Antioxidants, a radical solution?

Antioxidant responses of marine microalgae to ultraviolet radiation

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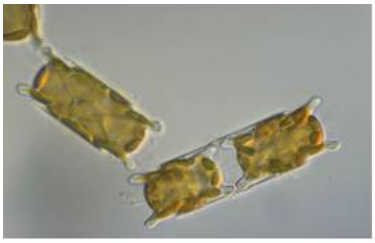
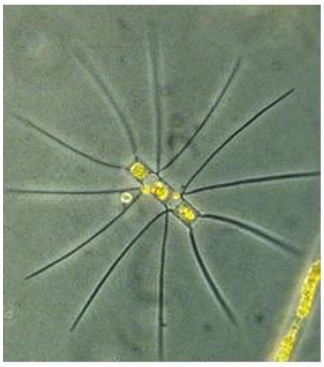
Chapter 1

Antioxidative responses of marine microalgae to ultraviolet radiation

General introduction






0. Introduction

Until just a few decades ago, the actual significance of marine microalgae to life on earth was highly underestimated. The proliferation of microalgae was thought to have a relatively small impact on human life, biodiversity and global nutrient cycles. Although algae share many properties with plants, such as their ability to photosynthesize, they live in a totally different environment. Nowadays, the realisation that algae have the capacity to influence practically all ecosystems including human life warrants extensive studies of their ecological and biogeochemical contribution. In addition, it is important to understand the physiological properties of microalgae and their responses to biotic and abiotic changes. The role of microalgae will be shortly discussed in the following three paragraphs. More extensive information can be found elsewhere (Graham and Wilcox 2000).

1. Marine microalgae

Virtually all sunlit waters around the world are inhabited by microalgae. Like plants, microalgae use photosynthesis to convert light into chemical energy, thereby fixing inorganic carbon into organic compounds. Herewith, microalgae are responsible for approximately 50% of earth's primary production (Zurzolo & Bowler 2001). Apart from the fixation of carbon dioxide, the process of photosynthesis also involves production and release of molecular oxygen (O_2). Furthermore, microalgae fulfil an important role in the cycling pathways of essential biochemical components and nutrients such as nitrogen, phosphorus, silicon and sulphur. Thus, algae hold a key position in the earth's biogeochemistry and are essential in supporting life on earth.

Since most microalgal species are primary producers, they form the base of the marine food chain. For protection against herbivory, phytoplankton species have evolved a variety of structures and forms such as extracellular spines, silicon and calcium carbonate shells, elongated shapes or gelatinous coatings. The fate of microalgae is such that generally most of their biomass is mineralised in the water column. However, a small portion may sink to the deep, dark zones of the ocean, where it remains biologically unavailable for thousands of years. As a result, billions of tons of atmospheric carbon dioxide have accumulated on the deep ocean floors during the past millions of years. Since this process causes overall oxygen production to exceed oxygen consumption, marine microalgae are for a great part responsible for maintaining a steady level of atmospheric carbon dioxide and molecular oxygen.

The combination of different environments, niches, habitats and trophic roles has diverged algae into a highly diverse group of organisms. Together, they comprise 36.000-50.000 described species, while at the same time estimates of 10.000.000 different species are given.

2. The rise of microalgae

It is generally believed that microalgae were the first compartmentalized (eukaryotic) cells on earth. They evolved 2.7 billion years ago when the atmosphere was rich in carbon

dioxide and poor in oxygen by which a broad range of chemical conversions was obstructed. Back then, life on earth only consisted of various groups of prokaryotes until the cyanobacteria obtained the ability to perform photosynthesis by which they utilized sunlight to fix the abundantly present carbon dioxide into organic molecules, thereby releasing oxygen as a by-product. Because the availability of oxygen was new to most organisms, it was tolerated only by a few organisms so that cyanobacteria were given the opportunity to produce more oxygen than could be utilized. In this way, an atmosphere was created in which the oxygen level could raise to around 21%. This formed the basis of three major changes for life on Earth:

1) Aerobic respiration became possible. Because of this, structural extension of the cell could be energetically supported and maintained. By using oxygen as a terminal electron acceptor much more energy could be released from a substrate compared to anaerobic respiration.
2) Biosynthesis of sterols. Sterols render cell membranes flexible, facilitating endosymbiotic incorporation of prokaryotes, which became persistently resident within the host cell. The combination of both, biosynthesis of sterols and aerobic respiration, seems to be responsible for the characteristic and required compartmentalization of key physiological processes in eukaryotic cells.
3) Generation of the stratospheric ozone layer. Hereby, life on the surface of the Earth became protected against the damaging effects of ultraviolet radiation (UVR; 200-400 nm), in particular UV-C (UVCR: 200-280 nm) and short wavelength UV-B (UVBR: 280 – 315 nm). This allowed algae to colonize surface waters as well as land surface that were previously kept sterile by UV radiation.

Even though the protective ozone layer prevents short wavelength UVR from penetrating the atmosphere, the hazardous UVBR and UV-A (UVAR: 315 – 400 nm) radiation still affect life on earth. In humans, UVR is responsible for several sun-related health problems such as sunburn, skin cancer and cataract (Madronich *et al.* 1998). In unicellular organisms like microalgae, effects can be deleterious as well. In contrast to multicellular life forms, most single cells cannot effectively shield themselves with special protective layers, have a disadvantageously surface/volume ratio and can be confronted with immediate death when its cellular machinery is damaged. Because of their geobiological importance, it is thus important to understand how microalgae deal with UVR and how they respond to increased UV radiation due to stratospheric ozone depletion and other features related with climate change, that affect irradiance exposure in marine environments.

3. Photosynthesis

Photosynthesis is earth's bio-assimilatory machinery, generating organic matter from inorganic compounds and sunlight. During photosynthesis, irradiance is absorbed by pigments and subsequently funneled to the photosynthetic reaction centers. To capture as

much solar energy as needed, chloroplasts are equipped with a very large surface area of thylakoid membranes in which huge quantities of pigment molecules are embedded. Chlorophyll *a*, the major light harvesting green pigment, is common to *all* photosynthetic eukaryotic cells. Because this molecule only absorbs a certain range of wavelengths, it is very often associated with accessory pigments which absorb energy that cannot be absorbed by chlorophyll *a*. The adjustable composition and amount of (accessory) pigments enables the organism to effectively trap a broad range of (changing) solar wavelengths falling onto the photosystems.

The excitation state of an electron is funneled towards the reaction centers of the photosynthetic apparatus. In the reaction centre of photosystem II (PSII), the captured energy is used to draw an electron from a water molecule which is passed on to a nearby acceptor molecule. Via a series of redox reactions, the electron is passed on to the protein complex present at the reaction centre of PSI during which protons are transported over the thylakoid membrane into its lumen. At the PSI reaction centre, the electron is re-exited by captured light energy to a still higher energy level. Via a second series or redox reactions, the electron is finally accepted by NADP to form NADPH. In addition, the electrochemical gradient of proton build up over the thylakoid membrane is used to support an ATP-ase driven phosphorylation of ADP into ATP (Fig. 1).

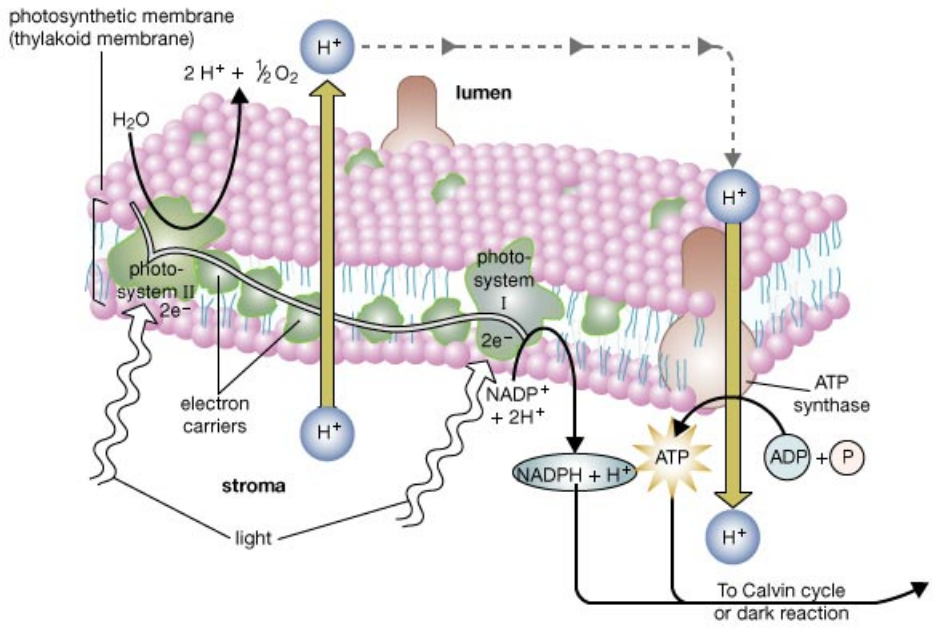


Figure 1: Light reaction of photosynthesis (from: Encyclopaedia Britannica, Merriam-Webster, inc. 2006).

Both NADPH and ATP are generated at the stromal side of the chloroplast where they are respectively consumed as a reducing and a bio-energetic agent in the Calvin Cycle. This cycle consists of a series of enzymatic reactions which catalyses fixation of carbon dioxide (C_1) to ribulose, 1-5, bisphosphate (RuBP; C_5) to form a C_6 compound which subsequently breaks down into 2 molecules of 3-phoshpoglycerate (C_3)- the building blocks of bioorganic molecules. Fixation of carbon dioxide is mediated by the enzyme ribulose, 1-5, bisphosphate carboxylase/oxygenase (Rubisco) which is also capable of oxidising RuBP. Finally, NADPH and ATP are used to regenerate a molecule of RuBP for the following cycle of carbon dioxide fixation.

4. Danger of excess irradiance and ultraviolet radiation

To live, microalgae have to be exposed to sunlight. Yet, excess photosynthetically active radiation (PAR; 400-700 nm) and UVR can be dangerous to microalgae. During exposure to excess irradiance, production of reduced equivalents (i.e. NADPH) can exceed their consumption by which electron transport chains (ETC) of the photosystems become overreduced. As a consequence, electrons will leak onto O_2, which will act as an alternative electron acceptor, thereby initiating the formation of reactive oxygen species (ROS; Mehler 1951, Asada *et al.* 1974, Gechev *et al.* 2006). Excess irradiance, and the accompanied formation of oxiradicals, may damage essential biomolecules which can cause viability loss and programmed cell death (Van de Poll *et al.* 2005, Gechev & Hille 2005). For instance:

1) ROS react with disulphide-bonds which form links between and within proteins. During this reaction, more radical moieties are generated which can initiate auto-oxidation of the protein.
2) Oxygen radicals are also able to attack the deoxyribose moiety of DNA what leads to the release of free bases. Hereby, the sugar backbone of the DNA molecule is left with a non-coding gap that can even lead to a strand break.
3) Most threatening is the ability of ROS to react with poly-unsaturated fatty acids (PUFA's) which make up a great part of the membrane structures. After this reaction, a carbonyl radical is formed which initiates a chain reaction of lipid peroxidation. As a result, membranes may become leaky, disintegrate and eventually will lose their integrity (Strid *et al.* 1994).

Environmental circumstances like the presence of UVR can stimulate the production of ROS (Mackerness 2000) and therewith increase the damaging effects of (excess) sunlight (Bischof *et al.* 2003). Hereby, absorbed UVAR can induce auto-oxidation of vital biomolecules like DNA and proteins, which may lead to interruption of metabolic pathways. In contrast, UVBR has direct detrimental effects on bioorganic molecules, thereby directly interfering with metabolic pathways and the process of photosynthesis. Especially Rubisco activity is susceptible to the damaging effects of UVR (Hazzard *et al.* 1997, Lesser *et al.* 1996, Bischof *et al.* 2002a). Eventually, inactivation of Rubisco or delayed supply of photosynthetic intermediates can lead to the obstruction of electrons flowing through the

photosystems, resulting in accumulation of reducing power and therewith enhancing ROS production (Halliwell 2006, Bischof *et al.* 2003).

5. Formation of radical oxygen species (ROS)

Aerobic life is inevitably accompanied by the participation of molecular oxygen in their metabolism. This especially applies to microalgae, which, besides the utilization of oxygen in mitochondria, also produce oxygen in the chloroplasts. Although the electron configuration implies reactivity of molecular oxygen towards other molecules, spin restriction (Pauli's exclusion principle) prevents this to occur by which it is relatively harmless to the cell (Apel & Hirt 2004, Taube 1965). To become biologically active, molecular oxygen can accept a single electron after which further reduction of the molecule to water occurs via a subsequent series of univalent electron transfer. It is the production of these oxygen intermediates, that is particularly hazardous to the cell (Mallick & Mohn 2000, Fridovich 1983). As described above, activation through single electron leakage especially occurs in chloroplasts with high concentrations of O_2 and a constant flow of electrons through photosystems, making them a principle site for ROS production (Pinto *et al.* 2003).

The first one-electron reduction leads to the generation of the superoxide radical ($O_2^{-\bullet}$; Yu 1994, Dat *et al.* 2000). This radical is only moderately reactive towards bioorganic molecules but could interfere with metabolic processes as it is able to reduce oxidized transition metal-ions (i.e. iron, cupper) present in many protein complexes. Consequently, redox-related processes get disrupted (Halliwell & Gutteridge 1984), which inevitably leads to overreduction of ETC's and enhanced $O_2^{-\bullet}$ production. Besides protein bound metal ions, $O_2^{-\bullet}$ is also able to reduce unchelated bivalent cations ($Fe^{3+/2+}$, $Cu^{2+/1+}$) which mediates electron transfer from one $O_2^{-\bullet}$ to another (Haber-Weiss/Fenton reaction) thereby generating hydrogen peroxide (H_2O_2). Both oxygen intermediates are moderately harmful but in the presence of unchelated bivalent cations, $O_2^{-\bullet}$ is also able to reduce H_2O_2 (Haber-Weiss/Fenton reaction) to form the biologically dangerous hydroxyl radical ($HO^{\bullet}$; Kehrer 2000, Cadenas 1989, Halliwell & Gutteridge 1984; Fig. 2).

Unlike its precursors, $HO^{\bullet}$ is one of the most reactive species known to chemistry and far too reactive to be controlled (Halliwell 2006, Fridovich 1978, 1998). Therefore, it is vital for any organism to prevent electrons from entering the Haber-Weiss/Fenton cycle and therewith to avoid $HO^{\bullet}$ formation. When not appropriately addressed, adverse effects can be expected, ranging from the temporary impairment of photosynthesis to viability loss, related with membrane damage due to lipid peroxidation (as described above; Van de Poll *et al.* 2006, Halliwell 2006).

6. Prevention of ROS formation

Since almost all organisms live in an oxygen rich atmosphere and most of the ROS target molecules are essential for life, strategies were developed to protect vital cell components against the damaging effects of oxygen. While some prokaryotes do this by simply avoiding contact with oxygen, eukaryote microalgae cannot do this as they require oxygen

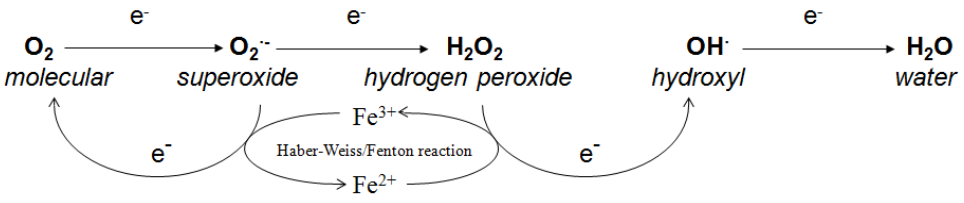


Figure 2: Formation of reactive oxygen species by single electron reduction. When molecular oxygen accepts a single electron, it becomes a superoxide radical. Successive steps of single electron reduction results in formation of respectively hydrogen peroxide, hydroxyl and eventually water. A bivalent cation catalyses transfer of electrons between the oxygen intermediates by concomitantly accepting and donating electrons. These reactions are also known as Haber-Weiss/Fenton reactions which play a crucial role in propagating the formation of reactive oxygen species.

for their metabolism. So, to avoid the potential destructive effects of oxygen, microalgae have developed mechanisms to protect them against the effects of high levels of visible, (PAR) and UV radiation.

6.1 UV absorbing compounds

Several algal species are known to synthesize PAR/UVR absorbing compounds. These include mycosporine-like amino acids (MAA's) which are mostly present at the periphery of the cell thereby preventing penetration of UVR further into the cell (Karentz *et al.* 1991a, Helbling *et al.* 1996, Buma *et al.* 2006). Yet, to be energetically feasible, MAA's are mostly produced by large microalgae as those cells have a smaller surface to volume ratio, thereby favouring effective screening (Garcia-Pichel 1994).

6.2 Pigmentation and xanthophyll cycling

When excessively absorbed light energy by the pigment antennae cannot be funnelled through the photosynthetic machinery, the energy flow towards photosystems can be tuned within a few days by adjusting the relative composition of the light harvesting and so-called photoprotective pigments (Photoacclimation; Falkowski & LaRoche 1991, Harris *et al.* 2005, Van de Poll *et al.* 2006). Then, on the regulatory level, excess excitation energy can be quenched as heat by activating the xanthophyll cycle (Demming-Adams & Adams 1992, Olaizola *et al.* 1994, Lavaud *et al.* 2002, Van de Poll *et al.* 2006). This cycle involves a certain group of carotenoids (xanthophylls), which can be interconverted enzymatically by which the molecular structures alternate between photosensitizing (harvesting) and photoquenching (protective) properties (Young & Frank 1996). In microalgae two different xanthophyll cycles can be found; either the diadino- diatoxanthin (DD/DT) cycle or the viola- anthera- zeaxanthin (VAZ) cycle of which the latter carotenoids (diatoxanthin, zeaxanthin) has the ability to quench excess excitation energy (Fig. 3). When a cell has regained its energy balance, the xanthophyll carotenoids are reconverted into their photosensitizing form (Young & Frank 1996).

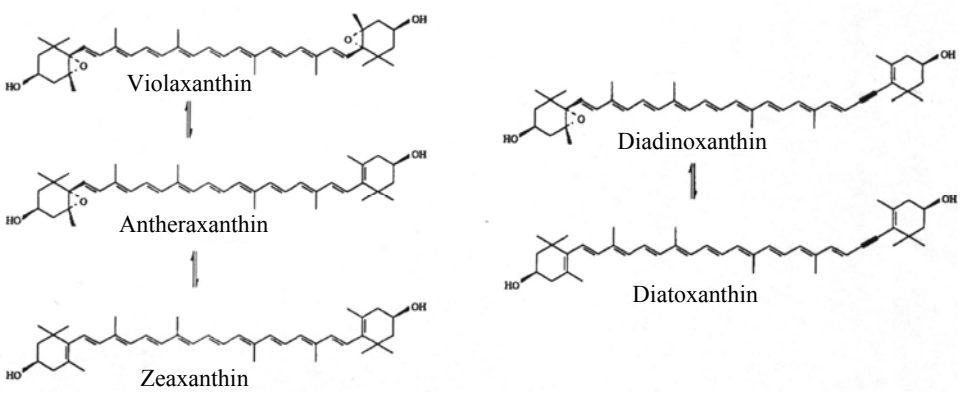


Figure 3: Molecule structures of carotenoids involved in two different xanthophyll cycles; the viola- anthera- zeaxanthin (VAZ) cycle and the diadino- diatoxanthin (DD/DT) cycle.

The epoxidized (photosensitizing) form is converted into the de-epoxidized (photoquencing) form by a de-epoxidase enzyme while the opposite reaction is catalyzed by an epoxidase enzyme. Regulation of epoxidase and de-epoxidase activity is thought to be coupled to the degree of acidification of the thylakoid lumen. During circulation of electrons through the photosystems, a proton motive force (PMF) is generated which drives the synthesis ATP out of ADP (see photosynthesis). When light absorption exceeds the rate of ADP regeneration, protons accumulate inside the thylakoid, leading to acidification of its lumen. The increased pH-gradient slows down the rate of proton translocation by which the flow of electrons through the photosystems decreases, thereby initiating ROS.

7. Scavenging of ROS

When the prevention of ROS formation is not adequate, the overreduced photosystems can become a major ROS source. Therefore, in order to counteract the production of ROS, cells are equipped with a complex network of enzymatic and non-enzymatic antioxidants which scavenge the reduced oxygen intermediates (Mallick & Mohn 2000, Asada 2006; Fig. 4). Because $HO^{\bullet}$ is too reactive to be effectively scavenged, the network is focused on neutralizing its precursors $O_2^{-\bullet}$ and H_2O_2 (Bartosz 1997, Halliwell 2006).

7.1 Superoxide dismutase

The first ROS formed, $O_2^{\bullet-}$, is scavenged by superoxide dismutase (SOD) which catalyzes the conversion of $O_2^{\bullet-}$ into H_2O_2 (Gregory & Fridovich 1973a, b; Klug & Rabani 1992; Fig. 4). This reaction has a 10 000-fold faster rate than spontaneous dismutation (Bowler *et al.*

1992; Fig. 4). Because SOD is the only enzyme capable of $O_2^{\bullet -}$ removal, and thereby prevents production of $HO^{\bullet}$, it holds a key position within the antioxidant network (Bowler *et al.* 1992).

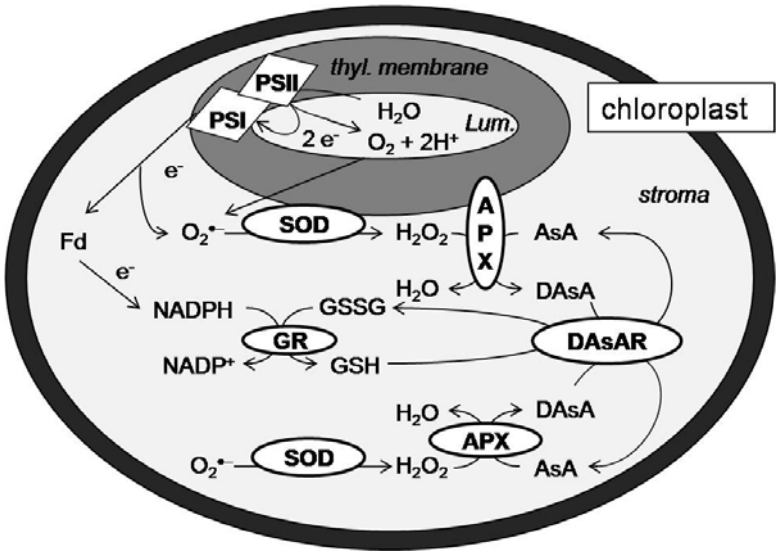


Figure 4: Antioxidant network in the chloroplast. When the electron flow from water to Ferredoxine (Fd), through Photosystem I and II (PSII and PSI) is obstructed, superoxide radicals ($O_2^{-\bullet}$) can be generated. Through a series of enzyme reactions (ovals), this radical is finally reconverted into water. Radicals generated at the site of membrane embedded complexes, are often scavenged by membrane (i.e. thylakoid) associated antixodants. In contrast, radicals generated within a hydrophilic environment (i.e. chloroplast stoma) are often scavenged by water soluble antioxidants. Abbreviations: SOD = superoxide dismutase; APX = ascorbate peroxidase; GR = glutathione reductase; AsA = ascorbate; DAsA = dehydroascorbate; DAsAR = dehydroascorbate reductase; H_2O_2 = hydrogen peroxidase; GSH = reduced glutathione; GSSG = oxidized glutathione; thyl membrane = thylakoid membrane; lum. = thylakoid lumen.

Its importance is also reflected in the amount of studies on microalgae describing the response of SOD on various ROS inducing stress effects (Table 1, section 9.2). The vast majority of these studies involve temperate marine microalgae whereas only a few report about responses of Antarctic species (Schriek 2000, Van de Poll *et al.* 2006).

Based on the metal co-factor, three major groups of SOD iso-enzymes (isozymes) are distinguished: iron-SOD (Fe-SOD), manganese SOD (Mn-SOD) and copper-zinc SOD (Cu/Zn-SOD). Until recently, only a few studies had determined SOD isozyme composition in microalgae (Asada *et al.* 1977, Okamoto & Colepicolo 1998). Because phospholipids are not permeable for the superoxide radical it is important that SOD is present within the compartments where the superoxide radical if formed (Takashashi & Asada 1983). Studies on plants have revealed that the three SOD isozymes are typically found in different

organelles; Fe-SOD in the stroma of the chloroplasts, Mn-SOD in the mitochondria and peroxisomes and Cu/Zn-SOD in the thylakoid membranes and cytosol (Slooten *et al.* 1995, Kliebenstein *et al.* 1998, Gómez *et al.* 2004, Wolfe-Simon *et al.* 2005). In eukaryotic algae, on the other hand, Cu/Zn-SOD is rarely detected and often replaced by Mn-SOD (Okada *et al.* 1979). Previously, the virtual absence of Cu/Zn-SOD was ascribed to early evolutionary processes (Asada *et al.* 1977, Wolfe-Simon *et al.* 2005, Lesser 2006). Yet, because results regarding SOD isozyme composition are not always unambiguous (Rao *et al.* 1996, Alscher *et al.* 2002) and because the number of studies is limited, SOD isozyme composition in microalgae needed further investigation.

7.2 Ascorbate peroxidase and Glutathione cycling

H_2O_2, formed by SOD activity, can diffuse out of the cell or be scavenged by a suite of other enzymes such as ascorbate peroxidase (APX; Shigeoka *et al.* 2002, Asada 2006). Because one of the major sources of H_2O_2 production is SOD, APX is mostly present at the sites of SOD activity. APX converts H_2O_2 into water during which it consumes ascorbic acid as a reducing substrate. Besides that, this low molecular weight (LMW) antioxidant also serves as an electron donor to regenerate other oxidized antioxidants, as well as a non-enzymatic ROS scavenger. Ascorbic acid itself can be regenerated though a series of different enzymatic reactions thereby using glutathione as a reducing substrate. Reduced glutathione (GSH) is a tripeptide (γ-glutamylcysteinylglycine) and is found in virtually all cell compartments. Like ascorbic acid, glutathione can also act as a non-enzymatic antioxidant. Because this LMW antioxidant forms the base of the ROS scavenging system, glutathione fuels the antioxidant network with reducing power (de Kok & Stulen 1993, Noctor *et al.* 2002). Oxidized glutathione (GSSG) is eventually reconverted into its reduced form by glutathione reductase (GR) which therefore is responsible for pumping the reducing power into the antioxidant network (Apel & Hirt 2004; Fig. 5).

Because glutathione plays an important role in ROS detoxification, changes in cellular pro- and antioxidants are reflected in the glutathione redox status (GRS; ratio of GSH over total amount of glutathione; De Kok & Stulen 1993, Blokhina *et al.* 2003). This has lead to the suggestion that glutathione might function as a ROS sensing and regulating agent (Wingate *et al.* 1988, Georgiou 2002, Noctor *et al.* 2002). Indeed there are indications in plants and microalgae that a decrease in GRS (as a result of ROS scavenging) causes upregulation of de-epoxidation activity of the xanthophyll cycle in order to constrain ROS production (Creissen *et al.* 1999, Xu *et al.* 2000). Also, there are studies reporting a negative relation between the change in GRS and the change in GR activity in plants, macro- and microalgae (Wingsle & Karpinski 1996, Karpinski *et al.* 1997, Shiu & Lee 2005).

8. Ecological consequences of climate change

Many studies have described the effects of excess irradiance including ultraviolet radiation (UVR) on photosynthesis. Judging from a wealth of *in situ* incubation studies primary production of phytoplankton in stratified surface waters is often inhibited by excess PAR. As a result of climate change phytoplankton irradiance exposure (intensity and spectral composition) may change significantly. For example, since stratification traps

phytoplankton in the upper water layer, excess irradiance exposure (PAR, UVAR, UVBR) may increase in those systems where stratification becomes more intense. This applies to areas characterized by reduced average wind speeds or increased input of melt water.

Regardless of the geographic location, UVR has been shown to substantially reduce primary production in most marine surface waters, with natural UVAR and UVBR often having similar relative proportions of inhibition (Helbling et al. 2001, Boucher and Prézelin 1996, Cullen et al. 1992). As a result of their detrimental action, UVR can reduce growth rates which can even eventuate in a complete cell cycle arrest of some species (Zhang *et al.* 2005, Rijstenbil 2003, Buma *et al.* 1996, 2000, Karentz *et al.* 1991b). Because of the breakdown of stratospheric ozone (Stolarski 1992), global UVBR radiation has been estimated to increase with at least 4% on a yearly basis (Madronich *et al.* 1998). So, an increased level of UVBR could significantly affect algal growth globally and therewith jeopardize fuelling of marine food chains.

Although there are several ways phytoplankton can deal with UVBR (effects), the actual response depends very much on species specific properties and their (evolutionary) irradiance history (Martínez 2007, Van de Poll *et al.* 2005, Helbling *et al.* 1992a). Because of this, increased UVBR could not only lead to decreased biomass, but also to changes in phytoplankton composition (Davidson & Belbin 2002, Villafane *et al.* 2004). In general, flagellates are more sensitive to UVR than diatoms (Villafane *et al.* 2004, Buma *et al.* 2001, Helbling *et al.* 1994) and small cells are more susceptible for UVR-induced DNA damage than large cells (Buma *et al.* 2001, Karentz *et al.* 1991b, Helbling *et al.* 2001b). As size and species-dependent nutritious value are crucial factors for grazers, any change in species composition could have a dramatic impact on food availability for organisms at higher trophic levels.

8.1 Consequences of Antarctic ozone depletion

Of all geographic regions, the stratospheric ozone layer over the Antarctic is affected the most. Hence, it is referred to as the Antarctic 'ozone hole' (Stolarski 1992). Under normal ozone column concentrations, the UVR-induced loss of integrated carbon fixation is estimated at 4.9% (Holm-Hansen *et al.* 1993). During springtime, over 50% of the ozone is lost which can lead to a 130% increase of UVR reaching the surface of the Earth, consisting particularly of the most damaging ultraviolet-B radiation (UVB; 280-315nm; Madronich *et al.* 1998, Smith *et al.* 1992). As a result, an extra reduction of integrated water column productivity of values up to 12% was estimated (Smith *et al.* 1992).

In contrast to temperate and tropical microalgae, Antarctic species are less likely to be adapted to high UV(B)R levels, since natural UVBR levels are relatively low (Helbling *et al.* 1992a, Villafañe *et al.* 2003, Martínez 2007). First of all, during winter, algae are exposed to extremely low intensities of solar radiation. So, at the start of spring, when sea ice starts to melt, they are acclimated to low irradiance conditions with virtually no UVR. Secondly, at the lower latitudes around the Antarctic, ambient UVBR is relatively low (compared to temperate, higher latitude regions) because of the low solar zenith angle (Boucher & Prézelin 1996a, Helbling *et al.* 1994). Therefore, through history Antarctic microalgae were not likely to be stimulated to invest in UV-protection mechanisms. On the other hand, it is likely that microalgae are exposed to higher levels of ROS as compared with algae inhabiting lower latitudes. As O_2 dissolves much better in cold seawater and

metabolism is generally slower, polar organisms are potentially more liable to ROS formation (Louanchi *et al.* 2001). Moreover, low temperature organisms contain more poly-unsaturated fatty acids (one of the main targets of ROS) which could potentially increase the risk of membrane disruption (Lesser 2006).

8.2 Influence of a fluctuating irradiance regime

For practical reasons, the majority of irradiance (including UVR) effect studies have been conducted under static conditions. Yet, in their natural environment, microalgae are almost always exposed to a fluctuating irradiance regime resulting from variations in intensity of incoming irradiance (daily and seasonally), cloud coverage, and vertical displacement through the water column (Helbling *et al.* 1994, Gautier *et al.* 1994, Lubin & Jensen 1995, Neale *et al.* 1998a, 2003). Vertical movement through the water column is strongly determined by wind-driven mixing of the upper water layer (Falkowski & Oliver 2007, Neale *et al.* 2003). As a result, cells have to deal with fast (hours to minutes) changing irradiance regimes ranging from excess to light limiting conditions. Due to climate change, regions (s.a. Antarctic Circumpolar Vortex) may become more deeply mixed by increased average wind speeds, thereby reducing irradiance intensities and exposure durations, but increasing irradiance dynamics.

When phytoplankton move through a shallow upper mixed layer (UML), cells are kept within the euphotic zone by which they are constantly exposed to fluctuating yet saturating amounts of PAR throughout a complete mixing cycle. In contrast to PAR, these cells do experience severe and quick changes in UVR as they may move through an UV-gradient from insignificant at the bottom of the UML to full exposure close to the water surface (Neale *et al.* 2003).

UVR effects depends on the balance between damage and repair. As UVR inhibits repair mechanisms, algae need a period with very low (or no) UV exposure to recover from the photoinhibiting damage (Neale *et al.* 1998a). Therefore, the effect of UVR on photosynthesis in a shallow UML is thought to depend very much on mixing speed (Neale *et al.* 1998a). In this case, slow mixing within the photic zone transports photoinhibited cells from near the water surface to deeper regions where recovery can occur. In a fast mixing cycle, the residence time at the bottom of the UML is shorter so that recovery may be less effective. So, cells exposed to a mixing regime need fast and readily available mechanisms to deal with the constantly changing irradiance conditions.

9. Microalgal responses to excess irradiance including UVR

As mentioned above, UVBR, as typically enhanced by ozone depletion, can depress growth significantly due to UVBR specific DNA damage, by which proteins involved in cell division cannot be transcribed properly (Gieskes & Buma 1997, Buma *et al.* 2000), and repair mechanisms cannot act efficiently. However, it has been found that microalgae can upregulate DNA repair activity (Buma *et al.* 1996, 2003), increase *de novo* synthesis of D1 photosystem II reaction centre protein (Ragni *et al.* 2008) and induce production of UV-absorbing compounds like MAA's (Buma *et al.* 2006, Helbling *et al.* 1996). Moreover, microalgae also have the ability to prevent and counteract irradiance induced generation of ROS.

9.1 Response of pigments and xanthophyll cycling

Studies regarding pigment responses of microalgae to high irradiance conditions have reported an increase in their protective/harvesting pigment ratio (Willemoës & Monas 1991, Mewes & Richter 2002, Lavaud *et al.* 2004, Van de Poll *et al.* 2005, 2007). Since growth rates can be strongly affected by UVR, the role of xanthophyll cycling in UVR protection is under discussion: protective/harvesting pigment ratios are not typically increased during UVR exposure (Zhang *et al.* 2005, Buma *et al.* 2000, Andreasson & Wängberg 2007). Furthermore, excess PAR enhances xanthophyll cycling by which its de-epoxidation state (DEPS; photoprotective xanthophyll/all xanthophyll) increases (Willemoës & Monas 1991, Mewes & Richter 2002, Van de Poll *et al.* 2005). Most studies did not find any UVR effects on xanthophyll cycling (Evens *et al.* 2001, Van der Poll *et al.* 2005, 2006). Yet, some observed UV induced decrease in the DEPS (Mewes & Richter 2002, Van de Poll *et al.* unpublished results) while Goss *et al.* (1999) found an increase.

When phytoplankton is exposed to wind induced mixing, cells have to deal with fast (hours to minutes) fluctuating irradiance conditions ranging from excess to limiting light conditions. Several studies have shown that cells obtain pigment characteristics of rather low irradiance acclimated cells (compared to static conditions with a similar irradiance dose) indicating that other protection mechanisms, like antioxidants, should become operative when residing under excess irradiance conditions (Van de Poll *et al.* 2007, Havelková-Doušová *et al.* 2004, Ibelings *et al.* 1994).

9.2 Antioxidant responses

There are many studies describing antioxidant responses to (increased) UVR in plants (Strid 1994, Willekens *et al.* 1994, Ledford & Niyogi 2005) and macroalgae (Aguilera *et al.* 2002a, b; Dummermuth *et al.* 2003, Shiu & lee 2005). In contrast, microalgae have been investigated rather poorly. Moreover, since those studies lack uniformity in exposure time, spectral composition, irradiance intensity, acclimation and growth condition, or species under consideration, it is virtually impossible to reach a satisfying consensus about their response (Refs see Table 1).

Table 1: Studies describing responses of superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), total amount of cellular glutathione ($Glut_{tot}$) and the glutathione redox status (GRS) to UVR. + represents an increase, - represents a decrease and 0 represents no response at all.

Publication	SOD	APX	GR	$Glut_{tot}$	GRS
Martínez 2007	+	0	+		
Bhargava et al. 2007	+	-	-	-	
Van de Poll *et al.* 2006	-				
Rijstenbil 2005	+	0	+		
Zhang *et al.* 2005	-				
Rijstenbil *et al.* 2002	+	0	+		-
Rijstenbil *et al.* 2001	+			+	-
Malanga *et al.* 1997, 1999	-				
Hazzard *et al.* 1997	0	+			
Lesser 1996a	+	+			
Lesser 1996b	+	+			
Malanga & Puntarulo 1995	+				

In addition, most studies were done under constant irradiance conditions, e.g. without addressing the more realistic, fluctuating light regimes. The scarcity of information and the absence of experimental uniformity becomes clearly visible when comparing antioxidant responses of different studies. Superoxide dismutase, a key enzyme in the antioxidant network, has been studied the most extensively (Table 1). The response of this enzyme to irradiance stress including UVR is ambiguous; some studies reported an increased activity while some found a down regulation or no change at all. SOD isozyme composition has been determined for a few microalgae only (Asada *et al.* 1977, Okamoto & Colepicolo 1998) but not in combination with environmental stressors such as UVR. Most surprisingly, merely two studies have been devoted to antioxidant activity and induction in Antarctic species of which only one describes the response of SOD to UVR (Van de Poll *et al.* 2006). Similarly, ascorbate peroxidase, glutathione reductase and glutathione show a wide variety of responses, but overall extremely little information existed about the functionality of these antioxidants in microalgae.

10. Outline of this thesis

As described above, no clear pictures existed of antioxidant responses in microalgae exposed to excess irradiance including UVR. Therefore, this thesis addressed three major questions:

1) **What is the role of antioxidants and xanthophyll pigments in excess irradiance protection, during and after photoacclimation?**
2) **How are microalgal antioxidant responses to excess irradiance, including UVR, related to habitat of origin, taxonomic origin, or cell size?**
3) **How are antioxidant responses involved in the acclimation process of microalgae of different taxonomic origin in a fluctuating irradiance regime?**

Since superoxide dismutase (SOD) holds a key position in the antioxidant network, this thesis first of all focused on responses of this particular scavenging enzyme. As SOD produces hydrogen peroxide, the coupling of SOD activity responses with other important antioxidant components were also investigated. In addition to ROS scavenging, microalgae can regulate the energy flow towards the primary site of ROS formation, the photosystems. Especially the interaction between antioxidants and the energy quenching xanthophyll cycle was of great interest since these pigments are able to constrain generation of ROS.

Considering their ecological significance, it was surprising that information on antioxidant responses, in particular SOD, in Antarctic microalgae was rare, while studies on responses related to (elevated) UVR were almost non-existing. This scarcity could possibly be explained by difficulties in obtaining sufficient biomass quantities for reliable SOD activity measurements and problems with performing activity measurements at low temperatures. In **chapter 2**, we compared and optimized existing cell harvesting methods and protein extraction procedures to obtain optimal biomass levels. Moreover, two

photospectrometric SOD enzyme assays were compared and adjusted to create a sensitive, reliably and feasible method for SOD measurements using the Antarctic diatom *Chaetoceros brevis*.

In **Chapter 3**, we used the renewed and optimalized method to investigate SOD activity responses of high irradiance acclimated *Chaetoceros brevis* when transferred to three different irradiance conditions: low irradiance, excess irradiance with UVR and excess irradiance without UVR. During four consecutive days, we followed SOD activity responses in conjunction with changes in pigment composition and membrane damage caused by ROS production. Herewith, we assessed the importance of SOD in photoacclimation processes as well as its protective role in damage control.

The Southern Ocean is characterized by deep, wind driven vertical mixing and low environmental iron concentrations. Therefore, algae have to deal with constantly changing irradiance conditions as well as iron deficiency. Acclimation to mixing requires the ability to rapidly respond to limiting and excess irradiance. Iron shortage, on the other hand, requires the ability to strategically distribute the limited amount of iron over the cell. So, as chlorophyll as well as photosystem I and Fe-SOD isozymes depend on the availability of iron, problems with acclimating to low as well as to high irradiance might occur. In **chapter 4** we transferred iron-limited *Chaetoceros brevis* cultures from a static to a fluctuating irradiance regime, and investigated responses of SOD activity in association with APX activity, pigment composition and viability.

Previous studies have suggested that antioxidative responses to high irradiance including UVR were related with geographic background, taxonomic background or cell size (described above). In **chapter 5** we examined these presumptions by exposing 15 species, ranging from small Antarctic flagellates to large temperate diatoms to excess irradiance including UVR.

In the previous chapters, laboratory set-ups were used to investigate microalgal responses to high irradiance including UVR. Yet, virtually nothing was known about microalgal responses under ambient solar irradiance conditions, let alone under fluctuating irradiance regimes. Therefore, the two final chapters described responses of temperate microalgae exposed to natural (irradiance) conditions in Patagonia, since Patagonian waters are characterized by strong wind mixing and high irradiance intensities. In **chapter 6**, natural occurring microalgal communities from the coastal waters of Patagonia (Argentina) were collected during a six weeks period and examined with respect to taxonomic composition, biomass and UVR-sensitivity. To investigate if antioxidants could explain the observed patterns as described in chapter 6, we investigated microalgal antioxidant responses under the physical conditions prevailing in Patagonia. In this study we exposed two distinct microalgal species from taxonomic groups dominating Patagonian coastal waters (diatoms, green flagellates) to ambient solar radiation including UVR and artificial mixing (**chapter 7)** Here, we assessed immediate (1 day), short term (3 days) and long term (7 days) responses on antioxidant and xanthophyll cycle activity and UVR sensitivity using two mixing speeds

Chapter 2

A comparison of quantitative and qualitative Superoxide dismutase assays for application to low temperature microalgae

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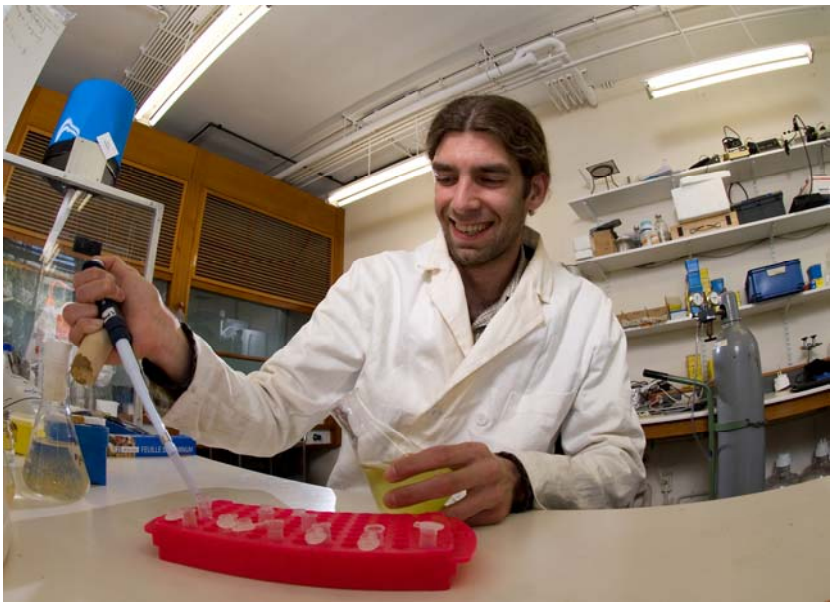

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Abstract

Antioxidant enzymes such as superoxide dismutase (SOD) play a key role in the removal of reactive oxygen species produced during visible and ultraviolet irradiance stress in microalgae and plants. However, little is known about the enzymatic antioxidative stress responses in ecologically important Antarctic marine microalgae. SOD in particular is difficult to analyze, possibly due to problems in obtaining sufficient quantities necessary for reliable and reproducible enzymatic assays.

The aim of the present work was to create a sensitive, easy-to-use and reliable method for SOD determination in Antarctic microalgal material by comparing and optimizing existing protein extraction procedures and SOD assays in the marine Antarctic diatom *Chaetoceros brevis*. Optimization was achieved in cell disruption (sonication) and protein extraction procedures, extraction buffers, SOD assay methods (Xanthine/Xanthine oxidase and NBT/riboflavin photometric quantitative methods and native gel electrophoresis qualitative method), and the assay temperature. Protein extraction was optimal at low sonication amplitudes after a few pulses, irrespective of the type of buffer used. Extraction efficiency varied highly between the tested buffers; most protein was extracted in the presence of 0.1% of Triton X-100. SOD activity was best quantified using the NBT/riboflavin method in combination with a buffer containing potassium phosphate and Triton X-100. Moreover, the NBT/riboflavin method was demonstrated to be the most reliable and sensitive method at low temperatures (5°C).

Introduction

Antarctic marine microalgae are known to experience high irradiance stress in situ. The extent to which microalgae are subjected to excess irradiance depends on factors like the intensity and spectral composition of incoming irradiance, position in the water column and the associated attenuation of irradiance (Helbling *et al.* 1994, Neale *et al.* 1998b, 2003). Natural solar ultraviolet radiation (UVR: 280 – 400 nm) can reduce water column productivity in Antarctic marine waters by more than 50% (Helbling *et al.* 1994, Boucher & Prezelin 1996a). Springtime Antarctic stratospheric ozone depletion causes an additional integrated productivity loss between 4 and 12% (Smith *et al.* 1992, Holm-Hansen *et al.* 1993, Helbling *et al.* 1994).

The detrimental effects of UVR-driven photoreactions to essential biomolecules can interrupt metabolic pathways and cause an overreduction of electron transport chains (ETC). When the consumption of reduced equivalents cannot match the production, electrons from ETC's can leak to molecular oxygen (O_2) and initiate formation of reactive oxygen species (ROS) (Mehler 1951, Asada *et al.* 1994, Gechev *et al.* 2006). ROS may damage photosystem II reaction center proteins by which photosynthesis could decrease and cause viability loss. (Van de Poll *et al.* 2005).

Microalgae have developed mechanisms that offer protection against high levels of solar radiation to prevent overreduction of ETC's. Yet, when these protection systems are not adequate, ETC's become overreduced and ROS will accumulate rapidly. As a response, cells can counteract ROS by using a sophisticated network of anti-oxidant enzymes which actively scavenge the various ROS intermediates (Mittler *et al.* 2000).

The first reactive oxygen species (ROS) formed is the superoxide anion ($O_2^{\bullet-}$). Through a series of uncontrolled univalent reductions, $O_2^{\bullet-}$ can be converted into the hydroxyl radical ($HO^{\bullet}$) which is among the most reactive oxygen species known to chemistry (Kehrer 2000). To prevent production of $HO^{\bullet}$ and oxidation of biomolecules, $O_2^{\bullet-}$ is converted into hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase (SOD; Gregory & Fridovich 1973a, b). SOD is not only the first line of defense but also the only enzyme capable of catalyzing this reaction. Therefore SOD holds a key position within the antioxidant network.

SOD has been shown to be present in all aerobic cells (Fridovich 1995). In plants, SOD varies in response to environmental stressors such as chilling (Lee & Lee 2000, Peltzer *et al.* 2002, Yuk *et al.* 2003), metal exposure (Iturbe *et al.* 1995), hypersalinity (Gomez *et al.* 2004, Parida *et al.* 2004) and UVR (Santos *et al.* 1999). In contrast, relatively little information is available on SOD activity in marine microalgae. Most of the studies are conducted with temperate marine microalgae, demonstrating alterations in SOD activity in response to metal exposure (Okamoto et al 1996, 1998, Li *et al.* 2005), salinity Jahnke & White 2003, Rijstenbil 2003), change in visible irradiance (lesser & Shick 1989a, Butow *et al.* 1997, Sigaud-Kutner *et al.* 2005) and UVR (Malanga & Puntarulo 1995, Lesser 1996a, b; Rijstenbil 2002, Zhang *et al.* 2005). Studies describing SOD responses in marine Antarctic microalgae are very scarce (Schriek 2000, Van de Poll *et al.* 2006), which is surprising because of the ecological significance of the mentioned excess irradiance responses.

The limited information on SOD activity in marine Antarctic microalgae is possibly related with the difficulties in obtaining sufficient biomass for reliable and reproducible

measurements. Methods so far used to measure SOD activity in microalgae are the Xanthine/Xanthine oxidase (X-XOD) (McCord & Fridovich 1969) and the NitroBlue Tetrazolium/Riboflavin (NBT/RF). The first one is used for photometric measurements only while the second is used also for Native PAGE (Beauchamp & Fridovich 1971). The commonly used X-XOD method (McCord & Fridovich 1969) requires such high quantities of biomass that obtaining reliable SOD assessments is virtually impossible for microalgal field material. In contrast, this method works fine for low temperature macroalgae as was demonstrated by Aguilera *et al.* (2002a, b) and Dummermuth *et al.* (2003). Own preliminary experiments with Antarctic microalgae showed, first of all, that standard cell disruption and protein extraction techniques were not satisfactory in terms of extraction efficiency. Secondly, the X-XOD assay did not seem suitable for application at the low temperatures required for analyzing Antarctic microalgal material. Therefore, it was the aim of the present work to find the optimal combination of cell disruption, protein extraction and SOD activity procedures at low temperature. To this end we tested a combination of various sonication procedures, protein extraction buffers and SOD assays. The material we used was derived from experiments with the common Antarctic diatom *Chaetoceros brevis.*

Materials & methods

Experimental procedures

Experiments were done using extracts of the marine Antarctic diatom *Chaetoceros brevis* (isolate CCMP 163). *C. brevis* was grown at 4°C and 25 µmol photons·m^{-2}·s^{-1} PAR (14L:10D cycle) in modified f/2 medium (Veldhuis & Admiraal 1987). Samples were harvested by filtration or centrifugation (see below for procedures) when the cultures contained approximately 1.10^5 cells·mL^{-1}. Samples were stored at –80°C and analyzed within 2 months. A comparison between filtration and centrifugation for harvesting biomass had shown that filtration onto 47 mm polycarbonate filters (2.0 µm pore size; Osmonics, USA) yielded 25% more material than centrifugation (1500 x g) for 20 min at 4°C (Falcan 6/300) (own unpublished results). Therefore, unless indicated otherwise (experimental series nr. 1), cells were harvested by filtration.

The following type of experiments were done: 1. effects of disruption procedures on protein extraction efficiency; 2. effect of four extraction buffers on protein extraction efficiency; 3. effects of four extraction buffers on SOD activity; 4. temperature dependence of SOD activity assays.

Cell disruption and protein extraction efficiency

To study disruption procedures, 48 tubes containing 50 mL of *C. brevis* culture were centrifuged after which supernatants were discarded. 24 cellular pellets were resuspended in 1.5 mL buffer A (Rijstenbil *et al.* 1994) containing 50 mM KH_2PO_4 (Merck, Darmstadt Germany), pH7 and 0.1 mM EDTA (Merck, Darmstadt Germany) and transferred to a 60 mL glass beaker. The other 24 cellular pellets were resuspended in buffer X (50 mM KH_2PO_4, pH7.8, 0.1 mM EDTA, 0.1% Triton X-100 (Aldrich, Steinheim Germany) and 2% PVP (Acros, New Jersey USA), modified from Gechev *et al.* (2003) and also transferred into a 60 mL glass beaker. To determine the effect of pulse frequency on protein extraction,

duplicates of 1 mL from both cell suspensions were sonicated applying a range between 1 and 5 pulses with 30 s intervals at a fixed amplitude of 25 µm (100W High Intensity Ultrasonic processor, Sonics and Materials Inc., Newtown, USA). The optimal disruption was determined for 1 mL duplicates by sonication at amplitudes between 30 nm and 90 nm with 2 pulses and 30 s intervals. Samples were kept on ice throughout the disruption procedures. Immediately after sonication the temperature of the extract was registered (Thermotime, Cresta, The Netherlands) and the protein content of the extracts were measured according to Bradford (1976) using BSA as protein standard.

