Selenium and zinc enriched bioproducts generated from wastewater as micronutrient feed supplements and biofertilizers

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Summary

Selenium (Se) is an essential micronutrient for humans and animals with a narrow window between deficiency and toxicity levels. Dietary Se intake of humans and animals is not adequate in some regions. On the other hand, Se toxicity also frequently occurs worldwide, due to water or soil contamination, as Se is widely applied in or released from industrial and agricultural activities. The trace element zinc (Zn) is also often present in too low concentrations in agricultural soils, but is also toxic at elevated concentrations.

Improvement of the dietary Se and Zn intake through enrichment of food and feed crops (named biofortification) is currently being explored as a possible solution for Se and Zn deficiency. Supplementation of feed and food products with Se and Zn is another solution. In biofortification, the application of conventional chemical Se/Zn fertilizers to increase the Se/Zn content in crops could result in secondary soil and water contamination due to the low utilization rate of Se/Zn and fast leaching. Slow-release Se/Zn-enriched fertilizers may therefore be beneficial. Moreover, the use of Se/Zn originating from primary mining for the production of Se/Zn enriched-feed/food supplements is not considered economically and environmental-friendly, taking into account that external Se/Zn is being used and the excess chemicals are then currently being discharged as waste. It may thus be beneficial from an economic and environmental point of view to produce slow release Se/Zn-enriched biofertilizers or Se/Zn-enriched feed supplements locally from Se/Zn-bearing water while partially cleaning the water. This may contribute to the worldwide drive for resource recovery and circular economy. Therefore, this thesis aimed to explore the potential of Se/Zn-enriched bioproducts produced from wastewater treatment processes by eco-technologies (phytoextraction, bioreduction and microalgae-based systems) as Se/Zn feed supplements and biofertilizers.

Chapter 1 and **Chapter 2** present the motivation, objectives and background information on the occurrence of Se and Zn in human and animal diets and their deficiency and toxicity for humans and animals. Current studies regarding micronutrient biofortification and the production of Se and Zn supplements to tackle micronutrient deficiency are discussed. This is followed by a discussion on the paradigm shift from waste treatment to resource recovery, highlighting the potential of

biobased technologies for micronutrient recovery from wastewater, while producing micronutrient-enriched feed/food supplements and biofertilizers.

Since Se can replace sulfur in amino acids and Zn can also be complexed by functional groups in proteins, protein-rich plants have an immense potential for the bioaccumulation/biofortification of these micronutrients. Thus, two aquatic plants (*Lemna* and *Azolla*) with substantial protein content were applied in **Chapter 3** to evaluate the possibility of Se and Zn bioaccumulation/removal from wastewater while producing micronutrient-enriched dietary proteins (for feed/food supplements) and biofertilizers. Nutrient-medium spiked with different concentrations of Se and Zn was used to mimic wastewater. Results of **Chapter 3** demonstrated that both *Lemna* and *Azolla* can accumulate high levels of Se and Zn, while they take up around 10 times more Se(IV) than Se(VI) from the medium. Besides, high transformation to organic Se forms and accumulation in plants after taking up Se(IV), together with the high protein content and fast growth rate, makes *Lemna* (also named duckweed later on) and *Azolla* good candidates for the production of Se- and Zn-enriched biomass, which can be used as crop fertilizers or protein-rich food/feed supplements or ingredients. Considering that a synergetic effect between Se and Zn in *Lemna*, but an antagonistic effect in *Azolla* was observed in **Chapter 3**, *Lemna* loaded with Se/Zn was selected for the subsequent experiments in **Chapter 4** and **Chapter 5**.

Subsequently, **Chapter 4** and **Chapter 5**, respectively, evaluated the valorization potential of the produced micronutrient-enriched duckweed as well as sludge generated in wastewater treatment processes containing single Se or Se combined with Zn as micronutrient biofertilizers. This was conducted in pot experiments using green beans (*Phaseolus vulgaris*). Micronutrient-enriched sludge dominated by the presence of Se in zero oxidation state (Se(0)) was found to be the preferred slow-release Se biofertilizer and an effective Se source to produce Se-enriched beans for Se-deficient populations. This was motivated by the higher Se bioavailability and lower organic carbon content released into the soil from micronutrient-enriched sludge, enabling a higher soil Se supply, as compared to micronutrient-enriched duckweed. The remarkably higher organic carbon content in the soil could result in Se immobilization. On the contrary, the Zn content in the seeds of beans was not successfully improved through the application of micronutrient-enriched biofertilizers in comparison with the control. This could be attributed to the lower Zn translocation rate

from plant roots to seeds, and the lower Zn amount applied into soils as Se/Zn-enriched biomaterials.

Additionally, microalgae have a great capacity to assimilate/remove excess nutrients from the corresponding growth medium (or wastewater) and metabolize them into valuable compounds such as protein, fatty acids, vitamins and carbohydrates. Microalgae are thus a potential protein source to substitute common animal and plant proteins (e.g. soybean). **Chapter 6** explored the potential of Se removal in high rate algae ponds (HRAPs) treating domestic wastewater, while producing high-value Se-enriched biomass that may be used as feed supplement (dietary protein) or biofertilizer. Results indicated that the wastewater treatment performance of the HRAPs was effective. The produced Se-enriched microalgae in HRAPs fed with domestic wastewater contained a high content of crude protein (48% of volatile suspended solids) and the selenoamino acid selenomethionine (SeMet) (91% of total Se). Besides, the essential amino acid content of the microalgae was comparable to that of soybean, an animal feed protein. This Chapter also highlighted that Se may potentially induce the production of the polyunsaturated fatty acids omega-3 (ω3) and omega-6 (ω6), and eicosapentaenoic (EPA) in microalgae, although further research is still needed to confirm this. Therefore, the production of Se-enriched microalgae in HRAPs may offer a promising alternative for upgrading low-value recovered resources into high-value feed supplements.

Chapter 7 aimed to evaluate the Se-enriched microalgae generated in Chapter 6 as a potential biostimulant to enhance plant growth and as a Se biofertilizer to improve the Se content of plants. Raw Se-enriched microalgal biomass and extracts thereof were applied in the production of green beans (*Phaseolus vulgaris*) through soil and foliar application. This study demonstrated that the application of raw Se-enriched microalgae biomass to soil (1-10%, soil application) and its extracts to leaves (1%, foliar spray) enhanced plant growth, which confirmed that Se-enriched microalgae acts as a biostimulant. Besides, a higher Se content in the plant and soil (for soil application) was achieved after the application of Se-enriched microalgae or extracts thereof. This indicated that Se-enriched microalgae cultivated during wastewater treatment can be valorized as a biostimulant and biofertilizer to improve both the seed yields and Se content of beans, leading to a higher market value of the beans.

Chapter 8 concluded and discussed the key findings of this thesis. It also highlighted the limitations of the study. The whole thesis contributes to offering an environmentally friendly and sustainable way for micronutrient biofortification/supplementation in Se/Zn-deficient areas, while recovering nutrients from wastewater.

Samenvatting

Selenium (Se) is een essentiëel micronutriënt voor mens en dier met een nauw bereik tussen deficiëntie en toxiciteit. De inname van Se via de voeding van mens en dier is in sommige regio's niet voldoende. Anderzijds komt Se-toxiciteit ook wereldwijd veel voor als gevolg van water- of bodemverontreiniging, aangezien Se veel wordt toegepast in of vrijkomt bij industriële en agrarische activiteiten. Het sporenelement zink (Zn) komt ook vaak in te lage concentraties voor in landbouwbodems, maar is bij verhoogde concentraties giftig.

Verbetering van de dieetopname van Se en Zn door verrijking van voedsel- en voedergewassen, biofortificatie, wordt momenteel onderzocht als een mogelijke oplossing voor Se- en Zn-deficiëntie. Suppletie van voeder en voedingsmiddelen met Se en Zn is een andere oplossing. Bij biofortificatie kan de toepassing van conventionele chemische Se/Zn-meststoffen om het Se/Zn-gehalte in gewassen te verhogen, leiden tot secundaire bodem- en waterverontreiniging vanwege de lage benuttingsgraad van Se/Zn en de snelle uitspoeling. Se/Zn-verrijkte meststoffen met vertraagde afgifte kunnen daarom nuttig zijn. Bovendien wordt het gebruik van Se/Zn afkomstig uit de primaire mijnbouw voor de productie van met Se/Zn verrijkte diervoeders en voedingssupplementen niet als economisch en milieuvriendelijk beschouwd, rekening houdend met het feit dat extern Se/Zn wordt gebruikt en de overtollige chemicaliën momenteel als afval worden geloosd. Het kan dus vanuit economisch en ecologisch oogpunt voordelig zijn om Se/Zn-verrijkte biomeststoffen met vertraagde afgifte of Se/Zn-verrijkte voedingssupplementen lokaal te produceren uit Se/Zn-houdende afvalwaters, terwijl het afvalwater gereinigd wordt. Dit kan bijdragen aan het wereldwijde streven naar terugwinning van hulpbronnen en circulaire economie. Daarom was dit proefschrift gericht op het onderzoeken van het potentieel van Se/Zn-verrijkte bioproducten, geproduceerd uit afvalwaterbehandelingsprocessen door middel van eco-technologieën (fyto-extractie, bioreductie en op microalgen gebaseerde methoden), als Se/Zn-voedingssupplementen en biofertilizers.

Hoofdstuk 1 en **Hoofdstuk 2** geven de motivatie, doelstellingen en achtergrondinformatie over het voorkomen van Se en Zn in de voeding van mensen en dier en hun deficiëntie en toxiciteit voor mens en dier. Huidige studies met betrekking tot de biofortificatie van micronutriënten en de productie van Se- en Zn-

supplementen om het tekort aan micronutriënten aan te pakken, worden besproken. Dit wordt gevolgd door een discussie over de paradigmaverschuiving van afvalverwerking naar terugwinning van hulpbronnen, waarbij het potentieel van biogebaseerde technologieën voor het terugwinnen van micronutriënten uit afvalwater wordt belicht, terwijl met micronutriënten verrijkte voeder-/voedingssupplementen en biomeststoffen worden geproduceerd.

Omdat Se het element zwavel in aminozuren kan vervangen en Zn ook kan worden gecomplexeerd door functionele groepen in eiwitten, hebben eiwitrijke planten een enorm potentieel voor de bioaccumulatie/biofortificatie van deze micronutriënten. Zo werden in **Hoofdstuk 3** twee waterplanten, *Lemna* en *Azolla*, met een substantieel eiwitgehalte, gebruikt om de mogelijkheid van bioaccumulatie/verwijdering van Se en Zn uit afvalwater te evalueren tijdens de productie van met micronutriënten verrijkte voedingseiwitten (voor voeder-/voedingssupplementen) en biomeststoffen. Nutriënten-medium verrijkt met verschillende concentraties Se en Zn werd gebruikt om afvalwater na te bootsen. De resultaten van **Hoofdstuk 3** toonden aan dat zowel *Lemna* als *Azolla* hoge niveaus van Se en Zn kunnen accumuleren, terwijl ze ongeveer 10 keer meer Se(IV) dan Se(VI) uit het medium opnemen. Bovendien maakt de hoge transformatie naar organische Se-vormen en hoge Se accumulatie in planten na het opnemen van Se(IV), samen met het hoge eiwitgehalte en de snelle groeisnelheid, *Lemna* (later eendenkroos genoemd) en *Azolla* goede kandidaten voor de productie van Se- en Zn-verrijkte biomassa, die kan worden gebruikt als gewasbemesting of als eiwitrijk voeder-/voedingssupplementen. Gezien een synergetisch effect tussen Se en Zn in *Lemna*, maar een antagonistisch effect in *Azolla* werd waargenomen in **Hoofdstuk 3**, werd *Lemna* verrijkt met Se/Zn geselecteerd voor de daaropvolgende experimenten in **Hoofdstuk 4** en **Hoofdstuk 5**.

Vervolgens hebben respectievelijk **Hoofdstuk 4** en **Hoofdstuk 5** het valorisatiepotentieel geëvalueerd van het geproduceerde eendenkroos en slib dat wordt gegenereerd in afvalwaterzuiveringsprocessen die enkelvoudig Se of Se gecombineerd met Zn bevatten als micronutriënten biomeststoffen. Dit werd uitgevoerd in potproeven met sperziebonen (*Phaseolus vulgaris*). Met micronutriënten verrijkt slib, gedomineerd door de aanwezigheid van Se in oxidatietoestand nul (Se (0)), bleek de preferentiële Se-biomeststof met langzame afgifte en een effectieve Se-bron te zijn om Se-verrijkte gewassen te produceren voor Se-deficiënte populaties. Dit werd

gemotiveerd door de hogere biologische beschikbaarheid van Se en het lagere organische koolstofgehalte dat in de bodem vrijkomt uit het met micronutriënten verrijkte slib, waardoor een hogere Se-aanvoer in de bodem mogelijk is in vergelijking met het met micronutriënten verrijkte eendenkroos. Het opmerkelijk hogere gehalte aan organische koolstof in de bodem kan leiden tot immobilisatie van Se. In tegenstelling, het Zn-gehalte in de zaden van bonen werd niet succesvol verbeterd door de toepassing van met micronutriënten verrijkte biomaterialen in vergelijking met de controle. Dit kan worden toegeschreven aan de lagere translocatie van Zn van plantenwortels naar zaden, en de lagere toegepaste hoeveelheid Zn in bodems als met Se/Zn verrijkte biomaterialen.

Microalgen hebben een groot vermogen om overtollige voedingsstoffen uit het overeenkomstige groeimedium (of afvalwater) te assimileren/verwijderen en deze om te zetten in waardevolle componenten zoals eiwitten, vetzuren, vitamines en koolhydraten. Microalgen zijn dus een potentiële eiwitbron ter vervanging van gewone dierlijke en plantaardige eiwitten (bijv. soja). **Hoofdstuk 6** onderzocht de mogelijkheid van Se-verwijdering door middel van intenrieve algenvijvers (HRAPs) die huishoudelijk afvalwater behandelen en tegelijkertijd waardevolle Se-verrijkte biomassa produceren die kan worden gebruikt als een mogelijk voedersupplement (voedingseiwit) of biomeststof. De resultaten gaven aan dat de performantie van de HRAPs op het gebied van afvalwaterbehandeling effectief was. De geproduceerde met Se verrijkte microalgen in HRAPs gevoed met huishoudelijk afvalwater bevatten een hoog gehalte aan ruw eiwit (48% vluchtige gesuspendeerde vaste stof) en het selenoaminozuur selenomethionine (SeMet) (91% van totaal Se). Bovendien was het essentiële aminozuurgehalte van de microalgen vergelijkbaar met dat van sojabonen, een diervoederproteïne. Dit hoofdstuk benadrukte ook dat Se mogelijks de productie van de meervoudig onverzadigde vetzuren omega-3 (ω3) en omega-6 (ω6) en eicosapentaeenzuur (EPA) in microalgen kan induceren, hoewel verder onderzoek nodig is om dit te bevestigen. Daarom kan de productie van met Se verrijkte microalgen in HRAPs een veelbelovend alternatief bieden voor het upgraden van laagwaardige afvalstromen tot hoogwaardige voedersupplementen.

Hoofdstuk 7 had als doel de met Se verrijkte microalgen die in Hoofdstuk 6 werden gegenereerd te evalueren als een potentiëel biostimulant om de plantengroei te bevorderen en als een Se biomeststof om het Se-gehalte van planten te verbeteren.

Ruwe Se-verrijkte microalgenbiomassa en extracten daarvan werden toegepast bij de productie van sperziebonen (*Phaseolus vulgaris*) via bodem- en bladtoepassing. Deze studie toonde aan dat de toepassing van ruwe Se-verrijkte microalgenbiomassa op de bodem (1-10%, bodemtoepassing) en de extracten ervan op bladeren (1%, bladspray) de plantengroei verbeterde, wat bevestigde dat met Se verrijkte microalgen werken als een biostimulant. Bovendien werd een hoger Se-gehalte in de plant en bodem (voor bodemtoepassing) bereikt na het aanbrengen van met Se verrijkte microalgen of extracten daarvan. Dit gaf aan dat Se-verrijkte microalgen die tijdens de afvalwaterzuivering worden gekweekt, kunnen worden gevaloriseerd als biostimulant en biomeststof om de opbrengst van zaden en het Se-gehalte van bonen samen te verbeteren, wat leidt tot een hogere marktwaarde van de bonen.

Hoofdstuk 8 concludeert en bediscussieert de belangrijkste bevindingen van dit proefschrift. Het benadrukt ook de beperkingen van het onderzoek. Dit proefschrift draagt bij aan de ontwikkeling van een milieuvriendelijke en duurzame manier voor biofortificatie en supplementatie van micronutriënten in Se/Zn-deficiënte gebieden, terwijl nutriënten worden teruggewonnen uit afvalwater.

Resumen

El selenio (Se) es un micronutriente esencial para los humanos y animales, con poco margen entre los niveles de deficiencia y toxicidad. Actualmente, la ingesta de Se por parte de humanos y animales no es adecuada en algunas regiones. Por otro lado, la toxicidad por Se también ocurre con frecuencia en todo el mundo, debido a la contaminación del agua o del suelo, ya que el Se se aplica o se libera ampliamente en actividades industriales y agrícolas. El oligoelemento zinc (Zn) también suele estar presente en concentraciones demasiado bajas en suelos agrícolas, pero también es tóxico en concentraciones elevadas.

Actualmente se está estudiando la mejora de la ingesta de Se y Zn mediante el enriquecimiento de cultivos alimenticios y piensos (denominado biofortificación) como una posible solución para la deficiencia de Se y Zn. La suplementación de piensos y productos alimenticios con Se y Zn es otra solución. En cuanto a la biofortificación, la aplicación de fertilizantes químicos convencionales de Se/Zn para aumentar el contenido de Se/Zn en los cultivos podría causar la contaminación secundaria del suelo y del agua debido a la baja tasa de utilización de Se/Zn y su rápida lixiviación. Por lo tanto, la aplicación de fertilizantes enriquecidos con Se/Zn de liberación lenta sería más adecuada. Además, el uso de Se/Zn procedente de la minería para la producción de piensos/complementos alimenticios enriquecidos con Se/Zn no se considera ni económico ni respetuoso con el medio ambiente, teniendo en cuenta que se está utilizando Se/Zn externo y que los productos químicos excedentarios actualmente se desechan como un residuos. Por lo tanto, sería más beneficioso, desde un punto de vista económico y ambiental, producir localmente biofertilizantes enriquecidos con Se/Zn de liberación lenta o suplementos alimenticios enriquecidos con Se/Zn a partir de aguaresidual que contenga Se/Zn, a la vez que se trata el aguaresidual. Esto contribuiría al impulso mundial de la recuperación de recursos y la economía circular. Por lo tanto, el objetivo de esta tesis fue explorar el potencial de los bioproductos enriquecidos con Se/Zn, producidos a partir del tratamiento de aguasresiduales mediante eco-tecnologías (fitoextracción, biorreducción y sistemas basados en microalgas) como complementos alimenticios y biofertilizantes enriquecidos en Se/Zn.

En el **Capítulo 1** y el **Capítulo 2** se presenta la motivación, los objetivos y antecedentes en cuanto a la presencia de Se y Zn en las dietas de humanos y animales, y su deficiencia y toxicidad para humanos y animales. Se discuten estudios actuales sobre la biofortificación de micronutrientes y la producción de suplementos de Se y Zn para abordar la deficiencia de micronutrientes. A esto le sigue una discusión sobre el cambio de paradigma del tratamiento de residuos a la recuperación de recursos, destacando el potencial de las biotecnologías para la recuperación de micronutrientes de las aguasresiduales, al tiempo que se producen suplementos alimenticios/piensos y biofertilizantes enriquecidos con micronutrientes.

Dado que el Se puede reemplazar el azufre en los aminoácidos y el Zn también puede formar complejos con grupos funcionales de las proteínas, las plantas ricas en proteínas tienen un inmenso potencial para la bioacumulación/biofortificación de estos micronutrientes. Por lo tanto, en el **Capítulo 3** se utilizaron dos plantas acuáticas (*Lemna* y *Azolla*) con un contenido sustancial de proteínas para evaluar la bioacumulación/eliminación de Se y Zn del aguaresidual, a la vez que se producían proteínas enriquecidas con micronutrientes (para piensos/complementos alimenticios). Se usó un medio de cultivo enriquecido con diferentes concentraciones de Se y Zn para simular el aguaresidual. Los resultados del Capítulo 3 demostraron que tanto *Lemna* como *Azolla* pueden acumular altos niveles de Se y Zn, mientras que absorben alrededor de 10 veces más Se(IV) que Se(VI) del medio. Además, la alta transformación a formas orgánicas de Se y la acumulación en las plantas después de absorber Se(IV), junto con el alto contenido de proteínas y la rápida tasa de crecimiento, hacen que *Lemna* (también llamada lenteja de agua en adelante) y *Azolla* sean buenas candidatas para la producción de biomasa enriquecida con Se y Zn, que se puede utilizar como biofertilizante para cultivos o como suplemento alimenticio/pienso rico en proteínas. Considerando el efecto sinérgico entre Se y Zn que se observó para *Lemna*, y antagónico para *Azolla*, se seleccionó *Lemna* enriquecida con Se/Zn para los siguientes experimentos del **Capítulo 4** y **Capítulo 5**.

Así, en el **Capítulo 4** y el **Capítulo 5** se evaluaó el potencial de valorización de la lenteja de agua, así como de los lodos de depuradora, con Se simple o Se combinado con Zn, como biofertilizantes de micronutrientes. Esto se llevó a cabo con experimentos en macetas utilizando judías verdes (*Phaseolus vulgaris*) como cultivo. El lodo enriquecido con micronutrientes, con Se en estado de oxidación cero (Se (0)),

resultó ser el biofertilizante de Se de liberación lenta más adecuado, siendo una fuente de Se eficaz para producir habas enriquecidas en Se para poblaciones deficientes en Se. Esto fue resultado de la mayor biodisponibilidad del Se y el menor contenido de carbono orgánico liberado al suelo en el caso de los lodos, lo que permitió un mayor suministro de Se al suelo, en comparación con la lenteja de agua enriquecida con micronutrientes. Esto se debe a que un contenido de carbono orgánico notablemente más alto en el suelo puede resultar en la inmovilización del Se. Por el contrario, el contenido de Zn en las semillas de judía verde no se mejoró mediante la aplicación de biofertilizantes enriquecidos con micronutrientes en comparación con el control. Esto podría atribuirse a la menor tasa de translocación del Zn de las raíces de las plantas a las semillas y a la menor cantidad de Zn aplicada al suelo como biofertilizante enriquecido en Se/Zn.

Por otro lado, las microalgas tienen una gran capacidad para asimilar/eliminar nutrientes del medio de cultivo (o de las aguas residuales) y metabolizarlos en componentes valiosos como proteínas, ácidos grasos, vitaminas y carbohidratos. Por lo tanto, las microalgas se están considerando actualmente como una fuente potencial de proteínas para sustituir las proteínas animales y vegetales habituales (por ejemplo, la soja). En el Capítulo 6 se investigó el potencial de eliminación de Se en lagunas de alta carga (HRAP por sus siglas en inglés) para el tratamiento de aguas residuales domésticas, y la producción de biomasa microalgal enriquecida con Se, que podría usarse como complemento alimenticio (proteína) o biofertilizante de alto valor añadido. Los resultados indicaron un tratamiento eficaz de las aguas residuales en las HRAP. Las microalgas enriquecidas con Se producidas en las HRAP alimentadas con agua residual doméstica contenían un alto contenido de proteína cruda (48% de sólidos volátiles en suspensión) y el selenoaminoácido SeMet (selenometionina) (91% del Se total). Además, el contenido de aminoácidos esenciales de las microalgas era comparable al de la soja, una proteína tìpica de la alimentación animal. Este Capítulo también mostró que el Se puede inducir la producción de ácidos grasos poliinsaturados omega-3 (ω3) y omega-6 (ω6) y eicosapentaenoico (EPA) en las microalgas, aunque se necesitaría más investigación para confirmarlo. Por lo tanto, la producción de microalgas enriquecidas en Se en HRAP se presenta como una alternativa prometedora para recuperar recursos de bajo valor y convertirlos en suplementos alimenticios de alto valor.

El **Capítulo 7** tuvo por objetivo evaluar las microalgas enriquecidas en Se generadas en el Capítulo 6 como bioestimulante para mejorar el crecimiento de las plantas y como biofertilizante de Se para mejorar el contenido de Se de las plantas. Se aplicaron tanto biomasa de microalgas enriquecida con Se (cruda), como extractos de la misma, en judías verdes (*Phaseolus vulgaris*) a nivel de suelo y foliar. Este estudio mostró que la aplicación de biomasa de microalgas enriquecidas con Se al suelo (1-10%) y sus extractos a las hojas (1%, aspersión foliar) mejoró el crecimiento de las plantas, lo que confirmó que las microalgas enriquecidas con Se actúan como un bioestimulante. Además, se logró incrementar el contenido de Se en la planta y el suelo (para la aplicación al suelo) tras la aplicación de microalgas enriquecidas con Se y de extractos de las mismas. Esto indica que las microalgas enriquecidas con Se, cultivadas mediante el tratamiento de aguas residuales, se pueden valorizar como bioestimulante y biofertilizante para mejorar tanto el rendimiento de semillas como el contenido de Se de las habas, incrementando el valor de mercado de las mismas.

En el **Capítulo 8** se muestran las conclusiones y se discuten los principales resultados de esta tesis. También se enumeran las limitaciones del estudio. Esta tesis contribuye al desarrollo sostenible y respetuoso con el medio ambiente de la biofortificación/suplementación de micronutrientes en áreas deficientes en Se/Zn, junto con la recuperación de nutrientes de las aguasresiduales.

List of abbreviations

BCF	Bioconcentration factor
BSA	Bovine standard albumin
CEC	Cation exchange capacity
COD_{tot}	Total chemical oxygen demand
COD_{sol}	Soluble chemical oxygen demand
DI	Deionized water
DM	Dry matter
DMDSe	Dimethyl diselenide
DMSe	Dimethyl selenide
DO	Dissolved oxygen
DW1	Se enriched duckweed application at 1.0 mg Se/kg soil
DW5	Se enriched duckweed application at 5.0 mg Se/kg soil
EC	Electrical conductivity
EDI	Estimated daily intake
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic
EXAFS	X-ray absorption fine structure
FAO	Food and Agriculture Organization
GI	Germination index
GP	Germination percentage
HPLC	High performance liquid chromatography
HRAPs	High rate algae ponds
HRAP-Se	High rate algae ponds with continuous Na_2SeO_3 spiking
HRAP-C	High rate algae ponds without Se spiking
HRI	Health risk index
HRT	Hydraulic retention time
ICP-MS	Inductively coupled plasma-mass spectrometry
IDA	Iodoacetic acid
Me-SeMet	Methylselenomethionine
MGT	Mean germination time
MTL	Maximum tolerable levels
MUFA	Monounsaturated fatty acids

OM	Organic matter
OPA	O-phthaldialdehyde
PUFA	Polyunsaturated fatty acids
RfD	Reference oral dose
ROS	Reactive oxygen species
SBM	Soybean meal
SDS	Sodium dodecyl sulfate
Se	Selenium
Se(IV)	Selenite
Se(VI)	Selenate
SeCys	Selenocysteine
$SeCys_2$	Se-cystine
SeMet	Selenomethionine
SeMetSeCys	Se-methyl-selenocysteine
SeZnDW1	Se and Zn enriched duckweed application at 1.0 mg Se/kg soil
SeZnDW5	Se and Zn enriched duckweed application at 5.0 mg Se/kg soil
SeZnSL1	Se and Zn enriched sludge application at 1.0 mg Se/kg soil
SeZnSL5	Se and Zn enriched sludge application at 5.0 mg Se/kg soil
SL1	Se enriched sludge application at 1.0 mg Se/kg soil
SL5	Se enriched sludge application at 5.0 mg Se/kg soil
SFA	Total saturated fatty acids
SP	Soluble phosphorus
SVI	Seedling vigor index
TC	Total carbon
TKN	Total Kjeldahl method
TN	Total nitrogen
TOC	Total organic carbon
TP	Total phosphorus
TSS	Total suspended solids
VSS	Volatile suspended solids
ω3	Omega-3
ω6	Omega-6
WHO	World Health Organization

XANES	X-ray absorption near edge structure
Zn	Zinc

Chapter 1 General introduction

1.1 Motivation

Selenium (Se) and zinc (Zn) are essential trace elements, playing a crucial role in the functioning of enzymes in humans and animals and protecting cells from damage by free radicals (Hatfield et al., 2014). Selenoproteins, i.e. proteins containing selenium, are also well known as antioxidants and catalysts for the production of the active thyroid hormone. Although Se is not essential for the growth and survival of plants, it is considered as a beneficial element for plants as well, which can enhance resistance to stress, whereas Zn is an essential element for plants, that can enhance plant growth (Feng et al., 2013; Subramanyam et al., 2019). The recommended daily Se and Zn intake in an adult human diet are 0.04–0.4 mg and 15 mg per person per day, respectively (FAO/WHO 2001). However, despite the importance of these trace elements, intake of Se and Zn by animals and humans in a wide range of countries, including e.g. Belgium, the United Kingdom and Keyna, is currently still quite low, resulting in Se and Zn deficiency and causing negative health effects, including increased risk of mortality, poor immune function, and cognitive decline (Broadley et al., 2006; Broadley et al., 2007; Rayman, 2012; Roekens et al., 1986). An estimated one billion people around the world are affected by selenium deficiency and more than 30% of the world's population is Zn-deficient because of low Se and Zn intake (Poblaciones & Rengel, 2017; Rayman, 2004). Besides, also farm animals (Dermauw et al., 2013) and pets (van Zelst et al., 2016) can be affected by Se and Zn deficiencies, leading to economic losses. Therefore, the Se and Zn content in the human and animal diet is a topic of interest to public health systems around the world (Lavu et al., 2012).

Food/feed biofortification (e.g. enrichment of food and feed crops with Se and Zn), food/feed supplements or dietary diversification are proposed as possible solutions to remediate these micronutrient deficiencies. For biofortification, inorganic Se forms (e.g., selenite and selenate) and Zn (Zn^{2+}) are generally added into soils to enhance the micronutrients in crops in order to achieve an optimal Se/Zn level in human and animal diets. However, the direct application of inorganic Se/Zn forms into soils could result in secondary soil and water contamination due to the fast leaching (high mobilization) of the fresh applied Se/Zn and the low utilization rate of Se/Zn by plants (Wang et al., 2018). It may thus be beneficial and environmental-friendly to produce slow-release Se/Zn-enriched organic biofertilizers through ecotechnologies in order to supply Se/Zn to soil for plant uptake. Likewise, many chemicals originating from primary mining (not

only Se/Zn but also other micro- and macro-nutrients) are used to cultivate microorganisms for the production of Se/Zn-enriched food/feed supplements, such as the production of Se-yeast (Rayman, 2004) and Se-algae (Gómez-Jacinto et al., 2020). This is not cost-effective and could even introduce new contaminations. The production of Se/Zn-enriched food/feed supplements or biofertilizers could be made more sustainable and environment-friendly by producing them using low-value nutrients in wastewaters as feedstock, while cleaning the wastewater.

Se and Zn excess in wastewaters frequently occurs, as Se and Zn are widely applied in or released from industrial and agricultural activities (Lim & Goh, 2005). These wastewaters may therefore serve as potential Se and Zn sources, and it may be beneficial to recover not only macronutrients, but also Se and Zn from these wastewaters through different biological technologies (e.g., phytoextraction, bioreduction or microalgae-based methods). Accordingly, bioproducts with high Se/Zn contents could be generated by these technologies, and these may be valorized as slow-release organic Se and Zn fertilizers or feed supplements. Besides, environmentally sustainable fertilizers and feed supplements may also be produced from wastewaters with a low Se/Zn content as feedstock, whereafter Se/Zn may be added to produce an added-value product. Such alternative micronutrient-enriched fertilizers or feed supplements will contribute to the sustainability of our food and feed production systems, as they can be produced on wastewater recovering nutrients during its secondary or tertiary treatment.

1.2 Objectives

The main objective of this thesis is to explore the potential of Se-enriched bioproducts generated from wastewater as environmental-friendly micronutrient feed supplements and biofertilizers. Specifically, it is aimed to:

- Develop sustainable and biobased methods which are able to remove and recover Se/Zn from aqueous solutions, meanwhile producing micronutrient-enriched biomaterials. Phytoextraction by aquatic plants, bioreduction by sludge and microalgae-based wastewater treatment are investigated in this thesis.
- Evaluate the produced Se/Zn-enriched duckweed and Se-enriched microalgae for potential use as micronutrient feed supplements by evaluating Se/Zn

accumulation, Se speciation, Se bioaccessibility and other nutritional compounds in these biomaterials (e.g., amino acids and fatty acids).

- Assess the applicability of the produced Se/Zn-enriched duckweed, sludge and microalgae as potential slow-release organic Se/Zn fertilizers or biostimulants for enhancement of Se/Zn accumulation by plants and stimulating plant growth in pot experiments. The kinetics of the micronutrient release from these biomaterials into soils and the uptake of the trace elements by crops are investigated.

1.3 Outline of the dissertation

The remainder of this thesis is composed of seven chapters and mainly focuses on Se because Se was less studied so far compared to Zn. Zn was included only in two chapters (interaction with Se), considering that Se and Zn are likely to co-exist in the environment and simultaneous occurrence of nutritional deficiencies of more than one micronutrient is more common. Multi-mineral enriched supplements or agronomic biofortification of crops are thus being explored as a possible way to alleviate multi-micronutrient deficiency (Mao et al., 2014).

Chapter 2 presents the background of the doctoral research and a literature review on the occurrence of Se and Zn in human and animal diets and their deficiency and toxicity for humans and animals. The current status on micronutrient biofortification and production of micronutrient feed/food supplements is discussed. This is followed by a discussion on the paradigm shift from waste treatment to resource recovery, highlighting the potential of biobased technologies for micronutrient recovery from wastewater, while producing micronutrient-enriched feed/food supplements and biofertilizers.

Chapter 3 evaluates the possibility to produce Se and Zn-enriched aquatic plants, *Lemna* (duckweed) and *Azolla,* as potential micronutrient-enriched feed supplements or biofertilizers while cleaning water. In Chapters 4 and 5, the potential to use single Se (Chapters 4) or simultaneous Se and Zn enriched (Chapters 5) duckweed (generated by phytoextraction from Chapter 3) and sludge (produced by a bioreduction process) as Se/Zn biofertilizers in the agronomic biofortification of green beans (*Phaseolus vulgaris*) was assessed, respectively. Other researchers have previously

reported the ability of sludge to precipitate Se from wastewater under anaerobic reducing conditions and thus the possibility to produce Se-enriched sludge (Staicu et al., 2015a; Staicu et al., 2015b). Therefore, we did not anymore focus on the production of the Se/Zn enriched sludge in this thesis, but only evaluated its potential use as biofertilizer in Chapters 4 and 5.

Chapter 6 investigates the possibility to produce Se-enriched microalgae in high-rate algae ponds treating domestic wastewater as high-value Se-enriched feed supplements, meanwhile cleaning the wastewater. Chapter 7 evaluates the Se-enriched microalgae obtained from Chapter 6 as potential Se biofertilizers and biostimulants for the enhancement of Se uptake by beans and growth of the beans. A scheme of the doctoral research is presented in Fig. 1.1.

A general discussion and conclusions drawn from this study are presented in Chapter 8, together with ideas and perspectives for future work.

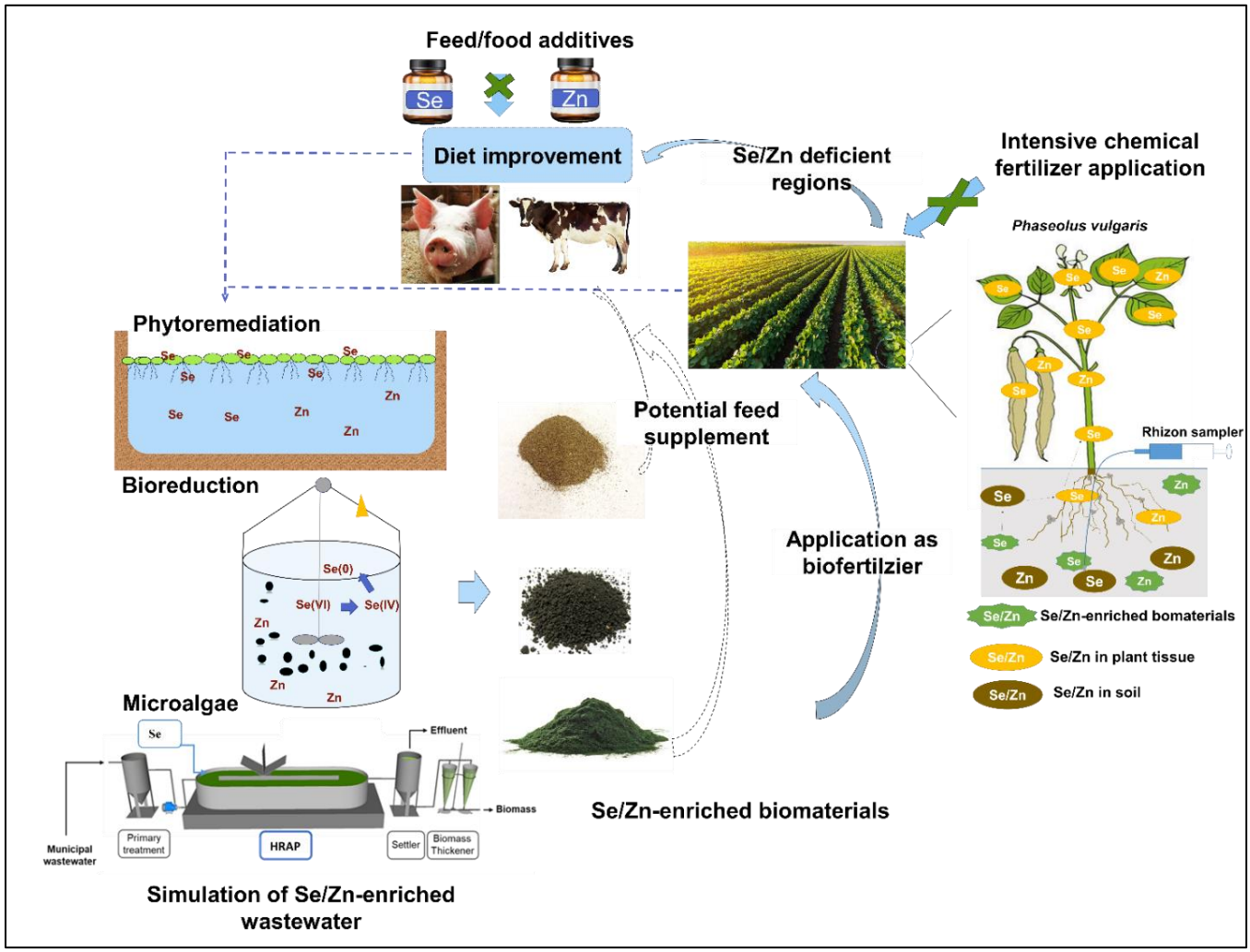

Figure 1.1. Schematic representation of the doctoral thesis research

Chapter 2 Literature review

2.1 Occurrence of Se

Selenium (Se) was identified as a new substance in 1817 by Jöns Jacob Berzelius when studying a method to produce sulfuric acid from sulfur-bearing rocks (Fernández-Martínez & Charlet, 2009). It is a metalloid element belonging to the oxygen group (Group 16 of the periodic table) and closely allied in chemical and physical properties with the elements sulfur and tellurium (Lenz & Lens, 2009). It exists in inorganic forms as selenate (SeO_4^{2-}), selenite (SeO_3^{2-}), selenide (Se^{2-}) and elemental Se (Se^0), and in organic forms such as selenocysteine (SeCys) and selenomethionine (SeMet). Due to this complex chemical behavior, Se is found in all natural materials on earth: soil, rocks, waters, air, plants and animals (Fordyce, 2007). During a long period, Se has been identified as a dangerous substance because of its toxicity (Fordyce et al., 2000). Afterwards, it has also been recognized as an essential trace element for human and animal health due to its crucial role in the functioning of enzymes for humans and animals (Fordyce, 2013; Rayman, 2000).

Selenium is released into the environment from both natural and anthropogenic sources. It is naturally found in the Earth's crust in an estimated concentration of 0.05–0.5 mg/kg (Tan et al., 2016). Rocks are the primary source of Se, which comprise approximately 40% of the total Se from in the Earth's crust (Wang & Gao, 2001). A greater Se concentration (0.06 mg/kg) is usually found in shales compared to that in limestones and sandstones (Fordyce, 2007). Coals and other organic-rich deposits can be rich in Se (typically from 1 to 20 mg/kg). Very high concentrations of Se have also been reported in some phosphatic rocks (≤ 300 mg/kg) and some black shales (300 mg/kg). Selenium is also often found as a minor component of sulfide mineral deposits (Fernández-Martínez & Charlet, 2009; Fordyce, 2007; Fordyce, 2013).

Human activities contribute to the introduction, mobilization and accumulation of Se in the environment (Winkel et al., 2015). Fordyce (2013) estimated that around 88000 Se tons/year are released globally to the environment from anthropogenic activities, which accounts for 50–65% of the total Se emissions. Industrial processes, in particular coal and petroleum combustion, are thought to be the main processes releasing Se into the atmosphere. Selenium is also mainly a by-product of the extraction of various metal elements (i.e. copper, zinc, uranium and lead) and of processing plants (i.e. sulfuric acid production) (Tan et al., 2016). Other anthropogenic activities, such as glass and

electronic production, utilization of rock phosphates as fertilizer and application of sewage sludge or Se-containing fertilizers to agricultural land for the enhancement of the Se concentration in food, also increase the Se content in the environment (Lemly, 1997).

The Se released by anthropogenic (e.g., coal combustion, mining and agricultural) and natural (e.g., biomethylation and volcanic eruptions) activities enters into nature and starts a cycle involving different Se species transformations (Fig. 2.1). Briefly, the released Se enters into water and soil in the form of Se(IV) and Se(VI) by leaching/discharging and weathering/precipitation processes, respectively. Se(IV) and Se(VI) in water or soil can be taken up by organisms (e.g., plants, animals and bacteria) and converted into organic Se. The organic Se in the organisms can be further methylated into volatile Se (e.g. Dimethyl diselenide (DMDSe) or Dimethyl selenide (DMSe)) and released into the atmosphere as H_2Se and SeO_2. Selenium enters into the atmosphere in the form of SeO_2 and H_2Se. The subsequent solubilization of SeO_2 in the atmosphere results in Se(IV) or Se(VI) precipitating with the rain and entering a new natural cycle.

Alternatively, Se(IV) and Se(VI) in the water and soil can also be reduced by microorganisms into Se(0) or even Se(-II), which are deposited in soils and sediments. These biogeochemical processes (e.g. weathering, leaching, rock–water connections and biological activities) mainly control the transport of Se from rocks (the main Se source) to other compartments in the environment, unevenly distributing it over the Earth (Fernández-Martínez & Charlet, 2009). This leads to widely varying Se concentrations in different geo-ecosystems, forming seleniferous or Se-deficient regions (Fernández-Martínez & Charlet, 2009). This uneven distribution is likely to affect the health of both humans and animals throughout the food chain (Tan et al., 2002).

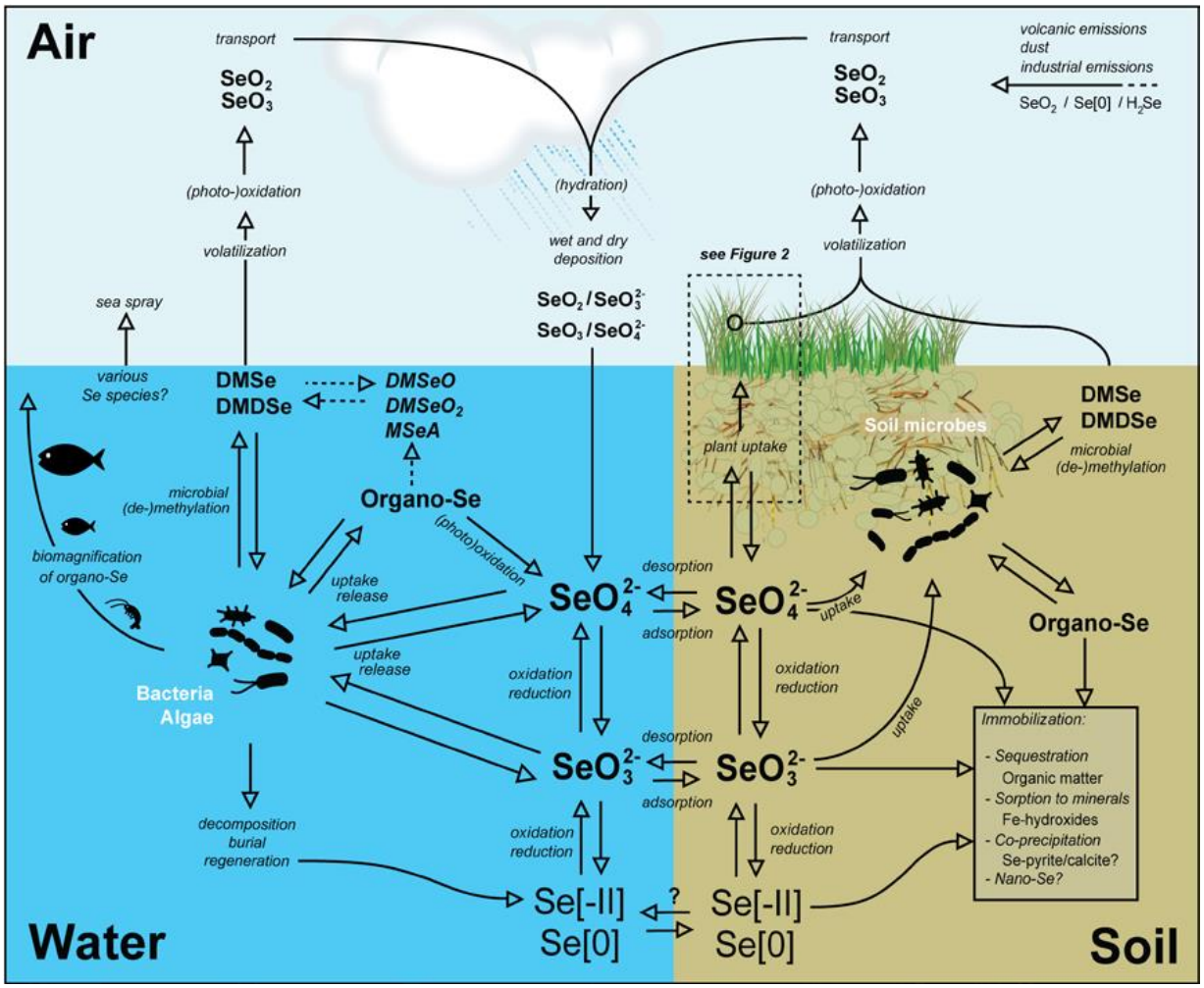

Figure 2.1. Schematic overview of Se transformations in natural systems (Winkel et al., 2015).

2.2 Se toxicity and deficiency

The range between beneficial and harmful Se concentrations is relatively narrow for animals and humans (Li et al., 2015a). Thus, both toxicity and deficiency have been reported over the world (Li et al., 2015a). In humans, chronic Se toxicity is observed above intake levels of 400 µg/day and Se deficiency occurs when the dietary intake of Se is below 40 µg/day (Winkel et al., 2012). More specifically, the tolerable upper intake levels are 90 µg/day for children of 1–3 years, 150 µg/day for children of 4–8 years, 280 µg/day for children of 9–13 years, and 400 µg/day for children >14 years and adults (Ngigi, 2019; National Academic of Sciences, 2000). Besides, for livestock, the toxic Se concentration in animal feed is 2–5 mg/kg dry forage. On the other hand, the minimum requirement is defined as 0.05–0.10 mg/kg (Gupta & Gupta, 2017). The

National Research Council (NRC, 2005) has published the following maximum tolerable levels (MTL) for animals: 5 mg Se/kg feed dry matter (DM) for cattle and sheep, 4 mg Se/kg feed DM for pigs, and 3 mg Se/kg feed DM for poultry. The MTL for horses and fish were derived from interspecies extrapolation and amount to 5 and 2 mg Se/kg DM feed, respectively (NRC, 2005).

Se intoxication events for animals and humans, such as selenosis in America, Canada, China, and Mexico, have occurred occasionally because Se has entered the food chain (Li et al., 2015a). These events were caused by excessive Se concentrations in soil and water. For instance, the discovered Se toxicity for humans and animals in the Enshi District, Hubei Province and in Ziyang County, Shaanxi Province in China was related to the exceedingly high Se concentrations in the local food and environment (Fordyce et al., 2000). For humans, Se toxicity (selenosis) could result in garlic breath, hair and nail loss, nervous system disorders, poor dental health and paralysis (Rayman, 2012). For animals, Se can cause alkali disease and blind staggers in livestock, and hooves loss in hooved animals (Fordyce, 2007; Tan et al., 2002). The alkali disease is characterized by dullness, lack of vitality, emaciation, rough coat, sloughing of the hooves, erosion of the joints and bones, anaemia, lameness, liver cirrhosis, and reduced reproductive performance (Reilly, 2006). Blind staggers result in impaired vision and blindness, anorexia, weakened legs, paralyzed tongue, labored respiration, abdominal pain, emaciation, and death (Fordyce, 2007). Hair loss and other abnormalities of farm animals have been observed in areas of Columbia as a result of Se toxicity (Johnson et al., 2009).

