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Impact of nutrient stress on antioxidant production in three species of microalgae

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ABSTRACT

Microalgae are a novel source of sustainable natural antioxidants with various applications, including food preservation. To optimize antioxidant production in microalgae, we investigated the influence of nutrient limitation on antioxidant content in three species, *Phaeodactylum tricornutum*, *Tetraselmis suecica* and *Chlorella vulgaris*. Microalgae were cultivated in batch culture under nutrient replete, P- and N-limited conditions. Total antioxidant activity of the biomass was measured using Trolox equivalent antioxidant capacity and square wave voltammetry. Additionally, contents of carotenoids, phenolics, tocopherols and ascorbic acid were measured. Nutrient limitation, particularly N-limitation, resulted in low antioxidant content. Both phenolic and carotenoid contents were significantly reduced in nutrient-limited cultures. In contrast tocopherols and ascorbic acid levels were higher in nutrient-limited cultures, particularly under P-limitation. Our results indicate that nutrient stress is not an effective strategy to enhance overall antioxidant content in microalgae, although it may be useful to enhance production of some vitamin antioxidants such as tocopherols or ascorbic acid.

1. Introduction

Antioxidants are widely used as additives in the food industry to prevent lipid oxidation [1]. Although the health benefits of antioxidants are sometimes questioned [2], antioxidants are nevertheless increasingly being commercialized as nutraceuticals and food supplements [3]. In the food industry, there is growing tendency to replace synthetic antioxidants, such as butylated hydroxytoluene of butylated hydroxyanisole, with natural alternatives. Important sources of natural antioxidants include, amongst others, rosemary, green tea and grape seeds [1].

Microalgae are a promising new source of natural antioxidants [4–10]. At present, two species of microalgae are already used commercially for the production of carotenoid antioxidants, i.e. *Dunaliella* for the production of beta-carotene and *Haematococcus* for the production of astaxanthin. In addition to carotenoids, microalgae also contain other potentially valuable antioxidants, such as tocopherols (vitamin E), ascorbic acid (vitamin C) or phenolic compounds. Tocopherols are widely used as additives to prevent oxidation of edible oils. Vitamin E contents in microalgae (0.01–0.3%) can be higher than those in virgin olive oil, a common source of natural tocopherols (0.02%; USDA National Nutrient Database for Standard Reference, 2013). Microalgae also contain high concentrations of vitamin C (0.1–1.5%), sometimes higher than the vitamin C content found in oranges (0.3–0.5%; USDA National Nutrient Database for Standard Reference, 2013). In terrestrial plants (e.g. in tea and grapes), phenolics are an important group of antioxidants [1]. Several recent studies showed that phenolic compounds also contribute significantly to antioxidant activity in microalgae [7,10,11].

The biochemical composition of microalgae often responds strongly to nutrient stress. In the past years, numerous studies have investigated the influence of nutrient stress on lipid accumulation in microalgae (e.g. [12]). Nutrient stress also results in the generation of free radical species in the cell and may therefore result in changes in the content of antioxidants. It is well-known that nutrient stress can induce accumulation of carotenoids in several species of microalgae such as *Dunaliella* [13], *Haematococcus* [14] or other chlorophytes like *Scenedesmus* [15]. Much less is known about the influence of nutrient stress on other classes of antioxidants in microalgae, although an increase in both alpha-tocopherol [16] and ascorbic acid [17] content has been observed in *Dunaliella salina* upon nitrogen stress.

In terrestrial plants, increased formation of polyphenols is observed when exposed to various forms of oxidative stress [18], including nutrient starvation [19]. Likewise, an increase in phlorotannins, a specific class of polyphenols, has been observed under limited nitrogen availability in the brown seaweed *Lobophora variegata* [20]. In microalgae,

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polyphenols have so far received little attention. Although there is no information available on the impact of nutrient stress on the phenolic content of microalgae, other types of environmental stress, such as exposure to metals [21] or UV stress [22], resulted in increased phenolic content.

For microalgae to become an economically viable source of antioxidants, it is important to maximize the content of antioxidants in the biomass. The aim of this study was to investigate to what extent nutrient stress can be used to modulate the content of antioxidants in microalgae. Therefore, we evaluated the response to nutrient stress of total antioxidant activity as well as the content of separate classes of antioxidants (carotenoids, phenolics, ascorbic acid and tocopherols) in microalgae. We compared the influence of both N and P-stress on antioxidant content. We carried out experiments with three species of microalgae to investigate whether the influence of nutrient stress on the content of antioxidants is similar in different microalgal species. We selected three species that were previously shown to possess a high overall antioxidant activity [10], i.e. the marine microalgae *Phaeodactylum tricornutum* and *Tetraselmis suecica*, and the freshwater *Chlorella vulgaris*.

