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Self-shading protects phytoplankton communities against H_2O_2-induced oxidative damage

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ABSTRACT: The aim of this study was to investigate whether increased phytoplankton growth rates and biomass caused by higher nutrient availability will result in lower oxidative stress in microalgae by reducing effective-light exposure, a process called self-shading. This hypothesis was tested by a 6 d mesocosm experiment carried out at the Zingst Marine Station, Germany (southern Baltic Sea, 8 to 9 psu) in June 2001. Out-door mesocosms filled with 1400 l of natural seawater sieved through a 100 µm plankton net were manipulated by daily additions of nitrate and phosphate (NP treatment), and nitrate, phosphate and silicate (NPSi treatment). Oxidative stress parameters monitored daily were H_2O_2 concentrations in the seawater and concentrations of superoxide dismutase (SOD) activity, catalase activity and lipoperoxidation products (thiobarbituric acid-reactive substances, TBARS) in the phytoplankton. Chlorophyll *a* (chl *a*) concentration, pH and photosynthesis (O_2 evolution) were measured throughout the experiment to describe the physiological status of the phytoplankton. Our data suggest that fast growth of nutrient-saturated microalgae strongly limits oxidative stress by self-shading. This was shown by significantly lower SOD and catalase activities in the nutrient treatments than in the control treatment without nutrient additions. No differences were observed between the 2 nutrient treatments (with or without Si), despite higher photosynthetic rates in the diatom-rich mesocosms (NPSi). Our findings may contribute to a better understanding of the close relationships between eutrophication, the occurrence of algal blooms and antioxidant activity in microalgae.

KEY WORDS: Oxidative stress · Eutrophication · Phytoplankton · Mesocosm experiment · Hydrogen peroxide · SOD · Catalase · Lipoperoxidation

INTRODUCTION

Long-term eutrophication in the Baltic Sea has led to the development of massive harmful algal blooms (Kononen 2001), shifts in phytoplankton species diversity (Leppäkoski & Mihnea 1996) and, based on an increasing number of reports, reproductional disturbances in higher trophic levels such as the M74 syndrome in salmonids (Bengtsson et al. 1999). Two natural compounds have been shown to be deficient in salmons suffering from M74: the vitamin thiamine and the carotenoid astaxanthin (Pettersson & Lignell 1999, Lundström et al. 1999), a powerful antioxidant (Palozza & Krinsky 1992, Woodall et al. 1997, Barros et al. 2001). Oxidative stress is recognized as a physiological condition established when the production of reactive oxygen species (ROS) exceeds the antioxidant defensive systems of organisms leading to higher oxidative damage in lipids, proteins and DNA (Halliwell 1987). In the present paper, we have concentrated on the incidence of oxidative stress in phytoplankton communities.

In photosynthetic organisms, high intensity light exposure is responsible for a significant fraction of ROS production, especially $O_2(^1\Delta_g)$ (singlet oxygen), H_2O_2

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and the superoxide radical, $O_2^{\bullet -}$ (Asada 1999, Karpinski 1999). Upon oxidative conditions, plants, macro- and microalgae, as many other living organisms, respond by increasing antioxidant defenses, notably enzymes such as superoxide dismutase (SOD) and catalase, and low molecular weight compounds like tocopherols, carotenoids and ascorbic acid (Halliwell 1987). It has also been shown that high H_2O_2 concentrations are extremely toxic to *Scenedesmus obliquus* (Chlorophyceae), limiting growth and photosynthetic activity in association with chlorophyll *a* (chl *a*) depletion (Mallick et al. 2002). Phytoplankton communities with higher cell densities may be less exposed to environmental oxidative challenge (high light) in the water column by self-shading, which would lead to lower concentrations of antioxidants in these microalgae. Anthropogenic activities in the Baltic Sea have also been introducing herbicides and heavy metal ions such as Fe^{2+}, Cu^{2+}, Cd^{2+} and Hg^{2+} to the environment (Breitholtz et al. 2001, Pempkowiak et al. 2001). Such toxic compounds are notoriously related to the induction of oxidative stress in many different organisms, including microalgae (Okamoto et al. 1999). Thus, in the high polluted Baltic Sea, oxidative stress may be an important process throughout the food web, including the primary producer level. The involvement of ROS metabolism in ecological processes has been little studied, especially concerning natural communities (Butow et al. 1996, 1997a). Most previous research deals with the effects of heavy metals and UV radiation on unialgal cultures (e.g. Sinha et al. 1998, Okamoto et al. 1999, Lage et al. 2001). In a laboratory experiment with the dinoflagellate *Peridinium gatunense*, Vardi et al. (1999) showed that catalase inhibited cell death, implicating H_2O_2 as the active ROS for apoptosis (programmed cell death). Our study considers the regulation of ROS by nutrient-mediated algal growth. The aim of this work was to investigate whether increased phytoplankton growth rates as a result of higher nutrient availability (nitrogen, phosphorus and silicate) will decrease oxidative stress and antioxidant activity by reducing effective light exposure through self-shading. This hypothesis was tested in a mesocosm experiment carried out in the southern Baltic Sea in summer (30 June to 5 July 2001) when high natural light irradiance levels would impose stressful conditions upon the phytoplankton. We manipulated natural phytoplankton by daily additions of nitrate and phosphate (NP treatment), and nitrate, phosphate and silicate (NPSi treatment). Measurements included total SOD and catalase activities, H_2O_2 production and thiobarbituric acid-reactive substances (TBARS, a marker for lipoperoxidation process). To our knowledge, this is the first mesocosm experiment focusing on microalgal antioxidant adaptations upon natural oxidative stress.

