Universitat de les Illes Balears

PHYTOPLANKTON CELL DEATH INDUCED BY SOLAR ULTRAVIOLET RADIATION

TESI DOCTORAL

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Abril 2008

Memòria presentada per Moira Llabrés Comamala per optar al títol de Doctora en el Programa de Ciències Marines organitzat per la Universitat de les Illes Balears (UIB), l'Institut Mediterrani d'Estudis Avançats (IMEDEA) i el Consell Superior d'Investigacions Científiques (CSIC).

Dissertation presented by Moira Llabrés Comamala for the PhD degree in the Programme of Marine Sciences, organized by the Institut Mediterràni d'Estudis Avançats (IMEDEA), Universitat de les Illes Balears (UIB), and Consejo Superior de Investigaciones Científicas (CSIC).

A TODA mi familia
y *amigos*

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Abstract

Increased levels of ultraviolet radiation (UVR) have been reaching the aquatic systems due to ozone loss with many damaging effects in phytoplankton organisms reported. However, the direct cell death induced by UVR in natural phytoplankton populations remained untested. In this thesis it has been examined if solar UVR cause direct cell death in natural phytoplankton organisms and it also has been evaluated its importance for the net growth balance of the phytoplankton populations.

This study has revealed that natural levels UVR causes important cell death in marine phytoplankton organisms from different areas of the Atlantic Ocean, the Southern Ocean and the Mediterranean Sea. It also has revealed the different sensitivity of phytoplankton communities to UVR, with the pico-sized phytoplankton being more vulnerable to ultraviolet radiation than larger phytoplankton.

During this study phytoplankton cell death was determined by the cell digestion assay (CDA), method designed to be applied for tropical and temperate environments. For the application of this method in phytoplankton organisms from the Southern Ocean, a modification of this method was proposed which consisted in the reduction of the incubation temperature.

This work identifies phytoplankton populations in Antarctic waters to be strongly controlled by UVR, with phytoplankton biomasses inhibited by up to 80-90%. We report evidence that the lethality of UVR in pico-phytoplankton communities of the Atlantic Ocean is strongly dependent on the doses of UVR received while for larger phytoplankton communities of Antarctica high UVR intensities, independently of the time of exposure, was the factor determining their sensitivity.

This study has also revealed the strong effect of hydroxyl (OH) radicals on phytoplankton cell death as an indirect effect of ultraviolet radiation. The transference of hydroxyl radical from the atmosphere to surface waters caused

strong phytoplankton cell death in communities from subtropical, temperate and Antarctic waters.

This work identifies *Prochlorococcus* as the phytoplanktonic organism most vulnerable to the effects of ultraviolet radiation with the highest decay rates and shortest half-lives under direct and indirect effects of solar radiation.

The work presented in this thesis remarks the importance of ultraviolet radiation as a factor inducing cell death in phytoplankton organisms and influencing on their net growth balance with the consequences derived for the microbial food web. This study is a contribution to the impact of the increasing UV-B radiation levels reaching the oceanic waters.

Resum

Nivells incrementats de radiació ultraviolada, a causa de la pèrdua d'ozó han estat arribant als sistemes aquàtics, de fet molts dels seus efectes perjudicals en els organismes de fitoplàncton han estat estudiats. En canvi, la mortalitat directa induïda per la radiació ultraviolada en les poblacions de fitoplàncton encara no havia estat analitzada. En aquesta tesi s'ha examinat si la radiació ultraviolada causa mortalitat directa en poblacions naturals de fitoplàncton i també s'ha evaluat la seva importància en el balanç del creixement net d'aquestes comunitats.

Aquest estudi ha demostrat que nivells naturals de radiació ultraviolada causen mortalitat important en comunitats de fitoplàncton de diferent àrees de l'Oceà Atlàntic, l'Oceà Sud i la Mar Mediterrània. També s'ha demostrat la diferent sensibilitat que presenten les comunitats de fitoplàncton front l'exposició a la radiació ultraviolada, essent el fitoplàncton més petit (pico-fitoplàncton) més vulnerable a la radiació ultraviolada que el fitoplàncton de major tamany.

Durant aquest estudi la mortalitat dels organismes de fitoplàncton es va determinar mitjançant el mètode de digestió cel.lular (CDA), mètode dissenyat per esser aplicat a aigües tropicals i temperades. Per poder aplicar aquest mètode en poblacions de fitoplàncton d'aigües antàrtiques es va proposar una modificació a aquest mètode que va consistir en la reducció de la temperatura d'incubació.

Aquest estudi identifica que el creixement de les poblacions de fitoplàncton d'aigües antàrtiques es troba controlat per la radiació ultraviolada amb inhibicions de biomasses per damunt del 80-90%. Reportem evidència que la letalitat de la radiació ultraviolada en comunitats de pico-fitoplàncton de l'Oceà Atlàntic és depenent de la dosi de radiació rebuda, en canvi per comunitats de fitoplàncton de major tamany, com a l'Antàrtida, la intensitat de la radiació, independent del temps d'exposició, és el factor determinant de la seva sensibilitat a l'ultraviolat.

Aquest estudi també ha examinat l'efecte del radical hidroxil (OH) en la mortalitat del fitoplàncton, com a efecte indirecte de la radiació ultraviolada. Hem demostrat que la transferència dels radicals OH des de l'atmosfera fins la superfície de l'oceà causa mortalitat en comunitats de fitoplàncton d'aigües subtropicals, temperades i antàrtiques.

Aquest treball identifica *Prochlorococcus* com a l'organisme de fitoplàncton més vulnerable als efectes de la radiació ultraviolada amb les majors taxes de decaïment i les vides mitjanes més curtes baix els efectes directes i indirectes de la radiació solar.

El treball presentat en aquesta tesi, assenyala la importància de la radiació ultraviolada com a factor que indueix mortalitat en els organismes de fitoplàncton, la seva influència en el balanç del creixement net de les poblacions, així com les conseqüències que es deriven a la cadena tròfica. Aquest estudi és una contribució a l'impacte dels nivells incrementats de radiació ultraviolada que arriben a les aigües oceàniques

General Introduction

Ozone depletion

Solar UV-B radiation on the Earth's surface has increased during the last two decades as a consequence of the degradation of the stratospheric ozone layer (Solomon 2004, McKenzie et al 2003). The trend described of an intensifying, but periodic, anthropogenic-induced loss in stratospheric ozone concentrations, with enhanced UV-B radiation was quite worrying. A worldwide network of ozone observation stations has documented continued ozone reductions over many areas of the globe. In the middle latitudes, since 1979, ozone levels have fallen about 5% per decade when averaged over the entire year (WMO Scientific Assessment, 1999). But depletion was generally worse at higher latitudes and most attention has been given to the ozone hole over Antarctica, with a minimum in the ozone values in October, coinciding with the austral spring. The ozone hole, defined geographically as the area wherein the total ozone amount is less than 220 Dobson Units, has steadily grown in size (up to 27 million sq. km.) and length of existence (from August through early December) since 1984. In October 2000, NASA instruments measured an area of the ozone hole over Antarctica of 29.4 million km2 (http://www.jpl.nasa.gov). Additionally, important ozone losses have also been measured in the Arctic and into the North Temperate Zones (EPA, US Environmental Protection Agency, NASA) with springtime ozone hole in the Arctic expected to grow larger during the coming decades as a result of global warming (Rex et al 2004).

The major factor responsible for the destruction of the ozone layer is anthropogenic emissions of chlorofluorocarbons (CFCs). These gases are photolysed in the stratosphere, thereby releasing reactive chlorine atoms that catalytically destroy ozone (Whitehead et al 2000). Other anthropogenic contributions to ozone depletion may include global changes in land use and the increased emission of nitrogen dioxide as a result of fertiliser applications (Bouwman, 1998).

In spite of the international efforts made to diminish the loss of stratospheric ozone (i.e. reduction of CFCs and other emissions in accordance with the Montreal Protocol signed in 1987), pre-1980 levels of ozone have not been recovered yet (Weatherhead and Andersen 2006). Predicting trends of ozone recovery have been questioned (Shindell et al 1998,Weatherhead and Andersen 2006) due to the emissions of other contaminants able to destruct ozone (such as nitrogen dioxide and new compounds produced for different usages) and the fact that global warming, caused by the accumulation of greenhouse gases in the atmosphere, is contributing to the looses of stratospheric ozone (Shindell et al 1998, WMO scientific assessment 2006). In fact, in October 2006, measurements taken from satellites (US National Oceanic and Atmospheric Administration, NOAA; National Aeronautics and Space Administration, NASA; European Space Agency, ESA) showed the largest average area of the ozone hole over Antarctic ever observed, indicating that the problem of ozone loss is still worrying.

Solar UV radiation effects

The decrease in stratospheric ozone concentrations results in an increase of UV-B radiation reaching the biosphere (Madronich 1992, Crutzen 1992, Smith et al 1992, Kerr & McElroy, 1993). Solar UV-B radiation is known to have a wide range of harmful effects, on freshwater and marine organisms, including phytoplankton (Vincent & Roy, 1993; Cullen & Neale, 1994). Numerous studies have pointed the different effects of UVR on phytoplankton communities, such as inhibition of photosynthesis, genetic damage, inhibition of respiration, deficient nutrient uptake, inhibition of cellular motility and the production of toxic photoproducts including reactive oxygen species (ROS) (Vincent and Neale 2000, Häder et al 1998, Helbling et al 2001). ROS react with biomolecules, such as proteins, lipids or DNA, generating oxidative stress (Mc Cord 2000, Freidovich 1999). One highly reactive ROS, primarily generated as a consequence of the destruction of ozone molecules by UV, is the hydroxyl

radical (OH•). It is also produced in the water from the photolysis of DOM, nitrate and nitrite (Mopper and Zhou 1990, Vaughan and Blough 1998).

Many works have identified environmental UV radiation as an important ecological stress that limits the production of phytoplankton and may influence its growth and distribution (Holm-Hansen et al. 1993, Neale 2001). Phytoplankton organisms display numerous photoprotection and reparation systems to avoid the harmful effects of UVR, such as mycosporine-like amino acids (MAAs), antioxidants like carotenoids, and mechanisms to repair damage to proteins and DNA (Roy 2000, Vincent & Roy 1993) which differed between species. It is assumed that photoprotection and repair systems of phytoplankton organisms would be enough to overcome cell damage caused by UVR, and consequently to avoid cell death. However, the capacity of phytoplankton photoprotection and repair systems to avoid cell death is poorly understood and this feature has not been quantified directly.

Phytoplakton losses and cell death

Despite the considerable contribution of marine phytoplankton to global primary production (e. g. Falkowski 1994) many aspects of the ecology and physiology of these organisms are poorly understood. For example losses of phytoplankton populations are often considered to be caused only by factors such as sedimentation and grazing (Walsh 1983) but only rarely cell mortality has been addressed. However, recent reports of high cell death and lysis in natural phytoplankton (Agustí 2004, Agustí et al. 1998, Agustí et al. 2001, Brussaard et al 1995) indicated that losses of phytoplankton by cell death and lysis were important in the ocean and pointed to cell death as a widespread process. Though, very little is known about the processes causing cell death in unicellular organisms as phytoplankton. Nutrient deficiency, virus infection and low light have been identified as the factors that may cause phytoplankton cell death in the ocean (Suttle 1992, Berges & Falkowski 1998, Geider et al 1993). A recent report, however, identified underwater UVR and PAR as the parameters explaining the vertical distribution of dead pico-phytoplankton cells

in the Central Atlantic (Agustí 2004). The highest phytoplankton lysis rates and percentages of dead cells have been reported at depths receiving the higher irradiances (Agustí 1998, Agustí 2004), suggesting solar radiation as an important stressor causing cell death in oceanic phytoplankton. However whether UVR is able to induce considerable cell death in oceanic phytoplankton has not been tested. Moreover, there is still a lack of studies approaching how UVR affect growth, biomass development and cell death on natural marine phytoplankton, with most of the studies about UVR effects on phytoplankton restricted to laboratory conditions.

Aim and outline of this thesis

The goal of this PhD thesis is to verify whether natural UVR levels in the ocean could generate direct cell death in phytoplanktonic organisms. Quantifying the UVR effect on mortality of natural phytoplankton communities will allow us to verify important aspects of the biology and ecology of the phytoplankton. The quantification of phytoplankton cell death induced by UVR will let to unambiguously establish differences in the UVR sensitivity of different taxonomic and size phytoplankton groups, and the differences as well between phytoplankton communities in the ocean. Also this analysis will permit to evaluate whether UVR may act as an important loss factor for marine phytoplankton in nature, allowing the identification of increased UVB radiation as a general stressor for present oceans.

The high incident ultraviolet radiation in tropical and subtropical regions along with the highly transparent waters of the oligotrophic ocean, allow a deep penetration of visible and ultraviolet radiation in these areas. So one important goal addressed in this work has been to determine the phytoplankton cell death induced by UVR in these waters, by analysing the UVR induced cell death in natural populations from the equatorial, tropical and temperate Central Atlantic Ocean (Chapters 1 and 3) and from the Mediterranean Sea (Chapter 2). Also, although UV radiation levels are lower at higher latitudes, high UV-B levels are reaching polar waters due to the stratospheric ozone

depletion, so UVR effect on growth and cell death of phytoplankton from Antarctic waters has also been addressed in chapter 5. To better address cell death induced by UVR in Antarctic phytoplankton, a modification of the method used to quantify living and dead cells in this thesis, the cell digestion assay (CDA, Agustí & Sánchez 2002) was required (Chapter 4). The cell digestion assay (CDA, Agustí & Sánchez 2002), a cell membrane permeability test, was described for its application in tropical and temperate species and required modifications for being applied in phytoplankton populations growing at cold and polar environments, that was addressed in Chapter 4.

Additionally, the possible lethal consequences of indirect and very toxic products of UVR, ROS, was tested by analysing whether atmospheric hydroxyl radical may induce direct cell death in phytoplankton from a variety of natural oceanic waters. This last objective has been addressed in chapter 6.

In summary, the main objective of this thesis is to improve understanding the effects of ambient solar ultraviolet radiation levels on natural phytoplankton cell death and evaluate their importance for the net growth balance of the phytoplankton populations.

And the following specific goals are pursued within each chapter:

Chapter 1. Test the lethal effect of solar UV radiation on pico-phytoplankton communities from the Atlantic Ocean, quantify the rates of mortality induced, and analyze whether the different groups forming the communities differ in their sensitivity to UVR.

C**hapter 2.** Assess the lethal effect of underwater UVA and UVB radiation on pico-cyanobacterial communities from the Mediterranean Sea, analyze the penetration of experimentally calculated UV lethal doses in cyanobacteria and elucidate the extent of potential population damage underwater.

Chapter 3. Extend the generality of the reported pico-phytoplankton cell mortality induced by ambient UVR and PAR levels along a large

number of pico-phytoplankton communities differing in nutrient availability, geographical location, and state of the community. Elucidate the degree of sensitivity observed in *Prochloroccocus, Synechococcus* and pico-eukaryotes and discuss the consequences of UVR-induced mortality of pico-phytoplankton for the functioning of the oligotrophic ocean.

Chapter 4. Modify the Cell Digestion Assay (CDA), the method used to identify living/dead cells, to allow its application in polar phytoplankton.

Chapter 5. Assess the effect of UV radiation in the growth and cell death of natural phytoplankton populations from Antarctica and elucidate whether UVR exerts a control in the balance of growth and cell death, controlling the biomass of phytoplankton in Antarctic waters.

Chapter 6. Assess the lethality of atmospheric hydroxyl radicals on natural phytoplankton communities from subtropical, temperate and Antarctic waters.

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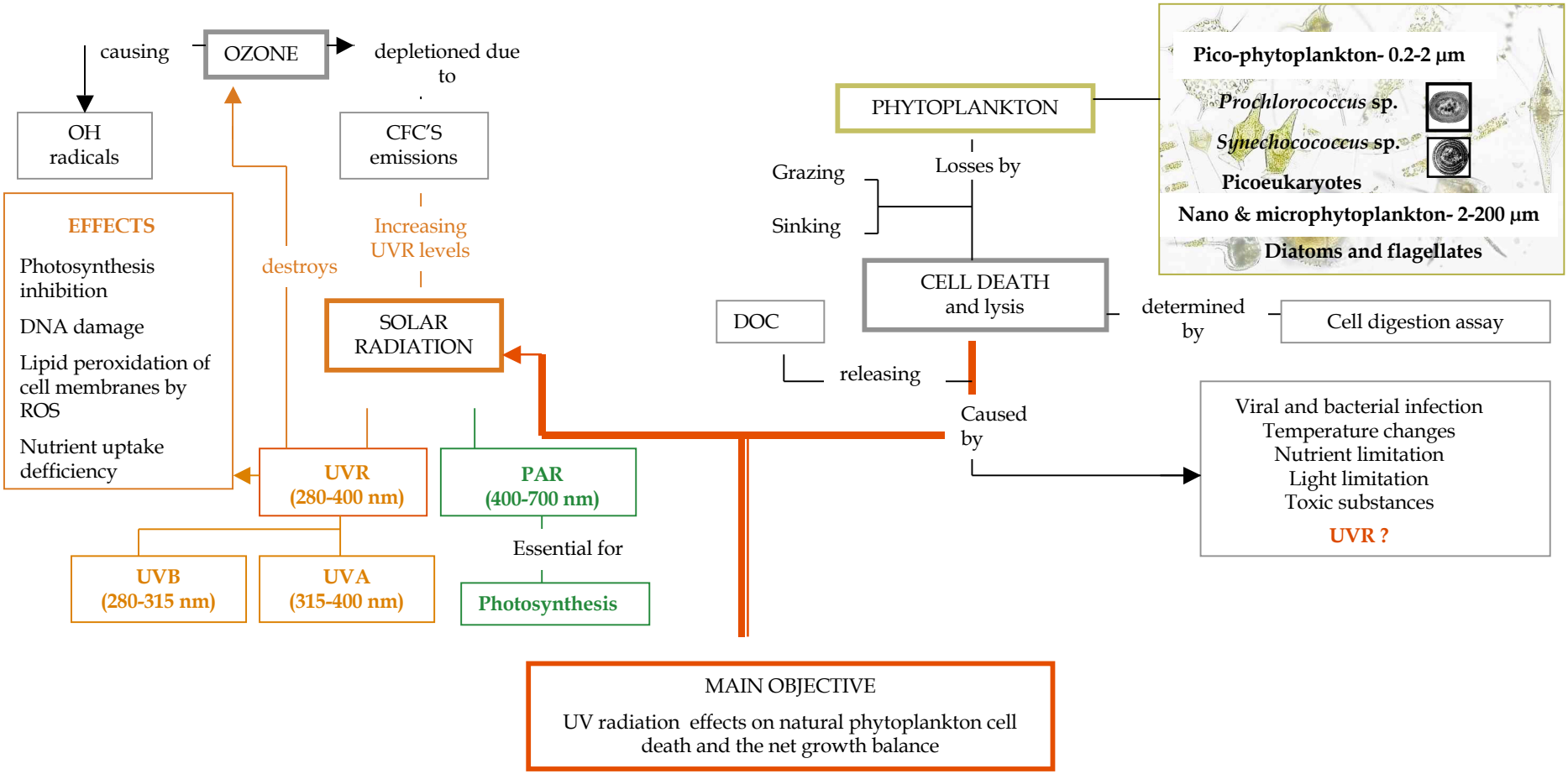

INTRODUCTION SCHEME. It summarizes the concepts needed to better understand the goal proposed in this thesis. Increased SOLAR UV RADIATION levels, reaching the marine systems due to ozone loss, are causing many harmful effects on aquatic organisms. UVR also destroys ozone generating toxic hydroxyl radicals (OH). PHYTOPLANKTON losses by CELL DEATH have been recently recognised important in the ocean, but very litte is known about the causes which are generating it. So the main objective proposed in this thesis is to determine UV radiation as a possible cause of natural phytoplankton cell death.

Chapter 1

Llabrés M. and Agustí S. 2006. **Picophytoplankton cell death induced by UV radiation: evidence for Atlantic communities**. *Limnology and Oceanography*. Vol. 51(1): 21-29.

Chapter 2

Synechococcus and *Prochlorococcus* cell death induced by UV radiation and the penetration of lethal UV doses in the Mediterranean Sea

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Submitted to Marine Ecology Progress Series, 2008

Abstract

Experiments performed with natural communities of picocyanobacteria from the Mediterranean Sea indicated that natural levels of underwater UVR induced important cell death in *Prochlorococcus* sp., although Mediterranean *Synechococcus* sp. appeared to be highly resistant. In treatments where UV-B radiation was excluded, *Prochlorococcus* experienced also high cell death showing short half -life times of 3.01 ± 0.1 h, indicating that UV-A radiation and PAR are able to induce important cell death as well. Underwater measurements indicate that penetration of UVR was considerable in the Mediterranean waters studied. The lethal doses of UVR required to decimate the picocyanobacteria populations to the half, $UVLRD_{50}$, calculated experimentally were related to underwater UVR penetration in the Mediterranean Sea measured during the cruise. UVR doses received daily underwater, equivalent to the experimental LRD_{50} values for *Prochlorococcus* sp. penetrated in the water column from 16 to 28 m depth in sunny days. For *Synechococcus* sp., however, the depth receiving daily $UVLRD_{50}$ was always shallower above 3-10 m depth. The differential sensitivity of the two genera and the considerable penetration of UVR underwater in the Mediterranean Sea suggest that solar radiation could be an important factor influencing the dynamics and distribution of cyanobacterial populations in the surface waters of this oligotrophic sea.

Introduction

Solar ultraviolet (UV, 280- 400 nm) radiation reaching the earth's surface increased due to the depletion of the stratospheric ozone layer as a result of anthropogenically released atmospheric pollutants such as chlorofluorocarbons (CFCs), chlorocarbons (CCs) and organo-bromides (OBs) (Blumthaler et al., 1990; Madronich et al., 1998; Weatherhead & Andersen, 2006). UV exerts a control over aquatic and terrestrial systems as a consequence of the damage that UVR could induce in organisms such as inhibition of photosynthesis, DNA damage, production of reactive oxygen species (ROS), etc. (e.g. Cullen et al., 1992; Vincent & Neale, 2000; Helbling et al., 2001). In order to diminish the UVR effects described above, phytoplankton have a variety of UVR photo-protection systems and different mechanisms to repair the cell damage caused by UVR exposure (Roy, 2000).

However, protective systems and repair pathways are not always sufficient and, in consequence, cell death could be induced by UVR exposure in phytoplankton organisms as demonstrated for picophytoplankton populations from the Atlantic Ocean (Llabrés & Agustí, 2006).

At present, the most harmful effects of UVR are attributed to UV-B radiation, (Rijstenbil, 2001; Buma et al., 2001), but also it has been described that UV-A radiation may induce photoinhibition, pigment degradation and changes in nitrogen metabolism (Kim & Watanabe, 1994; Döhler & Buchman, 1995; Forster et al., 1997). Although the energy yielded per photon is lower in UV-A radiation (315-400 nm) than in the UV-B radiation range, its damaging and inhibiting effects on growth and photosynthesis of aquatic organisms might also be considerable, since it penetrates deeper in the water column than UV-B radiation (Piazena & Häder, 1994). Water transparency to UV-A radiation is high in the oligotrophic regions of the oceans, where UV-A radiation can penetrate several meters into the water column (Tedetti & Sempere, 2006) being a potential source of damage for planktonic organisms. Studies describing the penetration of UVR underwater are still scarce, and only recently Morel et al. (2007) have described the UVR clearest oceanic waters located in the South Pacific gyre, indicating moreover that the penetration of UVR could be deeper than it was theoretically expected from laboratory measurements of pure water (Morel et al. 2007). The relation between UVR penetration, doses received and the damage to planktonic organisms is still not well explored.

In this study we analyzed the lethal effect of natural levels of underwater solar radiation on picocyanobacteria communities of the Mediterranean Sea, and tested whether UVR-A+PAR, after removing UVR-B, may induce cell death on these populations. The lethal UVR doses required to decimate the picocyanobacteria populations in the experiments were calculated. Underwater spectral UV radiation, and underwater PAR measurements were performed during the study and transformed to daily doses penetration to analyze the extent of potential UVR population damage for picocyanobacteria in the Mediterranean Sea.

