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# Effect of phosphorus limitation on Se uptake efficiency in the microalga *Nannochloropsis oceanica*

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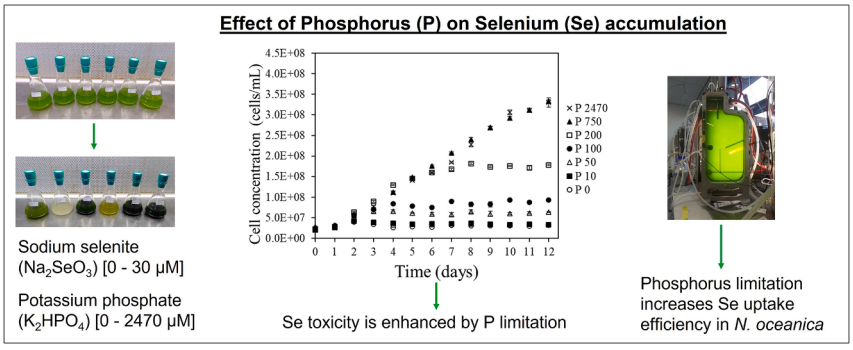
## HIGHLIGHTS

- P limitation increased Se uptake efficiency in *Nannochloropsis oceanica.*
- Higher Se uptake efficiency led to higher Se toxicity.
- Se accumulation was positively correlated with cell growth.
- P 200 and Se 5 were considered the optimal concentrations for Se-enrichment.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Microalgae are considered an efficient accumulator and promising source of Se for feed additive purposes. This study aimed at investigating, for the first time, the effect of phosphorus limitation on Se accumulation and uptake efficiency in *N. oceanica.* A range of phosphorus concentrations (0–2470 µM) were tested in either the presence or absence of sodium selenite (0, 5, 30 µM). Se accumulation was increased up to 16-fold and Se uptake efficiency was increased up to 3.6-fold under phosphorus growth-limiting concentrations. *N. oceanica* was then cultivated in a 1.8 L flat-panel photobioreactor in batch operation under two phosphorus growth-limiting concentrations (250 and 750 µM) where the accumulation of Se in the microalgal biomass, as well as its presence in the spent medium were analysed. This study is the first to investigate the effect of phosphorus limitation for increasing Se accumulation in microalgae, and to prevent the release of Se in wastewater.

*Abbreviations:* ICP-OES, Inductively coupled plasma - optical emission spectrometry; *N. oceanica*, Nannochloropsis oceanica; N, Nitrogen; NSW, natural seawater; OD, optical density; P, Phosphorus; PBR, photobioreactor; QY, Quantum yield; S, Sulphur; Se, Selenium; SeMet, selenomethionine; Tris, trishydroxymethylaminomethane.

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## 1. Introduction

Selenium (Se) is an essential element for numerous living organisms, including humans and fish (Young et al., 2010). Se deficiency is of global concern since it can lead to an increase in disease in both humans and animals (Wang et al., 2022). Humans receive Se from dietary sources including vegetables and farmed animals such as chicken and fish (Surai, 2007). The beneficial effects of Se in chickens include enhanced reproductive performance and higher Se accumulation in the egg white and yolk (Skřivan et al., 2006). Se is essential for fish growth and health since its deficiency causes growth retardation, higher mortality, and debility (Khan et al., 2017; Wang et al., 2022).

In the environment, Se is present in four different forms: selenate, selenite, elemental Se, and selenide (Young et al., 2010). The Se concentration in the soil is subjected to a biogeochemical cycle leading to high variations in Se distribution around the globe (Vriens et al., 2014). In aquatic ecosystems, Se is mostly present in two inorganic forms: selenite ($Na_2SeO_3$) and selenate ($Na_2SeO_4$) (Fernández-Martínez and Charlet, 2009). Se contamination occurs in aquatic ecosystems due to anthropogenic activities; such as agricultural activities and discharges of Se-contaminated wastewaters, and can result in the extinction of fish species (Bashir et al., 2020; Young et al., 2010). Se contamination has led to the implementation of maximum concentrations of Se in industry such as in aquaculture feed (aquafeed) in the European Union, where the legal limits are set to 0.5 $mg_{Se}/kg_{feed}$ for inorganic Se, and 0.2 $mg_{Se}/kg_{feed}$ for organic Se supplementation (EFSA, 2012). Similarly, there are maximum levels for Se concentration in wastewater effluents to protect the environment from Se toxic effects (FAO, 2004). Se in sludge should not exceed levels of 2 mg/kg of dry solids or 0.025 mg/L in wastewater effluents used for crop production (FAO, 2004). A major challenge remains in providing Se supplementation to fish whilst complying with the Se legislation limits to minimise Se effluent discharge. Currently, Se is supplemented in aquafeed in inorganic (e.g., sodium selenite) and organic (e.g., crustaceans, Se-enriched yeast, and Se-amino acids) forms (EFSA, 2012; Ferreira et al., 2022; Sele et al., 2018; Surai, 2007; Wang et al., 2022). Organic Se leads to higher Se accumulation in Atlantic salmon muscle when compared to inorganic Se. Thus, organic Se sources are recommended for Se supplementation (Sele et al., 2018). Microalgae are a valuable aquafeed ingredient rich in protein (containing essential amino acids), fatty acids, pigments, vitamins, and minerals (Brown, 2019; Ferreira et al., 2022) and they are able to accumulate and metabolise Se (Guimarães et al., 2022; Li et al., 2021; Umysová et al., 2009). A few studies have looked into Se-enrichment of microalgae and its promising applications in aquafeed, by feeding rotifers with Se-enriched *Isochrysis galbana*, *N. oceanica,* and *N. oculata* (Ahmadifard et al., 2022; Ghaderpour et al., 2021).

With the aim to develop efficient industrial processes for Se-enrichment of microalgae, it is important to consider that several factors affect Se accumulation in microalgae and determine the presence of unconsumed Se in the spent media (wastewater). Studies investigating Se accumulation in microalgae have reported both beneficial and toxic effects of this element on microalgal cell growth (Lazard et al., 2017; Zhao et al., 2019). An increase in cell growth has been observed in *Chlorella pyrenoidosa* when adding Se (<5 mg/L) to the medium. However, when adding Se at higher concentrations (>5 mg/L) toxic effects are observed, which lead to a decrease in cell growth (Zhao et al., 2019). When *N. oceanica* was cultivated with selenite (≥30 μM = 5.19 mg/L) and selenate (≥5 μM = 0.94 mg/L) in the medium this resulted in toxic effects such as decreased cell growth. To date, no positive effects have been observed with Se enrichment in *N. oceanica* (Guimarães et al., 2021a). The most influential factors affecting Se accumulation (and its toxicity) in microalgae are: the concentration of Se in the medium (Gojkovic et al., 2015; Guimarães et al., 2021a; Zhao et al., 2019; Zheng et al., 2017), the pH (when using selenite) (Riedel and Sanders, 1996), the microalgal species-specific response (Pastierová et al., 2009), the inorganic Se form used (such as selenite or selenate) (Guimarães et al., 2021a; Vriens et al., 2016), the sulphur (S) concentration (Fournier et al., 2010; Guimarães et al., 2022; Umysová et al., 2009), and the phosphorus (P) concentration (when using selenite) in the medium (Riedel and Sanders, 1996; Vriens et al., 2016).