Comparison of extraction buffers

To compare protein extraction efficiency, four extraction buffers were tested: Buffer A (Rijstenbil *et al.* 1994; 50 mM KH_2PO_4, pH7.8 and 0.1 mM EDTA. Buffer B (50 mM KH_2PO_4, pH7.8, 0.1 mM EDTA, 0.1% Triton X-100); Buffer X (50 mM KH_2PO_4, pH7.8 and 0.1 mM EDTA, 0.1% Triton X-100, 2% PVP and Complete protease inhibitor cocktail (Roche, Mannheim Germany)); and Buffer Y (Gechev *et al.* 2003; 50 mM KH_2PO_4, pH7.8 and 0.1 mM EDTA, 0.1% Triton X-100, 2% PVPP (Sigma, Steinheim Germany) and Complete protease inhibitor cocktail. For this experiment as well as experiments 3 and 4, approximately 2.10^6 cells were harvested by filtration (polycarbonate filters, 2.0 µm pore size; Osmonics, USA). The cells were disrupted by sonication (2 x 30 s, at A=30) at 4°C in 1.5 mL of one of the four buffers. All crude extracts were centrifuged for 20 min (20.000 x g) at 4°C after which the supernatants were collected (n=3). 150 µL of the supernatant was used for quantifying the protein concentration in triplicate according to Bradford (1976).

Comparison of 4 different protein extraction buffers on SOD activity

Aliquots of supernatants, obtained as described above, were used for determining SOD activity applying both the Xanthine/Xanthine oxidase (X-XOD) assay (McCord & Fridovich 1969) and the Riboflavin/NitroBlue Tetrazolium (RF/NBT) assay (Beauchamp & Fridovich 1971, Fryer *et al.* 1998). Both indirect methods involve the inhibition of respectively Cyt-c and NBT reduction. In the X-XOD method, SOD competes with cyt-c for $O_2^{-\bullet}$ generated by the Xanthine/Xanthine oxidase system. *SOD activity* is defined as the amount of sample required for 50% inhibition. Cyt-c reduction was measured for 3 min at 4°C in a 1.5 mL assay mix containing SOD buffer 1 (50 mM KH_2PO_4 and 0.1 mM EDTA at pH 7.8) 10 µM Cyt-c (Sigma), 50 mM Xanthine (Sigma, Steinheim Germany)) and XOD (Sigma, Steinheim Germany) at 550 nm on a Cary 3E UV/vis double beam spectrophotometer (Varian, Middelburg, The Netherlands) equipped with a temperature controlled cell attached to a water bath. The blank coloration slope was 0.025A.U/min. For activity determination in extracts, extract volumes of sample (V_{sample}) were added such that an inhibition (I) between 45-65% was achieved. The volume of sample needed to obtain 50% inhibition (V_{50}) was then calculated according to: $V_{50} = V_{sample}$*50% / I. Specific activity (*S.A)* was calculated as 1 / (V_{50}*[*protein*]). In the NBT/RF method, SOD competes with NBT for $O_2^{-\bullet}$ generated by the RF under illumination. NBT reduction was measured (in duplicate) in 5 reaction mixtures containing 0, 12.5 µL, 25 µL, 50 µL and 100 µL extract. The 1.5 mL reaction mixtures contained SOD buffer 2 (50 mM KH_2PO_4, pH 7.8, 0.1 mM EDTA, and 0.25% Triton X-100), 2 mM riboflavin (Sigma, Steinheim Germany) and 57 µM NBT (Sigma, Steinheim Germany). Reaction mixtures were incubated in the

dark for 30 min at 4°C. Since generation of superoxide radicals in the NBT/RF assay is driven by light, samples were subsequently illuminated from above for 15 min by 4 fluorescence tubes (Philips TLD/18W, 30cm distance) giving 199 µmol photons·m^{2}·s^{-1}. Afterwards, absorbance was measured at 560 nm on a Cary 3E UV/vis double beam spectrophotometer. Fifty percent inhibition was calculated by regression using the linear part of a natural semi-log curve after which the *S.A.* was calculated as described for the X-XOD method.

SOD activity was visualized using native 12% polyacrylamide gel electrophoresis (Native PAGE). Samples containing 15 µg protein were loaded onto a gel using 5 µL protein loading buffer. After electrophoresis (80 V), activity of SOD in the gel was visualized by the staining procedure described by Beauchamp & Fridovich (1971) and Parida *et al.* (2004). The gels were soaked in 35 mL staining buffer containing 0.25 mM RF, 5 mM NBT, and 0.5 mM TEMED (Amresco, Solon USA) for 30 min. in the dark at 4°C and subsequently illuminated from above for 15 min by 4 fluorescent tubes (18W each, 30 cm distance) giving 199 µmol photons·m^{2}·s^{-1}. SOD bands appeared as light bands on a blue/purple background. The reaction was stopped by rinsing with Milli Q.

SOD activity assays at different temperatures

Both quantitative assays were performed as described above at temperatures of 6°C, 12°C, 18°C, 24°C and 30°C. Regarding the X-XOD method, temperature dependence was determined by the activity of XOD required to obtain a blank slope of 0.025A.U./min. Measurements were done in triplicate. Generation of superoxide radicals in the NBT/RF assay is driven by light. To determine the effect of light intensity besides the effect of temperature, SOD measurements were performed under three different light intensities for each of the 5 different temperatures. Therefore, the reaction mixtures were incubated at the designated temperature in a water bath for 10 min prior to irradiation and kept within the water bath during illumination from above with 133, 166, and 199 µmol photons·m^{-2}·s^{-1}. The three irradiance intensities were applied by positioning the reaction mixtures at three different distances from the 4 fluorescent tubes. Irradiance was measured with a spherical light meter (QSL-100, Biospherical Instruments) in air. Measurements were done on 4 replicates.

Statistical analyses were done on all results using standard One way ANOVA significance tests. Error bars represent standard deviations.

Results

Disruption of cells

Applying an increasing range of sonication amplitudes resulted in enhanced extract temperatures regardless of the buffer used. Despite the fact that extracts were sonicated on ice, temperatures increased dramatically with increasing pulses, frequency and amplitude: up to 25 and 50°C at the highest pulse frequency and amplitude levels, respectively (Fig. 1). At the same time, the concentration of protein did not increase with increasing amplitude irrespective of the buffer used. Moreover, the protein concentration decreased significantly when applying sonication amplitudes of 60 µm or higher, probably due to the destructive effects of heat on protein integrity. Applying two sonication pulses of 30 seconds at 30 µm amplitude already resulted in obtaining a maximum amount of extracted protein.

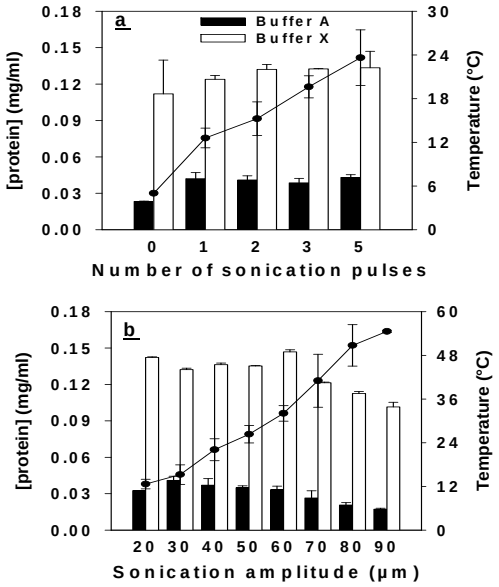

Figure 1: Effects of sonication on cell disruption efficiency and extract temperature. (**a**) effect of sonication pulses and (**b**) effect of sonication amplitude. Cell disruption procedures were tested in extraction buffer A (dark bars) and X (white bars) and expressed in amount of protein extracted. Extract temperature is indicated by the black dots.

Extraction buffer

Because of its membrane dissolving capacity, the presence of Triton X-100 in Buffer B, X and Y contributed significantly ($p<0.05$) to the protein extraction efficiency (Figs. 1, 2c). On average, Triton X-100 (Buffer X) yielded between 2 and 2.5 times more protein than the phosphate buffer (Buffer A) alone and was efficient without cell disruption by sonication

(Fig. 1a). Addition of the matrix forming molecules PVP and PVPP decreased the extraction efficiency of proteins significantly ($p<0.05$) by approximately 20% (Fig. 2c).

Protein extraction buffer and SOD activity

V_{50} values of the X-XOD method were nearly 10 times higher than the V_{50} values of the NBT/RF method, regardless of the buffer used (Figs. 2a, b). Differences between the V_{50} values within each assay were small when the four protein extraction buffers were compared, but were only significant ($p<0.05$) in combination with the NBT/RF method. Despite these differences, they were minor compared to the differences between the protein extraction efficiency of the four buffers. As a result, the large variation in specific SOD activities (SA) could rather be explained by differences in protein extraction efficiency between the four buffers (Fig. 2c), than by differences in V_{50} values (Figs. 3a, b). This was also confirmed by Native PAGE (Fig. 3c).

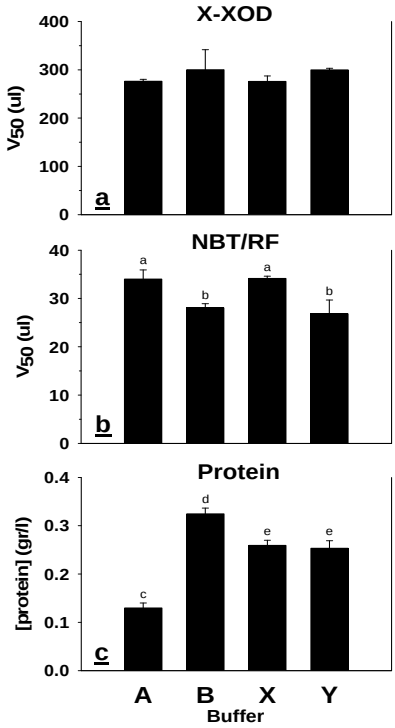

Figure 2: Effect of extraction buffers A (sole phosphate buffer), B (phosphate and Triton X-100), X (phosphate, Triton X-100 and PVP) and Y (phosphate, Triton X-100 and PVPP) on the amount of protein required for 50% inhibition (V_{50}) and protein extraction efficiency. (**a**) V_{50} values using the Xanthine-Xanthine oxidase (X-XOD) method. (**b**) V_{50} values using the Riboflavine/Nitroblue Tetrazolium (NBT/RF) method. (**c**) Effect of extraction buffer on protein extraction efficiency.
a: differs significantly from buffer B and Y ($p>0.0005$); b: differs significantly from buffer A and X ($p>0.0005$); c differs significantly from buffer B, X and Y ($p>0.0005$); d differs significantly from buffer A, X and Y ($p>0.0005$); e: differs significantly from buffer A and B ($p>0.0005$).

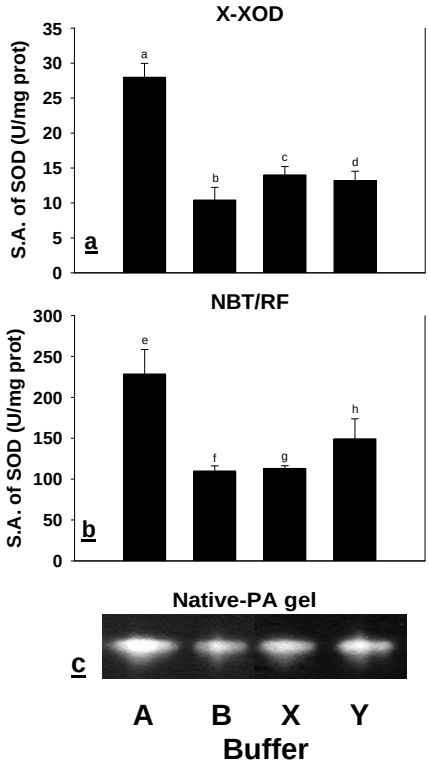

Figure 3: Effect of protein extraction buffers A (phosphate buffer), B (phosphate and Triton X-100), X (phosphate, Triton X-100 and PVP) and Y (phosphate, Triton X-100 and PVPP) on specific activity of SOD measured by the Xanthine-Xanthine oxidase (X-XOD) method (**a**), the Riboflavine/Nitroblue Tetrazolium (RF/NBT) method (**b**) and visualized on Native polyacrylamide (Native PA) gel (**c**). 20µg of protein was applied on gel. a differs significantly from buffer B, X and Y ($p>0.005$); b differs significantly from buffer A and X ($p>0.05$); c differs significantly from buffer A and B ($p>0.05$); d differs significantly from buffer A ($p>0.0005$); e differs significantly from buffer B, X and Y ($p>0.001$); f differs significantly from buffer A and Y ($p>0.05$); g differs significantly from buffer A ($p>0.0005$); h differs significantly from buffer A and B ($p>0.05$).

Temperature dependence

Because the X-XOD method uses the enzyme Xanthine oxidase (XOD) to generate superoxide radicals, this assay depends on temperature and requires a higher concentration of XOD at lower temperatures to obtain a similar activity slope as compared to the same assay at 25°C (Fig. 4a). In contrast, temperature marginally affected the NBT/RF method (Fig. 4b). Because superoxide radical formation is based on a photochemical reaction, this method was indeed found to be more dependent on irradiance intensity than on temperature (Fig. 4b). The SOD activity of *C. brevis* showed no significant dependency on temperature at all, regardless of the method used(X-XOD: slope=-0.3234, r^2=0.3628; NBT/RF: slope=-

0.1622, r^2=0.0041), although significant ($p<0.05$) variability was observed. This was confirmed by Native-PAGE (data not shown).

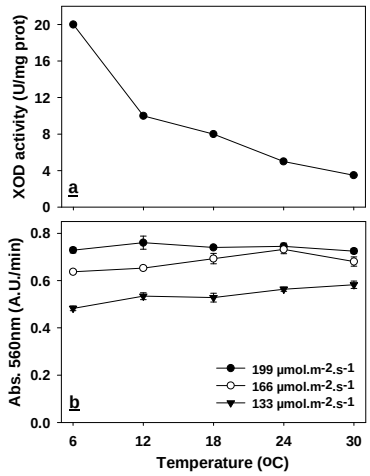

Figure 4: Temperature dependence of the SOD activity assay. Temperature effect on the Xanthine-Xanthine oxidase (X-XOD) method indicated as the amount of Xanthine oxidase (XOD) activity required to obtain a slope of 0.025A.U./min (**a**). Temperature effect on coloration of blanks using the Riboflavine/Nitroblue Tetrazolium (RF/NBT) method at 3 different irradiance levels (**b**).

Discussion

Studies on microalgal SOD responses, especially those on polar microalgal species are virtually non-existent. An important probable cause for this could be the difficulty in obtaining sufficient biomass required for the SOD activity assays. There are several ways to assess microalgal biomass, such as chlorophyll concentration, carbon concentration, or cell number, but most of them are subject to large variations caused by species specific differences and environmental circumstances. Therefore, in our study, we used protein concentration as a measure of biomass because it exhibits less variation when compared with other biomass parameters.

Specific activity of SOD is typically normalized to the amount of biomass like protein or cell concentration. Earlier studies showed that high protein concentrations or high cell numbers derived from large culture volumes (up to several hundred mL) were needed for a reproducible SOD assessment, regardless of the assay used (Lesser 1996b, Rijstenbil 2002, Sigaud-Kutner *et al.* 2002, Peers & Price 2004). Therefore, the low cell densities typically found in the field pose detection and accuracy problems to either protein harvest and/or SOD activity measurements. Thus, it is essential to optimize cell collecting and disrupting procedures. Because filtered cultures of *C. brevis* yielded 25% more protein than centrifuged cultures (data not shown) cells were standardly harvested by means of filtration, except in the case of the cell disruption experiments. Here, cells were harvested

by centrifugation after which the pellets were resuspended and joint together in the designated extraction buffer. In this way identical cell concentrations, as required for the cell disruption tests were obtained. This was not possible using filter derived material that showed a slight variability in cell concentration. For the cell disruption experiment, only buffers A (a simple phosphate buffer) and X (protease inhibitor, Triton X-100 and PVP), were compared which differed most in composition. Protein extraction was shown to be most effective using buffer X applying 2 sonication pulses at an amplitude of 25 µm (Fig. 1). Although buffer X yielded higher protein concentrations applying 2 pulses at an amplitude of 60 µm or 5 pulses at an amplitude of 25 µm, the temperature of the extract raised as well (up to 35 and 25°C respectively). In general, an increased number of pulses did not result in increased extraction efficiency. Moreover, an increase in sonication amplitude even reduced protein concentrations: in buffer A this was initiated by amplitudes over 40 µm whereas for buffer X this was initiated by amplitudes over 70 µm. Likely, increased extract temperatures at elevated amplitudes cause denaturation, followed by aggregation and precipitation, especially of low temperature proteins. In addition, because buffer X contains Triton X-100, denaturated proteins remain in solution, even at higher temperatures that would otherwise cause aggregation and precipitation.

Concerning the efficiency of the tested protein extraction buffers, buffer B was most efficient yielding 60% more protein than buffer A and 20% more than buffer X and Y. This can be explained by the presence of Triton X-100 which enables buffers B, X and Y to dissolve membranous structures to release membrane associated or enclosed proteins. It must be emphasized that the Triton X-100 strength needs to be well below 0.5% (in this study 0.1%) in order to avoid interference with Coomassie dye. In contrast to Triton X-100, the presence of PVP and PVPP had a negative effect on the amount of extracted protein. Their purpose is to indiscriminately abstract phenolic and polyphenolic impurities from protein extracts which could otherwise affect activity assays. However, PVP/PVPP not only precipitates interfering phenols but also proteins containing amino acids with phenolic residues (i.e. tyrosine, phenylalanine). On the other hand, although buffer B, X and Y yielded more protein, the presence of more chemical constituents (compared with Buffer A) could also affect SOD functioning and activity. Therefore, SOD assays were tested for all four buffers. These tests showed that the amount of extract required to obtain 50% inhibition (V_{50}) were rather similar (discussed below) when considering the four buffers (Figs. 2a, b). Evidently, SOD is (almost) entirely present in the cytosolic fraction of the cell. As a result, calculated specific SOD activities, typically normalized to protein, revealed lower values when applying more efficient protein extraction buffers, such as Buffers B, X and Y, simply because of higher protein concentrations obtained in these buffers (Fig. 2c). So, it is important to realize that Triton X-100 does not stimulate SOD extraction but it enhances overall protein extraction efficiency and therewith the accuracy of SOD activity calculation of low biomass samples.

Both qualitative SOD assays measure activity in an indirect way, using a reducible coloration agent as an activity reporter. This makes both methods highly susceptible for interference with non-SOD substances which are able to react with $O_2^{-\bullet}$ radicals or reduced as well as oxidized coloration agents. Because SOD activity is measured differently in both assays, the interference of non-SOD substances is also expressed in a different way. Since most non-enzymatic superoxide scavengers interfere with the SOD activity assay immediately after superoxides are generated, the cytochrome-c inhibition slope started very

irregular and was determined after 30 seconds following Xanthine oxidase addition. Therefore, despite the presence of reactive substances, these SOD activity values were found to have very low standard deviations. The RF/NBT method on the other hand measures SOD activity via the inhibition of NBT reduction against the sample concentration. Here, accumulated reduced NBT is measured by which these values not only represent SOD activity but include non-enzymatic scavenging of superoxide radicals as well. Up till now it seems unfeasible to distinguish between enzymatic and non-enzymatic inhibition of NBT reduction. Therefore, to minimize the contribution of non-enzymatic substances (i.e. metal ions, reducing equivalents), the amount of sample used is kept relatively low (compared to the X-XOD method). Also, because non-enzymatic superoxide scavengers react with the generated $O_2^{-\bullet}$ radicals immediately after its production, the reaction time is set on 15 minutes by which the enzymatic proportion is increased. As a result of both conditions, the contribution of the enzymatic superoxide scavenging activity is optimized while the inhibition of NBT reduction stays within its detection limits. Finally, (poly)phenolic compounds are able to produce $O_2^{-\bullet}$ or reduce NBT and thereby could strongly interfere with SOD activity. This would underline the significance of adding PVP or PVPP to the extraction buffer. However, this was not supported by our experiments, because the application of buffers X and Y (containing PVP or PVPP) in both SOD assays, did not significantly reduce V_{50} values. (Figs. 2a, b). Therefore, buffer B, containing Triton X-100 only, is most suitable for SOD activity measurements in *C. brevis*. Yet, it is advisable to test the need for PVP/PVPP when using other (microalgal) species.

The V_{50} values did not significantly differ between the four buffers, when using the X-XOD method. The V_{50} values for the NBT/RF method on the other hand varied slightly but significantly, with highest values for Buffers A and X. Generally, the X-XOD method required higher extraction volumes (275 - 300 µL) for a V_{50} inhibition than the NBT/RF method (27 – 34 µL). In terms of *C. brevis* protein this means that the X-XOD method required at least 36 µg protein to obtain 50% inhibition while the RF/NBT method was accurate when applying 3.5 µg protein or less. Thus, reliable and sensitive SOD activity measurements were more dependent on the assay used than on the applied protein extraction buffer. Because of the above mentioned difficulties in obtaining sufficiently high microalgal biomass, their SOD activity is best measured using the NBT/RF method. This method was also found to be less sensitive to temperature than the X-XOD method, although the observed lower XOD activity at lower temperatures could simply be counteracted by increasing the amount of XOD (Fig. 4). Surprisingly, regardless of the method used, the enzyme activity of *C. brevis* SOD did not reveal an optimum at lower temperatures pursuant to its growth optimum (data not shown).

Besides these two quantitative methods, SOD activity can also be detected by Native-PAGE, which is a qualitative method showing species specific SOD isozyme composition and their relative activity. This method is an excellent tool to visualize relative changes in SOD activity as well as pinpointing which isozyme species causes that change. Yet, the variety in isozyme composition makes it difficult for Native-PAGE to compare overall SOD (isozyme) activities between species. Also different species with similar values of SOD activity can show different intensities of SOD activity on a Native PAGE without obvious reasons. Because of both properties, Native-PAGE is primarily restricted to determine intraspecific, qualitative differences. Native-PAGE separating extracts of *C.*

brevis showed that applying an amount between 5 µg and 20 µg protein gave an optimal resolution to detect differences in activity (Fig. 5). The presence of one visible band indicates that one particular isozyme is predominant but that does not exclude the presence of more SOD isozymes. In conclusion, quantifying the band density cannot be used adequately as a measure for SOD activity.

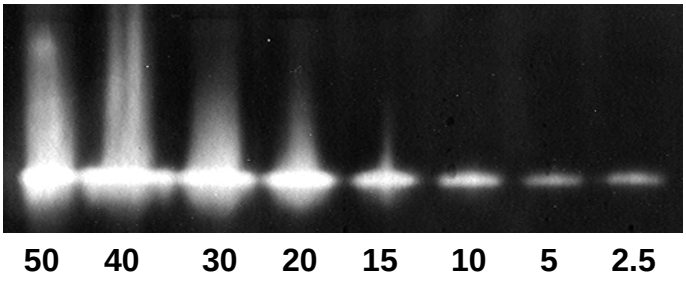

Figure 5: Enzymatic SOD activity applying on a 12.5% Native polyacrylamide gel. Numbers indicate the amount of protein applied (µg).

Comparing existing data on SOD activity in microalgae is difficult because of the broad range of microalgal species used, the use of different biomass extraction procedures and the application of different SOD assays. In addition, various studies normalize SOD activity to cell number or Chl.*a*, although most studies normalize to protein as initially proposed by McCord & Fridovich (1969). Virtually all studies involve temperate to tropical microalgal species (Lesser 1996a, b; Rijstenbil 2003, Sigaud-Kutner *et al.* 2005, Zhang *et al.* 2005) whereas only a few report about SOD activity in polar microalgae (Schriek 2000, Van de Poll *et al.* 2006). Values of SOD activities range between 2 U/mg prot and 2300 U/mg protein for studies that applied the X-XOD method (Butow *et al.* 1997, Peers & Price 2004), and between 10 U/mg prot and 620 U/mg prot for studies that applied the NBT/RF method [Okamoto *et al.* 1996, Sigaud-Kutner *et al.* 2002). So far, there does not seem to be an obvious trend with higher SOD activity values for the NBT/RF assay as compared with the X-XOD assay, as found in our study.

We also measured SOD activity in numerous other Antarctic (*Phaeocystis antarctica*, *Thalassiosira antarctica*, *Chaetoceros dichaeta*) and temperate (*Thalassiosira weissflogii* and *Emiliania huxleyii*) microalgal species, which were cultured under similar irradiance conditions as *C. brevis*. Moreover, a natural community of benthic diatoms from the Dutch Wadden Sea was tested as well (data not shown). In all these samples, the amount of biomass was insufficient to measure SOD accurately using the X-XOD method but instead could be measured using the NBT/RF method (values varying between 83 – 95 U/mg prot). So, besides *C. brevis,* the NBT/RF method can also be used for other polar as well as temperate microalgal species.

In conclusion, our study shows that the NBT/RF assay is the most reliable assay for measuring SOD activity in marine (Antarctic) microalgae within a large temperature range. The addition of Triton X-100 (< 0.5%) to standard phosphate buffers increases protein extraction efficiency, thereby increasing the accuracy of the SOD activity calculation. Studies using protein extraction buffer without Triton X-100 overestimate the SA of SOD at least 2 times compared to the actual value, according to our study. A constant systematic discrepancy between total and cytosolic protein could adjust existing SOD activity values but since there is no fixed ratio between total/cytosolic protein (because of difference in size, amount of metabolic enzymes, quantity of photosynthetic associated proteins) this seems unfeasible. Finally, with respect to future comparisons, the large differences in calculated SOD activities when using different protein extraction or SOD assays, corroborate the suggestion that SOD activity measurements in marine microalgae should be standardized.

Chapter 3

Oxidative stress responses in the marine Antarctic diatom *Chaetoceros brevis* (Bacillariophyceae) during photoacclimation

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Abstract

The enzyme superoxide dismutase (SOD) holds a key position in the microalgal antioxidant network. The present research focused on oxidative stress responses in the Antarctic diatom *Chaetoceros brevis* Schütt during transition to excess (including ultraviolet radiation) and limiting irradiance conditions. Over a four day period cellular responses of TBARS (a general oxidative stress indicator), SOD activity, photosynthetic and xanthophyll cycle pigments, PSII efficiency, and growth were determined. In addition, oxidative responses were measured during a daily cycle. Changing irradiance conditions significantly affected growth rates of *C. brevis*. PSII efficiency decreased significantly during periodic excess irradiance and increased under low irradiance conditions. Transition to excess irradiance increased the ratio of xanthophyll to light harvesting pigments, whereas the opposite was found for cultures transferred to low irradiance. This acclimation process was completed after two days in the new irradiance environment. SOD activity increased significantly after the first day regardless of the new irradiance environment but returned to pre-exposures values on the fourth day. We hypothesize that, SOD activity may be temporarily elevated in *C. brevis* after irradiance shifts, thereby reducing oxidative stress when photoacclimation is in progress.

Introduction

Reactive oxygen species (ROS) are produced as byproducts of photosynthetic electron transport and metabolism in photosynthetic organisms (Fridovich 1975, Cadenas 1989, Alscher *et al.* 1997). The chloroplasts with their high concentrations of O_2 and a constant flow of electrons through the photosystems are a principle site for ROS production (Pinto *et al.* 2003). When the consumption of the reduced equivalents cannot match their production, electron transport chains become overreduced and electrons leak onto O_2 by which ROS formation is initiated (Mehler 1951, Asada *et al.* 1974, Gechev *et al.* 2006). Environmental conditions such as the presence of ultraviolet radiation (UVR: 280–400 nm) stimulate radical formation (Hideg & Vass 1996). Reduction of O_2 occurs through a series of univalent electron transfer (Taube 1965). The first one-electron reduction generates the superoxide radical ($O2^{-\bullet}$). Addition of a second electron generates H_2O_2 either by spontaneous or an enzymatic reaction catalyzed by superoxide dismutase (SOD; Gregory & Fridovich 1973a, b; Halliwell & Gutteridge 1984,). Both reactive oxygen species are moderately harmful but in the presence of unchelated bivalent cations (i.e. $Fe^{2+/3+}$, $Cu^{+/2+}$), $O2^{-\bullet}$ is able to reduce H_2O_2 thereby generating the highly reactive hydroxyl radical ($HO^{\bullet}$; Kehrer 2000, Cadenas 1989, Halliwell & Gutteridge 1984). If the production of $HO^{\bullet}$ is not limited, adverse effects can be expected, ranging from the temporary impairment of photosynthesis to viability loss, possibly related with membrane damage due to lipid peroxidation (Van de Poll *et al.* 2006, Halliwell 2006)

Marine microalgae experience excess irradiance and oxidative stress conditions particularly near the water surface (Llabrés & Agustí 2006). In addition, they experience large fluctuations in quantity and quality of incoming irradiance due to changes in their position in the water column (Helbling *et al.* 1994, Neale *et al.* 1998a, 2003). Exposure to UVR causes additional stress and is responsible for significant decreases of the photosynthetic activity of Antarctic microalgae (Smith *et al.* 1992, Holm-Hanssen *et al.* 1993, Helbling *et al.* 1994). In Antarctic regions, the diurnal rhythm and the movement through the upper mixed layer are responsible for large variations in irradiance conditions. The presence of melting sea ice in spring and summer may further contribute to these large fluctuations: microalgae may be advected from the low irradiance under ice environment to stratified surface waters in the Marginal Ice Zone within a very short period of time (Lancelot *et al.* 1991).

To counteract or prevent ROS formation during excess *in situ* irradiance conditions Antarctic microalgae need flexible antioxidative mechanisms. For example, similar to plants, algae have the ability to tune the energy flow towards the photosystems by adjusting the composition of the light harvesting and photoprotective pigments (Falkowski & LaRoche 1991, Harris *et al.* 2005, Van de Poll *et al.* 2005). Additionally, excess excitation energy can be quenched as heat by activating the xanthophyll cycle (Olaizola *et al.* 1994, Lavaud *et al.* 2002, Van de Poll *et al.* 2006). When these defense systems are not sufficient, cells can counteract ROS with a sophisticated network of antioxidant enzymes, which scavenge the various ROS intermediates (Foyer *et al.* 1994, Asada 1999). The enzyme superoxide dismutase (SOD) holds a key position in this network because it is able to scavenge the superoxide radical by which proliferation of ROS is prevented (Wolfe-Simon *et al.* 2005). Although photosynthetic responses during irradiance transitions are well investigated in Antarctic microalgae, virtually nothing is known about their antioxidant

responses (Schriek 2000, Van de Poll *et al.* 2006). In temperate marine microalgae SOD activity and pigment composition were found to vary over the diurnal cycle as well as during prolonged growth in batch culture (Sigaud-Kutner *et al.* 2002, 2005). Furthermore, SOD activities in temperate microalgae and zooxanthellae fluctuated with season (Butow *et al.* 1997), salinity (Janke & White 2002, Rijstenbil 2003), irradiance (Lesser & Shick 1989b) and UVR (Lesser 1996a, b; Litchman *et al.* 2002, Rijstenbil 2002).

The aim of the present work was to investigate antioxidant responses during irradiance transitions in the Antarctic marine diatom *Chaetoceros brevis*. *C. brevis* was pre-cultured at 180 µmol·m^{-2}·s^{-1} and transferred to low and periodic excess irradiance (including UVR). Growth, PSII efficiency, pigment composition, and SOD activity were followed for four days after the transition. In addition, the possible variability in these responses during the diurnal cycle was investigated.

Materials & methods

Experimental design

Chaetoceros brevis (Schütt, Center for Culture of Marine Phytoplankton isolate 163) was cultured for three weeks in duplicate UV transparent 2.2 L polymethylmethacrylate boxes containing modified f/2 enriched seawater (Veldhuis & Admiraal 1987) of 34.5 PSU at 4°C. The pre-cultures received 180 µmol photons·m^{-2}·s^{-1} photosynthetically active radiation (PAR, initial condition) during a 16:8 h light dark-cycle. To avoid nutrient limitation, medium was replenished every ten days. The duplicate cultures were split into 4 UV transparent boxes (making 8 boxes in total) and supplied with 2 L fresh medium. After 3 more days of growth duplicate boxes, each originating from one pre-culture, were transferred to low irradiance (LL, 20 µmol photons·m^{-2}·s^{-1}), high irradiance conditions (HL, 1350 µmol photons·m^{-2}·s^{-1}) or high irradiance with UV (HL+UV, 1350 µmol photons·m^{-2}·s^{-1} PAR, 24.2 W·m^{-2} ultraviolet A (UVAR: 315 – 400 nm), and 2.1 W·m^{-2} ultraviolet B (UVB: 280 – 315 nm)). Two cultures remained at the original conditions (CTRL). High irradiance exposures (HL and HL+UV) were given for 4 hours per day after which the cultures were transferred back to the CTRL conditions. This treatment was repeated for 4 days. Samples for SOD activity, TBARS, PSII efficiency, growth, and pigment composition were taken from all cultures, directly after the HL and HL+UV exposures. On the fourth day, samples were obtained during the diurnal cycle: before the light period started, before transfer to HL+UV, after 4 h HL or HL+UV, 2 h after termination of the HL or HL+UV exposures, before the dark period, and before the beginning of the next light period. The entire experiment was repeated a few weeks later, giving a total of 4 replicates for each irradiance condition.

Irradiance

PAR was provided by Biolux fluorescence tubes (Osram, GmbH, Munich, Germany), whereas UVR was provided by 4 UVA 340 fluorescent tubes (Q-panel, Cleveland, Ohio, USA). PAR irradiance was measured with a spherical irradiance meter (QSL-100, Biospherical Instruments) in air. UVR was measured with a MACAM SR9910 double

monochromator scanning spectroradiometer (Macam Photometrics, Livingston, UK) in air. Cultures exposed to excess PAR irradiance (HL) were shielded from the UVR sources by a wooden cover**.**

Growth

On each experimental day a 2 mL sample from each replicate was fixed with 20 µL 37% formaldehyde (Merck, Darmstadt, Germany). Cell counts were performed with a Coulter XL-MCL flow cytometer (Beckman Coulter, Miami, FL, USA) as described by Van de Poll *et al.* (2005). Growth rate was determined by measuring the slope of the natural log (Ln) of cell number as a function of time during four days of the experiment. Growth rate of the initial culture was determined over four successive days before the start of the experiment.

SOD activity

Samples for SOD (70 mL) activity and TBARS (70 mL) concentration were filtered over 2.0 µm pore size polycarbonate filters (Ø 47mm; Osmonics Inc., USA) and stored at -80°C for one month. SOD analyses were done using the RF/NBT method modified for algal material, as described in Janknegt *et al.* (2007). Filters were thawed and resuspended in 0.75 mL buffer X containing 50 mM KH_2PO_4, pH 7.8, 0.1 mM EDTA, 0.1% Triton X-100 (Aldrich, Steinheim Germany), 2% PVP (Acros, New Jersey USA) and Complete protease inhibitor cocktail (Roche, Mannheim Germany). Subsequently, cells were disrupted by sonication (2 x 30 s, at A=30) at 4°C after which the extracts were centrifuged for 25 min (20.000 g) at 4°C. Supernatants were transferred to a clean 1.5 mL Eppendorf tube after which 150 µL was taken for duplicate protein quantification according to Bradford (1976). SOD activity was then determined using the Riboflavin/NitroBlue Tetrazolium (RF/NBT) method (Beauchamp & Fridovich 1971, Fryer *et al.* 1998). This assay is based on the competition between SOD and NBT for $O_2^{-\cdot}$ generated by the illuminated Riboflavin. SOD activity was defined as the amount of sample required to achieve 50% inhibition of the NBT reduction (V_{50}). NBT reduction was measured (in duplicate) in 4 reaction mixtures containing 0 (blank, no inhibition), 12.5, 25, and 50 µL extract. The 1.5 mL reaction mixtures contained SOD buffer (50 mM KH_2PO_4 pH 7.8, 0.1 mM EDTA, and 0.25% Triton X-100 (Sigma)), 2 mM riboflavin (Sigma) and 57 µM NBT (Sigma). Reaction mixtures were incubated in the dark for 30 min at 4°C and subsequently illuminated from above for 15 min by 4 fluorescence tubes (Philips TLD/18W, 30 cm distance) giving 199 µmol photons$\cdot m^{-2}\cdot s^{-1}$. Afterwards, absorbance was measured at 560 nm on a Cary 3E UV/VIS double beam spectrophotometer (Varian, Middelburg, The Netherlands). Fifty percent inhibition (V_{50}) was calculated by regression using the linear part of a natural semi-log curve. The specific activity of SOD (*S.A.*) was calculated according to 1 / (V_{50}*[*protein*]).

Pigments

Samples for pigment analysis were filtered over GF/F filters (Ø 25mm) and stored at -80°C for one month. Pigments were analysed as described by Van Leeuwe *et al.* (2006). In short, filters were handled under dim light, freeze-dried (48 h) after which pigments were extracted in 4 mL 90% cold acetone (v/v, 48 h, 5ºC). Pigments were resolved using HPLC (Waters 2690 separation module, 996 photodiode array detector) with a C_{18} 5 µm DeltaPak reversed-phase column (Milford, MA, USA) and identified by retention time and diode

array spectroscopy. Quantification was done using standard dilutions of Chlorophyll *a*, c_2, fucoxanthin, diadinoxanthin, diatoxanthin, and β carotene. Cell counts were done using a Coulter XL-MCL flow cytometer (Beckman Coulter, Miami, FL, USA) as described by Van de Poll *et al.* (2005) for calculation of cellular pigment concentrations.

Determination of Thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid reactive substances (TBARS) comprise a rough estimate of the presence of aldehydes. Yet, most reactivity originates from malondialdehyde (MDA), a product of ROS induced lipid peroxidation. TBARS analyses were done after Heath & Parker (1968): filters were resuspended in 0.8 mL MilliQ water and 0.8 mL Thiobarbituric acid (0.5% w/v, Sigma) in 20% Trichloroacetic acid (TCA). The mixture was heated for 30 min at 90ºC and immediately put on ice. After filter removal the supernatant was centrifuged (10 min, 20000 *g*). TBARS were detected by subtracting the nonspecific turbidity measured at 600 nm from the TBARS absorption maximum at 532 nm using a Cary 3E double beam UV/VIS spectrophotometer (Varian, Middelburg, The Netherlands). Extraction solvent was used as the blank.

PSII efficiency

PSII efficiency was measured each day after HL and HL+UV exposures and on the last day during the diurnal cycle. Samples for PSII efficiency were filtered over GF/F filters (Ø 11.3mm; Whatman, Maidstone, UK). Filtration was stopped before the filter dried after which the filter was transferred to a temperature controlled (4°C) seawater-filled cuvette. Chlorophyll fluorescence was measured with a PAM 2000 pulse amplitude modulated fluorometer (Walz, Effeltrich, Germany). Maximum quantum yield (F_v/F_m) was determined after 5 min of dark acclimation.

Statistical analyses

Differences in growth rates were analysed using One-way ANOVA. Other differences were tested for significance with a Repeated Measures ANOVA (RM ANOVA). Significant differences were further analysed using a LSD post-hoc test. Tests were performed using STATISTICA 7 for Windows.

Results

Growth

Under the pre-experimental conditions, where irradiance was the same as the experimental control, *Chaetoceros brevis* grew exponentially at an average rate of 0.45div.d^{-1} (Fig. 1). After transfer to the experimental irradiance conditions all cultures continued to grow exponentially. The control (CTRL) cultures grew fastest (0.42div·d^{-1}) followed by HL (0.34div·d^{-1}), HL+UV (0.28div·d^{-1}) and LL (0.12div·d^{-1}). The growth rates of the control and pre-experimental cultures were not significantly different while the growth rates of all experimental treatments were significantly different from each other (One-Way ANOVA, $p<0.0005$).

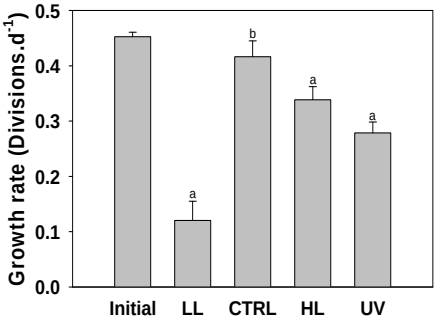

Figure 1: Growth rates of *Chaetoceros brevis* cultured at 180 µmol photons·m^{-2}·s^{-1} PAR (Initial) before and after transition to low irradiance PAR (LL, 20 µmol photons·m^{-2}·s^{-1}), high PAR irradiance (HL, 1350 µmol photons·m^{-2}·s^{-1}), and high PAR + UV irradiance (UV, 1350 µmol photons·m^{-2}·s^{-1} + 24 W·m^{-2} UVAR and 2.1 W·m^{-2} UVBR). The control cultures (CTRL) were kept at the same irradiance condition as the Initial culture. Bars show mean growth rates of four replicates, error bars indicate standard deviations. a: significant differences between all other irradiance conditions b: differs significantly from LL, HL and UV ($p<0.0005$).

PSII efficiency

At the start of the experiment (T=0), the maximum quantum yield (F_v/F_m) of the initial cultures was on average 0.411 (± 0.012 SD) (Fig. 2a). During the following four days, the F_v/F_m of the CTRL cultures remained on the same level (slope -0.0007; RM ANOVA, $p>0.07$). The diurnal rhythm of the CTRL condition showed a slight but significant decrease during the light period (RM ANOVA, $p<0.0005$) followed by a significant increase during the dark period (RM ANOVA $p< 0.0005$; Fig. 2b). The highest maximum quantum yield was measured under the LL condition. The F_v/F_m of LL increased significantly (RM ANOVA, $p<0.0005$) during the course of the experiment and leveled off around 0.7 (Fig. 2a). No significant changes were observed for LL during the diurnal cycle. Lowest values were found directly after HL and HL+UV exposure, resulting in a significant 60-70% decrease (RM ANOVA, $p< 0.0001$) of F_v/F_m. Only on the fourth day, F_v/F_m of HL+UV was significantly lower than HL (RM ANOVA, $p<0.01$).

The diurnal cycles on the fourth day showed significant recovery (RM ANVOVA, $p<0.0005$) two hours after the HL or HL+UV exposure, with Fv/Fm increasing to 89% and 79% of the CTRL values, respectively.

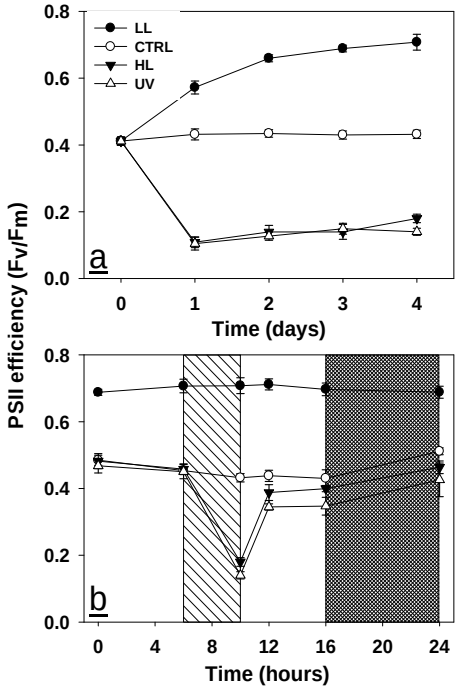

Figure 2: Photosystem II efficiency before (Initial, 180 µmol photons·m^{-2}·s^{-1} PAR) and after a shift to low PAR irradiance (LL 20 µmol photons·m^{-2}·s^{-1}), high PAR irradiance (HL, 1350 µmol photons·m^{-2}·s^{-1}), and high PAR + UV irradiance (1350 µmol photons·m^{-2}·s^{-1} + 24 W·m^{-2} UVAR and 2.1 W·m^{-2} UVBR). The control culture (CTRL) was kept at the same irradiance condition as the Initial irradiance condition. **a**) during four days of photoacclimation **b**) during the diurnal cycle on day four. LL cultures were kept at low irradiance for the entire photoperiod. During their diurnal cycle, HL and UV cultures were grown at CTRL conditions, transferred to the new irradiance environment (light dashed area) at t=6 h and returned to CTRL conditions at t=10 h. The dark dashed area (t=16 till t=24) represents the night period. The daily data points of all cultures were measured at t=10 h. Error bars represent standard deviations.

The following 12 h caused an additional increase of approximately 2% and 5%, respectively. The F_v/F_m of HL+UV remained significantly lower (RM ANOVA, $p<0.05$) than that of the HL exposed cultures until after the dark period. Yet, the recovery rates of both cultures (1.14 h^{-1}) were not significantly different.

Pigment analyses

After three weeks of pre-culturing at CTRL conditions, the ratio of photoprotective xanthophyll pigments to photosynthetic pigments (DD+DT/Fuco+Chl.*a*) was 0.25, and

remained on that level during the experiment (Fig. 3a). When transferred to LL, this ratio decreased significantly (RM ANOVA, $p<0.0005$) eventually dropping to 0.08. The diurnal cycle of this ratio did not show any variability for the LL condition (Fig. 3b). In contrast, when placed under HL and HL+UV, the ratio increased significantly (RM ANOVA $p<0.0005$) and reached values as high as 0.42.

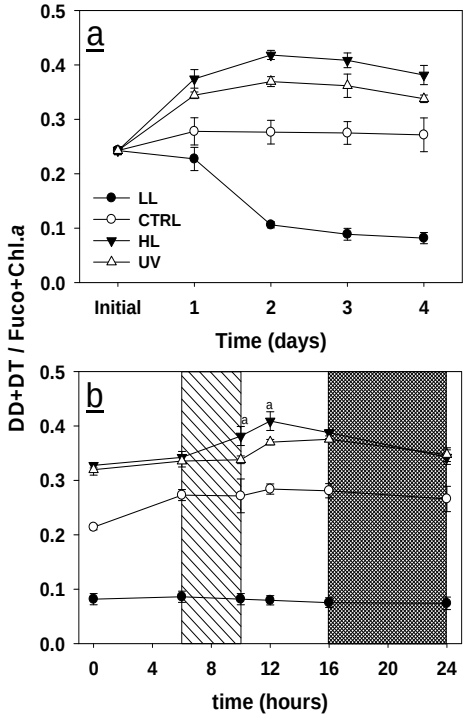

Figure 3: Light protection pigments (DD+DT) relative to light harvesting pigments (Fuco+Chl.*a*) before (Initial, 180 µmol photons·m^{-2}·s^{-1} PAR) and after a shift to low PAR irradiance (LL 20 µmol photons·m^{-2}·s^{-1}), high PAR irradiance (HL, 1350 µmol photons·m^{-2}·s^{-1}), and high PAR + UV irradiance (1350 µmol photons·m^{-2}·s^{-1} + 24 W·m^{-2} UVAR and 2.1 W·m^{-2} UVBR). The control culture (CTRL) was kept at the same irradiance condition as the Initial irradiance condition. **a**) during four days of photoacclimation **b**) during the diurnal cycle on day four. LL cultures were kept at low irradiance for the entire photo period. During their diurnal cycle, HL and UV cultures were grown at CTRL conditions, transferred to new irradiance environment (light dashed area) at t=6h and returned to CTRL conditions at t=10h. The dark dashed area (t=16 till t=24) represents the night period. The daily data points of all cultures were measured at t=10h. Error bars represent standard deviations a: significant UVR effect ($p<0.05$).

This increase was due to a significant decrease (RM ANOVA, $p<0.05$) in light harvesting pigments (Table 1). In the absence of UVR the protective capacity of cells was found to be significantly higher (RM ANOVA $p<0.005$) relative to their light absorption capacity. The

diurnal cycles of HL and HL+UV cultures showed significantly higher pigment ratio's (RM ANOVA $p<0.0005$) at the beginning of the light period compared with the CTRL condition. Furthermore, the protection over harvesting ratio of the HL culture increased immediately after HL exposure while the HL+UV culture did not show an increased ratio until two hours later. During the exposure period, the ratio of the HL culture increased mainly due to significant reduction of light harvesting pigments (RM ANOVA, $p<0.05$). This response was postponed in the HL+UV culture until after the exposure period (Table 1a, b).

Table 1: a) cellular pigment content of *Chaetoceros brevis* before (Initial, 180 µmol photons·m^{-2}·s^{-1} PAR) and after a shift to low PAR irradiance (Low light, 20 µmol photons·m^{-2}·s^{-1}), high PAR irradiance (High light, 1350 µmol photons·m^{-2}·s^{-1}), and high PAR + UV irradiance (High light+UVR, 1350 µmol photons·m^{-2}·s^{-1} + 24 W·m^{-2} UVAR and 2.1 W·m^{-2} UVBR). The control culture (CTRL) was kept at the same irradiance condition as the Initial irradiance condition. **b**) shows the cellular pigment content during their diurnal cycle, HL and UV cultures were grown at CTRL conditions, transferred to new irradiance environment at t=6h and returned to CTRL conditions at t=10h. Night period was between t=16 till t=24. The daily data points of all cultures were measured at t=10h. a) Chlorophyll *a* + fucoxanthin b) Diatoxantin + diadinoxantin. LL cultures were kept at low irradiance for the entire photoperiod. Values of pigments are means.$cell^{-1}$ ± SD shown for four replicas. T = time in days; t = time in hours. a: triplicate; b: duplicate.

a) Concentration Chlorophyll *a* + fucoxanthin per cell (x10^2 pg/cell).

Irradiance cond.	Initial	T = 1	T = 2	T = 3	T = 4	
Low light	1.78 ± 0.30	1.37 ± 0.18	1.41 ± 0.22[a]	1.79 ± 0.19	2.18 ± 0.43	
CTRL	1.78 ± 0.30	1.30 ± 0.12	1.25 ± 0.11	1.44 ± 0.18	1.41 ± 0.08	
High light	1.78 ± 0.30	1.16 ± 0.16	1.03 ± 0.15	1.21 ± 0.18	1.03 ± 0.08	
High light + UVR	1.78 ± 0.30	1.10 ± 0.23	1.10 ± 0.11	1.15 ± 0.07	1.27 ± 0.07	
	t = 0	t = 6	t = 10	t = 12	t = 16	t = 24
Low light	1.96 ± 0.34	2.10 ± 0.46	2.18± 0.43	2.16 ± 0.53	2.36 ± 0.61	2.59 ± 0.46
CTRL	1.23 ± 0.08	1.32 ± 0.15	1.41 ± 0.08	1.35 ± 0.09	1.52 ± 0.09	1.56 ± 0.15
High light	0.96 ± 0.08	1.09 ± 0.05	1.03 ± 0.08	1.08 ± 0.08	1.19 ± 0.20	1.29 ± 0.20
Hligh light + UVR	1.09 ± 0.10	1.19 ± 0.10	1.27 ± 0.07	1.34 ± 0.14	1.19 ± 0.18[b]	1.41 0.18

b) Concentration diatoxanthin + diadinoxanthin (x10 pg/cell).