2. Materials and methods

2.1. Microalgae culture conditions

The chlorophytes *C. vulgaris* (SAG strain 211-11B) and *T. suecica* (CCAP strain 66/4), and the diatom *P. tricornutum* (UGent strain Pt86) were cultured in 1 L borosilicate glass bottles (Duran, Schott AG, Mainz, Germany) using modified versions of Wright's Cryptophyte (WC) medium prepared with either deionized water for *C. vulgaris* or with synthetic seawater (30 g L^{-1} Homarsel, Zoutman, Belgium) for *T. suecica* and *P. tricornutum*. The medium was sterilized by autoclaving. Vitamins were added after autoclaving through sterile 0.2 μm PTFE filters. Cultures were illuminated using a 12:12 light:dark regime (125 μmol photons m^{-2} s^{-1} Philips Cool White fluorescent tubes) and mixed with moisturized filter-sterilized air (flow rate: 15 L h^{-1}). The inoculum was grown for 5 days in standard Wright's Cryptophyte medium prepared in salt water or freshwater (depending on the species), containing 1 mM N and 0.05 mM P. From these cultures, a 100 mL inoculum was added to 900 mL of the culture medium with different N and P-concentrations. The nutrient-replete treatments contained 5 mM N and 0.25 mM P (N:P molar ratio 20:1), the nitrogen-limited treatment 0.2 mM N and 0.05 mM P (N:P molar ratio 4) and the P-limited treatment contained 1 mM N and 0.01 mM P (N:P ratio 100). After 8 days of cultivation, the biomass was harvested by centrifugation, rinsed with demineralized water for *C. vulgaris* cultures or 0.5 M ammonium formate for *T. suecica* and *P. tricornutum* and then immediately freeze-dried. The dry biomass was stored frozen at −20 °C under nitrogen atmosphere until further analysis.

2.2. Monitoring of microalgal cultures

Microalgal growth was monitored daily, 2 h after the start of the light period by measuring optical density at 550 nm [23]. Quantum yield of photosystem II (F_v/F_m), which is a good indicator of nutrient stress, was also measured daily. To ensure that all samples had a similar biomass density when measuring F_v/F_m, the optical density (550 nm) of the culture was standardized at 0.1. F_v/F_m was measured using an Aquapen C100 (Photon Systems Instruments, Drasov, Czech Republic) after 5 min of dark adaptation of the microalgae. Microscopic observations were performed using an Olympus BX41 microscope equipped with Olympus DP-soft image processing software. Cell abundance was determined using an improved Neubauer counting chamber. Cell volume was calculated according to Hillebrand et al. [24] using cell measures obtained with the image analysis software. Biomass yield was determined by weighing the total amount of harvested biomass after freeze-drying. N and P-contents of the biomass samples were measured on the last day of the experiment. An amount of dry biomass was digested using alkaline persulfate digestion [25] and the N and P-contents in the digestate were measured as nitrate and phosphate using a microflow TechniconTM segmented flow analysis system (QuAAtro Seal Analytical, Bran+Luebbe, Germany) following the application notes of the manufacturer (Bran+Luebbe, application notes Q-031-04 rev. 1 and Q-035-04 rev. 4, 2005).

2.3. Analysis of total antioxidant activity

Extracts for measuring antioxidant activity were obtained using an ethanol/water (3/1, v/v) mixture as previously described [10]. Antioxidant activity was measured by the Trolox equivalent antioxidant capacity (TEAC) assay [10] and by means of square wave voltammetry [26]. Whereas TEAC measures the radical scavenging activity by both hydrogen atom transfer and electron transfer, square wave voltammetry only measures the ability of antioxidants to donate electrons. For the TEAC assay, $ABTS^{\bullet+}$ radical cation was generated by reaction of ABTS with potassium persulfate. The reaction mixture was allowed to stand in the dark for 16 h at room temperature and was used within two days. The $ABTS^{\bullet+}$ solution was diluted with deionized water to give an absorbance of 0.70 ± 0.05 at 734 nm. 50 μL of sample was mixed with 1.9 mL of diluted $ABTS^{\bullet+}$ solution and the absorbance of the resulting mixture was measured after 10 min incubation at room temperature. For square wave voltammetry (SWV), an Autolab PGSTAT12 (Metrohm Autolab B.V., Utrecht, The Netherlands) potentiostat was used for all measurements. The potentiostat was equipped with a glassy carbon working electrode (3 mm diameter), a titanium mesh counter electrode coated with a RuO_2/IrO_2 alloy (Magneto special anodes B.V., Schiedam, The Netherlands) and Ag|AgCl in 3 M KCl reference electrode. Tetrabutylammonium perchlorate was added to the extracts to increase the conductivity of the samples. The working electrode was conditioned for 60 s at a potential of 0.00 V. The measuring conditions were as follows: frequency 25 Hz, amplitude 20 mV, step size 5 mV. For both antioxidant assays, antioxidant activity was calibrated against the synthetic tocopherol analog Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (0.1–1.0 mM in ethanol/water 3/1, v/v).

2.4. Analysis of different classes of antioxidants

Phenolic content was measured in the same extracts as those used for assessing total antioxidant activity, and was determined by the Folin–Ciocalteu procedure as described by Goiris et al. [10]. For this, 200 μL extract was mixed with 1.5 mL of Folin–Ciocalteu reagent (previously diluted tenfold with distilled water) and allowed to stand at room temperature for 5 min. Next, 1.5 mL sodium bicarbonate solution (60 g L^{-1}) was added to the mixture. After incubation for 90 min at room temperature, the absorbance was measured at 750 nm. Total phenolics were calibrated against gallic acid standards and are expressed as mg gallic acid equivalent (GAE) g^{-1} biomass.

