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Selenium enrichment in the marine microalga *Nannochloropsis oceanica*

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ABSTRACT

Se-enriched ingredients have recently gained interest in the aquaculture industry as feed supplements due to their positive effects on fish health, growth, and potential effects on animal welfare. This study aims to assess which inorganic selenium (Se) species is suitable to produce Se-enriched *Nannochloropsis oceanica* (*N. oceanica*) biomass for aquafeed applications. The effective concentration for 50% growth inhibition (EC_{50}) and Se bioaccumulation of the two inorganic forms of Se, sodium selenite (Na_2SeO_3), and sodium selenate (Na_2SeO_4), were assessed at different concentrations after twelve days of cultivation. Toxicity results showed that selenate, EC_{50} = 32.93 μM, had a greater negative effect on cell growth than selenite, EC_{50} = 163.82 μM. Total intracellular Se was analysed by inductively coupled plasma - optical emission spectrometry (ICP-OES) and high resolution inductively coupled plasma mass spectrometry (HR-ICP-MS), which revealed that selenite was better accumulated by *N. oceanica*. Further investigation at 30 μM of selenite in the growth medium resulted in Se bioaccumulation with a minor effect on cell growth and reached a Se intracellular content of 0.131 $g_{Se}/kg_{biomass}$ after 12 days. Thus, 30 μM of selenite was selected for batch pilot-scale cultivation in a 1500 L tubular photobioreactor. Total Se accumulated in the biomass at pilot-scale was in the same order of magnitude compared with flasks (0.104–0.159 $g_{Se}/kg_{biomass}$). The results from this pilot-scale study are fundamental for a proof of concept from laboratory to pilot-scale production and they represent a critical bridging step for the potential use of Se-enriched *N. oceanica* in aquafeed.

1. Introduction

Selenium (Se) deficiency in fish can interfere with growth, cause greater fish mortality and hinder the fish immune response [1]. Se is a trace element naturally present in aquatic environments (both fresh and seawater) in two inorganic forms: selenite (SeIV) and selenate (SeVI) in the range of nanomolars (nM) to micromolars (μM) [2]. However, it is challenging to naturally meet Se dietary requirements in aquaculture, and efforts to mitigate this issue include adding mineral additives to aquafeed such as mineral mixes containing a higher supply of inorganic Se [3]. However, inorganic Se is not an effective aquafeed supplement since it is not easily absorbed by fish [3,4]. Thus, studies have focused on understanding the bioavailability of different Se sources used in aquafeed, including inorganic Se [5], organic Se (in the form of selenomethionine) [5], Se-enriched yeast [3], and Se-enriched microalgae [6,7]. Microalgae are considered an important aquafeed ingredient since they are the primary producers of (polyunsaturated fatty acids) PUFAs in the ocean and contain, carbohydrates, proteins, vitamins, essential amino acids, minerals and pigments which are essential to meet fish nutritional demands [8–10]. Studies have shown that microalgae play a crucial nutritional role in early development stages of finfish and for molluscs (such as oysters) during all stages of development [11,12].

The effect of Se on microalgae growth varies greatly and can be either beneficial or toxic [13]. Several studies on Se exposure in microalgae have shown that Se toxicological effects are mostly due to four main factors: the inorganic Se chemical species (e.g. selenite, selenate) [14,15]; the concentrations of Se in the media [16–18]; a reflection of a microalgae species-specific response [19], and the concentration of sulphur (S) present in the media [7]; since S and Se share chemical similarity, they are incorporated inside the cells through the same transporters, leading to an antagonistic uptake of S and Se [20–23].

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Table 1
Concentrations used for the selenite and selenate toxicity screening experiments.

Selenium (Se)		Sodium selenite (Na_2SeO_3)	Sodium selenate (Na_2SeO_4)
(μM)	(mg/L)	(mg/L)	(mg/L)
0	0.00	0.00	0.00
1	0.08	0.17	0.19
5	0.39	0.86	0.94
10	0.79	1.73	1.89
25	1.97	4.32	4.72
30	2.37	5.19	–
40	3.16	6.92	–
50	3.95	8.65	9.45
100	7.90	17.29	18.89
500	39.48	86.47	94.47
1000	78.96	172.94	188.94

Although several toxicological studies mention the effect of Se on growth, few studies have looked into the effect of the two inorganic species of Se on cell growth and accumulation under the same experimental conditions. In addition, no research has been conducted on pilot-scale production of Se-enriched marine microalgae of commercial interest such as *Nannochloropsis oceanica* (*N. oceanica*). *N. oceanica* is a key species in the aquafeed sector due to its nutritional value in pigments, fatty acids (up to 30.7%) [24], and eicosapentaenoic acid (EPA) content (2.3 to 5.6% DW) [24–28]. The aim of this paper is to determine for the first time which inorganic Se source is less toxic and more accumulated in *N. oceanica*, thus being suitable to produce Se-enriched biomass at pilot-scale.

