EFFECT OF NITROGEN STARVATION ON OPTICAL PROPERTIES, PIGMENTS, AND ARACHIDONIC ACID CONTENT OF THE UNICELLULAR GREEN ALGA PARIETOCHLORIS INCISA (TREBOUXIOPHYCEAE, CHLOROPHYTA)

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Spectral properties of cell suspensions, individual cells, and extracts of the unicellular green alga Parietochloris incisa (Reisigl) Shin Watan. grown under low light were studied. Long-term nitrogen (N) deprivation resulted in a decrease of chloroplast volume, appearance of numerous large cytoplasmic oil bodies, and the deposition of triacylglycerols with a high proportion of arachidonic acid. Chlorophylls \( a \) and \( b \) underwent a synchronous decline, whereas carotenoids (Car) showed a relative increase. Simultaneously, significant qualitative changes in the spectral properties of \( P. \) incisa individual cells, cell extracts, and cell suspensions were observed. To a large extent, the spectral changes observed in cell suspension could be attributed to a decrease in overall pigment content, leading to a gradual weakening of the so-called package effect and accumulation of additional amounts of Car over chl, most probably, in oil bodies. Several optical characteristics of cell suspensions could serve as sensitive indicators of N-deficiency in \( P. \) incisa. Furthermore, the absorption ratios, \( A_{476}/A_{676} \) and \( A_{650}/A_{676} \), showed close correlations with the Car-to-chl ratio and relative arachidonic acid (AA) content, respectively. The latter makes it possible to suggest that the increase in AA percentage in \( P. \) incisa proceeds in parallel with a decrease in cell chl content, accounting for the weakening of the package effect. N-replenishment resulted in complete recovery of cell optical properties. The possible significance of the changes in cell ultrastructure, pigments, lipids, and optical properties is discussed with special reference to the ability of algae to adapt to and survive under conditions of long-term nutrient deficiency.

Key index words: adaptation; arachidonic acid; carotenoid(s); chlorophyll; N-deficiency; Parietochloris incisa; spectroscopy; ultrastructure

Higher plants and microalgae are subjected to a variety of environmental stresses, including a deficiency of nitrogen (N), which affects many aspects of their physiology. The decreasing growth rate and declines in cell protein and chl observed during N-starvation indicate a restricted ability to maintain the photosynthetic functions (Thompson 1996, Young and Beardall 2003). Under such circumstances, some algae accumulate massive amounts of carbon in the form of carotenoids (Car) and lipids (Thompson 1996). Some lines of evidence suggest that the accumulation of Car under stress conditions represents a mechanism that protects cells from damage by light (Demmig-Adams et al. 1996, Niyogi 1999, Ledford and Niyogi 2005). It is generally accepted that algae acclimate to environmental conditions by alteration of their lipid metabolism and composition, which is thought to readjust the membrane systems to cope with both gradual and acute stresses (Thompson 1996). Under nutrient deprivation, particularly N-deprivation, some algal species actively synthesize triacylglycerols (TAG) as an efficient carbon sink (Thompson 1996, Guschina and Harwood 2006). The TAG of most oleaginous microalgae are composed mainly of saturated and monounsaturated fatty acids (Cohen and Khozin-Goldberg 2005). In contrast, the unicellular freshwater coccoid chlorophyte (Trebouxiophyceae) Parietochloris incisa was reported to be the richest plant source of the very-long-chain polyunsaturated arachidonic acid (AA, 20:4\( \omega \)6), with more than 90% of the total AA being deposited in TAG (Bigogno et al. 2002a,b, Khozin-Goldberg et al. 2002). Under N-starvation, AA accounts for 60% of total fatty acids and for more than 20% of the algal dry weight (Khozin-Goldberg et al. 2002). Since AA is an essential fatty acid in human nutrition, being the major fatty acid component of brain and retina phospholipids.

Abbreviations: AA, arachidonic acid; Car, carotenoid(s); STD, standard deviation; TAG, triacylglycerols

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as well as a precursor for the biologically active prostaglandins and leukotrienes (Koletzko and Braun 1991). *P. incisa* is being considered for mass-cultivation (Cheng-Wu et al. 2002).

