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ORIGINAL ARTICLE

Selenite as a Lipid Inductor in Marine Microalga *Dunaliella tertiolecta*: Comparison of One-Stage and Two-Stage Cultivation Strategies

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Abstract
Microalgaehave emerged as one of the most promising alternative sources of biofuels due to their high lipid accumulation ability. High lipid content is of pivotal importance for biodiesel production. In order to obtain high lipid content, modifications of culture conditions and development of an efficient lipid induction method are called for. In the present study, the possibility of using selenium in a form of sodium selenite as a lipid inductor in marine microalga *Dunaliella tertiolecta* was investigated during one- and two-stage cultivation modes. The effects of selenite on algal growth, pigment content, oxidative stress, and neutral lipid content were determined during both cultivation modes. The results revealed that the two-stage cultivation on 10.00–40.00 mg L^{-1} of selenite resulted in up to two-fold higher algal cell density compared to the one-stage cultivation. Selenite concentrations from 2.50 to 20.00 mg L^{-1} increased lipid peroxidation during both cultivation modes, emphasizing the selenite-induced oxidative stress accompanied by the increased lipid accumulation in microalgae cells. During one- and two-stage cultivation on 20.00 mg L^{-1} of selenite, lipid content increased 2.39- and 5.73-fold at days 9 and 14 of cultivation, respectively. Moreover, the highest obtained neutral lipid content during the two-stage cultivation was 5.40-fold higher than lipid content obtained during the one-stage cultivation. Collectively, these results suggest that the two-stage cultivation strategy, initiated with optimal culture conditions for biomass production and followed by the addition of selenite as a stress inductor, can be successfully deployed to enhance the lipid content in *D. tertiolecta.*

Keywords Reactive oxygen species · Oxidative stress · Nile Red · Pigments · Fluorescence

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Introduction

Under adverse growth conditions, microalgae can efficiently adjust their lipid metabolism through the synthesis of neutral lipids, mainly in the form of triglycerides, whose primary role is to store carbon and energy under stressful environmental conditions [1]. Through transesterification, triglycerides can be further transferred into various types of fatty acid methyl esters, constituents of biodiesel. The composition and quantity of lipids are species-dependent and can be affected by cultivation conditions [2]. A stress-based cultivation strategy is defined as a significant deviation from the optimal conditions necessary for the normal growth of microalgae. Such stress conditions not only generally lead to higher lipid contents in microalgae cells but also result in relatively low biomass productivity [3]. However, to ensure an economically sustainable process of biodiesel production, the focus must be placed on both biomass productivity and lipid content in microalgal cells [4]. To that end, a very promising approach named photoautotrophic two-stage cultivation strategy has been proposed by several authors [3, 5, 6]. The two-stage cultivation strategy represents the cultivation mode in which microalgae are firstly grown under nutrient-sufficient conditions (stage I). After producing sufficient biomass, the stress phase or induction of lipid accumulation in stage II is applied to produce a higher lipid content in microalgae cells [7, 8]. Currently, several stress-based strategies for microalgae lipid synthesis have been reported, including change in light intensity [9], increase or decrease of temperature and depletion of nutrient concentration [10], and change in oxygen (O_2) [11] or carbon dioxide (CO_2) concentration [12], pH [13], salinity [14], etc.

Furthermore, different studies have confirmed the beneficial effect of trace elements, especially copper (Cu), chromium (Cr), manganese (Mn), molybdenum (Mo), cobalt (Co), iron (Fe), and zinc (Zn), on microalgal growth performance and lipid content (Table S1). Although valuable, those studies are difficult to compare due to different concentrations, cultivation mode, monitored parameters, exposure time, and different microalgae strains.

Within this study, selenium (Se) was of particular interest due to its potential to act either as an essential micronutrient or as a toxicant, depending on the dose in biological and environmental systems [15]. It has also been documented as an essential micronutrient for at least 33 microalgae species, particularly for green algae [16]. However, only a few studies have been conducted to determine the impact of Se on *Dunaliella salina*, *Chlamydomonas reinhardtii*, *Chlorella* spp., and *Scenedesmus quadricauda* during the one-stage cultivation (Table S1). Those studies were mainly focused on the algal cell ultrastructure, photosynthetic process, and growth performance in the Se-enriched medium, but none of them was focused on lipid accumulation and consequently biodiesel production, despite the observed biomass increase. To the best of our knowledge, there are no studies that utilized the two-stage algal cultivation in the Se-enriched medium.

Among the oleaginous microalgae, green marine alga *Dunaliella tertiolecta* has many desirable features such as undemanding cultivation; high resistance to light, temperature, and salinity; relatively high growth rate and lipid content; and ability to remain in suspension [17]. Due to the high lipid content that can range from 16.70 to 71.00% of dry weight biomass, *Dunaliella* sp.–based biofuel production has already received considerable attention [18]. Additionally, Song et al. [19] showed that lipid content in *D. tertiolecta* exceeds 30% of its total dry weight when grown under optimal conditions, making this microalga highly suitable for such experimental design.

This research aimed to assess the dependence of neutral lipid production in *D. tertiolecta* upon exposure to different concentrations of sodium selenite (Na_2SeO_3), an inorganic form

of Se, under different cultivation regimes, thus highlighting the selenite potential as a neutral lipid inductor. Furthermore, the feasibility of the two-stage strategy and the impact on lipid accumulation was compared to the one-stage strategy. Since the ability of *D. tertiolecta* to tolerate selenite is currently completely unknown, analysis of lipid peroxidation was performed using the thiobarbituric acid reactive substances (TBARS) assay, while intracellular neutral lipid content was visualized and quantified using a modified Nile Red (NR) method. The present study reports valuable data regarding optimal selenite range for neutral lipid production in marine microalga *D. tertiolecta*, thus shedding light on its future utilization for biofuel production.

Materials and Methods

Chemicals

Sodium selenite (CAS No. 10102–18-8; Na_2SeO_3), ethanol (CAS No. 64–17-5), methanol (CAS No. 67–56-1), thiobarbituric acid (CAS No. 504–17-6; TBA), trichloroacetic acid (CAS No. 76–03-9; TCA), butylhydroxytoluene (CAS No. 128–37-0; BHT), NR (CAS No. 7385–67-3; NR), and triolein (Cas No. 122–32-7) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sterile f/2 medium was prepared according to the standardized protocol [20] using all chemicals obtained from Sigma-Aldrich.

Microalgae Cultures and Experimental Design

Marine microalga *D. tertiolecta* (SAG 13.86) was obtained from the Culture Collection of Algae (SAG), University of Göttingen, Germany. In the laboratory, culture was maintained photoautotrophically in a sterile f/2 medium [21]. The cultures were agitated in an orbital shaker (100 rpm) under a continuous light illumination of 7000 lx provided by white fluorescent lamps. The temperature of the culture was maintained at 25 ± 1 °C. The initial pH value was adjusted to 6.80 ± 0.20 and was monitored during the experiment.