2. Materials and methods

2.1. Cultivation medium, pre-cultivation

Nannochloropsis oceanica (*N. oceanica*) (CCAP 849/10) was cultivated in chloride medium (where all elements containing sulphate forms (SO_4^{2-}) were substituted by chloride (Cl^-) forms) as previously described [29]. The medium was filter sterilised (0.22 μM, Sartobran 300, Sartorius stedim, Germany). Pre-cultures were grown in 250 mL Erlenmeyer flasks containing a liquid volume of 150 mL. Cultures were kept in an orbital shaker incubator (100 rpm) with an incident light of 100 μmol m^{-2} s^{-1}, 18:6 h light:dark cycle, headspace with air enriched with 2.5% CO_2 at 25 °C.

2.1.1. Selenium treatment

Selenium (Se) stocks were prepared by dissolving sodium selenite (Na_2SeO_3) (Sigma Aldrich, UK) and sodium selenate (Na_2SeO_4) (Alfa Aesar, Germany) separately in de-mineralised water up to a concentration of 2 g/ L. Se stocks were added to the chloride medium during preparation.

2.2. Experimental set-up

2.2.1. Toxicity screening – sodium selenite and sodium selenate

Se toxicity was assessed by exposing *N. oceanica* to sodium selenite and sodium selenate (0, 1, 5, 10, 25, 50, 100, 500, 1000 μM) to characterise the effective concentration for 50% growth inhibition EC_{50} (Table 1). Additional selenite concentrations were then tested (0, 30, 40 μM) to assess the optimal concentration for pilot-scale production. Experiments were performed over 12 days in biological triplicates. A control with no Se (0 μM) was included in each screening series. An overview of the concentrations used can be found in Table 1. All experimental cultures had an initial cell concentration of ~2.4 × 10^7 cells/mL (OD_{750} of 0.5).

2.2.2. Pilot-scale production of selenium (Se) Se-enriched N. oceanica

Se accumulation was studied during batch cultivation in a 1500 L LGem tubular photobioreactor (PBR) (GemTube MK-1 1500s, LGem, The Netherlands), with a liquid volume of 1300 L, at AlgaePARC greenhouse facility (Wageningen University & Research) (Fig. 1). The pH was controlled at 7.5 by sparging CO_2 on demand, and the culture was mixed by using a combination of a liquid and air pump (approx. 55 L/min). Temperature in the culture ranged between 18 and 25 °C. *N. oceanica* biomass was sampled daily (up to 5 L a day) and the total sampling volume was maintained below 10% of the initial experimental volume (<130 L). *N. oceanica* was cultivated in natural seawater enriched with the same nitrate, phosphate, trace elements and Se stocks used for shake flask experiments. The pre-cultures were incrementally scaled up from Erlenmeyer flasks, to a 20 L flat panel PBR, to a 300 L tubular PBR, and finally transferred to the 1500 L tubular PBR. Microalgal cultures were inoculated on average with a starting OD_{750} between 0.4 and 0.5, to avoid photoinhibition. The PBRs were sterilised by adding 100 ppm of sodium hypochlorite for at least 24 h, followed by flushing the reactor twice with filtered tap water (Millipore Opticap® XL10 Durapore® 0.22 μM, Merck). At the end of each batch cultivation, approximately 1000 L was harvested from the PBR and the remaining 300 L in the PBR was used as inoculum for the next batch cultivation. The five consecutive batch reactor runs (16–26 days) described in this experiment occurred during Autumn and Winter (September 2019 – March 2020) (Fig. 1). The remaining microalgal biomass was firstly dewatered by a spiral plate technology centrifuge (EVODOS 25 SPT) and then lyophilised to 30% solids by freeze-drying (EKS 30-3, Zirbus). Freeze-drying was preferred for further processing to maintain the high nutritional composition of the microalgae and to avoid any heat effect on sensitive cellular components, such as fatty acids [24].

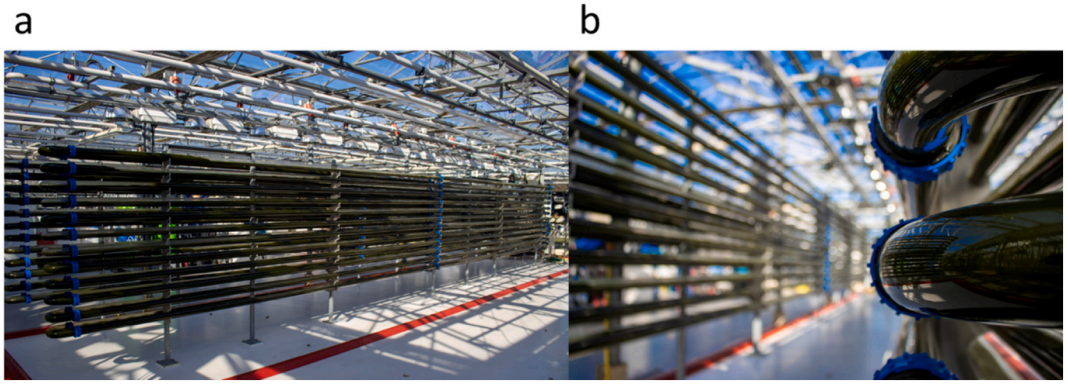

Fig. 1. LGem tubular photobioreactor (PBR) used for the pilot-scale production of *N. oceanica*. Se-enriched biomass: (a) 1500 L tubular PBR system. (b) close up of the glass tubes of the LGem system. Pictures were taken in AlgaePARC, Wageningen University and Research, The Netherlands.

