RESEARCH ARTICLE

Response of arctic snow and permafrost algae to high light and nitrogen stress by changes in pigment composition and applied aspects for biotechnology

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Abstract

Ten algal strains from snow and permafrost substrates were tested for their ability to produce secondary carotenoids and α-tocopherol in response to high light and decreased nitrogen levels. The Culture Collection of Cryophilic Algae at Fraunhofer IBMT in Potsdam served as the bioresource for this study. Eight of the strains belong to the *Chlorophyceae* and two strains are affiliated to the *Trebouxiophyceae*. While under low light, all 10 strains produced the normal spectrum of primary pigments known to be present in Chlorophyta, only the eight chlorophyceaen strains were able to synthesize secondary carotenoids under stress conditions, namely canthaxanthin, echinenone and astaxanthin; seven of them were also able to synthesize minor amounts of adonixanthin and an unidentified hydroxyechinenone. The two trebouxiophyceaen species of *Raphidonema* exhibited an unusually high pool of primary xanthophyll cycle pigments, possibly serving as a buffering reservoir against excessive irradiation. They also proved to be good α-tocopherol producers, which might also support the deactivation of reactive oxygen species. This study showed that some strains might be interesting novel candidates for biotechnological applications. Cold-adapted, snow and permafrost algae might serve as valuable production strains still exhibiting acceptable growth rates during the cold season in temperate regions.

Introduction

Snow algae are a group of freshwater algae representing several genera that are capable of thriving on semi-permanent to permanent snow and ice fields in polar and alpine regions (Kol, 1968). On snow substrates, a variety of different algal taxa can be found, generally called snow algae. Regarding their temperature requirements, one has to differentiate between psychrophilic and mesophilic snow algae. Psychrophiles (also termed cyrophiles) show temperature optima for their growth below 15 °C and die at higher temperatures (see also Hoham & Duval, 2001; Leya, 2004), while the mesophiles, formerly often addressed as nonobligate cryophiles, frequently represent permafrost algae and have a temperature optimum above 15 °C.

In spite of their considerable taxonomic diversity, snow algae share one main common property: their tolerance to the low temperature of their substratum, which reaches around 0 °C during the vegetative growth phase. At freezing temperatures, the algae are exposed to dehydration and osmotic stress. The first is a result of the high saturation deficiency of the air at the snow/air interface with water vapor; the latter is due to freezing of the intercrystalline water in snow and the enrichment of dissolved ions in the remaining thin water film that forms the living space for snow algae (Pomeroy & Brun, 2001). Light also exerts a considerable influence on the physiology of snow algae. Despite the high albedo of snow, light intensities in the upper layer of a snowfield are considerably elevated in comparison with uncovered permafrost soil. Because of the refraction of light, photon fluency rates within snow can reach values two or three times that of the incidence of photon irradiance on the snow surface (Gorton *et al.*, 2001). It is evident that snow algae have developed strategies to contend with these different stresses by adaptation of their cell morphology and metabolism. A number of snow algae

from the genera *Chlamydomonas* and *Chloromonas* (*Chlorophyceae*) are known to produce various secondary carotenoids in their vegetative and generative resting stages, such as the red-colored astaxanthin, known to be the main pigment causing the phenomenon of red snow (Bidigare *et al.*, 1993; Müller *et al.*, 1998; Remias *et al.*, 2005). In contrast, other typical snow algae such as *Raphidonema* spp. (*Trebouxiophyceae*) are unable to develop such orange- or red-colored cell types (Kol, 1968; Ettl & Gärtner, 1995).

Carotenoids play a major role in chloroplasts of all algae and higher plants to maintain photosynthesis, as they serve as structure stabilizers of pigment–protein complexes (Frank *et al.*, 1999). All carotenoids involved in photosynthesis are classified as primary carotenoids. It has been demonstrated that in the presence of light, mutants lacking primary carotenoids degenerate quickly due to oxidative stress (McCarthy *et al.*, 2004). Especially, green algae *sensu stricto* (*Chlorophyceae*) show a huge variety of secondary carotenoids, which are localized in cytoplasmic globules and are frequently esterified with fatty acids (Remias *et al.*, 2005). The synthesis of secondary carotenoids is regulated by a number of different environmental factors such as exposure to high light, nitrogen depletion, oxidative stress, pH, high salinity or a drastic change in temperature (Britton *et al.*, 1998; Ip & Chen, 2005). Common members of this pigment group are astaxanthin, canthaxanthin and echinenone. Their ecophysiological role is not yet fully understood; however, they are supposed to play a role in stress response such as shielding the photosystem against excessive irradiation (Cohen, 1999). α-Tocopherol is the main vitamin E compound and is produced by cyanobacteria and plants. Both carotenoids and tocopherols are lipophilic and have powerful antioxidant properties (Miki, 1991). These properties have enabled their use in human medication as well as agri- and mariculture (Edge *et al.*, 1997).

To date, pigment studies on snow algae have only analyzed the pigment patterns of resting stages from field samples (Bidigare *et al.*, 1993; Müller *et al.*, 1998; Remias *et al.*, 2005). Comparisons of pigments produced in cultures in the trophic stages (green and actively growing) under low light conditions with those produced after exposure to high levels of light and under low nitrogen levels have not yet been performed. This is also due to the fact that snow algae, and especially psychrophilic strains, are rare in most algal collections worldwide.

This study focuses on the trophic stages of 10 snow and permafrost algal strains from polar habitats to quantify the shift in secondary carotenoid and α-tocopherol synthesis in response to high light and decreased nitrogen levels. We hypothesize demonstration of alternative adaptation strategies between the *Chlorophyceae* and the *Trebouxiophyceae*. Also, especially potent strains with a high antioxidant production capacity had to be identified, which could make them valuable production strains in biotechnology. The effect of UV light was not studied because secondary carotenoids hardly absorb these wavelengths. An HPLC method was used to analyze both carotenoids and tocopherols in a single run (Remias & Lütz, 2007).