The optical properties of plants are able to provide information regarding the solar energy available for photosynthesis, responses to changes of other environmental conditions, and their nutritional status (Osborne and Raven 1986, Gitelson et al. 1996). For some algal species, remarkable changes in biooptical properties have been reported during their growth under N-deprivation (Cleveland and Perry 1987, Sosik and Mitchell 1991, Reynolds et al. 1997). Although the content and trends of pigment and biomass changes in the course of microalgae cultivation are routinely acquired through spectrophotometric measurements (Vonshak 1985), more comprehensive analysis is limited by complicated optics of the medium containing light-absorbing and light-scattering particles. To a large extent, the optical properties of cell suspensions of photosynthetic microorganisms depend on the size and shape of the cells interacting with radiation; refractive indexes of external and internal cell structures; and overall content, distribution, and local concentration of light-absorbing molecules within the cell (Laumker 1983, Osborne and Raven 1986, Naqvi et al. 2004b). Specifically, the absorption of light by suspensions containing cells with sizes exceeding the wavelengths of incident light is strongly affected by the package (“sieve”) effect. Because of the packaging (especially in the bands of strong pigment absorption), a considerable flattening of the spectrum occurs, and the quantity of absorbed light is reduced compared with that of the same amount of pigment in a true solution or a pigmented particle. Furthermore, the qualitative and quantitative analysis of pigmented cell suspensions is hampered because of the strong contribution by both nonselective and selective scattering. Nonselective scattering arises from light interaction with colorless structures and exhibits relatively uniform wavelength dependence (Koch 1981, Osborne and Raven 1986, Naqvi et al. 2004b). Selective scattering occurs in highly pigmented cell structures (such as thylakoid membranes of chloroplasts) when the refraction index changes sharply with the wavelength (see Naqvi et al. 2004b and references therein).

In this work, we investigated the effects of N-deficiency on ultrastructure, pigments, lipids, and optical properties of *P. incisa*. We attempted to investigate in detail the changes observed by analysis of pigment extracts, microspectrophotometry of individual cells, scattering-compensated spectra of cell suspensions, signature analysis, and modeling. As a result, we report that several characteristics of the optical spectra could serve as indicators of the physiological and nutritional status of *P. incisa* during starvation and recovery.

### MATERIALS AND METHODS

**Cultivation conditions.** A culture of *P. incisa* isolated from Mt. Tateyama in Japan (Watanabe et al. 1996) was grown on BG-11 medium (Stanier et al. 1971). At the outset of the experiments, cells were centrifuged, washed, and resuspended in fresh medium to reach a scattering-compensated absorbance of 0.2 at 676 nm. $A_{676}^{\text{comp}}$ (see below), equivalent to total chlorophyll concentration of ~3–4 μM. The two new batch cultures were grown—one on complete (+N) and one on N-free (−N) BG-11 medium, in 800 mL glass flasks placed on a rocker (100 rpm) at 25°C, under continuous illumination with daylight fluorescent lamps of 1.24 W·m$^{-2}$ (approximately, 6.20 μmol photons·m$^{-2}$·s$^{-1}$). After 60 d, both the +N and the −N cultures were 3-fold diluted with complete medium and examined for the next 18 d as shown in Figure 2. In other two experiments, the cultures were grown up to 63 d under illumination of 0.67 W·m$^{-2}$ (approximately 3.35 μmol photons·m$^{-2}$·s$^{-1}$). In these cases, in addition to recording cell-suspension absorption spectra, pigment, and fatty acid analyses, microspectrophotometry and EM investigations were carried out. The irradiance values were measured at the center of empty cultivation flasks with a Newport Optical Power Meter 1815c (Newport Corp., Irvine, CA, USA).

**Electron microscopy.** Algal cells were fixed in 0.5% glutaraldehyde in Millonig buffer at pH 7.4 (Millonig 1961) for 30 min at room temperature and then postfixed in 1% OsO$_4$ in the same buffer for 4 h at room temperature; the dehydration was carried out through a graded ethanol series and the cells were embedded in araldite (Korzhenevskaya et al. 1993). Ultrathin sections were made with an LKB-8800 ultratome. Sections mounted on copper grids were stained with basic lead citrate (Reynolds 1965) for 40 min and examined with a JEOL JEM-100B electron microscope (JEOL, Tokyo, Japan). Electron micrographs of *P. incisa* cells were taken from +N and −N cultures grown under 0.67 W·m$^{-2}$ illumination for 5 and 9 weeks. In each variant, >200 cells have been analyzed. As shown in Figure 1, ultrastructural features were characteristic for at least 70% of the cell populations.

**Microspectrophotometry.** Absorption spectra of *P. incisa* cells were measured by a customized Leitz MPV2 system (Ernst Leitz Wetzlar GmbH, Wetzlar, Germany), equipped with a 150 W high-pressure xenon lamp as a light source and a USB 2000 (grating #2) spectrometer (Ocean Optics, Dunedin, FL, USA) as described previously (Merzlyak et al. 2005). The measured area was selected by an iris diaphragm adjustable to a single cell size. The light passed through the sample was directed by the microscope optics to the spectrometer, and the spectra were recorded between 400 and 800 nm. Absorbance was calculated as follows:

$$A(\lambda) = -\log[I(\lambda)/I_0(\lambda)]$$

where $I(\lambda)$ and $I_0(\lambda)$ are the irradiances of a sample and reference (adjacent to the sample area), respectively.

**Spectrophotometry of cell suspensions.** The absorption spectra of cell suspensions were recorded in standard 1 cm quartz cuvettes on a Hitachi 150-20 spectrophotometer equipped with an external integrating sphere accessory (internal diameter: 150 mm, part 150-0901; Hitachi, Tokyo, Japan) with 2 nm sampling intervals and interfaced to a personal computer. All photometric values for cell suspensions are given for a path length of 1 cm.

