Research Article

Interactive effects of salinity, high light, and nitrogen starvation on fatty acid and carotenoid profiles in *Nannochloropsis oceanica* CCALA 804

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Flexible responses of lipid metabolism to changes in cultivation conditions characteristic of oleaginous microalgae of the genus *Nannochloropsis* render them a promising source of triacylglycerols for biodiesel (under nutrient-deprivation and high-light stress) and eicosapentaenoic acid (EPA; C20:5, *n*-3) (under nutrient sufficient conditions). We investigated the responses of fatty acid and pigment profile in the euryhaline *Nannochloropsis oceanica* CCALA 804 to the combined stresses of high light (HL), salinity (0, 27, and 40 g/L NaCl) and nitrogen deprivation. The growth in nitrogen-replete medium under HL triggered a rapid acclimation of the microalgae to the HL stress in a salinity-dependent manner associated with a moderate decrease of EPA proportion of total FA. Nitrogen starvation (i) slowed the biomass accumulation, (ii) enhanced the production of reserve lipids at the expense of chloroplast lipids, and (iii) triggered photoprotective responses of pigment apparatus in *N. oceanica*. Regardless of cultivation conditions, the stress-induced changes in pigments and fatty acid profile were highly coordinated. Nitrogen-starvation promoted total FA accumulation on the background of a marked decline in EPA and light-harvesting Car as well as up-regulation of violaxanthin cycle with concomitant rise in non-photochemical quenching. Zero-NaCl conditions appeared to be beneficial for biomass and EPA accumulation and alleviated, especially under nitrogen starvation, the effects of salinity stresses (the decline in biomass accumulation rate, content of light-harvesting carotenoids and EPA, and photosynthetic efficiency). Strategies of *N. oceanica* acclimation to stresses of different nature and their possible implications for the biotechnology of this species are discussed.

Keywords: Acclimation / Eicosapentaenoic acid / Eustigmatophyta / Salinity stress / Secondary carotenoids

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1 Introduction

Stress responses of lipid metabolism in the oleaginous microalgae of the genus *Nannochloropsis* (Eustigmatophyceae) draw increasing attention since the species of this genus are a promising feedstock for biofuel production and an efficient source of the valuable ω-3 long-chain polyunsaturated fatty acid (LC-PUFA) eicosapentaenoic acid (EPA; C20:5, *n*-3) [1]. Due to high EPA content in the chloroplast membrane lipids attained under nutrient-sufficient conditions, biomass of *Nannochloropsis* is widely used as a feed in aquaculture [2] and hold promise as a source of EPA for human nutrition [3].

Remarkable flexibility of lipid metabolism in *Nannochloropsis* microalgae exerted in response to abrupt changes in growth

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conditions makes it feasible to manipulate the quality of the microalga biomass in order to adjust its fatty acid (FA) composition for a desired application. Under favorable environmental conditions (or, in cultivation systems, under conditions facilitating the balanced growth), the photosynthetically fixed carbon is readily consumed for the construction of lipid building blocks of thylakoid membranes enriched with EPA. By contrast, under stressful conditions, the photosynthates are channeled to the biosynthesis of triacylglycerols (TAG), the major storage product, whereas carbohydrates change negligibly, e.g., by nitrogen (N) starvation [4, 5]. Under the conditions favoring rapid growth, *Nannochloropsis* species produce EPA as a major LC-PUFA of the chloroplast membrane galactolipids [6–8]. Under moderate stress and/or in the aged cultures the neutral reserve lipids TAG are accumulated containing only a few percent of EPA but rich in palmitic (16:0) and palmitoleic (16:1) acid [8, 9]. Under prolonged nitrogen starvation EPA is essentially absent from TAG. Furthermore, the changes in pigment composition observed in *Nannochloropsis* during growth under optimal conditions as well as under stress are coordinated with changes in total lipid (expressed as total FA) and carotenoid contents [10]. Therefore, insights into the interactive effects of different stressful factors affecting fatty acid profile and pigments status of *Nannochloropsis* has both important biotechnological and physiological implications.

Marine planktonic microalgae from the genus *Nannochloropsis* inhabit coastal waters and estuaries where they encounter abrupt fluctuations of salinity, light intensity, and nitrogen availability during the tidal cycle [11]. The eustigmatophytes cope with these stressors by means of coordinated metabolic adjustments including differential accumulation of osmoregulatory metabolites, redistribution of structural [7] and reserve lipids [12], and rearrangements in photosynthetic apparatus [10]. The salinity of *ca*. 0.6 M (27 g/L) NaCl is considered to be optimal for most *Nannochloropsis* species [13]. However, Pal et al. [9] demonstrated that lower salinities, e.g., characteristic of brackish water (13 g/L NaCl) are beneficial for accumulation of biomass and formation of membrane lipids with high percentage of EPA. On the contrary, high salinity in combination with high light and nitrogen starvation stresses is detrimental for neutral lipid (TAG) productivity and, in particular, for EPA accumulation. Furthermore, it was shown recently that the abrupt osmotic downshift to zero NaCl content (designated below as zero-saline conditions) enhances biomass and chlorophyll (Chl) *a* accumulation under different PAR irradiances [7].

