Li, J., Lens, P. N. L., Ferrer, I., Du Laing., G. (2021) Evaluation of selenium-enriched microalgae produced on domestic wastewater as biostimulant and biofertilizer for growth of selenium-enriched crops. Journal of Applied Phycology, 33(5), 3027–3039.

Evaluation of selenium-enriched microalgae produced on domestic wastewater as biostimulant and biofertilizer for growth of selenium-enriched crops

Jun Li[1,2], Piet N. L. Lens[3], Ivet Ferrer[2], Gijs Du Laing[1]

[1] Laboratory of Analytical Chemistry and Applied Ecochemistry, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium.

[2] GEMMA-Group of Environmental Engineering and Microbiology, Department of Civil and Environmental Engineering, Universitat Politècnica de Catalunya·BarcelonaTech, Jordi Girona 1-3, Building D1, 08034 Barcelona, Spain

[3] UNESCO-IHE Institute for Water Education, 2601 DA Delft, The Netherlands.

Corresponding Author: Jun Li

Jun.Li@UGent.be

Li, J., Lens, P. N. L., Ferrer, I., Du Laing., G. (2021) Evaluation of selenium-enriched microalgae produced on domestic wastewater as biostimulant and biofertilizer for growth of selenium-enriched crops. Journal of Applied Phycology, 33(5), 3027–3039.

Abstract

This study assessed selenium (Se)-enriched microalgae biomass produced in a pilot-scale raceway pond treating domestic wastewater and an extract thereof as biostimulant and biofertilizer. After producing the Se-enriched microalgae in a raceway pond treating domestic wastewater, the effect of Se-enriched microalgae biomass and an extract thereof on seed germination, growth and yield of beans (*Phaseolus vulgaris* L.) was studied by conducting a germination test as well as foliar and soil applications in pot experiments. Furthermore, the potential of the Se-enriched microalgae dry biomass and extract to elevate the Se concentration of beans, leading to a biofortified crop, was also assessed in a pot experiment. Presoaking seeds in the Se-enriched microalgae extract at low concentration (1%) enhanced their germination, as measured by the significant increase of seedling length and vigor index. Application of the Se-enriched microalgae extract as foliar spray was more effective in stimulating the growth of beans and increasing the Se concentration in the seeds compared to its application as soil drench. Foliar spray resulted in a 3.5 times increase of the dry biomass of the seeds (at 1%) and 1.8 times Se increment in the seeds (at 5%). Additionally, amendment of the soil with Se-enriched microalgae biomass (at 5%) enhanced the growth of beans (3.2 times for seeds) and Se concentration in the bean plants (1.8 times for seeds), simultaneously. These results indicate that microalgae cultivated in Se-rich wastewater could be used as a microalgae-based biofertilizer or biostimulant to improve the bean seeds yield as well as the Se content in the beans, leading to beans with a higher market value. This may also offer an environmental-friendly and sustainable approach to biofortify food crops in Se-deficient regions.

Keywords: Algae, Fertilizer, Biofortification, Beans, Selenium, Wastewater

1. Introduction

Selenium (Se) is an essential trace element of high importance for human and animal health because of its incorporation into selenoproteins, which have a wide range of functions from antioxidant and anti-inflammatory roles to the production of active thyroid hormone (Zhang et al. 2020; Rayman 2000). Despite the importance of Se, Se deficiency exists worldwide, resulting in negative health effects and even causing Se-deficiency diseases, e.g. endemic Keshan disease in China (Tan et al. 2002; Wu et al. 2015). It is estimated that approximately 15% of the World's population is still Se-deficient (Poblaciones and Rengel 2017) and even over 1 billion people may consume less Se than required for optimal protection against cancer and cardiovascular disease (Haug et al. 2007). The low dietary Se intake is generally associated with the consumption of food containing a low Se content, usually due to the low Se concentration in the soils on which the crops are grown. Biofortification, i.e. the dietary supply of Se through its enrichment in food and feed crops, is being explored as a possible solution for Se deficiency (Boldrin et al. 2013; Li et al. 2020). However, the adverse effects of applying conventional inorganic Se fertilizers on soils and the environment is leading to the exploration of alternative Se fertilizers.

Microalgae can be cultivated in wastewater and agricultural runoff, recovering excess nutrients, while reclaiming the wastewater (Garcia-Gonzalez and Sommerfeld 2016; Gan et al. 2019). The generated microalgae biomass with high nutrient content is not only considered as a valuable ingredient for food and animal feed (Li et al., 2021), but has also a potential as biofertilizer or biostimulant (Ronga et al. 2019). Nowadays, the use of microalgae in agricultural production as biofertilizer or biostimulant is attracting the interest of growers and agrochemical industries aiming to improve the sustainability of crop production (Grzesik and Romanowska-Duda 2014; Ronga et al. 2019; Calvo et al. 2014). Biostimulants are substances that are able to enhance physiological processes and stimulate the growth of plants under both optimal and stressful conditions after being applied to the plants or soils. Biofertilizers are bioproducts that deliver nutrients for plant growth, thus stimulating plant growth (Ronga et al. 2019).

Microalgae biomass contains several plant growth-promoting substances, such as phytohormones, vitamins, carotenoids, amino acids and antifungal substances

(Coppens et al. 2015), which could serve as potential biostimulant. A few studies have established an association between greater crop yields and the application of microalgae or microalgal cellular extracts as biostimulant or biofertilizer. For instance, the addition of the microalga *Chlorella vulgaris* to soil (2-3 g dry algae kg^{-1} soil) significantly increased the fresh and dry weight of lettuce (Faheed and Abd-El Fattah 2008). The application of 1.5 L ha^{-1} of *Spirulina* extract on the field has been found to significantly raise the number of grains in ear and shank length of wheat (*variety Akteur*) (Michalak et al. 2016). Similarly, the microalga *Acutodesmus dimorphus* dry biomass and its cellular extracts triggers faster germination and enhances the plant growth and floral production of Roma tomato (Garcia-Gonzalez and Sommerfeld 2016). The use of microalgae biomass as a slow-release biofertilizer resulted in a higher quality of tomatoes with increased carotenoid and sugar levels (Coppens et al. 2015).

When producing Se-enriched microalgae during wastewater treatment, the produced microalgae may not only be used as biofertilizer or biostimulant to improve crop growth, but also to biofortify the crops by enriching them with Se. Therefore, we explored whether Se-enriched microalgae biomass produced in raceway ponds from domestic wastewater and an extract thereof can be potentially used as an organic nutrient-rich biofertilizer or biostimulant in biofortification strategies to enhance the crop yield, meanwhile improving also the Se levels in the crops. This approach may be beneficial to save Se resources and avoid the introduction of chemicals contamination into the environment in biofortification schemes through the replacement of synthetic chemical fertilizers by Se-enriched microalgae biomass or its extract.

To this end, the influence of the produced Se-enriched microalgae dry biomass and an extract thereof on the germination, growth and yield of green beans (*Phaseolus vulgaris* L.) was assessed, along with the Se concentration in the beans. This is the first study to assess the application of Se-enriched microalgae biomass and an extract thereof as Se biofertilizer and biostimulant to biofortify green beans.

2. Materials and methods

2.1 Wastewater treatment and Se-enriched microalgae production in a pilot-scale raceway pond

Se-enriched microalgae were produced in a pilot-scale raceway pond located outdoors at the laboratory of the GEMMA Research Group (Universitat Politècnica de Catalunya, Barcelona, Spain) (Fig. 1). The system treated real municipal wastewater that received a screening pre-treatment before being pumped into a homogenization tank (1 m^3). The wastewater was then pumped from this tank into a primary settler (3 L of volume, 41 min of hydraulic retention time (HRT)) followed by a raceway pond (0.47 m^3 of volume, 1.54 m^2 of surface area, 0.3 m of depth) with continuous spiking of 500 µg L^{-1} Se, in the form of sodium selenite. The HRT of the raceway pond was 8 days in Spring (1 May – 15 June 2019) and 4 days in Summer (15 June – 31 July 2019), over an experimental period of 3 months. A secondary clarifier (3.3 L of volume, 46 min of HRT) followed to separate the microalgae biomass from the secondary effluent.

Qualitative microscopic evaluation of the biomass indicated that the microalgae biomass was dominated by *Chlorella* sp. (Fig. S1, Supplementary Information), which has been previously reported in studies using the same ponds during the same period of the year (Arashiro et al., 2019; Gutierrez et al., 2016).

Water samples after the primary (named influent) and secondary (named effluent) settler of the raceway pond (Fig. 1) were collected twice per week for analysis of the following parameters: pH, dissolved oxygen (DO), turbidity, total and volatile suspended solids (TSS and VSS), total and soluble chemical oxygen demand (COD_t and COD_s), total carbon (TC), total and soluble phosphorus (TP and SP), total nitrogen (TN), nitrite (NO_2^-), nitrate (NO_3^-), ammonium nitrogen (NH_4^+-N) and total Se.

The Se-enriched biomass from the secondary clarifier was collected weekly, thickened by centrifugation and washed twice with deionized (DI) water. The centrifuged paste was frozen at -80 °C overnight, lyophilized and then stored at -20 °C for the subsequential trial. After the entire production experiment, all lyophilized biomass was mixed thoroughly for subsequent experiments.

2.2 Preparation of liquid Se-enriched microalgae extract

The freeze-dried microalgae biomass (15 g) was ground by ball-milling (MM 400, Retsch, Haan, Germany) for 10 min at 30 Hz. The ground biomass was suspended into 90 mL DI water and 10 ml 10% sodium dodecyl sulfate (SDS). The final SDS concentration in the extract was 1%. SD had a significant effect on improving the microalgae extraction efficiency as shown in the previous experiment (Li et al., 2021) and other studies (Mota et al. 2018; Gómez-Jacinto et al. 2012). The suspension was stirred on a stirring plate for 10 min to allow the biomass to dissociate, and then incubated at 100 °C for 5 min to obtain the extract. The hot extract was cooled down at room temperature and centrifuged at 5000 rpm for 10 min to separate the microalgae extract from the biomass residue. The microalgae extract was stored at 4 ºC for further trials. The composition of the Se-enriched microalgae biomass and its extract is shown in Table 1.

2.3 Germination test with Se-enriched microalgae extract

The seeds of French green bean Vesperal (*Phaseolus vulgaris* L.) purchased from a seeds company (Roger De Baerdemaeker BVBA - Het Vlaams Zaadhuis, Lievegem, Belgium) were used for this study. This type of beans could produce seeds in approximately 50 days and the suitable growing season is May-July.

Seeds with uniform shape, size and weight were selected for the germination test. The germination test was conducted as described previously (Garcia-Gonzalez and Sommerfeld 2016; Hernández-Herrera et al. 2014) with minor modifications. Each treatment in Table 2 was replicated three times with 15 seeds per replicate. The seeds were surface-sterilized with 4% sodium hypochlorite solution for 10 min and subsequently rinsed twice with DI water prior to soaking in 10 mL of different concentrations of Se-enriched microalgae biomass extracts (Table 2) for 24 h. After the 24 h soaking, the seeds were placed on 42.5 mm Whatman no. 1 filter papers and then allowed to dry for 12 h at room temperature. The treated seeds were then transferred into 100 mm Petri plates containing moist filter paper (5 mL of DI water) and incubated in an incubator at 27 °C. The filter paper was kept moist by the regular

addition of DI water. Seeds germination was counted daily for one week. The seedling length was monitored every other day.

2.4 Beans production with foliar spray or soil drench of Se-enriched microalgae extract

Non-contaminated soil classified as sandy was collected at a depth of 0–20 cm from a field in Evergem (51°6´57" N, 3°39´40" E), Belgium. The physicochemical properties of the soil were presented in Table S1 (Supplementary Information). The soil was dried, homogenized and passed through a 2 mm sieve mesh. 0.5 kg of the soil was weighed and placed into 10 cm x 10 cm pots.