On the contrary, Se deficiency is also observed frequently worldwide and is even more widespread than Se toxicity. It is estimated that 0.5−1 billion people are directly affected by Se deficiency on a global scale, due to low Se dietary intake (Haug et al., 2007; Stonehouse et al., 2020). It has been demonstrated that Se deficiency can cause the Keshan disease and Kashin-Beck disease (endemic disease) with exceedingly low Se supplies in the food system, i.e. weakening of the heart and also atrophy and necrosis of cartilage tissue in the joints, which has been observed in the middle of China (Stone, 2009), Saudi Arabia, Czech Republic, Burundi, New Guinea, Nepal, Croatia, and Egypt (Wu et al., 2015). Low Se status has also been associated with a significantly increased risk of cancer incidence and mortality, cardiovascular risk, poor immune function, male infertility and lower reproduction (Fordyce, 2007; Haug et al.,

2007). In addition, Se deficiency may also be a factor in some other diseases. For instance, studies have found that the prevalences of iodine deficiency diseases were greater among populations with lower Se status than among those with higher Se status in Africa (Combs, 2001). This should probably be attributed to the fact that Se is essential for the metabolic production of thyroid hormone.

Se deficiency is known to adversely affect livestock health around the globe, which has been identified since the 1950s in several countries including south and north America, Africa, Australia, UK and New Zealand (Reilly, 2006). Selenium deficiency causes reproductive and immune response impairment of animals, growth depression (ill-thrift), and white-muscle disease, a myopathy of heart and skeletal muscle principally affecting cattle, sheep, poultry and horse (Rayman, 2000). Wolf et al. (1963) estimated that around 10–15 million sheep or 20 to 30% of the total stock were at risk of developing white muscle disease in New Zealand via an extensive international survery at that period.

Generally, Se deficiency in humans and animals is attributed to a low Se daily dietary intake, with this dietary intake varying considerably between countries/regions. As aforementioned, Se deficiency has mainly been identified in parts of the world which have notably a low content of Se in soil and water, as Se enters the food chain from the environment through crops and plants uptake (mainly local water or soil) (Haug et al., 2007). Therefore, the Se concentration in foods is determined by geological and geographical factors. Globally, the range of total Se concentration in soils is from 0.01 to 2.0 mg/kg (with a mean of 0.4 mg/kg) (He et al., 2010; Rayman, 2008). Some parts of the world (e.g. Denmark, Finland, New Zealand, eastern and central Siberia and a long belt extending from northeast to southwest China including parts of Heilongjiang, Jilin, Liaoning, Hebei, Shanxi, Shaanxi, Sichuan and Zhejiang Provinces and Inner Mongolia) have relatively low Se contents in their soils and, therefore, resulting in low amounts of Se in their food chains (Combs, 2001).

Table 2.1 summarizes the recommended daily Se intake and Table 2.2 overviews the status of daily Se intake in some countries. The two tables show that the recommended daily Se intake in some countries is not achieved yet, such as in some European countries (including Belgium) and parts of China. This demonstrates that the food systems of these countries do not provide sufficient Se for consumption. It may, thus,

be assumed that many individuals have a potential risk of Se deficiency, which can increase their risks to various diseases, including those of the heart and lungs, as well as cancer, and make them more vulnerable to infectious diseases due to poor functioning of their immune system. There is a clear need to enhance Se in food systems of these countries to remediate Se deficiency.

Table 2.1. Recommended daily Se intake for adult (µg/d)

Countries	Males	Females	Proposed year
Australia	85	70	1990
Belgium	70	70	2000
Netherlands	50-150	50-150	2000
Germany, Austria, Switzerland	30-70	30-70	2013
France	60	50	2001
Italy	55	55	1996
Ireland	55	55	1999
Japan	55-60	45	1999
Nordic countries	60	50	2014
USA and Canada	55	55	2000
United Kingdom	75	60	1991
Scientific Committee Food	55	55	2003
FAO/WHO	40	40	2001

Table adapted from: EC Scientific Committee on Food, (2003); Thomson, (2004); Rayman, (2004); and EFSA, (2014)

Table 2.2 Estimated selenium intake status of adults in some countries (µg/person per d)

Countries	Se intake
Australia	57-87
Belgium	28*-61
Austria	48
Germany	35*

Table 2.2 continued

Switzerland	70
France	29*-43
Italy	35*-42
Japan	104-199
New Zealand	55–80
Denmark	38*-47
Sweden	38*
Finland	
Before 1984	25*
After 1984 (Se biofortification)	67-110
USA	60-220
Canada	98-224
UK	29*-39*
Ireland	44-59
Slovakia	27*-43
Seriba	30*
Latvia	50
Czech Republic	10-25*
China	
Keshan disease area	7-11*
(e.g. a wild belt from Northeast China to southwest China)	
Moderate Se area	40-120
(e.g. Guangzhou)	
Selenosis area	750-4990
(e.g. Hubei and Shaanxi provinces)	

Table adapted from Combs, (2001); Rayman, (2004) and EFSA, (2014)

* indicates that this level does not meet the recommended requirement according to WHO (2001)

2.3 Approaches for addressing Se deficiency - biofortification

Addressing micronutrient deficiencies to reduce health-related issues can be achieved through various types of interventions, such as through food supplements, dietary diversification, Se biofortification, or increase of the digestibility of trace elements in products (Lavu et al., 2012; Li et al., 2020). For instance, sodium selenite has been supplemented in feeds in some areas with selenium deficiency in livestock in order to achieve optimal Se intake (EFSA, 2016).

Biofortification is one of the most promising strategies, widespread and accepted strategies, aimed at improving the lacking of a mineral (e.g. Se) content of the diet through it enrichment in food/feed crops, in particular the edible parts of plants, through soil or foliar application of mineral fertilizers (Sánchez et al., 2017). The agronomic approach of applying a fertilizer on the soil/foliar can improve the nutritional quality of the crop without genetic modifications (Storksdieck and Hurrell, 2009). It has been developed as a food-based method to help decreasing widespread deficiencies of minerals (e.g. Se). Although Se is not an essential trace element for plants, it presents chemical similarity to S, and both elements have the same carrier membranes and biochemical pathways of assimilation in plant uptake (Prado et al., 2017). Biofortification of Se fertilizers can therefore ensure its sufficient concentration in the edible parts of plants (Sarwar et al., 2020). Se biofortification of food crops is already successfully practiced in some countries (Se-deficient regions) to increase the Se concentration in staple grains and subsequent dietary Se intake, such as in Finland , by adding inorganic Se fertilizer to soils (Bañuelos et al., 2016). For instance, in Finland, a 3-folds increase of mean Se intake was observed after Se biofortification in the form of selenate within 2 years, and the concomitant human serum Se concentration was increased by 70% (Aro et al., 1995).

Since low concentrations of plant Se can decrease the dietary intake of Se, it is vital to increase Se uptake by plants and to produce plants with higher Se concentrations and bioavailability in their edible tissues (Bañuelos et al., 2017). This is the key issue for effectively developing a biofortification strategy. The Se biofortification efficiency depends on a number of factors associated with the Se concentration in plants (also called bioavailability) during biofortification, such as plant species, Se species and source (chemical Se fertilizer, natural source of Se or organic Se), soil pH and redox

conditions, soil texture and organic matter, and the presence of competitive ions (Fordyce, 2007).

Plant species: Table 2.3 summarizes Se concentrations in crops after Se fortification. Plants have been classified as hyperaccumulators (>1000 mg/kg, such as *Stanleya*), secondary accumulators (100–1000 mg/kg, such as *Brassica* species: broccoli), and non-accumulators depending upon Se accumulation inside their cells (Gupta & Gupta, 2017). Vegetables (e.g. *brassica* species: pak choi and cabbage) normally accumulate more Se than legumes (beans), followed by cereals (wheat and rice). The Se concentration accumulated in fruits is generally low, whereas high concentrations (ranging from 0.03–512 mg/kg) have been reported in Brazil nuts as a result of natural biofortification (Prado et al., 2017).

Table 2.3. Se concentration of some selected Se-enriched plants (crops, vegetable, and fruits) after Se fortification (Gupta & Gupta, 2017)

	Se-enriched parts	Accumulated Se (mg/kg)	Se dose for biofortification
Broccoli	Sprouts	467	60 mg/L
Kale	Sprouts	155	60 mg/L
Pak choi	Shoots	20–4000	2.5–40 mg/kg
Lettuce	Shoots	43	< 2.8 mg/L
Soybean	Seeds	75	130 mg/kg
Rice	Grains	1.3–3.3	2.850 mg/kg
Pear	Fruit	0.199	1 mg/L

Se application methods: Different application methods of Se-based fertilizer affect Se accumulation and transformation in plants. Foliar application is generally more efficient in enhancing the Se concentration in plants in comparison with soil application. Studies showed that the efficiency of Se foliar applications is on average 8 times more efficient than soil applied fertilizers (Ros et al., 2016). Besides, application of Se fertilizers at different plant growth stages can also result in a different biofortification efficiency. Wang et al. (2020b) demonstrated that foliar application of selenate or selenite at the pre-filling stage was superior in improving the Se concentration of wheat grains than that at the pre-flowering stage. Zhang et al. (2019) found that the foliar application of

selenite during the potato tuber bulking stage resulted in the greatest Se accumulation in tubers, compared to the application during the tuber initiation and maturation stages.

Se species and source: The uptake rates and mechanisms of selenite, selenate and organic Se are different. Some studies showed that selenite is adsorbed and taken up in a faster passive way and readily reduced to organic compounds in plants, while selenate is taken up in an active way and easily distributed from roots to shoots (Arvy, 1993; Gupta & Gupta, 2017). Selenate reduction occurs via substitution for sulfate in the ATP sulfurylase reductase system, which is an ATP-consuming process and rate-limiting step, resulting in lower selenate accumulation in plants compared to selenite (Van Hoewyk, 2013). However, Ros et al. (2016) showed that biofortification using selenate-based fertilizers has a high potential to increase Se uptake by crops and subsequently Se intake by animals and humans. This is attributed to the fact that selenate is not easily adsorbed into the soil matrix in comparison with selenite, resulting in higher bioavailable Se concentration in the soil, while selenite is readily adsorbed in the soil environment.

Soil pH and redox condition: soil pH and redox conditions have an important effect on Se availability since a combination of these factors determines the Se species present in a given soil environment. For instance, selenate is the predominant Se species in near-neutral pH environments under aerobic conditions, whereas selenite predominates at lower pH and redox potential. Selenate is much more mobile, and thus plant-available, in soils than selenite which is tightly bound to positively charged binding sites in soil (Eich-Greatorex et al., 2007). Besides, soil pH negatively correlates with the amount of Se adsorbed by soil (Li et al., 2015b). Most studies have demonstrated that relatively high pH values in soil solutions lead to a higher Se accumulation by plants in comparison with low pH soil (Li et al., 2016; Li et al., 2017). This is attributed to the fact that soil with low pH would exist a high amount of H^+, which will not compete for positively charged binding sites with selenite/selenate as acid radical anion (e.g., SeO_4^{2-} and SeO_3^{2-}) in soil, thus leading to a relatively high bioavailable Se in the soil solution.

Soil organic matter: Organic matter (OM) influence Se availability in different ways. On the one hand, OM has a significant capacity to remove Se from the soil solution, and immobilize Se by both biotic and abiotic mechanisms, thus reducing Se bioavailability.

On the other hand, OM can improve the soil structure and stimulate oxidizing conditions, thus enhancing Se bioavailability (Li et al., 2017). The release of OM-immobilized Se through mineralization will increase the bioavailable Se concentration in soil.

Competitive ions: The Se accumulation in plants can also be influenced by the presence of other ions, especially phosphate (PO_4^{3-}) and sulfate (SO_4^{2-}). Interactions between Se and other ions may occur in the soil or in the plant (Bingham, 1989). Li et al. (2008) studied the Se uptake in wheat under P and S-starved conditions and demonstrated that selenite uptake is an active process mediated partly by P transporters. Likewise, the Se uptake can be negatively influenced by the addition of sulfur (S) due to the chemical similarity between these two elements. Studies have demonstrated that selenate is taken up by sulfate transporters, thus the competition of the same transporters could inhibit Se uptake by plants when S is applied (Li et al., 2008). For instance, a decrease in Se concentration in the shoots and roots of corn (*Zea mays*) was observed when the S concentration in solution increased (Huang et al., 2008). Supplementation of S in the calcareous alluvial and yellow-brown soil reduces the Se contents in soybean (*Glycine max* L.) seeds (Deng et al., 2021).

2.4 Approaches for addressing Zn deficiency – biofortification

Similarly, Zn is also an important micronutrient because it plays an important role in crop production and human nutrition (Broadley et al., 2007; Sánchez et al., 2017). Approximately 10 % of human proteins require Zn to maintain their catalytic activity. Zinc is involved in the biosynthesis of proteins and scavenging of reactive oxygen species (Li et al., 2020b). It is deficient in 30 % of the soils used for agriculture in the world (Poblaciones & Rengel, 2017; Sánchez et al., 2017), and the WHO reports that about 33 % of the population is affected by Zn deficiency, in particular for developing countries. Zn deficiency affects organ functions such as epidermal, gastrointestinal, central nervous, immune, skeletal, and reproductive systems (Roohani et al., 2013). Zinc deficiency will also impair children's physical growth and development. It can result in a syndrome of anemia and increase the risk of pathogenic infections and diseases (Gibson, 2006). Children, pregnant and lactating women require more Zn, which thus have a higher risk of Zn deficiency (Roohani et al., 2013). It is estimated that more than half of the pregnant women and children in developing countries are suffering from Zn deficiencies (Maqbool et al., 2019).

Similar to Se, Zn deficiency is also mainly due to inadequate intake of dietary Zn in most situations. Increasing Zn levels in crops would lead to more Zn in humans. Zinc biofortification has therefore been approved as an effective strategy to increase the Zn concentration in crops, such as rice, maize and wheat (Sánchez et al., 2017). This has been extensively studied. For instance, some countries (e.g. China, Mexico, Indonesia and South Africa) have implemented the biofortification of maize or wheat flour with Zn (Gibson, 2006). Many comprehensive overviews about the current status, challenges and solutions of Zn biofortification for combating Zn deficiency have been clearly stated (Maqbool et al., 2019; Palmgren et al., 2008; Zaman et al., 2018).

Additionally, Se and Zn deficiency are likely to co-occur in the environment or human and animal nutrition (Darago et al., 2016; Ruz et al., 1999). Both the Se and Zn content in the human and animal diet is, therefore, a topic of interest to public health systems around the world. Multi-mineral agronomic biofortification of crops is thus being explored as a simple and effective way to alleviate micronutrient deficiency (Mao et al., 2014; Poblaciones & Rengel, 2017).

2.5 Micronutrient-enriched organic materials as Se/Zn biofertilizers for biofortification

Biomaterials (e.g. plant residues, sludge, and manures) that come from seleniferous and zinciferous areas potentially contain high levels of Se and/or Zn. These micronutrient-enriched materials may serve as potential micronutrient sources and can thus be re-utilized for Se or Zn biofortification of agricultural crops. If Se/Zn-enriched organic biomaterials are used to amend agricultural soils, the decomposition of organic biomaterials will gradually lead to the micronutrients released into the soil solutions, which would be bioavailable for crops uptake (Bañuelos et al., 2015). In this context, biofortification with these micronutrient-enriched biomaterials can thus be achieved, which is particularly beneficial for crops grown on micronutrient deficient soils (Bañuelos et al., 2016; Li et al., 2017). Some studies have investigated the possibility of using Se-enriched biomaterials as feedstock to improve the Se concentration in crops for biofortification purposes. For instance, the accumulation of Se in canola, grown on soil amended with 1.5 mg/kg seleniferous *Astragalus praelongus* E. and *Medicago saliva* L. tissues, was increased as the amount of application of these materials increased (Ajwa et al., 1998). Moreover, Se-enriched wheat and raya plants

straw were used to biofortify sorghum, maize and berseem (Dhillon et al., 2007), and results showed that the Se concentrations in the plant were consistent with the trend of soluble Se in soil.

The supplementation with Se-enriched organic materials in soils as biofertilizer may not only improve the Se concentration in the plants, but also result in value-added plant-based products, as plants can transform the Se taken up during growth into valuable organic Se species (e.g. SeMet, SeCys and MeSeCys), which have important assets in the nutrition of animals and humans. Bañuelos et al. (2015) reported that the Se concentration in the edible parts of broccoli and carrots was increased and that MeSeCys was the main accumulating Se species when the shoots of Se-enriched *Stanleya pinnata* were added to the soil as biofertilizer.

One of the main advantages of micronutrient-enriched organic materials is that they provide a long-lasting micronutrient source, slowly releasing the micronutrient along with the decomposition of the organic materials in the soil (Ajwa et al., 1998). However, the disadvantage is that the application of these materials can introduce additional organic matter into the soil, which can lead to the immobilization of other elements/nutrients in the soil, eventually decreasing the bioavailability for plant uptake (Stavridou et al., 2011).

It should be noted that Se/Zn biofortification via the application of Se/Zn-enriched organic materials may not be feasible in all Se/Zn-deficient areas. For instance, the Se-deficient region in Northeastern China, characterized by a high content of OM, are not suitable for supplementation with micronutrient-enriched organic materials, as the presence of too much organic matter in the soil will increase the retention of the released Se and Zn, reducing the bioavailability of Se/Zn in the soil. In contrast, some regional soil with strong leaching potential (i.e. high precipitation (rainfall) and humid climates) and low Se/Zn content can benefit from the addition of micronutrient-enriched organic materials since the added organic matter can act as a micronutrient reservoir to avoid the leaching of nutrients and their mobilization to the deeper soil layers (Wang & Gao, 2001).

2.6 Local “green” micronutrient-enriched bioproducts production by micronutrient removal from wastewater

In order to achieve optimal Se and Zn levels in the human diet, chemicals containing Se and Zn are commonly added to crops in deficient regions. Similarly, the production of micronutrient-enriched food/feed supplements is being explored as another solution for micronutrient deficiencies. However, for the biofortification process, plants usually can take up only a small amount of those applied elements (Tan et al., 2002). The residual trace elements are leached with rainwater or fixed and accumulated in the soil, thus posing a potential threat to the environment (Broadley et al., 2006; Wang et al., 2018). Micronutrient slow-release fertilizers, such as organic fertilizers (Bañuelos and Hanson, 2010) and chemical nano-Se (Wang et al., 2017), are currently being developed to overcome these limitations and risks. Moreover, chemical production processes currently used to produce inorganic fertilizers and food/feed supplements are usually not environmentally and economically sustainable. Given this, it may be beneficial to produce micronutrient-enriched fertilizers and food/feed supplements locally from micronutrient-containing wastewater using environmental-friendly techniques.

Bioremediation is an environmentally friendly method to recover micronutrients from wastewater. Of all treatment technologies, bioremediation approaches may have the lowest construction and operation costs for contaminant removal. Moreover, the macrophytes, *Lemna* and *Azolla* have fast growth rates, high tolerance/accumulation to extreme conditions, and can easily be harvested (Miranda et al., 2016; Sasmaz et al., 2015). Ohlbaum et al. (2018) found that the duckweed *Lemna minor* can efficiently remove 76% of Se from seleniferous soil leachates (with 74 µg Se/L). *Azolla filiculoides* grown in synthetic wastewater reduced the Se content up to 40 % after 5 days of treatment (Miranda et al., 2016). *Lemna* and *Azolla* have also a high potential to remove Zn (Sasmaz et al, 2015). Moreover, these two aquatic plants are rich in proteins, so they may also be considered as an alternative (micronutrient-enriched) protein source replacing animal proteins in food and feed systems, contributing to the sustainability of our food and feed production systems (Terry et al., 2000).

Similarly, microalgae have fast growth rates and a high protein content (50–60%, depending on nutrients availability). Many studies have proposed that microalgae could

be applied in wastewater treatment systems to efficiently clean water (Liu et al., 2016). They are low-cost technologies and can be successfully and easily implemented in locations where weather conditions are favorable for microalgae growth, e.g. high solar radiation and temperature (Arashiro et al., 2019). Besides, the installation and maintenance of algae ponds are also cheaper compared to conventional wastewater treatment systems, e.g. activated sludge systems (Arashiro et al., 2019). Most importantly, microalgae biomass could upgrade low-value products from wastewater and synthesize them into high-value compounds, such as protein, lipid and carbohydrate in their cells. Batch tests have already been done to study the Se removal by microalgae. For instance, Liu et al. (2016) developed a batch algae treatment system for Se removal and evidenced that the Se absorption efficiency by *Chlorella vulgaris* was 88%. However, there are no studies investigating the Se removal efficiency by microalgae in continuous or pilot-scale systems, meanwhile investigating the possibility of Se-enriched microalgae as Se biofertilizer or Se feed supplement.

Bioprecipitation or bioreduction, i.e. Se reduction by microorganisms, can also be used to remove Se from water, meanwhile producing more sustainable Se biofertilizers (Staicu et al., 2015a; Staicu et al., 2015b). In wastewater, Se is mainly present as Se oxyanions, namely selenite and selenate, which are soluble, bioavailable and toxic for the environment. However, elemental Se(0) is solid and less toxic in the water phase. Reduction of selenite and selenate in the water phase into solid-phase elemental Se(0) could thus be another sustainable and efficient method to remove Se from wastewater. Staicu et al. (Staicu et al., 2015a) described the reduction of selenite and selenate by anaerobic granular sludge, and Se nanoparticles (SeNPs) were obtained by the anaerobic reduction process. These SeNPs were stable in the solid phase and may thus be evaluated as a potential slow-release micronutrient biofertilizer. In recent years, the application of SeNPs has been proposed for Se biofortification. Previous studies have identified the potential of SeNPs to promote plant growth, increase Se uptake and improve plant quality (Domokos-Szabolcsy et al., 2012; Hussein et al., 2019). The beneficial effects of SeNPs have been shown for several plants, including tomato (Hernandez-Hernandez et al., 2019; Morales-Espinoza et al., 2019), pomegranate (Zahedi et al., 2019), wheat (Hu et al., 2018), rice (Wang et al., 2020a), garlic (Li et al., 2020c) and tobacco (Domokos-Szabolcsy et al., 2012).

Chapter 3 Production of selenium- and zinc-enriched *Lemna* and *Azolla* as potential micronutrient-enriched bioproducts

This chapter was redrafted after:

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Li, J., Lens, P.N.L., Otero-Gonzalez, L., Du Laing, G. 2020. Production of selenium- and zinc-enriched *Lemna* and *Azolla* as potential micronutrient-enriched bioproducts. *Water Research*. 172, 115522.

Abstract

Selenium (Se) and zinc (Zn) are essential micronutrients that are often lacking in the diet of humans and animals, leading to deficiency diseases. *Lemna* and *Azolla* are two aquatic plants with a substantial protein content, which offer the possibility of utilizing them to remove Se and Zn from wastewater while producing micronutrient-enriched dietary proteins and fertilizers. In this study, we explored interaction effects occurring between Se and Zn when these micronutrients are taken up by *Azolla* and *Lemna*. The two aquatic plants were grown on hydroponic cultures containing 0–5.0 mg/L of Se (Se(IV) or Se(VI)) and Zn. The Se and Zn content of the plants, growth indicators, bioconcentration factor (BCF) and Se/Zn removal efficiency from the water phase were evaluated. The results demonstrated that Se(IV) is more toxic than Se(VI) for both plant species, as evidenced by the remarkable decrease of biomass content and root length when exposed to Se(IV). Both aquatic plants took up around 10 times more Se(IV) than Se(VI) from the medium. Moreover, the Se accumulation and removal efficiency increased by 66–99% for Se(IV) and by 34–59% for Se(VI) in *Lemna* when increasing Zn dosage from 0 to 5.0 mg/L in the medium, whereas it declined by 13–26% for Se(IV) and 21–35% for Se(VI) in *Azolla*, suggesting a synergetic effect in *Lemna,* but an antagonistic effect in *Azolla*. The maximum BCF of Se in *Lemna* and *Azolla* were 507 and 667, respectively. The protein content in freeze-dried *Lemna* and *Azolla* was approximately 17%. The high tolerance and accumulation of Se and Zn in *Lemna* and *Azolla*, combined with their rapid growth, high protein content and transformation of inorganic to organic Se species upon Se(IV) exposure make *Lemna* and *Azolla* potential candidates for the production of Se(IV)- and Zn-enriched biomass that can be used as crop fertilizers or protein-rich food/feed supplements or ingredients. Accordingly, by growing the *Azolla* and *Lemna* on wastewater, a high-value product can be produced from wastewater while recovering resources.

Keywords: Aquatic plants; dietary protein; micronutrient fertilizer; nutrient accumulation; Se; Zn

3.1 Introduction

Se and Zn are essential for humans and animals. Certain human proteins require Se or Zn to maintain their catalytic activity. Se and Zn are involved in the biosynthesis of proteins and in scavenging of reactive oxygen species (Sánchez et al., 2017). For plants, Zn is also an essential element that can enhance plant growth, whereas Se is considered as a beneficial element that can enhance resistance to stress (Feng et al., 2013; Subramanyam et al., 2019). Despite the importance of these trace nutrients, Se and Zn intakes are still low in a wide range of countries (Broadley et al., 2006; Thomson, 2004), resulting in Se and Zn deficiencies. In order to achieve optimal Se and Zn levels in the human diet, Se and Zn inorganic fertilizers are commonly added to crops in deficient regions for enrichment of crops with Se and Zn. However, plants can not take up all of Se applied through conventional inorganic fertilizers, because of fast leaching and high mobilization of the applied inorganic Se and Zn (Broadley et al., 2006; Wang et al., 2018). Micronutrient slow-release fertilizers, such as organic fertilizers (Bañuelos et al., 2016; Bañuelos & Hanson, 2010) are therefore currently being explored.

On the contrary, excess of Se and Zn in the environment is also frequently observed. Se and Zn are likely to co-occur in waste streams or in the environment as a result of both industrial and agricultural activities, such as in petroleum refinery effluents (Wake, 2005), in the groundwater of uranium mill tailings repositories (Morrison et al., 2002), in leachates of Zn mining (Etteieb et al., 2020), and in agricultural runoff after application of Se and Zn fertilizers for improving crop yield and nutrition (Mao et al., 2014). Wastewaters loaded with both Se and Zn can serve as potential nutrient sources from which the nutrients may be valorized to produce slow-release organic fertilizers. Accordingly, it could be beneficial to produce micronutrient-enriched fertilizers or food/feed supplements locally from micronutrient-containing waters using environmentally friendly techniques.

Phytoextraction is an environmentally friendly method to recover micronutrients from wastewater. The aquatic plants *Lemna* and *Azolla* have fast growth rates, high tolerance to extreme conditions, and can be easily harvested, making them as potential plant species for natural wastewater/water treatment systems (Miranda et al., 2016; Sasmaz et al., 2015). Most the previous studies have investigated the Se or Zn removal by aquatic plants individually, while little studies consider the case of Se and Zn co-

occurrence in the environment. Hence, this chapter explored the case of simultaneous Se and Zn contamination in water and investigated the simultaneous effects of Se and Zn on floating aquatic plants that are rich in proteins. In addition, earlier studies only focused on the removal of contaminants by different biotechnologies (Tan et al., 2016), while the byproducts generated from the water treatment processes could be considered as a new contaminant for the environment (Luo et al., 2020). In this study, we will not only consider the removal of Se and Zn by biotechnology, but also propose the potential use of the resulting products (Se- and Zn-enriched *Lemna* and *Azolla*) as micronutrient-enriched food/feed supplements and biofertilizers. Especially, these two aquatic plants are rich in proteins, so they may also be considered as alternative (Se- and Zn-enriched) protein sources replacing animal proteins in food and feed systems. This may be particularly useful for Se, as Se is preferably accumulated as an organic form, i.e., as selenoaminoacids (Eiche et al., 2015). Such alternative proteins would contribute to the sustainability of our food and feed production systems, as they can be produced on wastewater as nutrient source during secondary or tertiary treatment.

Accordingly, in this study two aquatic plants, *Lemna* and *Azolla*, were planted in media with different dosages of Se and Zn to investigate: 1) the effect of two Se forms (selenite and selenate), supplied together with Zn, on the growth of the two plants; 2) the potential of the two plants to remove Se and Zn together, towards the potential use of the Se- and Zn-enriched *Lemna* and *Azolla* as micronutrient-enriched fertilizers and/or food/feed supplements; and 3) the potential interactions of Se and Zn on the uptake of these elements by those two plant species.

3.2 Materials and methods

3.2.1 Experimental materials

The aquatic plants *Lemna minuta* and *Azolla cristata* were collected from a natural freshwater canal in Delft (The Netherlands) and cultivated in modified Hoagland solution at pH 6 to acclimatize for seven days in a greenhouse (Hassan & Mostafa, 2016; Ohlbaum et al., 2018). The modified Hoagland solution contained: 472 mg/L $Ca(NO_3)_2{\cdot}4H_2O$, 202 mg/L KNO_3, 197 mg/L $MgSO_4{\cdot}7H_2O$, 9 mg/L $FeSO_4{\cdot}7H_2O$, 0.62 mg/L $MnSO_4{\cdot}7H_2O$, 1.14 mg/L H_3BO_3, 32 µg/L $CuSO_4{\cdot}5H_2O$, 12.8 µg/L $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 1.79 µg/L $NaWO_4{\cdot}2H_2O$, and 4 µg/L $CoCl_2{\cdot}6H_2O$. Afterwards, 1

g (wet weight) of each plant was transplanted into 150 mL of modified Hoagland solution with varied concentrations of sodium selenite (Na_2SeO_3), sodium selenate (Na_2SeO_4) and zinc chloride ($ZnCl_2$).

3.2.2 Experimental design

The concentrations of Se and Zn were ranged from 0 to 5.0 mg/L, including 0, 0.5, 2.5 and 5.0 mg/L, and also additionally 1.0 mg/L for Zn. Medium without Se and Zn served as control. All experiments were performed in triplicate, with a total of 210 pots for the 70 treatments (Table 3.1). The temperature in the greenhouse varied between 25 and 30 °C and light was provided with a minimum light intensity of 100 µmol/m^2/s photons. The whole plants were harvested after seven days of incubation, washed with deionized (DI) water and analyzed for dry weight, root length, total Se and Zn content, and protein content**.**

3.2.3 Analytical methods

3.2.3.1 Determination of Se and Zn concentration

The harvested whole plants were oven-dried at 60 °C until constant weight, homogenized and then digested using a microwave oven (CEM Mars 5, Matthews, NC, USA). Dry samples were weighed into a digestion vessel followed by the addition of 10 mL concentrated HNO_3. The digestion temperature was raised to 165 °C in 10 min and kept for 1 min, then raised to 175 °C in 2 min and maintained for 5 min. The digested solution was diluted with DI water and analyzed for total Se and Zn. Total Se was determined using an atomic absorption spectrophotometer coupled to a graphite furnace (GF-AAS, Thermo Elemental Solaar MQZ, GF95, Thermo Fisher Scientific, Waltham, MA, USA) and total Zn with flame atomic absorption spectroscopy (F-AAS, AAnalyst 200, Perkin Elmer, Waltham, MA, USA) as described by Ohlbaum et al. (Ohlbaum et al., 2018) and Mal et al. (Mal et al., 2016), respectively.

Table 3.1. Se(IV) or Se(VI) concentration and Zn concentration applied in each treatment with *Lemna* or *Azolla.* Each plant received 35 different treatments including 5 treatments of different Zn concentrations in the absence of Se (column 1), 15 treatments of different Se(IV) and Zn concentrations and 15 treatments of different Se(VI) and Zn concentrations (columns 2, 3 and 4).

Treatment	Content (mg/L)		Treatment	Content (mg/L)		Treatment	Content (mg/L)		Treatment	Content (mg/L)	
	Se	Zn		Se (IV or VI)	Zn		Se (IV or VI)	Zn		Se (IV or VI)	Zn
Control	0	0	Se0.5Zn0	0.5	0	Se2.5Zn0	2.5	0	Se5.0Zn0	5.0	0
Se0Zn0.5	0	0.5	Se0.5Zn0.5	0.5	0.5	Se2.5Zn0.5	2.5	0.5	Se5.0Zn0.5	5.0	0.5
Se0Zn1.0	0	1.0	Se0.5Zn1.0	0.5	1.0	Se2.5Zn1.0	2.5	1.0	Se5.0Zn1.0	5.0	1.0
Se0Zn2.5	0	2.5	Se0.5Zn2.5	0.5	2.5	Se2.5Zn2.5	2.5	2.5	Se5.0Zn2.5	5.0	2.5
Se0Zn5.0	0	5.0	Se0.5Zn5.0	0.5	5.0	Se2.5Zn5.0	2.5	5.0	Se5.0Zn5.0	5.0	5.0

3.2.3.2 Determination of protein content

The protein content was calculated by multiplying the total nitrogen (TN) concentration by 5.0. The conversion factor 5.0 was selected based on the literature (Brouwer et al., 2019; Brouwer et al., 2018; Zhao et al., 2014) and the analyzed amino acid profile of the two plants (Table 3.S1). TN was determined according to Van Ranst et al. (1999). Dry whole plant samples (0.100 g) were weighed and digested with 0.2 g Se catalyst and 10 mL concentrated sulfuric acid at 380 °C until the digestion solution turned clear. After acid digestion, the sample solution was cooled and ammonia was distilled and collected in boric acid. Then, a back titration was performed to measure the N concentration.

3.2.3.3 Determination of Se speciation

Se speciation analysis was determined according to Lavu et al. (Lavu et al., 2013; Lavu et al., 2012). Specifically, 0.1 g of whole plant samples and 40 mg of the enzyme protease XIV (Sigma Aldrich, St. Louis, MO, USA) were dispersed in 5 mL water in a 10-mL centrifuge tube. The mixture was shaken for 24 h at 37 °C and centrifuged for 30 min at 10000g. The supernatant was filtered through a 0.45-µm syringe PVDF membrane filter. The filtrate was analysed for total Se and Se speciation by inductively coupled plasma-mass spectrometry (ICP-MS, PerkinElmer DRC-e, Sunnyvale, CA, USA) and ICP-MS coupled to high performance liquid chromatography (Series 200 HPLC, Perkin Elmer, Sunnyvale, CA, USA), respectively. A Hamilton PRP-X100 anion exchange column (250 mm × 4.6 mm, 5 µm) was used as stationary phase in the HPLC instrument. The mobile phase was 10 mM citric acid with 5% (v/v) methanol, adjusted to pH 5.0. The standard solutions of the different Se species were prepared with sodium selenite (Na_2SeO_3), sodium selenate (Na_2SeO_4), Se-methionine (SeMet), Se-cystine ($SeCys_2$), Se-methyl-selenocysteine (SeMetSeCys), γ-glutamyl-methylselenocysteine and γ-glutamyl-selenomethionine.

3.2.4 Statistical analysis

Data were analyzed by ANOVA and Duncan's multiple comparison tests in SPSS 20.0. Graphs and tables were plotted by Excel 2016 and R-3.4.1. The following parameters were calculated:

$$Bioconcentration\ factor\ (BCF) = \frac{C_{plant}}{C_{medium}} \quad (1)$$

$$\text{Plant removal efficiency } (\%) = \frac{C_{plant} \times M_{plant}}{C_{mediun} \times V_{medium}} \times 100\% \quad (2)$$

Where C_{plant} (mg/kg dry weight) is the average Se or Zn concentration in the *Azolla* or *Lemna* biomass, C_{medium} (mg/L) is the total Se or Zn concentration in the corresponding medium, M_{plant} is the average biomass weight and V_{medium} is the volume of the medium (150 mL).

3.3 Results

3.3.1 Plant growth

The effect of Se and Zn on the growth of *Lemna* and *Azolla* was assessed based on the biomass production (i.e., dry weight, Fig. 3.1) and the root length (Fig. 3.2). A decrease of biomass production of both plant species was significantly associated with Se(IV) and Zn application (Fig. 3.1a and 3.1c) ($P < 0.01$). Increasing concentrations of Se(IV) from 0 to 5.0 mg/L reduced the dry weight of *Lemna* stepwise from 0.12 g to 0.05 g (Fig. 3.1a) ($P < 0.01$), while 0.5 mg/L of Se(IV) reduced the dry weight of *Azolla* from 0.10 g to 0.08 g ($P < 0.01$), and no further decrease was observed at higher Se(IV) concentrations (Fig. 3.1c). On the other hand, the exposure to up to 5.0 mg/L of single Se(VI) did not cause growth inhibition on *Lemna* (Fig. 3.1b) ($P = 0.24$). For *Azolla*, the effect of Se(VI) was similar to that of Se(IV) (Fig. 1d). In addition, the application of Se(VI) seemed to slightly counteract the inhibiting effect of Zn on the growth of *Lemna* (Fig. 3.1b). For example, the exposure to 1.0 mg/L of Zn in the absence of Se(VI) caused a remarkable decrease in the dry weight of *Lemna* from 0.12 g to 0.05 g ($P < 0.01$), while in the presence of 0.5–5.0 mg/L of Se(VI) and the same Zn concentration (1.0 mg/L), the dry weight of *Lemna* was approximately 0.11 g ($P = 0.92$).

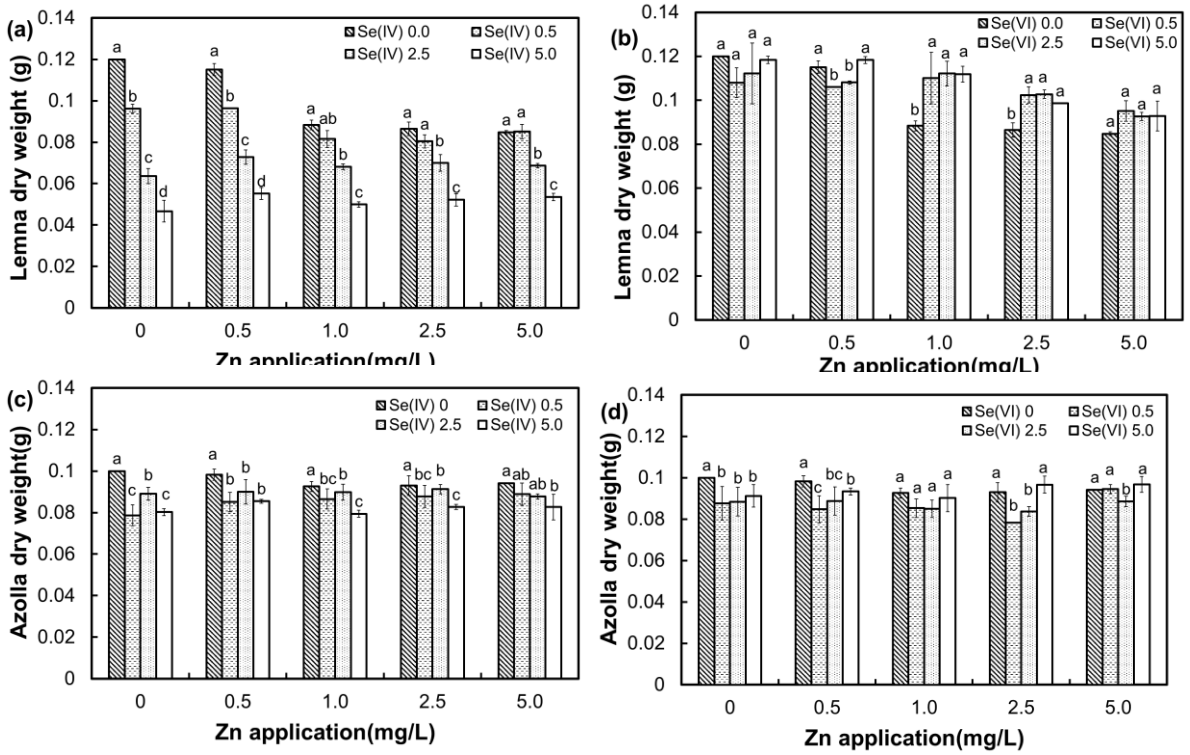

Figure 3.1. Dry weight of plants grown at different Se and Zn concentrations: (a) *Lemna* grown on Se(IV) and Zn, (b) *Lemna* grown on Se(VI) and Zn, (c) *Azolla* grown on Se(IV) and Zn and (d) *Azolla* grown on Se(VI) and Zn. Values are mean ± standard deviation (n=3). Different letters indicate statistically significant differences among treatments with the same Zn application according to Duncan's multiple comparison tests ($P < 0.05$).

The length of the roots of *Lemna* and *Azolla* decreased with increasing Se and Zn concentrations. An increment of the Se(IV) dosage from 0 to 5.0 mg/L significantly reduced the root length of *Lemna* from 1.5 to 0.5 cm ($P < 0.01$) and decreased the root length of *Azolla* from 3.7 to 1.4 cm ($P < 0.01$) (Fig. 3.2a and 3.2c). Additionally, the exposure to 5.0 mg/L of Se(VI) caused a decrease of 0.7 cm in both *Lemna* ($P < 0.01$) and *Azolla* ($P = 0.02$) roots (Fig. 3.2b and 3.2d). Similarly, an increasing Zn application from 0 to 5.0 mg/L in the absence of Se caused a noticeable decrease in the root length of *Lemna* from 1.5 to 0.7 cm ($P < 0.01$), while the growth of the root length of *Azolla* was not associated with the Zn application ($P = 0.08$). The dose-response data could be described satisfactorily by a log-logistic equation for *Lemna* (Fig. 3.2a and 3.2b). From the fitted equation, the effective concentrations of Se that caused a 50% inhibition (ED_{50}) of the root length of *Lemna* were estimated at 2.7 and 3.8 mg/L Se for the

application of Se(IV) and Se(VI), respectively. These results indicated that *Lemna* was more resistant to Se(VI) than to Se(IV).

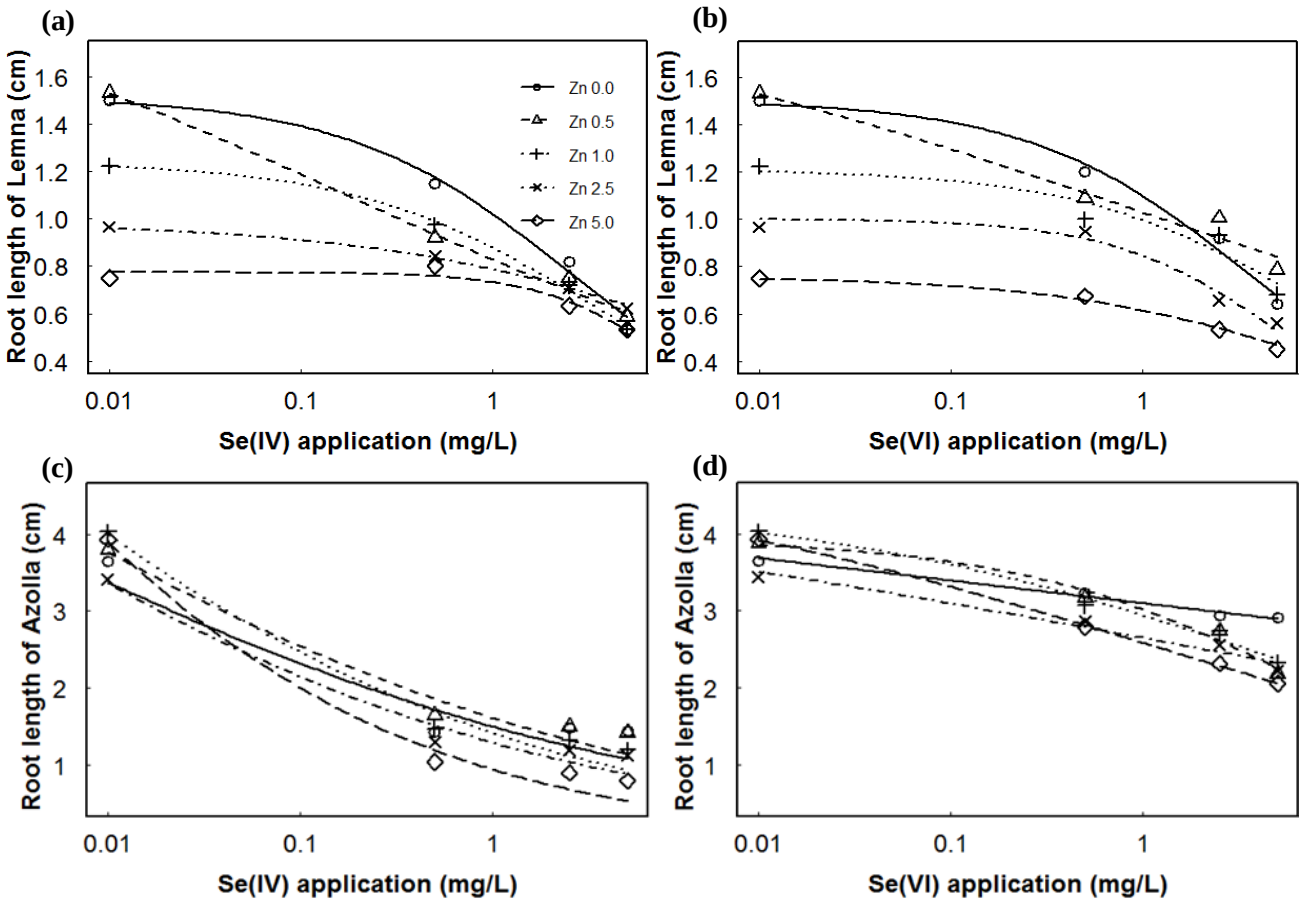

Figure 3.2. Root length of plants after exposure to different Se and Zn concentrations: (a) *Lemna* grown on Se(IV) and Zn; (b) *Lemna* grown on Se(VI) and Zn; (c) *Azolla* grown on Se(IV) and Zn; and (d) *Azolla* grown on Se(VI) and Zn. Lines are the fitted log-logistic curves. To allow log transformation, a small value (0.01) was added to the zero Se concentration.

3.3.2 Se concentration in *Lemna* and *Azolla*

The Se concentration in the plants differed significantly depending on the chemical form and concentration of the Se amendment (Fig. 3.3) ($P < 0.01$). For both *Azolla* and *Lemna*, the increasing of Se concentration was significantly related to the increasing Se dosage in the medium ($P < 0.01$). Both plants have a higher ability to take up Se(IV) compared with Se(VI), which is reflected by the higher Se content in the plants cultivated on the Se(IV)-enriched medium.

The presence of Zn in the medium affected the Se concentration in *Lemna* and *Azolla* differently (Fig. 3.3). In general, the Se concentration in *Lemna* gradually increased with increasing Zn application dose, while it declined in *Azolla*. Specifically, for the plants exposed to Se(IV), the maximum Se concentration in the plants was found at 5.0 mg/L Se(IV) with 5.0 mg/L Zn in *Lemna* (1665 mg/kg) and 5.0 mg/L Se(IV) without Zn in *Azolla* (1139 mg/kg) (Fig. 3.3a and 3.3c). Similarly, for the Se(VI) application, the highest Se concentration in *Lemna* was 168 mg/kg at 5.0 mg/L Se(VI) coupled with a 5.0 mg/L Zn dose, and the maximum Se concentration in *Azolla* was 196 mg/kg at 5.0 mg/L Se(VI) without Zn (Fig. 3.3b and 3.3d).

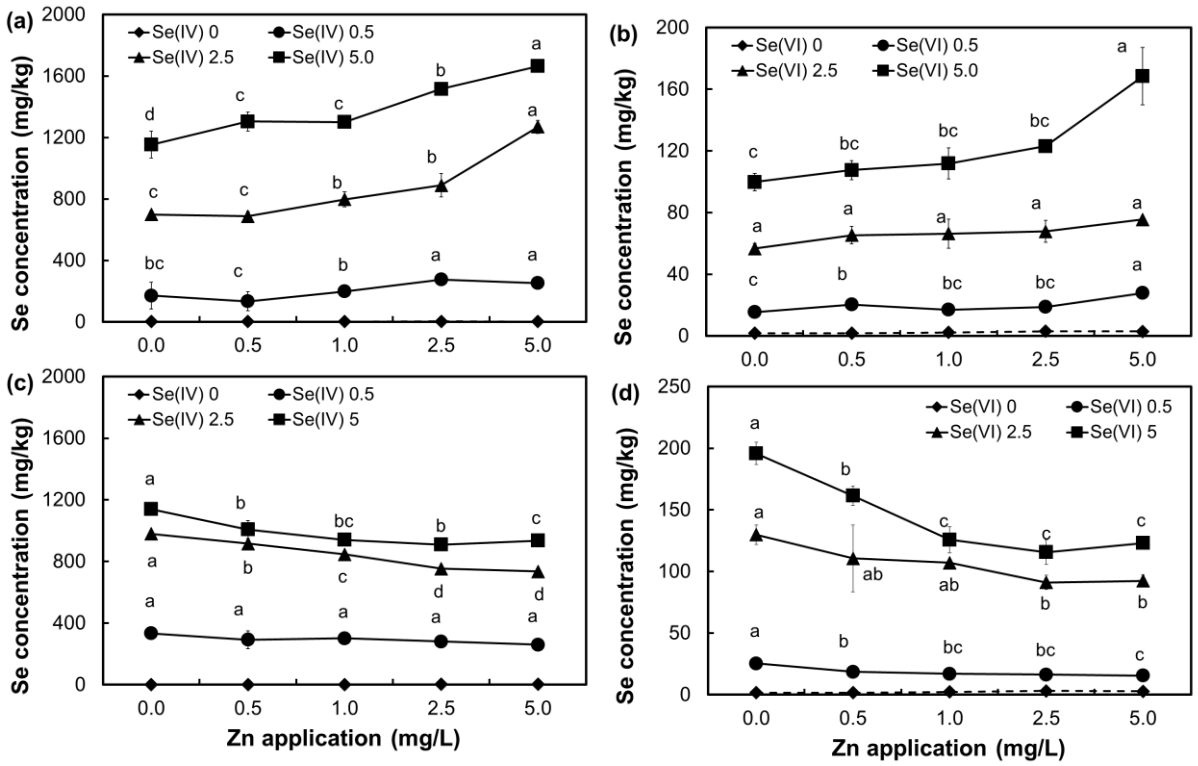

Figure 3.3 Se content in plants grown at different Se and Zn concentrations: (a) *Lemna* grown on Se(IV) and Zn, (b) *Lemna* grown on Se(VI) and Zn, (c) *Azolla* grown on Se(IV) and Zn and (d) *Azolla* grown on Se(VI) and Zn. Values are mean ± standard deviation (n=3). Different letters indicate statistically significant differences among treatments with the same Se application according to Duncan's multiple comparison tests *(P < 0.05)*.

