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Microalgae *Nannochloropsis oceanica* as a future new natural source of vitamin D₃

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Abstract

For the last two decades there has been a rise in awareness about the general low dietary intake of vitamin D₃. Fish have the highest natural content of vitamin D₃, which is suggested to originate from zooplankton and microalgae. However there are no studies reporting which microalgal species may be the source of vitamin D₃. In this study, four selected microalgal species were cultivated during exposure of artificial UVB. The effect of UVB dose on the growth and biochemical composition of the cells (vitamin D₃, PUFAs and carotenoids) was evaluated. Of the four species, exclusively *Nannochloropsis oceanica* was able to produce vitamin D₃ (up to 1±0.3 µg/g DM), and production was significantly enhanced by increasing the dose of the UVB. These findings suggest that *N. oceanica* exposed to artificial UVB could be used as a new natural source of vitamin D₃, either as direct source or through animal feed.

Keywords

7-dehydrocholesterol, carotenoids, fatty acids, microalgal cultivation, ultraviolet radiation

1. Introduction

Vitamin D belongs to a group of lipid soluble sterols with the two major forms - vitamin D₃ (cholecalciferol) and vitamin D₂ (ergocalciferol). Vitamin D₃ and its metabolites are found mainly in foodstuffs of animal origin, such as fish, eggs and meat, whereas vitamin D₂ can be found in wild mushrooms, and ultraviolet-B (UVB) exposed fungi and yeast (Jäpelt & Jakobsen, 2013). When consumed, vitamin D₃ is initially converted to 25-hydroxyvitamin D in the liver, and further in the kidney hydroxylated to the metabolically active form 1,25- dihydroxyvitamin D (Pilz et al., 2018). Besides the dietary intake, vitamin D₃ can be synthesized in skin cells during exposure to sun light. UVB light enables the conversion of naturally present 7-dehydrocholesterol (provitamin D₃) to vitamin D₃ (Pilz et al., 2018).

Vitamin D deficiency and insufficiency is a world-wide problem, which affect more than one billion people, and on a yearly basis e.g. around 13% of the European population is vitamin D₃ deficient (vitamin D₃ status < 30 nmol/L) and 40% vitamin D₃ insufficient (vitamin D₃ status < 50 nmol/L) (Cashman et. al, 2016a; Holick, 2017). The recommended daily intake is 10-20 µg vitamin D₃ per day in Europe and US (IOM, 2011; NNR, 2014; EFSA, 2016). The estimated intake of vitamin D₃ is only around 3–7 µg/day, which is far below daily recommendations (Cashman & Kiely, 2016b). An alternative to dietary intake of vitamin D is the production of vitamin D in human skin by UVB-exposure (Pilz et al., 2018). This production is limited in places above 35° latitude where the amount of UVB reaching the earth's surface depend on season (Webb, 2006). O'Neill et al. (2016) assessed vitamin D-effective UVB availability for several European countries using a validated UV irradiance model, which made use of the average UVB-measurements over a 10-year period. As an example in Copenhagen, Denmark (55 °N) the maximum UVB dose was shown to be in June and July at 5 kJ/m²/month, while from October to March no production of vitamin D is present (vitamin D winter months). In contrast in Southern Europe, represented by Athens, Greece (37 °N) the maximum was 9-10 kJ/m²/month in June and July, and only two vitamin D winter months (December and January), and as expected at Crete, Greece (35 °N), UVB-exposure is efficient for vitamin D production in human all year round. Apart from geographic latitude, vitamin D₃ synthesis in skin can be reduced for many other reasons like low outdoor activity due to unhealthy lifestyle, immobility of elderly people, or severe air pollution. Therefore, intake of vitamin D₃ through nutrition plays an essential role to maintain healthy vitamin D₃ levels.

Fish and fish products are major dietary sources of vitamin D₃, especially salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Jakobsen & Smith, 2017). However, the origin of

vitamin D in fish still remains unclear. The photochemical production of vitamin D₃ in fish is doubtful, due to the insufficient UV radiation in their natural habitats. Therefore, it is suggested that vitamin D₃ present in fish originates from zooplankton and microalgae (Takeuchi, Okano, Tanda & Kobayashi, 1991; Sunita Rao & Raghuramulu, 1996). Microalgae have been recognized as a potential source for various high-value ingredients with positive health effects, such as polyunsaturated fatty acids (PUFAs), pigments, vitamins, peptides and many other bioactive compounds. Microalgae can be found at the surface of the water bodies so it can be assumed that vitamin D₃ is synthesized due to the sun exposure. Microalgae should be able to synthesize 7-dehydrocholesterol in order to synthesize vitamin D₃ by UVB exposure, if they use the same metabolic pathway as vertebrates. Sterols in microalgae display enormous diversity, due to the large number of classes and their composition varies depending on growth stage, light spectrum and temperature (Véron, Billard, Dauget, & Hartmann 1996), which means it is hard to make any general conclusions about the ability of microalgae to synthesize vitamin D.