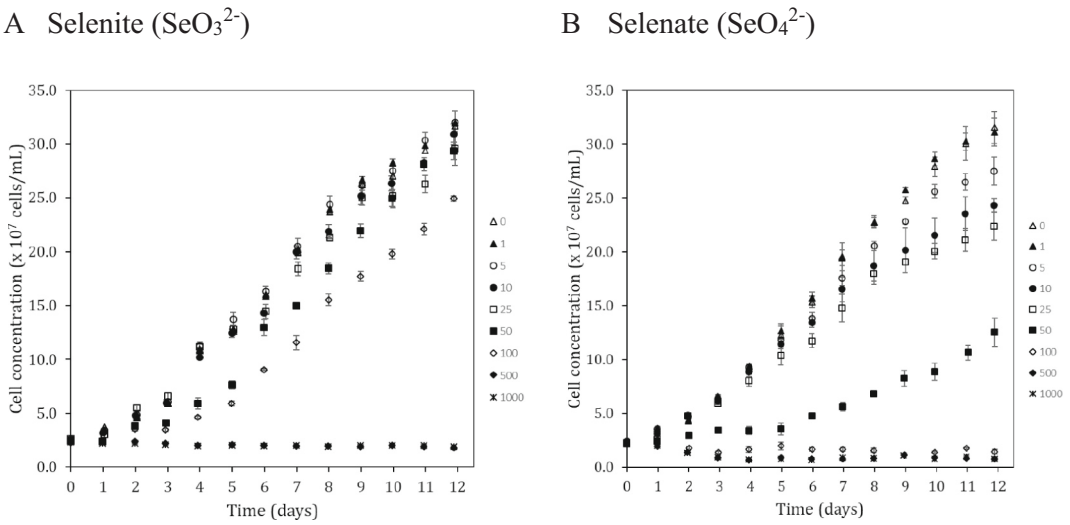

Fig. 2. Growth of *N. oceanica* when exposed to a range of Se concentrations (0, 1, 5, 10, 25, 50, 100, 500, 1000 μM) over 12 days. Two inorganic Se species were studied: a) sodium selenite (Na_2SeO_3) and b) sodium selenate (Na_2SeO_4). Samples were taken daily and each data point represents the average of three biological replicates (n=3).

2.3. Analytical methods

2.3.1. Biomass samples for cell growth determination

During all experiments, microalgae growth was monitored daily. Cell growth was determined by measuring *N. oceanica* optical density (OD) in triplicate with a UV-VIS-spectrophotometer (Hach Lange, DR 6000) (750 nM) and cell concentration was measured in triplicate with a cell counter (Beckman Multisizer™ 3 Coulter Counter) using a 50 μM aperture tube. Particles between 2 μM and 7 μM were considered to be *N. oceanica* cells. Dry weight was determined using the method previously described by Guimarães et al., [29]. Volumetric productivity (g/L/day) of each batch in the pilot-scale studies was calculated by subtracting the final dry weight from the initial dry weight and dividing it by the duration of the batch (days). Areal productivity (g/m^2/day) of each batch was calculated by multiplying the volumetric productivity by the culture volume in the PBR (1300 L) and dividing it by the aerial footprint of the LGem PBR (19 m^2).

2.3.2. Biomass samples for elemental analysis

N. oceanica flask cultures were harvested for elemental analysis on day 12 of the experiment. Cultures were centrifuged (2000 ×*g*, 15 min at 20 °C) and washed twice with ammonium formate (0.5 M) [29] to remove the excess of salts. After washing, the biomass pellets were lyophilised (Sublimator 2 × 3 × 3–5, Zirbus Technology, Germany). Samples for elemental analysis from the LGem reactor were taken at the end of each batch. For the last batch, samples were taken daily, and these were washed and centrifuged in the same manner.

2.3.3. Microwave-assisted acid digestion

Lyophilised biomass samples were microwave-assisted acid digested as previously described [29]. Each lyophilised microalgal sample (50 mg) was extracted with 10 mL dH_2O, 7.5 mL of hydrochloric acid (37%) and 2.5 mL of nitric acid (65%) in a microwave oven (milestone S.r.l. ETHOS 1). The total microwave run time was 40 min with a maximum temperature of 175 °C (maintained from 15 to 30 min). The maximum energy was 1400 W.

