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Selenium Incorporation to Amino Acids in *Chlorella* Cultures Grown in Phototrophic and Heterotrophic Regimes

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ABSTRACT: Microalgae accumulate bioavailable selenium-containing amino acids (Se-AAs), and these are useful as a food supplement. While this accumulation has been studied in phototrophic algal cultures, little data exists for heterotrophic cultures. We have determined the Se-AAs content, selenium/sulfur (Se/S) substitution rates, and overall Se accumulation balance in photo- and heterotrophic *Chlorella* cultures. Laboratory trials revealed that heterotrophic cultures tolerate Se doses ∼8-fold higher compared to phototrophic cultures, resulting in a ∼2−3-fold higher Se-AAs content. In large-scale experiments, both cultivation regimes provided comparable Se-AAs content. Outdoor phototrophic cultures accumulated up to 400 $\mu g\ g^{-1}$ of total Se-AAs and exhibited a high level of Se/S substitution (5−10%) with 30−60% organic/total Se embedded in the biomass. A slightly higher content of Se-AAs and ratio of Se/S substitution was obtained for a heterotrophic culture in pilot-scale fermentors. The data presented here shows that heterotrophic *Chlorella* cultures provide an alternative for Se-enriched biomass production and provides information on Se-AAs content and speciation in different cultivation regimes.

KEYWORDS: *Chlorella, microalgae, selenium, selenomethionine, selenocysteine, methylselenocysteine, phototrophic cultivation, heterotrophic cultivation*

INTRODUCTION

Selenium (Se) is an essential micronutrient for many living organisms and can be toxic at higher doses.[1−3] In mammals, including humans, Se is mainly incorporated into the active site of Se-proteins in the form of Se-cysteine (SeCys), many of which are detoxification and antioxidant enzymes.[4,5] Organically bound Se has anti-inflammatory, immunostimulating, antiaging, and anticarcinogenic activities. It is also involved in thyroid hormone metabolism and regulates reproduction abilities and development.[4−6]

Se is unevenly distributed in the environment. Its varying concentration in geo-ecosystems affects the health of humans and animals through the food chain.[7] Se intake also differs significantly in various regions. This depends not only on its amount in water or soil but also on its speciation and the local physicochemical conditions that determine Se bioavailability and bioaccessibility.[5,8,9]

The daily requirement of Se for humans is about 40 μg for adults.[3] In contrast, a dose of 800 μg is considered toxic, which makes the gap between deficiency and toxicity quite narrow.[3−5] Traditional food sources of Se are nuts, cereals, vegetables, seafood, and meat.[5,8] The content as well as bioavailability of Se in these foodstuffs is quite low so they need to be consumed in relatively large amounts to satisfy requirements of Se intake.[8,9] Lately, Se has also been supplied in the form of inorganic Se salts; however, these have numerous disadvantages due to their toxicity and low intake.[8,10] In this sense, biofortified foodstuffs, such as crops grown on Se-fertilized soils[9,11] or Se-enriched food supplements (e.g., selenized yeast or microalgae), have been used and represent a safe alternative of supplementation.[8,12] In some reports, only the amount of total or organic Se in the food products is shown,[13,14] but a few also focus on Se speciation, which along with Se species concentration differs vastly among plant species and due to Se-biofortification techniques.[15,16]

For example, in Se-enriched foods, a wide range and amount of Se species [in μg of Se species per 1 g of dry weight (DW)—$\mu g\ g^{-1}$ DW] were found: rice contained only ∼0.03 $\mu g\ g^{-1}$ DW of organic Se,[13] potato had mostly Se-methionine (SeMet) of ∼0.7 $\mu g\ g^{-1}$ DW,[16] broccoli had ∼150 $\mu g\ g^{-1}$ DW of methyl-Se-cysteine (MeSeCys),[15] and selenized yeast contained more than 3000 $\mu g\ g^{-1}$ DW of SeMet.[17,18] Recently, Se-enriched microalgae have received substantial attention as a cheap and easy means to produce a source of highly bioaccessible and bioavailable organically bound Se.[8] Numerous species of green microalgae, such as *Chlamydomonas reinhardtii*, *Dunaliella salina*, and *Scenedesmus quadricauda*, and various species of the genus *Chlorella* sp. have been studied with regard to Se bioaccumulation and metabolism.[3,19−21] The highest organic Se content was reported for *Chlorella pyrenoidosa* (∼340 $\mu g\ g^{-1}$ DW)[20] and *Chlorella vulgaris* (∼320 $\mu g\ g^{-1}$ DW)[1] grown phototrophically in laboratory

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experiments. For some of the species, only the SeMet content was determined, reaching up to ~300 $\mu g\ g^{-1}$ DW for *S. quadricauda*[19] and ~330 $\mu g\ g^{-1}$ DW for *Chlorella sorokiniana*.[22]

Chlorella sp. is well known for its balanced content of various important compounds: proteins, essential amino acids, fatty acids, vitamins, and microelements, all of which may be manipulated by cultivation conditions. This, along with its high productivity and ability to grow outdoors in large-scale plants, makes *Chlorella* a perfect functional food supplement.[23]

Microalgal cells take up the inorganic salts, selenite or selenate, via different transport systems. In the case of selenate, it is being additionally reduced intracellularly to selenite and further converted into organic bioavailable forms, mainly to selenoaminoacids (Se-AAs)—SeCys or SeMet—mostly via the sulfur (S) reductive assimilation pathway and incorporated into selenoproteins or into elemental Se.[3,4,24] If the intracellular Se concentrations are too high, Se-AAs may, via nonspecific incorporation, cause production of malformed proteins.[4] Thus, under such conditions, microalgae convert Se into volatile forms. SeCys is converted into dimethyldiselenide via the methylated intermediate MeSeCys, while SeMet is metabolized into dimethylselenide after its methylation. Specific methyltranferases, namely, selenocysteine methyltransferase and methylmethionine methyltransferase, are involved in these detoxification mechanisms.[3,4]

Most of the reports about Se-enriched *Chlorella* have focused on optimizing the laboratory-scale phototrophic cultivation (utilizing light as an energy source and CO_2 as a carbon source) to incorporate the maximum amount of Se into the biomass. Fewer reports have been dedicated to phototrophic large-scale production of the Se-enriched biomass. There is also a paucity of information about heterotrophic Se-enriched *Chlorella* cultivation. A heterotrophic regime of cultivation (without light and using glucose as a carbon and energy source) might represent an alternative method of cultivation in areas where climatic conditions are not favorable and sunlight is not sufficient for effective phototrophic cultivation. Moreover, *Chlorella* growth rates are higher under heterotrophic conditions, and the culture is able to reach higher cell densities.[26] Technology for the cultivation of Se-tolerant strains of *Chlorella*, which are able to grow heterotrophically, was developed in Centre Algatech (Czech Republic).[10,25] The addition of small doses of the Se-enriched *Chlorella* biomass to feedstock significantly improved physical and physiological parameters of farmed animals.[10] Although it was reported that heterotrophically produced Se-enriched *Chlorella* biomass was rich in organically bound Se, detailed information about forms has been missing.

To the best of our knowledge, there is little data available that compares the efficiency of the process, expressed in the rates of Se/S substitution in S-containing amino acids (AAs), between photo- and heterotrophic culturing regimes. Therefore, the objective of this research was to study this problem using highly sensitive high-performance liquid chromatography connected to inductively coupled plasma mass spectrometry (HPLC-ICP-MS) and gas chromatography in combination with atmospheric pressure chemical ionization high-resolution mass spectrometry (GC-APCI-HRMS). These techniques enabled us to determine Se-AAs accumulation and speciation in *Chlorella* grown in photo- or heterotrophic regimes on a laboratory scale and, based on the optimized conditions, to develop and perform large-scale cultivations in outdoor thin-layer cascades (phototrophic) or in a fermentor (heterotrophic).

MATERIALS AND METHODS

Cultivation Upscaling of Se-Enriched *Chlorella*. In the current research, we used different scales of microalgae cultivation:

(i) laboratory-scale experiments performed in volumes of up to 330 mL using artificial light to optimize certain variables for the larger-scale cultivations;
(ii) pilot-scale experiments as a "bridge" between laboratory- and large-scale cultivation performed with a volume up to 10 L to test upscaling; and
(iii) large-scale trials in 2200 L outdoor units that allow the generation of a large amount of biomass and any corresponding valuable product.