To our knowledge there are no studies reporting which microalgal species may be the source of vitamin D₃. In this study, four selected microalgal species from different classes, *Chlorella minutissima* from Chlorophyta, *Nannochloropsis oceanica* from Eustigmatophyta, *Arthrospira maxima* from Cyanophyta and *Rhodomonas salina* from Rhodophyta were cultivated and exposed to artificial UVB. Furthermore, the dependence on dose was investigated by varying the exposure time and distance from the light source. In addition to vitamin D₃ concentrations, also the effect of UVB light on the microalgal growth and changes in the biochemical composition of the cells were determined, due to the high nutritional importance of the omega-3-fatty acids and pigments in microalgal biomass.

2. Materials and methods

2.1 Microalgal production

Four microalgal species were purchased: *Chlorella minutissima* (UTEX 2219, UTEX Culture Collection of Algae, University of Texas, Austin, USA) *Nannochloropsis oceanica* (NIVA 2/03, Norwegian Institute for water research, Oslo, Norway) *Arthrospira maxima* (SAG 84.79, Culture Collection of Algae, Göttingen, Germany) and *Rhodomonas salina* (DTU Aqua, Technical University of Denmark, Lyngby, Denmark). The inoculum was prepared in Guillard F/2 medium (Guillard, 1962) for salt water species *N.oceanica* and *R. salina*; Zarrouk medium (Zarrouk,

1966) was used for *A. platensis* and Walne's medium (Walne, 1970) for cultivating *C. minutissima*.

The cultivation experiments were carried out in triplicates using 1 L and 5 L GS Schott bottles for up-scaling. The cultures were aerated with a mixture of carbon dioxide (5%) and sterile air. Cultivation temperature was 23 ± 1 °C and pH 7.5 ± 0.5 , except for *A. platensis* where pH was 9.5 ± 0.5 . pH monitoring was performed by Milwaukee MC-122-pH controller (Milwaukee Electronics, Szeged, Hungary) equipped with a solenoid valve to control CO₂ addition. The LED light (SunFlux A/S, Glostrup, Denmark) provided before UVB treatment had an intensity of 110-120 $\mu\text{mol photon/m}^2/\text{s}$ (measured on the outer side of the bottle; Universal Light Meter ULM-500, Heinz Walz GmbH, Effeltrich, Germany).

Cultures (in their exponential growth phase) were transferred from 5 L GS Schott bottles into transparent, rectangle-shaped, plastic containers (25 cm x 15 cm x 20 cm) in order to increase the surface of the culture for the UVB exposure.

2.2 UVB-treatment

For the UVB experiments UVB tube (D₃⁺ UV Flood DESERT, 24 W tubes, Arcadia, United Kingdom) with its main wavelength at 312 nm was used. For quantification of UVB irradiance dose over the microalgal cultures a handheld ILT 1400-BL photometer equipped with a SEL005/TLS312/TD detector (International Light Technologies, Peabody, MA, USA) was used within the three distances from the surface of the microalgal culture; 5 cm, 10 cm and 15 cm.

2.2.1 Experiment 1

In order to investigate ability of microalgae to synthesize vitamin D₃, as a preliminary experiment, all four microalgae species were exposed to UVB for 3, 6, 18 and 24 hours per day for 7 days, which corresponds 3 kJ/m²/day, 6 kJ/m²/day, 16 kJ/m²/day and 22 kJ/m²/day, respectively. The UVB-tube was placed at distance of 10 cm from the surface of the cultured microalgae. Growth was monitored every 24 hours by taking 5 mL of culture and measuring optical density at 750 nm. A control culture received no UVB exposure.