2.3.4. Mineral analysis in biomass samples

2.3.4.1. Sodium selenite-treated cultures. Samples treated with sodium selenite were measured by inductively coupled plasma - optical emission spectrometry (ICP-OES) (PerkinElmer Avio® 500) with operating conditions and calibration standards prepared from single elements as previously described [29]. Se was added to the combined mix containing phosphorus (P) and sulphur (S) to avoid precipitation. In sum, two mixes were prepared: combined mix 1: P, S, Se and combined mix 2: calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), sodium (Na), potassium (K), zinc (Zn).

2.3.4.2. Sodium selenate-treated cultures. Samples treated with sodium selenate were measured by high resolution - inductively coupled plasma - mass spectrometry (HR-ICP-MS) (Element2, Thermo Fisher Scientific). HR-ICP-MS was used since Se values were below detection limits of the ICP-OES. The nebulizer used for the ICP-MS was a concentric nebulizer made from PFA (Perfluoroalkoxy alkanes) and the spray chamber was cyclonic made and from quartz. The cones used were made of Nickel.

2.4. Statistical analysis

The data generated during this study was subjected to statistical analysis using one-way analysis of variance (ANOVA) to test the effect of Se on cell growth. When significant differences were found, post-hoc Tukey tests were applied. The statistical analysis was performed using SPSS version 25 with a significance level of $p < 0.05$. In order to perform the dose-response analysis, final obtained cell concentration values from the sodium selenate and selenite toxicity screening were normalised and plotted using Excel. The effective concentration for 50% growth inhibition (EC_{50}) determination was based on Geoffroy et al. [30], using the final cell concentration values adjusted to a four parameter logistic function using a dose-response model and defining the upper and lower limit using R [47] package *drc* [45]. Briefly, the lower limit was set between 0 and 2.4×10^7 cells/mL (the initial cell concentration) and the upper limit was set for the highest cell concentration obtained (3.3×10^8 cells/mL).

3. Results and discussion

3.1. Toxicity of selenium species in N. oceanica: selenite and selenate

Selenium (Se) can be either beneficial or toxic to microalgal cultures [13,31], however, the effect of Se in *N. oceanica* has not yet been described. In this study, various concentration of Se (0–1000 μM) (Table 1) were tested on *N. oceanica*. Cell growth was monitored to determine the effective concentration for 50% growth inhibition (EC_{50})

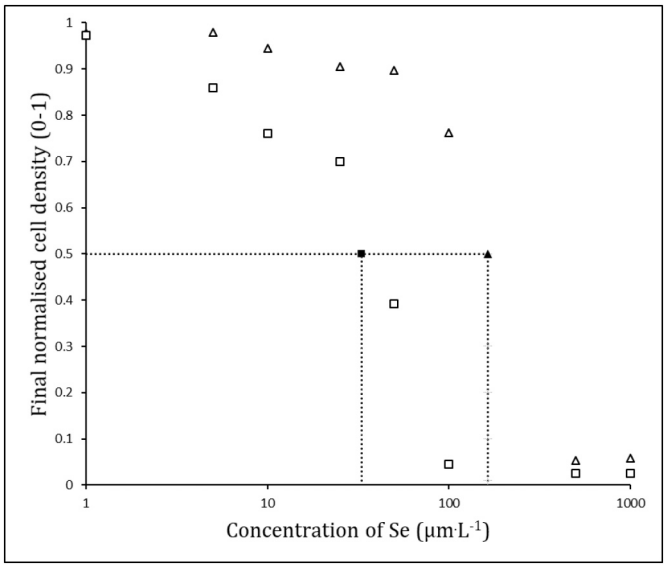

Fig. 3. Dose-response curve of the different Se concentrations according to the final cell concentration reached after 12 days. Squares represent values from sodium selenate, and triangles represent values from sodium selenite. The dotted lines represent the expected EC_{50} values for selenate (EC_{50} = 32.93 µM) and selenite (EC_{50} = 163.82 µM). Each point in the graph represents the average cell number of three biological replicates (n=3), except for Se 0 which is an average of the controls used during cultivation (n=6).

of Se (Fig. 2). Experiments lasted 12 days to observe the effect over several cell divisions of a culture during exponential and linear growth.

Toxic effects that resulted in no cell growth were observed at the highest Se concentrations tested (500 and 1000 µM), for both selenite and selenate. Moreover, for both selenite- and selenate-treated cultures (Fig. 2, A–B), concentrations ranging from 50 to 100 µM resulted in a significant reduction in cell concentrations ($p < 0.001$).

Differences in toxicity between the two Se species were observed at lower Se concentrations (1–50 µM). Selenate-treated cultures showed the highest toxicity, where the final cell concentration was significantly different from the control (0 µM) at the concentrations of 5 µM (p = 0.034), 10 µM (p = 0.001), and 25 µM ($p < 0.001$). Consequently, for selenate, non-toxic concentrations were only obtained at the lowest concentration tested, 1 µM (Fig. 2, B). For selenite-treated cultures, non-toxic concentrations were obtained within the concentration range 1–25 µM (Fig. 2, A). Overall, the toxicity screening successfully provided an assessment of toxic and non-toxic effects for both Se ionic species and it showed that selenate is more toxic to *N. oceanica* than selenite.