Methods

UV- radiation experiments were performed on Mediterranean communities sampled during two cruises, EUBAL-II (June 2002), a coastal study on board the R/V Mythilus, and BADE-I (September-October 2003) on board the R/V Pelagia, where an anticyclonic eddy located in the SW Mediterranean Sea was monitored. A total of three experiments were performed, one during EUBAL-II and two during BADE-I. Experiment 1 was done on 25 September 2003 with seawater sampled from 5 m depth at a station located at 37º 1'N, 2º 54'E, and experiment 2 was performed on 8 October 2003, with samples collected from 10 m depth at 37º 1'N, 1º 1'E, during BADE-I. Experiment 3 was conducted during the EUBAL-II cruise on 20 June 2002 with samples collected at 5 m depth at 39º 30' N, 2º 34'E, located in the Bay of Palma (Majorca Island, Spain). Seawater samples were incubated in duplicate quartz and black bottles (100 ml) in incubators on deck with sea-surface re-circulating water to maintain in situ temperature. Quartz bottles allowed all the radiation (UVR+PAR) to pass through and the black bottles shielded off all the solar radiation. In experiments 1 and 2, additional treatments were made to remove UV-B radiation. Quartz bottles were covered with a film (Mylar-D, foil) that absorbed UV-B but allowed penetration of UV-A and PAR (Fig. 1). The total duration of the experiments varied between 5 to 8 h and duplicate samples were taken from each treatment every 2 h, except for the last sample interval which was 3 or 4 h. Sampling intervals differed in experiment 3, as which subsamples were taken at shorter intervals of 15 min, 45 min, 3 h and 5 h from the beginning of the experiment.

Solar radiation received underwater in the incubation tanks was measured in experiments 1 and 2 using an PUVR 2500 Biospherical Instruments radiometer which measures ultraviolet radiation (UVR) at 7 wavelengths: 305, 313, 320, 345, 380, 395 nm. The instrument also has a Photosynthetically Active Radiation (PAR, 400-700 nm) sensor. Natural PAR and UVR were measured in the incubation tanks (sensor was 0.3 m below surface) every half hour during the experiments. UVR values obtained at the different wavelengths were integrated from 300-400 nm to calculate the whole incident UVR regime during the experiments. The same calculation was done for UV-A radiation (315-400 nm) and UV-B radiation (300-315 nm).

During BADE-I, underwater UV and PAR profiles were performed on 6 occasions by using the PUVR 2500 radiometer (Biospherical Instruments). Vertical profiles of pico-plankton abundance and cell viability were also done in 4 of the stations. Picoplankton samples were collected at 8 depths from the surface (5 m) to a maximum of 80 m depth.

The abundance of *Prochlorococcus* and *Synechococcus* cells in the samples from the experiments and the vertical profiles were determined on board by flow cytometric analysis of duplicated fresh samples using a FACSCALIBUR flow cytometer (Becton Dickinson). Picophytoplankton cell viability was determined by using the cell digestion assay (Agustí & Sánchez 2002), a membrane permeability test that discriminate living from dead cells. The abundance of living cells after the cell digestion was quantified on board by using flow cytometer as described in Llabrés and Agustí (2006).

Half-life time ($t_{1/2}$, time required to decline to one-half the initial cell density) and the lethal radiation dose (LRD_{50}, radiation dose required to decrease living cell abundance to one-half of its original value) were calculated for *Synechococcus* and *Prochlorococcus* , as described by Llabrés and Agustí (2006). UVA+B radiation doses were calculated by integrating ultraviolet radiation between 300 to 400 nm, while UV-A radiation doses were calculated by integrating radiation between 315 and 400 nm, and for both UVR and PAR by integrating the cumulative radiation received during the experiments until the time of sampling.

Underwater daily doses of UV-A, UV-B and total UV radiation were calculated from the water column profiles by integrating the radiation values obtained from the single wavelength measurements at each station to the different spectral bands, and integrated as well per day the instantaneous measurements assuming 12 h of day length.

Results

The percentage of transmitting radiation in quartz and plastic materials used in the experiments determined the radiation experienced by the samples. Quartz material allows total radiation (including UV-B radiation) to pass through with approximately 90% transmittance at all wavelengths (Fig. 1). The Mylar-D foil used

filtered out UV-B radiation exhibited a transmittance of 0% between 300 and 314 nm, increasing to 28% at 320 nm and allowing UV-A radiation to pass through at longer wavelengths (Fig. 1).

Maximum values of incident solar radiation received during the experiments were slightly higher on the 25th of September (experiment 1), than on the 8th of October (experiment 2), and resulted in slightly lower UV-B and UV-A radiation doses received during the experiment 2 (Table 1). PAR doses received during experiment 1 were however slightly higher than the PAR doses received by pycocianobacteria during experiment 2 (Table 1).

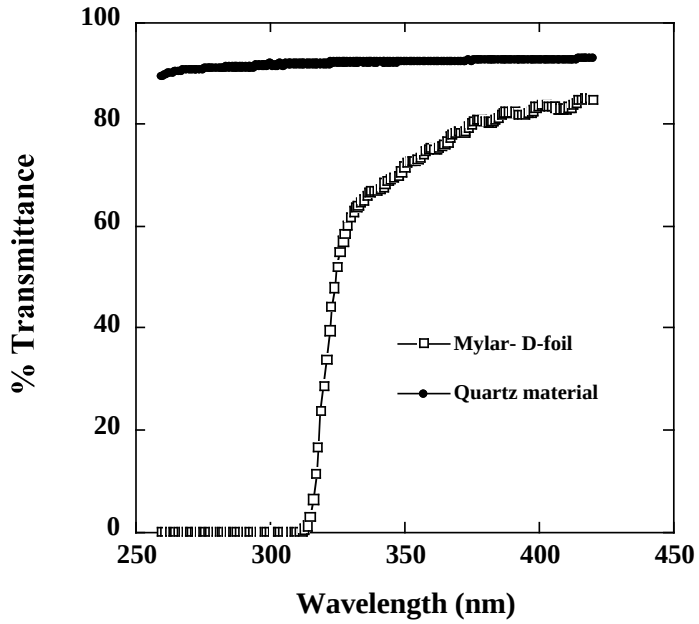

Figure 1. Percentage light transmittance through the quartz and Mylar-D film materials used for incubation under full solar radiation and to shield off UV-B radiation.

Water transparency varied slightly between the stations sampled (Table 1). The extinction coefficients were lower for the 305 nm (between 0.222 and 0.242 m^{-1}) and decreased with increasing the wavelength (Table 1). PAR extinction coefficients varied between 0.066 and 0.072 m^{-1} (Table 1), which represented a variability in the depth of the photic layer (1% of the surface light) between 64 and 70 m. The mix layer depth was shallower than the photic layer and varied slightly between 21.8 and 27.7 m (Table 1).

Table 1. Incident solar radiation during the experiments and underwater light environment at the time of the study in the Mediterranean Sea area sampled during the BADE-1 cruise. Downwelling extinction coefficients (K_d) for spectral UVR and for PAR, and the depth of the upper mixed layer (UPM, m) at 4 stations. Radiation doses represent (KJ m^{-2}) the UVR doses received during experiments 1 and 2 calculated for the different UVR wavelengths measured, and for integrated UVR-B and UVR-A bands, and PAR (mol photon m^{-2}).

	K_d (m^{-1})							**UPM (m)**
	305	313	320	340	380	395	PAR	
22/09/03	0.228	0.194	0.168	0.12	0.071	0.062	0.069	21.8
23/09/03	0.222	0.173	0.16	0.12	0.071	0.062	0.069	22.8
26/09/03	0.223	0.194	0.19	0.186	0.106	0.089	0.072	25.7
7/10/03	0.242	0.221	0.234	0.163	0.091	0.076	0.066	27.7

Radiation doses

	UV (KJ m^{-2})								**PAR**
	305	313	315	340	380	395	Integrated UVB	Integrated UVA	**(mol photon m^{-2})**
Exp. 1	0.24	1.70	2.07	6.21	8.66	8.33	12	568	60
Exp. 2	0.23	1.55	1.88	5.62	7.73	8.54	11	526	25

Picophytoplankton were an important component of the phytoplankton communities with *Synechococcus* sp. being more abundant at the surface than *Prochlorococcus* sp. (Fig. 2). During BADE-1, there were vertical differences in the distribution of picocyanobacteria, with *Prochlorococcus* sp. exhibiting a low abundance close to the surface, increasing until about 30-40 m depth where maximum abundance was reached (Fig. 2) and sharply decreasing in abundance below (Fig. 2). Unlike *Prochlorococus* sp., *Synechococococus* sp. followed a different distribution pattern, with two peaks in the abundance, one close to the surface and a

deeper one at about 40 - 50 m depth. Below this depth, *Synechococcus* sp, abundance decreased (Fig. 2).

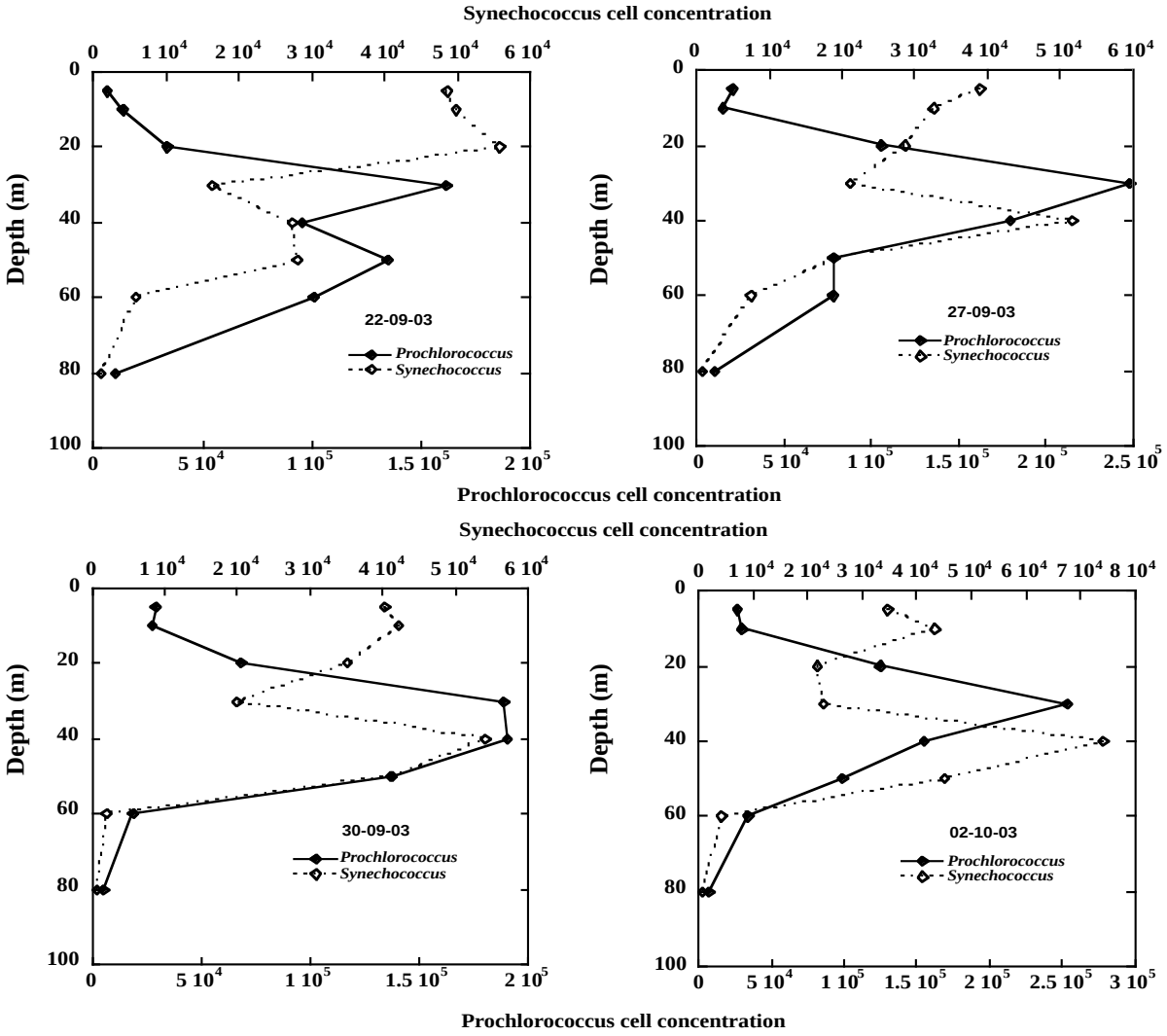

Figure 2. *Prochlorococcus* sp. and *Synechococcus* sp. dynamics in vertical distribution profiles observed in the eddy at different dates in the Mediterranean waters during September-October 2004.

In all experiments, the abundance of living *Prochlorococcus* sp. decreased remarkably following exposure to total solar radiation (Fig. 3). However, *Synechococcus* sp. only showed cell death in experiments 1 and 3, with a moderate decrease in living cells (Fig. 3). In experiment 3, where the frequency of sampling was shorter, the abundance of living *Prochlorococus* sp. strongly decreased after an

exposure to UV for only 45 min (Fig. 3). Dead cells, however, were still present in the total population, as indicated by the fact that total cell abundance did not decrease (Fig. 3). The abundance of the total population strongly decreased at the subsequent sampling interval (3 h of exposure) indicating that cell lysis of dead cells occurred (Fig. 3). The mortality of *Prochlorococcus* sp. was very high, with the abundance of the total population and that of living cells decreasing below the detection limit after an exposure to solar radiation of 4 h (experiments 1 and 3, Fig. 3) and 8 h (experiment 2, Fig. 3).

In treatments without UV-B radiation (UV-A+PAR), cell mortality was similar to that as under full solar radiation (UV-B+UV-A+PAR) for *Prochlorococcus* sp. (Fig. 4A, B). The decrease in the abundance of living cells of *Prochlorococcus* sp. in UV-A+PAR treatments was more pronounced in experiment 1, where the abundance was below the detection limit after 6 h of exposure (Fig. 4A) than in experiment 2 (Fig. 4B). Unlike *Prochlorococcus* sp., *Synechococcus* sp. living cell abundance did not decrease under UV-A+PAR treatments in experiment 1 (Fig. 4C), however, a decrease in the abundance of living *Synechococcus* sp. cells was observed at the end of experiment 2 (Fig. 4D). For both *Prochlorococcus* sp. and *Synechococcus* sp., there was no cell death detected in the dark controls (Fig. 4).

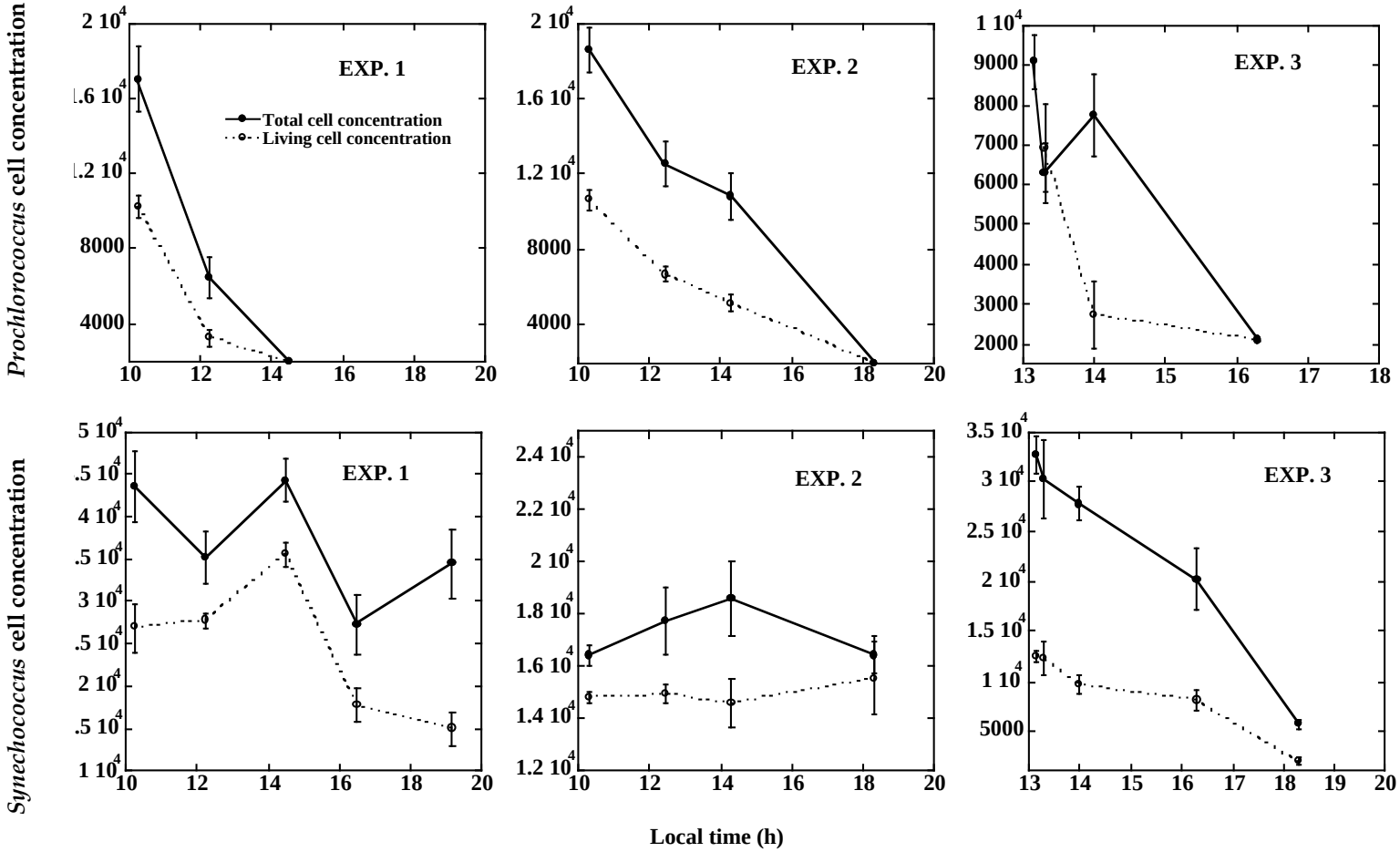

Figure 3. *Prochlorococcus* sp. and *Synechococcus* sp. Total and living cell concentrations of obtained under UV exposure during all the experiments carried out.

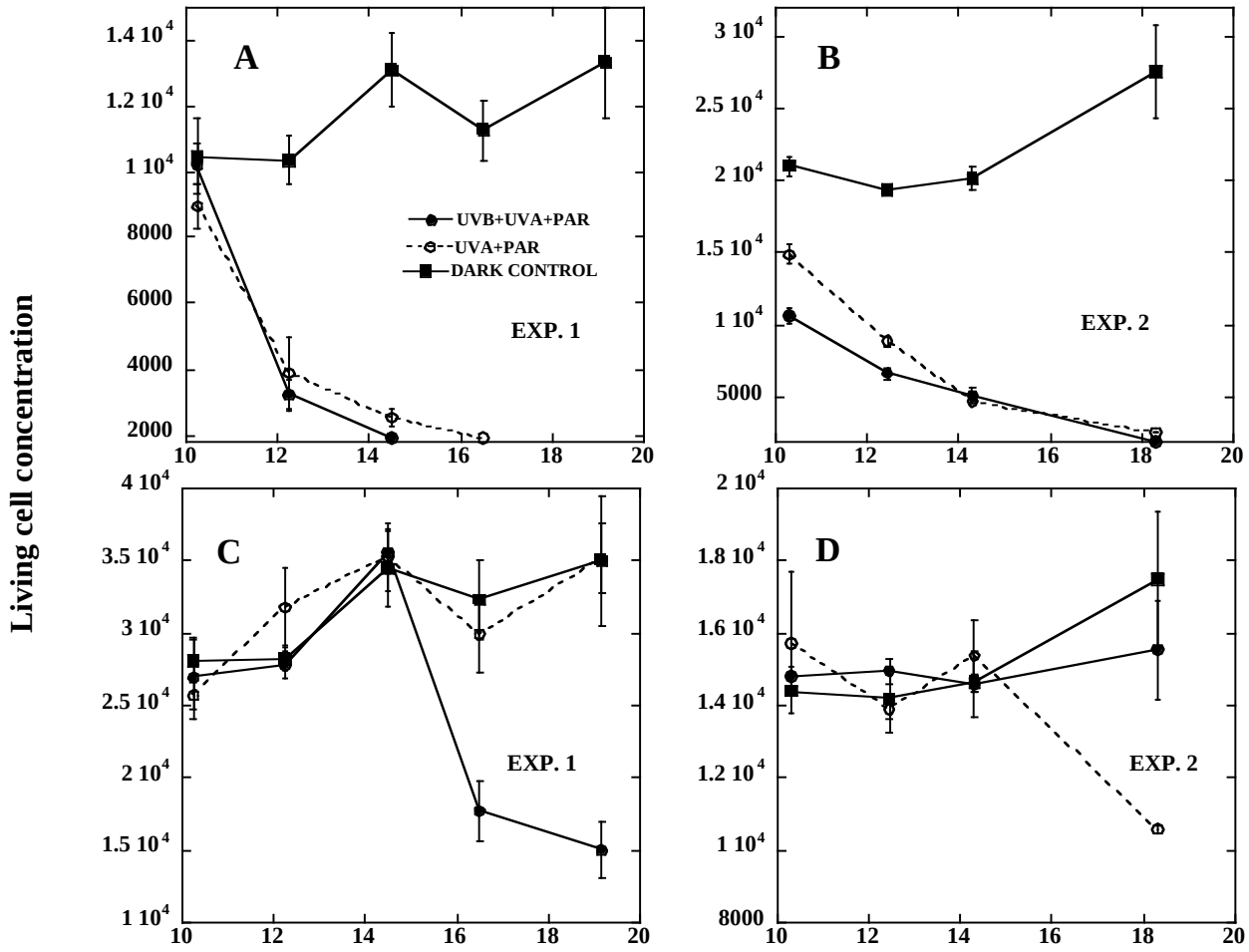

Figure 4. Living cell concentration of *Prochlorococcus* sp. (A, B) and *Synechococcus* sp. (C, D) obtained under full solar radiation, UV-A+PAR and in the dark control treatments during the experiments 1 and 2.

The high mortality observed for *Prochlorococcus* sp. caused very short half-life times for this taxa ranging between 1.8 and 3.3 h when exposed to total solar radiation (Table 2). Moreover, longer half-life times were obtained when shielding off UV-B radiation, with an average half-life of 3.0±0.1 h. The half-life times for *Synechococcus* sp. could be only calculated in some treatments of the experiments, since there was no detectable decay of *Synechococcus* sp. living cells in the other treatments (Table 2). Average half-life time value obtained for *Synechococcus* sp. for full solar radiation was 7.2 h (Table 2), much longer than that obtained for *Prochlorococcus* sp (2.4 h). When UV-B radiation was shielded off, the half-life time obtained for *Synechococcus* sp. in experiment 2 was 14.9 h, also much longer than the value obtained for *Prochlorococcus* sp. in the same experiment (Table 2).

Table 2. Half-life time values, t 1/2 (h), obtained for the three experiments under UV+PAR and UV-A+PAR treatments and lethal UV-A+B, UV-A and UV-B radiation doses (KJ m^{-2}) required to reduce the populations of *Synechococcus* sp. and *Prochlorococcus* sp. to 50% in experiments 1 and 2. Ndect = no detected mortality; (-) = no determined.

