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Density-dependent patterns of thiamine and pigment production in the diatom *Nitzschia microcephala*

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Abstract

In the present study we investigate how intraspecific (density-dependent) competition for nutrients by the diatom *Nitzschia microcephala* affects the level of oxidative stress in the algal cells as well as their production of pigments and thiamine. *N. microcephala* was grown in three different densities until the stationary growth phase was reached. Throughout the experiment, growth rate was negatively related to cell density. Superoxide dismutase activity, protein thiol, and diatoxanthin concentrations indicated increasing oxidative stress with increasing cell density, which was most probably caused by nutrient depletion of the medium. Pigment contents per cell (except for diatoxanthin) decreased with increasing cell density. *N. microcephala* was able to synthesize thiamine and its thiamine content per cell increased in concert with cell density. In comparison, the dinoflagellate *Amphidinium carterae* was unable to synthesize thiamine. These results suggest that cells of *N. microcephala* subjected to higher competition and lower growth rates have a lower carotenoid content and a higher thiamine content. If such responses would occur in nature as well, eutrophication (higher cell densities) may alter the quality of microalgae as food items for higher trophic levels not only by species shifts in the phytoplankton, but also by changes in the cellular nutritional value within species.

Keywords: *Nitzschia microcephala*; Bacillariophyta; Diatom; Intraspecific competition; Oxidative stress; Vitamin; Antioxidant; Thiamine; Carotenoids

1. Introduction

Eutrophication is a major problem in marine areas worldwide. One of the effects of higher N and/or P concentrations is increased algal growth and biomass (Smith et al., 1999). Increasing algal density modifies the cellular environment through (1) self-shading, (2) competition for nutrients, and (3) excretion of metabolic waste products and allelochemicals. This may affect the quality of microalgae as food items for higher trophic levels and thereby affect the whole system. For example, the Baltic salmon has developed a reproductional disturbance, the M74 syndrome, simultaneously with the acceleration of the eutrophication of the Baltic Sea after the 1960′s (Jansson and Dahlberg, 1999). This may cause over 90% mortality of the wild salmon fry in certain years (Bengtsson et al., 1999). Two natural compounds have been shown to be deficient in fish suffering from M74: the vitamin thiamine and the carotenoid astaxanthin (Karlsson et al., 1999; Pettersson and Lignell, 1999). It has been speculated that the development of M74 is a result of the large-scale changes in the Baltic Sea ecosystem coupled to eutrophication (Bengtsson et al., 1999). Eutrophication is held responsible for a doubling of the pelagic primary production in the Baltic Sea proper (Elmgren, 1989) and phytoplankton species shifts from diatoms to dinoflagellates and cyanobacteria in the southern Baltic Sea proper (Hajdu and Larsson, 2000; Finni et al., 2001).

The exposure of algae to environmental stress can increase the cellular levels of reactive oxygen species (ROS), such as the superoxide anion ($O_2\cdot^-$), hydrogen

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peroxide (H_2O_2), singlet molecular oxygen ($^1O_2$) and hydroxyl radicals (HO·), that disturb the steady-state balance of prooxidants and antioxidants in the cells (Bowler et al., 1992). Light stress is one of the most common sources of oxidative stress in algae (Collén and Pedersén, 1994, 1996). In the case of phytoplankton communities, increased growth of microalgae will reduce light levels in the water column through algal self-shading (Cloern, 2001), and may therefore reduce oxidative stress. On the other hand, greater competition for nutrients may increase oxidative stress (Falkowski and Raven, 1997). These two density-dependent factors are expected to regulate the synthesis of scavenging enzymes and certain carotenoid pigments that protect algal cells against oxidative stress.

If carotenoid levels decrease with increasing eutrophication as a result of cell crowding in phytoplankton communities, it may be hypothesized that the quality of phytoplankton as food for higher trophic levels will decrease. Carotenoids are transferred from algae to zooplankton grazers, and β-carotene is converted to astaxanthin by zooplankton crustaceans (Kleppel et al., 1985). In photosynthetic organisms, carotenoids act as accessory light-harvesting pigments, quenchers of triplet chlorophyll and quenchers of singlet oxygen (Siefermann-Harms, 1987; Young et al., 1997). In heterotrophic organisms they are thought to play an important role as antioxidants for protecting these organisms from injuries caused by ROS (Matsuno, 1989; Shimidzu et al., 1996).