2.2.2 Experiment 2

Species (*N. oceanica*) that produced vitamin D₃ following UVB exposure was tested further for the effect of dose, which was varied by exposure time and distance to the surface of the microalgal culture.

a. UVB exposure for 1, 3 and 6 days (24 h a day) at a distance of 15 cm above the surface of the culture, provided a dose of 15 kJ/m²/day.

b. UVB exposure for 5 days (24 h a day) at distance of 5 cm, 10 cm and 15 cm from the surface of the microalgal culture, provided a dose of 36 kJ/m²/day, 22 kJ/m²/day and 15 kJ/m²/day, respectively. A control culture received no UVB exposure.

2.3 Sampling

For chemical analysis 500 mL of culture was sampled. In order to achieve biomass separation, samples were centrifuged (refrigerated centrifuge, IEC Centra-GP8R, Buckinghamshire, England) at 8000 g for *A. maxima*, 6000 g for *N. oceanica* and *C. minutissima* and 4000 g for *R. salina*. Biomass was washed, freeze-dried (Beta 1-8, Martin Christ GmbH, Osterode, Germany) and stored at -20 °C until analysis.

2.4 Biochemical analysis

Total lipid content

Lipid extraction was performed according to Bligh & Dyer method (1959), with a reduced amount of solvent (30 mL of each). Approximately 1 g of the freeze-dried microalgal biomass was weighted into extraction glass. Extraction was carried out by subsequent addition of methanol, chloroform and water while stirring. In order to separate phases - methanol/water from chloroform/oil, samples were centrifuged at 1400 g for 10 min. Bligh & Dyer extracts were used for analyses of the total lipid content, fatty acid composition and tocopherols. Determination of total lipid content was done by weighing approximately 15 g of extract in beakers and keeping it overnight in a fume hood in order to evaporate chloroform.

Fatty acids

Fatty acid profile was determined based on the American Oil Chemist's Society (AOCS) official method Ce 1i-07 (Firestone, 2009) with slight modifications. Approximately 3 g of Bligh & Dyer extract was weighted in methylation glass tube and dried under the stream of nitrogen. A mixture of 100 µL of internal standard solution (C23:0), 100 µL of toluene and 200 µL of heptane with butylated hydroxytoluene (BHT) was added to extract. Methylation was performed in a microwave oven (Microwave 3000 SOLV, Anton Paar, Ashland, VA, USA) for 10 min at 100 °C and power of 500 watts. After methylation, 0.7 mL of heptane and 1 mL of saturated salt water (0.36 g NaCl/g H₂O, at 20°C) were added to the glass tube. The upper phase (heptane) was transferred into GC vials and analyzed by gas chromatography (HP-5890 A, Agilent Technologies, Santa Clara, CA, USA). Fatty acid methyl esters were separated by the GC column Agilent DB wax 127-7012 (10 m x 100 µm x 0.1 µm) (Agilent technologies, Santa Clara, CA,

USA). Standard mix of fatty acids methyl esters (Nu Check Prep 68D, Elysian, MN, USA) was used for fatty acid identification. Fatty acids were reported as area % of total fatty acids.

Pigments

Pigment analysis was done according to the method described by Safafar, Wagenen, Møller & Jacobsen (2015). Approximately 0.03 g of the freeze-dried microalgal biomass was placed in a 10 ml centrifugation tube. Methanol containing the internal standard and antioxidant BHT was added to the sample and they were placed in a sonication bath (Buch & Holm A/S, Herlev, Denmark) for 15 min at 5 ± 2 °C in order to extract pigments. Extraction was repeated three times and extracts were collected subsequently. Pigment analysis was performed by HPLC using Agilent 1100 Liquid Chromatograph with DAD 400-700 nm. Separation was carried out on a Zorbax Eclipse C8 column 150 mm x 46 mm x 3.5 µm (Phenomenex Inc. CA, USA). The mobile phase being a mixture of 70% methanol + 30% of 0.028 M tertiary butyl ammonium acetate in water used for gradient elution as described by Safafar et al. (2015) and at a flow rate of 1.1 mL/min and a run time of 40 min. DHI pigment standard mix (DHI LAB Products, Horsholm, Denmark) was used for identification of peaks, of which 18 of different carotenoids can be detected and 4 chlorophylls. BHT was detected at 280 nm, while detection of chlorophylls and carotenoids was done at 660 nm and 440 nm, respectively. Pigments are reported as mg/g dry biomass.

