



TESIS DOCTORAL

por compendio de publicaciones

Blue Cosmeceutical Applications of UV Screen and Antioxidant Compounds from Photosynthetic Organisms

Julia Vega Sánchez

Programa de Doctorado
Biotecnología Avanzada

Directores
Félix Diego López Figueroa
José Bonomi Barufi



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Málaga, diciembre 2023



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PROGRAMA DE DOCTORADO

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Blue Cosmeceutical Applications of UV Screen and Antioxidant Compounds from Photosynthetic Organisms

Comecéutica Azul: Aplicaciones de Compuestos Antioxidantes y
Absorbentes de Radiación UV a partir de Organismos Fotosintéticos

Julia Vega Sánchez

Departamento de Ecología y Geología. Área Ecología

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Málaga, diciembre 2023





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Memoria presentada por Julia Vega Sánchez para optar al grado de Doctora por la Universidad de Málaga con Mención Internacional en el Programa de Doctorado en Biotecnología Avanzada.



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Realizada bajo la tutorización de FÉLIX DIEGO LÓPEZ FIGUEROA y dirección de FÉLIX DIEGO LÓPEZ FIGUEROA Y JOSÉ BONOMI BARUFI (si tuviera varios directores deberá hacer constar el nombre de todos)

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A todos los que un día me enseñaron a pensar



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ABREVIATIONS

1064sk	Human fibroblasts
6-4PP	6-4 photoproducts
α_{ETR}	Photosynthetic electron transport rate efficiency
λ_c	Critical lambda
A	Absorptance
A-330	Asterina-330
Abs	Absorbance
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AC	Antioxidant capacity
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
BACs	Bioactive compounds
BCC	Basal cell carcinoma
BEPFs	Biological effective protection factors
CAT	Catalase
CCC	Counter-current chromatography
CFC	Chlorofluorocarbons
Chl	Chlorophyll
CPC	Centrifugal partition chromatography
CPDs	Cyclobutane pyrimidine dimers
DE	Dry extract
DF	Degrees of freedom
DGR	Daily growth rate
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
EAE	Enzyme assisted extraction

EC₅₀	Half maximal effective concentration
ECM	Extracellular matrix
EDR	Energy dissipation rate
ESAR	Effective solar absorpted radiation
EtOH	Ethanol
F_v/F_m	Maximal quantum yield
LED	Light-emitting diode
IPD	Inmediate pigments darkening
E_k	Saturated irradiance
E_{opt}	Optimal irradiance
ETR	Electron transport rate
ETR_{int}	Integrated ETR
ETR_{max}	Maximal ETR
FW	Fresh weight
G-361	Human malignant melanoma
GPx	Glutathione peroxidase
GR	Glutathione reductase
HACAT	Immortalized human keratinocytes
HEV	High energy visible
HPLC	High performance liquid chromatography
IMTA	Integrated multitrophic aquaculture
IR	Infrared
IR-A	Infrared A
IR-B	Infrared B
IR-C	Infrared C
MAAs	Mycosporine-like amino acids
MAE	Microwave assisted extraction
MED	Minimal erythema dose
MS	Mass spectrometry
M-ser	Mycosporine-serinol
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium <u>bromide</u>
MMPs	Metaloproteinases
NMR	Nuclear magnetic resonance

NMSCs	Non-melanoma skin cancers
NPQ	Non photochemical quenching
NPQ_{max}	Maximal NPQ
NUR	Nitrogen uptake rate
MeOH	Methanol
ODCs	Ozone depleting chemicals
P-334	Porphyrin 334
Pal	Palythine
PAM	Pulse amplitude modulated
PAR	Photosynthetic active radiation
PC	Phycocyanin
PE	Phycocerythrin
PPD	Persistent pigment darkening
PS	Photosystem
RLCs	Rapid light curve
ROS	Reactive oxygen species
SCC	Squamous cell carcinoma
SED	Standard erythema dose
SI	Selectivity index
SPF	Sun protection factor
SWE	Subcritical water extraction
SD	Standard deviation
SH	Shinorine
SNK	Student Newman Keuls posthoc test
SOD	Superoxide dismutase
TE	Trolox equivalents
UAE	Ultrasound assisted extraction
UHPLC	Ultra high performance liquid chromatography
UV	Ultraviolet
UVI	Ultraviolet index
UVR	Ultraviolet radiation
UV-A	Ultraviolet A
UVAPF	Ultraviolet A protection factor

UV-B	Ultraviolet B
UV-C	Ultraviolet C
Y(II)	Effective quantum yield
Y(NO)	Yield loss related to passive thermal dissipation
Y(NPQ)	Yield loss related to photoregulated process

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INTRODUCTION

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DISCUSSION

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ABSTRACT

The current lifestyle with a high sun exposure, due to the enjoying beach culture, recreational or working outdoor activities, and the increase of UV radiation in certain areas of the world due to the depletion of ozone layer and climate change, are contributing to the increase of skin and eyes problems caused by an excess of UV radiation exposure, such as sunburn, premature skin photoaging, immunosuppression, cataracts, or skin cancer.

It is well known that the use of sunscreens reduces the negative effects of UV radiation on our health, but the safety of certain UV-filters for our health and the environment has been questioned in the last years. UV-filters are responsible for the UV absorption or scattering and are divided in synthetic/chemical or mineral/physical. Some synthetic UV-filters have shown negative effects in human health, related to endocrine disruptions, photoallergies and oxidative stress. Moreover, accumulation of UV-filters in aquatic environments and organisms has been observed, causing negative impacts in the ecosystem, e.g. coral bleaching, reducing phytoplankton growth or hormone disorders in fish or mammals. Thus, areas with important marine ecosystems like Hawaii, Palau and some Caribbean islands have banned the use of certain synthetic UV-filters.

In order to prevent these damage consequences, it is necessary the search and development of alternative UV-filters that are natural, biodegradable and non-toxic. Fortunately, nowadays a large number of consumers are more concern about the possible negative impacts of UV-filters and other synthetic compounds normally used in cosmetic products, and they tend to prefer products containing natural products that are considered safer for the health and environment-friendly. In this context, algae, cyanobacteria and plants are daily submitted to different environmental stresses, so they have developed various defense and photoprotective mechanisms, including the synthesis and accumulation of both UV-screen and antioxidant compounds (e.g. carotenoids, polyphenols or mycosporine-like amino acids), that could be used as new sources of natural UV-filters in sunscreens.

Among bioactive compounds, mycosporine-like amino acids (MAAs) highlighted due to their UV-screen and antioxidant properties, as well as their high photo- and thermo-

stability and low toxicity. Therefore, the **first chapter** of the thesis it was reviewed published data about MAAs, including their biosynthesis, biomass production, extraction, characterization, identification, purification, and bioactivities. MAAs can be found in algae, cyanobacteria, or corals, but the highest concentrations are found in red macroalgae, mainly in the order Bangiales, as *Porphyra sensu lato*. There are different types of MAAs with different maximum absorptions (from 310 to 360 nm), the most common are: palythine (λ_{max} : 320 nm), asterina-330 (λ_{max} : 330 nm), palythanol (λ_{max} : 330 nm), shinorine (λ_{max} : 332 nm) and porphyra-334 (λ_{max} : 334 nm). The content of MAAs can vary depending on environmental factors, of which solar radiation and nitrogen availability are the most relevant. The use of MAAs as natural UV-filters required high biomass availability, that can be obtained from collection in high productive areas (local or invasive species) or culture under high nutrient conditions. Several authors have reported the culture of red algae (*Gracilaria cornea* or *G. vermiculophylla*, under integrated-multitrophic aquaculture (IMTA) systems with a high MAAs productivities. MAAs are easily extracted by maceration or sonication of the biomass in polar solvents (normally water, or combinations of water:ethanol/methanol), and are identified through HPLC, ESI-MS or NMR. Different HPLC methodologies have been found in the bibliography, some authors used C8 and other C18 columns, but most of them used acidified aqueous eluents. In our laboratory, we use a Luca C8 column (Phenomenex) with an isocratic flow (0.5 mL min^{-1}) of water with 1.5 % methanol and 0.15 % acetic acid. In recent years, different approaches have been followed to isolate MAAs, from simple chromatography columns, like silica-gel or ionic-exchange, to preparative UHPLC or liquid-liquid chromatography, being the latter industrially scaled. In addition to photoprotection, other bioactivities have been described in MAAs (*in vitro* and *in vivo*) such as antioxidant, anti-aging or immune-modulatory properties. All data presented in this review allow a broad comprehension of MAAs and show perspectives for their inclusion in cosmeceutical products.

As cyanobacteria and red macroalgae are the major producers of MAAs, and cyanobacteria can also present another photoprotective compound, named scytonemin, different species of these groups were analyzed in the **second chapter** of the thesis, looking for cosmeceutic properties, i.e. high content of MAAs, polyphenols and scytonemin, as well as antioxidant capacity. Biomass were extracted in different solvents (water, ethanol and two combinations of water:ethanol). In general, ethanol showed the lowest content of MAAs, phenols and antioxidant capacity. The highest concentrations

of MAAs were found in the red macroalgae *Porphyra umbilicalis* ($5.2 \pm 0.4 \text{ mg g}^{-1} \text{ DW}$), *Gelidium corneum* ($1.8 \pm 0.2 \text{ mg g}^{-1} \text{ DW}$ (dry weight)) and *Osmundea pinnatifida* ($1.5 \pm 0.2 \text{ mg g}^{-1} \text{ DW}$) in aqueous extracts. Regarding cyanobacteria, only *Lyngbya* sp. and *Scytonema* sp. presented MAAs (1.8 ± 0.2 and $0.6 \pm 0.1 \text{ mg g}^{-1} \text{ DW}$, respectively). The mycosporine-glutaminol (λ_{max} : 310 nm) was identified for the first time in *Scytonema* sp.. Scytonemin were determined spectrophotometrically in all cyanobacteria species, the highest content was observed in *Scytonema* sp. and *Lyngbya* sp. (2.2 ± 0.3 and $1.2 \pm 0.2 \text{ mg g}^{-1} \text{ DW}$, respectively). The highest content of polyphenols was observed in the aqueous extracts of *Scytonema* sp., *P. umbilicalis*, *N. commune* and *O. pinnatifida* (21.4 ± 0.1 , 13.2 ± 0.3 , 11.9 ± 0.7 and $11.4 \pm 0.3 \text{ mg g}^{-1} \text{ DW}$, respectively). In general, cyanobacteria extracts presented higher antioxidant activity than those of red macroalgae, e.g. the highest antioxidant capacity in cyanobacteria were observed in *Scytonema* sp reaching values of $36.2 \pm 0.6 \text{ } \mu\text{mol TE (trolox equivalents) g}^{-1} \text{ DW}$, whereas in red algae the highest antioxidant capacity were observed in *P. umbilicalis* that reached values of $12.7 \pm 0.5 \text{ } \mu\text{mol TE g}^{-1} \text{ DW}$. Positive correlations of antioxidant activity with different molecules, especially polyphenols, biliproteins and MAAs, were observed. Hydroethanolic extracts of some species incorporated in creams showed an increase in the photoprotection capacity in comparison with the base cream. However, despite the fact that the effective solar absorpted radiation (ESAR) increased from 33 to 51 % in case of erythema, the SPF only reach a value of 2 by adding 25 % of *P. umbilicalis* liquid extract to the base cream. This effect is because the photoprotection factors do not increase linearly with the absorption capacity. These species present potential applications in the cosmeceutical sector, although the incorporation in the creams should be improved. The addition of more concentrated extracts or purified MAAs could improve the photoprotection, or the combination of different natural compounds, e.g. MAAs and scytonemin, would present a broader-band absorption.

As environmental conditions can modify the internal composition of algae, in the **third chapter** of the thesis, the effects of different stress factors (e.g. light quality, nutrients availability or emersion conditions) in the photosynthesis and accumulation of bioactive compounds were studied in two red macroalga species: *Gracilaria armata* and *Neopyropia leucosticta*. Both species belong to groups that showed interesting results in the second chapter and are commercially culture: Gracilariales are exploited for their phycocolloids content (gelling agents) and nutritional values (commonly known as

Ogonori); and *Porphyra sensu lato* species are well known for their use as human food (known as Nori), with an economic value that is growing over the years.

G. armata was cultured under different radiation treatments (only PAR (P), PAR + UV-A (PA) and PAR+UV-A+UV-B (PAB)) and compared with specimens from their natural environment. The PAB treatment reduced the growth capacity in comparison with the P or PA treatment. The electron transport rate (ETR), as estimator of the photosynthetic capacity, did not show significant differences among treatment when it was measured *in situ*, whereas the ETR_{max} obtained from RLCs decreased in PAB treatment. The non-photochemical quenching (NPQ) increased under PAB treatment indicating photoprotection strategies. Total carbon increase in the PAB treatment, probably due to the thickening of the cell wall, being a potential photoprotection mechanism. However, other compounds decreased in the same treatment, even the photoprotective molecules (i.e. polyphenols and MAAs). Lipids, soluble proteins and photosynthetic pigments can be degraded under UV radiation, in this case related only to UV-B. Phenolic compounds and MAAs content were highest in algae from the nature. During the acclimation period (two months), algae decreased their content probably due to low stressful conditions at laboratorial cultivation. In the experimental treatments, no differences in the phenols content were observed, and the MAAs concentration were higher in the P treatment. As at the beginning of the experiment the photoprotective molecules were low, under UV radiation algae did not present enough photoprotective compounds to protect themselves and the available energy were possibly used into photo-repair mechanisms instead of synthesis of new molecules. Antioxidant capacity was correlated with some of the internal compounds and also decreased in the PAB treatment. *G. armata* increased the NPQ under high UVR conditions, whereas it was not able to increase photoprotective compounds (polyphenols or MAAs) probably to photodamage effects by UV radiation.

The effects of three different stress factors were studied in *N. leucosticta*, through two bi-factorial experiments: (1) the combination of PAR and PAR supplemented by UV-A, Violet and Blue radiation (PAR_{UVA-Vi-BI+}) and three nutrient concentrations (50, 250 y 500 μ M de NaNO₃), and (2) the combination of the same radiation quality and emersion/immersion conditions. The integrated ETR (ETR_{int}) showed an interactive response between the nutrient availability and light quality, i.e. in the PAR_{UVA-Vi-BI+} treatments the ETR_{int} were higher when the nutrients were high, suggesting a photoprotective effect of high nitrate concentration against the possible damage effect of

the UV-A radiation. The emersion condition provoked a decrease in the ETR_{int} due the lower effective quantum yield ($Y(II)$) during the emersion, that was recovered during re-hydration. The energy dissipation rate (EDR), firstly described in this work as an estimator of the energy loss, increased under nutrient limitation in the first experiment (without effect of the light quality), and under $PAR_{UVAVIBI+}$ in the second one (without effect of the emersion condition). This suggested a protection mechanism, avoiding that the excess of radiation reaches the photosystem and cause damages. The number of cells increased under nitrogen limitation and decrease under $PAR_{UVAVIBI+}$ treatment (separated effects of each factor were observed in the first experiment), whereas the protoplasm size was influenced by the interaction between nutrients availability and radiation quality, i.e. cells were smaller under low nutrients and only PAR. This indicates that the surface/volume (S/V) ratio of the cells increased under nutrient limitation favoring the nitrogen assimilation, but this ratio decreased in the $PAR_{UVAVIBI+}$ reducing that the damage rays reach the cells interior. In the second experiment, the protoplasm size increased under emersion conditions, as a way to difficult water evaporation during emersion. Biliproteins and mycosporine-like amino acids (MAAs) clearly increased under high nutrient availability. Biliproteins also decreased under $PAR_{UVAVIBI+}$ treatment at the end of both experiments, as they are very vulnerable to UV radiation. This can suggest a photoprotective role of the Violet-Blue radiation that stimulate the biliprotein synthesis and delay their degradation. Emersion did not show an effect on the biliproteins content, but seems to induce MAAs accumulation under $PAR_{UVAVIBI+}$ radiation in a short period time (3 days). Phenols content increased under $PAR_{UVAVIBI+}$ at the beginning of the first experiment as a photoprotection mechanism, although this effect disappears at day 7, when phenols were only affected by the nutrients concentration, i.e. higher values were observed under low N-availability. In the second experiment, phenols content was only affected by the radiation quality at the end of the experiment. The antioxidant capacity determined by ABTS showed an effect of the nutrients at the beginning (highest values were observed in the intermediate nutrients concentration), and an effect of the light quality at the end of the first experiment (highest values were found in algae submitted to only PAR). DPPH was only affected by the light quality (highest values under $PAR_{UVAVIBI+}$) at the beginning of the first experiment and at the end of the second experiment. Antioxidant capacity was positively correlated to antioxidant substances as biliproteins, polyphenols and MAAs in the first experiment. The application of short-term stress conditions (2-7 days) during culture, can be a strategy to increase the content of interesting compounds due to both UV screen properties and antioxidant capacity.

In the **fourth chapter** of the thesis, an isolation methodology of MAAs using high performance counter current chromatography (HPCCC) was developed. This technology is a type of liquid-liquid chromatography, that use two immiscible liquid phases as a “column” instead of solid support (e.g. HPLC), what confers some advantages like no irreversible absorption, high load capacity and recovery of the sample. MAAs from two red macroalgae (*Pyropia columbina* and *Gelidium corneum*) and one marine lichen (*Lichina pygmaea*) were isolated. The extract of *P. columbina* showed the highest MAAs content (31 mg g⁻¹ DE (dry extract)) containing two main MAAs, porphyra-334 (> 90%) and shinorine. *G. corneum* presented 6.3 mg MAAs g⁻¹ DE presenting mainly palythine and asterina-330. At last, *L. pygmaea* showed a MAAs concentration of 15.4 mg g⁻¹ DE and only one type of MAAs, mycosporine-serinol was found. Firstly, it was necessary the develop of the biphasic solvent system, that must provide a good partition coefficient of the target compound/s ($0.5 \leq K \leq 3.5$), a settling time lower than 30 s and a density difference higher than 0.08 g mL⁻¹. The selected biphasic solvent system consisted of ethanol, acetonitrile, saturated ammonium sulphate solution, and water (1:1:0.5:1; v:v:v:v). Then, different parameters of the equipment were optimized (i.e. the flow rate of the equipment was set at 3 mL min⁻¹ and the rotational speed of the column was the maximum (1600 rpm)). The sample loading was also optimized for each species: 1000 mg of *P. columbina* extracts were injected in a 6 mL loop, 200 mg of *G. corneum* extract were injected in 2 mL loop, and 1200 mg of *L. pygmaea* extract were injected in 6 mL loop. In order to increase the throughput of the process, multiple sequential injections with an elution-extrusion mode (EE) were performed: eight separation cycles were performed for *P. columbina* and *G. corneum*, whereas only three cycles were performed for *L. pygmaea* (due to less biomass availability). The separation process resulted in fractions enriched with palythine (2.3 mg), asterina-330 (3.3 mg), shinorine (14.8 mg), porphyra-334 (203.5 mg) and myc-serinol (46.6 mg), which had to be subsequently desalted by using precipitation with methanol and permeation on a Sephadex G-10 column. Isolated MAAs were identified by HPLC, MS, and NMR.

The **fifth chapter** was divided in three sections: (1) Characterization of aqueous and hydroethanolic extracts of two *Porphyra sensu lato* species, *P. linearis* from Spain and specimens of *P. columbina* from two sites of Chile (Valparaíso and Concepción) focusing on antioxidant and anti-aging properties; (2) Analysis of different plants looking for a new source of antioxidant and UV-screen compounds; (3) Design of cosmeceutical products with algae and plant extracts, analyzing their photoprotective capacity and photo- and thermostability. The three algae specimens showed similar extraction yields (around 20

%) and higher values in water than in hydroethanolic solvent. *P. linearis* showed the highest MAAs ($9.8 \pm 0.2 \text{ mg g}^{-1} \text{ DW}$), and both *P. columbina* showed similar values ($7.2 \pm 0.4 \text{ mg g}^{-1} \text{ DW}$). All of them presented porphyrin-334 and shinorine, although *P. columbina* Concep. showed a higher percentage of shinorine. Biliproteins and total N were also highest in *P. linearis*. Phenols content and ABTS values were higher in aqueous than in hydroethanolic solvent. Phenols showed similar values among samples (an average of $10.8 \text{ mg g}^{-1} \text{ DW}$) and the highest ABTS were observed in *P. columbina* Valp. In the DPPH assay, less differences between extraction solvents were observed, and the highest capacity was observed in the hydroethanolic extract of *P. linearis* ($15.4 \pm 0.8 \text{ } \mu\text{mol TE g}^{-1} \text{ DW}$). Catalase activity was only observed in the aqueous extract of *P. columbina* Valp. and *P. linearis*. All algae species showed collagenase activity close to 100 % in both aqueous and hydroethanolic extracts, showing a EC_{50} (half maximal effective concentration) of 2 mg mL^{-1} . Among plants, the highest potential (i.e. highest phenols, UV-screen and antioxidant compounds) were observed in ivy aerial roots, olive leaves, green coffee beans, liquorice root and flame of the forest leaves. All extracts (algae and plants) showed low toxicity in health human cell lines, and two of the plants (olive and liquorice) also showed potential results against melanoma cancer cell strains. Based on these results, different creams were designed combining *P. columbina* Valp. extract (from here named as *Pyropia*) and a mixture of plant extracts (MP: liquorice, green coffee and flame of the forest). The base cream (BKC) that contain vegetable oils (Karanja and Calophyllum) showed certain photoprotection (SPF of 4.2 and UVAPF of 2). The addition of the extracts to the creams increased the photoprotection (SPF of 23.7 and a UVAPF of 6.7), combining both *Pyropia* and MP. In addition, biological effective protection factors (BEPFs) against other effects of the UV radiation were also determined. The cream with all extracts showed a high photoprotection against immunosuppression and photocarcinogenesis, 32.5 and 27.0, respectively (both effects related with UV-B), whereas lower photoprotection were obtained against effects related with UV-A (e.g. 10.8 and 9.6 against singlet oxygen formation and photoaging, respectively). Extracts were stable against temperature and UV radiation, although the photoprotection factor of the creams decrease under UV radiation exposure (i.e. the SPF and UVAPF of the cream with all extracts decreased a 55.5 and 26.8 %, respectively). Physical filters were added in order to obtain a higher and broader band photoprotection, although only an increased in the UVAPF and biological effects related with UV-A were observed. The physical filters also increased the stability of the creams (i.e. the SPF and UVAPF of the cream with all extracts and physical filters decrease a 25.8 and 19.7 %, respectively).

respectively). The designed creams based on natural products showed promising results for the design of healthier and more environmentally-friendly sunscreens based on algae or plants extracts with booster effects.

RESUMEN

El actual estilo de vida, que nos lleva a estar sometidos a una alta exposición solar a causa de las actividades sociales, deportivas, recreativas e incluso laborales al aire libre; y el aumento de la radiación UV en ciertas zonas del planeta debido al agujero de la capa de ozono y al cambio climático, han provocado un aumento de los problemas oculares y de piel en la población como son las quemaduras solares, el fotoenvejecimiento prematuro de la piel, la inmunosupresión, las cataratas o el cáncer de piel.

Está comprobado que el uso de cremas solares reduce los efectos negativos de este tipo de radiación en nuestra salud; pero en los últimos años se ha puesto en duda la seguridad de ciertos filtros de radiación UV para la salud y el medio ambiente. Los filtros UV son responsables de la absorción o dispersión de los rayos UV, evitando así que penetren en nuestra piel. Generalmente se dividen en sintéticos/químicos o minerales/físicos. Algunos filtros UV sintéticos han mostrado efectos negativos tanto en la salud humana, (ej. alteraciones endocrinas, fotoalergias y estrés oxidativo) como en ambientes y organismos acuáticos, donde la acumulación de dichos filtros ha provocado impactos negativos en el ecosistema (ej. blanqueamiento de los corales, reducción del crecimiento del fitoplancton o trastornos hormonales en peces o mamíferos). Es por ello, por lo que zonas con ecosistemas marinos de alto valor como Hawái, Palau y algunas islas del Caribe han prohibido el uso de determinados filtros UV sintéticos.

Para prevenir estos efectos negativos, es necesario buscar y desarrollar nuevos filtros de radiación UV alternativos que sean naturales, biodegradables y no tóxicos. Afortunadamente, hoy en día, gran parte de los consumidores están concienciados por los posibles impactos negativos de los filtros UV y otros compuestos sintéticos utilizados normalmente en productos cosméticos, y tendemos a preferir el uso de productos naturales que se consideran más seguros para la salud y respetuosos con el medio ambiente. En este contexto, las algas, cianobacterias y plantas están diariamente sometidas a diferentes factores de estrés ambiental, por lo que han desarrollado diversos mecanismos de defensa y fotoprotección, incluida la síntesis y acumulación de compuestos que absorben la radiación UV y antioxidantes (ej. carotenoides, polifenoles

o aminoácidos tipo micosporina), que podrían usarse como nuevas fuentes de filtros UV naturales en cremas solares.

Entre los compuestos bioactivos, destacan los aminoácidos tipo micosporinas (MAAs del inglés mycosporine-like amino acids) por sus propiedades antioxidantes y capacidad de absorber la radiación UV, su alta foto-termo estabilidad, así como por su baja toxicidad. Por lo tanto, en el **primer capítulo** se hizo una revisión de los datos publicados sobre MAAs en algas rojas, incluida su biosíntesis, especies con mayores concentraciones, producción de biomasa, extracción, caracterización, identificación, purificación y bioactividades. Los MAAs se pueden encontrar en algas, cianobacterias o corales, pero las concentraciones más altas se encuentran en macroalgas rojas, principalmente del orden Bangiales, como *Porphyra sensu lato*. Existen diferentes tipos de MAAs con diferentes máximos de absorción (de 310 a 360 nm), los más comunes son: palythine (λ_{max} : 320 nm), asterina-330 (λ_{max} : 330 nm), palythanol (λ_{max} : 330 nm), shinorine (λ_{max} : 332 nm) y porphyra-334 (λ_{max} : 334 nm). El contenido de MAAs puede variar dependiendo de los factores ambientales, entre los cuales la radiación solar y la disponibilidad de nitrógeno son los más influyentes. El uso de MAAs como filtros UV naturales requiere una alta disponibilidad de biomasa algal, que puede obtenerse mediante recolección en áreas con altas productividades (especies locales o invasoras) o mediante cultivo. Varios autores han descrito el cultivo de algas rojas (*Gracilaria cornea* o *Agarophytum vermiculophyllum*) en sistemas de acuicultura multitrófica integrada (IMTA, del inglés Integrated Multitrophic Aquaculture) con altas productividades de MAAs. Los MAAs se extraen fácilmente mediante maceración o sonicación de la biomasa en disolventes polares (normalmente agua o combinaciones de agua-etanol/metanol), y se identifican mediante HPLC, ESI-MS o NMR. En la bibliografía se han encontrado diferentes metodologías de HPLC, algunos autores utilizaron columnas C8 y otros C18, pero la mayoría utiliza eluyentes acuosos acidificados. En nuestro laboratorio utilizamos una columna Luca C8 (Phenomenex) con un flujo isocrático ($0,5 \text{ mL min}^{-1}$) de agua con 1,5 % de metanol y 0,15 % de ácido acético. En los últimos años, se han realizado diferentes enfoques para aislar MAAs, desde simples columnas cromatográficas (ej. sílice o intercambio iónico), hasta métodos de UHPLC preparativos o cromatografía líquida-líquida, esta última puede escalarse industrialmente. Además de la capacidad de absorber la radiación UV, se han descrito otras bioactividades en los MAAs (*in vitro* e *in vivo*) como propiedades antioxidantes, antienvjecimiento o inmunomoduladoras. Todos los datos presentes en esta revisión

permiten una mayor comprensión de los MAAs y muestran perspectivas para su inclusión en productos cosmecéuticos.

Como las macroalgas rojas y las cianobacterias son las principales productoras de MAAs, y estas últimas también pueden presentar otro compuesto fotoprotector denominado escitonemina, en el **segundo capítulo** de la tesis se analizaron diferentes especies de estos grupos, buscando propiedades cosmecéuticas, es decir, alto contenido de MAAs, polifenoles y escitonemina, así como una buena capacidad antioxidante. Se realizaron extractos en diferentes solventes (agua, etanol y dos combinaciones de agua:etanol). En general, el etanol mostró el menor contenido de MAAs, fenoles y capacidad antioxidante. Las concentraciones más altas de MAAs se encontraron en medio acuosos y en las macroalgas rojas *Porphyra umbilicalis* ($5,2 \pm 0,4$ mg g⁻¹ PS), *Gelidium corneum* ($1,8 \pm 0,2$ mg g⁻¹ PS (peso seco)) y *Osmundea pinnatifida* ($1,5 \pm 0,2$ mg g⁻¹ PS). Respecto a las cianobacterias, sólo *Lyngbya* sp. y *Scytonema* sp. presentaron MAAs ($1,8 \pm 0,2$ y $0,6 \pm 0,1$ mg g⁻¹ PS, respectivamente). La micosporina-glutaminol (λ_{max} : 310 nm) fue identificada por primera vez en *Scytonema* sp. La escitonemina fue determinada espectrofotométricamente en todas las especies de cianobacterias. El mayor contenido se observó en *Scytonema* sp. y *Lyngbya* sp. ($2,2 \pm 0,3$ y $1,2 \pm 0,2$ mg g⁻¹ PS, respectivamente). El mayor contenido de polifenoles se observó en los extractos acuosos de *Scytonema* sp., *P. umbilicalis*, *N. commune* y *O. pinnatifida* ($21,4 \pm 0,1$, $13,2 \pm 0,3$, $11,9 \pm 0,7$ y $11,4 \pm 0,3$ mg g⁻¹ PS, respectivamente). En general, los extractos de cianobacterias presentaron una mayor actividad antioxidante que los de las macroalgas rojas (ej. la mayor capacidad antioxidante en cianobacterias se observó en *Scytonema* sp alcanzando valores de $36,2 \pm 0,6$ µmol ET (equivalentes trolox) g⁻¹ PS, mientras que en algas rojas la mayor capacidad antioxidante se observó en *P. umbilicalis* que alcanzó valores de $12,7 \pm 0,5$ µmol ET g⁻¹ PS). Se observaron correlaciones positivas de la actividad antioxidante con diferentes moléculas, especialmente polifenoles, biliproteínas y MAAs. Los extractos hidroetanólicos de algunas especies incorporados en cremas mostraron un aumento en la capacidad fotoprotectora en comparación con la crema base. Sin embargo, a pesar de que la radiación solar absorbida efectiva (ESAR del inglés effective solar absorpted radiation) aumentó del 33 al 51 % en caso de eritema, el SPF solo alcanza un valor de 2 al agregar extracto líquido de *P. umbilicalis* en un 25 %. Este efecto se debe a que los factores de fotoprotección no aumentan linealmente con la capacidad de absorción. Estas especies presentan potenciales aplicaciones en el sector cosmecéutico, aunque

se debería mejorar la forma de incorporación en las cremas. La adición de extractos más concentrados o MAAs purificados podrían mejorar la fotoprotección, o la combinación de diferentes compuestos naturales, ej. los MAA y la escitonemina presentarían una absorción de banda más amplia.

Como las condiciones ambientales pueden modificar la composición interna de las algas, en el **tercer capítulo** de la tesis se estudiaron los efectos de diferentes factores de estrés (ej. calidad de la luz, disponibilidad de nutrientes o condiciones de emersión) en la fotosíntesis y acumulación de compuestos bioactivos en dos especies de macroalgas rojas: *Gracilaria armata* y *Neopyropia leucosticta*. Ambas especies pertenecen a grupos que mostraron resultados interesantes en el segundo capítulo, y se cultivan comercialmente: las Gracilariales son explotadas por el contenido de ficocoloides (agentes gelificantes) y valor nutricional (comúnmente conocidos como Ogonori); y las especies de *Porphyra sensu lato* son conocidas mundialmente por su uso como alimento humano (conocido como Nori), con un valor económico que va creciendo con el paso de los años.

G. armata se cultivó bajo diferentes tratamientos de radiación lumínica (solo PAR (del inglés photosynthetic active radiation) (P), PAR+UV-A (PA) y PAR+UV-A+UV-B (PAB)) y se compararon con los especímenes de su entorno natural. El tratamiento PAB redujo el crecimiento en comparación con los tratamientos P o PA. La tasa de transporte de electrones (ETR, del inglés electron transport rate), como estimador de la capacidad fotosintética, no mostró diferencias significativas entre tratamientos cuando se midió *in situ*, mientras que la ETR_{max} obtenida de las curvas rápidas de luz (RLC, del inglés rapid light curves) disminuyó en el tratamiento con PAB. El amortiguamiento no fotoquímico (NPQ, del inglés non-photochemical quenching), como mecanismo de disipación de energía, aumentó con el tratamiento PAB, lo que sugiere mecanismos de fotoprotección. El aumento del carbono total en el tratamiento con PAB, se debe probablemente al engrosamiento de la pared celular que actúa como un mecanismo de fotoprotección. Sin embargo, otros compuestos disminuyeron en el mismo tratamiento, incluso las moléculas fotoprotectoras (ej. polifenoles y MAAs). Los lípidos, las proteínas solubles y los pigmentos fotosintéticos se pueden degradar fácilmente bajo la radiación UV; en este caso, solo la radiación UV-B los degradó. El contenido de compuestos fenólicos y MAAs fue mayor en las algas provenientes del medio natural. Durante el período de aclimatación (dos meses) las algas disminuyeron el contenido de moléculas fotoprotectoras, probablemente debido al menor estrés. En los tratamientos

experimentales no se observaron diferencias en el contenido de fenoles, y la concentración de MAAs fue mayor en el tratamiento P. Durante el experimento, las algas partieron de concentraciones bajas de moléculas fotoprotectoras, por lo que al someterlas a radiación UV las algas no presentaban suficientes compuestos para protegerse y la energía disponible se utilizó en mecanismos de fotoreparación en lugar de en la síntesis de nuevas moléculas. En cuanto a la capacidad antioxidante, se correlacionó con algunos de los compuestos internos y también disminuyó en el tratamiento con PAB. *G. armata* aumentó el NPQ bajo radiación UV, mientras que no fue capaz de aumentar los compuestos fotoprotectores (polifenoles o MAA) probablemente debido a los efectos dañinos de la radiación UV.

En *N. leucostica* se estudió el efecto de tres factores de estrés diferentes, a través de dos experimentos bifactoriales: (1) la combinación de PAR y PAR suplementado con radiación UV-A, Violeta y Azul ($\text{PAR}_{\text{UVAVIBI}+}$) y tres concentraciones de nutrientes (50, 250 y 500 μM de NaNO_3), y (2) la combinación de la misma calidad de luz y condiciones de emersión/inmersión. El ETR integrado (ETR_{int}) mostró una respuesta interactiva entre la disponibilidad de nutrientes y la calidad de la luz, es decir, los tratamientos $\text{PAR}_{\text{UVAVIBI}+}$ mostraron valores de ETR_{int} mayores cuando el contenido de nitrógeno era alto, sugiriendo un efecto fotoprotector de la alta concentración de nitratos contra el posible efecto dañino de la radiación UV-A. La condición de emersión provocó una disminución en el ETR_{int} debido al menor rendimiento cuántico efectivo ($Y(\text{II})$) durante la emersión, el cual se recuperó durante la rehidratación. La tasa de disipación de energía (EDR, del inglés energy dissipation rate), descrita por primera vez en este trabajo como un estimador de la disipación de energía en forma de calor y fluorescencia, aumentó por limitación de nutrientes en el primer experimento (sin efecto de la calidad de la luz), y por $\text{PAR}_{\text{UVAVIBI}+}$ en el segundo (sin efecto de la condición de emersión). Este es un mecanismo de protección, evitando que el exceso de luz llegue al fotosistema y cause daños. El número de células aumentó con la limitación de nitrógeno y disminuyó con el tratamiento $\text{PAR}_{\text{UVAVIBI}+}$ (se observaron efectos separados), mientras que el tamaño del protoplasma mostró interacción entre la disponibilidad de nutrientes y la calidad de la luz, es decir, las células son más pequeñas con niveles bajos de nutrientes y solo PAR. Esto indica que la relación superficie/volumen (S/V) de las células aumentó por limitación de nutrientes favoreciendo la asimilación de nitrógeno, pero esta relación disminuye en el $\text{PAR}_{\text{UVAVIBI}+}$ dificultando que los rayos dañinos lleguen al interior de las células. En el segundo experimento, el tamaño del protoplasma aumentó en condiciones de emersión, como una forma de dificultar la evaporación del agua durante la emersión.

Las biliproteínas y los MAAs aumentaron claramente con una alta disponibilidad de nutrientes, ya que ambos son compuestos nitrogenados. Las biliproteínas también disminuyeron con el tratamiento con PAR_{UVAVIBI}⁺ al final de ambos experimentos, ya que son muy vulnerables a la radiación UV. Este efecto solo se produjo al final del experimento, lo que puede sugerir un papel fotoprotector de la radiación Violeta-Azul que estimula la síntesis de biliproteínas y retrasa su degradación. La emersión no mostró efecto sobre el contenido de biliproteínas, pero parece inducir la acumulación de MAAs bajo radiación PAR_{UVAVIBI}⁺ en un periodo corto de tiempo (3 días).

El contenido de fenoles aumentó en el tratamiento PAR_{UVAVIBI}⁺ al inicio del primer experimento como mecanismo de fotoprotección, aunque este efecto desaparece en el día 7, donde los fenoles solo se vieron afectados por la concentración de nutrientes, es decir, se observaron valores más altos en los niveles bajos de nitrógeno. En el segundo experimento, el contenido de fenoles sólo se vio afectado por la calidad de la luz al final del experimento. La capacidad antioxidante determinada por ABTS mostró un efecto de los nutrientes al inicio (los valores más altos se observaron en la concentración de nutrientes intermedios) y un efecto de la calidad de la luz al final del primer experimento (los valores más altos se observaron solo bajo PAR). DPPH solo se vio afectado por la calidad de la luz (valores más altos bajo PAR_{UVAVIBI}⁺) al comienzo del primer experimento y al final del segundo experimento. La capacidad antioxidante se correlacionó positivamente con sustancias antioxidantes como biliproteínas, polifenoles y MAA en el primer experimento. La aplicación de condiciones de estrés a corto plazo (2-7 días) durante el cultivo puede ser una estrategia para aumentar el contenido de compuestos interesantes debido tanto a las propiedades de pantalla UV como a la capacidad antioxidante.

En el **cuarto capítulo**, se desarrolló una metodología de aislamiento de MAAs mediante HPCCC (del inglés high performance countercurrent chromatography). Esta tecnología es un tipo de cromatografía líquida-líquida, que utiliza dos fases líquidas inmiscibles como “columna” en lugar de un soporte sólido (ej. HPLC), lo que confiere algunas ventajas como la no absorción irreversible, alta capacidad de carga y recuperación de la muestra. Se aislaron MAA de dos macroalgas rojas (*Pyropia columbina* y *Gelidium corneum*) y un liquen marino (*Lichina pygmaea*). El extracto de *P. columbina* mostró el mayor contenido de MAA (31mg g⁻¹ ES (extracto seco)) con dos MAA principales, porphyra-334 (> 90%) y shinorine. *G. corneum* presentó 6,3 mg MAA g⁻¹ ES con palythine y asterina-330. *L. pygmaea* mostró una concentración de MAA de

15,4 mg g⁻¹ ES y solo un tipo de MAA, myc-serinol. En primer lugar, fue necesario el desarrollo del sistema bifásico de solventes, que debe proporcionar un buen coeficiente de partición del compuesto/s deseados ($0,5 \leq K \leq 3,5$), un tiempo de sedimentación inferior a 30 s y una diferencia de densidad superior a 0,08 g mL⁻¹. Tras la selección del sistema de solvente bifásico, que consistió en etanol, acetonitrilo, solución saturada de sulfato de amonio y agua (1:1:0,5:1; v:v:v:v), se optimizaron diferentes parámetros del equipo (ej. el caudal del equipo se fijó en 3 mL min⁻¹ y la velocidad de rotación de la columna fue la máxima (1600 rpm)). La carga de muestra también se optimizó para cada especie: se inyectaron 1000 mg de extracto de *P. columbina* en 6 mL de solvente, 200 mg de extracto de *G. corneum* en 2 mL de solvente y 1200 mg de extracto de *L. pygmaea* en 6 mL de solvente. Para aumentar el rendimiento del proceso, se realizaron múltiples inyecciones secuenciales mediante elución-extrusión (EE): se realizaron ocho ciclos de separación para *P. columbina* y *G. corneum*, mientras que solo se realizaron tres ciclos para *L. pygmaea* (debido a la mayor cantidad de inyección y menor disponibilidad de biomasa). El proceso de separación dio como resultado fracciones enriquecidas con palythine (2,3 mg), asterina-330 (3,3 mg), shinorine (14,8 mg), porphyra-334 (203,5 mg) y myc-serinol (46,6 mg), que tuvieron que ser posteriormente desaladas mediante precipitación con metanol y permeación en Sephadex G-10, lo que supuso una pérdida de los compuestos aislados. Los MAA aislados se identificaron mediante HPLC, MS y NMR.

El **quinto capítulo** de la tesis se dividió en tres secciones: (1) Caracterización de extractos acuosos e hidroetanólicos de tres especies de *Porphyra sensu lato*, centrándose en propiedades antioxidantes y antienviejimiento; (2) Análisis de diferentes plantas en busca de una nueva fuente de compuestos antioxidantes y con capacidad de absorber la radiación UV; (3) Diseño de productos cosmecéuticos con extractos de algas y plantas, analizando su capacidad fotoprotectora, foto-termoestabilizante. Las tres especies de algas mostraron rendimientos de extracción similares (alrededor del 20 %) y valores más altos en agua que en disolvente hidroetanólico. *P. linearis* mostró la cantidad de MAA más alta ($9,8 \pm 0,2$ mg g⁻¹ PS), mientras que ambas *P. columbina* mostraron valores similares ($7,2 \pm 0,4$ mg g⁻¹ PS). Todos ellos presentan porphyra-334 y shinorine, aunque *P. columbina* Concep. mostró un mayor porcentaje de shinorine. Las biliproteínas y el N total también fueron más altos en *P. linearis*. El contenido de fenoles y los valores de ABTS fueron mayores en el disolvente acuoso que en el hidroetanólico. Los fenoles mostraron valores similares

entre las muestras (un promedio de $10,8 \text{ mg g}^{-1} \text{ PS}$) y los valores más altos de capacidad antioxidante por ABTS se observaron en *P. columbina* Valp. En el ensayo DPPH se observaron menores diferencias entre los solventes de extracción, y la mayor capacidad se observó en el extracto hidroetanólico de *P. linearis* ($15,4 \pm 0,8 \text{ } \mu\text{mol ET g}^{-1} \text{ PS}$). La actividad catalasa sólo se observó en el extracto acuoso de *P. columbina* Valp. y *P. linearis*. Todas las especies de algas mostraron actividad anti-colagenasa cercana al 100% tanto en el extracto acuoso como en el hidroetanólico, mostrando una EC_{50} (concentración efectiva media máxima) de 2 mg mL^{-1} . Entre las plantas, el mayor potencial (es decir, mayor contenido de fenoles, protección UV y compuestos antioxidantes) se observó en las raíces aéreas de la hiedra, las hojas de olivo, los granos de café verdes, la raíz de regaliz y las hojas de llama del bosque. Todos los extractos (algas y plantas) mostraron baja toxicidad en líneas celulares sanas, y dos de las plantas (olivo y regaliz) también mostraron potenciales resultados contra el melanoma. En base a estos resultados se diseñaron diferentes cremas combinando extracto de *P. columbina* Valp. (de aquí en adelante llamada como *Pyropia*) y una mezcla de extractos de plantas (MP: regaliz, café verde y llama del bosque). La crema base (BKC) que contiene aceites vegetales (Karanja y Calófilo) mostró cierta fotoprotección (SPF de 4,2 y UVAPF de 2). La adición de extractos a las cremas aumentó la fotoprotección (ej. combinando ambos, *Pyropia* y MP, se obtuvo un SPF de 23,7 y un UVAPF de 6,7). Además de los conocidos SPF y UVAPF, también se determinaron los factores de protección biológica efectiva (BEPFs, del inglés biological effective protection factors) contra otros efectos de la radiación UV. La crema con todos los extractos mostró una alta fotoprotección contra la inmunosupresión y la fotocarcinogénesis, 32,5 y 27,0, respectivamente (ambos efectos relacionados con los rayos UV-B), mientras que se obtuvo una menor fotoprotección contra los efectos relacionados con los rayos UV-A (ej. 10,8 y 9,6 contra la formación de oxígeno singlete y fotoenvejecimiento, respectivamente). Los extractos fueron estables frente a la temperatura y la radiación UV, aunque el factor de fotoprotección de las cremas disminuyó bajo la exposición a la radiación UV (ej. el SPF y UVAPF de la crema con todos los extractos disminuyendo un 55,5 y un 26,8 %, respectivamente). Se agregaron filtros físicos con el fin de conseguir una mayor fotoprotección de banda ancha, aunque solo se observó un aumento en el UVAPF y efectos biológicos relacionados con la radiación UV-A. Los filtros físicos también aumentaron la estabilidad de las cremas (es decir, el SPF y UVAPF de la crema con todos los extractos y filtros físicos disminuyen un 25,8 y un 19,7 %, respectivamente). Las cremas diseñadas basadas en productos naturales mostraron resultados prometedores para el diseño de

protectores solares más saludables y respetuosos con el medio ambiente a base de extractos de algas o plantas, que además presentan otros efectos beneficiosos para la piel.



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GENERAL INTRODUCTION

At present, the continuous population growth and the environmental problems caused by human activities (e.g. habitat destruction, pollution or climate change) are generating new challenges that society must face, such as the search for new sources of raw materials that through a sustainable production, served as source of food, energy and bioactive compounds with applications in various fields such as food, feed, cosmetic, medicine or agriculture, that ensure food and water availability and security, energy supply and improve our health and well-being, while at the same time do not damage the natural environment. This is supported by the new approach for a sustainable blue economy in the European Union: Transforming the EU's Blue Economy for a Sustainable Future (EC COM 240, 2021), or by the Blue Transformation of the aquatic food systems promoted by the Food and Agriculture Organization of the United Nations (FAO, 2022a).

The anthropogenic impacts on Earth are grouped as Global Change, including climate change, air and water contamination, ocean acidification, biodiversity loss, deforestation, land degradation, or ozone layer hole. The depletion of the ozone layer has provoked an increase of the UV-B radiation that reach the Earth surface in some areas of the world. In 1989, the adoption of the Montreal Protocol regulated the production of chlorofluorocarbons (CFCs) and other ozone-depleting chemicals (ODCs), what is allowing the recovery of the ozone layer since 1991 (Williamson *et al.*, 2018; Neale *et al.*, 2021). However, climate change is affecting air circulation and contributing to less cloudiness and aerosols in terrestrial areas, what can provoke that UV-B radiation will increase during this century in certain areas of the world. In regions like southern-eastern Asia the UVI (Ultraviolet index, indicator of erythemal irradiance) can increase 20-40% in 2085-2095 compared to 2010-2020, or in central Europe and eastern part of North America the expected increase of UVI will be 5-10%, whereas in Antarctic the UVI can decreased 10-40% due to the recovery of the ozone layer (Bais *et al.*, 2019). In 2023, the ESA (European Space Agency) has determined that the ozone hole is one of the biggest on record (it is roughly three times the size of Brazil) and speculate that this effect could be associated with the eruption of the Hunga Tonga volcano in January 2022 (ESA, 2023).

Therefore, the increase UVR in certain areas of the world, together with the current lifestyle with a high sun exposure due to recreational or outdoor working activities and a enjoying beach culture, are contributing to the increase of skin and eyes problems caused by an excess of UVR, such as sunburn, immunosuppression, cataracts, premature skin photoaging or photocarcinogenesis (Abarca *et al.*, 2002; González *et al.*, 2008; Thomas *et al.*, 2012; D'Orazio *et al.*, 2013).

1. Ultraviolet radiation

The sun emits electromagnetic radiation in a wide range of wavelengths, including gamma rays, X-rays, microwaves, ultraviolet, visible light, infrared or FM and AM waves. There is an inverse relationship between wavelength and energy, i.e. shorter wavelengths have more energy. All high-energy rays (gamma and X) are reflected or absorbed by the atmosphere, and most of the UVR is also efficiently absorbed by the atmosphere. The ozone layer and the atmospheric oxygen absorb all UV-C (200-280nm) and most of UV-B (280-320nm), whereas only a small part of UV-A (320-400nm) is absorbed. Thus, the solar radiation that reach the Earth's surface consists of 9% UVR (290-400nm), of which 95% is UV-A and only 5% is UV-B, 42% of visible light (400-750nm) and the rest infrared radiation (750-2500nm) (Holick, 2016) (Figure I. 1).

According to the International Commission of Illumination (CIE from the French Commission Internationale de l'Eclairage), UVR is divided in UV-C (100-280nm), UV-B (280-315nm) and UV-A (315-400nm). However, in photobiology and photomedicine, the division of the different types of UVR are based on their biological effects, so UV-B rays comprise from 280 to 320nm and UV-A from 320 to 400nm. Recently, the UV-A has been divided in I (340-400nm) and II (320-340nm) (Figure I. 1).

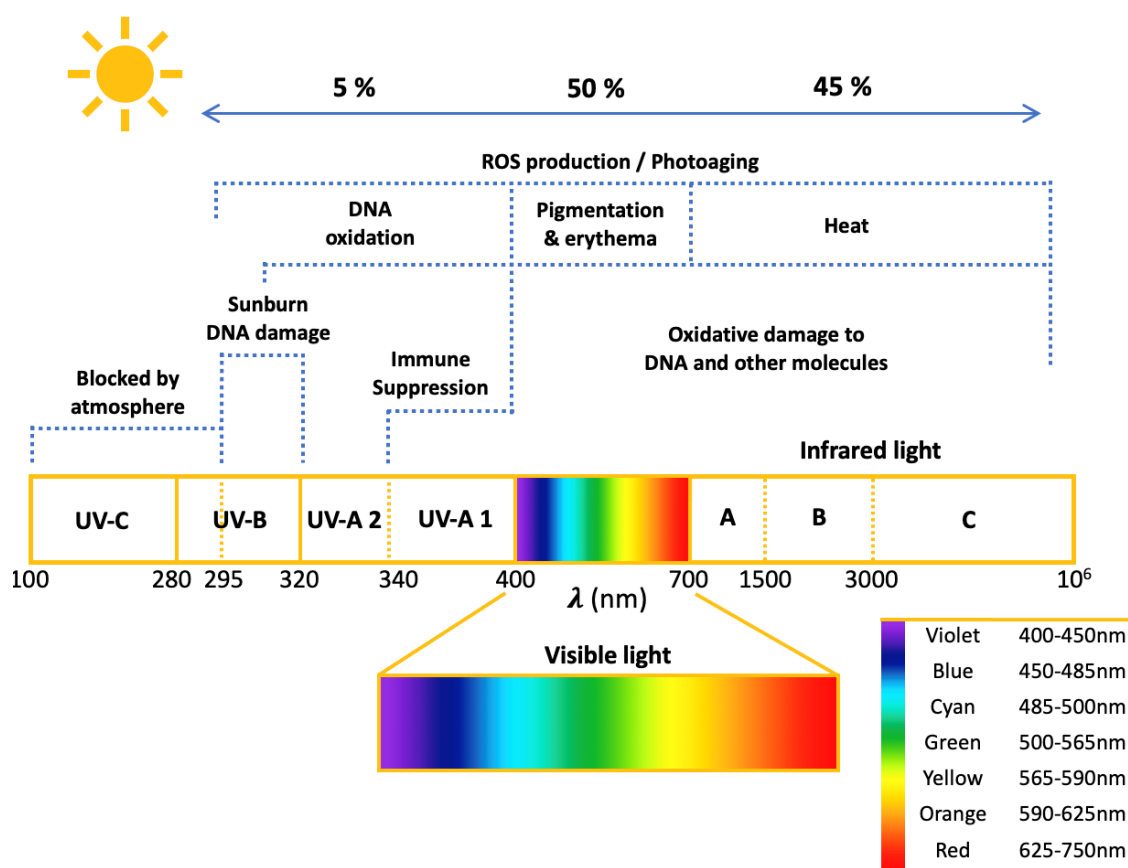


Figure I. 1. The solar radiation received in the Earth and its effects on the skin. UV radiation (5 %) is divided in: UV-C (100-280 nm) entirely blocked by the atmosphere. UV-B (280-320 nm) is the most dangerous, involved in sunburn and DNA mutations. UV-A provoke oxidative damage and immunosuppression, it can be divided in UV-A II (320-340 nm), more energetic and with similar effects to the UV-B radiation, and UV-A I (340-400 nm), mainly involved in pigmentation, erythema and oxidative damage. Visible light (50 %) is separated in different colors and can be involved in pigmentation, erythema and oxidative damage (mainly the short wavelengths: violet and blue). Infrared light (45 %) emits heat and can be involved in oxidative damage and is divided in A: near infrared (700-1500 nm), B: medium infrared (1500-3000 nm), and C: long infrared (3000-10⁶ nm). Adapted from Dupont *et al.* (2013).

The intensity of the radiation received may vary with latitude, altitude, season, time of the day, pollution or cloudiness, among other. In Málaga, recent dates (January 2020 to March 2023) obtained from the Meteorology Statal Agency of Spain (AEMET: Agencia Estatal de Meteorología) showed that the highest daily dose of global radiation (295-3500 nm), observed during summer months (June and July), reach values of 32000 kJ m⁻², whereas the lowest values observed during winter months (December-February) are around 4000 kJ m⁻². It is interesting to notice that temperature did not follow the same pattern as global radiation, but present a displacement (i.e. the maximal radiation is

observed during June/July, whereas the maximal temperature is observed in August). Regarding the UV index, it varies from 1-2 in winter to 9-10 during summer (Figure I. 2). UV index is an international scale that measure the intensity of the UVR in relation with sunburn. It is calculated as erythematic irradiance dose multiplied by 40. This index is divided into five bands: “low” (1-2), “moderate” (3-5), “high” (6-7), “very high” (8-10) and “extremely high” (≥ 11). The obtained values do not show the amount of UVR, but Aguilera *et al.* (2004) and the Photobiology Service of the Research Support Central Services from the University of Málaga (SCAI: Servicios Centrales de Apoyo a la Investigación), measured the solar radiation from 1997 to 2015, and observed that UV-A radiation reached maximal daily doses of 1300 kJ m^{-2} during summer, and minimal doses of 350 kJ m^{-2} during winter. In case of UV-B radiation, the highest daily dose reach values of 70 kJ m^{-2} in summer, and the lowest values were around 15 kJ m^{-2} during winter. The visible light showed maximal values of 14000 kJ m^{-2} during summer, and minimal doses around 3000 kJ m^{-2} . It was also observed that the ratio UV-A/Visible does not shown variation along the year, although the ratio UV-B/Visible or UV-B/UV-A vary during the year, with maximum values in summer.

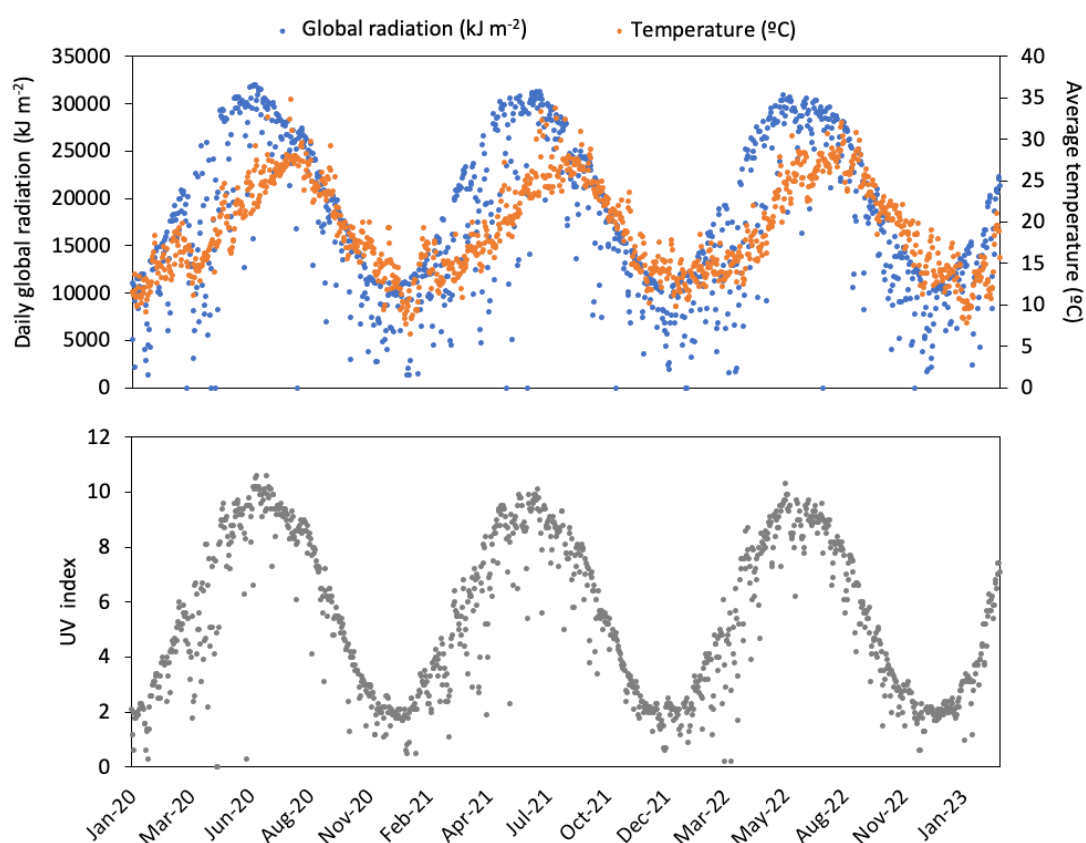


Figure I. 2. Daily irradiance of global radiation (295-1500 nm), average temperature and UVI index from January 2020 to January 2023 in the station of Malaga (36°25′49.8″N / 4°17′7.1″W). Data obtained from the Spanish Agency of Meteorology (AEMET: Agencia estatal de meteorología).

Solar global radiation is assumed to be constant over the years. However, a widespread reduction of 3-9 W m⁻² in the global radiation from 1950 to 1980 has been well documented, this phenomenon was called Global Dimming (Stanhill and Cohen, 2001; Wild *et al.*, 2012). However, since 1980s, an increase in the global radiation have been detected in many regions of the world, especially in industrialized areas, with an overall increase of 1-4 W m⁻² (Hatzianastassiou *et al.*, 2005). The global radiation over Spain showed a tendency to increase during 1985-2010 period, with a significant linear trend of +3.9 W m⁻² per decade, specifically in Malaga, the annual increase was +5.1 W m⁻² (Sanchez-Lorenzo *et al.*, 2013). These data are relevant since violet-blue light and infrared radiations can also provoke effects on the human skin (Mahmoud *et al.*, 2008; Sklar *et al.*, 2012; Tosini *et al.*, 2016; Bennet *et al.*, 2017; Nakashima *et al.*, 2017).

2. Effects of UV radiation on human skin

The skin is the largest organ of the body, forming a protective barrier against harmful microbes and the external environment. It is divided in epidermis, dermis and hypodermis. The epidermis is the outer and thinner layer of the skin, which protect against the environmental damage (e.g. pathogens, UV radiation, dehydration, or pollution). Epidermis can be divided in four different layers: stratum basale, stratum spinosum, stratum granulosum and stratum corneum. An additional layer, stratum lucidum, appears in areas of thick skin such as the heel and the palm of the hand. Keratinocytes are the predominant cells in the epidermis. They produce keratin, which provides strength to the skin, as well as forming the water-proof barrier of the skin. In addition, the epidermis contains other types of cells: melanocytes, responsible for the synthesis of melanin (pigment protector of the skin against the UVR), Langerhans' cells, which provide the primary defense against microbial invasion and Merkel cells, responsible to respond against the pressure exerted on the skin. The dermis is the middle and thickest layer (about 90 %) of the skin. It is a complex structure with blood vessels, hair follicles and sebaceous glands, among others. Fibroblasts are the main component of the dermis, which synthesize a dense extracellular matrix (ECM), composed of collagens, elastic fibers, proteoglycans and hyaluronic acid, among others. The main function of the dermis is to regulate the body temperature and supply nutrients and oxygen to the epidermis. The hypodermis is the deepest layer of the skin, and it is mainly compound by fatty acids which provide thermoregulation, store nutrition and protection against deep injuries (Graham *et al.*, 2019) (Figure I. 3).

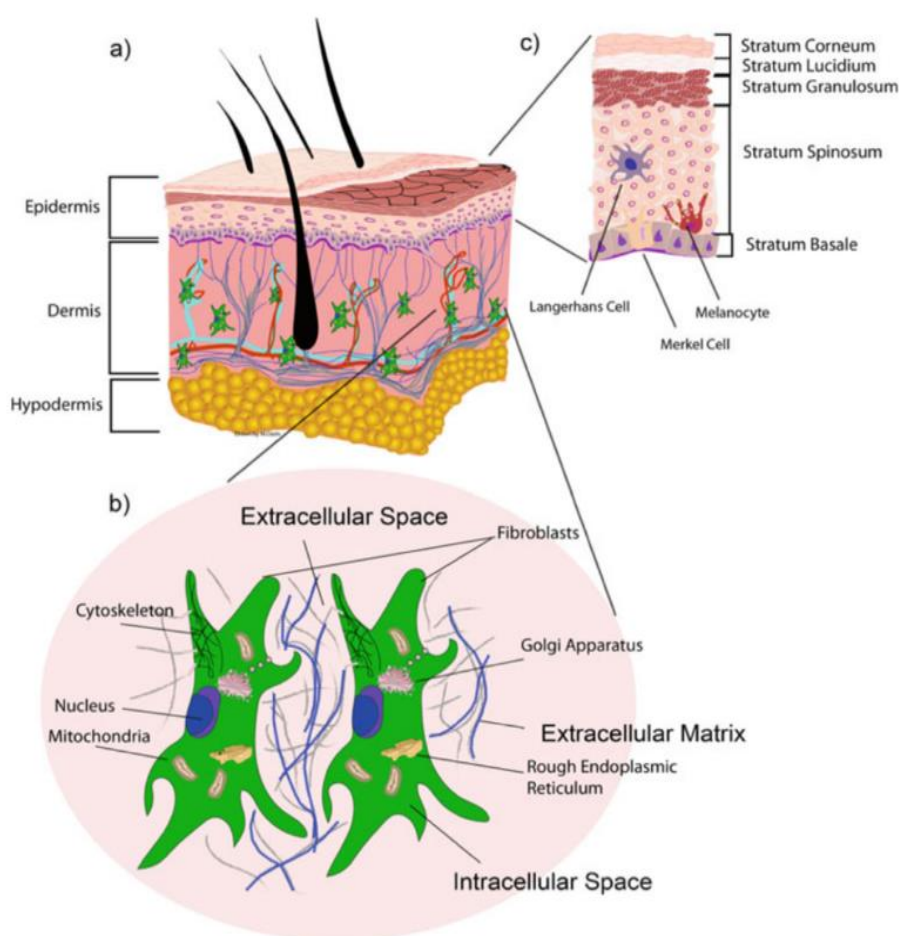


Figure I. 3. Transversal section of human skin. a) skin structure indicating the three layers: epidermis, dermis and hypodermis; b) details of the dermis, containing fibroblasts and different fibers that form the extracellular matrix; and c) epidermis details, representing the five described stratum and specialized cells (Langerhans cells, Merkel cells and melanocytes). Taken from Graham *et al.* (2019).

The UV-B radiation can be efficiently absorbed by DNA, RNA and proteins, therefore UV-B rays only penetrate the epidermis and upper dermis. The 70 % of UV-B is absorbed by the stratum corneum, the 20 % is absorbed in the basal layer of epidermis mainly by melanin and cellular DNA, and only a 10 % can reach the dermis. Regarding UV-A, about 70-80 % of the radiation is blocked in the epidermis by the melanin, the 20-30 % reach the dermis, and only 1 % reach hypodermis. In general, the UV-A II follow the same pattern as UV-B, whereas UV-A I reaches the deeper dermis. Visible and infrared radiation can achieve the hypodermis (Everett *et al.*, 1966; D'Orazio *et al.*, 2013; Holick, 2016) (Figure I. 4).

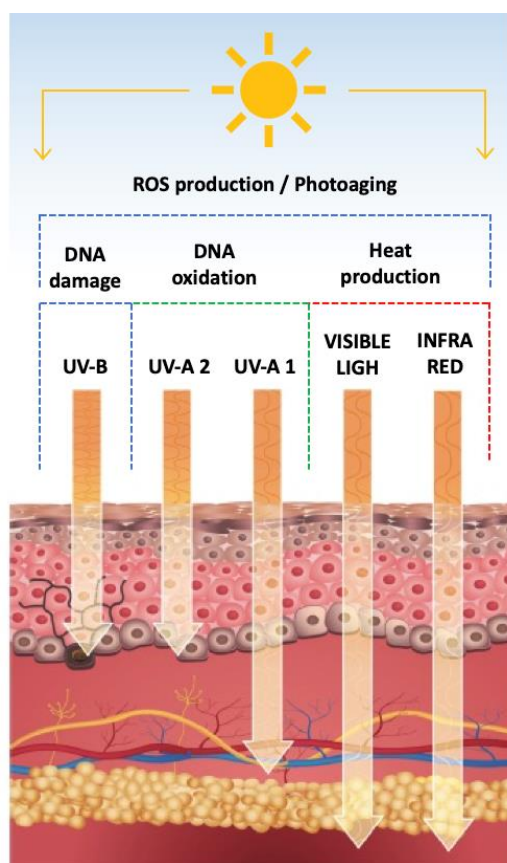


Figure I. 4. Penetration of the different wavelengths form solar radiation into the skin. UV-B (280-320 nm) and UV-A 2 (320-340 nm) penetrates in the epidermis and upper dermis, UV-A 1 (340-400nm) penetrates into the dermis, Visible (400-700 nm) and infrared (700-3000 nm) penetrate until the hypodermis. Moreover, consequences of each radiation quality to the human skin are indicated. Adapted from Krutmann *et al.* (2017).

DNA is the main chromophore for UV-B radiation. The most frequent mutations are the formation of dimers between consecutive pyrimidines bases, such as cyclobutane pyrimidine dimer (CPD's) or 6-4 photoproducts (6-4PP). CPD's are formed more often than 6-4PP (approximately in a ratio 3:1), and they are more mutagenic leading to a characteristic mutation named as "UV signature" that can be found in UV-induced cancer cells (Ichihashi *et al.*, 2003; Matsumura and Ananthaswamy, 2004). 6-4PP are less mutagenic because they can be efficiently repaired, i.e. 90 % of the 6-4PP are repaired at 3 h after irradiation. UV-A also can damage the DNA in a more indirect way, through the formation of reactive oxygen species (ROS). These radicals can induce crosslinks between DNA and adjacent proteins, break of the DNA strand, or cause mutations in DNA bases, among others. The 8-hydroxyguanine (8OH-G) is a mutagenic base (G to C) resulted from the interaction between ROS and guanine, and it is considered an

oxidative stress marker (Ichihashi *et al.*, 2003). There are important DNA repair mechanisms in all mammalian cells which avoid the potential mutagenic activity of UVR, such as direct repair, base excision repair (BER), mismatch repair or nucleotide excision repair (NER). NER is a highly conserved strategy to repair several DNA damages. CPDs and 6-4PP are primarily repaired by NER before replication. The NER involved 5 stages: (1) Recognition of the DNA lesion, (2) single strand incision at both sides of the lesion, (3) excision of damaged nucleotides, (4) de novo DNA synthesis to replace the DNA lesion, and (5) ligation (Ichihashi *et al.*, 2003; Matsumura and Ananthaswamy, 2004).

In addition to the well-known negative effects, UVR present important beneficial effects for the health. UV-B radiation is crucial for the synthesis of vitamin D, being the most important source of vitamin D for most people on Earth. This vitamin regulates the absorption of calcium and phosphorous from food, and it is essential for the development and maintenance of bones and muscles. Vitamin D deficiency is associated with an increased risk of osteoporosis in adults or rickets in children. An adequate content of vitamin D has shown to improve the immune system and reduce the risk of developing different types of cancers (Grant and De Grujil, 2003; Trummer *et al.*, 2016). Miyauchi and Nakajima (2016) determined the UV exposure time required for vitamin D synthesis according to the UV index. In Malaga (UVI: 2-3 in winter and 10 in summer), 25-40 min of daily UV exposure in face and hands will be necessary during winter, whereas during summer a daily exposure of 3-5 min will be enough. In addition, a recent review demonstrated that regular use of sunscreen does not compromise the vitamin D status in healthy individuals (Passeron *et al.*, 2019). Exposure to sunlight also regulates our circadian rhythm, essential to coordinate cellular functions in response to light and dark cycles. An unnatural exposure to light can weaken and/or disturb the circadian rhythm (van der Rhee *et al.*, 2016). Melatonin secretion is regulated by our circadian clock and exposure to sunlight. The precursor of melatonin, serotonin, is normally produced during the day and only converted to melatonin in darkness. This hormone is usually secreted at a daily rhythm with low concentrations during daytime and a peak around midnight. It helps to manage the sleep-wake cycle. When people are exposed to sunlight or very bright artificial light in the morning, their nocturnal melatonin production occurs sooner, and they enter into sleep more easily at night. Melatonin production also shows a seasonal variation relative to the availability of light, the hormone is produced for a longer period in winter than in the summer. Melatonin also has a potent antioxidant effect (Trummer *et al.*, 2016; van der Rhee *et al.*, 2016). In addition to the precursor of melatonin, serotonin is a neurotransmitter that modulates mood, anxiety, aggression,

pain, sexual behaviour, and sleep. Increased serotonin availability is associated with elevated mood and reduced anxiety (Gambichler *et al.*, 2002; Lambert *et al.*, 2002; Feldman *et al.*, 2004; Slominski *et al.*, 2005). UVR, mainly UV-A, also induce the release of nitric oxide (NO) into the circulation, leading to vasodilatation, reduced vascular resistance and a reduction in blood pressure (Juzeiene and Moan, 2012).

Regarding the negative effects of UVR, they can be differentiated in short and long-term effects (Matsumura and Ananthaswamy, 2004; Sklar *et al.*, 2012).

Short-term effects

- Erythema (or sunburn) is an inflammatory reaction in the skin that can be associated with redness, warm, sensibility or edema and when it is severe, blisters can appear (Hönigsmann, 2002). It is mainly caused by UV-B rays, although UV-A's, visible light and infrared radiation can also be implicated. The time course and intensity depend on the skin type, exposure time and wavelength of the rays, e.g. in fair skins can appear an immediate erythema and can last for 1-2 weeks, whereas in dark skins the erythema fade within 1-3 days (Sklar *et al.*, 2012).

- Tanning (or pigment darkening) can be differentiated in three phases: first, the immediate pigment darkening (IPD) occurs in the first minutes of UV-A exposure and can last until 2h. Second, the persistent pigment darkening (PPD) occurs with higher doses of UV-A and can last for 24h. Both are the result of oxidation and redistribution of pre-existing melanin. Third, the delayed tanning (DT) appears after 3-5 days of sunlight exposure and can persist during several days, weeks or even months, and is associated with the synthesis of new melanin. Both UV-A and UV-B are capable of inducing DT, but the tanning induced by UV-B is always preceded by erythema (Hönigsmann, 2002).

- Other short-term effects of the UVR exposure are immunosuppression or thickening of the epidermis (D'Orazio *et al.*, 2013).

Long-term effects

- Skin photo-aging. Overexposure to UV-A radiation increases the formation of reactive oxygen species (ROS), which at high concentrations stimulate the degradation of the dermal ECM components, e.g. collagen, elastin and glycoproteins. UVR can cause cross-linking of the collagen-elastin network of the dermis and increase the expression and secretion of matrix metalloproteinases (MMPs), capable of degrade the collagen present in the dermis. All these results in skin damage, such as deep wrinkles, dryness, roughness, irregular pigmentation (e.g. solar lentigines), elastosis or dilatation of blood

vessels (i.e. telangiectasia). These features are mainly observed in UV exposed areas (e.g. face, neck or arm) of fair-skinned people, who have been repeatedly exposed to sunlight during their lives (Masnec and Poduje, 2008; Gromkowska-Kępa *et al.*, 2021; Salminen *et al.*, 2022).

· *Skin carcinogenesis*. When the described mutations affect several oncogenes or tumor-suppressive genes, the cell cycle can be out of control and transformation of keratinocyte and melanocytes occurs (Matsumura and Ananthaswamy, 2004). Skin cancer can be divided into cancers derived from melanocytes (melanoma), or from the epidermally derived cells (non-melanoma skin cancers, NMSC), that are mainly divided in basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). There are strong evidences that all forms of skin cancer are related to UV exposure, it is estimated that around 65 % of melanoma and 90 % of NMSC are caused by UVR. The NMSCs are the most frequently diagnosed cancer in humans (aprox. 70 % are BCC and 25 % are SCC), both present good prognoses, especially if they are detected in their initial states. The BCC presents the lowest mortality, they usually grow slowly and rarely metastasize, whereas SCC shows a variable metastatic rate and provokes around 75 % of deaths due to NMSCs. On the other hand, melanoma is the deadliest form of skin cancer, and its incidence has increased in the last decades, especially in fair-skinned individuals living in warm and sunny areas. The UV exposure patterns influence the develop of different types of cancer, i.e. chronic cumulative UV exposure is more commonly associated with the develop of NMSCs, whereas melanoma is generally associated with intermittent UV exposure and a history of sunburn (Narayanan *et al.*, 2010; D'Orazio *et al.*, 2013; Watson *et al.*, 2016; Gracia-Cazaña *et al.*, 2020).

Skin cancer have become one of the most diagnosed types of cancers worldwide, with more than 1.5 million new cases estimated in 2020 of which approximately 325000 cases were melanoma. Melanoma incidence have been increasing in fair-skinned population during the last 50 years, and it is expected to increase a 50 % from 2020 to 2040 (Arnold *et al.*, 2018, 2022). The highest incidence of both types of skin cancer (melanoma and non-melanoma) is observed in Australia and New Zealand. Different factors have influenced this fact: the geographical location, the closeness to the ozone hole in the Antarctica have made that the people in these countries receive a higher dose of UV-B radiation, and the high percentage of fair-skinned population that migrate from northern European countries hundreds of years ago, that are more vulnerable to UVR (Buettner and Raasch, 1998; WCRF, 2023). In Europe, the highest incidence is observed

in Nordic countries, where population is exposed to UVR only during summer, whereas the distribution of NMSCs incidence is different, i.e. countries from central or south Europe with a high dose of UVR during all year (e.g. Spain, France, Italy, Germany or Croatia) present a higher incidence of NMSCs than melanoma (Figure I. 5).

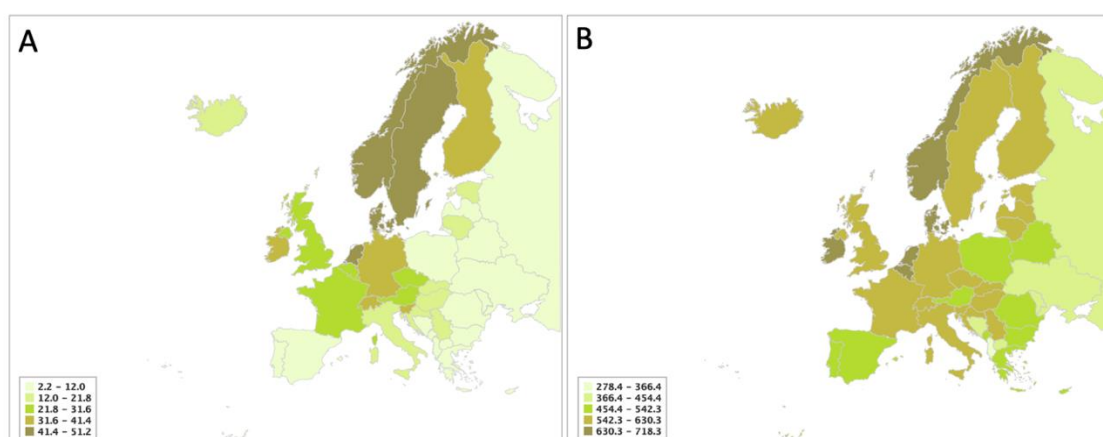


Figure I. 5. Incidence of melanoma (A) and non-melanoma (B) skin cancer in Europe. The incidence was calculated as ASR (aged-standardized rate) per 100000 habitants (European Cancer Information System, 2023).

In Spain, the incidence of skin cancer is growing 10 % each year (around 5000 new cases of melanoma and 70000 cases of non-melanoma skin cancer were diagnosed in 2022). In Andalusia, 13000 new cases of skin cancer, of which 700 are melanoma, were detected in 2022 (Asociación Española Contra el Cáncer, 2023), being Malaga one of the provinces with the highest mortality in both types (melanoma and non-melanoma) of skin cancer (Alcalá Ramírez del Puerto *et al.*, 2023).

Visible light and infrared radiation can also provoke some biological effects in the skin such as erythema, pigmentation, thermal damage or oxidative stress (Mahmoud *et al.*, 2008; Calles *et al.*, 2010; Nakashima *et al.*, 2017). Furthermore, it has been observed that certain photodermatoses (e.g. melasma, lichen planus pigmentosus or postinflammatory hyperpigmentation) present their action spectrum in the visible region, i.e. patients with the mentioned photodermatoses frequently complain about the worsening of their disease after sun exposure, even though they apply sunscreen regularly because normally they do not or weakly protect against visible light (Mahmoud *et al.*, 2010; Lyons *et al.*, 2021; Passeron *et al.*, 2021). There is also increasing evidence on the role of visible light (VL) in pigmentary disorders in dark skinned population and

immediate erythema in light-skinned individuals. This population is naturally better protected against UV-B radiation, but they are more prone to hyperpigmentation induced by VL and UV-A (Mahmoud *et al.*, 2010).

