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Arsenic accumulation in plants for food and phytoremediation: Influence by external factors

Arsenic accumulation in plants for food and phytoremediation:

Influence by external factors

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Abstract

Arsenic (As) appears in the environment as various As species, which may vary in plant uptake and toxicity. Moreover, As exposure may vary between habitat due to availability and speciation, both of which are influenced by redox potential. To decrease As uptake, addition of silicate may be a tool.

The aim of the study was to investigate how the external factors As availability, plant habitats, silicon and oxygen level, influenced the accumulation and speciation of As in plants for food and phytoremediation in a temperate region. The external factors were chosen due to their previously showed influence on As in plants.

The risks with dietary As was investigated by plant As accumulation and speciation in carrot, lettuce and spinach grown in alum shale and glassworks soils, and by the influence of silicon on As accumulation in lettuce in hydroponics. Suitable plant for As phytoremediation was investigated by analysing plants from various habitats, and by the O_2 influence on phytofiltration.

The results showed that vegetables accumulated more As in soils with higher As extractability, and the As extractability in the rhizosphere was higher than in bulk soil. Also, the As concentration in lettuce was higher in hydroponics than in soil, but silicon reduced the accumulation of As in lettuce in hydroponics. Also, the more toxic inorganic As were the main As species detected in vegetables, compared with the less toxic organic As. For phytoremediation, the results showed a low As accumulation in emergent and terrestrial plants. Submerged plants had had a higher shoot As concentration. In general, the habitat had a major influence on the As accumulation in plants. The results also showed that the submerged macrophyte *Elodea canadensis* accumulated more As in higher O_2.

In conclusion, consumption of vegetables cultivated in As polluted soils can result in an elevated intake of inorganic As, and *E. canadensis* is a promising candidate for As phytofiltration in a temperate region.

Keywords: *Arsenic, accumulation, availability, distribution, habitat, phytoremediation, rhizosphere, redox potential, speciation.*

List of papers

The following papers, referred to by their Roman numerals, are the basis of this thesis:

I. Bergqvist, C., Greger, M. 2012. Arsenic accumulation and speciation in plants from different habitats. Appl Geochem 27, 615–622.

II. Bergqvist, C., Greger, M. 2013. The effect of O_2 on the concentration and speciation of arsenic in sediment, water and *Elodea canadensis*. Manuscript.

III. Bergqvist, C., Greger, M. 2013. Phytostabilization of arsenic. In: Bundschuh, J., Hollaender, H., Ma, L.Q. (Eds). In-situ remediation of arsenic-contaminated sites. CRC Press, Boca Raton, FL. ISBN: 978-0415620857.

IV. Bergqvist, C., Herbert, R., Persson, I., Greger, M. 2013. Accumulation and speciation of arsenic (As) in vegetables cultivated in soils with different As availability. Manuscript.

V. Greger, M., Bergqvist, C., Sandhi, A., Landberg, T. 2013. Influence of silicon on arsenic uptake and toxicity in lettuce. Manuscript.

Papers I and III are reproduced with the permission of Elsevier. My contribution to the manuscripts includes the writing and planning of papers I - IV with the assistance of the co-authors, collecting field samples in paper I, and performing the laboratory work in papers I-II, IV. In paper IV, co-authors assisted with the XANES analysis and in paper II, I had some assistance with the laboratory work. In paper V, I participated in the writing of the introduction and discussion parts, and performed some of the lettuce analysis.

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Abbreviations

AAS	Aatomic absorption spectroscopy
AF	Accumulation faction (As plant : As soil ratio)
As	Arsenic
DMA	Dimethylarsinic acid
DW	Dry weigh
FW	Fresh weigh
HPLC	High-pressure liquid chromatography
MMA	Monomethyl arsenic acid
OM	Organic matter
PC	Phytochelatin
ROS	Reactive oxygen species
S/R	Shoot to root ratio
TeMA	Tetramethylarsonium ion
TMAO	Trimethylarsine oxide
XANES	X-ray absorption near-edge structure spectroscopy

1. Introduction

1.1 Arsenic in natural surroundings

Present in more than 200 minerals, arsenic (As) is represented as the twentieth most common basic element in the earth´s crust (Zhao et al. 2010). The As mineral composition which comprises more than 99 % of the world´s As, mainly consists of sulphurous and silicate minerals, while As forms solids with Al, Ca, Fe, Mg, Ni and S in soils (Bhumbla and Keefer, 1994). In soil pores and water, As is soluble in several different forms known as As species. The most common As species are presented in figure 1.

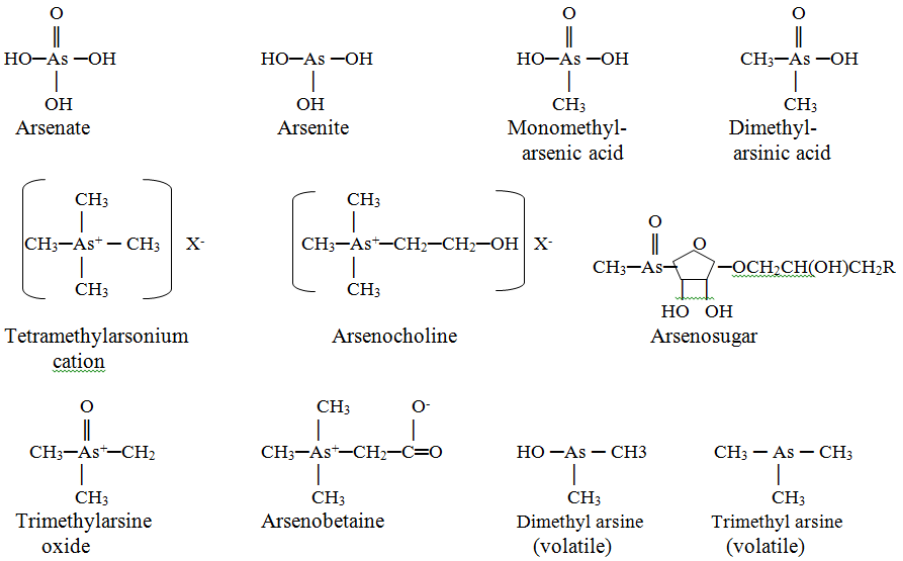

Figure 1. The most common As species found in nature. X = accompanying anion (Modified from Meharg and Hartley-Whitaker, 2002).

The oxidation states vary between −3, 0, +3, and +5 in the As species, but the predominating oxidation states in reducing conditions is -3 and in oxidizing conditions +5 (Moreno-Jiménez et al. 2012). Arsenate and arsenite are generally the predominating As species consisting of several forms. At pH 7 the general distribution of arsenate in descending order is, $HAsO_4^{2-}$ > $H_2AsO_4^-$ > $H_3AsO_4^0$. The general distribution of arsenite at pH 7 in descending order is, $HAsO_2^0$ = $H_3AsO_3^0$ > AsO_2^- = $H_2AsO_3^-$ > $HAsO_3^{2-}$ > AsO_3^{3-}

(Sadiq, 1997). Redox potential is usually the main determining factor of the ratio between arsenate and arsenite in soil. Generally, arsenite predominates under reducing conditions while arsenate predominates under oxidizing conditions (Sadiq, 1997). However, both biotic and abiotic factors may influence the arsenate–arsenite speciation, making predictions on As speciation solely based on redox potential uncertain (Ackermann et al. 2010).

The main factors influencing As speciation besides redox potential includes adsorption reactions, pH and biological activity (Bhumbla and Keefer, 1994). The effects on As speciation by pH and adsorption reactions cannot generally be summarized since their actions are highly dependent on the unique conditions at each site. Biological activity gives rise to an abundance of organic As species. The main organic As species found in soil and water, monomethyl arsenic acid (MMA) and dimethylarsinic acid (DMA), originate from the biological activity of microorganisms (Wood 1974) (Fig. 2). Arsenobetaine, which is the most common As species in marine animals is formed from arsenosugars originating in primary producers like algae, via the precursor arsenocholine (Francesconi and Edmonds, 1994). Also trimethylarsine oxide (TMAO) and tetramethylarsonium ion (TeMA) has been detected in marine animals (Hirahata et al. 2006).

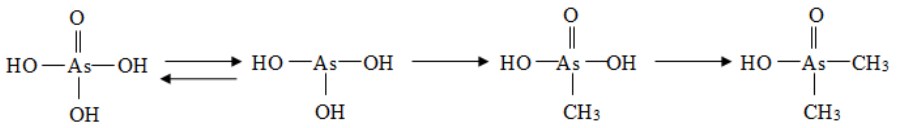

Figure 2. The transformations of the most common inorganic and organic As species are the result of both abiotic and biotic factors

1.2 Anthropogenic arsenic

Ever since the discovery of elemental As by the German alchemist Albertus Magnus (1193-1280), As has been increasingly released into the environment from human activities including mining, wood-impregnation, agriculture, fossil fuel treatment plants, glass production and military activities (Moreno-Jiménez et al. 2012). This has resulted in extensive As pollution in the environment. In the European Union, there may be up to 3 million sites polluted by anthropogenic activities and approximately 80000 of these are found in Sweden, with elevated levels of As in 25 % of these sites (EEA, 2007; Naturvårdsverket, 2009). Historically, diarsenicpentaoxide used in wood impregnation has represented most of the As use, and consequently

most of the pollution, in Sweden. However, the use of As in Sweden suddenly dropped after 2003 due to stricter rules towards the use of As in wood impregnation (Fig. 3).

