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Production of selenium-enriched microalgae as potential feed supplement in high-rate algae ponds treating domestic wastewater

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Abstract

This study assessed the selenium (Se) removal efficiency of two pilot-scale high-rate algae ponds (HRAPs) treating domestic wastewater and investigated the production of Se-enriched microalgae as potential feed supplement. The HRAP-Se had an average Se, NH_4^+-N, total phosphorus and COD removal efficiency of, respectively, 43%, 93%, 77%, and 70%. Inorganic Se taken up by the microalgae was mainly (91%) transformed to selenoamino acids, and 49–63% of Se in the Se-enriched microalgae was bioaccessible for animals. The crude protein content (48%) of the microalgae was higher than that of soybeans, whereas the essential amino acid content was comparable. Selenium may induce the production of the polyunsaturated fatty acids omega-3 and omega-6 in microalgae. Overall, the production of Se-enriched microalgae in HRAPs may offer a promising alternative for upgrading low-value resources into high-value feed supplements, supporting the drive to a circular economy.

Keywords: Selenium, Algae, HRAPs, Photobioreactor, Resource recovery, Wastewater treatment

1 Introduction

Selenium (Se) is an essential trace element, playing a crucial role in the functioning of a wide range of enzymes in humans and animals (Hatfield et al., 2014). Despite the importance of Se, dietary Se intake is still low in a wide range of countries, resulting in Se deficiency due to low Se concentrations in soils, waters and food/feed (Rayman, 2000). For instance, almost all European countries are classified as low-Se regions (Zhong & Cheng, 2017). Dietary Se deficiency can cause several negative effects on humans and animals, such as mastitis and poor growth rates of animals, thus leading to economic losses (Rayman, 2000). Therefore, Se supplementation of feed and food or fertilization of food/feed crops to overcome the Se deficiency receives much attention. On the contrary, wastewaters containing Se are frequently produced as a result of anthropogenic activities, e.g., municipal, agricultural and industrial activities, resulting in a heavy load of Se in the receiving water bodies (Li et al., 2020a; Tan et al., 2016). Those Se-bearing wastewaters may thus potentially serve as Se source to produce Se-enriched products by adopting eco-friendly techniques.

Nowadays, microalgae-based wastewater treatment technologies are attracting considerable attention, as they are low-cost, low-energy consuming and easily implemented in regions with high temperatures and sunlight exposure (Arashiro et al., 2019). Microalgae have a great capacity to remove/take up excess nutrients from the corresponding growth medium, as their cultivation requires high amounts of macro-nutrients (such as nitrogen and phosphorus) and micro-nutrients (such as Fe, Mn, Mg and Zn) (Arashiro et al., 2020a; Gan et al., 2019). Furthermore, microalgae are a potential source of protein-rich biomass and numerous other high-value compounds, e.g., fatty acids, lipids, pigments and vitamins (Arashiro et al., 2020a and b). Microalgae-based products are adding value to the market. In this context, the cultivation of microalgae on wastewater with nitrogen, phosphorus and organic matter removal does not only provide an option to treat wastewater, but also significantly reduces the cost and carbon footprint of conventional microalgae production systems that do not use wastewater as growth medium, by converting low-value resources in wastewater into value-added bioproducts (Silambarasan et

al., 2021). Nowadays, many bioproducts from microalgae biomass grown in wastewater have been explored. For instance, high-value phycobiliproteins have been obtained from microalgae cultivated in food-industry wastewater (Arashiro et al., 2020a), municipal wastewater and anaerobic digestion concentrate (Arashiro et al., 2020b); high-quality fatty acids, such as oleic acid for biodiesel production, have been extracted from microalgae biomass grown in sewage (Khan et al., 2019), piggery wastewater (Chen et al., 2020) and starch processing wastewater (Tan et al., 2019); and plant growth-promoting substances (e.g., phytohormones and polysaccharides) have been recovered together with high N and P contents from microalgae biomass cultivated in wastewater, enabling its use as biofertilizer (Supraja et al., 2020). Nevertheless, very few studies have been conducted on the production of Se-enriched microalgae from wastewater and assessing their potential as high-protein Se supplement for animal feed.

However, microalgae may have the ability to take up inorganic Se and incorporate it into amino acids forming selenoamino acids, such as selenomethionine (SeMet), Se-cystine ($SeCys_2$) and selenocysteine (SeCys), which are beneficial for animal and human health (Umysova et al., 2009). In this context, high-value microalgae biomass with high Se content may be generated during wastewater treatment processes and subsequently added to animal feed for alleviating Se deficiency issues in animals. The Se source for this process could be Se-rich wastewaters, but Se could also be added from a primary source to produce Se-rich microalgae on domestic wastewater, providing the macronutrients for microalgae growth, as feedstock. Accordingly, a higher-value animal feed product could be produced from wastewater, while recovering resources.

Therefore, in this study, microalgae were grown in two pilot-scale high-rate algae ponds (HRAPs) treating domestic wastewater with and without Se spiking in order to: (1) investigate the Se removal efficiency of pilot-scale HRAPs treating domestic wastewater; (2) evaluate the possible use of domestic wastewater as a nutrient source for microalgae growth in HRAPs to produce high-value Se-rich microalgae; and (3) assess the potential use of upgraded Se-rich microalgae as feed supplement

by examining their Se content and speciation, digestibility, biochemical properties and nutritional profile.

2 Materials and methods

2.1 Source of biomass and wastewater

The microalgae inoculum was collected from a demonstrative-scale photobioreactor treating agricultural runoff (90%) and domestic wastewater (10%) located outdoors at the Agròpolis experimental campus of the Universitat Politècnica de Catalunya-BarcelonaTech (Viladecans, Spain). Operational details of the photobioreactor and characteristics of the biomass were presented by García et al. (2018).

The wastewater used in this study was real domestic wastewater from a residential area close to the Universitat Politècnica de Catalunya·BarcelonaTech (Barcelona, Spain), as described by Arashiro et al. (2019). The experimental set-up was located outdoors. Domestic wastewater received a screening pretreatment before being pumped into a 1 m^3 homogenization tank that was continuously stirred to avoid solids sedimentation, followed by a 3 L primary settler (diameter: 18 cm, height: 30 cm) with a hydraulic retention time (HRT) of 41 min. The effluent from the primary settler (noted as primary effluent) was collected (Fig. 1) for the subsequent batch experiments or pumped into two parallel HRAPs (0.5 m^3 each) as influent of the continuous system. Each HRAP, constructed from PVC, had a surface area of 1.54 m^2, a water depth of 0.3 m, a working volume of 0.47 m^3 and a paddle-wheel constantly stirring the mixed liquor at an average velocity of 10 m/h. Two secondary clarifiers (effective volume 3.3 L working volume) followed the two HRAPs to separate the effluent and biomass. The scheme of the HRAPs is shown in Fig. 1.

The average values of the main parameters (e.g. pH, total suspended solids (TSS), volatile suspended solids (VSS), chemical oxygen demand (COD) and ammonium nitrogen (NH_4^+-N), among others) in the primary effluent that was pumped to the HRAPs and the secondary effluent from the HRAPs clarifiers through the entire experimental period (3 months) are presented in Fig. 4.

2.2 Se removal by microalgae in batch experiments

The mixed microalgae consortium was cultivated in a 3-L batch photobioreactor fed with the primary effluent for 2 weeks, which served as the microalgae inoculum for the subsequent batch experiments. A photon flux density of 120 μmol/m^2/s was provided by two cool-white fluorescent lamps with a 12 h/12 h of light/darkness photoperiod at 25 °C. The microalga biomass was continuously mixed with a magnetic stirrer. pH was continuously monitored with a pH sensor (HI1001, HANNA, U.S.A.) and maintained at 7.8 with a pH controller (HI 8711, HANNA, U.S.A.) by the automated addition of 0.1 M HCl and NaOH. This lab-scale set-up was located indoors.