Vitamin D

Vitamin D analysis was performed as previously described by Barnkob, Petersen, Nielsen & Jakobsen (2019). Briefly, 0.1 g of freeze-dried microalgae was weighed, added 8 ng *d6*-vitamin D₃ (Chemaphor, Ottawa, Canada) as internal standard and saponified over night at room temperature. The unsaponifiable matter was extracted with 20% ethyl acetate in *n*-heptane by liquid/liquid extraction, cleaned-up by a SPE-hybrid solid-phase extraction, and derivatized with 4-phenyl-1,2,4-triazole-3,5-dione (PTAD; Sigma-Aldrich, Steinheim, Germany) for 5 min in darkness. Quantification of vitamin D₃ and vitamin D₂ using a LC-MS/MS consisting of an Agilent 1200 Series HPLC and Agilent 6470 Triple Quadrupole MS (Santa Clara, CA) coupled with a C18 column (Ascentis Express, 2.1 mm x 10 cm, 2.7 µm; Supelco Analytical, Bellafonte, PA) and a gradient mobile phase of water:methanol including 2.5 mM ammonium phosphate. Limit of quantification (LOQ) for both vitamers was 4 ng/g dry biomass. The analyses for vitamin D were conducted in laboratory environment accredited to perform the analyses according to ISO17025 (2015).

2.5 Statistical analysis

All biological experiments were performed in three replicates. The results are given as the mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine significant difference in the vitamin D₃ content between the individual groups. Tukey's post hoc test was used to detect significant differences between groups where p-values < 0.05 were considered significant. Association between vitamin D₃ content and UVB dose was assessed with Pearson's correlation coefficient using the Data Analysis function in Excel (version 2010, Microsoft, Redmond, WA, USA). The Statistica v. 13.2 software (USA) was used for all other statistical analyses.

3. Results

The vitamin D₃ content in the four selected microalgal species for experiment 1 is shown in Table 1. *N. oceanica* was the only tested species that contained vitamin D₃ after UVB exposure. Results suggest that concentration of vitamin D₃ increases with the increase of the applied UVB dose. Pearson correlation coefficient was 0.88 indicating a strong positive linear relationship between provided UVB dose and vitamin D₃ content (data not shown).

Vitamin D₃ content in *N. oceanica* increased significantly ($p < 0.05$) over 6 days of constant UVB exposure in experiment 2a, providing a dose of 15 kJ/m²/day (Figure 1a). By decreasing the distance between the UVB lamp and the microalgal cultures from 15 cm to 5 cm, which corresponds 15 kJ/m²/day and 36 kJ/m²/day, respectively, there was a significant increase in D₃ concentration (Figure 1b). Furthermore, besides vitamin D₃, lower quantities of vitamin D₂ were also found in *N. oceanica*, as well as in microalgae *R. salina* (data not shown). Vitamin D₂ content was increasing with the increase of the UVB dose and the highest measured content was 0.27 ± 0.08 $\mu\text{g/g}$ dry matter after the UVB dose of 36 kJ/m²/day. *Rhodomonas salina* contained up to 0.20 $\mu\text{g/g}$ dry matter after exposure to UVB dose of 6 kJ/m²/day. All the other tested species showed to have vitamin D₂ content below the limit of quantification (< 0.004 $\mu\text{g/g}$ dry matter).

Effect of different doses of UVB on the cell growth and biochemical composition of *N. oceanica* was determined by monitoring growth on a daily basis and analyzing content of high value compounds. The measured content of total lipids, PUFAs, carotenoids and chlorophylls under different UVB exposure is displayed in Table 2. The total lipid content tended to increase by the increase of the UVB exposure, whereas PUFAs, chlorophylls and carotenoids content decreased. Surprisingly, the lipid content was significantly lower in the biomass treated with 3 kJ/m²/day and 6 kJ/m²/day compared to the untreated biomass. However, biomass that was exposed to the 16 kJ/m²/day of UVB had significantly higher lipid content compared to the untreated biomass.

Biomass treated with UVB of 6 kJ/m²/day and 16 kJ/m²/day showed significant decrease in the relative amount of PUFA compared to untreated biomass, however there was no significant difference between the biomass treated by 3 kJ/m²/day and 6 kJ/m²/day. Content of the highly sensitive eicosapentaenoic acid (EPA) in the *N. oceanica* biomass decreased significantly after exposure to UVB. Content in the untreated biomass was 21.06 ± 0.07 % of total fatty acids, compared to 16.93 ± 0.09 % of total fatty acids in the biomass treated by 16 kJ/m²/day (data not shown).