Carotenoids and chlorophylls were extracted by sonicating the biomass in a methanol/water (9/1, v/v) mixture, containing trans-β-apo-8′-carotenal as internal standard. Pigments were separated on a Waters Spherisorb ODS2 column (5 μm; 4.6 × 250 mm), set at 30 °C with a Waters Alliance 2695 chromatograph using the ternary gradient described in Wright et al. [27]. Eluted components were detected with a Waters 2996 PDA detector and quantified at 436 nm.

Ascorbic acid was quantified following the protocol of Brown et al. [28]. Freeze-dried biomass was mixed with MPA solution (aqueous solution of 30 g L^{-1} meta-phosphoric acid + 80 g L^{-1} acetic acid), in the presence of EGTA-glutathione to prevent oxidation of ascorbic acid during extraction. After extraction, samples were centrifuged and pH of the supernatant was adjusted with sodium acetate buffer (pH 6.2). The extracted ascorbic acid was derivatized using subsequent treatment

with ascorbic acid-oxidase and o-phenylene diamine. Chromatographic analysis of the derivatized samples was performed on a Waters Alliance HPLC by injecting 20 μL aliquots on an Alltech Altima C18 5 μm column (4.6 × 150 mm) and isocratic elution with a (2/8, v/v) mixture of methanol and 0.08 M potassium dihydrogen phosphate buffer (pH 7.8) at a flow rate of 1 mL min^{-1}. The fluorescence detector was set at an excitation wavelength of 355 nm and emission wavelength of 425 nm.

Tocopherol content was measured as described by Brown et al. [29]. For the extraction of tocopherols, equal amounts of biomass and ascorbic acid were mixed with alkaline ethanol (ethanol and 800 g L^{-1} KOH 10/1 v/v) and incubated for 30 min at 70 °C. After cooling the sample, the tocopherols were extracted from the liquid phase using hexane. After evaporating the hexane under a stream of nitrogen, the residue was re-dissolved in methanol and injected onto an Alltech Altima C18 5 μm column (4.6 × 150 mm). Tocopherols were eluted from the column with a mixture of water:methanol (2/98, v/v) at a flow rate of 1.5 mL min^{-1} and detected by a fluorescence detector (excitation 292 nm; emission 350 nm).

3. Results and discussion

3.1. Growth of the cultures

Biomass growth as estimated from optical density was in general lower in nutrient-limited when compared to the nutrient-replete cultures (Fig. 1). For *Chlorella* and *Tetraselmis*, growth was impaired by N as well as P-limitation while for *Phaeodactylum* growth was only impaired in the P-limited culture.

The biomass yield at the end of the experiment was significantly lower in the nutrient-limited cultures than in the nutrient-replete culture for all three species (Fig. 2). For *Chlorella* and *Tetraselmis*, the final biomass yield did not differ significantly between the N- and P-limited cultures. For *Phaeodactylum*, the final biomass yield was significantly lower in the N-limited than in the P-limited culture.

Data on cell abundance and cell volume (Fig. 2) suggest that differences in biomass yield were caused by both changes in cell abundance and changes in cell size. In *Chlorella* cultures, the lower biomass yield in the P-limited treatment was primarily caused by lower cell abundance, while it was due to a combination of lower cell abundance and smaller cells in the N-limited treatment. In the *Phaeodactylum* cultures, N- and P-limitation resulted in a decrease in cell abundance but cell volume was unaffected. In the *Tetraselmis* cultures, cell volume decreased in response to N- and P-stress but cell abundance was not affected. The decrease in cell size was more pronounced in the N-limited than in the P-limited *Tetraselmis* cultures. A decrease in cell volume but not cell numbers upon nutrient limitation has been reported before in *Tetraselmis* [30].

In nutrient-stressed cultures, biomass production often continues but the nutrient content of the biomass decreases. The nutrient content of the biomass is therefore often a better indicator of nutrient stress than the growth rate or the biomass yield. Biomass of the N-limited cultures had about a threefold lower N-content than biomass of the nutrient-replete cultures (Fig. 2). The N-content was also significantly reduced in the P-limited cultures. Similarly, the P-content was up to 5 times lower in the P-limited cultures than in the nutrient-replete cultures. Again, the P-content of the biomass was also slightly lower in the N-limited cultures. This reduced uptake of phosphorus under N-limitation has been described earlier for *Scenedesmus* [31].

The chlorophyll *a* content of the biomass was significantly lower in the nutrient-stressed cultures than in the nutrient-replete cultures (Fig. 2). The decrease in chlorophyll *a* content was most pronounced in the N-stressed cultures. This decrease in chlorophyll *a* content due