Irradiance cond.	Initial	T = 1	T = 2	T = 3	T = 4	
Low light	4.33 ± 0.85	3.08 ± 0.22	1.52 ± 0.15[a]	1.57 ± 0.67	1.75 ± 0.20	
CTRL	4.33 ± 0.85	3.61 ± 0.53	3.47 ± 0.48	3.99 ± 0.78	3.82 ± 0.25	
High light	4.33 ± 0.85	4.35 ± 0.75	4.31 ± 0.70	4.95 ± 0.78	3.91 ± 0.21	
High light + UVR	4.33 ± 0.85	3.79 ± 0.79	4.06 ± 0.49	4.17 ± 0.47	4.27 0.20	
	t = 0	t = 6	t = 10	t = 12	t = 16	t = 24
Low light	1.58 ± 0.19	1.78 ± 0.32	1.75 ± 0.20	1.69 ± 0.26	1.74 ± 0.25	1.88 ± 0.15
CTRL	2.64 ± 0.11	3.49 ± 0.17	3.82 ± 0.25	3.83 ± 0.38	4.15 ± 0.16	4.12 ± 0.33
High light	3.16 ± 0.27	3.74 ± 0.22	3.91 ± 0.21	4.44 ± 0.51	4.62 ± 0.80	4.41 ± 0.54
Hligh light + UVR	3.46 ± 0.25	3.99 ± 0.27	4.27 ± 0.20	4.96 ± 0.48	4.47 ± 0.67[b]	4.93 ± 0.64

UVR significantly affected de-epoxidation of diadinoxanthin (DD) to its energy dissipating form diatoxanthin (DT; RM ANOVA, $p<0.0005$); the de-epoxidation status (DES = DT/(DT+DD) of the HL culture was on average 73% (SD=2.7) while the DES after

HL+UV was on average 59% (SD=6.8). After two hours of recovery, the DES of both cultures returned to pre-exposure levels (Fig. 4b). Cultures growing at LL contained no detectable level of DT (Figs. 4a, b).

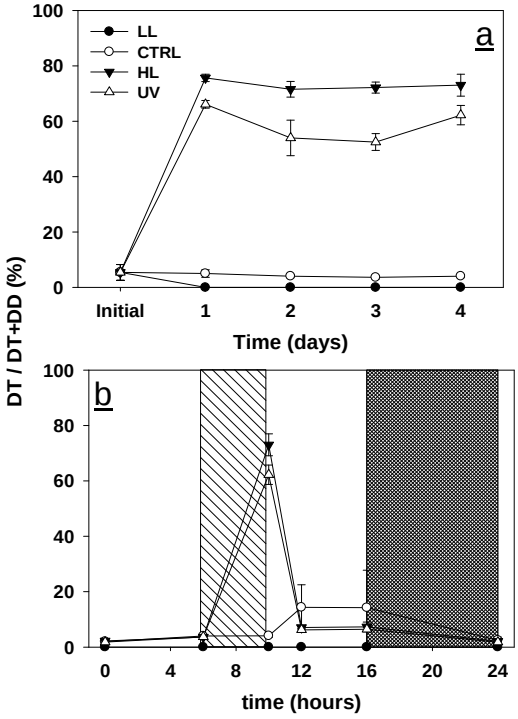

Figure 4: De-epoxidation status (DES = DT / DT+DD) of the photoprotective xanthophyll pigments before (Initial, 180 µmol photons·m^{-2}·s^{-1} PAR) and after a shift to low PAR irradiance (LL 20 µmol photons·m^{-2}·s^{-1}), high PAR irradiance (HL, 1350 µmol photons·m^{-2}·s^{-1}), and high PAR + UV irradiance (1350 µmol photons·m^{-2}·s^{-1} + 24 W·m^{-2} UVAR and 2.1 W·m^{-2} UVBR). The control culture (CTRL) was kept at the same irradiance condition as the Initial irradiance condition. **a**) during four days of photoacclimation **b**) during the diurnal cycle on day four. LL cultures were kept at low irradiance for the entire photoperiod. During their diurnal cycle, HL and UV cultures were grown at CTRL conditions, transferred to the new irradiance environment at t=6h and returned to CTRL conditions at t=10h (dashed area). The daily data points of all cultures were measured at t=10h. Error bars represent standard deviations, grey area represents dark period.

SOD activity

During the experiment, the SOD activity of the CTRL culture was on average 135 U/mg protein and did not change significantly over time (Fig. 5). Four hours after the irradiance transitions, LL, HL, and HL+UV cultures showed significantly increased SOD activities (RM ANOVA, $p<0.0005$), with values of 255, 350, and 368 U/mg protein, respectively. Although SOD activities decreased considerably on the second day, their values were still significantly higher (RM ANOVA, $p<0.005$) than those of the CTRL cultures. After the

third exposure period, HL, HL+UV, and CTRL contained similar SOD activity levels, in contrast to the LL culture, which still showed significantly (RM ANOVA, $p<0.05$) higher values. On the final day, all four cultures showed similar SOD activities, not significantly differing from the CTRL culture. Yet, during the diurnal cycle the LL culture contained significantly more SOD activity (average=167 U/mg protein, SD=29) than CTRL (average=106 U/mg protein, SD=15), HL (average=110 U/mg protein, SD=13) and HL+UV (average=116 U/mg protein, SD=18) cultures (RM ANOVA, $p<0.05$), which were all three similar to each other throughout the 24 h.

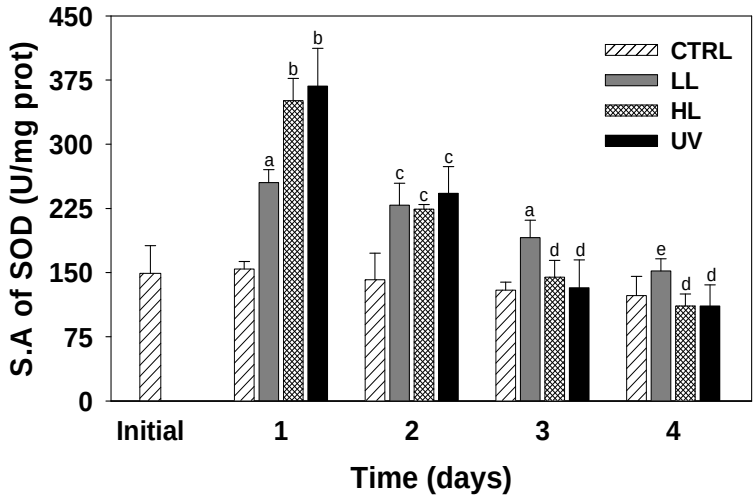

Figure 5: Specific activity (SA) of Superoxide dismutase (SOD) before (Initial, 180 µmol photons·m^{-2}·s^{-1} PAR) and after a shift to low PAR irradiance (LL 20 µmol photons·m^{-2}·s^{-1}), high PAR irradiance (HL, 1350 µmol photons·m^{-2}·s^{-1}), and high PAR + UV irradiance (1350 µmol photons·m^{-2}·s^{-1} + 24 W·m^{-2} UVAR and 2.1 W·m^{-2} UVBR). The control culture (CTRL) was kept at the same irradiance condition as the Initial irradiance condition. Error bars represent standard deviations. a: significant differences from all other irradiance conditions ($p<0.05$) on the same day; b: significant differences from CTRL and LL ($p<0.0005$) on the same day; c: significant differences from CTRL ($p<0.005$) on the same day; d: significant differences from LL ($p<0.05$) on the same day; e: significant differences from HL and UV ($p<0.05$) on the same day.

TBARS content

Four h after the irradiance transitions, the cellular TBARS content of all four cultures was similar to the Initial situation (T=0; Fig. 6a). During the following days, the cell specific amount of TBARS present in the CTRL, HL and HL+UV cultures remained at this concentration while TBARS in LL cultured cells increased. As a result, the LL cultures contained significantly more TBARS per cell on the fourth day than the other three cultures (RM ANOVA $p<0.05$). This trend persisted during the diurnal cycle on day 4 (Fig. 6b).

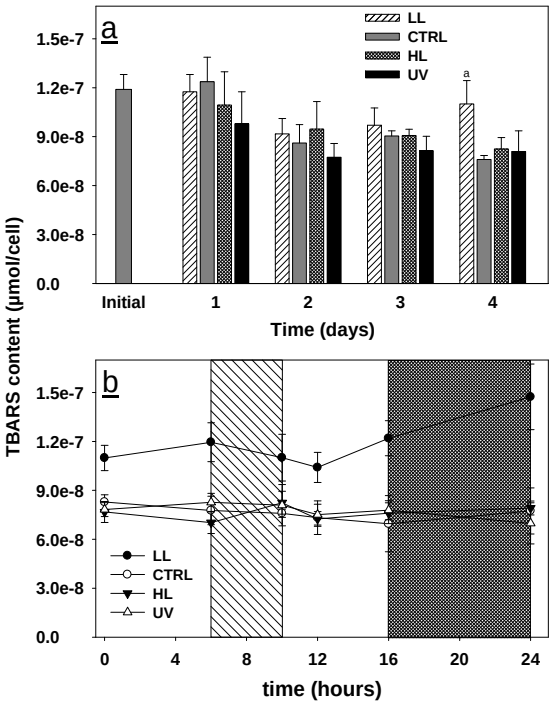

Figure 6: Cellular TBARS content before (Initial, 180 µmol photons·m^{-2}·s^{-1} PAR) and after a shift to low PAR irradiance (LL 20 µmol photons·m^{-2}·s^{-1}), high PAR irradiance (HL, 1350 µmol photons·m^{-2}·s^{-1}), and high PAR + UV irradiance (1350 µmol photons·m^{-2}·s^{-1} + 24 W·m^{-2} UVAR and 2.1 W·m^{-2} UVBR). The control culture (CTRL) was kept at the same irradiance condition as the Initial irradiance condition. **a**) during four days of photoacclimation **b**) during the diurnal cycle on day four. LL cultures were kept at low irradiance for the entire photoperiod. During their diurnal cycle, HL and UV cultures were grown at CTRL conditions, transferred to new irradiance environment (light dashed area) at t=6h and returned to CTRL conditions at t=10h. The dark dashed area (t=16 till t=24) represents the night period. The daily data points of all cultures were measured at t=10h. Error bars represent standard deviations. a differs significantly from all other irradiance conditions ($p<0.005$) on the same day.

Discussion

In this study we investigated oxidative stress responses in the marine diatom *Chaetoceros brevis* during photoacclimation. Prior to the experiment cells grew exponentially under saturating irradiance conditions (180 µmol·m^{-2}·s^{-1}, 16:8 h light dark and 4°C) at rates consistent with literature values (Van de Poll *et al.* 2005). Cultures transferred to the HL+UV regime were exposed to excess irradiance including ultraviolet A (UVAR: 315-400 nm) and B (UVBR: 280-315 nm) with a spectral ratio of 128:11.3:1 W·m^{-2} (PAR:UVAR:UVBR). This approximates natural Antarctic irradiance ratio's measured at Melchior station (80:13.6:1; Helbling *et al.* 1996) and Palmer station (450:12.5:1; Bouchard *et al.* 2005b). In this study, UVR resulted in a significant growth reduction of 15%, relative to excess PAR only (HL treatment) probably due to its stimulating effect on ROS

production. After transition to excess irradiance, light harvesting activity of HL and HL+UV cultures probably exceeded light processing activity, which over-reduced photosynthetic electron transport chains and subsequently enhanced ROS formation (Nishiyama *et al.* 2001, Karentz *et al.* 1991b).

The relatively low maximal quantum yield of CTRL cultures (Fv/Fm =0.4) indicated sub-optimal photosynthetic performance. Yet, the growth rate of the CTRL cultures was highest of all treatments, suggesting that the low Fv/Fm was not caused by photoinhibitory damage. Highest PSII efficiency was measured in LL cultures (average Fv/Fm 0.7), consistent with values obtained previously (Van de Poll *et al.* 2005, 2006). The PSII efficiency increase was stoichiometrically inverse to the decrease in the DD+DT/fuco+Chl.*a* ratio, which resulted from strong cellular decrease of xanthophyll pigments and a simultaneous increase in light harvesting pigments. In accordance with Post *et al.* (1985), this could not compensate for the decreased irradiance and resulted in lower growth rates.

The inverse relationship between DD+DT/fuco+Chl.*a* ratio and PSII efficiency also applied for cultures subjected to the HL treatment. This was also found in other studies on responses of diatoms to irradiance shifts (Willemoës & Monas 1991, Olaizola & Yamamoto 1994, Mewes & Richter 2002). Furthermore, field studies confirmed that transition from low to high irradiance resulted in an increased DD+DT/fuco+Chl.*a* ratio (Moline 1998). In accordance with Claustre and coworkers (1994), we found that the increased protection/harvesting ratio of HL cultures resulted almost entirely from a decrease in light harvesting pigments. Yet, the increased protection/harvesting ratios and de-epoxidation status could not prevent growth reduction in the HL and HL+UV cultures.

No apparent effect on PSII efficiency was observed directly after the UVR exposures, as found previously (Van de Poll *et al.* 2006, Lesser *et al.* 1996, Vassiliev *et al.* 1994). Furthermore, recovery rates of PSII efficiency over time were similar for HL and HL+UV cultures. However, the HL cultures showed 90% recovery, while PSII efficiency of HL+UV cultures remained 10% lower. This could indicate partial UVR induced photodamage to the PSII reaction centre D1 protein, as found elsewhere (Bouchard *et al.* 2005a, b; Nishiyama *et al.* 2001). In addition, the DD+DT/fuco+Chl.*a* ratio of HL+UVR exposed cells was significantly lower than that of the HL culture. This is contrary to other studies where UVR did not affect pigment ratios or even caused enhancement of xanthophyll pools and pigment ratios (Mewes & Richter 2002, Goss *et al.* 1999, Buma *et al.* 2000). The DES of the HL+UV culture was on average 10% lower than that of HL exposed cells, similar to values reported by Bischof *et al.* (2002a), Pfundel *et al.*(1992), and Mewes & Richter (2002). This reduction in xanthophyll cycle activity has been linked to the increased production of ROS (Bischof *et al.* 2002b). More specifically, Rijstenbil and coworkers (2003), and Mallick & Mohn (2000) suggested that diatoxanthin can act as an antioxidant by which the xanthophyll pigment is re-epoxidized to diadinoxanthin. Mewes & Richter (2002) proposed that UVR enhanced thylakoid membrane permeability, resulting in a lower ΔpH gradient, which increases the activity of diatoxanthin epoxidase.

The SOD activity of the CTRL culture remained unchanged throughout the experiment. This not only indicated that physiological stress conditions did not change but also that the cultures remained in the same growth phase during the experimental period (Malanga & Puntarulo 1995, Sigaud-Kutner *et al.* 2002). In contrast, the LL, HL, and HL+UV cultures showed elevated SOD levels right after the irradiance shifts. Evidently,

the change in irradiance environment stimulated production of superoxide radicals and subsequently its scavenging counterpart (Bowler *et al.* 1992, Malanga & Puntrulo 1995, Rijstenbil 2002), regardless of the new irradiance regime. After the first day, SOD activities of all three cultures decreased until they had returned to the CTRL value on the fourth day, while no significant additional UVR effect on SOD activity was found.

To our knowledge, no other study has reported an acclimation response in SOD activity to irradiance changes *per se*. Previous studies described SOD responses of temperate microalgae to elevated irradiance conditions after one exposure (Malanga *et al.* 1999, Rijstenbil 2002, Van de Poll *et al.* 2006), after an acclimation period (Rijstenbil 2003, 2001, Lesser 1996a, b; Lesser & Shick 1989b) or after a shift in growth-phase (Sigaud-Kutner *et al.* 2002, Malanga & Puntrulo 1995). Other studies described a UVR induced increase in SOD activity, although no effect or even decreased activity was also found (Van de Poll *et al.* 2006, Malanga *et al.* 1999, Zhang *et al.* 2005). All these studies suggest that there is no uniform response of SOD to excess irradiance including UVR. Genetic studies on plants and algae reveal that the expression levels of SOD encoding genes are regulated by the redox status of the cell (Bowler *et al.* 1992, Mittler *et al.* 2004, Kohen & Nyska 2002) and the intracellular location of $O2^{-\bullet}$ production (Wolfe-Simon *et al.* 2005). Because the cellular redox status depends on the amount of pro- and antioxidants, all genes involved in ROS scavenging influence each others expression level. Therefore, SOD genes could be down-regulated in one species (Wang *et al.* 2004) while up-regulated in another (Turpaev 2002, Mackerness *et al.* 1999, Mackerness 2000) as a response to oxidative stress.

The rapid decrease of SOD activity after the first day in HL and HL+UV treatment could be due to the gradual increase of the DD+DT/Chl.*a* ratio by which energy transfer towards the photosystems was diminished and also slowing down the formation of superoxide radicals. However since UVR is known to stimulate radical formation and affect the DD+DT/Chl.*a* ratio, this seems to explain the acclimation process only partially. Besides that, several studies have demonstrated UVR induced increases of other antioxidant compounds such as the water-soluble glutathione and ascorbate (Dai *et al.* 1997, Costa *et al.* 2002, Shiu & Lee 2005), or the lipid protecting α-tocopherol (Malanga & Puntarulo 1995, Malanga *et al.* 1997) which might be energetically more favourable than SOD. Exposure to HL and HL+UV did not change the level of oxidative damage measured as TBARS relative to CTRL but their growth rates were reduced significantly. Therefore it seems that not ROS induced damage is responsible for growth inhibition but rather other factors like a change in cellular metabolism as a response to irradiance transition (Falkowski & LaRoche 1991, Post *et al.* 1985). In contrast to the other irradiance conditions where TBARS remained unchanged, the cellular TBARS content of the LL culture gradually increased as from the second day after its irradiance shift. This could be due to an increased synthesis of light harvesting complex proteins and accommodating membrane lipids (Falkowski & LaRoche 1991, Evens *et al.* 2001). These metabolic changes (i.e. increased level of cellular $Fe^{2+/3+}$ content and the accompanied level of Haber-Weiss/ Fenton cycling) could therewith lead to an increased production of ROS and an increased activity of SOD. After this initial antioxidant response, other components of the network could take over, coinciding with a decrease in SOD capacity after day 1.

We suggest that SOD is an important ROS scavenging mechanism in *Chaetoceros brevis*, because the activity of SOD was rapidly (4 h) up-regulated in response to irradiance

transitions. Nevertheless, the increase in SOD activity was only short lived, returning to initial values once photoacclimation has progressed and other, perhaps more cost-effective or efficient ROS prevention mechanisms are installed.

Chapter 4

Excessive irradiance and antioxidant responses of an Antarctic marine diatom exposed to iron limitation and to dynamic irradiance.

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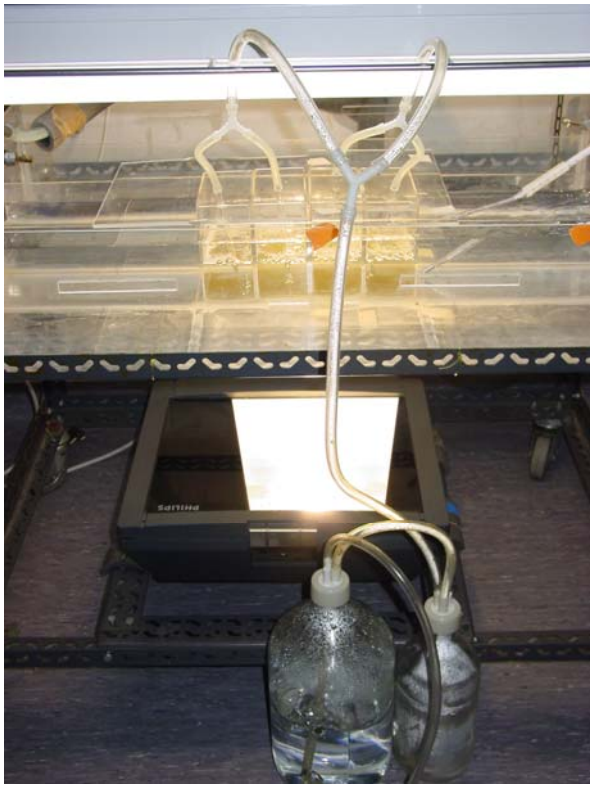

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Abstract

The synergistic effects of iron limitation and irradiance dynamics on growth, photosynthesis, antioxidant activity and excessive PAR (400-700 nm) and UVR (280-400 nm) sensitivity were investigated for the Antarctic marine diatom *Chaetoceros brevis*. Iron-limited and iron-replete cultures were exposed to identical daily irradiance levels, supplied as dynamic (20-1350 µmol photons·m^{-2}·s^{-1}) and constant (260 µmol photons·m^{-2}·s^{-1}) irradiance. After acclimation, growth, maximal quantum yield of PSII (Fv/Fm), pigment composition, and the activities of the antioxidant enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) were determined. Then, excessive irradiance sensitivity was assessed by monitoring pigment composition, Fv/Fm and viability loss during a single excessive PAR and UV treatment.

Iron limitation reduced growth rates, Fv/Fm dynamics, and cellular pigments pools. Cellular pigment concentrations were higher under dynamic irradiance than under constant irradiance but this difference was less pronounced under iron limitation compared to iron replete conditions. SOD and APX activities increased during dynamic irradiance under iron limitation, suggesting increased radical formation around PSII. Despite these physiological differences, no effects on growth were observed between constant and dynamic irradiance cultivation in iron-limited and iron-replete cells. The applied culturing conditions did not affect glutathione reductase activity in *C. brevis*. Fv/Fm and xanthophyll de-epoxidation dynamics during excessive irradiance were not different for iron-limited and replete cells and viability loss was not found during excessive irradiance. This study revealed photoacclimation differences between iron-limited and iron-replete *C. brevis* cultures that did not affect growth rates and excessive irradiance sensitivity after acclimation to constant and dynamic irradiance.

Introduction

The combination of iron limitation and deep wind driven vertical mixing can suppress primary productivity in vast parts of the Southern Ocean, leaving much of the dissolved nitrate, phosphate, and silicate unused for algal growth (De Baar *et al.* 2005, Boyd *et al.* 2007). These frequently co-occurring conditions both affect the photoacclimation state of algae. Vertical mixing in the water column can mediate strong irradiance fluctuations, from excessive irradiance near the water surface to complete darkness below the euphotic zone. These dynamics require contrasting acclimation pathways such as increased light harvesting under low irradiance as well as increased photoprotection when irradiance exceeds the photosynthetic requirements (MacIntyre & Geider 1996, Van de Poll *et al.* 2007). The effects of iron limitation on photoacclimation are well documented. When iron becomes limiting, algae respond by reducing the abundance of iron rich cellular components such as cytochrome b_6-f and PSI complexes (Raven 1990, Strzepek & Harrison 2004). This impairs the electron transport capacity during photosynthesis and leads to a strong reduction in cellular light harvesting pigments and decreased growth rates (Geider *et al.* 1993, Van Leeuwe & Stefels 1998).

How iron limitation affects photosynthesis and growth under dynamic irradiance remains unknown. Cytochrome b_6-f complexes are crucial in the buildup of a proton gradient across the thylakoid membrane, which activates protective heat dissipation via the xanthophyll cycle during excessive irradiance. Therefore, it was suggested that iron-limited algae were less able to cope with rapid irradiance fluctuations than those under iron replete conditions (Strzepek & Harrison 2004). Furthermore, Van Leeuwe & Stefels (1998) observed reduced xanthophyll de-epoxidation under high irradiance in iron-limited *Phaeocystis antarctica.* Therefore, a reduction in cytochrome b_6-f complexes may affect the efficiency of the xanthophyll cycle, and thus the protective down regulation of photosynthesis during excessive irradiance. Consequently, iron-limited algae could be more vulnerable to excessive irradiance due to increased formation of reactive oxygen species (ROS). ROS are typically formed as byproducts of electron transport and cellular metabolism. Environmental stress such as excessive PAR and UV exposure near the water surface can significantly enhance ROS formation, inhibiting photosynthesis and threatening viability (Van de Poll *et al.* 2005).

Cellular ROS concentrations are controlled by an elaborate antioxidant network. The superoxide radical is converted enzymatically to H_2O_2 by superoxide dismutase (SOD), which is subsequently neutralized to H_2O by ascorbate peroxidase (APX). Glutathione is used to regenerate ascorbate, which is the substrate of APX in the latter reaction. The redox status of glutathione is regulated by glutathione reductase (GR). Iron limitation was found to increase ROS production and superoxide dismutase (SOD) activity in marine diatoms (Peers & Price 2004). Nevertheless, a preliminary study indicated that iron limited algae were less sensitive to excessive PAR and UV radiation exposure than iron replete algae (Van de Poll *et al.* 2005). Furthermore, numerous studies demonstrated that iron-limited algae accumulated more protective xanthophyll cycle pigments relative to their light harvesting pigments compared to iron-replete cells (Geider *et al.* 1993). This emphasizes that the effect of iron limitation on the regulation of photosynthesis is not completely understood, let alone under dynamic irradiance as experienced in the field. Therefore, the responses of pigments, chlorophyll fluorescence and crucial components of the antioxidant

network were studied for the Antarctic diatom *Chaetoceros brevis* under simulated dynamic and constant irradiance for iron-limited and iron-replete conditions, followed by excessive (UV) irradiance treatments, during which Fv/Fm, xanthophyll cycling and viability were monitored.

Materials and methods

Cultivation and experimental design

Iron replete cultures of *Chaetoceros brevis* (CCMP 163) were grown as batch cultures in autoclaved sea water of 35 PSU enriched with f-2 nutrients (Guillard & Ryter 1962). Iron limited cultures were grown in sea water of 35 PSU, collected from the Southern Ocean, enriched with f-2 nutrients (without iron), and then run over a Chelex-100 column (Chelex, Rochester, NY, USA) to remove iron (except the silicate stock). The polycarbonate cultivation vessels of iron limited cells were washed with 1 N HCl and handled in a clean room to prevent iron contamination, whereas 10 µM EDTA (final concentration) was added to bind remaining iron. Iron-limited and replete cultures were grown at 4.5°C in a cooled culture cabinet under 75 µmol· photons·m^{-2}·s^{-1} photons PAR and a 16-8 h L-D cycle for several months during which the medium was regularly replaced. Acclimation to dynamic and constant irradiance was achieved by inoculating duplicate transparent polycarbonate erlenmeyers (2 L) with 30 mL culture, giving a total of 8 culture vessels. The dynamic irradiance set-up was as described in Van de Poll *et al.* (2007). Dynamic irradiance simulated vertical mixing over 4 h cycles, resulting in irradiance oscillations between 20-1350 µmol photons·m^{-2}·s^{-1} (Fig. 1, see results), whereas constant irradiance was kept at 260 µmol photons·m^{-2}·s^{-1} during the 16 h light period. The daily irradiance dose was similar for constant and dynamic irradiance. All cultures were maintained at 4.5°C by cryostat controlled water baths. After inoculation cell numbers were followed for 35 and 15 days, for iron-limited and replete cultures respectively. Iron-replete cultures were refreshed with new medium once. On the last day, samples for Fv/Fm, pigments, antioxidant activities and excessive irradiance sensitivity were obtained. After the experiments, the remaining iron-limited cultures were supplied with iron to test if this enhanced pigmentation, fluorescence and growth.

Growth

Two mL sub samples were obtained from the cultures for cell counts and immediately processed. Cell concentrations were determined on a Coulter MXL flow cytometer as in Van de Poll *et al.* (2006). The mean growth rate of each replicate was calculated from linear regression of 6–9 natural log transformed cell concentrations plotted against time.

Maximal quantum yield of PSII: Fv/Fm

To expand measuring capacity, two fluorometers were used to determine Fo and Fm after 5 min dark adaptation and the Fv/Fm was calculated as (Fm-Fo) / Fm. On the last day of the experiment, a dual modulated fluorometer with integrating sphere (Photosystem Instruments, Chech Republic, red excitation light; > 670 nm) was used to measure Fv/Fm of iron-replete and iron-limited cultures under dynamic irradiance. A pulse amplitude

modulation fluorometer (water PAM, Walz, Germany, blue excitation light; peak around 450 nm) was applied to measure Fv/Fm during excessive irradiance (simulated surface irradiance, SSI) in 5 mL sub samples, see below.

Pigment composition

Samples (75 mL) for pigment composition (one from each bottle, 2 replicates in total) were filtered on 25 mm GF/F (Whatman) by vacuum, frozen in liquid nitrogen and stored at -80ºC. Filters were freeze-dried (48 h) followed by pigment extraction in 3 mL 90% cold acetone (v/v, 48 h, 4ºC) after Van Leeuwe *et al.* (2006). Pigments were resolved by HPLC (Waters 2690 separation module, 996 photodiode array detector) with a C_{18} 5 µm DeltaPak reversed-phase column (Waters) and identified by retention time and diode array spectroscopy. For quantification, standards of chlorophyll *a*, fucoxanthin, diadinoxanthin, and diatoxanthin were used (DHI, Denmark). Cellular pigment concentrations were calculated from cell counts (flow cytometer), sample volume and extraction volume. Chlorophyll *c*1, 2 and β carotene were detected but not quantified in the present study.

Antioxidant activity: SOD activity

100-200 mL of culture was filtered on polycarbonate filters (47 mm, 2 µm pore size) by vacuum and stored at -80°C for SOD activity analysis. The Riboflavin/NitroBlue Tetrazolium (RF/NBT) method Beauchamp & Fridovich 1971), modified by Janknegt *et al.* (2007), was used to measure SOD activity. Filters were suspended in 0.75 mL buffer (50 mM KH_2PO_4, pH 7.8, 0.1 mM EDTA, 0.1% Triton X-100, 2% PVP, and Complete protease inhibitor cocktail). Cells were disrupted by sonication (2 * 30 s pulses, 4°C), and extracts were centrifuged (20.000 g, 4°C, 25 min), and transferred to a clean vial. SOD activity was defined as the amount of sample required for 50% inhibition of NBT reduction (V_{50}). NBT reduction was measured in reaction mixtures (1.5 mL) with 0, 12.5, 25, and 50 µL extract, SOD buffer (50 mM KH_2PO_4, 0.1 mM EDTA, and 0.25% Triton X-100), 2 mM riboflavin (Sigma), and 57 µM NBT (Sigma). After 30 min dark incubation (4°C) the reaction mixtures were illuminated from above for 15 min (Philips TLD/18W, 199 µmol photons$\cdot m^{-2}\cdot s^{-1}$). Afterwards, absorbance was measured at 560 nm on a Cary 3E UV/vis double beam spectrophotometer (Varian, Middelburg, The Netherlands). The V_{50} was calculated by regression using the linear part of a natural semi-log curve. The specific activity of SOD (U/mg protein) was calculated according to 1 / (V_{50}*[protein]). Protein concentrations in the extracts were determined in duplicate according to Bradford (1976).

Antioxidant activity: APX and GR activity

Sampling, filtration and extract preparation for ascorbate peroxidase (APX) and glutathione reductase (GR) activities was as for the SOD activity (see above). APX activity was determined spectrophotometrically by the consumption of ascorbic acid, for 3 min in quartz cuvette containing 2.4 mL buffer (50 mM KH_2PO_4, pH 7; 0.1 mM EDTA), 150 µL 10 mM ascorbic acid (Merck, Darmstadt Germany), and 400 µL cell extract. Reaction mixtures were incubated (5 min) at 4°C before 20 mM H_2O_2 was added. The rate of ascorbate consumption was measured at 290 nm on a Cary 3E UV/vis double beam spectrophotometer at 4°C. Specific activity (U) of APX was calculated according to: (-slope * $volume_{tot}$ / ε * $volume_{extr.}$) / [protein] of which ε = 2.8 $mM^{-1}\cdot cm^{-1}$.

GR activity was determined by its ability to convert oxidized glutathione (GSSG) into its reduced form (GSH) using NADPH as a reducing agent. NADPH consumption was measured spectrophotometrically at 340 nm for 3 min in a 3 mL quartz cuvette with 1 mL buffer, 150 µL, 10 mM GSSG (Merck, Darmstadt Germany), 300 µL cell extract and 50 µL, 2.5 mM NADPH (Merck, Darmstadt Germany). Before NADPH addition, the reaction mixture was incubated for 5 min at 4.5 °C. Specific activity of GR (in U/mg prot) was calculated according to: (-slope * $volume_{tot}$ / ε * $volume_{extr.}$) / [protein] of which ε = 6.22 $mM^{-1} \cdot cm^{-1}$.

Excessive irradiance sensitivity

350 mL portions of the cultures were transferred to 1N HCl washed quartz vessels and exposed to simulated surface irradiance (SSI: 448, 50, and 0,6 W m^{-2} PAR, UVAR, and UVBR, respectively) as in Van de Poll *et al.* (2005). Excessive irradiance effects on pigment composition and Fv/Fm were examined by taking samples before, directly after 20 min of SSI, and after 20 min of SSI followed by 60 min in low (10 µmol photons$\cdot m^{-2} \cdot s^{-1}$) irradiance. In addition, viability loss during prolonged SSI exposure (4 h) was investigated in a separate experiment using 25 mL of culture for each replicate. Subsamples for the viability assay were obtained every hour during 4 h SSI exposure (see below). During SSI exposure temperature was maintained at 4.5°C.

Viability loss during excessive irradiance

One mL sub samples were incubated for 30 min in darkness with 10 µL SYTOX (Molecular probes) solution that was 100 times diluted in MilliQ. SYTOX can enter and stain the DNA of cells with compromised membranes, a non-reversible condition that characterizes non-viable cells. SYTOX emits green fluorescence after excitation at 488 nm. The presence of cellular green fluorescence of SYTOX was assessed flow cytometrically. For each data point at least $30 \cdot 10^3$ individual cells were analyzed.

Statistics

Differences between groups of replicates were tested for significance with a two factor (iron replete, iron limited, and constant, dynamic irradiance) ANOVA and a LSD post hoc test.

Results

Fv/Fm during dynamic irradiance

On the last day of the experiment, Fv/Fm was significantly higher for iron- replete than for iron-limited cells. Moreover, iron-replete cells showed a stronger response in Fv/Fm under dynamic irradiance than iron-limited cells (Fig. 1). For iron-limited cells, changes in Fv/Fm were minimal during the irradiance dynamics.

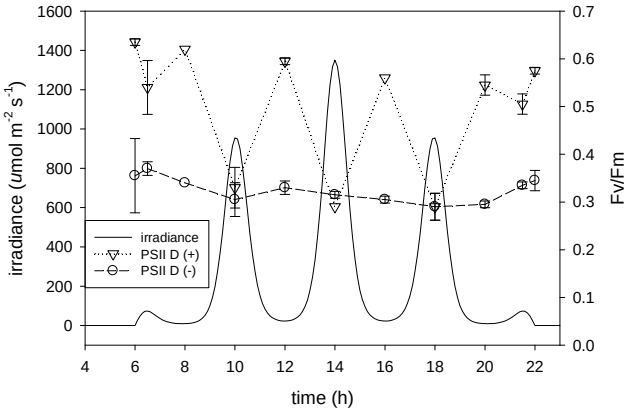


Figure 1: Irradiance and maximal quantum yield of PSII (Fv/Fm) of iron-limited (-) and replete (+) *Chaetoceros brevis* during the course of the dynamic (D) irradiance treatment. Mean and standard deviations are shown for two replicates.

Growth

Mean growth rates of iron-replete cultures (followed for 15 days) were significantly higher (~60%) than those of iron-limited cultures (followed for 35 days) (p= 0.0001, Fig. 2a). On the last day of the experiment all cultures were still in exponential growth. There was no significant difference in growth between static and dynamic irradiance under iron-limited and iron- replete conditions.

Pigment composition

Significant differences in pigment composition were found between iron- limited and iron-replete, and between dynamic and constant irradiance conditions. Total pigments (pg per cell) were 3 fold higher under iron-replete than iron-limited conditions for dynamic irradiance, whereas there was a twofold difference between iron- replete and iron-limited conditions under constant irradiance (Fig. 2b).

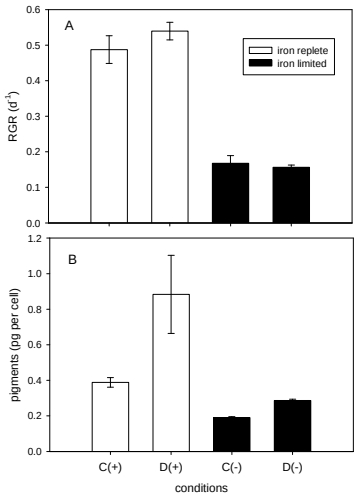


Figure 2: Relative growth rates (A) and cellular pigment concentrations (B) of iron-limited (-) and replete (+) *Chaetoceros brevis* grown under dynamic (D) and constant irradiance (C). Mean and standard deviations are shown for two replicates.

Cellular light harvesting (fucoxanthin and chlorophyll *a*) pigment contents were significantly higher for dynamic irradiance than for constant irradiance (p=0.01, Table 1). Cellular concentrations of both pigments were significantly lower during iron limitation (p=0.005). There was also a significant interaction between irradiance and iron availability for fucoxanthin (p=0.036) and chlorophyll *a* (p=0.037), showing different responses of the main light harvesting pigments under dynamic and constant irradiance during iron replete and limited conditions.

Table 1: Cellular concentrations of chlorophyll *a*, fucoxanthin, and the pool of diadinoxanthin and diatoxanthin of iron limited (-) and replete (+) *Chaetoceros brevis* grown under dynamic (D) and constant irradiance (C). The ratio between protective (diadinoxanthin, diatoxanthin) and light harvesting (chlorophyll a, fucoxanthin) is also shown (LP/LH). Mean and standard deviations are shown for two replicates.

Pigment	C (+)	D (+)	C (-)	D (-)
Chlorophyll a	0.130(0.007)	0.353(0.068)	0.048(0.002)	0.083(0.003)
Fucoxanthin	0.070(0.003)	0.198(0.037)	0.027(0.001)	0.053(0.004)
Diadino+diatoxanthin	0.093(0.015)	0.155(0.038)	0.058(0.008)	0.076(0.010)
Ratio (LP/LH)	0.470(0.079)	0.285(0.045)	0.783(0.139)	0.565(0.089)

The cellular pool of protective pigments (diadinoxanthin and diatoxanthin) was significantly different for irradiance and iron conditions. Iron-replete cells contained more

protective pigments than iron- limited cells, whereas cells grown under dynamic irradiance possessed a higher pool than those grown under constant irradiance. For the protective pigments there was no interaction between irradiance and iron conditions. The ratio of protective relative to light harvesting pigments was significantly higher for constant irradiance grown cells than for dynamic irradiance grown cells ($p=0.0001$), and was significantly higher for iron-limited cells compared to iron-replete cells. There was no interaction between irradiance and iron conditions for this ratio.

Antioxidant capacity

Significant differences in SOD and APX activities were not found between dynamic and constant irradiance grown cells under iron-replete conditions (Fig. 3). During iron limitation SOD and APX activity was increased 3 fold for cultures exposed to dynamic irradiance, but lower for those exposed to constant irradiance, compared to iron replete activity levels. APX activity followed a similar pattern as SOD activity, and the activity of both enzymes was significantly correlated (r^2: 0.88, n = 8, results not shown). Under iron limitation, APX activity was twofold enhanced under dynamic irradiance, but 10 fold reduced under constant irradiance. Significant differences in glutathione reductase were not found between any of the tested conditions (results not shown). The mean specific activity of glutathione reductase was 2.4 (±0.4) U per mg protein for 8 replicates.

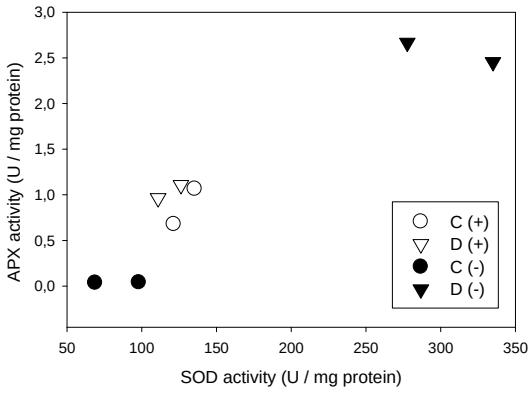


Figure 3: Activities of the antioxidant enzymes ascorbate peroxidase (APX) and superoxide dismutase (SOD) for *Chaetoceros brevis* grown under dynamic (D) and constant irradiance (C) under iron-limited (-) and replete (+) conditions. The activities were determined for two replicates.

Excessive irradiance sensitivity: Fv/Fm

Absolute F_v/F_m values were lower for the water PAM compared to those obtained with the PSI fluorometer, but the relative differences between the conditions were similar. As expected, there was a strong effect of iron on Fv/Fm: the highest Fv/Fm was found for iron-replete cells grown under dynamic irradiance. Fv/Fm was lower for iron-limited cells,

whereas significant differences between constant and dynamic irradiance cultivation were not observed (Figs. 4a, b). After 20 min SSI treatment Fv/Fm was significantly reduced to 23 and 58% of the pre-exposure values for iron-limited and replete conditions, respectively. Nearly complete recovery was found after 60 min in low irradiance for all cultivation conditions.

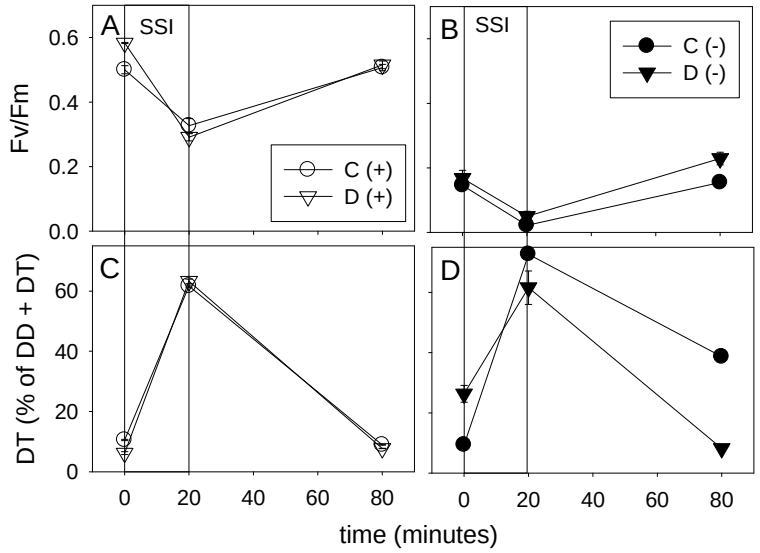


Figure 4: Dynamics of Fv/Fm (**a**, **b**) and the concentration of diatoxanthin relative to the diadino+diatoxanthin pool (**c**, **d**) during 20 min excessive irradiance exposure (SSI) and 60 min recovery in low irradiance for iron-replete (A, C) and limited (B, D) *Chaetoceros brevis* grown under dynamic (D) and constant irradiance (C). Mean and standard deviations are shown for two replicates.

Excessive irradiance sensitivity: Pigment composition

Apart from the de-epoxidation state of diadinoxanthin there were no significant changes in pigment composition after 20 min SSI and 60 min recovery in low irradiance (not shown). Before the SSI treatment 8-24% of the protective pigments was in the form of diatoxanthin (Figs. 4c, d). Directly after SSI this was 64-74%, whereas this was reduced to 8% after 60 min recovery in low irradiance, except for iron-limited cells that were grown in constant irradiance (39%).

Excessive irradiance sensitivity: Viability loss

The number of non-viable cells was low in iron-replete and iron-limited cultures before SSI exposure, with no difference between dynamic and static irradiance cultivation (mean 2.5±0.5% of cells non-viable, results not shown). The proportion of non-viable cells did not change significantly during 4 h SSI exposure (4 h SSI: mean 2.5±0.9% of cells non-viable).

Discussion

Chaetoceros brevis is a common open ocean species from the Southern Ocean with a low iron requirement due to its small size (5 µm) (Timmermans *et al.* 2001). Cultivation of this species under iron free conditions revealed some well described effects of iron limitation: a reduction in cellular pigments, a reduced ability to profit from irradiance, and highly reduced growth rates. During our experiment, we simulated vertical mixing by fluctuations between high (1350 µmol photons·m^{-2}·s^{-1}) and low irradiance (20 µmol photons·m^{-2}·s^{-1}) and compared growth and physiology of *C. brevis* with that under saturating, non fluctuating irradiance (260 µmol photons·m^{-2}·s^{-1}). Obviously, irradiance patterns that algae experience in the water column are more complex than those during our treatments. Despite the large range in irradiance, no differences in growth rates were observed between the constant and dynamic treatments under iron-limited and replete conditions. Nevertheless, significant physiological differences were found in pigment composition, Fv/Fm, and antioxidant activity. This showed that the algae had photoacclimated efficiently to both conditions, thereby mitigating potential effects of irradiance limitation and photoinhibition. Furthermore, this agrees with recent findings for iron-limited diatom assemblages from the North Pacific that displayed clear physiological responses after iron addition, whereas changes in productivity were not observed (Hopkinson & Barbeau 2008).

The temporal irradiance fluctuations experienced during the dynamic irradiance treatment increased cellular pigment content more than threefold compared to constant irradiance, under iron-replete conditions. This is consistent with previous results for *Thalassiosira weissflogii* and *Emiliania huxleyi* grown under the same conditions (Van de Poll *et al.* 2007), showing that dynamic irradiance acclimated algae displayed more low irradiance acclimation characteristics than those acclimated to constant irradiance of equal daily dose. Furthermore, these results imply that vertical mixing increases cellular iron demand. Moreover it suggests that algae have a higher iron requirement under natural irradiance conditions (dynamics caused by diurnal cycle, position in the water column and weather) than under the constant irradiance conditions normally used in the lab. As previously demonstrated, reduced light availability increased the cellular demand of iron rich components (Raven 1990, Sunda & Huntsman 1997, Strzepec & Price 2001).

Differences in cellular pigment content and Fv/Fm between dynamic and constant irradiance were significantly smaller under iron limitation, suggesting a reduced acclimation potential to prevailing irradiance conditions. However, the absence of effects on growth of iron-limited cells during dynamic and constant irradiance indicated that other phenomena compensated for this reduction in light harvesting. For example, the disproportionate down-scaling of light harvesting capacity during dynamic irradiance and iron limitation could be compensated by reduced self-shading of pigments, thereby increasing the light harvesting efficiency of the remaining pigments. Therefore, the interaction between iron and irradiance treatments could have originated from reduced self shading as was also suggested by Geider *et al.* (1993). Furthermore, relatively high, saturating irradiance was applied in both treatments, whereas vertical mixing can reduce the irradiance that algae experience, and impose light limitation when algae are mixed below the euphotic zone. Possibly, differences between dynamic and constant irradiance would be more pronounced during iron limitation and light limitation.

Previously, it was suggested that iron limitation enhanced excessive irradiance effects such as photoinhibition (Geider *et al.* 1993, Strzepek & Harrison 2004, Van Oijen *et al.* 2005). However, our research suggests that iron-limited *C. brevis* was not more vulnerable to excessive irradiance exposure than under iron-replete conditions. Nevertheless, the activities of the antioxidant enzymes SOD and APX were markedly enhanced for iron-limited cells grown under dynamic irradiance. The increased activities of both enzymes is indicative of enhanced superoxide formation during dynamic irradiance. We propose that increased cellular antioxidant activity is triggered by the periodic over-reduction of the photosynthetic electron transport chain during fluctuations in PSII excitation. Evidence for elevated ROS during iron limitation was reported previously for diatoms (Peers & Price 2004). Elevated ROS may be due to impairment of the PSI and cytochrome b_6-f complexes, which are particularly iron rich and their relative abundance can be reduced in response to iron limitation (Strzepek & Harison 2004). An imbalance between PSII and cytochrome b_6-f complexes could reduce the efficiency of the PSII electron cycle and therefore mediate increased over-reduction of PSII (Lavaud & Strzepek 2007). This scenario leads to enhanced superoxide radical formation, which requires increased scavenging by SOD and APX. In contrast to dynamic irradiance, a significant decrease in APX and SOD activities was found during iron limitation and constant irradiance. We hypothesize that the absence of irradiance fluctuations reduced PSII excitation pressure and therefore did not trigger elevated antioxidant activity. Because no growth reduction was found between dynamic and constant irradiance during iron limitation, it appeared that elevated antioxidant activity compensated the increased superoxide formation during the former condition. Elevated antioxidant activity can increase cellular iron demand because peroxidases like APX and superoxide dismutase (SOD) can contain iron cofactors. However, the iron cofactor in the latter enzyme can be substituted by manganese (Mn) under iron limitation in *T. weissflogii* (Peers & Price 2004). In our research SOD and APX activities were normalized to the protein content from the extracts. Normalization to chlorophyll *a* has been reported by Sunda *et al.* (2002), which would accentuate SOD and APX activity differences due to the large iron limitation mediated reduction in cellular chlorophyll *a*.

Iron-limited cells have a highly reduced light harvesting capacity, but this is accompanied by a much smaller reduction in the protective xanthophyll pigments pool. As a result, the ratio of protective relative to light harvesting pigments was higher during iron limitation of dynamic and constant irradiance grown cells. Previous experiments with low irradiance cultivated *C. brevis* showed pronounced viability loss during excessive irradiance. However, these cells had a 6 fold lower ratio of protective relative to light harvesting pigments than the cultures with the lowest ratio from the current experiment (iron-replete cells grown in dynamic irradiance) and a 16 fold difference with the iron-limited cells that were grown under constant irradiance

Consequently, *C. brevis* was relatively insensitive to the harmful effects of excessive irradiance under all of the current cultivation conditions, as shown by fast Fv/Fm recovery and the lack of viability loss after excessive irradiance.

Although the dynamic irradiance treatment covered almost the entire irradiance range that algae experience in the water column, Fv/Fm of iron-limited *C. brevis* showed surprisingly little response during the irradiance fluctuations. In contrast, Fv/Fm was strongly regulated under iron replete conditions during the dynamic irradiance regime, which coincided with xanthophyll (de-)epoxidation cycles (results not shown). The low

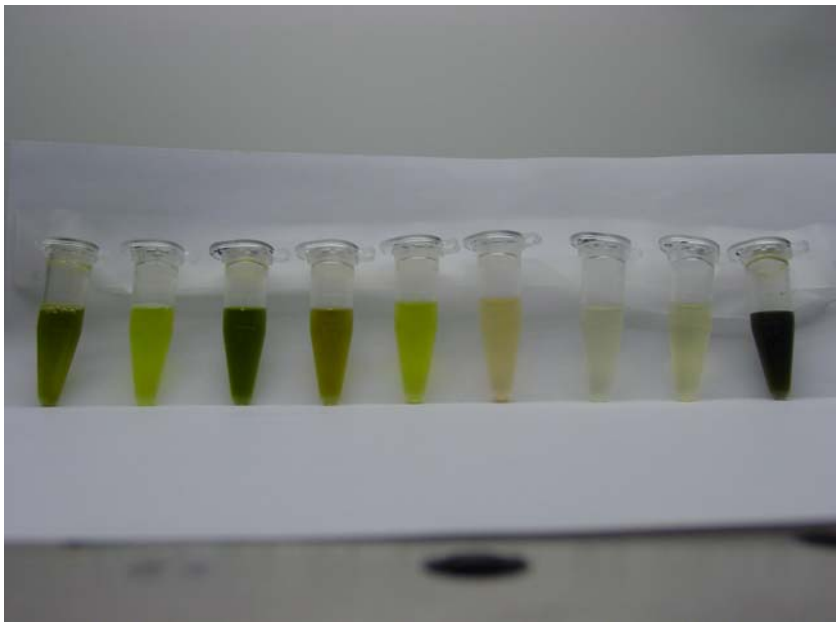
Fv/Fm under iron limitation at the start of the excessive irradiance exposure experiments coincided with low xanthophyll de-epoxidation. Therefore, low Fv/Fm values during iron limitation did not appear to be the result of xanthophyll cycle activity. The excessive irradiance exposure data showed no clear differences in xanthophyll cycle dynamics for iron-limited and replete cells after and during recovery from excessive irradiance. Furthermore, Fv/Fm recovery after 20 min simulated surface irradiance was not clearly different for iron replete and limited cells. Previous research on *Phaeocystis antarctica* reported reduced xanthophyll cycle activity under iron limitation during a dynamic irradiance treatment (Van leeuwe & Stefels 2007). Presumably, the proton gradient over the thylakoid membrane develops slower in iron-limited cells, thereby suppressing xanthophyll de-epoxidation. However, during our experiments we found no evidence for this and this issue should be verified in future research. The similar PSII response of iron limited and replete cells, and the lack of viability loss during excessive irradiance indicated that xanthophyll de-epoxidation could play a role in photo protection, regardless of iron availability.

