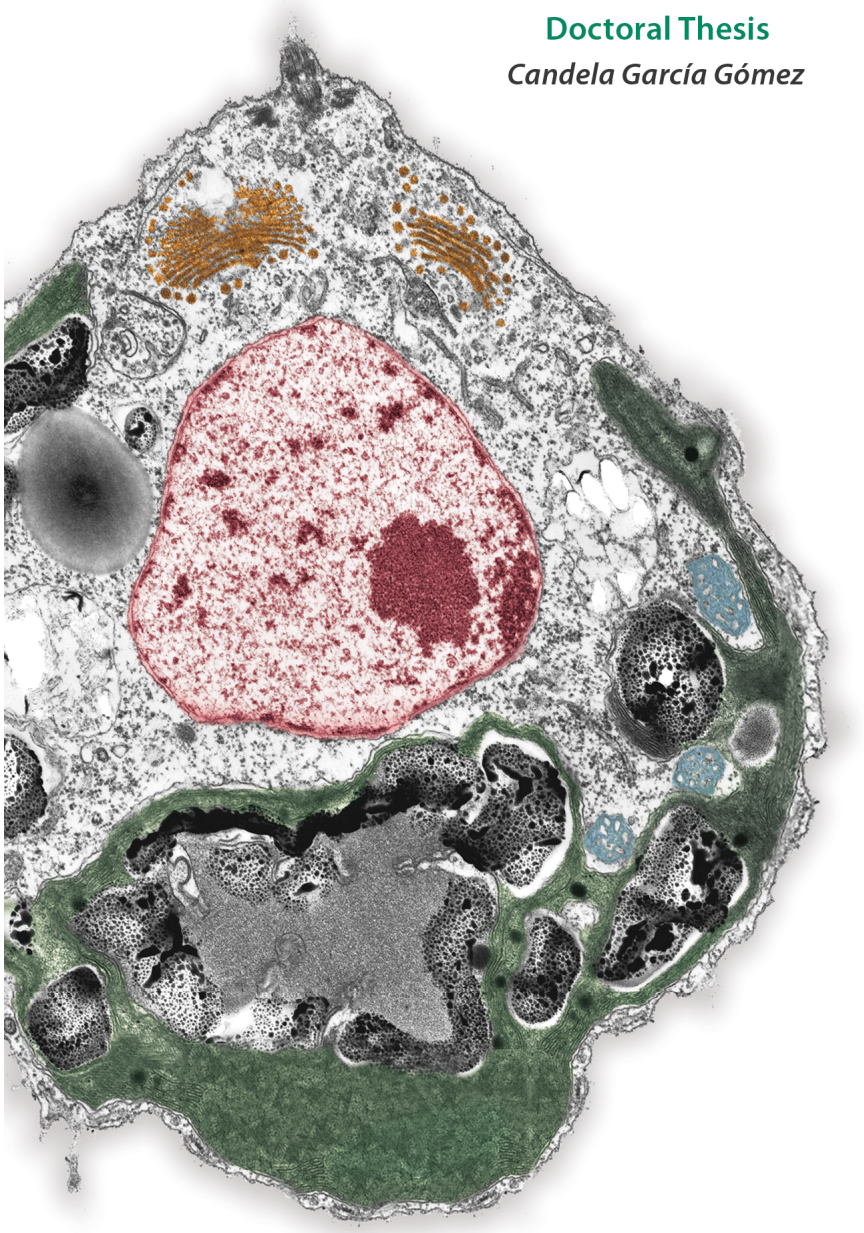
Resilience to UV radiation and high PAR in the marine Chlorophyte *Dunaliella tertiolecta* and the interaction of increased CO_2 in the context of global change

Doctoral Thesis

Candela García Gómez



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TESIS DOCTORAL

Candela García Gómez

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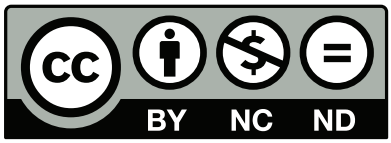

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Área de Ecología

Visado en Málaga a 9 de abril de 2014

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Memoria presentada para optar al grado de Doctor en Ciencias Biológicas

Fdo.: Candela García Gómez

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Dª. MARÍA SEGOVIA AZCORRA, Profesora Titular del Departamento de Ecología y Geología (Área de Ecología) de la Facultad de Ciencias de la Universidad de Málaga y D. FRANCISCO J. LÓPEZ GORDILLO, Profesor titular del Departamento de Ecología y Geología (Área de Ecología) de la Facultad de Ciencias de la Universidad de Málaga,

CERTIFICAN:
Que la presente memoria titulada "**Resilience to UV radiation and high PAR in the marine Chlorophyte *Dunaliella tertiolecta* and the interaction of increased CO_2 in the context of global change**" presentada por la Licenciada en Ciencias Biológicas Candela García Gómez, ha sido realizada bajo nuestra dirección y las publicaciones que la avalan no han sido utilizadas en tesis anteriores. Y considerando que representa trabajo de Tesis Doctoral, autorizamos su presentación y defensa para optar al grado de Doctor en Ciencias Biológicas.

Y para que así conste, a los efectos oportunos, firman el presente en Málaga, a 9 de Abril de 2014.

Fdo.: Dª. María Segovia Azcorra
Prof. Titular del Área de Ecología
Universidad de Málaga

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Área de Ecología

D. Carlos Jiménez Gámez, Director del Departamento de Ecología y Geología de la Facultad de Ciencias de la Universidad de Málaga,

CERTIFICA:

Que el trabajo de investigación "**Resilience to UV radiation and high PAR in the marine Chlorophyte *Dunaliella tertiolecta* and the interaction of increased CO_2 in the context of global change**" llevado a cabo por la Licenciada en Ciencias Biológicas Candela García Gómez, ha sido realizado en este Departamento.

Fdo.: D. Carlos Jiménez Gámez

Málaga, 9 Abril de 2014

Esta Tesis doctoral ha sido subvencionada por la Consejería de Innovación, Ciencia y Empresa, mediante una beca de formación de personal investigador FPI (convocatoria 2009) con cargo a los incentivos al proyecto de investigación de excelencia de la Junta de Andalucía "**Evaluación del aumento de CO_2 y radiación ultravioleta como factores de modificación de la biodiversidad y productividad del fitoplancton marino en el marco del cambio global**" (P08-03800) cuya investigadora principal es la Dra. María Segovia Azcorra.

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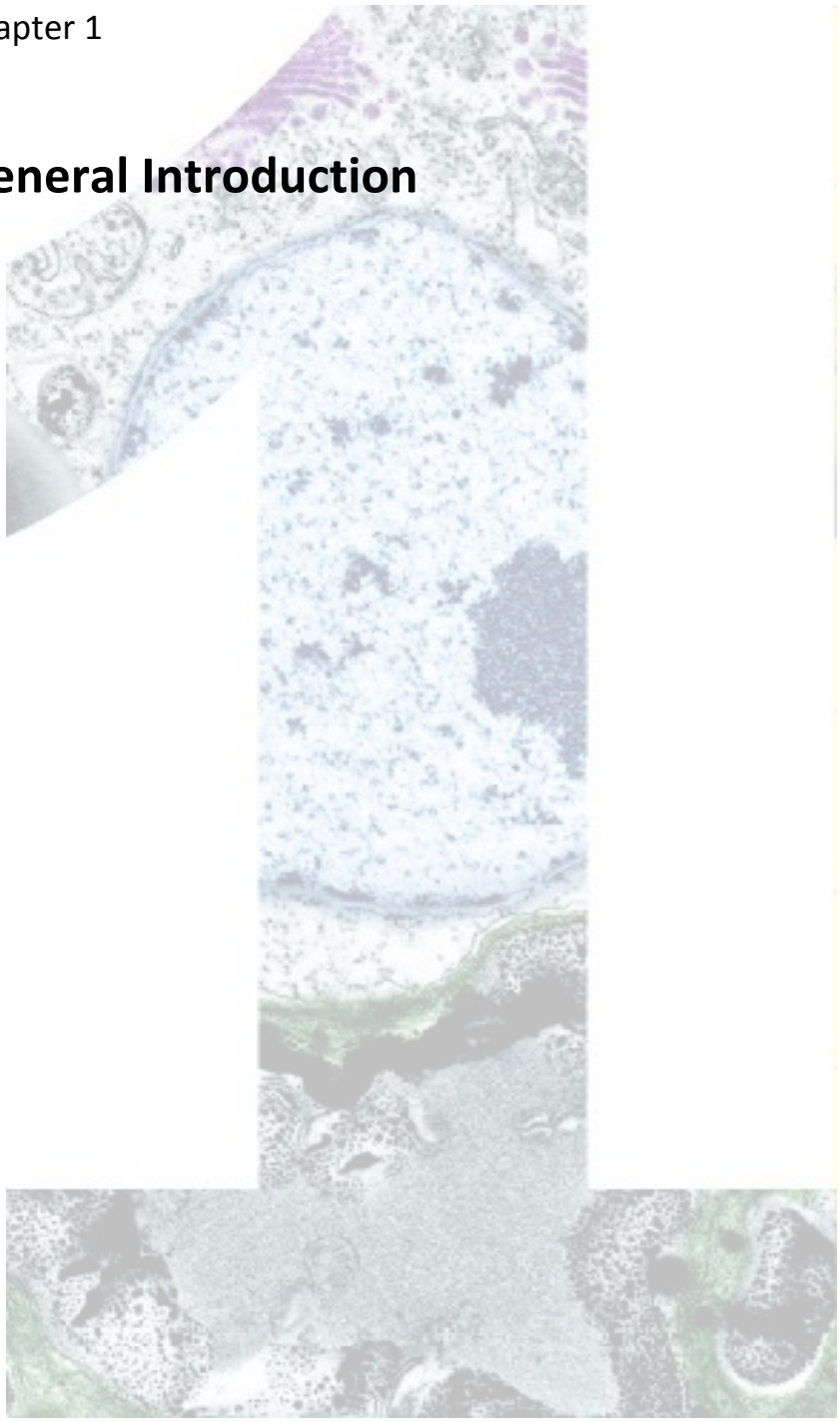
Chapter 1

General Introduction



Global change

The term “global change" encompasses planetary scale changes to atmospheric circulation, ocean circulation, climate, the carbon cycle, the nitrogen cycle, the water cycle and other cycles, sea-ice changes, sea-level changes, food webs, biological diversity, pollution, health and fish stocks. Civilization is a large driver of global change so the term includes population, economy, resource use, energy, development, transport, communication, land use and land cover, urbanization and globalization (definition given according to the International Geosphere-Biosphere Programme, IGPB). Therefore, the environment is experiencing a period of significant and fast change in climate as result anthropogenic activities. However, global change should not be confused with climate change, which is part of the above much wider concept.

In the geological past, the planet has experienced significant periods of fluctuations in some of the drivers (or modulators) attributed to global change, but the pace at which CO_2, global temperatures and exposure to ultraviolet radiation (UVR) are changing, has never been so fast. Concerning CO_2, it is predicted that its concentration in the atmosphere will reach 1000 ppmv by the end of this century (Stocker et al. 2013; Meehl et al. 2007). Oceans are absorbing part of this CO_2, producing a decrease in pH and changes in the dissolved inorganic carbon (DIC) equilibrium. If the atmospheric CO_2 concentration reaches the expected levels, it would cause a decrease in pH from 8.2 (preindustrial level) to 7.77 (Beardall et al. 2009). The CO_2 concentration would increase in the upper ocean layer from 9 to 28 µM (at 15 ºC in equilibrium with the atmosphere). This will produce in turn a 17% increase in bicarbonate ions (HCO_3^-) concentration and a 54% decrease in carbonate (CO_3^{2-}) concentration, compared to preindustrial levels (Beardall et al. 2009).

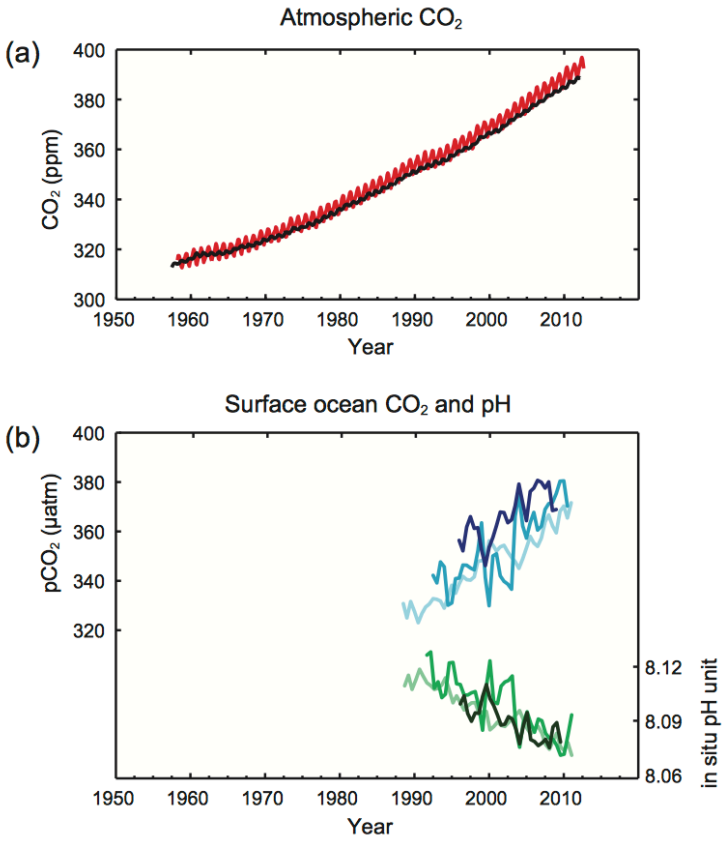


Figure 1.1 Multiple observed indicators of a changing global carbon cycle: (a) atmospheric concentrations of carbon dioxide (CO_2) from Mauna Loa (19°32'N, 155°34'W – red) and South Pole (89°59'S, 24°48'W – black) since 1958; (b) partial pressure of dissolved CO_2 at the ocean surface (blue curves) and in situ pH (green curves), a measure of the acidity of ocean water. Measurements are from three stations from the Atlantic (29°10'N, 15°30'W – dark blue/dark green; 31°40'N, 64°10'W – blue/green) and the Pacific Oceans (22°45'N, 158°00'W – light blue/light green). Taken form the IPCC Fifth Assesment Report, 2013.