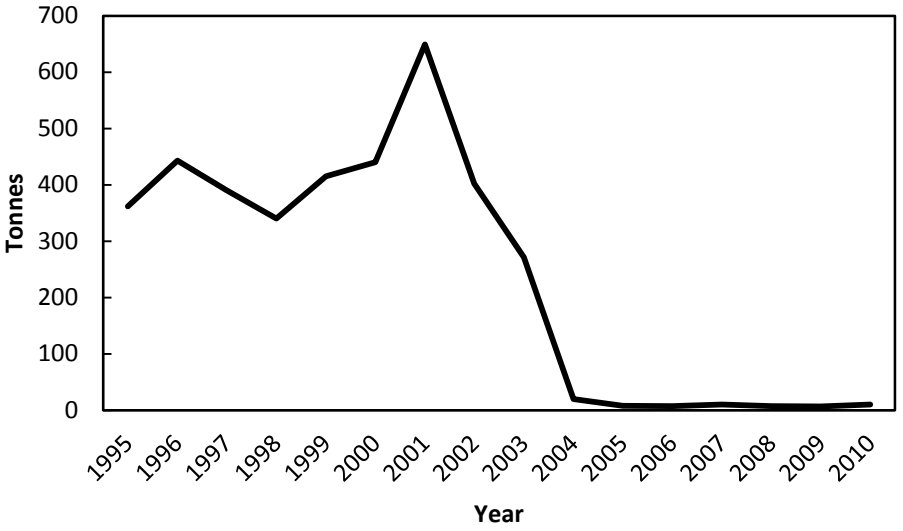

Figure 3. The use of As in products in Sweden between the years 1995 to 2010 (Produktregistret, 2010).

1.3 Arsenic toxicity to humans

Even at low concentrations, exposure to As may give rise to a variety of adverse symptoms, the most common ones being cancer in liver, lung, skin, bladder and kidney (Smith et al.1992). To counteract its negative effects, As is excreted via the urine. The basic steps of detoxification include absorption of inorganic As species in the intestine, methylation in the liver mainly to DMA and excretion via the urine (Suzuki et al. 2001). However, a negative side effect of the detoxification process is the production of the methylated intermediates such as methylarsonous acid ($CH_3As(OH)_2$) and dimethylarsinous acid ($(CH_3)_2AsOH$) in the human liver, which has trivalent oxidation states of the As atom. Trivalent methylated intermediates are responsible for many of the negative effects of As by an increased toxicity compared with the originally ingested inorganic As species in terms of cyto- and genotoxicity and enzymatic inhibition (Dopp et al. 2010).

Contrary to the general opinion of the negative effects of As, a few reports of positive actions of As to humans have been reported, for example in the formation of metabolites from methionine such as taurine and polyamines

(Nielsen, 1991). Generally, humans are reported to need small amounts of As to avoid problems like infertility, increased fetal mortality and growth inhibition (SGU, 2005).

1.4 Arsenic species toxicity

Organic As species, with the exception of the trivalent methylated intermediates mentioned above, are generally considered to be less toxic to living organisms than the inorganic species arsenite and arsenate (Meharg and Hartley-Whitaker, 2002). Of the inorganic As species, arsenite is considered to be more toxic than arsenate (Bhumbla and Keefer, 1994). Arsenobetaine and other organic As species found in marine organisms are generally considered non-toxic (Kaize et al. 1985). The determination of the amount of toxic As species is therefore of greater interest than total As concentration in food and water.

1.5 Availability of arsenic to plants

Arsenic in solution is readily available for uptake by plant roots or submerged shoots. In soil, however, the total As concentration does not always reflect the availability of As to plants and other organisms. The composition of the soil has a large influence on the availability of As. In soils containing iron, calcium and aluminum, the availability of As usually is low due to the formation of As carbonates and oxides/hydroxides (Sadiq, 1997). The geochemical form of As also determines availability, for example, As trioxide is highly available while the availability of arsenopyrite is low (Meunier et al. 2011).

Also the structure of the soil matters, for example, the availability of As in sandy soils is relatively high due to the low contents of clay which otherwise tends to bind As in the soil (Silva Gonzaga et al. 2012). The toxicity of As is consequently higher in sandy soils (Sheppard, 1992). The availability of As in clayey soils is generally low due to the large surface area for adsorption of As in these soils (Kumpiene el al. 2008). High organic matter (OM) generally increases the availability of As by forming aqueous complexes containing As and through electrostatic interactions with soil particles (Wang and Mulligan, 2009). However, the formation of As-OM complexes may also reduce availability in soils with high OM (Mikutta and Kretzschmar, 2011). Mineralization may also increase the availability of As in high OM soils due to the release of As previously bound in the OM, while the availability in soils with low OM remains relatively stable over time (Meunier et al. 2011).

In addition to soil structure and composition, other factors such as redox potential and pH affect the availability of As in soils. In general, a high redox potential promotes the predomination of arsenate which adsorbs strongly to aluminium and iron oxides leading to a lower availability, while a low redox potential promotes the formation of arsenite which has a low adsorption leading to a higher availability (Zhao et al. 2010). The effects of pH on As availability are usually complex. Generally, a high pH increases the availability of As due to the competition of binding sites with hydroxyl ions, however, a high pH may also favour the co-precipitation of As with calcium or sulfate, leading to a reduced As availability (Moreno-Jiménez et al. 2012). Also low pH may increase the availability of As, because arsenate may become fully protonated in pH<2.5 leading to higher mobility (Moreno-Jiménez et al. 2012).

1.6 Plant accumulation of arsenic

The accumulation of As by plants is influenced by a number of factors such as the As concentration in soil, As availability and redox potential. According to Baker (1981) plants can be divided into three groups depending on the response to increasing soil concentrations of an element, i.e. accumulators, indicators and excluders. The accumulators concentrates the element in the aboveground parts, the element concentrations in indicators reflects the external concentrations while the excluders prevents element uptake until the soil concentration gets too high (Baker, 1981) (Fig. 4).

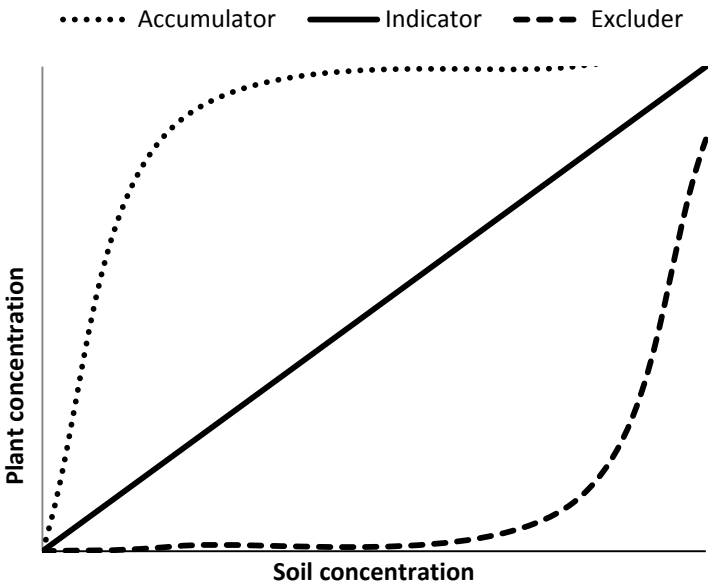

Figure 4. Element uptake in accumulator, indicator and excluder plants. Modified from Baker (1981).

The accumulation of As in terrestrial and emergent plants generally occurs via the roots, but also via shoots in areas with atmospheric depositions of As (De Temmerman et al. 2012). Aquatic macrophytes accumulate As from the water column as well as from the sediment (Azizur Rahman and Hasegawa, 2011) (Fig 5). For uptake via roots, plants are able to modify the rhizosphere to render elements available, for example by the exudation of organic acids, which has a major effect on the mobilization of elements bound to ion exchange sites in the rhizosphere (Marschner, 1995). The action of organic acids originating from plant roots may also increase As availability in soil leading to an increased plant As uptake (Moreno-Jiménez et al. 2012). For example, extraction of As from soil using plant specific organic acids showed a correlation between the As concentration in the plant and the As concentration in the soil, exemplifying the action of organic acids on As availability in soil (Silva Gonzaga et al. 2012). Also bacteria in the rhizosphere may help to increase the As availability, probably by solubilizing arsenates from insoluble $FeAsO_4$ and $AlAsO_4$ in the soil, resulting in a higher plant As uptake (Ghosh et al., 2011).

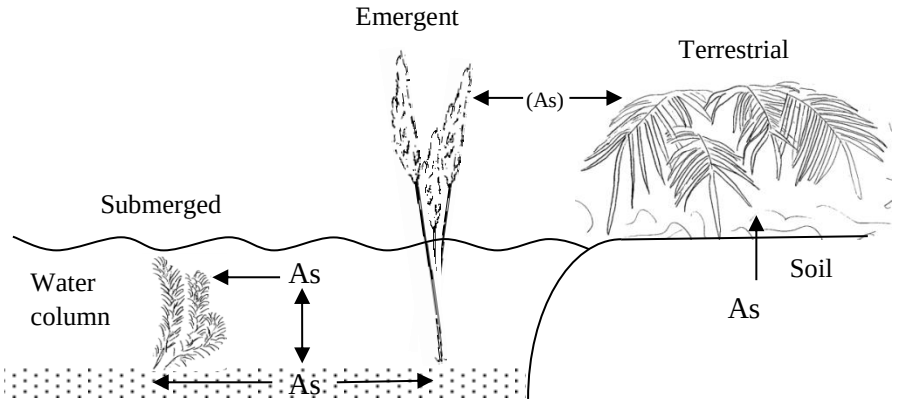

Figure 5. Uptake routes of As in terrestrial, emergent and submerged plants. The uptake from air is usually negligible due to the low As concentration in air in normal conditions.