Materials and methods

The Culture Collection of Cryophilic Algae (CCCryo) at the IBMT in Potsdam-Golm (Germany) served as the bioresource for the algal species used for this screening. All strains were originally cultured at 2 °C at the CCCryo and were clonal and axenic, i.e. bacteria free. Ten snow algal strains from different taxonomic groups were investigated in this study (Table 1). Six of them were collected from snowfields (strains 005-99, 011-99, 112-00, 138-01, 140-01 and 147-01) and four from permafrost substrates (strains 006-99, 039-99, 101-99 and 105-99).

Culture conditions and stress induction

Culture temperature was set to 10 °C during all the experimental stages to obtain sufficient growth rates for both psychrophilic and mesophilic strains. All cultures were grown under axenic conditions. Precultures in the mid-logarithmic growth phase were used to inoculate 1 L of sterile culture medium [Bold's basal medium (Bischoff & Bold, 1963) modified according to Starr & Zeikus (1987) and with triple-concentrated nitrate (9 mM nitrate), pH 5.5] in 2-L round-bottom flasks. The actively growing cultures in the green phase were incubated in a plant cabinet (Percival, model no. I-36 VLX; CLF Plant Climatics GmbH, Emersacker, Germany), illuminated with fluorescent tubes (type Universal/HIVision TL741; color temperature cool white, T = 4100 K; Philips GmbH, Hamburg, Germany) at a continuous photosynthetic active radiation (PAR) of 120–220 μmol photons m^{-2} s^{-1} (LI-COR light meter LI-250 with spherical sensor US-SQS/L, Heinz Walz GmbH, Effeltrich, Germany) and vented with sterile-filtered air. Samples for pigment analyses and dry mass determination were harvested during the logarithmic phase. All sampling and processing steps were carried out under dim light. The remaining culture volume was left standing until most cells had sedimented, and then the supernatant medium was decanted. For the second phase, the red phase, 250-mL wash bottles (outer diameter = 55 mm) were sterilized and 200 mL nitrogen-reduced (0.08 mM nitrate) Errötungsmedium, pH 5.5, (modified from Henning-Emmel, 1994) was added. The sedimented cell material of the green phase was transferred to new culture bottles, closed with wash bottle tops and returned to the plant cabinet. The PAR was adjusted to 150–300 μmol photons m^{-2} s^{-1} for the next 24 h. High light conditions were then applied by increasing the PAR to 800–960 μmol photons m^{-2} s^{-1}. All other parameters were

Table 1. Listing of arctic snow and permafrost algal strains used in this study

CCCryo strain no.	Class, phylum Taxon	Properties	Growth rate μ (day^{-1})	Origin
Chlorophyceae, Chlorophyta				
005-99	*Chloromonas nivalis* Hoham et Mullet	Psychrophilic T_{max} = above 18 °C T_{opt} = 13 °C	0.6	Spitsbergen icy snowfield
006-99	cf. *Chlorococcum* sp.	Psychrophilic T_{max} = above 18 °C T_{opt} = 8 °C	0.2	Spitsbergen permafrost
039-99	cf. *Chlorococcum* sp.	Psychrophilic T_{max} = above 18 °C T_{opt} = 8 °C	0.4	Spitsbergen permafrost
105-99	cf. *Chlorococcum* sp.	Mesophilic T_{max} = above 23 °C	ND	Spitsbergen permafrost
140-01	cf. *Chlorococcum* sp.	Mesophilic T_{max} = above 23 °C	ND	Spitsbergen snowfield
101-99	cf. *Chlamydocapsa* sp.	Psychrophilic T_{max} = above 18 °C T_{opt} = 14 °C	0.5	Spitsbergen permafrost
138-01	cf. *Chlamydocapsa* sp.	ND	ND	Spitsbergen snowfield
147-01	cf. *Chlamydocapsa* sp.	Psychrophilic T_{max} = 19 °C T_{opt} = 8–14 °C	ND	Spitsbergen snowfield
Trebouxiophyceae, Chlorophyta				
011-99	*Raphidonema sempervirens* Chodat	Psychrophilic T_{max} = 21 °C T_{opt} = 13 °C	0.1	Spitsbergen icy snowfield
112-00	*Raphidonema nivale* Lagerh.	Psychrophilic T_{max} = 22 °C T_{opt} = 12 °C	0.3	= CCAP 470/4, Mt Stuart, WA

cf. genus, (lat.: *confer*) taxonomic position yet unclear, genus name is provisional; not used in the running text and further tables/figures.
ND, temperature data not determined.
T_{max}, maximum temperature for cultivation of strain.
T_{opt}, optimum temperature for cultivation of strain, where highest growth rates are obtained (temperature data from Leya, 2004) and from latest studies (complete data not shown).
Data on growth rate (μ) were based on increase in cell numbers (unpublished data).

kept the same. Culturing was continued until the cells turned vividly yellow to orange in color (red phase). Some snow algal strains (CCCryo 005-99, 011-99 and 112-00) did not change their green appearance markedly (Fig. 1u). Red phase aliquots (R1 and R2) were harvested as described above. The two parameters, light and nitrogen, were varied for the induction of the red phase simultaneously to speed up carotenoid accumulation. Both nitrogen depletion as well as high light are known to induce secondary carotenoid synthesis (Harker *et al*., 1996; Fábregas *et al*., 2001). Immediately after harvest, samples for pigment extraction and dry mass determination were frozen at −20 °C, lyophilized overnight and again stored at −20 °C until further processing. The pigment values reported are based on one HPLC measurement per algal strain and treatment only. Initially, for each, two replicate samples were available, but several samples had to be combined due to low quantities. Hence, finally, for an equal treatment, two replicates were combined, and thus values represent a mean of two samples combined before measurement. Consequently, no standard variation could be reported. Variation of HPLC analyses ranges within an error of $< 0.1\%$.

