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MECANISMOS DE FOTOPROTECCIÓN EN ALGAS ROJAS

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Las algas proliferan en aguas someras o en la zona intermareal donde la irradiancia es variable. Por lo tanto, estos organismos presentan mecanismos de fotoprotección para evitar daños por el exceso de luz. Las rodófitas es uno de los grupos de organismos fototróficos más importantes que habita la zona intermareal. Sin embargo, se conoce poco sobre los mecanismos de fotoprotección y sobre la importancia de los pigmentos carotenoides en la la regulación de la actividad fotosintética en este grupo. Por lo tanto, primeramente se caracterizó el contenido de carotenoides en diferentes especies de algas rojas. Se encontró que existen diferentes perfiles de carotenoides y que el contenido de carotenoides tiene una relacion con el nivel taxonómico de las especies (un mismo perfil a nivel de orden). El perfil pigmentario estuvo relacionado principalmente con el contenido de luteína, zeaxantina y anteraxantina. Estos carotenoides, tienen una función fotoprotectora en plantas superiores y en varias divisiones algales. Específicamente, la zeaxantina es fundamental en el proceso de la disipación térmica del exceso de energía, por lo que se esperaban diferencias en la sensibilidad a alta luz entre especies con zeaxantina en comparación con especies con otros perfiles de carotenoides. Sin embargo, no se encontraron diferencias entre especies pero si se observó una diferencia entre la cinética de la disminución y recuperación de la eficiencia fotosintética. Esto sugiere la expresión de diferentes mecanismos fotoprotectores relacionados con el contenido de carotenoides. La importancia de los carotenoides en la fotoprotección en algas rojas se observó al analizar la regulación de la actividad del PSII entre especies con luteína o zeaxantina como carotenoide principal y entre tejidos fotosintéticos con diferentes aclimataciones. La conclusión principal del presente trabajo es que las algas rojas presentan diferentes estrategias para responder a cambios en la irradiancia. Hay mecanismos fotoprotectores en los cuales los carotenoides son fundamentales, como en las especies que presentan zeaxantina como carotenoide principal. La fotoprotección en estas especies parece estar relacionada con la disipación de energía en exceso y una regulación del recambio de la proteína D1 del PSII. En el caso de las especies con luteína como carotenoide principal, la regulación del recambio de D1 y probablemente la disipación de energía por centros de reacción inactivos y/o dañados, parecen ser más importantes. Estas diferencias en las estrategías fotoprotectoras representan ciertas ventajas ecológicas para las especies. Entre otros factores, el perfil pigmentario y las respuestas que regulan la eficiencia fotosintética podrían ser determinantes para la distribución de las diferentes especies con la profundidad. Asimismo, la composición de carotenoides en algas rojas indica la aparición o supresión de la síntesis de ciertos carotenoides y vías completas de síntesis en los diferentes grupos algales.

Palabras clave: Algas rojas, Carotenoides, Fotoprotección.

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PHOTOPROTECTIVE MECHANISMS IN RED ALGAE

Algae inhabit shallow waters or the intertidal zone where light is highly variable. Hence, they have evolved multiple photoprotective mechanisms to cope with the potentially damaging effects of light. The rhodophytes are one of the most important photosynthetic organisms that inhabit the intertidal zone. However, how does red algae get protection against high light conditions is still an open question. Specifically, the role of carotenoids in the photosynthetic regulation in rhodophytes is not known. Firstly, the carotenoid content of different red algal species was characterized and different carotenoid profiles were found. The taxonomic related differences (same profile at the order level) base principally in the content of certain carotenoids, such as lutein, zeaxanthin, and antheraxanthin that have a photoprotective function in higher plants and several algal divisions. Specifically, zeaxanthin has been reported to have a key function in the thermal dissipation of excess energy. Hence, differences in sensitivity to high light stress between species presenting zeaxanthin as the main carotenoid and species of the other two carotenoid profile groups were expected. However, any differences in susceptibility to high light exposure have been found. Instead, the carotenoid profile of the species showed a relationship with the kinetics of decrease and recovery of the photosynthetic efficiency. This suggests the expression of different mechanisms involved in the down-regulation of photosynthesis related to the carotenoid content. The differences in PSII regulation during light exposure among species with lutein or zeaxanthin as the main carotenoids and between photosynthetic tissues that showed different acclimation characteristics confirmed that carotenoids play an important role in photoprotection in red algae. In conclusion, different strategies to respond to irradiance changes are present in red algae. There are photoprotective mechanisms, in which carotenoids are involved as seen in species with zeaxanthin as the principal carotenoid. Photoprotection in these species seems to be related to excess energy dissipation and a regulation of D1 turnover depending on light exposure. In the case of species with lutein as the main carotenoid, regulation of D1 turnover and probably the involvement of inactivated and/or damages reaction centers in energy dissipation seem to be more important. Ecologically, these differences in photoprotective strategies imply certain advantages for the species. Besides other factors, the carotenoid profile and associated responses that regulate photosynthetic efficiency could be a determining aspect for the presence of different species in certain light environment. Moreover, the carotenoid composition of red algae has evolutionary implications suggesting the appearance or suppression of the synthesis of certain carotenoids and carotenoid biosynthesis pathways in the different algal groups.

Key words: Carotenoids, Photoprotection, Red algae.

SINOPSIS

Las algas rojas representan uno de los linajes algales principales compartiendo un origen común con los linajes de las Glaucophyta y Chlorophyta (Fig. 1; Stiller y Hall 1997, Bhattacharya y Medlin 1995, Delwiche 1999, Moreira et al. 2000, McFadden 2001, Palmer 2003). Además de las rodófitas modernas, el linaje de las algas rojas incluye a las Cryptophyta, Heterokontophyta, Dinophyta y Haptophyta. Esos últimos cuatro fila provienen de un evento endosimbiotico secundario en el cual un ancestro tipo rodófita estuvo involucrado (Fig. 1).

Las rodófitas son un filum eucariótico caracterizado principalmente por la ausencia de flagelas y la presencia de pigmentos accesorios como ficoeritrina, ficocianina y aloficocianina (Woelkerling 1990). Esos pigmentos son solubles en agua y están localizados en la superficie de los tilacoides en complejos proteicos llamadas ficobilisomas (Fig. 2A; Gantt 1979, Toole y Allnutt 2003). Asimismo, las algas rojas presentan únicamente la clorofila *a* mientras clorofila *b* y *c* son ausentes.

Las Rhodophyta son unas de las algas eucarióticas más primitivas. Su aparato fotosintético representa un estado intermedio entre el encontrado en cianobacteria y el presente en las eucariotas fotosintéticas (ver Fig. 4). Las cianobacterias y rodófitas contienen ficobilisomas como antena del fotosistema II (PSII) en lugar de las antenas transmembránicas con clorofila *a*/*b* (o clorofila *a*/*c*) que están presentes en plantas superiores y cromofitas (Green y Durnford 1996, Bassi et al. 1993, Durnford et al. 1999). Sin embargo, un complejo antena transmembránica (LHC) fue identificada en *Porphyridium cruentum* (Wolfe et al. 1994) y

Galdieria sulphuraria (Marquardt y Rhiel 1997). Ese complejo el cual esta asociado funcionalmente con el fotosistema I y no está presente en las cianobacterias.

La medición de la fluorescencia de la clorofila *a* es un método muy común en investigaciones relacionadas con la fotosíntesis, debido a que es no-destructivo, altamente sensible, rápido y fácil de aplicar y provee información importante sobre el aparato fotosintético. El método de la medición de la fluorescencia se basa en que la energía de la luz absorbida por moléculas de la clorofila *a* en el cloroplasto puede tomar tres vías: puede ser usada para la fotosíntesis, pueden ser disipada como calor o re-emitida como fluorescencia (Fig. 5). Esos tres procesos son competitivos por lo que el incremento en una de las vías resultaría en una disminución de las otras dos. Por lo tanto, mediante la medición del cambio de la fluorescencia de la clorofila se puede inferir sobre cambios en la eficiencia de la fotosíntesis y disipación térmica. El parámetro de fluorescencia F_v/F_m $[(F_m-F_o)/F_m]$ provee una estimación de la eficiencia quántica máxima del PSII. Los valores de ese parámetro pueden cambiar debido a cambios en la fotoquímica y disipación de energía (quenching no-fotoquímico- NPQ). Así que, una disminución en F_v/F_m puede ser causada por una disminución de la fracción de centros del PSII capaces de hacer fotosíntesis y/o por mecanismos de regulación (aumento de NPQ). La disminución de F_v/F_m está relacionada frecuentemente con la fotoinhibición. Sin embargo, este parámetro se ve afectado aismismo por procesos de los cuales algunos son rápidamente reversibles que no indican fotodaño y otros lentamente reversibles que pueden ser relacionados con fotodaño. Por lo tanto, en el presente trabajo, se usa el termino fotoinhibición como la disminución sostenida de la eficiencia del PSII de acuerdo a Lichtenthaler y Babani (2004).

La susceptibilidad de un organismo a la fotoinhibición depende de su capacidad de reparar los centros de reacción dañados durante condiciones de estrés por luz (Greer et al. 1986, Tyystjärvi et al. 1991). Asimismo, el tamaño de la antena y la capacidad para expresar mecanismos fotoprotectores como la disipación no-radiativa de energía de excitación, afecta la tasa de fotoinhibición del PSII (ver Krause 1988). Por tanto, la habilidad para fotoprotegerse durante exposiciones a altas irradiancias, así como la capacidad de las especies para la regulación de la fotosíntesis a diferentes condiciones de luz pueden determinar el límite superior de la distribución vertical de las macroalgas (Hanelt 1996, 1998).

Los organismos fotosintéticos han desarrollados varios mecanismos fotoprotectores para evitar los efectos dañinos de la luz (Fig. 8; resumido en Anderson et al. 1992, Osmond 1994, Niyogi 1999). Entre esos mecanismos, la disipación térmica del exceso de energía juega un papel importante (resumido en Horton et al. 1996). Este mecanismo está relacionado con carotenoides capaces de disipar la excitación de la Chl *a* como calor, los cuales son sintetizados por medio de un ciclo de xantofilas (XC) que está presente en plantas superiores y varios grupos algales (Demmig-Adams 1990, Franklin et al. 1992, Uhrmacher et al. 1995, Niyogi et al. 1997, Rodrigues et al. 2002, Gevaert et al. 2003). El XC es la interconversion rápida de violaxantina en zeaxantina vía anteraxantina bajo condiciones de estrés por luz y la reacción inversa en oscuridad o condiciones lumínicas no estresantes (Yamamoto et al. 1962). Aun no se ha demostrado un mecanismo análogo al XC en algas rojas. Sin embargo, en algunas especies de las rodófitas se han identificado los pigmentos relacionados con el XC (Brown y McLachlan 1982, Liang 1984) y la capacidad de interconversion de estos carotenoides (Liang 1984, Rmiki et al. 1996, Ursi et al. 2003).

Las algas rojas son cosmopolitas y la mayoría (98%) son marinas y crecen generalmente sobre un sustrato rocoso. En aguas templadas como en la costa de California, la mayoría de las especies vive en la zona del intermareal. En esta zona, la luz es altamente variable, por lo cual las algas están expuestas a cambios de irradiancia en escalas de segundos hasta horas. Por lo tanto, las algas en el intermareal tienen que tener la capacidad de colectar luz eficientemente y asimismo resistir condiciones de luz saturante.

Los reportes sobre la composición de carotenoides en algas rojas son muy diversos y muchas veces contradictorios y aparentemente no existe ninguna relación con la taxonomia (ver Anexo I), lo que hace difícil inferir sobre la importancia de estos pigmentos en la aclimatación y fotoprotección en este grupo algal. Como se protegen las rodófitas contra condiciones de alta luz y en específico que papel juegan los carotenoides en la regulación fotosintética no es conocido. Por lo tanto, el objetivo de este trabajo es la identificación de las estrategias fotoprotectoras en las rodófitas, especialmente las relacionadas con carotenoides.

En la primera parte de este trabajo (Capítulo I) se investigó la composición de carotenoides en diferentes especies de algas rojas y si existe una relación del perfil pigmentario entre especies de un mismo nivel taxonómico. Se analizó la composición pigmentaria de 65 especies de rodófitas pertenecientes a 12 órdenes y 18 familias. Los resultados mostraron que las algas rojas no presentan un único perfil de carotenoides. La diferencia principal está relacionada con la presencia o ausencia de carotenoides menores pero principalmente con la xantofila que representa el carotenoide dominante. El perfil más simple está presente en las Porphyridiales con zeaxantina y β-caroteno como únicos carotenoides. La mayoría de las

rodófitas tienen luteína como carotenoide mayor (grupo Lut) debido a que expresan preferencialmente la ruta de síntesis del α-caroteno en comparación a la ruta del β-caroteno. Esa última ruta de síntesis parece estar ausente o se expresa solo al nivel de β-caroteno (sin síntesis de zeaxantina) en algunos órdenes de las rodófitas. En contraste, en algunas especies la ruta de síntesis del β-caroteno está altamente expresada, por lo cual acumulan los pigmentos involucrados en el XC: violaxantina, anteraxantina y zeaxantina (grupo XC).

La agrupación de las rodófitas de acuerdo a su perfil pigmentario no coincide con su relación filogenética. Por ejemplo, especies del grupo XC están filogenéticamente más relacionadas con especies del grupo Lut que con especies del mismo grupo pigmentario. Las Ceramiales y Corallinales representan otro ejemplo de la inconsistencia entre el contenido de los pigmentos y la filogenia propuesta de las algas rojas. En las Ceramiales, el perfil pigmentario es consistente hasta el nivel de la familia mientras que las Corallinales presentan un perfil pigmentario común al nivel de la subfamilia.

La falta de una relación entre la filogenia y el perfil de carotenoides puede ser explicada por la hipótesis de que los pigmentos del XC y la ruta de síntesis del β-caroteno y α-caroteno estuvieron presentes en etapas tempranas de la evolución de las algas rojas, antes de la diversificación de los diferentes grupos de rodófitas. Por lo tanto se hipotetiza que todas las especies de algas rojas evolucionaron de un ancestro común, el cual presentó ambas rutas metabólicas y la capacidad de sintetizar pigmentos del XC. Después de la diversificación de las rodófitas, algunos grupos reprimieron o perdieron evolutivamente la capacidad de sintetizar ciertos carotenoides (ver Fig. 14). Especialmente la ruta de síntesis del α-caroteno

se perdió (o se reprimió) por lo menos dos veces en las Rhodophyta, en las Gracilariales y las Porphyridiales. Asimismo, la síntesis de pigmentos relacionados con el XC se perdió (o se reprimió) varias veces. La represión o perdida de la capacidad de sintetizar ciertos carotenoides como los relacionados con el XC indican que las algas rojas se protegen independientemente de la expresión del XC. Las rodófitas probablemente presentan otras respuestas fotoprotectoras las cuales les permiten colonizar ambientes como la zona del intermareal donde el estrés por irradiancias elevadas es alto.

Dado que carotenoides tienen papeles específicos en la fotoprotección, diferentes perfiles de carotenoides en especies de rodófitas podrían estar relacionados con su sensibilidad a altas irradiancias. Específicamente, ya que la zeaxantina (Zea) es un carotenoide clave en la fotoprotección, el alto contenido de Zea en ciertas especies de algas rojas sugiere una sensibilidad menor ante el estrés lumínico comparado con especies con otros perfiles pigmentarios. El análisis de la respuesta a exposición a alta irradiancia en 11 especies de rodófitas que presentaron tres diferentes perfiles de carotenoides (grupo Lut- luteína como carotenoide principal, grupo Zea- zeaxantina como carotenoide dominante y grupo Ant-anteraxantina como carotenoide principal) no mostró una relación entre el grado de la disminución de la eficiencia fotosintética, medida como F_v/F_m, a altas irradiancias y su recuperación y el perfil de carotenoides de las especies (Capítulo II). Eso indica que no toda la zeaxantina, presente en especies del grupo Zea, está involucrada en la disipación térmica o que la luteína (Lut) y anteraxantina (Ant) proporcionan el mismo nivel de fotoprotección como la zeaxantina en sus respectivos grupos pigmentarios.

Además, en contraste con reportes previos no se encontró una interconversión relacionada con el XC durante condiciones de estrés por luz en las rodófitas. Al parecer, la

fotoprotección relacionada con carotenoides en las algas rojas no depende de una interconversión rápida de estos pigmentos. Asimismo, las especies de las rodófitas del grupo Zea y del grupo Ant que contienen los pigmentos involucrados en el XC, no mostraron diferencias en sensibilidad a alta luz comparadas con especies del grupo Lut. Sin embargo, la reducción de la concentración de Zea y/o Ant con la profundidad en especies del grupo Lut y del grupo Ant, respectivamente, indica que la regulación de la cantidad de carotenoides capaces de disipar el exceso de energía parece ser una respuesta fotoprotectora importante.

En contraste con lo anterior, las cinéticas de la disminución y recuperación de F_v/F_m mostraron diferencias relacionadas con el perfil pigmentario de las especies. La cinética del cambio de la eficiencia fotosintética representa una combinación de diferentes mecanismos como son la regulación y recuperación rápida de F_v/F_m y eldaño al PSII. Esto último está relaciondo con la degradación de la proteína D1 del PSII. La reparación del PSII es un proceso relativamente lento debido a que requiere de la síntesis *de novo* de la proteína D1, por lo cual está relacionada con la fase lenta de la disminución y recuperación de la eficiencia fotosintética. Por lo tanto, las diferencias en las cinéticas de la reducción y recuperación de F_v/F_m entre los grupos pigmentarios indican diferencias en los mecanismos fotoprotectores y/o de fotodaño involucrados en la regulación de la eficiencia fotosintética. En el grupo Lut, por lo general, los procesos de activación rápida juegan un papel mayor en la disminución de F_v/F_m mientras en especies del grupo Zea y del grupo Ant, los mecanismos de activación y disipación lenta son más importantes en la regulación de la fotosíntesis. Además, la activación de procesos involucrados en la disminución rápida de

F_v/F_m en las especies con Zea estuvo relacionado con una tasa de disminución 20 veces mayor comparado con las especies de los otros grupos pigmentarios. Por lo tanto, la relación entre el perfil de carotenoides de las especies y las cinéticas de disminución y recuperación de la eficiencia fotosintética indica un papel de esos pigmentos en la fotoprotección en algas rojas.

Además de la disipación de energía a través de carotenoides, también se ha reportado que los fotosistemas inactivos y/o dañados puede jugar un papel en ese proceso. Por mediciones combinadas de los cambios en F_v/F_m y en la evolución de oxígeno la importancia de la fotoinactivación (reversible/irreversible) en la regulación de la eficiencia fotosintética puede ser determinada. Por lo tanto, se comparó el comportamiento de F_v/F_m y el de evolución de O_2 durante y después de exposición a alta luz en cuatro especies de algas rojas (Capítulo III). En general, el valor mínimo de ambos parámetros al final de la exposición lumínica fue similar mientras se observó diferencias en sus respectivas cinéticas. Con la excepción de una especie, F_v/F_m mostró una disminución inicial más rápida comparado con la evolución de oxígeno. Eso indica que en la caída de F_v/F_m durante condiciones de estrés por luz, estuvieron involucrados mecanismos de fotoprotección. Por otro lado, la recuperación inicial rápida de la evolución de O_2 indica que procesos como la inactivación reversible del PSII podría estar involucrados en la disminución de la eficiencia fotosintética en algas rojas.

Para identificar y caracterizar con más detalle los mecanismos involucrados en la fotoprotección en las rodófitas, se investigó la respuesta a diferentes intensidades de luz en dos especies, del grupo Lut y del grupo Zea (Capítulo IV). Asimismo, se ha analizó la respuesta en diferentes segmentos del talo (basal y apical), los cuales presentaron diferentes

aclimataciones a la luz. Las diferencias en aclimatación pueden causar diferencias en la respuesta fisiológica y también diferencias en el contenido de carotenoides y con esto en la fotoprotección.

Se observó que una disminución de F_v/F_m mucho menor después de la exposición a diferentes intensidades de luz en *Eucheuma isiforme*, una especie del grupo Lut, comparado con *Gracilaria damaecornis*, una especie del grupo Zea. Eso coincide con los resultados encontrados previamente (Schubert et al. 2006b, Schubert y García-Mendoza sometido). Las especies con Zea como carotenoide principal muestran una rápida disminución en F_v/F_m (Schubert y García-Mendoza, sometido). Como consecuencia, esas especies parecen estar mejor adaptadas a cambios rápidos en irradiancias comparadas con especies del grupo Lut. Asimismo, usando un inhibidor de la síntesis de proteínas en el cloroplasto (cloramfenicol), se determinó que el fotodaño y la síntesis de D1 estuvieron involucrados en la disminución y recuperación de F_v/F_m en ambas especies durante y después de exposiciones a bajas irradiancias, mientras la exposición a mayores irradiancias inhibió la reparación del PSII. Eso contrasta con la sugerencia de que la degradación de la proteína D1 aumenta con incremento de la intensidad de luz (Tyystjärvi et al. 1991, 1992, Öquist et al. 1992, Aro et al. 1993a). Sin embargo, otros autores han propuesto un recambio de D1 mas lento durante altas irradiancias (ver Critchley y Russell 1994). Asimismo, se ha propuesto que los centros de reacción del PSII pueden participar en la disipación de energía (Krause y Weis 1991).

Las diferencias en ambas especies también se reflejó en la respuesta de los segmentos del talo con diferentes aclimataciones de luz. La regulación del área de absorción del PSII es una de las respuestas aclimatativas más importantes en plantas superiores y algas. Las algas

rojas ajustan sus características de absorción de luz de acuerdo a las condiciones ambientales durante su crecimiento. Por lo tanto, las secciones basales de *E. isiforme* y *G. damaecornis* mostraron una mayor absorción *in vivo* comparada con la sección apical. Esto estuvo asociado con un contenido de pigmentos (Chl *a* y ficobilipigmentos) significantemente mayor en la sección basal. La sección basal de *G. damaecornis* mostró una absorción *in vivo* más alta (49%) comparada con la sección apical (24%). Sin embargo, eso no se reflejó en una regulación de la eficiencia fotosintética mayor en la parte basal. Al contrario, en la sección apical, la cual absorbió menos luz, se encontró una disminución mayor de F_v/F_m comparado con la sección basal. La disminución en el segmento apical aparentemente estuvo relacionada con fotodaño, el cual fue reparado durante la fase de la recuperación, pero también con un mecanismo de regulación con disipación lenta.

Las diferencias entre tejidos de *G. damaecornis* con diferentes aclimataciones estuvieron relacionadas con un mayor contenido de zeaxantina y β-caroteno en el segmento apical lo cual indica un papel de Zea en la regulación de la eficiencia fotosintética. El alto contenido de Zea en ese tejido podría resultar en una alta disipación de energía bajo condiciones de luz subsaturante y en una reversibilidad lenta de ese mecanismo como se ha propuesto anteriormente para mutantes de plantas superiores que acumulan Zea (Niyogi et al. 1998). Esa respuesta se ha observado también en plantas invernales que acumulan Zea y sobreexpresan el componente de relajación lenta del NPQ (Gilmore y Ball 2000, Demmig-Adams et al. 2006). El papel de la Zea en la regulación de la eficiencia fotosintética se ha propuesto también por otros autores (Falbel et al. 1994, Uhrmacher et al. 1995, Jahns y Miehe 1996). Asimismo, la sección apical presento un contenido mayor de β–caroteno

comparado con la sección basal. Ese pigmento así como la Zea presenta una actividad antioxidante, evitando fotodaño por la inactivación de los radicales de oxígeno y estados tripletes de las moléculas de la clorofila que producen estos radicales (Edge et al. 1997).

Por otro lado, la disminución menor de F_v/F_m en el segmento basal de *G. damaecornis* indica la ausencia de un mecanismo de regulación como el encontrado en el segmento apical. Eso podría estar relacionado con un contenido significativamente menor de Zea en la parte basal el cual presentó un alto contenido de Ant. Ese pigmento se ha relacionado asimismo con la disipación térmica (Goss et al. 1998, Gilmore y Yamamoto 2001). Sin embargo, el reemplazo de la Zea como carotenoide dominante por Ant aparentemente como estrategia de aclimatación a baja luz indica que es poco probable que Ant esté involucrado en fotoprotección en *G. damaecornis*. Asimismo, la proporción de carotenoides epoxidados fue mayor en la parte basal. En contraste, el contenido de carotenoides de-epoxidados fue mayor en la parte apical de *G. damaecornis*. Por lo tanto, la regulación de la concentración de carotenoides epoxidados/de-epoxidados parece ser una respuesta aclimatativa importante (por lo menos en especies que presentan esos carotenoides como *Gracilaria*). Eso es consistente con reportes previos sobre diferentes perfiles de carotenoides en especies de *Gracilaria* (Brown y McLachlan 1982, Ursi et al. 2003, Schubert et al. 2006a). Asimismo, se ha propuesto que la proporción de Zea a Ant está correlacionada con la irradiancia durante el crecimiento (Brown y McLachlan 1982). Una razón alta de Zea en relación a Ant se ha encontrado en material colectado en el campo y cultivados en invernaderos mientras organismos cultivados en el laboratorio con irradiancias bajas mostraron una relación inversa (Brown y McLachlan 1982, Liang 1984, Ursi et al. 2003). Por lo tanto, la

acumulación de Zea en las Gracilariales representa aparentemente una respuesta aclimatativa a condiciones de alta luz.

Respeto al recambio de la proteína D1 durante y después de las exposiciones lumínicas se detectaron diferencias entre los segmentos basales y apicales para *G. damaecornis*. En la sección apical, se observó un recambio de D1 principalmente durante la recuperación en luz tenue. Eso indica una respuesta aclimatativa a altas irradiancias respeto a la expresión de un mecanismo de regulación del PSII y la inhibición de la reparación de la D1 durante condiciones de estrés por luz. En contraste, la sección basal no presentó ese tipo de regulación, expresando reparación de D1 durante la exposición a luz.

Eucheuma isiforme, una especie del grupo Lut mostró una absorción *in vivo* ligeramente mas baja en la parte apical (38%) en comparación a la parte basal (43%). La respuesta de las partes basales y apicales a las exposición a diferentes intensidades de luz mostró un patrón inverso en *E. isiforme* en comparación con *G. damaecornis*. El segmento basal fue más susceptible a la exposición a diferentes intensidades de luz comparada con el segmento apical, el cual presentó un contenido mayor de todos los carotenoides. Las secciones apicales y basales mostraron un recambio de la D1 únicamente a irradiancias bajas, lo que indica una máxima reparación de PSII dañados en condiciones no estresantes de luz mientras a irradiancias altas, la reparación de D1 era inhibida probablemente para evitar un desperdicio de energía en la reparación de centros de reacción los cuales pueden ser dañados nuevamente durante condiciones continuas de alta luz.

La presencia de cloramfenicol mostró fotodaño en las partes apicales y basales de *E. isiforme* durante la exposición a bajas irradiancias. Sin embargo, ese daño se reparó inmediatamente en la parte apical así que no se observó una disminución en F_v/F_m mientras

el daño en la parte basal excedió la tasa de recambio de la D1. Una tasa lenta de la degradación de la D1 y una reparación lenta de PSII dañados durante estrés por luz se ha propuesto como un factor principal el cual hace organismos aclimatados a bajas irradiancias más susceptibles a fotoinhibición comparados con organismos aclimatados a alta luz (Aro et al. 1993a, Anderson et al. 1994).

Por lo tanto, el tejido apical de *E. isiforme* aclimatado a alta luz mostró una menor sensibilidad a la exposición a luz comparado con el tejido basal, aclimatado a baja irradiancia. Esa menor susceptibilidad del segmento apical al parecer está relacionada con una ligeramente menor absorción de luz, una capacidad mayor para la reparación de fotodaño y probablemente una mayor capacidad fotoprotectora relacionada con un contenido mayor de Zea y β-caroteno en comapración con el segmento basal.

En conclusión, las diferencias en la regulación de la eficiencia del PSII entre especies y entre tejidos fotosintéticos que mostraron diferentes aclimataciones a la luz confirman que los carotenoides juegan un papel importante en la fotoprotección en algas rojas. Especialmente la regulación de la concentración de Zea parece ser una respuesta aclimatativa importante. Por lo tanto, existen diferentes estrategias para responder a cambios en la irradiancia en las algas rojas, como se ha demostrado previamente también en plantas superiores (Murchie y Horton 1998). Hay mecanismos de fotoprotección relacionados con carotenoides como en *G. damaecornis* (grupo Zea) mientras en especies como *E. isiforme* (grupo Lut), la disipación de energía por centros de reacción inactivos y/o dañados puede ser mas importante. Dado la ausencia del XC, la cual interviene en la disipación térmica, en algas rojas, una acumulación de PSII inactivos que participan en la

disipación de energía podría ser un mecanismo fotoprotector importante para resistir en ambientes de altas irradiancias.

Uno de los papeles principales de los carotenoides es la transferencia de energía de excitación a la clorofila durante la colecta de luz pero también protegen el aparato fotosintético por desactivación de estados tripletes de la clorofila y radicales de oxígeno. Algunos carotenoides tienen la habilidad de disipar excesos de energía como calor. La síntesis a corto plazo de carotenoides fotoprotectores como Zea es a través de la de-epoxidación reversible de violaxantina y anteraxantina en el XC. La presencia de este ciclo en algas verdes y plantas superiores les permite tener una formación rápida de Zea en condiciones estresantes de luz pero también una remoción rápida de Zea en condiciones lumínicas no estresantes.

La presencia de una alta concentración de moléculas de Zea (formados por el XC o por β-caroteno) aumenta la probabilidad de la disipación de energía en forma de calor en el aparato fotosintético. Esto puede resultar en una disminución de la eficiencia fotosintética lo que bajo condiciones de luz no estresante representaría una desventaja. Por lo tanto, la capacidad de remover rápidamente la Zea puede ser un aspecto importante del XC.

Las rodófitas no presentan el XC pero un alto contenido permanente de carotenoides (p.ej. el contenido de Zea es 10 veces más alto comparado con mutantes de plantas superiores que acumulan zeaxantina; Hurry et al. 1997, Niyogi et al. 1998) y el papel que juega la Zea en la disipación de energía bajo estrés es importante en diferentes especies de algas rojas (ver Capítulo II y IV).

Se ha propuesto que no toda la Zea está involucrada en la disipación térmica debido a que no se han detectado diferencias en la sensibilidad a la fotoinhibición entre mutantes de *Arabidopsis* acumulando Zea y el genotipo silvestre (Hurry et al. 1997, Havaux et al. 2004). La Zea no involucrada en NPQ (hasta 35% del pool total) podría estar localizada en la pared del cloroplasto (Siefermann-Harms et al. 1978, Hurry et al. 1997) o libremente en la membrana tilacoide donde puede estar involucrada en la desactivación de radicales libres de O_2 (Baroli et al. 2003, Rissler y Durnford 2005). Como lo mismo se ha encontrado en cianobacterias donde la Zea en la pared celular tiene un rol fotoprotector (Resch y Gibson 1983, Jürgens y Weckesser 1985), se puede suponer que es similar en algas rojas.

Los procesos reguladores de la eficiencia fotosintética, como por ejemplo la disipación de excesos de energía en forma de calor, están fuertemente relacionados con la antena transmembránica del PSII (LHCII). Por lo tanto, los mecanismos involucrados en la disipación de energía que ocurren en las LHCII de las plantas superiores no son iguales en algas rojas pero si podrían ser más similares a los mecanismos en cianobacterias. En ese grupo, los carotenoides juegan un papel importante en la fotoprotección (Rakhimberdieva et al. 2004, 2007, Wilson et al. 2006, Kirilovski et al. 2007). Recientemente se ha encontrado una proteína asociada a carotenoides (orange carotenoid-protein; OCP) que está involucrada en la disipación térmica en las cianobacterias (Wilson et al. 2006, Rakhimberdieva et al. 2007). El carotenoide asociado al OCP varía entre especies. Zeaxantina está presente en algunas especies como *Anacystis nidulans* y *Lyngbya wholei* (Diverse-Pierluissi y Krogman 1988, Engle et al. 1991) mientras en otras especies se ha encontrado hidroxi-echinenona o sus derivados asociados al OCP (Wu y Krogman 1997, Kerfeld 2004a, b). Sin embargo, el mecanismo por lo cual la energía es disipada todavía no

es conocido. Existen diferentes posibilidades: Kirilovski et al. (2007) sugieren que el OCP es activado debido a la absorción de la luz por el carotenoide. Entonces, el OCP 'activado' interactúa con el núcleo del ficobilisoma, alterando su estructura, lo cual resulta en la disipación de energía (ver Fig. 8). Alternativamente, el carotenoide del OCP podría interactuar directamente con el aceptor terminal del ficobilisoma y disipar la energía absorbida.

Asimismo, recientemente se han descrito mecanismos de NPQ en cianobacterias mediados por un complejo proteico, IsiA (Iron-starvation-inducible protein) (Yeremenko et al. 2004, Ihalainen et al. 2005). Dos diferentes mecanismos relacionados con IsiA son propuestos: (1) luz azul convierte IsiA de una forma cual es eficiente en colectar la energía de la luz para la fotosíntesis a una forma alternativa que convierte excesos de energía en calor (Cadoret et al. 2004) y (2) una alta intensidad de luz induce un acoplamiento de ficobilisomas libres a IsiA, provocando una disminución de fluorescencia (Joshua et al. 2005).

La función de ciertos carotenoides está fuertemente relacionada con su ubicación en el aparato fotosintético y con su asociación a ciertas proteínas. Por lo tanto, la presencia de ciertos carotenoides con capacidad fotoprotectora en algas rojas no implica *per se* que actuarían como disipadores de energía. Se debería considerar donde están localizados y a que proteínas están asociadas para inferir sobre su papel en la disipación de energía. Sin embargo, existen muy pocos trabajos relacionados con la caracterización de los complejos proteicos asociados con pigmentos en algas rojas y su función. Por lo cual, no está conocido si en las rodófitas, proteínas asociadas a carotenoides están presentes que podrían actuar de manera similar como el OCP y/o el complejo IsiA en las cianobacterias.

Como se ha mostrado anteriormente, las algas rojas presentan diferencias en la composición de carotenoides, las cuales están relacionadas con la expresión diferencial de mecanismos fotoprotectores (Capítulo II y IV). Ecológicamente, esas diferencias en fotoprotección pueden implicar ciertas ventajas y restricciones para las especies. Como se ha encontrado en el presente trabajo, hay especies de rodófitas que dependiendo de su aclimatación a la luz presentan zeaxantina (alta luz) o anteraxantina (baja luz) como carotenoide principal (Capítulo I y IV). El perfil pigmentario en el cual Zea es acumulada parece estar relacionada con la expresión de mecanismos de regulación de la actividad del PSII a bajas irradiancias con una activación rápida y disipación lenta (Capítulo IV). Eso puede ser una ventaja en áreas someras con fluctuaciones de luz, debido a la respuesta rápida a cambios en la irradiancia relacionada con la Zea. La comparación entre la distribución vertical de especies de algas rojas y su respectivo perfil de carotenoides apoya esa idea. Especies del grupo Lut y del grupo Ant se han encontrado en la zona del intermareal hasta 15 m de profundidad mientras miembros del grupo Zea estaban restringidos a los primeros 8 m durante verano (Fig. 28A). Asimismo, especies de ese último grupo pigmentario no se han encontrado en invierno (Fig. 28B). Eso indica que un mecanismo fotoprotector de activación rápida pero de disipación lenta como el presente en el grupo Zea provee una ventaja durante condiciones de verano con altos niveles de irradiancia y un fotoperíodo largo pero puede ser una desventaja durante condiciones de bajas intensidades de luz y/o fotoperíodos cortos como en invierno.

En contraste, especies con luteína parecen ser exitosas en áreas someras con altas irradiancias pero también en áreas mas profundas con bajas intensidades de luz. Asimismo, en plantas supriores se han demostrado una correlación entre el contenido de

carotenoides y la distribución (Johnson et al. 1993) con especies adaptadas a sombra presentando un mayor contenido de luteína y una menor concentración de pigmentos del XC (Johnson et al. 1993).

Evidentemente, aparte del perfil de carotenoides y con esto la expresión de ciertas estrategias fotoprotectoras, existen varios factores que determinan la distribución vertical de algas rojas y de las macroalgas en general. Entre ellos, la morfología, por su efecto a la tasa fotosintética de las especies (Markager y Sand-Jensen 1992, Johansson y Snoeijs 2002), y la sensibilidad a luz ultravioleta (Hanelt et al. 1997, Bischof et al. 1998, 2002) pueden ser factores importantes que determinan la distribución de especies de rodófitas. Otro factor que afecta la zonación vertical aun en menor proporción puede ser el ciclo de vida de las especies (ver Markager y Sand-Jensen 1992).

Por lo tanto, factores tanto ecológicos como fisiológicos afectan la distribución vertical de las algas rojas, sin embargo, el perfil de carotenoides y la respuesta asociada que regula la eficiencia fotosintética puede ser un aspecto determinante para la presencia de especies en ciertos ambientes lumínicos.

Aparte de las implicaciones ecológicas, los resultados de presente trabajo también tienen implicaciones evolutivas. La composición de carotenoides en algas rojas es diversa y ambos vías de síntesis de carotenoides (a través de α- y de β-caroteno) están presentes. Eso puede ser relacionado con el modelo evolutivo de los diferentes plástidos en las eucariotas fotosintéticas (Capítulo I), por lo cual proponemos un modelo hipotético sobre la aparición de las vías de síntesis de carotenoides durante la evolución (Fig. 29).

El modelo se basa en la evolución de los plástidos y antenas colectores de luz propuesto por Durnford et al. (1999). El cloroplasto primario presentó complejos antena tipo ficobilisoma y tipo proclorofita (con clorofila *a*/*b*). El complejo antena tipo proclorofita fue reemplazado por un complejo antena transmembránico (preLHC) del cual se evolucionaron las antenas de los linajes de las algas rojas y algas verdes (Tomitani et al. 1999). Los pigmentos relacionados con el XC y la vía de síntesis de carotenoides del α-caroteno probablemente aparecieron en ese punto de la evolución, antes de la divergencia de los linajes de las algas rojas y algas verdes debido a la presencia de luteína y de carotenoides epoxidados como violaxantina y anteraxantina en ambos linajes (ver Capítulo I; García-Mendoza 2000).

La ocurrencia de carotenoides epoxidados indica que en ambos linajes, la zeaxantina epoxidasa la cual cataliza la síntesis de Zea hacia Ant y violaxantina está presente (Cunningham y Gantt 1998) y que esta enzima probablemente estuvo presente antes de la divergencia de las algas rojas y algas verdes (García-Mendoza 2000). En contraste, no se ha encontrado el homólogo de la enzima violaxantina de-epoxidasa que catalíza la conversión de violaxantina hacia Zea vía anteraxantina (XC) en las rodófitas.

En conclusión, los carotenoides juegan un papel en la fotoprotección en las Rhodophyta. Sin embargo, si las algas rojas presentan proteínas similares al OCP o un complejo IsiA no es conocido. La caracterización del sitio donde la disipación de exceso de energía en forma de calor toma lugar en ese grupo algal daría evidencia sobre la evolución de los mecanismos de fotoprotección en las eucariotas fotótrofas.