Growth of *N. oceanica* under different treatments was monitored over 7 days in experiment 1 (Figure 2). Biomass increment data showed that the increase in duration of UVB exposure resulted in lower biomass accumulation. The highest biomass increment was detected in untreated biomass for the first 4 days. However, after 4 days there was no significant difference in biomass accumulation for untreated and treated biomass, 3 kJ/m²/day and 6 kJ/m²/day, respectively. Cultures treated for 16 and 22 kJ/m²/day showed significantly lower biomass accumulation.

4. Discussion

It is already known that the conventional belief of vitamin D₃ as an ‘animal form of vitamin D’ is not valid anymore. Vitamin D₃ has been identified within the few plant species of Solanaceae family (Boland, Skliar, Curino & Milanesi, 2003), and in UVB-exposed leaves from tomato and bell pepper (Jäpelt & Jakobsen, 2013). This present cultivation study is the first that demonstrates the ability of microalgal species to produce vitamin D₃. As mentioned earlier, microalgae represent the primary source of vitamins in aquatic food chains. Since phytoplankton usually habitats the surface of the water, the presence of vitamin D₃ most likely originates from UV radiation of provitamin 7-dehydrocholesterol. However, these assumptions were not yet confirmed. Partly because of the challenge it is to quantify vitamin D₃ by a specific method and partly because the studies did not include specific strains.

4.1 Effect of UVB exposure on variation in vitamin D₃ content in *Nannochloropsis oceanica*

The results clearly showed that the vitamin D₃ concentration in the biomass of *N. oceanica* increased by the increase of the UVB dose provided. (Table 1, Figure 1). At the highest UVB dose tested at 36 kJ/m²/day the content was 1 ± 0.3 µg vitamin D₃/g dry matter (Figure 1b). There are only a few studies reporting the occurrence of vitamin D₃ in microalgae. Sunita Rao and

Raghuramulu (1996) examined mixed phytoplankton consisting of the green algae *Pediastrum*, *Scenedesmus*, *Crucigenia*, *Coelastrum*, *Chlorella*, and *Cosmarium*; blue green algae *Gomphosphania* and *Oscillatoria*; brown algae *Gomphonema*, *Synedra*, *Navicula*, and *Cyclotella*. They reported content of vitamin D₃ to be 0.8 µg/g dry matter in phytoplankton of fresh water species. Takeuchi et al. (1991) reported 0.15 µg/g of vitamin D₃ in dry matter of phytoplankton during the summer in Biwa Lake, Japan. And finally, Brown, Mular, Miller, Farmer & Trenerry (1991) studied content of vitamins in four microalgal species, *Tetraselmis*, *Pavlova*, *Stichococcus*, *Nannochloropsis*, and reported the content of vitamin D₃ below the detection limit in all four cases (< 0.35 µg/g dry matter).

To ensure a higher dietary intake of vitamin D₃ in the population voluntary or mandatory fortification of foods in margarine and milk products are used (Pilz et al., 2018). Due to difference in the dietary intake of these food groups among people a more optimal strategy will be to broaden the range of foods with a higher amount of vitamin D₃ through fortification and biofortification (Cashman & Kiely, 2016). Salmon is generally regarded as being high in vitamin D₃. Recently, salmon was reported to contain 2.3-7.3 µg/100g for farmed species and 6.7-26.6 µg/100g for wild caught (Jakobsen, Smith, Bysted & Cashman, 2019). Egg is another vitamin D₃ rich source with a content at 2.3 µg/100 g, but as for farmed fish the content in eggs depends on the vitamin D₃ content in feed, and for pork and beef the vitamin D₃ content also depends on the fat content (Danish Food Composition Database, 2019). The biofortification strategy by feeding may alternatively be performed by UVB exposure. For husbandry e.g. UVB-exposure of pigs in their indoor facilities increased the vitamin D₃ content in pork at least 10 times, up to 3 µg vitamin D₃/100g, while UVB exposure of mushrooms can create a content of vitamin D₂ up to 80 µg/g dry matter (Urbain, Valverde & Jakobsen, 2016; Barnkob et al., 2019). In the EU vitamin D enriched mushrooms are approved at a level of 20 µg vitamin D₂/100 g (Commission Implementing Regulation (EU) 2018/1011). The EFSA Panel on Nutrition, Novel Foods and Food Allergens (2020) concluded that the UVB exposed mushroom powder (containing 100-130 µg vitamin D₂/100 g) is safe to use for general population. The similar content of vitamin D₃ was found in *N. oceanica* (100 ± 30 µg/100 g). These findings call for a further investigation as this could be a new sustainable source for vitamin D₃. The microalgae *N. oceanica* is not yet approved for human consumption, therefore necessary approval e.g. in Europe as a novel food, may either be as a food or an ingredient. Microalgae might be used in fortified food (e.g. juice or bread) or as supplement, especially for vegetarians and vegans.