Sample preparation and pigment extraction

Freeze-dried algal samples were disrupted in a grinding mill (Mikro-Dismembrator, 2000 beats min^{-1}, 5 min; Sartorius AG, Göttingen, Germany). Samples, Teflon grinding jars and grinding beads made from agate were cooled in liquid nitrogen for 10 min before use. Afterwards, the material was completely suspended in 0.7–1.5 mL of dimethylformamide (HPLC grade; Scharlau Chemie S.A., Sentmenat, Spain). This solvent has a high capability of stable pigment conservation (Bergweiler & Lütz, 1986). Moreover, *c*. 1 mg

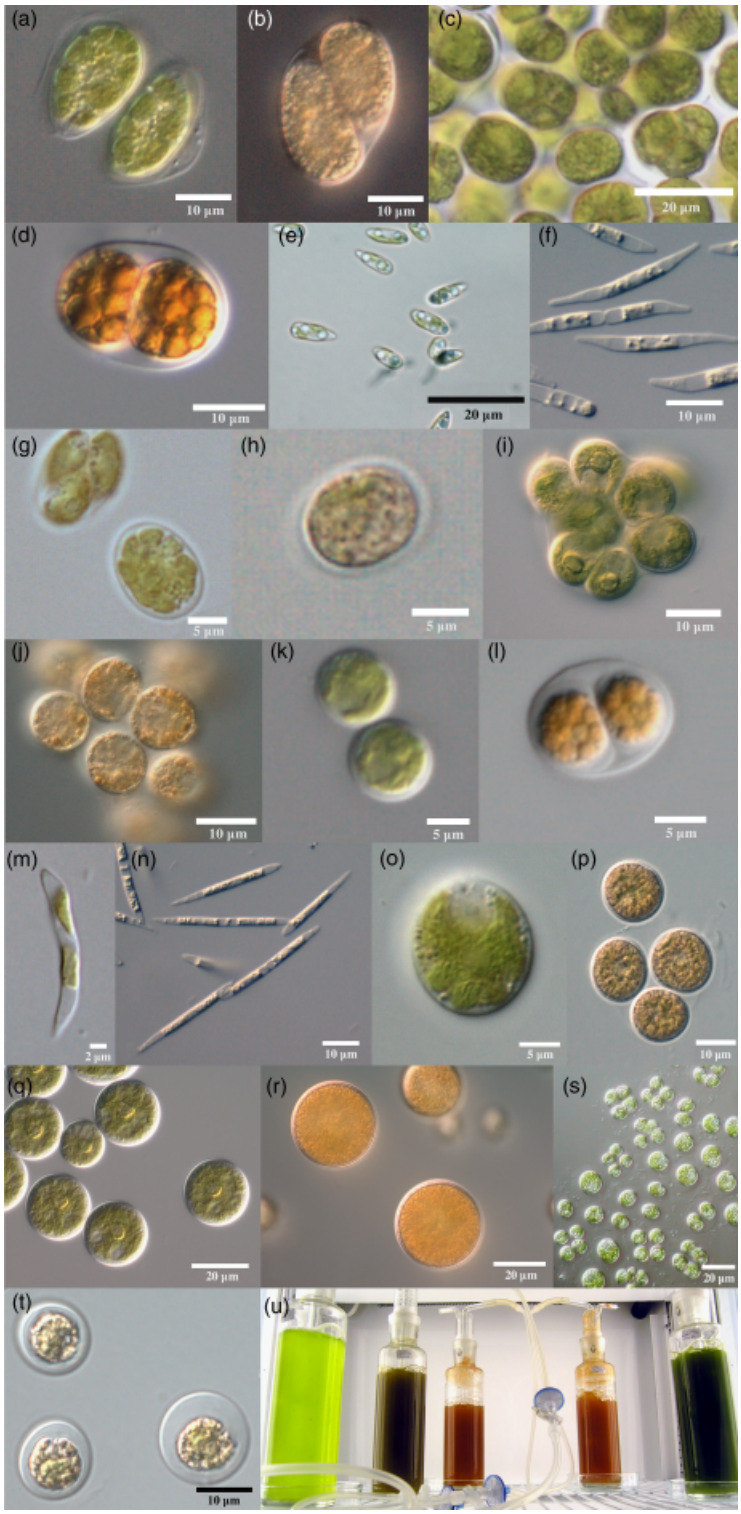

Fig. 1. Light microscopic photographs of the 10 different strains studied after cultivation under low light (G, green phase) and high light+low nitrogen conditions (R, red phase). (a) *Chloromonas nivalis* (CCCryo 005-99 G); (b) *C. nivalis* (CCCryo 005-99 R); (c) *Chlorococcum* sp. (CCCryo 006-99 G); (d) *Chlorococcum* sp. (CCCryo 006-99 R); (e) *Raphidonema sempervirens* (CCCryo 011-99 G); (f) *R. sempervirens* (CCCryo 011-99 R); (g) *Chlorococcum* sp. (CCCryo 039-99 G); (h) *Chlorococcum* sp. (CCCryo 039-99 R); (i) *Chlamydocapsa* sp. (CCCryo 101-99 G); (j) *Chlamydocapsa* sp. (CCCryo 101-99 R); (k) *Chlorococcum* sp. (CCCryo 105-99 G); (l) *Chlorococcum* sp. (CCCryo 105-99 R); (m) *Raphidonema nivale* (CCCryo 112-00 G); (n) *R. nivale* (CCCryo 112-00 R); (o) *Chlamydocapsa* sp. (CCCryo 138-01 G); (p) *Chlamydocapsa* sp. (CCCryo 138-01 R); (q) *Chlorococcum* sp. (CCCryo 140-01 G); (r) *Chlorococcum* sp. (CCCryo 140-01 R); (s) *Chlamydocapsa* sp. (CCCryo 147-01 G); (t) *Chlamydocapsa* sp. (CCCryo 147-01 R); (u) experimental setup for the red phase during incubation. (a), (b), (d), (f–k) and (m–t) in differential interference contrast modus; (c), (e) and (l) in bright field modus. Scale bars as indicated.

of calcium carbonate (Sigma-Aldrich Co., Vienna, Austria) was added to each sample to prevent any pigment damage by acids. Centrifugation was necessary (10 000 ***g***, 10 min, 4 °C) to pellet cell walls and glass fiber debris completely. The extracted pigments were stored at −20 °C for no more than 48 h. The supernatant was mixed with half of its volume of 50% methanol in dimethylformamide in order to make the sample more hydrophilic. Final centrifugation

