia* increased with nutrient enrichment in algae collected at 0.5 m depth in the non-enriched treatment and in transplanted specimens (to 100 $\%_{PAB}$) under non-enrichment treatments in those collected from 2.0 m depth. In brown algae, UV screen compounds (polyphenols) accumulate under high PAR and UVR and these compounds have strong antioxidant activity (Pavia et al. 1997, Connan et al. 2004, Cruces et al. 2012). This may suggest that this is probably more related to the nitrate availability than to solar irradiance conditions. Pavia & Toth (2000) indicate that the N content can enhance the accumulation of phenolic compounds in some brown algae. In fact, concentrations of phenolic compounds show phenotypic plasticity in response to changes in environmental parameters, such as salinity, nutrients, light quality and availability, and intensity of herbivores (Peckol et al. 1996, Pavia et al. 1997, Pavia & Toth 2000, Honkanen et al. 2002, Swanson & Druehl 2002, Amsler & Fairhead 2006). Moreover, *C. tamariscifolia* had higher antioxidant activity at 0.5 m depth in transplanted conditions (70 $\%_{PAB}$) without nutrient enrichment, and also in algae collected from 2.0 m depth in transplant conditions (100 $\%_{PAB}$) with nutrient enrichment.

As has been mentioned, the response of *E. elongata* collected from 0.5 m depth was dependent mostly on irradiance. However, the content of MAAs (UV-screening substance) of algae collected at 0.5 m depth depended on the interaction between irradiance and nutrients, as reported by Korbee-Peinado et al. (2004). Karsten et al. (1998) and Franklin et al. (2001) have shown that accumulation of MAAs depend on both quality and quantity of radiation, with higher accumulation of MAAs with high daily PAR doses and UV exposure. Korbee-Peinado et al. (2004) found that high ammonium concentrations significantly increased the content of MAAs in *Pyropia columbina* (as *Porphyra columbina*). In their study, an interaction between irradiance and nutrients was found. Similar results were found for other *Porphyra* species, *Grateloupia lanceola* and *Gracilaria* spp. (Korbee et al. 2005a, Huovinen et al. 2006, Barufi et al. 2011, Figueroa et al. 2012). In our study, the MAA total content decreased in algae transplanted from 100 $\%_{PAB}$ to 70 $\%_{PAB}$ and after nutrient enrichment, whereas no effect of nutrient was observed in algae collected from 2.0 m depth waters. It seems that the short-term effect of the nutrient addition is not enough to produce an increase of total MAA content under nitrogen-enriched conditions as has been reported in other algae (Barufi et al. 2011, Figueroa et al. 2012). However, the effect of nutrients was reflected by a preferential accumulation of some types of MAAs, but only in *E. elongata* collected from 2.0 m depth. The relative content of palythine increased in nutrient-enriched algae, which has been associated with higher antioxidant activities compared to shinorine (de la Coba et al. 2009).

3.6 In conclusion, *C. tamariscifolia* and *E. elongata* showed different physiological responses under dif- ferent nutrient and irradiance conditions. Few inter- active effects between these 2 physical stressors were found, suggesting major additive effects on the responses of both species. In fact, environmental variables acting in additive forms can act as more powerful stress factors (Martínez et al. 2012) leading to changes in the physiology of these macroalgae. Therefore, understanding the physiological conse- quences of the potential additive effects of these physical stressors on these dominant species is needed to predict future environmental fluctuations related to climate change.

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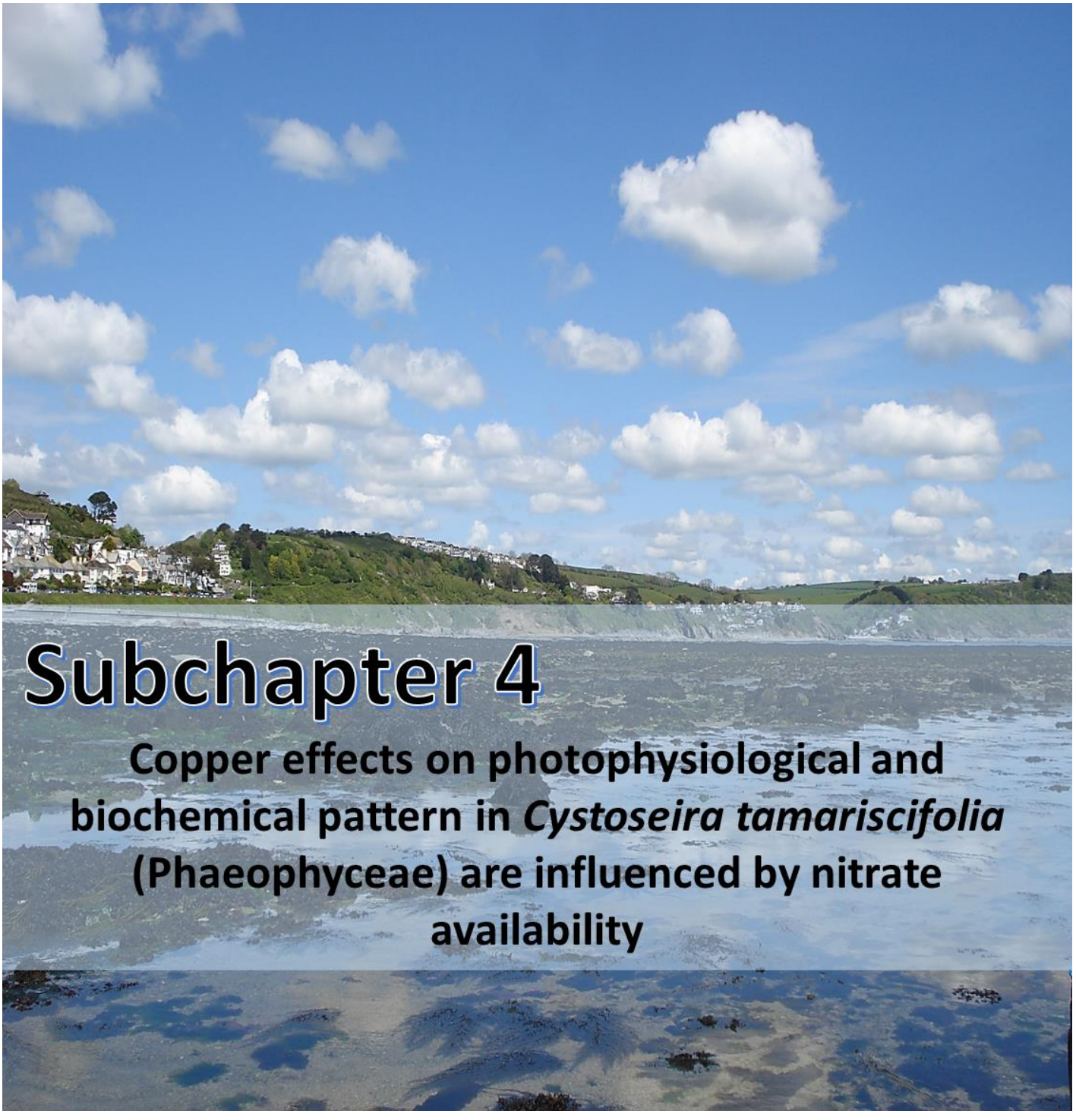

Subchapter 4

Copper effects on photophysiological and biochemical pattern in *Cystoseira tamariscifolia* (Phaeophyceae) are influenced by nitrate availability

Hannafore Point, Plymouth, UK. May 2014. Photograph by Paula S. M. Celis Plá

Copper effects on photophysiological and biochemical pattern in *Cystoseira tamariscifolia* (Phaeophyceae) are influenced by nitrate availability

4.1 ABSTRACT

Nitrate (5 and 50 μM) and Copper levels (Cu_T: nominal total Cu concentrations, control conditions, 0.5 and 2.0 μM) influenced the effects on photosynthetic rate determined as *in vivo* chlorophyll *a* fluorescence in *Cystoseira tamariscifolia* (Phaeophyceae), i.e., maximal quantum yield (F_v/F_m), maximal electron transport rate (ETR_{max}) and maximal non-photochemical quenching (NPQ), biochemical composition and antioxidant activity. After 14 d culture, the internal and external copper contents were affected by nitrate supply; under culture in high equivalent free Cu concentration [Cu^{2+}] or Cu_T (2.0 μM), the internal copper accumulation was significantly higher (two times) under low nitrate concentration. This result corresponds with about two times higher external copper concentration in the medium in high compared low nitrate treatments. Copper levels affected the internal content of total nitrogen content (Ni), being this affected by the nitrate availability. In 0.5 μM Cu_T grown algae, the Ni was higher in high nitrate supply, in contrast, the photosynthetic activity (F_v/F_m and ETR_{max}), Chl*a*, NPQ_{max} and total phenolic content decreased. However, under 2.0 μM Cu_T mainly in the short term (7 d culture), high nitrate treatment decreased Ni, F_v/F_m, total phenolic compounds, the level of phenols in the water and external copper content. In contrast, the internal copper content was lower under low nitrate supply. After 14 d in 2.0 μM Cu_T other variables did not show significant differences among the different nitrate concentrations, i.e., ETR_{max}, NPQ_{max}, Chl*a*, Chl*c*, fucoxanthin and total phenolic content in the water. Shikimic acid and phloroglucinol were the main detected polyphenols and they did not show significant differences in the interaction between copper and nitrate. However, shikimic acid was the highest in high copper concentration cultures and phloroglucinol was the highest under both high and low copper concentration with nitrate enrichment. In general, for *C. tamariscifolia* under 0.5 μM Cu_T, the physiological status seems to be more favourable in low nitrate level, than under 2.0 μM Cu_T with high nitrate level. The data show complex interaction effects between copper and nitrate on the ecophysiological variables, with high resistance to copper of *C. tamariscifolia* with no apparent damage effects after 14 d culture were found even at 2.0 μM Cu_T.

Key words: Antioxidant activity, Copper, *Cystoseira tamariscifolia*, *in vivo* chlorophyll *a* fluorescence, internal carbon and nitrogen, polyphenols

4.2 INTRODUCTION

Marine biota living in coastal waters and estuaries are under threat from exposure to elevated concentrations of metals and enrichment by nitrogen and phosphorus, that are derived from industrial and agriculture activities. (Brown and Newman 2003). While there is compelling evidence that macronutrients can influence the accumulation of metals in phytoplankton (Miao et al. 2005), the interaction between metals and nutrient enrichment is less well understood for macroalgae (Lee and Wang 2001), despite their ecological importance as the main primary producers and bioengineers in near-shore ecosystems. Some metals, such as copper (Cu), are essential micronutrients for chloroxygenic organisms, required for several important metabolic pathways and physiological processes (Yruela 2000). However, at elevated concentrations they are potentially toxic and can negatively affect development, growth and fertility of algae (Fernandes and Heriques 1991, Brown and Depledge 1998). In coastal and estuarine waters, ambient total dissolved Cu can reach concentrations of 10-100 nM, attributable to natural and anthropogenic origins. Physiological state effects of copper have been conducted in macroalgae, particularly in brown algae, as *Ascophyllum nodosum* and *Fucus vesiculosus* (Connan and Stengel 2011a and b) and *Fucus ceranoides* (Varna et al. 2013), as well as in *Ectocarpus siliculosus*, as well the relation between accumulation and the stimulation of antioxidant capacity (Sáez et al. 2015). The most toxicological studies used a single end-point measurement, i.e., growth or photosynthesis (Plotz 1991) and levels of exposure in laboratory far from the natural concentrations as mg L^{-1} instead of μg L^{-1} (Zolotukhina et al. 1991).

More recently, Brown and Newman (2003) studied relative effects of copper exposure (12 to 500 μg L^{-1}) in the red macroalgae *Gracilariopsis longissima* on both growth and physiology (*in vivo* chlorophyll *a* florescence, ion leakage, oxygen evolution and pigmentation). Biliprotein presented a significant reduction at the higher copper level (500 μg L^{-1}), whereas photosynthetic rate was first impaired at 250 μg L^{-1}. The observed uncoupling between growth and photosynthesis and low Cu concentrations as explained by the release of dissolved organic matter (DOC), resulting in less available energy for

growth (Brown and Newman, 2003). The photosynthetic activity in *Cystoseira tamariscifolia* was clearly dependent on the availability of nutrient (Celis-Plá et al. 2014a). High nitrate supply can reduce photoinhibition in seaweeds (Henley et al. 1991, Figueroa et al. 2009). In addition, other observations attributed effects of nitrogen in the decline in fluorescence measurements of maximal quantum yield (F_v/F_m) (Berges and Falkowski 1998, Young and Beardall 2003). Macronutrients as nitrate or phosphate had markedly influence in the rate of copper accumulation and toxicity in *Ulva fasciata* (Lee and Wang, 2001).

In this study, the interactive effects of copper and nitrate on the photosynthetic performance and biochemistry of *Cystoseira tamariscifolia* were investigated. The brown seaweed *C. tamariscifolia* occurs in both Atlantic and Mediterranean waters (Bunker et al. 2010, Gómez-Garreta et al. 2001). *Cystoseira* spp. are considered to grow mainly in high-quality waters according to the criteria of Water Framework Directive of the European Union (WFD, 2000/60/EC) and they are bioindicators of waters with high quality ecological status (Ballesteros et al. 2007, Arévalo et al. 2007). Recently published data on the effects of different environmental variables on photosynthesis and biochemistry of *C. tamariscifolia* show that a positive effect of nitrate enrichment has a positive effect on photosynthesis and photoprotection (Celis-Plá et al. 2014a, Figueroa et al. 2014a).

Hence, the hypothesis to be tested is that the negative effects of Cu on the physiology and biochemistry will be countered under nitrate enrichment due to expected increases in the photosynthetic activity and antioxidant capacity. Photosynthetic activity will be assessed from measurement of: maximal quantum yield (F_v/F_m), photosynthetic efficiency (αETR), irradiance at which saturation of electron transport rate (ETR) occurs (Ek). Maximal electron transport rate (ETR_{max}) as an estimate of algal productivity, and maximal non-photochemical quenching (NPQ_{max}) as an indicator of energy dissipation as well as photoprotection mechanisms (Celis-Plá et al. 2014b). The content of the following biochemical components was determined: total carbon (C) and nitrogen (N), photosynthetic pigments (chlorophylls and fucoxanthin), and phenolic compounds (substances with high antioxidant and photoprotection capacity) (Connan et al. 2004, Abdala-Díaz et al. 2006).

4.3 MATERIAL AND METHODS

Species and sampling

Thalli of *Cystoseira tamariscifolia* (Hudson) Papenfuss, (Phaeophyceae: Fucales) (Bunker et al., 2010) were collected, haphazardly, at low tide on May 14th 2014 from Hannafore Point, Cornwall, UK (50°36'N, 4°42'W) and transported in a cool box to the laboratory. Material was first cleaned of epiphytes under running seawater and then acclimated for two days in continuously aerated seawater in 10 L polyethylene tanks at 16 ± 3ºC. The irradiance of 100 µmol m^{-2} s^{-1} of photosynthetically active radiation PAR (λ=400-70 nm) by using cool white fluorescent lamps (OSMAR FH 21W/840HE, Luminox, Italy) on a 14:10 h light/dark cycle.

Experimental design

After the acclimation period, individual thalli (30 g wet biomass) were exposed in triplicate to three copper (Cu) levels, added as $CuSO_4 \cdot 5H_2O$: 0 (control, no added copper), 0.5 (equivalent to 127 µg L^{-1}), 2.0 µM (equivalent to 508 µg L^{-1}) Cu, and two nutrient levels: 5 µM (300 µg L^{-1} KNO_3) nitrate plus 0.5 µM (µg L^{-1} KH_2PO_4) phosphate and 50 µM (3150 µg L^{-1} KNO_3) nitrate plus 5 µM (µg L^{-1} KH_2PO_4). In both nutrient treatments, the relation N:P was the same i.e. N:P=10:1. The seawater medium was replenished on days 3, 7 and 10. Measurements of photosynthetic parameters and most biochemical analyses were carried out prior to the start of the experiment and after 7 and 14 d of exposure. Tissue Cu concentrations and the composition of phenolic compounds were determined only at the termination of the experiment. All samples were stored at -80°C until biochemical analyses were performed.

Photosynthetic activity

In vivo chlorophyll *a* fluorescence associated to Photosystem II was determined using a portable pulse amplitude modulated fluorometer Diving-PAM (Walz GmbH, Germany). Thalli were initially dark-adapted for 15 min before taking readings of F_o (basal fluorescence yield) and F_m (maximum fluorescence yield after a saturation light pulse of > 4000 µmol m^{-2} s^{-1}). The maximum quantum efficiency of PSII in the dark-adapted state is expressed as the ratio of variable to maximal chlorophyll fluorescence (F_v / F_m), derived

from ($F_m - F_o$) / F_o (Maxwell and Johnson, 2000). Other parameters were derived from Rapid Lights Curves (RLC) performed on apical tips in 10 mL incubation chambers. Electron transport rate (ETR) were determined after 20s exposures to twelve incremental increases in irradiance of actic halogen light (9.3, 33.8, 76, 145, 217, 301, 452, 629, 947, 1403, 2084 and 3444 µmol m^{-2} s^{-1}) provided by the halogen lamp of the Diving-PAM (according to Celis-Plá et al 2014b). The ETR was calculated according to Schreiber et al. (1995) as follows:

$$ETR\ (\mu mol\ electrons\ m^{-2}\ s^{-1}) = \Delta F/F'_m \times E \times A \times F_{II} \quad (1)$$

where $\Delta F/F'm$ is the effective quantum yield $\Delta F = Fm'-Ft$ (Ft = intrinsic fluorescence of the alga incubated in light; Fm' is the maximal fluorescence after a saturation pulse of algae incubated in light), E is the incident PAR irradiance expressed in µmol photons m^{-2} s^{-1}, A is the thallus absorptance as a fraction of incident irradiance that is absorbed by the alga (see Figueroa et al. 2003) and F_{II} is the fraction of chlorophyll related to PSII (400-700 nm) which is 0.8 in brown macroalgae (Grzymski et al. 1997, Figueroa et al. 2014b). The maximum electron transport rate (ETR_{max}) and the initial slope (α_{ETR}) of the ETR versus irradiance curve, which provides an estimation of photosynthetic efficiency, were obtained from the tangential function reported by Eilers and Peeters (1988). The saturation irradiance for ETR (Ek_{ETR}) was calculated from the intercept between ETR_{max} and α_{ETR}.

Non-photochemical quenching (NPQ) was calculated according to Schreiber et al. (1995) as:

$$NPQ = (Fm-Fm')/Fm' \quad (2)$$

Maximal NPQ (NPQ_{max}) and the initial slope of NPQ *versus* irradiance function (α_{NPQ}) were obtained from the tangential function of NPQ *versus* irradiance function according to Eilers and Peeters (1988).

Internal and external copper concentrations

At the end of the experiment and following removal of excess water, 40 mg fresh biomass (FW) samples of algae were either immediately frozen at −80◦C or washed twice for 15 min in Mili-Q water containing 10 mM EDTA to remove cell wall-bound Cu, thus

allowing distinction between total and intra-cellular (non-exchangeable) fractions (Hassler et al. 2004), and then frozen at −80◦C. Frozen biomass was freeze-dried for 24 h and digested in a microwave (MARSX-press; cycle of 34 min at 120–170◦C) using 2 mL of 70% (w/v) HNO_3. Digested samples were diluted to 5 mL with milli-Q water and Cu concentrations were determined by ICP-MS (Thermo Scientific, Hemel Hempstead, UK). External and internal calibrations of the instrument were achieved using Cu-certified standard solutions, and Itrium (193Ir) and Indium (115In), respectively. Certified reference material (*Fucus* spp. IAEA-140) was treated in the same way as experimental material and the Cu concentrations were within 12% of certified values.

Internal carbon and nitrogen content

Seaweed samples (1-2 g FW) were dried overnight in an oven at 60ºC and then maintained in a desiccator until analyses. Total internal C and N contents on a dry weight (DW) basis were determined using a CNHS-932 elemental analyzer (Leco Corporation, Michigan, USA).

Pigment content

Chlorophylls *a* (Chl*a*) and *c* (Chl*c*), and fucoxanthin (Fx) were extracted according to Seely et al. (1972). Approximately 0.02 g FW of material was placed in 1.5 mL glass test-tubes to which 800 µL of dimethyl sulfoxide (DMSO) was added. After 5 min, samples were diluted with distilled water in a ratio of 4:1 (DMSO: water) and absorption (A) determined spectrophotometrically (Jenway 7315) at specific wavelengths. Pigment concentrations are expressed as mg g^{-1} dry biomass (DW), after determining the FW to DW ratio of the tissue (8.1 for *C. tamariscifolia*), and calculated according to the following equations:

$$Chla = A_{665}/72.5 \quad (3)$$

$$Chlc = (A_{631} + A_{582} - 0.297A_{665})/61.8 \quad (4)$$

$$Fx = (A_{480} - 0.722(A_{631} + A_{582} - 0.297A_{665}) - 0.049A_{665})/130 \quad (5)$$

Phenolic compounds, antioxidant activity and phenolic compounds composition

Total phenolic content (PC), expressed as mg g^{-1} DW, and was determined using 0.25 g FW of material. Samples were pulverized in a mortar and pestle with sea-sand using 2.5

mL of 80% methanol. After storing the samples overnight at 4ºC, the mixture was centrifuged at 2253 g for 30 min at 4 ºC, and the supernatant then collected. Total PC was determined colorimetrically using Folin-Ciocalteu reagent (Folin and Ciocalteu 1927) and phloroglucinol (1,3,5-trihydroxybenzene, Sigma P-3502) as standard. The absorbance was determined at 760 nm using a spectrophotometer (Celis-Plá et al. 2014a). Concentrations of phenols released into the seawater medium (PCw) were determined after 7 and 14 d exposure from measurements of optical density at the maximum absorption (270 nm) for polyphenols in seawater and using phloroglucinol as the standard (Celis-Plá et al. 2014b). Results are expressed as a rate (mg g^{-1} DW d^{-1}).

Antioxidant activity of polyphenol extracts was measured according to Blois (1958), One hundred and fifty µL of DPPH (2,2-diphenyl-1-picrylhydrazyil), prepared in 90% methanol, was added to each extract and the reaction completed after 30 min incubation in darkness at room temperature (~20°C) and absorbance then read at 517 nm in a spectrophotometer (Jenway 7315). The calibration curve of DPPH concentrations [0-100 µM] was used to calculate the concentration of DPPH remaining in the reaction mixture. Values of DPPH concentration (mM) were plotted against seaweed extract concentration expressed as the EC_{50} value (oxidation index, mg DW mL^{-1}) required for scavenging 50% of the DPPH in the reaction mixture. Ascorbic acid was used as a control (Celis-Plá et al. 2014a).

The composition of phenolic compounds (PCC) was determined from the same extract used for measuring total phenolic content at the termination of the experiment. After extraction time, samples were centrifuged at 2253 g for 30 min at 4ºC and then the extracts were filtered (0.2 µm PVDF membrane filters). The PCC were determined using ultra high-performance liquid chromatography (DIONEX UltiMate 3000 UHPLC, Thermo Scientific Inc.) equipped with a UV detector set at 254-340 nm (DIONEX MWD-3000, Thermo Scientific Inc.). The chromatographic separation was obtained using a C-18 reverse phase column (Supelco, Sigma-aldrich 15 cm x 2.1 mm, 3 µm) protected by a C18 guard cartridge (Security Guard, Phenomenex Inc., USA). The mobile phase consisted of two components: Solvent A, acetonitrile and solvent B phosphoric acid 1% in Milli-Q water. The phenolic compounds were eluted using a gradient from 10% A for 2 minutes, 12% A for 3 minutes, 15% A for 1 minute, 30% A for 4 minutes, 35 % for 2 minutes, 50% A for 3 minutes, 35% A for 2 minutes, 3 minutes with 10% A and 80% B and finally an isocratic elution with 100% B for the next 3 minutes.

Statistical analysis

The effects of treatments on the physiological responses and biochemistry of *C. tamariscifolia* were assessed by analysis of variance (ANOVA). Three fixed factors were considered: Time with two levels (7 and 14 days), nitrate with two levels (5 and 50 μM of Nitrate and Phosphate at a ratio of 10:1) and copper with three levels (control, 0.5 μM: low copper level and 2.0 μM: high copper level). This design allows testing for interactive and additive effects of the variables, gaining important conclusions about the potential synergistic effects of stressors associated with environmental change. The Student Newman Keuls (SNK) post hoc test was performed if interactions were significant (Underwood 1997). Homogeneity of variance was tested using the Cochran test and by visual inspection of the residuals.

All data conformed to homogeneity of variance. Analysis were performed using SPSS v.21 (IBM, USA). In addition, Pearson correlation coefficients were calculated and tested between all measured dependent variables. The general variation patterns between variables measured in *C. tamariscifolia* were explored using a multivariate approach. A Principal Coordinates Analysis (PCO) was performed for this purpose on the basis of Euclidean distance using PERMANOVA+ for PRIMER6 package (Anderson et al. 2008). Such multivariate ordination was used because it allowed for investigating the variation of the content of all compounds at the same time by looking at the ordination plot. Each one of variables was represented by an arrow in the ordination plot pointing to the samples that showed the highest amount of that particular compound. Each replicate represented the content of all compounds calculated from the three thalli taken at one sampling date.

4.4 RESULTS

Photosynthetic activity

The maximum quantum yield of PSII (F_v/F_m) was significantly ($p<0.05$) affected by the interactions between time*copper (Cu) and Cu*nitrate (Figure 1, Table S1).

In the low N (N-) treatment, no significant differences amongst the different Cu treatments were observed whereas under N+, F_v/F_m decreased under 0.5 μM Cu^{2+} compared to the control or 2 μM Cu^{2+} (Figure 1, Table S1).

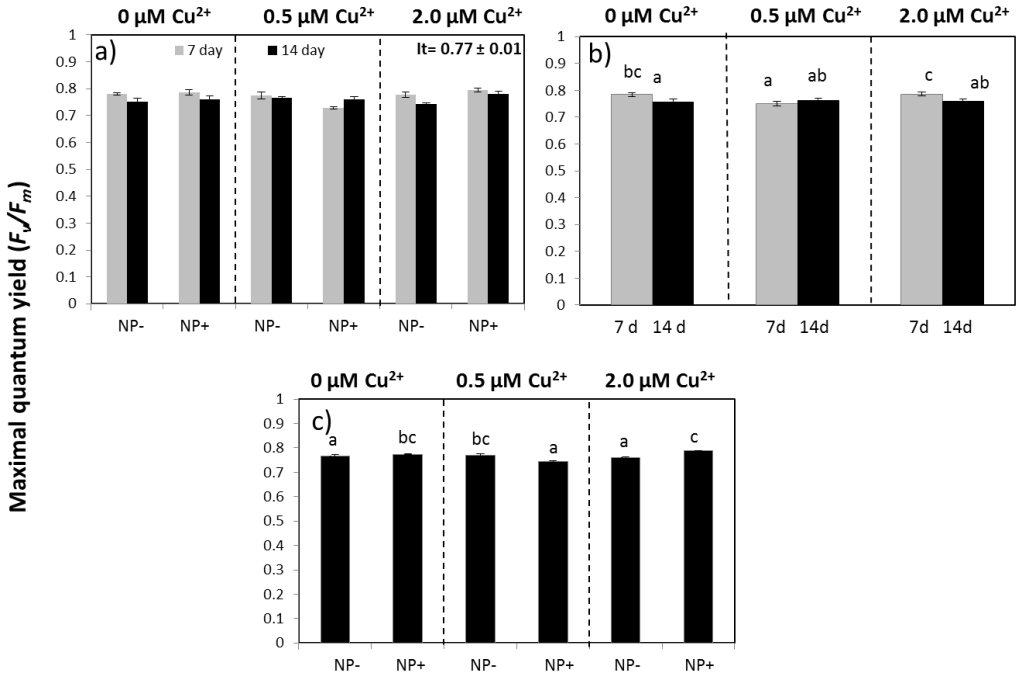

Figure 1. a) Maximal quantum yield (F_v/F_m) (mean values ± SE, n=3) of *Cystoseira tamariscifolia* in relation to Time: 7 d (black bars) and 14 d (grey bars), NP-(low nitrate and phosphate level) and NP+ (high nitrate and phosphate level) and Copper; 0 µM Cu^{2+} (Control), 0.5 µM Cu^{2+} (low copper concentration) and 2.0 µM Cu^{2+} (high copper concentration) treatments. Upper values in right box indicate initial time values (*It*: mean ± SE, n=3), b) pooled data for the interaction Time*Copper (mean values ± SE, n=6), and c) pooled data for the interaction Copper*Nitrate (mean values ± SE, n=6). Lower-case letters denote significant differences after a SNK test.

The pooled data between time and Cu factors showed that F_v/F_m was highest at 2.0 µM Cu^{2+} at 7d (Figure 1b, Table S1) and between copper and nitrate was highest at 2.0 µM Cu^{2+} at 14d (Figure 1c, Table S1).

The maximal production estimated as ETR_{max} was significantly influenced by interactions between time*copper and copper*nitrate (Figure 2a, b, c, Table S1). The pooled data between time and copper showed that the ETR_{max}, was higher in N- treatment at 0.5 µM Cu^{2+} and 7d compared to the control and decreased again at 2.0 µM Cu^{2+}. In contrast in N+ treatment no decrease was observed at 2 µM Cu^{2+} compared to 0.5 µM Cu^{2+} (Figure 2b, Table S1). The pooled data between copper and nitrate factors was higher at 0.5 µM Cu2+ at N- (Figure 2c, Table S1).

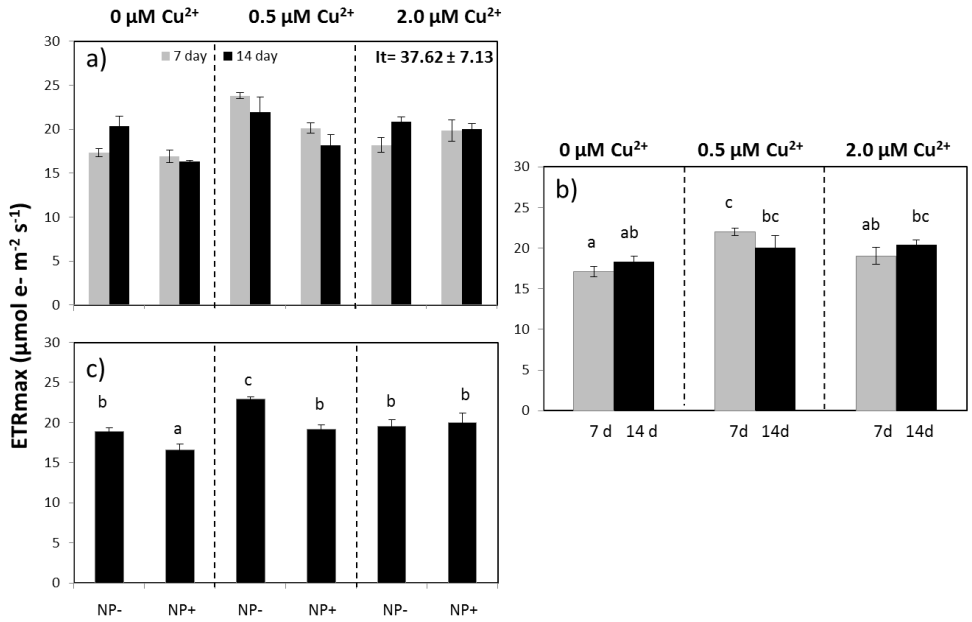

Figure 2. a) Maximal electron transport rate (ETR_{max}) (mean values ± SE, n=3) of *Cystoseira tamariscifolia* in relation to Time: 7 d (black bars) and 14 d (grey bars), NP-(low nitrate and phosphate level) and NP+ (high nitrate and phosphate level) and Copper; 0 µM Cu^{2+} (Control), 0.5 µM Cu^{2+} (low copper concentration) and 2.0 µM Cu^{2+} (high copper concentration) treatments. Upper values in right box indicate initial time values (*It*: mean ± SE, n=3), b) pooled data for the interaction Time*Copper (mean values ± SE, n=6), and c) pooled data for the interaction Copper*Nitrate (mean values ± SE, n=6). Lower-case letters denote significant differences after a SNK test.

The interaction between NPQ_{max} and all factors was significantly ($p<0.05$) (Figure 3, Table S1). In the control and 0.5 µM Cu^{2+} after 7 and 14 d culture, NPQ_{max} was higher under N- than in N+ treatment (Figure 3, Table S1). However, in high copper levels no significant differences with increase nitrate level were found (Figure 3, Table S1).

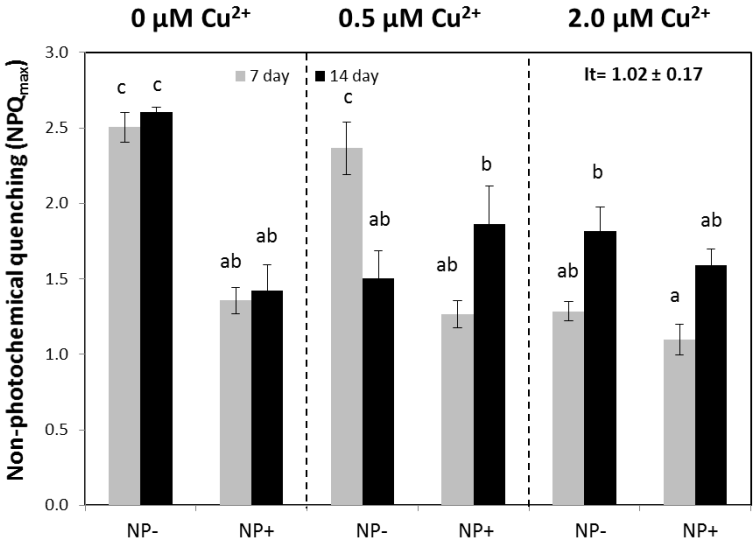

Figure 3. Maximal non-photochemical quenching (NPQ_{max}) (mean values ± SE, n=3) of *Cystoseira tamariscifolia* in relation to Time: 7 d (black bars) and 14 d (grey bars), NP-(low nitrate and phosphate level) and NP+ (high nitrate and phosphate level) and Copper; 0 µM Cu^{2+} (Control), 0.5 µM Cu^{2+} (low copper concentration) and 2.0 µM Cu^{2+} (high copper concentration) treatments. Upper values in right box indicate initial time values (*It*: mean ± SE, n=3). Lower-case letters denote significant differences after a SNK test.

The photosynthetic efficiency (α_{ETR}) was significantly different ($p<0.05$) by interaction between all factors. The α_{ETR} was higher in high copper levels at 7 d without enrichment treatment. (Tables 1 and S1). The saturated irradiance (Ek_{ETR}), was presents significantly ($p<0.05$) interaction between all factors (Tables 1 and S1). Ek_{ETR} was higher in high copper levels without nitrate enrichment at 7 d of the culture. Copper, nitrate and the interaction time significantly influenced the relationship between ETR_{max}/NPQ_{max} (production related to energy dissipation related to photoprotection)*nitrate (Tables 1 and S1).

Table 1. Photosynthetic efficiency (α_{ETR}), irradiance of saturation of ETR (Ek_{ETR}) and relation between maximal electron transport arte (ETR_{max}) and maximal non-photochemical quenching (NPQ_{max}) (mean values ± SE, n=3). In *Cystoseira tamariscifolia* in relation to Time (7 and 14 days), NP+ ((high nitrate and phosphate level) and NP- (low nitrate and phosphate level) and Copper; 0 µM Cu^{2+} (Control), 0.5 µM Cu^{2+} (low copper concentration) and 2.0 µM Cu^{2+} (high copper concentration) treatments. Initial time of the experimental period (*It*) is shown in the first column. Lower-case letters denote significant differences after SNK test.

			NP +		NP -	
		It	*7 d*	*14 d*	*7 d*	*14 d*
α_{ETR}	*0 µM (Control)*	0.27 ± 0.05	0.42 ± 0.04^{b}	0.34 ± 0.03^{b}	0.22 ± 0.02^{a}	0.21 ± 0.01^{a}
	0.5 µM		0.21 ± 0.01^{a}	0.40 ± 0.05^{b}	0.22 ± 0.02^{a}	0.22 ± 0.01^{a}
	2.0 µM		0.23 ± 0.01^{a}	0.23 ± 0.01^{a}	0.54 ± 0.04^{c}	0.20 ± 0.01^{a}
Ek_{ETR}	*0 µM (Control)*	189.59 ± 63.06	40.66 ± 2.69^{a}	71.30 ± 17.08^{ab}	95.81 ± 4.03^{ab}	64.63 ± 12.53^{a}
	0.5 µM		70.55 ± 13.76^{ab}	57.01 ± 13.10^{a}	68.45 ± 13.21^{ab}	100.45 ± 8.98^{ab}
	2.0 µM		73.09 ± 14.91^{ab}	68.15 ± 11.53^{ab}	59.92 ± 17.21^{a}	128.65 ± 19.19^{b}
ETR_{max}/NPQ_{max}	*0 µM (Control)*	38.15 ± 6.36	6.95 ± 0.41	12.52 ± 0.74	10.21 ± 0.91	16.01 ± 0.93
	0.5 µM		14.29 ± 1.41	18.61 ± 2.87	7.82 ± 0.46	11.86 ± 1.64
	2.0 µM		15.03 ± 2.01	10.29 ± 1.99	11.63 ± 0.95	12.71 ± 0.81

Internal and external cellular copper content

The total copper and internal cellular copper concentrations were significantly ($p<0.05$) affected by the supply of copper in the water (Figure 4, Table S2). After 14 d culture, total copper content showed maximal values in 2.0 µM Cu^{2+} with 262.4 ± 22.5 µg g^{-1} DW with and without nitrate whereas in the control conditions showed minimal values 69.9 ± 9.6 µg g^{-1} DW in the same nitrate levels (Figure 4, Table S2).

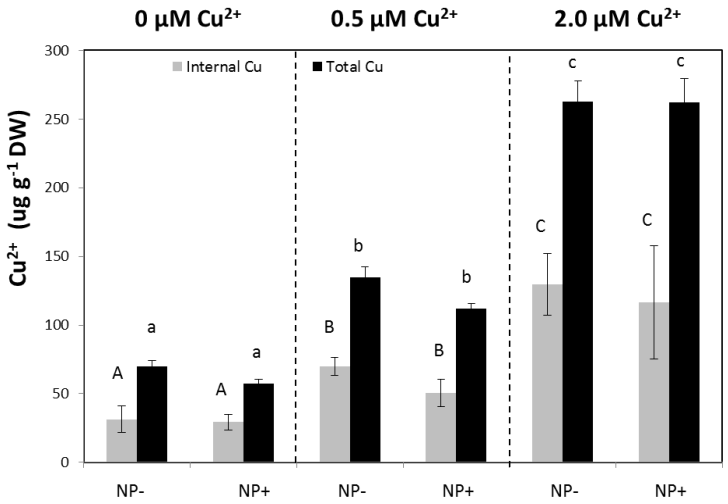

Figure 4. Total copper concentrations (black bars) and Intracellular copper concentration (grey bars) (mean values ± SE, n=3) of *Cystoseira tamariscifolia* the end of experimentation, NP-(low nitrate and phosphate level) and NP+ (high nitrate and phosphate level) and Copper; 0 µM Cu^{2+} (Control), 0.5 µM Cu^{2+} (low copper concentration) and 2.0 µM Cu^{2+} (high copper concentration) treatments. Shift case letters denote significant differences in total copper concentration and lower case letters denote significant differences in Internal copper concentration after SNK test.

The internal copper content of *C. tamariscifolia* was greatest in 2.0 µM Cu^{2+} with maximal values 129.7 ± 15.5 µg g^{-1} DW and minimal values in control conditions of the 29.3 ± 2.7 µg g^{-1} DW, these values were in both nitrate conditions (Figure 4, Table S2).

Nitrogen and carbon internal content

The nitrogen internal content was higher after 7-day culture in all treatments respect to the end the experimental period (Figure 5a, Table S3). Nitrogen internal content was significantly ($p<0.05$) affected by the interaction between time *copper and copper*nitrate (Figure 5b, c, Table S3).

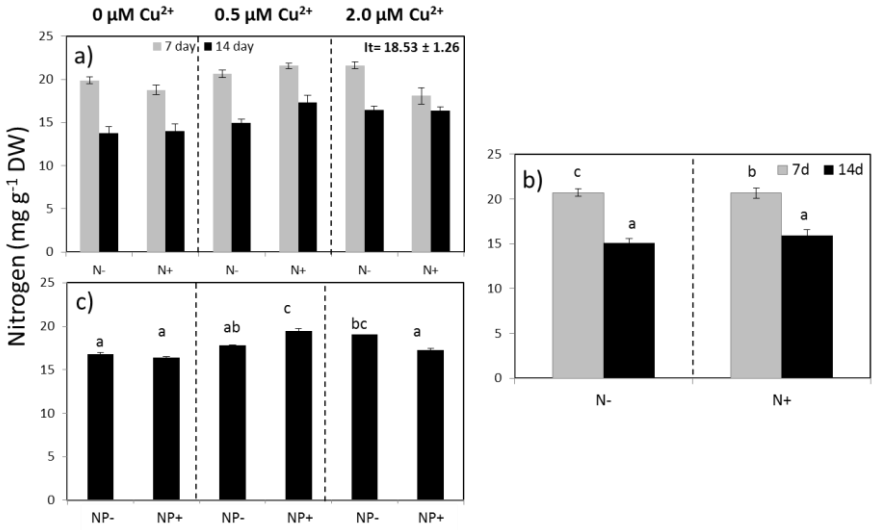

Figure 5. a) Nitrogen content (mg g^{-1} DW) (mean values ± SE, n=3) of *Cystoseira tamariscifolia* in relation to Time: 7 d (black bars) and 14 d (grey bars), NP-(low nitrate and phosphate level) and NP+ (high nitrate and phosphate level) and Copper; 0 µM Cu^{2+} (Control), 0.5 µM Cu^{2+} (low copper concentration) and 2.0 µM Cu^{2+} (high copper concentration) treatments. Upper values in right box indicate initial time values (*It*: mean ± SE, n=3), b) pooled data for the interaction Time*Nitrate (mean values ± SE, n=6) and c) Pooled data for the interaction Copper*Nitrate (mean values ± SE, n=6). Lower-case letters denote significant differences after a SNK test.

The pooled data between time and nitrate factors showed that the internal nitrogen was the highest at 7d in low nitrate treatment (Figure 5b) and between copper and nitrate was highest at 0.5 µM Cu^{2+} with nitrate enrichment (Figure 5c, Table S3). The internal carbon content did not change significantly over the period of the experiment (Table S3). C content of *C. tamariscifolia* showed maximal values 268.2 ± 13.7 mg g^{-1} DW and minimal values of the 244.6 ± 3.7 mg g^{-1} DW. The C: N ratio was influenced time and copper level (Table S3).

Pigment content

The interaction between Chlorophyll a (Chl*a*) and all factors content was significant ($p<0.05$) (Figure 6a, Table S4). The Chl*a* content was highest in 2.0 µM Cu^{2+} in both nitrate conditions, and it was highest in nitrate enrichment in middle copper treatment (Figure 6a, Table S4). Chlorophyll *c* (Chlc) and Fucoxanthin (Fx) contents presented significant ($p<0.05$) interaction with time and copper concentrations (Figure 6b, c, Table S4). Both pigments were highest at the end the experimental period in high copper concentration with and without nitrate treatments (Figure 6b, c, Table S4).

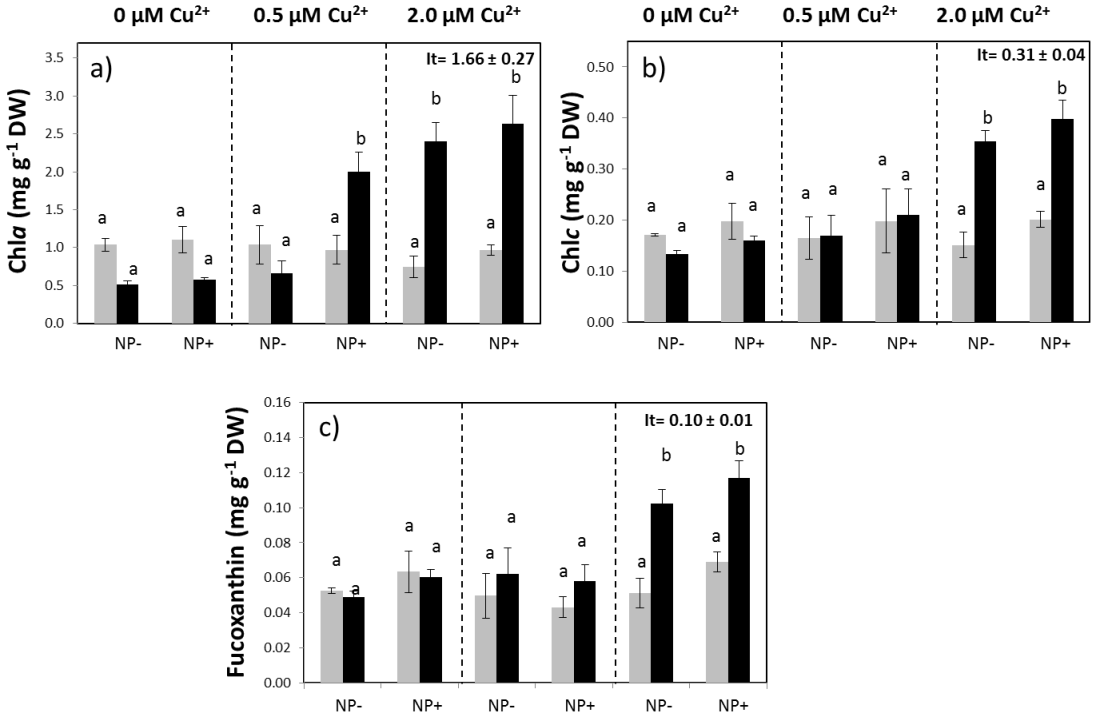

Figure 6. Pigment content; a) Chlorophyll *a* (Chl*a*) (mg g^{-1} DW), b) Chlorophyll *c* (Chl*c*) (mg g^{-1} DW) and c) Fucoxanthin (Fx) (mg g^{-1}DW) (mean values ± SE, n=3) of *Cystoseira tamariscifolia* in relation to Time: 7 d (black bars) and 14 d (grey bars), NP-(low nitrate and phosphate level) and NP+ (high nitrate and phosphate level) and Copper; 0 µM Cu^{2+} (Control), 0.5 µM Cu^{2+} (low copper concentration) and 2.0 µM Cu^{2+} (high copper concentration) treatments. Upper values in right box indicate initial time values (*It*: mean ± SE, n=3). Lower-case letters denote significant differences after a SNK test.

Polyphenolic compounds and antioxidant activity

Total phenolic content (PC) presented significantly ($p<0.05$) interaction among all factors (Figure 7a, Table S5). At 14 d culture, the total phenolic content increased at high copper level independent of the nitrate treatments (Figure 7a, Table S5). On a control with N+ and middle copper with N- treatments, PC was also higher at 14d (Figure 7a, Table S5).

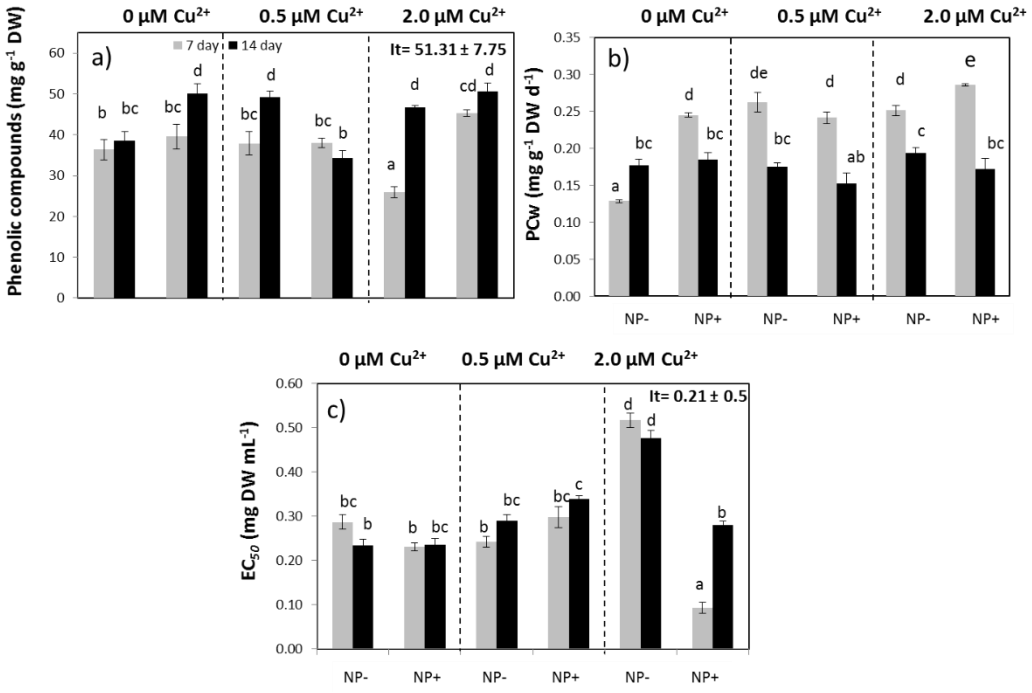

Figure 7. a) Total phenolic compounds (express as mg g^{-1} DW). b) Phenolic content in the water (PCw) (express as mg g^{-1} DW d^{-1}) and b) antioxidant activity ($EC_{50,}$ mg DW mL^{-1}) (mean values ± SE, n=3). Of *Cystoseira tamariscifolia* in relation to time: 7 d (black bars) and 14 d (grey bars), NP-(low nitrate and phosphate level) and NP+ (high nitrate and phosphate level), Copper; 0 µM Cu^{2+} (Control), 0.5 µM Cu^{2+} (low copper concentration) and 2.0 µM Cu^{2+} (high copper concentration) treatments. Upper values in right box indicate initial time values (*It*: mean ± SE, n=3). Lower-case letters denote significant differences after a SNK test.