Half-life time (t1/2)

	Synechococcus sp.		*Prochlorococcus* sp.	
Exp.	UV+PAR	UV-A+PAR	UV+PAR	UV-A+PAR
1	9.38	Ndect	1.77	2.90
2	Ndect	14.91	3.27	3.13
3	5.1	-	2.12	-
Mean ± st error	7.24 ± 2.14	> 14.91	2.38 ± 0.45	3.01±0.1

Lethal radiation doses (LRD_{50})

	Synechococcus sp.			*Prochlorococcus* sp.		
Exp.	UVA+B	(UV-B)	UV-A	UVA+B	(UV-B)	UV-A
1	686	(15.2)	Ndect	140	(3.2)	230
2	Ndect	(Ndect)	4537	279	(5.8)	205
	Mean ± st error			209± 69	4.5± 1.3	217 ± 13

The lethal radiation doses needed to reduce the living fraction of the population by half, LRD_{50}, for *Prochlorococcus* sp. exposed to full solar radiation varied between 140 and 279 KJ m^{-2} (Table 2). For *Synechococcus* sp. the LRD_{50} was 686 KJ m^{-2} for the full range of solar radiation in experiment 1. When UV-B radiation was shielded off, LRD_{50} for *Prochlorococcus* sp. was 217±13 KJ m^{-2}, much lower than that obtained for *Synechococcus* sp. with 4537 KJ m^{-2} in experiment 2 (Table 2).

Daily underwater UV radiation doses, calculated for the period of sampling of BADE-1 (September 22 to October 7, Fig. 5), indicated that the UV radiation doses, equivalent to the averaged LRD_{50} of 209 KJ m^{-2} calculated for *Prochlorococcus* sp. (Table 2), penetrated to a depth varying from 16 and 28 m in the water column. Moreover, for *Synechococcus* sp. the depth receiving daily UV doses equivalent to the maximum LRD_{50} of 686 KJ m^{-2}, resulting from experiment 1 (Table 2), was more shallow, being above 10 m depth (Fig. 5) during the same period of sampling.

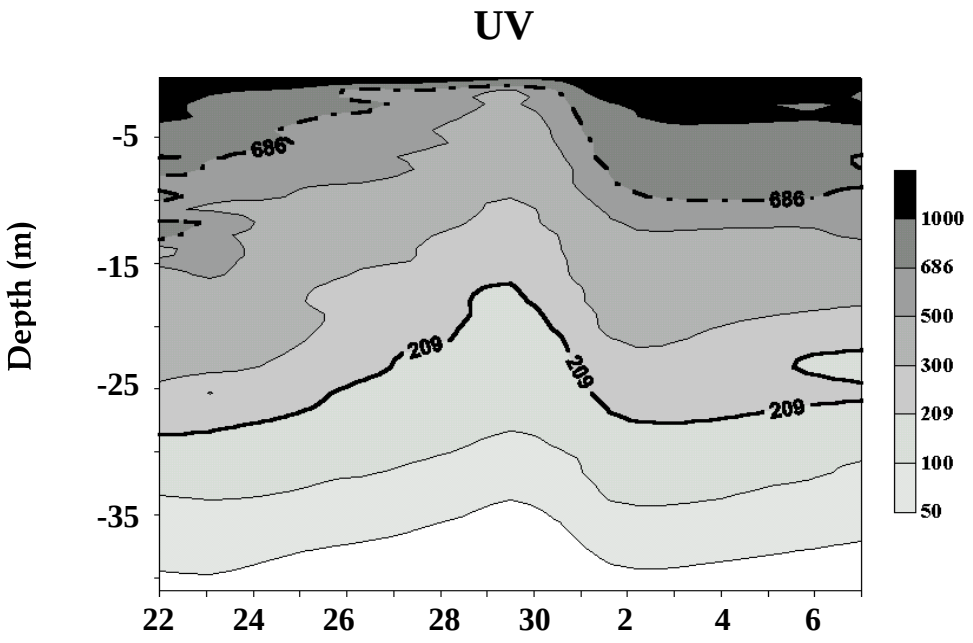

Fig. 5. Daily underwater UV radiation doses (KJ m^{-2} d^{-1}) calculated during the period of sampling during BADE-1 (September 22 to October 7). Solid lines represented the experimental UV-LRD_{50} calculated for *Prochlorococcus* sp. Dotted lines represented the experimental UV-LRD_{50} calculated for *Synechococcus* sp.

Discussion

Our results demonstrate that picocyanobacteria in the near-surface layers of the Mediterranean Sea may be severely affected by exposure to ambient levels of ultraviolet radiation causing cell mortality. The *Prochlorococcus* populations analysed here, as well as those from the Atlantic Ocean examined by Llabrés and Agustí (2006) experienced high cell death in some of the experiments indicating that the levels of photoprotection and repair systems were insufficient to repair the cell damage induced by solar radiation and, as a consequence, cells die off. Since no changes were observed in the living cell abundance of picocyanobacteria incubated in the dark (dark control treatment), the cell losses observed under the light treatments were strictly explained by solar radiation and not by other losses such as grazing.

Prochlorococcus sp. exhibited higher cell mortality when exposed to the treatments including UV-B radiation (full solar radiation) but our results also indicated, however, that UV-A+PAR might also induce considerable cell death. Despite UV-B radiation is considered to cause the most harmful effects in organisms,

including phytoplankton (Rijstenbil, 2001; Buma et al. 2001), adverse effects of UV-A radiation exposure are also well documented. Photosynthesis inhibition by UV-A radiation exposure is reported for both marine and freshwater phytoplankton (Kim & Watanabe, 1994; Helbling et al., 2001), as well as inhibition of the nitrogen metabolism, altering protein structure and functions (Döhler & Buchmann, 1995), other harmful effects as enhancement of lipid peroxidation (MDA production, Rijstenbil 2001) are also reported. In the other hand, UV-A radiation can counteract the adverse effects of UV-B radiation by activating repair processes (Quesada et al., 1995). The present results indicate, however, that exposure to high UV-A+PAR irradiance resulted in an important damage of *Prochlorococcus* sp. demonstrating the adverse effects of UVA radiation on these organisms. Recent work in which UV-A radiation was shielded off, *Prochlorococcus* sp. only exposed to PAR experienced less important cell losses than with UVA (Sommaruga et al., 2005), supporting our conclusion.

Similar to recent results from Atlantic communities (Llabrés & Agustí, 2006), Mediterranean *Synechococcus* sp. showed higher resistance to UV-B radiation indicating that the populations examined from these genera should have better photo-protection or repair systems than *Prochlorococcus* sp. The high sensitivity of pico-phytoplankton to solar radiation has been attributed to the small size of these cells (Llabrés & Agustí, 2006) that should be below the threshold size needed to accommodate sunscreen substances for effective cell protection (Garcia-Pichel, 1994). Differences observed in the sensitivity to UV radiation between *Synechococcus* sp. and *Prochlorococcus* sp. could be better attributed to different cellular properties such as morphology, pigment composition and DNA base content and sequence, as recent genomic studies describe some *Prochlorococcus* strains lacking important genes for DNA reparation (Hess at al. 2001; Dufresne et al., 2005). In contrast, for *Synechococcus*, have specific mechanisms of protection described for other cyanobacteria, as for example, can prevent inhibition of photosystem II by the capacity to rapid gene expression to exchange D1 proteins (Campbell et al., 1998).

The high cell death rates induced by solar radiation in *Prochlorococcus* sp. reported here were induced by high natural levels of solar radiation reaching the experiments equivalent to those able to reach shallow depths, around 0.3-0.5 m

underwater, indicating that only the in situ *Prochlorococcus* sp. populations placed at the upper sub-surface layer, could experience the fast death rates equivalent to those measured in the experiments. The decay rates of *Prochlorococcus* sp. induced by solar radiation in situ should, however, decrease as depth increased and levels of underwater UV decreased. However, longer exposition time of *Prochlrococcus* sp. to reduced levels of irradiance could lead to a similar loss of cells although occurring at a lower rate (Llabrés & Agustí 2006), which imply that UVR doses received are also important for the resulting cell death losses of the population (Llabrés & Agustí 2006). Our calculation of the depth receiving the daily UV radiation doses equivalent to the experimental UV LRD_{50}, represent the depth at which solar radiation could decimate *Prochlorococcus* sp. population to the half in a day.

The Mediterranean waters studied here were clear waters showing photic depths larger than 65 m depth, where UVR penetrated considerably in relation to PAR. For UVR penetration it is often calculated the depth of the layer receiving the 10% of the light at the surface, and this varied from 10 meters for the UV-B (at 305 nm) to 27 m for the UV-A radiation (at 380 nm), which are important when considering that the equivalent 10% layer for PAR corresponded to 34 m. The transparency in the UV-B and UV-A bands obtained for the Mediterranean Sea was equivalent to that reported for other oligotrophic areas as the waters around the Marquesas Islands, in the South Pacific (Morel et al. 2007, Tedetti et al. 2007), but lest clear that the clearest oceanic waters, where Kd coefficients reported are half the values reported here (Morel et al. 2007, Tedetti et al. 2007). Underwater penetration of UV radiation was transformed in this study to doses received underwater in a day, helping to relate the penetration of UV radiation with their potential damage to cyanobacterial populations. The underwater UVR layer able to represent considerable lethal effects for *Synechococcus* sp. populations was restricted to the upper surface, as indicated by the less than 10 m depth receiving the equivalent UV LRD_{50} calculated from the experiments. A different scenario was found for *Prochlorococcus* sp. for which the underwater doses able to decimate the populations to the half penetrated down to 16 m depth, in mid cloudy days, and to 28 m depth on sunny days. This is indicating that UV and solar radiation could exert a tight control over the populations of *Prochlorococcus* sp. in the water column of the Mediterranean Sea. In fact, *Prochlorococcus* sp. populations have

been described to show low abundant populations during the summer time, and tend to be less abundant at the surface waters than *Synechococcus* sp. (e.g. Vaulot et al. 1990; Alonso-Laita et al., 2005).

This UVR reactive layer calculated for phytoplankton will represent the potential maximum layer where LRD_{50} may actuate. The effective UVR doses received underwater by phytoplankton cells would depend also on the mixture regime of the water column. However, if we consider the depth of the UPM observed during this study, the layer at which the daily UV LRD_{50} for *Prochlorococcus* was found, was always within the mixing layer. Nevertheless for *Synechococcus,* potential mixing layer was deeper that the depth receiving the daily LRD_{50}. Thus, potential mixing of the upper water column may help to reduce the potential damage induced for UVR for *Synechococcus*, but will represent a minor aid for *Prochlorococcus*.

In conclusion, picocyanobacterial populations from oligotrophic Mediterranean waters could be severely affected by UVR causing cell mortality. Although UV-B radiation was an important lethal factor, most cell death could be attributed to UV-A+PAR in *Prochlorococcus* sp., which appears to be more sensitive to UVR than *Synechococcus* sp. The transparency of the water column in the Mediterranean Sea allowed a considerable penetration of UV radiation. The underwater UVR layer able to represent considerable lethal effects for *Synechococcus* sp. populations was limited to the upper surface, but represented a much larger layer, down to 28 m in sunny days, for *Prochlorococcus*. Our results point to the importance of UV and solar radiation as a cell loss factor of *Prochlorococcus* in the Mediterranean Sea that could control the dynamics and distribution of its populations.

Acknowledgements

This study was supported by grant REN-2002-10606E/MAR to S.A., funded by the Spanish Ministry of Education and Science, by the Netherlands Science Foundation, Earth and Life Sciences (NWO-ALW, project Nr. #812.03.001 to G.J.H.), and by the project EUROTROPH (Nutrients Cycling and the Trophic Status of Coastal Ecosystems) funded by FP5 of the European Union. M. Llabrés was supported by the project REN2001-5060-E /MAR. We thank the technical personnel and crew of the R/Vs Pelagia and Mythilus for their professional assistance. We are

grateful to E. Alou for sampling assistance and to C.M. Duarte for valuable comments on the manuscript.

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Chapter 3

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Chapter 4

Extending the Cell Digestion Assay to Quantify Dead Phytoplankton Cells in Cold and Polar Waters

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Submitted to Limnology & Oceanography methods, 2008

Abstract

The cell digestion assay (CDA) is an effective method to quantify living/dead cells in complex natural phytoplankton communities. The CDA involves the incubation of fresh samples at 35ºC for 45 minutes for the enzymatic digestion of dead cells. The relatively high incubation temperature has raised concerns as to the applicability of the CDA to phytoplankton samples from polar and cold environments, which could suffer a thermal shock during the process. Here, we examined the applicability of the CDA to phytoplankton growing at low temperatures, and developed modifications and recommendations to reliably use the CDA with polar and cold-water communities. We carried out different tests with polar and temperate phytoplankton species growing in cultures at cold (5ºC) and warmer (18ºC) temperatures, and with natural communities from the Arctic Ocean. Increased cell death when applying the standard CDA method was evident in some of the species tested, when compared with results obtained using alternative, staining membrane permeability tests. We confirmed that the use of a incubation temperature of 25ºC with Arctic communities, and species growing at 5 ºC in cultures results in the complete digestion of dead cells and no significant effect on the percentage of living cells. We, thus, recommend the use of 25ºC as the incubation temperature when applying the CDA to phytoplankton communities inhabiting cold environments.

Introduction

The study of phytoplankton population losses by cell death is increasingly getting importance in the ocean (e.g., Agustí et al. 1998; Kirchman 1999; Agustí and Sánchez 2002). Identification of living and death cells in natural phytoplankton has been prevented in the past by the lack of reliable methods (e.g., Jassby and Goldman 1974). Relatively recent advances in the field of cell biology have, however, provided new insights into the processes associated with cell death. These insights have, in turn, resulted in new concepts and the

development of new methods to quantify cell death (e.g., Darzynkiewick et al. 1994). In particular, the loss of the ability to maintain homeostasis, resulting in increased cell membrane permeability, has been identified as the event that characterizes dead cells (Wyllie et al. 1980; Ellis et al. 1991; Darzynkiewicz et al. 1994). Thus, membrane permeability test has been developed to identify dead cells. Most membrane permeability tests imply staining of cells resulting in different fluorescent labelling of living and/or dead cells. Ambiguities in the interpretation of fluorescent signals by incomplete staining or low brightly living/dying stained cells, and difficulties in the identification of phytoplankton cells against heterotrophic organisms after staining precluded the use of theses techniques to quantify living/dying cells in natural phytoplankton communities.

The cell digestion assay (CDA), a non-staining membrane permeability test (Darzynkiewicz et al. 1994), is an effective method to quantify dead cells in complex natural phytoplankton communities (Agustí and Sánchez 2002). Moreover, the enzymatic cell digestion assay has been applied to a variety of phytoplankton communities from different regions of the Atlantic Ocean (Agustí 2004; Alonso-Laita and Agustí 2006; Llabrés and Agustí 2006), the Mediterranean Sea (Agustí and Sánchez 2002; Alonso-Laita et al. 2005) and for freshwater phytoplankton communities in Florida lakes (Agustí et al. 2006).

The CDA implies incubation of fresh samples at 35 ^{o}C for 45 minutes for the enzymatic digestion of dead cells with Trypsin and DNAse I (Darzynkiewick et al. 1994; Agustí and Sánchez 2002). During the incubation time, the enzymatic cocktail penetrates inside dead cells with compromised membranes digesting them (Darzynkiewicz et al. 1994; Agustí and Sánchez 2002). The temperature assay of 35^{o}C has been tested to have no effect on the viability of the cells studied (Agustí and Sánchez 2002; Agustí et al. 2006). However, the CDA has been mostly applied to tropical or warm temperate phytoplankton communities. The assay has not yet applied to phytoplankton communities growing in cold and polar areas. For those communities, the incubation of fresh samples at 35^{o}C may represent a shock for the cells, due to the contrasting between growth temperature and that of the incubation, precluding the

application of the CDA to quantify phytoplankton cell death in cold and polar waters.

The main objective of this study was to analyze whether the cell digestion assay (Agustí and Sánchez 2002) is applicable to phytoplankton communities growing in cold and polar environments and, therefore, to provide the modifications and suggestions for the application of the CDA method to quantify the abundance of dead cells within phytoplankton communities growing at low temperatures. We first analyzed whether the assay temperature of 35ºC could influence the results obtained when applying the CDA to species growing in cold (5ºC) and polar waters. The efficiency of the CDA was tested here comparing the results obtained by applying this method with the viability results obtained using membrane permeability tests based in staining techniques. To assure the accuracy of the measurements tested, we provide new data and compile data from the literature of phytoplankton % living cells obtained by using both the CDA and staining techniques. We then optimize the assay, and search for an adequate incubation temperature to get the CDA applicable to phytoplankton communities growing in cold and polar environments.

Materials and procedures

Reagents- Dnase I (Deoxyribonuclease I from bovine pancreas, DN-25 Sigma-Aldrich Co), Trypsin (from bovine pancreas, T9201 Sigma-Aldrich Co), both prepared in HBSS medium (Hanks' balanced salt solution, H1387 Sigma-Aldrich Co) were used to determine cell viability by the cell digestion assay, and fluorescein diacetate (F7378, Sigma-Aldrich Co) was used to determine viability by staining.

LIVE-DEAD *Bac*light Bacterial Kit, L-7007 (to determine viability in diatoms by staining) was purchased from Molecular Probes. The *Bac*light ™ Viability Kit used contained component A with SYTO 9 dye (1.67 mM) and Propidium Iodide (1.67 mM) both in DMSO (Dimethyl sulfoxide) solutions; and

component B, with SYTO 9 dye (1.67 mM) and Propidium Iodide (1.83 mM), both in DMSO. Beads solution (to count samples at the flow cytometer) was prepared with 1µm green fluorescent latex microspheres, purchased from Polysciences Inc.

Phytoplankton cultures- In this study we used phytoplankton cultures growing in F/2 medium (rich in silica for diatoms), F/2-Si (without silica) and Prov for flagellates, with continuous light and at two different temperatures of 18º and 5ºC. The cultures included the temperate species *Thalassiosira* sp., *Dunaliella* sp., *Heterocapsa* sp., and *Chlorella* sp, the cold growing species *Thalassiosira nordenskioeldii* (CCMP 1017; 42º 40'N, 69º 36'W), and *Rhizosolenia setigera* (CCMP 1330; 41º 54'N, 70º 48'W) and the polar species *Heterocapsa arctica* (CCMP 445, isolated from Baffin Bay), *Chlamydomonas_cf* sp. (CCMP 681, isolated from Palmer station, Antarctica), *Polarella glacialis* (CCMP 1383, isolated from Mc Murdo Sound, Antarctica), and *Ochromonas* sp.(CCMP 1899, isolated from Mc Murdo Sound, Antarctica) obtained from Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Maine, USA). Temperate species grew at 18ºC and cold and polar species at 5ºC. Some species usually growing at 18ºC were also left to grow at 5ºC such as *Chlorella* sp., *Heterocapsa* sp., and *Thalassiosira* sp. The cell dynamics in the cultures was followed quantifying the total cell abundance every 2 days in species growing at 18ºC and every 5-7 days in species growing at 5ºC, because in the latter ones the growth rate was very slow.

Assesments of cell death

Cell digestion assay (CDA)- This non-staining membrane permeability test (Darzynkiewicz et al. 1994) was modified for phytoplankton cells by Agustí and Sánchez (2002). In this assay, cells are briefly exposed to the enzymes DNAase I and Trypsin, which enter the cytoplasm of cells with damaged plasma membranes resulting in their entire digestion, while having no measurable effect on the viability, morphology or function of live cells (Darzynkiewicz et al. 1994; Agustí and Sánchez 2002). The digestion of dead cells occurs as a

consequence of fragmentation and hydrolysis of DNA by DNAse I and peptide hydrolysis by Trypsin, which penetrates the damaged cells. The digested cells are undetectable by microscopic observation and lose any fluorescence signals and are removed from the population (Darzynkiewicz et al. 1994; Agustí and Sánchez 2002). The protocol was the following (scheme 1): two hundred µl DNAse (800 µg DNAse ml^{-1} HBSS) were added to replicated (2 or 3 replicates) 1 ml samples in assay tubes and incubated at 35ºC for 15 minutes. Then, 200 µl Trypsin 2 % (in HBSS) were added to the same samples and they were incubated at 35ºC for 30 minutes. Replicated culture samples to be used as blanks were incubated for the same time and temperature. At the end of the incubation samples were immediately quantified by using a flow cytometer or the light microscope. The cells counted after the cell digestion assay represented the living cells in the community, whereas the cells counted in untreated samples represented the total population (living and dying cells). The percentage of living (or viable) cells was calculated as the ratio between the concentration of cells after the enzyme digestion, and the cell concentration of untreated samples, which represented the total (dead plus living) cell population.

Staining methods--LIVE/DEAD BacLight™ Bacterial Viability Kit - The staining method used to determine viability in diatoms, as a function of the membrane integrity of the cell, was the BacLight ™ Kit (scheme 1), as diatoms showed clear and brightly staining with this method. The use of this staining method instead of FDA was chosen since living diatoms cells stained with FDA often result in low fluorescence signals (e.g. Agustí and Sánchez 2002, Garvey et al 2007) BacLight ™ Kit is a double staining method (it has two nucleic acid stains, Syto 9 and Propidium Iodide), which is considered more clear than a single staining, since both dead and living cells are simultaneously stained by two fluorochromes, avoiding ambiguity. When used alone, the SYTO 9 stain labels all cells in a population — those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates only cells with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. Duplicated 1 ml samples were stained with 0.2 µl

of BacLight ™ Kit, component B, for 10-15' in the dark. Finally samples were counted under the epifluorescence microscope. When illuminated with blue light, only cells with compromised membranes (dead cells) were stained by PI and fluoresced red, whereas
cells with intact membranes (live cells) were stained by SYTO 9 and fluoresced green (Lee and Rhee 1997; Decamp and Rajendran 1998). A total of 200-300 cells were counted per replicate so from the number of cells counted as green or red we calculated the percentage of living or dead cells as the ratio between green or red counts and the total cell population.

FDA- Fluoresceine diacetate (FDA), a substrate for the intracellular enzymes esterases, was used as a stain to quantify viability for other groups except diatoms (scheme 1). The product of FDA hydrolysis, fluorescein, yields a high green fluorescence when excited with blue light. FDA is hydrolyzed upon penetrating viable cells, leading to the accumulation of fluorescein within live cells, which is not possible in cells with compromised membranes (Diaper et al. 1992, Jochem 1999, Geary et al 1998, Rotman and Papermaster 1966). 40-80 µl, depending of the species, of FDA solution 2 mM (0.0416 g in 50 ml of acetone 100%) were added to duplicated 1 ml samples. After 10-15' in the dark, samples were counted under the epifluorescence microscope; longer staining times may imply the production of fluorescein by the dissolved esterases accumulated in the culture medium that may increase the fluorescent background in the sample. So a total of 200-300 cells per sample were counted and from the number of cells counted as green (live cells) we calculated the percentage of living cells as the ratio between green counts and the total cell population.

Cell counts- Once the CDA was applied. Cultures of *Thalassiosira* sp., *Dunaliella* sp., *Heterocapsa* sp., *Chlorella* sp. and *Chlamydomonas_cf sp.* were counted by flow cytometry (FACSCalibur Becton Dickinson) fitted with a 488 nm laser and a photomultiplier for forward-scattered light detection. An aliquot of a calibrated solution of 1 µm diameter fluorescent beads was added to the samples as an internal standard for the quantification of cell concentration. The fluorescent scattering signals of the cells and beads were used to detect the populations and

to differentiate them from the fluorescent beads. Culture phytoplankton samples with larger cell sizes, such as *Thalassiosira nordenskioeldii* and *Rhizosolenia setigera*, were counted under inverted light microscope (Axiovert 200, from Zeiss Co). Samples were quantified using 0.5 ml sedimentation cuvettes. A variable number of fields were counted until we had between 150-200 cells accumulated at list. *Heterocapsa arctica, Polarella glacialis, Ochromonas* sp. were first filtered onto black polycarbonate 0.8 µ filters and then counted by fields under epifluorescence microscope fitted with a blue light filter (Axiovert 200, Zeiss Co.).

Cell counts - Once the staining methods were applied. For the most part of species we put a drop of stained sample on a slide, then we covered it with a cover-slide and we proceeded to count the samples under the epifluorescence microscope (Zeiss Axioplan). This allowed us to observe cells in function of their autofluorescence and the features of the different stains as indicated above. In tests done with the flagellates *Heterocapsa arctica, Polarella glacialis, Ochromonas* the stained samples were filtered onto black polycarbonate filters and also counted under the epifluorescence microscope (Zeiss Axioplan).