“Detrás de cada logro hay otro desafio”

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ABBREVIATIONS

Ant: antheraxanthin

CAP: chloramphenicol, inhibitor of chloroplast-encoded proteins

DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea)

Ddx: diadinoxanthin

DMF: dimethylformamide

Dtx: diatoxanthin

LHC: light-harvesting complex

Lut: lutein

NPQ: non-photochemical quenching

OCP: orange carotenoid-binding protein

OUT: operational taxonomic unit

PAR: active photosynthetic radiation (400 – 700 nm)

PBS: phycobilisome

PSI: Photosystem I

PSII: Photosystem II

PUR: photosynthetic utilizable radiation

RC: reaction centre

Vio: violaxanthin

XC: xanthophyll cycle

Zea: zeaxanthin

GENERAL INTRODUCTION TO RED ALGAL PHYSIOLOGY

The red algae

Algae are a large and diverse group of unicellular to multicellular autotrophic organisms. They are considered "plant-like" because of their photosynthetic ability and “simple” because they lack the distinct organs of higher plants such as roots, leaves and vascular tissue.

The lineages of photosynthetic organisms evolved from a primary endosymbiotic event between an ancestral photosynthetic cyanobacteria and an eukaryotic cell. Three main algal lineages which present a common origin can be distinguished: Chlorophyta, Rhodophyta and Glaucophyta (Fig. 1; Stiller and Hall 1997, Bhattacharya and Medlin 1995, Delwiche 1999, Moreira et al. 2000, McFadden 2001, Palmer 2003). The red lineage includes modern rhodophytes, cryptophytes, heterokontophytes, dinophytes and haptophytes. The last four phyla arouse after a secondary endosymbiotic event in which a red algal type ancestor was involved (Fig. 1).

Algae are distinguished on a number of different characteristics. The color of the plastids (more correctly the combination of photosynthetic pigments that are present in the plastid) and the presence and type of flagella are the most important characteristics.

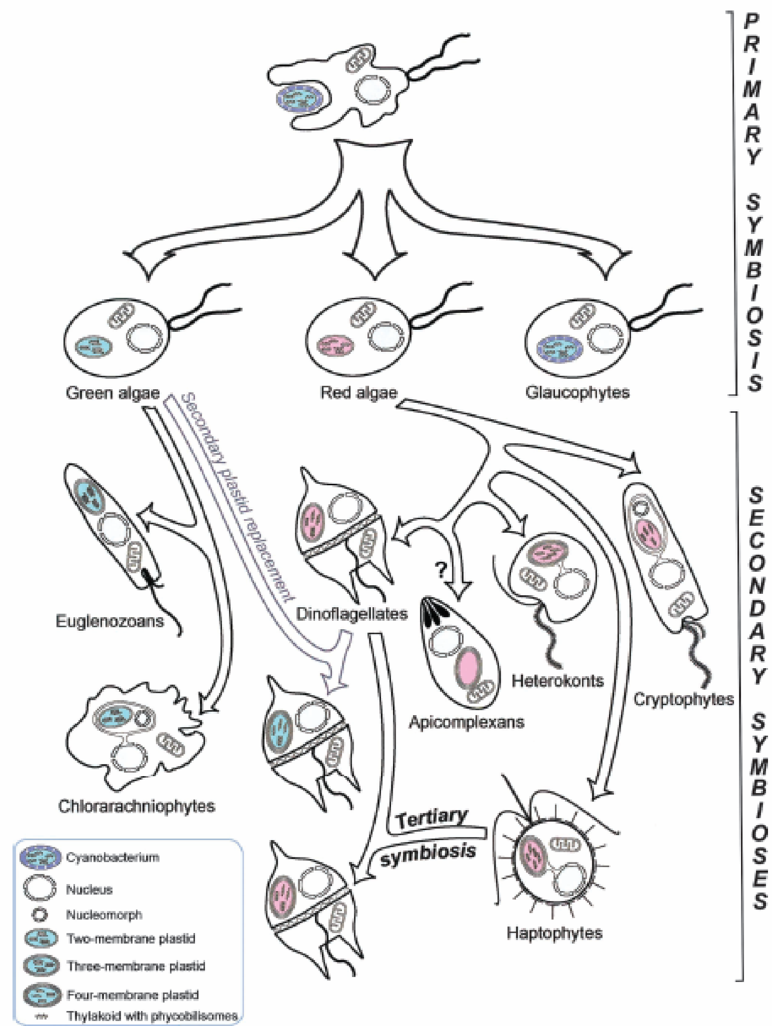

Figure 1. Primary and secondary endosymbiosis and the diversity of algae and their plastids (Palmer 2003).

Figura 1. Endosimbiosis primaria y secundaria y la diversidad de algas y sus plástidos (Palmer 2003).

There is controversy about the appearance of red algae. Tappan (1980) suggested that Rhodophyta exist since 2 billon years ago while Hori and Osawa (1987) estimated the age of the red algae to be 1.3-1.4 billion years.

The Rhodophyta are an eukaryotic phylum characterized principally by the absence of flagella and the presence of accessory photosynthetic pigments such as phycoerythrin, phycocyanin, and allophycocyanin (Woelkerling 1990). These pigments are hydrosoluble and located on the thylakoid surface in protein complexes called phycobilisomes (PBS) (Fig. 2A; Gantt 1979, Toole and Allnutt 2003). Moreover, red algae present only chlorophyll *a* while chlorophyll *b* and *c* are absent.

Another difference between red algae and other algal groups is the presence of unstacked thylakoids (Fig. 2B) while in other groups (except Glaucophyta) the thylakoids are stacked.

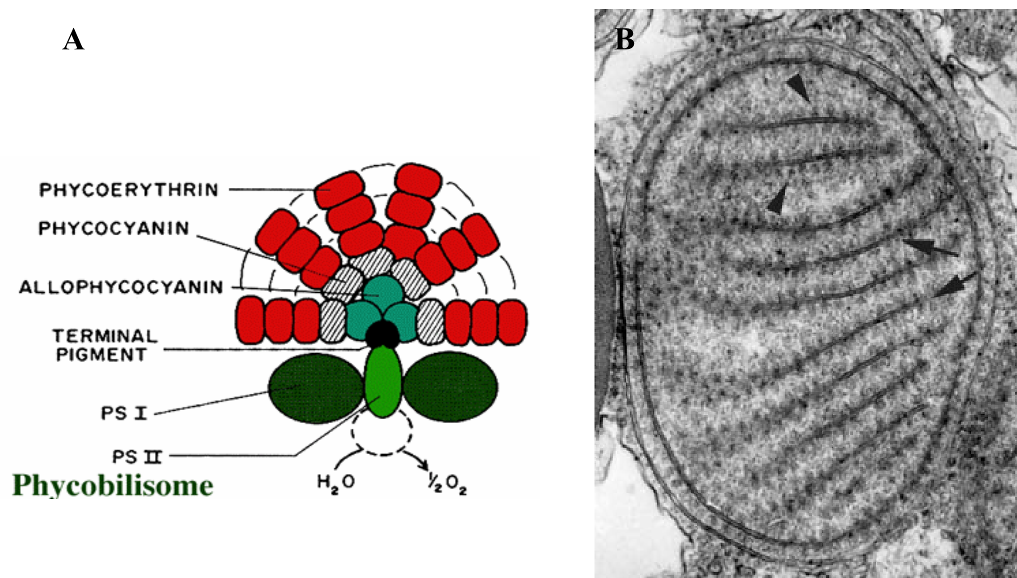

Figure 2. A) Organization of phycobiliproteins in the phycobilisome of red algae (Gantt 1990). B) Red algal chloroplast with unstacked thylakoids (arrows show phycobilisomes attached on the thylakoid membrane).

Figura 2. A) Organización de ficobiliproteínas en el ficobilisoma de algas rojas (Gantt 1990). B) Cloroplasto de algas rojas con tilacoides no apilados (las flechas muestran ficobilisomas asociados a la membrana tilacoide).

The morphology of the red algae is very variable and different thallus types exist. The simplest red algal thalli consist of free-living unicells, as in the case of *Porphyridium* and *Rhodella* (Fig. 3A). Red algal thalli can also be composed of cells arranged to form one- or two-layered parenchymatous sheets, as for example occurs in *Porphyra* (Fig. 3B). However, most Rhodophyta and specifically the Florideophyceae are filamentous or composed of packed aggregations of filaments, which in the latter case can produce thalli of considerable morphological complexity (Fig. 3C). Moreover, most of coralline algae, which secrete calcium carbonate and play a major role in building coral reefs, belong to this group.

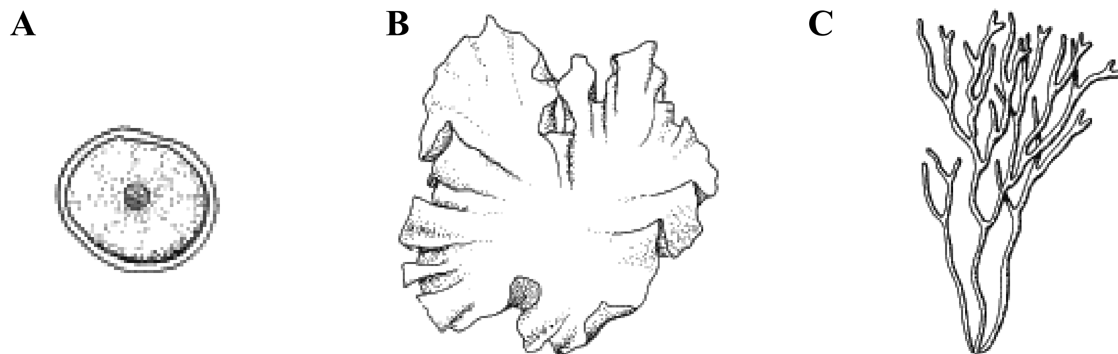

Figure 3. Different morphologies in Rhodophyta: A) *Porphyridium*, B) *Porphyra* and C) *Nemalion*.

Figura 3. Diferentes morfologías en Rhodophyta: A) *Porphyridium*, B) *Porphyra* y C) *Nemalion*.

The rhodophyte group presents between 2500 and 6000 marine species grouped in approximately 670 genera (Woelkerling 1990). Red algae dominate coastal areas of tropical

regions, middle latitudes and cold water regions (Lüning 1990). Only few species occurred in freshwater (about 150 species from 20 genera).

The red algal division consists of two classes, the Bangiophyceae and the Florideophyceae. It is supposed that Bangiophyceae is the most primitive class and consists of algae with unicellular and multicellular thalli. This class is divided in 5 orders: Porphyridiales, Rhodochaetales, Erythropeltidales, Compsogonales and Bangiales (van den Hoek 1995). The Florideophyceae class consists of multicellular species only. According to van den Hoek (1995), the Florideophyceae consist of 13 orders: Acrochaetiales, Palmariales, Nemaliales, Batrachospermales (only freshwater), Corallinales, Hildenbrandiales, Bonnemaisoniales, Gelidiales, Gigartinales, Gracilariales, Ahnfeltiales Rhodymeniales and Ceramiales.

The photosynthetic apparatus of red algae

Red algae are one of the most primitive eukaryotic algae. Their photosynthetic apparatus represents a transitional state between cyanobacteria and photosynthetic eukaryotes (Fig. 4). Both cyanobacteria and the red algae contain phycobilisomes that serve as the primary light-harvesting antenna for photosystem II (PSII) instead of the transmembranic chlorophyll *a*/*b* (or chlorophyll *a*/*c*)-binding proteins reported in higher plants and chromophyte algae (Green and Durnford 1996, Bassi et al. 1993, Durnford et al. 1999). However, the existence of a membrane-intrinsic light-harvesting complex (LHC) was demonstrated for *Porphyridium cruentum* (Wolfe et al. 1994) and *Galdieria sulphuraria* (Marquardt and Rhiel 1997). This complex, which is functionally associated with photosystem I (PSI), is not present in cyanobacteria.

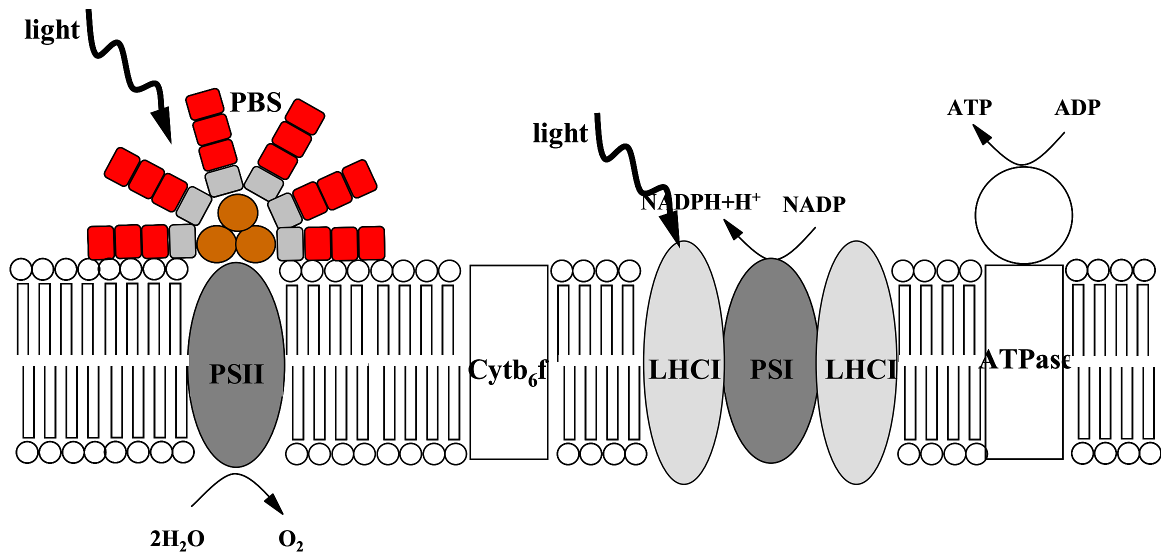

Figure 4. Schematic drawing of the photosynthetic apparatus of red algae. Shown is the phycobilisome antenna (PBS) of PSII, photosystem II (PSII), photosystem I (PSI), light-harvesting complex of PSI (LHCI), cytochrome b_6f ($Cytb_6f$) and ATPase. Photosynthetic accessory protein-pigment complexes in light-harvesting antennae are depicted in grey.

Figura 4. Esquema del aparato fotosintético de las algas rojas. Se muestran la phycobilisoma (PBS) como antena del PSII, el fotosistema II (PSII), el fotosistema I (PSI), el complejo antena colector de luz del PSI (LHCI), el citocromo b_6f ($Cytb_6f$) y la ATPasa (ATPase). Los complejos protéicos colectores de luz, asociados con pigmentos accesorios, son señalados en gris.

Red algal LHCs bind Chl *a* as the only Chl-type pigment and the carotenoids zeaxanthin and β-carotene (Wolfe et al. 1994, Marquardt and Rhiel 1997). The polypeptides composing LHCI are immunologically related to the polypeptides of Chl *a*/*b* (higher plants and green algae) and Chl *a*/*c* (chromophytes) LHCs (Wolfe et al. 1994).

All of the chlorophyll *a* and probably most of the carotenoids are attached to a few specific proteins. The PSI-LHC complex appears to have 82% of the Chl *a* while 6 to 8% are bound to two other complexes (CP43 and CP47) associated with PSII core antenna (Redlinger and

Gantt 1983). The role of carotenoids in the regulations of photosynthesis (as accessory pigments or quencher) in the Rhodophyta is not clear, except for β-carotene, which is active in PSI and probably in PSII (Redlinger and Gantt 1983).

The energy absorbed by phycobilisomes is transferred to the PSII chlorophyll antenna. The phycobiliproteins form an energy transfer chain in which the fluorescence emission of the preceding member overlaps the absorption of the next one (Glazer 1989, Gantt 1990). Therefore, phycoerythrin is present in the periphery, followed by phycocyanin, and then by allophycocyanin (see Fig. 2A). This corresponds energetically to an excitation chain, where the progression is from a higher to a lower energy level. Overall energy transfer efficiency from phycobilisomes to the reaction centre (RC) is not necessarily high (70-85%) compared with the other photosynthetic organisms due to relatively low transfer efficiency among phycobiliproteins (Mimuro 2004). Ratios of 3 to 4 PSII RCs per phycobilisomes (Kursar and Alberte 1983, Ley 1984, Cunningham et al. 1989) or 1.4 to 1.6 PSII per phycobilisomes (Toole and Allnutt 2003) have been reported for red algae.

Apparently, phycobilisomes transfer energy to both photosystems, even though the transfer to PSI is not necessarily clear. There are two possible pathways: a direct route from the phycobilisome to the PSI core complex, as proposed by Mullineaux et al. (1994, 1997), and an indirect route via PSII. Ley and Butler (1977) showed that about 50% of the energy absorbed by the phycobilisome is transferred to PSI when the PSII traps are open, and that this fraction increased up to 90 to 95% when the PSII traps are closed.

Chlorophyll fluorescence as a tool to monitor physiological responses to light stress

Chlorophyll fluorescence is a widespread method used in photosynthesis research. This is because it is non-invasive and highly sensitive, fast and easily measured, and it contains important information about the photosynthetic apparatus. At room temperature fluorescence is mainly emitted from PSII (see Krause and Weis 1991).

The principle underlying the chlorophyll fluorescence analysis based on that light energy absorbed by chlorophyll molecules in the chloroplast can undergo one of three fates: it can be used to drive photosynthesis (photochemistry), excess energy can be dissipated as heat (non-photochemical quenching; NPQ) or it can be re-emitted as fluorescence (Fig. 5). These three processes occur in competition, such that any increase in efficiency of one will result in a decrease in the yield of the other two. Hence, by measuring chlorophyll fluorescence quenching, information about changes in the efficiency of photochemistry and heat dissipation can be obtained. The term "fluorescence quenching" assumes that for every investigated sample a maximal fluorescence yield can be defined and it is observed when the other potentially quenching or deexcitation pathways (see Fig. 5) are minimized. Under normal physiological conditions, the photosynthetic apparatus of most organisms reaches a relatively stable state after dark-adaptation that is characterized by a full oxidized state of the PSII electron acceptor, quinone A (Q_A), and the absence of a transthylakoid proton gradient. Hence, in the dark-adapted state photochemical quenching is maximal and NPQ is minimal.

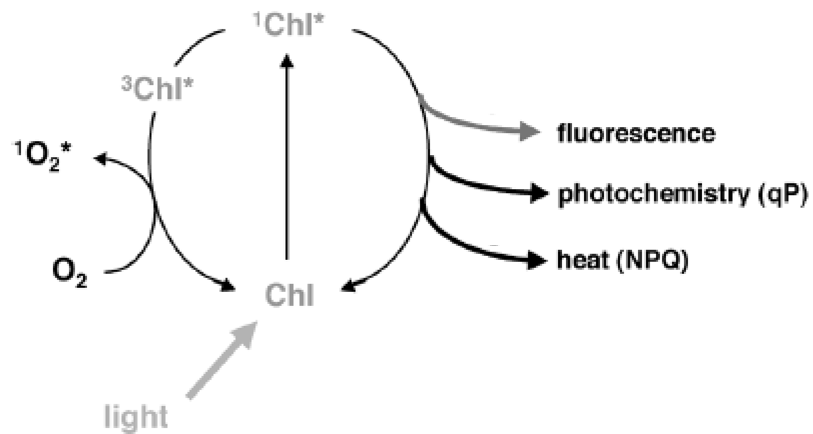

Figure 5. Possible fates of excited chlorophyll (^{1}Chl*) (Müller et al. 2001).

Figura 5. Posibles vías de la clorofila excitada (^{1}Chl*) (Müller et al. 2001).

A prerequisite for evaluation of fluorescence quenching during illumination is the reliable determination of the minimal and maximal fluorescence yields after dark-acclimation, F_o and F_m, respectively. F_o is the minimum fluorescence, i.e. when all reaction centers of PSII are active or "open", and F_m is the maximum fluorescence determined under strong light, i.e. when all PSII centers are "closed". Due to the extremely low measuring light intensity, F_o can be monitored continuously, without affecting the dark state, and a short pulse of saturating light to assess F_m can be applied for full reduction of Q_A and hence completely suppression of photochemical quenching (Fig. 6).

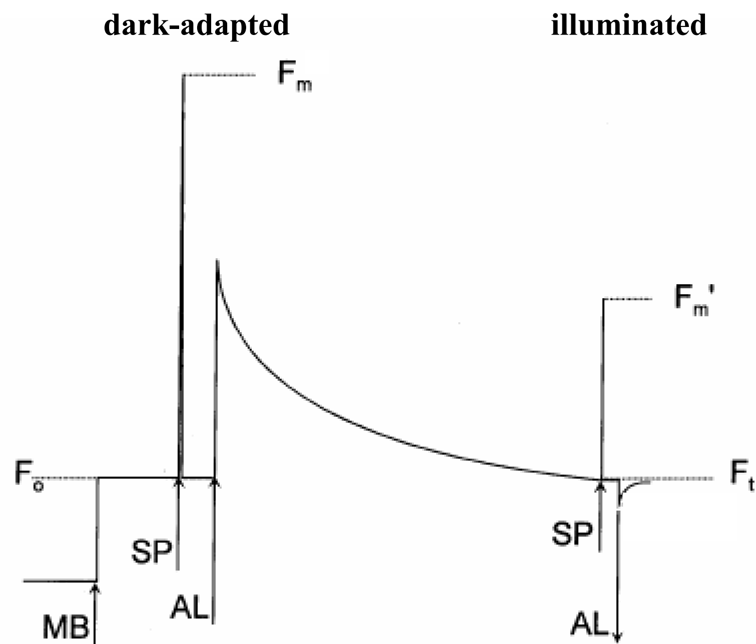

Figure 6. Fluorescence induction. A measuring light is switched on (↑MB) and the minimal fluorescence level (F_o) is measured. Application of a saturating flash of light (↑SP) allows measurement of the maximum fluorescence level (F_m). A light to drive photosynthesis (↑AL) is then applied. After a period of time, another saturating light flash (↑SP) allows measurement of the maximum fluorescence in the light (F_m'). The level of fluorescence immediately before the saturating pulse is termed F_t (taken from Maxwell and Johnson 2000).

Figura 6. Inducción de fluorescencia. Una luz de medición es encendida (↑MB) y el nivel mínimo de la fluorescencia (F_o) es medido. La aplicación de un pulso de luz saturante (↑SP) permite la medición de la fluorescencia máxima (F_m). Posteriormente se aplica una luz para iniciar fotosíntesis (↑AL). Después de un periodo de tiempo, otro pulso de luz saturante (↑SP) permite la medición de la fluorescencia máxima en luz (F_m'). El nivel de la fluorescencia inmediatamente antes del pulso saturante es llamado F_t (tomado de Maxwell y Johnson 2000).

The well established fluorescence parameter F_v/F_m provides an estimate of the maximum quantum efficiency of PSII photochemistry and it is given by the equation: $F_v/F_m = (F_m-F_o)/F_m$.

The values of this parameter can be changed by both photochemical and non-photochemical factors. A healthy terrestrial plant will almost have a dark-adapted F_v/F_m value close to 0.8. If an F_v/F_m measurement is made on a dark-adapted photosynthetic tissue and the tissue is then subjected to a period of high light, it is likely that a subsequent F_v/F_m measurement, made a few minutes after the high light-treatment would reveal a decrease in the value of this parameter. This decrease could result from a decrease in the fraction of PSII centers that are capable of photochemistry and/or down-regulation (increase in NPQ) that has not reversed between the end of the light-treatment and measurement of F_v/F_m.

A decline in F_v/F_m has often been interpreted as photoinhibition. This term is somewhat problematic because a decline in F_v/F_m may result from a number of different processes, some of which are readily reversible and not indicative for photodamage, and others which are slowly reversible and can be termed photodamage. Use of the word photoinhibition has led to confusion because in many studies, it has been used to describe decreases in F_v/F_m resulting from processes that include photoprotective non-photochemical quenching. Other authors reserve the term to indicate only slowly reversible F_v/F_m recovery that can be interpreted as photodamage. A more reliable definition of photoinhibition would be a sustained depression in PSII efficiency as proposed by Lichtenthaler and Babani (2004).

Photosynthesis and photoinhibition

Photosynthetic rates are affected by many abiotic factors besides light; also factors such as morphology, ontogeny and circadian rhythm affect photosynthesis. Red algal and macroalgal photosynthetic rates in general are strongly influenced by thallus morphology, particularly the surface-area: volume ratio and the proportion of non-photosynthetic tissue (Littler and Littler 1980, Littler and Arnold 1982, Littler et al. 1983, Lobban and Harrison 1994, Johansson and Snoeijs 2002). The sheet-like group had the highest mean net photosynthesis because all cells are photosynthetic active, have direct access to carbon supplies in the water and presents low self-shading. On a surface-area basis, filamentous algae should express higher photosynthetic rates, but clumping (which may have advantages in desiccation resistance etc.) decreases the effective surface area (Littler and Arnold 1982). Generally, mean net photosynthetic rates decreased from sheet-like, branched, thick blades, articulated calcareous to encrusting algae (Lobban and Harrison 2000). This consists with differences in light absorption according to the thallus morphology as reviewed by Ramus (1990).

Red algae can exist at a wide range of irradiances, but most species grow at relatively low light levels. Exceptions to this are a variety of intertidal rhodophytes, which can survive at full sunlight intensities (2000 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}) (Hodgson 1981). In shallow waters or in the intertidal zone there are daily changes in irradiance due to wave focusing, canopy movements, progressive tides, and suspended particles. These variations occur over time scales of seconds to hours (Schubert et al. 2001). Therefore, macroalgae in intertidal

environments present mechanisms to withstand this variability that are expressed depending on the vertical distribution of the species.

The vertical zonation of macroalgae shows a relationship between the maximum photosynthetic rate (P_{max}), dark respiration (R_d) and photosynthetic efficiency (α), and thallus morphology of the species, with higher values in thinner species (Johansson and Snoeijs 2002). Also, light requirement of the species has been related to its thallus morphology (Markager and Sand-Jensen 1992) while Johansson and Snoeijs (2002) found that the compensation irradiance (E_c) and saturating irradiance (E_k) were strongly related to water depth with lower values at greater depth and not to thallus morphology (Fig. 7).

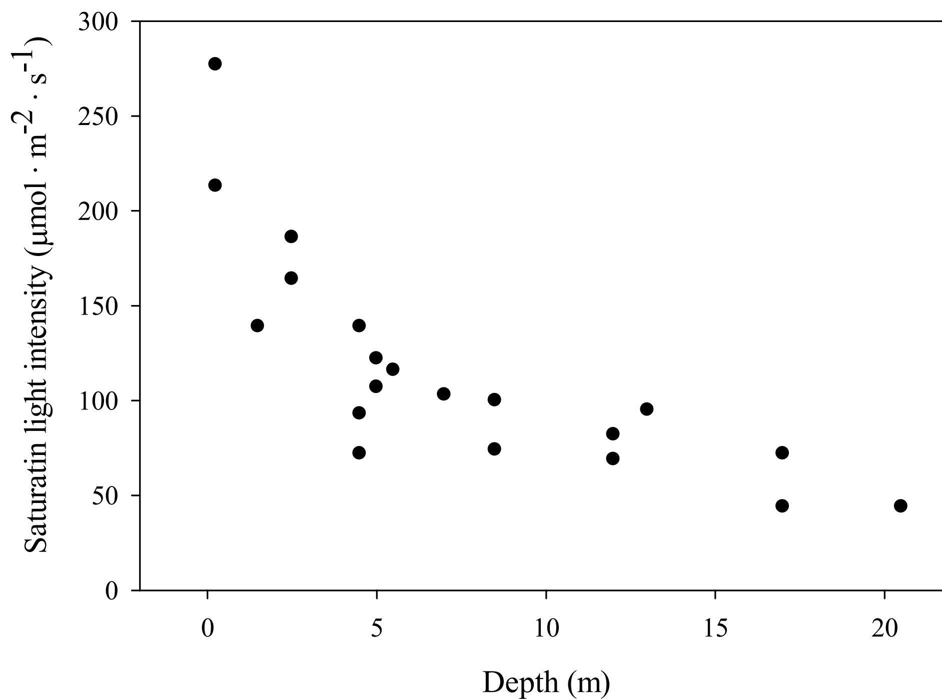

Figure 7. Relationship between saturation light intensity (μmol · m^{-2} · s^{-1}) and maximum abundance depth of different red algal species (data taken from Johansson and Snoeijs 2002).

Figura 7. Relación entre la irradiancia de saturación de la fotosíntesis (μmol · m^{-2} · s^{-1}) y la profundidad de la máxima abundancia de diferentes especies de algas rojas (datos tomados de Johansson y Snoeijs 2002).

Hence, light is an important factor for algal zonation. The ability to withstand variations in irradiance and to resist high light stress may be one of the factors determining the competitive ability of macroalgae at the upper limit of their distribution (Herbert 1990, Hanelt 1996, 1997). The exposure to high irradiances which exceed the energy requirement for photosynthesis causes a down-regulation of photosynthetic activity and can result in photodamage, a phenomenon called photoinhibition (cf. Powles 1984, Krause 1988, Long et al. 1994, Anderson et al. 1997).

Susceptibility to photoinhibition is dependent on the capacity of the organism to repair photodamaged reaction centers during light stress (Greer et al. 1986, Tyystjärvi et al. 1991). Also, the size of the light-harvesting antenna and the capacity of expression of photoprotective mechanisms, as for example the non-radiative dissipation of excitation energy, affect the rate of PSII photoinhibition (reviewed in Krause 1988).

Therefore, the ability to down-regulate photosynthetic activity during exposure to high light as well as the capacity of photosynthesis acclimation of individual species to different light regimes may determinate the upper depth distribution limit of macroalgae (Hanelt 1996, 1998). Thus, photoinhibition in algae grown in deeper waters is high upon exposure to light stress conditions (Sagert et al. 1997, Bischof et al. 1998, Jiménez et al. 1998, Hanelt 1998, Karsten et al. 1999, Cabello-Pasini et al. 2000).

Moreover, the acclimation to incident light can occur not only between different algal thallus but also between different regions of the same thallus depending on their light exposure (Calabrese and Piero Felicini 1973, Beach and Smith 1996a, b, Colombo et al. 2006).

Photoacclimation and photoprotection

The extremely high oxidizing potential needed for H_2O oxidation is the unique feature of PSII, one that requires regulation of light-harvesting. The pigments and proteins (mainly D1) of the RC can be photochemically damaged by formation of the triplet state of P680 and consequent singlet O_2 production or due to oxidation by $P680^+$ (reviewed in Horton et al. 1996). Repair involves disassembly, proteolysis, and introduction of newly synthesized D1 polypeptide (Chow 1994). When the rate of damage exceeds the rate of repair, inactive centers accumulate, which leads to a decline in photosynthesis. Furthermore, a damaged RC may generate oxidized Chl and free radicals and cause widespread damage to the thylakoid. Hence, photosynthetic organisms have evolved multiple photoprotective mechanisms to cope with the potentially damaging effects of light (Fig. 8; reviewed in Anderson et al. 1992, Osmond 1994, Niyogi 1999). Within the mechanisms to avoid and/or minimize photodamage, the thermal dissipation of excess absorbed light energy plays an important role (reviewed in Horton et al. 1996).

Acclimation (transcriptional and post-transcriptional) implies either rapid increments/decrements or the reorganization of already existing components within (PSI and PSII reaction centers) and nearby the photosynthetic membrane (PBS insertion/detachment). Short-regulation (post-transcriptional) to varying light conditions involves several regulatory mechanisms aimed to optimize photosynthetic activity and to prevent photodamage in the photosynthetic apparatus.

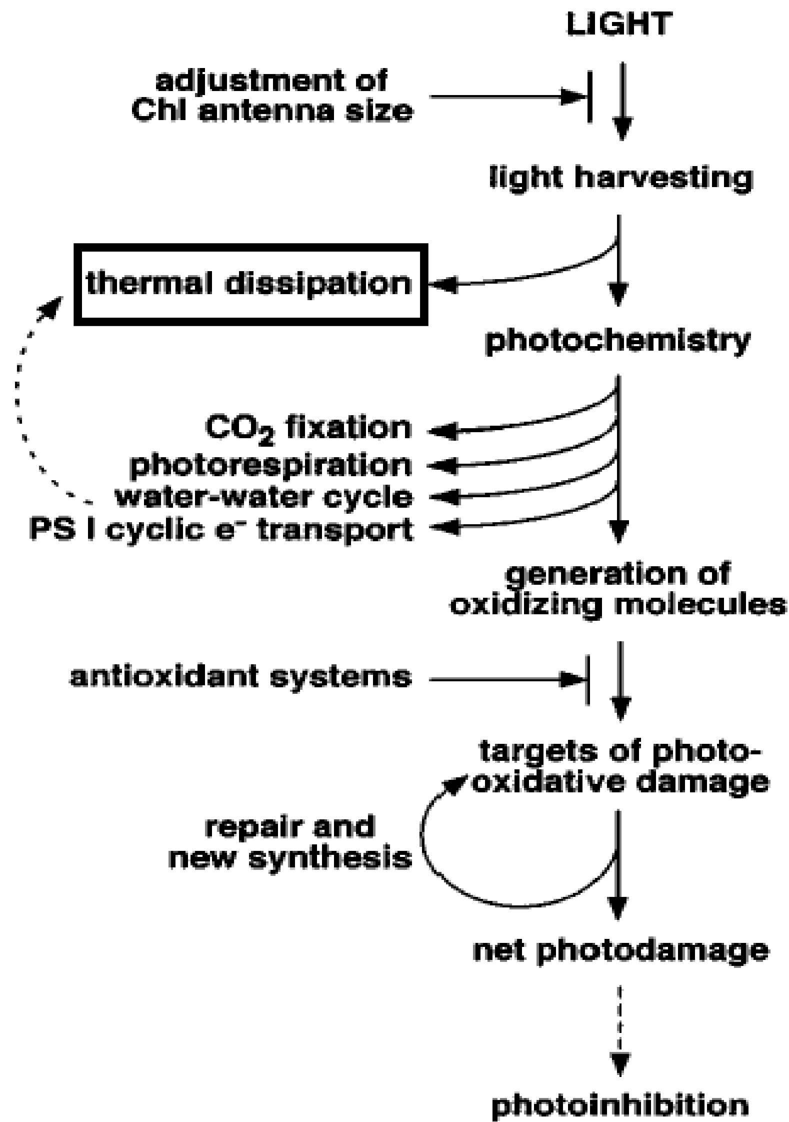

Figure 8. Schematic diagram of photoprotective processes occurring within chloroplasts (taken from Niyogi 1999).

Figura 8. Diagrama esquemático de los procesos de fotoprotección que ocurren en el cloroplasto (tomado de Niyogi 1999).

Also other short-term expressed molecular mechanisms such as spillover, state transitions, and cyclic electron flow around PSII help to regulate PSII function. Dynamic acclimation in the longer-term elicits both enhanced photon usage by the increase of the rate of photosynthetic electron transport to CO_2 and increase of D1 protein turnover, as well as the increase of non-radiative energy dissipation, partly achieved by certain carotenoids. All of the photoprotective strategies might act together, helping to balance energy supply with energy consumption under all light levels.

To prevent damage of the photosynthetic apparatus during high light exposure, photosynthetic organisms have evolved a variety of mechanisms which are principally associated to the transthylakoid proton gradient (ΔpH) formation. These mechanisms, such as the non-photochemical thermal energy dissipation, down-regulate the efficiency of PSII and are quickly reversible (Krause 1988). Carotenoids are responsible for the non-photochemical quenching of Chl *a* excitation by means of the xanthophyll cycle in higher plants and various algal groups (Demmig-Adams 1990, Franklin et al. 1992, Uhrmacher et al. 1995, Niyogi et al. 1997, Rodrigues et al. 2002, Gevaert et al. 2003). The xanthophyll cycle (XC) is the rapid conversion of violaxanthin (Vio) into zeaxanthin (Zea) via antheraxanthin (Ant) under light stress conditions and the back-conversion in darkness or under subsaturating light conditions (Yamamoto et al. 1962). An analogous mechanism has not yet demonstrated in red algae, even though in some rhodophyte species XC-related pigments (Brown and McLachlan 1982, Liang 1984) and the capability to xanthophyll interconversion have been reported (Liang 1984, Rmiki et al. 1996, Ursi et al. 2003).

A number of carotenoids have been found in red algae; however, it is believed that this group presents a simple carotenoid profile, with zeaxanthin and lutein as the only xanthophylls (Bjørnland and Aguilar-Martinez 1976, Goodwin 1980). On the other hand, a more diverse carotenoid composition of several red algae has been reported (Appendix Table X). Moreover, in Rhodophyta no relationship between carotenoid content and taxonomic position of red algal species has been found (Schöbel 2002). The contradictory reports and the inconsistency about presence or absence of certain carotenoids make it difficult to understand the importance of these pigments in the regulation of PSII activity in this algal group.

The involvement of ΔpH-triggered processes in photoprotection of Rhodophyta has been reported in the unicellular species *Rhodella violaceae* (Delphin et al. 1998, Ritz et al. 1999) and also suggested for multicellular species such as *Porphyra* (Bose et al. 1988). Furthermore, other processes as the formation of inactive, dissipative PSII centers which are reconverted rapidly to active centers in the recovery process can be involved in down-regulation of photosynthetic efficiency to prevent photodamage (Leitsch et al. 1994).

As mentioned above, red algae possess phycobilisomes instead transmembranic LHC-complexes as antennae for PSII. Excitation energy within the phycobilisomes is first transferred to the chlorophyll antennae of the PSII reaction centre, from which it can then follow two paths. From the PSII antennae it may go to the reaction center of PSII, leading to the electron transport reactions to PSI. Alternatively, excitation energy may go directly from the antennae of PSII to the antennae of PSI. If the PSII reaction centers are closed, the excitation energy can be transferred directly to PSI, a phenomenon called state transitions.

It has been reported in red algae, cyanobacteria and also in green algae and higher plants (Fork and Satoh 1986, Allen 2003). In cyanobacteria, the phycobilisome movement has also been related to NPQ by decreasing the transfer of excitation energy from PBS to PSII (Bissati et al. 2000, Joshua et al. 2005, Scott et al. 2006, Ma et al. 2007).

In higher plants and green algae the energy-dependent quenching of excess light energy takes place in the LHC. In contrast, it was believed that PBSs lack the capability to dissipate excess absorbed excitation energy since no quenchers were found in these complexes (Suter et al. 1984). However, recently, evidence for a carotenoid-related quenching of phycobilisome fluorescence has been described in cyanobacteria (Rakhimberdieva et al. 2004, 2007). This quenching is associated with a soluble orange carotenoid-binding protein (OCP) (Fig. 9; Wilson et al. 2006, Kirilovski et al. 2007).