The “true” scattering-compensated absorbance spectra $A(\lambda)$ were obtained from the measurements of extinction spectra $A_0(\lambda)$ of the cuvette placed at two fixed positions (S) as close as possible to $A^{\text{s}}(\lambda)$ and at a distance of ~1.1 cm from $A^{\text{s}}(\lambda)$ the input port of the integrating sphere as described previously (Merzlyak and Naqvi 2000). The results of the scattering measurements are presented for $S_1$ at 800 nm,
a wavelength where the contribution of absorption into transmitted light attenuation is negligible (Merzlyak and Naqvi 2000, Naqvi et al. 2004b).

The $A(\lambda)$ spectra containing both absorption [$A(\lambda)$], nonselective-scattering [$ANS(\lambda)$], and selective-scattering [$ASS(\lambda)$] fractions were simulated using the model of Naqvi et al. (2004b):

$$A^S(\lambda) = M(\lambda) = A_0 + A_1 A(\lambda) + A_2 ANS(\lambda) + A_3 ASS(\lambda)$$

where $M(\lambda)$ is the simulated spectrum, and $A_i$ are constants. The nonselective-scattering spectrum was assumed to be proportional to $\lambda^{-1}$; the spectrum of selective scattering was obtained from $A(\lambda)$ using the Kramer–Kronig relations as described in Naqvi et al. (2004b). The least-squares approach was used to find the optimum values of the fitting parameters, $A_i$, so as to minimize the sum of the residuals $\rho$ between the measured and the simulated spectra:

$$\rho = \sum_i [A^S(\lambda)_i - M(\lambda)_i]^2$$

in the whole spectral range measured (350–800 nm) or between 600 and 800 nm, models $M_1(\lambda)$ and $M_2(\lambda)$, respectively (Naqvi et al. 2004b). The fitted parameters ($A_i$) were obtained using Microsoft Excel (the Solver tool; Microsoft Corp., Redmond, WA, USA). The spectra of relative error, $E(\lambda)$, were calculated as follows:

$$E(\lambda) = [A^S(\lambda) - M(\lambda)]/A^S(\lambda)$$

Lipid extraction and pigment analysis. Cells were pelleted by centrifugation, transferred to a glass–glass homogenizer with a chloroform–methanol (10 mL, 2:1, v/v) mixture, and extracted to remove all pigment. The lipid fraction including chl and Car was separated according to Folch et al. (1957). The chloroform phase was used for further pigment and lipid analysis. Chl $a$ and $b$ and total Car (for the latter, molecular weight of 570 was accepted) were quantified using absorption coefficients for chloroform (Wellburn 1994). The pigment composition of the $P. incisa$ extract was also studied with the spectral reconstruction method (simulation of the absorption spectrum of an extract by a linear combination of the absorption spectra of its constituents) developed for analysis of Car of higher-plant leaf and algae extracts (Merzlyak et al. 1996a,b, Naqvi et al. 2004a). The extract spectra were fitted using the least-squares approach for finding the optimum values of the fitting parameters for chl $a$ and chl $b$ spectra [pure chl $a$ and $b$ obtained from Fluka (Fluka Chemie AG, Deisenhofen, Germany)].

Fig. 1. Ultrastructure of $Parietochloris incisa$ cells (a, c) and chloroplasts (b, d) after 5 weeks of growth on N-supplemented (a, b) and N-deficient (c, d) media at irradiance of 0.67 W·m$^{-2}$. Ch, chloroplast; CW, cell wall; M, mitochondria; OB, oil bodies; P, pyrenoid; SG, starch grains; Th, thylakoids.
After completion of the pigment analysis, the chloroform was evaporated, and the lipid residue was dissolved in methanol. Fatty acid methyl esters were prepared by transesterification of the lipids by refluxing for 1.5 h in methanol containing 5% concentrated sulfuric acid (Kates 1986) in the presence of 0.01% 2,6-di-tert-butyli-methylphenol as an antioxidant and heptadecanoic acid (C17:0) as an internal standard. Methyl esters were extracted with hexane and immediately used for GC analysis. The fatty acid methyl esters were separated isothermally at 190°C with a Chrom 5 chromatograph (Laboratori Pristroje, Praha, Czech Republic) equipped with flame-ionization detectors, 2 m glass columns (internal diameter: 4 mm) packed with 10% SP-2330 on 100/120 Supelcoport (Supelco Inc., Bellefonte, PA, USA); nitrogen at a flow rate of 30 mL·min⁻¹ was used as a carrier gas. Identification of fatty acids was done according to retention times of standards (Sigma-Aldrich Corp., St. Louis, MO, USA) and by hydrogenation using platinum oxide as a catalyst (Kates 1986).

Data treatment. All data were analyzed with Microsoft Excel™ software. Means ± standard deviations (STD) for n (number of samples) are given.