Phototrophic Cultivation. Unicellular green freshwater microalga *C. vulgaris* R117 (further denoted *Chlorella* R117; registered as *C. vulgaris* BEIJ., 2017/R117, CCALA 1107, Culture Collection of Autotrophic Organisms, Institute of Botany, Třeboň, Czech Republic) was used for phototrophic cultivations.

For the Se bioaccumulation laboratory tests, *Chlorella* R117 was cultivated in a batch mode for 4 days in glass columns (330 mL starting working volume) under continuous illumination at 28 °C. The cultures were grown in a modified inorganic medium, which was mixed by bubbling with air + 1.5% CO_2 with an initial biomass density of ~2 grams DW per liter (g DW L^{-1}).[27,28] Photosynthetically active radiation (PAR) of 120 μmol photons $m^{-2}\ s^{-1}$ was provided by a panel of horizontally placed, high-frequency cool fluorescent tubes (36 W/830 Lumilux, Osram, Germany). Light intensity was measured directly inside an empty cultivation column. Sterile distilled water was added to compensate for evaporation. The cultivation was started under sterile conditions, and cultures grown without Se addition served as controls.

The outdoor large-scale cultivation in thin-layer cascades (2200 L working volume) was performed in a fed-batch mode using an adjusted inorganic medium under a natural light/dark period in June 2018 (Cascade units 1 and 2 with Se and the Control unit (without Se)) and in September 2018 (Cascade unit 3 with the addition of Se).[8,27,29,30] The initial concentrations of biomass in Cascades 1, 2, and 3 and the Control were ~6.7, ~7.4, ~12.9, and ~7.8 g DW L^{-1}, respectively (Supporting Information). The biomass density was measured in triplicate and the analytical data once in June, whereas in the September trial, all variables were measured in triplicate. Growth curves were similar for all three units: in June on day 6 and in September on day 5, the cultures were kept in the retention tanks due to bad weather.[29,30] In this case, Se was not added and samples were not collected. PAR in laboratory trials was measured as 10 s averaged values using a light meter (LI-250A coupled to a cosine-corrected sensor LI-190SA, both Li-Cor). Light intensity and temperature for outdoor cultivation were provided by a weather station (Meteostation Warioweather Compact, model ME13).

Heterotrophic Cultivation. For the Se bioaccumulation laboratory tests, the strain of *C. vulgaris* G120 (further as *Chlorella* G120; registered as *C. vulgaris* BEIJ., 1996/H 14, CCALA 30001, Culture Collection of Autotrophic Organisms, Institute of Botany, Třeboň, Czech Republic) was grown heterotrophically at 25 °C in a batch mode in Erlenmeyer flasks (300 mL starting working volume) in the dark for 4 days using an orbital shaker with the initial biomass density of ~2 g DW L^{-1}. The medium for the batch cultivation contained the following compounds (g L^{-1}): glucose, 60; KNO_3, 12; $MgSO_4{\cdot}7H_2O$, 3.3; KH_2PO_4, 2. Micro- and trace elements were also added (mg L^{-1}): $CaCl_2$, 132; ferric-sodium chelatonate, 111; H_3BO_3, 18.5; $ZnSO_4{\cdot}7H_2O$, 8.6; $CoSO_4{\cdot}7H_2O$, 8.4; $CuSO_4{\cdot}5H_2O$, 7.4; $MnSO_4{\cdot}4H_2O$, 7; and $(NH_4)_6Mo_7O_{24}.4H_2O$, 6, and the pH was adjusted to 7.5.

The pilot-scale laboratory cultivation was performed in batch/fed-batch modes in a 9 L column fermentor (Gryf Ltd. Czech Republic). This was equipped with pH, dissolved oxygen, temperature, turbidity control probes, and peristaltic pumps for Se and nutrient addition.

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The culture was continuously mixed (400 rpm) and aerated (keeping the diluted oxygen to 2 ppm). The cultivation lasted for 4−6 days at 25 °C with the initial biomass density of ∼2 g DW L^{-1}. The cultivation media had the same composition as for the laboratory-scale screening with minor modifications: it had initially a higher glucose content of 80 g L^{-1} and additionally 8 g L^{-1} urea.

Optical Density and Dry Weight Determination. Optical density (OD_{750}) of the culture was measured at 750 nm using a spectrophotometer that was linearly related to biomass density. Growth rates were measured as an increase in the dry weight of biomass (g DW L^{-1}) that was estimated daily by centrifuging 2 mL of microalgae culture in preweighted Eppendorf tubes at 13 000*g* for 3 min, the sediment was dried at 105 °C for 8 h, and the weight of the biomass pellet was noted.[27]

Se Treatment. Se was added to the microalgae in the form of sodium selenite (Na_2SeO_3, Alfa Aesar, Karlsruhe, Germany) depending on the optical density of the cultures.[27]

In phototrophic laboratory cultures, Se was added to each cultivation column as a stock solution of Na_2SeO_3 twice a day to final concentrations of 0.5, 1, 4, and 8 mg of Se per 1 g of dry weight (further abbreviated as the mg Se treatment or mg Se g^{-1} DW).

Se was added to outdoor cultures (2200 L working volume) as a stock solution of Na_2SeO_3 twice a day calculated as mg Se per liter of microalgae culture (mg Se L^{-1}) estimated from biomass density when the Se dose was between 0.05 and 0.2 mg Se per g of biomass as described previously.[8,27] The total amount of Se added to cultures in Cascades 1, 2, and 3 (mg Se g^{-1} DW) and the procedure of Se treatments are described in detail in the "Results and Discussion" section.

In heterotrophic cultivation, Se was added to each cultivation flask as a stock solution of Na_2SeO_3 twice a day at several concentrations (0.5, 1, 4, 8, and 16 mg Se g^{-1} DW). A stock solution of Na_2SeO_3 was added twice a day at the concentration of 0.5 mg Se g^{-1} DW to the fermentor. All heterotrophic cultivations were performed under sterile conditions.

Glucose Concentration Assay. The glucose concentration was determined by a liquid chromatography system consisting of a prep pump (LabAlliance), a manual injector, a refractive index detector (Schamback RI2000), and a column (Watrex Polymer IEX Ca 8 μm, 250 × 8 mm). The consumption of glucose was taken as a measure of culture activity. The culture samples were purified by centrifugation, and 20 μL of supernatant was injected manually to the system. The degassed demineralized water was used as the mobile phase at a flow rate of 0.5 mL min^{-1}. The retention time of the glucose was 10.5 min. The column temperature was set to 80 °C using a thermostatic box (Con Brio, Czech Republic). The separation process was operated with Clarity lite 3.07.662 software, and the glucose concentration was calculated from a calibration curve.

Sample Collection and Preparation for the Analytical Procedures. An aliquot (between 10 and 50 mL) of the biomass suspension was collected in a plastic conical tube and centrifuged at 4000*g* for 5 min (Hettlich Rotofix 32A centrifuge) and then lyophilized (ScanVac CoolSafe 95-15 Freeze Dryer with a Vacuubrand RZ 2.5 vacuum pump) prior to analytical procedures.

In the biotechnological production experiments, the pellet was also washed with 2 volumes of water to remove toxic inorganic Se species from the surface of the microalgae. It was shown that the washing procedure did not influence Se-AAs content in the biomass.

On the last day of the outdoor large-scale cultivation in thin-layer cascades, the biomass was harvested by centrifugation, disintegrated, and spray-dried, as previously described.[8]

Quantification of Total Se. Total Se content in algal biomass was determined by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7700x ICP-MS) after mineralization of samples with nitric acid and hydrogen peroxide in a microwave digestion system (MWS 3+ Berghof, Germany).

The concentration of Se in cultivation media was determined by Agilent 7700x ICP-MS after dilution of samples with 5% nitric acid.

The amounts of total Se were calculated as μg g^{-1} DW for the biomass and g L^{-1} for the cultivation media, expressed as average ± standard deviation (SD) (three replicates).