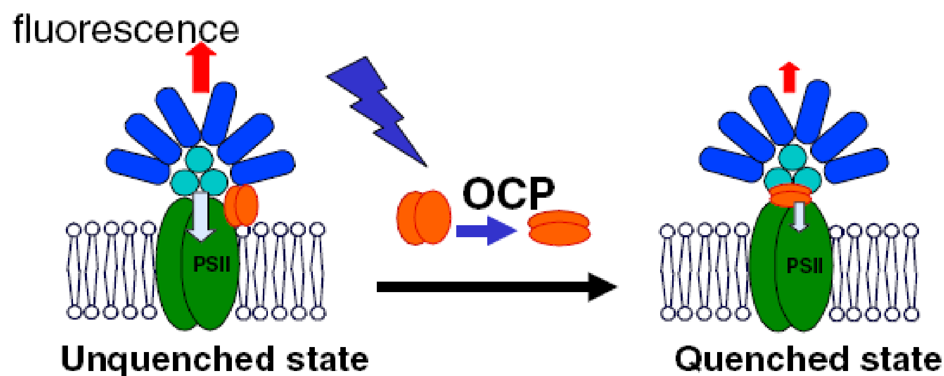

Figure 9. Working model of the function of the orange carotenoid-binding protein (OCP) in NPQ formation in cyanobacteria (taken from Kirilovski et al. 2007).

Figura 9. Modelo del funcionamiento de la proteína naranja asociada a carotenoides (OCP) en la formación de NPQ en cianobacterias (tomado de Kirilovski et al. 2007).

Ecological importance of red algae

Rhodophyta are cosmopolitan. They are found from the artic to the tropics. Most of red algal species (98%) are marine, living attached to rocks but they can also grow on other solid substrata, such as sea-walls, or more rarely on shells, seagrass or other algae. Muddy areas are generally inhospitable to red algae, although a few species such as *Gracilaria* spp., occasionally occur loose-lying or even embedded in mud.

Along the temperate waters of the California coast, the species of red algae far outnumber the species of green and brown algae. In temperate regions most of the species live in the intertidal zone. In the tropics, however, they are mostly subtidal, growing as epiphytes on seagrasses, within the crevices of rock and coral reefs, or occasionally on dead coral or sand. In some tropical waters, red algae can be found as deep as 268 m where only 0.001% (approx. 8 nmol $\cdot$ m^{-2} $\cdot$ s^{-1}) of the incident light is available for photosynthesis (Littler et al. 1985).

Red algae in the intertidal zone must compete for space with ascidians, oysters or zooxanthids in warm-water regions and with fucoid seaweeds, barnacles, and mussels on temperate shores (see Kaine and Norton 1990). Some species, however, are characteristically found growing on top of mussels or are epiphytes on other plants and seagrasses. The most distinctive zone of red algae is usually found close to or below the lower limit of barnacles, where a calcareous carpet of encrusting corallines begins and extends down to the subtidal zone (see Kaine and Norton 1990).

In the marine environment, the influences of two important factors, light and wave action, decrease logarithmically with depth, resulting in greater changes in shallow than in deep water. In temperate waters the highly favorable shallow parts are frequently dominated by

Phaeophyceae, relegating the red algae to undergrowth. In the absence of large brown algae, Rhodophyta may dominate at various levels. Some articulated corallines seem able to withstand breaking waves and may form a surf zone. Moreover, coralline red algae are extremely important in cementing together coral reefs. Although red algae often fail to dominate in biomass, they usually excel in terms of species number, increasing with depth in the northwest Atlantic, the Mediterranean, and the tropical Atlantic (Fig. 10; see Kaine and Norton 1990).

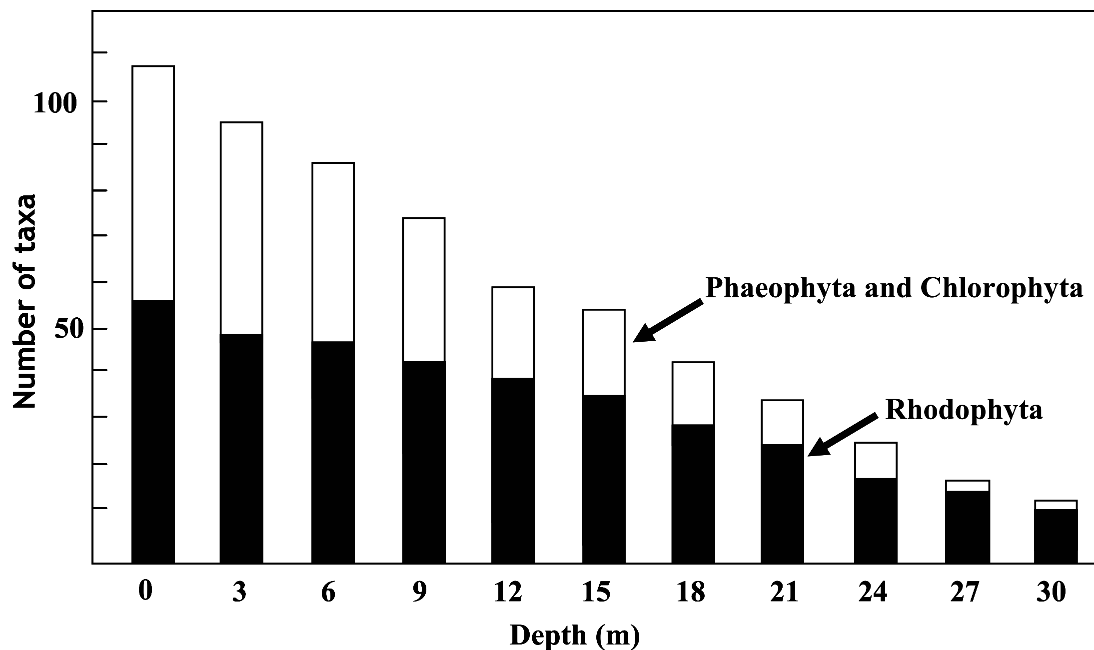

Figure 10. Number of taxa of red algae compared with the total of green, brown, and red algae at various depths (taken from Kaine and Norton 1990).

Figura 10. Número de taxas de algas rojas comparado con el numero total de algas verdes, cafés y rojas a diferentes profundidades (tomado de Kaine and Norton 1990).

The relative productivities of Rhodophyta in the intertidal region show higher value for the exposed compared to the sheltered site, quite close together in central California (Table I), mainly due to the abundance of coralline algae at the top of the subtidal zone (Kaine and Norton 1990). On a seasonal basis, red algae are most active in the winter and they are more important further south on the Pacific coast, Mexico (Table I).

Table I. The percentage of primary production (m^{-2}), attributable to red algae in some intertidal sites (from Kain and Norton 1990).
Tabla I. El porcentaje de la producción primaria (m^{-2}), atribuída a algas rojas en algunos sitios del intermareal (de Kain y Norton 1990).

Site	Remarks	%
Central California	Sheltered	29
	Exposed to waves	46
San Clemente Island, California	Spring	38
	Summer	24
	Fall	29
	Winter	55
Baja California, Mexico	Pacific side	91
	Gulf of California side	35

Scope and outline of the thesis

Red algae have a key role as primary producers in coastal areas. Thus, it is important to characterize the strategies used by this group to optimize photosynthetic performance.

How does red algae get protection against high light-conditions is still an open question. Specifically, the role of carotenoids in the photosynthetic regulation in rhodophytes is not known. Therefore, the aim of this work is to identify potential protective strategies, especially related with the carotenoid content of the species (presence of XC-related pigments, high content of lutein, antheraxanthin and/or zeaxanthin).

Moreover, from an evolutionary context, knowing the strategies for photoprotection and the molecular mechanism involved in the quenching of energy in rhodophytes is of primary importance.

The XC is a highly conserved photoprotective mechanism in algae and is present in six of the nine eukaryotic algal divisions (Larkum 2003). For red algae exist the general idea that the XC is absent (Stransky and Hager 1970, Larkum 2003). However, the evolutionary evidence indicates the contrary. The green lineage, from which evolved the higher plants, in that the XC is a fundamental photoprotective mechanisms (reviewed in Pfündel and Bilger 1994, Yamamoto 1999), presents a common origin with the red lineage (Wolfe et al. 1994, Tomitani et al. 1999, Moreira et al. 2000). In the same context there is strong evidence that the chromophyte chloroplast evolved from the lineage of the red algae by a second endosymbiotic event (Durnford et al. 1999, Green 2001, Ishida and Green 2002, Martin et al. 2002). This group, which is represented by different algal divisions, presents the diadinoxanthin cycle and some divisions also present the XC similar to green algae (Lohr and Wilhelm 1999). This indicates that the XC as photoprotective mechanism has to be

appeared before the divergence of the green and red lineage (Garcia-Mendoza 2000). Therefore, the knowledge about the presence/absence of the XC and/or its related pigments in red algae as ancestors of the Chromophyta, a dominant group in the ocean, gives information about the appearance of this photoprotective mechanism during evolution.

Thus, in the present work the following questions concerning red algal physiology were addressed:

1) Are there differences in carotenoid composition between red algal species and are they related to any taxonomic level?
2) Is the carotenoid content related with the sensitivity to high light stress in red algae?
3) Is there a differential expression of photoprotective mechanisms in red algal species with different carotenoid profile? Are there differences in the carotenoid content due to acclimation of the photosynthetic tissue and thus differential expression of PSII regulation?

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Chapter I

CAROTENOID COMPOSITION OF MARINE RED ALGAE

I.1. Abstract

Photosynthetic organisms possess carotenoids that function either as accessory, photoprotective or structural pigments. Therefore, the carotenoid profile provides information about certain photoacclimation and photoprotection responses. Carotenoids are also important chemosystematic markers since specific enzymes mediate each step of carotenoid biosynthesis. For red algae, diverse and often contradictory carotenoid compositions have been reported. As a consequence, it is difficult to infer the physiological importance of carotenoids in Rhodophyta. To characterize the relationship between carotenoid composition, rhodophycean phylogeny and the presence of potentially photoprotective pigments, we analyzed the carotenoid composition of 65 subtropical species from 12 orders and 18 rhodophyte families. Our results showed that red algae do not present a unique carotenoid profile. However, a common profile up to the level of order, with exception of the Ceramiales and the Corallinales, was found. The main difference between profiles is related to the xanthophyll that represents the major carotenoid. In some species lutein is the major carotenoid while in others it is substituted by zeaxanthin or antheraxanthin. The presence of this epoxy carotenoid together with the presence of violaxanthin that are xanthophyll cycle-related pigments were found in 4 of the 12 analyzed orders. The implications of the carotenoid pigment profiles are discussed and it

is suggested that the xanthophyll cycle related pigments appeared early in the evolution of eukaryotic phototrophs.

I.2. Introduction

Carotenoids play a key role in oxygenic photosynthesis, as accessory pigments for harvesting light or as structural molecules that stabilize protein folding in the photosynthetic apparatus (Siefermann-Harms 1987). Some carotenoids have protective functions, either as direct quenchers of reactive oxygen species (Edge et al. 1997) or playing a role in the thermal dissipation of excess of energy in the photosynthetic apparatus (Havaux and Niyogi 1999).

All photosynthetic eukaryotes are able to synthesize lycopene, a C_{40} polyene, which is the precursor for two different carotenoid synthesis pathways, the β,ε-carotene and the β,β-carotene pathway (Hirschberg et al. 1997). Xanthophylls are oxidation products of the carotenes and diversification of xanthophylls increases by the inclusion of allene or acetylene groups. Allenic and acetylenic carotenoids are highly represented in algae and at least 30 different carotenoids have been identified in this group (Jeffrey et al. 1997). The distribution of carotenoids having different molecular structures or the presence of specific biosynthesis pathways can be an index for algal classification (Mimuro and Akimoto 2003). Therefore, carotenoids are considered as important chemosystematic markers (Liaaen-Jensen 1977) and can be taxon-specific indicators (Mackey et al. 1998).

The presence or absence of certain carotenoids or their relative concentration also gives information about the possible acclimation or photoprotection responses of the organisms.

For instance, the presence of carotenoids involved in the xanthophyll cycle (XC) indicates the possibility that the organisms might express this photoprotection response. The XC is the rapid conversion of violaxanthin (Vio) into zeaxanthin (Zea) via antheraxanthin (Ant) under light stress and the reverse reaction in darkness or subsaturating light (Yamamoto et al. 1962). In some groups of the Chromophyta the XC consists in the interconversion of diadinoxanthin (Ddx) into diatoxanthin (Dtx). Both cycles are involved in the thermal dissipation of excess light that brings effective photoprotection to the photosynthetic apparatus (Demmig-Adams 1990, Lavaud et al. 2004).

The XC is a highly conserved photoprotective mechanism in algae. This cycle is present in 6 of the 9 eukaryotic algal divisions (Larkum 2003). In some groups the Vio to Zea cycle takes place and in some Chromophyta both cycles are present, although the Ddx to Dtx cycle is preferentially expressed (Lohr and Wilhelm 1999). The Cryptophyta, Glaucophyta and Rhodophyta do not possess the XC (Stransky and Hager 1970a, Larkum 2003). However, the presence of Vio and Ant in red algae (Aihara and Yamamoto 1968, Brown and McLachlan 1982, Liang 1984) and changes of the concentration of these pigments under different light conditions (Liang 1984, Rmiki et al. 1996, Ursi et al. 2003) suggest the presence of the XC in this group.

There are contradictory reports about the carotenoid content in red algae. It was believed that this group has a carotenoid profile, with zeaxanthin and lutein as the only xanthophylls (Bjørnland and Aguilar-Martinez 1976, Goodwin 1980). In contrast, more complex carotenoid profiles have been reported, with the presence of allenic and acetylenic carotenoids such as fucoxanthin and neoxanthin, respectively (Bjørnland and Aguilar-Martinez 1976, Czeczuga 1979, Schöbel 2002). The high diversity of carotenoids reported

for red algae and the inconsistency in the presence or absence of certain carotenoids makes it difficult to infer about the role of these pigments in the acclimation and photoprotection responses of this group. Moreover, it seems that there is no relationship between carotenoid content and taxonomic position of red algal species (Schöbel 2002). Therefore, in order to elucidate a possible relationship of carotenoid profile and phylogeny of red algae and to assess the presence of XC-related pigments in this group we characterized carotenoid content of different red algal species representative of several rhodophyte families. The carotenoid content found in different species is discussed in relation to the evolution of the Rhodophyta and of the other algal groups.

***I*.3. Materials and methods**

Algal material. Pigment content was analyzed in red algal species collected from different coastal locations near Ensenada, Baja California. The algae were collected from the inter-tidal zone and below a *Macrocystis pyrifera* kelp forest by SCUBA diving. Also, species from the Gulf of California were collected from the intertidal zone.

The algae were collected during different times throughout the year. Pigments were analyzed also in some species, cultivated by spore germination according to Collantes and Melo (1985) and maintained under controlled conditions to avoid epiphyte contamination. The species *Porphyridium purpureum* (strain POC1) was obtained from CICESE microalgae culture collection (http://acuicultura.cicese.mx/cepario.htm).

Pigment extraction. To avoid a contamination by the presence of epiphytes, the collected algae were meticulously cleaned with a brush and filtered seawater (0.45 µm) together with ultrasonication in a waterbath three times for 1 min. After this treatment, samples from the

organisms were frozen by immersion in liquid nitrogen and afterwards kept at –40º C until the analysis. The pigments were extracted with dimethylformamide (DMF) for 24 h at 4º C in darkness after grinding the tissue in liquid nitrogen. The extract was centrifuged (15600 g, 5 min) and the pigments were analyzed by High-Performance Liquid Chromatography (HPLC).

HPLC analysis. Pigment separation and quantification was performed as in Wright et al. (1991), using ethylacetate as solvent B and acetonitrile:water (9:1) as solvent A, delivered at a flow rate of 1.0 mL · min^{-1}. The analyses were performed at constant room temperature. To obtain a better resolution of the pigments lutein and zeaxanthin, the solvent delivery profile was modified (%B, time): 0%, 0 min; 0%, 2 min; 10%, 2.6 min; 35%, 13.6 min; 55%, 20 min; 55%, 25 min; 0%, 27 min. These modifications in the solvent delivery profile resulted in a baseline separation of lutein and zeaxanthin (see Fig. 11 and 12) with a resolution of 1.51. The HPLC equipment was a Shimadzu AV-10 system equipped with a Nucleosil C-18 reverse phase column (250 mm, 4.6 mm internal diameter and 5 µm particle size) and a guard column Eclipse XDB-C8 (12.5 mm, 4.6 mm internal diameter and 5 µm particle size). The detection wavelength was set at 447 nm. Control of the equipment and peak area calculation was performed with the Shimadzu EZChrom Chromatography Data System software (Shimadzu Scientific Instruments, Inc.).

For pigment identification, the retention time (Rt) of each peak was compared with the Rt of commercial and isolated pigment standards with which the HPLC was calibrated. The following standards were used: Chl *a*, Chl *b*, fucoxanthin, violaxanthin, zeaxanthin, lutein, β-cryptoxanthin, β-carotene, α-carotene (from DHI, Water and Environment; Denmark),

and antheraxanthin (Ant) that was isolated and purified from *Bossiella orbignyana* (Corallinales, Rhodophyta). The identification of this pigment was achieved by comparing with chromatographs of higher plants (data not shown) and by UV-VIS absorption characteristics (Perkin Lambda 40 spectrometer) of the isolated pigment after crystallization under N_2 gentle flow and re-dissolution in 100% ethanol. Purity and concentration determination of the standard was as in Jeffrey et al. (1997). In the cases in which pigments like fucoxanthin or Chl *b* were detected that indicated the possible presence of epiphytes, the analysis was repeated using another sample of the same species.

Cladistic analysis. The taxa were clustered together based on the number of their similarities, in this case, the similarity of their carotenoid composition. Preliminary analysis showed that the carotenoid composition of the species from the same order were similar; therefore for the cladistic analysis the orders were treated as operational taxonomic units (OTUs), except for Corallinales and Ceramiales (see Results) where the OTUs were subfamilies and families, respectively. The number of species of the same family and order could be considered as replicates for this taxonomic level. Therefore, with few exceptions, most of the orders were well represented since carotenoid profiles of more than three species of each family were determined. The presence of the carotenoids was used as character and included in a binary matrix for a cluster analysis. The average distance based on the dissimilarity between each OTU-pair was calculated using the Euclidian distance. The resulting distance matrix was used in a Neighbour-Joining cluster analysis, which was performed with the MEGA 3.0 software (Kumar et al. 2004).

I.4. Results

The retention time of some of the standards and the absorption characteristics of antheraxanthin (Ant), obtained by purification from *Bossiella orbignyana,* are shown in figure 11.

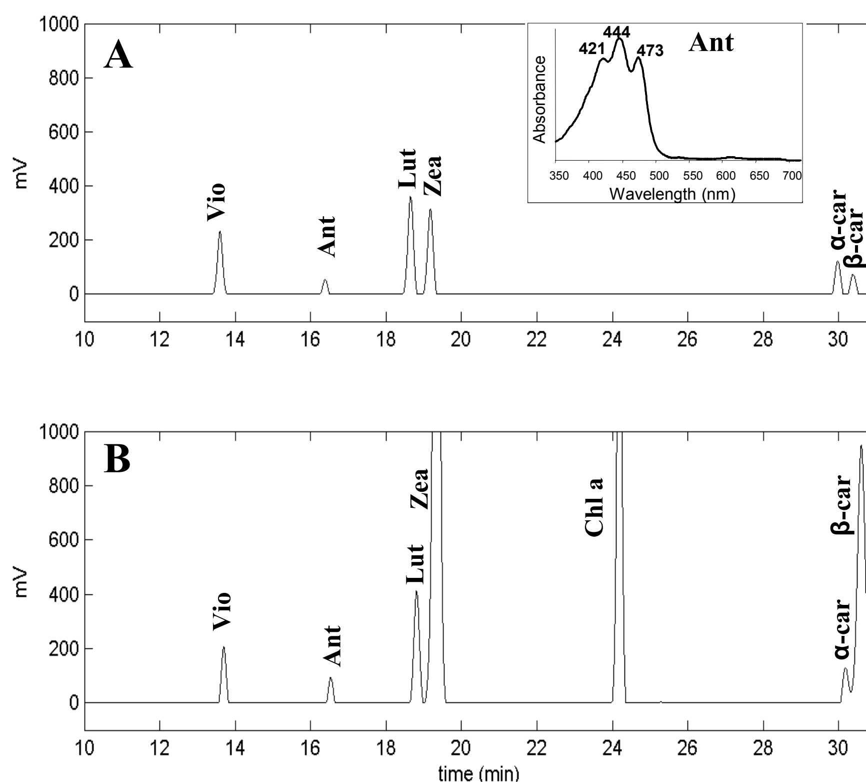

Figure 11. Identification of hydrophobic pigments in red algae. (A) Chromatograph of a mixture of 5 commercial carotenoid standards (Vio, violaxanthin; Lut, lutein; Zea, zeaxanthin; α-car, α-carotene; β-car, β-carotene) and antheraxanthin (Ant) purified from *Bossiella orbignyana*. The absorption characteristics of this pigment are presented in the inset. (B) Enrichment (peak spiking) of an extract of *Polysiphonia pacifica* with the carotenoid standard mixture.

Figura 11. Identificación de pigmentos hidrofóbicos en algas rojas. (A) Cromatograma de una mezcla de 5 estándares comerciales (Vio, violaxantina; Lut, luteína; Zea, zeaxantina; α-car, α-caroteno; β-car, β-caroteno) y anteraxantina (Ant) purificado de *Bossiella orbignyana*. Las características de absorción de ese pigmento se presentan en el recuadro interior. (B) Enriquecimiento de un extracto de *Polysiphonia pacifica* con la mezcla de estándares de carotenoides.

The maximum absorption peaks of Ant were at 421, 444, 473 nm and the III/II was 55% that confirmed the identity of the isolate. To verify the correct identification of the pigments, red algal extracts were enriched (co-chromatography) with a pigment standard mixture (Fig. 11).

Carotenoid signature in red algae was different among the analyzed species. Since the pigment profile could be result of a specific light-acclimation, we analyzed samples in duplicate or triplicate from species collected in different locations. Also, pigment analyses of the same species collected during different times of the year were performed. The variation in the relative concentration of major carotenoids was less than 10% of the total carotenoid content but in no case, the major carotenoid changed from one into another (data not shown).

Three main carotenoid profiles were identified in red algae. Most of the species presented lutein as the major carotenoid (Fig. 12A). In some other species Zea represented more of the 50% of the total carotenoid content (Fig. 12B). Xanthophyll cycle related carotenoids (Vio or Ant) were also detected in some species. The concentration of these pigments generally represented a low proportion of the total carotenoid content (Fig. 12B), but in some cases, Ant was the major carotenoid (Fig. 12C). The only ubiquitous pigments were α-carotene and β-carotene, even though, in some cases only α-carotene or β-carotene was detected (Fig. 12).

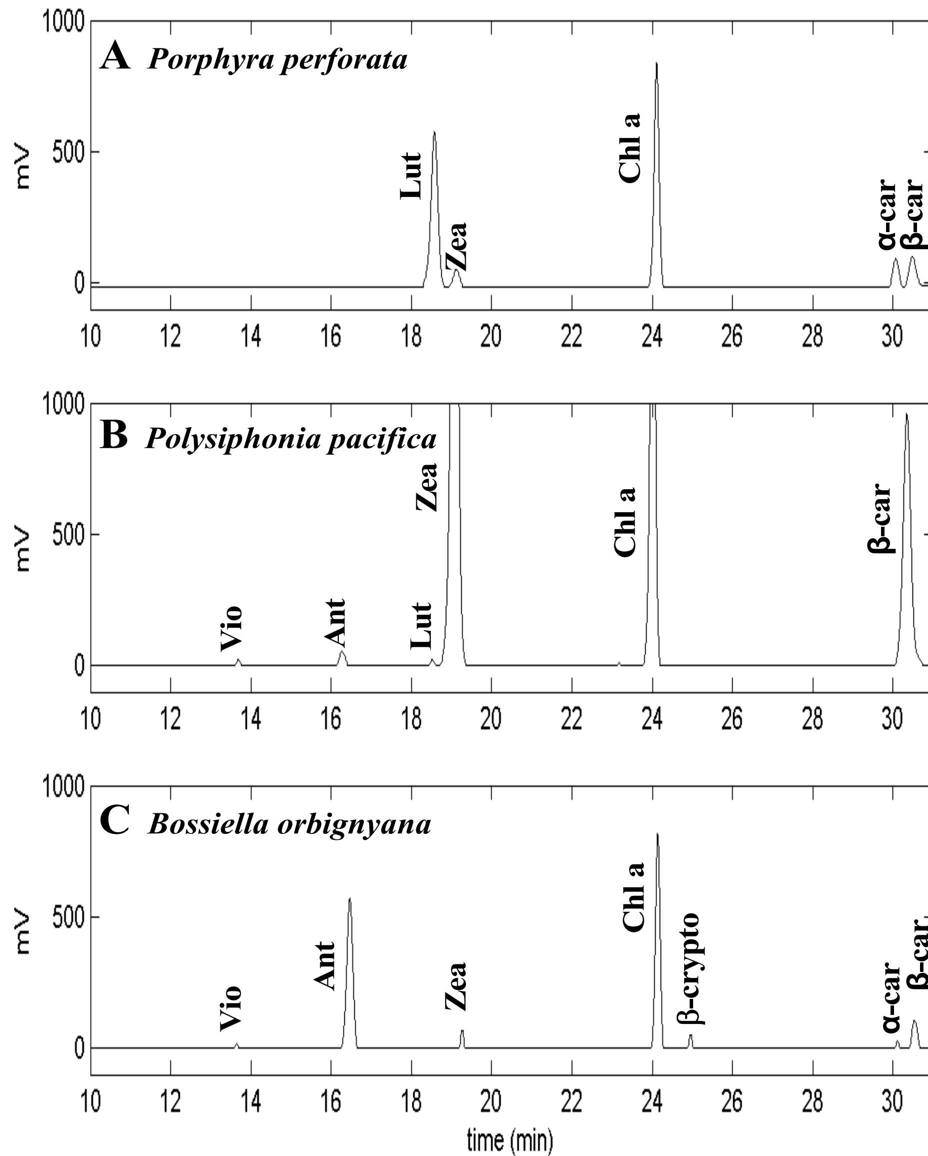

Figure 12. Principal carotenoid profiles detected in red algae. Chromatographs of extracts from A) *Porphyra perforata*, (B) *Polysiphonia pacifica*, and (C) *Bossiella orbignyana* (β-crypto, β-cryptoxanthin).

Figura 12. Principales perfiles de carotenoides detectados en algas rojas. Cromatogramas de extractos de A) *Porphyra perforata,* (B) *Polysiphonia pacifica* y (C) *Bossiella orbignyana* (β-crypto, β-criptoxantina).

The analyzed species were grouped (Table II) according to the systematics of Rhodophyta proposed by Silva (1998) and Harvey et al. (2003). The species presented a similar carotenoid profile up to the order level, with the exception of the Corallinales and Ceramiales (Table II).

Table II. Carotenoid composition of different rhodophyte species. The major carotenoid is highlighted.
Tabla II. Composición de carotenoides en diferentes especies de rodófitas. El carotenoide mayor es resaltado en negritas.

Order	Family	Specie	% of total carotenoids						
			Vio	**Ant**	**Lut**	**Zea**	**ß-cryp**	**a-car**	**ß-car**
BANGIOPHYCEAE									
Bangiales	Bangiaceae	*Bangia atropurpurea*			**73.5**	20.3		2.2	4.0
		Porphyra perforata			35.0	11.4		4.1	**49.5**
		P. perforata (conchocelis)			**50.8**	31.9	14.2	0.3	2.8
		P. suborbiculata			**65.5**	34.5			
		P. thurettii			**76.1**	23.9			
Erythropeltidales	Erythrotrichiaceae	*Smithora naiadum*	1.2	33.5		**62.4**			3.0
Porphyridiales	Porphyridiaceae	*Porphyridium cruentum*				**97.4**			2.6
FLORIDEOPHYCEAE									
Nemaliales	Chaetangiaceae	*Scinaia confusa*			**81.6**			10.7	7.7
		S. latifrons			**56.4**			29.6	14.0
Rhodymeniales	Rhodymeniaceae	*Fauchea galapagensis*			**51.2**			48.8	
		Rhodymenia californica			**96.4**			3.6	
Cryptonemiales	Halymeniaceae	*Grateloupia setchelii*			**70.7**	2.2		1.6	25.5
		Halymenia hollenbergii			**95.3**			4.7	
		Prionitis cornea			**75.8**	9.3		15.0	
		P. lyalii			**81.0**	18.4		0.5	0.1
	Kallymeniaceae	*Callophyllis pinnata*			**97.3**			1.2	1.5
Gelidiales	Gelidiaceae	*Gelidium coulterii*			**75.1**	24.5		0.3	
		G. johnstonii			**81.4**	11.5		4.7	2.4
		G. pusillum			**69.6**	16.6	7.7	6.1	
		G. robustum			**89.5**	9.8		0.6	0.2
		G. robustum [1]			**86.3**			13.7	
		Pterocladia capillaceae			**89.7**	4.2		3.5	2.6
Gigartinales	Gigartinaceae	*Chondracanthus canaliculatus*			**60.8**	9.5		0.4	29.3
		C. corymbiferus			**88.8**	1.6		3.6	5.9
		C. exasperatus			**84.1**	13.8	1.7	0.2	
		C. harveyanus			**94.0**	6.0			

Table II. continued

Order	Family	Specie	% of total carotenoids						
			Vio	**Ant**	**Lut**	**Zea**	**ß-cryp**	**a-car**	**ß-car**
	Gigartinaceae	*C. squarrulosus*			**87.6**	8.9	0.2	0.6	2.8
		C. volans			**85.8**	13.3			0.9
		Iridaea chordata			**86.6**	13.4			
		I. chordata [1]			**99.7**			0.3	
		Mazzaella affinis			**75.1**	20.7		3.0	1.2
		M. flaccida			**50.7**	16.8		7.3	25.2
		M. leptorhynchus			**82.4**	17.6			
	Phyllophoraceae	*Mastocarpus papillatus*			**80.8**	18.1		0.2	0.9
	Solieriaceae	*Eucheuma uncinatum*			**92.6**	4.2	0.6	0.9	1.7
		Opuntiella californica			**91.7**	2.5		3.1	2.8
Plocamiales	Plocamiaceae	*Plocamium cartilagineum*			**95.4**	0.7		2.1	1.7
		P. violaceae			**82.1**	8.6		3.8	5.4
Gracilariales	Gracilariaceae	*Gracilaria gracilis*	0.7	6.1		**76.8**	10.2		6.2
		G. textorii	2.3	32.3		**59.9**			5.5
		Gracilariopsis lemaneiformis	0.9	18.8		**78.6**	1.1		0.6
Ceramiales	Ceramiaceae	*Centroceras clavulatum*			**60.3**	12.8	6.4	3.5	17.1
		Microcladia coulteri			**79.6**	19.2			1.2
		Neoptilota densa			**92.2**			3.6	4.2
		N. hypnoides			**72.6**			1.8	25.6
		Ptilota filicina			**84.3**	8.8		7.0	
	Delesseriaceae	*Acrosorium ciliolatum*			**97.7**	0.8		0.9	0.6
		Asterocolax gardneri			**98.1**			1.9	
		Cryptopleura farlowiana			**93.2**	6.6		0.2	
		C. lobulifera			**97.2**			2.1	0.7
		Phycodrys amplissima			**86.7**			8.7	4.6
		Polyneura latissima			**49.4**			34.0	16.5
	Rhodomelaceae	*Laurencia gardneri*	8.0	1.9	18.9	**43.7**			27.5
		Osmundea spectabilis		4.3		31.8	5.2		**58.7**
		Polysiphonia hendryi		3.1	2.6	**89.9**			4.4
		P. pacifica	0.3	4.0	3.5	**57.0**			35.2

Table II. continued

Order	Family	Specie	% of total carotenoids						
			Vio	Ant	Lut	Zea	ß-cryp	a-car	ß-car
Corallinales	Corallinaceae								
	Corallinoideae	*Bossiella californica*	5.1	**69.5**		19.3			6.2
		B. orbignyana	1.8	**61.2**		8.5	5.2	2.2	21.2
		Corallina officinalis	3.1	**56.7**		32.3	4.6	0.3	2.9
		C. vancouverensis	1.0	**67.1**		13.9	10.5		7.5
		Jania tenella	3.5	**69.2**		12.4	5.2		9.7
	Lithophylloideae	*Lithophyllum margaritae*			**47.3**	2.8		5.8	44.0
		Lithothrix aspergillum			**48.2**	5.2	5.2	9.8	31.7
		Amphiroa zonata			**67.4**	8.6	6.7	3.9	13.5
	Hapalidiaceae								
	Melobesioideae	*Melobesia mediocris*			**77.5**	8.7		3.3	10.5

[1] low tidal species,

Vio, violaxanthin; Ant, antheraxanthin; Lut, lutein; Zea, zeaxanthin; β-cryp, β-cryptoxanthin; α-car, α-carotene; β-car, β-carotene

[1] especies de la parte inferior del intermareal,

Vio, violaxantina; Ant, anteraxantina; Lut, luteína; Zea, zeaxantina; β-cryp, β-criptoxantina; α-car, α-caroteno; β-car, β-caroteno

In the Ceramiales and Corallinales the carotenoid composition was different between species of different families or subfamilies, respectively. Therefore, for the cladistic analysis, the rhodophyte orders and in the case of the Ceramiales and Corallinales, families and subfamilies, respectively, were considered as taxonomic operational units. The analysis was focused on the presence or absence of the following carotenoids: zeaxanthin, lutein, antheraxanthin and violaxanthin. Three principal groups emerged from the cladistic analysis (Fig. 13).

The group with the simplest carotenoid composition was denominated as the Zea-group, with zeaxanthin and β-carotene only. This group was represented by the unicellular alga *P. cruentum* that belongs to the order Porphyridiales.

Most of the species belonged to the Lut-group. Lutein was the principal carotenoid in species of this group, while zeaxanthin was absent or present in minor concentration. The Lut-group was composed by the orders Nemaliales, Rhodymeniales, Gelidiales, Gigartinales Cryptonemiales, Bangiales, Plocamiales, Ceramiales and Corallinales (Fig. 13). Not all the families from the last two orders belonged to this group and their carotenoid composition is described separately. In some organisms of the Lut-group zeaxanthin was not detected (Table II). However, the amount of this pigment seemed to be related to the acclimation state of the alga. Organisms collected at the high tidal zone presented zeaxanthin while in organisms of the same species but from low tidal zone zeaxanthin was absent (Table II).

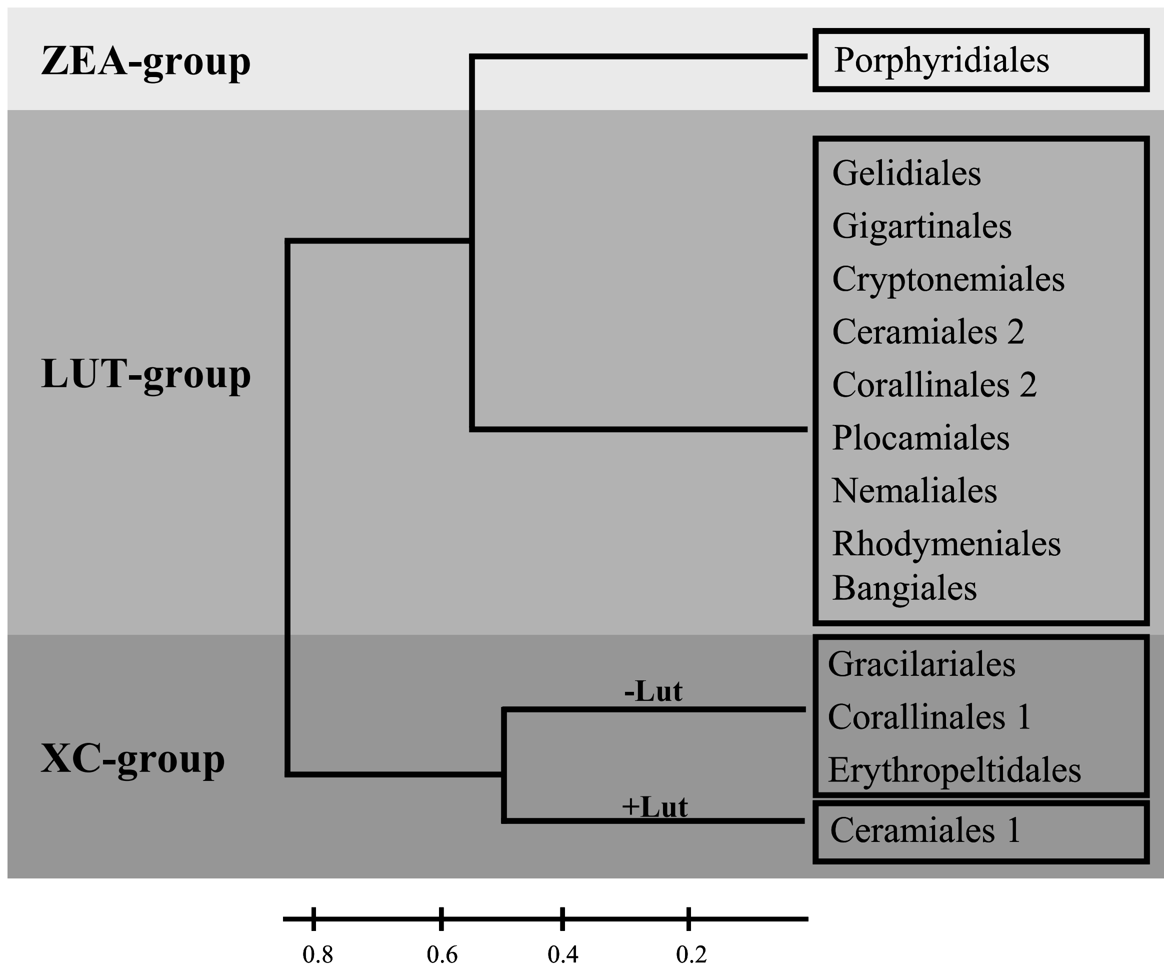

Figure 13. Grouping of rhodophyte orders superimposed on cladistic analysis based on the similarity of the carotenoid composition of the different analyzed species. Lut-group is represented by species with lutein as the major pigment. In the Zea-group, zeaxanthin was the major carotenoid while in the XC-group, the xanthophyll cycle-related pigments violaxanthin and antheraxanthin were detected (+, presence of minor carotenoid; -, absence of minor carotenoid).

Figura 13. Agrupación de órdenes de las Rhodophyta de acuerdo al análisis cladístico basado en la similitud de la composición de carotenoides en las diferentes especies analizadas. El grupo Lut está representado por especies con luteína como el pigmento principal. En el grupo Zea, zeaxantina fue el carotenoide mayor, mientras que en el grupo XC, se detectaron los pigmentos relacionados con el ciclo de las xantofilas, violaxantina y anteraxantina (+, presencia de carotenoide menor; -, ausencia de carotenoide menor).