RESULTS

Microscopy. Under the growth conditions we have employed, the population of P. incisa was heterogeneous and contained cells at different stages of division with a characteristic size of ~4–11 μm in diameter. Observations of P. incisa cells under a light microscope showed that initially and during cultivation, regardless of the presence of N, the culture was a mixture of single cells and, frequently, aggregates containing 4–20 (an average 6–8) cells.

Typical transmission electron micrographs (Fig. 1) show that P. incisa cells grown on full medium contained a large chloroplast, which occupied most of the cell cross-section. Numerous distinct thylakoids forming grana-like structures composed of 3–5 lamellae were observed inside the chloroplast, distributed over its whole volume. The stroma of the central part of a chloroplast contained a pyrenoid, a structure of medium electron density penetrated by 3–7 equidistant lamellae. Usually, the pyrenoid was surrounded by 3–15 oval-shaped starch grains ranging from 170 × 90 to 400 × 200 nm. The cells contained electron-dense intravacuolar inclusions and 130–160 nm cytoplasmic lipid globuli of medium electron density (hereafter referred to as oil bodies).

The ultrastructure of P. incisa cells grown on the N-free medium changed considerably. Specifically, the intravacuolar inclusions disappeared, and the number of intrapyrenoid lamellae as well as thylakoids in grana-like structures decreased. The contrast and osmiophility of the organelle membranes became lower; this process was especially expressed both in granal and stromal thylakoids. The latter, however, were not swollen and showed no detectable gaps. At the same time, the distinct pyrenoid was detected in the chloroplasts of the N-starved cells, and the starch grains became 3–6 times larger, reaching 1600 nm in length. Remarkably, the −N cells contained numerous and large oil bodies of high electron density, which at the terminal stages of N-starvation occupied most of the algal cell volume (Fig. 1d).

Time course of absorption and scattering changes. The extensive cell aggregation of P. incisa in culture made precise cell counting difficult; therefore, the growth of P. insica cultures was characterized by the changes in absorption and scattering. For this purpose, the chl absorption maximum at 676 nm in scattering-compensated spectra, $A_{676}$, and scattering at 800 nm, $A_{800}^{S}$, were used (Fig. 2, a and b). A series of +N P. incisa cell-suspension dilutions ($n = 18$) gave a tight linear correlation ($r^2 > 0.99$) between dilution factor and $A_{676}$ and $A_{800}^{S}$ across a wide range of values (0.03 ≤ $A_{676}$ ≤ 1.42, and 0.05 ≤ $A_{800}^{S}$ ≤ 0.085); for the data set $A_{676}/A_{800}^{S}$ ratios were close (3.56 ± 0.22).

Judging from $A_{676}$, during the first 2 weeks no difference was observed in chl content between +N and −N cultures growing at 1.24 W · m⁻². Later, in the presence of N, P. incisa showed a continuous chl increase over 60 d of cultivation, whereas under N-deficiency, $A_{676}$ displayed a noticeable decline.

![Fig. 2](image-url)
(Fig. 2a). At 60 d, the total chl concentrations comprised ~18.4 and 4.6 μM for +N and ~N cultures, respectively.

Scattering at 800 nm, $A_{800}^N$, showed a considerable increase during cultivation on N-deficient medium; this increase was lower in the N-supplemented culture (Fig. 2b). During growth in the presence of N, no significant changes in the $A_{676}/A_{800}^N$ ratio were observed (3.81 ± 0.29), whereas in the ~N culture, the ratio decreased considerably, reaching ~1 at 60 d.

After 60 d, ~N cells were reconstituted in full medium by washing and dilution. Following growth recovery, relatively small changes in $A_{800}^N$ were observed. However, $A_{676}$ and, consequently, the $A_{676}/A_{800}^N$ ratio increased close to that of the +N culture, reaching 3.9 at day 78.

**Fatty acid and AA content.** On complete medium (Fig. 2), the concentration of total fatty acids in *P. incisa* suspension increased 2-fold, reaching ~32 μM after 60 d, whereas by this time in the absence of N, the yield was approximately one order of magnitude greater. During growth under both irradiation conditions, the AA proportion of total fatty acids in +N cultures was relatively consistent at 27.4 ± 7 mol% ($n = 13$). N-deficiency brought about its continuous increase to 64.3 mol% after 60 d. The percentage of AA correlated with the concentrations of AA and total fatty acid with $r^2$ of 0.86 and 0.91, respectively. Eighteen days after the addition of N to the ~N culture, the AA proportion declined, reaching 29.9 mol%, or close to the +N control.

**Pigment analysis in extracts.** In the red region of the spectrum, the chloroform extract of +N cells exhibited a strong maximum at 666 nm and a shoulder near 651 nm, characteristic of chl $a$ and $b$, respectively (Fig. 3). In the blue region, the spectral reconstruction analysis revealed contributions of chl $a$ and $b$ between 350 and 550 nm, and three well-resolved peaks with maxima at 433, 457, and 485 nm. In comparison with the extract, the absorption spectrum of the cell suspension was much less resolved, and Car absorption in the blue region could hardly be distinguished from that of chl. In the red region of the spectrum, the chl $b$ band near 650 nm was much more expressed on the background of chl $a$ (Fig. 3a).