In summary, PSII dynamics were reduced and SOD and APX activities were enhanced when iron-limited cells were grown under dynamic irradiance, in comparison to iron-replete cells. However, growth rates of iron-limited and replete *C. brevis* were not different under dynamic irradiance compared to saturating, non-fluctuating irradiance. This indicates that the increased antioxidant activity compensated for decreased non photochemical quenching efficiency around PSII. Furthermore, xanthophyll cycle activity was still induced in iron-limited cells under excessive irradiance. Thus, the observed physiological changes secured maintenance of growth during dynamic irradiance and photoprotection against excessive irradiance.

Chapter 5

Short term antioxidative responses of 15 microalgae exposed to excessive irradiance including ultraviolet radiation

Paul J. Janknegt, C. Marco de Graaff, Willem H. Van de Poll, Ronald J. W. Visser, Jan W. Rijstenbil and Anita G. J. Buma



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Abstract

Short term photosensitivity and oxidative stress responses were compared for three groups of marine microalgae: Antarctic microalgae, temperate diatoms and temperate flagellates. In total, 15 low light acclimated species were exposed to simulated surface irradiance including ultraviolet radiation (SSI). Photosensitivity was assessed as the rate of recovery of Fv/Fm in the hours following SSI treatment. Before, during and after the SSI treatment, oxidative stress responses were assessed by following xanthophyll content and cycling, and activities of superoxide dismutase, ascorbate peroxidase and glutathione reductase, and glutathione redox status. When acclimated to low irradiance, antioxidant levels were not group specific. Superoxide dismutase activity was positively correlated with cell size, whereas in general, ascorbate peroxidase activity appeared to be lower and glutathione redox status appeared to be higher in Antarctic than in temperate species. After SSI exposure, the strong inhibition of PSII was followed by variable rates of recovery, yet 4 species remained photosynthetically inactive. SSI tolerance appeared neither related to geographic or taxonomic background nor to cell size. PSII recovery was enhanced in species with decreasing superoxide dismutase activity and glutathione redox status and increased xanthophyll cycle activity. We conclude that antioxidant responses are highly species specific and not related to geographic or taxonomic background. Furthermore, xanthophyll cycling seems more important than antioxidants. Finally, it can be hypothesized that glutathione could function as a stress sensor and response regulator.

Introduction

Marine microalgae can be exposed to high irradiance conditions such that the light harvesting capacity can exceeds light processing capacity. As a result, overreduced electron transport chains of the photosystems can leak electrons onto O_2 thereby generating reactive oxygen species (ROS; Mehler 1951, Asada *et al.* 1974, Gechev *et al.* 2006). The presence of ultraviolet radiation (UVR: 280-400 nm) has detrimental effects on metabolic pathways and therefore stimulates ROS accumulation (Bischof *et al.* 2003, Halliwell 2006).

ROS can cause severe damage in marine microalgae leading to decreased photosynthetic performance or even viability loss (Van de Poll *et al.* 2005). Marine microalgae possess a suite of mechanisms to prevent or counteract ROS accumulation. To restrain the production of ROS, cells regulate the energy flow towards the photosystems by adjusting the protective/harvesting pigment ratio (Falkowski & LaRoche 1991) and by activating the xanthophyll cycle (Demers *et al.* 1991, Olaizola *et al.* 1994, Demming-Adams & Adams 1996). Moreover, several larger (>20 µm) microalgal species are known to synthesize UVR absorbing compounds as mycosporine-like amino acids (MAAs) which are mostly present at the periphery of the cell (Karentz *et al.* 1991a, Helbling *et al.* 1996, Buma *et al.* 2006).

When the prevention mechanisms are insufficient, superoxide ($O_2^{\bullet-}$) is the first radical species formed in the photosystems (Yu 1994, Dat *et al.* 2000) and can be converted into hydrogen peroxide (H_2O_2). Both species are moderately harmful but in the presence of unchelated bivalent cations (i.e. $Fe^{2+/3+}$, $Cu^{1+/2+}$), $O_2^{\bullet-}$ is able to reduce H_2O_2, thereby generating the hydroxyl radical ($HO^{\bullet}$) (Kehrer 2000). $HO^{\bullet}$ is one of the most reactive species known to chemistry causing lipid peroxidation, DNA damage and protein oxidation (Halliwell 2006). Microalgae are thought to counteract ROS by means of their antioxidant network (Mallick & Mohn 2000, Asada 2006) in which the enzyme superoxide dismutase (SOD) catalyzes the conversion of $O_2^{\bullet-}$ into H_2O_2 (Gregory & Fridovich 1973*a*, *b*; Klug & Rabani 1992). Because SOD is the only enzyme capable of $O_2^{\bullet-}$ removal thereby preventing uncontrolled production of $HO^{\bullet}$, it holds a key position within the antioxidant network (Bowler 1992). Based on their metal co-factor, several SOD isozymes can be distinguished: Fe-, Mn- and Cu/ZnSOD, which occupy specific intracellular sites (Wolfe-Simon *et al.* 2005). Furthermore, SOD isozymes vary in amino acid composition, spatial structure and cellular content.

H_2O_2, formed by SOD activity, can diffuse out of the cell or be scavenged by a suit of other enzymes such as ascorbate peroxidase (APX; Shigeoka *et al.* 2002, Asada, 2006). During scavenging, APX oxidizes ascorbic acid, a low molecular weight antioxidant which is also used for regeneration of other oxidized antioxidants and non-enzymatic ROS scavenging. Ascorbic acid can be regenerated enzymatically, using glutathione as a substrate which can act as a non-enzymatic antioxidant as well (de Kok & Stulen 1993, Noctor *et al.* 2002). Oxidized glutathione (GSSG) is eventually reconverted into its reduced form (GSH) by glutathione reductase (GR). Therefore, GR is responsible for fueling the antioxidant network (Apel & Hirt 2004), controlling the glutathione redox status (GRS; ratio of GSH over total amount of glutathione).

Antioxidant responses to excess irradiance, including UVR, have been investigated extensively in plants (Strid 1993, Willekens *et al.* 1994, Ledford & Niyogi 2005) and

macroalgae (Aguilera *et al.* 2002*a*, *b*; Dummermuth *et al.* 2003, Shiu & lee 2005) while much less has been reported for microalgae. Data regarding microalgae are scattered and difficult to compare; experiments differ widely in set up, exposure time, spectral composition, irradiance intensity, acclimation and growth condition, or the species under consideration (single species or whole communities; Butow *et al.* 1997; Malanga *et al.* 1999, Lesser *et al.* 2002, Rijstenbil 2002, Sigaud-Kutner *et al.* 2002). Our aim was to elucidate if (irradiance induced) antioxidative responses were related with geographic or taxonomic background, or cell size. Therefore, 15 species, ranging from small Antarctic flagellates to large temperate diatoms were exposed to excess irradiance including UVR (SSI). To this end, several studies have demonstrated cell size related UVR vulnerability of phytoplankton (Karentz *et al.* 1991*b*, Laurion & Vincent 1998, Helbling *et al.* 2001b). Therefore, we examined the influence of specific cell size on irradiance stress (indicated by PSII recovery) and antioxidative processes (UV absorbing compounds, SOD activity and isozyme composition, APX activity, GR activity, GRS, xanthophyll cycling). Secondly, we compared temperate with polar species since polar microalgae are less likely to be adapted to increased levels of UVR due to their evolutionary history (Helbling *et al.* 1992a, Martínez 2007). Moreover, O_2 solubility is much higher in low temperature seawater, and low temperature organisms contain more poly-unsaturated fatty acids, both potentially increasing the risk of oxidative damage (Louanchi *et al.* 2001, Lesser 2006). Furthermore, since diatoms and phytoflagellates are thought to use different strategies to cope with excess irradiance we compared responses of distinct taxonomic groups (Davidson & Marchant 1994, Karentz 1994, Wagner *et al.* 2006).

Materials and methods

Culturing conditions

Experiments were conducted with the Antarctic species *Chaetoceros brevis*, *Chaetoceros dichaeta*, *Chaetoceros* sp., *Nitzschia frigida*, and *Pyramimonas* sp., and the temperate species *Odontella sinensis*, *Navicula salinarum*, *Navicula pelliculosa*, *Nitzschia ovalis*, *Thalassiosira weissflogii*, *Tetraselmis suecica*, *Prorocentrum micans*, *Fibrocapsa japonica*, *Emiliania huxleyi*, and *Porphyridium purpureum* (general information see Table 1). Of these 15 species 4 were benthic and 11 pelagic. It is important to notice that all cultures were maintained and grown for years under laboratory conditions without being exposed to UVR. Prior to the experiments, all cultures were grown in triplicate at 25 µmol photons·m^{-2}·s^{-1} photosynthetically active radiation (PAR) in 200 mL modified f/2 enriched seawater (Veldhuis & Admiraal 1987) at 34.5 practical salinity units (PSU). *Navicula salinarum* was cultured at a salinity of 25 PSU. Light was provided by Biolux fluorescent tubes (Osram) and the cultures were grown in a 16:8 h light-dark cycle, shaken manually each day, and medium was replenished every ten days. After three months, 150 mL of each culture was transferred to a UV transparent 2.2 L polymethylmethacrylate bottle containing 1500 mL of fresh medium, after which the cultures were placed back at 25 µmol photons·m^{-2}·s^{-1} PAR for 1 week.

Table 1: Cell size, pigment and culture isolate characteristics of 15 microalgal species. Specific cell size is indicated as surface/volume (S/V) ratio. Major harvesting pigments (Major harv. pig.) consisted of chlorophyll *a* (Chl.*a*), chlorophyll b (Chl.*b*) chlorophyll *b2* (Chl.*b2*) and fucoxanthin (Fuco). Major protective pigments (Major prot. pig.) consisted of diadinoxanthin (DD), diatoxanthin (dt), violaxanthin (V) antheraxanthin (A) and zeaxanthin (Z). Species were aquired from Goettingen university culture collection (SAGCC), Netherlands institute of ecology culture collection (CCY), university of Groningen culture collection (RUGCC), Bigelow laboratory for ocean sciences culture collection of marine phytoplankton (CCMP), Loras college freshwater diatom culture collection (FDCC), Norwegian polar institute culture collection (NPICC) and the royal Netherlands institute for sea research culture collection (NIOZCC).

Species	Class	S/V ratio	Major harv pig	Major prot pig	Isolate First author
Antarctic microalgae					
Chaetoceros brevis	Bacillariophyceae	1.22	Chl.*a*, Fuco	DD, DT	CCMP163 Schütt
Pyramimonas sp.	Prasinophyceae	1.21	Chl.*a*, Chl.*b*	VAZ	RUGCC, isolated from Weddell Scotia Confluence Schmayda
Chaetoceros sp.	Bacillariophyceae	1.05	Chl.*a*, Fuco	DD, DT	RUGCC, isolated from Weddell Scotia Confluence Ehrenberg
Nitzschia frigida	Bacillariophyceae	0.72	Chl.*a*, Fuco	DD, DT	NPICC Isolated from Barentz Sea by Tamelander Grunow
Chaetoceros dichaeta	Bacillariophyceae	0.31	Chl.*a*, Fuco	DD, DT	CCMP 1751 Mangin
Temperate diatoms					
Nitzschia ovalis	Bacillariophyceae	1.29	Chl.*a*, Fuco	DD, DT	CCY 0205 Arnott
Navicula pelliculosa	Bacillariophyceae	1.19	Chl.*a*, Fuco	DD, DT	CCMP 543 (Brébisson and Kützing) Hilse
Navicula salinarum	Bacillariophyceae	1.1	Chl.*a*, Fuco	DD, DT	FDCC L1262 Grunow
Thalassiosira weissflogii	Bacillariophyceae	0.82	Chl.*a*, Fuco	DD, DT	CCMP 1049 (Grunow) Fryxell and Hasle
Odontella sinensis	Bacillariophyceae	0.23	Chl.*a*, Fuco	DD, DT	CCY 0380 (Greville) Grunow
Temperate flagellates					
Porphyridium purpureum	Rhodophyceae	1.33	Chl.*a*	Not found	SAGCC 1380.1a (Bory de Saint-Vincent) Drew and Ross
Emiliania huxleyi	Haptophyta	0.95	Chl.*a*, Fuco	DD, DT	Strain L. isolated from Oslo fjord by Paasche (Lohmann) Hay and Mohler
Tetraselmis suecida	Prasinophyceae	0.70	Chl.*a*, Chl.*b*	VAZ	NIOZCC (Kylin) Butcher
Fibrocapsa japonica	Raphidophyceae	0.30	Chl.*a*, Fuco	VAZ	CCRUG W420 Toriumi and Takano
Prorocentrum micans	Dinophyceae	0.18	Chl.*a*, Chl.*b2*	DD, DT	T135MC1 Ehrenberg

Simulated surface irradiance

The polymethylmethacrylate bottles, containing optically dilute cultures, were exposed to 3 h of Simulated Surface Irradiance (SSI: 1580 µmol photons·m^{-2}·s^{-1} PAR, 42 W·m^{-2} UVAR and 0.47 W·m^{-2} UVBR) as in Van de Poll *et al.* (2005), five hours after the beginning of light period. These irradiance conditions are comparable with solar irradiance at noon at mid latitude (Van de Poll *et al.* 2005). The temperature was controlled by a water bath at 4 or 12°C. After the SSI treatment the bottles were transferred to the pre-exposure conditions (25 µmol photons·m^{-2}·s^{-1} PAR) for 21 h (including a dark period from t=13 till t=21) during which recovery was studied. SSI exposure and subsequent recovery was repeated with the three replicates of each species.

Sampling

Samples for superoxide dismutase (SOD) Native PAGE gel electrophoresis, SOD activity, ascorbate peroxidase (APX) activity, glutathione reductase (GR) activity, glutathione content, pigment composition, UV-absorbing compounds and Fv/Fm measurements were obtained before the SSI treatment (t=0), after 3 h of SSI treatment (t=3 h), and after 3 h (t=6 h) and 21 h (t=24 h) of recovery. Samples for SOD Native PAGE gel electrophoresis, SOD, APX, and GR activity (70 mL), and glutathione content (100 mL) were filtered over 2.0 µm pore size polycarbonate filters (47 mm; Osmonics Inc., USA) and stored at -80°C for a maximum of six months. Samples for pigment analysis and UV-absorbing compounds (30 mL) were filtered over GF/F filters (25 mm) and stored at -80°C for a maximum of six months. Samples for Fv/Fm measurements (30 mL) were processed immediately (see *PSII efficiency*).

PSII efficiency

Measurements for Fv/Fm, calculated as $(F_m-F_0)/Fm$, were performed according to Van de Poll *et al.* (2007) and Janknegt *et al.* (2007). In short: samples for Fv/Fm measurements were filtered over GF/F filters (11.3 mm; Whatman, Maidstone, UK) by mild vacuum. Filtration was stopped before the filter dried. The filter was transferred to a temperature controlled seawater-filled cuvette and chlorophyll fluorescence was measured with a PAM 2000 pulse amplitude modulated fluorometer after 5 min of dark acclimation (Walz, Effeltrich, Germany). Measurements were performed at either 4°C or 12°C depending on the cultivation temperature of the species.

SOD activity and isozyme composition

SOD analyses were done using the riboflavin/Nitro Blue Tetrazolium (RF/NBT) method modified for algal material, as described in Janknegt *et al.* (2007). In short, filters were disrupted by sonication in 0.75 mL protein extraction buffer X (50 mM KH_2PO_4, pH7.8; 0.1 mM EDTA; 0.1% Triton X-100; 2% PVP and complete protease inhibitor cocktail). After spinning down cell debris, supernatants were transferred to a clean 1.5 mL Eppendorf tube and 150 µL was used for duplicate quantification of the protein concentration according to Bradford (1976). SOD activity was defined as the amount of sample required to achieve 50% inhibition of NBT reduction (V_{50}). NBT reduction was measured (in duplicate) in 4 reaction mixtures containing 0 (blank, no inhibition), 12.5, 25, and 50 µL extract. Absorption was measured at 560 nm on a Cary 3E UV/vis double beam

spectrophotometer (Varian, Middelburg, The Netherlands) equipped with a temperature controlled cell attached to a water bath. Fifty percent inhibition (V_{50}) was calculated by regression using the linear part of a semi natural-log plot. The specific activity (S.A.) of SOD was calculated according to 1 / (V_{50}*[protein]).

SOD activity and isozyme composition was visualized by native-PAGE gel electrophoresis as described in Janknegt *et al.* (2007). Fifteen µg protein from each extract was loaded on a non-denaturating 12% polyacrylamide gel using 5 µL protein loading buffer. After electrophoresis (80 V), SOD activity was visualized by soaking the gel in 50 mL staining buffer containing 0.25 mM RF (Sigma, Steinheim Germany), 5 mM NBT (Sigma, Steinheim Germany), and 0.5 mM Tetramethylethylenediamine (TEMED; Amresco, Solon USA) for 30 min in the dark, at 4°C or 12°C depending on the species analyzed. After 15 min of illumination, SOD bands appeared as light bands on a blue/purple background. To determine isozyme composition based on their metal cofactor, samples were synchronically separated on three distinct native-PAGE gels. Of these, the first gel was soaked in normal staining buffer, the second was soaked in staining buffer containing 3 mM H_2O_2, inhibiting FeSOD and Cu/ZnSOD activity and the third was soaked in staining buffer containing 5 mM KCN which inhibits Cu/ZnSOD.

APX and GR activity

Supernatants for both APX and GR activity assays were prepared and protein concentrations were determined as described for the *SOD activity assay* (see above). APX activity was determined spectrophotometrically by measuring the consumption of its substrate, ascorbic acid, over a 3 min. interval, in a 3 mL quartz cuvette containing 2.4 mL buffer A (50 mM KH_2PO_4, pH7; 0.1 mM EDTA), 150 µL, 10 mM ascorbic acid (Merck, Darmstadt Germany), 400 µL cell extract and 30 µL, 20 mM H_2O_2 (Merck, Darmstadt Germany). Before H_2O_2 was added, the reaction mixture was incubated for 5 min. at the appropriate temperature. The rate of ascorbate consumption was measured at 290 nm on a Cary 3E UV/VIS double beam spectrophotometer. Specific APX activity (U/mg prot) was calculated according to: (-slope x $volume_{tot}$ / ε x $volume_{extr.}$) / [protein] of which ε = 2.8 $mM^{-1} \cdot cm^{-1}$. Determination of GR activity was based on its catalytic property to convert oxidized glutathione (GSSG) into its reduced form (GSH) thereby consuming NADPH as a reducing agent. NADPH consumption was measured spectrophotometrically by determining its decrease over a 3 min interval in a 3 mL quartz cuvette containing 1 mL buffer A; 150 µL, 10 mM GSSG (Merck, Darmstadt Germany), 300 µL cell extract and 50 µL, 2.5 mM NADPH (Merck, Darmstadt Germany). Before NADPH was added, the reaction mixture was incubated for 5 min in a cryostat at the appropriate temperature. The rate of NADPH consumption was measured at 450 nm on a Cary 3E UV/VIS double beam spectrophotometer. Specific activity of GR (in U/mg prot) was calculated according to: (-slope x $volume_{tot}$ / ε x $volume_{extr.}$) / [protein] of which ε = 6.22 $mM^{-1} \cdot cm^{-1}$.

Glutathione content

Filters were resuspended in 0.4 mL glutathione extraction buffer containing 50 mM sulfosalicylic acid, 1 mM EDTA, and 0.15% ascorbic acid after which the cells were disrupted by sonication (30 s, at A=30) at 4°C. Extracts were centrifuged for 25 min (20.000 x g) at 4°C and supernatants were transferred to a clean 1.5 mL Eppendorf tube.

The total amount of glutathione (TAG) was determined using the SIGMA Glutathione Assay Kit (CS0260; Sigma, Saint Louis, MI, USA); all chemicals were purchased from Sigma unless indicated otherwise. The kit is based on the principle that two molecules of reduced glutathione (GSH) react with the Ellman reagents 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to release 5-thio-2-nitrobenzoic acid (TNB) and oxidized glutathione (GSSG). By adding glutathione reductase, all GSSG is continuously reduced to two molecules of GSH by which the conversion of DTNB to TNB continues as well. As a result, the production rate of TNB is proportional to the concentration of the total amount of glutathione. The assay was performed in 96 wells microtiter plates (Cayman chemical) containing 150 µL working mixture 1 (100 mM KH_2PO_4, pH7; 100 mM EDTA, GR, 0.170 U/mL; 0.043 mg/mL DTNB), and 10 µL cell extract. After 5 minutes of incubation at room temperature, 50 µL of a 0.16 mg/mL NADPH solution was added. TNB production was then followed spectrophotometrically for 10 min at 405 nm using a Victor^{tm} 1420 multilabel counter plate reader (PerkinElmer, Waltham, MA, USA) reading at 1 minute intervals. To determine glutathione content in 10 µL algal extract, a reference line was included based on 0.5, 0.25, 0.125, 0.0625 and 0.0312 nmoles GSH (Sigma, Saint Louis, MI, USA) in 10 µL sample. The TNB production rate was calculated for 1 nmole of glutathione by extrapolation. The TAG (in nmol) in 1 mL algal sample was calculated according to: $slope_{sample}$ x dilution factor original sample / $slope_{1nmole\ GSH}$ x 0.01 mL sample). The amount of GSH was measured by the same principle as TAG but without adding GR and NADPH by which only GSH reacts with DTNB. This assay was performed in 96 wells microtiter plates containing 100 µL working mixture 2 (500 mM KH_2PO_4, pH7; 500 mM EDTA; 0.065 mg/mL DTNB), and 100 µL cell extract. After 10 min of incubation at RT the total production of TNB was determined spectrophotometrically at 405 nm. A reference line was included based on 2, 1, 0.5, 0.25, and 0.125 nmoles GSH in 100 µL sample. The amount of GSH in the reaction mixture was calculated in accordance to the reference line. The amount of GSH (in nmol) in 1 mL algal extract was calculated according to: GSH in reaction mixture x dilution factor original sample / 0.1 mL sample. The glutathione redox status (GRS) was expressed in sulphur atoms and calculated according to: GSH / (GSH + 0.5 GSSG) of which. GSSG = TAG – GSH.

Determination of pigments

Pigments were analyzed as described by Van Leeuwe *et al.* (2006). Filters were handled under dim light, freeze-dried (48 h), after which pigments were extracted in 4 mL 90% cold acetone (v/v, 48 h, 5ºC, darkness). Pigments were resolved using HPLC (Waters 2690 separation module, 996 photodiode array detector) with a C_{18} 5 µm DeltaPak reversed-phase column (Milford, MA, USA) and identified by retention time and diode array spectroscopy. Quantification was done using standard dilutions of Chlorophyll *a*, Chlorophyll *b*, Chlorophyll *b2,* fucoxanthin, diadinoxanthin, diatoxanthin, violaxanthin, antheraxanthin and zeaxanthin.

UV–absorbing compounds

UV-absorbing compounds were analyzed as described by Tartarotti & Sommaruga (2002). Filters were suspended in 2 mL, 25% aqueous methanol (v/v) at 45ºC for 2 h. Thereafter, extracts were filtered through a 0.2 µm disposable cellulose acetate filter (Whatman, Dassel,

Germany). The absorption of the filtrate was measured spectrophotometrically between 280 and 700 nm. Baseline correction was done by subtracting background absorption using 25% aqueous methanol as a blank. UV-absorbing compounds were expressed as absorption peak normalized to Chl.*a*.

Calculation of specific surface area

Specific cell size (cell surface area/volume; SA/V) was calculated from volume and surface area which were determined according to Hillebrand *et al.* (1999). Length and width of 15 cells of each species were measured using an Olympus IMT-2 inverted light microscope (Olympus, Centre Valley, PA, USA). Depth was only determined for *Odontella sinensis*, *Navicula salinarum*, *Nitzschia frigida*, *Nitzschia ovalis* and *Pyramimonas* sp.

Statistical analyses

Measurements were done on 3 replicates unless indicated otherwise. Statistical analyses were done using SPSS 12.0.1 for windows. Differences between species and responses to the different treatments (3 h SSI, 3 and 21 h recovery in low irradiance) were tested with One-Way ANOVA. Intraspecific differences were analyzed using paired-samples t-test. The influence of specific surface area, geographic region taxonomic background on SOD activity, APX activity, GR activity, GRS, Fv/Fm, pigment content and ratios were tested in an univariate nested ANOVA. In this analysis, species, as a random factor, was nested into the factors specific surface area, geographic region and taxonomic background. Here, geographic region and taxonomic background were introduced as fixed factors and specific surface area was tested as a covariance. Statistical error was determined as error type I. r^2 was obtained by plotting the covariance against the parameter in a regression analysis. Non-parametric Spearman's rho analysis was performed on mean values (n=3) of SOD activity, APX activity, GR activity, GRS, Fv/Fm, pigment content and ratios to test data for correlations.

Results

Photosystem II efficiency

In order to compare interspecific changes in Fv/Fm, values were normalized to the species specific initial value. The three hours of SSI exposure (t=3) caused a significant decrease of F_v/F_m in all cultures (t-test, $p<0.0005$; Fig. 1) down to 0.5 - 15% of the initial values (averaging 0.6 +/- 1.5).

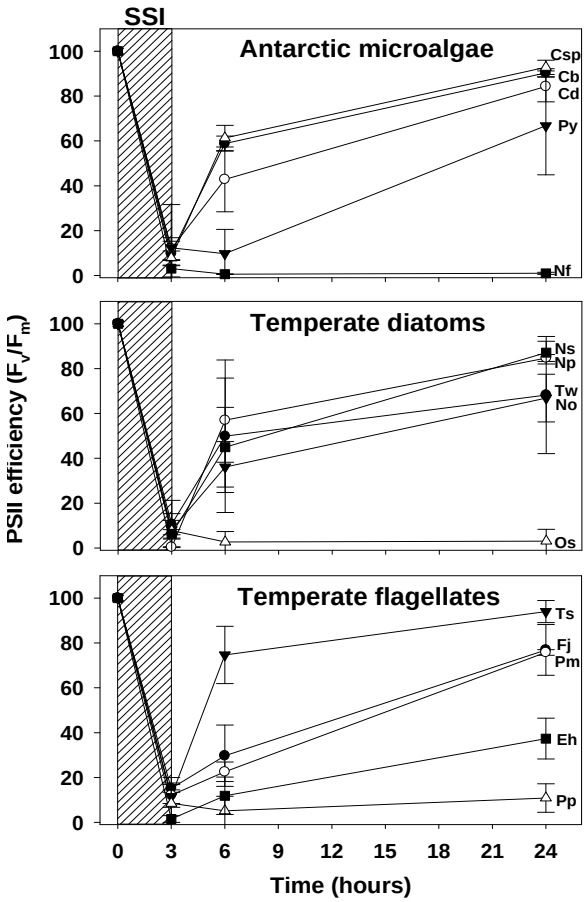

Figure 1: Photosystem II efficiency (Fv/Fm) of 15 microalgae when acclimated to low irradiance conditions (t=0; 25 µmol·m^{-2}·s^{-1} PAR), after 3 h exposure to SSI conditions (t=3; 1580 µmol photons·m^{-2}·s^{-1} PAR + 42 W·m^{-2} UVAR + 0.47 W·m^{-2} UVBR) and during the subsequent 21 h recovery period under low irradiance conditions (t=3 till t=24; 25 µmol photons·m^{-2}·s^{-1} PAR) which included a dark period from t=13 till t=21. Fv/Fm was expressed as percentage of the initial (t=0) value and error bars represent standard deviations (*n*=3). The dashed area (t=0 till t=3) represents the 3 h exposure period. Cb=*Chaetoceros brevis*, Cd=*Chaetoceros dichaeta*, Csp=*Chaetoceros* sp., Eh=*Emiliania huxleyi*, Fj=*Fibrocapsa japonica*, Nf=*Nitzschia frigida*, No=*Nitzschia ovalis*, Np=*Navicula pelliculosa*, Ns=*Navicula salinarum*, Os=*Odontella sinensis*, Pm=*Prorocentrum micans*, Pp=*Porphyridium purpureum*, Py=*Pyramimonas* sp., Ts=*Tetraselmis suecica* and Tw=*Thalassiosira weissflogii*.

Fv/Fm recovery during 21 h (t=3 till t=24) under low irradiance (25 µmol photons·m^{-2}·s^{-1}) was species specific After the first 3 h, Fv/Fm of *N. frigida*, *Pyramimonas* sp., *O. sinensis*, *E. huxleyi* and *P. purpureum* recovered poorly (< 12% of their initial F_v/F_m). In contrast, *P. micans* (23%), *F. japonica* (30%), *N. ovalis* (36%), *C. dichaeta* (43%), *N. salinarum* (45%), *T. weissflogii* (50%), *N. pelliculosa* (57%), *C. brevis* (59%), *Chaetoceros* sp. (61%) and *T. suecica* (74%) showed stronger Short term recovery of their Fv/Fm. After 21 h, Fv/Fm of *N. frigida*, *O. sinensis*, *E. huxleyi* and *P. purpureum* was still less than 38% of their initial values while all other species regained 67-94% of their initial F_v/F_m. No clear relation was observed in PSII responses based on size, taxonomic or geographic grouping.

Pigment analyses

The investigated species contained either the diato- diadinoxanthin cycle (DT/DD cycle) or the viola- anthera- zeaxanthin cycle (VAZ cycle), except for the red alga *P. purpureum* which possessed no xanthophyll cycle pigments (Table 1). After growing under low irradiance (25 µmol photons·m^{-2}·s^{-1}) for three months, the ratio of light protective relative to light harvesting pigments (LP/LH) ratio; DT+DD/Chl.*a*+Fuco or VAZ/Chl.*a*+Chl.*b*) reached values between 0.03 (*F. japonica*) and 0.13 (*C. brevis*; Table 2). Before and during the experiment, differences between these ratios could not be attributed to cell size, geographic background or taxonomic group although species containing the VAZ cycle had a significantly lower ratio than those equipped with the DT/DD cycle (One-Way ANOVA, $p<0.005$). In response to the SSI treatment, all species showed an increase in their LP/LH ratio (Table 2) which they achieved in two different ways. The species showing poor PSII recovery (*N. frigida*, *O. sinensis*, and *E. huxleyi*) exhibited decreased pigment concentrations in particular of the light harvesting pigments (Table 2). As a result, their LP/LH ratio increased with 68, 98 and 141%, respectively. All other cultures showed slight fluctuations in their harvesting pigment content and increased xanthophyll pigment concentrations thereby increasing the LP/LH ratio on average by 27%. *N. frigida*, *O. sinensis*, and *E. huxleyi* showed consistently declining pigment concentration during the recovery period (t=3 till t=24). All other species showed significantly higher LP/LH ratios after 21 h recovery in low irradiance (t-test, $p<0.05$; except for *Pyramimonas* sp and *N. ovalis*) than before the SSI treatment (on average 34% increased LP/LH ratio).

Xanthophyll cycle activity

Before, during and after the SSI treatment, (changes in) xanthophyll cycle activity depended neither on cell size nor on geographic region or taxonomic background. Throughout the experimental period (t=0 till t=24), *N. frigida*, *O. sinensis* and *P. purpureum* (which did not contain an active xanthophyll cycle) did not show changes in de-epoxidation status (DEPS: DT/DD+DT or zea- + antheraxanthin /viola- + anthera- + zeaxanthin) at all (Fig. 2). As a response to the SSI treatment, all cultures with an active xanthophyll cycle significantly (t-test, $p<0.005$) increased their DEPS, on average between 27 (*N. ovalis*) and 76% (*F. japonica*). DEPS decreased significantly (t-test, $p<0.005$) during recovery. Correlation analyses (Table 3) showed a positive and significant relation ($r=0.747$, $p<0.005$) between the increase in DEPS after the SSI treatment and 3h recovery of Fv/Fm.

Table 2: Ratio of light protective to light harvesting pigment (LP/LH) of 15 microalgae acclimated to low irradiance conditions (**Initial**; 25 µmol photons·m^{-2}·s^{-1} PAR), after 3 h exposure to SSI conditions (**after 3h SSI**; 1580 µmol photons·m^{-2}·s^{-1} PAR + 42 W·m^{-2} UVAR + 0.47 W·m^{-2} UVBR) and after a subsequent 21 h recovery period (**after 21h recovery**) under low irradiance conditions (25 µmol photons·m^{-2}·s^{-1} PAR) including a dark period from t=13 till t=21. All separate changes of light harvesting (LH) and light protective pigments (LP) are indicated as a percentage of the initial amount of pigment. Asterisk: increased significantly from Initial (t-test, p<0.05).

	Initial	***After 3h SSI***			***After 21h recovery***		
	LP/LH	LH	LP	LP/HP	LH	LP	LP/LH
Ant. microalgae							
Chaetoceros brevis	0.116	-3.6	4.7	0.125*	10.8	68.8	0.175*
Pyramimonas sp.	0.051	14.4	23.7	0.055*	-3.2	-2.3	0.052
Chaetoceros sp	0.127	7.0	17.4	0.139*	17.5	76.2	0.186*
Nitzschia frigida	0.051	-32.9	-16.0	0.074	-54.3	-22.7	0.090*
Chaetoceros dichaeta	0.113	-17.7	2.5	0.144*	-25.9	26.8	0.163*
Temp. diatoms							
Nitzschia ovalis	0.078	-4.9	16.3	0.095*	-9.4	6.0	0.090
Navicula pelliculosa	0.124	35.9	42.2	0.130	91.1	141.2	0.158*
Navicula salinarum	0.060	3.2	18.2	0.071	-4.8	22.1	0.075*
Thalassiosira weissflogii	0.056	-13.6	5.5	0.068*	-12.2	11.1	0.069*
Odontella sinensis	0.097	-48.8	-30.0	0.129	-93.9	-100	0
Temp. flagellates							
Porphyridium purpureum	n.a	-34	n.a.	n.a	-44.4	n.a.	n.a
Emiliania huxleyi	0.086	-47.5	-17.5	0.156	-95.9	-100	0
Tetraselmis suecica	0.032	23.1	33.4	0.035	6.3	34.7	0.041*
Fibrocapsa japonica	0.032	0.5	74.9	0.055*	-6.2	33.0	0.044*
Prorocentrum micans	0.098	21.9	34.4	0.108*	-6.8	31.4	0.138*

Table 3: Correlations between parameters superoxide dismutase (SOD) activity, de-epoxidation state of xanthophyll pigments (DEPS), ascorbate peroxidase (APX) activity, glutathione reductase (GR) activity, glutathione redox status (GRS) and short term recovery capacity of Fv/Fm (3 h PSII recov). Analysis were done using average values (*n*=3) of 15 microalgae when acclimated to low irradiance conditions (t=0; 25 µmol photons·m^{-2}·s^{-1} PAR) and after 3 h exposure to SSI conditions (t=3; 1580 µmol photons·m^{-2}·s^{-1} PAR + 42 W·m^{-2} UVAR + 0.47 W·m^{-2} UVBR). Responses (Δ) were analyzed (Spearman's rho correlation test) by correlating percentage changes during the SSI treatment. r: correlation coefficient between parameters, p: level of significance, asterisk: not significant.

Parameters		correlation data (*n*=15)	
X-axis	Y-axis	r	*p*
SOD (t=0)	3 h F_v/F_m recov.	-0.639	<0.05
SOD (t=3)	3 h F_v/F_m recov.	-0.714	<0.005
DEPS (t=3)	3 h F_v/F_m recov.	0.747	<0.005
APX (t=3)	3 h F_v/F_m recov.	-0.386	>0.05*
GR (t=3)	3 h F_v/F_m recov.	0.632	<0.05
GRS (t=3)	3 h F_v/F_m recov.	-0.614	<0.05
ΔGRS	3 h F_v/F_m recov.	-0.732	<0.005
ΔGRS	ΔGR	-0.593	<0.05
ΔGRS	DEPS	-0.613	<0.05
ΔSOD	ΔAPX	-0.700	<0.005

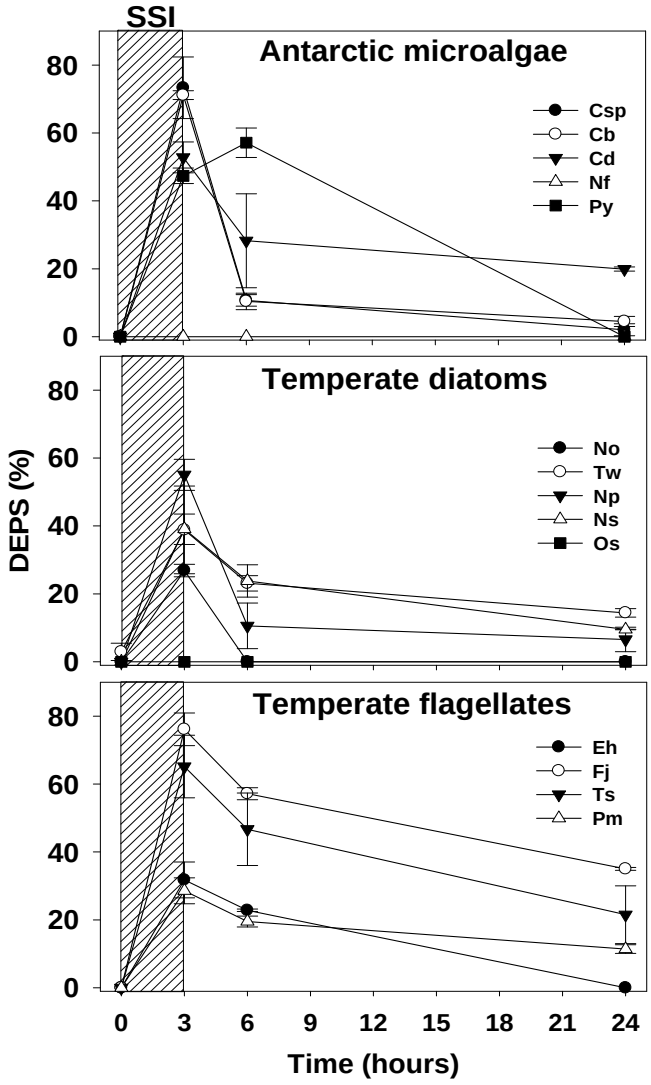

Figure 2: De-epoxidation state (DEPS = DT / DT+DD) of the photoprotective xanthophyll pigments of 15 microalgae when acclimated to low irradiance conditions (t=0; 25 µmol photons·m^{-2}·s^{-1} PAR), after 3 h exposure to SSI conditions (t=3; 1580 µmol photons·m^{-2}·s^{-1} PAR + 42 W·m^{-2} UVAR + 0.47 W·m^{-2} UVBR) and during the subsequent 21 h recovery period under low irradiance conditions (t=3 till t=24; 25 µmol photons·m^{-2}·s^{-1} PAR) which included a dark period from t=13 till t=21. *Porphyridium purpureum* did not have detectable amounts of xanthophyll pigments and therefore is not represented. Error bars represent standard deviations (*n*=3). The dashed area (t=0 till t=3) represents the 3h exposure period. Cb=*Chaetoceros brevis*, Cd=*Chaetoceros dichaeta*, Csp=*Chaetoceros* sp., Eh=*Emiliania huxleyi*, Fj=*Fibrocapsa japonica*, Nf=*Nitzschia frigida*, No=*Nitzschia ovalis*, Np=*Navicula pelliculosa*, Ns=*Navicula salinarum*, Os=*Odontella sinensis*, Pm=*Prorocentrum micans*, Pp=*Porphyridium purpureum*, Py=*Pyramimonas* sp., Ts=*Tetraselmis suecica* and Tw=*Thalassiosira weissflogii*.

Superoxide dismutase

Quantitative analyses of Superoxide dismutase (SOD) on non-denaturating PAGE gel showed a unique isozyme pattern for all species (except *Chaetoceros* sp. and *C. brevis*; Fig. 3). The composition differed both in number of detected bands and in their quantitative presence. The total brightness of the bands did not coincide with the quantity of SOD activity measured by the RF/NBT enzyme assays. The Native-page gel showed higher SOD activity for *N. ovalis* than for *O. sinensis* or *P. micans* while actual measurements showed the contrary. Within these patterns, no bands were found to be specific for cell size, taxonomic or geographic background. Despite the wide variety of isozymes, none of the bands was exclusively reserved for a certain taxonomic group (polar or temperate, diatoms or flagellates). Species of the genus *Chaetoceros* showed a high level of similarity (in contrast to *Navicula* or *Thalassiosira*) but their bands were also shared with other genera. Inhibition experiments indicated that MnSOD was most abundant for all species (results not shown). A majority of the species also contained one or two FeSOD isozymes but their appearance was much less pronounced than MnSOD. Except for *N. pelliculosa*, FeSOD was found in all diatoms and was absent in all flagellates. None of the species showed the presence of Cu/Zn-SOD.

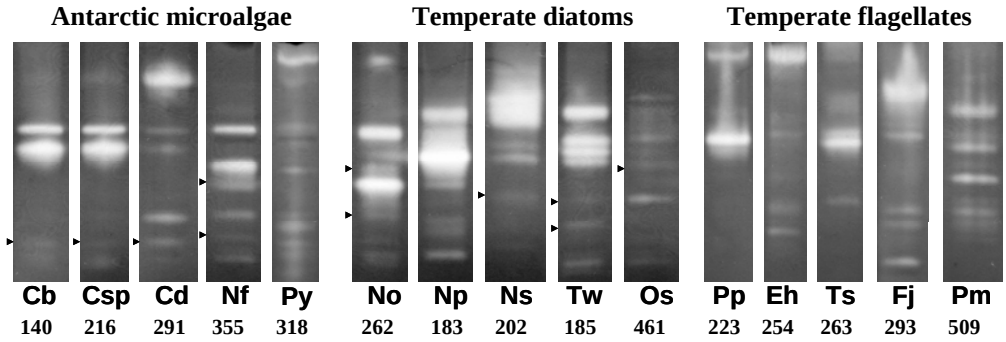

Figure 3: SOD isozyme composition of 15 microalgae visualized on a 12.5% Native polyacrylamide gel. Species were low irradiance acclimated (25 µmol photons·m^{-2}·s^{-1} PAR). Inhibition analysis identified MnSOD and FeSOD (indicated by the arrowheads). Numbers beneath species abbreviations represent SOD activity values as measured using the RF/NBT SOD activity assay. Cb=*Chaetoceros brevis*, Cd=*Chaetoceros dichaeta*, Csp=*Chaetoceros* sp., Eh=*Emiliania huxleyi*, Fj=*Fibrocapsa japonica*, Nf=*Nitzschia frigida*, No=*Nitzschia ovalis*, Np=*Navicula pelliculosa*, Ns=*Navicula salinarum*, Os=*Odontella sinensis*, Pm=*Prorocentrum micans*, Pp=*Porphyridium purpureum*, Py=*Pyramimonas* sp., Ts=*Tetraselmis suecica* and Tw=*Thalassiosira weissflogii*.

Quantitative SOD activity varied significantly (One-way ANOVA, $p<0.0005$) between the 15 species ranging from 140 (*C. brevis*) to 509 U/mg protein (*P. micans*; Figs. 3, 4). This variation was significantly (univariate nested ANOVA, $p<0.005$; $r^2=0.47$) related to cell size; the smaller the surface area/volume (SA/V) quotient, the higher the specific activity of SOD (Fig. 4a). To compare responses of different species, antioxidant activity was expressed as relative changes (Table 4).

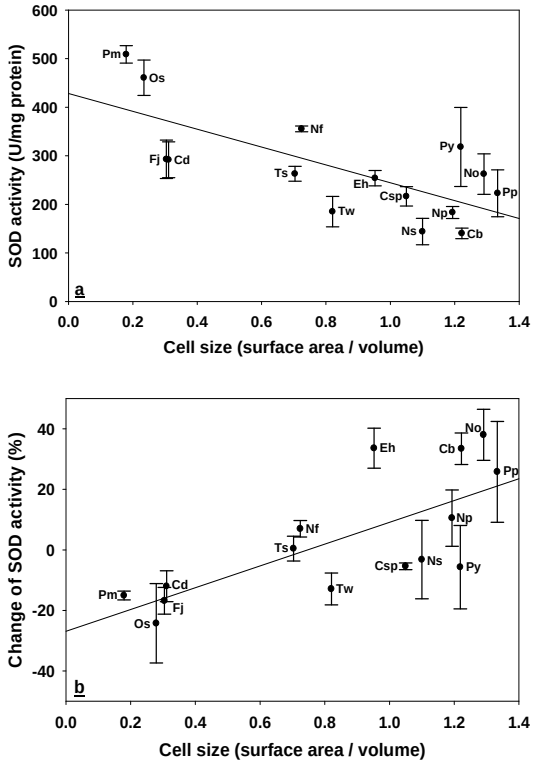

Figure 4: Cell size related activity of superoxide dismutase (SOD). **a**) activity of 15 microalgae when acclimated to low irradiance conditions (25 µmol photons·m^{-2}·s^{-1} PAR) is significantly related to cell size (univariate nested ANOVA, $p<0.005$; $r^2=0.47$). **b**) percentage change in SOD activity after 3 h exposure to SSI conditions (1580 µmol photons·m^{-2}·s^{-1} PAR + 42 W·m^{-2} UVAR + 0.47 W·m^{-2} UVBR) compared to initial (t=0) activity is significantly related to cell size (nested ANOVA, $p<0.01$; $r^2=0.41$). Error bars represent standard deviations of the percentage change (n=3). Cb=*Chaetoceros brevis,* Cd=*Chaetoceros dichaeta*, Csp=*Chaetoceros* sp., Eh=*Emiliania huxleyi*, Fj=*Fibrocapsa japonica*, Nf=*Nitzschia frigida*, No=*Nitzschia ovalis*, Np=*Navicula pelliculosa*, Ns=*Navicula salinarum*, Os=*Odontella sinensis*, Pm=*Prorocentrum micans*, Pp=*Porphyridium purpureum*, Py=*Pyramimonas* sp., Ts=*Tetraselmis suecica* and Tw=*Thalassiosira weissflogii*.

The (relative) response to the SSI treatment was significantly influenced by cell size (univariate nested ANOVA, $p<0.01$; $r^2=0.41$; Fig. 4b); species with the smallest specific surface area showed the largest decrease while species with the highest specific surface area showed the largest increase. Correlation analyses showed that the initial SOD activity (t=0) was significantly and negatively related with short term (3 h) recovery (Table 3); species with lower initial SOD activity showed higher recovery after SSI exposure. During the recovery period (t=3 till t=24), all 15 species showed a wide variety of responses (Table 4) which were not dependent on cell size, geographic background or taxonomic group.

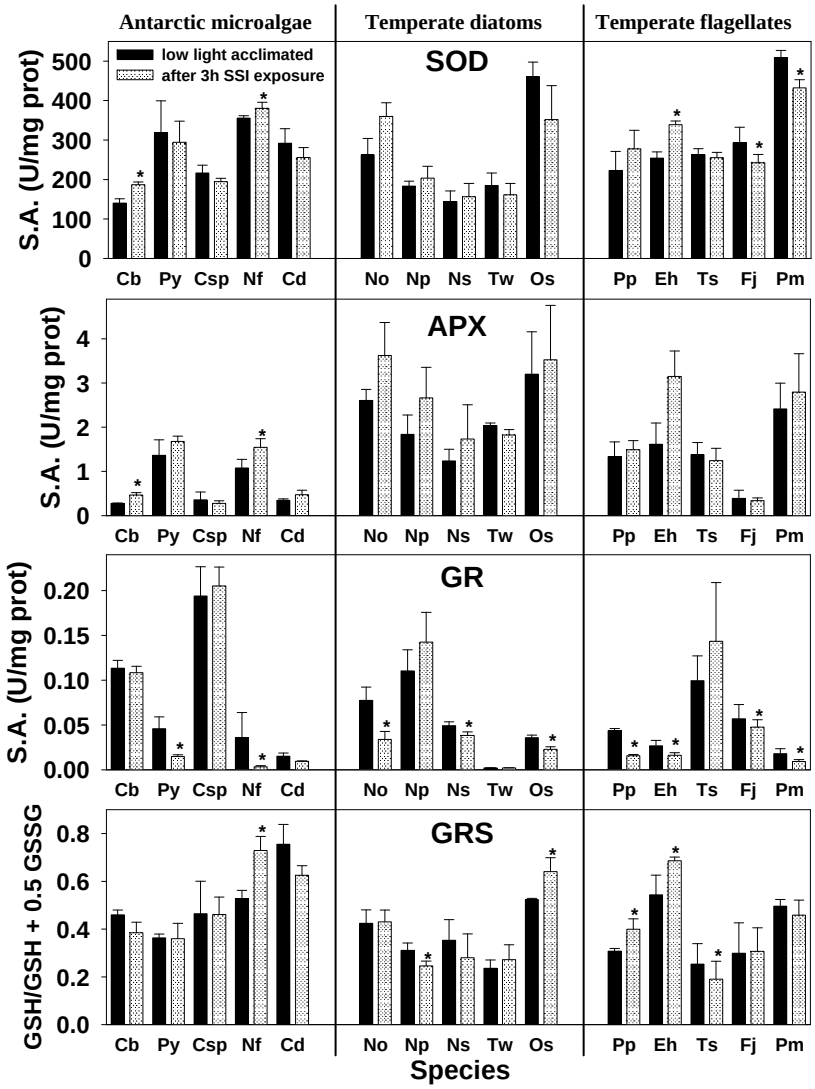

Figure 5: Activity of superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and glutathione redox status (GRS) of 15 microalgae when acclimated to low irradiance conditions (black bars; 25 µmol photons·m^{-2}·s^{-1} PAR) and after 3 h exposure to SSI conditions (dashed bars; 1580 µmol photons·m^{-2}·s^{-1} PAR + 42 W·m^{-2} UVAR + 0.47 W·m^{-2} UVBR). Error bars represent standard deviations (*n*=3). Asterisk: differs significantly from t=0 (t-test, $p<0.05$). Cb=*Chaetoceros brevis*, Cd=*Chaetoceros dichaeta*, Csp=*Chaetoceros* sp., Eh=*Emiliania huxleyi*, Fj=*Fibrocapsa japonica*, Nf=*Nitzschia frigida*, No=*Nitzschia ovalis*, Np=*Navicula pelliculosa*, Ns=*Navicula salinarum*, Os=*Odontella sinensis*, Pm=*Prorocentrum micans*, Pp=*Porphyridium purpureum*, Py=*Pyramimonas* sp., Ts=*Tetraselmis suecica* and Tw=*Thalassiosira weissflogii*.

