ITES
Institute of Terrestrial Ecosystems

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Swiss Federal Institute of Technology Zurich

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A thesis presented in partial fulfilment of a master of science in ecology

Investigating plant – antimony interactions: method development, plant tolerance, and plant uptake.

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December 31, 2007

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ABSTRACT

Antimony (Sb) is a toxic trace element that is non-essential for plants and animals. Antimony has become widespread in the environment through various industrial uses. In Switzerland, the largest source of Sb contamination is small arms ammunition, which contains 2 – 5% Sb. Some 6000 shooting ranges, representing ca. 6000 ha, have become contaminated with Sb, Pb, Cu and Ni. Sb is the most mobile contaminant on disused shooting ranges.

I aimed to develop a method for the assessment of plant – Sb interactions, and use it to test the responses of five common agricultural species, *Zea mays*, *Trifolium pratense, Helianthus annuus, Triticum aestivum* and *Brassica juncea*, to elevated Sb concentrations.

A comparison of various analytical techniques revealed that a modified *aqua regia* digestion, with Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) gave precise and accurate determinations of Sb in plant tissues. The detection limit for this method was ca. 2.5 Sb µg/kg in dried plant material.

A sterile agar technique, using specialised plant growth boxes, permitted the rapid growth of these species in the presence of precisely determined Sb concentrations. Unlike soil, agar does not bind significant amounts of Sb. Therefore, all the Sb in the system is available to interact with plant roots. Agar has the advantage over hydroponics that there is no risk of root damage, which may allow the direct passage of Sb into the root xylem.

I investigated the tolerance of the five crop species to elevated Sb and arsenic (As) concentrations in agar using a Root Length Index (RLI) experiment. At molar-equivalent concentrations, As was ca twice as toxic as Sb. The concentrations of Sb required to reduce the RLI of the plants by 50% was 631 µmol/l (77 mg/l) for *Triticum aestivum,* 829 µmol/l (101 mg/l) for *Zea mays,* 1247 µmol/ (152 mg/l) for *Trifolium pratense,* 2149 µmol/l (262 mg/l) for *Helianthus annuus* and >2463 µmol/ (300 mg/l)for *Brassica juncea*.

Using the results of the RLI study, I selected a concentration rage of 0 – 160 mg Sb / l agar, in which to grow the plants in the specialised growth boxes. At 160 mg/l, only the shoots of Trifolium showed a significant biomass reduction, while there was a general increase in the root : shoot ratio of all species.

Plants accumulated Sb in their shoots at concentrations between 0.6 and 9 times the Sb concentration of the agar in which they were grown. The root concentrations were tenfold higher. The shoot Sb concentrations followed the pattern *Trifolium pratense* > *Helianthus annuus* > *Brassica juncea* ≈ *Triticum aestivum* >> *Zea mays.*

Plants were grown on soil from the stop butt of a disused shooting range, which is the most heavily contaminated material, containing some 5000 mg/kg Sb. The shoots of all species had 50 – 100 mg/kg Sb, except *Zea mays*, which had just 5 mg/kg Sb.

My results show that the current land management practice of keeping disused shooting ranges under mowed pasture may result in elevated Sb concentrations in the plant tissue if clover is present. Feeding this cut pasture to livestock may endanger animal or human health. An alternative land management strategy would be to grow crops of Zea mays, which takes up very low concentrations of Sb. The biomass could be used as for bioenergy, thus producing an income of a contaminated land, without endangering ecosystem or human health.

ACKNOWLEDGEMENTS

I would like to express thanks to Prof. Rainer Schulin, Prof. Heinz-Ulrich Reyer and Dr. Brett Harvey Robinson, for their advice, assistance throughout the period of this work.

I am greatly indebted to Dr. Diane Burger and Viktor Stadelmann, for the assistance during the Sb analysis.

Tanks are also all the staff of Soil Protection group for their help and encouragement during this year.

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Foreword

This thesis describes investigations into the interactions of antimony (Sb) a scantly studied metalloid, with plants. Due to a lacuna of information about the analysis of Sb in plant tissues and plant-availability of Sb in soils, half of this thesis is devoted to developing a methodology for the study of plant Sb toxicity and uptake.

This thesis includes five Chapters; the first is a general introduction about antimony (Sb) in the environmental and a review of the literature on plant Sb-interactions. Chapters two and three detail the development of the methodology that was necessary to test plant-Sb interactions. They include the optimization of a suitable analytical method for the total Sb determination in plants tissue and the development of a "standard growing method" for the sterile growth of different plant species in agar. Chapter four discusses the tolerance of the selected crop species to Sb and. Chapter five discusses the uptake of Sb by plants and the consequent implications for the management of contained soil.

1 General introduction

1.1 Antimony in the environment

Antimony (Sb) is a rare element in the earth's crust (0.2 – 0.3 mg kg^{-1}). However, due to its industrial uses as components in fire retardants, semiconductors, and as an agent for metal hardening, it has become widespread in the environment. In the last decade, the global flux of Sb has increased tenfold, resulting in elevated Sb concentrations in soils and natural waters, where it may affect plants, animals and humans. Mining is one of the major sources of Sb contamination (Adriano 1986). In Switzerland, elevated soil Sb concentrations occur in the ca. 6000 shooting ranges scattered throughout the country (Gresch and Wettstein 2002). On average, new bullets and pellets consist of 1-7% Sb (Rooney 1999). Johnson et al. (Johnson, Moench et al. 2005) reported that Sb was the most soluble and mobile of all the trace element contaminant in Swiss shooting ranges. Compared to other trace elements, Sb is relatively mobile in soils, increasingly the likelihood of its entry into the food chain via plant uptake. Wersin, Johnson et al. (2002) measured elevated Sb concentrations in receiving waters near a Swiss shooting range.

The biogeochemical behaviour of Sb is in similar to the other group VI elements arsenic (As) and phosphorous (P). Therefore, Sb tends to occur with other chalcophyllic elements, especially As (Mason and Moore 1982). Sb is a metalloid that exists in oxidation states -III, 0, +III, and +V. In soils Sb usually occurs as Sb(III) (antimonite) and Sb(V) (antimonate) in reducing and oxidising conditions respectively. Common minerals are sulphides (in particular stibnite Sb_2S_3) and oxides (valentinite Sb_2O_3 and cervantite Sb_2O_4). In aerobic soils, Sb(III) rapidly oxidises to Sb(V). Using X-ray Absorption Spectrometry (XAS), Scheinost, Rossberg et al. (2006) showed that Sb shooting range soils was present as Sb(0) (unoxidised bullets) and Sb(V). No Sb(III) could be detected.

1.2 Antimony toxicity

Antimony is a non-essential element for both plants and animals. The toxicity of Sb depends on its molecular form; inorganic Sb compounds are more toxic than organic ones. Sb(III) compounds are ten times more toxic than Sb(V) compounds. The toxic effects of Sb result form irreversible binding to thiol-cotaining enzymes. Sb(III) shows affinity for red cells and thiol groups of cell constituents, while red cells are impermeable to Sb(V) (Guy, Jones et al. 1998). Gebel (1997) assessed genotoxicity of extracts of soils containing natural Sb and As sources with a sister chromatide exchange test. The genotoxicity observed waslow because Sb and As predominated in pentavalent, non genotoxic state. Antimony is toxic to humans at chronic uptake rates exceeding 100 mg/day, where it affects the liver and gastro-intestinal systems (Barren 1979). Ingestion of 11-75 mg/day is lethal for rats. Due to its toxicity and likely carcinogenic nature (Gebel 1997), human exposure to Sb is regulated by concentration limits at workplaces and for drinking water. The EU limit for Sb in drinking water is 5 $\mu g\ L^{-1}$ following an investigation by the Council of the European Communities (1976). To date, there are no EU or Swiss limits for antimony in soil and food.

1.3 Antimony bioavailability to plants

The solubility and speciation of Sb in soil determines its toxicity, availability for plant uptake, as well as its tendency to leach. Not all Sb in the soil interacts with plants. Solubility is a prerequisite for plant uptake or toxicity. The interaction of the soluble Sb with the plant is dependent on its chemical speciation, and the species of plant with which it is interacting. The solubility and speciation of Sb depend on many physical, chemical, microbial, and plant factors.

In soil, only some fraction of the total Sb concentration will be in soil solution, with the remainder bound to the soil matrix. The solubility of Sb in soil is a function of the soil's chemical and physical properties. Compared to other trace elements, Sb is relatively mobile in soils (Gresch and Wettstein 2002). Johnson et al. (2005) reported that Sb was the most soluble and mobile of all the trace element contaminants in Swiss shooting ranges under unsaturated condition.

Antimony oxyanions ($Sb(OH)_6$, SbO_3^- , $Sb(OH)_4^-$) carry a negative charge and therefore bind electrostatically to positively charged sites in the soil matrix, as occur in variably charged soils. This is measured by the soil's anion exchange capacity (AEC). In many temperate soils, the AEC is so small as to be insignificant. However, some highly weathered soils, and those that contain significant quantities of such volcanic minerals as allophane and imogolite, can have a significant AEC. The AEC is dependent on pH. Antimony oxyanions form specific chemical bonds with soil components. This results in their adsorption exceeding the AEC of the soil. Blay (2000) tested Sb(III) and Sb(V) sorption onto different components of the soil matrix (α-FeOOH, Fe_2O_3, kaolinite, montmorillonite and illite) and found that Fe(hydr)oxides were the strongest sorbents, most likely forming an innersphere complex with both Sb(III) and Sb(V). Sorption to clays was rather weak and most probably outer-sphere. Gal (2006) found with sequential extractions that As and Sb are strongly retained by the Fe-oxide fraction of soils in mining areas in Scotland and Italy.

Negatively charged tracer elements, such as $Sb(OH)_6^-$ $Sb(OH)_4^-$ tend to be more soluble at a higher pH. Sb may be displaced from exchange sites by other ions attracted from the soil solution. The extent of this competition for binding sites depends on both the type and concentration of the competing ion.

Antimony adsorption onto charged exchange sites is not the only mechanism governing its solubility in soil. The extent, soluble-insoluble partitioning, and mobility of soil organic matter may play a role in the solubility and environmental fate of Sb. Vegetation provides a continual source of organic matter in soil via plant exudates and the decomposition of litter. Antimony complexation by organic matter may either promote or reduce its solubility, depending on the solubility of the organic ligand. Ettler et al. (2007) found Sb to be specifically sorbed onto organic matter. Experiments by Steely et al. (2007) illustrated that insoluble humic acids removed Sb from soil solution.

Plant roots influence soil in their immediate vicinity, a zone known as the rhizosphere. The solubility and speciation of Sb in this zone may be distinct from the bulk soil. Plant roots excrete H^+ ions that exchange with nutrient base cations. Many plant species exude chelants, phytosiderophores, which mobilise Fe and perhaps other essential nutrients of low availability. Root exudations may acidify the rhizosphere by up to 2 pH units (Salisbury and Ross 1992). Such acidification invariably increases the solubility of non-essential trace element cations such as Cd^{2+} (Naidu, Bolan et al. 1994).

Plant root exudates and detritus provide a growth substrate for soil microflora, increasing microbial biomass in the rootzone. Soil fauna and microorganisms behave similarly to soil organic matter in that they possess binding sites for some trace elements (Robinson, Brooks et al. 1999). Exudates from rhizobacteria may change the speciation of Sb (Pedersen and Albinsson 1992). Roots improve soil aeration by extracting soil moisture and forming continuous channels for drainage and air exchange. However, increased metabolic activity can result in anaerobic conditions if more oxygen is consumed than can be re-supplied.

With the notable exception of the brassicaceae, the roots of most plants form symbiotic relationships with mycorrhizal fungi. These fungi solubilise and sequester nutrients and possibly other trace elements in the rhizosphere, and transport them toward, possibly into the root (Salisbury and Ross 1992). Many authors have demonstrated that mycorrhizal fungi enhance plant metal tolerance (Marschner 1995; Jentschke and Godbold 2000; Schutzendubel and Polle 2002). Mycorrhizas may absorb trace elements in their hyphal sheath and external mycelium. The fungal sheath may reduce the access of Sb to the plant roots due to hydrophobicity (Jentschke and Godbold 2000).

1.4 Antimony uptake by plants

There are large differences in the degree of measured Sb concentrations in plant shoot tissue for plants that occur in Sb-contaminated soil. Ainsworth et al. (1990) measured Sb concentrations of 300 mg/kg in the leaves of several grasses nearby an Sb smelter in North East England, where the soil Sb concentrations reached 400 mg/kg. Baroni et al. (2000) reported over 1000 mg/kg Sb in the basal leaves of *Achillea ageratum* growing at a tailing pond with a soil containing 9000 mg/kg Sb. Other species on the mine soil also had foliar Sb concentrations >100 mg/kg. In contrast to these high concentrations, there are reports of low Sb uptake from plants growing in heavily contaminated soils. Pratas et al. (2005) reported maximum stem concentrations of <5 mg/kg Sb for different tree and herb species growing in a Portuguese mine spoil with an average soil Sb concentration of 663 mg/kg. There are two possible routes for Sb to enter plant tissue: Surface deposition, or uptake from the soil via the roots.

1.5 Surface deposition

Antimony-rich dust may be deposited onto the surface of plants whereupon it could become incorporated into plant tissues, such as the waxy layers of leaves. So bound, dust often cannot be removed, even by vigorous washing (Hinton, Kopp et al. 1995). Surface deposition is always an issue with samples collected in the field, as it is not possible to control dust or industrial emissions.

There are only two reports of Sb uptake under greenhouse conditions, where surface contamination will not contribute to the plant's Sb burden. Davis et al. (1978) found in a sand culture experiment, with 100 mg/kg soluble Sb, that plant toxicity is not necessarily dependant on accumulation. Barley (*Hordeum vulgare*) yields were depressed by high concentrations of Sb in the nutrient solutions but these elements could not be detected in the plant shoots (<2 mg/kg). In contrast Hammel et al. (2000) conducted a pot experiment with 21 garden and crop plant species and soils spiked with Sb to give 70 mg/kg in soil solution. Here the plants accumulated Sb up to concentrations of 399 mg/kg and no toxicity symptoms were reported. This indicates that plant Sb accumulation, as with other trace elements, is species dependant.

1.6 Uptake of antimony via the plant roots

The translocation of Sb to the aerial parts of a plant requires its entry into the root xylem, via either the apoplastic or the symplastic pathways (Marschner 1995). The apoplastic pathway, consisting of cell walls and intercellular spaces, is discontinuous. It is interrupted by the endodermis, the innermost layer of cells of the cortex. In the radial and transverse walls of the endodermis, hydrophobic incrustations of suberin, known as Casparian strips, obstruct the passive transfer of solutes into the stele and thence into the root xylem.

However, the Casparian strips form an imperfect barrier (Crowdy and Tanton 1970). Soil organisms, pathogens or mechanical disturbance may create disruptions in the endodermis. Similarly, at the root apices, there is a small zone where soil solution can pass directly into the root xylem, because the Casparian bands have yet to develop in the newly formed root tissues (Harrison-Murray and Clarkson, 1973). Thus, small amounts of some Sb may enter the root xylem directly via the apoplastic pathway, without first having to traverse a cell membrane into the plant's symplast.

Trace elements, such as Sb, can only traverse the plasma membrane via embedded protein transporters. There are numerous transporters. For example, in *Arabidopsis thaliana*, 4589 genes code for membrane-spanning proteins, representing some 18% of the protein-coding genome (Ward 2001).

Passive transporters, or ion channels, permit ions with a specific size and charge to move across the cell membrane down their concentration gradient. Plant uptake requires that the target ion concentration in the cytoplasm be lower than the surroundings (Marschner 1995). Active transporters, requiring metabolic energy, move trace elements across membranes against their concentration gradient (Salisbury and Ross 1992). There are multiple transporters for essential elements, being either constitutive or inducible (Reid and Hayes 2003). Constitutive transporters are always operational; while a nutrient deficiency may induce the plant to activate additional transporters. Conversely, antiporters eject toxic elements from the cytoplasm. These may decrease plant trace uptake and increase the plant's tolerance to high concentrations of trace elements in the environment (Martinoia, Klein et al. 2002).

In their review of nutrient uptake by plants, Reid and Hays (2003) concluded that most membrane-transport proteins that mediate nutrient influx or efflux lack specificity. Therefore, Sb may be taken up via an essential nutrient transport system. The closest essential element that Sb resembles, in the form $Sb(OH)_6^-$ is HPO_4^{3-}. Given that Sb has similar chemical properties to As and P, plants may take it up by the same mechanism. It has been shown that P addition affects the uptake of As by competing for binding sites in the soil or transporters into the plants (Woolson, Axley et al. 1973; Gulz, Gupta et al. 2005).

1.7 Antimony translocation and storage within the plant

Plant water uptake drives trace element translocation from the roots to the shoots via the xylem (Salt 1995). In the aboveground portions, the highest concentrations are often found in the leaves as they are the major water sink prior to evaporation of the water. It is unlikely that phloem transport would redistribute Sb within the plant, since it is a non-essential element that is chemically distinct from other phloem-mobile elements.

1.8 Possible plant tolerance mechanisms of antimony

In plant tissues, nonessential trace elements, such as Sb, are potentially toxic. High concentrations of Sb may damage plant tissues via oxidative stress, blocking essential functional groups in biomolecules and displacement of essential nutrients in biomolecules (Schutzendubel and Polle 2002).

All plants have basic tolerance mechanisms. Some species and varieties can survive in soil with inordinately high concentrations of non-essential trace elements. Mostly, such plants are only tolerant to the trace elements that occur in the soil in which they grow Schat and Vooijs, (1997), indicating that there tolerance mechanisms are element specific. There are nine groups of tolerance mechanisms:

1) Root avoidance of patches of high Sb concentration
2) External sequestration by bacteria, mychorrizal fungi
3) Root exudates that render the Sb unavailable for plant uptake
4) Restriction of transport across plasmalemma into the cell
5) Active effluex into the apoplast
6) Chelation of the Sb in the cytosol by phytochelatins, metallothianenes or organic acids
7) Production of heat-shock proteins that repair cellular damage, particularly damage to the cell membrane.
8) Storage of Sb in the vacuole
9) Abscission of organs, such as leaves, with a high Sb load

The toxic effects of trace elements are larger when they are present as free ions. Therefore, most trace elements found in plant tissues are complexed with organic ligands (Hall 2002). In addition to low molecular weight organic acids, plants produce two classes of metal binding proteins, phytochelatins and metallothioneins (Cobbett and Goldsbrough 2002). Metallothioneins are low molecular weight, cystine-rich, metal binding proteins (Kagi 1991). Phytochelatins have the general structure $(\gamma\text{-Glu Cys})_n\text{-Gly}$ where n = 2-11 and are rapidly induced in plants by heavy metal treatments (Rauser 1995; Zenk 1996). Trace element cations that enter the cytoplasm are immediately chelated by “chaperone” ligands (Clemens 2001) and transported to trace element requiring cytostolic proteins or to organelles. The chaperone ligands are then freed to complex other trace element cations. Trace elements that are in excess to metabolic requirements are usually transported to the vacuole, where they are often complexed with phytochelatins and stored (Hall 2002).

Toxic concentrations of trace elements in cells may induce the production of heat-shock proteins (Lewis, Handy et al. 1999). Heat-shock proteins function in the production and repair of proteins under stress conditions (Hall 2002).

1.9 Implications of plant Sb uptake

Plant uptake of Sb may facilitate its entry into the food chain. This may occur if via physiological plant uptake, or via surface deposition of Sb-contaminated material. Most Sb-contaminated sites will eventually become vegetated, either through natural plant colonisation or through deliberate revegetation as part of a remediation programme. In this latter case, the plantings may be combined with soil conditioners to promote plant growth and limit Sb flux, either downward into receiving waters or upwards into the aerial portions of the plant where it may be consumed by herbivores.

1.10 Critical gaps in our knowledge of plant Sb uptake and aims of this study.

Thus far, most studies on plant-Sb uptake have occurred in the field situation. Here, the Sb measured in the aerial portions of the plants may have originated either from aerial deposition or physiological uptake. Most of the studies have investigated heavily-contaminated soils, such as disused mine sites, where Sb occurs at high concentrations (>500 mg/kg) along with several other elements. Moreover, there have not been any studies using commonly cultivated species such as sunflower, clover, mustard, and corn.

My study aims to elucidate the tolerance and uptake of Sb by these aforementioned plants. To achieve this aim, I will grow the plans in agar, spiked with various concentrations of Sb (V), because this oxidation state has the highest occurrence in waters and soil solution (Filella, Belzile et al. 2002). I used an agar system to eliminate surface contamination and the influence of confounding soil parameters that influence Sb bioavailability, such as particle size, organic matter content, and microbial flora. Since agar is transparent, it allows the easy measurement of root growth without destroying the plant. To achieve these aims, I first have to develop a reliable method to measure Sb in biological tissues, and determine the best protocol to grow plants in an agar medium.

2 Assessment of methods for the determination of antimony in plant samples

2.1 Introduction

Similar to other trace elements, the determination of Sb in plant tissues requires the digestion of the plant material (Nash, Maskall et al. 2000). There are many digestion methods for environmental samples for trace element determination. The applicability these methods depends on the nature of the material, the aims of the experiment, as well as its practically (Agemian and Chau 1976; Bajo 1978).