1.6.1 Distribution of arsenic in plants

For terrestrial and emergent plants, As is primarily, and most commonly accumulated in the roots, with low concentration in the shoot, for example in *Populus alba* and *Typha latifolia* (Di Lonardo et al. 2011; Afrous et al. 2011). The distribution of As may be higher to the shoots compared with the roots in some plant species, for example in the As hyperaccumulating fern *Pteris vittata* (Ma et al. 2001), and in some reports of non-hyperaccumulating plants like radish (*Raphanus sativus*) (Smith et al. 2008). In submerged plants, the As concentration in the shoot may be higher than the As concentration in the root, due to As accumulation by the shoots from the surrounding water column (Bergqvist and Greger, 2012).

1.6.2 Apoplasmic accumulation of arsenic

Arsenic enters the plant body through diffusion into the apoplasm from the soil solution or surrounding water body (Moreno-Jiménez et al. 2012). Accumulated As around the vascular bundles in plant roots and stems indicates a mechanism for As detoxification by apoplasmic depositions (Sridhar et al. 2011). Arsenic may be retained in the apoplasm through the passive binding to special active functional groups (Vithanage et al. 2012), but the exact mechanisms for this potential As detoxification mechanism is not clearly

established (Moreno-Jiménez et al. 2012). A large portion of the total As in plants may be situated in the apoplasm, for example >50 % in *Panax notoginseng* and >60 % in rice (*Oryza sativa*) of the total As, were found in the cell wall fractions during analysis (Yan et al. 2012; Bravin et al. 2008). In many instances, further entrance of As into the plant cells is restricted.

1.6.3 Cellular accumulation of arsenic

Symplasmic accumulation of As from the apoplasmic compartments into the cell cytoplasm occurs through cell membrane transporters used to transport nutrients like phosphate and silicon. Arsenite accumulation occurs through silicon aquaglyceroporins (Zhao et al. 2009), which also facilitate the transport of MMA and DMA (Azizur Rahman et al. 2011) (Fig. 6). The only known paths for arsenate accumulation in plants are through phosphate transporters (Moreno-Jiménez et al. 2012) (Fig. 6).

1.7 Toxicity of arsenic to plants

The direct toxic effects of the main As species includes an interference with phosphate metabolism by arsenate and enzyme inactivation by arsenite due to -SH bindings (Sharma, 2012). Plants exposed to either arsenate or arsenite produce reactive oxygen species (ROS) (Srivastava et al. 2007). The ROS are generated due to electron leakage during arsenate-arsenite reduction and the inhibition of key enzyme systems, which results in a number of damaging effects including membrane leakage, glutathione depletion and reduced photosynthetic activity (Sharma, 2012). Generally, arsenite is considered more toxic than arsenate, which in turn is more toxic than organic As, but some reports show a different order of toxicity, possibly due to differences in sensitivity to different As species between different plant species (Finnegan and Chen, 2012).

1.8 Plant defence mechanisms to arsenic

The first line of plant defence towards As is the retention of As in the rhizosphere. Examples of this includes the formation of iron plaque through the active release of O_2 from roots in flooded soils or the formation of iron oxide/hydroxides surrounding roots in aerated soils, which adsorbs As and reduce plant uptake (Moreno-Jiménez et al. 2012). Also reduced cellular

uptake is an efficient way to alleviate detrimental effects of As. Resistant plants suppress the high-affinity phosphate/arsenate uptake system leading to a reduced As uptake (Meharg and Hartley-Whitaker, 2002). Moreover, mycorrhizae may help to counteract the adverse effects of As for plants through selective uptake of phosphorous and efflux of As from the hyphae (Sharples et al. 2000). Inoculation of pea (*Pisum sativum*) with arbuscular mycorrhiza reduced the As uptake and increased both the nutritional and antioxidative status of the plant compared with un-inoculated plants (Garg and Singla, 2012). Similar results was shown for white clover (*Trifolium repens*) and ryegrass (*Lolium perenne*) inoculated with *Glomus mosseae*, which resulted in reduced plant As uptake and improved phosphorous status (Dong et al. 2008).

After cellular exposure by As, plants activate a number of defence mechanisms. The reduction of detrimental ROS is performed by enzymes like ascorbate peroxidase, catalase, superoxide dismutase glutathione reductase, guaiacol peroxidases and glutathione S-transferase as well as by non-enzymatic antioxidant molecules like ascorbate, glutathione and carotenoids (Sharma, 2012). Complexation of As by phytochelatins (PCs) have been shown in terrestrial plants like *Brassica juncea*, *Holcus lanatus*, *Pteris vittata* and *Silene vulgaris* (Mokgalaka-Matlala et al. 2009). Also in aquatic plants, As forms complexes with PCs. For example in *Wolffia globosa*, 74 % of the As was complexed with PCs and an inhibitor of PC-synthesis markedly increased the toxic effects by As to the plant (Zhang et al. 2012).

Figure 6 describes an overview of the general routes for As within root cells and tissues of plants. The major processes include the arsenate-arsenite reduction by gluthatione and the arsenite complexation with phytochelatins (PCs), a complex which is then transported to the vacuole or to the shoots (Tripathi et al. 2007). The transportation of the As-PC-complex into the vacuole is most likely facilitated by an unidentified ATP-binding cassette superfamily type transporter (Verbruggen et al. 2009). Arsenite may also be effluxed from the cells via members of the Nodulin26-like Intrinsic Proteins (NIP) subfamily of aquaporins in the plasma membrane (Bienert et al. 2008).

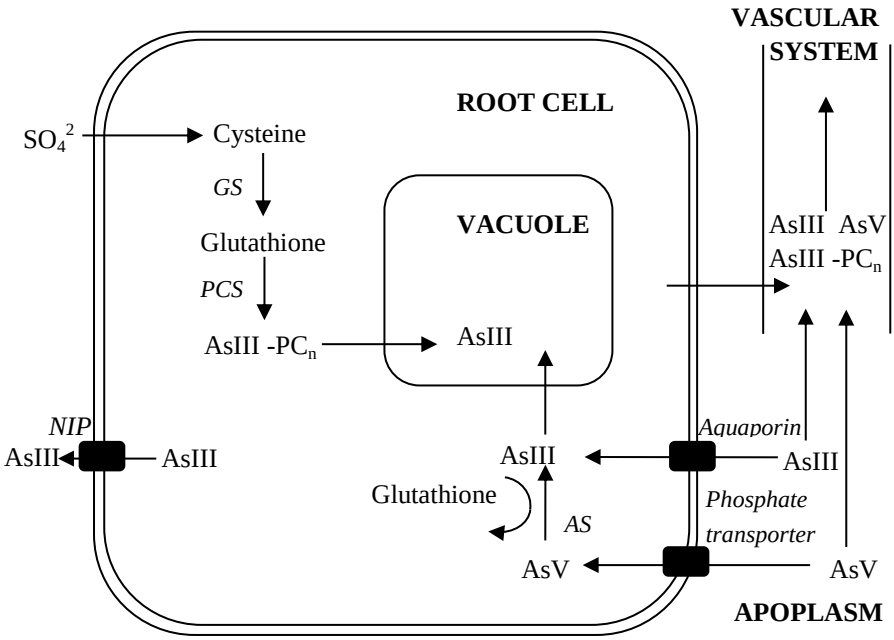

Figure 6. Uptake and detoxification in the root cell and transport of As in the vascular system of plants. AsV=arsenate, AsIII=arsenite. Glutathione synthetase (GS) produces glutathione from cysteine which is based on sulfate. Phytochelatins produced from glutathione by phytochelatin synthase (PCS) binds arsenite (AsIII -PC_n) and transports it into vacuoles or to the shoot as arsenite or as a phytochelatin-arsenite complex. NIP aquaporins may efflux arsenite. Arsenate is reduced to arsenite by arsenate reductase (AS) using glutathione as a reductant (Modified from Tripathi et al. 2007).

1.9 Influence of silicon on arsenic in plants

For the most higher plants, silicon is considered beneficial, but whether it is essential to plants is disputed because plants can survive without silicon (Epstein, 2009). A major part of the beneficial action of silicon to plants includes a decrease of pathogenic attacks and an increase in plant rigidity (Marschner, 1995). Besides such general beneficial effects, silicon may also alleviate As stress in plants by reducing As uptake. For example, silicon fertilization reduced the As accumulation in rice, probably by interfering with the cellular silicon transporters aquaporins Lsi1 and Lsi2, which also mediate the uptake of arsenite (Fig. 6) (Li et al. 2009). Possible interactions on the phosphate uptake system by silicon may in part also reduce arsenate uptake, since the phosphate uptake system also is responsible for cellular arsenate uptake (Guo et al. 2007). Silicon may also reduce As accumulation

in the apoplasmic compartment of plants, through interaction effects with the special active functional groups responsible for the passive binding of As in the apoplasm (Vithanage et al. 2012). For example in rice roots, silicon was mainly distributed in the endodermal cell walls, while As mainly localized in the vacuoles (Moore et al. 2011).

Silicon may also reduce the cellular toxicity of As within plant cells, by increasing the antioxidant activities which alleviate the negative effects of the reactive oxygen species (ROS) generated in plants exposed to As (Liu et al. 2009).