Bean seeds (*Phaseolus vulgaris* L.) were pre-cultivated in trays with wet vermiculite at 27 ºC for one week to achieve bean seedlings. Five of the bean seedlings were then transplanted into each pot. Potted plants were grown for 6 weeks indoors (at 24 ºC, 53% relative humidity and 100 µmol photons m^{-2} s^{-1} light intensity) with 80% of the water holding capacity. A total amount of 50 mL of different concentrations of the Se-enriched microalgae extract was applied to each pot every week by foliar spray or soil drench, except for the first and last week of the growth period. A 100 mL small spray bottle was used for the foliar application. The concentrations of the Se-enriched microalgae extract were 0%, 0.5%, 1%, 5% and 10%, which were derived from the previous germination test (in the range of 0–100%). This experiment was conducted in triplicate. During foliar application, the soil surface was covered with aluminum foil to prevent spray runoff from coming in contact with the potting soil and thus potentially being taken up by the roots. The bean plants were harvested, washed and separated into different tissues (root, stem, leaf and seed) for biomass and Se concentration analysis.

2.5 Beans production on soil amended with Se-enriched microalgae biomass

Different amounts (0 g (0%), 0.225 g (0.5%), 0.45 g (1%), 2.25 g (5%) and 4.5 g (10%)) of freeze-dried Se-enriched microalgae biomass were mixed thoroughly with 0.5 kg of sandy soil. Five bean seedlings were transplanted into each pot and grown indoors for 6 weeks by maintaining 80% of the water holding capacity as described above. Beans were harvested for determination of biomass weight and Se concentration analysis.

During the growth period, soil pore water was collected twice per week by using Rhizon soil moisture samplers (Eijkelkamp Agrisearch Equipment, Giesbeek, the Netherlands) and analyzed for its total Se concentration in order to evaluate the evolution of the Se release into the soil. pH and total organic carbon (TOC) of the soil pore water were measured before harvest.

2.6 Analytical methods

2.6.1 Characterization of the raceway pond wastewater

The parameters pH, DO, turbidity, TSS, VSS, COD_t, COD_s, TP, SP, NO_2^- and NO_3^- were analyzed according to standard methods (APHA-AWWA-WEF 2012). NH_4^+-N was measured according to the Solórzano method (Solórzano 1969). TC and TN were measured by a N/C-analyzer (multi N/C 2100S, Analytik Jena, Germany) as described by Arashiro et al. (2019). All analyses were conducted in triplicate. Selenium concentration in wastewater was measured using inductively coupled plasma-mass spectrometry (ICP-MS, ELAN DRC-e, PerkinElmer, Waltham, MA, USA) after being filtered by a 0.45-μm syringe PVDF membrane filter.

2.6.2 Characterization of microalgae biomass and its extract

pH and electrical conductivity (EC) of the microalgae extract were measured by using a pH (Orion Star A211, Thermo fisher scientific, Waltham, MA, USA) and electrical conductivity meter. For the determination of element concentrations in the microalgae extract and biomass (Table 1), 2 mL of the microalgae extract or 0.2 g of dry biomass were weighed into a digestion vessel followed by the addition of 8 mL or 10 mL of concentrated ultrapure HNO_3, respectively. The tubes were sonicated for 1 h, then placed in a microwave oven (CEM Mars 6, Matthews, NC, USA) for digestion with the following program: ramp to 180 °C in 25 min and holding for 20 min at 1200 W power. The digested samples were diluted to 50 mL with Milli-Q water and measured using inductively coupled plasma-optical emission spectrometry (ICP-OES, iCAP 7000, Thermo Scientific, Waltham, MA, USA) for all elements included in Table 1, except for Se, which was analyzed using inductively coupled plasma-mass spectrometry (ICP-MS, ELAN DRC-e, PerkinElmer, Waltham, MA, USA).

2.6.3 Determination of selenium concentration in beans and microalgae

For the determination of total Se in plants (beans and microalgae biomass), 0.2 g of dry samples were weighed into a digestion vessel followed by the addition of 10 mL of concentrated ultrapure HNO_3, and further digested as described in section 2.6.2. The digested samples were analyzed after dilution using ICP-MS. Internal standards (10 µg L^{-1} ^{103}Rh and ^{69}Ga) and an external multi-element standard solution were used during ICP-MS analysis. The certified reference materials white clover (BCR-CRM, 6.7 ± 0.25 mg Se kg^{-1}) and sea lettuce (BCR 279, 0.59 ± 0.04 mg Se kg^{-1}) were included in each analytical batch for quality control, resulting in recoveries of 97 (± 7)% and 106 (± 4)%, respectively.

2.6.4 pH and TOC analysis of soil pore water

The soil pore water collected through Rhizon samplers was analysed for pH and TOC. The pH was determined by using a pH-meter (Orion Star A211, Thermo fisher scientific, Waltham, MA, USA). 2.0 mL of soil pore water was diluted with Milli-Q water to obtain 20 ml volume for TOC measurement through a TOC-analyser (TOC-5000, Shimadzu, Tokyo, Japan), as described by Egene et al. (2018).

2.7 Statistical analysis

Statistical differences were identified with the ANOVA and Duncan's multiple comparison tests at 95% confidence interval level using SPSS 20.0. The Shapiro–Wilk test was used to assess the normality of the distribution of residuals in each data set before conducting ANOVA analysis. The germination percentage (GP) and germination index (GI) were calculated as described in Hernández-Herrera et al. (2014):

$$GP = \frac{Number\ of\ germinated\ seeds}{Total\ number\ of\ seeds} \times 100 \quad (1)$$

$$GI = \sum(\frac{G_t}{T_t}) \quad (2)$$

Where G_t is the number of seeds germinated on day t and T_t is the number of days.

The mean germination time (MGT) was estimated according to Ellis and Roberts (1981) and Hernández-Herrera et al. (2014):

$$MGT(d) = \frac{\sum(G_t \times T_t)}{\sum T_t} \quad (3)$$

The seedling vigor index (SVI) was determined by the following formula (Hernández-Herrera et al. 2014; Orchard 1977):

$$SVI = Seedling\ length\ (cm) \times GP \quad (4)$$

3. Results

3.1 Wastewater treatment and selenium removal in the raceway pond

The main parameters in the influent and effluent of the raceway pond over a period of 3 months are summarised in Table 3. The raceway pond system showed high organic matter and nutrients removal efficiencies. The average TSS removal efficienly was 86%. The COD_t and TC removal efficiencies were 70 and 67% throughout the whole experimental period, respectively, and the average TP and NH_4^+-N removal efficiencies were 77 and 93%, respectively. However, it should be noted that the removal efficiency of TN was lower (around 65%) in comparison with that of NH_4^+-N. . The average Se removal efficiency was 44%.

3.2 Effect of Se-enriched microalgae extract on seed germination and growth of bean seedlings

The germination percentage of bean seeds is shown in Fig. 2. Germination occurred in all treatments after 2 days. The Se-enriched microalgae extract showed a slight stimulatory effect on seed germination at low concentrations, but an inhibitory effect at higher concentrations. Specifically, the maximum GP among all treatments was found at the concentration of 1% microalgae extract. Conversely, 5% of microalgae extract had no significant impact on GP in comparison to the control, except for a significant decrease (by 33%, $P \leq 0.05$) at day 3. However, 10-75% of the microalgae extract significantly ($P \leq 0.05$) delayed the bean seed germination, showing a remarkable decline of GP the first 4 days after sowing. It should be noted that the undiluted

microalgae extract (100%) treatment significantly dropped off the GP during the entire germination period.

The microalgae extract had a significant ($P \leq 0.05$) effect on the growth of the bean seedlings (Fig. 3). Similar to its effect on the GP, the microalgae extract stimulated the growth of the bean seedlings at low concentrations (≤ 5%). The highest seedling length was observed for the bean seeds presoaked in 1% of the microalgae extract, which was around 2 times higher than that in the control. The addition of Se-enriched microalgae extract at the concentration range of 10-75% had no significant effect on the seedling length. However, 100% of Se-enriched microalgae extract application significantly ($P \leq 0.05$) decreased the seedlings length on day 2 in comparison with the control, although no obvious difference between them was noted on day 4 (Fig. 3).

Furthermore, seeds presoaked in 1% of microalgae extract showed the maximum GI and SVI (higher than the control treatment for SVI, $P \leq 0.05$), but the shortest MGT (Fig. 4). These results indicate that low concentrations of microalgae extract (≤ 1%) can act as biostimulant for bean seeds germination. Therefore, low concentrations (0.5 and 1%) of microalgae extract were selected for the subsequent pot experiments to further study their effects on the bean plant growth. Besides, taking into account that the function of the microalgae extract might be diluted by the soil or plant after being applied in pot experiments, slightly higher concentrations (5 and 10%) were selected for the subsequent experiments as well.

3.3 Effect of foliar and soil drench application of Se-enriched microalgae extract on the bean plant growth and selenium concentration in the plants

Application as a foliar spray was more effective in influencing the growth of bean plants than the soil drench application (Fig. 5). Bean plants treated with the foliar spray at 1% microalgae extract displayed a significant increase of 65 and 29% ($P \leq 0.05$) in the fresh and dry weight of the whole plants, respectively (Figs. 5a and 5c). An obvious increase in fresh and dry weight of the roots (113 and 51%, respectively) and seeds (364 and 252%, respectively) (Tables S2 and S3) was observed ($P \leq 0.05$). Basically, no significant difference in the total fresh and dry weight of the entire plant was

recorded when beans received the microalgae extract in the soil drench (Fig. 5b and 5d). Analysis of the significant difference in each tissue of the beans showed that 1% of the microalgae extract applied as soil drench resulted in a significant increase (approximately 13%, $P \leq 0.05$) in the fresh and dry weight of bean seeds (Tables S2 and S3). It should be noted that the relatively high concentration of microalgae extract (5 and 10%) did not inhibit the growth of beans through the foliar spray and soil drench application (Fig. 5).

Foliar spray and soil drench of the Se-enriched microalgae extract gradually increased the Se content in the bean plant (Fig. 6). Generally, foliar spray of Se-enriched microalgae extract provided a higher Se content in the leaves and seeds of the bean plants, while soil drench application resulted in a moderate Se content in the roots and stems of the bean plant. Increasing the application of microalgae extract from 0 to 10% by foliar spray increased the content of Se in the leaves, stems and seeds of beans by 6.2, 2.5 and 1.7 times, respectively (Fig. 6a), whereas no significant difference was found in the roots. On the other hand, the application of 10% microalgae extract as soil drench significantly increased the Se content in the roots and stems of the beans by 1.6 and 3.8 times, respectively, in comparison with the control (Fig. 6b), whereas the Se content in the seeds of beans was not significantly different among soil drench application treatments. This indicates a slow translocation of Se from the roots to seeds in bean plants.

3.4 Effect of Se-enriched microalgae biomass on the bean plant growth and selenium concentration in the plants and seeds

3.4.1 Bean plant growth and selenium concentration in the bean plants

Fig. 7 illustrates the fresh and dry weight of the beans grown in soil amended with Se-enriched microalgae biomass. The supplementation of Se-enriched microalgae biomass significantly stimulated the growth of the whole plant, except for the 0.5% Se-enriched microalgae amendment. The highest biomass yield was found at 1% Se-enriched microalgae addition, similarly to the foliar application of microalgae extract in the previous experiment. It gave an increase of 64 and 43%, respectively, in fresh and dry biomass of the whole plant. Besides, among all tissues, the roots and seeds of the

beans were more sensitive to the Se-enriched microalgae supplementation compared to the leaves and stems, as reflected in the significant increase of biomass in the roots and seeds for the 0.5-5% of Se-enriched microalgae supplementation, but absence of a considerable increase in the leaf and stem biomass (Table S4). Approximately 4 times higher seed yields (both fresh and dry weight) were obtained for the 1% Se-enriched microalgae amendment compared to the control.