Between 550 and 750 nm, absorption spectra (normalized at 666 nm) of extracts of ~N cells were very close in shape, resulting in very small deviation of their STD spectrum (Fig. 3b). Accordingly, the quantitative analysis did not reveal significant changes of chl $a/b$ ratio during cultivation independent of the presence of N and illumination conditions: the ratios comprised 1.74 ± 0.09 ($n = 23$) and 1.79 ± 0.06 ($n = 12$) for 0.67 and 1.24 W·m⁻², respectively. This finding strongly suggests the absence of significant changes in chl $a$ and $b$ spectral features and their contributions to absorption between 550 and 750 nm during *P. incisa* growth in the absence of N.

In the progress of N-starvation, relative absorption in the blue range showed a progressive increase, and the STD spectrum was characterized by maxima near 426 and 448 nm and a broad band centered near 478–482 nm (Fig. 3b); close bands were found in the difference spectra of extracts from ~N cells compared with control (Fig. 3b, inset). At all stages of growth of +N cultures under both light conditions, the Car/chl molar ratios were similar, comprising 0.42 ± 0.05. N-deficiency induced a considerable increase in the ratio, rising to ~1 by 60 d.

**Microspectrophotometry.** Absorption spectra of individual cells from +N and ~N cultures showed a high dispersion in recorded spectra (Fig. 4). This could in part be related to the low precision of the measurements due to the small size of cell and chloroplast,
and uncertainties in positioning of and effective path length for the measuring beam. On the other hand, the high STD could reflect heterogeneity of pigment content in the cell population (especially in the case of N-deficient cells). In the orange-red region, both types of cells possessed close and resolved spectral features of chl $a$ and of chl $b$. On average, in the red region, maximum absorbance by $-N$ cells was $\sim 1/3$ of that of control cells. In the blue region, distinct maxima near 437 and 467–471 nm were observed. N-deficient cells possessed higher relative absorption between 400 and 500 nm; the absorbance ratios $A_{457}/A_{678}$ were 1.27 and 1.48 for $+N$ and $-N$ cells, respectively. Two main maxima near 461 and 496 nm were seen in the difference spectrum between normalized spectra 4 and 3 (not shown).

**Modeling of cell-suspension spectra.** For $+N$ cells (not shown) and at the early stages of N-deprivation (Fig. 5, upper panel), the model $M_1(\lambda)$ provided a good simulation of the measured $P.\ incisa$ cell-suspension extinction spectra, $A^N(\lambda)$, with a relative error of $\leq 2\%$ in the range of 350–750 nm. A significant difference between the simulated and experimental spectra was observed after one week of N-starvation; this difference increased with time. After 60 d of N-starvation, the error of the measured $A^N(\lambda)$ spectrum approximation with $M_1(\lambda)$ model was as high as 15%, and the $E(\lambda)$ spectrum had a fine structure and showed both negative and positive maxima near 461 and 496 nm.
positive deviation in the bands of strong pigment absorption. The model $M_2(\lambda)$, in which $\rho$ was minimized for the spectral range of 600–800 nm, provided much better approximation to the $A^N(\lambda)$ spectrum in this band. However, a strong deviation was found in the blue region, and the relative error reached $\sim 50\%$ in three resolved sharp peaks near 440, 460, and 490 nm. After N-replenishment, the correspondence between measured and simulated spectra improved, and after 16 d, a good simulation of the spectrum with the model $M_1(\lambda)$ was observed (Fig. 5, upper panel).

Changes in cell-suspension absorption spectra. In comparison with the spectrum of the extract, absorption spectra of +N cell suspensions were much less resolved, and Car absorption in the blue region could hardly be differentiated from that of chl (Fig. 6). In the red region, the chl $b$ band was much more expressed on the background of chl $a$ than in the extracts (cf. Fig. 3). During growth of the +N culture, no changes in the shape of normalized absorption spectra were observed (not shown), and the STD spectrum was structureless and showed only a small increase toward short wavelengths. In contrast, remarkable changes occurred under N-deficiency (Fig. 6), and the STD spectrum for the N-deficient cells was characterized by distinct maxima in the red (635–650 and 690 nm) and blue (434, 460, and 482 nm) ranges. These changes were accompanied by increases in peak amplitude of the first derivative spectra and by the shift of the red edge maximum position from 688 to 685 nm (Fig. 6, inset). Simultaneously, the normalized absorption spectra showed a pronounced increase and appearance of spectral features between 400 and 500 nm. In the 520–560 nm band, the changes of the normalized absorption spectra were small. In accordance with the spectral changes found, the absorbance ratios $A_{476}/A_{676}$ and $A_{650}/A_{676}$ were similar at all stages in +N cultures but displayed considerable increase or decrease, respectively (Fig. 2, c and d), in the progress of N-starvation. The absorbance ratio $A_{690}/A_{676}$ (where wavelength of 690 nm corresponded to the second maximum in the STD spectrum) displayed behavior similar to $A_{650}/A_{476}$ (not shown). These N-deficiency-induced changes in the shapes of absorption spectra disappeared soon after N-replenishment. At the end of the experiment, the absorbance ratios $A_{476}/A_{676}$, $A_{650}/A_{476}$ (Fig. 6, c and d), and $A_{690}/A_{476}$ reached the values of the control (+N), and the normalized absorption spectrum was very close to that of the control (Fig. 6a).