Harvested biomass from the photobioreactor was thickened by gravity settling in Imhoff cones and then the cell number of the thickened biomass was counted by microscopy (BA310, Motic, China). The thickened biomass was added into 300 mL Erlenmeyer flasks containing 200 mL of the primary effluent to make cultures with an initial density of 1 x 10^6 cells/mL. Sodium selenite (Na_2SeO_3) or sodium selenate (Na_2SeO_4) was added to the Erlenmeyer flasks before inoculation at a Se concentration of 0, 10, 25, 50, 100, 200 and 500 μg/L. The cultures were mixed with magnetic stirrers and incubated for 7 days under the same light intensity and photoperiod as described above. All experiments were conducted in duplicate. pH and turbidity were monitored daily. 10 mL of medium was collected and filtered every other day for Se concentration analysis. After 7 days of incubation, the biomass was centrifuged and dried for total Se measurement.

2.3 Se removal in HRAPs and production of Se-enriched biomass

Experiments were carried out in an outdoor pilot plant (May 2019–July 2019) as described in detail by Arashiro et al. (2019) with some modifications. The microalgae species in the HRAPs were observed microscopically (BA310, Motic, China) every week. The medium was mainly composed of *Chlorella* sp. and *Scenedesmus* sp. The effluent from the primary settler (noted as primary effluent) was pumped into two parallel HRAPs: one with continuous spiking with Na_2SeO_3 (HRAP-Se) and another one without Se spiking as a control (HRAP-C).

The two HRAPs received the corresponding influents (53 L/day of wastewater and 6 L/day of Se stock solution (500 µg Se/L) for the HRAP-Se, and 59 L/day of wastewater for the HRAP-C) with an HRT of 8 days during the first 1.5 months. Afterwards, the HRT was adjusted to 4 days until the end of the experiment, by increasing the influent flow rates to twice the level previously mentioned. The flow rates of the Se spike and the wastewater in the HRAP-Se were monitored daily to accurately quantify the Se concentration in the influent. The effluent was collected daily for total Se analysis. The biomass in the secondary clarifiers was accumulated and collected every week.

2.4 Wastewater characterization in the HRAP systems

The wastewater treatment performance was monitored for 3 months. Samples from the influent, effluent and mixed liquor of the two HRAPs (Fig. 1) were collected twice per week for analysis of the following parameters: pH, dissolved oxygen (DO), turbidity, TSS, VSS, total and soluble COD (COD_{tot} and COD_{sol}), total and soluble P (TP and SP), nitrite (NO_2^-), and nitrate (NO_3^-); these parameters were analyzed according to standard methods (APHA-AWWA-WEF, 2012). NH_4^+-N was measured according to the Solórzano method (Solórzano, 1969). Total carbon (TC) and total nitrogen (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 over a 0.45-µm syringe PVDF membrane filter.

2.5 Nutritional parameters of the microalgae

The microalgae biomass collected in the secondary clarifier of the HRAPs at operational week 7 (from day 43 to 50) was rinsed with deionized (DI) water and centrifuged at 4200 rpm for 5 minutes. The centrifuged paste was frozen at -80 °C overnight and then lyophilized for 24 h. The freeze-dried biomass was stored in a -20 °C freezer for subsequent analysis and experiments.

2.5.1 Se speciation, Se bioaccessibility and total Se analysis

Selenium speciation of the freeze-dried microalgae was determined according to Li et al. (2020b). Besides, the bioaccessibility of Se in raw and bead milled microalgae for pigs was simulated in vitro in a two-step incubation based on the method described by Moheimani et al. (2018) and Vu et al. (2019) with minor modifications. Briefly, an amount of freeze-dried sample equivalent to 150 mg protein was weighed into a 100-mL centrifuge tube with 20 mL of simulated gastric juice (1 g pepsin dissolved into 500 mL of 0.075 M HCl) and one drop of 50 g/L thimerosal. The mixture was shaken in a reciprocating thermostatic shaking water bath at 37 °C for 4 h. After gastric digestion, the mixture was cooled down and the pH was adjusted to 7.5 using 0.2 M NaOH followed by adding 15 mL pancreatin solution (375 mg pancreatin dissolved into 250 mL phosphate buffer) to simulate small intestine digestion. The mixture was shaken in a water bath at 37 °C for 4 h, followed by adding 7.5 mL of 0.02 M phosphotungstic acid for deproteination, and afterwards centrifuged at 3000 rpm for 10 min. The supernatant was collected and filtered by a 0.45-μm syringe PVDF membrane filter for analysis of the Se content, which was considered to represent the digestibility in the gastric and intestine phase. Selenium bioaccessibility was determined by the ratio of Se obtained from the gastrointestinal digestion divided by the total amount of Se in the corresponding biomass.

For determination of the total Se concentration in the microalgae, 0.3 g freeze-dried sample was weighed into a digestion vessel followed by the addition of 10 mL concentrated pico-pure HNO_3. 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 digests were diluted to 50 mL with Milli-Q water for Se measurement using inductively coupled plasma-mass spectrometry (ICP-MS, ELAN DRC-e, PerkinElmer, Waltham, MA, USA). Internal standards (10 μg/L ^{103}Rh and ^{69}Ga) and an external multi-element standard solution were used during ICP-MS analysis. Certified reference materials white clover (BCR 402, 6.7 ± 0.25 mg Se/kg) and sea lettuce (BCR 279, 0.59 ± 0.04 mg Se/kg) were included in the analysis as quality control with recoveries of 97 (± 7)% and 106 (± 4%), respectively.

2.5.2 Macromolecular characterization and protein extraction by different cell disruption methods

Microalgae macromolecular composition (i.e., lipid, carbohydrate and crude protein) was determined and calculated over the VSS content. Lipids were extracted by chloroform and methanol (2:1) according to the Soxhlet extraction method (Folch et al., 1957). Carbohydrates were measured by phenol-sulphuric acid method with acid hydrolysis (Dubois et al., 1951) and determined by spectrophotometry (Spectronic Genesys 8, Helsingborg, Sweden). Total crude proteins were measured and quantified according to the total Kjeldahl nitrogen (TKN) method (Kjeldahl, 1883) with a TKN/protein conversion factor of 5.95 (Arashiro et al., 2019).

For different protein extraction method tests, 0.5 g freeze-dried microalgae biomass was dispersed and mixed into 25 mL PBS buffer solution. Five cell disruption methods for the microalgae suspension were investigated and compared: (a) freeze-thawing at -80 °C and 4 °C with 5 cycles; (b) combination of freeze-thawing and ultrasonication (Bandelin Sonouls HD2070, 20 kHz and 2 mm probe) for 30 min with 30 s on/off intervals at 70% amplitude; (c) high-pressure cell disruption (constant cell disruption systems with one-shot model, Northants, UK) at 2.4 kpsi; (d) ball milling (MM 400, Retsch, Haan, Germany) for 10 min at 30 Hz; and (e) bead milling (Powerlyzer 24, MO BIO Laboratories, Carlsbad, CA, USA) at 2000 rpm for 10 min. All experiments were performed in triplicate.