The interaction between content of phenols in the water (PCw) and all factors was significantly ($p<0.05$) (Figure 7b, Table S5). The phenolic compound levels in the water were higher after 7 d than after 14 d culture except in the control under N-treatment (Figure 7b). Under N+ treatment and after 7 d, and only under 2.0 µM Cu^{2+}, the PCw was higher than that in the control and 0.5 µM Cu^{2+} whereas after 14 d no significant effect of copper was detected (Figure 7b, Table S5). The antioxidant activity (as EC_{50}), was affected ($p<0.05$) by the interaction between all factors (Figure 7c, Table S5). EC_{50} was higher (lower EC_{50}) in high copper levels under N+ than N- treatment, in both times; at 7 and 14 d culture (Figure 7b, S5).

The phenolic compounds, i.e., Shikimic acid and phloroglucinol were found in all treatments after the experimental period (Table 2, S6). At initial time, we observed the presence of others compounds i.e. quinic acid, gallic acid and kaempferol. In the control

copper conditions, without nitrate enrichment, only gallic acid was found and benzoic acid with nitrate enrichment conditions (Tables 2 and S6). In high copper treatments without nitrate conditions, quercetin was found (Tables 2 and S6).

Table 2. Phenolic composition (µg mL^{-1}) mesure by Ultra-HPLC (mean values ± SE, n=3) of *Cystoseira tamariscifolia* after 14 days, Control (N-) and (N+); (control copper conditions low nitrate and high nitrate level), Cu 0.5 µM (N-) and (N+); (low copper concentration low nitrate and high nitrate level). Cu 2.0 µM (N-) and (N+); (high copper concentration low nitrate and high nitrate level) treatments. Initial time of the experimental period (*It*) is shown in the first column. Lower-case letters denote significant differences after SNK test. *nd*: not detected.

Phenolic Compounds	*It (µg ML^{-1})*	*After 14 days*					
		Cu 0 µM N-	Cu 0 µM N+	Cu 0.5 µM (N-)	Cu 0.5 µM (N-)	Cu 2.0 µM (N-)	Cu 2.0 µM (N+)
Shikimic acid	74.77 ± 4.72	31.84 ± 1.75	64.49 ± 0.87	56.83 ± 18.94	53.32 ± 14.46	61.76 ± 12.52	95.08 ± 30.93
Quinic acid	0.82 ± 0.17	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
Gallic acid	4.26 ± 1.10	2.07 ± 0.04	*nd*	*nd*	*nd*	*nd*	*nd*
Benzoic acid	*nd*	*nd*	24.68 ± 2.27	*nd*	*nd*	*nd*	*nd*
Quercetin	*nd*	*nd*	*nd*	*nd*	*nd*	13.32 ± 8.11	*nd*
kaempferol	3.88 ± 0.38	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
Phloroglucinol	11.97 ± 4.84	67.28 ± 14.68	70.03 ± 17.79	83.144 ± 16.63	145.07 ± 22.93	94.94 ± 25.89	119.01 ± 11.275

The shikimic acid and phloroglucinol did not show significant differences in the interaction between copper and nitrate. However, shikimic acid was the highest in high copper concentrations whereas phloroglucinol was the highest under both high and low copper concentration with nitrate enrichment (Tables 2 and S6).

Principal Coordinates Analysis

The Principal Coordinates Analysis (PCO) (Figure 8) revealed a positive correlation of the first axis (43.1% of total variation) with the internal PCw, N content, F_v/F_m and PC. In contrast, the internal C content, EC_{50}, Chl*c*, Chl*a*, fucoxanthin and ETR_{max} were negatively correlated with this axis. Taking into account the spatial distribution of the samples in relation to the studied factors, the time presented a high relationship with the mentioned axis (Figure 8). Moreover, the combination of the first two axes explained the 62.3% of the variation in these variables (Figure 8).

Moreover, the small angles between the arrows are indicative of high correlation between the variables they represent; thus, the plot gave an idea of the relationships between the variables included. In the Figure 9, PCO revealed a positive correlation of the first axis (68.1% of total variation) with the internal NPQ_{max}. In contrast, all the rest to the variables were negatively correlated with this axis whereas, the combination of the first two axes explained the78.5% of the variation in these variables (Figure 9).

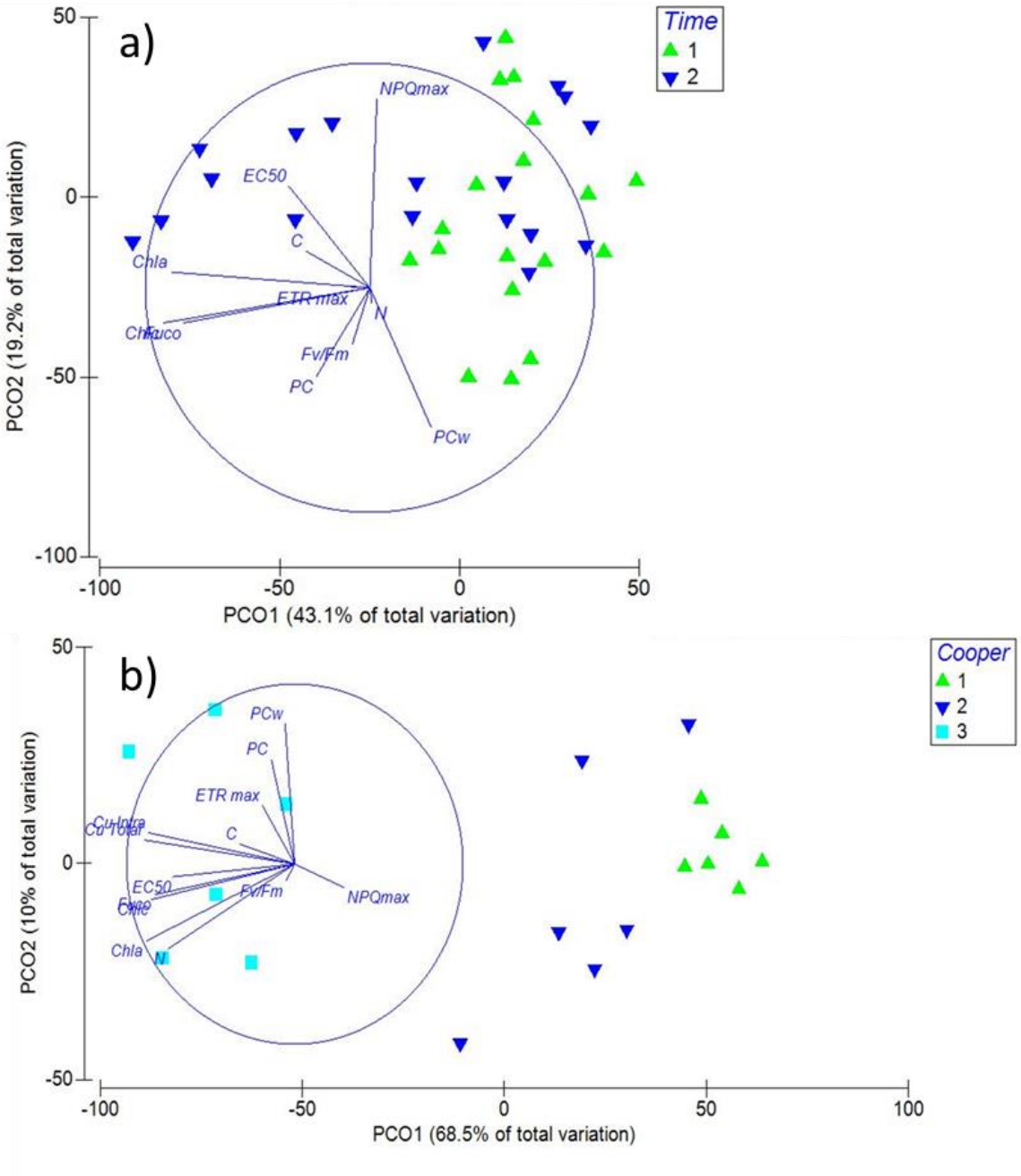

Figure 8. a) PCO diagram in relation to time (t1 and t2). Vectors overlay (Spearman rank correlation) indicates the relationship between the PCO axes and the ecophysiologycal variables; C and N internal conten, Chl*a* and Chlc: Chlorophyll *a* and *c*, respectively, Fuco: Fucoanthin, F_v/F_m: Maximal quantum yield, ETR_{max}: maximal electron transport rate, NPQ_{max}: Maximal non-photoquemichal quenching, PC: phenolic compounds, PCw: phenolic compound in the water and EC_{50}: antioxidant axtivity. b) PCO diagram in relation to copper (Control: 1, middle: 2 and high concentration: 3) with the same vectors overlay (Spearman rank correlation) and the ecophysiological variables.

Correlation analysis

In the control copper treatment, F_v/F_m was positively correlated to internal N (r=0.605, *P*=0.03, n=12), whereas ETR_{max} was not related to internal nitrogen (*r*=-0.286, *P*=0.36, n=12), but it was positively correlated to NPQ_{max} (*r*=0.610, *P*=0.03, n=12). Internal copper level was positively correlated to the external copper concentration in the water

(*r*=0.548, *P*=0.01, n=18) and to Ek_{ETR} (r=0.658, *P*<0.01, n=18), EC_{50} (r=0.848, *P*<0.01, n=18) and all photosynthetic pigments; Chl*a* (r=0.669, *P*<0.01, n=18), Chl*c* (r=0.752, *P*<0.01, n=18) and Fucoxanthin (r=0.737, *P*<0.01, n=18). External copper levels were positively correlated to all photosynthetic pigments; Chl*a* (r=0.754, *P*<0.01, n=18), Chl*c* (r=0.852, *P*<0.01, n=18) and Fucoxanthin (r=0.808, *P*<0.01, n=18). ETR_{max} was not significantly correlated to internal copper (r=0.367, *P*=0.13, n=18) or nitrogen (r=0.410, *P*=0.09, n=18), whereas photosynthetic efficiency (α_{ETR}) was negatively correlated to ETR_{max} (r=-0.503, *P*=0.03, n=18) and Ek_{ETR} was positively correlated to EC_{50} (r=0.596, *P*<0.01, n=18). The internal nitrogen was positively related to EC_{50} (r=0.497, *P*=0.03, n=18), Chl*a* (r=0.576, *P*=0.01, n=18) and Chl*c* (r=0.517, *P*=0.02, n=18).

4.5 DISCUSSION

Recent studies concur that brown macroalgal photosynthesis and biochemistry can be affected for different copper levels (Connan and Stengel 2011a and b, Ryan et al. 2012, Sáez et al., 2015). In our study, we show that any such responses will depend upon copper and nitrate availability such as, both the internal and external copper levels were affected by nitrate supply. After 14 d culture under high concentration of $CuSO_4 \cdot 5H_2O$ or Cu_T (2.0 µM or 500 µg L^{-1}) corresponding to 106.6 nM of free Cu^{2+}, the internal copper accumulation in *Cystoseira tamariscifolia* was significantly higher (2 times) under low than that under high nitrate culture. This result corresponds with about two times higher external copper concentration in the medium in high compared to low nitrate treatments. The copper levels affected the internal content of total nitrogen (Ni) in the alga and in addition as expected this was affected by the nitrate availability. Thus, nitrate modified the uptake of copper after 14 d grown algae under 2.0 µM Cu_T being the high nitrate treatment more favourable at physiological levels than low ones as indicated by the highest F_v/F_m values in high nitrate treatment.

One can expect that the different copper pattern in the nitrate treatments could be explained by the differences in the photosynthetic rate due to nitrate supply. However, under 2 µM Cu_T, both maximal quantum yield (*Fv/Fm*), as indicator of physiological state and photoinhibition, and maximal electron rate, as indicator of productivity (Figueroa et al. 2014a) did not present any significant differences with the nitrate treatment. Nitrate stimulates photosynthetic activity expressed as electron transport rate in red macroalgae (Korbee-Peinado et al. 2004, Huovinen et al. 2005, Bonomi-Barufi et al. 2011) and green

macroalgae (Cabello-Pasini et al. 2011). However, in contrast, F_v/F_m and ETR_{max} were higher under low than high nitrate supply both in the control and 0.5 μM Cu_T. The decrease of F_v/F_m and ETR_{max} under high nitrate availability was previously reported in *C. tamariscifolia* in experiments conducted in the field at different depths (Celis-Plá et al. 2014a). It was suggested that the input of high nitrate levels in the short term could produce a decrease in the photosynthetic rate by a transient unbalance between carbon and nitrogen metabolism affecting the electron transport rate as Turpin (1991) showed in microalgae. In other experiment, Celis-Plá et al. (2014b) showed in mesocosms under solar radiation that the highest ETR in *C. tamariscifolia* occurred in thalli with the lowest internal nitrogen level i.e. winter compared to summer grown algae. The similar response to nitrate on photosynthetic activity in *C. tamariscifolia* collected in the Mediterranean and North Sea have not necessary to be the same on copper tolerance since the response can be dependent on the natural exposure to copper in the collected area, as well as on the interactive effect of both factors (nitrate and copper).

C. tamariscifolia of Northern Sea seems to have the same photosynthetic pattern under the nitrate supply in spite of the fact that these specimens have to be acclimated to the higher levels of nitrate of Northern compared to Mediterranean Sea (Mercado et al. 2012). Nitrate concentration in the water affected the accumulation of other metal as Cadmium (Lee and Wang 2001) in the green alga *Ulva fasciata* i.e. the increase of nitrate produce a significant increase in Cd accumulation rates whereas the accumulation of Cr and Zn was not greatly affected by the ambient nitrate (10-100 μM). Zn uptake was enhanced in nitrate-enriched macroalgae whereas not data on copper were available (Lee and Wang 2001). Other macronutrients as ammonium had not any effect on metal accumulation in contrast to phosphate that produces a high accumulation of Cr (Lee and Wang 2001). The influence of major macronutrients on cationic and anionic metal accumulation seems to be highly metal specific according to Lee and Wang (2001). It would be interesting to study the effect of other macronutrients different to nitrate on the physiology and toxicity of metal in *C. tamariscifolia*.

Nielsen et al. (2003) showed in brown algae *Fucus serratus,* that photosynthetic activity was different in the same species according the acclimation of these species to their natural levels of copper. They found that the maximal quantum yield (F_v/F_m) decreased in non-tolerant algae (non-contaminated sites) whereas NPQ_{max} increase was the most pronounced in resistant algae (elevated copper concentration). Connan and Stengel (2011a), showed that lower copper content was not related to higher photosynthetic rate

as ETR_{max} but with the lower internal total nitrogen level. Thus, the lower internal content could indicate less favorable physiological activity and therefore the copper uptake is reduced. Other explanation is that the turnover of copper could be increased under high N supply and a possible higher release is produced than under low N supply and the level of external copper content increase. High levels of copper (5 mg L^{-1}) produced a decrease photosynthesis activity estimated as both F_v/F_m and $rETR_{max}$ in the brown algae *Ascophyllum nodosum* and *Fucus vesiculosus* after 15 d culture at normal salinity (Connan and Stengel, 2011b). At lower concentration of copper (250 μg L^{-1}) used by Connan and Stengel (2011b), copper reduced photosynthetic rate as measured by chlorophyll fluorescence and oxygen evolution in the red alga *Gracilariopsis longissima* (Brown and Newman, 2003). Copper is known to be the most toxic metal, particularly due to its effect on the photosynthetic apparatus both via immediate effects (Küpper et al. 2002, Pätsikkä et al. 2001) and by competition for binding sites with other ions (Lobban and Harrison 1994, Stauber and Florence 1987). Increasing NPQ up to 1 mg L^{-1} copper as observed previously on isolated thylakoids or membranes (Pätsikkä et al. 2001, Yruela et al. 2000) or in the thalli of the seaweed *Ectocarpus siliculosus* (Küpper et al. 2002). Nielsen and Nielsen (2010) suggested that, under similar contamination level, available copper (Cu^{2+}) did not induce dynamic photoinhibition.

The interaction by copper and nitrate produced significant differences in NPQ_{max}. After 14 d culture, NPQ_{max} values decreased from about 2.5 in the control to 1.75 under low nitrate treatments. However, under high nitrate supply no effect of copper on NPQ_{max} was found. It is important to indicate that NPQ_{max} values in the control were reduced by increasing nitrate supply as the ETR_{max}, thus high nitrate levels reduced both production and energy dissipation. Under these conditions, copper did not change the energy dissipation as NPQ_{max}. Connan and Stengel (2011b) showed drastic variations in NPQ_{max} with copper and salinity changes. They presented NPQ_{max} values as high as 10, typical for plants and most algae at high irradiances, although so high values has been reported in other brown seaweeds as *Pelvetia canaliculata* (Harker et al. 1999), *Saccharina latissima* (Gevaert et al. 2002) and *Macrocystis pyrifera* (Colombo-Pallotta et al. 2006) under moderate irradiances. The most pronounced increase of NPQ_{max} in copper tolerant *F. serratus* was related to the resistance mechanisms as the excitation energy being dissipated through xanthophyll dependent quenching mechanism in tolerant algae (Nielsen et al. 2003). In our study, no increase of NPQ_{max} due to increase of copper was found, but a decrease after 14 d culture under low nitrate supply whereas no variation was

found compared to the control under high nitrate supply. In any case the NPQ_{max} values as ETR_{max} were not significant different at the highest copper treatment (2.0 μM) showing a high resistance of this species to copper in both nitrate treatment, in spite of the internal level of copper was two times higher in low than that in high nitrate supply. The relationship between ETR_{max} (as indicator productivity) and NPQ_{max} (as indicator energy dissipation related to photoprotection) was significantly affected by time and nitrate. ETR_{max}/NPQ_{max} increased after 14d without nitrate enrichment. Figueroa et al. (2014b) showed the highest values of ETR_{max}/NPQ_{max} after 7 d in *C. tamariscifolia*, when increase the UVB radiation in 50μM nitrate enrichment.

The accumulation of pigments in responses to the interaction between copper and nitrate was clearly related to nitrate levels in the water. Under high nitrate, levels of Chl*a* increased at 0.5 and 2.0 μM Cu^{2+} whereas Chl*c* and fucoxanthin increased also under low nitrate levels but only under the highest copper concentration (2.0 μM Cu^{2+}). Pellegrini et al. (1993) showed the toxic effect of Cu in *Cystoseira barbata* involves a reduction of weight-growth and of pigment contents (chlorophyll *a* and carotenoids), the interactions e.g. Cd-Cu-Ca have effects in chlorophyll *a* and *c* contents, and Cu-Zn-Ca chlorophyll *a* content. In *Ectocarpus siliculosus,* Sáez et al. (2015), showed that the photosynthetic pigment content was affected by copper in different strains, but it was dependent on the acclimation of the strains to natural copper level. The Chl*a* decreased after 10 d culture under 2.0 μM Cu_T in algae collected from Scotland (pristine) and England (Cu-contaminated), whereas Chl*c* decreased only in pristine waters.

In the middle the experimental period, the content of total phenolic compounds decreased in higher copper conditions with low nitrate enrichment, whereas in high nitrate, was the highest. After 14 d, no differences in the phenol content were found among nitrate treatments indicating an acclimation pattern. It has been described that the variability in the phenolic content could be related with environmental factor such as herbivory, light, depth, salinity; nutrients, seasonality as well as intrinsic ones such as age, length or type of the tissues (see Amsler and Fairhead 2006). N can enhance the accumulation of phenolic compounds in some brown algae (Pavia and Toth 2000) as well as in *Ulva rigida* (Cabello-Pasini et al. 2011). The phenolic composition changed in nitrate enrichment or non-enrichment. After 14d, shikimic acid increased with enrichment nitrate levels and copper, on the other hand, phloroglucinol was higher in middle and high Copper levels with enrichment nitrate conditions.

Phlorotannins, a usual phenolic compound in brown seaweeds has a high capacity for binding external copper in *F. vesiculosus* and *A. nodosum* (Connan and Stengel, 2011a). In copper-enriched water, copper contents of the phenolic increased in contrast to other metals (Connan and Stengel, 2011a). Copper can also affect the level of phenols, both accumulation and exudation. An increase of copper concentration reduced total phenolic contents, changed phenolic composition increasing the proportion and positively affected the phenolic exudation (Connan and Stengel, 2011a). Antioxidant activity expressed as EC_{50} was influenced by the interaction of copper and nitrate. At 2.0 μM Cu_T the antioxidant activity was much higher in low than high nitrate grown algae. This suggest nitrate enrichment is more favourable under to stress condition since it can protect against oxidant radicals in more extent than that in algae grown in low nitrate levels. The internal copper was positively correlated to EC_{50}; this indicates that the protection against oxidant radicals is reduced by the increase of internal copper. The internal level of phenols was positively correlated to the external levels indicating a coupling between production and release of phenols to the water. However, Connan and Stengel (2011a) found that the high exudation of phenolic compounds into surrounding waters of the seaweed tips resulted in a significant reduction of total phenolic contents.

4.6 CONCUSIONS

Nitrate level in the water influenced the effects of copper on photosynthetic rate, biochemical composition and antioxidant activity. Low copper and high nitrate treatments decreased photosynthetic activity (F_v/F_m and ETR_{max}), NPQ_{max} and total phenolic content compared to low nitrate levels, whereas the Chl*a* increased. However, in high copper and nitrate at 7d of experimental period, N internal content, photosynthetic activity, PC and PCw decreased.

In general, at the end of the experimental period, middle copper levels seems to be more favorable in low nitrate conditions, respect to the physiological status of *C. tamariscifolia*. In contrast, in high copper levels with high nitrate levels, the physiological status in *C. tamariscifolia* was also favorable. These results suggest that interaction between copper and nitrate can give a high resistance to copper of *C. tamariscifolia* with no apparent damage effects after 14 d culture.

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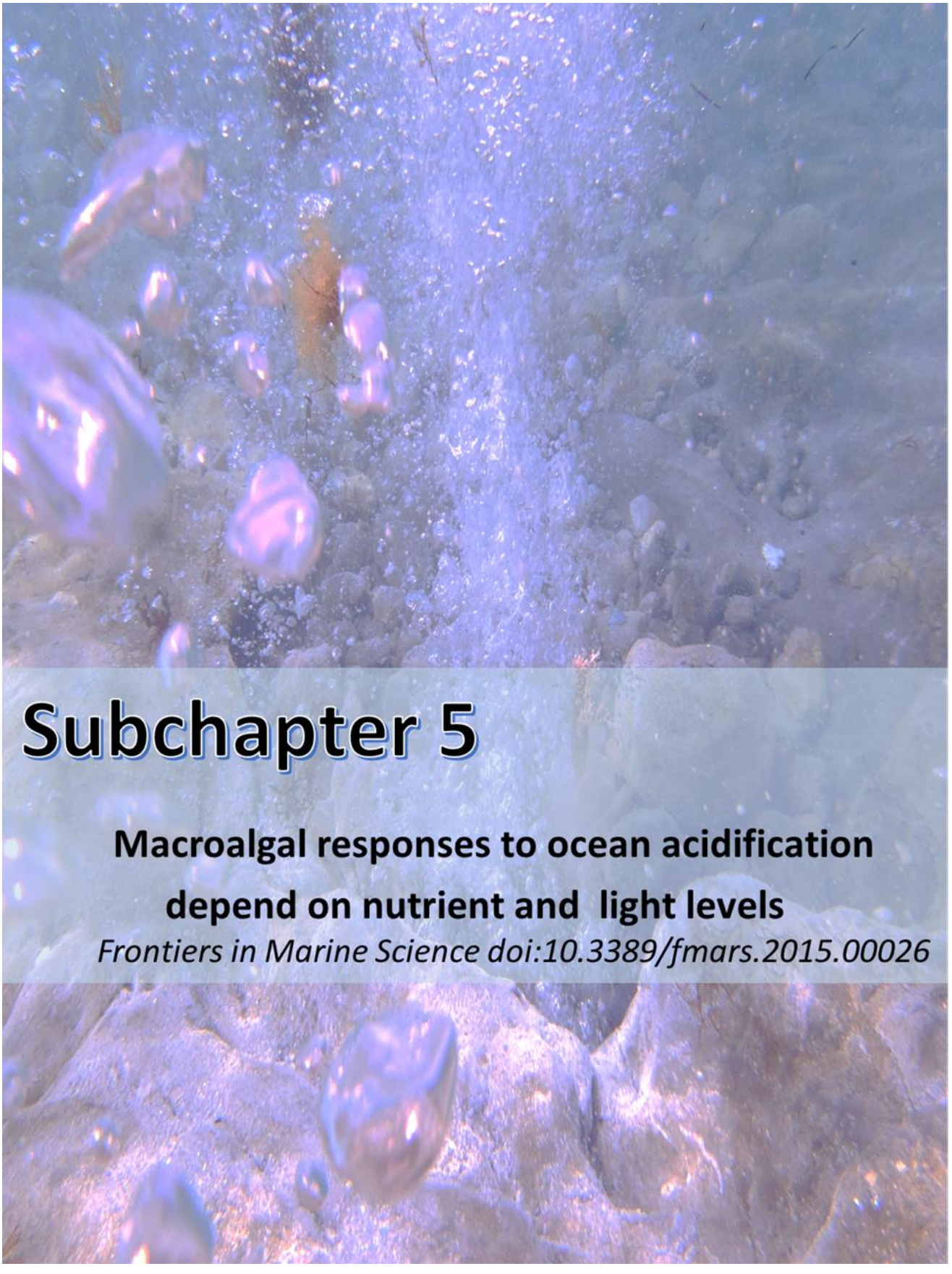

Subchapter 5

Macroalgal responses to ocean acidification depend on nutrient and light levels

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Natural pH gradient, Vulcano, Italy. March 2013. Photograph by Paula S. M. Celis Plá

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Macroalgal responses to ocean acidification depend on nutrient and light levels

Paula S. M. Celis-Plá [1, 2], Jason M. Hall-Spencer [3], Paulo Antunes Horta [4], Marco Milazzo [5], Nathalie Korbee [1], Christopher E. Cornwall [6, 7] and Félix L. Figueroa [1]*

[1] Department of Ecology, Faculty of Science, University of Málaga, Málaga, Spain, [2] Laboratory of Botany, Faculty of Pharmacy, University of Barcelona, Barcelona, Spain, [3] Marine Biology and Ecology Research Centre, Plymouth University, Plymouth, UK, [4] Department of Botany, University Federal of Santa Catarina, Florianopolis, Brazil, [5] Dipartimento di Scienze della Terra e del Mare, University of Palermo, Palermo, Italy, [6] Institute for Marine and Antarctic Studies, University of Tasmania, Hobart, TAS, Australia, [7] School of Earth and Environment and ARC Centre of Excellence for Coral Reef Studies, University of Western Australia, Crawley, WA, Australia

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Iris Eline Hendriks, University of the Balearic Islands, Spain

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****Correspondence:***
Paula S. M. Celis-Plá, Department of Ecology, Faculty of Science, University of Málaga, Campus teatinos s/n, 29016 Málaga, Spain
paulacelispla@uma.es

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5.1 Ocean acidification may benefit algae that are able to capitalize on increased carbon availability for photosynthesis, but it is expected to have adverse effects on calcified algae through dissolution. Shifts in dominance between primary producers will have knock-on effects on marine ecosystems and will likely vary regionally, depending on factors such as irradiance (light vs. shade) and nutrient levels (oligotrophic vs. eutrophic). Thus experiments are needed to evaluate interactive effects of combined stressors in the field. In this study, we investigated the physiological responses of macroalgae near a CO_2 seep in oligotrophic waters off Vulcano (Italy). The algae were incubated *in situ* at 0.2 m depth using a combination of three mean CO_2 levels (500, 700–800 and 1200 µatm CO_2), two light levels (100 and 70% of surface irradiance) and two nutrient levels of N, P, and K (enriched vs. non-enriched treatments) in the non-calcified macroalga *Cystoseira compressa* (Phaeophyceae, Fucales) and calcified *Padina pavonica* (Phaeophyceae, Dictyotales). A suite of biochemical assays and *in vivo* chlorophyll *a* fluorescence parameters showed that elevated CO_2 levels benefitted both of these algae, although their responses varied depending on light and nutrient availability. In *C. compressa*, elevated CO_2 treatments resulted in higher carbon content and antioxidant activity in shaded conditions both with and without nutrient enrichment—they had more Chl*a*, phenols and fucoxanthin with nutrient enrichment and higher quantum yield (F_v/F_m) and photosynthetic efficiency (α_{ETR}) without nutrient enrichment. In *P. pavonica*, elevated CO_2 treatments had higher carbon content, F_v/F_m, α_{ETR}, and Chl*a* regardless of nutrient levels—they had higher concentrations of phenolic compounds in nutrient enriched, fully-lit conditions and more antioxidants in shaded, nutrient enriched conditions. Nitrogen content increased significantly in fertilized treatments, confirming that these algae were nutrient limited in this oligotrophic part of the Mediterranean. Our findings strengthen evidence that brown algae can be expected to proliferate as the oceans acidify where physicochemical conditions, such as nutrient levels and light, permit.

Keywords: ocean acidification, macroalgae, photosynthesis, phenolic compounds, nutrient availability

5.2 Introduction

Ocean acidification due to increased atmospheric CO_2 levels is altering the concentrations of dissolved inorganic carbon (DIC) in surface waters; CO_3^{2-} levels are falling, which is expected to corrode marine carbonates, whilst CO_2 and HCO_3^- levels are rising which can stimulate photosynthesis (Connell et al., 2013; Cornwall et al., 2015). As some primary producers are better able to capitalize on increasing carbon availability than others, this is expected to alter marine communities (Hepburn et al., 2011; Connell et al., 2013; Koch et al., 2013; Gaylord et al., 2015). In the Mediterranean, surveys of coastal CO_2 seeps have repeatedly shown that coralline algae and sea urchins become less common as pH and CO_3^{2-} fall, whereas brown algae, such as *Cystoseira* spp., *Dictyota* spp., *Sargassum vulgare* and *Padina pavonica*, proliferate as CO_2 and HCO_3^- levels rise (Porzio et al., 2011; Baggini et al., 2014). The ways in which ocean acidification affects communities of primary producers are likely to vary regionally, depending on the species present and abiotic factors such as temperature, light and nutrient availability (Giordano et al., 2005; Brodie et al., 2014; Hofmann et al., 2014).

To begin to understand the influence of physicochemical factors on the responses of macroalgae to ocean acidification, we grew common types of brown algae (from the Families Fucales and Dictyotales) at CO_2 seeps in a multifactorial experiment in which we manipulated light (irradiance) and nutrient levels. At low light levels, macroalgae are thought to be more likely to rely on carbon uptake via diffusion than use energetically expensive carbon concentrating mechanisms (Raven and Beardall, 2014; Raven et al., 2014) which has led to the idea that any benefits of ocean acidification on growth would only be seen at lower light levels for the majority of species (Hepburn et al., 2011). However, ocean acidification also has the potential to damage photoprotective mechanisms which kick-in at high light levels (Pierangelini et al., 2014). Algae minimize damage from high irradiance by down-regulating photosystems-they also produce chemicals, such as phenolic compounds in the brown algae, which screen ultraviolet light and dissipate energy (Figueroa et al., 2014a). In oligotrophic waters, such as those of the Mediterranean, nutrient availability generally limits macroalgal growth (Ferreira et al., 2011), photosynthetic capacity (Pérez-Lloréns et al., 1996) and photoprotective mechanisms (Celis-Plá et al., 2014a).

Our study centers upon a highly oligotrophic region (the Tyrrhenian Sea) which is undergoing rapid changes in carbonate chemistry coupled with coastal eutrophication and increased land run-off (Oviedo et al., 2015). In this region, as with elsewhere in the world, canopy-forming brown algae have undergone a decline in abundance due to anthropogenic perturbation (Scherner et al., 2013; Strain et al., 2014; Yesson et al., 2015). Here, we investigate the interactive effects of increasing CO_2 levels and eutrophication on *Cystoseira compressa* and *Padina pavonica* using a pH gradient caused by volcanic seeps. These species were chosen because they are abundant around shallow Mediterranean CO_2 seeps (Baggini et al., 2014), because *Cystoseira* spp. are indicators of high water quality in the Mediterranean (Bermejo et al., 2013) and since *Padina* spp. tolerate loss of external calcification as CO_2 levels increase (Johnson et al., 2012; Pettit et al., 2015).

Macroalgal responses to ocean acidification may well depend upon their nutrient metabolism, which can vary widely between species (Hofmann et al., 2014; Hurd et al., 2014). Here, we compared interspecific physiological and biochemical responses to ocean acidification under different light and nutrient levels using standard methods for the study of multiple physical stressors in algae (Martínez et al., 2012; Celis-Plá et al., 2014a). Our hypothesis was that both brown algal species would benefit from ocean acidification in shaded conditions when nutrient levels were elevated. We expected that high light levels would inhibit photosystems, and that any benefits from high CO_2 would only occur if sufficient nutrients were available. If this were true we expected to observe increased photosynthetic activity (using electron transport rates and carbon content as a proxy) and increases in phenolic and antioxidant production in shaded nutrient-enriched treatments.

5.3 Materials and Methods

Experimental Design

Macroalgal incubations took place from 19 to 22 March 2013, along a CO_2 gradient near Vulcano, Italy (Figure 1; Boatta et al., 2013). *Cystoseira compressa* and *Padina pavonica* were collected at 0.5 m depth from a reference zone. Thalli (5 g fresh weight) were held in individual mesh cylinders (15 cm long × 5 cm in diameter) set 1 m apart and suspended at 0.2 m depth off a floating line that was anchored to the seabed perpendicular to the coast. This array was replicated at an ambient CO_2 site (*ca* 500 µatm CO_2), a medium CO_2 site (*ca* 700–800 µatm CO_2) and a high CO_2 site (*ca* 1200 µatm CO_2) (Table 1).

Each CO_2 zone had 12 replicates per treatment per species (nutrient enriched + ambient light or 100%$_{PAB}$, i.e., 100% of surface irradiance defined as PAB irradiance (PAR + UVR), nutrient enriched + shaded light or 70%$_{PAB}$, i.e., 70% of surface irradiance defined as PAB irradiance (PAR + UVR), non-enriched + ambient light or 100%$_{PAB}$, non-enriched shaded light

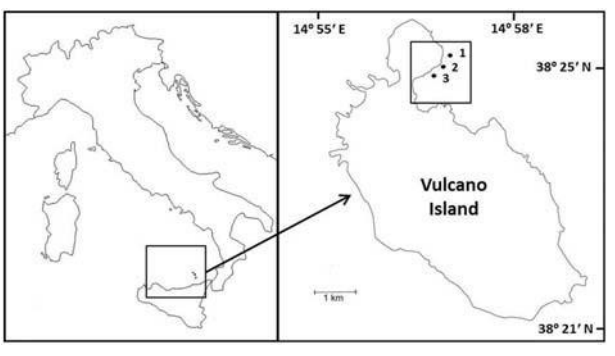

FIGURE 1 | Sample sites and location of experiments on *Cystoseira compressa* and *Padina pavonica* off Vulcano, Italy; (1) Ambient CO_2 site (*ca* 500 µatm CO_2), (2) Medium CO_2 site (*ca* 700–800 µatm CO_2) and (3) High CO_2 (*ca* 1200 µatm CO_2).

TABLE 1 | Seawater carbonate chemistry at three sites off Vulcano Island.

	Ambient CO_2	Medium CO_2	High CO_2
Salinity	38.19 ± 0.03	38.21 ± 0.04	38.23 ± 0.04
Temperature (°C)	14.94 ± 0.21	14.99 ± 0.13	15.06 ± 0.19
pH_{NBS}	8.11 ± 0.02	7.97 ± 0.04	7.86 ± 0.09
pCO_2 (µatm)	512 ± 29	779 ± 109	1250 ± 410
CO_2 (µmol kg^{-1})	18.8 ± 1.2	28.7 ± 4.1	46 ± 15.3
HCO_3^- (µmol kg^{-1})	2129 ± 20	2161 ± 23	2236 ± 36
CO_3^{2-} (µmol kg- 1)	181 ± 8.3	138 ± 9.3	119 ± 14.6
Total Alkalinity (µmol kg^{-1})	2527 ± 46	2499 ± 14	2569 ± 427
K Calcite	4.21 ± 0.19	3.22 ± 0.22	2.78 ± 0.34
K Aragonite	2.71 ± 0.13	2.07 ± 0.14	1.79 ± 0.22

Island, with an ambient CO_2, a Medium CO_2 and a High CO_2 site. Temperature (°C), Salinity and pH (NBS scale) were collected on different days in March 2013 (mean values ± SE, n = 5–14). Average total alkalinity (µmol kg^{-1}) was calculated from water samples collected at each site on 20th March 2013 (mean values ± SE, n = 3).

or 70%$_{PAB}$). Light levels were manipulated using a 1 mm^2 size pore mesh that reduced light levels to 70% of that of the unshaded treatments. The filter we used does not modify the light spectra (Aphalo et al., 2013). Mesh bags containing 100 g of a slow-release fertilizer comprising 17% N (NH_4^+ and NO_3^-), 17% P (P_2O_5) and 17% K (Multicote®, Haifa Chemicals, USA) were attached below nutrient enriched cylinders. For the non-enriched treatments, a bag with 100 g of sand was used as a control. The nutrient treatments were set 20 m apart from each other so that non-enriched treatments were unaffected.

Environmental Conditions

The seawater carbonate system was monitored at each site (Table 1). A 556 MPS YSI (Yellow Springs, USA) probe was used to measure salinity, pH and temperature (°C). The pH sensor was calibrated using NBS scale standard buffers. On 20th March 2013, water samples for total alkalinity (TA) were strained through 0.2 µm filters, poisoned with 0.05 ml of 50% $HgCl_2$, and then stored in the dark at 4°C. Three replicates were analyzed at 25°C using a titrator (Mettler Toledo, Inc.). The pH was measured at 0.02 ml increments of 0.1 N HCl.

Total alkalinity was calculated from the Gran function applied to pH variations from 4.2 to 3.0, from the slope of the curve HCl volume vs. pH. The pCO_2 and the saturation state of aragonite were calculated from pH_{NBS}, TA, temperature and salinity using the CO_2 SYS package (Pierrot and Wallace, 2006), using the constants of Roy et al. (1993) and Dickson (1990). Saturation state (K) is the ion product of calcium and carbonate ion concentrations as:

$$\Omega = [Ca^{2+}]\,[CO_3^{2-}]/K'sp \quad (1)$$

The apparent solubility product K'_{sp} depends on temperature, salinity, pressure, and the particular mineral phase (e.g. calcite and aragonite in this case).

Irradiance was monitored at the sea surface at two wavelength bands using PAR (QSO-SUN 2.5V) and UV-A (USB-SU 100, sealed in a water proof box (OtterBox3000). Water temperature was monitored using a HOBO logger (Onset Computer Corporation, Massachusetts, USA). The nutrient enrichment caused by the release of the fertilizer was assessed taking triplicate seawater samples at both enriched and non-enriched sites. Seawater was strained using portable GF/F filters (Whatman International. Ltd., Maidstone, UK) then transported to the laboratory inside an isotherm bag (4°C, in darkness), and kept at 20°C. Nitrate (NO_3^-) was determined using an automated analyzer (SanPlus^{++} System, SKALAR, Breda, Netherlands) applying standard colorimetric procedures (Koroleff, 1983).

Physiological and Biochemical Variables

Several physiological variables were obtained from the algae within each cylinder at the end of the experiment. These variables were also measured in *C. compressa* and *P. pavonica* from ambient CO_2 site (500 µatm) populations at 0.5 m depth. Carbon and nitrogen contents were determined using an element analyzer CNHS-932 model (LECO Corporation, Michigan, USA).

In vivo chlorophyll *a* fluorescence associated with Photosystem II was determined by using a portable pulse amplitude modulated (PAM) fluorometer (Diving-PAM, Walz GmbH, Germany). Macroalgal thalli were collected from natural populations (initial time) and after 4 days of incubation in the experiment (for each treatment or cylinder), and were put in 10 mL incubation chambers to obtain rapid light curves for each treatment. F_o and F_m were measured after 15 min in darkness to obtain the maximum quantum yield (F_v/F_m) being $F_v = F_m - F_o$, F_o the basal fluorescence of 15 min dark adapted thalli and F_m maximal fluorescence after a saturation light pulse of >4000 µmol m^{-2} s^{-1} (Schreiber et al., 1995). The electron transport rate (ETR) was determined after 20 s exposure in eight increasing irradiances of white light (halogen lamp provided by the Diving-PAM). The ETR was calculated according to Schreiber et al. (1995) as follows:

$$ETR\ (\mu mol\ electrons\ m^{-2}\ s^{-1}) = \Delta F/F'_m \times E \times A \times F_{II} \quad (2)$$

where $\Delta F/F'm$ is the effective quantum yield, being $\Delta F = Fm'-Ft$ (Ft is the intrinsic fluorescence of alga incubated in light and Fm^t is the maximal fluorescence reached after a saturation pulse of algae incubated in light), E is the incident PAR irradiance expressed in µmol photons m^{-2} s^{-1}, A is the thallus absorptance as the fraction of incident irradiance that is absorbed by the algae (see Figueroa et al., 2003) and F_{II} is the fraction of chlorophyll related to PSII (400–700 nm) being 0.8 in brown macroalgae (Figueroa et al., 2014a). ETR parameters as maximum electron transport rate (ETR_{max}) and the initial slope of ETR vs. irradiance function (α_{ETR}) as estimator of photosynthetic efficiency were obtained from the tangential function reported by Eilers and Peeters (1988). Finally, the saturation irradiance for ETR (Ek_{ETR}) was calculated from the intercept between ETR_{max} and α_{ETR}. Non-photochemical quenching (NPQ) was calculated according to Schreiber et al. (1995) as:

$$NPQ = (Fm-Fm')/Fm' \quad (3)$$

Maximal NPQ (NPQ_{max}) and the initial slope of NPQ vs. irradiance function (α_{NPQ}) were obtained from the tangential

function of NPQ vs. irradiance function according to Eilers and Peeters (1988).

Pigments were extracted from 20 mg fresh weight of thalli using 2 mL of 100% acetone and analyzed using an ultra-high-performance liquid chromatographer (Shimadzu Corp., Kyoto, Japan) equipped with a photodiode array detector to measure peaks in the range 350–800 nm. After extraction samples were centrifuged at 16200 g for 5 min (Sorvall Legend Micro 17, Thermo Scientific, Langenselbold, Germany) and then the extracts were filtered (0.22 µm nylon filters). The separation, was achieved with one column C-18 reversed phase (Shim-pack XR-ODS column; 3.0 x 75 mm i. d.; 2.2 µm particle size; Shimadzu, Kyoto, Japan) protected by a guard column TR-C-160 K1 (Teknokroma, Barcelona, Spain). The carotenoid composition was determined according to García-Plazaola and Becerril (1999) with some modifications (García-Plazaola et al., 2012), using commercial standards (DHI LAB Products). The mobile phase consisted of two components: Solvent A, acetonitrile: methanol: Tris buffer (0.1 M, pH 8) (84:2:14); and solvent B, methanol: ethyl acetate (68:32). The pigments were eluted using a linear gradient from 100% A to 100% B for the first 7 min, followed by an isocratic elution with 100% B for the next 4 min. This was followed by a 50 s linear gradient from 100% B to 100% A and an isocratic elution with 100% B for the next 3 min to allow the column to re-equilibrate with solvent A, prior to the next injection.

Total phenolic compounds were determined using 0.25 g fresh weight samples pulverized with a mortar and pestle with sand and 2.5 mL of 80% methanol. After keeping the samples overnight at 4°C, the mixture was centrifuged at 2253 g for 30 min at 4°C, and then the supernatant was collected. Total phenolic compounds were determined colorimetrically using Folin-Ciocalteu reagent and phloroglucinol (1,3,5-trihydroxybenzene, Sigma P-3502) as standard. Finally the absorbance was determined at 760 nm using a spectrophotometer (UV Mini-1240, Shimadzu) (Celis-Plá et al., 2014b). Total phenolic content was expressed as mg g–1 DW after determining the fresh to dry weight ratio in the tissue (5.2 for *C. compressa* and 4.5 *P. pavonica*, respectively). The results are expressed as average ± SE from three replicates of each treatment. Antioxidant activity was measured on polyphenol extracts according to Blois (1958); 150 µL of DPPH (2,2-diphenyl-1-picrylhydrazyil) prepared in 90% methanol were added to each extract. The reaction was complete after 30 min in darkness at ambient temperature (~20°), and the absorbance was read at 517 nm in a spectrophotometer (UVmini-1240, Shimadzu). The calibration curve made from DPPH was used to calculate the remaining concentration of DPPH in the reaction mixture after incubation. Values of DPPH concentration (mM) were plotted against plant extract concentration expressed as the EC_{50} value (oxidation index, mg DW mL^{-1}) required to scavenge 50% of the DPPH in the reaction mixture. Ascorbic acid was used as a control (Celis-Plá et al., 2014b).

Statistical Analysis

The effects of the *in situ* treatments on the physiological responses of *C. compressa* and *P. pavonica* were assessed using analysis of variance. Three fixed factors were considered: Site with three levels: ambient CO_2 site, medium CO_2 and high CO_2, Irradiance with two levels: 70 and 100% of surface irradiance (PAR + UVR irradiance), and two nutrient levels; enriched (N+) and non-enriched (N). This design allowed us to test interactive and additive effects of the variables on physiological responses after the 4 day experimental period. Student Newman Keuls tests (SNK) were performed on significant ANOVA interactions. Homogeneity of variance was tested using Cochran tests and by visual inspection of the residuals. All data conformed to homogeneity of variance. Analyses were performed by using SPSS v.21 (IBM, USA).

5.4 Results

Environmental Conditions

Cystoseira compressa and *Padina pavonica* were abundant at all three stations; *P. pavonica* was visibly less calcified at the site with the highest levels of CO_2. The seawater temperature was about15°C and the salinity was 38 at all stations; at the Ambient site, mean pH was 8.11, at the Medium CO_2 site (700–800 µatm), mean pH was 7.97 and at the High CO_2 site (1200 µatm), it was 7.86 (Table 1).

The average daily irradiance for the experimental period was 5360 kJ m^{-2} for PAR and 666 kJ m^{-2} for UVA. The nutrient enriched treatments had approximately 100 times the nitrate concentration of the ambient seawater; ambient vs. enriched ratios were 0.16 ± 0.04 vs. 106.17 ± 9.37 µM for the ambient site, 0.13 ± 0.01 vs. 106.33 ± 9.37 µM at the medium CO_2 site and 0.25 µM ± 0.01 vs. 106.42 ± 9.37 µM at the high CO_2 site (mean ± SE, $n = 3$).

Physiological and Biochemical Responses

The carbon content of *C. compressa* increased with increasing CO_2, whereas in *P. pavonica* showed interactive effects between all factors. Carbon, in *P. pavonica* showed maximal values 279.9 ± 6.5 with increased CO_2, in non-enrichment enriched treatments and minimal values 225.3 ± 2.4 mg g^{-1} DW with decreased CO_2, non-nutrient enriched and 70%$_{PAB}$ conditions (Table 2, Table S1). The nitrogen content of *C. compressa* was greatest in the high CO_2, nutrient enriched and 70%$_{PAB}$ treatment (Figure 2A, Figure S1); conversely, in *P. pavonica* the nitrogen content was highest at the reference site, ambient CO_2 treatment (Figure 2B, Figure S1). The ratio C:N of *C. compressa* did not show significant differences between treatments (Figure 3A, Figure S1), whereas the ratio in *P. pavonica* showed significant effects for CO_2 levels and nutrient enrichment showed maximal values (19.5 ± 5.8) with increased CO_2, non-nutrient enriched in 100%$_{PAB}$ conditions and minimal values 15.9 ± 0.5 in medium CO_2, nutrient enrichment and 70%$_{PAB}$ conditions (Figure 3B, Figure S1).