MATERIALS AND METHODS

Experimental design. A 6 d mesocosm experiment was carried out at the Zingst Marine Station, University of Rostock (Germany), southern Baltic Sea from 30 June to 5 July 2001. The mesocosms consisted of 9 transparent polyethylene enclosures (height 2.2 m, ∅ 0.9 m, 1400 l), double-sealed at the joints to avoid leaking. The enclosures were, with random placement, submerged in the water and attached to rubber rings on a floating raft with the upper edge about 40 cm above sea level. The mesocosms were filled with natural seawater sieved through a 100 µm mesh plankton net on 29 June in the afternoon and left to stabilize overnight. Three treatments were conducted in triplicate: (1) control treatment (no N, P or Si additions); (2) NP treatment (enriched with N and P); and (3) NPSi treatment (enriched with N, P and Si). The nutrient enrichment treatments followed the Redfield ratios of N:P = 16:1 and Si:N:P = 48:16:1 (on a molar basis), respectively. These ratios are stipulated as optimal for phytoplankton growth (Redfield et al. 1963). In the morning of each day, 4 µM $NaNO_3$ and 0.25 µM NaH_2PO_4 were added to the NP treatment, and 12 µM Na_2SiO_3, 4 µM $NaNO_3$ and 0.25 µM NaH_2PO_4 to the NPSi treatment. Bicarbonate (cf. Redfield ratio C:N:P = 106:16:1), as well as trace metals (Fe, Mn, Zn, Cu, Co and Mo salts), vitamin B_{12}, citrate and biotin in 0.5 % of f_2 medium concentrations (Guillard & Ryther 1962), were added to all 9 mesocosms daily. Sampling of seawater took place each morning at 08:00 h, before the nutrients were added. To avoid stratification, the mesocosms were stirred with a round plastic disc fixed to a rope 4 times each day; at 08:00 h before sampling, at around 08:30 h after the nutrient additions, at 13:00 and 17:00 h.

Water temperature and irradiance. Water temperature outside the mesocosms and irradiation at the water surface were obtained from the weather station of the Zingst Marine Station, with measurements every minute, 24 h d^{-1}. Water temperature in the mesocosms was measured on each experimental day at 13:00 h.

Nutrient concentrations. Water samples for nutrient concentrations (NO_2^-/NO_3^--N, NH_4^+-N, PO_4^{3-}-P and SiO_4^{4-}-Si) were taken daily from all 9 mesocosms, filtered (0.45 µm) in the field and frozen at –20°C. The nutrients were later analyzed at the Department of Systems Ecology, Stockholm University, Sweden.

Chl *a* measurements. Chl *a* concentrations were analyzed daily. A known volume of water was filtered through a GF/F Whatman™ filter. Chl *a* was extracted in methanol and the concentration was determined spectrophotometrically according to the Swedish Standard (NS 4767).

pH measurements. For the measurement of pH, 100 ml samples were collected in amber flasks 3 times

each day at 08:00, 13:00 and 17:00 h. To limit carbonic acid decomposition and significant pH variation during the measurement, the samples were kept in a thermostatic water bath. pH was measured with a pH electrode with temperature measurement (Pt 1000, Metrohm™) attached to a pH-meter (Model 713, Metrohm™), using the NBS scale and 2 point calibrated with Radiometer™ buffers pH 7 and 9.

