

Faculté des bioingénieurs

The impact of boron excess and vermicompost tea on the arsenic absorption by *Atriplex atacamensis* Phil.

Auteur : Ségolène Marcelis

Promoteur(s) : Prof. Stanley Lutts (UCL / ELIA)

Lecteur(s) : Dr. Sempo Grégory (PURVER®)
Dockx Adrien (UCL / ELIA)

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Table of contents

List of abbreviations	IV
Table of figures	V
Table of tables	VIII
Introduction	1
Chapter 1: state-of-the-art	3
1.1 Heavy metals contamination in the environment	3
1.1.1 GENERALITIES	3
1.1.2 SOURCES OF HEAVY METALS	3
1.1.3 CHEMISTRY AND BIOAVAILABILITY	4
1.1.4 TOXICITY AND GENERAL EFFECTS	4
1.1.5 RISK ASSESSMENT	5
1.2 The arsenic	6
1.2.1 GENERALITIES	6
1.2.2 ARSENIC SPECIATION AND MOBILITY	6
1.2.3 SOURCES OF ARSENIC IN THE ENVIRONMENT	7
1.2.4 ARSENIC TOXICITY	10
1.2.5 RESISTANCE TO ARSENIC TOXICITY	13
1.3 The boron	16
1.3.1 GENERALITIES	16
1.3.2 SOURCES AND FORMS OF BORON IN THE ENVIRONMENT	16
1.3.3 THE ROLE OF BORON IN PLANT METABOLISM	17
1.3.4 BORON UPTAKE AND TRANSLOCATION	18
1.3.5 BORON TOXICITY	19
1.3.6 TOLERANCE TO BORON TOXICITY	20
1.4 The phytoremediation	22
1.4.1 INTRODUCTION AND DEFINITION	22
1.4.2 DIFFERENT FORMS OF PHYTOREMEDIATION	23
1.4.3 FACTORS AFFECTING PHYTOREMEDIATION EFFICIENCY	25
1.4.4 SUITABLE PLANTS: FEW EXAMPLES	26
1.4.5 THE SPECIFIC CASE OF AS- AND B-PHYTOREMEDIATION	27
1.4.6 EFFICIENCY INDICATORS	28
1.4.7 WASTE MANAGEMENT	29
1.4.8 BENEFITS AND LIMITATIONS	29
1.5 Vermicomposting and vermicompost	30
1.5.1 DEFINITION OF VERMICOMPOSTING	30
1.5.2 PRODUCTION OF VERMICOMPOST	31
1.5.3 THE PUR VER® PROCESS	32
1.5.4 THE VERMICOMPOST AND ITS PROPERTIES	33
1.5.5 USES OF VERMICOMPOST	34
1.6 The genus <i>atriplex</i>	38

Table of contents

1.6.1 TAXONOMY, GEOGRAPHICAL DISTRIBUTION AND GENERALITIES.....	38
1.6.2 <i>ATRIPLEX ATACAMENSIS</i> PHIL.	40
1.7 Research objectives	45
Chapter 2: Materials and methods	47
2.1 Vermicompost tea analysis	47
2.1.1 GENERAL PARAMETERS	47
2.1.2 MINERAL ANALYSIS	47
2.1.3 NITROGEN CONTENT : NITRATE, AMMONIUM AND TOTAL NITROGEN	48
2.1.4 PROLINE CONTENT	48
2.1.5 FREE POLYAMINES QUANTIFICATION	49
2.1.6 ANTIOXIDANTS QUANTIFICATION	49
2.1.7 TOTAL PROTEIN QUANTIFICATION	52
2.1.8 SOLUBLE SUGARS QUANTIFICATION	52
2.1.9 PHYTOHORMONES ANALYSIS	52
2.1.10 STATISTICAL TREATMENT OF DATA	53
2.2 Analysis of the impact of boron excess and vermicompost on arsenic absorption by <i>atriplex atacamensis</i>	54
2.2.1 PLANT MATERIAL AND GROWING CONDITIONS.....	54
2.2.2 FRESH AND DRY WEIGHT, WATER CONTENT.....	56
2.2.3 MINERAL ANALYSIS (AS, B, P, S)	57
2.2.4 OSMOPROTECTANTS QUANTIFICATION.....	57
2.2.5 OXIDATIVE STRESS-RELATED PARAMETERS	58
2.2.6 STATISTICAL TREATMENT OF THE DATA.....	60
Chapter 3: Results analysis	61
3.1 Vermicompost tea analysis	61
3.2 The impact of boron and vermicompost tea on the arsenic absorption by <i>atriplex atacamensis</i>	64
3.2.1 MORTALITY PERCENTAGE	64
3.2.2 PLANT GROWTH	65
3.2.3 WATER CONTENT.....	66
3.2.4 IONS CONTENT: AS, B, P, S.....	67
3.2.5 PROLINE	71
3.2.6 GLYCINEBETAINE	72
3.2.7 MALONDIALDEHYDE	73
3.2.8 GLUTATHIONE.....	74
3.2.9 PHYTOCHELATINS	76
3.2.10 TOTAL PHENOLICS AND FLAVONOIDS	77
Chapter 4: Discussion	79
4.1 The impact of boron excess on the arsenic absorption by <i>atriplex atacamensis</i> phil.	80
4.1.1 PLANT WATER STATUS AND PLANT GROWTH	80
4.1.2 MINERAL NUTRITION	81
4.1.3 OXIDATIVE STRESS AND PLANT RESPONSE STRATEGY	84
4.2 The impact of vermicompost tea on the arsenic absorption by <i>atriplex atacamensis</i> phil.	86
4.2.1 PLANT GROWTH AND WATER STATUS.....	86

4.2.2 VERMICOMPOST TEA INTERESTING COMPOUNDS	87
4.2.3 MINERAL NUTRITION	87
4.2.4 EFFECT OF VERMICOMPOST TEA ON OXIDATIVE STRESS MANAGEMENT	88
<u>Conclusion and perspectives</u>	<u>91</u>
<u>References</u>	<u>93</u>
<u>Annex</u>	<u>101</u>
Annex a: s fraction used in gsht	101

List of abbreviations

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abscisic acid	ABA
arsenate	As(V)
arsenate reductase	AR
arsenite	As(III)
bioconcentration factor	BCF
cysteine	Cys
cytokinins	CK
dimethylarsinic acid	DMA
dry weight	DW
fresh weight	FW
glutamate	Glu
glutathione reductase	GR
glutathione transferase	GST
glycine	Gly
glycinebetaine	GB
jasmonic acid	JA
malondialdehyde	MDA
metallothionein	MT
monomethylarsinic acid	MMA
noduline 26-like intrinsic protein	NIP
oxidized glutathione	GSSG
phosphate transporters	PHTs
phytochelatins	PCs
reactive oxygen species	ROS
reduced glutathione	GSH
rhamnogalacturonan II	RGII
salicylic acid	SA
thiol group	-SH
total glutathione	GSht
translocation factor	TF
trimethylarsine oxide	TMAO
vermicompost leachate	VCL
vermicompost tea	VCT
water content	WC
world health organisation	WHO

Table of figures

<i>Figure 1 Chemical structure of some major arsenic compounds (Hasanuzzaman et al., 2015)</i>	6
<i>Figure 2 A simplified outline of major As reactions in the environment (Jones, 2007)</i>	7
<i>Figure 3 Major sources and routes of arsenic in soil and aquatic ecosystems (Hasanuzzaman et al., 2015).</i>	8
<i>Figure 4 Distribution of documented world problems with As in groundwater in major aquifers as well as water and environmental problems related to mining and geothermal sources. Areas in blue are lakes (Hasanuzzaman et al., 2015).</i>	9
<i>Figure 5 A simplified schematic diagram of As transport and metabolism in plants (Zhao et al., 2010). The thickness of arrow lines is indicative of the relative flux. In plant roots, arsenite (AsIII) and arsenate (AsV) are taken up by aquaporins (NIP) and phosphate transporters (PHTs) respectively. AsV is reduced to AsIII by arsenate reductases (AR) using glutathione (GSH) as a reductant. AsIII is complexed with phytochelatins (PCs) before sequestration in the vacuole via ATP-binding cassette (ABC) transporters. The transfer into the xylem is carried out by PHTs transporter and As efflux carrier. The As goes in leave by NIP were the same detoxification pathways as in root occur.</i>	13
<i>Figure 6 ROS detoxification pathway by GSH (Macdonald et al., 2003)</i>	14
<i>Figure 7 Schematic representation of the bis(rhamnogalacturonanII)-borate complex, showing the linkage through apiose groups in the cell wall (Power and Woods, 1997)</i>	18
<i>Figure 8 Structural representation of the bis(sorbitol)-2,3,20 ,30- borate complex, one of several isomers identified in phloem of sorbitol-rich plant species (Power and Woods, 1997)</i>	19
<i>Figure 9 Common remediation techniques for heavy metal-contaminated soils (Liu et al., 2018).</i>	22
<i>Figure 10 Uptake mechanisms in phytoremediation technology (Tangahu et al., 2011).</i>	23
<i>Figure 11 The mechanisms of pollutants uptake by plant through phytoremediation technologies (Tangahu et al., 2011).</i>	25
<i>Figure 12 Vermicompost in production state with high worms density (Black Diamond VermiCompost, n.d.)</i>	30
<i>Figure 13 Production lines of vermicompost of the PUR VER company ("Purver.be," n.d.)</i>	33
<i>Figure 14 Effect of vermicompost PUR VER on the tomatoes growth ("Purver.be," n.d.)</i>	36
<i>Figure 15 Effect of the vermicompost PUR VER on the basilica plants growth ("Purver.be," n.d.)</i>	36
<i>Figure 16 Geographical distribution of 19 Atriplex species in South America (yellow points represent A. atacamensis) (Brignone et al. 2016)</i>	38

Table of figures

<i>Figure 17 Rio Loa river near the Quillagua oasis, Atacama Desert, Northern Chile (2019).</i>	40
<i>Figure 18 Atriplex Atacamensis PHIL. along the Rio Loa river (Quillagua Oasis) in the Atacama desert, Northern Chile (2019).....</i>	41
<i>Figure 19 Atriplex atacamensis leaves</i>	42
<i>Figure 20 Female (1) and male (2) inflorescences of Atriplex atacamensis.....</i>	42
<i>Figure 21 Experimental schedule.</i>	54
<i>Figure 22 Young seedling and hydroponic culture of A. atacamensis</i>	55
<i>Figure 23 Experimental design.....</i>	55
<i>Figure 24 Harvesting plan.....</i>	56
<i>Figure 25 Mass of leaves and roots of Atriplex atacamensis after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively).Each value is the mean of 4 replicates and vertical bars are S.E.. The values with different letters are significantly different (p-value < 0.05).</i>	65
<i>Figure 26 Water content in leaves and roots of Atriplex atacamensis after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively).Each value is the mean of 4 replicates and vertical bars are S.E.. The values with different letters are significantly different (p-value < 0.05).</i>	66
<i>Figure 27 Arsenic content in leaves and roots of Atriplex atacamensis after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively).Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different (p-value < 0.05).</i>	67
<i>Figure 28 Boron content in leaves and roots of Atriplex atacamensis after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively).Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different (p-value < 0.05).</i>	68
<i>Figure 29 Phosphorous content in leaves and roots of Atriplex atacamensis after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively).Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different (p-value < 0.05).</i>	69
<i>Figure 30 Sulphur content in leaves and roots of Atriplex atacamensis after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1}))</i>	

in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different (p-value < 0.05). 70

Figure 31 Proline concentration in leaves and roots of Atriplex atacamensis after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different (p-value < 0.05), ND corresponds to no data. 71

Figure 32 Glycinebetaine concentration in leaves and roots of Atriplex atacamensis after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different (p-value < 0.05), ND corresponds to no data. 72

Figure 33 Malondialdehyde concentration in leaves and roots of Atriplex atacamensis after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different (p-value < 0.05). 73

Figure 34 Total glutathione concentration in leaves and roots of Atriplex atacamensis after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different (p-value < 0.05). 74

Figure 35 GSSG and GSH concentrations ratio in leaves and roots of Atriplex atacamensis after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different (p-value < 0.05). 75

Figure 36 Phytochelatins concentrations in leaves and roots of Atriplex atacamensis after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different (p-value < 0.05), ND corresponds to no data. 76

Figure 37 Total phenolics concentrations in leaves and roots of Atriplex atacamensis after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates

Table of tables

and vertical bars are S.E.. The values with different letters are significantly different (p-value < 0.05), ND corresponds to no data. 77

Figure 38 Flavonoids concentrations in leaves and roots of Atriplex atacamensis after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different (p-value < 0.05), ND corresponds to no data. 78

Figure 39 Fraction of S presents into GSht in leaves and roots of Atriplex atacamensis after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates. No statistics were performed on these data since it is calculated from mean values of different measurements. 101

Table of tables

Table 1 Composition of the Yoshida solution used for the hydroponic culture (Yoshida et al., 1976). 54

Table 2 PUR VER® vermicompost tea composition summary. Each value is the mean of 3 replicates. ND corresponds to not detected. 61

Table 3 PUR VER® vermicompost tea phytohormones content summary. Each value is the mean of 3 replicates. ND corresponds to not detected and SE means standard error. 62

Table 4 Mortality percentage of A. atacamensis after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). 64

Introduction

The Atacama Desert, in Northern Chile, is one of the most arid place in the world. It is characterized by extreme temperatures, water scarcity and salt contamination. Some regions like the Quillagua oasis are also highly contaminated by arsenic and boron. In addition to the geological origin of the soil and the geothermal activity in the Andean Cordillera, human activities are also the cause of the increase of contamination of the unique source of water, the Rio Loa river. Indeed, mining activity is the first economic activity in the Antafagosta Region of Chile. In addition to the high water consumption, mining activities release a lot of arsenic-contaminated waste in the environment. This pollution poses a serious health threat to the local population, the agriculture and the wildlife. Finally, all soils in this area exhibit a rather low fertility and a poor organic matter content.

Atriplex atacamenis is a xero-halophitic plant species endemic of this region. This plant is almost the only species able to survive in these very harsh conditions. Previous research has mentioned its potential for phytoremediation applications of arsenic-contaminated soils. Indeed, the Chilean salt bush resists to high concentrations of arsenic (up to 1,000 μM arsenate) and is able to accumulate it in its roots. Therefore, *A. atacamensis* could be used to stabilize arsenic polluted substrate and reduce its spread in the environment. The plant can also deal with an excess of boron but the behaviour of the plant simultaneously exposed to arsenic toxicity and boron excess has not yet been explored.

On the other hand, vermicompost is subject to growing curiosity in the scientific community. This consists in the digestion product of organic matter by earthworms. Vermicompost tea is produced by infusion of the solid fraction in water under fully controlled conditions. It contains interesting chemical compounds such as phytohormones, humic acids, antioxidants and minerals and could thus act as a biostimulant. Its beneficial effect on plant growth, biotic stress and abiotic stress, such as high temperatures or hydric stress has already been observed. However, the effect of vermicompost on metal stress is poorly documented.

The present study aims to highlight these gaps; its first objective is to provide the global composition of the vermicompost tea used, the second to identify the effect of boron excess and vermicompost tea on arsenic absorption by *A. atacamensis*.

Seeds of *A. atacamensis* collected in the region of the Quillagua oasis were grown in semi-controlled conditions in the greenhouses of UCLouvain. The experiment was carried out in hydroponic culture where heavy metals and the vermicompost were added in specific concentrations. The vermicompost tea used was kindly provided by the company PUR VER®. Then, physiological effects were quantified and analysed with biochemical techniques.

Introduction

The present work is divided into four sections. The first one discusses the state-of-the-art of the different subjects linked to the research: arsenic and boron, vermicompost, phytoremediation and finally *A. atacamensis*. Then the section materials and methods provides information about the technical aspects. The results are presented in the third section and discussed in the last one. This section summarizes the highlights and develops further horizons.

Chapter 1: State-of-the-art

1.1 Heavy metals contamination in the environment

1.1.1 Generalities

Although there is no consensus on the definition of heavy metals, it generally includes metal elements with a density higher than 5 g.cm^{-3} (Appenroth, 2010). The most commonly found heavy metals in terms of abundance are lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu), mercury (Hg), and nickel (Ni) (Wuana and Okieimen, 2011). Other less common metallic contaminants include aluminium (Al), cesium (Cs), cobalt (Co), manganese (Mn), molybdenum (Mo), strontium (Sr), and uranium (U) (Panagos et al., 2013).

Heavy metals occur naturally as “trace element” when concentrations are not higher than $1,000 \text{ mg.kg}^{-1}$; at this range of concentration, they are rarely toxic (Alloway, 2013). Most of these metals however do not undergo microbial or chemical degradation and accumulate in the environment where they persist for a long time and may cause contamination (Jovanović et al., 2015). At high concentration, heavy metals may pose risks and hazards to human by ingestion, contact with contaminated soil, through the food chain (risk of bioaccumulation and biomagnification) and drinking of contaminated ground water. It also may be toxic to plants, animals and ecosystems. Co, Cu, Iron (Fe), Mn, Mo, Ni, and Zn are essential metals element for plant growth and are required at low amounts to ensure optimal metabolism. However, at higher concentrations, they become toxic to plants as the other non-essential metals (Pb, Hg, ...) (Alloway, 2013; Wuana and Okieimen, 2011).

1.1.2 Sources of heavy metals

Heavy metals in soil derive from the geological parent material on which the soil has developed (lithogenic source) and various anthropogenic sources (Jovanović et al., 2015). Natural source is the main factor determining the heavy metals concentration in soils (Alloway, 2013). Contamination of soils becomes a worldwide problem and a serious environmental threat because of increasing human activities such as the use of fertilizers and pesticides, direct application of biosolids, sewage sludge and manure, wastewater irrigation, metal mining, milling process, industrial waste, coal combustion residues and air born sources, etc. (Nordberg et al., 2015; Sigel et al., 2013; Toth et al., 2016).

These human activities disrupt and accelerate the naturally slow geochemical cycle of metals and affect both agricultural and urban soils. Heavy metals from anthropogenic sources tend to be more mobile and bioavailable than lithogenic ones. For several heavy metals the anthropogenic emission into the atmosphere is one-to-three orders of magnitude higher than natural fluxes (Alloway, 2013; Wuana and Okieimen, 2011). Heavy metals can be transported on long distance through atmospheric deposition of aerosol particles, raindrops containing heavy metals, or gaseous forms of the elements. It is also transported by moving water and down-slope movement of metal-rich rock and contaminated mineral material or soil by gravity (Grant et al., 2013; Li et al., 2011). In many countries strict regulations are applied to reduce the contamination. However, in most developing countries where the emission are the highest there is little regulation (Alloway, 2013).

1.1.3 Chemistry and bioavailability

Heavy metals can change in their speciation, bioavailability, mobility, and toxicity. The available fraction involves free ions, soluble complexes and readily desorbable forms. It depends on factors such as pH, redox status, micronutrient levels, organic matter, available water content and temperature. These factors control the reactions of mineral precipitation and dissolution, ion exchange, adsorption, and desorption, aqueous complexation, biological immobilization and mobilization and plant uptake. Plants also influence the availability of heavy metals by the release of exudates from the roots and direct impact on the rhizospheric pH (Alloway, 2013; Lambrechts et al., 2014; Wuana and Okieimen, 2011). Different pollutants vary in their tendencies to form stable complexes with clay particles: while some heavy metals are easily present in the bioavailable form, others, such as Pb, are strongly adsorbed on clay particles and organic matter and may thus be present at high concentrations in the substrate without representing a major risk since they are not available for plant uptake (Mendez and Maier, 2008).

1.1.4 Toxicity and general effects

The toxicity of heavy metals mainly depends on their concentration and availability to living organisms. Furthermore, several elements are essential for the metabolism of cells at low concentration (like Co, Cu, Fe, Mn, Mo, Ni and Zn).

To evaluate toxicity of a given element, the dose-response relationship data is needed. The level of exposure and the duration are the key factors to determine the toxic effect of a substance (Appenroth, 2010). Heavy metals are known as carcinogens. Furthermore, many of

these substances can produce other toxic effects on the central nervous system, liver, kidneys, heart, lungs, skin, reproduction, and so forth (Panagos et al., 2013).

The most obvious plant reaction is the growth inhibition. The root system is the first part of the plant in contact with heavy metals and is therefore the first affected. Leaf chlorosis, disturbed water balance and reduced stomatal opening are characteristic effects of heavy metals contamination (Gallego et al., 2012; Xue et al., 2014). These symptoms are the typical ones of stress response. Heavy metals cause reactive oxygen species (ROS) formation, interact with proteins, other macromolecules and metabolites, disrupt the water balance, decrease the chlorophyll and carotenoid content and finally lead to the plant death (Appenroth, 2010; Kieffer et al., 2009).

1.1.5 Risk assessment

Risk assessment is an effective scientific tool which enables decision makers to manage contaminated sites in a cost-effective way while preserving public and environmental health (Wuana and Okieimen, 2011). Assessment of soil pollution with heavy metals involves sampling of representative soils in a problematic area, analysis of metals and related parameters, and comparison of soil pollution quantification with regulatory standards. The most used chemical indicator is the total/recoverable content in heavy metals. The indicator of (bio)available/extractable amount is closely related to plant uptake capacity (He et al., 2015). Heavy metals have been used by humans for thousands of years. Despite the fact that heavy metals toxicity is known for a long time, exposure continues and even increases in particular in developing countries (Panagos et al., 2013). Environmental contamination is a global challenge that requires joint efforts of governments, scientists, and citizens. Governmental regulations are essential in both source control and pollution remediation. Regulatory standards for heavy metal levels have been established for agricultural soils (He et al., 2015). Available technologies for remediation of heavy metal contaminated soils are mainly immobilization, soil washing and phytoremediation (Wuana and Okieimen, 2011). According to the World Health Organization (WHO), priority should be given to the pollutants on the basis of toxicity, environmental persistence, mobility, and bioaccumulation (Panagos et al., 2013).