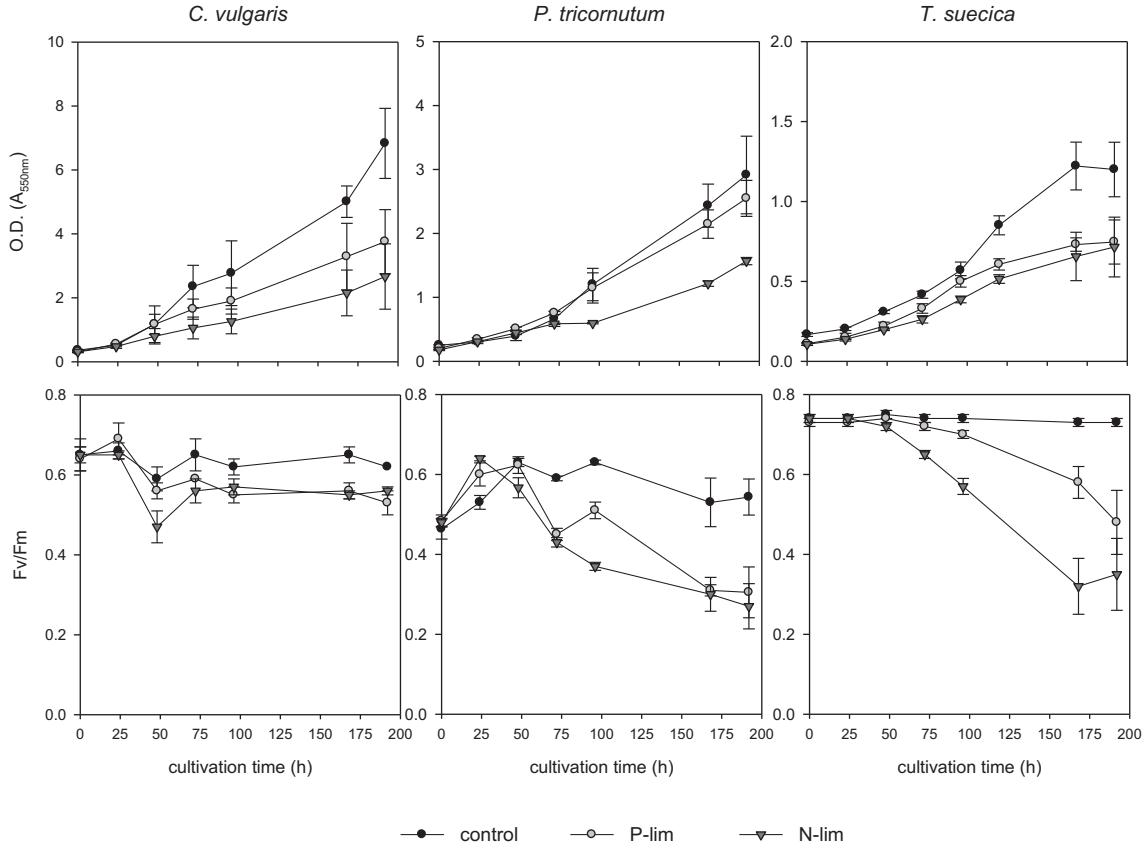

Fig. 1. Optical density (absorbance at 550 nm) and PSII quantum yield (F_v/F_m) as a function of time in nutrient replete, phosphorus limited or nitrogen limited cultures of *C. vulgaris*, *P. tricornutum* and *T. suecica*. Error bars correspond to standard deviation of triplicate cultures.

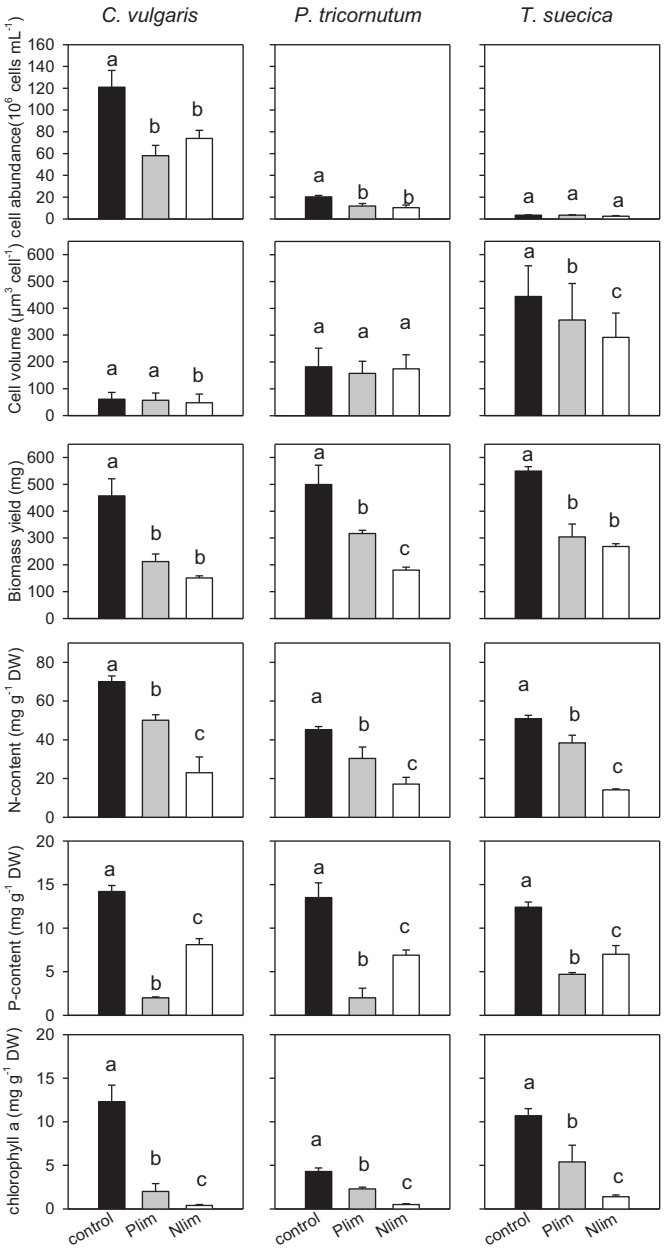

Fig. 2. Overview of culture parameters (cell density, cell volume and biomass yield), biomass composition (nitrogen, phosphorus and chlorophyll *a* contents) in three microalgal species, grown for 8 days in nutrient replete, phosphorus limited or nitrogen limited medium. Statistically different values ($p = 0.05$, one-way ANOVA, with Holm–Sidak posthoc test, $n = 3$) are indicated by a different letter for each microalgal species. Error bars correspond to standard deviation of triplicate cultures.

to nutrient stress is a well-known phenomenon, also known as 'bleaching' [32].