Se-AAs Quantification. Determination of the absolute amounts of Se-AAs (SeMet, SeCys, and MeSeCys) was performed by high-performance liquid chromatography inductively coupled plasma mass spectrometry (HPLC-ICP-MS), and gas chromatography atmospheric pressure chemical ionization high-resolution mass spectrometry (GC-APCI-HRMS) was used for the simultaneous quantification of methionine (Met), cysteine (Cys), SeMet, SeCys, and MeSeCys. The data obtained from GC-APCI-HRMS measurements were then used to calculate ratios of Se/S substitution in S-containing AAs, defined as

$$\mathrm{Se/S_{SeMet}} = C(\mathrm{SeMet})/(C(\mathrm{SeMet}) + C(\mathrm{Met})),$$
$$\mathrm{Se/S_{SeCys}} = C(\mathrm{SeCys})/(C(\mathrm{SeCys}) + C(\mathrm{Cys})),$$
and
$$\mathrm{Se/S_{MeSeCys}} = C(\mathrm{MeSeCys})/(C(\mathrm{MeSeCys}) + C(\mathrm{Cys}))$$

where "*C*" is a concentration.

Sample Hydrolysis. Prior to HPLC-ICP-MS and GC-APCI-HRMS analyses, samples were hydrolyzed by methanesulfonic acid (MA) with the optimized conditions as reported previously.[8] Briefly, lyophilized samples (from 5 to 25 mg) were weighed in 10 mL glass tubes, and then 1 mL of 3 M MA plus 20 μL of mercaptoethanol (ME) were added for the hydrolysis. The mixture was then vortexed and incubated at 95 °C for 24 h in an oven followed by an addition of 20 μL of ME. The samples were then diluted to 5 mL with LC-MS-grade water and centrifuged. The supernatant was then collected for derivatization prior to GC-APCI-HRMS or for HPLC-ICP-MS measurements.

HPLC-ICP-MS. HPLC separation was carried out by an Agilent 1100 HPLC system on a Hamilton PRPX-100 column (strong anion-exchange resin, 150 × 4.1 mm, 10 μm particle size). Species were separated in 10 mM ammonium citrate buffer at pH 4.5 with 2% methanol (v/v) and 0.02% ME (v/v) with a flow rate of the mobile phase of 1 mL min^{-1}. The injection volume was set to 100 μL. Detection was performed by Agilent 7700x ICP-MS on the ^{78}Se isotope operated in the helium collision mode. Dwell time was set to 2 s. The concentration of the Se-AAs was determined using an external calibration method. The method was validated by spiking of extracts with SeMet, SeCys, and MeSeCys, and recoveries were found between 95 and 103%.[8,28]

Sample Derivatization and GC-APCI-HRMS. Derivatization of samples for GC-APCI-HRMS measurements was done using 2,2,3,3,4,4,4-heptafluorobutyl chloroformate following the previously described protocol.[31] Gas chromatography (Bruker 456-GC) atmospheric pressure chemical ionization high-resolution mass spectrometry (Impact HD mass spectrometer, Bruker, Billerica, MA) and mass spectrometric data analysis were performed as described by Vu et al.[8] Limits of quantification of the method (μg mL^{-1}) for Met, Cys, SeMet, SeCys, and MeSeCys were 0.4, 0.7, 0.1, 0.5, and 0.2, respectively.

Photosynthesis Measurements. The photosynthetic activity of the cultures was measured using three techniques: saturation pulse analysis of fluorescence quenching to record rapid light-response curves (RLCs), fast fluorescence induction kinetics (OJIP curve), and photosynthetic oxygen evolution and respiration (POE/R).[32,33] Samples were measured under laboratory conditions after dilution to 0.2−0.3 g DW L^{-1} (corresponding to 5−7 mg Chl L^{-1}) with the growth medium and dark adaptation for 10 min in triplicate (n = 3). Details of RLCs, OJIP curves, POE/R, and chlorophyll concentration measurements are described in the Supporting Information.

Data Analysis and Statistics. Differences between the mean values were tested by one-way analysis of variance followed by post hoc comparison (Tukey HSD test). The normal data distribution was tested by the Kolmogorov−Smirnov test. The calculations were performed in Statistica for Windows software (version 13.2). The results were visualized using Sigma Plot 11.0 software.

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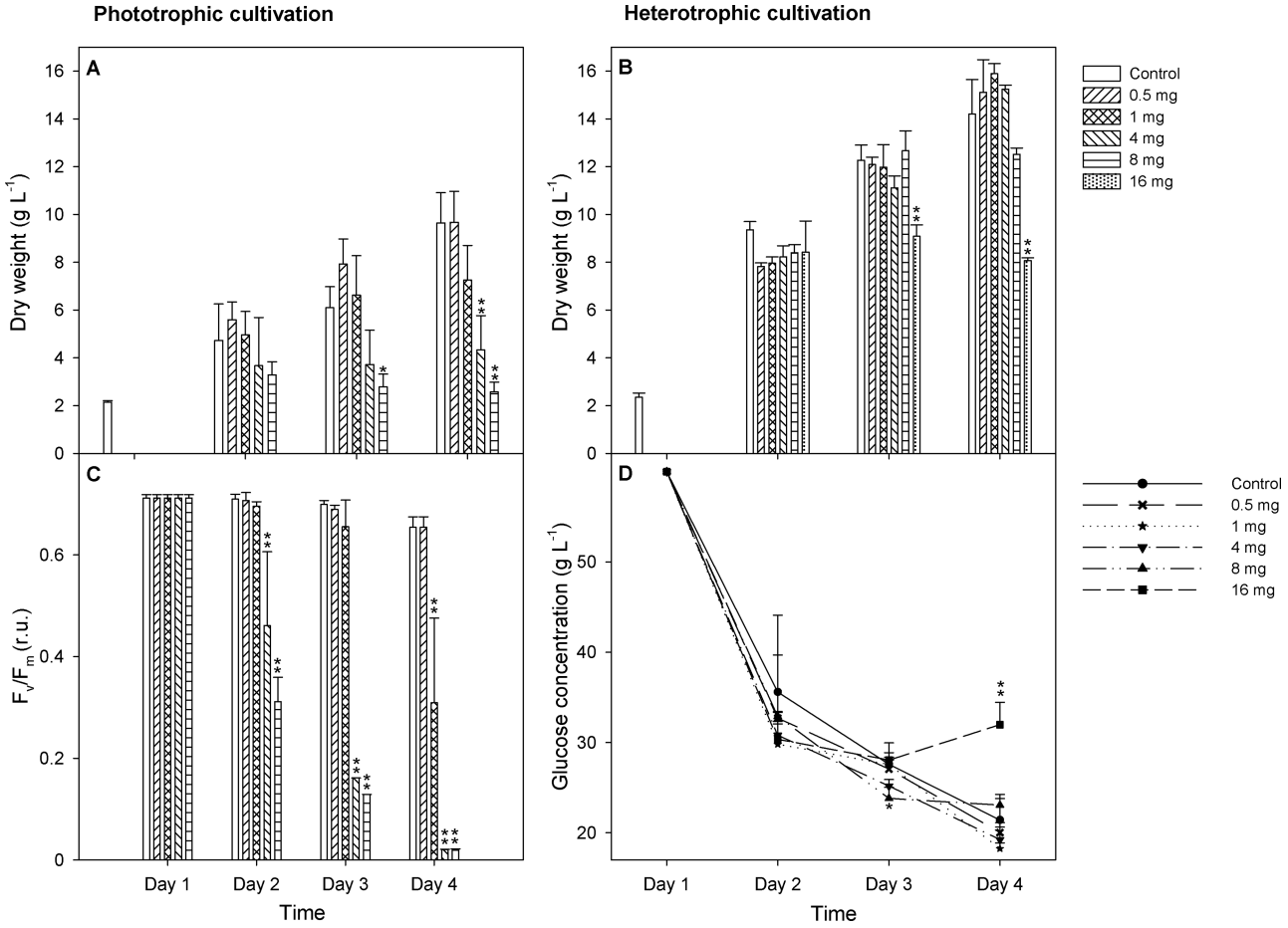

Figure 1. Growth (A, B), maximum photochemical performance of PSII, F_v/F_m values (C), and glucose consumption (D) of *Chlorella* cultures grown in phototrophic or heterotrophic regimes, which were treated with various selenite dosages (0, 0.5, 1, 4, 8, and 16 mg Se per 1 g of biomass added twice a day). Values were measured in triplicate and were expressed as mean ± SD. The asterisk denotes the statistical differences between the mean values of the groups from the control as obtained with the Tukey HSD test as a post hoc comparison (**$p < 0.01$, *$p < 0.05$).