3.3.3 Se accumulation and removal by *Lemna* and *Azolla*

The total Se accumulation in the plants and the Se removal efficiency are presented in Tables 3.2–3.5. In both plants, the total Se accumulation significantly increased with the increase of Se dose in the medium (Tables 3.2 and 3.4), whereas a decreasing trend was generally observed in the Se removal efficiency (Tables 3.3 and 3.5) ($P < 0.01$).

When both plants were exposed to Se(VI), the Se accumulation and Se removal efficiency were much lower compared to Se(IV) exposure. Specifically, the highest Se accumulation and Se removal efficiency were 15.9 and 17.3 µg/pot, and 3.6 and 3.0% in *Lemna* and *Azolla*, respectively, when exposed to the Se(VI) growth solution. However, when *Lemna* and *Azolla* were exposed to Se(IV) medium, the maximum Se accumulation and Se removal efficiency were 89.2 and 90.5 µg/pot, and 30.2 and 38.9%, respectively.

The exposure to Zn increased the Se removal efficiency and Se accumulation in *Lemna* (Tables 3.2 and 3.3), while having an inhibitory effect on the Se removal efficiency and Se accumulation in *Azolla* (Tables 3.4 and 3.5). For instance, for *Lemna* grown on 2.5 mg/L Se(IV), increasing the Zn dose from 0 to 5.0 mg/L remarkably increased the Se accumulation from 44 to 89 µg/pot and the Se removal efficiency from 12 to 24% ($P < 0.01$). In contrast, the addition of 5.0 mg/L of Zn to the growth medium of *Azolla* containing 2.5 mg/L Se(IV) caused a considerable decline of the Se accumulation from 88 to 66 µg/pot and the Se removal efficiency from 24 to 18% ($P < 0.01$).

3.3.4 BCF_{Se}

The BCF_{Se} in *Lemna* ranged from 231 to 552 for the Se(IV) addition and from 20.0 to 55.6 for the Se(VI) treatments. In *Azolla*, the range of the BCF_{Se} values was 182 to 667 for the Se(IV) application and 23.1 to 51.9 for the Se(VI) treatments (Table 3.6).

Table 3.2. Total Se accumulation in *Lemna* (µg/pot). Values are mean ± standard deviation (n=3). Different letters indicate statistically significant differences among treatments with the same Se application according to Duncan's multiple comparison test *(P < 0.05)*.

Se treatment (mg/L)	Se(IV)					Se(VI)				
	Zn 0.0	Zn 0.5	Zn 1.0	Zn 2.5	Zn 5.0	Zn 0.0	Zn 0.5	Zn 1.0	Zn 2.5	Zn 5.0
0.0	-	-	-	-	-	-	-	-	-	-
0.5	16.5 ± 0.3^{c}	12.2 ± 0.8^{d}	16.1 ± 0.2^{c}	22.6 ± 1.3^{a}	20.7 ± 1.0^{b}	1.7 ± 0.2^{c}	2.2 ± 0.2^{b}	2.0 ± 0.2^{bc}	1.9 ± 0.2^{bc}	2.7 ± 0.2^{a}
2.5	44.5 ± 1.0^{e}	50.0 ± 1.3^{d}	56.3 ± 0.2^{c}	65.0 ± 3.0^{b}	88.8 ± 3.7^{a}	7.0 ± 0.1^{ab}	7.6 ± 0.5^{ab}	8.0 ± 0.3^{a}	6.9 ± 0.6^{b}	5.8 ± 0.4^{c}
5.0	53.6 ± 1.9^{d}	73.9 ± 5.0^{b}	64.3 ± 3.0^{c}	77.6 ± 4.0^{b}	89.2 ± 2.12^{a}	11.9 ± 0.7^{b}	12.6 ± 1.1^{b}	12.5 ± 0.7^{b}	11.9 ± 0.2^{b}	15.9 ± 0.1^{a}

Table 3.3. Se removal efficiency by *Lemna* (%). Values are mean ± standard deviation (n=3). Different letters indicate statistically significant differences among treatments with the same Se application according to Duncan's multiple comparison tests (*P < 0.05*).

Se treatment (mg/L)	Se(IV)					Se(VI)				
	Zn 0.0	Zn 0.5	Zn 1	Zn 2.5	Zn 5.0	Zn 0.0	Zn 0.5	Zn 1.0	Zn 2.5	Zn 5.0
0.0	-	-	-	-	-	-	-	-	-	-
0.5	22.0 ± 0.4^{c}	16.2 ± 1.0^{d}	21.5 ± 0.3^{c}	30.2 ± 1.8^{a}	27.6 ± 1.4^{b}	2.2 ± 0.2^{c}	2.9 ± 0.3^{b}	2.7 ± 0.3^{bc}	2.5 ± 0.3^{bc}	3.6 ± 0.2^{a}
2.5	11.8 ± 0.3^{e}	13.3 ± 0.3^{d}	15.0 ± 0.1^{c}	17.3 ± 0.8^{b}	23.7 ± 1.0^{a}	1.9 ± 0.1^{ab}	2.0 ± 0.6^{ab}	2.1 ± 0.1^{a}	1.8 ± 0.2^{b}	1.5 ± 0.1^{c}
5.0	7.1 ± 0.3^{d}	9.8 ± 0.7^{b}	8.6 ± 0.4^{c}	10.3 ± 0.5^{b}	11.9 ± 0.3^{a}	1.6 ± 0.1^{b}	1.7 ± 0.1^{b}	1.7 ± 0.1^{b}	1.6 ± 0.1^{b}	2.1 ± 0.1^{a}

Table 3.4. Total Se accumulation in *Azolla* (µg/pot). Values are mean ± standard deviation (n=3). Different letters indicate statistically significant differences among treatments with the same Se application according to Duncan's multiple comparison tests (*P < 0.05*).

Se treatment (mg/L)	Se(IV)					Se(VI)				
	Zn 0.0	Zn 0.5	Zn 1.0	Zn 2.5	Zn 5.0	Zn 0.0	Zn 0.5	Zn 1.0	Zn 2.5	Zn 5.0
0.0	-	-	-	-	-	-	-	-	-	-
0.5	29.2±0.1^{a}	23.4±0.3^{c}	25.9±0.2^{b}	25.5±0.8^{b}	23.8±0.8^{c}	2.2±0.1^{a}	1.6±0.1^{b}	1.4±0.1^{b}	1.4±0.2^{b}	1.5±0.1^{b}
2.5	88.4±3.3^{a}	74.8±2.3^{b}	74.2±1.2^{b}	65.9±3.7^{c}	65.6±1.8^{c}	11.2±1.7^{ab}	12.4±0.1^{a}	9.4±0.9^{bc}	7.9±0.6^{c}	8.9±1.34^{c}
5.0	90.5±1.2^{a}	86.6±5.8 ^{a}	78.4±4.3^{a}	79. 9±3.3^{a}	80.7±2.9^{a}	17.3±1.3^{a}	15.1±0.5^{b}	15.3±1.4^{b}	11.6±0.8^{c}	11.2±0.1^{c}

Table 3.5. Se removal efficiency by *Azolla* (%). Values are mean ± standard deviation (n=3). Different letters indicate statistically significant differences among treatments with the same Se application according to Duncan's multiple comparison tests (*P < 0.05*).

Se treatment (mg/L)	Se(IV)					Se(VI)				
	Zn 0.0	Zn 0.5	Zn 1.0	Zn 2.5	Zn 5.0	Zn 0.0	Zn 0.5	Zn 1.0	Zn 2.5	Zn 5.0
0.0	-	-	-	-	-	-	-	-	-	-
0.5	38.9±0.1^{a}	31.2±0.4^{c}	34.6±0.2^{b}	34.0±1.0^{b}	31.8±1.0^{c}	3.0±0.1^{a}	2.2±0.1^{b}	1.9±0.1^{b}	1.9±0.3^{b}	2.0±0.1^{b}
2.5	23.6±0.9^{a}	20.0±0.6^{b}	20.0±0.3^{b}	17.6±1.0^{c}	17.5±0.5^{c}	3.0±0.5^{ab}	3.3±0.1^{a}	2.5±0.2^{bc}	2.1±0.2^{c}	2.4±0.4^{bc}
5.0	12.1±0.2^{a}	11.5±0.8^{a}	10.4±0.5^{a}	10.6±0.4^{a}	10.8±0.4^{a}	2.3±0.2^{a}	2.0±0.1^{b}	2.0±0.2^{b}	1.5±0.1^{c}	1.5±0.1^{c}

Table 3.6. Bioconcentration factor of Se (BCF_{Se}) determined for *Lemna* and *Azolla*

Se treatment (mg/L)	Se(IV)					Se(VI)				
	Zn 0.0	Zn 0.5	Zn 1.0	Zn 2.5	Zn 5.0	Zn 0.0	Zn 0.5	Zn 1.0	Zn 2.5	Zn 5.0
Lemna										
0.5	343	268	397	552	506	31.0	40.8	34.1	37.6	55.6
2.5	280	275	319	355	508	22.7	26.2	26.5	27.1	30.2
5.0	231	261	260	303	333	20.0	21.5	22.4	24.6	33.7
Azolla										
0.5	667	584	603	562	521	50.9	37.3	40.0	32.6	31.2
2.5	392	366	338	301	294	51.9	44.2	42.9	36.5	36.9
5.0	228	202	188	182	187	39.2	32.3	25.1	23.1	24.6

3.3.5 Zn concentration in *Lemna* and *Azolla*

A increased of Zn concentration in both plants was significantly associated with the increasing Zn dose in the culture solution (Fig. 3.4) ($P < 0.01$). The maximum Zn concentrations in *Lemna* and *Azolla* were 3144 and 1709 mg/kg, respectively, when exposed to the highest Zn amount (5.0 mg/L), but at different Se concentrations.

For the same Zn application, the Zn concentration in *Lemna* significantly increased with increasing Se(IV) dose ($P < 0.01$), while it remained almost constant with increasing Se(VI) concentrations (Fig. 3.4a and 3.4b). In contrast, the Zn concentration in *Azolla* generally declined with increasing amounts of Se(IV) and Se(VI) in the medium ($P < 0.01$), except for a slight increase observed at 5 mg/L Se(IV) (Fig. 3.4c and 3.4d). In the presence of 5 mg/L Zn, increasing the dose of Se(IV) from 0 to 5 mg/L raised the Zn content in *Lemna* from 1769 to 3144 mg/kg ($P < 0.01$), while it diminished the Zn content in *Azolla* from 1709 to 1530 mg/kg ($P = 0.09$).

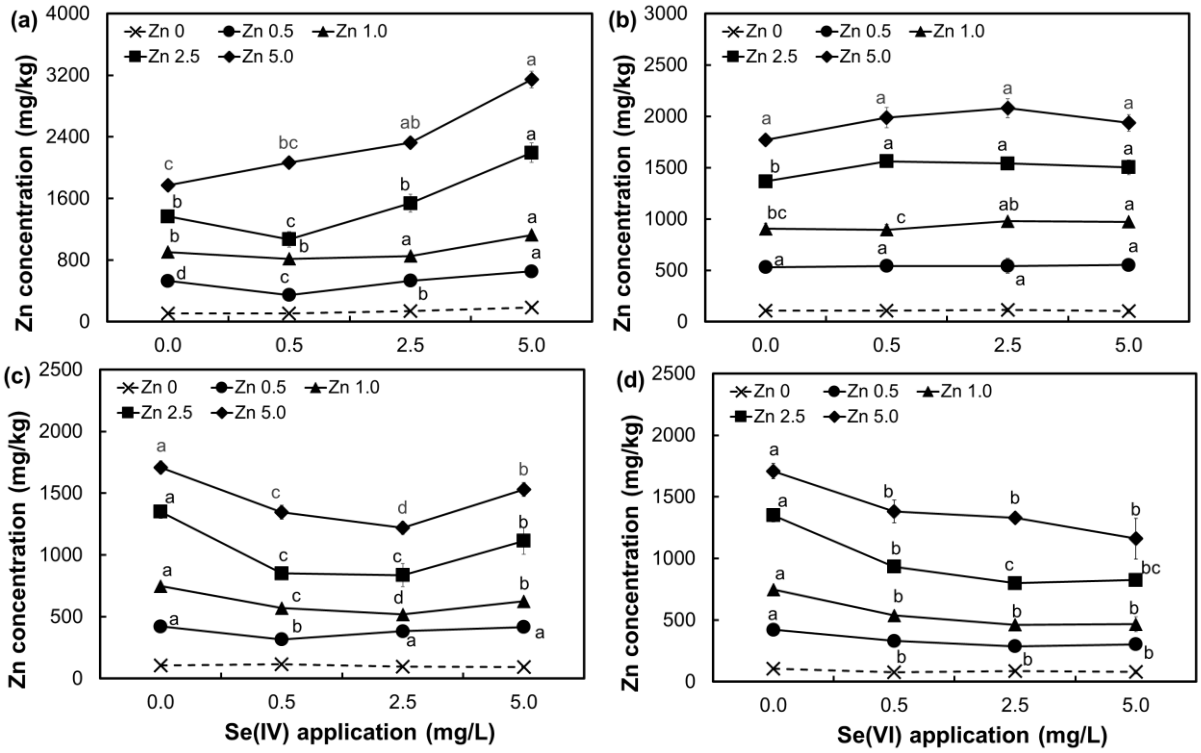

Figure 3.4. Zn content in plants after incubation on different Se and Zn concentrations: (a) *Lemna* grown on Se(IV) and Zn, (b) *Lemna* grown on Se(VI) and Zn, (c) *Azolla* grown on Se(IV) and Zn and (d) *Azolla* grown on Se(VI) and Zn. Values are mean ± standard deviation (n=3). Different letters indicate statistically significant differences among treatments with the same Zn application according to Duncan's multiple comparison tests *(P < 0.05)*.

3.3.6 Zn accumulation, removal and BCF_{Zn}

The total Zn accumulation, Zn removal efficiency and BCF_{Zn} by *Lemna* and *Azolla* are illustrated in Tables 3.S2–3.S6 (see Supplementary Information). The total Zn accumulation in both plants significantly raised with the increase in Zn application, while the Zn removal efficiency decreased remarkably ($P < 0.01$). The largest Zn accumulation and the highest Zn removal efficiencies in *Lemna* and *Azolla* were up to 196 µg/pot and 90.7%, and 151 µg/pot and 64.7%, respectively. The BCF_{Zn} was much higher than the BCF_{Se} in both plants (Table 3.S6). The maximum BCF_{Zn} reached up to 1310 and 843 in *Lemna* and *Azolla*, respectively.

3.3.7 Protein content

The maximum content of true protein in *Lemna* and *Azolla* was 162 and 170 mg/g when exposed to 2.5 mg/L Se(IV), respectively, whereas a protein content of 131 and 159 mg/g was observed when *Lemna* and *Azolla* were grown in the control medium. The inhibition effect of Se and Zn exposure was not reflected in the protein content, except a decrease of protein content in the presence of 5.0 mg/L Se(IV) for *Lemna* ($P = 0.04$) when increasing the Zn application to 5.0 mg/L (Fig. 3.5).

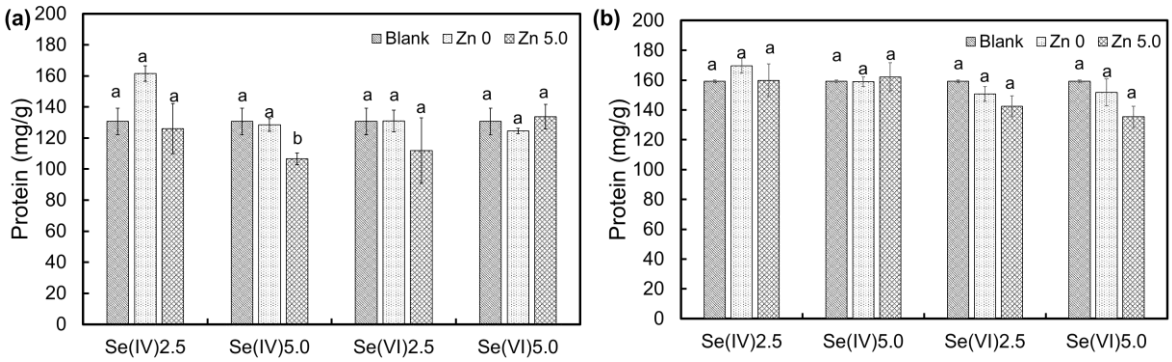

Figure 3.5. Protein content in *Lemna* and *Azolla* after incubation in the different dosages of Se and Zn medium: (a) *Lemna* and (b) *Azolla*. Values are mean ± standard deviation (n=6). Different letters indicate statistically significant differences among treatments with the same Zn application according to Duncan's multiple comparison tests *($P < 0.05$)*.

3.3.8 Se speciation in the plants

The plants grown in the presence of 2.5 mg/L of Se medium were selected for Se speciation determination. The recovery of Se in both plants grown in the Se(IV) containing medium ranged from 13% to 40% compared to the total Se content after protease hydrolysis, while it ranged from 63% to 92% in the presence of Se(VI) medium (Table 3.S7). When plants were grown in the Se(IV) medium, the production rate of Se amino acids was higher than when plants were grown in the medium with Se(VI) (Fig. 3.6, Table 3.S8).

Organic Se species such as SeMet, SeMetSeCys and $SeCys_2$ were the main Se species of identified Se in *Lemna* grown on Se(IV)-enriched medium (Fig. 3.6a), whereas inorganic Se in the form of selenate (approximately 50%) was the predominant Se species in *Lemna* grown on Se(VI)-containing medium (Fig. 3.6b, Table 3.S8). SeMet accounted for the highest proportion (5.9% of total Se) of identified Se species in *Lemna* grown in Se(IV), while SeMetSeCys predominated (4.6% of total Se) in the Se(IV) treatment with Zn.

In *Azolla,* the main species were organic SeMet and SeMetSeCys and inorganic selenate upon Se(IV) exposure, while selenate was detected in *Azolla* as predominant species (92% and 75% in the absence and presence of Zn, respectively) when exposed to Se(VI). Moreover, excepting selenate species in *Azolla*, SeMetSeCys was the most abundant Se species under Se(IV) exposure with Zn, while SeMet has the highest proportion under only Se(IV) exposure (Figs 6c and 6d, Table S8). Apart from the identified Se species, some unidentified Se species were found in both *Lemna* and *Azolla* grown in the presence of Se(IV) (Figs. 3.6a and 3.6c).

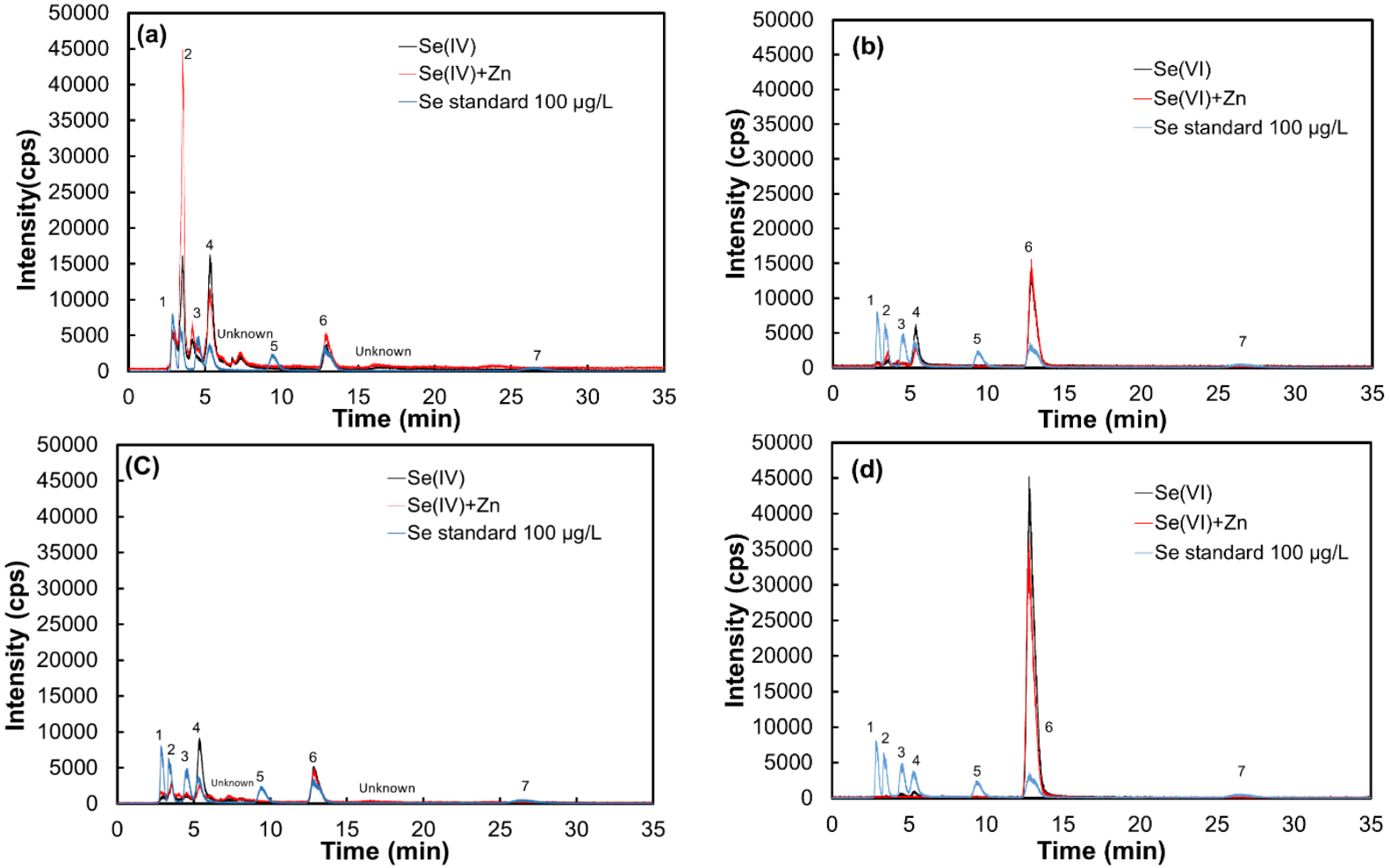

Figure 3.6. Chromatograms of Se speciation in *Lemna* and *Azolla* compared with that of a Se standard solution of 100 µg/L each species: (1) Se-cystine, (2) Se-methylselenocysteine, (3) selenite, (4) Se-methionine, (5) γ-glutamyl-methylselenocysteine, (6) selenate, and (7) γ-glutamyl-selenomethionine. (a) *Lemna* grown on Se(IV) and Zn, (b) *Lemna* grown on Se(VI) and Zn, (c) *Azolla* grown on Se(IV) and Zn and (d) *Azolla* grown on Se(VI) and Zn.

3.4 Discussion

3.4.1 Se uptake and toxic effects of Se and Zn on *Lemna* and *Azolla*

Changes in the growth rate of plants, as evidenced by biomass production and root length, are a direct indicator of plant toxicity in contaminated environments (Becker, 2013; Duan et al., 2010). In this study, the decrease of biomass production of both *Lemna* and *Azolla* was significantly associated (P<0.001) with increasing Se(IV) dosage in the medium (Figs. 3.1 and 3.2), while no inhibition effect was found at the highest dose of Se(VI) (P = 0.24 and 0.11 for *Lemna* and *Azolla*, respectively). The toxic effect of Se(IV) to aquatic plants has been reported previously. Zhong et al. (2016) reported negative impacts of Se(IV) on the chlorophyll fluorescence, starch content and fatty acid content of the duckweed *Landoltia punctata* after exposure to > 40 µmol/L Se(IV) (equivalent to 3.2 mg/L Se). Carvalho and Martin (2001) also recorded that dry biomass of the duckweed *Lemna obscura* Aust. decreased from 50 to 20 mg when the Se(IV) concentration was increased from 1 to 20 mg/L in the cultivation medium. The higher plant tolerance to Se(VI) compared to Se(IV) has also been observed in other plants such as sunflower and maize when cultivated in a hydroponic system (Garousi et al., 2016). The different toxicity of Se(IV) and Se(VI) on plants growth can be explained by the different mobility, bioavailability and metabolic uptake mechanisms (Li et al., 2008). Specifically, Se(VI) is taken up in an active way and easily distributed from roots to shoots (Arvy, 1993), while Se(IV) is absorbed in a faster passive way and quickly converted into organic forms of Se (Fig. 3.6). The organic Se is then incorporated into proteins by replacing sulfur in plant tissues, resulting in malformed selenoproteins (Arvy, 1993; de Oliveira et al., 2017). Moreover, Se-induced oxidative stress also contributes to plant toxicity (Van Hoewyk, 2013). Accordingly, the reduction of Se(IV) to organic Se forms (e.g., SeMet, $SeCys_2$) may produce additional organic Se metabolites such as selenodiglutathione, which is more toxic than Se(IV), inducing oxidative stress in plants (Van Hoewyk, 2013; Wallenberg et al., 2010).

The higher toxicity of Se(IV) can also be attributed to the higher Se uptake/accumulation in the plant tissues when the two plants were cultivated in Se(IV) containing medium compared to the Se(VI) treatment. The uptake of Se(IV) in both *Lemna* and *Azolla* was higher than that of Se(VI), resulting in Se concentrations in both plants more than 10-fold higher when they were grown in Se(IV)-supplemented

medium (Fig. 3.3). Similarly, Broyer et al. (1972) reported more accumulation of Se(IV) than Se(VI) by the hyper-accumulator *Astragalus crotolariae* after 7-12 weeks of hydroponic culture. Zhang et al. (2003) observed 4 times higher uptake of Se(IV) than Se(VI) by soybean *Glycine max*, while Arvy (1993) reported a similar uptake rate for the two Se species by bean plants (*Phaseolus vulgaris*) during a 3-h uptake experiment. The higher uptake of Se(IV) can be explained by the different metabolism of Se(IV) and Se(VI) in plants. Se(VI) is taken up by plants, reduced to Se(IV) and then converted into organic Se (Van Hoewyk, 2013). The Se(VI) reduction occurs via substitution for sulfate in the ATP sulfurylase reductase system, which is an ATP-consumption process and rate-limiting step (Salt et al., 2002; Van Hoewyk, 2013). The Se(VI) reduction rate is much slower than the uptake rate, resulting in Se(VI) saturation and lower Se content in plant tissues, which is supported by the high proportion of Se(VI) species in both *Lemna* and *Azolla* grown in the presence of Se(VI) (Fig. 3.6).

On the other hand, Versini et al. (2016) reported a lower uptake of Se(IV) compared to Se(VI) in the non-accumulator ryegrass grown in hydroponic cultures. Garousi et al. (2016) also observed that the root-to-shoot Se translocation and total Se uptake was lower in Se(IV)-treated plants (maize and sunflower) than in Se(VI)-treated plants. These discrepancies are likely caused by different external environmental factors, such as temperature, light intensity, Se exposure concentration, and medium composition — especially the content of macronutrient (e.g., sulfate and phosphate) or micronutrient elements — as well as the differences in plant species (Kikkert & Berkelaar, 2013). For instance, *Astragalus crotolariae* and soybean *Glycine max* are Se hyperaccumulators, whereas ryegrass, maize, and sunflower are non-accumulators. The Se hyperaccumulators differ from the non-accumulators in the capacity of reduction of the intracellular concentration of selenocysteine (SeCys) and SeMet (Terry et al., 2000). Non-accumulators incorporate most SeCys and SeMet into proteins with damaging effects to plant functions; while hyperaccumulators metabolize the SeCys primarily into various non-protein selenoamino acids, such as SeMetSeCys, Se-cystathionine and γ-glutamyl-methylselenocysteine to tolerate high concentrations of Se in their cells (Terry et al., 2000). The high tolerance keeps the cell membranes functional and improves the passive uptake of Se(IV), resulting in more accumulation of Se(IV) than that of Se(VI) by Se hyeraccumulators. Besides, Wang et al. (2019) evidenced that tomato (*Solanum lycopersicum* L.) has a higher uptake ability of Se(VI)

than Se(IV) at 0.0175-0.2998 mg/L Se exposure, while the opposite was observed when Se exposure was higher than 0.2998 mg/L, which partially confirms that the Se exposure concentration may affect the uptake ability of Se by plants.

3.4.2 Simultaneous uptake of Se and Zn

The Se concentration in *Lemna* increased with increasing Zn dosage in the medium, whereas it declined in *Azolla*. These results indicate that Zn promoted the Se uptake in *Lemna*, but inhibited the Se uptake in *Azolla*. Accordingly, the Zn concentration in *Lemna* also raised with raising Se dose in the culture medium, but it decreased in *Azolla*. These findings demonstrate that Se and Zn have synergetic effects in *Lemna*, but antagonistic effects in *Azolla*. The interactions between Se and other elements during plant absorption have been reported, but ambiguous conclusions have been drawn. In line with our results with *Azolla*, previous studies have demonstrated that Se absorption can decrease the uptake of certain elements. For example, Singh and Singh (1978) found that Se application reduced the Zn and Cu concentration in wheat (*Triticum aestivum*). Similarly, the antagonistic effects between Se and other elements such as Mg, K, P, Fe, Cu, and Zn have been demonstrated (Feng et al., 2009) in the fern *Pteris vittata* L. However, other studies showed that Se could improve Zn uptake, which supports part of our findings in *Lemna* (Fig. 3.4). For instance, Arvy (1992) demonstrated that *Helminthia echioides* grown under field conditions accumulated Se at concentration ranging from 2.05 to 7.90 mg/kg and that Se accumulation was positively correlated with Mn, Zn, Ni, Co, and Cd uptake. Hu et al. (2015) showed that Mn, Zn, Cu, Ni and Co in the stems of danshen (*Salvia miltiorrhiza*) were higher when Se(VI) was added to the soil. Similarly, the foliar application of Se(IV) in turnip (*Brassica rapa* var. *rapa* Linn.) positively affected the uptake of several elements such as Mg, Fe, Zn, Mn, and Cu (Li et al., 2018).

The increase of Se concentration in *Lemna* with increasing Zn addition (Fig. 3.3) could be partially explained by bioconcentration because of the decline of biomass (Li et al., 2015a). This is supported by the negative correlation between the Se concentration and biomass of *Lemna* exposed to different Zn dosages within each Se application dose, while this correlation was not observed in *Azolla* (Table 3.S9). Similarly, the increment of the Zn concentration in *Lemna* with increasing Se(IV) application may be related to the decrease of biomass (Table 3.S10), which is also evidenced by the

significant decrease of Zn accumulation (multiplying biomass by concentration) and Zn removal efficiency by *Lemna* when the Se application increased ($P < 0.01$) (Tables 3.S2 and 3.S3).

Additionally, Se is detoxified by methylation of $SeCys_2$ and SeMet to SeMetSeCys and methylselenomethionine (Me-SeMet), which cannot be incorporated into proteins, thereby avoiding toxicity and allowing a safe Se accumulation (Gupta & Gupta, 2017). In this study, SeMet accounted for the highest proportion of the identified Se species in *Lemna* grown in Se(IV), while SeMetSeCys predominated in the Se(IV) treatment with Zn (Fig. 3.6). The formation and accumulation of non-toxic SeMetSeCys species from toxic $SeCys_2$ could have been stimulated by the addition of Zn (probably by stimulating the expression of selenocysteine methyltransferase (SMT)) (Van Hoewyk, 2013), eventually resulting in the enhancement of Se accumulation and the higher Se concentration in *Lemna* in the treatment with Se and Zn.

Azolla, on the other hand, showed an antagonistic effect between the uptake of Se and Zn, which may be related to the higher metal tolerance ability of *Azolla* and the Se mediated detoxification of heavy metals. For *Lemna*, the supplementation of 5.0 mg/L Se(IV) or Zn decreased the dry weight of the biomass by 61% and 29%, respectively, while for *Azolla,* the reduction was only 20% and 6%, respectively (Fig. 3.1). The higher metal tolerance of *Azolla* suggests a detoxification mechanism was triggered by the exposure to Se and Zn. Accumulated metal ions are normally detoxified by phytochelatins (PC_S), which are synthesized from glutathione (GSH) during exposure to heavy metals. PCs form a complex with metal ions and sequester them into the vacuole (Yadav, 2010). Research has evidenced that Zn could induce the PCs synthesis in some plants (Tsuji et al., 2002). However, Hawrylak-Nowak et al. (2014) reported that Se reduced the PCs accumulation in the presence of Cd, due to Se interference with the S metabolism and replacement of S in amino-acids, forming Se-amino acids (SeMet and SeCys). Se-amino acids are subsequently incorporated into enzymatic proteins, including phytochelatin synthase as this contains cysteine, affecting their catalytic activity. Thus, the replacement of cysteine by SeCys in the phytochelatin synthase probably affects the biosynthesis and accumulation of PCs in the plant tissues (Malik et al., 2012; Wan et al., 2016). The decrease of PCs induced by Se could explain the decrease of the Zn concentration in *Azolla* when Se was supplied. In addition, increasing Se doses cause saturation of lipids and increases

membrane stiffness, resulting in lower membrane permeability and less accumulation of micro- and macronutrients in plants (Filek et al., 2010).

Contrasting interactions between the uptake of metal and metalloids by *Lemna* and *Azolla* have been reported for Cu and As. For example, Cu inhibited As uptake in *Azolla caroliniana,* while it stimulated As uptake in *Lemna minor* (Rofkar et al., 2014). Although the combined interactions of Se and Zn uptake in *Lemna* and *Azolla* were demonstrated in this study, the mechanisms are still unclear. Particularly, the reason why Zn and Se showed opposite effects on Se/Zn uptake between *Azolla* and *Lemna* should be further explored. Therefore, further studies using e.g. ^{77}Se isotope should be conducted to elucidate the mechanisms of micronutrient accumulation and Se species transformations in both *Lemna* and *Azolla* (Di Tullo et al., 2015).

3.4.3 Potential of *Lemna* and *Azolla* for wastewater treatment and production of micronutrient-enriched bioproducts

The species of *Lemna* and *Azolla* used in this study can tolerate and accumulate high Se levels, indicating that the two aquatic plants are optimal Se bioaccumulators. The maximum accumulation of Se in *Lemna* and *Azolla* was 1664 and 1139 mg/kg, respectively (Tables 3.2 and 3.4). These values are much higher than the Se accumulation in the duckweed *Landoltia punctate* (785 mg/kg), which was reported to tolerate up to 80 µmol/L Na_2SeO_3 (~6.3 mg/L Se) (Zhong & Cheng, 2016), and also higher than the Se accumulation in *Azolla caroliniana* (less than 1000 mg/kg) exposed to 1 to 10 mg/L Se(IV) (Hassan & Mostafa, 2016). Moreover, the Se and Zn accumulation in *Lemna* increased with increasing Zn exposure, which confirms that *Lemna* can efficiently remove Se and Zn together (Figs. 3.3 and 3.4).

The BCF provides information on the ability of a plant to accumulate contaminants from polluted water or soil. In general, a BCF larger than 1 suggests that a plant can be considered as a candidate with good phytoextraction efficiency (Li et al., 2015a). The BCF_{Se} values of *Lemna* and *Azolla* were all larger than 1 (Table 3.6). However, the Se removal efficiency by *Lemna* and *Azolla* was not as high as other indicators (e.g., Se content, BCF). The maximum Se(IV) removal efficiencies by *Lemna* and *Azolla* were 30% and 39%, respectively, and only 3% for Se(VI). This may be due to the small amount of biomass (1.0 g fresh weight) transferred into each treatment and to a limiting

water surface area in this study, as floating aquatic plants can only root on the water surface. Regarding the lower removal efficiency of Se(VI), it could be also partially attributed to the competition between Se(VI) and sulfate (0.83 mM) in the medium, resulting in a lower ability of the plant to take up and accumulate Se(VI). It should be noted that the sulfate concentration applied in the medium is a normal concentration in wastewater and suitable for plant growth (Li et al., 2008; Mechora et al., 2015). Therefore, the Se removal efficiencies could improve by increasing the biomass concentration or optimizing the geometry of the treatment tank and the chemical composition of wastewater.

Despite the currently low Se removal efficiencies, the high Se(IV) and Zn accumulation capacity (>1000 mg/kg Se and Zn) of *Lemna* and *Azolla*, together with the high tolerance to Se and Zn, the fast growth rate and the easy harvest make these plant species interesting alternatives for the production of micronutrient-enriched food/feed supplements or fertilizers. Therefore, the plants could also be grown in treatment ponds treating non-Se-rich wastewater (e.g., domestic wastewater), to which Se(IV) is added to obtain Se-enriched food/feed supplements or fertilizers. In that way, a high-value product is produced from the wastewater, while recovering resources. Particularly a high protein content is beneficial for food/feed supplements or ingredients. In this study, the true protein content in *Lemna* and *Azolla* were as high as 162 and 170 mg/g (Fig. 3.5), respectively, which is much higher than other plants, such as turnip (*Brassica rapa* var. *rapa Linn.*) and *Codonopsis lanceolata* (Li et al., 2018; Zhu et al., 2017). The crude protein content in turnip was 59.5-93.5 mg/g when 0-200 mg/L Se(IV) was sprayed on the leaves, and the protein content in *C. lanceolata* was 15.4-17.2 mg/g when 0.5-2.0 mg/kg Se(IV) was applied to the soil (Li et al., 2018; Zhu et al., 2017). Besides, it should be noted that the conversion factor 5 (conversion N to protein) applied in this study may potentially underestimate the protein content, due to some missing amino acids were not analyzed (e.g., cysteine and tryptophan) (Table 3.S1). Moreover, most of the identified Se species in both *Lemna* and *Azolla* were organic Se forms (around 88-90% and 54-76% of identified Se species, respectively) when grown on Se(IV) containing medium (Fig. 3.6), which are the preferred forms for Se supplementation in animal feed (Zhan et al., 2007). It has to be noted that the sum of identified Se species ranged from 3.2% to 12.2% when the plants were grown on Se(IV) containing medium (Table 3.S7). The low recoveries of Se indicated that Se(IV) is

easily metabolized and incorporated into different Se compounds, complicating their extraction and identification. Moreover, the inorganic Se content also seem to be relatively high when plants are exposed to Se(IV) and Se(VI), particularly for use in food or feed. Further product processing through fractionation of the obtained micronutrient-enriched *Lemna* and *Azolla* and/or applying lower Se dosages may help to remove excess inorganic Se. For use as micronutrient-rich feed or food supplement, a high Se concentration is preferred, but for use as feed or food ingredient, which will be consumed in higher amounts, a high protein content combined with a lower Se concentration is sufficient. Furthermore, the produced Se- and Zn-enriched bioproducts could be reused and applied as slow-release organic fertilizers in Se/Zn-deficient fields to improve the Se and Zn levels in the soil and the crops growing on these soils. The potential of Se- and Zn-enriched *Lemna* and *Azolla* for this application also requires further investigation, including pot or field trials with relevant crops.

3.5 Conclusions

This study investigated the accumulation and combined effects of Se and Zn in two aquatic plants, *Lemna* and *Azolla,* grown in hydroponic culture.

(1) The results demonstrated that Se(IV) is more toxic than Se(VI) for both plant species investigated, as evidenced by the considerable decrease of biomass content and root length when exposed to Se(IV) rich medium. Both aquatic plants took up around 10 times more Se(IV) than Se(VI) from the medium.
(2) The Se accumulation and removal efficiency by *Lemna* increased with increasing Zn dosage in the medium, whereas it declined in *Azolla*, suggesting a synergetic effect in *Lemna,* but an antagonistic effect in *Azolla*.
(3) Both *Lemna* and *Azolla* can tolerate and accumulate high levels of Se(IV) and Zn, which, combined with the observed transformation to organic species, high protein content and rapid plant growth, makes them good candidates for the production of Se- and Zn-enriched biomass that may be used as crop fertilizers or protein-rich food/feed supplements or ingredients

Supplementary Information

Table 3.S1. amino acid profile of *Lemna* and *Azolla*

	Lemna		*Azolla*	
Amino Acid	g/100g	± Stdev	g/100g	± Stdev
Aspartic acid	1.85	0.04	1.32	0.11
Glutamic acid	1.82	0.03	2.49	0.23
Asparagine	*N.D.*	*N.D.*	*N.D.*	*N.D.*
Serine	0.89	0.02	0.71	0.05
Glutamine	*N.D.*	*N.D.*	*<LOQ**	*<LOQ**
Histidine	0.42	0.00	0.26	0.01
Glycine	0.97	0.01	0.71	0.05
Threonine	0.90	0.02	0.68	0.06
Citrulline	*N.D.*	*N.D.*	*N.D.*	*N.D.*
Arginine	1.07	0.01	0.84	0.07
Alanine	1.23	0.02	0.87	0.08
Tyrosine	0.68	0.01	0.51	0.04
Valine	0.89	0.01	0.64	0.06
Methionine	0.30	0.01	0.16	0.02
Phenylalanine	0.98	0.00	0.67	0.06
Isoleucine	0.78	0.02	0.57	0.06
Ornithine	*N.D.*	*N.D.*	*N.D.*	*N.D.*
Leucine	1.50	0.01	1.07	0.09
Lysine	1.01	0.02	0.58	0.05
Hydroxyproline	*N.D.*	*N.D.*	*N.D.*	*N.D.*
Proline	1.04	0.01	2.23	0.20
Total amino acid	16.51	0.18	14.37	1.04
Total N	3.23	0.08	3.18	0.06
*Protein content	16.16	0.50	15.89	0.32
**Crude protein content	20.20	0.50	19.87	0.38

N.D.= not detected; *LOQ* = limit of detection
*calculated by multiplying the TN by 5.0 (this study)
**calculated by multiplying the TN by 6.25 (typical method)

Table 3.S2. Total Zn accumulation in *Lemna* for each pot (µg/pot). Values are mean ± standard deviation (n=3). Different letters indicate statistically significant differences among treatments with the same Zn application according to Duncan's multiple comparison tests (*P < 0.05*).

Se treatment	Se(IV)					Se(VI)				
(mg/L)	Zn 0.0	Zn 0.5	Zn 1.0	Zn 2.5	Zn 5.0	Zn 0.0	Zn 0.5	Zn 1.0	Zn 2.5	Zn 5.0
0.0	-	61.2±2.8^{a}	79.8±2.6^{a}	120±4.3^{a}	156±8.1^{c}	-	61.2±2.8^{a}	79.8±2.6^{c}	120±4.3^{b}	156±8.1^{b}
0.5	-	32.5±3.5^{c}	69.7±2.0^{b}	83.3±3.8^{c}	175±3.0^{a}	-	55.5±6.4^{a}	101±3.9^{b}	160±8.9^{a}	196 ±4.8^{a}
2.5	-	38.8±0.3^{b}	60.3±2.8^{c}	107±2.1^{b}	163±2.7^{bc}	-	63.2±3.3^{a}	107±1.8^{ab}	158±2.0^{a}	184±5.7^{a}
5.0	-	31.7±2.5^{c}	55.8±2.9^{c}	112±1.1^{b}	168±6.4^{ab}	-	68.0±3.5^{a}	111±4.3^{a}	154±4.8^{a}	185 ±7.0^{a}

Table 3.S3. Zn removal efficiency by *Lemna* for each pot (%). Values are mean ± standard deviation (n=3). Different letters indicate statistically significant differences among treatments with the same Zn application according to Duncan's multiple comparison tests (*P < 0.05*).

Se treatment	Se(IV)					Se(VI)				
(mg/L)	Zn 0.0	Zn 0.5	Zn 1.0	Zn 2.5	Zn 5.0	Zn 0.0	Zn 0.5	Zn 1.0	Zn 2.5	Zn 5.0
0.0	-	81.6±3.6^{a}	53.2±1.7^{a}	32.1±1.2^{a}	20.8±1.1^{c}	-	81.6±3.7^{a}	53.2±1.7^{c}	32.1±1.2^{b}	20.8±1.1^{b}
0.5	-	43.3±4.7^{c}	46.4±1.4^{b}	22.2±1.02^{c}	23.4±0.4^{a}	-	74.0±8.5^{a}	67.7±2.6^{b}	42.6±2.4^{a}	26.1±0.6^{a}
2.5	-	51.8±0.4^{b}	40.2±1.8^{c}	28.7±0.6^{b}	21.7±0.4^{bc}	-	84.2±4.4^{a}	71.7±1.2^{ab}	42.2±0.5^{a}	24.6±0.8^{a}
5.0	-	42.3±3.3^{c}	37.2±1.9^{c}	29.9±0.3^{b}	22.5±0.2^{ab}	-	90.7±4.7^{a}	74.0±2.9^{a}	41.2±1.3^{a}	24.7±0.9^{a}

Table 3.S4. Zn accumulation in *Azolla* for each pot (µg/pot). Values are mean ± standard deviation (n=3). Different letters indicate statistically significant differences among treatments with the same Zn application according to Duncan's multiple comparison tests (*P < 0.05*).

Se treatment	Se(IV)					Se(VI)				
(mg/L)	Zn 0.0	Zn 0.5	Zn 1.0	Zn 2.5	Zn 5.0	Zn 0.0	Zn 0.5	Zn 1.0	Zn 2.5	Zn 5.0
0.0	-	48.5±3.6^{a}	66.1±3.2^{a}	118 ±1.2^{a}	151±2.5^{a}	-	48.5±3.6^{a}	66.1±3.2^{a}	119±1.2^{a}	151±2.5^{a}
0.5	-	30.7±3.2^{c}	46.3±3.3^{c}	74.7±6.0^{c}	119±9.4^{c}	-	29.8±1.3^{b}	47.2±2.5^{b}	88.7±6.7^{b}	125±10.0^{b}
2.5	-	35.7±1.3^{bc}	49.0±3.0^{c}	72.2±4.6^{c}	107±5.1^{c}	-	26.5±0.7^{b}	39.7±1.3^{c}	66.7±0.4^{d}	128±5.8^{b}
5.0	-	36.8±2.4^{b}	59.2±2.8^{b}	95. 7±7.7^{b}	133±1.3^{b}	-	28.5±1.4^{b}	42.7±1.7^{bc}	79.7±0.4^{c}	114 ±10.5^{b}

Table 3.S5. Zn removal efficiency by *Azolla* for each pot (%). Values are mean ± standard deviation (n=3). Different letters indicate statistically significant differences among treatments with the same Zn application according to Duncan's multiple comparison tests (*P < 0.05*).

Se treatment	Se(IV)					Se(VI)				
(mg/L)	Zn 0.0	Zn 0.5	Zn 1.0	Zn 2.5	Zn 5.0	Zn 0.0	Zn 0.5	Zn 1.0	Zn 2.5	Zn 5.0
0.0	-	64.7±4.8^{a}	44.1±2.2^{a}	31.6±0.3^{a}	20.2±0.3^{a}	-	64.7±4.8^{a}	44.1±2.2^{a}	31.6±0.3^{a}	20.2±0.3^{a}
0.5	-	41.0±4.2^{c}	30.9±2.2^{c}	19.9±1.6^{c}	16.0±1.3^{c}	-	39.8±1.7^{b}	31.5±1.6^{b}	23.7±1.8^{b}	16.7±1.3^{b}
2.5	-	47.6±1.7^{bc}	32.7±2.0^{c}	19.3±1.2^{c}	14.3±0.7^{c}	-	35.3±0.94	26.4±0.8^{c}	17.8±0.1^{d}	17.1±0.8^{b}
5.0	-	49.0±3.1^{b}	39.4±1.8^{b}	25.5±2.0^{b}	17.8±0.6^{b}	-	38.0±1.8^{b}	28.5±0.7^{bc}	21.3±0.1^{c}	15.2±1.4^{b}

Table 3.S6. Bioconcentration factor of Zn (BCF_{Zn}) for *Lemna* and *Azolla* cultivated in different concentrations of Se and Zn.

Se treatment	Se(IV)					Se(VI)				
(mg/L)	Zn 0.0	Zn 0.5	Zn 1.0	Zn 2.5	Zn 5.0	Zn 0.0	Zn 0.5	Zn 1.0	Zn 2.5	Zn 5.0
Lemna										
0.0	-	1064	905	547	354	-	1064	905	547	354
0.5	-	696	818	428	413	-	1085	897	625	397
2.5	-	1069	853	616	465	-	1086	979	617	416
5.0	-	1310	1128	877	629	-	1106	973	602	387
Azolla										
0.0	-	843	748	540	342	-	843	748	540	342
0.5	-	633	571	340	269	-	662	538	373	276
2.5	-	766	519	335	244	-	577	462	320	266
5.0	-	833	625	446	306	-	609	467	331	232

Table 3.S7. Extraction efficiency of Se after protease hydrolysis and the identification rate of Se by anion exchange column

Treatments		Extraction efficiency (%)	Column recovery (%)
Se(IV)	*Lemna*	37	41
	Azolla	13	43
Se(IV)+Zn	*Lemna*	31	39
	Azolla	26	19
Se(VI)	*Lemna*	63	122
	Azolla	81	130
Se(VI)+Zn	*Lemna*	67	113
	Azolla	92	107

Table 3.S8. Se species concentration found in *duckweed* and *Azolla* grown on 2.5 mg/L Se at the absence of Zn or at the presence of 5.0 mg/L Zn (percentage of identified Se species comparison with total Se in plants)

Treatments		Se species concentration (mg/kg)					
		$SeCys_2$	SeMetCys	Se(IV)	SeMet	Se(VI)	Unknown
Duckweed	Se(IV)	5.4 (0.8%)	23.3 (3.3%)	5.6 (0.8%)	41.1 (5.9%)	9.9 (1.4%)	Detected
	Se(IV)+Zn	10.2 (0.8%)	58.3 (4.6%)	15.1 (1.2%)	34.7 (2.7%)	11.6 (0.9%)	Detected
	Se(VI)	1.4 (2.4%)	1.7 (3.1%)	1.8 (3.2%)	12.5 (22.1%)	28.1 (49.7%)	ND
	Se(VI)+Zn	1.0 (1.8%)	3.2 (5.6%)	2.0 (3.5%)	6.2 (11.0%)	30.1(53.5%)	ND
Azolla	Se(IV)	2.1 (0.3%)	6.0 (0.9%)	3.7 (0.5%)	24.5 (3.5%)	11.6 (1.7%)	Detected
	Se(IV)+Zn	2.9 (0.4%)	4.4 (0.6%)	1.8 (0.3%)	3.8 (0.5%)	10.9 (1.5%)	Detected
	Se(VI)	0.8 (0.7%)	ND	1.5 (1.3%)	2.1 (1.9%)	101 (91.7%)	ND
	Se(VI)+Zn	0.2 (0.3%)	ND	ND	ND	68.9 (75.5%)	Detected

Table 3.S9 Pearson's correlation coefficients between biomass and Se concentration in *Lemna* and *Azolla* within each Se application. Significant differences are indicated with * for *P-value <0.05* and ** for *P-value <0.01*.