Exposure to UVR radiation is an environmental factor that can modify atmospheric CO_2 sequestration in aquatic ecosystems by its direct effects on phytoplankton. The Antarctic ozone hole is expected to persist until at least 2050 (Mckenzie et al. 2011; Shanklin 2010) and although an ozone hole has been recently described over the Arctic in 2011, it seems to be ephemeral and hard to predict. The 2011 Arctic O_3 loss may be the largest ever observed, but it is still considerably less

than the 120–150 DU range of Antarctic losses reported (Strahan et al. 2013). Changes in UV radiation in the future are estimated by model simulations that are based on projected changes in ozone and clouds, which are the most important factors that are known to influence UVR. However, for estimations of UVB increase at the ocean surface, it is appropriate to consider simulations based on projected annually averaged changes in clear-sky erithemal irradiance (UV-Ery; Fig. 4 MacKenzie et al. 2011). These simulations show that UV-Ery is projected to return to its 1980 values in the early 2020s at northern latitudes, with a slower return in the southern hemisphere, especially over Antarctica.

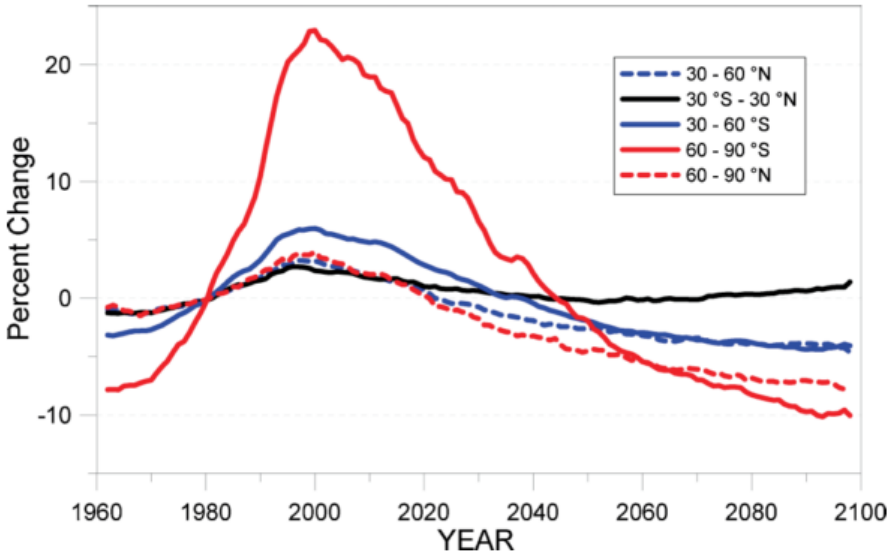


Figure 1.2 Time series of projected changes in annual mean of noon-time clear-sky erythemally-weighted UV over the period 1960 to 2100, relative to 1980, smoothed with a five-year running mean. Results are zonal means for several latitude belts. Figure taken from Makenzie et al. (2011).

However, these studies do not take into account the potentially important changes in cloudiness, surface reflectivity, and tropospheric aerosol loading due to or additional to climate change. When the effects of projected changes in clouds are

included, UV is projected not to return to its levels in 1980. Low latitude UV-Ery increases in response to the projected decreases in ozone due to the acceleration of the large scale atmospheric transport (specifically, the 'Brewer–Dobson' circulation). Although the magnitude of this increase in UV due to ozone loss is small (on average 2%) compared to the changes projected for the higher latitudes, the inclusion of clouds in the calculations results in an additional increase in UV-Ery of between 3 and 6% at low latitudes (see Fig. 5-MacKenzie et al. 2011). This additional increase in a region where UV-Ery is already high would increase the risk of adverse effects on ecosystems.

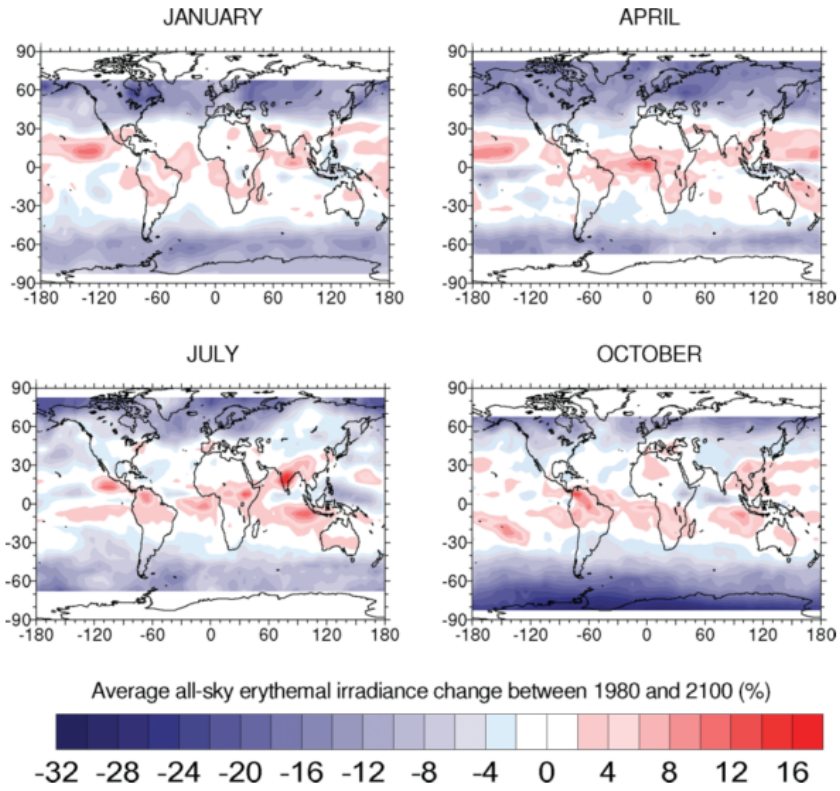


Figure 1.3 Multi-model average changes in surface erythemal irradiance from 1980 (1975–1985) to 2100 (2089–2099) under all-sky conditions for four months, calculated with a radiative transfer model using projections of ozone, cloudiness, temperature and solar radiation from 15 different CCM runs. Updated from ref. 99 and 100. Note the seasonally-dependent bands of missing data at high latitudes. Figure taken from Makenzie et al. (2011).

In addition to the mentioned above, global warming enhances stratification forcing the phytoplankton to be exposed to increasing irradiance by strengthening and shoaling of the thermocline (Boyd & Doney 2002; Doney 2006).

How global change affect phytoplankton is discussed within the next sections of this chapter, and in the context of the present work with regards to the marine unicellular chlorophyte *Dunaliella tertiolecta*.

Effects of global change on phytoplankton

Phytoplankton incorporate approximately 45 to 50 billion metric tons of inorganic carbon per year, removing a quarter of the CO_2 emitted to the atmosphere by anthropogenic activities (Häder 2011; Gao et al. 2012). In addition, phytoplankton populations are an important component of the global carbon cycle and they constitute the base of the marine food web (Field 1998). Thus, it is crucial to understand the possible effects of future changes in the global change scenario on phytoplankton.

Increased CO_2

Elevated CO_2 concentrations dissolved in seawater have impacts on phytoplankton productivity by directly affecting photosynthetic activity and the equilibrium of dissolved inorganic carbon (DIC) species in seawater. In the majority of the species a small direct effect of about 10% or less on the rate of photosynthesis at expected CO_2 levels has been found (Beardall et al. 2009). Some important exceptions to this have been found in some species exhibiting a greater increase in photosynthetic rate in response to elevated CO_2, i.e. *Emiliania huxleyi* (Rost & Riebesell 2004), *Phaeodactylum tricornutum* (Burkhardt et al. 2001) or in the halophyte *Dunaliella viridis* (Gordillo et al. 2003).

Most marine phytoplankton species show little effect on their photosynthetic rates because their photosynthesis is already saturated by carbon, due to the presence

of carbon concentrating mechanisms (CCMs) that actively take up inorganic carbon, either as CO_2 or HCO_3^- or both (Giordano et al. 2005). During photosynthesis, CO_2 is fixed in phytoplankton by the carboxylating enzyme rubisco (ribulose-1, 5-biphosphate carboxylase/oxygenase). However, for most species, rubisco is less than half saturated under current CO_2 levels since the typical half-saturation constant is around 20-70 µmol kg^{-1} of CO_2 and the concentration in sea water ranges between 8 and 25 µmol kg^{-1} (Riebesell 2004). This low affinity is aggravated by its dual role as an oxygenase, which produces a significant inhibition of CO_2 fixation by O_2. The limited CO_2 fixation by rubisco has been evolutively resolved by the operation of CCMs that provide a CO_2-rich environment around the enzyme, suppressing the oxygenase and saturating the carboxylase activity (Beardall & Raven 2004).

As part of these mechanisms, phytoplankton can convert HCO_3^- that occurs in large amounts in the ocean, into CO_2 using the enzyme external carbonic anhydrase (eCA). This enzyme also mediates the transport of CO_2 or HCO_3^- across membranes, either by passive (diffusion) or active mechanisms (Sültemeyer 1998). eCA has been found in many species of phytoplankton (Giordano et al. 2005) and its activity is commonly regulated by CO_2 concentration in the medium (Nimer et al. 1996; Burkhardt et al. 2001; Chen & Gao 2004), but also by UVR (Wu & Gao 2009). Usually CCMs are energetically costly mechanisms. The energy needed is derived mainly from photosynthesis, but also from mitochondria (Huertas et al. 2002). At high CO_2 levels (e.g. 1000 ppmv) these mechanisms are usually repressed and the energy spent in running them becomes available. In some species a downregulation of rubisco and/or respiration has also been reported (Beardall & Giordano 2002).

It is becoming increasingly evident that levels of CO_2 may also trigger a signal transduction pathway through cAMP that ultimately regulate gene expression (Matsuda et al. 2011); however, the differential expression of genes at high and low CO_2 has seldom been tackled beyond CCM-related genes and much on this topic remains to be elucidated.

Increased ultraviolet radiation (UVR)

The increase in PAR and UVR exposure has significant implications for primary producers because they are restricted to live within the euphotic zone, where solar UVR has a potentially negative impact. Additionally, as mentioned above, the inhibition of the surface waters mixing in tropic and mid-latitudes due to increased stratification will force phytoplankton to live at lower depths where solar radiation is higher (Doney 2006). UVR can act directly on cells by inducing degradation of some relevant biomolecules, or act indirectly through the induction of the production of cytotoxic compounds (Vincent & Neale 2000).

Exposure to an excess irradiance produces the over-reduction of the electron transport chain and electrons are driven to O_2, initiating the production and accumulation of reduced oxygen intermediates such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO^-). Damage induced by UVR to photosynthetic proteins produces the obstruction of electrons flowing through the photosystems, resulting in accumulation of reducing power (NADPH), enhancing reactive oxygen species production (Lesser 2006). Several studies reported that ROS are an important component of the stress response in marine organisms exposed to changes in environmental conditions, producing damage in lipids, proteins, and DNA (Vardi et al. 1999; Segovia 2008; van de Poll et al. 2009; Janknegt et al. 2009). The accumulation of ROS is known to activate cell death in metazoans (Cohen 1997; Leist & Nicotera 1997), vascular plants (Van Breusegem & Dat 2006), protozoa (Deponte 2008), yeast (Madeo et al. 2002), and phytoplankton (Vardi et al. 1999; Evans et al. 2006; Segovia 2008, Segovia & Berges 2009, Daherensouri et al., 2009; Bouchard et al., 2013).

The generation of ROS around PSII produces the degradation of proteins in the photosynthetic machinery. Proteins are one of the major targets of UVB because tryptophan, tyrosine, phenylalanine and histidine absorb in the range of 290–315 nm, with subsequent photooxidation of the peptidic bones. The damage produced primarily on the PSII site cause photoinhibition, decreasing photosynthetic activity,

growth and productivity (Helbling & Zagarase 2003; Häder 2011). In the core of the PSII complex the D1 and D2 reaction centres sub-units are located. They bind the chlorophyll, phaeophytin and plastoquinone co-factors involved in transmembrane light-induced charge separation (Rappaport & Diner 2008). Associated with this complex, there are distal light harvesting complexes, such as the Chl *a-b* light-harvesting complex (LHCII) sub-units. They contain bound pigments, whose function is to absorb photons of light and to pass the excitation energy to the reaction centres (Green & Durnford 1996). Most of the possible target sites for damage in the PSII complex, are associated with the D1 protein, and photoinactivation of the electron transport precedes the degradation of this protein (Prásil et al. 1992; Aro et al. 1993). The maintenance of PSII homeostasis consists of the operation of a PSII repair cycle which replaces damaged proteins (explained in detail in the "**Photosynthesis, photoprotection and repair** " section).

UVR and ROS also produce molecular modifications in the DNA. The most frequent photoproducts are covalent links between adjacent pyrimidines and the resulting structure is referred to as cyclobutane pyrimidine dimers (CPDs) and to 6-4 pyrimidine-pyrimidine products (PPs) (Löber & Kittler 1977). The photoproducts pose a threat to the viability and functional integrity of the cells because they can interfere with DNA replication and transcription. They constitute, quantitatively and qualitatively, important sources of damage in phytoplankton following the exposure to UVR (Buma et al. 2001; van de Poll et al. 2002, Boelen et al. 2001).

The effects of UVR on phytoplankton growth rates are species specific, thus UVR could induce changes in the community composition (Sommaruga 2003). Some reports identified underwater UVR and PAR as the factors explaining the vertical distribution of phytoplanktonic communities sampled in equatorial, tropical and temperate Central Atlantic Ocean in the Central Atlantic (Llabrés & Agustí 2006; Agustí 2004) and that of picocyanobacteria populations from oligotrophic Mediterranean waters (Llabrés et al. 2010). UVR has also been claimed to determine the vertical distribution of marine benthic macroalgae (Bischof et al. 2006).

Interactions between CO_2 and UV radiation

Change in several variables may not result in a simple additive response relative to that occurring by a given variable alone (Boyd & Hutchins 2012), but they can produce either synergistic or antagonistic effects (Folt et al. 1999). Accordingly, it has been observed that the effects of UVR are modulated by other environmental factors such as light availability, nutrient limitation and levels of dissolved CO_2 (Beardall et al. 2009).

Studies investigating the interactions between CO_2 and UVR have focused primarily on the effects on photosynthesis and growth, and a variety of responses have been reported. In some species elevated CO_2 did not affect the sensitivity to UVR exposure (Sobrino et al. 2005; Boelen et al. 2011), while other species showed a decrease in the sensitivity to UVR (Sobrino et al. 2008; 2009). However, increased sensitivity to UVR has been observed in species acclimated for longer time to elevated CO_2 (Sobrino et al. 2008). These authors related the down-regulation of the photosynthetic machinery under high CO_2 to the increase in UVR sensitivity observed. These results suggest that the interactions between elevated CO_2 and UVR may produce changes in the taxonomic composition of phytoplankton assemblages (Beardall et al. 2009). Accordingly, Gao et al. (2012) reported that increased CO_2 combined with high PAR stress will decrease the primary productivity of diatom dominated assemblages and observed a community shift away from diatoms, the main algal group that supports higher trophic levels and carbon export in the ocean.

All these results did not show a unique pattern of the interactive effects of UVR and increased CO_2, but confirmed that they depend on the species and that the effects of these environmental factors can be certainly synergistic. Hence, multivariable manipulative experiments are necessary to improve the predictions of phytoplankton community variations in a future global change scenario.