Selenite growth experiment was performed following OECD Guideline [21], with modifications. For the one-stage experiment, *D. tertiolecta* was cultured for 15 days in Erlenmeyer flasks containing 70 mL of different selenite concentrations: 0.31, 0.62, 1.25, 2.50, 5.00, 10.00, 20.00, and 40.00 mg L^{-1}. For the two-stage cultivation experiment, cells were firstly grown in 70 mL of f/2 medium until they reached the early stationary phase (day 9 of cultivation). After that, selenite concentrations previously mentioned for the one-stage experimental design were added. Negative controls were run in parallel on the 70 mL of f/2 medium. Besides, blank controls were prepared and contained the same selenite concentrations without microalgae cells. Each culture batch experiment was performed in triplicate, with an initial cell density of 1×10^4 cells mL^{-1}. Microalgal growth was monitored by optical density measurements using a spectrophotofluorimeter (Tecan Infinite M200 PRO, Switzerland, equipped with Magellan software version V 7.2). Cell number was calculated from the OD_{680} values using the regression equation (Eq. (1)):

$$\text{Number of cells (cells mL}-1) = 6 \times 10^6 \times \text{OD}_{680} + 259,587 \quad (1)$$

Analytical Procedures

Lipid Peroxidation

Lipid peroxidation products were measured by quantification of thiobarbituric acid reactive substances (TBARS) formed during the reaction between malondialdehyde and malondialdehyde-like substances with thiobarbituric acid under acidic conditions [22]. TBARS content was quantified according to Pancha et al. [8], with slight modifications. Briefly, 2 mL of algal culture was centrifuged, and the algal pellet was homogenized (Tehtnica Millmix 20, Slovenia) in 1 mL of ethanol: water solution (80:20, v:v) for 10 min at a frequency of 25 Hz. The homogenate was centrifuged at 9000 rpm, $r = 9.5$ for 10 min and 750 µL of supernatant was mixed with 750 µL of 0.65% TBA prepared in 20.00% TCA solution containing 0.01% BTA. Samples were mixed vigorously and heated at 100 °C for 25 min. After cooling at 4 °C, the absorbance of the supernatant was read at 450 nm, 532 nm, and 600 nm, and TBARS content was calculated using Eq. (2) [3]:

$$\mathrm{TBARS}[\mu\mathrm{mol} \times 10^6\mathrm{cells}^{-1}] = [6.45(\mathrm{OD}_{532} - \mathrm{OD}_{600}) - 0.56 \times \mathrm{OD}_{450}]10^6\mathrm{cells}^{-1} \quad (2)$$

Determination of Pigments

For the determination of total pigment content, 2 mL of algal culture was taken and methanol extraction was performed [8]. All pigment analyses were performed under subdued light conditions to prevent oxidative alteration of pigments [22]. Pigment content was determined using the following: Eq. (3), Eq. (4), and Eq. (5) [23]:

$$\text{Chlorophyll a;Chl-a}(\mu\mathrm{g\ mL}^{-1}) = 16.72A_{665.2} - 9.16A_{652.4} \quad (3)$$

$$\text{Chlorophyll b;Chl-b}(\mu\mathrm{g\ mL}^{-1}) = 34.09A_{652.4} - 15.28A_{665.2} \quad (4)$$

$$\text{Carotenoids}(\mu\mathrm{g\ mL}^{-1}) = (1000A_{470} - 1.63\ \text{Chl-a} - 104.9\ \text{Chl-b})/221 \quad (5)$$

Determination of Neutral Lipid

Neutral lipid quantification was performed using the Nile Red (NR) method, additionally adapted for this study, as explained below. The NR dye permeates all structures and selectively stains lipid droplets within the cell when yellow-gold fluorescence manifests. Compared to conventional methods, the NR method is advantageous since a small sample quantity is sufficient, requires no solvent extraction, and is not time-consuming [4]. The algal culture (2 mL) was centrifuged at 4200 rpm, $r = 16.4$ for 15 min, after which pellets were washed twice with an f/2 medium. Cells were mixed with 300 µL of 25.00% DMSO and 5 µL NR stain (0.50 mg mL^{-1} in acetone), followed by a 10-min incubation at 37 °C [24]. Cellular neutral lipids were quantified using the spectrophotofluorimeter (Tecan Infinite M200 PRO, Switzerland, equipped with Magellan software version V 7.2) with excitation and emission at wavelengths of 475 and 580 nm, respectively.

Cellular neutral lipid concentration was expressed as triolein equivalent according to Eq. (6) [25]:

$$\mu \text{ eg triolein mL}^{-1} = [\text{FL} - (\text{F}_\text{A} + \text{F}_\text{FSW})]\text{b} \quad (6)$$

Here F_L is a fluorescence of the cell suspension in f/2 medium after NR addition, F_A is an autofluorescence of the cells in f/2 medium before NR addition, F_{FSW} is the fluorescence of f/2 medium after NR addition, and b is a slope of the standard curve fluorescence (μg triolein mL^{-1}).

Fluorescence Microscopy

Oil droplets in algal cells were visualized by the fluorescence dye NR and imaged using a fluorescence microscope (Olympus® BX51 light binocular microscope equipped with the Microsoft® AnalySIS Soft Imaging System Software) and a high-pressure mercury lamp light source with a blue fluorescent filter [26].

Statistical Analysis

Data were statistically analyzed using GraphPad Prism 6.0 (GraphPad Software Inc., USA). The results were expressed as means of three replicates ± SD, and $p < 0.05$ was used as a cut-off value of statistical significance throughout the manuscript. One-way analysis of variance (ANOVA) and Tukey's post hoc test were performed to examine the significance of the difference between treatments. When the assumption for normality was violated, the Kruskal–Wallis one-way analysis of variance on ranks was performed.

Results

Effect of Sodium Selenite on the Growth of D. tertiolecta

The effect of selenite concentrations in the cascade range from 0.31 to 40.00 mg L^{-1} on the growth performance of *D. tertiolecta* during different cultivation strategies was investigated for up to 15 days. Growth curves of *D. tertiolecta* during one- and two-stage cultivation modes are shown in Fig. 1.

As shown in Fig. 1a, the one-stage cultivation under selenite concentrations of 10.00 and 20.00 mg L^{-1} significantly increased ($p < 0.01$) cell density (for 23.32 and 19.78% compared to the control group, respectively) on day 3. For the two-stage cultivation (Fig. 1b), significant increase ($p < 0.05$) of cell density was recorded in the 0.31 mg L^{-1} selenite treatment group (7.22 and 7.55% on days 14 and 15, respectively). The maximal cell density during one-stage algal cultivation (5.85×10^5 cells mL^{-1}) was achieved on day 15 at 0.63 mg L^{-1} of selenite, while in two-stage cultivation mode, the plateau (4.04×10^5 cells mL^{-1}) was reached 1 day earlier in the 0.32 mg L^{-1} treatment group. While low selenite concentrations (up to 1.25 mg L^{-1}) had no inhibitory effect on *D. tertiolecta* growth, 7 days of exposure to 10.00, 20.00, and 40.00 mg L^{-1} during the one-stage cultivation caused a significant decrease in cell density (43.19, 56.49, and 67.17%, respectively). Such a high toxicity trend was also observed during the early stationary phase. While *D. tertiolecta* exposed to 2.50 and 5.00 mg L^{-1} of selenite entered the stationary phase at day 7, cultures