P is an essential element found in structural phospholipids, DNA molecules, proteins, ATP, and other metabolites of any cell (Blank, 2012). P is therefore added to microalgae cultivation media and is directly associated with growth (Markou et al., 2014; Singh et al., 2018). In this context, P is the second most common nutrient, after nitrogen (N), that limits cell growth (Markou et al., 2012; Mühlroth et al., 2017; Singh et al., 2018). In microalgae, N, P, and S limitation has been investigated for the accumulation of storage products such as: starch in *T. subcordiformis* (Yao et al., 2013), carbohydrates/lipids in *Arthrospira platensis* (Markou et al., 2012), and lipids in *Chlorella kessleri* (Shrestha et al., 2020) and *N. oceanica* (Meng et al., 2019; Mühlroth et al., 2017). To date high and low affinity transport systems have been described for selenite transport in *E. huxleyi* (Araie and Shiraiwa, 2009). P limitation has also been applied to explore selenite accumulation in the freshwater microalga *Chlamydomonas reinhardtii*, where lower P concentrations in the media led to an increase in Se accumulation (Morlon et al., 2006; Riedel and Sanders, 1996; Vriens et al., 2016). A few studies have attributed this antagonistic effect of P limitation on Se accumulation to the fact that P shares similar mechanisms of uptake with selenite in *C. reinhardtii* (Morlon et al., 2006; Riedel and Sanders, 1996).

This suggests that P limitation could be used as a strategy to increase Se accumulation in microalgae, which is the aim of this study. However, P limitation has not been previously applied to Se-enriched microalgal production using marine microalgae species such as *N. oceanica.*

Previous work with *N. oceanica* has assessed sodium selenite ($Na_2SeO_3$) as the ideal inorganic Se form at a concentration of 30 μM in the medium for Se-enriched *N. oceanica* biomass production (Guimarães et al., 2021a). Furthermore, strategies such as sulphur limitation to increase Se accumulation have been explored. However, sulphur limitation is not applicable when using natural seawater due to the high sulphur concentrations naturally present (28 mM) (Guimarães et al., 2022). The P concentration in seawater is naturally low and must be added to the medium when cultivating microalgae such as *N. oceanica* in AlgaePARC at a scale of 1300 L (Guimarães et al., 2021a). Thus, P limitation would be an applicable strategy for cultivation strategies using both artificial or natural seawater. Furthermore, improving *N. oceanica* Se uptake efficiency at lower P concentrations would result in the use of less Se and P in the medium, and prevent the release of Se-enriched wastewater into the environment. The effect of P depletion and limitation on Se accumulation was investigated by growing *N. oceanica* at different P concentrations (untreated and Se-treated). In addition to P and Se accumulation, cell growth, photosynthetic performance, and cell diameter were monitored. Two growth-limiting P concentrations were selected to investigate the daily Se accumulation under P limitation in a 1.8 L flat-panel photobioreactor (PBR) in batch operation. The purpose of this study was to assess, for the first time, the effect of P limitation on the Se uptake of the marine microalga *N. oceanica* and how this strategy could reduce the presence of unused Se in the wastewater of the cultivation process.

## 2. Materials and methods

### 2.1. Microalgal species and culture medium

*N. oceanica* CCAP 849/10 was cultivated in chloride medium (Guimarães et al., 2021b) at a pH of 7.5. The chloride medium is an alteration of a medium from Janssen et al. (2018) where the elements which contained sulphate ($SO_4^{2-}$) forms have been substituted by chloride ($Cl^-$) forms to enable a single sulphate source in the medium. This change was implemented to allow for an easier manipulation of the medium formulation for experiments. The chloride medium contained 2.47 mM of phosphorus (P) in the form of $K_2HPO_4$. This P concentration

has been previously used to cultivate *N. oceanica* at flask and pilot-scale (1300 L) in AlgaePARC (Wageningen University, The Netherlands) (Guimarães et al., 2021a). 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; trishydroxymethylaminomethane (Tris-HCl) 20.00 mM. No vitamins were added. Stock and pre-cultures were always cultivated using chloride media in the absence of Se. The medium was filter sterilised (0.22 μm, Sartobran 300, Germany) prior to cultivation. The pH was controlled by using Tris-HCl buffer (20 mM) during flask cultivation. In the photobioreactors (PBRs) the pH was maintained by sparging $CO_2$ on demand. Stock and pre-cultures were routinely refreshed and kept in 250 mL Erlenmeyer flasks with a liquid volume of 150 mL in a shaker incubator (Infors, HT, Switzerland), at 100 rpm, 25 ± 1ºC, 100 μmol/$m^2$/s, 16:8 light:dark photoperiod and a headspace enriched with 50 % humidity and 2.5 % $CO_2$.

### 2.2. Experimental set-up

#### 2.2.1. Effect of phosphorus depletion and limitation

The effect of P on cell growth, quantum yield, and cell diameter was studied using P depletion (0 μM), P limitation (10, 50, 100, and 200 μM) and P repletion (750 and 2470 μM) in the medium. Natural seawater (Eastern Scheldt, The Netherlands) contains only trace amounts of P (<16 μM) which does not support industrial microalgae cultivation (data not shown). At pilot-scale, a P concentration of 2470 μM is supplemented to natural seawater to grow *N. oceanica* at high cell densities (Guimarães et al., 2021a), and this concentration was considered the P control condition, because it did not limit growth (Guimarães et al., 2021a). The chloride medium was modified to allow for changes in P concentration. P concentrations were changed by using a ratio of 1 mol of $K_2HPO_4$ to substitute 1 mol of KCl to maintain osmolarity (1:1 ratio) (Janssen et al., 2018). The control medium did not contain Se (0 μM of Se). Prior to inoculation cell clumps were removed by centrifugation (800 g, 2 min, 20 °C), and the remaining medium was replaced with experimental medium by washing the biomass twice (800 g, 15 min, 20 °C). Flasks were inoculated at an $OD_{750}$ of 0.5 (2.3 x $10^7$ cells/mL). Each condition was tested in 250 mL Erlenmeyer flasks in biological triplicates (n = 3) in the same conditions as the pre-cultures for 12 days. In total, 7 conditions were assessed. Flasks were rinsed three times with distilled water and heat-sterilised (120 °C, 20 min) prior to the experiments.