The protein content of the microalgae after each disruption was quantified by the Lowry method with minor modification (Lowry et al., 1951). In brief, 1.0 mL of cell suspension after disruption was vortex mixed with either 3 mL of 7.0% sodium dodecyl sulfate (SDS) solution or 3 mL DI water. The mixture was incubated at 100 ˚C for 5 min and cooled down before centrifuging at 4000 rpm for 10 min. 1.0 mL of the supernatant after centrifugation was collected and vortex mixed with 5.0 mL alkaline copper reagent. After 10 min, 0.5 mL Folin solution was added to react for 30 min in the dark. A spectrophotometer (Spectronic Genesys 8, Helsingborg, Sweden) was used to measure the protein content by measuring the absorbance at 750 nm. A calibration curve was prepared using bovine serum albumin (BSA).

248 2.5.3 Fatty acid and amino acid profiles analysis

249 Fatty acids of the microalgae were analyzed as described by Michiels et al. (2014).
250 Amino acids were analyzed by the lab of nutriFOODchem (Gent University, Belgium).
251 Briefly, the freeze-dried microalgae sample was hydrolyzed with 6 M HCl for 24 h.
252 After neutralization, the amino acids were derivatized in the injector of the HPLC,
253 separated on a C_{18} column and detected fluorometrically. Cysteine was derivatized in
254 the injector of the HPLC with iodoacetic acid (IDA) and o-phthaldialdehyde (OPA),
255 separated on a C_{18} column and detected fluorometrically. All samples were analyzed
256 in duplicate.

257 **2.6 Statistical analysis**

258 Descriptive statistics were performed using Sigma plot 13, Excel 2016 and SPSS
259 20.0. Results are expressed as mean ± standard deviation (SD).

260 **3 Results and discussion**

261 **3.1 Microalgae growth, Se accumulation and Se removal in batch experiments**

262 Fig. 2 shows the turbidity of the microalgae suspension when exposed to different Se
263 concentrations (0–500 µg Se/L). The highest turbidity in both selenite (Se(IV)) (885
264 NTU) and selenate (Se(VI)) (1614 NTU) treatments was observed at 50 µg/L of Se
265 exposure after 7 days of cultivation, being significantly ($p<0.05$) different from the
266 control, which demonstrates that low Se application may stimulate microalgae
267 growth. Fig. 2 further demonstrates that the turbidity significantly increased with
268 incubation time, and a similar turbidity value was observed for the control treatments
269 and the 500 µg Se/L selenite and selenate treatments, indicating that microalgae
270 growing on domestic wastewater treatment could tolerate such high concentrations of
271 Se.

272 Similarly, Reunova et al. (2007) reported positive impacts of selenite on the
273 unicellular alga *Dunaliella salina* (e.g., stimulation of cell growth) after exposure to
274 0.01 and 0.5 mg/L of Se dosed as sodium selenite in nutrient medium prepared in
275 32‰ seawater. Sun et al. (2014) found that Se(IV) concentrations lower than 75 mg

Se/L in BG11 medium promoted *Chlorella vulgaris* growth and acted as an antioxidant by inhibiting lipid peroxidation and formation of intracellular reactive oxygen species (ROS). Accordingly, the growth-stimulating effects of Se for microalgae in this study may be also related to the enhancement of the antioxidant activity in cells, as Se can increase the activity of antioxidant enzymes (e.g., glutathione peroxidases, superoxide dismutase and methionine sulfoxide reductase) and the synthesis of metabolites (such as phytochelatins and ascorbate), resulting in higher ROS scavenging capacity of cells (Sun et al., 2014), and eventually promoting microalgae growth.

The Se concentration in the microalgae differed significantly depending on the chemical form and concentration of the applied Se (Fig. 3). Generally, increasing the Se dosage in the wastewater resulted in a higher Se concentration in the microalgae biomass. The microalgae had a higher ability to take up Se(IV) compared with Se(VI), which is reflected in the around 3 times higher Se content in microalgae cultivated in the selenite amended wastewater compared to the Se(VI) amended wastewater (Fig. 3). The maximum Se content in the microalgae biomass was 67 and 24 mg/kg when exposed to 500 µg Se/L of Se(IV) and Se(VI), respectively. These values are much higher than the Se accumulation in the microalga *Spirulina platensis* (< 22 mg/kg) exposed to nutrient growth medium containing 500 µg Se/L of Se(IV) (Li et al., 2003), and also higher than the Se accumulation in the macroalga *Ulva australis* (around 20 mg/kg) exposed to 50 µM (equivalent to 4.0 mg/L) of Se(IV) or Se(VI) supplemented seawater after 7 days of incubation (Schiavon et al., 2016). Besides, the linear correlation ($R^2 > 0.99$) between the Se concentration in the microalgae and Se application dose indicates that the microalgae may still have the capacity to accumulate higher amounts of Se (Fig.3).

A higher Se accumulation, when exposed to Se(IV) compared to Se(VI), has also been observed in other algae species, such as *Chlamydomonas reinhardtii and Scenedesmus quadricauda* (Vitova et al., 2011; Vriens et al., 2016). The higher uptake of Se(IV) compared to Se(VI) may be attributed to the different uptake mechanisms and metabolism by microalgae, partially similar to those in plants. Se(IV) is mostly taken up in a low-affinity passive way and quickly converted into

organic Se forms (e.g., SeMet and $SeCys_2$) in algae (Li et al., 2020b; Schiavon et al., 2017). In contrast, Se(VI) is taken up in a high-affinity active way through the facilitation of a sulfur transporter, reduced to Se(IV) in cells and then converted into organic Se compounds. The Se(VI) reduction is an ATP-consuming process and the rate-limiting step, which eventually results in a lower Se uptake by microalgae (Schiavon et al., 2017). Besides, the lower Se(VI) uptake of microalgae could be partially attributed to the competition between Se(VI) and sulfate (164 mg/L) in the wastewater.

The efficiency of Se removal by the microalgae is presented in the Supplementary Information. A decreasing trend was observed in Se removal efficiency with increasing Se dose. Accordingly, when microalgae were exposed to Se(VI), the Se removal efficiency was much lower compared to Se(IV) exposure, which was associated with the lower Se(VI) uptake and accumulation in the microalgae cells (Fig. 3). The highest Se removal efficiency was 56 and 19% when microalgae were exposed to 10 μg/L of Se(IV) and Se(VI), respectively. Se(IV) was therefore selected for the subsequent pilot-scale experiment due to the higher Se accumulation ability.

3.2 Wastewater treatment efficiency in HRAPs

The temporal variation of the main parameters in HRAP-Se and HRAP-C over a period of 3 months is shown in Fig. 4. A summary of the average removal efficiencies for the main water quality parameters is calculated and presented in the Supplementary Information. Likewise, the variation of Se content in the influent and effluent of HRAP-Se over the monitoring period is shown in Fig. 5a. No significant differences were observed in the turbidity, TSS, VSS, total and soluble COD, NH_4^+-N, TN, TC, TP and SP removal efficiency throughout the entire experimental period between the HRAP-Se and HRAP-C (Fig. 4 and see in Supplementary Information). The HRAP systems showed high nutrients and organic matter removal efficiencies. Specifically, the average NH_4^+-N and turbidity removal efficiency reached 93% and 91%, respectively. The COD_{tot} and TC removal efficiency ranged between 70 and 66% in the HRAP-Se and HRAP-C throughout the whole experimental period. The average removal efficiencies of TP in HRAP-Se and HRAP-C were up to 77% and 72%, respectively. Despite the very high removal efficiency of NH_4^+-N in the HRAPs,

the TN removal efficiencies were lower (around 65%). This was attributed to the conversion of some NH_4^+-N into NO_3^--N and NO_2^--N (e.g. nitrification). In terms of the HRT influence, no significant differences in removal efficiencies between 8 d and 4 d were observed.