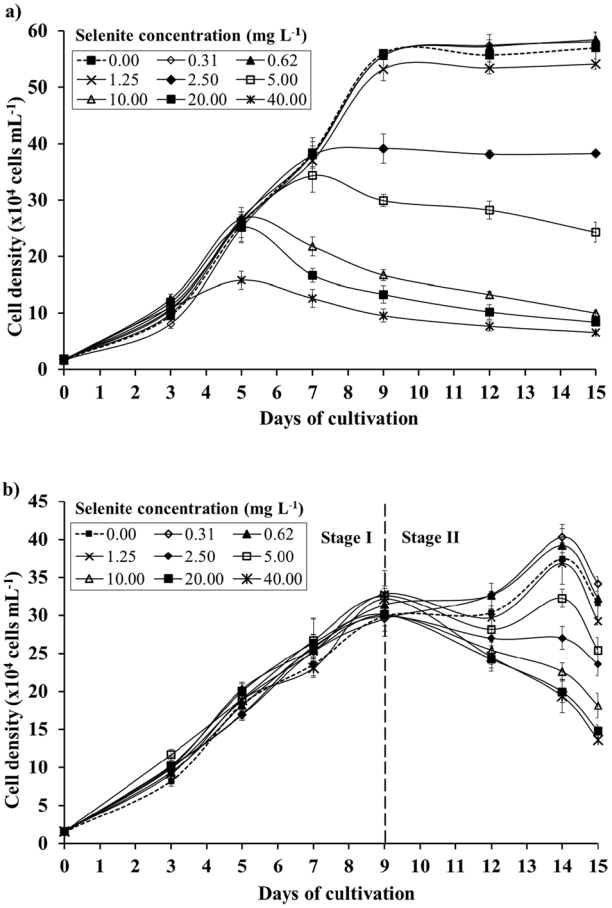


Fig. 1 Effect of different concentrations of selenite on the growth of *D. tertiolecta* during (**a**) one-stage cultivation and (**b**) two-stage cultivation. The time of selenite addition in the two-stage cultivation is indicated by a dashed line. Results are presented as mean ± SD of triplicates

on higher selenite concentrations (10.00, 20.00, and 40.00 mg L^{-1}) reached the stationary growth phase 2 days earlier (Fig. 1a). Conversely, during the two-stage cultivation, selenite concentrations of 5.00 and 10.00 mg L^{-1} caused a significant decrease in cell density on days 15 (19.50%) and 14 (39.53%), respectively, while 20.00 and 40.00 mg L^{-1} reduced cell density on day 14 of cultivation (46.67 and 56.75%, respectively). Furthermore, from the results obtained at the end of the two-stage cultivation, it can be observed that maximal algal cell densities at 10.00, 20.00, and 40.00 mg L^{-1} of selenite were approximately two-fold higher compared to the same selenite treatment groups during the one-stage cultivation (Fig. 1).

Effect of Selenite on Pigment Composition of D. tertiolecta

To understand the impact of selenite toxicity on chloroplast and to determine the correlation between the content of pigments and selenite exposure, concentrations of photosynthetic pigments chlorophyll a (Chl-a), chlorophyll b (Chl-b), and total carotenoid content (TCC) were also determined. As per Table 1, lower selenite concentrations (up to 5.00 mg L^{-1}) during the one-stage cultivation mode had a negligible effect on Chl-a, Chl-b content,

Table 1 Chlorophyll a (Chl-a), chlorophyll b (Chl-b), and total carotenoid content (TCC) in *D. tertiolecta* cultures supplemented with different selenite concentrations during (a) one-stage cultivation and (b) two-stage cultivation. Results are presented as mean ± SD of triplicates (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

Cultivation mode	Photosynthetic pigments	Days of exposure	Selenite concentration (mg L^{-1})								
			0.00	0.31	0.62	1.25	2.50	5.00	10.00	20.00	40.00
One-stage	Chl-a (μg mL^{-1})	5	1.04 ± 0.12	1.04 ± 0.05	1.00 ± 0.13	0.90 ± 0.07	0.89 ± 0.02	0.84 ± 0.06	0.81 ± 0.09	0.88 ± 0.13	0.14 ± 0.03***
		10	1.09 ± 0.14	1.10 ± 0.07	1.14 ± 0.13	1.08 ± 0.04	0.90 ± 0.05	1.01 ± 0.16	1.01 ± 0.12	0.90 ± 0.22	0.04 ± 0.02***
		15	0.94 ± 0.14	0.95 ± 0.07	0.87 ± 0.10	0.93 ± 0.15	0.70 ± 0.06	0.85 ± 0.12	0.60 ± 0.01	0.05 ± 0.01***	0.21 ± 0.11***
	Chl-b (μg mL^{-1})	5	1.18 ± 0.07	1.08 ± 0.07	1.37 ± 0.11	1.95 ± 0.36	1.89 ± 0.01	1.88 ± 0.52	2.43 ± 0.76**	2.74 ± 0.09***	0.10 ± 0.02*
		10	2.41 ± 0.23	2.52 ± 0.48	2.84 ± 0.31	2.79 ± 0.35	2.76 ± 0.29	2.94 ± 0.22	2.43 ± 0.39	1.81 ± 0.29	0.05 ± 0.00***
		15	2.21 ± 0.41	1.97 ± 0.29	1.96 ± 0.41	1.72 ± 0.27	1.43 ± 0.29	1.57 ± 0.34	1.04 ± 0.17*	0.05 ± 0.01***	0.28 ± 0.19***
	TCC (μg mL^{-1})	5	0.54 ± 0.08	0.52 ± 0.02	0.47 ± 0.05	0.25 ± 0.13	0.36 ± 0.02	0.34 ± 0.08	0.31 ± 0.18	0.23 ± 0.00	0.05 ± 0.01***
		10	0.45 ± 0.06	0.49 ± 0.08	0.43 ± 0.07	0.43 ± 0.03	0.35 ± 0.06	0.27 ± 0.06	0.38 ± 0.12	0.46 ± 0.22	0.01 ± 0.00*
		15	1.07 ± 0.15	1.10 ± 0.09	0.97 ± 0.14	0.95 ± 0.26	0.67 ± 0.04*	0.80 ± 0.17	0.58 ± 0.12***	0.03 ± 0.01***	0.03 ± 0.3***
Two-stage	Chl-a (μg mL^{-1})	5	0.70 ± 0.07	0.79 ± 0.06	0.74 ± 0.06	0.75 ± 0.12	0.78 ± 0.04	0.70 ± 0.07	0.78 ± 0.11	0.71 ± 0.06	0.74 ± 0.03
		10	0.85 ± 0.16	0.98 ± 0.14	0.98 ± 0.07	0.97 ± 0.01	1.09 ± 0.08**	1.04 ± 0.08	0.98 ± 0.10	0.94 ± 0.08	0.94 ± 0.06
		15	0.80 ± 0.07	0.74 ± 0.05	0.92 ± 0.02**	0.834 ± 0.00	0.89 ± 0.07*	0.68 ± 0.16**	0.56 ± 0.24***	0.37 ± 0.0***	0.24 ± 0.03***
	Chl-b (μg mL^{-1})	5	1.06 ± 0.19	0.89 ± 0.15*	0.96 ± 0.09	0.97 ± 0.13	1.09 ± 0.06	0.99 ± 0.06	1.07 ± 0.13	1.15 ± 0.17	0.79 ± 0.13***
		10	1.42 ± 0.17	1.23 ± 0.29	1.36 ± 0.09	1.29 ± 0.18	1.42 ± 0.18	1.21 ± 0.13	1.39 ± 0.27	1.51 ± 0.21	1.53 ± 0.09
		15	1.32 ± 0.06	1.53 ± 0.26**	1.32 ± 0.07	1.29 ± 0.15	1.32 ± 0.06	1.00 ± 0.24***	0.52 ± 0.03***	0.26 ± 0.04***	0.18 ± 0.02***
	TCC (μg mL^{-1})	5	0.30 ± 0.07	0.36 ± 0.05	0.34 ± 0.05	0.33 ± 0.06	0.33 ± 0.05	0.31 ± 0.04	0.34 ± 0.06	0.26 ± 0.07	0.33 ± 0.03
		10	0.69 ± 0.12	0.85 ± 0.02***	0.83 ± 0.04***	0.78 ± 0.05*	0.88 ± 0.03***	0.87 ± 0.04***	0.83 ± 0.09***	0.76 ± 0.07	0.74 ± 0.04
		15	0.93 ± 0.08	0.83 ± 0.03*	0.95 ± 0.05	0.79 ± 0.09***	0.74 ± 0.07***	0.44 ± 0.15***	0.30 ± 0.02***	0.19 ± 0.04***	0.12 ± 0.02***