In recent years, there are many evidences that other environmental factors (e.g. pollution, temperature, nutrition or stress) are involved in the development of skin cancer and other skin problems, what is known as skin exposome (Krutmann *et al.*, 2017; Gracia-Cazaña *et al.*, 2020; Passeron *et al.*, 2020; Vineis *et al.*, 2020). The term exposome describe the totality of exposures to which an individual is subjected during life. It includes both external and internal factors, as well as the responses of the human body to these factors (Krutmann *et al.*, 2017; Vineis *et al.*, 2020). Epidemiological studies have shown that the risk of skin cancer can be affect by different types of radiation (e.g. UV, visible or ionizing), temperature, air or water pollution, stress, nutrition, tobacco smoke, exercise, as well as determine microorganism (e.g. polyuomavirus or human papillomavirus) (Gracia-Cazaña *et al.*, 2020). Krutmann *et al.* (2017) suggested that skin aging exposome fall into the following categories: sun radiation: UVR, visible light and infrared radiation, air pollution, tobacco smoke, nutrition, stress, sleep deprivation, temperature, and cosmetics products (Figure I. 6). The exposome of other skin problems such as atopic dermatitis, rosacea, acne or actinic keratosis are also described (Dréno *et al.*, 2018; Morgado-Carrasco *et al.*, 2021; Stefanovic *et al.*, 2021; Molina-García *et al.*, 2022).

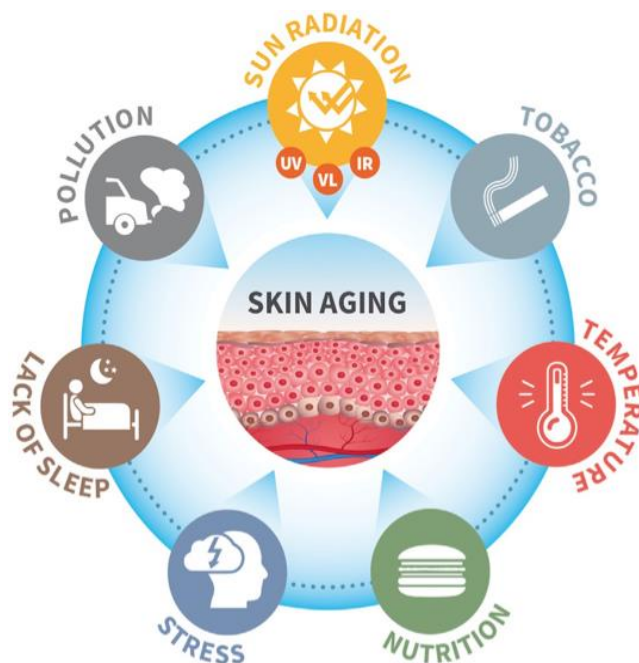


Figure I. 6. Example of the skin aging exposome proposed by Krutmann *et al.* (2017). Skin aging is related to sun radiation (UV radiation, visible light and infrared radiation), air or water pollution, tobacco smoke, high temperature, bad nutrition strategies, stress, or lack of sleep.

Fitzpatrick developed a way to classify the skin based on the color and how it reacts to sunlight exposure in terms of degree of burning and tanning (Fitzpatrick, 1988). It is commonly used to estimate the sun sensitivity in the population and helps to predict the risk of photodamage or skin cancer. The Fitzpatrick classification denotes six different skin phototypes (Table I. 1): Phototype I is the most vulnerable to UVR, that normally present a light or pale skin color, never get tan and always sunburn (e.g. redhead people), or phototype VI is the most protected against the UVR, that present a very dark skin color with black hair and eyes, never sunburn and are always tanned (e.g. African people). The minimal erythema dose (MED), define as the UV dose required to produce a perceptible erythema, is around $150\text{-}300\text{ J m}^{-2}$ in phototype I, whereas in phototype VI the MED is $900\text{-}1500\text{ J m}^{-2}$.

Table I. 1. Fitzpatrick skin type classification, taking into account the skin color, the hair and eyes types, frequency of sunburn and tanning, the minimal erythema dose (MED), the required sun protection factor (SPF), UV-A protection factor (UVAPF) and visible protection factor (VLPF). Adapted from Passeron *et al.* (2021).

Phototype	I	II	III	IV	V	VI
Skin color	 Light, pale, white	 White, fair	 Light brown	 Moderate brown	 Dark brown	 Very dark brown, black
Hair	Redhair, blond	Blond	Any	Brown	Dark brown	Black
Eyes	Blue, green, grey	Blue	Brown	Brown	Dark brown	Black
Sunburn	Always	Very easily	Easily	Ocasionalmente	Rarely	Never
Tanning	Never	Minimal	Gradual	Easy	Intense, fast	Always
MED (J m⁻²)	150-300	250-400	300-500	400-600	600-900	900-1500
SPF	50+ 					30
UVAPF	+++  >1/3					+++ >2/3
VLPF						+++

The sun protection factor (SPF) claim in all commercial sunscreens is determined based on the MED, and calculated as the ratio of the solar radiation necessary for a MED in the back of health volunteers with and without sunscreen application (ISO 24444, 2019). However, there are other biological effects related to the UVR exposure which are not considered in the evaluation of the protection of a sunscreen. Recently, the permanent pigment darkening (PPD) has been selected to calculate the UVA protection factor (ISO 24443, 2021). de la Coba *et al.* (2019) suggested the estimation of the protection factors against other biological effect, that were denominated as Biological effective protection factors (BEPFs). In addition to erythema, UV-B radiation is responsible for a beneficial effect, the synthesis of vitamin D, and other negative effects such as DNA damage, photocarcinogenesis, immunosuppression or the development of cataracts. Other effects mainly provoked by UV-A radiation are singlet oxygen formation

and photoaging. There are also effects provoked by a mixture of UV-B and UV-A such as photoisomerization of urocanic acid, lipid peroxidation or elastosis (Figure I. 7; Table I. 2).

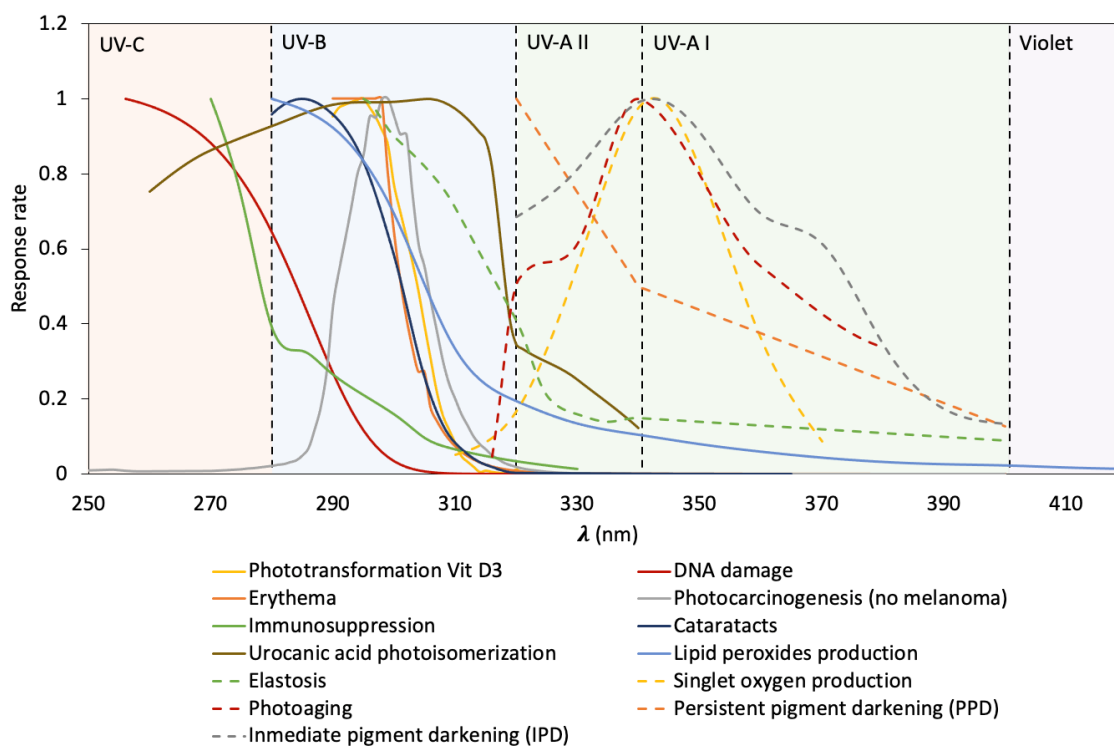


Figure I. 7. Action spectra of biological responses related to wavelength in the UV-C, UV-B, UV-A, and part the visible region. More information about the biological effects are found in Table I. 2.

Table I. 2. Characterization of the action spectra of the biological responses shown in Figure I. 7. Moreover, percentage of UV-B and UV-A, maximal lambda ($\lambda_{\max}$) and reference are also observed.

Biological effect	UV-B / UV-A (%)	$\lambda_{\max}$	Reference
Phototransformation Vit D3	100.0 / 0.0	294-295	(Webb <i>et al.</i> , 1989)
DNA damage	100.0 / 0.0	256	(Setlow, 1974)
Erythema	99.4 / 0.6	290-298	(McKinlay, 1987)
Photocarcinogenesis	99.2 / 0.8	298-299	(de Gruijl and Van der Leun, 1994)
Immunosuppression	93.5 / 6.5	270	(Fabo and Kripke, 1980)
Cataracts	99.5 / 0.5	284-286	(Baum and Pitts, 1997)
Urocanic acid photoisomerization	84 / 14	302 - 307	(Gibbs <i>et al.</i> , 1993)
Lipid peroxides production	74 / 26	280-281	(Morliere <i>et al.</i> , 1995)
Elastosis	62.4 / 37.6	295	(Wulf <i>et al.</i> , 1989)
Singlet oxygen formation	3.0 / 97.0	342-343	(Hanson and Simon, 1998)
Photoaging	2.4 / 97.6	339-340	(Bissett <i>et al.</i> , 1989)
Immediate pigment darkening (IPD)	1.4 / 98.6	341-343	(Irwin <i>et al.</i> , 1993)
Persistent pigment darkening (PPD)	0.0 / 100.0	320	(Moyal <i>et al.</i> , 2000)

3. Photoprotection: The current problem of UV-filters

The high incidence and mortality of skin cancer in many countries have led public and private organizations to become aware of this issue and carry out prevention and education campaigns that promote early skin cancer detection and photoprotection. Thanks to these, the incidence of skin cancer has been stabilized or reduced in many countries (e.g. Australia), people have realized about the need for photoprotection to avoid or prevent the damages caused UVR (González *et al.*, 2008; Sinclair and Foley, 2009; Stratigos *et al.*, 2012; Iannaccone and Green, 2014; Walker *et al.*, 2022). In Spain, these campaigns are conducted by the Spanish Academy of Dermatology and

Venerology, the Spanish group of Photobiology (GEF: Grupo Español de Fotobiología), “Fundación Piel sana” (<https://aedv.fundacionpielsana.es>) or the group “Soludable” (<https://soludable.hcs.es>). “Soludable” is an initiative that was born in Malaga and carry out several active programs to promote healthy sun protection habits. It is an interdisciplinary group that work in different areas: education, health, work, sports, gastronomy, or architecture, with the objective to improve the quality of life, reducing the burden of diseases associated with overexposure to UVR.

Photoprotection is based on avoid that UV rays reach our skin through different strategies, such as: reduction of the sun exposure during the hours with highest UVR intensity (e.g. from 12:00 to 16:00 in summer days); wearing appropriate clothes and hats; use of sunglasses or apply sunscreen (SPF > 30) in a correct way: reapplied it every 2 hours or after sweating or swimming and utilized the appropriate amount of sunscreen (2 mg cm⁻²), e.g. a teaspoon of cream is the recommended amount for covering the face (González *et al.*, 2008; Isedeh *et al.*, 2013).

It is noticeable that sunscreens have been very effective in preventing health problems cause by UVR (Green *et al.*, 1999, 2011; Sander *et al.*, 2020), so its use has increased and widespread over the years. They contained molecules that can absorb, reflect, or scatter UVR (named UV filters). There are two types of filters: synthetic filters (also known as chemical or organic), compounds that present at least one aromatic ring and/or carbonyl group providing them a high molar absorption capacity in the UV-B and/or UV-A region (e.g. ethylhexyl methoxycinnamate, homosalate, octocrylene or benzophenone-3); and mineral filters (also called physical or inorganic), molecules that in addition to absorb the UVR can reflect or scatter the UV rays (e.g., titanium dioxide or zinc oxide) (González *et al.*, 2008; Shaath, 2010). Other type of UV filters are the biological or natural ones, extracted from organisms. It is important to mentioned that nowadays there is no natural UV filters approved by any regulatory agency (i.e. European Commission (EC) or Food and Drugs Administration (FDA)), these natural compounds are normally use in sunscreens as boosters, improving the photoprotection by adding antioxidant, antiaging or immunomodulatory properties (e.g. carotenoids, vitamin C or E, flavonoids or polyphenols) (Aguilera *et al.*, 2023; Li *et al.*, 2023). UV filters can be also classified concerning their capacity to absorb UVR, in filters that absorb only UV-B (280-315 nm) or UV-A (315-400 nm), or both UV bands (known as broadband UV filters) (Table I. 3). Recently, an increasing number of research indicate that visible light, mainly the high energy visible (HEV; violet-blue 400-500 nm), and the near infrared

radiation (IRA; 760-1400 nm) can also damage human skin (Calles *et al.*, 2010; Nakashima *et al.*, 2017), so filters against these types of radiation are being investigated. Iron oxides have been shown to provide effective protection against the HEV. These compounds are normally used to provide colour to sunscreens, so the use of tinted sunscreen also provide protection against the visible light (Bernstein *et al.*, 2021) (Figure I. 8).

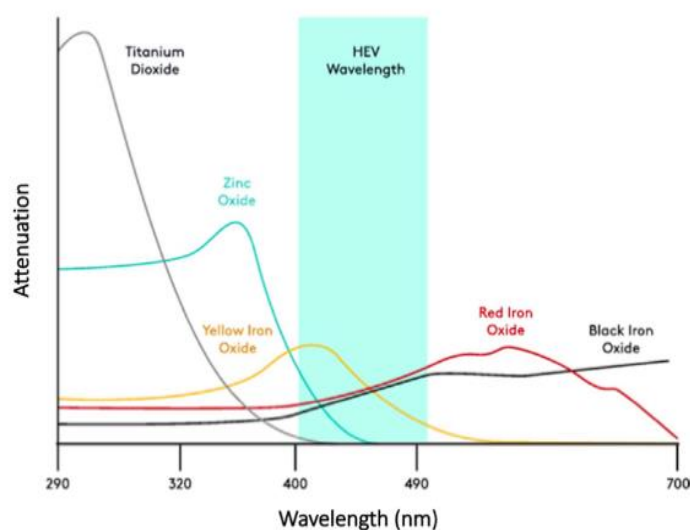


Figure I. 8. Radiation attenuation of both physical filters (TiO_2 and ZnO) and different iron oxides (yellow, red and black). In blue the high energy visible (violet-blue light). From Bernstein *et al.* (2021).

In Europe, sunscreens are considered cosmetic products (EC, 2009), which approved the use of 29 UV filters with specific limits of concentrations and warnings for the labelling of the products (Table I. 3). In addition, there are some specific guidelines for the formulations: the lowest photoprotection claim is SPF 6, the ratio of photoprotection UVA/UVB should be greater than 1/3, and the critical wavelength (λ_c is defined as the wavelength (from 290 to 400 nm) at which the product absorbed the 90% of the radiation) should be at least 370nm (EC, 2006).

Table I. 3. Most common UV-filters used in the European Union EU divided by type of filters (UV-B, UV-A or broad band) with the INCI (International Nomenclature Cosmetic Ingredient) name, the alternative name (if proceed), the common abbreviation, the maximal concentration (%) allowed by the EU, and the maximal absorption of each UV-filter. Adapted from González *et al.* (2008).

Type of UV filter	UV filter INCI	Alternative name	Common abbreviation	Max. concentration (%)	Maximal absorption	
Synthetic UV-filters	UV-B	Ethylhexyl triazone	Octyl triazone	EHT	5	314
		Ethylhexyl dimethyl PABA	Padimate O	EDP	8	290-310
		Ethylhexyl methoxycinnamate	Octinoxate	EHMC	10	311
		Ethylhexyl salicylate	Octisalate	EHS	5	307
		Homosalate	-	HMS	10	306
		Octocrylene	-	OCR	10	303
	UV-A	Phenylbenzimidazole sulfonic acid	Ensulizole	EHS	8	310
		Benzophenone-3	Oxybenzone	BP-3	6	288-325
		Butyl methoxydibenzoylmethane	Avobenzzone	AVO	5	360
		Terephthalylidene dicamphor sulfonic acid	Ecamsule	TDSA	10	345
Broad band	Methylene bis-benzotriazolyl tetramethylbutylphenol	Bisotrizole	MBBT	15	303-344	
	Drometrizole trisiloxane	Silatrizole	DTS	10	303-368	
	Titanium dioxide	-	TiO ₂	20	290-400	
Mineral UV-filters	Zinc oxide	-	ZnO	20	290-400	

Recently, the safety of certain UV filters for humans and the environment has been questioned (Huang *et al.*, 2021). Some synthetic UV filters can present risk for the human health, mainly related to endocrine disruptions, photoallergies or oxidative stress in the skin due to photoinstability of the filters when contact with sunlight (Wang *et al.*, 2016; Siller *et al.*, 2018; Santander Ballestín and Luesma Bartolomé, 2023). In 2022, the uses of oxybenzone and octocrylene were restricted concerning their potential for causing endocrine disruptions (EC, 2022), i.e. before the amending, oxybenzone could be use in a 10 %, and now the maximal allowed concentration is 6 % in face, hand and lip products, excluding propellants and pump spray products, in which the maximal allowed concentration is 2.2 %, and in other products is 0.5 %. Santander Ballestín and Luesma Bartolomé (2023) classified the UV-filters according to the toxicity effects based on EWG's (Environmental Working Group) sunscreen guide (Table I. 4).

Table I. 4. Toxicity range of some synthetic and mineral UV-filter based on EWG sunscreen guide. From Santander Ballestín and Luesma Bartolomé (2023).

UV-filter	Toxicity range (1-10)
Benzophenone-3	High danger: 7-10
Ethylhexyl methoxycinnamate	High danger: 7-10
Homosalate	High danger: 7-9
Ethylhexyl salicylate	High danger: 7-9
Octocrylene	Medium danger: 3-6
Titanium dioxide	Low danger: 1-2
Zinc oxide	Low danger: 1-2

On the other hand, sunscreens are considered an emerging contaminant in aquatic environments. The sunscreens can enter in the water through wastewater or directly from swimmers wearing sun protection and can accumulate in the aquatic environments causing negative effects on the ecosystem (Tovar-Sánchez *et al.*, 2020). Different organic UV-filters have been identified in surface waters (Tashiro and Kameda, 2013; Tsui *et al.*, 2014; Sánchez Rodríguez *et al.*, 2015; Carve *et al.*, 2021) and in several aquatic organisms, including species with commercial importance (e.g. mussels, clams, crabs, squids and fishes) (Gago-Ferrero *et al.*, 2012; Vidal-Liñán *et al.*, 2018; Prakash

and Anbumani, 2021). The accumulation of organic filter compounds can cause different biological and toxicological responses, such as the induction of coral bleaching, reducing phytoplankton growth, or hormone disorder in fish or mammals (Weisbrod *et al.*, 2007; Blüthgen *et al.*, 2012; Downs *et al.*, 2016; Tsui *et al.*, 2017; Araújo *et al.*, 2018; Nataraj *et al.*, 2020; Thorel *et al.*, 2020; Moeller *et al.*, 2021). Therefore, some areas with valuable marine ecosystem have taken regulatory action to ban some toxic sunscreen ingredients. For example, the State of Hawaii has banned the sale and distribution of any sunscreen containing benzophenone-3 (BP-3) and ethylhexyl methoxycinnamate (EHMC) to protect coral reefs. Other areas such as Key West (Florida), the Republic of Palau, or the Caribbean islands of Bonaire and Aruba, have also forbidden the use of certain UV filters (Levine, 2021). In this way, mineral UV filters are considered to provide the best and safest photoprotection since they can protect against all UVR (known as broad band UV filters), they do not degrade when exposed to UVR and they present limited penetration into the skin. However, they are less used in formulations because they provide a white and chalky texture that consumers do not appreciate. So, nowadays they are normally formulated as nanoparticles (< 100 nm diameter) to avoid the whitening effects on the skin and its use has increased. However, these compounds are able to generate ROS and release metal ions into aquatic environment, as well as the nanoparticles possibly can penetrate into the dermis (Miller *et al.*, 2010; Schneider and Lim, 2019; Santander Ballestín and Luesma Bartolomé, 2023).

The research about UV filters toxicity is still limited and very controversial to conclude their possible negative effects. In order to prevent the possible damage consequences, it is necessary the search and development of alternative UV filters that are natural, biodegradable and non-toxic for the environment and human health. In this context, nature can provide solutions that can protect against the damage UV rays. Terrestrial plants, algae or cyanobacteria are daily submitted to different environmental stress; thus, they have developed various defense and photoprotective mechanisms, including the synthesis and accumulation of both UV-screen and antioxidant compounds. These natural molecules could be used as new sources of natural UV filters in sunscreens (Saewan and Jimtaisong, 2015; Amador-Castro *et al.*, 2020; Sen and Mallick, 2021; Vega *et al.*, 2021; Li *et al.*, 2023).

4. **Blue biotechnology: Uses of algae in different fields**

Biotechnology is defined as the use of biology to develop new products, methods and organisms intended to improve human health and society. There are different types of biotechnology, divided into eight color-coded (e.g. red involves medical purposes, white refers to industrial processes, green is related to agriculture or blue covers processes with aquatic organisms among others). Taking into account that more than 70 % of the Earth's surface is covered by water, of which oceans hold about 96%, aquatic environments (mainly marine ones) are considered a great source of new raw material that are still unexplored, as present a wide diversity of species with enormous potential (Guillerme *et al.*, 2017; Rigogliuso *et al.*, 2023). Thus, blue biotechnology explores the potential uses of aquatic organisms (e.g. bacteria, cyanobacteria, fungi, algae, sponges) for application in different sectors such as agriculture, aquaculture, bioremediation, cosmetic, food, pharmaceutic or renewable energies, among others. These organisms, highlighting the algae, present a wide content of bioactive compounds, many of them still unknown. Thus, many researchers are investigating with these organisms, focusing on the understanding of their physiology, the search for novel molecules with potential applications, as well as the development/ optimization of culture technologies for their production (Michalak and Chojnacka, 2015; Brodie *et al.*, 2017).

Algae are a diverse group of aquatic, autotrophic and photosynthetic organisms, that can be found in a wide range of environments (e.g. freshwater, marine, hypersaline, or extremely cold) and with different sizes and morphologies (e.g. from microscopic phytoplankton to giant "forests" of Kelps). According to the size, algae are divided in micro- and macroalgae. Microalgae are microscopic and unicellular algae, that normally measure around 2-200 μm diameter. There are more than 50000 microalgae species, and around of 30000 have been studied (Richmond 2003). Some of them are: *Chlorella* spp. (Chlorophyta) which is usually consumed as food or feed supplement because of their high nutritional value (Liu and Chen, 2016), *Haematococcus lacustris* (previously *Haematococcus pluvialis*) and *Dunaliella salina* (Chlorophyta) for carotenoids production (astaxanthin and β -carotene, respectively) (Raja *et al.*, 2007; Li *et al.*, 2011), or *Microchloropsis gaditana* (previously *Nannochloropsis gaditana*) (Eustigmatophyceae) for its high lipid content (San Pedro *et al.*, 2014). Some authors include cyanobacteria (also known as blue-green algae) as microalgae due to their physiology, although they are not eukaryotic organisms. Cyanobacteria are the unique prokaryotes able to perform oxygenic photosynthesis, that have been responsible for the oxygenation of the Earth's

atmosphere since 2 billion years ago. Cyanobacteria can be found in different habitats, even terrestrial or extreme ones (e.g. tree barks, soil crusts, deserts, ice caps or thermal waters) (Mishra *et al.*, 2018). *Arthrospira* (Spirulina) *platensis* is one the best known cyanobacteria species, it is commercially used as food supplement for its high protein concentration, as well as vitamin B₁₂ and minerals content (Khan *et al.*, 2005). In contrast, macroalgae or seaweeds are multicellular or coenocytic organisms, which size can range from few centimeters to 50 m and can also present a very diverse morphology. Around 12000 species have been described, although only 200 are considered to present commercial value, and even fewer species (around 10) are cultivated intensively (FAO, 2018a). Macroalgae are divided according to the color due to pigment composition in three Phylla: (1) Chlorophyta or green algae (mainly in the Class Ulvophyceae ~ 2500 species) that present chlorophyll *a* and *b*, which give them their characteristic green color; (2) Ochrophyta, considering the Class Phaeophyceae (~ 2000 species of brown algae), that contain chlorophyll *a* and *c*, and their brown color is due the presence of the carotenoid fucoxanthin, and (3) Rhodophyta or red algae (~ 7000 species), that contain chlorophyll *a* and phycobiliproteins, mainly phycoerythrin and phycocyanin which give them the red color (Guiry and Guiry, 2023). Macroalgae have been traditionally collected or cultured for human consumption, such as the brown algae *Undaria pinnatifida* (Wakame), *Himanthalia elongata* (sea spaghetti), *Durvillea antarctica* (Cochayuyo) or *Saccharina japonica* (Kombu), the red algae *Chondrus crispus* (Irish moss), *Palmaria palmata* (Dulse) or *Porphyra* spp. (Nori), or the green algae *Ulva* spp. (sea lettuce), *Caulerpa* sp (sea grapes) o *Codium* sp (green sponge fingers). With the increasing knowledge about the chemical composition of algae and advancement in extractive technologies, additional applications were developed, such as the phycocolloids (e.g. agar, alginates or carrageenan) extractions from brown and red seaweeds that are used as texturing agents, emulsifiers and stabilizers in the food, feed, cosmetic or pharmaceutical industry (FAO and WHO, 2022) (Figure I. 9).

Some decades ago, algae gained a great biotechnological interest due to the high molecular diversity, and many researchers started to study their potential applications (Figure I. 9). In addition to the well-known used of algae as human food, they can be use in other sectors as a new or alternative source of raw material, such as animal feeding, agriculture, pharmaceutical, cosmetic, construction or renewable energy production (Cardozo *et al.*, 2007; Michalak and Chojnacka, 2014, Preprint; Stengel and Connan, 2015).

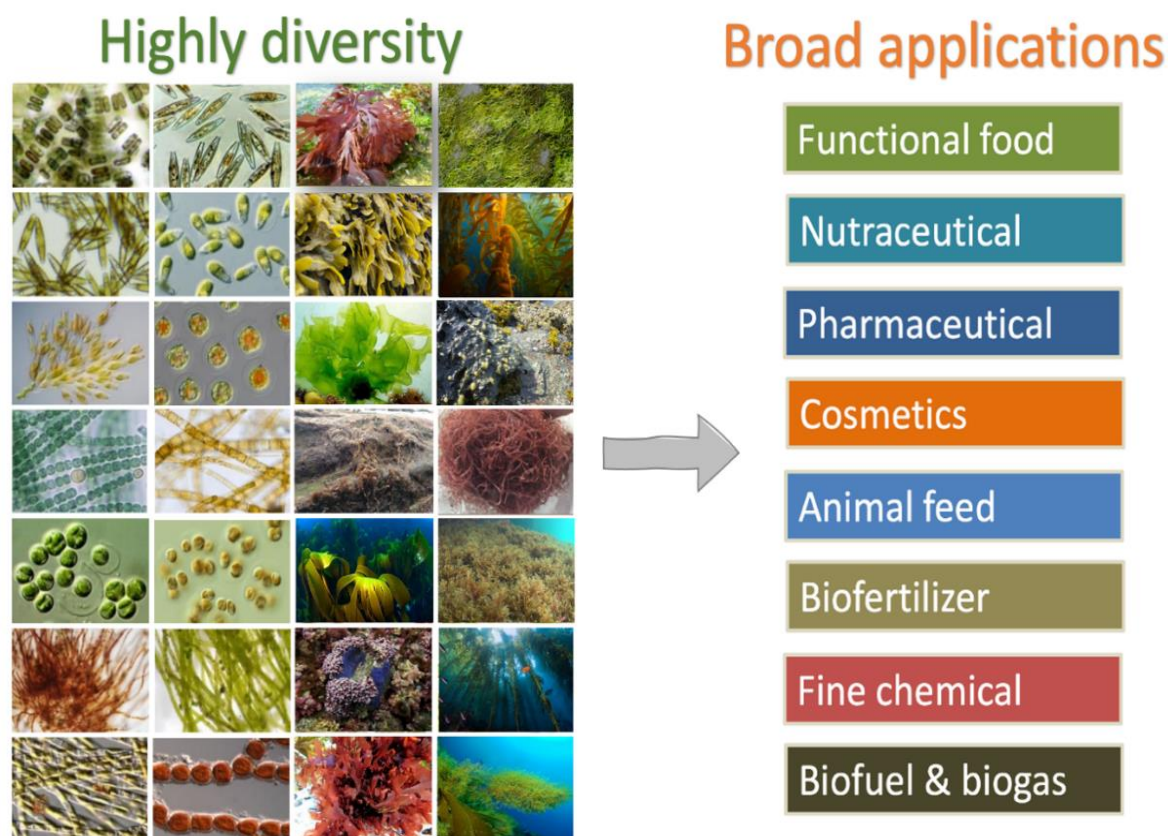


Figure I. 9. Diversity of algae species (cyanobacteria, micro- and macroalgae) and the different potential applications. Adapted from Gabriel Ación and EABA in “Jornadas sobre desalación y cultivos marinos en el marco de la Estrategia Andaluza de Economía Azul Sostenible”, Almería, 2023.

The main algal applications considered in this thesis are:

- **Human consumption.** Algae have been part of the human diet since ancient times. Traditionally, seaweeds have been consumed in many Asian countries. Species like *Laminaria*, *Saccharina*, *Undaria* (Wakame) (Phaeophyceae) or *Porphyra* (Nori) (Rhodophyta) have been and continue to be widely and frequently consumed in southeast Asia (Japan, China, Korea, Taiwan) as soup ingredients, salads, sushi or snacks. In north Atlantic countries (Ireland, Scotland, England or Iceland), algae have been also consumed historically, e.g. *Palmaria palmata* (Dulse) (Rhodophyta) was traditionally eaten in different forms (raw, boiled or fried). Their high nutritional value (high content of fiber, proteins and micronutrients and low lipids), as well as the recent studies about their beneficial properties (e.g. antioxidant, anti-aging, anti-inflammatory or anti-cancer) have made that, in the last years, their popularity increase also in other

countries (Stengel and Connan, 2015; Wells *et al.*, 2017; FAO, 2018b; Mendes *et al.*, 2022). Algae can be considered nutraceutic products as they present nutritional and good properties for the health (Figure I. 10). They are a nutritive and healthy alternative source of proteins for vegetarians, vegans or people who want to reduce the meat/fish consumption. Among microalgae and cyanobacteria, *Chlorella* and *Spirulina* are the most extended consume species, some of them can contain up to 70 % of dry proteins. They are normally consumed as food supplements, and are available in the market as capsules, tablets or powders (Vaz *et al.*, 2016; Matos *et al.*, 2017; Koyande *et al.*, 2019).

· **Cosmeceutics**. The cosmetic industry is continuously looking for new active principles and developing novel products. Nowadays, the cosmetic sector tends to use natural ingredients instead of synthetic ones in their formulations, as consumers are more concern about the negative effects of some synthetic compounds, and they prefer to use natural alternatives that are better and safer for their health and the environment. Algae are gaining much attention in this sector, due to their wide content of bioactive compounds with numerous beneficial properties for the skin health (e.g. gelling, emulsifier, moisturizing, anti-aging, antioxidant, immunostimulant or photoprotective properties) (Ariede *et al.*, 2017; Thiyagarasaiyar *et al.*, 2020; López-Hortas *et al.*, 2021; Pagels *et al.*, 2022; Dini, 2023; Fonseca *et al.*, 2023). Among the broad molecular diversity, some of them highlight, such as carotenoids, mycosporine-like amino acids, phenolic compounds, phycobiliproteins or polysaccharides. The term Cosmetic is defined by the European legislation as a substance or product intended to be applied in the external part of the human body (e.g. skin, hair, nails or lips) for cleaning, perfuming, beautifying, changing the appearance or protect. Many companies are developing multifunctional products, that in addition to cleaning or beautifying the skin, hair or nails, present other beneficial properties such as antioxidant or anti-aging. These products are known as Cosmeceutics, that they are combination of cosmetic and health (Figure I. 10). Recently, Thiyagarasaiyar *et al.* (2020) reviewed algae used in cosmeceutical applications and their bioactivities. Searching in the three major data base (Science Direct, Google Scholar and Pubmed), they observed that most of the algae studied for cosmeceutical applications are brown and red seaweeds (50 and 35 species, respectively), followed by green seaweeds and microalgae (18 and 19 species, respectively) and the main described bioactivities are antioxidant (39 %), antimicrobial (21 %) and anti-inflammatory (14 %).

· **Pharmaceutics (Drugs)**. Several researchers are looking for new molecules with potential applications in the treatment of different diseases (e.g. Alzheimer, Parkinson, diabetes or different types of cancers). The vast diversity of bioactive compounds in marine organism (specially algae) can serve as a new source of drugs discovery. Several compounds have been described as antioxidant, anti-inflammatory, anti-cancer, antibacterial or antiviral, among others (Thomas and Kim, 2011; Ngo *et al.*, 2012; Lindequist, 2016; Aziz *et al.*, 2021; Dai *et al.*, 2022). Some algae compounds, such as polyphenols or polysaccharides, have shown *in vitro* anti-proliferative potential against different types of cancer cells (e.g. colon, breast, lung), as well as immunomodulatory properties (Dellai *et al.*, 2013; Shi *et al.*, 2018; Abdala Díaz *et al.*, 2019; Zhang *et al.*, 2019). Oxidative stress has been linked to inflammatory processes and different chronic diseases, including some types of cancers, neurodegenerative disorders or metabolic syndromes (Sharifi-Rad *et al.*, 2020). Many algae compounds have been described as potent antioxidants or anti-inflammatory substances (e.g. carotenoids, fatty acids, polyphenols, polysaccharides), that can prevent the appearance of some diseases or reduce their pathologies (Pradhan *et al.*, 2020).

These three applications can be combined, developing nutraceutical, cosmeceutic, nutricosmetic and nutricosmeceutic products (Figure I. 10).

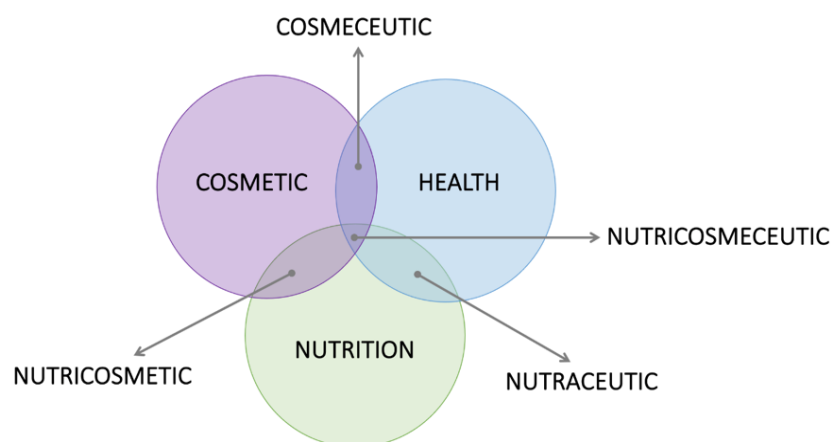


Figure I. 10. Approaches for studies combining cosmetic, health and nutrition can receive specific names. Cosmeceutic is the combination of cosmetic and health, Nutraceutical is used to refer about nutrition and health. Nutricosmetic considered the fusion of cosmetic and nutrition. Finally, the intersection of food, nutrition and health is called nutricosmeceutic.

Most of the algae are photosynthetic organisms, meaning they do not require organic compounds for growth. Instead, they convert sunlight into chemical energy and use the ATP and NADPH to assimilate CO₂, nitrate and sulphate to produce the organic matter needed to grow. In comparison with traditional crops cultures, algae present some advantages: they have high growth rates and productivities (e.g. microalgae like *Chlorella*, *Dunaliella* or *Tetraselmis* (Chlorophyta) or macroalgae like *Ulva* (Chlorophyta), *Porphyra* or *Gracilaria* (Rhodophyta) can present productivities of 20-40 g Dry weight (DW) m⁻² d⁻¹) (Masojídek *et al.*, 2011; Gómez Pinchetti *et al.*, 2012; Jerez *et al.*, 2016); they can be grown on non-productive lands and with different types of water (including seawater and wastewater). Thus, they do not compete for food resources or traditional agriculture, as well as they do not pollute the environment (they consume CO₂ while produce O₂) and consume relatively small amount of water (Acién *et al.*, 2016). Moreover, algae present some benefits for the ecosystem, i.e. they support several ecosystem services such as provisioning (food or medicinal resources), regulating (climate regulation or water purification), supporting (oxygen production or nutrient cycling) and cultural (recreation and ecotourism or aesthetic values) benefits (Figure I. 11).

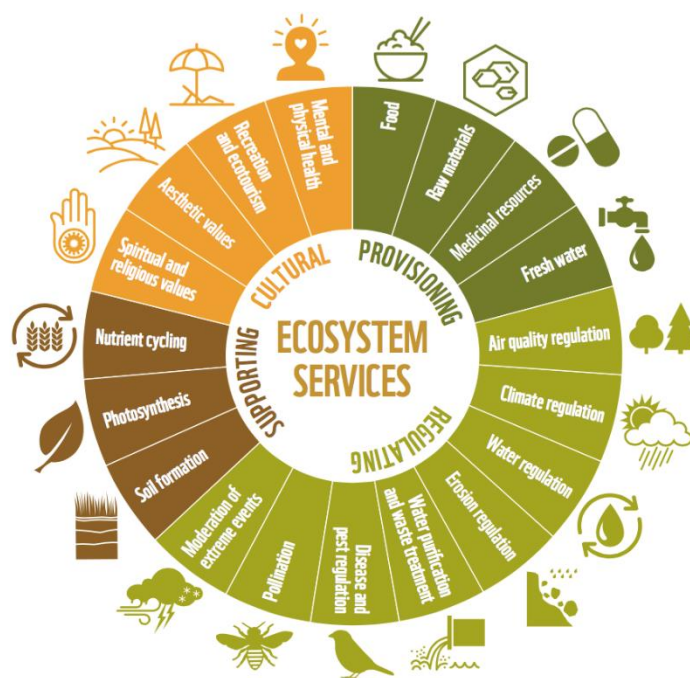


Figure I. 11. Ecosystem services from algae, that include provisioning, regulating, supporting and cultural (WWF, 2016).

The production of algae at big scale can be done through different technologies. Microalgae and cyanobacteria are normally cultured in open ponds systems or bioreactors (e.g. raceways, thin layer cascades or tubular) (Acién *et al.*, 2017), whereas macroalgae have been traditionally culture in on-shore farms or open ponds (Cai *et al.*, 2021), and recently, macroalgae are also culture in tanks, ponds or raceway systems (Abreu *et al.*, 2011b; Zertuche-González *et al.*, 2021). In addition, algae can be cultivated under integrated-multitrophic aquaculture (IMTA) systems. The most common system couples the production of algae and fish, in which algae consume the inorganic nutrients present in fishpond effluents, reducing the discharge of enriched nutrient water to the coast avoiding eutrophication. Furthermore, the algae growth under high nutrient availability can accumulate relevant content of bioactive compounds for different uses (e.g. feed, bioenergy or cosmetic) (Figure I. 12; Figure I. 13). Many macroalgae have been culture under this type of system obtaining high productivities of biomass and bioactive compounds, as well as high biofiltration capacity (Abreu *et al.*, 2011b; Figueroa *et al.*, 2012, 2022; Ben-Ari *et al.*, 2014; Barceló-Villalobos *et al.*, 2017; Peñuela *et al.*, 2018; Pliego-Cortés *et al.*, 2019).



Figure I. 12. In land facilities for micro- and macroalgae cultures: MICROALGAE: A) Out door race way (UAL, Almeria), B) Thin layer cascade (Algatech, Czech Republic), C) Vertical bags (IBYDA, UMA), D) Tubular photobioreactors (IBYDA, UMA); MACROALGAE: E) In door polyethyle tanks (BEA, ULPGC), F) Outdoor raceway (IBYDA, UMA), G) Large scale raceway (Israel), H) Tanks (Ensenada, México)

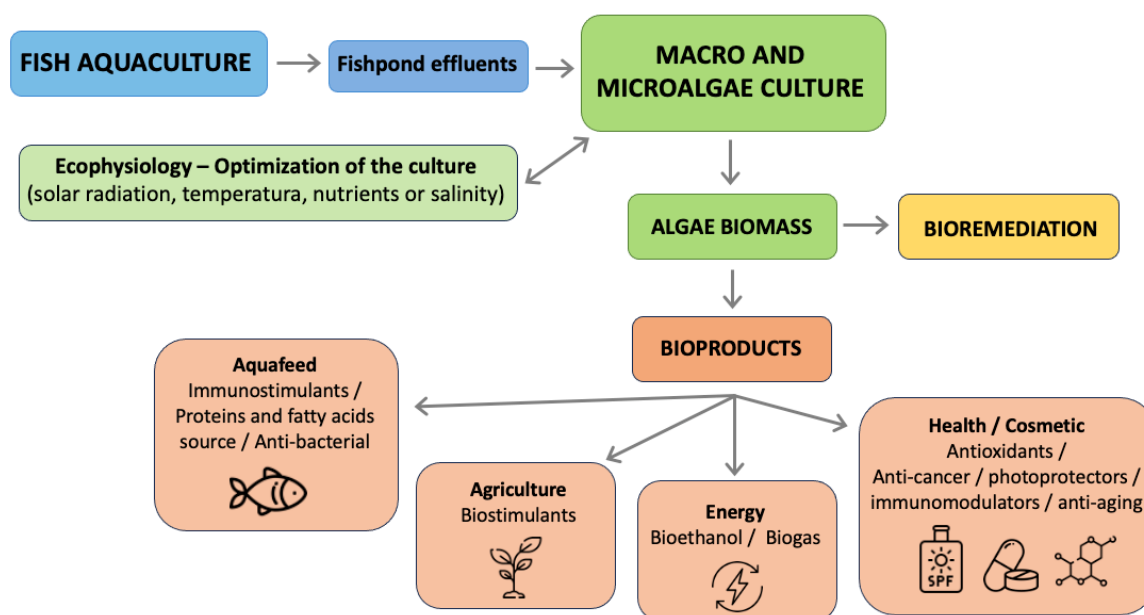


Figure I. 13. Scheme of an integrated multitrophic aquaculture (IMTA) system, in which fishpond effluents are used as nutrients source for macroalgae culture. This process can clean the water (Bioremediation) and produce biomass for different applications (e.g. aquafeed, agriculture, energy, health or cosmetic).

5. Protection mechanism in algae: Bioactive compounds with potential use in cosmeceutics

Algae are subjected to a variable and stressed environment, and they are exposed to extreme conditions both daily and seasonally. Some of the stress factors are: high solar radiation, changing temperature and salinity, nutrient supply, high hydrodynamics and even periods of desiccation. Thus, algae have developed different adaptation and acclimation strategies that can be divided in behavioral (e.g. molecular/cellular migration for self-shading), protection (e.g. antioxidant systems and photoprotective molecules) and repair mechanisms (e.g. photolyases) (Lyon and Mock, 2014; Lalegerie *et al.*, 2019; Rastogi *et al.*, 2020).

It is well known that environmental stresses increase the formation of reactive oxygen species (ROS), resulting in oxidative stress. ROS are forms of oxygen, sometimes combined with hydrogen, which are more reactive than the O_2 . The following compounds can be considered as ROS: singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide anion radical ($O_2^{\cdot-}$) or hydroxyl radical ($HO^{\cdot}$). The high ROS concentration can react with biomolecules, causing damage in various cellular components (e.g. lipids

peroxidation, proteins oxidation or damage of nucleic acids), and altering cellular structure and functions. Many algae present strong antioxidant systems to cope with the oxidative damage, such as antioxidant enzymes: Superoxide dismutase (SOD), that catalyzes the reaction between O_2^- and H_2O , producing O_2 and H_2O_2 ; Catalase (CAT), a very common and efficient enzyme, that catalyzes the decomposition of millions of H_2O_2 to H_2O and O_2 by each second; Ascorbate peroxidase (APX), that can eliminate the H_2O_2 using ascorbate as substrate, resulting in the oxidation of the ascorbate; Glutathione peroxidase (GPx), that catalyzes the reduction of lipid hydroperoxides to their corresponding alcohols and the free H_2O_2 into H_2O , using reduced glutathione as substrate; and Glutathione reductase (GR), responsible to maintain the supply of reduced glutathione. Another way to reduce ROS is the synthesis and accumulation of molecules with antioxidant capacity such as: glutathione, ascorbate, carotenoids, tocopherols or phenolic compounds (Dring, 2005; Rezayian *et al.*, 2019).

Some of the main stress conditions to which algae are exposed are the high irradiance PAR and the UVR. UVR can cause mutations in the DNA, production of ROS and degradation of important molecules (e.g. pigments, proteins or lipids), altering several important processes like photosynthesis, growth, nutrient uptake or sporulation. High irradiance of PAR can also lead to a high ROS production or even pigments bleaching, affecting the photosynthesis and compromising the growth. So, algae have developed specific protection mechanism against the high solar radiation, such as regulation of the size of the light-harvesting pigment antennae, energy dissipation mechanisms (i.e. non photochemical quenching), photorepair mechanisms (e.g. photolyases) and the accumulation of molecules that absorb or dissipate the UV rays (e.g. carotenoids, scytonemin, phenolic compounds or MAAs) (Falkowski and Chen, 2003; Häder and Sinha, 2005; Bischof *et al.*, 2006; Goss and Jakob, 2010). The size of the antennae is reduced under high light intensity avoiding the excess of energy reaching the photosystem that can provoke severe damages (Falkowski and Chen, 2003). Beside light absorption regulation, algae present ways of getting rid of the excess of energy that has already been absorbed, like the xanthophyll cycle present in brown and green algae (i.e. under high light conditions, violaxanthin is converted by de-epoxidation to antheraxanthin and then to zeaxanthin, which is able to reduce the excess of energy) (Goss and Jakob, 2010). Algae also present photolyases, enzymes capable of repair DNA damage caused by UVR, which are regulated by UV-A and blue light (350-450 nm) (Kavakli *et al.*, 2019).

Among the bioactive compounds with photoprotective properties in algae, the following must be highlighted:

Photosynthetic pigments or visible absorbing compounds

· Carotenoids are accessory pigments, that capture the light and transfer it to the chlorophylls for been used in the photosynthesis. As chlorophyll a, carotenoids are found in all photosynthetic organisms. Carotenoids are lipophilic molecules that absorb light in the range of 400-550 nm (Figure I. 14) and can be yellow, red or purple, depending on the type, e.g. β -carotene present a red-orange color. There are many types of carotenoids in algae. Takaichi *et al.* (2016) reviewed the distribution of carotenoids in algae, and observed that green algae mainly present β -carotene, violaxanthin and neoxanthin. Red algae principally contain β -carotene and zeaxanthin, and brown algae mostly present β -carotene, zeaxanthin, violaxanthin and fucoxanthin. Carotenoids can also play a photoprotective role, capturing the excess of energy from chlorophyll and dissipate it as heat (e.g. xanthophyll cycle). According to their structures, two types of carotenoids can be recognized: carotenes, which are formed by a hydrocarbon chain lacking oxygen molecules (e.g. α -carotene, β -carotene and lycopene), and xanthophylls, which are oxygenated derivatives of carotenes (e.g. lutein, violaxanthin, astaxanthin or zeaxanthin). In addition, carotenoids are normally distinguished between primary and secondary ones: primary compounds are those that are structural and functional components of the photosynthetic apparatus (e.g. xanthophylls), whereas secondary carotenoids are synthesized in large amounts under certain conditions via carotenogenesis (e.g. astaxanthin in *Haematococcus lacustris*) (Eonseon *et al.*, 2003; Guedes *et al.*, 2011). Carotenoids are also present in many fruits and vegetables. These compounds are important for vertebrates' health and development, that is normally incorporated in their diets through fruits of vegetables consumption. In addition, they provide the coloration of certain animals like flamingos, crustaceans, and some fish like salmon. Carotenoids are powerful antioxidants, and very important for the human eye healthcare, preventing the age-related macular degeneration. Commercially, the most important carotenoids are astaxanthin, canthaxanthin, lutein, zeaxanthin, β -carotene and lycopene (Christaki *et al.*, 2013; Maoka, 2020).

· Biliproteins are accessory photosynthetic pigments present in Rhodophyta, Cryptophyta and Cyanobacteria. There are different types of biliproteins with different

colors and maximum absorptions. The most common ones are phycoerythrin that absorbs in the range of 480-570nm and presents a red-pink color; and phycocyanin that absorbs light from 580 to 630 nm and has blue color (Figure I. 14). Some cyanobacteria present phycoerythrocyanin ($\lambda_{\text{max}} = 560\text{-}600\text{ nm}$) as an alternative form of phycoerythrin, and allophycocyanin ($\lambda_{\text{max}} = 620\text{-}650\text{ nm}$) is a mediator in the energy transfer between photosystems, passing the energy to photosystem I (Pagels *et al.*, 2019). They are water soluble proteins that have been used as fluorescent probes in immunofluorescence assay, food additives or natural colorants in ice-creams, candies, or soft drinks. In addition, different therapeutic properties have been described in these molecules, such as antioxidant, anti-cancer, neuroprotective, anti-inflammatory, antiviral or hepatoprotective (Sonani *et al.*, 2016; Pagels *et al.*, 2019; Chen *et al.*, 2022).

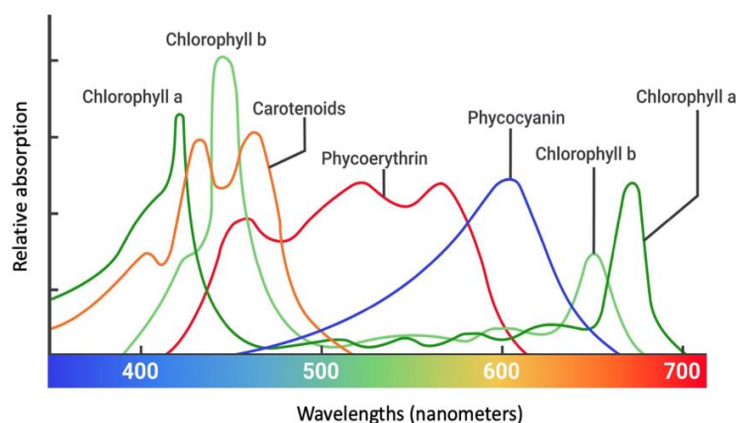


Figure I. 14. Absorption spectra of different pigments found in algae: chlorophyll a and b, total carotenoids, phycoerythrin and phycocyanin. Adapted from (Kukwa and Chetty 2021).

UV absorbing compounds

· *Scytonemin* is a lipophilic and yellow-brown pigment present in the polysaccharide sheath of some cyanobacteria, that act as protective mechanism against the UVR. It has been found in more than 300 cyanobacteria species belonging to the genera *Scytonema*, *Nostoc*, *Lyngbya*, *Calothrix* or *Chroococcidipsis*. This molecule mainly absorbs in the longer UV-A range (370-380 nm), but it can also absorb in the UV-C (250 nm) and UV-B range (280-320 nm) (Figure I. 15). Scytonemin has an *in vivo* maximum absorption (λ_{max}) at 370 nm, and an *in vitro* λ_{max} at 384 nm (Garcia-Pichel and Castenholz, 1991; Proteau *et al.*, 1993). Chemically, it is a dimer of indole and phenolic groups with a

molecular weight of 544 Da (Figure I. 16). Scytonemin can exist either in an oxidized green, or a reduced red form (Garcia-Pichel and Castenholz, 1991). Scytonemin presents potential application as sunscreen ingredients due to its capacity to absorb the UVR, protecting the skin in the UVA-Blue region. In addition, it has been described the antioxidant, anti-inflammatory and anti-proliferative properties of this molecule (Mishra *et al.*, 2015; Fuentes-Tristan *et al.*, 2019; Kang *et al.*, 2020).

· Mycosporine-like amino acids (MAAs) are also secondary metabolites, commonly found in red algae and cyanobacteria. They are small and water-soluble molecules mainly involved in the photoprotection. MAAs not only protect algae against the UVR, but also against other stress factors such as high temperature or salinity due to their antioxidant capacity and osmoregulatory properties. There are more than 25 types of MAAs, being the most abundant: palythine, asterina-330, shinorine or porphyra-334, with maximal absorption between 310-365 nm (Vega *et al.*, 2021) (Figure I. 15; Figure I. 16). MAAs are UV screen with antioxidant, anti-inflammatory and anticollagenase activities, and they have scientific and commercial interest for skin photoprotection (Wada *et al.*, 2015; Chrapusta *et al.*, 2017; Lawrence *et al.*, 2018; Kageyama and Waditee-Sirisattha, 2019; Vega *et al.*, 2021; Rosic *et al.*, 2023). More information on MAAs is included in Chapter 1.

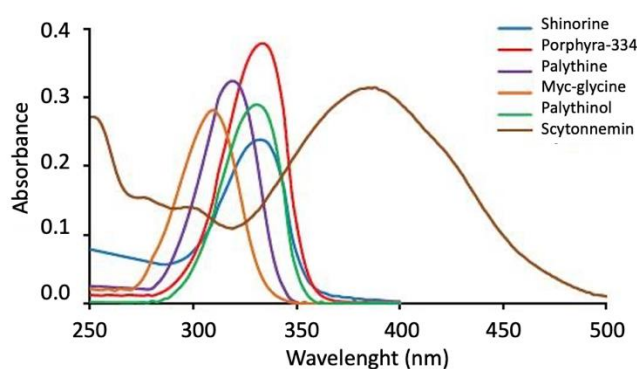


Figure I. 15. Absorption spectra of some common photoprotective compounds found in cyanobacteria, including mycosporine-like amino acids (shinorine, porphyra-334, palythine, myc-glycine, palythiol) and scytonemin (Rastogi and Madamwar, 2016).

· Phenolic compounds are secondary metabolites present in all types of algae, although brown algae normally present the highest content (Zubia *et al.*, 2007; Guinea *et al.*, 2012; Heffernan *et al.*, 2014; Tenorio-Rodriguez *et al.*, 2017). Structurally, phenolic compounds

are characterized by the presence of an aromatic ring with one or more hydroxyl groups. The structure can range from simple molecules (e.g. caffeic acid or phloroglucinol) to complex molecules with high molecular weights (e.g. some phlorotannins like phlorofucofuroeckol). There are different groups of phenolic compounds, such as phlorotannins, bromophenols, flavonoids or phenolic acids, among others (Figure I. 16). Phlorotannins are the group with the major interest for researchers due to their bioactivities, and they are only present in brown algae. Bromophenols and flavonoids can be found in green and red algae. These compounds act as protective agents that are synthesized in response to stress conditions like herbivory or UVR (Cotas *et al.*, 2020). Several bioactivities have been described in these compounds like antimicrobial, anticancer, antiobesity, antidiabetic, photoprotective, antioxidant, immunomodulatory, cardioprotective or neuroprotective, among others (Mateos *et al.*, 2020).

Other compounds

· Polysaccharides are macromolecules composed of repeated monosaccharides units bonded together through a glycosidic linkage. The chemical composition and molecular weight depend on the specie, and the presence of specific chemical structures (e.g. sulphate groups) can alter the biological properties. There are polysaccharides that form part the cell wall and other act as energy storage. The storage polysaccharides are composed of glucose units. Red and green algae present floridean starch and starch, respectively, whereas brown algae present laminarin. The structural polysaccharides are more diverse and complex. All macroalgae present cellulose or hemicellulose as the fibrillar part of their cell walls, that are normally combined with sulphated polymers and mucilages (phycocolloids). Red macroalgae can present agar (agarophyte species, e.g. *Gracilaria* spp. or *Gelidium* spp.) or carrageenan (carrageenophyte species, e.g. *Chondracanthus* spp. or *Kappaphycus* spp.), while green macroalgae produce ulvans, and brown algae produce alginates (e.g. Kelps) or fucoidans (e.g. Fucales). In addition, other types of polysaccharides can be found, such as porphyrans (in *Porphyra sensu lato* species) or xylans (green and red algae like Nemaliales and Palmariales) (Gomes-Dias *et al.*, 2022). The polysaccharide contents can vary from 4 to 70% of dry weight (Holdt and Kraan, 2011), and present wide applications due to their bioactivities (e.g. antioxidant, anticancer, antiviral, antibacterial or immunomodulatory), being the sulphated ones the most bioactive (Shao *et al.*, 2013; de Jesus Raposo *et al.*, 2015; Abdala Díaz *et al.*, 2019; Hentati *et al.*, 2020).

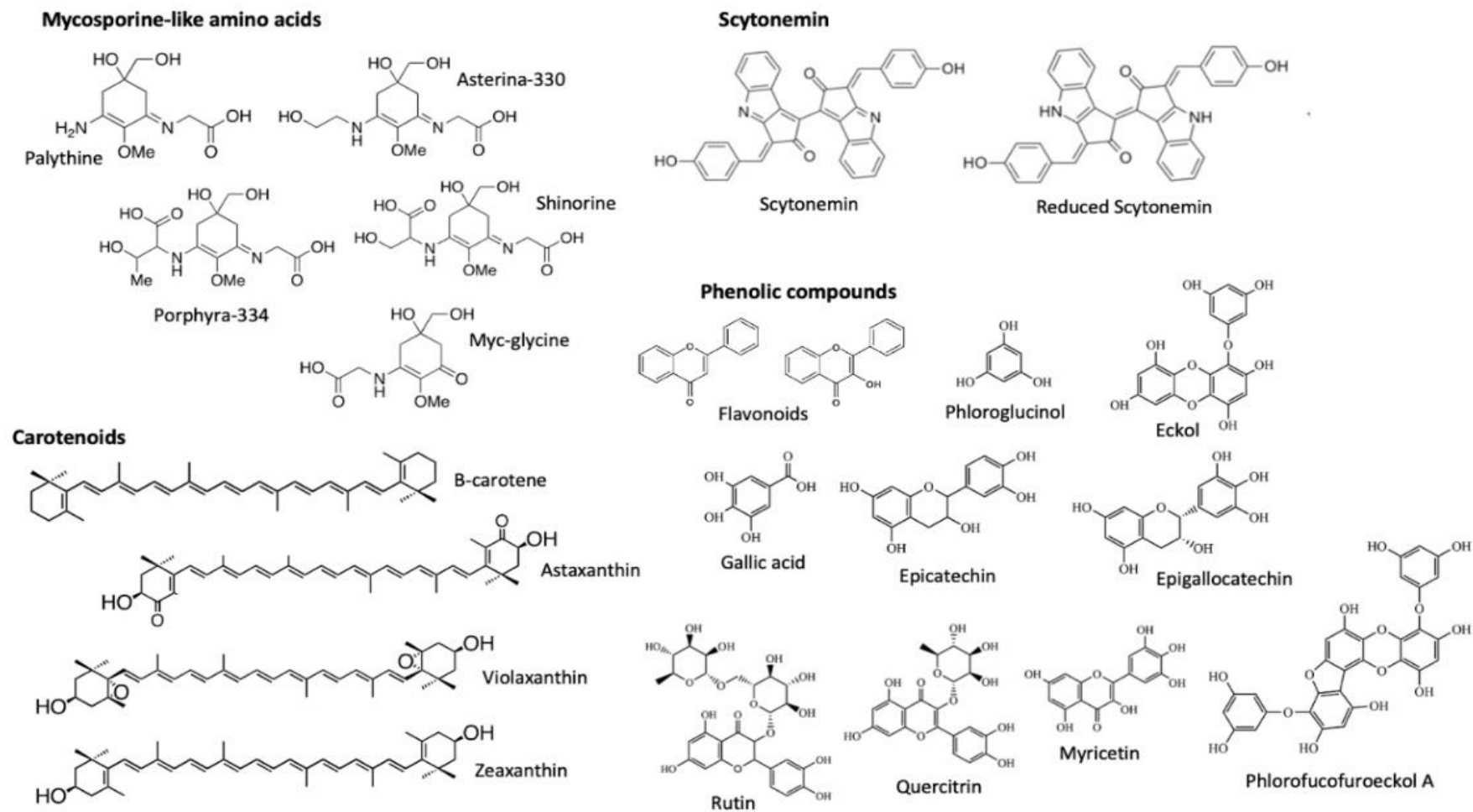


Figure I. 16. Molecular structures of some bioactive compounds found in algae, considering photoprotective roles: mycosporine-like amino acids, scytonemin, carotenoids and phenolic compounds (Rastogi *et al.*, 2015; Takaichi *et al.*, 2016; Santos *et al.*, 2019; Cotas *et al.*, 2020).

6. Seaweed aquaculture: the case of *Porphyra sensu lato*

The global aquaculture production, both marine and freshwater, have increased in the last years ranging from 36 Mt in 1997 to 112 Mt in 2017 with a great contribution of algae, which reached 35 Mt in 2017 (Naylor *et al.*, 2021). According to the FAO, the global seaweed production has increased from 10.5 Mt in 2000 to 35 Mt in 2020. In 2019, 97 % of the total production come from aquaculture. There are five genera that account for more than 95 % of total cultivated seaweed: *Laminaria/Saccharina* (35.4 %), *Kappaphycus/Eucheuma* (33.5 %), *Gracilaria* (10.5 %), *Porphyra/Pyropia* (8.6 %) and *Undaria* (7.4 %). There are significant differences in regional contribution to seaweed production, being Asian countries the greatest producers with more than 95 % (57 % on China and 28 % in Indonesia) (FAO and WHO, 2022) (Figure I. 17). In addition, seaweed aquaculture can offer several opportunities to mitigate and adapt to climate change. Via mitigation, seaweeds present a high carbon sequestration capacity (e.g. they can compensate the fossil fuels emissions of 1500 tons CO₂ Km⁻² year⁻¹), and can be a source of food with a reduce CO₂ footprint. Via adaptation, seaweeds can protect the shoreline by dissipation of wave energy or can ameliorate ocean acidification by increasing the pH through photosynthesis (Duarte *et al.*, 2017). Several species also present a high nutrients biofiltration capacity, disinishing the risk of eutrophication of natural ecosystems (e.g. *Ulva* sp and *Gracilaria* sp.) (Figueroa *et al.*, 2010, 2022; Abreu *et al.*, 2011a; Massocato *et al.*, 2022).

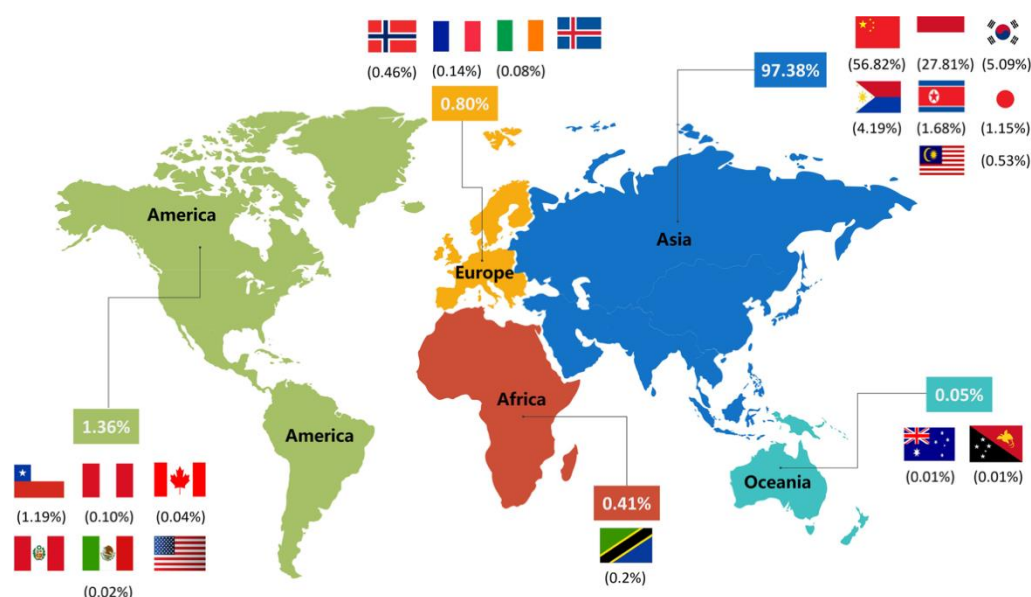


Figure I. 17. Production of seaweeds around the world in term of percentage (Zhang *et al.*, 2022).

Among seaweeds, red ones (Rhodophyta) present the widest diversity of species (around 6500) with the highest content of bioactive compounds (Holdt and Kraan, 2011; Stengel *et al.*, 2011; Michalak and Chojnacka, 2015; Stengel and Connan, 2015; Aziz *et al.*, 2021). Among them, the species of *Porphyra sensu lato* and the genus *Gracilaria* highlight in this thesis.

Porphyra sensu lato belong to the Bangiaceae family (class Bangiophyceae) and can be named as bladed bangiales. They are globally distributed in intertidal zones, although they are more abundant in cold temperate waters. They present monostromatic layers and are characterized by their elastic thalli and high resistance to desiccation, as they can spend several hours out of the water. Traditionally, two genera were recognized in the Bangiaceae family, the genus *Bangia* (filamentous bangiales) and *Porphyra* (foliose or bladed bangiales). Later, Sutherland *et al.* (2011), using molecular analyses, described fifteen genera of Bangiales, seven filamentous and eight foliaceous (e.g. *Porphyra*, *Boreophyllum*, *Wildemania* or *Pyropia*). Recently, Yang *et al.* (2020) through a global phylogenetic analysis redefined the genus *Pyropia* and separated it in *Phycocalidia*, *Neoporphyra*, *Neopyropia*, *Porphyrella* and *Pyropia*. These species present a marked seasonal pattern with a life cycle that alternate two phases: a macroscopic foliose gametophyte (n) and a microscopic filamentous sporophyte (2n), known as Conchocelis. The macroscopic gametophyte can be observed during winter, whereas the arrival of the summer (long photoperiod and warm temperatures) promotes the gametic reproduction. In this procedure, spermatia (male gametes) fertilizes the carpogonia (female gametes), forming diploid structures (zygotes) which will develop into small diploid cells groups, carposporophytic ones, which will produce and release diploid spores (zygotospores). These spores will originate the filamentous conchocelis phase, normally attached to mollusk shells. During the beginning of the winter, the filamentous phase will develop conchosporangia, where meiosis occurs, releasing haploid spores (conchospores), which will develop into a new foliose gametophytic thalli. *Porphyra* can also present asexual reproduction during stress conditions, in which vegetative cells directly generate spores (named archeospores) that form a new laminar thallus (Figure I. 18). The conchocelis phase is considered a way to avoid the unfavorable environmental conditions during summer (high temperature, radiation and grazing pressure) (Blouin *et al.*, 2011; López-Vivas *et al.*, 2015; Pereira *et al.*, 2019; Knoop *et al.*, 2020). Moreover, the conchocelis phase is where meiosis occurs, it means that this phase is necessary to increase the genetic variability.

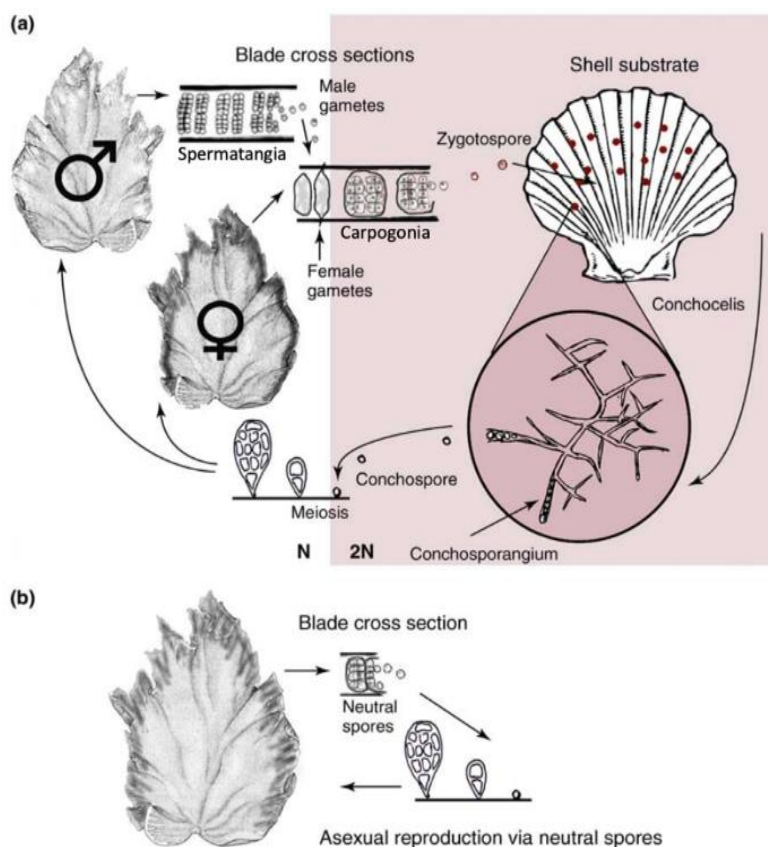


Figure I. 18. *Porphyra umbilicalis* life cycle showing the different strategies of species thalli propagation, including sexual (a), where male gametes fertilize the carpogonia forming zygospores (2n) which develop the conchocelis phase (in pink color). After meiosis, the conchosporangia release conchospores (n) that form new thallus; and the asexual reproduction (b), in which vegetative cells produce spores that can form a new thalli (Blouin *et al.*, 2011).

Porphyra sensu lato species present a great biotechnological interest. They present high resistance and resilience to environmental stress conditions, related to the accumulation of interesting secondary metabolites with potential applications. In spite of their complex life cycle, these algae can be culture both in land systems and on-shore farms, being one of the most cultivated species in the world. The species of *Porphyra sensu lato* that are usually culture are: *Pyropia yezoensis*, *Pyropia tenera* and *Pyropia haitanensis*. The cultivation technique of these species involves four major steps: 1) culture of conchocelis, 2) Seeding of conchospores in culture nets, 3) Transfer of nets to the sea, and 4) Harvesting. The culture biomass is mainly use for human food (worldwide known as Nori), although other applications could be developed due to the high content of high-added value compounds (e.g. antioxidant, anti-aging, photoprotective or

immunomodulatory) (Sahoo and Yarish, 2005; Blouin *et al.*, 2011; Groenigen *et al.*, 2022).

The genus *Gracilaria* (class Florideophyceae) is one of the major genera of Rhodophyta (199 species). They can be found from tropical to temperate latitudes, with a higher presence in tropical regions. Generally, they inhabit the intertidal and subtidal zones, between 0.5 to 10 m depth (Guiry and Guiry, 2023). Morphologically, the thallus of *Gracilaria* can be cylindrical, compressed or bladed, and normally are irregularly branched with a bushy appearance. It presents a triphasic life cycle. There are male and female gametophytes (n), that appear identical without the help of a microscope. After fertilization, cystocarps appear in the female gametophyte (now known as carposporophyte). The cystocarp release a large number of carpospores ($2n$) that will rise the tetrasporophyte ($2n$). The diploid tetrasporophyte, also morphologically similar to the haploid gametophytes, produce tetraspores through meiosis that will form both female and male gametophytes (Figure I. 19) (Mantri *et al.*, 2010).

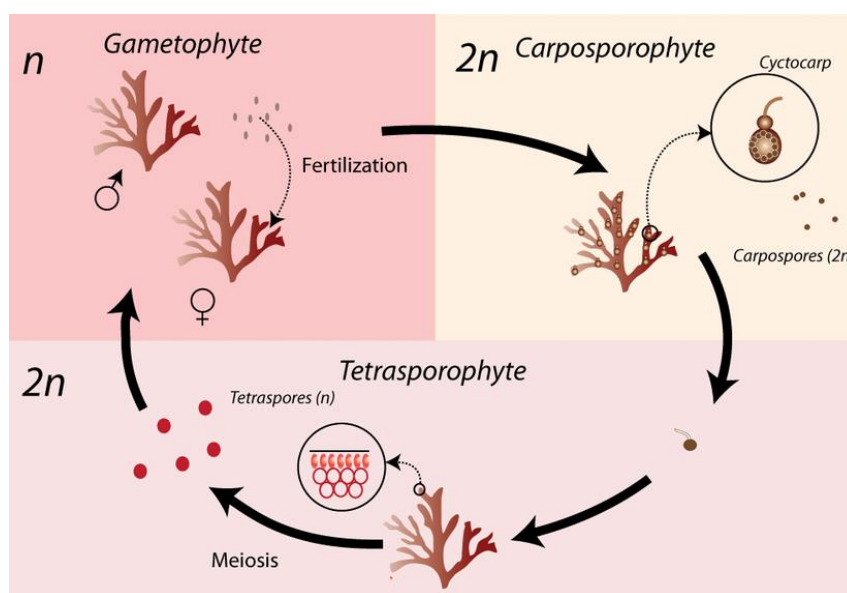


Figure I. 19. Summary of a *Gracilaria* life cycle. The male gametophyte (n) release spermatia (n) and fertilize the female gametophyte forming the carposporophyte ($2n$). Cystocarps release carpospores ($2n$) that will form the tetrasporophyte, that produce tetraspores (n) that will form male and female gametophytes (Bast, 2016).

Gracilaria species have been widely exploited by humans and have a great economic value. *Gracilaria* can be culture in three different ways: open water, ponds or tanks culture (including polyculture or IMTA systems). It is normally use the rope

cultivation technique, in which two approaches can be follow: The insertion of vegetative thalli within the ropes or the use of suspension spore cultivation to seed the nets (Sahoo and Yarish, 2005). These species are mainly use for phycocoloid extraction (i.e. agar) and human consumption (e.g. in Japan it is commonly known as oogonori). As *Porphyra sensu lato*, *Gracilaria* also present a wide tolerance of environmental stresses (e.g. euryhaline and eurytherme species have been reported), and can accumulate secondary metabolites with different potential application. Torres *et al.* (2019b) reviewed the chemical diversity and potential bioactivities or this genus, observing a considerable biological potential, mainly related with sulphated agarans, MAAs and biliproteins.

7. Blue cosmeceutics

Blue cosmeceutic is a concept that joins **Cosmeceutic**, i.e. the use of bioactive compounds in cosmetic products, that apport beneficial effects for the skin like antioxidant, anti-aging or anti-inflammatory; and **Blue** in two ways: (1) Blue biotechnology, i.e biotechnology of aquatic organisms and (2) Blue development, based on the principle of the blue, circular and ecological economy and the blue growth (Pauli, 2010; Smith-Godfrey, 2016; Eikeset *et al.*, 2018). Other approaches that use the blue concept are: blue revolution, blue transformation, blue transition, blue justice or blue acceleration (Nahuelhual *et al.*, 2019; Jouffray *et al.*, 2020; Bennett *et al.*, 2021; FAO, 2022a).

Since the last decade, the European policies are favoring the transition to a more sustainable and blue economy through different actions: The EU Bioeconomy Strategy aims to implement a sustainable and circular economy in Europe (adopted in 2012 and updated in 2018) (EC, 2018). The EU Blue Growth Strategy supports a sustainable growth in the marine and maritime sector, focusing on the potential of these sectors to contribute to a sustainable economy, create new jobs and promote innovation (EC COM 494, 2012). Recently, the European Green Deal action plan calls for a green transformation of our economy and societies, through different initiatives, with the final goal of reaching climate neutrality in 2050. Some of the initiatives are: reducing emissions from industry, transport and other sectors; promoting circular economy and sustainable consumption; creating a sustainable food system through the Farm to Fork strategy, preserving biodiversity or financing the green transition (EC COM 640, 2019). In addition, the Recovery Plan for Europe sets out to boost the green and digital transitions and make Europe's economy fairer, more resilient and more sustainable for

future generations (EC COM 442, 2020). The develop of a sustainable blue economy in Europe (EC COM 240, 2021) can help to achieve all these challenges. The blue economy sector, including fisheries, aquaculture, coastal tourism, or maritime transport, provides 4.5 million direct jobs, many in regions where there are few alternatives. This sector is a great segment of our economy, which over the past decade has taken significant steps to modernise and diversify (e.g. renewable energy, biotechnology, desalinization), thus providing new prospects and creating jobs. Reducing the impacts of human activities on the sea is a collective responsibility, and the blue economy can play a vital role in many aspects: (1) reduce the carbon footprint through the develop of renewable energies, (2) switch to a circular economy and reduce the waste, (3) preserve the biodiversity, (4) improve coastal resilience, (5) ensure a sustainable food production, and (6) improve management of the sea.

Aquaculture can be a valuable, low-impact source of food and feed when it is managed in a sustainable way. EU aquaculture meets high standards in terms of product quality and animal health, but there is still margin for improvement in terms of diversification, competitiveness, and environmental performance. The new strategic guidelines for a more sustainable and competitive aquaculture in EU (EC COM 263, 2021) supports circular practices to ensure good environmental performance (e.g. environmental monitoring of sites and waste management) and sets the need to promote the farming of algae, as way to contribute to the European Green Deal and sustainable Blue Economy in terms of decarbonisation, zero pollution, circularity or preservation of biodiversity. The FAO also proposes a roadmap 2022-2030 for the transformation to a more efficient, inclusive, resilient, and sustainable aquatic food system, named “Blue Transformation”, for a better production, nutrition and environment (FAO, 2022a).

Tigchelaar *et al.* (2022) reported that blue foods (obtained from aquatic environments, e.g. fish, invertebrates or algae) play an important role in nutrition security, economy, livelihoods and culture of many coastal communities. They generally present a high nutritional value (e.g. micronutrients or fatty acids), and generate lower emissions and impacts than many terrestrial animal-source foods. The European Green Deal and the Sustainable Blue Economy also support the blue foods as a protein source with a low-carbon footprint, specifically the Farm to Fork strategy highlight the important role of algae as a new source of alternative protein for a sustainable food system and global food security.

In addition to food, algae present other potential commercial applications: pharmaceuticals, bio-based plastics, cosmetics, plant fertilizers, biofuels or bioremediation. Recently, the European Commission published a communication entitled “Towards a Strong and Sustainable EU Algae sector”, in which recognize the importance of promoting the farming of algae (both macro and microalgae) as a renewable resource of raw materials, and mentioned that: “Although currently small, the European algae sector has the potential to become a significant part of the EU Blue Bioeconomy” (EC COM 592, 2022).