Natural phytoplankton communities- Some tests were done *in situ* in the Arctic Ocean on communities sampled during the ATOS-arctic cruise, June 29- July 27 on board the R/V BIO Hespérides. Communities were sampled from the ocean surface (5 m) using Niskin bottles inserted in a CTD-rosette system. Two communities were analyzed from two positions in the arctic. Community 1 was sampled at 80º 29'N, 16º 9'E and was dominated by diatoms of the genera *Thalassiosira* (represented by two species of different size) and *Chaetoceros* sp. Community 2 was sampled at 80º 23'N, 12º 11'E, and was also dominated by diatoms of the genera *Nitzschia* sp. and *Chaetoceros* sp.

In the two communities, the CDA incubated at 25ºC and the staining technique Bac-Light Kit used also to quantify the proportion of living cells were applied simultaneously. Just after sampling, 2 ml DNAse (400 µg DNAse ml^{-1} HBSS) were added to duplicated 10 ml samples in assay tubes and incubated at 25ºC for 15 minutes. Then, 2 ml Trypsin 1 % (in HBSS) were added to the same

samples and they were incubated at 25ºC for 30 minutes as indicated above. Duplicated samples to be used as blanks were incubated for the same time and temperature. At the end of the incubation samples were immediately quantified on board by using an inverted light microscope. Community 2 was inoculated in F/2 medium and preserved at 5ºC until the application of the test at the laboratory.

Parallel to the CDA, duplicated 2 ml samples were stained with 0.4 µl of BacLight ™ Kit, component B, for 10-15' in the dark. After this time, samples were counted under the epifluorescence microscope and living and dead cells were identified as described above. For both communities, only the most abundant phytoplankton species were analysed.

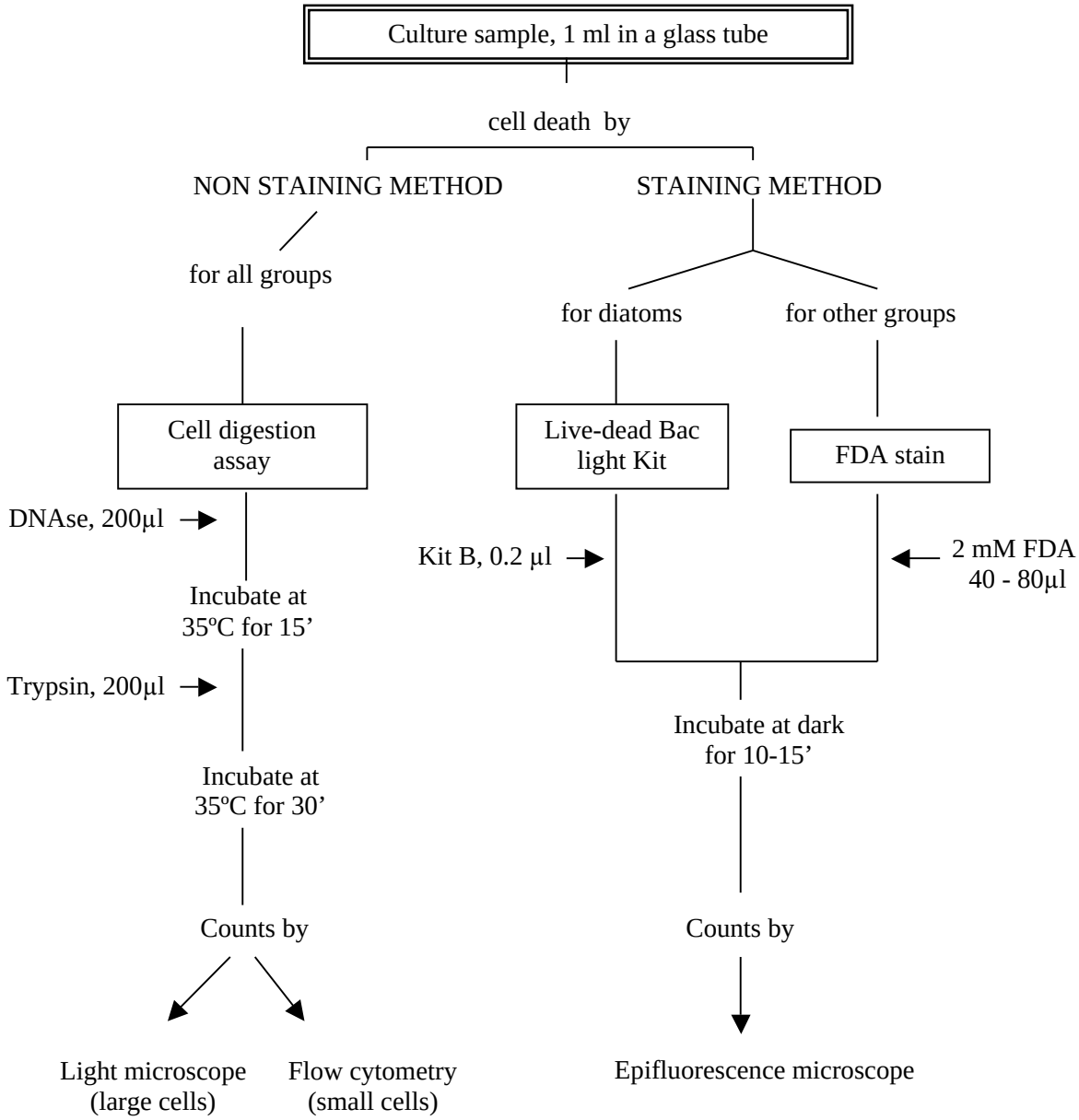
Assessment

Accuracy of cell death methods

The results from the cell digestion assay were compared to those obtained using the FDA and Bac-Light ™ Kit staining methods. For this comparison we compiled data from the literature of several studies in which cell death was determined simultaneously with the CDA and other staining methods. We found a total of 23 data from both in the field (Agustí et al. 2006) and in cultures (Agustí and Sánchez 2002; Agustí 2004). In order to complete the data obtained from the literature additional tests of viability were carried out with phytoplankton cultures in this study. The cultures used were *Thalassiosira* sp., *Dunaliella* sp., *Heterocapsa* sp., *Chlorella* sp. growing at 18ºC, including data from the stationary and exponential growth phases. In the tests the cell death of phytoplankton cultures was determined by the enzymatic cell digestion and by the staining methods Bac-Light ™ Kit for diatoms and FDA for the other groups.

The percentage of living cells values obtained for the comparison goes from 20 to 100% with most of the data presenting percentages above 50% (Fig. 1). A positive and significant relationship between the cell digestion method and the staining methods was found ($R^2 = 0.93$, $p < 0.001$, Fig. 1), with a slope value of 1 (1.02 ± 0.05, Fig. 1). The strong correlation observed indicated the similarity in

the results of viability using the different methods tested, allowing us to test whether the application of the CDA to phytoplankton growing in cold and polar temperatures may result in increased cell death.



Scheme 1. Steps followed to determine the effect of 35ºC incubation assay temperature of the CDA on species growing at 5ºC.

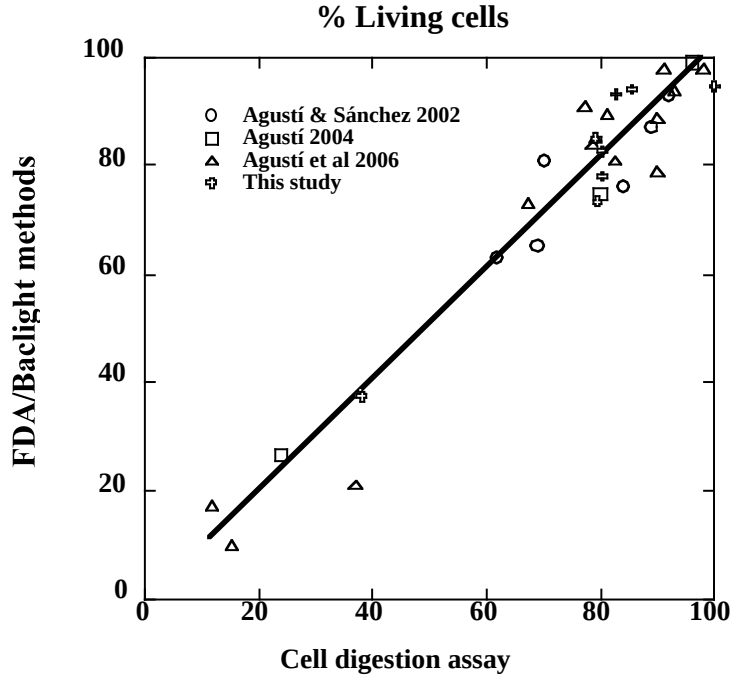


Fig. 1. Relationship between the percentage of living cells obtained applying the cell digestion assay (CDA) and the staining FDA or Baclight™ KIT methods. The different symbols represent data from the literature and from this study. The solid line represents the linear regression equation.

Effects of 35ºC incubation assay temperature on species growing in cold and polar environments

To determine if 35ºC (temperature of the CDA protocol) could affect species inhabiting cold and polar waters we performed some tests in which we compared the viability obtained by the CDA and by the staining method (see scheme 1). Several viability tests were performed in the diatoms *Thalassiosira nordenskioeldii*, *Rhizosolenia setigera*, *Thalassiossira* sp. (fig. 2) and in *Chlorella* sp., *Heterocapsa arctica*, *Chlamydomonas_cf* sp., *Polarella glacialis* and *Ochromonas* sp., all growing at 5ºC. In some of the tests done in diatoms we obtained similar percentages of living cells (Fig 2), with no significant differences between methods ($P > 0.05$) (Fig. 2). However, a significant lower percentage of living cells was obtained when applied the CDA in test 3 done in *Thalassiosira nordenskioeldii* (T-student test, $P = 0.04$), in test 2 done in *Rhizosolenia setigera* (T-

student test, P = 0.004) and in test 1 done in *Thalassiossira* sp (T-student test, P = 0.01, Fig.2). Furthermore, flagellates tested showed significant differences between methods (T-student test, P <0.05, Fig. 2), although *Heterocapsa arctica* showed higher percentages of living cells when applied the CDA (Fig. 2). These results suggest that incubating species from cold and polar waters at 35ºC may deviate the % of living cells obtained.

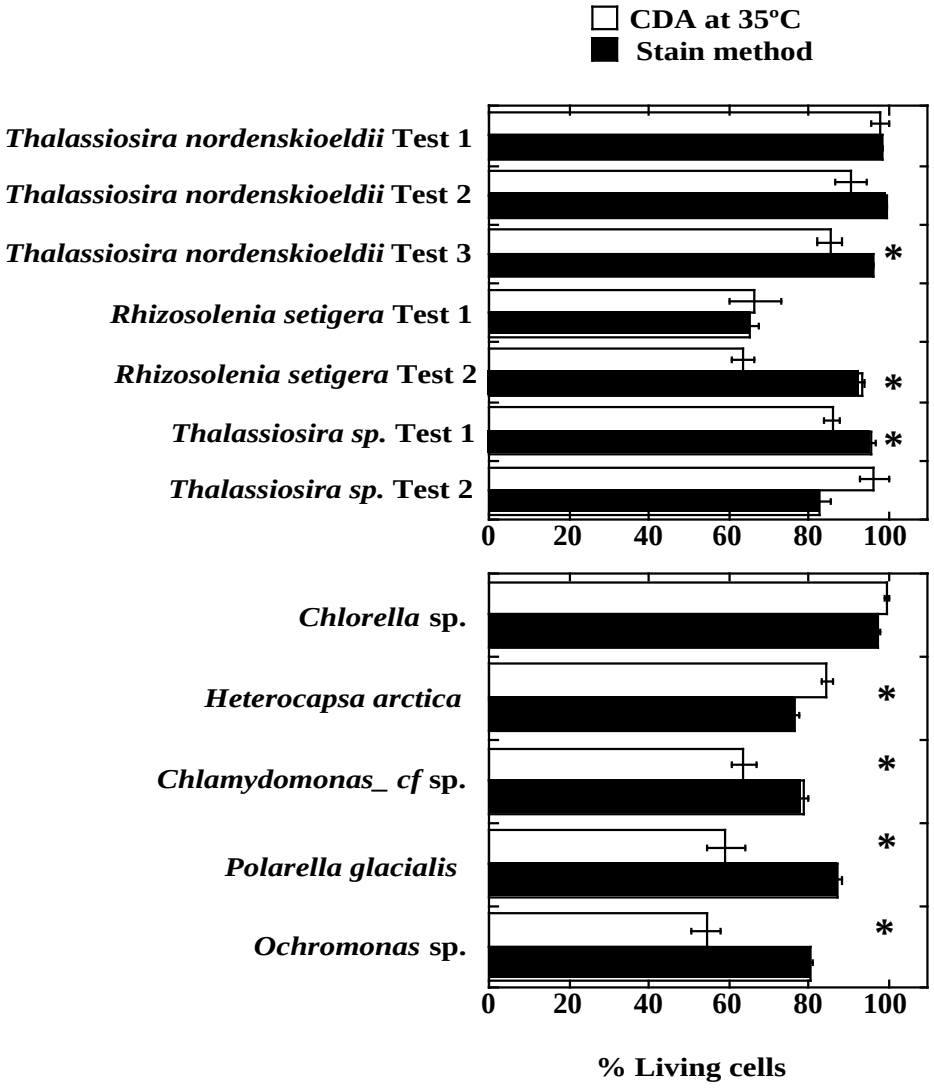


Fig. 2. Percentages of living cells obtained by the CDA at 35ºC (empty columns) and by staining methods (solid columns) applied to phytoplankton species growing at 5ºC. Asterisks indicate significant differences (Student test, P <0.05).

Feasibility of the cell digestion assay at different temperatures

One of the solutions to keep away from the problems described about the application of the CDA to cold growing phytoplankton species could be reducing the incubation temperature to reduce the thermal shock. Some comparisons on the effect of the CDA incubation temperature have been already described in the literature (Table 1), showing no differences between results obtained with cell digestion assay run at 35ºC and at 25ºC (Table 1). There were also no significant differences in the efficiency of the cell digestion assay when conducted at 20ºC or 37ºC (Table 1). A similar comparison was performed during this study (Scheme 1), with simultaneous CDA tests run at 35ºC and 25ºC in all the species growing at 5ºC in cultures (Scheme 1). We obtained similar percentages values, with no significant differences, between temperature treatments in most of the tests done ($P > 0.05$, table 1). However in some of the test performed, a significant lower percentage of living cells was achieved at 35ºC than at 25ºC, as observed for *Rhizosolenia setigera*, *Thalassiosira* sp., *Polarella glacialis* and *Ochromonas* sp. ($P < 0.05$; Table 1), for other polar species as Chlamydomonas sp. a lower percentage of living cells was also achieved at 35ºC than at 25ºC, although the difference was no significant (Table 1).

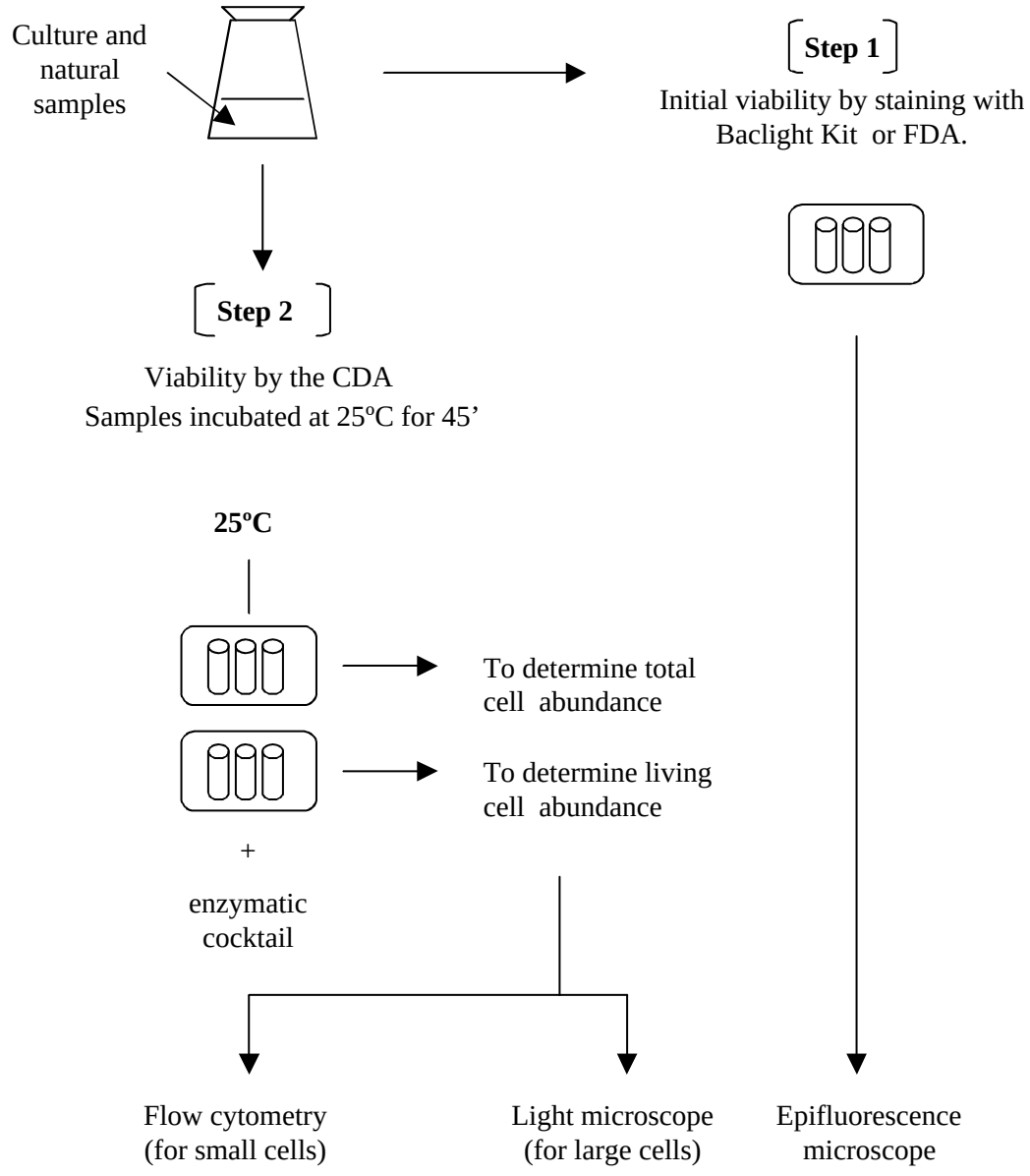
Table 1. Phytoplankton percentages of living cells (mean ± SE) obtained when running the CDA method at a variety of incubation temperatures (37ºC, 35ºC, 25ºC, and 20ºC) observed in this study and in data from the literature. Nd, means test not determined. The symbol (-) was used to indicate not significant (P > 0.05) differences between treatments and the symbol (+) was used to indicate significant (P <0.05) differences.

Species	Temperature of incubation				Significance	Reference
	37ºC	35ºC	25ºC	20ºC		
Scenedesmus sp.	Nd	65.2 ± 14.6	62.9 ± 18.1	Nd	−	Agustí et al. 2006
Scenedesmus sp.	Nd	75 ± 14.3	69.6 ± 13.8	Nd	−	Agustí et al. 2006
Flagellates	Nd	42.9 ± 5.5	53.4 ± 11.2	Nd	−	Agustí et al. 2006
Flagellates	Nd	45 ± 2.9	50 ± 9.6	Nd	−	Agustí et al. 2006
Pennate diatom	Nd	39.7 ± 4.4	42 ± 3.2	Nd	−	Agustí et al. 2006
Pennate diatom	Nd	58.4 ± 7.2	56.6 ± 11.3	Nd	−	Agustí et al. 2006
Thalassiossira sp.	74.5 ± 0.5	Nd	Nd	83.1 ± 6	−	Agustí & Sánchez 2002
Dunaliella sp.	55 ± 7.1	Nd	Nd	67 ± 2	−	Agustí & Sánchez 2002
Heterocapsa sp.	84.6 ± 9	Nd	Nd	94.6 ± 2.6	−	Agustí & Sánchez 2002
Phaeocystis sp.	66.2 ± 0.8	Nd	Nd	81 ± 6	−	Agustí & Sánchez 2002
Chlorella sp.	79.5 ± 7.5	Nd	Nd	91.5 ± 0.3	−	Agustí & Sánchez 2002
Phaeodactylum tricornutum	85.5 ± 1.3	Nd	Nd	90 ± 1.6	−	Agustí & Sánchez 2002
Synechococcus sp.	60.5 ± 4.5	Nd	Nd	63 ± 2.5	−	Agustí & Sánchez 2002
Thalassiosira sp	Nd	85.9 ± 2	94.5 ± 2	Nd	+	This study
Thalassiosira sp	Nd	93.3 ± 3.7	88.8 ± 1.4	Nd	−	This study
Thalassiosira nordenskioeldii	Nd	97.8 ± 2.1	92.8 ± 4.3	Nd	−	This study
Thalassiosira nordenskioeldii	Nd	90.4 ± 4.2	100 ± 0	Nd	−	This study
Thalassiosira nordenskioeldii	Nd	85.2 ± 3.3	95.8 ± 2.5	Nd	+	This study
Chlorella sp.	Nd	99.6 ± 0.3	92.6 ± 4.2	Nd	−	This study
Rhizosolenia Setigera	Nd	66.6 ± 6.8	69.7 ± 9	Nd	−	This study
Rhizosolenia Setigera	Nd	63.4 ± 2.9	90.2 ± 1	Nd	+	This study
Heterocapsa arctica	Nd	84.8 ± 1.4	80.2 ± 1.7	Nd	-	This study
Chlamydomonas_cf sp.	Nd	63.7 ± 3.1	72.1 ± 1.6	Nd	-	This study
Polarella glacialis	Nd	59.2 ± 4.9	96.8 ± 2.2	Nd	+	This study
Ocromonas sp.	Nd	54.3 ± 3.6	76.7 ± 1.7	Nd	+	This study

To determine if using 25ºC as the temperature of incubation for the cell digestion assay is efficient for phytoplankton growing at cold, we carried out some tests in which we determined the cell viability of phytoplankton cultures by the cell digestion assay at this temperature and by the staining method. The cultures tested were growing at 5ºC and they were *Thalassiosira* sp., *Thalassiosira nordenskioeldii*, *Chlorella* sp., *Rhizosolenia setigera*, *Heterocapsa* sp., *Heterocapsa arctica*, *Chlamydomonas_cf* sp, *Polarella glacialis*, *Ochromonas* sp. This comparison also included the tests performed on the natural communities from the Arctic examined. To carry out these tests we followed three basic steps (scheme 2). In Step 1 in replicated samples of phytoplankton cultures we determined the initial viability by the staining method (see scheme 1). In step 2 we determined the viability by the cell digestion assay (see scheme 1) but in this case the temperature of incubation was 25ºC not 35ºC (temperature of the protocol). Replicated samples were incubated at 25ºC, three samples were used to determine total cell abundance, and the other three were treated with enzymes to determine the living cell abundance (scheme 2). While samples for the enzymatic cell digestion were incubating, the samples proceeded from step 1 (treated with the staining method) were counted under the epifluorescence microscope (scheme 2). We have tested that stained samples could be maintained in dark and cold for two hours without loosing fluorescence or loose of cells. Once the period of incubation finished, samples that followed the cell digestion method were counted by flow cytometry or light microscope (scheme 2).