Other antioxidant parameters

At the beginning of the experiment (t=0), ascorbate peroxidase (APX) activity, glutathione reductase (GR) activity and the glutathione redox status (GRS; GSH / GSH + 0.5 GSSG) showed significant (One-Way ANOVA, $p<0.0005$) and large differences between the 15 microalgal species (Fig. 5). APX activity varied between 0.27 (*C. brevis*) and 3.14 U/mg prot (*O. sinensis*), GR activity varied between 0.002 (*T. weissflogii*) and 0.19 U/mg prot (*Chaetoceros* sp.), and the GRS varied between 0.13 (*T. weissflogii*) and 0.61 (*C. dichaeta*). At the beginning of the experiment, Antarctic species were found to have significantly lower ascorbate peroxidase (APX) activity (univariate nested ANOVA, $p<0.05$) and significantly higher GRS values (univariate nested ANOVA, $p<0.05$) than temperate species. None of these antioxidant parameters showed any cell size dependency. After SSI exposure, differences in APX and GRS as well as in GR were not explained by cell size, geographic background or taxonomic group. There was a positive relation between GR activity and Short term (3 h) recovery of Fv/Fm while APX activity and GRS showed negative relations with recovery (Table 3). The latter relation was particularly reflected in the response of the poorly recovering species *N. frigida*, *O. sinensis*, *P. purpureum* and *E. huxleyi* which significantly (t-test, $p<0.05$) increased their GRS while all other cultures showed a significant (t-test, $p<0.05$) decrease or no significant change at all (Fig. 5). Correlation analyses showed that the relative change in GRS had a significant and negative relation with the relative change in GR activity, DEPS of xanthophyll pigments and short term (3 h) recovery of Fv/Fm (Table 3). Also, the relative change in APX activity was significantly and positively correlated with the relative change in SOD activity. During recovery, changes in APX activity, GR activity and GRS values were species specific (Table 4) and independent of cell size, geographic background or taxonomic group.

UV-absorbing compounds

UV-absorbing compounds were found in the 3 large species *P. micans, F. japonica* and *C. dichaeta*. The first two species did not significantly change their UV-absorbing capacity during SSI exposure and recovery, which remained on average between 0.038 UV abs/Chl.*a* (*n*=12; sd=0.004) and 0.18 UV abs/Chl.*a* (*n*=12; sd=0.008), respectively. *C. dichaeta* did not increase its UV-absorbing compounds/Chl.*a* ratio as an immediate response to the SSI treatment but did significantly (t-test, $p<0.05$) increase its UV abs/Chl.*a* ratio from 0.093 (*n*=4; sd=0.014) to 0.128 (*n*=4; sd=0.007) after 3 h of recovery.

Discussion

In this study we show that transition of low light acclimated microalgae to high irradiance including UVR resulted in highly species-specific responses of photosystem II efficiency (F_v/F_m), pigment composition and antioxidant activities. Studies addressing irradiance induced physiological responses, often use malondialdehyde (product of lipid peroxidation) levels as a stress indicator (Rijstenbil 2002, Zhang *et al.* 2005; Buma *et al.* 2006). Yet

Table 4: Differences (%) in activity of superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR), and glutathione redox status (GRS) during 3 subsequent irradiance regimes. Low irradiance (25 µmol photons·m^{-2}·s^{-1} PAR) acclimated cultures were exposed to SSI conditions (1580 µmol photons·m^{-2}·s^{-1} PAR + 42 W·m^{-2} UVAR + 0.47 W·m^{-2} UVBR) for 3 h (**3h SSI**). SSI exposed cultures recovered under low irradiance conditions (25 µmol photons·m^{-2}·s^{-1} PAR) for 3 h (**3h rec**). Cultures recovered for an additional 18 h under low irradiance conditions including a dark period from t=13 till t=21 (**18h rec)**. ±: standard deviation (*n*=3). Cb=*Chaetoceros brevis*, Cd=*Chaetoceros dichaeta*, Csp=*Chaetoceros* sp., Eh=*Emiliania huxleyi*, Fj=*Fibrocapsa japonica*, Nf=*Nitzschia frigida*, No=*Nitzschia ovalis*, Np=*Navicula pelliculosa*, Ns=*Navicula salinarum*, Os=*Odontella sinensis*, Pm=*Prorocentrum micans*, Pp=*Porphyridium purpureum*, Py=*Pyramimonas* sp., Ts=*Tetraselmis suecica* and Tw=*Thalassiosira weissflogii*

	SOD			APX			GR			Glu		
	3h SSI	**3h rec**	**18h rec**	**3h SSI**	**3h rec**	**18h rec**	**3h SSI**	**3h rec**	**18h rec**	**3h SSI**	**3h rec**	**18h rec**
Antarctic microalgae												
Cb	33 ± 5	-4 ± 4	7 ± 4	67 ±18	-8 ± 10	7 ±20	-4 ± 4	75 ± 30	35 ± 22	-14 ± 2	-4 ± 6	29 ± 5
Py	-6 ± 14	2 ± 13	10 ± 15	85 ± 64	-17 ± 9	-3 ± 14	-74 ± 7	-3 ± 38	-5 ± 45	-25 ± 5	-10 ± 13	-27 ± 63
Csp	-5 ± 1	-4 ± 4	14 ± 8	-14 ± 22	35 ± 10	-9 ± 14	7 ± 8	13 ± 4	-1 ± 4	2 ± 18	-13 ± 7	31 ± 14
Nf	7 ± 3	4 ± 4	-3 ± 12	44 ± 8	-2 ± 10	15 ± 2	-55 ± 22	-13 ± 3	48 ± 99	50 ± 3	20 ± 10	-38 ± 5
Cd	-12 ± 5	4 ± 7	-10 ± 1	35 ± 17	11 ± 7	7 ± 6	-35 ± 12	49 ± 8	21 ± 12	-24 ± 6	26 ± 13	7 ± 1
Temperate diatoms												
No	38 ± 8	-10 ± 5	34 ± 4	38 ± 16	24 ± 21	17 ± 10	-56 ± 4	88 ± 33	25 ± 5	4 ± 6	- 1 ± 7	1 ± 3
Np	11 ± 9	5 ± 14	-13 ± 6	26 ± 13	6 ± 11	-15 ± 27	29 ± 8	- 12 ± 2	6 ± 13	-24 ± 2	- 4 ± 7	16 ± 17
Ns	-3 ± 12	24 ± 9	-10 ± 6	36 ± 31	1 ± 6	- 6 ± 14	-22 ± 2	-16 ± 5	29 ± 7	-30 ± 13	-16 ± 23	21 ± 33
Tw	-13 ± 5	-14 ± 15	-17 ± 3	-10 ± 4	23 ± 1	- 3 ± 2	- 5 ± 5	-12 ± 10	40 ± 23	17 ± 19	1 ± 1	- 1 ± 19
Os	-24 ± 13	28 ± 8	-6 ± 13	34 ± 12	17 ± 25	19 ± 11	-36 ± 5	16 ± 13	-11 ± 3	17 ± 19	-39 ± 6	40 ± 26
Temperate flagellates												
Pp	26 ± 17	-4 ± 12	11 ± 9	14 ± 13	22 ± 23	13 ± 18	-48 ± 19	- 9 ± 9	20 ± 8	37 ± 14	7 ± 16	-25 ± 13
Eh	34 ± 7	28 ± 1	-19 ± 1	106 ± 39	-72 ± 3	190 ± 19	-39 ± 3	-80 ± 2	469 ± 44	43 ± 28	-16 ± 8	- 2 ± 2
Ts	0.5 ± 4	-20 ± 1	9 ± 3	- 9 ± 10	-37 ± 14	22 ± 14	39 ± 41	33 ± 69	-7 ± 2	-28 ± 4	63 ± 3	- 6 ± 3
Fj	- 17 ± 4	-28 ± 10	34 ± 23	- 6 ± 24	25 ± 8	16 ± 6	-15 ± 11	11 ± 13	-17 ±15	30 ± 29	35 ± 17	57 ± 64
Pm	- 15 ± 1	-10 ± 2	23 ± 8	14 ± 9	-9 ± 13	9 ± 14	-48 ± 4	60 ± 16	- 9 ±19	-10 ± 10	18 ± 12	9 ± 22

changes in these stress metabolites are subject to rapid metabolic interference by which they are less suitable for this Short term study (Janknegt *et al.* 2008; Janero 1990). Although F_v/F_m recovery is a rather overall stress indicator, it does respond rapidly, accurate and liable to irradiance shifts and therefore, was more appropriate as a measure for photosensitivity than levels of stress metabolites in our study.

Three hours SSI exposure dramatically reduced the F_v/F_m of all cultures and simultaneously increased the DEPS of xanthophyll pigments. After 3 hours of recovery, most cultures showed a significant decrease in DEPS, accompanied by an increased F_v/F_m. *N. frigida*, *O. sinensis*, *P. purpureum* and *E. huxleyi*, on the other hand, did not show substantial PSII recovery, probably because of irreversibly damage (Bouchard *et al.* 2005*a*, *b*). The overall inverse relation between DEPS and Fv/Fm demonstrates the involvement of an active xanthophyll cycle in rapid and reversible PSII down regulation during light stress (Falkowski & LaRoche 1991, Goss *et al.* 1999, Mewes & Richter 2002). Its significance was further emphasized by the positive relation between the induction of DEPS during the SSI treatment and the subsequent recovery of Fv/Fm under low irradiance. The photoprotective role of the xanthophyll cycle was also apparent in changes of the pigment composition as all cultures had increased their light protective/light harvesting (LP/LH) ratio as a result of the SSI treatment (Table 2). These findings agree with other studies that confirmed the positive relation between high irradiance acclimation and increasing xanthophyll content as well as their DEPS (Buma *et al.* 2006, Van de Poll *et al.* 2006, Dimier *et al.* 2007). During recovery in low irradiance, all photosynthetically active cultures increased their LP/LH ratio, probably due to *de novo* synthesis of xanthophyll pigments. In contrast, the LP/LH ratio of *N. frigida*, *O. sinensis*, *P. purpureum* and *E. huxleyi* increased due to faster degradation of the light harvesting pigments than xanthophyll pigments. The difference in LP/LH ratio between species containing the VAZ pool and the DT + DD cycle has been reported before (Van Leeuwe *et al.* 2005, Wagner *et al.* 2006). Because there was no relation between type of xanthophyll cycle and PSII recovery, this could not explain the differences in SSI sensitivity.

Although not directly measured in this study, the photosensitivity of *N. frigida*, *O. sinensis*, *P. purpureum* and *E. huxleyi*, could be explained by uncontrolled generation of reactive oxygen species (ROS; Ishida *et al.* 1997, Shiu & Lee 2005). The synergistic effects of photoinduced damage and (the subsequent) production of ROS could inhibit photosynthesis and cause viability loss (Nishiyama *et al.* 2001, Gechev & Hille 2005, Van de Poll *et al.* 2005). To avoid damage from (photoinduced) oxygen radicals, cells monitor ROS formation and regulate antioxidant systems accordingly. Because glutathione plays an important role in H_2O_2 detoxification, changes in cellular pro- and antioxidants are reflected in the GRS (De Kok & Stulen 1993). Therefore, it has been suggested that glutathione acts as a ROS sensing and regulating agent (Wingate *et al.* 1988, Georgiou 2002, Noctor *et al.* 2002). From this perspective it is striking that the photosensitive species *N. frigida*, *O. sinensis*, *P. purpureum* and *E. huxleyi* significantly increased GRS after SSI exposure. Moreover, we found that a decrease in GRS coincided with an increase in DEPS. Previously, it has been reported that increased GRS inhibited xanthophyll cycle activity in plants and therewith increased photoinhibition and oxidative damage (Creissen *et al.* 1999, Xu *et al.* 2000). Thus, it can be speculated that a decrease in GRS caused upregulation of de-epoxidation activity of the xanthophyll cycle to constrain ROS production. This would avoid PSII reaction center damage and subsequently enhances Fv/Fm recovery after the SSI

treatment. Besides DEPS, we also found a negative relation between the change in GRS and the change in GR activity, its regenerating enzyme, as was reported for plants and macroalgae (Wingsle & Karpinski 1996, Karpinski *et al.* 1997, Shiu & Lee 2005).

Only a few studies have determined SOD isozyme composition in microalgae (Asada *et al.* 1977, Okamoto & Colepicolo 1998, Janknegt *et al.* 2007). Intensive studies on plants revealed the presence of three types of SOD (Mn, Fe and Cu/ZnSOD; Slooten *et al.* 1995, Kliebenstein *et al.* 1998, Gómez *et al.* 2004). In this study we only found Fe- and Mn-type SOD isozymes, of which the latter appeared to dominate in microalgae. Its prevalence in microalgae could be explained due to its association with the lumen side of photosystems and mitochondria; the major sites of superoxide formation (Wolfe-Simon *et al.* 2005). The absence of Cu/ZnSOD in (most) eukaryotic microalgae was reported previously, and was ascribed to early evolutionary processes (Asada *et al.* 1977, Wolfe-Simon *et al.* 2005, Lesser 2006). Yet, it cannot be completely excluded that the low amount of protein applied (16 µg) caused Cu/ZnSOD activity to remain beneath its detection limit. Furthermore, it must be noted that SOD inhibition studies are not always unambiguous. Besides *Chaetoceros* sp. and *C. brevis*, each species had a unique SOD activity pattern, probably due to the combined effects of intracellular enzyme distribution, the associated target signals, use of the co-metal, membrane solubility, quaternary structure and species-specific amino acid substitution (Fridovich 1975, Allen 1995, Rao 1996, Wolfe-Simon *et al.* 2005). Especially the latter property may cause the species specific SOD band patterns. Species singularity of the SOD isozyme patterns was also found by others (Asada *et al.* 1977, Okamoto & Colepicolo 1998, Lesser 2006).

Initial SOD activity was the only antioxidant parameter that correlated (negatively) with Short term F_v/F_m recovery after the SSI treatment. This relation was also apparent directly after SSI exposure. Moreover, we found that increased DEPS coincided with decreased SOD activity. We also showed that cell size related positively with SOD activity but negatively with the response to SSI exposure (Fig. 4, Table 3). Increasing cell size, increases molecular self-shading and decreases pigment-specific absorption of irradiance, which increases the requirement for thylakoid membranes and associated electron transport chains during growth under low irradiance conditions (Morel & Bricaud 1981, Falkowski & LaRoche 1991). Therefore, increased cell size probably coincides with an increased risk of $O_2^{\bullet-}$ formation, which enhances SOD activity requirement compared to smaller cells. The advantage of higher exposure intensity per photosystems with decreasing cells size during low irradiance, is disadvantageous during SSI exposure and may lead to an increased production of $O_2^{\bullet-}$ and SOD activity. Although we did not analyze the intracellular location of the SOD isozymes, cell size related change in SOD activity is probably associated with chloroplasts, as suggested by Malanga *et al.* (1997) and Rijstenbil (2002). These studies also suggested that larger cells are less able to shield off their interior. Therefore, increasing cell size would increase the susceptibility of other SOD containing organelles to hazardous radiation near the cell surface (i.e. mitochondria and peroxisomes), resulting in a decrease of overall SOD activity. In small cells, on the other hand, the photoabsorbing chloroplasts shield off the interior of small cells from hazardous radiation. As a result, their chloroplasts are also more subject to irradiance induced overreduction of ETC's and so, subject to increased ROS. Consequently, smaller cells would need to increase their chloroplastic SOD activity.

Although SOD activity was related to photosensitivity and cell size, there was no relationship between cell size and PSII recovery which could have several reasons. First of all, cellular characteristics like shape, morphology and cell wall influence irradiance transmission into the cell and therewith its SSI sensitivity (Karentz *et al.* 1991*b*, Laurion & Vincent 1998). Besides, UV-absorbing compounds in large microalgae (SA/V < 0.5 $\mu m^2/\mu m^3$), such as mycosporine-like amino acids (MAAs), may have contributed to SSI tolerance (Karentz *et al.* 1991*a*, Helbling *et al.* 1996, Laurion & Vincent 1998). Accordingly, we found that three of the four largest species, *P. micans, C. dichaeta* and *F. japonica*, contained UV-absorbing compounds and recovered well after SSI exposure, while *O. sinensis*, with no UV-absorbing compounds did not recover at all.

In contrast to our expectations, Antarctic microalgae showed lower APX activity and higher GRS than temperate species, indicating that the former experienced less oxidative stress when acclimated to low irradiance conditions. Although speculative, this might be due to their lower metabolic rate (Atkin & Tjoelker 2003) by which the rate of photosynthesis and the consumption of the reduced equivalents is lower as well. So, to tune the production of reducing power to the rate of their consumption, Antarctic microalgae require a lower amount of photosystems. As a result, there is less potential leakage of electrons by which polar microalgae are also less susceptible to ROS formation by which they need less antioxidant activity (Dohahue *et al.* 1997).

Photosensitivity to the SSI treatment decreased with an increasing level of DEPS and GR activity but with a decreasing level of SOD activity, APX activity (although not significant) and GRS (as discussed above). This contrasts with studies that suggest that stress tolerance increases with increasing levels of antioxidants activity (Donahue *et al.* 1997, Aguilera *et al.* 2002*b*). Responses of SOD and APX activity were probably positively correlated because of the SOD dependent generation of H_2O_2; a product which is scavenged by APX. H_2O_2 can also act as a signalling molecule, regulating antioxidant activity, including APX, at a genetic and mRNA level (Yoshimura *et al.* 2000, Neill *et al.* 2002, Ledford & Niyogi 2005).

The wide variety of antioxidant responses is probably due to the tightly regulated and balanced synergy of the antioxidant network (Kubo *et al.* 1999, Apel & Hirt 2004). Accordingly, several studies showed that external upregulation of SOD activity or GRS did not lead to a higher tolerance level against stress (Slooten *et al.* 1995, Mallick & Mohn 2000). Besides H_2O_2 and GRS, there are many regulation factors such as singlet oxygen, superoxide and the hydroxyl radical that can interfere with antioxidant activities (Allen 1995, Mallick & Mohn 2000, Laloi *et al.* 2004). Furthermore, the intracellular location of ROS formation is of importance as well by which antioxidant activity might increase at the site of production while it decreases at other locations (Willekens *et al.* 1994, Yosimura *et al.* 2000). Some microalgae respond by increasing the rate of photorespiration by which ROS formation is diverted towards the peroxisome (Apel & Hirt 2004). As the peroxisome scavenges H_2O_2 (without the use of a reducing agent) this leads to a decrease of APX and GR activity (Aquilera *et al.* 2002*a*).

We conclude that cell size, and regional and taxonomic background play a minor role in determining photosensitivity of microalgae. Like others, we found that differences in excess irradiance tolerance were highly species specific and depend on the synergy of protection mechanisms and cell characteristics rather than on cell size, geographical and taxonomic background (Laurion & Vincent 1998, Karentz *et al.* 1991*b*). Furthermore, our

study indicated that GRS plays a crucial role in the sensing and regulation system that counteracts photoinduced stress. Although speculative, the presence of ROS decreases GRS which in turn increases activity of GR and the activity of xanthophyll de-epoxidase. As a consequence, excess energy is dissipated as heat by which the formation of $O_2^{\bullet -}$ is constrained. Therefore, an increase of DEPS not only enhances the efficiency of PSII during recovery, it also reduces production of H_2O_2 which in turn down regulates the activities of SOD and APX. Thus, our study suggests that photosensitivity decreases with increasing xanthophyll cycle activity but decreasing levels of SOD.

Chapter 6

UVR-induced photoinhibition of summer marine phytoplankton communities from Patagonia

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Abstract

During austral summer 2006 experiments were carried out to evaluate the effects of ultraviolet radiation (UVR, 280–400 nm) on carbon fixation of natural phytoplankton assemblages from Patagonia (Argentina). Surface water samples were collected (ca. 100 m offshore) at mid morning using an acid-cleaned (1N HCl) dark container. The short-term impact of UVR (measured as radiocarbon incorporation) was immediately assessed by exposing samples to three artificial illumination treatments: PAR (400-700 nm), PAR+UVAR (315-700 nm) and PAR+UVAR+UVBR (280-700 nm). Pico-nanoplankton characterized the assemblages and taxon specific pigment fingerprinting combined with CHEMTAX and supplemented with microscopic observations showed varied proportions of diatoms, chlorophytes and cyanobacteria throughout January-February 2006. Photosynthetic efficiency, as assessed through assimilation numbers, was high (between 4.4 and 10.4 μg C (μg Chl.*a*)$^{-1}$ h^{-1}) and it was probably favored by the supply of inorganic nutrients from the Chubut River. UVR-induced photoinhibition appeared to be related to the taxonomic composition: in general, higher photoinhibition was observed when diatoms dominated whereas this was lower when samples were dominated by chlorophytes. Our data suggest that xanthophyll pigments might have provided only limited protection in these already high-light acclimated assemblages.

Introduction

Phytoplankton organisms rely on solar radiation to drive photosynthesis to synthesize carbohydrates. However, this requirement exposes them also to solar ultraviolet radiation (UVR, 280-400 nm) which is known to affect key molecules, organelles and metabolic processes (Vernet 2000, Villafañe *et al.* 2003, Häder *et al.* 2007). UVR can cause photoinhibition, a process that is variable among species and habitats (Villafañe *et al.* 2003). Moreover, the effect of UVR depends on nutrient and light history of the algae. For example, significant variability in UVR-mediated photoinhibition was observed when the dinoflagellates *Prorocentrum micans* and *Heterocapsa triquetra* were exposed to solar radiation under different nutrient regimes (Marcoval *et al.* 2007). Additionally, inhibition of photosynthesis during excessive PAR (Photosynthetically Active Radiation, 400-700 nm) and UVR exposure was influenced by acclimation to low and high- irradiance in two *Thalassiosira* species (Van de Poll *et al.* 2006).

Phytoplankton have developed a suite of physiological and biochemical responses that minimize the negative effects of UVR exposure (Roy 2000, Banaszak 2003). These include: (1) reduction of UVR exposure by migrating downward in the water column, as seen in the chlorophyte *Dunaliella salina* (Richter *et al.* 2007); (2) protection by synthesizing compounds such as mycosporine like amino acids (MAAs) as demonstrated for many species of cyanobacteria, dinoflagellates and diatoms (Banaszak 2003), and xanthophyll pigments that can potentially dissipate excessive energy as heat (Demming-Adams 1990, Demers *et al.* 1991, Sobrino *et al.* 2005) and, (3) repairing DNA and / or protein damage (Buma *et al.* 2003, Bouchard *et al.* 2005a).

The net UVR impact in natural microbial communities is the result of species specific differences in sensitivity as well as in acclimation potential. Due to seasonal shifts in species dominance and abundance, together with nutrient and irradiance levels it can be expected that the overall UVR impact on phytoplankton also shows seasonal variation. A study carried out with natural phytoplankton communities from coastal Patagonian waters demonstrated inter-seasonal variability of UVR effects, with high UVR-induced photoinhibition during spring and summer, and low levels during winter (Villafañe *et al.* 2004). However, biological weighting functions for this process showed that winter communities were more sensitive than those characterizing the summer. On the other hand, another study (Banaszak & Neale 2001) showed that phytoplankton in the Rhode River had a moderate sensitivity towards UVR that mainly varied on short-term rather than on seasonal time scales. In addition, a study in the Great Lakes demonstrated that, in spite of the large variations in nutrients and irradiance, phytoplankton sensitivity towards UVR was similar throughout the year (Furgal & Smith 1997). Finally, Gao *et al.* (2007) showed that the size structure of the community and cloudiness conditioned the overall impact of UVR on phytoplankton photosynthesis in a tropical marine site of Southern China. Thus responses to UVR stress are not only related to the differential sensitivity of the sampled assemblages, but also to the combination of UVR exposure with other environmental variables.

The focus of our study was to obtain knowledge on the variability of UVR effects on photosynthesis of summer phytoplankton communities of Patagonian coastal waters with a resolution of 2-3 days. To achieve this, we evaluated the shifts in phytoplankton community composition by means of HPLC pigment fingerprinting in combination with the

CHEMTAX program, as well as by classical microscopy and size fractionation. Then, we studied the effects of UVR on carbon incorporation using short-term artificial UVR exposures by means of a solar simulator. In this way, possible effects of variable exposure quantity and quality (as caused by season, time of day, atmospheric influences) were eliminated. In addition, we assessed the importance of photoprotective mechanisms (xanthophyll cycling) that would allow these communities to cope with the normally high radiation levels as registered in the area at this time of the year (Helbling *et al.* 2005).

Materials and Methods

Study area and sampling

This study was conducted at Bahía Engaño, Chubut, Argentina (43º 18.8´ S, 65º 02´ W, Fig. 1) during the period January 10 to February 19, 2006. The study site is located in close proximity to the Chubut River estuary, where geomorphology, biological and chemical characteristics were studied previously (Perillo *et al.* 1989, Villafañe *et al.* 1991, Helbling *et al.* 1992b, Sastre *et al.* 1994, Commendatore & Esteves 2004). Additionally, the assessment of the impact of UVR on natural phytoplankton assemblages was conducted before in this area (Barbieri *et al.* 2002, Villafañe *et al.* 2004, Helbling *et al.* 2005) however using natural exposures and larger time intervals between sampling.

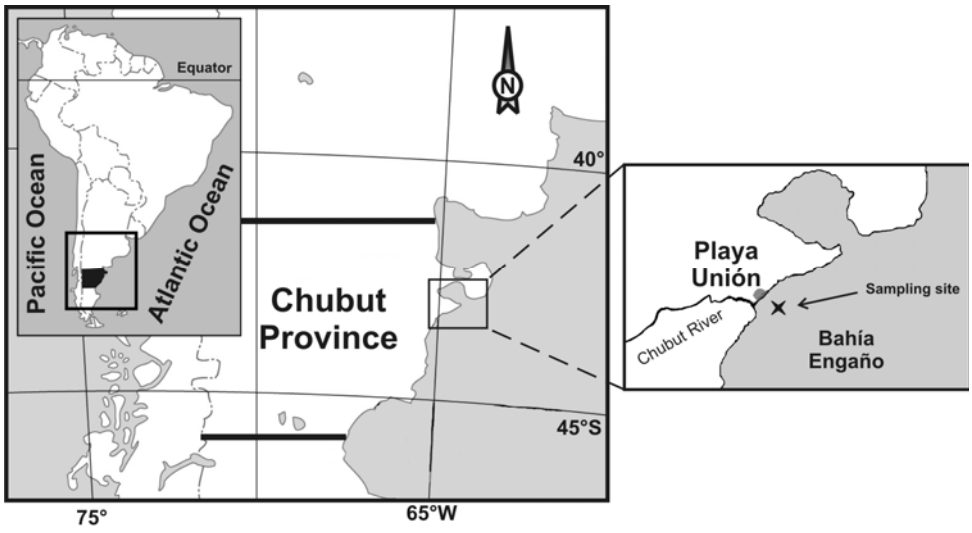

Figure 1: Map showing the study area and the relative position of the Chubut Province in South America.

To evaluate the variations in UVR-induced photoinhibition of phytoplankton assemblages, routine sampling for experimentation was carried out every 2-3 days. Surface water samples were collected (ca. 100 m offshore) at mid morning using an acid-cleaned (1N HCl) dark container and immediately taken to Estación de Fotobiología Playa Unión (EFPU; 5 min away from the sampling site) where the short-term effects of UVR upon phytoplankton photosynthetic rates were determined.

Experimentation

Samples were dispensed in 50-mL quartz tubes and inoculated with 5 μCi (0.185 MBq) of labeled sodium bicarbonate. The tubes were then attached to a black aluminum frame that was placed in a water bath inside a temperature controlled environmental chamber (20 °C). The samples were exposed for 2 h under a solar simulator (Hönle, Sol 1200, Germany). Three different radiation treatments (duplicate samples for each treatment) were implemented: (a) PAB treatment: Samples receiving PAR+UVAR+UVBR (280-700nm, unwrapped quartz tubes); (b) PA treatment: Samples receiving PAR+UVAR (315-700nm), tubes covered with Folex UV cut-off filter (Montagefolie, N°10155099) and, (c) P treatment: Samples receiving only PAR (400-700nm), tubes wrapped with Ultraphan UV Opak Digefra film (the transmission characteristics of filters and materials were previously reported elsewhere (Villafañe *et al.* 2003)). The lamp was covered with acetate film to avoid UV-C exposure of the samples. The irradiances used in the experiments were 183, 69.9 and 1.7 $W \cdot m^{-2}$ for PAR, UVAR and UVBR, respectively. It should be noted that PAR irradiance is lower and UV levels are slightly higher than the mean noon solar irradiances during the experimentation period (see Results); however, they were within the upper and lower limits of irradiances measured during the experimental period. After the incubation period, the samples were filtered onto Whatman GF/F filters (25 mm in diameter) and the filters were exposed to HCl fumes overnight. Then, the filters were dried and counted using a liquid scintillation counter (Holm-Hansen & Helbling 1995). Photosynthetic inhibition for each wavelength interval (i.e., carbon fixation in the PAB and PA treatments relative to that in the P control) over the incubation period was calculated as:

$$\text{UVBR inhibition} = [(C_P - C_{PAB}) - (C_P - C_{PA})] / (C_P) * 100$$

$$\text{UVAR inhibition} = (C_P - C_{PA}) / (C_P) * 100$$

where C_P, C_{PA}, and C_{PAB} are the carbon fixation values in the P, PA, and PAB treatments, respectively.

Pigment analyses

Duplicate aliquots of samples (100-250 mL) were filtered onto Whatman GF/F filters (25 mm in diameter) under dim light. The filters were immediately frozen in liquid nitrogen and stored at -80 °C until analysis in The Netherlands. Filters were handled under dim light, freeze-dried (48 h) and pigments extracted in 4 mL of 90% cold acetone (v/v, 48 h, 5 ºC). Then, pigments were resolved using HPLC (Waters 2690 separation module, 996 photodiode array detector) with a C_{18} 5 μm DeltaPak reversed-phase column (Milford, MA,

USA) and identified by retention time and diode array spectroscopy (Van Leeuwe *et al.* 2006). Quantification was done using standard dilutions of chlorophyll a, b, fucoxanthin, diadinoxanthin, diatoxanthin, zeaxanthin, lutein, antheraxanthin, prasinoxanthin, alloxanthin, peridinin, and violaxanthin.

The CHEMTAX matrix factorization program (Mackey *et al.* 1996) was used to assess phytoplankton class abundances. The initial ratio matrix was largely based on the Southern Ocean synthetic data set as presented in Mackey *et al.* (1997) using peridinin (dinoflagellates), fucoxanthin (diatoms), alloxanthin (cryptophytes), lutein (chlorophytes), and zeaxanthin (chlorophytes and cyanobacteria) as marker pigments. Prasinophytes might have been present in the samples, despite the absence of prasinoxanthin in our samples, since not all prasinophytes contain this group specific pigment. Yet, microscopy did not indicate the presence of prasinophyte: in contrast coccoid green algae were observed more often in the area. Therefore, only chlorophytes were included in the calculations. For all phytoplankton classes, the ratio limits were set to 10% so that only little variability was allowed for the output ratio matrix.

Chlorophyll (Chl.*a*) concentration was measured twice, once for pigment fingerprinting (as described before) and once for calculations of carbon assimilation and size fractionation, using fluorometry. For this later analysis, duplicate aliquots of 100 mL of sample were filtered onto a Whatman GF/F filter (25 mm in diameter) and the photosynthetic pigments extracted in absolute methanol (7 mL for at least 1 h). Chl.*a* concentration was calculated from the fluorescence of the extract, before and after acidification, using a fluorometer (Turner Designs model TD 700) (Holm-Hansen *et al.* 1965). Size fractionation was done by pre-filtering a sample with a Nitex® mesh (20 µm pore size) with subsequent Chl.*a* analyses as described above; in this way we had information of the total Chl.*a* concentration as well as on the Chl.*a* concentration in the < 20 µm size fraction. The fluorometer is routinely calibrated using pure Chl.*a* from *Anacystis nidulans* (Sigma C6144). As expected, HPLC-derived Chl.*a* concentration and fluorometer- derived Chl.*a* data were highly correlated (r^2=0.9, $P < 0.0001$, data not shown).

Additionally, scans of the methanolic extracts were obtained using a spectrophotometer (Hewlett Packard model HP 8453E) to estimate the amount of UV-absorbing compounds based on the peak at 337 nm (Helbling *et al.* 1996).

Taxonomic analyses

Samples for identification and enumeration of phytoplankton were placed in 125 mL brown bottles and fixed with buffered formalin (final concentration 0.4% of formaldehyde). A variable amount of sample (10-25 mL) was allowed to settle for 24 h in a Utermöhl chamber (Hydro-Bios GmbH, Germany) and species were enumerated and identified using an inverted microscope (Leica model DM IL) following the technique described in Villafañe & Reid (1995).

Atmospheric variables

Incident solar radiation over the study area was measured continuously using a broad band ELDONET radiometer (Real Time Computers Inc., Germany) that measures UVBR (280-315 nm), UVAR (315-400 nm) and PAR (400-700 nm) with a frequency of one reading per

second and storage the minute-averaged value for each channel. In addition, continuous monitoring of other atmospheric variables (i.e., temperature, humidity, wind speed and direction) was carried out using a meteorological station (Oregon Scientific model WMR-918).

Total ozone column concentrations were obtained from NASA (http://jwocky.gsfc.nasa.gov).

Statistics

The data were reported either as mean and half-range (as duplicate samples were incubated due to the effective area under the solar simulator) or as mean and standard deviations when triplicate analyses were performed; the non-parametric Kruskal Wallis test (Zar 1999) was used to test for significant differences between the samples exposed to different radiation treatments, using a 95% confidence limit.

We used multiple linear regression analysis to explain the variability observed in photosynthetic performance and UVR-induced photoinhibition. The variables that accounted for most of the variability were temperature, xanthophylls cycling, solar radiation and species abundance (as determined by CHEMTAX analysis).

Results

Atmospheric variables

Solar radiation during the sampling period showed a day-to-day variability in the daily doses due to cloud cover; nevertheless, there was a trend for decreasing radiation values after Julian day 29 (Figs. 2a, b). During the study period, daily doses for PAR and UVAR (Fig. 2a) varied between 6200 and 11200 and 960-1730 kJ·m^{-2}, respectively. Following the same trend, daily doses of UVBR ranged between 24.5 and 46.4 kJ·m^{-2}, with low values determined on Julian days 35-36 (Fig. 2b). The mean noon irradiances for the period were 311 (SD = 72.3), 49.4 (SD = 10.5) and 1.55 (SD = 0.3) W·m^{-2} for PAR, UVAR and UVBR, respectively (data not shown). Ozone values over Bahía Engaño were variable, but generally high, ranging from 239 to 323 Dobson Units (D.U.) during January-February (Fig. 2b). Ambient air temperature varied between 14.3 and 27.9°C, with a mean value of 19.4ºC (Fig. 2c).

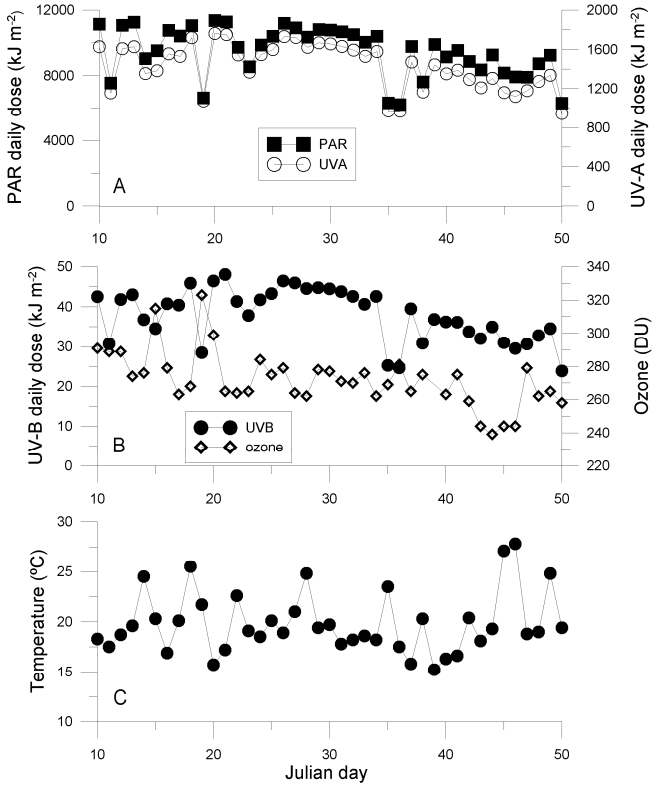

Figure 2: Solar radiation, ozone and temperature during the study period. **b**) Daily doses of Photosynthetically Active Radiation (PAR, 400–700 nm) and UVAR (315-400 nm) in kJ m^{-2}; **b**) Daily doses of UVBR (280-315 nm) and total ozone column concentrations (in Dobson Units, D.U.) over Playa Unión and, **c**) Mean daily ambient temperature (°C).

Pigments composition

Chl.*a* concentration (Fig. 3a) during most of the study period had values ranging between 5 and 15 µg Chl.*a*·L^{-1}. By the end of February, however, Chl.*a* concentration increased and reached values as high as 35 µg Chl.*a*·L^{-1}. The samples were generally dominated by pico-nanoplankton cells (< 20 µm) with more than 50% of Chl.*a* allocated in this fraction (Fig. 3a). Chl.*b*, characteristic of green algae showed small peaks during mid January (0.82 µg Chl.*b*·L^{-1}) and at the beginning of February (2.1 µg Chl.*b*·L^{-1}) (Fig. 3b). Zeaxanthin concentration varied between 0.19 and 0.72 µg·L^{-1}, whereas lutein reached a maximum concentration of 0.35 µg·L^{-1}) (Fig. 3c). Finally, fucoxanthin, diadinoxanthin and diatoxanthin concentrations were generally low (< 4 µg l^{-1}) but they increased towards the end of the sampling period, reaching values as high as 15.7, 2.76 and 1.14 µg·L^{-1}, respectively (Fig. 3d).

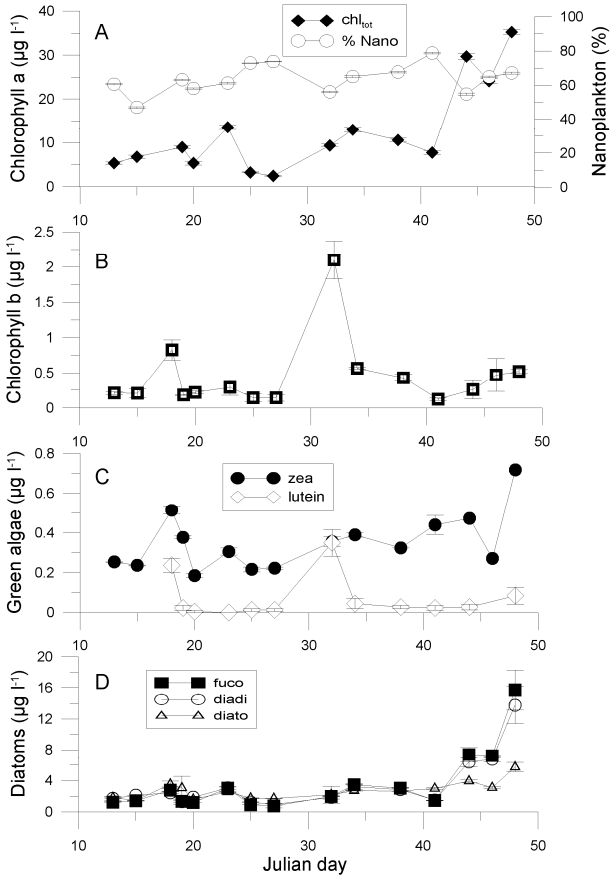

Figure 3: Total concentration of pigments (in µg·L^{-1}) throughout the study period. **a**) Chl.*a* and percentage of Chl.*a* in the nanoplankton fraction (<20 µm), **b**) Chlorophyll b; **C**) Zeaxanthin and lutein and, **d**) Fucoxanthin, diadinoxanthin and diatoxanthin. The vertical lines on top of the symbols indicate the half range.

Taxonomic composition

There was a general good agreement between CHEMTAX data and microscopic determinations of species composition. There was large variability in the relative proportion of phytoplankton class abundances over the study period as assessed with the CHEMTAX program (Fig. 4a). Dinoflagellates as well as cryptophytes abundances were low during the study period, ranging between 0.01 - 5.4% and 0.1 - 7.4% of total biomass, respectively. Chlorophytes and cyanobacteria (*Synechococcus* type) also showed high variability during the study period, ranging from 3.7 - 51.4% and 4.8 - 29.8% of total biomass, respectively. Diatoms were found to be the most abundant group, ranging from 35.8% (on Julian day 32) to 88.5% (on Julian day 46) of total phytoplankton biomass. Phytoplankton abundance in terms of cell numbers (Fig. 4b) was also variable.

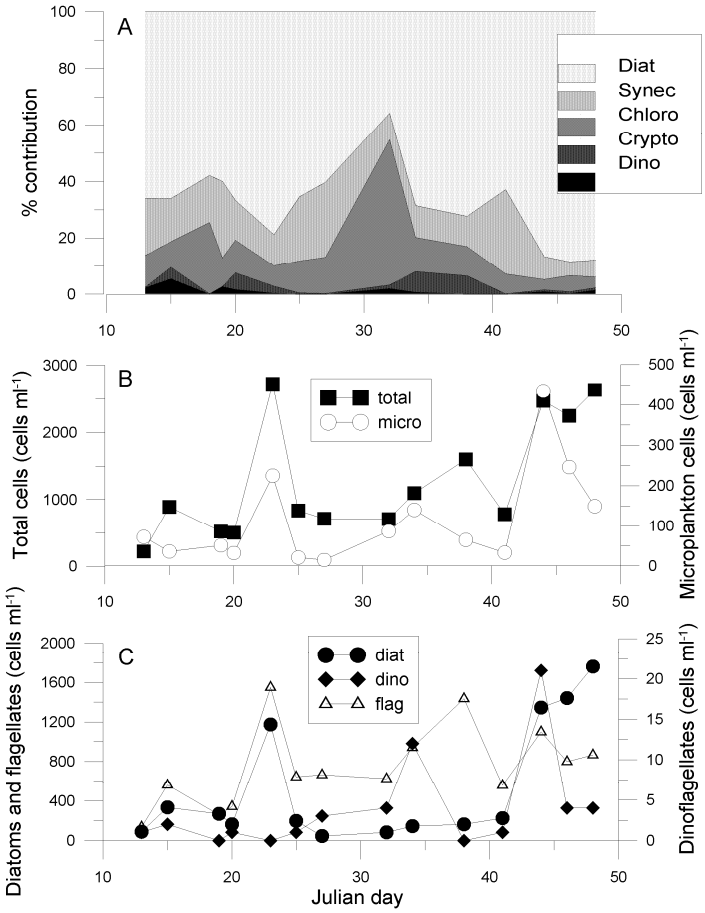

Figure 4: Taxonomic composition of the phytoplankton assemblages throughout summer 2006. **a**) Percent contribution of the major groups as resolved by CHEMTAX from the pigment data; **b**) Total phytoplankton and microplankton (> 20 µm) concentrations (in cells mL^{-1}) as assessed through microscopic analyses and, **c**) Diatoms, dinoflagellates and flagellates concentration (in cells mL^{-1}) as determined by microscopic analyses; note the different scale (y-axis) for dinoflagellates.

Their values ranged between 223 and 2725 cells·mL^{-1}, with a clear peak on Julian day 23, and also high values towards the end of the study period. The abundance of microplankton cells (> 20 µm) was generally low (< 200 cells·mL^{-1}) but small peaks in cell numbers were determined on Julian days 23 and 44 with 225 and 434 cells·mL^{-1}, respectively. Microscopic analysis revealed the conspicuous presence of picoplankton (including *Synecchococcus* like cells) throughout the study period (Fig. 4c) with concentrations as high as 1550 cells·mL^{-1}. The high diatom concentrations were due to the presence of small (< 20 µm) *Thalassiosira* species, although *Odontella aurita* and *Chaetoceros* spp. also contributed for an important part to total diatom abundance. The cell densities of dinoflagellates were always low, with few species represented in the samples such as *Gymnodinium* sp., *Protoperidinium* sp. and *Prorocentrum micans*.

UVR impact on carbon uptake

Assimilation numbers obtained during 2 h exposure to the solar simulator were variable and ranged between 4.4 and 10.4 µg C·(µg Chl.*a*)$^{-1}$·h^{-1} in the P treatment (Fig. 5a). Lower assimilation numbers were found in samples receiving additionally UVAR and UVBR Inhibition of photosynthesis (Fig. 5b) was detected in all samples, with UVAR and UVBR either contributing equally to the inhibition, or UVBR surpassing the contribution of UVAR. This latter case was especially evident at the beginning of the experimental period (Julian day 15) with 39% inhibition due to UVBR and 21% due to UVAR.

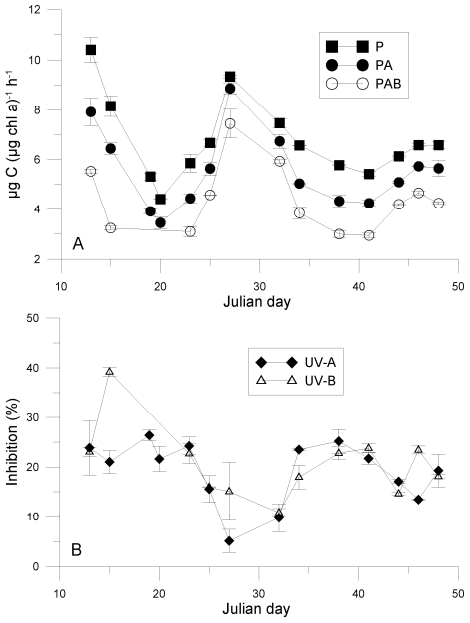

Figure 5: a) Phytoplankton assimilation numbers (in µC·(µg Chl.*a*)$^{-1}$·h^{-1}) throughout the study period for samples exposed to PAR only (P treatment), PAR+UVAR (PA treatment) and PAR+UVAR+UVBR (PAB treatment) (note that samples under the PAB treatment were lost on Julian days 19 and 20); **b**) Percentage inhibition of carbon fixation due to UVAR and UVBR. The lines on top of the symbols indicate the half range.

Photoprotective compounds

The potential protective role of xanthophylls was evaluated by considering both the pool of these pigments as well as the de-epoxidation status (diatoxanthin relative to diadinoxanthin + diatoxanthin – DT/(DT+DD) (Fig. 6). Xanthophyll cycling of chlorophytes (VAZ cycle) could not be calculated due to the fact that only traces of violaxanthin and antheraxanthin were detected in the HPLC chromatograms. The total pool of xanthophylls (dt+dd) was normalized to diatom (as the dominant dd+dt containing group) Chl.*a*, calculated from the fuco/Chl.*a* ratio used in the CHEMTAX input matrix. This derived ratio ((DD+DT)/Chl.*a*) was relatively high and ranged between 0.11 and 0.32. The extent of conversion of diadinoxanthin to diatoxanthin – DT/(DT+DD) throughout the study period was also relatively high with a mean value of 0.5 (SD = 0.12) and ranging between 0.29 to 0.69.

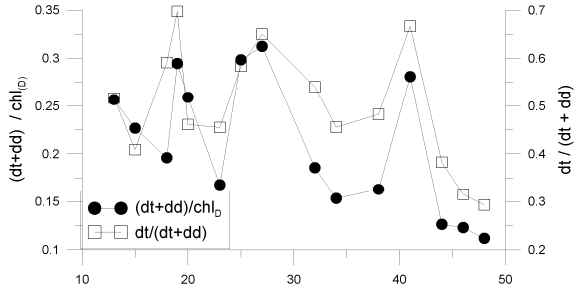

Figure 6: Total pool of xanthophyll pigments, expressed as the sum of diadinoxanthin and diatoxanthin normalized to diatom Chl.*a* - ((DT+DD)/Chl.$a_{(D)}$) assuming a fucoxanthin to Chl.*a* ratio of 0.45 (Mackey *et al.* 1997) and the extent of conversion from diadinoxanthin to diatoxanthin (DT/(DT+DD) throughout the study period.

Variability of photosynthetic efficiency and UVR-induced photoinhibition

The relative contribution of the main factors accounting for most of the variability observed in the photosynthetic performance of PAR exposed samples was done using a multiple linear regression analysis. The best model obtained (r^2 = 0.97, P < 0.0001) for the variability of assimilation numbers is shown in Fig. 7a and it had the following equation:

$$P_{ass} = 0.073\ T - 2.733\ DT/(DT+DD) + 0.05\ E \quad (\textbf{eq. 1})$$

where P_{ass} is the assimilation number of samples receiving only PAR, T is the ambient temperature, DT/(DT+DD) is the rate of conversion of the xanthophylls cycle, and E is the solar radiation dosis.

For UVR-induced photoinhibition (UVR_{inh}) throughout the study period (Fig. 7b) we obtained the best model ($r^2 = 0.96$, $p < 0.0001$) as:

$$UVR_{inh} = 0.459\ Diat - 0.015\ Chloro + 5.246\ Dino + 0.78\ Synec + 2.151\ Crypto - 2.098\ P_{ass} \quad (\textbf{eq. 2})$$

where: Diat, Chloro, Dino, Synec, and Crypto are the contributions of diatoms, chlorophytes, dinoflagellates, *Synechococcus* and cryptophytes (as determined by the CHEMTAX program, Fig. 4a) and P_{ass} is the assimilation number of samples receiving only PAR.

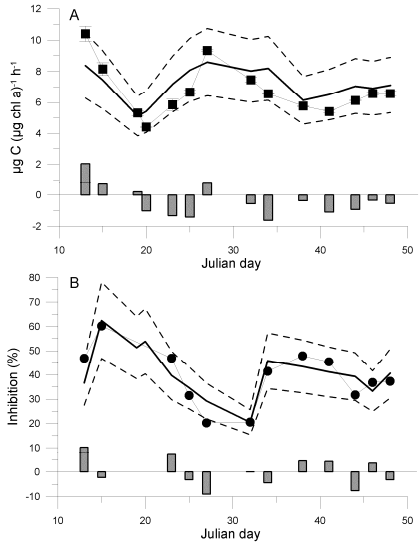

Figure 7: Output from the multiple linear regression models as compared to the data obtained for the assimilation numbers for (**a**) the PAR treatment in $\mu C \cdot (\mu g\ Chl.a)^{-1} \cdot h^{-1}$ and (**b**) for UVR inhibition. The thin lines and symbols are the experimental data while the thick lines are the modeled data; the broken lines represent the 95% limit. The vertical bars in each panel are the residuals from the models.

Discussion

In our study we investigated the UVR-induced photoinhibition in relation to phytoplankton composition from Patagonian coastal waters. To complement our previous studies carried out in the area (Buma *et al.* 2001, Helbling *et al.* 2001a, Villafañe *et al.* 2004) our approach here was to expose sea surface samples to constant temperature and UVR and PAR illumination. Thus we focused on responses due to changes in community structure and their associated sensitivity to UVR. However, samples collected at different times during the study period had their light history and environmental pre-acclimation that conditioned their overall UVR response. In the following paragraphs we will discuss the variability observed in UVR-induced photoinhibition as associated to changes in taxonomic composition and other environmental variables.