4.2 Origin of vitamin D₃ in *Nannochloropsis oceanica*

Presence of vitamin D₃ in *N. oceanica* indicates high levels of provitamin 7-dehydrocholesterol in this species, which is being converted to vitamin D₃ by UVB exposure. Sterols in microalgae display large diversity due to the wide phylogenetic heterogeneity and the long evolutionary distance among them. Microalgal sterol composition varies also upon different growth stage, temperature, light intensity and spectra (Véron, Billard, Dauguet & Hartmann, 1996). Interestingly, *N. oceanica* sterol biosynthetic pathway shares structure and sterol profiles features with both animals and plants (including green algae). The key enzyme catalyzing sterol side chain reduction (sterol-24(25)-reductase) is different from that of higher plants. Amino acid sequence analysis of the enzyme showed that is clustered with that of Choanoflagellates (assumed to be unicellular ancestor of animals) (Lu et al. 2014), which suggests the origin of *N. oceanica*. Cholesterol has been found to be the most abundant sterol in *N. oceanica*, 70-75% of the total sterol content, while only a minor amount of the phytosterols was found (Lu et al. 2014). In plants, cholesterol is normally a minor component among sterols, and it usually constitutes from 1% to 5% of the overall sterol composition, while phytosterols are the dominant sterols in plants (Carland, Fujioka & Nelson, 2010). This unusual feature most likely explains the ability of *N. oceanica* to produce vitamin D₃. Existing literature describing the presence and regulation of vitamin D₃ and 7-dehydrocholesterol in microalgae and plants has been reviewed (Jäpelt and Jakobsen, 2013), but knowledge is still very limited. However, the presence of cholesterol in some of the microalgal species raises the possibility that 7-dehydrocholesterol could be formed in these organisms. Since the other cultivated species were not able to synthesize vitamin D₃ after UVB exposure, it can be suggested that tested strains of *Chlorella minutissima*, *Arthrospira maxima* and *Rhodomonas salina* do not contain 7-dehydrocholesterol.

4.3 Changes in the microalgal biomass as a result of UVB exposure

Microalgal growth is considered an important parameter that integrates stress effects in several biochemical processes. Figure 2 shows that there was no significant difference in the biomass accumulation from day 4 between the cultures that received the two lower UVB doses, 3 kJ/m²/day or 6 kJ/m²/day, compared to the control. Cultures that have received UVB doses of 16 kJ/m²/day or 22 kJ/m²/day showed significantly lower biomass accumulation compared to the rest of the cultures. It can be observed that the time of adaptation (lag growth phase) was longer for the cultures that received higher UVB doses. However, all the cultures showed ability to grow or survive the given UVB doses since there was no decrease in the biomass accumulation in any of the cultures. There are a few studies on the effect of UVB on growth in microalgae (Suresh, Joshi & Viswanathan, 1998; Van de Poll, Eggert, Buma & Breemann, 2001; Kumar, Nanda,

Kumar & Chauhan, 2018). The results have mostly showed negative effect of UVB on the growth of algae, which is in the agreement with our results. Suresh et al. (1998) investigated effects of UVB on the growth and composition of cyanobacteria *Nostoc* spp. UVB dose of only 3 kJ/m²/day (for four days) resulted in the reduction of growth by 50%. Further increase in the UVB dose to 4 kJ/m²/day showed total inhibition in the growth of the culture. It was suggested that the total inhibition happened due to irreversible damage to photosynthetic apparatus (Quesada & Vincent, 1997). Based on the comparison of these studies with the current one, it can be noticed that *N. oceanica* exhibited high damage resistance towards UVB considering the drastically higher UVB doses applied.

