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Effect of sulphur on selenium accumulation and speciation in *Nannochloropsis oceanica*

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ABSTRACT

Sulphur (S) and selenium (Se) are chemically similar. Once Se is taken up, it substitutes S in S-containing amino acids. This study investigated the effect of S on selenite accumulation in the microalga *Nannochloropsis oceanica*. [0–28 mM] S concentrations and selenite concentrations of 0 and 30 μM were tested. S concentrations of ≤ 3 mM led to decreased cell growth whereas cultures with ≥ 4 mM were not growth limited. Se accumulation increased up to 8-fold when using S ≤ 2 mM and decreased with S 28 mM. The average relative abundance of organic Se species was selenomethionine (SeMet) 98.2 %, selenocystine ($SeCys_2$) 1.4 % and selenomethyl selenocysteine (SeMeSeCys) 0.4 %. Total fatty acids were not affected by S limitation or Se presence. This is the first study on the effect of S on selenite accumulation, organic Se speciation of *N. oceanica* and its potential as an organic Se-enriched food/feed ingredient.

1. Introduction

Selenium (Se) is a non-metal element occurring naturally in rocks, soil, and water (Fernández-Martínez & Charlet, 2009). Historically, Se was first discovered in 1817 by a Swedish chemist, Jöns Jakob Berzelius, who characterised an impurity that settled at the bottom of the sulphuric acid production chambers (Emsley, 2012; Fordyce, 2013; Lenz & Lens, 2009). In aquatic environments (fresh- and seawater), Se is mostly present in the form of selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) (Fernández-Martínez & Charlet, 2009). Se is considered an essential trace element for animals, including humans, and other microorganisms, such as some microalgae species (*Emiliania huxleyi*) (Araie & Shiraiwa, 2009; Lenz & Lens, 2009; Price, Thompson, & Harrison, 1987; Young et al., 2010).

Se and S share similar chemical properties and chemical behaviour (Fordyce, 2013; Lide, 2004; Tan, Nancharaiah, van Hullebusch, & Lens, 2016), also known as chemical similarity. The uptake of Se can lead to unspecific (and potentially deleterious) displacement of S in S-containing amino acids, leading to seleno-amino acids such as, selenocysteine (SeCys) and selenomethionine (SeMet) (Fordyce, 2013; Gómez-Jacinto, Navarro-Roldán, Garbayo-Nores, Vílchez-Lobato, Borrego, & García-Barrera, 2020; Gupta & Gupta, 2017; Tan et al., 2016; Young et al., 2010). Mammals and other vertebrates do not synthetise SeMet from inorganic Se precursors, instead, SeMet is obtained from the diet and it has been considered to be the most effective form of Se supplementation, estimated to account for>50 % of human dietary Se (Aguilar et al., 2008; Cummins & Martin, 1967; Gojkovic, Garbayo, Ariza, Márová, & Vílchez, 2015; Lyons, Papazyan, & Surai, 2007; Rayman, 2000; Stipanuk & Caudill, 2013). Furthermore, SeMet is considered the primary organic

Abbreviations: EPA, eicosapentaenoic acid; FA, fatty acid; FAMEs, fatty acid methyl esters; GC, gas chromatography; GC-FID, gas chromatography with flame ionization detection; HPLC, high-performance liquid chromatography; ICP-MS, inductively coupled plasma – mass spectrometry; ICP-OES, inductively coupled plasma – optical emission spectrometry; PL, polar lipids; *N. oceanica*, *Nannochloropsis oceanica*; OD, optical density; QY, quantum yield; S, Sulphur; Se, Selenium; SeMet, selenomethionine; $SeCys_2$, selenocystine; SeMeSeCys, selenomethyl selenocysteine; TAG, triacylglycerides; TEAC, tetraethyl ammonium chloride; TFA, total fatty acid.

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form of Se in aquatic food webs (Spallholz & Hoffman, 2002).

Recently, there has been an effort to investigate Se accumulation in microalgae, and Se speciation studies have revealed that microalgae can produce Se-amino acids, such as selenomethionine (SeMet) (Gojkovic, Vílchez, Torronteras, Vigara, Gómez-Jacinto, Janzer, Gómez-Ariza, Márová, & Garbayo, 2014; Gómez-Jacinto et al., 2020; Li et al., 2021). SeMet is an essential amino acid for fish health where fish are unable to synthetise SeMet and must ingest it through their food (Mechlaoui et al., 2019). Studies have reported higher Se retention in the muscle of Atlantic salmon fed with diets supplemented with organic rather than inorganic Se (Sele, Ørnsrud, Sloth, Berntssen, & Amlund, 2018). Organic Se supplementation also plays a role in fish antiviral responses (Wang, Lovell, & Klesius, 1997). Therefore, it is considered that organic Se supplementation can lead to a better accumulation of Se in the fish muscle, resulting in a reduction of Se in the aquaculture waste (Dauda, Ajadi, Tola-Fabunmi, & Akinwole, 2019). Thus, Se-enriched microalgae could play a role as an organic Se supplement in aquafeed.

Microalgae are considered an efficient accumulator and promising source of Se for feed additive purposes (Brányiková, Skřivanová, & Dlouhá, 2010; Gómez-Jacinto et al., 2020; Li, Guo, & Li, 2003). It has been observed that several factors play a role in Se toxicity and uptake in microalgae (Guimarães, de Boer, Gremmen, Drinkwaard, Wieggers, & Wijffels, 2021), and among others, the Se species used and the sulphur (S) concentration in the growth medium (Fournier, Adam-Guillermin, Potin-Gautier, & Pannier, 2010; Pastierová, Kramarová, Molnárová, & Fargašová, 2009). Generally, there is a negative effect of S present in the growth medium on the accumulation of Se. To date, different mechanisms have been described for S influence on selenite and selenate uptake in microalgae and plants, although the molecular mechanisms underlying the transport and accumulation of these two Se species are little elucidated (Gupta & Gupta, 2017; Vriens, Behra, Voegelin, Zupanic, & Winkel, 2016). It has been reported that in *Chlamydomonas reinhardtii*, selenate and S share specific transporters as observed for plants (Fournier et al., 2010; Gupta & Gupta, 2017). While for selenite, it has been observed that S can interfere with Se accumulation when using higher Se concentrations (μM) due to unspecific sulphate transporters (Morlon, Fortin, Adam, & Garnier-Laplace, 2006).

Although most studies have focused on sulphate and selenate, the effect of S on selenite accumulation has only been investigated in freshwater species (Fournier et al., 2010; Morlon et al., 2006; Williams, Ogle, Knight, & Burau, 1994). To the authors knowledge, no studies exist on the effect of S on Se uptake in marine microalgae, of the group of heterokonts. *N. oceanica* is a marine microalga, commonly used in aquaculture and investigated for its high total fatty acid (TFA) content (up to 53 % dry weight), including the presence of the essential omega-3 eicosapentaenoic acid (EPA) (Ashour, Elshobary, El-Shenody, Kamil, & Abomohra, 2019; Slocombe et al., 2015; Südfeld et al., 2021; Zanella & Vianello, 2020). In our previous study we have shown that in *N. oceanica* Se accumulation and toxicity is directly correlated to its concentration in the media, with selenite being the less toxic compared to selenate, and thus considered optimal Se species for Se accumulation. This was assessed by exposing *N. oceanica* to several concentrations of either selenite or selenate, and by determining their accumulation into the microalgal biomass, as well as their toxic effect on growth (Guimarães, de Boer, et al., 2021). However, whether S concentration has an effect on selenite accumulation in *N. oceanica* has never been investigated. A more in depth understanding of this interaction could allow for a higher Se bioaccumulation in the microalgal biomass, whilst utilising lower amounts of Se in the media.