The maximal quantum yield (F_v/F_m) was significantly different between CO_2 treatments, nutrient and irradiance in both macroalgae (Figure 4, Figure S2). In *C. compressa*, the F_v/F_m was greatest in 70%$_{PAB}$ treatments with high CO_2, and non-enriched enrichment (Figure 4A, Figure S2), but in *P. pavonica*

TABLE 2 | Carbon content, photosynthetic efficiency (α_{ETR}), maximal electron transport rate (ETR_{max}), expressed in µmol m^{-2} s^{-1}), irradiance of saturation of ETR (Ek_{ETR}), maximal non-photochemical quenching (NPQ_{max}) (mean values ± SE, $n = 3$) of *Cystoseira compressa* and *Padina pavonica* in relation to Irradiance (70%$_{PAB}$: low irradiance and 100%$_{PAB}$: ambient irradiance), Nutrients (Nutrient+ and Ambient Nutrient) and CO_2 (ambient CO_2 site: 500 µatm, Medium CO_2 site: 700–800 µatm and High CO_2: 1200 µatm) treatments.

		Cystoseira compressa					*Padina pavonica*				
		It	**Nutrient+**		**Ambient nutrient**		***It***	**Nutrient+**		**Ambient nutrient**	
			70%$_{PAB}$	***100%$_{PAB}$***	***70%$_{PAB}$***	***100%$_{PAB}$***		***70%$_{PAB}$***	***100%$_{PAB}$***	***70%$_{PAB}$***	***100%$_{PAB}$***
Carbon	*Ambient CO_2*	264.3 ± 8.4	266.3 ± 5.1	271.4 ± 2.7	261.7 ± 5.5	266.3 ± 7.6	274.0 ± 13.7	276.9 ± 5.9^{c}	254.8 ± 3.3^{bc}	225.3 ± 2.4^{a}	263.7 ± 0.8^{bc}
	Medium CO_2		253.2 ± 11.4	251.7 ± 3.4	252.2 ± 1.9	261.9 ± 5.2		240.0 ± 5.6^{ab}	243.0 ± 9.4^{ab}	226.8 ± 7.2^{a}	256.4 ± 7.4^{bc}
	High CO_2		276.2 ± 2.7	259.6 ± 8.6	273.2 ± 6.6	273.2 ± 6.6		271.1 ± 4.3^{c}	257.5 ± 4.4^{bc}	279.9 ± 6.5^{c}	275.7 ± 2.3^{c}
α_{ETR}	*Ambient CO_2*	0.31 ± 0.02	0.39 ± 0.02^{bc}	0.25 ± 0.06^{ab}	0.32 ± 0.07^{abc}	0.34 ± 0.01^{abc}	0.34 ± 0.01	0.28 ± 0.01^{b}	0.15 ± 0.02^{ab}	0.09 ± 0.02^{a}	0.27 ± 0.05^{b}
	Medium CO_2		0.32 ± 0.02^{abc}	0.34 ± 0.01^{abc}	0.20 ± 0.03^{a}	0.33 ± 0.01^{abc}		0.25 ± 0.02^{b}	0.23 ± 0.03^{b}	0.15 ± 0.03^{ab}	0.24 ± 0.03^{b}
	High CO_2		0.21 ± 0.02^{a}	0.40 ± 0.02^{bc}	0.43 ± 0.01^{c}	0.24 ± 0.03^{ab}		0.28 ± 0.02^{b}	0.19 ± 0.02^{ab}	0.23 ± 0.02^{b}	0.24 ± 0.02^{b}
ETRmax	*Ambient CO_2*	46.8 ± 5.3	55.5 ± 15.3^{a}	65.2 ± 13.1^{abc}	85.1 ± 9.2^{bc}	112.4 ± 12.9^{c}	51.8 ± 2.0	41.7 ± 1.7	49.0 ± 1.5	49.6 ± 3.8	57.9 ± 7.5
	Medium CO_2		60.1 ± 13.4^{abc}	86.2 ± 2.0^{bc}	67.0 ± 9.8^{abc}	73.8 ± 11.7^{abc}		42.4 ± 6.3	60.2 ± 6.9	48.6 ± 9.5	57.1 ± 3.7
	High CO_2		32.5 ± 7.9^{a}	101.1 ± 7.9^{bc}	95.1 ± 12.0^{bc}	63.7 ± 8.1^{abc}		42.5 ± 2.9	59.0 ± 3.8	64.4 ± 12.3	73.4 ± 5.5
Ek_{ETR}	*Ambient CO_2*	152.7 ± 6.8	139.9 ± 35.1	261.8 ± 33.8	281.5 ± 40.6	324.2 ± 31.3	150.1 ± 4.0	149.3 ± 8.3^{a}	339.5 ± 34.5^{cd}	416.7 ± 11.6^{d}	218.7 ± 17.9^{abc}
	Medium CO_2		187.1 ± 32.5	252.9 ± 5.0	337.6 ± 7.0	227.1 ± 42.8		174.9 ± 25.6^{ab}	262.2 ± 17.8^{abc}	329.9 ± 32.4^{cd}	319.7 ± 71.0^{bcd}
	High CO_2		154.1 ± 15.9	255.1 ± 33.1	221.9 ± 30.2	278.5 ± 62.7		156.1 ± 25.3^{a}	323.2 ± 29.9^{bcd}	224.8 ± 40.7^{abc}	313.6 ± 31.9^{bcd}
NPQmax	*Ambient CO_2*	1.61 ± 0.33	2.14 ± 0.58^{b}	0.48 ± 0.04^{a}	0.62 ± 0.02^{a}	3.24 ± 0.31^{c}	1.95 ± 0.39	1.91 ± 0.36^{bc}	0.31 ± 0.09^{a}	0.12 ± 0.02^{a}	1.97 ± 0.33^{bc}
	Medium CO_2		3.65 ± 0.33^{c}	1.40 ± 0.15^{ab}	0.47 ± 0.20^{a}	0.57 ± 0.10^{a}		3.32 ± 0.44^{d}	1.28 ± 0.33^{abc}	0.70 ± 0.13^{ab}	0.72 ± 0.01^{ab}
	High CO_2		2.21 ± 0.29^{b}	3.69 ± 0.19^{c}	1.84 ± 0.22^{b}	0.59 ± 0.17^{a}		2.39 ± 0.31^{c}	0.91 ± 0.18^{ab}	1.46 ± 0.52^{abc}	0.68 ± 0.13^{ab}

It: Initial time of the experimental period is shown in the first column. Lower-case letters denote significant differences after SNK test.

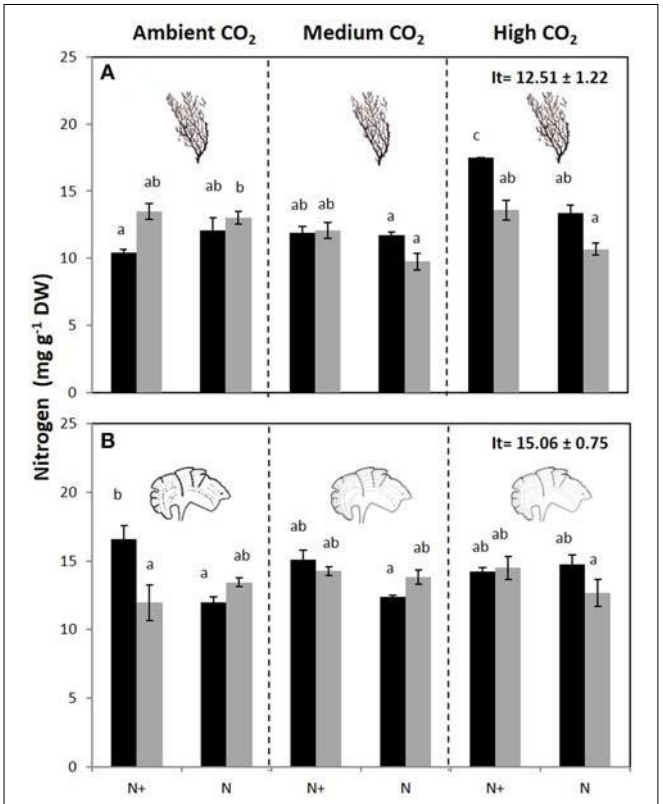

FIGURE 2 | Nitrogen content (mg g^{-1} DW) (mean ± SE, $n = 3$) in (A) *Cystoseira compressa* and (B) *Padina pavonica* at Ambient CO_2 (*ca* 500 µatm, left boxes), Medium CO_2 (*ca* 700–800 µatm, middle boxes) and High CO_2 (*ca* 1200 µatm, right boxes), in Irradiance and Nutrient treatments. Dark bars indicate 70%$_{PAB}$ (low irradiance), gray bars indicate 100%$_{PAB}$ (ambient irradiance). N+ and N indicate nutrient enriched and non-enriched treatments respectively. Upper values in right box indicate initial time values (It: mean ± SE, $n = 3$). Lower-case letters denote significant differences after an SNK test.

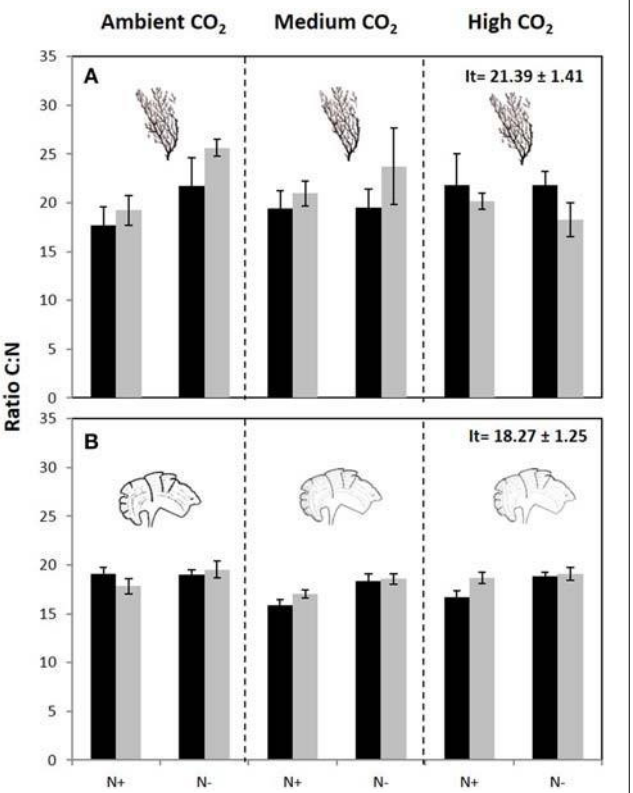

FIGURE 3 | C:N ratio (mean ± SE, $n = 3$) in (A) *Cystoseira compressa* and (B) *Padina pavonica* at Ambient CO_2 (*ca* 500 µatm, left boxes), Medium CO_2 (*ca* 700–800 µatm, middle boxes) and High CO_2 (*ca* 1200 µatm, right boxes), in Irradiance and Nutrient treatments. Dark bars indicate 70%$_{PAB}$ (low irradiance), gray bars indicate 100%$_{PAB}$ (ambient irradiance). N+ and N indicate nutrient enriched and non-enriched treatments respectively. Upper values in right box indicate initial time values (It: mean ± SE, $n = 3$).

this was greatest in the nutrient enriched treatments (Figure 4B, Figure S2). The α_{ETR} values also varied significantly between treatments in both species (Table 2, Table S2). In *C. compressa,* α_{ETR} was greatest in 70%$_{PAB}$ treatments at high CO_2 with non-enriched enrichment; in *P. pavonica* α_{ETR} was greatest in the high CO_2 conditions (Table 2, Table S2). ETR_{max} in *C. compressa* was highest in high CO_2, 70%$_{PAB}$ and non-nutrient enrichment, also in 100%$_{PAB}$ and nutrient enrichment, and also this was higher with decreased CO_2, 100%$_{PAB}$, in non-nutrient enrichment. In *P. pavonica*, ETR_{max} varied significantly depending on nutrient and irradiance, without interactions (Table 2, Table S2). In contrast, the Ek_{ETR} in *C. compressa* had one significant interaction among nutrient x irradiance. *P. pavonica* had significant interactions between CO_2 level, nutrient and irradiance. The Ek_{ETR} in *C. compressa*, was greatest in the 100%$_{PAB}$ treatments that had no CO_2 or nutrient enrichment, but in *P. pavonica* Ek_{ETR} was greatest in 70%$_{PAB}$ conditions (Table 2, Table S2). In both species, the maximal non-photochemical quenching (NPQ_{max}) was affected by the interaction of all factors. In *C. compressa*, NPQ_{max} increased significantly with increasing CO_2 conditions, under nutrient enriched and 100%$_{PAB}$, also increased in *ca* 700–800 µatm but in 70%$_{PAB}$. As well as, NPQ_{max} increased under ambient CO_2 conditions in 100%$_{PAB}$ in nutrient non-enriched. Finally, in *P. pavonica,* the NPQ_{max} was significantly higher in 70%$_{PAB}$ at 700 µatm CO_2 treatment with nutrient enrichment (Table 2, Table S2).

Nutrient enrichment increased Chl*a* significantly in *C. compressa*. In contrast, in *P. pavonica* significant differences were found for the following interactions: CO_2 level x nutrient, CO_2 level × irradiance and nutrient × irradiance (Table 3, Table S3). The same occurred for Chl*c* in *P. pavonica*; but there was no significant difference in *C. compressa* (Table 3, Table S3). The carotenoids, fucoxanthin and violaxanthin in *C. compressa* did not differ among factors (Table 3, Table S3) but in *P. pavonica* the fucoxanthin and violaxanthin contents were affected by the interaction of all factors. Fucoxanthin increased in 70%$_{PAB}$, non-enriched treatments in ambient CO_2 whereas violoxanthin levels were highest in 70%$_{PAB}$, *ca* 700–800 µatm CO_2, nutrient enriched treatment (Table 3, Table S3).

Phenolic content (PC) was affected by the interaction of all factors in both species (Figure 5, Figure S4). In *C. compressa*, PC

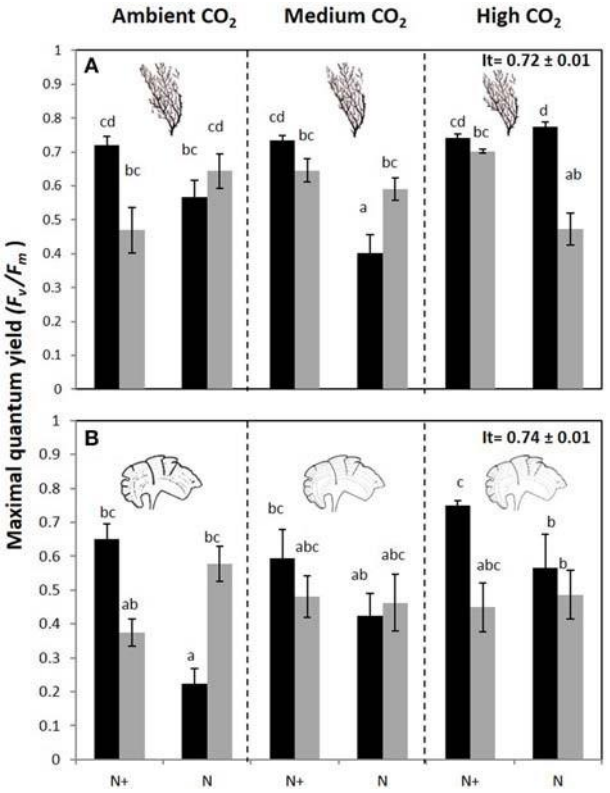

FIGURE 4 | Maximal quantum yield or F_v/F_m (mean ± SE, $n = 3$) in (A) *Cystoseira compressa* and (B) *Padina pavonica* at Ambient CO_2 (*ca* 500 µatm, left boxes), Medium CO_2 (*ca* 700–800 µatm, middle boxes) and High CO_2 (*ca* 1200 µatm, right boxes), in Irradiance and Nutrient treatments. Dark bars indicate 70%$_{PAR}$ (low irradiance), and gray bars indicate 100%$_{PAB}$ (ambient irradiance). N+ and N indicate nutrient enriched and non-enriched treatments respectively. Upper values in right box indicate initial time values (It: mean ± SE, $n = 3$). Lower-case letters denote significant differences after an SNK test.

was highest in CO_2 and nutrient enriched conditions (Figure 5A, Figure S4). In *P. pavonica* at 1200 µatm CO_2, PC was high in 100%$_{PAB}$ and nutrient enriched treatments and in 70%$_{PAB}$ treatments non-nutrient enrichment (Figure 5B, Figure S4). Antioxidant activity (EC_{50}) showed a significant interaction between CO_2 level × nutrient and CO_2 level × irradiance in *C. compressa*; however in *P. pavonica* the only significant difference found in antioxidant activity was between CO_2 level and irradiance. In *C. compressa* and *P. pavonica*, EC_{50} was lowest (i.e., it had higher antioxidant activity) in the high CO_2, 70%$_{PAB}$ light conditions and nutrient enriched treatments (Table 3, Table S4).

5.5 Discussion

Recent reviews surmise that ocean acidification is likely to increase macroalgal productivity due to beneficial effects of increased dissolved inorganic carbon (DIC) levels which can stimulate the growth of algae and allows them to divert more resources into anti-herbivore and photo-protective compounds (Harley et al., 2012; Brodie et al., 2014). Here we show that calcified and non-calcified macroalgae can indeed benefit physiologically from increases in DIC, but that the benefits, and the extent of the algal response, depend upon nutrient and light availability. Figure 6 summarizes our projections that brown macroalgal stands will both proliferate in the shallows (because of up-regulation of anti-herbivore and photo-protective compounds) and extend deeper due to a combination of ocean acidification and anthropogenic nutrient input, whereas other work on Mediterranean CO_2 seeps has established that sea urchins and coralline algae are adversely affected by acidification (Baggini et al., 2014). *In vivo* chlorophyll *a* fluorescence parameters (maximal quantum yield or F_v/F_m and maximal electron transport rate or ETR_{max}) and algal biochemical composition (Chl*a*, total phenolic compounds and antioxidant activity, %C) helps explain the dominance of phaeophytes at a variety of coastal Mediterranean CO_2 seeps. Increases in brown macroalgal cover at CO_2 seep sites are probably due to a combination of the direct stimulus of increased DIC for photosynthesis for species with inefficient carbon concentrating mechanisms (CCMs), and decreased grazing since sea urchins for example are excluded by hypercapnia (Calosi et al., 2013).

Other Mediterranean seep locations show similar trends to Vulcano, with increases in *Cystoseira* and *Padina* species at elevated CO_2 locations compared to reference locations (Johnson et al., 2012; Baggini et al., 2014). Work in other regions has also shown that ocean acidification can directly benefit some macroalgae, such as *Gracilaria lemaneiformis* in China (Zou and Gao, 2009) and mat-forming *Feldmannia* spp. in Australia (Russell et al., 2011), as well as canopy-forming phaeophytes such as *Nereocystis luetkeana* and *Macrocystis pyrifera* (Swanson and Fox, 2007; Roleda et al., 2012). We found that the benefits of increased DIC were even more pronounced when combined with increased nutrients. This is what we expected, given that macroalgae tend to be nutrient-limited in oligotrophic waters such as those of the Mediterranean Sea (Ferreira et al., 2011). Both our study species increased electron transport rates and the accumulation of photoprotectors when exposed to a Nitrogen Phosphorus Potassium fertilizer, but these were short-term experiments with macroalgae grown in isolation. We suspect that chronic eutrophication combined with ocean acidification may benefit more opportunistic algal groups, to the detriment of brown macroalgae based on research by Russell et al. (2009) and Falkenberg et al. (2013). In our study, *C. compressa* and *P. pavonica* had increased carbon content at elevated CO_2, which was augmented by increases in a range of other physiological parameters when nutrient levels were also increased. The F_v/F_m ratio was highest at increased CO_2 concentrations with no nutrient enrichment in *C. compressa*, but highest at increased CO_2 with nutrient enrichment for *P. pavonica* (Figure 4). The maximal photosynthetic activity (ETR_{max}) in *C. compressa* was reduced at high nutrient levels in shaded conditions but in fully lit conditions nutrients did not have significant effects under high DIC conditions. In other *Cystoseira* species, such as *C. tamariscifolia*, both F_v/F_m and ETR_{max} also decrease in nutrient enriched treatments in field experiments at various

TABLE 3 | Pigment contents; Chlorophyll *a* (Chla), chlorophyll *c* (Chl*c*), Fuxocanthin and Violaxanthin contents are expressed in mg g^{-1} DW and Antioxidant activity expressed as EC_{50} in mg DW mL^{-1} (mean values ± SE, $n = 3$) of *Cystoseira compressa* and *Padina pavonica* in relation to Irradiance (70%$_{PAB}$: low irradiance and 100%$_{PAB}$: ambient irradiance), Nutrient (Nutrient+ and Ambient Nutrient) and CO_2 (ambient CO_2 site: 500 µatm, Medium CO_2 site:700-800 µatm and High CO_2: 1200 µatm) treatments.

		Cystoseira compressa					*Padina pavonica*				
		It	**Nutrient+**		**Ambient nutrient**		***It***	**Nutrient+**		**Ambient nutrient**	
			70%$_{PAB}$	***100%$_{PAB}$***	***70%$_{PAB}$***	***100%$_{PAB}$***		***70%$_{PAB}$***	***100%$_{PAB}$***	***70%$_{PAB}$***	***100%$_{PAB}$***
Chla	*Ambient CO_2*	1.19 ± 0.17	1.41 ± 0.21	1.29 ± 0.27	0.86 ± 0.16	0.86 ± 0.36	0.80 ± 0.08	0.78 ± 0.05	0.70 ± 0.04	0.23 ± 0.03	0.67 ± 0.01
	Medium CO_2		1.61 ± 0.13	1.32 ± 0.11	1.09 ± 0.25	1.34 ± 0.28		0.95 ± 0.11	0.41 ± 0.08	0.57 ± 0.10	0.69 ± 0.02
	High CO_2		1.67 ± 0.18	1.65 ± 0.47	1.06 ± 0.25	1.42 ± 0.41		0.77 ± 0.40	0.32 ± 0.08	0.75 ± 0.03	0.75 ± 0.08
Chlc	*Ambient CO_2*	0.19 ± 0.09	0.45 ± 0.22	0.61 ± 0.23	0.52 ± 0.22	0.21 ± 0.07	0.86 ± 0.15	0.04 ± 0.02	0.09 ± 0.03	0.35 ± 0.10	0.04 ± 0.01
	Medium CO_2		0.08 ± 0.01	0.15 ± 0.06	0.04 ± 0.01	0.07 ± 0.01		0.09 ± 0.02	0.07 ± 0.01	0.35 ± 0.08	0.11 ± 0.06
	High CO_2		0.11 ± 0.04	0.52 ± 0.22	0.49 ± 0.27	0.39 ± 0.16		0.08 ± 0.01	0.46 ± 0.02	0.08 ± 0.01	0.08 ± 0.06
Fucoxanthin	*Ambient CO_2*	2.91 ± 0.13	0.44 ± 0.10	0.40 ± 0.12	0.38 ± 0.02	0.39 ± 0.05	0.83 ± 0.09	0.19 ± 0.04^{a}	0.26 ± 0.01^{ab}	1.32 ± 0.06^{c}	0.20 ± 0.04^{a}
	Medium CO_2		0.55 ± 0.12	0.47 ± 0.02	0.49 ± 0.13	0.52 ± 0.06		0.46 ± 0.10^{b}	0.18 ± 0.06^{a}	0.36 ± 0.06^{ab}	0.22 ± 0.01^{ab}
	High CO_2		0.66 ± 0.02	0.46 ± 0.04	0.39 ± 0.13	0.55 ± 0.21		0.27 ± 0.08^{ab}	0.29 ± 0.01^{ab}	0.28 ± 0.01^{ab}	0.26 ± 0.02^{ab}
Violaxanthin	*Ambient CO_2*	0.08 ± 0.01	0.07 ± 0.01	0.06 ± 0.02	0.06 ± 0.01	0.06 ± 0.01	0.16 ± 0.01	0.05 ± 0.01^{a}	0.05 ± 0.01^{a}	0.05 ± 0.02^{a}	0.05 ± 0.01^{a}
	Medium CO_2		0.08 ± 0.02	0.07 ± 0.01	0.12 ± 0.02	0.08 ± 0.01		0.15 ± 0.01^{b}	0.03 ± 0.01^{a}	0.06 ± 0.02^{a}	0.04 ± 0.01^{a}
	High CO_2		0.11 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.03		0.05 ± 0.01^{a}	0.06 ± 0.01^{a}	0.06 ± 0.01^{a}	0.02 ± 0.01^{a}
EC_{50}	*Ambient CO_2*	0.56 ± 0.02	0.93 ± 0.11	0.75 ± 0.07	0.94 ± 0.13	0.71 ± 0.06	0.75 ± 0.10	0.86 ± 0.13	0.84 ± 0.19	1.27 ± 0.14	1.00 ± 0.02
	Medium CO_2		0.89 ± 0.01	0.84 ± 0.07	0.73 ± 0.07	0.89 ± 0.09		0.80 ± 0.09	0.92 ± 0.18	0.64 ± 0.09	1.24 ± 0.06
	High CO_2		0.53 ± 0.09	0.77 ± 0.02	0.77 ± 0.09	1.19 ± 0.19		0.71 ± 0.15	0.82 ± 0.10	1.00 ± 0.13	0.87 ± 0.03

It: Initial time of the experimental period is shown in the first column. Lower-case letters denote significant differences after SNK test.

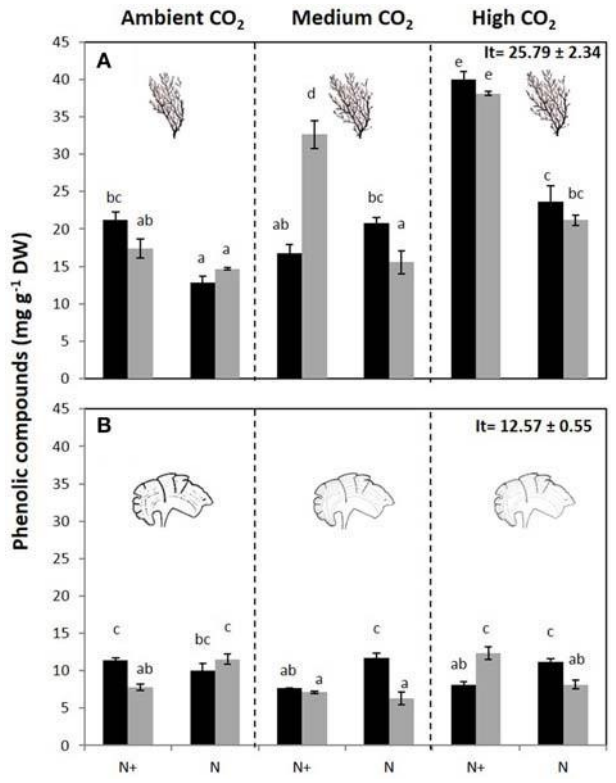

FIGURE 5 | Total phenolic compounds (mg g^{-1} DW) (mean ± SE, $n = 3$) in (A) *Cystoseira compressa* and (B) *Padina pavonica* from Ambient CO_2 (*ca* 500 µatm, left boxes), Medium CO_2 (*ca* 700–800 µatm, middle boxes) and High CO_2 (*ca* 1200 µatm, right boxes), in Irradiance and Nutrient treatments. Dark bars indicate 70%$_{PAB}$ (low irradiance), gray bars indicate 100%$_{PAB}$ (ambient irradiance). N+ and N indicate nutrient enriched and non-enriched treatments respectively. Upper values in right box indicate initial time values (It: mean ± SE, $n = 3$). Lower-case letters denote significant differences after an SNK test.

depths (Celis-Plá et al., 2014a). In another experiment, Celis-Plá et al. (2014b) found the highest ETR_{max} in *C. tamariscifolia* in thalli with the lowest internal nitrogen stores i.e., winter compared to summer grown algae. On the other hand, Ek_{NPQ} increased in all cases with the increased CO_2 as an acclimation to high light levels. On this basis, it is clear that the responses of coastal macroalgal communities to ocean acidification will depend on nutrient availability, and will be species-specific. Given these results we expect that in temperate waters, brown algae will benefit from increases in CO_2 if sufficient nutrients are available (Johnson et al., 2012). However, as with all ecology, we can expect that there will be a region-specific balancing act. We show here that in oligotrophic conditions brown macroalgae were unable to take full advantage of increased inorganic carbon availability. There is added complexity when we consider that many regions have experienced a die-back of canopy-forming brown algae due to excess nutrients or sedimentation (Strain et al., 2014); ocean acidification may exacerbate this problem since increased DIC may further benefit those algae that presently compete with fucoids and kelps in eutrophic conditions (Connell et al., 2013).

Light quantity and quality drive physiological processes in macroalgae (Hanelt and López-Figueroa, 2012), so we were not surprised to find that shading affected their responses to ocean acidification. We anticipated two outcomes of the effects of light: we expected ETR rates to be higher as the most obvious response to light, but we also expected low-light macroalgae to increase ETR rates and %C when transplanted to higher CO_2 concentrations. Our first expectations were met, as maximum quantum yield, photosynthetic efficiency, irradiance of saturation and non-photochemical quenching for chlorophyll fluorescence all increased at higher light levels and were, at times, amplified by increasing CO_2 and nutrient levels. The only instance where our second expectation was met was for *P. pavonica* under ambient nutrients, which had significantly higher %C (and non-significantly higher ETR_{max}) when transplanted to elevated CO_2 sites. Previous studies at the same sites found elevated ETR_{max} when comparing *P. pavonica* at an elevated CO_2 site compared to an ambient CO_2 site (Johnson et al., 2012). If the duration of our experiment had been longer, our transplanted *P. pavonica* may also have significantly increased their ETR_{max}. Our results emphasize the likelihood that ocean acidification will act upon primary production differently at different latitudes and depths, not always according to our expectations. This is important since increases in land nutrient run-off, due to changes in land use and/or rainfall, are altering light levels in coastal waters (Scherner et al., 2013).

One of the most important photoprotective mechanisms available to algae is an ability to dissipate excess thermal energy (Adams et al., 2006). Thermal dissipation measured as non-photochemical PSII fluorescence quenching (NPQ) is triggered by the trans-thylakoidal proton gradient (ΔpH) and zeaxanthin (ZEA) synthesis through the xanthophyll cycle (Gilmore et al., 1994) and is recognized as the most important photoprotective mechanisms in higher plants and several algal divisions (Rodrigues et al., 2002). Fucoxanthin and violaxanthin levels were not affected in *C. compressa* whereas in *P. pavonica* fucoxanthin and violaxanthin increased under 70%$_{PAB}$ conditions, nutrient enrichment and medium CO_2 levels. We used NPQ_{max} as an indicator of photoprotective energy dissipation efficiency (Celis-Plá et al., 2014b), and we also measured phenolic content and antioxidant activity (EC_{50}), both of which can be used as photoprotectors (Celis-Plá et al., 2014a). In *C. compressa* and *P. pavonica* NPQ_{max} was higher in all shaded treatments with nutrient enrichment, but not in the fully lit treatments, indicating higher photoprotection when nutrients were elevated and light was reduced. Phenols usually accumulated under higher irradiance and (for *C. compressa*) higher CO_2 treatments, as per past studies on kelp grown at high CO_2 (Swanson and Fox, 2007), or measured under higher irradiance (Connan et al., 2004). However, the effects of CO_2 on autotroph phenol production are not straight forward, as previous work has shown that both seagrass (Arnold et al., 2012) and the macroalga *Cystoseira tamariscifolia* (Figueroa et al., 2014b) decrease phenol production when CO_2 increased. In *C. compressa* and *P. pavonica*, antioxidant activity and EC_{50} were affected by the interactions between light levels and CO_2. EC_{50} tended to be higher in shaded, high CO_2

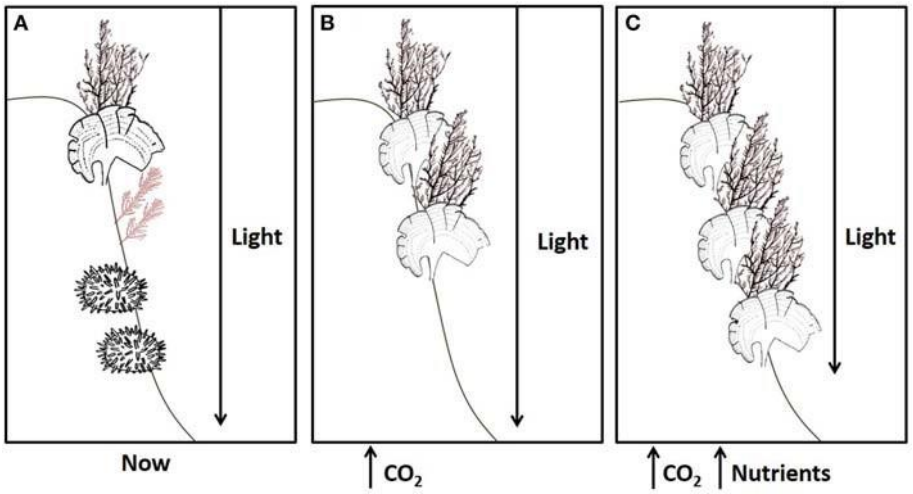

FIGURE 6 | The effects of ocean acidification on rocky shore communities will depend on nutrient levels. (A) A typical brown algal canopy with coralline algae and sea urchins at present-day levels of CO_2, nutrients and light. **(B)** Increased CO_2 in oligotrophic conditions has adverse impacts on most calcified organisms, such as coralline algae and sea urchins, yet dense brown algal stands persist at shallow depths despite corrosion of facultatively calcified algae, such as Padina. **(C)** Ocean acidification remains detrimental to most calcified organisms, but when this is combined with nutrient enrichment fleshy algae can flourish since they are able to take full advantage of increases in CO_2, extending deeper into the photic zone despite increased turbidity.

treatments with and without nutrient addition, suggesting a positive correlation with phenolic compounds and their use as antioxidants to prevent photodamage. Together, NPQ_{max}, phenol production and EC_{50} indicate that in elevated CO_2 conditions some species will have a higher capacity for photoprotection.

Macroalgae regulate their biochemical composition to changes in solar radiation (Bischof et al., 2006; Figueroa et al., 2014a,b). Whilst light obviously affects photosynthesis, other variables such pH, nutrients and the availability of different DIC species all have the potential to affect photosynthetic rates (Raven and Beardall, 2014). As interactions among such factors will determine the success of algal species and the amount of primary productivity in any time and place, it is crucial to know how the effects of ocean acidification are modified by other key drivers of photosynthesis. Research similar to our study, but with more species, in more locations and for longer durations, is clearly required before solid conclusions can be made with respect to the effects of ocean acidification on macroalgal productivity.

***5.6 In conclusion*, our study shows that ongoing ocean acidification can be expected to increase photosynthetic efficiency and algal productivity. The magnitude of these effects, and the species that benefit, will depend on light and nutrient levels. We show that *C. compressa* and *P. pavonica* are able to benefit from an increase in CO_2 levels, rapidly changing their physiology and biochemical composition over 3 day alterations in DIC, irradiance and nutrients. These factors had interactive effects on photosynthetic and photoprotective systems in both species and help explain why brown algae proliferate at CO_2 seeps. Longer-term growth studies involving algal interactions would be useful: we remain concerned that chronic eutrophication combined with ocean acidification may benefit more opportunistic algal groups to the detriment of canopy-forming brown macroalgae. As ocean acidification is not happening in isolation, but alongside a plethora of other anthropogenic changes, an understanding of the interactive effects of multiple stressors is critical to plan for global ocean change. We have shown that elevated CO_2 levels can enhance brown algal productivity, and may boost the kelp and fucoid forests of the planet, but the effects will depend upon interactions with other physicochemical parameters such as light and nutrient availability.**

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmars.2015.00026/abstract

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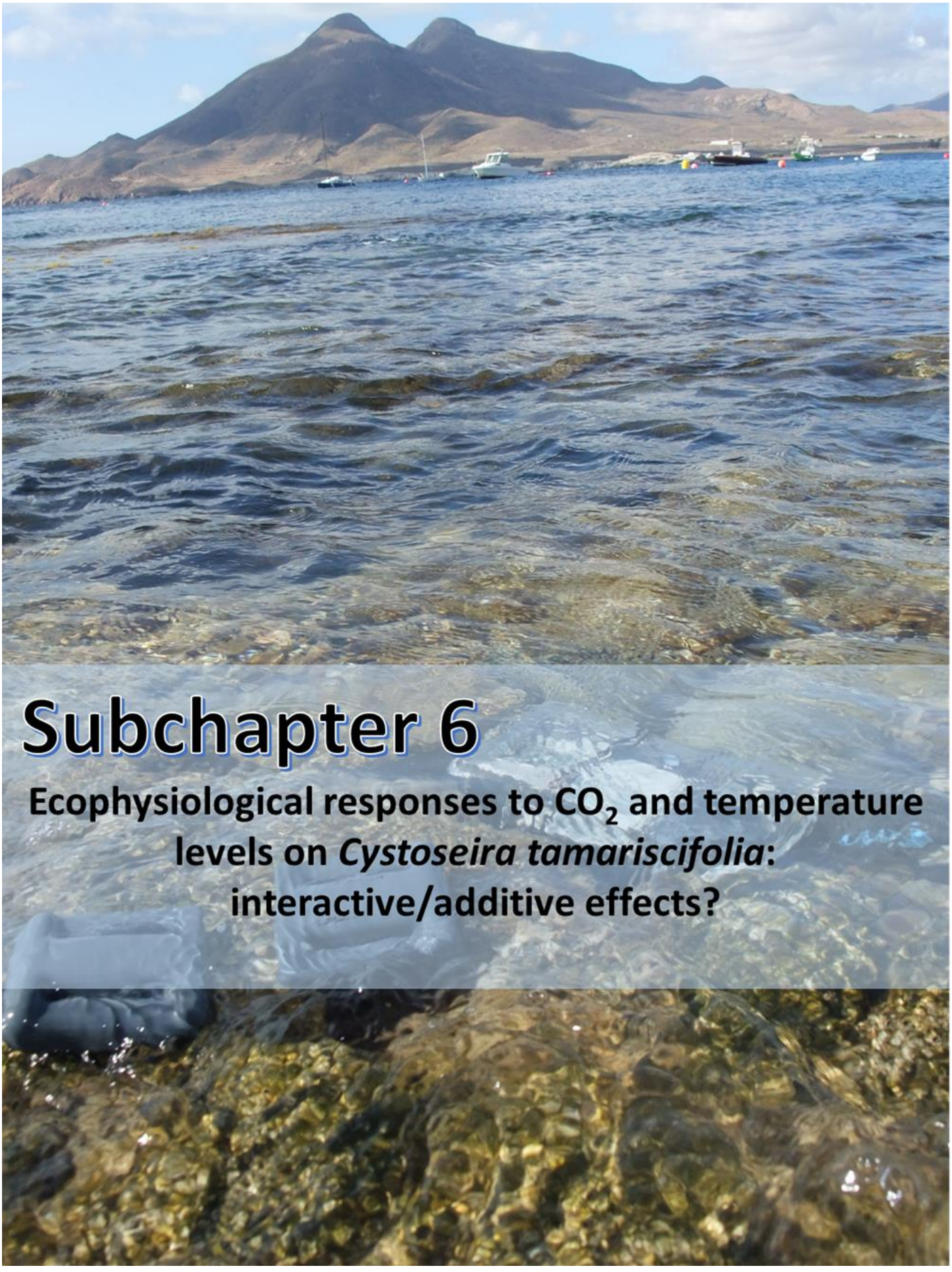
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.



Subchapter 6

Ecophysiological responses to CO_2 and temperature levels on *Cystoseira tamariscifolia*: interactive/additive effects?

Cabo de Gata-Níjar, Natural Park. Septiembre 2013. Photograph by Félix López Figueroa

Photophysiological responses to CO_2 and temperature levels on *Cystoseira tamariscifolia* (Phaeophyceae): interactive/additive effects?

6.1 ABSTRACT

This study investigates the interactive/additive effects of the combinations of two levels of CO_2 (*ca* 400-500 and 1000-1200 µatm) and temperature: ambient temperature (T) and ambient T +4ºC (T+4ºC) on the photophysiological responses of the macroalga *Cystoseira tamariscifolia* (Hudson) Papenfuss (Phaeophyceae) collected from two different areas of Alboran Sea in the western area of Mediterranean Sea, Cabo de Gata-Nijar Natural Park and Malaga (Southern Spain), ultraoligotrophic and oligotrophic waters, respectively. A suite of biochemical assays and estimation of photosynthesis as *in vivo* chlorophyll *a* fluorescence showed that increased CO_2 levels benefited algae of both collection sites. Growth increased in elevated CO_2 treatments and this was higher in algae harvested in ultra-oligotrophic than that from oligotrophic waters. Maximal electron transport rate (ETR_{max}) as estimator of photosynthetic capacity increased under elevated CO_2 level in ambient temperature in both algal population. A positive correlation between ETR_{max} with Chl*a* and Chl*c* was found. The antioxidant activity (i.e. lowest EC_{50}), carbon and nitrogen internal content, were higher in high CO_2 conditions and ambient temperature. The highest phenol content in algae from both sites was observed under enriched CO_2 and ambient temperature conditions being the concentration higher in algae from ultra-oligotrophic waters (1.5 - 3.0 %) than in oligotrophic waters (1.0 - 2.2 %). However, the contents of carotenoids (fucoxanthin, violaxanthin and β-carotene) were higher in oligotrophic than that from ultraoligotrophic algae after 28 d culture. However, the level of the main carotenoid Fucoxanthin respect to Chlorophyll (Fucoxanthin: Chl*a* ratio) was similar after 28 de culture in both collected algae, except under high temperature and high CO_2. In this last conditions the ratio was lower in oligotrophic than that in ultraoligotrophic collected algae indicating a decrease of photoprotection capacity in algae form oligotrophic waters. Nutrient levels in the grown site seem to affect the level of the main compounds for potential photoprotection i.e. phenols in ultraoligotrophic and carotenoids in oligotrophic waters.

Our study shows that ongoing ocean acidification can increase algal productivity (ETR_{max}), the antioxidant activity (EC_{50}) and the content of photoprotectors (PC and carotenoids) and the macroalgae acclimate to alterations in DIC and temperature ambient

as interactive pattern. Macroalgae from ultraoligotrophic presented more effective acclimation at biochemical level to the increase of both CO_2 and temperature since both Fucoxanthin: Chl*a* ratio and phenol levels and antioxidant activity increased under the increase of CO_2 and temperature.

Keywords: Antioxidant activity, carotenoids, *Cystoseira tamariscifolia, In vivo* chlorophyll a fluorescence, phenolic compounds, ocean acidification, temperature.

6.2 INTRODUCTION

Global-scale scenarios such as ocean warming and acidification manifest their influence at local scales and their impacts are increasing in the coastal ecosystems worldwide due the anthropogenic pressure as result of increase of human population (Harley et al. 2006, Halpern et al. 2008, Russell and Connell 2012, Smith and Schindler 2015). The temperature in the seawater has been increasing by 0.13°C per decade over the last 50 years, mainly due to the increase of CO_2 levels in the atmosphere (IPCC 2014). Ocean acidification due to increased atmospheric CO_2 levels is altering the concentrations of dissolved inorganic carbon (DIC) in surface waters; CO_3^{2-} levels are falling, which is expected be detrimental for the organisms calcified whilst CO_2 and HCO_3^- levels are rising which can stimulate photosynthesis and benefit seagrasses and non-calcified algae (Roleda et al. 2012, Connell et al. 2013, Ziveri et al. 2014).

These futures scenarios can affect species growing close to their absolute temperature tolerance limits (Harley et al. 2006). On the other hand, species with higher temperature tolerance will be better able to cope with global warming (Calosi et al. 2008). Brown macroalgal communities and seagrasses are affected by high CO_2 concentrations, either maintaining or accelerating their physiological processes as photosynthesis and nutrient uptake (Figueroa and Gómez 2001, Hall-Spencer et al. 2008, Porzio et al. 2011, Roleda et al. 2012). The changes in the structure of the community can be altered by the ocean acidification due to the decrease of growth rates in marine calcifies (Guinotte and Fabry 2008, Martin and Gattuso 2009), and increase in non-calcifying organisms (Kuffner et al. 2008, Connell and Russell 2010). Consequently, subsequent loss of habitat for many other species can be produced (Olabarria et al. 2012). In the Mediterranean, surveys of coastal CO_2 seeps have shown that brown algae, such as *Cystoseira* spp., *Dictyota* spp., *Sargassum vulgare* and *Padina pavonica*, proliferate as CO_2 and HCO_3^- levels (Porzio et al. 2011, Baggini et al. 2014, Celis-Plá et al. 2015).

Over-enrichment of water by nutrients such as nitrogen and phosphorus has been produced due to sewage discharge. The water quality is affected with two consequences or symptoms of eutrophication in the ocean as hypoxia and harmful algal blooms (Halpern et al. 2008). The effects of environmental degradation are clearly observed in littoral ecosystems of the Mediterranean Sea (Pérez and Vacelet 2014). The habitat-forming or engineer seaweeds are very important to maintain the diversity, structure, functioning and services marine coastal ecosystems in the Mediterranean Sea (Claudet and Fraschetti 2010, Coll et al. 2010, Lotze et al. 2006). The macroalgae of the order Fucales, with habitat forming species as *Cystoseira* spp. (Arévalo et al. 2007, Figueroa et al. 2014a) are suffering a general decline due habitat destruction or degradation both in the Mediterranean and Atlantic waters (Thibaut et al. 2005, Serio et al. 2006, Ferreira et al. 2011). In oligotrophic waters, as Mediterranean Sea, nutrient availability generally limits growth (McQuatters-Gollop et al. 2009, Ferreira et al. 2011), photoprotection mechanisms (Celis-Plá et al. 2014a) and photosynthetic capacity (Pérez-Lloréns et al. 1996, Figueroa et al. 2014a)

The interplay of factors can change the sign of the effect of a single factor (antagonistic effect) or, conversely, accentuate (synergistic effect). Therefore, the analysis of multiple factors acting at different rates and scales has recently become a "hot-point" of research (Xenopoulos et al. 2002, Doyle et al. 2005). Research carried out to study of the interactive effects both on the field and in outdoor experiential systems included variables as temperature, acidification, irradiance, etc. These studies can help to understand and assess the mechanisms of acclimation to global climate change (Villafañe et al. 2003, Wiencke et al. 2004, Figueroa et al. 2009, Figueroa and Korbee 2010, Martínez et al. 2015). Previous studies on the physiological state of macroalgae to stress, have been evaluated by the determination of photoinhibition, photoprotection, assimilation of nutrients, growth patterns, reproduction and morphogenesis (Häder and Figueroa 1997, Villafañe et al. 2003, Stengel et al. 2014, Figueroa et al. 2014b).

Our study centres upon a highly oligotrophic region (Alboran Sea) with input of nutrients by coastal upwelling in spring in the western part (Ramírez et al. 2005). In this region, as with elsewhere in the word, canopy-forming brown algae have undergone a decline abundance due to anthropogenic perturbation (Strain et al. 2014, Yesson et al. 2015). Here, we investigate the interactive effects of increasing CO_2 levels and temperature on *Cystoseira tamariscifolia* collected from two localities: Cabo de Gata-Nijar Natural Park belongs to Southeaster of the Alboran Sea and La Araña beach to

Southwester of the Alboran Sea, ultraoligotrophic and oligotrophic waters, respectively according to the nutrient characteristics (Table 1). The transparency determined by Secchi discs is higher in Cabo de Gata-Nijar (15-20 m) than that in La Araña (9-11 m) (Cortés et al., 2012). These species were chosen because they are abundant in shallow waters in Mediterranean Sea and because *Cystoseira* spp. are indicators of high water quality in Mediterranean. Water bodies of Cabo de Gata-Nijar are classified as high and of La Araña as good ecological status, respectively, based on the application of littoral community assemblages (CARLIT) according to European Water Frame work Directive (WFD2000/60/EC) (Bermejo et al. 2013).

In this study, we compared physiological and biochemical responses to ocean acidification under different temperatures in experimental system described by Stengel et al. (2014). Our hypothesis is that both brown macroalgae would benefit from increased CO_2 because they are sub-saturated for CO_2 related to its low CO_2 conductance (Mercado et al. 1998). On the other hand, the temperature increase can produce negative effects since *Cystoseira tamariscifolia* of Alboran Sea is located in the southern limit of the distribution of this species (Gómez-Garreta et al. 2001). In spite of the importance of these algal communities, the studies on the vulnerability and acclimation to increased temperature are scarce (Serio et al. 2006, Strain et al. 2014). Thus, we expect that increase of CO_2 levels will produce an increase of photosynthetic activity and antioxidant activity in *C. tamariscifolia* only under ambient temperature.