The third pigment group was composed of species that presented pigments involved in the xanthophyll cycle such as antheraxanthin and violaxanthin. Therefore, this group was designated as the XC-group (Fig. 13). With the exception of the Corallinales, zeaxanthin was the main carotenoid of the species belonging to this group. Antheraxanthin represented up to 77% of the total carotenoid content in the Corallinales of the XC-group (Table II). Two minor groups according to the presence or absence of lutein were formed within the XC-group (Fig. 13). The only species from the XC-group that possessed lutein (+Lut) belonged to the Rhodomelaceae, a family of the Ceramiales (Ceramiales1; Fig. 13).

As stated above, red algal species of the same order presented a common carotenoid profile, however, there were exceptions. Most of the Ceramiales families are included into the Lut-group (Ceramiales 2; Fig. 13), except for the Rhodomelaceae that are grouped with the species that presented XC-related pigments (Ceramiales 1; Fig. 13). The Corallinales represented another exception. In this order, differences in carotenoid composition were detected at the subfamily level. Species of the Corallinoideae subfamily (family Corallinaceae) showed the carotenoid composition of the XC-group, even though antheraxanthin and not zeaxanthin was the predominant carotenoid (Corallinales 1; Fig. 13). In contrast, species from the other subfamily of the Corallinaceae (Lithophylloideae) and from the Hapalidiaceae family of the Corallinales presented the most common pigment profile between the red algae with lutein as the major carotenoid and zeaxanthin in minor concentration (Corallinales 2; Fig. 13).

I.5. Discussion

Red algae do not possess a unique carotenoid profile. Differences of carotenoid composition between species are related to the presence or absence of specific minor carotenoids but mainly in the xanthophyll that represents the major carotenoid. A diverse carotenoid composition of red algae has been reported before (Liang 1984, Schöbel 2002). However, we did not find allenic or acetylenic carotenoids. Probably, the presence of fucoxanthin (Bjørnland and Aguilar-Martinez 1976, Czeczuga 1979, Schöbel 2002) and neoxanthin (Czeczuga 1979) was associated with epiphyte contamination of the samples. In this study, the most complex molecule detected was violaxanthin that results from the epoxidation of antheraxanthin. The detection of these two pigments in several species (Table II) confirmed the presence of epoxy carotenoids in the red algal group, which has been questioned by several authors (Stransky and Hager, 1970a, Vershinin and Kamnev 1996, Carnicas et al. 1999).

This is the first report that gives a taxonomical significance to the carotenoid content of red algae. We found a common carotenoid profile up to the level of order, with exception of the Ceramiales and the Corallinales. The simplest profile is present in the Porphyridiales with zeaxanthin and β-carotene as the only carotenoids (Table II, Stransky and Hager 1970b, Cunningham et al. 1989, Doan et al. 2003). Unicellular red algae possess the simplest carotenoid profile of all eukaryotic algae, comparable only with the Glaucophyta (van den Hoek et al.1995).

Several species possess lutein as the principal carotenoid (Lut-group; Fig. 13). This is the most common profile in red algae since it was found in species from 9 of the 12 analyzed

rhodophycean orders. In some species of the Lut-group zeaxanthin was present, but in low concentration (Fig. 13). Therefore, the α-carotene synthesis pathway is preferentially expressed in most of red algal species as compared to the β-carotene pathway. This last pathway seems to be absent or maintained at the β-carotene level without zeaxanthin synthesis in several rhodophyte orders (Table II). Probably the lack of Zea was related to the acclimation state of the algae. However, it is also probable that some rhodophyte species have lost the capability to synthesize this pigment, since no zeaxanthin has been reported in species from the Rhodymeniales (Table II; Liang 1984), the Nemaliales (Table II; Bjørnland and Aguilar-Martinez 1976, Liang 1984,) and the Palmariales (Sagert and Schubert 2000, Marquardt and Hanelt 2004).

On the other hand, there are some species that favor the β-carotene synthesis pathway, accumulating violaxanthin, antheraxanthin and zeaxanthin. These species form the XC-group and belong to the Gracilariales, Ceramiales (fam. Rhodomelaceae), Corallinales (subfam. Corallinoideae) and Erythropeltidales. The Gracilariales best represent the group of red algae with XC-pigments since these pigments have been detected in several species of this order (Table II; Brown and McLachlan 1982, Liang 1984, Rmiki et al. 1996, Ursi et al. 2003). Zeaxanthin was the major carotenoid in the Gracilariales (Table II) that coincides with previous reports (Brown and McLachlan 1982, Liang 1984, Rmiki et al. 1996), even though in some species of the genus *Gracilaria* antheraxanthin was reported as the principal carotenoid (Aihara and Yamamoto 1968, Brown and McLachlan 1982, Liang 1984, Ursi et al. 2003). The reason for this difference is not clear. It has been proposed that the proportion of zeaxanthin to antheraxanthin is correlated to the growing irradiance

(Brown and McLachlan 1982). A high zeaxanthin to antheraxanthin ratio was found in field-collected and greenhouse-grown material, while algae incubated in laboratory under low irradiance displayed the inverse relationship (Brown and McLachlan 1982, Liang et al. 1984, Ursi et al. 2003). The accumulation of zeaxanthin is probably an acclimation response to light stress condition in the Gracilariales. The Corallinales possessed antheraxanthin as the principal carotenoid. Since antheraxanthin synthesis is an intermediate step of the xanthophyll cycle, the accumulation of this pigment is unusual in photosynthetic organisms. In the Prasinophyta *Mantoniella squamata,* with a truncated XC, antheraxanthin is accumulated in minutes as a response to light stress (Goss et al. 1998). It is not known if accumulation of antheraxanthin in the Corallinales is associated to the XC. The only rhodophyte species in that an interconversion of xanthophyll cycle pigments has been reported are from the Gracilariales (Liang 1984, Rmiki et al. 1996, Ursi et al. 2003). However, it is unknown if these pigments have a photoprotective role in this group. Nevertheless, the presence of antheraxanthin and violaxanthin indicates that red algae can synthesize epoxy carotenoids that might play a role in photoprotection.

Carotenoid profiles and rhodophyte phylogeny. The rhodophyte grouping according to its pigment profile (Fig. 13) does not match to the phylogenetic relationships as proposed by Ragan et al. (1994) and Harper et al. (2001). For example, species of the XC-group, like the Gracilariales, are closer related with orders of the Lut-group, than with species of the same pigment group. The Ceramiales and Corallinales represent another example of the inconsistency between pigment content and phylogeny of red algae. The Ceramiales is a polyphyletic group with the Ceramiaceae as outgroup (Saunders et al. 1996, Choi et al. 2002). In contrast, in our cladistic analysis the Rhodomelaceae (Ceramiales 2; Fig. 13) was

outgrouped from the Ceramiales (Ceramiales 1; Fig. 13) since they possess XC-related pigments. In respect to the Corallinales, the species do not share a common carotenoid profile, not even at the subfamily level; even 18S rDNA data show that they are a monophyletic group (Saunders and Bailey 1997).

Failure of relationship between phylogeny and pigment profiles could be explained by the suggestion that XC-pigments (violaxanthin and antheraxanthin) and both, the β,β-carotene and the β,ε-carotene synthesis pathways were present early in the evolution of red algae, before the diversification of the different rhodophyte groups. Therefore, we suggest that all red algal species evolved from an ε-cyclase synthesizing ancestor with the capability to synthesize XC-related pigments and that some groups suppressed or lost the capability of synthesizing specific carotenoids through evolution (lack of XC-related pigments or lutein), as was postulated by others (Brown and McLachlan 1982, Liang 1984). This suggestion is further supported by the observation that orders from the two Rhodophyta classes, the Bangiophyceae and the Florideophyceae that diverged 800 Ma ago (Saunders and Hommersand 2004), belong to the Lut-group and the XC-group.

Apparently, most of rhodophyte species lost or suppressed the capability to synthesize certain carotenoids or even a carotenoid synthesis pathway (Fig. 14). Specifically, the β,ε-pathway was lost at least twice in the rhodophyte group, in the Gracilariales and in the Porphyridiales. Also, the synthesis of XC-related pigments was lost several times in the Rhodophyta.

The only species that conserved the ancient carotenoid profile belong to the family Rhodomelaceae of the Ceramiales. In these species the β,β-synthesis pathway is expressed

up to violaxanthin and the β,ε-pathways up to lutein (Fig. 14). Comparable to the Rhodomelaceae, species from the Corallinoideae possess an ancient carotenoid profile with the expression of both carotenoid biosynthetic pathways. Only lutein synthesis was lost in these species (Fig. 14). However, an ancient carotenoid profile was not maintained in all the families and subfamilies of the Ceramiales and Corallinales. The capability to synthesize XC-related pigments was lost after the divergence of the different families or subfamilies of these orders (Fig. 14).

The suppression or lost of the capability to synthesize certain carotenoids such as XC-related pigments indicate that red algae protect themselves independently of the expression of the xanthophyll cycle. Red algae present probably other photoprotective responses that allow them to colonize environments as the high intertidal zone, where the light stress is high.

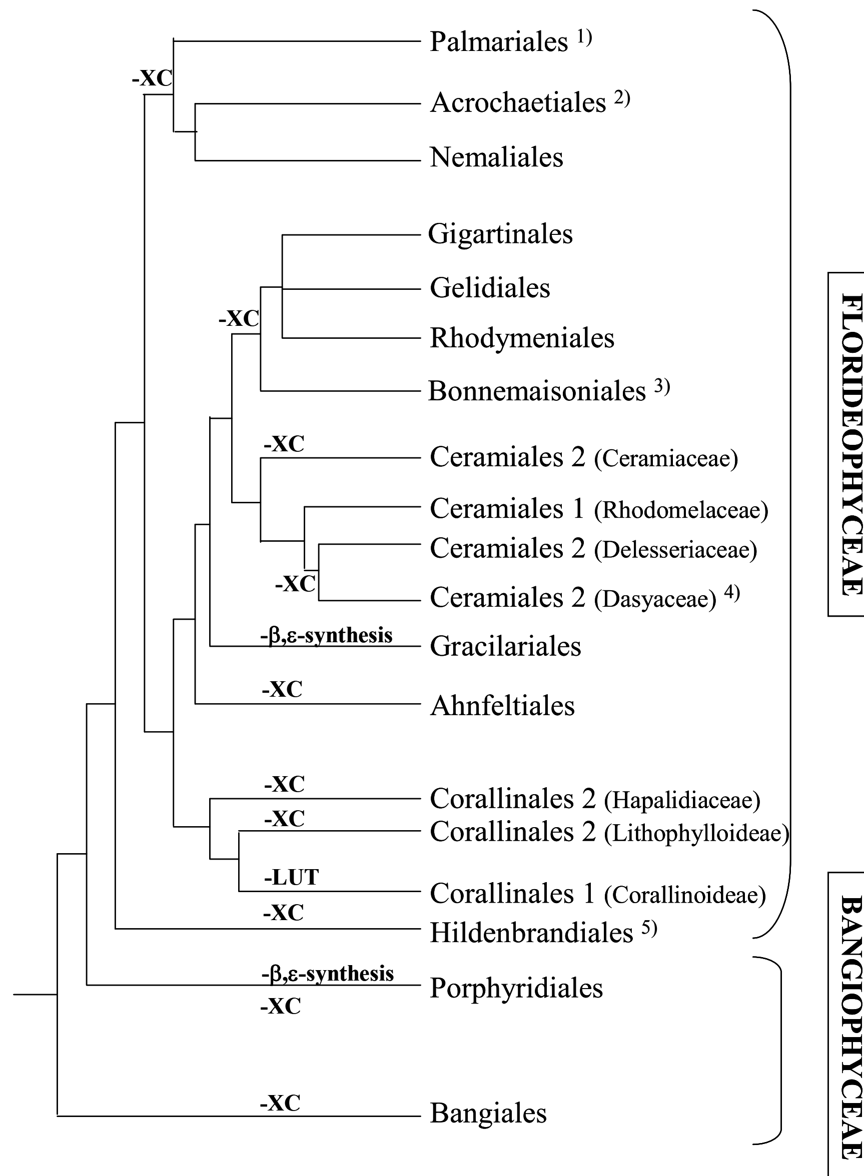

Figure 14. Lost or suppression of carotenoid synthesis in red algae. The missing carotenoids and carotenoid synthesis pathway (-XC, xanthophyll cycle-related pigments; –LUT, lutein; -β,ε-synthesis, β,ε-synthesis pathway) are superimposed to the phylogenetic tree proposed by Ragan et al. (1994) The carotenoid profiles in the Corallinales and Ceramiales showed differences at the subfamily and family level, respectively. Therefore, the phylogeny of the Corallinales according to Harvey et al. (2003) and the phylogenetic relationship in the Ceramiales according to Choi et al. (2002) were included in the tree. Since species from some orders represented in the tree were not analyzed in the present study, the carotenoid pigment profile reported by other authors is included: [1] Sagert and Schubert (2000), Marquardt and Hanelt (2004); [2] Strain (1966), Schagerl and Donabaum (2003); [3] Strain (1966), Bjørnland and Aguilar-Martinez (1976), Liang 1984; [4] Liang 1984; [5].Schöbel (2002).

Figura 14. Pérdida o supresión de la síntesis de carotenoides en algas rojas. Los carotenoides y vías de síntesis de carotenoides faltantes (-XC, pigmentos relacionados con el ciclo de las xantofilas; –LUT, luteína; -β,ε-síntesis, vía de β,ε-síntesis) son incluidos en el árbol filogenético propuesto por Ragan et al. (1994). Los perfiles de carotenoides en las Corallinales y Ceramiales mostraron diferencias al nivel de subfamilia y familia, respectivamente. Por lo cual, se incluyeron en el árbol la filogenia de las Corallinales de acuerdo a Harvey et al. (2003) y las relaciones filogenéticas de las Ceramiales de acuerdo a Choi et al. (2002). Debido a que especies de ciertos órdenes representados en el árbol no fueron analizadas en el presente trabajo, se incluyo el perfil de carotenoides reportados por otros autores: [1)] Sagert y Schubert (2000), Marquardt y Hanelt (2004); [2)] Strain (1966), Schagerl y Donabaum (2003); [3)] Strain (1966), Bjørnland y Aguilar-Martinez (1976), Liang 1984; [4)] Liang 1984; [5)].Schöbel (2002).

Red algal carotenoid composition and algal evolution. Carotenoid composition in red algae fits the model of plastid evolution of photosynthetic eukaryotes. Glaucophyta, red and green algal lineages diverged from the primeval photosynthetic organism after the primary endosymbiotic event (van de Hoek et al. 1995, Durnford et al. 1999). The red and green algal lineages share a common ancestor and molecular evidence shows that all the oxygenic phototrophs emerged from these two lineages (Durnford et al. 1999, Grzebyk et al. 2003). In red algae both the β,β-carotene and β,ε-carotene pathways are present, a characteristic that they share with green algae. Both groups also possess epoxy carotenoids such as antheraxanthin and violaxanthin, which is in agreement with the common origin of both lineages. The chromophyte chloroplast evolved from the red algal lineage through a second endosymbiotic event (ca. 1270 Ma; Durnford et al. 1999). The capacity of the Chromophyta to synthesize α-carotene and its derivates (Goodwin 1980, van den Hoek et al. 1995, Stolte et al. 2000) together with the presence of XC-related pigments is in accordance with its red algal origin.

We propose the appearance of the β,ε-carotene synthesis pathway and the zeaxanthin epoxidase, responsible for the synthesis of violaxanthin and antheraxanthin, early in the evolution of photosynthetic eukaryotes. The divergence of the Rhodophyta and Chlorophyta from the ancestral chloroplast implies an intermediate step in which the prochlorophyte type chlorophyll-*b*-binding antenna (Pcb) was replaced by a transmembrane light-harvesting antenna (preLHC; Tomitani et al. 1999). The lycopene ε-cyclase enzyme and the enzymes involved in xanthophyll cycling probably appeared at this evolutionary stage. Divergence of the red and green lineages prior to the development of epoxidation/deepoxidation reactions seems unlikely since a high degree of molecular convergence would have been required. Green and Kühlbrandt (1995) suggested that the most probable role of ancient light-harvesting complexes was not harvesting of light but photoprotection. Therefore, the appearance of the Zea epoxidase enzyme and Vio de-epoxidase at the early evolution of eukaryotic phototrophs is one of the prominent steps in the evolution of photoprotection.

J. Phycol. (submitted)

Chapter II

PHOTOINHIBITION IN RED ALGAL SPECIES WITH DIFFERENT CAROTENOID PROFILES

II.1. Abstract

The Rhodophyta present different carotenoid profiles. In the majority of the species lutein constitutes more than 50% of the total carotenoid content while in other species it is replaced by zeaxanthin or antheraxanthin. Given that carotenoids have specific roles in photoprotection, different carotenoid profiles of red algal species could be related to their capacity to cope with high light stress. Therefore, in the present work the sensitivity to light stress of red algal species with different carotenoid profiles was investigated. Photoinhibition of photosynthesis induced by high light stress and the subsequent recovery in dim light conditions was measured using maximal PSII quantum efficiency (F_v/F_m). The degree of decrease and recovery of F_v/F_m and their respective kinetics were related to the carotenoid profile of the species. Although no relationship between sensitivity to high light stress and the carotenoid profile was observed, there were clear carotenoid profile-related differences in the decrease and recovery kinetics. In species with zeaxanthin or antheraxanthin as the major carotenoid, F_v/F_m reduction and recovery was principally associated with slowly activated and relaxed processes. In contrast, in species with lutein as the major carotenoid rapidly activated processes appear to play a major role in the down-regulation of photosynthesis during light stress conditions. In these species, also the repair of D1 is important during light stress conditions. This could imply differential expression of

mechanisms involved in photoprotection in red algae that seems to be related to the carotenoid profile of the species.

II.2. Introduction

Light in shallow waters or in the intertidal zone is highly variable. Algae inhabiting these environments are exposed to changes in irradiance on the time scale of seconds to hours. Daily changes in irradiance are compounded with changes due to wave focusing, algal canopy movements, progressive tides, and suspended particles (Schubert et al. 2001). Therefore, intertidal macroalgae must be able to harvest light efficiently as well as to withstand excess energy in order to have an optimal photosynthetic performance. The exposure to high irradiances, which exceed the energy requirement for photosynthesis, causes a decrease in photosynthetic activity and photodamage, called photoinhibition (Krause 1988, Long and Humphries 1994).

Photoprotective mechanisms down-regulate the photosynthetic activity but if excitation energy exceeds the capacity for photoprotection, irreversible photodamage may result. The primary site of damage is the PSII reaction center, specifically the D1 protein, one of the key PSII-center polypeptides (Aro et al. 1993b, Osmond 1994). One of the most important photoprotective mechanisms is the thermal dissipation of excess energy (Adams et al. 2006). Thermal dissipation measured as non-photochemical PSII fluorescence quenching (NPQ) is triggered by the transthylakoidal proton gradient (ΔpH) and zeaxanthin synthesis through the xanthophyll cycle (Gilmore et al. 1994). The xanthophyll cycle (XC) is the conversion of violaxanthin into antheraxanthin and zeaxanthin in high light and the back-

conversion in darkness or under subsaturating light conditions (Yamamoto et al. 1962). The XC has been recognized as the most important short-term photoprotective mechanism that is present in higher plants and several algal divisions (Demmig-Adams 1990, Franklin et al. 1992, Demmig-Adams and Adams 1996, Niyogi et al. 1997, Rodrigues et al. 2002). Because carotenoids have an important role in photoprotection, the sensitivity to high light stress could be related to differences in the carotenoid content in red algal species. For example, species lacking XC-related carotenoids or having a reduced synthesis of zeaxanthin through this cycle could be highly sensitive to light stress (Niyogi et al. 1998, 2001).

Xanthophyll cycling has not yet been fully demonstrated in red algae, even though some species have both, violaxanthin and antheraxanthin (Brown and McLachlan 1982, Liang 1984, Schubert et al. 2006a) and the interconversion of these pigments under different light conditions has been reported (Liang 1984, Rmiki et al. 1996, Ursi et al. 2003). The importance of carotenoids in red algal photoprotection is difficult to appreciate because this group does not present a unique carotenoid composition. Different pigment profile groups have been identified in the Rhodophyta (Schubert et al. 2006a). The majority of the species contains lutein as the main carotenoid (Lut-group). In other species zeaxanthin is the predominant carotenoid (Zea-group) and in the Ant-group, antheraxanthin is the main carotenoid. Moreover, in the species from the latter two groups, violaxanthin is present. There is a taxonomic relationship based on pigment content with similar carotenoid profiles in species belonging to the same order, with the exception of the Ceramiales and Corallinales, in which similar profiles are found at the family and subfamily level, respectively (Schubert et al. 2006a). Due to this variability in carotenoid content and

specifically in photoprotective pigments it is expected that species with different carotenoid profiles will have a differential sensitivity and/or strategies to cope with high light stress. To test this hypothesis, we measured the photosynthetic performance, pigment content and the sensitivity to high light stress in several red algae with different carotenoid profiles.

***II*.3. Material and methods**

Plant material. Species of marine red macroalgae with different carotenoid profiles were collected in the high intertidal zone at Campo Kennedy (31° 42' 08" N, 116° 41' 05" W), Punta Morro (31° 51' 36" N, 116° 40' 12" W) and Ejido Erendira (31° 19' 07.9" N, 116° 26' 06.9" W) near Ensenada, Baja California, Mexico in spring and autumn 2006. Some rhodophyte species such as *Fauchea galapagensis*, *Gracilariopsis lemaneiformis* and *Calliarthron cheilosporioides* were collected at Campo Kennedy from the subtidal zone by SCUBA diving in March 2006. The species collected in autumn were used for the experiments in absence and presence of chloramphenicol, an inhibitor of protein synthesis.

The species were grouped according to their carotenoid profile: *Gelidium robustum*, *Chondracanthus volans*, *Chondracanthus canaliculatus*, *Fauchea galapagensis, Porphyra perforata* and *Lithothrix aspergillum* presented lutein as the principal carotenoid (Lut-group). *Gracilaria gracilis*, *Gracilariopsis lemaneiformis*, *Osmundea spectabilis* and *Pterosiphonia dendroidea* have zeaxanthin as the main carotenoid (Zea-group) while *Jania tenella, Bossiella orbignyana* and *Calliarthron cheilosporioides* have antheraxanthin as the principal carotenoid (Ant-group) (Table III).

To avoid contamination by the presence of epiphytes, the collected algae were meticulously cleaned with a brush and filtered seawater (0.45 µm) and subsequently, the discs or pieces

cut from the algae were maintained in filtered seawater (0.45 µm) with vigorous aeration and a constant temperature of 17° C (±1° C) until the experiments were performed within 2 or 3 days after collection. Light was provided by cool-white fluorescent tubes at 100 µmol · $m^{-2} \cdot s^{-1}$ with a 12 h light: 12 h dark photoperiod.

Photosynthesis-light response curves. Photosynthetic rates were measured as oxygen evolution in samples exposed to different light intensities. Dissolved oxygen evolved from the samples was measured polarographically (Yellow Spring Instruments model 5331) at 17° C in a closed incubation chamber, containing 15 mL of seawater. $NaHCO_3$ was added to a final concentration of 4 mM to avoid C limitation. The samples were maintained in darkness for 30 min before they were placed in the incubation chamber. After the measurement of respiration in darkness for 5 min, the samples were exposed to different light intensities provided by a tungsten lamp source (slide projector). The irradiance required at the onset of saturated photosynthesis (E_k), the photosynthetic efficiency (α) and the maximal photosynthetic activity (P_{max}) were derived by fitting the data to the exponential function proposed by Jassby and Platt (1976)

$$P=P_{max}[1-\exp(\alpha E/P_{max})]. \qquad (1),$$

High light treatment. For the photoinhibition experiments algal discs or pieces were dark-adapted for 30 min and afterwards placed in a home-made chamber with a continuous exchange of seawater to maintain a constant water temperature (17° C) during the experiments and to prevent nutrient depletion. The algal discs or pieces were placed

randomly and illuminated with white actinic light (1300 µmol · m^{-2} · s^{-1}) provided by a tungsten lamp source (slide projector) for different periods of illumination (5-60 min). For each period of illumination, a different piece was used to measure PSII maximum quantum efficiency (F_v/F_m), with the assumption that the high light-response of all pieces was similar. F_v/F_m recovery was measured in dim light (approx. 20 µmol · m^{-2} · s^{-1}) at 17° C with a maximum recovery time of 6 h after high light-illumination.

The degree of decrease during high light exposure and recovery in dim light was monitored by F_v/F_m changes in time and the results are expressed as a percentage of the value measured prior to the high light-treatment. Moreover, decrease and recovery of photosynthetic efficiency in species collected in September and October 2006 were determined by F_v/F_m changes in the absence and presence of chloramphenicol (CAP; Sigma Aldrich, Steinheim, Germany). The inhibitor was dissolved in ethanol and added to the seawater to a final concentration of 1 mg · mL^{-1}. After addition of the inhibitor the samples were incubated for 90 min in darkness before exposing them to high light as described above.

HPLC analysis. To determine possible changes in the pigment concentration, triplicate samples were taken before and after one hour of high light-exposure. The samples were frozen by immersion in liquid nitrogen and afterwards kept at –40º C until further analysis. Pigment extraction and quantification was conducted as described in Schubert et al. (2006a). The pigments of the algal samples were extracted in dimethylformamide (DMF) overnight at 4º C in darkness after grinding the previously frozen tissue. To clean the samples, the extracts were centrifuged (15600 g, 5 min) and the pigments were analyzed by

HPLC (High-Performance Liquid Chromatography) according to Wright et al. (1991) with a slightly modified solvent system program (Schubert et al. 2006a), using ethylacetate as solvent B and acetonitrile: water (9:1) as solvent A. The solvent delivery flow rate was 1.0 mL · min^{-1}. The analyses were performed at constant room temperature. The HPLC equipment was a Shimadzu AV-10 system equipped with a Nucleosil C-18 reverse phase column (250 mm, 4.6 mm internal diameter and 5 µm particle size) and a guard column Eclipse XDB-C8 (12.5 mm, 4.6 mm internal diameter and 5 µm particle size). The detection wavelength was set at 447 nm. Control of the equipment and peak area calculation was performed with the Shimadzu EZChrom Chromatography Data System software (Shimadzu Scientific Instruments, Inc.).

Fluorescence measurements. Measurements of the maximum efficiency of PSII charge separation (F_v/F_m) was done with a pulse amplitude modulated fluorometer (XE-PAM; Heinz Walz, Effeltrich, Germany). Maximum PSII efficiency was calculated as F_v/F_m with the variable fluorescence (F_v) as the difference between the maximum (F_m) and the minimum (F_o) fluorescence emission. F_o was the fluorescence in darkness excited only by the modulated measuring beam pulsed at 2 Hz (Xenon lamp with BG39 Schott filter) and F_m represents the maximum fluorescence measured in algal samples dark-adapted for 30 min by a short saturating light pulse. The PSII fluorescence signal was measured in pieces of algal thallus that were placed at a distance of 5 mm from the end of the fiber optic probe of the fluorometer. To reach F_m values, a 5-s far-red illumination period (~30 µmol · m^{-2} · s^{-1}, 735 nm) followed by dark-period of 5 min was applied. After these light pretreatments F_o was measured with white measuring light and F_m was determined with a 600 ms saturating

white light pulse (~6000 μmol · m^{-2} · s^{-1}). The fluorescence signals were detected with a RG9 Schott filter (>710 nm).

Data analysis. The decrease in maximum PSII efficiency and its recovery can be used as good indicators of the sensitivity of organisms to short-term environmental stress conditions (Hanelt 1992). Moreover, the analysis of the kinetics of reduction and recovery can indicate if the mechanisms involved in the change of PSII fluorescence are related either to the down-regulation or inhibition of the activity of this system (Hanelt 1998). Therefore, decrease and recovery rate constants were calculated by fitting the changes of F_v/F_m in tissue exposed to high light and during the recovery period to the models proposed by Hanelt (1998).

The inhibition phase is described by:

$$y_{inh.} = P_{fast} \cdot e^{(-k_{fast} \cdot t)} + P_{slow} \cdot e^{(-k_{slow} \cdot t)} \quad (2)$$

$P_{fast} + P_{slow} = 1$ at $t=0$ (t=time),

where P_{fast} and P_{slow} represent the proportions of the fast and slow decrease phase and k_{fast} and k_{slow} their respective rate constants.

The recovery phase is described by:

$$y_{rec.} = I_{max} - (P_{fast} \cdot e^{(-k_{fast} \cdot t)} + P_{slow} \cdot e^{(-k_{slow} \cdot t)}) \quad (3).$$

F_v/F_m decrease was expressed relative to the F_v/F_m measured before light stress. I_{max} represents the maximal value of F_v/F_m decrease (after 1 h of high light exposure).

Statistical analyses were performed by a one-way ANOVA using STATISTICA™.

II.4. Results

Photosynthetic characteristics. The photosynthetic rate was highly variable between species with the same carotenoid profile and also between different carotenoid groups with values between 33 and 177 µmol O_2 · (mg wet weight)$^{-1}$ · h^{-1} with the lowest values found in subtidal species (Table III). The slope of the photosynthesis-light-response curve (α) ranged from 0.3 to 6 µmol O_2 · (mg wet weight)$^{-1}$ · h^{-1} (µmol · m^{-2} · s^{-1})$^{-1}$ with the highest value in *F. galapagensis* collected from the subtidal zone (Table III). The saturating light intensity (E_k) also exhibited high variability with minimum values of 26 µmol · m^{-2} · s^{-1} measured in the subtidal *F. galapagensis* and 198 µmol · m^{-2} · s^{-1} measured in *G. gracilis* collected from the high intertidal zone (Table III). Therefore, there was no clear relationship between the carotenoid profile and photosynthetic characteristics of the investigated species. However, it should be considered that differences in photosynthesis between species may also be due to differences in morphology as shown by Johansson and Snoeijs (2002). Even though most investigated species showed a branched morphology the photosynthetic performance (P_{max}) per unit FW differed between species. These differences could be due to differences in the surface to volume ratio that is higher in more finely branched species providing a larger relative surface area for photosynthesis and carbon uptake and therefore a higher P_{max}, such as was the case for *G. gracilis* compared to *G. lemaneiformis* or *J. tenella* compared to *C. cheilosporioides* (Table III).

Moreover, a relationship between the collecting depth and photosynthetic characteristics was clearly observed for *C. cheilosporioides*. Organisms collected at 3 m and 6 m depth showed a 60% lower P_{max} as compared to organisms collected in the high intertidal zone.

Also, a decrease in respiration and photosynthetic efficiency with depth was observed for this species (Table III).

Table III. Photosynthetic characteristics of rhodophyte species collected in March 2006.
Tabla III. Características fotosintéticas de especies de rodófitas colectadas en marzo del 2006.

Species	P_{max}	Rd	α	E_k
Lut-group				
G. robustum	93 (±8)	9 (±4)	0.7 (±0.2)	147 (±47)
F. galapagensis	111 (±21)	46 (±11)	6 (±3)	26 (±20)
C. volans	86 (±23)	16 (±10)	2.3 (±1.2)	46 (±27)
C. canaliculatus	62 (±11)	11 (±7)	0.7 (±0.1)	84 (±10)
L. aspergillum	91 (±2)	20 (±7)	0.8 (±0.1)	121 (±8)
Zea-group				
G. gracilis	126 (±19)	20 (±3)	0.7 (±0.3)	198 (±91)
G. lemaneiformis	67 (±23)	32 (±8)	0.9 (±0.3)	95 (±73)
O. spectabilis	52 (±9)	15 (±9)	0.4 (±0.1)	127 (±24)
P. dendroidea	104 (±5)	21 (±7)	1.3 (±0.5)	91 (±39)
Ant-group				
J. tenella	177 (±47)	33 (±18)	2 (±0.3)	86 (±10)
C. cheilosporioides	86 (±5)	12 (±1)	0.7 (±0.2)	128 (±40)
C. cheilosporioides (3 m)	35 (±8)	8 (±3)	0.4 (±0.1)	82 (±2)
C. cheilosporioides (6 m)	33 (±5)	7.5 (±1)	0.3 (±0.03)	121 (±12)

Maximum photosynthetic rate (P_{max}) and respiration rate in darkness (Rd) in µmol O_2 · mg wet weight^{-1} · h^{-1}; initial slope at limiting irradiance levels (α) in µmol O_2 · mg wet weight^{-1} · h^{-1} (µmol · m^{-2} · s^{-1})$^{-1}$; light saturation point (E_k) in µmol · m^{-2} · s^{-1}. Values are means of three independent measurements ± SE.
Tasa fotosintética máxima (P_{max}) y tasa de respiración en oscuridad (Rd) en µmol O_2 · mg peso húmedo^{-1} · h^{-1}; pendiente inicial en niveles limitantes de irradiancia (α) en µmol O_2 · mg peso húmedo^{-1} · h^{-1} (µmol · m^{-2} · s^{-1})$^{-1}$; irradiancia de saturación (E_k) en µmol · m^{-2} · s^{-1}. Los valores son las medias de tres mediciones independientes ± DE.

Pigment characteristics. Red algal species present differences in their carotenoid content principally related with differences in the predominant carotenoid (Schubert et al. 2006a). Lutein concentration was between 51 and 98% of the total carotenoid content in species

from the Lut-group (Table IV). In this group zeaxanthin varied between 1 to 39% of the total carotenoid content and was not detected in *F. galapagensis*. In contrast, Zea was the predominant carotenoid with values ranging from 52 to 95% of the total carotenoid content for the species belonging to the Zea-group (Table IV). Lutein was poorly represented or absent in this group and trace amounts of these pigments were detected only in *P. dendroidea* (Table IV).

Table IV. Pigment composition in red algal species (in mmol pigment · mol Chl a^{-1}) before light treatment.
Tabla IV. Composición pigmentaria en especies de algas rojas (en mmol pigmento · mol Chl a^{-1}) antes del tratamiento con luz.

	Lutein	**Zeaxanthin**	**Antheraxanthin**	**α-carotene**	**β-carotene**
Lut-group					
G. robustum	431 (±179)	6 (±1.8)		3.3 (±1.9)	
F. galapagensis	170 (±21)			64 (±13)	
C. volans	294 (±195)	77 (±9)		9 (±5)	41 (±16)
C. canaliculatus	427 (±17)	97 (±9)			15 (±13)
L. aspergillum	292 (±77)	222 (±87)		7.2 (±4)	50 (±21)
Zea-group					
G. gracilis		494 (±64)	1.1 (±0.7)		22 (±12.5)
G. lemaneiformis		122 (±15)	5 (±0.3)		95 (±4)
O. spectabilis		182 (±10)			49 (±39)
P. dendroidea	0.1 (±0.01)	37 (±19)		4.3 (±3.6)	30 (±14)
Ant-group					
J. tenella		107 (±19)	260 (±34)		70 (±18)
C. cheilosporioides		64 (±40)	344 (±15)		11 (±13)
C. cheilosporioides (3 m)		29 (±16)	334 (±15)		19 (±7.5)
C. cheilosporioides (6 m)		4 (±0.5)	180 (±5)		92 (±14)

Values are means of three independent measurements ± SE.
Los valores son las medias de tres mediciones independientes ± DE.

Species from the Ant-group presented a similar carotenoid composition as species from the Zea-group but with antheraxanthin as the principal carotenoid (Table IV). Antheraxanthin concentration was between 60 and 88% of the total carotenoid content while the concentration of Zea was not higher than 25% (Table IV). In *C. cheilosporioides* a depth-related decrease of Ant and Zea was found. In organisms collected from the high intertidal Ant and Zea concentration was 82 and 15% of the total carotenoid content, respectively. These proportions decreased to 65 and 2%, in organisms collected from 6 m depth (Table IV).

Moreover, no changes in carotenoid concentration during light exposure specifically those related to xanthophyll cycling were found in the investigated species (data not shown).

F_v/F_m decrease and recovery. The maximum quantum efficiency (F_v/F_m) measured prior to the high light-treatment was similar between species from the Lut- and Zea-group, with values between 0.52 and 0.66. Coralline species of the Ant-group exhibited the lowest F_v/F_m. In these species F_v/F_m was between 0.33 and 0.46 with a depth-related increase in *C. cheilosporioides* (Table V).

After 1 h of high light exposure, F_v/F_m decreased in all species. The reduction of this parameter ranged between 44 and 12% of the initial value (Table V). With the exception of *F. galapagensis*, there was a significant recovery of F_v/F_m in all species after the light-stress treatment. The recovery of F_v/F_m after 6 h in dim light varied from 25 to 98% of the initial value (Table V). F_v/F_m reduction and recovery were not related to the pigment profile of the species (Table V). However, a depth-related sensitivity to high light was observed in *C. cheilosporioides* (Table V). Organisms collected from 3 m and 6 m depth presented a

significantly higher decrease and lower recovery of F_v/F_m as compared to organisms collected in the intertidal zone (ANOVA, p≤0.05).

Table V. F_v/F_m decrease after 1 h of high light exposure (1300 µmol · m^{-2} · s^{-1}) and its recovery after 6 h in dim light conditions in red algae species with different pigment profiles.
Tabla V. Disminución de F_v/F_m después de 1 h de exposición a alta luz (1300 µmol · m^{-2} · s^{-1}) y su recuperación después de 6 h en condiciones de luz tenue en especies de algas rojas con diferentes perfiles pigmentarios.

Species	Abbreviation	F_v/F_m	Decrease	Recovery
Lut-group				
G. robustum	Grob	0.53	31 (±6)	98 (±14)
F. galapagensis	Fgal	0.52	19 (±2)	15 (±5)
C. volans	Cvol	0.63	27 (±1.5)	65 (±2)
C. canaliculatus	Ccan	0.63	27 (±1)	85 (±5)
L. aspergillum	Lasp	0.52	19 (±5)	77 (±15)
Zea-group				
G. gracilis	Ggra	0.61	15 (±4)	58 (±9)
G. lemaneiformis	Glem	0.60	35 (±7)	76 (±9)
O. spectabilis	Ospe	0.66	21 (±3)	74 (±14)
P. dendroidea	Pden	0.57	44 (±7)	69 (±5)
Ant-group				
J. tenella	Jten	0.33	32 (±14)	85 (±4)
C. cheilosporioides	Cche	0.34	26 (±5)	63 (±17)
C. cheilosporioides (3 m)	Cche-3m	0.40	14 (±0.4)	30 (±5)
C. cheilosporioides (6 m)	Cche-6m	0.46	12 (±2)	25 (±4)

Values are normalized and expressed in percentage to the activity measured before light treatment (mean ± SE; n = 5).
Los valores fueron normalizados y expresados como porcentaje de la actividad medida antes del tratamiento de luz (media ± DE; n = 5).

The reduction and recovery of F_v/F_m during and after high light exposure showed a biphasic behavior in the analyzed rhodophyte species (Fig. 15) that could be described as the sum of a fast and a slow component (Hanelt 1998).