1.2 The Arsenic

1.2.1 Generalities

Arsenic (As) is a natural element classified as a metalloid. It has indeed both metallic and non-metallic properties because its electronegativity is not sufficient to give As a full metallic character. It is the third element in group 15 of the periodic table and its atomic mass is 74.91 g.mol^{-1} . Arsenic is ubiquitous in the environment: it is widely distributed and is the 20th most abundant element in the earth's crust. It has an average concentration in soil of $5\text{--}13 \text{ mg.kg}^{-1}$ (Chatterjee et al., 2017a). Arsenic is present in inorganic and organic forms and occurs in many minerals linked to other elements or as a pure elemental crystal. It is commonly bound to carbon, iron, oxygen, and sulphur and is concentrated in magnetic sulphides, iron ores and oxide complexes in soil and rock formations (Chatterjee et al., 2017a; Hasanuzzaman et al., 2015; James et al., 2017; Mitra et al., 2017)

1.2.2 Arsenic speciation and mobility

In nature, As exists as inorganic or organic species (Mitra et al., 2017). Arsenic is found in the oxidation states of +V (arsenate), +III (arsenite), 0 (arsenic), and -III (arsine). Arsenite species (H_3AsO_3 , H_2AsO_3^- , HAsO_3^{2-} , and AsO_3^{3-}) and arsenate species (H_3AsO_4 , H_2AsO_4^- , HAsO_4^{2-} , and AsO_4^{3-}) are the most frequent forms in natural systems. Arsenite species tend to predominate under reducing conditions, whereas arsenate species are more frequently found in aerobic soils and are more stable in the environment (Hasanuzzaman et al., 2015; James et al., 2017; Vromman et al., 2017). The two forms however often co-occur in both anoxic and oxic soils and water (Chatterjee et al., 2017a). The organic forms of As are mostly methylated and exist as monomethylarsinic acid (MMA), dimethylarsinic acid (DMA), and trimethylarsine oxide (TMAO) found at low concentrations in soils. Arsines are volatile compounds produced by microbial processes (Mitra et al., 2017). The structures of these compounds are illustrated on Figure 1.

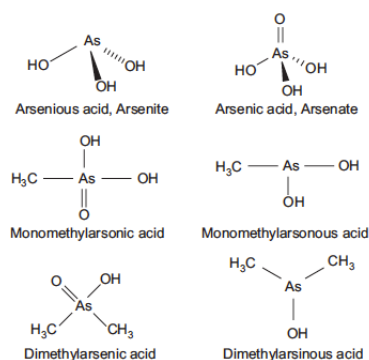


Figure 1 Chemical structure of some major arsenic compounds (Hasanuzzaman et al., 2015)

Parameters influencing speciation and mobility of arsenic compounds are : pH, redox potential (Eh), organic and other dissolved matter contents, soil texture and composition, biological processes (bacterial action), and the presence of other elements in the soil as Fe oxides (Chatterjee et al., 2017; Tapia et al., 2018). Arsenate species are the most mobile form in water, soil and sediment. Adsorption, desorption, precipitation, complexation, volatilization and methylation are chemical and biological processes undergo by As in soil (Hasanuzzaman et al., 2015). The Figure 2 summarize transformation processes of As.

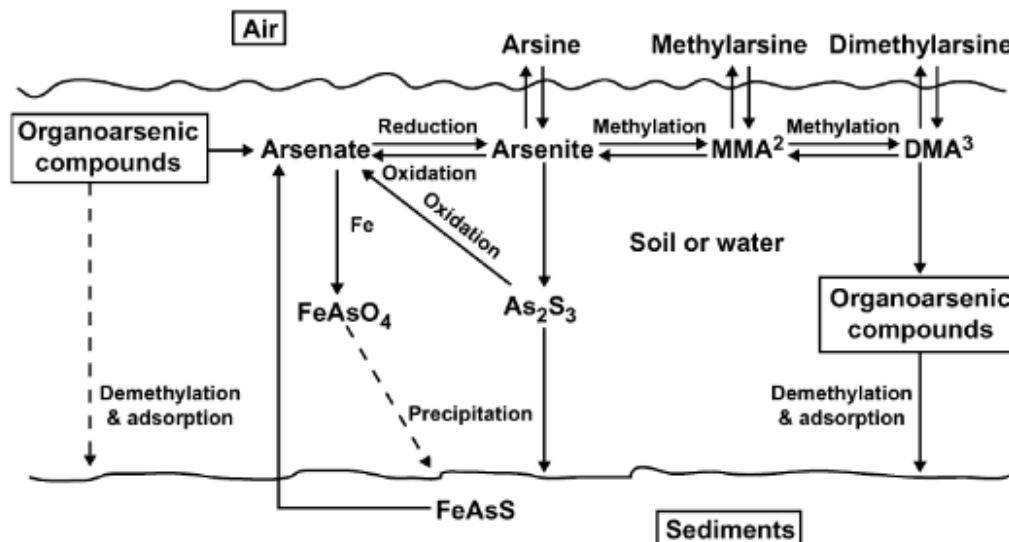


Figure 2 A simplified outline of major As reactions in the environment (Jones, 2007)

Arsenic in soil may be transported by wind erosion especially in zone with arid climate and flat geomorphology. It may also be transported in runoff, or can leach into the subsurface soil. Under reducing conditions, as those occurring during flooding or in underwater sediment, As adsorbed to iron and manganese oxides may be released. Microbes can also play a role in arsenic dissolution from sediment (Casanova et al., 2013a; James et al., 2017) and bacterial activity is thought to play a key role in methylation processes in the soil (Lomax et al., 2012; Ye et al., 2012).

1.2.3 Sources of arsenic in the environment

1.2.3.1 In general

As shown on Figure 3, the main sources of arsenic in the environment are geological (geogenic) and anthropogenic (human activities), the first one being predominant. The concentration depends on the parent materials. For example, in igneous rocks the recorded concentration varies from 1.5 to 5 mg.kg⁻¹ while in sedimentary rocks it ranges from 1.7 to 400 mg.kg⁻¹ (Hasanuzzaman et al., 2015). Weathering of rocks containing As has become a

considerably important natural source of inorganic As. Arsenates present in the soil can dissolve easily in groundwater and contaminate it. About one third of the atmospheric flux of arsenic is naturally derived, with volcanic eruption as the most significant source (Chatterjee et al., 2017a).

Since the last century, anthropogenic activities raised the level of arsenic contamination globally inducing soil pollution in some areas of the world (Chatterjee et al., 2017b). The major sources of anthropogenic arsenic contamination of water, soil, and air are mining industry, steel industry, petroleum and chemical industries, generation of commercial wastes (Abbas et al., 2018; Chatterjee et al., 2017a). Mining and smelting processes of metals like Au, Cu, Pb and Zn release large amount of As because this element is highly present in the sulphide ores of these metals. Furthermore, smelters also release flue gases and particulates containing toxic metals including As in the atmosphere and contribute to the contamination of nearby ecosystems (Giacomino et al., 2010; Robles-Arenas et al., 2006). The increasing metal mining and fossil fuel combustion lead to the spreading of large amount of arsenic (Hasanuzzaman et al., 2015). Other industrial and related processes such as electrolytic processes, wood and food preservation, urban wastes, medicinal and cosmetics uses, pesticides, insecticides and fungicides, sewage sludge, pigment, alloys, electronics, glass and mirror manufacture are also significantly contributing towards arsenic-related pollution (Gupta et al., 2017).

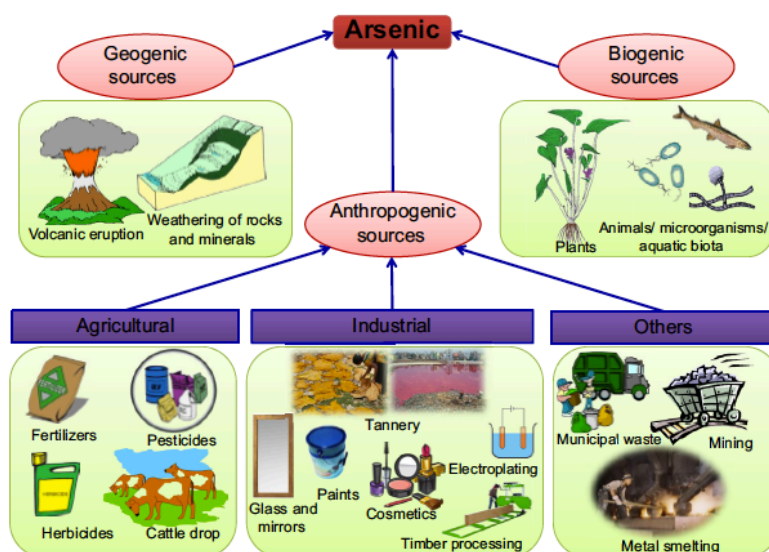


Figure 3 Major sources and routes of arsenic in soil and aquatic ecosystems (Hasanuzzaman et al., 2015).

Because of its presence in soil, water and air as in living matter and in form of solid, liquid or gas, As contamination becomes a widespread problem in many part of the world as illustrated on Figure 4. Contamination above the toxic level are reported in different countries such as Argentina, Bangladesh, Cambodia, Chile, China, Ghana, Hungary, India, Mexico, Nepal, New Zealand, the Philippines, Taiwan, the United States and Vietnam (Hasanuzzaman et al., 2015).

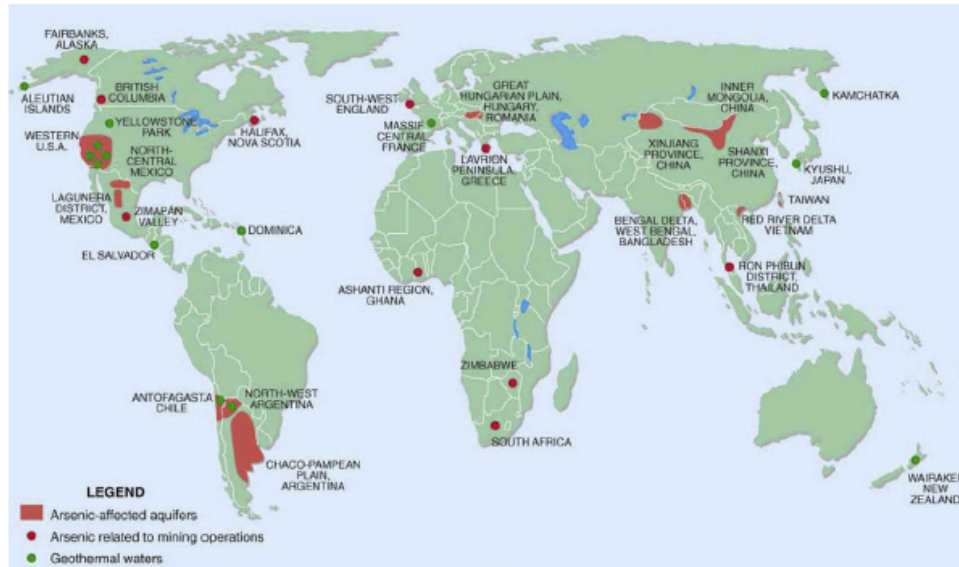


Figure 4 Distribution of documented world problems with As in groundwater in major aquifers as well as water and environmental problems related to mining and geothermal sources. Areas in blue are lakes (Hasanuzzaman et al., 2015).

1.2.3.2 The specific case of Chile

The present study focus on the case of Chile, more precisely the Pre-Andean zone of Northern Chile. This second region is called Antofagasta region. It is characterised by extremely arid conditions and water scarcity (Casanova et al., 2013b). The only water resource is the 440 km long Rio Loa. It supplies water for population (about 420,000 habitants) and the mining industry (the most important economic activity in the region). The Rio Loa basin is characterised by high As concentration in water and sediments. Measurements were taken near the city of Quillagua along the Rio Loa. In 2003, the average concentration in water was 2.35 mg.L^{-1} at this location (Romero et al., 2003). In 2016 the concentration was 2.5 mg.L^{-1} (Vromman et al., 2016b). This concentration is 100 times higher than the maximal concentration allowed by the WHO for drinking water. As is also present in soil with a concentration of 0.22 g.kg^{-1} . Quillagua oasis is heavily contaminated with As (Vromman et al., 2016b).

The high concentration of As is mainly associated with the Quaternary volcanic and geothermal activity in the Andes Cordillera (Casanova et al., 2013c; Lopez et al., 2012). The geothermal springs El Tatio has an As concentration up to 27 mg.L^{-1} in water. Erosion of volcanic rock is also a source of contamination. The very dry climate and strong evaporation added to the lack of any dilution from unaffected streams increased the high concentration. Sources of As in this region are therefore natural. However, Romero analysed the As concentration at several location along the Rio Loa and observed an enrichment downstream (Romero et al., 2003). Indeed, arsenic may also be spread in the environment because of human activities mainly in relation with mining industry (Vromman et al., 2016b). Mining activity in the Loa basin takes place in the intensively mineralised porphyry-Cu belt. Copper

mining activity releases SO_2 and As_2O_3 into the air. The mine wastes deposited in the past in the bed and on the banks of the river, inadequate treatment of the effluents from mining activities, as well as the As rich effluents from the water treatment plant represent other potential sources of As in this region. However, the poor quality of the water cannot be directly ascribed only to the intense mining activity (Romero et al., 2003).

1.2.4 Arsenic toxicity

1.2.4.1 Exposition

Although anthropogenic As contamination is widespread, it is mainly a cause for human health concern at a local scale. The heavy metals may be inhaled in the forms of dust, fumes or vapours, or ingested through food and water. The primary sources of chronic exposure for the majority of the world's population remain groundwater and food irrigated with the contaminated water. Arsenic contamination of groundwater is becoming a threat to human, animals and plant species because of its hazardous effects (Hasanuzzaman et al., 2015). Chile is part of aquifers with elevated As concentrations around the world. The WHO guideline indicates that $10 \mu\text{g.L}^{-1}$ is the maximum permissible limit of As concentration in drinking water. Water samples from aquifers in many parts of the world exceed this concentration level. In Chile 1 800 000 inhabitants are exposed to concentration above this limit and 500 000 persons are exposed to concentration up to $50 \mu\text{g.L}^{-1}$. Inorganic As exposure through food is highly variable across populations due to differentials concentrations in water used for irrigation or cooking. Groundwater irrigation is the main responsible for As contamination of the food chain through crops (Hasanuzzaman et al., 2015). Population close to metals mines and industries (pesticide, pigment and drug) has an increased As exposure (Jain and Chandramani, 2018; James et al., 2017). Contamination of rice, which is in several areas irrigated with poor quality water, is a well-known concern in Asian countries and constitutes the major source of human contamination through the food chain (Li et al., 2016; Lou-Hing et al., 2011; Saifullah et al., 2018).

1.2.4.2 Toxic effects of arsenic on human health

Chronic As toxicity is called arsenicosis by the WHO. It designates chronic disease caused by prolonged exposure of As in humans through drinking As-contaminated groundwater (Guha Mazumder and Dasgupta, 2011). Arsenic is one of the most dangerous elements. It has a wide range of health effects and is widely spread in the environment. Inorganic As compounds enter the human body via ingestion and inhalation routes. Absorption via dermal exposure has been considered negligible in the case of chronic exposure (James et al., 2017). Acute

arsenicosis takes a short time to manifest. The minimum duration for symptom manifestation is 8 months. In contrast, chronic arsenism may be asymptomatic for more than a decade. Several parameters affect the severity of toxicity : the chemical and physical form of As, the route of entry, the dose and duration of exposure, age and sex of the individual (Jain and Chandramani, 2018). Chronic As exposure produces various systemic manifestations. Pigmentation and keratosis are the specific skin lesions characteristic of chronic As toxicity. The major causes of morbidity reported by most of the investigators from different parts of the world are chronic lung disease, peripheral neuropathy, and chronic liver disease. There is a moderate to strong association between high As exposure and cardiovascular disorder. Pregnant women with chronic As exposure present a significantly higher frequency of stillbirth. Arsenic is also a cause of skin, lungs and urinary bladder cancers (Guha Mazumder and Dasgupta, 2011). The presence of pigmentary and/ or keratotic skin lesions and evidence of exposure are the two major criteria used for diagnosis (Jain and Chandramani, 2018).

1.2.4.3 Toxic effects of arsenic on plants.

Arsenic is a non-essential element but it can accumulate in plants to toxic levels (Zhao et al., 2009). The speciation of As influences its toxicity, but both arsenate and arsenite disrupt plant metabolism. Arsenite mainly affects the structure and the catalytic functions of proteins by binding their sulfhydryl groups. Arsenate interferes with phosphate metabolism and it disrupts functions such as oxidative phosphorylation, information storage (DNA, RNA metabolism), cellular signalling and ATP synthesis (Finnegan and Chen, 2012).

As a result, photosynthesis activity may be reduced through the decrease of chlorophyll production, disruption of photosystem II and the alteration of carbon fixation by Rubisco (Stoeva et al., 2005). Furthermore, respiration metabolism is also affected (Hasanuzzaman et al., 2015). Arsenic accumulation was also reported to have an impact on stomatal regulation which could also affect transpiration and hence the plant water status (Hettick et al., 2015; Mirza et al., 2014). For instance, water and osmotic stresses are highly frequent in As-treated plants. It induces the accumulation of osmoprotectants like proline, glycinebetaine (GB) and specific sugars within the plants to maintain water balance (Hasanuzzaman et al., 2015).

Arsenic toxicity in plant induces the generation of ROS such as H_2O_2 , $OH^\cdot$ and $O_2^\cdot$. These reactive species cause oxidative stress such as lipid peroxidation, protein oxidation, DNA damages and ultimately plant death (Farooq et al., 2016). For instance, malondialdehyde (MDA) is considered as an indicator of oxidative stress because it is an oxidized membrane lipid while carbonyl residues are relevant from protein oxidation (Hasanuzzaman et al., 2015). The overall consequences of As toxicity in plants consist in a decrease of root extension and proliferation, inhibition of shoot growth, reduction of fertility and germination disruption.

Globally, it reduces the fresh and dry biomass and as a consequence, the crop yield (Hasanuzzaman et al., 2015). Modification of plant morphology and phenology may also appear as a consequence of As impact on the plant hormonal status: ethylene and ABA, for example, frequently considered as typical stress hormones, may be overproduced in As-exposed plants.

1.2.4.4 Plant uptake of arsenic

The rate of As uptake and accumulation depends on the soil type, plant species and mechanism of uptake (Hasanuzzaman et al., 2015). Arsenate (As(V)) is a structural analogue of phosphate (Farooq et al., 2016) and may thus be taken up by plant roots through phosphate transporters (Gupta et al., 2009; Vromman et al., 2017). The mechanism is a cotransport of phosphate or arsenate and protons. Although most transporters have a higher affinity for phosphate than for arsenate, As(V) uptake may be more important in plants exposed to phosphate deficiencies (Zhao et al., 2010, 2009). Arsenite (As(III)) is transported into the plant through aquaporin channels in plant roots (the nodulin 26-like intrinsic protein or NIP) (Zhao et al., 2009). Plant aquaporins are complex network channels. They transport water and essential neutral molecules (including As(III)) across cell membranes (Farooq et al., 2016).

Methylated As species like MMA and DMA can also be taken up by plants through aquaporin channels or Si transporter (Lsi1 in rice). The uptake is less efficient than the one of inorganic As species (Farooq et al., 2016; Zhao et al., 2010). Several hydroponic studies agree that the order of As uptake by plant is AsIII > AsV > MMA > DMA (Finnegan and Chen, 2012). The Figure 5 summarizes the root uptake of the different arsenic species.

1.2.4.5 Arsenic translocation in plants

The translocation of As from root to shoot by xylem sap is limited because of the low mobility of the element. The explanation may be that arsenate is reduced to arsenite rapidly after being taken up by root and that the complexation of As(III) with thiols decreases its mobility (see below; Zhao et al., 2010, 2009). Although organic As compounds are less efficiently taken up than inorganic As species, their translocation from root to shoots is by far more efficient, often leading to As toxic accumulation in plants (Zhao et al., 2009). The Figure 5 shows the translocation of As through the xylem. Phloem transport does not seem efficient. The As concentration in the phloem sap is reduced mainly by the alkaline pH (Zhao et al., 2009). As a consequence, inorganic As distribution often presents a decreasing gradient from the base to the top of the plant. The translocation of organic species exhibits an inverse distribution and the order of translocation is opposite to the order of uptake: DMA > MMA > AsV > AsIII (Finnegan and Chen, 2012).