6.3 MATERIAL AND METHODS

Species and harvesting sites

Cystoseira tamariscifolia (Hudson) Papenfuss, (Phaeophyceae, Fucales) (Gómez-Garreta et al. 2001) were randomly collected on September 25th of 2013 in Cabo de Gata-Nijar Natural Park (36º51'N, 2º6'W), eastern (E) and in La Araña (36º42'N, 4º19'W), Málaga, western (W) of Alboran Sea (Figure 1, Celis-Plá et al. 2014a and b). Both macroalgae were collected in low tide from rocky shores (with a high rate of water renewal) and they were transported to the laboratory under cold conditions in order to avoid any damage in the biological material. Samples for biochemical analysis were frozen *in situ* by using liquid nitrogen.

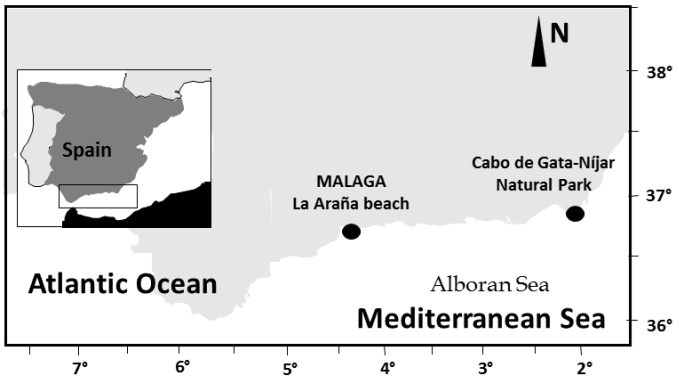


Figure 1. Samples sites of the collected *Cystoseira tamariscifolia* in the Mediterranean Sea (Alboran Sea), in South-west Malaga; La Araña becah and in South-east Cabo de Gata-Nijar Natural Park.

The waters of Cabo de Gata-Nijar Natural Park presented higher surface temperature, lower Chl*a* and nutrient concentration than that in La Araña beach (Figure 1). In addition, Pérez-Rodríguez (2000) reported attenuation constant values (K_d) lower in the eastern than western coast in comparison with the values reported for coastal areas of other regions. The highest nitrate and phosphate concentrations at the surface layer of the Alboran Sea have been registered in the coastal upwelling areas located of Málaga and Estepona in the western sector.

Table 1. Seasonal changes in physical and chemical features of the South-western (W) and South-eastern (E) waters of the Alboran Sea. Salinity (psu), nitrate, ammonium, phosphate, Silicate express as µM, N: P ratio and Chlorophyll *a* (µg L^{-1}) (mean values ± SE, n=180) according to Ramírez et al. (2005) and Mercado et al. (2007 and 2012). *nd*: no data.

Parameters	***Sectors Alboran***	***Spring***	***Summer***	***Autumn***	***Winter***
Tº	*W*	15.91±1.14	18.91±2.09	17.81±1.66	14.88±0.45
	E	13.91±0.16	22.16±2.21	18.30±1.30	14.89±0.36
Salinity	*W*	37.14±0.45	36.87±0.29	36.72±0.34	36.93±0.28
	E	38.22±0.01	36.99±0.22	37.72±0.27	37.42±0.21
Nitrate	*W*	1.59±1.44	0.58±1.07	0.62±0.77	1.52±1.07
	E	0.49±0.55	0.25±0.99	0.32±0.80	1.04±0.73
Ammonium	*W*	0.35±0.20	0.53±0.75	0.19±0.27	0.18±0.10
	E	*nd*	*nd*	*nd*	*nd*
Phosphate	*W*	0.15±0.09	0.12±0.08	0.14±0.01	0.14±0.05
	E	0.11±0.05	0.11±0.17	0.10±0.10	0.10±0.05
Silicate	*W*	1.87±0.78	1.0±0.81	1.15±0.49	1.70±0.92
	E	0.86±0.63	0.62±0.85	0.87±0.63	1.28±0.47
N:P molar ratio	*W*	16.0±21.3	4.3±6.6	7.4±10.9	13.4±12.3
	E	5.8±4.97	3.8±7.3	4.7±5.7	7.7±6.1
Chlorophyll *a*	*W*	1.45±0.99	0.92±0.69	1.21±0.94	1.22±1.14
	E	0.63±0.57	0.38±0.78	0.92±1.03	1.01±0.61

Comparatively, nitrate and phosphate concentrations at the eastern sector coast (i.e. Cabo de Gata-Nijar Natural Park) are lower by 30-40%, on average. Nitrate: phosphate molar ratio is lower than Redfield ratio (16:1) in the coastal areas of both sub-basins (Table 1, *fide* Ramírez et al. 2005, Mercado et al. 2007 and 2012). According to the oceanographic characteristics of the W and E sector, the waters can be classified as oligotrophic and ultra-oligotrophic, respectively according to the classification proposed by OCDE (1982).

Experimental design

The experiment was designed to examine interactive effects of the current pCO_2 (*ca* 400-500 µatm CO_2) and predicted future concentration for the year 2100 (*ca* 1000-1200 µatm CO_2) in a combination with two levels of temperature, ambient temperature and ambient temperature +4ºC. Seawater was enriched with 2 µM of nitrate (KNO_3) and 0.1 µM Phosphate (KH_2PO_4) i.e. Ratio N: P ratio of 20:1. The four treatments were designated as ambient temperature*ambient CO_2 (T*ACO_2), ambient temperature*High CO_2 (T*HCO_2), Ambient temperature +4ºC*High CO_2 (T+4ºC*HCO_2) and ambient temperature +4ºC*High CO_2 (T+4ºC*HCO_2).

The experiments were conducted in the Unit for Microbiology, Ecophysiology and Aquatic Organisms of Malaga University (UMEGOA), located in the Grice-Hutchinson experimental centre. By using a multi-tank system placed under semi-natural solar conditions, i.e. photosynthetically active radiation (PAR; 400-700 nm) was reduced by 35%; UVA (320-400 nm) and UVB (280-320 nm) by 39%, using a neutral green mesh, as reported by Stengel et al. (2014). The experimental system was composed of three open vessels (0.094 m^2 surface area, 14 L volume) per treatment, connected in parallel to a separate tank of 102 L capacity. The water flow between each box and its header tank was 0.84 ± 0.05 L min^{-1}, representing a turnover rate of $26 \pm 1\%$ h^{-1} (according to Stengel et al. 2014). The entire system was placed within 8 tanks of 1000 L with circulating freshwater, which were permanently cooled using 2 cooling units (Titan; Aqua Medic). More details of the experimental system according to Stengel et al. (2014).

Four thalli (25-30 g fresh weight) were randomly assigned in each box for two day of acclimation time under natural conditions. After this time, both *C. tamariscifolia* from Cabo de Gata-Nijar Natural Park (onwards from CG or ultra-oligotrophic waters) and La Araña beach (onwards from LA or oligotrophic waters), were submitted to the treatments

and maintained under these conditions for 28 days. Each treatment had three replicates per combination (T*ACO_2, T*HCO_2, T+4ºC*HCO_2 and T+4ºC*HCO_2, respectively).

Abiotic variables

The seawater-carbonated system was monitored at each week. Water samples for total alkalinity (TA) were filtered through 0.2 µm pore size filters and was measured by titrating. Three replicates, for treatments were analyzed at 25° C using a titration system (877 Titrino plus, Metrohm). The pH was measured at 0.02 ml increments of 0.1 N HCl. Total alkalinity was calculated from the Gran function applied to pH variations from 4.2 to 3.0, from the slope of the curve HCl volume versus pH. The pCO_2 and the saturation state of aragonite were calculated from pHNBS, TA, temperature and salinity with the free-access CO_2 SYS package (Pierrot et al. 2006). Using the constants of carbonic acid in seawater by Mehrbach et al. (1973), refit by Dickson and Millero (1987), and the first dissociation constant of boric acid in seawater by Lyman (1956) were used according to Celis-Plá et al. (2105).

Incident irradiance was monitored continuously in air using an UV-PAR Multifilter radiometer NILU-6 (Geminali AS, Oslo, Norway). The irradiances of UVA and UVB were calculated from the data of the different UV filters according to Høiskar et al. (2003). The nutrient enrichment was assessed taking triplicate seawater samples at all treatments. Seawater was filtered *in situ* using portable GF/F filters (Whatman International. Ltd., Maidstone, UK), transported to the laboratory inside an isotherm bag (4ºC, in darkness), and kept at -20ºC (Martínez et al. 2012). Nitrate (NO_3^-) and phosphate (P) were determined using an automated wet chemistry analyzer (SanPlus++ System, SKALAR, Breda, Netherlands) applying standard colorimetric procedures (Koroleff 1983).

Photophysiological and Biochemical variables

Several physiological and biochemical variables were obtained from the macroalgae at the initial time and during the experimental at the 7, 14, 21 and at the end the experimental period (28 days). These variables were also measurements in *C. tamariscifolia* from Cabo de Gata-Nijar Natural Park and La Araña beach.

(a) Growth

Fronds were blotted dry and weighted immediately before being transferred to the experimental tanks and after the experimental period (28 days). Growth was calculated as in Martínez et al. (2015),

$$Growth = (FW_{t=f} - FW_{t=0}) \cdot day^{-1} \quad (1)$$

Where $FW_{t=f}$ is fresh weight measured to final and $FW_{t=0\ is}$ fresh weight measured before the start of the experiment. Growth, tissue loss and mortality were plotted against the four experimental treatments to show physiological responses.

(b) Internal carbon and nitrogen content

Seaweed samples (1-2 g FW) were dried overnight in an oven at 60ºC and then maintained in a desiccator until analyses. Total internal C and N contents on a dry weight (DW) basis were determined using a CNHS-932 elemental analyzer (Leco Corporation, Michigan, USA).

(c) Photosynthetic activity as* in vivo *chlorophyll a fluorescence

In vivo chlorophyll *a* fluorescence associated with Photosystem II was determined by using a portable pulse amplitude modulated (PAM) fluorometer (Diving-PAM, Walz GmbH, Germany). Macroalgal thalli were collected from natural populations (initial time). In order to obtain rapid light curves (RLC) for each treatment, apical parts of *C. tamariscifolia* were introduce in 10 mL incubation chambers. F_o and F_m were measured after 15 minutes in darkness to obtain the maximum quantum yield (F_v/F_m) being $F_v=F_m-F_o$, F_o the basal fluorescence of 15 min dark adapted thalli and F_m maximal fluorescence after a saturation light pulse of > 4000 µmol m^{-2} s^{-1} (Schreiber et al. 1995). The electron transport rate (ETR) was determined after 20 s exposure in eight increasing irradiances of white light (halogen lamp provided by the Diving-PAM). The ETR was calculated according to Schreiber et al. (1995) as follows:

$$ETR\ (\mu mol\ electrons\ m^{-2}\ s^{-1}) = \Delta F/F'_m \times E \times A \times F_{II} \quad (2)$$

where $\Delta F/F'm$ is the effective quantum yield, being $\Delta F = Fm'-Ft$ (*Ft* is the intrinsic fluorescence of alga incubated in light and *Fm'* is the maximal fluorescence reached after a saturation pulse of algae incubated in light). *E* is the incident PAR irradiance expressed in µmol photons m^{-2} s^{-1}, A is the thallus absorptance as the fraction of incident irradiance that is absorbed by the algae (see Figueroa et al. 2003) and F_{II} is the fraction of chlorophyll related to PSII (400-700 nm) being 0.8 in brown macroalgae (Figueroa et al. 2014a). ETR parameters as maximum electron transport rate (ETR_{max}) and the initial slope of ETR versus irradiance function (α_{ETR}) as estimator of photosynthetic efficiency were obtained from the tangential function reported by Eilers and Peeters (1988).

(d) Phenolic compounds and antioxidant activity (EC_{50})

The content of total phenolic compounds (PC) was determined using 0.25 g fresh weight samples pulverized with a mortar and pestle with sea-sand and 2.5 mL of 80% methanol. After keeping the samples overnight at 4ºC, the mixture was centrifuged at 2253 g for 30 min at 4ºC, and then the supernatant was collected. PC were determined colorimetrically using Folin-Ciocalteu reagent and phloroglucinol (1,3,5-trihydroxybenzene, Sigma P-3502) as standard. Finally, the absorbance was determined at 760 nm using a spectrophotometer (UV Mini-1240, Shimadzu) (Celis-Plá et al. 2014a). Total phenolic content was expressed as mg g^{-1} DW after determining the fresh to dry weight ratio in the tissue (4.3 for *C. compressa* from Cabo de Gata-Nijar Natural Park and 5.6 for *C. tamariscifolia* from La Araña Beach, respectively). The results are expressed as average ± SE from three replicates of each treatment.

Antioxidant activity was measured on polyphenol extracts according to Blois (1958); 150 µL of DPPH (2,2-diphenyl-1-picrylhydrazyil) prepared in 90% methanol were added to each extract. The reaction was complete after 30 min in darkness at ambient temperature (~20°), and the absorbance was read at 517 nm in a spectrophotometer (UVmini-1240, Shimadzu). The calibration curve made from DPPH was used to calculate the remaining concentration of DPPH in the reaction mixture after incubation. Values of DPPH concentration (mM) were plotted against plant extract concentration expressed as the EC_{50} value (mg DW mL^{-1}) required to scavenge 50% of the DPPH in the reaction mixture. Ascorbic acid was used as a control (Celis-Plá et al. 2014a).

The content of polyphenols in the water (PCw) as indicator of phenol release in the seawater was determined by measuring the optical density in a spectrophotometer (UVmini-1240, Shimadzu) at the maximum absorption of polyphenols in the seawater, i.e. 270 nm (Celis-Plá et al. 2014a). The water samples were taken from waters in which *C. tamariscifolia* were growing. The concentration, expressed as mg g^{-1} DW d^{-1}, was obtained using phloroglucinol dissolved in seawater as standard.

(e) Photosynthetic pigments

Pigments were extracted from 20 mg fresh weight of thalli using 2 mL of 100% acetone and analysed using an ultra-high-performance liquid chromatographer (Shimadzu Corp., Kyoto, Japan) equipped with a photodiode array detector to measure peaks in the range 350-800 nm. After extraction, samples were centrifuged at 16200 g for 5 min (Sorvall Legend Micro 17, Thermo Scientific, Langenselbold, Germany) and then the extracts were filtered (0.22 µM nylon filters). The separation, was achieved with one

column C-18 reversed phase (Shim-pack XR-ODS column; 3.0 × 75mm i. d.; 2.2 um particle size; Shimadzu, Kyoto, Japan) protected by a guard column TR-C-160 K1 (Teknokroma, Barcelona, Spain). The carotenoid composition was determined according to García-Plazaola and Becerril (1999) with some modifications (García-Plazaola and Becerril 2001), using commercial standards (DHI LAB Products).

Statistical analysis

The general variation patterns between variables measured in *C. tamariscifolia* were explored using a multivariate approach. A Principal Coordinates Analysis (PCO) was performed for this purpose based on Euclidean distance using PERMANOVA+ for PRIMER6 package (Anderson et al. 2008). This procedure is an equivalent ordination to a PCA and calculates the percentage variation explained by each of the axes in the multidimensional scale. The overlay of the vectors onto the PCO was performed using Spearman correlation. The analyses were performed using PERMANOVA+ for PRIMER6 package (Anderson et al. 2008). Each one of variables was represented by an arrow in the ordination plot pointing to the samples that showed the highest amount of that particular compound and each point in the figure is a replicate.

The effects of the treatments on the photophysiological responses of *C. tamariscifolia* were assessed using analysis of variance. Four fixed factors were considered: Time with four levels: 7, 14, 21 and 28 d, Temperature with two levels; ambient temperature and ambient temperature +4ºC, pCO_2 with two levels; *ca* 400-500 and *ca* 1200-1300 µatm CO_2 and Origin of the population with two levels: Cabo de Gata-Nijar Natural Park and La Araña beach. To ensure the independence of each replica, each thalli was measured once and then was eliminated. This design allowed us to test interactive and additive effects of the variables on the physiological responses. Data used in the analyses were those obtained at during the experimental period. Student Newman Keuls tests (SNK) were performed after significant ANOVA interactions (Underwood 1997). Homogeneity of variance was tested using Cochran tests and by visual inspection of the residuals. All data conformed to homogeneity of variance. Analyses were performed by using SPSS v.21 (IBM, USA).

6.4 RESULTS

Environmental conditions

The seawater temperature during the experiment was about 19ºC; the salinity was 38psu at all treatments in ambient temperature, mean pH were 8.34-8.22 and 7.88 at ambient CO_2 and high CO_2, respectively (Table 2). The average daily-integrated irradiance for the experimental period was 4238 kJ m^{-2} for PAR, 329 kJ m^{-2} for UVA and 22 kJ m^{-2} for UVB. The nitrate and phosphate concentration of the seawater enriched ratios during the experimental period, were 1.27 ± 0.36 and 0.15 ± 0.01µM (mean ± SE, n = 8), respectively (Table 2).

Table 2. Seawater carbonate chemistry at the four treatment during the experimental period. The four treatments T*ACO_2, T*HCO_2, T+4ºC*ACO_2 and T+4ºC*HCO_2 treatments. Temperature (ºC), pH (NBS scale) (mean values ± SE, n=2232) and were collected during the experimental period each three hours in September and October 2013. Salinity, nitrate, phosphate and average total alkalinity (µmol kg^{-1}), were calculated from water samples collected every week for treatment in the same period (mean values ± SE, n=48).

	TACO_2*	*T*HCO_2*	*T+4ºC*ACO_2*	*T+4ºC*HCO_2*
Temperature (°C)	19.8 ± 0.01	20.1 ± 0.01	23.9 ± 0.02	23.9 ± 0.01
pH_{NBS}	8.34 ± 0.01	7.88 ± 0.01	8.22 ± 0.01	7.88 ± 0.01
Salinity	37.78 ± 0.04	37.98 ± 0.02	39.01 ± 0.05	39.19 ± 0.04
NO_3 (µM)	2.58 ± 0.52	1.06 ± 0.03	2.02 ± 0.51	1.41 ± 0.57
P (µM)	0.16 ± 0.01	0.13 ± 0.01	0.15 ± 0.02	0.15 ± 0.01
pCO_2 (µatm)	455.6 ± 11.9	1264.1 ± 30.2	509.8 ± 7.8	1274.8 ± 17.9
CO_2 (µmol kg^{-1})	13.6 ± 0.3	37.2 ± 0.8	15.1 ± 0.2	36.8 ± 0.5
HCO_3^- (µmol kg^{-1})	2340 ± 16.8	3239 ± 8.1	1891 ± 11.8	3107 ± 14.1
CO_3^{2-} (µmol kg^{-1})	217 ± 1.3	207 ± 2.5	302 ± 1.1	241 ± 3.3
Total Alkalinity (µmol kg^{-1})	2431 ± 11.99	3585 ± 14.16	3059 ± 13.45	3793 ± 5.31

Physiological and biochemical responses

The Principal Coordinates Analysis (PCO) (Figure 2) revealed a positive correlation of the first axis (41.3% of total variation) with the internal N content, ETR_{max}, F_v/F_m, PC, Chl*a* and Chl*c*. This suggest that these variables increased in the first week of culture of the experimental period (symbols in blue colour). The measurements in the middle the experimental time had not any variation (0 %, data no shown). In contrast, at the end the experimental period, the internal C content, EC_{50}, violaxanthin, fucoxanthin, β carotene and PCw, negatively correlated with this axis (symbols in red colour). Taking into account the spatial distribution of the samples in relation to the treatments, time presented a high relationship with the mentioned axis. Moreover, the combination of the first two axes explained the 66.6% of the variation in the response variables (Figure 2).

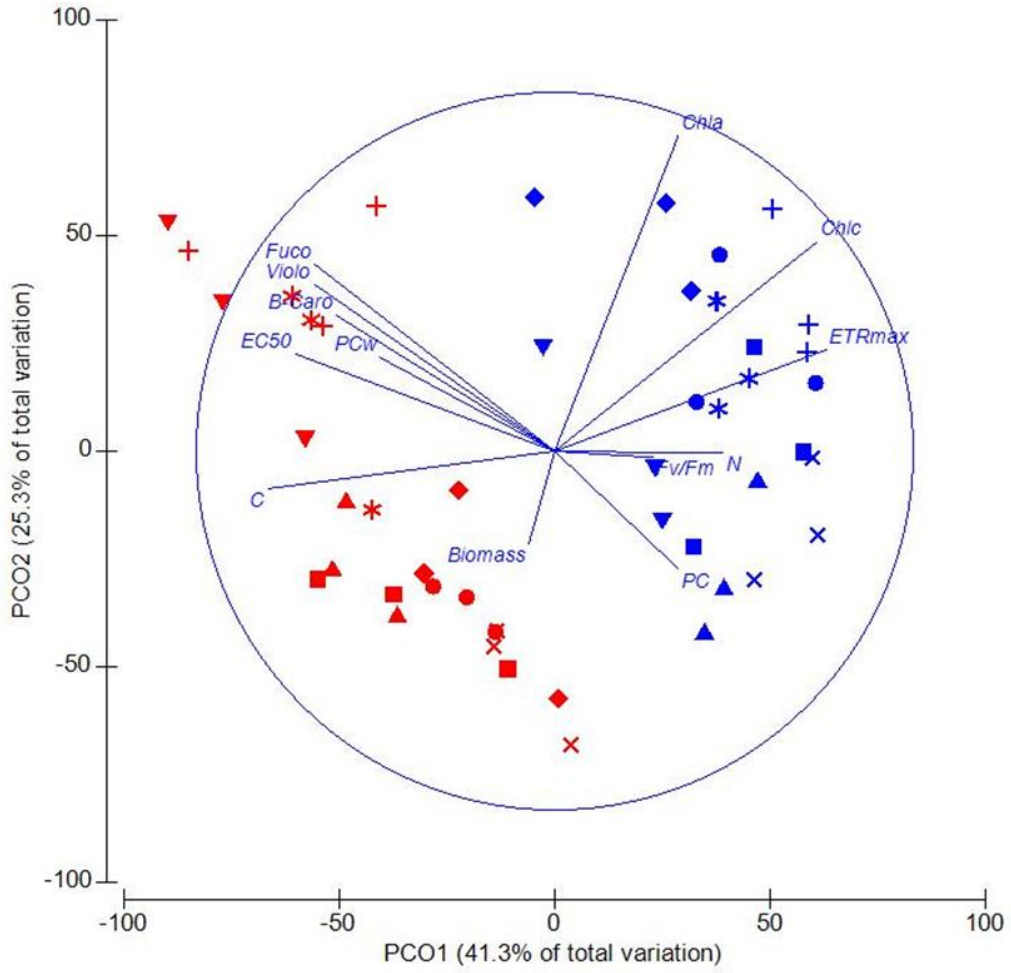


Figure 2. PCO diagram in relation to time (T1; in blue symbols and T4; in red symbols). Vectors overlay (Spearman rank correlation) indicates the relationship between the PCO axes and the ecophysiologycal variables; C and N internal conten, Chl*a* and Chl*c*: Chlorophyll *a* and *c*, respectively, Fuco: Fucoanthin, Viol: Violaxanthin, β-Caro: β Carotene, F_v/F_m: Maximal quantum yield, ETR_{max}: maximal electron transport rate, PC: phenolic compounds, PCw: phenolic compound in the water, EC_{50}: antioxidant axtivity and also Biomass the end the expermental period.

(a) Growth

The biomass of *C. tamariscifolia* had interactive effects between origin of the populations and CO_2 (P<0.01). The *C. tamariscifolia* (from CG and LA) increased in elevated CO_2 conditions (Figure 3, Table S1). However, the algae from ultraoligotrophic waters, the biomass was higher than algae from oligotrophic waters (Figure 3, Table S1).

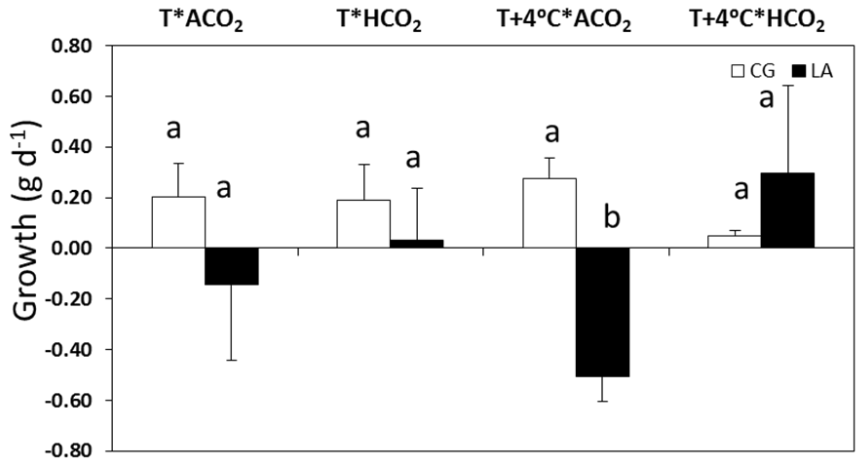


Figure 3. Growth (g d^{-1}) (mean ± SE, n=3) for *Cystoseira tamariscifolia* from both population of the origin at four treatments T*ACO_2 (ambient TºC*ambient CO_2), T*HCO_2 (ambient TºC*High CO_2), T+4ºC*ACO_2 (ambient T+4ºC*Ambient CO_2) and T+4ºC*HCO_2 (ambient T+4ºC*High CO_2) after the experimental period. Lower-case letters denote significant differences after a SNK test.

(b) Carbon and Nitrogen content responses

The carbon content of *C. tamariscifolia* (from CG and LA) increased with increasing CO_2 and ambient temperature. Carbon content was significantly different for all variables, this indicate additive effects. *C. tamariscifolia* (from CG and LA), showed maximal values 316.9 ± 4.98 and 308.3 ± 6.4 mg g^{-1} DW and minimal values 283.7 ± 4.4 and 267.9 8. ± 8.1 mg g^{-1} DW, respectively (Tables 3, S2). Nitrogen content have interactive effects between time, temperature and CO_2 conditions, indicating that nitrogen internal content increased in all treatments at the end the experimental period, respect to first week for macroalgae collected from both sites (Tables 3, S2). The ratio C: N has interactive effects between time and origin of the population. In both macroalgae (from CG and LA), C: N decreased at the end the experimental period respect to the first week (Tables 3, S2).

(c) Photophysiological variables

The maximal quantum yield (F_v/F_m) was significantly different between time, temperature and CO_2 conditions for macroalgae collected from both sites (Tables 3, S3). F_v/F_m at the first week, was greatest in ambient temperature and ambient CO_2 conditions in *C. tamariscifolia* (from CG and LA), but at the end the experiment, F_v/F_m increased in high CO_2 conditions of *C. tamariscifolia* (from LA) (Tables 3, S3).

Table 3. Carbon and Nitrogen internal content (mg g^{-1} DW), ratio C: N and Maximal quantum yield (F_v/F_m) (mean values ± SE, n=3) of *Cystoseira tamariscifolia*. In relation to Time (7, 14, 21 and 28 days), four treatments (T*ACO_2, T*HCO_2, T+4ºC*ACO_2 and T+4ºC*HCO_2) and population of origin of the macroalgae "Cabo de Gata-Nijar" (ultra-oligotrophic waters) and "La Araña" (oligotrophic waters).

		Cystoseira tamariscifolia									
		"Cabo de Gata-Nijar"					**"La Araña"**				
		It	*7 d*	*14 d*	*21 d*	*28 d*	*It*	*7 d*	*14 d*	*21 d*	*28 d*
Carbon	*T*ACO_2	287.1 ± 5.1	298.1 ± 2.3	314.1 ± 6.5	305.9 ± 6.6	289.8 ± 6.8	291.2 ± 3.5	294.7 ± 1.6	298.3 ± 8.9	282.4 ± 4.1	292.2 ± 2.7
	THCO_2		313.3 ± 9.1	316.9 ± 4.98	310.8 ± 4.4	309.3 ± 2.1		308.3 ± 6.4	301.5 ± 9.2	292.6 ± 6.7	301.4 ± 6.5
	*T+4ºC**ACO_2		298.5 ± 2.6	296.6 ± 7.4	284.7 ± 10.1	280.5 ± 1.6		281.9 ± 6.5	301.8 ± 0.52	274.2 ± 5.6	283.9 ± 5.2
	*T+4ºC**HCO_2		316.6 ± 2.3	311.4 ± 2.5	291.9 ± 2.1	283.7 ± 4.4		289.3 ± 7.7	296.4 ± 1.5	267.9 ± 8.1	270.1 ± 6.5
Nitrogen	*T*ACO_2	13.2 ± 0.7	14.5 ± 0.7	17.2 ± 2.3	18.9 ± 0.5	21.6 ± 0.7	8.1 ± 0.3	17.1 ± 1.4	20.9 ± 1.6	20.2 ± 0.5	20.6 ± 0.6
	THCO_2		15.6 ± 0.8	15.8 ± 0.3	16.9 ± 0.8	20.7 ± 0.6		18.1 ± 1.3	15.4 ± 0.5	17.6 ± 0.6	21.8 ± 0.6
	*T+4ºC**ACO_2		13.9 ± 0.7	15.4 ± 1.6	17.6 ± 0.7	23.6 ± 0.3		17.6 ± 1.6	21.2 ± 1.7	21.1 ± 1.2	24.5 ± 0.9
	*T+4ºC**HCO_2		15.2 ± 0.2	17.3 ± 0.3	18.8 ± 0.7	22.9 ± 1.4		18.3 ± 0.6	15.4 ± 0.5	20.3 ± 0.6	18.5 ± 1.4
C:N	*T*ACO_2	21.9 ± 1.6	21.9 ± 1.6	19.1 ± 3.1	16.2 ± 0.2	13.4 ± 0.2	36.2 ± 1.9	17.5 ± 1.3	14.4 ± 1.2	14.1 ± 0.3	14.2 ± 0.6
	THCO_2		20.4 ± 1.5	20.1 ± 0.7	18.5 ± 0.7	15.1 ± 0.4		17.4 ± 1.6	19.7 ± 1.1	16.6 ± 0.4	13.9 ± 0.2
	*T+4ºC**ACO_2		21.7 ± 1.2	19.7 ± 2.2	16.2 ± 0.5	11.9 ± 0.1		16.2 ± 1.0	14.4 ± 1.2	13.1 ± 0.5	11.6 ± 0.6
	*T+4ºC**HCO_2		20.6 ± 0.2	18.1 ± 0.4	15.6 ± 0.5	12.5 ± 0.9		15.9 ± 0.6	16.2 ± 0.2	13.3 ± 0.7	14.7 ± 0.8
F_v/F_m	*T*ACO_2	0.67 ± 0.01	0.72 ± 0.02	0.76 ± 0.03	0.67 ± 0.01	0.69 ± 0.02	0.72 ± 0.01	0.74 ± 0.01	0.75 ± 0.01	0.64 ± 0.01	0.67 ± 0.01
	THCO_2		0.71 ± 0.01	0.69 ± 0.02	0.73 ± 0.02	0.66 ± 0.02		0.69 ± 0.04	0.69 ± 0.04	0.68 ± 0.04	0.68 ± 0.01
	*T+4ºC**ACO_2		0.66 ± 0.01	0.72 ± 0.02	0.68 ± 0.01	0.73 ± 0.01		0.71 ± 0.01	0.68 ± 0.03	0.66 ± 0.01	0.67 ± 0.01
	*T+4ºC**HCO_2		0.68 ± 0.01	0.67 ± 0.01	0.62 ± 0.03	0.62 ± 0.01		0.71 ± 0.04	0.75 ± 0.04	0.65 ± 0.02	0.62 ± 0.02

The maximal electron transport rate (ETR_{max}) had interactive effects between all factors. ETR_{max} for macroalgae collected from both sites, were higher in high CO_2 conditions with ambient TºC (Figure 4a and b, S3). The highest ETR_{max} was observed under enriched CO_2 conditions and ambient temperature at the first two weeks whereas in the last two weeks the ETR_{max} decreased in all conditions, for *C. tamariscifolia* (from CG and LA) (Figure 4, Table S3).

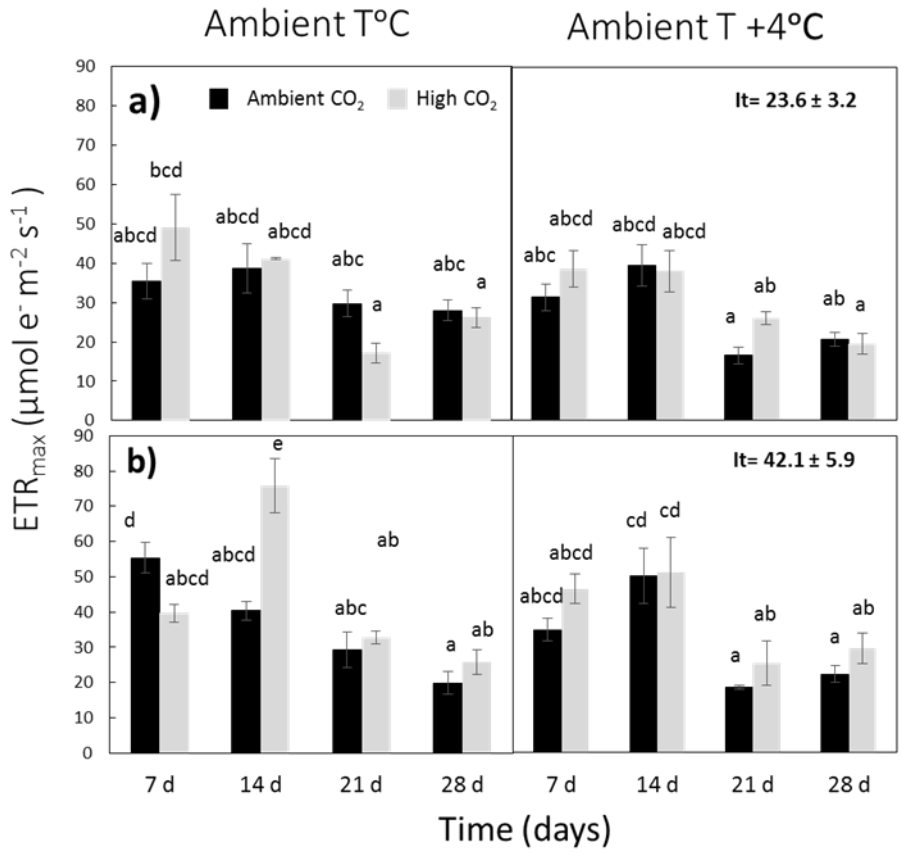


Figure 4. Maximal electron transport rate (ETR_{max} express as $\mu e^{-} m^{-2} s^{-1}$) (mean ± SE, n=3) for a) *Cystoseira tamariscifolia* from Cabo de Gata-Nijar Natural Park. b) *Cystoseira tamariscifolia* from La Araña beach at four treatments T*ACO_2 (ambient TºC*ambient CO_2), T*HCO_2 (ambient TºC*High CO_2), T+4ºC*ACO_2 (ambient T+4ºC*Ambient CO_2) and T+4ºC*HCO_2 (ambient T+4ºC*High CO_2) in relation to time. Upper values in right box indicate initial time values. Lower-case letters denote significant differences after a SNK test.

(d) Phenolic compound and antioxidant activity

Phenolic content (PC) was affected by time, temperature, CO_2 conditions and origin of population; this indicates additive effects in both macroalgae. *C. tamariscifolia* (from CG and LA), the phenols was highest in high CO_2 conditions independent of the temperature (Figure 5a, b and Table S5). In *C. tamariscifolia* (from CG), the polyphenols was *ca* 1.5 - 3.0 % whereas in *C. tamariscifolia* (from LA), the PC was *ca* 1.0 - 2.2 %

(Figure 5, Table S4), this suggest more phenolic production and photoprotection in the macroalgae collected from ultraoligotrophic waters than oligotrophic waters.

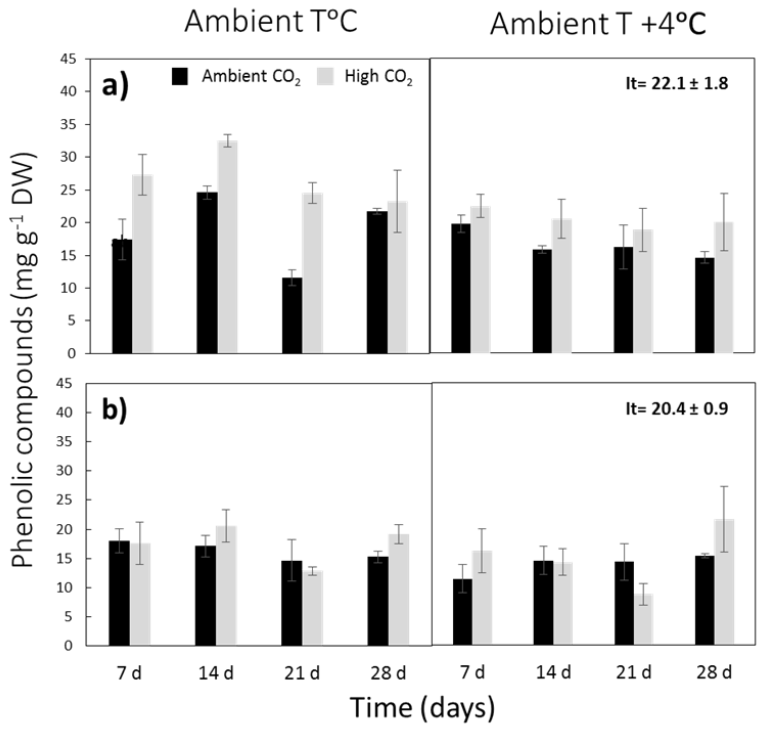


Figure 5. Total phenolic compounds (PC express as mg g^{-1} DW) (mean ± SE, n=3) for a) *Cystoseira tamariscifolia* from Cabo de Gata-Nijar Natural Park. b) *Cystoseira tamariscifolia* from La Araña beach at four treatments T*ACO_2 (ambient TºC*ambient CO_2), T*HCO_2 (ambient TºC*High CO_2), T+4ºC*ACO_2 (ambient T+4ºC*Ambient CO_2) and T+4ºC*HCO_2 (ambient T+4ºC*High CO_2) in relation to time. Upper values in right box indicate initial time values.

Antioxidant activity (EC_{50}) had interactive effects between time, CO_2 conditions and origin population (Figure 6, Table S4). In *C. tamariscifolia* (from CG and LA), the antioxidant activity were higher (i.e. lower EC_{50}) in high CO_2 and ambient temperature. In *C. tamariscifolia* (from CG), EC_{50} was higher in high CO_2 levels and high temperature (Figure 6, S4).

The phenolic compounds in the water (PCw), was affected for time, CO_2 conditions and origin populations (Figure 7, Table S4). PCw was higher in C. *tamariscifolia* from LA than *C. tamariscifolia* from CG. In this case, the polyphenols in the waters were highest in ambient CO_2 level independent of the temperature and origin of the macroalgae. This suggest, they had not additive effects, under this variable, because it was only affects for CO2 level (Figure 7, Table S4)

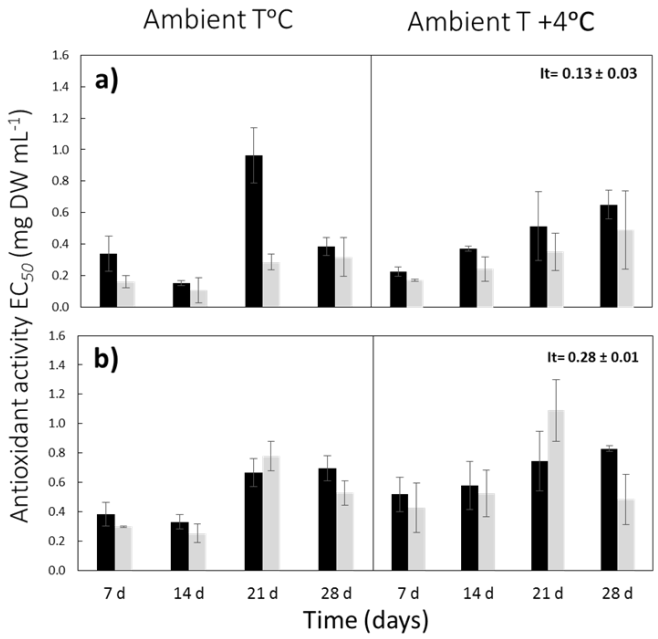


Figure 6. Antioxidant activity (EC_{50} express as mg DW mL^{-1}) (mean ± SE, n=3) for a) *Cystoseira tamariscifolia* from Cabo de Gata-Nijar Natural Park. b) *Cystoseira tamariscifolia* from La Araña beach at four treatments T*ACO_2 (ambient TºC*ambient CO_2), T*HCO_2 (ambient TºC*High CO_2), T+4ºC*ACO_2 (ambient T+4ºC*Ambient CO_2) and T+4ºC*HCO_2 (ambient T+4ºC*High CO_2) in relation to time. Upper values in right box indicate initial time values.

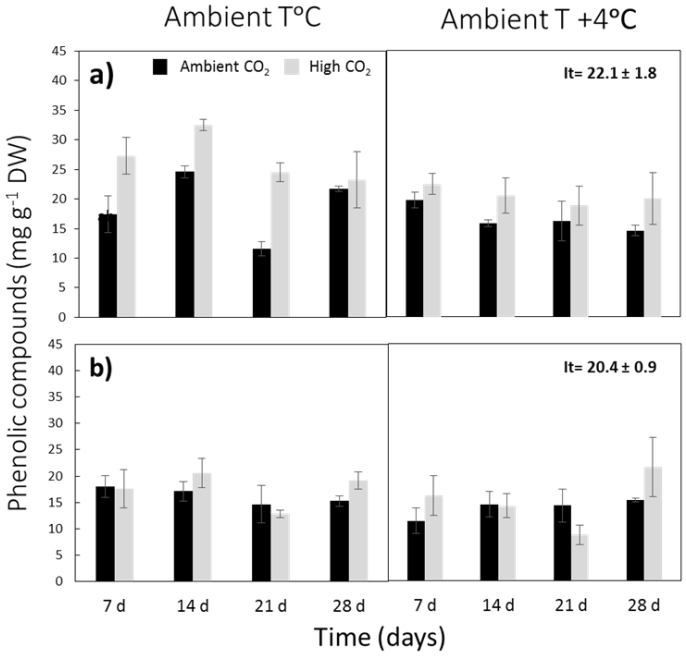


Figure 7. Polyphenols in the water (PCw express as mg g^{-1} DW d^{-1}) (mean ± SE, n=3) for a) *Cystoseira tamariscifolia* from Cabo de Gata-Nijar Natural Park. b) *Cystoseira tamariscifolia* from La Araña beach at four treatments T*ACO_2 (ambient TºC*ambient CO_2), T*HCO_2 (ambient TºC*High CO_2), T+4ºC*ACO_2 (ambient T+4ºC*Ambient CO_2) and T+4ºC*HCO_2 (ambient T+4ºC*High CO_2) in relation to time.

(e) Photosynthetic pigments

Chl*a* showed interactive effects between time, temperature and CO_2 levels (Tables 4, S4). In *C. tamariscifolia* from both sites, the greatest Chl*a* was content was observed in high temperature and high CO_2 conditions, and in ambient temperature and ambient CO_2 conditions, at the first week. However, in *C. tamariscifolia* from CG (ultraoligotrophic waters), Chl*a* at the end the experimental period, was higher ambient CO_2 conditions independent of the temperature. In addition, in *C. tamariscifolia* from LA was higher in ambient CO_2 levels in high temperature (Tables 4, S4). The Chl*c* content from macroalgae collected from both sites had interactive effects between time and CO_2 conditions. The highest Chl*c* content of *C. tamariscifolia* from both sites was reached in ambient temperature and high CO_2 levels (Tables 4, S4).

Significant quantitates of the fucoxanthin, violaxanthin and β-carotene (Tables 4, S4) in all treatments were detected but only traces of antheraxanthin, lutein and zeaxanthin (data no shown). The content of fucoxanthin was affected time, temperature and origin populations whereas violaxanthin was affected by the interaction between time, temperature and CO_2 levels (Tables 4, S4). The β-carotene content was significant affected by time, temperature and origin of populations (Tables 4, S4). Fucoxanthin and Violaxanthin contents, in *C. tamariscifolia* collected from both sites, were the highest at the end the experimental period in ambient CO_2 conditions independent of the temperature (Tables 4, S4). However, β-carotene was higher in high temperature and ambient CO_2 level (Tables 4, S4).

Table 4. Pigment contents: Chl*a*, Chl*c*, Fucoxanthin, Violaxanthin and β-carotene (µg g^{-1} DW) (mean values ± SE, n=3) of *Cystoseira tamariscifolia*. In relation to Time (7, 14, 21 and 28 days), four treatments ($T*ACO_2$, $T*HCO_2$, $T+4ºC*ACO_2$ and $T+4ºC*HCO_2$) and population of origin of the macroalgae "Cabo de Gata-Nijar" (ultra-oligotrophic waters) and "La Araña" (oligotrophic waters). Lower-case letters denote significant differences after SNK test.

		Cystoseira tamariscifolia									
		"Cabo de Gata-Nijar"					**"La Araña"**				
		It	*7 d*	*14 d*	*21 d*	*28 d*	*It*	*7 d*	*14 d*	*21 d*	*28 d*
Chla	$T*ACO_2$	1330 ± 26	2846 ± 214.1	916 ± 147.3	961 ± 171.7	1115 ± 18.1	1910 ± 21	3084 ± 247.6	1434 ± 278.1	1420 ± 232.7	2297 ± 130.3
	$T*HCO_2$		1790 ± 225.5	1019 ± 65.1	1036 ± 47.7	825 ± 74.6		2279 ± 309.5	1317 ± 188.1	1498 ± 237.9	2097 ± 427.9
	$T+4ºC*ACO_2$		1715 ± 276.2	637.2 ± 78.4	908.3 ± 180.3	1112 ± 115.6		2239 ± 284.7	1418 ± 59.3	1175 ± 152.1	2115 ± 271.7
	$T+4ºC*HCO_2$		2827 ± 400.2	1091 ± 171.5	633.5 ± 96.1	1063 ± 111.4		3513 ± 39.5	1603 ± 305.2	1237 ± 185.3	1140 ± 218.1
Chlc	$T*ACO_2$	102.9 ± 0.5	157.6 ± 23.7^{fg}	15.3 ± 1.7^{a}	26.4 ± 5.2^{a}	19.6 ± 6.4^{a}	143.6 ± 43.7	171.7 ± 2.6^{g}	74.2 ± 2.9^{abcde}	36.9 ± 10.3^{a}	75.7 ± 26.9^{abcde}
	$T*HCO_2$		117.1 ± 1.3^{def}	69.8 ± 0.3^{abcde}	11.7 ± 1.1^{a}	19.7 ± 7.8^{a}		167.1 ± 2.1^{g}	102.4 ± 12.1^{bcde}	57.5 ± 12.8^{abc}	31.1 ± 5.3^{a}
	$T+4ºC*ACO_2$		100.1 ± 21.9^{bcde}	27.3 ± 3.4^{a}	30.3 ± 2.9^{a}	21.1 ± 5.7^{a}		97.3 ± 1.2^{bcde}	45.1 ± 0.5^{ab}	67.1 ± 15.3^{abcd}	32.1 ± 0.5^{a}
	$T+4ºC*HCO_2$		106.6 ± 29.5^{cde}	10.1 ± 0.2^{a}	27.9 ± 2.8^{a}	14.8 ± 0.7^{a}		127.8 ± 24.8^{efg}	100.2 ± 28.1^{bcde}	49.6 ± 8.4^{abc}	32.4 ± 3.55^{a}
Fucoxanthin	$T*ACO_2$	206.3 ± 78.1	316.8 ± 102.5	386.7 ± 52.7	386.5 ± 71.3	426.5 ± 14.7	191.2 ± 31.5	352.8 ± 37.7	527.8 ± 112.1	536.1 ± 101.5	904.1 ± 82.2
	$T*HCO_2$		247.3 ± 30.1	429.2 ± 17.3	417.6 ± 14.1	321.5 ± 34.9		386.1 ± 89.1	443.7 ± 61.3	547.8 ± 84.3	801.1 ± 159.5
	$T+4ºC*ACO_2$		230.5 ± 37.5	246.9 ± 36.8	380.2 ± 77.1	434.1 ± 43.6		443.2 ± 81.1	573.8 ± 44.1	477.3 ± 89.9	830.1 ± 131.8
	$T+4ºC*HCO_2$		361.8 ± 65.2	437.7 ± 73.7	276.7 ± 33.4	437.3 ± 56.1		558.6 ± 110.4	626.1 ± 99.6	478.6 ± 58.3	401.9 ± 93.9
Violaxanthin	$T*ACO_2$	29.5 ± 6.5	65.3 ± 4.5	49.9 ± 10.5	57.2 ± 5.7	64.6 ± 0.7	46.4 ± 13.4	44.3 ± 15.7	68.9 ± 11.2	81.7 ± 14.1	118.2 ± 6.1
	$T*HCO_2$		23.8 ± 8.1	52.3 ± 7.3	57.7 ± 2.1	45.1 ± 5.1		33.3 ± 2.7	69.5 ± 8.7	82.6 ± 11.2	113.2 ± 21.6
	$T+4ºC*ACO_2$		26.7 ± 3.4	38.1 ± 4.8	52.4 ± 9.5	63.2 ± 5.1		22.7 ± 4.7	69.1 ± 3.1	65.1 ± 8.5	111.1 ± 13.6
	$T+4ºC*HCO_2$		51.4 ± 9.7	58.1 ± 6.1	38.7 ± 4.3	60.7 ± 7.6		90.4 ± 23.7	82.5 ± 20.8	72.5 ± 11.3	68.1 ± 12.6
β- carotene	$T*ACO_2$	21.5 ± 3.4	53.9 ± 3.7	56.4 ± 11.3	70.5 ± 3.2	74.6 ± 3.4	61.7 ± 15.6	63.4 ± 16.3	103.1 ± 13.5	113.1 ± 15.1	107.4 ± 7.8
	$T*HCO_2$		47.1 ± 15.9	83.2 ± 17.7	83.9 ± 10.1	60.1 ± 8.6		119.1 ± 23.8	94.1 ± 15.5	96.3 ± 7.6	107.6 ± 11.5
	$T+4ºC*ACO_2$		48.2 ± 10.1	69.5 ± 15.9	69.1 ± 2.1	91.1 ± 15.1		64.1 ± 5.8	88.8 ± 4.2	81.6 ± 2.4	113.8 ± 13.4
	$T+4ºC*HCO_2$		43.1 ± 4.1	63.9 ± 3.7	156.4 ± 7.6	73.6 ± 0.7		76.7 ± 12.1	92.2 ± 11.9	77.3 ± 11.5	78.4 ± 4.3

6.5 DISCUSSION

In this study, we show benefits of DIC increase on growth rate in *Cystoseira tamariscifolia* being the physiological responses more accelerated in ultraoligotrophic than in oligotrophic harvested algae.