This study is the first to evaluate the effect of S concentration on Se accumulation and to describe the Se speciation in *N. oceanica*, with the prospect of using this microalga as a food/feed ingredient. The effect of S-Se chemical similarity on Se (selenite) accumulation was investigated by growing *N. oceanica* at different concentrations of S (in the presence or absence of Se). The effect on cell growth and photosynthetic performance was analysed, along with the determination of the accumulation of Se and S. For selected S concentrations, Se speciation was determined, to understand whether Se is accumulated as an organic form in the microalgal biomass. We also analysed the fatty acid (FA) composition to identify if there is an impact of S limitation and Se treatment on the FA profiles of *N. oceanica*.

2. Materials and methods

2.1. Microalgal strain and culture medium

Nannochloropsis oceanica (*N. oceanica*) CCAP 849/10 was cultivated in chloride media (Guimarães, Gremmen, Wijffels, Barbosa, & D'Adamo, 2021) with 6.48 mM of sulphur (S) in the form of Na_2SO_4 at the amount of 0.92 g/L (Supplementary table - Table S1). This S concentration was based on a medium from (Janssen, Kastenhofer, de Hoop, Lamers, Wijffels, & Barbosa, 2018) used to cultivate *N. gaditana*. All the elements containing sulphate (SO_4^{2-}) forms were substituted by chloride (Cl^-) forms and sulphate was only added with Na_2SO_4. The final medium composition was: NaCl 444.90 mM; KNO_3 33.63 mM; Na_2SO_4 6.48 mM; K_2HPO_4 2.47 mM; $Na_2EDTA{\cdot}2H_2O$ 84.12 μM; $MnCl_2{\cdot}4H_2O$ 19.25 μM; $CoCl_2{\cdot}6H_2O$ 1.19 μM; $CuCl_2{\cdot}2H_2O$ 1.32 μM; $Na_2MoO_4{\cdot}H_2O$ 104.15 nM; $ZnCl_2$ 4.17 μM; NaFeEDTA 27.79 μM; $MgCl_2{\cdot}6H_2O$ 2.96 mM; $CaCl_2{\cdot}2H_2O$ 2.45 mM; $NaHCO_3$ 10.00 mM; Tris-HCl 20.00 mM. The media was filter sterilised (0.22 μm, Sartobran 300, Germany). Stock and pre-cultures were always cultivated using chloride media in the absence of Se in 250 mL-Erlenmeyer flasks containing 150 mL of liquid volume. Experimental and pre-cultures were kept in an incubator (Multitron, Infors HT, Switzerland) at: 25 ± 1 °C, continuous shaking at 100 rpm, relative humidity of 50 %, air enriched with 2.5 % CO_2, and incident light of 100 μmol m^{-2} s^{-1} on a 16 h:8h light: dark cycle. Experimental cultures had a starting optical density (OD) OD_{750} of 0.5 (~2.4×10^7 cells mL^{-1}) and were performed in biological triplicates.

2.2. Experimental set-up

2.2.1. Effect of sulphur concentration on growth and Se accumulation

N. oceanica cells were centrifuged (800*g*, 2 min, 20 °C) and washed twice (800*g*, 15 min, 20 °C) with fresh experimental medium prior to each experiment to remove cell clumps, the previous medium, and any remaining salts and S (Guimarães, Gremmen, et al., 2021). Different S concentrations [0, 1, 2, 3, 4, 5, 6.48, 28 mM] were used (Supplementary table – Table S1). For the growth conditions tested, S 0 (0 mM of S) was considered a deplete condition. S depletion was chosen to guarantee that S limitation was achieved in our culturing conditions. 1, 2, 3 mM of S were considered limiting [based on previously determined S accumulation by Guimarães et al., (2021b)]. The lower S concentrations [1–3 mM] were chosen to reach S limitation. 4, 5, 6.48, 28 mM of S were considered replete conditions and the highest S concentration was chosen to simulate natural seawater sulphate elemental composition (28 mM of S) (Giordano & Prioretti, 2016). Additionally, 6.48 mM of S was considered the control condition since it was used in previous experiments with *N. oceanica* (Guimarães, Gremmen, et al., 2021) (Supplementary table – Table S1).

For each S concentration studied, cells were grown in presence or absence of Se in the form of sodium selenite (untreated and Se-treated with 30 μM of Na_2SeO_3, respectively). The Se species and concentrations were based on sub-lethal concentrations previously described (Guimarães, de Boer, et al., 2021). NaCl was adjusted to maintain a conductivity of 60 ± 2 mS cm^{-1} (SevenCompact™, Conductivity S230, Mettler-Toledo AG, Switzerland). It was considered that 1 mM Na_2SO_4 = 3 mM of NaCl (Bochenek et al., 2013).

2.2.2. Effect of Se concentration on cell growth and Se accumulation under S limitation

N. oceanica cultures were grown in S 2 medium (2 mM of S) (Supplementary table – Table S1) with sodium selenite (Na_2SeO_3) at different

concentrations (0, 1, 5, 10, 25, 30, 50, 100, 500 μM of [Se]). Each flask was inoculated at an OD_{750} of 0.5 and were performed in biological triplicates (n = 3).

2.3. Biomass sampling

Biomass samples (1 mL) were taken daily to monitor OD, quantum yield (QY), and cell counts. Samples were diluted using media and the OD was measured per each biological replicate at 680 and 750 nm using a UV–VIS spectrophotometer (DR-6000, Hach Lange, Germany). All samples were first adjusted to an OD_{750} of 0.10–0.40 ± 0.01. The OD_{680} was used as an estimate for chlorophyll content. The OD_{750} was first subtracted from the OD_{680} to normalise for relative chlorophyll content and then divided by OD_{750} to normalise for biomass concentration (Janssen, Kastenhofer, et al., 2018). From these diluted samples, the cell number, cell volume and maximum dark-adapted quantum yield of the photosystem II (PSII) (Fv/Fm), were measured. Cell counting was performed with an automated cell counter (Beckman Multisizer™ 3 Coulter Counter®) with a 50 μm aperture tube. Particles of 2.00 to 7.00 ± 0.01 μm were considered to be *N. oceanica* cells. QY was measured in previously dark acclimated samples for 15 min by emitting a pulse of blue light (455 nm) (AquaPen AP100, Photon Systems Instruments) with QY values between 0.10 and 0.71 ± 0.01. Dry weight was determined on day 0 and day 12 as described by Guimarães et al. (2021). Dry weight measurements were used to calculate average productivity (g/L/day). At the end of the cultivation period (12 days), biomass samples were harvested by centrifugation (2000*g*, 15 min, 20 °C) and washed twice with ammonium formate (0.5 M). Microalgal biomass pellets were lyophilised (Sublimator 2 × 3 × 3–5, Zirbus Technology, Germany) and manually crushed to a homogeneous fine powder.

2.4. Microwave-assisted acid digestion

50 mg of microalgal lyophilised samples were microwave assisted acid digested as described by Guimarães et al. (2021). In short, samples were digested in an Aqua Regia acid mixture: 10 mL of dH_2O, 7.5 mL of hydrochloric acid (37 %), and 2.5 mL of nitric acid (65 %) (Merck, Germany) in a microwave oven (milestone S.r.L. ETHOS 1). A temperature program was followed with a five-min ramp to 100 °C, five-min to ramp to 130 °C, five-min to ramp to 175 °C, remained at 175 °C for fifteen min and then, cooled down for 10 min. The total time was 40 min and the maximum energy used was 1400 W. After the digestions, samples were washed and made up to a volume of 50 mL with deionised water.