Effects of high light (HL) stress in eustigmatophytes are of particular interest since these microalgae feature an unusual pigment composition. Thus, they contain Chl *a* as the only Chl along with violaxanthin and vaucheriaxanthin, in free or fatty acid-esterified form, as the major light-harvesting xanthophylls [14–17]. It was shown that acclimation to elevated salinities as well as to high light, and nitrogen starvation involves changes in Chl and carotenoid (Car) contents apparent as the increase in contribution of Car to spectral absorption of *Nannochloropsis* cell suspension [7, 10]. As a result, a considerable rise in Car/Chl along with accumulation of FA takes place similarly to that induced by nitrogen starvation [10], there were also reports about accumulation of secondary keto-carotenoids in the stressed *Nannochloropsis* cells [15].

To elucidate further the physiological mechanisms of acclimation of *Nannochloropsis* to the combined stresses, we investigated the effects of NaCl concentration, paying particular attention to the effects of cultivation in zero-saline media on FA and Car profiles, in the course of acclimation of *N. oceanica* to HL and nitrogen-starvation. Furthermore, the impact of zero NaCl content on the Car profile and acclimation of photosynthetic apparatus to HL has not been previously studied, to the best of our knowledge, in the N-depleted cultures of the algae of the genus *Nannochloropsis*.

2 Materials and methods

2.1 Strain, cultivation conditions, and experimental design

Nannochloropsis oceanica CCALA 804 was cultivated in artificial sea water (ASW)-based medium supplemented either with 0, 27, or 40 g/L NaCl [7, 9]. ASW with NaCl omitted was designated as Zero-Saline (ZS) medium; the medium supplemented with 27 or 40 g/L NaCl as Normal (NS) or High-Saline (HS) medium, respectively; the initial nitrate (KNO_3) content in all N-replete (+N) media was 1.5 g/L (Table 1). In the experiments on N starvation nitrate was omitted from the media (−N). Nitrate content as estimated by a nitrate test kit (Merck KGaA, Darmstadt, Germany) was depleted in the +N media within 4–5 days of the batch cultivation, prior the stationary phase.

Cultures were grown in 1-L glass columns (6 cm internal diameter) placed in a temperature-controlled water bath at 25°C and bubbled with a mixture of 2% CO_2 in air v/v. Continuous illumination was provided bilaterally by cool white fluorescent lamps external to the water bath at 700 $\mu mol\, m^{-2}\, s^{-1}$ PAR. Light intensity was measured at the center of an empty column with a LiCOR LI-850

Table 1. Composition modifications and designation of the ASW-based media used in this work

	Initial KNO_3 (g/L)	
NaCl (g/L)	1.5	0
0	ZS + N	ZS − N
27	NS + N	NS − N
40	HS + N	HS − N

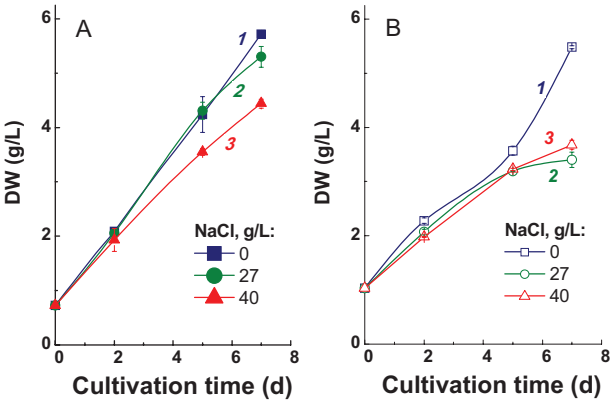

Figure 1. Biomass volumetric content of *N. oceanica* in (A) complete and N-free (B) ASW-based media containing (1) 0, (2) 27, or (3) 40 g/L NaCl under 700 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR.

quantum meter (Lincoln, USA). The initial semi-continuous cultures were diluted daily with fresh NS + N medium (to 15 g/L Chl *a*; *ca.* 0.2×10^9 cell/mL grown at 170 μmol PAR photons $m^{-2}\,s^{-1}$). At the onset of each experiment, cells were harvested by centrifugation ($1200 \times g$ for 5 min), washed twice in double-distilled water (DDW) and resuspended in the corresponding medium to the initial Chl *a* concentration and biomass content of 15 and 0.8 g/L, respectively. Under the specified conditions, at least three independent experiments were carried out for each treatment repeated in duplicate columns.

Estimation of biomass accumulation in the N-replete and N-free media was based on dry weight measurements [7]. Dry weight (DW) was determined as follows: 5-mL samples were washed with 35 mL of DDW and deposited on a pre-weighed 47-mm glass fiber paper filters (Sartorius Stedim Biotech, Goettingen, Germany). Filters were dried in a microwave oven to constant weight.

Contents of Chl *a* and total Car were measured spectrophotometrically in DMSO extracts [18] using a UV–Visible spectrophotometer (Cary 50 Bio Varian, Mulgrave, Australia).

2.2 Pigments analysis

Individual Car were extracted and analyzed using HPLC according to earlier published protocols [19]. Briefly, the HPLC apparatus was comprised by a Prostar 240 solvent-delivery module and Prostar 330 photodiode array detector (Varian Analytical Instruments, Walnut Creek, CA, USA) and a C18 reverse-phase column (5 mm, 250 mm Lichrosphere 100, Merck, Darmstadt, Germany). The system containing (A) acetonitrile : water (85:12 v/v) and (B) ethylacetate was used for gradient elution of pigments. A flow rate of 1 mL/min and a two-step linear solvent gradient from 0 to 30% B (18 min), then from 30 to 100% B (6 min), with a 6-min hold at the final concentration was used. Pigments were identified and quantified using pure pigment standards (β-carotene and zeaxanthin from Sigma–Aldrich, St. Louis, MO, USA; other from DHI, Hørsholm, Denmark).