O_2 evolution measurements. The O_2-evolution measurements were designed to measure the maximum photosynthetic and respiratory capacity of each phytoplankton sample. The O_2-measurements were carried out in an Illuminova™ light dispenser system ('Light Pipette'), equipped with a light source of 400 to 700 nm. The sample was incubated in a 2.6 ml incubation chamber with an O_2 electrode (Microelectrodes™) inserted. The measurements consisted of a 1200 s long computer-steered measuring sequence including 8 dark pulses and 7 light pulses of 80 s each. The % O_2 saturation and change in % O_2 saturation (photosynthetic rate) were recorded every 2 s. The light pulses increased in photon flux density during each sequence: 25, 50, 100, 400, 800, 1200 and 1600 µmol m^{-2} s^{-1}, respectively. The maximum O_2 evolution rates in light and darkness were selected and used to calculate gross photosynthetic activity.

H_2O_2 measurements. H_2O_2 concentrations in seawater samples were measured as luminol-dependent chemiluminescence with a luminometer (LKB Wallac 1250). During injection, 48 µl horseradish peroxidase stock solution and 16 µl luminol stock solution were mixed with 1 ml seawater from the sample. Five replicate measurements were made for each sample.

Oxidative stress parameters. From each mesocosm, 250 ml of 100 µm sieved seawater was concentrated on a Whatman Cyclopore™ polycarbonate membrane (porosity 0.2 µm, ∅ 47 mm). The concentrated phytoplankton was washed out into a 15 ml Falcon tube with 1.5 ml of 1 mM dithiothreitol (DTT) and 0.1 mM EDTA in 100 mM phosphate buffer pH 7.4. The suspensions were sonicated for 3 min in pulse mode (70% duty cycle) in an ice-water bath to avoid enzyme denaturation. A centrifugation step (3 min at 9000 × *g*) was included to eliminate debris. The supernatant was used for enzyme, TBARS and protein determinations. SOD activity was measured by the classic cytochrome *c* method, which employs a constant flux of superoxide radicals ($O_2^{\bullet-}$) generated *in situ* by the xanthine/xanthine oxidase system (Flohé & Ötting 1984). Cytochrome *c* reduction was followed at 550 nm for 3 min using various amounts of the supernatant. One SOD unit was defined as the enzyme concentration required for 50% inhibition of cytochrome *c* reduction at 25°C. Catalase activity was measured by following the absorbance decay of H_2O_2 at 240 nm as according to Beutler (1975). Protein concentrations were estimated by the method of Lowry et al. (1951) using bovine serum albumin as a standard. For TBARS analysis, continuous oxidation reactions were stopped by adding 8 mM butylated hydroxytoluene (ethanol solution) after sample homogenization. To detect the colored adducts, 300 µl of sample were incubated at 100°C for 15 min with 50 µl 100 mM phosphate buffer pH 7.4 and 0.25% thiobarbituric acid (TBA) in 0.25 M HCl and 1% Triton X-100. After reaching room temperature, the absorbances of the solutions were measured at 535 nm using malondialdehyde (MDA) as a standard (Fraga et al. 1988).

RESULTS

Weather conditions were sunny during the whole experiment, which suggests that self-shading was probably more important than if the weather would have been cloudy. Irradiance reached daily maximum values of 456 ± 37 µmol photons PAR m^{-2} s^{-1} (mean ± SD, n = 6 d) and daily mean values of 142 ± 24 µmol photons PAR m^{-2} s^{-1} (mean ± SD, n = 6 d). Water temperature in the mesocosms was 21.1 ± 0.8°C at 13:00 h (mean ± SD, n = 6 d) and during the night, water temperature was 1 to 2°C lower.