The quantum yield of photosystem II or F_v/F_m is a useful indicator of stress conditions in microalgae and this parameter is often reduced as a result of nutrient stress [33]. F_v/F_m remained stable in the nutrient-replete cultures of all species but declined significantly in nutrient-limited cultures (Fig. 1). This decline started already after 3 days and was particularly pronounced in the N- and P-limited *Phaeodactylum* cultures and the N-limited *Tetraselmis* culture. F_v/F_m is a parameter that requires only a few mL of living culture and a few minutes to measure. It is therefore a useful tool for routine monitoring of nutrient stress in microalgal cultures.

3.2. Total antioxidant activity

Two methods were used to quantify the overall antioxidant activity of the biomass: TEAC and SWV (Fig. 3). Antioxidant activity of the harvested biomass as determined by TEAC and SWV was 3–10 times higher in the nutrient replete cultures than in the nutrient-limited cultures. Nutrient stress resulted in a larger reduction of the antioxidant activity than P-stress.

In our previous study [10], total antioxidant activity of commercial samples from *Chlorella*, *Phaeodactylum* and *Tetraselmis* was higher in general. In that study, however, microalgae were cultured in outdoor photobioreactors that were exposed to natural sunlight while in this study artificial lights with a much lower intensity were used. At high

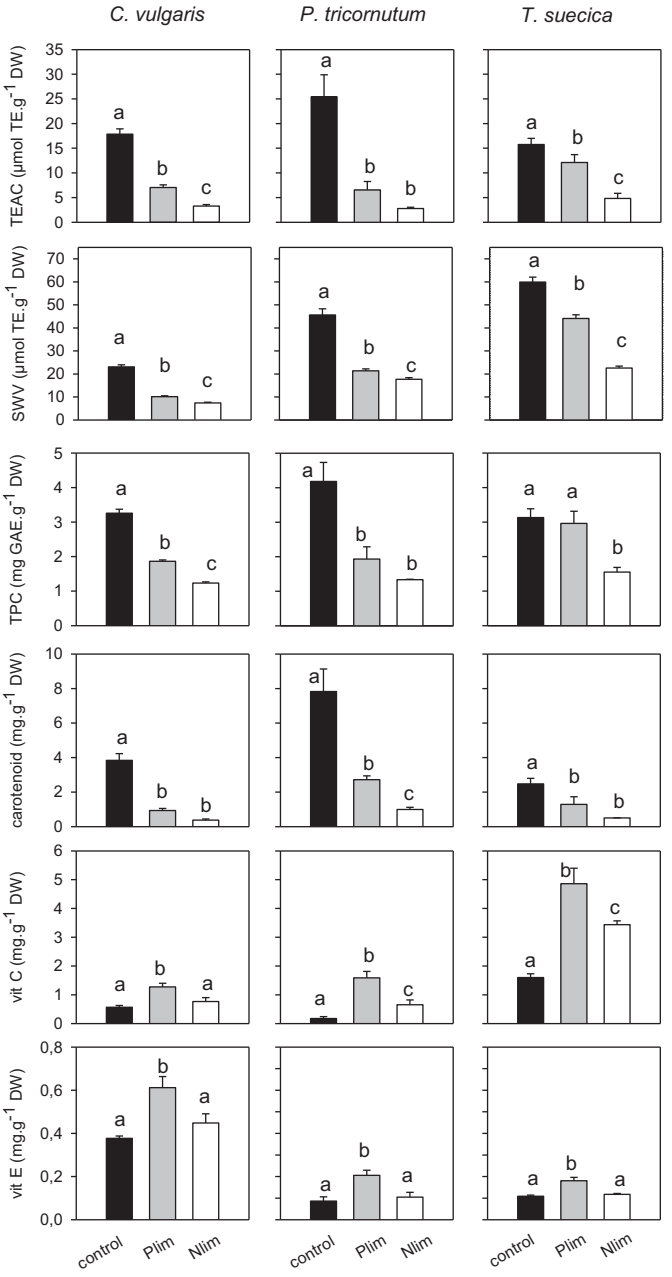

Fig. 3. Antioxidant activity (TEAC and SWV assay), total phenolic content (TPC), carotenoid content, vitamin C content and vitamin E content in three microalgal species grown for 8 days in nutrient replete, phosphorus limited or nitrogen limited medium. Statistically different values ($p = 0.05$, one-way ANOVA, with Holm–Sidak posthoc test, $n = 3$) are indicated by a different letter per antioxidant parameter for each microalgal species. Error bars correspond to standard deviation of triplicate cultures.

light intensities, saturation of photosystems may result in formation of reactive oxygen species. Microalgal cells can respond to this increased oxidative stress by producing more antioxidants, which could explain the higher antioxidant activities in the previous study.