Thiamine (vitamin B_1) is produced by bacteria and microalgae and is acquired by higher trophic level organisms (Chen et al., 1991; Niimi et al., 1997). It is an essential vitamin for many organisms because of its role in carbohydrate metabolism and neurology. Free thiamine (TF) is a precursor of thiamine diphosphate (TDP), which acts as a coenzyme for two types of enzymes, α-ketoacid dehydrogenases and transketolases that are involved in energy-generating systems in the cell (Krebs cycle and the oxidative pentose phosphate pathway). Increases in thiamine concentrations in nature have previously been correlated with algal blooms (Natarajan and Dugdale, 1966; Ohwada and Taga, 1973; Nishhijima and Hata, 1979). The role of thiamine monophosphate (TMP) in organisms is not fully clear, but in fish it may be a degradation product of TDP (Brown et al., 1998). Direct uptake of thiamine from water can be an important source for lower trophic level organisms, but the diet is probably more important among higher trophic level species such as fish (Niimi et al., 1997).

In the present study we investigate how intraspecific (density-dependent) competition for nutrients by the diatom *Nitzschia microcephala* affects the level of oxidative stress in the algal cells as well as their production of pigments and thiamine. Diatoms are a major phytoplankton group, which often decreases in abundance with eutrophication, being replaced by other groups such as dinoflagellates and cyanobacteria (Leppäkoski and Mihnea, 1996).

2. Results and discussion

2.1. Growth rates

Maximum mean cell densities (±S.D.) of *Nitzschia microcephala* were 1.2±0.1 million cells per ml in the experiment. A stationary growth phase was reached due to the exhaustion of nutrients at the carrying capacity level of the medium. Specific growth rates were highest, ca. 1.5 day^{-1}, at the start of the experiment. The density-dependence of μ throughout the experiment could be described by the regression model $\mu = -1.49D^2 + 0.43D + 1.50$ ($R^2 = 0.87$) with density (D) expressed in million cells per ml (Fig. 1). Thus, there was a strong negative correlation between cell density and growth rate as a result of crowding in *N. microcephala*. The initial density of the cultures did not affect this relationship. The mean (±S.D.) cell volume of *N. microcephala* was 122±26 μm^3 ($n = 50$). Cell volumes did not change with time and were independent of initial cell density.

2.2. Oxidative stress

Superoxide dismutase (SOD) activity increased and the concentration of thiol groups decreased during growth of *N. microcephala* (Fig. 2). This suggests that oxidative stress increased with higher intraspecific

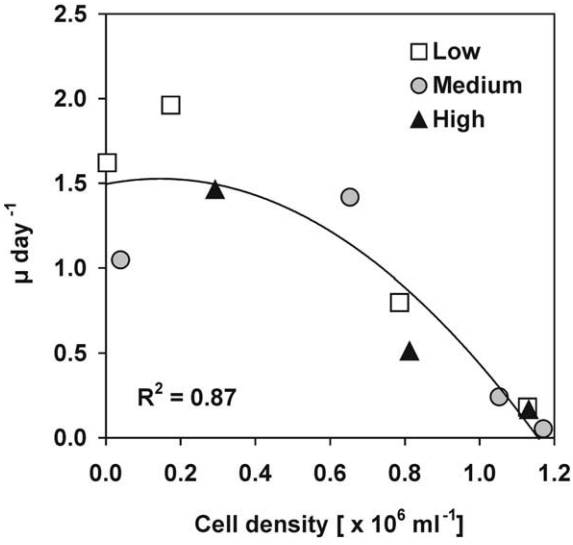

Fig. 1. Mean specific growth rates ($n = 3$) of *Nitzschia microcephala* related to mean cell density ($n = 3$) during the experiment. Symbols indicate the different initial cell densities: low = 100 cells per ml, Medium = 1000 cells per ml, high = 10,000 cells per ml.