Response to stress

Stress can be defined as the impact of biotic or abiotic factors that negatively affects the metabolic performance of an individual, and eventually deteriorates the population growth rate through the reduction of survival, growth or reproduction (Grime 1989; Vinebrooke 2004; Auerbach 1981). Hence, survival in a changing environment has required the development of sophisticated adaptation mechanisms of defence and response.

Signal transduction

In a permanently changing environment organisms have to be able to perceive external signals and react in an appropriate way. This process is carried out by a complex net of biochemical cascades that transmit the external signal to cellular DNA.

Mitogen-activated protein kinase (MAPK) signal transduction pathways are among the most widespread mechanisms of eukaryotic cell regulation. They can be activated by a variety of different stimuli acting through diverse receptor families and producing a coordinated activation of gene transcription, protein synthesis, cell cycle machinery, cell death, and differentiation. Mammalian cells possess at least six MAPK families, three of which have been characterized in detail: the extracellular signal-regulated kinases (ERKs), the stress-activated protein kinases (JunN-terminal kinases or JNKs) and the p38s (Kyriakis 2001). Plant MAPKs are more closely related to the mammalian ERK subfamily of MAPKs (Nakagami et al. 2005). Activation of MAPKs is carried out by their upstream kinases, MAPK kinases (MAPKKs), through the phosphorylation of a Thr and a Tyr residue in the Thr-X-Tyr activation motif of MAPKs. MAPK cascades play pivotal roles in regulating plant development and signalling plant responses to a variety of stress stimuli, including temperature, salinity, osmolarity, UVR and ROS (Rodriguez et al. 2010; Meng & Zhang 2013). Recently, it has been reported that phosphorylation of p38- and JNK-like MAPKs proteins occur in Artic kelps and intertidal macroalgae in response to high irradiance and UV stress (Parages et al.

2013; 2014) and in *Dunaliella viridis* p38 and JKN- like MAPKs, play a critical role in acclimation to hypertonicity and hyperosmotic stress (Jimenez et al. 2004; 2007).

Scavenging ROS

Photosynthetic microalgae produce oxygen in the chloroplast and use it in the mitochondria, and so they have mechanisms that protect vital cell components against the damaging effects of oxygen. These enzymes and compounds constitute a complex network, called the antioxidant system, by which ROS concentration is usually effectively and strictly controlled. The antioxidant system consists of several enzymes, such as catalase (CAT), ascorbate peroxidase (APX), secretory peroxidases (POX), glutathione reductases (GR), peroxiredoxins (Prx), and non-enzymatic compounds like tocopherols, ascorbic acid and flavonoids. This system scavenge the reduced oxygen intermediates (Petrov and Van Breusegem, 2012). Even though there are many reports describing these responses (P J. Janknegt et al. 2009; Wang et al. 2009; Lee & Shin 2003; Murik & Kaplan 2009; Lesser 2006; Hideg et al. 2013), it is very difficult to achieve a consensus about the final results observed, because of the particular characteristics of each analysed species.

Photosynthesis, photoprotection and repair

Photosynthesis is the biological conversion of light energy to chemical bond energy that is stored in the form of organic carbon compounds. However, it is reported that differences in photosynthesis do not necessary translate into similar differences in growth rates (Beardall & Raven, 2004).

Phytoplankton can receive light intensities that exceed photosynthetic requirements producing photoinhibition. Photoinhibition and viability loss are attenuated by mechanisms that allow for partial avoidance of photodamage. These are photoprotection processes, regulated in a short time scale (seconds to minutes), that limit the damage potential of photons that have been absorbed by photosynthetic pigments (Raven 2011). These mechanisms include non-photochemical quenching, by

increased fluorescence and thermal dissipation among others, such as xanthophyll cycles. There are also mechanisms that enhance photochemical quenching by the engagement of additional electron transport pathways, or increasing the rate of already functioning electron transport pathways, involving PSII and increased content of enzymatic and non-enzymatic scavenging of reactive oxygen species (Takahashi & Badger 2011).

In a different way, phytoplankton can induce changes in photophysiology in response to varying irradiance conditions by the process of photoacclimation. It occurs on time scales of hours or days and includes the adjustment of PSII reaction centres, pigment levels, rubisco activity and PSII repair. For example, plants and green algae change the structure and composition of their photosynthetic apparatus protecting PSII, as it appears to be the primary site of photoinhibitory damage (Horton et al. 1996). In chlorophytes and higher plants photosynthetic adjustment to irradiance stress involves an increase in the ratio of Chl a/b and a concomitant decrease in LHCII abundance. Thus, it has been suggested that irradiance regulates LHCII apoprotein content, either transcriptionally through the inhibition of cab mRNA accumulation, and/or post-translationally through the control of Chl synthesis (Laroche et al. 1991; Falkowski & LaRoche 1991; Escoubas et al. 1995).

Despite the mentioned process, some photodamage inevitably occurs. However, complete recovery from photoinhibition can be seen by *de novo* synthesis and replacement of proteins degraded in the PSII repair cycle (Prasil et al. 1992; Aro et al. 2005; Nath et al. 2013). When D1 protein is degraded, PSII suffers the photoinactivation of the electron transport, but D1 protein has an unusual light dependent turnover (Takahashi & Badger 2011). Hence, the extent of photoinhibition reflects a balance between damage and repair (Prásil et al. 1992; Aro et al. 1993). The degradation of D1 protein and the repair of PSII through protein replacement have been reported in microalgal cultures exposed to UVR (Xiong 2001). In phytoplankton communities, Bouchard et al. (2005) observed that high UVR exposures had more effect on D1 synthesis and PSII repair than on D1 degradation. It is known that, under

conditions of excess light for photosynthesis, the rate of PSII repair is depressed due to inhibition by ROS at the D1 protein translation level in chloroplasts (Nishiyama et al. 2006, Takahashi and Murata 2008; Takahashi & Badger 2011).

DNA repair mechanisms

In the view of the central part of UV radiation as a source of DNA damage since the beginning of biological life, it is no surprising that many organisms have evolved multiple and diverse mechanisms for DNA repair. In phytoplankton, the existence of DNA repair mechanisms has been demonstrated many times (Boelen et al. 2001; Buma et al. 2001; Yi et al. 2006).

The simplest, most efficient and most accurate DNA repair mechanism is photoreactivation, which consists in the specific monomerization of CPDs. During this process, the covalent bound of two adjacent pyrimidines forming a CPD is reversed, yielding native pyrimidine monomers (McCready & Marcello 2003). A specific enzyme that requires light in a particular range of wavelengths (near blue), called pyrimidine dimmer-DNA photolyase (PL), catalyses the damage reversal. The prefix “pyrimidine dimmer” is added to distinguish these enzymes from those that catalyse the repair of (6-4) photoproducts (6-4PPs) by an essentially identical mechanism. The family of genes referred to as the photolyase/cryptochrome genes encodes both types of enzymes. Photolyases are present in all three kingdoms of life: archaea, eubacteria and eukaryotes (Essen & Klar 2006) and the gene has been isolated in an extremophilic bacteria (Albarracín et al. 2014) and in microalgae (Cheng et al. 2007; Heijde et al. 2010; Brazard et al. 2012).

Other important processes are called “dark DNA repair” because, in contrast to photoreactivation, the energy required to perform them does not come directly from light. There are two primary categories: base excision repair (BER) and nucleotide excision repair (NER). BER and NER are complex multistep mechanisms and do not reverse the DNA damage directly; instead, they replace the damage base or nucleotide by a new one (Britt 2004; Seeberg et al. 1995). In BER, the crucial enzymes are DNA

glycosylases that initiates the multistep process. Like photolyases, they recognise only a particular class of base damage, a particular inappropriate base or a particular mispairing; however, some of these enzymes recognise more than one type of damaged base (Seeberg et al. 1995). There are at least nine *Arabidopsis* genes encoding bifunctional DNA glycosylases, and for seven of these AP lyase activity has been demonstrated *in vitro* (Córdoba-Cañero et al. 2009). One of these proteins is called repressor of silencing (ROS1) and possesses a bifunctional glycosylase/lyase activity. Because of this, its role in development programs by demethylation has been widely studied (Morales-Ruiz et al. 2006). However, Gong et al. (2002) obtained evidence of its implication in functional DNA repair.

A wide variety of distorting DNA lesions are removed by NER, including CPDs and 6-4PPs. This mechanism is highly conserved in eukaryotes and it is present in most organisms. One important protein involved at various steps in BER and NER is the proliferating cell nuclear antigen (PCNA; Umar et al. 1996). It is a processivity factor for δ / ε polymerases, essential for cellular DNA synthesis. Its abundance is cell cycle dependent, increasing during the S phase of the cell cycle. As in unicellular algae DNA replication, it is directly associated with cell proliferation. This protein has been used as a cell cycle marker and as an indicator of growth in phytoplankton populations (Carpenter et al. 1998). In normal conditions, cells express PCNA being indicative of cell proliferation. However, in UVR exposed cells it rather seems to be indicative of DNA damage, considering that high levels of PCNA expression have been reported after UVR (Masih et al. 2008).

Hence, the net effect of UVR exposure on phytoplankton primary production reflects a balance between damage and repair processes and the energetic costs of photoprotection developed by the organisms (Vincent & Neale 2000, Raven 2011).

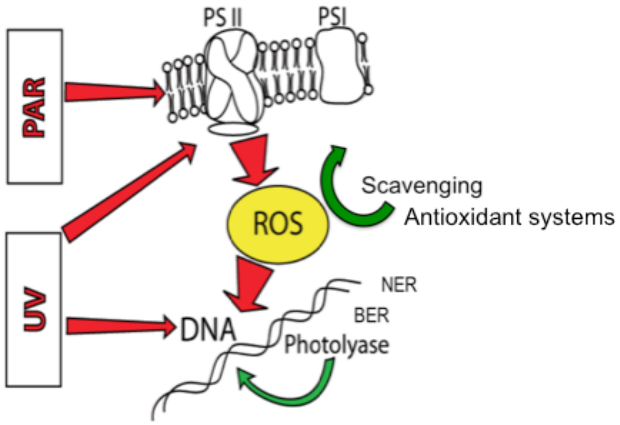


Figure 1.1: Diagram of high UV and PAR effects thought ROS accumulation.

Cell viability, cell death and caspase-like proteases

In addition to the mechanisms that avoid and repair damage, it is widely demonstrated that environmental stress can decrease cell viability, and promote different programmed cell death (PCD) types (Segovia 2008; Jiménez et al. 2009). The best known morphotype is the apoptotic one, consisting of morphological changes highly conserved, such as chromatin condensation and margination, and ordered DNA cleavage, while the cytoplasm and organelles remain unaffected (Segovia et al. 2003). Apoptosis is clearly different from necrosis that is characterized by a loss of membrane integrity, cell shrinkage, and final lysis. In animal cells, different kinds of programmed cell death have been characterised much more in detail, and in vascular plants, the pathways by which PCD occurs are also well established. However, the execution of PCD in non-metazoan organisms is morphologically different from apoptotic PCD in metazoans, and lacks a number of key molecular components of the apoptotic machinery. Accordingly, the different terms usually coined to describe the different cell morphotypes (apoptosis, paraptosis, necrosis-like, and others (Galluzzi & et al. 2012)) shall be used carefully in protists.

Massive cell death processes (in the absence of grazers and viruses) have been widely reported in cultured phytoplankton, covering practically all taxonomic groups (Berges & Falkowski 1998; Segovia et al. 2003; Segovia & Berges 2005; Segovia & Berges 2009; Jiménez et al. 2009; Vardi et al. 1999; Franklin et al. 2004; Segovia 2008; Bidle & Falkowski 2004; Franklin et al. 2012; Orellana et al. 2013). However, only few of them refer to UVR, and none of them to CO_2 or the interactive effects of UVR and CO_2. UVR produces increasing membrane permeability in phytoplankton species (Sobrino et al. 2005), hence loss of the ability to maintain homeostasis and finally cell death (Moharikar et al. 2006; Jiménez et al. 2009; Bouchard & Purdie 2010). Furthermore, cell death has been reported as a crucial process in ocean natural phytoplanktonic communities (Boelen et al. 2001; Agustí 2004; Llabrés & Agustí 2006; Llabrés et al. 2010), playing an important role in the oceanic carbon cycle, since the loss of organic carbon is a consequence of cell lysis. In this case, cell death constitute part of the fast turnover of the food web, and it is almost impossible to know which cell death type would be involved, or if it is an accidental death (lysis or simple necrosis).

The mechanisms by which cell death (programmed or not programmed) occurs, considering that cell death in unicells leads to the complete demise of the organism, are always intriguing, and there still are many unanswered questions. Among them, which is the proteolytic machinery involved and how it works (again, assuming that there is no grazing pressure). Caspases were first identified as the cysteine-proteases responsible for cell dismantling in undergoing apoptotic processes in metazoans. They are among the most specific proteases, having an unusual and stringent requirement for cleavage after aspartic acid in the first aminoacidic position (P1) (Thornberry 1999). The enzymatic activity that has been measured in phytoplankton up to date correspond to caspase-like activities. It is different than that of metacaspases found in fungi, vascular plants and unicellular eukaryotes(Deponte 2008), since metacaspases have preference requirements for lysine or arginine and not for aspartate in P1 (Vercammen et al. 2004; Vercammen et al. 2007; Tsiatsiani et al. 2011) (apparently,

metacaspases are not caspase or caspase-like proteins, but there is controversy about this, and it is not the topic of the present work (for details see Enoksson et al. 2010; Carmona-Gutierrez et al. 2010)). The exact nature of caspase-like activities is not known, although some authors have pointed to the serine protease family proteins or the vacuolar processing enzyme (Bonneau et al. 2008; Vartapetian et al. 2011; Hara-Nasimura et al. 2011), but they have traditionally been regarded as cell death proteases. However, the concept has widened up, and it is now known that their activity is essential during the normal physiology of the cells, cell survival, and most importantly, during cell stress, as demonstrated by several reports (Zeuner et al. 1999; Segovia & Berges 2005; 2009; Jiménez et al. 2009; Bouchard & Purdie 2011; Franklin et al. 2012; Berges & Choi 2013). Therefore, the presence of these proteases can be used as a marker not only for cell death, but also cell stress.

Thesis outline

It has been demonstrated that various components of global change can potentially have effects on phytoplankton. These effects are reported as species-specific, and so they may lead to shifts in community composition and biomass. Since the primary producers in freshwater and marine ecosystems constitute the base of food webs, providing energy for the primary and secondary consumers, these effects can propagate to higher trophic levels. Furthermore, they are key components of the global carbon cycle. Thus, it is highly relevant to analyse the different degree of sensitivity and the response of the species constituting the different phytoplankton communities to predict its global behaviour. As those responses generally are species-specific (Neale 2000; Litchman et al. 2002; Sobrino et al. 2008), it is important to know what are the key process underlying the response in a given species.