SWV provides quantitative data on the overall antioxidant activity of the biomass, based on the area under the curve, but can also provide qualitative information about the major antioxidants present, based on the peak potentials [26]. The SWV profiles (Fig. 4) show that the nutrient replete biomass samples have a higher total area under the curve, but also contain antioxidants with lower peak potentials than the nutrient-stressed cultures, which indicates that these samples contain more powerful antioxidants. In extracts of the control cultures, a distinct peak at a potential of 0.550 V can be distinguished in all three species but it is unclear with which antioxidant this peak corresponds. Further, in *P. tricornutum*, a peak at 0.715 V is present in the voltammogram of the biomass from the nutrient-replete medium. This peak can most likely be attributed to the presence of fucoxanthin, which has the same peak potential [26] and is an important primary carotenoid in *P. tricornutum*.

3.3. Content of different classes of antioxidants

Different classes of antioxidants responded in a different way to nutrient limitation. The content of carotenoids and phenolics in the biomass decreased in response to N and P-stress (Fig. 2). The content of carotenoids was significantly reduced in the N-limited as well as the P-limited cultures compared to the nutrient-replete cultures in all three species. In *Phaeodactylum*, the carotenoid content was more strongly reduced in the N-limited than in the P-limited treatments while for the other two species the response to N and P-stress was similar. For carotenoids, we also observed a change in the relative composition of the carotenoids. Carotenoids can be divided into photoprotective carotenoids that protect the cell against high light intensities and free radicals, and light-harvesting carotenoids that transfer absorbed light energy to chlorophyll to be used in photosynthesis. Photoprotective carotenoids include diatoxanthin and diadinoxanthin in *Phaeodactylum* and violaxanthin, antheraxanthin and zeaxanthin in *Chlorella* and *Tetraselmis*. The light-harvesting carotenoids are fucoxanthin in *Phaeodactylum* whereas lutein and neoxanthin act as accessory light harvesting carotenoids in *Chlorella* and *Tetraselmis* [34]. The ratio of photoprotective carotenoids over light-harvesting carotenoids increased in the nutrient-stressed cultures (Table 1). This increase was more pronounced in the N-limited when compared to the P-limited treatments.

The decrease in carotenoid content in nutrient-limited conditions observed in our experiment is in sharp contrast with the accumulation of carotenoids during nutrient stress in *Dunaliella* and *Haematococcus*. *Dunaliella* or *Haematococcus* are relatively unique in that they accumulate secondary carotenoids under stress conditions, often up to several % of the biomass [36]. Most species of microalgae, including those used in this study, mainly produce primary carotenoids and these primary carotenoids typically decrease upon stress [37]. Primary carotenoids are structural components of the photosystems, where they scavenge reactive oxygen species that are produced during photosynthesis or contribute to light absorption for photosynthesis, and these are not accumulated in high concentrations as our results demonstrate.

Like carotenoids, the phenolic content was also reduced in nutrient-limited treatments compared to the control treatment. Phenolics were reduced in the N-limiting treatments of all three species studied. Phenolics were also significantly reduced in the P-limited cultures of *Chlorella* and *Phaeodactylum* but not in *Tetraselmis*. To the best of our knowledge, no previous data on the influence of nutrient stress on the phenolic content of microalgae have been published. In terrestrial plants [19] as well as in macroalgae [20], the content of phenolics typically increases in response to nutrient stress. In microalgae, other stress factors such as UV stress [22] or metal stress [21] also cause an increase in the content of antioxidants. In microalgae however, the response of phenolic content to nutrient stress seems to be different than in other organisms. As knowledge about the role and identity of polyphenols in microalgae is limited, there is a need for further research on this class of metabolites.

The contents of ascorbic acid and tocopherols (Fig. 2) displayed a different response to nutrient stress when compared to polyphenols and carotenoids as they were generally higher in nutrient-limited treatments. In cultures of *Phaeodactylum* and *Tetraselmis*, ascorbic acid was increased in both N- and P-stressed cultures but in *Chlorella* only in the P-stressed culture. Tocopherols increased significantly only in the P-stressed culture for the three species. Other studies also reported a significant increase in ascorbic acid under N-limited conditions in *D. salina* [17]. Elevated tocopherol production under N-limited conditions was observed in *Nannochloropsis oculata* [16], and *D. salina* [17]. On the other hand, Carballo-Cardenas [38] found higher tocopherol content in *T. suecica* when nutrient deprived cells were given extra phosphorus and nitrogen.