Relations between light absorption by cells, Car-to-chl ratio, and AA percentage. The characteristic spectral features of normalized absorption of cell suspension occurring in the course of P. incisa N-starvation were linearly related to the changes in Car/chl ratio and AA percentage. Both spectra (Fig. 7) displayed a negative correlation in the red range (except for a narrow band in the chl maximum used for normalization). The correlation coefficient for the relationship $A(\lambda)/A_{476}$ versus AA percentage exceeded 0.9, in a broad band (600–665 nm), whereas that for the Car/chl ratio was lower. By contrast, between 350 and 500 nm, $r$ was positive and closely correlated with the Car/chl ratio. In both spectra, low correlations were determined in the green range, where only small changes in normalized cell absorption occurred (Fig. 6). The relationships between Car/chl ratio and AA content versus normalized absorption for wavelengths with high correlation coefficients (i.e., 480 and 650 nm, respectively) at both irradiation conditions are presented in Figure 7, b and c.

DISCUSSION

In the present work, the effect of N-starvation was investigated in long-term experiments carried out at
low light intensities, making it possible to study changes in spectral properties of *P. incisa* in detail. In +N cell suspensions, chl (as indicated by $A_{676}$) and scattering at 800 nm (proportional to a number of particles in suspension) increased linearly in time. During the first ~21 d of N-deficiency, $A_{676}$ values did not differ significantly from those of the +N culture; however, as the culture approached 60 d without N, those values decreased to initial values. In contrast, scattering of cell suspensions increased considerably, as did the Car/chl ratio. In the absence of N, both total fatty acids and the percentage of AA increased strongly relative to +N cells, consistent with previous observations (Bigogno et al. 2002a, b, Khozin-Goldberg et al. 2002).

The *P. incisa* +N cells possessed large chloroplasts with well-formed thylakoid systems and a moderate amount of starch grains, typical of chloroplasts with active photosynthetic function. N-starvation resulted in a reduction of chloroplast size and an alteration of thylakoid membranes. Even at advanced stages of N-deprivation, despite a decrease in the overall number of granae and their stacking, the integrity of the remaining thylakoids was retained, and chloroplasts did not show signs of a deep degradation. This finding suggests that the effect of even prolonged N-starvation is reversible in *P. incisa*, making it possible for the cells to recover their photosynthetic apparatus following N-replenishment. Irradiance is likely to be a factor in how long cells can survive N-starvation. Interestingly, the thylakoid organization in N-starved *P. incisa* cells resembled that observed during adaptation of *Dunaliella tertiolecta* Butcher to high-light conditions (Berner et al. 1989).

Under N-starvation, there was a vast increase of cytoplasmic lipid globuli (oil bodies), which eventually occupied most of the cell volume. The oil bodies became more electron dense, probably due to an increase in unsaturation of the lipids (Geyer 1973). The ultrastructural changes are compatible with the hypothesis that biosynthesis of polyunsaturated fatty acids (such as AA) serves as one of the main photoassimilate sinks under N-deficient conditions, when protein synthesis and hence growth capability are restricted, and helps the algae to survive under stressful conditions without irreversible loss of viability and photosynthetic function (Cohen and Khozin-Goldberg 2005).

The analysis of chloroform extracts did not suggest the presence of measurable quantities of products with spectral features characteristic of chl pheophytinization (Lichtenthaler 1987) or photooxidation (Merzlyak et al. 1996a, b) products. At all stages, *P. incisa* had similar chl $a/b$ ratios, regardless of the availability of dissolved inorganic N in the medium. Similarly, under steady-state N-limitation, no significant changes in the chl $a/b$ ratio were reported in *D. tertiolecta* (Sosik and Mitchell 1991, Young and Beardall 2003). The spectral reconstruction showed, in addition to chl $a$ and $b$, the presence of three well-resolved bands at 433, 457, and 485 nm characteristic of Car bands III, II, and I, respectively (Britton 1985, 1995, Lichtenthaler 1987). During 60 d on N-free medium, a considerable increase of absorption in the band of 400–500 nm was observed. The STD spectrum calculated for spectra (normalized at 666 nm) of the extracts was characterized by a weak band at 426 nm, a maximum near 448 nm, and a broad band at 478–482 nm attributable to Car. Similar spectral features were observed in the difference spectra (Fig. 3b, inset) recorded in the progress of N-starvation. Altogether, these findings indicate that during N-starvation, a considerable increase of Car over chl took place.
According to the microspectrophotometry, *P. incisa* cells possess similar spectral features between 550 and 750 nm, with a pronounced band of chl at near 650 nm, irrespective of the presence of N. On average, +N cells possessed ~3-fold chl absorption as compared with −N cells. Between 400 and 550 nm, the normalized absorption of −N cells was higher, and the difference spectrum between −N and +N cells contained spectral features attributable to Car.