8. Justification of the work

In this thesis, different steps have been followed for the development of cosmeceutical products based on photosynthetic organism extracts. In addition, blue approaches have been followed: the use of aquatic organisms, such as cyanobacteria and algae (blue biotechnology), which can be obtained through sustainable ways, e.g. IMTA systems, and can provide benefits for the ecosystem (e.g. decarbonization, low pollution or climate change mitigation) (Blue development).

The research group “Fotobiología y Biotecnología de Organismos acuáticos” (FYBOA, RNM-295) have been working in the physiology and biotechnology applications of algae during the last 20 years. Red algae (mainly *Porphyra sensu lato* and Gracilariales) had been largely investigated for their high content of MAAs and their culture possibilities. The research group presents 18 patents about MAAs, has published about 138 articles and 7 doctoral Theses on ecophysiology, photobiology and biotechnology of algae, especially on UV photoprotectors; and have developed a wide collaboration with the Federal University of Santa Catarina (Florianópolis, Brasil). In this Thesis, further research on the obtention and application of UV photoprotectors coming from red macroalgae, and its combination with other natural compounds, has been developed.

The diversity of photoprotective molecules among photosynthetic organisms is very high (Figure I. 20). Polyphenols are present in most of them (e.g. plants normally present flavonoids, and algae can present phlorotannins, bromophenols and flavonoids), whereas other molecules are characteristic of certain groups (e.g. MAAs are mainly found in cyanobacteria and red macroalgae). Human skin also presents UV photoprotectors such as melanin, urocanic acid or carotenoids, but they are not enough

for a full protection of the skin against damage effect of solar exposure. Thus, the use of broad band UV-filters is necessary to increase the photoprotection of the skin against the negative effects of solar exposure. One of the proposals of this thesis was to develop new natural UV-filters that reduce the impact on the environment and human health, since these natural biocompounds are biodegradable and they can be produced following sustainable approaches based in the blue and circular economy.

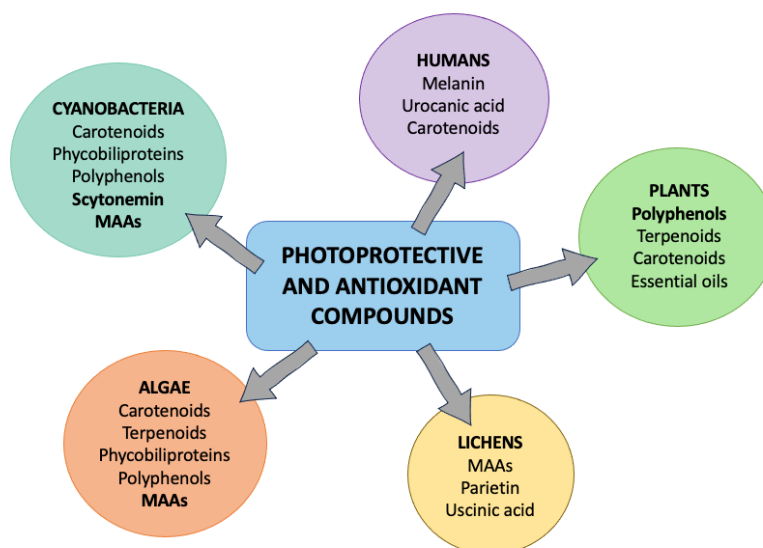


Figure I. 20. The diversity of UV screen substances with antioxidant capacity in different organisms including humans.

The different steps followed in this thesis are (Figure I. 21):

- 1. Selection of species** that present high content of bioactive compounds with potential application in the cosmeceutical sector (e.g. antioxidants and UV-screen properties), and with high biomass availability. The main bioactive molecules analyzed in this thesis are biliproteins, polyphenols and MAAs. The biomass can be obtained from high productive areas or from culture under IMTA systems. This selection has been done with the results obtained in this thesis and based on previous reports (Huovinen *et al.*, 2004; Figueroa *et al.*, 2006, 2012, 2022; Álvarez-Gómez *et al.*, 2016; Briani *et al.*, 2018; Navarro *et al.*, 2018; Schneider *et al.*, 2020b; Ashkenazi *et al.*, 2022; Moreira *et al.*, 2022)
- 2. Experimental approaches** to increase the content of bioactive compounds in selected species. Photobiological or stressed treatments can be done during culture to modify the internal biochemical compositions. Light treatments can present both

photosynthetic and photomorphogenic responses. Other stress factors like nutrients availability or emersion conditions can influence the accumulation of certain compounds. The experiments were design taking into account previous reports (Figueroa *et al.*, 1995; Korbee *et al.*, 2005b, 2005c; Barufi *et al.*, 2011; Álvarez-Gómez *et al.*, 2017, 2019b; Schneider *et al.*, 2022).

3. Extraction, identification and characterization of bioactive compounds.

Extracts are done in solvents compatible with the cosmetic application (e.g. water or ethanol), and different *in vitro* bioassays related to antioxidant, anti-aging and photoprotective are performed. (de la Coba *et al.*, 2009a; Chaves-Peña *et al.*, 2020; Castro-Varela *et al.*, 2022; Moreira *et al.*, 2022).

4. Isolation of MAAs by using more efficient and advance techniques than previous isolation in the research group (de la Coba *et al.*, 2009b)

5. Design of cosmeceutical products with antioxidant and photoprotective properties based on photosynthetic organism extracts. Evaluation of the efficacy (solar protection factor and other biological effects), photo-and thermostability of the creams, following different approaches than previous reports trying to obtain better results (Álvarez-Gómez, 2017; de la Coba *et al.*, 2019)

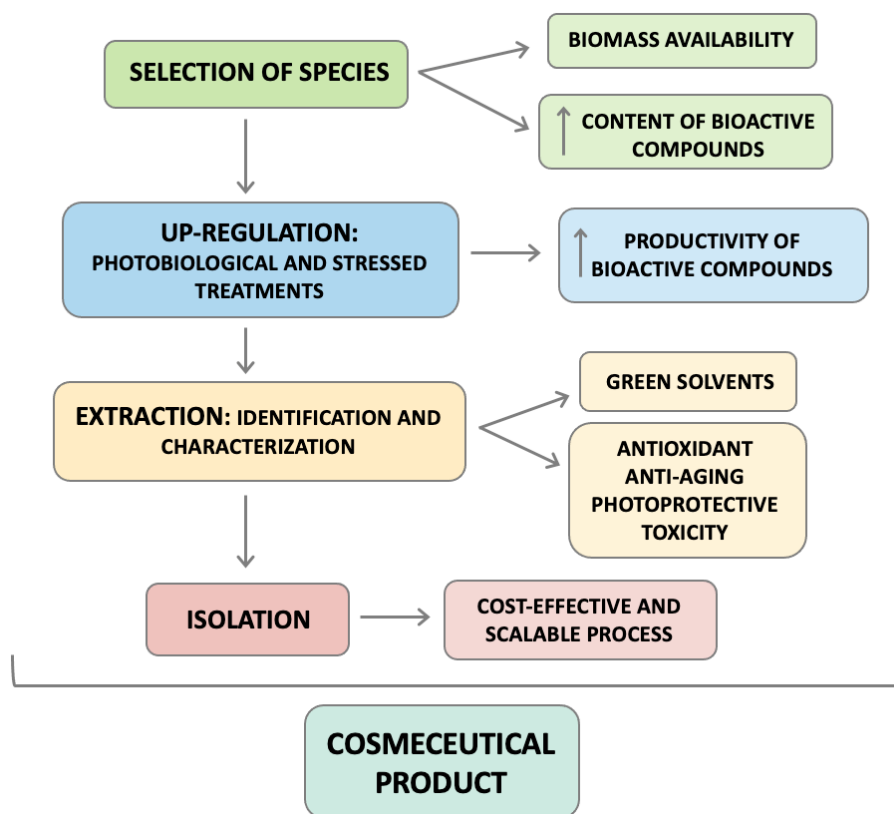


Figure I. 21. Different workpackges followed in this thesis for the development of cosmeceutical product with UV-screen and antioxidant properties based on photosynthetic organism.

HYPOTHESIS

The following hypothesis were defined for this thesis:

- Photosynthetic organisms (algae, cyanobacteria and plants) accumulate antioxidant and UV-screen compounds (e.g. polyphenols, MAAs and/or scytonemin), as a photoprotection mechanism, which are natural ingredients for cosmeceutical applications due to their beneficial properties for the skin.
- Intertidal red algae, as *Porphyra sensu lato* species, that live exposed to a very stressful environment, present a high content of UV-screen and antioxidant compounds (e.g. MAAs and polyphenols).
- Stressed conditions (UV radiation, nutrients availability or emersion conditions) affect the photosynthesis, biochemical composition and cellular structure of algae.
- The combination of UV radiation and high nutrients availability increases the amount of MAAs.
- Emersion condition in *Porphyra sensu lato* species provokes an increase of photoprotective and antioxidant compounds (e.g. MAAs and phenolic compounds).
- The supplementation of UVA-Violet-Blue radiation during *Porphyra sensu lato* culture increases the synthetesis of MAAs and biliproteins.
- MAAs are isolated through an efficient, cost-effective and scalable technique as counter-current chromatography.
- Algae extracts and isolated MAAs present antioxidant, anti-aging and photoprotective properties, and no toxicity.
- The addition of algal extracts combined with other natural compounds in cosmetic products improves the photoprotection against skin erythema (SPF) and other biological effects, such photocarcinogenesis, immunosuppression or anti-aging.

OBJECTIVES

General

The search for new natural ingredients for cosmeceutic products from photosynthetic organisms (algae, cyanobacteria and plants) that can protect the skin against the UV-Blue radiation, as well as provide antioxidant and anti-aging properties.

Specific

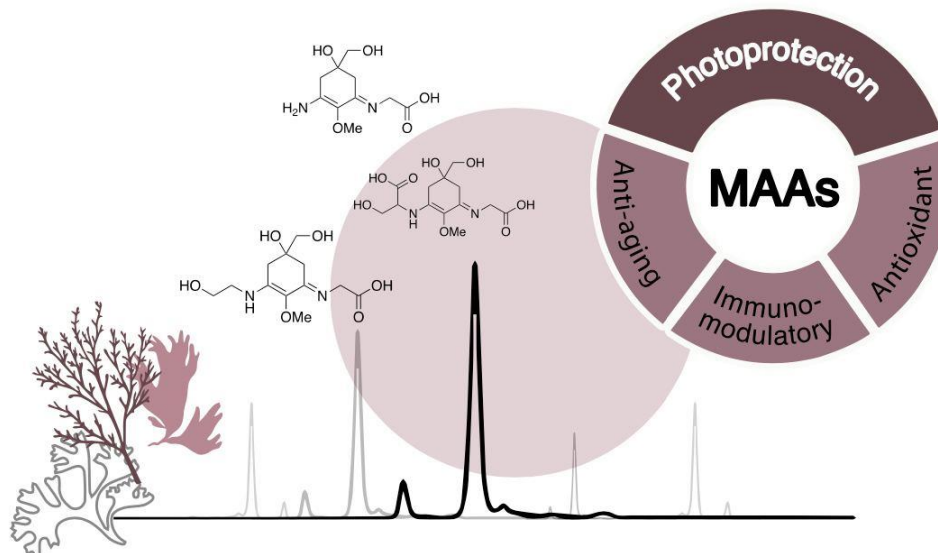
1. Identification of species (red algae, cyanobacteria and plants) with antioxidant and UV-absorbing compounds for the potential application in cosmeceutic products in a screening investigation.
2. Study the physiological and biochemical responses of two red macroalgae species (*Gracilaria armata* and *Neopyropia leucosticta*) against different stress factors (light quality, nutrients availability and emersion), to determine possible treatments that can increase the accumulation of interesting compounds (e.g. MAAs, biliproteins or phenolic compounds).
3. Isolation of MAAs through and efficient, cost-effective and scalable technology, for the obtention of natural photoprotectors that can be used in sunscreens as a natural alternative to the current UV filters.
4. Determine *in vitro* antioxidant, anti-aging potential and cellular toxicity of *Porphyra sensu lato* species, that can provide additional beneficial properties for the skin.
5. Inclusion of aqueous *Pyropia columbina* extracts in creams to determine solar protection factor related to erythema (SPF) and Biological effective protection factors (BEPFs), as well as photo- and thermostability of the creams.
6. Design cosmeceutic products with high SPF (30), antioxidant and anti-aging properties, based on algae and plants extracts and physical filters.



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CHAPTER 1

Mycosporine-Like Amino Acids from Red Macroalgae: UV-Photoprotectors with Potential Cosmeceutical Applications



A review published in Applied Science

Vega, J., Schneider, G., Moreira, B.R., Herrera, C., Bonomi-Barufi, J., and Figueroa, F.L. (2021). Mycosporine-Like Amino Acids from Red Macroalgae: UV-Photoprotectors with Potential Cosmeceutical Applications. *Applied Sciences* 11, 5112. <https://doi.org/10.3390/APP11115112>



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Mycosporine-Like Amino Acids from Red Macroalgae: UV-Photoprotectors with Potential Cosmeceutical Applications

Abstract

Macroalgae belong to a diverse group of organisms that could be exploited for biomolecule application. Among the biocompounds found in this group, mycosporine-like amino acids (MAAs) are highlighted mainly due to their photoprotection, antioxidant properties, and high photo and thermo-stability, which are attractive characteristics for the development of cosmeceutical products. Therefore, here we revise published data about MAAs, including their biosynthesis, biomass production, extraction, characterization, identification, purification, and bioactivities. MAAs can be found in many algae species, but the highest concentrations are found in red macroalgae, mainly in the order Bangiales, as *Porphyra* spp. In addition to the species, the content of MAAs can vary depending on environmental factors, of which solar radiation and nitrogen availability are the most influential. MAAs can confer photoprotection due to their capacity to absorb ultraviolet radiation or reduce the impact of free radicals on cells, among other properties. To extract these compounds, different approaches can be used. The efficiency of these methods can be evaluated with characterization and identification using high performance liquid chromatography (HPLC), associated with other apparatus such as mass spectrometry (MS) and nuclear magnetic resonance (NMR). Therefore, the data presented in this review allow a broad comprehension of MAAs and show perspectives for their inclusion in cosmeceutical products.

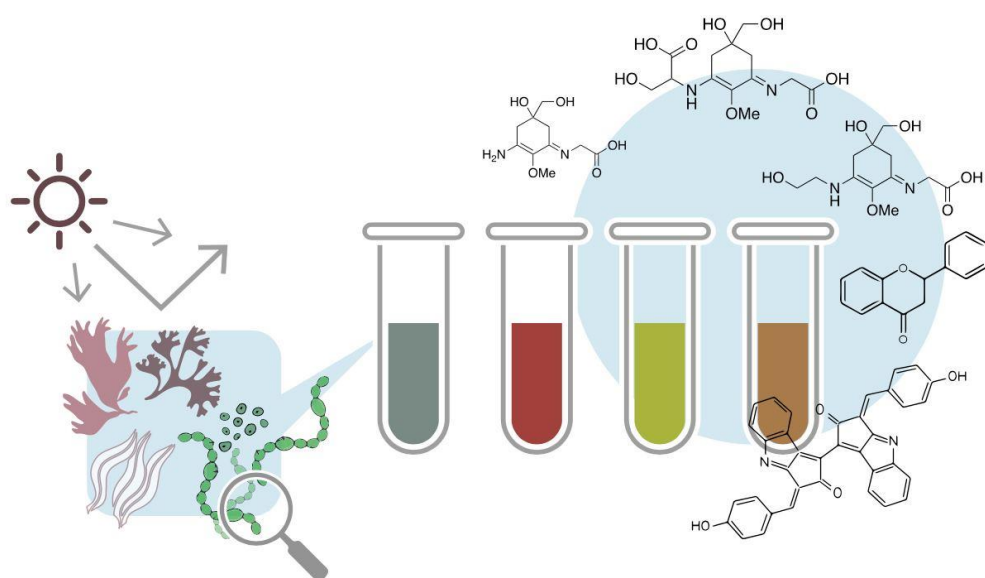
Keywords: antioxidants; cosmeceutic; extractions; integrated multitrophic aquaculture (IMTA), mycosporine-like amino acids (MAAs), purification; red macroalgae



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CHAPTER 2

Cyanobacteria and Red Macroalgae as Potential Sources of Antioxidants and UV Radiation-Absorbing Compounds for Cosmeceutical Applications



Article published in *Marine Drugs*

Vega, J., Bonomi-Barufi, J., Gómez-Pinchetti, J.L., and Figueroa, F.L. (2020). Cyanobacteria and Red Macroalgae as Potential Sources of Antioxidants and UV Radiation-Absorbing Compounds for Cosmeceutical Applications. *Mar Drugs* 18, 659. <https://doi.org/10.3390/md18120659>



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Cyanobacteria and Red Macroalgae as Potential Sources of Antioxidants and UV Radiation-Absorbing Compounds for Cosmeceutical Applications

Abstract

In recent years, research on natural products has gained considerable attention, particularly in the cosmetic industry, which is looking for new bio-active and biodegradable molecules. In this study, cosmetic properties of cyanobacteria and red macroalgae were analyzed. The extractions were conducted in different solvents (water, ethanol and two combinations of water:ethanol). The main molecules with antioxidant and photoprotective capacity were mycosporine-like amino acids (MAAs), scytonemin and phenolic compounds. The highest contents of scytonemin (only present in cyanobacteria) were observed in *Scytonema* sp. (BEA 1603B) and *Lyngbya* sp. (BEA 1328B). The highest concentrations of MAAs were found in the red macroalgae *Porphyra umbilicalis*, *Gelidium corneum* and *Osmundea pinnatifida* and in the cyanobacterium *Lyngbya* sp. *Scytonema* sp. was the unique species that presented an MAA with maximum absorption in the UV-B band, being identified as mycosporine-glutaminol for the first time in this species. The highest content of polyphenols was observed in *Scytonema* sp. and *P. umbilicalis*. Water was the best extraction solvent for MAAs and phenols, whereas scytonemin was better extracted in a less polar solvent such as ethanol:dH₂O (4:1). Cyanobacterium extracts presented higher antioxidant activity than those of red macroalgae. Positive correlations of antioxidant activity with different molecules, especially polyphenols, biliproteins and MAAs, were observed. Hydroethanolic extracts of some species incorporated in creams showed an increase in the photoprotection capacity in comparison with the base cream. Extracts of these organisms could be used as natural photoprotectors improving the diversity of sunscreens. The combination of different extracts enriched in scytonemin and MAAs could be useful to design broad-band natural UV-screen cosmeceutical products.

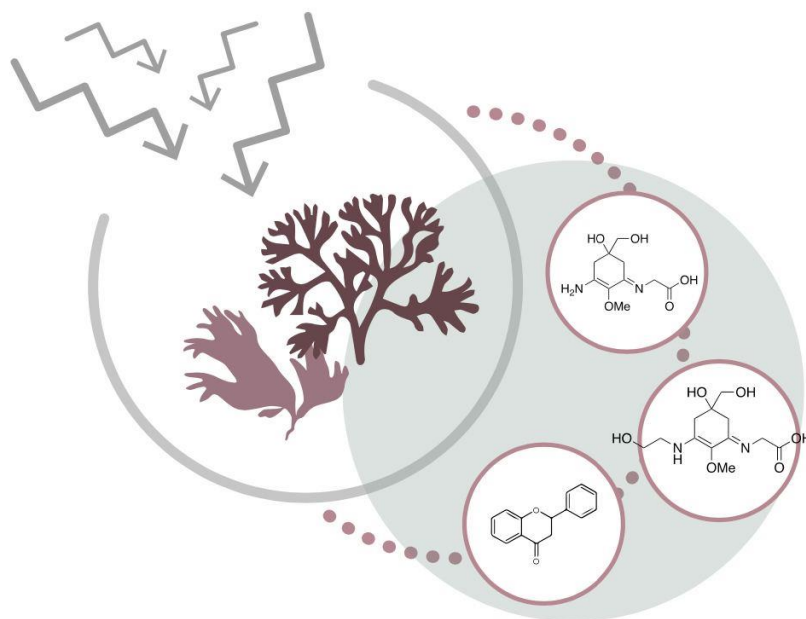
Keywords: antioxidant activity; cyanobacteria; mycosporine-like amino acids; scytonemin; photoprotection; red macroalgae; UV-screen



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CHAPTER 3

Physiological approaches to increase UV-absorbing compounds in red algae



This chapter includes two experimental approaches in two red algae species: *Gracilaria armata* and *Neopyropia leucosticta*.



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SUBCHAPTER 3.1

**Effects of ultraviolet radiation on photosynthesis,
biochemical composition, and antioxidant
capacity of *Gracilaria armata* (Gracilariales,
Rhodophyta)**



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Effects of ultraviolet radiation on photosynthesis, biochemical composition, and antioxidant capacity of *Gracilaria armata* (Gracilariales, Rhodophyta)

Abstract

Ultraviolet radiation can provoke important damages at molecular, cellular, or physiological levels in aquatic organism, mainly in macroalgae from the intertidal zone. These species have developed protection mechanism such as the accumulation of antioxidant and photoprotective compounds. Gracilariales are well-known for their phycocoloids contents (gelling agents), nutritional values (commonly known as ogonori) and the presence of bioactive compounds (biliproteins, polyphenols or mycosporine-like amino acids), as well as they are potentially cultivable. *G. armata* were cultured under different radiation treatments (only PAR, and PAR + UV-A (PA) and PAR+UVA+UV-B (PAB)) and compared with specimens from their natural environment. The PAB treatment reduced the growth capacity and the accumulation of certain compounds (biliproteins and polyphenols). The electron transport rate (ETR) as estimator of photosynthetic capacity was slightly affected by the PAB treatment, whereas the non-photochemical quenching (NPQ) increased under this treatment indicating photoprotection strategies. The mycosporine-like amino acids (MAAs) content were highest in algae from the environment. Under laboratory-controlled conditions the highest MAAs content was observed under P treatment. The antioxidant capacity correlated with some of the internal compounds and decrease in the PAB treatment. *G. armata* increased the NPQ under high UVR conditions, whereas it was not able to increase photoprotective compounds (polyphenols or MAAs) probably to photodamage effects by UV radiation.

Keywords: phycobiliproteins, Gracilariales, growth, mycosporine-like amino acids, photosynthesis

1. Introduction

Ultraviolet radiation (UVR) represents about 10 % of the total radiation emitted by the sun. The atmosphere absorbs most of the UVR, only UV-A (320-400nm) and a small part of UV-B (290-320 nm) can reach the Earth surface. This radiation, mainly UV-B, is extremely energetic due to its shorter wavelength and can provoke important damages to aquatic organisms, especially to macroalgae from the intertidal zone, which receive high inputs of UVR, mainly during daily periods when algae are uncovered by seawater. UVR can provoke negative effects at molecular, cellular, physiological or even ecological levels (Bischof *et al.*, 2006; Häder *et al.*, 2007; Schmidt *et al.*, 2009; Figueroa *et al.*, 2014b; Bonomi-Barufi *et al.*, 2020). DNA is a very UV-sensitive molecule, since it absorbs UV-B radiation, and is directly subjected to mutations like thymine dimers (e.g. cyclobutane pyrimidin dimers (CPDs) or 6-4 photoproducts). DNA can also be affected indirectly by degradative activity of reactive oxygen species (ROS) generated by UV-A radiation. Damage in the DNA can inhibit replication or affect genetic regulation or expression (Cadet *et al.*, 2005; Häder and Sinha, 2005; Rastogi *et al.*, 2010). Other molecules, as proteins, pigments or lipids, can be also affected by UVR, e.g. bonds between amino acids can be cleaved by UVR, affecting the appropriate protein folding and consequently influencing in their proper functions; furthermore, lipids can be degraded, through peroxidation due to the production of oxygen radicals by UVR, affecting the membrane structure of the cells. As consequence of the above-mentioned negative effects, several physiological processes are also affected, such as photosynthesis, survival, nutrient uptake, growth or sporulation (Michler *et al.*, 2002; Karsten, 2008; Kataria *et al.*, 2014). Despite that, some authors have observed positive effects of UV-A radiation on photosynthesis or growth in some macroalgae (Altamirano *et al.*, 2000; Gao and Xu, 2008; Ghedifa *et al.*, 2021).

Macroalgae have developed different mechanism to protect themselves from the harsh environment where they live, with a high exposure time to UVR. They present photorepair mechanism like photolyases, activated by radiation between 350-450 nm to reverse the formation of CPDs, and efficient antioxidant enzymes to protect against ROS, such as superoxide dismutase or catalase. They can also accumulate molecules with antioxidant and UV-absorbing properties like carotenoids, polyphenols or mycosporine-like amino acids (MAAs) (Poll *et al.*, 2002; Karsten, 2008; Lalegerie *et al.*, 2020a). The synthesis of these molecules is normally regulated by the exposure time to UVR and by its intensity and quality. For example, Schmitz *et al.* (2018) observed a higher content of

UV-absorbing compounds in species collected in tropical regions (with high solar irradiance), than in southern regions of Brazil, or Álvarez-Gómez *et al.* (2019b) observed a general increment of antioxidant activity, polyphenols and MAAs in three different red algae cultured under UVR.

Species from the genus *Gracilaria* (> 150 species) have been cultured for centuries in Asian countries (Santelices, 2014) and are well-known for their phycocolloids contents. They are the main source of agar-agar worldwide, which is used as gelling in food or cosmetics (Porse and Rudolph, 2017). In addition, *Gracilaria* spp. also have been used as human food or traditional medicine in many parts of the world. They are mainly consumed in Thailand, Hawaii, Japan or Korea (e.g. in Japan it is known as ogonori, and it is normally consumed in salads). More recently, these species have been tested for their used as feed for abalone, shrimps or fishes (Marinho-Soriano *et al.*, 2007; Silva *et al.*, 2015; Bansemer *et al.*, 2016; Vizcaíno *et al.*, 2016), and have gained an increasing interest as biofilter in integrated multitrophic aquaculture (IMTA) systems, since they present a high removal capacity of inorganic nutrients (Abreu *et al.*, 2011a; Figueroa *et al.*, 2022). Gracilariales also presented mycosporine-like amino acids (MAAs) in their internal composition, as most red algae species. MAAs are nitrogenous compounds mainly involved in photoprotection. These small and water-soluble molecules present antioxidant and UV-absorbing properties, protecting the cells against the damage effect of high exposure time to UV radiation, among other stress factors. These molecules are considered potential natural UV-filters to be used in cosmetic products with other beneficial properties for the skin such as antioxidant, anti-aging, photoprotective or wound-healing (Wada *et al.*, 2015; Chrapusta *et al.*, 2017; Lawrence *et al.*, 2017; Torres *et al.*, 2019b; Vega *et al.*, 2021).

Gracilaria armata (C.Agardh) Greville 1830, previously known as *Sphaerococcus armatus* or *Gigartina armata*, is distributed in tropical and subtropical areas around the world e.g. Spain, Italy, Mexico, Brazil, Morocco or Tunisia (Guiry and Guiry, 2017), although it has been found little research about its physiology or applicability. Only one previous study was found using this species (there named as *Gigartina armata*), in which its carbonic anhydrase activity was characterized (Bowes, 1969). The species *G. armata* was defined by Gargiulo *et al.* (1992).

The aim of this study was to analyze the physiological plasticity (photosynthesis and biochemical composition) of *G. armata* against variable radiation conditions. Firstly, algae collected from the environment were acclimated to laboratory-controlled conditions

(without UVR). After that, samples were exposed during one week to three different radiation treatments, with or without UVR. Effects of radiation variability were assessed to the intertidal *G. armata* measuring the photosynthetic activity using *in vivo* chlorophyll a fluorescence of photosystem (PS) II and the biochemical composition through different spectrophotometric methods.

2. **Material and methods**

2.1. **Biological material**

Gracilaria armata was collected in La Araña (Málaga, Spain; 36°42'41"N, 4°19'33"W) in March 2019. Algae were brought to the laboratory in a portable fridge and cleaned from debris and epiphytes. Part of the algal biomass was frozen and freeze dried (Cryodos, Telstar, Spain) to conduct further biochemical analyses described below, while other part was inserted in cultivation conditions at the laboratory. Algae were maintained in enriched seawater Provasoli medium (Provasoli 1968, Andersen 2005), renewed weekly, and under 50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (white LED light, 5000K, 54W, NU-8416, Nuovo), in a 12:12 light:dark cycle and with constant aeration. The salinity and temperature were 36 ± 2 and 25 ± 1 °C, respectively.

The molecular identification of the species was performed in the services of the University Institute of Marine Research (IN·MAR; University of Cadiz, Puerto Real, Spain). The DNA content was extracted using a commercial kit (DNeasy® Plant Mini Kit, QIAGEN, USA). The large subunit of the Ribulose Bisphosphate Carboxylate-Oxygenase (RuBisCO) (rbcL) was used as marker for the identification. After the amplification of the gene using the Polymerase Chain Reaction (PCR), the obtained DNA sequence were edited and ensembled using the software Genious 10.0.9 and the identification was made through the software BLASTn.

2.2. **Experimental design**

The experimental procedures can be divided in three main periods: 1) Collection of algal samples at the environmental conditions, 2) Acclimation period, and 3) Radiation shift experiment. Then, after collection, algae were maintained for one month (30 days) under laboratory culture conditions for acclimation (at general culture conditions mentioned in item 2.1) receiving only photosynthetic active radiation (PAR, $\lambda = 400\text{-}700$

nm) (Figure 3.1. 1: A). After this period, algae were transferred to different radiation treatments (radiation shift experiment), as follows: PAR (P), PAR supplemented with UV-A (PA, $\lambda = 320-700$ nm, Figure 3.1. 1: B) and with UV-A + UV-B (PAB, $\lambda = 280-700$ nm, Figure 3.1. 1: C). Spectra of the different treatments are shown in Figure 3.1. 1, as well as the spectrum of the solar radiation on a date (March 2016) seasonally similar to the collection date. PAR was obtained through a LED panel ($100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, white LED light, 5000K, 54W, NU-8416, Nuovo) and UV radiation by a fluorescence lamp (UVA340, Q-Lab Corporation, Canada). In order to obtain only UV-A radiation, an UV-B cut-off filter (130 LEE filters, Panavision Company, UK) was used. More information about the filters can be found in Oliveira *et al.* (2021). Irradiances and daily radiation doses used in this study are presented in Table 3.1. 1. The PAR irradiance used in the laboratory was measured using a spherical PAR sensor (US-SQS/L, Walz, Germany) connected to a data logger (LI-250A, LI-COR Biosciences, USA), whereas the UV irradiance were measured using a spectroradiometer (SMS-500, Sphere Optics, Germany). The sun spectrum and the dose received by the sun in a similar collection date (March 2016), was provided by the photobiology service of the Research Support Central Services (SCAI) at University of Málaga.

For the experiment of radiation shift, similar thalli pieces of PAR acclimated ($P_{\text{acclimated}}$) algae were distributed in 9 UV transparent polyvinyl methacrylate cylinders (Plexiglass XT-29080) (three replicates per treatment, 1 g of algae per flask) filled with 1 L of sea water. The experiment was conducted during 7 days, at 23 ± 1 °C, with a light:dark cycle of 12:12, a salinity of 36 ± 2 and with constant aeration. In the first day, Provasoli medium (containing $500 \mu\text{M NO}_3^-$ and $28 \mu\text{M PO}_4^{3-}$) was added to the seawater and in the third day a nitrate solution ($200 \mu\text{M NaNO}_3$) was added. At the end of the experiment, algae were weight, photographed (DMS 1000, Leica Microsystems, Germany) and freeze dried. Daily growth rate was calculated according to Lignell and Pedersen (Lignell and Pedersen 1989).

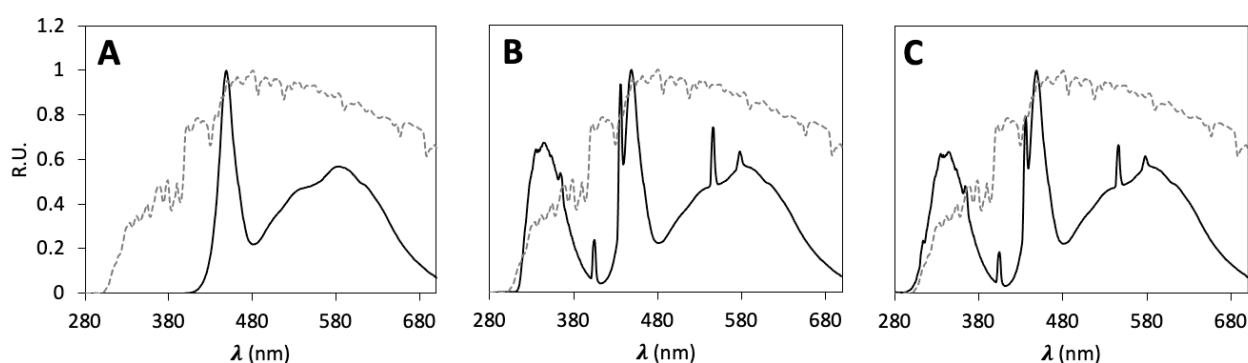


Figure 3.1. 1. Normalized spectra of the different radiation treatments received by *Gracilaria armata* at the laboratory: A) Pacclimated and PAR treatment (Pacclim and P); B) PAR + UV-A (PA); C) PAR + UV-A +UV-B (PAB). Dashed grey line represents the solar radiation received in the natural environment.

Table 3.1. 1. Irradiance values and daily radiation doses (kJ m^{-2}) received by algal thalli during the different radiation treatments. The values obtained in the natural environment (March 2016 in Malaga) are also shown (the intensity of solar radiation correspond to the maximal irradiance received during the central hours of the day (13:00)).

	Irradiances			Radiation doses (kJ m^{-2})			
	PAR ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	UV-A (W m^{-2})	UV-B (W m^{-2})	PAR	UV-A	UV-B	Total
Field / Sun	1700	46	2	6500	814	34	7348
P_{acclimated}	50	-	-	460	-	-	460
P	100	-	-	940	-	-	940
PA	100	7	-	940	302	-	1242
PAB	100	7.2	0.6	940	311	26	1277

2.3. Photosynthetic measurements

In vivo chlorophyll a fluorescence of photosystem II (PSII) was determined using a Mini-PAM-II (Walz, Germany). This analysis was performed to P_{acclimated} samples and to algae which were exposed to P, PA and PAB treatments at the end of the experimental period. The photosynthetic activity was measured *in situ* and *ex situ* as rapid light curves (RLCs).

During the *in situ* measurements, saturating pulses ($> 3000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) were applied to algal thalli during daily period (12:00), in order to quantify the steady-state fluorescence (F_t) and the maximal fluorescence (F_m' in light-acclimated samples).

The effective quantum yield ($Y(II)$) was quantified using the following formula: $Y(II) = (F_m' - F_t) / F_m'$. The electron transport rate (ETR) was calculated as $ETR = Y(II) \times E \times A \times F_{II}$, where E is the PAR irradiance ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$), A is the absorptance which was calculated as $A = 1 - (E_t / E_0)$ (E_t is the irradiance that pass through the thallus and E_0 is the emitted irradiance), and F_{II} is the fraction of chlorophyll a associated to PSII, being 0.15 the value in red algae (Johnsen and Sakshaug 2007).

Rapid light curves (RLCs) were also conducted. They represent how *in vivo* chlorophyll a fluorescence parameters vary with the increasing irradiance of the actinic red LED light using by the Mini-PAM-II (*ex situ* measurements). Firstly, algae were incubated in darkness for 15 min, to oxidize all the reaction centers before the beginning of the curves. Then, to begin the RLCs, the samples received a saturating pulse, which allowed the measurement of basal fluorescence (F_0) and maximal fluorescence (F_m), and then the calculation of maximum quantum yield, i.e. $F_v/F_m = (F_m - F_0) / F_m$. After that, algae were exposed during 20 seconds to twelve increasing irradiances (from 0 to 1500 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). Saturating pulses were performed after each incubation time, allowing the calculation of $Y(II)$, and consequently ETR (as described above in *in situ* data). From the values of RLCs, it was possible to obtain what was the *ex situ* ETR associated to the experimental PAR irradiance values at the RLC data (100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). Moreover, two different yield losses ($Y(NPQ) = (F_t / F_m') - (F_t / F_m)$ and $Y(NO) = F_t / F_m$) were quantified with fluorescence values obtained with RLCs, and the non-photochemical quenching (NPQ) was calculated as $Y(NPQ) / Y(NO)$. $Y(NO)$ and $Y(NPQ)$ at 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of actinic light were also determined from RLCs. The obtained ETR and NPQ vs. irradiance curves were fitted according to Eilers and Peeters (1988) and other parameters were obtained: photosynthetic efficiency of ETR (α_{ETR}), maximal ETR (ETR_{max}), saturated and optimal irradiance (E_k and E_{opt} , respectively) and maximal NPQ (NPQ_{max}). The E_k was subtracted to the E_{opt} values to know the range of intensity in which the photosynthesis is maintained in steady state.

2.4. Biochemical analysis

2.4.1. Total carbon and nitrogen

Total carbon (TC) and nitrogen (TN) were determined from dry biomass of algae using an elemental analyser CNHS (LECO-932, USA) in the Research Support Central

Services (SCAI, University of Málaga). C and N values were expressed as mg of element per dry weight of algal biomass and the C:N ratio was determined.

2.4.2. Total and soluble proteins

Total proteins were calculating using the nitrogen to protein conversion factor of 5.40 reported by Lourenço *et al.* (2002) for the red macroalga *Gracilaria domingensis*, phylogenetically close to *G. armata*.

Soluble proteins were determined through the Bradford method (Bradford, 1976). Briefly, 20 mg of dry weight (DW) were extracted in 1 mL of phosphate buffer (0.1M; pH:7) and incubated overnight at 4 °C. Extracts were centrifuged and supernatants were used for the assay. For that, 780 µl of phosphate buffer were mixed with 20 µl of the extracts and 200 µl of Biorad protein assay solution (Bio-rad, Germany). After 15 min of incubation in darkness, absorbance was measured at 595 nm. Bovine serum albumin (BSA) was used as standard.

2.4.3. Carbohydrates

Carbohydrates were measured according to Dubois *et al.* (1956) with some modifications. Five mg of DW were homogenized in 5 mL of H₂SO₄ 1M, mixed and incubated during 1 h at 100 °C. After cooling down, the mixture was centrifuged and 1 mL of the supernatant was mixed with 1 mL of phenol 5 % and 5 mL of concentrated sulphuric acid. After mixing and cooling down, absorbance was measured at 460 nm. Glucose was used as standard.

2.4.4. Lipids

Lipids were quantified by the sulpho-phospho-vainillin method (Mishra *et al.*, 2014). Briefly, 20 mg of DW were extracted in 1 mL of methanol and incubated overnight at 4 °C. After centrifugation, 100 µl of the supernatant were mix with 1 mL of concentrate sulphuric acid and incubated at 100 °C during 10 min. After cooling down, 2.5 mL of phospho-vainillin solution (0.6 g of vainillin, 100 mL of ethanol and 400 mL of H₃PO₄ 85 %) were added and incubated during 15 min at room temperature. Absorbance was measured at 530 nm. Triolein was used as standard.

2.4.5. Photosynthetic pigments

For chlorophyll a quantification, the same extract used for lipids determination was used. The extracts were measured spectrophotometrically at 663, 691 and 750 nm, and the content were quantified according to the formula described by Ritchie (2006).

For phycobiliproteins determination, the same extract used for soluble proteins analysis was used. The extracts were measured at 455, 564, 592, 618, 645 and 750 nm in the spectrophotometer and the quantification was made according to the formula described by Beer and Eshel (1985).

2.4.6. Phenolic compounds

Phenolic compounds were measured using the Folin-Ciocalteu method (Singleton and Rossi, 1965). Briefly, 20 mg of dry samples were extracted in 1 mL of methanol 80 % and incubated overnight at 4 °C. After centrifugation, 100 µl of the extracts were mixed with 700 µl of distilled water and 50 µl of Folin-Ciocalteu reagent (Sigma-Aldrich, USA). After vortexing, 150 µl of Na₂CO₃ 20 % was added and mixed again. The mixture was incubated for 2 h at 4 °C in darkness and absorbance was measured at 760 nm. Phloroglucinol was used as standard.

2.4.7. Mycosporine like aminoacids (MAAs)

MAAs were determined using HPLC (high performance liquid chromatography) according to Korbée-Peinado *et al.* (2004) with some modification reported by Chaves-Peña *et al.* (2020). Twenty mg of DW were incubated in 1 mL of methanol 20 % during 2 h at 45 °C. After that, samples were centrifuged and 700 µl of the supernatant were dried under vacuum. The dry samples were re-suspended in 700 µl of ultrapure H₂O, filtered through a 0.2 µm filter and injected in the HPLC (1260 Agilent InfinityLab Series, USA). MAAs were detected using a Luna C8 column (Phenomenex, Germany), applying an isocratic flow of 0.5 mL min⁻¹ and a mobile phase of 1.5 % methanol and 0.15 % acetic acid in ultrapure H₂O. The detection was made using a photodiode array detector at 330 nm. Secondary standards were used for the identification of MAAs, and the quantification was performed using the molar extinction coefficients (ϵ) of the different MAAs (U Karsten *et al.* 1998). Results were expressed as mg g⁻¹ DW.

2.5. Antioxidant capacity

Two different methods based on the free radical scavenging effect were used to determine the antioxidant capacity.

The ABTS assay was performed according to Re *et al.* (1999) with some modifications. The ABTS radical cations (ABTS^{•+}) were generated via a reaction of 7 mM ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and 2.45 mM K₂S₂O₈ in phosphate buffer (0.1M; pH:7). This reaction was stored for 12–16 h at room temperature to ensure the complete formation of the radical. ABTS^{•+} solution was diluted with phosphate buffer until the absorbance at 727 nm was around 0.75 ± 0.05 . The same extract prepared for soluble proteins quantification were used in this assay. For the reaction, 50 µL of the extracts were mixed with 950 µL of the diluted ABTS^{•+}. The mixture was incubated for 8 min at room temperature and darkness, and absorbances were measured at 727 nm.

The DPPH assay was made according to Brand-Williams *et al.* (1995) with some modifications. The same extracts prepared for phenols quantification were used in this assay. For the reaction, 200 µL of the extracts were mixed with 1 mL of the DPPH[•] (2,2-diphenyl-1-picrylhydrazyl) solution (0.06 mM of DPPH in methanol 80 %). After 30 min of incubation at room temperature and darkness, absorbances were measured at 517 nm.

For both methods, a standard solution of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as reference and the results were expressed as µmol TE (Trolox equivalent) g⁻¹ DW.

2.6. Statistical analysis

The software STATISTICA (V.7) was used for the statistical analysis. One-way ANOVAs were performed to detect significant effects of radiation treatments among samples in the different analyzed response-variables. A Student-Newman-Keuls (SNK) post-hoc test was performed to observe the different groups after a significant ANOVA. Student-t test was used to compare the ETR measured *in situ* and *ex situ*. Homogeneities of variance were tested using the Cochran test and via visual inspection of the residuals. Correlations among dependent variables were calculated using Pearson's correlation analysis.

3. Results

In general, UV radiation provoked a discoloration of the apical part of the algae as seen in Figure 3.1. 2. Algae in the acclimation period ($P_{\text{acclimated}}$) and under P treatment were similar, reddish and with no signal of discoloration. However, when UV radiation was added to the treatments (PA and PAB), apical parts of algae started to become yellowish. In the PAB treatment the loss of pigmentation seems more pronounced than in PA. In algae grown under UV-B radiation, less branchings were observed in comparison to the other algae.

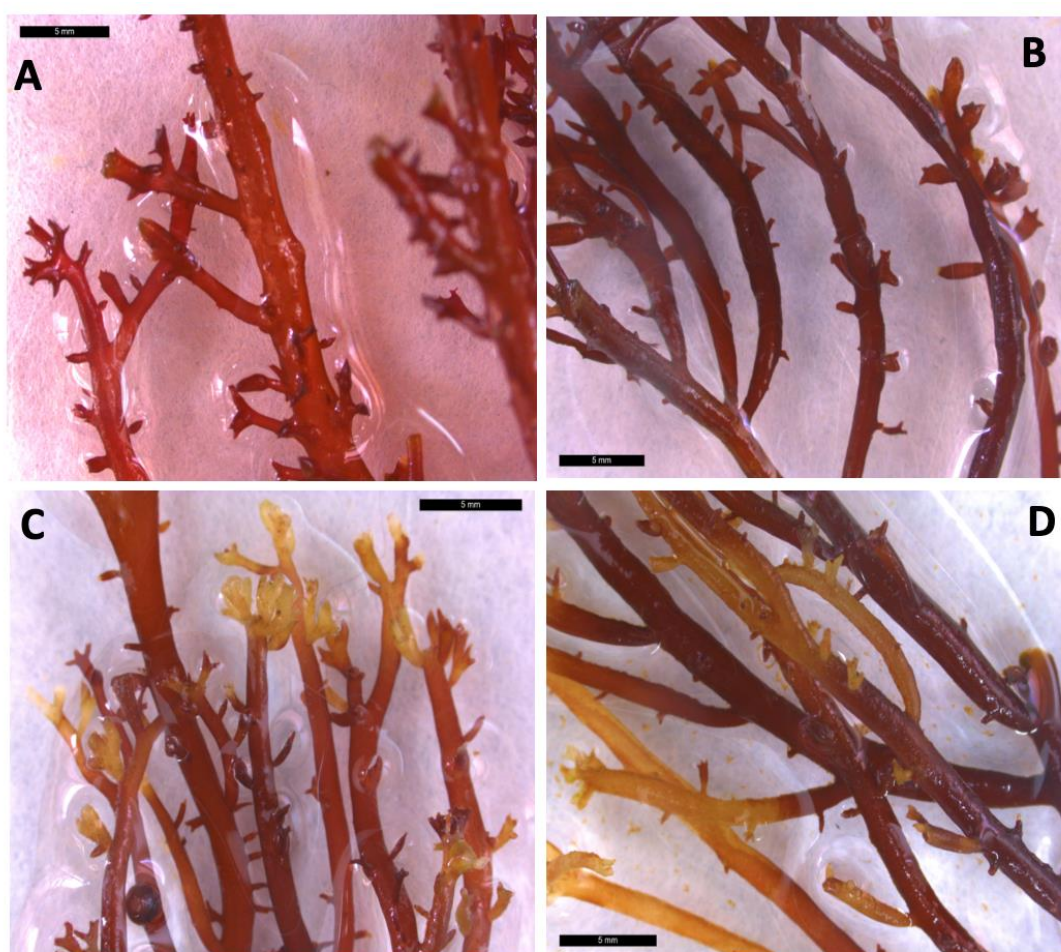


Figure 3.1. 2. Changes in morphology and color of *Gracilaria armata* cultivated under the different radiation treatments. A) Pacclimated; B) PAR (P), C) PAR + UV-A (PA); D) PAR + UV-A + UV-B (PAB). Scale = 5 mm.

The daily growth rates measured during the radiation shift experiment (exposure of *G. armata* to P, PA and PAB) are presented in Figure 3.1. 3. Algae under only PAR (P treatment) and under P + UV-A radiation (PA) presented the highest growth rate (1.6 ± 0.2 and 1.2 ± 0.4 % day⁻¹, respectively), without significant differences between them. Algae grown under PAB treatment showed the lowest growth rate (0.6 ± 0.2 % day⁻¹), showing significant differences between this treatment and the others (Table SM.3.1. 1).

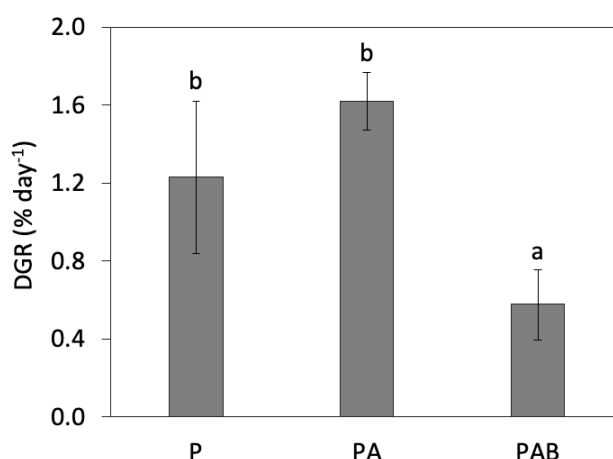


Figure 3.1. 3. Daily growth rate (DGR) measured as % day⁻¹ in *Gracilaria armata* cultivated under different radiation treatments: PAR (P), PAR + UV-A (PA) and PAR + UV-A + UV-B (PAB). Values are calculated as mean $\pm$ standard deviation (SD) (n = 3). Different letters indicate significant differences among treatments (ANOVA, SNK, p < 0.05).

A comparison between electron transport rate (ETR) calculated *in situ* and *ex situ* is shown in Table 3.1. 2. The *ex situ* values are the ones obtained after incubation at 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of the red LED light of the Mini-PAM during RLCs (similar irradiance than *in situ* measurements with white LED light). The ETR measured *in situ* was only significantly lower (Table SM.3.1. 1) in $P_{\text{acclimated}}$ algae (3.4 ± 0.1 vs 6.4 ± 0.1 $\mu\text{mol e}^{-} \text{m}^{-2} \text{s}^{-1}$), which were maintained under a low light intensity of 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. During *ex situ* measurements, the ETR values were also lower in $P_{\text{acclimated}}$ algae (1.4 ± 0.1 $\mu\text{mol e}^{-} \text{m}^{-2} \text{s}^{-1}$), which only showed significant differences with the highest values observed in the P treatment (2.7 ± 0.5 $\mu\text{mol e}^{-} \text{m}^{-2} \text{s}^{-1}$). In all samples, ETR values obtained *in situ* were higher than those obtained *ex situ*.

Table 3.1. 2. Electron transport rate (ETR) measured in situ and ex situ (during RLC at 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) in *Gracilaria armata* acclimated to PAR conditions in laboratory ($P_{\text{acclimated}}$) and submitted to the different radiation treatments: PAR (P), PAR + UV-A (PA) and PAR + UV-A + UV-B (PAB). Values are calculated as mean $\pm$ standard deviation (SD) ($n = 3$). Different letters indicate significant differences among treatments (ANOVA, SNK, $p < 0.05$). Asterisk (*) means significant differences between in situ and ex situ measurements.

	In situ	Ex situ
Pacclimated	$3.43 \pm 0.13\text{a}^*$	$1.41 \pm 0.06\text{a}$
P	$6.37 \pm 0.25\text{b}^*$	$2.66 \pm 0.45\text{b}$
PA	$6.51 \pm 0.73\text{b}^*$	$2.26 \pm 0.41\text{ab}$
PAB	$6.28 \pm 0.42\text{b}^*$	$1.95 \pm 0.41\text{ab}$

Figure SM.3.1. 1 represents the ETR, the non-photochemical quenching (NPQ), the effective quantum yield ($Y(\text{II})$), and both yield losses: $Y(\text{NPQ})$ and $Y(\text{NO})$ in function of the increasing irradiances of the red LED light of the Mini-PAM-II (*ex situ* measurements). ETR curves presented the maximal values around 100 and 250 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (depending on the treatment), followed by a decrease with increasing irradiances. Meanwhile, $Y(\text{II})$ data started in 0.4 ± 0.0 in all treatments and decreased with the increasing irradiances, achieving almost zero at the highest one. $Y(\text{NO})$ and $Y(\text{NPQ})$ increased until achieved different plateaus, that remained constant with the increasing irradiances. $Y(\text{NO})$ was around three times higher than $Y(\text{NPQ})$ in all treatments, i.e. at 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of the red LED light, the $Y(\text{NO})$ were 0.6 ± 0.1 and the $Y(\text{NPQ})$ were 0.2 ± 0.1 (average values). NPQ also increased with the increasing irradiance, reaching a plateau at 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of the red LED light.

Table 3.1. 3 shows the different parameters obtained after the mathematical fitting of ETR and NPQ vs. irradiance curves. F_v/F_m values of *G. armata* did not showed significant differences when the algae were submitted to different treatments (general mean 0.5 ± 0.1) (Table SM.3.1. 1). ETR_{max} values were significantly higher in the PA and P treatment (3.6 ± 0.2 and $3.4 \pm 0.1 \mu\text{mol e}^{-1} \text{m}^{-2} \text{s}^{-1}$, respectively) than that under PAB treatment or $P_{\text{acclimated}}$ species, that presented similar values (2.5 ± 0.4 and $2.6 \pm 0.0 \mu\text{mol e}^{-1} \text{m}^{-2} \text{s}^{-1}$, respectively). In contrast, the NPQ_{max} was significantly higher in $P_{\text{acclimated}}$ species and in the PAB treatment (0.6 ± 0.1 and 0.5 ± 0.1 , respectively) than in the other treatments (0.3 ± 0.1). The ratio $\text{ETR}_{\text{max}}/\text{NPQ}_{\text{max}}$ was significantly higher in the PA and P treatment (8.7 ± 2.2 and 8.8 ± 0.8 , respectively) compared to PAB treatment. Algal

photosynthetic efficiency of the ETR (α_{ETR}) and saturated irradiance ($E_{k\text{ETR}}$) did not show significant differences among the treatments (Table SM.3.1. 1). However, the $E_{\text{opt ETR}}$ values were significantly higher in PA treatment ($307.3 \pm 6.7 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) than in the others. The $E_{\text{opt}}-E_k$ values also showed significant differences, lowest values were obtained in the P and PAB treatments (104.4 ± 10.4 and $125.4 \pm 37.8 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), followed by $P_{\text{acclimated}}$ algae. The highest value was observed in PA treatment ($184.8 \pm 4.5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$).

Table 3.1. 3. Photosynthetic parameters obtained after the adjust of the different RLCs in *Gracilaria armata* acclimated to PAR conditions in laboratory ($P_{\text{acclimated}}$) and submitted to the different radiation treatments: PAR (P), PAR + UV-A (PA) and PAR + UV-A + UV-B (PAB). Values are calculated as mean $\pm$ standard deviation (SD) ($n = 3$). Different letters indicate significant differences among treatments (ANOVA, SNK, $p < 0.05$).

	$P_{\text{acclimated}}$	P	PA	PAB
F_v/F_m	0.54 ± 0.02	0.45 ± 0.07	0.45 ± 0.10	0.42 ± 0.09
α_{ETR}	0.04 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
ETR_{max}	2.60 ± 0.04^a	3.37 ± 0.14^b	3.63 ± 0.16^b	2.50 ± 0.35^a
$E_{k\text{ETR}}$	67.74 ± 2.77	89.43 ± 23.17	122.59 ± 2.23	87.77 ± 38.25
$E_{\text{opt ETR}}$	210.52 ± 24.56^a	193.86 ± 33.54^a	307.34 ± 6.72^b	213.21 ± 66.92^a
$E_{\text{opt}}-E_k$	142.78 ± 27.26^{ab}	104.42 ± 10.37^a	184.75 ± 4.48^b	125.44 ± 37.79^a
NPQ_{max}	0.60 ± 0.08^b	0.30 ± 0.07^a	0.33 ± 0.05^a	0.51 ± 0.05^b
$\text{ETR}_{\text{max}}/\text{NPQ}_{\text{max}}$	4.39 ± 0.65^b	8.70 ± 2.16^a	8.82 ± 0.82^a	5.19 ± 1.07^b

The biochemical composition of *G. armata* during experimental procedures is shown in Table 3.1. 4. The carbon was similar in almost all cases ($232.4 \pm 6.3 \text{ mg g}^{-1} \text{ DW}$), only a significant increase was observed in the PAB treatment ($257.7 \pm 5.0 \text{ mg g}^{-1} \text{ DW}$) (Table SM.3.1. 1). In relation to the nitrogen content, algae coming from the field presented the highest content ($22.6 \pm 1.6 \text{ mg g}^{-1} \text{ DW}$), which was significantly different when compared only to the lowest value observed during the acclimatation period ($P_{\text{acclimated}}$) and P treatment ($19.1 \pm 0.3 \text{ mg g}^{-1} \text{ DW}$) (Table SM.3.1. 1). The lowest C/N ratio was observed in algae collected from their natural environment (10.3 ± 0.5), showing significant differences with $P_{\text{acclimated}}$ algae (12.7 ± 0.7) and algae under PAB treatment (12.2 ± 0.4). Total proteins showed similar variation as observed to nitrogen content, i.e., algae from

the field with the higher content, similar to PA and PAB treated samples. In contrast, soluble proteins decreased during the experimental period. No significant differences were observed in algae collected from the field and $P_{\text{acclimated}}$ (9.2 ± 1.6 and 8.9 ± 0.7 mg g⁻¹ DW, respectively), whereas in the P, PA and PAB treatments the soluble proteins decreased (e.g. 4.9 ± 0.8 mg g⁻¹ DW with PAB treatment) (Table SM.3.1. 1). The lowest carbohydrates content of *G. armata* was observed in $P_{\text{acclimated}}$ algae, significant differences were only observed between these algae and those subjected to PA treatment, that presented the highest content (285.5 ± 17.4 mg g⁻¹ DW) (Table SM.3.1. 1). The lipids content increased under the culture conditions, i.e. values increased from 3.7 ± 0.7 in algae from the field, to 6.7 ± 1.2 mg g⁻¹ DW in the P or PA treatment, although during the PAB treatment algae reduce their lipids content (showing similar values that the obtained in the field; 2.3 ± 0.2 mg g⁻¹ DW). The highest content of phenolic compounds was observed in algae collected from the field (7.3 ± 0.4 mg g⁻¹ DW). The content decreased during the culture period in all treatments, the lowest values were reached in algae under PAB treatment (2.7 ± 0.2 mg g⁻¹ DW).

Table 3.1. 4. Content of total carbon, total nitrogen, total proteins, soluble proteins, carbohydrates, lipids, phenolic compounds, and C/N ratio of *Gracilaria armata* from their natural environment, acclimated to PAR conditions ($P_{\text{acclimated}}$) in laboratory and culture under the different radiation treatments: PAR (P), PAR + UV-A (PA) and PAR + UV-A + UV-B (PAB). Values are calculated as mean $\pm$ standard deviation (SD) ($n = 3$) and expressed as mg g^{-1} dry weight (DW), except the C/N ratio. Different letters indicate significant differences among treatments (ANOVA, SNK, $p < 0.05$).

	Carbon	Nitrogen	C/N	Total Proteins	Soluble Proteins	Carbohydrates	Lipids	Phenolic compounds
Field	232.35 ± 5.00^a	22.58 ± 1.59^b	10.31 ± 0.50^a	121.93 ± 8.56^b	9.24 ± 1.59^b	254.84 ± 23.06^{ab}	3.67 ± 0.74^a	7.31 ± 0.35^d
$P_{\text{acclimated}}$	238.33 ± 4.97^a	18.86 ± 0.62^a	12.65 ± 0.68^b	101.87 ± 3.32^a	8.94 ± 0.65^b	229.25 ± 9.64^a	4.22 ± 0.88^a	5.31 ± 0.35^c
P	225.65 ± 1.74^a	19.25 ± 1.31^a	12.15 ± 1.07^{ab}	103.96 ± 7.05^a	6.98 ± 0.72^a	256.33 ± 15.47^{ab}	6.64 ± 1.23^b	3.34 ± 0.47^{ab}
PA	235.09 ± 8.09^a	20.18 ± 1.16^{ab}	12.10 ± 0.90^{ab}	108.99 ± 6.26^{ab}	6.06 ± 0.70^a	285.47 ± 17.41^b	6.83 ± 1.18^b	3.83 ± 0.44^b
PAB	257.70 ± 5.00^b	21.16 ± 0.28^{ab}	12.18 ± 0.40^b	114.30 ± 1.55^{ab}	4.96 ± 0.82^a	259.85 ± 23.02^{ab}	2.33 ± 0.23^a	2.72 ± 0.23^a

The concentrations of chlorophyll *a* and phycobiliproteins (phycoerythrin and phycocyanin) are shown in Figure 3.1. 4. Similar values were observed in the chlorophyll *a* content of almost all *G. armata* thalli, and significant differences were only observed between the algae exposed to P and PAB treatment, that showed the highest (1.0 ± 0.2 mg g⁻¹ DW) and the lowest (0.5 ± 0.2 mg g⁻¹ DW) values, respectively (Table SM.3.1. 1). The phycoerythrin content was similar in algae collected from the field, P_{acclimated} and P treatment (0.8 ± 0.0 , 0.9 ± 0.1 and 0.7 ± 0.1 mg g⁻¹ DW, respectively), and a significant decreased was observed after the PA and PAB treatments (0.4 ± 0.2 and 0.2 ± 0.1 mg g⁻¹ DW). In the case of phycocyanin, a slight variation was observed only between samples from the field against those receiving some UV radiation (PA and PAB treatments), where the PC content was significantly lower.

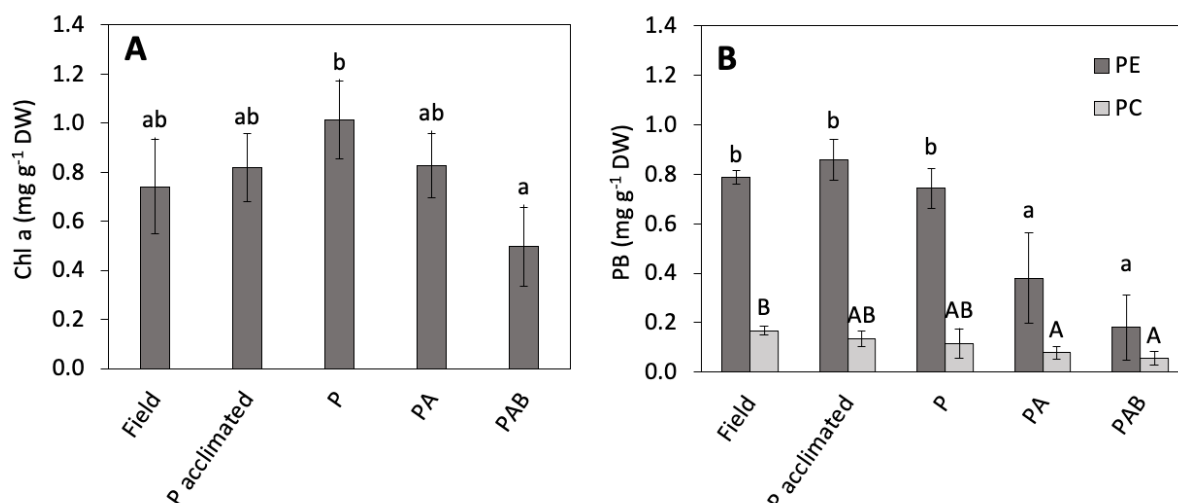


Figure 3.1. 4. Chlorophyll *a* (A) and phycobiliproteins (B) content of *Gracilaria armata* from their natural environment, acclimated to PAR conditions (P_{acclimated}) in laboratory and cultivated under different radiation treatments: PAR (P), PAR + UV-A (PA) and PAR + UV-A + UV-B (PAB). Values are calculated as mean $\pm$ standard deviation (SD) ($n = 3$) and expressed as mg g⁻¹ dry weight (DW). Different letters indicate significant differences among treatments (ANOVA, SNK, $p < 0.05$).

Total MAAs content is shown in Figure 3.1. 5. The highest content was observed in algae collected from the field (2.3 ± 0.2 mg g⁻¹ DW). After the acclimation period under low irradiance, MAA content drastically decreased (1.0 ± 0.3 mg g⁻¹ DW). Algae submitted to P treatment showed a higher MAAs content (1.7 ± 0.3 mg g⁻¹ DW) than those thalli grown under PA or PAB treatment (< 1 mg g⁻¹ DW). The MAA composition

was similar in all treatments: Porphyrin-334 was the predominant (> 65 %), followed by shinorine and palythol (30 %), whereas palythine presented the lowest content (2 %).

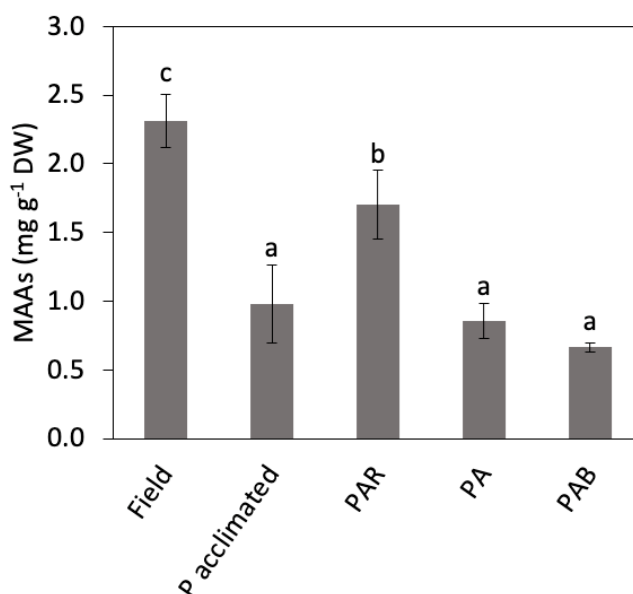


Figure 3.1. 5. Total mycosporine like amino acids (MAAs) content of *Gracilaria armata* from their natural environment, acclimated to PAR conditions ($P_{\text{acclimated}}$) in laboratory and cultivated under different radiation treatments: PAR (P), PAR + UV-A (PA) and PAR + UV-A + UV-B (PAB). Values are calculated as mean $\pm$ standard deviation (SD) ($n = 3$) and expressed as mg g⁻¹ dry weight (DW). Different letters indicate significant differences among treatments (ANOVA, SNK, $p < 0.05$).

The antioxidant capacities measured by two different methods based on the free radical scavenging activity are shown in Figure 3.1. 6. In general, aqueous extracts of *G. armata* submitted to ABTS assay showed higher antioxidant capacity than methanolic extracts utilized into DPPH assay. The highest antioxidant capacity was observed in algae collected from the field (ABTS: $26.3 \pm 4.3 \mu\text{mol TE g}^{-1} \text{ DW}$; DPPH: $5.4 \pm 0.5 \mu\text{mol TE g}^{-1} \text{ DW}$), and the lowest values in algae submitted to PAB treatment (ABTS: $15.1 \pm 2.0 \mu\text{mol TE g}^{-1} \text{ DW}$; DPPH: $3.1 \pm 0.2 \mu\text{mol TE g}^{-1} \text{ DW}$).

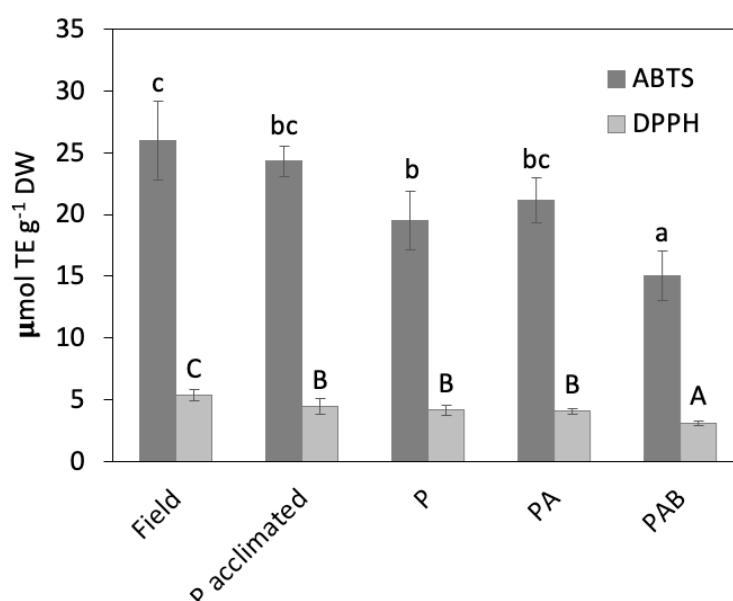


Figure 3.1. 6. Antioxidant capacity (AC) measured through two different assays (ABTS and DPPH) of *Gracilaria armata* from the field, acclimated to PAR conditions ($P_{\text{acclimated}}$) in laboratory and culture under three different treatments: PAR (P), PAR + UV-A (PA) and PAR + UV-A + UV-B (PAB). Values as calculated as mean $\pm$ standard deviation (SD) ($n = 3$) and expressed as $\mu\text{mol TE g}^{-1}$ dry weight (DW). Different letters indicate significant differences among treatments (ANOVA, SNK, $p < 0.05$).

Correlations between the different analyzed parameters are shown in Table SM.3.1.

2. Some of them must be highlighted. DGR was positively correlated with ETR_{max} and the ratio $\text{ETR}_{\text{max}}/\text{NPQ}_{\text{max}}$, whereas it was inversely correlated to the NPQ_{max} . The DGR also presented a positive correlation with some carbonated compounds (lipids and phenols) and both antioxidant capacity assays. ETR_{max} responses were also correlated with the bioactive compounds such as lipids, phenols, chlorophyll and both antioxidant methods. NPQ_{max} was positively correlated to C and N, whereas the correlation was inverse with the accumulation of bioactive compounds as pigments (chlorophyll and biliprotein) and antioxidant compounds as polyphenols and MAAs. Soluble proteins showed a positive correlation with pigments (chlorophylls and phycobiliproteins), MAAs and both antioxidant capacity assays. MAAs were positively correlated to other nitrogen compounds as phycoerythrin and soluble proteins. Phenols were correlated to antioxidant capacity measured through ABTS methods but not with AC obtained by DPPH method. In contrast, MAAs were not correlated with antioxidant capacities. Total nitrogen was not correlated to other N-compounds like soluble proteins, phycobiliproteins or MAAs. Finally, total carbon was positively correlated to NPQ_{max} but negatively

correlated to ETR_{max} and bioactive compounds as pigments (Chla and PE), lipids, soluble proteins, MAAs and antioxidant capacities.

4. **Discussion**

The objective of this paper was to evaluate the effects of UVR on the physiology and the biochemical composition of *Gracilaria armata*, in order to know possible acclimation and/or resistance strategies, as well as biotechnological uses of UVR to modify the content of bioactive compounds of the species. *G. armata* is a red alga with potential cultivation, as other Gracilariales (Abreu *et al.*, 2011a; Figueroa *et al.*, 2022). In this experiment, *G. armata* was exposed to a 30 % of UVR in relation to the total PAR received. This percentage was higher than the radiation received in nature, although the intensity of UVR was much lower in comparison with solar radiation (

Table 3.1. 1). Even so, the treatment conditions utilized here were efficient to help the understanding of radiation shift effects in *G. armata* responses.

Algae grew optimally in all treatments, only under PAB treatment the daily growth rate was significantly lower than in the other treatments. Other authors also observed a decrease in the growth under this type of radiation in *Gracilaria* spp. (Schmidt *et al.*, 2010; Ayres-Ostrock and Plastino, 2014; Zhu *et al.*, 2015). However, UV-A radiation did not provoke a negative effect on the growth as observed Schneider *et al.* (2020a) in *Osmundea pinnatifida* or Gao and Xu (2008) in *Gracilariopsis lemaneiformis* (formerly *Gracilaria lemaneiformis*). It has been described that UV-A can be used as light source for photosynthesis and present a positive effect on morphogenesis or growth in some species (Ji *et al.*, 2016).

The effects of UVR on photosynthesis have been studied by several researchers by using *in vivo* chlorophyll a fluorescence (Figueroa *et al.*, 2014b; Simioni *et al.*, 2014; Bao *et al.*, 2019; Bonomi-Barufi *et al.*, 2020). Growth rate was positively related to maximal electron transport rate (ETR_{max}) indicating coupling between growth and photosynthetic capacity. This relation has been observed by other authors in algae (Figueroa *et al.*, 2014b) In general, the photosynthetic activity is affected by UVR due to the denaturalization of important proteins, e.g. Rubisco, which implies in carbon fixation, that is normally the most affected process, or D1 and D2 proteins involved in the photosystem II structure. UVR can also produce lipid peroxidation affecting the thylakoid membrane

structure or degradation of the photosynthetic pigments (Casella *et al.*, 2016). Some authors observed a decreased of the photosynthetic capacity under UV-B radiation in other Gracilariales, such as *Gracilariopsis lemaneiformis* (Zhu *et al.*, 2015), *Gracilariopsis longissima* (Álvarez-Gómez *et al.*, 2017, 2019b) or *Gracilaria cornea* (Figueroa *et al.*, 2022). In this work, F_v/F_m values were not affected by the radiation treatments, whereas ETR_{max} values showed a slight decreased under PAB treatment. These results indicate that *G. armata* can maintain a good physiological state under all radiation treatments.

NPQ is the ratio between the two yield losses ($Y(NPQ)/Y(NO)$). ($Y(NO)$ is related to energy loss as heat or fluorescence, whereas $Y(NPQ)$ is related with the energy dissipation through photo-regulated mechanisms throughout carotenoids. Then, values of NPQ lower than 1 indicate that most of the energy is loss through thermal dissipation (aprox. 70%). NPQ_{max} values increased under the PAB treatment, what means that the photo-regulated dissipation mechanism increased under this treatment, as a photoprotection mechanism. However, these mechanisms remain to be determined, as the presence of the xanthophyll cycle in red algae have been discussed by several authors but not yet identified (Schubert *et al.*, 2011). There is only one evidence in Gracilariales (Ursi *et al.*, 2003)

A negative correlation was observed between NPQ_{max} and the DGR, ETR_{max} and most of the internal compounds, indicating that the increased of $Y(NPQ)$ provoke a reduction of the growth potential and the synthesis of internal compounds. This effect can be related with the use of the energy in photoprotection or photorepair mechanisms instead of growth or synthesis of compounds. The ratio ETR_{max}/NPQ_{max} which is a good indicator of the relation between production and photoprotection, was also lower in the PAB treatment, as observed Figueroa *et al.* (2014b) in *Ulva rigida* (Chlorophyta), *Ericaria selaginoides* (formely *Cystoseira tamariscifolia*) (Phaeophyta) and *Ellisolandia elongata* (Rhodophyta). The stationary phase of the photosynthesis, calculated as $E_{opt} - E_k$ was significantly larger in the PA treatment, what can be related to an acclimation to a higher light intensity.

In this study, two ways of measuring the photosynthetic activity were also compared. The ETR measured *in situ* were higher than *ex situ* (RLC). These differences have been also observed in other macroalgae species (Longstaff *et al.*, 2002; Figueroa *et al.*, 2021, 2022). An explanation can be the quality of the light to which algae were exposed during the measurements. The white light used in the experiments contributes

for wide range of wavelengths (enriched in blue, see figure 1), in comparison with the red LED light from the Mini-PAM-II used in the rapid light curves (RLCs). In this sense, more sections of radiation spectra can be absorbed by the photosynthetic pigments and increase the photosynthetic activity, transferring energy by resonance thorough antenna complex.

ETR_{max} was also related to the accumulation of photosynthetic pigments (chlorophyll and phycoerythrin), soluble proteins and lipids indicating a good physiological status. In addition to growth, the energy obtained under favourable conditions is used for the accumulation of different compounds, such as lipids, phenolic compounds, chlorophyll a or MAAs. However, during stressful periods, the energy is probably used for the activation of repair mechanisms against the UVR (potentially photolyases), reducing mainly the growth capacity. The UVR significantly increase the carbon content, probably structural C-compounds of the cell wall. This increment can be related to the thickening of the cell wall according to Schmidt *et al.* (2012, 2010), that acts as a protection response to UVR, reducing the penetration of this type of radiation inside the cell. The total N content did not decrease under UVR. However, N-based molecules such as soluble proteins or phycobiliproteins are known for being very sensitive to UVR. Soluble proteins showed a remarkable decrease in the PAB treatment, as well as the phycoerythrin content. A reduction of these molecules in algae expose to UVR have been observed by other authors (Schmidt *et al.*, 2012; Zhu *et al.*, 2015; Álvarez-Gómez *et al.*, 2019b). A possible explanation for the maintenance of total N content in this work can be the synthesis of structural proteins or the synthesis of N-compounds to be used in repair mechanism. Chlorophylls also decreased with UVR. In general, similar studies observed the same reduction of chlorophyll in samples expose to UVR (Schmidt *et al.*, 2012; Zhu *et al.*, 2015; Figueroa *et al.*, 2022), although other authors observed that chlorophyll can resist to the UVR but depending on the UV dose (Gómez and Figueroa, 1998; Flores-Moya *et al.*, 1999; Schneider *et al.*, 2020a, 2022). Lipids content also decreased with the UV-B radiation (i.e. algae from the field and under PAB treatment showed the lowest values), whereas the UV-A radiation showed similar values that the obtained under P treatment. Probably UV-B radiation induces lipid peroxidation. In general, macroalgae present low lipid content and there is few information about lipids regulation under stress conditions. However, both lipids or carbohydrates can be related to storage of energy, which could be available in the case of stressing situations.

Phenolic compounds are considered photoprotective molecules (Connan *et al.*, 2004; Zheng *et al.*, 2022). Their content can vary in response to environmental changes such as salinity, nutrients, UV radiation or herbivory (Abdala-Díaz *et al.*, 2006; Figueroa *et al.*, 2014a; Lomartire *et al.*, 2021). Polyphenols have effective photoprotection capacity related to high antioxidants capacities not only in brown alga (Zubia *et al.*, 2007, 2009), but also in several species of red macroalgae (Vega *et al.*, 2020a). It is expected that polyphenols content increased under UV radiation, although in this work a decrease of these compounds were observed under culture conditions, mainly in PAB treatment. It has also been described that under stressful conditions, phenols can be released from algal thalli (Koivikko *et al.*, 2005; Celis-Plá *et al.*, 2014).