Nutrient depletion did not occur in any of the treatments (Fig. 1), but nutrient dynamics during the experiment differed depending on treatment. Initial nutrient concentrations, after filling the mesocosms with natural seawater, were 0.25 ± 0.08 µM PO_4^{3-}-P, 0.21 ± 0.12 µM NO_2^-/NO_3^--N, 0.40 ± 0.12 µM NH_4^+-N, 1.16 ± 0.50 µM SiO_4^{4-}-Si (mean ± SD, n = 9 mesocosms). In the control treatment, PO_4^{3-}-P stayed constant throughout the experiment, NO_2^-/NO_3^--N increased while NH_4^+-N and SiO_4^{4-}-Si decreased. In the 2 nutrient treatments, all nutrients increased throughout the experiment, except for SiO_4^{4-}-Si which decreased in the NP treatment down to 0.30 ± 0.29 µM on Day 6.

No significant differences (ANOVA, 3 treatments, $p > 0.05$) were observed between the 9 mesocosms in physiological status (Fig. 2) or any of the biochemical markers for oxidative stress (Fig. 3) at the beginning of the experiment (Day 1), which suggests that all phytoplankton communities were at the same conditions before nutrients were added. However, the H_2O_2 concentrations and the SOD and catalase activities (CAT) of all 9 mesocosms on Day 1 were higher than on subsequent days (Fig. 3), around 0.15 µM H_2O_2, 3.2 U_{SOD} $µg^{-1}$ chl *a*, and 0.25 U_{CAT} mg^{-1} chl a, respectively. These results suggest that the phytoplankton was initially affected by mechanical stress caused by transportation and pumping of the water into the mesocosms.

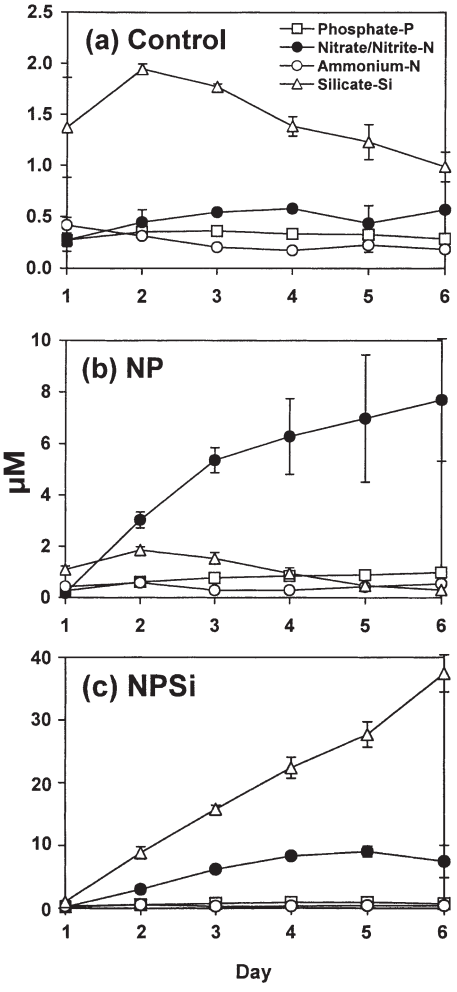

Fig. 1. Water nutrient concentrations during the 6 d mesocosm experiment. (a) Control treatment, (b) NP treatment, (c) NPSi treatment. Error bars represent the SE of the mean

The addition of inorganic nutrients induced differences in growth rate in the different treatments as reflected by chl *a* and protein concentrations (Fig. 2a,b). While on Day 1, all mesocosms had the same chl *a* concentrations (1.8 ± 0.4 µg chl *a* l^{-1}), already 24 h after nutrient additions, the chl *a* concentrations in the NP and NPSi treatments were about 75% higher than the control treatment. The algae in the nutrient treatments continued to grow fast throughout the experiment and on Day 6, chl *a* levels were about 10-fold those on Day 1. Similar patterns were found in the protein concentrations; although the absolute difference (in %) between control and nutrient treatments were not as large as for chl *a*. The relationship between protein and chl *a* concentrations could be described by a quadratic regression model with R^2 = 0.82. This means that chl *a* concentrations change more relative to protein concentrations at high concentrations, indicating that faster growing algae (NP and NPSi treatments) have a lower protein content than slower growing algae (control treatment). From Days 2 to 5, the NP mesocosms showed higher chl *a* and protein concentrations than the NPSi treatment, but this was reversed on Day 6. Microscopic analysis of the microalgae showed higher abundances of cyanobacteria, dinoflagellates and green algae in the control and NP treatments and higher abundances of diatoms in the NPSi treatment at the end of the experiment.