Summer time is considered the post–bloom condition in coastal Patagonian waters, with phytoplankton assemblages generally dominated by pico-nanoplankton cells (< 20 µm) (Buma *et al.* 2001, Helbling *et al.* 2001a, Barbieri *et al.* 2002, Villafañe *et al.* 2004). This dominance of pico-nanoplankton cells has been observed in Bahía Engaño throughout the

study period, as seen in both Chl.*a* and cell abundances (Figs. 3, 4). We further determined that the pico-nanoplankton cells were mostly small diatoms, chlorophytes and cyanobacteria (Fig. 4). This pico-nanoplankton abundance contrasts with the general pattern of dominance of microplankton species during the winter, where they cause blooms > 100 μg Chl.*a*·L^{-1} (Barbieri *et al.* 2002, Villafañe *et al.* 1991, Villafañe *et al.* 2004). These blooms are associated with calm weather conditions during the winter that allow for the stratification of the water column. In contrast, the prevalence of high winds during spring and summer favor the occurrence of deep upper mixed layers that preclude the growth of large cells (Villafañe *et al.* 2004) as also seen in Antarctic waters (Kopczynska 1992). It should be noted that during summer 2006 wind speed was exceptionally low (daily mean during January 2006 = 2 m·s^{-1}) as compared to the overall daily mean of 5 m·s^{-1} that was previously reported for this period (Villafañe *et al.* 2004, Helbling *et al.* 2005). Although wind data for February 2006 were not available, qualitative observations allow generalizing on the calm weather conditions as determined during January. Wind has been found to be a key variable shaping plankton communities in the Patagonia region (Villafañe *et al.* 2004, Helbling *et al.* 2005, Gonçalves *et al.* 2007). Moreover, wind is also responsible for the amount of time that cells are exposed to high radiation levels (i.e., at or near surface waters). When calm conditions prevail, algae near the surface become high-light acclimated whereas the opposite occurs when strong winds dominate. High-light acclimation in the diatom samples was supported by the high abundance of xanthophyll cycle pigments relative to Chl.*a* and by the relatively high conversion rates to diatoxanthin (mean = 0.5, Fig. 6). It has been previously determined that these compounds increase photoprotection during exposure to high PAR and UVR (Demers *et al.* 1991, Kudoh *et al.* 2003, Mohovic *et al.* 2006, Van de Poll *et al.* 2006, Dimier *et al.* 2007).

The high assimilation numbers (Fig. 5a) as compared to those determined in an earlier study carried out in coastal Patagonian waters - ~ 1 μg C·(μg Chl.*a*)$^{-1}$·h^{-1} (Helbling *et al.* 2001a) suggest a better photosynthetic performance of the phytoplankton assemblages sampled during the summer of 2006, even under the high radiation levels observed (Fig. 2). The high photosynthetic performance of phytoplankton during this period could be related to the input of nutrients via the outflow of the Chubut River. In fact, and although we did not measure nutrient concentration during summer 2006 it has been shown previously (Helbling *et al.* 1992b, Perez pers. comm.) that the river carries a heavy load of nitrogen, phosphorus and silicate due to supply of fertilizers used for agriculture upstream.

The multiple linear regression analysis indicated a positive relationship between temperature and assimilation number, which was expected due to the efficiency of enzymatic mechanisms (Falkowski 1981). In addition a negative relation was observed between to the rate of conversion of the xanthophyll cycle and assimilation numbers of PAR exposed samples. The high conversion of xanthophylls to their heat dissipating state suggests photosynthetic down regulation in these samples as a result of (previous) high light exposure, thereby depressing carbon incorporation rates while under the solar simulator. When cells were also exposed to UVR they always displayed some degree of photoinhibition due to both UVAR and UVBR (Fig. 5). We observed high variability occurring in a relatively short period of time, with photoinhibition ranging between 5-26% and 10-39% for UVAR and UVBR, respectively. These inhibition values are in the range as those previously reported for Patagonian coastal waters (Helbling *et al.* 2001a, Helbling *et al.* 2005, Barbieri *et al.* 2002, Villafañe *et al.* 2004). Similarly as to assimilation numbers,

multiple linear regression analysis indicated that the variability in UVR-induced photoinhibition was significantly related to the contribution of diatoms, chlorophytes, dinoflagellates, *Synechococcus* and cryptophytes in the samples and to the assimilation number of samples receiving only PAR. Furthermore, the negative relationship of P_{ass} and DT/(DT+DD) (eq. 1) was also supported by the positive relationship between the amount of diatoms and the UVR_{inh} (eq. 2) suggesting that when high-light acclimated diatoms dominated, the assimilation numbers decreased and photosynthesis was more inhibited by UVR. On the other hand, UVR_{inh} was negatively related to the amount of chlorophytes, suggesting that this group had a better performance under UVR exposure as compared to others present during the study period

Moreover, mycosporine like aminoacids (MAAs) were not detected in significant amounts throughout the study period (data not shown). The lack of these compounds was expected as during summer, the size structure of the communities did not favor their accumulation, because their useful concentration would be too high and osmotically disadvantageous (Garcia-Pichel 1994).

In conclusion, we have found that phytoplankton communities from Patagonia were sensitive to UVR stress, although the extent of the inhibition varied during the summer. Taxonomic composition played a key role in explaining the observed variability in UVR-induced inhibition throughout the study period. Other environmental variables, such as wind and nutrient input by the Chubut River presumably also played an important role conditioning the photosynthetic performance of cells and so their response to exposure to UVR.

Chapter 7

Antioxidative responses of two marine microalgae during static and dynamic natural ultraviolet radiation exposure

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Abstract

High light acclimated cultures of the marine microalgae *Thalassiosira weissflogii* and *Dunaliella tertiolecta* were exposed to static and dynamic natural ultraviolet radiation (UVR), the latter by applying artificial shallow mixing. Antioxidant (superoxide dismutase; SOD, ascorbate peroxidase; APX, glutathione cycling) responses were followed for one week during static and dynamic natural UVR and compared with PAR (Photosynthetically Active Radiation) only controls. In addition, changes in light harvesting and photoprotective pigment pools were followed. The overall impact of UVR was assessed by monitoring growth as well as UVR sensitivity, measured as UVR-induced inhibition of carbon assimilation under an indoor solar simulator. We hypothesized that enhanced UVR resistance during prolonged UVR exposure could at least partly be explained by increased antioxidant activity

Both species showed immediate antioxidant responses due to their transfer to the outdoor conditions. Furthermore, carbon assimilation and growth rates were reduced in both species in all outdoor treatments compared to initial conditions. *D. tertiolecta* was affected most by the outdoor exposures, with respect to antioxidant and pigment pools as well as growth rate and UVR sensitivity. Yet, in both species, static and dynamic UVR exposure did not alter antioxidant levels as compared with PAR only controls. In contrast, growth was most affected in the static UVR cultures. At the same time, the static UVR cultures showed significantly enhanced UVR resistance We therefore conclude that antioxidants play a minor role in the reinforcement of natural UVR resistance in *T weissflogii* and *D. tertiolecta*.

Introduction

Marine microalgae are subject to fluctuating irradiance conditions due to co-occurring changes in intensity of incoming irradiance (daily and seasonally), cloud coverage, and their changing position in the water column (Helbling *et al.* 1994, Lubin & Jensen 1995, Neale *et al.* 1998b, 2003). In a shallow upper mixed layer (UML; Neale *et al.* 2003) vertical mixing can mediate phytoplankton transport through a gradient of photosynthetic active radiation (PAR; 400-700 nm) ranging from saturating to excessive irradiance close to the water surface (Neale *et al.* 1998b, 2003). In contrast, ultraviolet radiation (UVR; 280-400 nm) is attenuated much faster and can be insignificantly low at the bottom of the UML by which shallow mixing has a stronger influence on UVR than on PAR exposure. As excess PAR and UVR can reduce carbon incorporation in different ways, irradiance-induced effects are subject to spectral quality and exposure duration. Also, UVR may inhibit repair pathways and therefore algae can benefit from periods with low UVR levels to recover from photodamage (Neale *et al.* 1998b). Therefore, the effect of UVR on photosynthesis in a shallow UML depends on the optical properties of water, and mixing depth and speed (Neale *et al.* 1998b, Helbling *et al.* 2003).

The detrimental action of excess irradiance including UVR is, among other things, mediated through enhanced reactive oxygen species (ROS; Mehler 1951, Asada *et al.* 1974) production. During excess irradiance exposure, light harvesting capacity can exceed photosynthetic capacity leading to overreduction of the photosystems and thereby to ROS generation (Gechev *et al.* 2006). ROS may damage DNA, phospholipids and proteins such as photosystem II reaction centers, and cause a decrease in photosynthetic efficiency or even viability loss (Van de Poll *et al.* 2005, Gechev & Hille 2005). Additionally, UVR is able to directly damage biological molecules (i.e. DNA, proteins), causing obstruction of metabolic pathways, therewith augmenting overreduction of the photosystems and production of ROS (Hideg & Vass 1996, Mackerness 2000, Bischof *et al.* 2003).

To prevent irradiance-induced formation of ROS, microalgae apply immediate regulatory mechanisms such as non photochemical quenching via xanthophyll cycling, and antioxidant action (Mewes & Richter 2002, Van de Poll *et al.* 2006, Janknegt *et al.* 2008). On a longer time scale photoacclimation occurs during which photosynthesis and its regulatory mechanisms are adjusted to the prevailing irradiance (Van de Poll *et al.* 2005, Falkowski & LaRoche 1991). When these systems are inadequate, overreduced photosystems initiate generation of the moderately dangerous superoxide ($O_2^{\bullet-}$) radicals. A second reduction step generates the mildly reactive hydrogen peroxide (H_2O_2) which, in the presence of $O_2^{\bullet-}$, can form the most destructive hydroxyl radical ($HO^{\bullet}$; Kehrer 2000). To counteract ROS accumulation, oxygen radicals are scavenged by a network of antioxidants which is aimed to prevent $HO^{\bullet}$ formation. In this network, $O_2^{\bullet-}$ is converted into H_2O_2 by superoxide dismutase (SOD; Gregory & Fridovich 1973a, b) and is subsequently scavenged by peroxidases like ascorbate peroxidase (APX; Shigeoka 2002, Asada 2006). Hereby, APX consumes ascorbic acid which is regenerated using glutathione as a reducing agent. Oxidized glutathione is reduced again by glutathione reductase (GR; De Kok & Stulen 1993, Noctor *et al.* 2002).

In contrast to constant irradiance conditions, the unpredictable nature of fluctuating light regimes makes is difficult for microalgae to adequately photoacclimate. Van de Poll *et al.* (2007) found a low irradiance type acclimation response in a diatom under a fluctuating

light regime with respect to pigment composition as compared with static conditions having an identical daily irradiance dose. As a result, cells could constantly be subjected to changing levels of (over) reduced photosystems and therewith to variable levels of ROS production.

Several laboratory studies have shown that microalgae adjust their antioxidant levels during photoacclimation to excess irradiance including UVR (Rijstenbil 2002, Bhargava *et al.* 2007, Janknegt *et al.* 2008). Yet, virtual nothing is known about antioxidant responses in marine microalgae under ambient UVR, let alone under more natural fluctuating irradiance regimes. In the present study we investigated antioxidant acclimation strategies of the diatom *Thalassiosira weissflogii* and the green phytoflagellate *Dunaliella tertiolecta* under static and dynamic natural UVR, the latter by applying artificial mixing. We chose a diatom and a phytoflagellate because representatives of these taxa have been demonstrated to fundamentally differ in dynamic irradiance responses as well as in excess irradiance vulnerability including UVR (Davidson & Marchant 1994, Van Leeuwe *et al.* 2005, Wagner *et al.* 2006). We assessed immediate (1 d), short term (3 d) and long term (7 d) responses during exposure to outdoor static UVR and simulated mixing regimes (60 min. and 10 min. cycle), using PAR only cultures as controls. Acclimation responses to static and dynamic conditions +/- UVR were followed for enzymatic and non-enzymatic antioxidants (SOD, APX, GR, glutathione levels). Also photoprotective and light harvesting pigment pools were measured. The overall impact of UVR was assessed by monitoring growth as well as UVR sensitivity, the latter measured as UVR-induced inhibition of carbon assimilation under an indoor solar simulator. We hypothesized that: I: antioxidant levels would be enhanced as a result of prolonged natural UVR exposures, compared with PAR only cultures; II: antioxidant levels would be lower in dynamic UVR as opposed to static UVR cultures; III: enhanced antioxidant activity could contribute to enhanced UVR resistance during prolonged natural UVR exposure.

Materials and Methods

Culturing conditions

Dunaliella tertiolecta (Dunal) Teodoresco (Chlorophyceae) and *Thalassiosira weissflogii* (Grunow) G. Fryxell et Hasle (Bacillariophyceae) were obtained from the Algal Culture Collection of Estación de Fotobiología Playa Unión. *T. weissflogii* and *D. tertiolecta* were grown at 250 µmol photons·m^{-2}·s^{-1} photosynthetically active radiation (PAR; 12/12 h l/d cycle) without UVR in f/2 enriched autoclaved sea water at a salinity of 34.5 PSU in a culture cabinet (Sanyo MLR 350) at 20°C for several weeks prior to the experiments. Cultures were kept in the exponential growth phase by regular dilution with fresh medium.

Experimental design.

Three types of experiments were performed: Experiment 1 (60 minute mixing cycle: from 3rd till 10th of February, 2006) and Experiment 2 (10 minute mixing cycle: from 10th till 17th of February, 2006) lasted seven days; Experimental series 3 were one-day experiments (*D. tertiolecta* experiments on January 31st and February 16th, 2006; *T. weissflogii* experiments on February 15th and 17th). For Experiment 3, only 10 minute mixing cycles were applied.

During all experiments, cultures of *D. tertiolecta* and *T. weissflogii* were subjected to mixed PAR (mixed PAR), mixed PAR+UVR (mixed PUV), static PAR (static PAR) and static PAR+UVR (static PUV) solar radiation regimes. At the start of experiment 1 and 2 cultures of *D. tertiolecta* and *T. weissflogii* were transferred to UV transparent 2.2 L polymethylmethacrylate (PMMA) bottles, diluted with 500 mL fresh medium and simultaneously exposed to the four irradiance conditions (hence giving a total of 8 experimental bottles: two species, four irradiance conditions each). During experiment 1 and 2, sampling volume (see below) was replenished with fresh medium once a day. As a result, cultures were growing under semi continuous batch conditions. The one day exposures of experiment 3 (one species per experimental day) were executed in UV transparent 2.2 L PMMA bottles, with cultures diluted with 500 mL fresh medium.

Irradiance conditions

The experiments were performed in an outside water filled basin (depth: 1.1 m, diameter: 5 m) to which sediment of the nearby Chubut river was added to enhance light attenuation. Sediment was resuspended twice a day during experimentation. Irradiance attenuation was checked three times a day, at three water depths: at 0.22, 0.44 and 0.8 m, and turbidity was adjusted when necessary. Bottles for the PAR only exposures were covered with 390 nm cut-off foil (Ultraphan, UV Opak Digefra, Germany, for transmission characteristics see Figueroa *et al.* 1997), while uncovered PMMA bottles transmitted the full solar spectrum (PUV). For each species, two bottles (PAR, PUV), were incubated 15 cm below the surface (Static) and two bottles (PAR, PUV) were moved through the water column between 15 and 80 cm depth by a home-made mixing simulator (dynamic). Note that static and dynamic irradiance conditions did not give identical daily PAR and UVR doses (see results, Table 1 and Discussion section). Sinusoidal vertical transport of the bottles was established by a DC motor (Maxon Motor, Switzerland). The speed of the motor and thus that of the bottles in the water was frequency controlled and adjusted to 60 min (Experiment 1) or 10 min (Experiment 2) mixing cycles.

Sampling

Pre-experimental samples (Initial) were taken for GR (120 mL), glutathione (160 mL), APX (120 mL), SOD (120 mL), pigments (100 mL), UV-sensitivity (100 mL) and cell counts (2 mL) (see below). During Experiments 1 and 2 samples were taken daily at 7:30 am for GR, glutathione, pigments, UV-sensitivity, and cell counts. This sampling time was chosen, because in this way we could follow longer term acclimation patterns without the interference of short term regulatory responses, which might have occurred during the daily exposure cycle. On the 3rd and the 7th day, additional samples were taken for APX and SOD activity. During experiment 3 samples for APX, SOD and GR activity, and glutathione were obtained at 9:00 am. However, here we also sampled at 1:00 pm and 5:00 pm, to collect additional data during the first day of the outdoor exposure. Samples for SOD, APX , and GR activity and glutathione content were filtered over polycarbonate filters (Ø 47 mm, 2.0 µm pore size; Osmonics Inc., USA), frozen in liquid nitrogen and stored at -80°C. Samples for pigment analysis were immediately filtered over GF/F filters (Ø 25 mm) in a darkened room, immediately frozen in liquid nitrogen and stored at -80°C. Samples for cell

counts were fixed with formalin (0.1%, V/V) and stored at 4°C in darkness. Samples for the assessment of UV-sensitivity were processed immediately (see below).

Measurement of UV sensitivity

To determine UV-sensitivity, we performed carbon assimilation experiments using an indoor solar simulator. Samples were collected from all outdoor cultures, transferred to quartz tubes (50 mL, in duplicate), spiked with ^{14}C sodium bicarbonate (3.75 µCi) and incubated under the solar simulator (Hönle, Sol 1200, Germany) for 1.5h in a water bath inside a temperature controlled chamber (20 °C). Half of the tubes was covered with 390 nm cut-off foil (receiving simulated PAR only) while the others remained uncovered (receiving simulated PUV). The lamp was covered with cellulose acetate film to avoid UV-C exposure. The applied radiation was 183, 69.9 and 1.7 $W \cdot m^{-2}$ for PAR, UVAR (315 – 400 nm) and UVBR (280 – 315 nm), respectively. After exposure, samples were filtered on 25 mm GF/F filters (Whatman), stored in scintillation vials, and exposed to concentrated HCl fumes overnight to remove unbound ^{14}C sodium bicarbonate. Radioactivity was quantified using liquid scintillation counting. ^{14}C incorporation, corrected for dark controls, and normalized to chlorophyll *a* concentration measured the beginning of the solar simulator experiment to provide carbon assimilation numbers. Chlorophyll *a* concentration was determined by filtering 50 mL culture on 25 mm GF/F filters followed by extraction in (100%) methanol (Holm-Hansen & Riemann 1978). The samples were sonicated (20 min) and extracted for at least 2 hours in darkness at 4ºC. After centrifugation (10 min, 3000 rpm), the Chl*a* concentration of the supernatant was determined by measuring the fluorescence of the extract before and after acidification, using a calibrated Turner Designs TD700 fluorometer. UV sensitivity was determined by calculating the relative difference in carbon assimilation between PAR-only and PUV exposure, expressed as percentage UVR-inhibition.

Cell counts and growth rates

Cell counts were performed on a Coulter MCL flow cytometer (Beckmann-Coulter) as described in Van de Poll *et al.* (2007). For estimation of increase in cell density, cell counts were corrected for the dilution factor. Growth rates were determined by measuring the slope of the natural log (Ln) of cell numbers as a function of time. Growth rates of the initial cultures were determined by measuring the cell concentrations over four successive days, prior to experimentation.

SOD activity

SOD analyses were done using the riboflavin/Nitro Blue Tetrazolium (RF/NBT) method modified for algal material, as described in Janknegt *et al.* (2007). Filters were disrupted by sonication in 0.75 mL protein extraction buffer X (50 mM KH_2PO_4, pH7.8; 0.1 mM EDTA; 0.1% Triton X-100; 2% PVP and complete protease inhibitor cocktail). After spinning down cell debris, supernatants were transferred to a clean 1.5 mL Eppendorf tube. SOD activity was defined as the amount of sample required to achieve 50% inhibition of the NBT reduction (V_{50}). NBT reduction was measured (in duplicate) in 4 reaction mixtures containing 0 (blank, no inhibition), 12.5, 25, and 50 µL extract. Absorption was measured at 560 nm on a Cary 3E UV/vis double beam spectrophotometer (Varian, Middelburg, The

Netherlands) equipped with a temperature controlled cell attached to a water bath. V_{50} was calculated by regression using the linear part of a natural semi-log curve. The specific activity (S.A.) of SOD was calculated according to 1 / (V_{50}*[protein]).

APX and GR activity

Supernatants for both APX and GR activity assays were prepared and protein concentrations were determined as described for the *SOD activity assay* (see above). APX activity was determined spectrophotometrically by measuring the consumption of its substrate, ascorbic acid, over a 3 min. interval, in a 3 mL quartz cuvette containing 2.4 mL buffer A (50 mM KH_2PO_4, pH7; 0.1 mM EDTA), 150 µL, 10 mM ascorbic acid (Merck, Darmstadt Germany), 400 µL cell extract and 30 µL, 20 mM H_2O_2 (Merck, Darmstadt Germany). Before H_2O_2 was added, the reaction mixture was incubated for 5 min. at 20°C. The rate of ascorbate consumption was measured at 290 nm on a Cary 3E UV/VIS double beam spectrophotometer. Specific APX activity (U/mg prot) was calculated according to: (-slope x $volume_{tot}$ / ε x $volume_{extr.}$) / [protein] of which ε = 2.8 $mM^{-1} \cdot cm^{-1}$. Determination of GR activity was based on its catalytic property to convert oxidized glutathione (GSSG) into its reduced form (GSH) thereby consuming NADPH as a reducing agent. The NADPH consumption was measured spectrophotometrically by determining its decrease over a 3 min interval in a 3 mL quartz cuvette containing 1 mL buffer A; 150 µL, 10 mM GSSG (Merck, Darmstadt Germany), 300 µL cell extract and 50 µL, 2.5 mM NADPH (Merck, Darmstadt Germany). Before adding NADPH, the reaction mixture was incubated for 5 min in a cryostat at the appropriate temperature. Specific activity of GR (in U/mg prot) was calculated by: (-slope x $volume_{tot}$ / ε x $volume_{extr.}$) / [protein] of which ε = 6.22 $mM^{-1} \cdot cm^{-1}$.

Glutathione content

Filters were suspended in 0.4 mL glutathione extraction buffer containing 50 mM sulfosalicylic acid, 1 mM EDTA, and 0.15% ascorbic acid hereafter cells were disrupted by sonication (30 s, at A=30) at 4°C. Extracts were centrifuged for 25 min (20.000 x g) at 4°C and supernatants were transferred to a clean 1.5 mL Eppendorf tube. The total amount of glutathione (TAG) was determined using the SIGMA Glutathione Assay Kit (CS0260; Sigma, Saint Louis, MI, USA); all chemicals were purchased from Sigma unless indicated otherwise. The assay was performed in 96 wells microtiter plates (Cayman chemical) containing 150 µL working mixture 1 (100 mM KH_2PO_4, pH7; 100 mM EDTA, GR, 0.170 U/mL; 0.043 mg/mL 5,5'-dithiobis-2-nitrobenzoic acid), and 10 µL cell extract. After 5 minutes of incubation at room temperature, 50 µL of a 0.16 mg/mL NADPH solution was added. 5-thio-2-nitrobenzoic acid (TNB) production was then followed spectrophotometrically for 10 min at 405 nm using a Victor^{tm} 1420 multilabel counter plate reader (PerkinElmer, Waltham, MA, USA) reading at 1 minute intervals. To determine the glutathione content in 10 µL algal extract, a reference line was included based on 0.5, 0.25, 0.125, 0.0625 and 0.0312 nmoles reduced glutathione (GSH, Sigma, Saint Louis, MI, USA) in 10 µL sample. The TNB production rate was calculated for 1 nmole of glutathione by extrapolation. The TAG (in nmol) in 1 mL algal sample was calculated according to: $slope_{sample}$ x dilution factor original sample / $slope_{1nmole\ GSH}$ x 0.01 mL sample). The amount of GSH was measured by the same principle as TAG but without adding GR and NADPH by which only GSH reacts with DTNB. This assay was performed in 96 wells microtiter

plates containing 100 µL working mixture 2 (500 mM KH_2PO_4, pH7; 500 mM EDTA; 0.065 mg/mL DTNB), and 100 µL cell extract. After 10 min of incubation at RT the total production of TNB was determined spectrophotometrically at 405 nm. A reference line was included based on 2, 1, 0.5, 0.25, and 0.125 nmoles GSH in 100 µL sample. The amount of GSH in the reaction mixture was calculated in accordance to the reference line. The amount of GSH (in nmol) in 1 mL algal extract was calculated according to: GSH in reaction mixture x dilution factor original sample / 0.1 mL sample. The glutathione redox status (GRS) was expressed in sulphur atoms and calculated according to: GSH / (GSH + 0.5 GSSG) of which GSSG = TAG – GSH.

Pigment

Pigments were analyzed as described by Van Leeuwe *et al.* (2006). In short, filters were handled under dim light, freeze-dried (48 h) after which pigments were extracted in 4 mL 90% cold acetone (v/v, 48 h, 5ºC). Pigments were resolved using HPLC (Waters 2690 separation module, 996 photodiode array detector) with a C_{18} 5 µm DeltaPak reversed-phase column (Milford, MA, USA) and identified by retention time and diode array spectroscopy. Quantification was done using standard dilutions of Chlorophyll *a*, Chlorophyll *b*, fucoxanthin, diadinoxanthin, diatoxanthin, violaxanthin, antheraxanthin and zeaxanthin. Cellular pigment concentrations were calculated from cell counts and extraction volume.

Irradiance

Irradiance (PAR, UVR) was continuously recorded as the average of one min intervals using a broadband ELDONET radiometer (Real Time Computers Inc.) located on the roof. Attenuation of UVR and PAR in the reservoir was determined with a USB diode array spectrophotometer (HR 2000CG-UV-NIR, Ocean Optics, Duneclin, USA) with a 4 m fiber optics and cosine diffuser. Attenuation coefficients were used to estimate the irradiance for the cultures during mixing and static solar radiation regimes. Irradiance measurements were done three times a day during experimental days at three water depths: 0.22, 0.44 and 0.8 m. By considering mixing depth and mixing speed, we estimated the irradiance that the mixed cultures experienced.

Data analysis and statistics.

Differences in irradiance conditions were tested using a paired t-test. Differences between *D. tertiolecta* and *T. weissflogii* were tested by performing a paired t-test. Since no significant differences were found between Experiments 1 and 2, as well as some experimental days, we distinguished immediate (data collected during one day, experiment 3), short term (using the average of data from day 1-3, with experiments 1 and 2 pooled) and long term (using the average of data from day 4-7, with experiments 1 and 2 pooled) responses. Intraspecific differences between response periods (immediate, short and long term) and irradiance treatments (static PUV, static PAR, mixed PUV, mixed PAR) for SOD activity, APX activity, GR activity, GRS, total glutathione content, pigment composition and growth, were tested by performing a multivariate ANOVA. When significant differences were found, an LSD post-hoc test was applied to specify these differences.

Results

Irradiance conditions

During the entire experimental period, the average daily outdoor radiation dose fluctuated between 4900 and 8200 kJ·m^{-2} PAR, 460 and 960 kJ·m^{-2} UVAR and 10 and 22 kJ·m^{-2} UVBR (Table 1). Attenuation coefficients (K_d) in the experimental basin were 0.91 (PAR), 2.74 (UVAR) and 3.95 (UVBR) so that the irradiance intensities at 0.15 m were reduced to 87, 66% and 55% respectively. At 0.8 m, irradiance intensities were reduced to 48, 11 and 4%, respectively, compared to surface irradiance. Regardless of the mixing speed, cultures subjected to the simulated mixing regime received on average 79% (PAR), 50% (UVAR) and 36% (UVBR) of the daily radiation dose compared with the static cultures. When comparing experiments 1 and 2, the average daily radiation doses between the two short term and the two long term periods did not differ significantly (data not shown). Therefore, the corresponding cultures were exposed to comparable radiation conditions during both experiments. This similarity was reflected in the response parameters of both experiments (UV sensitivity; SOD, GR and APX activity; cellular glutathione content and the glutathione redox potential) which showed highly corresponding trends in time. Therefore, average values of the two experiments were pooled.

Table 1: Calculated daily average outdoor doses (KJ·m^{-2}) experienced by the *Dunaliella tertiolecta* and *Thalassiosira weissflogii* PUV cultures for photosynthetically active radiation (PAR), ultraviolet A radiation (UVAR: 315 – 400 nm) and ultraviolet B radiation (UVBR: 280 – 315 nm) Immediate: day 1 between sunrise and 5:00pm . Short term: averaged over first 3 days of the outdoor exposures. Long term: averaged over days 4 – 7 of the outdoor exposures.

Response time	Condition	PAR	UVAR	UVBR
Pre-culturing	Indoors	2200	---	---
Immediate	*D. tertiolecta* Static	8163	958	22
	D. tertiolecta Mixed	4897	460	10
	T. weissflogii Static	6264	673	15
	T. weissflogii Mixed	5632	514	11
Short term	Static	7226	818	18
	Mixed	5984	555	12
Long term	Static	7599	845	19
	Mixed	6257	564	12

Growth

Before their transfer to the experimental basin, the growth rate of *D. tertiolecta* was higher than that of *T. weissflogii* (Table 2). During the first three days (short term) of exposure to outdoor irradiance mean growth rates of *D. tertiolecta* cultures remained at a similar level compared to initial values after which they all decreased significantly ($p<0.05$) by 37-66% (Table 2, 4). Except for the mixed PAR cultures growth rates of *T. weissflogii* were already reduced significantly ($p<0.05$) during the first three days (short term) by 14-26% and did not change significantly thereafter (Table 2, 4). Growth rates of both species were

significantly ($p<0.05$) affected by UVR (Table 2, 4). Eventually, *D. tertiolecta* was significantly ($p<0.05$) more affected by long term (7 d) outdoor irradiance exposure than *T. weissflogii*.

Table 2: Growth rates of *Dunaliella tertiolecta* and *Thalassiosira weissflogii*. Initial: acclimated to 250 µmol photons·m^{-2}·s^{-1} PAR, Short term: exposed for 3 days (1-3 d) to outdoor irradiance conditions. Long term: days 4-7 of outdoor irradiance. In brackets standard error of the linear regression of log transformed cell numbers.

	D. tertiolecta		*T. weissflogii*	
Initial	0.53 (0.040)		0.42 (0.035)	
	Short term	Long term	Short term	Long term
Mixed PAR	0.57 (0.088)	0.35 (0.008)	0.42 (0.037)	0.37 (0.180)
Mixed PUV	0.57 (0.075)	0.22 (0.052)	0.31 (0.031)	0.28 (0.021)
Static PAR	0.57 (0.062)	0.33 (0.229)	0.36 (0.012)	0.34 (0.066)
Static PUV	0.57 (0.085)	0.18 (0.094)	0.33 (0.036)	0.26 (0.055)

UVR sensitivity

During the entire experimental period, *D. tertiolecta* showed significantly ($p<0.05$) higher assimilation numbers than *T. weissflogii* when exposed to PAR under the solar simulator (Figs. 1a, b). However, *D. tertiolecta* showed a significantly ($p<0.05$) larger reduction in carbon assimilation on the short and long term than the corresponding cultures of *T. weissflogii.* (Figs. 1a, b; Table 4). After long term (7 d) outdoor exposure, assimilation numbers of all cultures of both species had decreased significantly ($p<0.005$) compared to pre-experimental values (Table 4). Although both species showed lowest assimilation numbers for the static PUV cultures, there were no significant differences between the 4 outdoor irradiance conditions for both species. *D. tertiolecta* was significantly ($p<0.05$) more UV sensitive (expressed as percentage UV-inhibition) than *T. weissflogii* (Figs. 1c, d; Table 4). In addition, UVR sensitivity of the static PUV cultures of both *T. weissflogii* and *D. tertiolecta* decreased significantly ($p<0.01$) over time, becoming significantly ($p<0.0001$) lower than the other (PUV mixed, PAR static and mixed) cultures. UV sensitivity decreased for both species following mixed PAR > static PAR > mixed PUV > static PUV.

Superoxide dismutase and ascorbic peroxidase

Species specific differences were observed in antioxidant enzyme activity and responses. When acclimated to pre-experimental irradiance (250 µmol·m^{-2}·s^{-1}), SOD activity of *D. tertiolecta* (103 U/mg protein, sd=3.0) was significantly ($p<0.05$) higher than *T. weissflogii* (68 U/mg protein, sd=10.6; Table 3). APX activity of *D. tertiolecta* (0.89 U/mg protein, sd=0.2) was significantly ($p<0.05$) lower than that of *T. weissflogii* (3.6 U/mg protein, sd=0.6; Table 3). During the first day of the outdoor exposure, no significant differences were found between samples taken during the daily cycle (9.00 am, 1.00 pm and 17.00 pm.) and therefore these data were pooled. Also, there were no significant differences in SOD and APX activities between the four radiation conditions during short and long term exposure. Therefore, these data were pooled as well. *T. weissflogii* showed no significant responses in SOD and APX activity regardless of radiation condition or exposure duration

(Table 3, 4). In contrast, *D. tertiolecta* showed a slight but significantly ($p<0.05$) enhanced SOD activity after three days which remained on this level until day 7 (Table 3, 4).

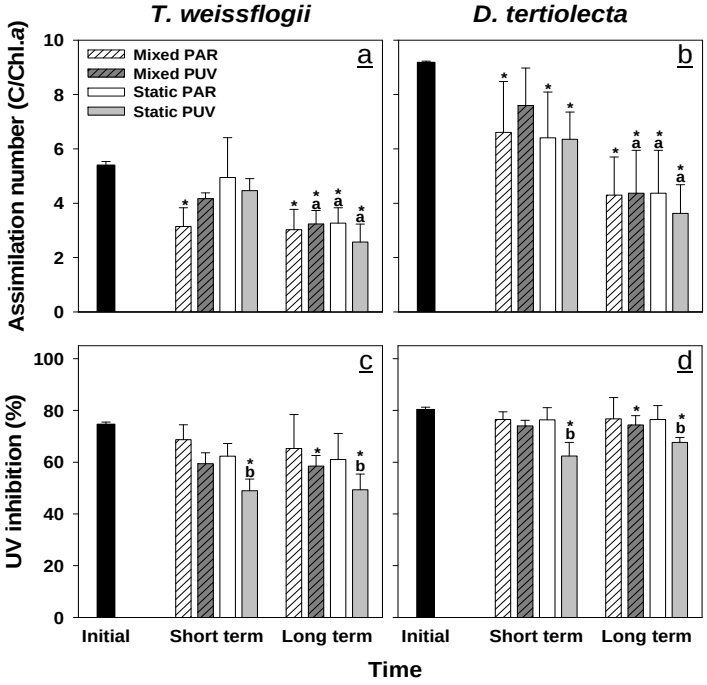

Figure 1:
Assimilation number (from solar simulator PAR condition only) and UVR-induced inhibition of ^{14}C incorporation of *Thalassiosira weissflogii* **a**), **c**) and *Dunaliella tertiolecta* **b**), **d**). Short term: cultures exposed up to 3 days. Long term: cultures exposed to outdoor irradiance from day 4 to day 7. Bars show mean values of three (Short term) or four (Initial and Long term) replicates, error bars represent standard deviations. * differs significantly from the Initial value ($p<0.05$) a: differs significantly from the previous exposure period ($p<0.05$). b: differs significantly from the other irradiance conditions within the same exposure period ($p<0.05$).

Table 3: Superoxide dismutase and ascorbate peroxidase activity (U/mg protein) of *Dunaliella tertiolecta* and *Thalassiosira weissflogii*. Initial: acclimated to 250 µmol photons·m^{-2}·s^{-1} PAR. Immediate: cultures exposed for one day (from sunrise till 5:00 pm) to outdoor irradiance conditions. Short term: exposed for 3 days (1-3 d) to outdoor irradiance conditions. Long term: days 4-7 of outdoor irradiance. Immediate APX responses of *D. tertiolecta* (See ⇒) showed significant differences between the four treatments and are indicated in the last two columns (treatment and Immed. APX). Hereby, S. PAR = static PAR culture, S. PUV = static PUV culture, M. PAR = mixed PAR culture and M. PUV = mixed PUV culture. In brackets standard deviation; Initial, Short term and Long term, n=4; Immediate APX (except for *D. tertiolecta*) n=12; Immediate SOD, n=6; Immediate APX activity responses of each of the four *D. tertiolecta* treatments, n=3. n/a = not available. * = differs significantly from initial value. [a] = differs significantly from other treatments.

	T. weissflogii		*D. tertiolecta*			
	SOD	APX	SOD	APX	treatment	Immed. APX
Initial	67.9 (10.6)	3.6 (0.60)	103.4 (3.0)	0.89 (0.21)	S. PAR	0.51*a (0.01)
Immediate	n/a	4.4 (1.20)	111.4 (19.8)	See ⇒	S. PUV	0.54*a (0.04)
Short term	66.9 (0.8)	3.9 (0.29)	110.9 (3.1)*	0.83 (0.11)	M. PAR	0.83 (0.13)
Long term	64.1 (9.9)	4.4 (0.38)	114.7 (2.5)*	0.91 (0.05)	M. PUV	0.88 (0.13)

Regarding APX activity, only static cultures of *D. tertiolecta* showed an immediate (1 d) significant ($p<0.05$) decrease by which they were significantly ($p<0.05$) lower than the cultures exposed to a mixed irradiance regime. After 3 days (3 d) their APX activities had returned to initial values and remained on this level until day 7 (Table 3, 4).

Glutathione and glutathione reductase

After acclimation to 250 $\mu mol \cdot m^{-2}.s^{-1}$ GR activity of *D. tertiolecta* (0.19 U/mg protein; sd=0.014) was significantly ($p<0.05$) higher than that of *T. weissflogii* (0.052 U/mg protein, sd=0.006; Figs. 2a, b). Both species contained comparable amounts of cellular glutathione (Figs. 2c, d; 18.1 nmol/10^6 cells, sd=2.1 and 15.9 nmol/10^6 cells, sd=1.6 respectively) and a similar glutathione redox status (GRS = GSH/GSH + 0.5 GSSG; Figs. 2e, f; 0.21, sd=0.024 and 0.20, sd=0.014 respectively). During the first day of the outdoor exposure, no significant differences were found during the daily cycle, and therefore these data were pooled. When exposed to outdoor radiation GR activity of *T. weissflogii* showed no response regardless of irradiance condition or duration of exposure (Fig. 2a; Table 4). Static *D. tertiolecta* cultures immediately (1 d) increased their GR activity reaching significantly ($p<0.05$) higher values than those of the mixed cultures. (Fig. 2b). Subsequently, both mixed cultures increased their GR activity significantly ($p<0.005$) to similar enzyme activities as the static cultures (3 d) response. During the following four days (7 d) all four cultures decreased their GR activity significantly ($p<0.05$) to initial values (Fig. 2b; Table 4).

Regardless of the outdoor irradiance conditions, all *T. weissflogii* cultures immediately (1 d) responded significantly ($p<0.0005$) by doubling their glutathione content (Fig. 2c). This was accompanied by a significant ($p<0.01$) decrease in their GRS (Fig. 2e). Throughout the following three days (short term), both parameters returned to their initial values and remained on this level for the rest of the experiments (7 d; Table 4). *D. tertiolecta* responded by immediately increasing the cellular amount of glutathione of both static cultures significantly ($p<0.05$) by which they were significantly ($p<0.05$) higher than both mixed cultures (Fig. 2d). Throughout time, the cellular glutathione content of all cultures dropped significantly ($p<0.01$) to comparable levels which were significantly ($p<0.005$) lower than the initial values (Table 4). During the first day outside, the GRS of all *D. tertiolecta* cultures decreased significantly ($p<0.005$) by which the static cultures had dropped to a significantly ($p<0.05$) lower level than the mixed cultures (Fig. 2f). During the short term response, the GRS of both static cultures increased again to similar values as the mixed cultures after which they remained on this same level, significantly lower than initial value (Fig. 2f; Table 4).

Pigments

T. weissflogii contained Chlorophyll *a* (Chl.*a*) and fucoxanthin (Fuco) as major photoharvesting pigments and the xanthophylls cycle pigments diatoxanthin and diadinoxanthin (DT/DD cycle). *D. tertiolecta* contained Chl.*a* and Chlorophyll *b* (Chl.*b*) as major light harvesting pigments and the xanthophyll cycle pigments violaxanthin, antheraxanthin and zeaxanthin (VAZ cycle). Before their transfer outdoors, *T. weissflogii* had a significantly ($p<0.05$) lower content of light harvesting pigments than *D. tertiolecta* (Figs. 3c, d; 1.91 pg/cell, sd= 0.16 and 2.57 pg/cell, sd=0.11, respectively) and significantly

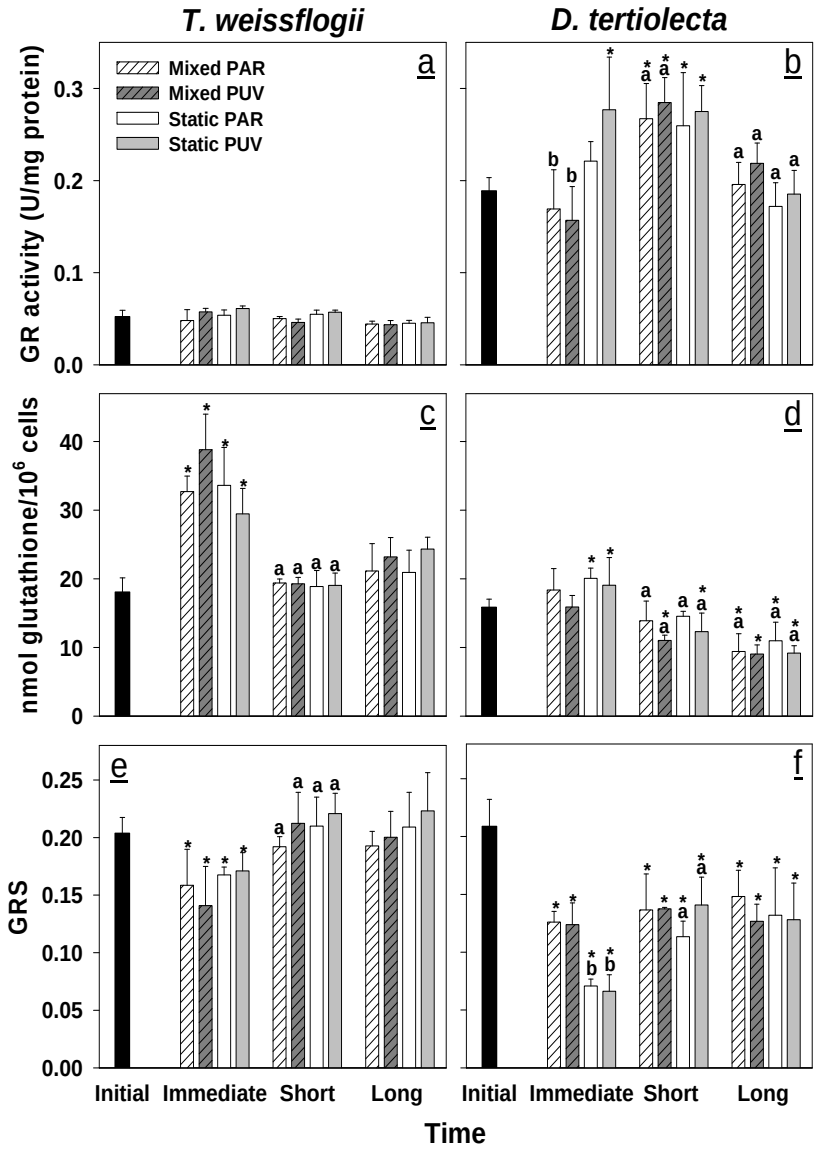

Figure 2: Antioxidant responses of *Dunaliella tertiolecta* and *Thalassiosira weissflogii*. **a**), **b**) Glutathione reductase (GR) activity. **c**), **d**) cellular amount of glutathione and **e**), **f**) glutathione redox status (GRS) Immediate: cultures exposed for one day (from sunrise till 5:00 pm) to outdoor irradiance conditions. Short term: cultures exposed for 3 days to outdoor irradiance conditions. Long term: cultures exposed to outdoor irradiance during the 4 subsequent days. Bars show mean values of three (Immediate and Short term) or four (Initial and Long term) replicates, error bars represent standard deviations. * differs significantly from the Initial value ($p<0.05$) a: differs significantly from the previous exposure period ($p<0.05$). b: differs significantly from the other irradiance conditions within the same exposure period ($p<0.05$).

($p<0.05$) more protective pigments (Figs. 3e, f; 0.44 pg/cell, sd= 0.08 and 0.27 pg/cell, sd=0.009 respectively). As a result, the ratio of protective over harvesting pigments (prot/harv ratio; Figs. 3a, b) of *T. weissflogii* (0.26, sd=0.01) was significantly ($p<0.0005$) higher than that of *D. tertiolecta* (0.10, sd=0.001). At the end of the long term period (7 d),

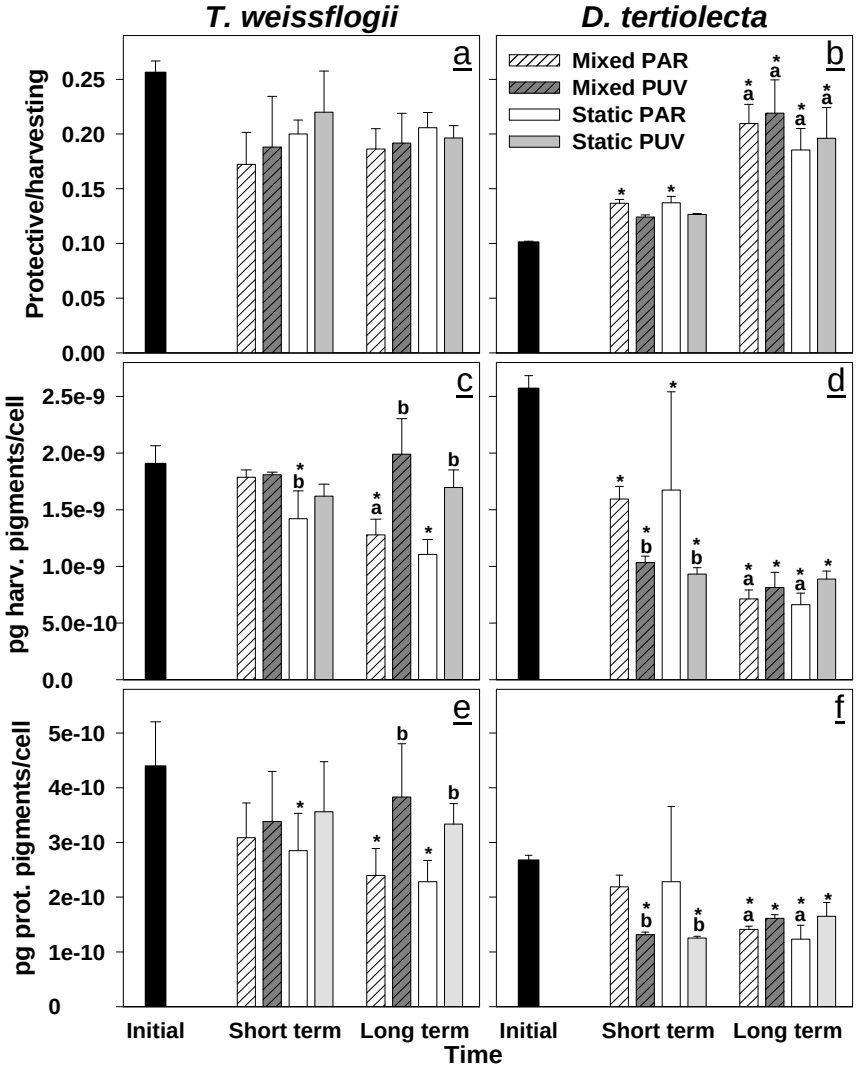

Figure 3: Pigment dynamics of *Thalassiosira weissflogii* and *Dunaliella tertiolecta.* **a**), **b**): Photoprotective over harvesting ratio. **c**), **d**): cell quota of harvesting and **e**), **f**): photoprotective pigments. Short term: cultures exposed for 3 days to outdoor irradiance conditions. Long term: cultures exposed to outdoor irradiance during the 4 subsequent days. Bars show mean values of three (Short term) or four (Initial and Long term) replicates, error bars represent standard deviations. * differs significantly from the Initial value ($p<0.05$) a: differs significantly from the previous exposure period ($p<0.05$). b: differs significantly from the other irradiance conditions within the same exposure period ($p<0.05$).

PAR-only exposed *T. weissflogii* had significantly ($p<0.05$) lower amounts of light harvesting and protective pigments as compared with PUV exposed cells (Figs. 3c, e; Table 4). However, the prot/harv ratios did not differ significantly between PAR and PUV cultures, and between short and long term exposure periods (Fig. 3a).During the first three days (short term) of outdoor exposure, all cultures of *D. tertiolecta*, significantly ($p<0.05$) decreased their cellular amount of harvesting and protective pigments with PUV exposed cultures containing significantly ($p<0.05$) less pigments than PAR-only exposed cultures (Figs. 3d, f). As a result of the long term (7 d.) exposure, PAR-only exposed cultures showed significant ($p<0.05$) decreases in their cellular pigment contents, reaching similar values as cultures subjected to PUV. As a result, prot/harv ratio of *D. tertiolecta* increased significantly ($p<0.01$; Fig. 3b; Table 4). Yet, no significant differences between the four irradiance conditions were found.

Table 4: Long term (4-7 d) effects on cellular parameters for both species, expressed as percent change relative to initial conditions. * differs significantly from initial value.

	T. weissflogii				*D. tertiolecta*			
	Mixed PAR	Mixed PUV	Static PAR	Static PUV	Mixed PAR	Mixed PUV	Static PAR	Static PUV
Growth	-12	-33	-19	-38	-34	-58	-37	-66
Ass no.	-44*	-33*	-39*	-54*	-50*	-53*	-52*	-61*
UV inhib	-7	-21*	-18	-33*	-5	-7*	-5	-16*
APX	+9	+26	+34	+20	-4	+5	-2	+7
SOD	-2	+10	+1	-7	+11*	+11*	+8*	+13*
GR	-16	-17	-14	-13	+4	+16	-9	-2
Glut/cell	+17	+28	+16	+35	-41*	-43*	-31*	-42*
GRS	-5	-2	+3	+10	-30*	-39*	-37*	-39*
Prot/harv	-28	-25	-20	-24	+105*	+115*	+82*	+92*
Harv/cell	-33*	+4	-42*	-11	-71*	-67*	-73*	-64*
Prot/cell	-46*	-13	-48*	-24	-47*	-40*	-54*	-38*

Discussion

Indoor mixing set-ups allow the accurate control of irradiance, but UVR/PAR ratios, the spectral quality within wavelength bands, as well as the irradiance levels fundamentally diverge from those occurring in the water column. Therefore we chose to perform our experiments under natural irradiance conditions, even though irradiance differences between experimental days were foreseen. Mixing speeds applied during our experiments (10 and 60 min.) are naturally occurring conditions in Patagonian coastal waters (Barbieri *et al.* 2002). During the 1 day experiments (experiment 3), weather conditions were rather variable. Yet, irradiance conditions during the two 7-day experiments (experiments 1 and 2) were highly stable allowing for the comparison between experimental treatments.

Previous investigations had shown that exposure of low irradiance acclimated cells to excess (artificial) sunlight can lead to significant viability loss (Van de poll *et al.* 2005, 2007). Therefore, in this study pre-cultivation was done at high irradiance (250 µmol

photons·m^{-2}·s^{-1}) to maximize photoprotection in order to guarantee survival after the transition to outdoor irradiance (Willemoës & Monas 1991, Mewes & Richter 2002, Buma *et al.* 2006). Due to this transfer, daily PAR doses increased 2-4 fold (Table 1), regardless of the irradiance treatments (static, mixed). In addition, both static and mixed cultures received on average more than 250 µmol photons·m^{-2}·s^{-1} between 07:45 and 19:15, even when mixed cultures were at the lower end of the mixing cycle. Therefore, our experimental irradiance conditions allowed comparison of excess irradiance effects without generating periods of irradiance limitation during the day. UVR intensities, on the other hand, were significantly lower at the bottom than at the surface of the mixing cycle. Therefore mixed cultures experienced periods of significantly low UVR irradiance as well as a lower daily UVR doses (Table 1). Thus, in the present experiments, antioxidant responses were compared for conditions of constant excess irradiance and shallow mixing, the latter characterized by fluctuating high to excess irradiance, without static and dynamic daily irradiance doses being identical (Table 1).