#### 2.2.2. Effect of phosphorus depletion and limitation and Se enrichment

The combined effect of P limitation and Se treatment on cell growth, Se accumulation, and Se uptake efficiency was assessed. For each P concentration studied above (0, 10, 50, 100, 200, 750, and 2470 μM) different Se treatments were applied to the medium. The treatments with Se consisted of adding sodium selenite ($Na_2SeO_3$) to the chloride media (5 and 30 μM) from a Se stock containing 2 g/L of sodium selenite. Two Se concentrations were used; a non-toxic concentration (5 μM) and a sub-lethal concentration (30 μM) based on a previous study with *N. oceanica* (Guimarães et al., 2021a). Each condition was tested in 250 mL Erlenmeyer flasks in biological triplicates (n = 3) in the same conditions as the pre-cultures for 12 days. In total, 14 conditions were assessed. For the PBR experiments Se 5 μM and two P concentrations (250 and 750 μM) were selected.

#### 2.2.3. Se uptake efficiencies in photobioreactor in batch cultivation

The selected conditions from the shake flask experiments were P 250 and P 750. Flask conditions were replicated in a 2 L batch-operated flat-panel PBR (Labfors 5, Infors HT, Switzerland) with a working volume of 1.8 L. The PBR had a light-path of 21 mm, a surface area of 0.08 $m^2$ and was heat sterilised before use (120℃, 20 min). The PBRs were mixed with filter-sterilised air at 1 L/min and $CO_2$ was injected on demand to maintain the pH at 7.5. The culture was cooled with a water jacket and maintained at 25℃ and illuminated on the culture side with 260 LED lights with a warm white spectrum. The ingoing light was set as a sinus light regime with a peak of 1200 μmol/$m^2$/s and an average light per day of 44 mol/$m^2$/day using a 16:8 light:dark cycle. *N. oceanica* cells were cultivated in two stages in the PBR; an initial growth phase of the pre-cultures without Se to adapt the cells to the cultivation conditions, and a second Se-cultivation period. During the initial growth phase the biomass was adapted to natural seawater (NSW) in the chloride media. The media were prepared as described above but with the exception that no calcium, magnesium, or sulphate were added since these were already present in the NSW. After the growth phase, the biomass was washed in NSW chloride media (800 g, 15 min, 20 °C) containing 5 μM of Se and the PBRs were re-inoculated with a starting biomass concentration of 0.60 ± 0.04 g/L (1.6 x $10^8$ cells/mL). The media were first acclimatised to the cultivation temperature (25 °C) and then a volume of medium equivalent to the required inoculum volume was removed from the reactor. The inoculum was added using a sterile syringe. After inoculation, a bicarbonate solution (2 mM, final medium concentration) was added.

### 2.3. Biomass sampling

Biomass samples were taken daily to determine cell growth by optical density (OD) at 450, 680, and 750 nm, cell concentration, and dry weight (DW). Samples were diluted to an $OD_{750}$ between 0.10 and 0.40 ± 0.01 using a UV–VIS spectrophotometer (DR-6000, Hach Lange, Germany) and then used for the cell concentration and photosynthetic efficiency measurements. Cell concentration measurements were performed with an automated cell counter (Multisizer™ 3 Coulter Counter®, Beckman coulter Inc., USA) with a 50 μm aperture tube. Particles of 2.00 to 9.00 ± 0.01 μm were determined to be *N. oceanica* cells. Photosynthetic efficiency was studied by monitoring dark adapted quantum yield (QY) after 15 min at 455 nm with values ranging from 0.10 to 0.71 ± 0.01 (AquaPen-C 100 fluorometer, Photon Systems Instruments, Czech Republic). Biomass samples (50 mL) for mineral analysis were collected from the flasks (after 12 days) and PBR cultures (daily). The biomass was washed twice with ammonium formate (0.5 M) (2000 g, 15 min, 20℃) for the removal of salts (Guimarães et al., 2021b). The biomass samples were lyophilised (Sublimator, 2 × 3 × 3–5, Zirbus Technology, Germany) and were kept at −20℃ until further analysis.

### 2.4. Supernatant sampling

During the PBR experiments supernatant samples were collected daily by centrifugation (2000 g, 15 min, 20℃). The supernatant samples were separated from the biomass samples prior to the biomass washing process and stored in 50 mL falcon tubes which were kept at − 4℃ until further analysis. Prior to mineral analysis supernatant samples were vortexed and re-centrifuged (2000 g, 15 min, 20℃) to remove any possible remaining cell debris.

### 2.5. Microwave-assisted acid digestion

Lyophilised biomass (50 mg) and supernatant (10 mL) samples were prepared by microwave-assisted acid digestion as described by Guimarães et al., (2021b). In short, biomass and supernatant samples were extracted in an Aqua Regia mixture with 10 mL of deionised water (only for biomass samples), 7.5 mL hydrochloric acid (37 %) and 2.5 mL of nitric acid (65 %) (Merck, Germany) in a microwave-oven with a high pressure rotor SK15 (ETHOS™ EASY, Milestone Srl, Italy). The total microwave run time was 40 min with a maximum temperature of 175℃ which was maintained for 15 min. The maximum energy used was 1400 W. After the digestions, the samples were washed in deionised water and

made up to a final volume of 50 mL.

### 2.6. Determination of total selenium and phosphorus

Inductively coupled plasma-optical emission spectrometry (ICP-OES) standards were prepared from single element standards (Certipur®, Merck, Germany) at different concentrations as described by Guimarães et al. (2021b). P was combined with Se in the ICP-OES standards. Se was measured at two concentration ranges; 0.1–1.0 mg/L and a lower concentration of 0.04–0.10 mg/L. Thus, the lowest Se detection limit was 0.04 $mg_{Se}$/L and values < 0.04 $mg_{Se}$/L were represented as below detection limit (BDL). An internal standard (yttrium) was added to the supernatant samples and standards. The ICP-OES (Avio® 500, PerkinElmer Inc., USA) operating conditions were as described by Guimarães et al. (2021b).