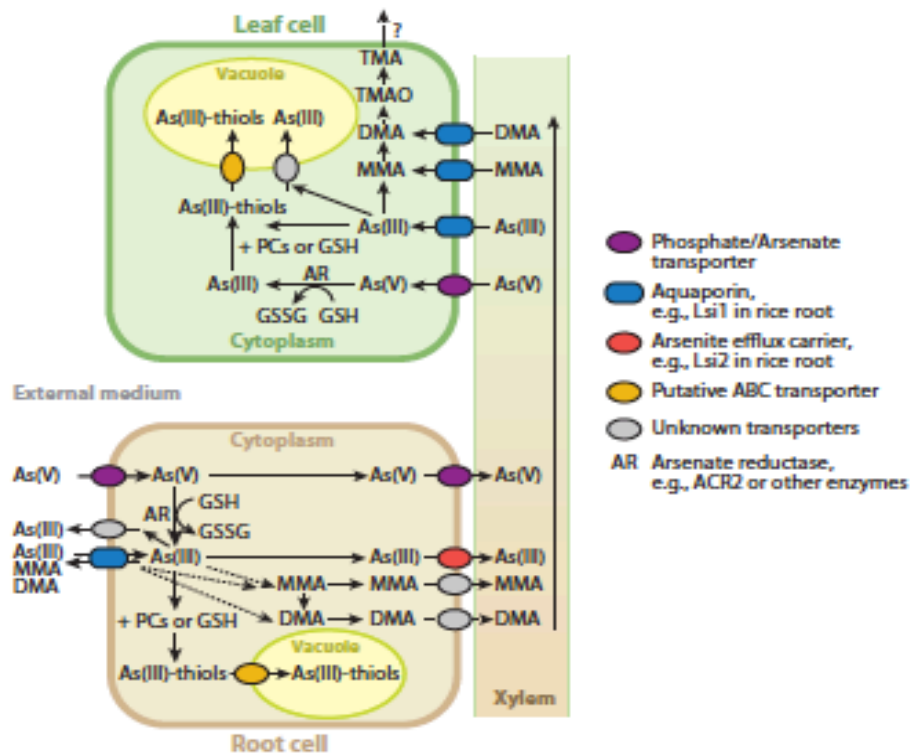


Figure 5 A simplified schematic diagram of As transport and metabolism in plants (Zhao et al., 2010). The thickness of arrow lines is indicative of the relative flux. In plant roots, arsenite (AsIII) and arsenate (AsV) are taken up by aquaporins (NIP) and phosphate transporters (PHTs) respectively. AsV is reduced to AsIII by arsenate reductases (AR) using glutathione (GSH) as a reductant. AsIII is complexed with phytochelatin (PCs) before sequestration in the vacuole via ATP-binding cassette (ABC) transporters. The transfer into the xylem is carried out by PHTs transporter and As efflux carrier. The As goes in leave by NIP were the same detoxification pathways as in root occur.

1.2.5 Resistance to arsenic toxicity

1.2.5.1 Arsenate reduction

Analysis of As speciation in plant tissues shows that the arsenite is predominant compared with arsenate even if the plant was exposed to arsenate. It means that arsenate is rapidly reduced to arsenite after being taken up in roots (Zhao et al., 2010, 2009). The reduction is mainly made by reductase enzymes and partly by glutathione (GSH). This arsenate reduction is accepted as the first step of detoxification mechanisms of As in plant tissues (Farooq et al., 2016). At first sight, this may appear as a paradox since As(III) is by far more toxic for living organisms than As(V) (Mirza et al., 2014; Vromman et al., 2018b).

1.2.5.2 Complexation with thiol ligands

Arsenic has a high affinity with sulfhydryl (-SH) groups. It is therefore not surprising that in plant tissues, As reacts with thiol-rich peptides such as GSH and phytochelatins (PCs) (Hasanuzzaman et al., 2015). Glutathione (γ -Glu-Cys-Gly) is a tripeptide formed by the binding of three amino acids: glutamate (Glu), cysteine (Cys) and glycine (Gly). Glutathione plays a key

role in many mechanisms of resistance against biotic and abiotic stress (Jozefczak et al., 2012). Glutathione is an important endogenous antioxidant: it is able to react with ROS in order to limit the impact of oxidative stress in plant cells. Oxidized glutathione (GSSG) is regenerated to GSH by glutathione reductase (GR) in the presence of NADPH as illustrated in Figure 6 (Farooq et al., 2016; Jozefczak et al., 2012; Zhao et al., 2010)

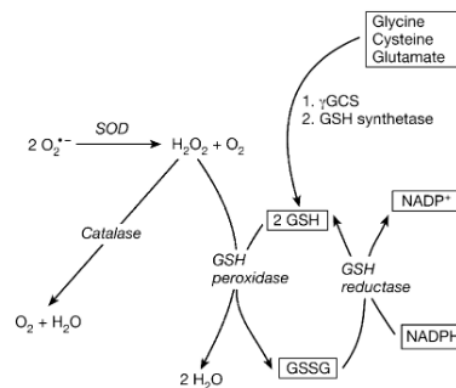


Figure 6 ROS detoxification pathway by GSH (Macdonald et al., 2003)

Secondly, due to the presence of a thiol group carried by the central cysteine, the GSH is also able to complex with numerous metals ions under the action of glutathione transferase (GST). This mechanism protect the other thiol groups mainly presents in enzymes and proteins and reduce the metabolic activity disturbance (Jozefczak et al., 2012). Finally, GSH is involved in the first step of detoxification of As because it contributes to reduce As(V) to As(III) (Jozefczak et al., 2012).

The GSSG/GSH balance is a good indicator of plant stress. Arsenic is a strong inducer of GSH production and recycling. However, the level of free GSH tends to be reduced under stress conditions because of its reaction with metals or its transformation in PCs (Jozefczak et al., 2012). Phytochelatins are polymers of 2 to 11 molecules of GSH. They complex with As mainly in form of As(III) due to their high number of thiol groups (-SH). The formed stable complex can be subsequently sequestered into vacuoles thus contributing to As detoxification (Hasanuzzaman et al., 2015; Jozefczak et al., 2012). It is why arsenic reduction is considered as the first detoxification step. Synthesis of PCs is under the complex regulation of phytochelatin synthase but the impact of As on enzyme activity and corresponding gene expression still has to be determined.

In order to sustain GSH and PCs synthesis, As-exposed plants need to efficiently regulate the absorption of sulphur (S). Beside these non-protein thiol-rich peptide (PCs), plants are also able to produce metallothionein (MT) consisting in protein also associating a huge number of cysteine residues (Cobbett and Goldsbrough, 2002). Although the role of MT in Cd and Cu binding has been demonstrated (Mirza et al., 2014; Suriyagoda et al., 2018), their role in As resistance is poorly documented.

1.2.5.3 Vacuolar and cell wall sequestration

Arsenic thiol complexes are transported by ABC-type transporters and stored irreversibly in vacuoles where As can no longer damage the plant. The vacuolar pH is about 5.5 which is favourable to the stability of the formed complexes. The transport across the tonoplast probably involves energy dependent mechanism because of the concentration gradient between the cytosol and the vacuole. The sequestration of As compounds complexed with PCs and GSH seems to be the major protection mechanisms observed in plants (Finnegan and Chen, 2012; Zhao et al., 2010, 2009).

Arsenic accumulation in the cell wall is also reported in some plant species (Vromman et al., 2018b). Cell wall binding is frequently mentioned as a tolerance strategy for positively-charged heavy metal ions (Sarma, 2011). In contrast, As accumulated in plant tissues is neutral (As(III)) or even negatively charged (As(V)) and the underlying mechanisms involved in apoplastic As accumulation is not fully understood.

1.2.5.4 Arsenic efflux

Following arsenic uptake and its reduction to arsenite, a rapid arsenite efflux from root tissues to the external medium may be observed in some cases (Tuli et al., 2010). It may be similar to the one of phosphate through ionic or NIP aquaporin channels some of which allow bidirectional passage of solutes by diffusion. Indeed, NIP aquaporins have been identified as As transporter across the cell membrane (Garbinski et al., 2019). Although arsenite can be re-absorbed, the rapid efflux would lead to decrease of As content in roots. The mechanism was observed in different plant species but its role in the detoxification process remains unclear (Farooq et al., 2016; Zhao et al., 2009).

1.2.5.5 Arsenic methylation

Methylated As species are commonly considered as less toxic than inorganic forms, although data are missing regarding the physiological impact of the various methylated As species on plant metabolism. Some traces of organic arsenocompounds may be found in plant tissues. According to Lomax et al. (2012), it is usually considered that these compounds mainly originate from the soil substrate where they are produced by bacteria. However, in different hydroponic culture systems, plants mainly fed with inorganic As compounds contain small amount of methylated As species such as MMA and DMA. This suggests that methylation of As compounds may occur in plants (Farooq et al., 2016; Ye et al., 2012; Zhao et al., 2010, 2009).

1.3 The Boron

1.3.1 Generalities

Boron (B) is a metalloid from the subgroup 3 of the periodic table and has intermediate properties between metals and non-metals. It is widely but not uniformly distributed in both lithosphere and hydrosphere. Boron is an essential micronutrient for plants and has a range of physiological effects on plants at low concentrations but becomes toxic at high concentrations (Camacho-Cristóbal et al., 2008; Uluisik et al., 2018). As a micronutrient, B has a narrow range of optimal concentrations (Macho-Rivero et al., 2017).

In soil solution B exists mainly as boric acid ($B(OH)_3$), which can be easily leached under high rainfall conditions leading to deficiency in plants. On the contrary, under low rainfall conditions, B cannot be sufficiently leached and therefore may accumulate to levels that become toxic to plants. It happens very often in arid and semiarid regions with high-boron concentrated groundwater. There, the accumulation of B in topsoil due to the evaporation of groundwater reaches toxic levels although B deficiency is more frequent than toxicity in field conditions (Camacho-Cristóbal et al., 2008; Nable et al., 1997).

1.3.2 Sources and forms of boron in the environment

1.3.2.1 Natural sources

Boron occurs naturally in soils. In most cases the primary sources of B consist in tourmaline and volatile emanations from volcanoes. Tourmaline is very resistant to chemical breakdown in the weathering zone and thus has a tendency to accumulate. Borosilicates are secondary B rocks and are also resistant to weathering and not readily available to plants. Most of the plant-available B comes from the decomposition of soil organic matter and from B adsorbed and precipitated onto the surfaces of soil fine mineral fractions. It is mainly boric acid that can be taken up by plants (Gupta et al., 1985; Nable et al., 1997).

1.3.2.2 Anthropogenic sources

Plants irrigation with contaminated water is the main source of high soil B levels resulting in toxicity. The soil properties determine the B retention in the soil solution. The B concentration of irrigation water should be controlled and adapted to the sensitivity of crops and to the absorption capacity of the soil. The contamination of irrigation water may be natural or derived from poor efficient desalinisation of sea water (Gupta et al., 1985; Nable et al., 1997).

In numerous areas in the world, desalinated sea water is indeed used for irrigation purposes and in several cases, it may contain high amount of B (up to 2 mg.L⁻¹) (Pinero et al., 2017).

Mining activity is another important source of B pollution. Water soluble B species can be released during the extraction process of ores. Furthermore, fly ashes containing B are also released from coal combustion used in electric power plant (Nable et al., 1997). Finally, boric acid and the sodium salts of boron are commonly used as an antiseptic, bactericide, cleaning agent, preservatives, fertilizers, insecticides and herbicides. The discharge of B derived products into the environment during production and end use has resulted in the accumulation of B in waste effluent and consequently in groundwater and natural aquatic systems. Boron is also used in industrial applications such as glass and porcelain manufacture, fiberglass insulation, ceramic glazes, and metal alloys (Nable et al., 1997; Uluisik et al., 2018).

1.3.3 The role of boron in plant metabolism

Boron is a necessary and beneficial microelement for plants. It is considered to have a primordial function in cell wall synthesis, cell wall structure and membrane integrity and function. In the cell wall, B cross-links two rhamnogalacturonan II (RGII) monomers to form peptic polysaccharide and thus provides stability to the cell wall matrix. However, there is increasing evidence for a possible role of B in other processes such as the maintenance of plasma membrane function and several metabolic pathways (Camacho-Cristóbal et al., 2008; Geng et al., 2007; Uluisik et al., 2018).

In the plant, boric acid and borate are in equilibrium due to the pH and is relatively unreactive and immobile in the plant once it's accumulated in leaves. Both intracellular species are capable to form complexes with different biological compounds having two hydroxyl groups in *cis*-configuration classified as *cis*-diols. This is the basis for binding of B to apiose which is the central component of the RGII complex present in plant cell walls (Camacho-Cristóbal et al., 2008; Reid et al., 2004). The major components of cell walls are cellulosic polymers, and matrix polymers such as hemicellulosic and peptic polysaccharides. The formation of borate esters with hydroxyl groups of carbohydrates or glycoproteins seems to be a mechanism for crosslinking cell wall polymers. Given these biochemical mechanisms, B can be considered as a key element in the formation of growth tissues (Blevins and Lukaszewski, 1998). The Figure 7 shows the cell wall structure containing boron.

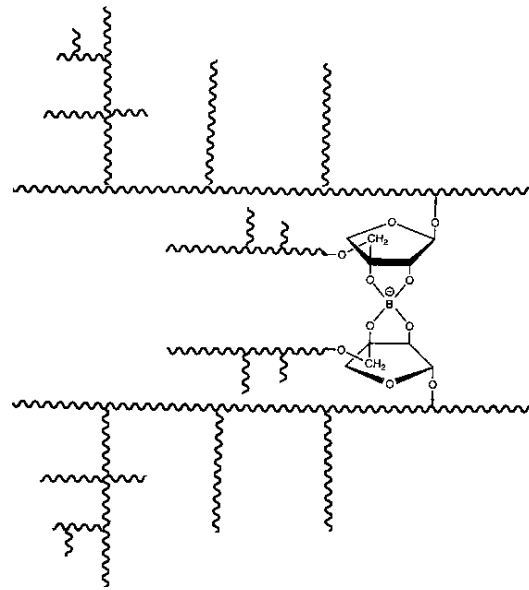


Figure 7 Schematic representation of the bis(rhamnogalacturonanII)-borate complex, showing the linkage through apiose groups in the cell wall (Power and Woods, 1997)

1.3.4 Boron uptake and translocation

Boric acid is a small uncharged molecule absorbed by plants. Depending on B availability, boric acid uptake by roots can be carried out by three different ways: by passive diffusion across the membrane; facilitated transport by aquaporins; and an energy-dependent high affinity transport system mediated via BOR transporters (Camacho-Cristóbal et al., 2008; Uluisik et al., 2018). Under conditions of adequate or excessive B availability, boric acid absorption by roots is mediated through a passive diffusion across the lipid bilayer. In fact, B has a high permeability through the cell membrane. Under B limitation, the small uncharged molecule can be transported by NIP aquaporins or through an active uptake by roots (Camacho-Cristóbal et al., 2008).

Once B has been absorbed by root cells, this micronutrient must be loaded into the xylem. The same mechanisms as for roots uptake occur depending again on the availability of boric acid. During this long distance transport from roots to shoots, B follows the transpiration streams and accumulates at the margins of mature leaves. The main part accumulates in the apoplast and contributes to the structure of cells walls (Camacho-Cristóbal et al., 2008; Matoh, 1997; Uluisik et al., 2018).

However, B can also be transported via the phloem. Phloem transport involved the formation of B-diol complexes with, for example sorbitol or mannitol as transport molecules as illustrated in Figure 8. This complex formation is based on the capacity of B to bind to cis-hydroxyl groups of sugar alcohols (Camacho-Cristóbal et al., 2008).

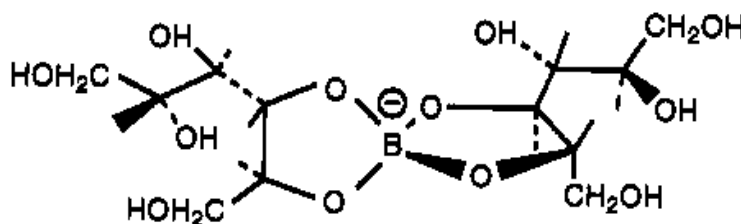


Figure 8 Structural representation of the bis(sorbitol)-2,3,20,30- borate complex, one of several isomers identified in phloem of sorbitol-rich plant species (Power and Woods, 1997)

1.3.5 Boron toxicity

The impact of B toxicity depends on the species. The typical visible symptoms are leaf burn, chlorotic and/or necrotic patches, often at the margins and tips of older leaves. These symptoms reflect the distribution of B in the plants because it accumulates at the end of the transpiration stream. The concentration is higher in leaves than in roots. The effects of high B concentration are reduced growth of shoots and roots, reduction of leaf area and lower leaf chlorophyll content and photosynthetic rates, inhibition of cell wall expansion and finally a decreased lignin and suberin levels (Camacho-Cristóbal et al., 2008; Nable et al., 1997). Boron toxicity delays germination and reduced the final germination percentage (Archana and Pandey, 2016).

Based on the ability of B to bind compounds with two hydroxyl groups in the cis-configuration, there are three main causes of boron toxicity: (i) alteration of cell wall structure; (ii) metabolic disruption by binding to the ribose of ATP, NADH and NADPH and (iii) disruption of cell division and development by binding to free ribose or the one within RNA (Camacho-Cristóbal et al., 2008).

In his study Reid et al. (2004) could not highlight a direct molecular interaction with the photosynthetic system. However, the necrotic lesions of leaves and the reduction of leaf area reduce the global photosynthetic activity of the stressed plants. Other authors, however, reported an obvious effect of B excess on PSII stability (Guidi et al., 2011, cited by Macho-Rivero et al., 2017).

Osmotic stress also results from excessive B accumulation as well as oxidative stress leading to an increase of membrane lipid peroxidation and antioxidant enzyme activity (Camacho-Cristóbal et al., 2008).

1.3.6 Tolerance to boron toxicity

1.3.6.1 Exclusion mechanisms

The first mechanism of tolerance to B toxicity consists in an exclusion process. It has been observed that tolerant genotypes of plants have a lower B concentration in roots, xylem and aerial part, even if the membrane permeability to B is the same than in sensitive plants. As mentioned above, B can be absorbed by passive and facilitated diffusion, but also by active transporters. Tolerant plants are able to remove B by an active efflux through aquaporins. A transporter (BOR₄) has also been identified as responsible for the efflux of B previously absorbed towards the external media (Herrera-Rodríguez et al., 2009; Nable et al., 1997; Reid and Fitzpatrick, 2009). Princi et al. (2016) demonstrated that the expression of the gene coding for this transporter was increased in resistant genotype of tomatoes in the presence of an excess of B but not in sensitive genotype.

According to Reid and Fitzpatrick (2009), an active transporter may also excrete the B from sensitive symplastic compartments into the leaf apoplast. In fact, in tolerant cultivars the leaf tissues are able to withstand higher concentrations of B (more than 2-fold higher than in sensitive cultivars). They have also shown that the transporter responsible for the efflux in roots was also expressed in leaves. The redistribution of boron in the apoplast and its excretion in trichomes at the leaf surface facilitates the leaching by rain. As a consequence, field-grown plants commonly show a lower B concentration than plants maintained in greenhouses, suggesting that rain efficiently helps to reduce the B toxicity by leaching of soil and plants leaves.

According to Pinero et al. (2017), external Ca also helps the plant to more efficiently regulate B absorption. According to this study, high ambient CO₂ also help the plants to cope with B excess, probably through a stimulation in photosynthesis. Indeed, Macho-Rivero et al. (2017) demonstrated that B excess leads to stomatal closure by increased concentration of abscisic acid (ABA) as a result of B-induced overexpression of the gene coding for NCED3 involved in ABA synthesis. Since stomatal closure limits photosynthesis, it might be argued that growing plants at high CO₂ concentration would lead to an increased water use efficiency. Beside ABA, the phytohormone kinetin (a synthetic cytokinin) may assume important function in plant response to B toxicity: Eser and Aydemir (2016) demonstrated that exogenous application of this protecting compound avoid B-induced decrease in chlorophyll content and limited the increase in H₂O₂ and MDA in wheat seedlings.

1.3.6.2 Internal tolerance mechanisms

Boron can bind readily with polysaccharides and various organic acids, particularly with the alcohol functional group. It can therefore be chelated to limit the free B. A similar process also happens in the rhizosphere and involves the excretion of organic compounds. Nevertheless, considering the low concentration of B in the roots, sequestration at this level is not thought to play a major role. This conjugation is the first step towards the excretion of B or its storage in vacuoles (Roessner et al., 2006). According to Yıldırım and Uylaş (2016), the last stage of B detoxification could involve cell apoptosis and foliar abscission. Indeed, the enzymatic activity of degradation of the cell wall increases in plants treated with B. Before leaf abscission, valuable nutrients may be recycled to young photosynthetically active leaves. Subsequent abscission of leaves accumulating excess B may thus be considered as a strategy of resistance. It is interesting to notice that halophyte plant species have been reported to be resistant to high B dose: this is especially the case for *Thelungiella halophila*, which is very close to the model plant species *Arabidopsis thaliana*: Lamdan et al. (2012) demonstrated that the plant is able to efficiently exclude B excess, but also synthesises B-binding polyhydroxyl metabolites such as malic acid, fructose, glucose, sucrose and citric acid to cope with internal high B concentration.

1.3.6.3 Adaptation to oxidative stress

The B excess induces an oxidative stress in the plant which leads to ROS formation. Besides the metabolism disruption, these compounds induce membrane damage by lipid peroxidation and protein degradation. The neutralisation of these compounds can be performed by enzymatic and non-enzymatic ways as previously indicated for the arsenic detoxification. It has been shown that B stress induces an increase of GSH and ascorbate content of the plant. Cytokinin, which may contribute to reduce B toxicity, act on a decrease in lipid peroxidation and H₂O₂ accumulation and an increase in proline concentration, an osmoprotectant putatively acting as a ROS scavenger (Eser and Aydemir, 2016). Similarly, Yıldırım and Uylaş (2016) used a transcriptomic approach to demonstrate that B excess trigger numerous gene regulating the oxidative status of stressed plants and that overexpression of genes coding for antioxidative enzyme should be regarded as an attempt to overcome the deleterious impact of B excess.