The pH reflected differences in primary productivity in the mesocosms between days and treatments, and also within days from the 3 measurements taken in the morning, early and late afternoon (Fig. 2c). In the morning, the pH was always lower than in the afternoon of the preceding day, while during the day, it increased in concert with photosynthetic activity. The pH of the nutrient-treated mesocosms increased throughout the experiment, but in the control treatment, it stayed 8.2 from Days 1 to 6. In the NPSi treatment, the pH increased more (up to 8.87 ± 0.13 on Day 6) than in the NP treatment (up to 8.44 ± 0.04 on Day 6). The diatom-rich mesocosms (NPSi) showed significantly higher photosynthetic activity throughout the whole experiment after Day 1 (ANOVA, $p < 0.05$), while the oxygen evolution rates in the control and NP treatments were not significantly different from each other (ANOVA, $p > 0.05$). From Days 2 to 6, oxygen evolution normalized to chl *a* was relatively constant in the NPSi treatment, but it decreased by about 50% in the control and NP treatments.

Oxidative challenge to the phytoplankton was apparently more intense for the control than for the nutrient treatments throughout the whole experiment, as shown by the oxidative stress parameters (Fig. 3). We normalized the activities of the 2 antioxidant enzymes (catalase and SOD) and MDA to chl *a* for reasons of comparison; although the same patterns were found when they were related to protein concentrations (data not shown). In the control treatment, there was increasing oxidative damage to lipids in the phytoplankton from Days 3 to 6 (Fig. 3b). Simultaneously, there was a decrease in the activity of the H_2O_2-decomposing enzyme catalase (Fig. 3c) but not in SOD, which generates H_2O_2 as a product while scavenging $O_2^{\bullet-}$ (Fig. 3d). Both nutrient treatments did not demonstrate increased oxidative damage to lipids or antioxidant enzyme adaptations to higher oxidative stress during the experiment. The only observed trend was, similar to the control treatment, a decrease in catalase activity from Days 3 to 6. A higher production of H_2O_2 by the phytoplankton in the control treatment might be shown by the 2- to 4-fold higher water H_2O_2 concentrations per µg chl *a*

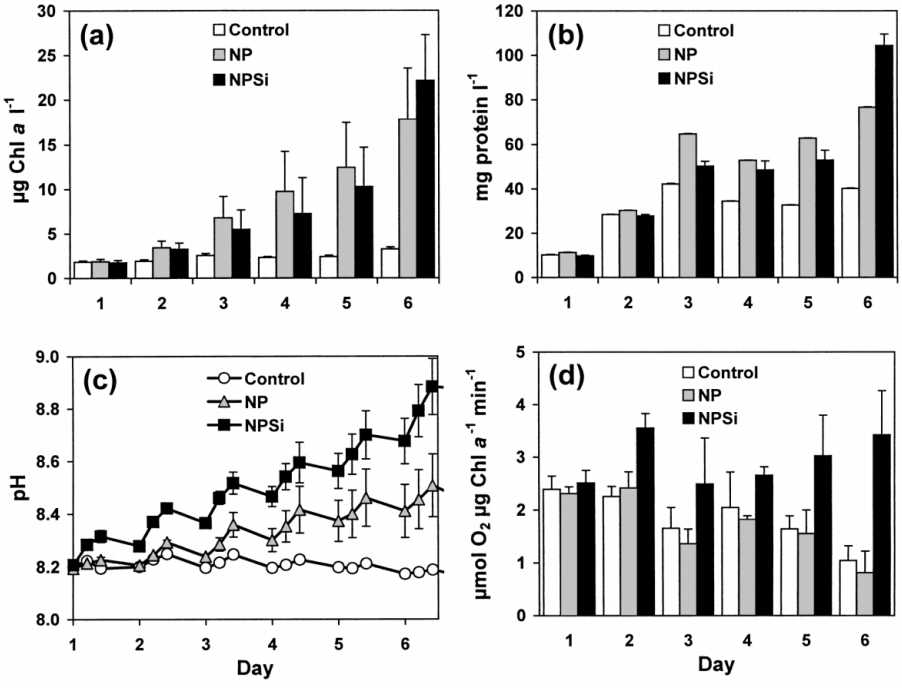

Fig. 2. Parameters describing the physiological status of the phytoplankton during the 6 d mesocosm experiment. (a) Chlorophyll *a* (chl *a*) concentrations, (b) protein concentrations, (c) pH, (d) oxygen evolution. Error bars represent the SE of the mean

than in the nutrient treatments (Fig. 3a). However, H_2O_2 in seawater can have other minor origins than production by the phytoplankton community. H_2O_2 concentrations per liter did not vary among the 3 treatments from Day 1 to 3; however, after Day 3, concentrations became slightly higher in the nutrient treatments than in the control treatment (highest in the NPSi treatment, up to 1.5 times the control treatment concentrations on Day 6).