The biological material used for this study is the unicellular Chlorophyta *Dunaliella tertiolecta* (CCAP 19/6B variety), Class Chlorophyceae, Order Volvocales. It is an obligate photoautotrophic alga that cannot use organic compounds. The genus

Dunaliella has been among the most studied members of Chlorophyceae, and species of this genus occur in habitats with a wide range of salinity, pH, light intensity and temperature (Polle et al. 2009). These organisms are capable of surviving in extreme and changing environments because they show a remarkable degree of acclimation (Berges & Falkowski 1998; Segovia & Berges 2005; Jiménez et al. 2004; 2007; 2009). Those features make *D. tertiolecta* a good candidate organism to study stress responses and the processes involved in survival.

The lack of knowledge on the response to high stress tolerance in species such as *D. tertiolecta*, to possible future extreme conditions, limits our prediction ability of future changes in communities. Therefore, this thesis addressed the following major questions:

1. What are the processes underlying the resilience of *D. tertiolecta* to chronic UVR exposure?
2. What is the role of oxidative burst in the response (survival *versus* mortality) of *D. tertiolecta* compared to a species that shows a different response pattern (such as the dinoflagellate *Gymnodinium sp*.) when exposed to UVR, either continuously or during photoperiod?
3. Does increased CO_2 interfere with the response to high irradiance (PAR and UVR) by interfering in ROS accumulation and/or essential repair mechanisms such as photolyase, PCNA, and ROS1 accumulation?
4. How do the interactive effects of UVR and CO_2 affect the transcriptome? Which are the families of genes dominantly expressed, or downregulated, and how this particular wide gene expression relates to the physiological performance of the cells?
5. Does the response obtained confer any biological advantage to *D. tertiolecta*?

To tackle these questions, four experimental studies were performed under different irradiance and CO_2 concentration conditions (see table 1.1). In **Chapter 2**, *D. tertiolecta* cells were maintained under chronic PAR and UVB (PAB) exposure for 6 days and the cell death or survival was compared to cells under PAR. For this purpose, we studied the cellular ultrastructure, the dynamics of DNA damage and the repair mechanisms. Additionally, the implication of MAPK-like and CL proteins in the response to stress was analysed. Those results showed that cells survived chronic UVR exposure by activation of DNA repair mechanisms by means of PCNA and ROS1-protein accumulation. Concurrently, we demonstrated that activation of MAPK-like proteins mediates the process and that caspase-like proteins are also involved during the response to stress.

In **Chapter 3**, the chlorophyte *D. tertiolecta* and the dinoflagellate *Gymnodinium sp*. were exposed to continuous and photoperiodic PAR plus UVA and UVB (PAB) for 6 days. Those conditions were chosen to simulate the long UVR exposure observed at high latitudes in summer (as an extreme condition), and a more typical situation observed in natural systems. In this experiment we compared the stress response of both phytoplankton species focusing the study on ROS accumulation and detoxification by scavenging, as well as on DNA damage and caspases-like enzymatic activities. Results obtained showed that *D. tertiolecta* was able to cope with continuous UVR exposure for 6 days, meanwhile *Gymnodinium* suffered from oxidative stress, sustained DNA damage and underwent cell death after less than 2 day of exposure to UVR even during a photoperiod.

The interactive effects of increased CO_2 and irradiance on the physiological performance of *D. tertiolecta* were studied simulating the solar radiation at different depths in **Chapter 4**. For this purpose, the photosynthetic response and the oxidative stress were assessed, in combination with the DNA repair mechanisms by evaluating photolyase gene expression and PCNA and ROS1 protein accumulation. We show that increased CO_2 allowed *D. tertiolecta* to cope with high PAR and UVR-induced damage

mainly by accumulating less ROS and increasing DNA repair mechanisms, partly by means of CO_2-induced upregulation of the Class II-CPD-photolyase gene.

In **Chapter 5**, *D. tertiolecta* was studied for 8 days under the combination of two factors: chronic PAR or PAB and atmospheric or high CO_2 conditions. In this case, comparing cell survival/viability, oxidative stress, photosynthesis and carbon fixation assessed the effects of the treatments on physiological activity. An approximation of the UVR and CO_2-induced response in the gene expression was done by using cDNA-Amplified Fragment Length Polymorphism (AFLP)-based transcript profiling, as well as a detailed study of the expression of genes related to photosynthesis and DNA repair. In this experimental study it was shown that UVR and CO_2 triggered the differential expression of a wide array of genes. Moreover, high carbon promoted a "low metabolic steady state" and the deleterious effect of UVR seemed to be attenuated, demonstrating that cells resilience to UVR is increased under elevated CO_2.

All these data are obtained under artificial conditions therefore caution must be taken when extrapolating these results to natural conditions. However, recent experiments performed with *D. tertiolecta* under semi-natural conditions (22 L microcosms, natural solar radiation) and increased CO_2, performed by our group, indicate that the role of the mechanisms analysed here are very similar. It can be concluded that *D. tertiolecta* shows remarkably high resilience to high PAR and UVR, which was even enhanced under high CO_2 conditions. This suggests that *D. tertiolecta* (and probably other chlorophytes) could take advantage over other phytoplankton species in the future global change scenario. In other words, in a winner *vs*. looser species situation forced by environmental factors derived from global change, chlorophytes are expected to be in the winner side.

Table 1.1: Summary of the experimental set-up and culture conditions.

	Growth Irradiance	**Pre-acclimation Irradiance**	**Treatments**
Chapter 2	100 µmol quanta $m^{-2}s^{-1}$ Continuous	NO pre-acclimation	P and PAB (continuous) PAR= 100 µmol quanta $m^{-2}s^{-1}$ 9.5 UVA: 0.45 UVB (Wm^{-2})
Chapter 3	100 µmol quanta $m^{-2}s^{-1}$ Photoperiod 12L:12D	NO pre-acclimation	PAB continuous and photoperiod 12L:12D PAR= 100µmol quanta $m^{-2}s^{-1}$ 9.5 UVA: 0.45 UVB (Wm^{-2})
Chapter 4	100 µmol quanta $m^{-2}s^{-1}$ Continuous	500 µmol quanta $m^{-2}s^{-1}$ Photoperiod 16L:8D	≠PAB (photoperiod 16L:8D)/ HC and LC I1100->21.4 UVA: 1.21 UVB (Wm^{-2}) I800-> 13.8 UVA: 0.83 UVB (Wm^{-2}) I400-> 6.6 UVA: 0.39 UVB (Wm^{-2}) I200-> 1.2 UVA: 0.07 UVB (Wm^{-2})
Chapter 5	100 µmol quanta $m^{-2}s^{-1}$ Continuous	100 µmol quanta $m^{-2}s^{-1}$ Continuous	P and PAB continuous/ HC and LC PAR= 100µmol quanta $m^{-2}s^{-1}$ 9.5 UVA: 0.45 UVB (Wm^{-2})

Chapter 2

Cell survival after UV stress in the unicellular chlorophyte *Dunaliella tertiolecta* is mediated by DNA repair and MAPKs phosphorylation

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Journal of Experimental Botany 63:695–709 (2012)

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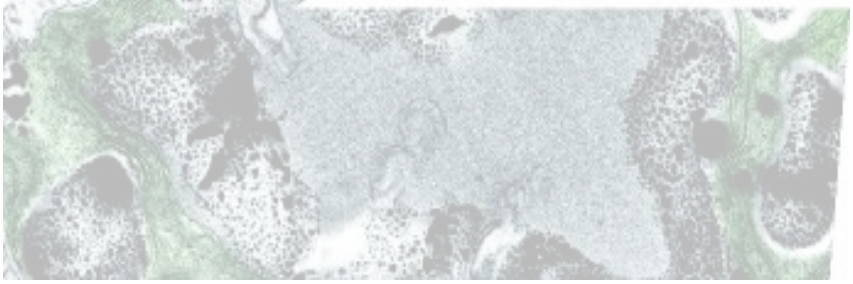

Abstract

Ultraviolet radiation induces damage in a variety of organisms, and cells may adapt by developing repair or tolerance mechanisms to counteract such damage, otherwise the cellular fate is cell death. We have studied in *Dunaliella tertiolecta* the effect of UVR-induced cell damage and the associated signalling and repair mechanisms by which cells are able to survive. UVR did not cause cell death as shown by the absence of SYTOX-green positive labelling cells. Ultrastructure analysis by TEM demonstrated that cells were alive but subjected to morphological changes such us starch accumulation, chromatin disaggregation and chloroplast degradation. This behaviour paralleled with a decrease in F_v/F_m and formation of cyclobutane–pyrimidine dimers (CPDs), showing a 10-fold increase at the end of the time course. There was a high accumulation of the repressor of transcriptional gene silencing-protein like (ROS1), as well as the cell proliferation nuclear antigen (PCNA) in UVR-treated cells, revealing the activation of DNA repair mechanisms. The degree of phosphorylation of JNK (c-Jun N-terminal kinase) and p38-like MAPKs was higher in UV-exposed cells, however the contrary occurred with the Ph-ERK (extracellular signal-regulated kinase). This confirms that both JNK and p38 need to be phosphorylated for triggering the stress response, as well as that cell division is arrested when an ERK is dephosphorylated. In parallel, both DEVDase and WEHDase caspase-like enzymatic activities were detected, even though the cells were not dead, suggesting that these proteases must be considered within a wider frame of stress proteins, rather than specifically involved in cell death in these organisms.

Chapter 3

Differential effect of ultraviolet exposure (UVR) in the stress response of the Dinophycea *Gymnodinium sp.* and the Chlorophyta *Dunaliella tertiolecta*: mortality *vs* survival

Josée Nina Bouchard, Candela García-Gómez, M. Rosario Lorenzo and María Segovia

Marine Biology 160, 10:2547–2560 (2013)
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Abstract

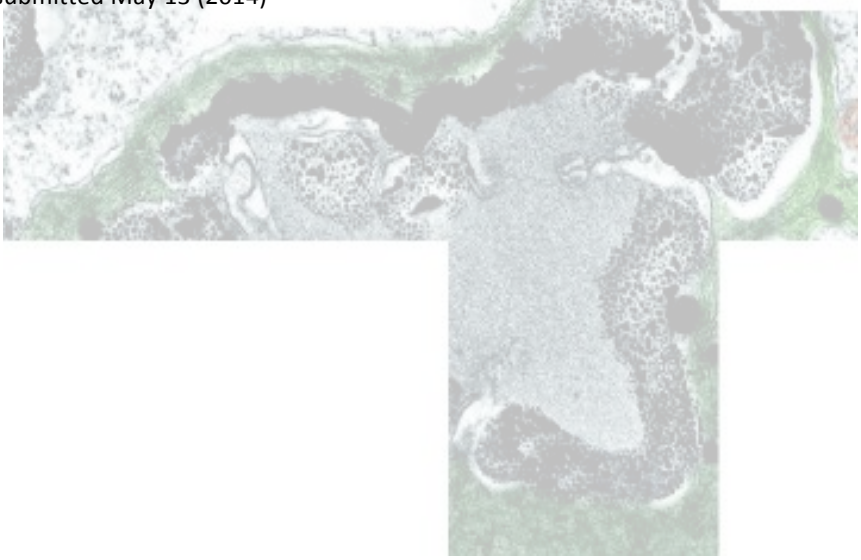
Dunaliella tertiolecta (Chlorophyta) and *Gymnodinium* sp. (Dinophyceae) cells were exposed to ultraviolet radiation (UVR) (PAR, UVA and UVB: PAB) for 6 days either continuously or during a photoperiod. Both UVR treatments were harmful to *Gymnodinium* but exposure to continuous PAB had the most dramatic effects. Although a number of lesions/damage could have happened during the first few hours of exposure to UVR, in less than 24 h, *Gymnodinium* lost its ability to detoxify ROS efficiently, photoinhibition occurred, thymine dimers formed in the DNA, caspase-like enzymatic activities DEVDase sharply increased and cells died as determined by SYTOX-green staining. Superoxide dismutase (SOD) activity did not significantly change with time and although the catalase (CAT) activity augmented in both treatments, cells still suffered from the UVR stress. Clearly, UVR was fatal to the dinoflagellate. For the chlorophyte, however, cell numbers increased regardless of the UVR treatment and mortality remained low (<20%). F_v/F_m showed an initial decrease but then remained constant for both light treatments. After 6 d of continuous PAB exposure, however, signs of stress (thymine dimers, oxidative stress) paralleled a drop in catalase activity. Results obtained here demonstrate that the dinoflagellate *Gymnodinium* was much more sensitive and was harmed more rapidly by UVR exposure than the chlorophyte *D. tertiolecta*. The increased tolerance to UVR exposure of the chlorophyte may provide advantages over other more sensitive phytoplankton species within the photic zone. We provide strong support in the present study for repair being an important component of UV resistance in this species.

Chapter 4

Elevated CO_2 alleviates high PAR and UV stress in the unicellular Chlorophyte *Dunaliella tertiolecta*

Candela García-Gómez, Francisco J. L. Gordillo, Armando Palma, M. Rosario Lorenzo, María Segovia

Photochemical and Photobiological Sciences, Submitted February 10 (2014), Resubmitted May 13 (2014)



Abstract

The effects of increased CO_2 and irradiance on the physiological performance of the chlorophyte Dunaliella tertiolecta were studied at different PAR and UVR (UVA+UVB) irradiances, simulating the solar radiation at different depths, under present (390 ppmv, LC) and predicted CO_2 levels for the year 2100 (1000 ppmv, HC). Elevated CO2 resulted in higher optimum and effective quantum yields (F_v/F_m and φPSII, respectively), electron transport rates (ETR) and specific growth rates (μ). Cell stress was alleviated in HC respect to LC as evidenced by a decrease in reactive oxygen species (ROS) accumulation. DNA damage showed a 42-fold increase in cyclobutane-pyrimidine dimers (CPDs) formation under the highest irradiance (1100 μmol quanta m^{-2} s^{-1}) in LC with respect to the lowest irradiance (200 μmol quanta m^{-2} s^{-1}). Photolyase (CII-PCD-PL) gene expression was upregulated under HC resulting in a drastic decrease in CPDs accumulation to only 25% with respect to LC. Proliferating cell nuclear antigen (PCNA) accumulation was always higher in HC and the accumulation pattern indicated its involvement in repair or growth depending on the irradiance doses. The repressor of silencing (ROS1) was only marginally involved in the response, suggesting that photoreactivation was the most relevant mechanism to overcome UVR damage. Our results demonstrate that future scenarios of global change result in alleviation of irradiance stress by CO_2–induced photoprotection in *D. tertiolecta*.