### 2.7. Se uptake efficiency calculation

Se uptake efficiency in the shake flasks (%) was calculated by multiplying the Se concentration (g/g) by the DW (g/L) achieved for that condition, divided by the concentration of Se added to the medium (g/L), and multiplied by 100 for the determination of a percentage value (Li et al., 2021; Liu et al., 2016). The Se uptake efficiency in the flasks (%) was calculated in the same manner but using the Se (mg/L) measured in the media.

### 2.8. Statistical analysis

The experimental flasks and PBR experiments were performed in three biological replicates (n = 3). The flask condition P 2470 Se 0 was used as a control condition and repeated for each experiment, and it consisted of nine biological replicates (n = 9). Growth, QY, cell diameter, and mineral data (Se and P accumulation) was analysed using SPSS (IBM SPSS Statistics 25). A one-way analysis of variance (ANOVA) followed by a post-hoc test (Tuckey) was used ($p < 0.05$).

## 3. Results and discussion

### 3.1. Effect of phosphorus depletion and limitation on microalgal growth

This study aimed at investigating P limitation as a method for increasing Se accumulation and uptake efficiency in *N. oceanica*. By increasing Se uptake efficiency in *N. oceanica*, this study also aimed to use less resources (Se and P) in the medium with a reduction of Se in the wastewater at the end of the production process. Thus, this study aimed to reduce the concentration of Se in the medium whilst keeping the same concentration of Se in *N. oceanica* biomass. Previous work with *N. oceanica* demonstrated that increased Se accumulation could lead to toxicity (reduced cell growth and biomass accumulation) (Guimarães et al., 2022).

To study the effect of P limitation on growth parameters and Se accumulation, batch experiments were performed in flasks with *N. oceanica* untreated and treated with selenite (Se 5 and 30 μM). *N. oceanica* was first cultivated at seven different P concentrations (0, 10, 50, 100, 200, 750, and 2470 μM) without selenite to understand the isolated effect of P on growth (Fig. 1, A-B-C). The control condition (2470 μM of P) was used previously for the cultivation of *N. oceanica* and *N. gaditana* at both laboratory and pilot-scale (Guimarães et al., 2021a; Janssen et al., 2018).

The results revealed that P ≥ 200 cultures did not exhibit any sign of

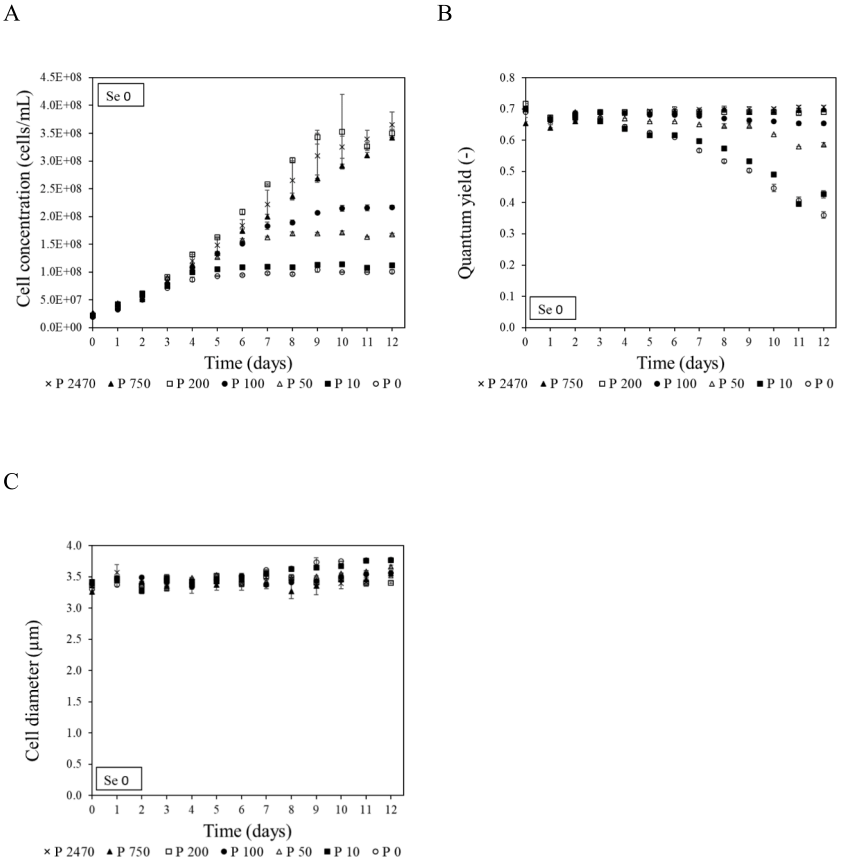


**Fig. 1.** Growth of *N. oceanica* when exposed to different phosphorus (P) concentrations (0, 50, 100, 200, 750, and 2470 μM) over 12 days. Cultures were not treated with selenium (Se) (0 μM); A) cell concentration (cells/mL), B) Quantum yield (-), and C) Cell diameter (μm).

P limitation on cell growth (Fig. 1, A) when compared to the control P 2470, and they grew as previously reported by Guimarães et al. (2021a). P ≤ 100 μM cultures grew significantly less when compared to the control cultures. Their growth curves showed that cells entered stationary phase after 7 days of cultivation (Fig. 1 A, $p < 0.05$). This was due to an insufficient supply of P in the medium. As P is an essential element for a living cell (Blank, 2012; Markou et al., 2012; Singh et al., 2018), P limitation leads to a decrease in cell growth by affecting structural cell processes (Shrestha et al., 2020; Yao et al., 2013). Quantum yield (QY) was used as a measure of photosynthetic performance. The QY of P ≤ 200 was significantly reduced when compared to the control (P 2470) (Fig. 1 B, $p < 0.05$). Overall, the QY dropped sharply for both P 0 and P 10 cultures. P 50 and P 100 cultures also showed a significant decrease in QY at the end of cultivation period, but it was less pronounced (Fig. 1 B, $p < 0.05$). Other studies have shown a decrease in QY due to limitation of P, S, N (Guimarães et al., 2022; Janssen et al., 2018; Wykoff et al., 1998), highlighting that these nutrients have an impact on photosynthetic performance.