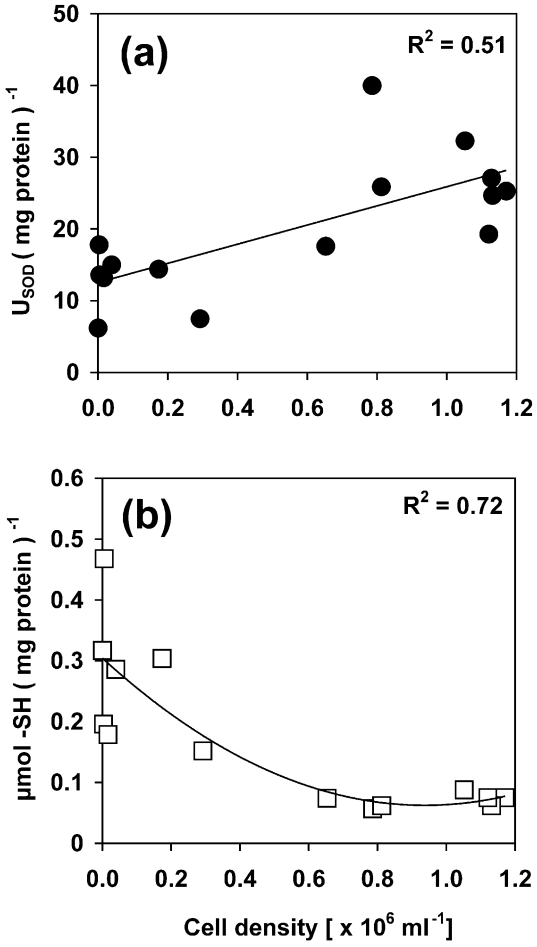

Fig. 2. Oxidative stress indicators for *Nitzschia microcephala* related to mean cell density ($n=3$) during the experiment: (a) Mean activity of SOD ($n=3$), (b) Concentration of thiol (–SH) groups.

competition for nutrients (higher cell density); more thiol groups became oxidized and the activity of the scavenging enzyme SOD increased. In marine environments, oxidative stress in algae is often caused by high light intensity and increased pH of the seawater as a result of algal photosynthesis (Falkowski and Raven, 1997). It is the imbalance between light uptake and carbon fixation in the algal cells that results in the production of oxygen radicals around photosystem I and increases the activity of SOD (Collén, 1994; Mtolera, 1995; Asada, 1999a,b). However, our results were opposite to this; the cultures of *N. microcephala* became very dense at the end of the experiment and cells were subject to self-shading while oxidative stress was much higher than at lower cell densities. Therefore, oxidative stress must have been caused by environmental constraints other than excess light. The growth of any algal culture in a closed vessel is accompanied by increasing nutrient depletion, increasing pH by photosynthetic carbon uptake with decreasing CO_2 levels, and increasing accumulation of a variety of cellular exudates. Nutrient stress (N, P) is known to create oxidative stress in algae and also carbon limitation will cause the production of superoxide ions around photosystem I (Falkowski and Raven, 1997; Asada, 1999a). Our experiment suggests that oxidative stress can increase in a dense algal bloom when intraspecific competition for nutrients (including inorganic carbon) occurs. Barros et al. (2003) performed an outdoor mesocosm experiment and showed that oxidative stress in natural phytoplankton communities decreased with increased cell densities when these communities were not nutrient-limited, and concluded that self-shading can protect phytoplankton communities against oxidative stress. From these results, together with our present results, we suggest that fast-growing nutrient-saturated phytoplankton blooms in nature probably suffer less from oxidative stress than nutrient-limited (dying) phytoplankton blooms. However, it should be kept in mind that in an experimental, closed container the nutrient concentration is constant at no or low cell growth or decreases with time when cells grow fast, while in nature there is mixing and a flux of nutrients occurs from inside and outside the system. In addition, high doses of cellular exudates, which could not be excluded from our experiment, may have unknown influences on the induction of oxidative stress in phytoplankton blooms.

2.3. Pigments

The quotes between fucoxanthin (fuco) and β-carotene (beta) and total chlorophylls (mol carotenoid per mol chlorophyll) were relatively constant throughout the experiment (mean ± S.D., $n=45$): 0.41 ± 0.05 for fuco and 0.028 ± 0.005 for beta. Maximum pigment concentrations per ml occurred around 0.8 million cells per ml for the major light-harvesting pigments chlorophyll *a* (chla), chlorophyll *c* (chlc) and fuco (Fig. 3a) and for the quencher beta (Fig. 4a). The cellular concentrations of the six major pigments in *N. microcephala* were strongly density-dependent (Figs. 3b, 4b). Chla, chlc, fuco, beta and diadinoxanthin (diadino) declined as the cultures entered stationary growth at the end of the experiment at ca. 1.2 million cells per ml. The xanthophyll cycle carotenoids diadino and diatoxanthin (diato) showed opposite patterns; while the cellular content of the epoxidated form diadino decreased similar to the other four pigments, that of the de-epoxidated form diato increased (Fig. 4b), although the levels of this latter pigment generally stayed very low. Goericke and Montoya (1998) showed strong positive correlations between growth rate and the chla concentration per cell in microalgae. This is in accordance with our results for *N. microcephala* as we found a negative correlation between cell density and the chla concentration per cell, as well as a negative correlation between cell density and growth rate. The chla content per cell in unicellular algae at very low irradiances (<10 µmol