Chapter 5

Differential gene expression and stress response to increased CO_2 and UVR in the unicellular chlorophyte *Dunaliella tertiolecta*

Candela García-Gómez, MTeresa Mata, Michael Vandorpe, Debbie Rombaut, Frank Van Breusegem and María Segovia

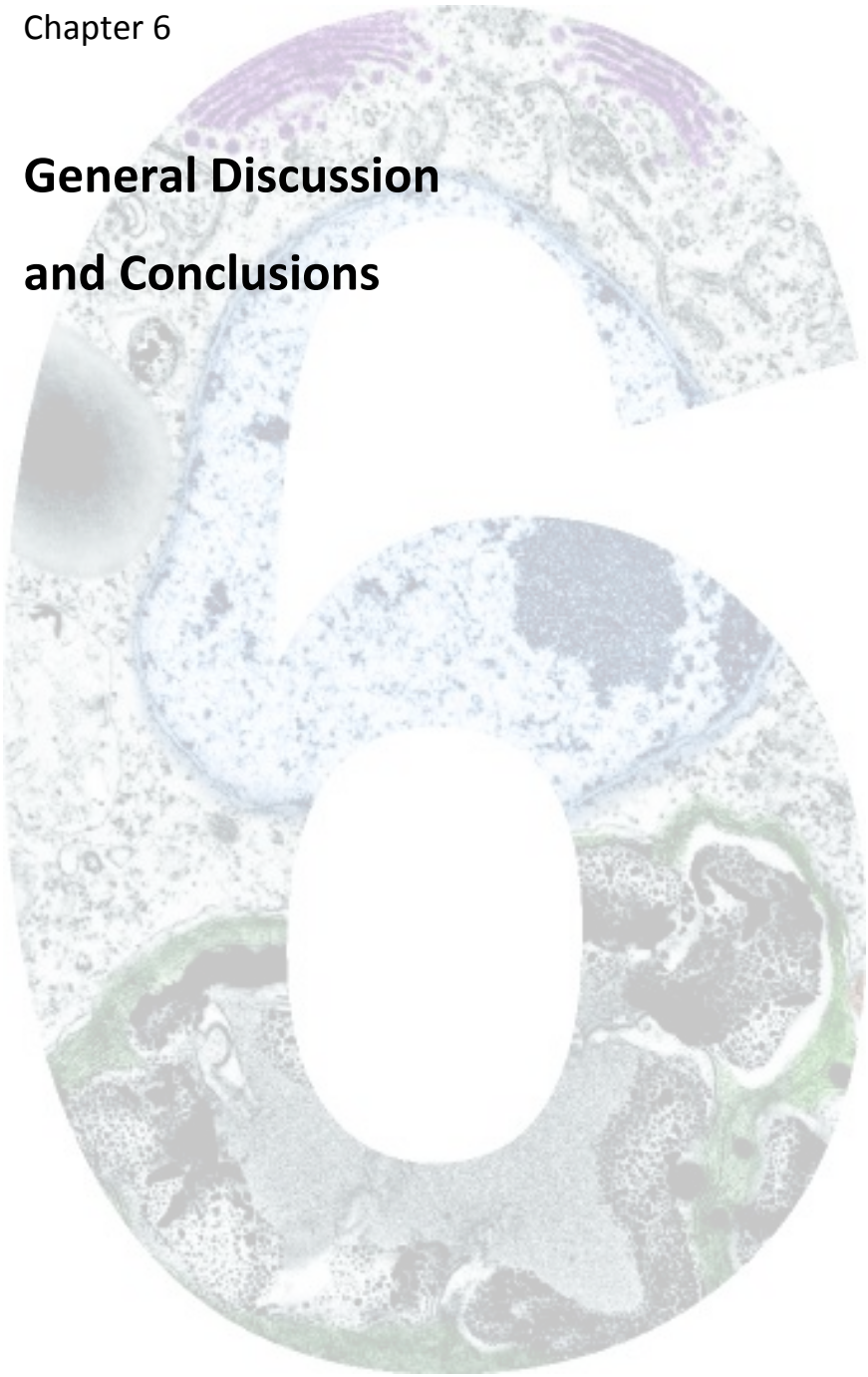
Manuscript for submission

Abstract

The interactive effects of increased CO_2 levels and ultraviolet irradiance (UVR) were studied on the stress response of the unicellular Chlorophyte *Dunaliella tertiolecta,* by means of transcriptome profiling analyses and the physiological performance shown by the cells. Cultures were exposed to PAR (P) and supplemental UVA+UVB (PAB) for 6 days under four different treatments: P-HC, PAB-HC, P-LC and PAB-LC. The seawater carbonate system was manipulated to achieve two different CO_2 levels, corresponding to the present (390 ppmV, LC) and to levels predicted for year 2100 (1000 ppmV, HC). HC promoted a "low metabolic steady state" demonstrated by low values (more than 2-fold) in cell viability, accumulation of reactive oxygen species (ROS), ^{14}C fixation, and reduced growth rates, when compared to LC. The deleterious effect of UVR seemed to be attenuated under HC, and only had a clear effect on Fv/Fm. UVR and CO_2 triggered the differential expression of a wide array of genes, grouped in 10 different functional categories, of which the dominant gene families were related to photosynthesis, protein synthesis and translation, and UVR response. PsbA (encoding D1 protein), the Chl *a-b* light-harvesting complex (LHCII) and external carbonic anhydrase (eCA) gene expression, were downregulated at PAB-HC and upregulated under PAB-LC. On the contrary, photolyase (PL) gene expression was upregulated under PAB-HC. These results suggest upregulation of such genes at PAB-LC being a consequence of activated repair, needed for the replacement of damaged proteins, which occurs under the usual active cellular metabolism at LC. The upregulated PL gene at PAB-HC points towards a low-steady–state metabolism (downregulated metabolism), which allows that the excess energy that is not used can be utilized for other processes such as repair. In addition, significant rates of cell death were not observed in any of the treatments throughout the experimental period. These data taken together demonstrate that cells resilience to UVR is increased under elevated CO_2. The ecological implications that this might have, needs further consideration.

Chapter 6

General Discussion and Conclusions



UVR is considered a prime factor able to cause detrimental effects on phytoplankton (Vincent & Roy 1993). It is particularly relevant when considering the increase in ultraviolet-B radiation reaching the ocean surface due to the depletion of stratospheric ozone layer, and aggravated by other global change conditions (McKenzie et al. 2011; Zepp et al. 2007). This work has demonstrated that *D. tertiolecta* is highly resilient to both UVR and high PAR during long-term exposure: it responds to stress, and it can unexpectedly survive for at least 6 days. To explain how a photoautotrophic unicellular alga is able to cope with the damaging effects of the two abiotic stressors used, *D. tertiolecta* was exposed to different treatments and periods of PAB. It always showed a remarkable ability to survive, and even to recover some functions once the stress finished. Moreover, the interference of rising CO_2, as a main driver of global change, has been assessed in the PAB survival and stress tolerance (experimental treatments summarised in Table 1.1 in Introduction). Despite that high PAR and UVR in any of the experiments did not produce significant cell mortality, the physiological response and the repair mechanisms triggered were not always the same.

In this work it has been observed that PAB exposure was always accompanied by a sharp decrease in photosynthetic parameters, because of the photoinhibition caused by the degradation in the photosynthetic machinery. However, the light history previous to the experiment and the type of treatment modulated the response of *D. tertiolecta*. When cells were pre-treated with a photoperiod of PAR (chapter 3) they suffered less photoinhibition when exposed to PAB, compared to cells pre-treated to continuous PAR (chapter 2 and 5). The results for oxidative stress were consistent with those, so that less photoinhibition was accompanied by a smaller accumulation of ROS under PAB (chapter 3). As discussed in previous chapters, several studies have described the enzymatic degradation of D1 protein in the PSII reaction centre after exposure to high irradiance (Roos & Vincent 1998, Takahashi & Badger 2011, Nath et al. 2013), with a consequent loss in photosynthetic rate. This is further supported by expression of PsbA and LHCII genes that showed values significantly higher in PAB than in PAR cultures, both pre-treated with continuous PAR (Chapter 5).

Moreover, the accumulation of DNA damage triggered by PAB was higher in cells pre-treated with continuous PAR (chapter 2), when compared with cells pre-treated with photoperiod and under similar treatments (Chapter 3 and 4). This must be attributed again to the deleterious effects of cumulative ROS.

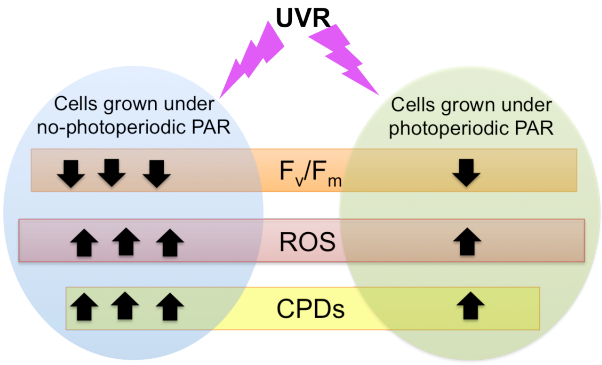


Figure 6.1: Schematic diagram of the different responses to UVR in *Dunaliella tertiolecta* grown under photoperiod and continuous PAR.

Although it is known that CPDs are only produced by UVR, the higher oxidative stress seen in cells previously treated with continuous PAR produced more damage in photosynthetic proteins and DNA. In turn, the accumulation of damage triggers the activation of repair mechanisms, as proved by the accumulation of proteins related to BER and NER pathways, such as PCNA and ROS1. In cells pre-acclimated to photoperiod (and to 5-fold higher irradiance; chapter 4) the accumulation of those proteins was not significantly different to initial values, even in cells under similar PAB treatments. Nevertheless, the accumulation of those proteins was crucial for DNA repair, hence, survival of cells pre-treated with continuous light, when exposed to continuous PAB (chapter 2).

These results agreed with those demonstrating that the sensitivity of phytoplankton to UVR is affected by the light history of the cells. MacDonald et al. (2003) observed that the response of *Synechococcus* cells to UVB depended strongly

on their light acclimation history. When cells grew under high PAR and after exposure to UVR, they did not show short-term inhibition of PSII or growth. In contrast, when cells grew under low PAR and then were exposed to the same amount of UVR, they suffered from short-term inhibition of PSII and growth. Similar results were obtained when Ivanov et al. (2000) exposed the cyanobacterium *Plectonema boryanum* to UVR. Acclimation of cells to moderate irradiance seem to induce a significant resistance to inhibition of PSII photochemistry compared to cells acclimated to low irradiance. In natural phytoplankton assemblages organisms from low light environments suffered more photoinhibition and DNA damage (Villafañe et al. 2004; Litchman & Neale 2005). In the same manner, Antarctic phytoplankton from deep mixed layers was sensitive to photoinhibition when exposed to near surface irradiance (Alderkamp et al. 2010).

The light history of the cells is influenced by the mixing regime prevailing in the water column. Therefore, exposure depends on the depth of the mixed layer, the rate of vertical mixing, and the water transparency. In phytoplankton communities Bouchard et al. (2005) observed that enhanced light harvesting capacity at low light can be achieved by an increase in the number of PSII reaction centres (D1 proteins) with relatively low absorption cross-sections (Behrenfeld et al. 1998). This acclimation strategy allows for a constant rate of photosynthesis over the maximum intensity of the photoperiod and prevents loss of photosynthesis under photoinhibitory irradiances (Behrenfeld et al. 1998).

It has been reported that ambient UVR levels produce species-specific phytoplankton mortality (Llabrés & Agustí 2006; Bancroft et al. 2007). These authors concluded, that UVR acts as a primary driver of the community structure of autotrophs affecting the dynamics of the microbial food web in oceanic waters. UVR has also been claimed to determine the vertical structure of seaweed benthic communities (Bischof et al. 2006). However, predictions on responses to UVR exposure are complex because of the interaction of factors, such as increasing CO_2, among others. Studies investigating the effects of CO_2 have been primarily focused on the effects in photosynthesis (Beardall et al. 2009; Rost & Riebesell 2004; Burkhardt et al. 2001) and

in the role of CCMs (Beardall & Giordano 2002; Sobrino et al. 2008; Sobrino et al. 2009; Huertas et al. 2002). Data demonstrated that differences in photosynthesis do not necessary translate into similar differences in growth rates (Beardall & Raven 2004) hence, CO_2 consequences on growth could be attributed to its effects on other physiological processes. In this regard, Gordillo et al. (2001) reported that the growth enhancement produced by increased CO_2 levels in the Chlorophyte *Ulva rigida* was dependent on the enhancement effect on nitrogen assimilation rates. In this species, under N sufficiency, high CO_2 led to higher growth rates without increasing the carbon fixed through photosynthesis (Gordillo et al. 2003). In contrast, in the cyanobacterium *Spirulina platensis* nitrogen assimilation was not affected by CO_2, and the authors related this response to the lack of response in the maximum growth rate (Gordillo et al. 1999). Similar results were obtained in *D. viridis* where an improvement in photosynthesis and growth rates by high CO_2, was observed. The strategy of acclimation involved the light harvesting machinery and the nutritional metabolism in an N supply dependent manner (Gordillo et al. 2003).

Recently, evidence has been obtained that there are genes regulated by both low and high CO_2 in phytoplankton. In this pathway triggered by changes in CO_2 concentration, the molecule 3'-5'-cyclic -adenosine monophosphate (cAMP) plays a critical role as a second messenger. This sensing-signalling pathway would contribute to increase interactions with other environmental signals via protein kinase/phosphatase activity and/or metabolic feedback (review in Matsuda et al. 2011). In the present work, we demonstrated that the CII-CPD-PL gene is high-CO_2 -inducible, and this effect was UVR dose dependent (Chapter 4 and 5). The upregulation of the CII-CPD-PL gene was observed in two different irradiation treatments: in chapter 5 cells were exposed to continuous PAB and the upregulation was observed from the beginning of the experiment. Otherwise, in chapter 4 cells were under a photoperiod of different PAB irradiances, and the upregulation was clear only in cultures treated with the highest irradiances, hence highest UVR dose. Additionally, in chapter 5 it was

observed that the same gene showed different expression patterns when two levels of CO_2 were applied.

The analyses performed in order to separate the effects of UVR and CO_2 resulted in two functional-based proportion of transcript fragments (Fig. 6.2). The differential expression obtained under two levels of CO_2 supports its role triggering signal transduction, as CO_2 cannot be only regarded for its effects as a substrate for photosynthesis. However, the specific mechanism acting under different environmental levels of CO_2 needs to be further elucidated. Furthermore, changes in variables may not result in a simple additive response (Boyd & Hutchins 2012), rather, it is expected that they will be synergistic or antagonist (Folt et al. 1999).