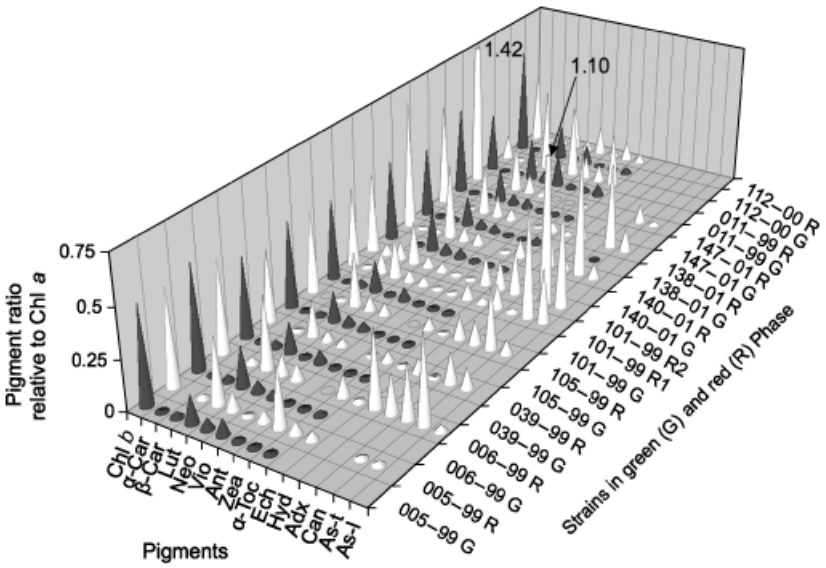

Fig. 2. Shift in pigment ratios relative to the chlorophyll *a* (= 1) content in the algal strains investigated after green phase (G, gray cones) and red phase (R, white cones) conditions. For strain 101-99, an additional sampling was performed after a prolonged red phase 2 (R2) of 12 days. For comparison, the values referring to freeze-dried dry mass are listed in Tables 2 and 3. Abbreviations as in Tables 2 and 3. As-I, astaxanthin isomers.

as above and filtration of the samples through a PTFE filter (0.45 μm; Nihon Millipore K.K., Tokyo, Japan) was necessary before HPLC separation.

HPLC analyses

Analyses of red phase samples were performed using an Agilent 1100 ChemStation system with RP C18 columns (Remias & Lütz, 2007). Because of a smaller number of peaks, the method for green phase samples was modified as follows: solvent A consisted of acetonitrile/methanol/Tris buffer (0.2 M, pH 8) (74/6/1), the pump flow rate was set to 0.9 mL min^{-1}. The gradient was as follows: 100% solvent A from the start to minute 7 and a linear gradient to 100% solvent B from minute 7 to minute 16. The run was completed after 30 min. The system was purged for 7 min with solvent A before a new sample was injected. Peaks were identified and quantified with calibration standards as follows: astaxanthin (Sigma-Aldrich Co.), canthaxanthin, echinenone, lutein, neoxanthin, violaxanthin, zeaxanthin (all DHI Water & Environment, Hørsholm, Denmark), antheraxanthin, adonirubin, adonixanthin, 13*Z*-astaxanthin, 3-hydroxyechinenone, 4′-hydroxyechinenone (all Carotenature, Lupsingen, Switzerland), α-tocopherol and β-carotene (Merck KGaA, Darmstadt, Germany). Both the accurate retention time as well as the peak spectra supported peak identification.

Results

Ten snow algal strains from different evolutionary lines within the green algae *sensu lato* (Chlorophyta) (Table 1) were screened. Figure 1 depicts the light microscopic pictures of each strain at the end of each culturing phase, i.e. the green phase (before stress application) and the red phase (after stress conditions). Figure 1u shows the experimental setup for the red phase during incubation. Figure 2 illustrates the shift in the pigment ratios between the green phase and the red phase relative to the chlorophyll *a* content for all the strains investigated. Tables 2 and 3 list the pigment concentration per freeze-dried algal dry mass at the end of the green and red culturing phases, respectively. In the green phase samples, chlorophyll *a*/*b* and lutein were the most abundant pigments in all strains. In the red phase, their amounts were generally reduced and sometimes surpassed by secondary carotenoids such as canthaxanthin. As all strains belong to the green algae, the well-known primary pigments of this group were found in each sample: chlorophyll *a* and *b*, antheraxanthin, β-carotene, lutein, neoxanthin, violaxanthin and zeaxanthin. These plastid pigments were detectable in both green and red phase samples. However, sometimes, antheraxanthin and zeaxanthin could not be measured because their amount is highly variable due to the de-epoxidation state of the xanthophyll cycle during harvest. Consequently, they were summarized together with violaxanthin (VAZ in Tables 2 and 3). A further plastidal carotenoid, α-carotene, occurred only in low amounts, and strain 140-01 of *Chlorococcum* sp. produced it only after the red phase treatment (Fig. 2). No secondary carotenoids were produced in any strain during the green phase, with the exception of strain 138-01 of *Chlamydocapsa* sp., which already contained trace amounts of canthaxanthin. Because of light and nutrient stress during the red phase treatment, the nonplastidal secondary carotenoids astaxanthin and canthaxanthin were, among others, accumulated in all strains of the *Chlorophyceae*, causing a yellow to orange pigmentation of the cells (Fig. 1). In contrast, the

Table 2. Pigment concentration in green phase samples in $\mu g\, g^{-1}$ freeze-dried dry mass

Taxon	Primary pigments						Xanthophyll cycle				
CCCryo strain no.	Chl *a*	Chl *b*	a-Car	b-Car	Lut	Neo	Vio	Ant	Zea	VAZ	a-Toc
Chloromonas nivalis (*Chlorophyceae*)											
005-99	17 489	8654	52	492	2408	967	1435	234	149	1818	107
Chlorococcum spp. (*Chlorophyceae*)											
006-99	12 757	6740	36	393	2548	728	450	34	46	530	267
039-99	23 149	9463	87	752	3584	219	1,365	100	109	1574	210
105-99	29 729	13 098	53	666	4348	1486	1205	98	–	1303	184
140-01	7314	3020	–	42	1610	381	350	74	83	507	156
Chlamydocapsa spp. (*Chlorophyceae*)											
138-01	26 060	9241	56	638	3890	1308	1042	116	T	1158	883
147-01	19 951	9129	12	117	3546	1040	998	142	173	1313	352

For strains 101-99, 011-99 and 112-00, no samples were available due to loss of material.
Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; a-Car, α-carotene; b-Car, β-carotene; Lut, lutein; Neo, neoxanthin; Vio, violaxanthin; Ant, antheraxanthin; Zea, zeaxanthin; VAZ, xanthophyll cycle pigments (Vio+Ant+Zea); a-Toc, α-tocopherol. T, traces (below HPLC calibration limit); –, not detected.