3.4. Implications for production of antioxidants using microalgae

Despite the fact that the content of ascorbic acid and tocopherols increased under nutrient stress, total antioxidant activity of the biomass decreased. This suggests that the contribution of these two vitamins to the overall antioxidant activity was limited. Carotenoids and phenolics displayed a similar response as total antioxidant activity, suggesting that these two classes of compounds are important contributors to the total antioxidant activity of the biomass. Whether they are both important cannot be concluded from this study. Previous studies, however,

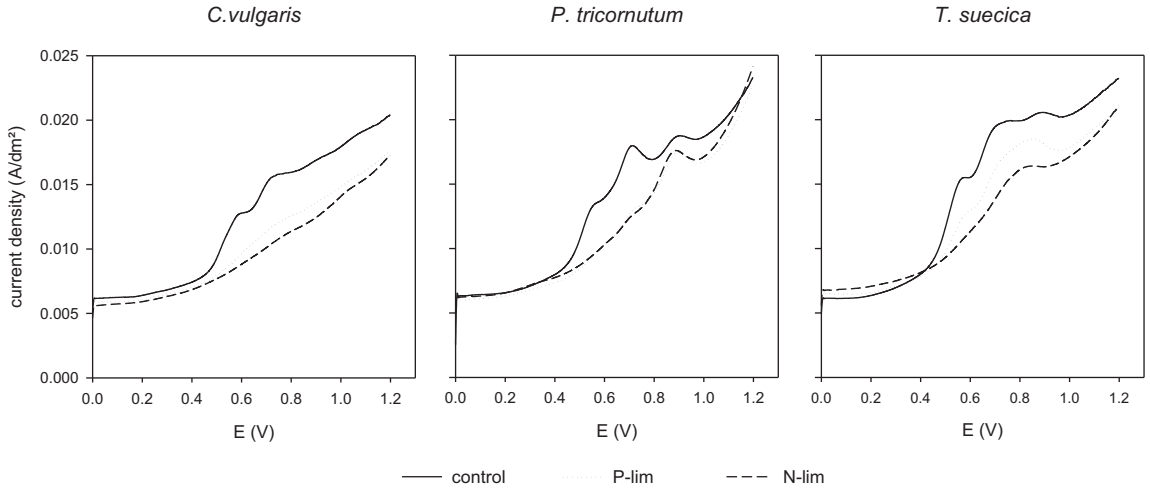

Fig. 4. Square wave voltammograms of ethanol/water extracts from three microalgal species grown for 8 days in nutrient replete, phosphorus limited or nitrogen limited medium.

Table 1
Overview of pigment composition in three microalgal species grown for 8 days under nutrient replete, phosphorus limited or nitrogen limited medium. Statistically different values ($p = 0.05$, one-way ANOVA, with Holm–Sidak posthoc test, $n = 3$) are indicated by a different letter for each microalgal species.

	Chlorella vulgaris			*Phaeodactylum tricornutum*			*Tetraselmis suecica*		
	Control	P-lim	N-lim	Control	P-lim	N-lim	Control	P-lim	N-lim
Carotenoids (mg g^{-1} DW)	3.8 ± 0.4^a	0.9 ± 0.1^b	0.4 ± 0.1^c	7.8 ± 1.3^a	2.7 ± 0.2^b	1.0 ± 0.1^c	2.5 ± 0.3^a	1.3 ± 0.4^b	0.5 ± 0.0^c
LP[1] (mg g^{-1} DW)	0.91 ± 0.05^a	0.83 ± 0.18^a	0.42 ± 0.03^b	0.78 ± 0.10^a	0.63 ± 0.05^b	0.23 ± 0.03^c	0.99 ± 0.15^a	0.91 ± 0.11^a	0.45 ± 0.03^b
LP/LH ratio[2]	0.04 ± 0.01^a	0.08 ± 0.02^b	0.19 ± 0.05^c	0.07 ± 0.01^a	0.14 ± 0.01^b	0.18 ± 0.01^c	0.04 ± 0.01^a	0.07 ± 0.01^b	0.11 ± 0.01^c
De-epoxidation state[3]	0.11 ± 0.01^a	0.33 ± 0.04^b	0.56 ± 0.04^c	0.01 ± 0.01^a	0.04 ± 0.01^b	0.06 ± 0.01^c	0.07 ± 0.02^a	0.17 ± 0.01^b	0.28 ± 0.01^c
Chlorophyll (mg g^{-1} DW)	13.8 ± 2.0^a	2.7 ± 0.6^b	0.5 ± 0.1^b	4.8 ± 0.4^a	2.4 ± 0.2^b	0.6 ± 0.1^c	13.2 ± 1.0^a	6.9 ± 2.3^b	1.9 ± 0.2^c

[1] Light protecting pigments diatoxanthin + diadinoxanthin (DT + DD) or violaxanthin + antheraxanthin + zeaxanthin (VAZ).
[2] Light protecting pigments / light harvesting pigments; (DT + DD)/ (Chl*a* + fucoxanthin) or VAZ / (Chl*a* + Chl*b*).
[3] De-epoxidation state: DT / (DD + DT) or (zeaxanthin + antheraxanthin) / VAZ [35].

have indicated that carotenoids and phenolics can both have a comparable contribution to total antioxidant activity [10].

The response of total antioxidant activity as well as the content of different classes of antioxidants was comparable in the three species studied. This suggests that our observations can be generalized to other species of microalgae. Microalgae such as *Haematococcus* and *Dunaliella* that massively accumulate secondary antioxidants, however, are likely to display a different response, but these species are extremophiles that are probably the exception rather than the rule. Although the overall response of antioxidants was similar, there were nevertheless obvious differences between the three species with respect to the content of specific classes of antioxidants: *Phaeodactylum* had a higher carotenoid content than the other species, *Tetraselmis* had a higher content of tocopherols and *Chlorella* had a high content of ascorbic acid.