The results of the wastewater treatment efficiency are in accordance with those of previous studies using HRAPs for wastewater treatment (Arashiro et al., 2019). The main mechanism of pollutants (e.g., N, P and C) removal by microalgae in HRAP is biomass assimilation, as microalgae can utilize them for the production of cellular components, such as the synthesis of proteins, nucleic acids and carbohydrates (Hoffmann, 1998). Furthermore, the high removal efficiencies of NH_4^+ and TN can also be partially attributed to ammonia volatilization and organic N settlement, respectively (Zhou et al., 2006). Likewise, previous studies have demonstrated that precipitation of phosphate with other ions, such as calcium and magnesium in HRAPs also contributes to P removal (Delgadillo-Mirquez et al., 2016; Zhou et al., 2006). For organic matter, the decomposition of complex organic carbon compounds by heterotrophic microorganisms in HRAPs (i.e. bacteria and fungi) has been proposed to partially facilitate organic C removal (Mohsenpour et al., 2021).

3.3 Selenium removal in HRAPs, Se bioaccessibility and speciations in Se-enriched microalgae

3.3.1 Se removal in HRAPs

As far as the Se removal efficiency is concerned, no significant difference between an HRT of 8 days (Se removal average 43%) and 4 days (Se removal average of 46%) was observed (Fig. 5a, and more in Supplementary Information). Liu et al. (2019) studied the Se removal efficiency by *Chlorella vulgaris* after exposure to different selenite concentrations in BG11 nutrient medium and found that approximately 51 and 90% of Se was removed upon 500 and 1000-3000 μg/L of Se exposure. This removal was mainly achieved through Se volatilization by facilitating Se methylation by algae upon exposure to highly toxic Se levels (also called Se detoxification mechanism). Besides, Liu et al. (2019) further studied the effect of *Chlorella vulgaris* biomass density on selenite removal under 1580 μg/L of Se

exposure after 3 days of cultivation and concluded that Se accumulation became the main Se removal mechanism at algal densities between 0.75 and 4.03 g dry weight/L, with an average Se removal of 49–62%, which is close to the Se removal efficiency observed in this study (43–46%). Likewise, it might be deduced that the Se removal in this study was mainly via microalgae Se accumulation, as reflected by the suitable biomass density (around 0.42 g DW/L in the HRAPs) and the lower Se exposure dosage (approximately 25–60 μg Se/L) without toxic effects. Additionally, the Se removal efficiency observed in this study was similar to those reported by Gerhardt et al. (1991), who found an average selenate removal of 45% in high-rate aerobic (algae)–anoxic (anaerobic bacteria) ponds treating agricultural drainage water over two years.

3.3.2 Selenium bioaccessibility of microalgae

Bioaccessibility measures the fraction of a substance released from products into the gastrointestinal tract by mimicking the gastric and intestinal digestion through *in vitro* tests (Vu et al., 2019). The digestion model in this study comprised a simulation of both the stomach and intestinal physiology of the pig. According to the results, the bioaccessibility of Se in the ball-milled sample was significantly higher than that in the raw sample (Fig. 5b). This result was expected, as the ball milling would disrupt microalgae cell walls and therefore enhance the Se release from biomass during the gastrointestinal digestion, indicating the importance of pretreatments (i.e. cell disruption) for improving nutrient bioaccessibility. 49 and 63% of the Se in the raw and ball-milled Se-enriched microalgae were solubilized under the gastrointestinal conditions and were thus potentially bioavailable, while the *in vitro* digestibility of Se in the raw and ball-milled microalgae grown in the HRAP-C was 69 and 95%, respectively. The lower digestibility of Se in the Se-enriched microalgae biomass may be attributed to the significantly higher total Se content in the Se-enriched biomass in comparison with the control microalgae, resulting in the incorporation of part of the extra Se in the less digestible microalgae fraction, such as in the hemicellulosic cell wall structure (Gómez-Jacinto et al., 2020).

A similar Se bioaccessibility (~49%) was found in Se-enriched *Chlorella vulgaris* (Vu et al., 2019), while it should be noted that Se bioaccessibility in Se-rich yeast, the most

popular supplement of organic Se, is generally higher in comparison with that of the HRAP-Se grown microalgae. For instance, Lavu et al. (2016) reported that approximately 70% of total Se was bioaccessible in SelenoPrecise tablet (Se-rich yeast); Reyes et al. (2006) observed that 89% of total Se in SEAS 6 (Se-rich yeast, Pharma Nord, Denmark) was extracted after gastrointestinal digestion; Thiry et al. (2013) demonstrated that 57% of Se from Se-yeast (LepiVits, Vincennes, France) was liberated through the in vitro gastrointestinal digestion. Moreover, a Se bioaccessibility of 81% was previously observed in the Se-enriched microalga *Chlorella sorokiniana* (Gómez-Jacinto et al., 2020), which is higher than that in the current study. The higher Se bioaccessibility observed in the above studies might be due to the different digestion methods (e.g. different amount and type of enzymes). Specifically, extra bile salts were adopted in the intestinal juice of the all studies referred to above, while they were not added in the current study. Bile salts assist in the solubilisation of fat, which may result in the dissolution of lipid-bound Se and thus a higher Se bioaccessibility. More enzymes (e.g., extra addition of amylase) and chemicals (e.g., NaCl) were also included in the intestinal juice used by Gómez-Jacinto et al. (2020) and Reyes et al. (2006) to simulate human gastrointestinal digestion in comparison with the juice used in this study. Taking into account that animals could also excrete bile and amylase, further assessment of Se bioaccessibility involving also bile salts and amylase is proposed for the Se-enriched microalgae tested in the current study. Besides, in this study, phosphotungstic acid was added into the extract after gastrointestinal digestion to precipitate undigested protein or peptide, which could correspondingly result in the precipitation of protein/peptide/macromolecule-bound Se and eventually lead to a lower Se bioaccessibility. Also results reported by Reyes et al. (2006) point in this direction, as they observed that 89% of total Se in SEAS 6 was extracted after gastrointestinal digestion, but only 34% of the extracted Se was free SeMet and most of the Se-species seemed to be peptide-bound. Additionally, the difference in microalgae species, Se concentrations or species in the growth medium and microalgae biomass could partially contribute to differences in results between this study and the study of Gómez-Jacinto et al. (2020).

3.3.3 Selenium speciation in Se-enriched microalgae

The chromatogram of Se species in microalgae grown in the HRAP-Se collected at the operational days 43–50 is shown in Supplementary Information. Se-methyl-selenocysteine (SeMetSeCys), Se-methionine (SeMet), Se(IV) and Se(VI) were observed in the sample. 95% of the accumulated Se in the microalgae was converted into organic Se forms. SeMet accounted for the highest proportion (91%) of the identified Se species, whereas the percentage of inorganic Se(IV) and Se(VI) was only 1.9% and 3.0%, respectively. This is consistent with many previous results. For instance, Gómez-Jacinto et al. (2020) found that 95% of the Se taken up by *Chlorella sorokiniana* was transformed into organic Se, and SeMet accounted for 79% of the total Se, when cultivated in Basal medium containing 50 mg/L Se (VI). Vu et al. (2019) demonstrated that SeMet and SeMetSeCys were the predominating Se species in Se-enriched *Chlorella vulgaris* upon selenite (2.25–4.5mg/L) exposure, while Umysova et al. (2009) reported that SeMet made up only 30–40% of the total Se in *Scenedesmus quadricauda* after selenite (10 mg/L) or selenate (20–50 mg/L) exposure.