The properties of antimony (Sb) and arsenic (As) make their retention and determination problematic. These group V metalloids commonly react with dissolution media differently to other heavy metals. For example, while a hydrofluoric acid-based digestion may be ideal for complete sample dissolution, volatilization of As and Sb as gaseous fluoride species can preclude this quantitative analysis (Bajo 1978). The choice of digest reagents requires careful consideration to ensure adequate matrix dissolution and prevent the formation of insoluble Sb-bearing precipitates. *Aqua regia* or hydrochloric acid-based digestions are widely used for As and Sb determination (Anderson, Davidson et al. 1998; Lintschinger, Michalke et al. 1998; Nash, Maskall et al. 2000). However, the loss of Sb and As as tri and penta–chloride species is possible, with these compounds volatilizing at comparable temperatures to the fluorides (Aylward and Findlay 1994).

Three processes are required for the accurate analysis of total Sb in environmental samples: (i) the total oxidation of the plant's organic matter thus requires an oxidising agent such as HNO_3, H_2O_2, H_2SO_4 or HCl (USEPA 1996). (ii) It is important that the oxidising agent does not form a volatile or insoluble complex. (iii) The newly formed Sb complex should be easily analysed from the machine.

Recently, microwave digestion has greatly improved the accuracy and precision of digestion for heavy metals analysis (Aylward and Findlay 1994; Tighe, Lockwood et al. 2004). Advantages include less sample used, reduced potential for cross-contamination, improved safety, and significant reductions in analyte loss by volatilization (Xing and Veneman 1998; Nash, Maskall et al. 2000), an important consideration for As and Sb digestion.

Some authors compare heating block and microwave digestion for the analysis of Sb and As in soil or peat samples and show the high efficient of microwave digestion (Krachler, Emons et al. 2002; Tighe, Lockwood et al. 2004). Martin J. Nash (2000) present a critical review on the methodologies for the determination of total Sb in aqueous and solid environmental samples, with emphasis on freshwaters, sediments material. However, in soil and sediments materials, the metals are bond in a silicate matrix and thus require a specific digestion system to extract these elements (USEPA 1996).

There is a lacuna of information dealing with the determination of total Sb in plant tissues. Here, I aimed to determine the efficiency of the different digestion systems and with analytical methods for Sb determination in plant tissues.

I compared four digestion methods of plants material for the recovery of Sb from a certified plant sample with standard additions of Sb. Subsequently, the samples were analyzed using three different analytical methods: HG-AFS (Hydride Generation Atomic Fluorescence Spectrometry), ICP-MS (Inductively Coupled Plasma Mass Spectroscopy), and GFAAS (Graphite Furnace Atomic Absorption Spectroscopy).

2.2 Material and Methods

2.2.1 *Sample set, reagents and equipment*

For each digestion method, I prepared a standard addition series, in triplicate, to give final solution concentrations of 0 µg/l + 5 µg/kg (Sb from plant material) , 20 µg/l + 5 µg/kg (Sb from plant material), 40 µg/l + 5 µg/kg (Sb from plant material), 80 µg/l + 5 µg/kg (Sb from plant material). About 200 mg of Virginia Tobacco Leaves (CTA-VTL-2) material were spiked with 1000 mg/l Sb standard solution up to the required concentrations. I obtained the 1000 mg/l Sb standard solution from Merck 1.70204.0500 Sb standard 1000±2 mg/l Sb_2O_3 in HCl 2M. For all extraction methods, reagent blanks were prepared and analysed. All the reagents were Merck Suprapure. Digestions were carried out in a microwave (MLS-1200 MEGA, with the rotor APCU-TR41, 5/70). Teflon digestion tubes were washed with 1M HNO_3 in Milli-Q water, then rinsed in Milli-Q water. Samples were filtered with Ashless Circles Filter MN640d 15 cm ø (Macherey-Nnagel GMBH & CO.KG).

2.2.2 *Microwave digestion methods*

Two hundred mg of plant material was accurately weighed into 50 ml Teflon tubes and digested using the methods described in Table 1. Subsequently, the samples were filtered with ashless filter paper MN640d 15 cm ø and diluted to 25 ml with 1M HNO_3.

The last digestion method described in Table 1, ’4. *Modified aqua regia*’, involve a subsequent treatment of the digested. This additional treatment included a cooling step after microwave digestion; subsequently, the digests was enriched with H_2SO_4 95-98% (0.5 ml). The digest were the headed at 180°C for 2-3 h and concentrated by evaporation to approximately 0.5 ml.

Digestion methods	Reagents	Microwave steps
1. *HNO_3*	12 ml HNO_3 65% 3 ml H_2O_2 30%	1. 1000 Watt (1 min) 2. 600 Watt (10 min) 3. ventilation (40 min)
2. *Modified HNO_3*	12 ml HNO_3 65% 3 ml H_2O_2 30%	1. 1000 Watt (10 min) 2. 600 Watt (10 min) 3. ventilation (40 min)
3. *Aqua regia*	12 ml HNO_3 65% 4 ml HCl 30%	1. 1000 Watt (10 min) 2. 600 Watt (10 min) 3. ventilation (40 min)
4. *Modified aqua regia*	2 ml HNO_3 65% 6 ml HCl 30% 2 ml H_2O_2 30% 8 ml Milli-Q H_2O	1. 188 Watt (12 min) 2. 450 Watt (6 min) 3. 300 Watt (12 min) 4. ventilation (30 min)

Table 1. Reagents and microwave steps for the digestion methods used.

2.2.3 GFAAS analysis

Analysis was performed with a VARIAN GTA120 Graphite Tube Atomiser equipped with an AA240 Zeema Atomic Absorbtion Spectrometer. The Graphite Tube was a VARIAN (CATED)-CTA 63-1000 12-00 and the lamp was VARIAN Sb ULTRAAA HC LAMP-SB MH-1504-1. The Matrix Modifiers used were 5 ml Palladium-Modifier Merck 1.07289.0050, 5 ml Magnesium-Modifier Merck 1.050813.0050 and 1 ml HNO3 Merck Suprapure. Calibrations was performed with Merck 1.70204.0500 Sb standard 1000±2 mg/l Sb_2O_3 in HCl 2M.

The analytical methods for the Graphite tube atomizers was a modified program of Varian (Rothery 1988).

Parameter	Amount
Wavelength	217.6 nm
Gap length	0.2 nm
Lamp power	10.0 mA
Maximum absorbance	1.4
Gain	85%
Measurements repetitions	3

Table 2. Instrument parameters for the analysis.

Step	Temperature	Time	Gas flow	Stage
n°	(°C)	(Sec.)	(l/min)	
1	85	5	3	Drying
2	95	40	3	
3	120	10	3	
4	700	5	3	Charing
5	700	1	3	
6	700	2	0	
7	2500	2.7	0	Analysis
9	2500	2	3	

Table 3. Graphite furnace operating conditions.

2.2.4 *HG-AFS analysis*

Analyses were performed with a hydride generator (PS Analytical 10.033) equipped with an Excalibur Fluorescence detector (PS Analytical 20.100). Calibrations were performed with an Sb-Standard (Merck 1.70302). As positive control, I used Multi-element-Standard 100 mg Sb/l (B.Kraft 21152). 2 ml sample were reduced with 0.4 ml solution potassium iodide (25%) ascorbic acid (5%), 5 ml HCl (37%) and 13.6 ml Milli-Q water. Dilution factor was 1:20.

2.2.5 *ICP-MS analysis*

During the measurement, I use the option “peak Hopping Node” and “Smart Rinse”.

Parameter	Amount
Power	1.4 Kw
Stab delay	60 s
Dwell time	10000 µs
Scan n° for each replicate	30
Measurement repetitions	5

Table 4. Instrument parameters for the ICP-MS.

2.2.6 *Statistical analyses*

I calculated the relative standard deviations, standard errors, and linear regression using Microsoft Excel.

2.3 **Results**

2.3.1 HNO$_3$ extraction

Fig. 1 and Table 5 show a low accuracy for the HNO3 extraction with all the analytical methods (HG-AFS, ICP-MS and GFAAS). The HNO$_3$ digestion recovered <50% of the Sb contained in the samples. Fig. 2 shows large differences between the standard deviation (RSD), a measure of the method's precision, between the different analytical methods. ICP-MS gave the best precision for Sb concentrations of 5 and 85 µg/l, with RSDs of 13% and 21% respectively. However, ICP-MS had the highest RSD (20%) for the 25 µg/l Sb. HG-AFS and GFAAS gave >50% RSD for Sb concentration of 5 µg/l.

2.3.2 Modified HNO$_3$ extraction

Fig. 3 and Table 6 shows that for the modified HNO$_3$ digestion, HG-AFS was the most accurate technique, with 76% Sb recovery. However, Fig.4 shows a high RSD, indicating a low precision, for the 5 and 85 expected Sb (µg/l), respectively with 36% and 22% RSD. GFAAS and ICP-MS give bad accuracy with lass then 70% retention of total Sb (µg/l). As it can be seen on the Fig. 4, modified HNO$_3$ digestion give low precision for all the analytical methods for the 5 Sb (µg/l). In higher Sb concentrations (25, 45, 85, µg/l), modified HNO$_3$ extraction gave good precision (less then 20% RSD) for the 25 and 45 µg/l concentrations.

2.3.3 Aqua regia extraction

Fig. 5 and Table 7 show a high accuracy with HG-AFS analysis methods for *aqua regia* digestion. The recovery of Sb was 82%. However, HG-AFS gave poor precision for the 5 µg/l concentration, with about 85% RSD (Fig. 6). Fig. 5 shows the accuracy for ICP-MS and GFAAS was <20%, with recoveries of just 76% and 62% respectively.

2.3.4 Modified aqua regia extraction

Fig. 7 and Table 8 show high accuracy for ICP-MS method with the modified *aqua regia* digestion, recovering 99% of the expected Sb concentration. In addition, the precision was relatively high with <15% RSD for all the Sb (µg/l) expected concentrations (Fig. 8).
HG-AFS and GFAAS recovered 57% and 70% respectively of the total Sb.

2.3.5 *(Figure and Table) HNO_3 extraction*

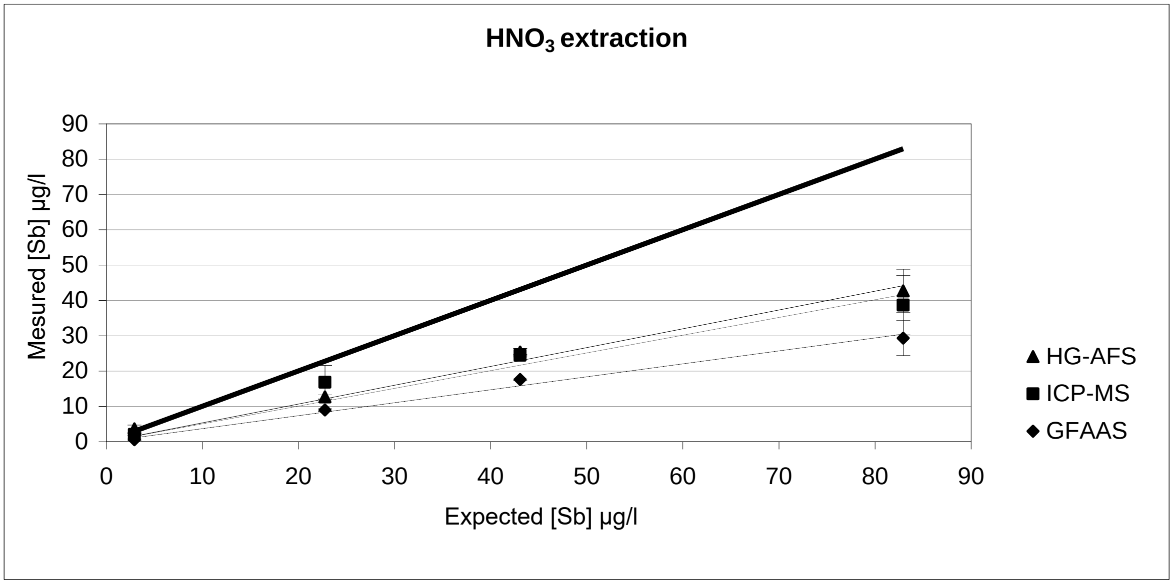

Figure 1. HNO3 digestion and analysis with HG-AFS, ICP-MS and GFAAS. Bars represent the standard error of the mean. The solid black line is y=x.

Analytical methods	**Equations**	**R^2**
Expected	y=x	$R^2=1$
HG-AFS	y = 0.53x	$R^2 = 0.99$
ICP-MS	y = 0.50x	$R^2 = 0.93$
GFAAS	y = 0.37x	$R^2 = 0.99$

Table 5. Equations parameter for the line on Fig. 1.

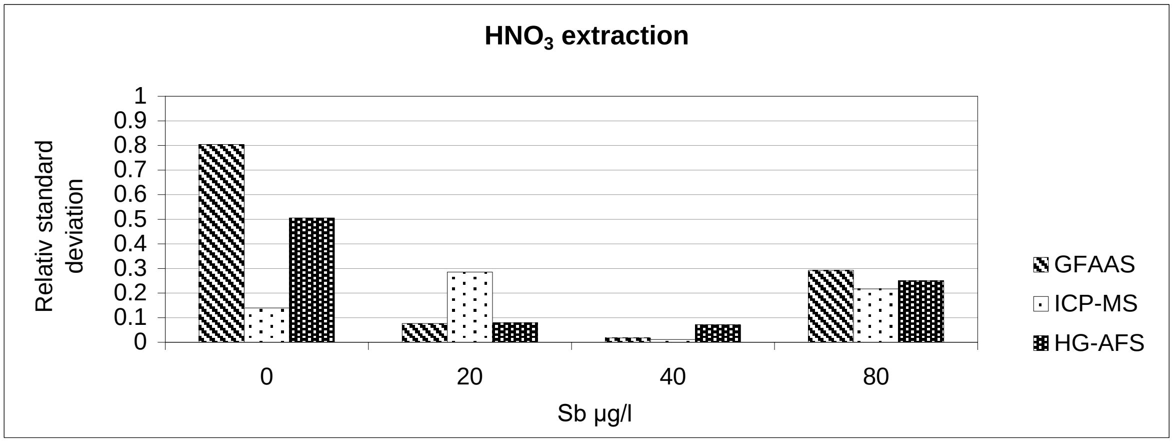

Figure 2. Relative standard deviations for HNO_3 digestion and analysis with HG-AFS, ICP-MS and GFAAS.

2.3.6 (Figure and Table) Modified HNO_3 extraction

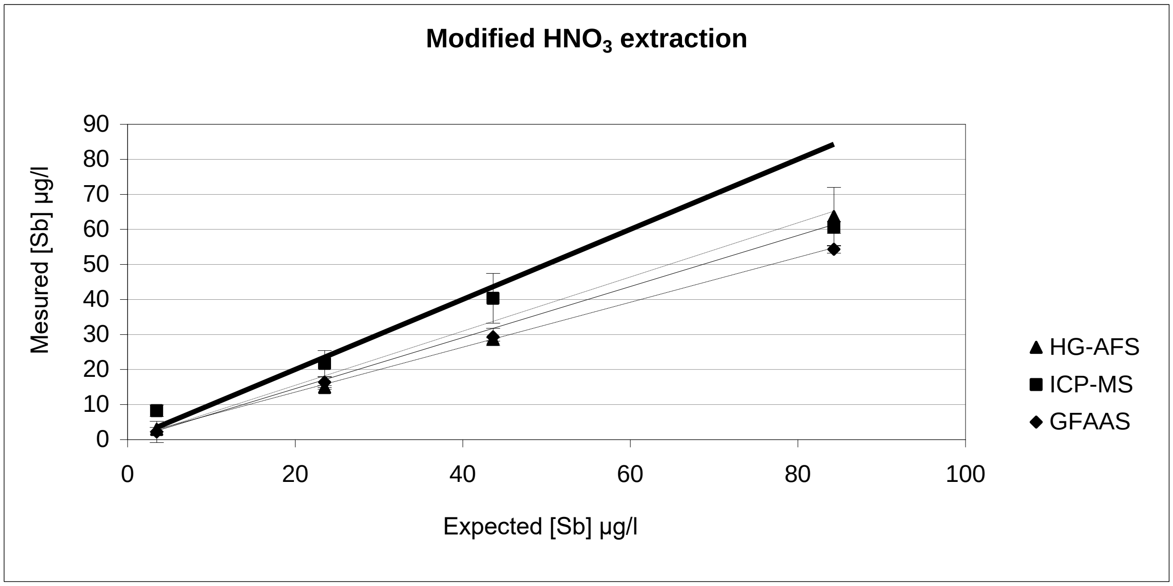

Figure 3. Modified HNO3 digestion and analysis with HG-AFS, ICP-MS and GFAAS. Bars represent the standard error of the mean. The solid black line is y=x.

Analytical methods	Equations	R^2
Expected	y=x	$R^2=1$
sHG-AFS	y = 0.76x	$R^2 = 0.99$
ICP-MS	y = 0.66x	$R^2 = 0.98$
GFAAS	y = 0.64x	$R^2 = 0.99$

Table 6. Equations parameter for the line on Fig. 3.

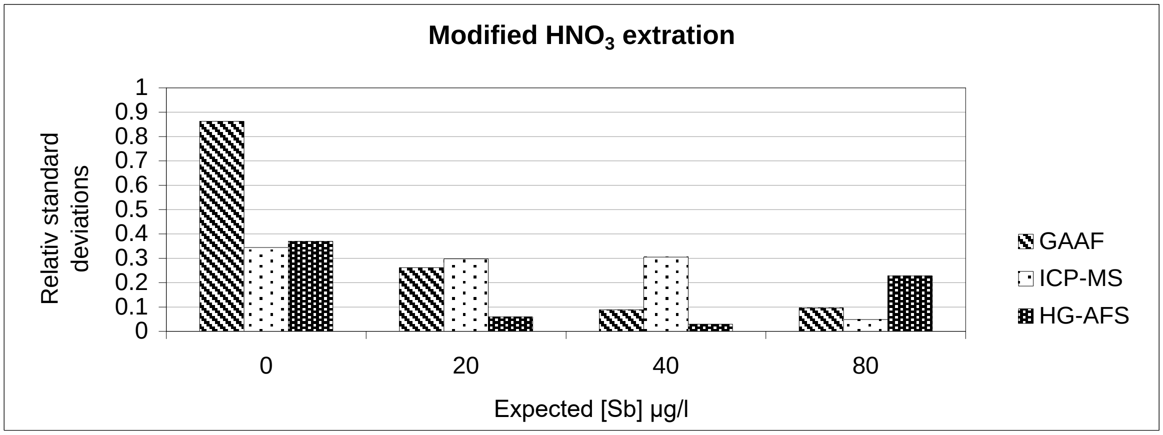

Figure 4. Relative standard deviations for modified HNO_3 digestion and analysis with HG-AFS, ICP-MS and GFAAS.

2.3.7 (Figure and Table) Aqua regia extraction

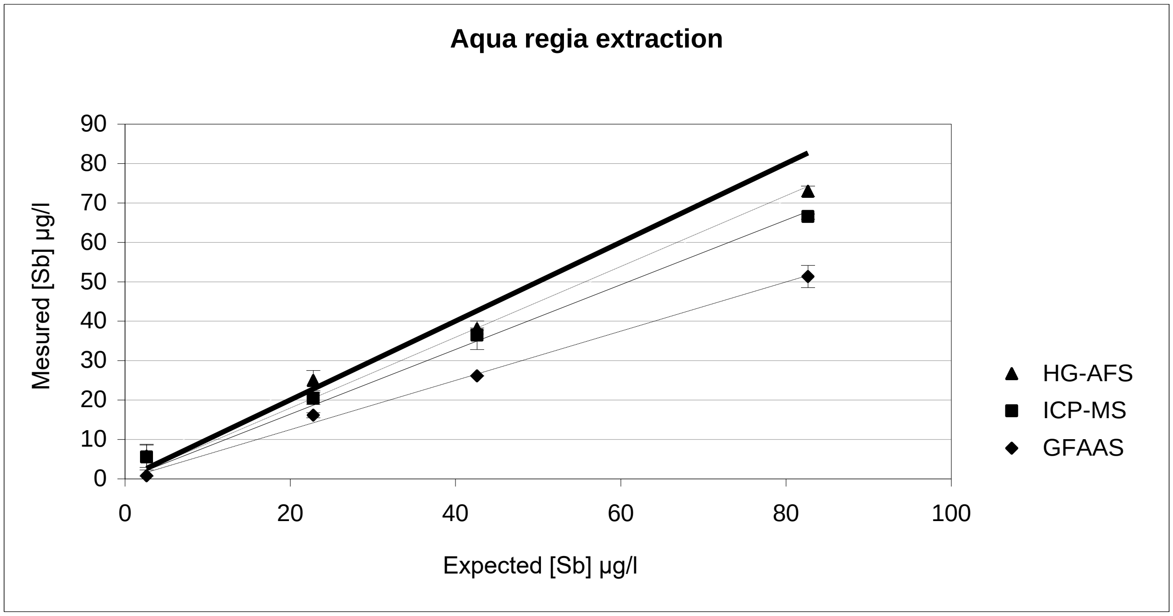

Figure 5. Relative standard deviations for the *aqua regia* digestion and analysis with HG-AFS, ICP-MS and GFAAS. The solid black line is y=x.