		Se application (mg/L)		
		0.5	2.5	5.0
Lemna	Se(IV)	-0.54*	-0.07	0.51*
	Se(VI)	-0.67*	-0.48*	-0.81**
Azolla	Se(IV)	-0.80*	0.12	-0.35
	Se(VI)	-0.01	0.49	-0.33

Table 3.S10. Pearson's correlation coefficients between biomass and Zn concentration in *Lemna* and *Azolla* within each Zn application. Significant differences are indicated with * for *P-value <0.05* and ** for *P-value <0.01*.

		Zn application (mg/L)			
		0.5	1.0	2.5	5.0
Lemna	Se(IV)	-0.46	-0.82**	-0.86**	-0.80**
	Se(VI)	-0.49	0.12	0.57*	-0.33
Azolla	Se(IV)	0.55	0.37	0.13	0.50
	Se(VI)	0.62*	-0.002	0.22	-0.26

Chapter 4 Valorization of Se-enriched sludge and duckweed generated from wastewater through ecotechnologies as micronutrient biofertilizers

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Abstract

The potential of high value-added biomaterials (Se-enriched sludge and duckweed) produced from synthetic wastewater as slow-release Se biofertilizers was evaluated by amending them to two soils with and without planted green beans (*Phaseolus vulgaris*). The Se concentration in the soil pore water with Se-enriched duckweed amendment was 3–15 times higher than that with the Se-enriched sludge amendment at the beginning of incubation. However, the Se concentration in the bean tissues was 1.1–3.1 times higher when soils were amended with Se-enriched sludge as compared to Se-enriched duckweed. This is attributed to different Se speciation (hexavalent Se form in the duckweed but zerovalent nano-Se form in the sludge) and organic carbon content accumulated and released between the two biomaterials, which may affect the bioavailability of Se present in the pore water. Selenium recovered from the wastewater was efficiently transformed to health-beneficial selenoamino acids (e.g. Se-methionine, 76–89%) after being taken up by beans without influence on beans growth. Besides, the Se-enriched sludge dominated by elemental nano-Se is considered as the preferred slow-release Se biofertilizer and an effective Se source to produce Se-enriched crops for Se-deficient populations, as shown by the higher Se bioavailability and lower organic carbon content. This could offer an environmental-friendly alternative to the application of conventional chemical Se fertilizers for biofortification, avoiding the problem of Se losses by leaching from chemical Se fertilizers while recovering resources. Accordingly, a high-value biofertilizer produced from wastewater is valorized.

Keywords

Selenium bioavailability, Se biofertilizer, Se-enriched biomaterials, Biofortification, Resource recovery, Wastewater.

4.1 Introduction

Biofortification is being explored as a possible solution for Se deficiency (Lavu et al., 2012; Li et al., 2010; Thavarajah et al., 2008). Selenium biofortification of food crops is practiced in Se-deficient regions of different countries, such as Finland, by adding inorganic Se fertilizer to soils (Bañuelos et al., 2016). Although the application of inorganic Se fertilizer can be an effective way to produce Se-enriched food and feed products, secondary contamination of soil and water can occur due to the low utilization rate of Se by crops (Wang et al., 2018). Therefore, other sources of Se, such as organic Se, could be useful as an alternative soil additive to produce Se-enriched crops. This approach would not only increase the organic matter content in soils, but also enhance Se uptake by plants due to the slower release of Se to the pore water (Wang et al., 2018). Moreover, the synchronization of the slow-release of Se from decomposing organic matter with crop uptake would also be beneficial to avoid Se loss and secondary pollution by leaching from soils.

A few Se-rich organic materials produced in seleniferous areas have been investigated as potential Se-sources to provide a certain amount of Se for plant uptake, e.g., Se-rich animal manure or plant residues collected from phytoremediation or biofortification sites (Ajwa et al., 1998; Bañuelos et al., 2016; Dhillon et al., 2007; Stavridou et al., 2011; Wang et al., 2018). For instance, the supplementation of 4.2 g Se-enriched wheat straw and pak choi into Eum-Orthic Anthrosol soil significantly increased the Se content in the shoot of pak choi from 0.16 mg/kg (in the untreated control) to 0.40 and 2.23 mg/kg, respectively (Wang et al., 2018). The total Se concentration in the edible parts of broccoli and carrot increased from 0.5 to 3.5 mg/kg, and from 0.3 to 2.3 mg/kg, respectively, after soil application of Se-enriched *Stanleya pinnata* (Bañuelos et al., 2015). Moreover, Ajwa (1998) showed the slow release of plant-available Se in soils amended with seleniferous organic materials, i.e., alfalfa (*Medicago sativa* L.), *Astragalus praelongus* and cattle manure. These materials can thus be used to increase the Se concentration in crops grown on Se deficient soils in a more effective and efficient manner.

On the other hand, excess of Se in the environment is also frequently observed. For instance, some wastewaters have a high Se content as a result of both agricultural and industrial activities (Lim & Goh, 2005). Nutrient-rich wastewater loaded also with Se,

such as aquaculture wastewater (Han et al., 2020) and agricultural drainage (CH2MHill, 2010), may thus serve as a potential Se source from which the Se may be recovered and valorized to produce slow-release organic biofertilizers. Products generated from Se-bearing wastewater normally have a high Se content, such as Se-enriched granular sludge (Staicu et al., 2015a) and aquatic plants after phytoremediation and phytoextraction (Li et al., 2020b). Thus, it is necessary to explore whether the Se contained in these products can be potentially used for biofortification purposes as an organic nutrient-enriched fertilizer to improve Se levels in soils. This approach would be beneficial to save Se resources and mitigate Se contamination, meanwhile also avoiding introduction of new Se contamination into the environment through the use of inorganic Se as fertilizer in biofortification. It would also contribute to the worldwide drive for resource recovery and circular economy.

The objectives of this study were, therefore, (1) to study the release of plant-available Se from Se-enriched duckweed and sludge produced through ecotechnologies after amending them into two types of soils during a long-term period; (2) to monitor the effect of soil amendment with these two Se-enriched biomaterials on green beans (*Phaseolus vulgaris*), a protein-rich crop regularly grown in moderate climates, in terms of their growth, production and Se accumulation; (3) to assess the potential of these two Se-enriched biomaterials as Se biofertilizers to improve the Se content in green beans; and (4) to preliminarily screen the suitable crop from five crops for Se accumulation.

4.2 Materials and methods

4.2.1 Soil collection and characterization

Two types of non-contaminated soils, classified as sandy and loamy, were collected at a depth of 0–20 cm from fields in Evergem (51°6´57" N, 3°39´40" E) and Wortegem-Petegem (50°50´20" N, 3°33´22" E), Belgium, respectively. The soils were dried, homogenized and passed through a 2-mm sieve mesh. The physicochemical properties of the soils were analyzed according to Van Ranst et al. (Van Ranst et al., 1999). The loamy soil had a higher electrical conductivity (EC), cation exchange capacity (CEC) and organic matter (OM) content than the sandy soil (Table 4.1).

Table 4.1. Physicochemical properties and (trace) element content of the tested sandy and loamy soil (mean ± standard deviation; n=3, except for texture).

	Loamy soil	**Sandy soil**
pH-KCl	6.14 ± 0.1	6.45 ± 0.0
pH-H_2O	6.92 ± 0.1	7.07 ± 0.1
EC (µS/cm)	175 ± 11	35 ± 1.7
CEC (cmol/kg)	7.38 ± 0.1	1.79 ± 0.2
OM (%)	7.88 ± 0.0	2.14 ± 0.0
Texture		
Sand (%)	53.7	91.5
Silt (%)	40.2	6.0
Clay (%)	6.1	2.5
Elements (mg/kg)		
Total Se	0.24 ± 0.0	0.10 ± 0.0
Available Se	0.013 ± 0.0	0.030 ± 0.0
P	1464 ± 52.1	300 ± 6.2
S	359 ± 11.2	76 ± 10.1
Cu	29.7 ± 0.5	2.69 ± 0.1
Zn	119 ± 3.4	8.47 ± 0.5
Ca	4285 ± 154.4	1103 ± 30.2
Mg	1866 ± 51.7	489 ± 31.0
Fe	10749 ± 136.2	3612 ± 104.2
Al	8420 ± 471.5	3772 ± 270.4

4.2.2 Crops screening pot experiment

Five crops, pak choi (*Brassica chinensis*), lettuce (*Lactuca sativa*), wheat (*Triticum aestivum*), maize (*Zea mays*) and beans (*Phaseolus vulgaris*), were screened for their capacity to bioaccumulate Se under different soil exposures. A quantity of Na_2SeO_4 stock solution equivalent to 0, 1.0, 3.0 and 5.0 mg Se/kg soil containing 100 mg/kg NPK fertilizer were sprayed onto 7.5 kg dried sandy soil for the growth of five crops. The treated soils were homogenized and divided into 0.5 kg in each pot to stabilize for two weeks for planting crops. All experiments were conducted in triplicate.

Seeds of the five crops were placed in Petri dishes filled with wet mineral vermiculite and incubated at 27 °C to stimulate germination. Four germinated seeds were transplanted into each pot containing the 0.5 kg treated soils and then grew in the indoor conditions with artificial light (2000 lux, 36w) placed 50 cm above of the plants (16 h of light and 8 h of darkness). The soil moisture during the growing period was maintained at approximately 80% of the water holding capacity by weighing the pots every day and refilling with an appropriate amount of deionized (DI) water.

The plants were harvested after 6 weeks of growth, cleaned with DI water, separated into different tissues (root, shoot, leaf and seed) and dried in an oven at 60 °C until constant weight. The dry biomass weight and total Se concentration of each tissue of the plants were determined.

4.2.3 Valorization of Se-enriched biomaterials as Se biofertilizers

4.2.3.1 Preparation of Se-enriched biomaterials (Se-enriched duckweed and sludge)

The duckweed was collected from a natural freshwater canal in Delft (The Netherlands) and cultivated in a Hoagland solution with 5.0 mg/L of Se added as Na_2SeO_4. The Se-enriched duckweed was harvested after 7 days of cultivation, oven-dried and stored for subsequent fertilization experiments. Further details on the Se-enriched duckweed production and Se removal can be found elsewhere (Li et al., 2020a; Li et al., 2020b). The Se-enriched sludge was generated as described by Staicu et al. (Staicu et al., 2015b). The sludge collected from a full-scale upflow anaerobic sludge blanket reactor treating pulp and paper wastewater in Eerbeek (The Netherlands) was added to an oxygen-free growth medium with 5.0 mg/L of Se in the form of Na_2SeO_4 and incubated for 14 days at 30 °C. Afterwards, the sludge enriched with Se was settled, separated and oven-dried. The supernatant was filtered and measured for total Se content in order to quantify the Se removal efficiency. The result showed that up to 93% of Se was removed from wastewater (Fig. 4.S1). The generated sludge samples were milled and passed through a 0.45-mm sieve mesh for further fertilization experiments. The Se-enriched duckweed and sludge contained 209 mg Se/kg dry weight and 314 mg Se/kg dry weight, respectively.

4.2.3.2 Selenium availability in the non-planted soils through perennial monitoring

The Se availability in the two soil types amended with the biomaterials was studied during a long-term incubation trial (around 15 months, 471 days). The Se-enriched biomaterials were applied to the soils in an amount equivalent to 1.0 and 5.0 mg Se/kg soil. The biomaterials were thoroughly mixed with 0.5 kg sandy or loamy soil, respectively, and placed into plastic bags within plastic pots (10 cm in height and 10 cm in diameter) during the entire incubation. Non-amended soils prepared similarly served as blank (noted as Blank). The following abbreviations are used indicating the amendments in this study: Se-enriched duckweed 1.0 mg Se/kg soil (DW1) and 5.0 mg Se/kg soil (DW5) and Se-enriched sludge 1.0 mg Se/kg soil (SL1) and 5.0 mg Se/kg soil (SL5).

Specifically, the entire incubation consisted of two monitoring periods by collecting soil pore water: 6 weeks at the beginning of the incubation period (first monitoring period) and 4 weeks at the end of the incubation period (second monitoring period, 13.5 months after the first incubation period). In each monitoring period, soil moisture was kept at 80% of the water holding capacity and the soil pots were placed indoor at room temperature and under natural light conditions. After the first monitoring period, the amended soils were air-dried, sealed in plastic bags and stored for 13.5 months at room temperature until the second monitoring period (Egene et al., 2018). In the first monitoring period, soil pore water was collected twice per week by using Rhizon soil moisture samplers (Eijkelkamp Agrisearch Equipment, Giesbeek, the Netherlands) and analyzed for total Se concentration, pH, total carbon (TC) and total organic carbon (TOC) (Egene et al., 2018). In the second monitoring period, deionized (DI) water was added to the pots to bring the soil moisture again to 80% of the water holding capacity. Soil pore water was extracted weekly using the Rhizon samplers and analyzed for total Se concentration.

4.2.3.3 Selenium bioavailability in planted soils

Similar to the non-planted experiment, an amount equivalent to 1.0 and 5.0 mg Se/kg soil of Se-enriched duckweed (DW1 and DW5) and sludge (SL1 and SL5) was applied to 0.5 kg loamy and sandy soils. For the control experiment, the same amount of non-enriched duckweed and sludge as in the Se-enriched biomaterials amendments was applied to the two types of soil (noted as control-DW1, control-DW5, control-SL1 and control-SL5). Soils without any biomaterial amendment served as blank soil (noted as

Blank). In each treatment, inorganic fertilizer (NPK) and the Se-enriched/control biomaterials were uniformly mixed with 0.5 kg sandy or loamy soil. Pots were placed indoors (at 24 ºC, 53% relative humidity and 100 µmol/m^2/s light intensity) with 80% of water holding capacity and pre-incubated for one week before transferring 4 seedlings of green beans (*Phaseolus vulgaris*) previously cultivated in trays with wet vermiculite for one week. All experiments were conducted in triplicate. The soil pore water was collected every week through Rhizon extraction for total Se measurement. The bean plants were harvested after 6 weeks of growth, washed and separated into different tissues (root, stem, leaf and seed) for biomass and Se concentration analysis. Se speciation analysis was performed on selected bean seeds (at the DW1 and SL1 amendment for sandy soil, and sandy soil blank) after lyophilization.

4.2.4 Analytical methods

4.2.4.1 Analysis of total Se concentration in plants

For the determination of total Se in plants, 0.3 g of dry samples were weighed into a digestion vessel followed by the addition of 10 mL of concentrated ultrapure 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 digested samples 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 to validate the accuracy of Se measurements. White clover samples (BCR-CRM, 6.7 ± 0.25 mg/kg) were included as certified reference materials in each analytical batch as quality control with recoveries of 97 (± 7)%.

4.2.4.2 Selenium speciation analysis

Selenium speciation in the plant samples (Se-enriched duckweed and Se-enriched bean seeds) was analyzed according to Lavu et al. (2012), Li et al. (2020b). The details are described in the section materials and methods of Chapter 3.

Selenium speciation in the solid sludge was determined by extended X-ray absorption fine structure (EXAFS) and X-ray absorption near edge structure (XANES) (Lenz et al., 2008). To prepare the samples for analysis, dried sludge samples and reference materials of different oxidation numbers were ground to fine powders, mixed with cellulose and pressed into pellets to allow a straightforward sample handling during the experiments. The amount of sludge and reference powders used for each sample was determined using the calculated optimum amount of Se per sample. The EXAFS/XANES spectra were collected over the Se-K (12.658 keV) absorption edge on the sludge sample at the DUBBLE beamline (BM26A) at the European Synchrotron and Radiation Facility (ESRF, Grenoble, France). Measurement for samples was carried out in transmission mode at room temperature. EXAFS data were processed using the Viper software package (Klementev, 2001). XANES data was processed by performing a pre- and post-edge normalisation, followed by linear combination analysis using the reference compound spectra.

4.2.4.3 pH, TC and TOC analysis of soil extract

The pH of the soil extracts was determined by using a pH-meter (Orion Star A211, Thermo fisher scientific, Waltham, MA, USA). 2.0 mL of Rhizon extract from each pot was diluted with Milli-Q water to obtain 20 ml volume for total carbon (TC) and total organic carbon (TOC) measurement through a TOC-analyser (TOC-5000, Shimadzu, Tokyo, Japan) as described by Egene et al. (2018).

4.2.5 Estimated daily intake and health risk assessment of the bean seeds

The estimated daily intake of Se (EDI, µg/kg/day) was calculated using equation (1):

$$EDI = \frac{C_{Se} \times C_{factor} \times D_{intake}}{B_{weight}} \quad (1)$$

Where: C_{Se} is the Se concentration in the bean seeds, C_{factor} represents the conversion factor from fresh weight to dry weight (0.075, calculated by dry weight/fresh weight in this study), D_{intake} and B_{weight} represent the daily intake of beans and the average body weight (BW), respectively. According to the daily food consumption issued by the European Food Safety Authority (EFSA), the chronic legume consumption

(D_{intake}/B_{weight}) is 0.55 g/kg BW per day for adults and 1.01 g/kg BW per day for children in Belgium (EFSA).

To assess the human health risk of Se-enriched bean seeds obtained in this study, it is necessary to calculate the level of human exposure to those seeds. The health risk index (HRI) for Se was calculated using equation (2):

$$HRI = \frac{EDI}{RfD} \quad (2)$$

Where: EDI is the estimated daily intake and RfD is the reference oral dose. According to the USEPA, the RfD value for Se is 5.0 µg/kg /day (IRIS, 2006). An estimated value of HRI <1 indicates no evident health risk to the exposed population. Otherwise, it may raise health risks (Sihlahla et al., 2019).

4.2.6 Statistical analysis

Descriptive statistics were performed using Sigma plot 13, Excel 2016 and SPSS 20.0. Results are expressed as mean ± SD. The different treatments were compared with a one-way analysis of variance (ANOVA) and Duncan's multiple comparison tests ($P < 0.05$).

4.3 Results

4.3.1 Crops screening for five plants

4.3.1.1 Plant biomass

The aboveground and underground biomass of pak choi and lettuce was significantly decreased by the Se(VI) amendment (Figs. 4.1a and 4.1b). Increasing applications of Se(VI) from 0 to 5.0 mg/kg reduced the dry weight of the shoot and root by 36% and 84% for pak choi, and 45 and 71% for lettuce, respectively. Additionally, for wheat, the exposure to high Se(VI) concentrations at 5.0 mg/kg did not cause any significant growth inhibition (Fig. 4.1c). The low dosage of Se(VI) applications even stimulated the wheat growth: the biomass of wheat shoot and root raised by 21% and 27%, respectively, in the 1.0 mg/kg Se(VI) amendment compared to the control. Similarly, for the maize, the root and stem biomass was increased under the low Se(VI) exposure, while significantly declined at the 5.0 mg/kg Se(VI) application compared to the control

(Fig. 4.1d). Exposure of beans to Se(VI) significantly stimulated the growth of each bean tissue except there was no significant effect on the growth of the root and stem at the 5.0 mg/kg of Se(VI) exposure in comparison with the control (Fig. 4.1e) Specifically, at 1.0, 3.0 and 5.0 mg/kg of Se(VI) treatments, the yield was improved by 14, 26 and 24% for the leaves, and 25, 31 and 16% for the seeds, respectively, compared to the control treatment.

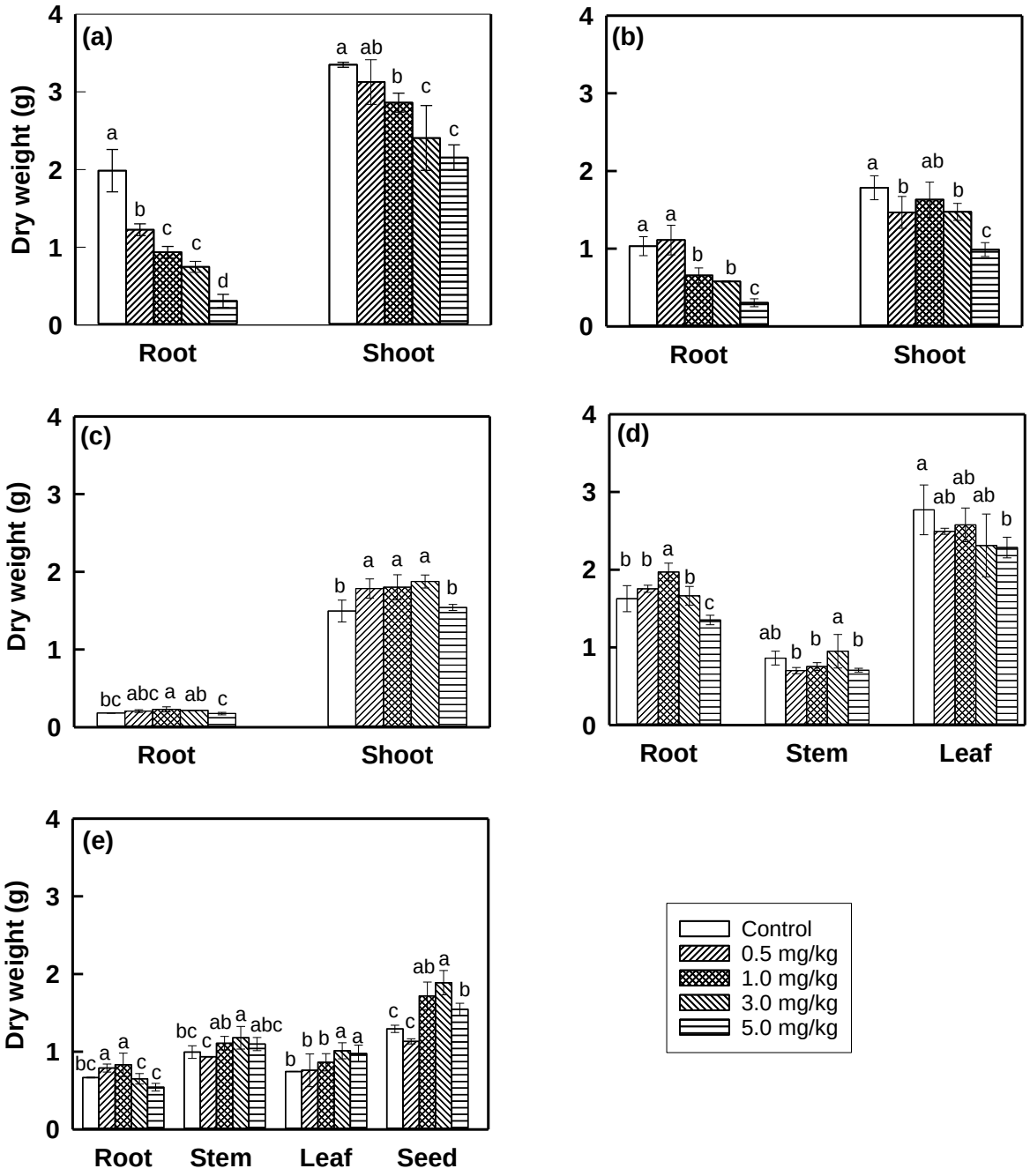

Figure 4.1. Dry weight of the five crops grown at different Na_2SeO_4 applications: (a) pak choi, (b) lettuce, (c) wheat, (d) maize and (e) beans. Values are mean ± standard

deviation (n=3). Different letters indicate statistically significant differences among treatments with the different Se applications according to Duncan's multiple comparison tests ($P < 0.05$).

4.3.1.2 Se content in plants

Se concentrations in all plants considerably raised with increasing dosages to the sandy soil (Fig. 4.2). Pak choi had the highest Se concentration among the five crops investigated, with a content of 732 and 335 mg/kg, respectively, for shoot and root at 5.0 mg/kg of Se(VI) treatment, respectively. The Se content in the shoots of pak choi was 2-4 fold higher than that in the corresponding roots (Fig. 4.2a). The Se content in the shoots of lettuce was 1.1-1.3 times higher than in the roots, but was 1.3-3.0 times lower than in the shoots of pak choi at the same Se amendments (Fig. 4.2b). Maize had an almost similar Se content to lettuce in the root and shoot at the 5.0 mg/kg of Se(VI) amendment, where the maximum Se concentration in the maize was 249 mg/kg in the roots, 136 mg/kg in the stems and 267 mg/kg in the leaves (Fig. 4.2d). The Se concentration was evenly distributed in each bean tissue (root, stem, leaf and seed) and was comparable to that of maize (Fig. 4.2e). Wheat presented the lowest Se concentration among all crops with 166 mg/kg for the shoots and 145 mg/kg for the roots under the highest Se dosage (Fig. 4.2c).

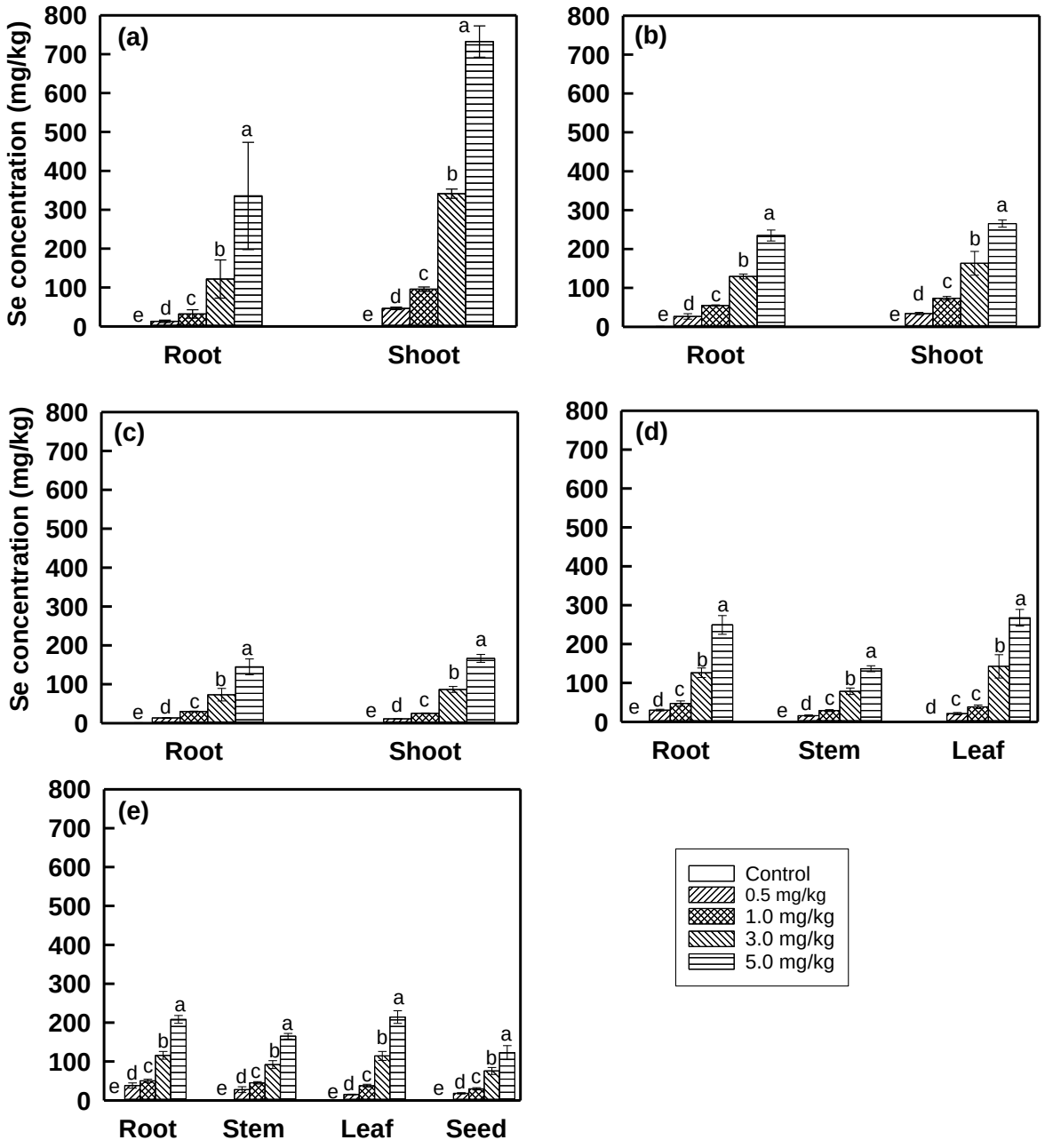

Figure 4.2. Se content in plants grown at different Na_2SeO_4 concentrations: (a) pak choi, (b) lettuce, (c) wheat, (d) maize and (e) beans. Values are mean ± standard deviation (n=6). Different letters indicate statistically significant differences among treatments with the different Se applications according to Duncan's multiple comparison tests ($P < 0.05$).

4.3.2 Characterization of the Se-enriched biomaterials

The Se species in the Se-enriched duckweed and sludge are illustrated in Fig. 4.3a and b, respectively. Se in the Se-enriched duckweed consisted of 80% selenate

(Se(VI)), 1.3% selenite (Se(IV)), 0.8% $SeCys_2$, 0.7% SeMetSeCys, 0.9% SeMet, and a small amount of an unidentified Se compound. In contrast, Se in the Se-enriched sludge was mainly present as the zerovalent form (94.2%) and the remaining Se species was selenite.

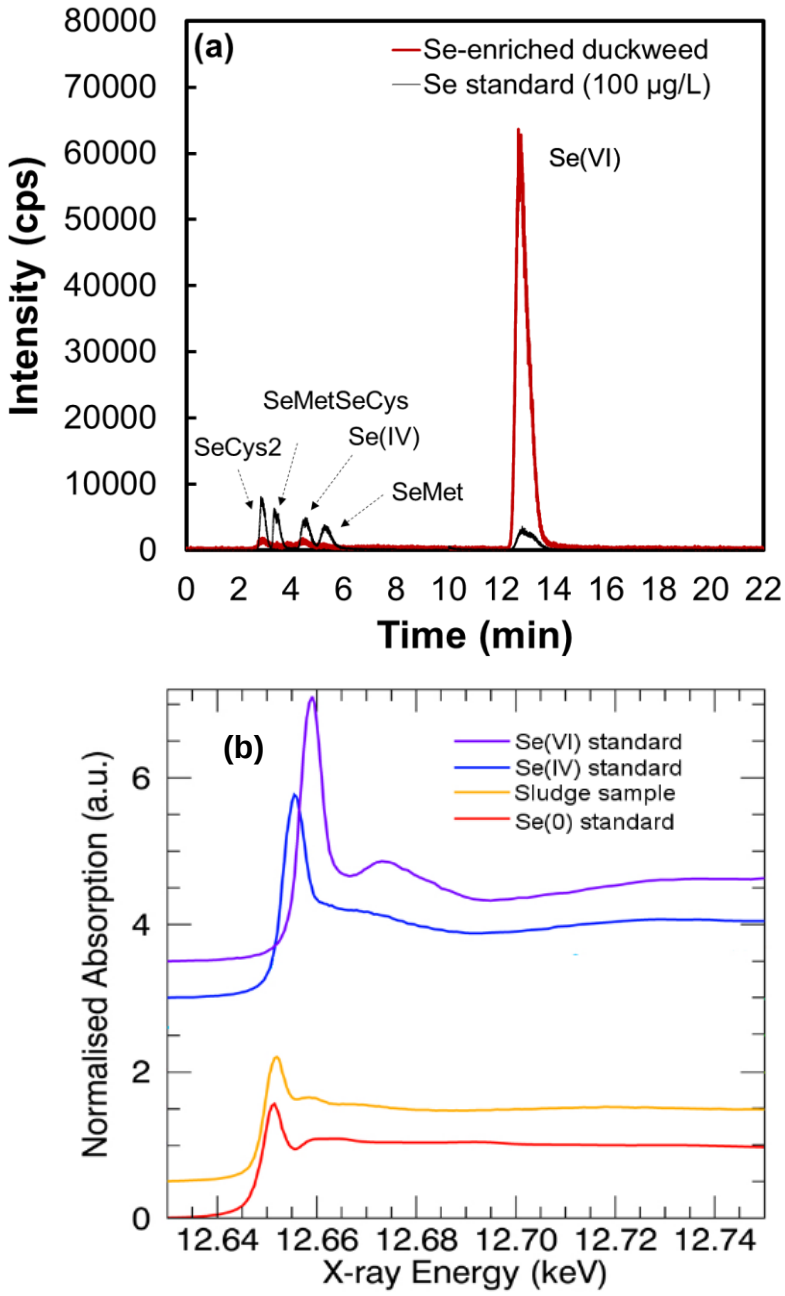

Figure 4.3. (a) Chromatogram of Se speciation in the Se-enriched duckweed compared with that of a Se standard solution of 100 µg/L of each species, and (b) XANES spectra of the Se species in the Se-enriched sludge (yellow) as compared to standards.

4.3.3 Se released to non-planted soils (availability) amended with Se-enriched biomaterials

Both Se-enriched biomaterials were effective for improving the availability of Se in the two types of soil. The pore water of soils amended with Se-enriched duckweed and anaerobic sludge contained a significantly higher amount of Se compared to the blank soil (Fig. 4.4). After 3 days of incubation (first monitoring period), the supplement of DW1 and DW5 increased the Se concentration in the pore water from the soil background values to 537 and 4375 µg/L for sandy soil, and to 413 and 1238 µg/L for loamy soil, respectively; whereas the supplement of SL1 and SL5 led to an increment of the Se content in the pore water to 65 and 322 µg/L for sandy soil, and 72 and 387 µg/L for loamy soil, respectively.

Generally, the Se concentration in the pore water of the two soils supplied with Se-enriched duckweed was significantly higher than that of the two soils supplied with Se-enriched sludge, except for the SL5 and DW5 application in the loamy soil after 14 days incubation of the first monitoring period (Fig. 4.4). Moreover, the Se concentration in the pore water of the sandy soil with the Se-enriched duckweed supplement was 1.1–5.0 times higher than that of the pore water of the loamy soil, while no significant difference was observed between the two soil types with Se-enriched sludge amendment before 42 days of incubation (first monitoring period) (Fig. 4.4).

The difference in the Se release pattern between Se-enriched duckweed and sludge continued to exist over time. The Se concentration in the pore water decreased along with the incubation time with the DW5 supplement in both soil types in the first monitoring period. Specifically, increasing the incubation time from 3 to 42 days reduced the Se content in pore water by 92% for the sandy soil and 89% for the loamy soil. Conversely, the Se concentration in the pore water of both soils supplied with Se-enriched sludge was stable. After one year of incubation (second monitoring period), the Se concentration in the pore water of the two soils supplied with Se-enriched duckweed was slightly lower compared to the 42 days incubation of the first monitoring period, while it was around 1.5–2.0 times higher for the Se-enriched sludge supplement in both soil types investigated (Fig. 4.4).

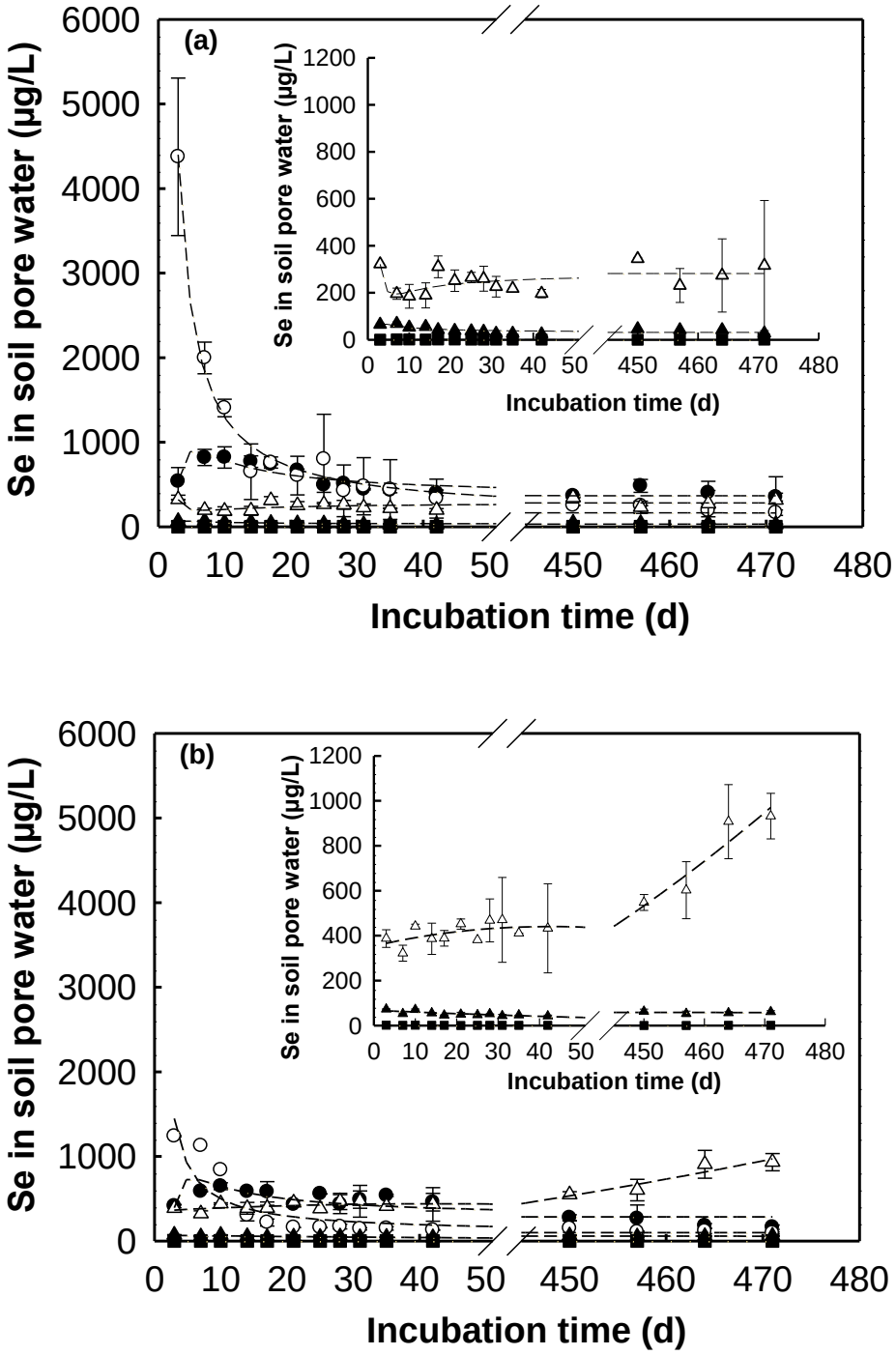

Figure 4.4. Evolution of Se concentration in the Rhizon extracts of non-planted soils amended with Se-enriched duckweed and sludge: (a) sandy soil, and (b) loamy soil. Values are mean ± standard deviation (*n* = 3), DW: duckweed amendment, SL: sludge amendment, ○ DW5, ● DW1, △ SL5, ▲ SL1 and ■ Blank.

4.3.4 Se released to planted soils (bioavailability) amended with Se-enriched biomaterials

4.3.4.1 Se content in the soil pore water

Similarly, as in the non-planted soils, amendment with Se-enriched duckweed or sludge significantly increased the Se concentration in the pore water of both soils compared to the blank (Fig. 4.5 and Table 4.S1). The effect of the Se dosage was also evident from the higher Se concentration in the soil pore water for the most Se-enriched biomaterials (DW5 and SL5). After 7 days of incubation, the supplement of DW1 and DW5 increased the Se concentration in the pore water from the soil background values (1.3 and 1.0 µg/L for the sandy and loamy soil, respectively) to 920 and 2330 µg/L for the sandy soil, and 595 and 1851 µg/L for the loamy soil, respectively; whereas the application of SL1 and SL5 led to an increment of the Se content in the pore water to 85 and 200 µg/L for the sandy soil, and 58 and 252 µg/L for the loamy soil, respectively.

The Se concentrations in the soil pore water with DW1 and DW5 amendments decreased by 70% and 80% for the sandy soil, and by 98% and 85% for the loamy soil from day 7 to day 42, respectively. Similarly, for the SL1 amendment, the Se concentration in the pore water decreased by 48% and 32% from day 7 to day 42 for the sandy and loamy soil, respectively. The decrease in Se pore water concentration for the SL1 amendment was less pronounced than the decrease observed for the Se-enriched duckweed amendment. However, the amendment of SL5 significantly increased the Se concentration in the pore water by 143% for the sandy soil after 42 days compared to 7 days, whereas it increased the Se concentration in pore water for the loamy soil in the middle of the planting phase (28 days) and remained stable afterwards (Fig. 4.5 and Table 4.S1).

Additionally, the Se concentration in the pore water of the two soil types supplied with Se-enriched duckweed was significantly higher than that of the soils supplied with Se-enriched sludge before 21 days of incubation, while no significant difference was observed afterwards, except for the application of SL1 and DW1 to the loamy soil (Table 4.S1).

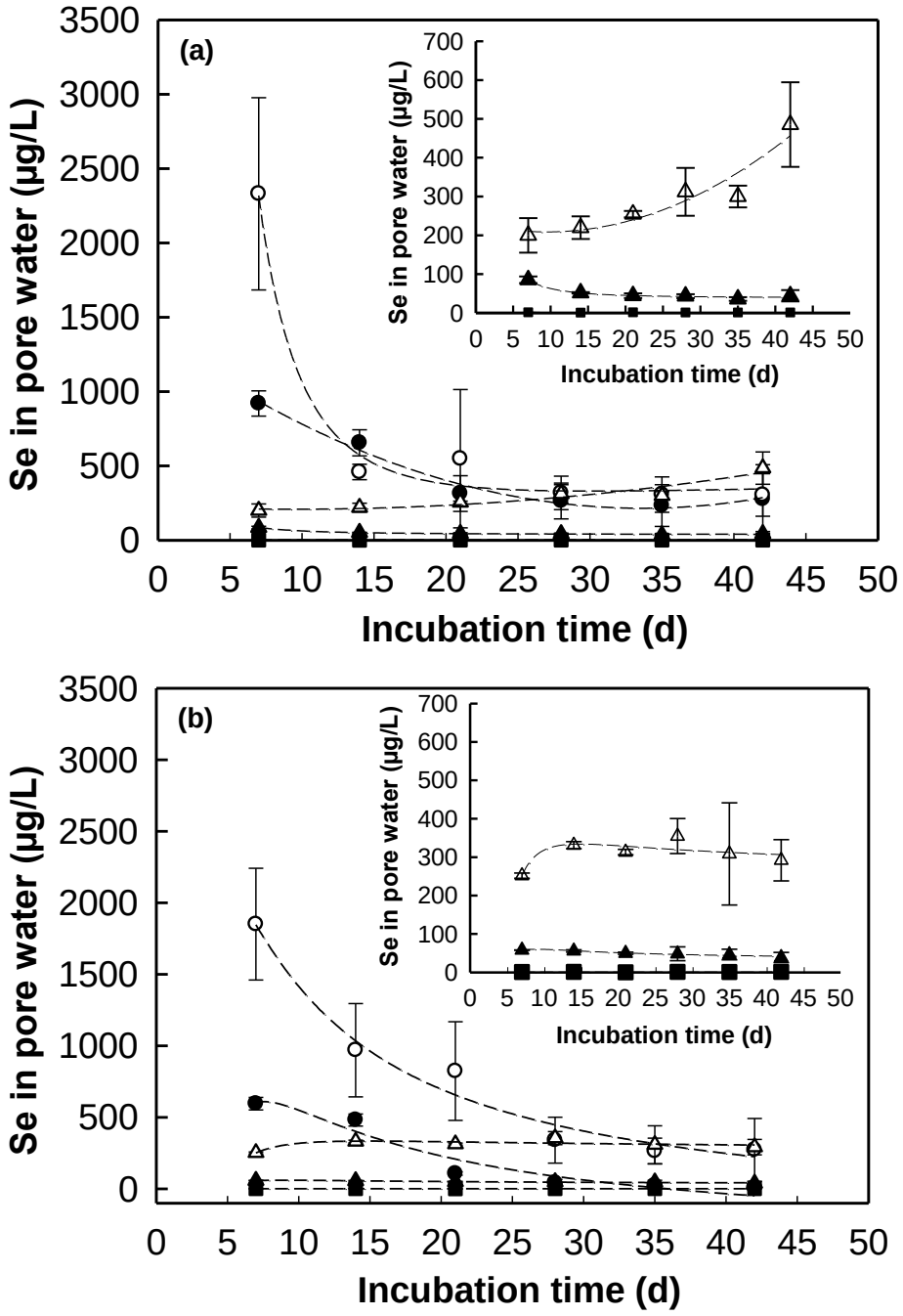

Figure 4.5. Evolution of Se concentration in the Rhizon extracts of soils amended with Se-enriched duckweed and sludge over the growth period of beans in (a) sandy soil, and (b) loamy soil. Values are mean ± standard deviation (n=3), ○ DW5, ● DW1, △ SL5, ▲ SL1 and ■ Blank.

4.3.4.2 Biomass of bean tissues

Table 4.2 shows the biomass of beans grown in soils amended with Se-enriched biomaterials, non-enriched biomaterials (the corresponding controls) and without any

biomaterials (Blank). There was no significant difference between the blank and the application of Se-enriched biomaterials except for the DW5 application in the sandy soil, which significantly declined the root biomass of the beans. However, when compared with the application of the same biomaterials without Se enrichment, the Se-enriched biomaterials seem to counteract the negative effect of the former. The amendment with non-enriched duckweed and sludge (control) significantly decreased the root biomass of beans in the sandy soil, and considerably reduced the seed and total biomass of beans in the loamy soil (Table 4.2). However, a higher biomass production was observed with the supplementation of Se-enriched biomaterials to both soils, indicating that Se released from the Se-enriched biomaterials could promote the growth of beans.

Table 4.2. Dry biomass of the roots, seeds and whole plant (sum of root, stem, leaf and seed) of beans grown in soils with different biomaterials application (Mean ± standard deviation, n = 3). Different lowercase letters indicate statistically significant differences between different Se dosages for the same type of Se-enriched biomaterial application and the same type of soil according to Duncan's multiple comparison tests ($P < 0.05$). Different uppercase letters indicate significant differences between different dosages for the same type of non-enriched biomaterials (control) application and the same type of soil ($P < 0.05$).

	Treatments	Root	Seed	Whole plant
Sandy soil	Blank	0.54 ± 0.01 b ^{A}	0.72 ± 0.53	4.21 ± 0.26 a ^{A}
	DW1	0.65 ± 0.04 a	0.79 ± 0.18	4.03 ± 0.32 a
	DW5	0.33 ± 0.02 c	0.57 ± 0.42	3.19 ± 0.38 b
	Control-DW1	0.48 ± 0.32 ^{A}	0.57 ± 0.23	4.14 ± 1.26 ^{A}
	Control-DW5	0.12 ± 0.04 ^{B}	0.06 ± 0.03	1.46 ± 0.05 ^{B}
	Blank	0.54 ± 0.01 ^{A}	0.72 ± 0.53	4.21 ± 0.26
	SL1	0.65 ± 0.13	0.53 ± 0.10	3.65 ± 0.60
	SL5	0.50 ± 0.05	0.40 ± 0.03	3.85 ± 0.30
	Control-SL1	0.36 ± 0.21 ^{AB}	0.69 ± 0.45	3.52 ± 1.5
	Control-SL5	0.20 ± 0.03 ^{B}	0.51 ± 0.31	2.80 ± 0.45
Loamy soil	Blank	0.48 ± 0.03 ^{A}	0.51 ± 0.24 ^{A}	3.70 ± 0.11 ^{A}
	DW1	0.37 ± 0.08	0.86 ± 0.39	3.79 ± 0.79
	DW5	0.49 ± 0.11	0.71 ± 0.41	4.12 ± 0.68
	Control-DW1	0.23 ± 0.09 ^{B}	0.25 ± 0.05 ^{AB}	2.72 ± 0.87 ^{B}
	Control-DW5	0.22 ± 0.00 ^{B}	0.12 ± 0.08 ^{B}	2.21 ± 0.11 ^{B}
	Blank	0.48 ± 0.03	0.51 ± 0.24 ^{A}	3.70 ± 0.11 ^{A}
	SL1	0.65 ± 0.13	0.55 ± 0.11	3.67 ± 0.37
	SL5	0.50 ± 0.05	0.41 ± 0.21	3.86 ± 0.37
	Control-SL1	0.30 ± 0.11	0.36 ± 0.12 ^{B}	2.87 ± 0.74 ^{AB}
	Control-SL5	0.23 ± 0.00	0.25 ± 0.02 ^{B}	2.61 ± 0.26 ^{B}

4.3.4.3 Se content in the different bean tissues

The amendment of Se-enriched duckweed and sludge significantly increased the Se concentration in the different tissues of beans (seed, leaf, stem and root) growing on both the sandy and loamy soil (Fig. 4.6). Increasing the amount of Se-enriched duckweed and sludge amendment from 1.0 to 5.0 mg Se /kg soil raised the Se content in the roots of beans from 17 to 52 mg/kg and from 27 to 117 mg/kg for the sandy soil, respectively, and increased the Se content in the roots of beans from 14 to 38 mg/kg and from 26 to 118 mg/kg for the loamy soil, respectively. The Se concentration in the beans amended with Se-enriched sludge was mostly 1.1–3.1 times higher than in those amended with Se-enriched duckweed. Besides, the highest Se content was observed in the roots among all tissues for both Se-enriched duckweed and Se-enriched sludge amendment, being 2–12 fold higher than those in the stem, leaf and seed.