Table 3. Pigment concentration in red phase samples in $\mu g\, g^{-1}$ freeze-dried dry mass

Taxon	Primary pigments						Xanthophyll cycle					Secondary carotenoids				
CCCryo strain no.	Chl *a*	Chl *b*	a-Car	b-Car	Lut	Neo	Vio	Ant	Zea	VAZ	a-Toc	Ech	Hyd	Adx	Can	Ast
Chloromonas nivalis (*Chlorophyceae*)																
005–99	1460	717	–	29	485	131	14	85	442	541	87	67	–	–	28	37
Chlorococcum spp. (*Chlorophyceae*)																
006-99	1396	654	–	44	513	142	112	1	T	113	153	24	546	277	388	674
039-99	3024	1101	–	29	557	153	110	1	16	127	155	35	215	4	461	320
105-99	1991	834	T	21	543	154	119	1	T	120	122	13	238	246	618	142
140-01	1188	796	10	20	461	91	82	–	10	92	111	–	280	80	768	90
Chlamydocapsa spp. (*Chlorophyceae*)																
101-99/R1	1338	537	14	140	282	78	63	16	12	91	105	42	171	28	781	365
101-99/R2	841	345	46	74	184	49	37	13	7	57	127	127	194	39	921	444
138-01	1996	1065	T	2	501	219	121	1	9	131	103	38	294	–	896	241
147-01	119	168	–	1	30	12	2	T	T	2	1	–	–	–	12	3
Raphidonema spp. (*Trebouxiophyceae*)																
011-99	1446	185	10	39	631	70	551	168	126	845	417	–	–	–	–	0
112-00	764	296	8	34	180	38	116	32	75	223	36	–	–	–	–	0

Abbreviations as in Table 2.
Ech, echinenone; Adx, Adonixanthin; Can, canthaxanthin; Ast, total astaxanthin.
The low pigment content per weight in strain 147-01 resulted from large quantities of extracellular biopolymers produced (Fig. 1s and t).
For strain 101-99, two values are shown, with sample R2 being cultured for an additional 12 days compared with sample R1

trebouxiophyceaen strains of *Raphidonema* (011-99 and 112-00) produced no secondary carotenoids at all; as a result, these strains did not markedly show a change in color. Strains 101-99 and 138–01 of *Chlamydocapsa* sp. produced the most canthaxanthin with levels up to 0.1% of their dry mass (Table 3). Echinenone was found in six out of eight chlorophyceaen strains. Only strains 140-01 and 147-01 of *Chlorococcum* sp. contained no echinenone, such as the two trebouxiophyceaen species (011-99, 112-00). Astaxanthin, another widespread secondary carotenoid, showed similar patterns of occurrence: *Raphidonema sempervirens* (011-99) and *Raphidonema nivale* (112-00) did not synthesize any, in contrast to strains 006-99 or 101-99 of *Chlorococcum* sp., which accumulated significant amounts (*c.* 0.05% of their dry mass after the red phase harvest). Only strain 006-99 of *Chlorococcum* sp. also included the minor isomeric 13*Z cis*-derivate of astaxanthin (As-I; Fig. 2), with about 92% of it in all-*trans* standard constitution. Unlike all other carotenoids that have been found in this study, 13*Z cis*-astaxanthin also absorbs effectively in the near-UV-A region (see also Remias & Lütz, 2007). Further secondary pigments were adonixanthin and a presumptive hydroxyechinenone, which were found in almost all red phase samples of the *Chlorophyceae*, but again not in the two

Table 4. Yield of pigments in μg day^{-1} L^{-1} culture volume

Taxon	Primary pigments						Xanthophyll cycle				Secondary carotenoids					
CCCryo strain no.	Chl *a*	Chl *b*	a-Car	b-Car	Lut	Neo	Vio	Ant	Zea	a-Toc	Ech	Hyd	Adx	Can	Ast	ΣSecCar
Chloromonas nivalis (*Chlorophyceae*)																
005-99	6.36	3.15	0.02	0.18	0.88	0.35	0.52	0.09	0.05	0.04	0.51	–	–	0.21	0.28	1.00
Chlorococcum spp. (*Chlorophyceae*)																
006-99	1.63	0.86	0.01	0.05	0.33	0.09	0.06	0.00	0.01	0.01	0.03	0.78	0.39	0.55	0.96	2.71
039-99	3.37	1.38	0.01	0.11	0.52	0.03	0.20	0.02	0.02	0.02	0.05	0.28	–	0.59	0.41	1.33
105-99	6.63	2.92	T	0.15	0.97	0.33	0.27	0.02	–	0.02	0.04	0.67	0.69	1.73	0.40	3.53
140-01	6.56	2.71	–	0.04	1.45	0.34	0.31	–	0.08	0.10	–	2.12	0.61	5.80	0.68	9.21
Chlamydocapsa spp. (*Chlorophyceae*)																
101-99/G+R1	5.69	2.07	T	0.07	1.03	0.28	0.36	0.01	0.02	0.10	0.29	1.18	0.19	5.38	2.51	9.55
101-99/R2	/	/	0.06	/	/	/	/	/	/	0.17	0.72	1.10	0.22	5.24	2.53	9.81
138-01	17.28	6.13	0.04	0.42	2.58	0.87	0.69	0.08	T	0.04	0.11	0.89	–	2.71	0.73	4.44
147-01	8.85	4.05	0.01	0.05	1.57	0.46	0.44	T	T	0.00	–	–	–	0.14	0.04	0.18
Raphidonema spp. (*Trebouxiophyceae*)																
011-99	5.03	1.54	–	0.08	1.12	0.28	0.86	0.12	0.13	0.21	–	–	–	–	–	–
112-00	16.21	9.14	0.03	0.42	2.87	1.02	1.64	0.23	0.43	0.11	–	–	–	–	–	–

Abbreviations as in Tables 2 and 3.