For the phytoplankton species growing at 5ºC analyzed, the percentages of living cells obtained by the cell digestion method run at 25ºC and by the stain method did not differ significantly ($P > 0.05$, Student-T test, Table 2). Only two of the tests showed significant differences (*Rhizosolenia setigera*, *Polarella glacialis,* $P = 0.02$, Table 2), in which the percentages of living cells obtained with CDA were higher, suggesting a small reduction in the enzymatic digestion of dead cells. For the five polar species examined in the natural communities sampled in the Arctic Ocean the results were similar, not significant differences were observed between the CDA run at 25ºC and the staining method ($P > 0.05$,

Student-T test, Table 2). The results obtained are indicating that the use of 25ºC as assay temperature to determine cell viability by the CDA in phytoplankton communities growing in cold and polar environments, avoid the problems derived by the large thermal difference observed when using the temperature of the standard protocol (35ºC).



Scheme 2. Steps followed to compare the cell viability obtained by the CDA using 25ºC as assay incubation temperature, with those obtained independently by staining methods applied in natural phytoplankton communities from the Arctic Ocean and in phytoplankton species growing in cultures at 5ºC.

Table 2. Percentages of living cells for phytoplankton cultures growing at 5ºC and for natural communities obtained by applying the CDA at the assay temperature of 25ºC, compared with the results obtained using alternative staining membrane permeability tests (as described in Scheme 2). Probability values were derived from Student-T test.

Species	Cell digestion assay	Staining method	Probability (p)	Origin
Thalassiosira sp.	94.5 ± 2.7	96.1 ± 1.3	0.55	Culture: exponential phase
Thalassiosira sp.	88.8 ± 1.4	82.9 ± 2.6	0.21	Culture: stationary phase
Thalassiosira nordenskioeldii	92.8 ± 4.3	98.7 ± 0.24	0.24	Culture: exponential phase
Thalassiosira nordenskioeldii	100 ± 0	99.5 ± 0.16	0.2	Culture: exponential phase
Thalassiosira nordenskioeldii	95.8 ± 2.5	96.2 ± 0.36	0.8	Culture: exponential phase
Chlorella sp.	92.6 ± 4.2	97.5 ± 0.5	0.32	Culture: exponential phase
Rhizosolenia setigera	69.7 ± 3	65.7 ± 5.2	0.58	Culture: exponential phase
Rhizosolenia setigera	90.2 ± 1	83.2 ± 1	0.02	Culture: stationary phase
Heterocapsa sp.	95 ± 1.7	93.5 ± 0.4	0.41	Culture: exponential phase
Heterocapsa arctica	80.2 ± 1.7	76.7 ± 0.8	0.13	Culture: stationary phase
Chamydomonas_cf sp.	72.1 ± 1.6	78.4 ± 1.6	0.06	Culture: stationary phase
Polarella glacialis	96.8 ± 2.2	87.4 ± 1.2	0.02	Culture: stationary phase
Ochromonas sp.	76.7 ± 1.7	83.6 ± 1.3	0.14	Culture: stationary phase
Large *Thalassiosira* sp. (Arctic)	54 ± 5.6	55.1 ± 4.4	0.8	Natural community: *in situ*
Small *Thalassiosira* sp. (Arctic)	73 ± 7.5	77 ± 1.5	0.6	Natural community: *in situ*
Chaetoceros sp. (Arctic)	74.2 ± 8.5	67.2 ± 13.4	0.7	Natural community: *in situ*
Nitzschia sp. (Arctic)	77.3 ± 1.9	81.2 ± 0.85	0.14	Natural community
Chaetoceros sp. (Arctic)	89 ± 3.2	86.1 ± 1.5	0.45	Natural community

Comments and recommendations

The tests and proves performed indicated that the quantification of living/dying cells in phytoplankton species growing in cold and polar waters could be approached by using the cell digestion assay, by simply reducing the incubation temperature. The tests performed indicated that some cold and polar phytoplankton species showed, when incubated at 35ºC (the standard assay temperature of CDA), increased cell death as a consequence of a thermal shock representing a reduction in the percentage of living cells between the 8 and 28%. Since significant increase in cell death was detected in some of the tests, the use of 35ºC incubation when applying the CDA to phytoplankton growing at low temperatures will be not appropriate. We recommend the use of 25ºC as

the temperature of incubation when applying the CDA to phytoplankton communities growing in cold and polar environments. The use of 25ºC for the enzymatic incubation was observed to show well accuracy when compared with staining viability tests, suggesting the completed digestion of dead cells during the assay run. However, we recommend users to perform in their polar or cold waters samples previous tests, consisting in the comparison of the results obtained using the CDA (incubated at 25ºC), and the results obtained using an independent staining technique, to check their agreement. The tests performed in this study extend the use of the cell digestion assay to a larger range of environments to quantify phytoplankton cell death.

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Chapter 5

Impacts of UV irradiance on growth, cell death and the standing stock of Antarctic phytoplankton

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Submitted to Global Change Biology, 2008

Abstract

We performed a series of experiments with Antarctic natural communities exposed to underwater natural levels of solar radiation in order to quantify the effect of ambient levels of ultraviolet radiation (UVR) on phytoplankton growth, cell death and their balance. Treatments in which UVR was excluded showed a high increase of biomass with chlorophyll a values reaching values as high as 22 μg l^{-1}, 9 times larger than initial values; in contrast chlorophyll a values remained low at the end of the experiments under treatments with full solar radiation. Phytoplankton growth rates were also inhibited by UVR, increasing up to 5 times in excluded UVR treatments. Antarctic phytoplankton communities also experienced important cell death with percentages of dead cells, within the communities, decreasing when UVR was filtered. Phytoplankton populations appeared to be strongly controlled by UV with biomasses inhibited by up to 80-90%. A positive relationship between the percent inhibition of phytoplankton biomass and the daily ambient UVR was obtained, implying an 80% biomass reduction when mean daily UVR doses exceeded 450 KJ m^{-2} d^{-1}. Increased UVR levels over Antarctica reduce phytoplankton growth rates and cause cell death, and consequently are reducing the phytoplankton stock, which should have important consequences for the food web in Antarctic waters.

Introduction

Stratospheric ozone levels, essential to protect organisms from excess ultraviolet radiation, significantly declined due to emissions of antropogenic chlorinated compounds until the mid-1990's (Staehelin et al. 2001), with the largest rates of depletion near the poles, particularly over Antarctica during the austral spring (Solomon, 1999). The loss of ozone over Antarctica has resulted in an important increase of UVB levels with negative impacts on Antarctic marine ecosystems (Smith et al 1992, Prézelin et al 1994, Buma et al. 2001). In spite of the efforts made to diminish the loss of stratospheric ozone

(i.e. reduction of CFC's and other adverse emissions since Montreal Protocol), pre-1980 levels of ozone have not-been recovered as yet, and the future trajectory toward this recovery involves considerable uncertainties (Weatherhead and Andersen, 2006). Thus, significantly enhanced UV-B ratiation (UVBR) reaches Antarctic surface waters, particularly during ozone hole episodes, and will likely continue to do so for several decades (Weatherhead and Andersen, 2006).

The increased UVBR over Antarctic waters has been reported to suppress oceanic productivity by 6-12 % (Smith et al 1992). Evidence of stress induced by UVR in Antarctic photosynthetic plankton has been reported on the basis of various indicators, as the inhibition of photosynthesis (Smith et al 1992, Prézelin BB et al 1994, Helbling et al 1994), the formation of cyclobutane pyrimidine dimmers in DNA (CPDs, Buma et al. 2001; Prézelin et al 1998), and the synthesis of micosporine-like aminoacids (MAA's, Karentz et al 1991, Villafañe et al 1995, Carreto et al 1990). The growth and survival of phytoplankton cells may be also strongly affected by the enhanced UVBR levels in Antarctica (Davidson et al. 1994), affecting the cell density and biomass of Antarctic populations. Studies examining the impact of increased UVBR levels on Antarctic phytoplankton population growth *in situ* are still few (Davidson and Belbin 2002, Nunez et al 2006), and the impact of UVR on phytoplankton cell mortality has not yet been tested. As a consequence, the effect of *in situ* UVR on the balance between the growth and death of natural phytoplankton populations has not been tested.

The main goal of this study is to investigate the effect of UV radiation in the demographic balance, between population growth and cell death of natural Antarctic phytoplankton populations. In addition, we examined the differences in the responses observed among phytoplankton groups in an effort to assess how UVR could affect the dominance of different groups, thereby shaping phytoplankton community structure in Antarctic waters.

Methods

Experimental design

Five UV experiments were carried out in Antarctic waters. Three experiments (experiments 1, 2 and 3) were carried out in the Spanish Antarctic Base Juan Carlos I (Livingston Island, South Shetlands Islands) during ICEPOS-1 expedition in January-February 2004 (Table 1). Two more experiments (experiments 4 and 5) were conducted on board the R/V Hespérides during the ICEPOS-2 cruise in January-February 2005 (Table 1).

The experiments consisted in incubating surface seawater sampled at different locations in 2 L recipients submersed in 2000 L incubators fed with running surface seawater to maintain temperature conditions and exposed to either natural solar radiation or radiation filtered off UV radiation. In 2004, the incubators were placed in the shore of South Bay (Livingston Island) with a pumping system to circulate surface water from the bay. In experiments performed during 2005 the incubators were placed on the deck of the vessel, in an area free of shade and with a circulating surface water (3 m depth) system. Replicated 2 L quartz bottles, that allow all the radiation to pass through, were used for the total solar radiation treatment and polycarbonate bottles, that filtered all UV radiation, were used for the treatment excluding UV radiation. Bottles were acid-cleaned and seawater samples were pre-filtered onto a 150 μm net (to exclude grazers) in the experiments performed in the Antarctic Base.

Surface water for the experiments was sampled using plastic carboys from a pneumatic boat, for experiments 2 and 3, and using Niskin bottles for experiments 1, 4 and 5. For experiment 1, surface seawater from Foster Port (Deception Island, South Shetlands Islands) was used, and experiments 2 and 3 used surface seawater sampled at South Bay (Livingston Island) (Table 1). Surface seawater for experiments 4 and 5 was sampled around the Antarctic Peninsula at 64º 44'S, 65º 42' W and 64º 03'S, 55º 50'W (Table 1).

During the time of the experiments performed in ICEPOS-1 solar radiation was automatically recorded by a meteorological station located in the Spanish Antarctic Base Juan Carlos I, in Livingston Island. The station

was provided with a Zipp & Zonen CM11 radiometer for the measurement of global solar radiation with sensitivity in the 305-2800 nm, and a Zipp& Zonen radiometer CUV3 for UVB+UVA (300-400 nm) measurements. All the sensors of the meteorological station integrated the radiation every half an hour from samplings of every second. During experiments performed in ICEPOS-2, solar and UV radiation were automatically measured by a Weatherlink Vantage Pro. Davis Co. meteorological station located on board R/V Hespérides. PAR was measured with the Solar radiation 6450 Davis sensor (from 400- 1100 nm) every 5 minutes. In addition, integrated UV (290-390 nm) values in all the wavelengths were obtained every 5 minutes with the UV 6490 sensor. UVR data were integrated every 30 minutes to be comparable with the data of 2004 meteorological station placed in Livingston Island.

UV and global radiation data were also transformed to daily doses (KJ m^{-2} d^{-1}) and the daily average radiation was calculated from the values obtained every day during the experiments.

Experiments sampling and analytical procedures

Every two days duplicated samples were taken from each replicated treatment to determine chlorophyll a, phytoplankton cell abundance and cell death parameters. A variable water volume (25 to 50 ml, depending on phytoplankton biomass) was filtered through Whatman GF/F filters for fluorometric analysis of chlorophyll *a* concentration (Parsons and others 1984). Phytoplankton abundance was determined using epifluorescence microscope. Samples for microscopic examination were preserved in glutaraldehyde (1 % final concentration), filtered onto 0.6 µm Nuclepore filters and kept frozen (-80^{o}C) until examination. Cells were counted at 200, 400 and 1,000 magnifications under a Zeiss epifluorescence microscope. The phytoplanktonic cells counted were differentiated into major taxonomic groups and grouped by cell size.

The proportion of dead cells in the phytoplanktonic communities was analyzed in experiments 2, 4 and 5 at the beginning and at the end of the

experiments. A cell membrane permeability test (cell digestion assay, Agustí and Sánchez 2002) was used to identify and count living and dying cells within the communities. The cell digestion assay is based on the brief exposure of the cells to the enzymes Trypsin and DNAse I, which enter the cytoplasm of cells with damaged plasma membranes (i.e. necrotic or advanced apoptotic cells) resulting in the entire digestion of the cells, while having little or no effect on the viability, morphology or function of live cells (Darzynkiewicz et al 1994, Agustí and Sánchez 2002). The digestion of dead cells is based on the fragmentation and hydrolysis of DNA by DNAse I, and peptide hydrolysis by Trypsin, which penetrates the damaged cells. The digested dead cells are undetectable by optical observation and lose any fluorescence signals and are, therefore, effectively removed from the population (Darzynkiewicz et al 1994, Agustí and Sánchez 2002). Live cells with intact membranes, are not affected by the enzyme cocktail so they remain in the sample and were counted after the assay.

Stock solutions of DNAse I (400 μg ml^{-1} in HBSS) and Trypsin (1% in HBSS) were prepared in HBSS medium (Sigma Co) and kept frozen at -65^{o}C until use. 5-10 ml of DNAse I stock solution were added to replicated 25-50 ml of experiment fresh samples followed by incubation for 15 min at 25^{o}C, temperature of the assay recommended for polar phytoplankton species (Llabrés & Agustí 2007). Then, 5-10 ml of Trypsin solution were added, followed by incubation for an additional 30 min at 25^{o} C. At the end of the incubation samples were filtered onto 0.6 μm Nuclepore filters, washed several times with 0.2 μm filtered seawater (to eliminate the enzymes) and fixed with glutaraldehyde (1 % final concentration). Filters were kept frozen (-80^{o}C) until to be counted under an epifluorescence microscope.

Parallel to samples run with the cell digestion assay for the quantification of living cells, duplicated samples to quantify the abundance of the total population (i.e. living and dying cells) were sampled and filtered as described above, preserved with glutaraldehyde (1 % final concentration), and kept frozen (-80^{o}C) until examination under epifluorescence microscope. To quantify the total population and the living/dead cells, both samples

were counted at 200, 400 and 1,000 magnifications under a Zeiss epifluorescence microscope, and classified into major taxonomic groups and grouped by cell size, as described above for regular counting.

Cells remaining after the enzymatic treatment, i.e. those having intact membranes, were considered to represent living or viable cells, whereas dead ones, with compromised membranes, were digested out by the enzymatic cocktail and were undetectable by the epifluorescence microscope. The fraction of dead phytoplankton cells in the sample was calculated by subtracting the abundance of living cells (after the enzyme treatment) from the counts of the untreated sample, and dividing the concentration of dead cells by the cell concentration in the untreated sample, which represent the total (dead plus living) cell concentration.

Net population growth rates were calculated for diatoms and flagellates from the cell abundances obtained at the beginning and at the final of the experiments. Inhibition percentages of diatom populations due to UV were calculated as the difference between growth rates obtained in the PAR and UVR+PAR treatments. Also inhibition percentages were calculated for the whole phytoplankton community as the differences between PAR and UVR+PAR treatments in terms of chlorophyll a biomass obtained at the final of the experiments. Statistical differences between treatments were determined by Student-t tests.

Table 1. Information about the experiments carried out in this study: date starting the experiment, date finishing the experiment, days of experiment, water origin, solar and UV radiation calculated as the average of daily radiation doses during the experiments and Chl a maximum reached at each experiment.

PARAMETERS	EXP. 1	EXP. 2	EXP. 3	EXP. 4	EXP. 5
Date starting experiment	17/01/04	26/01/04	11/02/04	6/02/05	11/02/05
Date finishing experiment	25/01/04	1/02/04	19/02/04	22/02/05	20/02/05
Days of experiment	8	6	8	15	9
Water origin	62º58'S	62º 39'S	62º 36'S	64º44S´	64º 03´S
	60º40'W	60º22´W	60º22´W	65º42W´	55º 50´W
Solar radiation (KJ m^{-2} d^{-1})	12287	13491	8090	5010	4709
UV radiation (KJ m^{-2} d^{-1})	650	684	420	241	223
Initial Chl a values (µg l^{-1})	-	0.82	1.01	1.64	3.8
Chl a maximum values (µg l^{-1})	-	14.3	22.6	7.6	3.5

Results

The experimental conditions differed greatly among the ICEPOS-1 and ICEPOS-2 experimental periods. The incident UV radiation was much greater during ICEPOS-1 (experiments 1 to 3) than that received during the ICEPOS-2 cruise (experiments 4 and 5, Fig.1).UVR was high throughout most of the ICEPOS-1 period, with maximum instantaneous UVR around 70 KJ m^{-2} (Fig. 1), compared to maximum values of 40 KJ m^{-2} and values regularly 30 KJ m^{-2} during ICEPOS-2 (Fig. 1). Accordingly, daily UVR doses were, on average, two to three times higher in experiments carried out during ICEPOS-1 (experiments 1, 2 and 3, Table 1) than in those conducted during ICEPOS-2 (experiments 4 and 5, Table 1).

The time series of phytoplankton abundance, as Chlorophyll *a* concentration, along the experiments clearly showed a suppression in response to UVR exposure (Fig. 2). These responses were strongest for experiments 2 and 3, where clear response of phytoplankton abundance to UVR exclusion was observed, reaching high chlorophyll *a* values of 15 and 23 µg l^{-1}after 6 and 8 days respectively, representing an increase of 9 and 5 times respectively over the treatments receiving total solar radiation. Phytoplankton abundance was clearly suppressed when exposed to UVR+PAR irradiance (Fig. 2) showing a small increase in chlorophyll *a* with values below 5 µg l^{-1} (Fig. 2). The increase in phytoplankton abundance when UVR was excluded was much more moderate in experiments performed during the ICEPOS-2 cruise (experiments 4 and 5, Fig. 2), consistent with the lower UVR during this experimental periods, with a maximum chlorophyll *a* concentration of 8 µg l^{-1} achieved in experiment 4 (Fig. 2), representing an increase of 1.7 times over the treatments receiving total solar radiation. Smaller changes were observed during experiment 5 (Fig. 2).

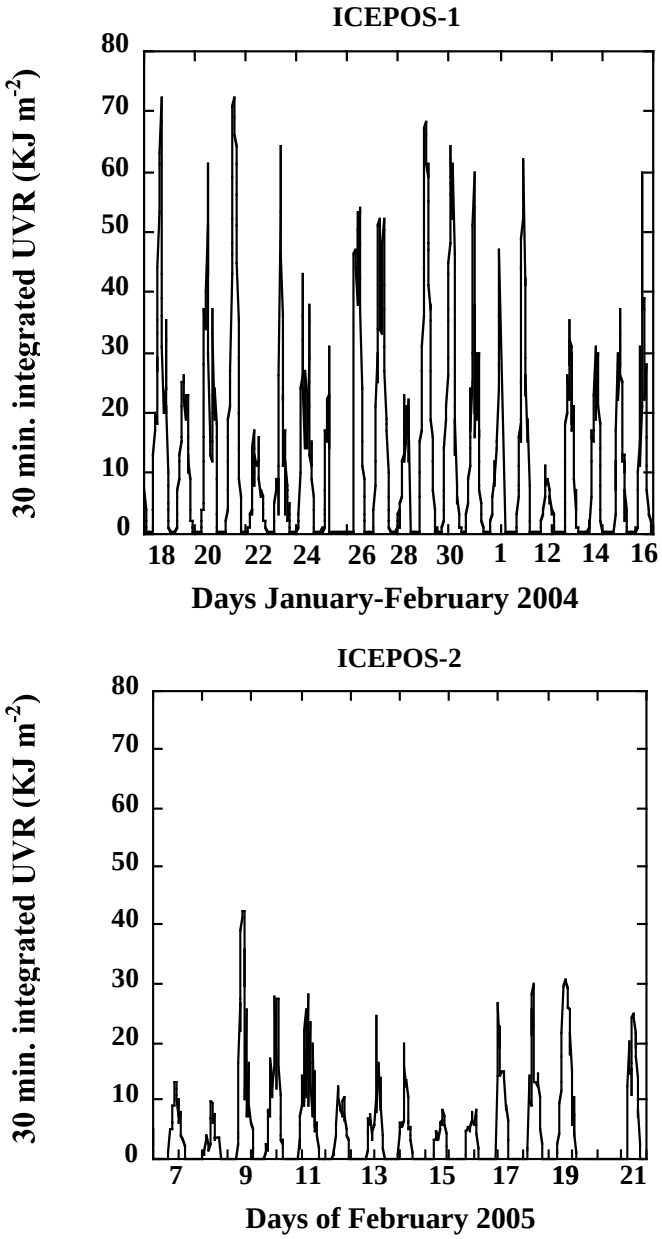

Fig. 1. Time course of UV radiation, integrated every 30 minutes (KJ m^{-2}) along the experimental periods of the ICEPOS-1 and ICEPOS-2 cruises

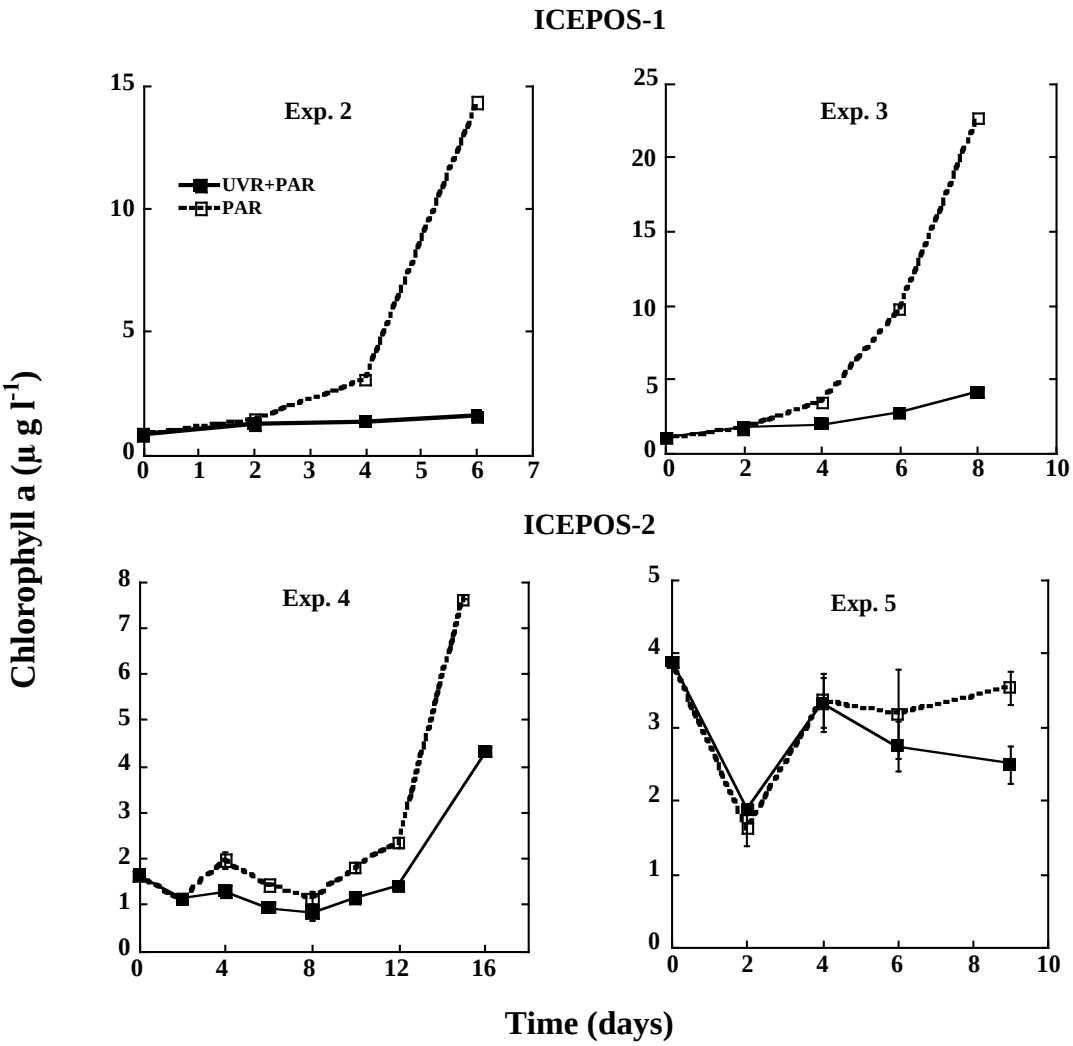

Fig. 2. Time course of Chlorophyll a (µg l^{-1}) under treatment with full solar radiation (UVR+PAR) and filtered UV radiation (PAR), in all the experiments carried out in ICEPOS-1 and ICEPOS-2 cruises.