Temperature increase has negative effect on growth rate only in algae from oligotrophic waters. Although the biomass in both *C. tamariscifolia* increased in elevated CO_2 conditions, biomass of algae collected from Cabo de Gata-Nijar Natural Park (ultraoligotrophic waters) was higher than algae collected from La Araña beach (oligotrophic waters).

This suggests interactive effects between origin of populations and CO_2 conditions, probability that algae collected in ultraoligotrophic waters maybe can capitalise carbon of the aquatic system when the nutrient is not limited in extreme. Recent reviews concur that non-calcareous macroalgal production and biomass may increase due to beneficial effects of ocean acidification on photosynthesis (Harley et al. 2012, Koch et al. 2014, Brodie et al. 2014). Reports on macroalgae from other regions have shown that ocean acidification can directly benefit the physiological state and growth (Baggini et al. 2014, Celis-Plá et al. 2015). Mostly in small size species such as *Gracilaria lemaneiformis* in China (Zou and Gao 2009) and *Feldmannia* spp. in Australia (Russell et al. 2011), as well as occasionally in canopy-forming phaeophytes such as *Nereocystis luetkeana* and *Macrocystis pyrifera* (Swanson and Fox 2007, Roleda et al. 2012).

In oligotrophic areas, brown macroalgae were unable to take full advantage of increased inorganic carbon availability by increasing their carbon content (Koch et al. 2014). There is added complexity when we consider that many regions have experienced a die-back of canopy-forming brown algae due to excess nutrients (Strain et al. 2014); ocean acidification may exacerbate this problem since increased DIC may further benefit those algae that presently compete with fucoids and kelps in eutrophic conditions (Connell et al. 2013). The highest carbon content was observed in *C. tamariscifolia* from both sites and incubated in elevated CO_2 level. This suggest that there is no additive effects because it was only affected for CO_2 level. The carbon content helps explain the dominance of these brown algae at a variety of coastal Mediterranean, probably due to a combination of the direct stimulus of increased DIC for photosynthesis (Mercado et al. 1998, Raven and Hurd 2012).

Carbon and nitrogen internal content and the maximal electron transport rate (ETR_{max}), as estimator of photosynthetic capacity, increased in high CO_2 conditions only

under ambient temperature in both algal populations. We found a positive correlation between ETR_{max} and chlorophyll; Chl*a* and Chl*c* (Table S6). Lüder et al. (2001), showed the same result in *Palmaria decipiens*, during the autumn, ETR_{max} and pigment content increased throughout the time. A positive correlation between ETR_{max} and internal carbon content was observed (Table S6), this suggest that carbon supply increased photosynthetic production expressed as ETR_{max}. In oligotrophic waters, such in the Mediterranean, nutrient availability generally limits macroalgal growth (Ferreira et al. 2011) and photosynthetic capacity (Pérez-Lloréns et al. 1996). After 28 d of incubation, internal nitrogen increased under T +4ºC of temperature in algae from both sites under natural CO_2 conditions. In contrast, after 7 day culture in thalli collected in winter (decreased temperature) the nutritional state seems to be more favorable than that of summer collected algae (Celis-Plá et al. 2014a). In this study, the highest ETR_{max} is reached at increased CO_2 and ambient water temperature conditions in algae from both sites, being the production higher in macroalgae from oligotrophic compared to ultra-oligotrophic waters. Johnson et al. (2012) showed a significant effect on the *in situ* photosynthetic responses of *Padina pavonica* with CO_2 enrichment (increases in $rETR_{max}$).

These benefits from increases in DIC in *C. tamariscifolia* from both localities are reflected in the values of photosynthetic parameters after 28 d culture. We found that the benefits of increased DIC were even more pronounced when combined with ambient temperature in the seawater. This result was expected since macroalgae tend to be nutrient-limited in oligotrophic waters such as those of the Mediterranean Sea (McQuatters-Gollop et al. 2009, Ferreira et al. 2011).

Phenol content was higher in algae from ultraoligotrophic waters i.e. 1.5 - 3.0 % than that form oligotrophic waters 1.0 - 2.2 %. A positive correlation between phenolic compounds and antioxidant activity expressed as EC_{50} (Table S6), indicates that phenolic compounds can prevent photodamage.

The higher concentration of phenolic compounds in algae from Cabo de Gata-Nijar can be related to the photoacclimation to high irradiance levels in coastal waters with high transparency. Figueroa and Gómez (2001) reported high penetration of PAR, UVA and UVB radiation with mean attenuation coefficient (K_d) values of 0.07, 0.105 and 0.220 m^{-1}. The increase of phenolic compounds under elevated CO_2 could increase the photoprotection of this species in future scenario of ocean acidification with seawater pH value of 7.78 corresponding to a 850 ppm CO_2 (IPCC 2014). Phenols usually accumulated under elevated CO_2 treatments, as per past studies on kelp grown at high

CO_2 (Swanson and Fox 2007). The content of photoprotectors (phenolic compounds) from both populations was higher under increased CO_2 conditions in ambient temperature. Celis-Plá et al. (2015) showed also that phenolic compounds in *Cystoseira compressa* and *Padina pavonica* accumulated in elevated CO_2 treatments with nutrient enrichment conditions, as interactive effects, in a field study with a natural pH gradient (Vulcano, Italy). However, the effects of CO_2 on autotrophic phenol production is not straight forward, as both seagrasses decreased the production of phenols when CO_2 increased, indicating these responses are species-specific (Arnold et al. 2012). In general, algae can minimize damage from high irradiance not only by down-regulating process in photosystems. Such energy dissipation (increase of non-photochemical quenching) but also by the production of UV photoprotectors and antioxidant compounds as phenols (Pérez-Rodríguez et al. 1998, Figueroa et al. 2014a) or other photoprotectors as mycosporine like amino acids in red macroalgae (Korbee-Peinado et al. 2004, Figueroa et al. 2012). However, ocean acidification also has the potential to damage these photoprotective mechanisms, which kick-in at high light levels (Pierangelini et al. 2014).

In addition, in oligotrophic ambient with natural input of the nutrient (i.e. La Araña beach) due to anthropogenic impact i.e. sewage discharges can increases the photoprotection capacity of seaweeds due to the increase in protein content or polyphenols (Arnold and Targett 2002). A positive correlation between EC_{50} and nitrogen internal content (Table S6) indicates again that nutrient level has a positive effect on photoprotection. We also found antioxidant activity trends to be higher (i.e. low EC_{50}) in high CO_2 treatments compared to ambient in ambient temperature in algae from both collected sites. In oligotrophic waters, the EC_{50} was higher in the first and second week whereas in ultra-oligotrophic waters the antioxidant activity was maintain high through the time during the experimental period. It is shown again, the algae submitted to more stress conditions in the natural environment i.e. ultra-oligotrophic versus oligotrophic presented the highest acclimation capacity. Intertidal macroalgae from Mediterranean and Atlantic coastal waters of southern peninsula presented higher capacity to respond to increased environmental stress compared to algae from subtidal or from other region with lower daily irradiance levels (Hanelt 1998, Hanelt and Roleda 2009, Gómez et al. 2004, Hanelt and Figueroa 2012). The acclimation pattern is governed by a high non-photochemical quenching, photoinhibition and the accumulation of UV screen photoprotectors (Pérez-Rodríguez et al. 1998, Abdala-Díaz et al. 2006, Figueroa et al. 2014a). The highest release of phenolic compounds in the water was observed in algae

from oligotrophic waters than ultraoligotrophic waters at the end of the experimental period in ambient CO_2 conditions independent of the temperature. The release of the phenolic compounds in brown macroalgae, are considerate as other mechanism of photoprotection, these can be expulsed to the seawater during periods of UVR stress (Swanson and Druehl 2002, Koivikko et al. 2005, Celis-Plá et al. 2014a).

In the collection site, the Fucoxanthin content was about 8% higher in algae harvested from ultraoligotrophic waters than that from oligotrophic whereas the ratio between the main carotenoid and chlorophyll (Fucoxanthin: Chl*a*) was still higher (about 33.0%). The high proportion of photoprotective carotenoid (Goss and Jakob 2010) respect to chlorophyll was expected since the penetration of both PAR and UVR (Figueroa and Gómez 2001) is higher in ultraoligotrophic compared to oligotrophic waters due to its lowest turbidity (Cortés et al. 2012). After 28 d however the highest increase was produced in oligotrophic collected algae except in T+4ºC*HCO_2. However after 28 d submitted to different CO_2 and temperature treatments, the Fucoxanthin: Chl*a* ratio was similar in algae collected from both site i.e. 0.38-0.39 except in T+4ºC*HCO_2 i.e. 0.41 in ultraoligotrophic and 0.35 oligotrophic collected algae. Thus, the increase of both CO_2 and temperature was less favourable for photoprotection mechanisms in oligotrophic than that in ultraoligotrophic harvested macroalgae.

A positive correlation between EC_{50} and fucoxanthin, violaxanthin and β-carotene (Table S6) was found in algae collected from oligotrophic waters and on the other hand the antioxidant activity had a less relationship with phenolic compounds in oligotrophic than that in ultraoligotrophic collected algae. This can suggest the relative importance of phenolic or the collection site i.e. different nutrient levels and bio-optical characteristics can affect carotenoids as photoprotectors. In oligotrophic collected algae, the decrease of phenolic compounds can be compensated by the carotenoids to maintain the photoprotection. The availability of nitrate affected the mechanisms for photoprotection in the dinoflagellate *Heterocapsa* sp., under high nitrate supply mycosporine like aminoacids dominated as photoprotectors whereas under low nutrient supply, the main photoprotectors were the carotenoids (diadinoxanthin - diatoxanthin cycle) (Korbee et al. 2010). Goss and Jakob (2010) indicated that the xanthophyll cycle represents an important photoprotection mechanism in plant cells. In this study we found that fucoxanthin had interactive effects between temperature and origin of population, while violaxanthin has interactive effects between temperature and CO_2 conditions and β-carotene content only was affect significantly for temperature factors. These responses of

the xanthophyll cycle could reflect a regulatory and photoprotective response that down-regulates the delivery of excitation energy into the electron-transport chain to match the rates at which products of electron transport can be consumed in these leaves (Demmig-Adams and Adams 2006).

6.7 CONCLUSIONS

In this study, we show that ongoing ocean acidification can increase algal productivity, antioxidant activity and photoprotectors compounds. We show that both *C. tamariscifolia* were able to acclimate to alterations in DIC and ambient temperature and that these had interactive effects on the photosynthetic and photoprotective systems in algae collected from both ultraoligotrophic and oligotrophic waters being the physiological responses of the ultraoligotrophic more rapid and persistent compared to that of algae from oligotrophic waters.

As Ocean acidification is not happening in isolation, but alongside a plethora of other anthropogenic changes, the study of the interactive/additive effects of multiple stressors is critical to plan for global ocean change. Elevated CO_2 levels can clearly enhance brown algal productivity, with implications for fucoid forests of the planet, but this will be contingent on other physicochemical parameters. Our study shows that ongoing ocean acidification had interactive effects with the temperature i.e. beneficial effects on growth rates, algal photosynthetic production (ETR_{max}) antioxidant activity (EC_{50}) and photoprotectors compounds (PC and xanthophyll cycle). We do not know if *C. tamariscifolia* from the two sites correspond to different ecotypes but at least the results indicate the importance of light and nutrient history of the macroalgae in the responses to climate change factors.

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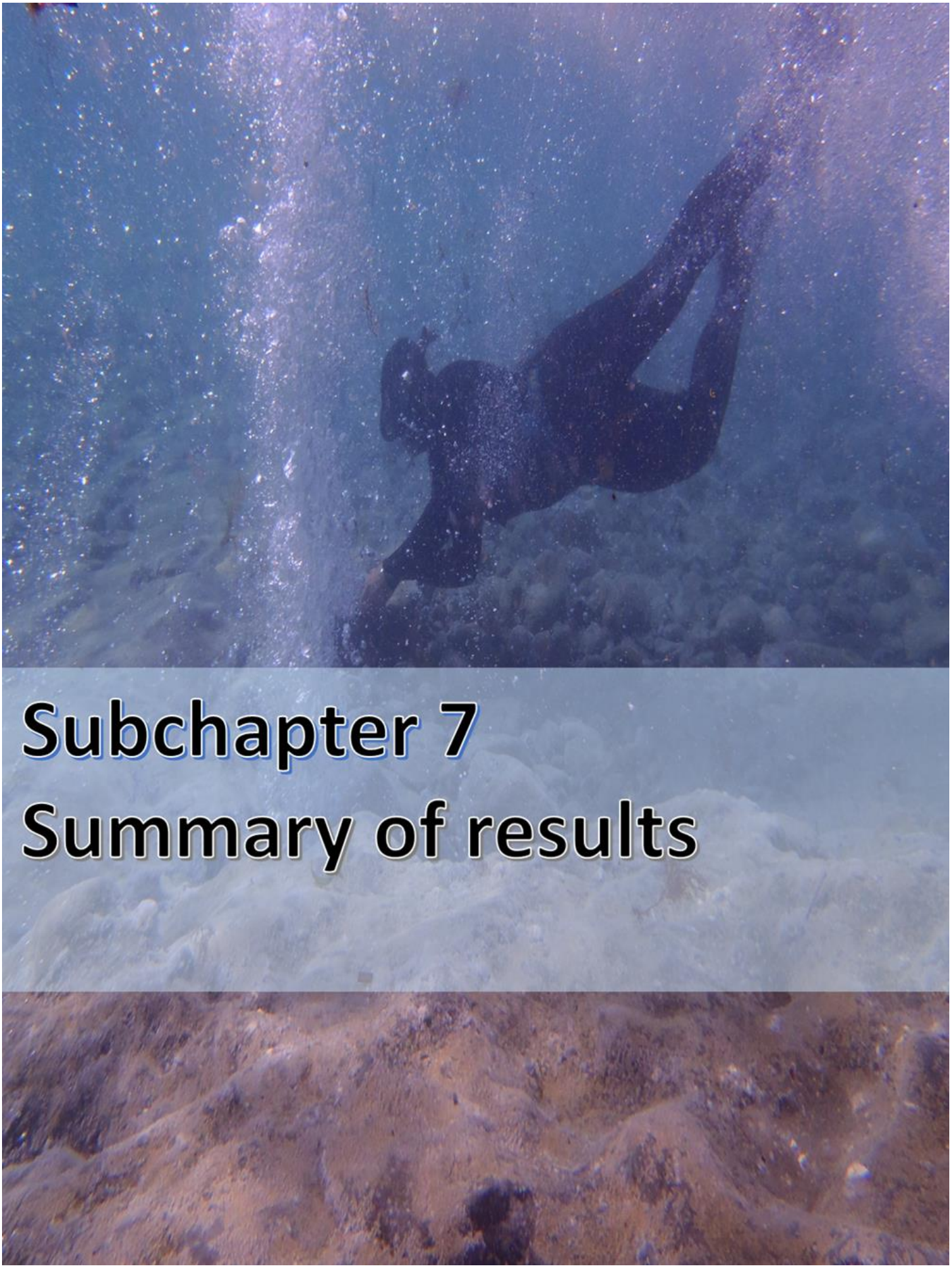


Subchapter 7
Summary of results

Natural pH gradient, Vulcano, Italy. May 2013. Photograph by Norah Brown

SUMMARY OF RESULTS

7.1 Subchapter 1:

Environmental conditions

Cystoseira tamariscifolia was abundant in all seasons, along with abundant *Ulva* spp. in spring and summer (Figure 1). The seawater temperature ranged from 14-23 ºC (Table 1) with a peak summer average daily irradiance of *ca*. 10165 kJ m^{-2} for PAR, 1051 kJ m^{-2} for UVA and 57.5 kJ m^{-2} UVB (Figure 2A-C). Seasonal nitrate (NO_3^-) concentrations ranged from 0.6-1.5 mg L^{-1} (Ramírez et al. 2005, Mercado et al. 2007, 2012). Seawater nitrate concentrations were approximately 2.5 times higher in winter and spring than in summer and autumn. Ammonium (NH_4^+) varied through the year from 0.1 to 0.5 mg L^{-1} and was 2.7 times higher in summer than in autumn and winter, 1.4 times higher than in spring. The phosphate (PO_4^{3-}) concentration varied little (0.12 to 0.15 mg L^{-1}) in all seasons. Chlorophyll *a* concentrations were highest in spring with 1.45 mg L^{-1} and lowest in summer with 0.92 mg L^{-1}, respectively (Table 1).

Morphological observations

Cystoseira tamariscifolia samples have olive green in colour, with a cylindrical frond and branches irregularly (Figure 3A). *C. tamariscifolia* was stained with toluidine Blue had a metachromatic reaction in the cell wall, indicating the presence of acidic sulphated polysaccharides. In the cytoplasm of cortical and subcortical cells, a large quantity of dark blue and yellow physodes was observed (Figure 3B, arrows). In the cortical cells, the physodes are located near in the surface of thallus (Figure 3B, arrows). When observed by transmission electron microscopy, the cortical cells had numerous chloroplasts (Figure 4A) and physodes (Figures 4A and C) with a thick cell wall (Figures 4A and C) that was embedded with phenolic compounds (Figure 4B arrows). Mitochondria were associated with the chloroplasts (Figure 4D) which had the typical internal organization of brown algae with thylakoids aggregated in bands (Figure 4D). Lipid droplets (plastoglobuli) were situated between the thylakoids (Figure 4D) and plasmodesmata cell connections were seen (Figure 4E).

Biochemical responses

The carbon and nitrogen content of *C. tamariscifolia* was significantly higher in winter and spring; the C: N ratio was significantly lower in winter (Figure 5, S1).The

amount of phenols was also significantly affected by season, being highest in spring when whereas antioxidant activity (EC_{50}) was significantly reduced (Figure 6, S1).

Physiological responses

F_v/F_m was not significantly affected by season although it tended to be higher in winter; maximal electron transport rate (ETR_{max}) was the highest in spring and photosynthetic efficiency (α_{ETR}) was significantly affected by season, being lowest in winter (Table 2, S2).

The irradiance of saturation of curve (Ek_{ETR}) was not significantly affected by season, but tended to be higher in winter and spring. The highest non-photochemical quenching (NPQ_{max}) occurred winter, although no statistically significant seasonal differences were found (Table 2, S2). The irradiance of saturation (Ek_{NPQ}) was significantly higher in autumn and the relationship between ETR_{max} (production) and NPQ_{max} (photoprotection) was highest in spring (Table 2, S2). A positive correlation was found between phenolic compounds and antioxidant activity, between antioxidant activity and nitrogen internal content, through all seasons, and positive correlation between EC_{50} and ETR_{max} and between photosynthetic efficiency (Table S3).

7.2 Subchapter 2:

Solar radiation and temperature

The daily-integrated irradiance in the air during the experimental period of PAR, UVA and UVB is represented in Fig. 1. The daily integrated irradiance in the period of 8th to 16th February 2011 was 204 kJm^{-2} of UVB, 4530 kJm^{-2} of UVA and 54266 kJm^{-2} of PAR whereas from 27th June to 5th July was 638 kJm^{-2} of UVB, 10276 kJm^{-2} of UVA and 99777 kJm^{-2} of PAR; about 3.1 times (UVB), 2.26 times (UVA) and 1.8 times (PAR) higher in summer than in winter (Fig.1). Most of the time the sky was cloudless in winter experiment except for days 4th and 5th (Fig. 1A, C) whereas, in summer experiment also thin clouds were observed on days 3rd to 5th (Fig. 1B, D). The average underwater temperature in the incubation vessels during the day in winter was maintained at 18^{o}C and 22^{o}C in summer and during the night at 12^{o}C in winter and 17^{o}C in summer (Fig.1E, F).

Physiological and biochemical responses

F_v/F_m was significantly ($p<0.05$) higher in winter than in summer both just after alga harvesting (Table 1) and after incubation for 3 and 7 days (Fig. 2A). F_v/F_m increased

during the experimental time only in winter whereas in summer a significant decrease was observed (Fig. 2A). The collection site did not affect the values of F_v/F_m in winter, whereas in summer F_v/F_m was higher in algae collected from RP than from RS.

ETR_{max} ($F_{1,8}$= 15.6, P<0.05) and α_{ETR} ($F_{1,8}$= 11.7, P<0.05) was higher in algae collected in summer than in winter (Table 1). In winter, α_{ETR} (Fig. 2B) and ETR_{max} (Fig. 3A) increased during the experimental time whereas in summer α_{ETR} (Fig 2B) and ETR_{max} (Fig. 3A) remained constant. ETR_{max} in the experimental period was higher in algae collected from RS than from RP, except for RP at 7d (Fig. 3A); whereas, α_{ETR} presented the same pattern only in winter and no significant differences (P<0.05) among the sites were found in summer period (Fig. 2B).

NPQ_{max} was higher in field algae collected in summer than in winter and no significant differences between RP and RS algae were found (Table 1). However, during the incubation period in the vessels, NPQ_{max} was not significantly different among location or season (Fig 3B).

In situ ETR in outdoor experiments presented a daily pattern both in winter (Fig. 4A, C) and in summer (Fig. 4B, D). In winter, the irradiance around noon was about 900 µmol m^{-2} s^{-1} whereas in summer was 1600 µmol m^{-2} s^{-1} (Fig. 4). In spite of the daily-integrated irradiance was about two times lower in winter than in summer, the ETR decreased at 7th day in the first season. However, in summer the decrease of ETR occurred at the 3rd but not at 7th day. The decrease of ETR in the experimental period in winter was higher in algae collected from RP than that from RS (Fig. 4A, C), whereas, in summer (Fig. 4B, D) it was the reverse. The period of the decrease of ETR was extended 4 hours in winter, whereas in summer it was 6 hours. However, in both seasons, the ETR reached similar values.

N internal content was higher and C:N ratio lower in algae collected in winter than in summer and no significant differences between RP and RS algae were found (Table 2). After 3 and 7 d incubation in cylindrical vessel, winter grown algae maintained the high levels of N and consequently the lower levels of C:N compared to summer ones. No significant differences in N and C:N ratio were found in algae collected from RP compared to RS after 3 and 7 d incubation in cylindrical vessels (Table 2).

Chl*a* and Chl$c_{1\pm}c_2$ concentrations were 1.6 ± 0.4 mg g^{-1} DW and 0.28 ± 0.04 mg g^{-1} DW, in field collected algae during summer time, respectively; whereas, in winter were 1.2 ± 0.2 mg g^{-1} DW and 0.17 ± 0.04 mg g^{-1} DW. After 3 and 7 d incubation in cylindrical

vessels, no significant differences were found in the chlorophyll concentrations (data not shown).

The content of phenolic compounds was higher ($F_{1,8}$= 5.8, P<0.05) in field collected algae in winter than in summer and during the experimental period the differences were maintained (Table 3). In winter, phenol concentration increased from 25 to 41 mg g^{-1} DW in RP algae from 3rd to 7th d incubation; in contrast, phenols of RS algae decreased from 41 to 27 mg g^{-1} DW. In summer, phenolic compounds did not change in RP (27-28 mg g^{-1} DW) and RS algae (27 to 22 mg g^{-1} DW) (Table 3).

The release of polyphenols expressed as mg g^{-1} DW after 3 d incubation was similar in algae collected from RP in both seasonal periods (Table 4), whereas after 7 d the release was higher in summer than in winter period, mainly in algae collected from RS. The release expressed, as percentage respect to the internal content was clearly higher after 7 d incubation in summer than in winter collected *C. tamariscifolia*. After 7d, the percentage of release was 3 and 5 times higher in summer collected RS and RP algae, respectively, than that in winter (Table 4).

In contrast, the antioxidant activity estimated as the oxidation index EC_{50} was higher in field collected algae in summer than that in winter. The antioxidant activity remained higher in algae collected in summer than that in winter through the incubation period (Table 3). Meanwhile only in winter, the antioxidant activity was higher in RS than in RP grown algae during the experimental period. In winter, the antioxidant activity decreased through the experimental time, whereas in summer no significant differences during the experimental period were found.

7.3 Subchapter 3:

Environmental conditions

Nitrate (NO_3^-), ammonium (NH_4^+) and phosphate (PO_4^{3-}) concentrations at the non-enriched site were1.34 ± 0.31 µM, 1.17 ± 0.35 µM and 0.09 ± 0.01 µM respectively. In contrast, concentrations at the nutrient enriched site were 107.51± 9.67 µM, 163.31 ± 6.10 µM and 24.52 ± 1.51 µM respectively (mean ± SE, n = 6). Hence, on average, the nutrient enriched treatment increased nitrate, ammonium and phosphate concentrations in the water column by 80, 139 and 272 times respectively. The average daily-integrated surface irradiance for the experimental period (20th to 21th on September 2012) was 5842 KJ m^{-2} for PAR, 673.3 KJ m^{-2} for UVA and 27.3 KJ m^{-2} for UVB. The attenuation

coefficients for PAR (Kd_{PAR}) and UVA (Kd_{UVA}) were 0.076 m^{-1} and 0.137 m^{-1}, respectively. The average seawater temperature at 0.2m (mean ± SE, N=1440) ranged between 24.42 ± 0.42 ºC (during the day) and 23.8 ± 0.19 ºC (at night).

Physiological response variables

Internal N content was higher in *C. tamariscifolia* than in *E. elongata* (Table 1, Fig. 2) ANOVA results showed that both species from 0.5 m depth presented significantly higher N content and lower C:N ratio under nutrient enriched treatment (Table 1, Fig. 2 and 3). However, the N content at 2.0 m depth was different for both species (Table 1, Fig.2). *C. tamariscifolia* specimens collected from 2.0 m depth showed similar N content to those from 0.5 m depth and C:N ratio increased under non-enrichment treatments (Fig. 2a and 3a). In contrast, *E. elongata* showed a significant interaction between nutrients and irradiance (Table 1). N content in the nutrient enriched treatment was lower under100%$_{PAB}$ treatments and C:N ratio higher in the same conditions (Fig. 2b and 3b).

F_v/F_m in *C. tamariscifolia* showed a significant interaction with nutrients and irradiance in algae collected at 2.0 m depth waters (Table 2). Specimens of *C. tamariscifolia* transplanted to 100%$_{PAB}$ presented higher F_v/F_m under non-enriched treatments (Table 3). Neither of the species collected at 0.5 m depth and *E. elongata* at 2.0 m depth waters showed significant differences (Table 2). In contrast, ETR_{max} of *C. tamariscifolia* showed significant differences among irradiance treatments (70%$_{PAB}$ and 100%$_{PAB}$) in 0.5 m depth (Table 2). This value was higher when they were transplanted to 70%$_{PAB}$ (Table 3). Conversely, specimens of both species collected in 2.0 m depth waters did not show any significant differences for both depths. The α_{ETR} in *C. tamariscifolia* showed a significant interaction with nutrients and irradiances in both depths (Table2). This value was lower under 70%$_{PAB}$ (transplant treatment) and non-nutrient enriched conditions. Besides, in both cases after the incubation in cylinders, they reached the initial α_{ETR} conditions as its natural habitat (Table 3). Meanwhile, in *E. elongata* the α_{ETR} showed two different significant results depending on the depth. Algae collected from 0.5 m depth showed significant increase at the nutrient enriched site and in 70%$_{PAB}$ treatment (Table 2 and 3). In contrast, in algae collected from 2.0 m depth waters, α_{ETR} showed higher values under the non-enriched treatment (Table 2, Table 3).

Ek_{ETR} in *C. tamariscifolia* collected from 0.5 m depth showed a significant interaction with nutrients and irradiance, thereby, 0.5 m depth algae under 70%$_{PAB}$ in non-enriched treatment showed higher Ek_{ETR} than that in the other three combinations of treatments (Table 3). Ek_{ETR} did not show any significant differences in algae collected from 2.0 m

depth waters (Table 2). Ek_{ETR} of both, 0.5 m depth and 2.0 m depth collected *E. elongata* showed significant differences with nutrients (Table 2). Nevertheless, the Ek_{ETR} values were opposite for the two depths. Thus, Ek_{ETR} values for algae collected from 0.5 m depth were higher in non-enriched treatments, whereas in algae from 2.0 m depth waters the values were higher in nutrients enriched treatments (Table 2).

NPQ_{max} in *C. tamariscifolia* showed significant differences due to nutrients treatments in algae collected from 0.5 m depth , and a significant interaction with nutrients and irradiance in those collected from 2.0 m depth waters was observed (Table 2). In algae from both depths, NPQ_{max} was higher in non-enriched treatments, whereas in algae collected from 2.0 m depth, the NPQ_{max} increased on $100\%_{PAB}$ conditions (Table 3). NPQ_{max} did not show any significant differences among treatments in *E. elongata* (Table 2) in contrast to *C. tamariscifolia,* which showed significant differences due to nutrients in both two depths (Table2). Thus, Ek_{NPQ} values in algae collected from 0.5 m depth were higher in enriched treatments, whereas in algae from 2.0 m depth, the values were higher under non-enriched treatments (Table 3). Finally, Ek_{NPQ} showed no significant differences among treatments in *E. elongata* (Table 2).

Pigment content

Chl*a* in *C. tamariscifolia* increased significantly, when algae from 0.5 m depth were exposed to lower irradiance ($70\%_{PAB}$ treatment). Similar results were found for Chl*c* in algae collected from 2.0 m depth (Table 4 and 5). Chl*c* content in *C. tamariscifolia* collected from 0.5 m was significantly higher in nutrient enriched treatment than in the non-enriched one (Table 4 and 5). Both chlorophylls (*a* and *c*), the initial contents were higher in algae collected from 0.5 m depth (Table 5). Chl*a* in *E. elongata* did not present any significant differences among treatments (Table 4 and 5).

PC content was significantly higher in nutrient enriched treatment in *E. elongata* collected from 0.5 m depth. In contrast, PE content did not show any differences after the experiment (Table 4 and 5).

The carotenoids fucoxanthin and violaxanthin in *C. tamariscifolia* showed a significant increase under nutrient enriched treatment at 0.5 m depth (Table 4 and 5). In contrast, carotenoid content in algae collected from 2.0 m depth was significantly higher under $70\%_{PAB}$ treatment (Table 4 and 5). Additionally, for antheraxanthin and β-carotene at the same depth was found significant interaction between nutrient and irradiance. Both compounds increased significantly at $70\%_{PAB}$ in non-enriched treatment site (Table 4 and 5). In *E. elongata*, fucoxanthin, antheraxanthin and β-carotene contents in algae collected

from 0.5 m depth, showed a significant increase in the 70%$_{PAB}$ irradiance treatment (Table 4 and5). Additionally, fucoxanthin content increased significantly in algae cultured under nutrient enrichment conditions (Tables 4 and 5). Zeaxanthin content did not show any differences after the *in situ* experiment (Tables 4 and 5) for both species.

Total phenolic compounds in *C. tamariscifolia* were significantly different among nutrient treatments at both, 0.5 m and 2.0 m depth (Table 6). Additionally, algae collected from 2.0 m depth had significant differences in both irradiance treatments (Table 6). Figure 4a shows that in algae collected from 0.5 m depth, the total phenolic compounds were higher in the nutrient enriched treatment. However, in *C. tamariscifolia* from 2.0 m depth, the differences in phenolic content were more evident under irradiance treatments than nutrient treatments and increased on 100%$_{PAB}$ (Fig. 4a). In *C. tamariscifolia* from 2.0 m depth, the increase of phenolic compounds was higher under 100%$_{PAB}$ than under 70%$_{PAB}$ whereas this increase was higher under non-enrichment than that under enrichment treatment (Fig.4a).

Antioxidant activity (EC_{50}) in *C. tamariscifolia* collected at 0.5 m depth showed a significant interaction between nutrient and irradiance (Table 6). In the non-enriched treatment the EC_{50} was higher (lower antioxidant activity) than in the other treatment combinations (Fig. 4b). In algae collected at 2.0 m depth significant differences were only found in nutrient treatments (Table 6); i.e. the EC_{50} was higher (lower antioxidant activity) in the nutrient enrichment treatment (Fig. 4b) than in the non-enriched treatment.

Total MAA content in *E. elongata* was higher in algae collected from 0.5 m depth than in those collected from 2.0 m depth (Fig. 5a). MAAs content from 0.5 m depth showed a significant increase under 100%$_{PAB}$ in nutrient enriched treatment (Table 7 and Fig. 5a). In contrast, in algae collected from 2.0 m depth waters, was significantly higher on 100%$_{PAB}$ for both nitrogen treatments (Table 7, Fig. 5a). The MAAs detected in this species were Shinorine (50-60%) and palythine (≈40%) as the most abundant, others MAAs such as asterina-330 was presented in traces. After the *in situ* experiment, algae collected from 2.0 m depth showed significantly higher contents of palythine under nutrient enrichment treatments, and in non-enriched treatment was Shinorine (Table 7, Figure 5b, c). In contrast, algae collected from 0.5 m depth did not show any differences (Table 7).

7.4 Subchapter 4:

Photosynthetic activity

The maximum quantum yield of PSII (F_v/F_m) was significantly ($p<0.05$) affected by the interactions between time*copper (Cu) and Cu*nitrate (Figure 1, Table S1). In the low N (N-) treatment, no significant differences amongst the different Cu treatments were observed whereas under N+, Fv/Fm decreased under 0.5 µM Cu^{2+} compared to the control or 2 µM Cu^{2+} (Figure 1, Table S1). The pooled data between time and Cu factors showed that F_v/F_m was highest at 2.0 µM Cu^{2+} at 7d (Figure 1b, Table S1) and between copper and nitrate was highest at 2.0 µM Cu^{2+} at 14d (Figure 1c, Table S1).

The maximal production estimated as ETR_{max} was significantly influenced by interactions between time*copper and copper*nitrate (Figure 2a, b, c, Table S1). The pooled data between time and copper showed that the ETR_{max} was higher in N- treatment at 0.5 µM Cu^{2+} and 7d compared to the control and decreased again at 2.0 µM Cu^{2+}. In contrast in N+ treatment no decrease was observed at 2 µM Cu^{2+} compared to 0.5 µM Cu^{2+} (Figure 2b, Table S1). The pooled data between copper and nitrate factors was higher at 0.5 µM Cu2+ at N- (Figure 2c, Table S1).

The interaction between NPQ_{max} and all factors was significantly ($p<0.05$) (Figure 3, Table S1). In the control and 0.5 µM Cu^{2+} after 7 and 14 d culture, NPQ_{max} was higher under N- than in N+ treatment (Figure 3, Table S1). However, in high copper levels no significant differences with increase nitrate level were found (Figure 3, Table S1).

The photosynthetic efficiency (α_{ETR}) was significantly different ($p<0.05$) by interaction between all factors. The α_{ETR} was higher in high copper levels at 7 d without enrichment treatment. (Tables 1 and S1). The saturated irradiance (Ek_{ETR}), was presents significantly ($p<0.05$) interaction between all factors (Tables 1 and S1). Ek_{ETR} was higher in high copper levels without nitrate enrichment at 7 d of the culture. Copper, nitrate and the interaction time significantly influenced the relationship between ETRmax/NPQmax (production related to energy dissipation related to photoprotection)*nitrate (Tables 1 and S1).

Internal and external cellular copper content

The total copper and internal cellular copper concentrations were significantly ($p<0.05$) affected by the supply of copper in the water (Figure 4, Table S2). After 14 d culture, total copper content showed maximal values in 2.0 µM Cu^{2+} with 262.4 ± 22.5 µg g^{-1} DW with and without nitrate whereas in the control conditions showed minimal values

69.9 ± 9.6 µg g^{-1} DW in the same nitrate levels (Figure 4, Table S2). The internal copper content of *C. tamariscifolia* was greatest in 2.0 µM Cu^{2+} with maximal values 129.7 ± 15.5 µg g^{-1} DW and minimal values in control conditions of the 29.3 ± 2.7 µg g^{-1} DW, these values were in both nitrate conditions (Figure 4, Table S2).

Nitrogen and carbon internal content

The nitrogen internal content was higher after 7-day culture in all treatments respect to the end the experimental period (Figure 5a, Table S3). Nitrogen internal content was significantly ($p<0.05$) affected by the interaction between time *copper and copper*nitrate (Figure 5b, c, Table S3). The pooled data between time and nitrate factors showed that the internal nitrogen was the highest at 7d in low nitrate treatment (Figure 5b) and between copper and nitrate was highest at 0.5 µM Cu^{2+} with nitrate enrichment (Figure 5c, Table S3). The internal carbon content did not change significantly over the period of the experiment (Table S3). C content of *C. tamariscifolia* showed maximal values 268.2 ± 13.7 mg g^{-1} DW and minimal values of the 244.6 ± 3.7 mg g^{-1} DW. The C: N ratio was influenced time and copper level (Table S3).

Pigment content

The interaction between Chlorophyll a (Chl*a*) and all factors content was significant ($p<0.05$) (Figure 6a, Table S4). The Chl*a* content was highest in 2.0 µM Cu^{2+} in both nitrate conditions, and it was highest in nitrate enrichment in middle copper treatment (Figure 6a, Table S4). Chlorophyll *c* (Chlc) and Fucoxanthin (Fx) contents presented significant ($p<0.05$) interaction with time and copper concentrations (Figure 6b, c, Table S4). Both pigments were highest at the end the experimental period in high copper concentration with and without nitrate treatments (Figure 6b, c, Table S4).

Polyphenolic compounds and antioxidant activity

Total phenolic content (PC) presented significantly ($p<0.05$) interaction among all factors (Figure 7a, Table S5). At 14 d culture, the total phenolic content increased at high copper level independent of the nitrate treatments (Figure 7a, Table S5). On a control with N+ and middle copper with N- treatments, PC was also higher at 14d (Figure 7a, Table S5). The interaction between content of phenols in the water (PCw) and all factors was significantly ($p<0.05$) (Figure 7b, Table S5). The phenolic compound levels in the water were higher after 7 d than after 14 d culture except in the control under N-treatment (Figure 7b). Under N+ treatment and after 7 d, and only under 2.0 µM Cu^{2+}, the PCw was higher than that in the control and 0.5 µM Cu^{2+} whereas after 14 d no significant effect of copper was detected (Figure 7b, Table S5). The antioxidant activity (as EC_{50}),

was affected ($p<0.05$) by the interaction between all factors (Figure 7c, Table S5). EC_{50} was higher (lower EC_{50}) in high copper levels under N+ than N- treatment, in both times; at 7 and 14 d culture (Figure 7b, S5).

The phenolic compounds, i.e., Shikimic acid and phloroglucinol were found in all treatments after the experimental period (Table 2, S6). At initial time, we observed the presence of others compounds i.e. quinic acid, gallic acid and kaempferol. In the control copper conditions, without nitrate enrichment only gallic acid was found and benzoic acid with nitrate enrichment conditions (Tables 2 and S6). In high copper treatments without nitrate conditions, quercetin was found (Tables 2 and S6). The shikimic acid and phloroglucinol did not show significant differences in the interaction between copper and nitrate. However, shikimic acid was the highest in high copper concentrations whereas phloroglucinol was the highest under both high and low copper concentration with nitrate enrichment (Tables 2 and S6).

Principal Coordinates Analysis

The Principal Coordinates Analysis (PCO) (Figure 8) revealed a positive correlation of the first axis (43.1% of total variation) with the internal PCw, N content, F_v/F_m and PC. In contrast, the internal C content, EC_{50}, Chl*c*, Chl*a*, fucoxanthin and ETR_{max} were negatively correlated with this axis. Taking into account the spatial distribution of the samples in relation to the studied factors, the time presented a high relationship with the mentioned axis (Figure 8). Moreover, the combination of the first two axes explained the 62.3% of the variation in these variables (Figure 8). Moreover, the small angles between the arrows are indicative of high correlation between the variables they represent; thus, the plot gave an idea of the relationships between the variables included. In the Figure 9, PCO revealed a positive correlation of the first axis (68.1% of total variation) with the internal NPQ_{max}. In contrast, all the rest to the variables were negatively correlated with this axis whereas, the combination of the first two axes explained the78.5% of the variation in these variables (Figure 9).

Correlation analysis

In the control copper treatment, F_v/F_m was positively correlated to internal N (r=0.605, P=0.03, n=12), whereas ETR_{max} was not related to internal nitrogen (r=-0.286, P=0.36, n=12), but it was positively correlated to NPQ_{max} (r=0.610, P=0.03, n=12). Internal copper level was positively correlated to the external copper concentration in the water (r=0.548, P=0.01, n=18) and to Ek_{ETR} (r=0.658, P<0.01, n=18), EC_{50} (r=0.848, P<0.01, n=18) and all photosynthetic pigments; Chl*a* (r=0.669, P<0.01, n=18), Chl*c* (r=0.752,

P<0.01, n=18) and Fucoxanthin (r=0.737, *P*<0.01, n=18). External copper levels were positively correlated to all photosynthetic pigments; Chl*a* (r=0.754, *P*<0.01, n=18), Chl*c* (r=0.852, *P*<0.01, n=18) and Fucoxanthin (r=0.808, *P*<0.01, n=18). ETR_{max} was not significantly correlated to internal copper (r=0.367, *P*=0.13, n=18) or nitrogen (r=0.410, *P*=0.09, n=18), whereas photosynthetic efficiency (α_{ETR}) was negatively correlated to ETR_{max} (r=-0.503, *P*=0.03, n=18) and Ek_{ETR} was positively correlated to EC_{50} (r=0.596, *P*<0.01, n=18). The internal nitrogen was positively related to EC_{50} (r=0.497, *P*=0.03, n=18), Chl*a* (r=0.576, *P*=0.01, n=18) and Chl*c* (r=0.517, *P*=0.02, n=18).

7.5 Subchapter 5:

Environmental conditions

Cystoseira compressa and *Padina pavonica* were abundant at all three stations; *P. pavonica* was visibly less calcified at the site with the highest levels of CO_2. The seawater temperature was about15 ºC and the salinity was 38 at all stations; at the Ambient site, mean pH was 8.11, at the Medium CO_2 site (700-800 µatm), mean pH was 7.97 and at the High CO_2 site (1200 µatm), it was 7.86 (Table 1).

The average daily irradiance for the experimental period was 5360 kJ m^{-2} for PAR and 666 kJ m^{-2} for UVA. The nutrient enriched treatments had approximately 100 times the nitrate concentration of the ambient seawater; ambient *vs* enriched ratios were 0.16 ± 0.04 *vs* 106.17 ± 9.37 µM for the ambient site, 0.13 ± 0.01 *vs* 106.33 ± 9.37 µM at the medium CO_2 site and 0.25 µM ± 0.01 *vs* 106.42 ± 9.37µM at the high CO_2 site (mean ± SE, n = 3).

Physiological and biochemical responses

The carbon content of *C. compressa* increased with increasing CO_2, whereas in *P. pavonica* showed interactive effects between all factors. Carbon, in *P. pavonica* showed maximal values 279.9 ± 6.5 with increased CO_2, in non-enrichment enriched treatments and minimal values 225.3 ± 2.4 mg g^{-1} DW with decreased CO_2, non-nutrient enriched and $70\%_{PAB}$ conditions (Table 2, S1). The nitrogen content of *C. compressa* was greatest in the high CO_2, nutrient enriched and $70\%_{PAB}$ treatment (Figure 2A, S1); conversely, in *P. pavonica* the nitrogen content was highest at the reference site, ambient CO_2 treatment (Figure 2B, S1). The ratio C:N of *C. compressa* did not show significant differences between treatments (Figure 3A, S1), whereas in *P. pavonica* showed significant effects

for CO_2 levels and nutrient enrichment (Figure 3B, S1). The C:N ratio , in *P. pavonica* showed maximal values (19.5 ± 5.8) with increased CO_2, non-nutrient enriched in $100\%_{PAB}$ conditions and minimal values 15.9 ± 0.5 in medium CO_2, nutrient enrichment and $70\%_{PAB}$ conditions (Figure 3B, S1).

The maximal quantum yield (F_v/F_m) was significantly different between CO_2 treatments, nutrient and irradiance in both macroalgae (Figure 4, S2). In *C. compressa*, the F_v/F_m was greatest in $70\%_{PAB}$ treatments with high CO_2, and non-enriched enrichment (Figure 4A, S2), but in *P. pavonica* this was greatest in the nutrient enriched treatments (Figure 4B, S2). The α_{ETR} values also varied significantly between treatments in both species (Table 2, S2). In *C. compressa,* α_{ETR} was greatest in $70\%_{PAB}$ treatments at high CO_2 with non-enriched enrichment; in *P. pavonica* α_{ETR} was greatest in the high CO_2 conditions (Table 2, S2). ETR_{max} in *C. compressa* was highest in high CO_2, $70\%_{PAB}$ and non-nutrient enrichment in $100\%_{PAB}$ and nutrient enrichment, and this was higher with decreased CO_2, $100\%_{PAB}$, in non-nutrient enrichment. In *P. pavonica*, ETR_{max} varied significantly depending on nutrient and irradiance, without interactions (Table 2, S2). In contrast, the Ek_{ETR} in *C. compressa* had one significant interaction among nutrient x irradiance. *P. pavonica* had significant interactions between CO_2 level, nutrient and irradiance. The Ek_{ETR}, in *C. compressa*, was greatest in the $100\%_{PAB}$ treatments that had no CO_2 or nutrient enrichment, but in *P. pavonica* Ek_{ETR} was greatest in $70\%_{PAB}$ conditions (Table 2, S2). In both species, the maximal non-photochemical quenching (NPQ_{max}) was affected by the interaction of all factors. In *C. compressa*, NPQ_{max} increased significantly with increasing CO_2 conditions, under nutrient enriched and $100\%_{PAB}$, also increased in *ca* 700-800 µatm but in $70\%_{PAB}$. As well as, NPQ_{max} increased under ambient CO_2 conditions in $100\%_{PAB}$ in nutrient non-enriched. Finally, in *P. pavonica,* the NPQ_{max} was significantly higher in $70\%_{PAB}$ at 700 µatm CO_2 treatment with nutrient enrichment (Table 2, S2).

Nutrient enrichment increased Chl*a* significantly in *C. compressa*. In contrast, in *P. pavonica* significant differences were found for the following interactions: CO_2 level x nutrient, CO_2 level x irradiance and nutrient x irradiance (Table 3, S3). The same occurred for Chl*c* in *P. pavonica*; but there was no significant difference in *C. compressa* (Table 3, S3). The carotenoids, fucoxanthin and violaxanthin in *C. compressa* did not differ among factors (Table 3, S3) but in *P. pavonica,* the fucoxanthin and violaxanthin contents were affected by the interaction of all factors. Fucoxanthin increased in $70\%_{PAB}$, non-

enriched treatments in ambient CO_2 whereas violoxanthin levels were highest in $70\%_{PAB}$, *ca* 700-800 µatm CO_2, nutrient enriched treatment (Table 3, S3).