and TCC. The greatest decrease of Chl-a content was recorded on 20.00 and 40.00 mg L^{-1} of selenite reaching around 90.00 and 80.00% decline on day 15. During the two-stage cultivation, this decrease was less steep—around 30.00% in the 10.00 mg L^{-1} treatment group, followed by 54.00% and 70.00% decline in 20.00 and 40.00 mg L^{-1} treatment groups, respectively. A similar trend was noted regarding Chl-b content in algae exposed to 10.00, 20.00, and 40.00 mg L^{-1} of selenite, indicating a significant decline during both cultivation modes (from 50.00 to 90.00%). A TCC content was also determined in correlation to the observed color change of the culture biomass during the experiments. Although the color change was observed in cultures grown at 10.00 mg L^{-1} and 20.00 mg L^{-1} of selenite (from green-colored controls to yellow-orange) on days 7 and 6 of the one-stage cultivation, a significant decrease of TCC was noted, reaching from 45.00% to almost 100.00% decline after 15 days of cultivation (Table 1). The 40.00 mg L^{-1} selenite treatment group remained colorless during the whole experiment. However, in the two-stage cultivation process after 10 days of cultivation, approximately 1.20-fold increase ($p < 0.001$) in TCC was observed in selenite treatment groups ranging from 0.31 to 10.00 mg L^{-1}, except for 1.25 mg L^{-1} treatment group where the increase was 1.10-fold higher ($p < 0.05$) than the control (Table 1). Despite this, after 15 days of cultivation, a significant decrease of TCC was observed in the selenite treatment groups ranging from 1.25 to 20.00 mg L^{-1} (Table 1). An exception was the 0.62 mg L^{-1} treatment group where TCC was similar to the control value. A slight increase in TCC along with a color change (yellow-orange) was observed after 10 days of cultivation in the 40.00 mg L^{-1} selenite treatment group. However, a significant decrease of TCC (Table 1) as well as discoloration by the end of the cultivation was noted at this highest concentration. Based on the obtained results, Chl a/Chl b and TCC/total Chl ratios were also calculated (Table 1). No significant changes in the Chl-a/Chl-b and TCC/total Chl ratios were observed during the one-stage cultivation. The only exception was the highest tested concentration (40.00 mg L^{-1}) where a 2.00-fold ($p < 0.001$) increase of these ratios was noted already on day 10 of the treatment. Conversely, pigment synthesis under two-stage cultivation significantly differs from one-stage cultivation. Lower selenite concentrations (0.31–5.00 mg L^{-1}) had a negligible effect on the Chl a/Chl b ratios after 15 days of cultivation compared to the control group, while higher concentrations (10.00–40.00 mg L^{-1}) caused a significant increase of this ratio. Regarding TCC/total Chl ratios under the two-stage cultivation, on day 10 of the cultivation, there was no significant difference between the control and all tested selenite treatment groups. A significant decrease in these ratios was observed in the 5.00–40.00 mg L^{-1} selenite treatment groups at the end of the experiment.

Selenite Induction of Lipid Peroxidation in D. tertiolecta

To quantify the oxidative stress under different selenite concentrations in both one- and two-stage cultivations of *D. tertiolecta*, the TBARS assay was utilized. As can be seen in Fig. 2, at lower selenite concentrations (up to 1.25 mg L^{-1}), TBARS content remained unchanged for both cultivation modes indicating no oxidative stress in *D. tertiolecta* cells. Contrarily, selenite concentrations from 2.50 to 20.00 mg L^{-1} significantly increased TBARS content during both cultivations, at the same time being negatively correlated to growth performance. The maximal TBARS content was observed in cells grown at 20.00 mg L^{-1} of selenite on day 9 during the one-stage mode (53.55 nmol g^{-1} fresh weight), and on day 14 of cultivation during the two-stage mode (81.59 nmol g^{-1} fresh weight).

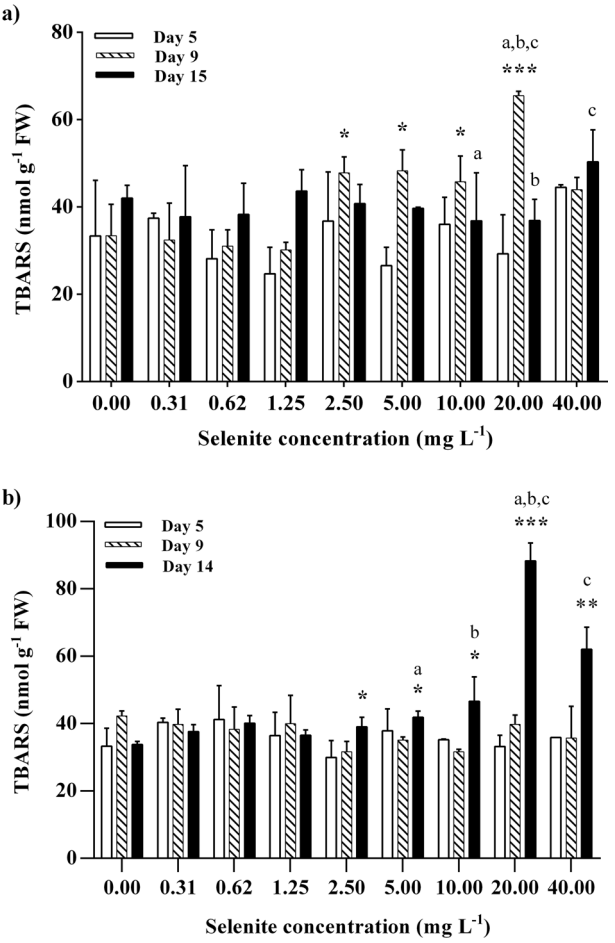


Fig. 2 Thiobarbituric acid reactive substances (TBARS) content in *D. tertiolecta* exposed to different selenite concentrations during (**a**) one-stage cultivation and (**b**) two-stage cultivation. Results are presented as mean ± SD of triplicates (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Mean values sharing common letters indicate significant differences