Analytical methods	Equations	R^2
Expected	y=x	R^2=1
HG-AFS	y=0.82x	R^2 = 0.99
ICP-MS	y=0.76x	R^2 = 0.99
GFAAS	y = 0.62x	R^2 = 0.99

Table 7. Equations parameter for the line on Fig. 5.

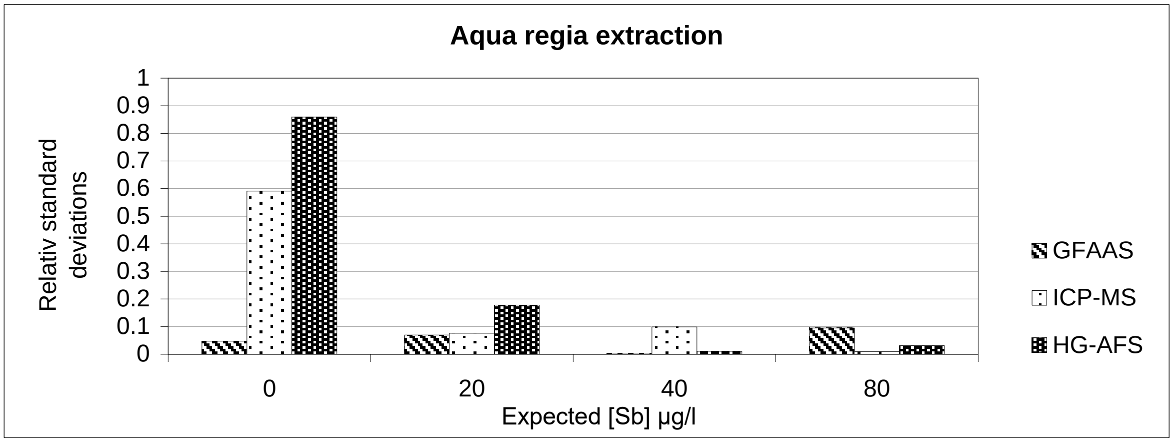

Figure 6. Relative standard deviations for the *aqua regia* digestion and analysis with HG-AFS, ICP-MS and GFAAS.

2.3.8 (Figure and Table) Modified aqua regia extraction

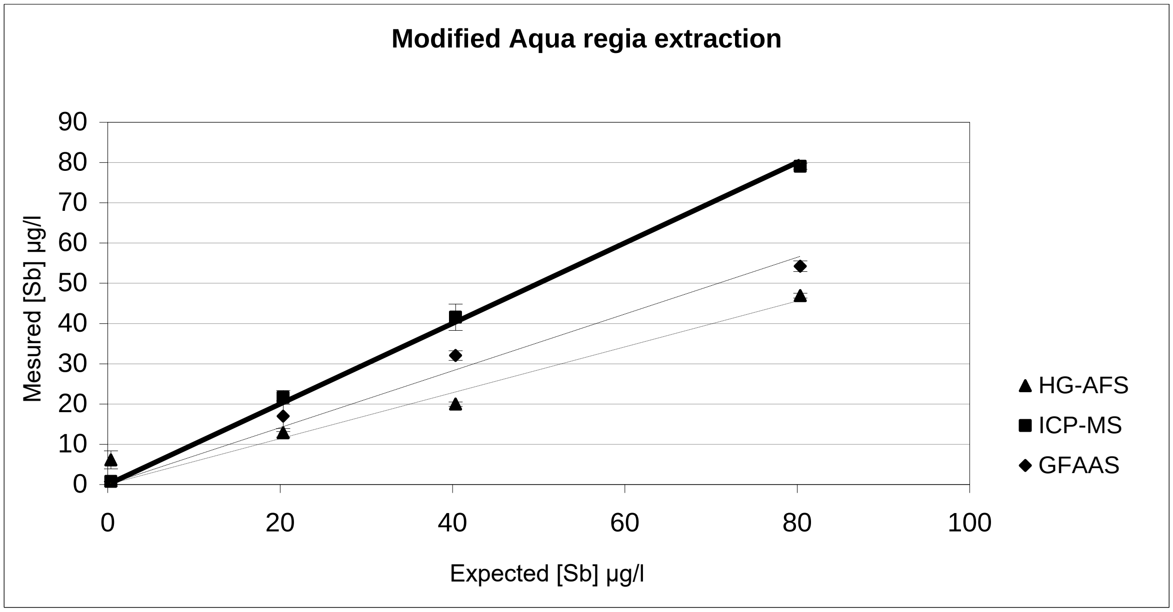

Figure 7. Modified *aqua regia* digestion and analysis with HG-AFS, ICP-MS and GFAAS. Bars represent the standard error of the mean. The solid black line is y=x.

Analytical methods	**Equations**	**R^2**
Expected	y = x	$R^2 = 1$
HG-AFS	y = 0.57x	$R^2 = 0.87$
ICP-MS	y = 0.99x	$R^2 = 0.99$
GFAAS	y = 0.70x	$R^2 = 0.98$

Table 8. Equations parameter for the line on Fig. 7.

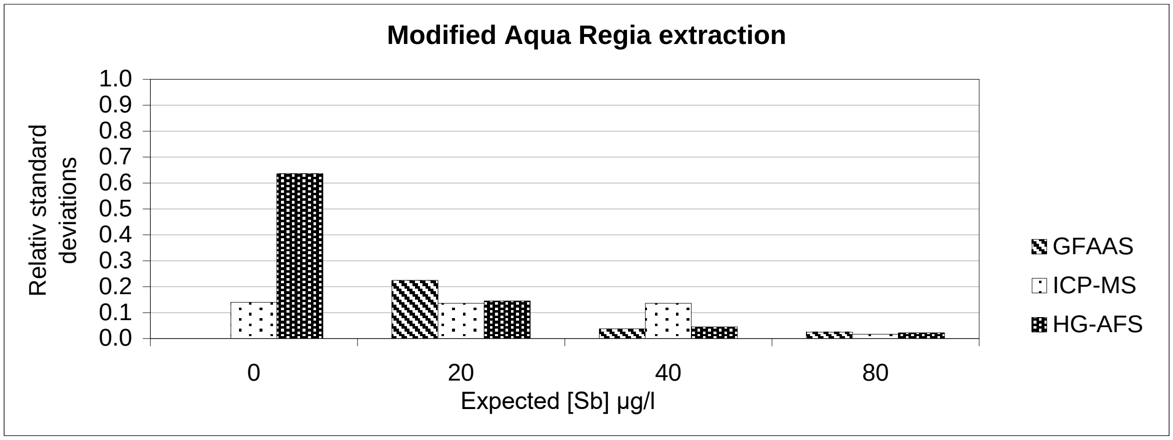

Figure 8. Relative standard deviations for the modified *aqua regia* digestion and analysis with HG-AFS, ICP-MS and GFAAS.

2.4 Discussion

Our result shows that only the modified *aqua regia* digestion method coupled with ICP-MS (section 2.3.8), gave high accuracy and high precision for total Sb determination in plant samples (Fig. 7 & 8). The *aqua regia* digestion method, coupled with HG-AFS (section 2.3.7), show acceptable accuracy (Fig. 5), however, the precision was low for at low concentrations (Fig. 6).

The modified HNO_3 extractions (section 2.3.1) and HNO_3 extraction (section 2.3.2) were unable to recover >80% of total Sb concentration, regardless of the analytical procedure. It is possible that Sb is not totally soluble in mixtures with high quantities of HNO_3, even though the Sb was added as a soluble compound. To avoid precipitation, in Modified HNO_3 extractions (section 2.2.2) the HNO3 was evaporated after digestion to eliminate the interference with Sb solubility. Nevertheless, this experiment resulted in low accuracy. On the other hand, in the *aqua regia*-based extractions (section 2.3.3 and 2.3.4) Sb was probably complexed with chlorine (Cl) as Sb (V) chloride ($SbCl_5$). These Sb complexes are soluble and are able to be detected using our spectroscopic methods. These result do not support the hypothesis that significant amounts of Sb are lost from solution as the tri and penta–chloride species during the digestion (Aylward and Findlay 1994).

My results show a low accuracy in all the digestion methods of total Sb analysis with GFAAS. This may be because the charing temperature necessary to purge the matrix (700°C), volatilised Sb. Antimony complexes with chlorine (Cl) have a low boiling point; Sb (III) chloride ($SbCl_3$) boils at 223°C and Sb (V) chloride ($SbCl_5$) boil at 140°C. There is a lacuna of information dealing with the physical properties of Sb complex with NO^{3-}. Probably volatilisation caused a significant loss of Sb in the samples digested with *aqua regia* or HNO_3.

2.5 Conclusions

ICP-MS coupled with the modified *aqua regia digestion* is a suitable method for total Sb analysis in plant samples. ICP-MS provide detection limits as low as 2.5 Sb µg/l, and can simultaneously determine many elements. It is thus likely that ICP-MS is the best method, since all Sb species are atomised in the plasma stream.

HG-AFS coupled with *aqua regia* extraction could be a suitable method for total Sb determination in plant samples. The detection limit was low (2 µg/l), however, the precision was low at lower concentrations. The high concentrations of strong oxidising reagents such as HNO_3 and HCl, may interfere with the reduction of Sb (section 2.2.2).

Henceforth, the determination of Sb in plant will be performed with the `*Modified aqua regia extraction*` coupled with ICP-MS analytical system, using the methods described in section 2.2

3 Development of an agar system to test plant Sb interactions

3.1 Choice of plants general materials in this study

This study concerns the following plant species: Maize *(*Zea mays *L.,)*, Sunflower *(Helianthus annuus L.)*, Clover *(Trifolium pratense L.)*, Indian mustard *(Brassica juncea L.)* and Wheat *(Tritium aestivum).* I choose these species because of their widespread use in agriculture and their rapid growth rate may result in high Sb uptake in the plants. More information about these species is given in Appendix 1.

Our experiments used antimonate Sb(V), because this is the most common chemical species in natural waters and soil solution (Chapter 1).

3.2 Introduction

In contrast to soil, agar is a homogeneous medium that can easily be sterilised. It supports bacterial, fungal and plant growth and can be spiked with Sb to give any concentration within the range of the element's solubility. Almost all plants can grow in agar (Butcher 1976).

I chose to use agar, rather than soil or hydroponics in this study because the total and soluble Sb concentrations could be controlled precisely.

In soil, Sb may be absorbed onto particles and bound to organic matter, which includes both soluble and insoluble fractions. Preparing homogeneous concentrations of Sb in soil is difficult, since soil consists of a mixture of varying sized particles, which absorb Sb to varying degrees. Soil microflora also affect the toxicity and uptake of trace elements, such as Sb, by plants.

In hydroponic systems, small breaks in the plant roots, caused by moving the plants from the germination media (usually perlite) into the hydroponic system may result in the direct entry of Sb into the root xylem. This would give a much higher uptake that what would occur in a soil. Similarly, in hydroponic systems, all Sb in the solution can sorb directly on the root, since there is no retardation on convective processes.

One of the major disadvantages of agar media for growing plants under sterile conditions is that the medium is non-porous. Consequently, gases diffuse only slowly though the medium and roots may be exposed to similar stresses as are encountered in waterlogged soils (Drew 1983;Jackson 1984). Under these conditions, roots grow may be impaired. Barrett-Lennard and Dracup (1988) proved that a porous agar medium improve roots growth of 80-90% in *Trifolium subterraneum.*

The composition of the agar should be amenable for plant growth. It should thus contain all the essential nutrients and not contain high concentrations of solutes, such as sugars, that make the medium osmotically unfavourable for root water uptake.

Concerning the optimal agar concentration for plant growth, there are large interspecific differences: the concentrations range varies between 0.5% w/v - >15.0% w/v. Pâques (1991) pointed out that there is a strong connection between culture medium hardiness, proliferation ratio, and hyperhydration of the plants. Normally, an increase in the agar concentration promotes a reduction in the occurrence of hyperhydration symptoms in plants. The process of hyperhydration results in plants taking up excess water and hence the abnormal structures of leaf and stem. The concentration of agar in the medium may also affect the formation of roots. Rahman et al. (1992) reported that rooting performance of rose decreased with increasing agar concentration (from 6.0 up to 15.0 w/v). The agar should physically support the roots, but also be thin enough to allow enough oxygen to diffuse into the root-zone. Unlike bacteria, plants translocate water from the roots to the aerial portions where it evaporates from the leaves. This can deform the agar and lead to cracks.

Most importantly, the plant – agar system must be free of alien microbes, especially fungi. Since such microbes occur on the coats of most seeds, the seeds need first to be sterilised, without damaging the embryo contained therein. Alien organisms must then be excluded from the agar system for the duration of the experiment.

All the seeds in their natural environments contain a wide range of microorganisms during their development. Some microorganisms invade the seeds from sub-epidermial infections while others occur only on the seed surface. The level of the internal and external contamination is variable and depends on many different factors and the specific features of the seeds. Surface sterilisation with sodium hypochlorite, has been widely practiced. Speakman and Krüger (1983) showed that NaClO was effective for removing the surface microbial contaminant with only small decrease of the seeds germination. However, even though such methods totally eliminate surface microorganisms, they seldom affect internally located microorganisms Sauer (1986) and Cuero (1986) found that the *Phycomicete* fungi and *Bacilluss* were eliminated and F*usarium* were much reduced. *Aspergillus*, *Penicillium* and S*accharomicyces* were less affected by sodium hypochlorite treatment while limited numbers of *Trichoderma cladosporium* appeared in the samples. The Ascomycota *Alternaria alternata* was also not killed by treatment with NaOCl (Ramakrishna 1991).

The soluble faction of Sb in the growing medium is an important point for ours study, because all Sb in solution can sorb directly on the root, since there is no retardation on convective processes (Chaper 1). This means that the soluble fraction is the bioavailable fraction as well.

The aims of this experiment were to determine the ideal treatment in order to achieve a good surface sterilisation of the seeds without affecting their germination and growth rates, and develop a method to grow plants in sterile conditions in agar. I also aimed to determine the plant – availability of Sb in agar, by separating the liquid from the gel fraction, and subsequently total Sb analysis.

3.3 Materials and methods

3.3.1 Step 1: seed sterilisation

I tested two sterilisation agents, 30% hydrogen peroxide (H_2O_2) and 13% sodium hypochlorite (NaClO) on the seeds of five species that may be used in further experiments throughout the project. These were:

- maize (*Zea mays L.*)
- clover (*Trifolium pratense L.*)
- sunflower (*Helianthus annuus L.*)
- Indian mustard (*Brassica juncea L. Czern.*)
- bread wheat (*Triticum aestivum L.)*

For each treatment, I immersed the seeds in the solution for 0, 2, 4, 8, 16, or 32 minutes.

For large seeds (maize, sunflower, wheat), fifty seeds per each plant were put in a separate beaker filled with each sterilisation agent. After two minutes, under laminar flow, ten seeds were taken out with tweezers, put in a sterile beaker and rinsed five times with sterile water (autoclaved), then stored for germination in a 60 mm Petri dish whose bottom was covered with two layers of filter paper. The same treatment was repeated after four, eight, sixteen and thirty-two minutes, for the two plants and the two sterilization media; the seeds were stored each time in a new, sterile Petri dish. The residence time was inexact due to the difficulty to take out rapidly the seeds with the tweezers. Therefore, I started to extract the seeds 15" before the time, and ended some 15" after the time.

For the smaller seeds (clover and Indian mustard), the process was modified due to the difficulty to take out rapidly the seeds with the tweezers. The fifty seeds were separated by putting ten seeds in each of five small beakers filled with H_2O_2, and the same was done with NaClO. After two minutes, the first beaker was emptied while stopping the seeds with tweezers, and then filled five times with sterile water to rinse the seeds, that were then stored for germination in a Petri dish containing wet paper filters. The same treatment was repeated for a different beaker after four, eight, and sixteen and thirty-two minutes, for the three plants and the two sterilization media; the seeds were stored each time in a new, sterile Petri dish.

3.3.2 Step 2: seed germination

Figure 2 shows the design of the germination test, with the Petri dishes stored in a climate chamber with the following parameters: 22°C, 75% relative humidity, 11'000 lux, 16h full light and 6h full dark.

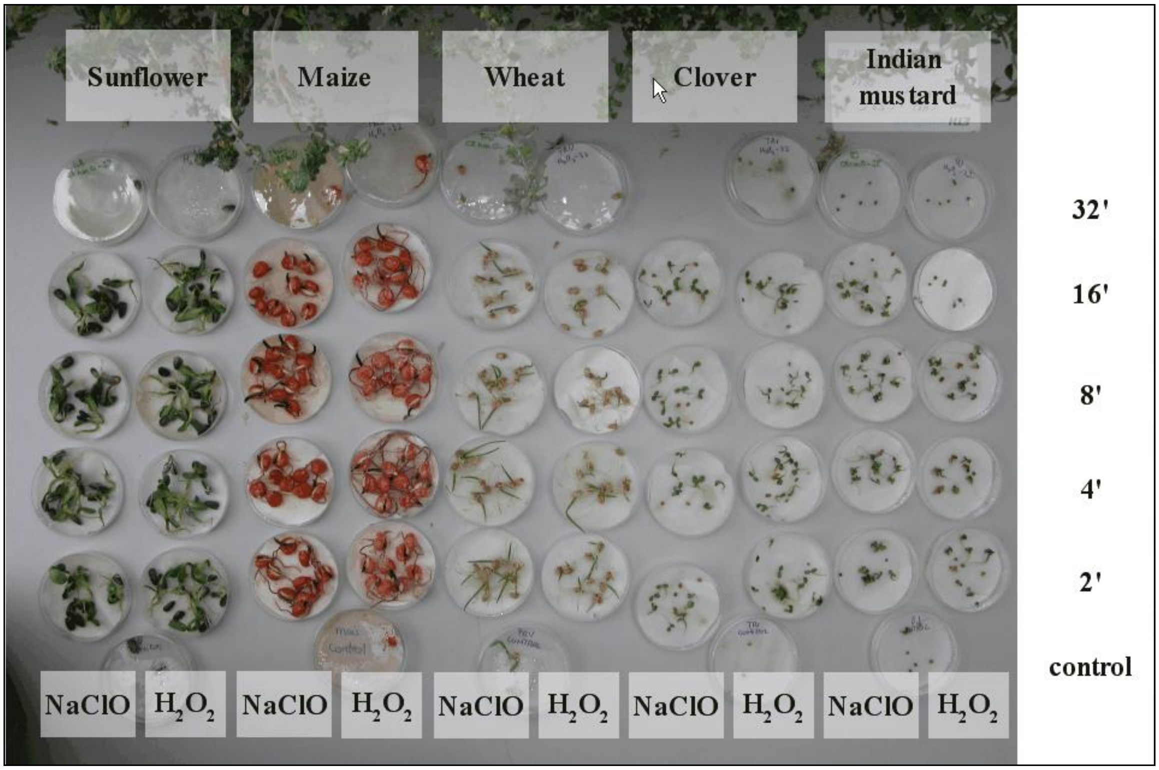

Figure 9. Shows the design of the germination test, with the Petri dishes.

The seeds were allowed to germinate then, the control samples (i.e. seeds without surface sterilisation) and the samples treated with H_2O_2 or NaClO during 32' were transferred into Petri dishes containing an agar substrate (see Step 3). The other samples were kept, and their root length (main root) was measured with a ruler.

3.3.3 Step 3: plant growing in agar

Control seeds and seeds treated during 32' with H_2O_2 or NaClO coming from the previous step of the experiment (see Step 2) were seeded in 60 mm Petri dishes containing 10 ml agar culture medium. The culture medium was made of 10 g/l agar dissolved in modified Hoagland's nutrient solution, and was autoclaved in order to ensure sterile growing conditions. I placed three seedlings in each Petri dish under laminar flow, and then sealed with Parafilm to prevent contamination.

3.3.4 Step 4: seed germination directly in agar (tubes)

I performed the following experiment with 50 ml pure polypropylene tubes (DigiTUBEs from SCP SCIENCE) and used polypropylene disposable watch glasses as lids; the tubes were then sealed with Parafilm. I filled the tubes with 25 ml of agar (prepared as in the previous experiment, see Step 3), and wrapped the external surface of the tube with aluminium foil to darken the agar growing medium. Due to mixing problems during agar preparation, some tubes contained thicker agar compared to others, but this does not seem to have affected seed germination and growth.

For the germination test, the seeds were sterilised with NaClO during 32'. Three replicates were prepared for each plant, each tube containing three seeds; a control tube containing three untreated seeds was also prepared for each plant species. Fig. 10 shows the experimental design. For the growth test, all healthy seedlings coming from the previous experiment and treated with NaClO for 32' were carefully removed from the Petri dishes and seeded in the tubes (one plant per tube).