The Se content in the bean plants depended on the amount of Se-enriched microalgae supplemented to the soil (Fig. 8). The Se content in all tissues of the bean plant raised gradually with the increasing dosage of Se-enriched microalgae amendment (Fig. 8). An increment of Se-enriched microalgae dosage from 0 to 10% increased the Se content of the beans stepwise from 1.05 to 4.15 mg kg^{-1} in the root, 0.12 to 0.34 mg kg^{-1} in the leaf, 0.09 to 0.42 mg kg^{-1} in the stem and 0.10 to 0.28 mg kg^{-1} in the seed.

3.4.2 Evolution of selenium content in the soil pore water during the growth period

The Se content in the pore water of the soil amended with Se-enriched microalgae during the entire growth period of the beans is shown in Fig. 9 and Table S5. Amendment with Se-enriched microalgae from 0 to 10% gradually and significantly increased the Se content in the soil pore water. Specifically, the addition of 0.5, 1, 5 and 10% of Se-enriched microalgae biomass increased the Se content in the soil pore water to 6.89, 9.20, 21.7, 30.9 μg L^{-1} after the first day, which was 3, 4, 9 and 13 times higher than that of the control, respectively. However, after 22 days of growth of the beans, the Se content in the pore water of the soil amended with Se-enriched microalgae at doses below 5% did not show statistically significant differences with the control (Table S5).

For the same amount of Se-enriched microalgae addition, the Se content in the soil pore water significantly declined along with the growth time during the first 22 days and was stable afterwards (Fig. 9 and Table S5). Increasing the growth time from 1 to 22 days reduced the Se content in the soil pore water by 74, 79, 83 and 68% for the 0.5, 1, 5 and 10% amendment dose, respectively.

3.4.3 pH and TOC in the soil pore water

The pH and TOC in the pore water of the soil amended with Se-enriched microalgae were measured at the time of harvest (Table 4). The addition of 10% of Se-enriched microalgae biomass noticeably increased the pore water pH and TOC ($P \leq 0.05$), while other applications did not result in a remarkable difference in comparison with the control, except for the TOC at 5% microalgal amendment. The highest TOC found at the highest application rate was 149 mg L^{-1}, which was 3 times higher than that of the control.

4. Discussion

4.1 Wastewater treatment performance of the raceway pond

The raceway pond presented a good wastewater treatment performance, as shown by the high nutrients and organic matter removal efficiencies, e.g., NH_4^+-N, TP and COD_t removals of 93, 77 and 70%, respectively (Table 3). These results are in accordance with the literature on raceway ponds for wastewater treatment (Arashiro et al. 2019; Gutierrez et al. 2016). The lower removal efficiency of TN in comparison with that of NH_4^+-N was mainly due to the conversion of some NH_4^+-N into NO_3^--N and NO_2^--N (e.g. nitrification), as shown by the higher contents of NO_3^--N and NO_2^- -N in the effluent compared to the influent (Table 3). This was also reported in a previous study using the same raceway pond system (Arashiro et al. 2019). A Se removal efficiency of 44% in the raceway pond was observed, which was similar to the ones reported by Gerhardt et al. (1991), who found an average selenate removal efficiency of 45% in high-rate aerobic (algae)–anoxic (anaerobic bacteria) ponds treating agricultural drainage water over two years.

4.2 Effect of Se-enriched microalgae extract on seeds germination

The results of the germination test demonstrated that presoaking seeds in low concentrations of Se-enriched microalgae extract could stimulate the seeds germination and growth of seedlings (< 5%), while presoaking seeds in the higher concentrations delayed the seeds germination (i.e. 10-100%) and decreased the seedlings length (i.e. 100%) (Figs. 2-4). Previous studies have also evaluated the beneficial effects of microalgae or macroalgae extracts on the germination and growth of different crops (Chiaiese et al. 2018). For instance, Gupta and Shukla (1969) studied

the effect of *Phormidium* species extracts on the growth of rice seedlings and demonstrated that presoaking rice seeds with algal extracts had a markedly beneficial impact on the development of both roots and shoots. The greatest effect was observed with 1 and 5% of algal extracts through ether and water extraction, respectively. Hernández-Herrera et al. (2014) evidenced that tomato seeds (*Solanum lycopersicum* L.) presoaked in 0.2% of *Ulva lactuca* L. and *Padina gymnospora* (Kützing) Sonder extracts showed an enhanced germination rate and greater plumule and radicle length. The study of Kumar and Sahoo (2011) also showed that the application of 20% of *Sargassum wightii* Grev. extract significantly enhanced the germination of wheat seeds (*Triticum aestivum* var. Pusa Gold) and seedlings shoot and root growth.

The significantly lower GP, GI and SVI observed for the bean seeds presoaked in a high concentration (e.g. 100%) of the microalgae extract (Figs. 2 and 4) could be explained by salinity stress, which can be deduced from the high EC (6.10 mS cm^{-1}) and salt content (e.g. Na^+, Ca^{2+}, Mg^{2+} and K^+) of the liquid microalgae extract (Table 1). The osmotic pressure caused by the high salt content would inhibit the seeds ability to imbibe water (Hernández-Herrera et al. 2014; Coppens et al. 2015), resulting in adverse effects on seeds germination and, eventually, seedling growth. Kaveh et al. (2011) evidenced that increasing salinity levels from 2.5 to 10 mS cm^{-1} (EC) delayed the germination percentage and rate, as well as the emergence percentage and rate of all tested tomato species. Likewise, Hernández-Herrera et al. (2014) elucidated that the negative effects of high concentrations of macroalgae extracts on the germination and growth of tomato could be a result of high salinity (around 4.00 mS cm^{-1}). Besides, ion toxicity could also explain the detrimental effects of the highly concentrated microalgae extracts on seeds germination and seedling growth. The high concentration of ions in the non-diluted microalgae extracts, such as Na^+ (616 mg L^{-1}), K^+ (476 mg L^{-1}) and Ca^{2+} (280 mg L^{-1}), can be toxic to the embryo and seedling development (Benlloch et al. 1994).

4.3 Effect of foliar and soil drench application of Se-enriched microalgae extract on bean plants growth and selenium accumulation

The results of this study indicated that the microalgae extract exhibits growth-stimulating activities on beans, as shown by the significant increase of fresh and dry

weight of plants at 1% of Se-enriched microalgae extract application by foliar spray (Fig. 5), which is partially consistent with other studies. Kumar and Sahoo (2011) found that 20% of macroalgae seaweed extract obtained by water boil extraction significantly increased the yield of wheat (*Triticum aestivum* var. Pusa Gold) by 22.86%, measured as dry weight of seeds. Application of 50% of *Chlorella vulgaris* extract by foliar spray resulted in an obvious increment of the yield in wheat (*Triticum aestivum* L. var. Giza 69) of more than 140% over the control (Shaaban 2001a). A substantial increase in the yield of eggplants was achieved by foliar spray of commercial Spirufert® fertilizer (*Spirulina platensis*) (Dias et al. 2016).

The stimulation effects of the microalgae extract on plant growth could be due to the presence of growth-promoting substances such as macro- and microelement nutrients (Table 1), amino acids, vitamins and phytohormones (e.g., cytokinins, auxins and gibberellins) that affect cellular physiology (e.g. cell division and cell elongation) in plants, leading to enhanced growth and crop yield (Hernández-Herrera et al. 2014). Another possibility is the presence of polysaccharides (e.g., carboxylated and sulfated polysaccharides or uronic acids) in the microalgae extract, which can improve plant growth in a similar way to hormones (Hernández-Herrera et al. 2014; Rolland et al. 2002).

Additionally, foliar application of the Se-enriched microalgae extract gradually increased the Se concentration in the leaf, stem and seed of plants, while soil drench application significantly enhanced the Se concentration in the root and stem of the plants (Fig. 6). It should be noted that the Se concentration in all tissues after the application of Se-enriched microalgae extract at doses below 1% showed no significant difference with the control (except for the leaf with foliar spray) (Fig. 6), whereas the highest fresh and dry biomass of beans was observed at 1% of Se-enriched microalgae extract application (Fig. 5). This indicates that the dose of microalgae extract and Se concentration in the extract needs to be carefully determined in order to balance out good nutrition (e.g. Se content) and obtain the highest yield of bean seeds.

4.4 Effect of Se-enriched microalgae biomass application on plant growth, selenium accumulation in plants and selenium concentration in the soil pore water

Similar to the results of bean plant growth upon application of microalgae extracts, the application of microalgae biomass (> 0.5%) also increased the fresh and dry biomass of the bean plants (Fig. 7) and the highest biomass yield was observed at 1% of microalgae biomass amendment. In line with these results, Shaaban (2001b) reported an increase in the dry weight of shoots and roots of maize (*Zea mays* L.) grown in a soil amended with the microalga *Chlorella vulgaris.* The best treatments were 150 and 200 kg algae Fed^{-1} (1 Feddan = 0.42 hectare) (Shaaban 2001b). As aforementioned in growth-stimulation by the microalgae extract, the stimulation effects of the microalgae biomass on beans growth was partially a result of the slow release of macro- and micro-nutrients from the microalgae biomass, particularly N and P, which have the same effects on plant growth as inorganic fertilizers (Mulbry et al. 2005). The applied microalgae biomass was composed of 48% proteins, 32% carbohydrates and 21% lipids (Li et al., 2021), thus enhancing the nitrogen and carbon content upon its application to the soil, leading to an increase in the soil microbial activity and potentially promoting plant growth. The presence of plant biostimulants (e.g. amino acids, polysaccharides and phytohormones) (Kumar and Sahoo 2011) contained in the microalgae biomass could also have contributed to the positive effects on the beans growth.

Besides, the Se concentration in the bean plants was improved in all tissues after microalgae biomass amendment (Fig. 8). However, it should be noted that the Se content in all tissues of beans at 1% of Se-enriched supplement did not significantly differ from that of the control and it was even slightly lower than that of the supplement at 0.5% dosage, which is partially similar to the results observed in foliar and soil drench application of Se-enriched microalgae extract (Fig. 6). This may be related to the greatest amount of biomass being produced at 1% of Se-enriched microalgae biomass/extract addition among all dosages (Figs. 5 and 7), which resulted in a biological dilution of Se in the plant tissues due to the significant biomass increase. This is supported by the higher Se accumulation in beans at 1% of Se-enriched microalgae biomass application in comparison with that of the control among all tissues, except for the stem ($P \leq 0.05$) (Table S6). Taking into account the biomass (Fig. 7) and Se content (Fig. 8) of the bean plants, 5% of dried Se-enriched microalgae biomass was selected as a recommended dosage in practice, which could not only increase the

Se concentration in the seeds of the beans, but also enhance the growth of the bean plants.

The Se content in the soil can indeed be increased by the application of Se-enriched organic materials (Fig. 9), which was also reported in other studies. For instance, Bañuelos et al. (2015) observed that the dose of Se-enriched *Stanleya pinnata* applied was positively correlated to the soluble and bioavailable Se content in soils. The application of Se-enriched wheat (*Triticum aestivum* L.) and raya (*Brassica juncea* L.) straw from 0 to 1% (ratio of straw weight to soil weight) increased the hot water-soluble Se (bioavailable Se) fraction in a sandy-loam soil from 18 μg kg^{-1} to 36 and 79 μg kg^{-1}, respectively (Dhillon et al. 2007). The significant decrease of Se in the soil pore water during the first 22 days of beans growth could be attributed to the fast adsorption of the released Se onto soil or organic matter and to Se uptake by bean plants (Li et al. 2017). Additionally, the Se-enriched microalgae biomass could also be considered as a potential slow-release Se bioproduct because only around 3% of Se in the biomass was released to the soil pore water on the first day after application of high concentrations (≥ 5%) of biomass (Table S7). The higher Se accumulation in the beans compared to the Se content in the soil pore water (on the first day) also evidenced that more Se was slowly released from the Se-enriched microalgae matrix and gradually supplied for uptake by the beans during the entire growth period (Table S7).