Phytoplankton communities were dominated by diatoms, represented by the genera *Thalassiosira*, *Eucampia*, *Chaetoceros, Fragillariopsis* and *Pseudonitzschia*. Other phytoplankton groups, mainly flagellated populations, were also present and were mostly dominated by Crytophyceae and other groups, such as *Phaeocystis* (free form). In all experiments the response observed in chlorophyll *a* concentration were consistent with those in phytoplankton cell concentration. Diatoms, which tended to dominate the biomass of phytoplankton communities in all experiments, increased their cell density when UVR was excluded between 9 to 21 times in experiments 1,

2, 3 and 4, with respect to the total radiation controls. However, in experiment 5 the cell density increased only 1.3 times when UVR was excluded relative to the total radiation control.

Phytoplankton net growth rates, calculated from changes in cell abundance, were strongly affected by UVR as indicated by the results of the different experiments in which the exclusion of UVR resulted in higher growth rates with respect to controls (Fig. 3). Growth rates of diatoms were inhibited by UVR in all the experiments (Fig. 3) showing high values of 0.8 and 0.5 d^{-1} in some of the experiments when UVR was removed (Fig. 3). In addition, in experiment 1 the growth rate reached by diatoms following UVR removal was moderate, but negative net growth rates were obtained in the total radiation control indicating strong UVR inhibition (Fig. 3). Flagellate net growth rates were often negative in the experiments. However in experiments 2 and 3, flagellates growth rates were positive and increased when UVR was excluded (Fig. 3).

The changes in the percentages of dead cells within the populations of diatoms and flagellates were calculated in experiments 2, 4 and 5 for controls and UVR excluded treatments (fig. 4). In general the percentages of dead cells were higher in the controls for both diatoms and flagellates (Fig. 4). The percentage of dead cells was significant lower for both diatoms and flagellates when UVR was removed in experiments 2 and 5 (Student t test, $p < 0.05$). There was a tendency, not significant (Student-T test, $p>0.05$, fig. 4), for the percent dead flagellate cells to decrease when excluding UVR in experiment 4 (fig. 4). These results suggested a general induction of cell death by UVR in diatoms, reflected in a larger pool of dead cells of 13.5 %, 22 % and 5.2% for diatoms (for experiments 2, 5 and 4, respectively), and 19.3% and 24% (experiments 2 and 5) for flagellates.

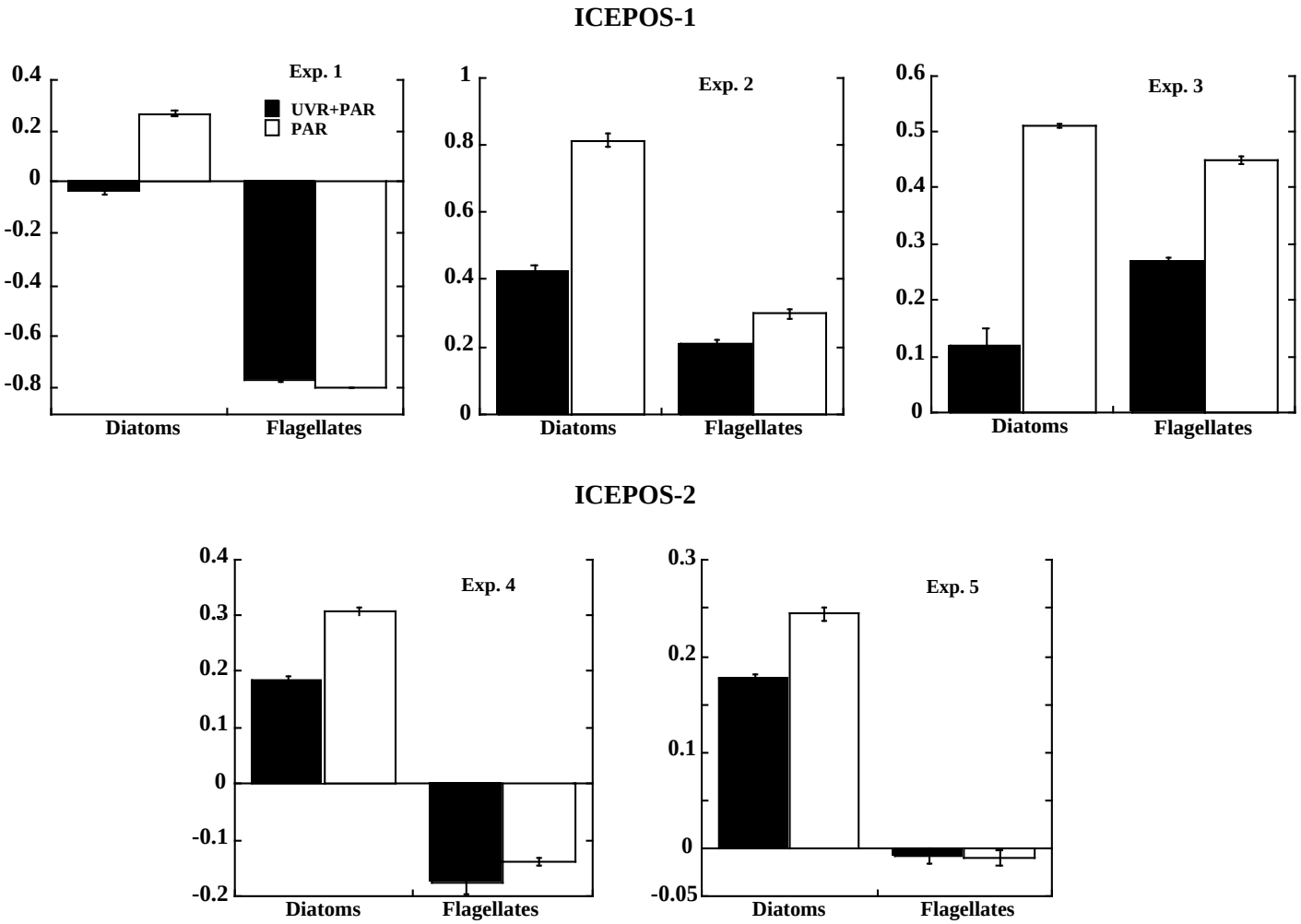

Fig. 3. Growth rates (d^{-1}, mean ± SE) of phytoplankton populations obtained under total solar radiation (UVR+PAR) and UVR removed (PAR) treatments in all the experiments carried out in this study.

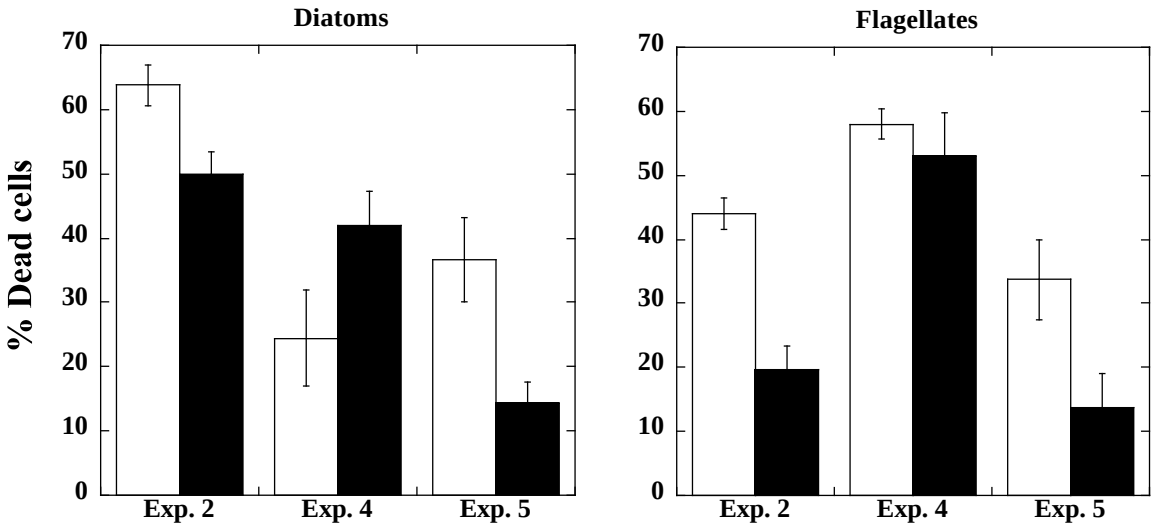

Fig. 4. Percentages of dead diatoms and flagellates (mean ± SE) obtained under total solar radiation (UVR+PAR) and UVR removed (PAR) treatments in all the experiments carried out in this study.

Differences in the average daily UVR doses received during the experiments explained the differences in the response of phytoplankton abundance to UVR across experiments, as indicated by the positive and significant relationship between the percentage of inhibition and the UV dose ($R^2 = 0.99$, 2nd order polynomial equation, Fig. 5A). Accordingly, the extent of inhibition of phytoplankton biomass increased with increasing UV radiation to represent more than 80% biomass reduction when mean daily doses exceeded 450 KJ m^{-2} d^{-1} (Fig. 5 A). A similar pattern, with a positive increase in the percent inhibition with increasing mean daily UV dose was observed for diatom abundance ($R^2 = 0.61$ Fig. 5B).

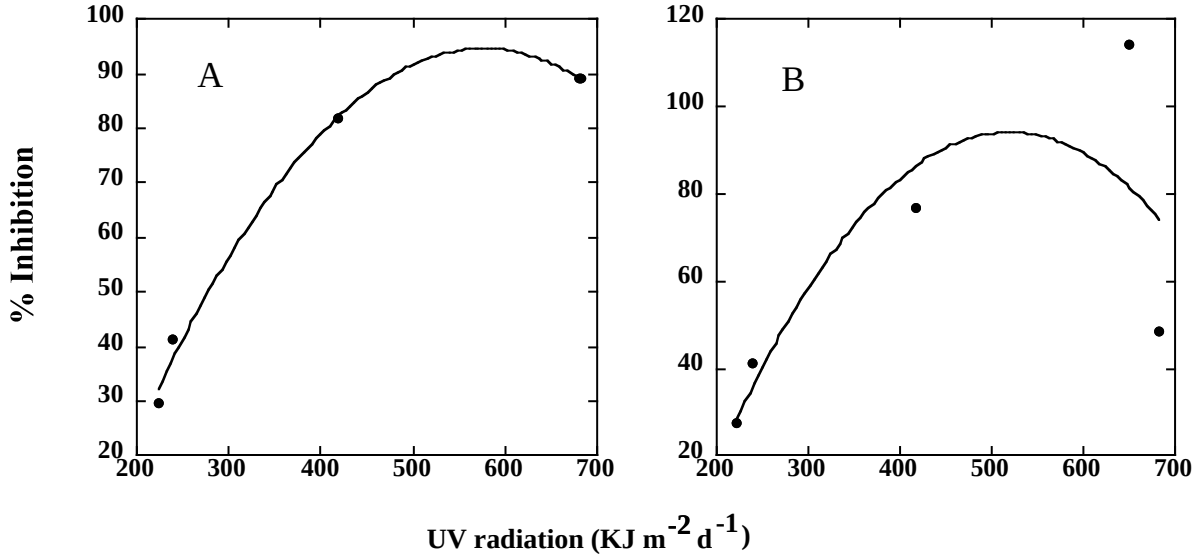

Fig. 5. A. The relationship between the percentage of inhibition in phytoplankton biomass (determined by chlorophyll a) and daily UV radiation (KJ m^{-2} d^{-1}). **B.** The relationship between percentage of inhibition in diatom concentration and daily UV radiation (KJ m^{-2} d^{-1}). Solid lines represent the fitted 2nd order polynomial.

Discussion

Our results clearly demonstrate that the high UVR levels over Antarctic waters strongly inhibit phytoplankton growth rates and enhance cell death, there by greatly suppressing phytoplankton biomass. Even relatively modest UVR (200 KJ m^{-2} d^{-1}) resulted in growth and biomass suppression, resulting in the widespread observation of impacts of UVR in phytoplankton biomass and growth across experiments, despite the three-fold variability in average daily UVR doses among experimental periods.

UVR undoubtedly inhibited phytoplankton biomass with chlorophyll a values remaining low at the end of the experiments under treatments receiving full solar radiation. In contrast, UVR exclusion resulted in high chlorophyll a values (as high as 22 µg l^{-1}) by the end of the experiments, revealing the strong effect of UVR in suppressing phytoplankton biomass in Antarctic waters. Similarly, when UVR was excluded, growth rates increased up to 5 times. Diatom growth rates reached rather high values when UVR were excluded (up to 0.8 d^{-1}), comparable to rates reported for

experimentally-induced blooms (Agustí and Duarte 2000), despite the low temperature of Antarctic waters, suggesting that the low growth rates of Antarctic phytoplankton in situ (e.g. Mura and Agustí 1996) may result from suboptimal conditions, such as UVR inhibition. UVR has been reported to suppress phytoplankton growth rates of Antarctic phytoplankton in cultures (Karentz et al 1991b). The few available experiments carried out with natural phytoplankton populations grown under UV ambient conditions also demonstrate growth inhibition (Davidson and Belbin 2002, Nunez et al 2006).

Here we show that ambient UVR not only suppresses Antarctic phytoplankton growth but also induces important cell death. In most of the experiments diatoms and flagellates experienced cell death resulting in percentages of dead cells decreasing when UVR was filtered out. In fact, an important cell death caused by UVR has also been described in phytoplankton from other marine environments, including tropical and temperate areas (Llabrés & Agustí 2006; Agustí & Llabrés 2007), but the role of UVR in causing cell death in natural phytoplankton from Antarctic waters has not been examined before. Comparable parameters, such as cell survival, were analysed before in culture studies with diatoms isolated from Antarctica (Karentz et al. 1991b, Davidson et al. 1994) and in natural communities, but these were restricted to the analysis of the decay in cell abundance (e.g. Davidson and Belbin, 2002). Our estimates of cell death induced by UVR were, however, based in direct counts of living/dead cells allowing the detection of dead cells in growing, not decaying, phytoplankton populations.

Diatoms dominated the phytoplankton community in the experiments reported here. The data available on flagellates do not allow to reach a clear conclusion as to their sensitivity to UVR relative to diatoms because in 3 out of 5 experiments flagellates did not respond even when UVR was filtered out. However, in the experiments in which a flagellate response was observed (experiments 2 and 3) diatoms presented significant higher percentages of inhibition to UVR than flagellates did (48% and 28% respectively, $p<0.05$, in experiment 2; 76% and 40% respectively, $p<0.05$, in

experiment 3). So our results do not support previous studies in which diatoms appeared to be less vulnerable to UVBR as compared with other phytoplankton organisms (Buma et al 2001, Bischof et al 1998, Karentz et al. 1991b), and which related their resistance to their large size (Karentz et al 1991b) and the presence of photoprotective compounds (MAA's) (Buma et al 2001). In contrast, diatoms showed very little response in MAA's induction under ultraviolet-B (280-320 nm) wavelengths (Riegger and Robinson, 1997) compared to colonies of the flagellate *Phaeocystis Antarctica*, in which a maximal induction of MAA's was reported (Riegger and Robinson, 1997) for the same wavelengths. Other feature affecting the sensitivity of diatoms to UVR could be the optical qualities, similar to quartz, of the siliceous cell walls (Karentz et al 1991b).

UVR was known to suppress phytoplankton growth, but our results show that it also induces cell death, thereby suggesting that the overall impact on the net balance of the phytoplankton populations must be greater than was considered. Indeed previous studies have revealed higher chlorophyll a and biomass values in treatments without UV radiation (Hernando et al. 2002, Hernando and Ferreyra 2005).

In our experiments environmental conditions were optimized, since mixing of the water column was eliminated, resulting in higher PAR levels. Shallow upper mixed layers and stability within the water column are conditions that allow cells to have enough light to saturate photosynthesis and to maintain turnover rates of repair mechanisms at PSII and to induce sufficient MAA synthesis (Bracher & Wiencke, 2000). The reduced mixing in the experiments should result in greater UVR doses than those received for Antarctic phytoplankton growing under strong mixing conditions of the water column. However, ice melting and increased air temperature in summer contribute to stabilize the water column and reduce mixing as described for South Bay, where a pycnocline at about 4 m depth was reported (Agustí and Duarte 2000). Hence, the percent inhibition of phytoplankton biomass by ambient UVR reported in this study (around 80-90%) should be close to those inhibiting phytoplankton biomass in this

Antarctic coastal location when UVR doses were high. Previous studies have calculated that primary production in Antarctic waters is UVR-inhibited by 6-12% (Smith et al. 1992), but our results indicated that inhibition of phytoplankton biomass and losses due to cell death should be added to the reported negative effects of UVR on Antarctic phytoplankton. Indeed, solar UVBR during the spring ozone depletion has been reported to reduce biomass production of other primary producers as Antarctic vascular plants, with an inhibition of more than 22% (F. S. Xiong and T. A. Day, 2001).

The strong positive relationship between the percent inhibition of phytoplankton biomass and ambient UVR doses derived here further provide evidence of the importance of the UV radiation effects on the natural phytoplankton communities. The UVR doses in Antarctica have been reported to be increased by about 30 to 50% due to stratospheric ozone depletion relative to pre-disturbance levels (Agustí 2007, Dahlback 2002). The steep UVR dose-biomass response relationship derived here suggests that an increase in UVR doses of 30 to 50% may result in an additional inhibition of phytoplankton biomass of up to 40% relative to pre-disturbance UVR doses. Because phytoplankton is the basis of the entire food web of Antarctica, a major suppression of phytoplankton biomass and growth must have clear impacts on the entire food web. The possible effect of phytoplankton suppression by UVR on the Antarctic food web has not, however, been assessed. However, the inferences drawn from the results presented here that Antarctic phytoplankton must have been strongly suppressed since the onset of stratospheric ozone depletion in the early 1980's is consistent with reports of a major concurrent decline in krill (Atkinson et al. 2004), the main consumer of phytoplankton. This observation suggests impacts of phytoplankton suppression due to enhanced UVR on the Antarctic food web should be explored.

In summary, the results indicated that Antarctic phytoplankton populations from the Antarctic Peninsula showed growth inhibition and enhanced cell death under ambient UVR doses. The decreasing growth and the increasing cell death influenced the net balance of the phytoplankton

populations that appear to be strongly controlled by UV, with phytoplankton biomasses suppressed by up to 80-90% in the presence of ambient UVR doses. These results show that springtime ozone depletion over Antarctica resulting in elevated levels of UVR should have reduced phytoplankton biomass and caused cell death, consequently reducing phytoplankton stocks and the associated Antarctic food web.

Acknowledgements

This is a contribution to the project ICEPOS (REN2002-04165-C03-02/ANT) funded the Spanish Ministry of Education and Science. M. Llabrés was supported by the special action REN2002-10871-E/ANT and by the EC project THRESHOLDS. We thank the BIO Hesperides crew and technical UTM personnel for professional assistance during the ICEPOS-2005 cruise, and help at the BAE Juan Carlos I, during the ICEPOS-2004 study. We are also grateful to R. Martínez and N. Carrillo for sampling assistance, to Isabel Reche for her help at ICEPOS-1 cruise and to C.M. Duarte for valuable comments on the manuscript.

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Chapter 6
OH radical effects on phytoplankton cell death

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Manuscript, 2008

Abstract

OH radicals are the main atmospheric oxidant globally, and they can be transferred to the surface ocean. In the present study, 7 experiments that simulate the transfer of OH radicals to seawater have been performed in the NE Atlantic and Antarctic waters. Seawater concentrations show a consistent diurnal variability that mimics that variability of atmospheric OH. The maximum diurnal seawater concentrations originated from air-water exchange, ranged from $1.8x10^{-17}$ to $5.1x10^{-16}$ mol L^{-1}, being significantly higher than those reported for photoproduction. OH radicals have important effects on phytoplankton survival as indicated by our results, with a clear correlation between phytoplankton cell mortality and OH concentration. The effect of OH radicals was higher for pico-phytoplankton organisms with *Prochlorococcus* showing the highest decay rate and the shortest half-life among the phytoplankton populations. The important effect of seawater OH radicals, introduced by air-water exchange, in phytoplankton cell death showed in this study contributes to better understanding the impact of ROS on natural marine phytoplankton, and many aspects related to the capacities of these organisms to overcome the oxidative stress along with the consequences of OH radicals in the marine systems should be addressed.

Introduction

OH radicals are the main atmospheric oxidant with an important role in atmospheric chemistry, especially in tropical regions where its abundance is higher (Cooper 1996, Brauers et al 2001). In addition, it has been recognized that OH radicals play an important role in the dynamics of dissolved organic matter (Mopper and Zhou 1990, Vaughan and Blough 1998). Also, there is a diffusive exchange of OH radicals between water and air, but that has not been quantified so far, due to the difficulties in OH determination. The ratio of concentrations of OH at equilibrium between the gas and dissolved phases is given by the Henry's Law constant. Unfortunately, there is a large discrepancy

in reported values for Henry's law constants of OH radicals (Lelieved and Crutzen 1991, Hanson et al. 1992).

In the atmosphere, OH radicals are mainly produced by a reaction initiated by UV photolysis of ozone, but other secondary sources exist (Brauers et al. 2001). Once produced in the atmosphere, a fraction of them will be transferred to the surface ocean, where may have consequences for aquatic organisms at this layer since OH radicals cause oxidative damage in lipids, proteins and DNA of cells and are considered as the most reactive of the oxygen species (ROS) (Kieber et al 2003, Mc Cord 2000, Freidovich 1999). Oxidative damage has been identified as an important cause of cell death in human cells and other organisms including photosynthetic ones (Cheng et al. 1995, Ueda and Shah 1992, Levine et al. 1996, Vardi et al. 1999). However, none study has directly addressed the lethal effects of OH radicals in natural phytoplankton growing in the ocean. The potential role of OH as a stressor of phytoplankton could be maximum near the air-water interface, since OH can be transferred to water from the atmosphere.

The objectives of this study are to elucidate the influence of seawater OH radicals, introduced by air-water exchange, on phytoplankton cell death and analyze whether different phytoplankton species from different oceanic regions varied in their susceptibility to OH.

Methods

Experimental design and locations: Experiments were carried out in the Atlantic Ocean (cruises COCA-2 and BADE-2) and in Antarctic waters (cruise ICEPOS-2). COCA-2 and ICEPOS-2 cruises were performed on board R/V BIO Hespérides and BADE-2 cruise on board R/V Pelagia. A total of 7 experiments were performed (table 1), one experiment in COCA-2, on May 2003, 5 experiments in BADE-2, on September-October 2004, and one experiment more in ICEPOS-2 on February 2005. In all the experiments surface (5 m) seawater was sampled using Niskin bottles inserted in a CTD-rosette system (see table 1 for more details on the sampling positions). Seawater samples were placed in 6 glass bottles (250 ml) and submerged on deck incubators on with sea-surface re-

circulating water, to maintain in situ temperature, and exposed to natural solar radiation. 3 bottles were used as controls and 3 for treatment in which atmospheric air was bubbled in the treatment bottles continuously during all the incubations period at an air flow rate of 10 ml min^{-1}. The bubbling process equilibrated the chemical composition of the gas phase species with that of the aqueous phase, analogously to the process occurring at the atmosphere-ocean interface, and therefore, OH radicals are transferred to water from the atmosphere.

Experiments were run for 0.5 - 2 days (Table 1) and were sampled periodically, with a more intense sampling during the day hours at intervals varying between 2.5 h to 7 hours (Table 1). In all the experiments the evolution of OH radicals, as well as that of the phytoplankton communities was analyzed.