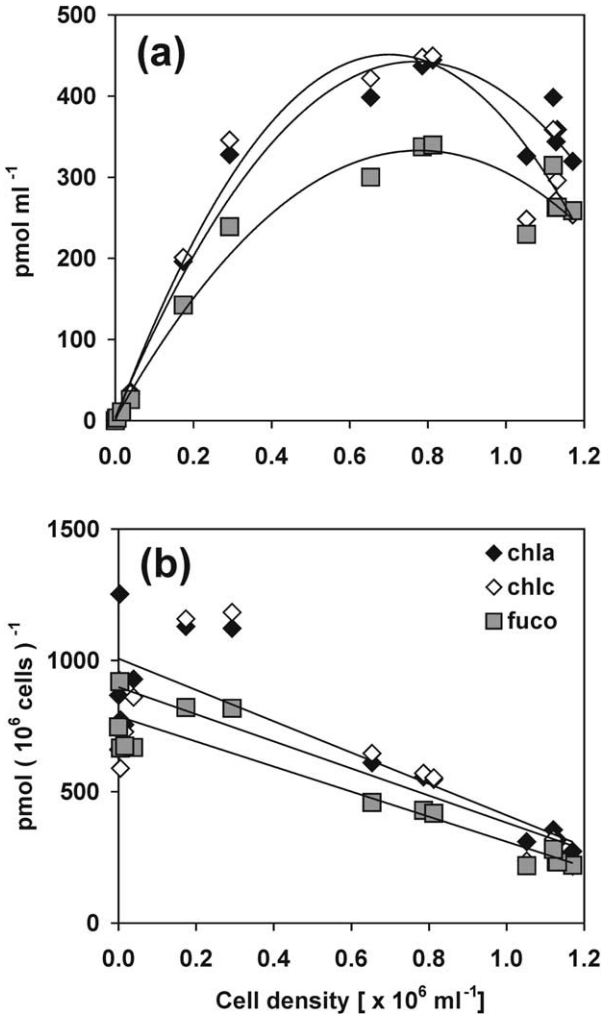

Fig. 3. Concentrations of the major light-harvesting pigments in *Nitzschia microcephala*, chlorophyll *a* (chla), chlorophyll *c* (chlc) and fucoxanthin (fuco) related to mean cell density ($n=3$) during the experiment: (a) mean pigment concentration per ml ($n=3$), (b) mean pigment content per million cells ($n=3$). R^2 values of significant ($P<0.05$) regression models were: (a) quadratic models, chla: 0.98, chlc: 0.96, fuco: 0.97, (b) linear models, chla: 0.78, chlc: 0.63, fuco: 0.88.