2.5. Elemental determination

Standards were prepared as described by Guimarães et al., (2021b) with Se added as an extra element, Merck (CertiPUR®). In short, single element standards were combined into two mixes to avoid precipitation of incompatible single element standards. Elements were mixed by performing matrix matching and preparing the standards in the expected concentration ranges; combined mix 1: P, S, Se and combined mix 2: Ca, Cu, Fe, Mg, Mn, Na, K, Zn. Dilutions were performed with a Hamilton diluter (Hamilton™ Microlab™ 600 Diluter) and 10 % aqua regia solution. Elemental determination was performed by inductively coupled plasma – optical emission spectrometry (ICP-OES) (PerkinElmer Avio® 500) with operational conditions as explained previously (Guimarães, Gremmen, et al., 2021), an Argon flame was used with a flow rate of 10 L min^{-1}. For instrumental conditions, see supplementary information Table S2.

2.6. Determination of Se speciation

Se speciation was carried out by using a high-performance liquid chromatography (HPLC) (model 1260 Infinity Quaternary LC, Agilent Technologies, Tokyo, Japan) coupled to a triple quadrupole ICP-mass spectrometry (MS) (Agilent 8800 model, Agilent Technologies, Waldbronn, Germany). Chromatographic separations of the chemical species were performed into a Phenomenex Luna C18 column (250 × 4.6 × 5 μm) using as mobile phase 0.75 % (v/v) tetraethyl ammonium chloride (TEAC), adjusted at pH 4.5 with diluted HCl. The Se signals were recorded in MS/MS mode utilizing a mixture of H_2 (2 mL min^{-1}) and O_2 (40 %). Instrumental conditions were optimized using a tuning aqueous solution containing Tl, Y, Co and Li at 1 μg L^{-1}. Ni skimmer and sampling cones were employed (sampling depth 10 mm). For instrumental conditions, see supplementary information Table S3. The standards used for the determination of Se speciation were the stock high purity, sodium selenite (Na_2SeO_3) (99 %, CAS number: 10102-18-8), selenocystine ($SeCys_2$) (95 %, CAS number: 29621-88-3), D,L-selenomethionine (D,L-SeMet) (99 %, CAS number: 1464-42-2), and selenomethyl selenocysteine (SeMetSeCys) (>95 %, CAS number: 863394-07-4), were purchased from Sigma–Aldrich (Steinheim, Germany).

For the preparation of standards, $SeCys_2$, D,L-SeMet, and SeMetSeCys were dissolved with ultrapure water adding 3 % HCl (v/v) for the complete dissolution of $SeCys_2$. Sodium selenate was prepared in 2 % (v/v) HNO_3. Stock solutions at 1000 mg L^{-1} were stored at 4 °C in darkness, while working standard solutions were prepared daily.

Se species were extracted using an ultrasonic homogenizer (Sonopuls, Bandelin electronic, GmbH & Co. Kg, Berlin, Germany). The sample extraction protocol was previously published by Gómez-Jacinto et al. (2020). Briefly, 40 mg of the lyophilized algae were sonicated at 25 % power for 2 min with 20 mg of protease and 5 mL of deionized water and then, the extract was centrifuged at 6000*g* for 5 min. This procedure was repeated again with 5 mL of ultrapure water. Finally, the extract was ultrafiltered using AMICON cut-off filters of 3 kDa (Millipore, Darmstadt, Germany). An aliquot of 500 μL of the extract was transferred into the filters and centrifuged at 4000*g* during 60 min at 4 °C. The extracts were dried under a N_2 gentle stream and finally reconstituted with 100 μL of mobile phase. The mobile phase, TEAC was obtained from Merck (Darmstadt, Germany).

2.7. Fatty acid analysis

Total fatty acid (TFA) and eicosapentaenoic acid (EPA) contents were analysed as described by Breuer et al. (2013) and León-Saiki et al. (2017). Ten mg of lyophilised biomass was used. The lipid extract was separated into two fractions (polar/apolar) using solid phase extraction columns as described by Remmers et al. (et al., 2017). The fatty acids (FAs) were methylated, and the FA methyl esters (FAMEs) measured using a gas chromatography (GC) with flame ionization detection (GC-FID). Tripentadecanoin (C15:0) and 1,2-didecanoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (C10:0) were used as the apolar and polar internal standards.

2.8. Statistical analysis

All the reported values in this work are the average of at least three biological replicates (n > 3) unless specified differently. Conditions S 2 and S 28 (untreated and Se-treated with 30 μM) were repeated for speciation experiments (n = 6). Condition S 6.48 (untreated and Se-treated with 30 μM), which represented the control conditions for each experiment, and consisted of nine biological replicates (n = 9). Growth, cell diameter, QY, chlorophyll absorbance and Se and S data were analysed using SPSS (IBM SPSS Statistics 25). One-way analysis of variance (ANOVA) and a post-hoc test (Tukey) with a 5 % level of significance was used ($p < 0.05$).

3. Results and discussion

3.1. Effect of sulphur on cell growth during limited and replete conditions

In the present study, the effect of S on Se accumulation was investigated by growing *N. oceanica* at different S concentrations [0–28 mM], and either untreated or treated with Se (30 μM), supplemented in the form of sodium selenite (Fig. 1). The S conditions were chosen to study Se uptake under; S limitation, our standard defined S medium concentration (6.48 mM), and the S concentration naturally found in seawater (28 mM). Se 30 μM was chosen based on our previous study (Guimarães, de Boer, et al., 2021). This allowed us to determine the impact of S and Se on cell growth, as a separate and combined effect.

In terms of S effect on cell growth, as expected, S concentrations of 0 and 1 mM led to no substantial growth in both untreated and Se-treated cultures, due to lack of sufficient amount of S that could sustain growth. S 2 and 3 untreated cultures grew significantly less ($p < 0.05$) when compared to the control (6.48 mM of S). The S limiting effect on cell growth has also been reported for other microalgae species, such as, among others, *Isochrysis galbana* T-ISO (Mulders et al., 2013), *Chlorella vulgaris* (Bránykiová et al., 2011), and *Emiliania huxleyi* (Bochenek et al., 2013). For S 4, 5, 28 cultures, no effect on growth was observed when compared to the control (Fig. 1, A-B) (Supplementary table - Table S5). This suggests that *N. oceanica* does not experience limitation in growth at S concentrations ≥ 4 mM, in the conditions tested.

In terms of the combined effect of the S and Se, no statistically significant differences in growth were observed for Se-treated cultures grown at concentrations of S 0, S 1, and S ≥ 4 (compared to the Se untreated cultures). Since S 0 and S1 lead to no growth, while S ≥ 4 cultures did not show growth limitation, we did not expect any differences between untreated and Se-treated cultures for those conditions. However, we observed a significant decrease in growth ($p < 0.05$) for S 2 and S 3 Se-treated, compared to the respective Se untreated cultures (Fig. 1). A reduction in biomass productivity was observed in Se containing cultures (4.4 and 1.2 % in S 2 and S 3, respectively) (Supplementary table - Table S4), compared to the control cultures (S 6.48 Se 30). An increase of Se toxicity has also been observed under S limiting conditions for *Arthrospira platensis* (Li et al., 2003), *Scenedesmus quadriculata* (Umysová et al., 2009) and *C. reinhardtii* (Fournier et al., 2010).