RESULTS AND DISCUSSION

Se incorporation and Se-AAs accumulation in microalgae biomass were studied in *Chlorella* R117 and G-120 strains grown under phototrophic and heterotrophic regimes, respectively. Four series of laboratory and outdoor trials were carried out: two laboratory experiments in both phototrophic and heterotrophic regimes, three heterotrophic pilot trials, and two outdoor large-scale trials.

Laboratory Experiments: Effect of Various Se Doses on Growth and Physiology, Se-AAs Accumulation, and Se/S Substitution in *Chlorella* Cultures. Microalgae have different abilities to take up and accumulate Se, principally depending on strain, cultivation conditions, and the chemical form of supplied Se.[4,10] In most of the biotechnological trials, microalgae have been treated either with selenate (SeO_4^{2-}) or with selenite (SeO_3^{2-}). Selenate has higher solubility and bioavailability, but it is considered to possess higher toxicity compared to selenite.[4] However, there are many uncertainties about the selection of the Se source for microalgae, as for many microalgal species one of the anions is more toxic than the other one.[3] In our study, we have selected sodium selenite as it was reported that *Chlorella* tolerates it better and reaches high levels of Se bioaccumulation after its application.[10,20] One of the notable features of our setup is that unlike most studies expressing the Se dose as the element concentration per volume of culture,[1,20] we calculated Se doses per actual DW of biomass in the culture.[27] We searched for the optimal Se concentrations in laboratory growth tests in phototrophic and heterotrophic regimes examining total Se and Se-AAs accumulation.

The phototrophic growth of *Chlorella* culture treated with a low dosage of 0.5 mg Se g^{-1} DW was comparable to the control culture (no Se added), reaching biomass density of about 10 g DW L^{-1} on day 4 (Figure 1A). The maximal photochemical efficiency of PSII (F_v/F_m) in the 0.5 mg Se treatment had a similar trend to that of the control culture (Figure 1C). In the 1 mg Se treatment, the F_v/F_m decreased significantly starting from day 4 (Tukey HSD test $p < 0.01$), and a reduction of growth was detected by the end of the experiment. A significant reduction of F_v/F_m was detected in the 4 and 8 mg Se treatments starting from day 2 (Figure 1C), and by day 4 the growth had been significantly inhibited (Tukey HSD test $p < 0.01$) and the F_v/F_m variable dropped almost to zero (Figure 1A,C). Simultaneously, as Se reached the inhibiting concentrations, the fluorescence kinetics variables V_j and V_i started to increase because of the reduced amount of required electrons for the cell metabolism (Figure S1). Another sign of Se toxicity was the fact that microalgae cultures started to become reddish[1,20] at concentrations of 4 and 8 mg Se g^{-1} DW on day 4 and with a specific garliclike smell.[3,27,34]

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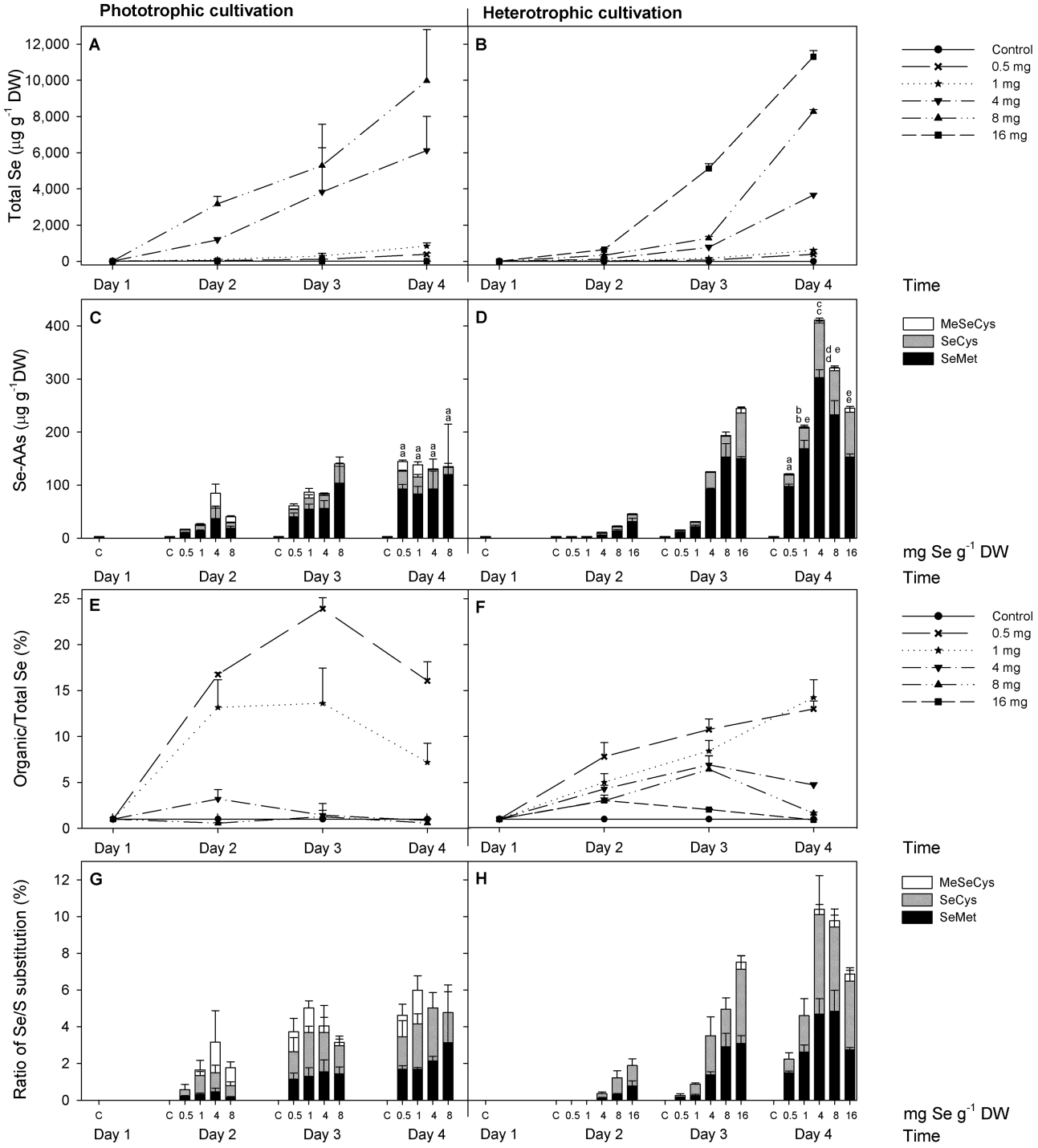

Figure 2. Total Se (A, B) and Se-AAs (C, D) content, the ratio of organically bound to total Se (E, F) and ratio of Se/S substitution (G, H) in *Chlorella* biomass grown in phototrophic or heterotrophic regimes and treated with various selenite dosages (0, 0.5, 1, 4, 8, and 16 mg Se per 1 g of biomass added twice a day). Values were measured in triplicate and expressed as mean ± SD. The letters above the columns (lower row, for SeMet; upper row, for SeCys) show the statistical difference in the mean values of the groups as obtained by analysis of variance (ANOVA) with the Tukey HSD test as a post hoc comparison (identical letters indicate nonsignificant results; different letters denote a statistically significant result).