4.3.4.4 Correlation of Se in the beans and Se in the soil pore water

Fig. 4.7 shows the correlation between the Se concentration in the seeds and roots of beans and the Se concentration in the pore water extracted from the sandy and loamy soils amended with Se-enriched duckweed and sludge after 7 days incubation (the first day of transplanting beans). The Se content in the soil pore water positively and significantly correlated with the Se concentration in both the seeds and roots. The high correlation coefficient (between 0.80 and 0.99) between the Se concentration in the tissues of beans and in the soil pore water indicates that the Rhizon extraction can properly assess the bioavailability of Se in soils.

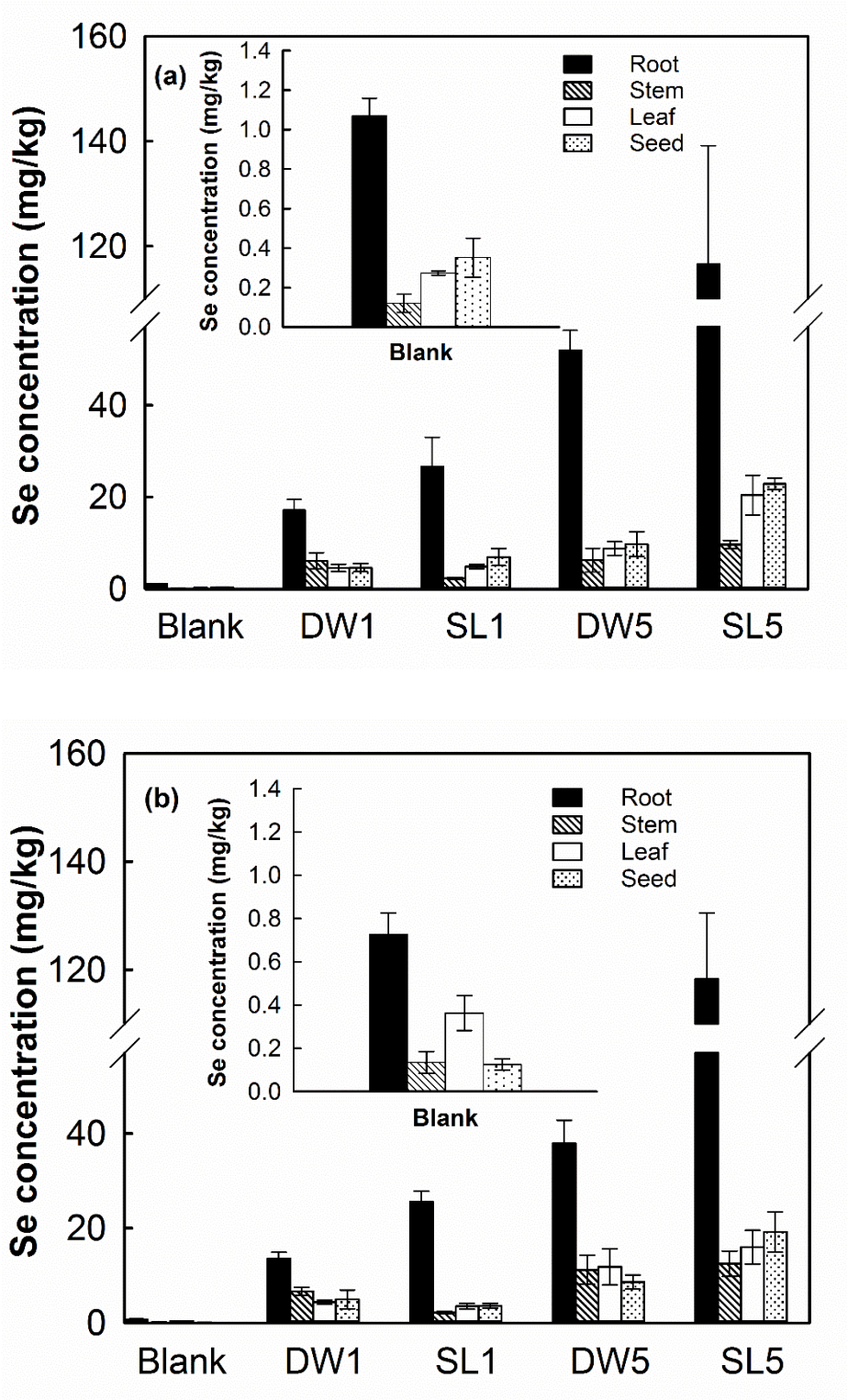

Figure 4.6. Se content in the different tissues of harvested beans (42 days growth) fertilized with Se-enriched duckweed and sludge in (a) sandy soil, and (b) loamy soil. Values are mean ± standard deviation (n=3).

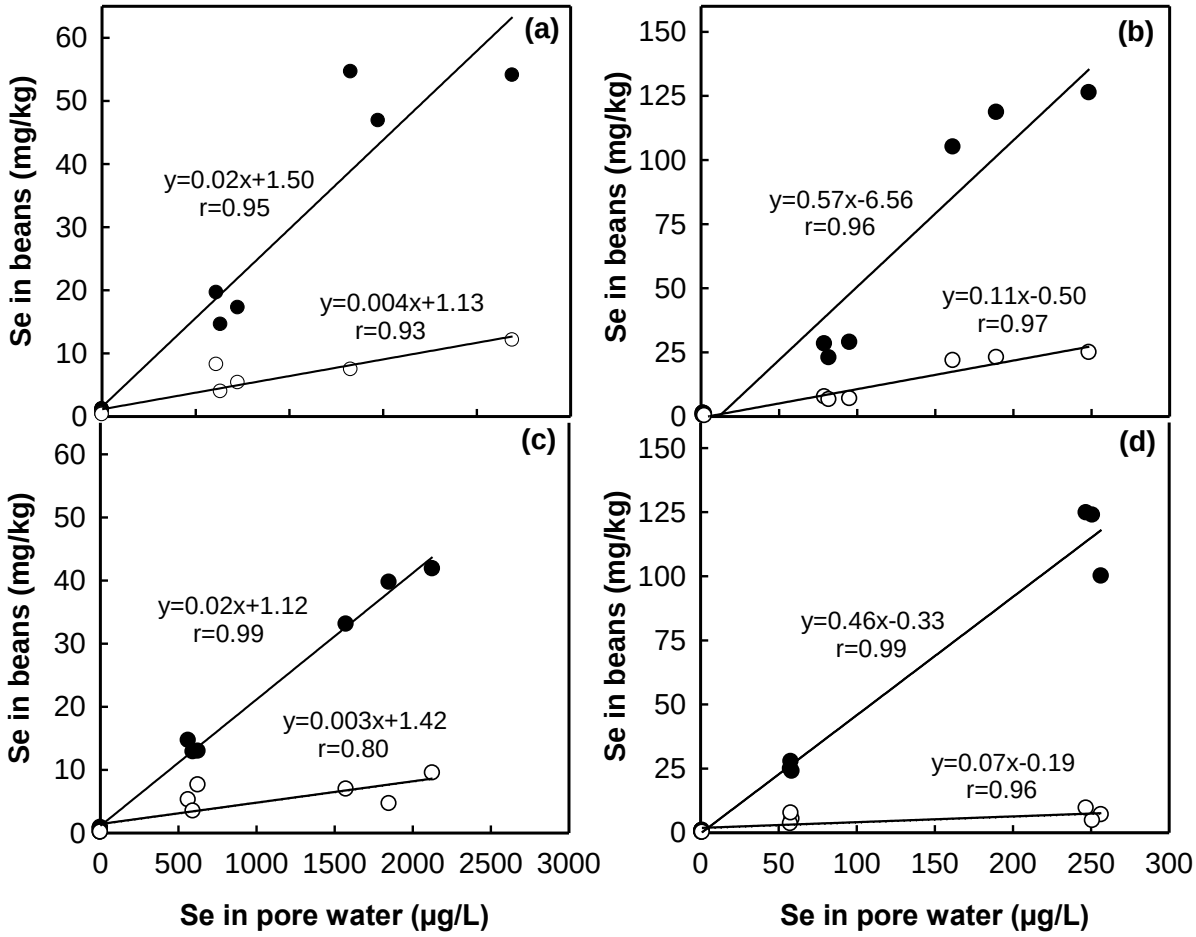

Figure 4.7. Correlation between the concentration of Se in the soil pore water (7 days incubation) and seeds (○) and roots (●) of beans: (a) sandy soil with Se-enriched duckweed supplement, (b) sandy soil with Se-enriched sludge supplement, (c) loamy soil with Se-enriched duckweed supplement, and (d) loamy soil with Se-enriched sludge supplement.

4.3.4.5 Se species in the seeds

The seeds of beans grown in the sandy soil with an amendment of 1.0 mg Se/kg Se-enriched duckweed (DW1) and sludge (SL1) and the blank soil were selected for the determination of Se speciation (Fig. 4.8 and Table 4.3). Organic Se (SeCys2, SeMetCys and SeMet) was the main Se species in all seeds samples. SeMet accounted for the highest percentage of the total Se in the seeds. The percentage of SeMet in the seeds with the amendment of DW1 (72%) was notably lower than that of the amendment of SL1 (85%). Besides, in the blank and DW1 amendment, Se(VI) was the dominant inorganic Se species (accounted for 13.7% and 12.2% of total Se, respectively). Compared to the Se-enriched duckweed amendment (13%), the

percentage of inorganic Se species of total Se in the bean seeds was much lower for the Se-enriched sludge amendment (1.7%) (Table 3). Apart from the identified Se species, unidentified Se peaks in the beans were observed both for the Se-enriched duckweed and sludge application (Fig. 4.8).

Table 4.3. Percentage of Se species in the seeds of beans grown in sandy soil amended with 1.0 mg Se/kg soil of Se-enriched duckweed (DW1) and sludge (SL1), and in the blank without amendment (Blank) comparison with total Se in bean seeds.

	Total Se	$SeCys_2$	SeMetCys	SeMet	Se(IV)	Se(VI)	Se species recovery
	(mg/kg)			(%)			
Blank	0.12	3.2	3.5	77.2	2.4	13.7	99
DW1	4.63	1.8	5.2	71.7	0.4	12.2	91
SL1	6.95	2.7	11.6	84.6	0.3	1.4	101

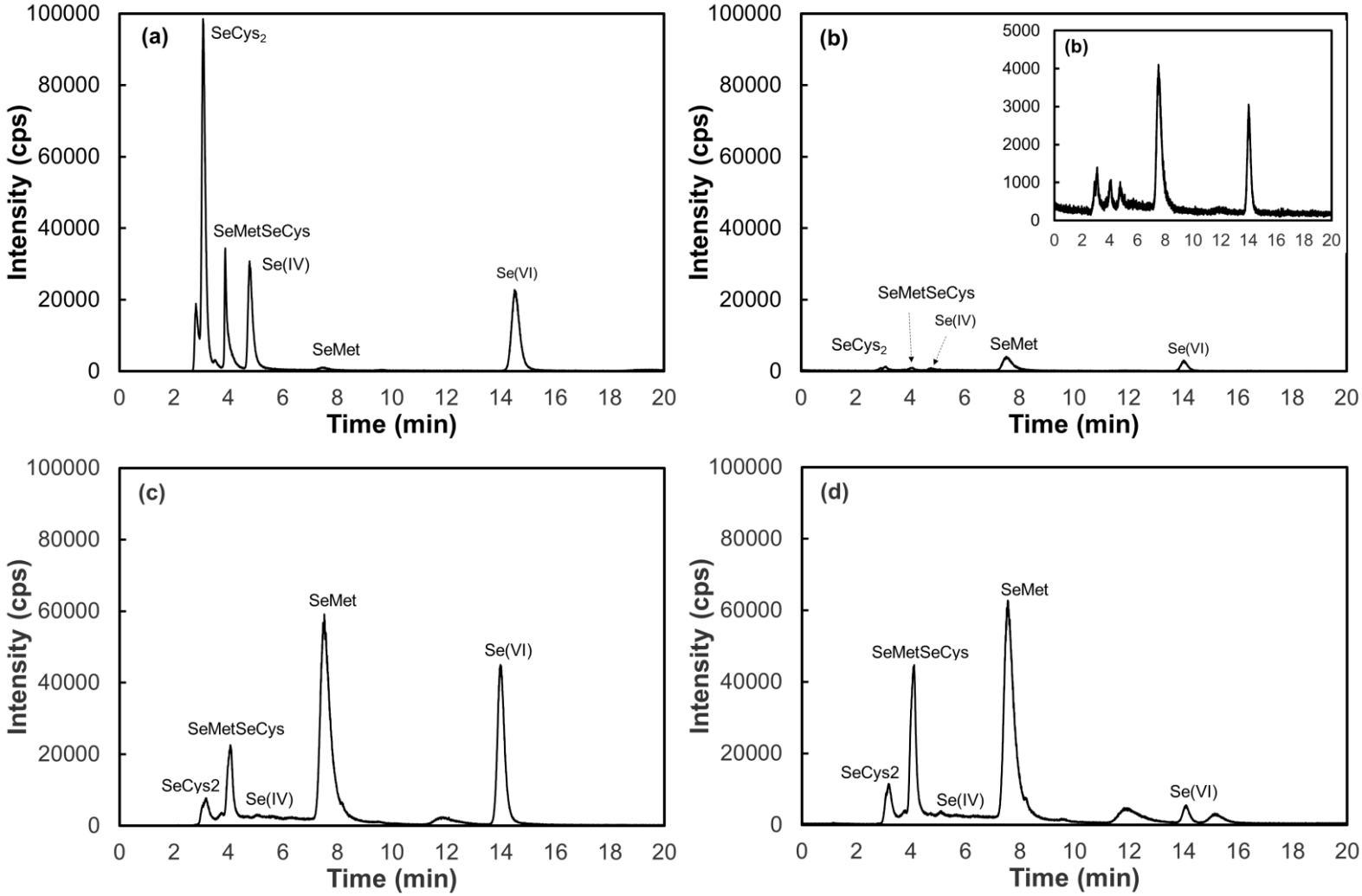

Figure 4.8. Se speciation in the harvested seeds of beans after 42 days grown in the sandy soil amended with 1.0 mg Se/kg of Se-enriched duckweed (DW1) and sludge (SL1): (a) Se standard solution of 10 µg/L, (b) Blank soil (higher magnification in insert), (c) DW1 amendment and (d) SL1 amendment.

4.3.5 pH, TC and TOC contents in the soil pore water

The pH, and the TC and TOC concentration significantly increased upon the amendment of Se-enriched biomaterials (Table 4.4). The highest TC content was found for the DW5 amendment for both the sandy and loamy soils, which was 7.2 and 3.2 times higher than that of the blank in the non-planted soil, and 4.8 and 2.9 times higher in the planted soil, respectively. However, no significant difference was observed between the amendment of Se-enriched sludge and the blank in the planted loamy soil. Moreover, the TC and TOC content in the Se-enriched duckweed amendments were 1.2–2.7 times higher than those of the Se-enriched sludge amendments for both planted and non-planted sandy and loamy soils. The values of pH, TC and TOC in the planted soils were slightly lower, in comparison with the non-planted soils.

Table 4.4. Characterization of Rhizon extracts after 42 days of Se-enriched biomaterials application in planted and non-planted soils.

		Sandy soil			Loamy soil		
	Treatments	pH	TC (mg/L)	TOC (mg/L)	pH	TC (mg/L)	TOC (mg/L)
Non-Planted	Blank	6.0 ± 0.2	33 ± 5.2	30 ± 5.9	6.5 ± 0.1	72 ± 5.0	70 ± 2.3
	DW1	6.8 ± 0.0	63 ± 8.4	57 ± 5.5	6.3 ± 0.3	110 ± 13.2	108 ± 15.8
	DW5	7.6 ± 0.2	236 ± 22.1	201±18.2	7.4 ± 0.1	233±113.8	212 ± 102.0
	SL1	7.8 ± 0.1	48 ± 4.3	47 ± 4.6	7.7 ± 0.1	68 ± 2.2	63 ± 10.3
	SL5	7.8 ± 0.0	136 ± 25.7	133 ± 24.8	7.8 ± 0.0	128 ± 39.2	111 ± 38.5
Planted	Blank	4.9 ± 0.5	36 ± 8.4	34 ± 5.6	6.2 ± 0.2	67 ± 16.5	67 ± 15.6
	DW1	5.4 ± 0.2	76 ± 4.4	75 ± 4.3	6.6 ± 0.3	77 ± 27.3	76 ± 24.9
	DW5	6.7 ± 0.2	172 ± 17.0	166 ± 17.2	7.1 ± 0.2	193 ± 65.4	181 ± 62.3
	SL1	7.0 ± 0.1	43 ± 10.3	41 ± 10.4	6.9 ± 0.2	52 ± 13.7	52 ± 12.4
	SL5	7.5 ± 0.1	79 ± 14.6	77 ± 14.8	7.6 ± 0.2	65 ± 15.2	58 ± 16.6

4.3.6 Evaluation of the Se-enriched biomaterials produced from wastewater as micronutrient fertilizers

All EDI values for the amendment of Se-enriched duckweed and sludge were lower than the oral reference dose of Se (5.0 μg/kg·day), while the value for the amendment

of chemical Na_2SeO_4 at a dose of 5.0 mg/kg (unpublished data from a previous experiment) was higher than 5.0 µg/kg·day (Table 4.5). Similarly, the estimated HRI in all treatments was below 1, except in the treatment of 5.0 mg/kg of Na_2SeO_4 (1.860). The EDI and HRI values of children were slightly higher than those of adults. These estimations suggest that normal consumption of Se-enriched beans produced from the amendment of Se-enriched duckweed and sludge would not pose a potential risk of excessive Se intake, but the beans grown on soil amended with 5.0 mg/kg Na_2SeO_4 might cause a significant health hazard for both adults and children.

Table 4.5. Estimated daily Se intake through seeds of beans grown in soil fertilized with Se-enriched biomaterials.

Treatments	EDI				HRI			
	Adult [µg/kg/day]		Children [µg/kg/day]		Adult		Children	
	Sandy	Loamy	Sandy	Loamy	Sandy	Loamy	Sandy	Loamy
Blank	0.01	0.01	0.03	0.01	0.003	0.001	0.005	0.002
DW1	0.19	0.21	0.35	0.38	0.038	0.041	0.070	0.076
DW5	0.40	0.36	0.74	0.65	0.080	0.071	0.148	0.130
SL1	0.29	0.15	0.53	0.27	0.057	0.030	0.105	0.054
SL5	0.94	0.79	1.73	1.45	0.188	0.158	0.346	0.291
Na_2SeO_4 1.0	1.23	-	2.26	0.00	0.246	0.000	0.452	-
Na_2SeO_4 5.0	5.06	-	9.30	0.00	1.013	0.000	1.860	-

Note: Na_2SeO_4 at a dose of 1.0 and 5.0 mg/kg was unpublished data from a previous experiment.

- : no data obtained in the corresponding treatment

4.4 Discussion

4.4.1 Se toxicity and accumulation in different crops

This study showed that pak choi had the highest ability of Se uptake among pak choi, lettuce, maize, wheat and bean, reflecting on the largest Se content in the shoot and

root (1.3–3.0 times higher than that in the lettuce), while wheat represented the lowest Se uptake ability (Fig. 4.2). Pak choi is a vegetable species of *Brassicaceae* commonly found in China, and has been shown as Se accumulator (Li et al., 2015a). *Brassicaceae* species such as *Stanleya pinnata* and *Brassica juncea* (indian mustard) can indeed accumulate and tolerate a high Se content and may potentially be a Se hyperaccumulator applied in seleniferous areas (White, 2016; Yawata et al., 2010). In contrast, cereals such as wheat, generally have a lower Se uptake ability, in comparison with legumes, some brassicas and fruits (Díaz-Alarcón et al., 1996; Wu et al., 2015), which corroborates to the findings of this study (Fig. 4.2).

The biomass yield obtained in this investigation showed that vegetables (pak choi and lettuce) have a lower tolerance to Se exposure among the five crops tested (Figs. 1a and b). The greatest reductions of root biomass under Se exposure among all crops were observed in pak choi and lettuce. In contrast, the application of Se stimulated the growth of wheat and bean in this study, even at the 5.0 mg/kg of Se application for the seeds and leaves of beans. The largest root biomass of these two crops was at the amendment 1.0 mg/kg Se, indicating that 1.0 mg/kg of Se is the optimal Se application for the two plants grown (Fig. 4.1) and these two crops have a higher tolerance for Se. In agreement with the findings in this research, other researchers have also demonstrated the dual effect of Se on crops (Guerrero et al., 2014; Wang et al., 2017). The optimal dose of Se stimulates crop growth, while excessive Se inhibits plant growth (Figs 4.1c-d).

Selenium taken up by plants will partly replace sulfur in amino acids and selenoaminoacids such as SeMet will subsequently be incorporated into proteins, which is beneficial for human and animal nutrition (Eiche et al., 2015; Schrauzer, 2000). Given that beans have the highest protein content in seeds as well as high Se tolerance ability among the five crops in this study, it was selected for the following experiment of Se-enriched biomaterials fertilization.

4.4.2 Selenium release from Se-enriched duckweed and sludge

The results showed that the amendment of soils with Se-enriched duckweed and sludge increased the availability of soluble Se in the soils, as evidenced by the increment of the Se concentration in the pore water with larger doses of enriched

biomaterials (Fig. 4.4). Similarly, Bañuelos et al. (2015) found that the increase of available soluble Se positively correlated with the amount of Se-enriched *Stanleya pinnata* applied. Dhillon et al. (2007) indicated that 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 the soil from 18 µg/kg to 36 and 79 µg/kg, respectively.

However, the available Se in soils amended with Se-enriched duckweed decreased by 90% over an incubation period of 42 days even in the absence of planted crops (Fig. 4.4). This may be partly attributed to the immobilization of the released Se in the soil solid phase (both inorganic and organic) and the adsorption of Se onto the extra organic matter introduced with the supplementation of the Se-enriched biomaterials (Li et al., 2017). Specifically, the organic content of the applied duckweed and sludge introduces organic compounds, including some organic acids, polysaccharides and lignin, which augment the soil organic matter (Wang et al., 2018). These organic compounds can bind soluble Se into stable compounds, resulting in Se immobilization (Ebrahimi et al., 2019; Li et al., 2017). The increase in organic compounds was confirmed by the significant increment of the TC and TOC concentration in the Rhizon extracts of both soils after the biomaterials application (Table 4.4). Research on Se species transformation in straw-amended soil further demonstrated that straw-derived organic carbon could accelerate the reduction of soluble Se (SOL-Se) to organic matter-bound Se (OM-Se) and residual Se (RES-Se), resulting in less soluble Se in soils (Arbestain, 2001). Wang et al. (2018) also found that most SOL-Se was reduced and transformed to RES-Se in a soil fertilized with Se-enriched wheat straw. Similarly, in this study, the decrease of Se in the pore water along the incubation time (Figs. 4.4 and 4.5) may be partly explained by the transformation of SOL-Se to other, unavailable Se fractions in soils, e.g. OM-Se and RES-Se.

On the other hand, the higher quantity of Se in the pore water of the Se-enriched duckweed amended soil (14% of the total applied Se after 3 days of DW1 amendment) compared to that of the Se-enriched sludge amended soil (2% of the total applied Se after 3 days of SL1 amendment) (Fig. 4.4) may be mainly ascribed to different Se species in the Se-enriched biomaterials. Specifically, inorganic Se(VI) represented a large proportion of the Se in duckweed (ca. 80%, Fig 4.3a), whereas elemental Se(0) dominated in the sludge (94.2%, Fig. 4.3b), especially nano-Se(0) (Staicu et al., 2015b).

Se(VI) is highly soluble and readily mobilized in soils and waters (Li et al., 2020a). In contrast, elemental Se(0) is stable and difficult to mobilize or oxidize in the environment (Hu et al., 2018), resulting in less and slower Se released to the soil pore water from the Se-enriched sludge amendment.

4.4.3 The potential of Se-enriched duckweed and sludge as Se biofertilizers

The increase in Se uptake by beans positively correlated with the application rates of Se-enriched duckweed and sludge (Fig. 4.7), implying that Se in the soil pore water released from the Se-enriched duckweed and sludge is the major source of Se in the tissues of beans. Interestingly, the higher Se concentration in the soil pore water with the Se-enriched duckweed amendment compared to that with the Se-enriched sludge supplement did not result in a higher Se concentration in the corresponding plant biomass (Figs. 4.5 and 4.6). This is probably due to the different Se species released to the soil solution from the two Se-enriched biomaterials (Fig. 4.1) and the different behavior of Se after being released into soils (Figs. 4.4 and 4.5), resulting in a different metabolism of Se in beans and consequently leading to a different Se uptake/accumulation ability. Specifically, the high amount of Se(VI) present in the duckweed (80%, Fig. 4.3a) is immediately released into the soil solution where it is quickly adsorbed by the soil matrix or extra organic matter (Li et al., 2016), thus leading to a significant decrease of the plant bioavailable Se concentration in the soil pore water along time, even lower than that in the sludge amendment after 28 days of growth (Fig. 4.5 and Table 4.S1). In contrast, the zerovalent form of Se in the Se-enriched sludge (Fig. 4.3b) may have been slowly released or oxidized to Se(IV) or Se(VI) in the soil solution. The slowly released Se in the soil pore water could continuously supply and satisfy the Se uptake requirement of the beans during the growth period, contributing to the higher Se uptake (Fig. 4.6). Furthermore, the Se(0) in the Se-enriched sludge may mostly exist as biogenic Se(0) nanoparticles with an average size of 166 nm (Staicu et al., 2015b), which has the excellent bioavailability and similar behavior to Se(IV) after being taken up by plants (El-Ramady et al., 2020; Moreno-Martin et al., 2020). This could also explain the higher Se concentration in bean tissues grown in Se-enriched sludge amendment soil. Besides, the speciation of Se accumulated in the seeds of beans fertilized with Se-enriched biomaterials further supports the different uptake patterns and different Se species released from Se-enriched duckweed and sludge (Fig. 4.8 and Table 4.3). Specifically, this study found

that a higher fraction of inorganic Se(VI) (13.3%) accumulated in the seeds of beans amended with Se-enriched duckweed, compared to only 1.6% of Se(VI) in the seeds fertilized with Se-enriched sludge (Table 3), which indirectly confirms that the form of Se released from Se-enriched duckweed was mainly Se(VI). Because Se(VI) taken up by plants is reduced into Se(IV) and then converted to selenaminoacids through a reduction process via substitution for sulfate in the ATP sulfurylase reductase system (Gupta & Gupta, 2017). This reduction process is an ATP-consuming process and rate-limiting step, resulting in the accumulation of Se(VI) in the beans with the Se-enriched duckweed amendment (Table 4.3). In contrast, the Se-enriched sludge may release Se(0) into soils and then be oxidized to Se(IV) by microorganisms (Sarathchandra & Watkinson, 1981; Winkel et al., 2015). It is also possible that the nano-Se(0) released in the soil solution was directly taken up and behaved a similar transformation to Se (IV) in plants (El-Ramady et al., 2020; Moreno-Martin et al., 2020). Se(IV) is quickly and easily converted to organic Se forms (e.g., SeMet and $SeCys_2$) in the beans after being taken up by the roots and translocated to the seeds.

The significantly higher amount of TOC in the soil pore water caused by the Se-enriched duckweed supplement as compared with the Se-enriched sludge amendment (Table 4.4) also partially contributed to the lower Se uptake by the beans (Fig. 4.6), as organic carbon in the soil pore water may bind with Se (Supriatin et al., 2016; Weng et al., 2011), and eventually lower the Se bioavailability (Li et al., 2017; Wang et al., 2018). Besides, soil pH also plays a role in the mineralization of soluble organic Se. Supriatin et al. (2016) demonstrated that soil pH is the primary factor to control the solubility of Se in dissolved organic carbon and confirmed that the solubility of Se-containing organic matter increases with pH. In this study, the higher pH of the pore water of the soils with Se-enriched sludge compared to the Se-enriched duckweed supplement (Table 4) may have resulted in a higher solubility of Se, hence more Se available for plant uptake and thus leading to a higher Se content in the beans (Fig. 4.6). These results indicated that Se-enriched sludge is considered as the preferred slow-release Se biofertilizer and an effective Se source to produce Se-enriched crops for Se-deficient populations

Additionally, the application of Se-enriched biomaterials did not show negative effects on the biomass production of beans, while the higher amount of non-enriched biomaterials application may have caused an abiotic stress, resulting in a decreased

biomass of beans (Table 4.2). Similarly, other researchers have demonstrated that the amendment of high amounts of compost, weeds or crops residues (such as the residues of *Chenopodium murale* and *Parthenium hysterophorus*) may release a wide range of inhibitors (e.g. tannins and phenols compounds) to the environment that may be toxic or cause stress to plants (Batish et al., 2007; Mushtaq et al., 2020; Singh et al., 2003), which is broadly associated with allelopathic interactions. Therefore, the presence of inhibitory compounds in the non-enriched biomaterials amended soils in this study may partially explain the significant reduction in the bean biomass upon non-enriched biomaterials supplementation. On the other hand, no significant reduction of bean biomass in the Se-enriched biomaterials application indicates that the Se released from the biomaterials could protect the plants against various types of external stresses (Handa et al., 2016). Various studies have revealed the direct effect of Se on the antioxidative defense system, which increases the potential of the plants to combat stressful conditions (Handa et al., 2016; Jozwiak & Politycka, 2019; Subramanyam et al., 2019).

Furthermore, the high amount of SeMet (around 80%, Table 4.3) present in the seeds of the beans suggests that these Se-enriched seeds obtained by the amendment of Se-enriched biomaterials may be of interest for animal or human nutrition in Se-deficient regions. SeMet is a selenoamino acid that is highly suitable for nutritional supplementation because it is more bioavailable, less toxic, and can provide higher Se concentrations in organs than inorganic Se (Gómez-Jacinto et al., 2020). SeMet is also one of the precursors of methyl selenol, a potent anticarcinogen that inhibits tumor invasion and angiogenesis (Gómez-Jacinto et al., 2020). Additionally, the HRI and EDI data suggest that Se-enriched beans produced from this study would not pose a potential risk of excessive Se intake for adults and children (Table 4.5). In contrast, the beans produced from the treatment with 5.0 mg/kg Na_2SeO_4 could cause a significant health hazard due to Se over-consumption. The result demonstrated that the Se recovered from wastewater through ecotechologies (phytoremediation and bioreduction) can be reused as biofertilizers to efficiently improve Se level in feeds/foods. High-value biofertilizers can thus be valorized, while recovering resources.

Even if the biofortification of beans through Se-enriched biomaterials produced from wastewater has been achieved in this study, additional studies should address the safety and other risks associated with the application of Se-enriched biomaterials from

ecotechnologies, such as their potential heavy metal content and pathogen load. Economic analysis of Se recovery from wastewater and reusing Se as biofertilizer is still necessary. Besides, the relatively lower Se bioavailability and the significant decrease of Se content in the soils amended with Se-enriched duckweed indicate that post-treatment of the duckweed harvested from wastewater treatment process (phytoremediation) should be further considered before application as biofertilizer, such as composting and extracting. Additionally, continuous cultivation under field conditions should be further studied to investigate the bioavailability of the Se released from the slow-release fertilizers in a long-term period and its effect on the environment and nutritional value of the crops.

4.5 Conclusions

Soil amendment with Se-enriched biomaterials produced from wastewater was shown to be a promising approach for Se biofortification, which was able to provide sufficient bioavailable Se and reduced environmental risks compared to the application of chemical Se fertilizers. Both the Se content in the tissues of beans and soil pore water were significantly enhanced by the Se-enriched duckweed and sludge amendment. Se in the Se-enriched duckweed enriched in Se-protein was released quicker to the soil than Se in the Se-enriched sludge enriched in nano-Se(0). However, the Se-enriched sludge (nano-Se(0)) was more efficient than the Se-enriched duckweed in increasing the Se content in beans, in particular the SeMet concentration of the seeds. The value-added Se-enriched sludge is thus considered as the preferred slow-release Se biofertilizer for reusing the recovered Se.

Supplementary Information

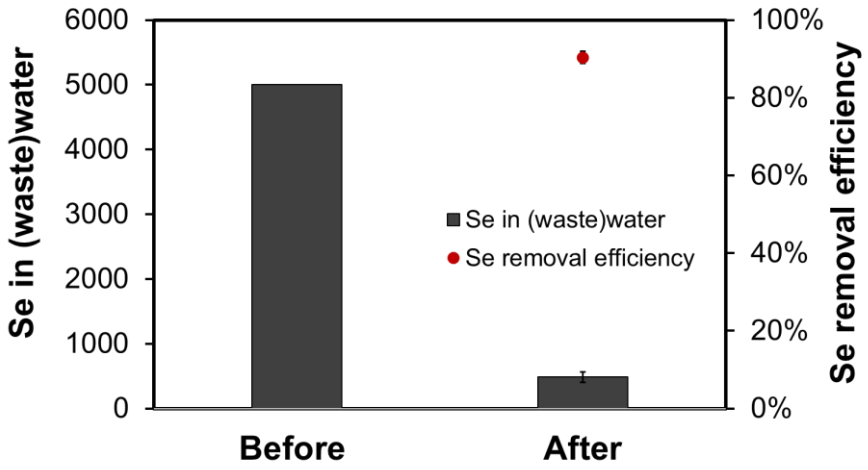

Figure 4.S1. Selenium content in the wastewater/medium at the beginning (Before) and end (After) of the experiment and Se removal efficiency by sludge.

Table 4.S1. Selenium concentrations (μg/L) in the pore water extracted from planted soils amended with Se-enriched duckweed and sludge. Mean ± standard deviation, n=3. Different lowercase letters indicate statistically significant differences between different incubation days according to Duncan's multiple comparison tests ($P < 0.05$). * indicates significant differences between different Se-enriched biomaterials.

		Day 7	Day 21	Day 28	Day 42
Sandy soil	DW1	920 ± 85.4 a*	314 ± 120.2 b*	264 ± 120.8 b*	278 ± 231.1 b
	SL1	85 ± 9.0 a*	45 ± 6.0 b*	43 ± 5.0 b*	44 ± 15.2 b
	DW5	2330 ± 646.1 a*	549 ± 466.0 b	319 ± 113.2 b	305 ± 143.2 b
	SL5	200 ± 44.0 c*	255 ± 8.3 bc	312 ± 62.1 b	485 ± 108.7 a
Loamy soil	DW1	595 ± 44.2 a*	107 ± 12.3 b*	42 ± 3.7 c	7.9 ± 1.3 d*
	SL1	58 ± 0.5 *	50 ± 3.0 *	48 ± 18.0	39 ± 13.3 *
	DW5	1851 ± 391.1 a*	823 ± 345.4 b*	341 ± 160.3 b	272 ± 220.2 b
	SL5	252 ± 7.0 c*	313 ± 7.0 ab*	355± 44.8 a	292± 53.9 bc

Chapter 5 Biofortification of green beans with Se and Zn-enriched duckweed and sludge

Abstract

The potential of biomaterials (duckweed and sludge) enriched with Se and Zn as micronutrient biofertilizers for simultaneously improving the Se and Zn content in green beans (*Phaseolus vulgaris*) was evaluated in pot experiments. Both the Se and Zn concentrations in the soil pore water increased upon amending both biomaterials. The concentration of Se released from SeZn-enriched duckweed rapidly decreased in the first 21 days and slowly declined afterwards, while it remained stable during the entire growth period upon application of SeZn-enriched sludge. The Zn content in the soil pore water gradually increased over time. In addition, the application of the SeZn-enriched biomaterials significantly increased the Se concentrations (in particular organic Se-methionine) in plant tissues including the seeds, without a negative impact on plant growth, except for a remarkable decrease in biomass production upon the high amount of SeZn-enriched duckweed application (SeZnDW5). This indicates that the SeZn-enriched biomaterials could be used as organic Se biofertilizers for Se-deficient soils, but an appropriate dose should be determined. In contrast, the Zn content of the beans was not noticeably improved by supplementation of SeZn-enriched biomaterials, so SeZn-enriched biomaterials may thus not be effective biofertilizers for Zn biofortification purposes.

Keywords: Selenium and Zn bioavailability, Se and Zn-enriched biomaterials, biofortification, biofertilizer, resource recovery

5.1 Introduction

Selenium and Zn are both essential micronutrients for humans and animals, playing an irreplaceable role in the functioning of enzymes (Hatfield et al., 2014). Multiple micronutrient (e.g. Se and Zn) deficiencies have been found worldwide, particularly in developing countries (Ngigi, 2019; Sazawal et al., 2018), which is mainly associated with the low dietary micronutrient intake in diets or the low diversity of foods (Mao et al., 2014). Improving the micronutrient content in plants, crops and foods is a possible solution for micronutrient deficiency. As plants and crops take up and accumulate micronutrients from the soil where they grow, multi-mineral agronomic biofortification of crops is thus being explored as a simple and effective way to alleviate micronutrient deficiency (Mao et al., 2014; Poblaciones & Rengel, 2017). On the other hand, discharged wastewaters may simultaneously contain excessive Se and Zn due to insufficient treatment (Lim & Goh, 2005). In this context, Se and Zn loaded in those wastewaters may serve as potential nutrient sources from which the nutrients may be recovered and valorized to produce slow-release micronutrient organic fertilizers. Accordingly, the main objective of this study was to explore the possibility of the two biomaterials (duckweed and sludge) generated from Se and Zn-containing water as potential micronutrient (Se and Zn) biofertilizers. The specific objectives include: (1) to monitor the evolution of the released micronutrients (Se and Zn) from the SeZn-enriched biomaterials in the soil pore water, (2) to evaluate the influence of the supplementation of the two biomaterials on the growth of green beans (*Phaseolus vulgaris*) on sandy and loamy soil and its micronutrient accumulation.

5.2 Materials and methods

5.2.1 Soil collection and characterization

Two types of soil (similar to those used in Chapter 4) were collected, and classified as sandy and loamy soil. The soil collection and characterization were detailed in the materials and methods section of Chapter 4.

5.2.2 Valorization of SeZn-enriched biomaterials as Se and Zn biofertilizers

5.2.2.1 Preparation of SeZn-enriched biomaterials (duckweed and sludge)

SeZn-enriched duckweed was produced as described previously (Li et al., 2020b) and in Chapter 4. Briefly, duckweed collected from a natural freshwater canal in Delft was cultivated in a Hoagland solution together with 5.0 mg/L of Se and 5.0 mg/L of Zn added as Na_2SeO_4 and $ZnCl_2$, respectively. The cultivated duckweed was harvested after 7 days of growth, oven-dried and ground for use in the subsequent fertilization experiment. The SeZn-enriched sludge was generated as described in Chapter 4, with minor modification. The sludge collected from a full-scale upflow anaerobic sludge blanket reactor treating pulp and paper wastewater in Eerbeek (The Netherlands) was added to a nutrient medium with 5.0 mg/L of Se and 5.0 mg/L of Zn in the form of Na_2SeO_4 and $ZnCl_2$ under anaerobic conditions. After 14 days of incubation at 30 °C, the sludge enriched with Se and Zn was separated from the supernatant, oven-dried and ground for further use. The obtained SeZn-enriched duckweed and sludge contained 103 mg Se/kg and 2289 mg Zn/kg dry weight, and 287 mg Se/kg and 563 mg Zn/kg dry weight, respectively.

5.2.2.2 Selenium and Zn bioavailability for planting green beans

An amount equivalent to 1.0 and 5.0 mg Se/kg soil of SeZn-enriched duckweed (4.9 and 24 g) and sludge (1.7 and 8.7 g) was applied to 0.5 kg of the soil. Accordingly, the amendments in this study were 1.0 mg Se/kg soil and 5.0 mg Se/kg soil of SeZn-enriched duckweed (noted as SeZnDW1 and SeZnDW5), and 1.0 mg Se/kg soil and 5.0 mg Se/kg soil of SeZn-enriched sludge (noted as SeZnSL1 and SeZnSL5). The soils without any biomaterials addition served as blank (noted as Blank). For the control experiment, the same amount of non-enriched duckweed and sludge as in the SeZn-enriched biomaterials amendments was applied to the two soil types (noted as control-SeZnDW1, control-SeZnDW5, control-SeZnSL1 and control-SeZnSL5). The biomaterials were mixed thoroughly with the soils and placed into 10 x 10 cm plastic pots before incubation.

Subsequently, 100 mL of chemical fertilizer solution including 600 mg/L of N, P_2O_5 and K_2O were added to each pot. Pots were incubated indoors at 24ºC, 53% relative humidity and 100 µmol/m^2/s light intensity by keeping 80% of the water holding capacity for one week. Afterwards, 4 seedlings of green beans (*Phaseolus vulgaris*) previously cultivated in trays with wet vermiculite for one week were transferred into each pot. All experiments were conducted in triplicate. The soil pore water was collected every week

through extraction using Rhizon soil moisture samplers for total Se and Zn measurement. pH, TC and TOC of the extracted soil pore water were determined before harvest. The bean plants were harvested after 6 weeks of growth, washed and separated into different tissues (root, stem, leaf and seed) for biomass, Se and Zn concentration analysis. Se speciation analysis was performed on selected bean seeds (at the SeZnDW1 and SeZnSL1 amendment for sandy soil, and sandy soil blank) after lyophilization.

5.2.3 Analytical methods

5.2.3.1 Total Se and Zn concentration in plants

0.3 g of the harvested plants were weighed into a digestion vessel followed by the addition of 10 mL concentrated ultrapure HNO_3. The tubes were sonicated for 1 h, then placed in a microwave oven (CEM Mars 6, Matthews, NC, USA) for digestion. The digestion temperature was raised to 165 °C in 25 min and kept for 20 min at 1200 W power. The digested solution was diluted with Milli-Q water and analyzed for total Se and Zn. Total Se and Zn were determined by 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 to validate the accuracy of Se and Zn measurements. White clover samples (BCR-CRM, 6.7 ± 0.25 mg Se/kg dry weight) and sea Lettuce (51.3 ± 1.3 mg Zn/kg dry weight) were included as certified reference materials for Se and Zn, respectively, in each analytical batch as quality control with recoveries of 107 (± 6%) and 97 (± 7)%.

5.2.3.2 Selenium speciation analysis

Selenium speciation in the SeZn-enriched duckweed and harvested seeds of beans was analyzed by ICP-MS coupled to high-performance liquid chromatography (Series 200 HPLC, Perkin Elmer, Waltham, MA, USA). Selenium speciation in the SeZn-enriched sludge was determined by extended X-ray absorption fine structure (EXAFS) and X-ray absorption near edge structure (XANES). Additional details on the analysis can be found in Chapter 4.

5.2.3.3 pH, TC and TOC analysis of the extracted soil pore water

pH, TC and TOC of the extracted soil pore water at the end of the experiment were analyzed as described in Chapter 4.

5.2.4. Statistical analysis

Descriptive statistics were performed using Sigma plot 13, Excel 2016 and SPSS 20.0. Results are expressed as mean ± SD. The different treatments were compared with a one-way analysis of variance (ANOVA) and Duncan's multiple comparison tests ($P < 0.05$).

5.3 Results and discussion

5.3.1 Characterization of SeZn-enriched biomaterials

The Se species present in the SeZn-enriched duckweed and granular sludge are shown in Figure 5.1a and 5.1b, respectively. Se in the SeZn-enriched duckweed was composed of 92% selenate (Se(VI)), 1.3% selenite (Se(IV)), 0.4% Se-cystine ($SeCys_2$), 3.7% methylselenocysteine (SeMetSeCys) and 4.9% Se-methionine (SeMet). Differently, the predominant Se species in SeZn-enriched sludge was the zerovalent form (72.9%) of Se, followed by Se(IV) (27.5%).

5.3.2 pH and TOC content of the extracted soil pore water

The amendment of SeZn-enriched duckweed and sludge notably increased the pH of the soil pore water extracted from the two soils (Table 5.1) ($P < 0.05$). Likewise, the TOC content of the soil pore water was increased with the amendment of SeZn-enriched duckweed, while it remained unchanged upon the application of SeZn-enriched sludge in both soils (Table 5.1). The maximum TOC concentration was observed upon the application of SeZnDW5, being 11 and 4 times higher than that of the blank in the sandy and loamy soil, respectively.

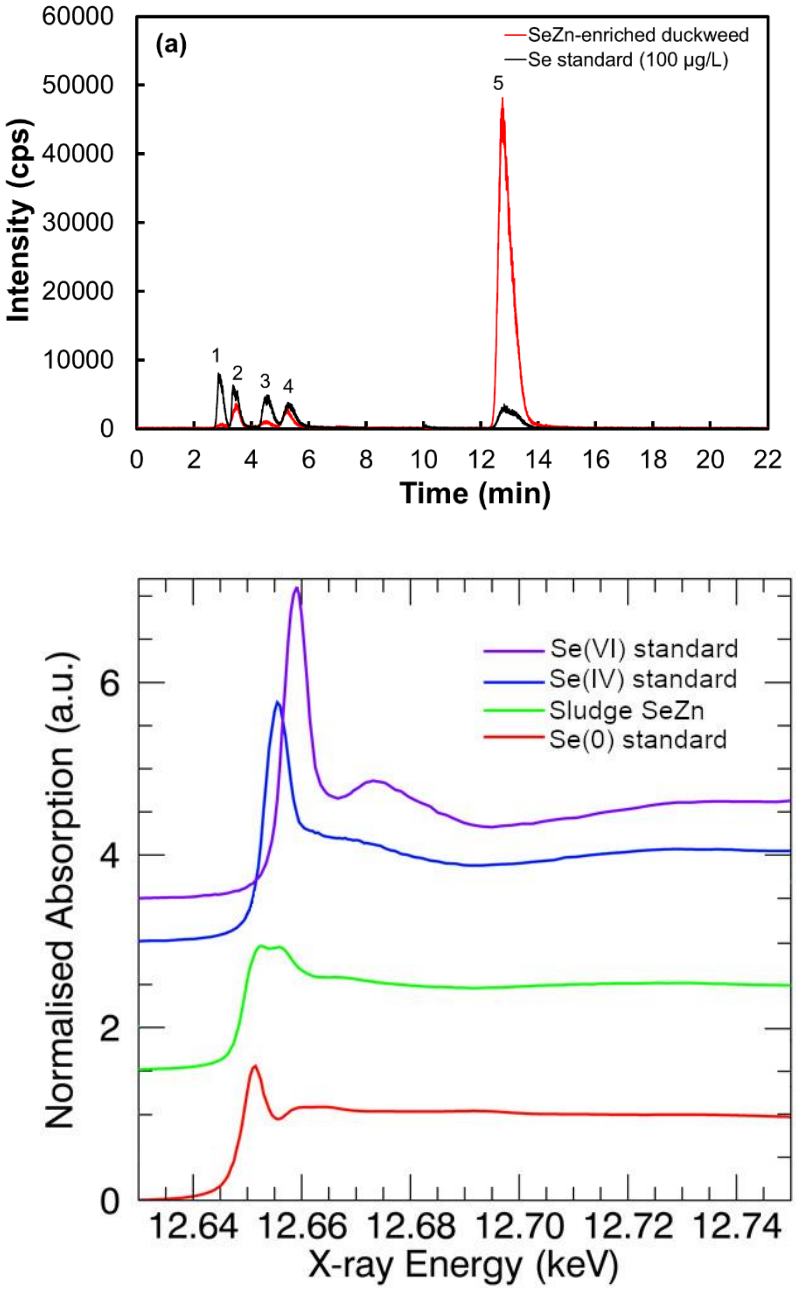

Figure 5.1. (a) Chromatogram of Se speciation in the SeZn-enriched duckweed compared with that of a Se standard solution of 100 µg/L of each species (1) $SeCys_2$, (2) SeMetSeCys, (3) selenite (Se(IV)), (4) SeMet, (5) selenate (Se(VI)), and (b) XANES spectra of the Se species in the SeZn-enriched sludge (green) as compared to standards.

Table 5.1. Characterization of Rhizon extracts after 42 days of SeZn-enriched biomaterials amended in the two soils. Different lower case or upper case letters in the same column, respectively, indicate statistically significant differences between different amounts of SeZn-enriched duckweed or sludge application according to Duncan's multiple comparison tests ($P < 0.05$). No letters indicate $P > 0.05$.

Amendments	Sandy soil		Loamy soil	
	pH	TOC (mg/L)	pH	TOC (mg/L)
Blank	4.9 ± 0.5 c B	34 ± 5.2 b	6.2 ± 0.2 b B	67 ± 16.2 b
SeZnDW1	5.8 ± 0.5 b	62 ± 11.4 b	6.3 ± 0.5 b	83 ± 24.5 b
SeZnDW5	7.8 ± 0.2 a	378 ± 53.3 a	6.9 ± 0.1 a	256 ± 43.4 a
SeZnSL1	6.9 ± 0.4 A	51 ± 9.7	7.1 ± 0.3 A	46 ± 10.6
SeZnSL5	7.5 ± 0.1 A	69 ± 13.5	7.3 ± 0.0 A	40 ± 7.2

5.3.3 Selenium content in the soil pore water during plant growth

Amendment with SeZn-enriched duckweed and sludge significantly increased the Se concentration in the pore water of both soils compared to the blank (Fig. 5.2). The effect of the Se dosage was also evident from the higher Se concentration in the soil pore water for the most Se-enriched biomaterials (SeZnDW5 and SeZnSL5). After 7 days of incubation (before planting), the supplement of SeZnDW1 and SeZnDW5 increased the Se concentration in the pore water from the soil background values (around 1.0 µg/L) to 2491 and 3789 µg/L for the sandy soil, and 2364 and 2540 µg/L for the loamy soil, respectively (Fig 5.2a and 5.2b). The supplement of SeZnSL1 and SeZnSL5 raised the Se content in the pore water to 49 and 175 µg/L for the sandy soil, and 30 and 190 µg/L in the loamy soil pore water, respectively (Fig 5.2c and 5.2d).