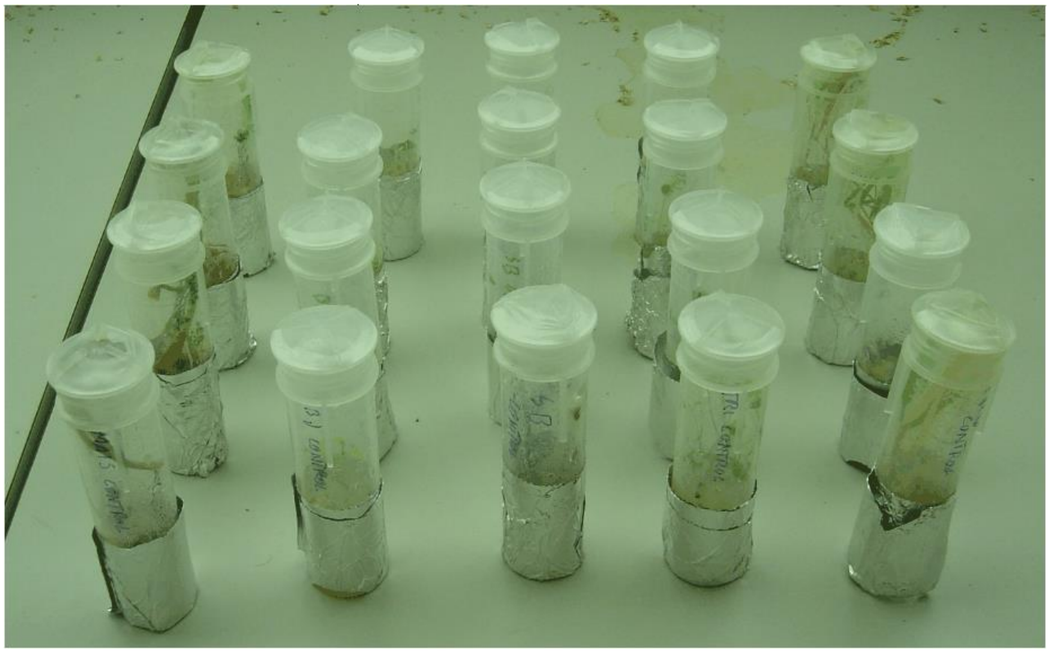

Figure 10. Experimental design for germination test.

3.3.5 Step 5: seed germination directly in agar (growth boxes)

I tested the growth of the selected species in large sterile transparent boxes. The boxes were obtained from Combiness$_{nv}$; dimensions were 90 mm diameter and 140 mm high. A permeable filter allows gas exchange between box and surrounding environment, without permitting the entry of dust or microorganisms.

3.3.6 Determination of Sb solubility in agar

A semisolid medium was obtained with 10 w/v Agar, and subsequently spiked separately with: 0 mg/l, 10 mg/l, 20 mg/l, 40 mg/l, 80 mg/l and 160 mg/l of Sb(V) as KH_2SbO_4. Three replicates were prepared for each treatment. The Medium was autoclaved for 21 min at 121 °C and 961 mbar. Total Sb was determined using methods developed in Chapter 2. After solidification, about three hundred mg of Agar was centrifuged into 50 ml Teflon tubes for 15 min at 9500 rpm. The supernatant was transferred and accurately weighed into 50 ml Teflon tubes and analysed using the methods described in chapter 1. Statistical analysis was performed with Microsoft excel.

3.4 Result and discussion

3.4.1 *Step 1: seed sterilisation*

I wanted to assess if our sterilisation protocol was effective at preventing fungal and/or bacterial infections during seed germination and plant growth in agar. No infection was visible in the Petri dishes; control seeds and seeds treated during 32' with H_2O_2 or NaClO were transferred in Petri dishes containing an agar substrate. After 4 days of germination for maize and sunflower, and 8 days of germination for wheat, clover and Indian mustard, a visual control of the samples gave the results reported in Table 9. Results in Table 10 refer to an observation of the germination test samples (i.e. samples treated during 2, 4, 8 and 16 minutes with H_2O_2 or NaClO.

Bacteria and fungi find in agar a good substrate for their growth, this explains why there were more infections observed in samples seeded in agar than in those germinating on filter paper. However, results in Table 9 show that NaClO prevented infections in all cases. Therefore, this reagent was a good choice for the next steps of this preliminary experiment.

Treatment	Wheat	Maize	Sunflower	Clover	Indian mustard
Control	All samples infected (bacteria)	All samples infected (bacteria)	All samples infected (fungi)	All samples infected (bacteria)	All samples infected (bacteria and/or fungi)
H_2O_2 32'	1 sample infected (fungi)	-	1 diseased sample	All samples infected (fungi)	-
NaClO 32'	-	-	-	-	-

Table 9. Results of the visual control performed on control samples and on 32'-treatment samples growing in agar.

Wheat	Maize	Sunflower	Clover	Indian mustard
-	-	Infection in NaClO 8' and 16'	Infection in H_2O_2 2'	-

Table 10. Results of the visual control performed on germination test samples germinating on paper filters.

3.4.2 Step 2: seed germination

I aimed to observe the influence of the sterilisation medium and residence time on seeds survival and germination, and the efficacy of our sterilisation technique in preventing bacterial and fungal infections. To do this, I let the seeds germinate in the Petri dishes and recorded some visual features to define the effect of the residence time on seed germination, i.e. whether the seeds suffer from damage if they bathe too long in the sterilisation medium; the results are summarized in Table 20, Table 21 and Table 22 (see Appendix, section 6.6). This was also useful to assess which sterilisation medium was most suitable for each plant species, if there was a correlation between the use of H_2O_2 or NaClO for a species and the germination of its seeds.

As a second test, I measured root length to determine the influence of the type of sterilization medium and the residence time on plant development (Table 12).

Table 20 shows that after 3 days almost all control sunflower, Indian mustard and wheat seeds were at a “green” germination stage (i.e. the shoot has already appeared), whereas for maize and clover just one seed reached this germination stage. Conversely, treated seeds of clover germinated more rapidly than untreated ones, and this was particularly true for those sterilized with NaClO. However, 4 day later this trend almost disappeared for Indian mustard and wheat, as almost all seeds (especially those treated with sodium hypochlorite) had reached the “green” stage (Table 21). Eight days later (Table 22) there was little difference between the treatments, as for 28 samples out of 40, >80% of the seeds have reached the “green” stage. The preliminary observation of data recorded in the tables shows no striking influence of the sterilisation medium or of the residence time on seeds germination.

Sunflower	Maize	Wheat	Clover	Indian mustard
Some problems with NaClO	Some problems with NaClO	No difference	Better germination with NaClO	Better germination with NaClO

Table 11. Effect of the sterilization medium on seed germination (results based on visual observation).

For a further comparison between the two treatments and the various sterilisation times, I measured the roots length (main root) of the seedlings coming from the 2', 4', 8' and 16' treatments. I wanted to assess whether the treatments have a significant detrimental effect on the development of the roots: to do this, I tested the significance of the data (i.e. the significance of the effect of sterilisation time and medium on roots' length) with a Student's t-test. The results are summarised in Table 12.

	Maize		**Clover**		**Sunflower**		**Indian mustard**		**Wheat**	
	H_2O_2	NaClO	H_2O_2	NaClO	H_2O_2	NaClO	H_2O_2	NaClO	H_2O_2	NaClO
4'	-	40%	-	-	-	-	-	-	-	-
8'	-	43%	-	-	-	-	-	-	-	-
16'	-	73%	-	-	-	-	80%	-	-	72%

Table 12. Significance of the effect of sterilization time on mean roots' length ('-' = no significant decrease; otherwise: % of roots' length decrease)

These data were calculated on the basis of the roots length index (RLI = L(time)/L(control)), where I took as control the root length of the seeds treated during 2', because the original controls have been used for another experiment. In almost all cases there was no significant decrease in roots length when increasing the sterilisation time. Just maize seems to suffer from sterilisation with NaClO; Indian mustard showed an important decrease after having been treated for 16' with H_2O_2, and the same happened to wheat with NaClO.

The sterilisation time did not affect any measured parameter of seed germination or roots development.

3.4.3 Step 3: plant growth in agar

The third step of this preliminary experiment consisted of testing whether the sterilisation of the seeds was effective and does not affect the growth of the plants; another goal was to verify if our protocol was efficient at ensuring sterile conditions for plant growing in agar. This protocol allows us to prepare a sterile growing environment for the plants.

	Maize		**Wheat**		**Sunflower**		**Clover**		**Indian mustard**	
	H_2O_2	NaClO	H_2O_2	NaClO	H_2O_2	NaClO	H_2O_2	NaClO	H_2O_2	NaClO
Infections	no	no	yes	no	yes	no	yes	no	no	no
Growth	+	-	+/-	+/-	+	-	-	+	-	+

Table 13. Effectiveness in preventing infections and influence on plants growth of the two sterilizations media.

Sodium hypochlorite was more effective than H_2O_2 in sterilising the seeds surface, as none of the samples treated with NaClO suffered from infection.

Concerning the influence of the sterilisation medium on plant growth, it changes for each plant species: maize and sunflower were more sensitive to NaClO, whereas clover and Indian mustard had more problems when treated with hydrogen peroxide.

Based on the results reported in Table 9, I decided to use NaClO for seeds sterilisation for the next step of this preliminary experiment.

3.4.4 *Step 4: seed germination directly in agar*

The next step aimed at developing an experimental design that allowed us to grow plants for a longer time in agar. I therefore needed a container where plants and roots could develop in the vertical plain, as well as in the horizontal plain, such as in the Petri dishes. Having more room for the plant meant also having more air. At the same time, I wanted to test if it was possible to skip the separated germination phase (germination of the seeds on paper filters) and make a direct germination on agar.

After one month of growth, all maize seeds but one germinated, and an infection developed in the control sample. Fig. 18 shows that these tubes were not very well adapted for maize plants, as they do not have enough space to grow properly. Due probably to vapour condensation and roots exudates, the upper agar layer became liquid. Indeed, the plants suffered from the deficient gas exchange between the tube and the climate chamber air and from the lack of space for roots and stem.

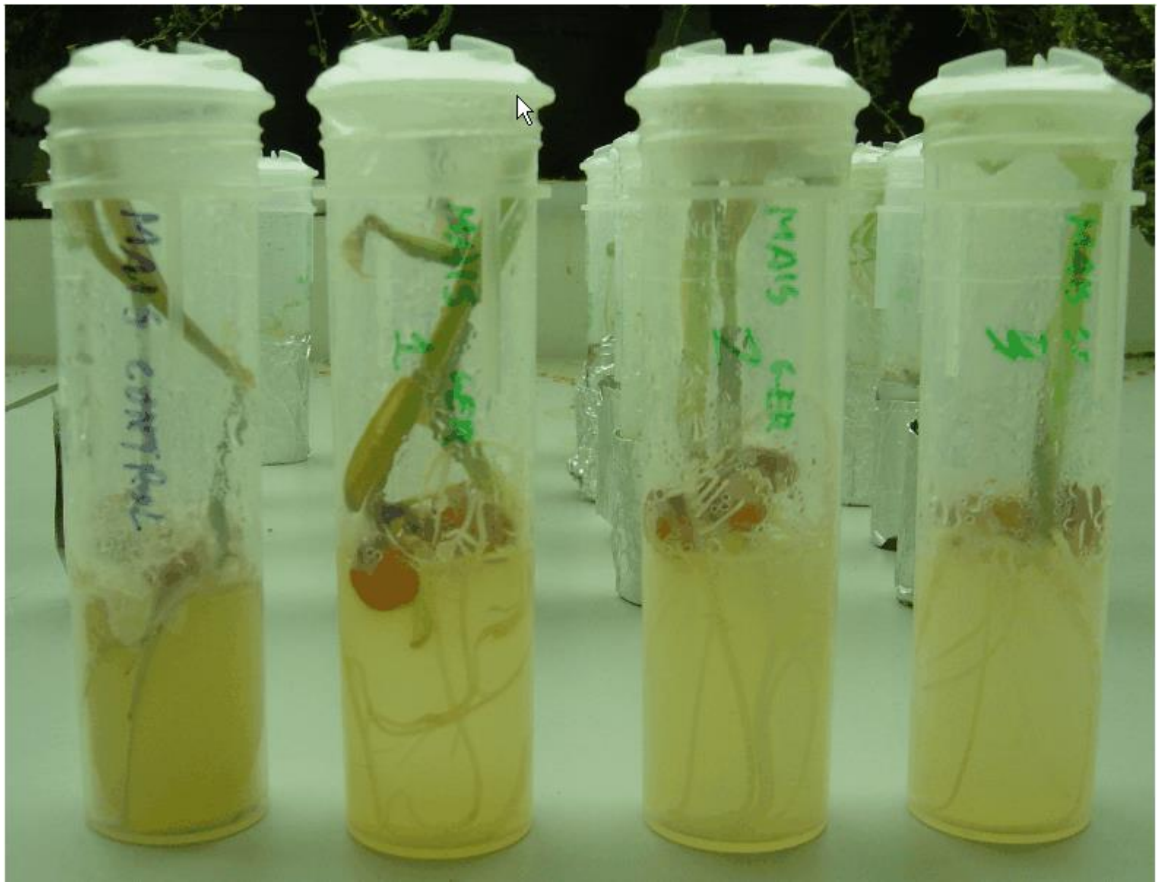

Figure 11. Example of *Zea mays* grow in tubes.

Note this system provides good growing conditions. However, there will be virtually no transpiration in such a system because the air is fully saturated with water vapour, which cannot escape from the container. Therefore, any uptake will be due only to active ion pumps in the roots, and phloem transport, since xylem transport, which depends on transpiration, will be nearly nonexistent. Since it is likely that Sb enters via the apoplastic pathway, and is not likely to be transported in the phloem, such a system would produce artificially low Sb uptake results, compared to those that may be obtained in the field.

Table 14 shows that the seeds in the agar germination experiment had a high germination rate also when seeded directly in agar, without previous germination on paper filters. In this way, I could avoid the separate germination step, eliminating a potential additional source of infection.

	Maize	**Indian mustard**	**Sunflower**	**Clover**	**Wheat**
Germination rate	11	11	12	11 infection in a treated sample	7 1 tube with rotted seeds

Table 14. Number of seeds that germinated (12 seeds per plant).

Concerning the growth experiment, I noticed that the plants could indeed grow for a longer time in the tubes than in the Petri dishes, but it appeared clearly that this solution was not the best either. This may have been due to:

- there was no circulation and air exchange
- there may have been a lack of nutrients
- the tube was not perfectly transparent, and this can affect light penetration and use by the plant for the photosynthesis
- there was not enough space for bigger plants to grow
- agar was not a suitable growing medium for roots on a long term basis (mainly due to the almost anoxic conditions).

3.4.5 Step 4: seed germination and growth in plant-growth boxes

Although the plant growth boxes were larger and more expensive than the aforementioned tubes, these boxes had three important advantages. There permeable filter allowed gas exchange with the ambient environment, thus facilitating plant transpiration and growth. The plastic is specifically designed for plant growth and therefore allows the passage of a greater proportion of photosynthetically active radiation the growth tubes. Thirdly the larger size of the boxes allowed some four weeks of growth without serious perturbation of the plant morphology. The larger volume of agar was also lese prone to cracking due to plant water uptake.

3.4.6 Determination of Sb solubility in agar

Fig. 12 shows that the detected Sb concentrations were directly proportional to the added Sb concentrations. The measurement even shows high precision, even if the standard errors increase proportionately with the rinse of the Sb concentration.

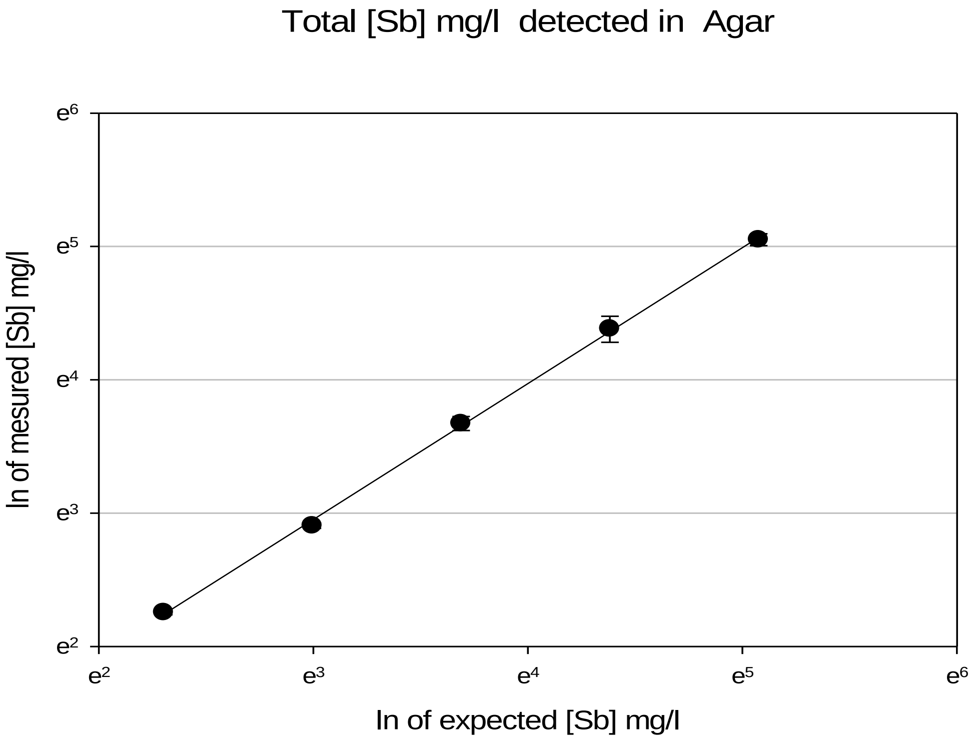

Figure 12. Total Sb concentration, measured in the liquid fraction of Agar. Error bars represent the standard error of the mean. The Linear regression respect the equations:

$y = 0.98x$; $R^2 = 0.99$.

[Sb] added to agar (mg/l)	Average of measured [Sb] in liquid phase (mg/l)	Relative standard deviation
0	0.35	0.19
10	9.55	0.32
20	18.29	0.67
40	39.38	3.54
80	80.11	13.55
160	156.31	12.07

Table 15. Expected and measures concentrations of total Sb in agar.

Fig. 12 and Table 15 shows that nearly all Sb in agar was present in the liquid fraction and little was bound to the agar matrix. This indicates that all the Sb present in the agar was able to interact with plant roots.

Sb is almost 100% soluble in agar. This result suggest us that the pollute should be full bioavailable for uptake by plants roots. Moreover, it is also possible that the metal form some kind of complex with agar that will be subsequently destroyed during the digestion process. Unfortunately, none information are available about these complexes agar-Sb in the literature. More investigation is needed to avoid this possible problems.

3.5 Conclusion

3.5.1 Agar method for my study

After these preliminary experiments, I selected a standard growth method for my subsequent experiments. I decided to use two different growth boxes. For the tolerance experiment (Chapter 4), I chose 150 mm sterile Petri dishes filled with 80 ml agar. These were transparent; therefore permitted me to observe the plants' root development (Fig. 13). For the uptake experiment (Chapter 5), I used the plant growth boxes (Fig. 14), which allowed plant transpiration and a growth period of ca. four weeks.

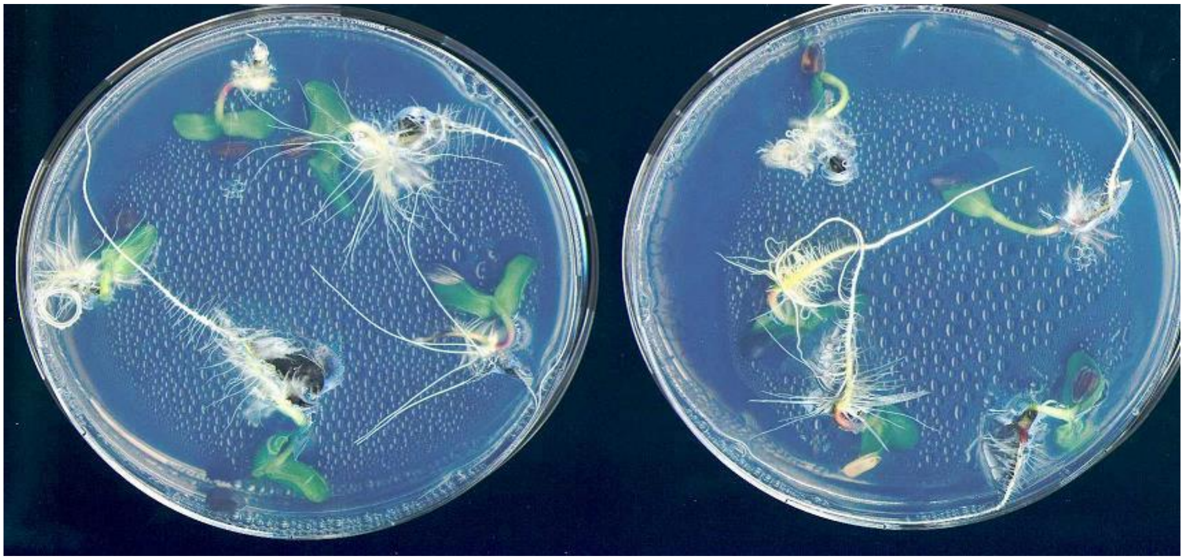

Figure 13. Example of *Helianthus annuus* grow in Petri disc.