In comparison to the phototrophic growth, heterotrophic *Chlorella* cultures tolerated higher Se concentrations. In the 0.5, 1, and 4 mg Se treatments, growth was comparable to that of the Control and reached its maximum of ∼15 g DW L^{-1} on day 4 (Figure 1B). In the 16 mg Se treatments, the cultures started to significantly reduce their growth from day 3 (Tukey HSD test $p < 0.01$), and the cultures in the 8 mg Se treatment were affected from day 4. The growth trends were similar to glucose consumption (a measure of culture activity) as a significant reduction was recorded on day 4 for the 16 mg Se treatment (Tukey HSD test $p < 0.01$, Figure 1D). Another sign of Se toxicity was the fact that the cultures treated with 4, 8, and 16 mg Se g^{-1} DW started to be reddish on day 4 and produced a specific, garliclike smell, as in the phototrophic experiments.

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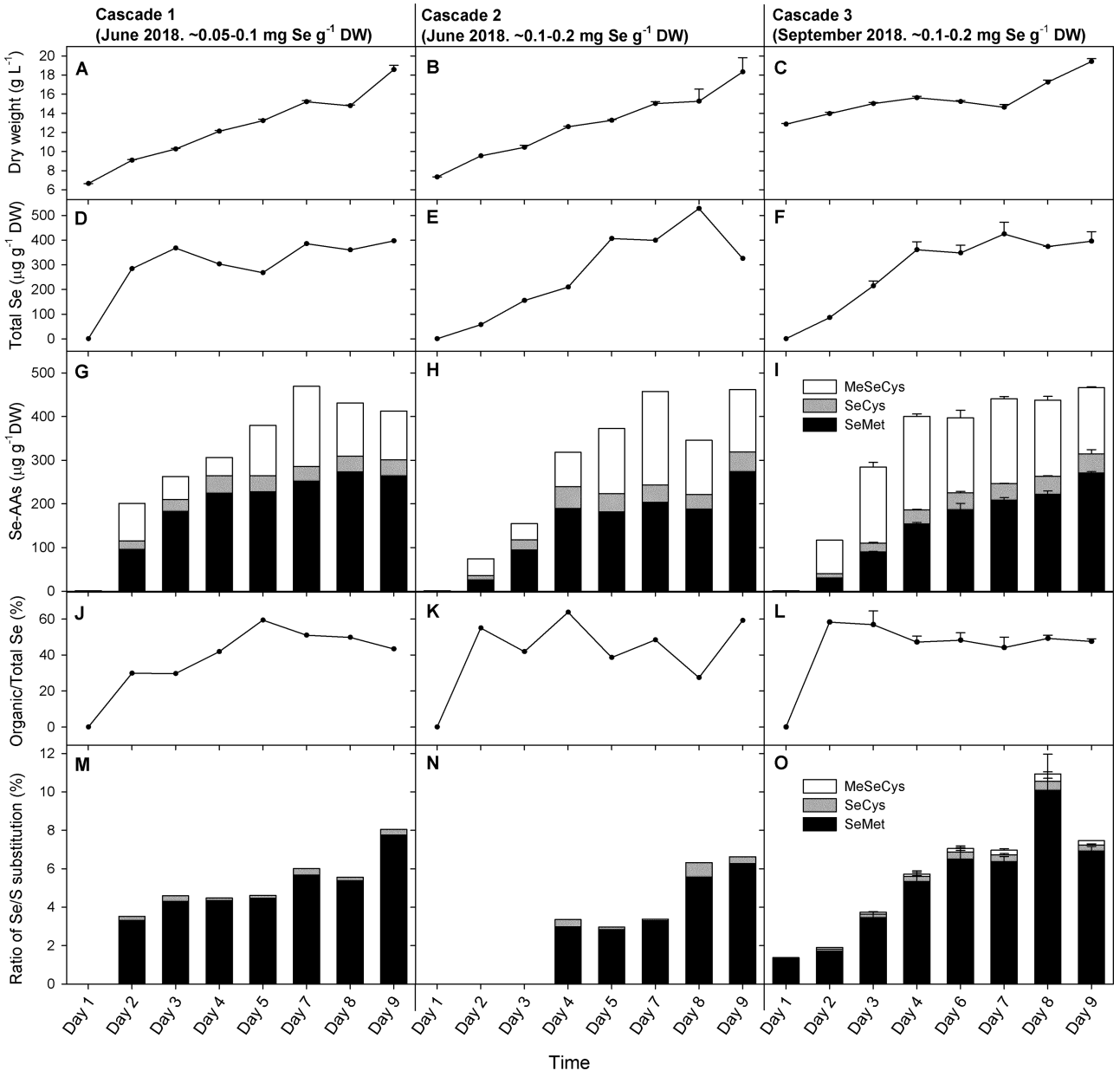

Figure 3. Growth (A–C), total Se (D–F), and Se-AAs (G–I) content, the ratio of organically bound to total Se (J–L), and the ratio of Se/S substitution (M–O) in *Chlorella* biomass grown in outdoor thin-layer cascades and treated with various Se dosages. For some of the measurements, values were recorded in triplicates (A, B, C, F, I, L, O) and were expressed as mean ± SD; for the rest of the measurements, there was only one repetition.

After assessing the growth experiments, we evaluated the total Se and Se-AAs accumulation. The total Se accumulated in the biomass was proportional to the Se dosage. In phototrophic experiments, it increased linearly with the time of cultivation and reached maxima of ~400, ~850, ~6000, and ~10 000 μg g^{-1} DW in the 0.5, 1, 4, and 8 mg Se treatments, respectively (Figure 2A). It is apparent that the total amount of accumulated Se increased with time even in the nongrowing culture, suggesting that adsorption may play a role in this process. This is in agreement with evidence that selenite is strongly bound to the organic surfaces.[1,4]

In the phototrophic regime, a majority of organic Se was bioaccumulated in the form of SeMet followed by SeCys and MeSeCys (Figure 2C). This proportion of the Se-AAs in Se-treated microalgae biomass was in agreement with previous studies.[3,8] In contrast to the total Se content, Se-AAs content in the biomass cultivated in the phototrophic regime was not proportional to the dosage of the applied selenite (Tukey HSD test, Figure 2). In all Se treatments, the maximal average contents were 80–120 μg g^{-1} DW for SeMet, 10–40 μg g^{-1} DW for SeCys, and 2–20 μg g^{-1} DW for MeSeCys on day 4. The ratios of Se/S substitution were similar for particular AAs: ~1.7–3% for SeMet and SeCys for all Se treatments (Figure 2G). For MeSeCys, occurring only at lower dosages, it was ~1.5%. Interestingly, SeMet concentrations were gradually increasing with cultivation time, whereas the levels of SeCys and MeSeCys were variable.

Despite the fact that the total content of Se-AAs was comparable regardless of the applied dosage, the dynamics of the Se-AAs accumulation differed with faster onset of

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accumulation in the 4 and 8 mg Se treatments on days 2 and 3, respectively. Surprisingly, even nongrowing cultures treated with these doses significantly increased their Se-AAs amount, as for example the total Se-AAs content increased ∼3 times between days 2 and 3 for the 8 mg Se treatment, while no growth was observed in this culture. The amount of organoselenium coming from the measured Se-AAs inside the pool of total Se in the biomass was the highest in the 0.5 and 1 mg Se treatments: up to ∼24 and ∼14% on day 3, respectively (Figure 2E); it started to decrease on the last day due to the high increase in total Se by the end of the experiment. This result is a crucial criterion for the selection of Se dosage for larger-scale cultivations.

In heterotrophic cultivation, the total Se accumulated in the biomass was proportional to the Se dosage and increased linearly with time similar to phototrophic cultures. It reached its maxima of ∼400, ∼600, ∼3700, ∼8300, and 11 300 μg g^{-1} DW for the dosages of 0.5, 1, 4, 8, and 16 mg Se g^{-1} DW, respectively (Figure 2B). During the whole cultivation period, the majority of organic Se was accumulated in the form of SeMet and less in the form of SeCys for all conditions, and by day 4, it had reached its maximum. Here, the accumulation of SeMet and SeCys was clearly dependent on the amount of the applied selenite (Tukey HSD test, Figure 2). The quantities of SeMet and SeCys increased in the range of dosages from 0.5 to 4 mg Se g^{-1} DW treatment (Figure 2D). The application of higher doses reduced the level of accumulation. The optimal dose of 4 mg Se g^{-1} DW yielded high concentrations: ∼300 μg g^{-1} DW and ∼100 μg g^{-1} DW for SeMet and SeCys, respectively, which is at least 3 times higher than the maxima obtained in the phototrophic cultures. In heterotrophic cultures, MeSeCys was present in a very low amount of ∼5 μg g^{-1} DW. In the 4 and 8 mg Se-treated cultures, the ratio of Se/S substitution was the highest: about 5% for SeMet and SeCys, whereas it was only ∼0.3% for MeSeCys at the end of the experiment (Figure 2H). The values for SeMet and SeCys were twice those obtained in the phototrophic cultures. Finally, the portion of organoselenium bound in Se-AAs in the total Se pool for the heterotrophic regime reached up to 14% for the 0.5 and 1 mg Se treatments (Figure 2F). In all other treatments, it was substantially lower.