4.5 Practical implications of using Se-enriched microalgae biomass and its extract as biostimulants and/or selenium biofertilizers

This study demonstrated that the Se-enriched microalgae biomass and its extract produced from Se-enriched domestic wastewater have the potential to be used as biostimulant and Se biofertilizer for enhancement of beans growth and Se biofortification. However, the potential loading of heavy metals, micropollutants and pathogens onto the biomass is still a main concern for the application of Se-enriched microalgae produced from domestic wastewater. The EU fertilizing products and amending regulation (2019) stipulates that contaminants in plant biostimulants (one of the product function categories of EU fertilizing products) must not exceed the following limit values (expressed as mg kg^{-1} dry matter): Cd 1.5, Cr (VI) 2.0, Hg 1.0, Ni 50, Pb 120 and As 40. The Cu and Zn content must not exceed 600 and 1500 mg kg^{-1} dry

matter, respectively. Pathogen loads must not exceed the following limits: *Salmonella* spp. absence in 25 g or 25 mL and *Escherichia coli* or *Enterococcaceae* 1000 CFU in 1 g or 1 mL.

In this study, the level of all heavy metals in the Se-enriched microalgae biomass was much below these limits, with the exception of Cr (Table 1). It should be noted that the regulation limited the Cr(VI) content instead of total Cr, since Cr(VI) is both toxic and carcinogenic, while other Cr species (e.g. Cr(0) and Cr(III)) are considered not toxic (Kimbrough et al. 1999). In most cases, Cr(III) is the dominating species in the environment and food (Kimbrough et al. 1999). The Cr species in the microalgae biomass in this study is possibly also dominated by Cr(III), but additional analyses are needed to confirm this.

Besides, the pathogen load in the Se-enriched microalgae biomass has been analyzed and it could be confirmed that *Salmonella* spp. and *Escherichia coli* were absent in the freeze-dried biomass, and most microorganisms were reduced after drying processes (Li et al., 2021). Eventhough the heavy metal contents and pathogen loads of the microalgae biomass did not exceed the European regulation of fertilizer products, more research is still needed to confirm environmental safety and assess human health risks related to the use of Se-enriched microalgae as biostimulant in crop production. Besides, further studies should be conducted to assess the effect of the Se-enriched microalgae and their extracts on Se accumulation in crops and growth of these crops under field conditions.

5. Conclusions

Se-enriched microalgae biomass was produced in raceway ponds treating domestic wastewater. Application of relatively low dosages of Se-enriched microalgae extract was beneficial for seed germination (≤ 1% dosage) and seedling growth (≤ 5% dosage) of beans, while high dosages (> 50%) significantly delayed the mean germination time. Foliar application of Se-enriched microalgae extract was more effective to stimulate the bean growth and increase the Se concentration in the seeds compared to soil drench application. 5% dosage of Se-enriched microalgae biomass could be used as a biostimulant enhancing the growth of bean plants, and as an organic slow-release

Se biofertilizer significantly improving the Se content in the beans, including the seeds. These results indicate that Se-enriched microalgae biomass and its extracts could potentially be used as an added-value biostimulant and Se biofertilizer replacing conventional mineral Se fertilizers in biofortification schemes. As the microalgae were generated during domestic wastewater treatment, the proposed approach contributes to resource recovery within the framework of the circular economy.

Acknowledgements:

This work has been financially supported by the Special Research Fund (BOF, grant number BOFCHN2017000801) from Ghent University as well as the Chinese Scholarship Council (CSC, grant number 201606300023). Ivet Ferrer is grateful to the Government of Catalonia (Consolidated Research Group 2017 SGR 1029).

Data availability

The datasets generated in the present study are available from the corresponding author on reasonable request.

Li, J., Lens, P. N. L., Ferrer, I., Du Laing., G. (2021) Evaluation of selenium-enriched microalgae produced on domestic wastewater as biostimulant and biofertilizer for growth of selenium-enriched crops. Journal of Applied Phycology, 33(5), 3027–3039.

References:

APHA-AWWA-WEF (2012) Standard methods for the examination of water and wastewater. 22nd edn. American Public Health Association, Washington, D.C. https://books.google.be/books?id=dd2juAAACAAJ

Arashiro LT, Ferrer I, Rousseau DPL, Van Hulle SWH, Garfi M (2019) The effect of primary treatment of wastewater in high rate algal pond systems: Biomass and bioenergy recovery. Bioresource Technology 280:27-36. https://doi.org/10.1016/j.biortech.2019.01.096

Bañuelos GS, Arroyo I, Pickering IJ, Yang SI, Freeman JL (2015) Selenium biofortification of broccoli and carrots grown in soil amended with Se-enriched hyperaccumulator *Stanleya pinnata*. Food Chemistry 166:603-608. https://doi.org/10.1016/j.foodchem.2014.06.071

Benlloch M, Ojeda MA, Ramos J, Rodriguez-Navarro A (1994) Salt sensitivity and low discrimination between potassium and sodium in bean plants. Plant and Soil 166 (1):117-123. https://doi.org/10.1007/BF02185488

Boldrin PF, Faquin V, Ramos SJ, Boldrin KVF, Ávila FW, Guilherme LRG (2013) Soil and foliar application of selenium in rice biofortification. Journal of Food Composition and Analysis 31 (2):238-244. https://doi.org/10.1016/j.jfca.2013.06.002

Calvo P, Nelson L, Kloepper JW (2014) Agricultural uses of plant biostimulants. Plant and Soil 383 (1-2):3-41. https://doi.org/10.1007/s11104-014-2131-8

Chiaiese P, Corrado G, Colla G, Kyriacou MC, Rouphael Y (2018) Renewable sources of plant biostimulation: Microalgae as a sustainable means to improve crop performance. Frontiers in Plant Science 9:1782. http://doi.org/10.3389/fpls.2018.01782

Coppens J, Grunert O, Van Den Hende S, Vanhoutte I, Boon N, Haesaert G, De Gelder L (2015) The use of microalgae as a high-value organic slow-release fertilizer results in tomatoes with increased carotenoid and sugar levels. Journal of Applied Phycology 28 (4):2367-2377. http://doi.org/10.1007/s10811-015-0775-2

Dhillon SK, Hundal BK, Dhillon KS (2007) Bioavailability of selenium to forage crops in a sandy loam soil amended with Se-rich plant materials. Chemosphere 66 (9):1734-4173. https://doi.org/10.1016/j.chemosphere.2006.07.006

Dias GA, Rocha RHC, Araújo JL, Lima JF, Guedes WA (2016) Growth, yield, and postharvest quality in eggplant produced under different foliar fertilizer (*Spirulina platensis*) treatments. Semina: Ciências Agrárias 37 (6):3893. https://doi.org/10.5433/1679-0359.2016v37n6p3893

Egene CE, Van Poucke R, Ok YS, Meers E, Tack FMG (2018) Impact of organic amendments (biochar, compost and peat) on Cd and Zn mobility and solubility in contaminated soil of the Campine region after three years. Science of The Total Environment 626:195-202. https://doi.org/10.1016/j.scitotenv.2018.01.054

Ellis RH, Roberts EH (1981) The quantification of aging and survival in orthodox seeds. Seed Science and Technology 9 (2):373-410. https://agris.fao.org/agris-search/search.do?recordID=XE8182678

Faheed FA, Abd-El Fattah Z (2008) Effect of *Chlorella vulgaris* as biofertilizer on growth parameters and metabolic aspects of lettuce plant. Journal of Agriculture & Social Science 4. https://www.semanticscholar.org/paper/Effect-of-Chlorella-vulgaris-as-bio-fertilizer-on-Faheed-Fattah/a33f6d3372cb73a34a37b095ecdd0482fa09fbd6

Gan X, Huang JC, Zhou C, He S, Zhou W (2019) Relationship between selenium removal efficiency and production of lipid and hydrogen by *Chlorella vulgaris*. Chemosphere 217:825-832. https://doi.org/10.1016/j.chemosphere.2018.11.075

Garcia-Gonzalez J, Sommerfeld M (2016) Biofertilizer and biostimulant properties of the microalga *Acutodesmus dimorphus*. Journal of Applied Phycology 28 (2):1051-1061. https://doi.org/10.1007/s10811-015-0625-2

Gerhardt MB, Green FB, Newman RD, Lundquist TJ, Tresan RB, Oswald WJ (1991) Removal of selenium using a novel algal-bacterial process. Research Journal of the Water Pollution Control Federation 63 (5):799-805. https://www.scopus.com/inward/record.uri?eid=2-s2.0-0026187699&partnerID=40&md5=58e17c02a6e2c6f9bc68153e467c00c8

Gómez-Jacinto V, García-Barrera T, Garbayo-Nores I, Vilchez-Lobato C, Gómez-Ariza J-L (2012) Metal-metabolomics of microalga *Chlorella sorokiniana* growing in selenium- and iodine-enriched media. Chemical Papers 66 (9):821-828. https://doi.org/10.2478/s11696-012-0186-7

Grzesik M, Romanowska-Duda Z (2014) Improvements in germination, growth, and metabolic activity of corn seedlings by grain conditioning and root application with cyanobacteria and microalgae. Polish Journal of Environmental Studies 23 (4):1147-1153. http://www.pjoes.com/Improvements-in-Germination-Growth-r-nand-Metabolic-Activity-of-Corn-Seedlings-r,89291,0,2.html

Gupta AB, Shukla AC (1969) Effect of algal extracts of *Phormidium* species on growth and development of rice seedlings. Hydrobiologia 34 (1):77-84. https://doi.org/10.1007/BF00040324

Gutierrez R, Ferrer I, Gonzalez-Molina A, Salvado H, Garcia J, Uggetti E (2016) Microalgae recycling improves biomass recovery from wastewater treatment high rate algal ponds. Water Research 106:539-549. https://doi.org/10.1016/j.watres.2016.10.039

Haug A, Graham RD, Christophersen OA, Lyons GH (2007) How to use the world's scarce selenium resources efficiently to increase the selenium concentration in food. Microbial ecology in health and disease 19 (4):209-228. https://doi.org/10.1080/08910600701698986

Hernández-Herrera RM, Santacruz-Ruvalcaba F, Ruiz-López MA, Norrie J, Hernández-Carmona G (2014) Effect of liquid seaweed extracts on growth of tomato seedlings (*Solanum lycopersicum* L.). Journal of Applied Phycology 26 (1):619-628. https://doi.org/10.1007/s10811-013-0078-4

Kaveh H, Nemati H, Farsi M, Jartoodeh SV (2011) How salinity affect germination and emergence of tomato lines. Journal of Biological and Environmental Science 5 (15):159-163. https://www.semanticscholar.org/paper/How-Salinity-Affect-Germination-and-Emergence-of-Nemati-Farsi/9e366e9aa2eda1c9fe05804171e594c5aa97e59c

Kimbrough DE, Cohen Y, Winer AM, Creelman L, Mabuni C (1999) A critical assessment of chromium in the environment. Critical Reviews in Environmental Science and Technology 29 (1):1-46. https://doi.org/10.1080/10643389991259164

Kumar G, Sahoo D (2011) Effect of seaweed liquid extract on growth and yield of *Triticum aestivum* var. Pusa Gold. Journal of Applied Phycology 23 (2):251-255. https://doi.org/10.1007/s10811-011-9660-9