SeMet, a type of selenoamino acid, is one of the major nutritional sources of Se for higher animals and humans, as these are unable to synthesize SeMet in their organs. Importantly, SeMet is more bioavailable, providing higher Se levels to tissues, than inorganic Se and is beneficial for human and animal health, which is thus claimed as the most suitable form of Se for nutritional supplementation (Gómez-Jacinto et al., 2020). Results in the current study indicate that microalgae cells are capable of accumulating and transforming less-valuable inorganic Se into more-valuable selenoamino acids efficiently.

3.4 Nutritional value of microalgae grown in HRAPs

3.4.1 Protein extraction and macromolecular characterization of microalgae

The total crude Kjeldahl-protein (TKN) content of the microalgae grown in both HRAPs was about 48% (Table 1), which is within the range reported in the literature for microalgae species (Arashiro et al., 2019). This is comparable to that of soybean (38% in full-fat soybeans, 48% for dehulled soybean meal and 44% for non-dehulled soybean meal) (Moheimani et al., 2018), which is currently the primary source of

460 protein for pigs around the world (Moheimani et al., 2018). Additionally, a slightly
461 higher content of carbohydrates and lipids was observed for the biomass grown in
462 the HRAP-Se than that grown in the HRAP-C. Specifically, the biomass grown in the
463 HRAP-C and HRAP-Se was composed of 21% and 32% carbohydrates, and 19%
464 and 21% lipids, respectively, indicating that Se may have the potential to stimulate
465 the biosynthesis of these compounds in microalgae, which is consistent with the
466 results of Gan et al. (2019). The increased carbohydrate levels in microalgae grown
467 in HRAP-Se may be attributed to the enhancement of photosynthesis. Indeed,
468 several studies have demonstrated that Se has the ability to improve the
469 photosynthetic performance of microalgae, as shown by the increase of
470 photosynthetic pigments content (e.g., carotenoid and chlorophyll) and the
471 enhancement of electron transport system activity (Zhong & Cheng, 2017). Similarly,
472 Se may stimulate the enzyme activity of fatty acid synthesis (FAS) in microalgae
473 cells, eventually resulting in higher lipid production in HRAP-Se. This stimulation
474 effect of Se to FAS has also been observed in yeast cells (Kieliszek et al., 2019).

475 The protein content obtained by SDS extraction was much higher than the protein
476 content obtained by DI water extraction (Table 1). The lowest protein content (12–
477 14% for SDS extraction) was observed for the biomass after cell disruption by freeze-
478 thawing, while ball and bead milling of the biomass favored the highest release of
479 protein, i.e. 46–48% upon SDS extraction. This result indicates that ball and bead
480 milling disruption in combination with SDS extraction results in the most efficient
481 solubilization and quantification of proteins in microalgae by the Lowry method, which
482 could provide a reference for protein extraction of microalgae.

483 3.4.2 Amino acids in the microalgae

484 Table 2 compares the amino acid content of microalgae grown in both HRAPs with
485 that of soybeans and soybean meal. The amino acid content of the microalgae grown
486 in both HRAPs was close to that of soybeans (with the exception of glutamic acid),
487 while it was slightly lower than that in soybean meal, except for glycine, threonine,
488 and alanine contents which were higher in the microalgae (Table 2). This result
489 showed that microalgae could be a source of some essential amino acids for
490 animals, such as lysine, threonine, methionine, cystine, isoleucine, histidine, valine,

arginine, phenylalanine and tyrosine, which must be provided in some animals' diets (Mahan and Shields, 1998). Eventhough the content of some essential amino acids (e.g., arginine, lysine and cystine) in the Se-enriched microalgae was slightly lower than that in the soybean, the result still shows the potential of using the produced microalgae as feed/food additive in animal diets, offering a valid alternative taking into account the higher land, water, nutrient and carbon footprint of conventional vegetable protein production (Matassa et al., 2016). However, the Se content in the Se-enriched microalgae should be particularly addressed when using it as a feed additive because European Food Safety Authority (EFSA) regulated that the total Se content in the complete feed can be maximum 0.5 mg Se/kg (EFSA, 2016). Besides, further study should quantify the digestibility of amino acids in the microalgae, as it is also an important factor affecting amino acids utilization efficiency.

3.4.3 Fatty acids in the microalgae

The composition and contents of total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in the microalgae are shown in Table 3. The detailed composition and contents are presented in the Supplementary Information. The data to some extent indicated that microalgae grown in the HRAP-C contained a higher percentage of total SFA and MUFA in comparison with those present in the HRAP-Se, while it had a lower percentage of PUFA omega-3 (ω3) and omega-6 (ω6) (Table 3). Specifically, SFA and MUFA accounted for 32.1 and 10.6% of the fatty acids for the biomass grown in HRAP-C, respectively, compared to 25.0 and 8.17% for biomass grown in the HRAP-Se, respectively. The percentage of PUFA ω6 and ω3 were 10.0 and 17.1% for the biomass grown in the HRAP-C, and 11.8 and 26.1% for the biomass grown in the HRAP-Se, respectively. Among PUFA ω3, eicosapentaenoic (EPA, C20:5) showed the most significant increase in concentration when Se was added to the growth medium (13.2% in versus 24.7% of the fatty acids in HRAP-C and HRAP-Se, respectively) (Supplementary Information). Although further research is still needed to confirm these results, this may to some extent indicate that Se has the potential of contributing to the synthesis of PUFAs. Moreover, previous studies have also shown the ability of Se to activate fatty acid unsaturation and stimulate the formation of

PUFA via inducing desaturase activity, such as ∆12 and ∆15 (Čertík et al., 2013; Kieliszek et al., 2019). Likewise, the increased percentage of PUFAs in microalgae cultivated in HRAP-Se of the current study could be due to the fact that Se may be involved in the induction of fatty acid ∆12, ∆15 and ∆5 desaturases. Noticeably, the elevated PUFA ω3 percentage of microalgae in the HRAP-Se, particularly for the EPA, could demonstrate this, as ∆15 and ∆5 are the main desaturases to catalyze PUFA ω3 and EPA formation (Ma et al., 2016).

PUFA ω3 and ω6, are considered essential fatty acids, being beneficial for human health and livestock nourishment (Moheimani et al., 2018). They have a positive effect on cardio-circulatory diseases, atherosclerosis, coronary disease, degenerative diseases and anticancer (Otleş & Pire, 2001). Besides, the proportion of the PUFA ω6 and ω3 of the biomass in this study is also higher than that of microalgae grown on anaerobically digested piggery effluent (8.7% for ω6 and 15.7% for ω3) (Moheimani et al., 2018). Besides, among the different PUFAs ω3 present in algae, eicosapentaenoic (EPA, C20:5) has the most important nutritional and health value. Its supplementation can be co-therapeutic (Doughman et al., 2007). The EPA proportion of the biomass grown in the HRAP-Se is higher than that of commercial products on the market, such as salmon (14% EPA) and fish (18% EPA) oil (Otleş & Pire, 2001), which is considered favorable for animal and human nutrition. The value-added biomass produced from HRAPs spiked with Se could thus offer a promising alternative source of valuable PUFAs.

Importantly, microalgae grown on wastewater can harbor a risk of pathogen transfer when they are consumed as feed, quantification of pathogen loads on the microalgae is therefore necessary in this study. The contents of selected manure-borne bacteria of fresh and dried HRAP grown biomass are shown in Supplementary Information. The results indicate that further downstream processing after harvest, such as drying, could reduce the bacterial loads of microalgae and avoid the pathogen risk, supporting the application of microalgae grown on domestic wastewater as a potential feed supplement. However, further risk assessment is still required, such as *in vivo* studies and quantification of other safety parameters (e.g., residues of mycotoxins, antibiotics, and nucleic acids).