The Se concentrations in the soil pore water significantly decreased along with the incubation time for the SeZn-enriched duckweed amendment, while they slightly declined for the SeZn-enriched sludge amendment. During the entire experiment (6 weeks), an approximate 93–98% decrease of the Se concentrations in the soil pore water was observed for the SeZn-enriched duckweed amendment, while the Se concentrations in the soil pore water decreased by 21–59% for the SeZn-enriched sludge amendment. It should be noted that the decrease of the Se concentration in the soil pore water with the amendment of SeZnDW5 was sharper than that of SeZnDW1

in the initial phase of the experiment (Fig. 5.2a and 5.2b), resulting in a lower Se concentration in the soil pore water with the SeZnDW5 application in comparison with the SeZnDW1 application after 21 days. The Se concentration in the soil pore water with SeZnDW5 supplement decreased by 97 and 90% in the first 21 days for the sandy and loamy soil, respectively, and remained almost stable in the remaining part of the experiment.

The Se concentration in the pore water of the two soil types supplied with SeZn-enriched duckweed was significantly higher than that of the soils supplied with SeZn-enriched sludge (Fig. 5.2a and 5.2b versus Fig. 5.2c and 5.2d). In addition, the Se concentrations in the sandy soil pore water were slightly higher than those in the loamy soil pore water, which could be the result of the different soil characteristics. The loamy soil has a higher content of organic matter (OM), CEC and EC, and a higher percentage of silt and clay, in comparison with the sandy soil (Table 4.1 in chapter 4). Generally, there are more oxygenic groups in soil OM, such as phenolic hydroxyl and carboxyl groups (Li et al., 2015b). These can complex or chelate soluble Se in soil, thereby decreasing the Se content in the soil pore water (Coppin et al., 2006; Li et al., 2015b).

The increased Se content in the soil (or pore water) upon Se-enriched biomaterials amendment has also been observed in our previous work (Chapter 4) and in other studies (Bañuelos et al., 2016; Wang et al., 2018). In line with our previous results (Chapter 4), the remarkable decrease of the Se content in the soil pore water with the amendment of SeZn-enriched duckweed along with the growth time could be attributed to (1) the plant uptake; (2) the immobilization of the released Se by the soil matrix; and (3) the adsorption of the extra OM introduced by the duckweed supplementation. The stable Se content in the soil pore water with the supplementation of SeZn-enriched sludge during the entire growth period could be due to the Se(0) species predominating in the sludge samples (Fig. 5.1), as Se(0) is highly stable in the environment (Hu et al., 2018; Zahedi et al., 2019).

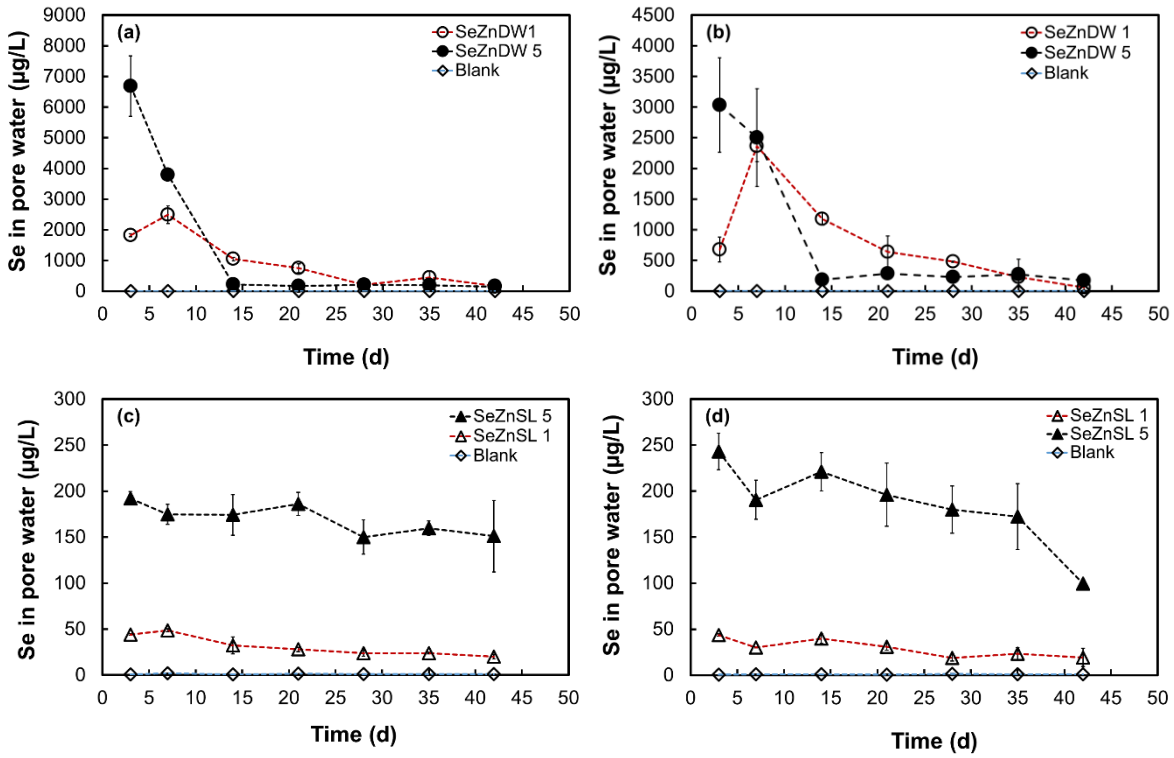

Figure 5.2. Evolution of the Se concentration in the pore water of the sandy and loamy soils with biomaterials supplementation: (a) sandy soil with SeZn-enriched duckweed supplementation, (b) loamy soil with SeZn-enriched duckweed supplementation, (c) sandy soil with SeZn-enriched sludge supplementation, and (d) loamy soil with SeZn-enriched sludge supplementation. Values are mean ± standard deviation (n=3).

5.3.4 Zinc content in the soil pore water during beans growth

Both SeZn-enriched biomaterials were effective for improving the availability of Zn in the two types of soil, which is reflected in a significantly higher amount of Zn in the pore water of soils amended with SeZn-enriched duckweed and sludge compared to that of the blank at the end of the experiment (Fig. 5.3). The application of SeZnDW5 showed the highest Zn content in pore water of both soils among all amendments in the first 3 days, indicating the fastest Zn release from this biomaterial (Fig.5.3a and 5.3b). Along with the growth time, the Zn content rapidly decreased within 21 days and slightly decreased thereafter for this amendment (SeZnDW5), resulting in a 65% (Fig. 5.3a) and 35% (Fig. 5.3b) decrease of the Zn content in the sandy and loamy soil, respectively, over the entire growth period. On the contrary, the Zn content in the pore water of both soils increased (approximately 4 times) along with the growth time for the amendment of SeZnDW1, which even had no significant difference in comparison with

that of the SeZnDW5 supplement after 21 days (Figs. 5.3a and 5.3b). Similarly, the supplement of SeZn-enriched sludge increased the Zn content in the soil pore water by 4–15 times from day 3 to day 42 (Figs. 5.3c and 5.3d). These results indicate that Zn is being slowly released from the SeZn-enriched biomaterials, except for the amendment of SeZnDW5. The reason for the noticeable decrease of the Zn content over time in the soil pore water after SeZnDW5 supplementation could be similar to that of the Se change (Fig. 5.2), i.e. the adsorption onto the extra organic matter (introduced by the high amount of SeZn-enriched materials application) (Table 5.1).

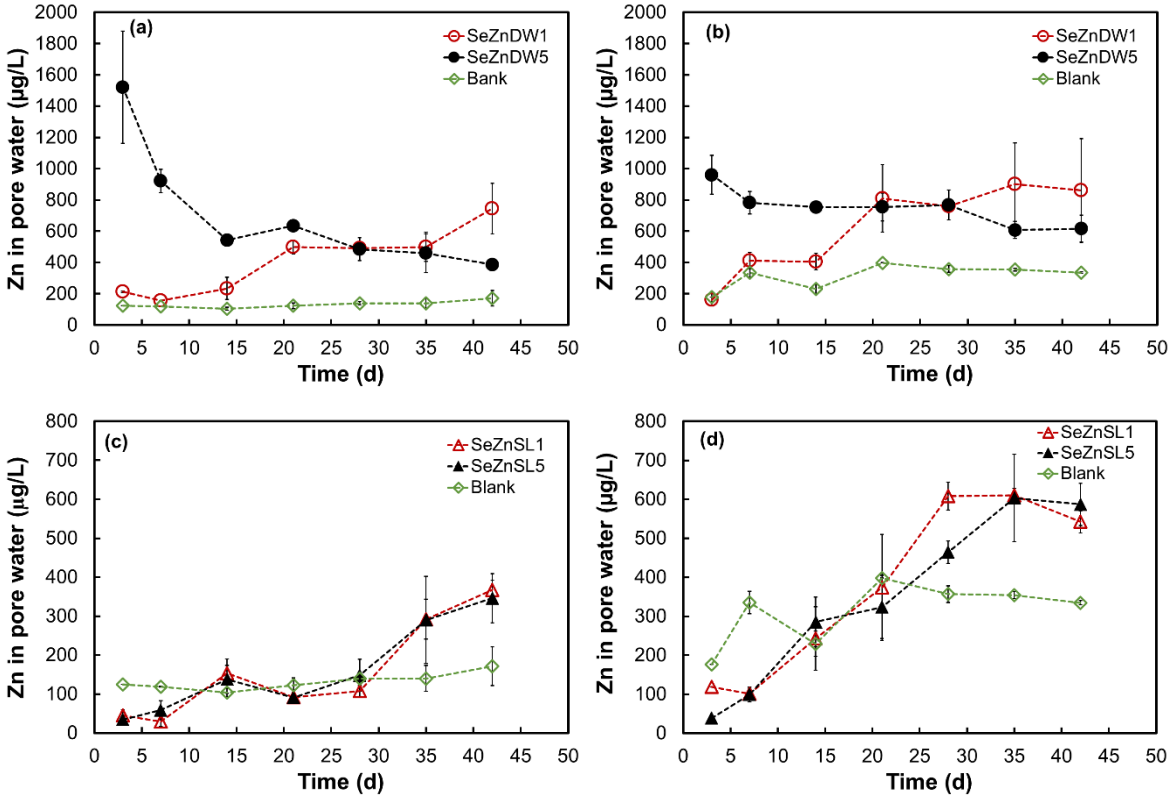

Figure 5.3. Evolution of Zn concentration in the pore water of sand and loamy soils amended with SeZn-enriched biomaterials: (a) sandy soil with SeZn-enriched duckweed supplementation, (b) loamy soil with SeZn-enriched duckweed supplementation, (c) sandy soil with SeZn-enriched sludge supplementation, and (d) loamy soil with SeZn-enriched sludge supplementation. Values are mean ± standard deviation (n=3).

Additionally, the Zn content of the pore water of soils supplied with SeZn-enriched duckweed was significantly higher than that of the soils supplied with SeZn-enriched sludge (Fig. 5.3 a and b versus Fig 5.3 c and d), which corresponds to the significantly higher Zn content in the SeZn-enriched duckweed (2289 mg Zn/kg dry weight)

compared to that in the SeZn-enriched sludge (563 mg Zn/kg dry weight). The Zn concentration in the pore water of the loamy soil was higher than that of the sandy soil (1.1–2.6 times and 1.1–5.6 times for the supplementation of SeZn-enriched duckweed and sludge, respectively), except for the SeZn-enriched duckweed application at the beginning, i.e. day 7 (Fig. 5.3).

5.3.5 Biomass of beans

Fig. 5.4 shows the biomass of beans grown in soils amended with Se-enriched biomaterials, non-enriched biomaterials (the corresponding controls), and without any biomaterials (Blank). The biomass yield of beans was related to the duckweed application dose ($P < 0.05$). Specifically, no significant influence on the dry biomass weight was observed for the sandy soil amended with SeZnDW1 and control-SeZnDW1 in comparison with the blank, while the duckweed amendment SeZnDW5 significantly decreased the dry weight of each beans tissue by 51-85% and an even over 85% decrease was noted for the control-SeZnDW5 (Figure 5.4a). Likewise, for the loamy soil, the amendment SeZnDW1 significantly increased the beans biomass of each tissue (up to 159%), while an over 48 and 72% decrease was observed for the amendment of SeZnDW5 and control-SeZnDW5, respectively (Figure 5.4b). On the other hand, there was no remarkable difference between the blank and the different amounts of SeZn-enriched sludge applied (Figure 5.4c and 5.4d). It should be noted that the Se and Zn present in the biomaterials seem to counteract the negative effects of the biomaterials themselves on the growth of beans, which was reflected in the slightly higher biomass observed for the SeZn-enriched biomaterials amendment compared to that of the control-biomaterials amendment (Fig. 5.4).

The remarkable decrease of the beans biomass upon amendment of SeZnDW5 might be due to the high amount of duckweed (24g in 0.5kg soil) applied to the soil. Some phytotoxic substances such as low molecular weight organic acids, phenols and other allelochemicals may be released at the early stage of the fresh duckweed decomposition, which may inhibit the growth of the beans (Dinh et al., 2020; Jin et al., 2020). Besides, a high amount of duckweed applied into soils may result in relatively high C/N ratios and then promote the use of mineral nitrogen from the soil for microorganism activities, resulting in “competition for nitrogen” between the soil microorganisms and the crop (Jin et al., 2020; Witt et al., 2000), thus reducing the

beans plant biomass yields. Moreover, accumulation of a large amount of duckweed in the soil may hinder the seeds/roots of beans to completely contact the soil, and seriously affecting the growth (Jin et al., 2020).

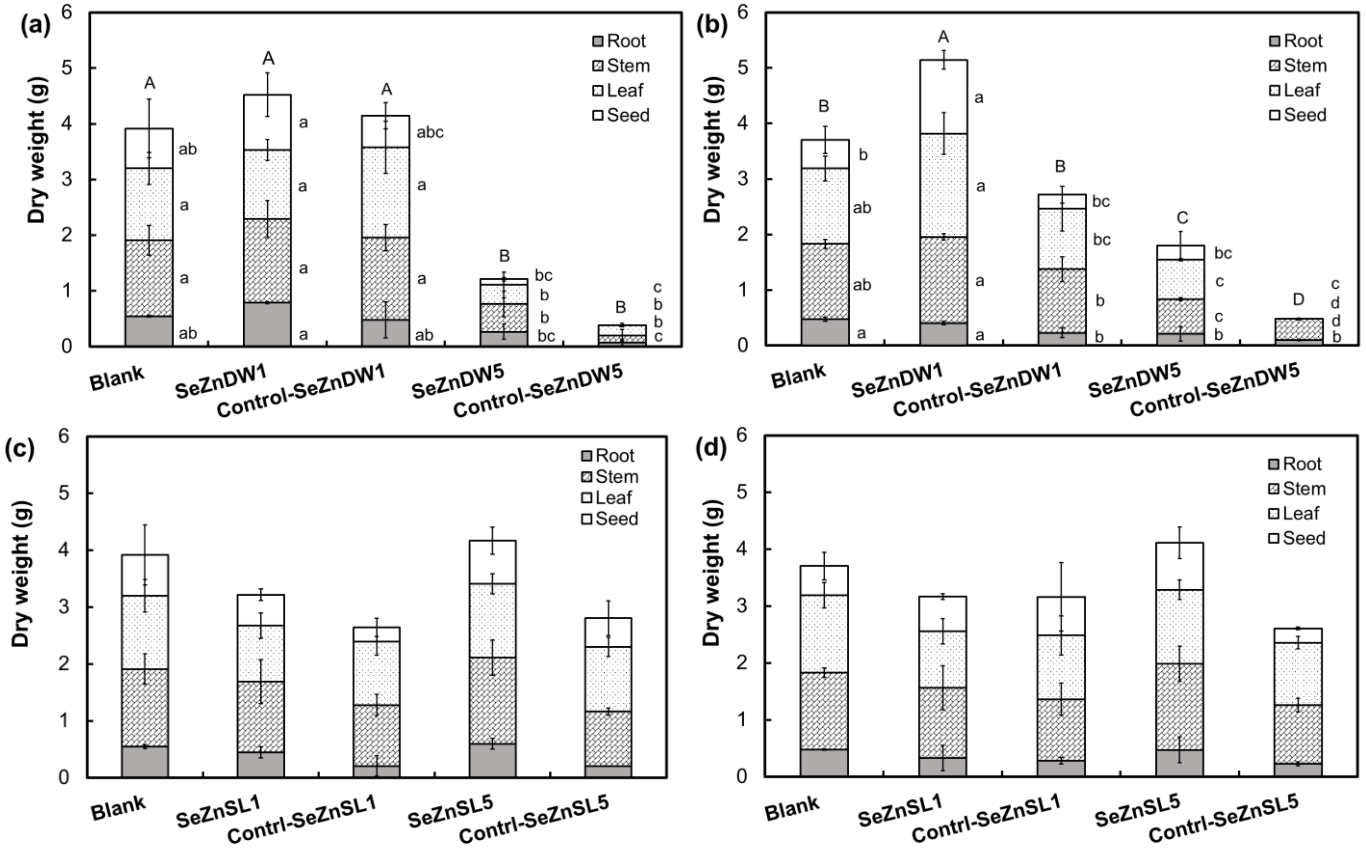

Figure 5.4. Dry biomass of the bean plant tissues grown on the sandy and loamy soils with SeZn-enriched biomaterials supplementation: (a) sandy soil with SeZn-enriched duckweed supplementation, (b) loamy soil with SeZn-enriched duckweed supplementation, (c) sandy soil with SeZn-enriched sludge supplementation, and (d) loamy soil with SeZn-enriched sludge supplementation. Values are mean ± standard deviation (n=3). Different lower-case letters indicate statistically significant differences between amendments within the same tissues according to Duncan's multiple comparison tests ($P < 0.05$). Different upper-case letters indicate the statistically significant differences in the total dry weight between amendments according to the Duncan's multiple comparison tests ($P < 0.05$).

5.3.6 Selenium content in the beans grown on soils amended with SeZn-enriched biomaterials

The amendment of SeZn-enriched duckweed and sludge remarkably increased the Se concentration in the different tissues of beans (seed, leaf, stem and root) in comparison with the blank (Fig. 5.5). More specifically, increasing the sludge amendment from SeZnSL1 to SeZnSL5 increased the Se concentration in the plant tissues by 3.1–4.3 times. However, the Se content in all tissues of the beans, except for the roots, decreased by 1.1–4.0 times when the SeZn-enriched duckweed amendment was increased from SeZnDW1 to SeZnDW5.

The Se concentration in all tissues of the beans amended with SeZnDW1 was 2.3–4.1 times higher than in those amended with SeZnSL1, except for in the stems (7–9 times) and similar values in the roots. However, the amendment of SeZnSL5 resulted in an approximately 1.6–3.0 times higher Se content in all beans tissues compared to the amendment of SeZnDW5. Selenium was mainly accumulated in the roots after being taken up by the bean plants, especially for the amendment of SeZnSL1 and SeZnSL5, while Se was more rapidly translocated from the roots to aboveground biomass when SeZnDW1 was amended.

Similarly, our previous work (chapter 4) and some other studies (Bañuelos et al., 2015; Bañuelos et al., 2016) also indicated that micronutrient (Se)-enriched supplements can successfully increase the Se content in crops and plants species after being applied into soils, such as the supplementation of the Se-enriched hyperaccumulator *Stanleya pinnata* biomass to sandy-loam soil. However, the higher Se content in the tissues of beans upon amendment of SeZnDW1 compared to SeZnDW5 was attributed to the negative effects of the high amount of duckweed (SeZnDW5) on the plant growth and nutrients accumulation, which was indicated by the remarkable decrease of beans biomass (Fig. 5.4). The different translocation rate of Se from the roots to aboveground biomass (stem, leaf, and seed) between the supplement of SeZn-enriched duckweed and sludge may be due to the different Se species dominating in the two biomaterials. Specifically, 92% Se(VI) being present in the SeZn-enriched duckweed (Fig. 5.1a) may result in a high amount of Se(VI) released in the soil pore water. Se(VI) is highly soluble and readily translocated from underground to aboveground plant parts after being taken up by plants (Peng et al., 2019). In contrast, the zerovalent form (72.9%) of Se

was predominant in the SeZn-enriched sludge, which may be released as such or converted into Se(IV) under oxic conditions and mainly accumulate in the roots.

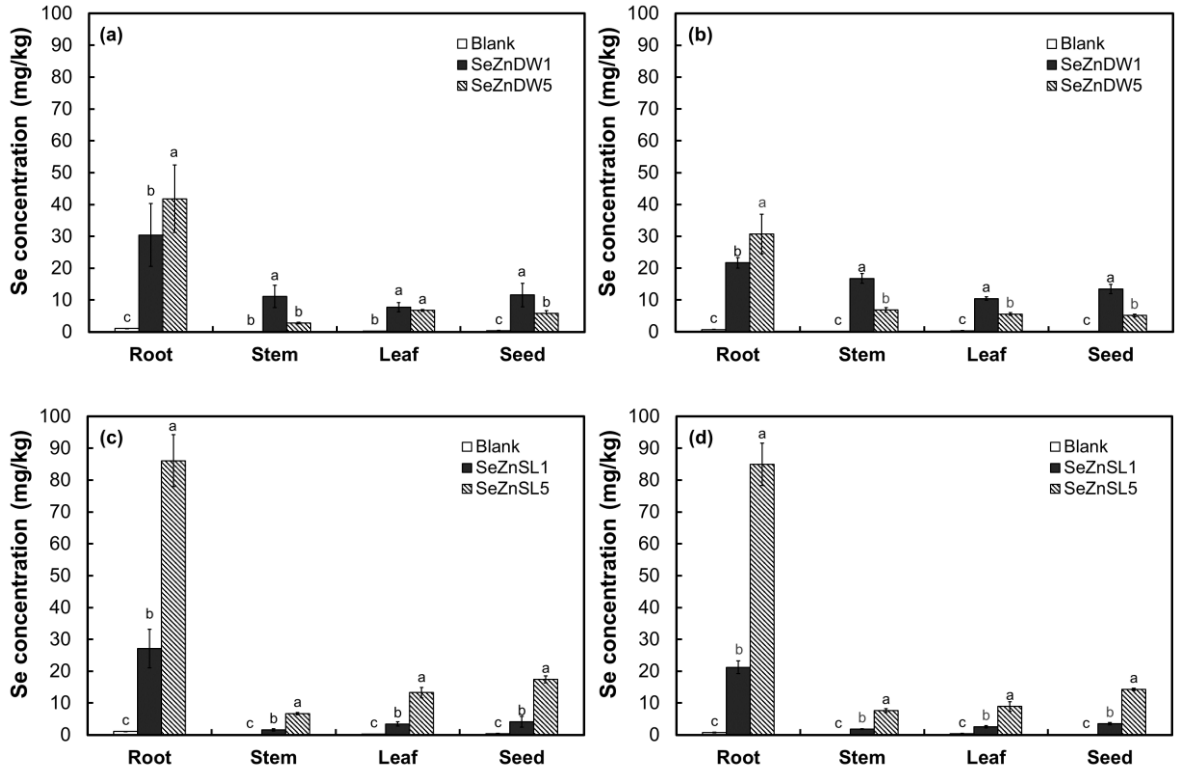

Figure 5.5 Se concentration in the different tissues of the harvested beans grown on sandy and loamy soils amended with SeZn-enriched biomaterials: (a) sandy soil with SeZn-enriched duckweed supplementation, (b) loamy soil with SeZn-enriched duckweed supplementation, (c) sandy soil with SeZn-enriched sludge supplementation, and (d) loamy soil with SeZn-enriched sludge supplementation. Values are mean ± standard deviation (n=3). Different letters indicate statistically significant differences between the different doses within the same tissues according to Duncan's multiple comparison tests ($P<0.05$).

Additionally, the Se concentration ranged from 5.1 to 13.5 mg/kg and 3.5 to 17 mg/kg in the seeds of beans with the supplementation of SeZn-enriched duckweed and sludge, respectively. In this regard, the US Food and Drug Administration recommends a daily dietary allowance (RDA) of 70 µg Se/day for human diets. If we assume that a daily serving size of the Se-enriched beans seeds is 96 g fresh weight (or 7.2 g dry weight due to its 92.5% water content calculated in this study), consumption of the highest Se containing bean seeds (13.5 and 17 mg Se/kg as observed in SeZnDW1

and SeZnSL5) would result in the ingestion of 97.2 and 122.4 µg Se. This means that the recommended daily Se requirements would be safely met and not substantially exceeded.

5.3.7 Zinc content in the beans grown on soils amended with SeZn-enriched biomaterials

Fig. 5.6 illustrates the Zn content in different tissues of the beans grown on sandy and loamy soil amended with SeZn-enriched duckweed (Fig. 5.6a and 5.6b) and sludge (Fig. 5.6c and 5.6d). An increasing Zn content in the different bean tissues was associated with the amendment of SeZn-enriched duckweed, while contrary results were observed with the amendment of SeZn-enriched sludge in all bean tissues except for the roots. Specifically, increasing the SeZn-enriched duckweed application from the blank to SeZnDW5 increased the Zn content in the roots stepwise from 168 to 780 mg/kg for the sandy soil and from 541 to 931 mg/kg for the loamy soil (Fig. 5.6a and 5.6b). On the other hand, the application of SeZn-enriched sludge declined the Zn concentrations by 20–34% in the stems, leaves and seeds. Besides, no significant difference of the Zn content in the roots between the bank and the amendment of SeZn-enriched sludge was observed (Fig. 5.6c and 5.6d).

Zn mainly accumulated in the roots after being taken up by the beans with the amendment of SeZn-enriched duckweed, as shown by the higher ratio of Zn content between the roots and aboveground tissues (3–11 times for the sandy soil, 5–13 times for the loamy soil). This indicates that Zn was not easily translocated in the plants, which is consistent with other studies (Fontes et al., 2014; Mao et al., 2014; Montalvo et al., 2016). For instance, Mao et al. (2020) found that Zn applied to the soil in the form of Zn sulfate at 232.7 kg/ha did not increase the Zn concentrations in the edible parts of wheat, maize and soybean, due to the limited transportation ability of Zn by the phloem to the grain/seed.

The decrease of the Zn content in the bean tissues with the supplementation of SeZn-enriched sludge could be attributed to the competition between Zn and other ions (e.g. Se) and the increase of soil pH. Specifically, the higher amount of Se in the soil pore water (released from the SeZn-enriched sludge) (Fig. 5.3) and in the plants (Fig. 5.5) may inhibit the uptake and translocation of Zn by beans. The antagonistic effect of Se

and Zn on plant uptake has also been observed in other plants, such as wheat, cabbage and potato (Mao et al., 2014; Singh & Singh, 1978). Besides, pH is the most important factor in determining Zn solubility (Montalvo et al., 2016). Increasing pH values could increase the complexation of Zn with dissolved organic matter (e.g. humic and fulvic acids), resulting in the lower bioavailability of Zn (Dinh et al., 2020). In this study, the pH of the soil pore water was remarkably increased by the supplementation of SeZn-enriched biomaterials (Table 5.1), which may therefore partially explain the lower Zn content in the bean tissues.

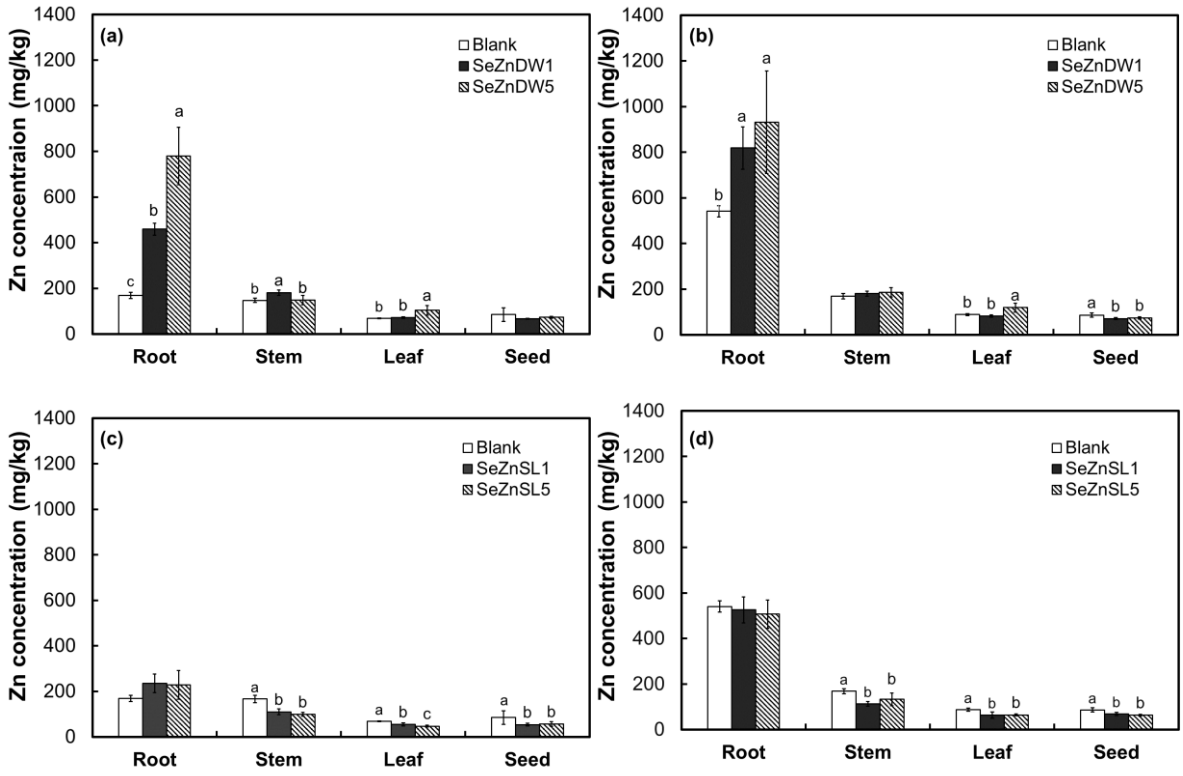

Figure 5.6. Zn concentration in the different tissues of beans grown on sandy and loamy soils amended with SeZn-enriched biomaterials: (a) sandy soil with SeZn-enriched duckweed supplementation, (b) loamy soil with SeZn-enriched duckweed supplementation, (c) sandy soil with SeZn-enriched sludge supplementation, and (d) loamy soil with SeZn-enriched sludge supplementation. Values are mean ± standard deviation (n=3). Different letters indicate the statistically significant differences among amendments within the same tissues according to Duncan's multiple comparison tests ($P<0.05$).

The supplementation of the SeZn-enriched biomaterials did not successfully improve the Zn content in the seeds of beans (Fig. 5.6), and the biofortification purpose of Zn is thus not achieved. This could be attributed to the lower Zn translocation rate between plant roots and seeds, and the application of a too low Zn dose, particularly for the SeZn-enriched sludge application. As aforementioned, the lower translocation rate of Zn in plants was reflected in the remarkably higher Zn content in the bean roots after SeZn-enriched duckweed application, with no difference in aboveground biomass among all treatments (Fig. 5.6a and 5.6b). On the other hand, the Zn content in the SeZn-enriched sludge was only 563 mg/kg (vs 2289 mg/kg in SeZn-enriched duckweed), and the applied amounts of SeZnSL1 and SeZnSL5 were 1.7 and 8.7 g/pot, which may not be able to provide sufficient Zn for plant uptake. This suggests that the Zn content in the sludge should be reconsidered during its production.

5.3.8 Se species in the beans

The seeds of beans grown on the sandy soil without (blank) and with the amendment of SeZnDW1 and SeZnSL1 were selected for the measurement of Se speciation (Fig. 5.7 and Table 5.2). The recovery of Se in all seeds ranged from 82 to 99% of the total Se content after protease hydrolysis.

Organic Se (SeCys2, SeMetCys and SeMet) was the main Se species in all seed samples. SeMet accounted for the highest percentage of the total Se in the seeds for the blank (77.2%), SeZnDW1 (68.5%) and SeZnSL1 (82.3%). Besides, the percentage of inorganic Se species in the seeds with the SeZnDW1 amendment and blank was much higher compared to that of the SeZnSL1 amendment. Particularly for Se(VI), 13.7, 10.6, and 2.4% of the total Se was detected in the seeds of the blank, SeZnDW1 and SeZnSL1 amendment, respectively.

Organic Se species are beneficial for dietary intake by humans and animals, as they are more bioavailable and less toxic (Gómez-Jacinto et al., 2020) compared to inorganic Se species. SeMet and SeCys can be incorporated at the active sites of a wide range of selenoproteins involved in major metabolic pathways, such as thyroid hormone metabolism, antioxidant defense, and immune function (Malagoli et al., 2015). Therefore, the high percentage of organic Se species present in the seeds of the beans indicates that Se in the SeZn-enriched duckweed and sludge could be taken up and

transformed into more valuable Se species. These results demonstrated the potential of SeZn-enriched biomaterials produced from wastewater as Se biofertilizers.

Table 5.2. Percentage of Se species in the seeds of beans grown on the sandy soil amended with SeZnDW1 and SeZnSL1 and without amendment (Blank) relative to total Se in bean seeds.

	Total Se	$SeCys_2$	SeMetCys	SeMet	Se(IV)	Se(VI)	Se species recovery
	(mg/kg)	(%)					
Blank	0.1	3.2	3.5	77.2	2.4	13.7	99%
SeZnDW1	11.6	1.1	2.1	68.5	0.3	10.6	82%
SeZnSL1	4.1	1.4	4.5	82.3	0.4	2.4	91%

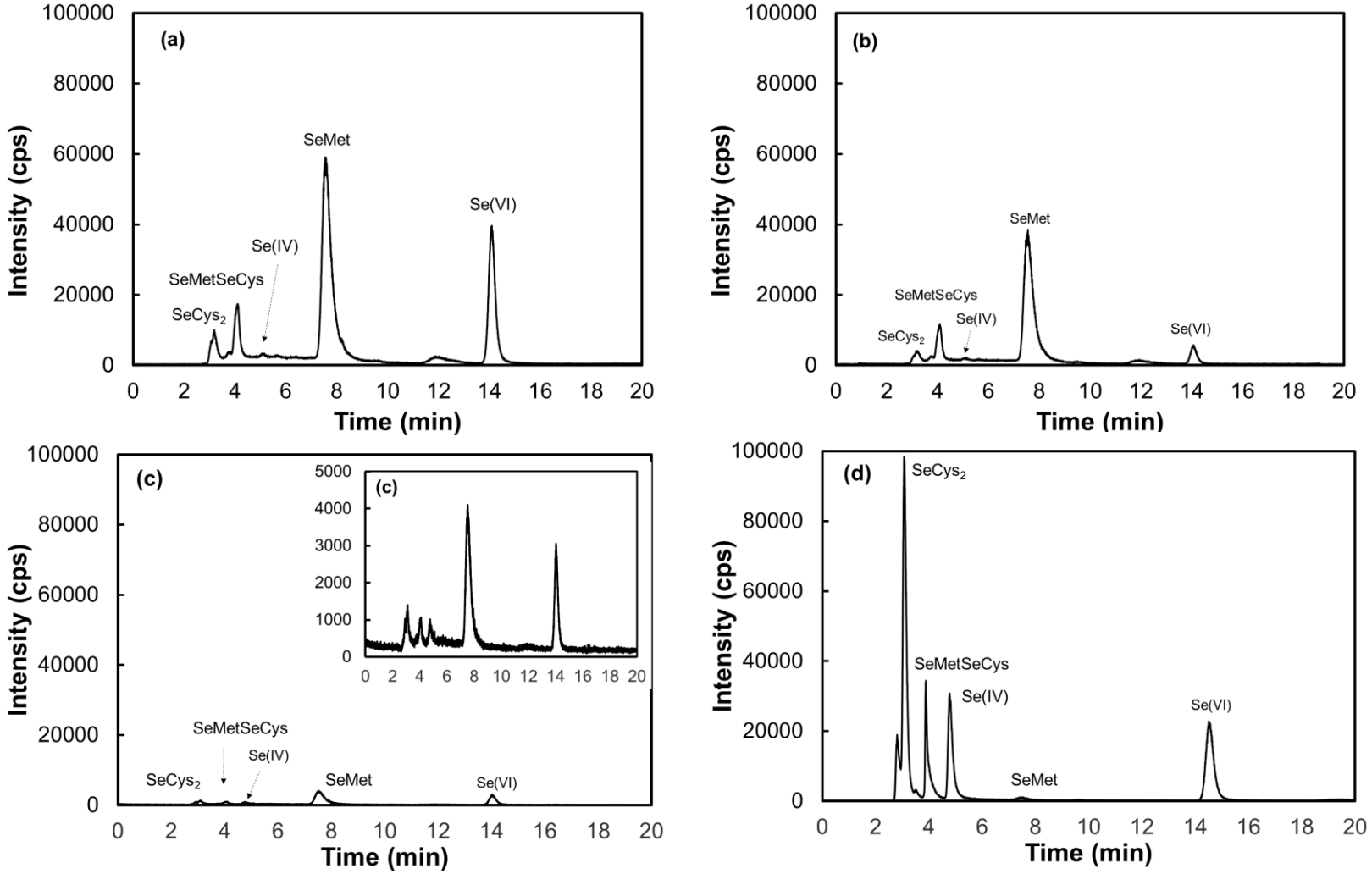

Figure 5.7. Se speciation in harvested seeds of beans after 42 days of growth on sandy soil amended with (a) SeZnDW1 and (b) SeZnSL1 and the (c) Blank soil (higher magnification in insert) together with (d) Se standard solution of 10 µg/L of each Se species.

5.4 Conclusions

The potential of SeZn-enriched biomaterials produced from simulated wastewater as Se biofertilizers has been proven, as illustrated by the remarkably higher Se content in the soil pore water and plant tissues upon amendment of soil with these biomaterials. The highest dose of SeZn-enriched duckweed (SeZnDW5) inhibited the plant growth and noticeably raised the pH and TOC content of soils, confirming that appropriate amounts of biomaterials should be applied. SeZn-enriched sludge is considered as the preferred Se biofertilizer in comparison with SeZn-enriched duckweed, as the Se slowly released from the SeZn-enriched sludge remained stable in the soil pore water, which could slowly provide sufficient Se for plant uptake and transformation to selenoamino acids (SeMet). On the other hand, the agronomic application of the SeZn-enriched duckweed and sludge may not be an effective alternative for Zn biofortification, as reflected by the lower Zn content in the bean seeds. This should be optimized by increasing Zn content in the SeZn-enriched biomaterials.

Chapter 6 Production of Se-enriched microalgae as potential feed supplement in high rate algae ponds treating domestic wastewater

This chapter was redrafted after:

Li, J., Otero-Gonzalez, L., Michiels, J., Lens, P.N.L., Du Laing, G., Ferrer, I., 2021. Production of Se-enriched microalgae as potential feed supplement in high rate algae ponds treating domestic wastewater. ACS Sustainable Chemistry & Enginnering (Under Review).

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 HRAPs were operated for 3 months under two hydraulic retention times (8 and 4 days) with corresponding average organic loading rates of 66 and 127 mg COD/L·day, respectively. The HRAP-Se had a selenium loading rate of 6.28 µg Se/L·day, while the HRAP without Se spiking served as control (HRAP-C). The wastewater treatment efficiency of the HRAPs, the Se content and speciation, in vitro digestibility, nutritional value, and pathogen load of the microalgae grown in the HRAP were evaluated. The HRAP-Se had an average Se, NH_4^+-N, total phosphorus, total COD, total carbon removal efficiency of, respectively, 43%, 93%, 77%, 70% and 67%. Inorganic Se taken up by the microalgae was mainly (91%) transformed into valuable selenomethionine (SeMet), and 49–63% of Se in the Se-enriched microalgae was bioaccessible for animals through in vitro digestibility tests. Besides, the crude protein content (around 48%) of the microalgae was higher than that of conventional plant-based protein sources in feed (soybeans and soybean meals), whereas the essential amino acid content of the microalgae was comparable to that of soybeans. Fatty acid profile analysis demonstrated that Se may induce the production of the polyunsaturated fatty acids omega-3 and omega-6 in microalgae, particularly for eicosapentaenoic (EPA). Microbiological analysis indicated that downstream drying processes of microalgae could avoid pathogen contamination. Although more research is still further needed to confirm the results, this study showed how that the production of Se-enriched microalgae in HRAPs may offer a promising alternative for upgrading of low-value recovered resources into high-value feed supplements, supporting the drive to a circular economy.

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

6.1 Introduction

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 (Arashiro et al., 2020; Gan et al., 2019). Furthermore, microalgae are a potential source for the production of protein-rich biomass and numerous other high-value compounds, e.g. fatty acids, pigments and vitamins (Markou et al., 2018). Microalgae based products have found their way into the market as alternative micronutrient-rich food/feed supplements and protein sources replacing animal proteins. The cultivation of microalgae on wastewater with nitrogen, phosphorus and organic matter removal does not only assist with the treatment of wastewater, but also significantly reduces the cost and carbon footprint of conventional microalgae production that does not use wastewater as a growth medium, meanwhile converting low-value resources in wastewater into high value-added bioproducts (Borowitzka & Moheimani, 2013; Silambarasan et al., 2021).

Furthermore, Se supplementation of feed and food to overcome the Se deficiency received much attention in recent years. Microalgae may also have the ability to take up inorganic Se and incorporate it into amino acids forming selenoamino acids, such as selenomethionine (SeMet) and Se-cystine ($SeCys_2$), which are beneficial for animal and human health (Umysova et al., 2009; Winkel et al., 2015). In this context, cultivation of microalgae in Se-containing wastewater could generate not only high-value microalgae biomass (e.g. protein, fatty acids, pigments and vitamins) but also high Se enriched biomass, which may be reused for Se deficiency. The Se source in wastewater could be Se-rich wastewaters, but if too little would be present in those wastewaters (e.g., domestic wastewater), Se could also be added from an external source to produce a Se-enriched product without the need to apply additional macronutrients for microalgae growth. Accordingly, a higher-value 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 with 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-enriched microalgae, and (3) assess the potential use of upgraded Se-enriched microalgae as feed supplement by examining the Se content and speciation, digestibility, biochemical properties and nutritional profile.

6.2 Materials and methods

6.2.1 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. 6.1) 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 (10 L) followed the two HRAPs to separate the effluent and biomass. The scheme of the HRAPs is shown in Fig. 6.1.

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. 6.2 and Table 6.S1 in supplementary information.

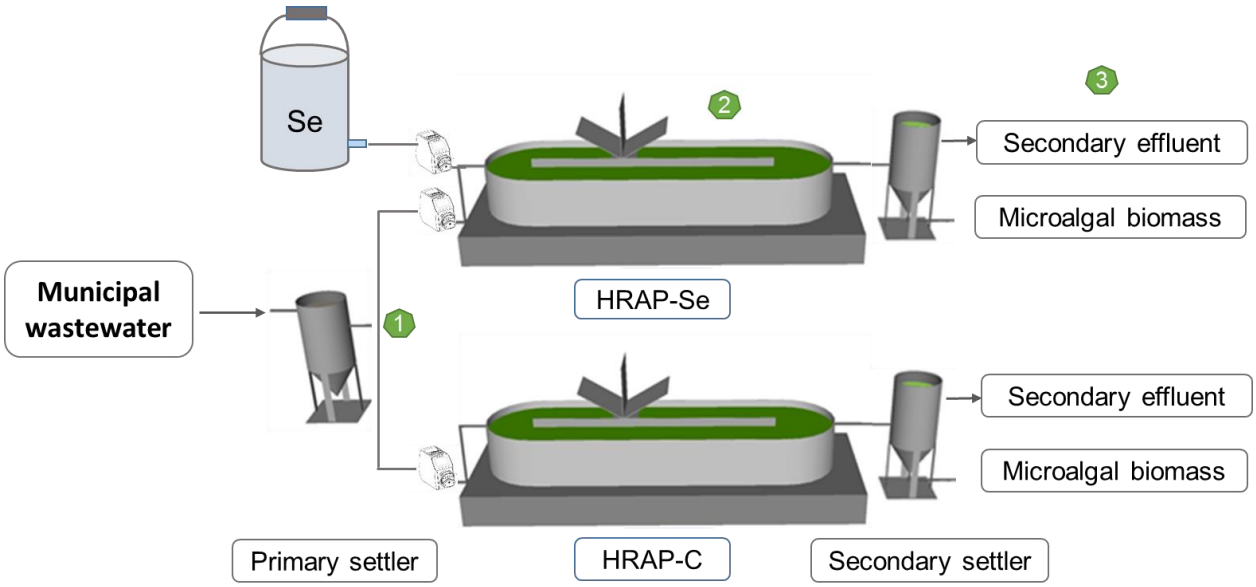

Figure 6.1. Scheme of the HRAPs treating domestic wastewater. HRAP-Se is the line with Se spiking and HRAP-C is the line without Se spiking, which served as control. Sampling points are 1: primary effluent (also called influent of the HRAPs), 2: mixed liquor of the HRAPs, 3: secondary effluent.

6.2.2 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.

6.2.3 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, which were 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, and the influent flow rates were twice the previously mentioned. The flow rates of Se spiking and 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.

6.2.4 Wastewater characterization in HRAPs systems

The wastewater treatment performance was monitored for 3 months. Samples from the influent, effluent and mixed liquor of the two HRAPs (Fig. 6.1) 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 by a 0.45-µm syringe PVDF membrane filter.

6.2.5 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 the subsequent analysis and experiments.

6.2.5.1 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. Se 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 samples were 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.

6.2.5.2 Macromolecular characterization and protein extraction by different cell disruption methods

Microalgae macromolecular characterization (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-thaw at -80 °C and 4 °C with 5 cycles; (b) combination of freeze-thaw 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 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 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 a dark place. A spectrophotometer (Spectronic Genesys 8, Helsingborg, Sweden) was used to measure the protein content at the absorbance of 750 nm. A calibration curve was prepared using bovine serum albumin (BSA).

6.2.5.3 Fatty acid and amino acid profiles analysis

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

6.2.6 Pathogenic bacterial content

Pathogen loads (aerobic bacteria, coliforms, *E. coli*, *Listeria* and *Salmonella*) of fresh, oven-dried (at 70 ºC until constant weight) and freeze-dried microalgae were evaluated according to the method of Montville and Matthews (2008) with three replicates. For fresh microalgae analysis, the biomass was collected, rinsed with DI water, and analyzed immediately

6.2.7 Statistical analysis

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

6.3 Results and discussion

6.3.1 Microalgae growth, Se accumulation and Se removal in batch experiments

Fig. 6.2 shows the turbidity of the microalgae suspension when exposed to different Se concentrations (0–500 µg Se/L). The highest turbidity in both selenite (Se(IV)) (885 NTU) and selenate (Se(VI)) (1614 NTU) treatments was observed at 50 µg/L of Se exposure after 7 days of cultivation, being significantly ($p<0.05$) different from the control, which demonstrates that low Se application may stimulate microalgae growth. Fig. 6.2 further demonstrates that the turbidity significantly increased with incubation time, and a similar turbidity value was observed for the control treatments and the 500 µg Se/L selenite and selenate treatments, indicating that microalgae growing on domestic wastewater treatment could tolerate such high concentrations of Se.

Similarly, Li et al. (2003) reported that sodium selenite has either stimulating (0.5 mg Se /L) or toxic (500 mg Se/L) effects on *Spirulina platensis* growing in Zarrouk medium. Reunova et al. (2007) reported positive impacts of selenite on the unicellular alga *Dunaliella salina* (e.g., stimulation of cell growth) after exposure to 0.01 and 0.5 mg/L of Se dosed as sodium selenite in nutrient medium prepared in 32‰ seawater. Conversely, the inhibition of cell growth together with an increasing number of destroyed cells and cells with damaged organoids were observed after exposure to Se concentrations higher than 1.0 mg/L. 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 (Schiavon et al., 2017b; Sun et al., 2014), and eventually promoting microalgae growth.

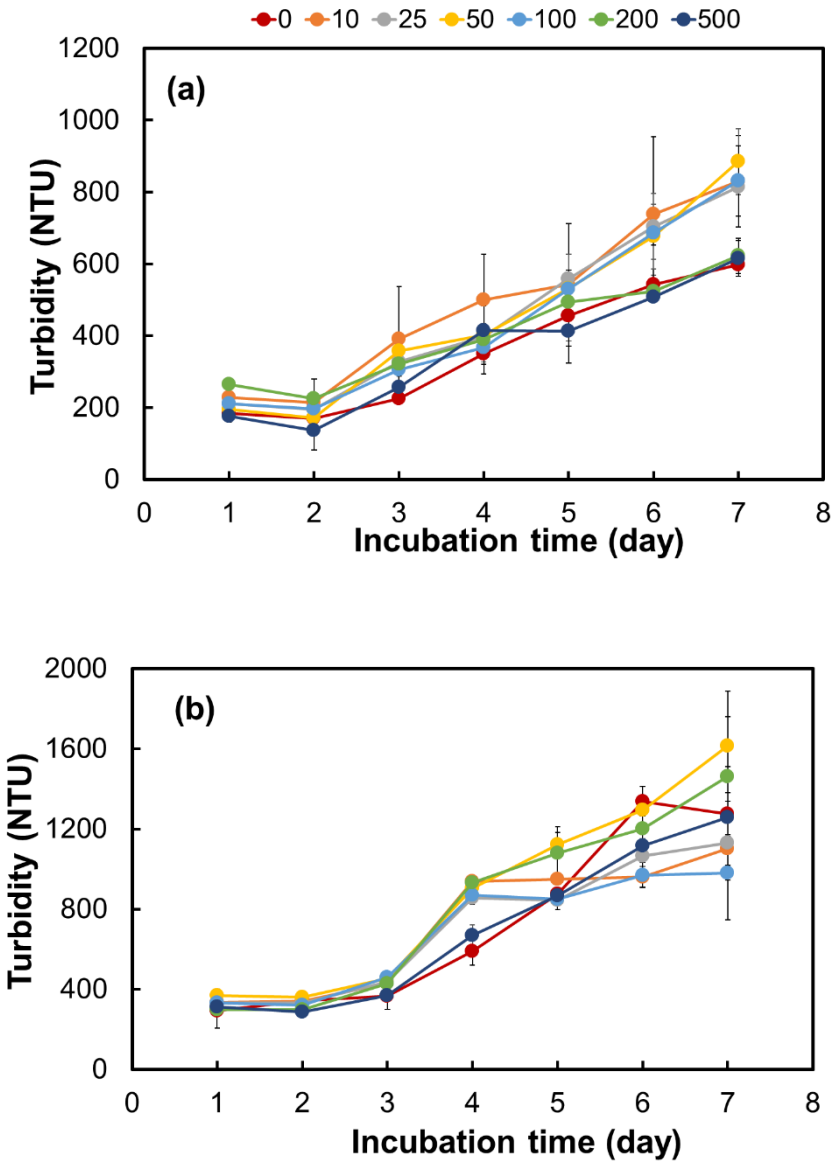

Figure 6.2. Biomass growth, measured as turbidity (NTU), during batch incubation in domestic wastewater supplemented with varying Se concentrations (µg/L), (a) selenite and (b) selenate. Values are mean ± standard deviation (n=3).