Large-Scale Phototrophic Production of Se-Enriched *Chlorella* Biomass in Outdoor Thin-Layer Cascades. For further cultivations in both pilot and large scales, it was decided to use 0.5 mg Se g^{-1} DW dose for two reasons.

(i) In the 0.5 mg Se treatment, the same growth rates were measured as in the control cultures in both photo- and heterotrophic regimes. In phototrophic cultivation, we found similar Se-AAs accumulation and rates of Se/S substitution as for other applied doses, providing similar results as the dose 0.5 mg Se g^{-1} DW in the heterotrophic regime.

(ii) The biomass treated with a low dose of 0.5 mg Se g^{-1} DW had a more favorable ratio between the organoselenium bound in the measured Se-AAs and total Se. This is beneficial for long-term cultivations and at the same time reduces the risk of high content of inorganic and potentially toxic Se forms found in higher doses. For example, in the heterotrophic biomass treated with 4 mg Se g^{-1} DW, we detected the highest Se-AAs accumulation, but the portion of organoselenium was found to be only ∼5% due to the high total Se values.

Accordingly, we planned Se incorporation in large-scale trials using outdoor thin-layer cascades. We carried out two independent trials of Se treatments in *Chlorella* cultures: Cascade units 1 and 2 with Se added were run in parallel with the Control unit in June, and trial 2 in Cascade 3 with Se added was run in September 2018. All cascades were operated at volumes of 2200 L. The average irradiances were 582 and 697 μmol photons m^{-2} s^{-1} during the trials in June and September, respectively (the course of ambient irradiance and temperature from sunrise to sunset is shown in Figure S2). The fluorescence variables at the I-step and J-step were high at the start of the experiment as the cultures were not light acclimated—high V_j and V_i showing high plastoquinone pool reduction, and then, it varied depending on the ambient irradiance (Figure S3C,D). The daily temperatures fluctuated largely from 10−15 °C up to 34 °C at midday during both trials (Figure S2C,D).

Cascade 1 was initially treated with a dosage of 0.5 mg Se g^{-1} DW once a day, based on the results of laboratory experiments (Figure 3). After two Se additions (15.7 and 24 g of Na_2SeO_3 equivalent to ∼7 and ∼11 g of Se per cascade), the microalgae culture started to produce a significant garliclike smell as described previously,[3,4,27,34] and a reddish foam was apparent. The measurement of photosynthetic activity indicated the stress caused to the culture by Se. The maximum photochemical yields of PSII, F_v/F_m, relative maximum electron transport rate $rETR_{max}$, and maximum oxygen evolution remarkably decreased on day 2 in Cascade 1 compared to the Control: F_v/F_m: 0.71 vs 0.75; ETR_{max}: 121 vs 252; maximum oxygen evolution: 27 μmol O_2 μg (Chl)$^{-1}$ h^{-1} vs 191 μmol O_2 μg (Chl)$^{-1}$ h^{-1} (Figure S3A,B,E). This treatment was evidently inhibiting and so we were compelled to lower the Se dose. From day 3, the photosynthetic activities of the culture in Cascade 1 recovered back to the control level (Figure S3A). During further cultivation trials, we added 5 g of Na_2SeO_3 twice a day to Cascade 1, corresponding to 0.05−0.1 mg of Se per g DW, after taking into account the increasing biomass. After the reduced Se addition, the volatile compounds were produced during cooler days with lower irradiation (Figure S2), as the Se was not incorporated into the biomass. Because of low atmospheric pressure occurring on some days, the absence of wind, lower temperature, and irradiance, volatile compounds were released to the environment from the cultures. It was observed that their production was significantly decreased in photosynthetically active, well-growing cells. On day 4, there was a significant temperature decrease that remarkably influenced photosynthesis (measured as the maximum photochemical yield of PSII, F_v/F_m, and the maximum electron transport rate ETR_{max} (Figure S3A,B)). The ambient temperature was variable, and not always optimal, during the trial, which was reflected in the courses of measured variables (Figure S3). Nevertheless, *Chlorella* R117 is a robust strain tolerating a wide temperature range, and, therefore, the biomass density reached about 20 g DW L^{-1} on day 9 in the Cascade 1 and 2 cultures as well as the Control (Figures 3A−C and S4), with the only course of the growth curves proving slightly different (Figure S4). The growth of the culture in Cascade 3 was slower as it was cultivated in September when the day was shorter (Figure S4).

To better reproduce the dosage set by the laboratory experiments, the cultures in Cascades 2 and 3 were operated with addition of 10 g of Na_2SeO_3 twice a day (Figure 3), corresponding to 0.1−0.2 mg Se g^{-1} DW during the

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cultivation, taking into account the changing concentration of the biomass. Total Se concentration was different for individual cultures because of the way of Se additions as described above: it rapidly reached ~300 μg g^{-1} DW in the Cascade 1 culture on day 2 and stayed in the range between 270 and 400 μg g^{-1} DW until the end of cultivation (Figure 3D); however, for cultures in Cascades 2 and 3, it steadily reached about 400 μg g^{-1} DW on days 5 and 4, respectively, and remained in the range between 330 and 530 μg g^{-1} DW until harvesting (Figure 3E,F).

In outdoor large-scale cultures, higher rates of Se-AAs accumulation were detected as compared to laboratory experiments. The concentrations were in the range of 180−280 μg g^{-1} DW for SeMet, about 30−50 μg g^{-1} DW for SeCys, and 40−215 μg g^{-1} DW for MeSeCys starting from day 4 of cultivation for all of the cascades (Figure 3G−I). The biomass cultivated in thin-layer cascades showed a substantially higher ratio of Se/S substitution, in particular AAs (Figure 3M−O), compared with the laboratory cultures (Figure 2G). They were in the range of 5−10% for SeMet, 0.1−0.7% for SeCys, and 0−0.2% for MeSeCys (Figure 3M−O). The fraction of organo-selenium coming from the measured Se-AAs inside the pool of total Se in the biomass reached the maximum of 60% (Figure 3J−L). The difference in the dynamics of Se-AAs accumulation was observed between the cascades due to different Se treatments: the Se-AAs levels rapidly reached a plateau in the Cascade 1 culture, and a gradual increase was recorded for Cascades 2 and 3. The ratio of organically bound to total Se in the biomass had various trends in the cultures: in the Cascade 1 culture, this variable grew continuously, whereas for the cultures in Cascades 2 and 3 it grew rapidly, and after reaching a plateau, the ratio remained in the range of ~30−60% for all cascades (Figure 3J−L).

Total Se and Se-AAs content as well as the portion of organic Se and rates of Se/S substitution in harvested and processed biomass is available in Figure S5.