Li J, Ferrer I, Gijs Du L, Otero-Gonzalez L, Loi G, Lens PNL (2020) Selenate and selenite uptake, accumulation and toxicity in *Lemna minuta*. Water Science and Technology 81 (9):1852-1862. https://doi.org/10.2166/wst.2020.214

Li J, Otero Gonzalez L, Michiels J, Lens PNL, Du Laing G, Ferrer I (2021) Production of selenium-enriched microalgae as potential feed supplement in high-rate algae ponds treating domestic wastewater. Bioresource Technology 333: 125239. https://doi.org/10.1016/j.biortech.2021.125239

Li Z, Liang D, Peng Q, Cui Z, Huang J, Lin Z (2017) Interaction between selenium and soil organic matter and its impact on soil selenium bioavailability: A review. Geoderma 295:69-79. https://doi.org/10.1016/j.geoderma.2017.02.019

Michalak I, Chojnacka K, Dmytryk A, Wilk R, Gramza M, Roj E (2016) Evaluation of supercritical extracts of algae as biostimulants of plant growth in field trials. Frontiers in Plant Science 7:1591. https://doi.org/10.3389/fpls.2016.01591

Mota MFS, Souza MF, Bon EPS, Rodrigues MA, Freitas SP (2018) Colorimetric protein determination in microalgae (Chlorophyta): association of milling and SDS treatment for total protein extraction. Journal of Phycology 54 (4):577-580. https://doi.org/10.1111/jpy.12754

Mulbry W, Westhead EK, Pizarro C, Sikora L (2005) Recycling of manure nutrients: use of algal biomass from dairy manure treatment as a slow release fertilizer. Bioresource Technology 96 (4):451-458. https://doi.org/10.1016/j.biortech.2004.05.026

Orchard T (1977) Estimating the parameters of plant seedling emergence. Seed Science and Technology 5:61-69

Poblaciones MJ, Rengel Z (2017) Combined foliar selenium and zinc biofortification in field pea (*Pisum sativum*): accumulation and bioavailability in raw and cooked grains. Crop and Pasture Science 68 (3):265. https://doi.org/10.1071/cp17082

Rayman MP (2000) The importance of selenium to human health. The Lancet 356 (9225):233-241. https://doi.org/10.1016/s0140-6736(00)02490-9

Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019 laying down rules on the making available on the market of EU fertilising products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32019R1009

Rolland F, Moore B, Sheen J (2002) Sugar sensing and signaling in plants. Plant Cell 14 Suppl (Suppl):S185-205. https://doi.org/10.1105/tpc.010455

Ronga D, Biazzi E, Parati K, Carminati D, Carminati E, Tava A (2019) Microalgal biostimulants and biofertilisers in crop productions. Agronomy 9 (192):1-22. https://doi.org/10.3390/agronomy9040192

Shaaban MM (2001a) Green microalgae water extract as foliar feeding to wheat plants. Pakistan Journal of Biological Sciences 4 (6):628-632. https://doi.org/10.3923/pjbs.2001.628.632

Shaaban MM (2001b) Nutritional status and growth of maise plants as affected by green microalgae as soil additives. Journal of Biological Sciences 1 (6):475-479. https://doi.org/10.3923/jbs.2001.475.479

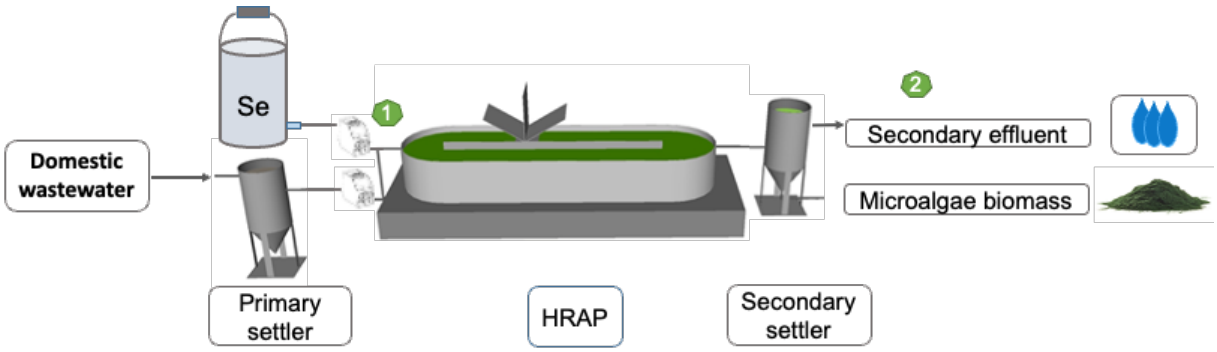
Solórzano L (1969) Determination of ammonia in natural seawater by the phenol-hypochlorite method. Limnology and Oceanography 14 (5):799-801. https://doi.org/10.4319/lo.1969.14.5.0799

Tan Ja, Zhu W, Wang W, Li R, Hou S, Wang D, Yang L (2002) Selenium in soil and endemic diseases in China. Science of The Total Environment 284 (1):227-235. https://doi.org/10.1016/S0048-9697(01)00889-0

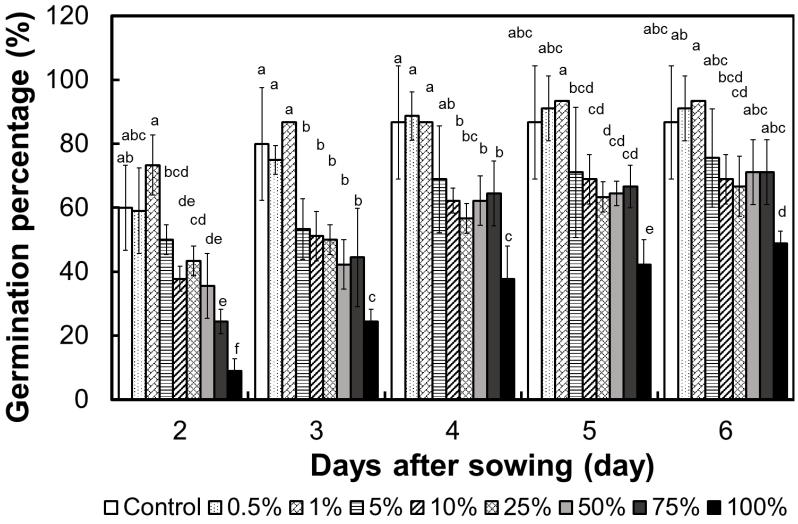
Wu Z, Bañuelos GS, Lin Z-Q, Liu Y, Yuan L, Yin X, Li M (2015) Biofortification and phytoremediation of selenium in China. Frontiers in Plant Science 6 (136). https://doi.org/10.3389/fpls.2015.00136

Zhang J, Saad R, Taylor EW, Rayman MP (2020) Selenium and selenoproteins in viral infection with potential relevance to COVID-19. Redox Biology 37:101715. https://doi.org/10.1016/j.redox.2020.101715

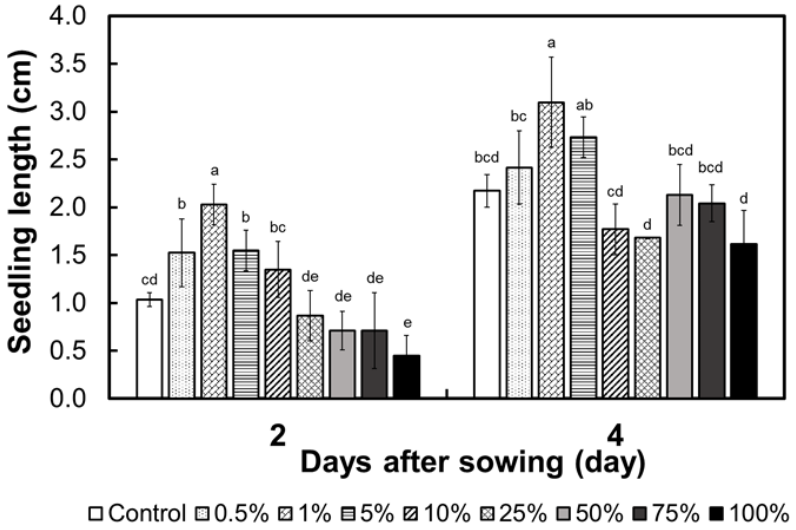
Li, J., Lens, P. N. L., Ferrer, I., Du Laing., G. (2021) Evaluation of selenium-enriched microalgae produced on domestic wastewater as biostimulant and biofertilizer for growth of selenium-enriched crops. Journal of Applied Phycology, 33(5), 3027–3039.

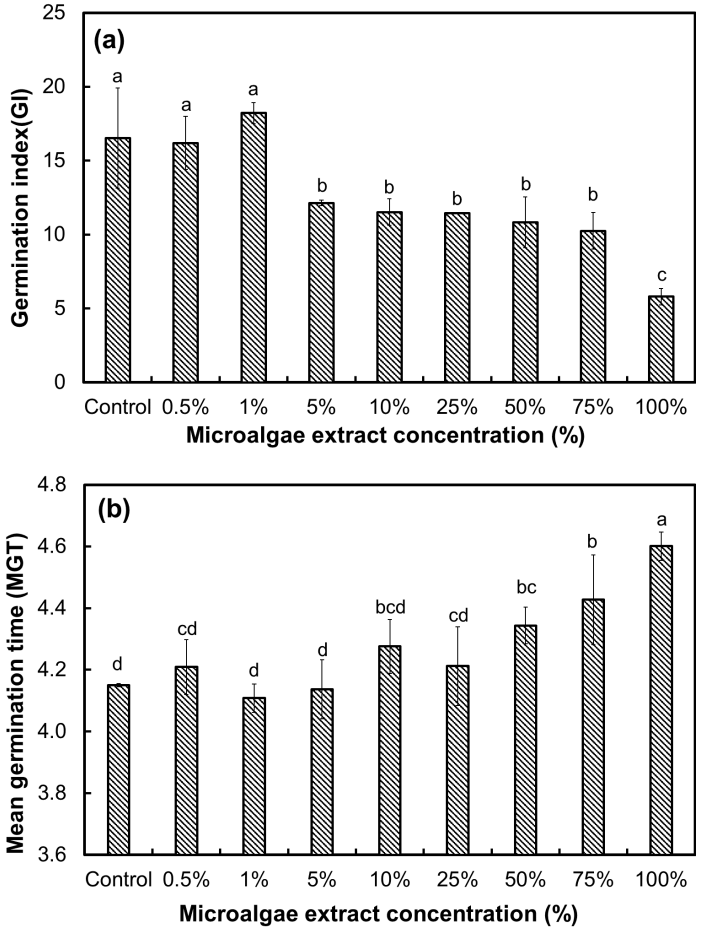


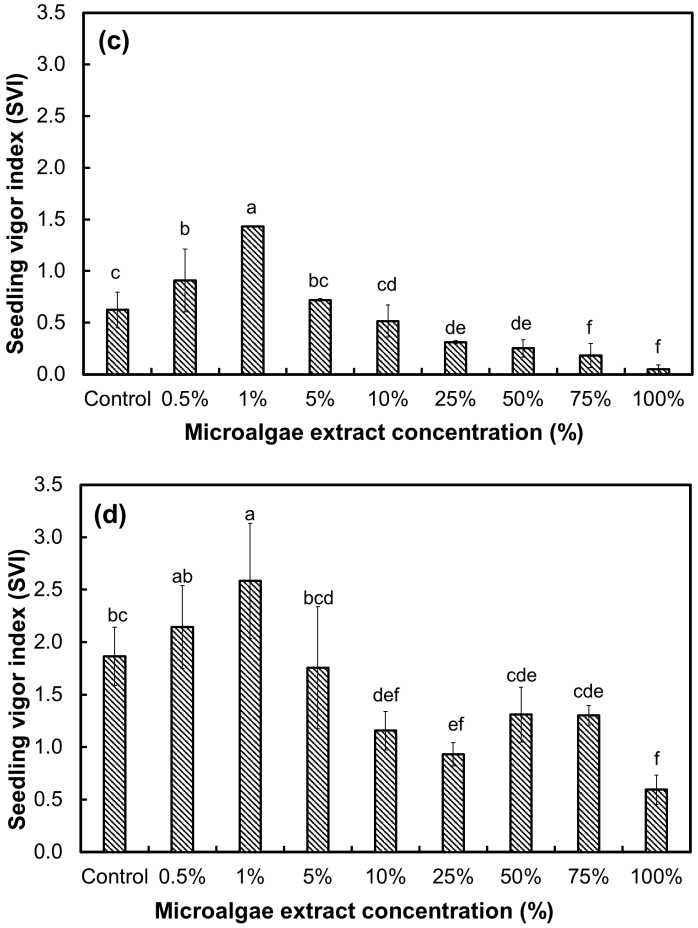
Li, J., Lens, P. N. L., Ferrer, I., Du Laing., G. (2021) Evaluation of selenium-enriched microalgae produced on domestic wastewater as biostimulant and biofertilizer for growth of selenium-enriched crops. Journal of Applied Phycology, 33(5), 3027–3039.