Enhanced UVB exposure on microalgae may result in damaging cells and on the other hand may exhibit protection or defense mechanisms to prevent the cell death (Xue, Zhang, Zhang, An & Wang, 2005). Microalgae face high levels of stress when exposed to UVB exposure and were usually found to respond with reversible photoinhibition (Franklin, Osmond & Larkum, 2003; García-Gómez, Parages, Jiménez, Palma, Mata & Segovia, 2012). Therefore, UVB radiation may affect primarily photosynthesis but also growth, pigmentation, general metabolism, and nitrogen fixation (Xue et al. 2005). These effects are the results of either direct impact of UVB on membrane proteins, DNA, enzymes and pigments or indirect impact of UVB by the formation of reactive oxygen species. Kumar, Tyagi, Nath Jha, Srinivas & Singh (2003) studied protective mechanisms against UVB damage in microalgae and reported the presence of photoreactivation mechanism, which allows survival under natural conditions, even under continuous exposure to UVB. The “safe” dose of UVB will depend upon the species, but if growth is being observed under UVB exposure it suggests the presence of the photoreactivation mechanisms in the cells. In addition, repair-capacity increases when UVB exposure is accompanied by low intensity visible light (below 300 $\mu\text{mol photon/m}^2/\text{s}$) because it is assumed that the visible light can enhance protection against photo-damage. Several microalgal species are able to produce UV-absorbing compounds, which may protect cells from unwanted effects of UVB (Pangestuti, Siahaan & Kim, 2018). Some of the most known compounds are water soluble mycosporine-like amino acids and pigment scytonemin. Further studies are needed to confirm whether such compounds were formed in *N. oceanica*. Besides UV absorbing compounds, another result of the UVB exposure may be the accumulation of detoxifying enzymes such as superoxide dismutase, catalase and quenching agents like carotenoids. However, in the present study the content of carotenoids decreased with increasing exposure to UVB. Even though carotenoids may be

synthesized in microalgae due to the UVB exposure, at the high UVB doses, such as above 3 kJ/m²/day, they can be rapidly oxidized in order to protect the cells against the photo-damage.

Microalgae are of industrial interest because of their e.g. rapid growth and ability to synthesize large amounts of lipids, high-value PUFAs and carotenoids. These high value compounds were highly affected by the UVB exposure. Lipid content of *N. oceanica* significantly decreased after applying UVB dose of 3 kJ/m²/day and 6 kJ/m²/day, but interestingly UVB dose of 16 kJ/m²/day resulted in the significant increase in lipid content. It is known that some microalgal species can accumulate lipids as a result of environmental stress, which could be the reason why the lipid content increased after the exposure to such a high dose of UVB. Microalgae have the ability to overproduce lipids under stress condition such as high salt concentrations, high light intensities, or nutrient limitation (Sun, Ren, Zhao, Ji & Huang, 2018). However, that means that the two other tested UVB doses do not represent the environmental stress for *N. oceanica*, which is also supported by the growth trend data (Figure 2). Furthermore, it can be assumed that the decrease in the lipid content of the cultures exposed to the lower UVB doses was a result of a biosynthetic shift towards production of carbohydrate and protein. However, more experiments need to be carried out to confirm these assumptions.

Our study revealed decrease in PUFAs, carotenoids and chlorophylls content compared to the control. PUFAs content decreased 9% when exposing cultures to 3 kJ/m²/day and 6 kJ/m²/day compared to the control. After exposing the culture to 16 kJ/m²/day, PUFAs content decreased 18%. Kumar et al. (2018) examined the effect of UVB light on the content of carotenoids and fatty acids in microalga *Chlorella sorokiniana*. The given UVB dose was 0.04 kJ/m²/day for four days. They reported a decrease in the lipid content of 19% compared to control. Similar to the current study, they have observed an increase in monounsaturated fatty acids (MUFAs) and a decrease in PUFAs levels, which is most likely the result of lipid oxidation. Also, another suggestion is that for PUFA synthesis high levels of the adenosine triphosphate (ATP) molecules are required, but under UVB exposure, ATP is being used by the different mechanisms in order to protect the cell against intracellular oxidative stress (Talero, García-Mauriño, Ávila-Román, Rodríguez-Luna, Alcaide & Motilva, 2015). Carotenoids and chlorophylls showed the same pattern as the PUFAs, with the maximum decrease of 76% and 81%, respectively. Suresh et al. (1998) reported a chlorophyll content reduction of 26% and 35% when the *Nostoc* spp. cultures were exposed to UVB at 3 kJ/m²/day and 4 kJ/m²/day, respectively. A significant decrease in carotenoid contents was also observed in UV treated biomass with 31.4% decrease compared to control.