2.3 Chlorophyll fluorescence measurement

Induction curves of Chl fluorescence (Suppl. Fig. 1) were recorded and analyzed using a Fluorpen FP100s portable Pulse Amplitude Modulated fluorometer (Photon Systems Instruments, Drasov, Czech Republic) as described earlier [20]. Non-photochemical quenching (NPQ) was calculated as $NPQ = F_m/F_m' - 1$ ([21], see also suppl. Table 1).

2.4 Fatty acid analysis

Transmethylation of fatty acids was performed by incubating freeze-dried biomass in dry methanol containing 2% v/v H_2SO_4 at 80°C for 1.5 h under argon atmosphere with continuous stirring. Heptadecanoic acid (C17:0) (Fluka, Buchs, Switzerland) was added as an internal standard. FAME were quantified on a Trace GC Ultra (Thermo, Milan, Italy) equipped with a flame ionization detector (FID) and programmed temperature vaporizing (PTV) injector as previously described [9].

2.5 Statistical treatment

The results of three independent experiments are presented in the figures. Where appropriate, averages and standard errors of the mean were calculated and displayed. All correlations are significant at $p<0.001$ level.

3 Results

3.1 Growth of the cultures

Growth curves of *N. oceanica* are shown in Fig. 1. In N-replete media, the culture grown in zero-saline (ZS + N) medium attained the highest average biomass accumulation rate in terms of cell dry weight (DW) increase ($0.82\,g\,L^{-1}\,day^{-1}$; curve 1 in Fig. 1A), a similar biomass accumulation rate ($0.76\,g\,L^{-1}\,day^{-1}$; curve 2 in Fig. 1A) was shown by the culture in the NS + N medium. A lower biomass accumulation rate of $0.64\,g\,L^{-1}\,day^{-1}$ (curve 3 in Fig. 1A) was displayed by *N. oceanica* in the highly-saline N-replete (HS + N) medium. Remarkably, in the N-free medium lacking NaCl (ZS–N; curve 1 in Fig. 1B), the biomass accumulation kinetics of *N. oceanica* was similar to that in N-replete medium. There was no significant difference between the patterns of biomass accumulation in the NS–N (curve 2′ in Fig. 1B) or HS–N (curve 3′ in Fig. 1B) conditions. As a result, following average rates of biomass accumulation were recorded in all cultures grown in N-free media (0.78, 0.49, and $0.53\,g\,L^{-1}\,day^{-1}$ for the ZS – N,

NS – N, and HS – N cultures, respectively). Remarkably, accumulation of biomass by the ZS + N and ZS – N cultures did not level off within 7 days of cultivation whereas the growth of the NS and HS cultures generally slowed down after the 2nd day of cultivation suggesting the onset of stationary phase (curves 2,2′ and 3,3′ in Figs. 1A and B).

3.2 The changes in fatty acid content and composition

The rapid biomass accumulation of the cultures in N-replete media was accompanied by a small increase in total fatty acid (TFA) percentage of DW (curve 1 in Fig. 2A). In the cells grown in the NS + N medium, TFA content increased after the 5th day of cultivation (curve 2 in Fig. 2A). High salinity (HS + N) in combination with HL induced the increase in TFA proportion of DW which took place earlier and occurred at a higher rate in comparison to the cultures grown in the less saline media (curve 3 in Fig. 2A). In N-depleted media, the kinetics of the changes in TFA (% DW) of the culture subjected exposed to N-starvation under zero-saline conditions (ZS – N; curve 1′ in Fig. 2A) was slower than that of NS – N and HS – N and similar to that in the NS + N culture (curve 2 in Fig. 2A). The highest and most rapid increase in TFA proportion of DW was recorded in the NS – N and HS – N cultures which gained close final TFA percentages of 35–40% DW (curves 2′ and 3′ in Fig. 2A).

Generally, as expected for cultivation under HL favoring accumulation of TAG, a decline in the proportion of EPA (% of TFA) of *N. oceanica* with concomitant increase in the proportions of 16:0 and 18:1 was recorded in all cases studied (Fig. 2B). Under the N-replete conditions, the effect depended on salinity level: the higher was the NaCl content in the medium, the more profound and rapid was the decline in EPA proportion (curves 1–3 in Fig. 2B). The highest retention of EPA was recorded in the ZS + N cultures (from 23 to 18% at the culture initiation and 7 day, respectively). In the HS + N cultures EPA declined by a half (to *ca.* 12% of TFA) by the 2nd day of cultivation but recovered to a certain degree later on (curve 3 in Fig. 2B). The cultures grown in saline media (NS + N and HS + N) attained a similar final EPA proportion of ca. 15% (curves 2 and 3 in Fig. 2B). Under N-starvation conditions EPA proportion decreased sharply (to 7–10% of TFA) within the first 2 days of cultivation and did not change significantly thereafter regardless of the medium salinity (curves 1′ and 2′ in Fig. 2B). Notably, EPA tended to increase in the cells grown in the ZS – N medium (curve 1′ in Fig. 2B).

The trends of volumetric contents of TFA and EPA calculated on the base of the biomass accumulation (Fig. 1) and the changes of TFA content therein (Fig. 2A and B) are presented in Fig. 2C and D, respectively. As it obvious from Fig. 2C, the volumetric TFA content increased almost linearly to 1200–1600 mg/L in a salinity-dependent manner (Fig. 2C).