Cell diameter was monitored during the cultivation period and remained constant for all conditions between 3 and 4 μm during the 12 days of cultivation (Fig. 1, C). Lower P concentration led to an increase in cell diameter. P ≤ 50 cultures had a significantly higher cell diameter when compared to the control (Fig. 1 C, $p < 0.05$). An increase in cell diameter due to P limitation has been previously reported in *N. gaditana* when cells were cultivated with 104 μM of $NaH_2PO_4$ (Fattore et al., 2021) and in *N. oceanica* when grown under P starvation (P = 0 μM) (Shi

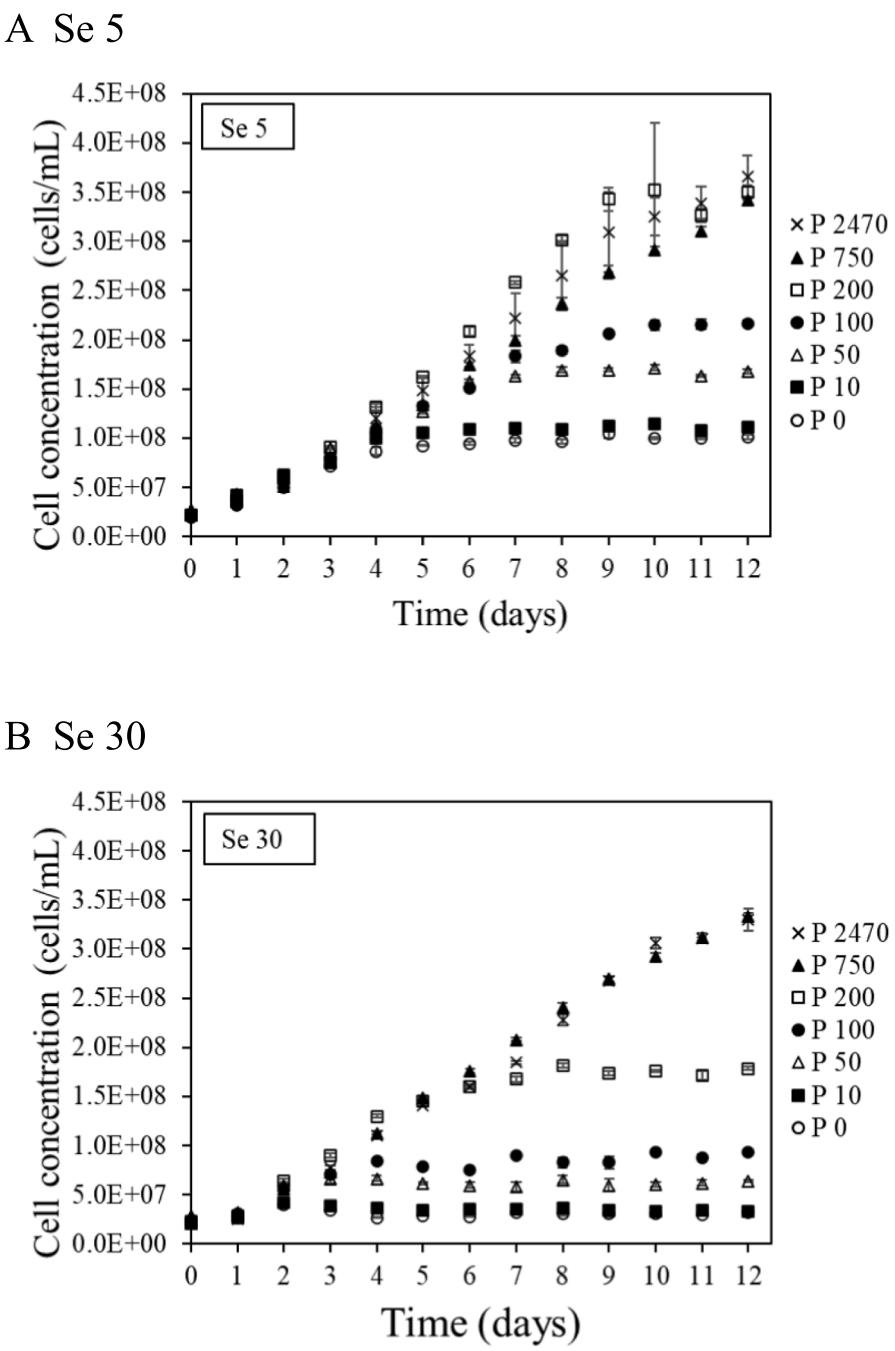


**Fig. 2.** Growth of *N. oceanica* when exposed to different phosphorus (P) concentrations (0, 50, 100, 200, 750, and 2470 μM) over 12 days. Cultures treated with selenium (Se) (5, 30 μM) under the same S concentrations; A) Cell concentration with 5 μM Se (cells/mL) and B) Cell concentration with 30 μM of Se in the media (cells/mL).

et al., 2020).

### 3.2. Effect of selenium enrichment on microalgal growth under phosphorus limitation

Batch experiments were performed on Se-treated cultures using sodium selenite (5, 30 μM) with the seven different P concentrations used above (Fig. 2, A-B) to understand if any toxic Se effect would be increased by P limitation. The Se 30 concentration was selected since it resulted in an industrially relevant Se incorporation in the biomass (0.13 $g_{Se}/kg_{biomass}$), thus representing the control condition (Guimarães et al., 2021a). The lower Se concentration (Se 5) was chosen by estimating the Se required in the medium to allow for a similar biomass incorporation, assuming higher Se uptake efficiency with P limitation. Overall, by increasing Se uptake efficiency this study aimed to reach a relevant Se accumulation in the biomass (e.g., 0.13 $g_{Se}/kg_{biomass}$), whilst reducing the presence of Se in the waste medium, after cultivation at the end of the process.

Growth analysis demonstrated that the P 2470 Se 30 control grew significantly less than the P 2470 Se 0 control (Fig. 2, B, $p < 0.05$), and inferred that Se 30 had an adverse effect on cell growth. This was also observed in a recent study, where the selenite Effective Concentration for 50 % growth inhibition was established ($EC_{50}$ = 163.82 μM) under the same experimental conditions, and Se 30 μM was deemed to be a sublethal concentration (Guimarães et al., 2021a).

P ≥ 750 (untreated and Se-treated) cultures did not show any sign of P limitation when compared to the Se-treated control (P 2470, Se 30) (Fig. 2, B). A detrimental effect on cell growth was instead observed for P ≤ 200 cultures treated with Se 30 (Fig. 2 B, $p < 0.05$). For Se untreated cultures, only P ≤ 100 cultures showed cell growth impairment, suggesting that Se became toxic upon P limitation (Fig. 1, A). A detrimental effect on cell growth was also observed for P ≤ 200, Se 5 cultures. Nevertheless, P ≤ 200, Se 5 cultures showed less pronounced effects on cell growth when compared to P < 200, Se 30 cultures (Fig. 2, A). Overall, these results highlighted that the toxic effect of selenite was enhanced by P limitation, which could have been attributed to higher Se accumulation in the microalgal cells. This increase in selenite toxicity has also been observed in other studies where media components such as S and P were limiting (Guimarães et al., 2022; Umysová et al., 2009), suggesting that P limitation allows for more accumulation of Se, which may become toxic to the cell (Riedel and Sanders, 1996; Umysová et al., 2009). It has been reported that selenite uptake in *C. reinhardtii* was significantly increased when the phosphate concentration in the medium was reduced (<20 μM) due to shared phosphate and selenite transporters (Riedel and Sanders, 1996). Thus, it is fundamental to measure Se accumulation to assess the effect on Se uptake efficiency.