In relation to MAAs, different contents and patterns have been observed in Gracilariales, although in most of the cases the main MAAs is porphyra-334, and the content is very variable depending on the environmental and culture conditions. For example, in *Gracilaria tenuistipitata*, porphyra-334 was the predominant MAAs (> 90 %) and reached values of 2.2 mg g⁻¹ DW under high nitrogen input and UVR (Barufi *et al.*, 2011). On the other hand, *Gracilaria cornea* presented similar proportions of porphyra-334 and asterina-330, reaching values of 5.0 mg g⁻¹ DW under high nitrogen and solar radiation (Álvarez-Gómez *et al.*, 2016; Figueroa *et al.*, 2022). In this work, *G. armata* mainly present porphyra-334 followed by shinorine and palythanol and reached values of 2.3 mg g⁻¹ DW in nature. MAAs are UV-absorbing compounds, so it was expected that its content should have increased under UVR (as polyphenols), although the UVR / PAR ratio were different in laboratorial conditions in comparison to environmental conditions, and this aspect can explain the reason to the absence of MAAs increases under PA or PAB treatments. Hoyer *et al.* (2001) indicated three different groups of algae based on their MAAs content: (1) species without the capacity to synthesize MAAs, (2) species with a basic concentration of MAAs, that can change depending on radiation conditions, and (3) species that present a relatively high MAAs content, that is less affected by the radiation conditions. Later, the same author (Hoyer *et al.* 2002) described other three groups based on the stimulation of the MAAs synthesis under different radiation treatments: (A) species that increased their MAAs content under full spectrum (UV-A and UV-B), (B) species that stimulate the synthesis of MAAs under PAR + UV-A, whereas UV-B did not lead the accumulation of MAAs, and (C) species that also exhibit higher MAAs content after treatments with PAR + UV-A, whereas a full spectrum (UV-B) provoke a strong decline of MAAs content. *G. armata* decreased its MAAs content during the acclimation to the laboratory conditions (low PAR intensity) in comparison with values

obtained in the natural conditions, probably due to the decrease of the light intensity and the removal of the UVR. However, during the different radiation treatments, MAAs content increased under PAR conditions which received the double or irradiance than the utilized during acclimation, in comparison to PA or PAB. In this context, *G. armata* belongs to the second group, as a species that can modulate its MAAs content depending on the radiation conditions and probably also belong to the group A because the highest MAAs content was observed in nature. It is relevant to mention that the species already showed high quantity of MAAs in nature, even being collected in March (final period of the winter), when the radiation availability in the field is lower than the observed in summer at the collection area. If the samples were obtained in the summer, maybe the MAAs quantities could be even higher in the field, although this remain to be evaluated.

The increase of MAAs content under P treatment can be due to the addition of nutrients. It has been demonstrated that MAA synthesis is regulated by both UVR and nitrate (Korbee *et al.*, 2005a; Huovinen *et al.*, 2006; Barufi *et al.*, 2012; Figueroa *et al.*, 2022). In fact, *G. tenuistipitata* cultured under laboratory conditions for 7 days had the ability to accumulate high amounts of MAAs following a nitrate concentration dependent manner under PAR+UVR (Barufi *et al.*, 2011). In this study, the intensity of the UV-B radiation was higher than the one used by (Barufi *et al.*, 2011) (0.6 vs 0.42 W m⁻²). *G. armata* seems to be more sensitive to UVR than *G. tenuistipitata*, and even with high input of nitrate (500 µM), the species was not able to increase its MAAs content. Probably the high percentage of UV-B in relation to the total radiation received would difficult the photorepaired mechanism, avoiding that the energy is used in the synthesis of MAAs. Another difference between both species is the NPQ values, *G. tenuistipitata* presented higher NPQ values than *G. armata*, that is considered a photoprotection mechanism (Figueroa *et al.*, 2014b, 2022). MAAs content can be affected in short or long term, i.e. hours (Taira *et al.*, 2004), days (Korbee-Peinado *et al.*, 2004; Álvarez-Gómez *et al.*, 2019b; Figueroa *et al.*, 2022), and months (Barceló-Villalobos *et al.*, 2017; Yamamoto *et al.*, 2023). For example, (Álvarez-Gómez *et al.* (2019b) observed an increase of MAAs culture under UVR during 8 days in three red algae species (*Gracilaria cornea* (formely *Hydropuntia cornea*), *Gracilariopsis longissima* and *Halopithys incurva*), whereas after 14 days the MAAs content decreased back to similar amounts as the initial values. In this way, the MAAs amounts of *G. armata* also varied according to the time of experimental period, and this factor must be taken into account when defining strategies for MAAs synthesis stimulation.

The antioxidant capacity was also related to the DGR and ETR_{max} . Most of the analyzed molecules presented antioxidant capacity, although not all of them showed a positive correlation. The antioxidant capacity was measured from the extracts, that are a pool of different compounds that can influenced the results. Antioxidant capacities have been described in both N-based compounds, MAAs and biliproteins (de la Coba *et al.*, 2009b; Wada *et al.*, 2015; Sonani *et al.*, 2016; Chen *et al.*, 2022), and polyphenols (Cotas *et al.*, 2020; Zheng *et al.*, 2022). In *G. armata* the antioxidant capacity showed a slight decreased in the PAB treatment, probably due to the denaturalization of antioxidant compounds.

In conclusion, *G. armata* presents certain capacity of resistance to UVR radiation, however lower than other Gracilariales species, as some photoprotective substances such as MAAs were not upregulated with UV availability. UV-B produces a decrease in growth, ETR_{max} and the content of certain compounds such as lipids, phenolic compounds or biliproteins. The content of MAAs increased in P treatments, although in the treatments with UVR the content remained the same as $P_{acclimated}$ algae. Certainly, UV-B radiation caused a damage in algae, although without the action of the photoprotective compounds as MAAs or polyphenols the photodamage would have been much higher than the detected. In addition, photoprotection through photoregulated energy dissipation processes increased in PAB treatments. It is relevant to highlight that metabolism of the species were diversely regulated according to radiation availability, which is relevant considering a biotechnological approach for utilization of some of *G. armata* bioactive molecules.

SUPPLEMENTARY MATERIAL

Table SM.3.1. 1. Results of the one-way ANOVA (analysis of variance) of the analyzed dependent variables measured in *Gracilaria armata*, being the variation source the light quality treatments (only PAR -P-, PAR+UV-A -PA- and PAR+UV-A+UV-B). SS: sum of squares, DF: degrees of freedom, MS: means of squares ($p < 0.05$).

Dependant variables	SS	DF	MS	F-ratio	p-value
DGR	1.664	2	0.8320	12.0740	0.01
ETR _{in situ}	19.689	3	6.5631	33.4117	>0.01
ETR _{ex situ}	2.513	3	0.8378	6.0867	0.02
Fv/Fm	0.026	3	0.0086	1.4231	0.31
α_{ETR}	0.000	3	0.0000	1.8358	0.22
ETR _{max}	2.809	3	0.9364	22.2207	>0.01
Ek	4645.998	3	1548.6660	3.0782	0.09
Eopt	23827.195	3	7942.3983	5.0823	0.03
Eopt - Ek	10457.552	3	3485.8505	6.0651	0.02
NPQ _{max}	0.192	3	0.0641	15.0590	>0.01
ETR/NPQ	0.192	3	0.0641	15.0590	>0.01
C	1742.817	4	435.7042	15.4209	>0.01
N	27.173	4	6.7933	5.6230	0.01
C/N	9.283	4	2.3208	4.4066	0.03
Total proteins	792.370	4	198.0924	5.6230	0.01
Soluble proteins	40.562	4	10.1405	10.8977	>0.01
Carbohydrates	4781.012	4	1195.2530	3.5200	0.05
Lipids	45.495	4	11.3736	13.1703	>0.01
Phenolic compounds	40.581	4	10.1453	70.1196	>0.01
Chl a	0.420	4	0.1050	4.2240	0.03
PE	1.042	4	0.2605	19.9109	>0.01
PC	0.024	4	0.0060	4.9329	0.02
MAAs	5.691	4	1.4227	35.6343	>0.01
ABTS	218.345	4	54.5862	11.0456	>0.01
DPPH	7.886	4	1.9715	11.5032	>0.01

DGR: Daily growth rate (%); ETR: electron transport rate ($\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$); Fv/Fm: Maximal quantum yield, α : photosynthetic efficiency; Ek: Saturated irradiance ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$); Eopt: Optimal irradiance ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$); NPQ: non-photochemical quenching, C: carbon ($\text{mg g}^{-1} \text{DW}$), N: nitrogen ($\text{mg g}^{-1} \text{DW}$), Chl: chlorophyll ($\text{mg g}^{-1} \text{DW}$), PE: phycoerythrin ($\text{mg g}^{-1} \text{DW}$), PC: phycocyanin ($\text{mg g}^{-1} \text{DW}$), MAAs: mycosporine-like amino acids ($\text{mg g}^{-1} \text{DW}$), ABTS and DPPH: antioxidant capacity methods ($\mu\text{mol TE g}^{-1} \text{DW}$)

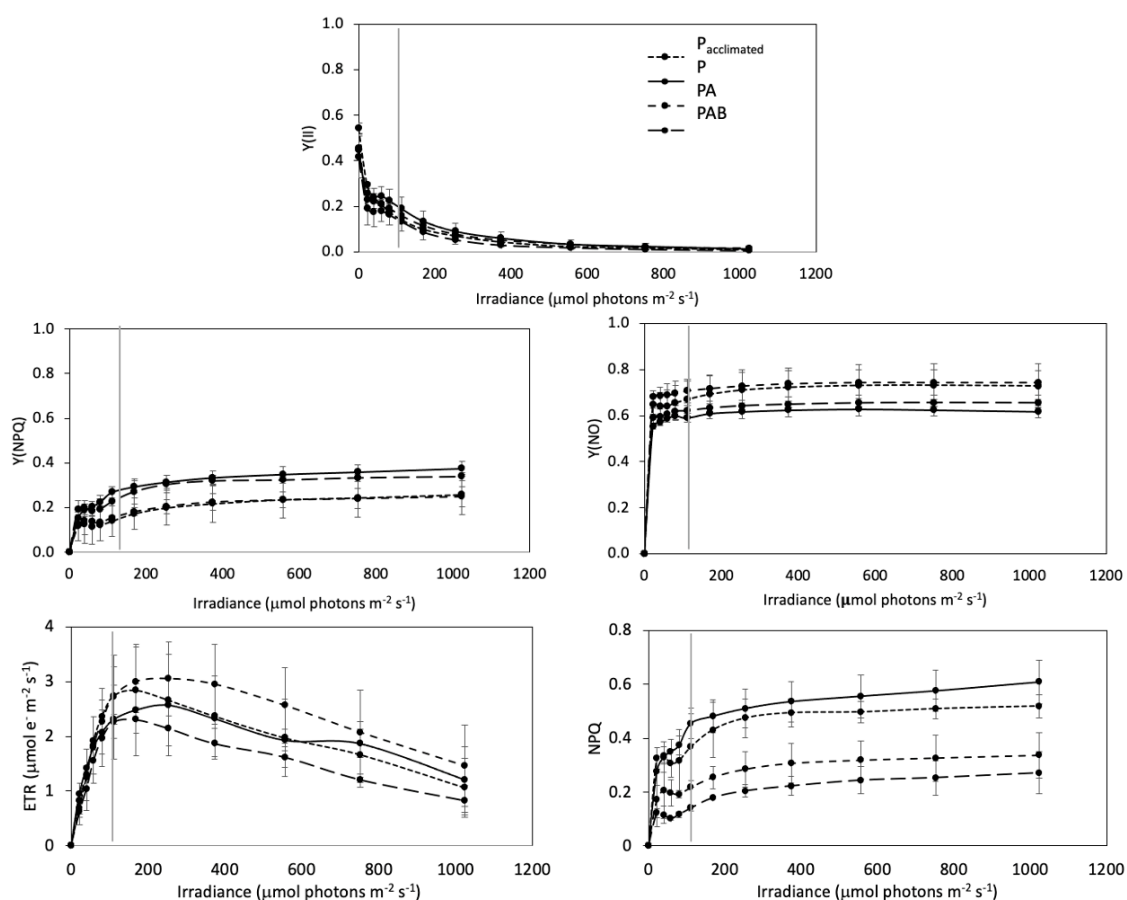


Figure SM.3.1. 1. Parameters obtained from the RLCs (rapid light curves) of *Gracilaria armata* acclimated to PAR conditions in laboratory ($P_{\text{acclimated}}$) and submitted to the different radiation treatments: PAR (P), PAR + UV-A (PA) and PAR + UVAB (PAB). Effective quantum yield ($Y(\text{II})$), two yield losses ($Y(\text{NPQ})$ and $Y(\text{NO})$), electron transport rate (ETR) and non-photochemical quenching (NPQ). Values are calculated as mean $\pm$ standard deviation (SD) ($n = 3$). A vertical line marks the values obtained after incubation at 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of the red LED light of the Mini-PAM II (similar intensity to the used with the White LED light during the experiment).

Table SM.3.1. 2. Pearson correlation coefficients of the different parameters measured in *Gracilaria armata* submitted to the three radiation treatments: PAR (P), PAR + UV-A (PA) and PAR + UVAB (PAB).

	F_v/F_m	ETR _{in situ}	ETR _{ex situ}	ETR _{max}	NPQ _{max}	ETR _{max} /NPQ _{max}	C	N	C/N	S prot.
DGR	0.5521	0.2674	0.2246	0.8117**	-0.8233**	0.9030**	-0.6009	-0.6630	-0.1186	0.4320
F_v/F_m		0.2966	0.4429	0.5410	-0.5724	0.5642	-0.7540*	-0.3687	-0.4769	0.1211
ETR _{in situ}			0.6183	0.2868	-0.0169	0.4543	0.0302	0.1007	-0.3443	-0.2320
ETR _{ex situ}				0.5491	-0.3144	0.4387	-0.5174	-0.1781	-0.6499	0.4058
ETR _{max}					-0.8299**	0.7329*	-0.7590*	-0.4032	-0.4494	0.6672*
NPQ _{max}						-0.8015**	0.8476**	0.7343*	0.1729	-0.7484*
ETR _{max} /NPQ _{max}							-0.6307	-0.7292*	-0.2617	0.4500
C								0.5792	0.4938	-0.7228*
N									-0.0617	-0.5493
C/N										-0.1556
S prot.										
Carboh.										
Lipids										
Phenols										
Chla										
PE										
PC										
MAAs										
ABTS										

Table SM.3.1. 2. Continue

	Carboh.	Lipids	Phenols	Chla	PE	PC	MAAs	ABTS	DPPH
DGR	0.4843	0.7877*	0.8128**	0.5577	0.3288	0.2158	0.3595	0.7677*	0.7596*
F_v/F_m	0.1425	0.7301*	0.7919*	0.5004	0.5392	0.4904	0.4796	0.3861	0.4484
ETR _{in situ}	-0.2181	0.2275	0.3017	0.2384	0.2996	0.4391	-0.0202	-0.0179	0.1694
ETR _{ex situ}	-0.4693	0.5690	0.2909	0.6190	0.8229**	0.8809**	0.5118	0.2259	0.5709
ETR _{max}	0.2806	0.8831**	0.7452*	0.8196**	0.6429	0.4578	0.3850	0.9093*	0.7914**
NPQ _{max}	-0.3328	-0.8248**	-0.6793*	-0.8500**	-0.6672*	-0.3052	-0.6904*	-0.8722*	-0.7343**
ETR _{max} /NPQ _{max}	0.3297	0.8073**	0.7353*	0.6390	0.5443	0.4424	0.5901	0.5919	0.8065**
C	-0.1748	-0.8871**	-0.6487	-0.8100**	-0.7998**	-0.5488	-0.7670*	-0.6879*	-0.7651*
N	-0.1482	-0.5757	-0.4409	-0.4215	-0.4189	-0.3353	-0.7884*	-0.4093	-0.6744*
C/N	-0.1402	-0.6403	-0.5194	-0.3337	-0.5591	-0.7983**	-0.2608	-0.1414	-0.4379
S prot.	0.0016	0.5915	0.1723	0.7830*	0.6892*	0.3278	0.6705*	0.7168*	0.7280*
Carboh.		0.3660	0.5606	-0.0166	-0.2436	-0.2538	-0.1380	0.3802	0.2096
Lipids			0.8598**	0.7244*	0.7014*	0.6546	0.6068	0.7166*	0.8727**
Phenols				0.4545	0.3700	0.4347	0.2926	0.6154	0.5915
Chla					0.8882**	0.4482	0.6645	0.7854*	0.6319
PE						0.7533*	0.8091**	0.4823	0.6151
PC							0.5990	0.1199	0.5952
MAAs								0.3114	0.6289
ABTS									0.6197

DGR: Daily growth rate; F_v/F_m : Maximal quantum yield0. ETR: Electron transport rate; NPQ: Non-photochemical quenching; C: Total carbon; N: Total nitrogen; S prot.: Soluble proteins; Carboh.: Carbohydrates; Chla: Chlorophyll a; PE: Phycoerythrin; PC: Phycocyanin; MAAs: Mycosporine-like amino acid



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SUB-CHAPTER 3.2

Short-term effects of UVA-Violet-Blue radiation, nutrient concentrations, and desiccation on the photosynthesis and accumulation of bioactive compounds in *Neopyropia leucosticta*



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Short-term effects of light quality, nutrient concentrations and emersion on the photosynthesis and accumulation of bioactive compounds in *Neopyropia leucosticta* (Rhodophyta)

Abstract

Intertidal macroalgae have developed adaptation and acclimation strategies protecting against stressful environments. The red algae, *Porphyra sensu lato* are well known for their use as human food, but they also present potential applications in cosmeceutics. This study evaluated the effect of three different stress factors, through two bi-factorial experiments, the combination of PAR and PAR supplemented by UV-A, Violet and Blue radiation ($PAR_{UVAVIBI+}$) and three nutrient concentrations or emersion/immersion conditions. Interactive effect of $PAR_{UVAVIBI+}$ and nutrient concentrations on the daily integrated electron transport rate ($_{int}ETR$) was observed, suggesting a photoprotective effect of nitrate against the possible damage effect of the UV-A radiation. The emersion produced a decrease in the *in situ* ETR followed by a recovery during re-hydration. The energy dissipation rate (EDR), firstly described in this work, increased under nutrient limitation and under $PAR_{UVAVIBI+}$ radiation, as a photoprotection mechanism. The surface/volume (S/V) ratio of the cells increased under nutrient limitation favoring the nitrogen assimilation, although it could be a risk of photodamage under UV-A radiation. Biliproteins and mycosporine-like amino acids (MAAs) clearly increased under high nutrient availability as it was expected due to both are N-compounds. Phenolic compounds were higher under $PAR_{UVAVIBI+}$ at day 2, indicating a photoprotective mechanism, whereas this effect disappeared at day 7. Emersion seems to induce MAAs accumulation under $PAR_{UVAVIBI+}$ radiation. Antioxidant capacity determined by ABTS was positively correlated to antioxidant substances as biliproteins, polyphenols and MAAs. The application of short-term stress conditions (2-7 days) during culture, can be a strategy to increase the content of interesting compounds due to both UV screen properties and antioxidant capacity.

Keywords: biliproteins, blue and violet light, emersion, integrated electron transport rate, mycosporine-like amino acids, ultraviolet A radiation

1. Introduction

Intertidal macroalgae have developed different adaptation and acclimation strategies to protect themselves against a harsh and highly variable environment (e.g. periods of desiccation, high solar radiation, hydrodynamics or changing temperature and salinity), such as strong antioxidant defense systems (e.g. catalase or superoxide dismutase enzymes), the synthesis of secondary metabolites with antioxidant, photoprotective or anti-herbivory properties (e.g. phenolic compounds, carotenoids or mycosporine-like amino acids), photorepair mechanisms (e.g. photolyases) and energy dissipation mechanism (i.e. non photochemical quenching) (Lalegerie *et al.*, 2020a; Figueroa *et al.*, 2021). Beside these, the synthesis of primary metabolites, that are mainly implicated in physiological and structural functions (e.g. amino acids, proteins, lipids or polysaccharides), also play an important role in the protection against environmental factors (Stengel and Connan, 2015; Nunes *et al.*, 2017; Rezayian *et al.*, 2019; Olsson *et al.*, 2020).

Macroalgae have acquired a great biotechnological interest in the last years, due to the wide diversity of bioactive compounds with several potential applications. Among the bioactive compounds found in macroalgae, three of them are highlighted in this study: biliproteins, phenolic compounds and mycosporine-like amino acids (MAAs). Biliproteins as accessory pigments are involved in the light absorption for photosynthesis in some algae species (Rhodophyta, Cyanobacteria and Cryptophyta). These compounds have been used as fluorescent probes in immunofluorescence assay, food additives or natural colorants in ice-creams, candies, or soft drinks. In addition, different therapeutic properties have been described in these molecules, such as antioxidant, anti-cancer, neuroprotective, anti-inflammatory, antiviral or hepatoprotective (Sonani *et al.*, 2016; Chen *et al.*, 2022). Phenolic compounds are secondary metabolites, that can be found in plants and algae. Among algae, brown algae normally present the highest content (Zubia *et al.*, 2007; Guinea *et al.*, 2012; Heffernan *et al.*, 2014; Tenorio-Rodriguez *et al.*, 2017). These compounds act as protective agents that are synthesized in response to stress conditions like herbivory or UV radiation. Several bioactivities with potential pharmaceutical properties have been described in these compounds: antioxidant, anti-inflammatory, antimicrobial, antiviral, antidiabetic, anticancer or neuroprotective (Cotas *et al.*, 2020; Lomartire *et al.*, 2021). Mycosporine-like aminoacids (MAAs) are secondary metabolites, mainly found in red algae and cyanobacteria and they are involved in photoprotection. These molecules have been considered potential compounds to be

used as natural UV filters in sunscreens, due to their capacity to absorb the UV radiation, their no-toxicity and biodegradable properties. In addition, different beneficial properties for the skin have been described in these molecules, such as antioxidant, anti-inflammatory, wound-healing or anti-aging (Wada *et al.*, 2015; Chrapusta *et al.*, 2017; Vega *et al.*, 2021).

The physiology and biochemical composition of algae can be influenced by the environmental factors such as light quality, nutrients availability or desiccation. Modify or control these factors during algae culture can influence the growth or induce the accumulation of specific compounds. Light quality can affect photosynthesis through both photosynthetic process (Korbee *et al.*, 2005a; Schneider *et al.*, 2020a) and the regulation of some physiological responses by the action of non-photosynthetic photoreceptors in photomorphogenic process (e.g. cryptochromes or phytochromes) (Rüdiger and López-Figueroa, 1992; Kianianmomeni and Hallmann, 2014). Thus, different authors have analyzed the effect of different light qualities in algal culture. Some of them cultivated algae under broad band light colors, using white light lamps with different filters (Figueroa *et al.*, 1995; Franklin *et al.*, 2001; Korbee *et al.*, 2005a; Barufi *et al.*, 2015), and other researchers studied the photomorphogenic responses of different light qualities, by adding a small light supplementation by using LEDs (5-20 %) of UV-A, blue, green, or red radiation to a saturating photosynthetic irradiance produced by amber light (Schneider *et al.*, 2020a, 2022; Ghedifa *et al.*, 2021). Most reports observed that UV-A radiation and blue light increased the accumulation of certain compounds such as MAAs or biliproteins in different red algae species, or that supplementation of UV-A radiation (as green or red light) can increase growth, but reduce the photosynthetic rate. In this work, the combination of UV-A, violet and blue radiation is studied (320-450 nm), since all of them could be potentially detected by the same photoreceptor, the cryptochrome, and in addition possible photodamage by UVA radiation can be also detected (Petersen *et al.*, 2021).

In relation to the nutrient availability, high nitrogen conditions increased the photosynthetic capacity, growth, as well as the accumulation of N-compounds (e.g. proteins, phycobiliproteins or MAAs) (Korbee-Peinado *et al.*, 2004; Barufi *et al.*, 2011; Figueroa *et al.*, 2022). The culture of red algae under IMTA systems (high nitrogen supply through fishpond effluents) have been analyzed by some researchers, both inland or open water systems (Hernández *et al.*, 2005; Figueroa *et al.*, 2008; Abreu *et al.*, 2011b; Bermejo *et al.*, 2019; Ashkenazi *et al.*, 2022) obtaining promising results with

high nitrogen uptake rates and biomass productivity, and the accumulation of certain compounds with commercial application such as biliproteins or MAAs.

As mentioned before, intertidal algae face periods of desiccation, although not all of them tolerate it in the same way. According to Contreras-Porcia *et al.* (2017) and Lalegerie *et al.* (2020a) “desiccation tolerance is known as the ability to withstand a significant water loss and a fast recovery during re-hydration, and not the ability to retain water”. There are tolerant species, those that live in the upper intertidal zone, e.g. *Porphyra* sp. can lose more than 90 % of the internal water content, follow by a fast recovery after de-hydration (Ji and Tanaka, 2002; Contreras-Porcia *et al.*, 2011). By contrast, those species living in the lower intertidal zone are more sensitive to desiccation, e.g. *Gelidium rex* does not recover after re-hydration (Flores-Molina *et al.*, 2014). Desiccation can provoke different morphological, biochemical or physiological responses in macroalgae. For example, Contreras-Porcia *et al.*, (2011) observed morphological changes in *Pyropia columbina* (formerly *Porphyra columbina*) during low tides desiccation, showing that dehydrated fronds become dark purple, tightly folded, stiff and brittle, as a strategy to prevent the rupture of the plasma membrane.

Bladed *bangiales* (*Porphyra sensu lato*) are common species globally distributed in intertidal zones, although they are more abundant in cold temperate waters. These species present one cell layers and are characterized by their elastic thalli, high resistance to desiccation and a seasonal cycle (i.e. a macroscopic phase, folious blades, can be observed during winter, whereas the summer (long photoperiod and warm temperatures) promotes the sporulation and the microscopic filamentous conchocelis phase). *Neopyropia leucosticta*, previously *Pyropia leucosticta* (and earlier as *Porphyra leucosticta*) is commonly found in the intertidal Mediterranean coast, sharing habitats with other macroalgae as *Rissoella verrucullosa* (endemic to the Mediterranean Sea), *Nemalion helminthoides* (Rhodophyta) or *Ulva* sp. (Chlorophyta) (Flores-Moya *et al.*, 2021). *Porphyra sensu lato* are commercially known as nori or laver and are among the most cultured macroalgae in the world (mainly concentrated in Asian countries), with an economic value that is growing over the years (Cai *et al.*, 2021; FAO, 2022b). Besides their known used as human food (e.g. nori blades for sushi), *Porphyra sensu lato* could be source of bioactive compounds with relevant industrial applications, such as mycosporine-like amino acids (MAAs) for their photoprotective properties (Vega *et al.*, 2021; Rosic *et al.*, 2023), or sulphated polysaccharides called “porphyrans” that have

been described as anti-cancer, antioxidant or immunomodulatory (Chen and Xue, 2019; Li *et al.*, 2020; Wu *et al.*, 2020).

Considering that algal metabolism must be fitted to so harsh environmental variable conditions, and that *N. leucosticta* is a relevant species for biotechnological applications, our study aimed to evaluate the effects of three physiological regulating parameters (UVA-Violet-Blue radiation, nutrients concentration and emersion), through two bi-factorial experiments in laboratorial conditions, combining UVA-Violet-Blue radiation and nutrient concentrations and emersion/immersion conditions, and analyzing different responses such as the photosynthetic capacity, cell morphology and biochemical composition.

2. Material and Methods

2.1. Algae biomass

Neopyropia leucosticta (Bangiales, Rhodophyta) thalli were collected in January 2021 from Lagos (Malaga, Southern Spain; 36°44'N; 4°01'W). Algae were transported to the laboratory in a portable fridge at 4 °C and cleaned to remove sediments and epiphytes. Algae were maintained in polyvinyl methacrylate cylinders (1 L seawater) at an algal density of 10g fresh biomass (FB) L⁻¹ at 20 °C temperature, salinity of 36, irradiance of photosynthetic active radiation (PAR, λ = 400-700 nm) of 150 μ mol photons m⁻² s⁻¹ (White LED light, 5000K, 54W, NU-8416, Nuovo) with a photoperiod of 12:12 hours (light:dark) and under low nutrients concentration (50 μ M NaNO₃ and 2.7 μ M of C₃H₇Na₂O₆P added each week), before the starting of the experiments.

2.2. Experimental design

In this study, two different bi-factorial experiments were performed on *N. leucosticta*. The first one considered the effects of two light qualities: PAR and PAR supplemented by UV-A, Violet and Blue radiation (PAR_{UVAVIBI+}), and nutrient availability (three different concentrations); and the second one, the effects of the same two light qualities and emersion/immersion conditions. All treatments were performed in triplicate.

The radiation treatment was the same in both experiments: PAR (λ = 400-700 nm), provided by a white LED light (5000K, 54W, NU-8416, Nuovo), and PAR supplemented

with UV-A ($\lambda = 320\text{-}400\text{ nm}$), violet ($\lambda = 400\text{-}450\text{ nm}$) and blue light ($\lambda = 450\text{-}500\text{ nm}$). The supplementation was obtained combining a fluorescent UV-A lamp (UVA340, Q-Lab Corporation, Canada) with a Lee-130 filter (de Oliveira *et al.*, 2021) to cut-off the UV-B radiation, and an UVA-Violet-Blue LED lamp (Aralab, Portugal), that present peaks at 370, 406 and 446 nm (Figure 3.2. 1: A; Figure SM.3.2. 1). This combination of UV-A, Violet and Blue radiation was applied since it is known the photocontrol by non photosynthetic UVA/Blue photoreceptor (Cryptochromes) in the synthesis of different bioactive compounds in macroalgae, including *Porphyra* spp. (López-Figueroa and Niell, 1989; López-Figueroa, 1991; Rüdiger and López-Figueroa, 1992; Figueroa *et al.*, 1994, 1995).

To simulate a daily cycle of natural radiation, PAR irradiance increased from 150 to 300 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ during the central period of the day (13-17 pm). The supplemented radiation was also provided during this period at the irradiances of 8 W m^{-2} of UV-A and 70 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of violet-blue light, corresponding to a supplementation of 15 % of UV-A and 30 % of violet-blue radiation (Figure 3.2. 1: B). The white light used for the PAR already have a peak in blue (446 nm), and the PAR_{UVA+VIB+} treatment was supplemented with more blue (446 nm) and other two peaks 365 nm (UV-A) and 404 nm (violet). The irradiance of PAR was measured using a radiometer (LI-1000 Data Logger, LI-COR Biosciences, USA) with a flat sensor (LI-190R quantum sensor, LI-COR Biosciences, USA). The spectrum of the different lamps and the irradiance of the UV radiation was measured by using a spectroradiometer (SMS 500, Sphere Optics, Germany). The daily integrated irradiances received by algae in each treatment are shown in **Table 3.1. 1**. The supplemented radiation (UV-A and Violet-Blue) represents an increase in the daily dose of 4 % for UV-A radiation, and 15 % for Violet-Blue radiation. The calculated effective radiation doses against five different action spectra: photosynthesis (Haxo and Blinks, 1950), chloroplast photoinhibition (Jones and Kok, 1966), DNA damage (Setlow, 1974), general damage in plants (Caldwell, 1971) and lipid peroxidation (Morliere *et al.*, 1995) (Figure SM.3.2. 2) are shown in Table 3.2. 2.

In the first experiment, three different nutrient concentrations were tested: 500 + 27.7, 250 + 13.8 and 50 + 2.7 μM of NaNO_3 and $\text{C}_3\text{H}_7\text{Na}_2\text{O}_6\text{P}$, respectively. Provasoli medium (Provasoli, 1968; Andersen, 2005) was added in all cylinders and only NaNO_3 and $\text{C}_3\text{H}_7\text{Na}_2\text{O}_6\text{P}$ were modified for the different treatments. After five days of the experimental period, half of nitrate and phosphate compared to initial values were added.

To achieve algal emersion, a net was placed at the bottom of the cylinder and during the central hours of the day (13-17 pm), the net was raised so that algae were out of the water during this period. In those cylinders with no emersion, a net was also placed at the bottom of the cylinders and it was also raised, although algae were maintained covered by the water in the upper part of the cylinder.

During the experiments, algae were maintained at a concentration of 5 g L⁻¹, at 20 °C and salinity of 36. Both experiments were conducted for seven days. In the second experiment Provasoli medium was also added to all cylinders with the normal nitrate and phosphate concentration (500 µM of NaNO₃ and 27.7 µM C₃H₇Na₂O₆P). In the fifth day of the experiment, 50 µM of nitrate and 2.7 µM phosphate were also added.

Different parameters were assessed during both experimental periods. Nitrogen uptake rate (NUR) was analyzed from seawater samples. Photosynthesis was followed by *in vivo* chlorophyll a fluorescence of photosystem II (PSII). Biochemical composition and antioxidant capacity were determined by using different spectrophotometric and chromatographic procedures at day 2 (500 mg of fresh biomass were collected) and at the end (day 7) of the experimental period.

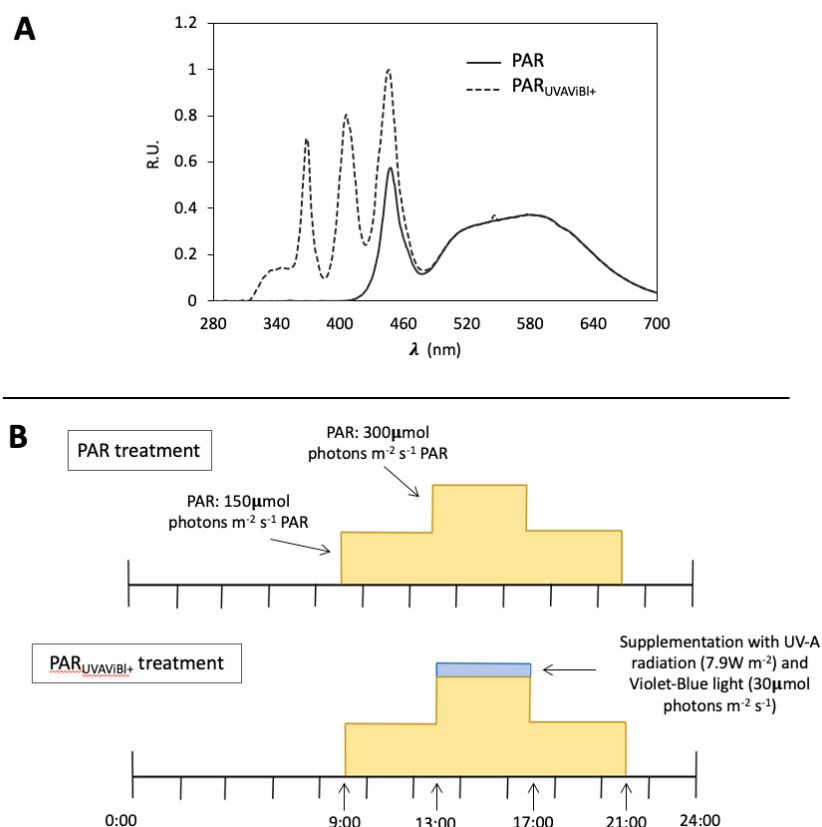


Figure 3.2. 1. Experimental design of the radiation treatment used in both experiments with *Neopyropia leucosticta* evaluating: (1) the effect of supplemented light treatment with UV-A, violet and blue radiation (P_{UVAViBI+}) and nutrient concentrations (500, 250 and 50 μM NaNO₃), and (2) the effect of P_{UVAViBI+} and emersion/immersion condition. A) Spectra of the two radiation treatments used in the experiments. B) Design of the radiation treatments. PAR was provided from 9:00 to 21:00. During central hours of the day (13:00 to 17:00), PAR irradiance increased, and samples also received supplementation of UVA-Violet-Blue radiation.

Table 3.2. 1. Daily radiation doses received ($\text{kJ m}^{-2} \text{ day}^{-1}$) in each light treatment (PAR and $\text{PAR}_{\text{UVAVB}+}$) during the experimental period. The doses were also calculated for different fractions of the full spectra received in each treatment.

	PAR	$\text{PAR}_{\text{UVAViBI}+}$
UV-B (280-320)	0.22	1.00
UV-A (320-400)	0.86	54.71
Violet+Blue (400-500)	359.88	577.13
PARwithout Vi+BI (500-700)	1031.40	1033.22
PAR (400-700)	1391.28	1610.35
TOTAL	1392.36	1666.1

Table 3.2. 2. Daily effective radiation doses received ($\text{kJ m}^{-2} \text{ day}^{-1}$) related to different biological responses (photosynthesis, DNA damage, chloroplast photoinhibition, general damage in plants and lipid peroxidation) driven by UVR and PAR, in each radiation treatment during the experimental periods. The doses were also calculated for different fractions of the full spectra received in each treatment.

	Photosynthesis		DNA damage		Chloroplast photoinhibition		General damage in plants		Lipid peroxidation	
	P	P _{UVAViBI+}	P	P _{UVAViBI+}	P	P _{UVAViBI+}	P	P _{UVAViBI+}	P	P _{UVAViBI+}
UV-B (280-319)	0.00	0.00	0.03	0.08	0.13	0.50	0.08	0.23	0.10	0.31
UV-A (320-399)	0.00	0.00	0.00	0.00	0.11	7.11	0.00	0.27	0.03	1.69
Violet-Blue (400-499)	64.47	95.30	0.00	0.00	0.00	0.32	0.00	0.02	0.00	0.05
PAR_{without Vi+BI} (500-700)	639.61	641.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PAR (400-700)	704.08	720.53	0.00	0.00	0.00	0.32	0.00	0.02	0.00	0.05
TOTAL	768.55	815.83	0.03	0.08	0.24	8.25	0.08	0.54	0.12	2.11

2.3. Nitrate uptake rate (NUR)

For nutrients analysis, 10 mL of water were collected from the culture cylinders at day 0, 2, 5 and 7 for the first experiment, and at day 0, 3, 5 and 7 for the second experiment. At day 5, water was sampled before and after the addition of new nutrient input. Water samples were filtered through 0.45 μm and frozen at $-20\text{ }^{\circ}\text{C}$ until analysis. Total nitrate contents were analyzed by segmented flow analyzer (SFA) using a Seal Analytical autoanalyzer QuAatro following the methods described by Grasshoff *et al.* (1999). The detection limits of the inorganic nutrients were $0.05\mu\text{M}$. The nitrate uptake rates (NUR) were calculated according to the following formula and expressed as $\mu\text{mol NaNO}_3\text{ L}^{-1}\text{ day}^{-1}$.

$$\text{NUR} = (C_1 - C_2) / t$$

where C_1 and C_2 are the initial and final concentrations expressed as $\mu\text{mol NaNO}_3$, and t is the time expressed as days.

2.4. Photosynthetic activity by using *in vivo* chlorophyll a fluorescence

The photosynthetic activity of *N. leucosticta* was estimated through the *in vivo* chlorophyll a fluorescence associated to photosystem II (PSII), using a Mini-PAM-II (Walz GmbH, Germany). Two different approaches were followed:

2.4.1. *In situ* measurements

The *in vivo* chlorophyll a fluorescence was measured *in situ*, i.e under the growth conditions in the culture chambers, during different days and periods of the day. In the first experiment (light quality and nutrients), it was monitored during days 0, 2, 5 and 7, and at 8:30, 10:00, 14:00 and 18:00. In the second experiment (light quality and emersion) it was determined during days 0, 3, 6 and 7 at 8:30, 10:00, 14:00, 16:00 and 18:00. For that, saturating pulses ($> 3000\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$) were applied to algal thalli (three replicates per cylinder) during darkness of the daily photoperiod (8:30 am) and at different times during the light cycle, in order to quantify the basal (F_0), steady-state (F_t) and the maximal fluorescence (F_m measured in samples at darkness or F_m' in

light-acclimated samples). Optimal quantum yield (F_v/F_m) and the effective quantum yield ($Y(II)$) were calculate as follows:

$$F_v/F_m = (F_m - F_0) / F_m$$

$$Y(II) = (F_m' - F_t) / F_m'$$

Electron transport rate (ETR; $\mu\text{mol electrons m}^{-2} \text{ s}^{-1}$) values were calculated using the following formula:

$$\text{ETR} = Y(II) \times E_{\text{PAR}} \times A \times F_{II}$$

where E_{PAR} is the irradiance in the PAR region of the spectra ($\lambda = 400\text{-}700 \text{ nm}$) and expressed as $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, A is the absorptance that was calculated as $A = 1 - (E_t / E_0)$ (E_t is the irradiance that pass through the thallus and E_0 is the emitted irradiance), and F_{II} is the fraction of chlorophyll a associated to PSII, in red algae is 0.15 (Johnsen and Sakshaug, 2007). The ETR values obtained for each day were integrated using the trapezoidal rule, allowing the calculation of intETR (integrated ETR).

2.4.2. Rapid light curves (RLC) and Yield losses

Before the starting of the rapid light curves (RLCs), algae were incubated in darkness for 15 minutes, in order to oxidize the reaction centers. During RLCs, algae were incubated during 30 seconds at increasing irradiances (from 0 to $1500 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) of the actinic red light of the Mini-PAM-II. After incubation at each intensity, a saturating pulse was performed. Different parameters were calculated for each intensity of the RLCs: F_v/F_m , $Y(II)$, ETR, two different yield losses: $Y(\text{NPQ})$ calculated as $(F_t / F_m') - (F_t / F_m)$ and $Y(\text{NO})$ calculated as F_t / F_m , and non-photochemical quenching (NPQ) calculated as $Y(\text{NPQ}) / Y(\text{NO})$. Similarly to (Figueroa *et al.* 2021), it was calculated the amount of absorbed energy used in photochemistry (ETR) and amount of absorbed energy that is dissipated (yield losses), named as energy dissipation rate (EDR) and calculated as ETR, but replacing $Y(II)$ values for the sum of both yield losses ($Y(\text{NPQ}) + Y(\text{NO})$), and it was expressed as $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

$$\text{EDR} = (Y(\text{NO}) + Y(\text{NPQ})) \times E \times A \times F_{\text{II}}$$

The obtained ETR vs. irradiance, curves were fitted according Eilers and Peeters (1988). The following RLC descriptive parameters were obtained: ETR_{max} , photosynthetic efficiency (α_{ETR}) and saturated irradiance ($E_{k_{\text{ETR}}}$).

Previously to the experiments, a RLC and an *in situ* light curve ($\text{LC}_{\text{in situ}}$) were performed, in order to determine the appropriate PAR irradiances during experiments (Figure SM.3.2. 3). For the $\text{LC}_{\text{in situ}}$, algae were incubated for one minute to increasing irradiances of the white LED light (from 0 to 700 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). The E_k values obtained were 130 and 175 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ from RLC and $\text{LC}_{\text{in situ}}$, respectively. So, an irradiance of 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ was selected.

2.5. Microscopic analyses

Microscopic images were made on algal thalli immerse in 20 % glycerine, using the Olympus VS120 (Tokio, Japan) virtual microscopy slide scanning. The processing of the obtained images was done with the software ImageJ. The following parameters were calculated from the surface view: (a) number of cells, (b) percentage occupied by the protoplasm, and (c) average size of the protoplasm.

2.6. Biochemical analyses

All biochemical analyses were performed from the fresh weight (FW), except total carbon, nitrogen and sulphur content that need dry weight (DW). The ratio FW/DW was calculated as 5.5 and all results were expressed as $\text{mg g}^{-1} \text{DW}$. Biochemical analyses were performed at day 0, 2 and 7 for the first experiment, and at day 0, 3 and 7 for the second experiment (except total carbon, nitrogen and sulphur content in the second experiment, that was only determined at day 7).

2.6.1. Total internal carbon, nitrogen and sulphur

Total carbon, nitrogen and sulphur content were determined from dry biomass using an elemental analyzer (CNHS LECO-932, Michigan, USA) in the Research Support Central Services (SCAI, University of Malaga).

2.6.2. Photosynthetic pigments

For phycobiliproteins determination, 50 mg of FW were homogenized in 1.5 mL of phosphate buffer (pH 6.5, 0.1M) using pistil, mortar, and sand as abrasive. After overnight incubation at 4 °C, samples were centrifuged (Heraeus Labofuge 400R, Thermo Scientific, Waltham, MA, USA) and measured by using a spectrophotometer (UV-2600, Shimadzu, Duisburg, Germany) at the following wavelengths: 455, 564, 592, 618, 645 and 730 nm. Quantification was made using the chromatic formulas described Beer and Eshel (1985) for phycoerythrin (PE) and phycocyanin (PC).

In the case of chlorophyll determination, 50 mg of FW were homogenate in 1.5 mL of 90 % acetone saturated with magnesium carbonate using pistil, mortar, and sand as abrasive. After incubation (4 °C overnight) and centrifugation, extracts were spectrophotometrically measured at the following wavelengths: 664, 691 and 750 nm and total of chlorophyll a (Chl a) was quantified according to Ritchie (2006).

2.6.3. Phenolic compounds

Phenolic compounds were measured using the Folin-Ciocalteu method according to Singleton and Rossi (1965) with some modifications. Fifty mg of FW were extracted in 1.5 mL of methanol 80 % using pistil, mortar, and sand as abrasive, and leave overnight at 4 °C. After centrifugation, 100 µL of the extracts was mixed with 700 µL of $\text{d}_2\text{H}_2\text{O}$ and 50 µL of Folin-Ciocalteu reagent (Sigma-Aldrich, San Luis, MO, USA). The mixture was vortexed and 150 µL of NaCO_3 20 % was added to the mixture and vortexed again. Samples were incubated during 2 h at 4 °C and measured by a spectrophotometer described above at 760 nm. Phloroglucinol (Sigma-Aldrich, San Luis, MO, USA) was used as standard.

2.6.4. Mycosporine like amino-acids (MAAs)

MAAs was determined using a uHPLC according to Korbee-Peinado *et al.* (2004) with some modifications of Chaves-Peña *et al.* (2020), i.e. resuspension of the samples in water instead of methanol. Fifty mg of FW were incubated in 1.5 mL of 20 % methanol in water during 2 h at 45 °C in a water bath. After that, samples were centrifuged and 700 µL of the supernatant were dried under vacuum (Speed-Vac SPD210, Thermo Scientific, Waltham, MA, USA). The dried samples were re-suspended in 700 µL of ultrapure H_2O , filtered through a 0.2 µm filter and injected in the uHPLC (1260 Agilent

InfinityLab Series, Santa Clara, CA, USA). MAAs were detected using a Luna C8 column (Phenomenex, Aschaffenburg, Germany), applying an isocratic flow of 0.5 mL min⁻¹ and a mobile phase of 1.5 % methanol and 0.15 % acetic acid in ultrapure H₂O. The detection was made using a photodiode array detector at 330 nm. Isolated MAAs through HPLC (Vega *et al.* 2023) were used as standards. Quantification was performed using the molar extinction coefficients (ϵ) of the different MAAs (Karsten *et al.*, 1998b). Results were expressed as mg g⁻¹ DW.

2.7. Antioxidant capacity

Two different methods based on the free radical scavenging effect were used to determine the antioxidant capacity.

The ABTS assay was performed according to Re *et al.* (1999) with some modifications. The ABTS radical cations (ABTS^{•+}) were generated via a reaction of 7 mM ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; Sigma-Aldrich, San Luis, MO, USA) and 2.45 mM K₂S₂O₈ in phosphate buffer (0.1M, pH:7). This reaction was stored for 12–16 h at room temperature to ensure the complete formation of the radical. ABTS^{•+} solution was diluted with phosphate buffer until the absorbance at 727 nm was around 0.75 ± 0.05. The same extracts prepared for phycobiliproteins quantification were used in this assay. For the reaction, 50 µL of the extracts were mixed with 950 µL of the diluted ABTS^{•+}. The mixture was incubated for 8min at room temperature and darkness, and absorbances were measured at 727 nm.

The DPPH assay was made according to Brand-Williams *et al.* (1995) with some modifications. The same extracts prepared for phenols quantification were used in this assay. For the reaction, 200 µL of the extracts were mixed with 1 mL of the DPPH[•] (2,2-diphenyl-1-picrylhydrazyl; Sigma-Aldrich, San Luis, MO, USA) solution (0.06 mM of DPPH in methanol 80 %). After 30 min of incubation at room temperature and darkness, absorbances were measured at 517 nm.

For both methods, a standard solution of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Sigma-Aldrich, San Luis, MO, USA) was used as reference and the results were expressed as µmol TE (Trolox equivalent) g⁻¹ DW.

2.7. Statistics

The software STATISTICA (V7) were used for the statistical analysis. Initially, data were checked for normality (Kolmogorov-Smirnov test) and homogeneity of variances (Cochran test). Then, two-way analyses of variance (ANOVA, $p < 0.05$) were used for the comparison of the treatments in the different analyzed variables. In the case of the first experiment, the factors were radiation (two levels: PAR and PAR+UVAVB) and nutrients availability (three levels: 500, 250 and 50 $\mu\text{M NO}_3$). For the second experiment, the factors were also radiation (same two levels) and emersion/immersion conditions (two levels: emersion (EMER) and immersion (IMMER)). These analyses were conducted separately for each time period. Newman-Keuls post-hoc test was used to identified homogenous groups after significant interaction in the ANOVA. Student-t test was used to compared obtained values with initial ones ($p < 0.05$). Pearson's correlation analysis was used to observed positive or negative correlations between dependent variables ($p < 0.05$ or 0.01).

3. Results

An increment of algal biomass during the experimental period was not observed, i.e the algal weight remained constant. However, during the acclimation period (two weeks), algae grew 1.2 % day^{-1} . The nutrient uptake rates (NUR) during both experimental periods are presented in the Table SM.3.2. 1. In the first experiment (light quality and nutrients), significant differences in the NUR were only observed among nutrients concentrations (no effect of the light quality was obtained) in both analyzed periods (Table SM.3.2. 3). The highest NUR were observed in the treatment with the highest nutrients availability. During the first period of the experiment (0-2 days), the highest NUR were 245.4 $\mu\text{mol NaNO}_3 \text{ L}^{-1} \text{ day}^{-1}$, whereas in the last period (5-7 days) with a lower nutrients addition, the highest NUR were 111.4 $\mu\text{mol NaNO}_3 \text{ L}^{-1} \text{ day}^{-1}$. During the second experiment (light quality and emersion/immersion), significant interaction between treatments were observed in the first periods of the culture (0-3 days), whereas in the last period (5-7 days) no significant differences were obtained (Table SM.3.2. 4). Significant differences were only observed in algae culture with $\text{P}_{\text{UVAVIBI+}}$ under immersion and emersion conditions (146.7 and 101.4 $\mu\text{mol NaNO}_3 \text{ L}^{-1} \text{ day}^{-1}$, respectively).

In situ electron transport rate (ETR) during the experimental period in both experiments is shown in Figure SM.3.2. 4. In all cases, there is an increment of *in situ*

ETR in the middle of the day, followed by a decrease at the afternoon. In order to compare the different treatments, the integrated daily ETR (ETR_{int}) of the different treatments were calculated for each day (Figure 3.2. 2). In case of the first experiment, no significant interaction between the studied factors were observed during the first day of the experiment, whereas significant effects in the treatments of each factor separately was observed (Table SM.3.2. 3). Algae under $PAR_{UVAViBI+}$ treatment presented a lower ETR_{int} ($0.27 \text{ mol e}^- \text{ m}^{-2}$), than algae under PAR treatment ($0.28 \text{ mol e}^- \text{ m}^{-2}$). Algae submitted to the lowest nutrient concentration ($50 \mu\text{M}$) showed the lowest ETR_{int} ($0.26 \text{ mol e}^- \text{ m}^{-2}$), in comparison with the other nutrient concentrations. In the rest of the experiment (day 2, 6 and 7) significant interaction between variables was obtained (Table SM.3.2. 3). At day 7, the lowest ETR_{int} was obtained in the treatments with low nutrients ($50 \mu\text{M}$; $0.19 \text{ mol e}^- \text{ m}^{-2}$), followed by the $PAR_{UVAViBI+}$ and $250 \mu\text{M}$ nitrate treatment ($0.24 \text{ mol e}^- \text{ m}^{-2}$), and for the rest of the treatments (PAR 500, PAR 250 and $PAR_{UVAViBI+}$ 500) without significant differences among them ($0.31 \text{ mol e}^- \text{ m}^{-2}$). In relation with the second experiment, significant differences between factors separately (no significant interaction) were observed in the first day of the experiment (Table SM.3.2. 4). Higher ETR_{int} values were observed in algae under $PAR_{UVAViBI+}$ radiation ($0.35 \text{ mol e}^- \text{ m}^{-2}$) than in algae under only PAR ($0.32 \text{ mol e}^- \text{ m}^{-2}$). Higher values were also observed in those algae with emersion (EMER: $0.35 \text{ mol e}^- \text{ m}^{-2}$) than in algae under no emersion (IMMER; $0.32 \text{ mol e}^- \text{ m}^{-2}$). In the third day of the experiment, significant interaction among factors was obtained (Table SM.3.2. 4), differences were only observed between the $PAR_{UVAViBI+}$ and IMMER ($0.36 \text{ mol e}^- \text{ m}^{-2}$) treatment and the others ($0.32 \text{ mol e}^- \text{ m}^{-2}$). At the end of the experiment (day 5 and 7), the interaction between factors disappeared (Table SM.3.2. 4), and significant differences were only observed between immersed or emerged conditions. Algae under immersed condition showed a highest $intETR$ (EMER: $0.29 \text{ mol e}^- \text{ m}^{-2}$; IMMER: $0.33 \text{ mol e}^- \text{ m}^{-2}$).

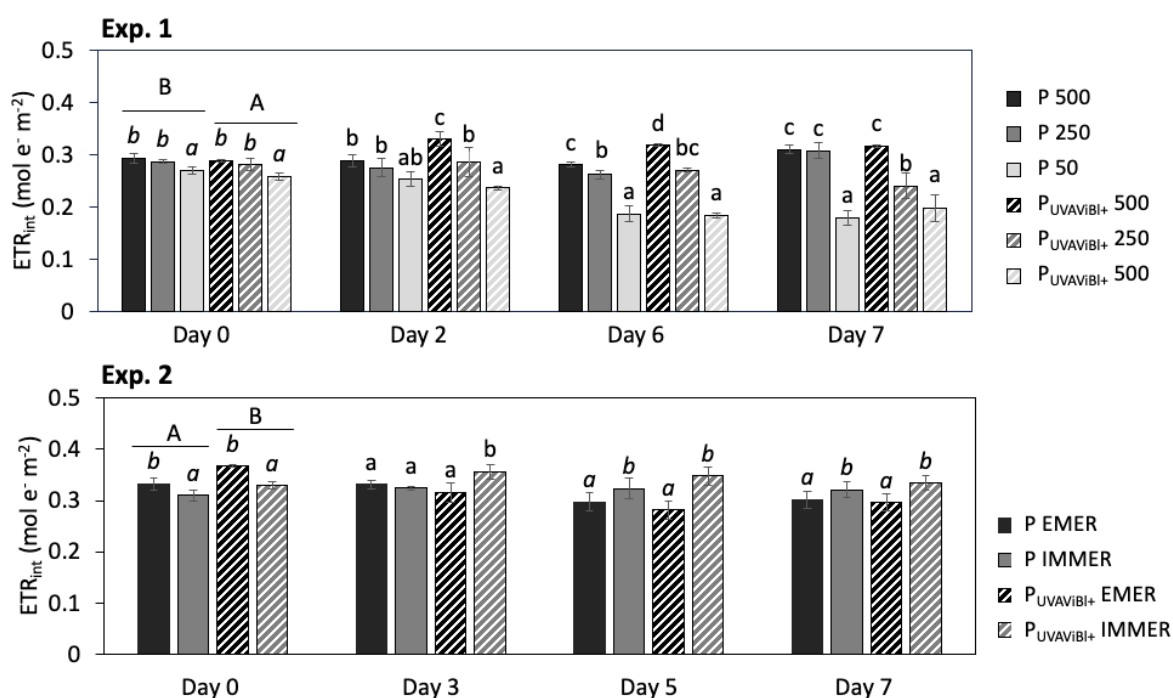


Figure 3.2. 2. Integrated electron transport rate (ETR_{int}) of *Neopyropia leucosticta* calculated with the in situ measurements show in figure SM.4 in both experiments with *Neopyropia leucosticta*: Exp. 1) light quality and nutrients (500, 250 and 50 μM of NaNO_3) and Exp. 2) light quality and emersion/immersion (EMER/IMMER) conditions. Values are expressed as mean $\pm$ standard deviation (SD) ($n = 3$). Different letters indicate significant differences among treatments: lower-case letters indicate significant interaction between variables (radiation and nutrients or radiation and emersion/immersion), capital letters indicate significant differences between radiation treatments (PAR and PAR_{UVAVIBI+}) and italic letters indicate significant differences among nutrients concentrations (50, 250 and 500) or emersion/immersion conditions (EMER and IMMER). ANOVA, SNK ($p < 0.05$).

In Table 3.2. 3, different values obtained from the RLCs at end of the experimental periods are shown. In case of the first experiment, F_v/F_m values showed interaction between factors (Table SM.3.2. 3). The lowest value was obtained in the treatment PAR_{UVAVIBI+} 50 (0.56) and the highest value was observed in the PAR 500 (0.7). Significant difference with the initial value (0.65) were only observed in the PAR_{UVAVIBI+} 50 treatment (0.56). The ETR_{max} values only showed significant differences among nutrients concentrations (Table SM.3.2. 3). The ETR_{max} varied from 2.15 $\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$ in the treatments with low nutrients (50), to 2.97 $\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$ in the treatments with high nitrogen (500). Significant differences with the initial value (3.83 $\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$) were observed in the treatments PAR 50, PAR_{UVAVIBI+} 250 and PAR_{UVAVIBI+} 50 (2.30, 2.17 and 2.01 $\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$, respectively). α_{ETR} and E_{kETR} values did not shown significant

differences among the treatments (Table SM.3.2. 3) or with the initial value. The average values of α_{ETR} and $E_{k_{ETR}}$ were 0.03 and 110.84 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, respectively. In the second experiment, no significant differences were observed in any of the variables (Table SM.3.2. 4). Significant differences with the initial values were only observed in certain cases, e.g. F_v/F_m value in the PAR EMER treatment showed a significant increased, the ETR_{max} value showed a significant decrease in the PAR_{UVAVIBI+} IMMER treatment, or the $E_{k_{ETR}}$ showed a significant decreased in the PAR EMER treatment.

Table 3.2. 3. Parameters obtained from the rapid light curves (RLCs) measured at day 7 of both experiments with *Neopyropia leucosticta*: Exp. 1) light quality and nutrients (500, 250 and 50 μM of NaNO_3) and Exp. 2) light quality and emersion/immersion conditions. Maximal quantum yield (F_v/F_m), maximal electron transport rate (ETR_{max}), photosynthetic efficiency of ETR (α_{ETR}) and saturated irradiance of ETR ($E_{k_{ETR}}$). Values are expressed as mean $\pm$ standard deviation (SD) ($n = 3$). Different letters indicate significant differences among treatments: lower-case letters indicate significant interaction between variables (radiation and nutrients or radiation and emersion/immersion), capital letters indicate significant differences between radiation treatments (PAR and PAR_{UVAVIBI+}) and italic letters indicate significant differences among nutrients concentrations (50, 250 and 500) or emersion/immersion conditions (EMER and IMMER). ANOVA, SNK ($p < 0.05$). Asterisk (*) showed significant differences between initial values and the obtained in the different treatments. Student's t-test ($p < 0.05$).

		F_v/F_m	ETR_{max}	α_{ETR}	$E_{k_{ETR}}$
Exp.1	Initial	0.65 $\pm$ 0.05	3.83 $\pm$ 1.07	0.03 $\pm$ 0.01	129.29 $\pm$ 19.89
	PAR 500	0.70 $\pm$ 0.01 ^c	3.03 $\pm$ 0.48 ^b	0.02 $\pm$ 0.00	139.94 $\pm$ 23.62
	PAR 250	0.66 $\pm$ 0.01 ^b	2.82 $\pm$ 0.73 ^{ab}	0.03 $\pm$ 0.01	110.73 $\pm$ 4.34
	PAR 50	0.66 $\pm$ 0.03 ^b	2.30 $\pm$ 0.15 ^{a*}	0.02 $\pm$ 0.01	103.09 $\pm$ 25.82
	PAR_{UVAVIBI+} 500	0.66 $\pm$ 0.01 ^b	2.92 $\pm$ 0.31 ^b	0.03 $\pm$ 0.01	122.11 $\pm$ 41.82
	PAR_{UVAVIBI+} 250	0.64 $\pm$ 0.01 ^b	2.17 $\pm$ 0.36 ^{ab*}	0.02 $\pm$ 0.01	109.86 $\pm$ 42.38
	PAR_{UVAVIBI+} 50	0.56 $\pm$ 0.03 ^{a*}	2.01 $\pm$ 0.32 ^{a*}	0.03 $\pm$ 0.00	79.28 $\pm$ 4.55 [*]
Exp.2	Initial	0.67 $\pm$ 0.01	3.95 $\pm$ 0.42	0.03 $\pm$ 0.00	128.76 $\pm$ 8.68
	PAR EMER	0.69 $\pm$ 0.01 [*]	3.08 $\pm$ 0.42	0.03 $\pm$ 0.00	112.16 $\pm$ 8.68 [*]
	PAR IMMER	0.69 $\pm$ 0.02	2.64 $\pm$ 0.87	0.02 $\pm$ 0.00 [*]	132.15 $\pm$ 54.27
	PAR_{UVVIBI+} EMER	0.68 $\pm$ 0.02	2.60 $\pm$ 0.20 [*]	0.02 $\pm$ 0.01	135.34 $\pm$ 54.50
	PAR_{UVAVIBI+} IMMER	0.68 $\pm$ 0.02	2.57 $\pm$ 0.75 [*]	0.02 $\pm$ 0.01	122.40 $\pm$ 19.38

Table SM.3.2. 2 show the ETR, the energy dissipation rate (EDR), and the non-photochemical quenching (NPQ) obtained during RLCs at 75, 150 and 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. In general, a higher EDR than ETR is observed in all treatments of both experiments (e.g. in the PAR 500 treatment at 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, the ETR and EDR values were 2.5 and 8.5, respectively), and the values of the three variables increased with the increasing irradiances (e.g. in the PAR 500 treatment at 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, the ETR values increased from 1.3 to 3.0, the EDR values increased from 3.5 to 19.0 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, and the NPQ values increased from 0.5 to 0.9). In the first experiment, ETR and EDR only showed significant differences among the different nutrient concentrations at 150 and 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, (no interaction between factors; Table SM.3.2. 3). Higher ETR values were observed in the treatments with high nitrogen than in the treatments with low nitrogen (e.g. at 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ETR values varied from 3.03 to 1.98 $\mu\text{mol e}^{-} \text{m}^{-2} \text{s}^{-1}$). The opposite occurs with the EDR, higher values were obtained in the treatments with low nitrogen (e.g. at 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, the EDR values varied from 20.1 to 19.0 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). In relation to NPQ values, a significant effect of the radiation treatments was observed for the three light irradiances (no significant interaction between factors; Table SM.3.2. 3). Higher NPQ values were observed in PAR_{UVAVIBI+} treatment than that under PAR treatments (e.g. at 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, the NPQ was 0.83 under PAR treatment and 1.27 under PAR_{UVAVIBI+}). In the second experiment, ETR and EDR values showed significant effects related to the radiation treatments at 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Table SM.3.2. 4). ETR values were higher in the PAR treatment (2.4 $\mu\text{mol e}^{-} \text{m}^{-2} \text{s}^{-1}$), than in the PAR_{UVAVIBI+} treatment (1.7 $\mu\text{mol e}^{-} \text{m}^{-2} \text{s}^{-1}$). The NPQ values did not shown significant differences among treatments (e.g. at 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, the average NPQ were 0.74). The ratio ETR/EDR at different irradiances in the RLCs are shown in Figure 3.2. 3. In general, the ratio decreased with the increasing irradiance (e.g. in the PAR 500 treatment the ratio varied from 0.36 to 0.16). In the first experiment, significant effect of the nutrient concentration was observed under 150 and 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (no significant differences were obtained for 75 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). The highest ratio was observed with the highest nitrogen level (500), and the lowest ratio with the lowest nitrogen (50) (e.g. at 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), decreasing the ratio from 0.16 to 0.11. In the second experiment, significant differences between the radiation treatments were only observed at 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, lower values were observed in the PAR_{UVAVIBI+} treatments (0.19) than in the PAR treatment (0.3).

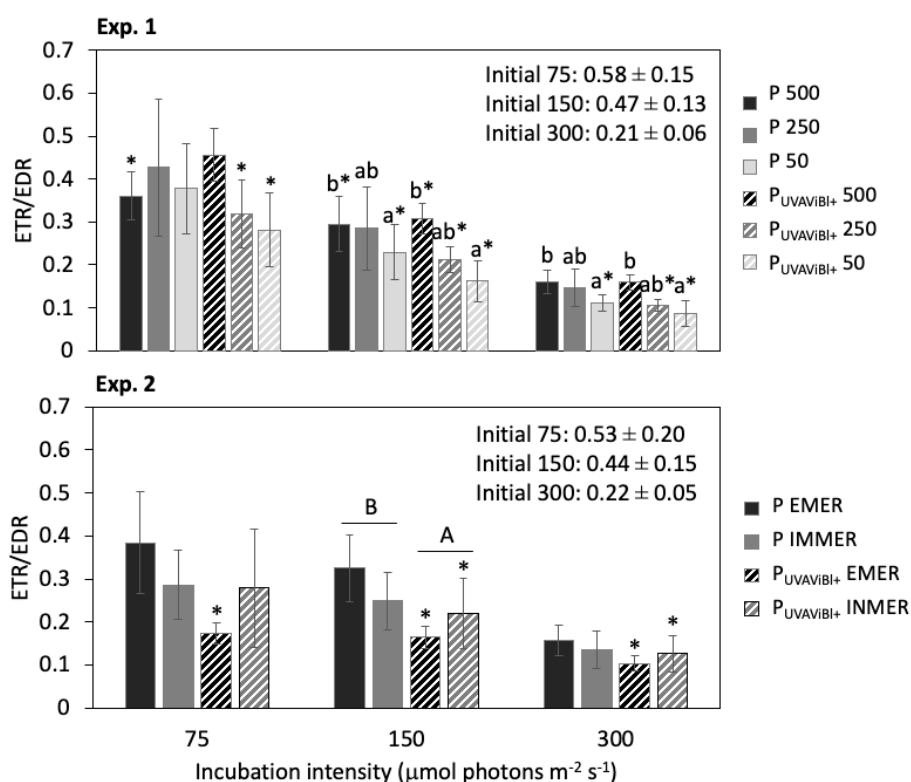


Figure 3.2. 3. Ratio between the electron transport rate (ETR) and the electron dissipation rate (EDR) obtained from the rapid light curves (RLCs) at 75, 150 and 300 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of both experiments with *Neopyropia leucosticta*: Exp. 1) light quality and nutrients (500, 250 and 50 μM of NaNO_3) and Exp. 2) light quality and emersion/immersion (EMER/IMMER) conditions. Values were expressed as mean $\pm$ standard deviation (SD) ($n = 3$). Different letters indicate significant differences among treatments: lower-case letters indicate significant interaction between variables (radiation and nutrients or radiation and emersion/immersion), capital letters indicate significant differences between radiation treatments (PAR and PAR_{UVAVIBI+}) and italic letters indicate significant differences among nutrients concentrations (50, 250 and 500) or emersion/immersion conditions (EMER/IMMER). ANOVA, SNK ($p < 0.05$).

Microscopic images of *N. leucosticta* thalli at the end of the experimental period are shown in Figure 3.2. 4. Clear spaces represent cell wall while internal content of each cell is colored. Some groups of small cells derived from mitotic division can be observed in all images. In general, the thalli remained with a red-brownish color. In the treatment with high N and only PAR, the red color was more patent, whereas in the treatment with low N and UVR, the color changed to yellowish (Figure 3.2. 4). Different parameters were calculated from those photos: number of cells in the analyzed area, percentage of the protoplasmatic area and average size of the protoplasm (Table 3.2. 4). In the first experiment, the cell number showed a significant effect of both studied factors separately (Table SM.3.2. 3). The number of cells decreased with the increasing nitrogen content,

i.e. the cell content varied from 147.6 in the high nitrogen treatment to 253.3 in the low nitrogen content. The cell number was also affected by the PAR_{UVAiBI+} radiation, i.e lower number was obtained in the PAR_{UVAiBI+} treatment (182.0) than in the PAR treatments (213.2). The protoplasmatic area and the protoplasm size showed interaction between the analyzed factors. The lowest protoplasmatic area (34.9 %) was observed in PAR 50, PAR 250 and PAR_{UVAiBI+} 50 treatments, the other treatments showed an area of 40.0 %. In the case of the protoplasm size, the lowest value was obtained in the PAR_{UVAiBI+} 50 treatment (15.0 μm), and the highest in the PAR 500 treatment (26.0 μm). In relation to the second experiment, significant effect of the radiation treatment was observed in the cell number and in the protoplasm size, whereas the protoplasmatic area was affected by desiccation (Table SM.3.2. 4). The number of cells increased under PAR_{UVAiBI+} treatments (159.2 vs 183.6). The protoplasmatic area increased under desiccation (38.6 vs 35.18). The protoplasm size decreased under PAR_{UVAiBI+} (32.69 vs 25.51).

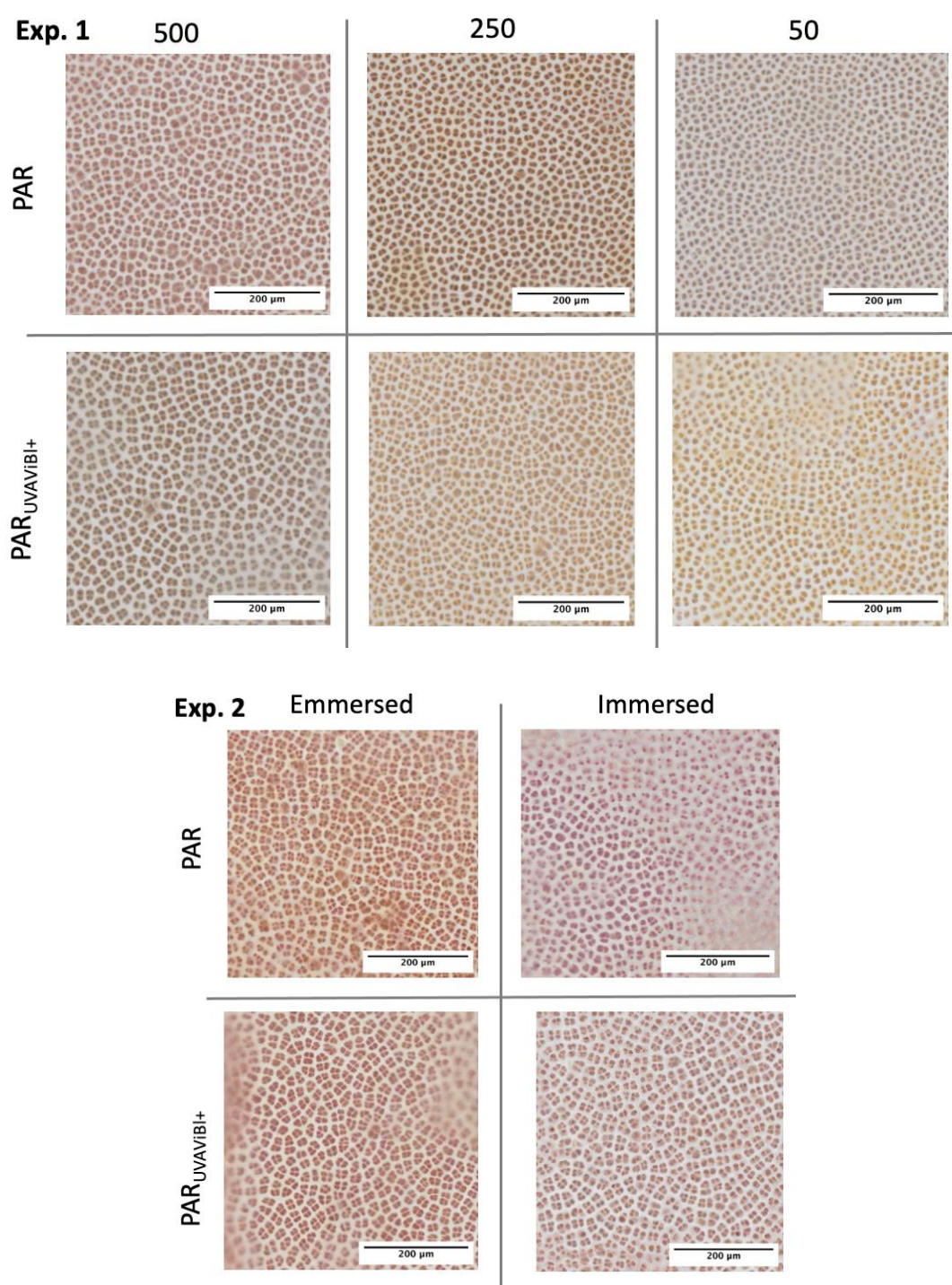


Figure 3.2. 4. Microscopic photos of *Neopyropia leucosticta* thalli at the end of the experimental period (day 7) for both experiments: Exp. 1) light quality and nutrient concentrations (500, 250 and 50 μM of NaNO_3); and Exp. 2) Light quality and emersion/immersion (EMER/IMMER) conditions. Images are representatives of a total of 3 analysed samples (3 images were performed of each sample).

Table 3.2. 4. Parameters measured from microscopic images obtained at the end of the experimental period in both experiments with *Neopyropia leucosticta*: Exp. 1) light quality and nutrient concentrations (500, 250 and 50 μM of NaNO_3); and Exp. 2) light quality and emersion/immersion (EMER/IMMER) conditions. Number of cells present in the analyze area, protoplasmatic area (%) and protoplasm average size (μm^2). Values are expressed as mean $\pm$ standard deviation (SD) ($n = 3$). Different letters indicate significant differences among treatments: lower-case letters indicate significant interaction between variables (radiation and nutrients or radiation and emersion/immersion), capital letters indicate significant differences between radiation treatments (P and PUVA+BLUE) and italic letters indicate significant differences among nutrients or emersion/immersion conditions (EMER/IMMER). ANOVA, SNK ($p < 0.05$).

	Treatment	n° cells	Protop. area (%)	Protop. average size (μm^2)
Exp. 1	PAR 500	165.89 $\pm$ 2.34 ^{aB}	40.49 $\pm$ 2.22 ^b	26.00 $\pm$ 2.28 ^d
	PAR 250	202.78 $\pm$ 32.67 ^{bB}	33.65 $\pm$ 2.24 ^a	18.24 $\pm$ 3.31 ^c
	PAR 50	271.06 $\pm$ 27.92 ^{cB}	32.01 $\pm$ 2.38 ^a	12.70 $\pm$ 0.60 ^a
	PAR_{UVAVIBI+} 500	129.25 $\pm$ 1.21 ^{aA}	39.74 $\pm$ 0.17 ^b	32.85 $\pm$ 0.30 ^c
	PAR_{UVAVIBI+} 250	181.06 $\pm$ 25.09 ^{bA}	38.77 $\pm$ 2.31 ^b	22.82 $\pm$ 4.43 ^b
	PAR_{UVAVIBI+} 50	235.44 $\pm$ 24.74 ^{cA}	32.17 $\pm$ 1.45 ^a	15.02 $\pm$ 1.02 ^{ab}
Exp. 2	PAR EMER	113.50 $\pm$ 2.00 ^A	37.57 $\pm$ 2.31 ^{ab}	34.28 $\pm$ 2.24 ^{bB}
	PAR IMMER	134.50 $\pm$ 10.17 ^A	36.91 $\pm$ 1.46 ^{ab}	29.69 $\pm$ 2.25 ^{aB}
	PAR_{UVAVIBI+} EMER	163.11 $\pm$ 17.02 ^B	39.72 $\pm$ 0.75 ^b	26.39 $\pm$ 2.04 ^{bA}
	PAR_{UVAVIBI+} IMMER	154.75 $\pm$ 12.25 ^B	35.02 $\pm$ 0.23 ^a	24.85 $\pm$ 1.61 ^{aA}

Table 3.2. 5 show elemental carbon, nitrogen and sulphur contents and the radio C/N, and Table 3.2. 6 show the concentration of phenolic compounds and the antioxidant capacity measured through two different radical scavenging methods (ABTS and DPPH). In the first experiment, the C content only showed significant differences between radiation treatments in the day 7 (Table SM.3.2. 3). Lower C values was obtained in the PAR_{UVAVIBI+} treatments (32.9 %) in comparison to PAR treatments (34.25 %). At day 2, no significant differences were observed among treatments although the C content increased in most of the treatment in comparison with the initial values. At day 7, only the PAR 500 treatment showed a significant increase compared to the initial value. In relation to the N content, a separately effect of nutrients concentration and radiation treatment was obtained in day 2 (Table SM.3.2. 3). The highest nitrogen content was obtained in the treatment with highest nutrient level (500; 2.76 %) and in the P treatments

(2.42 %). At day 7, significant effects were observed among the nutrient concentrations (same as day 2) (Table SM.3.2. 3). An increase of the N content in comparison with the initial value were observed in all treatments. The C/N ratio was also affected by the nutrient concentration in both days (Table SM.3.2. 3), the lowest ratio was obtained in the treatments with highest nutrient availability. Considering the sulphur content of algal thalli, no significant differences were found (Table SM.3.2. 3). The phenols content showed a significant effect of the radiation treatments at day 2 (Table SM.3.2. 3). Higher value was obtained in the P_{UVA+BLUE} treatments (11.9 mg g⁻¹ DW) than in the P treatments (9.0 mg g⁻¹ DW). However, at day 7 a significant effect of the nutrient concentrations was obtained (Table SM.3.2. 3). Lowest values were obtained in the treatment with lowest nutrient concentration (50; 9.5 mg g⁻¹ DW). The antioxidant capacity measured through ABTS method, showed significant effects of the nutrient concentration at day 2 and radiation treatments at day 7 (Table SM.3.2. 3). At day 2, highest values were obtained in the intermediate content of nutrients (250; 23.9 µmol TE g⁻¹ DW) and in day 7 higher values was obtained in the P treatments (22.2 µmol TE g⁻¹ DW) than in the P_{UVA+BI+} treatments (16.8 µmol TE g⁻¹ DW). The antioxidant capacity through the DPPH methods only showed a significant effect of the light treatments at day 2 (Table SM.3.2. 3), in which higher values was obtained under P_{UVA+BI+} treatments (11.5 µmol TE g⁻¹ DW) than under P treatments (6.5 µmol TE g⁻¹ DW).

