Antioxidants, a radical solution?
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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 (O₂). 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).

\[ \text{Figure 1: Light reaction of photosynthesis (from: Encyclopaedia Britannica, Merriam-Webster, inc. 2006).} \]
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-phosphoglycerate (\(C_3\)) - 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 O₂ 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 ($\text{O}_2^-$; 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, copper) present in many protein complexes. Consequently, redox-related processes get disrupted (Halliwell & Gutteridge 1984), which inevitably leads to overreduction of ETC’s and enhanced $\text{O}_2^-$ production. Besides protein bound metal ions, $\text{O}_2^-$ is also able to reduce unchelated bivalent cations ($\text{Fe}^{3+/2+}$, $\text{Cu}^{2+/1+}$) which mediates electron transfer from one $\text{O}_2^-$ to another (Haber-Weiss/Fenton reaction) thereby generating hydrogen peroxide ($\text{H}_2\text{O}_2$). Both oxygen intermediates are moderately harmful but in the presence of unchelated bivalent cations, $\text{O}_2^-$ is also able to reduce $\text{H}_2\text{O}_2$ (Haber-Weiss/Fenton reaction) to form the biologically dangerous hydroxyl radical ($\text{HO}^*$; Kehrer 2000, Cadenas 1989, Halliwell & Gutteridge 1984; Fig. 2).

Unlike its precursors, $\text{HO}^*$ 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 $\text{HO}^*$ 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.
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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 (diantoxanthin, 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).
Chapter 1

Violaxanthin  

Antheraxanthin  

Zeaxanthin

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• is too reactive to be effectively scavenged, the network is focused on neutralizing its precursors O2•− and H2O2 (Bartosz 1997, Halliwell 2006).

7.1 Superoxide dismutase

The first ROS formed, O2•−, is scavenged by superoxide dismutase (SOD) which catalyzes the conversion of O2•− into H2O2 (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 \( \text{O}_2^- \) removal, and thereby prevents production of \( \text{HO}^* \), 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 (\( \text{O}_2^- \)) 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 antioxidants. In contrast, radicals generated within a hydrophilic environment (i.e. chloroplast stroma) are often scavenged by water soluble antioxidants. Abbreviations: SOD = superoxide dismutase; APX = ascorbate peroxidase; GR = glutathione reductase; AsA = ascorbate; DAsA = dehydroascorbate; DAsAR = dehydroascorbate reductase; \( \text{H}_2\text{O}_2 \) = 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 ($\gamma$-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, Villafañe et al. 2004). In general, flagellates are more sensitive to UVR than diatoms (Villafañe 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, Martinez 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 O₂ 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 polyunsaturated 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


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).

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<td>Lesser 1996b</td>
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<td>Malanga &amp; Puntarulo 1995</td>
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Table 1: Studies describing responses of superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), total amount of cellular glutathione (Gluttot) and the glutathione redox status (GRS) to UVR. + represents an increase, - represents a decrease and 0 represents no response at all.
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 optimized 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.