### 3.3. Effect of phosphorus limitation on selenium accumulation and uptake efficiency

The P and Se content in the biomass after 12 cultivation days in the shake flasks were analysed to understand the effect of P limitation on Se accumulation in *N. oceanica* (Fig. 3) (see supplementary materials). The P content of the control (2470 μM) cultures was on average 8.93 $g_P/kg_{biomass}$ (Fig. 3, A). Overall, a trend was observed in which higher concentrations of P in the media resulted in a higher P content in the biomass. Similarly, lower P concentrations led to lower P content in the biomass (Fig. 3, A). This trend was also observed in *Nannochloropsis salina* (Sforza et al., 2018).

Se accumulation varied according to the different Se and P concentrations tested. As expected, Se accumulation in Se untreated cultures (Se 0) was below the detection limit (BDL) (<0.04 $mg_{Se}/L$) (Fig. 3, A). The highest P concentration (P 2470) led to the lowest Se accumulation (0.03 $g_{Se}/kg$ and 0.12 $g_{Se}/kg$ for Se 5 and Se 30 respectively). A decrease in selenite uptake with increasing P concentrations in the media has also been reported in *C. reinhardtii* (Vriens et al., 2016). Overall, under P limitation, Se accumulation was significantly higher for P ≤ 200 μM cultures (Fig. 3, B, $p < 0.05$) and it increased up to 5- and 16-fold

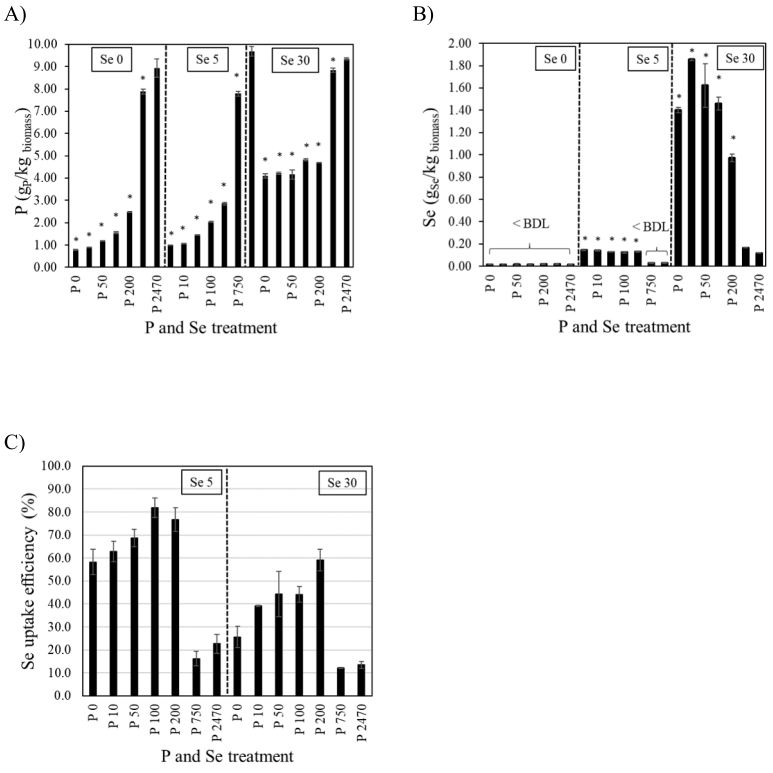


**Fig. 3.** Phosphorus (P) and selenium (Se) accumulation, and Se uptake efficiency in *N. oceanica* when exposed to Se 5 and Se 30 after 12 days of cultivation. The dotted line separates the Se 0, Se 5, and Se 30 treatments from each other denoting the same trend; as P concentration increases, Se uptake efficiency decreases. A) P accumulation in the biomass ($g_P/kg_{biomass}$), B) Se accumulation in the biomass ($g_{Se}/kg_{biomass}$), and C) Se uptake efficiency (%). Values below detection limit (BDL) (<0.04 $mg_{Se}/L$) are represented in the graph with < BDL. A dry weight outlier was removed from the P 100 Se 30 condition.

(0.15 $g_{Se}$/kg and 1.86 $g_{Se}$/kg), for Se 5 and Se 30 respectively. The highest Se accumulation in the biomass was achieved with P 10 and Se 30 (Fig. 3, B), however this led to severe growth impairment (Fig. 2, B), which can be attributed to the increase in Se accumulation in the biomass. The most promising P condition was P 200, which led to both an increase in Se accumulation and a limited decrease in cell growth (Fig. 2). Se accumulation for P 200 cultures increased by 5- and 8-fold, 0.13 $g_{Se}/kg_{biomass}$ and 0.97 $g_{Se}/kg_{biomass}$for Se 5 and Se 30, when compared to their respective control cultures (P 2470) (Fig. 3, B). An antagonistic effect of P limitation has also been observed in *C. reinhardtii* where lower P concentrations in the media led to higher selenite accumulation (Morlon et al., 2006; Riedel and Sanders, 1996). Overall, these results confirm that the Se concentration in the media affects the final Se content in the biomass which can lead to toxic effects (Guimarães et al., 2021a). Nevertheless, the results obtained with P 200 Se 5 demonstrate that P limitation resulted in a negligible loss of biomass productivity