In relation to the second experiment, the carbon, nitrogen and sulfur content showed a significant interaction between variables (Table SM.3.2. 4), and the lower C value was obtained in the PAR_{UVA+BI+} IMMER treatment (29.7 %) and higher in P IMMER treatment (35.8 %). The lowest N-value was obtained in PAR_{UVA+BI+} IMMER treatment (2.5 %) and the highest at PAR IMMER treatment (3.9 %). The C/N ratio only showed significant effect of the radiation treatments (Table SM.3.2. 4), where higher values was obtained in the PAR_{UVA+BI+} treatments (11.6) in comparison to P treatments (9.7). The highest S value was obtained in PAR IMMER treatment (1.2 %) and the lowest in PAR_{UVA+BI+}-IMMER treatment (0.2 %). The phenolic compounds and DPPH antioxidant capacity only showed a significant effect of the radiation treatments at day 7 (Table SM.3.2. 4). In the case of phenolics, a higher content was found in algae under PAR_{UVA+BI+} treatments (15.8 mg g⁻¹ DW) than under P treatments (13.7 mg g⁻¹ DW). The ABTS did not show significant differences (Table SM.3.2. 4).

Table 3.2. 5. Total internal carbon, nitrogen and sulphur content (%), and C/N ratio of both experiments with *Neopyropia leucosticta*: A) light quality and nutrient concentrations (500, 250 and 50 μM of NaNO_3); and B) light quality and emersion/immersion (EMER/IMMER) conditions. Values are expressed as mean $\pm$ standard deviation (SD) ($n = 3$). Different letters indicate significant differences among treatments: lower-case letters indicate significant interaction between variables (radiation and nutrients or radiation and emersion/immersion), capital letters indicate significant differences between radiation treatments (PAR and $\text{PAR}_{\text{UVA-ViBI+}}$), italic letters indicate significant differences among nutrients concentrations or emersion/immersion (EMER/IMMER conditions. ANOVA, SNK ($p < 0.05$). Asterisks (*) indicate significant differences between initial values and the obtained in the different treatments by Student's t-test ($p < 0.05$).

	Treatment	C		N		C/N		S	
Exp. 1	Initial	33.02 $\pm$ 0.59		1.65 $\pm$ 0.23		20.3 $\pm$ 2.99		1.08 $\pm$ 0.19	
		Day 2	Day 7	Day 2	Day 7	Day 2	Day 7	Day 2	Day 7
	PAR 500	35.92 $\pm$ 1.55*	35.14 $\pm$ 0.29 ^{B*}	2.99 $\pm$ 0.09 ^{Bb*}	2.88 $\pm$ 0.29 ^{b*}	12.03 $\pm$ 0.28 ^{a*}	12.31 $\pm$ 1.35 ^{a*}	1.47 $\pm$ 0.3	1.18 $\pm$ 0.19
	PAR 250	35.46 $\pm$ 0.28*	33.86 $\pm$ 0.38 ^B	2.23 $\pm$ 0.08 ^{Bb*}	2.41 $\pm$ 0.56 ^{ab*}	15.92 $\pm$ 0.47 ^{b*}	14.69 $\pm$ 3.96 ^a	1.27 $\pm$ 0.12*	1.08 $\pm$ 0.20
	PAR 50	37.65 $\pm$ 0.65*	33.76 $\pm$ 1.02 ^B	2.03 $\pm$ 0.01 ^{Ba*}	2.23 $\pm$ 0.70 ^a	18.54 $\pm$ 0.42 ^c	16.22 $\pm$ 5.01 ^b	1.32 $\pm$ 0.01*	1.14 $\pm$ 0.28
	$\text{PAR}_{\text{UVA-ViBI+}}$ 500	34.06 $\pm$ 2.47	33.37 $\pm$ 1.84 ^A	2.54 $\pm$ 0.27 ^{Ab*}	2.90 $\pm$ 0.36 ^{b*}	13.57 $\pm$ 2.24 ^{a*}	11.64 $\pm$ 1.75 ^{a*}	1.26 $\pm$ 0.33	1.04 $\pm$ 0.41
	$\text{PAR}_{\text{UVA-ViBI+}}$ 250	35.49 $\pm$ 1.63*	32.92 $\pm$ 1.02 ^A	2.10 $\pm$ 0.03 ^{Ab*}	2.22 $\pm$ 0.46 ^{ab}	16.94 $\pm$ 0.81 ^b	15.22 $\pm$ 2.88 ^a	1.21 $\pm$ 0.21	1.11 $\pm$ 0.09
	$\text{PAR}_{\text{UVA-ViBI+}}$ 50	35.64 $\pm$ 1.72*	32.46 $\pm$ 1.93 ^A	1.98 $\pm$ 0.19 ^{Aa*}	1.50 $\pm$ 0.22 ^a	18.18 $\pm$ 2.38 ^c	21.80 $\pm$ 2.22 ^b	1.26 $\pm$ 0.38	1.02 $\pm$ 0.14
Exp. 2	Initial	31.64 $\pm$ 0.61		2.33 $\pm$ 0.78		14.64 $\pm$ 5.01		0.77 $\pm$ 0.09	
		Day 7		Day 7		Day 7		Day 7	
	PAR EMER	32.70 $\pm$ 1.34 ^{ab}		3.26 $\pm$ 0.36 ^b		10.09 $\pm$ 0.82 ^A		0.29 $\pm$ 0.15 ^{a*}	
	PAR IMMER	35.84 $\pm$ 2.54 ^{b*}		3.88 $\pm$ 0.05 ^{b*}		9.25 $\pm$ 0.69 ^A		1.24 $\pm$ 0.31 ^{b*}	
	$\text{PAR}_{\text{UVA-ViBI+}}$ EMER	33.30 $\pm$ 2.52 ^{ab}		3.04 $\pm$ 0.87 ^b		11.44 $\pm$ 2.47 ^B		0.54 $\pm$ 0.62 ^a	
	$\text{PAR}_{\text{UVA-ViBI+}}$ IMMER	29.67 $\pm$ 1.62 ^a		2.47 $\pm$ 0.25 ^a		11.68 $\pm$ 1.10 ^B		0.20 $\pm$ 0.21 ^{a*}	

Table 3.2. 6. Phenols content (mg g⁻¹ DW), and antioxidant capacity measured through ABTS and DPPH method (μmol TE g⁻¹ DW) of both experiments with *Neopyropia leucosticta*: Exp. 1) light quality and nutrient concentrations (500, 250 and 50 μM of NaNO₃); and Exp. 2) light quality and emersion/immersion (EMER/IMMER) conditions. Values are expressed as mean ± standard deviation (SD) (n = 3). Different letters indicate significant differences among treatments: lower-case letters indicate significant interaction between variables (radiation and nutrients or radiation and emersion/immersion), capital letters indicate significant differences between radiation treatments (PAR and PARUVAViBI+), italic letters indicate significant differences among nutrients concentrations or emersion/immersion (EMER/IMMER) conditions. ANOVA, SNK (p < 0.05). Asterisks (*) indicate significant differences between initial values and the obtained in the different treatments by Student's t-test (p < 0.05).

		Phenols		ABTS		DPPH	
Exp. 1	Initial	11.6 ± 4.44		23.49 ± 0.69		8.53 ± 0.70	
		Day 2	Day 7	Day 2	Day 7	Day 2	Day 7
	PAR 500	8.73 ± 0.63 ^A	9.95 ± 1.13 ^a	17.77 ± 2.06 ^a	23.49 ± 2.97 ^B	7.08 ± 2.81 ^A	12.52 ± 1.09 [*]
	PAR 250	9.45 ± 2.23 ^A	10.94 ± 1.75 ^{ab}	23.97 ± 4.9 ^b	21.36 ± 2.18 ^B	8.49 ± 7.49 ^A	11.74 ± 4.41
	PAR 50	8.91 ± 0.89 ^A	13.22 ± 1.28 ^b	20.90 ± 1.66 ^{a*}	21.74 ± 3.91 ^B	3.75 ± 1.53 ^{A*}	12.37 ± 2.26 [*]
	PAR _{UVAViBI+} 500	11.6 ± 1.95 ^B	9.09 ± 1.15 ^a	16.30 ± 4.61 ^{a*}	15.18 ± 0.62 ^A	9.64 ± 5.1 ^B	12.59 ± 1.6 [*]
	PAR _{UVAViBI+} 250	11.29 ± 0.49 ^B	10.73 ± 1.56 ^{ab}	23.84 ± 2.22 ^b	15.17 ± 2.61 ^{A*}	12.13 ± 0.51 ^B	15.61 ± 2.38 [*]
	PAR _{UVAViBI+} 50	12.88 ± 2.08 ^B	10.43 ± 0.87 ^b	18.69 ± 2.51 ^{a*}	20.23 ± 3.36 ^{A*}	12.87 ± 0.99 ^B	9.92 ± 5.85
Exp. 2	Initial	11.31 ± 2.05		18.6 ± 1.6		2.06 ± 1.40	
		Day 3	Day 7	Day 3	Day 7	Day 3	Day 7
	PAR EMER	8.92 ± 2.93	15.07 ± 1.67 ^A	17.98 ± 1.49	20.60 ± 0.66 [*]	7.51 ± 1.94 [*]	11.33 ± 1.70 ^{A*}
	PAR IMMER	11.41 ± 3.44	12.38 ± 1.10 ^{A*}	17.87 ± 4.94	23.36 ± 3.18	5.82 ± 3.35	10.95 ± 1.38 ^{A*}
	PAR _{UVAViBI+} EMER	12.84 ± 5.25	15.72 ± 0.50 ^{B*}	19.80 ± 3.19	20.98 ± 1.10	6.60 ± 3.95	15.04 ± 1.69 ^{B*}
	PAR _{UVAViBI+} IMMER	10.10 ± 3.9	15.94 ± 0.99 ^{B*}	19.35 ± 2.25	21.50 ± 1.77	11.42 ± 6.51 [*]	13.17 ± 7.27 ^{B*}

Photosynthetic pigments of *N. leucosticta* submitted to the two experimental procedures are shown in Figure 3.2. 5. In case of the first experiment, pigments clearly increased during the experimental process and showed a variation caused by significant effects due to nutrient concentrations applied during the experiment (Table SM.3.2. 3). The values increased with the increasing nutrient concentrations. For phycoerythrin and phycocyanin content, a significant effect of the nutrient concentrations was observed for day 2, whereas at day 7 a significant effect of the radiation treatments was also observed (Table SM.3.2. 3). At day 2, values of phycoerythrin varied from 4.3 to 11.1 mg g⁻¹ DW. At day 7, PAR_{UVAVIBI+} showed lower values than PAR treatments (6.1 and 8.2 mg g⁻¹ DW, respectively). In the case of chlorophyll *a* content, a significant effect of the nutrients was obtained in both days (Table SM.3.2. 3). The content varied from 1.3 to 2.0 mg g⁻¹ DW at day 2. In the second experiment, a significant effect of radiation treatments was only observed for phycoerythrin content at day 7, in which a lower content was obtained in the PAR_{UVAVIBI+} (18.2 mg g⁻¹ DW) treatments in comparison with PAR treatments (24.7 mg g⁻¹ DW). The other two pigments remained constant, although the chlorophyll *a* showed a significant increase in comparison with the initial values.

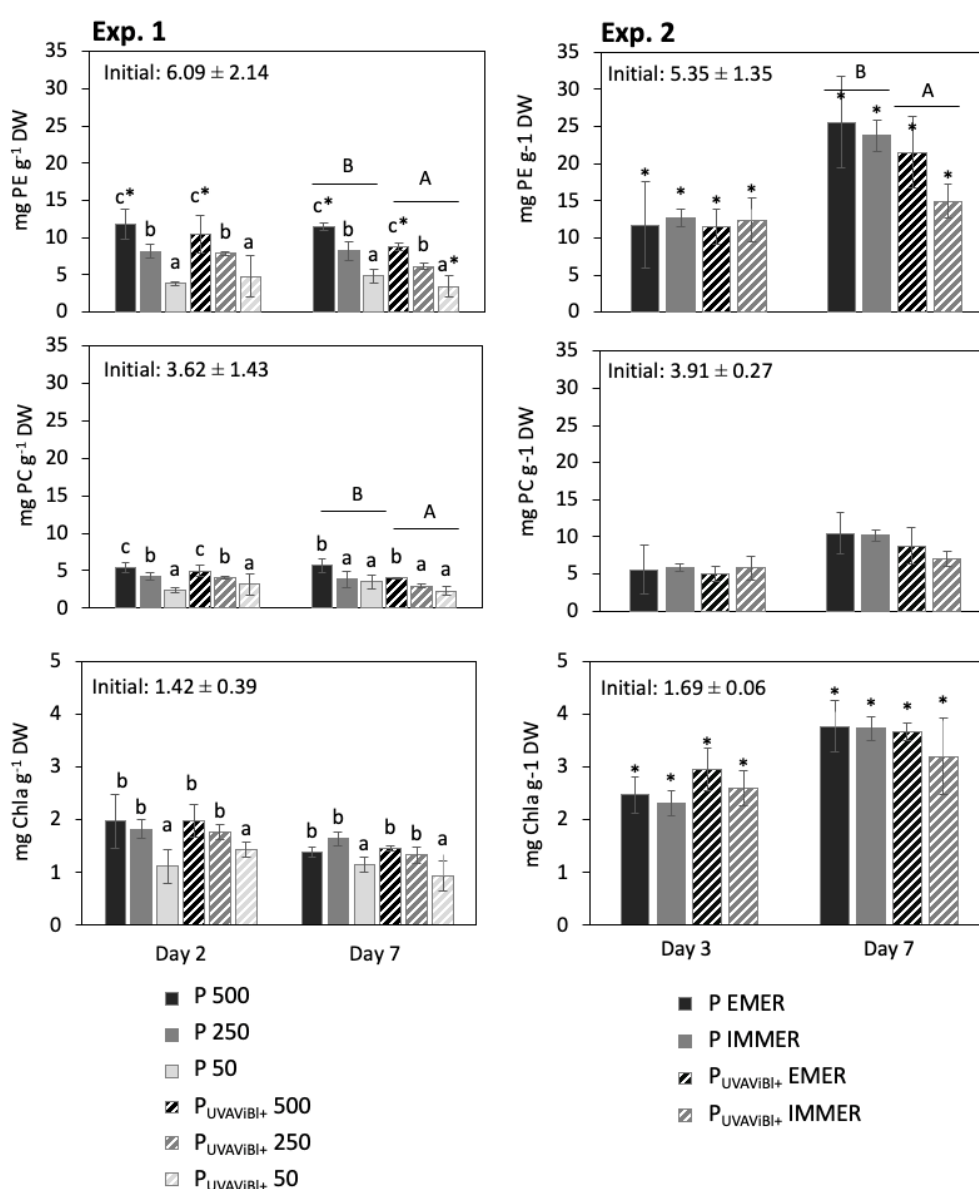


Figure 3.2. 5. Photosynthetic pigments (phycoerythrin, phycocyanin and chlorophyll a) measured in both experiments with *Neopyropia leucosticta*: Exp. 1) light quality and nutrient concentrations (500, 250 and 50 μM of NaNO_3) and Exp. 2) light quality and emersion/immersion (EMER/IMMER) conditions. Values are expressed as mean $\pm$ standard deviation (SD) ($n = 3$). Different letters indicate significant differences among treatments: lower-case letters indicate significant interaction between variables (radiation and nutrients or radiation and emersion/immersion), capital letters indicate significant differences between radiation treatments (PAR and PARUVAVIBI+), italic letters indicate significant differences among nutrients or emersion/immersion (EMER/INMER) conditions. ANOVA, SNK ($p < 0.05$). Asterisks (*) indicate significant differences between initial values and the obtained in the different treatments. Student's t-test ($p < 0.05$).

Finally, the MAAs contents of *N. leucosticta* are shown in Figure 3.2. 6. In both experiments, the content of MAAs increased throughout the time. In case of the first experiment, a clear effect of nutrients concentration was obtained (Table SM.3.2. 3). The content of MAAs increased in the treatments with high N-availability. MAAs content varied from 0.8 to 2.7 mg g⁻¹ DW at day 2, and from 3.3 to 6.7 mg g⁻¹ DW at day 7. In the second experiment, an interaction among variables was obtained (Table SM.3.2. 4). In day 3, the highest MAAs values was obtained in the PAR_{UVAVIBI+} EMER treatment (5.1 mg g⁻¹ DW). In the end of the experiment, in day 7, the highest values were obtained in PAR IMMER and PAR_{UVAVIBI+} EMER treatments, followed by PAR EMER and PAR_{UVAVIBI+} IMMER treatment (9.4, 8.1, 6.7 and 4.3 mg g⁻¹ DW, respectively).

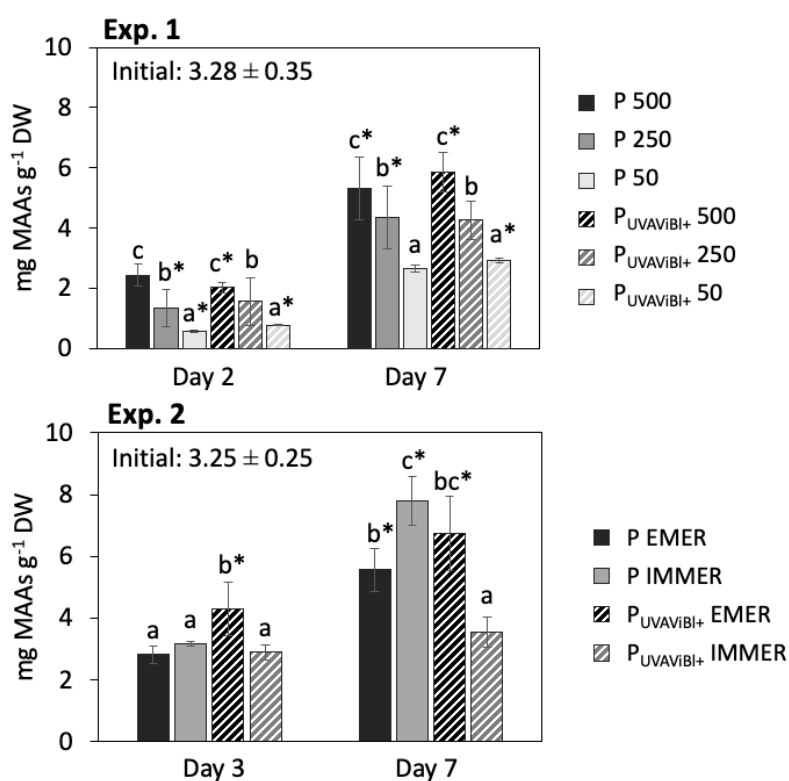


Figure 3.2. 6. Mycosporine-like amino acids (MAAs) measured in both experiments with *Neopyropia leucosticta*: Exp. 1) light quality and nutrients (500, 250 and 50 μM of NaNO₃) and Exp. 2) light quality and emersion/immersion (EMER/INMER) conditions. Values are expressed as mean ± standard deviation (SD) (n = 3). Different letters indicate significant differences among treatments: lower-case letters indicate significant interaction between variables (radiation and nutrients or radiation and emersion/immersion), capital letters indicate significant differences between radiation treatments (PAR and PAR_{UVAVIBI+}), italic letters indicate significant differences among nutrients or emersion/immersion (EMER/IMMER) conditions. ANOVA, SNK (p

< 0.05). Asterisk (*) showed significant differences between initial values and the obtained in the different treatments. Student's t-test ($p < 0.05$).

Pearson's correlation analysis for experiments conducted with *N. leucosticta* are shown in Table SM.3.2. 5. In the first experiment, some photosynthetic parameters ($_{int}ETR$, F_v/F_m and ETR_{max}) were positively correlated among them and with most of the internal compounds (N, phenols, PE, PC, Chla and MAAs). Nitrogen content correlated positively with the nitrogenous compounds (PE, PC, Chla and MAAs). Carbon content also showed a positive correlation with the phycobiliproteins content (PE and PE). Antioxidant capacities of algal extracts with DPPH assay were correlated with the phenols content, while those obtained from the ABTS assay were correlated with the PC content. In the second experiment, less significant correlations were found. Carbon content correlate with N and S content, as well as, PE, PC and MAAs. Nitrogen content correlated with the nitrogenous compounds (PE, PC and MAAs) and sulfur content, and correlates negatively with the antioxidant capacity (by DPPH). ABTS assay data showed positive correlation with PE and PC of *N. leucosticta*, while antioxidant capacity estimated by DPPH was positively correlated with phenols.

4. Discussion

Photosynthesis is dependent on irradiance, light quality, and nutrient availability. Nitrogen assimilation is necessary to produce the proteins involved in photosynthesis (and other processes), while at the same time this process demand ATP and NADPH produced in the photosynthetic electron transport chain. In this study, both maximal quantum yield (F_v/F_m) and electron transport rate (ETR) decreased under low nutrient conditions, clearly related to the lower substrate source for photosynthesis and the inability to synthesize important proteins like photosynthetic pigments that harvest the light. F_v/F_m is an indicator of the physiological state of photosynthetic organism (Maxwell and Johnson, 2000) and ETR can be related to productivity (Jerez *et al.*, 2016; Figueroa *et al.*, 2022). Low values of both F_v/F_m and ETR are consequence of stress conditions (e.g. low nutrient availability) producing photoinhibition. So, as it is expected, the increase in nitrogen content is related to a high pigment content that allow a higher light absorption, what increase the electron sink and consequently increase the photosynthetic rate (Figueroa *et al.*, 2021). According to the different action spectra showed in Figure SM.3.2. 2, UV radiation can affect photosynthesis and other processes, through chloroplast inhibition, DNA and proteins damage, or lipid peroxidation. Among

them, chloroplast photoinhibition and lipid peroxidation were the most affected by UV-A radiation. This can explain the reduction in the integrated ETR under the PAR_{UVAVIBI+} treatment in some cases (e.g. the day 0 or the PAR_{UVAVIBI+} 250 at day 7) during the first experiment. However, the interaction between both studied factors (light quality and nutrients) during the experimental period in the F_v/F_m and ETR values, suggest that high nitrogen availability can help to reduce or delay the negative effect provoke by UV-A radiation. This positive effect has been previously reported by Figueroa *et al.* (2009), Barufi *et al.* (2011) and Bonomi-Barufi *et al.* (2020) in *Ulva lactuca* and *Gracilaria tenuistipitata*, respectively. Additionally, the violet-blue light increased the amount of photons available for photosynthesis (Lüning and Dring, 1985) in the PAR_{UVAVIBI+} treatment, that can explain the significant increase in the $_{int}ETR$ observed in the PAR_{UVAVIBI+} 500 at day 2 and 6 and in the algae cultured under PAR_{UVAVIBI+} during the first day of the second experiment. This supplemented radiation has a positive effect on photosynthesis only during a short period, whereas the positive effect of high nutrient availability is maintained during a longer time. The photosynthetic increase with the supplemented light can be also due to photomorphogenic responses of these wavelength through the action of cryptochromes as it has been shown by other authors (Schneider *et al.*, 2020a; Ghedifa *et al.*, 2021).

On the other hand, emersed conditions caused a decrease in the photosynthetic capacity ($_{in situ}ETR$) after 3h of emersion, followed by a recovery after the re-hydration (Figure SM.3.2. 4). This pattern has been observed in different species, such as *Porphyra columbina*, *Mastocarpus papillatus* or *Mazzaella laminarioides* (Bell, 1993; Contreras-Porcia *et al.*, 2011; Flores-Molina *et al.*, 2014). Zou and Gao (2002) observed that the photosynthetic capacity of *Porphyra haitanensis* decreased under emersion due to limitation of the atmospheric CO₂, demonstrating that higher CO₂ concentrations will enhance the photosynthetic capacity. In contrast, many macroalgae have shown an increase in the photosynthetic rate in moderate emersion conditions, like *Pyropia yezoensis* (formerly *Porphyra yezoensis*), *Ishige okamurae*, *Chondracanthus intermedius* or *Pterocladia capillacea* (formerly *Pterocladia capillacea*) (Rhodophyta) (Ji and Tanaka, 2002) due to the removal of the water layer on the surface of the thallus and the higher diffusion rate of CO₂ in air than in water (10000 times higher). Nevertheless, prolonged desiccation periods produce negative effects for photosynthesis due to a further decrease of water content. Water is necessary for the proper function of the photosystem (PS) II and the electron flow between the two photosystems, whereas the cyclic electron flow around PS I do not need water and plays a significant physiological

role during desiccation and re-hydration, i.e. Gao *et al.* (Gao and Wang, 2012) observed that the cyclic electron flow improve the recovery after severe desiccation in *P. yezoensis*.

The ETR_{max} values determined during RLCs showed a similar pattern that the obtained in the $intETR$ calculated *in situ*, although the values were very different. The ETR_{max} values obtained incubating algae under the red LED light of the Mini-PAM (that reach $1200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) were much lower than that ETR values obtained *in situ* at $300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ of white LED light (3.3 vs $14.8 \mu\text{mol e}^{-} \text{m}^{-2} \text{s}^{-1}$, respectively in the PAR 500 treatment). These differences have been observed by other species of macroalgae (*Ulva lactuca*, *Ulva rigida* and *Gracilaria cornea*) and microalgae *Desmodesmus abundans* (formerly *Chlorella fusca*) (*Chlorella*) (Longstaff *et al.*, 2002; Jerez *et al.*, 2016; Figueroa *et al.*, 2021, 2022). Figueroa *et al.* (2022) suggested that this difference can be due to the light quality of the incubation light. Solar or white light present a broader spectrum than the red, blue or the white halogen lights of the PAM devices, thus chlorophyll and accessory pigments can absorb light from different wavelengths, reaching a higher photosynthetic capacity.

From the RLCs can be observed how much energy is dissipated and in which way. The term energy dissipation rate (EDR), proposed for the first time in this work, is related to the amount of energy that is dissipated and it is calculated using both yield losses: $Y(NO)$ and $Y(NPQ)$, instead of the $Y(II)$ (used for ETR calculation). The dissipation can be performed through two mechanisms: $Y(NPQ)$, that is related with the dissipation as fluorescence and heat through photoregulated mechanisms (e.g. xanthophyll cycle) and $Y(NO)$, that is refer to the passively dissipation as heat and/or fluorescence, mainly due to close PSII reaction centers. In all treatments, most of the obtained energy is dissipated, although differences in the ratio ETR/EDR (production/dissipation) were observed among treatments. The non-photochemical quenching (NPQ) is the ratio between both yield losses ($Y(NPQ)/Y(NO)$), values higher than 1 indicate that the main dissipation mechanism is the photoregulated one. The results indicate that under high intensities, the ETR/EDR decreased and the NPQ increased, showing that algae are not able to use all the available energy, and as photoprotection mechanism the photoregulated dissipation mechanism increased. The ratio ETR/EDR was affected by nutrient availability as was observed with ETR values since both values are related, i.e. if ETR values decrease, EDR values increased. However, the NPQ was only affected by the UVAVB radiation, increasing the values under this type of radiations and indicating

that a photoregulated dissipation mechanism is activated under this condition. When emersion and UVA, Violet and Blue were combined, a decrease in ETR/EDR was observed, probably due to the effect of light quality (no effect of the emersion conditions were observed).

Other authors have used the ratio ETR/NPQ as indicator of the relation between energy used on photosynthesis and dissipation quenching (Figueroa *et al.*, 2014b). The authors shown that this ratio decreased under increased stress condition, e.g. increase the exposure UV-B radiation. ETR/EDR is suggested as a better indicator than ETR/NPQ since they are expressed in the same energy units and they represent the ratio of energy used for photosynthesis (ETR) and energy dissipated (EDR). Hendrickson *et al.* (2004) proposed a simpler alternative method for quantifying the partitioning of excitation energy between photochemistry, thermal/fluorescence dissipation or photoregulated dissipation. They calculated the energy used or dissipated as the product between YII, Y(NO) or Y(NPQ) and the absorbed irradiance and expressed it as irradiance unit ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$). Figueroa *et al.* (2021) also calculated the energy used and dissipated but taking into account the absorbed irradiance and the fraction of Chl *a* associated to PSII (FII). The EDR was proposed in this work, as a form to calculate the general energy dissipation, taking into account both yield losses, Y(NO) and Y(NPQ).

In addition to the effects of the different factors on photosynthesis, the microscopic photographs showed that light, nitrate and emersion can induce morphological cell changes. Under low nitrogen conditions the surface/volume (S/V) ratio increased (n° cells increased and the protoplasm average size decreased), what would favor a high nitrogen incorporation (Figueroa *et al.*, 1995). The opposite is observed in algae under PAR_{UVA+VIBI+} radiation, where the number of cells decreased, and their size increased, what mean a reduction of the S/V ratio. These morphological changes can be attributed to a photoprotection strategy, decreasing potentially the entrance of UV-A radiation in the inner part of the cells. The increase of cell size of *N. leucosticta* under blue light has been previously observed by (Figueroa *et al.* 1995), who observed that the content of PE was much higher under blue light than that under red light, although growth rate in term of area was much lower in blue light and consequently the PE productivity decreased. In this work, we also observed a positive correlation between the protoplasm average size and the accumulation of certain compounds like phycoerythrin, phenols or MAAs. It is also expected that UV radiation cause an increase of the intercellular matrix due to the synthesis of carbonated compounds (such as polysaccharides) as protection

mechanism to reduce the penetration of UV rays in the cells (Schmidt *et al.*, 2009, 2010; Álvarez-Gómez *et al.*, 2017), although the obtained results did not show a clear effect of the PAR_{UVAVIBI+} radiation on the protoplasmatic area, and the total C content did not increase under these treatments. Emersion condition increased the protoplasm size, that can be a strategy to reduce the S/V ratio, avoiding the water evaporation. As mentioned before, it is expected that under UV radiation the S/V ratio decrease (as happen in the first experiment), but in the second experiment the opposite occurred: S/V increase (n° cell increase and protoplasm average size decreased). Escassi *et al.* (2002) observed that the potassium influx into the cells, influenced the thallus area, so the osmotic regulation related to emersed or immersed conditions can interfere in the obtained results.

The total carbon values of 30-35 % are in accordance with the obtained by other authors (Holdt and Kraan, 2011; Cho and Rhee, 2019), its content showed a little increase in the treatments with only PAR probably due to the major content of several compounds (e.g. biliproteins or phenols). The nitrogen content clearly increased in the treatments with high nitrogen availability. In this study carbon was not enriched and thus C/N increased in the treatment with low nutrients. Stoichiometry imbalance as the increase of C/N from 12.0 in the treatments with 500 μ M of nitrate to 18.5 in the treatments under 50 μ M nitrate can have negative physiological effects, reducing not only photosynthetic rate but also photoprotection. It is known the role of increased inorganic dissolved carbon on photoprotection due to increase of dissipating excess of light energy (Qiu and Liu, 2004; Li *et al.*, 2016). It has been observed that desiccation conditions induce nitrogen release, followed by a rapid incorporation during re-hydration (Kim *et al.*, 2013). Thomas *et al.* (1987) observed that desiccation appears to enhance the uptake of nitrate and ammonia in four intertidal species (*Pelvetiopsis limitata*, *Fucus distichus*, *Ulva intestinalis* (formerly *Enteromorpha intestinalis*) and *Mastocarpus papillatus* (formerly *Gigartina papillate*), whereas phosphate uptake did not show and enhancement by desiccation (Hurd and Dring, 1991). Kim *et al.* (2008) found that *Porphyra* species followed different patterns in the nitrogen recovery during re-hydration depending on the vertical distribution, i.e. species from the upper-littoral showed a very fast recovery, while lower- or sub-littoral species could not recover. Thus, normally species that are subjected to periods of desiccation showed high internal N content than the others. In the emersion experiment, similar N content were observed among treatments, only immersed algae under PAR_{UVAVIBI+} showed a lower content. This can

suggest that the emersion could act as a photoprotection mechanism, through a more rapid N incorporation. Although no significant difference among treatments were observed in nitrogen uptake rate.

Algae color changed under the different treatments. Under low nitrogen, thalli become more brownish mainly due to biliproteins degradation. These pigments are considered nitrogen reservoirs, so under low nitrogen conditions, these molecules break down, supplying the lack of nitrogen for the basal metabolism (Zhao *et al.*, 2017). Biliproteins are very vulnerable to high exposure of PAR and UV radiation, whereas blue light can induce their synthesis and accumulation in the cells (Korbee *et al.*, 2005a; Schneider *et al.*, 2020a). In this work, the combination of Violet-Blue light with UVA radiation seems to reduce or delay the negative effect of the UV-A radiation on the phycobiliproteins content, as a slight significant effect of the radiation treatment was only observed after 7 days of culture.

Generally, the content of bioactive compounds (phenols, MAAs and phycobiliproteins) are correlated with the photosynthetic activity ($_{int}ETR$ and ETR_{max}) since a higher amount of energy is available for been used to synthesize new molecules inside the cells. This positive correlation was observed in the first experiment. As polyphenols act as protective agents in algae, it is expected the increment of these molecules under UV radiation. This effect was observed at day 2, although after 7 days of culture, the effect of the radiation disappeared, and the effect of the nitrogen availability became more prominent. Phenols contents increased in the treatment with low nitrogen, nitrogen limitation stimulates the synthesis of C-compounds such as lipids, carotenoids or phenols. Other authors also observed this response, Sharma *et al.* (Sharma *et al.*, 2012) reviewed the effect of nitrogen starvation in the lipids content from different microalgae species. Lamers *et al.* (2012) also observed carotenoid accumulation in *Dunaliella salina* induced by nutrient limitations.

Mycosporine-like amino acids clearly increased with high nitrogen availability as it has been previously described in red algae, including *Porphyra* species (Korbee-Peinado *et al.*, 2004; Korbee *et al.*, 2005d; Barufi *et al.*, 2011) with variations along the culture time (Korbee *et al.*, 2005d; Álvarez-Gómez *et al.*, 2019b; Figueroa *et al.*, 2022). UV-A radiation stimulated the accumulation of MAAs (Karsten *et al.*, 1999; Korbee-Peinado *et al.*, 2004; Álvarez-Gómez *et al.*, 2019b; Schneider *et al.*, 2020a; Ghedifa *et al.*, 2021; Figueroa *et al.*, 2022), although in this study no differences between PAR and PAR_{UVAVIB+} treatments were observed when was combined with different nutrients levels.

The emersion condition seems to stimulate the synthesis of MAAs in combination with PAR_{UVAVIB+} radiation at day 2, whereas at day 7 the unique difference was observed between PAR_{UVAVIB+} under IMMER and the other treatments. This difference can be related with the lowest N content in this treatment. This is the first work that study the MAAs content in relation to the emersion/immersion conditions. A difference between this study and other studies that observed MAAs stimulation with UV radiation, is that we have used a LED of UV-A radiation with maximal emissions at 365nm in combination with the Q-panel fluorescent lamp. Previous works only used the Q-Panel lamp with maximal emission at 340nm and with small content of UV-B (in this work we cut off the UV-B), closer to the maximal absorptions of MAAs and maybe can stimulate more optimally the synthesis of MAAs. However, Huang *et al.* (2018) increased the level of fucoxanthin and MAAs in two microalgae (*Cylindrotheca costerium*, formerly *Nitzschia closterium*, and *Isochrysis zhangjiangensis*) using a UV-A light with maximum absorption at 365nm. Another explanation is that *N. leucosticta* is an alga that live in a very stressful environment and normally present a high constitutive content of MAAs in nature (Hoyer *et al.*, 2002), that probably need higher radiation doses or more intense desiccation events.

Regarding the antioxidant capacity, both methodologies used are based on the free-radical scavenging capacity, although there are some differences between them. ABTS is a water-soluble reagent, so it is used for the analysis of hydrophilic compounds such as biliproteins, proteins or polysaccharides, whereas DPPH is only soluble in organic solvents (e.g. methanol, ethanol, chloroform or hexane) and can measured apolar compounds such as phenols, lipids or chlorophylls. In this work the ABTS correlated with phycobiliproteins content and DPPH with phenols content. The antioxidant capacity is considered a protection mechanism against stressful conditions (where there are ROS production) (Dring, 2005), whereas in this work the PAR_{UVAVIB+} treatments only provoke a small increase in the DPPH antioxidant capacity at day 2 for the nutrients experiments and at day 7 for emersion experiment. Other authors have observed an increase in the antioxidant capacity (measured through the ABTS method) in three red algae (*Gracilaria cornea*, *Gracilariopsis longissima* and *Halopithys incurva*) culture under UV radiation in comparison algae culture under only PAR (Álvarez-Gómez *et al.*, 2019b). No effect of the emersion/immersion conditions in the antioxidant capacity were observed. Contreras-Porcia *et al.* (Contreras-Porcia *et al.*, 2011) observed an increase of ROS production and antioxidant enzymes (e.g. catalase (CAT), ascorbate peroxidase (AP)

and glutathione reductase (GR)) during emersion, followed by a decrease to normal values during re-hydration.

The stimulation of certain compounds in a short period (2-7 days) can be a strategy to increase the productivity of interesting molecules with potential applications during culture. In addition to the food use, *Porphyra sensu lato* species present a high interest for cosmeceutical application, mainly due to the high content of MAAs, that provide photoprotection capacity, as well as antioxidant or anti-aging properties (Vega *et al.*, 2021; Rosic *et al.*, 2023). One of the limitations for the use of *Porphyra sensu lato* as cosmeceutic ingredient is that the obtained biomass is preferably used for human consumption. The stimulation of the production of high-added value compounds, such as MAAs, for the cosmeceutic industry can be a way to diversify the industrial use of *Porphyra sensu lato*.

5. Conclusions

In conclusion, the short-term stressed conditions studied in this work can modify the biochemical composition of *N. leucosticta*, maintaining the photosynthetic capacity. The effects of the combination of UV-A (320-400 nm with a peak at 365 nm), and violet-blue light (400-500 nm) is studied for the first time in this work. The interactive effect of UVA-Violet-Blue radiation and nutrients concentration suggested that the high nutrient availability play an important role in photoprotection against the UV-A radiation. It has been observed that the violet-blue light could reduce or delay the negative effects caused by the UV-A radiation in photosynthesis due to the accumulation of photoprotectors with antioxidant capacity. The term electron dissipation rate (EDR) and the ratio ETR/EDR are described for the first time in this work, as a way to know the amount of energy used in photochemistry and dissipated. It is also the first time that emersion/immersion conditions have been related to the MAAs concentration, under a short period the UVA-violet-blue radiation and emersion condition seem to increase the MAAs content. The studied stressed conditions could be scaled up in inland culture facilities, and the supplementation of violet-blue light can be used as a way to minimize the negative effects caused by solar radiation.

SUPPLEMENTARY MATERIAL

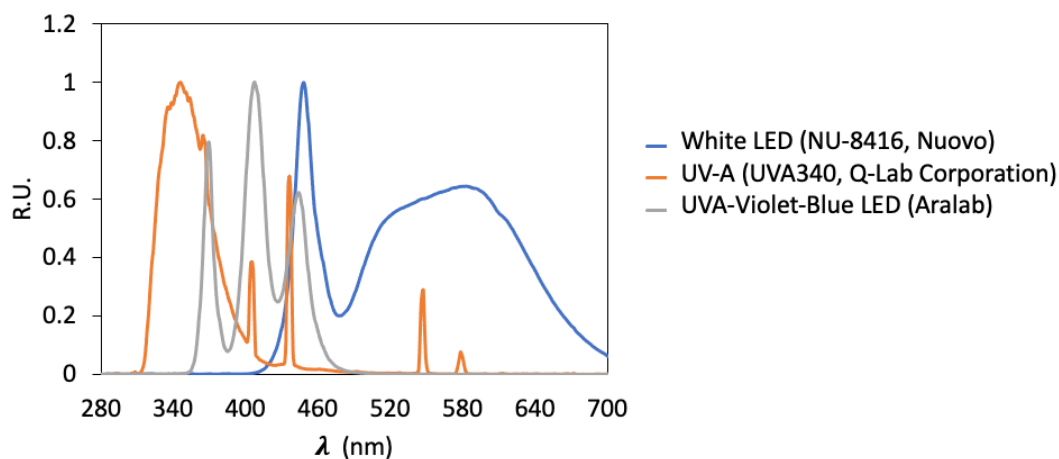


Figure SM.3.2. 1. Spectra of the different radiation sources used to define the radiation treatments during both experiments. PAR was obtained with White LED light (NU-8416, Nuovo), while PAR_{UVAVIBI+} was provided by the same white LED light source complemented with a UV-A fluorescent lamp (UVA340, Q-Lab Corporation) and a UVA-Violet-Blue LED lamp (Aralab).

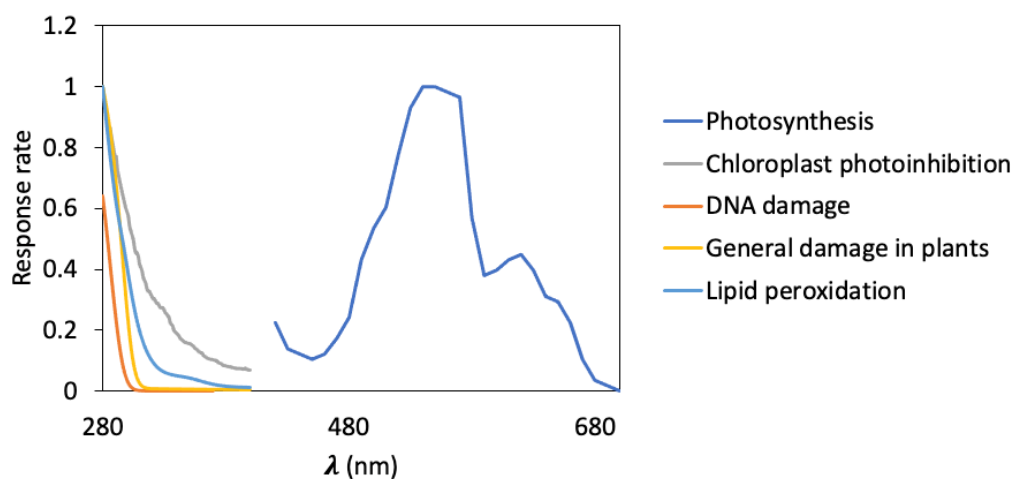


Figure SM.3.2. 2. Normalized action spectra of positive (photosynthesis) and negative (DNA damage, chloroplast photoinhibition, general damage in plants and lipid peroxidation) biological responses driven by UVR and PAR and utilized for calculation of biological effective radiation doses.

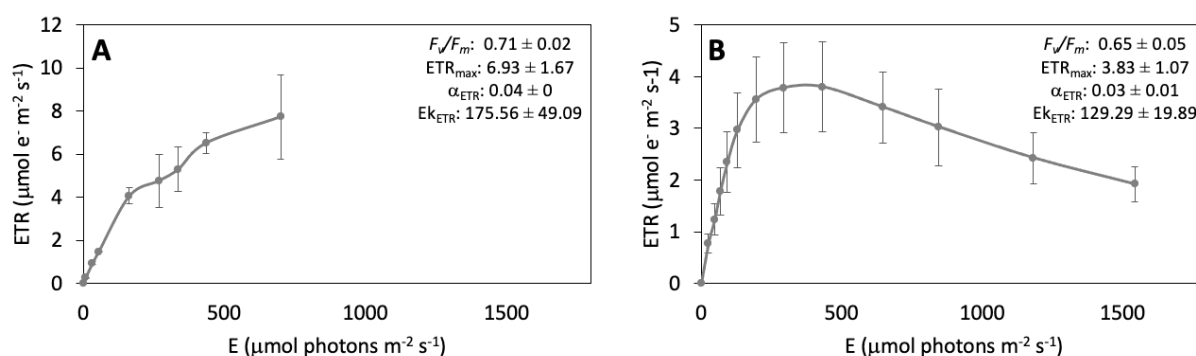


Figure SM.3.2. 3. In situ light curve ($LC_{in situ}$) and rapid light curve (RLC) of *Neopyropia leucosticta* before the starting of the experiments. The electron transport rate (ETR) is plotted against increasing irradiance intensities (White LED light for $LC_{in situ}$, and red LED light for RLC). Maximal quantum yield (F_v/F_m), maximal ETR (ETR_{max}), photosynthetic efficiency of ETR (α_{ETR}) and saturated irradiance of ETR (E_{kETR}) were also calculated. Values are expressed as mean $\pm$ standard deviation (SD) ($n = 3$).

Table SM.3.2. 1. Nitrate uptake rate ($\mu\text{mol NaNO}_3 \text{ L}^{-1} \text{ day}^{-1}$) by algae in different periods of both experiments with *Neopyropia leucosticta*: Exp.1) light quality and nutrient concentration (500, 250 and 50 μM of NaNO_3) and Exp.2) Light quality and emersion/immersion conditions (EMER/IMMER) conditions. At day 5th, half of the initial nutrients were added in the first experiment, and 50-2.7 μM of NaNO_3 and $\text{C}_3\text{H}_7\text{Na}_2\text{O}_6\text{P}$, respectively, were added in the second experiment.

	Treatment	0 - 2	5 - 7
Exp. 1	P 500	249.3 $\pm$ 1.2 ^c	110.7 $\pm$ 13.2 ^c
	P 250	125.0 $\pm$ 0.0 ^b	56.6 $\pm$ 10.2 ^b
	P 50	25.0 $\pm$ 0.0 ^a	11.8 $\pm$ 0.1 ^a
	P _{UVAViBI+} 500	241.5 $\pm$ 8.6 ^c	112.1 $\pm$ 22.1 ^c
	P _{UVAViBI+} 250	124.0 $\pm$ 0.9 ^b	55.3 $\pm$ 12.5 ^b
	P _{UVAViBI+} 50	25.0 $\pm$ 0.0 ^a	11.8 $\pm$ 0.0 ^a
Exp. 2	P EMER	122.1 $\pm$ 22.3 ^{ab}	123.2 $\pm$ 5.8
	P IMMER	128.1 $\pm$ 10.3 ^{ab}	120.1 $\pm$ 8.9
	P _{UVAViBI+} EMER	101.4 $\pm$ 0.8 ^a	118.8 $\pm$ 7.2
	P _{UVAViBI+} IMMER	146.7 $\pm$ 0.0 ^b	125.0 $\pm$ 0.0

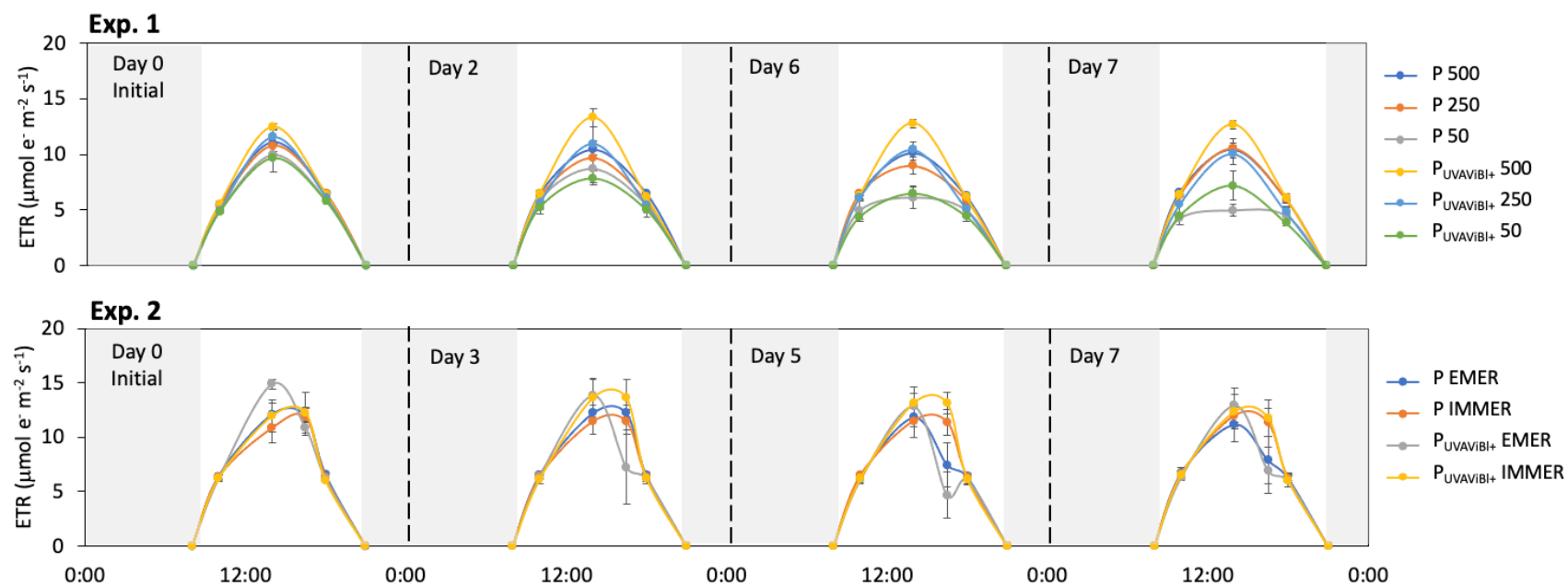


Figure SM.3.2. 4. Electron transport rate measured in situ ($\text{ETR}_{\text{in situ}}$) at different times of the day for day 0, 2, 6 and 7 of both experiments with *Neopyropia leucosticta*: Exp.1) Light quality and nutrient concentrations (500, 250 and 50 μM of NaNO_3); and Exp.2) Light quality and emersion/immersion (EMER/IMMER) conditions. White colour represents the period with lights on and grey colour the period with lights off (night). Values are expressed as mean $\pm$ standard deviation (SD) ($n = 3$).

Table SM.3.2. 2. Electron transport rate (ETR; $\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$), energy dissipation rate (EDR; $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and non-photochemical quenching (NPQ) obtained from the rapid light curves (RLCs) at 75, 150 and 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of both experiments with *Neopyropia leucosticta*: Exp.1) Light quality and nutrient concentrations (500, 250 and 50 μM of NaNO_3); and Exp.2) Light quality and emersion/immersion (EMER/IMMER) conditions. Values are expressed as mean $\pm$ standard deviation (SD) ($n = 3$). Different letters indicate significant differences among treatments: lower-case letters indicate significant interaction between variables (radiation and nutrients or radiation and emersion/immersion), capital letters indicate significant differences between radiation treatments (PAR/PAR+UVAVB) and italic letters indicate significant differences among nutrients concentrations (50, 250 and 500) or emersion/immersion conditions (EMER/IMMER). ANOVA, SNK ($p < 0.05$). Asterisk (*) showed significant differences between initial values and the obtained in the different treatments. Student's t-test ($p < 0.05$).

		ETR ₇₅	ETR ₁₅₀	ETR ₃₀₀	EDR ₇₅	EDR ₁₅₀	EDR ₃₀₀	NPQ ₇₅	NPQ ₁₅₀	NPQ ₃₀₀
Exp. 1	Initial	1.96 $\pm$ 0.38	3.44 $\pm$ 0.66	3.78 $\pm$ 0.89	3.56 $\pm$ 0.38	7.58 $\pm$ 0.66	18.27 $\pm$ 0.89	0.40 $\pm$ 0.06	0.49 $\pm$ 0.07	0.73 $\pm$ 0.10
	PAR 500	1.25 $\pm$ 0.15*	2.50 $\pm$ 0.42 ^b	3.04 $\pm$ 0.46 ^b	3.48 $\pm$ 0.12	8.53 $\pm$ 0.41 ^a	19.01 $\pm$ 0.47 ^a	0.47 $\pm$ 0.09 ^A	0.66 $\pm$ 0.16 ^A	0.85 $\pm$ 0.25 ^A
	PAR 250	1.40 $\pm$ 0.41	2.41 $\pm$ 0.67 ^{ab}	2.80 $\pm$ 0.77 ^{ab}	3.36 $\pm$ 0.34	8.62 $\pm$ 0.67 ^{ab}	19.26 $\pm$ 0.77 ^{ab}	0.27 $\pm$ 0.20 ^A	0.35 $\pm$ 0.22 ^A	0.46 $\pm$ 0.27 ^A
	PAR 50	1.29 $\pm$ 0.28*	2.04 $\pm$ 0.47 ^{a*}	2.21 $\pm$ 0.34 ^{a*}	3.45 $\pm$ 0.23	8.99 $\pm$ 0.47 ^{b*}	19.85 $\pm$ 0.34 ^{b*}	0.82 $\pm$ 0.26 ^{A*}	1.00 $\pm$ 0.25 ^{A*}	1.18 $\pm$ 0.26 ^{A*}
	PAR _{UVAViBI+} 500	1.49 $\pm$ 0.15	2.59 $\pm$ 0.22 ^b	3.02 $\pm$ 0.30 ^b	3.28 $\pm$ 0.12	8.43 $\pm$ 0.22 ^a	19.03 $\pm$ 0.3 ^a	0.81 $\pm$ 0.15 ^{B*}	1.01 $\pm$ 0.22 ^{B*}	1.29 $\pm$ 0.24 ^{B*}
	PAR _{UVAViBI+} 250	1.13 $\pm$ 0.22*	1.93 $\pm$ 0.23 ^{ab*}	2.09 $\pm$ 0.25 ^{ab*}	3.58 $\pm$ 0.18	9.10 $\pm$ 0.23 ^{ab*}	19.96 $\pm$ 0.25 ^{ab*}	0.91 $\pm$ 0.15 ^{B*}	1.03 $\pm$ 0.18 ^{B*}	1.17 $\pm$ 0.22 ^{B*}
	PAR _{UVAViBI+} 50	1.02 $\pm$ 0.25*	1.53 $\pm$ 0.39 ^{a*}	1.75 $\pm$ 0.55 ^{a*}	3.67 $\pm$ 0.21	9.50 $\pm$ 0.39 ^{b*}	20.3 $\pm$ 0.55 ^{b*}	1.08 $\pm$ 0.49 ^{B*}	1.24 $\pm$ 0.56 ^{B*}	1.35 $\pm$ 0.60 ^B
Exp. 2	Initial	1.87 $\pm$ 0.47	3.29 $\pm$ 0.79	3.96 $\pm$ 0.78	3.65 $\pm$ 0.47	7.73 $\pm$ 0.79	18.09 $\pm$ 0.78	0.45 $\pm$ 0.12	0.51 $\pm$ 0.1	0.67 $\pm$ 0.06
	PAR EMER	1.51 $\pm$ 0.33	2.69 $\pm$ 0.47 ^B	3.00 $\pm$ 0.57	4.00 $\pm$ 0.33	8.34 $\pm$ 0.47 ^{A*}	19.06 $\pm$ 0.57	0.43 $\pm$ 0.12	0.61 $\pm$ 0.14	0.74 $\pm$ 0.18
	PAR IMMER	1.22 $\pm$ 0.26	2.18 $\pm$ 0.49 ^{B*}	2.62 $\pm$ 0.77	4.30 $\pm$ 0.26	8.85 $\pm$ 0.49 ^{A*}	19.43 $\pm$ 0.77	0.60 $\pm$ 0.24	0.81 $\pm$ 0.26	0.95 $\pm$ 0.24
	PAR _{UVAViBI+} EMER	0.81 $\pm$ 0.10*	1.55 $\pm$ 0.21 ^{A*}	2.07 $\pm$ 0.33*	4.70 $\pm$ 0.10*	9.47 $\pm$ 0.21 ^{B*}	19.98 $\pm$ 0.33*	0.56 $\pm$ 0.07	0.71 $\pm$ 0.10*	0.83 $\pm$ 0.11*
	PAR _{UVAViBI+} IMMER	1.17 $\pm$ 0.45	1.96 $\pm$ 0.59 ^{A*}	2.44 $\pm$ 0.74*	4.34 $\pm$ 0.45	9.07 $\pm$ 0.59 ^{B*}	19.6 $\pm$ 0.74*	0.69 $\pm$ 0.13*	0.84 $\pm$ 0.17*	0.96 $\pm$ 0.19*

Table SM.3.2. 3. Results of the two-way ANOVA (analysis of variance). The variation sources were radiation (PAR/PAR+UVAVB) and nutrients concentration (50, 250 or 500 μM of NaNO_3). Dependent variable included: NUR (nitrate uptake rate; %), integrated electron transport rate (ETR_{int} ; $\text{mol e}^- \text{m}^{-2}$), maximal quantum yield (F_v/F_m), maximal ETR (ETR_{max} ; $\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$), photosynthetic efficiency of ETR (α_{ETR}), saturated irradiance of ETR (Ek_{ETR} ; $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), ETR at 75, 150 and 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ during rapid light curves (RLCs); energy dissipation rate (EDR) at 75, 150 and 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ during RLCs, , ETR/EDR at 75, 150 and 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ during RLCs, n° cells, protoplasmatic area (%), protoplast average size (μm^2), internal carbon, nitrogen and sulphur content (%), C/N, phenols content ($\text{mg g}^{-1} \text{DW}$), antioxidant capacity (ABTS and DPPH, $\mu\text{mol TE g}^{-1} \text{DW}$), phycobiliproteins (phycoerythrin -PE- and phycocyanin -PC-; $\text{mg g}^{-1} \text{DW}$), chlorophyll a (Chla; $\text{mg g}^{-1} \text{DW}$), and mycosporine-like amino acids (MAAs; $\text{mg g}^{-1} \text{DW}$). Significant differences were obtained at $p < 0.05$. SS: sum of squares, MS: means of squares

Dependent variables	Time	Source of variation	SS	MS	F	p
NUR	Day 0-2	Radiation	39.3505	39.3505	3.1140	0.10
		Nutrients	146161.8839	73080.9419	5783.2585	<0.01
		Radiation * Nutrients	54.4831	27.2415	2.1558	0.16
	Day 5-7	Radiation	0.0041	0.0041	0.0000	0.99
		Nutrients	29880.5855	14940.2927	97.1743	<0.01
		Radiation * Nutrients	5.6914	2.8457	0.0185	0.98
ETR_{int}	Day 0	Radiation	0.0002	0.0002	4.8208	0.05
		Nutrients	0.0023	0.0011	22.7075	< 0.01
		Radiation * Nutrients	0.0000	0.0000	0.4184	0.67
	Day 2	Radiation	0.0006	0.0006	2.1779	0.17
		Nutrients	0.0125	0.0063	23.5350	< 0.01
		Radiation * Nutrients	0.0024	0.0012	4.5746	0.03
	Day 5	Radiation	0.0009	0.0009	15.8630	< 0.01
		Nutrients	0.0413	0.0206	353.6085	< 0.01
		Radiation * Nutrients	0.0013	0.0006	10.7457	< 0.01
	Day 7	Radiation	0.0009	0.0009	3.2661	0.10
		Nutrients	0.0489	0.0244	84.0875	< 0.01
		Radiation * Nutrients	0.0064	0.0032	11.0801	< 0.01
F_v/F_m	Day 7	Radiation	0.0136	0.0136	70.6200	< 0.01
		Nutrients	0.0141	0.0070	36.5500	< 0.01
		Radiation * Nutrients	0.0046	0.0023	11.8400	< 0.01
ETR_{max}	Day 7	Radiation	0.5502	0.5502	2.9327	0.11

			Nutrients	2.0421	1.0210	5.4425	0.02
			Radiation * Nutrients	0.2317	0.1159	0.6177	0.56
α_{ETR}	Day 7	Radiation	0.0000	0.0000	0.0230	0.88	
		Nutrients	0.0000	0.0000	0.0542	0.95	
		Radiation * Nutrients	0.0000	0.0000	0.4528	0.65	
E_{KETR}	Day 7	Radiation	903.2000	903.2000	1.1268	0.31	
		Nutrients	4766.0000	2383.0000	2.9729	0.09	
		Radiation * Nutrients	424.8000	212.4000	0.2650	0.77	
ETR 75	Day 7	Radiation	0.0427	0.0427	0.6309	0.44	
		Nutrients	0.1435	0.0717	1.0602	0.38	
		Radiation * Nutrients	0.2600	0.1300	1.9217	0.19	
ETR 150	Day 7	Radiation	0.3987	0.3987	2.1845	0.17	
		Nutrients	1.7447	0.8723	4.7790	0.03	
		Radiation * Nutrients	0.3487	0.1744	0.9552	0.41	
ETR 300	Day 7	Radiation	0.7002	0.7002	3.0510	0.11	
		Nutrients	3.3279	1.6640	7.2508	0.01	
		Radiation * Nutrients	0.3741	0.1871	0.8151	0.47	
EDR 75	Day 7	Radiation	0.0283	0.0283	0.6289	0.44	
		Nutrients	0.0968	0.0484	1.0747	0.37	
		Radiation * Nutrients	0.1721	0.0861	1.9106	0.19	
EDR 150	Day 7	Radiation	0.4021	0.4021	2.2058	0.16	
		Nutrients	1.7542	0.8771	4.8111	0.03	
		Radiation * Nutrients	0.3484	0.1742	0.9556	0.41	
EDR 300	Day 7	Radiation	0.6956	0.6956	3.0571	0.11	
		Nutrients	3.3110	1.6555	7.2759	0.01	
		Radiation * Nutrients	0.3608	0.1804	0.7928	0.47	
NPQ 75	Day 7	Radiation	0.7645	0.7645	11.3817	0.01	
		Nutrients	0.4657	0.2329	3.4668	0.06	
		Radiation * Nutrients	0.1219	0.0609	0.9073	0.43	
NPQ 150	Day 7	Radiation	0.7988	0.7988	9.0497	0.01	
		Nutrients	0.5666	0.2833	3.2096	0.08	
		Radiation * Nutrients	0.1527	0.0764	0.8651	0.45	
NPQ 300	Day 7	Radiation	0.8731	0.8731	7.8132	0.02	
		Nutrients	0.6149	0.3074	2.7511	0.10	



		Radiation * Nutrients	0.2260	0.1130	1.0112	0.39
ETR/EDR 75	Day 7	Radiation	0.0059	0.0059	0.6181	0.45
		Nutrients	0.0189	0.0095	0.9892	0.40
		Radiation * Nutrients	0.0393	0.0196	2.0538	0.17
ETR/EDR 150	Day 7	Radiation	0.0079	0.0079	2.1870	0.16
		Nutrients	0.0337	0.0168	4.6801	0.03
		Radiation * Nutrients	0.0068	0.0034	0.9468	0.42
ETR/EDR 300	Day 7	Radiation	0.0023	0.0023	3.0320	0.11
		Nutrients	0.0110	0.0055	7.2775	0.01
		Radiation * Nutrients	0.0013	0.0006	0.8270	0.46
Nº cells	Day 7	Radiation	4415.3893	4415.3893	8.5589	0.01
		Nutrients	33793.6674	16896.8337	32.7532	< 0.01
		Radiation * Nutrients	208.2323	104.1161	0.2018	0.82
Protop. Area	Day 7	Radiation	10.3273	10.3273	2.6856	0.13
		Nutrients	193.3442	96.6721	25.1393	< 0.01
		Radiation * Nutrients	29.9198	14.9599	3.8903	0.05
Protop. av. size	Day 7	Radiation	3183.7917	3183.7917	15.2276	< 0.01
		Nutrients	24614.6681	12307.3341	58.8642	< 0.01
		Radiation * Nutrients	515.9412	257.9706	1.2338	0.33
Carbon	Day 2	Radiation	7.3700	7.3700	3.0210	0.11
		Nutrients	8.6600	4.3300	1.7740	0.21
		Radiation * Nutrients	3.8600	1.9300	0.7910	0.48
	Day 7	Radiation	8.0600	8.0600	5.1600	0.04
		Nutrients	4.2600	2.1300	1.3600	0.29
		Radiation * Nutrients	0.5100	0.2600	0.1600	0.85
Nitrogen	Day 2	Radiation	0.2033	0.2033	9.9140	0.01
		Nutrients	1.9180	0.9590	46.7620	< 0.01
		Radiation * Nutrients	0.1290	0.0645	3.1440	0.08
	Day 7	Radiation	0.3878	0.3878	1.8233	0.20
		Nutrients	3.1606	1.5803	7.4298	0.01
		Radiation * Nutrients	0.4457	0.2229	1.0477	0.38
C/N	Day 2	Radiation	2.4151	2.4151	1.2267	0.29
		Nutrients	95.7322	47.8661	24.3122	< 0.01
		Radiation * Nutrients	2.8806	1.4403	0.7316	0.50



	Day 7	Radiation	14.7034	14.7034	1.5015	0.24
		Nutrients	149.6011	74.8005	7.6386	0.01
		Radiation * Nutrients	33.0627	16.5313	1.6882	0.23
Sulphur	Day 2	Radiation	0.0585	0.0585	0.8568	0.37
		Nutrients	0.0494	0.0247	0.3619	0.70
		Radiation * Nutrients	0.0217	0.0109	0.1591	0.85
	Day 7	Radiation	0.0259	0.0259	0.4501	0.51
		Nutrients	0.0030	0.0015	0.0261	0.97
		Radiation * Nutrients	0.0291	0.0146	0.2532	0.78
Phenols	Day 2	Radiation	37.7260	37.7260	15.8347	< 0.01
		Nutrients	1.7140	0.8570	0.3597	0.71
		Radiation * Nutrients	3.3890	1.6950	0.7113	0.51
	Day 7	Radiation	7.4650	7.4650	4.2710	0.06
		Nutrients	16.1050	8.0520	4.6070	0.03
		Radiation * Nutrients	5.3720	2.6860	1.5370	0.25
ABTS	Day 2	Radiation	7.2530	7.2530	0.6853	0.42
		Nutrients	143.2930	71.6470	6.7696	0.01
		Radiation * Nutrients	3.3510	1.6750	0.1583	0.86
	Day 7	Radiation	128.0940	128.0940	16.2363	< 0.01
		Nutrients	22.5430	11.2720	1.4287	0.28
		Radiation * Nutrients	36.4020	18.2010	2.3071	0.14
DPPH	Day 2	Radiation	117.3610	117.3610	10.7442	0.01
		Nutrients	15.5770	7.7890	0.7130	0.51
		Radiation * Nutrients	37.0520	18.5260	1.6961	0.22
	Day 7	Radiation	1.0940	1.0940	0.0963	0.76
		Nutrients	19.2560	9.6280	0.8471	0.45
		Radiation * Nutrients	30.3320	15.1660	1.3343	0.30
PE	Day 2	Radiation	0.2730	0.2730	0.0844	0.78
		Nutrients	139.0610	69.5310	21.4656	< 0.01
		Radiation * Nutrients	3.6520	1.8260	0.5637	0.58
	Day 7	Radiation	19.3255	19.3255	21.6810	< 0.01
		Nutrients	105.1105	52.5552	58.9610	< 0.01
		Radiation * Nutrients	1.3446	0.6723	0.7540	0.49
PC	Day 2	Radiation	0.0179	0.0179	0.0297	0.87

Chla	Day 7	Nutrients	17.0184	8.5092	14.0842	< 0.01
		Radiation * Nutrients	1.2357	0.6179	1.0227	0.39
		Radiation	6.8521	6.8521	13.6294	< 0.01
		Nutrients	12.1663	6.0832	12.1000	< 0.01
		Radiation * Nutrients	0.4987	0.2494	0.4960	0.62
		Radiation	0.0359	0.0359	0.4078	0.54
	Day 2	Nutrients	1.5880	0.7940	9.0288	< 0.01
		Radiation * Nutrients	0.1143	0.0572	0.6498	0.54
		Radiation	0.1016	0.1016	3.9720	0.07
	Day 7	Nutrients	0.6678	0.3339	13.0480	< 0.01
		Radiation * Nutrients	0.1106	0.0553	2.1610	0.16
		Radiation	0.0003	0.0003	0.0011	0.97
MAAs	Day 2	Nutrients	10.5061	5.2531	18.6006	< 0.01
		Radiation * Nutrients	0.5867	0.2934	1.0388	0.38
		Radiation	0.3674	0.3674	0.7726	0.40
	Day 7	Nutrients	34.0662	17.0331	35.8120	< 0.01
		Radiation * Nutrients	0.4255	0.2128	0.4473	0.65
		Radiation	0.0003	0.0003	0.0011	0.97

Table SM.3.2. 4. Results of the two-way ANOVA (analysis of variance). The variation sources were radiation (PAR/PAR+UVAVB) and emersion condition (Emersed or Immersed). Dependent variable included: NUR (nitrate uptake rate; %), integrated electron transport rate (ETR_{int} ; $\text{mol e}^- \text{m}^{-2}$), maximal quantum yield (F_v/F_m), maximal ETR (ETR_{max} ; $\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$), photosynthetic efficiency of ETR (α_{ETR}), saturated irradiance of ETR ($E_{k_{ETR}}$; $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), ETR at 75, 150 and 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ during rapid light curves (RLCs); energy dissipation rate (EDR) at 75, 150 and 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ during RLCs, , ETR/EDR at 75, 150 and 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ during RLCs, n° cells, protoplasmatic area (%), protoplast average size (μm^2), internal carbon, nitrogen and sulphur content (%), C/N, phenols content ($\text{mg g}^{-1} \text{DW}$), antioxidant capacity (ABTS and DPPH, $\mu\text{mol TE g}^{-1} \text{DW}$), phycobiliproteins (phycoerythrin -PE- and phycocyanin -PC-; $\text{mg g}^{-1} \text{DW}$), chlorophyll a (Chla; $\text{mg g}^{-1} \text{DW}$), and mycosporine-like amino acids (MAAs; $\text{mg g}^{-1} \text{DW}$). Significant differences were obtained at $p < 0.05$. SS: sum of squares, MS: means of squares

Dependant variables	Time	Source of variation	SS	MS	F	p
NUR	Day 0-2	Radiation	3.2726	3.2726	0.0217	0.89
		Emersion	1974.6226	1974.6226	13.1179	<0.01
		Radiation * Emersion	1149.8681	1149.8681	7.6389	0.03
	Day 5-7	Radiation	0.2002	0.2002	0.0049	0.95

		Emersion	6.9769	6.9769	0.1700	0.69
		Radiation * Emersion	65.5669	65.5669	1.5979	0.24
ETR _{int}	Day 0	Radiation	0.0021	0.0021	26.4023	< 0.01
		Emersion	0.0027	0.0027	33.4978	< 0.01
		Radiation * Emersion	0.0002	0.0002	2.0570	0.19
	Day 2	Radiation	0.0002	0.0002	1.3039	0.29
		Emersion	0.0008	0.0008	4.9645	0.06
		Radiation * Emersion	0.0017	0.0017	10.8396	0.01
	Day 5	Radiation	0.0019	0.0019	5.3340	0.05
		Emersion	0.0089	0.0089	25.0120	< 0.01
		Radiation * Emersion	0.0016	0.0016	4.3660	0.07
	Day 7	Radiation	0.0032	0.0032	6.2080	0.04
		Emersion	0.0063	0.0063	12.3560	0.01
		Radiation * Emersion	0.0000	0.0000	0.0100	0.92
Fv/Fm	Day 7	Radiation	0.0003	0.0003	0.7400	0.42
		Emersion	0.0000	0.0000	0.0000	0.97
		Radiation * Emersion	0.0001	0.0001	0.3200	0.59
ETR _{max}	Day 7	Radiation	0.2274	0.2274	0.5907	0.46
		Emersion	0.1625	0.1625	0.4221	0.53
		Radiation * Emersion	0.1266	0.1266	0.3290	0.58
α	Day 7	Radiation	0.0000	0.0000	0.4152	0.54
		Emersion	0.0000	0.0000	0.9867	0.35
		Radiation * Emersion	0.0000	0.0000	0.6723	0.44
Ek	Day 7	Radiation	135.2000	135.2000	0.0850	0.78
		Emersion	37.3000	37.3000	0.0234	0.88
		Radiation * Emersion	813.4000	813.4000	0.5111	0.50
ETR 75	Day 7	Radiation	0.4174	0.4174	4.3212	0.07
		Emersion	0.0029	0.0029	0.0303	0.87
		Radiation * Emersion	0.3209	0.3209	3.3224	0.11
ETR 150	Day 7	Radiation	1.3792	1.3792	6.3835	0.04
		Emersion	0.0085	0.0085	0.0394	0.85
		Radiation * Emersion	0.6382	0.6382	2.9536	0.12
ETR 300	Day 7	Radiation	0.9361	0.9361	2.3905	0.16
		Emersion	0.0000	0.0000	0.0000	1.00

		Radiation * Emersion	0.4215	0.4215	1.0765	0.33
EDR 75	Day 7	Radiation	0.4160	0.4160	4.3195	0.07
		Emersion	0.0027	0.0027	0.0278	0.87
		Radiation * Emersion	0.3195	0.3195	3.3179	0.11
EDR 150	Day 7	Radiation	1.3656	1.3656	6.3710	0.04
		Emersion	0.0085	0.0085	0.0394	0.85
		Radiation * Emersion	0.6388	0.6388	2.9802	0.12
EDR 300	Day 7	Radiation	0.9097	0.9097	2.3107	0.17
		Emersion	0.0001	0.0001	0.0001	0.99
		Radiation * Emersion	0.4235	0.4235	1.0756	0.33
NPQ 75	Day 7	Radiation	0.0341	0.0341	1.4285	0.27
		Emersion	0.0689	0.0689	2.8830	0.13
		Radiation * Emersion	0.0013	0.0013	0.0537	0.82
NPQ 150	Day 7	Radiation	0.0155	0.0155	0.4845	0.51
		Emersion	0.0803	0.0803	2.5129	0.15
		Radiation * Emersion	0.0040	0.0040	0.1256	0.73
NPQ 300	Day 7	Radiation	0.0063	0.0063	0.1836	0.68
		Emersion	0.0832	0.0832	2.4297	0.16
		Radiation * Emersion	0.0052	0.0052	0.1516	0.71
ETR/EDR 75	Day 7	Radiation	0.0359	0.0359	3.5702	0.10
		Emersion	0.0000	0.0000	0.0045	0.95
		Radiation * Emersion	0.0312	0.0312	3.1058	0.12
ETR/EDR 150	Day 7	Radiation	0.0271	0.0271	5.9862	0.04
		Emersion	0.0004	0.0004	0.0781	0.79
		Radiation * Emersion	0.0132	0.0132	2.9222	0.13
ETR/EDR 300	Day 7	Radiation	0.0032	0.0032	2.3773	0.16
		Emersion	0.0000	0.0000	0.0000	1.00
		Radiation * Emersion	0.0014	0.0014	1.0774	0.33
Nº cells	Day 7	Radiation	3660.4311	3660.4311	26.7665	< 0.01
		Emersion	119.8061	119.8061	0.8761	0.38
		Radiation * Emersion	646.5561	646.5561	4.7279	0.06
Protop. Area	Day 7	Radiation	0.0480	0.0480	0.0238	0.88
		Emersion	21.5776	21.5776	10.6838	0.01
		Radiation * Emersion	12.2136	12.2136	6.0474	0.04

Protop. av. size	Day 7	Radiation	121.5933	121.5933	28.8920	< 0.01
		Emersion	28.1611	28.1611	6.6914	0.03
		Radiation * Emersion	6.9893	6.9893	1.6607	0.23
Carbon	Day 7	Radiation	23.2200	23.2200	5.3830	0.05
		Emersion	0.1800	0.1800	0.0420	0.84
		Radiation * Emersion	34.3400	34.3400	7.9610	0.02
Nitrogen	Day 7	Radiation	1.1438	1.1438	10.0230	0.01
		Emersion	0.0852	0.0852	0.7470	0.41
		Radiation * Emersion	1.8557	1.8557	16.2620	< 0.01
C/N	Day 7	Radiation	10.6757	10.6757	5.4181	0.05
		Emersion	0.2730	0.2730	0.1386	0.72
		Radiation * Emersion	0.8934	0.8934	0.4534	0.52
Sulphur	Day 7	Radiation	0.4711	0.4711	3.5959	0.09
		Emersion	0.2774	0.2774	2.1177	0.18
		Radiation * Emersion	1.2568	1.2568	9.5939	0.01
Phenols	Day 2	Radiation	5.0810	5.0810	0.3219	0.59
		Emersion	0.0480	0.0480	0.0031	0.96
		Radiation * Emersion	20.6060	20.6060	1.3054	0.29
	Day 7	Radiation	13.3370	13.3370	10.1740	0.01
		Emersion	4.5930	4.5930	3.5030	0.10
		Radiation * Emersion	6.3480	6.3480	4.8430	0.06
ABTS	Day 2	Radiation	8.2050	8.2050	0.7850	0.40
		Emersion	0.2300	0.2300	0.0220	0.89
		Radiation * Emersion	0.0890	0.0890	0.0085	0.93
	Day 7	Radiation	1.6470	1.6470	0.4430	0.52
		Emersion	8.0420	8.0420	2.1620	0.18
		Radiation * Emersion	3.7850	3.7850	1.0170	0.34
DPPH	Day 2	Radiation	16.4553	16.4553	0.9014	0.37
		Emersion	7.3557	7.3557	0.4030	0.54
		Radiation * Emersion	31.8934	31.8934	1.7472	0.22
	Day 7	Radiation	74.4370	74.4370	25.7101	< 0.01
		Emersion	2.4030	2.4030	0.8300	0.39
		Radiation * Emersion	4.8510	4.8510	1.6756	0.23
PE	Day 2	Radiation	0.1650	0.1650	0.0132	0.91



PC	Day 7	Emersion	2.5810	2.5810	0.2064	0.66
		Radiation * Emersion	0.0240	0.0240	0.0019	0.97
		Radiation	124.2210	124.2210	7.0431	0.03
		Emersion	52.7640	52.7640	2.9916	0.12
		Radiation * Emersion	16.6100	16.6100	0.9417	0.36
		Radiation	0.2450	0.2450	0.0676	0.80
	Day 2	Emersion	0.7987	0.7987	0.2205	0.65
		Radiation * Emersion	0.1300	0.1300	0.0359	0.85
		Radiation	17.9424	17.9424	4.7555	0.06
	Day 7	Emersion	3.0865	3.0865	0.8181	0.39
		Radiation * Emersion	1.8388	1.8388	0.4874	0.50
		Radiation	0.4291	0.4291	3.7725	0.09
	Day 2	Emersion	0.1980	0.1980	1.7409	0.22
		Radiation * Emersion	0.0279	0.0279	0.2453	0.63
		Radiation	0.3055	0.3055	1.4527	0.26
	Day 7	Emersion	0.1959	0.1959	0.9313	0.36
		Radiation * Emersion	0.1363	0.1363	0.6481	0.44
		Radiation	2.2335	2.2335	6.4164	0.04
MAAs	Day 2	Emersion	1.8461	1.8461	5.3036	0.05
		Radiation * Emersion	2.4396	2.4396	7.0086	0.03
		Radiation	10.0936	10.0936	10.0468	0.01
	Day 7	Emersion	0.9752	0.9752	0.9707	0.35
		Radiation * Emersion	31.7315	31.7315	31.5844	< 0.01
		Radiation				

Table SM.3.2. 5. Pearson's correlation coefficient (r) obtained between the different analyzed parameters at the end (day 7) of both experiments with *Neopyropia leucosticta*: A) bi-factorial experiment of light quality and nutrients concentration, and B) bi-factorial experiment of light quality and emersion/immersion conditions. Green color means positive significant correlation. Red color means negative significant correlation. *p < 0.01; **p < 0.05.