The normalized scattering-free absorption spectra of +N cells were essentially the same throughout the experiment, with only a small increase at short wavelengths. In contrast, significant changes in the shapes of the spectra of N-deficient cell suspensions were recorded: the chl absorption band in the red range became gradually narrower, and the relative absorption in the blue range increased considerably. The STD spectrum for normalized absorption of N-deficient cells contained two chl bands peaking near 690 and 630–650 nm in the red, and three bands at ~436, 458, and 484 nm in the blue, in which both chl and Car contribute to the absorption. As a result of the N-deficiency, relative chl absorption peaking near 436 nm increased and became more resolved, as in the spectra of individual cells. At advanced stages of N-starvation, the ratio $A_{650}/A_{676}$ in cell suspensions decreased from 0.70–0.75 to 0.57, approaching the $A_{460}/A_{676}$ ratio recorded in the spectra of individual cells (0.46–0.51). Notably, in the region of 400–500 nm, an increase of relative absorption by suspensions of −N cells was greater than in the spectra of chloroform extracts and individual cells. Collectively, the results of the spectral analysis of cell extracts and individual cells, a marked increase in the Car/chl ratio along with N-starvation, and a high correlation between $A_{480}/A_{676}$ and Car/chl ratios indicate that under N-deficiency, Car strongly contribute to the optical properties of *P. incisa* cell suspensions.

It appears that to a large extent the spectral changes recorded during N-starvation could be explained in terms of changes in the package effect as previously reported for the marine chlorophyte *D. tertiolecta* (Sosik and Mitchell 1991) and the diatom *Thalassiosira pseudonana* Hasle et Heimdal (Reynolds et al. 1997) growing under N limitation. The absorption spectrum of +N cell suspensions contained features of the package effect: it was flat, and the bands of absorption by individual pigments were poorly resolved as compared with the absorption of individual cells and pigment extracts. Apparently, the packaging of *P. incisa* is increased due to cell aggregation. As with other green microalgae (Duyssens 1956, Das et al. 1967, Naqvi et al. 2004b), for suspensions of +N *P. incisa*, the absorbance ratio in the maxima of chl $a$ absorption in the Soret band to that in the red, $A_{436}/A_{676}$, was close to 1.3. As a result of N-starvation and, thus, limited capacity for protein biosynthesis, pigment content decreased, and pigment absorption features of the cells appeared more resolved throughout the visible range; the $A_{650}/A_{676}$ ratios underwent a continuous decrease, approaching those of individual cells; and the $A_{436}/A_{676}$ ratio increased, eventually reaching 2.3. Toward the end of the experiment, this ratio reached 1.56 in the −N culture grown at irradiance of 0.67 W·m$^{-2}$. In this respect, it is of interest to note that in *Chlorella* species, according to theoretical predictions (which do not take into account heterogeneity of pigment distribution in the cell), the ratio $A_{436}/A_{676}$ in the absence of packaging is between 2 (Duyssens 1956) and 3 (Latimer 1983).

During cultivation on N-free medium, *P. incisa* underwent remarkable changes in cell ultrastructure, pigments, and fatty acid content. In many green algae, the N-deficiency-induced decrease in chl content likely affected light absorption and utilization by these organisms (Osborne and Raven 1986). As in some other species of microalgae (Osborne and Raven 1986, Sosik and Mitchell 1991), during N-deficiency, no significant changes in the chl $a/b$ ratio have been observed in *P. incisa*. This finding is in keeping with observations that during N-starvation the light-harvesting pigment–protein complex composition is conserved (Osborne and Raven 1986). In spite of the low accuracy of microspectrophotometry, our measurements showed that in the absence of N, cell absorbance at 676 nm was considerably lower than that of N-sufficient cells. Apart from the changes in pigment content and composition, the package effect in Chlorophyta is strongly influenced by the transparency and stacking degree of the thylakoid membranes (Berner et al. 1989). Similar effects in *P. incisa* cannot be excluded since considerable changes in the number and stacking of the thylakoid membranes were found in this alga under N-deficiency. Therefore, it is possible to suggest that the reduction in the chloroplast volume and cell chl content, and the changes in the ultrastructure brought about a decrease in the amount of light absorbed by individual cells and cell suspensions in the red region of the spectrum, diminishing the risk of photooxidative events.