1.4 The phytoremediation

1.4.1 Introduction and definition

Numerous in-situ and ex-situ remediation techniques have been developed to contain, clean up, or restore heavy metal-contaminated soils (Houben, 2013). The most widely used are surface capping, soil flushing, electrokinetic extraction, solidification, vitrification, and phytoremediation. These techniques can be divided into different categories as shown in Figure 9 (Liu et al., 2018).

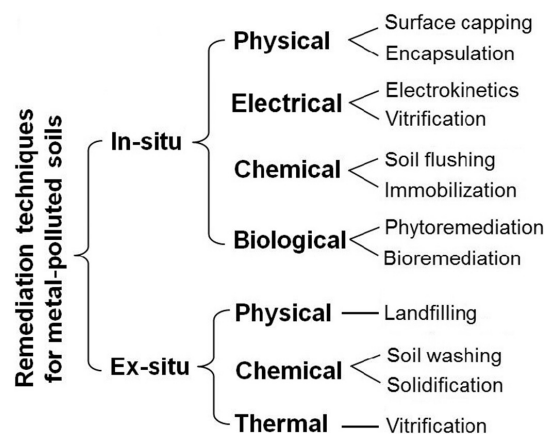


Figure 9 Common remediation techniques for heavy metal-contaminated soils (Liu et al., 2018).

Phytoremediation is a plant-based technology. It uses plants and associate microorganisms to clean up a contamination of soils, sediments, and water. It helps to reduce the concentrations or toxic effects of contaminants in the environment or help in their immobilization in order to avoid dispersion of pollution (Pilon-Smits, 2005; Vromman, 2018).

Phytoremediation is an emerging technology that may be an alternative to physical, chemical and thermal expensive and destroying procedures. On the contrary, phytoremediation improves the physical and chemical properties of the soil and increases its biological quality. This technology is a cost-effective, efficient, novel, eco-friendly, and solar-driven technology with good public acceptance (Ali et al., 2013; Liu et al., 2018; Tangahu et al., 2011).

Phytoremediation is a field of active current research. It explores and uses plants with metal-accumulating capacities. Some of these plants are known as hyperaccumulators and accumulate exceptional high level of toxic elements (Brooks et al., 1998). Most hyperaccumulators are however producing a low level of biomass and remain rather short with a poor root system: although they constitute an interesting material for identification of physiological and molecular properties involved in heavy-metal and metalloid resistance, they probably do not constitute the most interesting material for field application (Sarwar et al., 2017).

Other plants are able to accumulate heavy metals to a lower extent, but maintain high relative growth rate and thus contribute to remove substantial amounts of pollutants from the soil. These plants clean up environment and improve its quality. Phytoremediation takes advantage of uptake capacity of plant roots and of translocation, bioaccumulation, and contaminant degradation abilities of the entire plant (Ali et al., 2013; Tangahu et al., 2011).

This technology can be used for removal of heavy metals and radionuclides but also for organic pollutants such as polynuclear aromatic hydrocarbons, polychlorinated biphenyls, and pesticides (Ali et al., 2013).

1.4.2 Different forms of phytoremediation

According to the type of contaminant and the considered plant, there are different mechanisms involved in the phytoremediation process. Phytostabilization, rhizodegradation, rhizofiltration, phytodegradation, and phytovolatilization are involved in the removal of organic pollutants. For inorganic pollutants, phytostabilization, rhizofiltration, phytoaccumulation and phytovolatilization are mechanisms involved in the phytomanagement of contaminated sites. As shown on Figure 10, they do not exclude each other's and it may even be argued that the most efficient approach for phytomanagement of contaminated sites constitutes in the optimal association of complementary procedures (Tangahu et al., 2011).

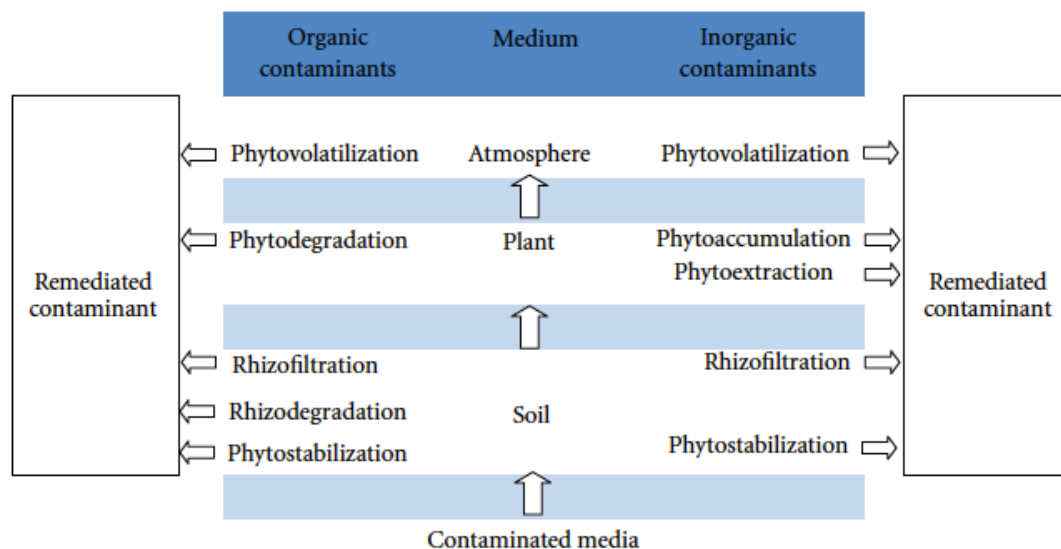


Figure 10 Uptake mechanisms in phytoremediation technology (Tangahu et al., 2011).

Phytoextraction is the uptake of contaminants from soil or water by plant roots and their translocation to and accumulation in the aerial harvestable part of the plant. The translocation process to the aboveground plant body is important because it allows the harvest of biomass and further treatment or recovery of metals which is not feasible with the root biomass (Ali

et al., 2013; Tangahu et al., 2011). The fate of heavy-metal contaminated biomass still remains an unresolved question which is currently under investigation (see below).

Phytostabilization is the stabilization of contaminants in contaminated soils. This method prevents their migration to groundwater or their entry into the food chain by reducing the mobility and bioavailability of pollutants in the environment. Root absorption and accumulation in plant tissues, adsorption onto roots, exudate precipitation and complexation in the root zone or metal valence reduction and precipitation are processes through which plants can immobilize heavy metals in the rhizosphere. The plants used for phytostabilization are resistant to heavy metals, produce a large amount of root biomass and are not supposed to absorb and accumulate pollutants within the shoots (Houben, 2013). Consequently, this should not appear as a permanent solution because it does not remove the pollutants from the soil but immobilize them in the soil (Ali et al., 2013; Liu et al., 2018; Tangahu et al., 2011). It has been proven that phytostabilization is effective for reducing the mobility of Pb, As, Cd, Cr, Cu, and Zn in contaminated soils. It is a good strategy to mitigate the ecological risk in contaminated sites with poor natural vegetation due to the contamination. This technique is frequently used in combination with chemical amendments to reduce the bioavailability and biotoxicity of heavy metals and allow the development of the plants (Liu et al., 2018). Other amendments consisting in hydrous Fe oxides and inorganic clay minerals may also be used (Houben, 2013).

Phytovolatilization is the uptake of pollutants from soil by plants, their transformation into gaseous species and their release into the atmosphere through transpiration of aerial parts of the plant body (Ali et al., 2013; Tangahu et al., 2011). For inorganic pollutants, phytovolatilization commonly requires the integration of the metal in organic compounds and has been reported for Hg and Se (Pilon-Smits, 2005).

Phytodegradation is the degradation and detoxification of organic pollutants by the plant metabolic activity with the help of enzymes. Because heavy metals are not biodegradable, phytodegradation is limited to organic contaminants (Ali et al., 2013; Tangahu et al., 2011).

Rhizodegradation is the degradation of organic pollutants into simpler molecule contaminants by microorganisms in the rhizosphere. By secreting exudates, the plant creates a nutrient-rich environment and stimulates the metabolic activity of microbes around the roots (Ali et al., 2013; Tangahu et al., 2011).

Rhizofiltration is the adsorption or precipitation of contaminants on plant roots. They can also be absorbed into it to minimize their movement in the soil (Ali et al., 2013; Tangahu et al., 2011). Some specific devices may also be adopted to clean up contaminated domestic water

by constructed wetlands and semi-aquatic plants producing mucilage in their roots. It may constitute an interesting material for this purpose (Lutts et al., 2016).

All these processes are illustrated in the following Figure 11.

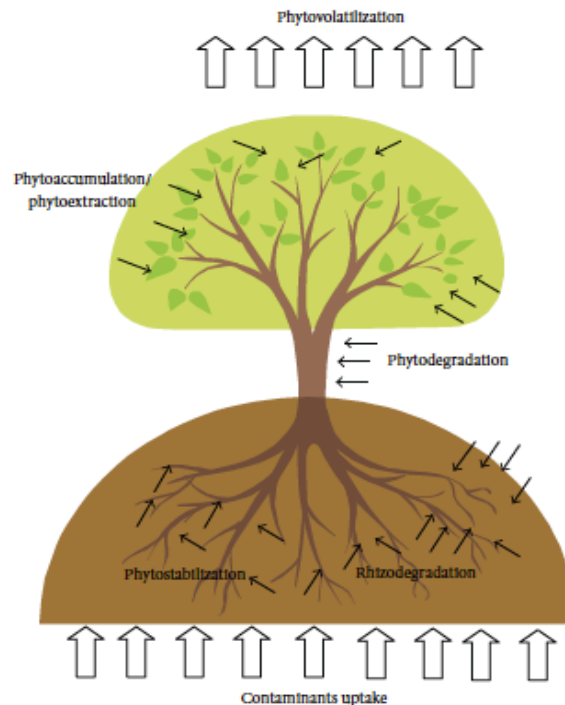


Figure 11 The mechanisms of pollutants uptake by plant through phytoremediation technologies (Tangahu et al., 2011).

1.4.3 Factors affecting phytoremediation efficiency

Phytoextraction is the mainly used phytoremediation technic and seems to be the most promising for commercial applications. There are several factors which can affect the efficiency of phytoextraction of heavy metals (Ali et al., 2013; Tangahu et al., 2011).

The plant species affect the uptake of the contaminants. Suitable plant species that hyperaccumulate heavy metals are identified, but as mentioned earlier, hyperaccumulation is not always compatible with maintenance of biomass production.

A suitable plant for phytoextraction should have the following characteristics (Ali et al., 2013; Lambrechts et al., 2011b; Pilon-Smits, 2005; Sarwar et al., 2017):

- High growth rate and perennial phenology
- Production of an important aboveground biomass
- Widely distributed and highly branched root system
- More accumulation of the target heavy metals from soil

- Translocation of the accumulated heavy metals from roots to shoots
- Tolerance to the toxic effects of the target heavy metals
- Good adaptation to prevailing environmental and climatic conditions
- Resistance to pathogens and pests
- Easy cultivation and harvest
- Repulsion to herbivores to avoid food chain contamination

A very important limiting factor in field conditions is the bioavailability of the pollutant, which could be defined as the proportion of the pollutant available to move from the soil to living organisms. Bioavailability may, in some cases, be extremely low; as an example, Pb phytoavailability is frequently lower than 1%. Heavy metals may be strongly bound to clay particles or organic matters and not accessible for root absorption, thus limiting the capacity of cultivated plants to remove pollutants (Ruttens et al., 2006). Numerous soil properties directly affect bioavailability, among which pH, organic matter and phosphorus content. Speciation of heavy metals in the root zone may also be directly influenced by physical properties and biological activity of the rhizosphere and this may directly influence root absorption (Tangahu et al., 2011). This is especially the case for As since, as previously mentioned, this element exist at different oxidation stage and may be absorbed by the plant with different mechanisms depending on its oxidation state (Vromman et al., 2018b).

Specific tools may be adopted when pollutant bioavailability is too low and consist in “induced phytoextraction”: in this case, chelating agents are used to increase availability of the target pollutant in order to induce its extraction by the roots. A wide range of chelating agents are now proposed for this purpose which contrast which each other in terms of selectivity. The most widely used chelating agent is still EDTA but this compound may be itself toxic for the plants and, when presents in the tissue, may chelate essential Ca. The modalities of EDTA-HM complex absorption still remaine poorly known, and it is not clear if the complex itself is absorbed. Another risk is that the use of chelates strongly increased the mobility of the pollutant leading to contamination of aquifer before the plant has enough time to absorb it (Lambrechts et al., 2011b, 2011a). Besides EDTA, other chelates (including biodegradable compounds) are now tested but all of them are devoted to cationic pollutant and only mercapto-succinate appears as a putative chelate for induced phytoextraction of As.

1.4.4 Suitable plants: few examples

Phytoremediation require the use of “metallophytes” plants. They are specifically adapted to and thrive in heavy metal-rich soils. These plants can act both as accumulators or excluders.

Metal excluders accumulate heavy metals from the soil into their roots, but restrict their translocation into their aerial parts. These plants have a low potential for phytoextraction but are more suitable for phytostabilization purposes (Houben, 2013). The accumulator type of plant is putatively tolerant to the metals and survives despite the uptake and translocation of pollutants in its aboveground part. The plants biodegrade and biotransform the contaminants into less toxic compounds in their tissues and finally accumulate it in the shoots. Hyperaccumulators can concentrate heavy metals to levels far exceeding those present in the soil (Ali et al., 2013; Tangahu et al., 2011).

There is no scientifically defined standard to identify hyperaccumulating plants. Nevertheless, the recommended criteria are based on concentration for different metals and metalloids in the dried foliage of plants growing in their natural environment: 100 mg kg⁻¹ for Cd, Se and Ti ; 300 mg kg⁻¹ for Co, Cu and Cr; 1000 mg kg⁻¹ for Ni, Pb and As. Compared to crops or common plants, hyperaccumulators achieve 100-fold higher shoot metal concentration (Ali et al., 2013). Metal-hyperaccumulator plants count more than 400 species. The majority are members of the brassicaceae family (Ali et al., 2013).

Halophyte plant species generally dispose of an advantage regarding the tolerance to several environmental factors including heavy metals. They have been suggested to be naturally better adapted to cope with environmental stress because of a more efficient performance of several basic biochemical tolerance mechanisms (Lutts and Lefèvre, 2015). These plants are able to synthesize osmoprotectants in order to maintain a favourable water potential gradient and to protect cellular structures. Additionally, in a recent study, it was found that the presence of heavy metals may trigger the osmoprotectant onversynthesis like glycinebetaine or proline. These mechanisms allow the plant to tolerate chilling, freezing, heat, drought, salts and heavy metals. Thus, it can be safely assumed that halophytes like *Atriplex sp.* and heavy metal-tolerant plants possess specific and general functioning mechanisms of tolerance toward a wide range of abiotic factors (Lefèvre et al., 2009, 2010; Lutts et al., 2004; Zhou et al., 2018). Halophyte plant species offers a great potential for phytoremediation research. They tolerate heavy metal stress through tree different ways: phytoextraction, phytostabilization and phytoexcretion. Phytoexcretion is a kind of phytoextraction based on the ability of some species to excrete excess of salts and heavy metals on the leaves surface (Manousaki and Kalogerakis, 2011).

1.4.5 The specific case of As- and B-phytoremediation

Numerous species have been identified in As-contaminated areas (Meharg and Hartley-Whitaker, 2002). In several cases, an amendment with available P is required to allow optimal

plant growth on As-contaminated substrate (Chen et al., 2007; Mirza et al., 2014). Sultana et al. (2015) performed efficient phytoremediation of polluted soils using *Echinocloa* plant species. Under flooded conditions, plant tends to develop an iron plaque at the root surface which may hamper translocation of As to the aerial part since As is efficiently adsorbed on iron oxides (Vromman et al., 2013). Some ligneous plant species, especially those growing in mangroves such as *Tamarix parviflora* and *Eucalyptus camaldulensis* appear as suitable candidate for phytomanagement of As-contaminated soils (Alkorta et al., 2004). Association with mycorrhiza and biochar amendments provide interesting strategies to improve As phytoextraction (Vithanage et al., 2017). The fern species *Pteris vitata* is now considered as a model plant species for As hyperaccumulation: more than 95% of the absorbed As accumulates in the aerial parts, among which 60 to 90% as As(III) compounds. Despite such fascinating level of resistance associated with a good level of biomass production, attempts to use this form for phytoextraction remain partly unsuccessful until now, mainly because of its limited range of ecological adaptation (Zhang et al., 2004).

Boron removal using plants still remains quite poorly documented. *Lemna gibba* was used by Türker et al. (2017) and were able to remove 19-63% of B from a contaminated irrigation water: in this filtration process, B removal followed a second order kinetic model and the material was still able to grow at high B concentration. Rámila et al. (2015) identified a promising species as an extremely tolerant plant for B phytoremediation: *Puccinellia frigida* is native from North Chile where it colonizes banks of hydrothermal spring releasing huge amounts of B in the environment. This plant is able to cope with more than 4000 mg.kg⁻¹ available B in the soil and to accumulate up to 4900mg.kg⁻¹ DW within the shoots without exhibiting detrimental symptoms. In a complementary study, Rámila et al. (2016) analysed the expression of genes suspected to play a major role in B resistance and found the expression of a gene coding for an efflux transporter at the leaf level, suggesting that this transporter may be involved in apoplastic sequestration of absorbed B, supporting the previous hypotheses that B excluded from cytosol may be accumulated within the plant cell wall polymers.

1.4.6 Efficiency indicators

The efficiency of phytoextraction needs to be quantified. The first way is by calculating the bioconcentration factor (BCF). BCF is calculated by dividing the concentration of metal in the harvested tissues by the concentration of metal in the soil. The second way is by calculating the translocation factor (TF) which express the efficiency of the plant in translocating the accumulated metal from its roots to shoots. TF is calculated by dividing the concentration of metal in shoot by the concentration of metal in roots. Both BCF and TF are important

parameters to determine the hyperaccumulator character of a plant. BFC and TF greater than 1 indicate the potential for being used in phytoextraction purposes (Ali et al., 2013). It has, however, to be considered that shoot part on one hand, and root part, on the other hand, may be affected by external pollutants in contrasting way in terms of growth; hence concentration is also influenced by growing process leading to accumulation or, on the contrary, dilution process, so that the same TF calculated on the basis of quantity (amount) and not only concentration might be interesting to consider.

1.4.7 Waste management

After being harvested, the plants can be burned. This method allows the energy recovery as well as the volume reduction of biomass. The ashes can be either disposed properly in specialized dumps or if economically feasible, processed for recovery of metals (Ali et al., 2013; Liu et al., 2018).

1.4.8 Benefits and limitations

Phytoremediation is an alternative to traditional technologies. It has several benefits compared to these ones (Ali et al., 2013; Liu et al., 2018; Tangahu et al., 2011):

- Low cost
- Effective for a wide range of contaminants
- In situ technology, less disruptive for the soil properties
- Suitable for very large field contamination
- Solar-driven and environmental friendly method with possibility of energy recovery
- Aesthetically pleasant and good public acceptance

On the other hand, it also has limitations:

- Time-consuming method
- Require a high biomass production
- The bioavailability of metals depends on the root system and the soil properties
- Depends on environmental conditions
- Limited to soil with low to moderate levels of metal contamination
- Risk of food chain contamination
- Treatment of secondary waste

1.5 Vermicomposting and vermicompost

1.5.1 Definition of vermicomposting

Since the work of Darwin (1881), the importance of earthworms in the breakdown of organic matter and the release of the nutrients that it contains has been known. They are able to process many different biodegradable organic wastes such as crop residues, animal, municipal and agro-industrial wastes that pose major problems in disposal and treatment (Edwards and Arancon, n.d.; Kiyasudeen et al., 2016a; Sharma et al., 2005). Vermicomposting is one of the most efficient ways to convert these organic wastes into useful and valuable products: earthworm biomass and bio-fertilizer (vermicompost). Indeed, the final product of vermicomposting can be used as a source of organic matter for soil amendment, as sources of nutrients for soil fertilization or as growing media constituents for soilless cultivation. Therefore, the use of earthworms may be an answer as an ecologically sound, economically viable and socially acceptable technology to help the sustainable management of organic wastes (Kiyasudeen et al., 2016a; Sharma et al., 2005).

Vermicomposting increases the decomposition process by 2–5 times compared with thermophilic composting. The earthworms act as mechanical blenders and fragment the organic waste substrates. The physical and chemical modifications stimulate the microbial activity and facilitate further decomposition (Kiyasudeen et al., 2016a).

According to Atiyeh et al (2000). “Vermicomposting is a non-thermophilic biological oxidation process in which organic material are converted into vermicompost which is a peat like material, exhibiting high porosity, aeration, drainage, water holding capacity and rich microbial activities, through the interactions between earthworms and associated microbes”.



Figure 12 Vermicompost in production state with high worms density (“Black Diamond VermiCompost,” n.d.)

1.5.2 Production of vermicompost

The first step in vermiculture is to select suitable feed materials for the earthworms because it determines the onset and rate of production. These can be nitrogen rich material but the C:N ratio should not be higher than 40 (Sharma et al., 2005). Suitable organic wastes for vermicomposting are (Edwards and Arancon, 2005):

- Farm waste: chicken, turkey, duck, horses, cattle, pig and rabbit
- Industrial/urban waste: brewery, potato, paper, sewage, restaurant, food market and yard waste (leaves and grass)

There are three ecological varieties of earthworms. Epigeics are surface dwellers and feed on organic matter on the soil surface. Endogeic earthworms are mainly in the mineral layer of soil and burrow predominantly. Anecic earthworms predominantly make vertical burrows. The epigeics and anecics have been harnessed for use in the vermicomposting process (Sharma et al., 2005). There are six earthworm species that have been identified as potentially the most useful species to process vermicomposting by the breakdown of organic wastes. In temperate regions, these are *Eisenia fetida* (one of the most commonly used world-wide), *Dendrobaena veneta*, and *Lumbricus rubellus*. *Eudrilus eugeniae*, *Perionyx excavatus*, and *Perionyx hawayana* have been identified from the tropics. These species are able to grow in a wide range of different organic wastes (Edwards and Arancon, 2005).