Effects of Selenite on Neutral Lipid Accumulation in D. tertiolecta

In this study, the NR method was successfully implemented for the detection and quantification of neutral lipids in *D. tertiolecta* exposed to selenite. As can be seen in Fig. 3a–d, lower selenite concentrations (up to 2.50 mg L^{-1}) had no impact on the neutral lipid content for the entire duration of the one-stage cultivation. However, cultivation at a higher concentration range (5.00–40.00 mg L^{-1}) significantly increased the neutral lipid content (Fig. 3e–h). Regarding the two-stage cultivation mode, an increase in the neutral lipid content was observed at almost all tested selenite concentrations (the exceptions were the two lowest tested concentrations, 0.31 and 0.62 mg L^{-1}). Furthermore, the maximal neutral lipid content obtained during the one-stage cultivation (5.48 μeg triolein L^{-1}) was recorded on day 9 in cultures exposed to 20.00 mg L^{-1} of selenite and was 2.40-fold higher compared to the control group. For the two-stage cultivation, the maximal neutral lipid content (29.63 μeg triolein L^{-1}) was recorded in the 20.00 mg L^{-1} selenite treatment group at day 5 upon selenite addition and was 5.37-fold higher compared to the control group (5.17 μeg triolein L^{-1}). By comparing one- and two-stage cultivations, the highest neutral lipid

Fig. 3 Content of total neutral lipids in *D. tertiolecta* at different selenite concentrations during the one-stage and two-stage cultivations: (**a**) 0.31 mg L^{-1}, (**b**) 0.62 mg L^{-1}, (**c**) 1.25 mg L^{-1}, (**d**) 2.50 mg L^{-1}, (**e**) 5.00 mg L^{-1}, (**f**) 10.00 mg L^{-1}, (**g**) 20.00 mg L^{-1}, and (**h**) 40.00 mg L^{-1}. Results are presented as mean ± SD of triplicates (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Asterisks indicate a significant difference between selenite treatment group (gray columns) and negative control (black columns), while significant differences among selenite treatment groups are marked with common letter(s)

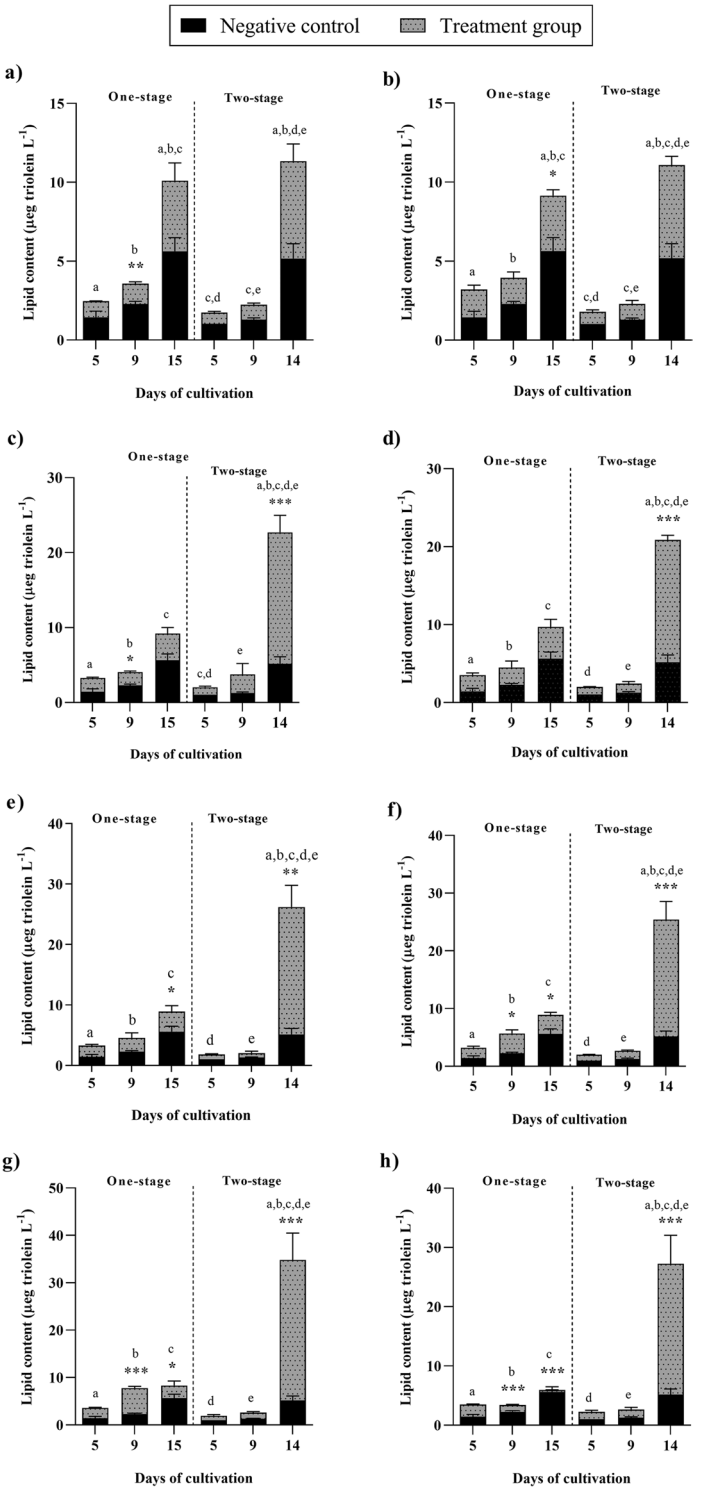
content was recorded during the two-stage cultivation (20.00 mg L^{-1} selenite treatment group, day 14) and was 5.40-fold higher than the highest obtained neutral lipid content in one-stage cultivation (20.00 mg L^{-1} selenite treatment group, day 9). Except for the high neutral lipid content obtained during the two-stage cultivation, reduction of cell density at 20.00 mg L^{-1} of selenite on day 14 in the two-stage cultivation was observed and was 1.63-fold lower compared to the same selenite treatment group on day 9 in the one-stage cultivation.

Fluorescence Microscopy of Neutral Lipids

To visualize neutral lipid content inside NR-stained *D. tertiolecta* cells, fluorescence microscopy was conducted. Microscopy images (Fig. 4) suggest a dose-dependent increase of neutral lipid accumulation for both one- and two-stage cultivation modes. On day 5 of one-stage cultivation, microalgal cells were mostly stained in red in all selenite treatment groups (Fig. 4a, d, g, j, m). However, on day 9 of cultivation, selenite-treated cells contained numerous neutral lipid globules stained in yellow (Fig. 4b, e, h, k, n). Besides, higher selenite concentrations not only increased the size of cytoplasmic lipid droplets but also their number. The highest number and the largest size of lipid droplets were recorded on day 9 in the 20.00 mg L^{-1} selenite treatment group (Fig. 4k). However, on day 15, the decrease in the number and size of lipid droplets was recorded within all selenite treatments. Regarding the two-stage cultivation, on days 5 and 9 of exposure to all tested concentrations, microalgal cells were mostly stained in red. The largest number of yellow-colored lipid bodies was observed on day 15 of two-stage cultivation on 20.00 mg L^{-1} of selenite.