5. Conclusion and perspectives

Microalga *Nannochloropsis oceanica* was able to naturally produce high levels of vitamin D₃ by exposure to artificial UVB. *Chlorella minutissima*, *Arthrospira maxima* and *Rhodomonas salina* were not able to produce vitamin D₃ and the UVB exposure had inhibitory effect on their growth. For *N. oceanica* vitamin D₃ production was significantly enhanced by increasing the dose of the UVB exposure and there was a linear relationship between the dose and the vitamin D₃ production. High UVB doses were shown to have an adverse effect on the growth and content of PUFAs, chlorophylls and carotenoids. These findings suggest that *N. oceanica* exposed to artificial UVB could be used as a new natural source of vitamin D₃.

Microalgae are an extremely diverse group of organisms with a long evolutionary history, and therefore, it is still hard to make any conclusions about microalgae and vitamin D₃ production in general. Based on this study, for microalgae *N. oceanica*, UVB dose of 6 kJ/m²/day may be suggested as a compromise, where biomass will contain sufficient amounts of vitamin D₃, PUFAs and carotenoids.

These findings may present a new opportunity for a biorefinery concept for microalgae in which both PUFAs, vitamin D and pigments could be obtained. Though, more research needs to be conducted in order to determine the presence of vitamin D₃ in other microalgal species considering geographic factors including the areas with the high light intensities over the year that might provide the UVB dose that can naturally trigger the production of vitamin D₃. Another important task to be considered is evaluating both bioaccessibility and bioavailability of vitamin D₃ from *N. oceanica* in order to discuss the potential role of microalgae as a new natural source of vitamin D₃ in human diet.

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LIST OF FIGURES:

Figure 1. Changes in content of vitamin D₃ (µg/g dry matter) in microalgae *Nannochloropsis oceanica* treated with UVB (**a**) dose of 15 kJ/m²/day over different number of days (**b**) at different distances between the culture and the UVB source: 5 cm (36 kJ/m²/day), 10 cm (22 kJ/m²/day) and 15 cm (15 kJ/m²/day), after 5 days. The standard deviations are presented as bars (n=3). Different letters above the bars represent significant differences (p < 0.05).

Figure 2. Effect of different dose of UVB on growth of *Nannochloropsis oceanica*. The standard deviations are presented as bars (n=3).

LIST OF TABLES:

Table 1. Content of vitamin D₃ (µg/g dry matter) in tested microalgal species treated with different doses of UVB (kJ/m²/day) for 7 days. Values are expressed as mean ± SD of n = 3. Different letters in the same row represent significant differences (p < 0.05).

Table 2. Concentration of the lipid, PUFA, carotenoid and chlorophyll of the microalga *Nannochloropsis oceanica* treated with different doses of UVB for 7 days. Values are expressed as mean ± SD of n = 3. Different letters in the same column represent significant differences (p < 0.05).

571 Table 1.

Vitamin D ₃ (µg/g dry matter)				
UVB dose (kJ/m ² /day)	<i>Nannochloropsis</i> <i>oceanica</i>	<i>Rhodomonas</i> <i>salina</i>	<i>Arthrospira</i> <i>maxima</i>	<i>Chlorella</i> <i>minutissima</i>
0	< 0.004	<0.004	<0.004	<0.004
3	0.09 ± 0.01 ^c	<0.004	<0.004	<0.004
6	0.29 ± 0.00 ^b	<0.004	<0.004	<0.004
16	0.42 ± 0.04 ^a	<0.004	<0.004	<0.004
22	0.48 ± 0.05 ^a	<0.004	<0.004	<0.004

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574 Table 2.

UVB dose (kJ/m ² /day)	Total lipids (%)	PUFA (% of total FA)	Carotenoids (mg/g)	Chlorophylls (mg/g)
0	31.4 ± 1.4 ^b	27.1 ± 0.2 ^a	10.2 ± 0.9 ^a	13.7 ± 0.4 ^a
3	23.9 ± 1.3 ^c	24.6 ± 0.4 ^b	6.7 ± 0.2 ^b	8.8 ± 0.4 ^b
6	25.0 ± 0.6 ^c	24.2 ± 0.1 ^b	3.3 ± 0.2 ^c	4.7 ± 0.1 ^c
16	36.9 ± 1.8 ^a	22.1 ± 0.4 ^c	2.4 ± 0.1 ^d	3.4 ± 0.2 ^d

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Figure 1a.

Figure 1b.

Figure 2.