Pilot-Scale Cultivation for Production of the Se-Enriched *Chlorella* Biomass in the Heterotrophic Regime. Most of the previous studies found in the literature have been focused on the development of phototrophic cultivation of microalgae for Se-enriched biomass production.[3,4] Limited information has been available on Se assimilation by microalgae cultivated under heterotrophic conditions. Doucha et al. showed that Se was accumulated in *Chlorella* biomass under the heterotrophic regime but without specifying amounts in distinct organic species.[10]

One series of experiments was aimed at performing pilot-scale heterotrophic cultivation in a fermentor to study Se and specifically Se-AAs accumulation. Based on the laboratory-scale screening, we found that addition of 0.5 mg Se g^{-1} DW of biomass twice a day (1 mg of Se per gram DW per day in total) did not have any inhibiting effect on microalgae growth, although high amounts of Se-AAs were accumulated (Figures 1B and 2D). As the initial trials showed that the fed-batch regime led to a higher accumulation of Se-AAs (about 85 μg g^{-1} DW) compared to the batch mode (about 60 μg g^{-1} DW), we used the former regime in cultivation trials. By the end of cultivation (day 6), a biomass density of ~25 g DW L^{-1} had been achieved (Figure 4A) and a total Se content of about 2000 μg g^{-1} DW (Figure 4B). Of the total Se content in the biomass, about 430 μg g^{-1} DW (22% of the total Se) was found incorporated in SeMet and about 115 μg g^{-1} DW (about 6% of the total Se) in SeCys (Figure 4C). The ratio of Se/S

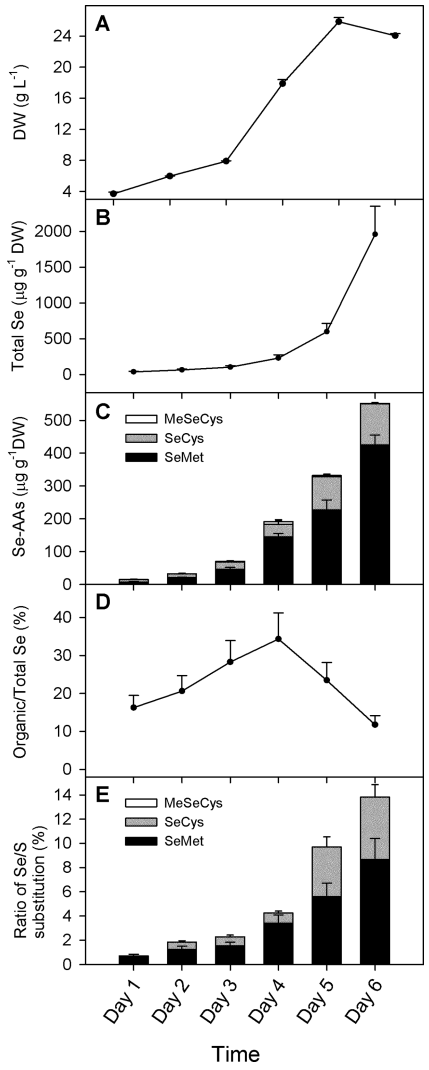

Figure 4. Growth (A), total Se (B), and Se-AAs content (C), the ratio of organically bound to total Se (D) and the ratio of Se/S substitution (E) in *Chlorella* biomass grown in the pilot scale in a heterotrophic fermentor. The amount of Se added was 0.5 mg per 1 g of biomass twice a day. Values were measured in triplicate and were expressed as mean ± SD.

substitution was approximately 9% for SeMet and 5% for SeCys on day 6 (Figure 4E). The MeSeCys concentration was very low with a maximal amount of about 8 μg g^{-1} DW on day 4 (Figure 4C). The maximum fraction of organoselenium found in Se-AAs inside the pool of the total Se in the biomass was up to 36% on day 4 (Figure 4D). The accumulation of Se in the heterotrophic culture was different from the outdoor phototrophic cultures in several aspects. First, the amount of total Se in the biomass was 4 times higher in heterotrophic cultures due to a faster growth rate, which then allowed the addition of higher Se doses. Second, the total amount of accumulated Se-AAs was significantly higher in heterotrophic cultures compared to the phototrophic ones. About 1.5 times and 3 times higher SeMet (t-test, $p < 0.01$) and SeCys concentrations (t-test, $p < 0.01$), respectively, were found in heterotrophic cultures at the end of cultivation compared to phototrophic ones. On the contrary, no accumulation of MeSeCys was found in the heterotrophic regime, although in the phototrophic regime, it formed a significant proportion of

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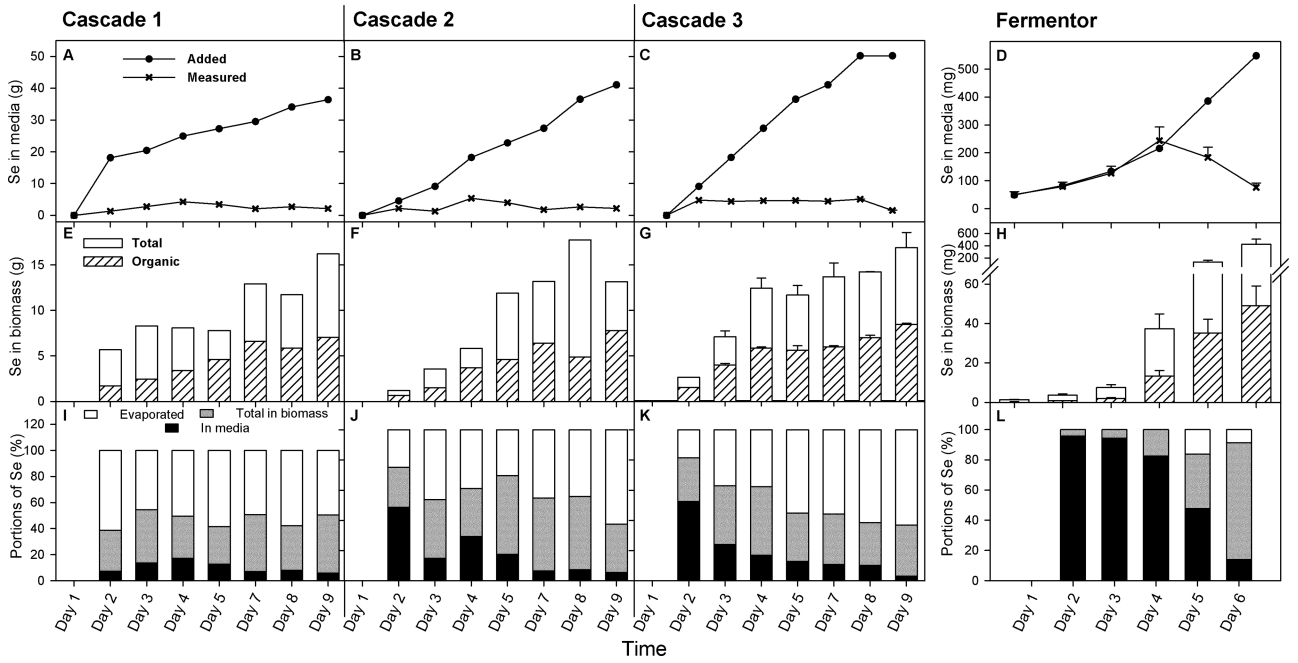

Figure 5. Se concentrations are shown as lines with circles representing the amount of Se added to the medium at individual time periods; lines with crosses are actual measured concentrations in the medium after cell harvesting (A−D). Se concentrations in biomass samples of individual trials: organically bound Se in Se-AAs constitutes a part of the total Se amount (E−H). Distribution of Se during the cultivation process in phototrophic large-scale or heterotrophic pilot-scale cultures (I−L). Percentage of the total Se found in biomass and media was calculated from analyses, and the difference to 100% was attributed to evaporated Se.

all of the Se-AAs (~50%). Finally, the ratio of Se/S substitution was similar for SeMet in both of the regimes, whereas for SeCys it was ~7 times higher in the heterotrophic culture. The ratio of organoselenium bound in Se-AAs inside the pool of the total Se in the biomass was about 1.5-fold lower in the heterotrophic regime as compared to phototrophic cultures. In contrast to the laboratory experiments in the heterotrophic regime, the pilot-scale cultivation had a higher amount of Se-AAs and ratio of Se/S substitution, which can be explained by the faster growth, resulting in a higher biomass production.