Physical parameters are also important to ensure the fast growth of earthworms. The place should be slightly dark with 80 – 90% moisture in bed. The temperature can range between 20 to 30 °C and the pH between 5 and 9. The ammonia content must be lower than 0.5 mg.g⁻¹ and the salt content as well. In fact, there is a sharp cut-off point between toxic and non-toxic level of ammonia and inorganic salts. Aerobic conditions are also needed (Edwards and Arancon, 2005; Sharma et al., 2005).

The process of vermicomposting can be divided into different steps. First, earthworms feed on organic waste particles (from 100 to 300 mg.g⁻¹ body weight/day). The particle size is reduced under the physical action of the earthworm gut. They only use a small portion of the ingested material (10 – 15%) for their body synthesis and excrete a large part of the consumed waste in a half-digested form. The digestion is made by the microorganisms, enzymes and hormones present in the earthworm gut. It is the reason why the digested material decomposes rapidly and becomes vermicompost (Kiyasudeen et al., 2016a; Sharma et al., 2005).

The benefits of using the vermicomposting technology are that the reaction is non-exothermic and that the burrowing activity of the earthworms allow the aeration and the mixing of

substrate without any human intervention (Kiyasudeen et al., 2016a). It exists different forms of vermicomposting systems. They can be very simple and low cost methods such as windrows, waste heaps or containers, but can also become more complex and completely automated continuous flow reactors (Kiyasudeen et al., 2016a).

The vermicompost tea (VCT) is another form of the product. This is an aqueous extract from vermicompost that is produced by mixing solid vermicompost with water in a similar way than tea infusion during 1 to 7 days. It is assumed that the microbial biomass activity and plant nutrients can be transferred from vermicomposts into the tea. They are different methods used to make tea. The solid/water ratio can vary and some process are aerated and others are not. The microbial activity and diversity are reduced by one-third compared with the solid vermicompost. The aeration during the tea production has also a positive impact on the pH, nitrate, dehydrogenase enzyme activity and microbial biomass. It is probably because dissolved oxygen supports microbial activity (Arancon et al., 2007; Edwards and Arancon, 2005).

There are other derivatives of vermicompost. Vermiwash is a liquid that is collected after the passage of water through a column of worms. It is used as a foliar spray and seems to have properties of fertilizer and biocide. Vermicompost leachate (VCL) comes from the excess water that leaches out of the vermicomposting system. When collected, it can be used as liquid fertilizer because it contains high concentrations of nutrients and humic and fulvic acids (Kiyasudeen et al., 2016b).

1.5.3 The PUR VER® process

In this study the vermicompost tea used is produced by the company PUR VER. This company produces vermicompost since 2014 and is a pioneer in industrial vermiculture in Europe (Meir and Sempo, 2018). They used the top-feed windrow technology. This is a continuous flow operation designed as a layered windrow. The bed is placed first and inoculated with worm. They always stay in the upper part of the bed and consume the fresh food, then drop their casting near the bottom of the windrow. This creates 3 layers in 50 cm substrate: the fresh food at the top, a partially digested layer in the middle and the finished product at the bottom. The process is continuous which means that the consumed substrate must be replaced repeatedly by a thin layer of fresh material (Kiyasudeen et al., 2016a).

The company produces the vermicompost in five production lines as shown on Figure 13. The process is covered in order to control the physical parameters such as temperature and moisture at optimal level. There is a warming/cooling system that passes in the bed to

maintain the temperature around 25°C. The food is also well controlled and prepared in advance by mixing waste from brewery, coffee-industry and chicory producer in well studied proportion. By buying the input wastes, PUR VER insures the quality and the standardization of the product. The production process takes from 2 to 3 month between the fresh food and the end product. The vermicompost produced at the bottom is recovered with the help of automatic broom. Then it is screened before the conditioning. The solid product is free of odours and worms with a dark brown color and a fine particulate structure. Recently the company decided to develop the production of VCT. This is the product that will be analyse and use in this experiment (Meir and Sempo, 2018).

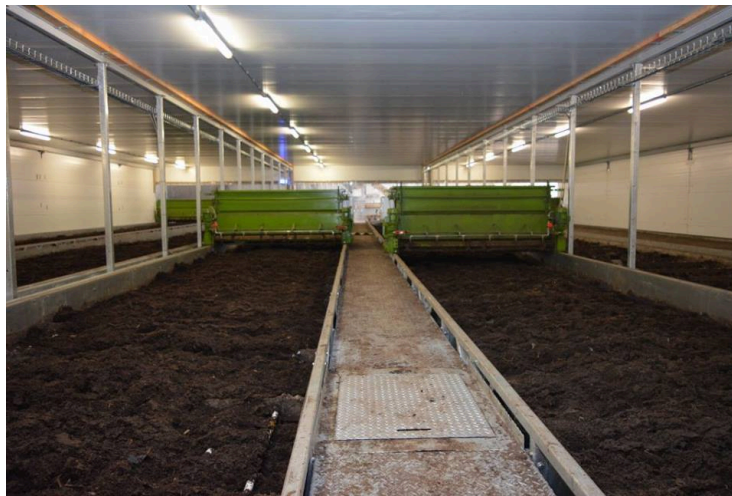


Figure 13 Production lines of vermicompost of the PUR VER company ("Purver.be," n.d.)

1.5.4 The vermicompost and its properties

From a physical point of view, vermicompost is a dark stable and homogenous organic material with a fine particulate structure. It has a high porosity that gives aeration, drainage and a good water-holding capacity. The finely divided structure also gives adsorption and nutrient retention properties (Atiyeh et al., 2000; Edwards and Arancon, 2005; Kiyasudeen et al., 2016a; Sharma et al., 2005).

Vermicompost contains a high and divers microbial and enzymatic activity due to the passage through the earthworm gut (Atiyeh et al., 2000; Edwards and Arancon, 2005; Kiyasudeen et al., 2016a; Sharma et al., 2005).

Biochemical changes in the degradation carried out by microorganisms through the enzymatic digestion process are the most interesting ones. The microbial respiration reduces the carbon content which results in a low C:N ratio. Furthermore, the mineralisation process increases the concentration in N, K, P, Ca and Mg in more bioavailable forms that are retained by the

fine structure of the vermicompost and make it readily available for the plants. The process also reduces the pH and the electrical conductivity. Earthworms and associated biota produce plant growth hormones, humic and fulvic acids that are key compounds in plant growth regulation. Finally, the vermicompost is an odour-free humus-like substance considered as the black diamond for the second green revolution (Albanell et al., 1988; Atiyeh et al., 2000; Edwards et al., 2006; Edwards and Arancon, 2005; Kiyasudeen et al., 2016a; Sharma et al., 2005).

1.5.5 Uses of vermicompost

1.5.5.1 Soil fertility

Addition of vermicompost in soil increases plant growth and productivity. As explained above, vermicompost contains enzymes, hormones and nutrients such as nitrates, exchangeable phosphorus, soluble potassium, calcium, and magnesium in plant available forms. The micro-structure of the vermicompost allows the retention of these nutrients as well as the development of beneficial microbes. The addition of vermicompost also improves the soil structure resulting in improved air-water relationship in the soil, favourably affecting plant growth. Both physico-chemical and biological properties of vermicomposts improve the plant growth. It acts as a soil conditioner and a slow-release fertilizer (Kiyasudeen et al., 2016b).

1.5.5.2 Plant growth

Different experimentations confirm that the addition of vermicompost enhance seed germination, seedling development, growth and productivity as well as flowering and fruiting process (Arancon et al., 2007; Atiyeh et al., 2000; Kiyasudeen et al., 2016a; Sharma et al., 2005). This is especially the case for a range of greenhouse and field horticultural crops including tomatoes, peppers, strawberries, raspberries, grapes, marigolds and petunias (Arancon et al., 2007). The greater bioavailability of mineral nutrients may be an explanation for this beneficial effect on growth and probably helps to overcome the stress. However, investigation have demonstrated that the plant growth is independent of nutrient transformations and availability (Kiyasudeen et al., 2016b, 2016a). Benazzouk et al. (2019) recently demonstrated that VCL is an efficient priming agent for seeds of *Brassica napus* that could even be used for rejuvenation of aged seed lots.

High performance liquid chromatography and gas chromatography mass spectroscopy analyses of VCT showed the presence of significant amounts of indole-acetic-acid (IAA), gibberellins and cytokinins. Indeed, investigations revealed that plant hormones and plant-

growth regulating substances such as auxins, gibberellins, cytokinins, ethylene and abscisic acid are produced by earthworm gut associated microorganisms. They enrich vermicompost with plant growth hormones which are adsorbed into humic acid substances making them stable and allowing them to persist a long time in the soil and be absorbed by the plant (Kiyasudeen et al., 2016b).

Edwards et al. (2006) reported that VCT increased the tomato growth. The tea made from the solid vermicompost could contain soluble mineral nutrients, beneficial microorganisms, humic and fulvic acids, plant growth hormones and plant growth regulators. These compounds present in the solid vermicompost are probably extracted into the tea during the brewing process. Since all of the tomato plants grew in an optimal nutrient condition, microorganisms and plant growth regulators such as hormones, humic acids and fulvic acids are the most probable mechanisms for growth increases of tomatoes (Edwards et al., 2006).

In a recent work, Benazzouk et al. (2019b) applied VCL on tomato grown in nutrient solution and showed that this fertilizer drastically changed the phytohormonal profile of the plant. Although numerous studies reported the presence of phytohormones in vermicompost, this is the first study establishing a link between exogenous and endogenous plant growth regulators. It might be hypothesized that phytohormonal effect could be direct, through the absorption and translocation by the plant of exogenous hormones, or indirect, through an influence of exogenous compounds on synthesis of endogenous hormones. Beside classical phytohormones, specific compounds such as polyamines were reported by these authors to play a key role in plant growth and development and in response to abiotic stresses as well (see below).

The member of the company PUR VER realised some experimentation to assess the beneficial effect of their product on plant growth. An organic vegetable market gardener using the PUR VER vermicompost reported that the zucchini yield increased from 4.5 kg/plant to 6.5 kg/plant. The company collaborates with research centers in order to determine the optimal percentage of vermicompost to mix with soil. They found that 15% seems to be the best one. As shown on the Figure 14 and Figure 15 the product greatly increases the plant development ("Purver.be," n.d.)



Figure 14 Effect of vermicompost PUR VER on the tomatoes growth ("Purver.be," n.d.)

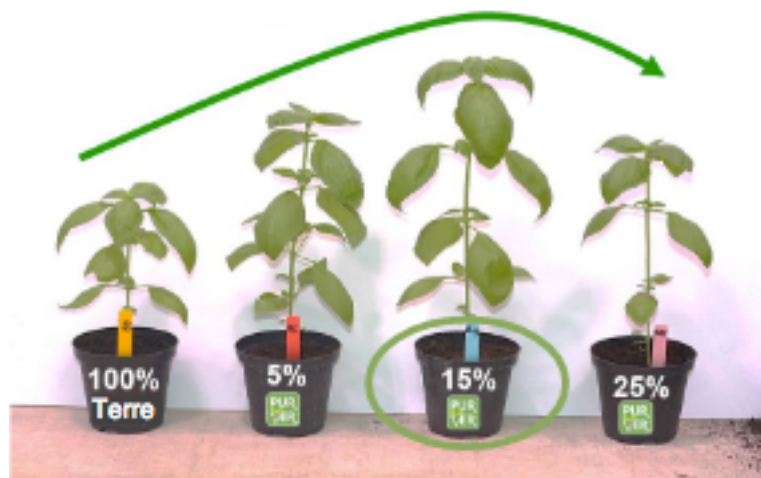


Figure 15 Effect of the vermicompost PUR VER on the basilica plants growth ("Purver.be," n.d.)

1.5.5.3 Biotic stress

Vermicompost has a rich microbial diversity and a wide range of antagonistic bacteria. These can act as bio-control agent and suppress plant diseases (Arancon et al., 2007; Edwards and Arancon, 2005; Sharma et al., 2005).

This property has already been reported in the literature for the traditional thermophilic compost. The suppression of diseases is clearly microbial since suppression properties of vermicomposts are lost after its sterilization. A possible mechanism for disease suppression may be based on competition for nutrients and energy between pathogens and other microorganisms. Antagonistic mechanism may also be an explanation since pathogens and other microbes are in competition for infection sites (Edwards et al., 2006).

Edwards et al. (2006) reported several diseases suppression such as *Pythium* on radishes and *Rhizoctonia* on cucumbers in the greenhouse. In field experimentation he reported the suppression of *Verticillium* wilt on strawberries, *Phomopsis* and powdery mildew on grapes and bacterial rot on cucumbers. He also noted the effect of VCT on the suppression of *Verticillium* wilt affecting tomato plants. This suggests that microorganisms present in the solid vermicompost pass to tea (Edwards et al., 2006).

1.5.5.4 Abiotic stress

Chinsamy et al. (2014) tested the effect of VCL on tomato seedlings under high temperature and water stress conditions. The results showed that the growth performance was better in presence of VCL under temperature higher than the optimal one. The experimentation under water stress showed significant results suggesting that VCL can allow the production of quality tomatoes by using less water resources. Vermicompost leachate seems to be a suitable soil amendment to significantly improves the crop yield under abiotic stress (Chinsamy et al., 2014). Benazzouk et al. (2018) provided experimental evidences that vermicompost helps tomato to cope with high NaCl doses through an increase in proline and stomatal conductance of stressed plants. It also helps the plant to more efficiently regulate Na⁺ absorption and provided a better salinity resistance when applied before stress exposure than concomitantly with stress treatment.

Chand et al. (2012) evaluate the influence of different combinations of nickel (Ni) and lead (Pb) applied with and without vermicompost, on growth and yield, as well as on the concentration and accumulation of Ni, Cr and Pb by *Mentha arvensis*. The results showed that plants stressed by Ni and Pb with vermicompost produced 40 and 52 % higher fresh herbage and root yield, respectively over control where it exhibits negative effect. Concentration and uptake of heavy metals (Pb, Ni, Cr) and micronutrients (Zn, Cu, Mn) were higher when Ni and Pb were applied in combination with vermicompost (Chand et al., 2012).

Pati and Makhopadhyay (2009), found that the extractable As from the soil was lowered by the application of vermicompost and farmyard manure. He also demonstrated the reduction of the As uptake by the rice species *Oryza sativa* L. by the influence of vermicompost and farmyard manure. Hence, the incorporated organics seemed to have some moderating effects in releasing As in the soil-plant system (Pati and Makhopadhyay, 2009).

These different studies suggest that vermicompost can also have a positive impact on the abiotic stress resistance of plants. Furthermore, vermicompost by its high content in nutrients, can help the plants to deal with mineral deficiency.

1.6 The genus *Atriplex*

1.6.1 Taxonomy, geographical distribution and generalities

The genus *Atriplex* belongs to the Amaranthaceae family (formerly called Chenopodiaceae family), which is distributed worldwide. It counts more than 400 species with different morphology, biological cycles and ecological adaptations. They are herbs, small shrubs or subshrubs and usually inhabit arid or semi-arid environments. They are also present in environments with saline and/or alkaline soils. Tolerance to drought, salinity, heavy metals and temperature are important characteristics of these species which often present a C₄ metabolism. It's the reason why it is commonly denominated saltbush (Benzarti et al., 2013; Brignone et al., 2016; Fernández et al., 2016; Lutts et al., 2004; Tapia et al., 2013).

Atriplex sp. are geographically distributed in all continents. They are mainly found in North America, South Australia, South Central Asia, West and South East America, and the Mediterranean basin in habitats that combine relatively high soil salinity and aridity (Benzarti et al., 2013). The genus *Atriplex* counts 55 species in South America with 45 native species and 10 adventive species (Brignone et al., 2016). It grows mainly in Argentina and Chile but some species are also found in Bolivia, Ecuador, Paraguay, Peru, Uruguay and Venezuela (see Figure 16). *Atriplex* is one of the most important families in North Chile (region of Antofagasta). This region exhibits a high concentration of As and salts in soil and water that is mainly associated with the Quaternary volcanic and geothermal activity in the Andes Cordillera. Atacama desert is characterized by its extremely arid climate which simulates development of this genus (Brignone et al., 2016; Fernández et al., 2016; Tapia et al., 2013).

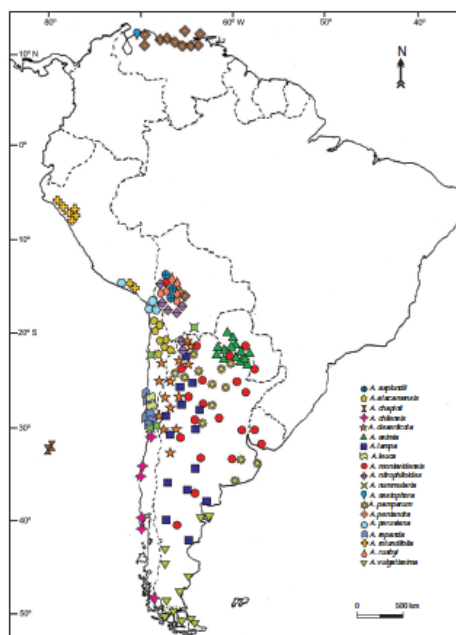


Figure 16 Geographical distribution of 19 *Atriplex* species in South America (yellow points represent *A. atacamensis*) (Brignone et al. 2016)

Soil salinization becomes a widespread issue mainly in arid and semi-arid regions of the world. It causes increasing threat to agricultural expansion and productivity. *Atriplex sp.* contain proteins among other economically valuable compounds. Certain species of the *Atriplex* genus also have a high biomass productivity. Therefore, some *Atriplex sp.* can be suitable to produce food for livestock in regions with this particular climate where green forage production is the main limitation of animal production, especially at the end of the growing season (Benzarti et al., 2013; Fernández et al., 2016).

Furthermore, *Atriplex* genus has the ability to uptake salts from saline soils and sequester it into the salt glands at the leaf surface. This characteristic allows them to be used for regeneration and revegetation of saline arid and semi-arid area. The ability to accumulate soluble salts in leaves is associated with the drought tolerance mechanism of the genus *Atriplex* (Martinez et al., 2005; Ben Hassine et al., 2008). It is the reason why these species are considered as a high potential candidate for reducing soil salinity in drylands, if harvested and collected (Benzarti et al., 2013). Studies show that the xerohalophyte species of the genus *Atriplex* display high capacity of osmotic adjustment to cope with water shortage, and also activate numerous protective mechanisms against desiccation, Na^+ accumulation and ROS (Ben Hassine and Lutts, 2010; Martínez et al., 2003; Martínez et al., 2004). This is, at least partly, related to the capacity of these species to synthesize efficient osmocompatible solutes, especially proline and glycinebetaine (Ben Hassine et al., 2008; Martínez et al., 2005). Specific hormones such as abscisic acid and ethylene may be involved in a contrasting way in the response to salinity and water stress (Ben Hassine and Lutts, 2010). Abscisic acid may also influence salt excretion in the trichomes through the stimulation of polyamine synthesis which will directly stimulate the expression process (Ben Hassine et al., 2009).

Finally, soil contamination by heavy metals is also a serious environmental problem and has significant impact on human health. The clean-up of contaminated soils remains a challenge. Halophytes plants like some of *Atriplex sp.* exhibit a high level of heavy metal accumulation (Glenn et al., 2001; Salo et al., 1996). Because of their high biomass production associated with a deep root system, these plants have been suggested as promising candidates for further management of heavy-metal-polluted areas (Benzarti et al., 2013; Lutts and Lefèvre, 2015). Lefèvre et al. (2009) provided experimental evidences that salinity reduces Cd absorption by the roots of *Atriplex halimus* but also the proportion of Cd excreted in the trichomes: a more efficient Cd tolerance within tissues may be the consequence of a protective mechanism resulting from an increase in proline and endogenous free polyamines. Cd and Zn resistance in *A. halimus* are not only due to trichomes accumulation and Lefèvre et al. (2010) demonstrated that undifferentiated cell lines maintained in vitro and selected to water deficit induced by polyethylene glycol are able to tolerate high doses of heavy metals.

1.6.2 *Atriplex atacamensis* PHIL.

1.6.2.1 Geographical distribution

Atriplex atacamensis Phil., also called Cachiyuyo, is a native species of northern Chile (Desert of Atacama). It extends from the latitude 19°S up to 23°S. It grows from the sea level up to an altitude of 3000 meters (Rosas, 1989; Tapia et al., 2013).

As some other plants from the genus *Atriplex*, it is a xerohalophyte plant species. It means that it can grow under extremely harsh conditions characterized by high salt levels and poor water availability. Furthermore, *Atriplex atacemensis* grows mainly along Rio Loa river and in localized arsenic-contaminated area along it (Figure 17). These zones carries heavy metals from the surrounding open-cast mining sites (Vromman et al., 2011).