1.10 Dietary arsenic

Drinking water constitutes the most prevalent mode of As intake in humans. Millions of people on all continents, and especially in South East Asia, are continuously exposed to As- contaminated drinking water (Nordstrom, 2002). Based on the risk for developing cancer during a lifetime exposure to As, the limit for As drinking water is set to 10 µg l^{-1} in the EU (Commission Directive 2003/40/EC). This limit is also recommended by the WHO (World Health Organization) and common throughout the world.

Dietary intake of As from food may also provide a significant input of As in humans. There is no worldwide consensus in regards to the limit for As in food. However, the European Food Safety Authority (EFSA) has based on epidemiological studies, suggested limit values between 0.3–8 µg of inorganic As kg^{-1} bodyweight per day, due to a 1 % increased risk of developing lung, skin, bladder cancers and skin lesions, respectively, at higher intake levels (EFSA, 2009).

Cultivation of vegetables in highly polluted As soil is not probable due to the phytotoxic effects of As. Generally, crop failure and crop retardation are the main effects of high As concentration in the soil (Mäkelä-Kurtto et al. 2007). However, vegetables cultivated in low to medium As polluted soil, for example home garden vegetables cultivated in Bangladesh, may contribute to As intake from diet (Rahman, et al. 2012).

Rice (*Oryza sativa*) is generally considered as the main contributor of As from food. The concentration of As in rice is usually low, < 0.4 mg kg^{-1} (DW), but the normal consumption of approximately 200 g (DW) of rice common in Asian diets, may result in a relatively high As intake (Zhu et al. 2008). Also a variety of vegetables can accumulate As in their edible parts (Baig and Kazi, 2012). Vegetables cultivated in low As polluted soil (<10 mg As kg^{-1}) normally contains low levels of As (<1 mg As kg^{-1} DW)

(Bhattacharya et al. 2010), while vegetables cultivated in high As polluted soil (3-400 mg As kg^{-1}) generally contains up to 5 mg As kg^{-1} (DW) (Vamerali et al. 2011). Hydroponic cultivation usually results in higher As accumulation, for example > 30 mg As kg^{-1} (DW) in radish (Smith et al. 2009). Arsenic has also been shown to bio-accumulate in animal products like milk and meat when the livestock was exposed to As (Bundschuh et al. 2012). However, the problem with rice is more comprehensive than other food products due to its nature as staple food. Also, poverty may often escalate the problems with dietary As. For example, the consumption of the lower-priced brown rice, which has a higher As concentration than white rice, is higher compared with white rice in rural Bengal (Halder et al. 2012).

1.11 Phytoremediation of arsenic

In the European Union, there may be up to 3 million polluted sites, many containing As, with 250000 of those in urgent need of restoration (EEA, 2007). Here, phytoremediation could be applied as an alternative to traditional remediation techniques like chemical treatments and land filling (Sarma, 2011). Phytoremediation can be defined as the removal, degradation or immobilization of pollutants using plants (Ward and Singh, 2004). There are four main types of phytoremediation; phytoextraction, phytostabilization, phytofiltration and phytovolatilization (Sarma, 2011) (Fig. 7).

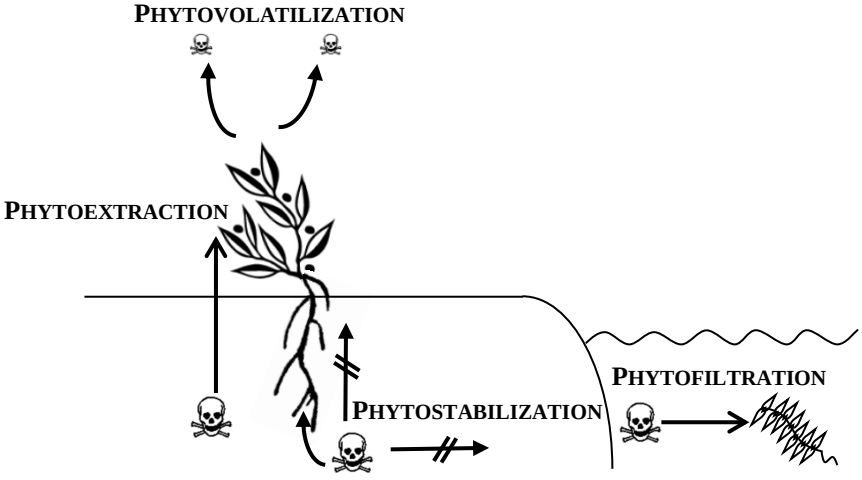

Figure 7. The four main types of phytoremediation; phytoextraction, phytovolatilization, phytostabilization and phytofiltration.

The main advantages of phytoremediation compared with traditional remediation techniques like land filling and chemical treatments includes cost-effectiveness and a more eco-friendly approach, while the limitations includes a long time-span, efficiency problems and phytotoxicity in sites with multiple contaminants (Sarma, 2011). Further beneficiary effects of phytoremediation includes the potential of additional economic values, besides the remediation, like the production of bioenergy, wood, ecological services and dust control (Robinson et al. 2009). Plants especially interesting in regards to phytoremediation are the hyperaccumulators. Hyperaccumulators are plants with the ability of accumulating >1000 mg kg^{-1} (DW) of metals or metalloids in the shoots (Brooks et al. 1977). The only detected terrestrial plant species capable of As hyperaccumulation are 12 species of ferns from the family Pteridaceae (Zhao et al. 2009). However, several aquatic macrophytes have been reported to accumulate >1000 mg As kg^{-1} (DW) (Favas et al. 2012; Robinson et al. 2006).

The phytoremediation techniques which may be suitable for As remediation are phytoextraction, phytostabilization and phytofiltration.

1.11.1 Phytoextraction

To perform a successful As phytoextraction, plants with the ability to extract relatively large amounts of As in the shoots in combination with a large biomass are ideal, since the two major factors determining phytoextraction efficiency are the plant to soil concentration ratio and the biomass production (Rascio and Navari-Izzo, 2011). For As phytoextraction, the As hyperaccumulating ferns belonging to the family Pteridaceae are of special interest (Zhao et al. 2009). However, these hyperaccumulating ferns are tropical, and not suitable for As phytoextraction in temperate regions. Other problems with the use of hyperaccumulators are that they usually are element selective which limits the use in multiple contaminated sites, have low biomass and slow growth rates limiting the speed of removal (Rascio and Navari-Izzo, 2011). More suitable candidates for general phytoextraction include trees like poplars (*Populus sp.*) and willows (*Salix sp.*), which have relatively high accumulation properties, high biomass and the ability to grow in a wide range of climatic conditions (Bhargava et al. 2012). However, to this date, no successful As phytoextraction from a contaminated site has been reported.

1.11.2 Phytostabilization

The goal of phytostabilization is to immobilize pollutants in plant roots and onto soil particles, and thereby creating a self-sustaining, vegetative cap,

preventing pollutant dispersal from an area in a long-term perspective (Mendez and Maier, 2008). Phytostabilization may be the only realistic form of remediation in areas where the level of contamination is high. The selection of plant species used for phytostabilization should focus on native plant species, to prevent the introduction of potentially invasive plant species (Mench et al. 2010). The characteristics for plants suitable for use in phytostabilization include a high tolerance to pollutants in the soil and a low accumulation of pollutants in shoots (Butcher, 2009). Trees are often regarded as good candidates for phytostabilization due to their ability for deep rooting and high rates of evapotranspiration which reduces the pollutant mobility (Pulford and Watson, 2003). To promote plant growth in soils with poor quality, amendments like organic matter may have to be supplied to initialize phytostabilization (Moreno-Jiménez et al. 2010). To prevent an increased mobility of As which may result from adding organic matter amendments, additions of for example iron-based amendments with reduces the mobility of As (Kumpiene el al. 2008), may be necessary for a successful phytostabilization.

1.11.3 Phytofiltration

Phytofiltration may be easily implemented as a successful As remediation technique since constructed wetlands for storm- and wastewater already are in use. The cost scenario for establishing As phytofiltration may therefore be more beneficial compared with traditional As removal techniques from water including physical procedures like reversed osmosis to a range of inorganic and organic As adsorbents like ferrihydrite, peat and clay minerals, and As immobilization using bacteria (Ng et al. 2012). Submerged macrophytes are able to accumulate relatively large amounts of As directly from the water, due to a thin cuticle and a high biomass production, even under limited nutritional conditions (Xue et al. 2012). Submerged macrophytes from Portugal have been shown to contain 300 - 500 mg As kg^{-1} and *Callitriche lusitanica* > 2000 mg As kg^{-1} (Favas et al. 2012). Even dead macrophytes may be able to accumulate As from water, as seen with the accumulation of Cd from water (Fritioff and Greger, 2007).

2. Aim

This work was set out to investigate how a number of external factors influenced As accumulation and speciation in plants for food and phytoremediation (in a temperate region). The external factors were chosen based on theory of factors which have a major effect on the As accumulation and speciation in plants. The overall aim of this thesis was to evaluate how the external factors influenced the efficiency of phytoextraction and phytofiltration and the risk of dietary As in crops. Specifically, the analyses were focused on the following factors and questions:

1. *Plant habitat*. How did the habitat of submerged, emergent and terrestrial plants influence the As accumulation properties in plants suitable for As phytoremediation?

2. *Oxygen level*. How did low, medium and high O_2 levels influence the As speciation in submerged plants and the efficiency of phytofiltration?

3. *Arsenic availability from soil*. How was As availability from soil associated with As accumulation by vegetables, and how did plant root organic acids influence the bioavailability of As in the rhizosphere?