If microalgae are to be used for commercial production of antioxidants, the overall antioxidant yield of the culture is a more relevant parameter than the content of antioxidants in the biomass. The antioxidant yield can be calculated by multiplying biomass yield and antioxidant activity per unit of biomass. The total antioxidant yield is much higher in nutrient-replete conditions than in nutrient-limited conditions because both biomass production and antioxidant content of the biomass are maximum when nutrients are non-limiting. The antioxidant yield was 2–6 times lower in P-limited conditions than in nutrient-replete conditions. For N-limitation, the antioxidant yield was even 7–25 times lower. Cultivation under nutrient-replete conditions will mainly result in a high yield of carotenoids and phenolic antioxidants. For production of tocopherols or ascorbic acid, nutrient-limited culture conditions are more favorable. Because nutrient limitation results in an increase in tocopherol and/or ascorbic acid content but a decrease in biomass productivity, the degree of nutrient stress will need to be optimized in order to maximize the yield of these antioxidants.

When microalgae are nutrient-limited, the flow of electrons from the photosystems to the electron transport chain is impaired and reactive oxygen species are formed [39]. The impairment of the photosystems is illustrated by the decrease in F_v/F_m upon nutrient limitation. The higher de-epoxidation state of the xanthophylls also points to a higher production of reactive oxygen species [40]. Therefore, an increase in antioxidant content is expected under nutrient limited conditions. The fact that total antioxidant content of the biomass decreased when nutrients were limiting may suggest that cells are less protected against these reactive oxygen species. However, this is not necessarily the case, as under nutrient-limiting conditions, the number of photosystems is greatly reduced, illustrated by the reduction in chlorophyll *a* content of the biomass in our experiment. While each photosystem may produce more reactive oxygen species under nutrient-limiting conditions, the total production of reactive oxygen species in the cell is not necessarily higher because the number of photosystems is greatly reduced. In our experiments, the chlorophyll *a* content of the biomass decreased much more strongly than the antioxidant content. This resulted in a larger number of antioxidants per unit of chlorophyll *a* under nutrient-limited conditions, especially in the chlorophytes (Fig. 5). In other studies, the content of carotenoid antioxidant is often expressed per unit of chlorophyll *a* rather than per unit of biomass [41]. Like in our study, these studies generally report an increase in carotenoids per unit chlorophyll *a* under nutrient-limited conditions. We also observed an increase in the de-epoxidation state of xanthophylls in nutrient-stressed conditions, which was explained before by an increased rate of xanthophyll cycling under these conditions [35].

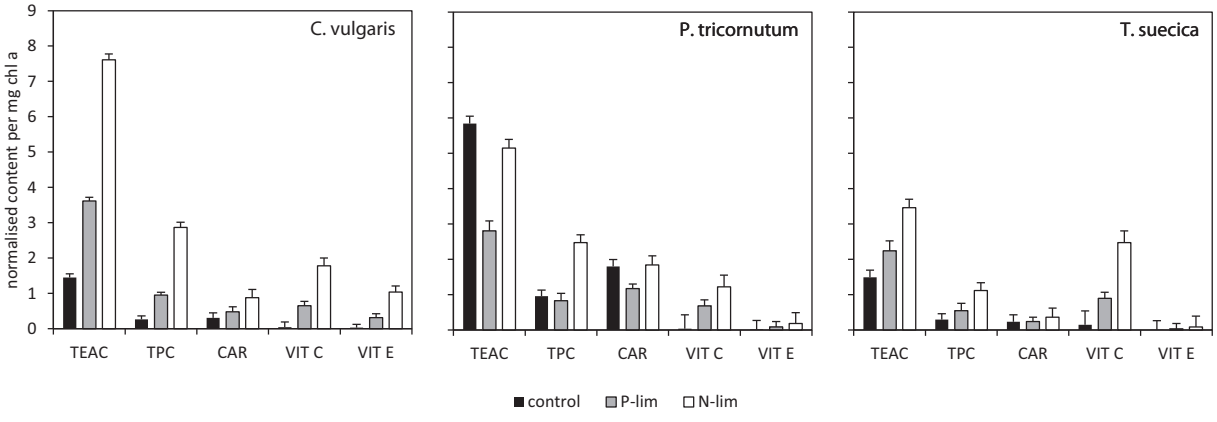

Fig. 5. Antioxidant activity (TEAC; µmol TE mg^{-1} chlorophyll *a*), total phenolic content (TPC; mg GAE mg^{-1} chlorophyll *a*), carotenoid content (CAR; mg mg^{-1} chlorophyll *a*), vitamin C content (VIT C; mg mg^{-1} chlorophyll *a*), and vitamin E content (VIT E; mg mg^{-1} chlorophyll *a*) in three microalgal species grown in nutrient replete, phosphorus limited or nitrogen limited medium. Statistically different values ($p = 0.05$, one-way ANOVA, with Holm–Sidak posthoc test, $n = 3$) are indicated by a different letter for each microalgal species. Error bars correspond to standard deviation of triplicate cultures.

4. Conclusions

For the three species of microalgae studied, nutrient limitation (N as well as P) resulted in a decrease in total antioxidant activity of the biomass and a decrease in the content of carotenoids and phenolic compounds. The content of ascorbic acid and tocopherols, however, was higher in nutrient-limited conditions than when nutrients were non-limiting. These results indicate that cultivation under nutrient-replete conditions should be applied when microalgae are used for production of antioxidants for nutritional or chemical applications.

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