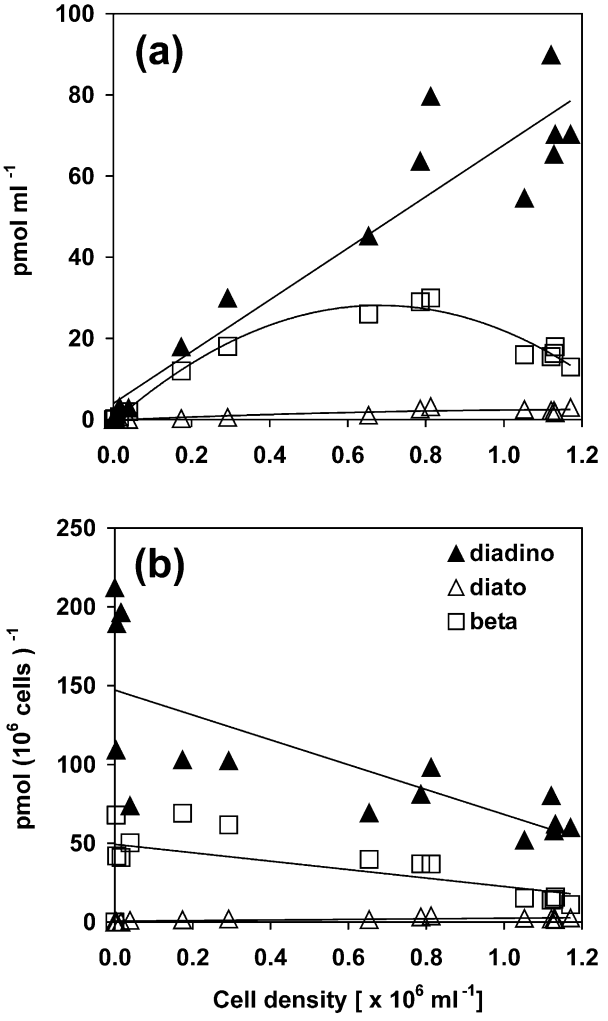

Fig. 4. Concentrations of the major photoprotective carotenoids in *Nitzschia microcephala*, β-carotene (beta), diadinoxanthin (diadino) and diatoxanthin (diato) related to mean cell density ($n=3$) during the experiment: (a) mean carotenoid concentration per ml ($n=3$), (b) mean carotenoid content per million cells ($n=3$). R^2 values of significant ($P<0.05$) regression models were: (a) beta (quadratic model): 0.98, diadino (linear model): 0.90, diato (linear model): 0.83, (b) linear models, beta: 0.36, diadino: 0.54, diato: 0.53.

photons m^{-2} s^{-1}) is often slightly depressed, rising to a maximum value at some low to moderate light level (ca. 20–50 μmol photons m^{-2} s^{-1}), and thence decreasing as a log-normal with increased irradiances (Falkowski and Raven, 1997). However, chla levels per cell in *N. microcephala* decreased by ca. 65% as cell density increased from ca. 100 to 1.2 million cells per ml (and irradiance decreased). In our experiment, the decreases in chla, chlc and fuco could thus both be an adaptation to very low light levels inside the cultures or a result of intraspecific competition for nutrients. Fuco follows the same pattern as chla, probably because it is present in the same antenna pigment (Siefermann-Harms, 1987).

As cell density increased we found an opposite trend for cellular concentrations of diadino (decreasing) and diato (increasing). This may be an indication for the transfer of diadino into diato in the diadino-diato xanthophyll cycle, an epoxidation and de-epoxidation mechanism for carotenoid-mediated dissipation of excess excitation energy (Olaizola and Yamamoto, 1994; Young et al., 1997; Meyer, 2000). In high light conditions diadino is de-epoxidated into diato and vice-versa in low light conditions. Our results suggest that *N. microcephala* would use this mechanism, but as the trends are opposite to light in our experiment, it is probably not a matter of photoprotection. Most likely diatoxanthin acts as a quencher of excitation energy created by oxidative stress from localized (cellular) nutrient deficiency. The increasing trend of the concentration of diadino per ml, opposite to those of the

chlorophylls, fuco and beta may indicate de novo synthesis of diadino in our experiment.

2.4. Thiamine

The initial levels of the three forms of thiamine in the cell cultures were ca. 1.5 pmol per ml of TF and TDP and ca. 0.2 pmol per ml of TMP for both the diatom *N. microcephala* and the dinoflagellate *Amphidinium carterae* (Fig. 5a and b). In *A. carterae* there was an initial increase in the biologically active form TDP from ca. 1.5 to ca. 3.0 pmol per ml, which indicates the cellular uptake of thiamine from the F/2 medium (Fig. 5a). TDP reached a maximum in *A. carterae* at a cell density of ca. 0.08 million cells per ml, while in *N. microcephala* TDP still increased at 1.2 million cells per ml (Fig. 5b). TF and TMP per ml showed little change with cell density in *A. carterae*, however these two compounds increased in *N. microcephala* with increased cell density. These results suggest that the dinoflagellate is not able to produce thiamine, and that the thiamine necessary for its growth was only supplied by the growth medium, while the diatom is able to produce thiamine. At low cell densities, both species had a high thiamine content per cell originating from the growth medium, but above ca. 0.02 million cells per ml for *A. carterae* and 0.6 million cells per ml for *N. microcephala*, trends depending on the presence or absence of thiamine production were observed (Fig. 5c and d). In *A. carterae*, all three thiamine species decreased with increasing cell density, suggesting that TF, TMP and TDP were biologically diluted in the growing cells. In *N. microcephala*, all three thiamine species increased with increasing cell density.