4. *Silicon influence*. Did silicon influence the accumulation, distribution and speciation of As in the vegetable lettuce (*Lactuca sativa*)?

3. Comments on the materials and methods

3.1 Plants

The geography of Sweden results in a short vegetation season and a cold climate. The most suitable candidates for understanding As accumulation in Sweden are therefore local ecotypes. However, also Slovakian plants were collected for comparison. Self-sown plants in areas with natural or anthropogenic As were collected for analysis. The plants were grouped as submerged, emergent and terrestrial in order to make comparisons between the different habitats in terms of As accumulation and speciation. Factors like As concentration in soil, longitude-latitude and soil composition influence the As accumulation in plants. It may be noted that the examples from different habitats were collected at one location and that other factors might have influenced the AS accumulation than only the habitat, for example when comparing a submerged plant in the south of Sweden with a terrestrial plant from the north (Paper I).

The crop species, carrot, lettuce and spinach, were selected on the basis of being common vegetables with a relatively short time span from sowing to harvest. An important observation during the cultivation of lettuce was that the accumulation of As was higher in lettuce cultivated in the natural vegetation season compared with the lettuce cultivated in the winter time (paper IV).

Results from paper I indicated that submerged plants were able to accumulate high levels of As. The submerged macrophyte *Elodea canadensis* was selected for further studies because it is common around the world, grows fast and competes successfully against other submerged macrophytes, as well as previous promising results using *E. canadensis* for As phytofiltration (Greger et al. 2010). The concentration of As in the experiments with *E. canadensis* was chosen as to represent conditions in a natural pollutant situation, and below phytotoxic concentrations (Paper II).

3.2 Arsenic-species extraction

The procedure of extracting of As species from plant material was constantly modified throughout the experiments. The basis for the extraction protocol was developed by Mir et al. (2007). Extraction of As species was performed

using MeOH:H_2O(1:1)-solution followed by a 0.1 M HCl-solution in all experiments. The preparation of the plant material was, however, modified throughout the experiments. The plants in paper I were dried in room temperature before As species extraction while the plants in papers II and III were extracted as fresh plant material. In paper V, some of the early experiments were performed with dried plant material while the later experiments were performed with fresh plant material. The relative abundance of arsenate in the terrestrial and emergent plants analysed in paper I compared with the vegetables analysed in paper IV, could be due to the extraction procedure because the plant parts were air-dried in room temperature before As extraction in paper I. The air drying could have resulted in the oxidation of some of the arsenite into arsenate (paper I), which was the reason for the modification of the method.

The attempts to improve the extraction efficiency of the As species were continuous throughout the experiments as it ranged between 3 - >100 %. Low extraction efficiencies may be due to a number of factors, like physical and chemical As bonding to the plant matrix, immobility of As in the vascular tissues and insoluble forms of As in the plant (Mir et al. 2007). The reasons for the problems of getting all the As in the plant material in solution were not investigated in detail but it is likely that the particle size of the plant material exposed to the extraction solutions had an influence. For example, it was hard to fragmentize woody plants enough leaving relatively large fragments of plant material after the extraction. Presumably some of these fragments contained As. The idea that the particle size was involved in the extraction efficiency was strengthened further after seeing that the introduction of a finer dispersing tool doubled the extraction efficiency of As from *Elodea Canadensis* compared with using the coarser dispersing tool (paper II). However, the varying extraction efficiencies were sometimes puzzling since extractions from the same plant species exhibited different extraction efficiencies despite seemingly identical extraction procedures (papers I-II, IV-V).

Silicon may also influence the extraction efficiency. Addition of silicon along with arsenite significantly decreased the extraction efficiency of As from lettuce shoots compared with only arsenite addition (paper V). However, the extraction efficiency was not significantly altered in arsenate treated roots or shoots or in lettuce roots treated with arsenite (paper V). Since almost half of the As in lettuce was located in the apoplasm (paper V), the reason for the decreased extraction efficiency in arsenite treated lettuce shoots could be related to changed adsorption properties in the cell walls. Silicon additions to plants increased the secondary cell wall components (Yamamoto et al 2012), which could influence the adsorption of As in the cell walls as well as the solubility of As during As extraction, resulting in differences in the efficiency of As extraction.

3.3 Arsenic-species analysis

The methodology for separating and detecting the As species arsenate, arsenite, MMA and DMA was developed from an initial, relatively inaccurate method towards more precise measurements. First, after the separation of the individual species in the HPLC (high-pressure liquid chromatography), the eluent from the HPLC column outlet was manually extracted in fractions followed by analysis for As concentration in the AAS (atomic absorption spectroscopy). However, this method was tedious and unreliable. Instead, the HPLC was connected directly to the pump facilities in the hydrid generator (VGA-77) and pumped into the AAS. The separation of the As species was dependent on the specificity of the column, the eluent and the flow rate of the system. The first anionic column tested (Ionpac AG9-HC, Dionex), with $NaCO_3$ eluent was not specific enough since arsenite was not separated from DMA. Modifications of the eluent in terms of pH and composition (methanol+acetonitril, methanol+H_2O, methanol+$NaCO_3$) were not able to separate arsenite from DMA. The general conclusion from the eluent-modifications was that methanol was not a suitable eluent during As species separation since the adsorption abilities in the column of some of the As species were negatively affected by methanol resulting in a lack of concentrated peaks during detection. The second anionic column tested (Hamilton PRP X-100 anion exchange column, 250mm x 4.6 mm), was able to achieve some separation of arsenite from DMA with the 30 mM phosphate buffer (pH 6) as eluent, but the species were still overlapping. After modifying the eluent to 20 mM phosphate buffer (pH 5.8) the peaks finally separated from each other. A flow rate of 1 ml min^{-1} was used to obtain optimal separation of the peaks (Fig. 8). The interaction effects of the matrix were eliminated by the addition of chemical standards to each sample in the analysis. The detection limit for arsenate was 9 µg L^{-1}, for arsenite 1.5 µg L^{-1}, for MMA 3 µg L^{-1} and for DMA 7 µg L^{-1}.

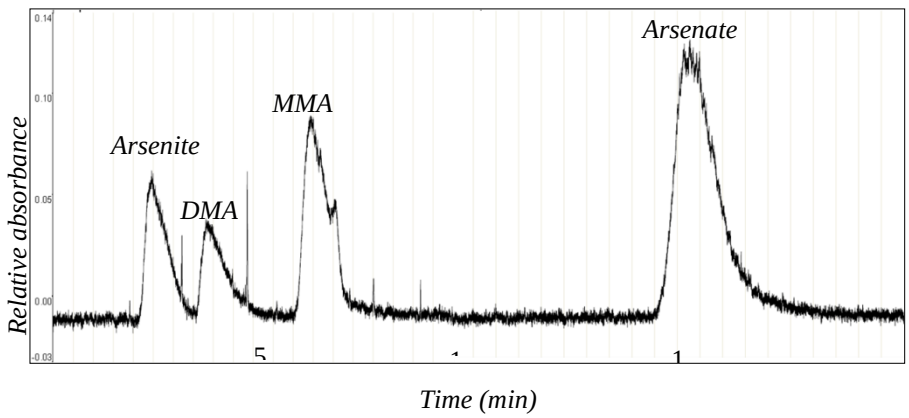

Figure 8. Separation of As standard peaks using the Hamilton PRP X-100 (250mm x 4.6 mm) anion exchange column. Detection was performed with AAS vapour generation technique.

Some of the samples analysed with HPLC-AAS was also analysed with X-ray absorption near-edge structure (XANES) spectroscopy (Fig. 9). The intention of using the (XANES) spectroscopy was to supplement the determination of As species with the HPLC-AAS. For As species analysis, the XANES spectroscopy supplied relative data on the As species, providing an excellent tool for verification of the quantitative data acquired by the HPLC-AAS measurements. The usefulness of XANES spectroscopy to complement As-species analyses on HPLC-AAS has previously been reported (Mir et al. 2007).

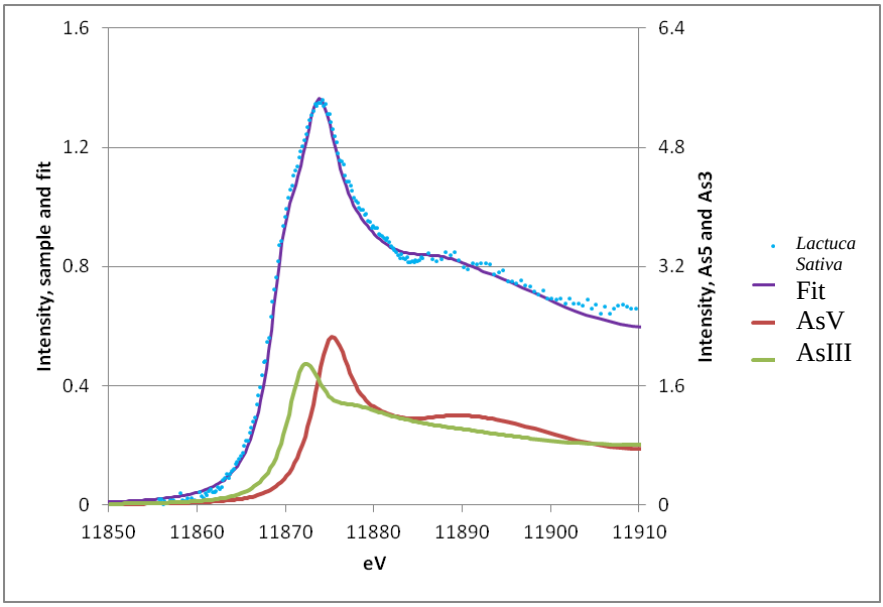

Figure 9. Example of XANES spectroscopy. This sample spectrum was fitted with other spectra of linear combinations of the arsenate (AsV) and arsenite (AsIII) species with different ratios (1:9, 1:1 and so on) to determine the concentration of arsenate and arsenite in the samples.