Intraspecific differences

Acclimation to the outdoor conditions did not lead to significantly different carbon assimilation numbers as determined in the solar simulator under PAR-only, when comparing the four irradiance treatments. This indicates that cultures that were acclimated to outdoor PUV did not exhibit a chronically obstructed photosynthetic machinery (MacIntyre *et al.* 1996) as compared with the cultures acclimated to outdoor PAR-only. Similarly, shallow mixing did not chronically affect photosynthetic performance as compared with the static cultures. In contrast, prolonged exposure to outdoor UVR (static as well as mixed cultures) strongly affected growth rates and UVR-sensitivity of both species. These results suggest that prolonged elevated excess irradiance induces increasing levels of photoprotection, therewith preventing photosystem damage and reduced carbon incorporation, however at the expense of growth, as found elsewhere (lesser 1996b, Zudaire & Roy 2001, Hernando *et al.* 2002). In support of this, static PUV cultures, that received the highest outdoor UVR-doses, were significantly more resistant to solar simulated UVR than the other three cultures. This indicates that UVR protection was effectively induced in static PUV cultures, however at strongly depressed growth rates. Mixed PUV cultures on the other hand exhibited somewhat higher growth rates, yet their UV resistance was only slightly higher than those of the PAR exposed cultures. In other words, the dynamic regimes increased growth rates, but decreased UVR resistance, possibly related with overall lower UVR levels as compared with the static PUV cultures (Table 1)

Strikingly, exposure to outdoor UVR or irradiance fluctuations did not induce distinct antioxidant responses between the four treatments in either *T. weissflogii* or *D. tertiolecta* on the longer term (3-7 d). This implies that prolonged outdoor excess PAR rather than fluctuating irradiance or outdoor UVR affected antioxidant levels, if at all (see below). Many studies have stressed the importance of xanthophyll cycling for quenching of excess irradiance energy (Young & Frank 1996, Niyogi 1999). Yet, protective over harvesting (prot/harv) ratios were not significantly different between irradiance treatments, despite the treatment-related changes in cellular pigment quantities. So, similar to the antioxidants, pigment ratios rather responded to the excess outdoor PAR than to additional UVR exposure or fluctuating irradiance. Therefore, irradiance induced differences in UV-

sensitivity or growth rates could not be explained by explicit antioxidant or pigment responses. Clearly, other mechanisms must have been responsible for the observed increase in UVR resistance in the static PUV cultures. These could involve upregulation of repair processes (Karentz *et al.* 1991b), increased protein turnover (Young & Frank 1996), production of UV-absorbing compounds (Karentz *et al.* 1991a, Hazzard *et al.* 1997, Laurion & Vincent 1998), *de novo* synthesis of D1 proteins (Ragni *et al.* 2008), increased level of lipid protecting α-tocopherol (Malanga & Puntarulo 1995, Malanga *et al.* 1997) or rerouting of metabolic pathways (Apel & Hirt 2004, Aguilera *et al.* 2002, Foyer & Noctor 2000).

Interspecific differences.

Cell size or surface to volume (S/V) ratio may partly determine excess irradiance or UVR responses in marine microalgae (Karentz *et al.* 1991b, Laurion & Vincent 1998, Helbling *et al.* 2001a). However, in our study, *T. weissflogii* and *D. tertiolecta* had a similar cell volume (367 and 368 μm^3, respectively) and S/V ratio's (0.82 and 0.69 respectively) and therefore cell size characteristics had a minimal impact on the observed responses. The phytoflagellate *D. tertiolecta* was found to be more sensitive to outdoor excess irradiance including UVR than the diatom *T. weissflogii* when considering carbon assimilation (Fig. 1b; Table 4), UVR resistance (Fig. 1d; Table 4) and growth rate (Table 2, 4). It must be noted that growth rates of *D. tertiolecta* during pre culturing (250 µmol photons$\cdot m^{-2} \cdot s^{-1}$) and the short term (3 d) outdoor exposures were maintained at a high level of 0.53 – 0.57 d^{-1} before the strong reduction (4-7 d, Table 2) occurred.. Other authors also found rapid acclimation of *D. tertiolecta* to high PAR levels (360-1000 µmol photons$\cdot m^{-2} \cdot s^{-1}$), with respect to growth rate (Havelková-Doušová *et al.* 2004, Kristin *et al.* 2007). Havelková-Doušová and coworkers (2004) showed increased growth (up to 0.61 d^{-1}) with increasing PAR dose during a one week period. However growth was slightly reduced at the highest total irradiance dose, being lower than our outdoor conditions (max. of 1750 µmol photons$\cdot \mu m^{-2} \cdot s^{-1}$). This could indicate that prolonged exposure to PAR intensities exceeding 1000 µmol photons$\cdot m^{-2} \cdot s^{-1}$ leads to growth inhibition in *D. tertiolecta*. In contrast, growth rates of *T. weissflogii* decreased immediately but to a lesser extent as compared with *D. tertiolecta*. Also growth rates of *T. weissflogii* were not further reduced after 3 days.

During pre-culturing, *D. tertiolecta* showed higher SOD and GR activity compared with the diatom. Considering that both species showed similar values for GRS and cellular glutathione content, this indicated that glutathione consumption by *D. tertiolecta* was much higher than by *T. weissflogii*. This implied enhanced levels of ROS in *D. tertiolecta* compared to *T. weissflogii* (27, 55-56). APX activity, on the other hand, was nearly four times higher in *T. weissflogii* than in *D. tertiolecta*. Yet, because microalgae use various pathways to remove hydrogen peroxide (catalase, glutathione peroxidase, non-enzymatic antioxidants, diffusion), these results do not imply that *T. weissflogii* experienced enhanced hydrogen peroxide production.

Immediate antioxidant responses during the first day after their transfer indicated enhanced levels of oxidative stress in both species. On a longer term, *D. tertiolecta* increased its SOD and GR activity, decreased its GRS and progressively reduced its glutathione quota throughout the experimental period in contrast to *T. weissflogii*. As SOD

is a $O_2^{\bullet-}$ scavenger and glutathione plays an important role in H_2O_2 detoxification (Alscher 1989, De Kok & Stulen 1993), observed changes suggest prolonged and increased levels of ROS production during short (3 d) and long (7 d) term exposure periods. The stoichiometrically inverse relation between GR and GRS has been found before in plants and macroalgae (Karpinski *et al.* 1997, Shiu & Lee 2005) and was ascribed to a putative regulatory role of glutathione in controlling (antioxidant) gene expression (Wingate *et al.* 1988, Georgiou 2002, Noctor *et al.* 2002). *T. weissflogii*, on the other hand, did neither significantly change its glutathione parameters, nor its levels of SOD and APX activity during short (3 d) and long (7 d) exposure to outdoor irradiance. These responses show that antioxidants are especially important directly after an irradiance shift. As they did not correspond with the differences in growth rates or reduced photosynthetic activity under the solar simulator, antioxidant responses were not found to explain interspecific differences in irradiance sensitivity in our study.

Similar to the antioxidants, *T. weissflogii* did neither show differences in its prot/harv pigment ratio during short (3 d) and long (7 d) term exposure to outdoor irradiance conditions. The prot/harv pigment ratio of *D. tertiolecta*, on the other hand, increased gradually to similar values as *T. weissflogii* during exposure to outdoor irradiance. Yet, this increase was not achieved by increasing the cellular amount of protective pigments but by dramatically decreasing the cellular amount of light harvesting and protective pigments, on average 3.5 and 2 fold, respectively. So, despite its increased prot/harv ratio and antioxidant responses, *D. tertiolecta* still showed the largest adverse responses to the outdoor irradiance exposure.

It must be emphasized that photoprotection properties, as described above, might potentially vary during the day as a result of photoregulation processes. In accordance, other studies described diurnal variation in antioxidant and pigment levels as a response to increased irradiance (Dupont *et al.* 2004, Janknegt *et al.* 2008). Yet, our study focused on photoacclimation responses (variation in photoprotective pools) and not on diurnal photoregulation. So, to avoid influence of photoregulation, samples were collected before algal cultures were exposed to (excess) solar irradiance.

Our results imply that both species might use different photoprotection strategies during acclimation to increased irradiance conditions. It has been suggested that taxon specific photoprotection strategies depend on the deployment of additional energy dissipation mechanisms (Meyer *et al.* 2000, Van Leeuwe *et al.* 2005, Wagner *et al.* 2006). In contrast to diatoms, green flagellates depend less on their xanthophyll cycle but contain supplementary quenching mechanisms such as rapid redistribution of energy via state transitions and cyclic PSI electron transport. Antioxidant and pigment responses indicate that cyclic PSI electron transport could play a role in energy dissipation in *D. tertiolecta*, since circulation of electrons around PSI inevitably leads to formation of superoxide radicals (Ishida *et al.* 1997, Apel & Hirt 2004, Kristin *et al.* 2007). Consequently, *D. tertiolecta* showed high SOD activities during pre-culturing conditions (compared to *T. weissflogii*) which increased during outdoor exposure conditions. Furthermore, in contrast to *T. weissflogii*, *D. tertiolecta* only started to increase its prot/harv ratio when exposed to outdoor irradiance conditions indicating that this species uses additional quenching mechanisms besides its xanthophyll cycle. So, it seems the pre-experimental irradiance level had already induced maximal photoprotection potential in *T. weissflogii* whereas *D. tertiolecta* was only moderately protected against outdoor irradiance conditions. As a

consequence, *T. weissflogii* could handle a long (7 d) exposure period of outdoor irradiance relatively well at the expense of growth whereas *D. tertiolecta* could grow relatively well during a short (3 d) exposure period at the expense of effective long (7 d) term protection mechanisms. In support of this, others also found that diatoms are more resistant than flagellates to high outdoor irradiance including UVR (Helbling *et al.* 1994, Buma *et al.* 2001, Villafañe *et al.* 2004).

We conclude first of all that simulated shallow mixing may offer protection against harmful UVR, but may increase UVR vulnerability, independent of mixing speed. Secondly, the green phytoflagellate *D. tertiolecta* was more sensitive to excess irradiance including UVR than *T. weissflogii* as a result of insufficient photoprotection strategies. Thirdly, antioxidants were primarily deployed as an initial response after the irradiance shift to excess outdoor PAR. Finally, antioxidant and pigment responses could not explain the observed differences in growth and UV sensitivity in both species.

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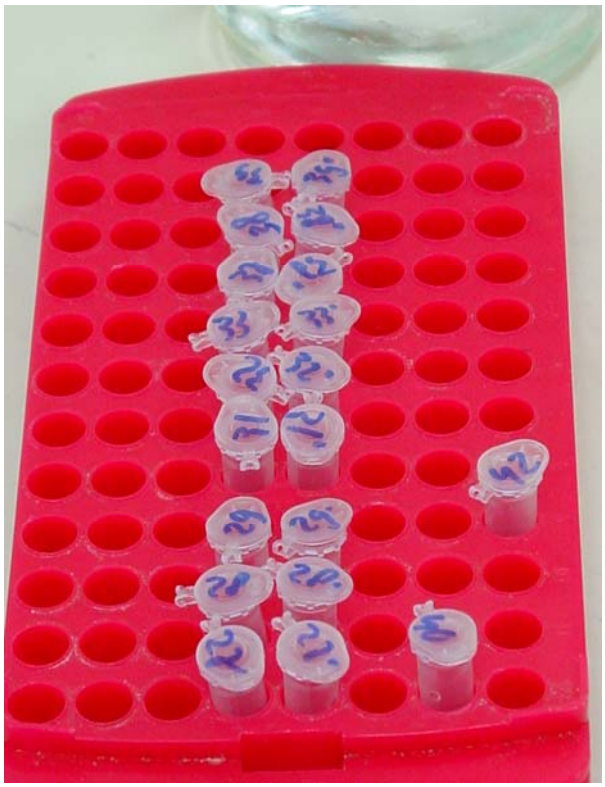

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Summary

In this thesis I described antioxidant responses of marine microalgae to excess irradiance including ultraviolet radiation (UVR). Marine microalgae, including phytoplankton, comprise an essential group of organisms: first of all they form the basis of open ocean food chains and secondly they are able to withdraw (anthropogenic) carbon dioxide from the atmosphere. Many studies have already described the effects of excess irradiance including ultraviolet radiation (UVR) on photosynthesis, judging from a wealth of *in situ* incubation studies showing inhibition of primary production in sunlit surface waters. In addition, incident PAR levels may induce excess irradiance responses for example by *de novo* synthesis of photoprotective pigments (xanthophylls). Ultraviolet radiation (280 – 400 nm) has been shown to substantially reduce primary production in most marine systems, with natural UVAR (315 – 400 nm) and UVBR (280-315 nm) often having similar relative proportions of inhibition. As a result of climate change phytoplankton irradiance exposure (intensity and spectral composition) may change significantly. For example, since stratification traps phytoplankton in the upper water layer, excess irradiance exposure (PAR, UVAR, UVBR) may increase in those systems where stratification becomes more intense. This applies to areas characterized by reduced average wind speeds or increased melt water input. In contrast, other regions (s.a. Antarctic Circumpolar Vortex) may become more deeply mixed by increased average wind speeds, thereby reducing irradiance intensities and exposure durations, but increasing irradiance dynamics.

During shallow as well as deep mixing conditions, phytoplankton cells have to deal with periods of excess irradiance conditions. During these exposure periods, the amount of absorbed energy cannot match the consumption of reduced equivalents hereby causing overreduction of the photosystems. Consequently, the flow of excited electrons through electron transport chains becomes hampered. This may lead to leakage of electrons onto oxygen, thereby producing reactive oxygen species (ROS), especially in the chloroplasts. ROS are able to damage essential biomolecules such as DNA, RNA, proteins, and membranous phospholipids which can cause viability loss. With respect to the polar regions, the springtime reduction of stratospheric ozone causes significant increases in (biologically active) UVBR. UVBR has direct detrimental effects on bioorganic molecules, thereby directly interfering with metabolic pathways and the process of photosynthesis. As a result, the presence UVBR stimulates the production of ROS. As photosynthetically active radiation fuels the electron transport chains with excited electrons, UV(B)R-mediated production of ROS is particularly dangerous in conjunction with (excess) PAR. As a consequence, (increased) UVBR can cause significant reduction of marine primary productivity. In contrast to UVBR, UVAR causes indirect damage to the cell; after absorbance by photosensitizing molecules, the energy can be transferred onto bioorganic molecules thereby initiating auto-oxidation of these compounds leading to function loss. Because phytoplankton productivity provides the basis of open ocean ecosystems and, moreover, a feedback for anthropogenic carbon emissions, it is essential to understand how stratification, mixing and ozone depletion affect phytoplankton performance. Excessive irradiance effects depend on photoacclimation, a process by which algae balance photosynthesis and photoprotection during irradiance changes. Because of genotypic constraints in photoacclimation potential, the irradiance climate in the upper mixed layer may regulate phytoplankton species composition and productivity. Finally, the nutritional value of a species is not only species-specific but is also affected by environmental (stress)

conditions. Thus, altered (excess) irradiance could have a significant impact on food availability for organisms at higher trophic levels.

As the detrimental effects of excess irradiance (including UV(B)R) are particularly mediated through the production of ROS, microalgae have developed several mechanisms to protect themselves against (generation of) those oxygen intermediates. This thesis especially focused on two of these mechanisms First, their ability to tune the energy flow towards the photosystems by quenching excessively absorbed light as heat via the xanthophyll cycle. And second, their ability to deploy their sophisticated network of enzymatic and non-enzymatic antioxidants to scavenge ROS at the site of their production. In this network, the enzyme superoxide dismutase (SOD) holds a key position as it is a unique component and prevents uncontrolled proliferation of ROS formation.

Chapter 1 described the global role of microalgae, and the state of knowledge with respect to effects of climate change on irradiance regimes, the consequences of enhanced UVBR related with stratospheric ozone reduction, and the molecular tools microalgae can deploy to cope with changing environmental conditions. In that respect, the role of the xanthophyll cycle had so far been documented rather well, although much controversy existed on the possible role of xanthophyll cycle activity in prevention of UVR damage. Microalgal antioxidant responses, on the other hand, had been studied poorly and information was scattered because of differences in experimental set-up. Information on antioxidant function in Antarctic microalgae was almost non-existing which was surprising given the enhanced UVBR levels observed in Antarctic regions, as well as other irradiance modifications related with climate change (changes in mixing depth and stratification). Thus, at the start of the here presented research project, virtually nothing was known about intraspecific (in response to excess irradiance, UVR, enhanced UVBR and dynamic irradiance exposure) and interspecific antioxidant responses in marine microalgae in general, and polar marine microalgae in particular. As a result, this thesis focussed both on intraspecific as well as interspecific excess irradiance responses. More specifically, the following research questions were addressed *in concreto*:

1) **What is the role of antioxidants and xanthophyll pigments in excess irradiance protection, during and after photoacclimation? (intraspecific responses, addressed in Chapters 3 and 4)**
2) **How are microalgal antioxidant responses related to habitat of origin, taxonomic origin, or cell size? (interspecific responses, addressed in Chapter 5)**
3) **How are antioxidant responses involved in the acclimation process of microalgae of different taxonomic origin in a fluctuating irradiance regime? (intra- and interspecific responses, addressed in Chapters 6 and 7)**

Virtually nothing was known about (inter- and intraspecific) SOD variability in Antarctic marine microalgae. This absence of information could be related with problems in obtaining sufficient quantities of biomass (including SOD) necessary for reproducible activity measurements in field samples. Moreover, earlier SOD assays had been developed using activity measurements at room temperature and it was not clear which method was

most suitable for modification to "polar" assay temperatures. In **chapter 2**, I compared and optimized existing protein extraction procedures and SOD activity assays to create a sensitive, easy-to-use and reliable method for Antarctic microalgal material. Different cell harvesting and disruption methods, several protein extraction and assay buffers, and three SOD activity assays were compared using the marine Antarctic diatom *Chaetoceros brevis* as test organism. Results showed that protein extraction was optimal at low sonication amplitudes after a few pulses, irrespective of the type of buffer used. Most protein was extracted in the presence of 0.1% Triton X-100. SOD activity was best quantified using the NBT/riboflavin method in combination with a buffer containing potassium phosphate and Triton X-100. Moreover, the NBT/riboflavin method was demonstrated to be the most reliable and sensitive method at low temperatures (5°C). It was concluded that optimization of SOD activity measurements was increased by using a Triton X-100 containing extraction buffer after which SOD activity was measured with the NBT/riboflavin method.

The modified SOD assay was used to investigate SOD as well as other antioxidative responses in the common Antarctic diatom *Chaetoceros brevis* during and after photoacclimation to new irradiance regimes **(Chapters 3 and 4:** intraspecific responses**)**. C**hapter 3** described responses of SOD activity, photosynthetic and xanthophyll pigments, PSII efficiency, cellular concentration of Thiobarbituric acid reactive substances (TBARS, indicator of lipid peroxidation caused by oxidative stress) and growth when high light acclimated *C. brevis* was transferred to a range of irradiance conditions, including ultraviolet radiation (UVR). The result showed that changing irradiance conditions significantly reduced growth rates of *C. brevis* regardless of the new irradiance condition. PSII efficiency decreased significantly during periodic excess irradiance (including UVR) and increased under low irradiance conditions. Acclimation of pigment composition was completed after two days irrespective of the new irradiance condition. At the same time SOD activity increased significantly after the first day regardless of the new irradiance environment but returned to pre-exposures values on the fourth day. Under enhanced irradiance conditions (including UVR) TBARS levels remained constant throughout the experiment. We concluded that SOD activity may be temporarily elevated in *C. brevis* after an irradiance shift, in order to facilitate a metabolic change which could be accompanied by increased ROS formation. Thus, an initial increase in SOD activity could allow acclimation to a new irradiance environment without getting significantly damaged.

The Southern Ocean is characterized by deep, wind driven vertical mixing and low environmental iron concentrations. Here, Antarctic microalgae are not solely exposed to occasional excess irradiance stress, but also to constantly changing irradiance conditions and low iron availability. Therefore, we investigated antioxidant en xanthophyll responses in *C. brevis* during fluctuating irradiance and iron deficiency. In **chapter 4**, iron limited and iron replete cultures of *C. brevis* were exposed to identical daily irradiance levels, supplied as dynamic and constant irradiance. After an acclimation period of two weeks, we assessed growth, PSII efficiency, pigment composition, and antioxidant activities which included SOD, ascorbate peroxidase (APX) and glutathione reductase (GR) activities, the latter two involved in H_2O_2 (product of SOD) scavenging. On the last experimental day, we then tested irradiance sensitivity by subjecting the cultures to excessive irradiance (including UVR) after which we determined xanthophyll cycle activity, PSII efficiency and viability loss. Results of this experimental series showed that iron limitation reduced growth rates, PSII efficiency dynamics and cellular pigment pools, implying that iron deficiency

significantly affected photosynthetic performance. When comparing dynamic and constant irradiance conditions, growth rates did not differ while at the same time, significant differences were found in pigment composition, PSII efficiency, and antioxidant activity (iron limited cells only). Apparently, *C. brevis* had photoacclimated efficiently to both conditions (constant and dynamic), thereby mitigating potential effects of irradiance limitation and photoinhibition. While iron replete cultures did not show altered antioxidant activities, iron limited cultures had increased their SOD and APX activity during dynamic irradiance conditions. This suggested that the combination of iron limitation and fluctuating irradiance stress increased radical formation around PSI which could be related with an inability to rapidly adjust PSII efficiency under iron limitation. Consequently, the electron transport chains would then be more subject to overreduction, increasing ROS production and thereby antioxidant requirement. PSII efficiency and xanthophyll de-epoxidation dynamics during the excessive irradiance test were not different for iron limited and replete cells and viability loss was not observed during excessive irradiance. In conclusion, chapter 4 showed that increased antioxidant activity could be required when cells are exposed to iron limitation during mixing, a rather natural situation for microalgae originating from the Southern Ocean like *C. brevis*.

Species-specific differences in antioxidant responses were so far hard to unravel, since earlier studies done differed widely in set up, exposure time, spectral composition, irradiance intensity, acclimation and growth condition, or species under consideration**.** So, to gain more insight in interspecific variability, we exposed fifteen identically cultivated marine microalgae (apart from the experimental temperature difference between polar and temperate algae) to simulated surface irradiance (SSI) for 3 h (**chapter 5)**. Of these microalgae, PSII efficiency (a general measure of photosensitivity), pigment composition and antioxidant capacity were determined before and after exposure, and compared with respect to habitat origin (polar temperate), cell size and taxonomic background (diatoms, flagellates). When acclimated to low irradiance, no correlation was found between (structural) antioxidant levels and taxonomic background. In contrast, SOD activity was positively correlated with cell size whereas APX activity was lower and glutathione redox status (GRS) was higher in Antarctic than in temperate species. The strong PSII reduction after SSI exposure was followed by recovery in all species but 4, who remained photosynthetically inactive for the duration of the experiments. In addition, SSI tolerance was neither related to geographic or taxonomic background nor to cell size. Yet, PSII recovery increased with decreasing SOD and GRS and increasing xanthophyll cycle activity. Strikingly, GRS correlated negatively and significantly with GR activity, DEPS of xanthophyll pigments and recovery of PSII efficiency. As a result we postulated that glutathione may act as a stress sensor and response regulator, inducing xanthophyll de-epoxidation. In conclusion, this chapter showed that although a high interspecific variability in antioxidant activities exists, the variability is not related with habitat origin, taxonomic background, or cell size.

In the previous chapters, laboratory set-ups were used to investigate excess irradiance responses including UVR. Yet, virtually nothing was known about antioxidant responses under ambient solar irradiance conditions, let alone under fluctuating irradiance regimes. The last two chapters described responses of temperate microalgae exposed to natural (irradiance) conditions in Patagonia (Argentina), since Patagonian waters are characterized by strong wind induced mixing as well as high irradiance levels. In **chapter 6**,

we first evaluated the effects of UVR on carbon fixation and algal community dynamics of natural phytoplankton assemblages from Patagonian coastal waters during the austral summer of 2006. To this end, we collected surface water samples at mid morning over a 7 week period and analyzed changes in phytoplankton species composition over the sampling period. The results showed that especially pico- and nanoplankton (<20 μm) dominated the assemblages. Within these assemblages, the different taxonomic groups were determined by both pigment fingerprinting which was analyzed with HPLC combined with the matrix factorization program CHEMTAX as well as with microscopic observations. Both methods showed variable proportions of small diatoms, chlorophytes and cyanobacteria throughout January-February 2006. Photosynthetic efficiency, as assessed through assimilation numbers under PAR only conditions, was high (between 4.4 and 10.4 μg C (μg Chl.*a*)$^{-1}$ h^{-1}), probably favored by the supply of inorganic nutrients from the nearby Chubut River. The short-term impact of UVR (measured as inhibition of radiocarbon incorporation) was assessed immediately after sampling by exposing samples to three irradiance treatments under a solar simulator: PAR, PAR+UVAR and PAR+UVAR+UVBR. These measurements indicated that solar simulated UVR-induced photoinhibition was related with taxonomic composition: in general, higher photoinhibition was observed when diatoms dominated whereas photoinhibition was lower when samples were dominated by chlorophytes. Although this implies that chlorophytes are less sensitieve to UVR, it does not rule out that irradiance history plays an important role as well. Additional analysis of xanthophyll cycle activity furthermore indicated that xanthophyll pigments provided only limited UVR protection in these already high-light acclimated assemblages.

As indicated in chapter 6, UVR sensitivity appears to be roughly related to taxonomic background; diatoms were more sensitive to artificial UVR than chlorophytes, Yet although diatoms showed higher photoinhibition, they did dominate the natural taxonomic composition under increased natural UVR conditions. Moreover, other studies have also shown that, in general, diatoms are more resistant (regarding their viability) to ambient UVR than nanophytoflagellates. To investigate whether antioxidants could explain these observations, we investigated microalgal antioxidant responses under the physical conditions prevailing in Patagonia. C**hapter 7** described outdoor experiments with a temperate diatom (*Thalassiosira weissflogii*) and a phytoflagellate (*Dunaliella tertiolecta*) exposed to ambient solar radiation including UVR under static and two artificial shallow mixing conditions. In these experiments we assessed immediate (1 day), short (3 days) and long term (7 days) antioxidant (SOD, APX, glutathione cycling) responses as well as growth, UVR sensitivity and pigmentation. Regardless of the mixing speed, UVR exposure as well as shallow mixing did not alter antioxidant levels. Yet, despite the lack of intraspecific variation, both species did show immediate antioxidant responses due to the experimental transitions from the pre-experimental indoor low irradiance conditions, to the outdoor ambient irradiance conditions. On a longer time scale, *D. tertiolecta* showed a short term increase in glutathione reductase activity, a permanent decrease in glutathione and a permanent increase in SOD. In *T. weissflogii*, on the other hand, antioxidant levels returned to their pre-experimental values after which they remained stable. Under pre-culturing conditions, carbon assimilation and growth rates were highest in *D. tertiolecta*. Yet, both parameters reduced in both species when transferred to the outside conditions, in particular in static UVR cultures, which simultaneously showed significantly enhanced UVR resistance. After a 7 day period, *D. tertiolecta* was affected most by the ambient treatments.

This is contradictory to observations of chapter 6 which could be related to differences in irradiance history or species specific, size related responses. Also, the protective over harvesting pigment ratios of *D. tertiolecta* were elevated while in *T. weissflogii* these ratios remained unchanged. Apparently, *T. weissflogii* was already better equipped for excess irradiance exposure than *D. tertiolecta*. As a result, this study revealed first of all that mixing does offer protection against UVR but also increases UVR vulnerability, independent of mixing speed. Secondly, we could confirm that diatoms would be less sensitive to natural UVR than phytoflagellates irrespective of irradiance dynamics. Finally, our results supported the general notion that diatoms prevail in highly irradiated stratified water, whereas phytoflagellates would prefer deeply mixed systems. Yet, the observed species specific differences could not be explained by differences in antioxidant activities or pigmentation.

Concluding remarks

Our research was performed to investigate antioxidant responses of marine microalgae to excess irradiance including UVR. Although methods were available for measuring non-enzymatic and enzymatic antioxidants, oxidative stress and viability, it was essential to first determine, adopt and optimize methods, to make them suitable for obtaining accurate and reproducible information with respect to marine polar microalgae. In this thesis we tested, compared and optimized extraction and analytical SOD procedures. Given the high variability in SOD data when comparing the different methods, standardization of SOD (and perhaps other antioxidant) assays is strongly recommended for studies addressing antioxidant responses of microalgae, in order to facilitate future comparison of results. By using standardized procedures throughout this entire thesis, we were able to compare all responses of our investigated species. From this, we are able to postulate that, overall, microalgal antioxidants are especially deployed as an immediate (hours) response to enhanced irradiance including UVR. Over prolonged acclimation periods (days to weeks), antioxidant levels tend to return to their initial, structural levels. Antioxidants seem to offer protection against irradiance changes which lead to an immediate generation of ROS. During this initial transition period, cells are then able to induce metabolic changes and install more permanent protection systems. Therefore, antioxidants seem to function as a first line of defense, protecting biomolecules and organelles against ROS by which they facilitate irradiance induced metabolic transitions. These transitions often lead to less efficient use of absorbed energy and therewith result in a reduced photosynthetic performance and growth rates. So, antioxidants may prevent severe damage or viability loss during photoacclimation to high irradiance.

From this thesis it became clear that microalgal antioxidant responses are highly species specific; not two single species responded similarly. This could be explained by the complex relationships between the components within the antioxidant network, the difference in contribution of the antioxidant components, the putative regulatory role of certain components (i.e. glutathione) or their products (i.e. H_2O_2) and their interaction with other protection mechanisms (i.e. xanthophyll cycle). For example, increased ROS production can lead to increased SOD activity by which the generation of H_2O_2 is increased as well. As a consequence, a cell could increase its APX activity, increase other peroxides,

reroute metabolic pathways by which more catalase activity and less APX activity is required, or increase its glutathione metabolism to scavenge ROS non-enzymatically. Besides that, a cell is also able to decrease ROS scavenging activity by which the increased concentration of oxygen intermediates could regulate antioxidant capacity (i.e. gene expression, mRNA translation, enzyme activity) in order to change existing antioxidant levels, initiate other ROS scavenging/prevention systems (i.e. xanthophylls cycle) or induce more permanent protection mechanisms (i.e. protective pigments, MAA's). Thus, antioxidants are clearly not individual, stand-alone components but they are embedded in a complex network which is carefully balanced between pro-oxidants and ROS constraining mechanisms. Because of this complexity, external upregulation of a single component does not automatically lead to improved protection against ROS. Secondly, because of this balance, the amount of antioxidative power is not linearly correlated with UVR sensitivity in an algal cell. For example, initial SOD activity seemed to be correlated with PSII recovery rate, yet in a negative way (chapter 5).

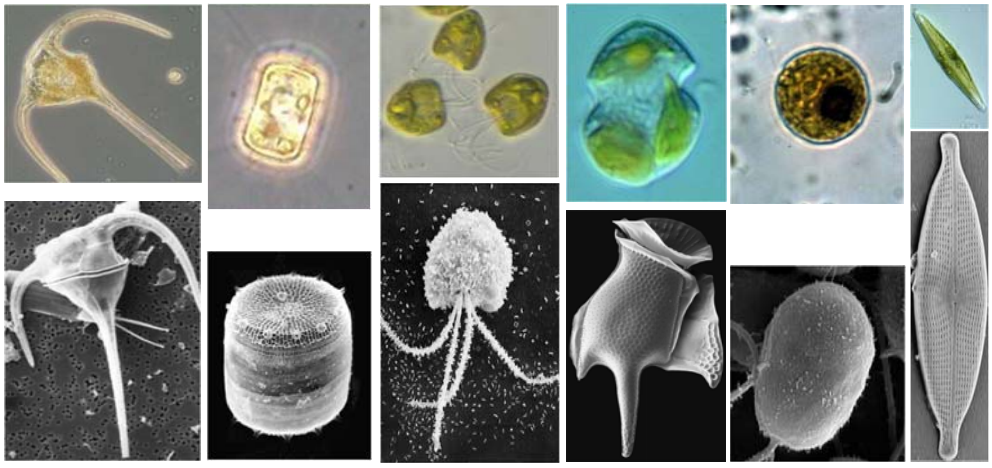
Although UVR vulnerability was found to differ between representative of taxonomic groups, antioxidant responses did not explain this phenomenon as they did not correspond with the irradiance induced differences in growth rates or reduced photosynthetic activity. This could be attributed to species specific responses to high irradiance including UVR, including antioxidants but also a broad spectrum of other mechanisms like induction of MAAs, enhanced D1 turnover use of low molecular weight ROS scavengers (i.e. α-tocopherol, ascorbate) or differences in metabolic changeovers. In conclusion, this thesis has provided evidence that antioxidants play an important role in the immediate response of microalgae against excess irradiance. Furthermore, a fluctuating irradiance regime or the presence of UVR does not induce particular antioxidant responses. Also, antioxidant responses do not explain differences in irradiance sensitivity, as we found that increased antioxidant levels did not lead to increased UVR- tolerance.

Fotobiologie voor **dummies**

Microalgen en hun antioxidant reacties op excessieve hoeveelheden licht en ultraviolette straling

Mariene microalgen

Bij het woord algen wordt al snel gedacht aan slijmerig en draderig spul op sloten of groene drab op vijvers. En dat is geheel terecht: op warme en zonnige dagen groeien algen als kool en kunnen ze een verstikkende deken op het wateroppervlak vormen. Echter, algen komen in veel meer verschijningsvormen en plaatsen voor. Vooral de oceanen en zeeën herbergen een keur aan soorten en maten. Zo zijn er grote zeewieren (macroalgen of kelpen) die dertig meter lang kunnen worden en als slappe bomen met het water meedeinen. Echter, de groep van zoutwater (mariene) algen bestaat voornamelijk uit eencellige soorten die ook wel microalgen of (fyto)plankton worden genoemd. Met het blote oog zijn ze onzichtbaar maar onder de microscoop kom je de meest uiteenlopende creaturen tegen (Fig. 1).



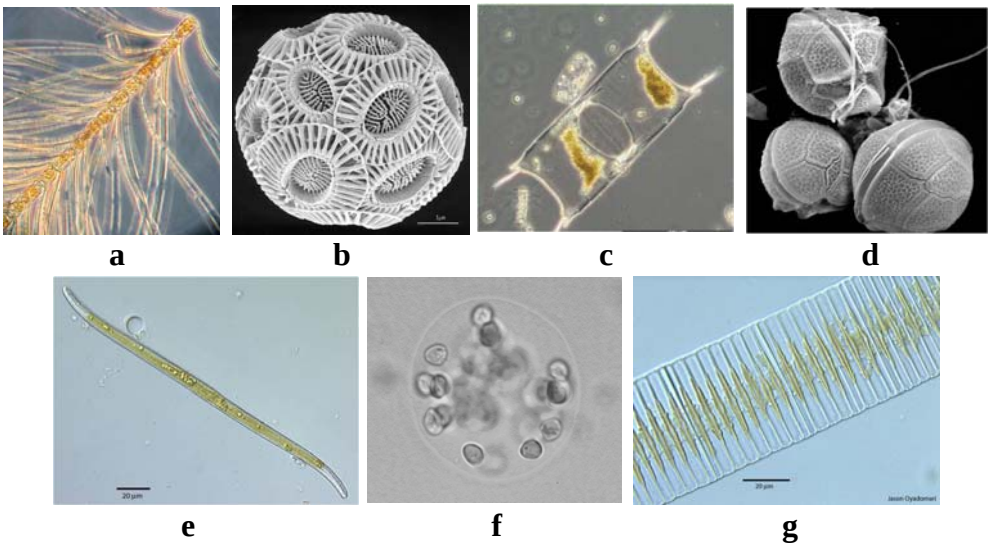
Figuur 1: Mariene microalgen komen in vele verschillende verschijningsvormen voor. **Boven**) eencellige algen gezien door een lichtmicroscoop (www.io-warnemuende.de, www.io-warnemuende.de, www.shigen.nig.ac.jp, http://starcentral.mbl.edu, www.answers.com, www.keweenawalgae.mtu.edu). **Onder**) zelfde algen als boven maar dan gezien m.b.v. elektronenmicroscopie (www.io-warnemuende.de, www.whoi.edu, www.aad.gov.au. www.botany.unimelb.edu.au, www.answers.com, www.lbm.go.jp).

Tot voor kort werd de rol van microalgen voor het leven op aarde danig onderschat en hun invloed op de mensheid werd als marginaal bestempeld. Dat kwam vooral doordat het leven van de microalg zich in een totaal andere omgeving afspeelt dan het onze. Onderzoeksinteresse ging dan ook grotendeels uit naar planten en dieren die veel dichter bij ons staan. Tegenwoordig wordt daar heel anders over gedacht en realiseert men zich dat microalgen invloed uitoefenen op en in vrijwel ieder ecosysteem, inclusief dat van de mens.

Net als planten gebruiken ook microalgen licht en CO_2 om te groeien en geven ze tegelijkertijd zuurstof af aan de omgeving. Er wordt geschat dat ongeveer 50% van de zuurstofproductie op aarde voor rekening komt van deze microalgen! De opgenomen CO_2 wordt gebruikt om allerlei essentiële biomoleculen te maken die nodig zijn om onder andere celwanden, DNA en eiwitten op te bouwen. Door hun vermogen om organische

bouwstenen te maken uit van licht en CO_2, vormen microalgen een uitstekende voedselbron voor het leven in zeeën en oceanen. Ze worden dan ook begraasd door vele mariene dieren variërend van minuscule koud-water kreeftjes (krill) tot grote tropische koralen. Daarnaast bezetten microalgen een onmisbare positie in de kringloop van zowel organische (zoals zwavel, koolstof, stikstof) als anorganische elementen (zoals ijzer, koper, mangaan). De combinatie van verschillende leefomgevingen, omzettingscapaciteiten en milieufactoren hebben er toe geleid dat de microalgen een zeer diverse groep vormt; tot nu toe zijn er tussen de 36.000 en 50.000 soorten beschreven en er wordt zelfs geschat dat het werkelijke aantal kan oplopen tot 10.000.000.

Door hun eigenschap om zonlicht als energiebron te gebruiken, kunnen microalgen feitelijk in alle zeeën en oceanen voorkomen; van de warme tropen tot aan de ijskoude poolgebieden. Mede hierdoor vormen ze de voedingsbasis van elk ecosysteem in de mariene omgeving. Om zich te beschermen tegen begrazing, hebben microalgen een scala aan structuren en strategieën ontwikkeld variërend van extracellulaire stekels, glazen hulzen, kalkschalen, houtbepantsering en langgerekte vormen tot samenlevingsverbanden in ketens en slijmbollen (Fig. 2).



Figuur 2: Beschermingsstrategieën van microalgen tegen begrazing. **a**) extracellulaire stekels (www.io-warnemuende.de). **b**) celwand van kalkschalen (www.biol.tsukuba.ac.jp). **c**) celwand van glas (www.io-warnemuende.de). **d**) bepantsering met cellulose (hout) platen (www.aad.gov.au). **e**) aannemen van een langgerekte vorm (www.keweenawalgae.mtu.edu). **f**) samenleven in een slijmbol (http://dissertations.ub.rug.nl). **g**) samenleven in ketenverband (www.keweenawalgae.mtu.edu).

Als een microalg ten prooi valt aan vraat of gewoon sterfte, wordt deze voor het overgrote deel verteerd door de grazers of afgebroken door bacteriën, waardoor zijn onderdelen weer terug worden gebracht in de biologische kringloop. Een klein deel blijft echter onverteerbaar voor bacteriën (zoals bijvoorbeeld de glazen huls of de kalkschaal met

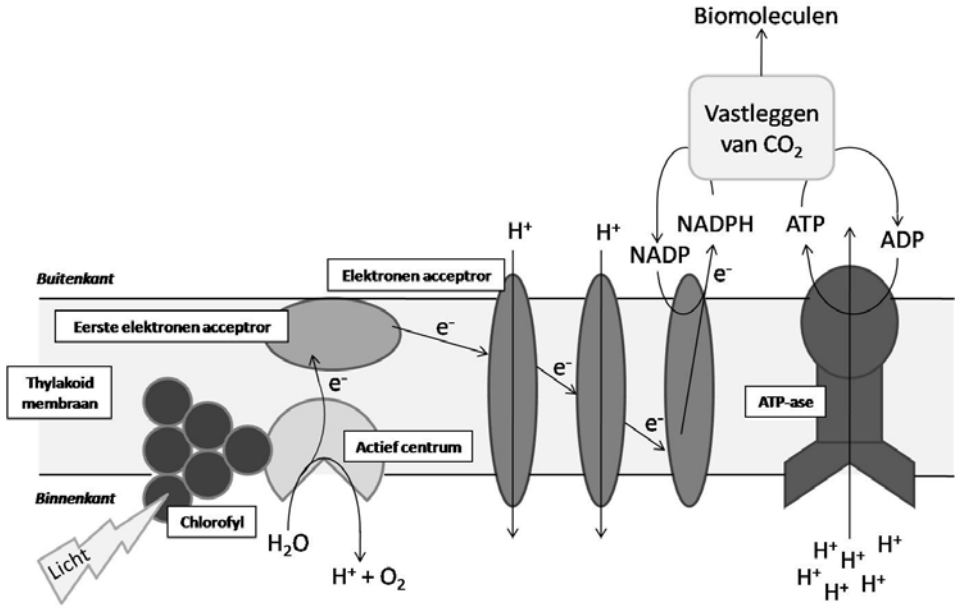
allerlei biomoleculen erin verwerkt) en zinkt naar de diepe en donkere zones van de oceaan waardoor het voor zeer lange tijd niet terugkeert in de biologische kringloop. Als bacteriën meer tijd en vooral zuurstof hadden, zouden ze ook de laatste onverteerde delen kunnen afbreken. Maar omdat de behuizing van veel algen relatief zwaar is (glas is bijvoorbeeld veel zwaarder dan water) zinken overblijfselen van microalgen zo snel de diepte in, dat deze verdwijnen voordat bacteriën er iets mee kunnen uitrichten. Deze overblijfselen kunnen dan voor duizenden jaren in deze dieptes blijven zitten zonder dat ze terugkeren in de biologische kringloop. Omdat microalgen CO_2 uit de atmosfeer hebben gebruikt om deze biomoleculen te bouwen, is het resultaat hiervan dus eigenlijk dat die CO_2 (in de vorm van biomoleculen) met de dode microalg wordt meegesleurd naar donkere, soms zuurstofloze dieptes. Hierdoor ligt er voor miljarden tonnen aan atmosferische CO_2 opgeslagen op de bodem van de oceanen. Omdat er met elke vastgelegd CO_2 molecuul een zuurstof molecuul wordt geproduceerd, is het gevolg van dit proces dat er netto meer zuurstof wordt geproduceerd dan verbruikt. Zodoende zijn mariene microalgen voor een groot deel verantwoordelijk voor de handhaving van de atmosferische CO_2 en het zuurstofniveau. En aangezien CO_2 een broeikasgas is, is dit proces erg belangrijk voor de warmteregulatie van de aarde. Microalgen vormen dus een onmisbare schakel in het tegengaan van het broeikaseffect.

Fotosynthese

Fotosynthese is het proces waarbij CO_2 (anorganische of 'dode' koolstof) uit de lucht wordt gebruikt om biomoleculen (organische of 'levende' koolstof) op te bouwen met behulp van lichtenergie. Dit gebeurt in de chloroplast (bladgroenkorrel) die is opgebouwd uit membraanzakjes, de thylakoiden. In de membranen van deze zakjes (de thylakoidmembranen) zit het fotosynthetisch apparaat dat er voor zorgt dat lichtenergie wordt omgezet in biologisch bruikbare energie (Fig. 3).

Tijdens fotosynthese wordt licht geabsorbeerd door pigmentmoleculen (chlorofyl) wat vervolgens naar eiwitten met de actieve centra van het fotosynthetisch apparaat wordt gesluisd. Hier wordt de energie gebruikt om watermoleculen te splitsen in zuurstof, die de cel uit diffundeert, en hoog energetische elektronen, die opgevangen worden door een eerste elektronenacceptor. Deze acceptor geeft de elektronen door aan een volgende acceptor en via een keten van aaneengesloten acceptormoleculen (de elektronen transportketen of ETK) belanden ze bij de laatste acceptor die ze overdraagt aan de terminale elektronenacceptor (NADP dat dan NADPH wordt). Tijdens de gang van elektronen door de ETK komt er per overdracht steeds een bepaalde hoeveelheid energie vrij die wordt gebruikt om protonen het thylakoidzakje binnen te pompen. Hierdoor ontstaat er een concentratieverschil in protonen tussen de binnen- en buitenkant van de thylakoid en is de lichtenergie in feite vastgelegd als een concentratiegradiënt over de thylakoidmembraan. De protonen kunnen dan via een ATP-ase (biologische dynamo), met hun concentratieverschil mee, weer terug naar buiten stromen waarbij ze de dynamo aandrijven. De energie die deze dynamo hierbij opwekt, wordt opgeslagen in bio-energetische moleculen (ATP). ATP reist vervolgens af naar de plaats waar nieuwe biomoleculen worden gebouwd om daar energie te leveren voor het vastleggen van CO_2. De eerder aangemaakte NADPH brengt hier zijn opgevangen elektronen naar toe om de

vastgelegde CO_2 verder om te zetten in bruikbare biomoleculen (zoals suikers). De lege ATP (=ADP) en NADPH (=NADP) gaan dan weer terug om nieuwe energie en elektronen op te halen zodat een volgende CO_2 vastgelegd kan worden om wederom een biomolecuul op te bouwen. Het is hierbij van essentieel belang dat zowel de ATP als NADPH constant geleegd worden. Gebeurt dit niet, dan loopt het protonengradiënt te hoog op waardoor er geen protonen meer het thylakoidzakje in kunnen en lopen de ETK's vast wat weer leidt tot hapering van de fotosynthesemachinerie met alle gevolgen van dien (verderop beschreven).



Figuur 3: Fotosynthese vindt plaats in het membraan van de thylakoidzakjes die zich in de chloroplast bevinden. Hierbij wordt lichtenergie geabsorbeerd door chlorofyl die het weer doorsluist naar het actieve centrum. Deze gebruikt de lichtenergie om water te splitsen in zuurstof (O_2) en elektronen (e^-). De vrijgekomen elektronen worden opgevangen door de eerste elektronenacceptor. Dit molecuul geeft ze weer aan een volgende elektronenacceptor die ze op zijn beurt ook weer doorgeeft aan de volgende elektronenacceptor. Aan het eind worden de elektronen opgevangen door de terminale elektronenacceptor NADP (en wordt dan NADPH) Tijdens het doorgeven van de elektronen via deze keten van elektronenacceptoren (ook wel elektronen transportketen genoemd), worden protonen van buiten naar binnen gepompt. Door het concentratieverschil willen ze weer terug naar buiten en dat kan alleen via APT-ase; een moleculaire dynamo. De dynamo wordt aangedreven door de stroom van protonen waarbij de ATP-ase bio-energetische moleculen produceert (ATP). De gemaakte NADPH en ATP worden vervolgens gebruikt om CO_2 vast te leggen waaruit weer biomoleculen kunnen worden opgebouwd. De lege NADP en ADP gaan dan terug naar de thylakoidmembraan om weer elektronen en energie op te vangen om een volgende CO_2 vast te leggen.

Het gevaar van ultraviolette straling

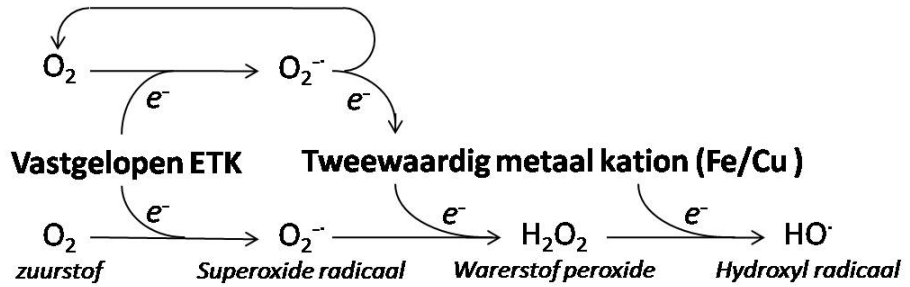
Omdat microalgen voor hun energiebehoefte afhankelijk zijn van zonlicht, zitten ze vaak dicht bij het wateroppervlak. Echter een excessieve hoeveelheid zonlicht kan schadelijk zijn voor de algencel. In zo'n situatie wordt er meer NADPH en ATP aangemaakt dan dat er

gebruikt wordt waardoor de volle moleculen zich ophopen en er gebrek ontstaat aan lege equivalenten. Aangezien de elektronen hierdoor niet meer kunnen worden overgedragen op NADP, resulteert dit in het vastlopen van de elektronenstroom in de ETK. In deze situatie biedt zuurstof zich aan als alternatieve elektronenacceptor waardoor de aanmaak van zuurstofradicalen of reactieve oxiradicalen (ROS, Fig. 4) wordt geïnitieerd. Deze agressieve zuurstofintermediairen hebben het vermogen om te reageren met essentiële biomoleculen als DNA, eiwitten en membraanlipiden (waaruit de thylakoid is opgebouwd) met als gevolg dat de levensvatbaarheid van de cel achteruit gaat tot de dood er zelfs op kan volgen.

Wanneer een microalg zich aan het wateroppervlak bevindt, wordt deze niet alleen blootgesteld aan fotosynthetisch bruikbare straling, maar ook aan biologisch gevaarlijke ultraviolette straling (UVR). Deze hoogenergetische straling heeft het vermogen om vitale biomoleculen te beschadigen waardoor UVR metabolische processen kan dwarsbomen, waaronder de werking van het fotosynthetische sleutelenzym Rubisco. Deze metabolische achteruitgang heeft tot gevolg dat NADPH en ATP minder gebruikt worden waardoor de ETK's volstromen met energie en elektronen en zuurstof zich weer aanbiedt als alternatieve elektronenacceptor. Dus naast directe schade aan belangrijke biomoleculen, stimuleert UVR ook extra aanmaak van ROS wat desastreuze gevolgen kan hebben voor de cel.

Reactieve zuurstoffen

Als een zuurstofmolecuul een elektron opneemt van de vastgelopen ETK, ontstaat er eerst het superoxide radicaal (Fig. 4). Deze reactie vindt vaak meerdere malen plaats waardoor er veel superoxide moleculen tegelijk ontstaan. Het superoxide kan dat extra elektron afgeven aan een tweewaardig metaal kation; een metaalion dat meerdere ladingen kan hebben waardoor deze makkelijk een elektron kunnen opnemen en afgeven zoals bijvoorbeeld ijzer (Fe^{2+}/Fe^{3+}) of koper (Cu^{+}/Cu^{2+}). Door deze eigenschap kunnen de tweewaardige mataal kationen niet alleen elektronen opnemen van superoxide radicalen (waardoor het radicaal weer een gewoon zuurstofmolecuul wordt), maar deze ook weer doorgeven aan een andere superoxide molecuul waardoor waterstofperoxide ontstaat.