During cultivation, we also observed a significant increase ($p < 0.05$) in cell diameter for cultures grown on S 0, S 1 (untreated and Se-treated) compared to the respective controls (Fig. 1, C-D). Cultures grown under the remaining S concentrations had an average cell diameter of 3.51 ± 0.06 μm. We believe this increase in cell diameter is due to a cessation of cell division. The increase in cell diameter has also been reported in *C. reinhardtii* under S depletion where, under a S 'stress' response, cells increase their cell diameter due to an increase in storage compounds, such as triacylglycerides and starch (Cakmak et al., 2012).

Daily maximum dark-adapted QY of the PSII (Fv/Fm) was also monitored to address whether Se and S concentration had an effect on photosynthetic efficiency. QY can be used as an indicator of photosynthetic efficiency, which in turn, gives an indication of the presence of any 'stress' factors. 'Stress' factors can include nutrient depletion (Janssen, Driessen, Lamers, Wijffels, & Barbosa, 2018), high light

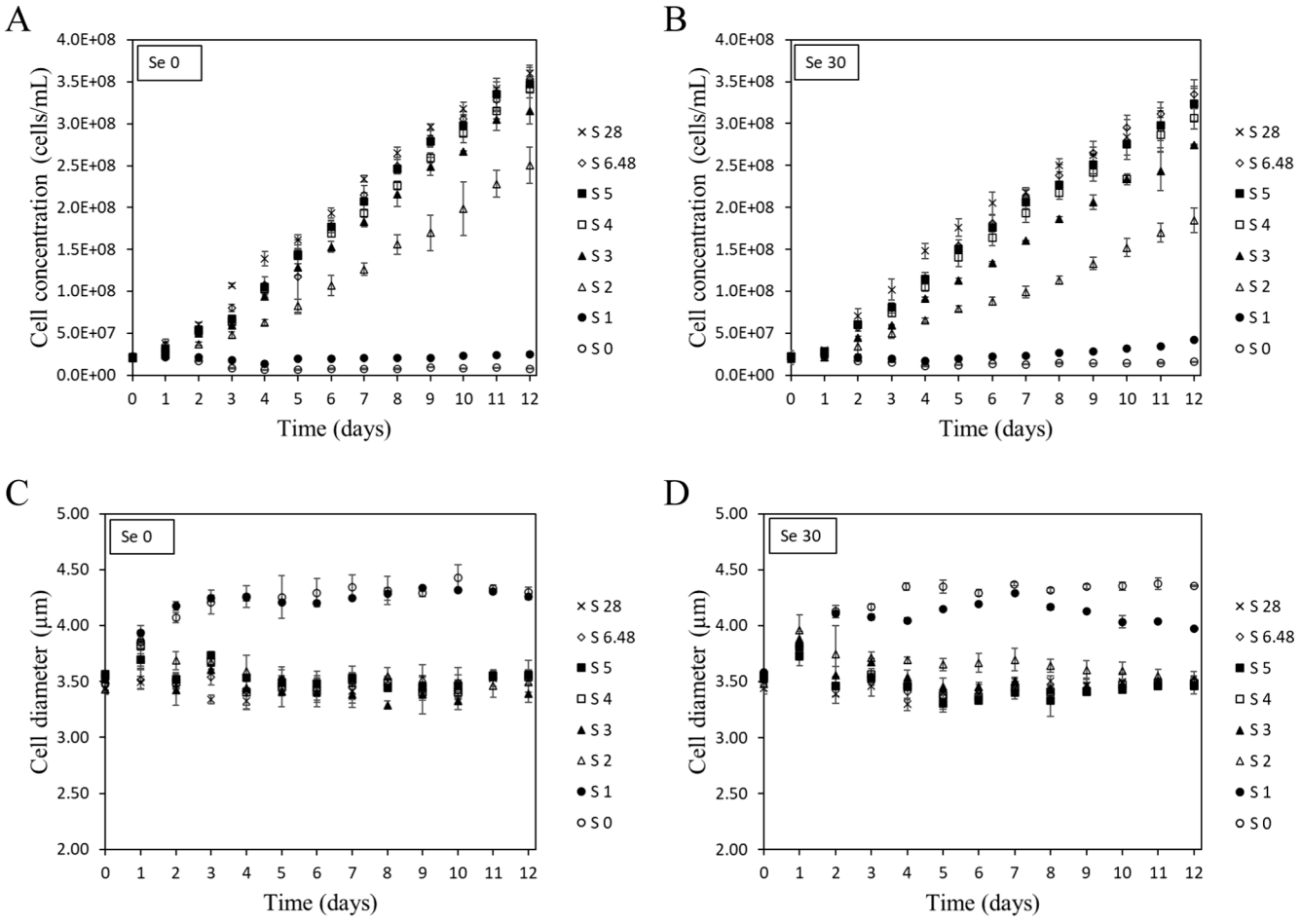

Fig. 1. Growth and average cell diameter of *N. oceanica* when exposed to different sulphur (S) concentrations (0, 1, 2, 3, 4, 5, 6.48, 28 mM) over 12 days (n = 3), (n = 9 for the controls 6.48 Se 0, 6.48 Se 30). Cultures were untreated and selenium (Se)-treated under the same S concentrations; A-C) untreated (Se = 0 μM) and B-D) with 30 μM of Se in the media.

(Franco, Buffing, Janssen, Lobato, & Wijffels, 2012), or Se exposure (Zhong & Cheng, 2017). We observed that the QY drastically decreased from 0.71 to 0.17 for S 0 cultures (for both untreated and Se-treated) (Fig. 2, A-B), which is in line with the lack of growth (Fig. 1, A-B) imposed by absence of S. A similar decrease in QY has also been observed in S deprivation studies in *Tetraselmis subcordiformis* (Yao, Ai, Cao, Xue, & Zhang, 2012) and *Dunaliella salina* (Yuan, Li, & Zhao, 2019). S 1 cultures untreated or Se-treated showed a significant ($p < 0.05$) but less pronounced decrease in QY when compared to control, S 6.48, at the end of the cultivation period (Fig. 1, A-B).

For S 2 and S 3, only the Se-treated cultures showed a decrease in QY that was significant at the end of cultivation period (Fig. 2, B), reflecting an increase of Se toxicity under S limiting conditions (Fournier et al., 2010). No other statistically significant differences in QY were observed for the remaining S $\geq$ 4 concentrations (untreated and Se-treated), which were in the range of 0.68–0.71. It has previously been reported that values of 0.6–0.7 are considered healthy *Nannochloropsis* cultures (Janssen et al., 2018). This indicates that when S is present in sufficient amounts, Se treatment has little or no effect on the photosynthetic performance of *N. oceanica*.

We also looked into the chlorophyll absorbance (OD_{680}) for further insight into the effect of Se (Fig. 2, C-D). S 0 cultures, untreated and Se-treated, revealed a step decrease in chlorophyll absorbance, similarly to what was observed for QY. S 1 culture, untreated and Se-treated, also showed a decrease in chlorophyll absorbance. Although there was some decrease in chlorophyll absorbance no other significant differences were observed for the remaining tested conditions S $\geq$ 2, at the end of cultivation. Overall, these results indicated that the absence of S is a 'stress' factor for the microalgal cells, which hindered photosynthetic efficiency and cell growth. Furthermore, the addition of Se under severe S limitation (S 1) elevated the 'stress' effect.

3.2. Sulphur and selenium accumulation

To understand whether selenite accumulation is affected by S, we performed a mineral analysis of *N. oceanica* biomass for the conditions tested. Thus, Se and S accumulation was analysed after twelve days of cultivation (Fig. 3). There was no significant difference in S accumulation between control conditions (5.70 $g_S/kg_{biomass}$ for S 6.48 Se untreated and 5.66 $g_S/kg_{biomass}$ for S 6.48 Se-treated). Furthermore, the Se accumulation in these control cultures (0.14 $g_{Se}/kg_{biomass}$) was in line with what was achieved in our previous study (0.13 $g_{Se}/kg_{biomass}$) (Guimarães, de Boer, et al., 2021).