Phenolic content (PC) was affected by the interaction of all factors in both species (Figure 5, S4). In *C. compressa*, PC was highest in CO_2 and nutrient enriched conditions (Figure 5A, S4). In *P. pavonica* at 1200 µatm CO_2, PC was high in $100\%_{PAB}$ and nutrient enriched treatments and in $70\%_{PAB}$ treatments non-nutrient enrichment (Figure 5B, S4). Antioxidant activity (EC_{50}) showed a significant interaction between CO_2 level x nutrient and CO_2 level x irradiance in *C. compressa*; however, in *P. pavonica* the only significant difference found in antioxidant activity was between CO_2 level and irradiance. In *C. compressa* and *P. pavonica*, EC_{50} was lowest (i.e. it had higher antioxidant activity) in the high CO_2, $70\%_{PAB}$ light conditions and nutrient enriched treatments (Table 3, S4).

7.6 Subchapter 6:

Environmental conditions

The seawater temperature during the experiment was about 19ºC, the salinity was 38psu at all treatments in ambient temperature, mean pH were 8.34-8.22 and 7.88 at ambient CO_2 and high CO_2, respectively (Table 2). The average daily-integrated irradiance for the experimental period was 4238 kJ m^{-2} for PAR, 329 kJ m^{-2} for UVA and 22 kJ m^{-2} for UVB. The nitrate and phosphate concentration of the seawater enriched ratios during the experimental period, were 1.27 ± 0.36 and 0.15 ± 0.01µM (mean ± SE, n = 8), respectively (Table 2).

Physiological and biochemical responses

The Principal Coordinates Analysis (PCO) (Figure 2) revealed a positive correlation of the first axis (41.3% of total variation) with the internal N content, ETR_{max}, F_v/F_m, PC, Chl*a* and Chl*c*. This suggest that these variables increased in the first week of culture of the experimental period (symbols in blue colour). The measurements in the middle the experimental time had not any variation (0 %, data no shown). In contrast, at the end the experimental period, the internal C content, EC_{50}, violaxanthin, fucoxanthin, β carotene and PCw, negatively correlated with this axis (symbols in red colour). Taking into account the spatial distribution of the samples in relation to the treatments, time presented a high relationship with the mentioned axis. Moreover, the combination of the first two axes explained the 66.6% of the variation in the response variables (Figure 2).

(a) Growth

The biomass of *C. tamariscifolia* had interactive effects between origin of the populations and CO_2 ($P<0.01$). The *C. tamariscifolia* (from CG and LA) increased in elevated CO_2 conditions (Figure 3, Table S1). However, the algae from ultraoligotrophic waters, the biomass was higher than algae from oligotrophic waters (Figure 3, Table S1).

(b) Carbon and Nitrogen content responses

The carbon content of *C. tamariscifolia* (from CG and LA) increased with increasing CO_2 and ambient temperature. Carbon content was significantly different for all variables, this indicate additive effects. *C. tamariscifolia* (from CG and LA), showed maximal values 316.9 ± 4.98 and 308.3 ± 6.4 mg g^{-1} DW and minimal values 283.7 ± 4.4 and 267.9 8. ± 8.1 mg g^{-1} DW, respectively (Tables 3, S2). Nitrogen content have interactive effects between time, temperature and CO_2 conditions, indicating that nitrogen internal content increased in all treatments at the end the experimental period, respect to first week for macroalgae collected from both sites (Tables 3, S2). The ratio C: N has interactive effects between time and origin of the population. In both macroalgae (from CG and LA), C: N decreased at the end the experimental period respect to the first week (Tables 3, S2).

(c) Photophysiological variables

The maximal quantum yield (F_v/F_m) was significantly different between time, temperature and CO_2 conditions for macroalgae collected from both sites (Tables 3, S3). F_v/F_m at the first week, was greatest in ambient temperature and ambient CO_2 conditions in *C. tamariscifolia* (from CG and LA), but at the end the experiment, F_v/F_m increased in high CO_2 conditions of *C. tamariscifolia* (from LA) (Tables 3, S3).

The maximal electron transport rate (ETR_{max}) had interactive effects between all factors. ETR_{max} for macroalgae collected from both sites, were higher in high CO_2 conditions with ambient TºC (Figure 4a and b, S3). The highest ETR_{max} was observed under enriched CO_2 conditions and ambient temperature at the first two weeks whereas in the last two weeks the ETR_{max} decreased in all conditions, for *C. tamariscifolia* (from CG and LA) (Figure 4, Table S3).

(d) Phenolic compound and antioxidant activity

Phenolic content (PC) was affected by time, temperature, CO_2 conditions and origin of population; this indicates additive effects in both macroalgae. *C. tamariscifolia* (from CG and LA), the phenols was highest in high CO_2 conditions independent of the temperature (Figure 5a, b and Table S5). In *C. tamariscifolia* (from CG), the polyphenols was *ca* 1.5 - 3.0 % whereas in *C. tamariscifolia* (from LA), the PC was *ca* 1.0 - 2.2 %

(Figure 5, Table S4), this suggest more phenolic production and photoprotection in the macroalgae collected from ultraoligotrophic waters than oligotrophic waters.

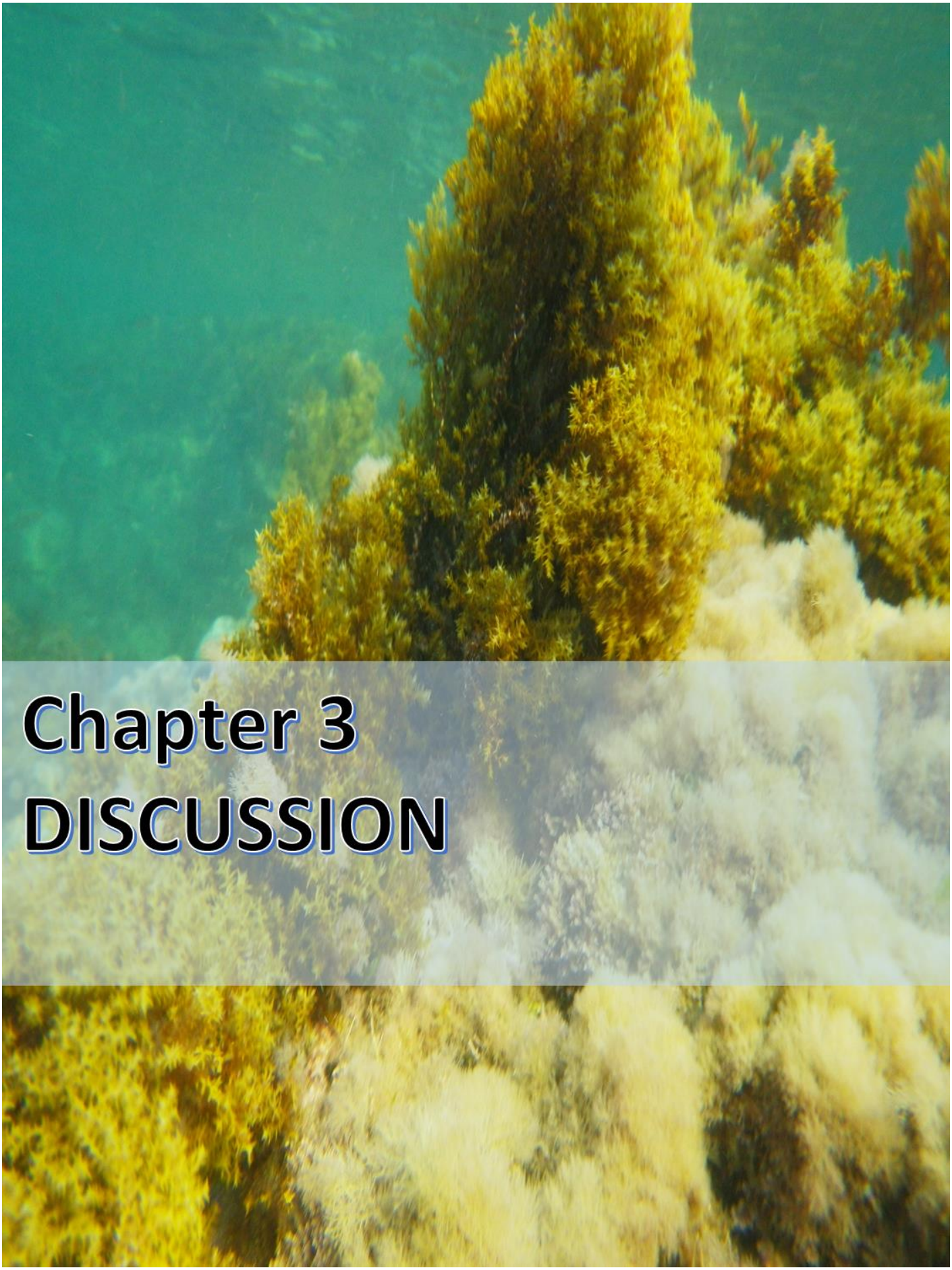
Antioxidant activity (EC_{50}) had interactive effects between time, CO_2 conditions and origin population (Figure 6, Table S4). In *C. tamariscifolia* (from CG and LA), the antioxidant activity were higher (i.e. lower EC_{50}) in high CO_2 and ambient temperature. In *C. tamariscifolia* (from CG), EC_{50} was higher in high CO_2 levels and high temperature (Figure 6, S4).

The phenolic compounds in the water (PCw), was affected for time, CO_2 conditions and origin populations (Figure 7, Table S4). PCw was higher in C. *tamariscifolia* from LA than *C. tamariscifolia* from CG. In this case, the polyphenols in the waters were highest in ambient CO_2 level independent of the temperature and origin of the macroalgae. This suggest, they had not additive effects, under this variable, because it was only affects for CO2 level (Figure 7, Table S4)

(e) Photosynthetic pigments

Chl*a* showed interactive effects between time, temperature and CO_2 levels (Tables 4, S4). In *C. tamariscifolia* from both sites, the greatest Chl*a* was content was observed in high temperature and high CO_2 conditions, and in ambient temperature and ambient CO_2 conditions, at the first week. However, in *C. tamariscifolia* from CG (ultraoligotrophic waters), Chl*a* at the end the experimental period, was higher ambient CO_2 conditions independent of the temperature. In addition, in *C. tamariscifolia* from LA was higher in ambient CO_2 levels in high temperature (Tables 4, S4). The Chl*c* content from macroalgae collected from both sites had interactive effects between time and CO_2 conditions. The highest Chl*c* content of *C. tamariscifolia* from both sites was reached in ambient temperature and high CO_2 levels (Tables 4, S4).

Significant quantitates of the fucoxanthin, violaxanthin and β-carotene (Tables 4, S4) in all treatments were detected but only traces of antheraxanthin, lutein and zeaxanthin (data no shown). The content of fucoxanthin was affected time, temperature and origin populations whereas violaxanthin was affected by the interaction between time, temperature and CO_2 levels (Tables 4, S4). The β-carotene content was significant affected by time, temperature and origin of populations (Tables 4, S4). Fucoxanthin and Violaxanthin contents, in *C. tamariscifolia* collected from both sites, were the highest at the end the experimental period in ambient CO_2 conditions independent of the temperature (Tables 4, S4). However, β-carotene was higher in high temperature and ambient CO_2 level (Tables 4, S4).



Chapter 3
DISCUSSION

Cabo de Gata-Níjar, Natural Park. September 2013. Photograph by Paula S. M. Celis Plá

3. DISCUSSION

In this study, functional indicators suggested by Figueroa and Korbee (2010) have been used to evaluate the vulnerability and acclimation capacity to environmental variables related to stress conditions and climate change: (1) *in vivo* chlorophyll fluorescence as indicator of photosynthesis (yields, production and energy dissipation), (2) stoichiometric C:N ratio as indicator of nutritional state and (3) polyphenols, mycosporine-like aminoacids and carotenoids as biochemical indicators of the acclimation mechanism to stress conditions due to both theirs both photoprotection and antioxidant capacities.

Chlorophyll *a* fluorescence has proven to be a powerful tool for the analysis of spatial and temporal variation in quantum efficiency and photosynthetic performance, and for the analysis of short-term and responses to several environmental factors in higher plants (Schreiber et al. 1995, Baker 2008) and both micro and macroalgae (Suggett et al. 2011, Hanelt and Figueroa 2012). A great number of studies have proven this a powerful tool for the elucidation of fundamental processes in photosynthesis (Maxwell and Johnson 2002, Wilhelm et al. 2004). In addition, there are broad number of reports that use the chlorophyll *a* fluorescence signal for the study the macroalgae photosynthetic performance and/or stress with both physiological and ecological approaches (Hanelt 1998, Häder et al. 2002, Longstaff et al. 2002, Gómez et al. 2004, Figueroa et al. 2006, Enríquez and Borowitzka 2011, Gómez and Huovinen 2011). The capability of photoinhibition of photosynthesis has been related to depth distribution of macroalgae (Hanelt 1998, Gómez and Huovinen 2011). The photoinhibition of photosynthesis is a decrease of the maximum quantum yield of PSII (F_v/F_m) and it is used as an indicator of physiological status of macroalgae (Schreiber et al. 1986). The photoinhibition of photosynthesis can be produced under high solar irradiance (PAR and UVR) depending on the daily changes in irradiance, vertical light attenuation, or the combination of both (Häder and Figueroa 1997, Wiencke et al. 2000). Photosynthetic activity was simultaneously determined as O_2 evolution rates and PAM-fluorescence) in algae exposed to different light quality conditions (Figueroa et al. 2003) including UV radiation (Figueroa et al. 1997, Flores-Moya et al. 1998). It is generally well recognized as the major breakthrough study in using fluorescence as a means to examine photosynthetic yields and capacities (Genty et al. 1989). Thus, ETR (PSII) was found to be linearly related to the rate of CO_2 fixation as gross photosynthesis (Genty et al. 1989, Flameling and Kormkamp 1998, Carr and Björk 2003), with the proportionality coefficient being

the electron requirement for CO_2 fixation. The ratio ETR/GP is about 5) although under high irradiances or nutrient depletion the ratio ETR/GP is higher than the expected value of 5 (Figueroa et al. 2003).

Similar observations relation between oxygen and fluorescence have been found in higher plants (see Baker and Oxborough 2004) and they confirmed that PSII photochemical efficiency appeared to be strongly correlated with photosynthetic CO_2 fixation. Electrons transported by PSII can follow several competing pathways: the majority of the electrons are normally used to reduce CO_2 to carbohydrates and nitrate assimilation , allowing the synthesis of other cellular macromolecules like proteins, lipids or nucleotides, but some of them might be lost by alternate cellular processes (alternate electron cycling) or dissipated (non-photochemical quenching) (Ralph et al. 2011). A liner relation between electron transport rate and gross photosynthesis by oxygen evolution was only found under irradiances below Ek for gross photosynthesis, thus, under higher irradiance than Ek, an increase in ETR is produced without the correspondent increase of gross photosynthesis (Figueroa et al. 2003). This stationary phase is explained by oxygen consumption process as Mehler reaction and cyclic photosynthetic chain around both PSII and PSI (Flameling and Koromkamp 1998, Figueroa et al. 2003, Carr and Björk 2003).

In addition to F_v/F_m and ETR, non-photochemical quenching (NPQ) is other relevant fluorescence parameter. It is originates in the light-harvesting antenna (LHC) of PSII being one of the most important mechanism for the fast regulation of photosynthesis in higher plants as well as in algae related to protons gradient in the chloroplast i.e. energy dissipation (Szabo et al. 2005, Demmig-Adams and Adams 2006, Lavaud 2007). In addition, NPQ is related to xanthophyll cycles (Enríquez and Borowitzka 2011).

The physiological status of the seaweeds can be estimated through of the stoichiometric ratios (according to Figueroa and Korbee 2010). Carbon: nitrogen (C:N) ratios can give information about the nutritional status of the macroalgae and can be used to evaluate any possible damage to internal compounds that may be caused by stressors such as high irradiance, UVR and high temperature (Sterner et al. 1997). The nitrogen availability limitation, affects a lot of process in macrophytes not only the photosynthetic capacity (Pérez-Lloréns et al. 1996) but also the content of proteins (Henley et al. 1991, Vergara et al. 1995), and photoprotection mechanisms (Korbee et al. 2005, Huovinen et al. 2006).

The process of stress acclimation leads to a number of photoprotective mechanisms. We have analysed as biochemical indicators, photoprotectors and the antioxidant activity. In brown macroalgae, UV screen compounds, with antioxidant capacity are the polyphenols (Pavia et al. 1997). The content of polyphenols can change in function of the spatial and temporal variations in the environmental factors such light and temperature (Arnold and Targett 1998, Connan et al. 2004) as the availability of nitrogen (Ragan and Glombitza 1986, Arnold et al. 1995). In red macroalgae, the main photoprotectors with antioxidant capacity are the mycosporine like amino acids (Karsten et al. 1998, Carreto et al. 2005, De la Coba et al. 2009). The main function of MAAs is photoprotection, especially for its ability to absorb short wavelengths of UVA and UVB regions of the spectra. This coupled with high photo-stability make them effective agents protective against UV radiation (Korbee et al. 2006).

The carotenoids due to their antioxidant capacities (Takaichi 2011) and their state of the photoprotective system can also serve as an integrating indicator of the overall functional and developmental state of plants (Demmig-Adams and Adams 2006, Schubert et al. 2011).

The functional indicators as suggested by Korbee and Figueroa (2010) have previously been applied in three Doctoral Thesis and published in different papers (García-Sánchez et al. 2012, Martínez et al. 2012a, Quintano et al. 2013, García-Sánchez et al. 2014, Figueroa et al 2014a, b, Korbee et al. 2014, Celis-Plá 2014a, b, Betancor et al. 2014, Betancor et al. 2015, Celis-Plá et al. 2015). The three doctoral Thesis were conducted in the frame of a coordinated project entitled "Ecological status and vulnerability of aquatic ecosystem to climate changes: biological, ecological and functional indicators monitoring the adaptation response of macrophytes to the environmental stress" (ECOLIFE CGL08-0565, 2009-2011). One thesis was conducted in the habitat forming species of Cantabric Sea i.e. *Gelidium corneum* which are suffering a reduction in frond density, weight loss and yellowing related to increase of PAR and UVA irradiances in the subtidal areas of Basque Country Coast (Quintano 2014). A second thesis was conducted in algae and marine angiosperms from Mediterranean, confined in the Mar Menor lagoon (Murcia) compared to the algae growing in the coast (Cabo de Palos) (García-Sánchez 2015). The third study was conducted in the Atlantic coast waters of Canary Islands paying special attention to habitat-forming species of the genus *Cystoseira* and other species of Fucales as *Fucus spiralis*, which is being reduced in the intertidal area (Betancor 2013). This thesis has been conducted in habitat-forming

species of Alboran Sea. As *Cystoseira tamariscifolia* growing both in the western oligotrophic area (Malaga coast), and ultraoligotrophic eastern area (Almeria) in the frame of the above-cited project and other research project entitled "Interactive effect of acidification and global change variables (UV radiation and temperature) on the ecophysiology of marine macrophytes of Andalusian Mediterranean" (INTERACID RNM-5750, 2011-2014).

There is still scarce information on the effects of climate change variables, on the structure of the population and primary production of *Cystoseira* communities. In spite of they are considered bioindicators of the high-quality waters according to the criteria of Water Framework Directive of the European Union (WFD, 2000/60/EC) according to Ballesteros et al. (2007), Arévalo et al. (2007) and Bermejo et al. (2013). In addition, several *Cystoseira* species are listed as strictly protection under the Berne Convention (Annex I 1979) and all the Mediterranean species of the genus *Cystoseira*, except *C. compressa* have been listed under Annex II of the Barcelona convention (2010) (Thibaut et al. 2014).

Thus, in this study a novel aspect was to analyze the interaction of solar radiation with other climate variables such as temperature change and other anthropogenic disturbances such as the availability of nutrients or heavy metals (copper) in coastal waters and thus give information about the ecophysiological responses of this group of macroalga related with different stressors. Always taking into account that the interplay of factors can change the sign of the effect of a single factor (antagonistic effect) or, conversely, accentuate (synergistic effect).

As previous studies, Häder et al. (1996) found in *Cystoseira* spp. that the photosynthetic quantum yield was not depressed at midday when UV irradiance was cutoff. Abdala-Diaz et al. (2006) investigated the annual and daily fluctuations of phenolic compound concentration in the tissue of *C. tamariscifolia*. These algae showed an annual cycle of phenolic compound concentration in the apical thalli, which was positively correlated with incident irradiance in ultraoligotrophic waters in the eastern part of Alboran Sea. This investigation show the increase in phenolic compounds, was greater in the first half of the year, respect to the second half of the year. Loss of phenolic compounds from the tissue to the surrounding water was increased in function of the dose-integrated irradiance (at noon in summer time).

Historical declines of *Cystoseira* species have been reported in many regions of the Mediterranean Sea (Bouderesque 2003, Gianni et al. 2013), but the scarce of studies of

Cystoseira spp. respect to the increase of temperature due to climate change (Serio et al. 2006, Strain et al. 2014). There is still no clear link between physiological responses to geographic distribution in *Cystoseira* spp. due to the scarce physiological studies in the species of this genus (Orfanidis 1991, Mercado et al. 1998, Celis-Plá et al. 2014a, b, Figueroa et al. 2014a, b). At the moment, the regression of the *Cystoseira* populations have been attributed to stressors such as direct degradation or destruction of habitat, overgrazing, eutrophication or chemical pollution (Sala et al. 1998, Cormaci and Furnari 1999, Benedetti-Cecchi et al. 2001, Thibaut et al. 2005, Airoldi and Beck 2007, Mangialino et al. 2008). Rodríguez and Polo (1986), investigated about the effects of sewage pollution in the structure and dynamics of the community of *Cystoseira mediterranea.*

Experiments performed with *Ulva rigida*, *Cystoseira tamariscifolia* and *Corallina elongata* collected in the western part of Alboran Sea (Figueroa et al. 2014a, b, Stengel et al. 2014). Indicate that the degree of response among the species to simultaneous changes in nutrients, radiation and temperature can become quite different and consequently the competitive performances will result modified, which in turn will affect the community structure. A low affinity for C was reported for carbonic anhydrase in *C. tamariscifolia* (Mercado et al. 1998); therefore, the direct uptake of CO_2 when the alga is exposed to the air favored the photosynthetic activity. In *Cystoseira crinita*, Sales and Ballesteros (2012) reported higher growth rate from the northwestern Mediterranean in summer than that in winter period.

Ballesteros et al. (2007) designed a non-destructive methodology (CARLIT), that permit the use to the most *Cystoseira* species (and other seaweeds of the order Fucales that dominate the intertidal zone in most temperate to subtropical areas) as biological indicators. They are very sensitive and vulnerable to natural or anthropogenic disturbances and have very slow recovery rates (Thibaut et al. 2005, Arévalo et al. 2007). This method permit the continuous monitoring of the coastline allows the location of small sewage outfalls and other environmental problems at a reduced scale, which is extremely important in the establishment of accurate management plans.

In addition the evaluation of the physiological and biochemical responses under in situ and outdoor controlled conditions allow us to define the level of substances with biotechnological application as carotenoids as polyphenols.

The acclimation and vulnerability of *C. tamariscifolia* to stress and climate change factors can be explained by the functional responses (photosynthesis, stoichiometry and

biochemistry) to the different question as we ask through the different chapters in studies conducted *in situ* and with experimental approach both in outdoor and laboratory controlled conditions.

3.1 What is the vulnerability and capacity of acclimation of Cystoseira tamariscifolia through the seasonal period? (Subchapter 2.1)

Before any experimental approach to evaluate the effects of climate change factors, it is necessary to know the functional responses in the present environmental conditions. This is an adequate temporal scale as seasonal variations through several years or comparing physiological responses in different seasonal periods at appropriate spatial scale. The first approach, seasonal study on *C. tamariscifolia* growing in the rocky shores of intertidal system (oligotrophic waters of La Araña beach in Málaga), was presented in subchapter 2.1.

The expected physiological responses are as follows: algae present the highest production in spring since this is the most favorable season: optimal light conditions. i.e., high irradiance but not photoinhibitory and nutrient conditions, i.e., the highest nutrient levels in seawater is reached in springtime (see hydrographic characteristic reported in Table 1 and 2 in the Chapter 1, introduction) whereas in the less favorable environmental condition was reached in summer time. The acclimation mechanisms are activated in summer time as increased of phenolic compounds, antioxidant activity and energy dissipation (high non-photochemical quenching).

In the seasonal study (Subchapter 2.1), we found the highest photosynthetic activity (ETR_{max}) and maximal photosynthetic efficiency (F_v/F_m) during the springtime in rocky shore *Cystoseira tamariscifolia* and they were related to the highest nutrient level in the seawater and internal content in this season. In addition, a more favorable temperature is expected in spring compared to summer since the optimal temperature for growth in this species is around 19-20^{o}C (observed in springtime) according to Gómez-Garreta et al. (1994) and García-Sánchez (2015). In algae collected from rocky shores under increased the PAR and UV irradiance (i.e. summer time), the photosynthetic activity (ETR_{max} and F_v/F_m) decreased, i.e., photoinhibition of photosynthesis activity respectively. On the other hand, the highest nitrogen and carbon internal contents were detected in spring and the lowest in summer, thus, a relation between photosynthetic efficiency and production and favorable stoichiometric C:N ratio was observed i.e., values of 13-15 in winter and

spring and 16-20 in autumn and summer. This suggest that nutritional state seems to be more favorable in winter or spring than in summer or autumn for this macroalgae. In any case, the C:N ratios are higher than that observed in other species growing in waters with higher nutrient levels (Duarte 1992, Kaehler and Nesh 1996, Lourenco et al. 2000) indicating that the production of *Cystoseira tamariscifolia* in Alboran Sea could be limited by the low level of nitrate and phosphate in the Mediterranean. We observed that the C:N ratio in *C. tamariscifolia* of Atlantic waters in the summer time (Southern England, UK) was about 14 (Subchapter 2.4) and in spring in Northern Portugal was 13 according to García- Sánchez (2015), higher than that observed in *C. tamariscifolia* from Alboran sea in summer time (Subchapter 2.1).

In oligotrophic waters the nutrient content are limiting and consequently, photosynthetic activity can be reduced. The cover by *C. tamariscifolia* of intertidal rocky shores in springtime was higher than that in summer period (Figure 1 in subchapter 2.1). The reduction of cover is summer period can be related to the photoinhibition by the elevated solar irradiance and possible thermal stress. In addition, the acclimation mechanisms in summer demand energy for photoprotection reducing the available energy for growth (Raven 2011). In summer, antioxidant activity increases with the increase of C:N ratio, as it was found by Ibrahim and Jaafar (2011). Thus, in the less favorable nutritional conditions, the antioxidant activity increases too i.e., in summer time. This response is an evidence of the high vulnerability, because the antioxidant activity was higher but phenolic compounds, a photoprotection indicator, was lower than that in wintertime. This suggest, that the high antioxidant activity in summer time can be due by other compounds e.g., carotenoids or xanthophyll cycle or due to phenolic release is accelerated in summer time, reducing the internal but contributing, in certain scale, to photoprotection (Swanson and Druehl 2002, Koivikko et al. 2005, Polo et al. 2014, Celis-Plá et al 2014a). In brown algae, phenols are stimulated by high light levels (Pavia and Brock 2000) without nutrient limitation. Thus, peaks in phenol content are often not found in summer since nitrate concentrations become very low in this season and nutrient limitation or starvation is produced (Pavia and Toth 2000, Cabello-Pasini et al. 2011). We suggest that the photoprotection mechanism in *C. tamariscifolia*, is regulated through the released of the phenols and it is not excluded the activation of other photoprotectors as carotenoids.

The internal concentration of phenolic compounds presented positive correlation with the internal nitrogen content in spite of they are not N compounds as it is the case

of other N compounds with photoprotection capacity i.e., mycosporine like amino acids and biliproteins in red macroalgae (López-Figueroa and Rüdiger 1991, Korbee-Peinado et al. 2004). As conclusion, algae in spring are less vulnerable due to the light is more favorable, temperature and nutrient environmental conditions but we observed that the algae in summer could acclimate to increased stress conditions by the increase in the antioxidant activity.

3.2 What is the vulnerability and capacity of acclimation of Cystoseira tamariscifolia, from to emersion/immersion microhabitat and incubated in an experimental system under out-door conditions? (Subchapter 2.2)

On the spatial scale, in the intertidal area of La Araña beach (oligotrophic waters) two *Cystoseira* population were detected, the above cited rocky shores grown algae and other population of submerged algae in small ponds connected to the open sea (rockpool algae). Rocky shores and rockpool population are very close in the coastal area, but they are exposed to different light, temperature and nutrient history since rocky shore algae are exposed to the air certain time during the daily cycle, whereas rockpool algae are always submerged. The physiological responses in these two populations were analyzed in two seasons in which extreme environmental characteristic are expected as; winter and summer (see Subchapter 2.2).

The functional responses of this two different grown algae collected in two seasons (summer and winter) was evaluated as experimental approach transferring the algae to cylindrical vessels and exposed, to outdoor controlled solar conditions. Algae collected in winter were incubated to an average water temperature of 18^{o}C and in summer time at 22^{o}C simulating natural temperature conditions. The experiment were conducted under non-limited nitrate conditions (5 to 50μM Nitrate injection).

The expected results are as follows: algae form rocky shores present higher production than that from rockpools because the increased CO_2 supply during the emersion periods is favorable since the photosynthetic activity of *C. tamariscifolia* since it is subsaturated in CO_2 with a low conductance for CO_2 and low carbonic anhydrase activity (Mercado et al. 1998). Since Nitrogen was not limited during the experimental period, the physiological responses will be mainly affected by the natural solar irradiance and temperature conditions.

The photosynthetic activity (i.e. ETR_{max}) in initial time was higher, as expected, in rocky shores than in rockpools collected algae but only wintertime. In rockpools collected algae, ETR_{max} was higher in summer than that in wintertime. Sales and Ballesteros (2012), reported higher growth rate in *Cystoseira crinita* from the northwestern Mediterranean in summer than in winter period. Moreover, Celis-Plá et al. (2014b, subchapter 2.3) found higher ETR_{max} in *C. tamariscifolia* collected from shallow waters than algae collected from depth waters in summer after *in situ* experimental period. After 7 d incubation under nitrate enriched conditions, both ETR_{max} and photosynthetic efficiency (α_{ETR}) were maintained higher in summer than that in winter (Celis-Plá et al. 2014a)

In contrast, the maximal quantum yield as indicator of photoinhibition (i.e. F_v/F_m) was lower in summer time respect to the wintertime in algae collected from both sites. These responses, indicated photoinhibition for *C. tamariscifolia* in summer time and high vulnerability respect to the winter time as it was suggested in the seasonal study (Subchapter 2.1).

In addition to ETR_{max} obtained from the fitting of ETR versus irradiance function of rapid light curves conducted with halogen lamp supply by the Diving–PAM, ETR was determined during the daily cycle under solar radiation (*in situ* ETR). In summer time, *in situ* ETR was higher in algae collected than that winter in both rocky shores and rockpools collected algae. i.e., higher productivity under *in situ* condition. *In situ* ETR under solar radiation was higher than ETR_{max} fitted from rapid light curves. The *in situ* data is expected to explain better the physiological state than ETR_{max} since *in situ* ETR is an actual productivity and ETR_{max} is a potential production. ETR_{max} can be reached at higher irradiance than that reached in the natural conditions during the daily cycle. Similar results have been observed comparing ETR in solar and artificial light sources in red macroalga as *Porphyra leucosticta* (Figueroa et al. 1997) and in green macroalga *Ulva lactuca* (Longstaff et al. 2002) or the green microalga *Chlorella fusca* (Jerez 2015). A high photoinhibition (decreased of ETR) was detected in *C. tamariscifolia* at noon in summer time at 3 d but after 7d culture no photoinhibition was detected indicating an acclimation of this species to high irradiance and recovery in the afternoon values was found. Intertidal macroalgae form southern Spain presented high dynamic photoinhibition determined as decreased percentage of F_v/F_m but in addition, they reached full recovery in the afternoon time (Häder et al. 1996, Pérez-Rodríguez et al. 1998, Flores-Moya et al. 1998, Figueroa and Viñegla 2001b, Häder et al. 2002, Viñegla et al. 2006).

The internal nitrogen content was higher in wintertime than summer time as it was observed during the seasonal study in Subchapter 2.1. C:N ratio in the field algae was 13.9 in winter and 15.7 in summer time. This indicates more reservoir of N for these algae in wintertime compared to summer time probably because the decrease of nitrate in the water column in summer due to the reduction of coastal upwelling compared to spring time (Ramírez et al. 2005) and secondly to a possible use of N source in summer to maintain metabolic fitness. Interestingly after 7 d in spite of the N enriched conditions of the culture, internal N was maintained higher after 7 d culture in winter that in summer collected algae indicating that N is not accumulated in summer-collected specimens.

The photoprotectors (i.e. phenolic compounds) in *C. tamariscifolia* collected from both sites were higher in winter time than that summer time. This is an unexpected results since phenolic compounds are describe to increase under increased of PAR or UV irradiance (Pavia et al. 1997, Connan et al. 2006, Abdala-Díaz et al. 2006). Phenolic content was three times higher in *C. humilis* growing at 0.05-0.1 m compared 3-5 m depth (Betancor et al. 2015). As it was observed in the seasonal study (subchapter 2.1), phenol content was correlated to internal nitrogen levels, this indicates less vulnerability in algae collected in winter than summer time since phenolic compounds are positively related to antioxidant activity. Phenolic compounds are related to secondary metabolism but in *C. tamariscifolia* the direct positive relation between photosynthetic activity and internal nitrogen seem to be calcified as primary metabolic substances as carbohydrates produced by the assimilation of CO_2. The positive effect of nitrate supply and phenols was previously reported in the brown macroalgae *Ascophyllum nodosum* and *Fucus vesiculosus* (Pavia and Toth 2000). The phenolic compound in the seawater was also different depending on the grow site, after 7d in summer time, phenolic compound content was higher in algae collected from rocky shores than from rockpools. The emerged condition in the collection site can positively affect the capacity to incorporate the nitrate as it has been reported in intertidal (Dring and Brown 1982) and consequently phenolic compound increase compared to rockpool algae. The possible higher nitrate incorporation can be suggested, since internal nitrogen content after 7 d in summer time was higher in rocky shores compared to rockpools algae. The highest nitrogen internal indicates more photoprotection capacity as it has been reported in red macroalgae (Figueroa et al. 2012).

C. tamariscifolia presented higher antioxidant capacity in thalli collected in summer than in winter and in thalli collected from rocky shores than from rockpools in spite of the lower content of internal phenols. The phenols and the antioxidant activity have been

related to algal zonation (Connan et al. 2004); it is shown that phenolic compound content was higher in intertidal compared the low intertidal or sublittoral zone. A higher antioxidant activity (i.e. lower EC_{50}) was found in summer time submitted to high solar irradiances and low internal N content, this responses is an evidence of the high vulnerability under stress full conditions and can be associated to other photoprotection mechanism, in summer time e.g. as carotenoid accumulation. In addition, the non-photochemical quenching was higher in summer than in winter-collected algae indicating increase of energy dissipation in summer as a photoprotection mechanism.

Gómez and Huovinen (2010) found that the synthesis of phlorotannins was induced by UV radiation only in summer, the induction was fast (3 days) and they found a positive relationship between the contents of insoluble phlorotannins and the suppression of photoinhibition and DNA damage. As it has been suggested in this study, the photoprotective role of phlorotannins appears to respond to an interplay between solar radiation stimulus (UV), seasonal acclimation and intrinsic morpho-functional processes as for example photosynthetic activity as affected by the S/V ratio. Gómez and Huovinen (2010), reported than in summer time, soluble phlorotannins are induced and the content of insoluble phlorotannins (more related to growth) is maintained constant. In this study, we have not discriminated between soluble and insoluble, but we have determined a third fraction, the released phenols as reported by Koivikko et al. (2005) in *Fucus vesiculosus*. In general, cell-wall bound phlorotannins (insoluble phenols) are in the cells at lower concentration than soluble phlorotannins and in addition, it is the unique fraction affected by nutritional conditions. Thus, as we indicated above, phenol content is sensible to nutrient availability. The grazing of herbivory animals (Koivikko et al. 2005) stimulates the exudation or release of phenols. In our study, release can be stimulated by a physical factor (UV radiation) increased in summer time instead of herbivory. Release of phenolic compounds were increased by increase temperature (+4^{o}C) (Figueroa et al. 2014a). We suggest that the depletion of internal nitrogen can related the stimulation of release but in contrast, Koivikko et al (2005) observed that a lack of nutrient had no effect on exudation.

3.3 Are there interactive effects of irradiance and nutrient availability on photophysiological responses of Cystoseira tamariscifolia and Ellisolandia elongata, collected from two different depths? (Subchapter 2.3)

In the first two subchapters (2.1 and 2.2) functional responses to environmental variations through seasonal periods and in algae collected in rock shores and rockpools

in oligotrophic waters of La Araña beach (Málaga) in two seasonal period (summer and winter) in coastal sea with a good ecological status (according to Bermejo et al. 2013) were studied. In the subchapter 2.3, evaluation of the irradiance effects on functional indicators was evaluated *in situ* in the other area of Alboran sea included in this Thesis (Cabo de Gata-Nijar Natural Park, Almeria) with ultraoligotrophic waters (see Tables 1 and 2 in the chapter 1) and high ecological status according to Bermejo et al. (2013).

The capacity to acclimate in the short term to natural solar irradiance was evaluated, by transplant approach in summer time by using algae collected form 0.5 and 2.0 m and incubated in surface waters to 100% and 70% of surface irradiance experiment. In addition, to C. *tamariscifolia*, the red calcareous macroalga, *Ellisolandia elongata*, was studied. In this experiment, we tested interactive effects between light, nutrient and two different depths for both algae. The photosynthetic activity (i.e. ETR_{max}) in *C. tamariscifolia* collected from both 0.5 and 2.0 depth waters was higher in shade conditions without nutrient enrichment. These differences could be explained by the high transparency in the coastal waters of Cabo de Gata-Nijar Natural Park (Pérez-Rodríguez 2000). High PAR and UVR can cause photoinhibition, which can be defined as the light dependent decline in photosynthetic capacity and maximal photosynthetic efficiency therefore the dominance of photodamage versus photo repair processes (Osmond 1994, Gómez et al. 2004). In contrast, the F_v/F_m was higher in both depths under full light and non-nutrient enriched conditions. This is unexpected results since F_v/F_m as indicator of photoinhibition would have to be higher in shade conditions and without nutrient limitation. In *E. elongata* collected from both 0.5 and 2.0 m depth after short-term experimental period, the photosynthetic activity (i.e. ETR_{max}), was higher in shade conditions with nutrient enrichment. This suggest, less vulnerability in algae collected from 2.0 depth in different light conditions, respect to the algae collected from shallow waters and full light conditions. F_v/F_m as expected was higher shaded that in full light conditions but under non-enriched nutrient condition. The elevated NPQ_{max} indicates high photoprotection capacity. Sun type pattern is shown by using photosynthetic parameters as indicators as high Ek_{ETR} values (200-220 µmol photons m^{-2} s^{-1}) in both depths waters conditions (initial conditions). These values were lower than that reported by Celis-Plá et al. (2014a, subchapter 2) and Figueroa et al. (2014b) in *C. tamariscifolia* growing in coastal area of Mediterranean Sea, but exposed to emersion conditions in contrast to the subtidal species of Cabo de Gata-Nijar.

The nitrogen internal content, as indicator of the nutritional status in *C. tamariscifolia* was highest in algae collected from shallow waters with nutrient enrichment. The response to nutrient enrichment was very rapid. In *E. elongata*, internal N was higher in algae collected from depth in shaded conditions and shallow in full light conditions, under enrichment nutrient treatments. The C:N ratio in *C. tamariscifolia* was highest in shaded conditions without nutrient enrichment in algae collected from 2.0 m depth. C:N ratio was much higher in 2m incubated algae i.e., 20-30 compared to algae incubated at 0.5 m i.e., 12-20 . The higher C:N ratio in algae from Cabo de Gata-Nijar compared to algae form La Araña confirms the ultraoligotrophic conditions in Cabo de Gata-Nijar. In *E. elongata* the C:N ratio increase without nutrient enrichment in both depth independent of the light conditions. This C:N ratio varied as function of N availability and in general in shade condition the ratio was stoichiometrically less favorable indicating certain nutritional stress.

The photoprotection in *C. tamariscifolia* by phenolic compounds was higher in shallow water (0.5 m) under nutrient enrichment conditions independent of the light; in contrast, in depth waters (2.0) the photoprotectors content was higher in non-enrichment conditions independent of the light conditions. This could be explained because of the high irradiance in shallow waters, phenolic compounds can be released preventing the photodamage as a photoprotection strategy (Abdala-Díaz et al. 2006, Celis-Plá et al. 2014a-subchapter 2). The antioxidant activity was higher in shallow waters in full light conditions without nutrient enrichment, this indicate stressful conditions, and in addition in algae from depth waters, the antioxidant activity was higher in full light and non-enrichment conditions. In general, phenolic content was correlated to nitrate as it has been shown above in different seasonal (subchapter 2.1) and sites (subchapter 2.2). Nitrate has a clear influence on the accumulation of phenolic compounds also in algae for ultraoligotrophic waters as it is shown in the subchapter 2.3.

In the case of the *E. elongata*, mycosporine like amino acid content, as photoprotective compounds, was unexpectedly higher in shaded conditions with nutrient conditions. The content of MAAs as UV-screen substance has been reported to be higher in algae exposed to elevated irradiances as algae from shallow waters (intertidal) compared algae from subtidal system (Franklin et al. 1999, Hoyer et al. 2002, Helbling and Zagarese 2003, Carreto and Carignan 2011). It is possible that in shallow waters MAAs can be reduced photodamage by the excess of solar irradiance. The higher accumulation under nitrogen enrichment has already reported in other red algae (Korbee

et al. 2005, Huovinen et al. 2006, Bonomi et al. 2011, Figueroa et al. 2012, Navarro et al. 2014). Karsten et al. (1998) and Franklin et al. (2001) have shown that accumulation of MAAs depended on both quality and quantity of radiation, with higher accumulation of MAAs on high daily PAR doses and UV exposure.

The carotenoids were less influenced by irradiance or nutrients with the exception of violaxanthin that had higher content after nutrient enrichment. In *C. tamariscifolia*, violaxanthin had higher content in the simulated deeper irradiance. However, antheraxanthin and β-carotene were significantly affected by the interaction of factors irradiance and nutrients. In addition, in *E. elongata* collected from 0.5 m depth, an effect of irradiance was found. These responses for both species are similar to those described by Demmig-Adams & Adams (1996) as the response of the xanthophyll cycle and light absorption that could reflect a regulatory and photoprotective response. Goss & Jakob (2010), indicate that the xanthophyll cycle represents an important photoprotection mechanism in plant cells. This suggests the relation between higher photosynthetic rates and a higher activity of the xanthophyll cycle.

3.4 How the effects of copper and nutrient enrichment does effects on photophysiology and biochemistry in Cystoseira tamariscifolia? (Subchapter 2.4)

In the subchapter 2.3, it was shown the important role of nitrate of the physiological responses in the natural gradient of light (depth) in *C. tamariscifolia* grown in ultraoligotrophic waters. It was indicated that the algal communities studied in this Thesis are located in the southern limit of the geographical distribution governed mainly by the temperature ranges. In the subchapter 2.4, the interactive effects of nitrate with heavy metal was analyzed in *C. tamariscifolia* from the limit north of the temperature (Atlantic Ocean ~16ºC). Copper was selected since it is one of the heavy metal that is polluting coastal waters as consequence of human activities (Brown and Newman 2003). The algae were collected in the coastal area of Plymouth (UK) and transplanted to the laboratory system. Nitrate (5 and 50 μM) and Copper levels (Cu_T: nominal total Cu concentrations, control conditions, 0.5 and 2.0 μM) influenced the effects on photosynthetic rate determined as *in vivo* chlorophyll *a* fluorescence in *Cystoseira tamariscifolia* (Phaeophyceae), i.e., maximal quantum yield (F_v/F_m), maximal electron transport rate (ETR_{max}) and maximal non-photochemical quenching (NPQ), biochemical composition and antioxidant activity. In 0.5 μM Cu_T grown algae, the Ni was higher in high nitrate

supply, in contrast, the photosynthetic activity (F_v/F_m and ETR_{max}), Chl*a*, NPQ_{max} and total phenolic content decreased. However, under 2.0 µM Cu_T mainly in the short term (7 d culture), high nitrate treatment decreased Ni, F_v/F_m, total phenolic compounds, the level of phenols in the water and external copper content. The maximal quantum yield (F_v/F_m) was higher under both high nutrient and copper concentrations. However, the maximal electron transport rate (ETR_{max}) was higher in middle cooper and without nitrate levels. Thus, complex interactive responses between copper and nitrate were shown. *C. tamariscifolia* of Northern Sea seems to have the same photosynthetic pattern under the nitrate supply and 0.5 µM Cu_T in spite of the fact that these specimens have to be acclimated to the higher levels of nitrate of Northern (Howarth et al. 1996) compared to Mediterranean Sea (Mercado et al. 2012).

The non-photochemical quenching (NPQ_{max}) as photoprotection indicators, was higher in control (no copper addition) and nitrate conditions, simulating the natural levels in Hannafore point, Atlantic Ocean. Connan and Stengel (2011b) showed drastic variations in NPQ_{max} with copper and salinity changes.

We found higher copper internal and external cell in high copper concentration independent of the nutrient conditions, this indicate the maximal stress conditions incorporate the copper in the cell in *C. tamariscifolia* in responses to the interactive effects to the both variables.

In other experiment, Celis-Plá et al. (2014a - subchapter 2.2) showed in mesocosms under solar radiation that the highest ETR_{max} in *C. tamariscifolia* occurred in thalli with the lowest internal nitrogen level i.e. winter compared to summer grown algae. Similar response to nitrate on photosynthetic activity in *C. tamariscifolia* collected in the Mediterranean and North Sea have not necessary to indicate the same on copper tolerance since the response can be dependent on the natural exposure to copper in the collected area, as well as on the interactive effect of both factors (nitrate and copper). Connan and Stengel (2011a) showed that lower copper content was not related to higher photosynthetic rate as ETR_{max} but with the lower internal total nitrogen level. Thus, the lower internal content could indicate less favorable physiological activity and therefore the copper uptake is reduced. The internal nitrogen content was higher in the middle copper concentration and enrichment conditions. The phenolic compounds in *C. tamariscifolia* after exposure copper concentrations was higher in high copper levels independent of the nutrient conditions.

Phenolic released was highest in high copper levels and with nitrate enrichment conditions and positive correlation with antioxidant activity. Thus, the highest antioxidant activity was observed under both high Cu and N levels. Chl*a, c* and fucoxanthin contents were higher in high coper levels independent of the nitrate conditions. Pellegrini et al. (1993) showed the toxic effect of Cu in *Cystoseira barbata* involves a reduction of weight-growth and of pigment contents (chlorophyll a and carotenoids), the interactions e.g. Cd-Cu-Ca have effects in chlorophyll a and c contents, and Cu-Zn-Ca chlorophyll a content.

In this study, it is shown that functional indicators, (1) physiological ETR_{max}, F_v/F_m and NPQ_{max}, (2) nutritional indicator (C:N) and (3) biochemical indicator of stress were sensible to Copper and nitrate variations and they respond as interactive effect of both heavy metal and nutrients. The data show complex interaction effects between copper and nitrate on the ecophysiological variables, with high resistance to copper of *C. tamariscifolia* with no apparent damage effects after 14 d culture were found even at 2.0 μM Cu_T. In general, at the end of the experimental period, middle copper levels seems to be more favorable in low nitrate conditions, respect to the physiological status of *C. tamariscifolia*. In contrast, in high copper levels with high nitrate levels, the physiological status in *C. tamariscifolia* was also favorable. These results suggest that interaction between copper and nitrate can give a high resistance to copper of *C. tamariscifolia* with no apparent damage effects after 14 d culture.

3.5 Are there interactive effects between irradiance and nutrient levels in a natural pH gradient of Cystoseira compressa and Padina pavonica? (Subchapter 2.5)

In recent years, there has been a significant effort to predict the future impact of climate changes on seaweed communities (Graham et al. 2007, Halpern et al. 2008, Wernberg et al. 2010). Two main approaches have been followed: (1) experimental approaches designed as factorial experiments incubating macroalgae for days or months at different growth temperatures, according to the future predicted scenarios and evaluating the interactive responses with other variables as; acidification, UV radiation, and nutrient availability amongst others (Hoppel et al. 2008, Porzio et al. 2011). In addition, (2) field studies of seaweeds growing at their temperature limit for growth and reproduction, monitoring the temporal and spatial variation of temperature and other variables (Viejo et al. 2011, Martínez et al. 2012b). Most investigations have been

conducted on individual species separately, rather than communities (Olabarria et al. 2013), although it has been reported that community level impacts might be less noticeable (Kroeker et al. 2010).