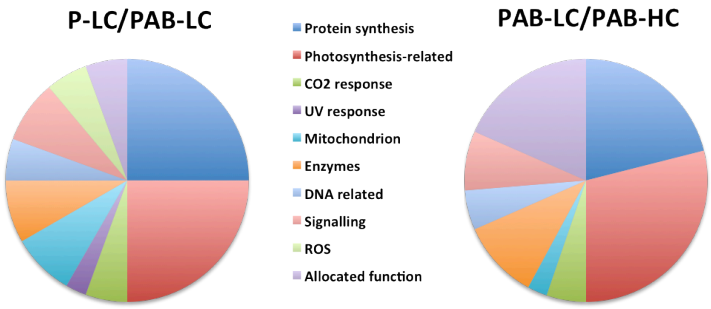


Figure 6.2: Functional-based proportion of transcript fragments in *D. tertiolecta* analysed by means of cDNA-AFLP (Chapter 5).

It is clear that algae with different responses to inorganic carbon concentration will react differently to future changes in atmospheric CO_2 concentrations. Most of the diatoms studied and the haptophyte *Phaeocystis globosa* (the species that often dominates the phytoplankton communities in temperate and polar oceans) live close to inorganic carbon saturation levels for photosynthesis at present (Tang et al. 2001; Riebesell 2004). CO_2 perturbation experiments in pelagic mesocosms with natural plankton communities showed a decrease in the abundance of diatoms when pCO_2 levels were raised (Riebesell 2004). In contrast, coccolithophorids at present CO_2

concentrations have a relative photosynthesis rate of about 50% in comparison to the photosynthesis at saturation levels (Riebesell et al. 2000; Rost et al. 2003). Hence, coccolithophorids could benefit from the enhanced CO_2 levels. However, as mentioned above, higher photosynthesis rates does not necessarily mean a higher growth rate (Beardall & Raven 2004). Falkowski & Oliver (2007) and Riebesell (2004) predicted an increase in coccolithophorids and a decrease in diatoms biomass caused by an increase in stratification that will result in a decrease in the average nutrient availability and, therefore, a reduction in the abundance of large cells. On the contrary, Litchman et al. (2006) predicted a shift towards larger sizes. What has been demonstrated is that elevated CO_2 levels increase malformed coccoliths and incomplete coccospheres which make coccolithophorids prone to predation and photoinhibition, by decreasing the radiation screened out by the coccoliths (Riebesell et al. 2000; Zondervan et al. 2001). In accordance, some studies found that high CO_2 enhanced the detrimental effects of UVR because of the reduced calcification (Wu & Gao 2009; Gao et al. 2012; Xu & Gao 2012).

However, other studies reporting on changes in sensitivity to UVR due to CO_2 concentration showed opposite results. A decrease in sensitivity to UVR under elevated CO_2 conditions has been found in the haptophyte *Nannochloropsis gaditana*. However *Nannochloris atomus* showed no variations on the sensitivity to UVR under the same conditions (Sobrino et al. 2005). The diatom *Thalassiosira pseudonana* acclimated to high CO_2 was more sensitive to photoinhibition by UVR than those under atmospheric levels, but acclimation to UVR partially counteracted the increased susceptibility observed under elevated CO_2 conditions (Sobrino et al. 2008). Similar results of decreasing growth rate and increasing photoinhibition were obtained for the diatoms *Phaeodactylum tricornutum*, *Thalassiosira pseudonana* and *Skeletonema costatum* grown at high CO_2 concentration and under varying levels of solar radiation. On the other hand, Boelen et al. (2011) observed that elevated CO_2 does not significantly affect the photophysiological performance of the dinoflagellate *Karenia brevis*.

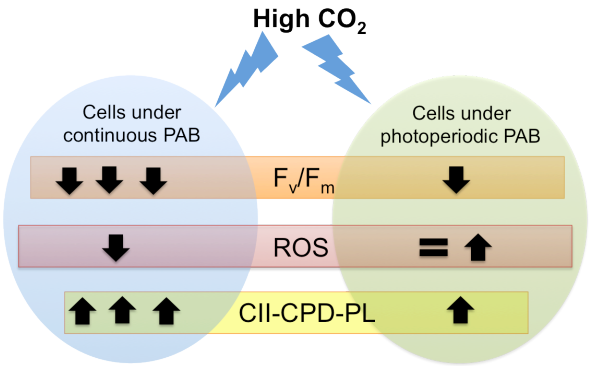


Figure 6.3: Schematic diagram of the different response to high CO_2 of *Dunaliella tertiolecta* under photoperiod and continuous PAB.

These studies did not verify the potential effect of UVR but showed that differences in UVR sensitivity related to external CO_2 concentrations can affect taxonomic composition in microalgae communities. Conforming to this, a recent study reported a significant decrease in primary production in natural phytoplankton diatom-dominated assemblages under increased pCO_2, compared to those under ambient pCO_2, when exposed to upper surface layer light intensities. Other consequence observed in increased pCO_2 assemblages was that diatoms became less abundant and haptophytes increased in relative abundance. Hence, the phytoplankton community shifted and diatoms abundance sharply decreased (Gao et al. 2012).

In this work we have shown that when CO_2 is at ambient levels, *D. tertiolecta* cells survived chronic UVR exposure by activation of DNA repair mechanisms by means of proliferating cellular nuclear antigen (PCNA) and repressor of silencing 1 (ROS1) protein accumulation, and that activation of mitogen activated protein kinase (MAPK)-like proteins mediated the process (Chapter 2). By comparing predictive models (demonstrating that when repair is absent or slow, responses depend on cumulative exposure, and when repair is present, responses are irradiance-dependent (Neale & Kieber 2000) with experimental data, it was confirmed that repair is a key element in

D. tertiolecta resilience (Chapter 3), in which the activation of antioxidant enzymes has a priority role to detoxify and protect the cells by scavenging ROS. The net effect of UVR exposure on phytoplankton survival reflects a balance between damage, repair processes, and the energetic cost of photoprotection (Vincent & Neale 2000).

On the other hand, it seems that *D. tertiolecta* coped better with UVR stress under rising CO_2. Although the physiological response observed depended on other variables different from pCO_2. When pre-acclimation to CO_2 was accompanied by high PAR irradiance (500 µmol quanta m^{-2} s^{-1}) and photoperiod, cells exposed to PAB under photoperiod and high CO_2 accumulated less ROS and increased DNA repair by means of upregulation of the Class II-CPD-photolyase (CII-CPD-PL) gene (Chapter 4). Nevertheless, pre-acclimation to CO_2 together with moderate (100 µmol quanta m^{-2} s^{-1}) continuous PAR irradiance produces, has a main physiological effect, the downregulation of the overall metabolism, as demonstrated by values obtained in carbon fixation, ROS and cell viability. When cultures were exposed to continuous PAB, high CO_2 cells that underwent this low metabolic state did not show stress effects. Meanwhile, low CO_2 –acclimated cells showed increased ROS accumulation and decreased C fixation, but did not die (Chapter 5).

Given that future scenarios of global change could produce detrimental effects on diatoms, the current dominant phytoplankton group, other groups could benefit. It is difficult to predict which species would be winners after the changes, and our results from artificial light treatments must be used with caution.

Our results also suggest that the ability to repair and the consequent tolerance to high dose of UVR could provide advantageous traits to *D. tertiolecta* and other species with similar mechanisms over other phytoplanktonic organisms (namely diatoms) in the photic zone. Furthermore, considering the global change predictions, the CO_2–induced enhancements in the ability to resist and repair UVR damage demonstrated in this work in *D. tertiolecta* further support this statement.

Conclusions

1. *D. tertiolecta* is able to withstand stressful chronic UVR for 6 days without undergoing mortality.

2. The activation of DNA repair mechanisms by means of photoreactivation, and sometimes excision repair, allow the cells to survive to short/medium-term exposure to UVR. Long-term continuous damage accumulation drives the cells into a physiological arrested state permitting that most of them survive.

3. MAPK-like and caspase-like proteins mediate the process of acclimation to chronic UVR. This provide evidence that caspase-like proteins are also involved in the response to stress and do not only act as cell death proteases during intrinsic (non-accidental and/or necrotic) cell death events.

4. *D. tertiolecta* does not follow the general pattern regarding size-depending damage. Despite that it is generally assumed that large cells have slower kinetics of photoinhibition than small cells, the dinoflagellate *Gymnodinium sp.* was much more sensitive to UVR exposure than the smaller *D. tertiolecta*.

5. The effect of UVR on *D. tertiolecta* varied greatly depending on the type of exposure. When cells were exposed under photoperiod they recovered from stress; however, continuous exposure led to a more intense stress and metabolic drop. In *Gymnodinium sp.*, the deleterious effects caused by UVR exposure occurred in a much more rapid manner under continuous exposure to PAB than under photoperiod.

6. The antioxidant superoxide dismutase was not at first line of defence under continuous PAB exposure in *D. tertiolecta*; catalase showed a principal role in ROS detoxification.

7. Stress caused under high PAR irradiance and UVR was alleviated in high CO_2 acclimated cells, allowing *D. tertiolecta* to cope better with excess PAR and UVR.

8. Elevated CO_2 resulted in higher photosynthetic activity, but it is difficult to identify if it accounted for less ROS accumulation (through increased demand of excitons by carbon assimilation) or if higher activity of detoxifying enzymes (catalase and superoxide dismutase) decreased ROS accumulation allowing higher photosynthetic rates.

9. Increased CO_2 induced upregulation of the Class II-CPD-photolyase gene in *D. tertiolecta* exposed to high PAR and UVR, resulting in a drastic decrease in DNA damage.

10. A wide array of genes related to photosynthesis, protein synthesis/ translation, and UVR response were differentially expressed, evidencing active repair during the exposure to stress.

11. The response triggered, both at the genetic and physiological levels, is an adaptive advantage, consequence of a low-steady-state metabolism acquired after long-term elevation of CO_2

12. A downregulated metabolism allows that the excess energy that is not used can be utilised for other processes, such as repair, finally conferring the cells with mechanisms for acclimation and adaptation to stressful environments, leading to better fitness and survival rates.

13. High CO_2 enhanced resilience to high irradiance stress. This reveals traits that may be advantageous to potential changes in marine ecosystem as a result of global change.

Resumen

1. Introducción General

Como resultado las actividades antropogénicas el medio ambiente está experimentando un período de cambios significativos en el clima, denominado **cambio global**. Se ha pronosticado un aumento de la concentración atmosférica de CO_2, alcanzando 1000ppmv al final de este siglo (Stocker et al. 2013; Meehl et al. 2007). El océano absorberá parte de éste CO_2 causando una disminución del pH a 7.77, un incremento del 17% en la concentración de bicarbonato y una disminución del 54% en la concentración de carbonato (Beardall et al. 2009).

La radiación ultravioleta (RUV) puede modificar la tasa de secuestro de CO_2 atmosférico ya que ejerce efectos directos en el fitoplancton. Se prevé que altas dosis de RUV alcancen la superficie del océano (Bischof et al. 2006; Häder 2011), dado que los agujeros de la capa de ozono persistirán hasta 2050 (Mckenzie et al. 2011; Shanklin 2010). Además, el calentamiento global produce un aumento de la estratificación de la columna de agua forzando al fitoplancton a estar mas expuesto a la radiación (Boyd & Doney 2002; Doney 2006).

El **fitoplancton** incorpora aproximadamente 50 mil millones de toneladas métricas de carbono al año, eliminando un cuarto del CO_2 emitido por la actividad humana (Hader 2011; Gao 2012). Ante un **aumento de CO_2** a niveles esperados en un futuro próximo la mayoría de las especies fitoplanctónicas muestras un efecto de un 10% o menos en la tasa de fotosíntesis, aunque existen excepciones (Beardall 2009). Esto se debe a que presentan mecanismos de concentración de carbono y a la actividad de la enzima carbónica anhidrasa (CA) que convierte HCO3- en CO_2 (Giordano et al. 2005). La actividad de dicha enzima se encuentra regulada por la concentración de CO_2 en el medio (Nimer et al. 1996; Burkhardt et al. 2001; Chen & Gao 2004) y por los niveles de RUV (Wu & Gao 2009). Por otro lado, cada vez hay mas pruebas de que el CO_2 activa señales de traducción a través de AMPc, que a su vez regula la expresión de genes (Matsuda et al. 2011).

La **RUV** tiene efectos directos sobre el fitoplancton ya que induce la degradación de biomoléculas relevantes, y además actúa indirectamente a través de la producción de especies reactivas de oxígeno (ROS; Vincent & Neale 2000). Numerosos estudios han demostrado el papel de las ROS ante diferentes estreses, produciendo daños en lípidos, proteínas y ADN (Vardi et al. 1999; Segovia 2008; van de Poll et al. 2009; Janknegt et al. 2009). Además la producción de ROS alrededor del PSII produce la degradación de proteínas de la maquinaria fotosintética (mayoritariamente de la proteína D1; Prásil et al. 1992; Aro et al. 1993), causando fotoinhibición, disminuyendo la actividad fotosintética, el crecimiento y la producción (Helbling & Zagarase 2003; Hader 2011). La RUV y las ROS también producen modificaciones en el ADN, siendo las lesiones mas frecuentes los dímeros ciclobutano pirimidina (CPDs; Lober & Kittler 1977). El alcance de estos efectos es diferente en cada especie fitoplanctonica, por lo que se ha demostrado que la RUV puede producir cambios en la composición de las comunidades (Neale 2001) y en la distribución vertical de las mismas (Llabrés & Agustí 2006; Agustí 2004; Llabrés et al. 2010).

Además, hay que tener en cuenta que el cambio en un grupo de variables no resulta en la simple adición de los efectos de cada variable de forma independiente (Boyd & Hutchins 2012). De este modo, se ha observado que en algunas especies el CO_2 no afecta a la sensibilidad a RUV (Sobrino et al. 2005; Boelen et al. 2011), mientras que en otras disminuye (Sobrino et al. 2008; 2009) o aumenta dicha sensibilidad (Sobrino et al. 2008).

En un medio en constante cambio los organismos perciben señales ambientales y responden a ellas. La traducción de señales vía **quinasas activadas por mitógenos (MAPKs)** es uno de los mecanismos de regulación mas común en eucariotas. Son activadas por gran variedad de estímulos y producen la actvación coordinada de transcripción de genes, síntesis de proteínas, maquinaria del ciclo celular, muerte y diferenciación. Estudios recientes han demostrado la implicación de las proteínas p38 y JNK MAPKs en respuesta a estrés de diferentes naturalezas (Jimenez et al. 2004; 2007; Parages et al. 2013; 2014).

Las microalgas fotositéticas presentas mecanismos que protegen a los componentes celulares frente a las ROS. Sin embargo, es difícil hacer un consenso de las respuestas observadas dadas las características particulares de cada especie analizada.

La **fotosíntesis** es la conversión biológica de la energía lumínica en energía química que se almacena en la forma de compuestos orgánicos de carbono. Sin embargo, diferencias en la fotosíntesis no se traducen necesariamente en diferencias similares en las tasas de crecimiento (Beardall y Raven, 2004).