4. Results and Discussion

This work aimed at investigating factors affecting the risks of dietary As intake from vegetables as well as influencing As phytoremediation. Arsenic availability to plants, As species toxicity and the possible effects of silicon applications, is of special interest when determining dietary risks with As. For phytoremediation, the plant habitat and the influence of O_2 on As accumulation in submerged plants is of special interest.

4.1 Arsenic in diet

Food is the main contributor of As to populations not exposed to As in drinking water (Zhao et al. 2010), and the consumption of dietary plants cultivated in As media may result in an eleveated As intake. Based on the lowest limit value of As for food, established by the European Food Safety Authority (EFSA), of 0.3 µg As kg^{-1} bodyweight per day, a 60 kg person should not be exposed to more than 18 µg of inorganic As per day from food (EFSA, 2009).

4.1.1 Arsenic availability to vegetables

Cultivation of crops in As containing soil is common throughout the world, for example in soil originating from alum shale soil (paper IV), and in fertile paddy fields of South East Asia (Zhao et al. 2010). Whether or not a vegetable cultivated in As-containing soil will accumulate potentially harmful amounts of As or not is influenced by several factors, but one of the major ones is the availability of As to the plant. Generally, the higher As extractability from soil, the higher the As content in the plant (Giri et al. 2012). As exemplified by using the As extractability from soil with HCl (hydrochloric acid), which can be used to estimate plant available As (Martínez-Sánchez et al. 2011), a correlation between the As concentration in lettuce and As extractability from soil was shown (paper IV). A correlation between acid (including HCl) extractable As and plant As has previously also been shown for plants growing in mine areas in Spain (Anawar et al. 2008), as well as under varying concentrations of As in soil for the same cultivar of rice (Giri et al.

2012), indicating the suitability of HCl as an indicator of plant available As. The lettuce in paper IV was cultivated in two soils; one alum shale soil with almost twice as high As concentration in soil as the other, comparing glassworks soil (paper IV). The results showed that both the As concentration in lettuce and the extractable As in the glassworks soil was approximately 15 times higher compared with the alum shale soil. The higher accumulation of As in plants grown in the glassworks soil is notable, since the alum shale soil containing 142 mg As kg^{-1} (DW), did not result in alarming concentrations of As in the edible parts (1.41±0.17 mg kg^{-1} DW). According to the Swedish environmental protection agency, 142 mg As kg^{-1} (DW) in soil, is characterised as being a "serious" level of As pollution (Naturvårdsverket, 2002). However, the higher the As concentration in the alum shale soil as compared with the glassworks soil was evidently not having a major influence on the As accumulation in lettuce. Instead, the higher As accumulation in lettuce cultivated in glassworks soil was probably related to the higher plant available As in the glassworks soil (paper IV).

The availability of As in solution is usually higher compared with soil. This could present a problem for commercial cultivations of vegetables, which is often performed on mineral wool with nutrients added via solution. The use of As polluted water for the nutrient solution could result in As accumulation in the vegetables, even at a relatively low water As concentration. The potential problem for commercial vegetable cultivation with the high As availability from solution, can be exemplified with the hydroponic cultivation of lettuce for four days compared with a soil cultivation of lettuce for four days (table 1). The total As concentration was approximately 100-200 times lower in the hydroponic solution compared with the soil, but resulted in a much higher As concentration in lettuce in the hydroponic cultivation compared with soil cultivation (table 1). Previous results have also shown differences between the As accumulation in plants growing in mine tailings compared with the same plant species in hydroponics, probably in part due to differences in As availability (Stoltz and Greger, 2002).

Table 1. Arsenic concentration in lettuce cultivated in hydroponics and soil for 4 days. Mean ±SE. (mg kg^{-1} DW). n=3.(Paper IV).

Growing medium		Total As	Available As	Shoots (mg As kg^{-1})		Roots (mg As kg^{-1})	
Soil	Alumshale	142.2 (mg As kg^{-1})	2.2±0.2 (mg As kg^{-1})	0.16	± 0.03	7.3	± 0.8
	Glassworks	77.7 (mg As kg^{-1})	35.3±0.8 (mg As kg^{-1})	0.23	±0.04	35.5	±7.5
Hydroponics	Arsenate	0.75 (mg As L^{-1})	0.75 (mg As L^{-1})	12.20	±1.82	568	±184
	Arsenite	0.75 (mg As L^{-1})	0.75 (mg As L^{-1})	7.01	±0.53	552	±127

Plant availability of As from soil may also be influenced by the vegetables themselves in the rhizosphere, for example by the exudation of organic acids. These acids may affect the mobilization of nutrients which also cause the release of As into the soil solution (Moreno-Jiménez et al. 2012; Silva Gonzaga et al. 2012). For lettuce cultivated in alum shale and glassworks soils, the extractability of As increased in the rhizosphere soil compared with the bulk soil (paper IV). The extractability in the rhizosphere soil was approximately doubled compared with the bulk soil and the doubling in extractability was regardless of high or low initial extractability (paper IV). One important factor of the increased extractability in the rhizosphere was probably succinic acid, which was present at more than 20 times higher concentration than other organic acids in the root exudates of lettuce (paper IV). Succinic acid could have similar actions on As availability from soil as dimercaptosuccinic acid (DMSA), which is a well known As chelator (Pickering et al. 2000), due to their chemical similarity (Fig. 10).

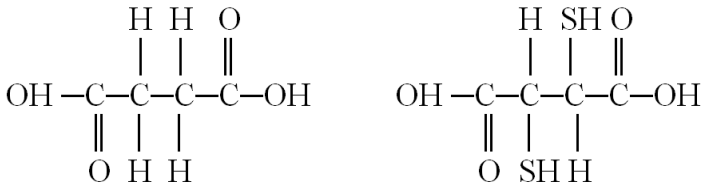

Fig. 10. Structural formulas of succinic acid and dimercaptosuccinic acid (DMSA), showing their similarities.

The influence of redox potential on the availability of As to plants is usually minor, since the vast majority of crops is cultivated in aerated soils, i.e. in soils with high redox potential, were the tightly bound, low plant-available arsenate predominates, as seen in the agricultural alum shale soil where only arsenate was detected (paper IV). Generally, a lower redox potential results in a predomination of arsenite which has a lower adsorption in soils, resulting in a higher As availability, while a higher redox potential results in a predomination of arsenate with a strong adsorption to iron and aluminium oxides resulting in a lower availability (Zhao et al. 2010). One example of crop cultivation in low redox soils is rice, that is regarded as the cereal with the highest As-content, which is result of a higher fraction of plant available As in the soil (Signes-Pastor et al. 2012).

4.1.2 Arsenic speciation in vegetables

Due to the differences in toxicity between the different As-species, knowledge of the predominating As-species in the plants is fundamental when determining the risk of consuming vegetables containing As. The inorganic As species are the carcinogenic As species regulated by EFSA (EFSA, 2009), i.e. no more than 18 µg As per day from food for a 60 kg person. The predominating As species detected in all plant analyses were the inorganic As-species arsenate and arsenite (papers I-II, IV-V). Arsenite was the predominating As species both in the roots and shoots of vegetables cultivated in moderately As polluted soil (paper IV), results which correspond to the general opinion that As is stored in the vacuoles as arsenite (Moreno-Jiménez et al. 2012). However, in vegetables cultivated in the highly polluted glassworks soil (paper IV) and in lettuce cultivated in hydroponics (paper V), arsenate was predominating. The predomination of arsenate in vegetables from the highly polluted glassworks soil and hydroponics could relate to the phytotoxicity of the soil and the hydroponic solution, due to the high As concentration. Plants growing in phytotoxic conditions may lose the activity of the arsenate reductase resulting in a higher proportion of arsenate compared with healthy plants (Mattusch et al. 2000).

As stated above, only inorganic As was detected in the edible parts of carrot (*Daucus carota*) and spinach (*Spinacia oleracea*) cultivated in two different soils, and lettuce (*Lactuca sativa*) cultivated in three different soils, all with elevated levels of As (paper IV). For the soil with the highest As concentration (514 mg As kg^{-1} DW), the ingestion of 13-49 g (FW), which is well within the range of normal consumption for carrot, lettuce and spinach, would result in the intake of 18 µg of As (table 2). For lettuce cultivated in hydroponics for four days, 29 g (FW), would result in the intake of 18 µg of As (table 2). However, addition of silicon to the hydroponic solution lowered the As concentration in lettuce, meaning that a consumption of 67 g (FW), would result in an equal exposure (table 2) (an extended discussion about silicon and As in crops will follow in section 4.1.3 below). The crops collected from As contaminated agricultural field, oats (*Avena sativa*) and alfalfa (*Medicago sativa*) (paper I), had concentrations similar to that of rice which is considered to pose a health risk upon consumption (approximately 0.3 mg As kg^{-1} DW) (Zhao et al. 2010). For the oats in this survey, less than two portions of oatmeal or muesli corresponds to the intake of 18 µg of As, and for alfalfa which is a common dried fodder for example for horses, the intake can reach mg-values every day (table 2). Due to the low As concentration, consumption of berries (*Rosa villosa, Rosa rugosa, Rubus caesius*) and apple (*Malus domestica*) grown in As polluted soil, did not constitute a serious problem for As intake in this survey (table 2).