A

	ETR _{int}	F _v /F _m	ETR _{max}	α _{ETR}	E _k ETR	ETR/EDR ₇₅	ETR/EDR ₁₅₀	ETR/EDR ₃₀₀	NPQ ₇₅	NPQ ₁₅₀	NPQ ₃₀₀	n° cell
ETR _{int}	1,0000	,5375*	,6544**	,0510	,4060	,4502	,6548**	,7171**	-,6052**	-,5581*	-,4722*	-,7853**
F _v /F _m		1,0000	,6171**	-,0158	,5135*	,4353	,6655**	,6633**	-,5122*	-,4407	-,3387	-,3822
ETR _{max}			1,0000	,1658	,5808*	,6670**	,8847**	,9514**	-,4066	-,3368	-,2256	-,4575
α _{ETR}				1,0000	-,6853**	,6737**	,4303	,1968	,1957	,2612	,2953	-,0910
E _k ETR					1,0000	-,0535	,2976	,5325*	-,4072	-,4127	-,3539	-,3032
ETR/EDR ₇₅						1,0000	,8861**	,7356**	-,2199	-,1513	-,0504	-,3978
ETR/EDR ₁₅₀							1,0000	,9371**	-,3817	-,2954	-,1714	-,5184*
ETR/EDR ₃₀₀								1,0000	-,5269*	-,4544	-,3351	-,5301*
NPQ ₇₅									1,0000	,9863**	,9542**	,1626
NPQ ₁₅₀										1,0000	,9875**	,1266
NPQ ₃₀₀											1,0000	,0440
n° cell												1,0000
Prot. area												
Prot. Size												
C												
N												
S												
Phenols												
ABTS												
DPPH												
PE												
PC												
Chla												
MAAs												

Table SM.3.2. 5. Continue

A

	Prot. area	Prot. size	C	N	S	Phenols	ABTS	DPPH	PE	PC	Chla	MAAs
ETR _{int}	,5581*	,7127**	,2879	,5442*	,0355	,5334*	,2423	,1528	,8504**	,6202**	,7258**	,8383**
F _v /F _m	,4722*	,4123	,6308**	,6592**	,1532	,5525*	,5347*	,1572	,8012**	,7919**	,5319*	,5129*
ETR _{max}	,3630	,4512	,4859*	,5746*	,0114	,4422	,3758	-,0970	,7156**	,6466**	,4634	,6383**
α _{ETR}	-,1684	-,0152	,0068	-,1890	-,4137	-,0484	,1486	-,1013	-,0199	,0565	-,1876	,0965
E _{KETR}	,4717*	,4014	,3172	,5861*	,3638	,3681	,1104	,0898	,5361*	,4226	,4552	,4127
ETR/EDR ₇₅	,1132	,3404	,2264	,2934	-,1188	,2941	,4053	,1536	,4332	,4421	,3579	,4349
ETR/EDR ₁₅₀	,3303	,4822*	,4263	,4846*	-,0164	,4764*	,4219	,1005	,6950**	,6516**	,5087**	,6615**
ETR/EDR ₃₀₀	,3847	,5194*	,3745	,5642*	-,0041	,5116*	,3426	,0832	,7417**	,6651**	,5555**	,6757**
NPQ ₇₅	-,0208	-,0708	-,2106	-,3035	-,0662	-,5414*	-,3702	-,2499	-,5574*	-,4768*	-,6454**	-,2647
NPQ ₁₅₀	,0148	-,0275	-,1426	-,2347	-,1210	-,4641	-,2644	-,2622	-,4825*	-,3812	-,6431**	-,1943
NPQ ₃₀₀	,0879	,0691	-,0900	-,1377	-,1268	-,3920	-,1788	-,2467	-,3859	-,2859	-,5866*	-,0847
n° cell	-,7527**	-,9455**	-,1594	-,4492	-,0729	-,4483	,0972	-,2921	-,7239**	-,5186*	-,4048	-,8475**
Prot. Area	1,0000	,8633**	,1575	,6652**	,0553	,4753*	-,0563	,2939	,6280**	,4069	,4094	,7265**
Prot. Size		1,0000	,1199	,5879*	,0609	,3999	-,0509	,2817	,6751**	,4589	,4195	,8512**
C			1,0000	,2946	,2317	,2664	,4525	-,3869	,6044**	,6334**	,0971	,3438
N				1,0000	-,2630	,4300	,4657	,2115	,6661**	,5787*	,6528**	,5145*
S					1,0000	,1721	-,0675	,0097	,1414	,1291	,0006	,0806
Phenols						1,0000	,4103	,5189*	,7153**	,7280**	,3775	,5249*
ABTS							1,0000	,0367	,4055	,5298*	,2933	,1545
DPPH								1,0000	,1604	,1301	,3360	,1767
PE									1,0000	,9035**	,5727*	,7688**
PC										1,0000	,3539	,5272*
Chla											1,0000	,4879*
MAAs												1,0000

Table SM.3.2. 5. Continue

B

	ETR _{int}	Fv/F _m	ETR _{max}	alfa _{ETR}	Ek _{ETR}	ETR/EDR ₇₅	ETR/EDR ₁₅₀	ETR/EDR ₃₀₀	NPQ ₇₅	NPQ ₁₅₀	NPQ ₃₀₀	n° cells
ETR _{int}	1,0000	-,1783	,0990	-,5107	,3855	,1134	,0879	,1807	,2248	,1977	,2594	,0064
Fv/F _m		1,0000	-,1389	-,2432	,2024	-,1638	-,1081	-,1403	,0560	,1763	,1813	,0546
ETR _{max}			1,0000	,5284	,2089	,7629**	,8072**	,8701**	-,7673**	-,7014*	-,6017*	,0135
Alfa				1,0000	-,6874*	,5168	,4780	,3133	-,3981	-,4068	-,4049	-,1828
Ek					1,0000	-,0791	,0104	,2539	-,2704	-,2104	-,1414	,3420
ETR/EDR ₇₅						1,0000	,9710**	,9235**	-,4296	-,3419	-,2403	-,3274
ETR/EDR ₁₅₀							1,0000	,9482	-,5763*	-,4810	-,3673	-,3813
ETR/EDR ₃₀₀								1,0000	-,5582	-,4534	-,3224	-,2084
NPQ ₇₅									1,0000	,9747**	,9209**	,0998
NPQ ₁₅₀										1,0000	,9775**	,0851
NPQ ₃₀₀											1,0000	,0942
N° cells												1,0000
Area												
Av. Size												
C												
N												
S												
Phenols												
ABTS												
DPPH												
PE												
PC												
Chla												
MAAs												

Table SM.3.2. 5. Continue

B

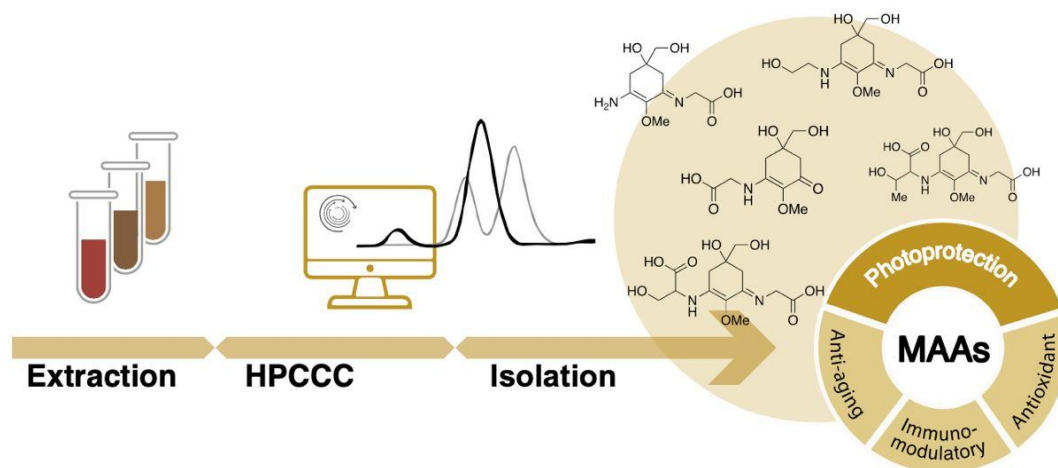
	Protot. area	Protot. size	C	N	S	Phenols	ABTS	DPPH	PE	PC	Chla	MAAs
ETR _{int}	-,2746	,1032	,0696	-,2783	-,0357	,2526	,0002	,3116	-,0620	,0537	-,5396	-,4726
F _v /F _m	-,1550	-,0791	,1983	,1029	,2989	-,4286	,0725	-,4022	,0702	,2016	,2545	,3775
ETR _{max}	,4666	-,0035	-,1819	,1607	-,1454	-,5127	,3132	-,1965	,1847	,1302	,0208	-,1115
Alfa	,1396	,1906	-,4401	,1460	-,2885	-,4106	,2398	-,2574	,0096	-,0551	-,0052	,0035
Ek	,3657	-,2909	,3864	-,0307	,1021	,0396	,0439	,1348	,1462	,1417	,0197	,0138
ETR/EDR ₇₅	,0496	,3174	-,1839	,0600	-,0787	-,5351	,5000	-,2066	,3610	,3799	-,0003	-,2427
ETR/EDR ₁₅₀	,1443	,3792	-,0442	,1793	-,0154	-,6480*	,4986	-,3645	,4478	,4570	,0512	-,1862
ETR/EDR ₃₀₀	,2458	,2135	-,0473	,1284	,0191	-,4959	,3798	-,2162	,3714	,3595	,0799	-,2043
NPQ ₇₅	-,5743	-,1616	-,2033	-,3234	,2193	,6477*	-,4315	,4402	-,3538	-,2779	,0150	-,0971
NPQ ₁₅₀	-,5253	-,1138	-,1542	-,2723	,2987	,5459	-,3943	,3259	-,2529	-,1869	,1634	-,0182
NPQ ₃₀₀	-,4127	-,0832	-,1231	-,2263	,3412	,4895	-,3425	,2669	-,1400	-,0964	,2009	-,0212
N° cells	,4740	-,9304**	-,4831	-,5204	-,4066	,2442	-,0153	,5857*	-,4274	-,4441	-,0713	-,3497
Area	1,0000	-,2086	,0514	,1313	-,2481	-,1833	,2970	-,0475	,3530	,1418	,1872	,0486
Av. Size		1,0000	,6003*	,6060*	,3791	-,2433	,1715	-,6236*	,6705*	,6044*	,1734	,4922
C			1,0000	,7195**	,5989*	,0009	,0931	-,5123	,6661*	,6671*	,1371	,6485*
N				1,0000	,7436**	-,0891	-,0005	-,6816*	,5853*	,5925*	,2136	,7049*
S					1,0000	,1728	-,3482	-,4640	,2970	,4117	,3335	,5063
Phenols						1,0000	-,5007	,6504*	-,3256	-,3474	-,2145	,0221
ABTS							1,0000	-,0528	,6338*	,6717*	-,2799	-,1085
DPPH								1,0000	-,4638	-,4611	-,4607	-,5246
PE									1,0000	,9357**	,1374	,3945
PC										1,0000	,0249	,3075
Chla											1,0000	,3578
MAAs												1,0000

ETR_{int}: integrated electron transport rate (mol e⁻ m⁻²); F_v/F_m: maximal quantum yield, ETR_{max}: maximal electron transport rate (μmol e⁻ m⁻² s⁻¹); α_{ETR}: photosynthetic efficiency; Ek_{ETR}: saturated photosynthetic irradiance (μmol photons m⁻² s⁻¹); ETR/EDR: ratio of electron transport rate and electron dissipation rate at 75,150 and 300 μmol photons m⁻² s⁻¹, NPQ: non-photochemical quenching at 75,150 and 300 μmol photons m⁻² s⁻¹; n° cell: number of

cells; Prot. area: protoplasmatic area (m^2), Prot. size: protoplasmatic size (μm); C: internal carbon content (%); N: internal nitrogen content (%); S: internal sulphur content (%); Phenols: mg g^{-1} DW; ABTS: antioxidant capacity thorough ABTS method ($\mu\text{mol TE g}^{-1}$ DW); DPPH: antioxidant capacity through DPPH method ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$); PE: phycoerythrin (mg g^{-1} DW); PC: phycocyanin (mg g^{-1} DW); Chla: chlorophyll a (mg g^{-1} DW); MAAs: mycosporine-like amino acids (mg g^{-1} DW).

CHAPTER 4

Isolation of Mycosporine-Like Amino Acids from Red Macroalgae and a Marine Lichen by High-Performance Countercurrent Chromatography: A Strategy to Obtain Biological UV-Filters



Article published in *Marine Drugs*

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Isolation of Mycosporine-Like Amino Acids from Red Macroalgae and a Marine Lichen by High-Performance Countercurrent Chromatography: A Strategy to Obtain Biological UV-Filters

Abstract

Marine organisms have gained considerable biotechnological interest in recent years due to their wide variety of bioactive compounds with potential applications. Mycosporine-like amino acids (MAAs) are UV-absorbing secondary metabolites with antioxidant and photoprotective capacity, mainly found in organisms living under stress conditions (e.g., cyanobacteria, red algae, or lichens). In this work, five MAAs were isolated from two red macroalgae (*Pyropia columbina* and *Gelidium corneum*) and one marine lichen (*Lichina pygmaea*) by high-performance countercurrent chromatography (HPCCC). The selected biphasic solvent system consisted of ethanol, acetonitrile, saturated ammonium sulphate solution, and water (1:1:0.5:1; v:v:v:v). The HPCCC process for *P. columbina* and *G. corneum* consisted of eight separation cycles (1 g and 200 mg of extract per cycle, respectively), whereas three cycles were performed for *L. pygmaea* (1.2 g extract per cycle). The separation process resulted in fractions enriched with palythine (2.3 mg), asterina-330 (3.3 mg), shinorine (14.8 mg), porphyra-334 (203.5 mg) and mycosporine-serinol (46.6 mg), which were subsequently desalted by using precipitation with methanol and permeation on a Sephadex G-10 column. Target molecules were identified by HPLC, MS, and NMR.

Keywords: countercurrent chromatography, isolation, marine lichen, mycosporine-like amino acids, photoprotection, red macroalgae

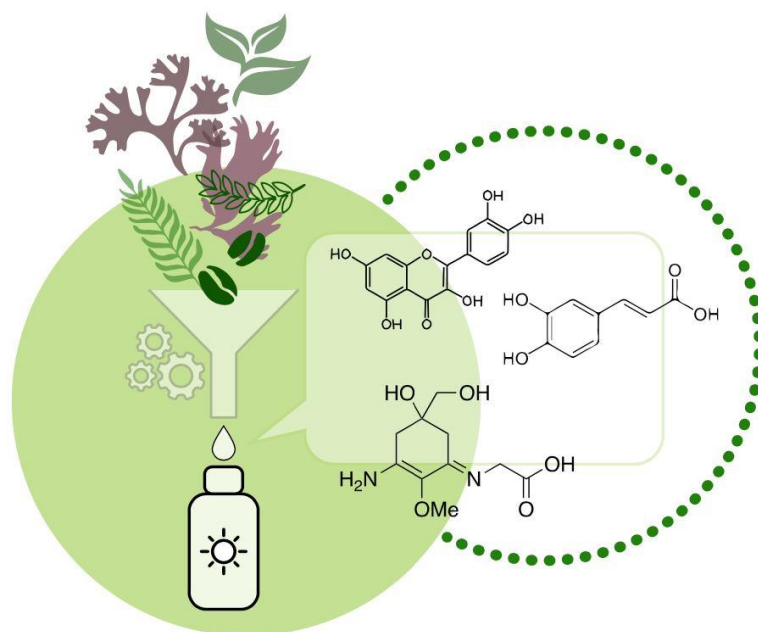


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CHAPTER 5

Design of cosmeceutical products with algae and plants extracts enriched in UV-screen with antioxidant and anti-aging properties

- Potencial cosmeceutic uses of extracts from *Porphyra*-like species (Rhodophyta)
- Looking for new antioxidants and UV screen compounds in diverse plants
- Design and analysis of cosmeceutic products: Determination of photoprotection factors and stability





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Design of cosmeceutical products with algal and plant extracts enriched in UV-screen compounds with antioxidant and antiaging properties

Abstract

Nowadays, consumers are more concern about the possible negative effects of some synthetic compounds in cosmetic products. Algae and plants are gaining interest in this context, as they present molecules with antioxidant and UV-absorbing properties. The objective of this study was the use of algae and plants extracts for the development of cosmeceutical products with antioxidant and photoprotective properties. Firstly, the potential cosmeceutical applications of two *Porphyra sensu lato* species and ten plants were analyzed. *Porphyra linearis* showed the highest MAAs and biliproteins content (10.2 and 11.3 mg g⁻¹ DW, respectively). The highest phenol content was observed in the aqueous extract of *P. linearis* (11.8 mg g⁻¹ DW). Regarding antioxidant capacity, the highest ABTS value was observed in the aqueous extraction of *Pyropia columbina* Valp. Catalase activity were only observed in the aqueous extract of *P. columbina* Valp. and *P. linearis*. All algal species showed collagenase activity close to 100 % in both aqueous and hydroethanolic extracts. The plants with highest potential (highest phenols, UV-screen and antioxidant compounds) were ivy aerial roots, olive leaves, green coffee beans, liquorice root and flame of the forest leaves. All extracts (algae and plants) showed low toxicity in health cell lines, and two of the plants (olive and liquorice) also showed interesting results against melanoma. The addition of algae (*P. columbina* Valp.) and plant (liquorice, green coffee and flame of the forest) extract to the creams increased the photoprotection, e.g. by adding both extract, creams showed a SPF of 23.7 and an UVAPF of 6.7. Other biological effective protection factors (BEPFs) were also determined. Extracts were stable against temperature and UV radiation, although the photoprotection factor of the creams decrease under UV radiation exposure. The addition of physical filters to the formulations, only increased the photoprotection in the UV-A region, and not in UV-B, although it increased the photostability of the creams.

Key words: Antioxidant, Biological effective protection factors, Collagenase, Cosmeceutic, Mycosporine-like amino acids, Photoprotection, Polyphenols, *Pyropia*, Sun protection factor

1. Introduction

The cosmetic industry is continuously growing and evolving, looking for new bioactive compounds and developing novel formulations. Although natural products for medical purpose or as skin-care products have been used since ancient times, it is gaining a growing interest in recent years (Ramawat *et al.*, 2009). Nowadays, consumers are more concern about the negative effects of some synthetic compounds in the human health and the environment. So, they tend to prefer natural alternatives that present low toxicity and are environmentally friendly (Amberg and Fogarassy, 2019; Bom *et al.*, 2019). Natural products obtained from cyanobacteria, algae or plants present a high content of compounds with potential bioactivities, such as antioxidant, antibacterial, antitumor, anti-aging or photoprotective capacities, that can be used in the cosmetic or pharmaceutical sector, grouped as cosmeceutical (Ariede *et al.*, 2017; Charles Dorni *et al.*, 2017; Fonseca *et al.*, 2023).

According to the European legislation, cosmetic is defined as a substance or product intended to be applied in the external part of the human body (skin, hair, nails, lips, external genitals, teeth, and mucous membrane of the mouth) for cleaning, perfuming, beautifying, changing the appearance or protect. It is important to mention that in Europe sunscreens are considered cosmetic products (EC, 2009), but with more strict regulation on the efficacy and security than other products. For example, it is mandatory to determine the sun protection factor *in vivo*. By other side, in United States sunscreens are regulated as drugs (FDA, 2004). The term cosmeceutic, although is not recognize under the law, is used to define the cosmetic products with bioactive ingredients, that claim to enhance both health and beauty of the skin. Many cosmeceutical products are developed for the prevention of the premature skin aging (mainly caused by the exposure to UV radiation or pollution (Krutmann *et al.*, 2017)). The use of topical compounds with antioxidant properties, DNA repair systems or stimulation of collagen synthesis are potential treatments (e.g. vitamins, hydroxy acids, peptides, growth factors or even natural compounds like green tea or *Gingko biloba*) against the photoaging (Draelos, 2007; Martin and Glaser, 2011).

The effects of UV radiation on our health are well established. In fact, UV exposure can be beneficial for our health, i.e. UV-B is necessary for the synthesis of the active vitamin D (involved in the immune system and bone metabolism), contributes to the circadian rhythm, as well as to the stimulation of serotonin and endorphin production affecting our mood (Juzeniene and Moan, 2012; Trummer *et al.*, 2016; van der Rhee *et*

al., 2016). However, overexposure to UV rays cause negative impacts in our health (e.g. sunburn, immunosuppression, photoaging or skin cancer). UV-B rays are the most dangerous radiation type, since they cause mutations in the DNA that can develop skin cancer, whereas UV-A radiation is considered less harmful, but it produces reactive oxygen species that mainly provoke the premature photoaging of the skin (Matsumura and Ananthaswamy, 2004; Narayanan *et al.*, 2010; D'Orazio *et al.*, 2013; Holick, 2016).

Sunscreens can protect against UV damage effects because they contain compounds involved in the absorption, dissipation, or scattering of the UV radiation, reducing the irradiance that penetrates in the different layers of the skin. UV filters are mainly divided in synthetic or chemical, which absorb the UV radiation and dissipate its energy as heat (e.g. avobenzone, octinoxate or octocrylene), and mineral or physical, that can also reflect and scatter the UV radiation (e.g. titanium dioxide and zinc oxide) (González *et al.*, 2008; Shaath, 2010). In the last years, it has been demonstrated that visible light, mainly the high energy visible (HEV; violet-blue; 400-500 nm), also provoke negative effects in the skin through oxidative stress (Nakashima *et al.*, 2017); and that infrared radiation can increase the temperature of the skin above 40 °C, what can provoke some clinical effects (e.g. erythema or premature skin-aging) due synergic negative effects with UVR (Cho *et al.*, 2009). The current UV-filters present limited protection against visible light (i.e. only ZnO can attenuate wavelengths until 450 nm), although iron oxides, normally used to provide color to sunscreen, have been shown to protect against the visible light (Bernstein *et al.*, 2021).

Recently, an increasing number of studies have described that certain UV filters can provoke negative effects on human health (mainly photoallergies or hormone disorders) and the natural environment, especially aquatic ecosystems (Tovar-Sánchez *et al.*, 2020; Santander Ballestín and Luesma Bartolomé, 2023). In aquatic environments, sunscreens are considered an emerging contaminant. Different organic UV-filters have been identified in surface waters (Carve *et al.*, 2021) and in several aquatic organisms (e.g. phytoplankton, mussels, clams, fish), causing negative impacts (e.g. induce coral bleaching, reduce phytoplankton growth or hormone endocrine disruptions in fish or mammals) (Prakash and Anbumani, 2021). Therefore, some areas with a valuable marine ecosystem like Hawaii have banned the sale, distribution and use of sunscreens containing certain UV-filters to protect the environment.

The research and development of new natural alternatives that are biodegradable and non-toxic are still a great challenge. In this context, the natural photoprotection

mechanisms observed in many organisms can be studied and used as new sources of natural UV filters for sunscreens. Intertidal algae, that live fixed to the substrate and are exposed to a very harsh environment, have developed different adaptation and acclimation strategies to protect themselves against the damage UV radiation, such as strong antioxidant defense systems and the accumulation of UV-absorbing compounds (e.g. mycosporine-like amino acids or polyphenols) (Lalegerie *et al.*, 2020a). *Porphyra sensu lato* species live in the upper part of the intertidal zone, being exposed to a high solar radiation, a great range of temperature and salinity, as well as periods of desiccation. Thus, they must present a high antioxidant capacity and one of the highest contents of mycosporine-like amino acids (MAAs), among red algae (Korbee-Peinado, 2003; Huovinen *et al.*, 2004; Navarro *et al.*, 2018; Schneider *et al.*, 2020b; Sun *et al.*, 2020; Moreira *et al.*, 2022). MAAs are secondary metabolites involved in photoprotection and mainly present in red algae and cyanobacteria. They are able to absorb UV radiation energy and dissipate it as heat without the production of oxidative photoproducts (Carreto and Carignan, 2011; Vega *et al.*, 2021). In addition to the UV absorbing capacity, other beneficial properties for the skin have been described in MAAs, like antioxidant, anti-inflammatory or anti-aging (de la Coba *et al.*, 2009b, 2019; Hartmann *et al.*, 2015b; Wada *et al.*, 2015; Becker *et al.*, 2016; Lawrence *et al.*, 2018), thus they are potential candidates for the cosmeceutical industry. *Porphyra sensu lato* species present other molecules like biliproteins or polyphenols also with potential good properties for the skin (Mateos *et al.*, 2020; Chen *et al.*, 2022).

In addition to algae, terrestrial plants are a great and diverse source of novel bioactive compounds. Several reviews and articles have been published about the potential use of different plants as UV-absorbing, anti-inflammatory, anti-photoaging or antioxidants, demonstrated by different *in vivo* and *in vitro* studies (Radice *et al.*, 2016; Torres *et al.*, 2020). Plants present several secondary metabolites including alkaloids, carotenoids, flavonoids, lignans, phenols, tannins, quinones, saponines and methylxanthines that can be produced as acclimation to different environmental conditions (Dinkova-Kostova, 2008).

Torres *et al.* (2020) reviewed the most extensively studied plants with photoprotective properties, such as *Polypodium leucotomos* (currently named as *Phlebodium aureum*) extract, green tea, pomegranate, resveratrol, curcumin, and silymarin. For example, *P. aureum* (commonly known as calaguala) is a fern native from the tropical and subtropical region of America, whose leaves extract is commercially

used by the company Cantabria Labs (Madrid, Spain), named as Fernblock[®], due to the antioxidant, immunomodulatory, tumor suppressive and anti-aging properties (Parrado *et al.*, 2016, 2020; Aguilera *et al.*, 2021). The beneficial properties are mainly attributed to the polyphenol content (e.g. caffeic and ferulic acid) (Parrado *et al.*, 2016). It was observed that the oral administration of *P. aureum* extract was less likely to get sunburn in the solar-exposed skin denominated as oral photoprotection (Nestor *et al.*, 2015; Kohli *et al.*, 2017; Parrado *et al.*, 2018). Pomegranate (*Punica granatum*) extracts are also used as oral supplements and/or incorporated to commercial skin care products, due to its potent antioxidant activity and anti-inflammatory properties. The extracts are rich in phenols, specifically anthocyanins, catechins and tannins. It confers photoprotection through inhibition of the free radicals produced by UV radiation exposure, preventing DNA damage or collagen breakdown (Baccarin *et al.*, 2015; Saewan and Jimtaisong, 2015; Parrado *et al.*, 2018; Henning *et al.*, 2019).

Among the plants analyzed in this study, many of them have shown potential *in vitro* cosmeceutical application. Coffee (*Coffea* spp.), one of the most consumed beverages in the world, is known as a source of antioxidant compounds (e.g. caffeine or chlorogenic acid) with health benefits like reduction of diabetes, cardio- or neuroprotective properties (Ludwig *et al.*, 2012; Tajik *et al.*, 2017; O'Keefe *et al.*, 2018). Regarding photoprotection, some authors observed potential applications: Chiari *et al.* (2014) observed a synergistic effect combining green coffee oil with the synthetic UV filter octinoxate, leading to an increase of 20 % of the SPF values. Koo *et al.* (2007) observed that caffeine application after UV exposure reduce the photodamage effects in mouse skin. In addition, Bessada *et al.* (2018) and Rodrigues *et al.* (2023) reviewed the beneficial properties for the skin described in the coffee or its compounds, e.g. anti-aging, anti-inflammatory or antimicrobial.

Liquorice (*Glycyrrhiza glabra*) roots have been traditionally used to treat many diseases like respiratory disorders, epilepsy, fever, stomach ulcers, sexual debility, or skin diseases, among others (Mamedov and Egamberdieva, 2019). More than 400 compounds have been isolated from liquorice, being Glycyrrhizinic acid the major bioactive compound that constitute around 10-25 % (Cerulli *et al.*, 2022). Several cosmeceutical applications have been described from liquorice, such as skin lightening, antioxidant, anti-wrinkle, hair care, hair growth, anti-acne or photoaging (Cerulli *et al.*, 2022), although very few information have been found about photoprotection (Xuan *et al.*, 2017; Park *et al.*, 2021)

Olive (*Olea europea*) leaves are considered a by-product of the olive oil industry, so many researchers have studied their potential bioactivities (Nunes *et al.*, 2016; Otero *et al.*, 2021), with a specific focus on the cosmetic sector (Melguizo-Rodríguez *et al.*, 2022; Nunes *et al.*, 2022). Regarding photoprotection, Kimura and Sumiyoshi (2009) observed that the orally administration of oleuropein (the main polyphenol found in olive) protect against the damage effects of UV-B radiation. Machała *et al.* (2022) also obtained promising results of olive leaves as photoprotective agent under *in vitro* assays with skin fibroblast. Da Silva *et al.* (2019) added 3-5 % of olive leaves extract to a cream with synthetic UV-filter (octocrylene) and increased of SPF values from 22 to 56. Ivy is a widely known plant with the ability to climb and adhere to different solid substrates. (Xia *et al.*, 2010) studied nanoparticles present in the aerial roots of ivy as UV-blocking compounds and demonstrated that ivy nanoparticles were more efficient in blocking UV light, less toxic and easily biodegradable than a commercial physical UV-filter (TiO₂ nanoparticles).

Considering the need to develop natural UV-filters that are safer for the health and the environment, and that algae and plants present photoprotective molecules in their internal composition, in this study three different approaches were followed:

- Characterization of aqueous and hydroethanolic extracts from two species of *Porphyra sensu lato*, focusing on the presence of UV-absorbing and antioxidant compounds, and analyze their antioxidant and anti-aging properties.
- Evaluation of different plant extracts (collected or commercially available) looking for a new source of antioxidants and UV-absorbing compounds.
- Development of cosmeceutical products with algae and plant extracts, as well as other natural compounds, and analyze their photoprotective capacity and photo- and thermostability.

2. **Material and methods**

2.1. **Biological material** (algae and plants)

Two *Porphyra sensu lato* species were analyzed in the first part of this study. *Pyropia columbina* (Montagne) W.A.Nelson 2011 specimens were collected in two different sites of Chile: Cocholgüe beach, Concepción, (36°35'37"S / 75°58'43"W) and Maitencillo beach, Valparaíso, (32°38'55"S 71°26'30"W). *Porphyra linearis* Greville 1830 were

sampled in Tarifa (Cádiz, Spain, 36°00'42"N / 05°35'59"W). All thalli were collected in February 2021. Algae were transported to the laboratory in a portable fridge at 4 °C, cleaned to remove rest of sand and sediments, frozen at -20 °C and freeze-dried (LyoQuest, Telstar, USA). Samples were grounded with a coffee grinder and kept in darkness and dried with silica-gel until extractions.

In the second part of this study, different plants were analyzed (Table 5. 1). Plant biomass (fresh) were grounded using a coffee grinder and kept at -10 °C until its analysis.

The biological material (algae and plants) was submitted to different procedures as detailed in the Figure 5. 1 and explained below.

Table 5. 1. Summary of the different plants used in the study, specifying the common name, scientific name, part of the plant used, collection place and CAS number (if it exists).

Common name	Scientific name	Part of the plant	Collection place	N° CAS
Ginger	<i>Zingiber officinale</i>	root	Commercial	84696-15-1
Ivy	<i>Hedera helix</i>	leaves	Malaga, Spain	84082-54-2
Ivy	<i>Hedera helix</i>	root	Malaga, Spain	
Orcanette	<i>Alkanna tinctoria</i>	root	Commercial	85251-58-7
Olive	<i>Olea europea</i>	leaves	Malaga, Spain	8060-29-5
Green coffee	<i>Coffea</i> sp.	seed	Commercial	327-97-9
Liquorice	<i>Glycyrrhiza glabra</i>	root	Commercial	68916-91-6
Calaguala	<i>Phlebodium aureum</i>	leaves	Málaga, Spain	
Urukum	<i>Bixa orellana</i>	seed	Commercial	8015-67-6
Flame of the forest	<i>Spathodea campunulata</i>	leaves	Florianopolis, Brasil	
Calaguala (capsules)	<i>Phlebodium aureum</i>	leaves	Commercial	

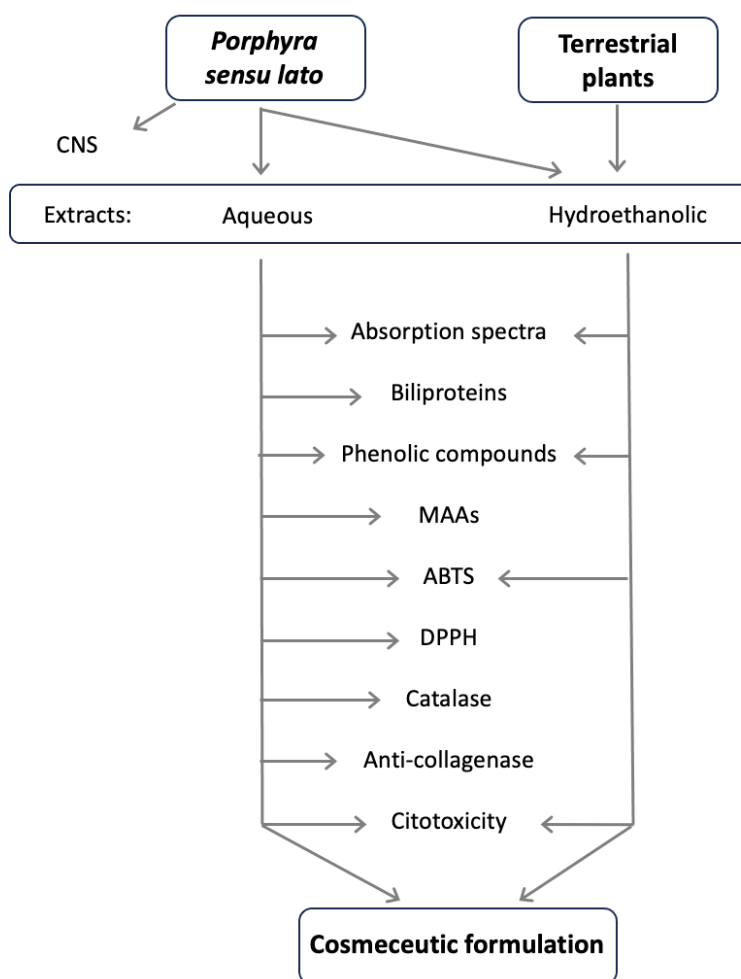


Figure 5. 1. Scheme of the different analyzed performed, from the algal or plant extraction procedures and analyses, aiming to integrate them into a cosmeceutic formulation.

2.2. Total carbon, nitrogen and sulphur content

From dry algal biomass, total carbon, nitrogen and sulphur content and the C/N ratio were determined using an elemental analyzer (CNHS LECO-932, Michigan, USA) in the Research Support Central Services (SCAI, University of Málaga).

2.3. Extraction of bioactive compounds

2.3.1. Extraction procedure

For the extraction of the biomass from different *Porphyra sensu lato* species, two different extraction solvents were used: aqueous (distilled H_2O ; H_2O_d), and hydroethanolic 50 % (H_2O_d :ethanol; 1:1), whereas in the case of the plants, only the

hydroethanolic 50 % solvent was used. One g of dried algae was homogenized in 50 mL of the solvent (concentration of 20 mg mL⁻¹), and 12 g of the fresh biomass were homogenized in 100 mL (concentration of 120 mg mL⁻¹). Both extracts were homogenized using ultra-turrax (T25, IKA, Germany). Extracts were incubated in a water bath during 2h at 45 °C with constant agitation. After incubation, extracts were centrifuged (10 min, 4000 rpm and 4 °C; Labofuge 400R, Heraeus) and the supernatants were used for the different analysis. One part of some extracts was frozen at -80 °C and freeze-dried for the calculation of the extraction yield, and determination of the catalase activity, collagenase inhibitory capacity and cytotoxicity. The extraction yield was calculated using the following formula $Y = (W_{DE} / W_{DB}) * 100$, where W_{DE} is the weight of the dry extract and W_{DB} is the weight of the dry biomass.

2.3.2. Absorption spectrum

Absorbance or optical density of the different extracts (algae and plants) were measured using an UV-visible spectrophotometer (UV Mini-1240, Shimadzu, USA) from 280 to 700 nm.

2.3.3. Phycobiliproteins

For phycobiliproteins content in algal extracts, aqueous extracts were measured spectrophotometrically at 455, 564, 592, 618, 645 and 750 nm, and the total of phycoerythrin and phycocyanin were calculated applying the formula described by Beer and Eshel (1985).

2.3.4. Phenolic compounds

Phenolic compounds were quantified from the different extracts (algae and plants) using the Folin-Ciocalteu method according to Singleton and Rossi (1965). For the reaction, 100 µL of the algal extracts were mixed with 700 µL of H₂O_d and 50 µL of Folin-Ciocalteu reagent (Sigma-Aldrich, USA). After vortexing, 150 µL of 20 % Na₂CO₃ were added to the mixture and vortexed again. After an incubation period of 2h at 4 °C, absorbance was measured spectrophotometrically at 760 nm. Phloroglucinol was used as standard.

2.3.5. *Mycosporine-like amino acids (MAAs)*

In algal extracts, MAAs were identified and quantified through high performance liquid chromatography (HPLC) as described Korbbee-Peinado *et al.* (2004) with modification of Chaves-Peña *et al.* (2020). Aqueous extracts were filtered through 0.2 μm filters and analyzed using an Agilent UHPLC system (1260 Agilent InfinityLab Series, USA) with a photodiode array detector (DAD). MAAs were detected using a Luna C8 column (Phenomenex, Germany), applying an isocratic flow of 0.5 mL min^{-1} and a mobile phase of 1.5 % methanol and 0.15 % acetic acid in ultrapure H_2O . The obtained peaks were identified using isolated MAAs (palythine, asterina-330, shinorine and porphyra334) obtained through liquid-liquid chromatography (Vega *et al.* 2023). The quantification was performed using the molar extinction coefficients (ϵ) of the different MAAs (Karsten *et al.*, 1998b). Results were expressed as mg g^{-1} DW.

2.4. *In vitro* assays in the extracts

2.4.1. *General antioxidant capacity (non-enzymatic antioxidants)*

General antioxidant capacity was analyzed through two different free radicals scavenging methods:

The ABTS methodology was performed according to Re *et al.* (1999) with some modifications in both algae and plant extracts. Seven mM of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and 2.45 mM of $\text{K}_2\text{S}_2\text{O}_8$ were mixed in phosphate buffer (0.1M; pH:6.5) for the formation of the ABTS radical cation (ABTS^+). This mixture was stored for 12-16h at room temperature to ensure the complete formation of radical. Before the assay, the stock solution of ABTS^+ was diluted with phosphate buffer until the absorbance at 727 nm was around 0.75 ± 0.05 . For the reaction, 50 μL of the extracts were mixed with 950 μL of diluted ABTS^+ . After 8 min of incubation, absorbance was measured at 727 nm.

The DPPH assay was made according to Brand-Williams *et al.* (1995) with some modifications only in the case of algae extracts. Two hundred μL of the extracts were mixed with 1 mL of 0.06 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) in methanol 80 %. After 30 min of incubation at room temperature and darkness, absorbance was measured at 517 nm.

For both methods, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as standard and the results were expressed as $\mu\text{mol TE (Trolox equivalent) g}^{-1}$ DW.

2.4.2. Catalase activity (enzymatic antioxidant)

Catalase activity was determined from algal extracts following the decay of hydrogen peroxide absorbance at 240 nm, as described by Moreira *et al.* (2022). Twenty mg of the lyophilized extracts were dissolved in 1 mL of dH_2O . Fifty μL of the extracts were mixed with 1 mL of phosphate buffer (50 mM, pH:7) containing 20 mM of hydrogen peroxide. Absorbance was measured each 15s during 7 min. One unit of catalase was defined as the amount of enzyme that hydrolyzes 1 $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1}$, at 25 °C. The results were expressed as U. CAT per milligram of soluble protein. The determination of the soluble protein content in the extracts was performed according to Bradford (1976). Briefly, 100 μL of aqueous extracts were mixed with 700 μL of PBS and 200 μL of protein assay solution (Biorad, Germany). The mixture was vigorously shaken, and absorbance was measured at 595 nm. Albumin bovine serum was used as standard.

2.4.3. Collagenase inhibitory activity

The inhibition of the collagenase activity was determined from algae extracts as described by Moreira *et al.* (2022). Collagenase from *Clostridium histolyticum* (Sigma-Aldrich, USA) was dissolved in tricine buffer (50 mM, pH 7.5 with 10 mM CaCl_2 and 400 mM NaCl) at a concentration of 1.6 U mL^{-1} . FALGPA (Furylacrololyl-LEU-GLY-PRO-ALA) was used as substrate and dissolved in tricine buffer at a concentration of 1 mM. For the reaction, 20 mL of the collagenase solution were incubated during 20 min with 50 μL of the different algal extracts (5 mg mL^{-1}). After that, 70 μL of tricine buffer and 100 μL of FALGPA solution were added. Absorbance was measured in a microplate reader (Micro Plate Reader Synergy HTX, BIOTEK, USA) at 0 min and after 5 min of incubation at room temperature. Epigallocatechin gallate (EGCG) was used as positive control. Results were expressed as inhibition percentage and for one of the species the half maximal effective concentration (EC_{50}) was calculated.

2.4.4. Cytotoxicity on healthy and tumoral cells

The cytotoxicity of algae and plant extracts was determined against three different skin cell lines: human fibroblasts (1064sk, CIC-UGA, ES), immortalized human keratinocytes (HACAT, ATCC, USA), and human malignant melanoma (G-361, ATCC, USA). HACAT cells were cultured in RPMI-1640 (Bio-Whittaker). 1064sk and G-361 cells were cultured with DMEM (Biowest) medium. Both mediums were supplemented with 10 % fetal bovine serum (Biowest), 1 % penicillin-streptomycin and 0.5 % of amphotericin B. Cells were maintained in an atmosphere-controlled incubator with 5 % CO₂ and 37°C.

Citotoxicity was determined using the MTT assay, based on the reduction of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) with yellow color to its formazan insoluble form (purple color) due to the activity of the mitochondrial succinate dehydrogenase in living cells (Mosmann 1983). Cells were incubated with different extracts concentrations (0.009-10 mg mL⁻¹) during 72 h in 96-well plates. After that time, 10 µL of the MTT solution (5 mg mL⁻¹ in phosphate buffered solution) and 100 µL of culture medium were added to each well. After 4 h of incubation, the produced formazan crystals were solubilized with 150 µL of 0.04 M HCl in 2-propanol and absorbance was measured at 550 nm using a microplate reader. The EC₅₀ were calculated and expressed mg mL⁻¹. The selectivity index (SI) were calculated as $EC_{50non\ cancer\ cells} / EC_{50cancer\ cells}$. SI values > 10 considered that the extract/compound present potential to be further investigated due to its toxicity (Peña-Morán *et al.* 2016), whereas Bézivin *et al.* (2003) considered lower values (> 3).

2.5. Development of cosmeceutic formulations

A water-in-oil (W/O) base cream formulation was elaborated according to the requirements of natural cosmetics, by adding two commercial oils from plants, karanja (Aroma-Zone) and calophyl (Camassia), with potential bioactivities, and one of the analyzed algae (*P. columbina* Vapl., in the last section of the chapter named as *Pyropia*) and/or some of the analyzed plants (liquorice roots, green coffee beans and flame of the forest leaves). The selection of the species was done based on the best results previously obtained (in case of the algae the biomass availability also influenced the selection).

Five different approaches were followed in the design of the creams (Table SM.5. 1):

A) Combination of different oils for the base cream.

- B) Addition of the *Pyropia* extracts to the formulation in different percentages (5, 10 and 15 %).
- C) Addition of a mixture of plants (MP: 40 % liquorice, 40 % green coffee and 20 % flame of the forest) extracts to the formulation in different percentages (5, 10, 15 and 20 %).
- D) Combination of the *Pyropia* and MP extracts in the formulation (50 % each one).
- E) Addition of physical filters for the formulation and its combination with the *Pyropia* and MP extracts.

2.5.1. Analysis of the ingredients

New extracts were done for the develop of the cosmeceutical products as described in section 2.3.1, but in a higher volume and using a blender. Extracts were concentrated, frozen at -80 °C, freeze-dried and kept in dark and with silica gel until their use. For the analysis, dry extracts were dissolved in their extraction solvents (aqueous for *Pyropia* and hydroethanolic for plants and) at a concentration of 1 mg mL⁻¹. The mixture of plants (MP) and the combination of *Pyropia* + MP were also prepared at 1 mg mL⁻¹ and analyzed. The UV-spectrum was determined as in section 2.3.2, the phenols content as in section 2.3.4 and the antioxidant capacity (ABTS) as in section 2.4.1. For the analysis of commercial oils (karanja and calophyl), they were dissolved in hexane at a concentration of 10 mg mL⁻¹. The UV spectrum was measured as in section 2.3.2. The antioxidant capacity was determined through the DPPH method as described in section 2.4.1, although with some modifications, i.e. DPPH were dissolved in hexane instead of methanol 80 %.

2.5.2. Thermo and photostability of the extracts

The thermo- and photostability of the *Pyropia* extracts, the mixture of plant extracts (MP) and the combination of *Pyropia* + MP were determined. For that, the dry extracts were dissolved in H₂O_d (the hydroethanolic solvent was not used to avoid a fast evaporation) at a concentration of 1 mg mL⁻¹. For the thermostability, eppendorfs with 1 mL of the extracts were place in a block heater (SBH130D/3, Stuart) during 1h at different temperature: 45, 60 and 80 °C. For the photostability, 8 mL of the extracts were placed in small petri dishes and irradiated with a UV lamp (3 x UVA340, Q-Lab Corporation, Canada) during 30 and 60 min. The spectrum and UV-intensities obtained from the Q-

Panel UVA340 were measured using a spectroradiometer (SMS 500, Sphere Optics, Germany) and shown in Figure 5. 2.

After the incubation time, UV spectra, phenolic compounds, and antioxidant capacity through ABTS method were measured again.

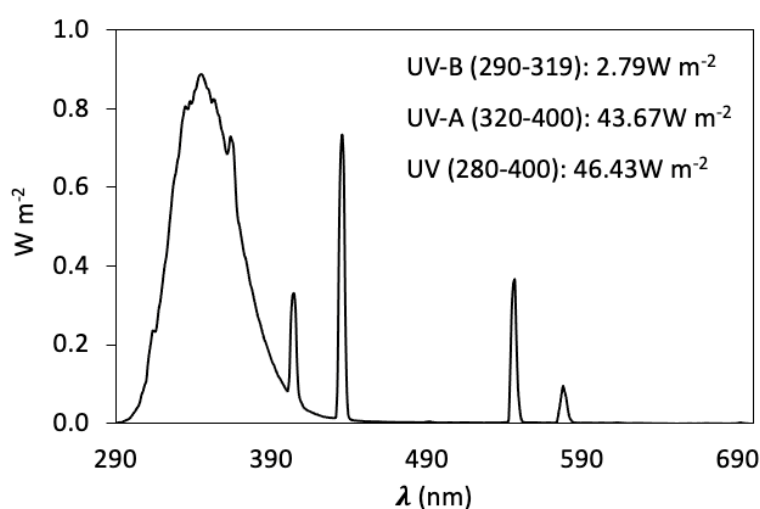


Figure 5. 2. Spectra of the light source (3 x UVA340, Q-Lab Corporation, Canada) used for the irradiance of the plates (Q-panel 340).

2.6. Photoprotection assay of the creams

Different photoprotection factors were determined: Sun protection factor (SPF), UVA protection factor (UVAPF) and Biological effective protection factors (BEPFs).

For all photoprotection factors determination, creams were spread in PMMA (polymethyl metacrilate) plates as suggest the ISO 24443 (2012) and Pissavini *et al.* (2018). PMMA plates (Schönberg, Hamburg, Germany) presented a roughness (Ra) of 6 μm and a surface of 50 x 50 mm. Creams were spread at a concentration of 1.2 mg cream cm^{-2} (32.5 mg in the total surface of the plate) using a fingertip gloves (previously saturated with the cream) and for no more than 1 min. After 15 min of incubation at room temperature and darkness, transmittance through the plates were measured using a spectrophotometer (UV-2600, Shimadzu, Duisburg, Germany) with an integrated sphere (ISR-2600Plus, Shimadzu, Duisburg, Germany) (ISO 24443 2012; Pissavini *et al.* 2018). The transmittance (T) values were converted to absorbance (A) values as $A = -\log(T)$. Three plates were spread for each cream and two measured per plate were done.

The sun protection factor was calculated using the erythematic action spectrum (McKinlay 1987) as described Pissavini *et al.* (2018) with some modifications. These authors suggested the use of two type of plates (molded and sandblasted) (Figure SM.5. 1) and three replicates per type of plate, as a previous study found that a single plate type was insufficient to allow the accurate calculation of the *in vitro* SPF for multiple emulsion types (e.g. lotions, creams, sprays, milk, gel, fluid milk), although we only use one type of plate (sandblasted). They also use a robot to automate the spread of the creams in the plates, while we spread the creams manually. The determination of the initial SPF value was done using the following formula:

$$SPF = \frac{\int_{\lambda=290}^{\lambda=400} E(\lambda) I(\lambda) d(\lambda)}{\int_{\lambda=290}^{\lambda=400} E(\lambda) I(\lambda) 10^{-Abs(\lambda)} d(\lambda)}$$

where,

$E(\lambda)$ = Erythematic action spectra (0-1)

$I(\lambda)$ = spectral irradiance of a sunny midday in summer in Malaga ($W m^{-2}$)

$d(\lambda)$ = wavelength step (1nm)

$Abs(\lambda)$ = absorbance values (0-1)

After the calculation of the initial value, the plates were irradiated with a UV source. For the irradiation, Pissavini *et al.* (2018) used a UV solar simulator according to the limits described in the ISO 24444 (2010) for the determination of the SPF *in vivo*, and calculate the irradiation dose as $D (J cm^{-2}) = 0.25 \times SPF$. However, we used the source described in the section 2.5.2 and irradiate all the plates during different times until a maximum of 2 hours (the calculated irradiation dose did not reach 120 min in any case). After the irradiation time, the final SPF was calculated again using the same formula mentioned below.

The UVAPF was calculated as described the ISO 24443 (2012) for the determination of the UVA protection *in vitro*. Firstly, the SPF was determined *in vitro* using the same formula described below (without irradiation). After this, it was determined the “C” value,

in order to adjust the calculated *in vitro* SPF to the labelled SPF in commercial sunscreens (determined *in vivo*). The C value must lie between 0.8 and 1.6.

$$\text{SPF in vivo} = \text{SPF in vitro} = \frac{\int_{\lambda=290}^{\lambda=400} E(\lambda) I(\lambda) d(\lambda)}{\int_{\lambda=290}^{\lambda=400} E(\lambda) I(\lambda) 10^{-\text{Abs}(\lambda)C} d(\lambda)}$$

The UVAPF was calculated in a similar way to the SPF, but using the persistent pigment darkening (PPD) action spectrum ISO 24443 (2012) and using the following formula:

$$\text{UVAPF} = \frac{\int_{\lambda=290}^{\lambda=400} \text{PPD}(\lambda) I(\lambda) d(\lambda)}{\int_{\lambda=290}^{\lambda=400} \text{PPD}(\lambda) I(\lambda) 10^{-\text{Abs}(\lambda)C} d(\lambda)}$$

After the calculation, plates were also irradiated, and the radiation doses were calculated as $D \text{ (J cm}^{-2}\text{)} = \text{UVAPF} \times 1.2$. For the irradiation it should be used a UV source with an irradiance (290-400nm) of 40-200 W m^{-2} , and a ratio UV-A/UV-B between 8 and 22 (ISO 24443 2012). We used the UV source described in section 2.5.2, and as mentioned before we irradiated the plates during different times until a maximum of 2 hours (the calculated irradiation dose did not reach the 2 hours in any case). After the irradiation time, the final UVAPF were calculated using the same formula.

This procedure was followed for the commercial sunscreens, but in case of the creams made by us it was not possible as we did not have the labelled or *in vivo* SPF. Thus, we considered the “C” value used for the UVAPF determination equal to 1 in all cases.

The critical lambda (λ_c) were also calculated as the wavelength (from 290 to 400 nm) at which the creams absorbed 90 % of the radiation.

Other biological effective protection factors (BEPFs) were calculated as described de la Coba *et al.* (2019), against different action spectrum: photocarcinogenesis (de Gruijl and Van der Leun, 1994), immunosuppression (Fabo and Kripke, 1980), elastosis (Wulf *et al.*, 1989), singlet oxygen formation (Hanson and Simon, 1998) and photoaging (Bissett *et al.*, 1989). The action spectra of the different biological effects can be

observed in the Introduction of the thesis. The following formula was used, where Act. Sp can be the different action spectrum described above.

$$\text{BEPFs} = \frac{\int_{\lambda=290}^{\lambda=400} \text{Act.Sp}(\lambda) I(\lambda) d(\lambda)}{\int_{\lambda=290}^{\lambda=400} \text{Act.Sp}(\lambda) I(\lambda) 10^{-\text{Abs}(\lambda)} d(\lambda)}$$

2.7. Thermo- and photostability of the creams

To determine the photostability of the creams. Selected creams were spread in the PMMA plates as described in section 2.6 and irradiated with the same UV lamp (3 x UVA340, Q-Lab Corporation, Canada) mentioned below during different periods of time (Figure 5. 2). After the irradiation time, transmittance through the plate was measured again as described in section 2.6. The dose and erythema dose emitted at each period are shown in Table 5. 2. In addition, the irradiation time of this lamp required for the standard erythema dose (SED; 100 J m⁻²) and minimal erythema dose (MED) using an average of phototype 2 and 3 (300 J m⁻²), were also calculated, and were 6.20 and 18.60 min respectively. Thermostability of the creams were done as described in 2.5.2.

Table 5. 2. Exposure time to UV radiation (min) of the PMMA plates with the creams, the dose (kJ m⁻²) and the erythema dose (J m⁻²) received in each period of irradiation.

Exposure time to UVR (min)	Dose (KJ m-2)	Erythema dose (J m-2)
15	41.79	241.92
30	83.58	483.84
60	167.15	967.67
120	334.30	1935.34

2.8. Statistical analysis

The software STATISTICA was used for the statistical analysis. Two-way ANOVAs (analysis of variance) were used to analyze significant differences caused by the factors extraction solvent and species or *Porphyra sensu lato* in the extraction yield, phenols content, antioxidant capacity (ABTS and DPPH) and cytotoxicity of HACAT cells. One-way ANOVAs were used to analyze the differences in the biliproteins and MAAs content, and cytotoxicity of 1064SK cells of *Porphyra sensu lato* species, the phenols content, antioxidant capacity and cytotoxicity of plant extracts, the photoprotection factors of the

different creams and photo- and thermostability of the creams. Student t-test was used to compare when there were only two samples (catalase) and between the samples and the initial values. Correlations among measured variables in aqueous and hydroethanolic extracts of *Porphyra sensu lato*, and hydroethanolic extracts of plants were determined using Pearson's correlations analysis.

3. Results

3.1. Potencial cosmeceutic uses of extracts from *Porphyra sensu lato* species

The yields of the aqueous and hydroethanolic extractions are observed in Figure 5. 3. Significant differences between extraction solvents and among species separately were observed (no significant interaction) (Table SM.5. 2). Aqueous extractions showed the highest yield (19.2 vs 16.1 %). Small differences were observed among species, and *P. columbina* Concep. showed lower values (16.4 %) than the other species (18.3 %).

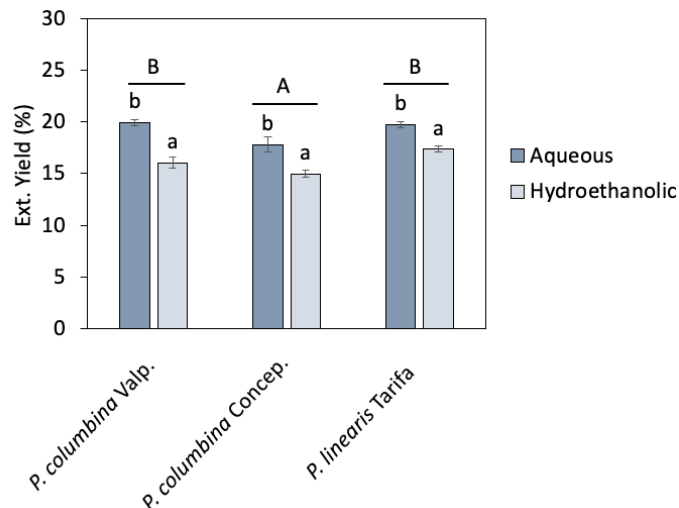


Figure 5. 3. Extraction yield (%) of aqueous (H_2O_d) and hydroethanolic 50 % (H_2O_d :ethanol; 1:1) extracts of two *Porphyra sensu lato* species: *Pyropia columbina* collected in Valparaiso (Chile), *Pyropia columbina* collected in Concepcion (Chile) and *Porphyra linearis* collected in Tarifa (Spain). Results are expressed as mean $\pm$ standard deviation (SD) ($n = 3$). Different letters indicate significant differences between variables: Capital letters indicate significant differences among species, and lower-case letters indicate significant differences between extraction solvents (ANOVA, SNK, $p < 0.05$).

The absorption spectra of algae extracts are observed in Figure 5. 4. Regarding the UV spectra, all analyzed algae showed similar peaks between 320 and 340 nm. In the case of the visible spectrum, evident differences between the extraction solvents were observed. The aqueous extract showed two main peaks with maximum absorption at 500 and 565 nm, whereas the hydroethanolic extract showed a linear absorption with the highest absorption around 420 nm.

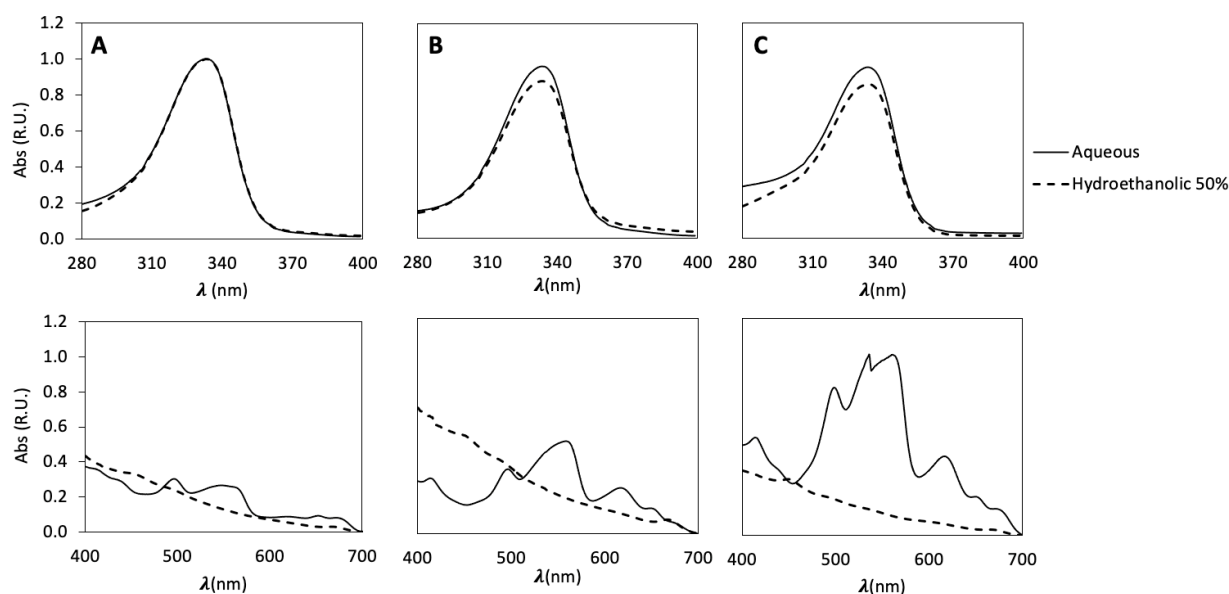


Figure 5. 4. Absorbance or optical density in the UV (280-400 nm) and visible region (400-700 nm) of aqueous (H_2O_d) and hydroethanolic 50 % (H_2O_d :ethanol; 1:1) extracts of two *Porphyra sensu lato* species: (A) *Pyropia columbina* collected in Valparaiso (Chile), (B) *Pyropia columbina* collected in Concepción (Chile) and (C) *Porphyra linearis* collected in Tarifa (Spain). Absorbance values were divided by the maximum of all the plant extracts in order to obtain values between 0 and 1. Values are expressed as the mean of three replicates ($n = 3$).

The three algae presented relevant content of MAAs in their aqueous extracts, although *P. linearis* was highlighted, reaching $10.0 \pm 0.5 \text{ mg g}^{-1} \text{ DW}$ (Figure 5. 5: A; Table SM.5. 2). All samples presented two predominant MAAs (porphyra-334 and shinorine), although different contents were observed. *P. columbina* Valp. presented 92.8 % of P-334, 5.7 % of shinorine and 1.5 % of palythine. *P. columbina* Concep. showed 66.4 % of P-334, 32.8 % of shinorine and < 1% of palythine. The other species, *P. linearis*, contained 92.6 % of P-334 and 7.4 % of shinorine (Figure 5. 5:B).

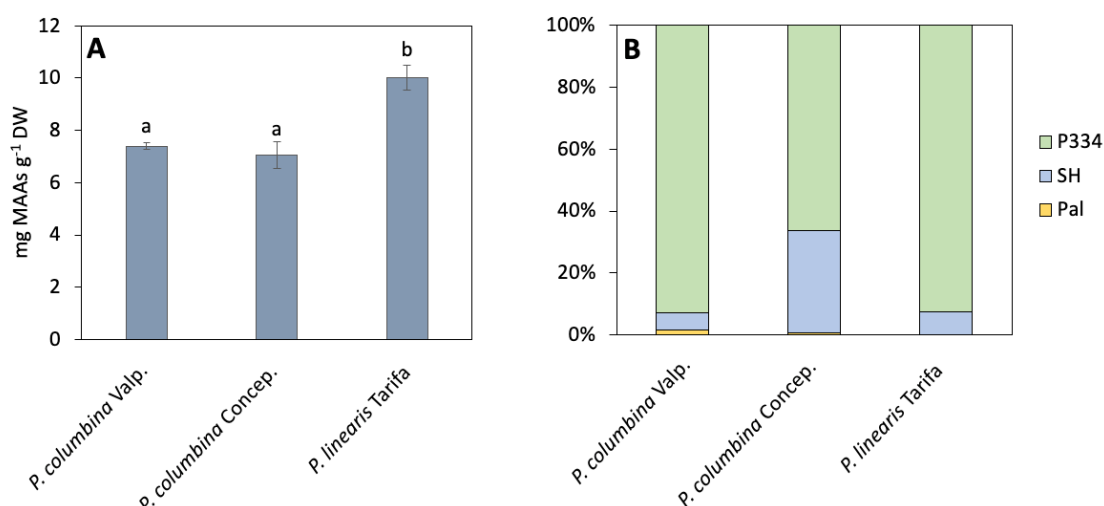


Figure 5. 5. Total mycosporine-like amino acids (MAAs) (A) and relative content (%) of each MAAs in aqueous extracts of two *Porphyra sensu lato* species: *Pyropia columbina* collected in Valparaíso (Chile), *Pyropia columbina* collected in Concepción (Chile) and *Porphyra linearis* collected in Tarifa (Spain). Results are expressed as mean $\pm$ standard deviation (SD) ($n = 3$). Different letters indicate significant differences between species (ANOVA, SNK ($p < 0.05$)).

The phycobiliproteins content is observed in Figure 5. 6. In this case, *P. linearis* showed the highest content of these pigments (8.3 ± 0.1 and 3.2 ± 0.2 mg g⁻¹ DW of phycoerythrin and phycocyanin, respectively) (Table SM.5. 2). *P. columbina* Concep. presented 3.9 ± 0.1 mg g⁻¹ DW of phycoerythrin and 1.7 ± 0.0 mg g⁻¹ DW of phycocyanin. *P. columbina* Valp. showed the lowest phycobiliproteins concentration (1.7 ± 0.2 and 0.02 ± 0.0 mg g⁻¹ DW of phycoerythrin and phycocyanin, respectively). The highest ratio PE/PC were observed in *P. columbina* Valp. (85). The other two species showed similar ratios, 2.29 and 2.59 for *P. columbina* Concep. and *P. linearis*, respectively.

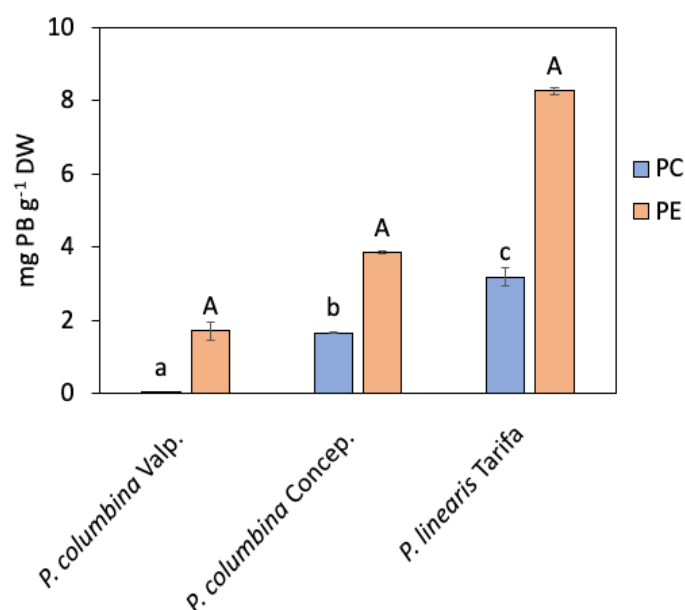


Figure 5. 6. Phycobiliproteins (phycoerythrin -PE- and phycocyanin -PC-) content of three *Porphyra sensu lato* species: *Pyropia columbina* collected in Valparaíso (Chile), *Pyropia columbina* collected in Concepción (Chile) and *Porphyra linearis* collected in Tarifa (Spain). Results are expressed as mean $\pm$ standard deviation (SD) ($n = 3$). Different letters indicate significant differences between species (lower case letter: phycocyanin; capital letters: phycoerythrin) (ANOVA, Newman-Keuls post hoc, $p < 0.05$).

Table 5. 3 indicates the total internal carbon, nitrogen and sulphur content. The carbon percentage was similar in the three samples, without significant differences among them. The nitrogen content was highest in *P. linearis* (5.2 %) and the lowest content was observed in *P. columbina* Val (3.1 %). No differences were observed in the sulphur content. The highest C/N ratio was observed in *P. columbina* Valp. (11.7).

Table 5. 3. Total internal carbon, nitrogen and sulphur content of two *Porphyra sensu lato* species: *Pyropia columbina* collected in Valparaíso (Chile), *Pyropia columbina* collected in Concepción (Chile) and *Porphyra linearis* collected in Tarifa (Spain). Results are expressed as mean $\pm$ standard deviation (SD) ($n = 3$). Different letters indicate significant differences between species (ANOVA, SNK, $p < 0.05$).

	%C	%N	%S	C/N
<i>P. columbina</i> Valp.	35.75 $\pm$ 0.50	3.06 $\pm$ 0.23a	1.44 $\pm$ 0.2	11.71 $\pm$ 1.04b
<i>P. columbina</i> Concep.	36.01 $\pm$ 1.0	4.43 $\pm$ 0.1b	1.67 $\pm$ 0.04	8.13 $\pm$ 0.41a
<i>P. linearis</i> Tarifa	37.26 $\pm$ 0.78	5.23 $\pm$ 0.1c	1.89 $\pm$ 0.02	7.13 $\pm$ 0.01a

The phenolic contents are observed in Figure 5. 7. Significant interaction between variables was observed after the ANOVA (Table SM.5. 2). In general, higher contents were observed in aqueous extraction than in the hydroethanolic ones. The lowest values were observed in the hydroethanolic extract of *P. linearis* ($7.5 \pm 0.3 \text{ mg g}^{-1} \text{ DW}$), and the highest value was obtained in the aqueous extract of the same specie ($11.3 \pm 0.4 \text{ mg g}^{-1} \text{ DW}$). Table 5. 4 show the antioxidant capacity (non-enzymatic and enzymatic) of the different algal extracts. Higher values were obtained using the ABTS than DPPH. In the ABTS assay, significant interaction was observed between extraction solvents and species (Table SM.5. 2). Aqueous extractions showed the highest values in this assay. The highest antioxidant capacity was observed in the aqueous extraction of *P. columbina* Valp. ($15.4 \pm 0.8 \text{ } \mu\text{mol TE g}^{-1} \text{ DW}$), followed by the hydroethanolic extraction of the same species ($12.2 \pm 0.6 \text{ } \mu\text{mol TE g}^{-1} \text{ DW}$). The lowest value was obtained in the hydroethanolic extract of *P. columbina* Concep. ($4.0 \pm 0.5 \text{ } \mu\text{mol TE g}^{-1} \text{ DW}$). In the DPPH assay significant interaction between variables was also observed (Table SM.5. 2). Hydroethanolic extract of *P. linearis* showed the highest value ($13.0 \pm 0.2 \text{ mg g}^{-1} \text{ DW}$). On the other hand, catalase activity was only detected in aqueous extract of *P. columbina* Valp. and *P. linearis* (0.2 ± 0.0 and $0.3 \pm 0.1 \text{ Ucat mg SP}^{-1}$), with significant differences between them.

The capacity to inhibit the enzyme collagenase by the different extracts is observed in Figure 5. 8. All extracts were able to inhibit almost 100 % of that enzyme, with no significant differences among species of solvents (Table SM.5. 2). As all of them showed similar results, one of them was chose to calculate the EC_{50} . *P. linearis* showed an EC_{50} of $2 \text{ mg extract mL}^{-1}$.

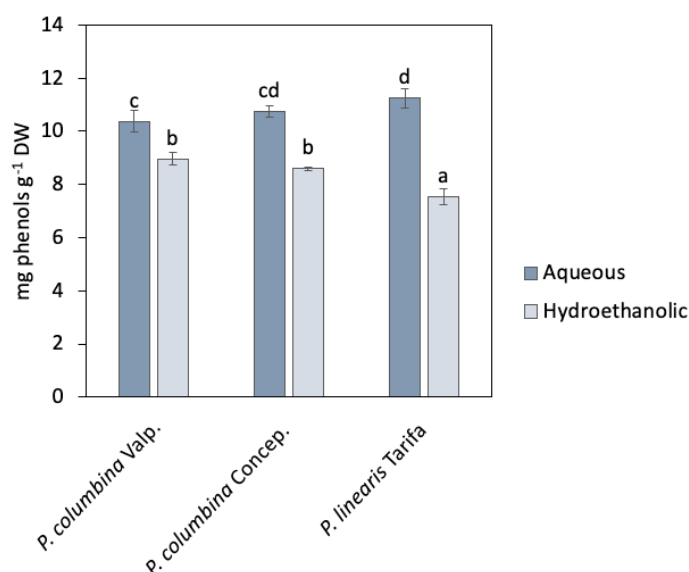


Figure 5. 7. Phenolic contents (mg g⁻¹ DW) of aqueous (dH₂O) and hydroethanolic 50 % (dH₂O:ethanol; 1:1) extracts of two *Porphyra sensu lato* species: *Pyropia columbina* collected in Valparaiso (Chile), *Pyropia columbina* collected in Concepcion (Chile) and *Porphyra linearis* collected in Tarifa (Spain). Values are expressed as mean $\pm$ standard deviation (SD) (n = 3). Different letters indicate significant interaction between variables, species and solvents (ANOVA, SNK, p < 0.05).

Table 5. 4. General antioxidant capacity (non-enzymatic) measured through two methods (ABTS and DPPH) (μ mol TE g⁻¹ DW) and catalase activity (Ucat mg SP⁻¹) of aqueous (dH₂O) and hydroethanolic 50 % (dH₂O:ethanol; 1:1) extracts of two *Porphyra sensu lato* species: *Pyropia columbina* collected in Valparaiso (Chile), *Pyropia columbina* collected in Concepcion (Chile) and *Porphyra linearis* collected in Tarifa (Spain). Values are expressed as mean $\pm$ standard deviation (SD) (n = 3). Different letters indicate significant interaction between variables, species and solvents (ANOVA, SNK, p < 0.05). * Indicate significant differences in the catalase activity (Student-t test, p < 0.05).

Species	Antioxidant capacity				Catalase (Ucat mg SP ⁻¹)	
	ABTS (μ mol TE g ⁻¹ DW)		DPPH (μ mol TE g ⁻¹ DW)			
	Aqueous	Hydroeth.	Aqueous	Hydroeth.	Aqueous	Hydroeth.
<i>P. columbina</i> Valp.	15.36 $\pm$ 0.83d	12.22 $\pm$ 0.49c	7.30 $\pm$ 2.86a	9.34 $\pm$ 1.47a	0.19 $\pm$ 0.04	-
<i>P. columbina</i> Concep.	10.62 $\pm$ 0.51bc	3.97 $\pm$ 0.48a	10.84 $\pm$ 0.56ab	7.03 $\pm$ 0.54a	-	-
<i>P. linearis</i> Tarifa	11.77 $\pm$ 2.02c	8.94 $\pm$ 0.40b	8.21 $\pm$ 1.53a	12.96 $\pm$ 0.24b	0.25 $\pm$ 0.07*	-

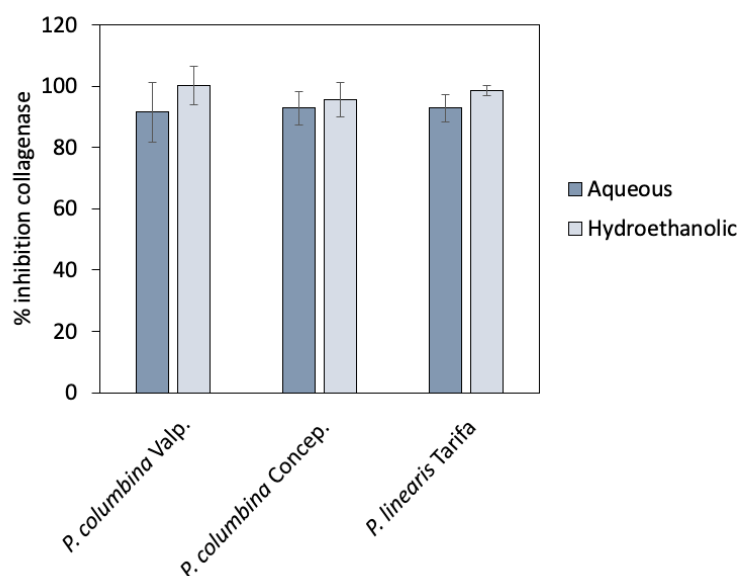


Figure 5. 8. Inhibition of collagenase activity by aqueous (dH₂O) and hydroethanolic 50 % (dH₂O:ethanol; 1:1) extracts of two *Porphyra sensu lato* species: *Pyropia columbina* collected in Valparaíso (Chile), *Pyropia columbina* collected in Concepción (Chile) and *Porphyra linearis* collected in Tarifa (Spain). Values are expressed as mean ± standard deviation (SD) (n = 3). No significant differences were observed (ANOVA, SNK, p < 0.05).

The survival (%) of keratinocytes, fibroblasts and melanoma cells incubated in different concentrations of the extracts is shown in (Figure SM.5. 2). There were variations into cell survival exposed to different concentrations of algal extracts. Most of the tested extracts showed a survival lower than 5 % at maximum concentration (10 mg mL⁻¹). Using those values, the half maximal effective concentration (EC₅₀) was calculated (Table 5. 5). Significant interaction between variables was obtained in HACAT cells (Table SM.5. 2). The highest EC₅₀ was observed in the hydroethanolic extract of *P. columbina* Concep. (5238.4 ± 480.0 µg mL⁻¹), followed by the aqueous extraction of the same species (3831.6 ± 68.1 µg mL⁻¹). The other two species presented a higher EC₅₀ in the aqueous extraction. The lowest EC₅₀ were observed in the hydroethanolic extract of *P. linearis* (467.9 ± 17.3 µg mL⁻¹). Not all species were tested with 1064sk cells. The highest EC₅₀ were observed in the aqueous extract of *P. columbina* Concep., follow by the aqueous extract of *P. linearis* (8527.4 ± 903.7 and 6198.2 ± 383.5 µg mL⁻¹). The lowest EC₅₀ were obtained in the hydroethanolic extract of *P. linearis* (1684.0 ± 303.2 µg mL⁻¹) (Table SM.5. 2). The melanoma cell strain was only tested against the aqueous extract of *P. columbina* Concep. showing an EC₅₀ of 8999.8 ± 1361.5 µg mL⁻¹. A selectivity index lower than 1 was observed with both health cell strains.

Table 5. 5. Half maximal effective concentration (EC₅₀) of the aqueous and hydroethanolic extract of two *Porphyra sensu lato* species: *Pyropia columbina* collected in Valparaíso (Chile), *Pyropia columbina* collected in Concepción (Chile) and *Porphyra linearis* collected in Tarifa (Spain), against three skin cell lines (keratinocytes -HACAT-, fibroblast -1064sk- and malignant melanoma -G-361-), and the selectivity index (SI) between HACAT/G-361 and 1064SK/G-361. Values are expressed as mean $\pm$ standard deviation (SD) (n = 3). Different letters indicate significant differences between variables (significant interaction were observed in HACAT cells) (ANOVA, SNK, p < 0.05).