For the analysis of thiamine we developed a new method with high accuracy and the possibility to separately quantify TF, TMP and TDP in small samples

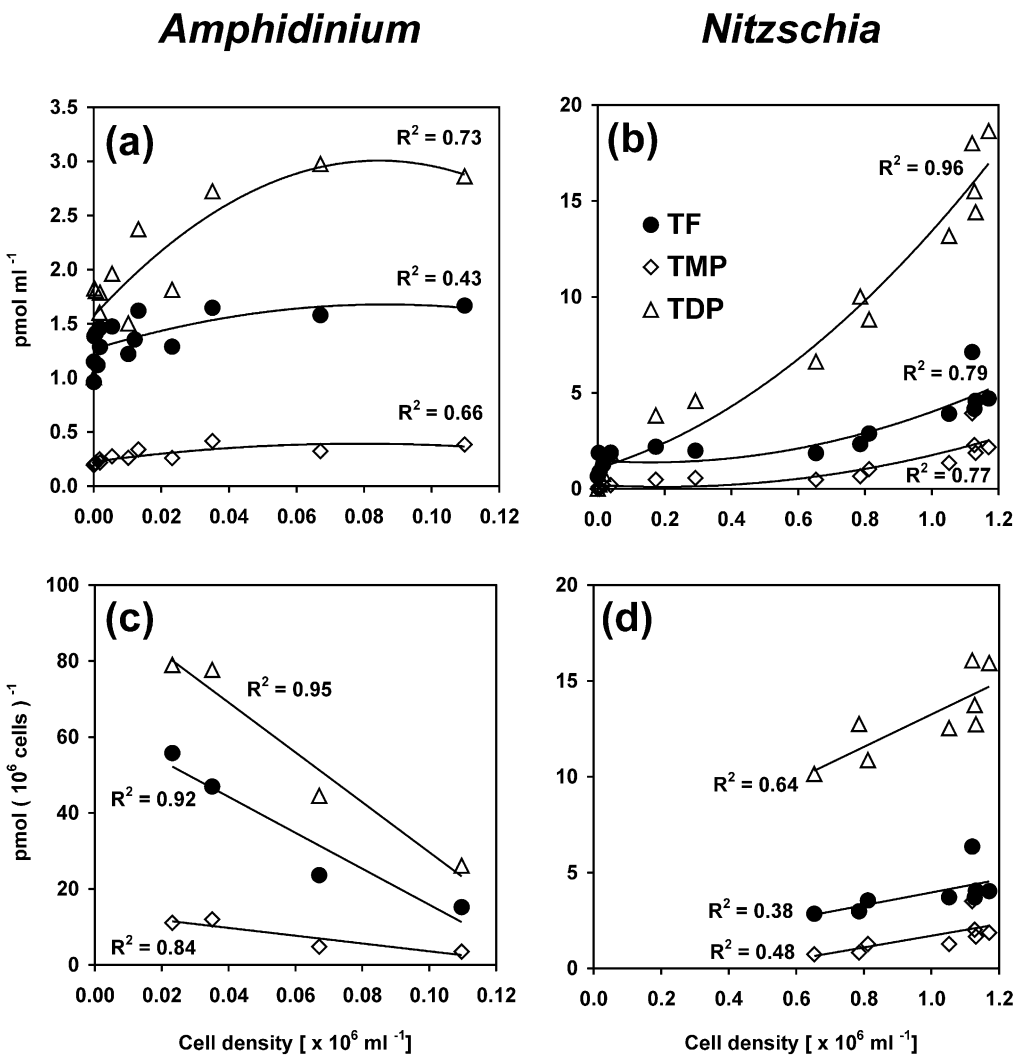

Fig. 5. Free thiamine (TF), thiamine monophosphate (TMP) and thiamine diphosphate (TDP) in *Amphidinium carterae* and *Nitzschia microcephala* related to mean cell density ($n=3$) during the experiment: (a,b) mean concentrations per ml ($n=3$), (c,d) mean content per million cells ($n=3$). R^2 values of significant ($P<0.05$) quadratic (a,b) or linear (c,d) regression models are shown.