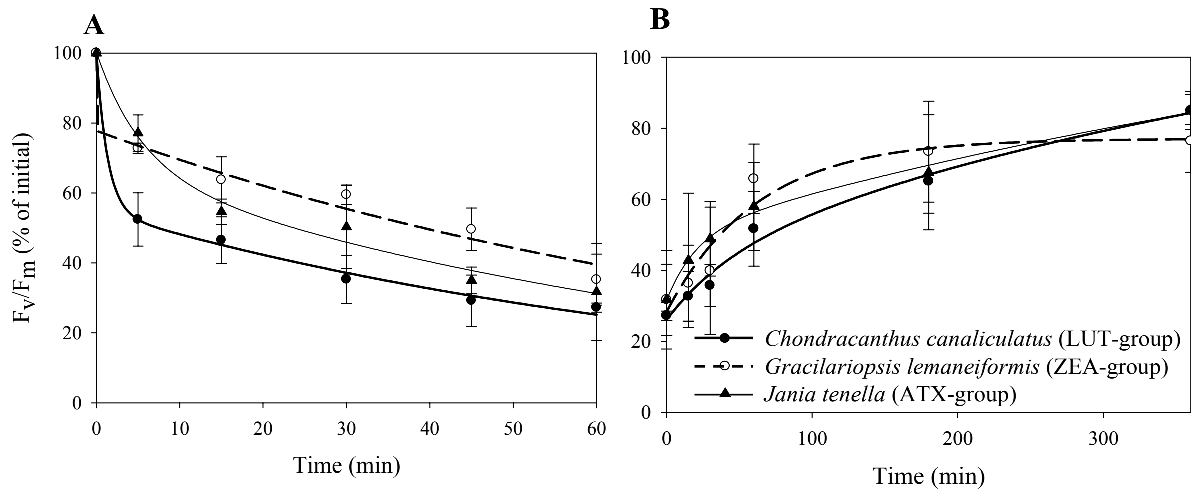

Figure 15. Kinetics of F_v/F_m in red algal species with different carotenoid profiles. A) F_v/F_m decrease during exposure to high light (1300 µmol · m^{-2} · s^{-1}, 1 h) and B) F_v/F_m recovery in dim light conditions (20 µmol · m^{-2} · s^{-1}, 6 h). F_v/F_m is expressed as the percentage to the value measured before the light treatment (mean ± SE, n = 5). The lines represent the fit of the data to the models proposed by Hanelt (1998) (see Material and methods, $R^2 \geq 0.99$).

Figura 15. Cinéticas de F_v/F_m en especies de algas rojas con diferentes perfiles de carotenoides. A) Disminución de F_v/F_m durante exposición a alta luz (1300 µmol · m^{-2} · s^{-1}, 1 h) y B) recuperación de F_v/F_m en condiciones de luz tenue (20 µmol · m^{-2} · s^{-1}, 6 h). F_v/F_m es expresado como porcentaje del valor medido antes del tratamiento con luz (media ± DE, n = 5). Las líneas representan el ajuste de los datos a los modelos propuestos por Hanelt (1998) (ver Materiales y métodos, $R^2 \geq 0.99$).

The proportion of the fast (P_{fast}) and slow (P_{slow}) components during decrease of F_v/F_m showed a dependence based on the carotenoid profile group. The response of species from the Lut-group was heterogeneous, but in general they showed a high P_{fast} (40 to 75%) as compared with the species from the other carotenoid profile groups (Fig. 16A). Decrease kinetics in species from the Zea- and Ant-group showed a small P_{fast} (20 to 35%) and consequently a high P_{slow} (Fig. 16A). Additionally, differences in the fast decrease rate

(k_{fast}) were detected with the highest values found in the Zea-group. In this group k_{fast} was ten to twenty times higher than in other carotenoid profile groups (Fig. 16B).

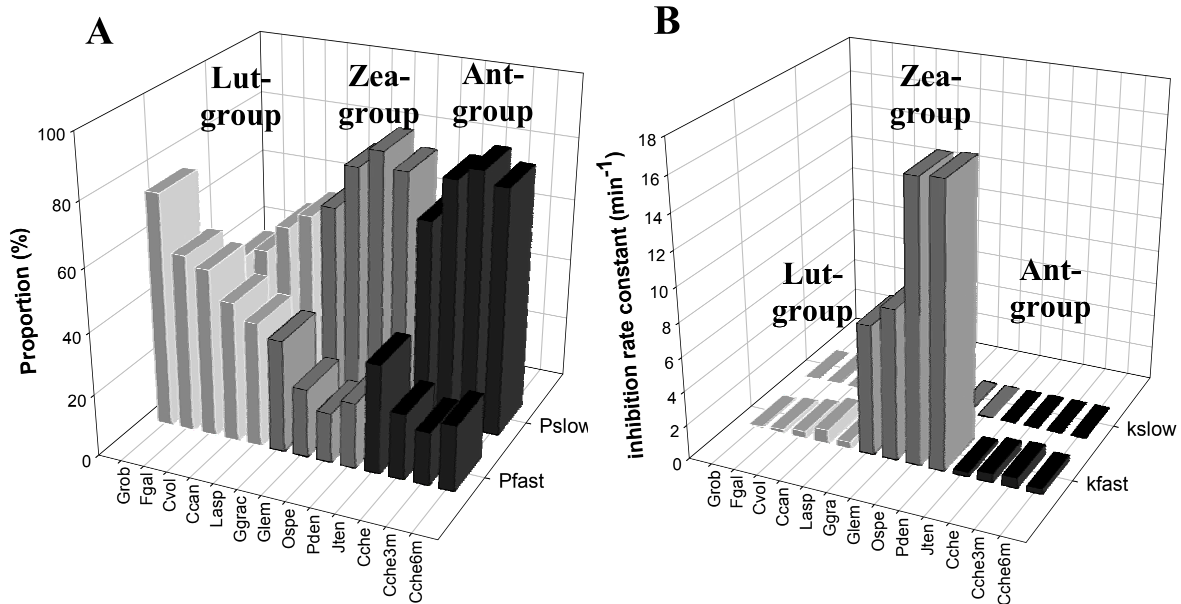

Figure 16. Components of the F_v/F_m decrease at high light exposure (1300 μmol · m^{-2} · s^{-1}, 1 h) calculated from the mathematical model according to Hanelt (1998) in the different pigment groups (Lut-group- bright grey, Zea-group- dark grey, Ant-group- black). P_{fast} and P_{slow} are the proportions of the fast and the slow F_v/F_m decrease (A) and k_{fast} and k_{slow} are their respective rate constants (B).

Figura 16. Componentes de la fase de disminución de F_v/F_m para la exposición a alta luz (1300 μmol · m^{-2} · s^{-1}, 1 h) calculados del modelo matemático de acuerdo a Hanelt (1998) en diferentes grupos pigmentarios (grupo Lut- gris claro, grupo Zea- gris oscuro, grupo Ant- negro). P_{fast} y P_{slow} son las proporciones de la disminución rápida y lenta de F_v/F_m (A) y k_{fast} y k_{slow} son sus tasas respectivas (B).

Similar to the decrease kinetics, the F_v/F_m recovery kinetics also showed differences between carotenoid profile groups (Fig. 17). Species from the Lut-group presented species-specific differences in the recovery kinetics. For example, *L. aspergillum* exhibited a high

fast recovery (P_{fast}-72%) and consequently a low P_{slow} while *G. robustum*, *C. volans* and *C. canaliculatus* showed the opposite pattern (Fig. 17A). Species from the Zea-group had similar proportions of P_{fast} and P_{slow} while the members of the Ant-group exhibited a low P_{fast} (6-20%) and a high P_{slow} (80-94%) (Fig. 17A). Moreover, species from the Ant-group showed a significantly higher fast rate constant compared to the other pigment groups (Fig. 17B).

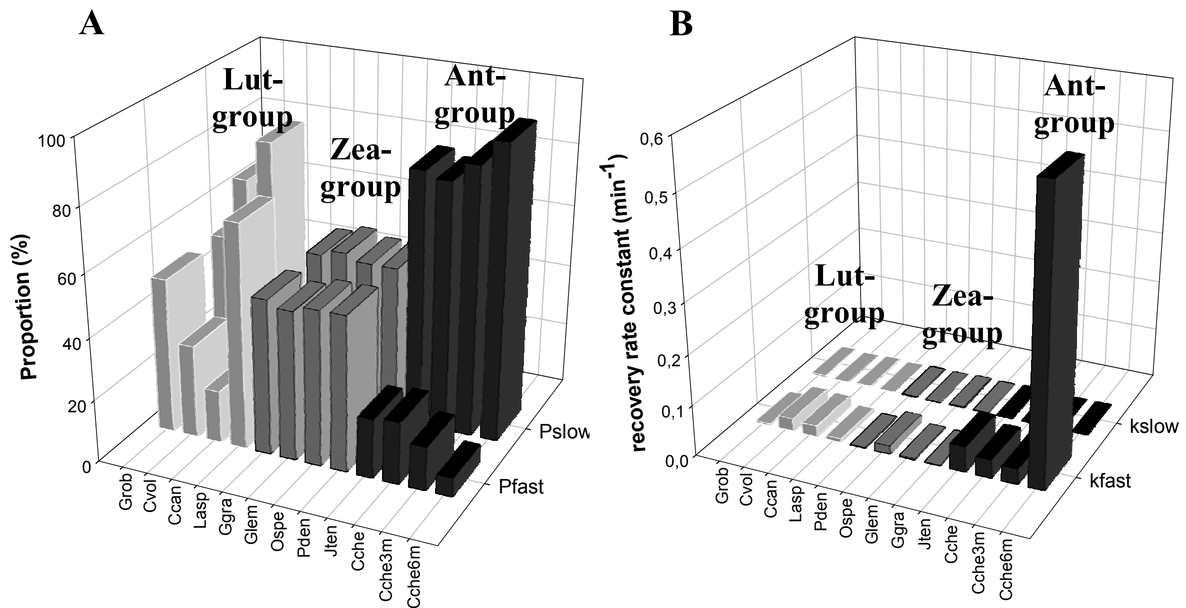

Figure 17. Components of the recovery phase at dim-light exposure (20 μmol · m^{-2} · s^{-1}, 6 h) calculated from the mathematical model according to Hanelt (1998) in the different pigment groups (Lut-group- bright grey, Zea-group- dark grey, Ant-group- black). P_{fast} and P_{slow} are the proportions of the fast and the slow recovery phase (A) and k_{fast} and k_{slow} are their respective rate constants (B).

Figura 17. Componentes de la fase de recuperación para la exposición a luz tenue (20 μmol · m^{-2} · s^{-1}, 6 h) calculados del modelo matemático de acuerdo a Hanelt (1998) en diferentes grupos pigmentarios (grupo Lut- gris claro, grupo Zea- gris oscuro, grupo Ant- negro). P_{fast} y P_{slow} son las proporciones de la fase rápida y lenta de la recuperación (A) y k_{fast} y k_{slow} son sus tasas respectivas (B).

The depth-related sensitivity to light stress of *C. cheilosporioides* was not reflected in the decrease kinetics but was seen in the recovery behavior of F_v/F_m (Fig. 16). The slow component of the F_v/F_m recovery increased with depth (Fig. 16A). Also, k_{fast} of subtidal algae was higher compared to high intertidal organisms (Fig. 16B).

D1 repair during high light exposure. Generally, the slow component of F_v/F_m decrease and recovery is associated to the repair of photodamaged PSII centers while the fast component is related to the down-regulation of PSII activity caused by the expression of photoprotective mechanisms. To determine the relative importance of these processes in red algae with different carotenoid profiles, the effect of CAP on F_v/F_m reduction and recovery during and after light stress was assayed.

CAP (an inhibitor of the synthesis of proteins encoded in chloroplasts) had a differential effect on species from different carotenoid profile groups. Species from the Lut-group such as *P. perforata*, *C. canaliculatus* and *C. volans* showed a significantly lower F_v/F_m in the presence of CAP after 1 h of high light exposure (Table VI). Moreover, F_v/F_m recovery after 6 h in dim light was significantly lower in *C. volans* and *C. canaliculatus* (Table VI). On the other hand, CAP did not have a noticeable effect on F_v/F_m decrease and recovery in species of the Zea- and Ant-group (Table VI).

Table VI. F_v/F_m decrease after 1 h of high light exposure (1300 µmol · m^{-2} · s^{-1}) and its recovery after 6 h in dim light conditions in absence and presence of CAP.
Tabla VI. Disminución de F_v/F_m después de 1 h de exposición a alta luz (1300 µmol · m^{-2} · s^{-1}) y su recuperación después de 6 h en condiciones de luz tenue en ausencia y presencia de CAP.

Species	F_v/F_m		Decrease		Recovery	
	Control	CAP	Control	CAP	Control	CAP
Lut-group						
C. canaliculatus	0.65	0.59	34 (±2)	21 (±3)*	60 (±3)	51 (±3)*
C. volans	0.62	0.59	30 (±11)	17 (±1)*	74 (±7)	61 (±5)*
P. perforata	0.57	0.51	20 (±3)	13 (±2)*	88 (±3)	89 (±0.1)
Zea-group						
G. gracilis	0.60	0.54	33 (±9)	32 (±5)	104 (±2)	101 (±5)
G. lemaneiformis	0.47	0.48	38 (±8)	40 (±14)	82 (±3)	78 (±9)
O. spectabilis	0.63	0.56	38 (±2)	37 (±7)	90 (±4)	89 (±14)
Ant-group						
B. orbignyana	0.42	0.39	36 (±12)	33 (±11)	67 (±11)	65 (±10)
J. tenella	0.40	0.38	47 (±12)	42 (±1)	86 (±5)	86 (±10)

Values are normalized and expressed in percentage to the activity measured before light treatment (mean ± SE; n = 5). *Significant differences between treatments ($p \leq 0.05$).
Los valores fueron normalizados y expresados como porcentaje de la actividad medida antes del tratamiento de luz (media ± DE; n = 5). *Diferencias significativas entre tratamientos ($p \leq 0.05$).

II.5. Discussion

In the marine habitat, macrophytes are exposed to considerable diurnal changes of solar irradiance and therefore they must possess effective mechanisms to prevent photodamage. Carotenoids can play an important role in photoprotection by their ability to quench reactive oxygen species (Edge et al. 1997) or in the thermal dissipation of excess of energy in the photosynthetic apparatus (Havaux and Niyogi 1999). Within the carotenoids, zeaxanthin is one of the key carotenoids involved in the photoprotective response to high light stress (Demmig-Adams 1990). The high content of zeaxanthin in species from the Zea-group would suggest a lower sensitivity to high irradiances of these species compared with species from the other pigment-profile groups. However, the degree of F_v/F_m reduction

and recovery in the investigated red algal species did not show a clear relationship with their prevailing carotenoid profile. That implies that not all zeaxanthin present in species from the Zea-group is involved in dissipation of excess energy as heat or that lutein and antheraxanthin proportionate the same level of photoprotection as zeaxanthin in the Lut- and Ant-group, respectively.

In respect to the first idea, it has been suggested that not all Zea is involved in energy dissipation since no differences in the sensitivity to high light were detected between zeaxanthin-accumulating mutants of *Arabidopsis* and wild type genotypes (Hurry et al. 1997, Havaux et al. 2004). Zeaxanthin not involved in thermal dissipation (up to 35% of the total pool) might be located in the chloroplast envelope (Siefermann-Harms et al. 1978, Hurry et al. 1997) or free in the lipid phase of the membrane, where it could be involved in the quenching of free O_2 radicals (Baroli et al. 2003, Rissler and Durnford 2005). On the other hand, a high content of antheraxanthin and lutein in the Ant- and Lut-group, respectively, might also represent an efficient photoprotective strategy since these carotenoids are capable of functioning as direct quenchers of energy from excited Chl. Although lutein and antheraxanthin may be weaker quenchers of excess light energy compared to zeaxanthin since they present higher singlet energy states (Frank et al. 2000, 2001), it has been shown that they are involved in thermal dissipation of excess light energy in different organisms (Niyogi et al. 1997, Goss et al. 1998, Gilmore and Yamamoto 2001, Garcia-Plazaola, et al. 2003).

Carotenoid-related photoprotection in red algae seems to be linked to the steady state concentration of these pigments since short-term interconversion of them was not detected. In contrast to previous reports (Liang 1984, Rmiki et al. 1996, Ursi et al. 2003) but

consistent with a recent report (Anderson et al. 2006), no xanthophyll cycling was observed as response to light stress. Furthermore, red algal species from the Zea- and Ant-group that contain XC-related pigments did not show differential sensitivities to high light when compared to the Lut-group. Therefore, the regulation of the amount of carotenoids capable of dissipation of excess energy seems to be an important long-term photoprotective response as shown by the depth-related reduction of zeaxanthin in species from the Lut-group (Schubert et al. 2006a) and in the present study by a depth-related reduction of zeaxanthin and antheraxanthin in relation to the depth-associated increase in sensitivity to high light in *C. cheilosporioides* from the Ant-group. Even though the sensitivity of photosynthesis to light stress seemed not to be related to the carotenoid profile of the species, the comparison of the reduction and recovery kinetics of different species showed pigment-group specific differences in the induction and relaxation of down-regulatory processes.

F_v/F_m reduction and recovery kinetics are the combination of different mechanisms such as the fast down-regulation and recovery of PSII activity (seen in P_{fast} component of the F_v/F_m reduction and recovery kinetics) and PSII photodamage. PSII damage is concentrated normally at the level of D1 protein degradation. PSII repair is a relatively slow process ($t_{½}$≈60min) as it requires *de novo* synthesis of D1 protein and has been related to the slow reduction and recovery phase (Krause 1988, Leitsch et al. 1994, Jahns and Miehe 1996).

The differences in F_v/F_m decrease and recovery kinetics between pigment groups indicate differences in photoprotective mechanisms and/or photodamage involved in the down-regulation of photosynthetic activity. In the Lut-group fast activated processes generally played a major role in the decrease of photosynthetic efficiency. D1 damage and repair was

also involved in PSII regulation in these species. This indicates that in contrast to the Zea- and Ant-group, where no effect of the inhibition of chloroplast protein synthesis on F_v/F_m was detected (Table VI), D1 turnover might be important during light stress and recovery in the Lut-group.

In the species from the Zea-group slowly activated and relaxed mechanisms, apparently not related to D1 turnover, were also important in the down-regulation of photosynthesis. The species with zeaxanthin as the principal carotenoid presented rapidly activated processes (a k_{fast} ten to twenty times higher as compared to the other groups) that might play an important role in the down-regulation of photosynthetic activity during high light conditions. In red algae, rapidly activated photoprotective processes have been related to the fast fluorescence quenching dependent on the formation of a pH gradient across the thylakoid membrane (Delphin et al. 1996, 1998, Ritz et al. 1999). Moreover, in recent works on *Gracilaria domingensis,* a member of the Zea-group, a very fast induction of non-photochemical quenching compared to another red algal species belonging to the Lut-group has been observed (Anderson et al. 2006, Schubert et al. 2006b).

High levels of Zea can result in more energy dissipation in steady state under subsaturating light conditions and in a slower reversibility of NPQ as proposed in Zea-accumulating mutants (Niyogi et al. 1998) that would also be reflected in a slower recovery of F_v/F_m. This response has been observed also in overwintering higher plants which accumulate zeaxanthin and overexpress the slowly relaxing component of NPQ referred as qI (Gilmore and Ball 2000, Demmig-Adams et al. 2006). This could be seen in *G. gracilis* that exhibited at least 4 times more Zea as the other species of the group (Table IV) and correspondently the highest F_v/F_m decrease and the lowest recovery (Table V). Therefore, the high k_{fast} for

the F_v/F_m decrease during light exposure and the higher importance of slowly reversible processes in recovery in species of the Zea-group could be related with NPQ that is induced very fast and that remains engaged for a longer period of time after a decrease in incident light (probably similar to qI described in higher plants). This would explain F_v/F_m kinetics in this carotenoid group.

Similar to the species from the Zea-group, slowly activated and relaxed processes play the major role in down-regulation of photosynthetic activity in members of the Ant-group. Both groups seem to rely on this type of photoprotection response to down-regulate PSII activity probably achieved through expression of an effective and slowly relaxing quenching mechanism related with a high accumulation of zeaxanthin and antheraxanthin. However, the incomplete recovery in all investigated species after 6 h in dim light conditions also indicates photodamage that was repaired during exposure and subsequently short-term recovery only in organisms of the Lut-group since CAP did not have any effect on F_v/F_m decrease and recovery in the other carotenoid-profile groups (Table VI).

Photoprotection mechanisms and its effect on PSII regulation are compared to ones described for higher plants. However, the model described for higher plants most probably does not apply for red algae. Thermal dissipation during high light stress is dependent on ΔpH and photoprotective carotenoids within the LHCII antenna complex of PSII (e.g. Ruban and Horton 1999, Bassi and Caffari 2000). In higher plants and green algae carotenoids related to the xanthophylls cycle play an important role in photoprotection (e.g. Demmig-Adams 1990, Franklin et al. 1992, Demmig-Adams and Adams 1996, Niyogi et al. 1997). This applies also for other algal groups, evolutionarily closer related to the red

algae such as brown algae and diatoms (Durnford et al. 1999) that also present the xanthophyll cycle as photoprotective mechanism (e.g. Olaizola and Yamamoto 1994, Rodrigues et al. 2002). However, red algal antenna arrangement is different from higher plants and they do not present an active xanthophyll cycle. Red algae do not possess transmembranic LHCII complexes but instead phycobilisomes (PBS), a feature that they share with cyanobacteria. In this former group, phycobilisome movement due to state transitions has been reported to be involved in photoprotection (Joshua et al. 2004, 2005, Ma et al. 2007). Furthermore, recently in cyanobacteria carotenoid-related quenching has been found to be associated with a carotenoid-binding protein (OCP) that may be directly responsible for energy quenching through interaction with the PBS core (Rakhimberdieva et al. 2004, 2007, Wilson et al. 2006, Kirilovski et al. 2007). Energy quenching in red algae must resemble the one present in cyanobacteria. However, the role of state transitions on photoprotection and the possible presence of carotenoid-binding proteins (or the association of specific carotenoids to photosynthetic protein complexes that might be involved in energy quenching) has not been investigated in red algae. Therefore, due to the lack of LHCII in Rhodophyta, the role of the carotenoids such as lutein, zeaxanthin and antheraxanthin which have a function in non-radiative dissipation in other algal groups and higher plants is still an open question.

However, the relationship between the carotenoid profile of the species and the reduction and recovery kinetic of F_v/F_m found in this study indicates an involvement of carotenoids in photoprotection in the red algae even though the mechanisms are still unknown.

Chapter III

COMPARISON OF THE HIGH LIGHT RESPONSE OF DIFFERENT RED ALGAL SPECIES MEASURED AS OXYGEN EVOLUTION AND CHLOROPHYLL FLUORESCENCE

III.1. Abstract

There are different processes involved in the decrease of the photosynthetic efficiency during light stress conditions. Some of these processes are reversible and activated rapidly, providing photoprotection to the photosynthetic apparatus while others are related to irreversible photoinactivation by photodamage. Changes in variable to maximum chlorophyll fluorescence (F_v/F_m) and also changes in photosynthesis measured as oxygen evolution can be used to evaluate the decrease in photosynthetic efficiency during high light exposure. The first method gives information about down-regulation of the photosynthetic efficiency and the comparison with oxygen measurements can give insights in the involvement of photoinactivation (reversible or irreversible due to photodamage). In the present study, differences in the maximum decrease of F_v/F_m compared to O_2 evolution activity, and moreover, differences in their respective kinetics have been found. Generally, with exception of one species, F_v/F_m showed a faster initial decline compared to the O_2 evolution. This indicates that in the decrease of F_v/F_m down-regulatory mechanisms were involved. However, due to the incomplete recovery of both, F_v/F_m and oxygen evolution activity during recovery, also photodamage seemed to be occurred. Moreover, the high fast initial recovery of the O_2 evolution activity indicates the presence of processes such as reversible PSII inactivation that could be involved in photoprotection in red algae.

***III*.2. Introduction**

Macroalgae are exposed to considerable variations in photon irradiance, comprising diurnal changes as well as changes due to tide and to weather conditions. In general, the photosynthetic rate is related to these variations in light intensity and usually there is an excess of energy reaching the photosynthetic apparatus. Therefore different mechanisms have been evolved to protect the alga from such excess energy. Formation and activation of these mechanisms result in a decrease in the maximum photosynthetic efficiency. This decrease results from two processes. There is photoprotective down-regulation of PSII activity; however, at certain photon irradiance when the photoprotective capacity is exceeded, photoinactivation occurs (Krause 1988).

Photoprotection refers to a light dependent, often rapidly reversible decline in photochemistry while photoinactivation refers to a slower reversible decline, often related to photodamage. Two of the methods that can be used to evaluate this decrease in photosynthetic efficiency are changes in variable to maximum chlorophyll fluorescence (F_v/F_m) and changes in photosynthesis measured as oxygen evolution (Henley et al. 1991, Hanelt et al. 1992). Fluorescence as well as oxygen production is used as a common tool to investigate the response upon high light stress in algae.

Light stress conditions cause a decrease of the photosynthetic efficiency and as a result, both variable fluorescence of PSII and oxygen yield decrease. A linear correlation between the fluorescence ratio F_v/F_m and gross photosynthetic oxygen production at different levels of high light stress was shown in different red algal species (Hanelt et al. 1992). However, in the arctic red alga *Palmaria palmata* the results of fluorescence and oxygen measurements showed clear differences since the oxygen production rate commenced to

decrease not before the F_v/F_m level decreased 60% of the control before light exposure (Hanelt and Nultsch 1995). In contrast, a more pronounced decrease in photochemical efficiency when measured as fluorescence emission has been reported in brown algae (Rodrigues et al. 2000).

The measurement of oxygen evolution during high light treatment does not include processes that can interfere in photosynthetic capacity such as photorespiration. Therefore, combined oxygen and fluorescence measurements can insure that the observed O_2 production decrease caused by high irradiance is really result of a decrease in photochemical efficiency and that interference by other processes can be ruled out. Moreover, changes in the chlorophyll fluorescence characteristics can provide insights into relative contributions of processes of photoprotection and photodamage in declining photosynthetic efficiency. While initial fluorescence (F_o) may increase or decrease, depending on the process occurring, it has been demonstrated that high light treatment leads to a reduction in maximum (F_m) and variable fluorescence (F_v=F_m-F_o) (Powles 1984, Krause 1988).

In the present study, photosynthetic oxygen production and chlorophyll fluorescence *in* vivo were measured in marine red algae under high irradiance conditions to obtain insights in processes involved in the down-regulation of photosynthetic efficiency and also to determine if there is linear correlation between both measuring parameters as reported previously.

***III.3*. Materials and Methods**

Plant material. Species of marine red macroalgae (*Chondracanthus volans*, *Porphyra perforata*, *Lithothrix aspergillum* and *Jania tenella*) were collected in the high intertidal zone at Punta Morro (31° 51' 36" N, 116° 40' 12" W) and Punta Cabras (31° 19' 48" N, 116° 27' 36" W) in October 2005. To avoid a contamination by the presence of epiphytes in the organisms, the collected algae were meticulously cleaned with a brush and filtered seawater (0.45 μm) and afterwards discs (0.9 cm diameter) or pieces cut from the algae were incubated under vigorous aeration in filtered seawater (0.45 μm) until the experiments. Lighting was by cool-white fluorescent tubes at 100 μmol photons · m^{-2} · s^{-1}. Photoperiod was 12 h light: 12 h dark at a constant temperature of 15° C. The experiments were performed within 2 or 3 days after the collecting.

Pigment analysis. To determine the pigment concentration, triplicate samples were taken before high light exposure. The samples were frozen by immersion in liquid nitrogen and afterwards kept at –40º C until further analysis. Pigment extraction and quantification was conducted as described in Schubert et al. (2006a). The pigments of the algal samples were extracted in dimethylformamide (DMF) overnight at 4º C in darkness after grinding the previously frozen tissue. To clean the samples, the extracts were centrifuged (15600 g, 5 min) and the pigments were analyzed by HPLC (High-Performance Liquid Chromatography) according to Wright et al. (1991) with a slightly modified solvent system program (Schubert et al. 2006a), using ethylacetate as solvent B and acetonitrile: water (9:1) as solvent A. The solvent delivery flow rate was 1.0 mL · min^{-1}. The analyses were performed at constant room temperature. The HPLC equipment was a Shimadzu AV-10

system equipped with a Nucleosil C-18 reverse phase column (250 mm, 4.6 mm internal diameter and 5 µm particle size) and a guard column Eclipse XDB-C8 (12.5 mm, 4.6 mm internal diameter and 5 µm particle size). The detection wavelength was set at 447 nm. Control of the equipment and peak area calculation was performed with the Shimadzu EZChrom Chromatography Data System software (Shimadzu Scientific Instruments, Inc.).

Photosynthesis-irradiance-curves measured by O_2 evolution. Photosynthetic oxygen evolution from the tissue samples were measured polarographically (Yellow Spring Instruments model 5331) at 15° C in an incubation chamber, containing 15 mL of seawater to which $NaHCO_3$ was added to a final concentration of 4 mM. The alga was darkened 30 min before it was placed in the incubation chamber. Before the oxygen measurements, the oxygen concentration of the seawater was lowered to 50% of the saturation level by flushing with gaseous nitrogen. After a preceding dark-period of 5 min to measure respiration rate, the sample was exposed to different light intensities provided by a tungsten lamp source (slide projector), each 3 min, varied by neutral density filters. The irradiance required at the onset of saturated photosynthesis (E_k), the irradiance compensation point (E_c), the photosynthetic efficiency (α) and the maximal photosynthetic activity (P_{max}) were derived by fitting the data to the exponential function proposed by Jassby and Platt (1976),

$$P=P_{max}[1-\exp(\alpha E/P_{max})] \quad (6),$$

using a statistical package based on the Marquardt algorithm (Regression; Blackwell Scientific Publications, Oxford, UK).

High light treatment. For high light experiments, the algae discs or pieces were dark-adapted for 30 min and then placed in a home-made chamber with a continuous exchange of seawater ensuring a nearly constant water temperature (15° C) and salinity and preventing nutrient depletion during the experiments. The discs or pieces were placed randomly and illuminated with 1300 μmol photons · m^{-2} · s^{-1} of white actinic light provided by a tungsten lamp source (slide projector) for different periods of illumination (5-60 min). For each period of illumination, a different piece was used, on the assumption that the response of each piece is similar. Photorecovery was performed by leaving the pieces that had been light exposed for 1 h, for different periods (15-420 min) in dim light (approx. 20 μmol · m^{-2} · s^{-1}) at 17° C. The periods of light exposure and subsequently recovery were monitored by fluorescence and oxygen evolution measurements and the results were expressed as a percentage of the activity prior to the high light treatment. All points are mean of 3-5 independent measurements.

Oxygen evolution measurements. The samples were monitored at different time points during light exposure and subsequently recovery by determining the oxygen evolution activity, measured as described above. The time required for each O_2 evolution measurement was 5 min, with an irradiance of 200 μmol • m-2 • s-1. All points are means of three independent measurements.

Fluorescence measurements. The light response during exposure and recovery was monitored by *in vivo* chlorophyll fluorescence measured with a pulse-amplitude modulation fluorometer (XE-PAM, Walz, Effeltrich). Changes in the ratio of variable to maximum fluorescence, F_v/F_m, of algae temporarily acclimated to darkness were used as a measure of

decrease and recovery of photochemical efficiency. $F_v=F_m-F_o$, in which F_o is the minimum fluorescence, i.e. when all reaction centers of PSII are active or “open”, and F_m is the maximum fluorescence determined under strong light, i.e. when all PSII centers are “closed”. Thallus pieces were placed at a distance of 5 mm from the end of the fiber optic probe of the fluorometer. After application of a 5-s far-red pulse (~30 µmol · m^{-2} · s^{-1}, 735 nm), the sample was darkened for 5 min. Then, F_o was measured with white measuring light pulses, and F_m was determined with a 600 ms saturating white light pulse (~6000 µmol · m^{-2} · s^{-1}). During the periods of light exposure and subsequently recovery, photosynthetic efficiency of each sample was measured after different times of illumination or recovery by the fluorescence method described above.

To analyze the differences in the reaction kinetics of decrease and recovery of oxygen evolution and F_v/F_m, mathematical models proposed by Hanelt (1998) were applied.

The phase of decrease is described by:

$$y_{inh.} = P_{fast} \cdot e^{(-k_{fast} t)} + P_{slow} \cdot e^{(-k_{slow} t)} \text{ and} \qquad (4),$$

$$P_{fast} + P_{slow} = 1 \text{ at } t=0,$$

where P_{fast} and P_{slow} represent the proportions of the fast and slow decay phase and k_{fast} and k_{slow} their respective rate constants.

The recovery phase is described by the model:

$$y_{rec.} = I_{max} - (P_{fast} \cdot e^{(-k_{fast} t)} + P_{slow} \cdot e^{(-k_{slow} t)}) \qquad (5),$$

for F_v/F_m decrease expressed in percentage, where I_{max} represents the maximum value of decrease (in % at 1 h of high light illumination).

Statistical analysis. Statistical comparison between both measuring assays was performed by the student t-test and the significance was set at $p \leq 0.05$.

III.4. Results

Photosynthetic-irradiance-curves. The photosynthetic light-response curves showed differences between the investigated species (Fig. 18). Especially *P. perforata* differed significantly from the other species, with the highest P_{max} and α (2-4 times higher as in the other species) and the lowest light compensation point (E_c) (Fig. 18, Table VII). However, dark-respiration rates (R_d) and light compensation points (E_k) were relatively similar between species with values between 14 and 37 µmol $O_2 \cdot g\ FW^{-1} \cdot h^{-1}$ for R_d and 89 to 164 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for E_k (Table VII).

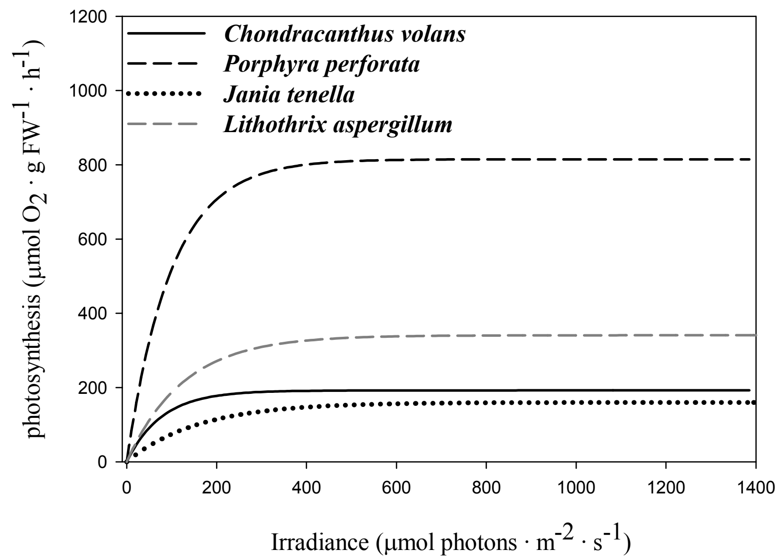

Figure 18. Light-response curves on a wet weight basis for different red algal species. O_2 evolution was measured in seawater containing 4 mM NaHCO3 at 15° C under different light intensities. Curves represent fitting (see text for details) of three measurements.

Figura 18. Curvas de fotosíntesis-irradiancia normalizadas por peso húmedo para diferentes especies de algas rojas. La evolución de O_2 fue medida en agua de mar con 4 mM NaHCO3 a 15° C bajo diferentes intensidades de luz. Las curvas representan el ajuste de tres mediciones independientes (ver texto para detalles).

Table VII. Photosynthetic parameters of different rhodophyte species. P_{max} = light saturation net photosynthetic rate in µmol $O_2 \cdot g\ FW^{-1} \cdot h^{-1}$. R_d = respiration rate in darkness in µmol $O_2 \cdot g\ FW^{-1} \cdot h^{-1}$. α = initial slope at limiting irradiance levels in µmol $O_2 \cdot g\ FW^{-1} \cdot (\mu mol \cdot m^{-2} \cdot s^{-1})^{-1}$. $E_k = P_{max}/\alpha$ = light saturation point in µmol $\cdot m^{-2} \cdot s^{-1}$. $E_c = R_d/\alpha$ = compensation irradiance in µmol $\cdot m^{-2} \cdot s^{-1}$. Each point is the mean of three independent measurements ± standard deviation.

Tabla VII. Parámetros fotosintéticos de diferentes especies de rodófitas. P_{max} = tasa fotosintética máxima en µmol O_2 · g peso húmedo$^{-1} \cdot h^{-1}$. R_d = tasa de respiración en oscuridad en µmol O_2 · g peso húmedo$^{-1} \cdot h^{-1}$. α = pendiente inicial a niveles limitantes de irradiancia en µmol O_2 · g peso húmedo$^{-1} \cdot (\mu mol \cdot m^{-2} \cdot s^{-1})^{-1}$. $E_k = P_{max}/\alpha$ = irradiancia de saturación en µmol $\cdot m^{-2} \cdot s^{-1}$. $E_c = R_d/\alpha$ = irradiancia de compensación en µmol $\cdot m^{-2} \cdot s^{-1}$. Cada punto representa la media de tres mediciones independientes ± DE.

Species	**P_{max} (±SE)**	**R_d (±SE)**	**α (±SE)**	**E_k (±SE)**	**E_c (±SE)**
C. volans	**194** (±14)	**34** (±12)	**2,3** (±0,7)	**89** (±28)	**14,5** (±2,7)
P. perforata	**817** (±210)	**36,7** (±15)	**8,3** (±3,7)	**105** (±28)	**3,6** (±0,5)
L. aspergillum	**342** (±20,5)	**29,4** (±12,5)	**2,7** (±0,6)	**130** (±34)	**10,5** (±2,7)
J. tenella	**161,5** (±37,9)	**14,1** (±1,0)	**1,0** (±0,2)	**164** (±50)	**14** (±3,5)

Pigment characteristics. Between the investigated species, *C. volans* and *L. aspergillum* showed a relatively similar Chl *a* concentration while *J. tenella* presented only half of the Chl *a* content of the former species (Table VIII). In contrast, *Porphyra perforata* exhibited about twice of Chl *a* compared to the other species.

Moreover, the red algal species presented differences in their carotenoid content principally related with differences in the predominant carotenoid (see Chapter I; Schubert et al. 2006a). *Chondracanthus volans*, *Porphyra perforata* and *Lithothrix aspergillum* belong to the Lut-group due to the presence of lutein as the main carotenoid while *Jania tenella* presented antheraxanthin as the principal carotenoid and belongs therefore to the Ant-group (Table VIII).

Table VIII. Pigment composition in red algal species (Chl *a* in mol · g FW^{-1}, carotenoids in mmol pigment · mol Chl a^{-1}) before light treatment.
Tabla VIII. Composición pigmentaria en especies de algas rojas (Chl *a* en mol · g peso húmedo^{-1}, carotenoides en mmol pigmento · mol Chl a^{-1}) antes del tratamiento con luz.

Species	Chl *a*	Lut	Zea	Ant	α-car	β-car
C. volans	1,1 ± 0,2	230,7 ± 21	52,9 ± 9,5		7,3 ± 3	59,8 ± 13
P. perforata	2,8 ± 0,2	210 ± 36	16,4 ± 5		29,4 ± 17	71,7 ± 15
L. aspergillum	1,3 ± 0,5	140 ± 4	15,9 ± 7		8,2 ± 6	38,9 ± 17
J. tenella	0,6 ± 0,06		21,5 ± 9	324,6 ± 49		79,3 ± 42

High light response measured as O_2 evolution activity and fluorescence emission. The time course of decrease and recovery of the photochemical efficiency monitored by O_2 evolution

and fluorescence emission in the investigated red algal species was compared. Strong white light illumination caused a decrease in O_2 evolution and F_v/F_m in all samples.