Regarding S accumulation, *N. oceanica* cultivated without S (S 0) and S 1 resulted in a significant ($p < 0.05$) decrease in S accumulation for both untreated and selenite-treated cultures (Fig. 3). Although S 2 revealed limited growth, it did not cause any significant change in S accumulation compared to the control condition S 6.48. This could be due to the increase in sulphate transport into the cell under S limiting conditions (Yildiz, Davies, & Grossman, 1994). In *C. reinhardtii,* it has been observed that sulphate deprivation leads to an increase in sulphate transport into the cell (Yildiz et al., 1994) due to an increased capacity/affinity for SO_4^{2-} (Zhang et al., 2004). No increase in S accumulation was observed for the remaining tested conditions (S $\geq$ 2).

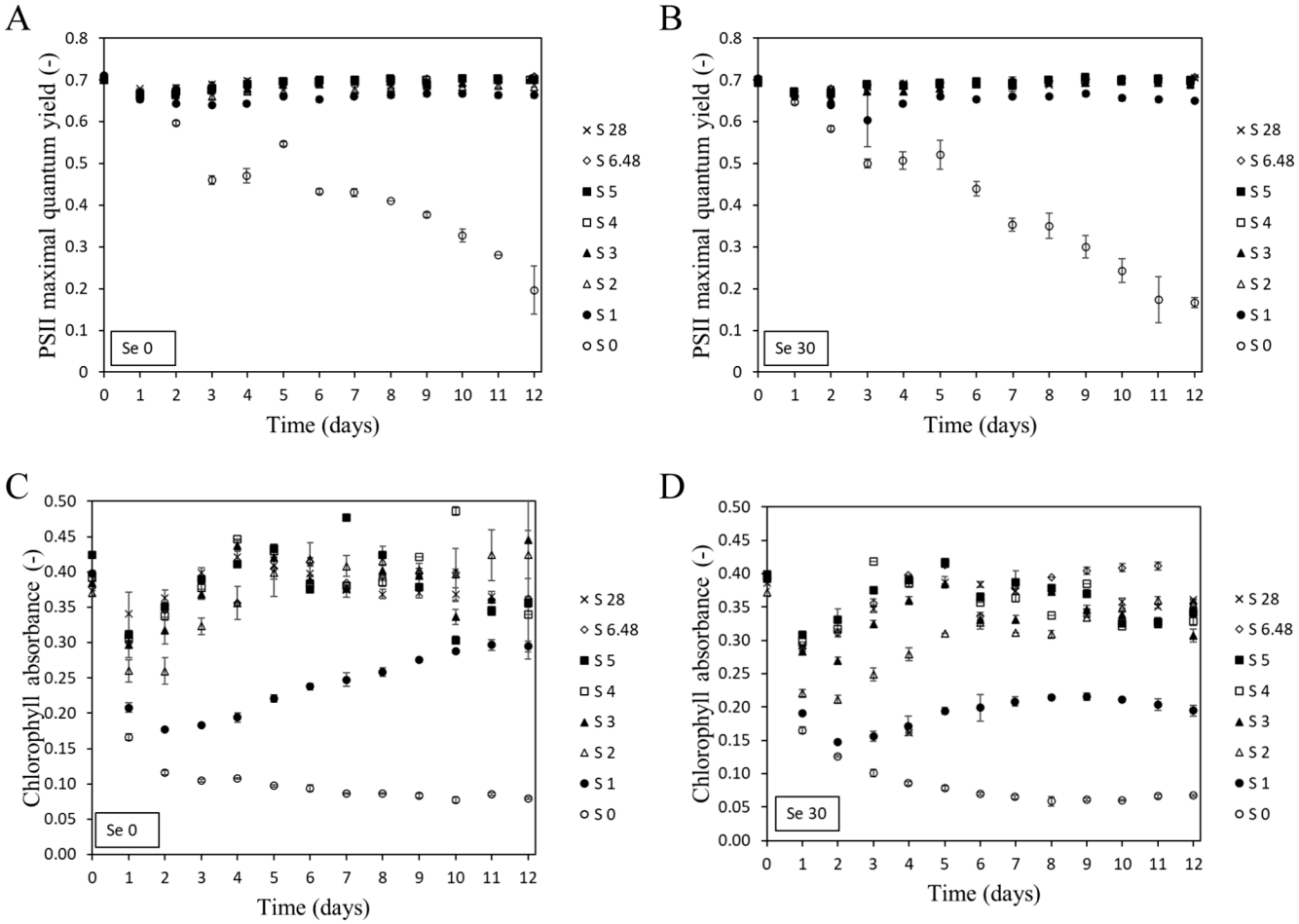

Fig. 2. Average PSII maximum quantum yield and chlorophyll absorbance of *N. oceanica* when exposed to different sulphur (S) concentrations (0, 1, 2, 3, 4, 5, 6.48, 28 mM) over 12 days. Cultures were grown untreated and selenium (Se)-treated under the same S concentrations: A-C) untreated (Se = 0 μM), B-D) with 30 μM of Se in the media (n = 3), (n = 9 for the controls 6.48 Se 0, 6.48 Se 30).

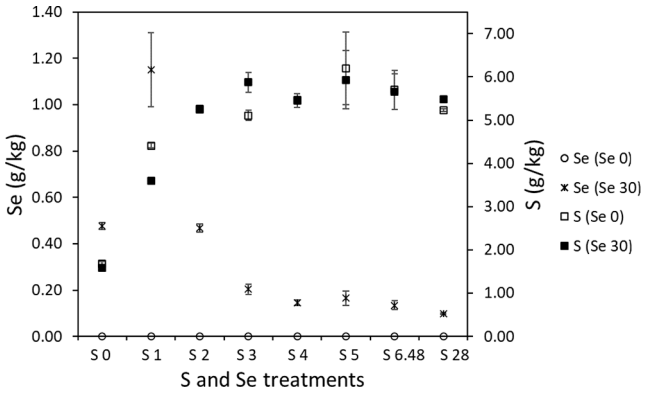

Fig. 3. Sulphur (S) and selenium (Se) accumulation in microalgal cultures after twelve days of batch cultivation (n = 3, n = 9 for controls 6.48 Se 0, 6.48 Se 30). *N. oceanica* cultures were exposed to different S concentrations [0–28 mM], and were either untreated or treated with Se (30 μM), supplemented in the form of sodium selenite.

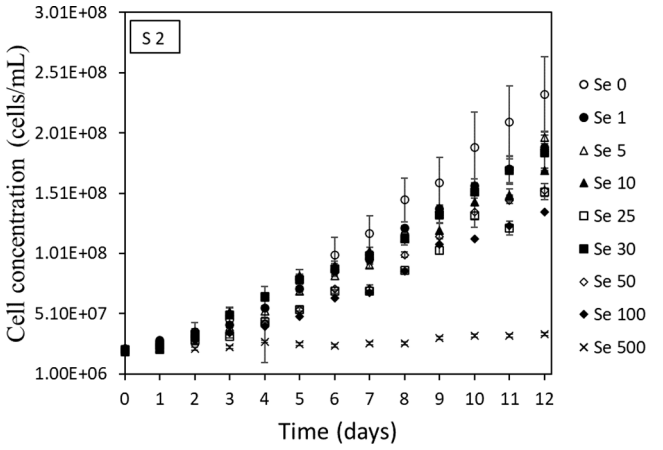

Fig. 4. Growth of *N. oceanica* when cultures in sulphur (S) limiting conditions (2 mM) and exposed to different selenium (Se) concentrations (0, 1, 5, 10, 25, 30, 50, 100, 500 μM) (n = 3), (n = 6 for S 2 Se 0, S 2 Se 30, S 2 Se 0, S 28 Se 30).