Similar to our findings, other Se comparative studies showed higher toxicity of selenate compared to selenite in the freshwater microalgae *Selenastrum capricornutum* (*S. capricornutum*) [14] and *Chlamydomonas reinhardtii* (*C. reinhardtii*) [15].

Selenite concentrations in the range of 25–50 µM seemed promising since no effect on cell growth was observed at 25 µM and only a small effect on cell growth was observed at 50 µM (Fig. 2, A). Therefore, we tested two intermediate concentrations of selenite (30, 40 µM). Se exposure caused a significant decrease in cell growth for 30 µM Se (2.37 mg_{Se}/L), (p = 0.032) and for 40 µM Se (3.16 mg_{Se}/L), (p = 0.001) (Supplementary data – Fig. S1). Although some studies report a beneficial effect on microalgal growth when exposed to low concentrations of selenite, for example *Arthrospira platensis* (0.5–40 mg/L) [16], *Chlorella vulgaris* (*C. vulgaris*) (≤75 mg/L) [17], and *Chlorella pyrenoidosa* (*C. pyrenoidosa*) (≤40 mg/L) [32], we did not observe any positive effects on growth. This could be due to a microalgal species-specific response to Se [19].

3.2. Evaluation of effective concentration for 50% growth inhibition EC_{50}

The toxicological effects of both inorganic Se species in *N. oceanica* were assessed by analysing the effective concentration for 50% growth inhibition (EC_{50}), the no observed effect concentration (NOEC), and the

Table 2
Overview of EC_{50} values for several microalgal species, both fresh and seawater: *Chlorella pyrenoidosa* (*C. pyrenoidosa*), *Chlamydomonas reinhardtii* (*C. reinhardtii*), *Chlorella sorokiniana* (*C. sorokiniana*), *Chlorella vulgaris* (*C. vulgaris*), *Desmodesmus quadricauda* (*D. quadricauda*), *Desmodesmus subspicatus* (*D. subspicatus*), *Haematococcus pluvialis* (*H. pluvialis*), *Nannochloropsis oceanica* (*N. oceanica*), *Pseudokirchneriella subcapitata* (*P. subcapitata*), *Selenastrum capricornutum* Printz (*S. capricornutum*), *Scenedesmus quadriculata* (*S. quadriculata*), when exposed to selenium (Se) in two oxidation states (sodium selenate and sodium selenite). Values in bold refer to the EC_{50} values obtained experimentally in this study.

Microalgal species	EC_{50} [µM]				Exposure time (days)	Cultivation type (batch/continuous)	Source
	Sodium selenite		Sodium selenate				
	($mg \cdot L^{-1}$)	($\mu mol \cdot L^{-1}$)[a]	($mg \cdot L^{-1}$)	($\mu mol \cdot L^{-1}$)[a]			
C. pyrenoidosa	–	–	0.79	10	n.a.	C	[35]
C. pyrenoidosa	15.37	194.7	–	–	3	B	[32]
C. reinhardtii	–	–	0.032	0.4	4	B	[7]
C. reinhardtii	–	–	0.245	3.1	4	B	[7]
C. reinhardtii	–	–	0.355	4.5	4	B	[30]
C. reinhardtii	1.1	14	–	–	4	B	[38]
C. reinhardtii	6.3	80	–	–	4	B	[33]
C. sorokiniana	–	–	45[b]	570[b]	5	B	[37]
C. vulgaris	73.2	927.1	322.7	4086.9	14	B	[19]
D. quadriculata	84.5	1070	115.5	1463	14	B	[19]
D. subspicatus	54.7	693	167	2115	14	B	[19]
H. pluvialis	24.02	304	–	–	15	B	[6]
N. oceanica	**12.94**	**163.82**	**6.22**	**32.93**	**12**	**B**	**Present study**
P. subcapitata	302	3824.7	212.5	2691.2	14	B	[19]
S. capricornutum	143	1811	61.5	779	1	B	[14]
S. capricornutum	100	1266	–	–	2	B	[14]
S. capricornutum	96	1216	–	–	4	B	[14]
S. capricornutum	65	823	40	507	6	B	[14]
S. quadricauda	4	50	33	418	4	B	[36]

Non-applicable (n.a.).
[a] ($\mu mol \cdot L^{-1}$) values are expressed in elemental Selenium with a molecular weight of 78.96 (g/mol).
[b] Data from Gojkovic was previously reported as sodium selenite (238.2) but was recalculated to elemental Se.
Table adapted from [7,36,37].

Table 3
Selenium (Se) accumulation for both sodium selenite and sodium selenate-treated microalgal cultures after twelve days.