(Pinto et al., 2002). Niimi et al. (1997) also distinguished between the different forms of thiamine in field samples of phytoplankton, but other published analyses of microalgae report total thiamine content only. Our data show that the biologically active form TDP increases most during cell growth in *N. microcephala* while TF and TMP probably act as stock and synthesis/degradation product, respectively. *N. microcephala* was able to produce thiamine, but *A. carterae* was not. The inocula were not axenic, but as the increase in thiamine levels in the *A. carterae* cultures was very small compared to that in *N. microcephala*, we assume that those in *A. carterae* reflected uptake from the growth medium and that the contribution by bacterial production was negligible. It has previously been shown that the ability to produce thiamine is species-specific in microalgae. In culture studies, thiamine addition to the medium was required by 6 out of 46 marine microalgae representing 7 algal classes (Provasoli and Carlucci, 1974; Turner, 1979). It has also been shown that several species of diatoms have the highest production of thiamine in comparison with other microalgal species (Carlucci and Bowes, 1970a; De Roech-Holtzhauer et al., 1991). Carlucci and Bowes (1970b) found, similar to our results, that *A. carterae* was unable to synthesize thiamine. They also found that *A. carterae* could utilize the thiamine produced by the diatom *Phaeodactylum tricornutum* when both species were grown in the same culture. This implies that thiamine can be excreted to the medium by diatoms, which probably is of great importance in nature for the survival of phytoplankton organisms lacking the synthetic pathway for thiamine.

We observed that thiamine concentrations (per ml) increased in concert with microalgal density in *N. microcephala*. Increases in water thiamine concentrations in the field have been found to be correlated to seasonal and spatial variation of phytoplankton blooms (Ohwada and Taga, 1973). Thiamine production has also been found to be enhanced by eutrophic conditions and concentrations of 400–2000 ng l^{-1} have been reported from eutrophic lakes (Carlucci and Bowes, 1972). Brown et al. (1999) showed that the thiamine content (in μg g^{-1} alga) significantly increased at the onset of the stationary growth phase. This is in agreement with our results that the thiamine content per cell in the diatom *N. microcephala* increased with increasing cell density and decreasing growth rates.

2.5. Possible implications for natural conditions

In nature the species diversity of phytoplankton is usually very high and it should be kept in mind that we investigated only one species. Our results would imply that diatom cells in a eutrophied environment with a high degree of crowding (blooms) are less nutritious food items in terms of carotenoids for higher tropic levels. This is especially important in the case of β-carotene, which acts as a precursor for vital animal antioxidants such as astaxanthin. However, *N. microcephala* was a good producer of thiamine and the thiamine content per cell increased with increasing cell density and decreasing growth rates. This implies that diatom cells in a eutrophied environment with high intraspecific competition are better food items in terms of thiamine for higher tropic levels. However, in nature species shifts occur as a result of eutrophication. These shifts often promote dinoflagellates, cyanobacteria and some other phytoplankton groups at the cost of diatoms (Leppäkoski and Mihnea, 1996; Snoeijs, 1998). In the light of our experiment, this would result in lower availability of thiamine in aquatic systems as a result of eutrophication if the by eutrophication promoted phytoplankton organisms would be unable to synthesize thiamine.

3. Experimental

3.1. Materials

Nitzschia microcephala (diatom) was isolated from the brackish Baltic Sea (Askö, Sweden, 6.5 psu). *Amphidinium carterae* (for comparison of thiamine production) was obtained from the Plymouth Marine Laboratory (UK). The microalgae were pre-cultivated for four weeks in F/2 medium (Guillard and Ryther, 1962), with a salinity of 6.5 psu for *N. microcephala* and 35 psu for *A. carterae* at 20 °C in an 80 μmol photons m^{-2} s^{-1} 12:12 h light:dark regime. On day 0 of the experiment, 500-ml Erlenmeyer flasks were filled with 250 ml F/2 medium and 100, 1000 or 10,000 cells per ml were added. No further nutrients were added during the experiment. Fifteen replicate flasks were prepared for each cell density and species. The flasks were closed with sterile cotton stoppers and cultured in the same conditions as the pre-cultivation. Each day the flasks were shaken and randomly replaced. Three replicate flasks of each cell density and each species were sampled on days 0, 2, 4, 6 and 8 at 4–6 h into the light cycle.

3.2. Determination of cell density

For cell counts, 10 ml of each culture were fixed with acid Lugol's iodine solution. Cell density of *N. microcephala* was determined in a Bürker counting chamber using light microscopy (Nikon™ type 104). Cell density of *A. carterae* was determined in a 10 ml counting chamber (Utermöhl, 1931) using an inverted microscope (Olympus™ IMT). Growth rates, μ (day^{-1}), were calculated from the cell counts as $\mu = (\ln (N_2/N_1))/(d_2 - d_1)$, where N_2 and N_1 are cell numbers on days d_2 and d_1, respectively (Guillard, 1973).