The Se concentration in the microalgae differed significantly depending on the chemical form and concentration of the applied Se (Fig. 6.3). 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. 6.3). 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. 6.3).

A higher Se accumulation, when exposed to Se(IV) compared to Se(VI), has also been observed in other algae species. Vriens et al. (2016) reported 10 times more accumulation of Se(IV) than Se(VI) by the microalga *Chlamydomonas reinhardtii* when grown in 100 µM of Se (equivalent to 8.0 mg/L) nutrient growth medium for 24 h. The wild type of microalgae *Scenedesmus quadricauda* took up 2 times more Se(IV) than Se(VI) after exposure to 50 mg/L Se in mineral medium (Vitova et al., 2011). However, some other reports indicated a reversed result, for instance, Simmons and Wallschager (2011) found that *Chlorella vulgaris* had around 5-fold preference for Se(VI) uptake over Se(IV) upon 10 µg/L of Se exposure in 10% Bold's basal medium. These discrepancies are likely due to the different algal genus and species (Schiavon et al., 2017a; Simmons & Wallschlager, 2011).

In this study, 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 (de Oliveira et al., 2017; Li et al., 2020b; Schiavon et al., 2017a). 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 (Arvy, 1993; Li et al., 2008). 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., 2017a; Van Hoewyk, 2013).

The efficiency of Se removal by the microalgae is presented in Fig. 6.S1 of the supplementary information (SI). A decreasing trend was observed in the Se removal efficiency with the increase of the 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. 6.3). 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.

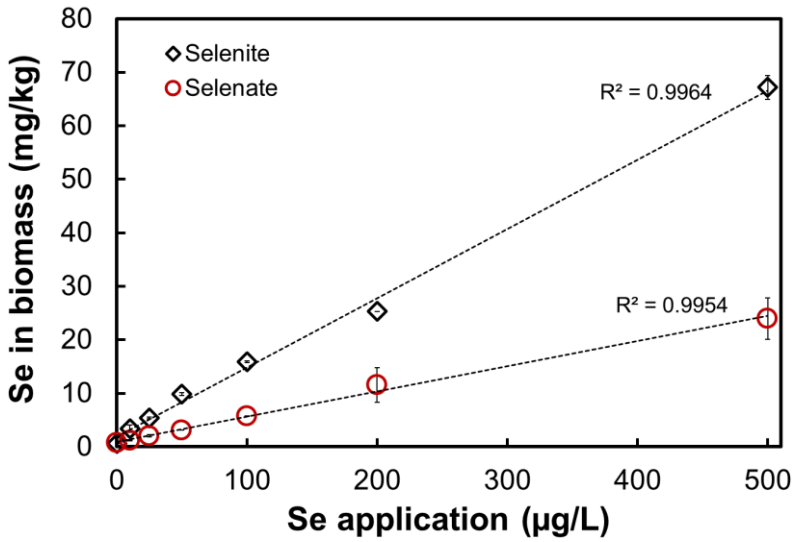

Figure 6.3. Se concentration in the microalgal biomass grown in wastewater with different selenite and selenate concentrations. Values are mean ± standard deviation (n=3).

6.3.2 Wastewater treatment efficiency and Se removal in HRAPs

The temporal variation and average values of the main parameters in HRAP-Se and HRAP-C over a period of 3 months are shown in Fig. 6.4 and Table 6.S1. A summary of the average removal efficiencies of the main water quality parameters is calculated and presented in Table S2. Likewise, the variation of Se content in the influent and effluent of HRAP-Se over the monitoring period is shown in Fig. 6.5. 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. 6.4 and Table S1-2). 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_{tol} 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), which has also been observed in a previous study using the same HRAPs (Arashiro et al., 2019). 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; Gutierrez et al., 2016).

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. 6.5). 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 under high toxic Se exposure (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. (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.

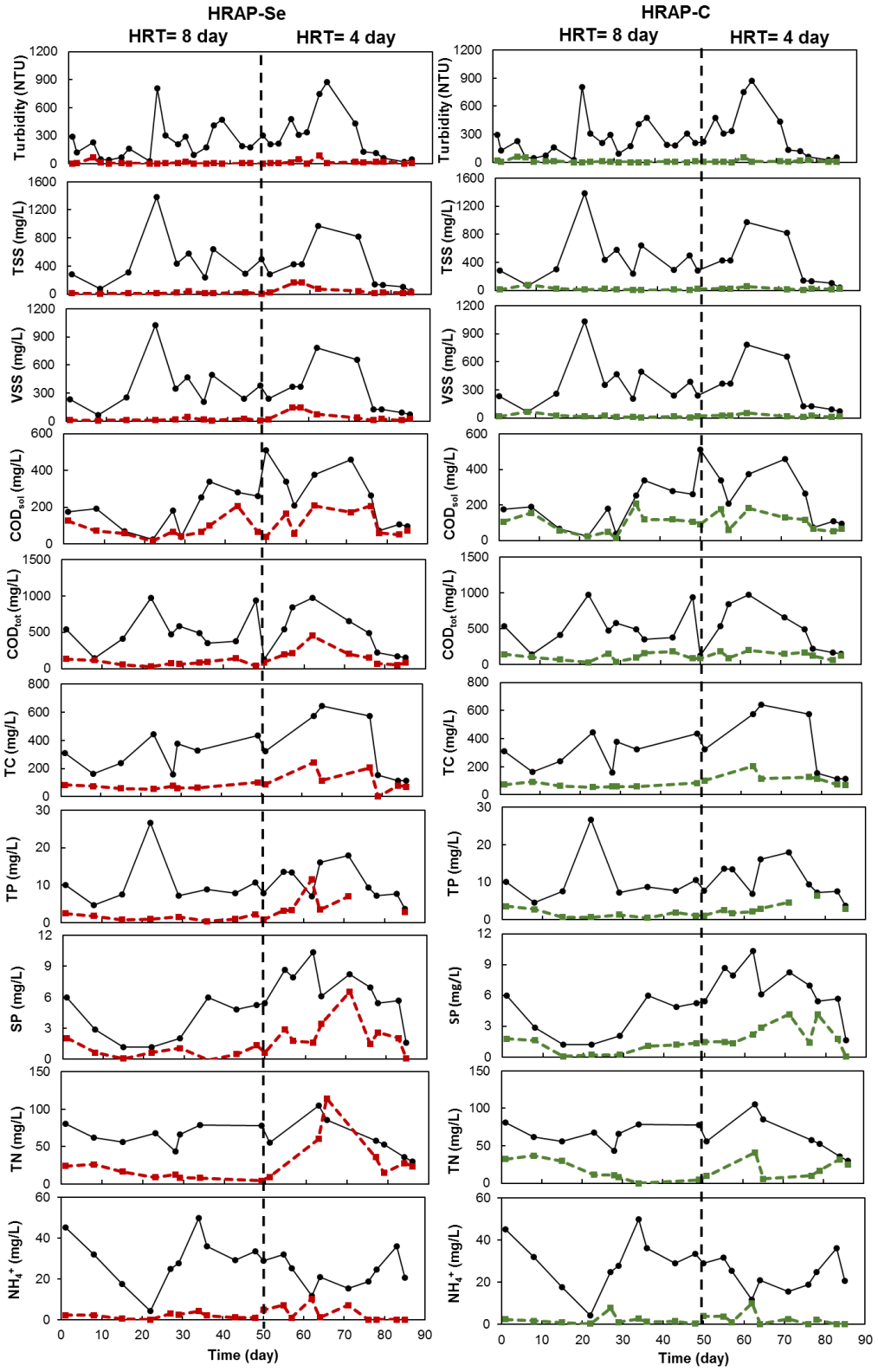

Figure 6.4. Influent (●) and effluent (■) concentration of turbidity, total suspended solids (TSS), volatile suspended solids (VSS), total carbon (TC), total and soluble chemical oxygen demand (COD_{tot} and COD_{sol}), total and soluble P (SP and TP) and NH_4^+-N monitored in the HRAP-Se (with Se spiking, left) and HRAP-C (without Se spiking, right) systems over the experimental period.

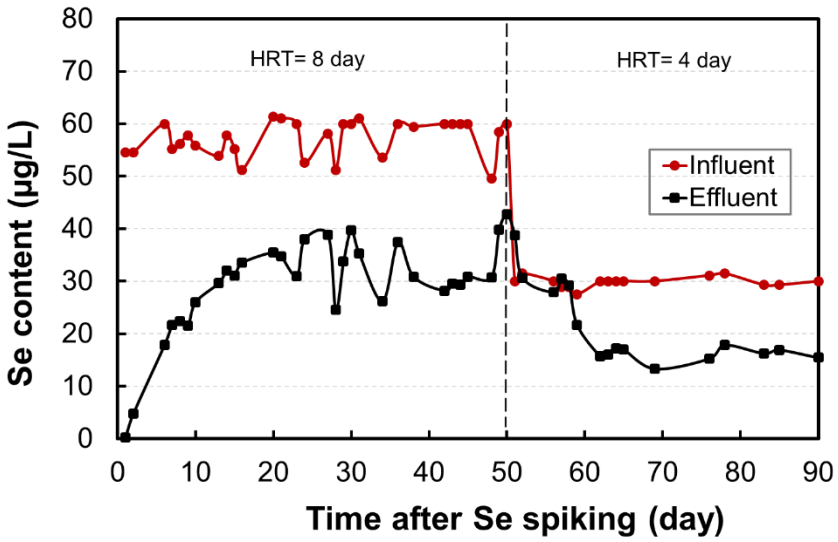

Figure 6.5. Influent and effluent concentrations of total Se in the HRAP-Se with continuous selenite spiking during the experimental period. HRT was reduced from 8 to 4 days after 50 days.

6.3.3 Nutritional value of microalgae grown in HRAPs

6.3.3.1 Selenium species in Se-enriched microalgae

Fig. 6.6 shows the chromatogram of Se species in microalgae grown in the HRAP-Se collected at the operational days 43–50. 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 some 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 selenate. Vu et al. (2019) demonstrated that SeMet and SeMetSeCys were the predominated 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.

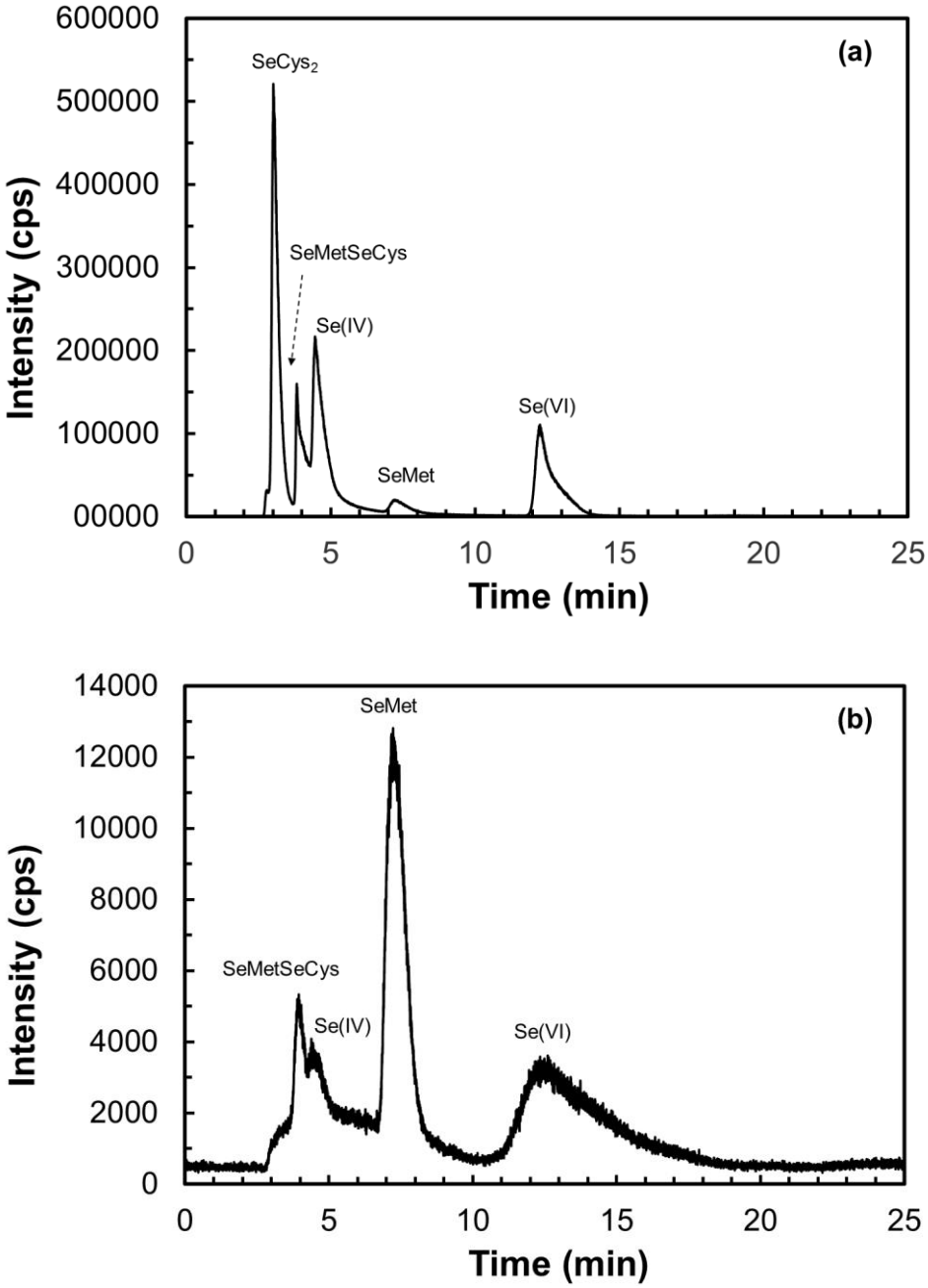

Figure 6.6. Chromatograms of Se speciation of (a) Se standard solution containing 100 µg/L of each Se species and (b) an extract of Se-enriched microalgae (diluted 20 times) grown in the HRAP-Se at the operational week 7.

SeMet, a type of selenoamino acid, is one of the major nutritional source of Se for higher animals and humans, as these are unable to synthesize SeMet in their organs (Schrauzer, 2003). Importantly, SeMet is more bioavailable to provide higher Se concentrations in 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). Our results indicate that microalgae

cells are capable of accumulating and transforming less-valuable inorganic Se into more-valuable selenoamino acids efficiently. Accordingly, the microalgae enriched with SeMet produced in this study might be a potential and preferable alternative Se source for feed supplementation without utilizing other external nutrients for microalgae growth in domestic wastewater.

6.3.3.2 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. 6.7). 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 (control) 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)*, which is significantly higher than that in Se-enriched yeast (~21%) and commercial Se-supplement (~32%) (Vu et al., 2019), while it should be noted that a relatively higher Se bioaccessibility in the Se-supplement SelenoPrecise (Se-enriched yeast, ~70%) was found by Lavu et al. (2016). The large difference in Se bioaccessibility of Se supplements between these two studies is mainly due to the different calculation methods. Vu et al. (2019) calculated the Se bioaccessibility based on the total Se in the biomass (the amount of Se dissolved in the gastrointestinal extract divided by the total amount of Se in the biomass), while Lavu et al. (2016)

determined this according to the Se concentration in the suspension (Se in the gastrointestinal extract divided by the amount of Se in the suspension), thus leading to a higher Se bioaccessibility. Moreover, a Se bioaccessibility of 81% was also previously observed in the Se-enriched microalga *Chlorella sorokiniana* (Gómez-Jacinto et al., 2020), which is higher than that in our study. This discrepancy might be due to the difference in microalgae species, Se concentrations or species in the growth medium, and the digestion methods (e.g. different amount and type of enzymes). For instance, more enzymes and chemicals were included in the intestinal juice by Gómez-Jacinto et al. (2020) to simulate human gastrointestinal digestion, such as amylase and bile salts, compared to those in our study. Bile salts can assist the digestion of fat, which may result in some undissolved Se (e.g., hydrophobic lipid-bound Se) in microalgae dissolved in the gastrointestinal extract, leading to a higher Se bioaccessibility.

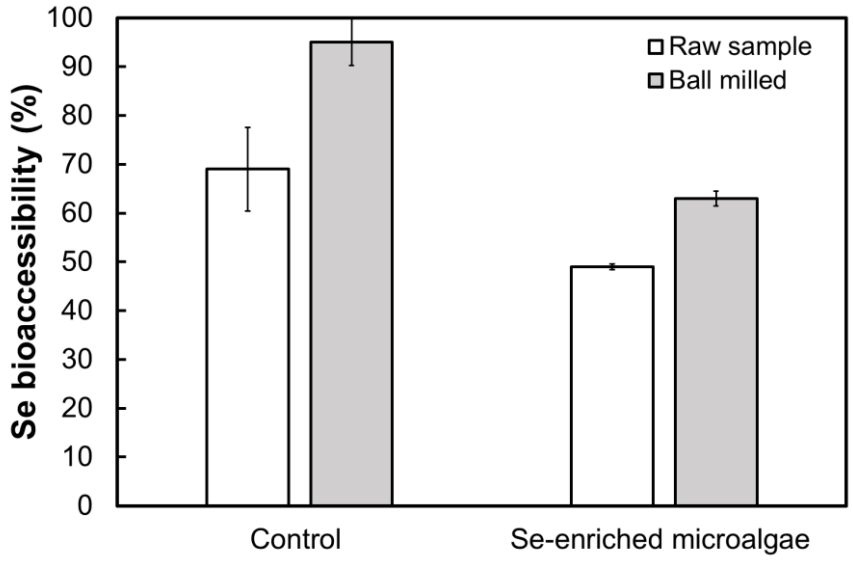

Figure 6.7. Bioaccessibility of Se in the raw and ball-milled microalgae grown in both the HRAP-C (Control) and HRAP-Se (Se-enriched microalgae). Values are mean ± standard deviation (n=3).

6.3.3.3 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 6.1), which is within the range reported in the literature for microalgae species (Arashiro et al., 2019; Rasouli et al., 2018). 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 protein for pigs around the world (Moheimani et al., 2018). Additionally, a slightly higher content of carbohydrates and lipids was observed for the biomass grown in the HRAP-Se than that grown in the HRAP-C. Specifically, the biomass grown in the HRAP-C and HRAP-Se was composed of 21% and 32% carbohydrates, and 19% and 21% lipids, respectively, indicating that Se may have the potential to stimulate the biosynthesis process in microalgae.

Table 6.1. 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.

For protein extraction, the protein content obtained by SDS extraction was much higher than the protein content obtained by DI water extraction (Table 6.1). The lowest protein content (12–14% for SDS extraction) was observed for the biomass after cell disruption by freeze-thawing, while ball and bead milling of the biomass favored the highest release of protein, i.e. 46–48% upon SDS extraction. This result indicates that ball and bead milling disruption in combination with SDS extraction results is the most efficient solubilization and quantification of proteins in microalgae by the Lowry method, which could provide a reference for protein extraction of microalgae.

6.3.3.4 Amino acids in the microalgae

Table 6.2 compares the amino acid content of microalgae grown in both HRAPs with that of soybeans and soybean meal. The amino acid content of the microalgae grown in both HRAPs was close to that of soybeans (with the exception of glutamic acid), while it was slightly lower than that in soybean meal, except for glycine, threonine, and alanine contents which were higher in the microalgae (Table 6.2). This result showed that microalgae could be s source of some essential amino acids for 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 to the high 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 regulated that the maximum Se total content in the complete feed is 0.5 mg Se/kg (EFSA, 2016). Besides, further study should quantify the digestibility of amino acids in microalgae, as it is also an important factor affecting amino acids utilization efficiency.

Table 6.2. Amino acid contents of microalgae grown in HRAPs at day 50 compared with soybeans and soybean meal (SBM) for pigs. Values are mean ± 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	--	--

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

N.D.= not detected

-- = no data shown

6.3.3.5 Fatty acids in the microalgae

The composition and content of total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in the microalgae are shown in Table 6.3. The detailed composition and content is listed in Table 6.S3. 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 6.3). 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. Although further research is still needed to confirm these results because of the fewer sample points we collected, this may to some extent indicate that Se has the potential of contributing to the synthesis of PUFAs and the production of value-added biomass, as PUFAs, especially ω3 and ω6, are considered essential fatty acids and 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).

Among the different PUFAs ω3 present in algae, eicosapentaenoic (EPA, C20:5) has the most important nutritional and health value (Becker, 2013). EPA supplementation can be co-therapeutic (Doughman et al., 2007). In this study, EPA was dominant in the biomass grown in both the HRAP-C and HRAP-Se, accounting for 13.2 and 24.7% of the fatty acids, respectively (Table 6.S3). Interestingly, 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). This is favorable in animal and human nutrition. HRAPs may thus contribute to offering a promising alternative source of the valuable EPA in PUFA ω3.

Table 6.3. 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).

	HRAP-C		HRAP-Se	
	[% relative fat]	[mg/100 g]	[% relative fat]	[mg/100 g]
Total SFA	32.1 ± 0.1	1359 ± 7.9	25.0 ± 0.1	2215 ± 4.4
Total MUFA	10.6 ± 0.1	450 ± 4.1	8.17 ± 0.0	773 ± 3.1
Total PUFA ω-6	10.0 ± 0.0	426 ± 2.8	11.8 ± 0.0	1048 ± 10.0
Total PUFA ω-3	17.1 ± 0.1	726 ± 4.1	26.1 ± 0.1	2310 ± 12.0

6.3.4 Pathogenic bacterial content

Microalgae grown on wastewater can harbor a risk of pathogen transfer when they are consumed as feed. Therefore, for the effective protection of human and animal health, microbiological regulations must be met (Montville & Matthews, 2008). Table 6.4 shows the content of selected manure-borne bacteria of fresh and dried HRAP grown biomass. A concentration of over 10^7 CFU/g of aerobic bacteria was found in the fresh microalgae samples, however, these were absent in the dried biomass (both oven-dried and freeze-dried). The coliform, *E. coli* (at a concentration > 10^5 CFU/g) and *Salmonella* were detected in the fresh microalgae samples grown in the HRAP-C, while the population of *Listeria* was below 25 CFU/g (tolerance level < 100 CFU/g (Montville & Matthews, 2008) in all types of samples. Table 6.4 indicates 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. This is similar to the finding of Moheimani et al. (2018), who studied the pathogen loads of dried microalgae cultivated on anaerobically digested piggery effluent. However, further risk assessment on the implementation of Se-enriched microalgae grown in domestic wastewater as feed supplement is still required, such as *in vivo* studies and quantification of other safety parameters (e.g., residues of mycotoxins, antibiotics, and nucleic acids).

Table 6.4. Pathogen loads on fresh (CFU/g wet weight) and dry (CFU/g dry weight) microalgae biomass grown in the control (HRAP-C) and Se spiked (HRAP-Se) microalgae ponds treating domestic wastewater.

		Aerobic bacteria	Coliforms	*E. coli*	*Listeria*	*Salmonella*
		[CFU/g]				
Fresh	HRAP-Se	$2.35*10^7$	$2.60*10^6$	N.D.	< 25	Absence
biomass	HRAP-C	$9.93*10^7$	$4.52*10^7$	$2*10^5$	< 25	Presence
Oven-dry	HRAP-Se	N.D.	N.D.	N.D.	< 25	Absence
biomass	HRAP-C	N.D.	N.D.	N.D.	< 25	Absence
Freeze-dry	HRAP-Se	N.D.	N.D.	N.D.	< 25	Absence
biomass	HRAP-C	N.D.	N.D.	N.D.	< 25	Absence

N.D.= not detected

6.4 Conclusions

This study investigated the potential of microalgae to remove selenite and selenate from domestic wastewater and to recover and upgrade low-value resources into high-value products in HRAPs systems. This study clearly highlighted that HRAPs-grown microalgae are good candidates to upgrade nutrients and carbon dioxide into Se-enriched microalgae biomass that can be used as valuable feed supplements. The main findings are:

(1) Microalgae mainly accumulate organic SeMet (91%) in their cells after taking up inorganic Se from the solution, indicating the ability of microalgae to transform inorganic Se into selenoamino acids.

(2) Se release upon gastrointestinal digestion of microalgae was quite high and additional processing of the microalgae through ball-milling treatment further improved the digestibility significantly.

(3) Se may potentially assist the production of lipids and carbohydrates by microalgae, but further research with more analysis is still needed to confirm this. The nutritional properties of Se-enriched microalgae were comparable to commercially available soybean meals in terms of protein and amino acids

content, while the fatty acid content of Se-enriched microalgae surpassed that of high-quality commercial fish oil.

(4) Downstream drying processes of microalgae could avoid pathogen contamination.

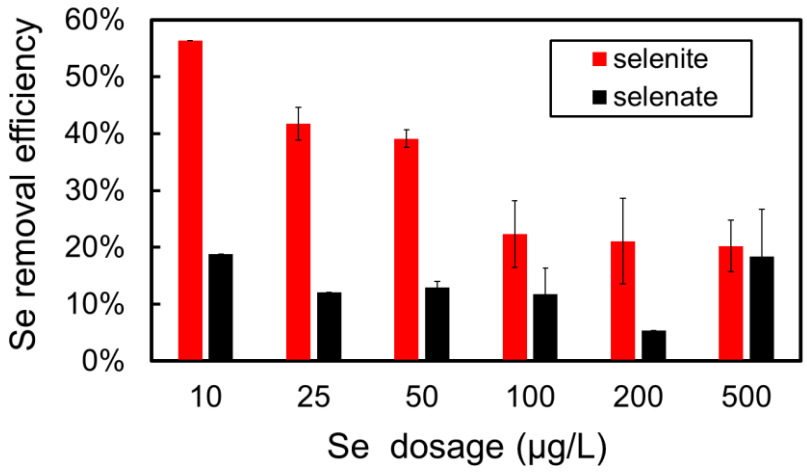

Figure 6.S1. Se removal efficiency of microalgae grown in the batch system after 7d cultivation

Table 6.S1. Summary of the main parameters (average ± standard deviation) monitored in the primary effluent (the influent of HRAPs) and the effluent of HRAPs throughout the entire experimental period.

	Primary effluent (=influent of HRAPs)	Effluent of HRAP-Se	Effluent of HRAP-C
pH	7.8 ± 0.2	8.7 ± 0.6	8.7 ± 0.5
Turbidity (NTU)	271 ± 241.9	14 ± 20.0	12 ± 16.0
TSS (mg/L)	424 ± 341.7	37 ± 48.0	22 ± 18.8
VSS (mg/L)	345 ± 254.6	35 ± 42.3	21 ± 15.1
COD_{tot} (mg/L)	497 ± 279.3	123 ± 97.2	117 ± 50.1
COD_{sol} (mg/L)	223 ± 139.7	97 ± 63.1	99 ± 54.6
TC (mg/L)	331 ± 176.2	97 ± 57.6	90 ± 39.0
TP (mg/L)	10 ± 5.5	2.9 ± 2.9	2.3 ± 1.6
SP (mg/L)	5.3 ± 2.7	1.6 ± 1.6	1.6 ± 1.2
TN (mg/L)	64 ± 19.8	26 ± 28.2	18 ± 13.1
NH_4^+-N (mg/L)	27 ± 2.9	2.9 ± 2.8	2.4 ± 2.6
NO_3^--N (mg/L)	0.4 ± 0.9	1.3 ± 3.6	1.8 ± 6
NO_2^--N (mg/L)	0.8 ± 2.0	4.6 ± 3.7	5.8 ± 5.1
SO_4^{2-}-S (mg/L)	55 ± 55.4	47 ± 17.4	49 ± 17.6

Table 6.S2. Summary of the removal efficiency (%) of the main water quality parameters measured in influent and effluent of the two HRAPs (HRAP-Se and HRAP-C) (average ± standard deviation) for the entire experimental period as well as the two subperiods with a HRT of 8 days and 4 days, respectively.

	Entire experimental period		HRT= 8d		HRT= 4d	
	HRAP-Se (%)	HRAP-C (%)	HRAP-Se (%)	HRAP-C (%)	HRAP-Se (%)	HRAP-C (%)
Turbidity	91 ± 9.6	90 ± 19.7	92 ± 9.0	89 ± 24.2	89 ± 10.3	91 ± 12.4
TSS	86 ± 16.0	90 ± 14.1	94 ± 3.7	96 ± 2.5	80 ± 14.1	87 ± 6.4
VSS	88 ± 12.1	92 ± 6.9	93 ± 3.9	95 ± 3.0	82 ± 14.7	90 ± 8.4
COD_{tot}	70 ± 20.4	66 ± 23.1	74 ± 23.4	70 ± 22.0	64 ± 17.1	63 ± 24.7
COD_{sol}	49 ± 23.9	47 ± 23.7	46 ± 23.6	40 ± 26.0	52 ± 25.1	53 ± 20.9
TC	67 ± 17.6	65 ± 20.2	73 ± 13.9	73 ± 15.4	60 ± 19.7	59 ± 22.6
TP	77 ± 18.1	72 ± 25.5	84 ± 12.6	65 ± 20.4	70 ± 20.9	67 ± 29.2
SP	71 ± 21.6	72 ± 18.8	75 ± 21.8	77 ± 16.5	68 ± 22.1	69 ± 20.4
TN	65 ± 24.8	67 ± 27.3	76 ± 12.0	70 ± 21.9	53 ± 29.8	64 ± 32.6
NH_4^+-N	93 ± 6.2	92 ± 7.7	94 ± 3.6	92 ± 9.3	91 ± 8.9	93 ± 6.2
Total Se	44 ± 6.5	N.D.	43 ± 7.3	N.D.	46 ± 4.3	N.D.

N.D.: Not determined

Table 6.S3. Fatty acid composition and content of the microalgae grown in HRAPs the control (HRAP-C) and Se spiked microalgae ponds (HRAP-Se). Values are mean ± standard deviation (n=3).

	HRAP-C		HRAP-Se	
	[% relative fat]	[mg/100 g]	[% relative fat]	[mg/100 g]
C10:0	0.62 ± 0.00	26.4 ± 0.06	0.91 ± 0.14	80.5 ± 11.51
C12:0	0.77 ± 0.00	32.7 ± 0.43	0.44 ± 0.00	38.6 ± 0.35
C14:0	4.48 ± 0.00	190 ± 1.18	4.44 ± 0.00	393 ± 2.50
C15:0	0.60 ± 0.00	25.7 ± 0.18	0.32 ± 0.01	28.7 ± 0.65
C16:0	21.4 ± 0.10	908 ± 4.81	17.2 ± 0.03	1524 ± 13.44
C17:0	0.80 ± 0.01	33.8 ± 0.45	0.25 ± 0.01	22.2 ± 0.65
C18:0	2.34 ± 0.02	99.4 ± 1.12	1.05 ± 0.02	92.9 ± 1.02
C20:0	0.23 ± 0.02	9.66 ± 0.89	0.09 ± 0.00	8.06 ± 0.06
C22:0	0.38 ± 0.00	15.9 ± 0.04	0.15 ± 0.00	13.2 ± 0.71
C24:00	0.42 ± 0.03	17.9 ± 1.03	0.16 ± 0.01	13.9 ± 0.71
Total SFA	32.1 ± 0.10	1359 ± 7.94	25.0 ± 0.13	2215 ± 4.38
C14:1	0.08 ± 0.01	3.27 ± 0.32	0.07 ± 0.00	6.10 ± 0.39
C17:1	0.25 ± 0.00	10.7 ± 0.02	0.28 ± 0.01	25.2 ± 0.77
c9C18:1	6.40 ± 0.05	271 ± 2.85	6.25 ± 0.00	553 ± 4.14
c11C18:1	3.74 ± 0.03	159 ± 1.75	1.52 ± 0.01	134 ± 0.01
C20:1	0.15 ± 0.01	6.19 ± 0.23	N.D.	N.D.
C24:1	N.D.	N.D.	0.05 ± 0.00	4.52 ± 0.04
Total MUFA	10.6 ± 0.07	450 ± 4.08	8.17 ± 0.03	773 ± 3.01
C18:2ω-6	6.5 ± 0.02	276 ± 1.70	6.60 ± 0.02	584 ± 5.81
C18:3ω-6	0.34 ± 0.01	14.3 ± 0.37	0.50 ± 0.00	43.8 ± 0.56
C20:2ω-6	0.18 ± 0.01	7.62 ± 0.32	0.07 ± 0.00	6.23 ± 0.17
C20:3ω-6	0.18 ± 0.00	7.56 ± 0.21	0.21 ± 0.00	18.6 ± 0.58
C20:4ω-6	2.74 ± 0.01	116 ± 0.91	4.42 ± 0.00	391 ± 3.15
C22:5ω-6	0.08 ± 0.00	3.38 ± 0.04	0.05 ± 0.00	4.77 ± 0.04
Total PUFA ω-6	10.0 ± 0.04	426 ± 2.82	11.8 ± 0.03	1048 ± 10.32
C18:3ω-3	3.62 ± 0.02	153 ± 1.32	1.23 ± 0.05	109 ± 3.46
C20:4ω-3	0.12 ± 0.00	5.20 ± 0.02	0.12 ± 0.02	10.4 ± 1.75
C20:5ω-3	13.2 ± 0.03	558 ± 2.83	24.7 ± 0.03	2182 ± 13.72
C22:5ω-3	0.09 ± 0.01	3.81 ± 0.22	0.04 ± 0.00	3.29 ± 0.20
C22:6ω-3	0.13 ± 0.01	5.63 ± 0.24	0.06 ± 0.00	5.52 ± 0.15
Total PUFA ω-3	17.1 ± 0.05	726 ± 4.15	26.1 ± 0.06	2310 ± 11.97

N.D.= not detected

Chapter 7 Production of Se-enriched microalgae in raceway ponds treating domestic wastewater as biostimulant and biofertilizer

Abstract

This study assessed the production of Se-enriched microalgae in a pilot-scale raceway pond treating domestic wastewater as biostimulant and biofertilizer. The effect of Se-enriched microalgae extracts and dry biomass on seed germination, growth and yield of beans (*Phaseolus vulgaris*) was studied by conducting a germination test as well as foliar and soil applications in pot experiments. The potential of Se-enriched microalgae extracts and dry biomass as Se biofertilizers to elevate the Se concentration of beans was assessed. Presoaking seeds in the Se-enriched microalgae extracts at low concentration (1%) enhanced their germination, as measured by the significant increase of seedling length and vigor index. Application of Se-enriched microalgae extracts 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% application) and 1.8 times of Se increment in the seeds (at the 5% application). Additionally, amendment of the soil with Se-enriched microalgae dry biomass (at 5%) enhanced the growth of beans (3.2 times for seeds) and increased the Se concentration in the bean plants (1.8 times for seeds), simultaneously. These results indicate that Se recovered through microalgae cultivation in wastewater can be recycled as a microalgae-based biofertilizer and biostimulant to improve both the bean seed yields and Se content, leading to a higher market value of the high-value beans. This may also offer an environmentally friendly and sustainable way for Se biofortification in Se-deficient areas.

Keywords: Algae, biofertilizer, biostimulant, crop, selenium, wastewater

7.1 Introduction

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 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 is the possible solution for Se deficiency (Boldrin et al., 2013; Li et al., 2020a). However, the adverse effects of applying conventional inorganic Se fertilizers on soils and the environment is leading to the exploration of alternative Se biofertilizers.

Microalgae can be cultivated in wastewater and agricultural runoff, recovering excess nutrients, including Se, while reclaiming the wastewater (Gan et al., 2019; Garcia-Gonzalez & Sommerfeld, 2016). The generated microalgae biomass with high nutrient content are not only a valuable ingredient for food and animal feed, but have also a potential as biofertilizers or biostimulants (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 (Calvo et al., 2014; Grzesik & Romanowska-Duda, 2014; Ronga et al., 2019). Biostimulants and biofertilizers are compounds and bioproducts that are able to stimulate the growth and development of several crops under both optimal and stressful conditions after being applied to the plants and soils (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 microalgal cellular extracts as biostimulant or microalgae biomass as biofertilizer, respectively. For instance, the application of 1.5 L/ha of the *Spirulina* extract obtained by supercritical fluid extraction 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). The addition of the microalga *Chlorella vulgaris* biomass to soil (2–3 g dry algae/kg soil) significantly increased the fresh and dry weight of lettuce (Faheed & Abd-

El Fattah, 2008). Similarly, the microalga *Acutodesmus dimorphus* dry biomass and its cellular extracts could trigger faster germination and enhance the plant growth and floral production of Roma tomato (Garcia-Gonzalez & Sommerfeld, 2016). The use of microalgae as a slow-release biofertilizer results in a higher quality of tomatoes with increased carotenoid and sugar levels (Coppens et al., 2015).

Considering that Se can be present in wastewater and that microalgae have the potential to efficiently remove Se from wastewater (Han et al., 2020; Tan et al., 2016), Se-enriched microalgae can thus be produced during the wastewater treatment process without adding other essential nutrients for microalgae growth. It is, therefore, necessary to explore whether the Se-containing microalgae generated from wastewater can be potentially used for biofortification purposes as an organic nutrient-rich biofertilizer to improve the Se levels in plants/soils and meanwhile to enhance plant growth and crop yield as biostimulant. This approach would be beneficial to save Se resources and avoid the introduction of chemicals contamination into the soil or environment through the replacement of synthetic chemical fertilizers by Se-enriched microalgae biofertilizers.

This study aimed to investigate the potential of Se-enriched microalgae from raceway ponds treating wastewater as biostimulant and biofertilizer. To this end, the influence of Se-enriched microalgae extracts and dry biomass on the germination, growth and yield of green beans (*Phaseolus vulgaris)* was assessed, along with the Se content in the beans. This is the first study to assess the application of Se-enriched microalgae biomass and its extracts as biostimulant and Se biofertilizer for green beans production.

7.2 Materials and methods

7.2.1 Se-enriched microalgae production

Se-enriched microalgae were produced as described in our previous study (Chapter 6). Briefly, microalgae were cultivated in an outdoor pilot-scale high rate algae pond (HRAP) located at the laboratory of the GEMMA Research Group (Universitat Politècnica de Catalunya, Barcelona, Spain) during 3 months. The system treated real municipal wastewater that received a screening pre-treatment before being pumped into a homogenization tank. The wastewater was pumped from this tank into a primary settler followed by a high rate algal pond (nominal volume of 0.5 m^3) with continuous

spiking of 500 µg/L Se, in the form of sodium selenite. The microalgal biomass was dominated by *Chlorella sp*. and *Scenedesmus sp.* A secondary clarifier separated the microalgae biomass from the secondary effluent. The Se-enriched biomass collected from the secondary clarifier was thickened by centrifugation and washed twice with deionized (DI) water. The centrifuged paste was frozen at -80 °C overnight, lyophilized and stored at -20 °C for subsequent experiments.

7.2.2 Preparation of liquid Se-enriched microalgae extracts

Freeze-dried 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%. SDS has a significant effect on improving the microalgae extraction efficiency as shown in our previous experiment (Chapter 6). 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 cell extracts from the biomass residue. The cell extracts were stored at 4 ºC for further trials. The composition of the Se-enriched microalgae biomass and the extracts is shown in Table 7.1.

Table 7.1. Characteristics of the Se-enriched microalgae biomass and the extract

	Biomass	Extracts
pH	N.D.	5.71
EC (mS/cm)	N.D.	6.10
Elements	[mg/kg dry matter]	[mg/L]
Total Se	29 ± 0.6	0.67 ± 0.0
P	13935 ± 323.5	445 ± 5.5
S	6995 ± 148.4	378 ± 4.3
Zn	520 ± 5.1	1.45 ± 0.0
Cu	145 ± 3.0	1.36 ± 0.0
Ca	51215 ± 1478.3	280 ± 4.8
Mg	8199 ± 175.2	230 ± 4.0
Na	1640 ± 56.7	616 ± 15.0
K	4799 ± 195.5	476 ± 9.5
Ni	15 ± 0.5	1.01 ± 0.0
Cr	31 ± 3.0	<LOQ
Cd	<LOQ	<LOQ
Pb	19 ± 0.3	<LOQ
Hg	0.32 ± 0.1	<LOQ
As	4.4 ± 0.3	N.D.
Co	<LOQ	<LOQ

N.D.: Not determined

< LOQ: values lower than the limited of quantification

7.2.3 Bioassay for germination test

Bean seeds (*Phaseolus vulgaris*) with uniform shape, size and weight were selected for the germination test. The germination test was conducted as described previously (Garcia-Gonzalez & Sommerfeld, 2016; Hernández-Herrera et al., 2013) with minor modifications. Each treatment (Table 7.2) 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 the different concentrations of Se-enriched microalgae cell extracts (Table 7.2) 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. Bud length was monitored every other day.

Table 7.2. Concentration of Se-enriched microalgae cell extracts in each treatment

Treatment	Concentration (%)	Preparation	Final SDS concentration (%)
Control	0.0	10 mL of DI water	0
T1	0.5	0.05 mL cell extract in 9.95 mL DI water	0.005
T2	1.0	0.1 mL cell extract in 9.9 mL DI water	0.01
T3	5.0	0.5 mL cell extract in 9.5 mL DI water	0.05
T4	10	1 mL cell extract in 9 mL DI water	0.1
T5	25	2.5mL cell extract in 7.5mL DI water	0.25
T6	50	5 mL cell extract in 5 mL DI water	0.5
T7	75	7.5 mL cell extract in 2.5 mL DI water	0.75
T8	100	10 mL cell extract	1.0

7.2.4 Microalgae extracts as biostimulant through foliar spray and soil drench application

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 described previously (Chapter 4). The soil was dried, homogenized and passed through a 2 mm sieve mesh. 0.5 kg of the soil was weighed and placed into a 10 cm x 10 cm pot.

Bean seeds (*Phaseolus vulgaris*) 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/m^2/s light intensity) with 80% of the water holding capacity. A total amount of 50 mL of different concentrations of the Se-enriched microalgae extracts was applied to each pot by foliar spray or soil drench every week, except for the first and last week of the growth period. The concentrations of the Se-enriched microalgae cell extracts were 0%, 0.5%, 1%, 5% and 10%, which were derived from the previous germination test (from 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 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.

7.2.5 Microalgae biomass application as biofertilizer

Different amount (0 g (0%), 0.225 g (0.5%), 0.45 g (1.0%), 2.25 g (5%) and 4.5 g (10%)) of freeze-dried Se-enriched microalgae biomass were mixed thoroughly with 0.5 kg 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 in Chapter 4. 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 TOC of the soil pore water were measured before harvest.

7.2.6 Analytical methods

For the determination of total Se in plants (beans and microalga 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. 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 for Se measurement using inductively coupled plasma-mass spectrometry (ICP-MS, ELAN DRC-e, PerkinElmer, Waltham, MA, USA). Internal standards (10 µg/L 103Rh and 69Ga) and an external

multi-element standard solution were used during ICP-MS analysis. White clover samples (BCR-CRM, 6.7 ± 0.25 mg/kg) and sea lettuce (BCR 279, 0.59 ± 0.04 mg Se/kg) were included as certified reference materials in each analytical batch as quality control with recoveries of 97 (± 7)% and 106 (± 4)%, respectively.

pH and 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 each element content in the microalgae extract and biomass (in Table 7.1), 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 HNO3, respectively. The tubes were sonicated for 1 h, then placed in a microwave oven (CEM Mars 6, Matthews, NC, USA) for digestion. The digestion programme was the same as aforementioned for the determination of total Se in plants. The digested samples were measured using inductively coupled plasma-optical emission spectrometry (ICP-OES, iCAP 7000, Thermo Scientific, Waltham, MA, USA) for all elements analysis in Table 7.1, except for Se. Se was determined by ICP-MS.

The pH of the soil extracts was determined by using a pH-meter. 2.0 mL of Rhizon extract from each pot 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. (Egene et al., 2018).

7.2.7 Statistical analysis

Statistical differences were identified with the ANOVA and Duncan's multiple comparison tests in SPSS 20.0. The germination percentage (GP) and germination index (GI) were calculated as described in Hernández-Herrera et al. (2013):

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

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

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 & Roberts, (1981) and Hernández-Herrera et al., (2013):

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

The seedling vigor index (SVI) was determined by the following formula (Hernández-Herrera et al., 2013; Orchard, 1977):

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

7.3 Results and discussion

7.3.1 Effect of Se-enriched microalgae cell extracts on seed germination and growth of bean seedlings

The germination percentage of bean seeds is shown in Fig. 7.1. Germination occurred in all treatments after 2 days. The Se-enriched microalgae cell extract showed a slightly 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 cell extract. 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 at the first 4 days after sowing. It should be noted that undiluted microalgae extract (100%) treatment significantly dropped off the GP during the entire germination period.

The microalgae extracts had a significant ($P \leq 0.05$) effect on the growth of the bean seedlings (Fig. 7.2). Similar to its effect on the GP, the microalgae extracts 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 extracts at the 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. 7.2).

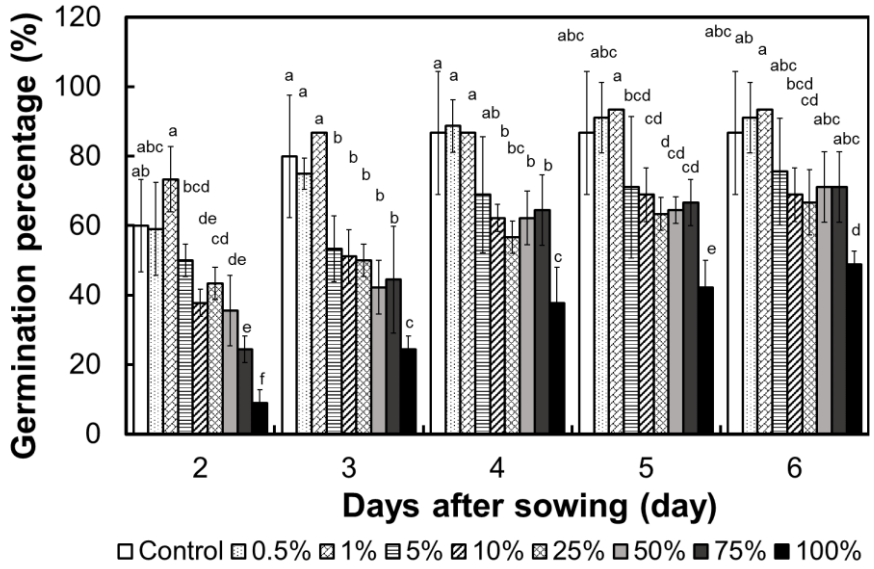

Figure 7.1. Effect of Se-enriched microalgae extracts treatment on the bean seeds germination percentage (GP). Values are mean ± standard deviation (n=3). Different letters indicate statistically significant differences between treatments within the same day according to Duncan's multiple comparison tests ($P \leq 0.05$).

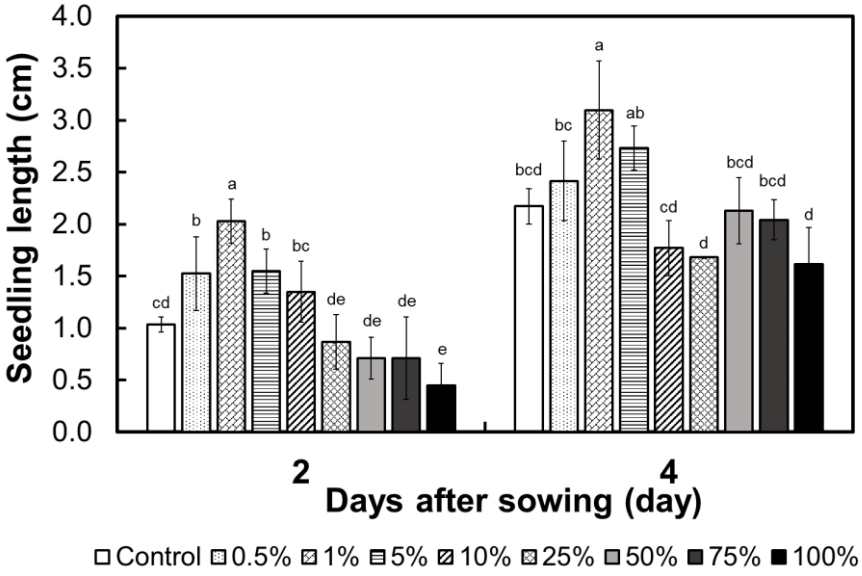

Figure 7.2. Effect of Se-enriched microalgae extracts on the length of bean seedlings. Values are mean ± standard deviation (n=3). Different letters indicate statistically significant differences between treatments within the same day according to Duncan's multiple comparison tests ($P \leq 0.05$).

Furthermore, seeds presoaked in 1% of microalgae extract showed the maximum GI and SVI (significantly higher than the control treatment for SVI, $P \leq 0.05$), but the

shortest MGT (Fig. 7.3). 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 extracts 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 extracts 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.