The increase in the Car/chl ratio during N-starvation in *P. incisa*, presumably to enhance photoprotection, is consistent with previous reports on other microalgae (Thompson 1996, Young and Beardall 2003 and references therein) presumably to enhance photoprotection. Within thylakoid membranes, the protection by Car is related to the deactivation of chl excited states, quenching of singlet oxygen, interception of free radicals, and dissipation of the excessive absorbed light (Demmig-Adams et al. 1996, Niyogi 1999, Ledford and Niyogi 2005). In addition, extrathylakoid Car in plastoglobuli (Merzyk and Solovchenko 2002) and extraplastidial Car in oil bodies (Thompson 1996) are able to exert photoprotection by trapping harmful radiation.
in the blue range. It should be noted that the induction of carotenogenesis was more expressed in the N-deficient \textit{P. incisa} cells under higher irradiance: in this case, the molar Car content exceeded that of chl, and Car strongly contributed to absorption between 400 and 500 nm.

Similarly to suspensions of subchloroplast particles, chloroplasts, and cells of some algal species, the model assuming a linear combination of $A(\lambda)$, $A^{\text{NS}}(\lambda)$, and $A^{\text{SS}}(\lambda)$ allowed for a satisfactory simulation of measured extinction spectra and of \textit{P. incisa} cell suspensions grown in the presence of N. In this respect, we speculated (Naqvi et al. 2004b) that the interrelationship between absorption and scattering in a suspension of pigmented particles holds if each particle behaves as a single giant chromophore. When applied to cells containing different pools of pigments, each situated in a different refractive environment and making its own contribution to the overall absorption and scattering, this assumption does not hold. This was the case in N-deprived \textit{P. incisa} cells as well as in the green alga \textit{Haematococcus pluvialis} Flot., which in response to strong light accumulated high amounts of the secondary Car, astaxanthin, in lipid globules (Naqvi et al. 2004b). Considerable deviations were observed in the blue range of the error spectrum, with distinct spectral features of Car more apparent with the model $M_2(\lambda)$, in which the parameters of the fit were limited for the band of chl absorption in the red range. Therefore, there is reason to believe that Car located outside chloroplast thylakoids contribute to the optical properties and are involved in photoprotective mechanisms of \textit{P. incisa} at advanced stages of N-starvation.

The ability to survive under extreme environmental conditions may depend on the cell’s ability to modify its membrane composition and adjust the desaturation level of its fatty acids (Whiteman and Codd 1986, Thompson 1996). The capability to store very-long-chain polyunsaturated fatty acids in TAG as a buffering capacity would allow the organism to swiftly adapt to the rapidly changing environment. Bigogno et al. (2002b) have further speculated that the unique ability of \textit{P. incisa} to simultaneously accumulate AA and TAG allows this organism to utilize TAG as a reservoir of AA, for rapid membrane construction following sharp and fast changes in environmental conditions (Khozin-Goldberg et al. 2005). In addition, this could explain the ability of this organism to retain much of its photosynthetic activity at low temperatures.

Following prolonged N-deficiency (60 d), N-replenishment resulted within 14–16 d in values of $A_{476}/A_{676}$, $A_{530}/A_{676}$, $A_{650}/A_{676}$, and $A_{676}/A_{500}$ similar to that of the N-sufficient initial culture, suggesting complete recovery of pigment content and cell optical properties. It could be also noted that the changes of $A_{476}/A_{676}$ and $A_{650}/A_{676}$ during recovery proceeded more or less simultaneously, suggesting a synchronous adjusting of the proportion of chl to Car and chl-content-dependent “packaging” to the levels of intact cells.

Collectively, the results of the study emphasize ultimate linkage between metabolism of carbon and N (Thompson 1996, Raven et al. 2004). In particular, in \textit{P. incisa} the lower the effect of the packaging of N-dependent chl and pigment–protein complexes, the higher the accumulation of Car and storage lipids. The spectral region sensitive to and closely correlated with the changes in the relative content of AA was found in the orange-red region, in the band of chl absorption. These observations suggest that the increase in AA percentage proceeds in parallel with a decrease in cellular chl content that results in weakening of the package effect. A close positive correlation between relative absorbance of cell suspensions and Car/chl ratio was observed in a broad band between 400 and 500 nm, in which the contribution of Car into light absorption progressively increases along with the duration of N-starvation. With the absorbance ratio $A_{480}/A_{676}$, a precise estimation of the Car/chl ratio across a wide range of Car/chl ratios (from ~0.4 to 1.1) was observed irrespective of light irradiation conditions.

The results of the study indicate that in spite of complicated optics of turbid and light-absorbing systems, valuable information on nutritional status and physiological state of microalgae could be obtained as a result of nondestructive absorption spectrophotometry. The data presented show that the absorbance ratios $A_{476}/A_{676}$, $A_{500}/A_{676}$, $A_{690}/A_{676}$, and $A_{676}/A_{500}$ as well as the derivative spectra of cell suspensions, undergoing considerable changes during N-starvation and recovery, could serve as sensitive indicators of N-deficiency in \textit{P. incisa}. Moreover, the results of this study suggest that under certain circumstances it becomes feasible to monitor AA accumulation by simple spectrophotometric analysis, avoiding tedious and time-consuming chromatographic analysis. In conclusion, it should also be noted that nutritional deficiency proved to be a useful model for investigating of the effect of pigments on cell optical properties.

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