Balance of Se Intake during the Cultivation. The final experiment evaluated the balance of Se incorporation into the *Chlorella* biomass during the photo- and heterotrophic cultivation processes. The total Se amount added to the cultivation systems was calculated based on the sodium selenite additions and represents the sum of all applied doses over time (Figure 5A−D). In phototrophic large-scale cultivation, this amount was more or less linearly increasing over time in all of the cascades, reaching the total amount of 30−50 g per unit; only Cascade 1 was nonlinear at the beginning of cultivation due to the higher initial additions (Figure 5A−C). The total amount of Se that remained in the medium at the end of the cultivation on day 9 was about 2 g, representing 3−6% of the total Se added (Figure 5A−C,I−K). The concentration of Se in media fluctuated widely during the cultivation process from 3.2 to 48% of the total selenium added. It was especially high during the first three days after the start of cultivation, but for most of the time, it was up to 10% (Figure 5I−K). The amount of Se incorporated in the biomass increased over the period of cultivation, reaching about 15 g by the end of cultivation in all cascades (Figure 5E−G). When this amount was expressed proportionally to the total Se added, the value fluctuated from 26 to 52% during cultivation and it formed 32−44% at the end of the cultivation process (Figure 5I−K). Having determined the Se distribution, it can be assumed that the rest of the Se added to the cultivation system was transformed to volatile compounds such as dimethylselenide and dimethyldiselenide and evaporated. From the total balance, it is apparent that at the end of the cultivation, the amount of volatile Se compounds formed 50−63%, and this fluctuated over individual time points from 18 to 63% (Figure 5I−K).

In summary, we can conclude that 94−96% of the Se dose was metabolized by microalgal cells through calculating the content of Se-AAs and potentially released volatile Se compounds that are formed intracellularly. The high rates of volatile Se compound formation in the phototrophic outdoor mass cultures are in agreement with the high concentrations of MeSeCys, a precursor of the volatile form dimethyldiselenide, present in the *Chlorella* biomass (see above).

Under heterotrophic conditions, the overall Se distribution was different (Figure 5D,H,L). In this case, high actual Se concentration in the media was detected during the whole cultivation process, ~50−94% of the total amount applied, eventually dropping to ~13% at the end of the cultivation (Figure 5D,L). The majority of the applied dose was incorporated into the biomass (~77%). In contrast to phototrophic outdoor cultures, only a small portion was evaporated in the volatile forms (~9% at the end of fermentation; Figure 5L), which is in agreement with the low concentration of MeSeCys, a precursor of dimethyldiselenide, detected in the heterotrophic biomass. This suggests that selenocysteine methyltransferase, the enzyme that converts SeCys to MeSeCys, is not very active during heterotrophic cultivation.

Potential Increase of Organic Se Content in Se-Enriched *Chlorella*. Our study has shown several important

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facts about the enrichment procedure of the *Chlorella* biomass by nutritionally valuable Se-AAs in pilot- and large-scale cultivations. In the ideal case in outdoor large-scale cultivations, the cultures should grow comparably to the controls and have similar or higher values of Se-AAs accumulation and ratios of Se/S substitution as in the laboratory-scale experiments. These prerequisites were successfully achieved in our study in both photo- and heterotrophic regimes. Despite the fact that the amounts of Se-AAs accumulated in *Chlorella* biomass were higher than the majority of Se-enriched foodstuffs,[8] we can assume that there still exists a potential for optimization of the cultivation process to enhance its affectivity.

With regard to the phototrophic mass cultures, our data clearly show that the maximum Se-AAs accumulation occurs at the early stage of cultivation (on days 4−6). Based on this result, we can assume that an even shorter Se treatment of a pregrown culture of higher cell density may result in the same degree of Se-AAs enrichment but in a shorter time, thereby reducing cultivation costs. In the heterotrophic cultures, the maximum Se-AAs accumulation was detected during active growth. Therefore, it is likely that the prolonged fed-batch cultivation would have resulted in a higher biomass yield and even higher Se-AAs accumulation. Maximal biomass densities obtained in our experiments were roughly 25 g DW L^{-1}; however, it is reported that heterotrophic *Chlorella* cultures can reach densities up to 100 g DW L^{-1}.[10,35] Finally, in both of the regimes, a lower dose of Se (e.g., continuous supply of Se solution to the cultivation units using peristaltic pumps) might improve Se bioaccumulation as the microalgae will be able to metabolize lower and potentially less toxic inorganic Se concentrations during their active growth phase.[36]

The predominant Se-AA generated in both regimes was SeMet: maximal contents were ∼275 μg g^{-1} DW and ∼430 μg g^{-1} DW for phototrophic and heterotrophic regimes, respectively. While the second most abundant form in phototrophic biomass was MeSeCys ∼215 μg g^{-1} DW, it was SeCys in heterotrophic cultures (∼115 μg g^{-1} DW). The overall organic Se content might be even higher, as it was shown by other studies that a significant Se portion can be distributed between lipids and other organic molecules.[20] To conclude, our data show that both photo- and heterotrophically produced *Chlorella* provide biomass with one of the highest contents of Se-AAs. The only known higher accumulation of Se-AAs was determined in yeast, accumulating up to ∼3200 μg g^{-1} DW of SeMet.[17,18] On the other hand, our previous study has shown that the disintegrated and spray-dried *Chlorella* biomass possesses one of the highest Se-AAs bioaccessibility of all tested products.[8] The data produced in this study allowed us to calculate the recommended daily intake of Se to be equivalent to about 0.61 g of phototrophic Se-enriched *Chlorella* biomass per person.[8] This is a much smaller amount than the required intake from naturally available Se-rich foods (∼130 g), commercial Se-enriched food supplement (∼5.1 g),[8] and biofortified Se products (1.12 g of florets or 7.2 g of sprouts for broccoli, ∼300 g of potatoes, and ∼1300 g of rice in g of fresh weight of each item of product per person per day).[13,15,16] Only selenized yeast could be consumed in smaller amounts (∼0.18 g), but organic Se from this source is less bioaccessible (∼20 vs ∼50% for Se-enriched *Chlorella*).[8]

Moreover, some organic Se species have not just nutritional importance. For example, MeSeCys[15] and methylselenol, derived from SeMet,[3,37] were reported to have anticancer activity.[15] Our study has shown that phototrophically grown Se-enriched *Chlorella* is a good source of MeSeCys and SeMet, whereas heterotrophically grown biomass is rich in SeMet. Moreover, *Chlorella* biomass contains proteins (up to 58% of dry matter), carbohydrates (12−17%), lipids (14−22%), essential amino acids, unsaturated fatty acids, and carotenoids (mainly lutein and violaxanthin), as well as some vitamins and minerals.[38]

Interestingly, production costs of *Chlorella* biomass largely depend on the cultivation system, operation volume, and downstream processing,[35] roughly ranging between 10 and 40 Euros per 1 kg, which makes it definitely higher when compared to natural sources of Se. However, when compared to the total amount of organic Se, it is higher in Se-enriched *Chlorella* than in naturally Se-rich or Se-biofortified food, except selenized yeast.[8,13,15−18] In terms of organic Se bioaccessibility, nowadays *Chlorella* biomass is one of the best among available foods and feeds, competing in this sense even with selenized yeast.[8]

Moreover, in this respect, our finding about the high accumulation of Se-AAs in the heterotrophic regime is very relevant as it allows the possibility of a permanent cultivation process, which can be further scaled up, to reduce production costs.[36]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.9b06196.

Fluorescence variables measured in phototrophic cultures treated with different selenium concentrations in the laboratory scale (Figure S1); weather report (Figure S2), selected fluorescence variables (Figure S3), and growth curves (Figure S4) of *Chlorella* cultures grown in outdoor thin-layer cascades; total selenium and selenoamino acids content during downstream processing of harvested *Chlorella* biomass grown in outdoor thin-layer cascades (Figure S5) (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AAs, amino acids; DW, dry weight; GC-APCI-HRMS, gas chromatography in combination with atmospheric pressure chemical ionization high-resolution mass spectrometry; HPLC-ICP-MS, high-performance liquid chromatography connected to inductively coupled plasma mass spectrometry; ICP-MS, inductively coupled plasma mass spectrometry; ME, mercaptoethanol; MA, methanesulfonic acid; MeSeCys, methylselenocysteine; OJIP curve, fast fluorescence induction kinetics; POE/R, photosynthetic oxygen evolution and respiration; RLC, rapid light-response curve; Se, selenium; Se-AAs, selenoamino acids; SeCys, selenocysteine; SeMet, selenomethionine; S, sulfur

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