Discussion

A large number of studies characterized Se as a natural trace element metabolically required by a variety of microalgal species, but it may induce toxicity at high doses [27, 28]. In this study, for both one-stage and two-stage cultivation modes, the stimulatory effect of selenite on *D. tertiolecta* growth performance was detected. The observed difference in the time of onset of stimulatory effect between one- and two-stage cultivation modes can be explained by the fact that the two-stage cultivation adaptation phase starts on day 5 upon selenite addition. One of the possible explanations of the stimulatory effect of selenite on *D. tertiolecta* growth performance in both modes is an increased activity of selenoproteins. It results in more effective removal of reactive oxygen species (ROS), and subsequently in the increase of the algal growth rate [7, 29]. Growth stimulation upon selenite exposure has already been reported for numerous microalgal species, such as *Dunaliella salina* [30], *Chlorella pyrenoidosa* [31], *Amphiprora hyalina*, *Alexandrium minutum*, and *Platymonas subcordiformis* [32]. Reunova et al. [30] noted that selenite concentrations ranging from 0.01 to 0.50 mg L^{-1} stimulated *D.*


Negative control
Treatment group
a)
b)
c)
d)
e)
f)
g)
h)
One-stage
Two-stage
Lipid content (µeg triolein L-1)
Days of cultivation
5
9
15
14

I)

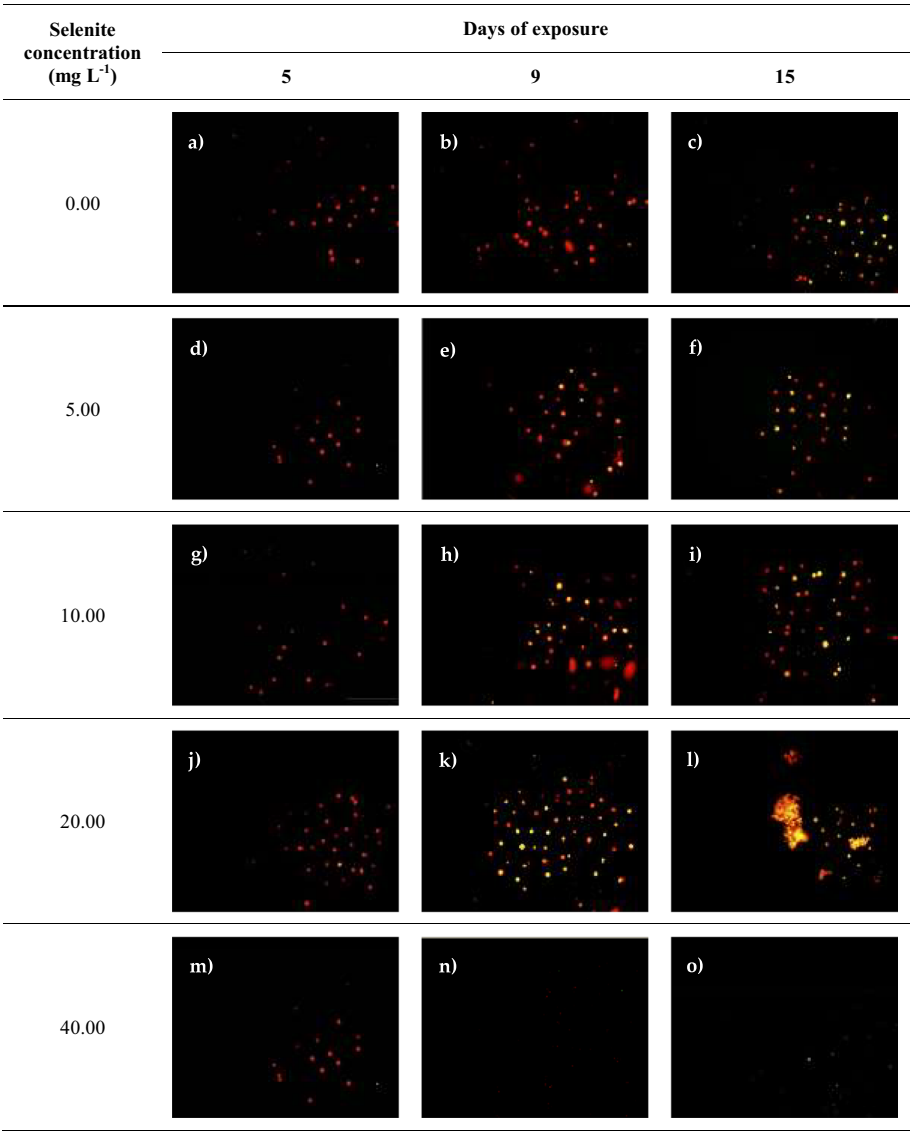


Fig. 4 Fluorescence microscopy pictures of Nile Red stained lipids in *D. tertiolecta* cells exposed to selenite under (I) one-stage and (II) two-stage cultivation modes. Microalgal cells are stained in red, while intracellular lipid droplets are stained in yellow, scale bar = 100 μm

salina growth at day 14 of one-stage cultivation. However, the highest tested selenite concentrations (5.00 and 10.00 mg L^{-1}) were toxic after 7 and 4 days of the experiment, respectively [30], which is in agreement with our study implying similar sensitivity of both microalgae species, *D. salina* and *D. tertiolecta*, to selenite.

II)

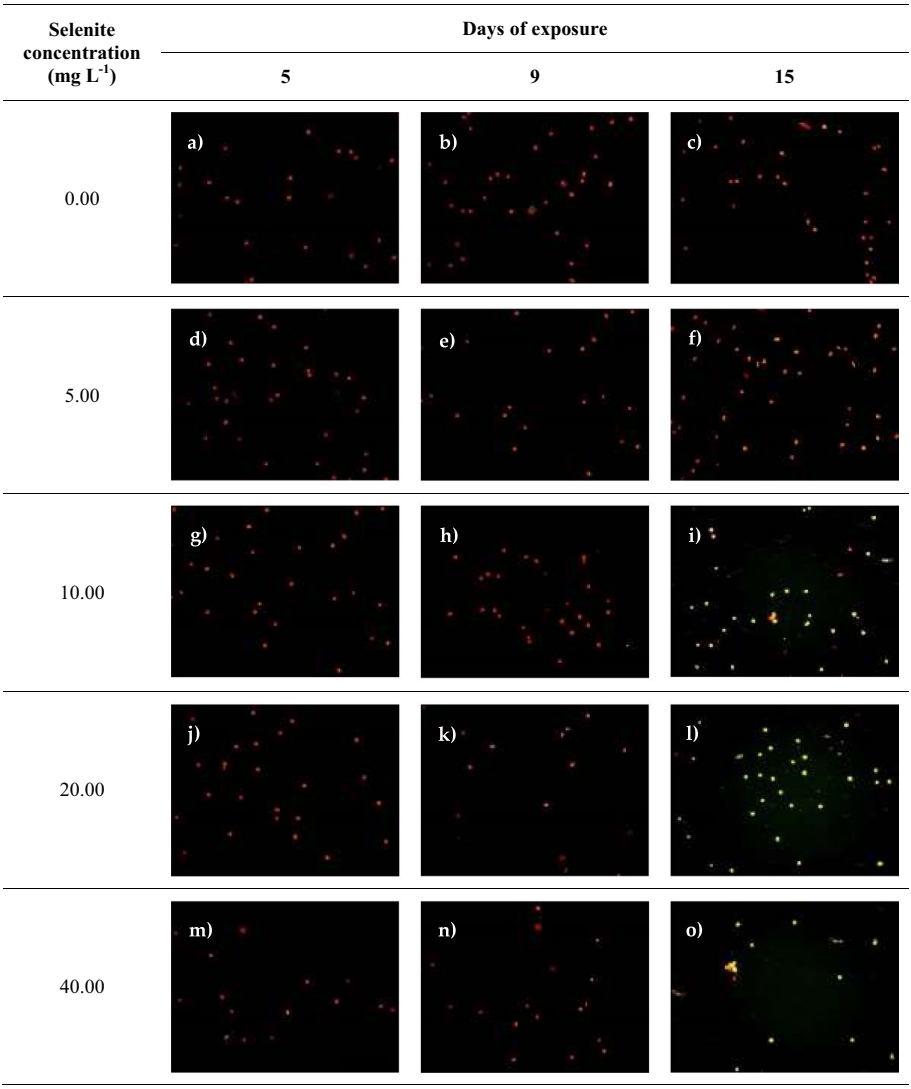


Fig. 4 (continued)