The changes in the EPA content followed different trends depending on N availability in the medium. In the N-replete media an increase in EPA volumetric content at a nearly constant rate (curves 1–3 in Fig. 2D) took place along with the culture growth and concomitantly with TFA. Only in the ZS + N the rate of EPA accumulation decreased slightly after 5 days of cultivation (curve 1 in Fig. 2D). As expected, the N-depleted cultures demonstrated a lower EPA accumulation (curves 1′–3′ in Fig. 2D) although the EPA content tended to increase exclusively in the ZS – N culture (curves 1′ in Fig. 2D).

The analysis of FA composition showed that the most profound changes in FA profile were comprised by an increase in the palmitate (16:0) and oleate (18:1) relative proportions on the background of the decline in EPA proportion; the extent of these changes generally depended on the salinity and N availability (Fig. 3) with 16:0 remaining the major FA (ca. 30% TFA at time 0). Under our experimental conditions, the accumulation of 16:0 and 18:1 was slightly higher in the N-starved cultures whereas EPA proportion changes more significantly. More EPA was retained under zero-saline conditions by day 7 of HL

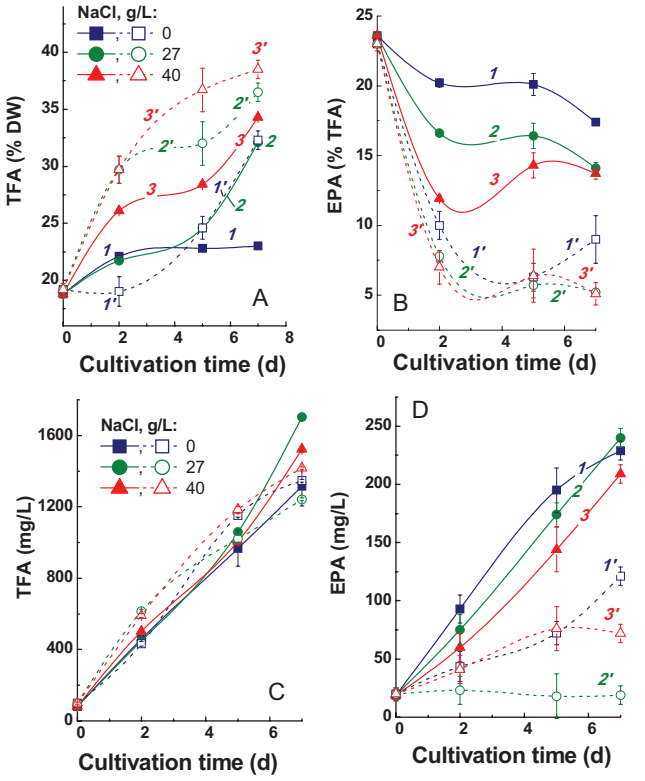

Figure 2. Time-course of changes in (A) TFA percentage of dry weight, (B) EPA percentage of TFA, and volumetric content of (C) TFA and (D) EPA in the cells of *N. oceanica* grown in complete (1–3) and N-free (1′–3′) ASW-based media containing (1, 1′) 0, (2, 2′) 27 or (3, 3′) 40 g/L NaCl.

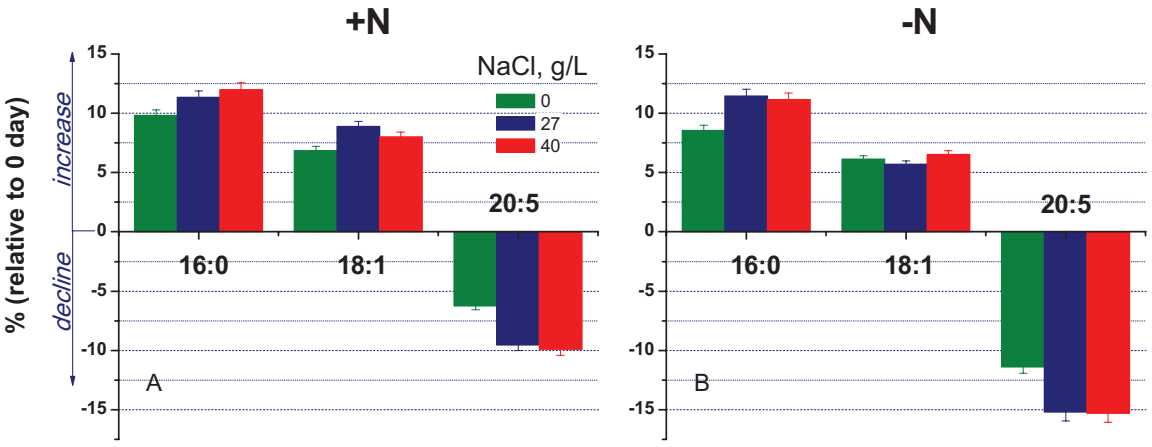

Figure 3. Changes in the proportions of palmitic (16:0), oleic (18:1), and eicosapentaenoic (20:5 *n*-3) acids in the fatty acid profile of *N. oceanica* cells after 7 days of growth in (A) complete and N-free (B) ASW-based media (the NaCl content is specified at the diagram).

exposure; the highest EPA retention was observed in the ZS + N medium (Fig. 3A).