Figure 17 Rio Loa river near the Quillagua oasis, Atacama Desert, Northern Chile (2019)

1.6.2.2 Morphological description

Vegetative system

Atriplex atacamensis (Figure 18) is a persistent shrub strongly woody and branched with internodes of 3 to 20 mm. It erects up to 3 meters tall. The leaves (Figure 19) are 10 to 25 mm in length and 8 to 15 mm in width and alternate with small axillary leaves. They have a short petiole. Leaves are oval and oblong, the end base is obtuse or truncated, the margin of the limb is wavy and sometimes whole. The leaves have a thick cuticle covered with trichomes harboring salt crystals. It gives the white or grey colour on the leaves. These contribute to the salt resistance and also reduce transpiration and temperature at the leaf surface by reflecting much of the incident light. Finally, the plant has a deep and well-developed root system that is able to collect large amounts of groundwater (Rosas, 1989).



Figure 18 *Atriplex Atacamensis* PHIL. along the Rio Loa river (Quillagua Oasis) in the Atacama desert, Northern Chile (2019).



Figure 19 *Atriplex atacamensis* leaves

Reproductive system

Like many *Atriplex* sp., *Atriplex atacamensis* is monoecious but has an important degree of dioecia. The female inflorescence (Figure 20.1) is a glomeruli and counts 2 to 10 flowers. The male inflorescence (Figure 20.2) is a glomeruli of 4 to 6 mm composed of many flowers. The female flowers have an oval and welded beige bracts. The seeds are 1.3 to 1.5 mm in diameter and have a brown integument and are protected by the bracts female flowers'. The spheroidal pollen has a diameter of 21 to 25 μm (Rosas, 1989).



Figure 20 Female (1) and male (2) inflorescences of *Atriplex atacamensis*

1.6.2.3 Adaptation to environmental abiotic stresses

Atriplex atacamensis is a halophyte plant species and it can deal with high salt concentration in soil. Its area of origin (Atacama desert) is probably one of the most deleterious for plant development. It is a salt desert with extreme high soil electrical conductivity. Mean temperature is very high, and relative humidity and soil water content are very low. Moreover, the soil often contains toxic elements released by surrounding mining areas, including As.

Atriplex atacamensis is developed in arid regions where it rains less than 50 mm per years. Therefore, it is obvious that the plant can naturally resist to very low water supply probably thanks to the osmotic adjustment using proline and/or glycinebetaine. Besides this physiological mechanism, the C₄ photosynthesis of the plant probably helps it to tolerate the extremely dry climate. C₄ photosynthetic pathway makes the plant particularly successful in areas with high light, low water, and low nutrients. C₄ plants turn CO₂ into carbohydrate as available light increases on the contrary of C₃ plant that stop at a light level well below that of full sunlight. In addition the CO₂ compensation point is remarkably low (Kellogg, 2013; Rosas, 1989).

Atriplex atacamensis is an opportunity for forage production as well as vegetation cover to protect and supply resources to wildlife and microflora in the hyper-arid region of the Atacama Desert in the Northern Chile. Furthermore, the studied area, along the Rio Loa river (oasis of Quillagua), is highly contaminated by As because of natural and anthropogenic sources. In fact, the intensive mining activity generates a pollution of the surrounding environment. In water, the As concentration reaches 2.5 mg.L⁻¹ and it is highly saline. A high concentration of B was also recorded and associated to near-neutral pH values. The coarse and sandy soil contains about 220 mg.kg⁻¹ of arsenic (Vromman et al., 2016b).

Previous studies elucidated different aspect of the *Atriplex Atacamensis* physiological behaviour in presence of heavy metals. In 2011, Vromman et al. showed that *Atriplex atacamensis* can cope with high external concentration of As and accumulates it mainly in its roots. She also showed that an increase of the translocation rate from roots to shoots induces an increase of plant mortality (Vromman et al., 2011).

According to Vromman et al (2016a). the addition of NaCl in the As-contaminated nutrient solution reduced the concentration in roots and increase the arsenic translocation from the root to the shoot. She concluded that *Atriplex Atacamensis* is an interesting candidate for the phytomanagement of As-contaminated soils and that soil salinity improve the resistance of plant against this pollutant (Vromman et al., 2016a). This author also performed speciation analysis and demonstrated that 55% of total extractable As was in the redox state As(III) and

45% as As(v). Moreover, more than half of the total As accumulated in the cell walls and additional NaCl did not modify As distribution between apoplasm and symplasm. This study also confirmed that As and salinity induce synthesis of proline and glycinebetaine and also trigonelline. Non-proteins thiols are involved in the plant tolerance to accumulated As, especially at the root level.

A field analysis was performed and showed that mature plants of *Atriplex atacamensis* were able to grow on the contaminated studied area and accumulated moderate amounts of As in the shoots. The plant accumulates more As in the leaves than in the stems but also accumulates As in the seeds. The halophyte character of the plant was confirmed because of high Na accumulation in the shoots. Vromman et al. (2016b) also noticed a low amount of phosphate (P) in the plant and suggested a competition between As and P for the P transporters. The funded high amount of sulphur may be relayed to the high synthesis of PCs and GSH which are protection compounds (Vromman et al., 2016b).

Finally, she confirmed the link with phosphate supply. A phosphorus starvation increased the As concentrations in roots, but decreased it in shoots. This suggests that the P deficiency reduces the As translocation from roots to shoots. P also influence the production of protective compounds and the lipid peroxidation process (Vromman et al., 2017).

Vromman et al. (2017) also highlighted that:

- Arsenate addition increased total GSH content
- Arsenate also induced an increase in S accumulation and non-protein thiol

These data strongly highlight the ability of *Atriplex Atacamensis* to deal with high concentration of As. Tapia et al. (2013) confirmed that *A. atacamensis* resist As pollution and accumulates the metalloid mainly in the roots. He recommends the plant to generate a plant cover in the Atacama Desert region in order to prevent the dispersion of this metalloid via wind and leaching. This means that *Atriplex Atacamenis* can be considered as a candidate for phytoremediation purposes.

Beside the presence of As, an excess of boron and a low fertility of the soil in this area greatly hamper plant growth and development. The interaction between As and these parameters were however never studied until now.

1.7 Research objectives

Previous studies have demonstrated the potential of the xero-halophyte chilian plant species *Atriplex atacamensis* for phytomanagement of As-contaminated soils, specifically in Northern Chile where this specific pollution is mainly due to the nature of the parent rocks. In addition, the soil of this environment also shows a B enrichment for similar geological reason and therefore soils simultaneously present high doses of As and B. Moreover, soils in this area frequently exhibit a low fertility and a poor content in organic matter. Until now, few studies address the possible beneficial effect of vermicompost tea in heavy metals stress management.

The global aims of the thesis were: i) to determine the influence of B excess on As absorption by the plant and ii) to assess the effect of vermicompost tea used as a natural amendment on plant responses to these environmental constraints.

The first part focuses on the biochemical analysis of the vermicompost tea in order to understand why it may have a significant effect on the plant physiology as reported several times in the literature. It is also a way to assess the tea production process efficiency of the company PUR VER®.

The second part consists in the study of the impact of boron excess on the As absorption by the plant *Atriplex atacamensis*. In this section, we observe if the presence of B influences the behaviour of the plant regarding As management. At the same time, we assess the effect of vermicompost tea on the As, B or combined stress management of the plant *A. atacamensis* using morphophysiological parameters.

Chapter 2: Materials and methods

2.1 Vermicompost tea analysis

Vermicompost tea was kindly provided by PUR VER®. Liquid fraction was produced through the incubation of solid vermicompost in water. The received product was kept at room temperature until analysis.

2.1.1 General parameters

The pH and electrical conductivity (EC) of the VCT were measured by a pH meter Mettler Toledo (FE20/EL20) and a conductimeter (WTW LF92 + probe Tetracon 96), respectively. The density was determined by weighing 1 ml of VCT. The dry matter residue (expressed in %) was estimated after incubation in an oven at 70°C during 48h.

2.1.2 Mineral analysis

The following elements were quantified in the VCT: Na, Ca, K, Fe, Zn, Mg, Cu, P, As, B. Samples (1ml) of VCT were digested in 4 ml of HNO₃ (35%) and evaporated to dryness on a sand bath at 80°C. The resulting minerals were incubated with a mix of 37% HCl and HNO₃ (3:1, v/v). It evaporated slightly during few minutes until total dissolution of the residues in 10 ml HCl 0.1N. The samples were then filtrated on a Whatman N°2 filter paper. Elements were quantified by inductively coupled plasma-optical emission spectroscopy (type MPX; Varian). Blank controls were prepared following the same procedure but without VCT. The concentrations of ions (C) were calculated with the equation:

$$C = \frac{Cp * df * 0,01}{V} [\text{mg. L}^{-1} \text{VCT}]$$

Where Cp is the concentration measured, df the dilution factor and V the initial volume of VCT analysed (0.001 L).

Chapter 2: Materials and methods

2.1.3 Nitrogen content : nitrate, ammonium and total nitrogen

2.1.3.1 Total nitrate

Total nitrate was quantified colorimetrically according to Cataldo et al. (1975). A mix of salicylic acid and sulfuric acid (1:5, v/v) was added to 0.2 ml of VCT and samples incubated during 20 min at room temperature. Then 19 ml of NaOH (1M) were added. The absorbance was read at 410 nm on a Beckman spectrophotometer. Nitrate concentration was calculated from a standard curve established with NaNO_3 . Blank controls were prepared following the same procedure but without VCT. All analyses were performed in triplicates.

2.1.3.2 Ammonium

Ammonium quantification required two reactive solutions according to Weatherburn (1967). First the solution A was a mix of 2.5 g of NaOH and 26.85 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in 200 ml deionized water. Then 5 ml of NaClO were added and the solution was completed to 500 ml. The solution B consists in a mix of 10 g of phenol and 50 mg of sodium nitroprusside in 500 ml of water. All chemicals were purchased from Sigma Aldrich (Belgium). Three samples were prepared with 1 ml of VCT and 2.5 ml of both solutions A and B. Then, the samples incubated for 30 min at 30°C. The absorbance was read at 625nm. A standard curve was established with NH_4Cl . Blank controls were prepared following the same procedure but without VCT.

2.1.3.3 Total nitrogen

Total nitrogen quantification was performed with the kit LCK 138 ($1\text{-}16 \text{ mg.L}^{-1} \text{ TN}$) (Hach Lange). The principle is that inorganically and organically bonded nitrogen is oxidized to nitrate by digestion with peroxo-disulphate. Then, the nitrate ions react with 2,6-dimethylphenol in a solution of sulphuric and phosphoric acid to form a nitrophenol. The mineralisation was performed with LT 200 (Hach Lange) and the measurement with the spectrophotometer: DR3900 (Hach Lange).

2.1.4 Proline content (Bates et al., 1973).

Samples (1 ml of VCT) were mixed with 10 ml of sulfosalicylic acid 3% and incubated at 70°C for 30 min. Then 2 ml of ninhydrine solution and 2 ml of acetic acid were added to 2 ml of the supernatant. The ninhydrine solution was prepared with 1.25g of ninhydrine, 30 ml of acetic acid and 20 ml of H_3PO_4 (6M). The samples were incubated again at 90°C for 1h. The reaction was stopped by rapid cooling on ice. Two ml of toluene were added and the samples were vortexed during 10 sec. The colored upper part was collected and the absorbance was read at

520nm in quartz cuvettes. The standard curve was prepared from a concentrate solution of proline and sulfosalicylic acid 3%.

2.1.5 Free polyamines quantification

Free polyamines were quantified according to Quinet et al. (2014). This method consists in an extraction of polyamines with HCl followed by a dansylation. Three repetitions of samples with 1 ml of VCT were mixed with HCl. The dansylation was performed with 1,7-diaminoheptane, saturated sodium carbonate and dansyl chloride in acetone (20mg/ml). After incubation, the excess dansyl chloride was removed with a solution of proline and acetone was evaporated under nitrogen flux. The PAs were then extracted with ethyl acetate with a sufficient incubation time in order to obtain a good separation between organic and aqueous phases. The upper organic phase was collected and dried under nitrogen. Dried extract was dissolved in methanol and filtered through 0.45 μm microfilters. Sample were injected on a Nucleodur C18 Pyramid column. Analyses through high performance liquid chromatography coupled with a fluorescence detector (HPLC-FLD) were performed on a Shimadzu HPLC system.

2.1.6 Antioxidants quantification

2.1.6.1 Total phenolics

Total phenolics were quantified according to Singleton and Rossi (1965). Three repetitions of samples with 1 ml of VCT were mixed with 2 ml of methanol 80% (v:v) and centrifuged at 10,000 g during 10 min at 4°C. Then, 1.58 ml of water and 100 μl of Folin-Ciocalteu reagent were added to 250 μl of the methanolic extract. The samples were vortexed and left to stand for 6 minutes. Then 300 μl of sodium carbonate 2% (v:v) were added. After 2h the absorbance was read at 765 nm. The standard curve was prepared from a methanolic solution of gallic acid (200 $\mu\text{g}/\text{ml}$) and the blank contained only methanol 80% (v:v). Both followed the same procedure as the samples.

2.1.6.2 Flavonoids

Flavonoids were estimated according to Dewanto et al. (2002). Three repetitions of samples with 1 ml of VCT were mixed with 2 ml of methanol 80% (v:v) and centrifuged at 10,000 g during 10 minutes at 4°C. At 250 μl of the methanolic extract, 1.25 ml of water and 75 μl of sodium nitrite 5% (v:v) were added. The samples were vortexed and left to stand for 6 min. Then 150 μl of aluminium chloride 10% (v:v) were added. The samples were vortexed and left

to stand for 5 min again. After incubation, 500 μl of NaOH (1M) were added and the samples was completed with water to a final volume of 2.5 ml. The absorbance was read at 510 nm. The standard curve was prepared from a methanolic solution of catechine (200 $\mu\text{g}.\text{ml}^{-1}$) and the blank contained only methanol 80% (v:v).

2.1.6.3 Anthocyanin

The method of Giusti and Wrolstad (2001) was used for anthocyanin estimation. Three repetitions of samples with 1 ml of VCT were mixed with 1.5 ml of a solution of methanol:water:acetic acid (80:19.5:0.5) and vortexed vigorously. Samples were left to stand 30 min in ice and then centrifuged at 10,000 g during 15 min at 4°C. Then, the samples were divided in two batches. A first batch was composed of 200 μl of each sample to which were added 1.8 ml of a solution at pH 1 prepared with HCl and KCl (0,1M). The second batch also contained 200 μl of each one to which were added 1.8 ml of a solution at pH 4.5 prepared with acetic acid and sodium acetate (0. 2M). Then for each batch the absorbance was read at both 510 and 700 nm (a_{510} and a_{700}). The blank are prepared with the methanol:water:acetic acid (80:19,5:0,5) solution and solution at pH 1 or pH 4.5.

The anthocyanins quantification is calculated with the equation:

$$A = (a_{510} - a_{700})_{pH1} - (a_{510} - a_{700})_{pH4.5}$$

With A the total absorbance and a the absorbances measured with the spectrophotometer. The anthocyanins concentration (C) is then calculated as:

$$C = \frac{A * Mw * df * 1000}{\epsilon * 1} [\text{mg}.\text{L}^{-1} \text{ VCT}]$$

where Mw is the molecular weight of anthocyanins (449.2 $\text{g}.\text{mol}^{-1}$ for cyanidine). Df is the dilution factor (equal to 10 in this case) and ϵ the molar extinction coefficient (26900 $\text{l}.\text{mol}^{-1}.\text{cm}^{-1}$).

2.1.6.4 Glutathione

Glutathione quantification was performed according to Cereser et al. (2001) and is divided in two steps: the quantification of the reduced glutathione (GSH) and the quantification of the total glutathione (GSht). For the GSH, one sample with 1 ml of VCT was mixed with 4 ml of methaphosphoric acid 6% (MPA) and vortexed. From this solution 1 ml is taken and centrifuged at 10,000 g during 7 min at 4°C. For the GSht, one sample with 1 ml of VCT was mixed with 1 ml of dithiothreitol 25 mM (DDT) and 500 μl of Tris Buffer (0.1M; pH 8.5) and vortexed. The DTT was used to reduce all the glutathione present. The sample was left to

stand in ice for 30 min. Then 8 ml of MPA 4% were added and the sample was vortexed. From this solution 1 ml was taken and centrifuged at 10,000 *g* during 7 min at 4°C. For both GSH and GSht 100 µl of the samples were taken and mixed with 100 µl of O-phthalaldehyde (OPA 5%) solution and left to stand for 5 min. The solution was prepared with 50 mg of OPA, 0.5 ml of methanol and 9,5 ml of sodium tetraborax 0.1M, pH 9.9. Then 800 µl of sodium phosphate (500mM; pH 7) were added to the samples.

Extracts were filtered through 0.45 µm microfilters (Chromafil PES-45/15, Macherey-Nagel) prior to injection and OPA derivatives were separated on a reversed-phase HPLC column with an acetonitrile-sodium acetate gradient system and detected fluorimetrically. Five µL of sample were injected into a Shimadzu HPLC system (Shimadzu, 's-Hertogenbosch, The Netherlands) equipped with a Nucleodur C18 Pyramid column (125 x 4.6 mm internal diameter; 5 µm particle size) (Macherey-Nagel, Düren, Germany). Derivatives were eluted in acetonitril gradient in a 50 mM sodium acetate buffer pH 6.2 at 30 °C at a flow rate of 0.7 ml min⁻¹. Fluorimetric detection was performed with a spectra system Shimadzu RF-20A fluorescence detector at 420 nm after excitation at 340 nm. GSH was quantified using nine-point calibration curves with custom-made external standard solutions ranging from 0.0625 to 50 µM and every ten injections, a check standard solution was used to confirm the calibration of the system. The recovery was determined using GSH as an internal standard.

The concentration in the oxidized form of glutathione (GSSG) was calculated by the subtraction of the concentration of GSH to GSht and divided by 2.

$$GSSG = \frac{GSht - GSH}{2} [\mu\text{moles. L}^{-1} \text{ VCT}]$$

2.1.6.5 Ascorbate

Ascorbate was quantified according to Okamura (1980). Ascorbate is present in two forms: the reduced one (AsA) and the oxidized one (DAsA). The AsA can reduce the Fe³⁺ to Fe²⁺ by complexing with dipyridil which gives the red colour to the solution and can be measured with the spectrophotometer. To quantify both oxidized and reduced form, the reduced as well as the total content of ascorbate was measured. In order to measure the total AsA, the DAsA must be reduced with dithiolthreitol (DTT). The reaction is stopped with *N*-ethylmaleimide (NEM). The measurements of total AsA and AsA allow to calculate the DAsA content.

For both measurements, three samples of 1 ml of VCT were mixed with 4 ml trichloroacetic acid (TCA) 5% (v:v) and left to stand on ice for 15 min. Then, the samples were centrifuged at 10,000 *g* during 5 min at 4°C. For the AsA quantification, 0.2 ml of the three supernatants were mixed with 0.4 ml of phosphate buffer (0.2M; pH 7.4) and 0,4 ml of water. Then they were

mixed with 1 ml of TCA 10% (v:v), 0.8 ml of H_3PO_4 42,5% (v:v), 0.8 ml of dipyridyl 2% (v:v) and finally 0.4 ml of FeCl_3 3% followed by vigorous vortexing.

For the total AsA quantification, 0.2 ml of the three supernatants were mixed with 0.4 ml of phosphate buffer (0.2M; pH 7.4) and 0.2 ml of DTT (10 mM). The samples were left to stand during 5 min then 0.2 ml of NEM 0,5% (v:v) were added and the samples were left to stand for 1 min. Then they were mixed with 1 ml of TCA 10% (v:v), 0.8 ml of H_3PO_4 42;5% (v:v), 0.8 ml of dipyridyl 2% (v:v) and finally 0.4 ml of FeCl_3 3% followed by vigorous vortexing.

All the tubes were incubated 1h at 37°C. The standard curve was prepared from a solution of AsA (1M) and TCA 5% and followed the same procedure as the samples. The absorbance of both batches as well as the standard curve were read at 525 nm

2.1.7 Total protein quantification (Bradford, 1976)

Three repetitions of samples with 1 ml of VCT were mixed with phosphate buffer (100mM; pH 7,4) and centrifuged at 12,000 *g* during 15 min at 4°C. Then 25 μl of the supernatant were placed in a cuvette and 1.25 ml of Biorad reagent (diluted 5 times) were added. The absorbance was read at 595 nm. The standard curve is prepared from a solution of albumin (500mg/l) mixed with phosphate buffer and followed the same procedure as the samples.

2.1.8 Soluble sugars quantification (Yemm and Willis, 1954)

Three repetitions of samples with 1 ml of VCT were mixed with 2 ml of ethanol 70% (v:v) and left to stand on ice for 5 minutes. The samples were centrifuged at 8,000 *g* during 10 min at 4°C. The pellets were re-dissolved again in 1ml ethanol 70% (v:v) and processed in the same way one more time. The supernatants were pooled and the volume was adjusted to 3.5 ml with ethanol 70% (v:v). Then 200 μl of each samples were mixed with 1ml of anthrone and incubated at 100 °C for 10 min. The reaction was stopped by putting the samples in the ice. Finally, the samples were vortexed and the absorbance was read at 625 nm. The standard curve was prepared from a solution of glucose (500mg.L⁻¹) mixed with ethanol 70% (v:v) and followed the same procedure as the samples. The blank consisted in ethanol 70% (v:v) and anthrone.