Table 2. Arsenic concentration (mg As kg^{-1} DW) in soil and edible parts of vegetables, fodder and berries and amounts of plant material that contains 18 µg As, the lowest limit for As in food for a 60 kg person. Mean ± SE. (modified from papers I and IV)

Vegetables (edible part)	Soil (mg As kg^{-1} DW)	As (mg kg^{-1} DW)		Consumption (g) to reach 18 µg As
Avena sativa (oats)	170	0.29	±0.05	62 (DW)
Allium cepa (onion)	30.8	nd		-
Daucus carota (carrot)	514	27.3	±13	13 (FW)
”	142	0.32	±0.03	1125 (FW)
Lactuca sativa (lettuce)	514	11.4	±0.09	32 (FW)
”	142	1.41	±0.17	255 (FW)
”	77.7	21.2	±4.2	17 (FW)
“ (hydroponics)	0.75 (mg L^{-1})	12.2	±1.8	29 (FW)
“ (hydroponics + Si)	0.75 (mg L^{-1})	5.38	±1.42	67 (FW)
Malus domestica (apple)	24.3	nd		-
Medicago sativa (alfalfa)	142	0.37	±0.14	49 (DW)
Rosa villosa (rose hip)	30.9	nd		-
Rosa rugosa (rose hip)	8.6	nd		-
Rubus caesius (dewberry)	17.2	0.63	-	570 (FW)
Spinacia oleracea (spinach)	514	7.03	±2.93	51 (FW)
”	142	0.91	±0.38	396 (FW)

In contrast to the inorganic As regulated by EFSA, the organic As is generally considered less toxic to humans (Meharg and Hartley-Whitaker, 2002), thereby posing less of a threat for ingestion by food. Organic As represented by MMA was detected in some of the analysis performed in this study, for example in some arsenate treated lettuce grown in hydroponics (paper V), and in five out of six perennial plant species collected in As polluted areas in Sweden (paper I). The level of MMA was relatively low, usually < 10 % of the total As (Papers I and V).

The origin of organic As in plants has not been clearly established. Some argue that plants themselves methylate the As (Raab et al. 2007), while recent results suggest that plants are unable to methylate inorganic As; micro-

organisms are instead responsible for the methylated As species which are subsequently taken up by plants (Lomax et al. 2012). The production of MMA in non-sterile, but not in sterile, growth media containing As supports the idea of biological production of MMA by microorganisms (Paper V). It is possible that the plants in paper I were not able to methylate As and the detected MMA were taken up from the surroundings. All of the plants analysed for As species in paper I were perennial and had consequently been growing several vegetative seasons in As polluted media, giving them time to accumulate MMA above the detection limit for analysis. The accumulation of MMA over several vegetative seasons suggests that MMA remains stable over time to build up in concentration. In contrast, the plants in papers III-IV were only exposed to As polluted media for days or weeks, a timespan which was possibly not enough to accumulate organic As above the detection limits. This suggests that plants do not possess the ability to methylate As and that the accumulation of methylated As from microorganisms needs time to accumulate to noticeable levels in plants. Another possibility is that plants indeed are able to methylate As (Raab et al. 2007), but that the methylation of As and the consequent accumulation of organic As by plants is a slow process. It is also possible that methylation occurs in young metabolically active parts, which was not seen when the whole plant was analysed as one. However, methylation by plants has been detected after a relatively short time span, for example for methyl-Hg which was detected after only three days after Hg exposure in young parts in *Ipomoea aquatica* (Göthberg and Greger, 2006). Since methylation of Hg was seen only after three days, the long experiment times without detected organic As in this study, further supports the idea that plants do not methylate As. The general results from these studies, based on the low toxicity and the low relative amounts of organic As, suggests that inorganic As, rather than organic As should be main focus for As studies in food.

4.1.3 Influence of silicon on arsenic accumulation by vegetables

Silicon may influence the accumulation of As in vegetables. A way to minimize As accumulation into crops in As-polluted agricultural land can be silicon applications. For example, the As accumulation in both shoots and roots of arsenate treated hydroponic cultivations of lettuce was reduced, after silicon addition (paper V). Reduced As accumulation in rice has also been shown after silicon fertilization (Li et al. 2009). In arsenite treated hydroponic cultivations of lettuce, the As accumulation increased in the shoots and decreased in the roots after silicon addition (paper V). The addition of silicon did not result in any changes in the ratio between arsenite and arsenate in either roots or shoots, compared with the non-silicon-treatments, for either arsenate or arsenite treated plants (paper V), suggesting that silicon does not influence the enzymes responsible for arsenate/arsenite metabolism. The

reduction of arsenate accumulation upon silicon treatment has previously been reported, possibly as an indirect influence on the phosphate uptake system (Guo et al. 2007). Reduced arsenate accumulation could also be the result of silicon induced secondary cell wall modifications (Yamamoto et al 2012), which could influence the apoplasmic adsorption of As by interacting with the passive binding of As to active functional groups in the apoplasm (Vithanage et al. 2012). The decreased accumulation by lettuce roots of arsenite (paper V), could relate to molecular competition in the shared silicon/arsenite uptake routes that result in lower arsenite accumulation upon silicon addition. The increased accumulation of arsenite in lettuce shoots probably does not relate to cellular As accumulation but to apoplasmic accumulation, as seen in the increased proportion of As situated in the cell wall fraction from 38 to 47 % in the shoots (paper V). The influence of silicon on cell wall modifications (Yamamoto et al 2012), with the concomitant modification of the As binding functional groups in the apoplasm (Vithanage et al. 2012), could be involved in the increased apoplasmic As accumulation in lettuce shoots after silicon addition (paper V).

The influence of silicon on the modifications of the cell wall, for example on the amount of lignin and the content of sugar in the cellulose (Yamamoto et al 2012), may also have influenced the analysis of As. In lettuce shoots treated with silicon and arsenite, a higher percentage of the As was found in the pellet fraction, the plant material left after As species extraction, compared with non-silicon treated plants (Paper V). It is likely that the silicon treatment resulted in a stronger binding of As to the walls in arsenite treated lettuce, for example by an increased number of functional groups for As binding, as indicated by the increased As in the cell wall fraction mentioned above, thereby reducing the amount of As extracted from the pellet (Paper V).

4.2 Phytoremediation of arsenic

Techniques which can be used for As phytoremediation are phytoextraction, phytostabilization and phytofiltration. Phytoremediation can have great potential for As remediation. The general As accumulation and speciation behaviour in plants, and specifically interesting plant species in this regard is important for getting an idea of the potential of phytoremediation in the temperate regions. In addition, the influence of redox potential on As speciation and availability can be important for phytofiltration efficiency.

4.2.1 Arsenic accumulation by terrestrial and emergent plants

The As concentration in the shoots was low and generally lower than the As concentration in the roots for the analysed terrestrial and emergent plants (Papers I,IV). The low As accumulation and translocation corresponds to the general idea of a limited As uptake and translocation in plants to reduce the toxic effects of As in the shoots (Wang et al. 2002). Most plants had an As concentration in the shoots below 10 mg As kg^{-1} (DW), with a few examples of an As concentration in the shoots up to 70 mg As kg^{-1} (DW), for example *Allium ursinum*, *Capsella bursa-pastoris* and *Stachys sylvatica* (Paper I). One exception was *Cirsium palustre*, with an As concentration in the shoots > 600 mg As kg^{-1} (DW), but that result was probably explained by the extremely high As concentration in the soil (approximately 100 g As kg^{-1} (DW)), which is also shown by the low accumulation factor (AF) (0.01), which was lower than for most other analysed plants (Paper I). The As concentration in the roots was almost exclusively higher than the As concentration in the shoots, with a few exceptions, for example *Empetrum nigrum* which had a higher As concentration in the shoots than the roots (paper I).

For phytoextraction purposes, the low As accumulation and translocation, suggested no suitability for terrestrial and emergent plants. Even after a closer look on the over 120 plant species analysed for As accumulation, the results showed a low As concentration in the shoots and/or a low As plant:As soil ratio < 1 (Paper I). For example, to remediate the surface (0 - 20 cm) and subsurface (20 - 60 cm) soil with phytoextraction below the ecological investigation level (20 mg kg^{-1} DW) as proposed by Niazi et al. (2012), the best candidate from these studies, *Pinus sylvestris* (paper I), would need a considerable time. The estimated time to reach below 20 mg kg^{-1} (DW) from both the alum shale soil (147 mg kg^{-1} DW) and the moderately As polluted glassworks soil (77.7 mg kg^{-1} DW) would take several thousand years, considering an average biomass increase for *Pinus sylvestris* of 1.75 Mg ha^{-1} $year^{-1}$ (Geudens et al. 2004). Also, the analysis of As accumulation showed a linear increase of the As concentration in the plants with increasing As concentration in the soil for terrestrial and emergent plants (paper I), in a general similar pattern as that of indicator plants (Fig. 4). A linear increase of the As concentration in the shoots with the As concentration in the soil does not suggest any suitability for temperate emergent and terrestrial plants for either phytoextraction, since such plants should have a As shoot:As soil ratio > 1, or for phytostabilization, since such plant should have a As root:As soil ratio > 1 and As shoot:As root ratio < 1 (Nouri et al. 2011).