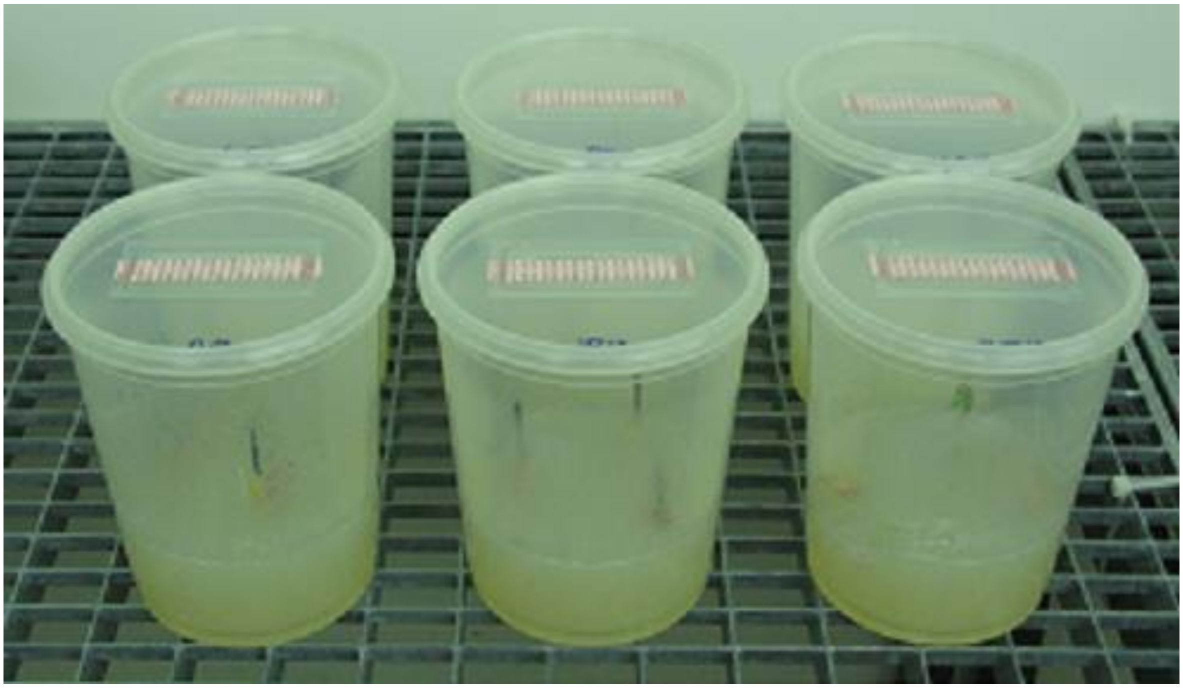

Figure 14. example of *Zea mays* grow in boxes.

I will sterilise the seeds surface by immersion into NaOCl 13% for 32 minutes. After this, the seeds will be washed for 5 times in abound sterile deionised water. Seeds will be germinated in sterile conditions in Petri dishes containing moist sterile filter papers. Germination will occur in darkness at 22 °C. After germination on filter papers, only the germinated seeds will be transferred in agar.

The nutrient medium will be a modified Hoagland solutions (pH 6±0.1) made with the following salt: 0.1 mmol KH_2PO_4, 0.5 mmol KNO_3, 0.4 mmol $Ca(NO_3)_2$, 0.2 mmol $MgSO_4$, 0.01 $FeSO_4$, 0.01 mmol H_3BO_3, 2 µmol $MnSO_4$, 2 µmol $ZnSO_4$, 0.2 µmol $CuSO_4$, 0.1 µmol Na_2MoO_4 and 0.02 mmol NaCl. Semisolid medium will be obtained with 10 w/v agar, and subsequently spike separately with: 0 mg/l, 10 mg/l, 30 mg/l, 100 mg/l and 300 mg/l of pollutant metal. I will add 10 w/v agar. The Medium will be autoclave for 21 min at 121 °C and 961 mbar. The climate chambers will have: photo period of 16h, day night temperatures of 14/ 22 C°, relative humidity 75% and a light intensity 11 000 lux.

4 Plant tolerance to Sb and As: a root length index study

4.1 Introduction

Both antimony (Sb) and its sister element arsenic (As) are toxic to animals and humans (Gebel 1997). However, there is little information on the toxicity of Sb to plants (Chapter 1). In contrast, As is a priority pollutant (USEPA 1979), therefore its biogeochemical behaviour, metabolism in plants and uptake by plants are object of intensive studies (Meharg and Hartley-Whitaker 2002) and are well characterised. Whereupon, As, being well characterised and chemically similar to Sb, makes a good benchmark element with which one can compare Sb. Therefore, As, being well characterised and chemically similar to Sb, makes a good benchmark element with which one can compare Sb.

As discussed in Chapter 1, Sb and As have similar chemical properties to P (phosphorus) and, it has been shown that As addition affects the uptake of P by competing for plant transporters (Woolson, Axley et al. 1973; Gulz, Gupta et al. 2005). In addiction, As(V) arsenate interferes with essential cellular processes such as oxidative phosphorylation and ATP synthesis (Tripathi, Srivastava et al. 2007). It is reasonable to assume that Sb(V) antimonate could be show similar behaviours and toxicity symptoms to As(V) (Kabata-Pendias 2000).

4.1.1 Root length index and EC50 as an indicator of plant tolerance to Sb

The Root length index (RLI) is a system for measuring the toxicity of a soil, or in our case agar, borne agent. It is based on the hypothesis that root length in a contaminated media is an indication of the plants tolerance. The RLI is the treatment / control root length quotient. The RLI is most useful for species that do not have a large natural variation in root development, because high standard deviations in root lengths in the control cannot be corrected. The RLI may provide misleading results if the action of the toxic agent affects other plant functions at lower concentrations than it affects root length.

The EC50 is a definitions frequently used in the toxicology. It corresponds to the concentrations of a toxic substance that is lethal to 50% of the individuals in a population. The EC50 value depends on the toxicity of the substance and the sensitivity of the population in question. In my study, I have adapted the EC50 to an RLI50, which represents the concentration of the toxic agent that reduces the RLI to 50% of the control.

The aim of this experiment was to investigate the toxicity of Sb(V) (antimonate) and compare it with that of As(V) (arsenate). In particular, I sought to find the relevant concentration range in which to work for the plant uptake experiments. A rapid Petri dish experiment eliminates the need to prepare many growth boxes where the concentration of Sb either kills the plant, or does not provoke a plant response.

4.2 Materials and methods

The plants were growing on agar, containing all essential plant nutrients, in Petri dishes; details of the preparations are described in Chapter 3. Eighty ml of agar was added to each dish. The agar was spiked with $KSb(OH)_6$ or Na_2HAsSO_4 to give As(V) and Sb(V) concentrations of: 0, 10, 30, 100 and 300 mg/l. These equate to molar concentrations of 0 µmol/l, 82.1 µmol/l, 246.3 µmol/l, 821 µmol/l, 2463 µmol/l, for Sb and 0 µmol/l, 133.4 µmol/l, 400.4 µmol/l, 1334.7 µmol/l, 4004.2 µmol/l, for As. In this Chapter, I report only the molar concentrations, because they provide a better indication than weight rations on the biological influence of the solute. There were three replicates for each treatment, with 5 germinated seeds each Petri dish. I grew the plant for 14 days in the climate chamber (Chapter 2). The plants were washed thoroughly with distilled water to remove any attached agar. The root lengths of all plants were measured using a ruler. For each Petri dish, I calculated the average root length of all living plants. The RLI of each Petri dish was calculated by dividing the average root length of the seedlings in the treatment with the average root length of the control. For the RLI50 calculations of Sb and As, linear regression was calculated with the program SigmaPlot, and subsequently, RLI50 were obtained throughout the resulting equations: $x=(-m+-q)/-y$; where x= ln [Sb or As] µmol/l and y= RLI.

Significant differences between the treatments were determined using a one-way ANOVA followed by LSD test performed with Microsoft Excel.

4.3 Results and discussion

Table 13 shows that Sb was always less toxic than As in all treated species. This result indicate that the toxic effect of antimonate on root grow is not comparable with that of arsenate. The order of tolerance for Sb is Brassica *juncea* > *Helianthus annuus* > *Trifolium pratense* > *Zea mays* > *Triticum aestivum* and that of As is *Brassica juncea* > *Helianthus annuus* > *Zea mays* > *Triticum aestivum* > *Trifolium pratense*.

Table 13 shows small difference between the As RLI50 of the treated species, but this may have been due to the fact that often the RLI50 at or below the lowest As treatment (133.4 µmol/l), thus disguising interspecific differences.. The great difference between the Sb RLI50, indicate species dependent response of the plants to Sb in the agar solution. Interestingly, the two monocotyledonous species, *Zea mays* and *Triticum aestivum* showed similar root behaviours with increasing Sb concentrations. Both these species bellowing to the *Poaceae (Gramineae)* family. It is possible that similar uptake and detoxification processes are involved in both species.

Plant spices	**Sb**	**As**
Zea mays	829	142
Brassica juncea	>2463	160
Trifolium pratense	1247	<133
Helianthus annuus	2149	146
Triticum aestivum	631	139

Table 16. RLI50 of Sb and As for all species. The data are given in µmol/l of polluting

metal (Sb or As).

Table 16 shows that the dicotyledonous species (*Brassica juncea, Trifolium pratense* and *Helianthus annuus)* were more tolerant to Sb than the monocot species.

Wysocki (2003) showed that phytochelatins (PC) contribute to Sb tolerance in *Triticum aestivum*. Recent reports have suggested the involvement of PC in the tolerance of As in many different plant species (Hartley-Whitaker 2001). None other studies have been carried out for PC Sb tolerance in the others tested plants species

Brassica juncea, *Helianthus annuus* and *Trifolium pratense* are dicotyledonous plants that belong to the *Brassicaceae Asteraceae* and *Fabaceae* families respectively. Table 13 and Fig. 11 shows large differences for RTL50 and RLI between the three species. This may be explained by the genetic differences between the species

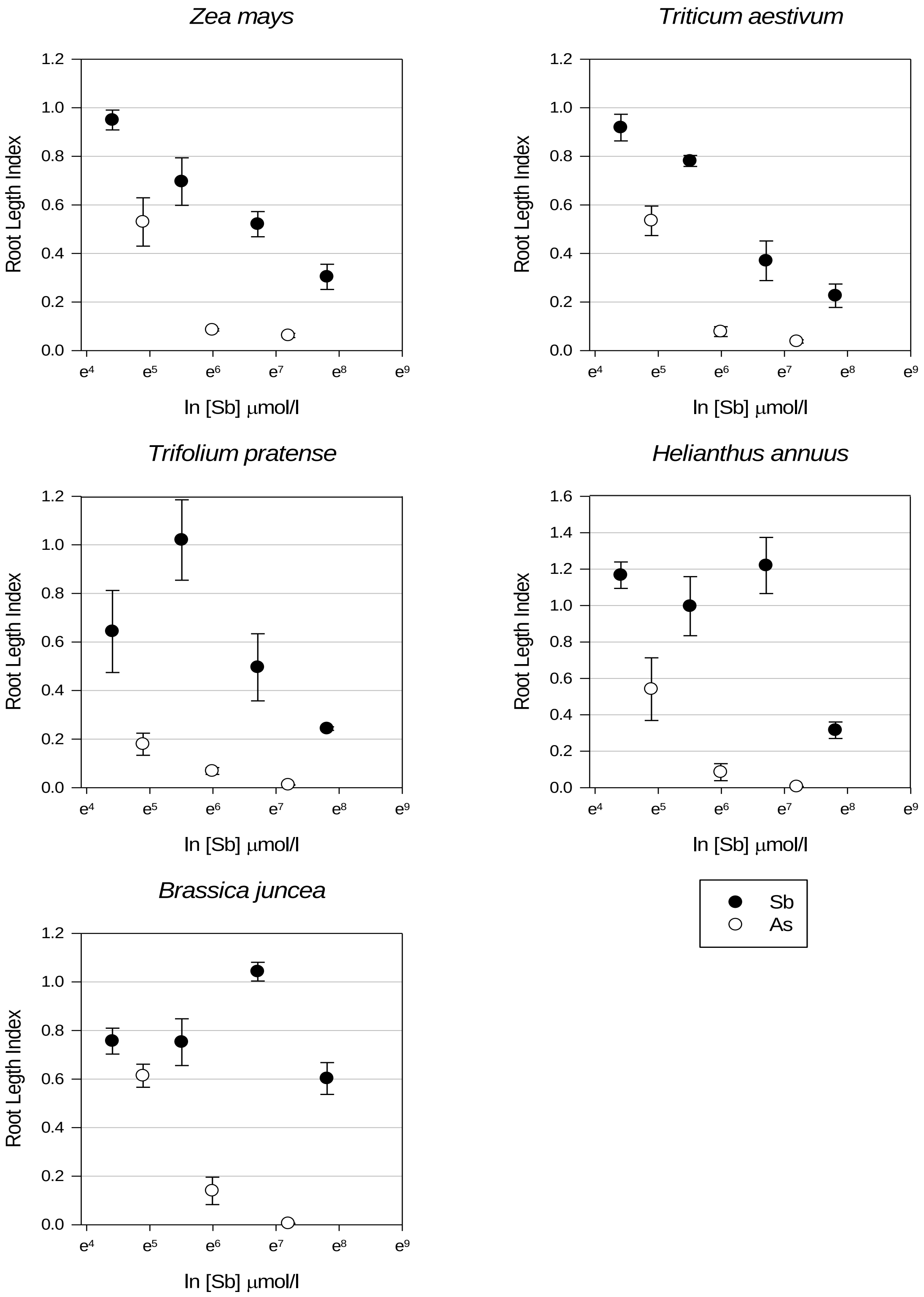

Figure 11*:* effect of Sb and As on the RLI for all tested species grew in agar. Error bars represent the standard error of the mean. The highest concentrations for As are not shown because geminated seeds died immediately

Fig. 11 shows that only he highest Sb concentration (2463 µmol/l) strongly affected root grow. However, the roots of *Brassica juncea* were not even affected by this concentration. Since my experiments were for just two weeks, I do not have enough information to determine the long-term affect of Sb on plant health and reproduction.

4.4 Conclusions

In the absence of sufficient experimental data for Sb, it has been common practice to extend the observed behaviour of As (uptake and tolerance mechanisms) to Sb on the basis of the chemical similarities that exist between the two elements. However, my results bring into question the validity of such parallels. In addiction, antimonate toxicity to roots is strongly species dependent. This study did not consider the influence of Sb on plant biomass, therefore, more investigations are needed on this topic.

Based on the results of this study, I will use an Sb concentration range of 0 – 160 mg/l. I chose to exclude the 300 mg/l because most plants succumbed at this concentration.

5 Plant uptake of Sb from agar and polluted soil

5.1 Introduction

Based on the results of Chapter 4, I determined that Sb is less toxic than As, and that the concentration range of interest in the agar system is 0 – 160 mg/l. The next step in this study was therefore to investigate how Sb affects plant biomass production, and how much Sb is taken up into the roots and the shoots of the plant. I therefore aimed to compare the Sb tolerance and uptake of my five selected species in the aforementioned Sb concentrations, using the agar growth system developed in Chapter 3, and the Sb determination technique developed in Chapter 2.

In a second step, I grew the plants in a polluted soil, obtained from a shooting range near the city Lucern, with the aim of comparing the Sb uptake of plants in agar with those grown in soil.

5.2 Materials and methods

5.2.1 Plant growth in agar boxes

The agar preparation an seed sterilization techniques are described in Chapter 3. For this experiment I used large sterile boxes, which allowed me to grow the plants for four weeks

Eighteen plant boxes were filled with 300 ml of plant-growth agar, containing Sb (as KH_2SbO_4) at concentrations of 0, 10, 20, 40, 80, and 160 mg/l. Three replicates were prepared for each treatment. In each box, I placed three seeds for *Zea mays*, *Helianthus annuus*, and *Triticum aestivum*, while I placed five seeds for *Brassica juncea* and *Trifolium pratense*. Sufficient seeds were required to produce enough biomass for Sb analysis (Chapter 2). Plants were grown in the growth chamber described in Chapter 3, for four weeks.

Plants shoots were excised. The roots were removed from the agar and washed thoroughly in distilled water to remove any attached agar. Plant material was placed in aluminium containers and dried at 80°C until a constant weight was obtained. All samples were weighed, and analysed using the methods described in Chapter 2.

Significant differences were determined using one-way ANOVA followed by *LDS* test performed with Microsoft Excel.

5.2.2 Plant grow in a polluted soil

Sampling of contained soil occurred on the stop butt (15 m x 100 m) of a 300 m military shooting range (MSR) at the Allmend (47° 01'42.85" N, 8° 18' 36.8" E), near the city of Lucerne, Switzerland.. The average annual rainfall is 1171 mm, with an average annual temperature of 8.8°C, as measured at a nearby meteorological station in Luzern. The soil pH was 6.9. The median sand, silt, and clay fractions were 59%, 26% and 15%, and the median organic carbon content was 7.3%. Cylinder pots were 100 mm height and 500 mm large. The containers were completely full with well-homogenised soil. I placed three seeds for *Zea mays*, *Helianthus annuus*, and *Triticum aestivum*, while I placed ten seeds for *Brassica juncea* and *Trifolium pratense*. Sufficient seeds were required to produce enough biomass for Sb analysis (Chapter 2). Plants were grown in the growth chamber described in Chapter 3, for four weeks.

Plants shoots were excised. The roots were removed from the soil and washed thoroughly in distilled water to remove any attached soil. Plant material was placed in aluminium containers and dried at 80°C until a constant weight was obtained. All samples were weighed, and analysed using the methods described in Chapter 2.

5.3 Results and discussion

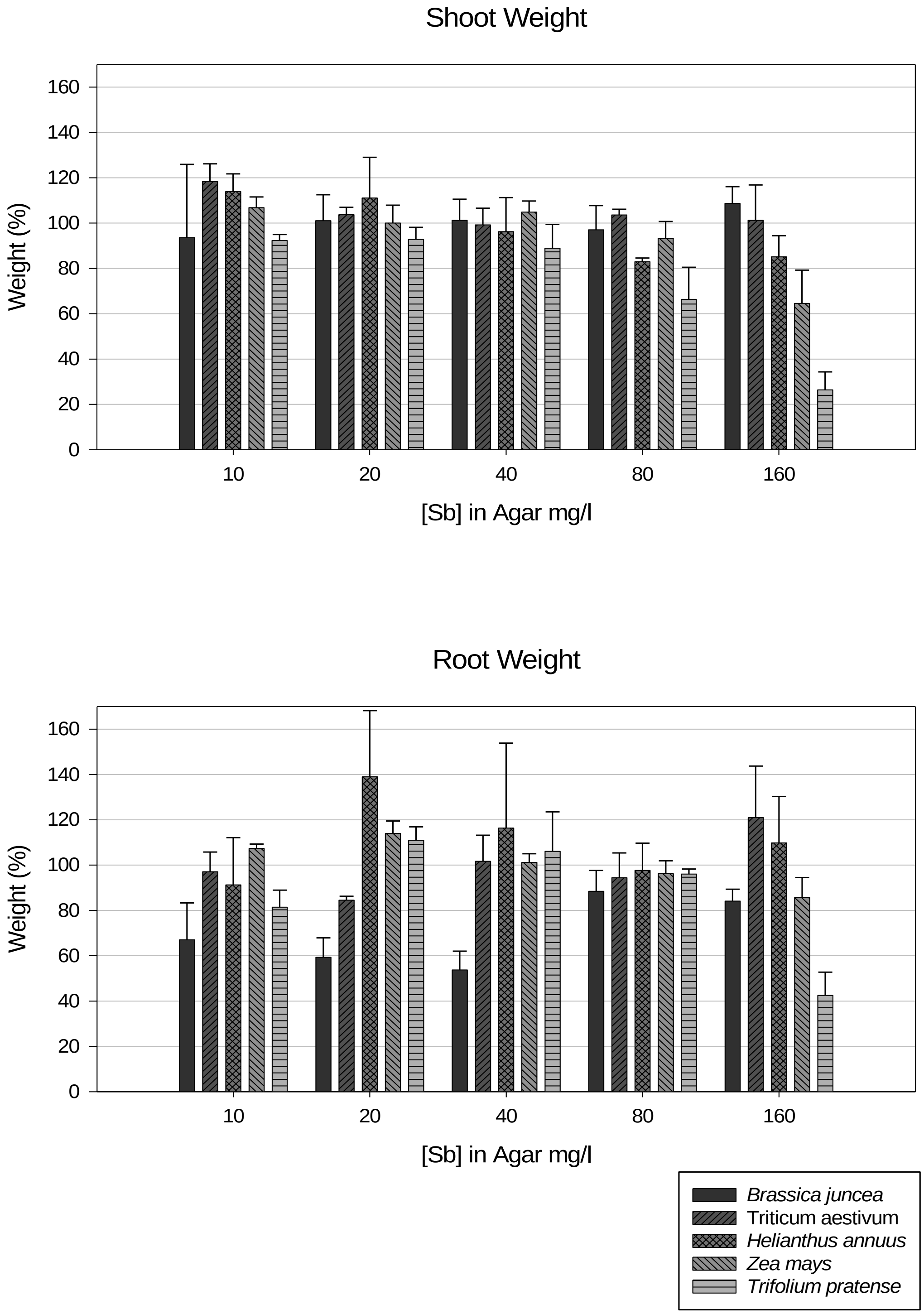

Figure 15. Root and shoot weight in percentage for all the investigate species grew in agar. Error bars represent the standard error of the mean.