An antagonistic effect was observed between S and selenite accumulation, with less S supplementation leading to a significantly higher Se uptake (Fig. 3). The increase in selenite uptake due to sulphate limitation has also been reported in *Chlamydomonas reinhardtii* (Morlon et al., 2006). Likewise, we hypothesise that sulphate has an effect on selenite accumulation in *N. oceanica*. Se uptake was 8-fold and 3-fold higher ($p < 0.05$) for cells grown in S 1 and S 2, respectively, when compared to the respective control cultures (Fig. 3). The order of Se uptake rate and accumulation was: S 28 Se 30 (0.10 g/kg Se) (11.2 %) < S 6.4 Se 30 (0.14 g/kg Se) (17.0 %) < S 2 Se 30 (0.47 g/kg Se) (48.0 %) < S 1 Se 30 (1.15 g/kg Se) (82.3 %). Similar results were reported for *Selenastrum capricornutum* (Williams et al., 1994) and *C. reinhardtii* in S-limited and Se-treated cultures (Fournier et al., 2010).

Se-treated cultures with the highest S concentration used (S 28) had the lowest Se accumulation (Fig. 3), which further emphasises the antagonistic effect between S and Se. For the remaining cultures (S ≥ 3 and S < 28) no significant difference in Se accumulation was observed. Overall, we identified S 2 as the most promising S concentration, since there is a significant increase in Se accumulation with only a reduction of 4.4 % in productivity (Supplementary Table S3).

Nutrient limitation (N, S) has been proposed as a way to optimise storage compounds, such as FAs, as well as biofuels such as hydrogen, but this comes at a trade-off, with a reduction in biomass productivity (Melis, Zhang, Forestier, Ghirardi, & Seibert, 2000; Ran et al., 2019; Su et al., 2011). Thus, a compromise between cell growth and Se accumulation must be achieved, for Se-enriched microalgae biomass production. We have concluded that S limitation (2 mM) can be used as an effective way to increase Se accumulation in *N. oceanica*, with a limited effect on growth.

3.3. Se incorporation under sulphur limited conditions

As revealed above S limitation (2 mM) can be used as an effective way to increase Se accumulation. To increase Se accumulation we exposed *N. oceanica* to a range of Se concentrations [0–500 μM] under a limiting S condition (S 2) (Fig. 4). The Se concentration range was selected to maximise Se accumulation and understand Se toxicity under S limited conditions.

Se-treated cultures with concentrations ≥ 10 μM grew significantly less ($p < 0.05$) when compared to the control (S 2, Se 0). This suggests that the increase of Se in the medium, leads to toxicity and a reduction in cell growth. This finding is in line with what was previously reported for *N. oceanica* (Guimarães, de Boer, et al., 2021), *Chlorella pyrenoidosa* (Zhong & Cheng, 2017), and *C. reinhardtii* (Ene Morlon et al., 2005) when treated with different Se concentrations. Our study reveals Se toxicity occurs in the micromolar range which has also been reported by (Fournier et al., 2010).

We also analysed Se and S accumulation in the S limited cultures (Table 1). S accumulation was not affected within the range of Se concentrations investigated. On the other hand, we observed that with an increase in Se in the medium there was an increase in intracellular Se accumulation, which was also observed in our previous work (Guimarães, de Boer, et al., 2021). The maximum Se accumulation was 2.60 $g_{Se}/kg_{biomass}$ (Table 1), was 1.2-fold higher than what was previously observed in S replete conditions (2.12 $g_{Se}/kg_{biomass}$) under the same Se treatment (100 μM of Se) (Guimarães, de Boer, et al., 2021).

From this analysis we could also observe that cultures treated with Se concentrations ≤ 10 μM did not show a significant increase in Se accumulation. Treatments with higher Se concentrations (>25 μM), resulted in higher Se accumulation (Table 1), coupled to a decrease in growth, thus suggesting an increase in Se toxicity (Fig. 4). Since Se 25 and Se 30 μM resulted in similar Se accumulation rates, Se 30 μM was chosen to compare with our previous work. Overall, the Se 30 treatment was the most promising tested condition since an optimum was achieved between S limitation, Se accumulation, and cell growth and we propose this condition (S 2 Se 30) for future studies.

3.4. Effect of S concentration on Se speciation

We also performed a speciation analysis, in order to understand whether Se can be found incorporated on S-containing amino acids, and whether S concentration has any effect on the Se speciation in *N. oceanica*. Three concentrations of S (2, 6.48, 28 mM) were chosen from the previous experiments and speciation analysis was performed by HPLC-

Table 1
Sulphur (S) and selenium (Se) accumulation in microalgal cultures after twelve days of batch cultivation. ([a]) represents the cultures that were bleached and could not be measured by ICP-OES.

	S			Se		
	g/kg			g/kg		
S 2 Se 0	5.18	±	0.03	0.01	±	0.00
S 2 Se 1	5.16	±	0.22	0.02	±	0.00
S 2 Se 5	5.16	±	0.06	0.08	±	0.00
S 2 Se 10	5.01	±	0.18	0.23	±	0.02
S 2 Se 25	5.26	±	0.03	0.45	±	0.02
S 2 Se 30	5.26	±	0.06	0.47	±	0.02
S 2 Se 50	5.30	±	0.10	1.08	±	0.10
S 2 Se 100	5.29	±	0.13	2.60	±	0.26
S 2 Se 500	[a]	±	[a]	[a]	±	[a]

ICP-MS (Table 2, Fig. 5). These conditions were chosen to represent: S limitation (2 mM of S), our standard defined S medium concentration (6.48 mM of S), and the S concentration naturally found in seawater (28 mM of S).

Our results showed that the dominant organic Se species in *N. oceanica* is SeMet (>97 %) (Fig. 5). This has also been observed in other microalgae species such as *Chlorella sorokiniana* (Gómez-Jacinto et al., 2020). This is the first time the Se speciation has been reported in an Eustigmatophyte. The average abundance of the organic Se species in the cells was first SeMet (98.2 %), followed by $SeCys_2$ (1.4 %) and SeMeSeCys (0.4 %). Inorganic species are under detection limits in almost all samples (Fig. 5). *N. oceanica* accumulates the highest proportion of SeMet of the microalgae tested to date.

In previous studies, it was found that a mixed microalgae consortium (SeMet > inorganic Se(IV) > Se(VI) (SeMet 91 %, inorganic Se(IV) 1.9 % and Se(VI) 3.0 %) (Li et al., 2021), *C. sorokiniana* SeMet > SeMeSeCys > $SeCys_2$ > SeVI (SeMet 84 %, SeMeSeCys 11 %, SeCys 24 %) (Gómez-Jacinto et al., 2020) and *C. vulgaris* SeMet > MeSeCys > SeCys (SeMet 56 %, MeSeCys 28 %, SeCys 15 %) (Mylenko et al., 2020).

Furthermore, as previously stated, SeMet is the most interesting Se form for nutritional supplementation and bioavailability in humans and animals (Gojkovic et al., 2015; Lyons et al., 2007). Thus, knowing that Se in *N. oceanica* is metabolised and bioaccumulated into amino acids (>97 % SeMet) can instigate the use of *N. oceanica* as an effective Se-enriched ingredient for both food and aquafeed.