3.3. Analysis of oxidative stress indicators

For superoxide dismutase (SOD) assays, analyses of protein thiol (-SH) groups of proteins and total protein concentrations, 10 ml of each culture were centrifuged at 10 °C and 4000 rpm for 10 min The pellets were immediately frozen in liquid N_2 and stored at −86 °C until analysis. The pellets were sonicated for 2 min with 1 ml of phosphate buffer (0.1 M, pH = 7.4). The crude extract was centrifuged at 4 °C and 10,000 rpm for 10 min and the supernatant was used for the analyses. Determination of total SOD activity was carried out by the cytochrome *c*-based assay (McCord and Fridovich, 1968), which employs the hypoxanthine/xanthine oxidase system to generate superoxide anions *in vitro*. SOD competes with cytochrome *c* for $O_2\cdot^-$ generated by the system. Absorption of cytochrome *c* was observed on a spectrophotometer at 550 nm for 60 seconds to follow the reduction reaction using various amounts of the supernatant. One unit of SOD activity (U_{SOD}) is defined as the amount of SOD causing 50% inhibition of cytochrome *c* reduction at 25 °C (McCord and Fridovich, 1968). Protein concentrations were estimated by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Thiol groups were measured by the method described by Murphy and Kehrer (1989) using reduced gluthathione as a standard. The concentration of thiol (-SH) groups of proteins was used as a measure of oxidative stress; with higher oxidative stress, more thiol groups become oxidized and less thiol groups occur in the cells.

3.4. Analysis of carotenoids

For carotenoid analyses, 40 ml of each culture were filtered over a Whatman™ GF/F filter. The filters were immediately frozen in liquid N_2 and stored at −86 °C until analysis. The filters with the algal cells were sonicated in cold methanol for 1 min and centrifuged at 4 °C for 4 min at 3000 rpm following the extraction method of Wright and Shearer (1984). The final injection solution consisted of 800 ml of extract and 200 ml ammonium acetate. Before injection, the solution was filtered through a 0.45 mm PTFE/PP filter. For pigment separation the reversed-phase HPLC method of Wright and Jeffrey (1997) was used with a slightly modified solvent system program [(time,%A,%B,%C): (0 min, 100, 0, 0), (2 min, 0, 100, 0), (17 min, 0, 20, 80), (18,5 min, 0, 20, 80), (22 min, 0, 100, 0), (24 min, 100, 0, 0), (30 min, 100, 0, 0)]. The flow rate was 1 ml min^{-1}. A volume of 25–200 μl was injected into the HPLC system using a HPLC autoinjector (SparkHolland™, Promis II). A Spherisorb 5 ODS column, 250×4.60 mm, 5 μm particle size, Phenomenex™ was used with an Opti-guard (1×4.6 mm, RP-18) as guard column. Pigment detection was performed with a variable wavelength UV detector (Milton Roy™ spectroMonitor 3100) at 436 nm coupled to a multiple solvent delivery system (Milton Roy™ CM 4000). For chromatogram analysis, the program Chrom-card™ (version 1.21 for Windows) was used. Pigment quantification was achieved with the help of standards for β,β-carotene (beta) and chlorophyll *a* (chla) obtained from Sigma™ and chlorophyll c_2 (chlc) diadinoxanthin (diadino), diatoxanthin (diato) and fucoxanthin (fuco) obtained from DHI Water and Environment™, Denmark.

3.5. Analysis of thiamine

For thiamine analyses 40 ml of each culture was filtered over a Whatman™ GF/F filter. The filters were immediately frozen in liquid N_2 and stored at −86 °C until analysis. Free thiamine (TF) and its phosphate esters, thiamine monophosphate (TMP) and thiamine diphosphate (TDP) were extracted and analysed according to the method described by Pinto et al. (2002). The HPLC analyses were performed using a Reprosil-Pur™ NH_2 column (5 μm particle size, 250 mm×4.6 mm I.D.) from Corricon™ (Knivsta, Sweden) on a Jasco™ Model 821-FP scanning fluorimetric detector equipped with a Merck™ D7000 interface module, a Merck™ L-7250 autosampler, a Peltier™ sample cooler L-7250 and a Merck™ Model L7100 pump. The detector was set at excitation 375 nm and emission 450 nm. The injection volume was 100 μl and the flow rate 1 ml min^{-1}. The mobile phase was methanol and phosphate buffer 100 mM pH 7.4 in a ratio of 43:57 v/v. The chromatograms were integrated using the software HSM-LaChrom™ D-7000.

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