DISCUSSION

The purpose of this study was to relate the occurrence of oxidative stress in natural phytoplankton communities to conditions typical of eutrophication. We mimicked such conditions by creating 2 different phytoplankton blooms from 1 initial natural community by nutrient additions in mesocosms. The constituent species each have their characteristic ROS pro-

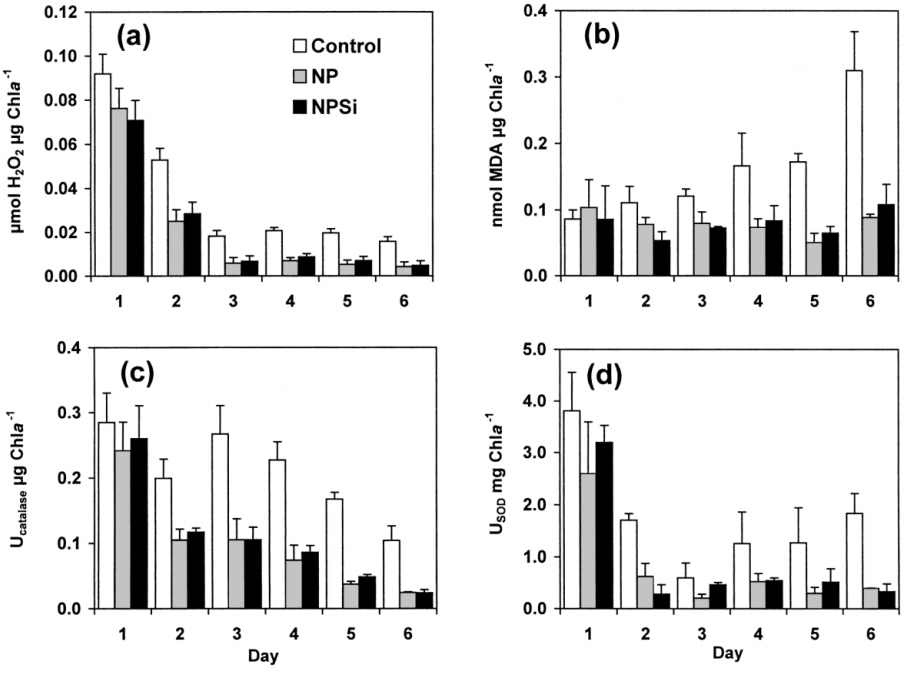

Fig. 3. Parameters describing oxidative stress during the 6 d mesocosm experiment. (a) H_2O_2 concentrations in seawater, (b) thiobarbituric acid-reactive substances (TBARS) measured as malondialdehyde (MDA) in phytoplankton, (c) catalase activity in phytoplankton, (d) superoxide dismutase (SOD) activity in phytoplankton. Error bars represent the SE of the mean

duction levels, antioxidant capacities and responses upon oxidative challenges (Sies 1993). Even closely related species can be very different in this respect (Oda et al. 1997). We showed in our study that even on a community level SOD and catalase activities can respond to decreasing or increasing oxidative stress, as was corroborated by H_2O_2 and lipoperoxidation products determination during the experiment.

Typical conditions creating oxidative stress in phytoplankton are high light intensities, carbon deficiency, high pH, nutrient limitation and heavy metal contamination (Butow et al. 1994, 1998, Falkowski & Raven 1997, Mallick & Mohn 2000). Despite that pH significantly increased in the 2 nutrient treatments, with subsequent CO_2 limitation, oxidative stress did not seem to be intensified, but rather reduced. Carbon limitation in our experiment was prevented by daily additions of bicarbonate. The carbon equilibrium of seawater is pH-dependent and CO_2 becomes less abundant with higher pH. The lack of increased oxidative stress with increased pH in our experiment suggests that the phytoplankton communities were able to utilize HCO_3^-, a feature shared by many microalgal species (Badger & Price 1992). Although natural phytoplankton biomass in the southern Baltic Sea is apparently limited by low water nutrient concentrations (as shown by higher growth when nutrients are added), nutrient depletion did not occur during our experiment and therefore, we assume that nutrient stress was not a major issue in any of our treatments. However, it can not be excluded that the decrease in catalase activity in the control treatment may have been caused by nutrient limitation. An argument against this is that catalase activity decreased in the nutrient treatments as well. The general reduction of catalase activity (all 3 treatments) during the experiment could also be a consequence of a gradual adaptation to a lower environmental stress imposed by the mesocosm conditions.