Se produciendo **fotoinhibición** cuando el fitoplancton recibe intensidades de luz que exceden los requerimientos fotosintéticos. La fotoinhibición y la pérdida de viabilidad son atenuados por mecanismos que permiten la evasión parcial del daño solar. Por otro lado, mediante el proceso de **fotoaclimatación** el fitoplancton puede inducir cambios fotofisiológicos en respuesta a diferentes condiciones irradiancia.

A pesar de estos procesos, inevitablemente se produce fotodaños. Sin embargo, se ha observado la recuperación completa gracias a la síntesis de novo y el reemplazo de las proteínas degradadas, proceso denominado **ciclo de reparación de PSII** (Prasil et al 1992; Aro et al 2005; Nath et al 2013). En condiciones de exceso de luz, la tasa de reparación de PSII es reducida debido a la inhibición de traducción de la proteína D1 por ROS (Nishiyama et al 2006, Takahashi y Murata 2008; Takahashi y Badger 2011).

También existen mecanismos de **reparación del ADN** como la fotoreparación de CPDs mediante enzimas llamadas fotoliasas (McCready y Marcello 2003), y la "reparación oscura" que sustituye bases o nucleótidos dañados en los procesos denominados reparación por escisión de nucleótido o base (NER y BER; Britt 2004).

Está ampliamente demostrado que el estrés ambiental puede reducir la viabilidad celular, y promover diferente clases de **muerte celular programada (PCD)** (Segovia 2008; Jiménez et al 2009). Prácticamente en todos los grupos taxonómicos de fitoplancton se han observado procesos de muerte celular masiva en cultivos. Sin embargo, sólo unos pocos de ellos se asocian a la RUV, y ninguno de ellos al CO_2 o a los efectos interactivos de ambos. Además, se ha demostrado que la muerte celular es un

proceso crucial en las comunidades fitoplanctónicas naturales (Boelen et al 2001; Agustí 2004; Llabrés y Agustí 2006; Llabrés et al 2010).

La naturaleza exacta de las actividades de las **caspasas** no se conoce, pero se han considerado tradicionalmente como proteasas de muerte celular. Sin embargo, el concepto se ha ampliado y ahora se sabe que su actividad es esencial durante la fisiología normal de las células, supervivencia celular, y lo más importante, durante el estrés celular (Zeuner et al. 1999; Segovia y Berges 2005; 2009; Jiménez et al. 2009; Bouchard y Purdie 2011; Franklin et al. 2012; Berges y Choi, 2013). Por lo tanto , la participación de estas proteasas se puede utilizar como un marcador no sólo para la muerte celular, sino también estrés.

Objetivos

La falta de conocimiento sobre la alta tolerancia al estrés en especies como *D. tertiolecta* limita nuestra capacidad de predicción sobre posibles cambios en las comunidades ante condiciones extremas previstas en el futuro. Por lo tanto, esta tesis aborda las siguientes cuestiones principales:

1. ¿Cuáles son los procesos que subyacen a la capacidad de recuperación de *D. tertiolecta* a la exposición crónica a RUV?

2. ¿Cuál es el papel de las ROS en la respuesta (supervivencia *versus* mortalidad) de *D. tertiolecta* en comparación con una especie que muestra un patrón de respuesta diferente (como el dinoflagelado *Gymnodinium sp*.) cuando se expone a RUV, ya sea continua o durante fotoperiodo?

3. ¿Interfiere el aumento de CO_2 en la respuesta a la alta radiación solar (PAR y RUV), al influir en la acumulación de ROS y/o mecanismos de reparación esenciales como la encima fotoliasa y la acumulación delas proteínas PCNA y ROS1?

4. ¿Cómo afectan al transcriptoma los efectos interactivos de UVR y CO_2? ¿Cuáles son las familias de genes expresados predominantemente y cómo esta amplia expresión de genes se relaciona con el desempeño fisiológico de las células?

5. ¿La respuesta obtenida confiere alguna ventaja biológica a *D. tertiolecta*?

2. La supervivencia celular después de estrés por RUV en la clorófita unicelular *Dunaliella tertiolecta* está mediada por la reparación del ADN y la fosforilación de las MAPKs

La radiación ultravioleta induce daño en gran variedad de organismos, aunque las células puede adaptarse para contrarrestar tales daños mediante el desarrollo de mecanismos de reparación o de tolerancia, de lo contrario el destino celular es la muerte celular. Los cultivos de *D. tertiolecta* se mantuvieron a 16 ºC, bajo agitación continua y burbujeo, y una irradiancia de 100 µmol quanta m^{-2} s^{-1}, hasta que llegaron a la mitad de la fase de crecimiento logarítmica. En este momento cada tratamiento comenzó, aplicándose PAR (P) o PAR + UVA + UVB (PAB). Las tasas de flujo UV fueron proporcionados por lámparas QPanel-340 (9.5 Wm^{-2} UVA y 0,45 Wm^{-2} UVB, no ponderado). La exposición a P y PAB crónico se mantuvo durante 6 días y la muerte celular o la supervivencia se compararon con la de las células en PAR. Se estudiaron los efectos de daño celular inducido por la RUV y los mecanismos de señalización y de reparación asociados por el cual las células son capaces de sobrevivir. Para este fin, se estudió la ultraestructura celular, la dinámica de daño del ADN y los mecanismos de reparación. Además, se analizó la implicación de las proteínas MAPK y caspasas en la respuesta al estrés. Sorprendentemente, la RUV no causó muerte celular como se mostró en la ausencia de células positivas teñidas por la sonda sytox-green . El análisis con microscopía electrónica de transmisión (TEM) de la ultraestructura demostró que

las células estaban vivas, pero sujetas a cambios morfológicos tales como la acumulación de almidón, la desagregación de la cromatina y la degradación de cloroplastos. Este comportamiento fue paralelo a una disminución en F_v/F_m y al aumento en la formación de dímeros de ciclobutano pirimidina (CPDs), mostrando las células en PAB valores al final del experimento 10 veces mayores que las células en P. En los cultivos tratados con RUV se observó una alta acumulación de la proteína represora del silenciamiento (ROS1), así como el antígeno nuclear de proliferación celular (PCNA), revelando la activación de mecanismos de reparación del ADN. El grado de fosforilación de JNK (c-Jun N-terminal quinasa) y MAPK p38 fue mayor en las células expuestas a RUV, sin embargo, el proceso contrario se observó con la Ph-ERK (señal extracelular - quinasa regulada). Esto confirmó que es necesario que tanto la JNK y p38 sean fosforiladas para la activación de la respuesta al estrés, así como que la división celular es detenida cuando se desfosforila la ERK. Paralelamente, se detectaron actividades enzimáticas de caspasa, tanto DEVDasa y WEHDase, a pesar de que las células no estaban muertas, lo que sugiere que estas proteasas deben considerarse dentro de un marco más amplio de las proteínas de estrés, en lugar de implicado específicamente en la muerte celular en estos organismos .

Por lo tanto, estos resultados mostraron que las células sobrevivieron a la exposición UVR crónica por la activación de mecanismos de reparación del ADN por medio de la acumulación de las proteínas PCNA y ROS1. Al mismo tiempo, se demostró que la activación de las proteínas MAPK media en el proceso y que las proteínas caspasas también están involucrados en la respuesta al estrés.

3. Efecto diferencial de la exposición a ultravioleta (RUV) en la respuesta al estrés del dinoflagelado *Gymnodinium sp.* y la clorófita *Dunaliella tertiolecta*: mortalidad *vs* supervivencia

Monocultivos de *Dunaliella tertiolecta* (Clorofita) y *Gymnodinium sp*. (Dinoflagelado) fueron expuestos a RUV (PAR, UVA y UVB: PAB) durante 6 días, con dos regímenes diferentes de irradiancia: continua o fotoperiodo. Estas condiciones se eligieron para simular la larga exposición a RUV observada en verano en las latitudes altas (como una condición de extrema), y una situación más típica observado en los sistemas naturales. Los cultivos se mantuvieron bajo un ciclo 12h luz (PAR 400 - 700nm):12h oscuridad con una irradiancia de 100 a 120 µmol quanta m^{-2} s^{-1}. Las células de *D. tertiolecta* se cultivaron a 16 °C con una agitación suave y burbujeo, mientras que las células de *Gymnodinium sp*. fueron cultivadas a 25 °C sin burbujeo o agitación. Después, ambas especies se pusieron bajo dos tratamientos de luz: 1) PAR + RUV (UVA y UVB) bajo fotoperíodo (fotoperíodo PAB) y, 2) RUV y PAR continua (sin periodo de oscuridad) (Continuo PAB). Las tasas de flujo UV fueron proporcionados por lámparas QPanel- 340 que simulan las condiciones naturales de radiación solar que llega a la zona fótica (9,5 Wm^{-2} de UVA y 0,45 Wm^{-2} de UVB, sin ponderar).

En este experimento se comparó la respuesta de estrés de ambas especies de fitoplancton que se centran el estudio en la acumulación de ROS y la desintoxicación por actividad de encimas específicas, así como en el daño del ADN y las actividades enzimáticas de las caspasas. Los resultados obtenidos mostraron que ambos tratamientos de RUV fueron perjudiciales para *Gymnodinium,* pero la exposición a PAB continuo tuvo efectos más dramáticos. Un gran número de lesiones y daños ocurrió durante las primeras horas de exposición a la RUV, y en menos de 24h, *Gymnodinium* perdió su capacidad para eliminar ROS de manera eficiente, se observó foto-inhibición, la detectó la acumulación de daños en el ADN en forma de dímeros de timina, las actividades enzimáticas de caspasas (como DEVDasa) aumentaron considerablemente y las células murieron, como fue determinado por la tinción SYTOX-green. La actividad de la encima superóxido dismutasa (SOD) no cambió significativamente durante el experimento y, aunque la actividad de la catalasa (CAT) aumentó en ambos tratamientos, las células todavía sufría estrés por RUV. Claramente, se pudo concluir que la RUV fue fatal para el dinoflagelado. Sin embargo, en los cultivos de la clorofita el

número de células aumentó independientemente del tratamiento de RUV y la mortalidad se mantuvo baja (menor al 20 %). F_v/F_m mostró una disminución inicial pero luego se mantuvo constante en ambos tratamientos de luz. Finalmente, después de 6 días de exposición PAB continuo se observaron signos de estrés como la acumulación de dímeros de timina y el estrés oxidativo, en paralelo una caída en la actividad de la catalasa. Los resultados obtenidos aquí demostraron que el dinoflagelado *Gymnodinium* fue mucho más sensible y sufrió daños más rápidamente por la exposición a RUV que la clorofita. *D. tertiolecta* fue capaz de hacer frente a la exposición a RUV continua durante 6 días, mientras tanto Gymnodinium sufrió estrés oxidativo, daño en el ADN y la muerte celular después de menos de 2 días de exposición a la radiación UV, incluso durante fotoperíodo . El aumento de la tolerancia a la exposición a RUV del clorofita puede proporcionar ventajas dentro de la zona fótica sobre otras especies de fitoplancton más sensibles. En este estudio se proporcionaron evidencias del papel relevante de la reparación como un componente importante de la resistencia a la RUV en esta especie.

4. El aumento de CO_2 alivia el estrés por alto PAR y RUV en la clorófita unicelular *Dunaliella tertiolecta*

En este experimento se estudiaron los efectos interactivos del aumento de CO_2 y la irradiancia en el rendimiento fisiológico de la clorofita *Dunaliella tertiolecta.* Se eligieron diferentes irradiancias PAR y RUV (UVB+UVA), como una simulación de la radiación solar a diferentes profundidades, y se aplicaron niveles de concentración de CO_2 obtenidos en la actualidad y niveles pronosticados para el año 2100.

Las células se mantuvieron a 16 °C, bajo agitación continua, burbujeo y con una irradiancia de 120 µmol quanta m^{-2} s^{-1} . Antes de los experimentos, cuando los cultivos alcanzaron la fase de crecimiento logarítmica se procedió a la pre-aclimatación a CO_2, para lo que se diluyeron los cultivos a una concentración de 0,2 10^6 células mL^{-1} y se

mantuvieron durante 72 horas bajo fotoperíodo de 16:8h luz:oscuridad con una irradiancia de 500 µmol quanta m^{-2} s^{-1}. Esta pre-acimatación se llevó a cabo a condiciones de pCO_2 atmosférico (390 ppmv) o en condiciones de alto pCO_2 (1000 ppmv) (se denominó "LC" a los cultivos a 390 ppmv, y " HC " a los cultivos enriquecidos en CO_2 a 1.000 ppmv). Después de la pre-aclimatación se expusieron los cultivos a diferentes irradiancias de PAR, UVA y UVB con una proporción que simuló el espectro solar. Las tasas de flujo de PAR fueron elegidas para representar el 100% , 73 %, 36 % y 18 % de la radiación solarque alcanza la superficie del océano, tal y como es experimentada por el fitoplancton a diferentes profundidades de la zona eufótica. Esto correspondió a 1100, 800 , 400 y 200 µmol quanta PAR m^{-2} s^{-1} (llamados I1100, I800, I400 y I200). Las irradiancias de RUV no ponderados (en Wm- 2) asociados a estas tasas de flujo PAR fueron 21,37 UVA:UVB 1.21 para I1100, 13,8 UVA:UVB 0.83 para I800, 6,6UVA:UVB 0,39 para I400, y 1,23 UVA:0.07 UVB para I200.

Con este propósito, se evaluaron la respuesta fotosintética y el estrés oxidativo, en combinación con los mecanismos de reparación del ADN mediante la evaluación de la expresión génica de la fotoliasa y la acumulación de proteína PCNA y ROS1. El tratamiento de CO_2 elevado resultó en mayores tasas de rendimiento cuántico óptimo y efectivo (F_v/F_m y ϕPSII, respectivamente), de transporte de electrones (ETR) y las tasas de crecimiento (µ). El estrés celular de los cultivos en HC se alivió con respecto a los de LC, como fue patente en la disminución de la acumulación en las especies reactivas de oxígeno (ROS). Bajo la irradiancia más alta (I1100) el daño del ADN mostró un aumento de 42 veces en la formación de los dímeros ciclobutano pirimidina (CPD) con respecto a LC a la irradiación más baja (I200). La expresión del gen de la fotoliasa (CII-PCD-PL) se upregulated en HC resultando en una disminución drástica de la acumulación de CPDs, mostrando sólo un 25% de lo observado en LC. La acumulación de la proteína PCNA fue siempre mayor en HC y el patrón de acumulación indicó su participación en la reparación o el crecimiento en función de las dosis de irradiancia. Sin embargo, la acumulación de la proteína ROS1 evidenció que estaba involucrada sólo marginalmente en la respuesta, lo que sugiere que la fotorreactivación era el

mecanismo más relevante para superar el daño por RUV. Los resultados demostraron que el aumento de CO_2 permitió a *D. tertiolecta* hacer frente a alto PAR y al daño inducido por la RUV. Esto se debió principalmente a la menor acumulación de ROS y al aumento de los mecanismos de reparación del ADN, en parte por medio de la regulación positiva inducida por el CO_2 del gen de la fotoliasa. Esto permite pronosticar que, en *D. tertiolecta,* escenarios futuros de cambio global podrían resultar en el alivio del estrés por irradiancia a través de la fotoprotección inducida por CO_2.