ΣSecCar, total secondary carotenoids; /, no sample material available.

Yield of primary pigments was based on culture duration during green phase; yield of secondary pigments was based on culture duration during red phase; yield of α-tocopherol was based on culture duration during green phase+red phase.

trebouxiophyceaen strains of *Raphidonema*. In *Chloromonas nivalis* (005-99) and in the *Chlamydocapsa* strain 147-01, these oxy-carotenoids could not be detected either. The largest production of adonixanthin plus hydroxyechinenone showed the *Chlorococcum* strain 006-99 with $> 0.08\%$ pigment per dry mass.

To detect a further change in the pigment content during a prolonged red phase, strain 101-99 of *Chlamydocapsa* was harvested after an additional 12 days of stress incubation (R2) compared with sample R1. This step led to an additional decrease of 35–40% in all primary carotenoids and chlorophylls on a dry mass basis, with the exception of α-carotene, which increased from 14 to 46 μg g^{-1} (Table 3). Also, the concentration of antheraxanthin was reduced by about 14%. β-Carotene was affected, with even more than 47% loss. In contrast, all secondary carotenoids as well as α-tocopherol concentrations increased in the R2 sample of *Chlamydocapsa* strain 101-99. The largest relative increase was shown by echinenone, which was found to be threefold that of the R1 sample. The comparison of yields in *Chlamydocapsa* sp. (101-99) before (R1) and after (R2) the prolonged 12-day red phase in Table 4 showed the same trend as in Table 3: the yield of all primary pigments generally decreased (with the exception of α-carotene), while all secondary carotenoids and α-tocopherol showed much higher amounts. However, strain 101-99 of *Chlamydocapsa* revealed an exceptionally high ratio of β-carotene to chlorophyll *a* during the red phase. In all strains, the ratio of α-tocopherol to chlorophyll *a* was mainly increased after transition from the green to the red cell stages (Fig. 2), although absolute concentrations were higher in the green phase samples and varied between 110 and 350 μg g^{-1} freeze-dried dry mass in contrast to 90–150 μg g^{-1} in red-pigmented cells (Tables 2 and 3). This was a consequence of a partial decomposition of chlorophyll *a* in all strains during the red phase. Only strain 011-99 of *R. sempervirens* exhibited extraordinarily high levels of this antioxidant in the red phase samples (417 μg g^{-1}).

The overall pigment yield per day and liter culture volume is given in Table 4. Because the production of primary pigments usually ceased or even decreased with the start of the red phase, their yield is calculated from the total length of the green phase. The synthesis of secondary carotenoids began with the red phase and its values refer to the full length of that phase. α-Tocopherol values are given for the total length of the experiments because their production was independent of it. Each strain gave a very individual yield. Regarding secondary carotenoids, *C. nivalis* and three *Chlorococcum* strains (006-99, 039-99, 105-99) gave lower yields than the four *Chlamydocapsa* strains and strain 140-01 of *Chlorococcum*. The highest amounts for α-tocopherol were yielded by *R. sempervirens*. Together with strain 112-00, these two *Trebouxiophyceae* also yielded the highest amounts of Xanthophylls. Lutein was the most frequent primary carotenoid in green as well as red phase cells, with higher absolute values in the green phase samples (Tables 2 and 3). The best yields of this pigment were achieved by the *Chlamydocapsa* strain 138-01 (2.58 μg day^{-1} L^{-1}) and *R. nivale* (2.87 μg day^{-1} L^{-1}). The same applied to β-carotene, with equal yields of

Table 5. Concentration of commercially valuable substances in green phase and red phase samples

Taxon	Lutein		β-Carotene		α-Tocopherol	
CCCryo strain no.	Green phase	Red phase	Green phase	Red phase	Green phase	Red phase
Chloromonas nivalis (*Chlorophyceae*)						
005-99	15.6	7.4	3.2	0.5	0.7	1.3
Chlorococcum spp. (*Chlorophyceae*)						
006-99	18.1	2.9	2.8	0.3	1.9	0.9
039-99	23.3	1.1	4.9	0.1	1.4	0.3
105-99	28.5	4.8	4.4	0.5	1.2	0.9
140-01	30.1	8.0	0.8	0.0	2.9	0.3
Chlamydocapsa spp. (*Chlorophyceae*)						
101-99/R1	22.9	1.9	1.4	1.0	3.9	0.7
101-99/R2	/	1.3	/	0.5	/	0.9
138-01	22.7	1.4	3.7	1.7	5.2	1.9
147-01	23.0	1.0	0.8	0.4	2.3	1.9
Raphidonema spp. (*Trebouxiophyceae*)						
011-99	16.5	11.5	1.2	0.7	4.3	7.6
112-00	37.4	9.2	5.5	0.2	6.4	1.1

Abbreviations as in Tables 2–4.

Values are stated in mg 100 g^{-1} fresh mass to be able to draw comparison to other values from literature. For strain 101-99, two values are shown, with sample R2 having been cultured for an additional 12 days.

0.42 μg day^{-1} L^{-1} in both strains. *Raphidonema nivale* was the most efficient producer of primary pigments and xanthophylls among all the strains tested; only strain 138-01 of *Chlamydocapsa* performed slightly better with respect to chlorophyll *a* and α-carotene.

Table 5 lists the metabolites of commercial interest: lutein, β-carotene and α-tocopherol. Under stress conditions (red phase), nearly the concentrations of all three substances decreased in almost all the strains. α-Tocopherol concentrations increased only in *C. nivalis* (005-99) and *R. sempervirens* (011-99).