4 Conclusions

This study highlighted that HRAPs-grown microalgae are good candidates to upgrade nutrients in wastewater and carbon dioxide into Se-enriched microalgae biomass that can be used as feed supplements, as shown by the comparable nutritional properties of Se-enriched microalgae and soybean meal, the high amount of SeMet (91%) accumulation in their cells, and the relatively high Se digestibility of the (ball-milled) microalgae. However, the Se bioaccessibility in the microalgae may still be further improved, e.g., by further testing and optimization of pretreatments. Moreover, standardization of assessment methods for bioaccessibility may enable more accurate comparison of bioaccessibility data reported by different researchers.

E-supplementary data for this work can be found in e-version of this paper online.

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Figures captions

Figure 1. Scheme of the HRAPs treating domestic wastewater. HRAP-Se is the line with Se spiking and HRAP-C is the line without Se spiking, which served as control. Sampling points are 1: primary effluent (also called influent of the HRAPs), 2: mixed liquor of the HRAPs, 3: secondary effluent.

Figure 2. Biomass growth, measured as turbidity (NTU), during batch incubation in domestic wastewater supplemented with varying Se concentrations (µg/L), (a) selenite and (b) selenate. Values are mean ± standard deviation (n=3).

Figure 3. Se concentration in the microalgal biomass grown in wastewater with different selenite and selenate concentrations. Values are mean ± standard deviation (n=3).

Figure 4. Influent (●) and effluent (■) concentration of turbidity, total suspended solids (TSS), volatile suspended solids (VSS), total carbon (TC), total and soluble chemical oxygen demand (COD_{tot} and COD_{sol}), total and soluble P (SP and TP), total nitrogen (TN), and NH_4^+-N monitored in the HRAP-Se (with Se spiking, left) and HRAP-C (without Se spiking, right) systems over the experimental period.

Figure 5. Se removal by high rate algae pond (HRAP) and Se bioaccessibility in the produced microalgae: (a) Influent and effluent concentrations of total Se in the HRAP-Se with continuous selenite spiking during the experimental period (HRT: hydraulic retention time), and (b) Bioaccessibility of Se in the raw and ball-milled microalgae grown in both the HRAP-C (Control) and HRAP-Se (Se-enriched microalgae). Values are mean ± standard deviation (n=3).

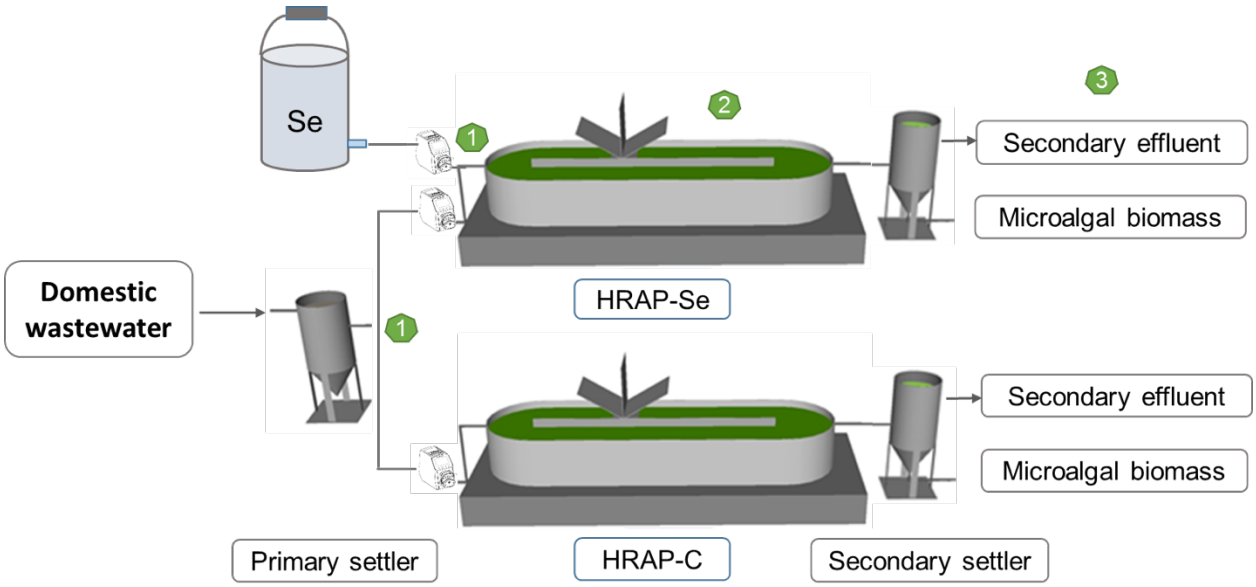
Tables Captions

Table 1. Protein content of microalgae subjected to different cell disruption techniques and biochemical composition (%) of microalgae grown in the HRAPs. Results are reported as percentage of the total volatile suspended solids (VSS). Values are mean ± standard deviation (n=3).

Table 2. Amino acid contents of microalgae grown in HRAPs at operational week 7 compared with soybeans and soybean meal (SBM) for pigs. Values are mean ± standard deviation (n=3).

Table 3. Fatty acid composition and content of the microalgae grown in the control (HRAP-C) and Se spiked microalgae pond (HRAP-Se). SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; ω3 and 6: omega-3 and 6. Values are mean ± standard deviation (n=3).

Figures



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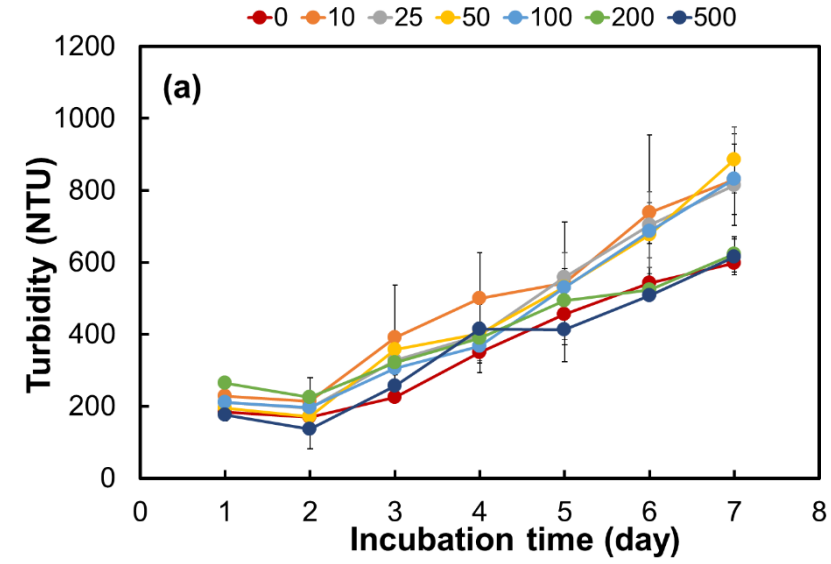


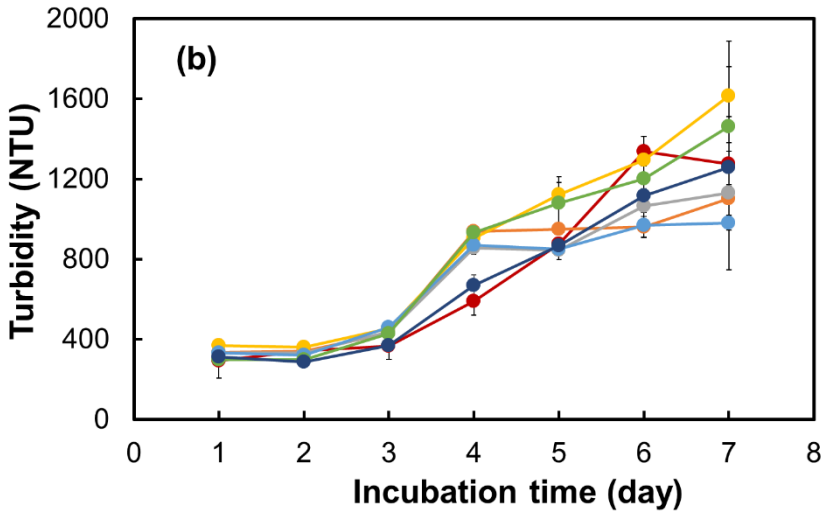


Figure 2. Biomass growth, measured as turbidity (NTU), during batch incubation in domestic wastewater supplemented with varying Se concentrations (μg/L), (a) selenite and (b) selenate. Values are mean ± standard deviation (n=3).