OH determination: To determine OH radical concentrations in the atmosphere and water, parallel to the glass bottles in which phytoplankton abundance and viability was being determined, atmospheric air was bubbled in Mili-Q water with erioglaucine 0.01 mM, for the same time and the same sampling intervals. Changes in the erioglucine absorbance measured at 620 nm were followed with a spectophotometer (Shimadzu ISR-240A). OH radical concentrations in water were estimated as explained elsewhere (Molot et al. 2003). Briefly, the absorbance of erioglucine after a time period t (At) will be:

$$A_t = A_0 K_e t$$

where A_0 is the absorbance of erioglaucine at initial time, and *Ke* is the decay rate of erioglaucine related to the aqueous OH concentrations ($[OH]_W$, M) by,

$$[OH]_W = Ke / ke$$

where $ke = 1.11\ 10^{11}\ M^{-1}\ s^{-1}$. Therefore, OH concentrations are measured from the measurement of the decay of absorbance of erioglaucine. The limit of detection for OH radicals with the procedures used was of $3\ 10^{-18}$ mol L^{-1}. The

reaction times of OH radicals are so fast that air and water can never be in equilibrium (Lelieveld and Crutzen 1991). However, the OH fugacity in water ($[OH]_G$, atm), which is the hypothetical gas phase concentration in equilibrium with the measured OH concentration, can be estimated by

$$[OH]_G = [OH]_W H$$

where H (atm M^{-1}) is the temperature corrected Henry's law constant. It is common in the literature (Spivakovky et al. 2000) to report gas phase concentrations as molecules cm^{-3} instead that in SI units (atm). In the present work the H values and their temperature dependence used are those reported by Hanson et al. (1992). Indeed, for Antarctic waters, H is three fold lower than for the NE Atlantic cruises.

Plankton abundance quantification: The method to quantify the abundance of phytoplankton varied with the community found. Pico-phytoplankton from the Atlantic Ocean was quantified by flow cytometric techniques. A FACSCalibur (Becton-Dickinson) flow cytometer, fitted with a 488 nm laser and a photomultiplier for forward scattered light detection was used. An aliquot of a calibrated solution of 1µm diameter green fluorescent beads (Polysciences Inc.) added to the samples was used as an internal standard for the quantification of cell concentrations. Bead concentration in the standard solution was calculated by filtering duplicate aliquots onto black Nuclepore filters and counting the beads under an epifluorescence microscope. The red, green and orange fluorescence emissions and the forward and side scattering of the cells and beads were used to detect different cell populations and to differentiate them from the fluorescent beads. In the experiments performed during COCA-2 duplicate fresh samples were quantified on board. Samples from BADE-2 cruise experiments were first fixed with glutaraldehyde (1% final concentration) and then preserved in liquid nitrogen until their analysis at the laboratory.

To determine nano and micro-phytoplankton abundance, samples were preserved in glutaraldehyde (1 % final concentration), filtered onto 0.6 μm Nuclepore filters and kept frozen (-80ºC) until examination. Cells were counted at 200, 400 and 1,000 magnifications under a Zeiss epifluorescence microscope. The phytoplanktonic cells counted were differentiated into major taxonomic groups and grouped by cell size.

The abundance of living pico, nano and micro-phytoplankton organisms was analyzed by using the cell digestion assay (Agustí and Sánchez 2002), a cell membrane permeability test used to differentiate living from dead cells. Stock solutions of DNAse I (400 μg ml^{-1} in HBSS, Hanks' Balanced Salts) and Trypsin (1% in HBSS) were prepared in HBSS medium (Sigma Co) and kept frozen at -65ºC until use. For pico-phytoplankton analyzed during the experiments run in the cruise COCA-2, 200 μl DNAse I stock solution were added to 1 ml duplicate fresh samples in assay tubes and incubated at 35ºC for 15 min. Then, 200 μl trypsin (1% in HBSS) were added and incubated at 35ºC for 30 min. At the end of the incubation, the samples were placed on ice to stop the enzyme reaction and the abundance of pico-cyanobacteria cells remaining after the assay was quantified flow cytometrically as described above. For nano and micro-phytoplankton, 2 and 5 ml of DNAse I stock solution were added to 10 ml (for ICEPOS experiment) and 25 ml (for experiments in the Atlantic cruise BADE) respectively and incubated at 35ºC for 15 min. In ICEPOS experiment the temperature of incubation was 25ºC instead 35ºC temperature of the assay recommended for polar phytoplankton species (Llabrés & Agustí, 2007). Then, 2-5 ml of Trypsin stock solution were added, followed by incubation for additional 30 min at 35º C. At the end of the incubation samples were filtered onto 0.6 μm Nuclepore filters, washed several times with 0.2 μm filtered seawater (to eliminate the enzymes) and fixed with glutaraldehyde (1 % final concentration). Filters were kept frozen (-80ºC) until to be counted under an epifluorescence microscope as we described for total cell abundance. Pico-phytoplanktonic cells of *Synechococcus sp*. were observed in the samples from the Antarctic experiment and counted as well in the filters.

The cells remaining after the enzymatic treatment, i.e., those having intact membranes, were considered living or viable cells, whereas dead cells with compromised membranes were digested by the enzyme cocktail and were therefore undetectable by the flow cytometer. The fraction of living phytoplankton cells in the samples was calculated by dividing the concentration of living cells after the enzyme treatment by the cell concentration in the untreated sample representing the total (dead plus living) cell concentration. The number of dead cells was calculated by subtracting the number of living cells from the total cell abundance. Student *t*-test was used to test whether differences between taxa were significant.

Results

OH occurrence in seawater and the atmosphere: The hydroxyl radical values measured in seawater in this study varied between experiments, based on the geographical position and the time of the day (Table 1). Maximum hydroxyl radical values ranged from $1.8x10^{-17}$ to $5.1x10^{-16}$ mol L^{-1} (Table 1) which are indicating a high transfer from the atmosphere to the water. The higher maxima of OH radicals were obtained in experiments carried out in COCA-2 cruise, in the Atlantic Ocean (21ºN 25º 59'W) and in ICEPOS-2 cruise, in the Antarctic Ocean, with values of $5.1x10^{-16}$ mol L^{-1} and 2.4×10^{-16} mol L^{-1}, respectively, with maxima occurring generally at the late afternoon (Table 1).

To the best of our knowledge, there is a lack of field studies reporting simultaneous gas and dissolved phase OH concentrations. It is possible to estimate which would be the atmospheric concentration in equilibrium with the measured OH seawater concentration by using equation 3, which is in fact a measure of the dissolved OH fugacity. Figure 1 shows the latitudinal variability of OH seawater fugacities (given as molecules cm^{-3} instead of atmospheres of pressure) with values up to 8 times higher in the tropics than at high latitudes.

Table 1. Maximum OH radical (mol L^{-1}) measured in the water for all the experiments carried out, local time in which maximums were detected, position of sampling and duration of the experiments.

Experiments	Position	Local time	Maximum OH (mol L^{-1})	Duration
COCA-2	Atlantic Ocean 21ºN 25º 59'W	13:00	5.1 x10^{-16}	30 h (1.2 d)
BADE-2 Exp. 1	Atlantic Ocean 20º 1'N 19º 16'W	20:30	1.9 x 10^{-16}	50.5 h (2.1 d)
BADE-2 Exp. 2	Atlantic Ocean 20º 9'N 19º 5'W	15:00	1.8 x 10^{-17}	12 h (0.5 d)
BADE-2 Exp. 3	Atlantic Ocean 21º 47'N 26º 42'	19:00	9.4 x 10^{-17}	35 h (1.4 d)
BADE-2 Exp. 4	Atlantic Ocean 23ºN 29º 23'W	19:00	8 x 10^{-17}	11 h (0.45 d)
BADE-2 Exp. 5	Atlantic Ocean 24º 35'N 31º 14'W	15:00	9.1 x 10^{-17}	34.5 h (1.4 d)
ICEPOS-2	Antarctic Ocean 62º 44'S 60º 32'W	16:15	2.4 x 10^{-16}	44 h (1.8 d)

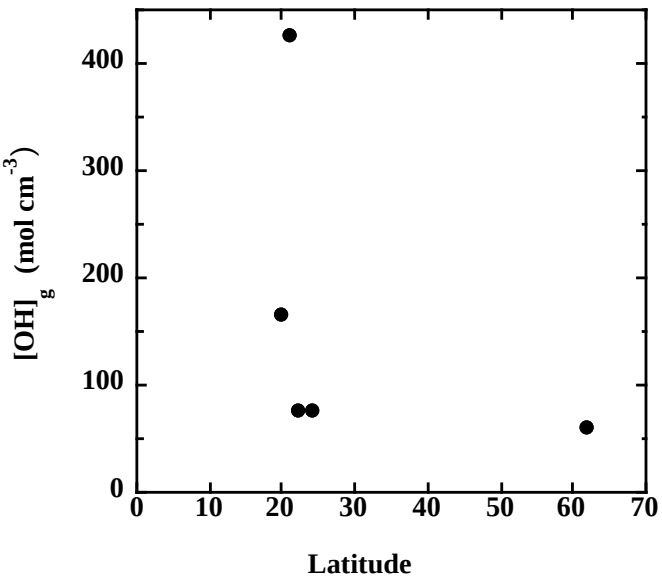

Figure 1. Seawater OH fugacity (molecules cm^{-3}) versus latitude for the diurnal maximum

Phytoplankton abundance and decay: Because experiments were carried out in different locations and season we did not always analyse the response of the same phytoplankton communities. For example in the NW Africa upwelling area, in where water was sampled for experiment 2 of BADE-2 (20^{o} 9'N 19^{o} 5'W, table 1), and in the experiments from Antarctic waters, *Prochlorococcus* abundance was very low or absent and its response could not be followed. Similarly, the abundance of diatoms and nanoflagellates were low in the most oligotrophic waters of the Atlantic Ocean (Table 1) precluding the analysis of the effect of OH radicals on these organisms.

The diel variation in the hydroxyl radical concentrations was followed in all the experiments by the observation of peaks (Fig 2). A high reduction in the total and living cell concentration was observed for pico-cyanobacterial communities exposed to hydroxyl radicals associated to the peaks measured (Fig. 2), reaching values below the detection limits at the end of some experiments. The decreasing pico-phytoplankton cell concentration was detected after the hydroxyl peaks were detected (Fig. 2). For diatom and flagellate populations the living cell concentration also declined under treatment in respect to the controls at the final of the experiments but in minor level than for pico-cyanobacteria (Fig. 2).

The populations did not decrease in the controls although in some experiments *Prochlorococcus* cell abundance slightly decreased in the controls as well indicating other stressors during the experiment (e.g. grazing pressure).
Phytoplankton populations analyzed in the experiments showed a negative exponential relationship with hydroxyl radical concentrations in seawater (Fig. 3), with decreasing cell concentration as hydroxyl radical concentration increased (Fig. 3). However for Antarctic flagellates during the ICEPOS experiment no relationship was observed between cell concentration and hydroxyl radical (Fig. 3).

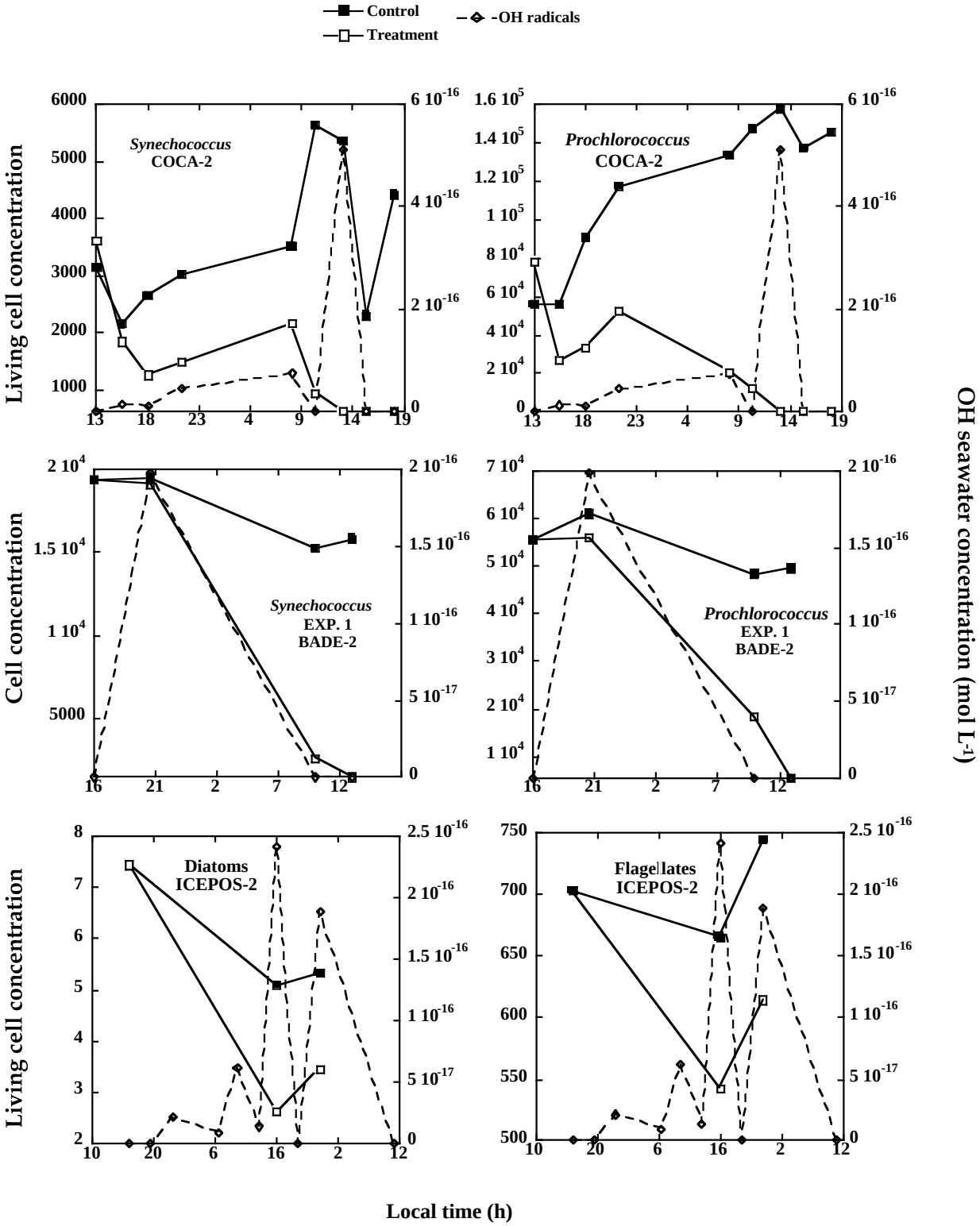

Figure 2. Phytoplankton cell decay under control and OH treatments during some experiments carried out. Continuous lines represent phytoplankton cell concentration, dashed lines represent OH radicals peaks measured in the water along the experiments.

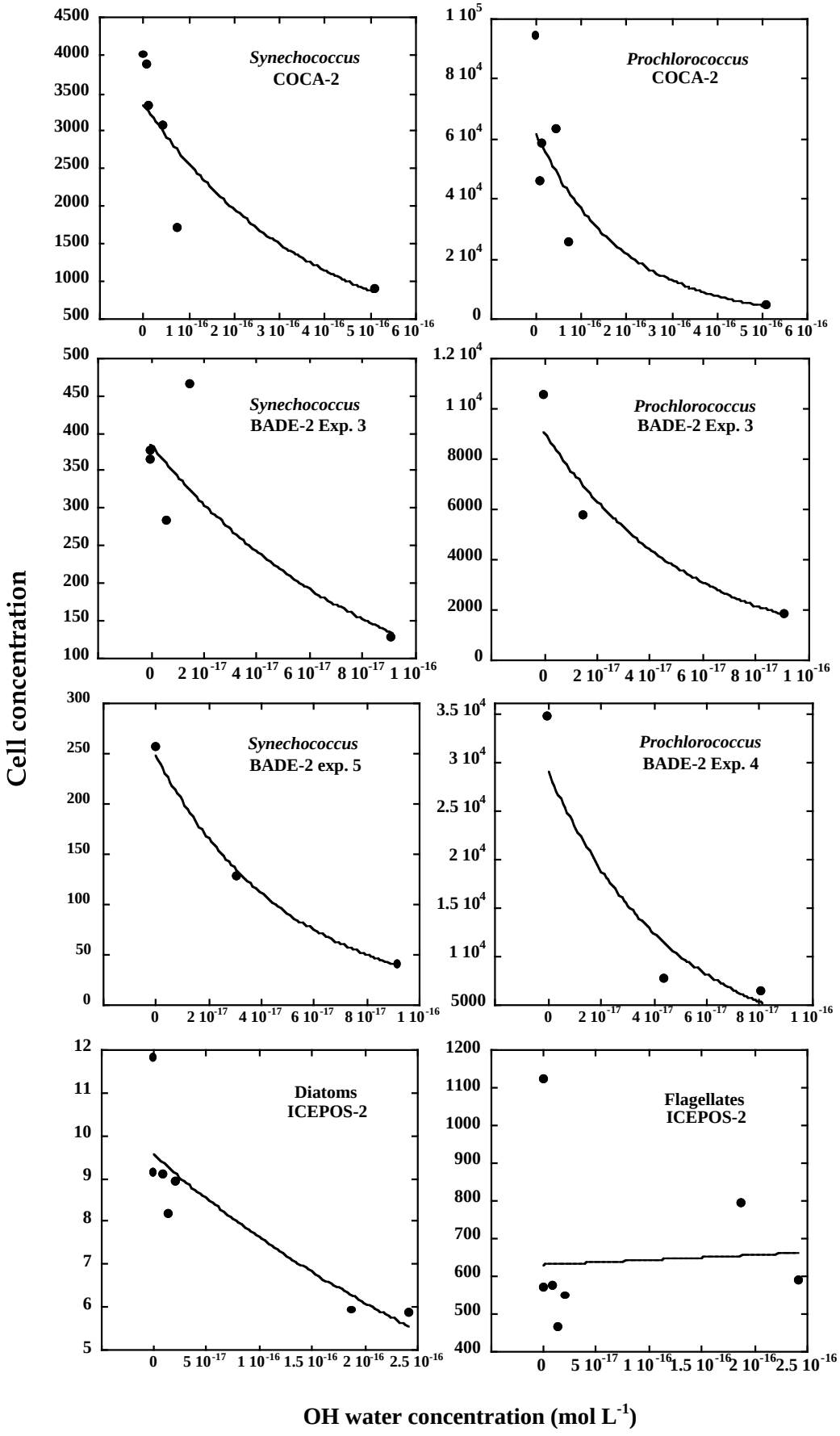

Figure 3. Relationship obtained between phytoplankton cell concentration and OH concentration measured in the waters in the experiments carried out.

Then again, the percentage of dead phytoplankton cells under OH radical treatments was higher than in controls at the end of the experiments (Fig. 4) despite a decrease in the population abundance was not observed. Under OH radicals treatments cell death induced was higher in *Synechococcus* and *Prochlorococcus* with all the population dead at the end of some experiments (Fig. 4). However, diatoms and flagellates also showed higher percentage of dead cells under treatments than in controls although the increase in the percentage of dead cells was not so high as for pico-phytoplankton communities (Fig. 4).

To compare the response between phytoplankton communities to hydroxyl radicals, decay rates were determined under treatments and the average between all the experiments was calculated (Fig. 5). We observed important differences among groups, with *Prochlorococcus* showing the highest decay rate, with an averaged value of 0.14 ± 0.02 h^{-1} (mean ± standard error) followed by *Synechococcus* (Fig. 5). Diatoms and flagellates showed lower decay rates than pico-cyanobacteria being the flagellates the group with the lowest decay rate, 0.003± 0.0037 h^{-1} (mean ± standard error, Fig. 5). Thus, half-life times for *Prochlorococcus* were the shortest among the phytoplankton populations, with an average value of 5.2 ± 0.7 (mean ± standard error) hours. Conversely, *Synechococcus* needed 11.7 ± 3.4 (mean ± standard error) hours to decimate its population to a half. Diatoms showed an averaged half-life time of 35.7 ± 2.7 (mean ± standard error) hours and flagellates presented the longest half-lives, of 93.6 hours.

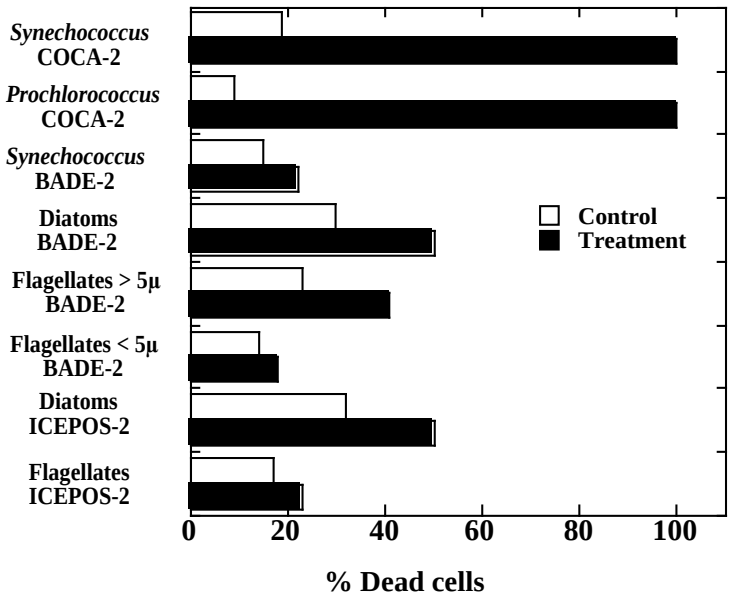

Figure 4. % Dead cells of phytoplankton communities under controls and OH radical treatment obtained at the end of the experiments.

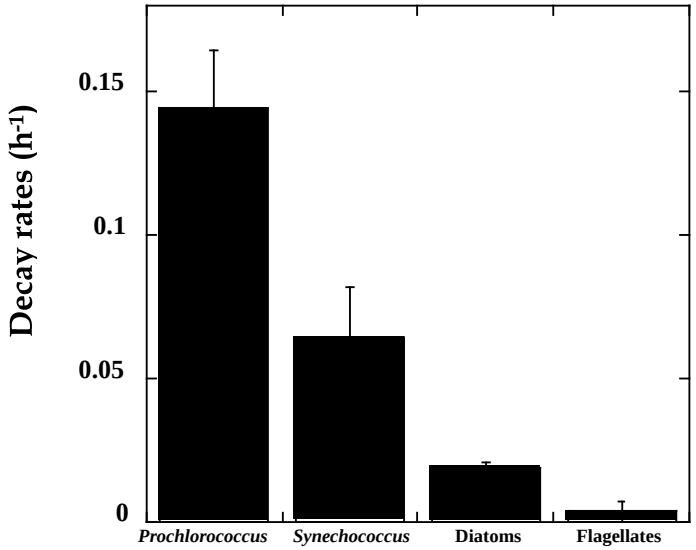

Figure 5. Averaged decay rates (h^{-1}) obtained for *Prochlorococcus, Synechococcus,* diatoms and flagellates in the experiments under OH radical treatment.

Discussion

Hydroxyl radicals react fast in water and the atmosphere, and thus it is difficult to measure its concentration. Aquatic concentrations are always measured by OH generation by irradiation (Mopper and Zhou 1990, Qian et al. 2001) or transferring them from the atmosphere (as done here). Therefore, the water column concentrations of OH radicals reported elsewhere are those that would occur if seawater would be under solar radiation. These concentrations have been described to be in the order of 10^{-19} to 10^{-17} for different oceanic regions, being those in Antarctica at the lower range (Mopper and Zhou 1990, Qian et al. 2001). The maximum OH concentrations obtained in this study were higher, by a factor of 2 to 10 than those values previously reported. This indicated that transfer from the atmosphere can lead to higher surface OH concentrations than due to photoproduction.

This is the first time diurnal cycles of seawater concentrations of OH radical are measured, since the previous attempts could only provide the OH concentration when seawater was irradiated (midday concentration). OH radical values obtained in the experiments showed a daily variation with maxima occurring at the late afternoon. This diurnal cycle mimics that known for atmospheric OH, which presents a maximum during the day, and minimums during night time (Brauers et al. 2001). Conversely, the diurnal averages were one order of magnitude lower than the maximums observed and thus comparable to the values reported previously.