Species	Solvent	Cell strains			SI
		HACAT	1064SK	G-361	
<i>P. columbina</i> Valp.	Aqueous	1651.64 $\pm$ 240.64 ^c	-	-	-
	Hydroethanolic	592.52 $\pm$ 155.25 ^a	-	-	-
<i>P. columbina</i> Concep.	Aqueous	3831.57 $\pm$ 68.11 ^d	8527.42 $\pm$ 903.70 ^c	8999.87 $\pm$ 1361.49	0.43 / 0.94
	Hydroethanolic	5238.42 $\pm$ 479.92 ^e	-	-	-
<i>P. linearis</i> Tarifa	Aqueous	1057.29 $\pm$ 79.55 ^b	6198.23 $\pm$ 383.55 ^b	-	-
	Hydroethanolic	467.89 $\pm$ 17.27 ^a	1683.97 $\pm$ 303.20 ^a	-	-

Table SM.5. 3; Table SM.5. 4 showed correlations among the different analyzed parameters in the *Porphyra* species for aqueous and hydroethanolic extracts respectively. In aqueous extracts, positive correlations were obtained between the extraction yield, the ABTS and catalase activity. The different analyzed molecules (phycocyanin, phycoerythrin, MAAs and phenols) showed positive correlations among them. Biliproteins and phenols correlates also with the survival of HACAT cells, and phenols also correlate with catalase activity. Catalase activity also showed positive correlation with the survival of HACAT cells. In relation with the hydroethanolic extracts, the extraction yield showed a negative correlation with the phenols content and a positive correlation with the DPPH method. Phenols correlated negatively with the DPPH. Phenols and ABTS correlates with the survival of HACAT cells.

3.2. Looking for new antioxidants and UV screen compounds in diverse plants

Several plants were analyzed in this study (Table 5. 1). Some of them were commercially available (e.g. ginger root, liquorice root or green coffee beans) and other were cultured / collected (e.g. ivy leaves and roots, or olive leaves). Commercial

capsules based on calaguala extract were also measured as positive control. The extracts presented different colors, e.g. ginger extract present a very fair yellow, the extracts from leaves (ivy, olive or calaguala) and the green coffee present a greenish color, the urukum showed an orange color, or the orcanette a dark purple color (Figure 5. 9).

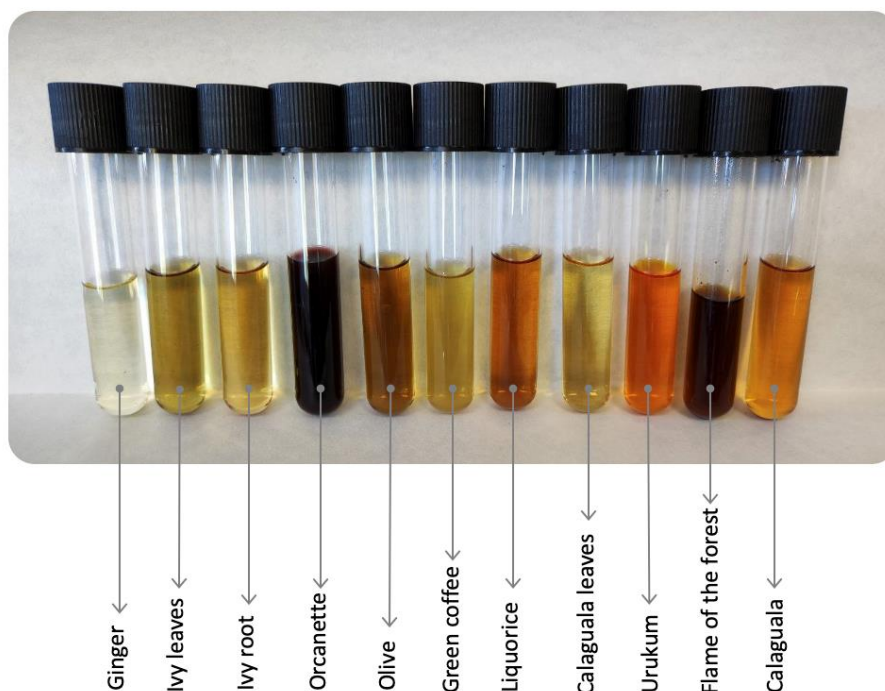


Figure 5. 9. Plant hydroethanolic extracts utilized in this study.

The absorption spectra (290-500 nm) of plant extracts are observed in Figure 5. 10. All of them presented UV absorption capacity, although different spectra were observed. Some of them presented maximum absorption ($\lambda_{\max}$) in the UV-B region (290 nm), such as ginger, ivy root, orcanette, olive, liquorice, urukum or calaguala capsules (Table 5. 6), while others presented $\lambda_{\max}$ in the UV-A band, like ivy leaves (330 nm), green coffee (328 nm), calaguala leaves (326 nm) or flame of the forest (331 nm) (Table 5. 6). The highest absorption capacity was observed in green coffee, followed by liquorice and flame of the forest (Table 5. 6).

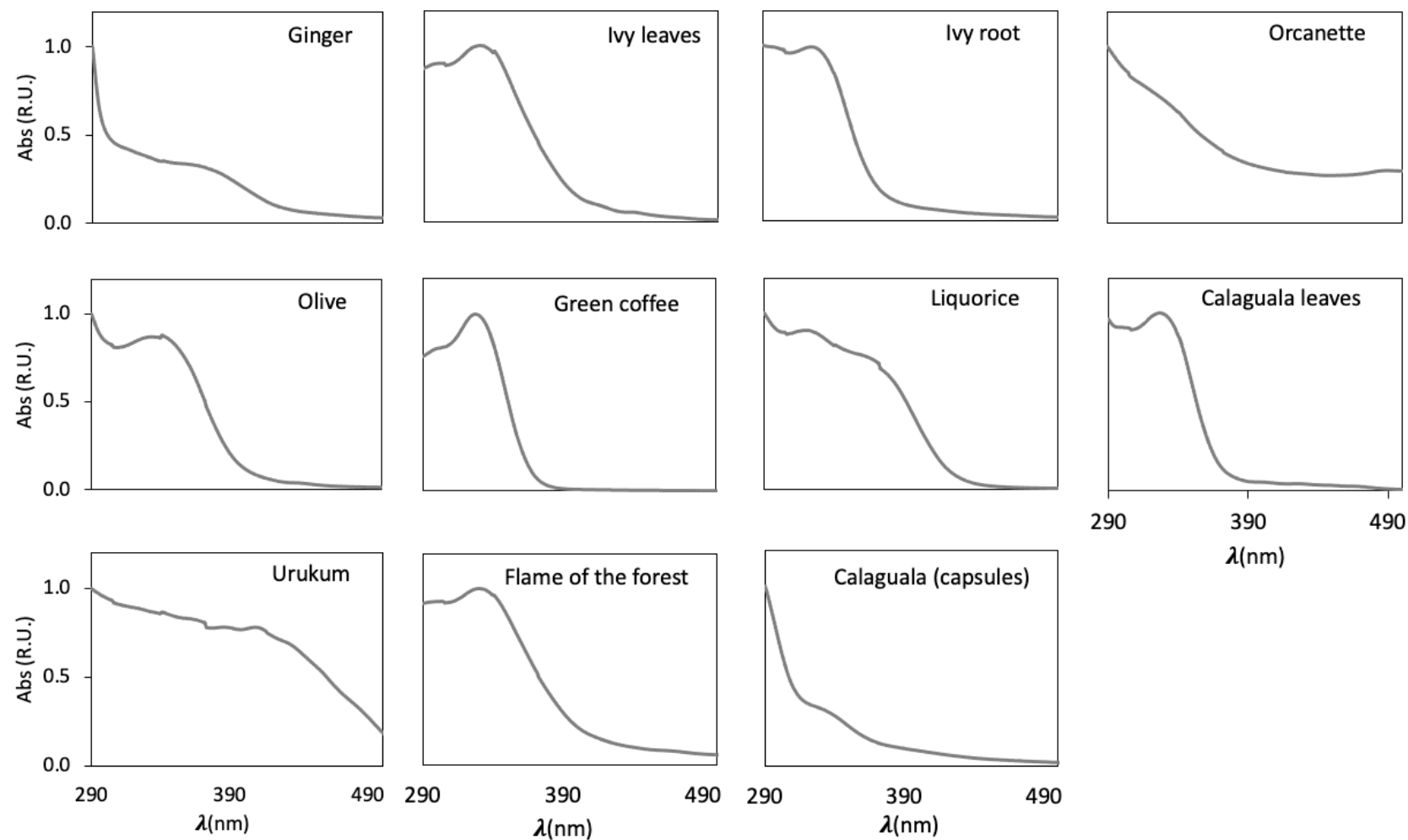


Figure 5. 10. Absorbance (290-500 nm) spectrum of the hydroethanolic extracts of several plants. The absorbance values were divided by the maximum of each extract, ensuring that in any extract the values are fitted between 0 and 1. Values are expressed as the mean of three replicates ($n = 3$).

Table 5. 6. Parameters obtained from the absorbance spectra of the hydroethanolic extracts: Maximal absorbance (Abs_{max}) and lambda maxima (λ_{max}) in the range of 290-400 nm. Previously to determine both parameters, absorbance values were divided by the maximum of all the plant extracts in order to obtain values between 0 and 1.

Plant	Abs _{max}	λ _{max}
Ginger	0.03	290
Ivy leaves	0.09	330
Ivy root	0.07	290
Orcanette	0.10	290
Olive	0.11	290
Green coffee	1.00	328
Liquorice	0.51	290
Calaguala leaves	0.04	326
Urukum	0.08	290
Flame of the forest	0.36	331
Calaguala (capsules)	0.32	290

The phenols content and antioxidant capacity of the different plant extracts are observed in Figure 5. 11. The highest phenols content was obtained in ivy root (39.2 ± 1.6 mg g⁻¹ FW), followed by flame of the forest, calaguala capsules and green coffee (17.4 ± 1.3 , 15.9 ± 0.5 and 13.0 ± 0.5 mg g⁻¹ FW, respectively). The lowest amount of the phenols was observed in urukum and ginger (1.8 ± 0.2 mg g⁻¹ FW in both extracts), without significant differences between them. In relation to the antioxidant capacity, the highest values were observed in calaguala capsules and flame of the forest (199.4 ± 4.6 and 192.9 ± 9.8 μmol TE g⁻¹ DW), without significant differences between them. Followed by green coffee, olive, ivy root and liquorice (122.1 ± 7.8 , 105.0 ± 5.5 , 92.1 ± 3.9 and 80.2 ± 1.0 μmol TE g⁻¹ DW). The lowest antioxidant capacity was also observed in ginger and urukum (5.6 ± 0.1 and 20.2 ± 1.1 μmol TE g⁻¹ DW, respectively) (Table SM.5. 2). Positive correlations between ABTS and phenol contents were observed among hydroethanolic extract of the plants ($r = 0.5039$, p-value < 0.01)

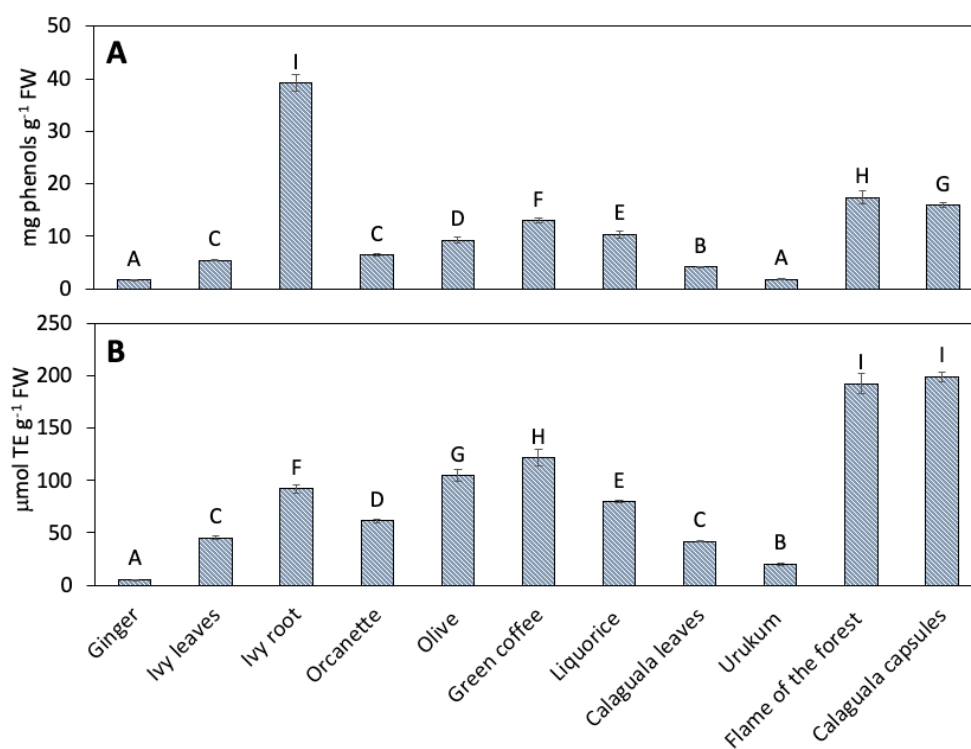


Figure 5. 11. Phenolic contents (mg g⁻¹ FW) (A) and antioxidant capacity measured through ABTS method (μmol TE g⁻¹ FW) (B) of the hydroethanolic extraction of several plants. Values are expressed as mean ± standard deviation (SD) (n = 3). Different letters indicate significant differences between variables (significant interaction were observed in HACAT cells) (ANOVA, SNK, p < 0.05).

According to the previous results, five plant extracts were selected: ivy root, olive, green coffee, liquorice and flame of the forest. The extraction yield and cytotoxicity were determined from these species. The highest yield was obtained in liquorice (30.8 %), followed by flame of the forest, green coffee and olive (24.4, 23.0 and 21.2 %, respectively). The lowest extraction yield was observed in ivy root (9.0 %) (Table 5. 7). The toxicity of the extracts against health and tumoral cell lines is observed in Table 5. 8. The lowest EC₅₀ in 1064sk cells were obtained in flame of the forest, green coffee and ivy root extracts (869.3 ± 52.3, 1159.1 ± 58.4 and 1630.2 ± 423.3 μg mL⁻¹, respectively), followed by liquorice (12282.4 ± 1882.6 μg mL⁻¹). Olive presented the highest EC₅₀ (20330.2 ± 1564.1 μg mL⁻¹). In relation with the G-361 cell line, the lowest EC₅₀ were obtained in ivy root, liquorice and flame of the forest (600.4 ± 7.7, 993.9 ± 16.3 and 1035.7 ± 63.1 μg mL⁻¹, respectively), followed by olive and green coffee (4579.2 ± 318.5 and 11880.2 ± 392.9 μg mL⁻¹, respectively). The highest selectivity index was obtained in liquorice (12.4), followed by olive and ivy root (4.4 and 2.7, respectively). The lowest

were obtained in green coffee and flame of the forest (0.1 and 0.8, respectively) (Table SM.5. 2).

Table 5. 7. Extraction yield (%) of hydroethanolic extract of ivy root, green coffee, olive, liquorice and flame of the forest.

Extraction yield (%)	
Ivy root	8.96
Olive	21.20
Green coffee	22.92
Liquorice	30.84
Flame of the forest	24.38

Table 5. 8. Half maximal effective concentration (EC_{50}) of hydroethanolic extracts of ivy root, green coffee, olive, liquorice and flame of the forest extracts on the growth of human health cell line (human fibroblast: 1064sk) and a carcinogenic cell line (melanoma: G-361), and the selectivity index (SI) between them (1064SK/G-361). Values are expressed as mean $\pm$ standard deviation (SD) ($n = 3$). Different letters indicate significant differences between variables (significant interaction) (ANOVA, SNK, $p < 0.05$).

	1064SK	G-361	SI
Ivy root	1630.18 $\pm$ 423.32 ^a	600.42 $\pm$ 7.70 ^a	2.72
Olive	20330.24 $\pm$ 1567.13 ^c	4579.17 $\pm$ 318.46 ^b	4.44
Green Coffee	1159.12 $\pm$ 58.44 ^a	11880.17 $\pm$ 392.93 ^c	0.10
Liquorice	12282.36 $\pm$ 1880.59 ^b	993.86 $\pm$ 16.26 ^a	12.36
Flame of the forest	869.29 $\pm$ 52.34 ^a	1035.65 $\pm$ 63.11 ^a	0.84

3.3. Design and analysis of cosmeceutic products: photoprotection factors and stability

The phenolic contents and the antioxidant capacity of the selected extracts and mixtures of extracts used for the design of the cosmeceutic products are shown in Table 5. 9. Among extracts, green coffee and flame of the forest presented the highest phenolic content (314.4 ± 53.3 and 271.3 ± 9.6 mg g⁻¹ DE, respectively) and antioxidant capacity (1118.8 ± 20.7 and 1359.7 ± 18.4 μmol TE g⁻¹ DE, respectively), followed by liquorice (218.0 ± 35.5 mg phenols g⁻¹ DE and 901.7 ± 37.3 μmol TE g⁻¹ DE). The *Pyropia* extracts presented the lowest values (58.5 ± 2.4 mg phenols g⁻¹ DE and 87.6 ± 1.9 μmol TE g⁻¹ DE of antioxidant capacity). The mixture of plants (MP) presented 19507 ± 6.9 mg g⁻¹ DE of phenols and 991.7 ± 46.0 μmol TE g⁻¹ DE of antioxidant capacity. When MP is combined with *Pyropia* extracts in a 50 % each one, the values decreased (127.1 ± 6.8 mg phenols g⁻¹ DE and 539.7 ± 23.4 μmol TE g⁻¹ DE of antioxidant capacity). The absorption spectra of the different extracts and mixtures are observed in Figure 5. 12: A. Green coffee presented the highest absorbance and mainly in UV-B, followed by flame of the forest. Liquorice presented a broad spectrum that comprise UV-B and UV-A. *Pyropia* presented a peak in the UV-A region (λ_{max} : 334 nm). The antioxidant capacity of the commercial oils used in the design creams is also shown in Table 5. 9, and the spectrum is observed in Figure 5. 12: B. The antioxidant capacity of calophyl and karanja were 4.8 ± 0.4 and 3.2 ± 0.5 μmol TE g⁻¹ oil. Calophyl presented a higher absorption in the UV-B band, whereas karanja presented a broader spectrum including UV-B and UV-A.

Table 5. 9. Phenol contents and antioxidant capacity (AC) of the different algae and plants extracts, the combinations of them and the commercial oils used in the cosmetic products. Mixture of plants (MP: 40 % liquorice, 40 % green coffee and 20 % flame of the forest). Pyropia + MP (50 % each one). Values are expressed as mean $\pm$ standard deviation (SD) ($n = 3$). Different letters indicate significant differences between variables (significant interaction) (ANOVA, SNK, $p < 0.05$).

Extracts	Phenols (mg g ⁻¹ DE)	AC (μ mol TE g ⁻¹ DE)
<i>Pyropia</i>	58.52 $\pm$ 2.43 ^a	87.63 $\pm$ 1.87 ^a
Liquorice	217.97 $\pm$ 35.50 ^c	901.67 $\pm$ 37.34 ^c
Green coffee	314.40 $\pm$ 52.31 ^d	1118.75 $\pm$ 20.74 ^e
Flame of the forest	271.33 $\pm$ 9.56 ^d	1359.73 $\pm$ 18.38 ^f
Mixture of plants (MP)	195.71 $\pm$ 6.90 ^c	991.71 $\pm$ 46.04 ^d
<i>Pyropia</i> + MP	127.12 $\pm$ 6.82 ^b	539.66 $\pm$ 23.43 ^b

Oils	AC (μ mol TE g ⁻¹ oil)
Calophyl	- 4.83 $\pm$ 0.39
Karanja	- 3.22 $\pm$ 0.46

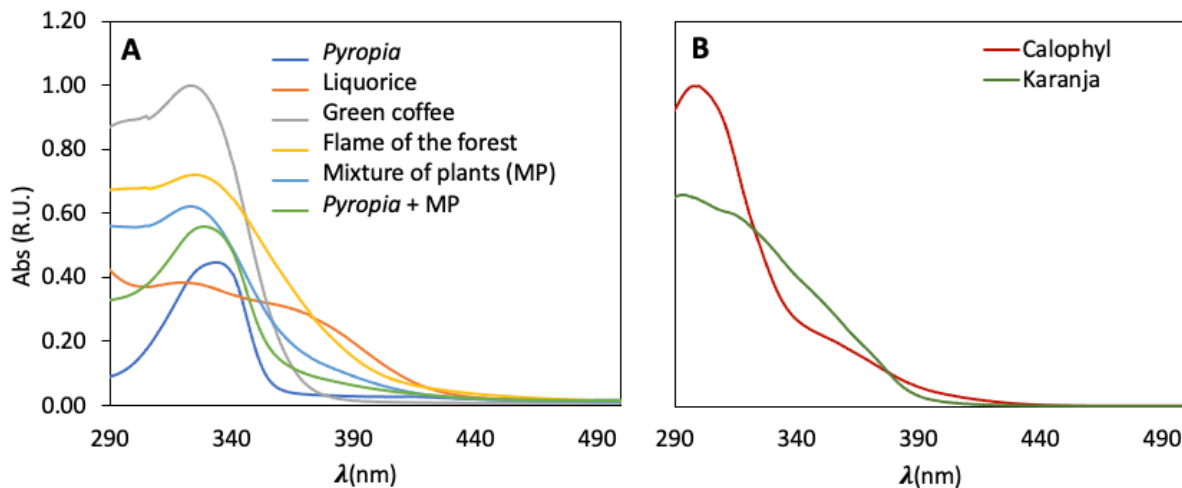


Figure 5. 12. Absorbance or optical density from 290 to 500 nm of the algae (*Pyropia*) and plants extracts (Liquorice, Green coffee and flame of the forest), and combinations used in the cosmetic products: mixture of plants (MP: 40 % liquorice, 40 % green coffee and 20 % flame of the forest) and *Pyropia* + MP (50 % each). Values are expressed as the mean of three replicates ($n = 3$). Absorbance values were divided by the maximum of all the extracts or mixture in order to obtain values between 0 and 1.

The photo and thermo stability of the *Pyropia* extract, MP and *Pyropia* + MP are observed in Figure SM.5. 3. In relation to thermostability, no degradation of polyphenols was observed after incubation at different temperatures in any case. Regarding the antioxidant capacity, a small decrease was observed in the case of *Pyropia* extract, the values decreased from 87.0 to 66.6 $\mu\text{mol TE mL}^{-1}$ after 1 h at 80 °C. The area under the curve (AUC) of the UV-Vis spectrum (290-500 nm) did not show any differences under the different temperatures. In relation to photostability, no significant differences were observed after UVR in the phenols content. In the antioxidant capacity, a slight increased were observed in *Pyropia* extract and *Pyropia* + MP after 60 min of UVR. In case of MP, decrease in the antioxidant capacity was observed after 30 min of UVR (Table SM.5. 2).

Photoprotection factors of commercial sunscreen were measured through the described methodology in order to validate the method (Table 5. 10). Depending on the brand, the calculated SPF values are closer or not to the labelled ones. The calculated SPF values from the Avène sunscreens showed higher values than the labelled ones, i.e. Avène 20 showed a SPF of 34.6 and the Avène 50 showed a SPF value of 103.6. However, in Biarritz 50 and Babaria 50 the calculated SPF were very similar to the labelled ones. The “C” value varied from 0.8 in the Aderma sunscreen, that showed the highest difference between the *in vitro* and the labelled SPF, to 1 in Biarritz sunscreen, in which the *in vitro* and the labelled SPF was very similar.

According to the labels all the sunscreen tested presented protection against UV-A rays (the industry is not obliged to mention the number of the UVAPF). Our results showed UVAPF values adjusted that varied from 9.9 in Biarritz SPF 50 to 20.5 in Babaria SPF 50. The reference cream from the ISO: 24443 (2012) showed a calculated SPF of 22 and an adjust UVAPF of 11.3. The ratio SPF/UVAPF varied from 1.4 in the reference cream and 2.1 in the Avène SPF20 to 5.1 in the Biarritz SPF50. The critical lambda (λ_c) were higher than 370 nm in all cases, varying from 370 in Biarritz SPF 50 to 379 in Babaria SPF 50 and Aderma SPF 50. In this study, other BEPFs have been calculated (Table SM.5. 5). Photoprotection factors against photocarcinogenesis and immunosuppression (both related with UV-B rays) presented the highest values, reaching a protection factor of 140 against immunosuppression in the Aderma SPF 50. The other photoprotection factors calculated are related to UV-A rays (elastosis, singlet oxygen formation and photoaging). The highest values were observed against singlet oxygen formation that reached a value of 91 in the Aderma SPF 50. The absorbance spectra of the commercial sunscreens are shown in Figure 5. 13. Two absorption regions

can be observed in most of them, in the UV-B band and another in the UV-A band. Commercial sunscreens were submitted to photo- and thermostability, and no degradation was observed. Only slight differences were observed in the cream Avène SPF20 at 45 °C (Figure SM.5. 4).

Table 5. 10. Determination of two photoprotection factors *in vitro* from commercial sunscreens. Sun protection factors (SPF) were calculated according to Pissavini *et al.* (2018). UV-A protection factor (UVAPF) were calculated as ISO 24443 (2012), indicating the UVAPF_{in vitro}, the factor C and the UVAPF_{in vitro} adj. The ratio SPF/UVAPF and the critical lambda (λ_c) were also calculated. Values are expressed as mean $\pm$ standard deviation (SD) (n = 3).

	SPF _{in vitro}	UVAPF _{in vitro}	Factor C	UVAPF _{in vitro} adj	SPF/UVAPF	λ_c
Avène SPF 20	34.62 $\pm$ 5.28	12.97 $\pm$ 3.13	0.85	8.53 $\pm$ 1.98	2.34	377
Avène SPF 30	51.18 $\pm$ 4.09	17.41 $\pm$ 0.92	0.86	12.56 $\pm$ 0.67	2.39	377
Avène SPF 50	103.62 $\pm$ 31.31	30.92 $\pm$ 5.79	0.83	19.94 $\pm$ 3.56	2.51	378
Biarritz SPF 50	50.83 $\pm$ 11.39	9.87 $\pm$ 0.85	1	9.87 $\pm$ 0.85	5.07	370
Babaria SPF 50	56.34 $\pm$ 8.85	21.81 $\pm$ 2.45	0.98	20.53 $\pm$ 2.33	2.44	379
Aderma SPF 50	124.30 $\pm$ 17.44	36.05 $\pm$ 3.52	0.8	19.81 $\pm$ 1.95	2.52	379
Heliocare Ultra SPF 50	73.40 $\pm$ 13.52	16.99 $\pm$ 1.91	0.9	13.44 $\pm$ 1.52	3.72	374
Reference ISO 24443 (2012) – SPF 16	21.98 $\pm$ 4.71	12.47 $\pm$ 1.61	0.91	10.26 $\pm$ 1.35	1.56	378

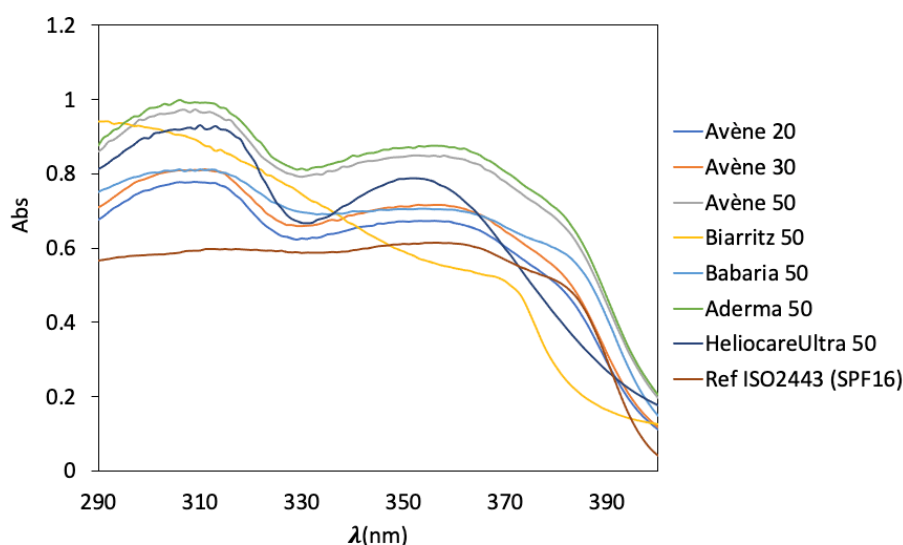


Figure 5. 13. Absorbance spectra of commercial sunscreens from 290 to 400 nm. Values are expressed as the mean of three replicates ($n = 3$). Absorbance values were divided by the maximum of all of them in order to obtain values from 0 to 1.

Absorbance spectra of the design creams are observed in Figure 5. 14. The base cream with karanja and calophyl oil (BKC) showed an increase in the absorbance values mainly in the UV-B region, but also in the UV-A region. When physical filters (PF) were also added, the absorbance increase in the UV-A and visible region. By adding *Pyropia* extract, the absorbance increased between 290 and 350 nm, whereas when the mixture of plants (MP) was added the absorbance showed a broader increase (until 450 nm). Mixing both extracts (*Pyropia* and MP) in a 10 % each one (total 20 % of extract) the absorbance increased comparing with the addition of only one of the extracts in a 10 % (similar happen in the cream with physical filters).

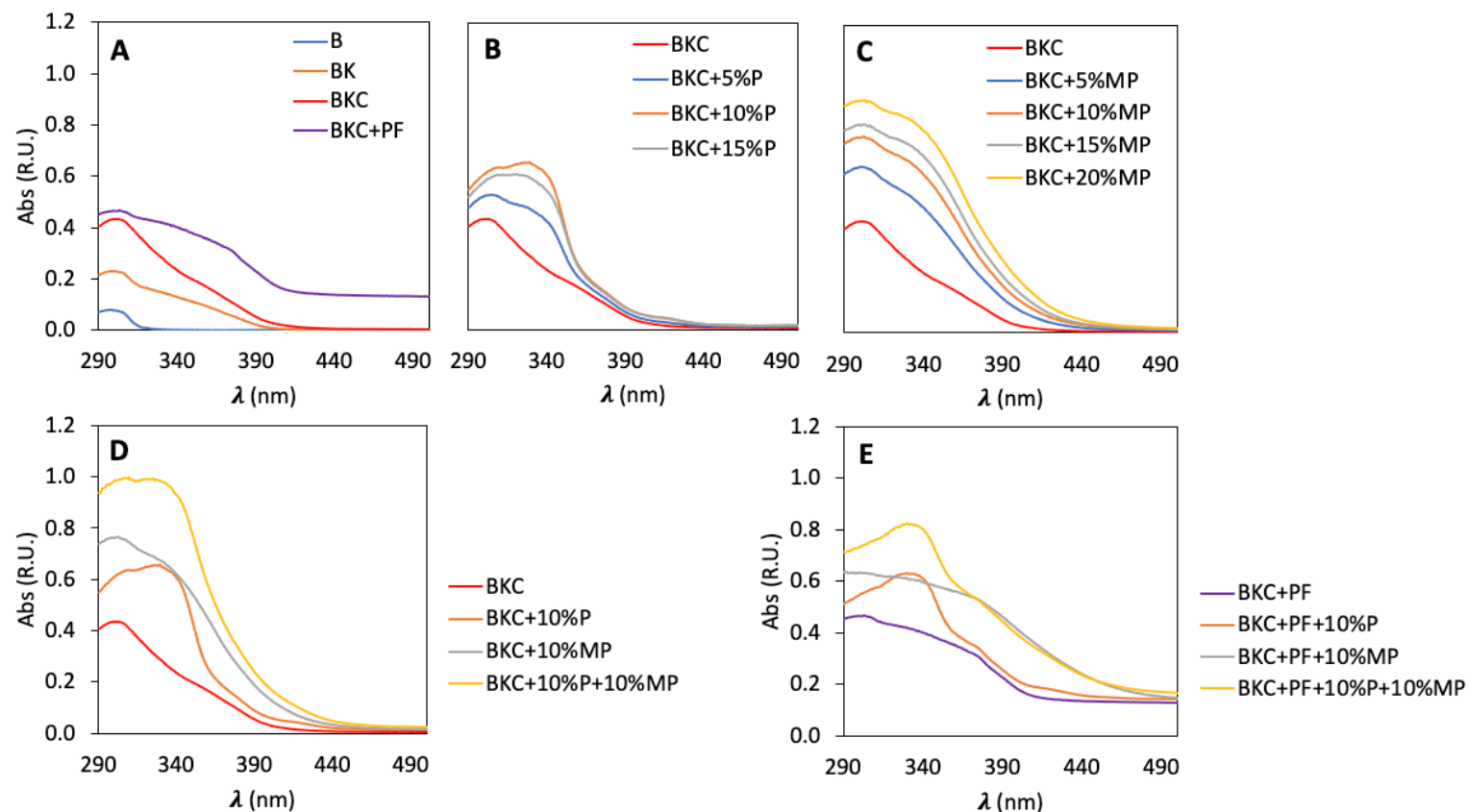


Figure 5. 14. Absorbance spectra of the designed creams. A) Base creams, with and without physical filters: B: Base with coconut oil; BK: Base with coconut and karanja oil; BKC: Base with karanja and calophyl oils; BKC + PF: BKC with physical filters, B) BKC with different percentages of *Pyropia* extract (5, 10 and 15 %), C) BKC with different percentages of the mixture of plants (MP; 40 % green coffee, 40 % liquorice, 20 % flame of the forest) extract (5, 10, 15 and 20 %), D) BKC with the combination of 10 % of MP and 10 % *Pyropia* extract, and E) BKC + PF with 10 % *Pyropia* extract, 10 % MP and the combination of both. Values are expressed as the mean of three replicates ($n = 3$). Absorbance values were divided by the maximum of all of them in order to obtain values from 0 to 1.

The photoprotection factors obtained from the design creams are observed in Table 5. 11. The base cream that only presented coconut oil (B) showed very low values in the different photoprotection factors (< 1.5). Adding both oils (BKC) the SPF was 4.2 ± 0.7 and adding the physical filters (BKC + PF) was 5.8 ± 0.8 , no significant differences were observed among these creams. However, the UVAPF in the BKC + PF (4.0 ± 0.5) showed a significant difference with BKC (1.0 ± 0.0). Regarding the others photoprotection factors, only those related with UV-A rays (elastosis and photoaging) showed significant differences between the BKC + PF and BKC. In the relation to the creams with different percentages of *Pyropia* extract, the SPF values varied from 6.8 ± 0.4 to 9.8 ± 2.3 , adding 5 and 15 % of extract respectively. No significant differences were observed between the different percentages in any of the photoprotection factors. Comparing with BKC, significant differences were only observed in some cases (SPF, UVAPF and elastosis) between BKC + 10-15 % of *Pyropia* extract. Regarding the creams with MP extracts, the SPF values varied from 16.7 ± 1.0 to 31.9 ± 7.0 , adding 5 and 20 % respectively. In all photoprotection factors, no significant differences were observed between adding 5 and 10 % of MP, whereas significant differences were observed with 15 and 20 % of MP. A significant increase in the photoprotection values were observed between BKC + 5-10 % MP (depending on the photoprotection factor) and BKC. It is important to highlight that BKC + 15 % MP showed significant differences with the BKC + PF. When both type of extracts were mixed (BKC + 10 % *Pyropia* + 10 % MP), the photoprotection values were similar to the one obtained with BKC + 15 % MP in most cases (without significant differences). The creams with physical filters did not show significant differences with the creams without physical filters in the photoprotection factors related to UV-B (SPF, photocarcinogenesis and immunosuppression), whereas significant differences were observed in those related to UV-A (UVAPF, elastosis, singlet oxygen formation and photoaging), i.e. the UVAPF of the BKC + PF + 10 % *Pyropia* + 10 % MP presented a UVAPF increased to 14.1 ± 2.8 in comparison with the same without physical filter (6.7 ± 1.3). Regarding the ratio SPF/UVAPF, it increased when the different extracts were added, the highest ratio (3.63) were observed in the BKC + 10 % *Pyropia* + 10 % MP. Finally, the critical lambda varied from 308 in the B cream to 382 in the BKC + PF + 10 % MP cream. In general, the λ_c increased when the physical filters were added.

Table 5. 11. Different photoprotection factors (PPFs) of the designed base creams. Sun protection factor (SPF), UVA protection factor (UVAPF), different biological effective protection factors (BEPFs) related to different biological response driven by UVR (photocarcinogenesis, immunosuppression, elastosis, singlet oxygen and photoaging), ratio SPF/UVAPF and critical lambda (λ_c). Values are expressed as mean $\pm$ standard deviation (SD) ($n = 3$). Different letters indicate significant differences between variables (significant interaction) (ANOVA, SNK, $p < 0.05$).

	SPF	UVAPF	SPF/UVAPF	λ_c
B	1.18 $\pm$ 0.01 ^a	1.00 $\pm$ 0.00 ^a	1.18	308
BK	2.16 $\pm$ 0.15 ^{ab}	1.49 $\pm$ 0.05 ^{ab}	1.45	364
BKC	4.24 $\pm$ 0.64 ^{abc}	2.00 $\pm$ 0.15 ^{abc}	2.12	365
BKC + PF	5.83 $\pm$ 0.80 ^{abcd}	4.04 $\pm$ 0.48 ^{de}	1.46	376
BKC + 5% P	6.87 $\pm$ 0.41 ^{abcd}	2.78 $\pm$ 0.11 ^{abcd}	2.48	363
BKC + 10% P	8.64 $\pm$ 2.05 ^{abcd}	3.17 $\pm$ 0.37 ^{bcd}	2.83	363
BKC + 15% P	9.84 $\pm$ 2.30 ^{bcd}	3.40 $\pm$ 0.37 ^{bcd}	2.90	363
BKC + 5% MP	10.66 $\pm$ 1.02 ^{cd}	4.10 $\pm$ 0.25 ^{de}	2.60	370
BKC + 10% MP	13.62 $\pm$ 1.76 ^d	4.97 $\pm$ 0.42 ^{ef}	2.85	372
BKC + 15% MP	22.22 $\pm$ 5.74 ^e	7.31 $\pm$ 1.14 ^g	3.04	373
BKC + 20% MP	31.87 $\pm$ 7.04 ^f	10.05 $\pm$ 1.33 ^h	3.17	374
BKC + 10% P + 10% MP	23.70 $\pm$ 7.17 ^e	6.67 $\pm$ 1.28 ^{fg}	3.63	370
BKC + PF + 10% P	9.77 $\pm$ 1.59 ^{bcd}	6.03 $\pm$ 0.76 ^{fg}	1.66	376
BKC + PF + 10% MP	12.07 $\pm$ 1.23 ^{cd}	9.39 $\pm$ 1.01 ^h	1.29	382
BKC + PF + 10% P + 10% MP	23.30 $\pm$ 5.92 ^e	14.11 $\pm$ 1.46 ⁱ	1.65	380

Table 5. 11. Cont.

	Photocarino.	Immunosup.	Elastosis	Sing. Oxygen	Photoaging
B	1.34 ± 0.01 ^a	1.28 ± 0.01 ^a	1.02 ± 0.00 ^a	1.00 ± 0.00 ^a	1.00 ± 0.00 ^a
BK	2.51 ± 0.22 ^{ab}	2.40 ± 0.19 ^a	1.51 ± 0.05 ^{ab}	1.66 ± 0.07 ^{ab}	1.55 ± 0.05 ^{ab}
BKC	5.44 ± 0.99 ^{abc}	5.10 ± 0.90 ^{ab}	2.05 ± 0.15 ^{ab}	2.33 ± 0.22 ^{ab}	2.15 ± 0.18 ^{abc}
BKC + PF	6.42 ± 0.88 ^{abc}	6.29 ± 0.86 ^{ab}	4.00 ± 0.47 ^{cd}	4.73 ± 0.63 ^{abc}	4.42 ± 0.57 ^{bcde}
BKC + 5% P	8.13 ± 0.57 ^{abcd}	8.13 ± 0.57 ^{ab}	2.73 ± 0.10 ^{bc}	3.63 ± 0.20 ^{ab}	3.24 ± 0.16 ^{abcd}
BKC + 10% P	9.57 ± 2.38 ^{abcd}	9.87 ± 2.55 ^{ab}	3.05 ± 0.33 ^{bcd}	4.47 ± 0.79 ^{abc}	3.86 ± 0.59 ^{abcd}
BKC + 15% P	10.72 ± 2.77 ^{abcd}	11.02 ± 2.96 ^{abc}	3.27 ± 0.33 ^{bcd}	4.43 ± 0.71 ^{abc}	4.22 ± 0.60 ^{abcde}
BKC + 5% MP	13.69 ± 1.48 ^{cd}	13.67 ± 1.52 ^{abc}	3.98 ± 0.22 ^{cd}	5.95 ± 0.48 ^{bcd}	5.05 ± 0.37 ^{cde}
BKC + 10% MP	17.54 ± 2.55 ^{de}	17.66 ± 2.62 ^{bc}	4.75 ± 0.38 ^{de}	7.93 ± 0.92 ^{cde}	6.38 ± 0.66 ^{de}
BKC + 15% MP	28.83 ± 8.44 ^f	30.16 ± 9.28 ^d	6.81 ± 0.98 ^f	13.13 ± 3.04 ^f	10.24 ± 2.05 ^f
BKC + 20% MP	41.57 ± 10.58 ^g	43.99 ± 11.8 ^e	9.22 ± 1.14 ^g	19.49 ± 3.87 ^g	14.81 ± 2.54 ^g
BKC + 10% P + 10% MP	27.14 ± 10.54 ^f	32.50 ± 10.85 ^d	6.15 ± 1.10 ^{ef}	10.79 ± 2.89 ^{ef}	9.64 ± 2.48 ^f
BKC + PF + 10% P	9.32 ± 1.51 ^{abcd}	9.52 ± 1.56 ^{ab}	5.72 ± 0.69 ^{ef}	8.83 ± 1.44 ^{de}	7.13 ± 1.03 ^e
BKC + PF + 10% MP	12.97 ± 1.11 ^{bcd}	12.87 ± 1.15 ^{abc}	9.30 ± 0.96 ^g	10.55 ± 1.24 ^{ef}	10.05 ± 1.15 ^f
BKC + PF + 10% P + 10% MP	22.31 ± 5.45 ^{ef}	22.72 ± 5.59 ^{cd}	13.43 ± 1.43 ^h	19.21 ± 4.60 ^g	16.94 ± 2.81 ^g

The addition of the extracts to the design creams gives them a brownish color that vary in the intensity depending on the type of extract, i.e. the combination of *Pyropia* and MP extract showed the darkest color. The addition of physical filters bleached the color of the creams, becoming to a more yellow-brownish color.

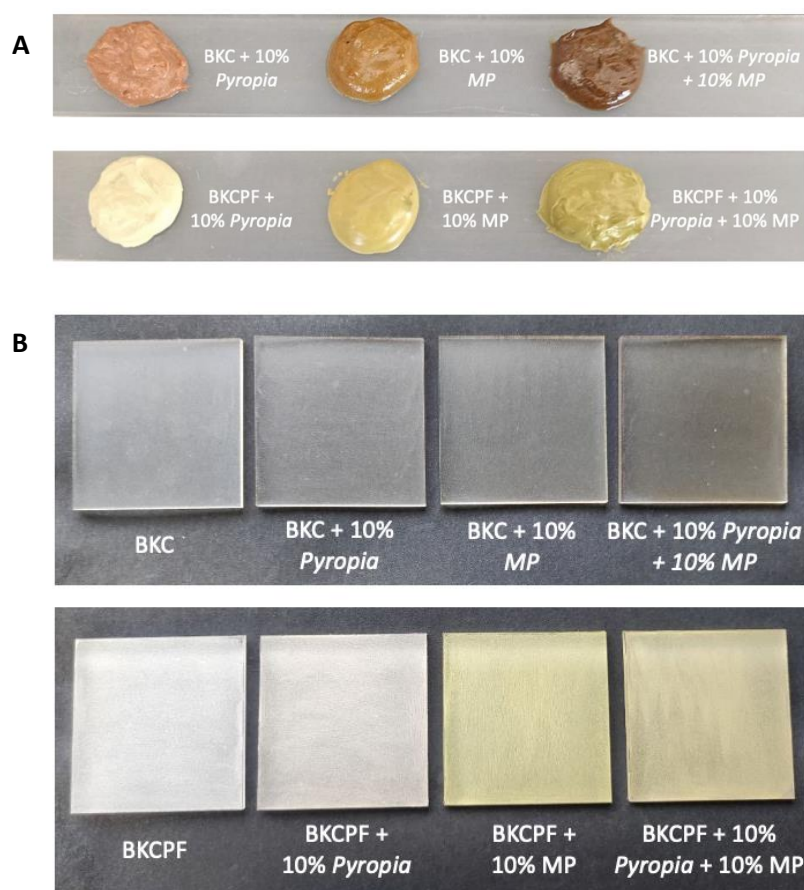


Figure 5. 15. Some of the designed creams containing algae and plants extracts used in this study. A) Samples of the design creams with *Pyropia* extract, mixture of plants (MP) extracts and the combination of both (*Pyropia* + MP), in combination or not with physical filters (PF). B) The design creams spread in polymethyl methacrylate (PMMA) plates.

Some of the designed creams were submitted to photostability (BKC, BKC + 10 %*Pyropia*, BKC + 10 % MP, BKC + 10 % *Pyropia* + 10 % MP, BKC + PF, BKC + PF + 10 %*Pyropia*, BKC + PF + 10 % MP, BKC + PF + 10 % *Pyropia* + 10 % MP). Figure SM.5. 5 shown the absorbance spectra of the creams submitted to 15, 30, 60 and 120 min of UVR, that correspond to 2.4, 4.8, 9.7 and 19.4 SED, respectively. The SPF and UVAPF values during the different radiation time periods are observed in Figure 5. 12,

and the percentage of decay of the SPF and UVAPF values are observed in Table SM.5.

6. BKC showed the lowest photostability during UVR exposure. SPF value decrease a 46 % during the first 15 min and 65 % during 120 min, whereas the UVAPF values decrease 25 % during the first 15 min and 45 % during 120 min. When the different extracts were added a better photostability were found, in the BKC + 10 % *Pyropia* + 10 % MP, the SPF values decrease a 37 % during the first 15 min and 55 % during the 120 min, and the UVAPF decrease 12 % in the first 15 min and 27 % during 120 min. The addition of physical filters to the formulation showed a much better photostability. The BKC + PF showed a decay of 20 % during the first 15 min and 34 % during 120 min, and the UVAPF decreased a 15 % and 28 % during 15 and 120 min. The BKC + PF + 10 % *Pyropia* + 10 % MP showed a SPF decrease of 13 % and 26 % at 15 and 120 min, and the UVAPF at 15 min did not decrease and at 120 min decrease a 20 %.

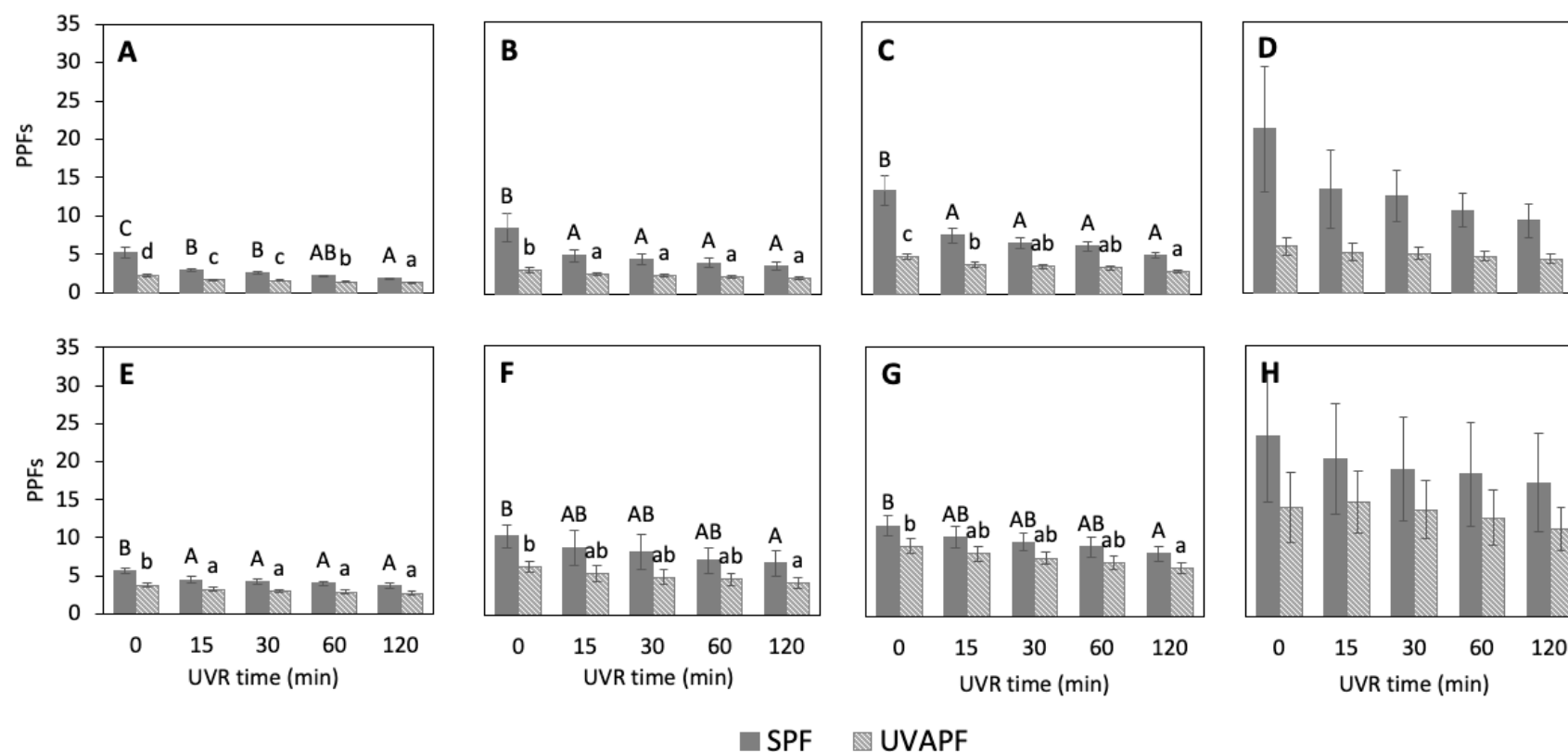


Figure 5. 16. Changes in the SPF and UVAPF values of the different creams during the UV exposure time (15, 30, 60 and 120 min). A) BKC; B) BKC + 10% *Pyropia*; C) BKC + 10% MP; D) BKC + 10% *Pyropia* + 10% MP; E) BKC + PF; F) BKC + PF + 10% *Pyropia*; G) BKC + PF + 10% MP; H) BKC + PF + 10% *Pyropia* + 10% MP. Values are expressed as mean $\pm$ standard deviation (SD) ($n = 3$). Different letters indicate significant differences in the SPF values (capital letters) or UVAPF values (lowercase letter) (ANOVA, SNK, $p < 0.05$)

4. Discussion

Cosmeceutical products with algae and plant extracts were developed. The original plan was the design of creams with natural UV-filter based on extracts from *Porphyra sensu lato* species due to the presence of UV-absorbing compounds, as MAAs and polyphenols, antioxidant capacity (enzymatic and non-enzymatic) and anti-aging potential. However, the unique addition of *Pyropia* extract to the base cream showed very low photoprotective values (Vega *et al.*, 2020b; Moreira *et al.*, 2022), as the MAAs peak is very narrow (320-345 nm) and not cover the entire erythemal or persistent pigment darkening (PPD) action spectrum used for the determination of the SPF and UVAPF. The unique addition of polyphenols to creams also resulted in low values (Gager *et al.*, 2020; Hermund *et al.*, 2022). Thus, other natural compounds were analyzed (plant extracts and vegetable oils) in order to combine them with the *Porphyra sensu lato* extracts and enhance the photoprotection properties of the designed creams. The combination of different compounds could present a broad band absorbing properties, as well as a more potent antioxidant capacity.

Before the design of the creams, different algae and plant extracts were analyzed. In algae, water achieve a high yield as has been observed in other algae species (Farvin and Jacobsen, 2013; Hwang and Do Thi, 2014; Moreira *et al.*, 2022). Other authors obtained similar (Hwang and Do Thi, 2014; Moreira *et al.*, 2022) or higher yields (Farvin and Jacobsen, 2013). The solvent used influence the type of molecules to be extracted and consequently in the extract's bioactivities. Aqueous solvents are used for the extraction of soluble polysaccharides, proteins, biliproteins, MAAs or phenols, among others, whereas more non-polar solvents (e.g. ethanol, methanol or acetone) are used for chlorophylls, carotenoids or lipids extractions. In plants, hydroethanolic solvent were used in order to extract compounds with different polarities, as we did not know what type of molecules could be present. The obtained yields were based on fresh biomass, and consequently lower yields were observed in comparison with algae (based on dry biomass). Other authors obtained extraction yield values of 7-9 % in flame of the forest leaves (dry biomass) or 20 % in ethanolic extract of green coffee (Díaz-Hernández *et al.*, 2022; Świątek *et al.*, 2022). The extraction yields could be increase by using alkaline or enzymatic hydrolysis, microwave or high pressure extractions (Michalak and Chojnacka, 2014; Sosa-Hernández *et al.*, 2018; Naseri *et al.*, 2020; Picot-Allain *et al.*, 2021; Díaz-Hernández *et al.*, 2022; Spain *et al.*, 2022).

The elemental analysis in the three analyzed *Porphyra sensu lato* species showed a carbon (C) content higher than 35 %. Other red algae species rich in agars or carragenans such as *Gelidium* spp., *Gracilaria* spp. or *Chondracanthus* spp. presented similar or lower values (Holdt and Kraan, 2011; Álvarez-Gómez *et al.*, 2016; Vega *et al.*, 2020b, 2020a; Moreira *et al.*, 2022). This relevant content of C can be due to the presence of porphyrans, a sulfated polysaccharides with pharmacological properties (Jiang *et al.*, 2012; Ueno *et al.*, 2018; Fu *et al.*, 2019). In general, *Porphyra* species are known for present a high proteins concentration, that is related to their N contents (Cian *et al.*, 2014; Cho and Rhee, 2019; Venkatraman and Mehta, 2019).

The aqueous absorption spectra of the *Porphyra sensu lato* species showed the presence of mycosporine-like amino acids (MAAs) and biliproteins. Contreras-Porcia *et al.* (2011) obtained similar biliproteins values in *P. columbina* (1.8 mg g⁻¹ DW), and other authors obtained values that varied from 1.8 (summer) to 5.5 mg g⁻¹ FW (winter) in *P. umbilicalis* collected in New Hampshire (USA) (Sampath-Wiley *et al.*, 2008), or 5 mg g⁻¹ FW in *Pyropia elongata* collected in winter (Venice, Italy) (Sfriso *et al.*, 2018), demonstrating that the environmental factors influenced the accumulation of these compounds. The drying and extraction process also influenced the obtained results (Amorim *et al.*, 2020; Nguyen *et al.*, 2020; Castro-Varela *et al.*, 2022). Castro-Varela *et al.* (2022) showed a high PE extraction by using high pressure homogenization in the red algae *Sarcopeltis skottsbergii*. Several bioactivities have been described in biliproteins such as antioxidant, anticancer or anti-inflammatory (Pagels *et al.*, 2019; Chen *et al.*, 2022). Similar results in the MAAs content were observed in other *Porphyra sensu lato* species with values that varied from 5.2 to 10.6 mg g⁻¹ DW and being porphyra-334 and shinorine the predominant MAAs (Huovinen *et al.*, 2004; Briani *et al.*, 2018; Schneider *et al.*, 2020b; Vega *et al.*, 2020b; Moreira *et al.*, 2022). Some authors have analyzed the photoprotective properties of MAAs on mouse skin and demonstrated that the presence of MAAs can prevent UV-induced skin damage (de la Coba *et al.*, 2009a; Mercurio *et al.*, 2015; Ying *et al.*, 2019). Lawrence *et al.* (2018) also observed this effect using palythine on human keratinocytes. In addition to photoprotection, other bioactivities have been described in these molecules such as antioxidant, wound-healing or anti-photoaging (Wada *et al.*, 2015; Chrapusta *et al.*, 2017; Vega *et al.*, 2021). The hydroethanolic extract of the different plants also showed UV-absorbing compounds, although it is not clear what molecules are implied. Polyphenols such as flavonoids are the molecules more related to photoprotection, since these compounds present two major peaks in the UV region at 240-285 nm and 300-550 nm (Robards and Antolovich,

1997). Rutin and quercetin are the flavonoids most reported in plants with photoprotective properties (Li *et al.*, 2022; Monsalve-Bustamante *et al.*, 2023).

Phenolic compounds are secondary metabolites generally involved in defense mechanism (against UV radiation, herbivores or pathogens). Among algae, brown seaweeds presented the highest content of these compounds. Red and green macroalgae can also present them, although normally in lower quantities (Jiménez-Escrig *et al.*, 2001; Zubia *et al.*, 2007; Schneider *et al.*, 2020b; Vega *et al.*, 2020a). Similar results to the obtained in this work were observed by other authors (Tala and Chow, 2014; Machu *et al.*, 2015; Schneider *et al.*, 2020b; Moreira *et al.*, 2022), who obtained values that varied from 8 to 18 mg g⁻¹ DW. Generally, plants are great sources of polyphenols, reaching values higher than 100 mg g⁻¹ DW (Pérez-Jiménez *et al.*, 2010; Checkouri *et al.*, 2020; Mueed *et al.*, 2023). The broad bioactivities of phenolic compounds have been reviewed by different authors, e.g. antidiabetic, cardiometabolic, antiobesity, neuroprotective, anticancer, antimicrobial and modulator of gut microbiota, among others (Al Jitan *et al.*, 2018; Mateos *et al.*, 2020).

The obtained general antioxidant values of *Porphyra sensu lato* species are in accordance with the obtained by other authors in red algae, that varied from 5 to 50 µmol TE g⁻¹ DW (Álvarez-Gómez *et al.*, 2016; Schneider *et al.*, 2020b; Vega *et al.*, 2020b). In plants, the antioxidant capacity is normally higher than in red algae (Ivanova *et al.*, 2005; Kirca and Arslan, 2008; Song *et al.*, 2010). Catalase and other antioxidant enzymes (e.g. superoxide dismutase or glutathione peroxidase) are the first protection mechanism against the oxidative stress in the cells, more specifically catalase is a very efficient enzyme, able to neutralized millions of hydrogen peroxide molecules per seconds (Dring, 2005). Different methodologies and expression units were used by other authors in catalase activity, what difficult the comparisons. Sampath-Wiley *et al.* (2008) measured the catalase activity in *Porphyra umbilicalis* through O₂ evolution rate using electrodes and obtained values that vary from 50 to 80 µmol O₂ mg proteins⁻¹ h⁻¹. Contreras-Porcia *et al.* (2011) measured the catalase activity in *P. columbina* species and observed values that varied from 10 to 95 µmol min⁻¹ mg proteins⁻¹. Regarding anti-aging properties, all *Porphyra sensu lato* species presented capacity to inhibit the enzyme collagenase. This enzyme is a matrix metalloproteinase (MMPs), as elastase or hyaluronidase, involved in the degradation of the fibers present in the extracellular matrix (ECM) of the dermis, such as collagen, fibronectin or proteoglycans, contributing to photoaging (Pittayapruerk *et al.*, 2016). UV radiation exposure increase the production of MMPs, so the use of products

with this bioactivity can reduce or delay the premature photoaging. Moreira *et al.* (2022) also observed a high collagenase activity in *Porphyra linearis* and *Phycocalidia acanthophora* and obtained a strong correlation with the MAAs content. Hartmann *et al.*, (2015b) already reported the collagenase activity of three isolated MAAs: palythine, shinorine and porphyra-334. Positive correlations between antioxidant capacity and the content of different bioactive compounds (phycoerythrin, phenolic compounds and MAAs) would be expected. However, in *Porphyra sensu lato* species, ABTS and DPPH did not showed correlation with any of these compounds. The few analyzed species and the similar obtained results can difficult the obtention of correlations. Nevertheless, the analyzed plants showed positive correlation between phenols and antioxidant capacities.

Among the hydroethanolic plant extracts analyzed, the best results were observed in flame of the forest leaves, green coffee beans, liquorice root, olive leaves and ivy root. The flame of the forest is a plant native from Africa with an ancient history of medicinal used, that currently found as ornamental plant in many parts of the world. Many bioactivities have been described in this plant such as antimalarial, antidiabetic, antibacterial, anticancer, anti-inflammatory, antioxidant or cardioprotective, among others (Padhy, 2021). However, few information has been found about the antioxidant capacity and phytochemical composition of this plant. Kumar and Thampi (2015) determined the presence of polyphenols (i.e. flavonoids and tannins), alkaloids, terpenoids or anthraquinones in methanolic and aqueous extracts of the leaves. Santos *et al.* (2020) observed higher antioxidant capacity and total phenols content in the leaves than in the flowers of this plant, although obtained lower values than the obtained in this work ($44.4 \pm 0.0 \mu\text{M TEAC mg}^{-1} \text{ DW}$ measured through ORAC method or $6.3 \pm 0.0 \text{ mg phenols g}^{-1} \text{ DW}$). These authors also identified different phenols and flavonoids: caffeic acid, ferulic acid, rutin and vitexin. Very few information has been found about photoprotective properties in this plant. In a similar way to us, (Patil *et al.*, 2009) observed a potential capacity to absorbed UV radiation in methanolic extracts of the flower of this tree, with maximum absorption at 200-240 nm and medium absorption at 240-325 nm.

Regarding coffee, Muñoz *et al.* (2020) compared the phenols content and antioxidant capacity of green and roasted coffee and obtained similar results. This author obtained lower values of phenols ($15 \text{ mg L}^{-1} = 0.25 \text{ mg g}^{-1} \text{ FW}$) and higher values of antioxidant capacity ($21 \mu\text{mol Trolox L}^{-1} = 330 \mu\text{mol g}^{-1} \text{ FW}$) than the obtained in this work. In the case of liquorice, Sohail *et al.* (2018) obtained similar phenols content (1% FW) and lower antioxidant values ($11 \mu\text{mol TE g}^{-1} \text{ FW}$) than the obtained in this work,

analyzing an ethanolic liquorice extract. However, Quintana *et al.* (2019) obtained much higher values using supercritical extraction (180 mg phenols g⁻¹ extract and 555 µmol TEAC g⁻¹ extract). Liquorice have shown beneficial properties for skin care application, but no UV-absorbing compounds have been described in the literature.

In olive leaves, Talhaoui *et al.* (2016) observed higher phenols content than the obtained in this work, that varied from 46 to 83 mg g⁻¹ DW (depending on the olive variety). Şahin *et al.* (2018) also observed higher phenols content (37 mg GAE g⁻¹ DW), but similar antioxidant capacity (25 mg TEAC g⁻¹ DW = 100 µmol TE g⁻¹ DW). Few information has been found about the composition of ivy. Pop *et al.* (2017) obtained lower phenols content than the obtained in this work in ivy leaves, 1 mg g⁻¹ FW, that were mainly chlorogenic acid and isoquercitrin, and observed an antioxidant capacity of 122.5 µg mL⁻¹ (IC₅₀), whereas Žugić *et al.* (2014) obtained phenols content 15 mg g⁻¹ dry extract and an antioxidant capacity of 107.3 µg mL⁻¹ (IC₅₀). For the aerial roots of the ivy, no information about biochemical composition have been found.

Regarding the other plants considered in our study, ginger showed very low results, whereas Stoilova *et al.* (2007) obtained high phenols content and antioxidant capacity in an ethanol extract of ginger (871 mg g⁻¹ DW; IC₅₀ DPPH: 0.64 µg mL⁻¹). A methanolic extract of orcanette root showed a higher phenols content (48.3 ± 1.2 mg g⁻¹ DW), than the obtained in this work (6.5 mg g⁻¹ DW), and an antioxidant capacity expressed as IC₅₀ of 30.9 ± 2.4 µg mL⁻¹ (Jaradat *et al.*, 2018). Franklin *et al.* (2023) analyzed the aqueous extract of urukum and observed that this type of extract present norbixin (a carotenoid), and an antioxidant capacity of 147.5 µmol g⁻¹ DW, a higher value than the observed in this study (61.6 µmol TE g⁻¹ DW). The antioxidant capacity and phenols content of chokeberry fruits was analyzed by Oszmiański and Lachowicz (2016), who observed values that varied from 5 to 25 mg 100 g⁻¹ DW of phenols (where highlighted flavanone and anthocyanins) and values of antioxidant capacity that ranged from 20 to 80 µmol Trolox 100 g⁻¹ DW.

Most natural compounds presented toxic molecules under certain doses, so it is important to analyze its possible adverse toxic effects in health cell lines. In addition, as we are looking for a cosmetic application, the cytotoxic activity against carcinogenic cells in the skin, like malignant melanoma, is also of interest. A higher half maximal inhibitory concentration (IC₅₀) means a lower toxicity. In general, *Porphyra sensu lato* extracts presented low toxicity and no anti-tumor activity against malignant melanoma. There is

few information about cytotoxicity or anti-tumor activity of seaweed extracts in cell lines. Senevirathne *et al.* (2010) and Moreira *et al.* (2022) also found no cytotoxicity *Porphyra sensu lato* species in health cell lines. Harb *et al.* (2023) studied the cytotoxicity of beach-cast algae of Brazilian coast and observed no toxicity in any of the species. However, the toxicity and anti-tumor potential of certain compounds coming from algae have been widely studied for their therapeutic application, such as polysaccharides (Ghannam *et al.*, 2018; Abdala Díaz *et al.*, 2019; Mazepa *et al.*, 2022; Ju *et al.*, 2023).

Regarding plants, Świątek *et al.* (2022) also observed low toxicity in flame of the forest leaves extract in health cell lines ($IC_{50} > 1000 \mu\text{g mL}^{-1}$), and anti-proliferative potential against some cancer cell lines (e.g. human hypopharyngeal squamous cell and colon carcinoma). No studies about the anti-melanoma activity in flame of the forest leaves have been found, although the flowers of this plant showed an IC_{50} of $8 \mu\text{g mL}^{-1}$ against melanoma (Mbosso Teinkela *et al.*, 2016). Essafi *et al.* (2019) and Makowska-Wąs *et al.* (2017) studied the antiproliferative activity of olive leaves extract and observed good results against breast and prostate cancer cells. In contrast to our good result against melanoma, Makowska-Wąs *et al.* (2017) did not observed activity against melanoma. In relation to green coffee, similar results were observed by Díaz-Hernández *et al.* (2022) and Amigo-Benavent *et al.* (2017), in which no toxicity were observed in different carcinogenic cell lines. Nazari *et al.* (2017) reviewed toxicological effect of liquorice, e.g. Khazraei-Moradian *et al.* (2017) observed that a concentration of $50\text{--}100 \mu\text{g mL}^{-1}$ increased the apoptosis in murine colon carcinoma, but not in human colon cancer and health cell lines. About ivy root, only the study of Xia *et al.* (2010) was found, who studied the cytotoxicity of ivy root nanoparticle aqueous suspension in HeLa cells and observed very low apoptosis. According to our results, liquorice root and olive leaves presented selectivity index (SI) higher than 3, indicating a great potential to be used as anti-proliferative agent against melanoma.

The develop of cosmeceutical formulations based on natural cosmetic and with biological UV filters are still a great challenge. In this study, the creams were designed using karanja (*Millettia pinnata*) and callophyl (*Callophylum inophyllum*) commercial oils with potential bioactivities, e.g. photoprotective, antioxidant or anti-microbial (Al Muqarrabun *et al.*, 2013; Gupta and Gupta, 2020; Ácsová *et al.*, 2021; Grygier *et al.*, 2022). It is important to notice that there are many websites that attribute beneficial properties to natural products without any scientific testing. Couteau *et al.* (2021) found on websites that provide handmade sunscreen recipes with the SPF values of many

vegetable oils, e.g. karanja oil - 20, avocado oil - 15, coconut oil - 8, olive oil - 8 or jojoba oil - 4; and criticized the dissemination of false information about natural UV-filters and its dangerousness because they are not subjected to any testing. More recently, Couteau *et al.* (2022) analyzed the SPF values of some vegetable oils and obtained very low values, e.g. karanja oil showed a SPF of 1.1. Ácsová *et al.* (2021) also observed low photoprotective values in vegetable oils, with the exception of calophyll that showed *in vivo* values of 4.1. In this study, the designed creams with 7 % of karanja oil and 7 % of calophyll oil showed a SPF of 4.2.

In general, the calculated *in vitro* SPF of the commercial sunscreens are higher than the labelled SPF (determined *in vivo*), what means that the methodology followed seems to overestimate the photoprotection factors. This difference can be due to the fact that we only use one type of plate and that the manual spread increases the variability among plates, i.e. in this case, it would be recommended to do more replicates (the ISO 24443 (2012) suggest 9 replicates), but the cost of the plates (around 2 euros each one) difficult it. However, the calculated *in vitro* SPF of the commercial sunscreen Biarritz SPF50 that contained physical UV-filters and algae extracts (without chemical UV-filters) was very similar to the labelled SPF. According to the formulation, our design sunscreen is more similar to the latter one, so it can be considered that the values obtained *in vitro* for our creams can be reliable. The determination of the SPF *in vivo* is necessary to commercialize a cream that claim photoprotection (EC, 2009). It is a precise method, although it is time consuming, complex (i.e. it requires human volunteers) and expensive process. Thus, the *in vitro* methodology is a faster and cheaper alternative to obtain information about the photoprotection properties of new UV-filters and formulations, as well as it is possible to determine photoprotection against other biological effects (e.g. photocarcinogenesis, immunosuppression, elastosis or photoaging) and not only against erythema. Nowadays, there is not an ISO (International standard organization) for the determination of the *in vitro* SPF (as there are for the *in vivo* SPF (ISO 24444, 2019) or *in vitro* UVAPF (ISO 24443, 2021)), but there is research that have validate the methodology (Pissavini *et al.*, 2018).

According to the European Commission (EC, 2009), a cosmetic product can be considered a sunscreen if contain at least one approved UV-filter and present a minimal SPF of 6. Most of the designed creams showed higher values, reaching SPF values of 32 by adding 20 % of the mixture of plant extracts, although only those creams with physical filters could be considered sunscreens. The addition of physical filters increased

the photoprotection, mainly in the UV-A region, although not as expected. The physical filters seem to form a barrier that absorbed in the UV-B and UV-A region and hide the effect of the extracts, i.e. by adding physical filters to the BKC + 10 % *Pyropia* + 10 % MP the SPF did not change, whereas the UVAPF increased from 6.7 to 14.1. There are many types of physical filters and their incorporation to the formulations are not easy, so it is necessary to try with other types of physical filters, e.g. physical filters as nanoparticles provide a better incorporation and a better texture.

In addition to efficacy and toxicity, it is necessary to evaluate the photo- and thermostability of the extracts and creams. Extracts were fully stable (regarding UV-screen, phenolic compounds content, and antioxidant capacity) at different temperature (even 80 °C) during 1h and UV radiation exposure (until a dose of 334.3 kJ m⁻²). MAAs are stable molecules, although de la Coba *et al.* (2019) observed a progressive absorbance decrease in isolated MAAs at 85 °C at alkaline pH (1.5-4.5 h). Polyphenols can be extracted at 60-80 °C, so they are regarded as thermostable molecules (Antony and Farid, 2022). However, creams showed a decay of the photoprotection factors when were irradiated with UV radiation. As the extracts have shown stability against temperature and UV radiation, the decrease in photoprotection could be due to the oxidation of the oils used in the formulation. Otherwise, the photostability of the creams were evaluated in the PMMA plates, i.e. a very thin layer, what could provoke the faster degradation of certain compounds related with photoprotection. The addition of the physical filters increased the photostability of the creams, probably because the physical filters increase the viscosity (personal observation) and protect the other compounds of the creams. Similarly, the addition of starch to formulations can enhance the stability and even provide a synergistic effect in photoprotection (Daudt *et al.*, 2015; Marto *et al.*, 2016), i.e. Marto *et al.* (2016) doubled the photoprotection properties of a formulation containing physical filters and green coffee oil, by using the pickering emulsion concept (Guzmán *et al.*, 2022), in which creams are stabilized by solid particles (starch) instead of surfactants. Thus, the pickering emulsions can be an alternative to avoid the use of surfactants that can present adverse effects (e.g. irritancy), and reduce the amount of UV-filters included in sunscreens. The designed creams were also subjected to thermostability, but many creams were separated in the two phases (those without physical filters) and the others increased their viscosity (probably due to water evaporation) what difficult the spread in PMMA plates and was not possible to determine the photoprotection factors again.

The develop of new UV-filters is not an easy task. The new product must be approved by the European Commission, what it is a large and high-cost process. In addition, the accreditation of an extract as a new UV-filter is not possible since it is a pool of molecules, i.e. it would be possible the accreditation of isolated MAAs. It is important to mentioned that no natural UV-filters are currently approved by the European Commission. Most of these substances are considered boosters for their beneficial properties (e.g. antioxidant, immunostimulant, anti-aging, antimicrobial), although several natural compounds are potential candidates for new UV-filters due to their high photoprotective efficacy.

On the other hand, to certify a cosmetic product in the European Union it is necessary to undergo all the required testing defined by the (EC, 2009): 1) Physical, chemical and microbiological testing. Some of the most common analyzed parameters are viscosity, pH, phase separation or organoleptic properties. Regarding microbiological analysis, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans* and *Escherichia coli*, are the most common investigated pathogens with a limit of 1000 CFU (colony forming unit) per g or mL; 2) Challenge testing, that determine the preservation efficacy after an artificial contamination with bacteria (e.g. *Staphylococcus aeruginosa*) or fungi (e.g. *Aspergillus brasiliensis*) 3) Stability against temperature, humidity and UV light; and compatibility with the packaging is also testing.

In addition to UV-filters, sunscreens contain other ingredients that can produce allergies and damages in aquatic environments as preservatives (2-bromo-2-nitropropane 1-3 diol) antioxidants (BHT, EDTA), parabens (propyl and butyl parabens), fragrances (limonene, benzyl salucilate) and microplastcis (dimethicone, carbomer acylates polymers) (Osterwalder *et al.* 2014). In the last years, the interest in natural products have increased (both in the industry and consumers). In the market, it can be found cosmetic products labelled as natural, organic or ecological. However, there is not an EU regulation of this type of cosmetic, but there are certifications emitted by private companies as Ecocert and Cosmebio (France), Bioinspecta (Switzerland), BDIH (Germany), Cosmetici Biologici (Italy), Natrue (Belgium) or Bio-Vida Sana (Spain) among others. All these different companies and the lack of an EU regulation, generate confusion among consumers about what is natural and what need to be. Thus, it is necessary a public EU regulation on natural cosmetics and specifically in the field of sunscreens and natural UV filters.

5. Conclusions

Porphyra sensu lato species presented antioxidant and anti-aging (anti-collagenase) activity as well as no toxicity in health cell lines (keratinocytes and fibroblasts). Among plants, flame of the forest, liquorice and green coffee were selected for the development of the cosmeceutic products as presented high phenols content and antioxidant capacity, as well as the presence of UV-absorbing compounds. The combination of aqueous extracts from algae (*Pyropia*) and hydroethanolic extracts of the mentioned plants increased the photoprotection of cosmeceutic formulations against erythema and other biological effects related with the UV radiation (i.e. reaching SPF values of 20-25, and UVAPF values of 6-7). The extracts presented optimal photo and thermostability, although the creams lost efficacy in photoprotection after UV radiation exposure. The addition of physical filters increased the stability of the creams against UV radiation, and improve the photoprotection in the UV-A region. Promising results were obtained in the development of cosmeceutical formulations based on natural cosmetic, by using natural products as UV-filters what is safer for humans and the environment.

SUPPLEMENTARY MATERIAL**Table SM.5. 1.** Design of the different cosmeceutic formulations based on natural cosmetic, that contain the analyzed algae and plant extracts, as well as other natural compounds (oils and physical filters).

		B	BK	BKC	BKC + Ext.	BKC + PF	BKC + PF + Ext.
PHASE A							
Emulgents	Ritamulse	7.0	7.0	7.0	7.0	7.0	7.0
	VE	2.0	2.0	2.0	2.0	2.0	2.0
	Miristil miristrate	2.0	2.0	2.0	2.0	2.0	2.0
Oils	Soy wax	1.0	1.0	1.0	1.0	1.0	1.0
	Fractionated coconut oil	20.0	13.0	6.0	6.0	6.0	6.0
	Calophyl oil	-	-	7.0	7.0	7.0	7.0
	Karanja oil	-	7.0	7.0	7.0	7.0	7.0
	Isopropil miristrate	2.0	2.0	2.0	2.0	2.0	2.0
	Dicaprylyl ether	1.0	1.0	1.0	1.0	1.0	1.0
Vitamin	Vitamin E	1.0	1.0	1.0	1.0	1.0	1.0
Physical filters	TiO ₂	-	-	-	-	5.0	5.0
	ZnO	-	-	-	-	2.5	2.5
		36.0	36.0	36.0	36.0	44.0	44.0
PHASE B							
	Distilled water	37.3	37.3	37.3	37.3	29.8	29.8
	Glycerine	5.0	5.0	5.0	5.0	5.0	5.0
	Xanthan gum	0.2	0.2	0.2	0.2	0.2	0.2
		42.5	42.5	42.5	42.5	35.0	35.0
PHASE C							
	Cosgard	1.0	1.0	1.0	1.0	1.0	1
	Olor	0.5	0.5	0.5	0.5	0.5	0.50
		1.5	1.5	1.5	1.5	1.5	1.5
PHASE D							
	Distilled water	20.0	20.0	20.0	0.0 – 15.0	20.0	0.0 – 15.0
	Powder extract	-	-	-	5.0 – 20.0	-	5.0 – 20.0
		20.0	20.0	20.0	20.0	20.0	20
TOTAL		100	100	100	100	100	100

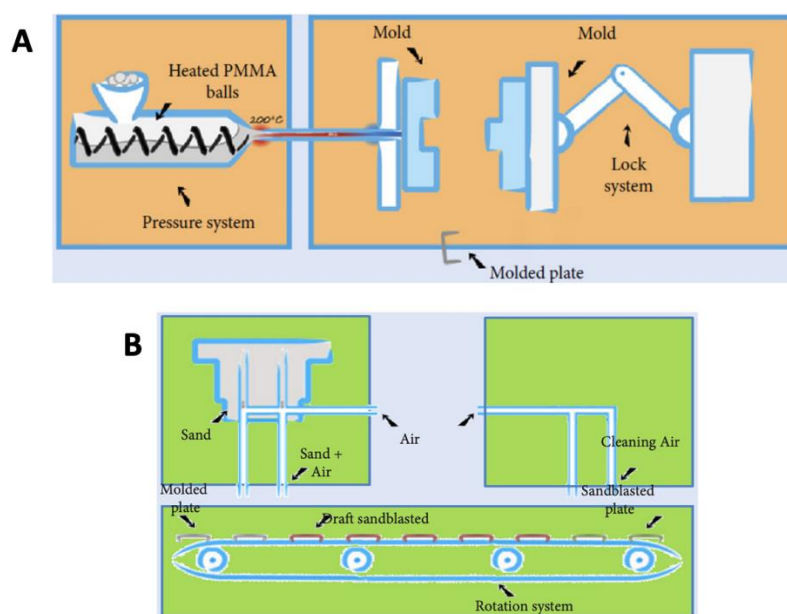


Figure SM.5. 1. Manufacture process of both type of plates: (A) moulded and (B) sandblasted. (Technical sheet of Helioscreen).