Discussion

In this study, a set of so far unstudied polar algal strains were subjected to two different culture regimes to identify shifts in their pigment and α-tocopherol patterns. The study focused on the identification of strains that have the potential to be used in biotechnology as alternative production strains of antioxidants in photobioreactors. Although the yields of valuable substances (Tables 4 and 5) and the growth rates of the algae (Table 1) are reported, these data were not obtained from an optimized experimental setup, as this preliminary study did not aim to identify the best production parameters.

Factors and strategies of antioxidant syntheses

Under low light conditions during the green phase, the normal spectrum of primary pigments was formed in all strains. The impact of high light and nitrogen starvation during the red phase resulted in the production and accumulation of secondary carotenoids in the eight strains belonging to the *Chlorophyceae*, usually resulting in a yellow to orange cell pigmentation (Fig. 1). The nonplastidal oxycarotenoids found in all these strains were astaxanthin, canthaxanthin, echinenone, adonixanthin and a putative hydroxyechinenone. Only strain 005-99 of *C. nivalis* lacked the two latter ones. The exact chemical constitution of Hyd remains unclear, although it seems to be common in many species of *Chlorophyceae*. Apparently, it has not yet been reported because it generally occurs in lower amounts compared with other secondary carotenoids. The most similar absorption spectra have 3-hydroxyechinenone and 4′-hydroxyechinenone. The latter has been reported from an aerophytic *Chlorococcum* species (Zhang *et al.*, 1997), although it is much more lipophilic than Hyd, which distinguishes between antheraxanthin and lutein, suggesting that the two oxygen atoms are in a steric constitution that makes this carotenoid more hydrophilic. In contrast to the other chlorophyceaen algae of this study, *C. nivalis* and strain 147-01 of *Chlamydocapsa* synthesized only low amounts of canthaxanthin and astaxanthin. Obviously, the red phase culture conditions did not elicit a physiological response in these species to induce a massive accumulation of secondary carotenoids, or these species generally have a limited ability to synthesize these pigments.

Several theories about the role of secondary carotenoids in green algae have been published (Czygan, 1964, 1968, 1970; Bidigare *et al.*, 1993). One obvious aspect is their relation to nitrogen starvation. In this case, the cells can redirect their biochemical reduction potential away from proteins to nitrogen-free metabolites such as fatty acids or

carotenoids. Because the amount of storable intraplastidal primary carotenoids is limited by the physiological demand of the photosynthetic apparatus, the strategy to synthesize nonphotosynthetic secondary carotenoids and their export into the cytoplasm is activated instead. There, these carotenoids, which possess hydroxyl groups, such as astaxanthin, can be esterified with the nitrogen-free fatty acids. The resulting carotenoidesters as well as the other secondary carotenoids accumulate in extraplastidal lipid globules, which can be detected by light microscopy as spherical, colored bodies in the resting stages of many green algal species (e.g. Fig. 1b, d and l) (Weiss, 1983). With an increase in the culture time, secondary carotenoid concentrations continuously increase (compare Fig. 2, strain 101-99 R1 and R2). This process ceases when the carotenoid concentration within the lipid globules reaches the maximum solubility, thereby preventing cell damage due to carotenoid crystallization. Cell proliferation also stops and the thickening cell walls of the developing resting stages make further cell growth and the availability of additional storage space impossible. This is an interesting aspect for biotechnology, as from commercial aspects, it has to be weighed between costs for prolonging the culture time and increasing the yield of valuable carotenoids. Monitoring of the carotenoid contents would help to determine the most efficient harvest time. Also, nitrogen-free culture media should be used for the red phase, instead of the nitrogen-reduced *Errötungsmedium* used in this study, to enhance the synthesis of the valuable nitrogen-free metabolites required, the secondary carotenoids and also the fatty acids involved in this process.

Light stress is the second major factor that may induce massive production of secondary carotenoids . High irradiation enhances carotenoid synthesis (Bohne & Linden, 2002). Excessive light damages the photosynthetic apparatus, but cytoplasmic secondary carotenoids accumulating around the chloroplast can protect it from too much PAR. Along with snow algae, which have evolved this strategy to protect the chloroplast, well-studied mesophilic species such as *Haematococcus pluvialis* and *Trentepohlia aurea*, which live in exposed spots such as bare rocks, also developed a similar mechanism (Abe *et al.*, 1999; Lorenz & Cysewski, 2000). It has to be pointed out that the ability of secondary carotenoids to protect against excessive PAR is limited to wavelengths between 390 and 600 nm. As a consequence, most carotenoids cannot function as UV-screening compounds. However, strain 006-99 of *Chlorococcum* in our study and also *C. nivalis* and *H. pluvialis* are known to contain a native *13Z cis*-isomer of astaxanthin, which has an additional absorption shoulder at 375 nm (Remias & Lütz, 2007). This isomer ensures additional protection at least in the near-UV-A range, which is consistent with the fact that these three species live in habitats with very high irradiance levels.

An adaptation strategy to high light stress other than producing secondary carotenoids was found in the two *Raphidonema* species. These two trebouxiophyceaen strains were not able to produce such carotenoids, and to our knowledge, this is the first report where this fact was experimentally proven. *Raphidonema sempervirens* (011-99) was apparently light stressed, as indicated by a very low chl *b*/chl *a* ratio of 0.13. It had the highest amount of α-tocopherol in all the species investigated (417 $\mu g\, g^{-1}$ lyophilized dry mass). This confirms studies by Estevez *et al.* (2001) and Trebst *et al.* (2002), who found that as long as the stress conditions do not exceed a critical limit, the level of α-tocopherol is generally increased under light stress and low temperature. The high amount of α-tocopherol in *R. sempervirens* seems to act as the main antioxidant as this compound supports the deactivation of reactive oxygen species derived under high light stress (Munné-Bosch, 2005). In addition, no species other than *R. sempervirens* had a larger pool of xanthophyll cycle pigments (V+A+Z/chl $a = 0.59$). *Raphidonema nivale* (112-00) developed no secondary carotenoids either, but instead had a large xanthophyll cycle capacity (V+A+Z/chl $a = 0.29$). Physiologically, it remains unclear why these species do not invest the reductive power gained by the photosynthesis during the red phase into nitrogen-free metabolites such as carotenoids, which can also be produced on starvation media or, for example, in a nutrient-poor snowfield. Such species, which are not able to shade their chloroplast against excessive PAR by secondary carotenoids in the cytoplasm (Bidigare *et al.*, 1993; Remias *et al.*, 2005), seem to compensate for this fact partly by the activity of a well-developed xanthophyll cycle.