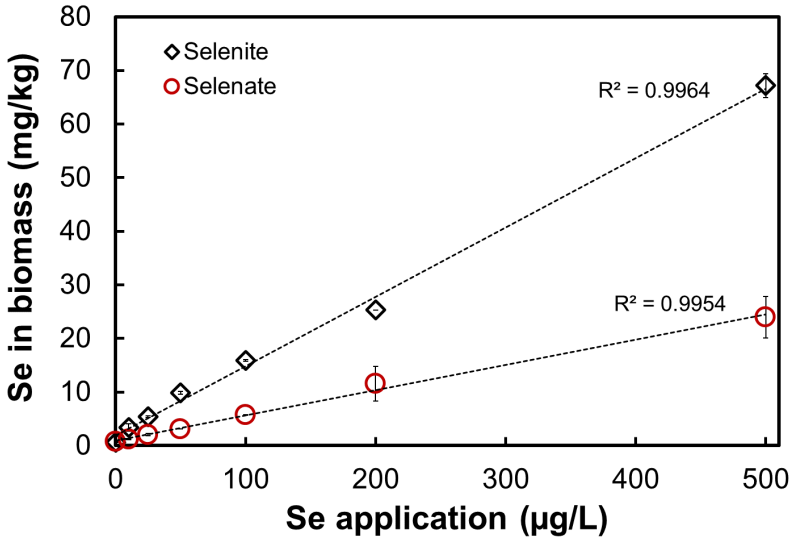


Figure 3. Se concentration in the microalgal biomass grown in wastewater with different selenite and selenate concentrations. Values are mean ± standard deviation (n=3).

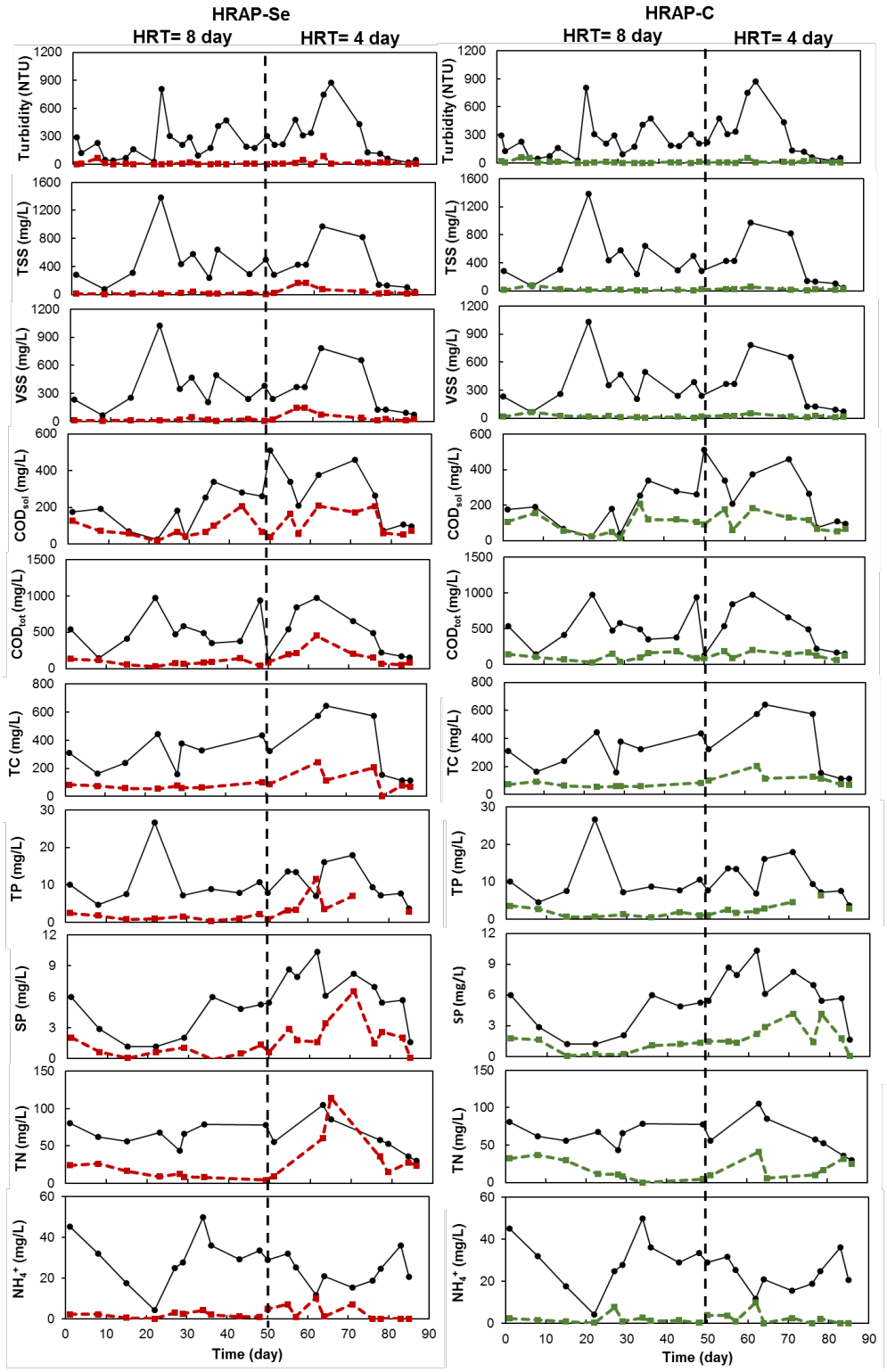
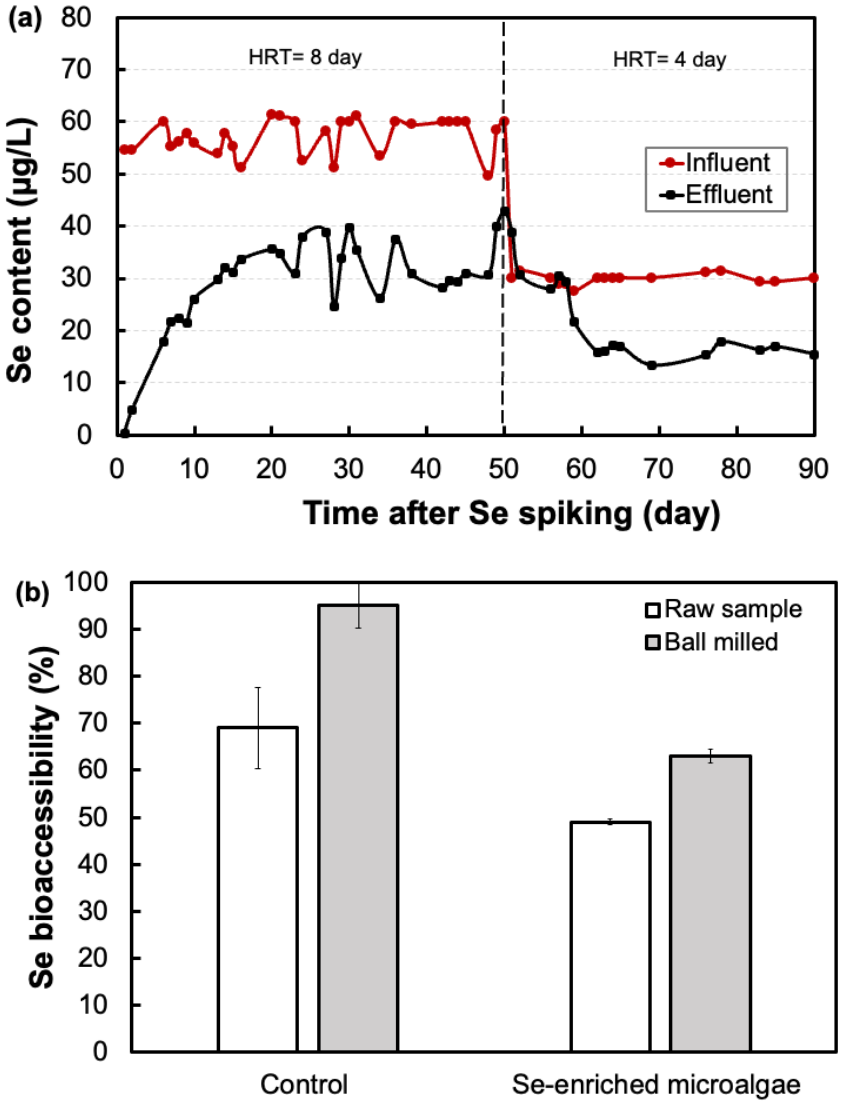