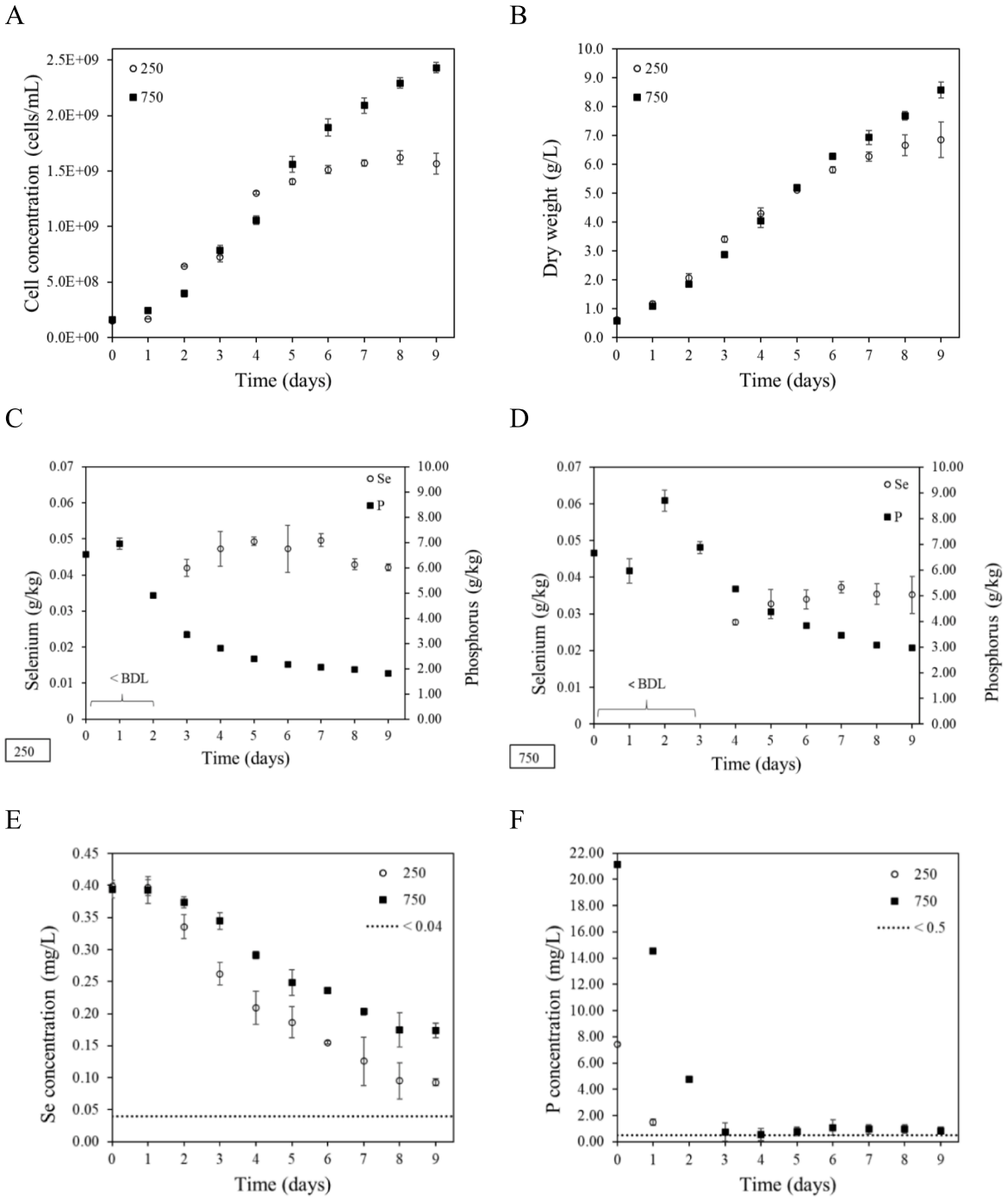


**Fig. 4.** Biomass growth (cell concentration and dry weight), selenium (Se), and phosphorus (P) accumulation in the biomass and Se and P left in the cultivation media during the batch production of *N. oceanica* for 9 days in PBRs. Batch cultures were exposed to two different P concentrations (250 and 750 μM) and were treated with Se (5 μM); A) Cell concentration with P 250 and 750 μM, B) Biomass concentration (dry weight – g/L) with P 250 and 750 μM, C) Se and P concentration in the biomass with P 250 μM, D) Se and P concentration in the biomass in P 750 μM, E) Se remaining in the cultivation media (mg/L), and F) P remaining in the cultivation medium (mg/L). The Se in the biomass was below detection limit on day 0 until day 2 and day 3 for P 250 and P 750 μM respectively (which is why these values are not represented in the graph) (Fig. 4, C, D). Values below detection limit (BDL) (<0.04 $mg_{Se}$/L) are represented in the graph with < BDL.

(0.18 g/L/day) when compared to the Se-treated control P 2470 Se 30 (0.20 g/L/day). Furthermore, P 200 Se 5 resulted in a Se accumulation of 0.13 $g_{Se}/kg_{biomass}$ which was similar to that obtained in the control condition, Se 30P 2470 (0.12 $g_{Se}/kg_{biomass}$), which required 6-fold more Se and 12-fold more P in the initial medium. The uptake efficiencies varied according to the Se and P concentrations tested.

The uptake efficiency of P 100 and P 200 Se 30 cultures reached 70 % and 59 % respectively. These were the highest uptake efficiencies among the tested conditions for Se 30, whereas the P 2470 control condition led to an uptake of only 14 % (Fig. 3, C) (see supplementary materials). However, this increase in Se uptake efficiency also led to Se toxicity, as demonstrated by the negative effect on cell growth (Fig. 2, B, Fig. 3, C).

When using Se 5, the uptake efficiency of Se for P 100 and P 200 cultures reached 82 % and 77 % respectively, representing the highest uptake efficiencies achieved among all the tested conditions (Fig. 3, C). This represented a substantial increase compared to the P 2470 Se 5 control condition, where Se uptake efficiency was only 23 %.

Overall, the Se uptake efficiency obtained with P 200 Se 5 enabled significant Se accumulation whilst simultaneously using less Se in the medium. This increase in uptake efficiency is essential for the utilisation of Se in the media and for limiting the release of Se in wastewater. Other studies have also investigated Se removal efficiency in *C. pyrenoidosa* and *C. vulgaris* and considered microalgae a promising Se filtering system (Liu et al., 2016).

Overall, these results confirm that P limitation can increase Se uptake efficiency and the results obtained with Se 5 demonstrated that higher Se accumulation was possible using lower concentrations of Se and P in the media, avoiding toxic-Se effects on cell growth. Overall, these results have demonstrated for the first time the potential of improving Se accumulation and uptake efficiency by applying P limitation. Thus, Se 5 is a promising concentration for further *N. oceanica* studies.

### 3.4. Batch cultivation in photobioreactors under P limiting conditions and daily Se uptake

A batch experiment in bench-scale flat-panel photobioreactors (PBRs) (1.8 L) was performed to investigate the daily Se uptake efficiency under two P concentrations (250 and 750 μM). Based on the flask experiment, P concentrations of 250 and 750 μM were selected which were lower than the control P concentration, but were still able to support cell growth (Fig. 2A). Batch cultivation was chosen as it enabled the effect of P limitation on Se accumulation to be investigated during the different stages of cell growth (exponential phase and stationary phase).