Regarding the effect of S on Se speciation, there is a proportional increase in SeMet with the increase in Se accumulation (S 2 Se 30) (Fig. 5). Similarly, with S 28 Se 30, where we have observed a decrease in Se accumulation, there is also a proportional decrease in SeMet. Thus, the percentage of SeMet in the overall Se organic fraction remains unchanged (>97 % SeMet). For the other seleno-amino acid species (SeCys2, SeMeSeCys) we could not observe this trend. This suggests that there may be a preferred route to metabolise Se into SeMet, in *N. oceanica.*

Finally, we compared the sum of the organic Se species (SeMet > $SeCys_2$ > SeMeSeCys > Se) with the total Se detected by ICP-MS to understand the percentage of organic Se in the cells. The percentage of organic Se varied with the S concentration used: 62.62 %, 87.92 %, 85.66 % (2, 6.48, 28 mM of S, respectively) (Table 2). Overall, this highlights that S limitation is an efficient way of increasing Se accumulation, but it comes at a cost of Se bioavailability in *N. oceanica*. Furthermore, S concentrations present in laboratory cultivated media and natural seawater (S 28) result in a high Se bioavailability (>85 %) which has not been demonstrated before.

3.5. Effect of Se on fatty acid profile and content

The effect of Se treatment and the combined effect of S limitation and Se treatment on the FAs profile has not been investigated in *Nannochloropsis* spp. before. We analysed FAs accumulation in *N. oceanica* for the three S concentrations selected above (2, 6.48 and 28 mM), untreated and Se-treated (30 μM).

Our results show there was no significant reduction in TFA content for all the conditions tested, indicating that Se exposure does not trigger any effect on FA accumulation (Fig. 6). However, S 2 conditions led to a significant decrease in the polar lipid fraction (PL) and a significant increase in the neutral lipid fraction, triacylglycerides (TAG) (Fig. 6, $p < 0.05$). Other studies have shown that S deficiency led to an increase in FAs and an increase in TAG under S depletion in *Chlamydomonas reinhardtii* (Matthew et al., 2009; Mizuno et al., 2013).

Only one study exists related to Se and EPA accumulation, whereby the presence of Se in the medium resulted in an increase in the proportion of EPA in TFAs in a microalgal consortia for wastewater treatment at pilot-scale (containing mostly *Chlorella* sp.) (Li et al., 2021). To the authors knowledge no study exists on the combined effect of S limitation and Se treatment on EPA content in any microalgae. We also

Table 2
Selenium (Se) speciation in *N. oceanica* after twelve days of cultivation with different sulphur (S) concentrations. Conditions tested were: S 2 mM Se 0 μM, S 2 mM Se 30 μM, S 6.48 mM Se 0 μM, S 6.48 mM Se 30 μM, S 28 mM Se 0 μM, S 28 mM Se 30 μM. Se accumulation measured by ICP-MS and ICP-OES show that there were minor differences when using the two different required methodologies.

S [mM], Se [μM]	$SeCys_2$ (μg Se/g)			SeMeSeCys (μg Se/g)			SeMet (μg Se/g)			Se (VI) (μg Se/g)			Total (Se) as sum of species (μg/g)			Total (Se) ICP-MS (μg Se/g)			Total (Se) ICP-OES (μg Se/g)		
	avr		sd	avr		sd	avr		sd	avr		sd	avr		sd	avr		sd	avr		sd
S 2, Se 0	0.01	±	0.01	n.d.	±	n.d.	7.11	±	0.01	n.d.	±	n.d.	7.12	±	0.02	19.23	±	6.07	<0.01		–
S 2, Se 30	2.71	±	0.01	0.45	±	0.01	280.50	±	0.01	0.10	±	0.01	283.76	±	0.01	453.14	±	13.17	466.73	±	17.66
S 6.48, Se 0	0.10	±	0.01	0.00	±	0.00	0.56	±	0.01	n.d.	±	n.d.	0.67	±	0.01	0.96	±	0.03	<0.01		–
S 6.48, Se 30	1.44	±	0.01	0.30	±	0.01	147.43	±	0.01	0.00	±	0.00	149.17	±	0.01	169.67	±	6.36	135.07	±	20.17
S 28, Se 0	0.05	±	0.01	0.03	±	0.01	5.13	±	0.08	n.d.	±	n.d.	5.22	±	0.10	7.47	±	0.00	<0.01		–
S 28, Se 30	2.65	±	0.02	0.88	±	0.02	111.49	±	0.02	n.d.	±	n.d.	115.02	±	0.05	134.27	±	5.71	97.95	±	3.71

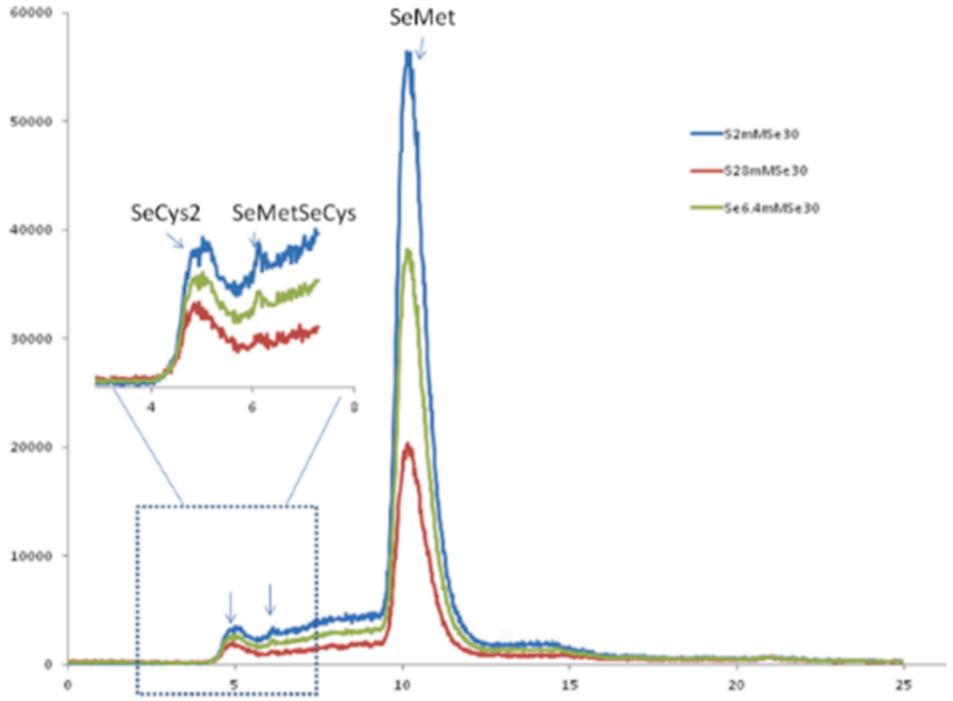

Fig. 5. Superimposed chromatograms of the selenium (Se) speciation within the three sulphur (S) treatments (S 2, 6.48, 28 mM) containing Se (30 μM).

looked at the omega-3 eicosapentaenoic acid (EPA) content, S limitation led to a decrease in EPA in PL and total EPA. A significant decrease in EPA was observed for S limited (S 2) cultures compared to the control (S 6 Se 0) (Fig. 6, B). This is well in line with the reduction of PL fraction, as under normal growth conditions EPA is mainly present in the membranes (Janssen, Lamers, de Vos, Wijffels, & Barbosa, 2019). Likewise, S 28 led to the highest percentage of EPA in PL (4.6 % DW) and total EPA (4.9 % DW), and it was not statistically different than control cultures (S 6 untreated and Se-treated), for all these conditions, the increase in EPA was statistically higher when compared to S 2 cultures. These findings highlight that there is a decrease in EPA due to the 'stress' factor of S limitation. Se treatment does not have an effect on EPA.