Depending on the concentration and duration of the exposure in the environment, selenium can also act as a toxicant by inhibiting algal growth [15]. Such a growth inhibitory effect during exposure to higher concentrations of selenite was also observed in this study. A possible explanation of selenite-induced toxicity in *D. tertiolecta* during both cultivation modes is the formation of malformed proteins. A similar effect of Se was previously found in other microalgae species (*Spirulina platensis* [33], *Chlamydomonas reinhardtii* [34]), due to chemical similarity between Se and sulphur (S) present in both free amino acids and proteins. This results in the formation of seleno-analogues of the sulphur amino acids,

which ultimately leads to inhibition of cell division and growth reduction [35, 36]. Another possible explanation is selenite's ability to react with sulfhydryl groups and inhibit sulfhydryl enzymes in the Krebs cycle, thus leading to growth reduction [31]. Nevertheless, from the results obtained in this study, it can be seen that maximal algal cell densities during the two-stage cultivation on higher selenite concentrations (10.00 and 20.00 mg L^{-1}) were twofold higher compared to the one-stage cultivation. This could be explained by achieving a late stationary phase during the two-stage cultivation due to a culture strategy. During the first phase of the two-stage cultivation, microalgae were grown under nutrient-sufficient conditions to obtain maximal biomass production. Furthermore, in the second phase of the two-stage cultivation, stress is imposed to trigger an increased accumulation of algal lipids. The second phase of the two-stage cultivation begins when the maximal biomass concentration in the first phase is obtained (in this study on day 9 of cultivation) which enabled better biomass adjustment to the stress inductor [32]. Consequently, it can be concluded that only the two-stage cultivation of microalgae *D. tertiolecta* with selenite as a stress inductor enabled a higher cell culture density, i.e., biomass production. Such observation favors the two-stage cultivation for achieving higher biomass content and improved lipid productivity which contributes to cost-effective and environment-friendly algal lipid production.

To establish the connection between the content of pigments and selenite exposure, chlorophyll a, chlorophyll b, and total carotenoids were determined during both one- and two-stage cultivation processes, as can be seen in Table 1. Except for individual concentrations of photosynthetic pigments, it was also important to determine Chl-a/Chl-b and TCC/total Chl ratios as they represent an indirect indication of oxidative stress and reduced photosystem II activity [8]. From the obtained results, it can be deduced that lower selenite concentrations (0.31–5.00 mg L^{-1}) had a negligible effect on the pigment content of *D. tertiolecta*, while higher selenite concentrations (10.00–40.00 mg L^{-1}) caused an overall reduction of the content of the photosynthetic pigments during the one-stage cultivation, consequently leading to the reduction of carbon fixation [37]. This ultimately resulted in a decrease in biomass yield (Fig. 1). Such observation corresponds with calculated Chl-a/Chl-b and TCC/total Chl ratios, where the only significant increase occurred at the highest tested concentration (40.00 mg L^{-1}) after 10 days of cultivation due to the high acute toxicity. However, at the end of the cultivation process (15 days), the Chl a/Chl b ratio remained unchanged at lower selenite concentrations (0.31–5.00 mg L^{-1}), while higher concentrations ($\geq$ 10.00 mg L^{-1}) caused a significant increase in this ratio indicating oxidative stress. Regarding the two-stage cultivation process, sparse literature data dealing with the correlation between stress induction methods and photosynthetic pigment content is available [8]. When compared to the one-stage, the overall inhibitory effect of selenite on photosynthetic pigment content in the two-stage cultivation was reduced. Chl-a and Chl-b content reduction in the two-stage cultivation at 10.00 and 20.00 mg L^{-1} of selenite was l.76- and 1.21-fold lower, respectively, compared to the corresponding group in the one-stage process. However, one should note an increase of TCC after 10 days of the two-stage cultivation for all tested concentrations, except for 40.00 mg L^{-1}, which corresponded with no observable change in TCC/total Chl ratio and observed color change in 10.00 and 20.00 mg L^{-1} treatment groups. This could be explained by the already mentioned two-stage culture strategy where adaptation to a stress inductor enabled carotenoid synthesis. A change of culture biomass color from green to yellow-orange upon higher selenite concentrations exposure supports the premise of carotenoid synthesis after 10 days of cultivation, which represents a defense strategy of microalgae. Besides, several primary carotenoids, such as β-carotene, act as secondary metabolites and therefore accumulate under stress conditions

[38]. A similar finding was reported by Zheng et al. [39] as they observed a red pigment in *Haematococcus pluvialis* cells after 10 days of selenite exposure, suggesting a substantial accumulation of astaxanthin, a xanthophyll carotenoid. However, a decrease of pigments in the two-stage cultivation at the end of the experiment was also observed with a significant increase in the Chl a/Chl b ratio. The reduction of the photosynthetic pigment content due to selenite exposure can be explained by the fact that higher selenite concentrations cause a generation of ROS (see the "Selenite Induction of Lipid Peroxidation in *D. tertiolecta*" section) and therefore induce a state of oxidative stress in algal cells [40]. Further on, because of the chemical similarity between Se and S, it can be assumed that upon depletion of all the S from the culture medium, the uptake of Se is promoted due to reduced competition between Se and S. This leads to the disruption of the photosynthetic electron transport chain causing a significant decrease in primary production of biomass. Previous studies have also shown that during the one-stage cultivation, selenium concentrations above 175.00 mg L^{-1} induced lipid peroxidation and caused a decrease of photosynthetic pigment content in *S. platensis* [41].

Understanding of the relationship between stress factors caused by excessive ROS formation and lipid production is of great importance since stress-based strategies represent one of the leading methods for lipid accumulation in microalgal cells [42]. To quantify the oxidative stress under different selenite concentrations, a TBARS lipid peroxidation assay was utilized. From the obtained results, it can be concluded that selenite concentrations from 2.50 to 20.00 mg L^{-1} in both cultivation modes increased TBARS content thus indicating lipid peroxidation in algal cells. This can be explained by the fact that increasing selenite concentrations resulted in excess ROS production, which affects cellular processes, causing lipid peroxidation (Fig. 2), and finally resulting in cell death due to the initiation of programmed cell death pathway (Fig. 1). Similar results were already reported during the one-stage cultivation of *C. pyrenoidosa* at selenite concentrations higher than 40.00 mg L^{-1}, which resulted in increased lipid peroxidation that reached the maximum at 80.00 mg L^{-1} [15]. Chen et al. [41] also recorded selenite-induced lipid peroxidation in *S. platensis*, with the maximum values recorded at 250.00 mg L^{-1}. Such results suggest that the occurrence of lipid peroxidation in microalgal cells also depends on species sensitivity that could differ even within the same genus. Furthermore, from the obtained results, it can be concluded that the maximal TBARS content obtained during the two-stage cultivation is 1.52-fold higher compared to the value obtained during the one-stage cultivation. Such a difference in TBARS content among cultivation modes indicates that the subsequent addition of selenite during the two-stage cultivation exhibits a higher level of stress. Since the addition of selenite commenced on day 9 of cultivation, microalgal cells were already at the end of their exponential (log) phase, i.e., higher cell density ultimately led to the more stress-induced response. In the one-stage cultivation, the addition of selenite as a stress inducer occurred at the beginning of the cultivation when microalgal cells were in the lag phase of growth; thus, their adaptation to stress was prolonged. However, higher TBARS content in two-stage cultivation corresponds well with the increased carotenoid content as an already mentioned result of the microalgae defense strategy.