Previous studies have also tested 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 *Phormiudium* 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 lactuea* and *Padina gymnospra* 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* extracts significantly enhanced the germination of wheat seeds (*Triticum aestivum*) and seedling 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 extracts could be explained by salinity stress, which can be deduced from the high EC (6.10 mS/cm) and salt content (e.g., Na, Ca, Mg and K) of the liquid microalgae extracts (Table 7.1). The osmotic pressure caused by the high salt content would inhibit the seeds' ability to imbibe water (Coppens et al., 2015; Hernández-Herrera et al., 2013), 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 (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. (2013) 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). 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), K^+ (476 mg/L) and Ca^{2+} (280 mg/L), can be toxic to the embryo and developing seedlings (Benlloch et al., 1994).

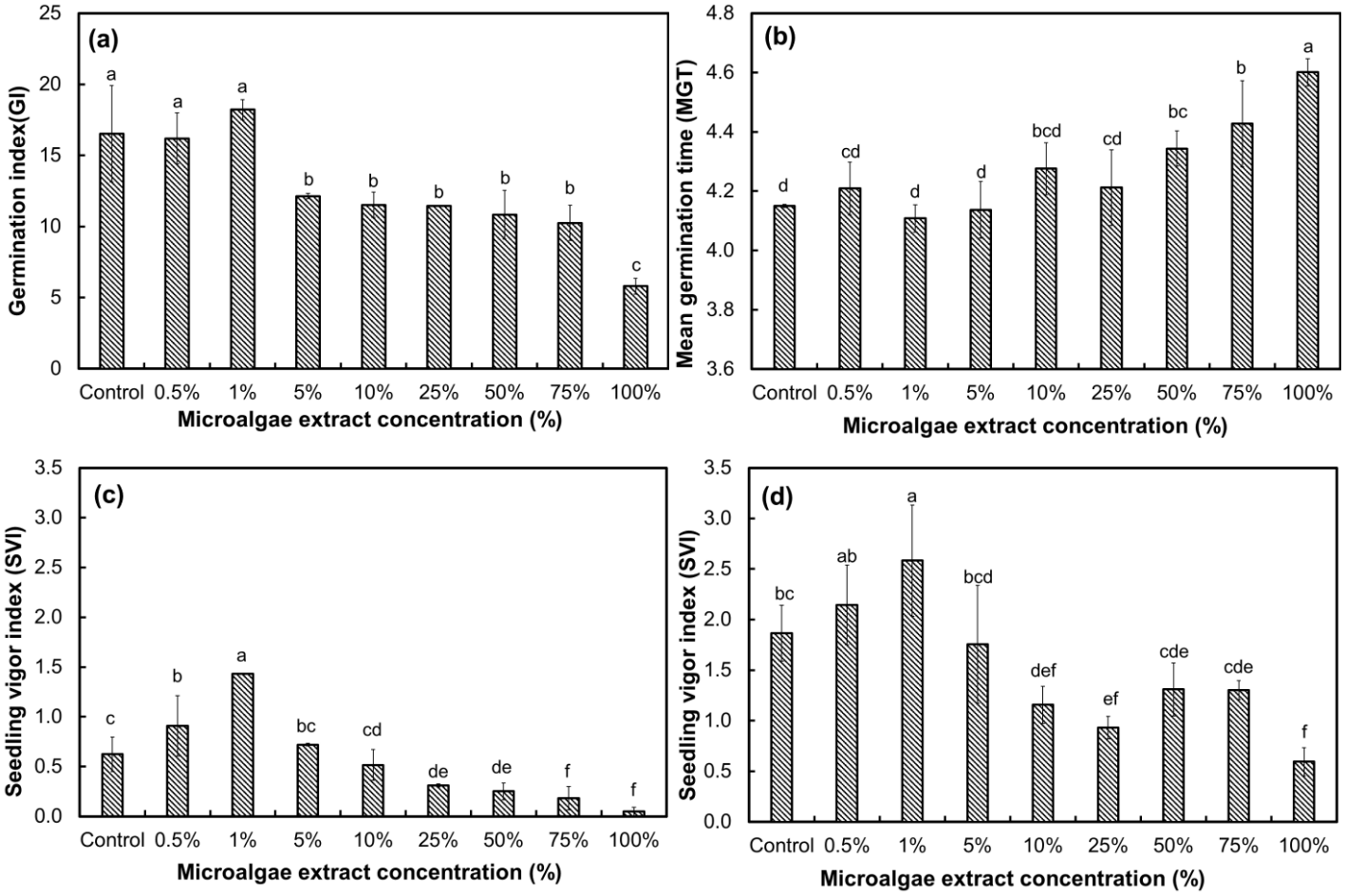

Figure 7.3. Effect of Se-enriched microalgae extracts on the germination parameters of bean seeds: (a) germination index (GI), (b) mean germination time (MGT), (c) seedling vigor index (SVI) at day 2, and (d) SVI at day 4. Values are average ± standard deviation (n=3). Different letters indicate statistically significant differences between treatments according to Duncan's multiple comparison tests ($P \leq 0.05$).

7.3.2 Effect of foliar and soil drench application of Se-enriched microalgae extracts on the bean plant growth and Se concentration in the bean plants

7.3.2.1 Bean plant growth

Application as a foliar spray was more effective in influencing the growth of bean plants than the soil drench application (Fig. 7.4). 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. 7.4a and 7.4c). An obvious increase in fresh and dry weight of the roots (113 and 51%, respectively) and seeds (364 and 252%, respectively) (Tables S7.2 and S7.3 in supplementary information) was observed ($P \leq 0.05$). Basically, no significant difference in the total fresh and dry weight of the entire plants was recorded when beans received the microalgae extract in the soil drench (Figs. 7.4b and 7.4d). 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 7.S2 and 7.S3). It should be noted that the relatively high concentration of microalgae extracts (5 and 10%) did not inhibit the growth of beans through the foliar spray and soil drench application (Fig. 7.4).

These results indicated that the microalgae extract exhibits growth-stimulating activities on beans, which are partially consistent with other studies. Kumar and Sahoo (2011) found that 20% of macroalgal seaweed extracts 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. Foliar spray of 50% of *Chlorella vulgaris* extracts obtained by freeze-thaw extraction resulted in an obvious increment of the yield in wheat (*Triticum aestivum* L.) 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 microalgae extracts on plant growth could be due to the presence of growth-promoting substances such as macro- and microelement nutrients (Table 7.1), 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., 2013). Another possibility is the presence of polysaccharides (e.g. carboxyled and sulfated polysaccharides or uronic acids) in the microalgae extracts (Rachidi et al., 2020), which can improve plant growth in a similar way to hormones (Hernández-Herrera et al., 2013; Rolland et al., 2002).

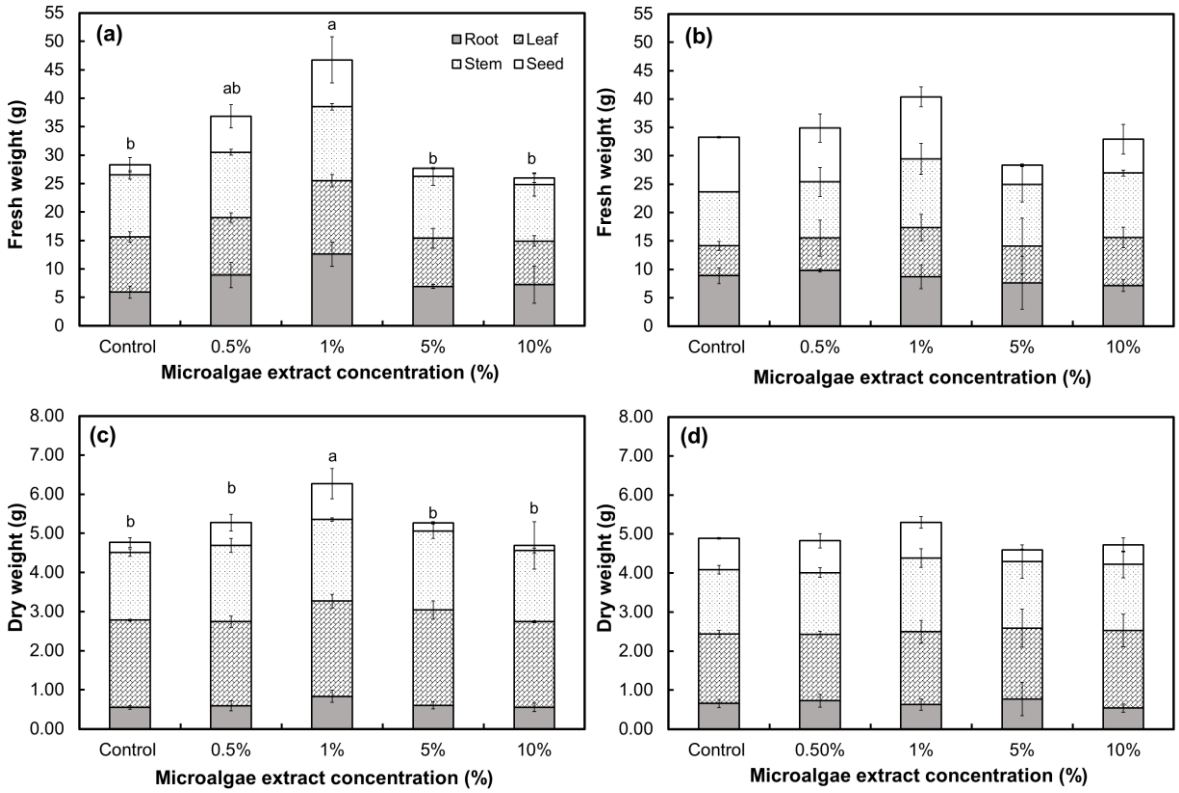

Figure 7.4. Effect of Se-enriched microalgae extracts applied as foliar spray (a, c) and soil drench (b, d) on fresh weight (a, b) and dry weight (c, d) of bean tissues. Values are average ± standard deviation (n=3). Different letters indicate statistically significant differences between different treatments according to Duncan's multiple comparison tests ($P \leq 0.05$).

7.3.2.2 Se concentration in the plants and seeds

Foliar spray and soil drench of the Se-enriched microalgae extracts gradually increased the Se content in the bean plant (Fig. 7.5). Generally, foliar spray of Se-enriched microalgae extracts 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 extracts 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. 7.5a), whereas no significant difference was found in the roots. On the other hand, the application of 10% microalgae extracts 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. 7.5b), whereas, the Se content in the seeds of beans was not significantly different between soil drench application treatments. This indicates a slow translocation of Se from the roots to seeds in bean plants.

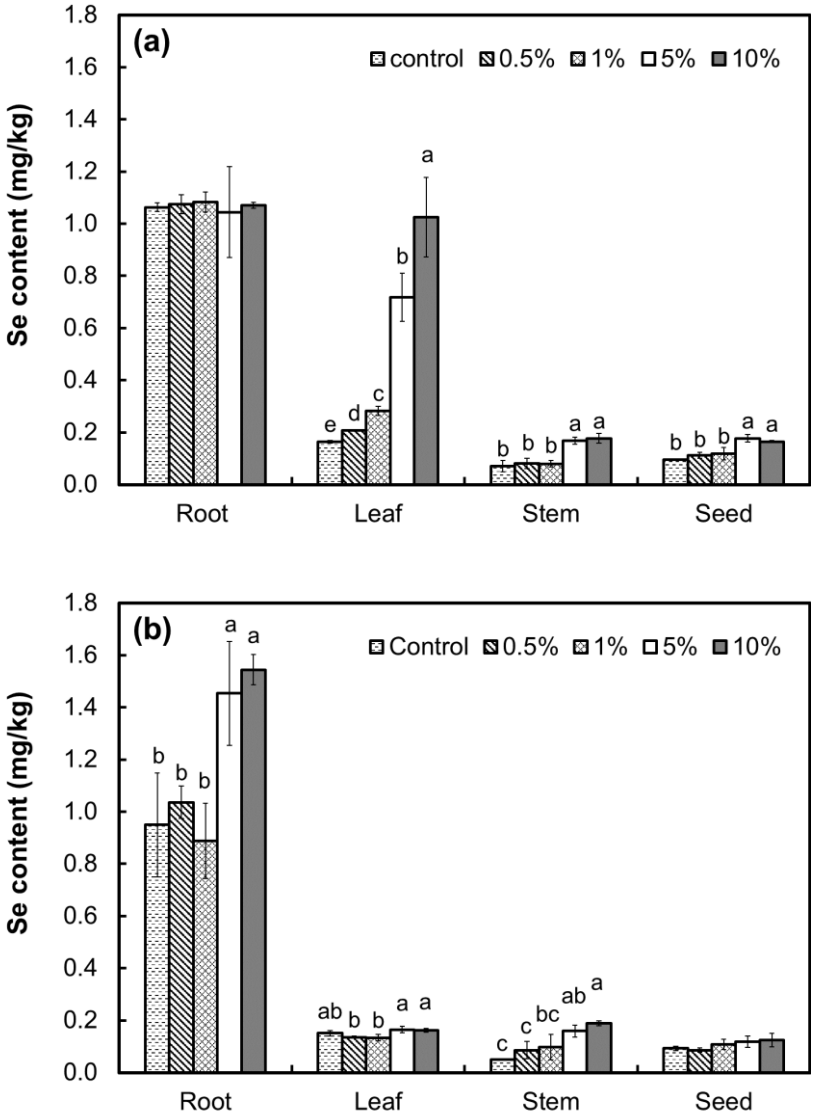

Figure 7.5. Effect of Se-enriched microalgae extracts applied as (a) foliar spray and (b) soil drench on the Se concentration in bean tissues. Values are average ± standard deviation (n=3). Different letters indicate statistically significant differences between treatments in the same tissue according to Duncan's multiple comparison tests ($P \leq 0.05$).

It should be noted that the Se concentrations in all tissues after the application of Se-enriched microalgae extracts at doses below 1% showed no significant difference with the control (except for the leaves with foliar spray) (Fig. 7.5), whereas the highest fresh and dry biomass of beans was observed at 1% of Se-enriched microalgae extract application (Fig. 7.4). This indicates that the optimum ratio of the Se content in the microalgal extracts and the dose of microalgae extracts themselves needs to be re-defined in order to balance out good nutrition (e.g. Se) and high yield of bean seeds.

7.3.3 Effect of non-extracted Se-enriched microalgal biomass on bean plant growth and Se concentration in plants and seeds

7.3.3.1 Bean plant growth

Fig. 7.6 illustrates the fresh and dry weight of the beans grown in soil amended with non-extracted Se-enriched microalgae biomass. The supplementation of Se-enriched microalgae 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% in, 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 microalgal supplementation compared to the leaves and stems, as reflected in the significant increase of biomass in the root and seed for the 0.5-5% of Se-enriched microalgae supplementation, but absence of a considerable increase in the leaves and stems biomass (Table 7.S4). Approximately 4 times higher seed yields (both fresh and dry weight) were obtained for the 1% of Se-enriched microalgae amendment compared to the control.

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 microalgae *Chlorella vulgaris.* The best treatments were 150 and 200 kg algae/Fed (1 Feddan = 0.42 hectare) (Shaaban, 2001b). As aforementioned in growth-stimulation of microalgae extracts, 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 fertilizer (Mulbry et al., 2005). The applied microalgae biomass is

composed of 48% protein and 32% carbohydrates (Chapter 6), thus enhancing the nitrogen and carbon content upon its application to the soil, leading to an increase of soil microbial activity and potentially promoting plant growth. The presence of plant biostimulants (e.g., amino acids, polysaccharides and phytohormones) (Kumar & Sahoo, 2011) contained in the microalgae could also have contributed to the positive effects on the beans growth.

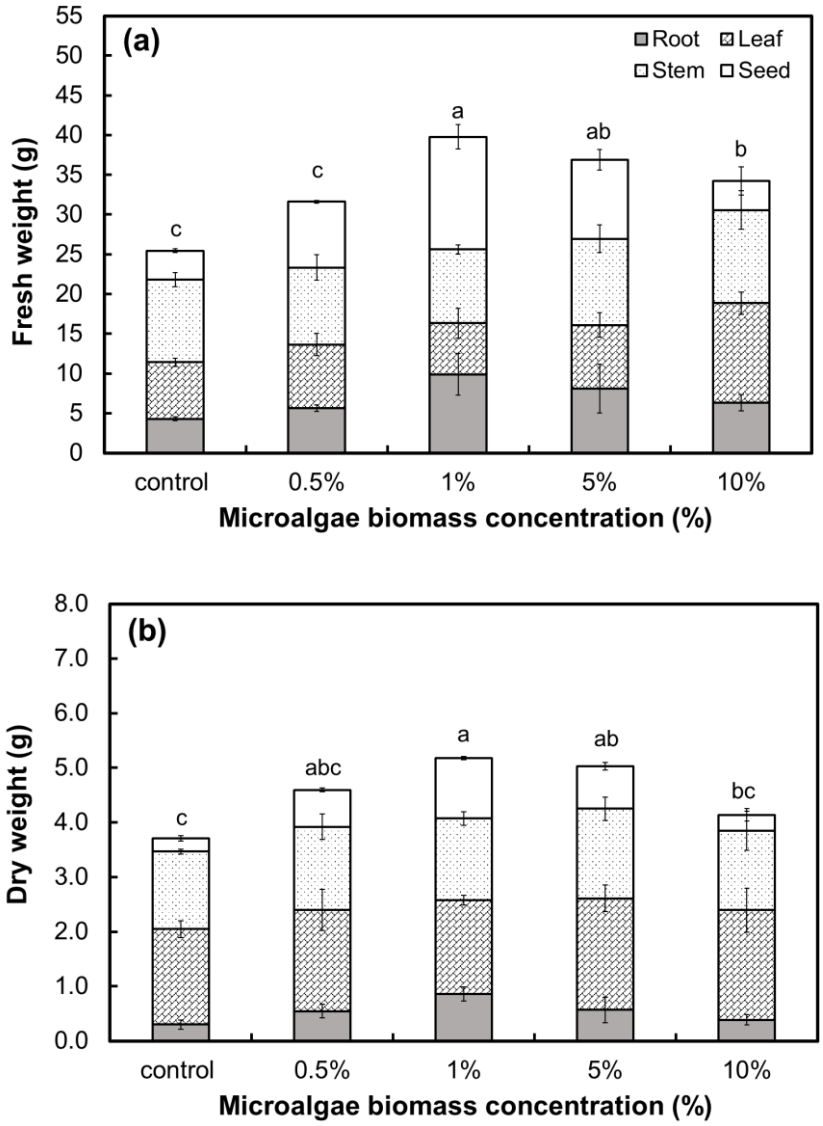

Figure 7.6. Effect of non-extracted Se-enriched microalgae biomass on the (a) fresh and (b) dry weight of beans. Values are average ± standard deviation (n=3). Different letters indicate statistically significant differences between treatments in the same tissue according to Duncan's multiple comparison tests ($P \leq 0.05$).

7.3.3.2 Se content in the bean plants

The Se content in the bean plants depended on the amount of Se-enriched microalgae supplemented to the soil (Fig. 7.7). The Se content in all tissues of the bean plant raised gradually with the increasing dosage of Se-enriched microalgae amendment (Fig. 7.7). 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 in the root, 0.12 to 0.34 mg/kg in the leaf, 0.09 to 0.42 mg/kg in the stem and 0.10 to 0.28 mg/kg in the seed.

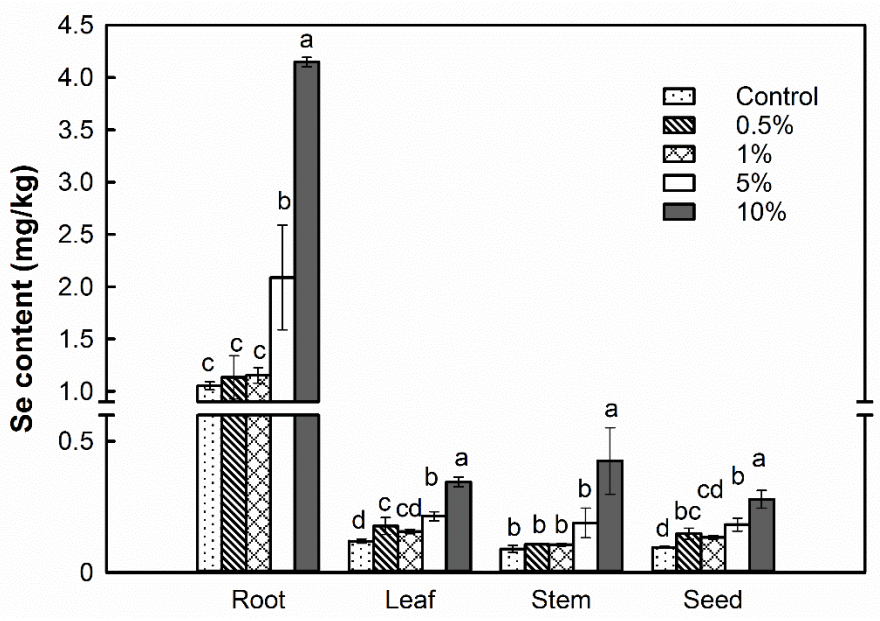

Figure 7.7 Effect of Se-enriched microalgae biomass on the Se concentration in the different tissues of beans. Values are mean ± standard deviation (n=3). Different letters indicate statistically significant differences between treatments in the same tissue according to Duncan's multiple comparison tests ($P \leq 0.05$).

It should be noted that the Se content in the 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. This may be related to the greatest amount of biomass being found at 1% of Se-enriched microalgae addition among all dosages, which resulted in a biological dilution of Se in the plant tissues due to the significant biomass increase. This is supported by the obviously higher Se accumulation in beans at 1% of Se-enriched microalgae application in comparison with that of the control among all tissues, except for the stem ($P \leq 0.05$) (Table 7.S5).

Taking into account the biomass (Fig. 7.6) and Se content (Fig. 7.7) 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.

7.3.3.3 Evolution of Se 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. 7.8 and Table 7.S6. 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 increased the Se content in the soil pore water to 6.89, 9.20, 21.7, 30.9 µg/L 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 difference with the control (Table 7.S6).

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. 7.8 and Table 7.S6). 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.

The Se content in the soil can indeed be increased by the application of Se-enriched organic materials, 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 to 36 and 79 µg/kg, 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).

Se-enriched microalgae biomass could be considered as a slow-release Se biofertilizer 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 7.S7). The higher Se accumulation in the beans compared to the Se content in the soil pore water (first day) also evidenced that extra Se was slowly released from the Se-enriched microalgae matrix and gradually supplied for uptake by the beans during the entire growth period (Table 7.S7). These results demonstrated that the Se-enriched microalgae produced from Se-containing domestic wastewater have potential to be used as a slow-release Se biofertilizer and biostimulant for enhancement of beans growth and Se uptake. However, the potential loading of heavy metals, micropollutants and pathogens onto the biomass is still a main concern for the application Se-enriched microalgae produced from domestic wastewater as biofertilizer.

The EU fertilizer regulation (2019) stipulates that contaminants in an organic fertilizer must not exceed the following limit values (expressed as mg/kg 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 300 and 800 mg/kg 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 the safety limits, with the exception of Cr. It should be noted that the fertilizer 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). Accordingly, the Cr species in the microalgae biomass in this study may possibly also be dominated by Cr (III), but more analysis is needed to confirm this. Besides, the pathogen load in the Se-enriched microalgae biomass used in this study have been characterized in our previous study (in Chapter 6). *Salmonella* spp. and *Escherichia coli* were absent in the freeze-dried biomass, and most microorganisms were reduced after drying. Eventhough the heavy metal contents and pathogen loads of the microalgal biomass did not exceed the fertilization regulation, more research is still needed in terms of environmental safety analysis and risk assessment of the Se-enriched microalgae as biofertilizer on the long term. Besides, further studies should

be conducted to assess the effect of Se-enriched microalgae and their extracts on Se accumulation and growth of beans under field conditions.

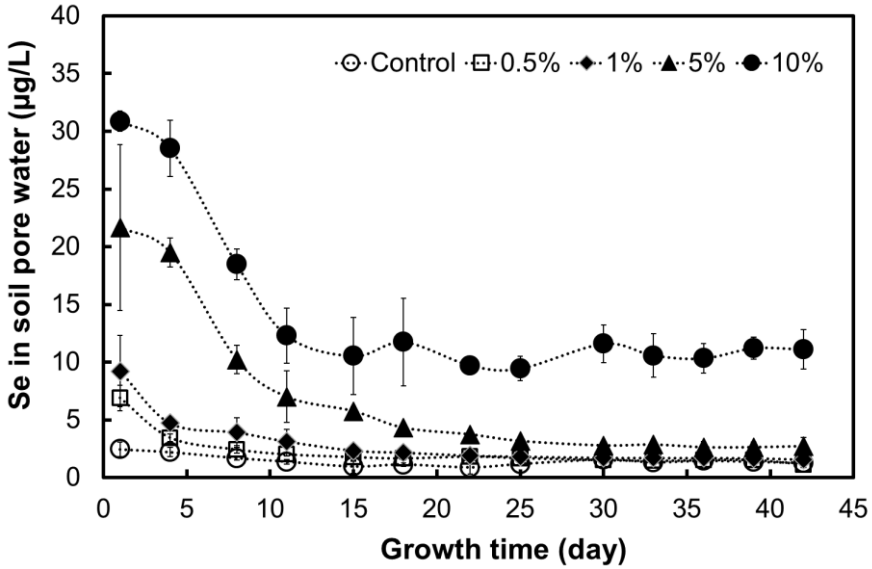

Figure 7.8. Evolution of the Se concentration in the pore water of soil amended with Se-enriched microalgae over the growth period of beans. Values are mean ± standard deviation (n=3).

7.3.3.4 pH and TOC in soil extracts

The pH and TOC in the pore water of the soil amended with Se-enriched microalgae were measured at the time of harvest (Table 7.3). The addition of 10% of Se-enriched microalgae noticeably increased the pore water pH and TOC ($P \leq 0.05$), while other applications had no remarkable difference in comparison with the control, except for the TOC for the 5% microalgal amendment. The highest TOC found at the highest application rate was 149 mg/L, which was 3 times higher than that of the control.

Table 7.3. pH and TOC content in the pore water of the soil amended with different amounts of Se-enriched microalgal biomass at the time of harvesting the beans. Values are mean ± standard deviation (n=3)

	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)	50 ± 7.7 ^{c}	47 ± 12.6 ^{c}	39 ± 6.6 ^{c}	65 ± 2.8 ^{b}	149 ± 8.7 ^{a}

7.4 Conclusions

Application of relatively low dosages of Se-enriched microalgae extracts 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 extracts 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 can be used as a biostimulant enhancing the plant growth and Se content in the seeds, and as an organic slow-release Se biofertilizer significantly improving the Se content in the beans, including seeds. These results indicate that Se-enriched microalgae biomass and their extracts could potentially be used as an added-value biostimulant replacing conventional Se fertilizer. As these were generated during domestic wastewater treatment, this contributes to resource recovery.

Supplementary Information

Table 7.S1. Effect of microalgae extracts on germination parameters of bean seeds: germination index (GI), mean germination time (MGT) and seedling vigor index (SVI). Values are average ± standard deviation (n=3). Different letters within columns indicate statistically significant differences between treatments according to Duncan's multiple comparison tests ($P \leq 0.05$).

Microalgae extract	Parameter			
	GI	MGT (days)	SVI at 2d	SVI at 4d
Control	16.5 ± 3.4 ^{a}	4.15 ± 0.0 ^{d}	0.62 ± 0.2 ^{c}	1.86 ± 0.3 ^{bc}
0.5%	16.2 ± 1.8 ^{a}	4.21 ± 0.1 ^{cd}	0.91 ± 0.3 ^{b}	2.14 ± 0.4 ^{ab}
1%	18.2 ± 0.7 ^{a}	4.11 ± 0.0 ^{d}	1.43 ± 0.0 ^{a}	2.58 ± 0.6 ^{a}
5%	12.1 ± 0.2 ^{b}	4.14 ± 0.1 ^{d}	0.72 ± 0.0 ^{bc}	1.76 ± 0.6 ^{bcd}
10%	11.5 ± 0. 9 ^{b}	4.28 ± 0.1 ^{bcd}	0.52 ± 0.2 ^{cd}	1.16 ± 0.2 ^{def}
25%	11.4 ± 0.0 ^{b}	4.21 ± 0.1 ^{cd}	0.31 ± 0.0 ^{de}	0.93 ± 0.1 ^{ef}
50%	10.8 ± 1.7 ^{b}	4.34 ± 0.1 ^{bc}	0.25 ± 0.1 ^{de}	1.31 ± 0.3 ^{cde}
75%	10.3 ± 1.3 ^{b}	4.43 ± 0.2 ^{b}	0.18 ± 0.1 ^{f}	1.30 ± 0.1 ^{cde}
100%	5.8 ± 0.6 ^{c}	4.60 ± 0.1 ^{a}	0.05 ± 0.0 ^{f}	0.60 ± 0.1 ^{f}

Table 7.S2. Effect of Se-enriched microalgae extract treatment 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 ± 1.1 ^{b}	9.7 ± 0. 9 ^{b}	10.9 ± 0.8	1.8 ± 1.3 ^{b}	8.9 ± 1.4	5.2 ± 0.8	9.5 ± 0.1	9.6 ± 0.2 ^{ab}
0.5%	8.9 ± 2.2 ^{ab}	10.1 ± 0.9 ^{ab}	11.5 ± 0.5	6.3 ± 2.1 ^{ab}	9.8 ± 0.3	5.7 ± 3.2	9.9 ± 2.5	9.5 ± 2.5 ^{ab}
1%	12.6 ± 2.2 ^{a}	12.9 ± 1.1 ^{a}	13.0 ± 0.6	8.2 ± 4.0 ^{a}	8.7 ± 2.1	8.7 ± 2.4	12.1 ± 2.7	11.0 ± 1.7 ^{a}
5%	6.9 ± 0.4 ^{b}	8.5 ± 1.7 ^{b}	10.9 ± 1.6	1.4 ± 0.2 ^{b}	7.6 ± 4.7	6.5 ± 4.9	10.8 ± 3.1	3.4 ± 0.2 ^{c}
10%	7.2 ± 3.3 ^{b}	7.7 ± 0.9 ^{b}	9.9 ± 2.0	1.1 ± 0.8 ^{b}	7.1 ± 1.0	8.5 ± 1.8	11.4 ± 0.5	5.9 ± 2.6 ^{bc}

Table 7.S3. Effect of Se-enriched microalgae extract treatment 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 ± 0.0 ^{b}	2.23 ± 0.0	1.72 ± 0.1	0.26 ± 0.1 ^{bc}	0.66 ± 0.1	1.78 ± 0.1	1.64 ± 0.1	0.81 ± 0.0 ^{a}
0.5%	0.59 ± 0.1 ^{b}	2.15 ± 0.2	1.95 ± 0.2	0.58 ± 0.2 ^{ab}	0.73 ± 0.2	1.70 ± 0.1	1.58 ± 0.1	0.82 ± 0.2 ^{a}
1%	0.83 ± 0.2 ^{a}	2.43 ± 0.2	2.09 ± 0.0	0.92 ± 0.4 ^{a}	0.63 ± 0.1	1.87 ± 0.3	1.89 ± 0.2	0.92 ± 0.2 ^{a}
5%	0.60 ± 0.1 ^{b}	2.44 ± 0.2	2.02 ± 0.2	0.20 ± 0.0 ^{bc}	0.77 ± 0.4	1.81 ± 0.5	1.70 ± 0.4	0.30 ± 0.0 ^{b}
10%	0.55 ± 0.1 ^{b}	2.19 ± 0.0	1.82 ± 0.1	0.13 ± 0.6 ^{c}	0.54 ± 0.1	1.99 ± 0.4	1.69 ± 0.3	0.50 ± 0.2 ^{b}

Table 7.S4. Effect of non-extracted Se-enriched microalgae biomass application to soil 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 ± 0.2 ^{c}	7.11 ± 0.5 ^{b}	10.4 ± 0.9	3.65 ± 0.2 ^{c}	0.30 ± 0.1 ^{b}	1.75 ± 0.2	1.42 ± 0.0	0.25 ± 0.1 ^{c}
0.5%	5.64 ± 0.4 ^{c}	8.01 ± 1.4 ^{b}	9.69 ± 1.6	8.28 ± 0.2 ^{b}	0.54 ± 0.1 ^{b}	1.85 ± 0.4	1.52 ± 0.2	0.68 ± 0.0 ^{b}
1%	9.89 ± 2.6 ^{a}	6.44 ± 1.9 ^{b}	9.27 ± 0.6	14.2 ± 1.5 ^{a}	0.86 ± 0.1 ^{a}	1.72 ± 0.1	1.49 ± 0.1	1.10 ± 0.0 ^{a}
5%	8.09 ± 3.1 ^{ab}	8.03 ± 1.5 ^{b}	10.8 ± 1.7	9.95 ± 1.3 ^{b}	0.57 ± 0.2 ^{b}	2.04 ± 0.2	1.64 ± 0.2	0.78 ± 0.1 ^{b}
10%	6.35 ± 1.0 ^{ab}	12.5 ± 1.4 ^{a}	11.7 ± 2.4	3.67 ± 1.7 ^{c}	0.39 ± 0.1 ^{b}	2.01 ± 0.4	1.46 ± 0.4	0.29 ± 0.1 ^{c}

Table 7.S5. Effect of non-extracted Se-enriched microalgae biomass application to soil on Se accumulation (µg/pot) 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 ± 0.1 ^{c}	0.21 ± 0.0 ^{d}	0.13 ± 0.0 ^{b}	0.02 ± 0.0 ^{c}
0.5%	0.63 ± 0.2 ^{c}	0.32 ± 0.0 ^{c}	0.15 ± 0.0 ^{b}	0.10 ± 0.0 ^{b}
1%	0.99 ± 0.1 ^{b}	0.27 ± 0.0^{cd}	0.15 ± 0.0 ^{b}	0.15 ± 0.0 ^{a}
5%	1.11 ± 0.1 ^{b}	0.44 ± 0.1 ^{b}	0.31 ± 0.1 ^{b}	0.14 ± 0.0 ^{a}
10%	1.47 ± 0.2 ^{a}	0.65 ± 0.1 ^{a}	0.58 ± 0.2 ^{a}	0.07 ± 0.0 ^{b}

Note: Se accumulation (µg/pot) was calculated by multiplying the Se concentration in tissues (µg/g) by the dry weight of corresponding tissues (g).

Table 7.S6. Selenium concentrations (µg/L) 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 ± 0.6 ^{D}	1.69 ± 0.2 ^{D}	0.95 ± 0.6 ^{C}	0.87 ± 0.6 ^{D}	1.33 ± 0.1 ^{B}	1.26 ± 0.1 ^{B}
0.5%	6.89 ± 1.1 a ^{C}	2.42 ± 0.3 b ^{CD}	1.77 ± 0.3 bc ^{C}	1.82 ± 0.3 bc ^{C}	1.44 ± 0.3 bc ^{B}	1.13 ± 0.2 c ^{B}
1%	9.20 ± 3.1 a ^{C}	3.91 ± 1.3 b ^{C}	2.18 ± 0.4 b ^{C}	1.91 ± 0.2 b ^{C}	1.70 ± 0.1 b ^{B}	1.55 ± 0.5 b ^{B}
5%	21.7 ± 7.2 a ^{B}	10.2 ± 1.3 b ^{B}	5.37 ± 0.7 bc ^{B}	3.74 ± 0.2 c ^{B}	2.86 ± 0.1 c ^{B}	2.73 ± 0.8 c ^{B}
10%	30.9 ± 0.9 a ^{A}	18.5 ± 1.3 b ^{A}	10.5 ± 3.3 c ^{A}	9.73 ± 0.6 c ^{A}	10.6 ± 1.9 c ^{A}	11.1 ± 1.7 c ^{A}

Table 7.S7. Mass balance of Se in the Se-enriched microalgae biomass, soil pore water and plant system.

Treatments	Applied Se *	Se in pore water ** (first day)	Se in pore water (last day)	Se accumulation by plant***	Se in pore water (first day) / applied Se	Se in plants / applied Se
	[µg/pot]				%	%
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 accumulation in each plant tissue.

Chapter 8 General discussion, conclusions and future perspectives

The main objective of this PhD dissertation was to explore the possibility of producing micronutrient-enriched biomaterials on wastewater as feedstock through ecotechnologies, and using these materials as feed supplement (Chapters 3 and 6) or biofertilizer and biostimulant (Chapters 4, 5 and 7). The used production methods are phytoextraction using duckweed and *Azolla*, bioreduction by anaerobic sludge, and microalgae-based wastewater treatment methods.

8.1 Production of micronutrient-enriched bioproducts as potential feed/food supplements

8.1.1 Production and valorization of Se/Zn-enriched duckweed as potential feed supplements

Lemna (Duckweed) and *Azolla*, two aquatic plants with a substantial protein content, were selected to evaluate the possibility to produce Se/Zn-enriched dietary proteins and fertilizers while removing Se/Zn from wastewater. The interaction effects occurring between Se (Se(IV) and Se(VI)) and Zn when these micronutrients are taken up simultaneously by *Lemna* and *Azolla* were assessed as well. The results demonstrated that both plant species could accumulate (around 10 times) more Se(IV) than Se(VI). This is in agreement with what was previously observed for other plants, such as sunflower and maize, when being cultivated in hydroponic systems. A synergetic effect between Se and Zn was observed in *Lemna*, but an antagonistic effect in *Azolla.* This was concluded from the significant increase of the removal efficiency of Se and its accumulation in *Lemna* when increasing the Zn dosage, but the opposite being observed in *Azolla*.

A high content of true protein (approximately 17%) in freeze-dried *Lemna* and *Azolla* and high Se/Zn accumulation in the two plant tissues (up to 1664 mg/kg for Se and 3144 mg/kg for Zn) were observed. Besides, the ability of *Lemna* and *Azolla* to take up and transform inorganic Se(IV) in the growth medium into organic Se (e.g., SeMet, $SeCys_2$, and SeMetSeCys) in their tissues was validated. Organic Se species are considered beneficial in human and animal nutrition. These results together with the fast growth rate of the plants make *Lemna* and *Azolla* potential candidates for the production of Se/Zn-enriched biomass that can be used as crop fertilizers or protein-rich food/feed supplements or ingredients.

However, it should be noted that the Se/Zn removal efficiency by *Lemna* and *Azolla* may require further improvement. This may be attributed to the small amount of biomass used at the start of the experiments and the limited water surface area to volume ratio applied in this study. The latter is an important parameter as it concerns floating aquatic plants. Therefore, further study should reconsider the biomass used and the wastewater surface area to volume ratio. Besides, the challenge of dewatering aquatic plants between harvest and their use as feed supplements or fertilizers should also be considered from energy- and cost-saving perspectives.

8.1.2 Production and valorization of Se-enriched microalgae as potential feed supplements

Batch lab-scale and continuous pilot-scale (HRAPs) experiments have been conducted to study the production of Se-enriched microalgae biomass as potential Se-enriched feed supplement and biofertilizer (Chapter 6). Based on the results of the batch test, Se(IV) was preferred over Se(VI) to be supplied to the continuous HRAPs systems for the production of Se-enriched microalgae, as microalgae biomass could accumulate remarkably more Se(IV) than Se(VI). Furthermore, the HRAPs fed with domestic wastewater were operated for 3 months under two HRTs (4 days and 8 days). The HRAPs had a good wastewater treatment performance, with an average COD, NH_4^+-N, and total phosphorus removal efficiency of, respectively, 70%, 93%, and 77%. This is in line with previous studies (Arashiro et al., 2019; Gutierrez et al., 2016). The Se removal efficiency by HRAP systems under the two HRTs was around 44% at the dose of around 50 μg Se/L for both 4 d and 8 d HRT operation.

Nutritional analyses evidenced the potential of the produced Se-enriched microalgae as feed supplements or alternatives for animal protein, as both the content of protein (around 48%) and the occurrence of essential amino acids in the Se-enriched microalgae were comparable to those of conventional plant-based protein sources used in feed (soybeans). Moreover, Se-enriched microalgae biomass was shown to contain a higher content of fatty acids beneficial for human and animal consumption, such as omega-3, omega-6, and EPA. Moreover, the predominance of selenoamino acids (SeMet, accounting for 91% of the total Se) found in the Se-enriched microalgae grown in the HRAP demonstrates the ability of microalgae to upgrade low-value inorganic Se to high-value products. However, *in vitro* digestibility tests indicated that

only 49% and 63% of the incorporated Se are bioaccessible for animals in raw and ball-milled Se-enriched microalgae, respectively. This may be due to the robustness of the microalgae cell wall, which cannot be easily disrupted, eventually resulting in the low Se bioaccessibility. In fact, this is currently also the bottleneck for the reuse of microalgae for other purposes, e.g. as biofuel. Therefore, future research should also focus on improving disruption of the cell wall of the microalgae in a cost-effective and energy-efficient manner for improved digestion by animals.

8.2 Valorisation of the produced micronutrient-enriched bioproducts as Se/Zn biofertilizers

8.2.1 Agronomic biofortification of *Phaseolus vulgaris* with Se/Zn-enriched duckweed and sludge

Pot experiments using green beans (*Phaseolus vulgaris*) were conducted to assess the potential of two micronutrient-enriched biomaterials (sludge and duckweed) as slow-release Se and Zn biofertilizers (Chapter 4 and 5). The biomaterials were previously generated using single Se or simultaneous Se and Zn-bearing water as feedstock. The results demonstrated that Se contained in the Se-enriched duckweed was released quicker into soils than Se contained in the Se-enriched sludge. However, the Se contained in the Se-enriched sludge was more bioavailable for plant uptake than the Se contained in duckweed, particularly with respect to the final concentration of the selenoamino acid SeMet (Se-methionine) in the bean seeds. This is due to (1) the different Se species and organic matter content present in the two Se-enriched biomaterials. Specifically, elemental nano-Se was the predominant Se species in the Se-enriched sludge, which is relatively stable, while Se(VI) was the main Se form in the Se-enriched duckweed, which has a higher mobility; (2) the different Se immobilization rate and transformation in soils after Se is released from the two biomaterials.

This thesis thus concluded that the micronutrient-enriched sludge is considered as the preferred slow-release Se biofertilizer for Se-deficient areas, in comparison to micronutrient-enriched duckweed. Besides, the main Se aminoacid species (Se-methionine, 76–89%) detected in the bean seeds together with the estimated daily intake (EDI) and health risk index (HRI) indicated that the bean seeds produced by

biofortification using Se-enriched duckweed/sludge as fertilizer could contribute to achieving the recommended daily Se intake for human diets and would likely not pose a potential risk of excessive Se intake.

On the other hand, Zn released from the biomaterials was not readily transferred from underground to aboveground plant parts, especially to the seeds. Therefore, the biofortification of Zn through the application of Se/Zn-enriched biomaterials as fertilizers in this thesis was not completely successful. Besides, it should be highlighted that higher amounts of duckweed (>11 g in 0.5 kg soil) application resulted in negative effects on plant growth (i.e. lower biomass yield), which is thus not recommended in practice. Some pre-treatment (e.g., composting, pyrolysis, or extracting) of the micronutrient-enriched duckweed may be needed before application as Se/Zn biofertilizer.

8.2.2 Valorization of Se-enriched microalgae and their extract as potential Se biofertilizers and biostimulants

The use of Se-enriched microalgal biomass and their extract as potential Se biofertilizer and biostimulant to simultaneously enhance plant growth and Se uptake was assessed through soil and foliar application via pot experiments (Chapter 7). Green bean (*Phaseolus vulgaris*), a protein-rich crop regularly grown in moderate climates, was targeted. We concluded that the foliar application of Se-enriched microalgae extracts is recommended in practice, in comparison with the application of Se-enriched microalgae extract as soil drench, because the foliar application of Se-enriched microalgae extracts could obviously enhance the plant yield and meanwhile improve the Se content in the tissues of beans (seeds, leaves, and stems). Besides, the results also verified the possibility of Se-enriched microalgal biomass as a potential Se biofertilizer, which was shown by the remarkable increase of plant biomass and Se concentration in bean tissues after application of Se-enriched microalgae biomass (1% and 5%), compared to that of the control. This could lead to a higher market value of the beans. Overall, this thesis has valorized the use of the high value-added Se-enriched microalgae biomass and their extracts generated from domestic wastewater as biostimulant and biofertilizer.

8.3 Future perspectives

8.3.1 Production of micronutrient-rich bioproducts

The Se/Zn-enriched duckweed and sludge used as biofertilizer were generated from a micronutrient-enriched medium mimicking wastewater in this thesis. The physicochemical properties of real Se/Zn-contaminated water being used (partially purified wastewater) may be different from those of the Se/Zn-enriched media prepared in the laboratory. This could possibly lead to producing bioproducts having different physicochemical properties. Thus, the production of Se/Zn-enriched duckweed and sludge under real wastewater conditions needs to be further confirmed. In this context, potential environmental and health risks related to the possible presence of heavy metals, pathogen loads, and organic micropollutants in the bioproducts generated from waste should be particularly addressed. Besides, the enhancement of Se removal by duckweed and microalgae is still needed in future studies, such as optimization of growth conditions (e.g. optimal temperature and light), selecting specific duckweed/microalgae species with high Se accumulation ability. Apart from the use of real Se/Zn-enriched wastewater, wastewater with low Se/Zn content (e.g. domestic wastewater) could also be considered to act as nutrients source to produce the bioproducts while spiking Se and Zn. Additionally, pilot studies are also recommended for upscaling and verifying the results obtained in our study, in particular for duckweed, as the performance of the plants is expected to be different under different conditions (i.e. different geometry of growth tanks, different biomass density, and different ambient conditions).

In chapter 6, we did not perform replications of the Se-HRAPs due to the limitation of the facility (two HRAPs) and we did not analyze nutritional parameters of biomass produced at different time points. Accordingly, some findings in our study, e.g. Se inducing more omega-3 (ω3) and omega-6 (ω6) formation, require additional data collection for further validation. Thus, analysis of more biomass samples or more replications of the HRAPs (if applicable) are suggested for future studies.

Additionally, although the potential of the Se/Zn-enriched duckweed and microalgae as value-added feed supplements was confirmed by measuring different nutritional parameters (i.e. amino acids, fatty acids, and organic Se species), the digestibility in

animals (pig) assessed through *in vitro* studies may still be complemented by *in vivo* studies as the real bioavailability after ingestion may still be different in living animals.

8.3.2 Biofortification

Along with the incubation time, the considerable decrease of Se or Zn concentration in the pore water of soils amended with Se/Zn-enriched duckweed and microalgae was observed in this thesis. However, the explanation of these results is based on crop yield and agronomic parameters, such as absorption, transformation between different Se/Zn species in the soil matrix, Se and Zn uptake and translocation of Se and Zn within the plant tissues. The specific mechanisms affecting the availability of the released Se and Zn from bSe/Zn-enriched biomaterials in soils should still be explored and verified in future studies. This could include measurement of Se/Zn in different fractions, e.g., the exchangeable, Fe/Mn oxide-bound, and organic matter-bound fractions of soils fertilized with Se/Zn-enriched biomaterials. This could contribute to understanding the dynamics, fate, and transformation of Se/Zn in the soil after being released from these biomaterials and to assessing the possible factors affecting the plant uptake.

Micronutrient-enriched duckweed was less efficient in increasing the Se/Zn concentrations in beans and even inhibited the plant growth at high application doses, compared to the micronutrient-enriched sludge (Chapter 4 and 5). Moreover, the Se/Zn in the soil pore water after being released from the duckweed was not stable in the first three weeks after the soil application. All these results indicate that stabilization of the produced micronutrient-enriched duckweed is definitely needed before being added to soils as a biofertilizer, contributing to improving the utilization efficiency of the trace elements. Post-treatment of the Se/Zn-enriched duckweed (such as composting and anaerobic digestion) after being harvested from wastewater is also needed to reduce its organic carbon content and phytotoxic substances (i.e. low-molecular weight organic acids), consequently mitigating its effects on plant growth at higher application doses.

Besides, a long term experiment with continuous planting should be performed in soils amended with micronutrient-enriched bioproducts to further investigate the evolution of Se/Zn in the soil pore water and its effects on the crops and soil environment on the

longer term. The potential leaching and retaining of Se/Zn in the soil with micronutrient-enriched biomaterials application should be further investigated on a longer-term. Besides, the effects of the fertilization of Se/Zn-enriched biomaterials on soil quality including microorganism activities are still needed. Finally, a field trial study with supplementation of the micronutrient-enriched bioproducts is recommended to compare and validate the results of the lab study on a larger scale.

8.3.3 Feasibility analysis

The analysis of economic, environmental and social impacts, including safety and health issues, for the production and use of the micronutrient-enriched biomaterials with wastewater as feedstock is still needed. Health-related risk from pathogen loads and the potential presence of heavy metals and micropollutants should be carefully evaluated on the long term and compared with the legal frameworks of fertilizer legislation. Conducting a life cycle assessment (LCA) would be beneficial. Moreover, a cost-benefit analysis has to be done based on the results of pilot studies to evaluate the profit margin of production (recovery), processing, and use of these micronutrient-enriched bioproducts produced from wastes. The market potential of these new micronutrient-enriched bioproducts should also be assessed.

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Conferences and Workshops

The 11th International Symposium on Selenium in Biology and Medicine and The 5th International conference on Selenium in the Environment and Human Health. August 2017

- Stockholm, Sweden (Poster presentation)

The 15th International Conference on the Biogeochemistry of Trace element. May 2019

- Nanjing, China (Oral presentation)

3rd IWA Resource Recovery Conference 2019. September 2019

- Venice, Italy (Poster presentation)

The 6th International Conference on Selenium in the Environment and Human Health. November 2019

- Xi'an, China (Oral presentation, award one of the best presentations)

Workshops

Algal biotechnology techniques and opportunities for the sustainable bioeconomy

- Stuggart, Germany

Workshop on business case development in the field of resource recovery, energy and product from wastewater.

- Prague, Czech Republic

Workshop on sustainability, societal aspects, policy and stakeholder involvement

- Delft, The Netherlands

Workshop on modelling tools

- Barcelona, Spain

Workshop on creative problem-solving in resource recovery technology

- Aachen, Germany