In summary, at lower S concentrations, where there is an increase in Se accumulation there is no effect on TFA but a significant decrease in total PL and EPA, with increase in total TAG. Thus, besides the effect on cell growth and Se accumulation, the effect of nutrient limitation on other products of interest should also be investigated in future Se-enriched microalgae studies. The use of higher S concentrations, such as using natural seawater, could lead to more EPA content at a trade-off of lower Se accumulation. *N. oceanica* is considered a good source of TFAs, including the omega-3 FA EPA. A thorough process design incorporating medium formulation could lead to the use of *N. oceanica* as a multi-functional ingredient (Se and other minerals, FAs, seleno- and other essential amino-acids) for feed purposes.

4. Conclusions

Our study was the first to investigate the effect of S concentrations on Se uptake in *N. oceanica.* Overall, we found that S depletion and limitation resulted in an increase of up to 8-fold in Se uptake. Thus, our research demonstrates that S limitation is an effective tool for Se-enriched microalgae production. Additionally, the remaining S replete conditions ($\geq$4 mM) had no effect on Se accumulation, except for the highest concentration (28 mM) which resulted in a decrease in Se accumulation. Furthermore, three concentrations were chosen to investigate Se speciation (2, 6.48 and 28 mM of S) since these represented S limitation, our standard defined medium S concentration, and S concentration found in natural seawater. The results of these experiments demonstrated for the first time that Se in *N. oceanica* is metabolised and bioaccumulated into seleno-amino acids. The average abundance of the organic Se species in the cells was first SeMet (98.2 %), followed by $SeCys_2$ (1.4 %) and SeMeSeCys (0.4 %) (SeMet > $SeCys_2$ > SeMeSeCys > Se). Furthermore, we found that the proportion of SeMet in *N. oceanica* remained > 97 % in the organic fraction of the total Se accumulation. This reveals the potential of using *N. oceanica* as an effective Se-enriched ingredient for both food and aquafeed regardless of the S concentration used in the media. In addition, although S limitation does not affect TFA content it can result in a decrease in EPA content. Thus, when applying this process at pilot-scale, an integrated approach should focus on the effects on cell growth, Se accumulation, Se speciation and products of interest to allow for an optimal production process.

5. Ethics Statement

This research paper contains original research, and no human or animal rights are applicable to this study.

CRediT authorship contribution statement

Bárbara O. Guimarães: Conceptualization, Methodology, Formal analysis, Visualization, Investigation, Writing – original draft, Writing – review & editing. **Belén Villarreal-Toribio:** Investigation, Formal analysis. **Tamara García-Barrera:** Methodology, Supervision, Writing – review & editing. **Ana Arias-Borrego:** Methodology, Investigation, Formal analysis, Writing – review & editing. **Pieter Gremmen:** Conceptualization, Methodology, Writing – review & editing. **René H. Wijffels:** Supervision, Writing – review & editing, Funding acquisition. **Maria J. Barbosa:** Supervision, Writing – review & editing. **Sarah D'Adamo:** Conceptualization, Supervision, Writing – review & editing, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial

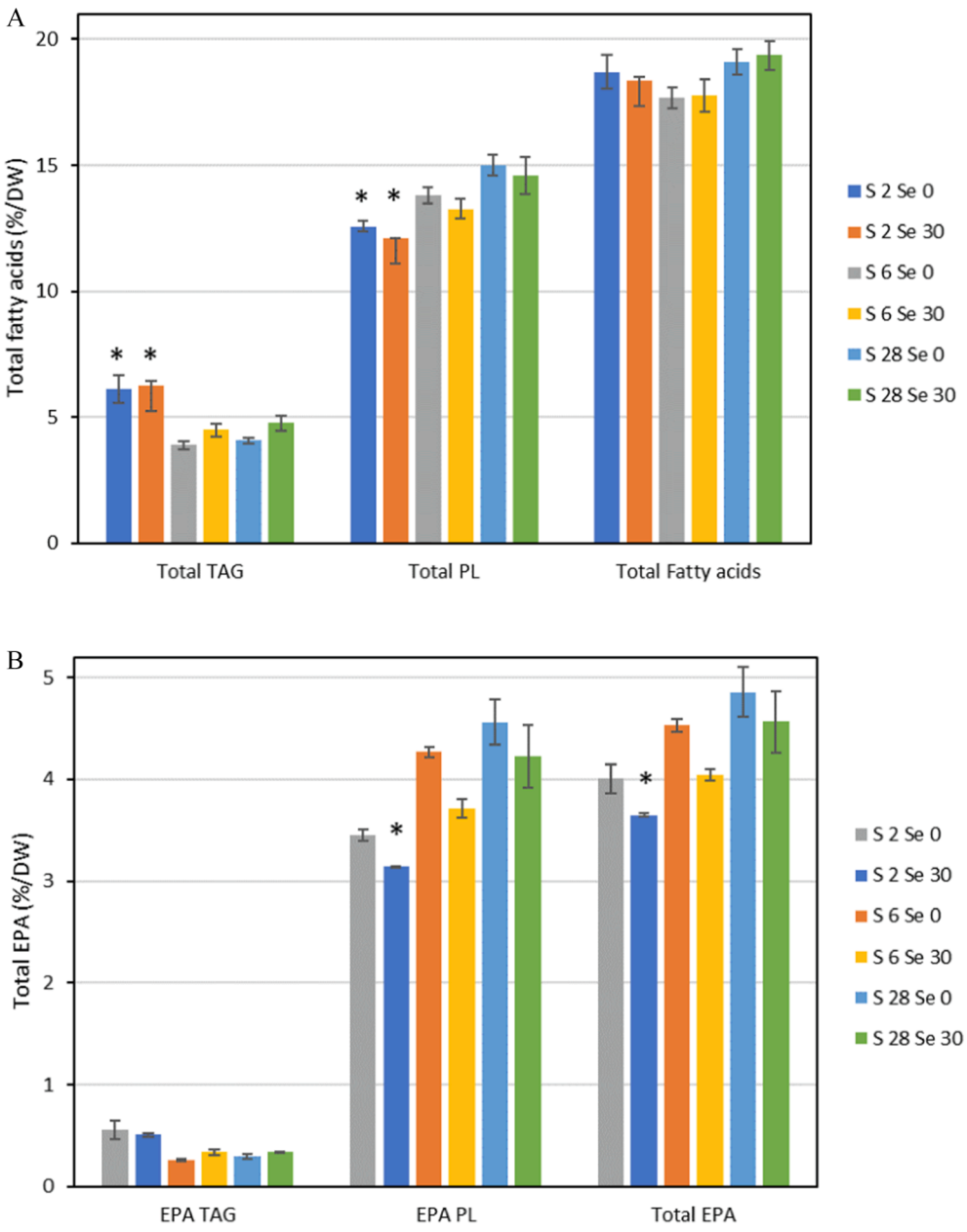

Fig. 6. Fatty acid profile of *N. oceanica* after twelve days of cultivation (n = 3) of the different sulphur (S) concentrations. Cultures were untreated and selenium (Se)-treated under the same S concentrations. Conditions tested were: S 2 mM Se 0 μM, S 2 mM Se 30 μM, S 6.48 mM Se 0 μM, S 6.48 mM Se 30 μM, S 28 mM Se 0 μM, S 28 mM Se 30 μM. A) Triacylglycerol (TAG), B) Polar lipids (PL).

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2022.105215.

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