3.3 The dynamics of chlorophyll and total carotenoid content

The time-course of changes in the contents of Chl, total Car per DW unit as well as their ratio is plotted in Fig. 4. A sharp decrease in the pigment content occurred within the first 2 days of cultivation under HL in all cases (Fig. 4A and B). Under the N-replete conditions, the lag was followed by a prominent rise in the pigments' contents (curves 1–3 in Fig. 4A and B). Consequently, the Car/Chl ratio, a sensitive marker of stress in *Nannochloropsis* [10], did not change significantly (Fig. 4C); only the HS + N culture displayed a steep rise in Car/Chl ratio within the first 2 days of cultivation (curves 2 in Fig. 4C). In the N-starved cultures, Chl and total Car contents decreased monotonously and leveled off by the 7th day of cultivation; the rate of Chl decrease (curves 1′–3′ in Fig. 4A) was higher than that of Car (curves 1′–3′ in Fig. 4B) resulting in the sharp increase in Car/Chl during the first 5 days of cultivation shown in Fig. 4B (curves 1′–3′). The rise of Car/Chl was followed by a drop which was the most prominent in the ZS–N culture indicative of lower stress intensity in this culture (Fig. 4C, curves 1′–3′).

It is important to note that, under all the stressful conditions studied, the changes of total Car contents exhibited a strong linear (r^2>0.97) relationship with the changes in Chl (Fig. 5A) suggesting that the changes in the pigment contents occur in a highly synchronous manner and there is a limited build-up of secondary Car – the Car that are localized outside chloroplast thylakoid membranes and do

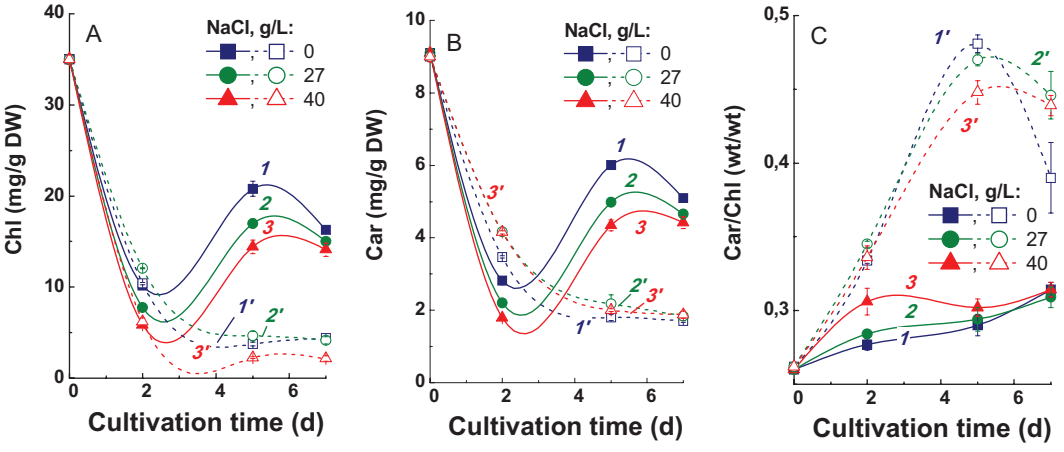

Figure 4. Time-course of changes in (A) chlorophyll and (B) total carotenoid contents per unit dry weight as well as (E) carotenoid-to-chlorophyll ratio in the cells of *N. oceanica* grown in N-replete (1–3) and N-free (1′–3′) ASW media containing (1, 1′) 0, (2, 2′) 27, or (3, 3′) 40 g/L NaCl.

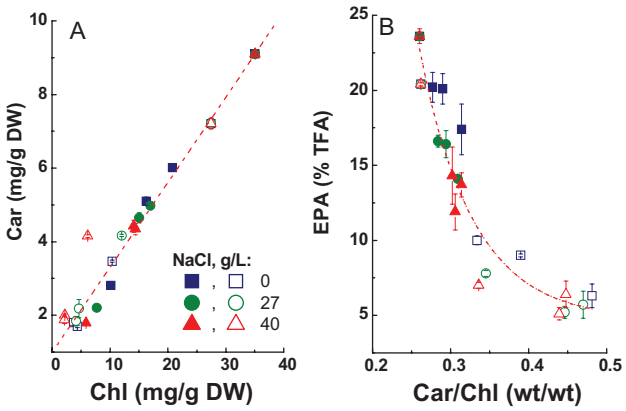

Figure 5. Relationships between the changes (A) in carotenoid and chlorophyll content and (B) EPA percentage of TFA and carotenoid-to-chlorophyll ratio in the in the cells of *N. oceanica* grown in complete (closed symbols) and N-free (open symbols) ASW-based media containing (squares) 0, (circles) 27, or (triangles) 40 g/L NaCl.

not participate in photosynthesis [22]. At the same time, a strong nonlinear relationship (r^2>0.95) was found between EPA percentage of TFA and Car/Chl ratio (Fig. 5B) presumably reflecting the obvious relationship between the decomposition of the EPA-enriched lipids constituting the thylakoid membranes and photosynthetic pigments contained therein during the acclimation of *N. oceanica* to the stress.