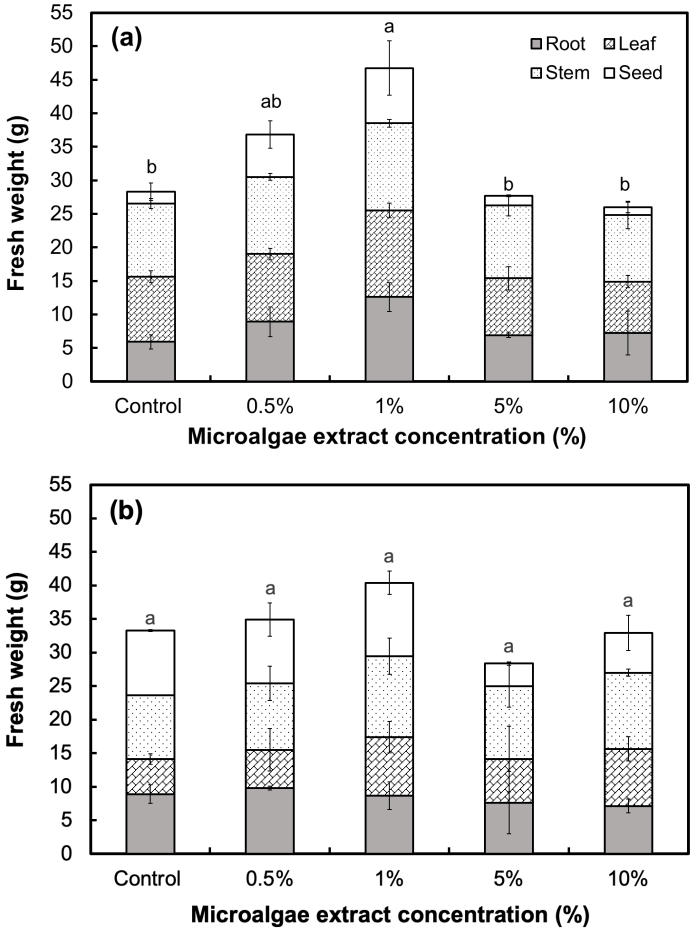


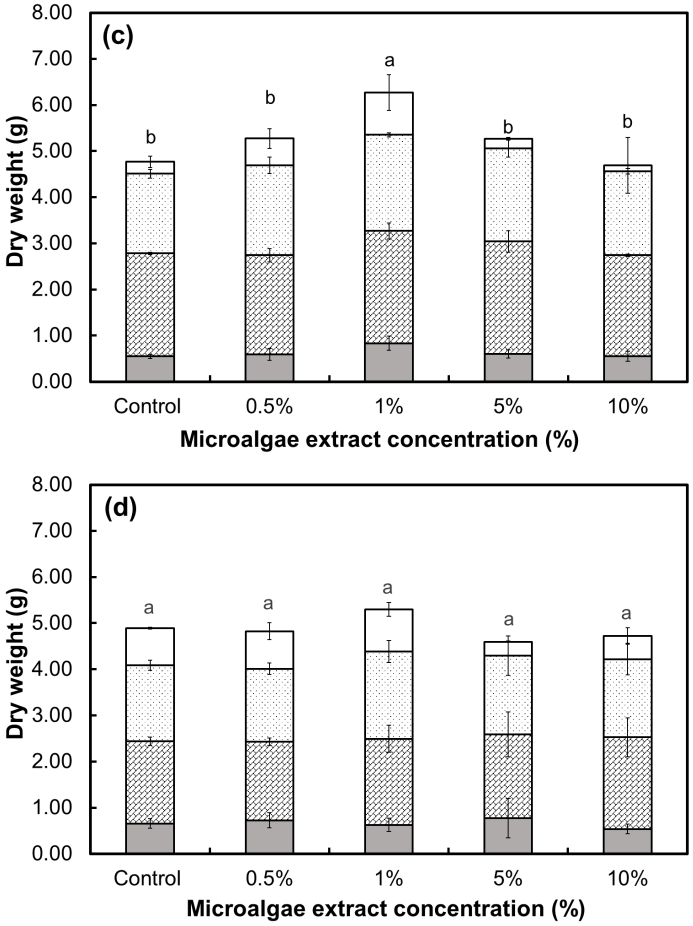
Li, J., Lens, P. N. L., Ferrer, I., Du Laing., G. (2021) Evaluation of selenium-enriched microalgae produced on domestic wastewater as biostimulant and biofertilizer for growth of selenium-enriched crops. Journal of Applied Phycology, 33(5), 3027–3039.



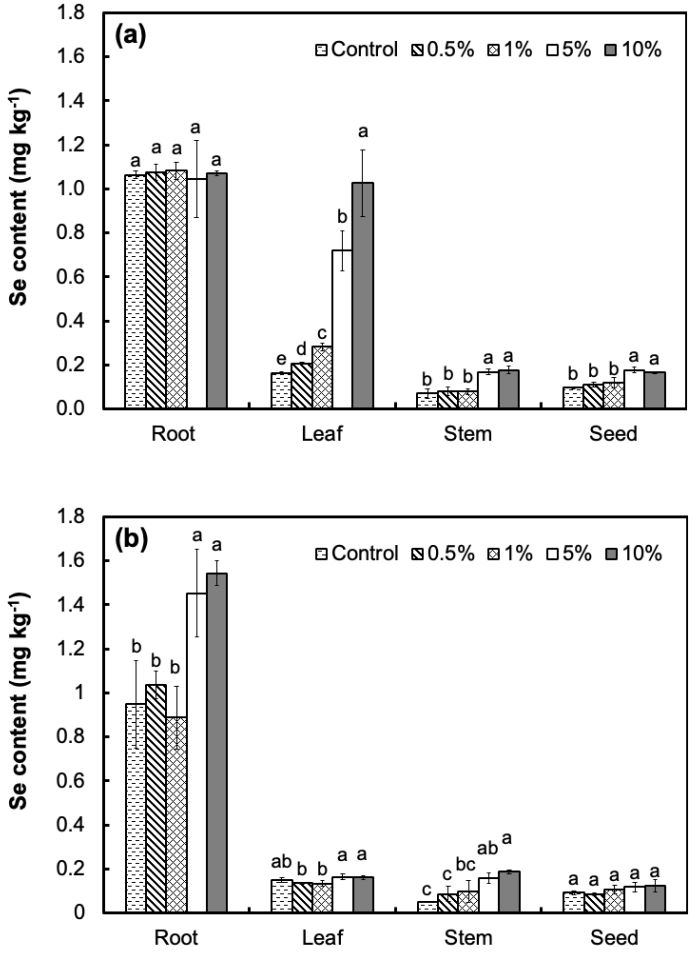

(a)
Germination index(GI)
25
20
15
10
5
0
a
a
a
b
b
b
b
b
c
Control
0.5%
1%
5%
10%
25%
50%
75%
100%
Microalgae extract concentration (%)
(b)
Mean germination time (MGT)
4.8
4.6
4.4
4.2
4.0
3.8
3.6
d
cd
d
d
bcd
cd
bc
b
a
Control
0.5%
1%
5%
10%
25%
50%
75%
100%
Microalgae extract concentration (%)

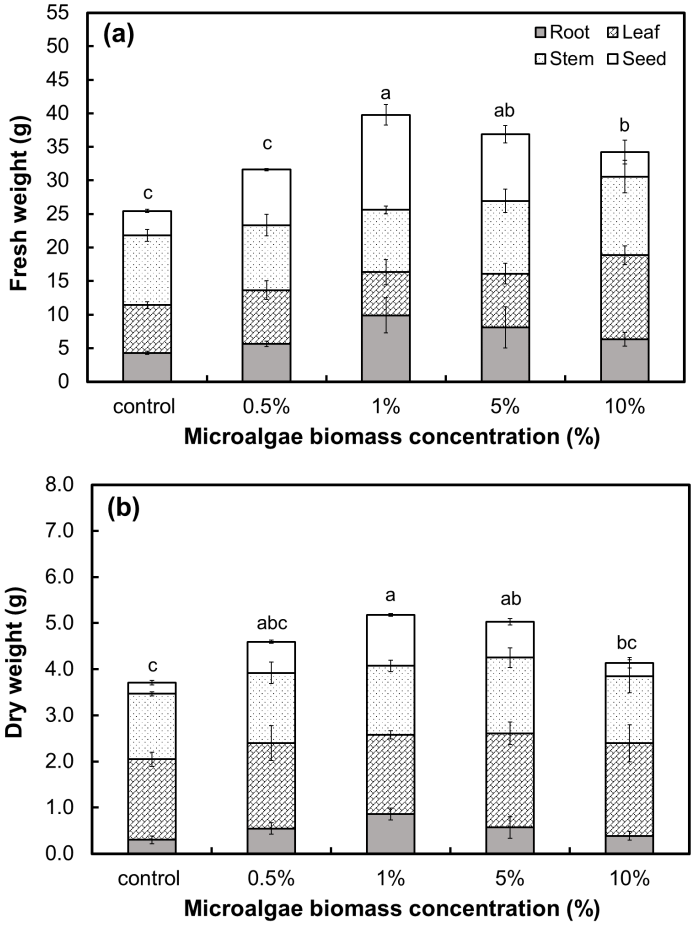

(c)
Seedling vigor index (SVI)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
c
b
a
bc
cd
de
de
f
f
Control
0.5%
1%
5%
10%
25%
50%
75%
100%
Microalgae extract concentration (%)
(d)
Seedling vigor index (SVI)
bc
ab
a
bcd
def
ef
cde
cde
f
Control
0.5%
1%
5%
10%
25%
50%
75%
100%
Microalgae extract concentration (%)


(a)
Root
Leaf
Stem
Seed
Fresh weight (g)
Control
0.5%
1%
5%
10%
Microalgae extract concentration (%)
(b)
Fresh weight (g)
Control
0.5%
1%
5%
10%
Microalgae extract concentration (%)

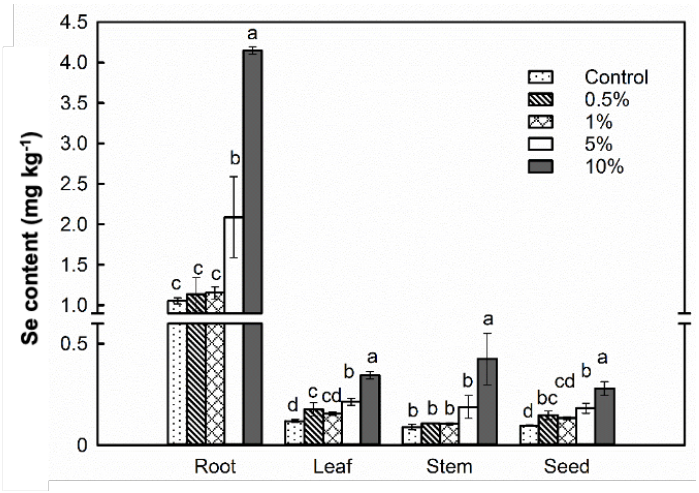

(c)
Dry weight (g)
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
a
b
b
b
b
Control
0.5%
1%
5%
10%
Microalgae extract concentration (%)
(d)
Dry weight (g)
a
a
a
a
a
Control
0.5%
1%
5%
10%
Microalgae extract concentration (%)

Li, J., Lens, P. N. L., Ferrer, I., Du Laing., G. (2021) Evaluation of selenium-enriched microalgae produced on domestic wastewater as biostimulant and biofertilizer for growth of selenium-enriched crops. Journal of Applied Phycology, 33(5), 3027–3039.