5. Expresión génica diferencial y respuesta al estrés por aumento de CO_2 y RUV en la clorófita unicelular *Dunaliella tertiolecta*

En este experimento se estudiaron los efectos interactivos de aumento de los niveles de CO_2 y la RUV sobre la respuesta al estrés de la clorófita unicelular *Dunaliella tertiolecta*, por medio de análisis del rendimiento fisiológico mostrado por las células y de perfiles del transcriptoma. El sistema de burbujeo fue manipulado para lograr dos niveles diferentes de CO_2, correspondiente a la concentración presente (390 ppmV, LC) y a los niveles para el año 2100 (1000 ppmV, HC). Las células fueron pre-aclimatados a estas condiciones pCO_2 durante 72h, y después fueron expuestas a cuatro tratamientos diferentes:1) radiación fotosintética activa (PAR) continua y LC (P-LC); 2) RUV suplementaria continuo (PAR+UVA+UVB) y LC (PAB-LC); 3) PAR continuo y HC (P-HC) y 4) de UVR suplementario continua (PAR+UVA+ UVB) y HC (PAB-HC) durante 6 días. Después de que el final del curso 6 días de tratamiento, los cultivos se transfirieron a condiciones de recuperación en sólo PAR durante 2 días. La irradiancia PAR fue a 120 µmol quanta m^{-2} s^{-1} y las tasas de flujo de UVR fueron 9,5 Wm^{-2} UVA y 0,45 Wm^{-2} UVB (sin ponderar).

En este caso, se evaluaron los efectos de los tratamientos sobre la actividad fisiológica mediante la comparación de la supervivencia celular/viabilidad, el estrés oxidativo, la fotosíntesis y la fijación de carbono. Se realizó una aproximación de la

respuesta inducida en la expresión de genes por la RUV y el CO_2 mediante el uso de AFLP, basado en los perfiles de transcripción. También se realizó un estudio detallado de la expresión de genes relacionados con la fotosíntesis y la reparación del ADN. Los resultados mostraron que HC promovió un "estado de metabolismo bajo" demostrado por valores más de 2 veces mas bajos de viabilidad celular, de acumulación de especies reactivas de oxígeno (ROS), de fijación de ^{14}C, y en las tasas de crecimiento reducidas, en comparación con LC. El efecto nocivo de la RUV, que parecía ser atenuada con por HC, sólo tuvo un efecto claro en F_v/F_m. La RUV y el CO_2 activaron la expresión diferencial de una amplia gama de genes, agrupados en 10 categorías funcionales diferentes, de las cuales las familias de genes dominantes fueron los relacionados con la fotosíntesis, la síntesis de proteínas y la traducción, y la respuesta de RUV. La expresión génica de PsbA (que codifica la proteína D1), los genes del complejo captador de luz de clorofila *ab* (LHCII) y de la anhidrasa carbónica externa (eCA) se downregularon en PAB-HC y upregularon bajo PAB-LC. Por el contrario, la expresión del gen de la fotoliasa (PL) se upreguló bajo PAB-HC. Estos resultados sugieren que la regulación positiva de estos genes en PAB-LC es una consecuencia de la activación de la reparación, necesaria para la sustitución de las proteínas dañadas, que se produce en el metabolismo celular activo habitual en LC. El gen PL upregulated en puntos PAB-HC hacia un metabolismo bajo estado estacionario (el metabolismo downregulated) que permite que el exceso de energía que no se utiliza, se puede utilizar para otros procesos, como la reparación. Además, no se observaron en ninguno de los tratamientos tasas significativas de muerte celular durante todo el período experimental. En este estudio experimental se ha demostrado que la radiación UV y CO_2 activan la expresión diferencial de una amplia gama de genes. Por otra parte, el alto carbono promovió un "estado de metabolismo bajo" y atenuó el efecto nocivo de la radiación UV. Estos datos en conjunto demuestran que la resistencia de las células a la radiación UV se incrementa bajo niveles elevados de CO_2. Las implicaciones ecológicas que esto podría tener, necesita una mayor consideración.

6. Discusión general

La RUV es considerada un factor capaz de causar efectos perjudiciales sobre el fitoplancton (Vincent & Roy 1993). Esto es particularmente relevante cuando se considera el aumento de la radiación ultravioleta-B que alcanza la superficie del océano debido la desgtrucción de la capa de ozono, y agravada por otras condiciones de cambio global (McKenzie et al. 2011; Zepp et al. 2007). Este trabajo ha demostrado que *D. tertiolecta* es muy resistente tanto a la exposición a largo plazo a RUV y a alto PAR: responde al estrés, y puede sobrevivir de forma inesperada por lo menos durante 6 días. Para explicar cómo un alga unicelular fotoautótrofa es capaz de hacer frente a los efectos dañinos de los dos factores de estrés abiótico utilizados, *D. tertiolecta* fue expuesta a diferentes tratamientos y períodos de PAB. Siempre mostró una notable capacidad para sobrevivir, e incluso para recuperar algunas de las funciones una vez que el estrés hubo terminado. Por otra parte, se ha evaluado la interferencia del aumento del CO_2, como principal motor del cambio global, en la supervivencia a PAB y la tolerancia al estrés (tratamientos experimentales resumen en la Tabla 1.1 en la Introducción). A pesar de que el alto PAR y la RUV en ninguno de los experimentos produjeron mortalidad celular significativa, la respuesta fisiológica y los mecanismos de reparación activados no fueron siempre los mismos.

Está ampliamente demostrado que la exposición a la radiación UV disminuye la productividad primaria y biomasa del fitoplancton (Neale et al. 1998; Banaszak y Neale 2001; Leavitt et al. 2003; Bancroft et al 2007). En este trabajo se ha observado que la exposición PAB siempre fue acompañada por una fuerte disminución de los parámetros fotosintéticos, debido a la fotoinhibición causada por la degradación en la maquinaria fotosintética. Sin embargo, la historia lumínica anterior al experimento y el tipo de tratamiento moduló la respuesta de *D. tertiolecta.* Cuando las células fueron tratadas previamente con PAR en fotoperíodo (capítulo 3) sufrieron menos

fotoinhibicion por PAB, en comparación con las células pretratadas con PAR continuo (capítulo 2 y 5). Los resultados para el estrés oxidativo fueron consistentes con estos, de modo que la menor fotoinhibición fue acompañada por una menor acumulación de ROS bajo PAB (capítulo 3). Por otra parte, la acumulación de daño en el ADN provocado por PAB fue mayor en las células pretratadas con PAR continuo (capítulo 2), en comparación con las células pretratadas con fotoperíodo y bajo tratamientos similares (Capítulo 3 y 4). Esto debe atribuirse de nuevo a los efectos deletéreos de la acumulación de ROS. Estos resultados concuerdan con los que demuestran que la sensibilidad a la RUV del fitoplancton se ve afectada por la historia lumínica de las células (MacDonald et al. 2003; Ivanov et al. 2000).

Sin embargo, las predicciones sobre las respuestas a la exposición RUV son complejas debido a la interacción de otros factores, tales como el aumento de CO_2, entre otros. Recientemente, se han obtenido pruebas de que hay genes regulados por tanto CO_2 de alta y baja en el fitoplancton. En el presente trabajo, se ha demostrado que el gen de la PL es CO_2-inducible, y este efecto fue dependiente de la dosis de RUV (Capítulo 4 y 5). Además, en el capítulo 5 se observó que el mismo gen mostró diferentes patrones de expresión cuando se aplicaron dos niveles de CO_2. La expresión diferencial obtenida por AFLP bajo dos niveles de CO_2 corrobora su papel como señal desencadenante de transducción, afirmando que el CO_2 no puede ser considerada sólo por sus efectos como un sustrato para la fotosíntesis. Sin embargo, el mecanismo específico que actúa debe ser estudiada posteriormente.

Ha sido ampliamente demostrado que las diferencias en la sensibilidad a la RUV relacionados con las concentraciones externas de CO_2 pueden afectar la composición taxonómica de las comunidades de microalgas (Sobrino et al. 2005; 2008; Wu & Gao 2009; Boelen et al. 2011; Gao et al. 2012; Xu & Gao 2012). En este trabajo se ha mostrado que cuando el CO_2 no está presente, las células de *D. tertiolecta* sobrevivieron a la exposición crónica a RUV por la activación de mecanismos de reparación del ADN por medio de la acumulación de las proteínas PCNA y ROS1, y que la activación de las proteínas MAPK median el proceso (Capítulo 2). Mediante la comparación de modelos

de predicción se confirmó que la reparación es un elemento clave en la resiliencia de *D. tertiolecta* (Capítulo 3), en el que la activación de las enzimas antioxidantes tiene un papel prioritario para proteger las células contra las ROS.

Por otro lado, pareció que *D. tertiolecta* hace frente mejor al estrés por UVR bajo concentraciones de CO_2incrementadas. Aunque la respuesta fisiológica observada dependía de otras variables diferentes de la pCO_2. Cuando la preaclimatación a CO_2 fue acompañada por una alta irradiancia PAR (500 µmol quanta m^{-2} s^{-1}) y con fotoperíodo, las células expuestas a PAB bajo fotoperiodo y alto CO_2 acumularon menos ROS y la reparación del ADN aumento por medio de la regulación positiva de gen de la fotoliasa (capítulo 4). Sin embargo, la preaclimatación a CO_2 junto con irradiancia continua PAR moderado (100 µmol quanta m^{-2} s^{-1}) tuvo un efecto fisiológico principal, la ralentización del metabolismo en general, como lo demuestraron los valores obtenidos en la fijación de carbono, ROS y viabilidad celular. Cuando estos cultivos se expusieron a PAB continuo, las células a alto CO_2, que fueron las que mostraron este estado metabólico bajo, no mostraron efectos del estrés. Mientras tanto, las células aclimatadas a bajo CO_2 mostraron aumento de la acumulación de ROS y disminución de la fijación de carbono, pero no murieron (Capítulo 5).

Dado que futuros escenarios del cambio global producirán efectos perjudiciales en las diatomeas, actualmente el grupo de fitoplancton dominante, otros grupos podrían verse beneficiados. Es difícil predecir qué especies serían “ganadoras” después de los cambios, y nuestros resultados obtenidos con tratamientos de luz artificial se debe utilizar con precaución. Nuestros resultados sugieren que la capacidad de reparar y la consiguiente tolerancia a altas dosis de RUV podrían proporcionar características ventajosas a *D. tertiolecta* (y otras especies con mecanismos similares) con respecto a otros organismos de fitoplancton de la zona fótica. Por otra parte, teniendo en cuenta las predicciones del cambio global, la fotoprotección inducida por el CO_2 demostrada en *D. tertiolecta* en este trabajo también apoya esta declaración.

Conclusiones:

1. *D. tertiolecta* es capaz de soportar la exposición a radiación UV crónica durante 6 días sin sufrir mortalidad.

2. La activación de los mecanismos de reparación del ADN por medio de la fotorreactivación, y en algunas ocasiones de la reparación por escisión, permite que las células sobrevivan a la exposición a RUV a corto/medio plazo. La acumulación a largo plazo de daños conduce a las células a un estado fisiológico detenido, permitieron el que la mayoría sobrevivan.

3. Las proteínas MAPK y caspasas median el proceso de aclimatación a la RUV crónica. Esto proporciona evidencias de que las caspasa también están involucrados en respuesta al estrés y no sólo actúan como proteasas de muerte celular.

4. *D. tertiolecta* no sigue el patrón general en cuanto a daños dependiendo del tamaño. A pesar de que en general se supone que las células grandes tienen cinéticas de la foto-inhibición más lentas que las células pequeñas, el dinoflagelado *Gymnodinium sp*. fue mucho más sensible a la exposición a la RUV que *D. tertiolecta*, a pesar de ser más pequeña.

5. El efecto de la RUV sobre *D. tertiolecta* varia mucho dependiendo del tipo de exposición. Cuando las células fueron expuestas a fotoperiodo se recuperaron del estrés. Sin embargo, la exposición continua llevó a un estrés más intenso y una caída metabólica. En *Gymnodinium sp*., los efectos deletéreos causados por exposición a la RUV se produjeron de una manera mucho más rápida bajo la exposición continua a PAB que bajo fotoperiodo.

6. La encima antioxidante superóxido dismutasa no fue la primera línea de defensa en *D. tertiolecta* bajo la exposición a PAB continuo; sino que la encima catalasa mostró un papel principal en la detoxificación de las ROS.

7. El estrés causado por la alta irradiancia PAR y la RUV se alivió en las células aclimatadas a alto CO_2, lo que permitió a *D. tertiolecta* lidiar mejor con el exceso de PAR y RUV.

8. El tratamiento de elevado CO_2 se tradujo en una mayor actividad fotosintética, pero es difícil determinar si fue debido a una menor acumulación de ROS (a través de una mayor demanda de excitones por la asimilación de carbono) o a una mayor actividad de las enzimas desintoxicantes (catalasa y superóxido dismutasa) disminuyendo la acumulación de ROS y permitiendo una mayor tasa fotosintética.

9. En *D. tertiolecta* expuesta a alto PAR y RUV el aumento CO_2 indujo la regulación positiva del gen de la fotoliasa, lo que resultó en una disminución drástica de los daños en el ADN .

10. Una amplia gama de genes relacionados con la fotosíntesis, la síntesis/traducción de proteínas y la respuesta a RUV fueron expresados diferencialmente, lo que evidencia la reparación activa durante la exposición al estrés.

11. La respuesta activada, tanto a nivel genético como fisiológico, es una ventaja adaptativa, consecuencia de un estado de metabolismo bajo adquirido después de la exposición a largo plazo a elevado CO_2.

12. Un metabolismo ralentizado permite que el exceso de energía que no se utiliza se puede utilizar para otros procesos, tales como la reparación, otorgando finalmente a las células con mecanismos de aclimatación y adaptación a

ambientes estresantes, lo que lleva a mejores tasas de rendimiento y de supervivencia.

13. El tratamiento de alto CO_2 produce una mayor resistencia al estrés por alta irradiancia. Esto revela características que pueden ser ventajosas para los posibles cambios en el ecosistema marino como consecuencia del cambio global.

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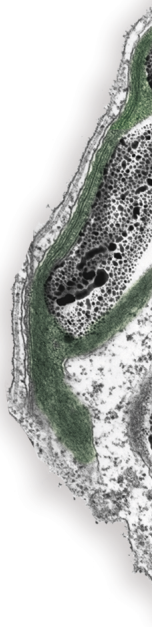

Resilience to UV radiation and high PAR in the marine Chlorophyte *Dunaliella tertiolecta* and the interaction of increased CO_2 in the context of global change

Doctoral Thesis

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