Since one of the features of the Nile Red dye is the possibility to shift in the fluorescence emission from red to yellow correlating with the level of hydrophobicity of lipid, the NR method can be used to discriminate polar, non-polar (or neutral) lipids, or even single lipids. Within this study, the potential of different selenite concentrations to increase lipid accumulation in microalgae *D. tertiolecta* was investigated. Also, the NR method offers numerous advantages such as easier and cheaper usage, requiring a much smaller amount of equipment and sample, and offering quick response in comparison

with conventional methods for lipid content determination (gravimetric measurements) [43]. As shown in Fig. 3a, a statistically significant increase in the neutral lipid content in microalgae *D. tertiolecta* on higher tested selenite concentrations (5.00–40.00 mg L^{-1}) during both cultivation modes was observed and could be explained by the fact that during optimal growth conditions, microalgae use nutrients for biomass development, but when exposed to stress conditions (in this case elevated selenite concentrations), they change their metabolism from biomass to lipid production [44]. Among trace elements, iron (Fe) [20, 28, 35], zinc (Zn) [11, 23, 25, 35], manganese (Mn) [27, 35], and molybdenum (Mo) [35] are mostly used to enhance lipid content in microalgae. Several researchers demonstrated that Fe^{3+} addition in the growth medium has the potential to increase both growth rate and lipid content in several microalgal species (*Nannochloropis oculata*, *Scenedesmus obliquus*, *Monoraphidium* sp.) [33, 45, 46]. However, although mentioned trace elements are effective inducers of lipid accumulation, it should be emphasized that lipid productivity is in most cases dose-dependent. In that manner, Dou et al. [33] noticed a decrease in lipid content in *N. oculata* cells during exposure to high concentrations of Zn^{2+}, Mn^{2+}, and Mo^{6+}. Indeed, the decrease of the neutral lipid content in *D. tertiolecta* exposed to 40.00 mg L^{-1} of selenite in this study negatively affected growth performance in both one- and two-stage experiments. This can be explained by the fact that two major consequences of cellular responses to stress-based strategies are a decline in biomass and oxidative injury, which ultimately results in a decreased yield of neutral lipids [32]. Therefore, it can be concluded that the selection of an appropriate method of microalgal lipid induction is of great importance. Furthermore, 5.40-fold higher neutral lipid content obtained during the two-stage cultivation in regard to the one-stage can be explained by the fact that the two-stage cultivation mode is intended to favor biomass production in the first stage and lipid production in the second stage of cultivation. Similar to the present study, Ra et al. [34] reported the contribution of the second phase (cultivation under low-salt conditions) in the cultivation of four microalgal species (*I. galbana*, *N. oculata*, *D. salina*, and *D. tertiolecta*).

Furthermore, the results of fluorescence microphotography (Fig. 4) were in strong agreement with neutral lipid content measured by NR fluorescence (Fig. 3). Red-stained microalgal cells on day 5 during one-stage cultivation indicate that lipid synthesis has not yet started (Fig. 4a, d, g, j, m). However, numerous neutral lipid globules stained in yellow observed on day 9 point out that selenite serves as a stress factor during microalgal lipid synthesis (Fig. 4b, e, h, k, n). Furthermore, the highest obtained number and the largest size of lipid droplets on day 9 in the 20.00 mg L^{-1} selenite treatment group during one-stage cultivation is in accordance with previous findings regarding TBARS content and neutral lipid quantification (see the "Selenite Induction of Lipid Peroxidation in *D. tertiolecta*" and "Effects of Selenite on Neutral Lipid Accumulation in *D. tertiolecta*" sections). A decrease in the number and size of lipid droplets on day 15 during one-stage cultivation confirms the fact that prolonged stress period decreased overall biomass and TAG formation rate. The highest amount of yellow-colored lipid bodies was observed on day 15 of two-stage cultivation in the 20.00 mg L^{-1} selenite treatment group, indicating lipid synthesis. Considering the strong agreement between the results obtained from quantification (Fig. 3) and visualization (Fig. 4) of neutral lipid content in algal cells, we can conclude that selenite as a stress inducer represents a great potential for induced lipid production, at the same time pointing out the two-stage cultivation strategy as a more productive way to improve the lipid content. Taking both neutral lipid content and cell density into account, it should be noted that reduction of cell density in 20.00 mg L^{-1} selenite treatment group at day 14 in two-stage cultivation is 1.63-fold lower, compared to the same selenite treatment

group at day 9 in the one-stage cultivation, thus favoring the two-stage strategy with selenite as a neutral lipid inductor in sustainable lipid production.

Conclusion

The present study reveals the impact of the trace element selenium in the form of sodium selenite on *D. tertiolecta* growth, pigment content, lipid peroxidation, and neutral lipid accumulation during one- and two-stage cultivation modes. The beneficial effect of selenite on *D. tertiolecta* growth was observed during both one-stage (10.00 and 20.00 mg L^{-1} selenite treatment groups) and two-stage (0.31 mg L^{-1} selenite treatment group) cultivation. However, twofold higher algal densities were observed from 10.00 to 40.00 mg L^{-1} of selenite during the two-stage cultivation compared to the one-stage mode. Under both cultivation strategies, higher selenite concentrations led to ROS formation resulting in increased TBARS content in *D. tertiolecta* cells and consequently higher accumulation of neutral lipids. The maximal obtained neutral lipid content during one- and two-stage cultivation was obtained in 20.00 mg L^{-1} selenite treatment group on days 9 and 14 of cultivation, respectively. However, the two-stage cultivation resulted in a 5.40-fold higher neutral lipid content. The obtained higher cell density and neutral lipid content in the two-stage cultivation show that this approach is more advantageous than the one-stage cultivation. Findings from this study contribute to elucidating the impact of selenite on *D. tertiolecta* culture attributes and characteristics during both one- and two-stage cultivation modes. In summary, this study gave input for future usage of the two-stage cultivation techniques and selenite as an efficient neutral lipid inductor to be implemented in biodiesel production from microalgae. However, further studies regarding carotenoid synthesis and lipid productivity in high-scale experiments are needed.

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Author Contribution MGP: conceptualization, methodology, visualization, writing—original draft. SB: conceptualization, validation, writing—review and editing. LČ: conceptualization, validation, writing—review and editing. AS: conceptualization. NTP: validation, writing—review and editing. MDS: resources, funding acquisition. ISP: validation, writing—review and editing. RČR: resources, funding acquisition, writing—review and editing. All authors read and approved the final manuscript.

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Data Availability The data underlying this article are available in the article. Raw data files will be shared on reasonable request to the corresponding author.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Competing Interests The authors declare no competing interests.

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