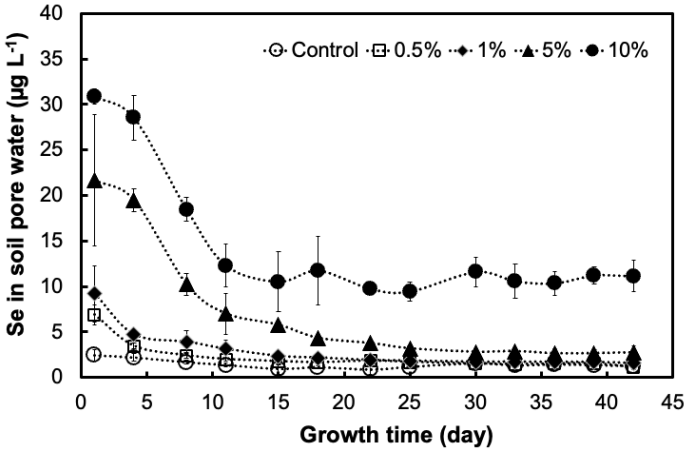



(a)
Root
Leaf
Stem
Seed
Fresh weight (g)
55
50
45
40
35
30
25
20
15
10
5
0
c
c
a
ab
b
control
0.5%
1%
5%
10%
Microalgae biomass concentration (%)
(b)
Dry weight (g)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
c
abc
a
ab
bc
control
0.5%
1%
5%
10%
Microalgae biomass concentration (%)

Li, J., Lens, P. N. L., Ferrer, I., Du Laing., G. (2021) Evaluation of selenium-enriched microalgae produced on domestic wastewater as biostimulant and biofertilizer for growth of selenium-enriched crops. Journal of Applied Phycology, 33(5), 3027–3039.



Li, J., Lens, P. N. L., Ferrer, I., Du Laing., G. (2021) Evaluation of selenium-enriched microalgae produced on domestic wastewater as biostimulant and biofertilizer for growth of selenium-enriched crops. Journal of Applied Phycology, 33(5), 3027–3039.



Tables

Table 1 Characteristics of the Se-enriched microalgae biomass and extract (average ± standard deviation, n=3).

	Biomass	Extract
pH	N.D.	5.71
EC (S m^{-1})	N.D.	0.61
Elements	[mg kg^{-1} dry matter]	[mg L^{-1}]
Se	29 ± 0.6	0.67 ± 0.02
P	13935 ± 323	445 ± 5
S	6995 ± 148	378 ± 4
Zn	520 ± 5	1.45 ± 0.04
Cu	145 ± 3	1.36 ± 0.01
Ca	51215 ± 1478	280 ± 5
Mg	8199 ± 175	230 ± 4
Na	1640 ± 175	616 ± 15
K	4799 ± 195	476 ± 9
Ni	15 ± 0.46	1.01 ± 0.02
Cr	31 ± 3	<LOQ
Cd	<LOQ	<LOQ
Pb	19 ± 0.25	<LOQ
Hg	0.32 ± 0.07	<LOQ
As	4.4 ± 0.32	N.D.
Co	<LOQ	<LOQ

N.D.: Not determined

< LOQ: values lower than the limit of quantification

Table 2 Concentration of Se-enriched microalgae biomass extracts in each treatment. SDS: sodium dodecyl sulfate.

Treatment	Concentration (%)	Preparation	Final SDS concentration (%)
Control	0	10 mL DI water	0
T1	0.5	0.05 mL extract in 9.95 mL DI water	0.005
T2	1.0	0.1 mL extract in 9.9 mL DI water	0.01
T3	5.0	0.5 mL extract in 9.5 mL DI water	0.05
T4	10	1 mL extract in 9 mL DI water	0.1
T5	25	2.5mL extract in 7.5mL DI water	0.25
T6	50	5 mL extract in 5 mL DI water	0.5
T7	75	7.5 mL extract in 2.5 mL DI water	0.75
T8	100	10 mL extract	1.0

Table 3 Summary of the main parameters (average ± standard deviation, n=24) monitored in the influent and the effluent of the raceway pond throughout the entire experimental period: total and volatile suspended solids (TSS and VSS), total and soluble chemical oxygen demand (COD_t and COD_s), total carbon (TC), total and soluble P (TP and SP), total nitrogen (TN), ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), nitrite (NO_2^--N) and selenium (Se).

	Concentration			Removal efficiency
	Influent	Effluent	Unit	%
pH	7.8 ± 0.2	8.7 ± 0.6	-	-
Turbidity	263 ± 220.7	14 ± 20.0	NTU	91 ± 9.6
TSS	424 ± 341.7	37 ± 48.0	mg L^{-1}	86 ± 16.0
VSS	345 ± 254.6	35 ± 42.3	mg L^{-1}	88 ± 12.1
COD_t	497 ± 279.3	123 ± 97.2	mg L^{-1}	70 ± 20.4
COD_s	233 ± 136.3	99 ± 63.5	mg L^{-1}	49 ± 23.9
TC	342 ± 175.7	97 ± 57.6	mg L^{-1}	67 ± 17.6
TP	10 ± 5.5	2.9 ± 2.9	mg L^{-1}	77 ± 18.1
SP	5.3 ± 2.7	1.6 ± 1.6	mg L^{-1}	71 ± 21.6
TN	64 ± 19.8	26 ± 28.2	mg L^{-1}	65 ± 24.8
NH_4^+-N	28 ± 11.4	2.2 ± 2.0	mg L^{-1}	93 ± 6.2
NO_3^--N	0.4 ± 0.9	1.3 ± 3.6	mg L^{-1}	-
NO_2^--N	0.8 ± 2.0	4.6 ± 3.7	mg L^{-1}	-
Se	48 ± 13.5	26 ± 9.6	μg L^{-1}	44 ± 6.5

Table 4 pH and total organic carbon (TOC) content in the pore water of the soil amended with different amounts of Se-enriched microalgae biomass at the time of beans harvesting. Values are mean ± standard deviation (n=3). Different letters indicate statistically significant differences between treatments according to Duncan's multiple comparison tests ($P \leqslant 0.05$)

	Control	0.5%	1%	5%	10%
pH	5.2 ± 0.3 ^{c}	6.2 ± 0.4 ^{ab}	6.0 ± 0.6 ^{abc}	5.3 ± 0.5 ^{bc}	6.5 ± 0.7 ^{a}
TOC (mg L^{-1})	50 ± 8 ^{c}	47 ± 13 ^{c}	39 ± 7 ^{c}	65 ± 3 ^{b}	149 ± 9 ^{a}

Li, J., Lens, P. N. L., Ferrer, I., Du Laing., G. (2021) Evaluation of selenium-enriched microalgae produced on domestic wastewater as biostimulant and biofertilizer for growth of selenium-enriched crops. Journal of Applied Phycology, 33(5), 3027–3039.

Supplementary Information

Evaluation of selenium-enriched microalgae produced on domestic wastewater as biostimulant and biofertilizer for growth of selenium-enriched crops

Jun Li[1,2], Piet N. L. Lens[3], Ivet Ferrer[2], Gijs Du Laing[1]

[1] Laboratory of Analytical Chemistry and Applied Ecochemistry, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium.

[2] GEMMA-Group of Environmental Engineering and Microbiology, Department of Civil and Environmental Engineering, Universitat Politècnica de Catalunya·BarcelonaTech, Jordi Girona 1-3, Building D1, 08034 Barcelona, Spain

[3] UNESCO-IHE Institute for Water Education, 2601 DA Delft, The Netherlands.

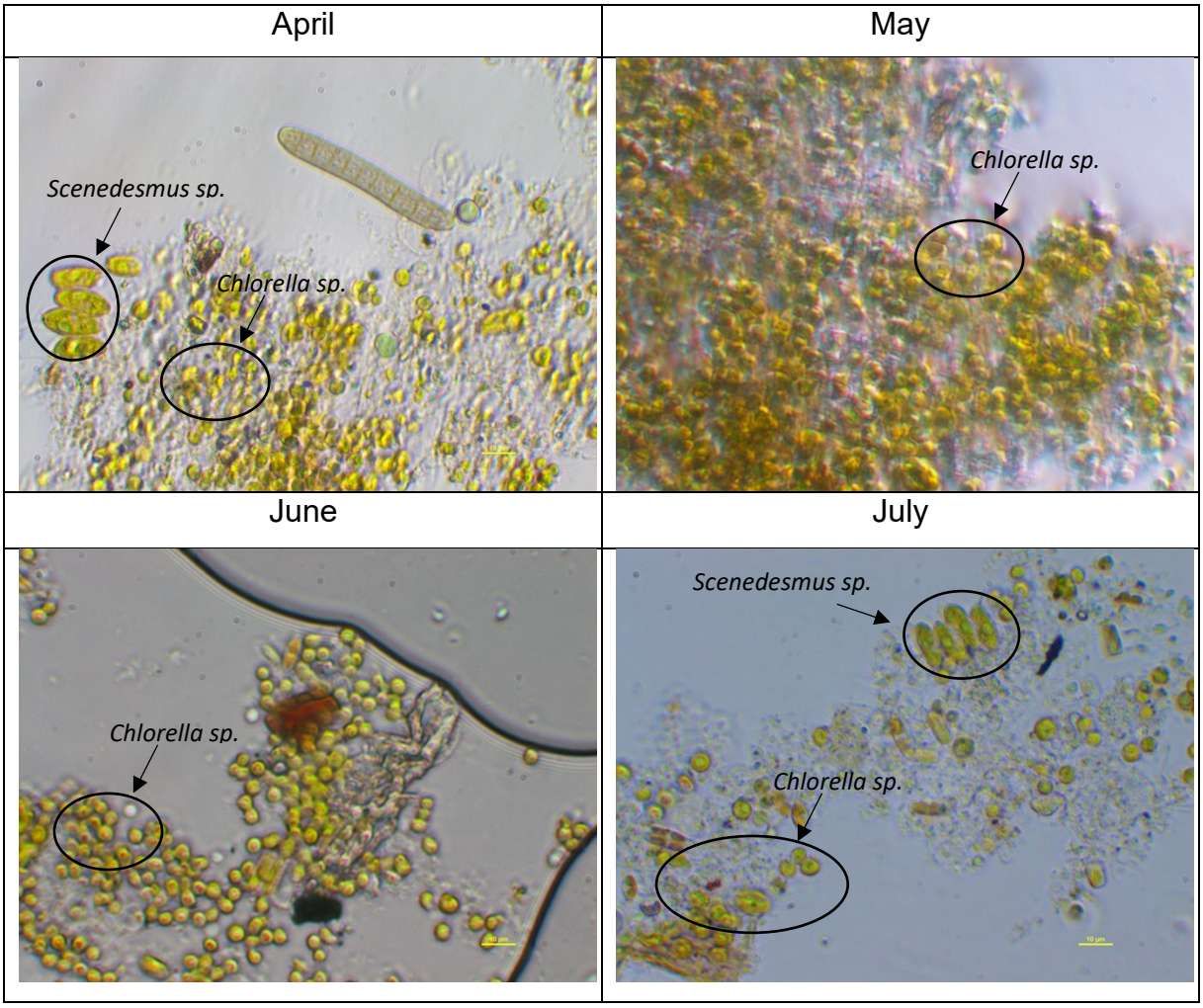


Figure S1 Optic microscope images of the microalgae biomass composition over the experimental period (scale 10 um).