Se	Selenite			Selenate		
(μM)	(g/kg)		std. dev	(g/kg)		std. dev
0	<0.01			0.001	±	0.001
1	0.014	±	0.001	0.002	±	0.000
5	0.037	±	0.003	0.009	±	0.001
10	0.086	±	0.004	0.017	±	0.000
25	0.249	±	0.004	0.043	±	0.002
30	0.131	±	0.017	[b]	±	[b]
40	0.315	±	0.069	[b]	±	[b]
50	0.671	±	0.014	0.085	±	0.002
100	2.115	±	0.077	[a]	±	[a]
500	[a]	±	[a]	[a]	±	[a]
1000	[a]	±	[a]	[a]	±	[a]

[a] Se determination was not possible for 100 μM (selenate only), 500 and 1000 μM treated cultures since these were bleached after exposure to both sodium selenite and sodium selenate.
[b] Only selenite was tested for 30 μM and 40 μM.

lowest observed effect concentration (LOEC) (Fig. 3). The EC_{50} of selenate, EC_{50} = 32.93 μM Se (2.60 mg_{Se}/L) ($p < 0.001$), was four times more toxic than selenite, EC_{50} = 163.82 μM Se (12.94 mg_{Se}/L) ($p < 0.001$), and it reveals its adverse effect in *N. oceanica*. The NOEC of selenate, NOEC = 1 μM Se, was also lower than for selenite, NOEC = 25 μM Se. The LOEC for selenate, LOEC = 5 μM Se ($p = 0.034$), is again lower than that of selenite, LOEC = 30 μM Se (p = 0.001). Overall, these values confirm the higher toxicity of selenate over selenite with the same concentrations and cultivation conditions. Regarding selenite, the LOEC (30 μM) and NOEC (25 μM) values for *N. oceanica* are similar to values obtained for *C. reinhardtii*, 50 and 10 μM, respectively [33].

3.3. Comparison of EC_{50} values across microalgal strains

Table 2 presents an overview of EC_{50} values reported for other microalgae when treated with selenate or selenite. The selenate EC_{50} of 32.93 μM obtained for *N. oceanica* (this study - Fig. 3) is higher compared to those obtained for *C. reinhardtii*: 0.4 or 3.1 μM [7] or 4.5 μM [34], or 10 μM for *C. pyrenoidosa* [35], suggesting a better tolerance for *N. oceanica* to this element. However, other studies reported higher EC_{50} values for microalgae strains such as *C. vulgaris* (4086.9 μM) [19], *S. quadriculata* (418 μM) [36], *S. capricornutum* (507 μM) [14], and *Chlorella sorokiniana* (*C. sorokiniana*) (570 μM) [37], indicating higher Se tolerance for these species.

The selenite EC_{50} values for *N. oceanica* (163.82 μM) (Fig. 3) were similar to the ones reported for *C. pyrenoidosa* (194.7 μM) [32], lower compared to what has been reported for *H. pluvialis* (304 μM) [6] and *S. capricornutum* (823 μM) [14], 2- to 12-fold higher than *C. reinhardtii* (14 μM and 80 μM, respectively) [33,38], and 3-fold higher than *S. quadriculata* (50 μM) [36]. Overall, this suggests that *N. oceanica* is more tolerant to selenite than *C. reinhardtii* and *S. quadriculata,* has a similar Se tolerance as *C. pyrenoidosa,* and is less tolerant to Se than *H. pluvialis* and *S. capricornutum*.

Overall, this broad range of reported levels of Se toxicity (Table 2) emphasises the necessity for species-specific studies. Moreover, other elements in the medium composition may interfere with Se uptake, which also needs to be investigated in more depth. For example, the competitive uptake of sulphur (S) may have an important role in the toxicity and bioaccumulation of Se [15,16].

Overall, the toxicological differences observed in our study confirm that the toxic effect of Se depends on the concentration of Se in the medium, the inorganic Se chemical species, and the *N. oceanica* species-specific response, which has also been observed in other studies [15,16,19,37]. Moreover, it was observed that increasing intracellular Se negatively affected growth [7,38]. Our study emphasises the need for an assessment of Se toxicity and bioaccumulation in the same study.

3.4. Selenium bioaccumulation

Se was measured in both selenite- and selenate-treated *N. oceanica* cultures, after 12 days (Table 3). However, Se analysis was not possible for 100 μM (selenate only), 500 μM, and 1000 μM Se-treated cultures since no growth was observed at the highest Se concentrations tested for both selenite and selenate (cells were bleached) (Fig. 2). Control groups (without Se) had a maximum amount of total Se <0.001 $g_{Se}/kg_{biomass}$ for selenate and <0.01 $g_{Se}/kg_{biomass}$ for selenite cultures.

N. oceanica selenate-treated cultures had a maximum Se accumulation of 0.085 $g_{Se}/kg_{biomass}$ (at 50 μM) (Table 3). Moreover, the increase of selenate concentrations in the media resulted in more Se accumulation in the biomass. Selenate-treated cultures in the range from 5 to 50 μM had significantly ($p < 0.05$) more Se than control cultures (no added Se), indicating Se accumulation is proportionate to the Se concentration in the media [39].