The values of maximum reduction were significantly higher when measured as oxygen evolution compared to fluorescence emission in *C. volans, J. tenella* and *L. aspergillum* while in *P. perforata* the decline measured as chlorophyll fluorescence was significantly higher compared with the value of the oxygen evolution activity (Fig. 19).

Beside the differences in the decrease and recovery values, also differences in their respective kinetics depending of the measuring assay were found (Fig. 19, 20). In *C. volans*, the values of the decline in photosynthetic efficiency measured as oxygen evolution and F_v/F_m were similar at the end of exposure but the initial decline of O_2 evolution was much faster (Fig. 19A). Moreover, the oxygen evolution activity reached maximum reduction values already at approx. 15 min with no changes until the end of the light exposure.

The decay kinetics of F_v/F_m and O_2 evolution measured in *P. perforata* were similar with a half maximum response at 9.5 min and 12 min, respectively (Fig. 19B). However, the photosynthetic efficiency assayed as oxygen evolution showed a significantly lower decline at the end of exposure (60% of the initial value) compared to F_v/F_m measurements (42% of the initial value).

Jania tenella exhibited a much faster initial decay of photosynthetic efficiency when measured as F_v/F_m with a half maximum response at 4 min compared to 17 min for O_2 evolution (Fig. 19C). In contrast, the photosynthetic efficiency when measured as oxygen evolution showed a higher decline at the end of the light exposure (15% of the initial value) compared to a F_v/F_m decrease to 38% of the initial value.

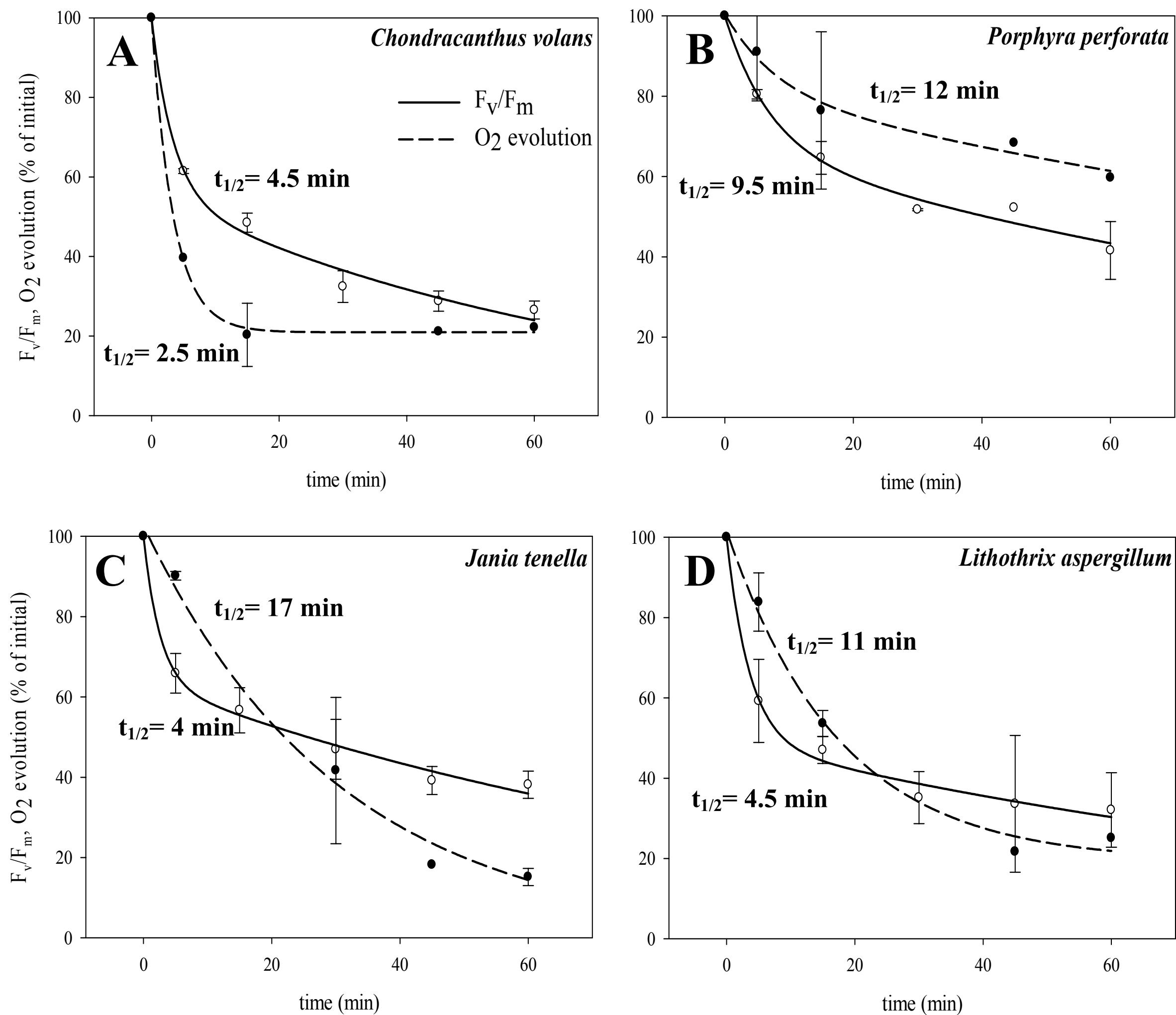

Figure 19. Comparison of decrease kinetics of photosynthetic efficiency in different red algal species at exposure to strong white light illumination (1300 µmol · m^{-2} · s^{-1}, 1 h) when assayed as chlorophyll *a* fluorescence emission (white circles, solid line) and O_2 evolution (black, circles, dashed line). Data were fitted using the mathematical model proposed by Hanelt (1998) and the time necessary to reach half of the maximal response ($t_{1/2}$) was included. Values are expressed in percentage to the activity found before light treatment (means ± standard deviation, n = 3 samples).

Figura 19. Comparación entre las cinéticas de la disminución de la eficiencia fotosintética en diferentes especies de algas rojas expuestas a alta irradiancia (1300 µmol · m^{-2} · s^{-1}, 1 h), medida como fluorescencia de clorofila *a* (círculos blancos, línea sólida) y evolución de O_2 (círculos negros, línea interrumpida). Los datos fueron ajustados al modelo matemático propuesto por Hanelt (1998) y el tiempo para alcanzar el 50% de la respuesta máxima fue incluído. Los valores son expresados en porcentaje de la actividad medida antes del tratamiento de luz (media ± DE, n = 3).

The same pattern as in *J. tenella* was found in *L. aspergillum* where a faster initial decline in F_v/F_m compared to O_2 evolution was observed related to a $t_{1/2}$ of 4.5 min and 11 min, respectively (Fig. 19D).

After high light exposure (6 h in dim light conditions), in most species a recovery up to 70 or 80% of the initial value was found, with slight higher values for the photosynthetic efficiency measured as O_2 evolution compared to F_v/F_m (Fig. 20). The only exception was *P. perforata* where the O_2 evolution recovered almost to 100% (Fig. 20B).

With respect to the recovery kinetics, in *C. volans* the O_2 evolution activity recovered much faster compared to F_v/F_m with a half-maximum response at 25.5 min and 199 min, respectively (Fig. 20A). On the other hand, in *P. perforata,* F_v/F_m reached the half-maximum response faster (44 min) compared to the O_2 evolution activity (68 min) (Fig. 20B).

Similar to *C. volans*, *J. tenella* exhibited a faster initial recovery of the O_2 evolution activity ($t_{1/2}$= 26.5 min) compared to the kinetics observed for F_v/F_m with a half-maximum response at 174 min (Fig. 20C). In contrast, *L. aspergillum* showed relatively similar recovery kinetics of the measuring assays (Fig. 20D).

Thus, depending on the measuring assay, the reduction and recovery of the photosynthetic efficiency was higher or lower. Moreover, significantly differences in the decline and recovery kinetics between F_v/F_m and O_2 evolution have been found that seem to be species-related.

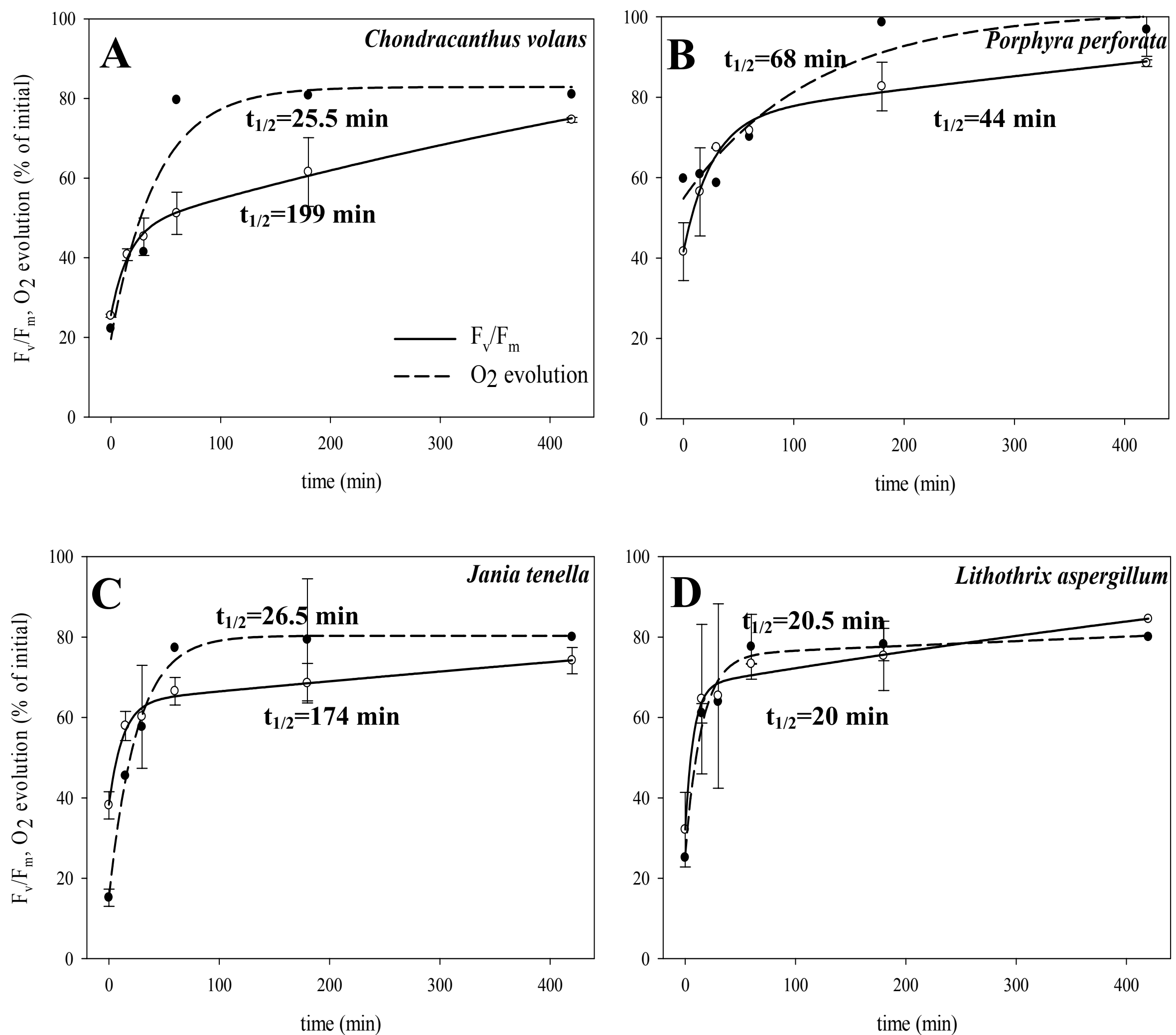

Figure 20. Comparison of recovery kinetics of photosynthetic efficiency in different red algal species in dim light conditions (~20 µmol · m^{-2} · s^{-1}, 1 h) when assayed as chlorophyll *a* fluorescence emission (white circles, solid line) and O_2 evolution (black, circles, dashed line). Data were fitted using the mathematical model proposed by Hanelt (1998) and the time necessary to reach half of the maximal response ($t_{1/2}$) was included. Values are expressed in percentage to the activity found before light treatment (means ± standard deviation, n = 3 samples).

Figura 20. Comparación entre las cinéticas de la recuperación de la eficiencia fotosintética en diferentes especies de algas rojas en condiciones de luz tenue (~20 µmol · m^{-2} · s^{-1}, 1 h), medida como fluorescencia de clorofila *a* (círculos blancos, línea sólida) y evolución de O_2 (círculos negros, línea interrumpida). Los datos fueron ajustados al modelo matemático propuesto por Hanelt (1998) y el tiempo para alcanzar el 50% de la respuesta máxima fue incluído. Los valores son expresados en porcentaje de la actividad medida antes del tratamiento de luz (media ± DE, n = 3).

***III*.5. Discussion**

The observed differences between species with respect to their photosynthetic characteristics, especially for *P. perforata*, probably result from the normalization by wet weight. *Porphyra perforata* presents a thin flat-like thallus of one cell layer so that it can be supposed that all cells are involved in photosynthesis. For this kind of algae, a direct correlation between pigment content and photosynthesis is likely due to proportionality of absorptance and photosynthesis (see Ramus 1990). However, species as *C. volans* and the coralline algae *J. tenella* and *L. aspergillum* present ticker, more optically complex tissues where not all cells are involved in photosynthesis. For these species it is more reliable to normalize to wet weight, dry weight or area (see Ramus 1990). The normalization of the photosynthesis-irradiance curves by the Chl *a* concentration of the species resulted in relatively similar photosynthetic characteristics (data not shown) due to higher Chl *a* content of *P. perforata* compared to the other species.

In the present study, no linear correlation between F_v/F_m and oxygen production was observed at exposure of the red algal species to light stress conditions. Instead, differences in the degree of decrease and recovery and their respective kinetics have been found. Generally, the decrease of F_v/F_m could be due to the increase in slow-relaxing, photoprotective energy dissipation, competitive to the photochemical events causing a decrease in the yield of photosynthesis (i.e. also in the initial slope of the photosynthetic response curves) that is not visible in O_2 measurements. On the other hand, the decrease of P_{max} which is reflected by a decrease of oxygen evolution activity is related to inactivation of PSII reaction centres, either by reversible inactivation triggered by the formation of a proton gradient and related with photoprotection or by irreversible damage of PSII centres

(Krause 1988, Leitsch et al. 1994). Thus, uncoupling of reaction centres from the electron transport chain, an increasing number of inactive, damaged or slowly reopening reaction centres or a limitation of processes involved in the Calvin cycle could cause a decrease in O_2 evolution activity.

The higher initial decrease of F_v/F_m as compared to oxygen evolution activity during high light treatment observed in three of the four investigated red algal species indicates that there are processes involved in quenching of F_v/F_m that not directly affect PSII oxygen evolution, as dissipation of excess energy as heat that affects the photosynthetic efficiency but not P_{max} (Leverenz et al. 1990, Henley 1993, Hanelt et al. 1995). Previously, in *P. perforata*, a large decrease of variable fluorescence produced by high light exposure has been reported which was not accompanied by reduction of O_2 evolution rate (Bose et al. 1988). Part of the initial decrease of F_v/F_m in this species has been reported to be related with the formation of a proton gradient and thus it is less pronounced in presence of an inhibitor of the transthylakoid proton gradient (Bose et al. 1988). The oxygen evolution activity showed a lag phase during the first 20 min in *P. perforata* (Herbert and Waaland 1988, Herbert 1990) that is eliminated by chloramphenicol and therefore it appears to present an initial, rapid translation rate of high light-sensitive proteins followed by a slower rate (Herbert 1990). Hence, the importance of D1 turnover during and after high light exposure in this species has been shown previously (see Chapter II).

The higher and faster decline of the oxygen production compared to F_v/F_m in *C. volans* could be result of processes that consume oxygen such as photorespiration. As reported previously by Hanelt et al. (1992) in several red algal species, photorespiration increased during high light treatment and resulted in a ¼ decrease of the P_{max}-level. In contrast, it was

suggested that photorespiration in *P. perforata* had apparently not a significant photoprotective effect (Herbert and Waaland 1988). Moreover, quickly reversible PSII inactivation as proposed by Leitsch et al. (1994) could be involved in the fast decay of the O_2 production as observed in *C. volans* due to its fast recovery that makes it unlikely that the strong initial decay during light exposure was related to photodamage.

In conclusion, the combined measurement of oxygen production and fluorescence emission during and after high light exposure in different red algal species indicates that in the decline of F_v/F_m, down-regulatory mechanisms were involved, that resulted in a faster reduction of F_v/F_m compared to the O_2 evolution activity. However, in the slow component of the F_v/F_m decrease also photodamage seemed to be involved due to the incomplete recovery of both, F_v/F_m and oxygen evolution activity during short-term recovery (70-80% of the initial value). Moreover, the high fast initial recovery of the O_2 evolution activity indicates the involvement of processes such as reversible PSII inactivation in photoprotection in red algae. However, complementary experiments about the importance of D1 turnover and reversible PSII inactivation related with the formation of a proton gradient are necessary to insure the correct interpretation of the data.

Chapter IV

IMPORTANCE OF CAROTENOID COMPOSITION IN THE ACCLIMATIVE RESPONSE TO HIGH LIGHT EXPOSURE IN TWO RED ALGAL SPECIES

IV.1. Abstract

Previous works showed that within the red algal group different carotenoid profiles are presented and that there exist a relationship between the pigment profile and the F_v/F_m decrease and recovery kinetics at light stress conditions, indicating expression of photoprotective mechanisms related to the carotenoid content of the species. In the present study, the response to exposure at different light intensities in two species, representatives of different carotenoid profile groups, were compared. Especially, the response of thallus segments, apical and basal section, of the same species were compared and related to differences in the carotenoid content due to different light-acclimations to evaluate the role of carotenoid content in photoprotection. The differential responses to cope with light stress in both species were reflected by comparing the light response in thallus segments with different light-acclimations. In *Gracilaria damaecornis*, a zeaxanthin-containing species, the basal section (low light-acclimated) showed much less F_v/F_m decrease, especially at low light intensities, compared to the apical section (high light-acclimated) that seemed to be related to a much higher zeaxanthin and β-carotene content in the apical segment. Interestingly, in this species, a change in the carotenoid profile depending on light-acclimation were found. Apparently, high zeaxanthin content was related to high light-acclimation while under low irradiance, the tissue presented antheraxanthin as the main

carotenoid. This, together with a strong quenching of F_v/F_m indicates an involvement of zeaxanthin in the down-regulation of photosynthesis. On the other hand, in *Eucheuma isiforme*, a lutein-containing species, the opposite pattern was found. As expected, the basal segment was more susceptible to different light intensities compared to the apical segment, accompanied with a higher content of all carotenoids in the apical segment. However, here zeaxanthin, if involved in photoprotection, seemed to be related to another mechanism, not involved in slow-relaxing chlorophyll fluorescence quenching as observed in *G. damaecornis*.

***IV*.2. Introduction**

In the marine habitat, especially in shallow water or in the intertidal zone, macroalgae are exposed to considerable daily changes in solar irradiance due to wave focusing, canopy movements, progressive tides, and suspended particles. Thus, macroalgae living in intertidal environments must have both, the capacity to efficiently harvest light energy at low irradiances and to be able to withstand excess energy. The ability to withstand variations in irradiance and to resist high light stress may be one of the factors determining the competitive ability of macroalgae at the upper limit of their distribution (Herbert 1990, Hanelt 1996, 1997).

When exposed to light stress, photosynthetic organisms show a light-dependent reduction in photosynthetic efficiency (light-limited photosynthesis) occurring when photons are absorbed in excess of those which can be used for photosynthesis. This phenomenon is related to the photoinactivation of photosystem II (PSII) electron transport. In parallel, the

repair of photodamaged PSII centers via *de novo* synthesis of the D1 protein is activated under high light conditions (Krause 1988, Aro et al. 1993b).

Higher plants and macroalgae have a number of protective mechanisms that serve to ameliorate light stress (Demmig-Adams and Adams 1992, Niyogi 1999). Dissipation of excess light energy as heat is one of the most important photoprotective strategies that down-regulates PSII activity. This strategy is related to the presence of specific carotenoids that have the capability to dissipate excess light energy as heat (Demmig-Adams and Adams 1996b, Havaux and Niyogi 1999). One of the key carotenoids involved in photoprotection is zeaxanthin (Demmig-Adams 1990), but also antheraxanthin and lutein have been related with energy dissipation (Niyogi et al. 1997, Goss et al. 1998, Gilmore and Yamamoto 2001, Garcia-Plazaola et al. 2003). Short-term synthesis (min) of the photoprotective carotenoid zeaxanthin is achieved through the xanthophyll cycle (XC), which consists in the interconversion of violaxanthin to zeaxanthin via antheraxanthin in saturating light conditions and its back-conversion in darkness or subsaturating light conditions (Yamamoto et al. 1962).

Down-regulatory mechanisms as the XC in higher plants and most algae take place in the antenna complex of PSII (Färber et al. 1997, Jahns et al. 2001, Phillip et al. 2002). Red algae present a different PSII antenna arrangement and apparently do not have the xanthophyll cycle (see Chapter II; Stransky and Hager 1970b, Larkum 2003). Instead, they present phycobilisomes as antenna for PSII as found also in cyanobacteria. Therefore, the strategies and/or mechanisms to overcome with light stress presented in the Rhodophyta might not be similar to higher plants. Probably, a high steady state concentration of zeaxanthin or lutein as found in several red algal species (Schubert et al. 2006a) represent

an efficient photoprotective strategy since these carotenoids are capable to functioning as direct quenchers of excess light energy from excited Chl (Demmig-Adams 1990, Niyogi et al. 1997).

On the other hand, photosynthetic organisms are able to overcome photodamage by the rapid and efficient repair of PSII under light stress conditions (Ohad et al. 1984, Aro et al. 1993a). This repair requires protein synthesis. Rapid turnover of the D1 polypeptide in reaction centre of PSII plays a major role in maintaining PSII integrity in high light, especially for 'sun' acclimated plants (reviewed by Long et al. 1994, Ohad et al. 1994). The rate of photodamage is proportional to the intensity of light (Tyystjärvi and Aro 1996, Nishiyama et al. 2004), whereas the rate of repair is the highest only at a certain light intensity (Allakhverdiev and Murata 2004). When photosynthetic cells are exposed to low light, the rate of repair is higher than the rate of photodamage and, thus, apparently photodamage does not occur. However, when cells are exposed to saturating light, the rate of photodamage exceeds the rate of repair, resulting in the reduction of PSII activity (Greer et al. 1986). The molecular mechanisms involved in the PSII repair cycle have been thoroughly characterized in higher plants. In contrast, there is scare information related to PSII dynamics of photodamage and repair in red algae.

Red algae present different carotenoid profiles (Schubert et al. 2006a). In some species lutein is the predominant carotenoid while zeaxanthin occurs in minor concentration. In other species zeaxanthin or antheraxanthin have been found as the principal carotenoids. Species with different carotenoid profiles apparently have different PSII down-regulatory mechanisms since they present different kinetics of the decline of the maximum quantum

efficiency of PSII (F_v/F_m) under light stress and during recovery in dim light (Schubert and García-Mendoza, submitted).

There are several approaches to identify and to characterize the importance of the mechanisms involved in the regulation of photosynthetic activity and photoprotection. One of the most important is to reinforce the expression of these mechanisms by acclimation of the organism to different (sometimes extreme) conditions. In higher plants it is well known that the most important regulation response is the change of the size and number of PSII (Falkowski and LaRoche 1991) and in relation to photoprotection, the XC pool as well as NPQ increases in high light-acclimated organisms (Demmig-Adams and Adams 1996a, Eskling and Åkerlund 1998, Rodrigues et al. 2002, Colombo-Pallotta et al. 2006).

In the present work, our experimental goal was to characterize the high light-response of differential light-acclimated thallus segments of a single organism. Differences in acclimation have been reported to cause differences in the high light-response of the tissue (Enríquez et al. 2002) and also differences in carotenoid content and therefore photoprotection (e.g. Collomba-Pallotta et al. 2006)

Therefore, acclimation characteristics and the response upon exposure to different light intensities were assessed to determine the role of photoprotection especially that related to carotenoids and D1 turnover in two red algal species with different carotenoid profiles.

IV.3. Material and methods

Plant material. Two red algal species with lutein or zeaxanthin as the main carotenoid were used for this study. *Eucheuma isiforme*, belonging to the first group, and *Gracilaria damaecornis*, representative for the zeaxanthin group, were collected at 1-2 m depth near

the beach during September 2007 in Santa Clara, Yucatan, Mexico. Both species present a basal growth pattern. They had a coarsely-branched morphology with similar length and thallus diameter of the branches. The collected algae were maintained in outdoor tanks at the Instituto de Ciencias del Mar y Limnología (ICMyL, UNAM) facilities in Puerto Morelos. The algae were maintained with continuous flow of seawater until the experiments that were performed within 2 days after collection.

Thallus optical properties. Thallus light absorption was measured using the opal glass technique developed for intact plant leaves by Shibata (1959). Fragments of algal thalli were mounted into 3 mL cuvettes filled with filtered seawater. Thallus sections were held against the wall of the cuvette with a holder specifically developed to avoid thallus misplacement during the spectroscopic determinations. Absorbance values (i.e. optical density, OD) were determined at 1 nm intervals between 350 and 750 nm in an Aminco DW2 (USA) spectrophotometer controlled by an OLIS (USA) data collection system. Bleached thallus segments were used as a reference to correct for non-pigment absorption. Algal tissue were bleached by submerging the samples (30 min) in filtered seawater with 15% bleach (vol:vol). The absorption spectra were also corrected by subtracting absorbance at 750 nm to exclude residual scattering; however, in all cases, there were low levels of back- and residual scattering. The percentage of light absorbed by pigments (absorptance; A) was calculated using the equation:

$$A = 100 \cdot (1-10^{-OD}) \quad (6).$$

Two descriptors were used: (1) average light absorptance for the range between 400 and 700 nm (PAR range) called A_{PAR}, and (2) average light absorptance for the range between 400 and 700 nm (PAR range) corrected by the lamp emission spectrum to obtain the photosynthetic utilizable radiation (PUR) called A_{PUR}. This correction was made by multiplying the absorptance spectrum with the lamp emission spectrum measured with a fiber optic spectrometer (USB4000, Ocean Optics Inc., Dunedin, USA). After the analysis, the thallus segments was frozen (-70° C) for pigment content analysis.

Pigment light absorption efficiency related to total pigment content ($a^*_{pigments}$, $m^{-2} \cdot mg^{-1}$ [pigments]) and chlorophyll *a* absorption efficiency ($a^*_{Chl\ a}$, $m^{-2} \cdot mg^{-1}$ Chl [*a*]) were calculated according to Enríquez and Sand-Jensen (2003) and Enríquez (2005). The light absorption efficiency was calculate as

$$a^*=(OD/\rho) \cdot \ln 10 \qquad (7),$$

with OD as the absorbance in the PAR range ($a^*_{pigments}$) or at 677 nm ($a^*_{Chl\ a}$) and ρ representing the pigment concentration (total pigment content or Chl *a* concentration).

Pigment concentration was measured spectrophotometrically on the same fragments used for light absorption measurements. Phycobilipigment extraction was done by maintaining the extracts in sodium phosphate buffer (0.1 M, pH 6.8) for 2 h in darkness at 4° C after grinding the tissue with liquid nitrogen. Subsequently, the extract was centrifuged (15600 g, 2 min) and the supernatant was recovered for phycobilipigment determination. The pellet was resuspended with dimethylformamide (DMF) to extract lipophylic pigments overnight

in darkness at 4°C. The phycobilipigment content was calculated according to Kursar et al. (1983) and the total chlorophyll *a* of algal extracts was calculated using the equation of Porra (2002).

Light treatments. The algal samples were dark-adapted for one hour in the absence or presence of chloramphenicol (CAP; Sigma Aldrich, Steinheim, Germany), a chloroplast-protein synthesis inhibitor, at a final concentration of 1 mg · mL^{-1}. Subsequently, the samples were placed in a home-made acrylic chamber with continuous stirring and constant water temperature (28 ± 0.1° C). The algal samples were illuminated with white actinic light (250, 500, 1000, 1500 and 2000 µmol · m^{-2} · s^{-1}) provided by a halogen lamp for one hour. PSII maximum quantum efficiency (F_v/F_m) was measured before and during the light treatment and during a recovery period of 4 h at 25 µmol · m^{-2} · s^{-1}.

Fluorescence measurements. Measurements of the maximum efficiency of PSII charge separation (F_v/F_m) was done with a pulse amplitude modulated fluorometer (Diving-PAM; Heinz Walz, Effeltrich, Germany). The samples were dark-incubated for one hour and afterwards a 5 s far-red pulse was applied followed by further 5 min dark-incubation to ensure complete relaxation of the electron transport chain. PSII maximum quantum efficiency was calculated as F_v/F_m with the variable fluorescence (F_v) as the difference between the maximum (F_m) and minimum (F_o) fluorescence emission.

Pigment analysis. Pigment composition was measured in five samples from the basal and apical segments of both species. Samples were taken from algae maintained for 1 h in darkness before the light exposure experiments. The samples were frozen with liquid N_2 and kept at -70° C until further analysis. Pigment extraction and quantification was

conducted as described in Schubert et al. (2006a). The pigments of the algal samples were extracted in dimethylformamide (DMF) overnight at 4° C in darkness after grinding the previously frozen tissue. The pigment samples were analyzed by HPLC (High-Performance Liquid Chromatography) according to Schubert et al. (2006a). The HPLC equipment was a Shimadzu AV-10 system equipped with a Nucleosil C-18 reverse phase column (250 mm, 4.6 mm internal diameter and 5 μm particle size) and a guard column Eclipse XDB-C8 (12.5 mm, 4.6 mm internal diameter and 5 μm particle size). The detection wavelength was set at 447 nm.

Statistical analyses were performed by one-way ANOVA using the software Statistica 6.0.

_IV_.4. Results

Pigments and thallus optical properties. As *Eucheuma isiforme* and *Gracilaria damaecornis* present a basal growth pattern, younger parts of the algal thallus (basal tissue) are exposed to lower light intensities than the older apical sections. Therefore, the basal tissue is photoacclimated to low light conditions and apical tissue is acclimated to higher light conditions. This difference was reflected as in the pigment contents as in the thallus absorption characteristics.

The investigated species presented different pigment profiles. Lutein and β-carotene were found in high concentration in *E. isiforme* but also zeaxanthin and α-carotene were present. Differences in the carotenoid content per Chl *a* were mainly associated with the lower chlorophyll *a* content of the apical section (Table IX).

The total carotenoid content was 80% higher in the apical segment (Table IX) due to a significantly higher concentration of α-carotene and β-carotene (Fig. 21A). Moreover, the Lut:Zea ratio decreased from eight in the basal section to two in the apical section.

Table IX. Pigment content (PE- phycoerythrin, PC- phycocyanin), pigment specific absorption coefficients ($a^*_{pigments}$, absorption coefficient for total pigment content; $a^*_{Chl\ a}$, Chl *a* specific absorption coefficient) and absorptance for PAR range (A_{PAR}) and for photosynthetic utilizable radiation (A_{PUR}).

Tabla IX. Contenido de pigmentos (PE- ficoeritrina, PC- ficocianina), coeficientes específicos de absorción ($a^*_{pigments}$, coeficiente específico de absorción para el contenido total de pigmentos; $a^*_{Chl\ a}$, coeficiente específico de la Chl *a*) y valores de absorptancia para el rango del PAR (A_{PAR}) y para la radiación utilizable para la fotosíntesis (A_{PUR}).

	E. isiforme		***G. damaecornis***	
	Apical	**Basal**	**Apical**	**Basal**
PE ($mg \cdot m^{-2}$)	63 ± 11	156 ± 46	40 ± 13	220 ± 11
PC ($mg \cdot m^{-2}$)	4 ± 0.6	13.4 ± 5.7	3.2 ± 0.6	32.4 ± 10
Chl *a* ($mg \cdot m^{-2}$)	1.3 ± 0.4	9.8 ± 3.2	3.6 ± 1.9	8.1 ± 4
Carotenoids ($mol \cdot mol\ Chl\ a^{-1}$)	2.2 ± 0.7	0.42 ± 0.06	0.75 ± 0.14	0.34 ± 0.06
$a^*_{Chl\ a}$ ($m^{-2} \cdot mg\ Chl\ a^{-1}$)	0.93 ± 0.4	0.13 ± 0.05	0.22 ± 0.13	0.15 ± 0.02
$a^*_{pigments}$ ($m^{-2} \cdot mg\ pigment^{-1}$)	0.011 ± 0.004	0.006 ± 0.003	0.009 ± 0.005	0.004 ± 0.001
A_{PAR} (%)	57 ± 4	61 ± 5	41 ± 3	71 ± 4
A_{PUR} (%)	38 ± 2	43 ± 3	24 ± 2	49 ± 2

Gracilaria damaecornis presented a different carotenoid profile as compared to *E. isiforme*, with violaxanthin, antheraxanthin, zeaxanthin, and β-carotene. Similar to *E. isiforme*, the apical section showed a 55% higher carotenoid concentration compared to the basal section (Table IX), here related to a significantly higher zeaxanthin and β-carotene content in the apical segment (Fig. 21B).

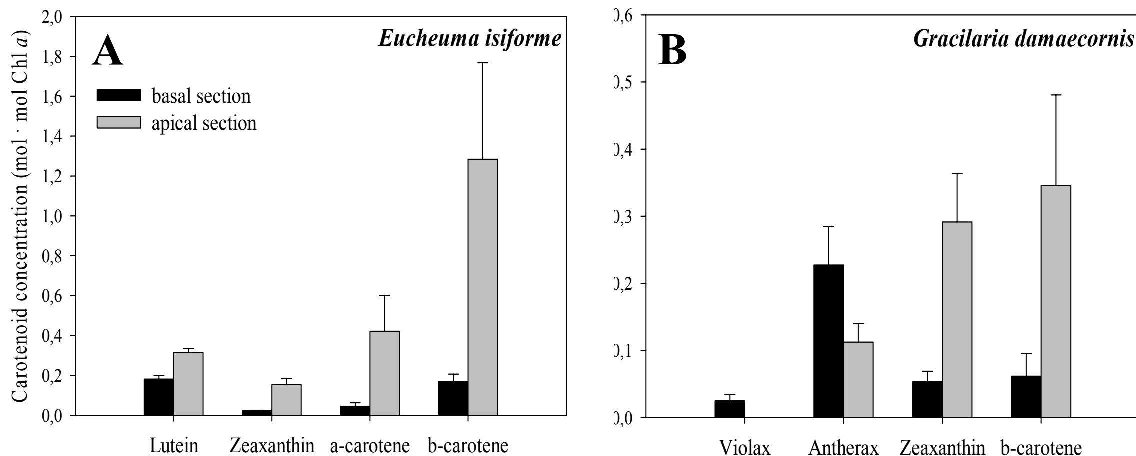

Figure 21. Carotenoid composition in *Eucheuma isiforme* (A) and *Gracilaria damaecornis* (B) expressed in mol carotenoid · mol Chl a^{-1} (Violax- violaxanthin, Antherax- antheraxanthin).

Figura 21. Composición de carotenoides en *Eucheuma isiforme* (A) y *Gracilaria damaecornis* (B) expresados en mol carotenoide · mol Chl a^{-1} (Violax- violaxantina, Antherax- anteraxantina).

There was an important response observed in this species: de-epoxidated carotenoid concentration (zeaxanthin + β-carotene) was higher in the apical as in basal segment. Here, the proportion of epoxidated carotenoids (antheraxanthin + violaxanthin) to the total carotenoid content was 73% higher in the basal than in the apical section (15%).

Phycobilipigment content were similar in *G. damaecornis* and *E. isiforme* but differences between apical and basal sections of each species were detected. The concentration of phycobilipigments was two (*E. isiforme*) to five times (*G. damaecornis*) higher in the basal as compared to the apical section (Table IX).

The differences in Chl *a* and phycobilipigment content between apical and basal sections were reflected in the *in vivo* absorption. Basal sections of both species showed similar

absorption that was higher compared to apical sections (Fig. 22A). In contrast, the apical segment of *E. isiforme* showed a higher absorption as compared to the apical segment of *G. damaecornis* (Fig. 22A). These differences were also reflected in the absorptance values (A_{PAR}, A_{PUR}) (Table IX).

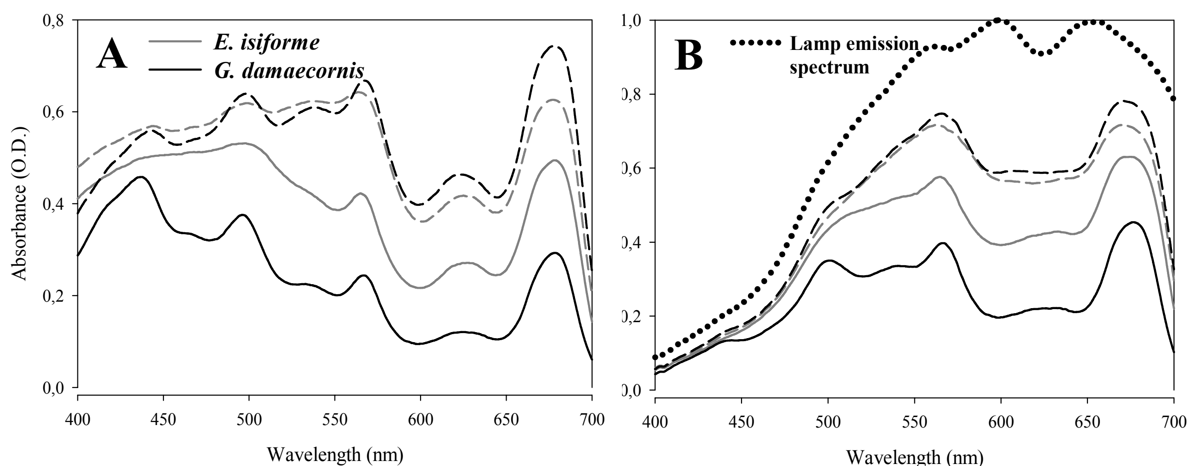

Figure 22. *In vivo* absorption (A) and absorptance (A_{PUR}) spectra (B) for the apical (solid line) and basal sections (broken line) of *E. isiforme* (grey line) and *G. damaecornis* (black line). Absorptance spectra were corrected by the emission spectrum of the lamp used for exposure (dashed line) (B).

Figura 22. Espectros de absorción *in vivo* (A) y de absorptancia (A_{PUR}) (B) para las secciones apicales (línea sólida) y basales (línea interrumpida) de *E. isiforme* (línea gris) y *G. damaecornis* (línea negra). Los espectros de absorptancia fueron corregidos por el espectro de la emisión de la lámpara de exposición (línea punteada) (B).