3.4 Changes in the carotenoid profile and thermal dissipation of the absorbed light energy

The Car profile of *N. oceanica* was comprised by violaxanthin (Vio), antheraxanthin (Anth), zeaxanthin (Zea), vaucheriaxanthin (Vau), and its fatty acid esters (VE) as well as β-carotene (Fig. 6). Seven-day cultivation under HL in the N-replete media exerted a relatively small effect on the Car composition of the algae, the most conspicuous changes included *ca*. 10-% decrease in the proportion of Vio, the major light-harvesting xanthophyll of *Nannochloropsis* [14, 15] under high salinity (Fig. 6D) in comparison with the

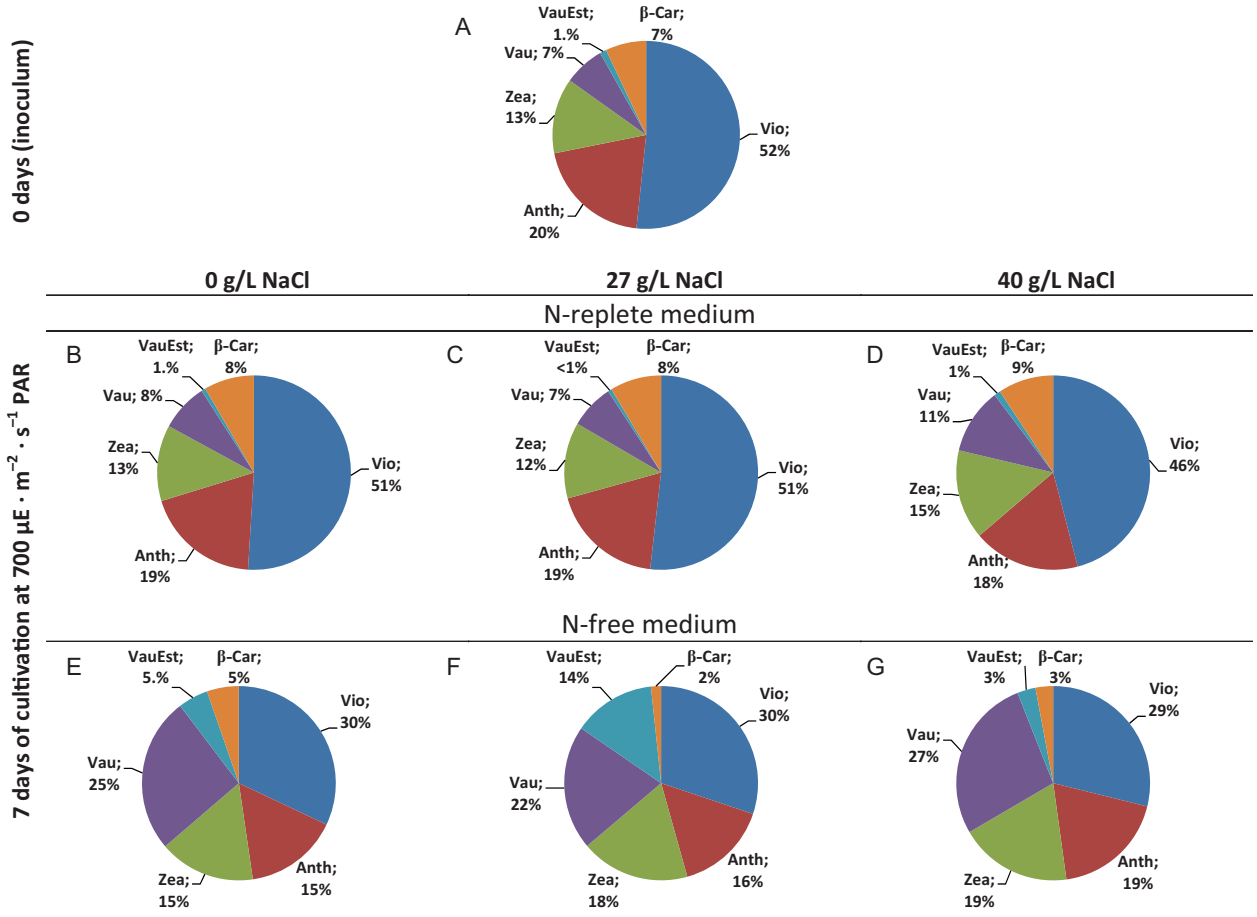

Figure 6. Carotenoid profile of *N. oceanica* cells at inoculation (0 day, A) and after 7 days of growth in (B–D) complete and N-free (E–G) ASW-based media containing (B, E) 0, (C, F) 27, or (D, G) 40 g/L NaCl.

inoculum (Fig. 6A) suggesting the decrease in light-harvesting antenna size.

More prominent changes were detected in the Car profile of the N-starved cells (Fig. 6E–G). In particular, the proportion of Vio comprised ca. one half of that recorded at the day 0 indicating more profound shrinking of the antenna in comparison with N-sufficient cultures. A notable decline in β-carotene in the cells grown in saline media lacking N (NS – N and HS – N, Fig. 6F and G) could be a manifestation of a decline in the amount of reaction centers [14]. At the same time the proportion of Vau increased threefold to fourfold and reached, on an average, 25% of total Car regardless of NaCl content in the medium. The most conspicuous relative increase was displayed by Vau esters (up to 14-fold in the NS – N cultures, Fig. 6F) but their absolute amount remained modest (<1.5 mg/g DW) further supporting the suggestion of low accumulation of secondary Car.

The extent of Vio de-epoxidation (DE, Fig. 7) did not rise significantly under N-replete conditions remaining at the level of 10–15%. Correspondingly, the level of non-photochemical quenching (NPQ) in these cultures did not exceed *ca*. 0.4 till the end of the experiment. By contrast, a considerable rise of DE was detected (to 25–30%) after 5 days of cultivation under N-starvation conditions which was accompanied by a gradual increase in NPQ; the higher was the salinity of the medium, the higher was the NPQ level attained by the corresponding culture by 7th day of N-starvation (Fig. 7).