Figure 4. Influent (●) and effluent (■) concentration of turbidity, total suspended solids (TSS), volatile suspended solids (VSS), total carbon (TC), total and soluble chemical oxygen demand (COD_{tot} and COD_{sol}), total and soluble P (TP and SP), total

768 nitrogen (TN), and NH_4^+-N monitored in the HRAP-Se (with Se spiking, left) and HRAP-
769 C (without Se spiking, right) systems over the experimental period.



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772 **Figure 5**. Se removal by high rate algae pond (HRAP) and Se bioaccessibility in the
773 produced microalgae: (a) Influent and effluent concentrations of total Se in the HRAP-
774 Se with continuous selenite spiking during the experimental period (HRT: hydraulic
775 retention time), and (b) Bioaccessibility of Se in the raw and ball-milled microalgae
776 grown in both the HRAP-C (Control) and HRAP-Se (Se-enriched microalgae). Values
777 are mean ± standard deviation (n=3).

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Tables

Table 1. Protein content of microalgae subjected to different cell disruption techniques and biochemical composition (%) of microalgae grown in the HRAPs. Results are reported as percentage of the total volatile suspended solids (VSS). Values are mean ± standard deviation (n=3).

		HRAP-C		HRAP-Se	
		H_2O-Lowry[a]	SDS-Lowry[b]	H_2O-Lowry[a]	SDS-Lowry[b]
Protein content after application of different cell disruption techniques	Freeze-thawing	3.2 ± 0.0	14 ± 0.7	2.4 ± 0.1	12 ± 2.5
	Sonication	9.4 ± 0.7	18 ± 1.4	10 ± 0.3	16 ± 0.8
	High-pressure cell disruption	7.5 ± 0.5	24 ± 5.7	10 ± 0.4	32 ± 1.7
	Ball milling	4.5 ± 0.3	46 ± 1.8	5.9 ± 0.4	48 ± 1.2
	Bead milling	10 ± 2.3	47 ± 4.8	7.6 ± 1.9	48 ± 6.3
Macromolecular composition	Kjeldahl-protein [c]	47.6		48.4	
	Carbohydrates	20.5		31.9	
	Lipids	18.7		20.9	

[a] Disrupted microalgae cell suspension was incubated at 100 °C for 5 min with DI water followed by Lowry protein measurement.

[b] Disrupted microalgae cell suspension was incubated at 100 °C for 5 min with SDS solution followed by Lowry protein measurement.

[c] Total protein content was calculated by multiplying the total Kjeldahl nitrogen by 5.95.

792 **Table 2.** Amino acid contents of microalgae grown in HRAPs at operational week 7
793 compared with soybeans and soybean meal (SBM) for pigs. Values are mean ±
794 standard deviation (n=3).

Amino acid	HRAP-C	HRAP-Se	Soybeans	Soybean meal
		[g/100g DW]		
Aspartic acid	3.11 ± 0.05	2.57 ± 0.03	3.89	4.88
Glutamic acid	3.65 ± 0.09	3.06 ± 0.04	6.05	7.87
Asparagine	*N.D.*	*N.D.*	--	--
Serine	1.48 ± 0.03	1.25 ± 0.01	1.67	2.14
Glutamine	0.15 ± 0.01	*N.D.*	--	--
Histidine	0.60 ± 0.00	0.56 ± 0.01	0.88	1.26
Glycine	2.05 ± 0.01	1.60 ± 0.03	1.52	1.89
Threonine	1.78 ± 0.04	1.49 ± 0.02	1.42	1.76
Citrulline	*N.D.*	*N.D.*	--	--
Arginine	1.91 ± 0.04	1.74 ± 0.03	2.45	3.17
Alanine	3.03 ± 0.05	2.15 ± 0.03	1.59	1.92
Tyrosine	1.25 ± 0.02	1.02 ± 0.02	1.20	1.55
Valine	1.81 ± 0.04	1.39 ± 0.04	1.73	1.93
Methionine	0.60 ± 0.01	0.51 ± 0.02	0.55	0.60
Phenylalanine	1.68 ± 0.03	1.38 ± 0.02	1.74	2.26
Isoleucine	1.45 ± 0.03	1.18 ± 0.04	1.60	1.96
Ornithine	0.19 ± 0.00	*N.D.*	--	--
Leucine	2.79 ± 0.06	2.18 ± 0.04	--	--
Lysine	1.76 ± 0.01	1.49 ± 0.02	2.23	2.76
Hydroxyproline	*N.D.*	*N.D.*	--	--
Proline	1.66 ± 0.02	1.54 ± 0.01	--	--
Cysteic acid	0.25 ± 0.00	0.25 ± 0.00	0.59	0.68
Total	31.24 ± 0.52	25.76 ± 0.41	--	--

795 Data of soybeans and soybean meal from Moheimani et al. (2018).

796 N.D.= not detected

797 -- = no data shown

Li, J., Otero-Gonzalez, L., Michiels, J., Lens, P. N. L., 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.

798

799 **Table 3**. Fatty acid composition and content of the microalgae grown in the control
800 (HRAP-C) and Se spiked microalgae pond (HRAP-Se). SFA: saturated fatty acids;
801 MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; ω3 and 6:
802 omega-3 and 6. Values are mean ± standard deviation (n=3).

	HRAP-C		HRAP-Se	
	[% relative fat]	[mg/100 g]	[% relative fat]	[mg/100 g]
Total SFA	32.1 ± 0.1	1359 ± 8	25.0 ± 0.1	2215 ± 4
Total MUFA	10.6 ± 0.1	450 ± 4	8.17 ± 0.0	773 ± 3
Total PUFA ω-6	10.0 ± 0.0	426 ± 3	11.8 ± 0.0	1048 ± 10
Total PUFA ω-3	17.1 ± 0.1	726 ± 4	26.1 ± 0.1	2310 ± 12

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