To date, few short-term (<1 year) experiments have been performed and reveal mixed responses depending on the algal species and the culture conditions applied (Porzio et al. 2011, Zou et al. 2011, Cornwall et al. 2012). In macroalgae, doubling CO_2 level caused an increase in photosynthetic activity of 52-130% depending of the species (Gao et al. 1993, Kübler et al. 1999, Riebesell et al. 2007). Overall, the sensitivity of algae to acidification is expected to be complex, due to the interactions between the effects of pH and CO_2 in the enhancement of photosynthesis. Although increasing ocean CO_2 concentration may enhance rates of photosynthesis and growth (particularly in species without carbon concentrating mechanisms), such increase may be limited by the availability of other limiting nutrients (Raven et al. 2005).

In the previous subchapters, the physiological responses by using functional indicators has been shown in *C. tamariscifolia* growing; in the natural habitat of the southern limit distribution in oligotrophic (subchapter 2.1), ultraoligotrophic waters (subchapter 2.3), in outdoor experiments of oligotrophic grown specimens (Subchapter 2.2) and in an indoor laboratory study from algae collected in the northern limit of distribution (Subchapter 2.4).

Due the important role of nitrate and the interest to study in the field or in mesocosms the impact of climate change factors on this key species, two studies were designed. One in the field by using the natural pH gradient to evaluate the interactive effects of acidification, nutrient and light on the ecophysiology of *C. compressa* (subchapter 2.5). We could not use *C. tamariscifolia* in this study as we wish because the last species does not grow in Vulcano island coastal water where pH gradient exists (according to distribution reported by Gómez-Garreta et al 2001). The second study was conducted in a mesocosms in Málaga University (UMEGOA, unit of microbiology, ecophysiology and genetic of aquatic organism) analyzing additive/interactive responses of CO_2, temperature and nutrients on physiological status (subchapter 2.6) by using functional indicators as Figueroa and Korbee (2010) suggested.

In the natural pH gradient in the Mediterranean Sea (Vulcano Island, Italy the interactive effects between light, CO_2 and nutrient levels was studied in addition in the calcareous alga *Padina pavonica*. Both *C. compressa* and *P. pavonica* were benefited as physiological level by the increases of DIC (dissolved inorganic carbon), but that the

benefits, and the extent of the algal response, depends upon nutrient and light availability. In Figure 6 (subchapter 2.5) it is summarize our projections that brown macroalgal stands will both proliferate in the shallows y extend deeper due to the greater availability of DIC and nutrients due to a combination of ocean acidification and anthropogenic nutrient input.

The photosynthetic activity responses (i.e. ETR_{max}), in *C. compressa* and *P. pavonica* were higher in full light in ambient nutrient conditions. Maximal quantum yield (F_v/F_m), in *C. compressa* was higher in high CO_2, shaded with nutrient conditions; in contrast, in *P. pavonica* was higher in non-enrichment levels. In macroalgae, doubling CO_2 level caused an increase in photosynthetic activity of 52-130% depending of the species (Gao et al. 1993, Kübler et al. 1999, Riebesell et al. 2007). Overall, the sensitivity of algae to acidification is expected to be complex, due to the interactions between the effects of pH and CO_2 in the enhancement of photosynthesis. Although increasing ocean CO_2 concentration may enhance rates of photosynthesis and growth (particularly in species without carbon concentrating mechanisms), such increase may be limited by the availability of other limiting nutrients (Raven et al. 2005). Mercado et al. (1998) reported a relationship between carbon concentration mechanisms (CCM) and light energy availability in intertidal macroalgae, but not with inorganic carbon availability: intertidal algae with emersion periods presented higher photosynthetic rates (Mercado et al. 1998) and carbon uptake (Flores-Moya et al. 1998) due to the higher availability of CO_2 than submerged algae. Seasonal changes in temperature, nutrient availability and light are also likely to interact with the effect of CO_2 on metabolic processes in algae (Martin and Gattuso 2009, Mercado and Gordillo 2011, Hofmann et al. 2013). As calcification, photosynthesis, nutrient uptake, growth, and other metabolic processes are affected by temperature, light and nutrient availability, changes in these parameters are likely to have a strong influence on the enzymatic response of macroalgae to increasing CO_2. Therefore, outdoor mesocosms studies are useful for monitoring CO_2 effects over time during natural temperature, nutrient and light fluctuations.

The nitrogen internal content, in *C. compressa* was higher in high CO_2 (18 mg g^{-1} DW), shaded and enrichment conditions; in contrast, in *P. pavonica* was higher in ambient CO_2 (16.5 mg g^{-1} DW) in shaded and nutrient enrichment conditions. Lourenzo et al (2002) showed in *Padina gymnospora* the total nitrogen internal content values of the 2.41 mg g^{-1} DW and they explained that the total nitrogen showed relatively low values in some species in oligotrophic waters, mainly in brown alga.

The phenolic compounds increase in *C. compressa* in high CO_2 levels with nutrient enrichment independent of the light conditions. As in the previous subchapter, it is shown that phenolic compound is directly relate to photosynthetic production and it is accumulated under favorable condition of light and nutrients (enriched levels).

The effects of CO_2 on phenol production are different compared to previously observations in seagrasses (Arnold et al. 2012), *C. tamariscifolia* (Figueroa et al. 2014a) and other brown algae (Betancor et al. 2014, Figueroa et al 2014a). Phenolic compounds, antioxidant activity and calcification were studied in the calcareous alga *Padina pavonica* and non-calcareous alga *Lophora variegata* during a submarine eruption produced one nautical mile off shore El Hierro Island (Canary Island, Spain) from October 2011 to March 2012. During the eruptive phase, pH dropped of ca. 1.22 units relative to standard condition and *P. pavonica* suffered decalcification and loss of phenolic compounds and antioxidant activity whereas photosynthetic acclimation was as sun type alga i.e., increase of ETR_{max}, Ek, and decrease of α_{ETR}. However, in *L. variegata,* phenolic compounds and antioxidant activity were reduced without any evident change in photosynthetic pattern. In the post-eruptive phase with pH of *ca.* 8.23, the level of phenolic compounds, antioxidant activity and photosynthetic activity and calcification state recovered reaching the same values as in the algal populations no affected by the eruption (reference algal communities).

Phenol content decreases when CO_2 increased also in a mesocosms study with the macroalga *Cystoseira tamariscifolia* (Figueroa et al. 2014a). The decrease of total internal compound was related to high release under acidification conditions. The increased temperature (+4^oC) provoked a decrease however only in algae grown under ambient CO_2 conditions. Thus, the internal level of phenols remain constant with the increasing temperature under acidification conditions allowing maintaining the antioxidant capacity. In the study conducted in Vulcano Island, the temperature was not increased as in the mesocosms study conducted by Figueroa et al (2014a), thus the physiological responses are related to pH and light gradient. The concentration of phenolic compounds (expressed as phloroglucinol equivalent) in the seawater (experimental vessel) under ambient temperature was higher than under increased temperature. The release of phenolic compounds from *C. tamariscifolia* thalli at noon has been suggested to be a photoprotective mechanism under high irradiance conditions (Abdala-Díaz et al. 2006, Celis-Plá et al 2014a). In the green macroalga *Dasycladus vermicularis*, phenolic compounds (trihydroxycoumarins) with antioxidant capacity are

released under stress conditions reducing the photoinhibition and increasing the recovery capacity of the photosynthetic yield and productivity (Pérez-Rodríguez et al. 1998).

In *C. compressa* and *P. pavonica*, antioxidant activity and EC_{50} were affected by the interactions between light levels and CO_2. EC_{50} tended to be higher in shaded, high CO_2 treatments with and without nutrient addition, suggesting a positive correlation with phenolic compounds and their use as antioxidants to prevent photodamage. Together, NPQ_{max}, phenol production and EC_{50} indicate that in elevated CO_2 conditions some species will have a higher capacity for photoprotection.

These results suggest an increase of algae biomass or growth in the futures scenarios. The phenolic compounds, usually accumulated under higher irradiance and (for *C. compressa*) higher CO_2 treatments, as per past studies on kelp grown at high CO_2 (Swanson and Fox, 2007), or measured under higher irradiance (Connan et al. 2004).

3.6 How is changed the physiological pattern and biochemical composition in an experimental mesocosms study according to two levels of CO_2 and temperature of Cystoseira tamariscifolia grown in ultraoligotrophic and oligotrophic coastal waters? (Subchapter 3.6)

We tested the interactive or additive effects between temperature, CO_2 and origin of population of *C. tamariscifolia* from two populations of the Mediterranean Sea growing in ultra and oligotrophic waters. The expected levels of increased CO_2 and temperature for the end of this century according to Business as usual (IPPC 2014) scenario were simulated in mesocosms described by Stengel et al. (2014).

The expected results are as follows: functional indicators related to photosynthesis and internal nitrogen will be increased by CO_2 and nutrient enrichments under ambient temperature. Stress condition related to high temperature and low nitrate availability produce an increase of biochemical responses against the stress. It is expected more active responses to stress and climate change factors in ultraoligotrophic compared to oligotrophic grown algae since they naturally submitted to high stress conditions.

The photosynthetic activity (i.e. ETR_{max}) was higher in CO_2 high levels for both sites collected algae. This indicate more production in this treatment and less vulnerability in these macroalgae. After experimental period (28d), the maximal quantum yield (F_v/F_m) was higher in *C. tamariscifolia* from ultraoligotrophic waters under increased temperature (+4ºC) and ambient CO_2 levels. In contrast, for oligotrophic waters F_v/F_m was higher in

high CO_2 levels and ambient temperature. Thus, the interactive effect of temperature and CO_2 was different in algae collected under different light and nutrient history grown conditions. We do not know if *C. tamariscifolia* from the two sites correspond to different ecotypes but at least the results indicate the importance of light and nutrient history of the macroalgae in the responses to climate change factors.

Intertidal macroalgae from Mediterranean and Atlantic coastal waters of southern peninsula presented higher capacity to respond to increased environmental stress compared to algae from subtidal or from other region with lower daily irradiance levels (Hanelt 1998, Hanelt and Roleda 2009, Gómez et al. 2004, Hanelt and Figueroa 2012). The acclimation pattern is governed by a high non- photochemical quenching, photoinhibition and the accumulation of UV screen photoprotectors (Pérez-Rodríguez et al. 1998, Abdala-Díaz et al. 2006, Figueroa et al. 2014b).

The highest release of phenolic compounds in the water was observed in algae from oligotrophic waters than ultraoligotrophic waters at the end of the experimental period in ambient CO_2 conditions independent of the temperature. The release of the phenolic compounds in brown macroalgae, are considerate as other mechanism of photoprotection, these can be expulsed to the seawater during periods of UVR stress (Swanson and Druehl 2002, Koivikko et al. 2005, Celis-Plá et al. 2014a).

The nitrogen and Carbon internal content for both sites was higher in high CO_2 levels and ambient temperature, this indicate less vulnerability for this treatments and good state for this macroalgae. We found benefits of DIC (dissolved inorganic carbon) increase on growth rate in *Cystoseira tamariscifolia* being the physiological responses more accelerated in ultraoligotrophic than in oligotrophic harvested algae. Temperature increased has negative effect on growth rate only in algae from oligotrophic waters. Although the biomass in both *C. tamariscifolia* increased in elevated CO_2 conditions, biomass of algae collected from ultraoligotrophic waters was higher than algae collected from oligotrophic waters. This suggest *C. tamariscifolia* had interactive effects between origin of populations and CO_2 conditions, probability that algae collected in ultraoligotrophic waters maybe can capitalize carbon of the aquatic system when the nutrient is not limited in extreme. Reports on macroalgae from other regions have shown that ocean acidification can directly benefit the physiological state and growth (Baggini et al. 2014, Celis-Plá et al. 2015-subchapter 2.5). In addition, in non-calcareous macroalgal production and biomass may increase due to beneficial effects of ocean acidification on photosynthesis (Harley et al. 2012, Koch et al. 2014, Brodie et al. 2014).

The phenolic compounds were higher in algae from ultraoligotrophic waters in high CO_2 waters (i.e. 1.5 - 3.0 %) than that form oligotrophic waters in high CO_2 waters (1.0 - 2.2 %) in ambient temperature. The higher concentration of phenolic compounds in algae from ultraoligotrophic waters can be related to the photoacclimation to high irradiance levels in coastal waters with high transparency. Figueroa and Gómez (2001a) reported high penetration of PAR, UVA and UVB radiation with mean attenuation coefficient (K_d) values of 0.07, 0.105 and 0.220 m^{-1} in this area. The increase of phenolic compounds under elevated CO_2 could increase the photoprotection of this species in future scenario of ocean acidification with seawater pH value of 7.78 corresponding to a 850 ppm CO_2 (IPCC 2014).

A positive correlation between phenolic compounds and antioxidant activity expressed as EC_{50} (Table S6), indicates that phenolic compounds can prevent photodamage. The antioxidant activity, in *C. tamariscifolia* from ultraoligotrophic waters to be directly correlation with the phenolic compounds because it was higher when increase CO_2 levels. In contrast, in *C. tamariscifolia* from oligotrophic waters, antioxidant activity was higher in high CO_2 levels with ambient temperature, only in the first period.

At the initial time, Fucoxanthin content was about 8% higher in algae harvested from ultraoligotrophic waters than that from oligotrophic whereas the ratio between the main carotenoid and chlorophyll (Fucoxanthin: Chl*a*) was still higher (about 33.0%). The high proportion of photoprotective carotenoid (Goss and Jakob 2010) respect to chlorophyll was expected since the penetration of both PAR and UVR (Figueroa and Gómez 2001a) is higher in ultraoligotrophic compared to oligotrophic waters due to its lowest turbidity (Cortés et al. 2012). After 28 d, however, the highest increase was produced in oligotrophic collected algae except in T+4ºC*HCO_2. After 28 d, algae submitted to different CO_2 and temperature treatments, the Fucoxanthin: Chl*a* ratio was similar in algae collected from both sites i.e. 0.38-0.39 except in T+4ºC*HCO_2 i.e. 0.41 in ultraoligotrophic and 0.35 oligotrophic collected algae. Thus, the increase of both CO_2 and temperature was less favourable for photoprotection mechanisms in oligotrophic than that in ultraoligotrophic harvested macroalgae. These responses of the xanthophyll cycle could reflect a regulatory and photoprotective response that down-regulates the delivery of excitation energy into the electron-transport chain to match the rates at which products of electron transport can be consumed in these leaves (Demmig-Adams and Adams 2006).

In this study, the decrease of maximal quantum yield and electron transport rate , the increase of phenolic compounds and antioxidant activity or the increase of C:N ratio are produced in stress conditions and thus they are validated as stress indicator. In addition, it is possible to evaluate the direction of the physiological response i.e., positive or negative to expected changes in climate change factors under other anthropogenic impacts as eutrophication (increased nitrate levels in the water columns) or pollution by heavy metals. However, on the other hand, the increase of phenolic compounds are produced also under increased photosynthetic activity showing a link between antioxidant and algal production. This is not an estrange result since a high photosynthetic activity is related to a high oxygen production which can be produced oxidative stress. Non-photochemical quenching, oxygen consumption through Mehler reaction and increased antioxidant activities are down regulation mechanisms to survive under promoted oxygenic scenario. Thus, phenolic accumulation under increased nitrate and CO_2 levels or the release of phenols under increased irradiance in *C. tamariscifolia* shows us that this species has effective biochemical mechanisms to acclimate for the expected variations in climate change factors although this is limited by temperature. As it was suggested, the algae growing under more stress conditions i.e., ultraoligotrophic compared to oligotrophic waters seem to have more amplified physiological responses to the variations of physical-chemical variables

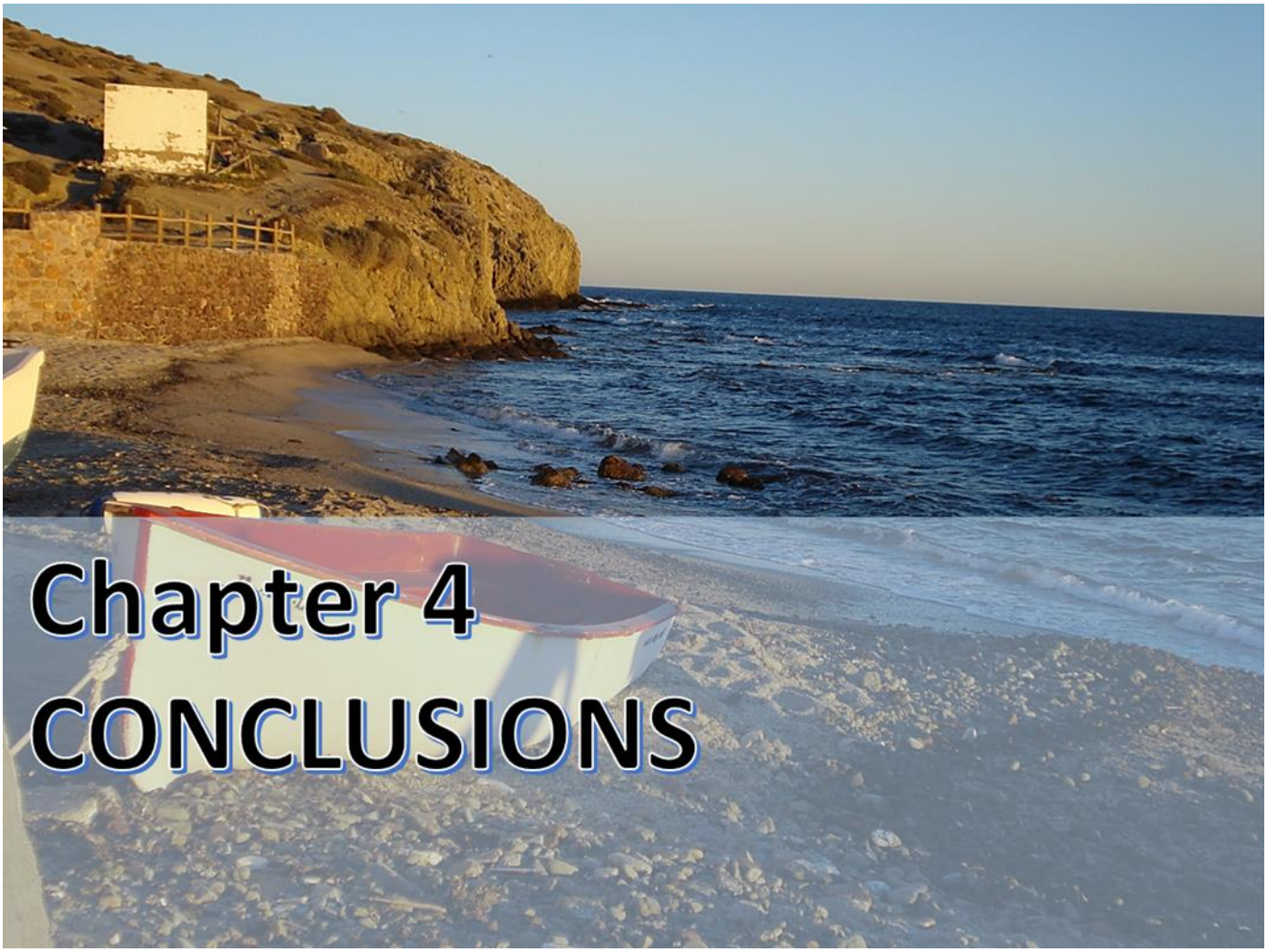
In addition, to physiological-biochemical approach, it would be very interesting to have physiological molecular approach to analyze the expression of enzymes related to stress. The sensing of stress signals and their transduction into appropriate responses is crucial to the acclimation and survival of intertidal macroalgae. These stress response mechanisms must be rapid as well as optimally regulated. If not, intertidal macroalgae would be seriously damaged or even die during periods of low tide. It has been extensively described that in animal cells and in yeast these stress mechanisms involve the activation of a well-regulated signaling network, in which the central role is played by a specific group of cytoplasmic phosphoproteins called MAP kinases (Mitogen Activated Protein Kinases). All eukaryotic cells possess multiple MAPK pathways. Scientific evidences accumulated during the last years indicate that the external information is transferred to the cell nucleus through a series of phosphorylation/dephosphorylation reactions of the MAPKs that lead to the activation/deactivation of specific groups of genes (Kyriakis and Avruch 2001). *C. tamariscifolia* showed clear protein cross-reactivity with very specific antibodies raised

against the phosphorylated forms of p38 and JNK (Parages et al. 2014), which are established players in the mammalian stress response. The observed response of the MAPK-like proteins varied depending of the species of macroalgae and on the growth conditions, e.g. low and high C, low and high N, and low and high temperature (Parages et a. 2014). Previous findings have demonstrated the existence and activation of p38-like and JNK-like MAPK cascades in other macroalgae (Parages et al. 2012, 2013, 2014) in response to stress. In the case of *C. tamariscifolia*, rapid response to increasing temperature occurred in some cases without further activation of the stress–response mechanism mediated by MAPKs. The results demonstrated that the level of active (phosphorylated) p-38- and JNK-like in this species was altered when a change in temperature occurred (+4°C), but activation of MAPKs varied depending on C and N availability. The response to stress in low nitrate conditions was principally mediated by JNK activation, and that response in high carbon (700 ppm) occurred mainly through p38 activation. In all cases a significant drop of ERK phosphorylation occurred, which might indicate that the temperature increase (+4ºC) was above the optimal for growth of *C. tamariscifolia.* Thus by analysing the expression of genes related to stress; it has been also concluded that the temperature increase of 4ºC will be unfavourable for *C. tamariscifolia.* Connell et al. (2008) concluded that turf expansion at high CO_2 and elevated temperature would not just negatively affect growth of calcareous algae, but also inhibiting kelp recruitment.

As ocean acidification is not happening in isolation, but alongside a plethora of other anthropogenic changes, the study of the interactive/additive effects of multiple stressors is critical to plan for global ocean change. Elevated CO_2 levels can clearly enhance brown algal productivity, with implications for fucoid forests of the planet, but this will be contingent on other physicochemical parameters. Our study shows that ongoing ocean acidification had interactive effects with the temperature i.e. beneficial effects on growth rates, algal photosynthetic production (ETR_{max}) antioxidant activity (EC_{50}) and photoprotectors compounds (PC and xanthophyll cycle).

The oligotrophication produced in certain areas of Mediterranean Sea will be unfavorable for *Cystoseira tamariscifolia* communities in a climate change scenario. The data on vulnerability and acclimation to climate change factors of *Cystoseira tamariscifolia, Ellisolandia elongata* and *Padina pavonica* presented in this study can help the management of macroalgal communities, mainly in protected areas. In addition, the physiological and biochemical data will help to predict the effects of climate change

on bioactive compounds with antioxidant capacity and their potential biotechnological uses as phenolic compounds, mycosporine like amino acids and carotenoids.



Cabo de Gata-Níjar, Natural Park. Septiembre 2013. Photograph by Félix López Figueroa

CONCLUSIONS

Effects of stress and climate change factors on photosynthetic functional indicators

1. *C. tamariscifolia* from Mediterranean oligotrophic waters presented the highest photosynthetic production and maximal quantum yield in spring period due to the irradiance, temperature and nutrient environmental levels were the most physiologically favorable. Algae in summer time present acclimation

2. The photosynthetic activity (ETR_{max}) was higher in rocky shore than that in rock pool grown algae in winter time. This was explained by a more CO_2 supply during emerged period and nitrate incorporation after rehydration in rock shore algae compared to always immersed

3. *In situ* ETR under solar radiation was higher than ETR_{max} fitted from rapid light curves. *In situ* ETR is expected to explain better the physiological state than ETR_{max} since *in situ* ETR is an actual productivity whereas ETR_{max} represents a potential production.

4. *C. tamariscifolia* and *Ellisolandia elongata* from Mediterranean ultraoligotrophic waters collected at 0.5 and 2.0 m acclimated to increased irradiance and the photosynthetic responses were modulated by nutrient availability. Sun type pattern is shown by using photosynthetic parameters as indicators as high Ek_{ETR} values in both depths.

5. In *C. tamariscifolia* from Atlantic Ocean, functional indicators were sensible to interactive effects of copper and nitrate. In general, low copper levels seems to be more favorable on physiological state under low nitrate conditions whereas in high copper levels it was under high nitrate levels. The results suggest that interaction between copper and nitrate can give a high resistance to copper of *C. tamariscifolia* with no apparent damage effects after 14 d culture.

6. *Cystoseira compressa* and *Padina pavonica* in an *in situ* pH natural gradient benefited at physiological level by the increases of dissolved inorganic carbon, but that the benefits, and the extent of the algal response depends upon nutrient and light availability.

7. *C. tamariscifolia* collected from Mediterranean ultra and oligotrophic waters shows interactive and additive effects of CO_2 and temperature on physiological state. Photosynthetic activity (ETR_{max}) increased in high CO_2 high levels but increased temperature provoked a fragmentation and loss of biomass only in oligotrophic grown algae whereas algae from ultraoligotrophic waters presented the most accelerated physiological acclimation to stress conditions.

Effects of stress and climate change factors on nutritional state (C:N ratios)

8. In *Cystoseira tamariscifolia* from oligotrophic waters, C:N and antioxidant activity were positively related , indicating a link between less favorable nutrient status and oxidative stress conditions

9. In *C. tamariscifolia* from ultraoligotrophic waters, the internal nitrogen content was highest in algae collected from shallow waters with nutrient enrichment whereas in *E. elongata* occurred in algae collected from both depths. In *C. tamariscifolia*, the internal nitrogen content was positively correlated to phenolic compounds and antioxidant activity independent of the irradiance. In *E. elongata*, mycosporine like amino acid content was increased under enriched nutrient conditions.

10. Internal nitrogen and Carbon content in both ultra and oligotrophic grown algae was increased in high CO_2 levels under ambient temperature.

Effects of stress and climate change factors on biochemical indicators (photoprotectors and antioxidant activity)

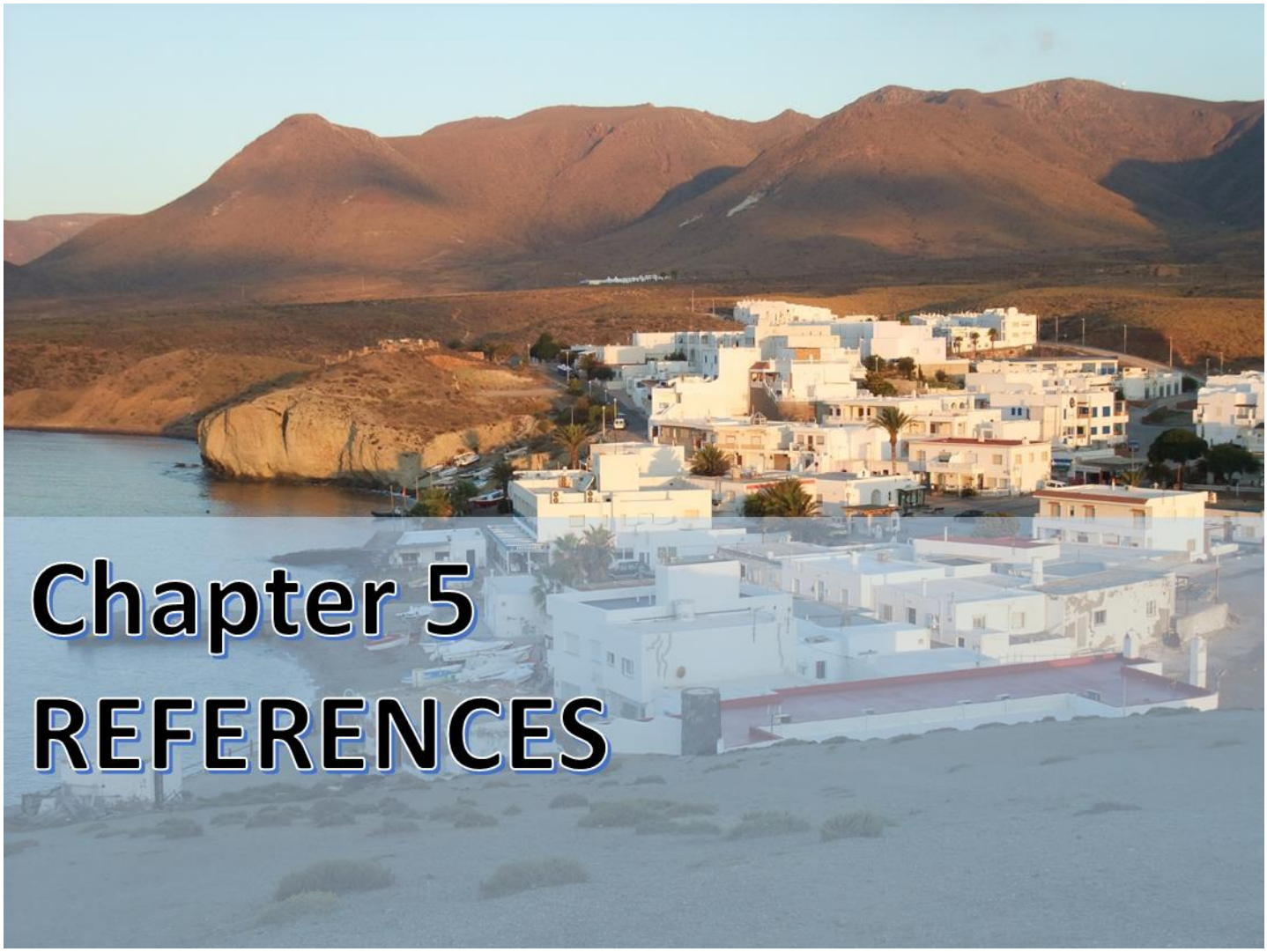
11. Phenolic compounds in *C. tamariscifolia* of Mediterranean oligotrophic waters, under non stress conditions in winter time, were positively correlated to internal nitrogen content, thus phenolic compound seem to link to primary metabolism. The acclimation mechanisms are activated in summer time as increased of phenolic compounds, antioxidant activity and energy dissipation (high non-photochemical quenching).

12. The release of phenolic compounds in *C. tamariscifolia* from Atlantic Ocean was highest in high copper levels and with nitrate enrichment conditions and it was positively correlated with antioxidant activity.

13. The phenolic content in *C. compressa* and *P. pavonica* increased in high CO_2 but the response was modulated by the irradiance and nutrient levels. In elevated CO_2 antioxidant, activity was affected by the interactions between light levels and CO_2 and positive correlation with phenolic compounds and the increase of NPQ_{max} suggest that photoprotection capacity can be increased in climate change scenario.

14. The higher concentration of phenolic compounds and antioxidant activity in algae from ultraoligotrophic waters, in high CO_2 with ambient temperature can be related to the photoacclimation to high irradiance levels in coastal waters with high transparency. The increase of phenolic compounds under elevated CO_2 could increase the photoprotection of *C. tamariscifolia* in future scenario of ocean acidification.

15. The stress conditions are indicated by the increase of antioxidant activity and high C:N ratio but the level of phenols are not always related to stress condition but phenol content was also positively related to internal nitrogen and photosynthetic activity. In addition, high antioxidant activity was also observed under high photosynthetic production (ETR_{max}) probably related to the production reactive oxygen substances.



Chapter 5
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Cabo de Gata-Níjar, Natural Park. Septiembre 2013. Photograph by Félix López Figueroa

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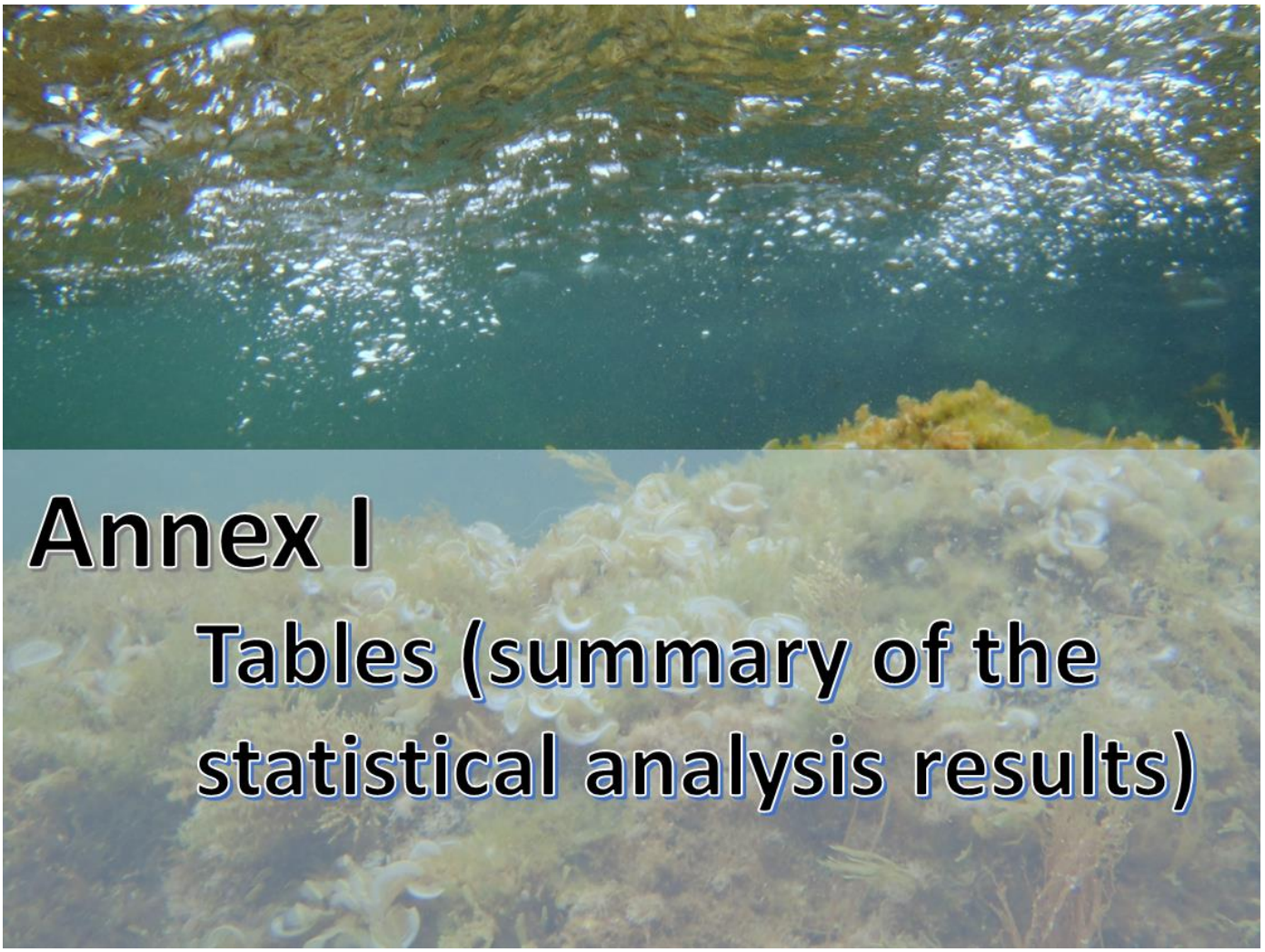


Natural pH gradient, Vulcano, Italy. March 2013. Photograph by Paula S. M. Celis Plá

Supplementary Information

Subchapter 1:

Table S1. Effect of year and season on the carbon, nitrogen, C:N ratio, phenolic compounds and antioxidant activity (EC_{50}) of *Cystoseira tamariscifolia* collected in 2012-14 on a rocky shore near Málaga, southern Spain. Significant differences at $P< 0.05$ are shown in bold.

		Cystoseira tamariscifolia			
		df	**MS**	***F***	***P***
Carbon	*Year*	1	81.83	0.40	0.53
	Season	3	**928.75**	**4.52**	**<0.01**
	*Year*Season*	3	201.34	0.98	0.41
	Res	64	205.41		
Nitrogen	*Year*	1	15.11	1.59	0.21
	Season	3	**163.24**	**17.14**	**<0.01**
	*Year*Season*	3	12.49	1.31	0.28
	Res	64	9.52		
Ratio C:N	*Year*	1	9.83	1.54	0.22
	Season	3	**81.43**	**12.76**	**<0.01**
	*Year*Season*	3	11.12	1.74	0.17
	Res	64	6.38		
Phenolic compounds	*Year*	1	188.25	2.04	0.16
	Season	3	**1763.93**	**19.11**	**<0.01**
	*Year*Season*	3	**1576.40**	**17.07**	**<0.01**
	Res	64	92.33		
EC_{50}	*Year*	1	0.00	0.47	0.49
	Season	3	**0.04**	**4.31**	**<0.01**
	*Year*Season*	3	0.02	1.86	0.15
	Res	64	0.01		

Table S2. Seasonal effects on *Cystoseira tamariscifolia* photosynthesis on a rocky shore near Málaga in 2013-14; maximal quantum yield (F_v/F_m), maximal electron transport rate (ETR_{max}), photosynthetic efficiency (α_{ETR}), irradiance of saturation of ETR (Ek_{ETR}), maximal non-photochemical quenching (NPQ_{max}), irradiance of saturation of NPQ (Ek_{NPQ}) and relationship between ETR_{max}/NPQ_{max} . Significant differences at $P< 0.05$ are shown in bold.

		Cystoseira tamariscifolia			
		df	**MS**	***F***	***P***
Fv/Fm	*Season*	3	0.00	0.19	0.90
	Res	32	0.00		
ETRmax	*Season*	3	**680.4**	**3.8618**	**0.02**
	Res	32	176.2		
α_{ETR}	*Season*	3	**0.03**	**5.61**	**<0.01**
	Res	32	0.01		
Ek_{ETR}	*Season*	3	42939.9	1.91	0.15
	Res	32	22502.1		
NPQ_{max}	*Season*	3	0.18	0.82	0.49
	Res	32	0.21		
Ek_{NPQ}	*Season*	3	**62901.2**	**3.23**	**0.04**
	Res	32	19464.4		
ETR_{max}/NPQ_{max}	*Season*	3	691.3	0.90	0.45
	Res	32	767.9		

Table S3. Pearson correlations between the variables; nitrogen (N), carbon (C), C:N ratio (CN), phenolic compounds (PC), antioxidant activity (EC_{50}) and maximal electron transport rate (ETR_{max}). Significant differences at $P< 0.05$ are shown in bold.

	N	*CN*	*PC*	EC_{50}	ETR_{max}
C	0.518	-0.268	0.395	0.231	0.163
	0.000446	**0.0865**	**0.00956**	0.141	0.301
	42	42	42	42	42
N		-0.939	0.26	0.513	-0.149
		3.46E-20	0.0966	**0.00051**	0.347
		42	42	42	42
CN			-0.244	-0.497	0.217
			0.119	**0.000804**	0.167
			42	42	42
PC				-0.106	0.326
				0.502	**0.0351**
				42	42
EC_{50}					0.371
					0.0155
					42

Subchapter 4:

Table S1. ANOVA results after experiment testing for the effect of Time (T), Copper (Cu^{2+}) and Nitrate (N) on the photosynthetic parameters; maximal quantum yield (F_v/F_m) photosynthetic efficiency (α_{ETR}), maximal electron transport rate (ETR_{max}), irradiance of saturation of ETR (Ek_{ETR}), maximal non-photochemical quenching (NPQ_{max}) and ETR_{max}/NPQ_{max} of *Cystoseira tamariscifolia.* Significant differences at $P<0.05$ are shown in bold.

		Cystoseira tamariscifolia			
		df	**MS**	***F***	***P***
F_v/F_m	*Time (T)*	1	**0.0016**	**6.40**	**<0.01**
	Copper (Cu^{2+})	2	**0.0009**	**3.73**	**<0.03**
	Nitrate (N)	1	0.0001	0.45	0.51
	TCu^{2+}*	2	**0.0015**	**5.85**	**<0.01**
	*T*N*	1	0.0010	4.10	0.05
	*Cu^{2+}*N*	2	**0.0022**	**8.68**	**<0.01**
	TCu^{2+}*N*	2	0.0003	1.14	0.34
	Res	24	0.0003		
α_{ETR}	*Time (T)*	1	**0.0135**	**5.55**	**<0.01**
	Copper (Cu^{2+})	2	0.0049	2.03	0.15
	Nitrate (N)	1	**0.0137**	**5.63**	**<0.01**
	TCu^{2+}*	2	**0.0529**	**21.68**	**<0.01**
	*T*N*	1	**0.0501**	**20.54**	**<0.01**
	*Cu^{2+}*N*	2	**0.0765**	**31.37**	**<0.01**
	TCu^{2+}*N*	2	**0.0300**	**12.28**	**<0.01**
	Res	24	0.0024		
ETR_{max}	*Time (T)*	1	0.54	0.22	0.65
	Copper (Cu^{2+})	2	**33.02**	**13.28**	**<0.01**
	Nitrate (N)	1	**30.99**	**12.46**	**<0.01**
	TCu^{2+}*	2	**10.41**	**4.19**	**<0.01**
	*T*N*	1	9.59	3.85	0.06
	*Cu^{2+}*N*	2	**13.39**	**5.39**	**<0.01**
	TCu^{2+}*N*	2	2.45	0.99	0.39
	Res	24	2.49		
Ek_{ETR}	*Time (T)*	1	1669.08	3.17	0.09
	Copper (Cu^{2+})	2	623.49	1.18	0.32
	Nitrate (N)	1	**4703.16**	**8.92**	**<0.01**
	TCu^{2+}*	2	819.11	1.55	0.23
	*T*N*	1	823.42	1.56	0.22
	*Cu^{2+}*N*	2	11.00	0.02	0.98
	TCu^{2+}*N*	2	**3834.10**	**7.27**	**<0.01**
	Res	24	527.31		
NPQ_{max}	*Time (T)*	1	0.21	3.66	0.07
	Copper (Cu^{2+})	2	**0.83**	**14.24**	**<0.01**
	Nitrate (N)	1	**3.04**	**51.90**	**<0.01**
	TCu^{2+}*	2	**0.32**	**5.49**	**<0.01**
	*T*N*	1	**0.48**	**8.17**	**<0.01**
	*Cu^{2+}*N*	2	**0.79**	**13.47**	**<0.01**
	TCu^{2+}*N*	2	**0.56**	**9.57**	**<0.01**
	Res	24	**0.06**		
ETR_{max}/NPQ_{max}	*Time (T)*	1	21.35	3.39	0.08
	Copper (Cu^{2+})	2	63.99	10.15	**<0.01**
	Nitrate (N)	1	64.61	10.25	**<0.01**
	TCu^{2+}*	2	17.10	2.71	0.09
	*T*N*	1	58.64	9.30	**<0.01**
	*Cu^{2+}*N*	2	13.69	2.17	0.14
	TCu^{2+}*N*	2	17.24	2.74	0.09
	Res	24	6.30		

Res: Residual

Table S2. ANOVA results after experiment testing for the effect of the Copper (Cu^{2+}) and Nitrate (N) treatments on the Total copper and internal copper cellular contents in *Cystoseira tamariscifolia.* Significant differences at $P<0.05$ are shown in bold.

		Cystoseira tamariscifolia			
		df	**MS**	***F***	***P***
Total Copper	*Copper (Cu^{2+})*	2	62272.63	50.33	**<0.01**
	Nitrate (N)	1	635.72	0.51	0.49
	*Cu^{2+}*N*	2	189.86	0.15	0.86
	Res	12	1237.35		
Internal copper	*Copper (Cu^{2+})*	2	17819.04	42.52	**<0.01**
	Nitrate (N)	1	0.43	0.00	0.97
	*Cu^{2+}*N*	2	206.91	0.49	0.62
	Res	12	419.10		

Res: Residual

Table S3. ANOVA results after experiment testing for the effect of Time (T), Copper (Cu) and Nitrate (N) on Carbon, Nitrogen and Ratio C:N contents of *Cystoseira tamariscifolia*. Significant differences at $P<0.05$ are shown in bold.

		Cystoseira tamariscifolia			
		df	**MS**	***F***	***P***
Nitrogen	*Time (T)*	1	**192.05**	**182.43**	**<0.01**
	Copper (Cu^{2+})	2	**13.40**	**12.73**	**<0.01**
	Nitrate (N)	1	0.34	0.33	0.57
	TCu^{2+}*	2	3.27	3.11	0.06
	*T*N*	1	**9.80**	**9.31**	**<0.01**
	*Cu^{2+}*N*	2	**9.01**	**8.56**	**<0.01**
	TCu^{2+}*N*	2	1.04	0.98	0.39
	Res	24	1.05		
C:N	*Time (T)*	1	**97.38**	**29.99**	**<0.01**
	Copper (Cu^{2+})	2	**21.89**	**6.74**	**<0.01**
	Nitrate (N)	1	4.32	1.33	0.26
	TCu^{2+}*	2	2.50	0.77	0.47
	*T*N*	1	1.93	0.60	0.45
	*Cu^{2+}*N*	2	7.90	2.43	0.11
	TCu^{2+}*N*	2	0.67	0.21	0.81
	Res	24	3.25		
Carbon	*Time (T)*	1	263.52	1.50	0.23
	Copper (Cu^{2+})	2	125.36	0.71	0.50
	Nitrate (N)	1	134.56	0.77	0.39
	TCu^{2+}*	2	379.30	2.16	0.14
	*T*N*	1	5.92	0.03	0.86
	*Cu^{2+}*N*	2	107.22	0.61	0.55
	TCu^{2+}*N*	2	71.51	0.41	0.67
	Res	24	175.61		

Res: Residual

Table S4. ANOVA results after experiment testing for the effect ofTime (T), Copper (Cu^{2+}) and Nitrate (N) on the photosynthetic pigments; Chl*a*, Chl*c* and Fucoxanthin of *Cystoseira tamariscifolia*. Significant differences at $P<0.05$ are shown in bold.

			Cystoseira tamariscifolia		
		df	**MS**	***F***	***P***
Chla	*Time (T)*	1	**2.124**	**18.19**	**<0.01**
	Copper (Cu^{2+})	2	**2.349**	**20.12**	**<0.01**
	Nitrate (N)	1	**0.870**	**7.45**	**<0.01**
	TCu^{2+}*	2	**3.644**	**31.19**	**<0.01**
	*T*N*	1	**0.505**	**4.33**	**0.04**
	*Cu^{2+}*N*	2	0.264	2.26	0.13
	TCu^{2+}*N*	2	**0.494**	**4.23**	**0.02**
	Res	24	0.117		
Chlc	*Time (T)*	1	**0.029**	**8.12**	**<0.01**
	Copper (Cu^{2+})	2	**0.042**	**11.84**	**<0.01**
	Nitrate (N)	1	0.012	3.48	0.07
	TCu^{2+}*	2	**0.048**	**13.44**	**<0.01**
	*T*N*	1	0.000	0.00	0.99
	*Cu^{2+}*N*	2	0.000	0.09	0.92
	TCu^{2+}*N*	2	0.000	0.01	0.99
	Res	24	0.004		
Fucoxanthin	*Time (T)*	1	**0.004**	**14.90**	**<0.01**
	Copper (Cu^{2+})	2	**0.004**	**15.35**	**<0.01**
	Nitrate (N)	1	0.000	2.01	0.17
	TCu^{2+}*	2	**0.002**	**9.15**	**<0.01**
	*T*N*	1	0.000	0.00	0.99
	*Cu^{2+}*N*	2	0.000	1.56	0.23
	TCu^{2+}*N*	2	0.000	0.03	0.97
	Res	24	0.000		

Res: Residual

Table S5. ANOVA results after experiment testing for the effect of Time (T), Copper (Cu^{2+}) and Nitrate (N) on total phenolic content, PCw and antioxidant activity (EC_{50}) by *Cystoseira tamariscifolia.* Significant differences at $P< 0.05$ are shown in bold.

		Cystoseira tamariscifolia			
		df	**MS**	***F***	***P***
Phenolic compounds	*Time (T)*	1	**537.00**	**43.97**	**<0.01**
	Copper (Cu^{2+})	2	16.08	1.32	0.29
	Nitrate (N)	1	**132.44**	**10.84**	**<0.01**
	TCu^{2+}*	2	**69.23**	**5.67**	**<0.01**
	*T*N*	1	**125.92**	**10.31**	**<0.01**
	*Cu^{2+}*N*	2	**297.53**	**24.36**	**<0.01**
	TCu^{2+}*N*	2	**139.39**	**11.41**	**<0.01**
	Res	24	12.21		
PCw	*Time (T)*	1	**0.29**	**138.03**	**<0.01**
	Copper (Cu^{2+})	2	**0.05**	**22.50**	**<0.01**
	Nitrate (N)	1	**0.02**	**9.51**	**<0.01**
	TCu^{2+}*	2	**0.06**	**28.51**	**<0.01**
	*T*N*	1	**0.06**	**29.26**	**<0.01**
	*Cu^{2+}*N*	2	**0.05**	**23.37**	**<0.01**
	TCu^{2+}*N*	2	**0.02**	**9.30**	**<0.01**
	Res	24	0.00		
EC_{50}	*Time (T)*	1	**0.01**	**13.62**	**<0.01**
	Copper (Cu^{2+})	2	**0.03**	**42.05**	**<0.01**
	Nitrate (N)	1	**0.08**	**126.36**	**<0.01**
	TCu^{2+}*	2	**0.01**	**11.66**	**<0.01**
	*T*N*	1	**0.02**	**29.91**	**<0.01**
	*Cu^{2+}*N*	2	**0.11**	**169.16**	**<0.01**
	TCu^{2+}*N*	2	**0.01**	**17.05**	**<0.01**
	Res	24	0.00		

Res: Residual

Table S6. ANOVA results after experiment testing for the effect of Copper (Cu^{2+}) and Nitrate (N) on the phenolic compound components; Shikimic acid and Phloroglucinol of *Cystoseira tamariscifolia.* Significant differences at $P< 0.05$ are shown in bold.