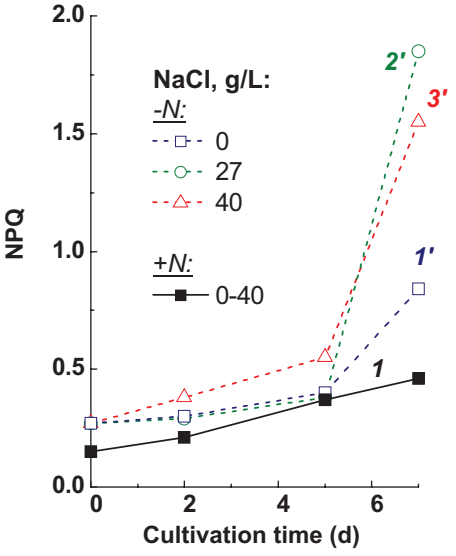

Figure 7. The time-course of changes of NPQ level in the cells of *N. oceanica* grown in N-free media containing (squares) 0, (circles) 27, or (triangles) 40 g/L NaCl in comparison with the cells grown in N-replete media (closed symbols). A single trend is shown for all the cultures grown in the N-replete media at different salinities since the NPQ values recorded in these cultures did not differ significantly.

4 Discussion

The capability of the marine eustigmatophyte *N. oceanica* of growth in zero-saline media was demonstrated recently [7] and confirmed in the present work (Fig. 1). Remarkably, the accumulation of biomass in N-lacking media was commensurate to that of the culture grown on N-sufficient media (*cf.* curves 1 in Fig. 1A and B). Pal et al. [7] suggested that enhanced growth of *N. oceanica* following osmotic downshift, especially under higher PAR, is associated with adjustments of the pigment apparatus and membrane lipid composition of chloroplasts facilitating photosynthetic carbon fixation and its allocation to proteins and structural lipids rather than osmolytes and inert carbon reserves.

For the acclimation to changing salinity, *N. oceanica* employs a strategy evolved to survive in surface water layers. This strategy involves a number of mechanisms including the capability of rapid adjustment of cellular lipid metabolism and of swift modification of the distribution and degree of unsaturation of membrane lipids [7]. In particular, the content of chloroplast lipids often decreases under high salinity stress [12] as well as photosynthetic pigment content [10, 23]; in *Nannochloropsis* these responses could be exacerbated by additional stressors such as high light intensity and/or nitrogen deficiency [9]. Generally, cultivation of *N. oceanica* under HL was accompanied by a decline in EPA associated largely with the chloroplastic lipids on the background of the increase in palmitate and oleate (Fig. 3) reflecting the accumulation of storage TAG [7]. The enhanced biosynthesis of TAG probably served as a sink for excessive photosynthates mitigating the risk of photo-oxidative damage under conditions limiting cell division (N deprivation). Indeed, a tight relationship was found between the decrease of EPA and the gradual decline in Chl and Car under all experimental conditions studied in this work (Fig. 4A and B) suggesting that similar mechanisms participate in the acclimation of *N. oceanica* to HL, extreme salinity, and N starvation as well as to combinations of these stressors. The responses of pigment and lipid metabolism involved in these mechanisms are executed in a highly coordinated manner.

Remarkably, the cells grown on the ZS media displayed the smallest decline in the percentage and the highest volumetric content of EPA together with the lowest accumulation of 16:0 and 18:1 (Fig. 3). Pal et al. [9] have previously reported the enhancement in growth and EPA content in parallel with the decreased TFA content by lowering the salinity to 50% of that in ASW, indicating the prevalent formation of chloroplast membrane lipids over the storage TAG. Accordingly, we recorded low TFA accumulation in the cells of the microalga under the N-replete conditions (Fig. 2A). However, the enhancement of growth rate under lower salinities resulted in similar volumetric TFA productivity values for all conditions studied (Fig. 2C). This finding is also important for the choice of cultivation

conditions for *Nannochloropsis* since these microalgae gain an increasing importance as promising organisms for EPA and biodiesel production due to their environmental plasticity [1, 2, 24]. The ability to thrive in a freshwater environment described in [7, 9] and confirmed here further extends the possibilities of the application and might have valuable commercial implications for utilization of *Nannochloropsis* species in algal- and fish-growing facilities where cost-efficient cultivation on marine water is not feasible.

Previously we found that acclimation of *N. oceanica* to high-light and salinity stresses induced the increase of light absorption by the algal cells in the blue-green part of the spectrum suggesting the increase in Car content [7]. Similar spectral changes were recorded in the chlorophytes *Parietochloris incisa* [18] and *Desmodesmus* sp. [25]. To further elucidate the physiological meaning of the observed changes in pigment composition, individual Car species were analyzed (Fig. 6). Seven days of cultivation under HL under N-replete conditions brought about only a modest alteration of Car profile with *ca.* twofold increase in Vau as the most striking change. By contrast, the N-starving culture displayed a strong decrease of β-carotene and Vau indicative, together with the decline in Chl, of the shrinking of the light harvesting antenna, a HL response common for microalgae. Since Vau is believed to be, together with Vio, a major light-harvesting xanthophyll in *Nannochloropsis* [15] and in the absence of a sizable accumulation of secondary Car we hypothesized that the light-harvesting antenna in the stressed *N. oceanica* was not only down-regulated under the stressful conditions but underwent a remodeling as well. One may speculate that the light-harvesting pigment–protein complexes with increased Vau/Vio ratio are more stable and/or somehow facilitate thermal dissipation of the light energy absorbed in excess. This suggestion is indirectly supported by the elevated NPQ levels recorded in the N-starving cells. It remains to be elucidated to which extent the changes in the FA pertinent to membrane lipids recorded in the stressed *N. oceanica* (Figs. 2 and 3; see also [7]) are related with the rearrangement of its Car profile under the stress. At the same time, the most significant rise of NPQ was recorded after a prolonged stress exposure (Fig. 7). Therefore, the contribution of photoinhibitory quenching (qI) to the overall quenching could be significant in this case taking into account a notable rise of Fo under the stress at day 7 (Suppl. Table 1). Further experiments on recording kinetics of the dark recovery of variable fluorescence are required to estimate the contribution of qI and to distinguish it from the quenching due to remodeling of the photosynthetic apparatus and de-epoxidation of xanthophylls.