Table S1 Physicochemical properties and (trace) element content of the tested sandy soil (mean ± standard deviation; n=3, except for texture).

	Sandy soil
pH-KCl	6.45 ± 0.00
pH-H_2O	7.07 ± 0.06
EC (µS/cm)	35 ± 1.73
CEC (cmol/kg)	1.79 ± 0.22
OM (%)	2.14 ± 0.02
Texture	
Sand (%)	91.5
Silt (%)	6.0
Clay (%)	2.5
Elements (mg kg^{-1})	
Total Se	0.10 ± 0.01
Available Se	0.030 ± 0.00
Total P	300 ± 6
Total S	76 ± 10
Total Cu	2.69 ± 0.14
Total Zn	8.47 ± 0.48
Total Ca	1103 ± 30
Total Mg	489 ± 31
Total Fe	3612 ± 104
Total Al	3772 ± 270

Table S2 Effect of Se-enriched microalgae extracts applied as foliar spray and soil drench on fresh weight (g) of bean tissues at different doses. Values are average ± standard deviation (n=3). Different letters within columns indicate statistically significant differences between treatments according to Duncan's multiple comparison tests ($P \leq 0.05$).

Treatment	Foliar spray				Soil drench			
	Root	Leaf	Stem	Seed	Root	Leaf	Stem	Seed
Control	5.9 ± 1.06 ^{b}	9.7 ± 0.89 ^{b}	10.9 ± 0.75	1.8 ± 1.29 ^{b}	8.9 ± 1.39	5.2 ± 0.79	9.5 ± 0.07	9.6 ± 0.15 ^{ab}
0.5%	8.9 ± 2.20 ^{ab}	10.1 ± 0.85 ^{ab}	11.5 ± 0.50	6.3 ± 2.05 ^{ab}	9.8 ± 0.27	5.7 ± 3.17	9.9 ± 2.54	9.5 ± 2.49 ^{ab}
1%	12.6 ± 2.16 ^{a}	12.9 ± 1.08 ^{a}	13.0 ± 0.57	8.2 ± 4.04 ^{a}	8.7 ± 2.06	8.7 ± 2.35	12.1 ± 2.72	11.0 ± 1.73 ^{a}
5%	6.9 ± 0.36 ^{b}	8.5 ± 1.73 ^{b}	10.9 ± 1.57	1.4 ± 0.15 ^{b}	7.6 ± 4.65	6.5 ± 4.91	10.8 ± 3.10	3.4 ± 0.22 ^{c}
10%	7.2 ± 3.27 ^{b}	7.7 ± 0.93 ^{b}	9.9 ± 2.03	1.1 ± 0.79 ^{b}	7.1 ± 1.00	8.5 ± 1.81	11.4 ± 0.51	5.9 ± 2.61 ^{bc}

Table S3 Effect of Se-enriched microalgae extracts applied as foliar spray and soil drench on dry weight (g) of bean tissues at different doses. Values are average ± standard deviation (n=3). Different letters within columns indicate statistically significant differences between treatments according to Duncan's multiple comparison tests ($P \leq 0.05$).

Treatment	Foliar spray				Soil drench			
	Root	Leaf	Stem	Seed	Root	Leaf	Stem	Seed
Control	0.55 ± 0.04 ^{b}	2.23 ± 0.03	1.72 ± 0.10	0.26 ± 0.12 ^{bc}	0.66 ± 0.10	1.78 ± 0.09	1.64 ± 0.11	0.81 ± 0.02 ^{a}
0.5%	0.59 ± 0.13 ^{b}	2.15 ± 0.15	1.95 ± 0.18	0.58 ± 0.21 ^{ab}	0.73 ± 0.17	1.70 ± 0.08	1.58 ± 0.12	0.82 ± 0.19 ^{a}
1%	0.83 ± 0.16 ^{a}	2.43 ± 0.18	2.09 ± 0.04	0.92 ± 0.38 ^{a}	0.63 ± 0.14	1.87 ± 0.29	1.89 ± 0.24	0.92 ± 0.15 ^{a}
5%	0.60 ± 0.09 ^{b}	2.44 ± 0.23	2.02 ± 0.19	0.20 ± 0.03 ^{bc}	0.77 ± 0.43	1.81 ± 0.49	1.70 ± 0.43	0.30 ± 0.01 ^{b}
10%	0.55 ± 0.11 ^{b}	2.19 ± 0.03	1.82 ± 0.06	0.13 ± 0.61 ^{c}	0.54 ± 0.10	1.99 ± 0.42	1.69 ± 0.34	0.50 ± 0.18 ^{b}

Table S4 Effect of Se-enriched microalgae biomass soil application on the fresh and dry weight of beans (g). Values are average ± standard deviation (n=3). Different letters within columns indicate statistically significant differences between treatments in the same tissue according to Duncan's multiple comparison tests ($P \leq 0.05$).

Treatment	Fresh weight				Dry weight			
	Root	Leaf	Stem	Seed	Root	Leaf	Stem	Seed
Control	4.31 ± 0.24 ^{c}	7.11 ± 0.53 ^{b}	10.4 ± 0.89	3.65 ± 0.21 ^{c}	0.30 ± 0.09 ^{b}	1.75 ± 0.16	1.42 ± 0.04	0.25 ± 0.05 ^{c}
0.5%	5.64 ± 0.44 ^{c}	8.01 ± 1.38 ^{b}	9.69 ± 1.59	8.28 ± 0.16 ^{b}	0.54 ± 0.12 ^{b}	1.85 ± 0.38	1.52 ± 0.23	0.68 ± 0.04 ^{b}
1%	9.89 ± 2.62 ^{a}	6.44 ± 1.87 ^{b}	9.27 ± 0.57	14.2 ± 1.53 ^{a}	0.86 ± 0.13 ^{a}	1.72 ± 0.09	1.49 ± 0.12	1.10 ± 0.03 ^{a}
5%	8.09 ± 3.07 ^{ab}	8.03 ± 1.55 ^{b}	10.8 ± 1.74	9.95 ± 1.30 ^{b}	0.57 ± 0.23 ^{b}	2.04 ± 0.24	1.64 ± 0.21	0.78 ± 0.07 ^{b}
10%	6.35 ± 1.03 ^{ab}	12.5 ± 1.42 ^{a}	11.7 ± 2.41	3.67 ± 1.75 ^{c}	0.39 ± 0.10 ^{b}	2.01 ± 0.40	1.46 ± 0.36	0.29 ± 0.11 ^{c}

Table S5 Selenium concentration (μg L^{-1}) in the pore water extracted from soil amended with non-extracted Se-enriched microalgae biomass. Mean ± standard deviation, n=3. Different lowercase letters indicate statistically significant differences between different incubation days according to Duncan's multiple comparison tests ($P < 0.05$). Uppercase indicates significant differences between different doses.

	Day 1	Day 8	Day 15	Day 22	Day 33	Day 42
Control	2.45 ± 0.64 ^{D}	1.69 ± 0.20 ^{D}	0.95 ± 0.57 ^{C}	0.87 ± 0.55 ^{D}	1.33 ± 0.06 ^{B}	1.26 ± 0.06 ^{B}
0.5%	6.89 ± 1.10 a ^{C}	2.42 ± 0.33 b ^{CD}	1.77 ± 0.34 bc ^{C}	1.82 ± 0.30 bc ^{C}	1.44 ± 0.29 bc ^{B}	1.13 ± 0.24 c ^{B}
1%	9.20 ± 3.09 a ^{C}	3.91 ± 1.27 b ^{C}	2.18 ± 0.35 b ^{C}	1.91 ± 0.21 b ^{C}	1.70 ± 0.06 b ^{B}	1.55 ± 0.45 b ^{B}
5%	21.7 ± 7.18 a ^{B}	10.2 ± 1.25 b ^{B}	5.37 ± 0.73 bc ^{B}	3.74 ± 0.24 c ^{B}	2.86 ± 0.11 c ^{B}	2.73 ± 0.75 c ^{B}
10%	30.9 ± 0.86 a ^{A}	18.5 ± 1.32 b ^{A}	10.5 ± 3.34 c ^{A}	9.73 ± 0.62 c ^{A}	10.6 ± 1.89 c ^{A}	11.1 ± 1.73 c ^{A}

Table S6 Effect of Se-enriched microalgae biomass soil application on the selenium accumulation (μg pot^{-1}) in different tissues of beans. Values are average ± standard deviation (n=3). Different letters within columns indicate statistically significant differences between treatments in the same tissue according to Duncan's multiple comparison tests ($P \leq 0.05$).

Treatments	Se accumulation			
	Root	Leaf	Stem	Seed
Control	0.32 ± 0.09 ^{c}	0.21 ± 0.02 ^{d}	0.13 ± 0.02 ^{b}	0.02 ± 0.00 ^{c}
0.5%	0.63 ± 0.24 ^{c}	0.32 ± 0.04 ^{c}	0.15 ± 0.01 ^{b}	0.10 ± 0.01 ^{b}
1%	0.99 ± 0.15 ^{b}	0.27 ± 0.01^{cd}	0.15 ± 0.01 ^{b}	0.15 ± 0.00 ^{a}
5%	1.11 ± 0.15 ^{b}	0.44 ± 0.06 ^{b}	0.31 ± 0.09 ^{b}	0.14 ± 0.02 ^{a}
10%	1.47 ± 0.18 ^{a}	0.65 ± 0.10 ^{a}	0.58 ± 0.24 ^{a}	0.07 ± 0.02 ^{b}

Note: Se accumulation (μg pot^{-1}) was calculated by multiplying the Se concentration in tissues (μg g^{-1}) by the dry weight of the corresponding tissues (g).

Table S7 Selenium mass balance calculated from the amount of Se applied through Se-enriched microalgae biomass, the Se content in the soil pore water and the Se accumulated in the plant.

Treatment	Applied Se *	Se in pore water ** (first day)	Se in pore water (last day)	Se accumulated by the plant***	Se in pore water (first day) / applied Se	Se in plants / applied Se
	[μg pot^{-1}]	[μg pot^{-1}]	[μg pot^{-1}]	[μg pot^{-1}]	%	%
0.5%	6.53	0.91	0.15	1.20	9	18
1%	13.1	1.21	0.20	1.56	7	12
5%	65	2.86	0.36	2.00	4	3
10%	130	4.08	1.47	2.76	3	2

Note:

$* \; Applied\ Se = Se\ concentration\ in\ the\ microalgae \times weight\ of\ microalgae\ biomass\ applied\ in\ each\ pot$

$** \; Se\ in\ pore\ water = Se\ concentration\ in\ the\ soil\ pore\ water \times water\ content\ of\ the\ soil$

***Se accumulation in the plant is the sum of Se accumulated in each plant tissue.

Li, J., Lens, P. N. L., Ferrer, I., Du Laing., G. (2021) Evaluation of selenium-enriched microalgae produced on domestic wastewater as biostimulant and biofertilizer for growth of selenium-enriched crops. Journal of Applied Phycology, 33(5), 3027–3039.