The results revealed that cells grew significantly less with P 250 compared to P 750 (Fig. 4, A-B, $p < 0.05$). The P 250 cultures entered stationary phase on day 4 since the initial P concentration was not sufficient to support growth during the whole cultivation period in the PBRs. This effect on growth was not observed in P 750 cultures which grew exponentially up to day 5 and linearly until the end of cultivation (day 9). In the PBRs an increase of Se accumulation was observed in the biomass due to P limitation in the media (Fig. 4, C-D), which confirmed what was previously observed in the flask experiments (Fig. 3, B). The pre-cultures used for this experiment had no prior exposure to Se, thus, the Se in the biomass was below the detection limit (BDL, < 0.04 $mg_{Se}$/L) on day 0 until day 2 and day 3 for P 250 and P 750, respectively (Fig. 4, C, D).

Once growth reached the stationary phase (≥day 4 for P 250 and ≥ day 6 for P 750) no further increase in Se accumulation was observed (Fig. 4, A-D). On average, Se accumulation in P 250 (≈0.05 $g_P$/kg) (day 3 - day 9) was higher than in P 750 (≈0.03 $g_P$/kg) (day 4 - day 9). However, average biomass productivity was 0.69 and 0.89 g/L/day for P 250 and P 750, respectively (Fig. 4, B). P 250 PBRs had a lower biomass productivity, reflecting the lower concentration of P provided in the cultivation medium. It has previously been reported that the P concentration in the media is directly correlated to cell growth (Markou et al., 2014; Singh et al., 2018). Both conditions were limiting in P as the P in the biomass decreased from 6.59 $g_P/kg_{biomass}$ (in the beginning of the experiment) to 1.81 and 2.97 $g_P/kg_{biomass}$ for P 250 and P 750 respectively. The P decrease in the biomass was representative of P being limited in the cultivation medium (Fig. 4, A). Overall, for both PBRs, the Se uptake efficiency increased when P started to become limiting.

In P 250 and P 750 the Se uptake efficiency reached up to 77 % and 56 % respectively by the end of the cultivation period (Fig. 4, C-D) (see supplementary materials). The differences observed in Se uptake efficiency were due to lower amounts of P available in the P 250 condition during the exponential growth phase. Similarly, an increase of Se uptake upon P limitation has been reported in *C. reinhardtii* (Riedel and Sanders, 1996; Vriens et al., 2016). P limitation could induce an upregulation of P transport, and incidentally, of Se into the cells. This could be due to the fact that selenite and phosphate share similar transporters, as hypothesised for certain land plants and green microalgae such as *C. reinhardtii* (Araie et al., 2011; Gupta and Gupta, 2017; Morlon et al., 2006).

The supernatants after cell removal were also analysed. On day 0, the Se concentration in the medium was 0.4 mg/L and by end of the cultivation it was 0.09 and 0.17 $mg_{Se}$/L for P 250 and P 750 respectively (Fig. 4, E-F). The P concentration in the media followed a clear decreasing trend (Fig. 4, E-F) until it was under ICP-OES detection limit (<0.5 $mg_P$/L). The legislated thresholds for residual Se and P in wastewater effluents are 0.025 mg/L in wastewater effluents used for crop production (FAO, 2004) and 0.02 $mg_P$/L (Melia et al., 2017) which are close to the residual concentrations measured in this study for both P 250 and P 750 conditions. Although there was a limitation by the detection limit of the ICP-OES instrument, the results clearly demonstrate the potential of using P limitation to reduce Se and P in the wastewater at the end of the cultivation period. Both P 250 and P 750 allowed for lower Se in the wastewater by increasing Se uptake efficiency which is fundamental to scale-up this microalga in a Se-enriched medium. Overall, this study is the first to evaluate the effect of P limitation on Se uptake efficiency in the marine microalga *N. oceanica* and has demonstrated that P limitation in both cases (P 250 and P 750) successfully increased Se accumulation during the cultivation process. As observed with P 250 the P concentration must be adjusted to the specific cultivation process to result in the least biomass productivity loss. Once growth was no longer exponential, no more Se accumulation was observed suggesting that Se accumulation is correlated with cell growth.

## 4. Future perspectives

*N. oceanica* is a robust strain and its biomass is produced and sold for aquaculture, as greenwater treatment for fish, larval tanks, and for rotifers. Its interest for aquaculture applications is growing (Li et al., 2020), and the utilisation of this biomass for fish feed has been evaluated in recent studies in tilapia and shrimp, with positive effects on growth performance and omega-3 fatty acids (Adissin et al., 2020; Lupatsch and Gbadamosi, 2018) and it was also recently positively evaluated for outdoor growth in open ponds (Li et al., 2020). Moreover, recently it has been revealed that Se-enrichment in *N. oceanica* led to the incorporation of organic selenium (>97 % SeMet of total organic selenium) (Guimarães et al., 2022). Therefore, Se-enrichment in *N. oceanica* has the potential to become an organic source of Se supplementation in aquafeed. This study has made advances on the understanding of factors affecting Se accumulation and uptake efficiency in *N. oceanica*. Se-enriched *N. oceanica* has been successfully cultivated at pilot-scale (1300 L) (Guimarães et al., 2021a). *N. oceanica* can lead to an increase in Se accumulation up to 8-fold (Guimarães et al., 2022). In this study, it has been demonstrated that P limitation can be applied to microalgae production processes which use both artificial and natural seawater since it is a component that is only present in trace amounts in natural

seawater and must be added to the medium. In conclusion, P limitation is a promising way of increasing Se uptake efficiency in *N. oceanica* with a negligible loss in biomass productivity and leads to minimal Se and P left at the end of the Se-enriched microalgal production process. Future work should focus on further reducing the Se left in the wastewater medium to allow for the implementation of this process in the microalgae industry whilst complying with legislations limits.

## 5. Conclusion

This study was the first to demonstrate that P limitation can lead to an increase of up to 16-fold in Se accumulation (1.86 $g_{Se}/kg_{biomass}$) and 3.6-fold in Se uptake efficiency (82 %) in *N. oceanica,* compared to P non-limiting concentrations. Flask cultures with 200 μM of P and 5 μM of Se resulted in a Se accumulation of 0.13 $g_{Se}/kg_{biomass}$ whilst using 6-fold less Se and 12-fold less P than the initial control medium. Photobioreactor cultivation using P limitation also led to higher Se accumulation and to minimal Se and P left at the end of the production process ($<$0.17 $mg_{Se}$/L).

## CRediT authorship contribution statement

**Bárbara O. Guimarães:** Conceptualization, Methodology, Formal analysis, Visualization, Investigation, Writing – original draft, Writing – review & editing. **Youp Van der Graaf:** Investigation, Formal analysis. **Isabelle Kunert:** Investigation, Formal analysis. **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 financial 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 data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.128239.

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