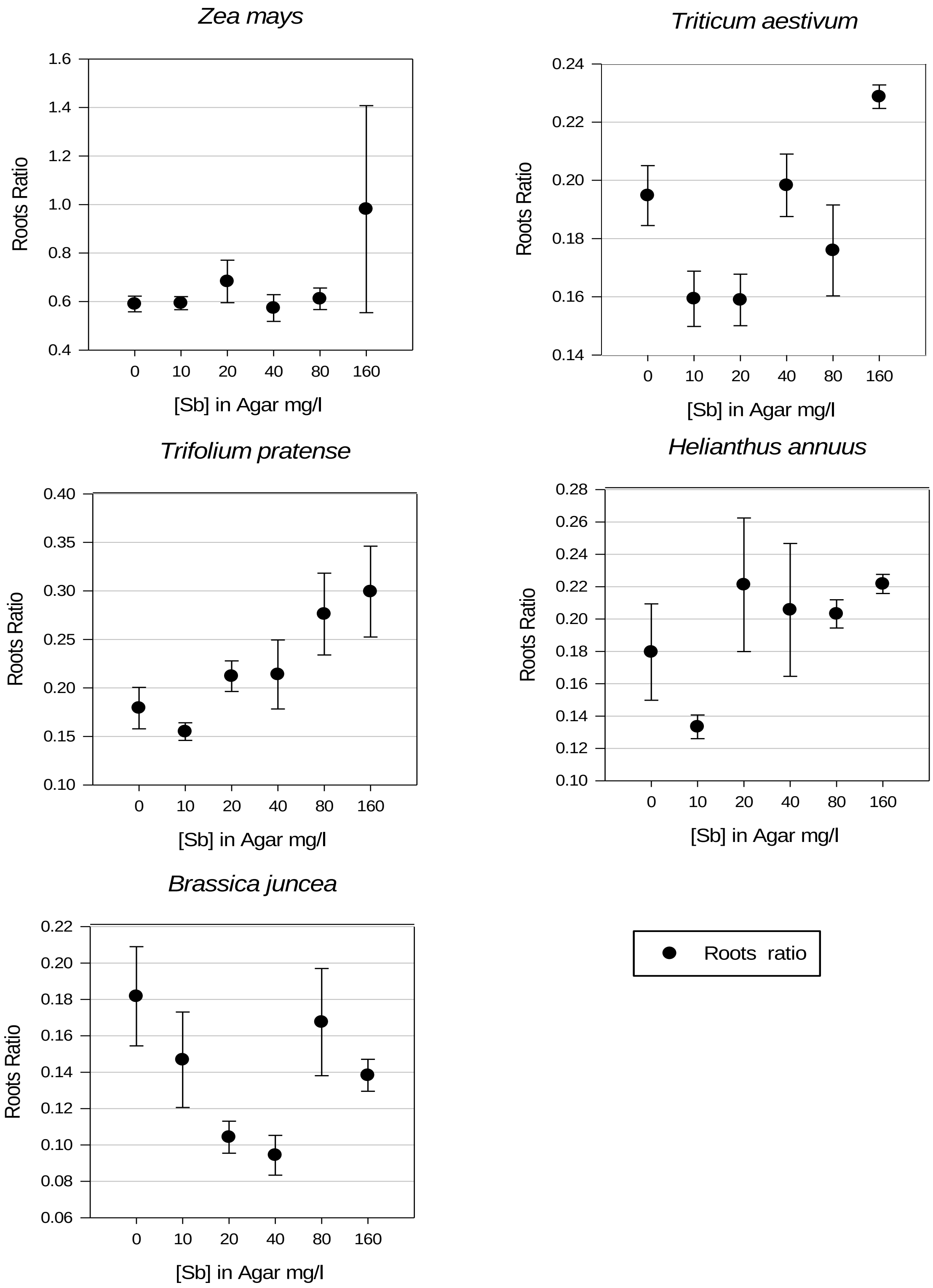

Figure 16. Root: shoot ratios for all species grown in agar. Error bars represent the standard error of the mean.

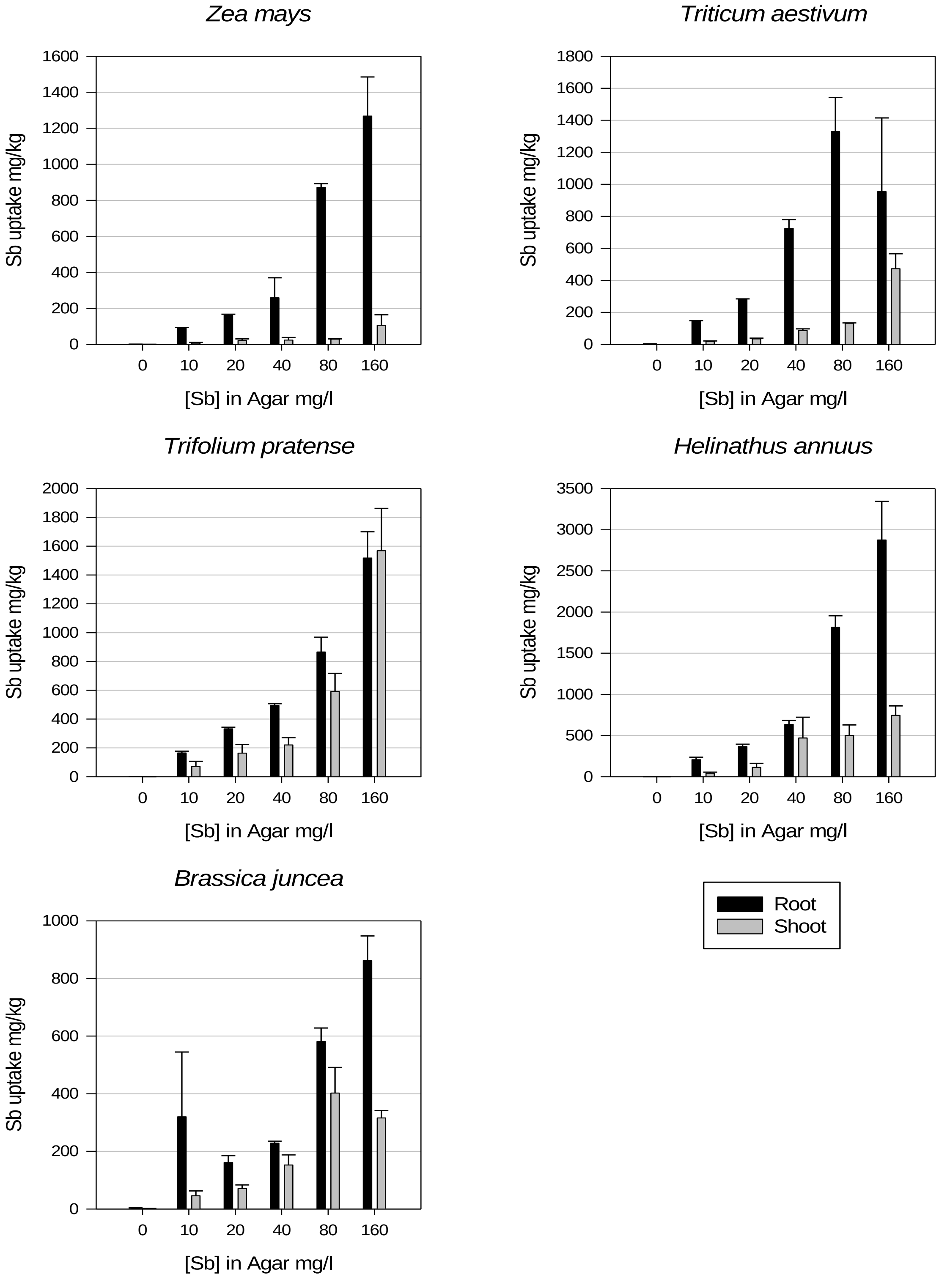

Figure 17. Total Sb concentrations in roots and shoots for all the investigate species grew in agar. Error bars represent the standard error of the mean**.**

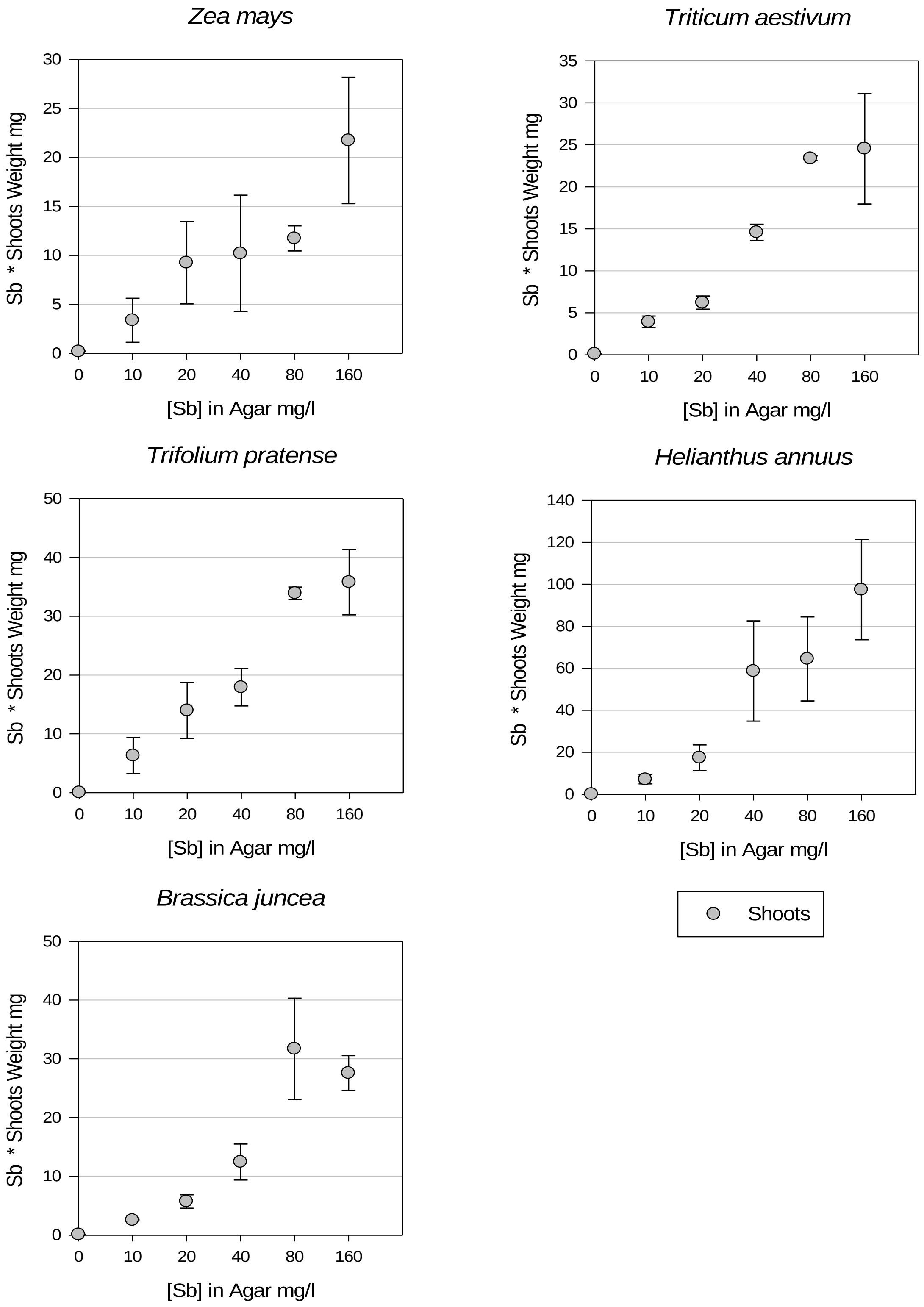

Figure 18. Total Sb content in the plant shoots. Bars represent the standard error of the mean.

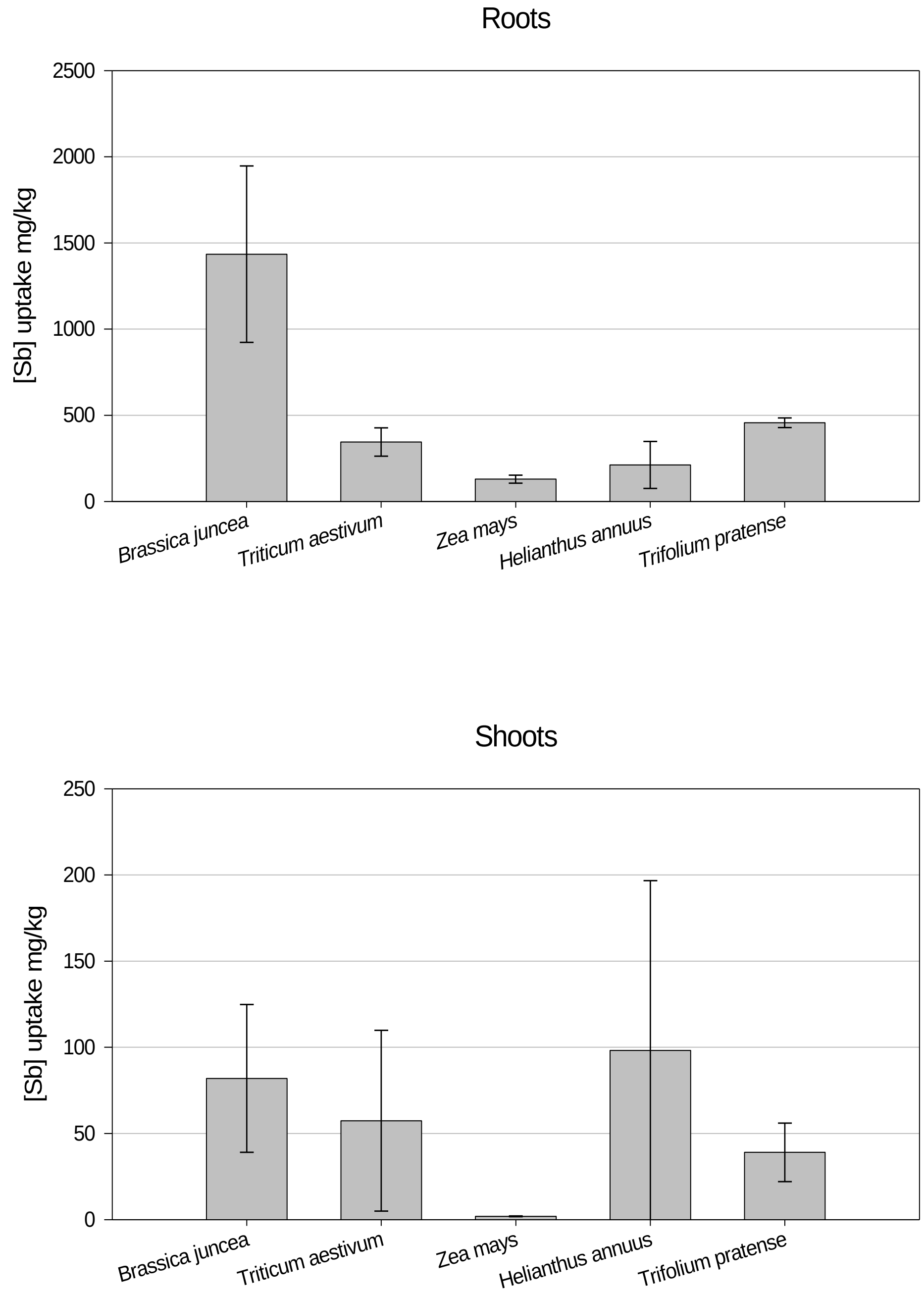

Figure 19. Total Sb concentrations in roots and shoots for all the investigate species grew in Lucerne shooting range soil. Error bars represent the standard error of the mean.

Species	Bioacc. Coeff. Roots	R^2	Bioacc. Coeff. Shoots	R^2
Zea mays	8.4	0.95	0.6	0.93
Triticum aestivum	8.7	0.44	2.6	0.94
Helianthus annuus	18.8	0.98	5.3	0.82
Brassica juncea	5.8	0.82	2.7	0.59
Trifolium pratense	10.0	0.96	9.1	0.97

Table 17. Average bioaccumulation coefficients for each species, along with the R^2 value of the linear regression line.

Species	Best case (g)	Worst case (g)
Zea mays	14055	944
Triticum aestivum	5179	211
Helianthus annuus	2357	134
Brassica juncea	2177	316
Trifolium pratense	1395	63

Table 18. Value of plant biomass (g) that have to be ingested by human to shown chronic poisoning symptom. In the best case the plats are grow on a soil containing 10 mg/l of soluble Sb. In the worst case the plants are grow on a soil containing 160 mg/l of soluble Sb.

Species	Root : shoot ratio agar	Root : shoot ratio from literature	Substrate literature	Literature reference
Zea mays	0.59±0.03	0.22	Soil	(Shaozhong 2002)
Triticum aestivum	0.18±0.01	0.28-0.59	Soil	(Liu 2004)
Helianthus annuus	0.18±0.03	0.8	Soil	(Whitehead 1963)
Brassica juncea	0.17±0.01	Not foud	-	-
Trifolium pratense	0.18±0.03	0.35(a) 0.2(b)	Hydroponic(a) Sand(b)	(Wild 1974)

Table 19. Comparisons of the root : shoot ratio for plants grew in agar (control treatment), with root ratio found in the literature.

5.3.1 Plant tolerance to Sb in agar

All plants survived in all treatments and grew throughout the experiment. The average shoot weight in the control treatments for *Zea mays*, *Triticum aestivum*, *Helianthus annuus*, *Trifolium pratense* and *Brassica juncea* were 0.42, 0,16, 0.15, 0.10, and 0.08 g on a dry matter basis. The average root weights for these species were 0.25, 0.03, 0.03, 0.02, 0.02.

Fig. 19 shows the biomass of the shoots and the roots in the treatments as a percentage of the control. With the exception of *Trifolium pratense,* which had a significantly lower root biomass in the 160 mg/l treatment, the root biomasses of all species in all treatments were not significantly different from the control. This is in contrast with the result of Chapter 4, where significant reduction of root RLI was detected for *Zea mays*, *Triticum aestivum* and *Trifolium pratense.* However, the RLI only measures the root length, anf not the root biomass. Therefore, it may be possible that Sb toxicity in the roots caused a change in root morphology, ie shorter thicker roots, without affecting root biomass.

The shoot weights of *Zea mays* and *Trifolium pratense* were significantly lower in the 80 and 160 mg/kg Sb treatment. This is consistent with the findings in Chapter 4, that these were the most sensitive species.

There was a significant and positive correlation between Sb concentration in agar and the root : shoot biomass ratios for *Trifolium pratense* and *Triticum aestivum* (Fig. 15). The root : shoot ratios of most plant species decrease when high concentrations of toxic agents are present in the substrate (Ross 1994). Here, the lack of root toxicity, and the increase in the root: shoot biomass ratio in *Trifolium pratense* and *Triticum aestivum* implies either toxicity occurs mostly in the aerial portions, or that the plant preferentially allocated assimilate into the roots, upon exposure to Sb. This latter response may occur because Sb reduces the roots' ability to accumulate essential nutrients or water. This hypothesis could be tested by measuring the nutrient status of the plant tissues in the Sb treatments. In addition, previous studies have shown that root: shoot ratios decrease when plants are grown in waterlogged soils or hypoxic nutrient solution (Limpinuntana 1979; Kriedermann 1984). Here, this is not the case. In most cases, the root : shoot ratio in the control is already low compared to plants literature values (Table 19), and this may indicate a weakness of the agar technique for such studies.

Agar media for growing plants under sterile conditions is non-porous, consequently gases diffuse only slowly though the medium and roots are exposed to similar stresses as encountered in waterlogged soils (Drew 1983 ; Jackson 1984). Barrett-Lennard (1988) found a strong reduction in the root : shoot ratio when *Trifolium subterraneum* was grown in agar.

Compared to other common soil contaminants, namely As, Ni, Cu, Zn, Cd and Pb, Sb is less toxic to plants (Kabata-Pendias 2000). At 40 mg/L (330 µmol) in agar, there were no toxic effects on any of the plants tested. Result obtained on *Helianthus annuus* from Tandy (2006) show that Cu and Pb reduce shoot and root biomass, were as Zn had a significant adverse only on root grow; the metals concentration was of 1000 mmol/l in hydroponic conditions.

If toxicity occurred in the plant shoots, then Sb must be taken up into the aerial portions of the plant. Fig. 17 shows the Sb concentrations in the roots and shoots of the plants. For all species there were significantly higher Sb concentrations in the roots than in the shoots. Root and shoot Sb concentrations were significantly and positively correlated with the agar Sb concentration. The relationship between the Sb concentration in agar and that in plant tissue concentration was linear for all species. Thus, these plants behave as "indicator" species, according the scheme proposed by Baker (1981). The linear response of these plants to Sb implies that there are no biological mechanisms controlling the entry of Sb into the root and translocation into the stem. This is perhaps unsurprising, as Sb is a non-essential element that is not widespread in the environment. Therefore, it is unlikely that plants would have evolved specific mechanisms to deal with elevated Sb concentrations in soils. The linear response also indicates that it is that Sb, unlike As, does not enters the plant via the pathway of an essential nutrient such as phosphate. Tschan (2007, personal communication) showed that phosphate did not affect Sb uptake by *Helianthus annuus* and *Triticum aestivum* from nutrient solutions.