Regarding biotechnological production processes, the following can be summarized. Valuable primary pigments such as β-carotene and lutein are best harvested from unstressed cultures. To yield the maximum secondary carotenoids, suitable algal strains should be subjected to stress by complete nitrogen starvation and high light. Biomass should be harvested in the late culture phase. When Xanthophylls or α-tocopherols are the focus of a mass production, algal species from different taxonomic groups (e.g. *Trebouxiophyceae*), which are unable to produce secondary carotenoids, might be a valuable alternative bioresource.

Many other factors such as salinity, temperature, pH or the presence of reactive oxygen species play an additional role in the production of secondary carotenoids (Liu & Lee, 2000; Steinbrenner & Linden, 2001). Light stress can also trigger the production of more antioxidants such as free proline and phenolic compounds, as shown for *C. nivalis* exposed to UV irradiation (Duval *et al.*, 2000). Many differences in the adaptation strategies between species seem to depend on their individual environmental adaptations

and genetic capabilities (Lohr *et al.*, 2005) and are unidentified so far. An extended screening of further snow and permafrost algal species for secondary carotenoids under controlled culture conditions would therefore be of great interest, for basic as well as applied research.

The potential of snow and permafrost algal strains in biotechnology

The results of this study have shown that various snow and permafrost algal strains are valuable bioresources for different interesting pigments and α-tocopherol. Especially, lutein and α-tocopherol were synthesized in considerable amounts by individual strains (Table 5). Lutein accumulated in the highest amounts in unstressed samples of *R. nivale* (112-00) (37.4 mg 100 g^{-1} fresh mass) and *Chlorococcum* strain 140-01 (30.1 mg 100 g^{-1} fresh mass), and thus, for example, equaled or even exceeded the concentration of this valuable pigment in various fruits and vegetables used for human consumption. Spinach, kale and broccoli were found to be good sources (Huck *et al.*, 2000), with lutein plus zeaxanthin concentrations of *c.* 20 mg 100 g^{-1} fresh mass in kale and *c.* 12 mg 100 g^{-1} cooked fresh mass in spinach (Chug-Ahaja *et al.*, 1993; Mangels *et al.*, 1993; Müller, 1997). Comparison of our results with studies on other green algae is complicated due to the fact that often concentrations are stated in other units and/or it is not declared whether data refer to fresh or dry mass. Del Campo *et al.* (2000) found up to 38 mg lutein L^{-1} culture (*c.* 740 mg 100 g^{-1} fresh mass) in *Chlorococcum citriforme* (strain SAG 62.80). In *Botryococcus braunii* (*Chlorococcales*), the lutein content accounts for up to 29% of the total carotenoid content (Grung *et al.*, 1989).

In our study, α-tocopherol accumulated considerably in strains 138-01 of *Chlamydocapsa* (5.2 mg 100 g^{-1} fresh mass) during the green phase and *R. sempervirens* strain 011-99 (7.6 mg 100 g^{-1} fresh mass) during the additional red phase, whereas concentrations in rye (1.4 mg 100 g^{-1} edible fraction), chicken eggs (1.9 mg 100 g^{-1} edible fraction) and carp (0.6 mg 100 g^{-1} edible fraction) were much lower (Deutsche Forschungsanstalt für Lebensmittelchemie, 1987). In UV studies on an Antarctic *Chlorella* strain, Estevez *et al.* (2001) detected 0.65 pmol α-tocopherol per 10^4 cells in the untreated log phase of the culture (this corresponds to *c.* 10–14 mg α-tocopherol per 100 g fresh mass calculated on the basis of various fresh mass values of different species from our studies). This value is slightly higher than our measurements.

The highest concentration of β-carotene was found in the green phase sample of *R. nivale* (strain 112-00) with 5.5 mg 100 g^{-1} fresh mass, followed by strain 039-99 of *Chlorococcum* with 4.9 mg 100 g^{-1} fresh mass. Del Campo *et al.* (2000) detected 6.1 mg β-carotene L^{-1} culture (*c.* 119 mg 100 g^{-1} fresh mass?) for *C. citriforme* (strain SAG 62.80), which lies within the range of our values. Estevez *et al.* (2001) found 0.21 pmol β-carotene in 10^4 cells in the untreated log phase of the *Chlorella* strain studied (amounting to *c.* 37–56 mg 100 g^{-1} fresh mass as calculated above), which is about 10 times higher than in our algae.

When comparing pigment and α-tocopherol concentrations, it has to be noted that we did not optimize culture conditions to obtain the highest biomass levels and concentrations of these substances, as this was not the primary aim of this study. Therefore, regarding the algae mentioned above, our strains were comparable in their productive capacity. However, compared with other food sources for humans, we showed that snow algae could be taken into account when looking for alternative sources for these essential nutrients. One even more important aspect emerges when discussing production facilities in outdoor photobioreactors for Central Europe on an industrial scale. The productivity of microalgal production plants greatly relies on optimal culture conditions, where especially temperature and light are decisive. Species such as *H. pluvialis*, *Dunaliella salina*, *Arthrospira platensis* (syn. *Spirulina platensis*) and *Chlorella vulgaris*, the main algal producers currently used, prefer a culture temperature of around 20 °C. These conditions are found only within a short period of the year – for *c.* 4 months in the summer period. During the rest of the year, culture conditions for these mesophilic algae are suboptimal, and often the production process ceases, especially during the cold winter months when less light is available. Psychrophilic and mesophilic snow and permafrost algae might be a valuable alternative or flanking add-on to algal production plants, as they still exhibit satisfactory growth rates, even at temperatures < 15 °C and under low light conditions.

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Authors' contribution

All authors have contributed equally to this article.

Statement

Culture experiments were carried out by Leya and Rahn. Sample preparation and analyses were performed by Lütz and Remias. Leya, Remias and Lütz prepared the manuscript.

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