It should be noted that the salinity stress *per se* did not exert a measurable effect on Car composition of the cells grown in N-replete media. On the other hand, in the N-starving cells the salinity stress did not alter the proportions of major light harvesting Car but increased the proportions of Anth and Zea. Taking into account that eustigmatophytes are characterized by operational violaxanthin cycle, this might imply the enhanced de-epoxidation of Vio into Zea. It is difficult to determine precisely the extent of Vio de-epoxidation in the case of eustigmatophytes because it is hardly possible to differentiate the pools of Vio involved in light harvesting and in the dissipation of the excess light energy *via* operation of the xanthophyll cycle. Nevertheless, our estimations of the engagement of the violaxanthin cycle under the stressful conditions based on HPLC pigment assay were in accord with the levels of non-photochemical quenching (NPQ) as assessed by means measurement of variable fluorescence of Chl in the cells of *N. oceanica*. Still, direct measurements of epoxidation/de-epoxidation (as well as variable fluorescence recovery) kinetics are needed to elucidate the significance of violaxanthin cycle in the build-up of NPQ under the stress.

Indeed, the salinity stress under HL conditions exerted somewhat limited effect on thermal dissipation of the light energy absorbed by the microalgal cells grown on the N-replete media ($NPQ < 0.4$) which is in accord with the results obtained by Martínez-Roldán et al. [23] for *Nannochloropsis* sp. On the contrary, N deprivation led to a conspicuous rise of NPQ after five days of cultivation in salinity-dependent manner (Fig. 7). Importantly, the magnitude of this rise was inversely related to the salinity level: the smallest increase in NPQ was recorded in the culture grown in ZS media (Fig. 7, curve 1). In view of these findings one can assume that zero-saline conditions facilitate the efficient photochemical utilization of the absorbed light energy hence the culture grown in ZS media feature higher content of light-harvesting pigments per unit DW (Fig. 4a) or per cell [9].

The presence of xanthophyll (mainly Vau) fatty acid esters (VE) is characteristic of the Car profile of *Nannochloropsis* [15]. Like non-esterified Vau, VE may present in the pigment–protein complexes of the thylakoid membranes [14] but do not participate in light harvesting in *Nannochloropsis* [16]. It is also likely that VE are associated with cytoplasmic lipid droplets similarly to fatty esters of astaxanthin in the chlorophyte *Haematococcus pluvialis* [26, 27]. The free-to-esterified Vau ratio varies in different *Nannochloropsis* strains from ca. 1:1 to 1:4 [15] but under our experimental conditions VE content remained very low (ca. 1% of total Car) during cultivation of the cells in N-replete media (Fig. 6B–D). Nitrogen deprivation increased VE content to ca. 5–14% of total Car in salinity-independent manner (Fig. 6E–F). In other microalgae xanthophyll esters massively accumulating under stress can protect the cells from photooxidative damage [28]. It is unlikely that VE fulfill a similar function in *N. oceanica* since their content is too low (less 15% of total Car), so the physiological meaning of the limited accumulation of VE under N starvation remains unclear. It is conceivable that the enhancement of VE formation during N-starvation results just from the increased availability of the substrates for VE biosynthesis – free FA and

Vau molecules originating from the thylakoids dismantling in the course of acclimation of the microalgae to HL.

Collectively, the results obtained in this work suggest that the reduction of the light-harvesting antenna and the adjustment of chloroplast membrane composition are important factors of stress tolerance of *N. oceanica*, in addition to the earlier described mechanisms [7]. Other mechanisms include the up-regulation of thermal dissipation of the absorbed light energy and channeling of the excessive photosynthates to the biosynthesis of reserve lipids. Depending on the combination of the stresses, different mechanisms are engaged allowing the cells to withstand nitrogen starvation and extreme salinities on the background of high irradiance. Thus, the xanthophyll cycle was up-regulated only by high salinity in combination with N deprivation; apparently it is not required during growth in N-replete media.

Finally, an important finding of the present work is that, in line with our previous results [7], zero-saline conditions under HL enhance the culture growth, regardless of the presence of N. Zero-saline conditions also seem to alleviate the deteriorative effect of the stresses on EPA-enriched chloroplast lipids and photosynthetic apparatus in *N. oceanica*. The enhanced biomass accumulation under N-starvation in ZS cultures could be associated with (i) the lower N demand for the biosynthesis of nitrogenous osmolytes and stress-responsive metabolites, such as proline and spermidine [7], and (ii) a lower requirement for carbon flux shuffling for the production of the major sugar mannitol that was determined by metabolite analysis in the previous study and, hence, more carbon and nitrogen availability to sustain biomass production.

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