For phytostabilization, as opposed to phytoextraction, a low shoot accumulation of As is desirable, i.e. a As root:As soil ratio > 1 and As shoot:As root ratio < 1< 1 (Nouri et al. 2011). Also other qualities, like high evapotranspiration and deep rooting which prevents pollutant mobility, as in the case of

trees, are interesting for phytostabilization. However, of all terrestrial and emergent plants analysed, no tree species were identified as good candidates for phytostabilization, since three out of four trees had As shoot:As root ratio > 1 (*Picea abies*, *Pinus sylvestris* and *Sorbus aucuparia*) and the fourth (*Betula pubescens*) > 0.5 (paper I). However, other plant species like *Rhododendron tomentosum* and *Veronica beccabunga* were identified as possible candidate for phytostabilization due to the high As root:As soil ratio (>2.5) and low As shoot:As root ratio (<0.2) (paper I).

4.2.2 Arsenic accumulation by submerged plants

The correlation between the As concentration in the soil and the As concentration in the plant, as seen for emergent and terrestrial plants, was not seen for submerged plants between the As concentration in the sediment and the As concentration in the plant, either for roots, shoots, or the whole plant (paper I). This was likely depending on the submerged macrophytes ability to accumulate As directly from the water due to the absence of a cuticle (Xue et al. 2012). The submerged plants also had a high accumulation factor (AF) both in the shoots (> 1) and the roots (>10) compared to the relatively low AF for terrestrial and emergent plants (paper I). The lack of correlation between the As concentration in the sediment and the As concentration in the plant and the high AF for submerged plants were probably the result of As adsorption on the surfaces and apoplasm of roots and shoots along with iron oxides as seen with other submerged macrophytes (Robinson et al. 2006). Translocation of As from roots to shoots or from shoots to roots may also help to explain the lack of correlation between the As concentration in the sediment and the As concentration in the plant and the high AF for submerged plants.

The general strategy of plants to avoid the detrimental effects of As is the reduction of arsenate to arsenite followed either by efflux from the cell or storage in the vacuole (Bienert et al. 2008). *Elodea canadensis* did not seem to follow this general behaviour of As response in plants, since most of the As in both *E. canadensis* and in the surrounding water was detected as arsenate (paper II). This suggests that As was adsorbed on the plant surfaces and in the apoplasm of *E. canadensis*, as previously has been reported for some submerged plants (Robinson et al. 2006). The accumulation of As in the apoplasm from the surrounding water column can be a relatively quick process, as exemplified by As uptake in lettuce during the first 30 min from a hydroponic solution (paper V). After a four day hydroponic cultivation of lettuce with the same As concentrations as above, approximately 35-40 % of the As was found in the cell wall fraction, both in shoots and roots (paper V). The As content in *E. canadensis* cell walls was, however, not analysed. The quick accumulation of As in the apoplasm from the water column is favour-

able in phytoremediation when As in constantly removed from flowing water. Also, since *E. canadensis* has the ability for growth even in winter time (Bowmer et al. 1995), it could function as a "biological filter" for As accumulation during a longer period of the year (paper II). Submerged plants using photosynthetic energy for As efflux or storage in the vacuole, for example *Hydrilla verticillata* and *Ceratophyllum demersum* (Xue and Yan 2011; Xue et al. 2012), will probably not function well in the winter time, as opposed to the proposed ability of *E. canadensis*.

4.2.3 The influence of redox potential on phytofiltration

The redox potential is generally low in the submerged habitats and a low redox potential influences the availability of As. For example, the availability of As to plants commonly increases in low redox potential due to the lower adsorption of arsenite to soil and sediment particles, while the opposite occurs in high redox potential due to the higher adsorption of arsenate to soil and sediment particles (Zhao et al. 2010).

In the submerged macrophyte *E. canadensis* cultivated for 96 h in As polluted water, the plant As accumulation properties generally decreased at high O_2 in the water (paper II). Medium O_2 was shown to result in low As release from the sediment along with a relatively high plant accumulation, compared with the low and high O_2 treatments (paper II). It is possible that the differences in plant As accumulation between the different O_2 treatments was due to an influence of the O_2 on the plant reduction of arsenate to arsenite and storage in the vacuole, as shown by an increased arsenate:arsenite ratio with increasing O_2 (paper II). Another possible influence on plant As accumulation in different O_2, could relate to morphological and physiological changes like increased aerenchyma formation and shoot growth and stimulated photosynthesis, which are common responses to oxygen deficiency stress in submerged plants (Voesenek et al. 2006). It is not unlikely that such responses also may influence the accumulation of As. The presence of *E. canadensis* also increased the oxidation of arsenite to arsenate in the water, probably relating to a promotion of the bacterial community by *E. canadensis*, which in turn was responsible for the oxidations of arsenite (paper II).

4.2.4 The use of *Elodea canadensis* in phytofiltration

The high accumulation of As by submerged plants is of particular interest in terms of phytofiltration. Also, the relatively short time span to accumulate As from water into submerged plants suggests possible applications for successful As phytofiltration. For example, already after four days, *E. canadensis* accumulated > 100 mg As kg^{-1} (DW) in the shoots (paper II). Based on

dense populations of *E. canadensis* (approximately 750 g FW m^{-3}) (Kornijów et al. 2005), the plants from these studies can accumulate approximately 5 µg As L^{-1} from moderately As-polluted water (45 µg As L^{-1}) already after four days, representing a removal of approximately 11 % of the As from the water (paper II).

The feature of optimal As accumulation in *E. canadensis* in medium O_2 levels may favour As phytofiltration. It is well-established in constructed wetlands to control the O_2 levels, for example by regulating water depth and flow, and providing shallow water flow areas and height differences, to promote nitrification, denitrification and mineralization of organic matter. Also plant density may control both the water flow and increase the O_2 via photosynthesis. Such factors can be optimized to promote optimal plant As removal from water.

4.2.5 Phytoremediation in agriculture

Phytoremediation may be a cost-effective alternative to remove As from large areas like agricultural land, for example As containing agricultural alum shale soil. The best candidate for phytoextraction from these studies, *P. sylvestris* (paper I), would remove approximately 16 ug As kg^{-1} $year^{-1}$ from the agricultural alum shale soil (147 mg kg^{-1} DW). This speed of removal would have little influence of the reduction of the total As concentration in the soil, but since the availability of As in the alum shale soil was low (paper IV), the removal of As by *P. sylvestris* from the plant available As pool could reduce the As accumulation in crops planted in the soil after the harvest of *P. sylvestris*. Also in practical field experiments, the remediation times using phytoextraction are long, even using the most promising candidates for As phytoextraction, the hyperaccumulating ferns. In an area with relatively high As levels (up to 900 mg As kg^{-1} DW), it was estimated that it would take up to 400 years to remediate the area using *Pteris vittata* (Niazi et al. 2012). Similarly, previous field studies using *P. vittata* have also shown less promising results due to a low biomass production (Kertulis-Tartar et al. 2006; Salido et al. 2003). However, these field experiments were performed in sites with relatively high As levels and with multiple contaminants. The limitations of phytoextraction in terms of phytotoxicity in sites with multiple contaminants and efficiency problems (Sarma, 2011), do not necessarily apply to fertile agricultural land with relatively low levels of As, where optimal growth can be achieved. In addition, bioenergy can be produced as previously has been shown in moderately metal-polluted agricultural land (Greger and Landberg 1999).

Based on the promising results as discussed above (4.2.2-4.2.4), As phytofiltration may also be used in agricultural practices. Artificial irrigation using

As-polluted water is a widespread problem, especially in South East Asia, which may result in elevated levels of As in crops. An initial phytofiltration of water to be used for irrigation may decrease the As in crops. This could reduce the As ending up in food in a cost-efficient manner compared with other As removal techniques from water (Ng et al. 2012).

5. Conclusions

It is apparent from this work that there is a risk that consumption of carrot, lettuce and spinach cultivated in As containing soil can result in the intake of inorganic As above the suggested limit set for inorganic As in food by the European Food Safety Authority (EFSA). From our analysis it can be concluded that soils with a high As extractability are likely to result in higher concentrations of As in vegetables compared with soils with low As extractability. Silicon fertilization may reduce As in vegetables, as the accumulation of both arsenate and arsenite in lettuce was lower with than without silicon.

Our studies also shows that for the efficiency of As phytoremediation, the habitat is of greater importance than the plants species specific characteristics, even if exceptions exists, for example As hyperaccumulators. We further show that the accumulation of As in the shoots of terrestrial and emergent plants is generally low, while the accumulation of As in submerged plants generally is higher, probably relating to the availability of As to the plants. An increased O_2 level in submerged conditions increase the As accumulation in the submerged macrophyte *E. canadensis*.

To previous findings we can also add that successful phytoextraction of As in temperate regions is not likely due to too low As accumulation in plant shoots, for example the reduction of As in the agricultural alum shale soil to 20 mg kg^{-1} (DW) would take thousands of years with the best phytoextraction candidate from these studies, *P. sylvestris*. The work also demonstrate that phytofiltration can be successful, especially if using *E. canadensis* in dense populations which can remove up to 11 % of the As from moderately As polluted water (45 ug As L^{-1}) already after four days.

6. Future prospects

The methylating ability of As by plants would be interesting to elucidate in the future. The setup should include long-term cultivations, at least longer than six weeks as performed in paper IV and by Lomax et al. (2012), who claim that plants are unable to methylate As. Sterile compared with non-sterile conditions, should be applied to determine if organic As originate in plants, in microorganisms or in both.

To follow up the results showing that *E. canadensis* is a promising candidate for phytofiltration, an outdoor experiment over the whole year to remove As from water could be set up. It would be interesting to elucidate the capacity of *E. canadensis*, and other submerged macrophytes, to remove As from water as well as the efficiency of As removal between different seasons of the year.

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