When the absorption spectra were corrected by the emission spectrum of the lamp used for the light exposure to obtain the photosynthetically usable radiation (PUR), significantly higher values for the basal sections were found (Table IX, Fig. 22B). Also, apical A_{PUR} was significantly higher (ANOVA, $p < 0.05$) in *E. isiforme* (38%) as compared to *G.*

damaecornis (24%) (Table IX). Moreover, the differences in absorption and absorptance between basal and apical segments of *E. isiforme* and *G. damaecornis* was accompanied by higher specific absorption coefficients ($a^*_{Chl\ a}$ and $a^*_{pigments}$) for the apical sections (Table IX), indicating a higher pigment absorption efficiency of these sections. Such enhancement in pigment absorption efficiency may counterbalance the reduction in thallus absorptance associated with the loss of pigments.

Light treatments. The investigated red algal species differed in their regulation of PSII activity, measured as F_v/F_m, when exposed to different light intensities. *Gracilaria damaecornis* showed a fast F_v/F_m decrease to 50% of the initial value at low light intensities up to 200 µmol · m^{-2} · s^{-1} but afterwards a more slowly decrease while *E. isiforme* exhibited a more uniform F_v/F_m decline (Fig. 23A). Moreover, the last species showed a significantly lower F_v/F_m reduction (ANOVA, $p< 0.05$) at all light intensities when compared to *G. damaecornis* (Fig. 23A), even though the former species exhibited a significantly lower A_{PUR} compared to *E. isiforme* (24% and 38%, respectively).

In contrast to the differences between species found at exposure to different light intensities, the recovery values of F_v/F_m after 4 h in dim light conditions (~25 µmol · m^{-2} · s^{-1}) was similar between species (Fig. 23B).

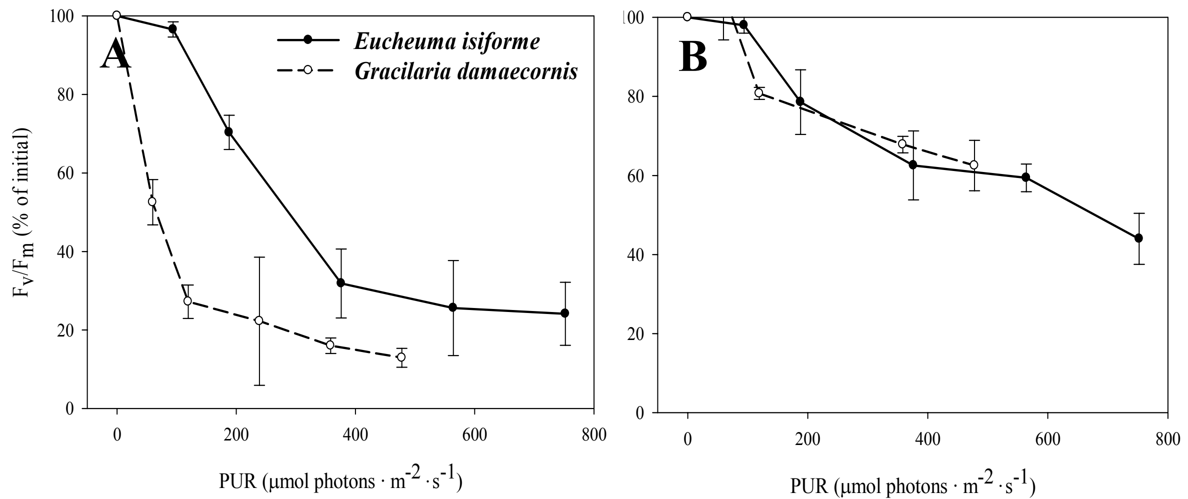

Figure 23. F_v/F_m decrease and recovery during and after exposure to different light intensities. A) F_v/F_m decrease after 1 h of light exposure and B) F_v/F_m recovery in dim light conditions (4 h at ~25 µmol photons · m^{-2} · s^{-1}) after light exposure. F_v/F_m was measured in apical sections of the thallus of *E. isiforme* (black circles, solid line) and *G. damaecornis* (white circles, broken line). For comparison purposes F_v/F_m was related to the specific PUR of the samples (see Material and methods).

Figura 23. Disminución y recuperación de F_v/F_m durante y después de la exposición a diferentes intensidades de luz. A) Disminución de F_v/F_m después de 1 h de exposición y B) recuperación de F_v/F_m en condiciones de luz tenue (4 h a ~25 µmol fotones · m^{-2} · s^{-1}) después del tratamiento con luz. F_v/F_m fue medido en las secciones apicales del talo de *E. isiforme* (círculos negros, línea sólida) y de *G. damaecornis* (círculos blancos, línea interrumpida). Para la comparación, F_v/F_m fue relacionado al PUR específico de las muestras (ver Materiales y métodos).

Moreover, comparing the apical and the basal sections of the thallus, differences in F_v/F_m in both species were found. The dark-adapted F_v/F_m was significantly higher in the basal sections of each species (0.6 ± 0.04 for *E. isiforme* and 0.5 ± 0.08 for *G. damaecornis*) as compared to the apical section (0.43 ± 0.06 for *E. isiforme* and 0.31 ± 0.08 for *G. damaecornis*). Furthermore, the apical section of *G. damaecornis* presented a significantly higher F_v/F_m decline at all light intensities compared to the basal segment (ANOVA, p<

0.05) (Fig. 24A). Moreover, the recovery of F_v/F_m after 4 h in dim light conditions showed higher values for the basal section (Fig. 24B).

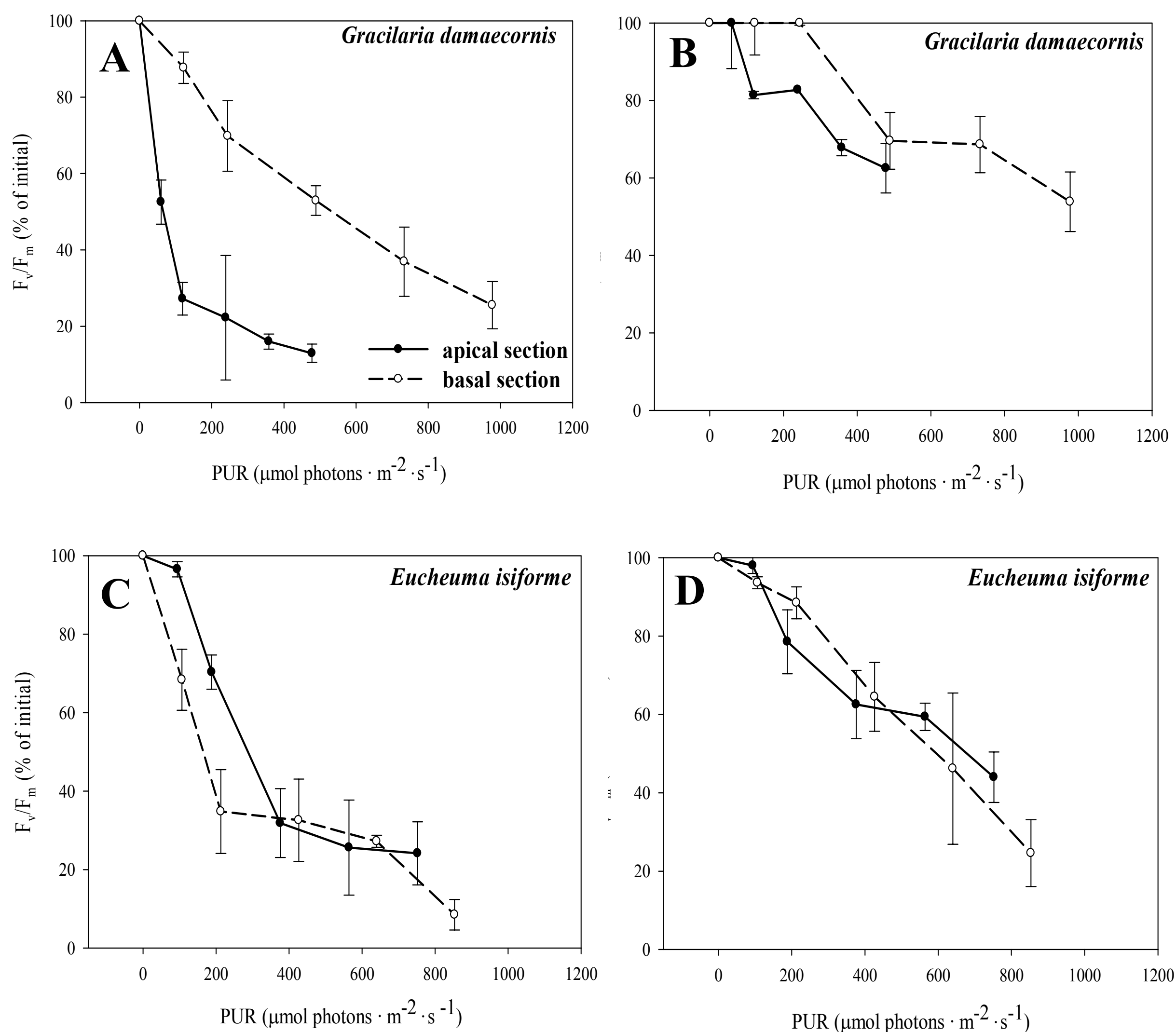

Figure 24. Decrease of F_v/F_m during exposure for 1 h to different light intensities (A, C) and its recovery after 4 h in dim light conditions (~25 µmol photons · m^{-2} · s^{-1}) (B, D) in apical (black circles, solid line) and basal sections (white circles, broken line) of *G. damaecornis* (A, B) and *E. isiforme* (C, D). The different light intensities are expressed as PUR.

Figura 24. Disminución de F_v/F_m durante 1 h de exposición a diferentes intensidades de luz (A, C) y su recuperación después de 4 h en condiciones de luz tenue (~25 µmol fotones · m^{-2} · s^{-1}) (B, D) en secciones apicales (círculos negros, línea sólida) y basales (círculos blancos, línea interrumpida) de *G. damaecornis* (A, B) y de *E. isiforme* (C, D). Las diferentes intensidades de luz son expresadas como PUR.

E. isiforme showed the opposite pattern as *G. damaecornis*. Here, the basal section exhibited a higher F_v/F_m decrease at lower light intensities as compared to the apical section. However, after 4 h of recovery, apical and basal section reached similar F_v/F_m values for all light treatments (Fig. 24C, D).

CAP effect. Chloramphenicol, an inhibitor of chloroplast-encoded protein synthesis caused a differential effect on F_v/F_m reduction and recovery in the apical and basal sections of both species. A significantly lower F_v/F_m in the presence of CAP was detected in the apical section of *G. damaecornis* only when exposed to 250 µmol · m^{-2} · s^{-1} meanwhile the CAP effect in the basal section was shown at light exposure up to 1000 µmol · m^{-2} · s^{-1} (Fig. 25A, B). The opposite pattern was found after 4 h of recovery at dim light conditions with D1 turnover after exposure up to 1500 µmol · m^{-2} · s^{-1} in the apical section while in the basal section D1 repair was observed only after exposure up to 500 µmol · m^{-2} · s^{-1} (Fig. 25C, D). In *E. isiforme*, the presence of CAP caused significant lower F_v/F_m values at irradiances below 500 µmol · m^{-2} · s^{-1} in the basal and apical section (Fig. 26A, B). However, during recovery, a CAP effect in the apical section was found only upon exposure at 250 µmol · m^{-2} · s^{-1} meanwhile in the basal section no effect was observed (Fig. 26C, D).

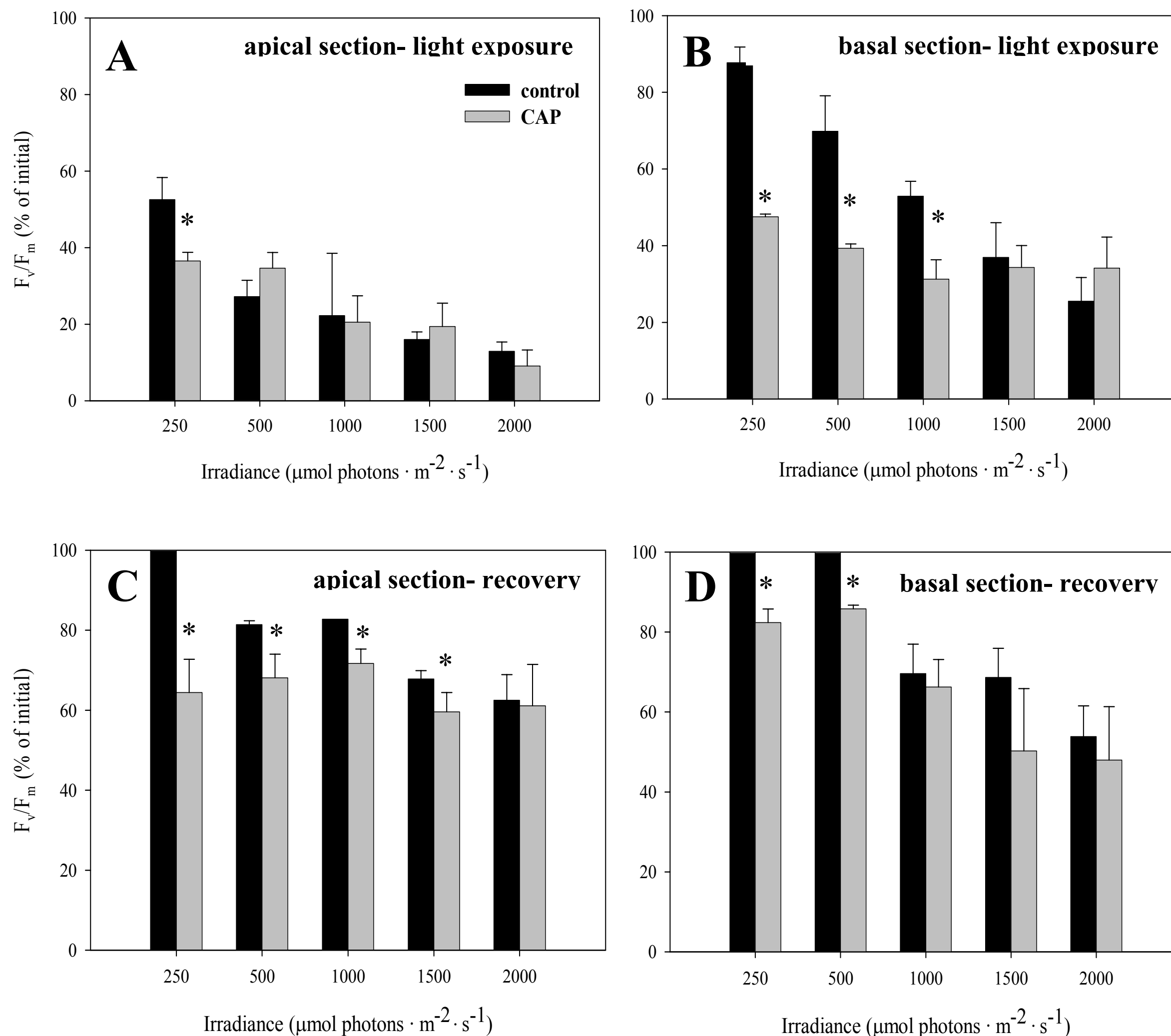

Figure 25. Decrease of F_v/F_m after exposure to different light intensities (A, B) and its recovery in dim light conditions (4 h at ~25 µmol photons · m^{-2} · s^{-1}) (C, D) in apical (A, C) and basal section (B, D) of *G. damaecornis* in absence and presence of CAP (n=5). * Significant differences between control and CAP-treated samples (ANOVA, p<0.05).

Figura 25. Disminución de F_v/F_m después de exposición a diferentes intensidades de luz (A, B) y su recuperación en condiciones de luz tenue (4 h a ~25 µmol fotones · m^{-2} · s^{-1}) (C, D) en la sección apical (A, C) y basal de *G. damaecornis* (B, D) en presencia y ausencia de CAP (n=5). * Diferencias significativas entre control y muestras tratadas con CAP (ANOVA, p<0.05).

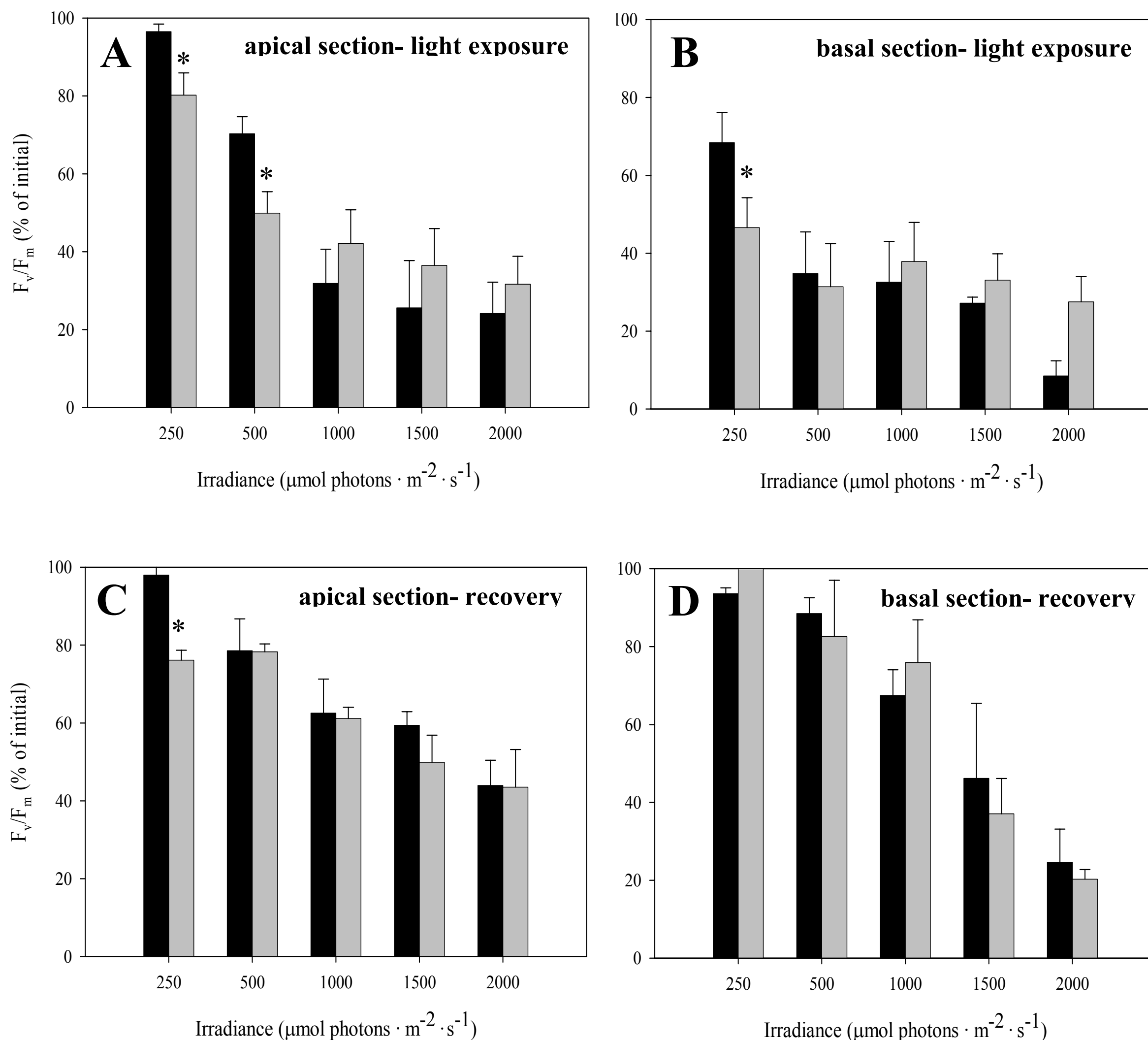

Figure 26. Decrease of F_v/F_m after exposure to different light intensities (A, B) and its recovery in dim light conditions (4 h at ~25 µmol photons · m^{-2} · s^{-1}) (C, D) in apical (A, C) and basal sections (B, D) of *E. isiforme* in absence and presence of CAP (n=5). * Significant differences between control and CAP-treated samples (ANOVA, $p<0.05$).

Figura 26. Disminución de F_v/F_m después de exposición a diferentes intensidades de luz (A, B) y su recuperación en condiciones de luz tenue (4 h a ~25 µmol fotones · m^{-2} · s^{-1}) (C, D) en la sección apical (A, C) y basal de *E. isiforme* (B, D) en presencia y ausencia de CAP (n=5). * Diferencias significativas entre control y muestras tratadas con CAP (ANOVA, $p<0.05$).

As both species showed a CAP effect in their apical and basal sections at the lowest light intensity used, the effect in F_v/F_m reduction and recovery in time in the presence of this inhibitor was further investigated.

The presence of CAP upon light exposure to 250 µmol · m^{-2} · s^{-1} resulted an approx. 20% higher F_v/F_m decrease compared to the control in the apical section of *G. damaecornis* while this effect was much more pronounced in the basal section (about 40% higher reduction in F_v/F_m) (Fig. 27A, B). However, during recovery, the CAP effect increased over the recovery period of 4 h in the apical section accounting for up to 40% differences in F_v/F_m values between control and CAP-treated sample (Fig. 27A). On the other hand, the basal section showed a relatively consistent CAP effect during recovery accounting for 20% of differences in F_v/F_m values between control and CAP-treated sample (Fig. 27B).

In contrast, the CAP effect on different thallus segments of *E. isiforme* after light exposure was similar (20% of difference between control and CAP-treated sample) (Fig. 23C) while during recovery period a significantly effect of D1 repair inhibition was observed only in the apical section (Fig. 27D).

In summary, clear differences in the regulation of PSII activity in response to different irradiances were found comparing red algal species with different carotenoid profiles. These differences resulted from the expression of a slow-relaxing quenching mechanism in high light-acclimated *G. damaecornis* that was not observed in *E. isiforme*. This quenching mechanism in *G. damaecornis* apparently is related to zeaxanthin because it was not expressed in low light-acclimated tissue of the species that presented much lower zeaxanthin but higher antheraxanthin content compared to the high light-acclimated tissue.

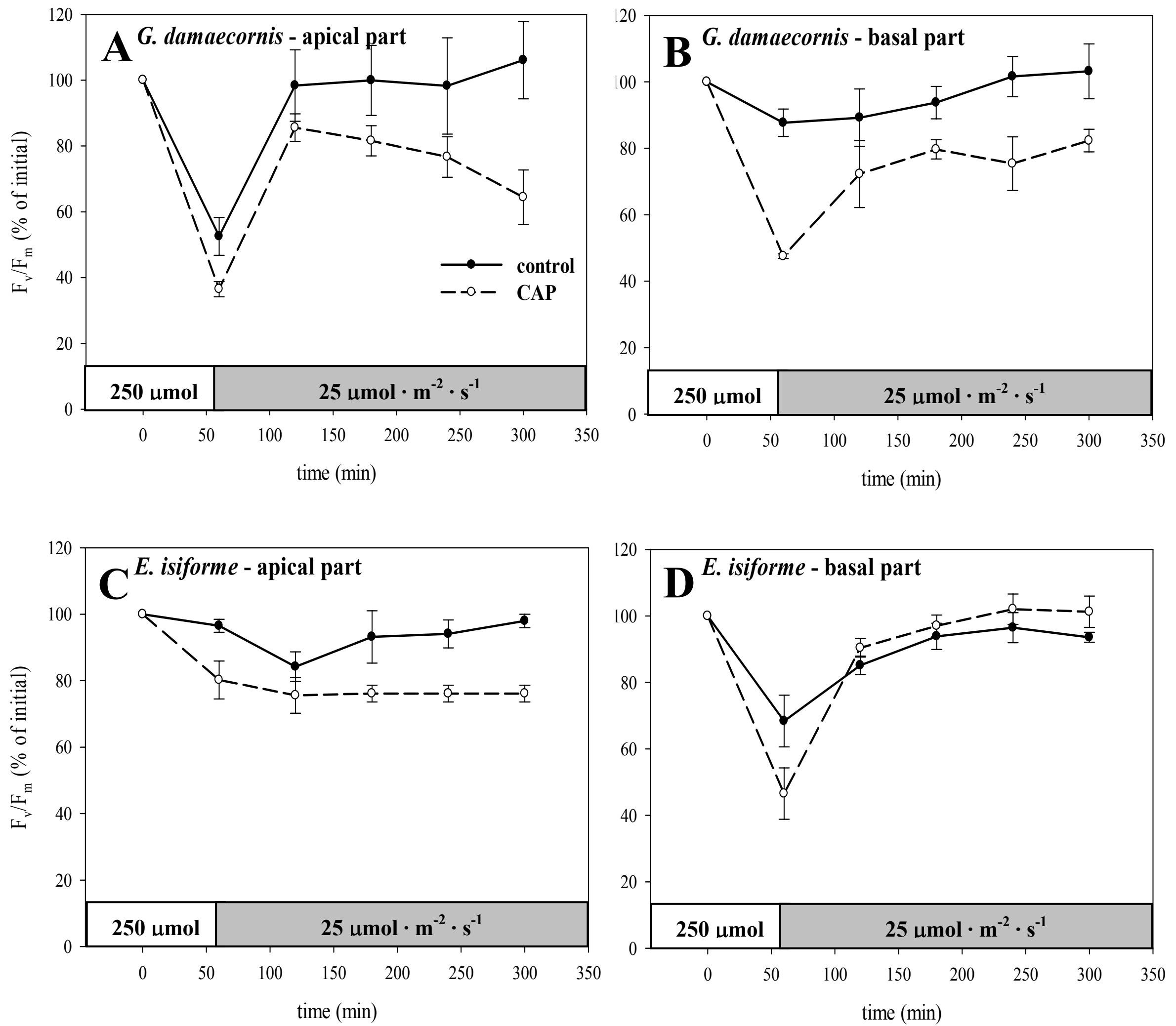

Figure 27. Decrease and recovery of F_v/F_m at exposure to 250 µmol photons · m^{-2} · s^{-1} in apical and basal sections of *G. damaecornis* (A, B) and *E. isiforme* (C, D) in absence (solid line) and presence of CAP (broken line).

Figura 27. Disminución y recuperación de F_v/F_m durante y después de la exposición a 250 µmol fotones · m^{-2} · s^{-1} en secciones apicales y basales de *G. damaecornis* (A, B) y de *E. isiforme* (C, D) en ausencia (línea sólida) y presencia de CAP (línea interrumpida).

***IV*.5. Discussion**

In the present work, the differences in PSII regulation among species and between photosynthetic tissues that showed different acclimation characteristics confirmed that carotenoids play an important role in photoprotection in red algae. Specifically, the regulation of the zeaxanthin concentration (either synthesized from β-carotene or from antheraxanthin) relative to Chl *a* seems to be an important acclimative response for PSII activity regulation. Several acclimation responses take place to maximize photosynthetic work. Regulation of PSII absorption cross section area is one of the most important responses in higher plants and algae (Falkowski and LaRoche 1991). Red algae adjust light absorption characteristics according to growth conditions. The basal segment of *G. damaecornis* presented a higher *in vivo* absorption (49% of the incident light) compared to the apical segment (24% of the incident light). This was associated with a significantly higher pigment content of both Chl *a* and phycobilipigments in the basal section (Table IX). Therefore, the adjustment of the absorption cross-section seems to be an important acclimation response of *G. damaecornis*. However, the higher *in vivo* absorption of the basal section was not reflected in a higher PSII down-regulation. On the contrary, a higher decrease of PSII activity was found in the apical section which absorbed less light compared to the basal section (Fig. 24A). This decrease in the apical segment apparently was related to photodamage that was repaired mainly during recovery but also to a slow-relaxing quenching mechanism. High zeaxanthin content as found in this tissue could result in high energy dissipation under subsaturating light conditions and in a slower reversibility of NPQ, resulting in a lower short-term recovery of the apical segment (Fig. 24B), as

proposed in a previous work (Schubert and Garcia-Mendoza, submitted) and for zeaxanthin-accumulating mutants (Niyogi et al. 1998). This response has been observed also in overwintering higher plants which accumulate zeaxanthin and overexpress the slowly relaxing component of NPQ referred as qI (Gilmore and Ball 2000, Demmig-Adams et al. 2006). The involvement of zeaxanthin in the down-regulation of PSII has also been proposed by other authors (Falbel et al. 1994, Uhrmacher et al. 1995, Jahns and Miehe 1996). Moreover, the apical section also presented a higher content of β–carotene compared to the basal section (Fig. 21B). This pigment as well as zeaxanthin presents antioxidant activity, preventing photodamage by inactivation of oxygen radicals and triplet states of chlorophyll molecules that produce these radicals (Edge et al. 1997).

On the other hand, the lower F_v/F_m decrease in the basal segment (Fig. 24A) indicates the absence of the quenching mechanism found in the apical segment. This could be related to the significantly lower zeaxanthin content of this tissue, which instead presented high antheraxanthin content (Fig. 21B). This pigment has also been related to energy dissipation (Goss et al. 1998, Gilmore and Yamamoto 2001) but apparently, the replacement of Zea as the dominant carotenoid by antheraxanthin in *G. damaecornis* represents a low light-acclimation strategy so that it is unlikely that Ant is involved in photoprotection in this species. Hence, the proportion of epoxidated carotenoids was higher in the basal tissue. In contrast, de-epoxidated carotenoid content was much higher in the apical tissue of *G. damaecornis*. It was believed, that epoxidated carotenoids were absent in red algae (Stransky and Hager, 1970a, Vershinin and Kamnev 1996, Carnicas et al. 1999). However, we demonstrated that this type of carotenoid was widely distributed among several species

of Rhodophyta following a taxonomical pattern (Schubert et al. 2006a). In the present work, we further demonstrated that the regulation of the concentration of epoxy/de-epoxy carotenoids seems to be an important acclimation response to growth light conditions (at least in the species possessing these carotenoids such as *Gracilaria*). This is consistent with previous reports about different carotenoid profiles in *Gracilaria* species (Brown and McLachlan 1982, Ursi et al. 2003, Schubert et al. 2006a). Hence, it has been proposed that the proportion of Zea to Ant is correlated with the growth irradiance (Brown and McLachlan 1982). A high Zea:Ant ratio was found in field-collected and greenhouse-grown material, while algae incubated in the laboratory under low irradiance displayed the inverse relationship (Brown and McLachlan 1982, Liang 1984, Ursi et al. 2003). Therefore, in the Gracilariales zeaxanthin accumulation apparently seems to represent an acclimation response to high light, related to a strong and slow-relaxing quenching mechanism. Moreover, there were differences between apical and basal segments with respect to D1 turnover during and after light exposure. In the apical section, D1 turnover was observed mainly during recovery after exposure up to 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Fig. 25B). This could indicate a high light-acclimatory response that seems to rely on the expression of quenching mechanisms and the inhibition of D1 repair in high light conditions. In contrast, the basal segment seemed to lack this kind of regulation, expressing D1 turnover after exposure at light intensities up to 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Enriquez et al. (2002) suggested that for the apical, high light-acclimated section of the turtle grass *Thalassia testudinum* a complete PSII recovery may be unnecessary during exposure, because leaves are exposed during most of the daylight period to supersaturating

irradiance. They proposed that the increase in the fraction of closed PSII could be a photoacclimatory leaf response to supersaturating light conditions. This last response will increase the energy dissipation since inactive PSII centers do no reduce PQ pool and can dissipated the energy safely (Krause and Weis 1991)

Eucheuma isiforme, a lutein-containing species, exhibited a slight lower *in vivo* absorption in the apical (38%) compared to the basal section (43%). This characteristic was shared with *G. damaecornis.* However, in contrast to *G. damaecornis*, the difference in light absorption coincided with an expected lower F_v/F_m in the basal compared to the apical segment upon exposure to lower light intensities (Fig. 24C). Moreover, apical and basal sections showed D1 turnover only at low light intensities (Fig. 26A, B). This indicates the maximization of photochemical efficiency by the repair of photodamaged PSII at non-light stress conditions meanwhile at higher irradiances D1 repair was inhibited probably to prevent a waste of metabolic energy in the repair of reaction centres that could been damaged again during continuous light stress conditions.

The comparison of the CAP effect at exposure to 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in *E. isiforme* revealed that at this light intensity, photodamage occurred in both segments. However, in the apical segment this damage was immediately repaired so that no F_v/F_m decrease was observed while the D1 turnover in the basal segment could not keep pace with the damage (Fig. 27A, B). A slow rate of D1 protein degradation and a slow repair cycle of the photodamaged PSII centers at high light for low light-acclimated organisms have been proposed as the main factor that makes them more susceptible to photoinhibition compared with high light-grown organisms (Aro et al. 1993a, Anderson et al. 1994).

Thus, our results showed that in *E. isiforme* the high light-acclimated apical tissue of the thallus showed a lower susceptibility to light exposure, specifically at lower light intensities, compared to the low light-acclimated basal tissue. This lower susceptibility of the apical segment seems to be related with a slight lower light absorption, a higher capacity to repair photodamage during and after light exposure, and probably with a higher photoprotective capacity related to its higher zeaxanthin and β-carotene compared to the basal segment.

As there were differences in the light response of basal and apical sections of the same thallus in both investigated species, there were also clear differences in the light response between species. *Gracilaria damaecornis* presents a strong down-regulatory mechanism that seems to relax very slowly, and which was not present in the younger segment of the thallus meanwhile *E. isiforme* responded with a lower decline of F_v/F_m, more pronounced in the basal segment. This coincides with the documented short-term differential responses to high light in red algal species that present different carotenoid profile (Schubert et al. 2006b, Schubert and García-Mendoza submitted). Species with Zea as the main xanthophyll show a fast F_v/F_m decrease (Schubert and García-Mendoza, submitted). As a consequence, they seem to be better adapted to sudden changes in irradiances as compared to species with Lut as the main carotenoid.

In relation to D1 turnover, the effectiveness of chloramphenicol in increasing photodamage or preventing recovery in both investigated species was observed only during and after low photon exposure. In fact, D1 turnover was only observed at exposure to low light intensities while it was inhibited at exposure to higher irradiances that resulted in F_v/F_m decrease

below 50% of the initial value. This is in contrast to suggested increasing D1 protein degradation with increasing light intensities (Tyystjärvi et al. 1991, 1992, Öquist et al. 1992, Aro et al. 1993a). On the other hand, it has been suggested that D1 turnover may be slowed down during light stress conditions (for review see Critchley and Russell 1994).

Moreover, it has been proposed previously that at photon exposure higher than the growth irradiance, damaged PSII reaction centres appear to accumulate rather than being rapidly degraded and repaired, and to act as centres of energy dissipation (Krause and Weis 1991). Energy dissipation has also been proposed to occur via PSII centres which regain function in the absence of D1 protein synthesis (reviewed by Krause and Weis 1991). Although a mechanistic basis for this process is unknown, this situation has been described in higher plants and in *Ulva rotundata* (e.g. Somersalo and Krause 1990, Franklin 1994, Schnettger et al. 1994) under conditions of low temperature acclimation, where protein turnover and xanthophyll de-epoxidation is slow, or when these processes are chemically inhibited. *Chlorodesmis fastigiata*, which appears to lack a functional xanthophyll cycle as it is also the case in red algae, also exhibits this characteristic at normal growth temperature (Franklin and Larkum 1997). Moreover, in a previous work (see Chapter III), the comparison of the decrease and recovery of photosynthetic efficiency during high light exposure assayed as F_v/F_m and oxygen evolution showed that in rhodophytes, photoinactivation, apparently not related to photodamage, is present.

In conclusion, different strategies to respond to irradiance changes are presented in red algae as it has been demonstrated previously in higher plants (Murchie and Horton 1998). There are photoprotective mechanisms, in which carotenoids are involved as seen in *G. damaecornis*. Photoprotection in this species seems to be related to excess energy

dissipation and a regulation of D1 turnover depending on light exposure. In the case of *E. isiforme*, regulation of D1 turnover and maybe the involvement of inactivated and/or damaged reaction centers in energy dissipation seem to be more important. Given the absence of xanthophyll cycle interconversion in the Rhodophyta as one the most important response mechanism to high light, a rapid accumulation of inactivated PSII that might function as quenchers could be an important photoprotective mechanism to withstand fluctuating light environments.

GENERAL DISCUSSION

Carotenoids and photoprotection in red algae

Photosystem II regulating processes, for example the thermal dissipation (qE) as one of the most important mechanisms, is strongly related to the light-harvesting complex (LHC) composition and macrodomain arrangement that will control protein-pigment and protein-protein interactions. Therefore, the mechanisms involved in energy quenching that occurs in the LHCs of higher plants are not the same in red algae; they should be much more similar to cyanobacteria.

In this last group, carotenoids play an important role in photoprotection (Rakhimberdieva et al. 2004, 2007, Wilson et al. 2006, Kirilovski et al. 2007). Since energy dissipation related to carotenoids has not been investigated in red algae, the comparison with characterized models (higher plants and cyanobacteria) has to be done although underlying controlling processes could be different.

One of the main roles of carotenoids is the transfer of excitation energy to chlorophyll during light harvesting but they also protect the photosynthetic apparatus by quenching triplet chlorophyll states and singlet oxygen. Some carotenoids have the ability to deactivate excited chlorophyll and dissipate the excess energy as heat. Short-term synthesis of photoprotective carotenoids such as zeaxanthin is achieved through the reversible de-epoxidation of violaxanthin and antheraxanthin in the xanthophyll cycle (XC). The presence of this cycle in green algae and higher plants allows them to have a rapid formation of zeaxanthin upon exposure to excessive light levels but also the rapid removal of zeaxanthin upon return to non-excessive light conditions. The presence of a high

concentration of zeaxanthin molecules (formed either by XC-interconversion or β-carotene) that could act as quenchers increases the probability of heat production in the photosynthetic apparatus. This can result in a decrease of the photochemical efficiency that under non-saturating light conditions represents a disadvantage. Therefore, the capability for a rapid removal of zeaxanthin might be one important aspect of the XC.

Red algae do not possess the XC but a sustained high content of carotenoids (e.g. zeaxanthin content is 10 x higher as compared to zeaxanthin-accumulating mutants; Hurry et al. 1997, Niyogi et al. 1998) and the involvement of zeaxanthin in energy dissipation under light stress is important in several red algal species (see Chapter II and IV). It has been suggested that not all Zea is involved in energy dissipation since no difference in the sensitivity to photoinhibition were detected between zeaxanthin-accumulating mutants of *Arabidopsis* and wild type genotypes (Hurry et al. 1997, Havaux et al. 2004). Zeaxanthin not involved in thermal dissipation (up to 35% of the total pool) might be located in the chloroplast envelope (Siefermann-Harms et al. 1978, Hurry et al. 1997) or free in the lipid phase of the membrane, where it could be involved in the quenching of free O_2 radicals (Baroli et al. 2003, Rissler and Durnford 2005). As the same has been found in cyanobacteria where zeaxanthin in the outer membrane and cell wall (Resch and Gibson 1983, Jürgens and Weckesser 1985) presumably carries out photoprotective function, it is likely that it might work the same way in red algae.