2.1.9 Phytohormones analysis

Abscisic acid (ABA), auxins, salicylic acid (SA), jasmonates (JA), brassinosteroids (BR), benzoic acid (BzA), cytokinins (CK) and their metabolites were extracted and quantified from VCT according to Dobrev and Kamínek (2002) and Dobrev and Vankova (2012): 100 mg fresh

sample were homogenized with 0.5 ml extraction mixture (methanol: formic acid: water, 15:1:4, by volume) and placed to cold (-20°C). The following internal standards (10 pmol per sample) were added: $^{13}\text{C}_6$ indole-3-acetic acid (Cambridge Isotope Laboratories, Tewksbury, MA), $^2\text{H}_4$ -SA (Sigma-Aldrich), $^2\text{H}_3$ -phaseic acid (PA), $^2\text{H}_6$ -ABA (both NRC-PBI, Saskatoon, Canada), $^2\text{H}_5$ -jasmonic acid (JA; C-D-N Isotopes Inc., Pointe-Claire, Canada), $^2\text{H}_5$ -*trans*Z, $^2\text{H}_5$ -*trans*ZR, $^2\text{H}_5$ -*trans*Z7G, $^2\text{H}_5$ -*trans*Z9G, $^2\text{H}_5$ -*trans*ZOG, $^2\text{H}_5$ -*trans*ZROG, $^2\text{H}_5$ -*trans*ZRMP, $^2\text{H}_3$ -DHZ, $^2\text{H}_3$ -DHZR, $^2\text{H}_3$ -DHZ9G, $^2\text{H}_6$ -iP, $^2\text{H}_6$ -iPR, $^2\text{H}_6$ -iP7G, $^2\text{H}_6$ -iP9G, $^2\text{H}_6$ -iPRM (all CK standards OlchemIm, Olomouc, Czechia; the system of CK abbreviations adopted and modified according to Kamínek et al. (2000), $^2\text{H}_3$ -castasterone, $^2\text{H}_3$ -epibrassinolide, $^2\text{H}_2$ -GA1, $^2\text{H}_2$ -GA4, $^2\text{H}_2$ -GA7, $^2\text{H}_2$ -GA8, $^2\text{H}_2$ -GA19 and $^2\text{H}_2$ -GA20 (all GAs standards OlchemIm, Olomouc, Czechia). After 1h extraction at -20°C the pellet was separated by centrifugation (15,000 g, 15 min) and re-extracted with 0.5 ml of the same extraction buffer at -20°C for 30 min. Both mixed supernatants were purified using the dual-mode solid-phase method as described by Dobrev and Kamínek (2002). Two phytohormone fractions were obtained: fraction A, with acidic and neutral compounds (auxins, BRs, ABA, SA, JA), and fraction B, with basic compounds (CK). Hormonal quantification was performed by HPLC (Ultimate 3000, Dionex) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP; Applied Biosystems) as described previously by Djilianov et al. (2013) using isotope dilution method with multilevel calibration curves ($r^2 > 0.99$). Data processing was carried out with Analyst 1.5 software (Applied Biosystems).

2.1.10 Statistical treatment of data

The data were analysed by calculating the means and the standard error by the help of Microsoft Excel (2016). This allows to have a good overview of the average composition of the VCT.

Chapter 2: Materials and methods

2.2 Analysis of the impact of boron excess and vermicompost on arsenic absorption by *Atriplex atacamensis*

2.2.1 Plant material and growing conditions

The used seeds of *A. atacamensis* belong to the research group of vegetal physiology (GRPV) of the Catholic University of Louvain and were collected at Quillagua in northern Chile (UTM 19K: 442015 E, 7609153 N). The seeds were germinated in a greenhouse on loam and sand (1:1) substrate. They were regularly moistened and covered by a glass plate in order to promote germination (see Figure 22). The plants grew up until they reached approximately 5 cm. They were then transferred to individual pots (4x4cm) containing loam and sand (1:1). After 2 weeks they were fixed in plugged holes on polystyrene plates that were floating in containers of 1.2 L of Yoshida solution (hydroponic culture see Figure 22). The composition of the solution is detailed in the Table 1. The pH was set at 5.7.

Table 1 Composition of the Yoshida solution used for the hydroponic culture (Yoshida et al., 1976).

MACRO			
Source	Quantité(g /5L)	Quantité finale(mg/L)	Concentration finale
NH ₄ NO ₃	457	114.25	1.43mM
NaH ₂ PO ₄ .2H ₂ O	201.5	50.37	323μM
K ₂ SO ₄	357	89.25	512μM
CaCl ₂ .2H ₂ O	443	110.75	750μM
MgSO ₄ .7H ₂ O	1620	405	1.64mM
MICRO			
MnSO ₄ .H ₂ O	7.73	1.93	11.4μM
Na ₂ MoO ₄ .2H ₂ O	0.13	0.033	0.14μM
H ₃ B ₃	14.3	3.58	57.8μM
ZnSO ₄ .7H ₂ O	1.10	0.275	0.96μM
CuSO ₄ .5H ₂ O	0.40	0.100	0.4μM
FER			
Fer-EDTA	4.7	15.7	42.7μM

After three weeks of adaptation, the Yoshida solution have been renewed and 100 ml of PUR VER® VCT were added in the half of the containers. One week after the addition of VCT, the treatments of As and B were applied. The experimental schedule is illustrated on Figure 21.

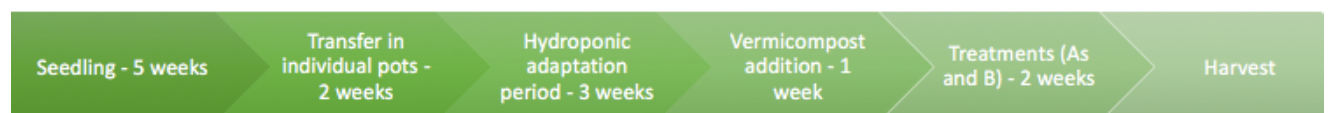


Figure 21 Experimental schedule.



Figure 22 Young seedling and hydroponic culture of *A. atacamensis*

The concentration of arsenic (HAsNa_2O_4) in the container reached $500 \mu\text{mol.L}^{-1}$ and the concentration of boron ($\text{Na}_2\text{B}_4\text{O}_7$) reached 500 mg.L^{-1} . The different treatments applied for a duration of two weeks were: i) the control, ii) As treatment iii) B treatment and iv) a combination of As and B. They were applied simultaneously to plants that received VCT and to those which did not receive it. The experimental design is illustrated on the Figure 23. Each treatment counted 8 containers of 6 plants (48 plants/treatment). During all the hydroponic culture, the level of solution was regularly checked and completed with deionized water.

Water and Vermicompost							
Control		As		B		As + B	
...	...	...	...	...	...	...	...
...	...	...	...	...	...	...	...
...	...	...	...	...	...	...	...
...	...	...	...	...	...	...	...

Water							
Control		As		B		As + B	
...	...	...	...	...	...	...	...
...	...	...	...	...	...	...	...
...	...	...	...	...	...	...	...
...	...	...	...	...	...	...	...

Figure 23 Experimental design.

All the experimentation took place in the greenhouse of the Catholic University of Louvain. The photoperiod was 16h with natural lighting, which was supplemented with red/blue LED of 325 watts that provided a light intensity around $100 \mu\text{moles/m}^2\text{s}$. The temperature was maintained around $22^\circ\text{C} \pm 2^\circ\text{C}$ and the relative humidity was around 60% (semi-controlled conditions).

The rate of mortality was estimated at the end of the treatment, plant with all leaves fully necrotic was considered as dead. The dead plants were separately harvested and pooled for each treatment, leaves and stems were then separately weighed and incubated in an oven at

70°C for 72h but no further analysis were performed on it. For the living plants, 4 plants per treatment were randomly chosen: leaves were counted, weighed, and placed in the oven to dry at 70°C 72h. The stems were also harvested and treated in the same way. The remaining living plants were harvested and pooled by treatment: leaves were quickly frozen in liquid nitrogen and stored at -80°C until analysis. The roots of individual plants growing in a same container were impossible to separate. The total pool of roots was then collected as a whole for each container (roots of 6 plants together). Root systems was quickly rinsed in deionized water, weighed and cut in small pieces. For each treatment, four samples of c.a. 1g FW were incubated in an oven as stated before. The remaining root sample were quickly frozen in liquid nitrogen and stored at -80°C. Figure 24 summarizes the harvest procedure. Further analysis were only performed on leaves and roots.

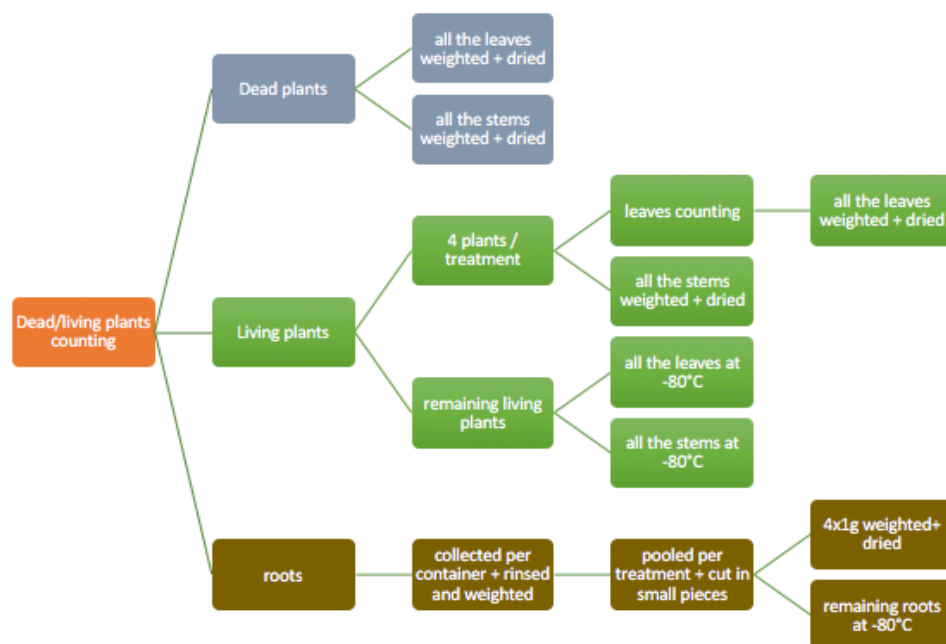


Figure 24 Harvesting plan

2.2.2 Fresh and dry weight, water content

As mentioned above, some plants were weighted during the harvest in order to determine the fresh weight (FW). Then they were dried at 70°C for 72h. Then the plants were weighed again in order to measure the dry weight (DW). This allows to calculate the water content (WC) with the equation:

$$WC = \frac{FW - DW}{FW} * 100 [\%]$$

2.2.3 Mineral analysis (AS, B, P, S)

Mineral analysis was performed in triplicates on 50 to 100 mg DW of plant tissues. Procedure for mineralisation and analytical estimation of ion was similar to the procedure used for the VCT (see before).

The minerals content per kilogram of dry matter (C) was calculated with the equation:

$$C = \frac{Cp * df * 0.01}{M} \text{ [mg. kg}^{-1} \text{ DM]}$$

Where Cp is the concentration measured in ppm, df the dilution factor and M the weight of plant dry material used.

2.2.4 Osmoprotectants quantification

2.2.4.1 Proline (Bates et al., 1973)

Three repetitions of samples with 100 to 200 mg of leaf/root powder preliminary frozen in liquid nitrogen were ground in cold mortar and mixed with 10 ml of sulfosalicylic acid 3%. Analytical procedure was then similar to procedure used for VCT analysis (see before).

The concentration (C) of proline per gram of fresh matter is calculated with the equation:

$$C = \frac{Cp * 0.01}{M} \text{ [}\mu\text{mol. g}^{-1}\text{FM]}$$

Where Cp is the concentration derived from the absorbance and the standard curve and M the weight of plant fresh matter used in gram.

2.2.4.2 Glycinebetaine (Grieve and Grattan, 1983)

The quantification of glycinebetaine was performed in two batches: the first one was used to quantify the total amount of quaternary ammonium and the second one for the choline quantification.

For both batches, three repetitions of samples with 1 g of leaf/root powder preliminary frozen in liquid nitrogen were mixed with 10 ml of water and agitated at 25°C for 24h at 100 rpm. The samples were filtered through Whatman filter paper. Then for the first batch, 250 µl were mixed with 250 µl of H₂SO₄ (1M) and left to stand in the ice. For the second batch, 250 µl of K₃PO₄ (0.2M, pH 6.8) were added to 250 µl of the samples and left to stand in the ice too.

Chapter 2: Materials and methods

After 1h 200 μl of KI-I_2 were added and the samples were vortexed. They were incubated in the fridge (0 -4 °C) for 16h and then centrifuged at 10,000 g for 15 min at 0°C. The supernatant was removed from each sample and was dried with pressurized air. Then 1.5 ml of 1,2-dichloroetane were added, the samples were vortexed and left to stand at 4°C in the dark for 1h. Then the supernatant was collected and the step was repeated 3 times until 4,5 ml of 1,2-dichloroetane were collected. The absorbance was read at 365 nm in quartz cuvette.

The standard curve was prepared from a concentrated solution of anhydrous betaine in H_2SO_4 (0,5M). The blank consisted in H_2SO_4 (0.5 M).

The concentration was calculated with the equation:

$$C = \frac{Cp*2*10}{M} [\mu\text{g. g}^{-1}\text{FM}]$$

Where Cp is the concentration derived from the absorbance and the standard curve and M the weight of plant fresh matter used in gram.

2.2.5 Oxidative stress-related parameters

2.2.5.1 Malondialdehyde (Heath and Packer, 1968)

The amount of lipid peroxidation was estimated as the amount of MDA and was assessed in triplicates on fresh samples of 100 to 200 mg of leaf/root powder preliminary frozen in liquid nitrogen and homogenized in 5 ml of 5% (v:v) trichloroacetic acid and glycerol 1.25% (TCA solution). The homogenates were centrifuged at 12,000 g for 10 min at 4°C and filtered through Whatman filter paper. A 2 ml aliquot of thiobarbituric acid 0.67% (v:v) (TBA solution) was added to 2 ml of supernatant. The blank was prepared by mixing 2 ml of TCA solution and 2 ml of TBA solution. Then the samples and the blank were heated at 100°C for 30 minutes and quickly cooled on ice. Samples were centrifuged at 12,000 g for 1 min and the absorbance was read at 532 nm (a_{532}). The non-specific absorption at 600 nm was subtracted (a_{600}). The concentration of MDA (Cp) was calculated using an extinction coefficient (ϵ) of $155 \text{ mM}^{-1}\text{cm}^{-1}$ and the absorption (A) in the equations:

$$A = a_{532} - a_{600}$$
$$Cp = \frac{A}{\epsilon} [\text{mmol. L}^{-1}]$$

And the concentration in the sample (C) was calculated with the equation:

$$C = \frac{Cp*df*V*1000}{M} [\mu\text{moles. g}^{-1}\text{ FM}]$$

Where df is the dilution factor (2), V the initial volume (0.005 L) and M the the weight of fresh matter of the sample in gram.

2.2.5.2 Glutathione

Quantification of GSH and GSSG was performed as previously described for VCT analysis according to Cereser et al. (2001). Tissue samples (c.a. 200 mg FW) were ground in liquid nitrogen mixed with 4 ml of methaphosphoric acid 6% (MPA) and vortexed. From this solution 1 ml was taken and centrifuged at 10,000 g during 7 min at 4°C. For the GSht, tissue powder samples were mixed with 1 ml of dithiothreitol 25 mM (DDT) and 500 μ l of Tris Buffer (0.1M; pH 8.5) and vortexed. The DTT was used to reduce all the glutathione present. The samples were left to stand in ice for 30 minutes. Then 8 ml of MPA 4% were added and the samples were vortexed. From this solution 1 ml was taken and centrifuged at 10,000 g during 7 minutes at 4°C. Remaining part of the procedure was as described for VCT.

The concentrations of GSH and GSht (C) in the samples were calculated from the measured concentration (C_p) with the equation:

$$C = \frac{C_p \cdot v}{M} [\mu\text{moles. g}^{-1} \text{ FM}]$$

Where v is the extraction volume (0.004 L for GSH and 0.0095 L for GSht) and M the weight of fresh matter of the sample in gram.

The concentration in the oxidized form of glutathione (GSSG) was calculated by the subtraction of the concentration of GSH to GSht and divided by 2.

$$GSSG = \frac{GSht - GSH}{2} [\mu\text{moles. g}^{-1} \text{ FM}]$$

2.2.5.3 Total non-protein thiols and phytochelatins (Hartley-Whitaker et al., 2001)

Each sample (50 to 150 mg of leaf/root powder preliminary frozen in liquid nitrogen) was mixed with 2 ml of a solution of sulfosalicylic acid 5% and diethylenetriaminepentaacetic acid (DTPA at 6.3 mM). The samples were centrifuged at 10,000 g during 30 min at 4°C and 300 μ l of supernatant were mixed with 630 μ l of K_2HPO_4 (0.5 M) and the absorbance was read at 412 nm (A_1). Then 25 μ l of a solution of 5,5-dithiobis 2-nitrobenzoic acid (DTNB; 10mM) with K_2HPO_4 (0.143 mM) and DTPA (6.3 mM) were added in the cuvettes and the absorbance was read again at 412 nm after 2 minutes (A_2). The standard curve was prepared from a solution of cysteine (200 μ M) and a solution of sulfosalicylic acid 5% and DTPA at 6.3mM. The blank

contained only the solution of sulfosalicylic acid 5% and DTPA 6.3 mM. Both followed the same procedure as the samples.

The absorbance (A) was calculated by the equation:

$$A = A_2 - A_1$$

The final concentration (C) of the sample was calculated by:

$$C = \frac{C_p * 0.002}{M} [\mu\text{moles. g}^{-1} \text{ FM}]$$

With C_p the concentration derived from the absorbance and the standard curve and M the fresh weight of the sample in gram.

The phytochelatin (PCs) concentrations were calculated from the results obtained for total non-protein thiols (NPT) and reduced glutathione (GSH) as followed:

$$GSH = GSH_t - GSSG [\mu\text{moles. g}^{-1} \text{ FM}]$$

$$PCs = NPT - GSH [\mu\text{moles. g}^{-1} \text{ FM}]$$

2.2.5.4 Total phenolics and flavonoids

Phenolics and flavonoids were extracted from frozen plant samples ground in the presence of liquid nitrogen and extracted in 2 ml of methanol 80% (v/v). Samples were then centrifuged at 10,000 *g* during 10 minutes at 4°C. Total phenolics and flavonoids were then colorimetrically determined according the methods of Singleton and Rossi (1965) and Dewanto et al. (2002), respectively, as previously described for analysis of VCT.

2.2.6 Statistical treatment of the data

The results were analysed with the version 3.5.2 (2018) of the statistical software R. Variance analysis with two factors (ANOVA 2) were performed to determine if the treatment and/or the watering had an impact on the measured parameters. The application conditions of the ANOVA were checked by the Shapiro-Wilk test for normality of data and the Harley test for homoscedasticity of variance. The third condition is the independence of the samples which is always respected (related to the procedure of treatments and watering). Depending on the results obtained for the application conditions, ANOVA could be applied with a Turkey test or if the conditions were not respected non-parametric tests were used (e.g. Kruskal-Wallis).

Chapter 3: Results analysis

3.1 Vermicompost tea analysis

The following Table 2 presents the summary of compounds found in the VCT. The pH is close to neutral. The main ions detected are K, Ca and Na as well as S. Nitrogen is mainly present in form of nitrate. The VCT contains proline as osmoprotectant. It also contains antioxidants such as phenolics, flavonoids, anthocyanins, glutathione and ascorbate in detectable quantities. Proteins and especially sugars are present.

Table 2 PUR VER® vermicompost tea composition summary. Each value is the mean of 3 replicates. ND corresponds to not detected.

Analysed parameters	Results
pH	7,39
Electrical conductivity	2.35 [mS/cm]
Density	1010 [g/L]
Dry matter residue	3 ± 0 [%]
Na	89,51 ± 0,69 [mg/L]
Ca	132,04 ± 11,79 [mg/L]
K	199,82 ± 5,86 [mg/L]
Fe	2,67 ± 0,2 [mg/L]
Zn	0,64 ± 0,04 [mg/L]
Cu	ND
Mg	27,62 ± 1 [mg/L]
P	5,72 ± 0,53 [mg/L]
As	< 0,2 [mg/L]
B	1 ± 0,12 [mg/L]
S	92,48 ± 2,07 [mg/L]
Nitrates	5,89 ± 0,03 [mmol/L]
Ammonium	17,38 ± 0,13 [μmole/L]
Total nitrogen	92,4 ± 0,3 [mg/L]
Proline	12,16 ± 0 [μmole/L]
Free polyamines	ND
Total phenolics	0,985 ± 0,43 [mg/L]
Flavonoids	3,42 ± 0,16 [mg/L]
Anthocyanins	0,58 ± 0 [mg/L]
Total glutathione	480,51 [μmole/L]
Total ascorbate	65,59 ± 2,26 [μmole/L]
Total proteins	5,3 ± 0,09 [mg/L]
Soluble sugars	24,69 ± 0,03 [mg/L]

The major classes of phytohormones were identified in the VCT (Table 3), except gibberellins which were all below the limit of detection. Total cytokinins (CK) represent more than 20 pmol.ml^{-1} and the major part (43.5%) is represented by trans-zeatin and trans-zeatin-riboside. Dihydrozeatin-type and *cis*-zeatin type exhibited similar concentrations in the VCT and the *cis*Z free form, which is poorly active from a biological point of view, is present at a significant concentration of 1.63 pmol.ml^{-1} while the isopentenyl free form (iP) remain at a lower concentration (less than 0.8 pmol.ml^{-1}). For each class of CK, several types of conjugated forms were detected, especially glucoside and riboside forms.