The concentration of Sb accumulated in the roots and shoots was species dependent. Table 17 shows the average bioaccumulation coefficients for each species, along with the R^2 value of the linear regression line. The bioaccumulation coefficient is defined here as the plant tissue / agar concentration quotient. The shoot Sb concentrations followed the pattern *Trifolium pratense* > *Helianthus annuus* > *Brassica juncea* ≈ *Triticum aestivum* >> *Zea mays.* The highest shoot concentrations occurred in Trifolium (>1500 mg/kg), which was also the plant with the highest reduction in shoot biomass (Fig. 17).

With exception of *Zea mays,* in all cases, the bioaccumulation coefficient was >1, indicating that there was a higher Sb concentration in the plant tissues than in the agar. When relating this result to soil, it must be remembered that only some fraction of the total Sb in the soil is soluble, so the amount accumulated in the shoots of a plant growing in a real soil is likely to be 2.5 – 9 times the Sb concentration in soil solution.

Fig. 19 shows the root and shoot concentrations of the species grown in soil from the stop-butt of a shooting range, which contained 4760 mg/kg Sb. The shoot concentrations of *Brassica juncea*, *Triticum aestivum*, *Helianthus annuus* and *Trifolium pratense* were in the range 50 – 100 mg/kg, equivalent to my 50 mg/l Sb agar treatment. Similar to the results in the agar experiment, the Sb concentration in the shoots if *Zea mays* were low, < 5 mg/kg. Unfortunately, I do not have the concentration of soluble Sb in this stop butt soil, so I cannot directly compare my agar results with those from the shooting range soil. The neutral pH of this soil would tend to favour the solubility of the negatively-charged Sb oxyanion (Chapter 1). It is unlikely that there will be contaminated soils with a higher Sb concentration than the soil from the stop butt. Therefore, the plant Sb concentrations found here are likely to represent a maximum.

5.3.2 Implications of Sb uptake by plants

The high tolerance of Sb by plants means that these plants may take up high concentrations of Sb without showing any negative growth effects, and thus facilitate the entry of this toxic element into the food chain. Table 18 shows a "worst case" scenario, where I have calculated how much plant material needs to be consumed daily for the ingestion of 100 mg Sb / day, a value shown to be toxic to humans (Barren 1979). Clearly, *Trifolium pratense* presents the greatest risk, where just 60 g of plant material are required to induce toxicity symptoms. If the contaminated soil is under pasture, then it is conceivable that stock would consume more than this amount of clover. However, these same calculations, using the plant values from the stop-butt soil, show that a much larger mass of plant material needs to be consumed to result in toxicity to animals.

If the animal consumes sub-toxic quantities of Sb, then Sb may accumulate in the flesh or internal organs of the animal, and present a human health risk when the animal is eaten. There is no information on the accumulation of Sb in animals.

The high bioaccumulation coefficient of these plants may make them suitable for the phytoextraction of Sb from contaminated soils (Salt et al., 1995), provided the Sb could be brought into soil solution. However, there are many problems surrounding this technology (Nowack et al., 2006). In any case, a simple calculation reveals that millennia would be required to reduce the Sb concentration in a shooting range soil to acceptable levels.

Plants grown on Sb-contaminated sites such as shooting ranges should, ideally, take up minimal amounts of Sb into the shoots. My study shows that the current practice of keeping disused shooting ranges under mowed pasture may result in high Sb concentrations in the pasture, if clover is present. This could present an animal or human health risk, if the mowed pasture is used as stock fodder.

An alternative land use may be maize production, either for stock fodder or for bio energy. This would have several advantages over mowed pasture. Maize has a relatively low Sb bioaccumulation coefficient. Since it is relatively sensitive to high Sb concentrations in the substrate, it is unlikely to grow on the most contaminated areas and therefore even less likely to accumulate high concentrations of Sb in the shoots. Unlike pasture, maize is large plant (see Appendix) and the edible parts are encased in a sheath. Therefore, unlike pasture, the likelihood of Sb entering the cobs via dust deposition is low.

Maize is also ideally suited for bioenergy production, because of its high biomass, which can be converted to ethanol via fermentation. Since a bioenergy crop would not be consumed, any Sb or other heavy metals that enter the shoots via plant uptake or dust deposition are relatively unimportant. An additional benefit of bioenergy production on contaminated land is that it does not take fertile agricultural land out of production, a phenomenon that has lead to a sharp increase in food process (Economist 7th December, 2007). Bioenergy production could provide an economic return, via ethanol production, Kyoto carbon credits, and subsidies, from the some 4000 ha (Salzmann 2007) of disused shooting ranges in Switzerland.

5.4 General conclusion

Antimony is less toxic and less accumulated by plants compared to other common soil contaminants, namely As, Ni, Cu, Zn, Cd and Pb. The growth of *Brassica juncea*, *Triticum aestivum*, *Zea mays*, and *Helianthus annuus* was unaffected by soluble Sb concentrations of 160 mg/l. While *Trifolium pratense* could tolerate up to 40 mg/l Sb. At these higher concentrations, all species except *Zea mays* took up significant amounts of Sb into the shoots, representing a potential exposure pathway for this toxic element to enter the food chain.

There is no possibility to use any of the plant species tested to extract Sb from contaminated soils, a process known as phytoextraction (Salt et al., 1995), because the time required to reduce the Sb levels in soil to acceptable levels is in the order of a millennia.

A good option for the management of Sb contaminated shooting range soils in Switzerland may be growing crops of maize to produce bioenergy.

5.5 Future work

Determination of soluble Sb fraction of contaminate soil analyzed in section 5.2.2 is needed to compare directly uptake of Sb in agar and in real pulled soil. In addiction, is necessary to measure the total concentration of nutrient element in shoot and root, to point out eventually interaction like antagonism or synergism between them, since both antagonistic and synergistic can develop imbalanced reactions, that may cause a real chemical stress in plants. Antagonism occurs when the combined physiological effect of two or more elements is less than the sum of their independent effects, and synergism occurs when the combined effects of these element is greater. These interactions may also refer to the ability for one element to inhibitor simulates the absorption of other element in plants.

6 Appendix

6.1 Brassica juncea (L.) Czern.

Dicotyledonous pant that belong to the Brassicaceae (Cruciferae) family.
Chromosome number: 2n = 36

6.1.1 Description

Erect, annual to biennial herb up to 160 cm tall, often unbranched, sometimes with long ascending branches in upper part, almost glabrous to scattered hairy, slightly glaucous; taproot sometimes enlarged (root mustard). Leaves alternate, pinnately lobed but upper ones often simple; stipules absent; all leaves with short petiole; blade ovate to lanceolate or with up to 2 side lobes on each side and a large end lobe, up to 30 cm × 10 cm, margin irregularly toothed. Inflorescence initially an umbel-like raceme but soon strongly elongating, up to 60 cm long. Flowers bisexual, regular, 4-merous; pedicel ascending, 5–12 mm long; sepals oblong, 4–6 mm long, green; petals obovate, 6–10 mm long, clawed, bright yellow; stamens 6; ovary superior, cylindrical, 2-celled, stigma globose. Fruit a linear silique 2.5–7.5 cm × 2–3.5 mm, often constricted between the seeds, with a conical beak usually longer than 6 mm, dehiscent, up to 20-seeded. Seeds globose, 1–1.5 mm in diameter, finely reticulate, pale to dark brown.

6.1.2 Ecology

Brown mustard is reported to tolerate annual precipitation of 500–4000 mm and temperatures of 6–37°C and is therefore suited to the tropical lowlands as well as relatively cool conditions. It grows best on fertile, well-drained loamy soils with a pH of 5.5–6.8, rich in organic matter. At high temperatures it will quickly flower and yields are lower, but production is still possible. For seed production, brown mustard is tolerant of adverse conditions including moisture stress, high or low pH, salt and insect damage.

6.1.3 Distribution

Brown mustard has been cultivated in Asia and Europe for thousands of years for its leaves and seeds. Presently, vegetable types of *Brassica juncea* are cultivated throughout southern and eastern Asia. Variation is greatest in China. Brown mustard is grown as a leafy vegetable in West and southern Africa, known as ‘laulau’ in Nigeria, ‘mpiru’ in Malawi and ‘tsunga’ in Zimbabwe. In many African countries it has been introduced and became naturalized. However, its exact distribution in Africa is difficult to indicate because of confusion with other *Brassica* species, especially *Brassica carinata* A.Braun. Oilseed types are particularly important in southern Asia, China, North America and Europe, but are not or only rarely found in Africa. *Brassica juncea* is important as a source of mustard in

Europe and North America, and it is occasionally planted for this purpose in Africa, e.g. in Réunion and Mauritius.

6.1.4 Cultivation

Statistics on the production and trade of seed oil and mustard of *Brassica juncea* are difficult to find as they are often combined or confused with those of rape seed (*Brassica napus* L. or *Brassica rapa* L.). Brassica oil is the third-most important vegetable oil after only soya bean and oil palm. Brown mustard as a vegetable is only marketed locally even in those parts of Asia where it is an important vegetable. In Africa it is mainly encountered in southern Africa and is quite rare elsewhere. In Zambia and Zimbabwe, where it is referred to as 'rape', it is popular, but no reliable statistics are available on the area under cultivation, production or produce traded.

6.1.5 Uses

Brassica juncea has many uses: it yields a seed oil, crushed seed is used in the production of mustard and it has a variety of vegetable uses. It is used as forage and medicinally. In Africa and many parts of Asia the leaves are eaten as a vegetable; they are often shredded, cooked and served as a side dish with the staple food. Older leaves and leaves affected by drought are bitter. When they have to be used, consumers renew the cooking water once. Young tender leaves, called 'mustard greens' are used in salads, mixed with other salad greens. In Asia brown mustard leaves are used in pickles or offered as frozen or canned vegetables. Sprouted seeds are used as a garnish or to add a spicy note to salads. In East Asia a variety of vegetable types have been developed that are comparable to that of *Brassica oleracea* L. 'Tai Tau Choi' has an enlarged root and is prepared and eaten like turnips, while 'Cha Tsoi' has peculiar swollen stems with knobby bulges that are preserved in brine and pressed flat until most of the sap is removed. In Asia, Europe and America, *Brassica juncea* is grown mainly for its seed used in the fabrication of brown mustard or for the extraction of vegetable oil. It has been introduced for this purpose locally in Africa, e.g. in the Mascarene Islands. In much of Europe *Brassica juncea* has replaced *Brassica nigra* as the main source of commercial mustard seed. Its mustard is spicier than the yellow type made from *Brassica nigra*. Mustard oil is one of the major edible oils in Bangladesh, India and Pakistan, appreciated for its special taste and pungency. In adjacent parts of the former Soviet Union it is used as a substitute for olive oil. In Western countries its use as edible oil is restricted because of the high erucic acid content. The oil is also used as hair oil and as lubricant. The oil of cultivars bred for extra high erucic acid content is used for industrial purposes. A peculiar use of mustard oil is to retard the fermentation process when making cider from apples. The seeds are also used in birdseed mixtures. The remaining seed meal is high in protein, but the high glucosinolate content makes it unacceptable for human or for monogastric animal consumption.Brown mustard is reported to have anodyne, aperient, diuretic, emetic and rubefacient properties. It is a folk remedy for arthritis, foot ache, lumbago and rheumatism. In China the seed is used as medicine against tumours. Ingestion may impart a body odour repellent to mosquitoes. Leaves applied to the forehead are said to relieve headache. The leaves are eaten in soups to treat bladder inflammation or haemorrhage. In Korea the seeds are used to treat abscesses, colds, lumbago, rheumatism and stomach disorders. Brown mustard oil is used against skin eruptions and ulcers. In Tanzania the roots have been given to cows to promote milk production.

Information source: Plant Resource of Tropical Africa; www.prota.org

6.2 Trifolium pratense (L.)

Dicotyledonous pant that belong to the *Fabaceae* family.
Chromosome number: 2*n* = 14, 28.

6.2.1 Description

Perennial, sometimes biennial, legume, sparingly pilose to glabrous, sometimes densely pilose [forma *pilosum* (Griseb.) Hayek]; stems erect or ascending, 1–5 cm long; leaves of basal rosette all long-petioled, those of stem moderately long-petioled to nearly sessile; leaflets oval or elliptic to cuneate-obovate, 1–3 cm long, 0.5–1.5 cm broad, subentire; stipules oblong-oval to oval-triangular, the free part broadly triangular, abruptly tapering to an erect setaceous tip; peduncles short or absent; heads mostly terminal, sessile, short-peduncled, usually closely subtended by the stipules of the upper pair of leaves, dense, subglobose to ovoid, 1.2–3 cm long; flowers sessile, 10–15 mm long, rosy purple to creamy-white (forma *leucochraceum* Aschers. & Prantl), erect; calyx-tube campanulate, narrower at base, 10-nerved, pubescent including the inner margin of the throat,.The teeth filiform from a triangular base, sparsely hirsute, porrect, the upper about equaling the tube, the lower almost twice as long; corolla about twice the length of the calyx; pods oblong-ovoid, circumscissile; seeds ovoid, asymmetrical, yellowish to purplish.

6.2.2 Ecology

Native on wet to dry meadows, open forests, forest margins, field borders, and paths. Grows best on well-drained loam soil, but also adapted to wetter soils. Most soils that produce good crops of corn, tobacco or small grains will also produce a good crop of red clover. Loams, silt loams, and even fairly heavy soils are better than light sandy or gravelly soils. Some of these soils may need lime or fertilizer, or both. Red clover is most productive on soil that is within a pH range of 6.6 to 7.6. It also needs P and K to produce good yields; amounts needed can be determined by soil tests. Ranging from Boreal Moist to Wet through Subtropical Moist Forest Life Zones, red clover is reported to tolerate annual precipitation of 3.1 to 19.2 dm (mean of 91 cases = 8.6 dm), annual mean temperature of 4.9 to 20.3°C (mean of 91 cases = 10.6°C), and pH of 4.5 to 8.2 (mean of 84 cases = 6.3). Maximum yields obtained at pH >6 with adequate calcium. A photoperiod of at least 14 hours seems necessary for the double-cut type to flower, 16–18 hours for 'Mammoth' .

6.2.3 Distribution

Native to north Atlantic and central Europe, the Mediterranean region, Balkans, Asia Minor, Iran, India, Himalayas, Russia from Arctic south to east Siberia, Caucasus, and the

Far East. It spread to England ca 1650 and was carried to America by British colonists. Widely introduced and cultivated.

6.2.4 Cultivation

In northeastern United States and Canada, and at higher elevation in southeastern and western United States, red clover grows as a biennial or short-lived perennial; at lower elevations in southeastern United States, it grows as a winter annual, and at lower elevation in western United States and Canada, it grows under irrigation as a biennial. Most red clover is spring seeded in a crop of fall- or spring-sown small grain. In the early spring the soil alternately freezes and thaws, thus covering the seed with soil. The small grain holds weeds in check while the clover is getting started. At lower elevations in southeastern and western United States, red clover is sown ca Oct. 15, no later than Dec. 15. In these areas it is most frequently sown without a companion crop. In south-eastern United States, late-summer seedlings can be successful on a seedbed, fallowed to prevent weed growth. Grass is extensively seeded with red clover. Clover-grass mixtures are usually superior to clover. In vitro and vivo experiments show that some lines of red clover perform better with ryegrass (*Lolium multiflorum*). Clover-grass yields better hay that cures more rapidly than pure clover hay. Animals are more likely to bloat on pure clover than on clover-grass pasture. Timothy has a high yield, and is ready to cut for hay with the red clover. Sow the grass in the early fall in the small-grain crop; sow the red clover in the small grain-grass in the spring. When the grain is harvested, remove the straw and stubble, as they tend to smother the clover and favor disease. Clover-hay yields from fields where the straw and stubble have been left are only about one-half as large as the yields from fields where they have been removed immediately after combining. Small-grain companion crops compete with red clover for mineral nutrients, moisture, and light. This competition can be reduced by grazing or clipping the small grain in late winter or early spring, just before stems begin growth., Grazing or clipping after clover stems have begun to branch will reduce small-grain yield.

6.2.5 Uses

Extensively grown for pasturage, hay and green manure, considered excellent forage for livestock and poultry. Compared with alfalfa, red clover has about two-thirds as much digestible protein, slightly more total digestible nutrients, and slightly higher net energy value. The best approximation to vegetable boullion I ever made consisted of red clover and chicory flowers, boiled vigorously with wild onion and chives. Red-clover flowers are reported to possess antispasmodic, estrogenic, and expectorant properties. The solid extract is used in many food products, usually at <20 mg/kg, but in jams and jellies, it may be 525 mg/kg.

Information source: James A. Duke, 1983, Handbook of Energy Crops, unpublished, (www.hort.purdue.edu)

6.3 Helianthus annuus (L.)

Dicotyledonous pant that belong to the *Asteraceae* family.
Chromosome number: 2*n* = 34

6.3.1 Description

Erect annual herb up to 4(–5) m tall, long-hairy; taproot strong, up to 3 m deep with numerous lateral roots 60–150 cm long in the top 40–60 cm of the soil; stem erect, but slightly to sharply curved below the flower head in mature plants, 3–6 cm in diameter, terete but with ridges, branched in many wild types, unbranched in most cultivated types, woody and angular at maturity and often becoming hollow. Leaves opposite in lower part of plant, higher ones arranged spirally, simple; stipules absent; petiole long; blade of lower leaves cordate, of higher ones ovate, 10–30 cm × 5–20 cm, apex acute or acuminate, margin toothed, hairy on both sides with glandular and non-glandular hairs, veins prominent and forming a reticulate pattern. Inflorescence a terminal head 10–50 cm in diameter, sometimes drooping when mature; receptacle flat to concave, 1–4 cm thick; involucral bracts arranged in 3 rows, ovate to ovate-lanceolate, ciliate. Ray florets sterile, showy, deciduous, corolla ligulate, elliptical, c. 6 cm × 2 cm, usually yellow; disk florets bisexual, numerous, arranged in spiral whorls from the centre of the head, c. 2 cm long, subtended by a pointed palea, pappus scales 2, chaff-like, deciduous, corolla tubular, 5-lobed, brown or purplish, stamens 5, filaments flattened, free, anthers long, fused into a tube, ovary inferior, pubescent, style long with nectaries at its base, stigma with 2 curved lobes. Fruit an obovoid achene 7–25 mm × 4–15 mm × 3–8 mm, flattened, slightly 4-angled with rounded base and truncate tip, white, cream, brown, purple, black or white-grey with black stripes. Seed with thin seed coat adnate to the fruit wall. Seedling with epigeal germination; hypocotyl 6–8 cm long, epicotyl c. 0.5 cm long, hairy; cotyledons stalked, leafy, 2.5–3 cm long, glabrous.

6.3.2 Ecology

Sunflower is cultivated mainly between 20–55°N and 20–40°S, in relatively cool temperate to warm subtropical climates. In the tropics it can be grown in the drier regions, up to 1500–2500 m altitude, but sunflower is unsuitable for humid climates. Temperatures for optimum growth are 23–27°C. When grown in hotter climates, oil content is lower and the composition of the oil changes with less linoleic and more oleic acid. Temperatures for germination should not be below 4–6°C and maximum temperatures during growth not above 40°C. Young sunflower plants with 4–6 leaves may withstand short periods of frost down to –5°C. Most sunflower cultivars show day-neutral or quantitative long-day responses to photoperiod. Long photoperiods increase plant height. Water requirement is 300–700 mm during the growing period, depending on cultivar, soil type and climate. More than 1000 mm rain increases the risk of lodging and disease incidence. Sunflower is capable of extracting more soil moisture than most other field crops. Dry weather after seed set is important for adequate ripening of the crop. A wide range of soils from sandy to clayey are suitable for sunflower cultivation, provided they are deep, free draining and not acid; suitable pH ranges from 5.7 to 8.1. The tolerance of sunflower of saline soils is only slightly better than that of soya bean and comparable to that of wheat.

6.3.3 *Distribution*

Wild *Helianthus annuus* spread from its origin in the south-western United States to most other regions of North America in association with human migration in prehistoric times. According to archaeological evidence, modern single-headed sunflowers are derived from types first domesticated in central North America more than 5000 years ago. European explorers of the 16th century found tall and large-headed sunflowers widely used as food and as a source of oil. Sunflower became popular in Europe as a novel ornamental soon after its first arrival from Mexico in the botanic garden of Madrid around 1510. Its potential as an oilseed crop for higher latitudes became apparent in the 18th century in Russia, and by 1880 sunflower was grown on some 150,000 ha mainly in the Ukraine and Caucasus regions for the manufacture of edible vegetable oil. In the Soviet Union of the 1930s more than 3 million ha of sunflower were harvested annually against 0.5 million ha in the remainder of Europe, particularly Hungary and the Balkan Peninsula. Breeding programmes in the Soviet Union developed high-yielding and oil-rich sunflower cultivars, which played a crucial role in the expansion of sunflower production in Europe and elsewhere between 1920 and 1970. Modern sunflower production in North and South America (mainly the United States, Canada and Argentina) developed from sunflower types re-introduced by immigrants from Eastern Europe and Russia at the end of the 19th century and from Russian cultivars brought in after 1960. The application of F_1-hybrid seed technology in combination with dwarf and semi-dwarf plant habits, high oil content of the seed and host resistance to diseases and pests have been major factors leading to the spectacular increase of sunflower production since 1980 in Argentina, India, China, Turkey, the European Union (e.g. France, Spain) and South Africa. Sunflower production in tropical Africa is expanding mainly in the highlands of eastern and southern countries. Occasionally sunflower escapes and becomes naturalized, also in tropical Africa.