N. oceanica selenite-treated (100 μM) cultures had a maximum Se accumulation of 2.115 $g_{Se}/kg_{biomass}$ (Table 3). This value is 4-fold higher than what was previously observed in selenite-treated *C. pyrenoidosa* (231 μM–40 mg/L) (0.435 $g_{Se}/kg_{biomass}$) [18] and selenite-treated *H. pluvialis* (13 mg/L) (0.646 $g_{Se}/kg_{biomass}$) [6], highlighting the potential of using *N. oceanica* as a Se-enriched biomass source. As observed in selenate-treated cultures, selenite-treated cultures significantly accumulated ($p < 0.05$) more Se from 25 μM (0.249 $g_{Se}/kg_{biomass}$), 30 μM (0.131 $g_{Se}/kg_{biomass}$), Se 40 μM (0.315 $g_{Se}/kg_{biomass}$) up to 100 μM.

Overall, Se accumulation was 4 to 8-fold higher when treating *N. oceanica* cultures with selenite rather than selenate. This was to some extent in line with the published literature where selenite was preferentially taken up by *C. vulgaris* [40] and selenite was found to be accumulated up to ten times more efficiently than selenate by *C. reinhardtii*, due to different transport mechanisms [15]. In our study, selenite was the least toxic and the most accumulated Se inorganic form in *N. oceanica*, which contradicts previous observations where the form of Se that is most accumulated is also the most toxic to microalgae [13]. This emphasises the need for more research in Se uptake and accumulation and its species-specific effect. Additionally to Se, for selenite treated cultures, other major and minor elements (S, P, Ca, Cu, Fe, Mg, Mn, Na, K, Zn) were measured at the end of each cultivation (Supplementary data – Table S1). The elements were present in a decreasing concentration of P > S > K > Mg > Ca > Fe > Na > Se > Zn > Mn > Cu > Co ≈ Mo [41], where Se is between Na and Zn in all conditions, except the control (Se 0 μM) and the lowest selenite concentrations (1, 5 μM), where Se is least accumulated. This suggests that the presence of Se does

Table 4
Overview of all the cultivation runs of Se-enriched *N. oceanica* in AlgaePARC.

Batch	Total cultivation	Volumetric productivity			Areal productivity			Final dry weight			Selenium content		
(n°)	(days)	(g/L/day)			(g/m^2/day)			(g/L)			($g_{Se}/kg_{biomass}$)		
1	17	0.248	±	0.002	16.968	±	0.142	3.420	±	0.026	–	±	–
2	26	0.180	±	0.005	12.290	±	0.346	4.533	±	0.049	0.104	±	0.002
3	16	–	±	–	–	±	–	5.027	±	0.795	0.110	±	0.008
4	18	0.203	±	0.002	13.880	±	0.157	3.840	±	0.063	0.109	±	0.010
5	24	0.192	±	0.003	13.151	±	0.189	5.000	±	0.089	0.159	±	0.015

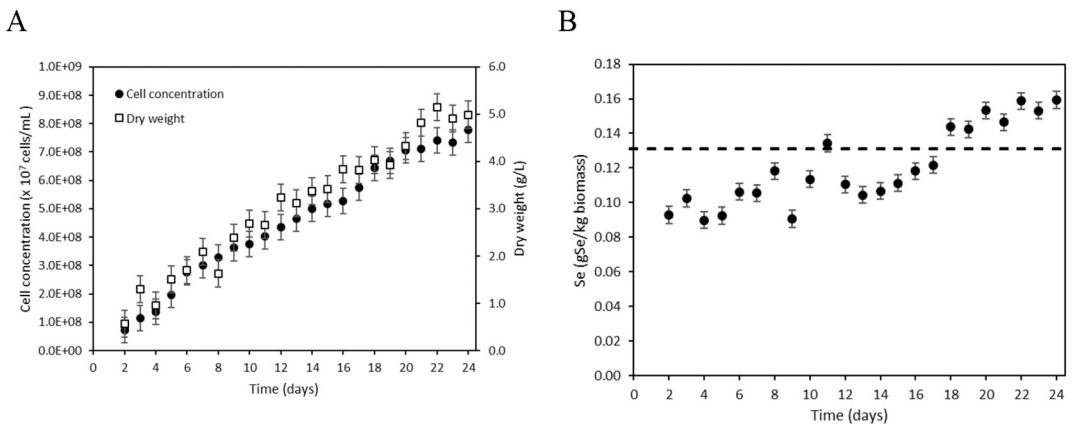

Fig. 4. Pilot-scale cultivation of *N. oceanica*. a) Cell growth during cultivation in the LGem tubular system over a period of 24 days up to a biomass concentration of 5 g/L. Average productivity was 0.21 g/L/day. b) Daily total selenium (Se) accumulation ($g_{Se}/kg_{biomass}$) in *N. oceanica*. The dotted line represents the Se concentration achieved during the flask cultivation using 30 μM of sodium selenite in the growth medium. Each data point represents the average of three technical replicates.

not influence or correlate with the uptake of other minerals in the conditions tested. However, a significant decrease of Mg was observed for the Se concentrations from 25 μM onwards ($p < 0.05$). This suggests that the decrease in growth caused by the toxic effect of Se is also reflected in a decrease in Mg, which is a component of chlorophyll.