Table SM.5. 2. ANOVA (Analysis of variance) summary table of the different analyzed variables. In A) Potential cosmeceutic uses of extracts from *Porphyra sensu lato* species, the variable source were the species and the extraction solvent. In B) Looking for new antioxidant and UV screen compounds in diverse plants, the variable source were the plant species. In C) Design and analysis of cosmeceutic products: determination of photoprotection factors and stability, the variable sources were: the species in case of the different extracts, the temperature in case of thermostability of the extracts and commercial sunscreens, the type of cream in case of the design creams, and the time in case of photostability of the design creams. SS: sum of squares, DF: Degrees of freedom, MS: mean square, F: F-value, and p: p-value. $p < 0.05$.

		SS	DF	MS	F	p
A) <i>Porphyra sensu lato</i> species						
Ext. Yield	Solvent	41.552	1	41.552	143.04	< 0.01
	species	15.037	2	7.518	25.88	< 0.01
	Solvent*species	1.833	2	0.916	3.15	0.08
Carbon	Species	2.612	2	1.306	2.10	0.27
Nitrogen	Species	4.7973	2	2.3987	99.32	< 0.01
Sulphur	Species	0.19989	2	0.09995	7.32	0.07
C/N	Species	23.2529	2	11.6264	28.12	0.01
MAAS	species	42.8510	2	21.4255	76.81	< 0.01
PC	species	15.06989	2	7.53495	400.99	< 0.01

PE	species	67.0655	2	33.5327	1463.38	< 0.01
	Solvent	26.474	1	26.474	317.63	< 0.01
Phenols	species	0.293	2	0.147	1.76	0.21
	Solvent*species	4.061	2	2.030	24.36	< 0.01
	Extract	79.755	1	79.755	84.78	< 0.01
ABTS	Species	126.847	2	63.423	67.42	< 0.01
	Extract*Species	13.525	2	6.763	7.19	0.01
	Solvent	4.443	1	4.443	1.99	0.18
DPPH	species	16.483	2	8.241	3.69	0.06
	Solvent*species	57.542	2	28.771	12.89	< 0.01
	Solvent	145.8	1	145.8	4.11	0.07
Anti-collagenase	species	9.6	2	4.8	0.13	0.88
	Solvent*species	28.1	2	14.0	0.40	0.68
	Solvent	236576	1	236576	4.39	0.06
EC ₅₀ HACAT	species	20596035	2	10298018	190.94	< 0.01
	Solvent*species	36356450	2	18178225	337.05	< 0.01

B) Plants

ABTS	Species	122893.2	10	12289.3	574.96	< 0.01
Phenols	Species	3397.704	10	339.770	704.92	< 0.01
EC ₅₀ HACAT	Species	91743578 0	4	229358945	185.63	< 0.01
EC ₅₀ G-361	Species	27494400 3	4	68736001	1321.26	< 0.01

C) Design creams**Extracts**

Phenols	Species	131422.5	5	26284.5	37.66	< 0.01
ABTS	Species	3091880	5	618376	793.54	< 0.01

Extracts thermostability

Phenols_P	Temperature	59.30	2	29.65	1.13	0.38
Phenols_MP	Temperature	253.4	2	126.7	4.58	0.06
Phenols_P + MP	Temperature	2.24	2	1.12	0.01	0.99
ABTS_P	Temperature	3027.37	2	1513.68	51.10	< 0.01
ABTS_MP	Temperature	242	2	121	0.04	0.96
ABTS_P + MP	Temperature	6262	2	3131	3.77	0.09
AUC_P	Temperature	2.589	2	1.294	0.34	0.72
AUC_MP	Temperature	2.78	2	1.39	0.31	0.74
AUC_P + MP	Temperature	2.47	2	1.24	0.15	0.86

Commercial creams thermostability

Avène20_SPF	Temperature	229.61	2	114.81	29.80	< 0.01
Avène20_UVAPF	Temperature	9.810	2	4.905	37.77	< 0.01
Avène30_SPF	Temperature	65.11	2	32.55	0.40	0.69
Avène30_UVAPF	Temperature	2.491	2	1.246	0.28	0.76
Avène50_SPF	Temperature	204.14	2	102.07	2.50	0.16
Avène50_UVAPF	Temperature	23.644	2	11.822	6.57	0.03
Biarritz50_SPF	Temperature	96.26	2	48.13	1.62	0.27
Biarritz50_UVAPF	Temperature	0.9751	2	0.4875	1.89	0.23
Design creams						
SPF	Cream	4501.818	14	321.558	22.78	< 0.01
UVAPF	Cream	739.505	14	52.822	52.34	< 0.01
Elastosis	Cream	643.636	14	45.974	56.35	< 0.01
Photoaging	Cream	1269.005	14	90.643	38.44	< 0.01
Photocarcinogenesis	Cream	6965.62	14	497.54	21.22	< 0.01
Immunosuppression	Cream	8359.05	14	597.07	16.88	< 0.01
Singlet oxygen	Cream	1917.377	14	136.955	34.25	< 0.01
Design creams phototability						
Base_SPF	Time	22.1609	4	5.5402	50.66	< 0.01
Base_UVAPF	Time	1.79573	4	0.44893	71.41	< 0.01
Base + P_SPF	Time	46.6483	4	11.6621	11.49	< 0.01
Base + P_UVAPF	Time	2.00642	4	0.50161	8.52	< 0.01
Base + MP_SPF	Time	127.5498	4	31.8874	28.90	< 0.01
Base + MP_UVAPF	Time	6.2641	4	1.5660	20.44	< 0.01
Base + P + MP_SPF	Time	206.560	4	51.640	3.05	0.07
Base + P + MP_UVAPF	Time	4.4791	4	1.1198	1.67	0.23
Base + PF_SPF	Time	9.5895	4	2.3974	9.94	< 0.01
Base + PF_UVAPF	Time	3.4331	4	0.8583	8.50	< 0.01
Base + PF + P_SPF	Time	92.3584	4	23.0896	6.80	0.01
Base + PF + P_UVAPF	Time	26.0293	4	6.5073	8.56	< 0.01
Base + PF + MP_SPF	Time	43.222	4	10.805	10.53	< 0.01
Base + PF + MP_UVAPF	Time	26.1081	4	6.5270	12.69	< 0.01
Base + PF + P + MP_SPF	Time	81.413	4	20.353	0.18	0.94
Base + PF + P + MP_UVAPF	Time	22.703	4	5.676	0.17	0.95

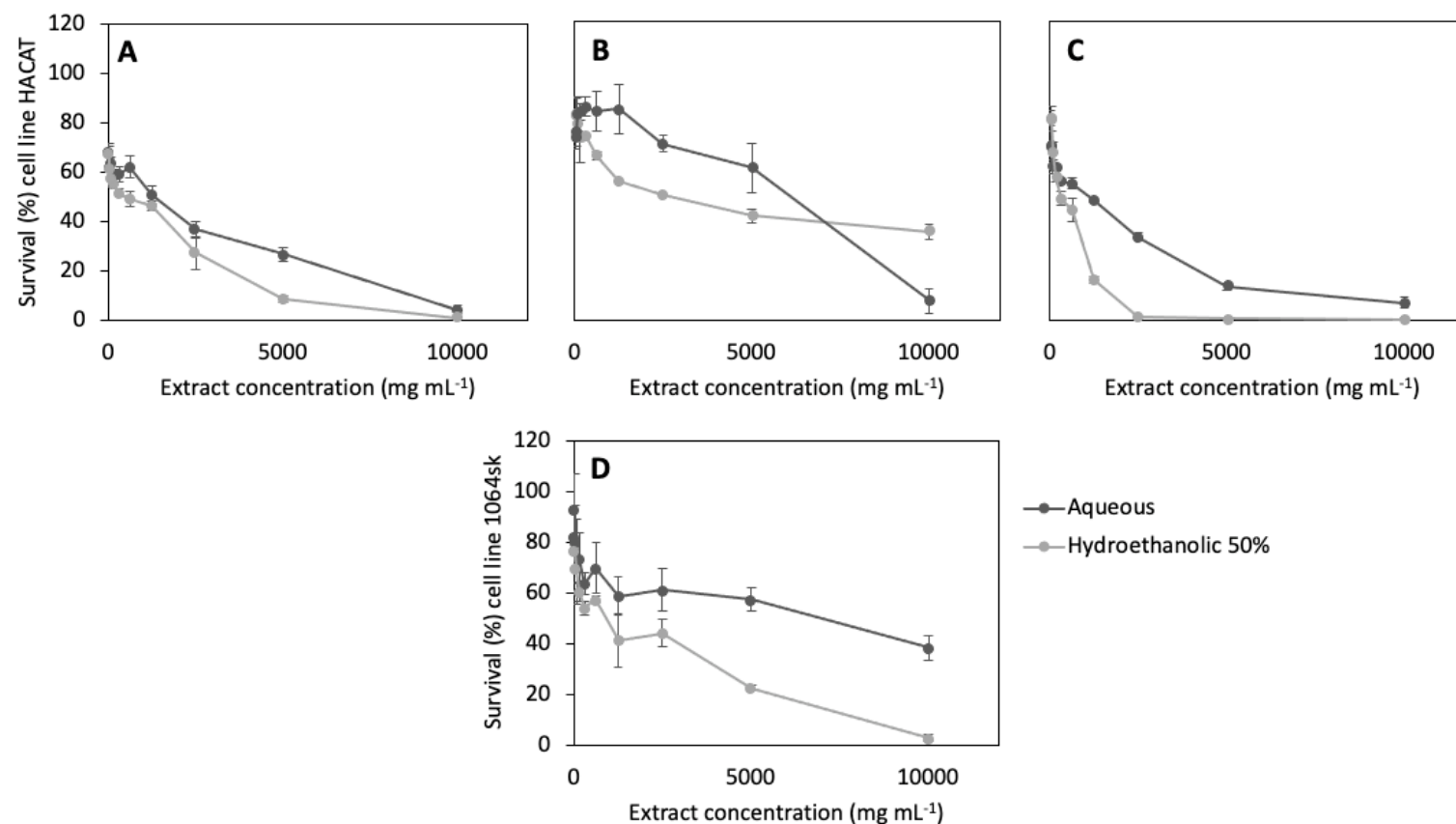


Figure SM.5. 2. Survival (%) of two different health human cells (Keratinocytes - HACAT- and Fibroblast -1064sk-) after exposure to several concentrations of aqueous and hydroethanolic (dH₂O:ethanol; 1:1) extracts of three *Porphyra sensu lato* species: *Pyropia columbina* collected in Valparaíso (Chile) (A). *Pyropia columbina* collected in Concepción (Chile) (B) and *Porphyra linearis* collected in Tarifa (Spain) (C and D). Values are expressed as mean $\pm$ standard deviation (SD) (n = 3)

Table SM.5. 3. Pearson correlation coefficients (r) of the different parameters measured from aqueous extracts of two *Porphyra* sensu lato species: *Pyropia columbina*, collected in Valparaíso (Chile) and other collected in Concepción (Chile) and *Porphyra linearis* collected in Tarifa (Spain). Green and red colors mean positive and negative correlations, respectively. Colors are in scale according to the p-value. darkest colour mean a lower p-value (< 0.01). ** p < 0.01. * p < 0.05. PC: phycocyanin; PE: phycoerythrin; MAAs: Mycosporine-like amino acids, ABTS and DPPH: antioxidant capacity methodologies, Collag.: Collagenase inhibitory activity, and Surv. HACAT: EC₅₀ of HACAT cell line.

	PC	PE	MAAs	Phenols	ABTS	DPPH	CAT	Collag.	Surv, HACAT
Yield	-0.0715	0.1034	0.4718	-0.1572	0.7358*	-0.5832	0.8820**	-0.0476	0.6049
PC		0.9735**	0.8055**	0.7903*	-0.6213	0.2119	0.1893	0.0894	0.6409
PE			0.9054**	0.7662*	-0.4997	0.0659	0.3819	0.0747	0.7871*
MAAs				0.6192	-0.1530	-0.2146	0.7159*	-0.0428	0.9371*
Phenols					-0.5690	-0.0971	0.1646	0.0693	0.6045
ABTS						-0.3387	0.4122	-0.1806	0.0337
DPPH							-0.7122*	0.1999	-0.4296
CAT								-0.0710	0.8265**
Collag.									-0.0189

Table SM.5. 4. Pearson correlation coefficients (r) of the different parameters measured from hydroethanolic extracts of two *Porphyra* sensu lato species: *Pyropia columbina*, specimens collected in Valparaíso (Chile) and other collected in Concepción (Chile) and *Porphyra linearis* collected in Tarifa (Spain). Green and red colors mean positive and negative correlations, respectively. Colours are in scale according to the p-value. Darkest colors mean lower p-value (< 0.01). ** p < 0.01. * p < 0.05.

	Phenols	ABTS	DPPH	Collag.	Surv. HACAT
Yield	-0.6870*	0.5233	0.9397**	0.3167	-0.1567
Phenols		0.1331	-0.6766*	-0.0363	0.7320*
ABTS			0.4859	0.4682	0.7222*
DPPH				0.3083	-0.2276
Collag.					0.3780

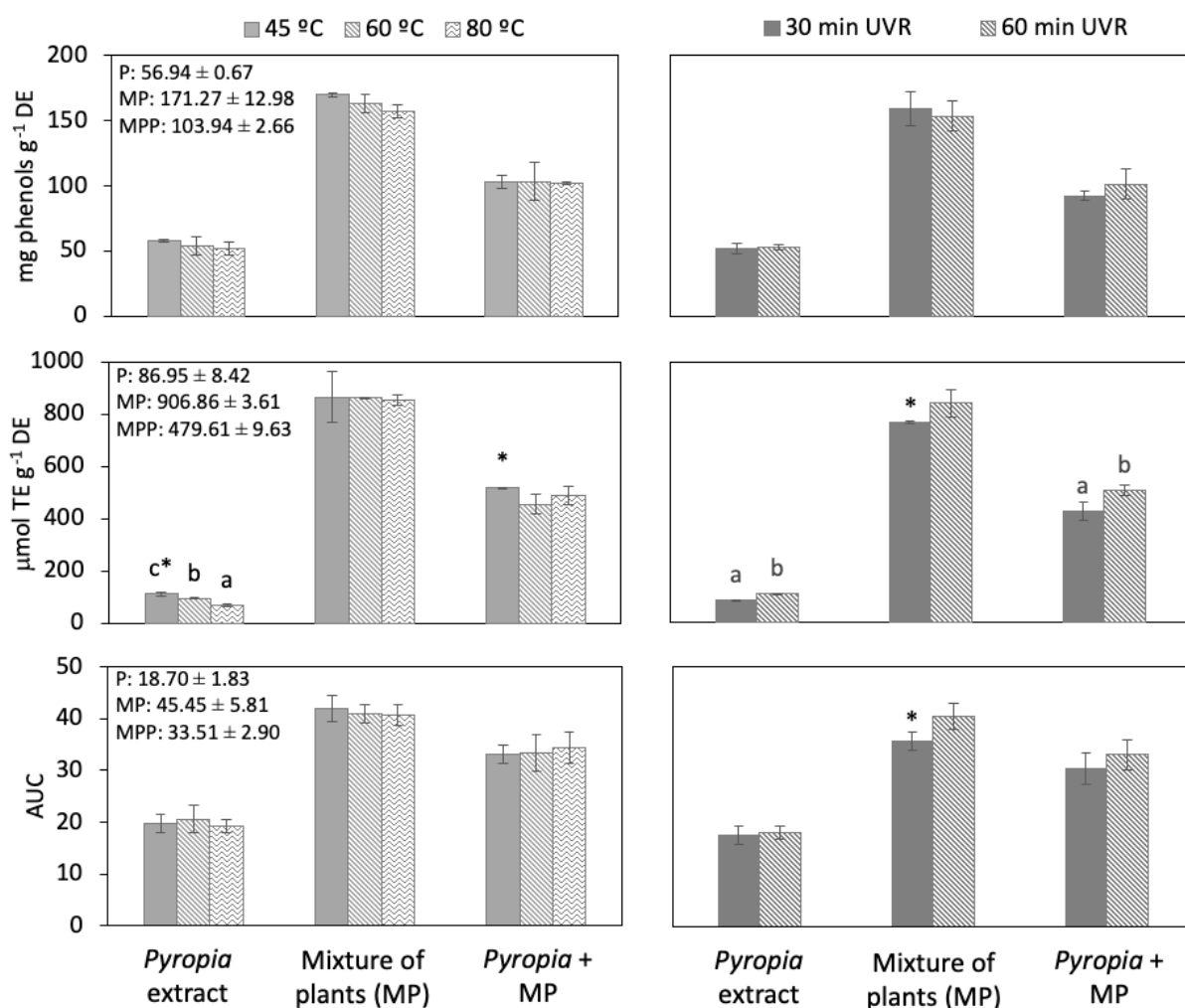


Figure SM.5. 3. Phenolic compounds (mg mL⁻¹), antioxidant capacity measured through ABTS method (μmol TE mL⁻¹) and area under the curve (AUC) of the UV-PAR spectrum (290-500 nm) of the *Pyropia* extracts, mixture of plants (MP: 40 % liquorice, 40 % green coffee and 20 % flame of the forest), and mixture of plants + *Pyropia* (50 % each one) incubated at different temperatures (45, 60 and 80 °C) during 1h, and irradiated under UVR during 30 and 60min. Different letters indicate significant differences among temperatures or irradiation times (ANOVA, SNK, Student-t test, $p < 0.05$). * Asterisks mean significant differences between initial values and 45°C of 30min UVR (Student-t test, $p < 0.05$).

Table SM.5. 5. Biological effective protection factors (BEPFs) related to different biological responses driven by UV: photocarcinogenesis, immunosuppression, elastosis, singlet oxygen and photoaging. Values are expressed as mean $\pm$ standard deviation (SD).

	Photocarcino.	Immunosup.	Elastosis	Sing. oxygen	Photoaging
Avène SPF 20	35.32 $\pm$ 9.15	35.43 $\pm$ 12.48	11.42 $\pm$ 2.38	25.34 $\pm$ 6.33	23.23 $\pm$ 4.14
Avène SPF 30	52.04 $\pm$ 4.44	53.56 $\pm$ 4.94	14.79 $\pm$ 0.67	38.93 $\pm$ 3.33	35.79 $\pm$ 3.02
Avène SPF 50	103.10 $\pm$ 30.98	114.99 $\pm$ 40.14	25.76 $\pm$ 4.13	78.23 $\pm$ 24.31	68.87 $\pm$ 21.76
Biarritz SPF 50	81.79 $\pm$ 21.45	115.94 $\pm$ 32.29	8.71 $\pm$ 0.62	28.02 $\pm$ 6.00	19.13 $\pm$ 3.1
Babaria SPF 50	57.97 $\pm$ 9.15	62.14 $\pm$ 10.44	18.79 $\pm$ 1.91	41.33 $\pm$ 6.41	38.54 $\pm$ 5.93
Aderma SPF 50	125.52 $\pm$ 18.30	140.22 $\pm$ 22.55	29.69 $\pm$ 2.62	91.01 $\pm$ 13.13	83.68 $\pm$ 11.9
Heliocare Ultra SPF 50	71.15 $\pm$ 12.56	89.04 $\pm$ 20.54	14.90 $\pm$ 1.46	46.67 $\pm$ 9.48	36.20 $\pm$ 6.60
Reference ISO 24443:2012 – SPF 16	21.92 $\pm$ 4.81	22.61 $\pm$ 5.09	10.28 $\pm$ 1.11	24.98 $\pm$ 5.77	23.7 $\pm$ 5.29

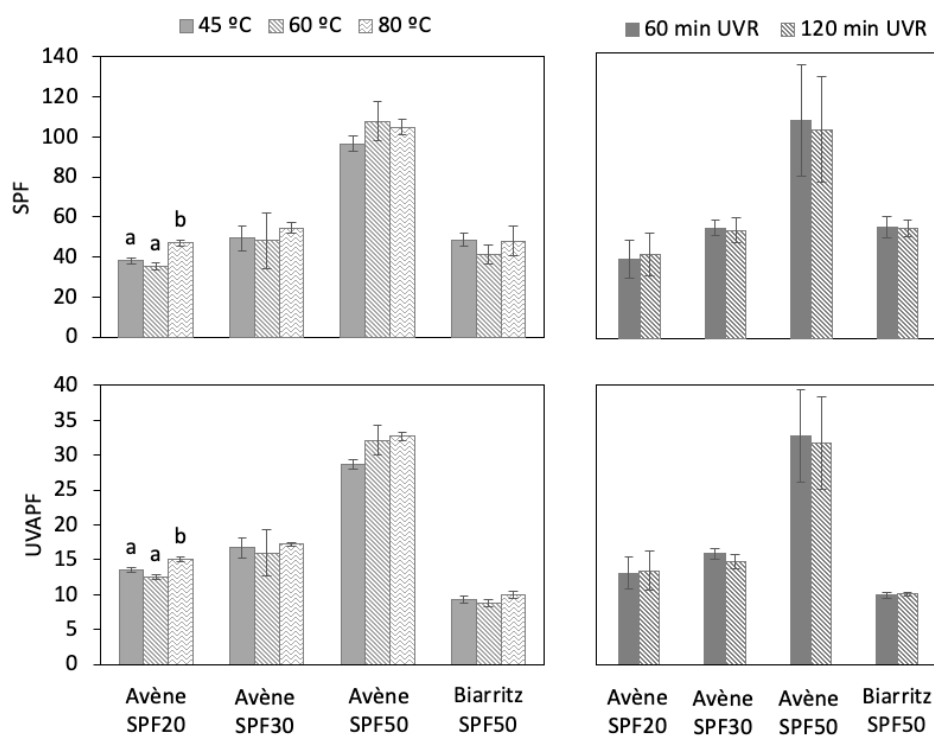


Figure SM.5. 4. SPF and UVAPF values of four commercial sunscreens after incubation at three different temperatures (45, 60 and 80 °C) and expose to UVR during 60 and 120 min. Different letters indicate significant differences among temperatures or irradiation times (ANOVA, SNK, Student-t test, $p < 0.05$).

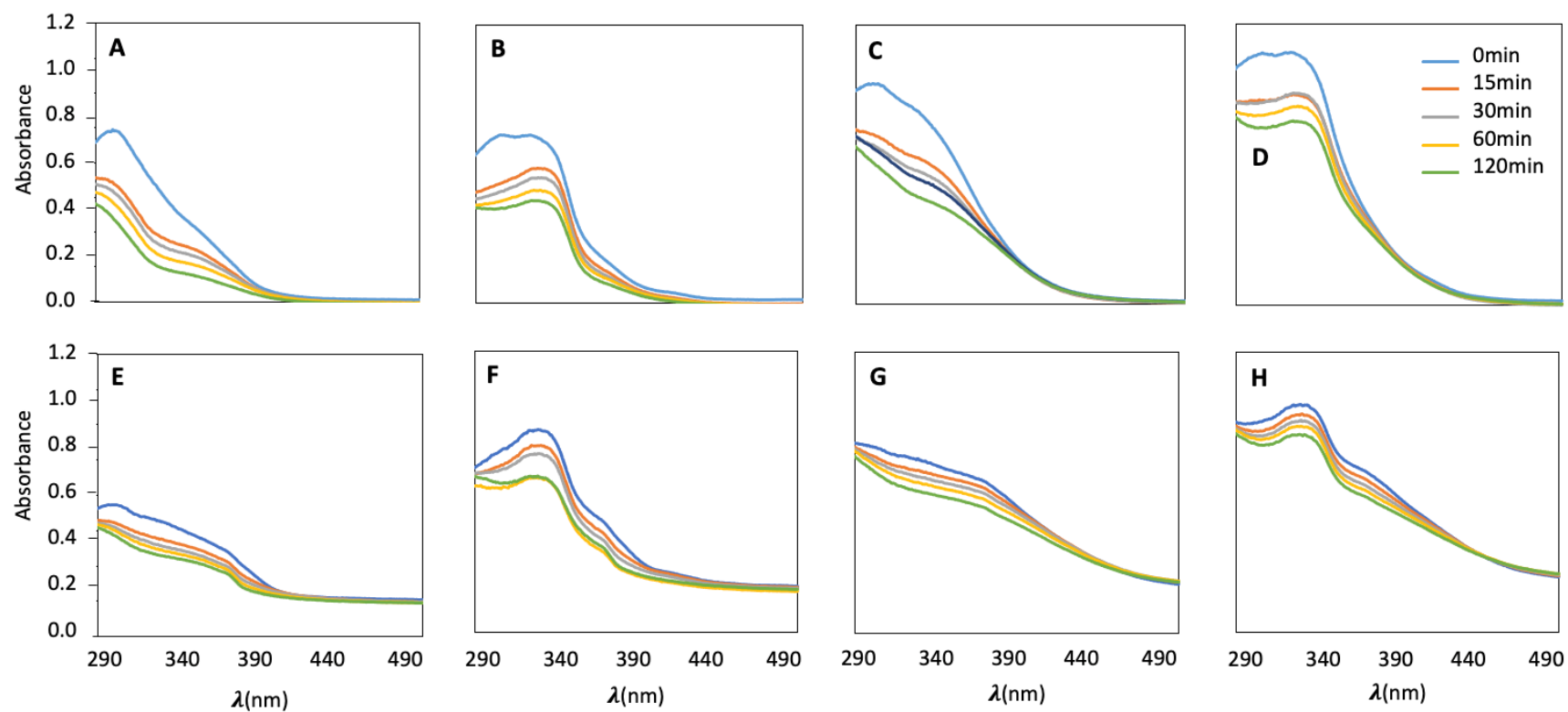


Figure SM.5. 5. Absorbance spectra of different creams submitted to photostability. Creams were irradiated with a UV lamp during 0, 15, 30, 60 and 120min. A) BKC; B) BKC + 10% *Pyropia*, C) BKC + 10% MP; D) BKC + 10% *Pyropia* + 10% MP; E) BKC + PF; F) BKC + PF + 10% *Pyropia*, G) BKC + PF + 10% MP; H) BKC + PF + 10% *Pyropia* + 10% MP. Values are the mean of three replicates.

Table SM.5. 6. Decay percentage during the exposure time (15, 30, 60 and 120 min) to a UV lamp of SPF and UVAPF values of the different creams: BKC, BKC + 10% *Pyropia*, BKC + 10% MP; BKC + 10% *Pyropia* + 10% MP; BKC + PF, BKC + PF + 10% *Pyropia*, BKC + PF + 10% MP, BKC + PF + 10% *Pyropia* + 10% MP. Values were calculated from the average SPF and UVAPF values.

	Min	SPF	UVAPF		Min	SPF	UVAPF
BKC	15	45.68	25.27	BKC + PF	15	19.80	14.50
	30	51.19	30.53		30	25.61	20.55
	60	57.73	37.06		60	28.91	23.94
	120	65.25	44.65		120	33.67	28.28
BKC + 10%P	15	41.46	17.27	BKC + PF + 10%P	15	14.71	12.87
	30	46.96	22.50		30	19.92	20.46
	60	52.90	28.01		60	30.62	24.81
	120	57.65	33.95		120	33.98	32.60
BKC + 10%MP	15	43.52	21.24	BKC + PF + 10%MP	15	12.75	10.47
	30	50.77	27.49		30	18.06	16.68
	60	53.84	30.51		60	23.05	22.71
	120	62.26	39.57		120	30.80	30.72
BKC + 10%P + 10%MP	15	36.63	12.38	BKC + PF + 10%P + 10%MP	15	12.55	-5.21
	30	40.72	15.07		30	18.47	2.01
	60	49.41	21.39		60	20.96	9.27
	120	55.52	26.88		120	25.79	19.71

GENERAL DISCUSSION

The main objective of this thesis was the development of cosmeceutical products based on natural components with UV screen and antioxidant properties. Algae are normally exposed to a very stressful environment, so they should present a high content of protective molecules (e.g. UV screen, antioxidant or anti-herbivory) (Lalegerie *et al.*, 2020a). Some of the most interesting molecules with potential cosmeceutical applications are the secondary metabolites known as mycosporine like-amino acids (MAAs). More than 30 types of MAAs have been identified with different maximum absorptions, being the most abundant palythine (λ_{max} : 320 nm), asterina-330 (λ_{max} : 330 nm), shinorine (λ_{max} : 332 nm) and porphyra-334 (λ_{max} : 334 nm). In the last 10 years, a great number of reviews and book chapters have been published about MAAs, describing their biology, chemistry and/or bioactivities (mainly focusing on skin photoprotection), among others (La Barre *et al.*, 2014; Chrapusta *et al.*, 2017; Lawrence *et al.*, 2017; Navarro *et al.*, 2018; Kageyama and Waditee-Sirisattha, 2019; Geraldes and Pinto, 2021; Vega *et al.*, 2021; Rosic *et al.*, 2023). In the first chapter of the thesis, published data about MAAs were reviewed, focusing on the possible use of these compounds as biological UV-filters in cosmeceutical products and covering the necessary steps to achieve it: screening of species with high MAAs content, MAAs production strategies, extraction, quantification, and purification technologies and described bioactivities. MAAs are very interesting molecules for cosmeceutical applications due to their multifunctional characteristics, i.e. UV-screen, antioxidant, antimicrobial, anti-inflammatory, DNA protection or anti-aging (de la Coba *et al.*, 2009b, 2009a; Hartmann *et al.*, 2015b; Wada *et al.*, 2015; Lawrence *et al.*, 2018). The structure of this thesis follows most of the steps described in the first chapter. In addition to MAAs, biliproteins, polyphenols and scytonemin are also interesting molecules with potential applications in the cosmeceutic sector due to the antioxidant and UV absorbing properties (Amador-Castro *et al.*, 2020; Cotas *et al.*, 2020; Pathak *et al.*, 2020; Chen *et al.*, 2022).

As red algae and cyanobacteria are among the major producers of MAAs, in the second chapter a screening of intertidal red algae from the coast of Málaga and Cádiz and cyanobacteria coming from the Spanish Bank of Algae (BEA: Banco Español de Algas) were done, looking for species with the highest content of UV-screen and antioxidant compounds. Among red algae, *Porphyra umbilicalis* highlighted as has been observed in other studies (Navarro *et al.*, 2018; Schneider *et al.*, 2020b). Species from the Bangiaceae family, where *P. umbilicalis* belongs, live in the upper part of the intertidal zone, exposed to very harsh conditions, such as high solar radiation or periods of desiccation, and normally present the highest MAAs content, reaching concentrations of 10-12 mg g⁻¹ DW (Navarro *et al.*, 2018; Schneider *et al.*, 2020b). Other species with high MAAs content belong to the orders Gracilariales, Ceramiales, Gigartinales or Gelidiales (Vega *et al.*, 2021). The cosmeceutic application of algae requires high biomass availability, being bladed Bangiales and Gracilariales among the most cultivated algal species worldwide (FAO 2022b). In this screening, *Gracilariopsis longissima* did not show very high content of MAAs or phenolic compounds, but other Gracilariales from the field have shown relevant MAAs content (> 1.5 mg g⁻¹ DW) (Briani *et al.*, 2018; Torres *et al.*, 2019a), and their culture can present high biomass productivities and under certain conditions (e.g. high nutrients availability and UV radiation) can increase the accumulation of specific bioactive compounds (e.g. MAAs or biliproteins) (Barceló-Villalobos *et al.*, 2017; Álvarez-Gómez *et al.*, 2019b; Bonomi-Barufi *et al.*, 2020; Figueroa *et al.*, 2022), e.g. *Gracilaria cornea* culture outdoor and with high nitrogen input can reach MAAs values of 5 mg g⁻¹ DW (Figueroa *et al.*, 2022). *Porphyra sensu lato* specimens are well known for their use as human food (commonly known as Nori and use in sushi or soups), and the genus *Gracilaria* are traditionally exploited for phycocolloids extraction and for human consumption (known as Ogonori). The cosmeceutical application of algae will have to compete with their traditional uses, although algae production is increasing over the years, e.g. *Porphyra* spp. production have increased from 430 to 2200 tons per year in the period 2000-2020 (FAO, 2022b), what can allow the diversification of algae applications.

Among cyanobacteria, *Scytonema* sp. highlighted for the presence of a MAA (Myc-glutaminol) with maximal absorption in the UV-B region (λ_{max} : 310nm) and normally not found in red algae. As far as we know this is the first report of myc-glutaminol (identified by HPLC and confirmed by MS) in *Scytonema* sp. In addition to MAAs, cyanobacteria can also present another photoprotective molecule, scytonemin. This molecule is a lipophilic and yellow-brown pigment found in the extracellular sheath of some

cyanobacteria, that mainly absorbs in the UV-A I region (λ_{max} : 384nm) (Garcia-Pichel and Castenholz, 1991; Proteau *et al.*, 1993). Thus, the combination of both molecules, MAAs and scytonemin, can protect against UV-B and UV-A radiation, i.e. MAAs that absorbed in the UV-B region (λ_{max} : 310nm) such as myc-glutaminol; MAAs that absorbed in the UV-A II such as shinorine (λ_{max} : 332nm) or porphyrin-334 (λ_{max} : 334nm); and scytonemin (λ_{max} : 386nm) that protect against the UV-A I and high-visible light. The last molecule would be very interesting against photoaging as it is related to UVA-I and blue light. Scytonemin was detected in all cyanobacteria species spectrophotometrically by using the chromatic equations of García-Pichel and Castenholz (1991). However, its identification through HPLC was not achieved due to methodological problems. Besides the lack of commercial standard of scytonemin add difficulties to the identification. It is interesting to notice that even though scytonemin present great potential applications as UV screen substance (Rastogi *et al.*, 2015; Fuentes-Tristan *et al.*, 2019; Pathak *et al.*, 2020), nowadays there is not too much scientific information about its production conditions, extraction, identification or bioactivities, neither commercial products with this compound. In contrast, a lot of scientific information about bioactivities and applications of MAAs can be found, as well as some products based on red algal extracts enriched in MAAs can be found in the market, e.g. Helionori® (Gelyma company) is an active principle based on *P. umbilicalis* extract enriched in MAAs, that can protect the skin against UV-A rays, by preventing the formation of sunburn cells, and protecting DNA and cell membranes; ALGA MARIS® (Laboratories Biarritz) is a sunscreen that combines physical filters and *Gelidium corneum* extract (Alga-Gorria®), which provide antioxidant capacity and protection against skin aging.

As we did not success with the identification of scytonemin through HPLC, and its lipophilic character difficult the extraction and the compatibility with the natural cosmetic, we decided to look for another potential source of UV-screen and antioxidant compounds in plants. After the analysis of 10 plant species, ivy roots (the aerial ones), liquorice root, olive leaves, green coffee beans and flame of the forest leaves were selected according to both UV screen and antioxidant capacity potential applications. No specific molecules were identified in plants, although according to the bibliography, plants are rich in polyphenols (e.g. flavonoids, phenols, tannins), alkaloids, carotenoids or terpenoids (Dinkova-Kostova, 2008; Pichersky and Raguso, 2018). We worked with hydroethanolic solvents in order to extract both polar and non-polar compounds.

The internal composition of algae can vary with the environmental conditions, so photobiological and stressing treatments can be done during algal culture in order to increase the content of the antioxidant and photoprotective molecules. Among red algae, it is observed that bladed Bangiales and Gracilariales highlight for their content of bioactive compounds and culture capabilities as both are commercially cultivable species as it was indicated above, so in the third chapter, one species of each group was submitted to different experimental conditions: *Gracilaria armata* and *Neopyropia leucosticta*, both collected in the intertidal coasts of Málaga. *G. armata* thalli were exposed to UV radiation in two different treatments (UV-A and UV-A+B), and *N. leucosticta* blades were submitted to UVA-Violet-Blue radiation, different nutrient availabilities, and emersion conditions.

UV radiation (specially UV-B) can provoke important damages in aquatic organisms (e.g. DNA mutations, proteins denaturalization, reduction of growth or photoinhibition, among others), whereas in low quantities can also stimulate the accumulation of antioxidant and photoprotective compounds like MAAs or polyphenols (Bischof *et al.*, 2006; Karsten, 2008; Kataria *et al.*, 2014). UV-A is considered less deleterious, and even can provoke positive effects in algae culture, e.g. in growth or photosynthetic capacity (Altamirano *et al.*, 2000; Gao and Xu, 2008; Ghedifa *et al.*, 2021), probably due to the promotion of secondary photosynthetic pigments as carotenoids.

G. armata acclimated to laboratory conditions (i.e. only PAR) were exposed to artificial UV radiation (UVA340, Q-Lab Corporation) with the objective to increase MAAs and polyphenols content, but no increment was observed. The highest content of photoprotective molecules were observed in nature (2.3 mg g⁻¹ DW). Other Gracilariales showed an increase of MAAs and polyphenols under supplementation with UV radiation in laboratory conditions (Cruces *et al.*, 2018; Álvarez-Gómez *et al.*, 2019b; Torres *et al.*, 2019a; Bonomi-Barufi *et al.*, 2020) and under solar radiation (Barceló-Villalobos *et al.*, 2017; Figueroa *et al.*, 2022). *G. armata* was able to increase the NPQ as a photoprotective mechanism, but it could not increase the accumulation of photoprotective compounds. *G. armata* had been acclimated to only PAR radiation during a couple of months, and the content of MAAs and polyphenols had significantly decreased, probably due to a less stressful conditions. It is necessary to take into account that the net accumulation of MAAs is a balance between synthesis and photodegradation (Álvarez-Gómez *et al.*, 2019b). MAAs content normally increases with nitrogen enrichment but until certain levels, e.g. in *Gracilaria tenuistipitata* the MAAs

content increased until 500 μM of nitrate available, and then started to decrease (Barufi *et al.*, 2011). The UVR dose also affects the MAAs accumulation, e.g. MAAs increased under UV radiation after 7 days of culture, but in extended period as 14 days no differences between PAR and PAR+UVR was found including a depletion of MAA content under both radiation treatments (Álvarez-Gómez *et al.*, 2019b). In our study, the low initial content of photoprotective molecules could make *G. armata* incapable of resisting to the UV radiation, and the energy was used in photorepair mechanisms (e.g. photolyases) instead of in the synthesis of new photoprotective molecules. This species seems to be more vulnerable to UV radiation than other Gracilariales such as *Gracilaria conferta*, *Gracilaria cornea*, *G. vermiculophylla*, *G. longissima* or *G. tenuistipitata*, *G. chilensis* (Figueroa *et al.*, 2010, 2022; Barufi *et al.*, 2012; Cruces *et al.*, 2018; Álvarez-Gómez *et al.*, 2019b; Bonomi-Barufi *et al.*, 2020).

High nitrogen availability clearly increases the internal content of nitrogenous compounds, and its combination with UV radiation have shown an interactive response in some algae species (Korbee-Peinado *et al.*, 2004; Barufi *et al.*, 2012; Beardall *et al.*, 2014; Álvarez-Gómez *et al.*, 2017). The effect of different light colors has been also studied in different red algae species (e.g. *Porphyra leucosticta*, *Gracilaria birdiae*, *Gracilaria gracilis*, *Osmundea pinnatifida* or *Gracilaria cornea*), showing that in most of the cases UV-A and blue increase the accumulation of biliproteins and MAAs (Franklin *et al.*, 2001; Korbee *et al.*, 2005a; Barufi *et al.*, 2015; Schneider *et al.*, 2020a, 2022; Ghedifa *et al.*, 2021). This stimulation can be mediated by photoreceptors, i.e. the cryptochromes increase the activity of the nitrate reductase, increasing the nitrogen uptake and for instance nitrogenous compounds such as biliproteins and MAAs (Aparicio *et al.*, 1985; López-Figueroa and Rüdiger, 1990, 1991; Corzo and Niell, 1992). Phytochrome is normally detected by red/far red, although recently it has been observed that in algae it can be activated by other wavelengths as blue, green and yellow light (Kianianmomeni and Hallmann, 2014; Rockwell *et al.*, 2014).

Thus, in this chapter, we analyzed the combination of different nutrient concentrations and white light supplemented with UV-A and high energy visible radiation (violet and blue), in order to observed possible interactive effects. In general, nitrogen showed clearer effects than light quality, e.g. the content of phenolic compounds and MAAs were only affected by nutrients availability. An interactive response observed in the ETR_{int} suggested that the high N availability can act as a photoprotective mechanism as it has been reported in other algae (Korbee-Peinado *et al.*, 2004; Huovinen *et al.*,

2006; Barufi *et al.*, 2011, 2012). The delay effect of the light quality on biliproteins concentration, that are normally very vulnerable to UV radiation, can suggest a beneficial effect of the violet-blue radiation, stimulating the biliproteins synthesis.

In nature, most Bangiales live in the upper part of the intertidal zone and are subjected to emersion conditions during low tides, i.e. they can spend several hours out of the water. So, another experiment combining the same light quality and emersion/immersion conditions was done. Kim *et al.* (2008) observed that the species exposed to emersion presented a higher N content than those species that are always under water. During emersion, algae decreased the photosynthetic capacity due to the loss of water content (up to 90 % in some species), followed by a fast recovery in those species adapted to suffer emersion like *Porphyra sensu lato* species (Contreras-Porcia *et al.*, 2011; Flores-Molina *et al.*, 2014). In our experiment, a decrease in the photosynthetic capacity was observed during emersion, followed by a total recovery. However, the effect of the light quality was more significant in this experiment than the emersion condition, i.e. no clear changes due to emersion were observed. Probably the emersion was not as pronounced as in the nature, in this experiment the Y(II) values decreased from 0.7 to 0.2 under emersion, whereas other studies have shown Y(II) values close to 0 under emersion in *Porphyra yezoensis* (Gao and Wang, 2012).

It is important to notice that *Porphyra sensu lato* species present a constitutive high level of MAAs, i.e. the content of MAA are always high with a low impact of the environmental conditions (e.g. UV radiation), whereas other species such as *Palmaria decipiens* or *Gracilaria tenuistipitata* present a facultative content of MAAs, that can increase under UV radiation exposure. Hoyer *et al.* (2001) differentiate algae in three groups according to their MAAs content and capacity to synthesize them: (1) species with relatively high MAAs content, that are less affected by radiation conditions, (2) species with a basic MAAs concentration, that can increase depending on the radiation conditions, and (3) species without MAAs. Thus, to observed differences in the MAAs content under certain conditions in *Porphyra sensu lato* species, it is necessary to previously reduce the amount of MAAs (i.e. nitrogen limitation).

It is remarkable that the analyzed treatments can be scaled up. In inland facilities, big lamps with UVA-violet-blue light can be place above tanks or raceways systems during some hours of the day or night. The emersion condition can be also carried out during culture: the commercial culture of Nori is normally subjected to emersion conditions, e.g. in on-shore systems, algae are subjected to the tides. This condition

improves the nitrogen assimilation, and drastically reduces the amounts of epiphytes and pathogens (Sahoo and Yarish, 2005).

Antioxidant capacity is related with different molecules, e.g. biliproteins, carotenoids, polysaccharides, phenolic compounds, terpenoids, proteins, fatty acids, although this thesis have been focused in three of them: biliproteins, phenolic compounds and MAAs. Table D. 1 summarizes the three mentioned bioactive compounds and the antioxidant capacity measured through ABTS method, of the red algae species analyzed in this thesis and other red alga species analyzed in collaboration with other research groups. Among the screening studies, *Porphyra sensu lato* species highlighted. Diverse screening studies of MAAs through the world have been conducted in coastal waters from Europe (Korbee-Peinado, 2003; Álvarez-Gómez *et al.*, 2016; Guihéneuf *et al.*, 2018; Lalegerie *et al.*, 2019; Orfanoudaki *et al.*, 2019b; Schneider *et al.*, 2020b), polar areas (Karsten *et al.*, 1998b), Chile (Huovinen *et al.*, 2004; Jofre *et al.*, 2020) or Brazil (Briani *et al.*, 2018). In all of them, Bangiales presented the highest levels of MAAs. Other species with interesting content of MAAs are: *Bostrychia scorpioides*, a red alga commonly found in saltmarshes, also showed a high MAAs content (10 mg g⁻¹ DW) (Orfanoudaki *et al.*, 2021), although in this species, MAAs probably act as a osmoregulator instead of photoprotector. *Gracilaria cornea* culture outdoor (i.e. exposed to solar radiation) and under high N availability that simulate fishpond effluents reached high values of MAAs (5 mg g⁻¹ DW) and antioxidant capacity (Figueroa *et al.*, 2022). This species is native from Mexico but has been culture during several years in the Spanish bank of algae in the Canary Islands. Ashkenazi *et al.* (2022) also analyze the MAAs and antioxidant capacity of red algae (*Gracilaria conferta* and *Hypnea musciformis*) maintained in IMTA systems, and observed interesting MAAs content. In contrast, *Halopithys incurva* has low MAAs content, but is one of the red algae with highest polyphenols content, closed to some brown algae (Vega *et al.*, 2020a). Other species with potential applications are: *Osmundea pinnatifida*, the genus *Chondracanthus* or *Rissoella verruculosa*. The last species is endemic from the Mediterranean and has been less studied but presented high MAAs and polyphenol contents. Correlations between the bioactive compounds (polyphenols and MAAs) and the antioxidant capacity were done, although only polyphenols showed a positive correlation ($r = 0.7388$; $p < 0.05$). In spite of MAAs did not show a positive correlation, these substances have been associated to antioxidant capacity (de la Coba *et al.*, 2009b; Wada *et al.*, 2015; Torres *et al.*, 2018). As extracts are a pool of molecules, the sum of the bioactive compounds (BACs) were calculated (phenols + MAAs) and showed a positive correlation with the

antioxidant capacity ($r = 6862$; $p < 0.05$). The highest sum of BACs was observed in *G. cornea* culture outdoor and with high N availability ($60.9 \text{ mg g}^{-1}\text{DW}$), followed by *R. verruculosa* ($28 \text{ mg g}^{-1}\text{DW}$) and *Porphyra sensu lato* species (e.g. *N. leucosticta* showed around $20 \text{ mg g}^{-1}\text{DW}$). The ratio polyphenols/MAAs vary among species, all *Porphyra sensu lato* species showed a ratio lower than 2, indicating that they present similar values of both bioactive compounds. In contrast, other red algae (e.g. *G. cornea*, *O. pinnatifida* or *R. verruculosa*) normally present much higher content of polyphenols than MAAs.

Table D. 1. Biliproteins, phenolic compounds and mycosporine-like amino acids (MAAs) content (mg g⁻¹ DW), the sum of the bioactive compounds (BACs: polyphenols + MAAs) and the general antioxidant capacity (AC) measured through the ABTS method (μmol TE g⁻¹ DW) of several red algae species (some of them from this thesis and other from collaboration with others research groups). The algae species present the collection place and date of the algae species, as well as the published word (if proceed).

	Species	Collection place	Collection date	Biliproteins	Polyhenols	MAAs	BACs	AC
	<i>Bangia atropurpurea</i>	La Caleta, Tarifa, Cádiz (Spain) ¹	02 - 2018	-	-	5.57 ± 0.85	-	20.35 ± 1.47
	<i>Ceramium virgatum</i>	La Caleta, Tarifa, Cádiz (Spain) ¹	02 - 2018	-	2.27 ± 0.08	0.22 ± 0.04	2.49	10.07 ± 0.01
	<i>Chondracanthus acicularis</i>	La Araña, Málaga (Spain) ²	02 - 2018	-	6.55 ± 0.40	3.05 ± 0.79	9.60	14.44 ± 0.41
	<i>Gracilaria cornea</i>	CC, Málaga (Spain) ³	02 - 2018	-	5.73 ± 0.53	0.51 ± 0.07	6.24	6.04 ± 0.08
	<i>Ellisolandia elongata</i>	La Caleta, Tarifa, Cádiz (Spain) ¹	02 - 2018	-	2.10 ± 0.06	0.49 ± 0.06	2.59	5.24 ± 0.09
	<i>Ellisolandia elongata</i>	La Araña, Málaga (Spain) ²	02 - 2018	-	1.98 ± 0.04	0.48 ± 0.02	2.46	3.27 ± 0.21
(Schneider et al. 2020b)	<i>Feldmannophycus rayssiae</i>	La Araña, Málaga (Spain) ²	02 - 2018	-	4.74 ± 0.20	4.05 ± 0.35	8.79	8.53 ± 0.31
	<i>Gelidium spinosum</i>	La Araña, Málaga (Spain) ²	02 - 2018	-	3.00 ± 0.07	1.70 ± 0.27	4.70	6.44 ± 0.24
	<i>Gelidium microdon</i>	La Caleta, Tarifa, Cádiz (Spain) ¹	02 - 2018	-	3.83 ± 0.09	2.51 ± 0.20	6.34	6.16 ± 0.45
	<i>Gracilaria multipartita</i>	La Araña, Málaga (Spain) ²	02 - 2018	-	6.98 ± 0.27	1.29 ± 0.27	8.27	11.52 ± 0.65
	<i>Osmundea pinnatifida</i>	La Araña, Málaga (Spain) ²	02 - 2018	-	10.34 ± 0.71	1.53 ± 0.28	11.87	10.67 ± 0.36
	<i>Porphyra umbilicalis</i>	La Caleta, Tarifa, Cádiz (Spain) ¹	02 - 2018	-	18.20 ± 0.32	10.24 ± 1.73	28.44	49.99 ± 8.72
	<i>Pyropia elongata</i>	La Araña, Málaga (Spain) ²	02 - 2018	-	8.68 ± 0.50	3.52 ± 0.93	12.20	11.94 ± 0.98
	<i>Sphaerococcus</i> sp.	La Caleta, Tarifa, Cádiz (Spain) ¹	02 - 2018	-	2.32 ± 0.09	0.00 ± 0.00	2.32	11.06 ± 0.30
	<i>Porphyra umbilicalis</i>	La Caleta, Tarifa, Cádiz (Spain) ¹	02 - 2019	3.61 ± 0.10	9.54 ± 2.11	5.2 ± 0.40	14.74	11.99 ± 0.75
(Vega et al. 2020b)	<i>Gracilariopsis longissima</i>	Puerto Real, Cádiz (Spain) ⁴	03 - 2019	0.52 ± 0.03	2.52 ± 0.81	0.42 ± 0.10	2.94	4.59 ± 0.55
	<i>Gelidium corneum</i>	La Caleta, Tarifa, Cádiz (Spain) ¹	10 - 2019	1.58 ± 0.18	4.71 ± 0.67	1.84 ± 0.23	6.55	6.52 ± 0.54
(Chp. 2)	<i>Osmundea pinnatifida</i>	La Araña, Málaga (Spain) ²	02 - 2019	0.25 ± 0.07	11.99 ± 0.91	1.51 ± 0.20	13.50	10.59 ± 1.18
	<i>Ceramium virgatum</i>	La Araña, Málaga (Spain) ²	02 - 2019	0.99 ± 0.02	5.39 ± 1.32	0.97 ± 0.17	6.36	7.68 ± 0.41

GENERAL DISCUSSION

(Moreira et al. 2022)	<i>Phycocalidia acanthophora</i>	Florianopolis (Brasil) ⁵	08 - 2020	2.31 ± 0.54	5.44 ± 0.74	5.60 ± 3.20	11.04	4.67 ± 0.49
	<i>Porphyra linearis</i>	A Coruña (Spain) ⁶	04 - 2021	13.94 ± 0.32	10.6 ± 3.35	8.00 ± 2.70	18.60	9.44 ± 0.32
	<i>Chondracanthus teedei</i>	Imbutuba (Brasil) ⁷	08 - 2020	0.51 ± 0.04	1.65 ± 0.63	1.70 ± 0.30	3.35	3.93 ± 1.12
	<i>Gracilaria cornea</i>	CC, Gran Canaria ⁸	06 - 2020	8.29 ± 0.42	6.37 ± 1.3	1.80 ± 0.20	8.17	7.30 ± 1.13
	<i>Osmundea pinnatifida</i>	La Araña, Málaga (Spain) ²	05 - 2021	0.06 ± 0.00	7.32 ± 0.76	2.10 ± 0.40	9.42	2.23 ± 0.27
	<i>Plocamium cartilagineum</i>	La Caleta, Tarifa, Cádiz (Spain) ¹	03 - 2021	0.06 ± 0.01	2.86 ± 0.10	2.00 ± 0.30	4.86	6.12 ± 0.14
	<i>Plocamium cartilagineum</i>	La Herradura, Granada (Spain) ⁹	04 - 2021	2.13 ± 0.14	4.90 ± 0.89	3.85 ± 0.50	8.75	8.03 ± 0.99
(Orfanoudaki et al. 2021)	<i>Bostrychia scorpioides</i>	Algeciras, Cadiz (Spain) ¹⁰	07 - 2019	-	-	10.20 ± 0.63	-	-
(Figueroa et al. 2022)	<i>Gracilaria cornea</i>	CC, Gran Canaria ⁸	08 - 2017	2.00 ± 0.76	55.90 ± 26.51	5.00 ± 0.80	60.90	56.59 ± 6.16
(Ashkenazi et al. 2022)	<i>Gracilaria conferta</i>	CC, Haifa ¹¹	06 - 2019	2.98 ± 0.97	-	1.80 ± 0.70	-	9.20 ± 1.00
	<i>Hypnea musciformis</i>			5.39 ± 2.20	-	3.30 ± 0.50	-	17.98 ± 1.24
Chapter 3.1	<i>Gracilaria armata</i>	La Araña, Malaga (Spain) ²	03 - 2019	0.96 ± 0.03	7.32 ± 0.35	2.31 ± 0.20	9.36	26.30 ± 4.27
Chapter 3.2	<i>Neopyropia leucosticta</i>	Lagos, Málaga (Spain) Lab-CC ¹²	02 - 2021	12.44 ± 3.24	10.94 ± 1.75	4.45 ± 1.81	15.39	21.36 ± 2.18
Not published	<i>Chondracanthus acicularis</i>	San Fernando, Cádiz (Spain) ¹³	06 - 2020	-	6.31 ± 0.83	0.85 ± 0.03	7.16	3.49 ± 0.50
	<i>Halophithys incurva</i>	Santander (Spain) ¹⁴	05 - 2022	-	17.47 ± 0.74	< 0.01	17.47	41.81 ± 2.68
Chapter 5	<i>Pyropia columbina</i>	Concepción (Chile) ¹⁵	02 - 2021	5.52 ± 0.08	10.76 ± 0.21	7.05 ± 0.52	17.81	10.62 ± 0.51
	<i>Pyropia columbina</i>	Valparaiso (Chile) ¹⁶	02 - 2021	1.73 ± 0.24	10.38 ± 0.42	7.40 ± 0.14	17.78	15.36 ± 0.83
	<i>Porphyra linearis</i>	La Caleta, Tarifa, Cádiz (Spain) ¹	03 - 2021	8.45 ± 0.33	11.25 ± 0.36	10.01 ± 0.48	21.26	11.77 ± 2.02
Not published	<i>Porphyra umbilicalis</i>	Laxe, A Coruña (Spain) ¹⁷	05 - 2022	5.80 ± 1.67	14.56 ± 1.11	7.58 ± 0.27	22.14	14.75 ± 1.89
	<i>Neopyropia leucosticta</i>	Málaga (Spain) ¹⁸	05 - 2022	9.22 ± 2.03	11.56 ± 1.93	9.90 ± 0.03	21.46	8.63 ± 1.06
	<i>Neopyropia leucosticta</i>	Lagos, Málaga (Spain) ¹²	05 - 2022	5.27 ± 0.56	11.56 ± 1.93	6.70 ± 0.06	18.26	16.82 ± 0.92
	<i>Neopyropia leucosticta</i>	Maro, Málaga (Spain) ¹⁹	05 - 2022	4.05 ± 0.56	10.25 ± 1.54	6.25 ± 0.15	16.50	12.78 ± 1.90

<i>Rissoella verruculosa</i>	Lagos, Málaga (Spain) ¹²	05 - 2022	1.03 ± 0.11	25.11 ± 4.32	3.80 ± 0.03	28.91	56.96 ± 3.05
<i>Rissoella verruculosa</i>	Maro, Málaga (Spain) ¹⁹	05 - 2022	1.00 ± 0.23	22.15 ± 1.04	5.00 ± 0.31	27.15	± 1.87

CC: Culture conditions; **1.** 36°00'41.7''N / 05°35'59.3''W; **2.** 36°42'41.3''N / 04°19'38.2''W; **3.** Experimental Centre Grice-Hutchinson, Universidad de Malaga, Málaga (Spain) 36°40'10.4''N / 4°28'41''W; **4.** 36°30'17.7''N / 06°09'53.1''W; **5.** 27°34'25''S / 48°25'16''W; **6.** 42°24'22''N / 8°41'06''W; **7.** 28°20'17''S / 48°42'12''W; **8.** Spanish bank of algae, Gran Canaria (Spain), 27°59'29''N / 15°22'7.2''W; **9.** 36°44'17''N / 3°45'15''W; **10.** 36°10'25.3''N / 5°26'22.9''W; **11.** Israel Oceanographic and Limnological Research Center, Haifa (Israel), 32°49'34.8''N / 34°57'27.1''E; **12.** 36°44'27''N / 4°0'33''W; **13.** 36°27'28.3''N / 6°13'49.4''W; **14.** 43°23'27''N / 4°17'21.7''W; **15.** 36°35'37''S / 75°58'43''W; **16.** 32°38'55''S / 71°26'30''W; **17.** 43°13'18''N / 9°00'16''W; **18.** 36°40'56.2''N / 4°26'31.3''W; **19.** 36°44'42''N / 3°47'55''W

The obtention of isolated molecules through a fast, cheap, scalable, and environmental-friendly process is a determining step for the commercial application of a compound. Extracts are a pool of molecules, in which our target compound can be in very low quantities (e.g. MAAs are around 1 % of the algal dry biomass) and can present synergistic or antagonists effects among them. The isolation process aims to separate the desirable compound from the others present in the extract. Purified substances can be added to the final product and can present a much higher bioactivity. In chapter 4, it was developed an isolation process using high performance counter-current chromatography (HPLCC) a type of liquid-liquid chromatography, that is cost-effective and industrially scalable (Sutherland *et al.*, 2013). Five different MAAs (shinorine, porphyra-334, palythine, asterina-330 and myc-serinol) were isolated using this technique, although with some difficulties. The liquid-liquid chromatography requires the develop of a biphasic solvent system, i.e. two immiscible liquids, of which one acts as stationary phase and the other as the mobile phase of the “column”. As MAAs are aqueous compounds, it was necessary the develop of an aqueous biphasic solvent system, for that it is required to increase the density of one of the phases, and it was done by adding a saturating solution of ammonium sulphate. Thus, the isolated MAAs presented a high content of salt, and had to be desalted. In addition, MAAs are very small molecules, what difficult the separation from the ammonium sulphate. Liquid-liquid chromatography is a very attractive technology for the industry as it presents some advantages: lack of irreversible absorption, low solvent consumption, possibility to use crude or particulate samples, high load capacity, and practically full recovery of the sample and solvents. It has been used successfully for the separation of apolar or lipophylic compounds, such as carotenoids, terpenoids, fatty acids or flavonoids (Marston and Hostettmann, 2006; Fábryová *et al.*, 2019; Gong *et al.*, 2020; Bárcenas-Pérez *et al.*, 2021; Li *et al.*, 2022). However, this technology required a very deep chemical knowledge about the target compound and lot of time to develop the biphasic solvent system and optimize the procedure. There are other technologies available in the market that could provide promising results at industrial scale and are more affordable, such as the flash chromatography. Thus, further efforts to optimize methodology for purification of MAAs are necessary, since purified MAAs are potential candidates for been considered new natural UV-filters approved by the European Commission (although this a long and complex process, that pharmaceutical companies should conduct). In addition, purified MAAs can be used as standards for chemical

analysis, nowadays there is a great demand to obtain commercial standards (e.g. for MAAs identification and quantification through HPLC).

As *Porphyra sensu lato* species have highlighted among the different performed analysis. In chapter 5, other species (*Pyropia columbina* from Chile and *Pyropia linearis* from Cadiz) were analyzed, and two new analyses were performed (catalase and anti-collagenase activity). Catalase, as one of the most efficient antioxidant enzymes that convert H_2O_2 into H_2O and O_2 , and collagenase, an enzyme that degrade the collagen and contributed to the skin aging. The extracts showed catalase and anti-collagenase activity. Few information has been found about anti-collagenase activity of algae extract, it has been only observed in some red (e.g. *Phycocalidia acanthophora*, *Porphyra linearis*, *Chondracanthus teedei*, *Osmundea pinnatifida* or *Plocamium cartilagineum*) and brown algae (e.g. *Sargassum plagyophyllum* and *Ericaria amentacea*) (Riani Mansauda *et al.*, 2018; Moreira *et al.*, 2022; Mirata *et al.*, 2023). In red algae this activity can be attributed to MAAs, as *Porphyra sensu lato* species showed the highest activity (Moreira *et al.*, 2022) and Hartmann *et al.* (2015) determined the anti-collagenase activity of isolated MAAs.

Although it is though that natural compounds do not present toxicity, most of them can be toxic in certain doses, so it is important to determine the possible toxicity before the development of a product. The three *Porphyra* and the five selected plant extracts showed low toxicity in health cell lines (fibroblasts and keratinocytes). In addition, two plants (liquorice root and olive leaves) also showed potential application against the development of melanoma in human cells.

Finally, cosmeceutic formulations based on natural cosmetics were developed with algae (*P. columbina*) and a mixture of plant extracts (liquorice, green coffee and flame of the forest). *P. columbina* were selected based on biomass availability at the moment of the design of the creams. Based on previous analysis, the addition of only algae extracts to the creams did not show a high photoprotection factor (i.e. $\text{SPF} > 10$) (Vega *et al.*, 2020b; Moreira *et al.*, 2022).

It would have been of great interest the determination of some bioactivities and toxicity of the isolated MAAs in the previous chapter, as well as their inclusion in creams. However, the problems observed during the isolation process (i.e. high amount of ammonium sulfate) diffculted the obtention of enough isolated compounds for the this analysis.

Few authors have studied the inclusion of purified MAAs in lotions. de la Coba *et al.* (2019) reached a SPF value of 8.4 by adding a combination of isolated MAAs: Porphyrin-334 at 4.1 % and Myc-serinol at 2.9 %. The obtained values were similar to the obtained with the combination of two chemical filters in similar percentages (9.5). This limitation is due to the narrow peak of MAAs and that most of them absorbed in the UV-A region without covering the entire erythemal action spectrum used for the SPF or the persistent pigment darkening used for the UVAPF.

The designed creams were formulated by mixing both algae and plant extract in order to try to reach a high SPF and UVAPF (e.g. SPF > 20). In addition, the extracts were added as powder in order to be more concentrated and obtain a higher effect. The base cream, which contains vegetable oils (karanja and calophyl) that can also absorb part of the UV radiation, reached a SPF of 4 and a UVAPF of 2. The combination of both extracts in a 10% each one (total of 20% of the cream) allowed a SPF of 24, and a UVAPF of 6.7. Regarding other biological effective protection factors (BEPFs), this cream showed a high photoprotection against photocarcinogenesis (27.1) and immunosuppression (32.5), both related with UV-B radiation. In the BEPFs related with UV-A, the protection against the singlet oxygen formation showed the highest value (10.8). Physical filters, which are the unique UV-filters allowed in natural cosmetics, were added to the creams with the objective to increase the photoprotection and provide a broad band UV absorption. However, the SPF (based in erythema) did not increase as expected, whereas the UVAPF and other BEPFs related with UV-A (e.g. elastosis, singlet oxygen formation and photoaging) values were doubled with the physical filters. In addition, the λ_c also increased from 370 to 380nm in the creams that contained physical filters. However, it is important to notice that the addition of plants to the creams showed better results than algae, the incorporation of the mixture of plants in a 20% increased the SPF until 32.

A limitation of our products was the photostability. In spite of the extracts showed photostability against the UV (until an erythemal dose of 1935.34 J m⁻²), the SPF and UVAPF of the creams decreased after UV irradiation. The addition of physical filters also provided a better stability to the creams. With this work, we have improved the previous results, but certainly, more research is necessary to obtain cosmetic formulations based in natural products that reach SPF of 30-50, as well as better incorporation of the physical filters in the creams and more proofs that ensure the stability of the creams. In addition, the application of the principle of natural cosmetics difficult the development of stable

formulations. The list of natural ingredients is limited, i.e. there are about 22000 ingredients for cosmetic, of which only around a 10 % are natural, and some natural products not work as well as their synthetic equivalents or are harder to work with. Currently, there is not an EU regulation for natural cosmetic, although, recently, it was developed and ISO that establish the technical definitions and criteria for natural and organic cosmetic ingredients and products (ISO 16128, 2016)

During the last decade, the protection against UV-A (UVAPF) radiation gained importance in the industry and population, as it is the main responsible for the premature photo-aging of the skin, and now most of the sunscreens claim photoprotection in the UV-A region. It is recommended that the UVAPF would be at least 1/3 of the SPF. Most of our designed creams showed a good UVAPF. Recently, it has been demonstrated the effects of high-visible light (violet-blue) on the skin (hyperpigmentation, photoaging and oxidative stress), so cosmetic industries are starting to develop new filters and claim high-energy visible defenses, although as far as we know there is no regulation for this label.

The SPF determined the protection of a sunscreen against erythema, and the UVAPF against the persistent pigment darkening. However, as mentioned above, there are other biological effects of the UV radiation such as elastosis, photoaging, immunosuppression, photocarcinogenesis or cataracts, among others. Thus, the develop of new protection factors, as the suggested by de la Coba *et al.* (2019), is necessary.

According to the European legislation, a sunscreen must contain at least one approved UV-filter. The algae or plants extracts are not considered UV-filters, so they would act as boosters improving the photoprotection and providing other beneficial effects for the skin like antioxidant or anti-aging. In the market, there are more and more sunscreen products that claim other benefits, mainly antioxidant, due the presence of natural products. One of the most known case is the extract of the tropical fern *Phlebodium aureum* (previously named as *Polypodium leucotomos*). The company Cantabria Labs has several patents of this fern extract, named as Fernblock®. It is used as booster in their sunscreen products, enhancing the photoprotection due to the ability to inhibit the generation and release of ROS, protect the DNA and modulate immune and inflammatory responses, among others (Gonzalez *et al.*, 2011; Parrado *et al.*, 2016, 2020; Aguilera *et al.*, 2021). Natural products can be also used as oral supplements, enhancing the photoprotection. The oral administration of Fernblock® has shown

different molecular and cellular effects that are driven into long-term inhibition of photoaging and carcinogenesis (Gonzalez *et al.*, 2011; Parrado *et al.*, 2018), called as “Oral photoprotection”.

The creams designed in this study based in algal and plant extracts follow the concept of blue cosmeceutic (Figure D. 1): (1) the use of extracts from marine algae, that present bioactive compounds with beneficial properties for the skin health; (2) the sustainable production of these organism (e.g. IMTA systems), (3) their no toxicity, and (4) biodegradable character, following the concept of blue and circular economy. Plants are also a great source of new natural photoprotectors, although the production of algae present some advantages in comparison with terrestrial plants, such as: they do not require fertile soil, they can use different types of water sources (e.g. wastewaters or saltwater), the water can be recycled (e.g. by using re-circulating systems) and they can present high productivities. Furthermore, the extracts studied in this thesis could also be used as oral photoprotection (i.e. supplements that enhance the protection against UV radiation thanks to the antioxidant or anti-aging properties).

Thus, with this approach we are providing an alternative for the traditional UV-filters that are found in most commercial sunscreens and can provoke negative effects in our health and aquatic ecosystems. The combination of traditional UV-filters with natural products that can enhance the photoprotection, and in addition provide other beneficial effects for the skin such as antioxidant, anti-aging or anti-carcenogenic, is a way to reduce the used of traditional UV-filters (i.e. percentage use in sunscreens) and satisfy the necessity of natural products that are environmental-friendly and safer for human health.

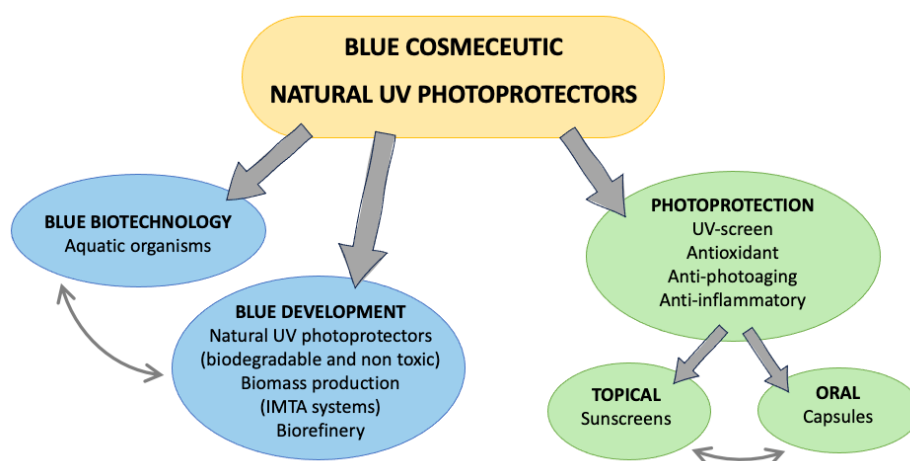


Figure D. 1. The Blue cosmeceutic involves Blue biotechnology and Blue development. Natural compounds with UV-screen, antioxidant, anti-photoaging and anti-inflammatory properties can be use in both topical and oral photoprotectors.

In the last years, algae have gained interest in the economic and political sector of Europe. The European Commission in a recent communication establishes the importance of algae to achieve several objectives of the European Green Deal (e.g. decarbonization, zero pollution, circularity, sustainable food system or preservation and restoration of biodiversity and ecosystems). Algae not only can provide sustainable food and feed, but also pharmaceutical, nutraceutical or cosmeceutical products, plant biostimulants or bio-based packaging, among others. Despite the recognize potential of algae biomass, there is still significant knowledge gaps in dimension, capability and structure of the algae production in Europe (Araújo *et al.*, 2021; EC COM 592, 2022). Araújo *et al.* (2021) studied the current status of algae production industry in Europe, and observed a growing trend in seaweed aquaculture, demonstrated by the high number of companies producing algae, being Spain, France and Ireland the countries with the largest number of macroalgae companies (Araújo *et al.*, 2021; Vazquez Calderon and Sánchez López, 2022). In 2019, the algae production was 270.000 tons, whereas it is expected to increase to 8 million tons in 2030, what will reach 3-9 billion euros across all segments (being food, feed or biostimulants the largest). This increase can generate 85.000 new jobs with a high increased of the number of companies. In addition, it is important to mentioned that the economical value of the algal products are very diverse depending on the applications, with a much higher value of cosmeceutical or pharmaceutical products compared to the phycocolloid sector (EC SWD 361, 2022).

The European Commission also encourage Member States to introduce algae cultivation into

National/regional plans. At regional levels in Spain, Canary Islands already have a strategy for the development of Blue Economy (2021-2030)(BOC 152, 2021), and Andalucía is developing the strategy within an open consulting process with experts in different fields. In both strategies, the algae sector presents an important role.

FUTURE PERSPECTIVES

This thesis has increased the knowledge about the search, production, and application of new natural ingredients in the cosmeceutic sector, mainly focused in mycosporine-like amino acids (MAAs) and opens new perspectives and challenges:

- Improvement of the algal biomass productivity by scaling up the cultures in outdoor facilities. The use of IMTA systems would reduce the cost and the environmental impact of the cultured and the use of photobiological treatments (LED radiation sources) can increase the productivity of the industrial relevant biocompounds (i.e. UV-screen compounds).
- Development of more efficient extraction methodologies by using advanced techniques such as high pressure, ultrasonication, or alkaline/acid or enzymatic hydrolysis. This would be always developed using green solvents without toxicity and compatible with natural cosmetic.
- Continuity of the research in natural UV-filters that absorbed in the UV-A I and high energy visible (HEV; violet-blue from 400 to 500nm): Scytonemin and carotenoids.
- Development of encapsulation protocols of extracts or isolated MAAs that protect the bioactive compounds (i.e. avoiding the premature degradation), but without hiding its effects or allowing the compounds release in the required moment (i.e. under UV radiation exposure).
- Determination of bioactivities of isolated MAAs, and development of further analysis such as: inhibition of elastase and hyaluronidase, antioxidant enzymes (SOD, GR, GPX, APX, and POX), inhibition of ROS or DNA protection.
- Development of Blue Cosmeceutic projects cooperating with companies.
- Application of a biorrefinery approach, in which after the extraction of MAAs and other bioactive compounds for the cosmeceutic application, the waste biomass could be used for other application (e.g. extraction of polysaccharides or use as biofertilizer).
- Search for others MAAs applications like the photoprotection in plants or materials, for example:
 - 1) The foliar application of natural UV filters (e.g. MAAs) on leaves could be an ecofriendly strategy to reduce the photoinhibition of valuable plants in agriculture, reducing the loss of photosynthetic capacity and productivity.

This proposal is based on the photoprotection against chloroplast photoinhibition. The effective radiation of chloroplast photoinhibition is the same as the maximum absorption of some MAAs like Porphyra-334 or shinorine (Figure D. 2).

- 2) Many materials that are designed for outdoor application contain UV-filters in their surface to avoid degradation due to solar radiation. This UV-filters could be replaced for new natural alternatives that are not dangerous to the environment.

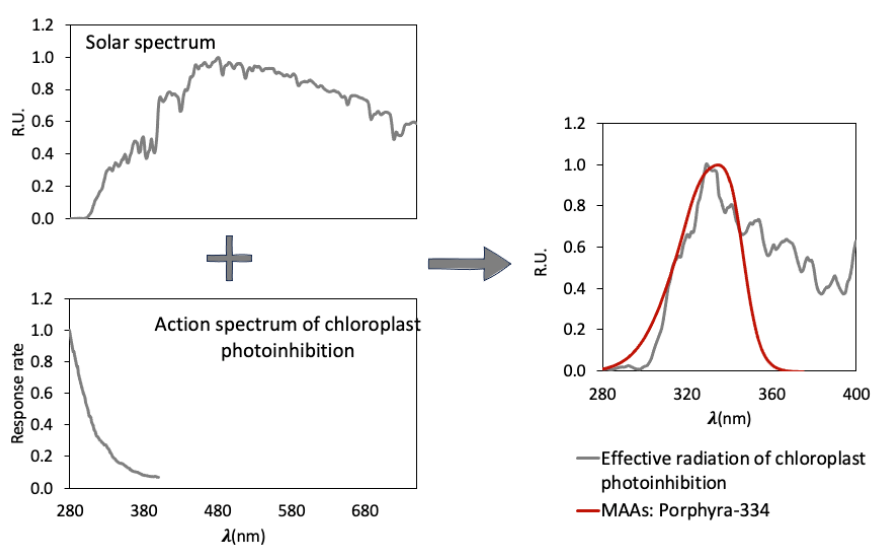


Figure D. 2. Similarities between the effective solar radiation of chloroplast photoinhibition (calculated by multiply the solar spectrum and the action spectrum of chloroplast photoinhibition) and the MAAs spectrum (e.g. Porphyra-334).

GENERAL CONCLUSIONS

1. The intertidal red alga *Porphyra umbilicalis* highlighted in the screening study due to its highest phenols and MAAs content and antioxidant capacity.
2. Among cyanobacteria, only *Scytonema* sp. and *Lyngbya* sp. presented MAAs in their internal composition.
3. The highest antioxidant and photoprotective properties in plants were found in ivy roots, olive leaves, liquorice root, green coffee beans and flame of the forest leaves.
4. *Gracilaria armata*, acclimated to laboratory culture conditions (without UV radiation), was not able to increase their photoprotective compounds (MAAs and phenols) after exposure to UV radiation, although it increased the NPQ as a way to dissipate the excess of energy. The highest MAAs and phenols content in this species were observed in nature.
5. *Neopyropia leucosticta* showed an interactive response between nutrients availability and light quality in the ETR_{int}, suggesting a photoprotective role of the high nitrogen availability.
6. *N. leucosticta* increased the content of N-compounds (MAAs and biliproteins) under high nitrogen availability. Biliproteins decreased under P_{UVA-Vi-BI+} treatment at day 7.
7. *N. leucosticta* increased the phenols content in the treatments P_{UVA-Vi-BI+} at day 2, whereas the phenols increased in the treatment with low nitrogen at day 7.
8. *N. leucosticta* increased the S/V ratio of the cells under N limitation favoring the nitrogen assimilation, whereas under P_{UVA-Vi-BI+} the S/V ratio decrease avoiding that the damage rays reach the inner of the cells.
9. *N. leucosticta* under emersion conditions showed a decreased of the Y(II) followed by a recovery during re-hydration, what is translate in a reduction of the ETR_{int} in this treatment.
10. *N. leucosticta* under emersion conditions increased the protoplasmatic size as a way to difficult water evaporation.
11. *N. leucosticta* did not showed a clear effect of the emersion condition on the phenols and MAAs content, and antioxidant capacity.

12. Five different MAAs were isolated through HPCCC, although the desalting process reduces the yield of the procedure.
13. Aqueous extracts of different *Porphyra sensu lato* species showed catalase and anti-collagenase activity.
14. Aqueous extracts of *Pyropia* and hydroethanolic extracts of different plants (ivy root, liquorice root, olive leaves, green coffee beans and flame of the forest leaves), showed low toxicity, and two of the plants (liquorice and olive leaves) showed potential results against melanoma cancer cell strain.
15. The inclusion of aqueous extracts of *Pyropia* and hydroethanolic extracts of a combination of plants (liquorice, green coffee and flame of the forest) in a cosmetic formulation increase the SPF, UVAPF and other biological effects related with UV-B and UV-A. The addition of physical filters only increased the photoprotection in the UV-A region.
16. Extracts showed thermo- and photostability, although the designed creams loss photoprotection after UV irradiance.

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ANNEXES

PUBLICATIONS DURING THE DEVELOPMENT OF THIS THESIS (the articles that support the thesis are marked in bold).

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