		Cystoseira tamariscifolia			
		df	**MS**	***F***	***P***
Shikimic acid	*Copper (Cu^{2+})*	2	1508.04	1.09	0.37
	Nitrate (N)	1	1951.11	1.42	0.26
	*Cu^{2+}*N*	2	665.78	0.48	0.63
	Res	12	1377.85		
Phloroglucinol	*Copper (Cu2+)*	2	3914.1	3.74	0.05
	Nitrate (N)	1	4796.7	4.59	0.05
	*Cu^{2+}*N*	2	1313.9	1.26	0.32
	Res	12	1045.2		

Res: Residual

Subchapter 5:

Table S1. ANOVA results after *in situ* experiment testing for the effect of Site, Nutrient and Irradiance on the Carbon and Nitrogen contents of *Cystoseira compressa* and *Padina pavonica*. Significant differences at $P< 0.05$ are shown in bold.

			Cystoseira compressa			***Padina pavonica***		
		df	**MS**	***F***	***P***	**MS**	***F***	***P***
Carbon	*Site (S)*	2	**576.60**	**5.01**	**0.02**	**2613.28**	**28.63**	**<0.01**
	Nutrient (N)	1	3.36	0.03	0.87	60.71	0.67	0.42
	Irradiance (E)	1	57.25	0.50	0.49	241.03	2.64	0.12
	*S*N*	2	68.94	0.60	0.56	**923.14**	**10.11**	**<0.01**
	*S*E*	2	35.05	0.30	0.74	**496.31**	**5.44**	**<0.01**
	*N*E*	1	420.25	3.65	0.07	**2333.70**	**25.57**	**<0.01**
	*S*N*E*	2	181.11	1.57	0.23	**506.53**	**5.55**	**<0.01**
	Res	24	115.11			91.28		
Nitrogen	*Site (S)*	2	**17.81**	**19.55**	**<0.01**	0.93	0.63	0.54
	Nutrient (N)	1	**17.66**	**19.39**	**<0.01**	**14.57**	**9.87**	**<0.01**
	Irradiance (E)	1	**4.80**	**5.27**	**0.03**	4.83	3.27	0.08
	*S*N*	2	**12.73**	**13.97**	**<0.01**	0.84	0.57	0.57
	*S*E*	2	**21.02**	**23.07**	**<0.01**	2.76	1.87	0.18
	*N*E*	1	2.26	2.48	0.13	**9.18**	**6.22**	**0.02**
	*S*N*E*	2	2.75	3.02	0.07	**13.44**	**9.11**	**<0.01**
	Res	24	0.91			1.48		
C:N	*Site (S)*	2	0.97	0.07	0.93	**6.12**	**5.11**	**<0.01**
	Nutrient (N)	1	33.31	2.41	0.13	**16.37**	**13.66**	**<0.01**
	Irradiance (E)	1	9.13	0.66	0.42	1.99	1.66	0.21
	*S*N*	2	29.07	2.11	0.14	1.01	0.85	0.44
	*S*E*	2	29.20	2.12	0.14	1.81	1.51	0.24
	*N*E*	1	2.37	0.17	0.68	0.16	0.13	0.72
	*S*N*E*	2	4.94	0.36	0.70	2.44	2.04	0.15
	Res	24	13.80			1.20		

Res: Residual

Table S2. ANOVA results after *in situ* experiment testing for the effect of Site, Nutrient and Irradiance on the photosynthetic parameters; maximal quantum yield (F_v/F_m), photosynthetic efficiency (α_{ETR}), maximal electron transport rate (ETR_{max}), irradiance of saturation of ETR (Ek_{ETR}), maximal non-photochemical quenching (NPQ_{max}) of *Cystoseira compressa* and *Padina pavonica.* Significant differences at $P< 0.05$ are shown in bold.

			Cystoseira compressa			***Padina pavonica***		
		df	**MS**	***F***	***P***	**MS**	***F***	***P***
F_v/F_m	*Site (S)*	2	**0.02**	**5.05**	**<0.01**	0.03	2.73	0.09
	Nutrient (N)	1	**0.08**	**17.37**	**<0.01**	**0.08**	**6.16**	**0.02**
	Irradiance (I)	1	**0.04**	**9.49**	**<0.01**	0.04	2.77	0.11
	*S*N*	2	**0.03**	**6.80**	**<0.01**	0.00	0.09	0.91
	*S*I*	2	**0.04**	**8.19**	**<0.01**	0.04	3.18	0.06
	*N*I*	1	**0.03**	**6.40**	**0.02**	**0.25**	**19.72**	**<0.01**
	*S*N*I*	2	**0.08**	**17.64**	**<0.01**	**0.05**	**3.95**	**0.03**
	Res	24	0.00			0.01		
α_{ETR}	*Site (S)*	2	0.003	0.89	0.42	0.004	1.61	0.22
	Nutrient (N)	1	0.001	0.16	0.69	0.006	2.38	0.14
	Irradiance (I)	1	0.001	0.14	0.71	0.001	0.24	0.63
	*S*N*	2	0.008	2.14	0.14	0.001	0.55	0.58
	*S*I*	2	**0.013**	**3.63**	**0.04**	0.005	2.15	0.14
	*N*I*	1	0.004	0.96	0.34	**0.070**	**27.79**	**<0.01**
	*S*N*I*	2	**0.067**	**18.51**	**<0.01**	**0.011**	**4.47**	**0.02**
	Res	24	0.004			0.003		
ETR_{max}	*Site (S)*	2	207.5	0.59	0.56	344.0	2.94	0.07
	Nutrient (N)	1	**2334.4**	**6.63**	**0.02**	**790.7**	**6.75**	**0.02**
	Irradiance (I)	1	**2859.1**	**8.13**	**<0.01**	**1141.2**	**9.74**	**<0.01**
	*S*N*	2	**1298.4**	**3.69**	**0.04**	208.1	1.78	0.19
	*S*I*	2	4.25	0.01	0.99	26.5	0.23	0.80
	*N*I*	1	**2581.7**	**7.34**	**<0.01**	62.8	0.54	0.47
	*S*N*I*	2	**2709.7**	**7.70**	**<0.01**	22.4	0.19	0.83
	Res	24	351.9			117.2		
Ek_{ETR}	*Site (S)*	2	2323.2	0.66	0.53	2184.9	0.67	0.52
	Nutrient (N)	1	**44102.2**	**12.45**	**<0.01**	**43711.9**	**13.46**	**<0.01**
	Irradiance (I)	1	**19246.6**	**5.43**	**0.03**	**26418.7**	**8.13**	**<0.01**
	*S*N*	2	2519.1	0.71	0.50	4438.1	1.37	0.27
	*S*I*	2	10595.1	2.99	0.07	**13590.8**	**4.18**	**0.03**
	*N*I*	1	**22495.3**	**6.35**	**0.02**	**79505.7**	**24.48**	**<0.01**
	*S*N*I*	2	3494.6	0.99	0.39	**22613.5**	**6.96**	**<0.01**
	Res	24	3541.4			3248.4		
NPQ_{max}	*Site (S)*	2	1.07	5.11	0.01	0.57	2.26	0.13
	Nutrient (N)	1	**9.73**	**46.53**	**<0.01**	**5.00**	**19.95**	**<0.01**
	Irradiance (I)	1	0.23	1.11	0.30	**4.04**	**16.11**	**<0.01**
	*S*N*	2	**6.27**	**30.02**	**<0.01**	**1.80**	**7.19**	**<0.01**
	*S*I*	2	**1.96**	**9.37**	**<0.01**	**1.43**	**5.72**	**<0.01**
	*N*I*	1	**3.78**	**18.08**	**<0.01**	**9.66**	**38.53**	**<0.01**
	*S*N*I*	2	**9.84**	**47.06**	**<0.01**	**1.43**	**5.71**	**<0.01**
	Res	24	0.21			0.25		

Res: Residual

Table S3. ANOVA results after *in situ* experiment testing for the effect of Site, Nutrient and Irradiance on the photosynthetic pigments; Chl*a*, Chl*c*, fucoxanthin and violoxanthin of *Cystoseira compressa* and *Padina pavonica*. Significant differences at $P < 0.05$ are shown in bold.

			Cystoseira compressa			***Padina pavonica***		
		df	**MS**	***F***	***P***	**MS**	***F***	***P***
Chla	*Site (S)*	2	0.384	1.69	0.21	0.012	0.86	0.43
	Nutrient (N)	1	**1.331**	**5.85**	**0.02**	0.016	1.19	0.29
	Irradiance (I)	1	0.009	0.04	0.84	**0.067**	**4.92**	**0.04**
	*S*N*	2	0.044	0.19	0.83	**0.188**	**13.78**	**<0.01**
	*S*I*	2	0.044	0.19	0.83	**0.153**	**11.20**	**<0.01**
	*N*I*	1	0.263	1.15	0.29	**0.674**	**49.41**	**<0.01**
	*S*N*I*	2	0.035	0.15	0.86	0.009	0.68	0.52
	Res	24	0.228			0.014		
Chlc	*Site (S)*	2	0.244	2.16	0.14	0.005	0.68	0.51
	Nutrient (N)	1	0.073	0.64	0.43	0.008	1.13	0.30
	Irradiance (I)	1	0.002	0.02	0.88	0.005	0.71	0.41
	*S*N*	2	0.110	0.98	0.39	**0.110**	**15.29**	**<0.01**
	*S*I*	2	0.076	0.67	0.52	**0.107**	**14.86**	**<0.01**
	*N*I*	1	0.108	0.95	0.34	**0.239**	**33.17**	**<0.01**
	*S*N*I*	2	0.164	1.45	0.25	0.005	0.70	0.51
	Res	24	0.113			0.007		
Fucoxanthin	*Site (S)*	2	0.048	1.38	0.27	**0.166**	**19.19**	**<0.01**
	Nutrient (N)	1	0.016	0.47	0.50	**0.251**	**29.03**	**<0.01**
	Irradiance (I)	1	0.003	0.08	0.77	**0.542**	**62.60**	**<0.01**
	*S*N*	2	0.005	0.14	0.87	**0.301**	**34.75**	**<0.01**
	*S*I*	2	0.000	0.00	1.00	**0.218**	**25.18**	**<0.01**
	*N*I*	1	0.067	1.92	0.18	**0.300**	**34.59**	**<0.01**
	*S*N*I*	2	0.021	0.60	0.56	**0.399**	**46.05**	**<0.01**
	Res	24	0.035			0.009		
Violoxanthin	*Site (S)*	2	0.003	3.39	0.05	0.002	3.97	0.03
	Nutrient (N)	1	0.000	0.06	0.81	**0.005**	**7.82**	**<0.01**
	Irradiance (I)	1	0.001	1.03	0.32	**0.005**	**8.92**	**<0.01**
	*S*N*	2	0.001	1.51	0.24	0.002	3.05	0.07
	*S*I*	2	0.000	0.45	0.64	**0.005**	**7.79**	**<0.01**
	*N*I*	1	0.000	0.17	0.68	0.001	1.10	0.31
	*S*N*I*	2	0.001	1.35	0.28	**0.004**	**7.10**	**<0.01**
	Res	24	0.001			0.001		

Res: Residual

Table S4. ANOVA results after *in situ* experiment testing for the effect of Site, Nutrient and Irradiance on the phenolic compounds and antioxidant activity (EC_{50}) of *Cystoseira compressa* and *Padina pavonica*. Significant differences at $P< 0.05$ are shown in bold.

			Cystoseira compressa			***Padina pavonica***		
		df	**MS**	***F***	***P***	**MS**	***F***	***P***
Phenolic	*Site (S)*	2	**597.34**	**140.24**	**<0.01**	**14.46**	**13.78**	**<0.01**
compounds	*Nutrient (N)*	1	**782.49**	**183.71**	**<0.01**	**5.11**	**4.87**	**0.04**
	Irradiance (I)	1	1.89	0.44	0.51	**12.32**	**11.73**	**<0.01**
	*S*N*	2	**122.05**	**28.65**	**<0.01**	**3.80**	**3.62**	**0.04**
	*S*I*	2	**52.62**	**12.35**	**<0.01**	**9.42**	**8.97**	**<0.01**
	*N*I*	1	**77.18**	**18.12**	**<0.01**	**12.06**	**11.49**	**<0.01**
	*S*N*I*	2	**135.71**	**31.86**	**<0.01**	**32.91**	**31.35**	**<0.01**
	Res	24	4.26			1.05		
EC_{50}	*Site (S)*	2	0.00	0.04	0.96	0.04	0.87	0.43
	Nutrient (N)	1	0.06	2.24	0.15	**0.24**	**5.32**	**0.03**
	Irradiance (I)	1	0.04	1.27	0.27	0.07	1.45	0.24
	*S*N*	2	**0.13**	**4.68**	**0.02**	0.02	0.45	0.64
	*S*I*	2	**0.22**	**7.69**	**<0.01**	**0.17**	**3.85**	**0.04**
	*N*I*	1	0.03	1.14	0.30	0.00	0.05	0.82
	*S*N*I*	2	0.01	0.50	0.62	0.12	2.63	0.09
	Res	24	0.03			0.05		

Res: Residual

Subchapter 6:

Table S1. ANOVA results after experimental period testing for the effect of Temperature, CO_2 levels and Origin on the Growth of *Cystoseira tamariscifolia*. Significant differences at $P < 0.01$ are shown in bold.

		Cystoseira tamariscifolia			
		df	**MS**	***F***	***P***
Growth	*Tempertaure (Tº)*	1	0.01	0.26	0.62
	pH (pH)	1	0.20	5.28	0.04
	Origin (O)	1	**0.40**	**10.54**	**<0.01**
	*Tº*pH*	1	0.06	1.67	0.21
	*Tº*O*	1	0.00	0.01	0.93
	*pH*O*	1	**0.56**	**14.47**	**<0.01**
	*Tº*pH*O*	1	0.27	7.03	0.02
	Res	16	0.04		

Table S2. ANOVA results after experimental period testing for the effect of Time, Temperature, CO_2 levels and Origin on the Carbon, Nitrogen and Ratio C:N of *Cystoseira tamariscifolia*. Significant differences at $P < 0.01$ are shown in bold.

		Cystoseira tamariscifolia			
		df	**MS**	***F***	***P***
Carbon	*Time (T)*	3	**1545.19**	**15.59**	**<0.01**
	Temperature (TºC)	1	**3987.39**	**40.23**	**<0.01**
	pH (pH)	1	**1013.35**	**10.23**	**<0.01**
	Origin (O)	1	**3221.33**	**32.50**	**<0.01**
	*T*TºC*	3	250.40	2.53	0.07
	*T*pH*	3	135.65	1.37	0.26
	*TºC*pH*	1	331.16	3.34	0.07
	*T*O*	3	237.48	2.40	0.08
	*TºC*O*	1	3.05	0.03	0.86
	*pH*O*	1	428.84	4.33	0.04
	*T*TºC*pH*	3	120.43	1.22	0.31
	*T*TºC*O*	3	175.61	1.77	0.16
	*T*pH*O*	3	26.52	0.27	0.85
	*TºC*pH*O*	1	225.40	2.27	0.14
	*T*TºC*pH*O*	3	28.69	0.29	0.83
	Res	64	99.10		
Nitrogen	*Time (T)*	3	**132.09**	**42.71**	**<0.01**
	Temperature (TºC)	1	13.05	4.22	0.04
	pH (pH)	1	19.16	6.20	0.02
	Origin (O)	1	**60.15**	**19.45**	**<0.01**
	*T*TºC*	3	1.76	0.57	0.64
	*T*pH*	3	10.73	3.47	0.02
	*TºC*pH*	1	1.38	0.44	0.51
	*T*O*	3	**17.41**	**5.63**	**<0.01**
	*TºC*O*	1	2.14	0.69	0.41
	*pH*O*	1	**22.73**	**7.35**	**<0.01**
	*T*TºC*pH*	3	**12.78**	**4.13**	**<0.01**
	*T*TºC*O*	3	3.73	1.21	0.31
	*T*pH*O*	3	4.60	1.49	0.23
	*TºC*pH*O*	1	9.18	2.97	0.09
	*T*TºC*pH*O*	3	4.19	1.35	0.26
	Res	64	3.09		
C:N	*Time (T)*	3	**139.08**	**43.78**	**<0.01**
	Temperature (TºC)	1	**35.94**	**11.31**	**<0.01**
	pH (pH)	1	17.78	5.60	0.02
	Origin (O)	1	**124.65**	**39.24**	**<0.01**
	*T*TºC*	3	1.97	0.62	0.60
	*T*pH*	3	4.87	1.53	0.21
	*TºC*pH*	1	9.07	2.85	0.10
	*T*O*	3	**22.38**	**7.05**	**<0.01**
	*TºC*O*	1	2.53	0.80	0.38
	*pH*O*	1	9.88	3.11	0.08
	*T*TºC*pH*	3	6.02	1.90	0.14
	*T*TºC*O*	3	2.49	0.78	0.51
	*T*pH*O*	3	4.42	1.39	0.25
	*TºC*pH*O*	1	1.97	0.62	0.43
	*T*TºC*pH*O*	3	1.95	0.62	0.61
	Res	64	3.18		

Res: residual

Table S3. ANOVA results after experimental period testing for the effect of Time, Temperature, CO_2 levels and Origin on the photosynthetic parameters; maximal quantum yield (F_v/F_m) and maximal electron transport rate (ETR_{max}), of *Cystoseira tamariscifolia*. Significant differences at $P< 0.01$ are shown in bold.

		Cystoseira tamariscifolia			
		df	**MS**	***F***	***P***
F_v/F_m	*Time (T)*	3	**0.01**	**7.07**	**<0.01**
	Temperature (TºC)	1	**0.01**	**5.73**	**0.02**
	pH (pH)	1	**0.01**	**4.89**	**0.03**
	Site (S)	1	0.00	0.02	0.88
	*T*TºC*	3	0.00	0.08	0.97
	*T*pH*	3	0.00	1.50	0.22
	*TºC*pH*	1	0.00	0.25	0.62
	*T*S*	3	0.00	0.91	0.44
	*TºC*S*	1	0.00	1.16	0.29
	*pH*S*	1	0.00	1.51	0.22
	*T*TºC*pH*	3	**0.01**	**4.11**	**<0.01**
	*T*TºC*S*	3	0.00	0.83	0.48
	*T*pH*S*	3	0.00	1.17	0.33
	*TºC*pH*S*	1	0.00	2.22	0.14
	*T*TºC*pH*S*	3	0.00	0.42	0.74
	Res	64	0.00		
ETRmax	*Time (T)*	3	**3288.77**	**53.01**	**<0.01**
	Temperature (TºC)	1	**545.34**	**8.79**	**<0.01**
	pH (pH)	1	**487.13**	**7.85**	**<0.01**
	Site (S)	1	**982.73**	**15.84**	**<0.01**
	*T*TºC*	3	28.11	0.45	0.72
	*T*pH*	3	68.02	1.10	0.36
	*TºC*pH*	1	9.21	0.15	0.70
	*T*S*	3	**223.85**	**3.61**	**0.02**
	*TºC*S*	1	2.13	0.03	0.85
	*pH*S*	1	149.46	2.41	0.13
	*T*TºC*pH*	3	**314.65**	**5.07**	**<0.01**
	*T*TºC*S*	3	92.60	1.49	0.22
	*T*pH*S*	3	**239.97**	**3.87**	**<0.01**
	*TºC*pH*S*	1	21.68	0.35	0.56
	*T*TºC*pH*S*	3	**297.97**	**4.80**	**<0.01**
	Res	64	62.04		

Table S4. ANOVA results after experimental period testing for the effect of Time, Temperature, CO_2 levels and Origin on the Pigment content of *Cystoseira tamariscifolia*. Significant differences at $P<$ 0.01 are shown in bold.

		Cystoseira tamariscifolia			
		df	**MS**	***F***	***P***
Chla	*Time (T)*	3	10.48	77.68	**<0.01**
	Temperature (TºC)	1	0.21	1.58	0.21
	pH (pH)	1	0.02	0.12	0.73
	Site (S)	1	8.24	61.05	**<0.01**
	*T*TºC*	3	0.16	1.18	0.33
	*T*pH*	3	0.36	2.70	0.04
	*TºC*pH*	1	**1.50**	**11.12**	**<0.01**
	*T*S*	3	0.24	1.80	0.16
	*TºC*S*	1	0.02	0.15	0.70
	*pH*S*	1	0.03	0.23	0.64
	*T*TºC*pH*	3	**1.86**	**13.79**	**<0.01**
	*T*TºC*S*	3	0.29	2.13	0.11
	*T*pH*S*	3	0.14	1.06	0.37
	*TºC*pH*S*	1	0.06	0.47	0.50
	*T*TºC*pH*S*	3	0.12	0.92	0.44
	Res	64	0.13		
Chlc	*Time (T)*	3	**0.05**	**106.16**	**<0.01**
	Temperature (TºC)	1	**0.01**	**13.82**	**<0.01**
	pH (pH)	1	0.00	0.44	0.51
	Site (S)	1	**0.02**	**48.25**	**<0.01**
	*T*TºC*	3	**0.00**	**6.82**	**<0.01**
	*T*pH*	3	**0.00**	**4.47**	**<0.01**
	*TºC*pH*	1	0.00	0.50	0.48
	*T*S*	3	0.00	2.18	0.10
	*TºC*S*	1	0.00	0.84	0.36
	*pH*S*	1	0.00	1.52	0.22
	*T*TºC*pH*	3	0.00	2.72	0.04
	*T*TºC*S*	3	0.00	0.72	0.54
	*T*pH*S*	3	0.00	1.50	0.22
	*TºC*pH*S*	1	0.00	1.51	0.22
	*T*TºC*pH*S*	3	0.00	3.47	0.02
	Res	64	0.00		
Fucoxanthin	*Time (T)*	3	**176424.94**	**9.93**	**<0.01**
	Temperature (TºC)	1	5253.55	0.30	0.59
	pH (pH)	1	7376.06	0.41	0.52
	Site (S)	1	**931182.81**	**52.39**	**<0.01**
	*T*TºC*	3	34794.66	1.96	0.13
	*T*pH*	3	58662.92	3.30	0.03
	*TºC*pH*	1	3967.73	0.22	0.64
	*T*S*	3	47447.94	2.67	0.04

	*TºC*S*	1	27.50	0.00	0.97
	*pH*S*	1	25556.62	1.44	0.23
	*T*TºC*pH*	3	27326.97	1.54	0.21
	*T*TºC*S*	3	**67590.85**	**3.80**	**<0.01**
	*T*pH*S*	3	25161.18	1.42	0.25
	*TºC*pH*S*	1	18101.53	1.02	0.32
	*T*TºC*pH*S*	3	21129.89	1.19	0.32
	Res	64	17774.30		
Violaxanthin	*Time (T)*	3	**5140.16**	**15.48**	**<0.01**
	Temperature (TºC)	1	302.50	0.91	0.34
	pH (pH)	1	0.17	0.00	0.98
	Site (S)	1	**14113.21**	**42.50**	**<0.01**
	*T*TºC*	3	481.14	1.45	0.24
	*T*pH*	3	982.29	2.96	0.04
	*TºC*pH*	1	2014.00	6.07	0.02
	*T*S*	3	**1475.01**	**4.44**	**<0.01**
	*TºC*S*	1	1.50	0.00	0.95
	*pH*S*	1	347.27	1.05	0.31
	*T*TºC*pH*	3	**2129.20**	**6.41**	**<0.01**
	*T*TºC*S*	3	872.85	2.63	0.06
	*T*pH*S*	3	711.05	2.14	0.10
	*TºC*pH*S*	1	68.35	0.21	0.65
	*T*TºC*pH*S*	3	433.69	1.31	0.28
	Res	64	332.04		
β- Carotene	*Time (T)*	3	**2471.83**	**6.57**	**<0.01**
	Temperature (TºC)	1	1994.36	5.30	0.02
	pH (pH)	1	22.32	0.06	0.81
	Site (S)	1	**17547.20**	**46.61**	**<0.01**
	*T*TºC*	3	522.64	1.39	0.25
	*T*pH*	3	1037.13	2.75	0.04
	*TºC*pH*	1	1200.28	3.19	0.08
	*T*S*	3	114.44	0.30	0.82
	*TºC*S*	1	1266.31	3.36	0.07
	*pH*S*	1	75.75	0.20	0.66
	*T*TºC*pH*	3	70.71	0.19	0.90
	*T*TºC*S*	3	125.41	0.33	0.80
	*T*pH*S*	3	932.83	2.48	0.07
	*TºC*pH*S*	1	3.35	0.01	0.93
	*T*TºC*pH*S*	3	817.63	2.17	0.10
	Res	64	376.49		

Res: residual

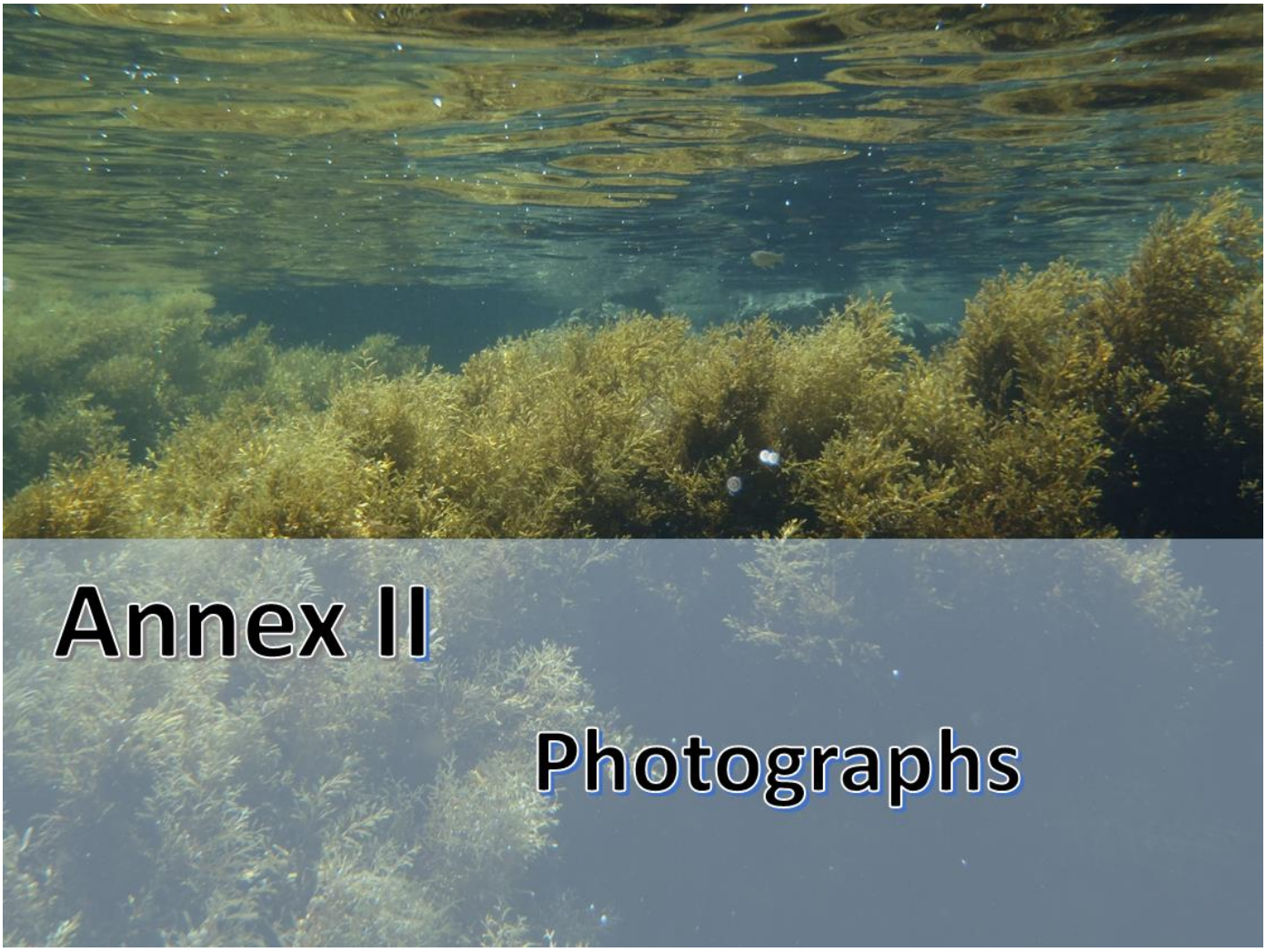
Table S5. ANOVA results after experimental period testing for the effect of Time, Temperature, CO_2 levels and Origin on the phenolic compound, phenolic compounds in the water and antioxidant activity (EC_{50}) of *Cystoseira tamariscifolia*. Significant differences at $P< 0.01$ are shown in bold.

		Cystoseira tamariscifolia			
		df	**MS**	***F***	***P***
Phenolic compounds	*Time (T)*	3	**103.65**	**4.89**	**<0.01**
	Temperature (TºC)	1	**272.75**	**12.86**	**<0.01**
	pH (pH)	1	**314.86**	**14.84**	**<0.01**
	Site (S)	1	**586.94**	**27.67**	**<0.01**
	*T*TºC*	3	45.79	2.16	0.10
	*T*pH*	3	6.82	0.32	0.81
	*TºC*pH*	1	25.67	1.21	0.28
	*T*S*	3	25.22	1.19	0.32
	*TºC*S*	1	24.88	1.17	0.28
	*pH*S*	1	129.44	6.10	0.02
	*T*TºC*pH*	3	27.31	1.29	0.29
	*T*TºC*S*	3	35.95	1.70	0.18
	*T*pH*S*	3	43.45	2.05	0.12
	*TºC*pH*S*	1	26.98	1.27	0.26
	*T*TºC*pH*S*	3	16.15	0.76	0.52
	Res	64	21.21		
Polyphenols in the water	*Time (T)*	3	**3.76**	**20.29**	**<0.01**
	Temperature (TºC)	1	0.04	0.24	0.63
	pH (pH)	1	**2.59**	**13.97**	**<0.01**
	Site (S)	1	**3.01**	**16.23**	**<0.01**
	*T*TºC*	3	0.22	1.21	0.31
	*T*pH*	3	0.41	2.19	0.10
	*TºC*pH*	1	0.30	1.61	0.21
	*T*S*	3	0.16	0.86	0.47
	*TºC*S*	1	0.11	0.61	0.44
	*pH*S*	1	0.65	3.48	0.07
	*T*TºC*pH*	3	0.03	0.17	0.92
	*T*TºC*S*	3	0.62	3.33	0.03
	*T*pH*S*	3	0.40	2.14	0.10
	*TºC*pH*S*	1	0.03	0.15	0.70
	*T*TºC*pH*S*	3	0.28	1.48	0.23
	Res	64	0.19		
EC_{50}	*Time (T)*	3	**0.75**	**17.13**	**<0.01**
	Temperature (TºC)	1	0.24	5.41	0.02
	pH (pH)	1	**0.32**	**7.24**	**<0.01**
	Site (S)	1	**1.08**	**24.60**	**<0.01**
	*T*TºC*	3	0.06	1.31	0.28
	*T*pH*	3	0.01	0.30	0.82
	*TºC*pH*	1	0.03	0.61	0.44
	*T*S*	3	0.02	0.40	0.76
	*TºC*S*	1	0.08	1.91	0.17
	*pH*S*	1	0.12	2.62	0.11
	*T*TºC*pH*	3	0.07	1.65	0.19
	*T*TºC*S*	3	0.08	1.85	0.15
	*T*pH*S*	3	**0.18**	**4.14**	**<0.01**
	*TºC*pH*S*	1	0.01	0.32	0.57
	*T*TºC*pH*S*	3	0.01	0.22	0.88
	Res	64	0.04		

Res: residual

Table S6. Pearson correlations between the variables (n=96); nitrogen (N) and carbon (C), phenolic compounds (PC), antioxidant activity (EC_{50}), phenolic compounds in the water (PCw), Chlorophyll *a* (Chla), Chlorophyll *c* (Chl*c*), Fucoxanthin (Fuco), Violaxanthin (Viol), β-carotene (β-caro), maximal quantum yield (*Fv/Fm*) and maximal electron transport rate (ETR_{max}). Significant differences at $P < 0.05$ are shown in bold.

	N	C:N	PC	EC50	PCw	Chla	Chlc	Fuco	Viol	β-caro	Fv/Fm	ETRmax
C	-0.322	0.553	0.506	-0.491	-0.421	0.187	0.186	-0.0823	-0.108	0.0256	-0.00974	0.276
	0.0014	**5.3E-09**	**1.42E-07**	**3.76E-07**	**2.E-05**	0.068	0.0695	0.425	0.294	0.805	0.925	**6.49E-03**
N		-0.945	-0.201	0.391	0.383	-0.146	-0.462	0.444	0.393	0.321	-0.135	-0.277
		2.31E-47	**0.0495**	**8.25E-05**	**1.17E-04**	0.155	**2.22E-06**	**5.85E-06**	**7.56E-05**	**1.41E-03**	0.191	**6.35E-03**
C:N			0.328	-0.481	-0.452	0.211	0.446	-0.396	-0.356	-0.258	0.117	0.292
			1.10E-03	**6.92E-07**	**3.80E-06**	**0.0389**	**5.40E-06**	**6.62E-05**	**3.73E-04**	**0.0112**	0.255	**3.92E-03**
PC				-0.79	-0.349	-0.00731	0.0638	-0.0823	-0.0903	-0.0124	-0.0223	0.154
				1.22E-21	**4.97E-04**	0.944	0.537	0.425	0.382	0.905	0.829	0.133
EC50					0.52	-0.131	-0.259	0.215	0.235	0.112	-0.156	-0.315
					5.48E-08	0.202	**0.0108**	**0.0355**	**0.0213**	0.277	0.13	**1.75E-03**
PCw						-0.131	-0.285	0.302	0.218	0.198	-0.156	-0.329
						0.203	**4.84E-03**	**2.80E-03**	**0.0328**	0.0532	0.128	**1.06E-03**
Chl*a*							0.501	0.317	0.306	-0.0153	-0.0982	0.0726
							1.99E-07	**0.00167**	**0.0024**	0.882	0.341	0.482
Chl*c*								-0.283	-0.234	-0.277	0.095	0.378
								5.22E-03	**0.0215**	**6.37E-03**	0.357	**1.46E-04**
Fuco									0.853	0.444	-0.225	-0.162
									3.05E-28	**0.00000589**	**0.0274**	0.114
Viol										0.482	-0.258	-0.202
										6.61E-07	**0.0113**	**0.0483**
β–caro											-0.252	-0.187
											0.0133	0.0677
Fv/Fm												0.466
												0.00000173



Natural pH gradient, Vulcano, Italy. May 2013. Photograph by Paula S. M. Celis Plá

Subchapter 1:

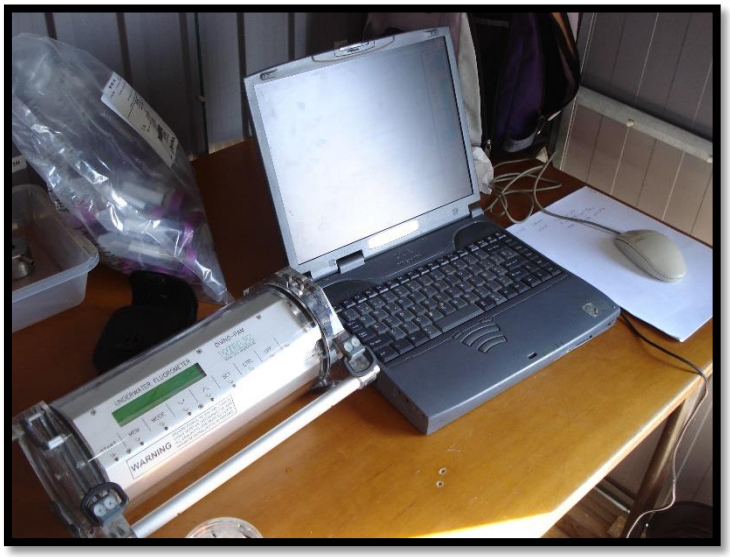
Light measurements in La Araña beach

Sampling for C:N ratio of *Cystoseira tamariscifolia*

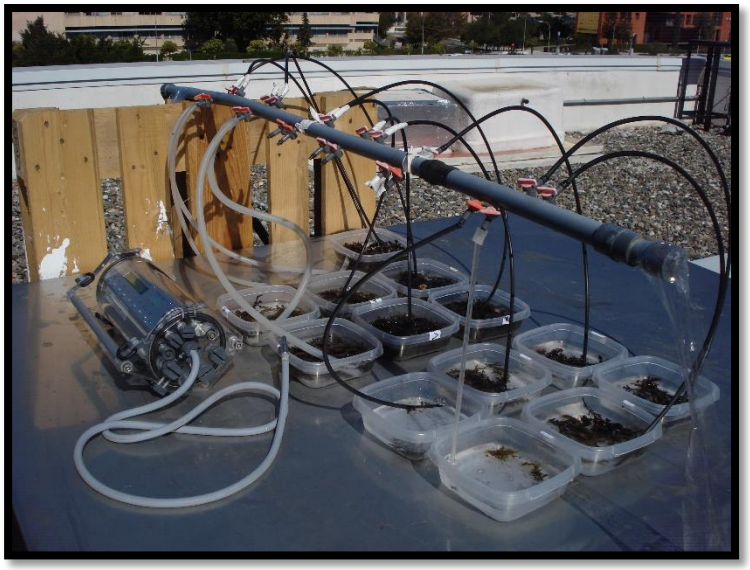
Cystoseira tamariscifolia from La Araña beach

Subchapter 2:

Methacrylate UV transparent vessel
(Plexi- glass XT- 29080) with *C. tamariscifolia*
In outdoor system in Malaga University

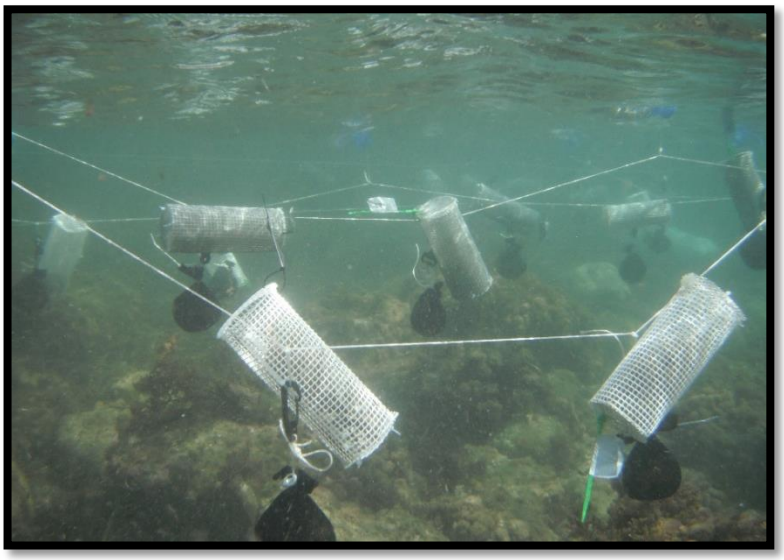


Diving Pam fluorometer with
connect to personal computer



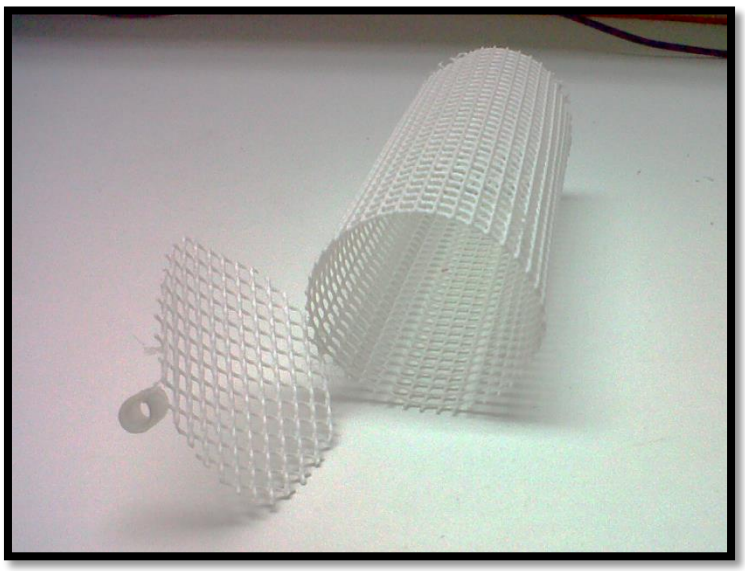
Experiment of exposure and
recovery with *C. tamariscifolia*

Subchapter 3:



Floating line system for each macroalgae in Cabo de Gata-Níjar Natural Park

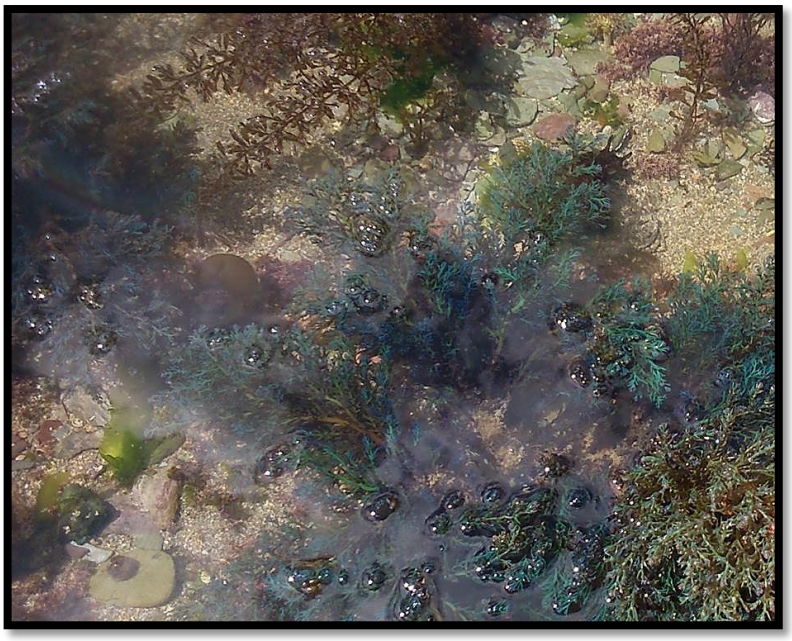
PAR and UVA sensors, Polycarbonate boxes



Cylinder for floating line system

Subchapter 4:

In door system in
Plymouth University



C. tamariscifolia from
Hannafore Point, UK

Low tidal in
Hannafore Point, UK

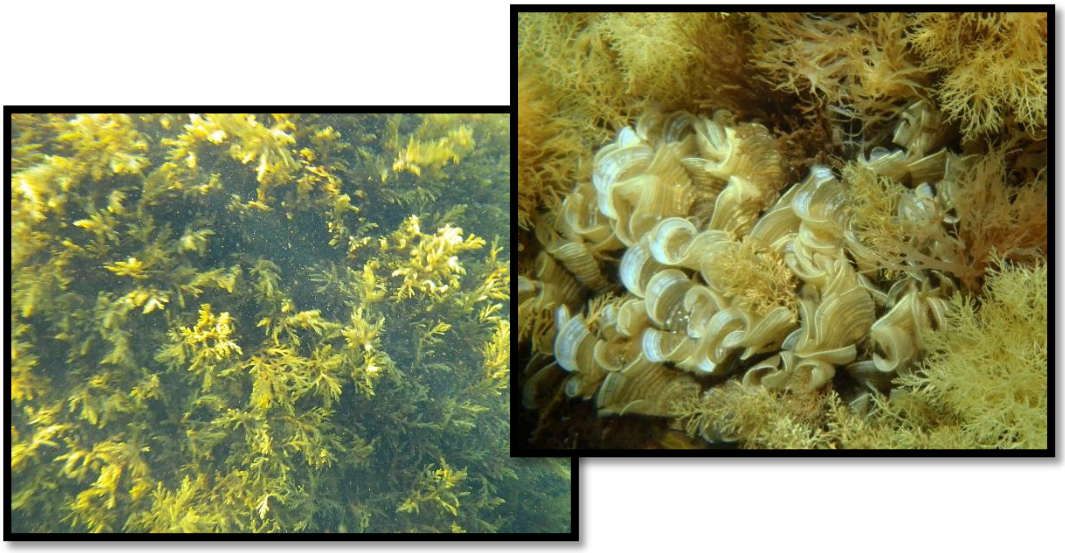
Subchapter 5:

Upper view of the pH gradient in Vulcano Island, Italy

CO_2 bubbles in low pH site

Sampling in the natural pH gradient with Diving PAM

Padina pavonica and *Cystoseira compressa* in natural pH (ambient CO_2) gradient in Vulcano



Site of the middle pH and in front volcano of the pH gradient

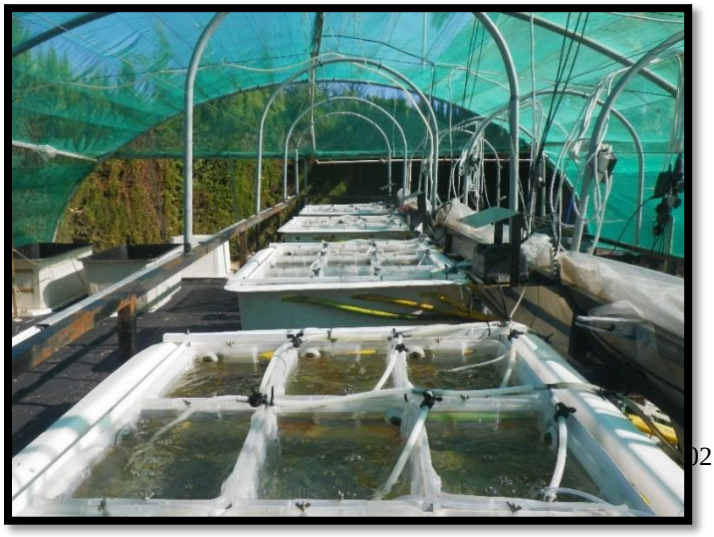
Team Vulcano Island

Subchapter 6:

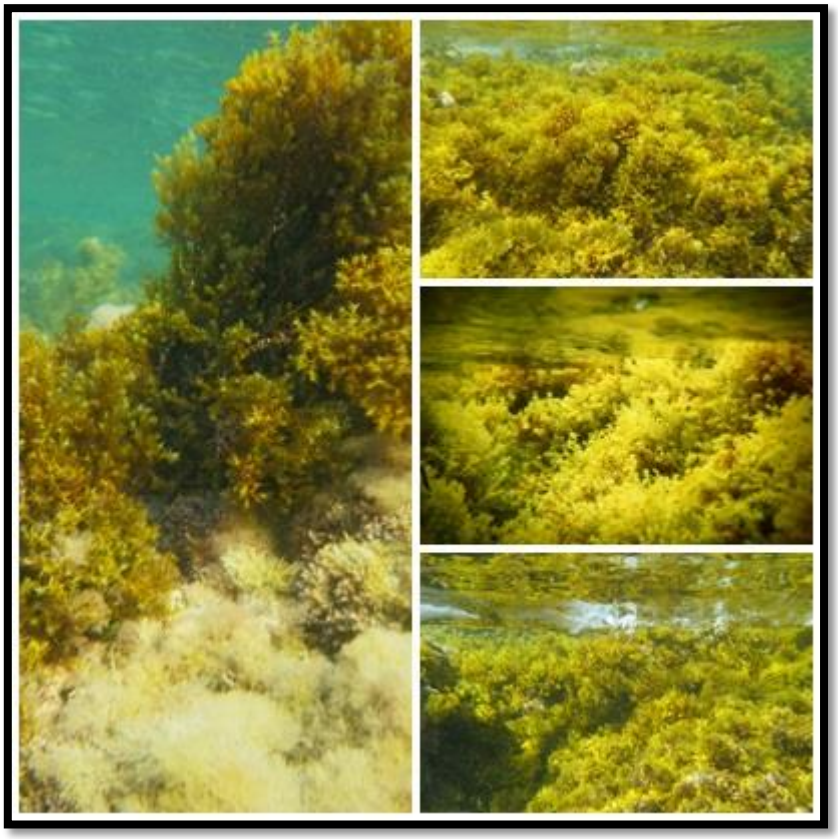
Mesocosms located in the Grace-Hutchinson experimental Centre

C. tamariscifolia
in the mesocosms system

Upper view of the
Mesocosms systems



Ulva spp, *Cystoseira tamariscifolia* and *Ellisolandia elongata* in oligotrophic waters



Cystoseira tamariscifolia and *Ellisolandia elongata* in ultraoligotrophic waters