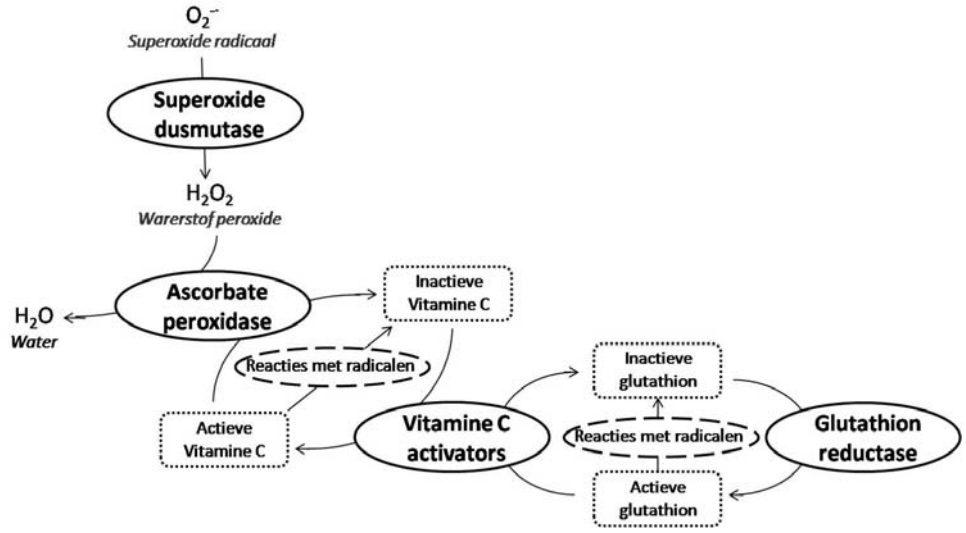
Figuur 4: Het ontstaan van zuurstofradicalen. Door bijvoorbeeld UVR-geïnduceerde schade aan belangrijke biomoleculen, kan het voorkomen dat de aanmaak van lege terminale elektronenacceptoren stil komt te liggen. Hierdoor lopen elektronen transportketens vast en kiezen de elektronen voor een alternatieve terminale acceptor: zuurstof. Dit leidt tot de vorming van het superoxide radicaal. Deze radicalen kunnen het opgenomen elektron afstaan aan een tweewaardig metaal kation (waardoor het weer zuurstof wordt, bovenste kringetje) dat het vervolgens doorgeeft aan een ander superoxide radicaal waardoor er waterstofperoxide ontstaat. Als ook deze nog een elektron ontvangt van een tweewaardige metaal kation, ontstaat er het zeer agressieve hydroxyl radicaal.

Superoxide en waterstofperoxide zijn gematigd reactief maar omdat het tweewaardige metaal kation elektronen blijft doorgeven en niet alleen aan superoxides, maar ook aan de nieuwgevormde waterstofperoxides, ontstaat er uiteindelijk het hydroxyl molecuul: het meest reactieve molecuul denkbaar in de biologie dat met alles reageert! Het doorgeven van elektronen met behulp van tweewaardige kationen wordt de Haber-Weiss/Fenton cyclus genoemd. Dit moet dus koste wat kost vermeden worden; om te overleven is het tenslotte van eminent belang dat de alg voorkomt dat het hydroxyl radicaal gevormd wordt.

Bescherming tegen ROS

Antioxidanten

Om zich te beschermen tegen de destructieve werking van reactief zuurstof, beschikken microalgen over een netwerk van antioxidanten (= anti-reactief zuurstof). Dit is een samenspel tussen enzymatische en niet-enzymatische radicaalruimers die er samen zorg voor dragen dat de reactieve zuurstoffen worden omgezet in water (Fig. 5). Omdat het hydroxyl radicaal zo snel en heftig reageert, kan deze niet afgevangen worden door antioxidanten. Daarom is het netwerk erop geënt om de precursors van het radicaal, superoxide en waterstofperoxide, weg te nemen voordat ze via de Haber-Weiss/Fenton cyclus het hydroxyl radicaal kunnen genereren.



Figuur 5: Samenhang van een aantal componenten in het antioxidant-netwerk. Het eerstgevormde radicaal, superoxide, kan door het enzym Superoxide dismutase omgezet worden in waterstofperoxide. Aangezien dit ook een reactieve zuurstof is, wordt deze verder omgezet tot water door het enzym Ascorbaat peroxidase. Voor deze reactie is echter wel actieve vitamine C nodig; een niet-enzymatisch antioxidant dat ook zelf radicalen onschadelijk kan maken door ermee te reageren. Het inactieve vitamine C kan weer omgezet worden in de bruikbare vorm door vitamine C activerende enzymen (activators). Deze activators hebben op hun beurt actieve glutathion nodig dat, net als vitamine C, ook een non enzymatisch antioxidant is en dus ook zelf met radicalen kan reageren. Vervolgens kan het inactieve glutathion weer worden omgezet in z'n actieve vorm door het enzym glutathion reductase.

Aan het begin van het enzymatische deel van het netwerk staat het enzym superoxide dismutase (SOD). Dit enzym is verantwoordelijk is voor de omzetting van het superoxide radicaal naar waterstofperoxide. Hierbij voorkomt het enzym niet alleen dat het superoxide de cel beschadigt, maar ook dat elektronen niet worden afgegeven aan de tweewaardige kationen. Het enzym houdt dus de vorming van het hydroxyl radicaal tegen. Omdat alleen superoxide dismutase deze reactie kan katalyseren, bezet dit enzym een sleutelpositie in het antioxidant-netwerk. Hoewel waterstofperoxide zelf ook een reactieve zuursof is, is deze net als superoxide relatief mild van aard. Bovendien zit er in de nabijheid van superoxide dismutase altijd een scala aan vervolgenzymen, zoals ascorbaat peroxidase (APX), dat waterstofperoxide verder afbreekt tot water. Naast de enzymatische antioxidanten heeft de cel ook beschikking over non-enzymatische antioxidanten, zoals het water oplosbare vitamine C (voor bescherming van onder andere biomoleculen in het cytosol), het vetoplosbare vitamine E (voor bescherming van onder andere thylakoidmembranen) en glutathion. Deze laatste kan net als vitamine C en vitamine E zelf radicalen onschadelijk maken, maar zorgt er vooral voor dat opgebruikte vitamine C en vitamine E weer geactiveerd kunnen worden. Daarmee wordt glutathion gezien als de brandstof van het antioxidant-netwerk. Glutathion reductase (GR), zet het opgebruikte glutathion weer om in zijn actieve vorm en is dus grotendeels verantwoordelijk voor de 'brandstof' toevoer.

Energie afvloeiende pigmenten

Een tweede mechanisme dat bescherming biedt tegen (excessief) lichtgeïnduceerde aanmaak van ROS, is het reguleren van de (licht)energietoevoer naar het fotosynthetisch apparaat. Microalgen zijn namelijk in staan om binnen een paar dagen de hoeveelheid lichtabsorberende pigmenten naar beneden te schroeven en lichtafwerende pigmenten bij te maken. Echter, omdat dit enkele dagen duurt, kunnen microalgen ook een tweede pigment regulatiemechanisme activeren dat onmiddellijk in stelling gebracht kan worden: de xanthophyl-cyclus, die het surplus van opgevangen lichtenergie als warmte kan laten afvloeien. Deze cyclus bestaat uit een aantal gespecialiseerde pigmentmoleculen (xanthophyllen) die enzymatisch kunnen schakelen tussen een energieabsorberende en energieaflatende structuur. Hierbij zorgt de aflatende structuur niet alleen voor minder absorptie, maar is het ook in staat om energie af te vloeien die door voornamelijk chlorofyl is opgevangen. In de groep van microalgen komen twee cycli voor: de diadino-diatoxanthine (DD/DT) cyclus en de viola- anthera- zeaxanthine (VAZ) cyclus. In geval van weinig licht staan de meeste xanthophyl moleculen in hun lichtabsorberende configuratie (diadino- of violaxanthine, afhankelijk van welke cyclus aanwezig is) maar mochten algen blootgesteld worden aan excessieve lichtintensiteiten, dan wordt het merendeel van de xanthophylpigmenten omgezet in diato- of zeaxanthine welke de energieaflatende eigenschappen bezitten.

De regulatie van de enzymactiviteiten lijkt gekoppeld te zijn aan het protonengradiënt dat opgebouwd wordt tijdens fotosynthese. Als CO_2, door bijvoorbeeld licht of UVR-geïnduceerde schade, niet snel genoeg wordt vastgelegd, worden NADPH en ATP niet snel genoeg geleegd waardoor de ETK's vastlopen maar het protonengradiënt tegelijkertijd wel stijgt. De ophoping van protonen aan de binnenkant van het thylakoidzakje is een teken voor de cel dat er meer energie binnenkomt dan dat er gebruikt kan worden en dat de absorptie van lichtenergie dus teruggeschroefd moet worden. Door

het protonengradiënt positief te koppelen aan de enzymactiviteit, worden de lichtabsorberende moleculen automatisch enzymatisch omgezet naar hun lichtaflatende structuur waardoor er minder energie naar het fotosynthetisch apparaat wordt gesluisd. Zodra de energiebalans is hervonden, worden ze weer terug omgezet naar hun energieabsorberende structuur.

Ecologische consequenties van een veranderend klimaat

Excessief licht

Veel studies hebben al aangetoond dat blootstelling van microalgen aan excessieve hoeveelheden licht leidt tot een daling van de productie van biomoleculen. Door onder andere de vorming van ROS raakt de cel (en het fotosynthetisch apparaat) zo beschadigd dat er minder energie verwerkt kan worden voor de opbouw van biomoleculen en er tegelijkertijd meer energie gestoken wordt in herstelwerkzaamheden. Hierdoor is blootstelling aan het volle zonlicht dus erg stressvol voor microalgen. De productieafname is vooral goed meetbaar bij microalgen die verblijven in gelaagd oppervlaktewater. Door instraling van de zon is de bovenste waterlaag warmer dan de waterlagen eronder, waardoor deze op de koude onderlaag blijft liggen en zich er geheel niet mee mengt ("stratificatie"). Omdat microalgen voor hun verplaatsing grotendeels afhankelijk zijn van de beweging van water, kunnen ze door de gelaagdheid dus niet in de diepere waterlagen geraken (waar ze ook minder last hebben van hoge lichtintensiteiten) en zitten zodoende gevangen in de oppervlaktelaag. Windenergie zorgt ervoor dat deze bovenste laag wel gemengd wordt waardoor microalgen zich niet constant in het volle zonlicht hoeven ophouden. Echter, het is afhankelijk van de windsnelheid tot hoe diep de menging is en dus tot in hoeverre de microalgen in gunstigere lichtomstandigheden terecht kan komen. Kortom, wind en watertemperatuur zijn belangrijke factoren die invloed uitoefenen op de groei van microalgen. Door klimaatverandering groeit de kans dat er meer stratificatie van zeeën en oceanen optreedt met name daar waar windsnelheden afnemen of waar veel smeltwater wordt ingebracht. In dit laatste geval kan het koude zoete smeltwater niet mengen met de lagen daaronder, waardoor microalgen worden blootgesteld aan excessieve lichtcondities.

UV straling en het Antarctisch ozongat

Ongeacht de plek op aarde, ultraviolette straling zorgt overal voor een substantiële daling van de productie van biomoleculen. Door de schade die het aanricht aan het fotosynthetisch apparaat en vele andere metabolische processen, kan UVR zelfs zorgen voor een complete groeistilstand van bepaalde soorten (door aantasting van het delingsproces). Als gevolg van de aantasting van de ozonlaag, neemt de hoeveelheid UV-straling jaarlijks schrikbarend toe (gemiddeld met 4%). Door de stijging van deze biologisch schadelijke straling, zou de groei van microalgen in zeeën en oceanen significant af kunnen nemen waarmee dus ook de brandstoftoevoer naar de voedselketens in gevaar zou komen.

De afbraak van de ozonlaag is het meest heftig boven Antarctica. Tijdens de Antarctische lente neemt de hoeveelheid ozon met ongeveer 50% af wat kan leiden tot een 130% toename van de intensiteit van UVR. En hier speelt ook nog een ander probleem. Door de lage hoek van de zon bereikt relatief weinig UV straling het aardoppervlak

(vergeleken met tropische gebieden waar de zon recht boven hangt) waardoor Antarctische microalgen, uit historisch perspectief, nooit gestimuleerd zijn om te investeren in beschermingsmechanismen tegen UVR. De kans is dus groot dat Antarctische microalgen, in tegenstelling tot tropische soorten, niet beschikken over een adequaat antwoord op de extreme UVR toename met alle gevolgen van dien.

Invloed van fluctuerende lichtcondities

Onderzoek naar de gevolgen van excessief licht en UVR zijn veelal gedaan onder statische condities; het is praktischer om een alg(enculture) stil te laten staan ten opzichte van de lichtbron dan om het te laten bewegen. Door deze experimentele opzet rapporteren veel onderzoeken dus resultaten als gevolg van blootstelling aan een continu gelijke hoeveelheid licht. Echter, onder natuurlijke omstandigheden is een microalg bijna altijd onderhevig aan fluctuerende lichtcondities; zelfs in de meest gestratificeerde situatie wordt een microalg nog altijd geconfronteerd met een steeds weer veranderend lichtklimaat. Deze lichtfluctuaties worden voornamelijk veroorzaakt door veranderingen in het binnenkomend daglicht (door dag-en-nachtcyclus of seizoensritme), de graad van bewolking en de verticale beweging van de algencel in de waterkolom. De laatste oorzaak kan voor zeer snelle intensiteitfluctuaties zorgen waardoor microalgen gedwongen worden om de hele tijd snel in te spelen op deze veranderende lichtsituaties welke kunnen variëren van excessieve to limiterende lichtcondities. Als eerder genoemd, is het op en neer bewegen door het water voornamelijk het gevolg van windgedreven menging van bovenste waterlaag. Door de klimaatverandering kunnen in bepaalde regionen de windsnelheden afnemen waardoor de mengdiepte minder wordt. Maar het tegenovergestelde kan ook het geval zijn waardoor een toegenomen windsnelheid de mengdiepte vergroot zoals het geval in de oceanen rondom Antarctica. Hierdoor nemen de gemiddelde lichtintensiteit en de duur van de blootstelling af, maar loopt de stress van het constant aanpassen aan fluctuerend licht ook hoog op. Het snelle aanpassen komt met een energieprijs die niet iedere soort zomaar kan ophoesten.

Als de bovenste waterlaag vrij ondiep is, kan het voorkomen dat een cel de hele tijd in een hoge lichtcondities blijft (hier wordt groei dus nooit beperkt door gebrek aan licht), ondanks dat de lichtcondities constant veranderen. Echter, doordat UVR sneller uitdooft in water dan fotosynthetisch bruikbaar licht, heeft de cel wel te maken met snelle veranderingen in het ultraviolette straling. De cel kan UVR-geïnduceerde schade repareren op het moment dat deze niet meer is blootgesteld aan UV-straling. De snelheid van reparatie hangt af van de soort en de hoeveelheid aangerichte schade die vooral in het begin van de blootstelling snel kan toenemen. Aangezien het UV-effect de resultante is van bruto UV-schade (die vooral in het begin van de blootstelling groot is) en reparatie op UV-loze dieptes (die langzaam op gang komt), hangt het uiteindelijk UV-effect erg af van de mengsnelheid; hoe sneller de menging, hoe korter er reparatie plaats kan vinden.

Reacties van antioxidanten en pigmenten

Pigmenten

Veel studies hebben aangetoond dat microalgen, blootgesteld aan excessieve lichtcondities, de hoeveelheid beschermende pigmenten kunnen laten toenemen ten opzichte van de

hoeveelheid absorberende pigmenten. Dit gaat vaak gepaard gaat met het activeren van de xanthophyl-cyclus. UVR daarentegen heeft nog geen (eenduidig) effect laten zien; de verhoudingen tussen de pigmenten alsook de activiteit van de xanthophyl-cyclus kan afnemen, gelijk blijven en zelfs toenemen tijdens blootstelling aan UVR. Een aantal studies heeft aangetoond dat microalgen die onderhevig zijn aan menging veelal eigenschappen aannemen van laag licht aangepaste microalgen; naar verhouding een toegenomen hoeveelheid absorberende pigmenten ten opzichte van beschermende pigmenten. Hierdoor lijkt het er op dat andere mechanismen dan pigmenten in werking moeten treden bij een tijdelijke verhoging van de lichtintensiteit zoals wellicht antioxidanten.

Antioxidanten

Tot recentelijk was er minimaal onderzoek verricht naar antioxidant-reacties van microalgen op excessieve lichtcondities (inclusief UVR). Daar komt nog eens bij dat er tijdens deze onderzoeken geen gelijke onderzoeksomstandigheden zijn gebruikt. Ze variëren zelfs zodanig in hun proefopzet (verschillende soorten, lichtintensiteiten, spectrale samenstellingen, duur van de blootstelling) dat er niet eens een consensus bereikt kon worden over hoe microalgen reageren op excessieve lichtcondities (inclusief UVR). De onderzoeken die waren uitgevoerd richtten zich vooral op het enzym SOD, waarschijnlijk omdat dit het sleutelenzym is in het antioxidant-netwerk. Hiernaast valt op dat er feitelijk niets gepubliceerd was over de specifieke reacties van Antarctische soorten. Aangezien deze algen van grote ecologisch waarde zijn, is dat vreemd en omdat deze regio het meest te maken heeft met verhoogde UV-straling, was er waarschijnlijk meer aan de hand dan gebrek aan onderzoeksinteresse.

De vragen van dit proefschrift

Zoals boven beschreven, bestond er geen eenduidige opvatting over antioxidant-reacties van microalgen op excessieve lichtcondities al dan niet in samenhang met UVR. Wel was bekend dat verschillende soorten verschillende reacties kunnen vertonen en dat ze na verloop van tijd zo zijn aangepast dat ze minder gevoelig zijn geworden voor het hoge lichtcondities. Gezien de prominente rol van microalgen in de ecologie van zeeën en oceanen, was het van belang meer inzicht en duidelijkheid te krijgen over hun reacties op de veranderende klimatologische omstandigheden. Daarom zijn we, in **hoofdstuk 1** van dit proefschrift, gekomen tot de volgende drie onderzoeksvragen:

1) **Wat is de rol van antioxidanten en xanthophylpigmenten bij de bescherming van microalgen tijdens en na hun aanpassing aan excessieve lichtcondities inclusief UVR?**
2) **In hoeverre zijn deze antioxidant-reacties gerelateerd aan de oorspronkelijke leefomgeving (historische achtergrond), taxonomische achtergrond of celgrootte van de microalg?**
3) **Hoe zijn antioxidanten betrokken bij de aanpassing van taxonomisch verschillende microalgen wanneer deze blootgesteld worden aan fluctuerende lichtcondities?**

De antwoorden van dit proefschrift

Ondanks dat superoxide dismutase (SOD) een sleutelpositie inneemt in het antioxidant netwerk, was er feitelijk niets over bekend voor wat betreft de ecologisch belangrijke Antarctische microalgen. Dit gebrek zou te maken kunnen hebben met problemen in het verkrijgen van genoeg biologisch materiaal om een reproduceerbare SOD-activiteitsmeting te kunnen verrichten in veldmonsters. Daarnaast waren eerdere SOD-meetprotocollen ontwikkelend voor metingen bij kamertemperatuur waarbij het onduidelijk was welke geschikt zou kunnen worden gemaakt voor metingen nabij het vriespunt. In **hoofdstuk 2** heb ik bestaande eiwitextractie- en SOD-meetprotocollen met elkaar vergeleken en geoptimaliseerd, gebruikmakend van de Antarctische diatomee *Chaetoceros brevis* als test organisme. Uit de resultaten bleek dat het meeste eiwit werd geëxtraheerd door algen bloot te stellen aan een paar korte ultrasone geluidspulsen bij een lage amplitude in samenhang met de toevoeging van 0.1% Triton X-100 aan de extractiebuffer. SOD activiteit was het beste meetbaar door gebruik te maken van de NBT/riboflavine methode, vooral ook omdat deze het meest betrouwbaar en gevoelig bleek bij lage temperaturen.

Het aangepaste SOD-meetprotocol werd in de volgende twee hoofdstukken 3 en 4, gebuikt om te onderzoeken hoe SOD van de Antarctische diatomee *Chaetoceros brevis* reageert tijdens en na z'n aanpassing aan nieuwe, veranderde, lichtcondities. **Hoofdstuk 3** beschrijft de reacties van hoog licht aangepaste *C. brevis* op zijn overplaatsing naar een variëteit aan andere lichtcondities inclusief UVR. Naast de respons van SOD-activiteit, werd ook gekeken naar de respons van de absorberende en beschermende pigmenten, de xanthophyl-cyclusactiviteit, efficiëntie van energie doorsluizing, de concentratie beschadigde lipiden (veroorzaakt door ROS) en groei. Uit dit onderzoek bleek dat een veranderde lichtconditie leidt tot een significante afname van de groei, ongeacht de nieuwe lichtomstandigheid. Onder lichtlimiterende condities nam de efficiëntie in energie doorsluizing toe terwijl deze juist afnam wanneer *C. brevis* werd blootgesteld aan tijdelijk verhoogde lichtcondities (inclusief UVR). Ongeacht de nieuwe lichtomstandigheid duurde het twee dagen vooraleer de nieuwe pigmentsamenstelling ingesteld was. In de tussentijd schoot de SOD activiteit onder alle omstandigheden op de eerste dag omhoog waarna het vier dagen later weer was teruggezakt naar de oorspronkelijke waarde. Tijdens het gehele onderzoek leidde een verhoging van de lichtcondities (inclusief UVR) niet tot een verandering in de hoeveelheid beschadigde lipiden. Hieruit kon geconcludeerd worden dat na verandering van de lichtcondities de SOD-activiteit voor een tijdelijke periode wordt verhoogd om een metabolische aanpassing mogelijk te maken die gepaard zou kunnen gaan met een verhoogde aanmaak van ROS. Kortom, verhoging van de SOD-activiteit zou de metabolische aanpassing aan de nieuwe lichtconditie mogelijk kunnen maken zonder dat *C. brevis* noemenswaardige schade oploopt.

Twee belangrijke kenmerken van de Zuidelijke Oceaan zijn de diepe windgedreven menging en de opmerkelijk lage concentratie ijzer, benodigd voor de elektronenacceptoren in de ETK. Hierdoor zijn Antarctische microalgen blootgesteld aan steeds weer veranderende lichtcondities waarbij ze tegelijkertijd lijden aan chronisch ijzertekort. Om inzicht te krijgen hoe Antarctische microalgen zich hierop aanpassen, hebben we de antioxidant- en xanthophylreacties van *C. brevis* op deze omstandigheden onderzocht. Om een goede vergelijking te kunnen maken, hebben we in **hoofdstuk 4** ijzerrijke en ijzergelimiteerde cultures blootgesteld aan fluctuerende en constante lichtcondities waarbij

de dagelijkse hoeveelheid ontvangen licht voor beide cultures gelijk was. Na twee weken onderhevig te zijn geweest aan deze omstandigheden, hebben we de groei, efficiëntie in energie doorsluizing, pigmentsamenstelling en de antioxidant-activiteiten van zowel SOD als ascorbaat peroxidase (APX) als glutathion reductase (GR) bepaald. Daarnaast hebben we op de laatste dag getest hoe gevoelig de algen waren voor excessieve lichtcondities waarbij we tegelijkertijd de xanthophyl-cyclusactiviteit hebben bepaald. Uit al deze metingen bleek dat ijzerlimitatie zorgde voor een afname in de hoeveelheid pigmenten (ongeacht de eigenschap van het pigment), een afname in de groeisnelheid en gebrek aan dynamiek in de efficiëntie van energie doorsluizing (bij de fluctuerende lichtcondities). Het komt er dus op neer dat ijzergebrek zorgt voor een gebrekkige werking van het fotosynthetische apparaat. Wat verder opviel was dat de groeisnelheden tussen de fluctuerende en constante lichtcondities niet verschilden terwijl er wel significante verschillen waren gevonden in pigment samenstelling, efficiëntie in energie doorsluizing en antioxidant activiteiten (alleen bij ijzergelimiteerde cultures). Klaarblijkelijk heeft *C. brevis* zich efficiënt (maar verschillend) aangepast aan beide lichtcondities waarbij de nadelige effecten goed opgevangen worden en niet zichtbaar zijn in hun groei. Tijdens dit onderzoek liet alleen de ijzergelimiteerde cultures die onderhevig waren aan fluctuerende lichtcondities een verhoging zien van hun SOD- en APX-activiteiten wat suggereert dat alleen de combinatie van beide stressfactoren aanleiding geeft tot een verhoogde ROS-productie. Dit zou te maken kunnen hebben met het onvermogen van de ijzergelimiteerde algen om snel hun efficiëntie in energie doorsluizing bij te stellen wat nodig zou zijn om de verminderde hoeveelheid werkzame elektronenacceptoren zijn (door het ijzertekort) te ontlasten. Dit zou kunnen resulteren in een verhoogde doorsluizing van energie naar het fotosyntheseapparaat tijdens periodes van excessieve lichtcondities, waardoor deze overbelast raakt wat leidt tot een toename van de ROS-productie. Tijdens de licht gevoeligheidstest op de laatste dag waren er geen verschillen gevonden tussen ijzerrijke en ijzergelimiteerde cultures. Kortom, uit dit hoofdstuk kan geconcludeerd worden dat verhoogde antioxidant activiteit nodig is tijdens fluctuerend licht en ijzerlimiterende omstandigheden; een vrij natuurlijke situatie voor microalgen uit de Zuidelijke Oceaan zoals *C. brevis*.

Omdat voorgaande onderzoeken naar antioxidant activiteiten zoveel variatie bevatten, was het tot op heden moeilijk om deze met elkaar te vergelijken om soortspecifieke verschillen vast te stellen. Om toch meer inzicht te krijgen in de variabiliteit tussen soorten, hebben we 15 verschillende soorten microalgen op identieke wijze voorgekweekt bij limiterende lichtomstandigheden en ze vervolgens voor drie uur blootgesteld aan zeer excessieve lichtcondities (inclusief UVR). **Hoofdstuk 5** beschrijft hoe we van al deze soorten de efficiëntie in energie doorsluizing (als algemene maat voor de lichtgevoeligheid), pigmentsamenstelling en antioxidant-activiteiten hebben vastgesteld, voor en na de blootstellingperiode. Hierbij hebben gekeken naar soortspecifieke verschillen als ook of de gevonden variatie gerelateerd was aan de oorspronkelijke leefomgeving (polen, gematigde gebieden), celgrootte en taxonomische achtergrond (diatomee, flagellaat). Na maanden van voorkweken bij lichtlimiterende omstandigheden was er geen enkele correlatie tussen de hoeveelheid antioxidanten en hun taxonomische achtergrond. Daarentegen werd het wel duidelijk SOD-activiteit toeneemt naarmate de celgrootte ook toeneemt, en dat APX-activiteit lager en glutathion redox status (GRS; hoeveelheid actief t.o.v. de totale hoeveelheid glutathion) hoger is in Antarctische dan in gematigde soorten.

Bij elke soort was een bepaalde mate van ineenstorting van de efficiëntie in energie doorsluizing waar te nemen ofschoon de gevoeligheid voor de extreem excessieve lichtcondities aan geen enkele achtergrond of celgrootte was gerelateerd. Hierbij viel echter wel op dat het herstel van de efficiëntie in energie doorsluizing toenam naarmate de activiteit in SOD en GRS afnam en de xanthophyl-cyclusactiviteit toenam. Ook was het verrassend te zien dat naarmate de GRS toenam, de GR- en xanthophyl-cyclusactiviteit, en de efficiëntie in energie doorsluizing afnam. Deze resultaten suggereren dat glutathion zou kunnen werken als sensor die tegelijkertijd bepaalde systemen reguleert, zoals de xanthophyl-cyclus- en GR-activiteit, dit alles om de schadelijke effecten van ROS-formatie tegen te gaan. Kortom, dit hoofdstuk toont aan dat er een grote soortspecifieke variabiliteit bestaat in antioxidant-activiteit en dat deze, overall, niet gerelateerd zijn aan de oorspronkelijke leefomgeving, celgrootte of taxonomische achtergrond. Wel lijkt de mate van werking van bepaalde antioxidanten gerelateerd te zijn aan het vermogen om te herstellen.

In de voorgaande hoofdstukken werden er laboratoriumexperimenten uitgevoerd om antioxidant activiteiten te onderzoeken. De laatste twee hoofdstukken gaan over de reacties van gematigde microalgen die blootgesteld zijn aan constante en fluctuerende natuurlijke lichtcondities in Patagonië (Argentinië). De kustwateren van Patagonië worden gekenmerkt door zowel een sterke windgedreven menging van het de bovenste waterlaag als een zeer hoge zonnestraling. Om te onderzoeken wat voor effect deze omstandigheden hebben op de UV-gevoeligheid van de natuurlijk voorkomende microalgen, hebben we in de zuidelijke zomer van 2006 natuurlijke planktongemeenschappen uit de Patagoonse kustwateren verzameld en onderzocht op hun reacties op kunstmatige UV-straling. Hiervoor beschrijven we in **hoofdstuk 6** het effect van kunstmatige UV-straling op het vastleggen van CO_2 en wat de invloed hierbij is van de soortensamenstelling van de natuurlijk algengemeenschap. Ook werd er gekeken hoe de dynamiek was van de microalgensamenstelling over een periode van 7 zomerse weken. Tijdens deze periode werden er in de loop van de ochtend monsters genomen van het water aan de oppervlakte, ongeveer honderd meter uit de kust, en hieruit bleek dat de samenstelling vooral overheerst werd door hele kleine soorten microalgen (<20µm). In deze gemeenschappen werden de verschillende taxonomische groepen bepaald door hun pigment 'vingerafdruk' (elke groep heeft z'n eigen pigment profiel) dat gesplitst en geanalyseerd werd met behulp van het computerprogramma CHEMTAX. Daarnaast werd de soortensamenstelling ook vastgesteld met behulp van de microscoop. Beide methodes lieten soortgelijke resultaten zien die uitwezen dat de relatieve hoeveelheid kleine diatomeeën, chlorophyten en cyanobacteriën varieerde gedurende januari en februari 2006. Om vast te stellen hoeveel koolstof (aangeboden als een opgeloste CO_2 variant) de gemeenschappen vast konden leggen, werden deze blootgesteld aan een niet limiterende hoeveelheid fotosynthetisch bruikbaar licht. Hieruit bleek dat de gemeenschappen relatief grote hoeveelheden koolstof wisten vast te leggen. De algen verkeerden dus in een goede conditie wat waarschijnlijk kwam door de toevoer van nutriënten uit de nabij gelegen Chubut rivier. Na de monstername werd meteen de gevoeligheid voor kunstmatige UV-straling vastgesteld. Hiervoor werden de algenmonsters blootgesteld aan verschillende lichtcondities uit de zonnesimulator: fotosynthetisch bruikbaar licht met UVR en zonder UVR. Uit deze metingen bleek dat, in aanwezigheid van UVR, de gemeenschap die gedomineerd werd door diatomeeën, minder koolstof vast konden leggen dan gemeenschappen gedomineerd door chlorophyten. In combinatie met de

pigment gegevens bleek dat de xanthophylpigmenten maar heel weinig bescherming boden tegen UV-straling, waarschijnlijk omdat de algen al volledig waren aangepast aan hoog licht. Bovendien lijkt het erop dat er naast xanthophylpigmenten nog andere systemen actief waren als bescherming tegen UVR. Kortom, vooral de algencompositie bleek gerelateerd aan de mate van UV-gevoeligheid en gaf een mogelijke verklaring voor de geobserveerde variatie.

Alhoewel het voorgaande hoofdstuk suggereerde dat diatomeeën gevoeliger zijn voor UVR, voert deze groep wel vaak de boventoon in de natuurlijke algengemeenschappen. In schijnbare tegenspraak hiermee laten andere onderzoeken zien dat diatomeeën, in het algemeen, beter bestendig zijn tegen natuurlijke UVR dan fytoflagellaten (waar de chlorophyten uit hoofdstuk 6 ook onder vallen). Om te zien of deze taxonomische variaties toe te schrijven zijn aan verschillen in antioxidant activiteit, hebben we twee soorten microalgen blootgesteld aan Patagonische lichtomstandigheden. In **hoofdstuk 7** wordt beschreven hoe we buitenexperimenten hebben gedaan met de gematigde diatomee *Thalassiosira weissflogii* en de fytoflagellaat *Dunaliella tertiolecta*. Hierbij zijn de twee soorten voorgekweekt bij zeer milde laboratoriumcondities (zonder UVR) waarna ze zijn blootgesteld aan natuurlijk zonlicht met UVR en zonder UVR (geblokkeerd door een UV-filter) waarbij beide omstandigheden werden aangeboden als constante en fluctuerende lichtcondities. De fluctuerende lichtcondities werden gecreëerd door microalgencultures in een troebele waterbak op en neer te bewegen waardoor er een ondiep mengklimaat werd nagebootst. Tijdens de zeven dagen durende experimenten hebben we gekeken naar de onmiddellijke reacties (tijdens de eerste buitendag), korte termijn reacties (gedurende de drie daaropvolgende buitendagen) en lange termijn reacties (gedurende de laatste 4 buitendagen). Hierbij zijn antioxidant (SOD, APX, glutathion-cyclus) activiteiten, groei, UV-gevoeligheid en pigmentsamenstelling doorgemeten. Uit de resultaten kwam naar voren dat UV-straling noch fluctuerende lichtcondities (ongeacht de mengsnelheid) effect had op de antioxidant-activiteiten. Hoewel er geen variatie was tussen de verschillende lichtcondities binnen de soort, lieten beide soorten wel een onmiddellijke antioxidant-reacties zien tijdens de overgang van milde laboratoriumcondities naar de extremere buitenomstandigheden. Op korte termijn liet *D. tertiolecta* een korte termijn stijging van de GR-activiteit, permanente daling van de hoeveelheid glutathion en een permanente stijging van SOD-activiteit zien. Bij *T. weissflogii* keerden de antioxidant-waarden na de eerste dag meteen weer terug naar hun oorspronkelijke laboratoriumwaardes waarop ze de gehele experimentele periode bleven staan. Onder de laboratoriumcondities groeide *D. tertiolecta* het snelst en legde deze ook de meeste koolstof vast. Echter, eenmaal naar buiten gebracht, namen beide parameters in beide soorten af, en dan vooral in de cultures die waren blootgesteld aan constant licht met UVR. Tegelijkertijd nam de UV-bestendigheid in deze cultures toe. Na de periode van zeven dagen was *D. tertiolecta* het meest gestrest door de buitenomstandigheden. Dit is tegenstrijdig met de resultaten van hoofdstuk 6, echter, dit zou te maken kunnen hebben met het verschil in lichtgeschiedenis (die bepaalt in hoeverre je al gewapend bent voordat je wordt blootgesteld aan UVR), soortspecifieke verschillen of gedrag gerelateerd aan celgrootte. Daarnaast viel op dat de hoeveelheid beschermende pigmenten ten opzichte van de lichtabsorberende pigmenten toenam in *D. tertiolecta* terwijl deze in *T. weissflogii* gelijk bleven. Klaarblijkelijk was *T. weissflogii* van tevoren beter uitgerust tegen het excessieve buitenlicht (inclusief UVR) dan *D. tertiolecta*. Kortom, dit hoofdstuk laat zien dat menging bescherming biedt tegen UV-

straling maar dat daardoor ook de UV-gevoeligheid toeneemt, ongeacht de mengsnelheid. Daarnaast konden we bevestigen wat eerdere onderzoeken ook al hebben aangetoond, namelijk dat diatomeeën minder gevoelig zijn voor natuurlijke UV-straling dan fytoflagellaten. Tot slot ondersteunden onze resultaten de eerdere bevindingen dat diatomeeën goed kunnen gedijen in ondiep gelaagd water met hoge lichtintensiteiten terwijl fytoflagellaten juist voorkeur hebben voor diep gemengd water. Echter, deze soortspecifieke variaties kunnen niet verklaard worden door verschillen in activiteiten van antioxidanten of door pigmentsamenstelling.

De lering van dit proefschrift

Voordat we antioxidanten konden gaan meten was het van essentieel belang om eerste een bruikbaar en reproduceerbaar meetprotocol op te stellen voor het bepalen van de SOD-activiteit. Per slot van rekening is dat het sleutelenzym in het antioxidant-netwerk en is het dus belangrijk om over zeker deze schakel betrouwbare informatie te verkrijgen, vooral als je kijkt naar de microalgen uit de poolgebieden. Gezien de hoge mate van variabiliteit in de bekende SOD-data die mede veroorzaakt worden door het gebruik van verschillende methodes, pleiten wij voor een standaardisatie van het te gebruiken SOD-meetprotocol. Hierdoor zou je een veel eenduidiger antwoord kunnen krijgen op antioxidant activiteiten van microalgen als reaktie op veranderende lichtomstandigheden. Omdat we deze standaardisatie tijdens het onderzoek van dit proefschrift in praktijk hebben gebracht, waren we in staat om alle reacties van alle onderzochte soorten met elkaar te vergelijken. Hierdoor kunnen we stellen dat, overall gezien, microalgen hun antioxidanten vooral aanwenden als onmiddellijke reactie op verandering van de lichtomstandigheden, UVR meegerekend. Na verloop van tijd (dagen tot weken) keren de antioxidantwaardes weer terug naar hun oorspronkelijke, structureel aanwezige capaciteit. Antioxidanten lijken dus microalgen bescherming te bieden tegen lichtveranderingen die leiden tot onmiddellijke aanmaak van ROS. Tijdens deze overgangsperiode kunnen de cellen dan de benodigde metabolische veranderingen doorvoeren en meer permanente beschermingssystemen in werking stellen. Kortom, antioxidanten lijken een eerste verdedigingslinie die de cel moet beschermen tegen ROS waarmee ze de cel helpen bij de lichtgeïnduceerde overgang van hun metabolisme. Zo voorkomen antioxidanten dat de microalg ernstige of zelfs dodelijke schade oploopt tijdens z'n aanpassing aan de hogere lichtcondities.

Uit dit proefschrift blijkt dat de antioxidant-activiteiten van microalgen in hoge mate soortspecifiek zijn; geen twee algen vertoonde een soortgelijke respons. Dit zou verklaard kunnen worden door 1) de ingewikkelde verhoudingen tussen de verschillende componenten van het antioxidant-netwerk, 2) het verschil in de bijdrage van de verscheidene componenten, 3) de vermeende regulatorrol van bepaalde componenten zoals glutathion en 4) hun interactie met andere beschermingmechanismen zoals de xanthophyl-cyclus. Zo zie je bijvoorbeeld dat een toename van ROS-productie kan leiden tot een verhoogde SOD-activiteit waardoor de hoeveelheid waterstofperoxide ook toeneemt. Als gevolg hiervan kan een cel z'n APX-activiteit omhoog schroeven of de activiteit van ander peroxides (allemaal gespecialiseerd in het afbreken van waterstofperoxide) verhogen. Ook kunnen hele metabole routes worden verlegd zodat er alleen op plaatsen met een hoge antioxidant capaciteit (vooral in de vorm van het enzym catalase) ROS wordt geproduceerd

waardoor er dus minder APX nodig is, of waardoor de cel z'n glutathionmetabolisme verhoogt om ROS niet-enzymatisch op te ruimen. Daarnaast kan een cel zelf z'n gehele antioxidant activiteit tijdelijk verlagen waardoor de toegenomen concentratie ROS de antioxidant-capaciteit kan regelen. Deze zuurstofintermediairen zijn namelijk in staat gen expressie, RNA transcriptie, translatie en modificatie alsook een scala aan enzymactiviteiten te beïnvloeden. Dit verandert vervolgens de bestaande capaciteit van antioxidanten of wordt gebruikt om andere, meer permanente mechanismen, in stelling te brengen zoals de aanmaak van meer xanthophylpigmenten. Antioxidanten zijn dus niet een verzameling losse componenten maar liggen ingebed in een ingewikkeld netwerk dat zorgvuldig de balans bewaakt tussen pro-oxidanten (ROS) en ROS-inperkende mechanismen. Het extern verhogen van een bepaalde component (door van buitenaf extra glutathion toe te dienen of door SOD transcriptie te stimuleren) zal dus niet automatisch leiden tot een betere bescherming tegen ROS. Sterker nog, het zou zelfs eerder de balans kunnen verstoren en daarmee de cel gevoeliger maken voor ROS-productie.

Alhoewel de UV-gevoeligheid varieerde tussen de verschillende representanten van taxonomische groepen, konden antioxidanten dit verschil niet verklaren aangezien de antioxidant-activiteiten geenszins correleerden met de lichtgeïnduceerde verschillen in groei of fotosynthetische activiteit. Dit zou kunnen komen door de soortspecifieke reacties van microalgen op de verhoogde lichtcondities en UV-straling. De verschillen hebben we kunnen aantonen in de antioxidant- en pigmentactiviteiten maar deze zouden ook kunnen liggen in een scala aan ander mechanismes zoals de aanmaak van UV-werende moleculen, het in werking stellen van herstelmechanismen, de aanmaak van andere niet-enzymatische antioxidanten (zoals vitamine C en E) of zelfs in verschil in metabolische aanpassing.

Uiteindelijk kunnen we uit dit proefschrift concluderen dat antioxidanten een belangrijke rol spelen bij de onmiddellijke reactie van microalgen op excessieve lichtcondities inclusief UVR. Veder kan er geconcludeerd worden dat noch fluctuerende lichtcondities noch de aanwezigheid van UVR leidt tot specifieke antioxidant-reacties. Daarnaast geven antioxidant-reacties ook geen verklaring voor het feit dat de ene soort gevoeliger is voor lichtveranderingen dan de andere. Immers, een toegenomen antioxidant-capaciteit leidde niet tot een verhoogde UV-bestendigheid

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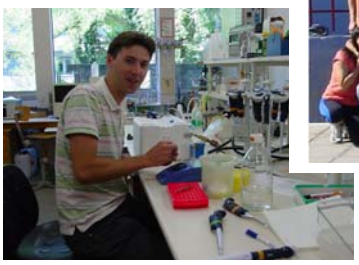
Een woord van **dank**





Het overkomt iedereen en vaak wel een paar keer per dag. En zo overkwam het mij ook ergens in de lente van 2002: het gevoel dat je naar de wc moet. Op zich niets bijzonders, zou je denken maar op die bewuste dag struinde ik visieloos door de TL-verlichte gangen van het Biologisch Centrum op zoek naar de dichtstbijzijnde wc. Geheel toevallig eindigde ik zo bij de afdeling van mariene biologie waar ik normaal gesproken nooit kwam. De wc bleek echter bezet en zodoende wierp ik een verveelde blik op de naastgelegen aanplakmuur om te zien of er nog wat interessants te lezen was. Ah, een advertentie voor een AIO-positie… Tot mijn grote verbazing las deze vacature alsof ik in een spiegel keek. De baan combineerde laboratoriumwerk met avontuur en microbiologie met fotosynthese. Mijn visieloze toestand was gelijk opgeheven: dat is wie ik ben, dit is wat ik wil.

Mariene biologie, de tak van wetenschap die onderzoek doet naar leven in zeeën en oceanen; een immens werkterrein dat zich uitspreidt langs de zeven continenten. Het meest onbekende werelddeel is Antarctica en daar begint dan ook het eerste half jaar van mijn aio-periode. Dit was een zeer heftige en geweldige ervaring die vooral is vormgegeven door de mensen waarmee ik de expeditie op het kale continent heb doorgebracht. *Anouk Piquet*, dank voor al je werk, inititaief, stimulans en tomeloze inzet om het onderzoek in goede banen te leiden. Also many thanks to *Paul 'Thomo' Thomson*; I loved your humor and your optimism, *Nina Cadman*, I really enjoyed our conversations in the minicosm van when we had to work there for hours in a row, and *Tessa Vance*; your boldness and striking comments were often hilarious. *Andrew 'Clobbs' Davidson*, you are fantastic as a person and as a project leader. Our four day walk through the Vestfold Hills around New Years Eve is unforgettable. Also, I will never forget you gave up your helicopter seat for me so that I could enjoy the marvelous view over the Vestfold Hills from the air, instead of you. Thank you all for a memorable stay at Davis Station.

Terug op het Biologisch Centrum wil het onderzoek niet van de grond komen. Maar dan, op een goede dag, staat er een Bulgaarse jongeman voor de deur; klein van stuk maar met een groots wetenschappelijk zelfvertrouwen. 'I heard you need help with SOD measurements', sprak hij tot mij. Dit moment markeert nog altijd de vonk die mijn promotieonderzoek deed ontbranden. Anderhalf jaar ploeteren en ladingen frustrerende data deden de wanhoop stijgen en de nachtrust dalen. *Tsanko Gechev* reikte mij tenslotte het ontbrekende puzzelstukje aan waardoor het onderzoek vlam vatte. Tsanko, thank you very much for showing up on one unexpected morning. You were of eminent importance for showing me the right direction. From that moment on, this thesis started to bloom.

Zo blij als een kind ben ik en vol enthousiasme en met karrenvrachten aan overmoed begon ik de meest fabelachtige onderzoeken op te zetten. Het ene experiment was nog grootser dan de ander; binnen vier jaar had ik elke oceaan doorgemeten en was er geen geheim meer te ontrafelen.....op papier althans. Nog even met *Willem van de Poll* doornemen en de wereld zou verlost worden van onwetendheid. Echter, Willem was niet altijd even enthousiast als ik en vond mijn plannen veelal iets te ehhhhh..... ambitieus. Willem, temmer van mijn ongebreidelde onderzoeksdrift, dompteur van mijn experimenten, ik wil je hierbij ontzettend bedanken voor al jouw tijd, moeite en overredingskracht die jij in dit proefschrift hebt gestopt. Jij wist mijn bombastische experimenten terug te snoeien tot realistische proporties en hebt mij geleerd dat eenvoud niet alleen de duidelijkste maar uiteindelijk ook de meeste antwoorden levert. Daarnaast was het zeer plezierig om met jou samen te werken. Je slechte grappen en zorgvuldige planning maakte het laboratorium een prima plek om wetenschap te bedrijven. Willem, het was echt super.

Voor veel vragen over antioxidanten kon ik op het Biologisch Centrum de antwoorden vinden, maar als ik er echt niet meer uitkwam, kon ik terecht in Zeeland bij het orakel van Yerseke. *Jan Rijstenbil*, mijn onderzoek had veel parallellen met dat van jou waardoor ik jouw expertise en kennis kon gebruiken om mijn vastgelopen onderzoeken of manuscripten vlot te trekken. Ondanks dat het een behoorlijke treinreis was van Groningen naar Kruiningen/Yerseke is het elke minuut waard geweest. Ik ging altijd weer vol wijsheid en goede moed terug naar Groningen waar jouw aanwijzingen naadloos in mijn onderzoek pasten. Het bespaarde me uiteindelijk vele dagen werk en daar wilde ik met alle graagte wel acht uur voor in de trein zitten. Heel veel dank hiervoor.

Als sluitstuk van mijn onderzoek heb ik genoten van de gastvrijheid van *Walter Helbling* en *Virginia Villafãne* tijdens onze expeditie naar Patagonië. In the backyard of the Estación de Fotobiología de Playa Unión we experienced scientific research in Argentinean style and I loved it. Our combined effort and your knowledge of the local costums resulted in innovative experiments, by which we gained a lot of information. I really appreciate your great scientific skills and your useful comments. Walter and Virginia, thank you for your hospitality, the feast in your shakra and your drive all the way to Comodoro Rivadavia to see the penguins. Also thanks to *Rodrigo Conçalves*, *Elena Barbieri* and *Regina Flores* for making our work comfortable, providing us with mate and spending great evenings with us in and around Playa Union.

Marco de Graaff, ik ben nog altijd zeer blij dat je als student bent meegegaan naar Patagonië. Tijdens ons verblijf heb ik veel steun gehad aan jouw werklust, initiatief en je gezellige persoon. Ik zal nooit vergeten dat je zo koppig was dat je de kapotte DIVE dwong om weer fatsoenlijk aan het werk te gaan; met zoveel eigenwijze vastberadenheid liet je het arme apparaat geen keus dan weken achtereen netjes te blijven draaien. Ik had het na een half uur allang opgegeven, maar jouw weerbarstigheid redde op dat moment mijn onderzoek! Ook in het Harense laboratorium heb ik vele plezierige dagen met je doorgebracht. De grote hoeveelheden werk die je hebt verzet, heeft het realiseren van mijn proefschrift in een stroomversnelling gebracht waar ik je nog steeds erg dankbaar voor ben.

Hierbij wil ik verder graag de leescommissie bedanken voor het beoordelen van mijn proefschrift. *Kai Bischof*, I started my PhD project with measuring SOD samples for you which you used for publication and which earned me my first authorship. It is very nice to realize that you are also involved in finishing my PhD project. *Winfried Gieskes*, jouw ongelooflijk opgewekte instelling en gemoedelijkheid waren een baken van positivisme waardoor het altijd heerlijk was om even bij je aan te komen. *John Beardall*, thank you for reading the manuscript and your feedback on this thesis.

De afgelopen zes jaar heb ik deel uitgemaakt van de vakgroep mariene biologie en ik ben deze zeer gaan waarderen. Het beslaat prachtige vakgebieden en bestaat uit het komen en gaan van vele kleurrijke mensen. *Peter Boelen*, je was een erg gezellige kamergenoot maar als ik toch zo kon praten als jij….. *Maria van Leeuwe* en *Jacqueline Stefels*, wat was het heerlijk om op jullie poes Jerry en het huis te passen. Het gaf me een instant vakantiegevoel als ik van het BC weer naar 'huis' fietste. *Steven Ferber*, het waren mooie borrels die we samen gaven met als hoogtepunt de Highland games met barbecue. Ik heb erg veel plezier aan onze samenwerking beleefd. *Jos de Wiljes*, dank voor de verhalen over Groningen uit vervlogen tijden en je keuze voor muziek die nu ook door mijn boxen schalt. En *Ronald Visser*, naast dat je twee gouden handen hebt en de gave om uit bijna niets een opstelling te 'MacGyveren', ben ik je bovenal dankbaar voor het eindeloos

repareren van mijn fiets. Oh ja, en natuurlijk voor die 'paar' monstertjes die je hebt doorgemeten. *Henk-Jan Hoving* en *Jos Mieog*, dankzij jullie heb ik mijn wetenschappelijk journalistieke ambities kunnen ontplooien. Fantastisch dat ik jullie onderzoek heb mogen gebruiken als springplank. *Jeanine Olsen*, *Wytze Stam* en *Wim Wolff*, dank voor de samenwerking in het dagelijks bestuur. *Jim Coyer* regardless of day or time, you were always present in the Biological Centre. *Sandra Nauwelaerts* en *Louis Flinn*, een jaar was te kort.

Vooral tijdens de pauzes kwam de gezelligheid van mariene biologie levendig tot uiting. Veel onzinnig gewauwel, mooie verhalen, en pittige discussies met *Wim Klaassen, Henk de Groot, Jan Roggeveld, Steven van Heuven, Sandra Hazelaar, Anne-Carlijn Alderkamp, Karin de Boer, Han van der Strate, Verena Brauer, Ika Neven, Deniz Haydar, Jeroen Creuwels, Galice Hoarau, Malia Chevolot, Jan Veldsink, John Videler* en *Eize Stamhuis, Klemens Eriksson*, *Andreas Zipperle*, *Stella Boele, Loes Venekamp* (dank voor al je culture werk), en *Gezien van Roon* en *Joukje de Vries* (dank jullie voor alle lastige administratieve zaken). Jullie waren geweldige collega's waarmee ik zeer aangenaam heb mogen werken. Daarnaast hebben de studenten *Sasja Sjollema* en *Astrid Hoogstrate* onze mannenkamer nog tijdelijk opgevrolijkt. Dank jullie allemaal voor een onvergetelijke tijd.

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