In our experimental conditions, the major factor differing between the treatments was phytoplankton biomass and thereby, irradiance to the individual cells by different degrees of self-shading, especially because the weather was very sunny during the experiment. Therefore, we identify self-shading of the phytoplankton community in the nutrient treatments as the proximate factor causing the lower SOD and catalase activities per unit phytoplankton biomass (suggesting lower cellular production of H_2O_2), compared to the control treatment. The causes of oxidative stress may be very different for different algal species and our results present the combined responses of many species. Butow et al. (1997b) found that stressful irradiance is the overriding cause of increased SOD activity in the dinoflagellate *Peridinium gatunense*. Contrarily, Twiner & Trick (2000) suggested that oxidative stress in microalgae does not have to be photosynthetically derived through excess light. They assumed that production of H_2O_2 by the raphidophyte *Heterosigma akashiwo* is an enzymatic process linked to iron availability. However, it is noteworthy that iron release from heme and FeS cluster-enzymes is a process proven to be triggered by ROS attack, which are mainly produced by chloroplasts in photosynthetic organisms under light stress (Laulhere & Briat 1993, Becana et al. 1998).

The increase in TBARS in the control treatment may be related to a higher production of H_2O_2 in the microalgae caused by 2 concomitant events: higher $O_2^{\bullet-}$ production and lower catalase activities. In photosynthetic organisms, H_2O_2 is predominantly produced by imbalance between photosynthesis and carbon fixation or by dismutation of $O_2^{\bullet-}$ by SOD (Asada 1999). However, it should be kept in mind that extracellular H_2O_2 concentrations also depend on spontaneous H_2O_2 production and non-enzymatic decomposition in the seawater (Collén & Pedersén 1994). The difference in TBARS between the control and nutrient treatments may also be a consequence of the dilution of TBARS by *de novo* production of fatty acids, rather than diminished oxidative stress. However, malondialdehyde and other aldehydes (TBARS) are mostly generated by lipoperoxidation of unsaturated fatty acids, as arachidonic and linolenic acids, and therefore, such an approach is unreliable without proper evaluation of phytoplankton fatty acid composition.

Several oxyradical reactions were probably involved in low (control treatment) and high (nutrient treatments) microalgal self-shading conditions in our experiment. With low self-shading, the effective light incidence probably resulted in higher production of mainly H_2O_2, but also $O_2^{\bullet-}$. The decrease in catalase activity led to higher availability of H_2O_2 to generate more reactive ROS, like the hydroxyl radical ($OH^{\bullet}$) by the Fenton reaction (Halliwell 1987, Fridovich 1998). This, in turn, caused significant exacerbated levels of lipid oxidative damage (TBARS). On the other hand, in high self-shading conditions more balanced activities of SOD and catalase throughout the whole experiment limited $OH^{\bullet}$ generation and consequently lipoperoxidation was less. Catalase activity also decreased in the nutrient treatments throughout the experiment, probably because the algal cells grew very fast, investing more in growth (cell division) than in defence enzyme synthesis. It may be argued that there was no subsequent increase in TBARS in the nutrient treatments. Probably, the available catalase in these treatments was high enough to keep up the redox status in the algae.

Extrapolating our results to nature would mean that eutrophication may result in lower oxidative stress in phytoplankton blooms by reducing effective-light ex-

posure (self-shading). This is probably true only for fast-growing nutrient-saturated phytoplankton (as in our experiment), while nutrient-limited (dying) phytoplankton blooms may increase oxidative stress (Butow et al. 1994). An implication of decreased oxidative challenge for algal cells in a eutrophied environment is that this may affect the quality of microalgae as food items for higher trophic levels in terms of antioxidant components, particularly low molecular weight compounds, such as ascorbic acid, tocopherols and carotenoids, and thereby, affect the whole system.

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