6.3.4 *Cultivation*

Average annual world production of sunflower seed over the period 2002–2004 was about 26.2 million t, equivalent to 9.8 million t oil, from 21.4 million ha in 66 countries. The Russian Federation (4.3 million t) is the largest producer, followed by Ukraine (3.7 million t), Argentina (3.6 million t), China (1.9 million t), France (1.5 million t), Romania (1.4 million t), USA (1.2 million t), India (1.1 million t), Hungary (1.0 million t), South Africa (800,000 t), Spain (780,000 t) and Turkey (750,000 t). Countries in tropical Africa with sizable sunflower production are Tanzania (28,000 t), Sudan (18,000 t), Kenya (12,000 t), Angola, Mozambique and Zambia (each about 11,000 t). Most sunflower oil is consumed in the countries of origin and only 30% reaches the international market; the European Union absorbs about two-thirds of it. Important exporting countries are Argentina, the United States and Hungary. The 9–10 million t of sunflower presscake are also of considerable commercial value. The oil represents about 75% and the meal 25% of the total value of sunflower oilseed production. Most of the sunflower meal is traded on domestic markets, except for the 1.0–1.5 million t imported annually into the European Union from Argentina. Non-oilseed production of sunflower represents only 5–10% of the total production.

6.3.5 Uses

Sunflower seed yields an edible oil of excellent quality due to a high proportion of unsaturated fatty acids, near absence of toxic substances, light colour, and good taste and flavour. The oil is used mainly as cooking and salad oil and in the manufacture of margarine, sometimes as a pure sunflower product, but more often in blends with other vegetable oils. Inferior grades of sunflower oil find application as drying oils for paints and varnishes, and in the manufacture of soap. The main by-product of sunflower oil extraction is a protein-rich meal used as livestock feed. For this purpose, the meal is commonly blended with soybean meal. Defatted sunflower meal is also suitable for human consumption and has been used as a partial substitute for wheat flour in baking bread and cakes. When oil is extracted industrially, the stalk and flower head of sunflower are processed into cellulose and fibre mats. The indigenous peoples of North America have had a long tradition of preparing bread-like products from ground sunflower seeds. The seeds (botanically fruits) of non-oil cultivars, which are larger and often black and white striped, are consumed directly. Generally, the largest 25% fraction of the seeds are consumed as salted and roasted snacks, the medium 30–50% fraction as hulled kernels in various confectionery and bakery products, and the smallest seeds are birdseed and pet food. Sunflower is sometimes cultivated as a forage crop. In comparison with maize, it requires a shorter growing season, is more drought tolerant and produces lower yields but a silage of often slightly superior quality. Sunflower is also grown as an ornamental garden and pot plant and is an important bee plant.

Information source: Plant Resource of Tropical Africa; www.prota.org

6.4 Zea mays (L.)

Monocotyledonous pant that belong to the *Poaceae (Gramineae)* family.
Chromosome number: 2*n* = 20

6.4.1 Description

Robust annual grass up to 4(–6) m tall; root system consisting of adventitious roots, developing from the lower nodes of the stem near the soil surface, usually limited to the upper 75 cm of the soil, but single roots sometimes penetrating to a depth of over 2 m; stem (culm) usually single and simple, solid. Leaves alternate, simple; leaf sheaths overlapping, auricled at the top; ligule c. 5 mm long, colourless; blade linear-lanceolate, 30–150 cm × 5–15 cm, acuminate, margins smooth, midrib pronounced. Male and female inflorescences separate on the same plant; male inflorescence (‘tassel’) a terminal panicle up to 40 cm long, lateral branches with paired spikelets 8–13 mm long, one sessile, the other on a short pedicel, each spikelet with 2 glumes and 2 florets, each floret with an ovate lemma, a thin palea, 2 fleshy lodicules and 3 stamens; female inflorescence a modified spike, usually 1–3 per plant in leaf axils about half way up the stem, composed of a thick spongy axis with paired sessile spikelets in 8–20 longitudinal rows and enclosed by 8–13 modified leaves (spathes), spikelet with 2 glumes and 2 florets, lower floret sterile, consisting solely of a short lemma and palea, upper floret with a short, broad lemma and palea, a single superior ovary and a long threadlike style and stigma (‘silk’) up to 45 cm in length and emerging from the top of the inflorescence, receptive throughout most of its length. Fruit a caryopsis (grain), usually obovate and wedge-shaped, variously coloured from white, through yellow, red and purple to almost black, up to 1000 together in an infructescence (‘cob’) enclosed by modified leaves up to 45 cm × 8 cm.

6.4.2 Ecology

Maize is adapted to a wide range of environments, but it is essentially a crop of warm regions where moisture is adequate. The bulk of the crop is grown in tropical and subtropical regions. In West and Central Africa the Guinea savanna zone offers the best ecological conditions for maize. The mid-altitude regions of East and southern Africa are also suitable for maize production. In Ethiopia, for instance, maize is mainly grown at 1000–2400 m altitude. Maize is generally less suited to semi-arid or equatorial climates, although drought-tolerant cultivars adapted to semi-arid conditions are now available. The crop requires an average daily temperature of at least 20°C for adequate growth and development; the optimum temperature for growth and development is 25–30°C; temperatures above 35° reduce yields. Frost is not tolerated. Maize requires abundant sunlight for optimum yields. The time of flowering is influenced by photoperiod and temperature; maize is considered a quantitative short-day plant. Maize is less drought-resistant than sorghum, pearl millet and finger millet. In the tropics it does best with 600–900 mm well-distributed rainfall during the growing season. It is especially sensitive to drought and high temperatures around the time of flowering. Maize can be grown on a wide range of soils, but performs best on well-drained, well-aerated, deep soils containing adequate organic matter and well supplied with nutrients. The high yield of maize is a heavy drain on soil nutrients and maize is therefore often grown as a first crop in the rotation. It can be grown on soils with a pH of 5–8, but 5.5–7 is optimal. It does not tolerate waterlogging and is sensitive to salinity. Since a young crop leaves much of the ground uncovered, soil erosion and water losses can be severe and attention should be paid to adequate soil and water conservation measures.

6.4.3 Distribution

Maize was domesticated in southern Mexico around 4000 BC. Early civilizations of the Americas depended on maize cultivation. When the Europeans arrived in the Americas, maize had already spread from Chile to Canada. Maize was reported for the first time in West Africa in 1498, six years after Columbus discovered the West Indies. The Portuguese brought floury grain types from Central and South America to São Tomé, from where they spread to the West African coast. Portuguese and Arab traders introduced Caribbean flint maize types into East Africa in the mid 1500s, from where they spread to southern Africa. Through the trans-Saharan trade, the Arabs introduced the flinty types that had been brought to northern Africa into sub-Saharan Africa. The flinty types still predominate in northern parts of West Africa while the floury types prevail in the southern parts, with some variation from this pattern. Maize had become a staple food in East and southern Africa by the 1930s. Maize has an extremely wide distribution. It is grown from latitude 58°N in Canada and Russia, throughout the tropics, to latitude 42°S in New Zealand and South America, and in areas below sea level in the Caspian Plain up to areas as high as 3800 m in Bolivia and Peru. It is grown in all countries of Africa, from the coast through savanna regions to the semi-arid regions of West Africa, and from sea-level to the mid- and high-altitudes of East and Central Africa.

6.4.4 Cultivation

According to FAO estimates, the average world production of maize in 1999–2003 amounted to 611 million t/year from 139 million ha. The main producing countries are the United States (243 million t/year in 1999–2003, from 28 million ha), China (117 million t/year from 24 million ha), Brazil (38 million t/year from 12 million ha), Mexico (19 million t/year from 7 million ha), France (15 million t/year from 2 million ha), Argentina (15 million t/year from 3 million ha) and India (12 million t/year from 7 million ha). South Africa produced 9.4 million t/year from 3.6 million ha. Maize production in tropical Africa in 1999–2003 was 26.6 million t/year from 21.2 million ha. The main producing countries in tropical Africa are Nigeria (4.7 million t/year from 4.2 million ha), Ethiopia (2.9 million t/year from 1.6 million ha), Tanzania (2.6 million t/year from 1.6 million ha), Kenya (2.5 million t/year from 1.6 million ha) and Malawi (2.0 million t/year from 1.5 million ha). From 1961–1965 to 1999–2003 the annual maize production in tropical Africa increased from 9.1 to 26.6 million t/year, and the harvested area from 10.2 to 21.2 million ha. Average world export of maize amounted to 80.1 million t/year in 1998–2002, with the United States (47.5 million t/year), Argentina (10.3 million t/year), France (7.9 million t/year) and China (7.4 million t/year) as main exporters. Export of maize from tropical Africa was only 307,000 t/year, with Zimbabwe (143,000 t/year), Tanzania (42,000 t/year) and Uganda (25,000 t/year) as main exporters. The main importers were Japan (16.3 million t/year) and South Korea (8.3 million t/year). Maize imports into tropical Africa were 1.8 million t/year.

6.4.5 Uses

Maize grain is used for three main purposes: as a staple food, as feed for livestock and poultry, and as a raw material for many industrial products. In tropical Africa nearly all maize grain is used for human food, prepared and consumed in many ways. It may be eaten fresh on the cob and simply roasted, but the grain is usually ground and the meal is boiled into porridge or fermented into beer. In tropical Africa maize is mainly consumed as thick porridge (‘ugali’ in East Africa, ‘sadza’ in Zimbabwe). It is commonly eaten with cooked vegetables and, when available, meat. A thin porridge (‘uji’ in East Africa, ‘ogi’ in Nigeria, ‘koko’ in Ghana) is also commonly eaten especially as weaning food. In Ethiopia local beer (‘tella’) and spiritual liquor (‘arakie’) are prepared from maize grain malt. Popcorn is a popular snack. The main industrial products obtained from maize are breakfast products such as cornflakes, starch, sugar and oil. The main product is starch that is used for human consumption or made into syrup, alcohol, but also among others as laundry starch and as a source material for many chemical products. Most industrial products are obtained by the wet-milling process, in which the grain is first steeped in water, after which the germ and bran are separated from the endosperm. The various products are subsequently obtained by physical or chemical processes, and e.g. sugars from maize now account for half of the sugars used in human nutrition. Dry milling produces grits, consisting of coarsely ground endosperm from which most of the bran and the germ have been separated. The germ yields an oil that can be refined for human consumption, widely used as cooking or salad oil and in margarines. It is the second most widely consumed vegetable oil in the United

States and is also made into soap or glycerine. The residues from the production of starch or oil, together with the bran, are used in animal feeds (corn gluten meal and corn gluten feed). Unripe cobs are consumed as vegetable or green maize, boiled or roasted. Young female inflorescences ('baby cobs') are a fancy vegetable in Western countries and in Asia. Mature maize plants are used for animal feed. Silage maize is one of the leading crops in industrialized Western countries, where special cultivars and production technologies have been developed. The stalks are used for fuel, fodder and thatching and as compost. The fibre in the stems and the inner leaves surrounding the cob are made into paper. These cob leaves are often used to wrap foods, and may also be made into cloth or mats, and be used for mattress filling. Ash of the burnt stem is sometimes a substitute for salt. The cob is made into pipe-bowls. In southern Africa the incinerated cob is included in a snuff. Maize has a range of uses in traditional African medicine. Urino-genital problems are treated with prescriptions based on the whole or parts of the maize plant, especially a decoction of the styles, which is also used to treat jaundice. A leaf maceration is drunk to treat fever. Charcoal made from the culms is included in medicines to treat gonorrhoea; an infusion from the burnt cob is used to wash wounds.

Information source: Plant Resource of Tropical Africa; www.prota.org

6.5 Triticum aestivum (L.)

Monocotyledonous pant that belong to the *Poaceae (Gramineae)* family.
Chromosome number: 2*n* = 42

6.5.1 Description

Annual, tufted grass up to 150 cm tall, with 2–5(–40) tillers; stem (culm) cylindrical, smooth, hollow except at nodes. Leaves distichously alternate, simple and entire; leaf sheath rounded, auricled; ligule membranous; blade linear, 15–40 cm × 1–2 cm, parallel-veined, flat, glabrous or pubescent. Inflorescence a terminal, distichous spike 4–18 cm long, with sessile spikelets borne solitary on zigzag rachis. Spikelet 10–15 mm long, laterally compressed, 3– 9-flowered, with bisexual florets, but 1–2 uppermost ones usually rudimentary, sometimes only 1 of the florets bisexual; glumes almost equal, oblong, shorter than spikelet, thinly leathery, keeled towards the tip, apiculate to awned; lemma rounded on back but keeled towards the tip, leathery, awned or blunt; palea 2-keeled, hairy on the keels; lodicules 2, ciliate; stamens 3; ovary superior, tipped by a small fleshy hairy appendage and with 2 plumose stigmas. Fruit an ellipsoid caryopsis (grain), at one side with a central groove, reddish brown to yellow or white.

6.5.2 Ecology

Bread wheat can be grown from within the Arctic Circle to near the equator, but it is most successful between 30–60°N and 27–40°S. Optimum temperatures for development are 10–24°C, with minima of 3–4°C and maxima of 30–32°C. An average temperature of about 18°C is optimal for yield. Temperatures above 35°C stop photosynthesis and growth, and at 40°C the heat kills the crop. Wheat does not grow well under warm conditions with high relative humidity, and in the tropics it is best grown at higher elevations (1200–3000 m) or in the cooler months of the year. Bread wheat requires at least 250 mm water during the growing season for a good crop; it can be grown in areas that receive 250–750 mm rain annually. The sensitivity to daylength differs among genotypes, but most are quantitative long-day plants; they flower earlier at long daylengths, but they do not require a particular daylength to induce flowering. Soils best suited for bread wheat production are well aerated, well drained, and deep, with 0.5% or more organic matter. Optimum soil pH ranges between 5.5 and 7.5. Wheat is sensitive to soil salinity.

6.5.3 Distribution

Bread wheat arose in the corridor extending from Armenia in Transcaucasia to the south-west coastal areas of the Caspian Sea in Iran. Hybridization of a wild *Aegilops* species (*Aegilops tauschii* Coss., with the D-genome) with emmer, an old type of cultivated wheat belonging to *Triticum turgidum* L., gave rise to the hexaploid wheats, but it is unknown whether bread wheat or spelt wheat (*Triticum spelta* L.) appeared first. The earliest archaeological finds of spelt wheat are from the southern Caspian area and are dated at around 5000 BC. Finds of bread wheat are difficult to distinguish from durum wheat (*Triticum turgidum*), but one thinks that those found in the Caucasus, on the anatolian plateau (Turkey), in Central Europe and in Central Asia from the fifth millennium onwards belong to bread wheat. The D-genome conferred to bread wheat and spelt wheat the adaptation to cold winters and humid summers, allowing them to conquer temperate Eurasia, whereas the Mediterranean remained the area of emmer and durum wheat. By the third millennium BC, bread wheat had reached China. In 1529, the Spanish took it to the New World. Bread wheat was introduced into tropical Africa by Arab traders, missionaries and colonial settlers. It is not known exactly when it reached Ethiopia. It was brought from northern Africa to West Africa, where it was already known around 1000 AD. In the early 20th century it was introduced into Kenya and eastern DR Congo. Bread wheat today is grown in almost all parts of the world. In tropical Africa, it is mainly produced in Nigeria, Sudan, Ethiopia, Kenya, Tanzania, Zambia and Zimbabwe.

6.5.4 Cultivation

According to FAO estimates, the average world production of wheat grain (bread wheat and durum wheat together) in 1999–2003 amounted to 576 million t/year from 209 million ha. Worldwide, bread wheat constitutes more than 90% of the area under the cultivated wheats. The main wheat producing countries are China (96.8 million t/year from 25.2 million ha), India (71.0 million t/year from 26.4 million ha), the United States (56.9 million t/year from 20.6 million ha), the Russian Federation (39.4 million t/year from 21.7 million ha) and France (35.1 million t/year from 5.0 million ha). Wheat production in tropical Africa in 1999–2003 was 2.5 million t/year from 1.6 million ha, the main producing countries being Ethiopia (1.4 million t/year from 1.1 million ha), Kenya (272,000 t/year from 137,000 ha), Sudan (254,000 t/year from 124,000 ha), Zimbabwe (237,000 t/year from 43,000 ha), Zambia (87,000 t/year from 13,000 ha), Tanzania (82,000 t/year from 60,000 ha) and Nigeria (75,000 t/year from 53,000 ha). In Ethiopia close to 50% of the wheat production consists of bread wheat, the other 50% of durum wheat. From 1961–1965 to 1999–2003 the world production of wheat increased from 248 to 576 million t/year, whereas the harvested area remained stable at around 210 million ha. In the same period the wheat production in tropical Africa increased from 960,000 to 2.5 million t/year, and the harvested area from 1.2 to 1.6 million ha.

6.5.5 Uses

Bread wheat flour is made into numerous products including bread (leavened or flat; baked, steamed or deep fried), pastries, crackers, biscuits, pretzels, noodles, farina, breakfast foods, baby foods and food thickeners. It is also used as a brewing ingredient in certain beverages (white beer). Leavened breads are the most popular use of wheat in almost all parts of the world. Increased bread consumption is often linked to increasing urbanization and higher per capita income. Bread wheat utilization has also been adapted to local cuisine. In Ethiopia, for instance, the flour is used to prepare ‘injera’ (pancake-like unleavened bread), porridge and soup. The grain is eaten as a snack and during social gatherings as ‘nifro’ (boiled whole grain often mixed with pulses), ‘kollo’ (roasted grain) and ‘dabo-kollo’ (ground and seasoned dough, shaped and deep fried).

Industrial uses of wheat products centre on the production of glues, alcohol, oil and gluten. By-products of flour milling, particularly the bran, are used almost entirely to feed livestock, poultry or prawns. Wheat germ (from wheat embryos) is sold as a human food supplement. Straw is fed to ruminants or used for bedding material, thatching, wickerwork, newsprint, cardboard, packing material, fuel and as substrate for mushroom production. In many dry parts of the world it is chopped and mixed with clay to produce building material.

Information source: Plant Resource of Tropical Africa; www.prota.org

6.6 Additional result for the secton 3.4.2

“residence time”	**feature**	**Mais**		**Clover**		**Sunflower**		**Indian mustard**		**Wheat**	
		H_2O_2	NaClO	H_2O_2	NaClO	H_2O_2	NaClO	H_2O_2	NaClO	H_2O_2	NaClO
2'	A		4			1				1	
	B	4	3	3	6	2	1	4	1	2	
	C	3	2	2	1	6	5	1	7	7	8
4'	A	2	3			3		5	1	7	
	B	7		8	7	2	3				1
	C					5	7	3	8	3	8
8'	A	2	9	1				2		6	4
	B	4		5	9	3	4	1	1	1	2
	C	1				5	1	6	8	3	4
16'	A	2	3			1	2	1	1	4	5
	B	4		6	10	7	3	1	2	3	3
	C	1				1	2		5	1	2
32'	A	3	8			2		1	1	4	3
	B	5		8	10	4	4	3		3	1
	C			1		3	1	4	4	1	4
control	A	3		1							
	B	4		5						2	
	C			1		8		10		8	

Table 20. germination state of the seeds after 3 days. (Description of the symbols: A = not germinated or at an early stage; B = “white germination”; C = “green germination”)

“residence time”	**feature**	**Mais**		**Clover**		**Sunflower**		**Indian mustard**		**Wheat**	
		H_2O_2	NaClO	H_2O_2	NaClO	H_2O_2	NaClO	H_2O_2	NaClO	H_2O_2	NaClO
2'	A	4		1		1	1	2	1		
	B	4	6	3	3	1	1	1			
	C		3	2	4	7	5	4	8	9	8
4'	A	1	1	1		2		5	1	4	1
	B	5	3	3	5	3	1	1		2	
	C	4		5	3	5	7	3	8	4	8
8'	A	1	4			2	1	1	1	4	2
	B	5	4	1	2		1	1		2	
	C	3		5	7	5	5	6	8	4	8
16'	A	1	4			1	2	1	1	6	2
	B	6		4	6	2	1	2	1		
	C	2		2	3	5	5		8	3	7
32'	A	2	5			2				5	4
	B	6	2	2	8	4	3	2			1
	C	1	1	6	2	3	2	5	7	5	4
control	A	3		1							
	B	4		3		1					
	C			4		7		10		9	

Table 21. Germination state of the seeds after 4 days. (Description of the symbols: A = not germinated or at an early stage; B = “white germination”; C = “green germination”)

“residence time”	**feature**	**Mais**		**Clover**		**Sunflower**		**Indian mustard**		**Wheat**	
		H_2O_2	NaClO	H_2O_2	NaClO	H_2O_2	NaClO	H_2O_2	NaClO	H_2O_2	NaClO
2'	A									1	
	B	1					1	4	1		
	C	8	10	7	8	9	4	5	9	9	9
4'	A			1							
	B		5					1			
	C	10	5	9	8	10	8	9	10	10	8
8'	A									1	
	B	3		1						3	
	C	7	10	6	9	7	7	10	9	6	10
16'	A							1			
	B	3	2					3	1	6	1
	C	7	8	7	10	9	8	1	9	4	9

Table 22. Germination state of the seeds after 8 days. (Description of the symbols: A = not germinated or at an early stage; B = “white germination”; C = “green germination”; the data for the 32' and control seeds are not reported because these seeds were used for the second part of the preliminary experiment)

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