Near-saturating and saturating intensities of white light induce NPQ in higher plants, eukaryotic algae (green, brown and red) and cyanobacteria. In these organisms, part of this quenching is associated with an increase in the thermal dissipation of absorbed energy in

the PSII antenna complex induced by a transthylakoid proton gradient. The formation of this quenching is accompanied by the accumulation of de-epoxidated xanthophylls (reviewed in Demmig-Adams 1990) and conformational changes (most probably, aggregation) of PSII peripheral antenna (LHCII) (Ruban et al. 1992, Horton et al. 1996).

In the red algae *Rhodella violaceae* and *Porphyridium cruentum*, near-saturating white light illumination also induces a large ΔpH-dependent quenching (Delphin et al. 1996, 1998). The molecular mechanism involved in this type of quenching has to be elucidated since red algae do not have LHCII or the XC.

Obviously, it is difficult to explain the quenching processes seen in red algae with the higher plant model (see above), rather photoprotective strategies must resemble the ones described for cyanobacteria. In this group, it was found recently that a carotenoid-binding protein (OCP) is involved in energy dissipation (Wilson et al. 2006, Rakhimberdieva et al. 2007). This type of energy quenching is not dependent on the presence of a transthylakoid ΔpH (Wilson et al. 2006).

The OCP was first described by Holt and Krogman (1981) and later found in several different cyanobacterial species both in natural blooms and in laboratory cultures (reviewed in Kerfeld 2004a, b). The OCP, a 35 kDa protein, contains a single non-covalently bound carotenoid (Holt and Krogman 1981, Wu and Krogman 1997, reviews: Kerfeld, 2004a, b). The carotenoid associated with the OCP varies among organisms. Zeaxanthin is present in some species such as *Anacystis nidulans* and *Lyngbya wholei* (Diverse-Pierluissi and Krogman 1988, Engle et al. 1991). In other species, hydroxyechinenone or its derivates have been found to be associated to the OCP (Wu and Krogman 1997, Kerfeld 2004a, b).

The mechanism by which the energy is dissipated is still unknown. There are several possibilities. Kirilovski et al. (2007) suggested that the OCP is a light sensor and an activator of allosteric changes in the phycobilisomes. Absorption of light by the carotenoid ‘‘activates’’ the OCP. Then, the ‘‘activated’’ OCP, through interaction with the phycobilisome core, could mediate an alteration of the phycobilisome structure leading to a quenched state (see Fig. 9). Alternatively, the carotenoid of the OCP could directly interact with a phycobilin chromophore (most probably the terminal acceptor) and dissipate the absorbed energy.

Moreover, in cyanobacteria NPQ mechanisms mediated by the chlorophyll-protein complex IsiA (Iron-starvation-inducible protein) were recently described (Yeremenko et al. 2004, Ihalainen et al. 2005). Two different mechanisms involving IsiA were suggested: (1) blue-light converts the IsiA from one form that is efficient in harvesting light energy for photosynthesis to an alternative form that converts excess energy into heat (Cadoret et al. 2004), and (2) strong light induces a coupling of free phycobilisomes to IsiA provoking a large fluorescence quenching (Joshua et al. 2005).

Hence, the function of certain carotenoids is strongly related to their location within the photosynthetic apparatus and their association to certain proteins. Thus, the presence of carotenoids with photoprotective capacity in red algae does not *per se* imply that they would act as quenchers. It should be considered where they are located and to which proteins they are associated to infer about their role in energy dissipation. However, there is minimal work related to the characterization of protein-pigment complexes in red algae and their functional role. Therefore, it is not known if there are carotenoid-binding proteins in red algae that could act similar to the OCP and/or chlorophyll-complexes as the IsiA found

in cyanobacteria. Revealing the molecular quenching mechanism in red algae is a fundamental aspect in the investigation of the evolution of photoprotection mechanisms in phototrophs (see below).

Ecological implications

Light is an important factor for algal zonation (Lüning 1981). Some species proliferate in high-irradiance environments, and others are confined to deep waters (low-irradiance environments). The ability to withstand variations in irradiance and to resist high light stress may be one of the factors determining the competitive ability of macroalgae at the upper limit of their distribution (Herbert 1990, Hanelt 1996, 1997).

Red algae present differences in the carotenoid composition that are related to the differential expression of photoprotective mechanisms (see Chapter II and IV). Ecologically, these differences in photoprotection might imply certain advantages and restrictions for the species. As found in the present study, there are red algal species that depending on their light acclimation present zeaxanthin (high light) or antheraxanthin (low light) as the main carotenoid (see Chapter I and IV). The carotenoid profile in which zeaxanthin is accumulated seems to be related to the expression of fast activated/slow-relaxing down-regulation of PSII activity already at low light intensities. This might represent an advantage in light-fluctuating shallow areas because of their ability to respond very quickly lo irradiance changes (see Chapter IV). The vertical distribution of red algal species and their respective carotenoid profile support this idea. Species from the Ant- and Lut-group were found from the intertidal zone to 15 m depth while members of the Zea-

group were restricted to the first upper 8 m during summer (Fig. 28A). Moreover, species of this last carotenoid profile group were not found in winter (Fig. 28B).

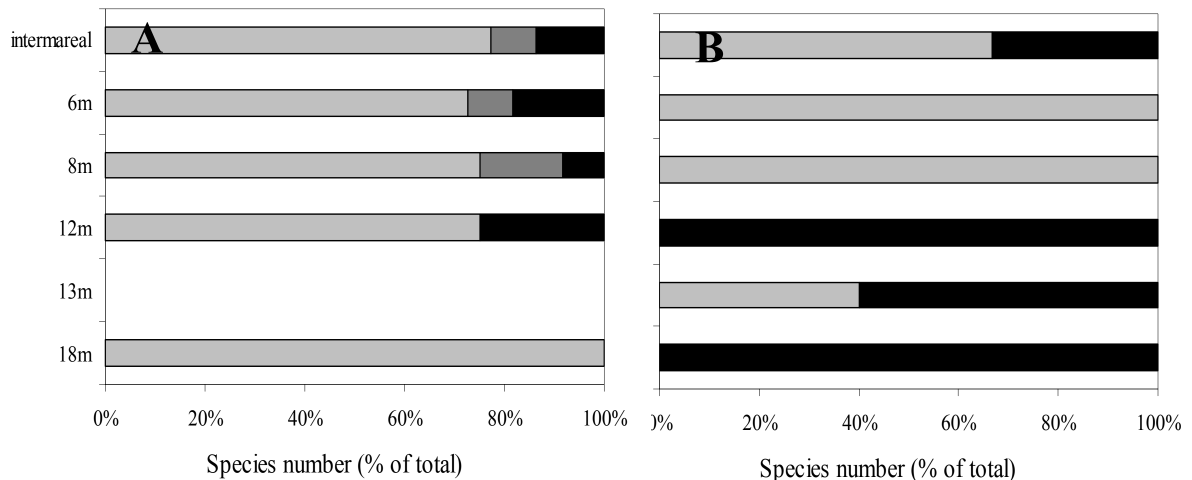

Figure 28. Vertical distribution of red algal species with different carotenoid profiles at Todos Santos island (Lut-group, black, Zea-group, dark grey, Ant-group, light grey) during summer (A) and winter (B) (unpublished data from Dr. Isai Pacheco Ruiz).

Figura 28. Distribución vertical de especies de algas rojas con diferentes perfiles de carotenoides en la isla Todos Santos (grupo Lut- negro, grupo Zea- gris oscuro, grupo Ant- gris claro) durante verano (A) e invierno (B) (datos no publicados del Dr. Isai Pacheco Ruiz).

This might indicate that a rapid, efficient but slow-relaxing photoprotective mechanism as present in the Zea-group provides an advantage during summer conditions with high irradiance levels and long photoperiod but might be disadvantageous during conditions of low light intensity and/or short photoperiods as in winter.

On the other hand, lutein-containing species seem to be successful in shallow areas with high irradiances but would also be successful in deeper areas with lower light intensity. The

species that have this carotenoid profile, apparently do not express a fast activated/slow-relaxing down-regulatory mechanism as found for the Zea-group (see Chapter III and IV). Also, in higher plants a correlation between carotenoid content and distribution has been demonstrated with shade-adapted species presenting higher lutein and lower xanthophyll cycle pigments content (Johnson et al. 1993).

Evidently, besides the carotenoid profile and therefore expression of certain photoprotective strategies, there are several other factors that determine the vertical distribution of red algae and macroalgae in general.

Among them, morphology that could be related to differential photosynthetic rates (Markager and Sand-Jensen 1992, Johansson and Snoeijs 2002), and sensitivity to ultraviolet radiation (Hanelt et al. 1997, Bischof et al. 1998, 2002) are important factors that determine the distribution of red algal species. Another factor that influences the vertical zonation although in minimal proportion could be the lifecycle of the species (see Markager and Sand-Jensen 1992).

Hence, ecological and physiological factors influence the vertical distribution of red algae, but carotenoid profile and associated responses that regulate photosynthetic activity could be one determining aspect for the presence of species in certain light environment.

Evolutionary implications

The rhodophyte phylogeny proposed by Ragan et al. (1994) and Harper and Saunders (2001) does not match with the carotenoid profile of the species (see Chapter I). However, the absence of this relationship could be explained by the suggestion that XC-pigments (Vio and Ant) and both the β,β-carotene and the β,α-carotene synthesis pathways were

present early in the evolution of red algae, before the diversification of the different rhodophyte groups. Apparently, most of rhodophyte species lost or suppressed the capability to synthesize certain carotenoids or even a carotenoid synthesis pathway through evolution (lack of XC-related pigments or Lut). Hence, these findings imply that Rhodophyta must have mechanisms different than the XC to protect them against light stress.

The carotenoid composition in red algae is diverse and both main synthesis pathways are present on this group. This can be related to the proposed model of evolution of the different plastids of photosynthetic eukaryotes (Chapter I). Thus, we propose a hypothetical model about the evolution of the carotenoid-synthesis pathways (Fig. 29).

The model is based on the plastid and light-harvesting antennae evolution proposed by Durnford et al. (1999). The primeval chloroplast presented a phycobilisome-containing (PBS) and a prochlorophyte type chlorophyll a/b antenna complex (Pcb) that was replaced by a transmembrane light-harvesting complex (preLHC) from which antennae of the red and green algal lineages evolved (Tomitani et al. 1999). The appearance of xanthophyll cycle-related pigments and the β,ε-synthesis pathway probably occurred at this evolutionary stage before the divergence of red and green algal lineages due to the presence of epoxy-carotenoids such as violaxanthin and antheraxanthin in both lineages (see Chapter I; García-Mendoza 2000).

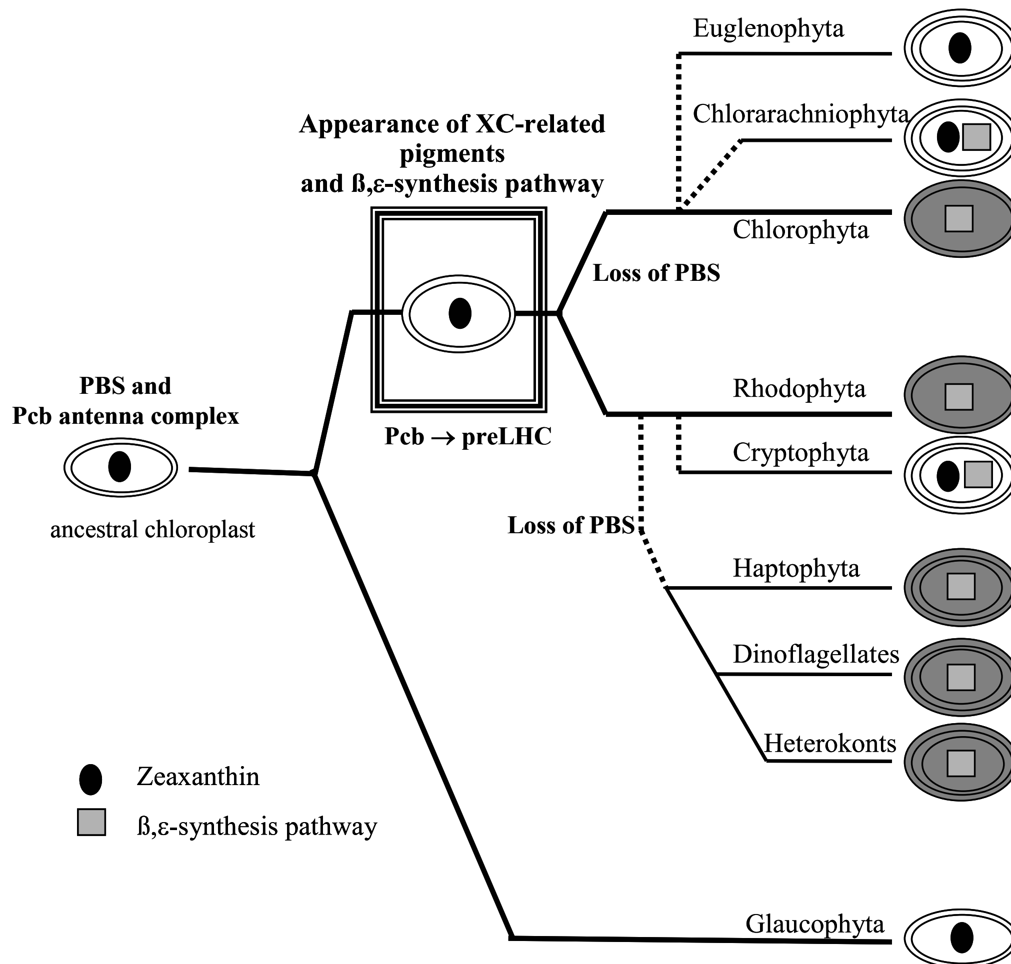

Figure 29. Hypothetical model of the evolution of carotenoid biosynthetic pathways in algae (PBS – phycobilisome antenna, Pcb - prochlorophyte type chlorophyll *a*/*b* antenna complex, preLHC - transmembrane light-harvesting complex). The lines around the chloroplast represent a single membrane bilayer. Dashed lines reflect the lateral transfer of plastids through secondary endosymbiosis. The presence of XC-related pigments (violaxanthin, antheraxanthin and zeaxanthin) in modern algae divisions is highlighted by shaded plastids.

Figura 29. Modelo hipotético de la evolución de las vías de síntesis de carotenoides en algas (PBS- antena tipo ficobilisoma, Pcb- complejo antena tipo proclorofita con clorofila *a*/*b*, preLHC- complejo antena transmembránico). Las líneas alrededor del cloroplasto representan una única membrana doble. Las líneas punteadas reflejan la transferencia lateral de los plástidos por endosimbiosis secundaria. Los plástidos en gris indican la presencia de pigmentos relacionados con el XC (violaxantina, anteraxantina y zeaxantina) en divisiones algales modernas.

The occurrence of epoxy-carotenoids indicates that in both lineages the zeaxanthin epoxidase that catalyze the synthesis from zeaxanthin to antheraxanthin and violaxanthin is present (Cunningham and Gantt 1998) and that this enzyme probably was presented before the divergence of red and green algae as also proposed by other authors (García-Mendoza 2000). On the other hand, no red algal homologue of the enzyme, catalyzing the conversion from violaxanthin to zeaxanthin via antheraxanthin (XC), the violaxanthin de-epoxidase has been found yet.

The red algal photosynthetic apparatus, specifically the light-harvesting complexes of PSI and PSII represents an intermediate between cyanobacteria, the ancestors of the chloroplasts, and higher plants (for details see Introduction). As in higher plants, in cyanobacteria the presence of a transmembranic chlorophyll-binding light-harvesting antenna (IsiA) facilitates the formation of NPQ. Hence, interesting questions arise regarding the mechanism of NPQ processes in oxygenic phototrophs. There is no evidence for a pH-dependent regulation of NPQ in *Prochlorococcus* and *Synechocystis* using uncouplers (Bailey et al. 2005) that typically block the formation of NPQ in higher plants. In addition, PsbS, a protein essential for the formation of NPQ in higher plants (Li et al. 2000) has not been found in cyanobacteria and red algae. On the other hand, the carotenoid-binding protein (OCP) of cyanobacteria like PsbS protein of plants lacks chlorophylls but bind carotenoid: 3′-hydroxyechinenone or zeaxanthin upon protonation (Aspinal-O‘Dea et al. 2002, Dominici et al. 2002).

Karapetyan (2007) proposed that the water-soluble OCP interacting with water-soluble extra-membrane light-harvesting complex (phycobilisomes) was replaced in the course of evolution by the membrane bound PsbS. This took place after phycobilisomes were lost and

the membrane-bound light-harvesting complex (LHCII) appeared. Both carotenoid-binding proteins (PsbS and OCP) function as dimers, indicating the necessity of conformational changes of proteins in the course of non-photochemical quenching.

It is not known if red algae present OCP-like proteins and/or an IsiA-like complex. Characterization of the site in which energy quenching takes place in red algae would give evidence about the evolution of photoprotective mechanisms in eukaryotic phototrophs due to Rhodophyta evolved by a primary endosymbiosis event, share a common origin with the green algal lineage, and chromophytes evolved from a red algal ancestor by a secondary endosymbiotic event.

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APPENDIX

Carotenoid composition in red algae

In red algae, diverse and often contradictory carotenoid compositions have been reported (Table X).

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Table X. Carotenoid composition in red algae.
Table X. Composición de carotenoides en algas rojas.

Class	*Order*	*Family*	*Species*	**Lutein**	**Zeaxanthin**	**Antheraxanthin**	**Violaxanthin**	**Lutein-epoxide**	**Lycopne**	**α-cryptoxanthin**	**β-cryptoxanthin**	**α-carotene**	**β-carotene**	**References**
Bangiophyceae	***Bangiales***	***Bangiaceae***	*Bangia atropurpurea*	+	+								+	4, 14
			Porphyra sp.	+	+							+	+	21
			Porphyra dioica	+	+							+		28 *
			P. leucosticta	+	+									28 *
			P. perforata	+	+							+	+	30
			Porphyra umbilicalis	+	+									28 *
	Erythropeltidales	***Erythrotrichiaceae***	*Erythrotrichia carnea*		+								+	30
			E.carnea	+	+								+	2
			Smithora naiadum	+	+								+	30
	Goniotrichales	***Goniotrichiaceae***	*Chroodactylon ornatum*		+								+	11, 30
	Porphyridiales	***Porphyridiaceae***	*Porphyridium aerugineum*		+						+		+	11, 32
			P. purpureum		+								+	12
			P. purpureum		+						+		+	31, 32
			Rhodella violacea		+									15, 28
			Rhodosorus marinus		+								+	2, 31
Florideophyceae	***Acrochaetiales***	***Acrochaetiaceae***	*Rhodochorton purpureum*	+	+							+	+	31
			Audouinella sp.		+							+	+	27

Table X. continued.

Class	Order	Family	Species	Lutein	Zeaxanthin	Antheraxanthin	Violaxanthin	Lutein-epoxide	Lycopene	α-cryptoxanthin	β-cryptoxanthin	α-carotene	β-carotene	References
Florideophyceae	**Ahnfeltiales**	**Ahnfeltiaceae**	*Ahnfeltiopsis concinna*	+	+							+	+	21
			Ahnfeltia tobuchiensis		+					+			+	33
	Gigartinales	**Cystocloniaceae**	*Cystoclonium purpureum*	+								+	+	22
		Endocladiaceae	*Endocladia muricata*	+	+							+	+	29
			Chondrus crispus	+								+		27
			C. crispus	+	+							+	+	22
		Furcellariaceae	*Furcellaria lumbricalis*	+										27
		Hypneaceae	*Hypnea sp.*	+	+							+	+	21
			H. chordacea	+	+							+	+	21
			H. musciformis		+			+			+	+	+	13 *
			H. musciformis	+	+							+	+	21
		Phyllophoraceae	*Gymnogongrus disciplinalis*	+	+							+	+	21
			G. turquetii	+								+	+	22
			Mastocarpus stellatus	+								+		27
			M. stellatus	+	+								+	4
			M. stellatus	+								+	+	22
			Phyllophora crispa	+									+	33

Table X. continued.

Class	Order	Family	Species	Lutein	Zeaxanthin	Antheraxanthin	Violaxanthin	Lutein-epoxide	Lycopene	α-cryptoxanthin	β-cryptoxanthin	α-carotene	β-carotene	References
Florideophyceae	**Gigartinales**	**Phyllophoraceae**	*Schottera nicaeënsis*	+								+	+	8
		Solieriaceae	*Eucheuma denticulatum*	+	+							+	+	3
			Kappaphycus alverezii	+	+							+	+	3, 21, 28
	Cryptonemiales	**Acrosymphytaceae**	*Acrosymphyton purpuriferum*	+	+		+				+	+	+	13 *
		Dumontiaceae	*Dumontia contorta*	+										27 *
			D. contorta	+								+	+	22
			Akalaphycys liagroides	+	+							+	+	21
			Cryptosiphonia woodii	+	+							+	+	29
			Farlowia compressa	+								+		29
			F. mollis	+	+							+	+	29
		Halymeniaceae	*G. hawaiiana*	+				+				+		27
			Grateloupia doryphora	+	+							+	+	29
			G. filicina	+	+			+				+	+	14
			G. filicina	+	+							+	+	29
			G. proteus	+	+							+	+	14
			Halymenia floresia	+								+	+	3
			H. formosa	+	+							+	+	21

Table X. continued.

Class	Order	Family	Species	Lutein	Zeaxanthin	Antheraxanthin	Violaxanthin	Lutein-epoxide	Lycopene	α-cryptoxanthin	β-cryptoxanthin	α-carotene	β-carotene	References
Florideophyceae	**Cryptonemiales**	**Halymeniaceae**	*Norissia setchelii*	+	+				+			+	+	29
			Prionitis australis	+	+							+	+	29
			Prionitis lanceolata	+	+							+	+	29
			Prionitis lyalii	+	+							+	+	29
			Callophyllis marginifructa	+	+							+	+	29
		Rhizophyllidaceae	*Portieria hornemanii*	+	+							+	+	21
	Gelidiales	**Gelidiaceae**	*Pterocladia lucida*	+	+							+	+	30
			Pterocladiella caerulescens	+	+							+	+	21
			Pterocladiella capillaceae		+							+	+	8
			P. capillaceae	+	+							+	+	30
			G. coulterii	+	+							+	+	29
	Nemaliales	**Nemaliaceae**	*Nemalion helminthoides*	+								+	+	2, 4, 14
			S. confusa	+	+							+	+	30
			Actinotrichia sp.	+								+	+	21
			Galaxaura spp.	+	+							+	+	30
		Liagoraceae	*Galaxaura obtusata*	+	+							+	+	30
			Cumagloia andersonii	+	+							+	+	29
	Palmariales	**Palmariaceae**	*Devaleraea ramentacea*	+								+	+	22

Table X. continued.

Class	Order	Family	Species	Lutein	Zeaxanthin	Antheraxanthin	Violaxanthin	Lutein-epoxide	Lycopene	α-cryptoxanthin	β-cryptoxanthin	α-carotene	β-carotene	References
Florideophyceae	**Palmariales**	**Palmariaceae**	*Palmaria palmata*	+										27
			P. palmata	+									+	25
			P. palmata	+								+	+	4
			P. decipiens	+	+							+	+	16
			P. decipiens	+								+	+	22
			R. leptophylla	+								+	+	21
			Maripelta sp.	+								+	+	21
		Faucheaceae	*Botryocladia skottbergii*	+								+	+	21
		Champiaceae	*Lomentaria clavellosa*	+			+							27
	Rhodymeniales	**Rhodymeniaceae**	*Cordylecladia erecta*	+	+							+	+	3
	Bonnemaisoniales	**Bonnemaisoniaceae**	*Bonnemaisonia hamifera*	+	+							+	+	4, 14
			Asparagopsis armata	+	+							+	+	30
			A. taxiformis	+								+	+	21
			A. taxiformis	+	+							+	+	30
	Batrachospermales	**Batrachospermaceae**	*Batrochospermum sp.*	+	+							+	+	30, 31
			B. gelatinosmum	+	+							+	+	26
	Hildenbrandiales	**Hildenbrandiaceae**	*Hildenbrandia rubra*	+	+							+		27 *****
	Plocamiales	**Plocamiaceae**	*Plocamium cartilagineum*	+	+		+				+	+	+	13 *****

Table X. continued.

Class	Order	Family	Species	Lutein	Zeaxanthin	Antheraxanthin	Violaxanthin	Lutein-epoxide	Lycopene	α-cryptoxanthin	β-cryptoxanthin	α-carotene	β-carotene	References
Florideophyceae	**Plocamiales**	**Plocamiaceae**	*P. cartilagineum*	+										27 *
			P. cartilagineum	+								+	+	22
	Ceramiales	**Rhodomelaceae**	*Acanthophora pacifica*		+	+							+	21
			A. spicifera		+	+					+		+	1, 21
			Brongniartella byssoides	+	+		+					+		27 *
			Chondria sp.	+	+								+	30
			Erythrocystis saccata	+	+								+	29
			Laurencia filiformis	+	+								+	30
			L. nidifica	+	+							+	+	21
			L. nidifica	+	+								+	29
			L. obtusa		+			+			+	+	+	13 *
			L. obtusa	+	+		+	+		+	+	+	+	18 *
			L. obtusa	+	+								+	30
		Rhodomelaceae	*L. pacifica*	+	+								+	29
			L. rigida	+	+								+	30
			Melanamansia dietrichiana	+	+							+	+	30
			M. glomerata	+								+	+	21
			Neorhodomela larix	+	+								+	29
			Neosiphonia tongatensis	+	+								+	29
			Odonthalia floccosa	+	+								+	29
			O. spectabilis	+	+								+	29

Table X. continued.

Class	Order	Family	Species	Lutein	Zeaxanthin	Antheraxanthin	Violaxanthin	Lutein-epoxide	Lycopene	α-cryptoxanthin	β-cryptoxanthin	α-carotene	β-carotene	References
Florideophyceae	**Ceramiales**	**Rhodomelaceae**	*P. bipinnata*	+	+								+	29
			Polysiphonia brodiaei		+						+		+	4
			P. brodiaei	+	+						+		+	14
			P. elongata		+							+		27 *
			P. lanosa	+	+								+	2
			P. panicultata	+	+								+	29
			P. scopulorum var.villum	+	+							+	+	30
			P. stricta		+						+		+	4, 14
			P. urceolata		+								+	22
			Pterosiphonia baileyi	+	+								+	29
			P. dendroidea	+	+							+	+	29
		Ceramicaeae	*Callithamnion acutum*	+	+							+	+	29
			C. corymbosum	+			+							27 *
			C. pikeanum	+	+							+	+	29
			Centroceras clavulatum	+								+	+	21
			C. clavulatum	+	+							+	+	29
			Ceramium eatonianum	+	+							+	+	29
			C. virgatum	+	+			+		+		+	+	4, 14
			C. virgatum		+		+	+			+		+	13 *
			Griffithsia sp.	+	+							+	+	30

Table X. continued.

Class	Order	Family	Species	Lutein	Zeaxanthin	Antheraxanthin	Violaxanthin	Lutein-epoxide	Lycopene	α-cryptoxanthin	β-cryptoxanthin	α-carotene	β-carotene	References
Florideophyceae	**Ceramiales**	**Ceramicaeae**	*G. pacifica*	+	+							+	+	29
			Microcladia borealis	+	+							+	+	30
			M. coulteri	+	+							+	+	29
			N. densa	+	+							+	+	29
			Pterothamnion plumula	+										27 *
			P. plumula	+						+		+	+	2, 5
			Spyridia filamentosa	+	+							+	+	21, 30
			Tiffaniella snyderiae	+	+							+	+	29
			Wrangelia penicillata		+								+	21
		Dasyaceae	*Dasya baillouviana*		+		+	+			+	+	+	13 *
			D. iridescens	+	+							+	+	21
			C. farlowiana	+	+							+	+	30
			C. lobulifera	+	+							+	+	29
			C. ramosa	+								+	+	22
			C. violaceae	+	+							+	+	29
			Delesseria sanguinea	+								+		27
			D. sanguinea	+								+	+	14
			Hymenena flabelligera	+	+							+	+	29
			H. kylinii	+	+							+	+	29
			Martensia sp.	+	+							+	+	21
			Membranoptera alata	+										27 *
			Myriogramme multiloba	+	+							+	+	29

Table X. continued.

Class	Order	Family	Species	Lutein	Zeaxanthin	Antheraxanthin	Violaxanthin	Lutein-epoxide	Lycopene	α-cryptoxanthin	β-cryptoxanthin	α-carotene	β-carotene	References
Florideophyceae	**Ceramiales**	**Dasyaceae**	*Nienburgia andersoniana*	+	+							+	+	29
			Pantoneura fabriciana		+								+	22
			P. austrogeorgica	+								+	+	22
			P. quercifolia	+								+	+	22
			P. rubens	+								+	+	22
		Delesseriaceae	*P. rubens*	+			+							27 *
			Polyneura latissima	+	+							+	+	29
			B. orbignyana	+	+							+	+	29
			B. plumosa	+	+							+	+	29
			C. tuberculosum	+	+								+	29
			Corallina elongata		+								+	23 *
			C. officinalis		+		+							27 *
			C. officinalis		+								+	23 *
			C. officinalis var.chilensis	+	+								+	29
			H. roseum	+	+							+	+	29, 30
			Jania sp.		+								+	23 *
			J. capillacea	+	+							+	+	21
			J. rubens	+	+		+	+		+	+	+	+	17 *
			L. neofarlowii	+									+	29
	Gracilariales	**Gracilariaceae**	*Gracilaria birdiae*		+	+	+		+		+		+	3, 32
			G. bursapastoris		+	+							+	7, 21
			G. bursapastoris	+	+								+	9

Table X. continued.

Class	Order	Family	Species	Lutein	Zeaxanthin	Antheraxanthin	Violaxanthin	Lutein-epoxide	Lycopene	α-cryptoxanthin	β-cryptoxanthin	α-carotene	β-carotene	References
Florideophyceae	**Gracilariales**	**Gracilariaceae**	*G. canaliculata*		+	+							+	21
			G. coronopifolia		+	+	+				+		+	7
			G. coronopifolia		+	+							+	21
			G. domingensis		+	+	+		+				+	3, 28
			G. foliifera		+	+	+				+		+	7
			G. gracilis		+	+							+	24
			G. multipartita		+	+							+	24
			G. pacifica		+	+	+				+		+	7
			G. salicornia		+	+	+				+		+	7
			G. tenuistipata	+	+								+	10
			G. tikvahiae		+	+	+				+		+	7
			G. turgida		+	+	+				+		+	7
			G. lemaneiformis		+	+					+		+	7
			G. longissima		+								+	24
			Hydropuntia edulis		+								+	33
			H. edulis		+	+					+		+	1

* biological contamination (reports of neoxanthin and/or fucoxanthin)

* contaminación biológica (reportes de neoxantina y/o fucoxantina)

Chlorophyll fluorescence measurements in red algae

Chlorophyll fluorescence analysis is a useful monitor of photosynthesis, because it is non-invasive, sensitive, and scalable over large ranges of time, light and distance. However, as the fluorescence measurement protocols and interpretations originally were developed for higher plants, the interpretation of the fluorescence signals from red algae should be done with precaution because of several differences in the photosynthetic apparatus of this group compared to higher plants that affect the fluorescence signal.

An important feature of red algae compared to other algal groups and higher plants is a much higher PSI/PSII ratio (Cunningham et al. 1989, Abe et al. 1994), resulting in a lower fluorescence yield by energy-spillover from PSII to PSI that can be enhanced by phycobilisome movement (see Zhang et al. 2007). Moreover, the presence of phycobilisomes as antenna of PSII and associated related responses (state transitions, phycobilisome movement) influence PSII emission. In contrast, in higher plants, state transitions have relatively minor influences on PSII fluorescence (Krause and Weis 1991).

State 1–State 2 transitions (‘state transitions’) represents a rapid mechanism for reconfiguring the photosynthetic light-harvesting apparatus in response to changing conditions. The phenomenon was first described in a red alga (Murata 1969) and a green alga (Bonaventura and Myers 1969). Although green plants have a very different light-harvesting apparatus compared to cyanobacteria and red algae, state transitions are conceptually similar in both groups of organisms. Illumination conditions which lead to excess excitation of PSII compared with PSI induce a transition to State 2, in which more absorbed excitation energy is diverted to PSI. When PSI is over-excited relative to PSII this induces a transition to State 1, in which more energy is transferred to PSII. Thus state

transitions appear to act as a mechanism to balance excitation of the two photosystems under changing light regimes (reviewed by van Thor et al. 1998, Allen and Forsberg 2001). In general, the interpretation of the fluorescence signal based on the supposition that the antenna are in State 1 condition (association of LHCII to PSII) that in most cases is induced by dark-incubation. However, in phycobilisome-containing organisms the State 1 condition by dark-adaptation alone is not ensured. In fact, for cyanobacteria, a State 2 condition in darkness has been reported (Fork and Satoh 1986) and in red algae conditions between State 1 and State 2 have been observed in darkness (Ley and Butler 1976, Satoh and Fork 1983, Biggins and Bruce 1989). Therefore, a pretreatment with the application of far-red light that excites PSI and subsequently 5 min dark-incubation before measurement of F_o and F_m is recommended so that the State 1 condition is achieved. Moreover, in cyanobacteria, a relationship between the F_o level and phycocyanin content has been described (Campbell et al. 1998). In the present study, not such relationship has been found (data not shown). However, a proper filter set to avoid phycobilisome emission in the measurement is recommended. In this study a XE-PAM system used was equipped with a BG39 Schott filter in the excitation source (Xenon Lamp) and with a RG9 filter (>710 nm, Schott) in the detector. With this filter combination phycobilisome fluorescence contribution is minimal in the measured signal.

As mentioned above, red algal fluorescence signals should be treated with precaution. They can be interpreted according to the higher plant model only as long as fluorescence yields (maximum, F_m; and minimum, F_o) are not affected by changes in the absorption cross section area of PSII (phycobilisome movement, state transitions) during different measurements. Therefore, when phycobilisome content not vary during measurements and

the association of the PBS to PSII (State 1 condition) is ensured, changes in F_v/F_m can be related to regulation processes of PSII activity (photoinactivation and/or photodamage of PSII and non-photochemical downregulation of PSII activity) as proposed by the higher plant model.

In contrast, thermal dissipation definitively can not be explained by the higher plant model, since for the expression of this process the antenna complexes are fundamental. Therefore, in the present study, NPQ values (as indicator for photoprotective energy dissipation) determined for different red algal species were not included due to the strong differences in fluorescence induction compared to the higher plant model (Fig. 30A, B) indicating other mechanisms involved, such as phycobilisome movement.

As seen in figure 30B, the fluorescence did not show any relaxation upon change to darkness and also rose during light exposure reaching values similar or above F_m, determined before light treatment.

This indicates in some species an incorrect determination of F_m and changes in the absorption cross section area of PSII. As NPQ calculation based on the correct determination of the maximum fluorescence (NPQ= $(F_m\text{-}F_m')/F_m'$) this results in negative values for NPQ. For the correct measurement of F_m in cyanobacteria, termination of the measurement with the addition of DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea)] which closes PSII reaction centers, causing a rapid rise in fluorescence, has been proposed (Campbell et al. 1998). However, the changes in the absorption cross section area of PSII during light exposure make it still not advisable to interpret the measurements according to the higher plant model.

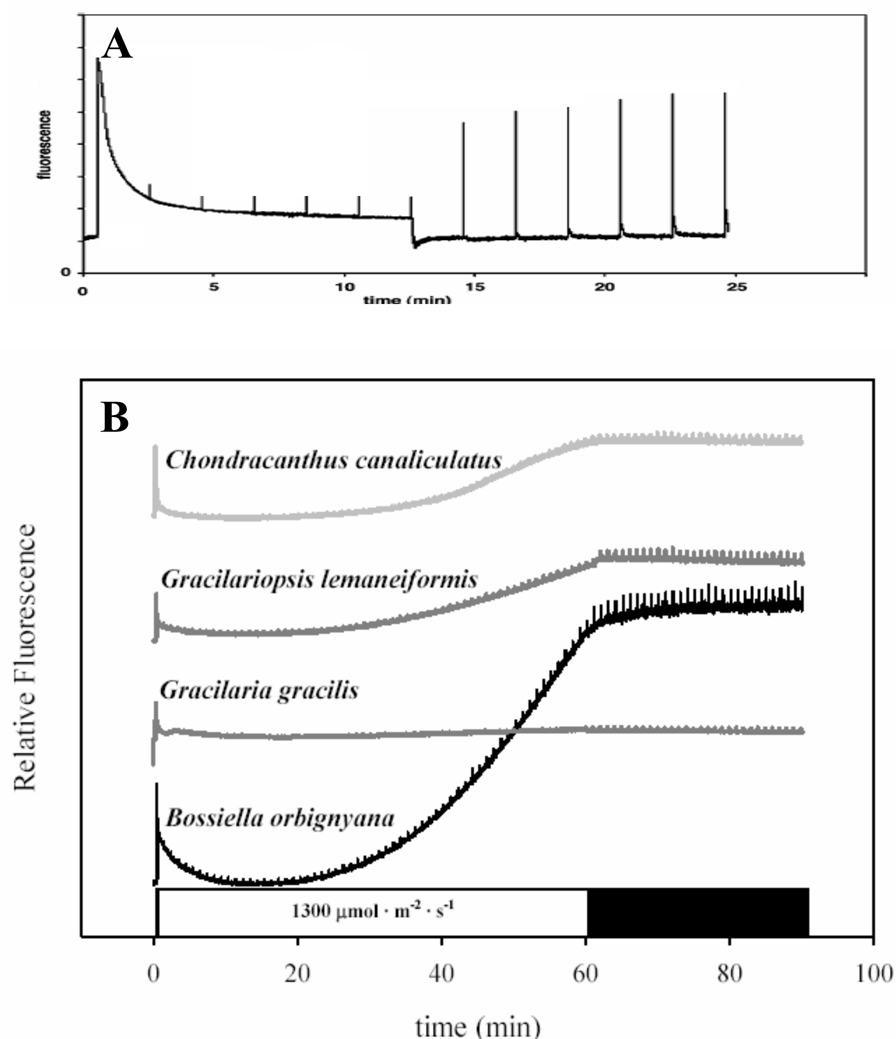

Figure 30. Fluorescence induction curves A) in a higher plant (*Arabidopsis*) (taken from Müller et al. 2001) and B) in different red algal species. The species were exposed for 1 h at 1300 µmol · m^{-2} · s^{-1} and subsequently maintained for 30 min in darkness. A saturating pulse was applied every minute. The species belonged to different carotenoid-profile groups (Lut-group in light grey, Zea-group in dark grey, and Ant-group in black).

Figura 30. Curvas de inducción de fluorescencia A) en una planta superior (*Arabidopsis*) (tomado de Müller et al. 2001) y B) en diferentes especies de algas rojas. Las especies fueron expuestas por 1 h a 1300 µmol · m^{-2} · s^{-1} y posteriormente fueron mantenidas en oscuridad por 30 min. Un pulso de luz saturante fue aplicado cada minuto. Las especies pertenecen a diferentes grupos de perfiles de carotenoides (grupo Lut- gris claro, grupo Zea- gris oscuro y grupo Ant-n negro).