To produce Se-enriched biomass, a compromise needs to be reached between Se accumulation and the effect on growth during cultivation. Therefore, we consider selenite to be the most suitable Se source for the production of Se-enriched *N. oceanica.* A selenite concentration of 30 μM, which is just above the NOEC (25 μM), was selected for the pilot-scale experiments.

3.5. Pilot-scale production of selenium (Se) Se-enriched N. oceanica

To test the feasibility of pilot-scale production, Se-enriched *N. oceanic*a was cultivated in a 1500 L photobioreactor for 101 days in five consecutive cultivation batches (Table 4). Table 4 gives an overview of growth and the final Se accumulation obtained. Overall, each cultivation batch lasted between 16 and 26 days. The average volumetric productivity of all batches was approximately 0.21 g/L/day, which is in the same order of magnitude as the value achieved in an outdoor study with *N. oceanica* in tubular PBRs (0.33 g/L/day) during Spring/Summer in Portugal [42]. The average areal productivity reached in this study was 14.07 g/m^2/day (Autumn/Winter, The Netherlands). Similar results were found in a study with *Nannochloropsis* sp. in vertical outdoor photobioreactors (10.6–24.4 g/m^2/day, during Summer in The Netherlands) [43]. Similarly, a study with *N. oceanica* obtained an average areal productivity of 10.7 g/m^2/day during Spring/Summer in Portugal [42]. Overall, this emphasises that the cultivation of Se-enriched *N. oceanica* did not affect its volumetric or areal productivity.

The total amount of Se accumulated at the end of each batch was determined. However, Se determination was not possible for batch 1 since the biomass was not pre-washed with ammonium formate and only centrifuged. Overall, at the end of the batch (2–4) Se accumulation did not vary significantly. However, batch 5 had significantly more Se than all other batches ($p = 0.001$). This could be due to biological variability between the different batches, which occurred between Autumn and Winter.

Selenium accumulation in the biomass was measured daily in batch 5 (Fig. 4, B). In this batch *N. oceanica* was cultivated for 24 days (Fig. 4, A) to a final biomass concentration of 5 g/L. There was no sampling on day zero (inoculation day) and day 1 since the tubular PBR was still being mixed to a uniform culture. Se inclusion increased during cultivation, reaching 0.110 ± 0.015 $g_{Se}/kg_{biomass}$ on day 12 and 0.159 ± 0.015 $g_{Se}/kg_{biomass}$ on day 24 (Fig. 4, B). Overall, Se accumulation at pilot-scale was similar to the Se accumulation observed in flasks after 12 days of cultivation (0.131 ± 0.017 $g_{Se}/kg_{biomass}$), suggesting a stable and growth-dependent Se accumulation (Fig. 4, dotted line). Our results are also in the same order of magnitude as the values achieved with the freshwater microalgae *Chlorella* (0.251 $g_{Se}/kg_{biomass}$) [44].

4. Conclusion

This study is the first to assess toxicological effects of Se and the production of Se-enriched biomass using *N. oceanica*. The dose-response effect was evaluated for sodium selenate, EC_{50} = 32.93 μM (2.60 mg_{Se}/L) and sodium selenite, EC_{50} = 163.82 μM (12.94 mg_{Se}/L), revealing that selenite is less harmful for the growth of *N. oceanica*. Selenite is more efficiently taken up than selenate, thereby showcasing its potential to be used as an inorganic source for the production of Se-enriched *N. oceanica* biomass. During pilot-scale experiments, the presence of selenite had a minimal effect on the growth of *N. oceanica* and resulted in selenium accumulation between 0.104 and 0.159 $g_{Se}/kg_{biomass}$. These results are in line with what was observed during laboratory scale trials (0.131 ± 0.017 $g_{Se}/kg_{biomass}$). Overall, we demonstrated the capability of *N. oceanica* to accumulate Se, validated this at pilot-scale and provided the proof of concept for using this species as a Se-enriched biomass for aquafeed.

Statement of informed consent, human/animal rights

No conflicts, informed consent, or 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. **Kieke de Boer**: Investigation, Writing - Review & Editing. **Pieter Gremmen**: Conceptualization, Methodology, Writing - Review & Editing. **Anemoon Drinkwaard**: Investigation, Writing - Review & Editing. **Rick Wieggers**: Methodology, Investigation. **René H. Wijffels**: Conceptualization, Supervision, Writing - Review & Editing, Funding acquisition. **Maria J. Barbosa**: Conceptualization, 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 personal or financial interests or relationships that could have influenced the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2021.102427.

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