Air and water concentrations of OH radicals can never be at equilibrium (Lelieveld and Crutzen 1991) due to the fast reaction of OH radicals in air and seawater, being these reaction rates faster than diffusive air-water exchange (Lelieveld and Crutzen 1991). However, our estimates of the dissolved OH fugacity clearly showed a latitudinal variability, with values up to 8 times higher in the tropics than at high latitude, similar to latitudinal trends predicted for atmospheric boundary layer OH (Spivakovsky et al. 2000). Therefore, the present results provide a consistent view of OH radicals in contact with the atmosphere for a 60 degrees latitudinal range. However, the fugacities predicted here are 10^4 times lower than atmospheric concentrations (and thus

fugacities) of OH radicals. Considering that accommodation of OH radicals at the air-water interface is virtually instantaneous (Roeselova et al. 2004, Vieceli et al. 2005), the lower fugacity of water concentrations with those described for the air is related to faster reaction rates of OH in seawater than at the atmosphere.

If concentrations of seawater OH from photoproduction are considered (Mopper and Zhou 1991, Qian et al. 2001), the difference in atmospheric and seawater fugacities is 10 fold higher, thus indicating a large lower-atmosphere, upper surface ocean gradient in OH concentrations that drives an important air-water exchange of OH radicals.

This air-water exchange of OH radicals has important effects on phytoplankton survival as indicated by our results. The relationship observed in the experiments between phytoplankton cell abundance and OH radical concentration shows the strong decrease of cell concentration as the concentration of OH radicals increased, with some populations disappearing in some experiments. OH radicals caused, thus, an important cell death in the variety of phytoplankton communities examined as reflected by the high cell decay rates obtained. In cases in which the decay was low, the lethality of OH for phytoplankton was also observed by the increased percentages of dead cells obtained during the experiments. The important phytoplankton cell death induced by OH peaks is expected since the OH radical is considered the most reactive of the oxygen species (ROS). It reacts with biomolecules, such as proteins, lipids or DNA, modifying or destroying their functionality, consequently generating oxidative stress (Kieber et al. 2003, Mc Cord 2000, Freidovich 1999) Oxidative stress generated by ROS is one of the causes of cell ageing and cell death in most organisms, either in humans, animals and plants (Cheng et al. 1995, Ueda and Shah 1992, Jabs 1999, Levine et al. 1996). Conversely, studies addressing cell death under oxidative stress in aquatic organisms are still scarce. Vardi et al (1999), however, demonstrated that the oxidative stress caused by ROS induced programmed cell death both in cultures and natural populations of the phytoplanktonic species *Peridinium gatunense* (Vardi et al 1999). Moreover, in cultures, increased percentages of dead cells

were well related to the accumulation of ROS in cells of *P. gatunense* (Vardi et al 1999).

OH radical effects on phytoplankton cell mortality varied, however, largely among the populations found. Averaged decay rates observed for *Prochlorococcus* and *Synechococcus* due to hydroxyl radical were higher than those obtained for diatoms and flagellates, showing the higher sensitivity of the pico-sized phytoplankton ($P < 0.05$). *Prochlorococcus* was the most vulnerable within the phytoplankters to OH radical ($P < 0.05$), in concordance with other results identifying *Prochlorococcus* as the most sensitive to other stresses induced by solar radiation, as direct mortality induced by natural UVR (Llabrés and Agustí 2006, Agustí and Llabrés 2007).

Diatoms and flagellates were also affected by hydroxyl radical, although in a minor level, and although flagellates showed no relationship between cell concentration decay and OH radical concentration, differences between diatoms and flagellates to OH sensitivity are not significant ($P > 0.05$).

The results indicated high variability in the survival capacity of phytoplanktonic groups. Mallick & Mohn (2000) described that the capacity to affront the oxidative stress, as the production of antioxidants in algae, were both species and stress specific. Thus, the different sensitivity to OH radicals observed here between phytoplankton groups could be related to potential different antioxidant mechanisms they may have as a defence against toxicity. So according to the results obtained, a major display of antioxidants mechanisms should be expected for the most resistant groups, flagellates and diatoms, which may be addressed.

Also the higher sensitivity to OH described for pico-phytoplankton could be associated to their small cell size. A recent study related plant size, including phytoplankton, to survival capacity (Marbá et al. 2007) and small cell size have been described to impose a limit to UVR survival in phytoplankton (Llabrés and Agustí 2006, Agustí and Llabrés 2007).

The phytoplankton cell mortality rates measured during the experiments will be equivalent to that experienced by phytoplankton growing at the upper oceanic layer, where the air-water exchange may occur. The upper surface layer

has described to be a particular microenvironment, with a specific chemistry and high photochemical production reactions, moreover the strong atmosphere-ocean exchange (Hardy 1997). These properties have been useful to identify this layer as a potential extreme environment for planktonic organisms (Hardy 2005), as we demonstrated here. However, under high wind speed conditions, the layer under the influence of atmospheric OH radicals may much larger.

In summary, in this study we showed that there is a high exchange of OH radicals between atmosphere and seawater leading to events of high OH radical concentrations in the surface upper layer. So as we have show in this work the exchange of OH radicals between the atmosphere and the ocean surface is inducing strong cell death on marine phytoplankton, and this feature represents a novel contribution to the impact of ROS on natural marine algae. We also demonstrated that OH radicals caused direct cell death in phytoplankton as expected from its strong oxidant capacity, with pico-phytoplankton resulting the most vulnerable to its effects. Few studies have directly related the increased concentrations of OH with cell mortality in phytoplankton. Many aspects related to the capacities of these organisms to overcome the oxidative stress and with the consequences of the strong effects of OH radicals from the atmosphere in the marine systems should be addressed.

Acknowledgements

This is a contribution to the project COCA (REN 2000-1474-CO2), funded by the Spanish Ministry of Science and Technology to S. Agustí, BADE (grant REN-2002-10606E/MAR funded by the Spanish Ministry of Education and Science to S. Agustí and project Nr. #812.03.001 funded by the Netherlands Science Foundation, Earth and Life Sciences to G.J.H.), ICEPOS (REN2002-04165-C03-02/ANT) funded the Spanish Ministry of Education and Science, and DEPANT (CGL2004-20013-E) funded by the Spanish Ministry of Education and Science to J. Dachs. M. Llabrés was supported by project REN2001-5060-E /MAR, the special action REN2002-10871-E/ANT and by the EC project THRESHOLDS. We thank the BIO Hesperides crew and technical UTM

personnel for professional assistance during the COCA-2 and ICEPOS-2005 cruise. Also, we are grateful the technical personnel and crew of the R/Vs Pelagia for their professional assistance during the BADE-2 cruise.

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General Discussion

This work demonstrated that UV radiation causes cell death in phytoplanktonic organisms. This is an important contribution to the phytoplankton ecology due to the few studies addressing cell death in phytoplankton and the lack of knowledge on the factors inducing it. UVR has been identified here as an important factor able to cause phytoplankton cell death, which is specially pertinent when considering the increasing ultraviolet-B radiation levels reaching the ocean surface due to global decreases in stratospheric ozone (Crutzen 1992, Smith et al. 1992, Kerr & McElroy, 1993). Although numerous studies have reported many effects of UVR radiation in phytoplankton populations triggering cell death (Roy 2000, Vincent and Neale 2000, Banaszak 2003), the direct cell death induced by UVR in these photosynthetic organisms had never been demonstrated before. This work also reveals that solar UVR may cause direct cell death in phytoplankton organisms from a variety of oceans, as the Atlantic, Mediterranean Sea and Antarctic waters (chapters 1, 2, 3 and 5). The strong cell death induced by UVR in pico-phytoplankton populations examined in the Atlantic Ocean and in the Mediterranean Sea (chapters 1, 2 and 3) point to the high sensitivity of these organisms to high solar UV radiation. Also, the highest decay rate detected for *Prochlorococcus* in this study revealed that *Prochlorococcus* is the phothosynthetic organism with the major sensitivity to UVR.

Calculations done in this study as half-life and lethal radiation doses have been revealed as important tools to differentiate phytoplankton sensitivity to UVR, allowing better and unambiguous comparisons between communities. Thus, *Prochlorococcus* presented the shortest half-life times and required the lowest lethal radiation doses within the phytoplanktonic organisms. Average half-life times calculated for *Prochlorococcus* exposed to natural UVR in this study rounded the 3 hours (Table 1), which is remarkably low, while for *Synechococcus* and Pico-eukaryotes average half-life times were longer, 11 and 8.8 hours respectively (Table 1) which approximate the day light duration.

Antarctic phytoplankton communities also experienced cell death induced by UVR (chapter 5), although the effect was not as severe as for pico-phytoplanktonic communities from tropical and temperate latitudes, and much longer half-life times were obtained for Antarctic communities (table 1). The sensitivity of diatoms and flagellates from Antarctica to UVR was expressed by a significant inhibition of the net population growth rates.

Lifespan for photosynthetic organisms has been described to be size-dependent, with lower lifespan expected as cell size decrease (Marbà et al. 2007). So phytoplankton organisms when exposed to UVR could probably experience larger cell death than predicted by this relationship (Marbà et al. 2007). In fact results obtained within this study revealed that lifespan for phytoplankton organisms is strongly reduced by adding UVR as a factor of mortality (Table 1). According to the calculations done in table 1, half-lives obtained for pico-phytoplankton under UVR exposure were, although lower, also related to size, with the largest reduction in half-life times obtained for *Prochlorococcus*, the smallest photosynthetic organism. Conversely, *Synechoccus* although its size is smaller than pico-eukaryotes presents slightly longer half-lives front UVR exposures (Table 1) than the pico-eukaryotes.

However, UVR half-lives obtained for larger phytoplankton organisms under UVR were not lower, when averaged, than the potential lifespan derived from their cell size (Table 1). Diatoms and flagellates half-lives under UVR were not clearly size-dependent (Table 1). Diatoms and flagellates here were communities integrated by organisms of different shapes and sizes, in definitive with a higher structural complexity, as reflected in the large SE of the UVR half-lives. Also the major morphological and genetic complexity of nano and microplankton may allow the development of a variety of capacities to overcome stressors as UVR, independently of the cell size.

Phytoplankton Community	Cell diameter size (μm)	Life span (h)	Averaged UVR half-life (h)	Min-Max UVR half-life (h)
Prochlorococcus	0.6	22	3 ± 1	1.7 - α
Synechococcus	1	36	11 ± 5.7	4.9 - α
Pico-eukaryotes	2	57	8.8 ± 7.5	2.6 - α
Flagellates	5-15	104 - 213	816 ± 412	11 - α
Diatoms	20-40	241 - 379	1827 ± 913	19 - α

Table 1. Comparison of life spans predicted for phytoplankton communities (calculations based on Marbà et al. 2007) and averaged, minimum and maximum half-life times obtained under UVR exposure in this study for the same phytoplankton communities. Life span was calculated from the equation ln (2)/D, where D was the cell mortality rate. Cell mortality rates were calculated from the regression equation $D= 0.0009\ M^{-0.22}$ (Marbà et al. 2007) in which M represented the individual mass of the phytoplankton organisms, as grams dry weight (W_c). Phytoplankton mass was estimated from biovolume estimates by using the equation $W_c=0.47\ V^{0.99}$ (Reynolds 2006) Volume of phytoplankton organisms (V) was calculated from cell sizes, by measurements at the microscope. *Prochlorococcus* and *Synechococcus* cell sizes were obtained from the literature (Morel et al. 1993). UVR half-lives in this study were obtained from the equation ln (2)/k, in which k was the cell decay rate obtained in the phytoplankton communities exposed to UVR within all the experiments and calculated as the slope of the linear regression between the natural logarithm of cell abundance and time. Min. values of UVR half-life times obtained for diatoms and flagellates correspond to only one data in which a decay of these organisms was detected. α as the max. UVR half-life value means no mortality.

Although increased in the actuality, due to the ozone loss, the exposure to certain levels of UV is natural for organisms living in the Earth. Millions of years of evolution have allowed the species to develop mechanisms to minimize the harmful effects of UV radiation, which will be efficient on certain radiation levels and in determined doses of exposure. So an important evolution has been done in the genetics of organisms to have protection against UV radiation. For example, reparation systems are present in all kind of cells, as much eukaryotes as prokaryotes, and have evolved until becoming very important in mammal cells; as for example human cells are predicted to suffer 500.000 damages a day in its DNA molecule but also is predicted they can repair their DNA around 8-15 times a day (Lodish et al. 2004, Browner et al 2004).

The high sensitivity of pico-phytoplankton to UVR obtained in this work, particularly for *Prochlorococcus*, is in concordance with studies predicting that UVR sensitivity should be related to cell size (García-Pichel, 1994). The small size of these pico-plankters is below the theoretical threshold size needed to accommodate enough sunscreen substances (García-Pichel, 1994). Also,

Prochlorococcus contains a limited genome lacking important genes for DNA reparation (Hess at al. 2001, Dufresne et al. 2005) that may preclude these species to resist UVR.

The second notable estimation of this work is the lethal radiation doses needed to decimate the phytoplankton populations to the half (LRD_{50}). The experiments performed using gradients of shading demonstrated that UVR doses were important as Irradiance it was. Populations were decimated to the same degree of accumulated doses, as much these doses resulted from short exposition periods at high irradiances as by a longer exposition time to reduced irradiances (Chapter 1). LRD_{50} were very low for *Prochlorococcus*, with an averaged value between experiments of 276 KJ m^{-2}. Conversely, higher values were needed to decimate *Synechococcus* and Pico-eukaryotic communities, with averaged values of 1044 and 783 KJ m^{-2} respectively. These lethal doses of *Prochlorococcus* will be equivalent to 0.3 days of UV exposure in the tropical and temperate areas, being 1.2 days for *Synechococcus*, which inform of the strong effect UVR may have on the daily variation of the populations. Lethal doses of pico-eukaryotes were also low, equivalent to 0.9 days, if growing in the tropical and temperate areas, and 1.7 days if growing under Antarctic UVR levels. These data indicated the small scale of time at which losses by UVR are generated on the pico-plankton.

LRD_{50} obtained for flagellates and diatoms from Antarctica are derived from only one experiment, and were 2445 and 10706 KJ m^{-2} respectively (Chapter 5). These doses will be equivalent to a minimum of 5 to a maximum of 16 days of exposure for flagellates and between 23 and 96 days for diatoms, which will imply that the role of UVR for the diatom dynamics in Antarctica will be irrelevant. In fact, the results from the experiments performed with the Antarctic communities indicated that UVR intensity was more important than accumulated doses of UVR for the total duration of the experiment. The inhibition percentage of phytoplankton biomass was well related to the average day Irradiance (Fig. 5, Chapter 5), but not to the accumulated doses. So during the Antarctic experiments, sunny and high UVR days were required to damage

diatoms and flagellated cells, but prolonging the experiments under low UV irradiance would have no consequences.

This study has also demonstrated the lethality of atmospheric hydroxyl radicals induced in natural phytoplankton communities (chapter 6). The exchange of OH radicals between atmosphere and seawater lead events of high OH radical concentrations to reach the surface upper layer inducing strong cell death on phytoplankton populations. The variety of tropical, temperate and Antarctic communities examined indicated that the higher sensitivity was observed for the pico-sized phytoplankton. *Prochlorococcus* was again the organism most vulnerable to the strong oxidant capacity of OH radical within the phytoplankters coinciding with its major sensitivity to direct UVR as we have explained above. OH radicals induced also cell death in *Synechococcus*, pico-eukaryotes, flagellates and diatoms with decay rates decreasing in concordance with increasing size and cell complexity of the different phytoplankton organisms, as we have described above for the direct effects of UVR. Although in this case differences between diatoms and flagellates were not significant. Half-lives obtained under OH radical effects for *Prochlorococcus* and *Synechococcus*, averaged values of 5.2 hours and 11.8 hours respectively, which are close to the half-lives calculated in this study for direct UVR (Table 1). This is indicating that OH radical, as an indirect effect of UVR, may be considered as an important factor causing phytoplankton cell death. The results provided represent a novel contribution to the impact of ROS on natural marine micro-algae since direct effect of OH radicals on phytoplankton cell death had never been measured before.

The significant cell death caused by UV radiation in phytoplankton organisms, reported in this study, point to the importance of the harmful effects of the ultraviolet radiation in the aquatic systems, in an environment extremely affected by the increased UV-B radiation levels due to the ozone loss. Actual predictions about ozone full recovery, based on the rhythm of CFC's disappearance in the atmosphere, is not expected to be reached before 2050-2065 (Weatherhead and Andersen 2006). However, as a consequence of emissions of other contaminants able to destroy ozone along with the negative

effect of global warming to the ozone recovery, these predictions are questioned. So as predicting trends in ozone recovery are unconvincing, high UV-B levels are expected to continue reaching the ocean waters and affecting marine ecosystems adversely. Thus, as demonstrated in this work, lethal effects on phytoplankton organisms due to UV-B radiation, with their consequences in the microbial food web, will be occurring for many decades. So the evidence of increasing UV-B radiation levels in oceanic waters urgently needs a quantification of its impact in the ecosystems. The impacts of increasing UV-B radiation levels in marine organisms have not been well evaluated yet. A recent study suggested that the influence of UV-B at the aquatic ecosystem level may be more pronounced on trophic level structure, than on biomass levels per se (Häder et al. 2007). However this assessment do not consider the important losses of phytoplankton cells and biomass presented here. The high increased UV-B levels in polar regions have motivated, however, few estimates of an overall impact in polar ecosystems (Agustí 2007, Dahlback 2002) that will be stressed by the extreme climate conditions to develop life in these areas. In fact, results from this study (Chapter 5) reveal that UV-B radiation is controlling significantly the Antarctic phytoplankton abundance and therefore is avoiding to reach the biomasses expected due to the high nutrient concentration present in Antarctic waters. Most calculations pondering the impact of UV-B consequences in the total production of aquatic ecosystems have been based on inhibition of photosynthesis and therefore on the reduction of phytoplankton production due to UVR (Smith et al 1992, Vincent and Neale 2000, Villafañe et al. 2004). However our results indicate that inhibition of phytoplankton biomass and losses due to cell death should be added to the reported negative effects of UVR on Antarctic phytoplankton. So UVR may be considered an important factor of phytoplankton losses, as well other significant losses such as grazing are considered.

All results obtained within this work reveal, in summary, the importance of ultraviolet radiation as a factor inducing cell death in phytoplankton organisms and influencing on their net growth balance. Concluding remarks are exposed in the following section.

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Conclusions

1. Solar UV radiation induces important cell death in pico-phytoplankton communities from the Atlantic Ocean. The degree of lethality of UVR in the pico-phytoplankton communities of the Atlantic Ocean was strongly dependent on the doses of UVR received.

2. Pico-phytoplankton communities present different sensitivities to solar radiation, being *Prochlorococcus* sp. the most sensitive among groups, with averaged half-lives of 3 hours. *Synechococcus* sp. appears to be the most resistant to UVR within pico-phytoplankters with averaged half-lives of 11 hours.

3. *Prochlorococcus* from the Mediterranean Sea also experienced high cell death under UVR. *Prochlorococcus* presents high decay rates even when UV-B radiation is excluded indicating a high effect of UVA and PAR in its cell mortality. *Synechococcus,* however, was highly resistant.

4. The high transparency of Mediterranean waters lead to a deeper penetration of UVR, resulting in experimentally calculated lethal doses (LRD50) for *Prochlorococcus* extending to 16 - 28 m depth, although for *Synechococcus* was above 10 m.

5. The high sensitivity of pico-phytoplankton to ambient levels of solar radiation appear to be a general phenomenon in the oligotrophic Ocean since natural populations of *Prochlorococcus*, *Synechoccoccus*, and Eukaryotes from the equatorial, tropical and temperate Central Atlantic Ocean and from the Mediterranean Sea declined when exposed to total solar radiation.

6. *Prochlorococcus*, *Synechococcus* and pico-eukaryotes communities present contrasting capacities to survive under high solar radiation. *Prochlorococcus* decay rates were dependent on the health condition of the original population, with lower decay rates when the % living cells was high. *Synechococcus* decay rates vary with latitude, being those from equatorial and inter-tropical regions more sensitive. Pico-eukaryotes sensitivity to solar radiation varied, however,

with the trophic degree, with the populations from low chlorophyll a concentration waters being less sensitive.

7. The cell digestion assay (CDA, Agustí & Sánchez 2002) applied to determine cell death in tropical and temperate phytoplankton needed a modification to be applied in phytoplankton communities from the Southern Ocean. The reduction of the assay temperature to 25ºC allowed its application in Antarctic waters.

8. Solar UV radiation also induces cell death in phytoplankton organisms from Antarctic waters but in minor degree than for pico-phytoplankton communities from the Atlantic Ocean and the Mediterranean Sea.

9. Phytoplankton populations in Antarctic waters appear to be strongly controlled by UVR, a positive relationship has been found between ambient UV irradiance and the percentage of inhibition in the phytoplankton biomass. Phytoplankton biomasses in Antarctic waters were inhibited by up to 80-90%.

10. The transference of hydroxyl radicals, formed by UVR, from the atmosphere to the surface ocean causes strong phytoplankton cell death in communities from subtropical, temperate and Antarctic waters. The higher sensitivity to OH among groups was observed for the pico-sized phytoplankton, being *Prochlorococcus* the population most affected.

11. *Prochlorococcus* appears to be the phytoplankton organism most vulnerable to solar radiation as demonstrated by its highest decay rates and shortest half-lives under direct and indirect effects of solar radiation.

12. UVR phytoplankton sensitivity is higher in the pico-sized plankton communities than in the larger communities, such as those from Antarctic, identifying cell size as an important factor determining phytoplankton sensitivity to UVR.

AGRADECIMIENTOS

Y parecía que no llegaría este momento…..

Quiero expresar mi más profundo agradecimiento a Susana, quien me introdujo en este "mundo" de la Ciencia, quien me ha guiado a lo largo de esta tesis y quien ha compartido conmigo su conocimiento científico y entusiasmo. Sin su apoyo este trabajo no hubiera sido posible. También mi agradecimiento a Carlos por darme la oportunidad para continuar en el mundo científico y por sus sabios consejos.

Muy en especial quiero agradecer a mis padres, hermanos (Bea, Gabi, Merce, Marta) & Co quienes siempre han estado siempre animándome en esta aventura.
A Gaspy, por el apoyo y afecto que me has dado siempre que lo he necesitado, por tu confianza y por aguardar siempre en mis idas y venidas, gràcies bitxo!!

A Patricia, quien me hizo entrar en contacto con la ciencia, y quien además de amiga se convirtió en compañera durante estos años. Gracias Pat, por estar siempre para ayudar y por hacer el día a día tan divertido.

También mi agradecimiento a todos los amigos y compañeros de faena, quienes hicieron la vida a bordo mucho más amena y divertida (Na Maria, Eva, Regino, Natalia, Antonio, Jordi, Isabel, Sabino, Juan, Laura,Txetxu …..), también en Esporlas (Juan Carlos, Rocio, Neus, Joselito, Pedro, Sebastien, Alexia, Raquel, Cris, Iris y toda la comunidad de becarios & Co.) Gracias a todos!
También quiero agradecer a gente con la cual he colaborado en diferentes proyectos (Gerhard, Dianne, Harriet, Anya, Dave, Jordi, Pep, Dolors, Javier, y otros), a toda la dotación del Bio-Hespérides, Pelagia, Surveyor y también a todos los UTM quienes siempre me han ayudado y hecho la vida más fácil tanto a bordo como en la base antártica.

A todos mis amigos que ajenos al mundo científico me han mostrado también su apoyo lo cual me ha ayudado muchísimo.

!!MUCHÍSIMAS GRACIAS A TODOS!!

Este trabajo de investigación es también una contribución a los proyectos COCA, ICEPOS, THRESHOLDS y BADE, fundados por el Plan Nacional de I+D español y por el Dutch Earth and Life Sciences (ALW-NWO).

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