Free abscisic acid (ABA) was detected in significant amounts ($> 3 \text{ pmol.ml}^{-1}$) but the proportion of the *cis* and *trans* isomers could not have been detected with the used analytical procedure. The product of ABA oxidation (dihydrophaseic and phaseic acids) were detected at even higher concentration than the free form. The concentrations of salicylic acid (SA) and its immediate precursor, the benzoic acid (BzA), were extremely high, while the concentration of jasmonic acid (JA) and JA-Ileu remained low.

Total auxin was also detected and free indole acetic acid was present at a concentration close to 10 pmol.ml^{-1} . At the opposite, its conjugated forms (IAA-Asp and IAA-Glu) remained rather low. The immediate precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid, was detected at a low concentration but its malonyl form remained below the limit of detection.

Table 3 PUR VER® vermicompost tea phytohormones content summary. Each value is the mean of 3 replicates. ND corresponds to not detected and SE means standard error.

Analyzed phytohormones ^a	Mean concentrations [pmol/ml]	SE
<u>cytokinins</u>		
transZ	2.94	0.32
transZR	1.47	0.21
transZ7G	1.23	0.17
transZ9G	0.84	0.07
transZOG	0.74	0
transZROG	0.53	0.06
transZRMP	1.01	0.08
total transZ type	8.76	
DHZ	3.65	0.21
DHZ7G	0.51	0.06
total DHZ-type	4.16	
<i>cis</i> Z	1.63	0.15
<i>cis</i> ZR	1.55	0.1
<i>cis</i> Z7G	0.51	0.08
<i>cis</i> ZOG	0.23	0.04
<i>cis</i> ZROG	0.34	0.05
<i>cis</i> ZRMP	0.07	0
total <i>cis</i>Z type	4.33	

iP	0.74	0.05
iPR	0.45	0.04
iP7G	0.44	0.07
iP9G	1.01	0.11
iPRMP	0.24	0.02
total iP type	2.88	
total cytokinins	20.13	
<u>ABA</u>		
ABA libre	3.05	0.18
DPA	4.12	0.03
PA	3.07	0.4
ABA-GE	4.83	0.6
9OH-ABA	0.78	0.03
total ABA	15.85	
SA	564.2	32.1
BzA	1108.4	100.9
PAA	6.13	0.51
JA	1.05	0.14
JA-Ileu	0.98	0.09
cisOPDA	ND	
<u>Auxin</u>		
IAA	9.54	0.29
IAA-Asp	0.21	0.04
IAA-Glu	0.37	0.14
OxIAA	2.03	0.29
total auxin	12.15	
ACC	7.84	1.02
Gibberellins	ND	

^a transZ: trans-zeatin, transZR: trans-zeatin riboside, transZ7G: trans-zeatin-7-glucoside, transZ9G: trans-zeatin-9-glucoside, transZOG: trans-zeatin-O-glucoside, transZROG: trans-zeatin riboside -O-glucoside, transZRMP: trans-zeatin riboside monophosphate, DHZ: dihydrozeatine, DHZ7G: dihydrozeatine-7-glucoside, cisZ: cis-zeatin, cisZR: cis-zeatin riboside, cisZ7G: cis-zeatin-7-glucoside, cisZOG: cis-zeatin-O-glucoside, cisZROG: cis-zeatin riboside -O-glucoside, cisZRMP: cis-zeatin riboside monophosphate, iP: isopentenyl adenine, iPR: isopentenyl adenosine, iP7G: isopentenyl adenine-7-glucoside, iP9G: isopentenyl adenine-9-glucoside, iPRMP: isopentenyl adenosine monophosphate, ABA: abscisic acid, DPA: dihydrophaseic acid, PA: phaseic acid, ABA-GE: ABA-glucose ester, 9OH-ABA: 9-hydroxy-ABA, SA: salicylic acid, BzA: benzoic acid, PAA: phenylacetic acid, JA: jasmonic acid, JA-Ileu: JA-isoleucine, cisOPDA: JA precursor, IAA: indole-3-acetic acid, IAA-Asp: IAA-aspartate, IAA-Glu IAA-glutamate, OxIAA: oxo-IAA, ACC: 1-aminocyclopropane-1-carboxylic acid.

Chapter 3: Results analysis

3.2 The impact of boron and vermicompost tea on the arsenic absorption by *Atriplex atacamensis*

3.2.1 Mortality percentage

The recorded mortality percentages are presented in Table 4. Statistical analysis was not performed on these data because of the absence of repetition. Plants exposed to B and As exhibited the highest mortality percentage. In contrast, all plants remained alive in response to the As treatment. Exposure to vermicompost tea decreased mortality in plants exposed to B excess but increased it in plants exposed to the mixed treatment.

Table 4 Mortality percentage of A. atacamensis after two weeks of treatment (control, As (500 μ mol.L-1), B (500 mg.L-1), As (500 μ mol.L-1) and B (500 mg.L-1)) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively).

	water	vermicompost
C	0.00	2.08
As	0.00	0.00
B	52.08	37.50
As+B	54.17	89.58

3.2.2 Plant growth

The considered treatments had no impact on the leaf fresh masses (Figure 25). A non-significant decrease was however suggested in plants exposed to B excess, both in the absence or in the presence of As. In the absence of vermicompost tea, the root masses also remained unaffected by the treatments. Addition of vermicompost tea tended to increase the root masses, except in the cases of plants simultaneously exposed to As+B.

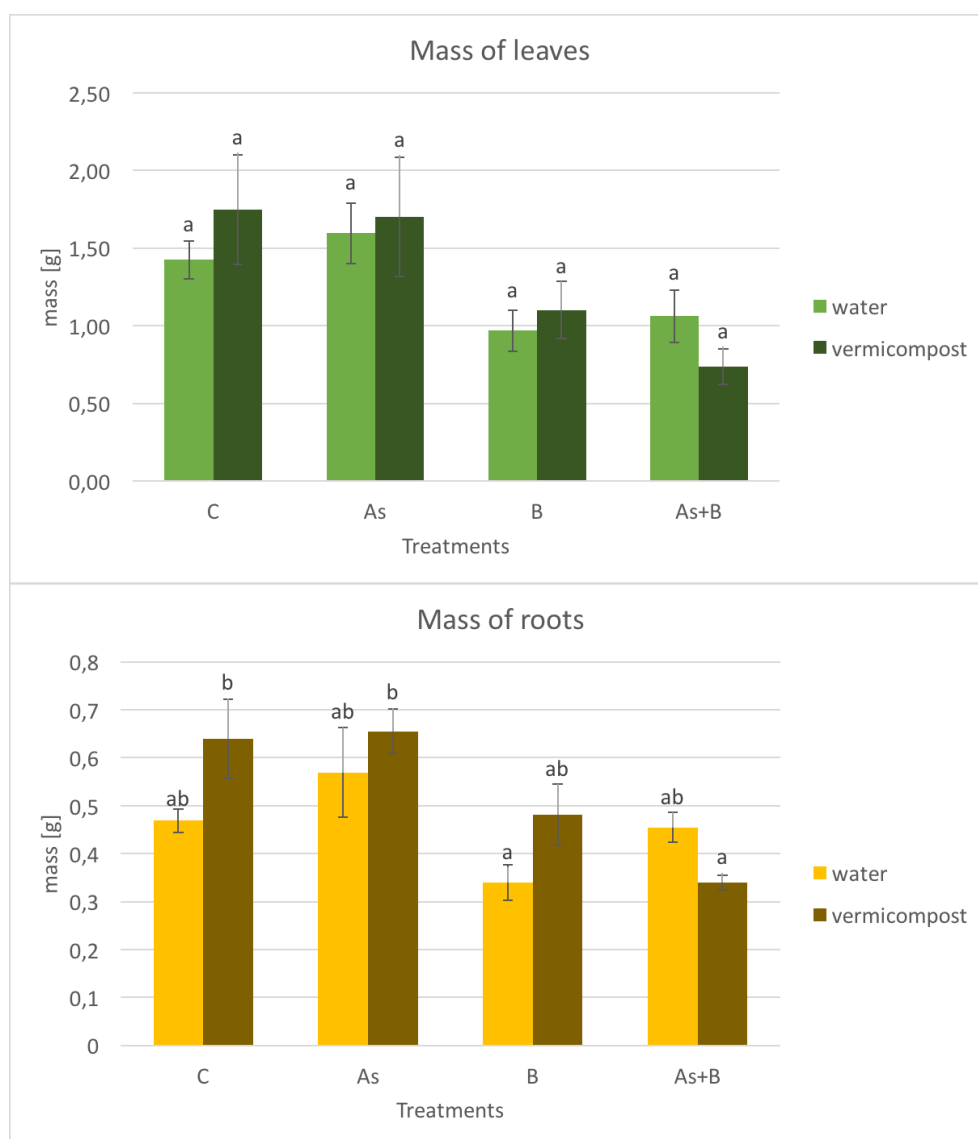


Figure 25 Mass of leaves and roots of *Atriplex atacamensis* after two weeks of treatment (control, As ($500 \mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As ($500 \mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 4 replicates and vertical bars are S.E.. The values with different letters are significantly different ($p\text{-value} < 0.05$).

3.2.3 Water content

The leaf water content was not significantly affected by the treatment (Figure 26). In the absence of vermicompost tea, the root water content was higher in plants exposed to As than in those exposed to the mixed treatment (As+B). In this latter case, however, exposure to vermicompost tea significantly improved the root water content.

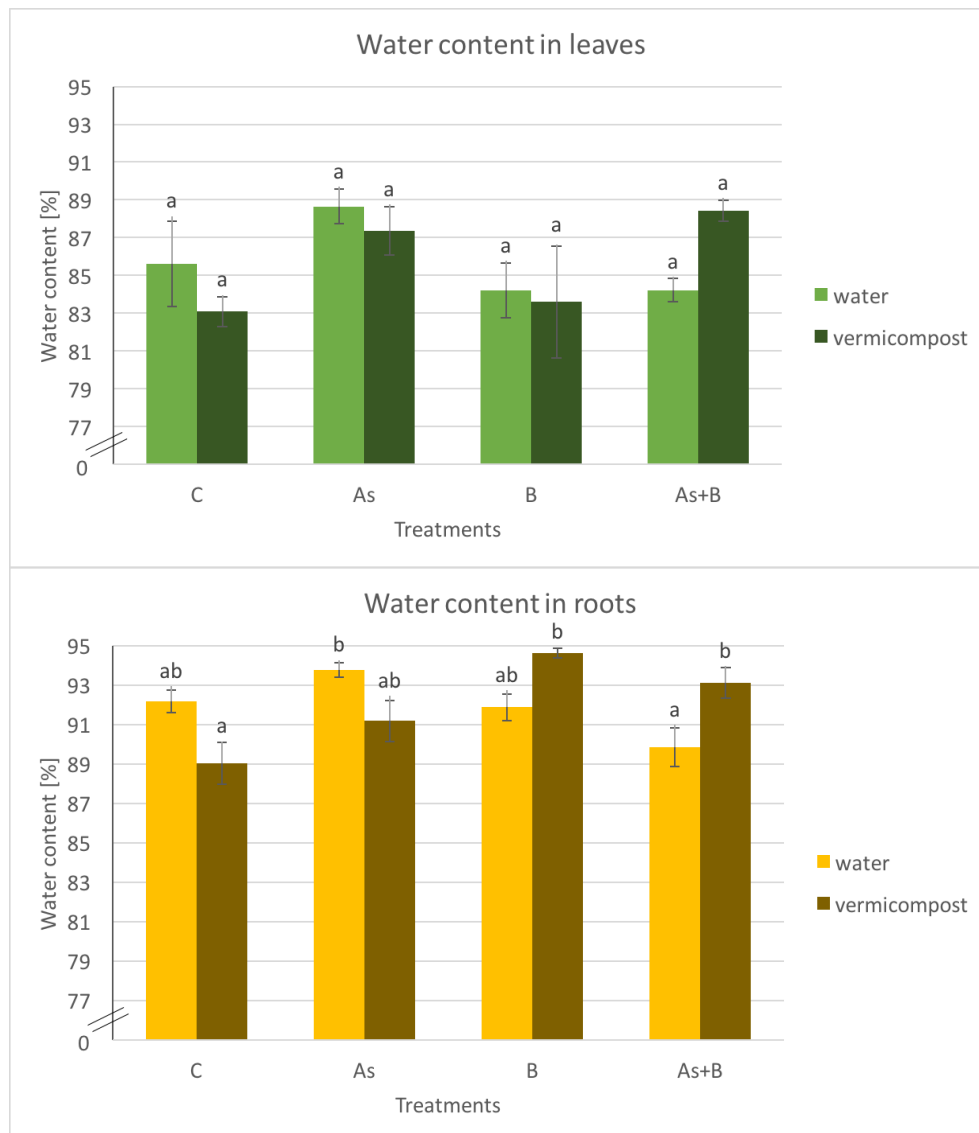


Figure 26 Water content in leaves and roots of *Atriplex atacamensis* after two weeks of treatment (control, As ($500 \mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As ($500 \mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 4 replicates and vertical bars are S.E.. The values with different letters are significantly different ($p\text{-value} < 0.05$).

3.2.4 Ions content: AS, B, P, S

As expected, As was not detected or was below the detection limit in plants that were not exposed to As toxicity. In leaves, plant exposed to As in the absence of B excess exhibited higher As content than plants exposed to mixed toxicity and vermicompost tea had no significant impact on the parameter. Root As content was also lower in plants exposed to As+B comparatively to exposure to As alone, but in this case, vermicompost tea significantly decreased the root As accumulation, especially in the mixed treatment. The range of As accumulation in the roots was ten times higher than in the leaves (Figure 27).

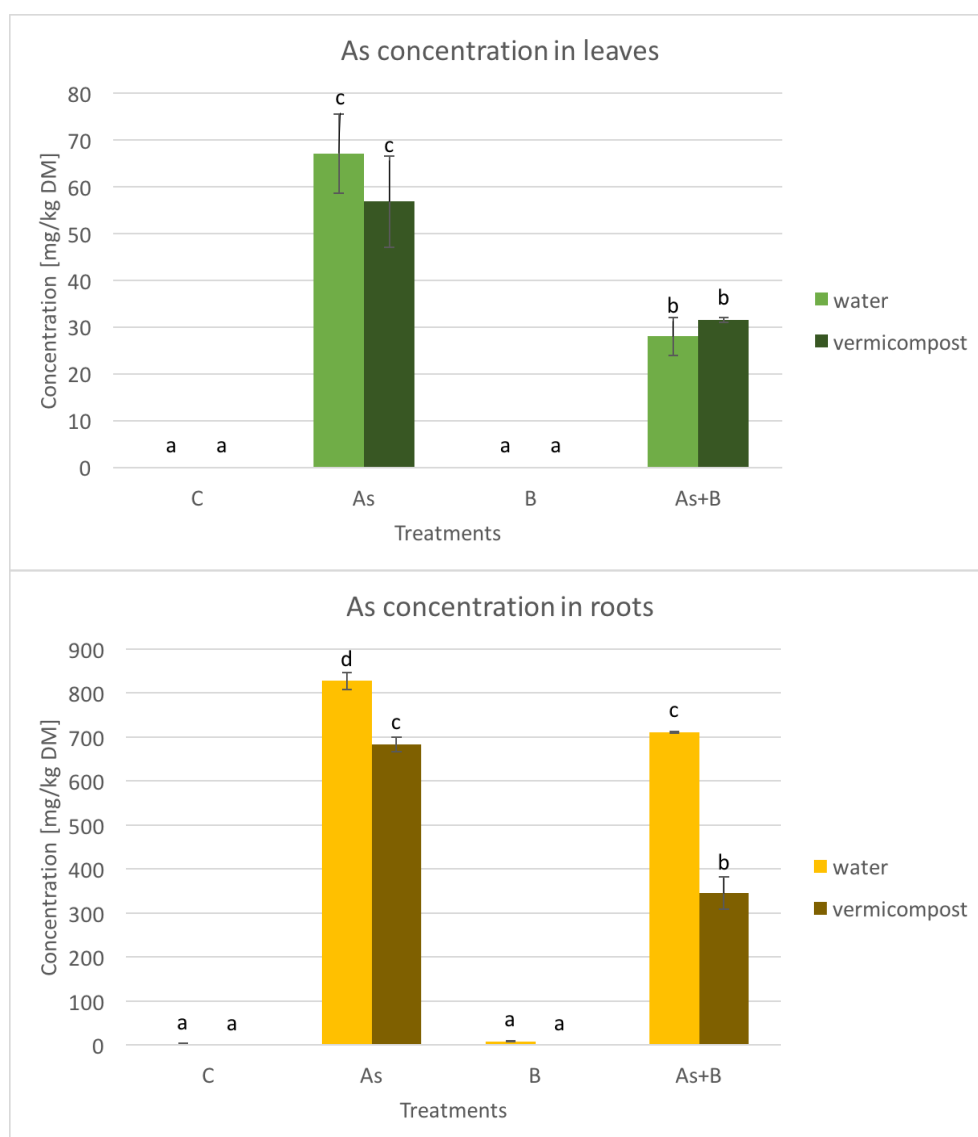


Figure 27 Arsenic content in leaves and roots of *Atriplex atacamensis* after two weeks of treatment (control, As ($500 \mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As ($500 \mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different ($p\text{-value} < 0.05$).

In plants exposed to control conditions or to As toxicity, B (Figure 28) was detected at normal physiological concentrations in both roots (37.04 mg/kg DM) and leaves (70.73 mg/kg DM). In leaves, exposure to B excess induced a strong increase of the B concentration which was more marked in plants exposed to As+B than in plants exposed to B excess alone. Vermicompost tea increased leaf B accumulation in plants exposed to B excess but reduced it in plants exposed to As+B. Root B concentration was also higher in plants exposed to As+B than in those exposed to B alone and vermicompost tea significantly reduced root B in both cases. It is noteworthy that B concentration was higher in the leaves than in the roots.

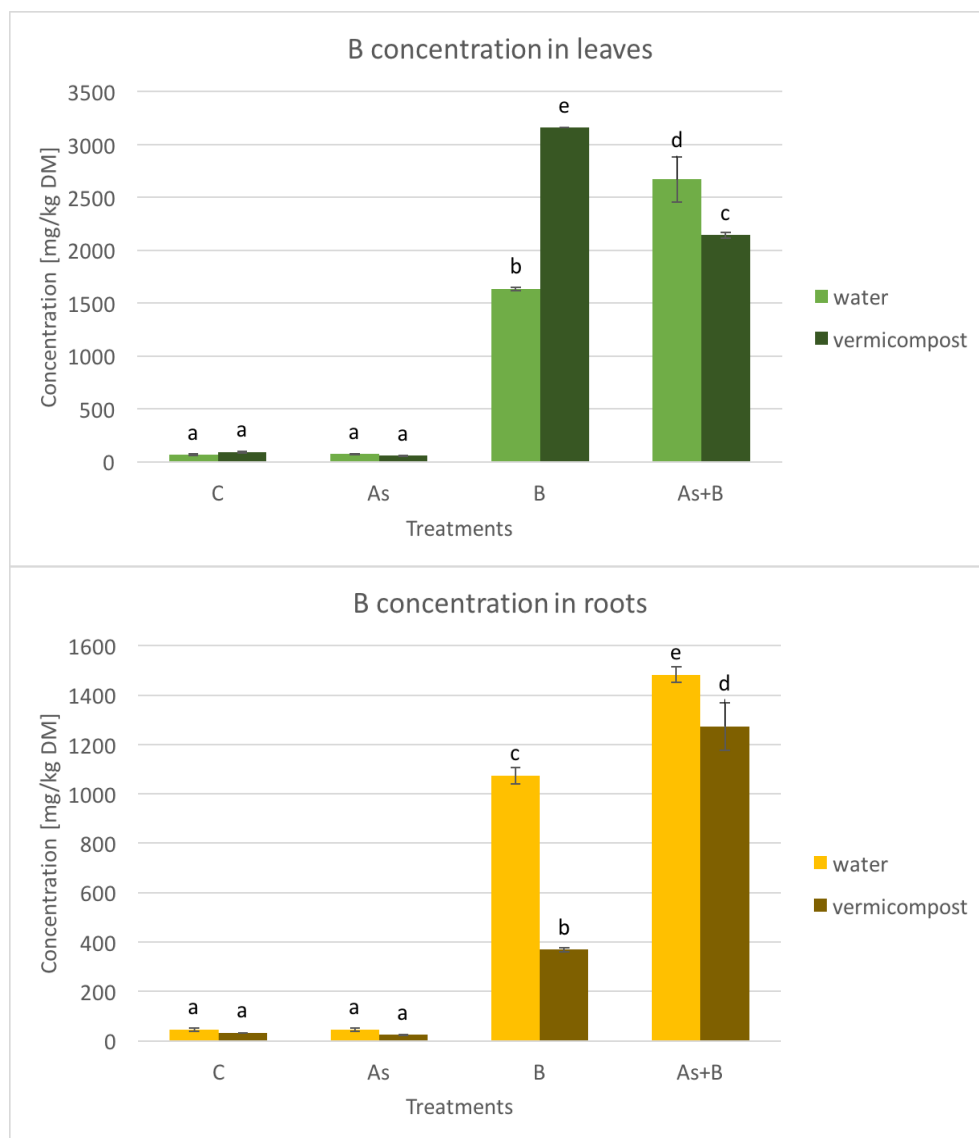


Figure 28 Boron content in leaves and roots of *Atriplex atacamensis* after two weeks of treatment (control, As ($500 \mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As ($500 \mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different ($p\text{-value} < 0.05$).

In the absence of vermicompost tea, leaf P concentration (Figure 29) was the highest in plants exposed to As toxicity and the lowest in those exposed to As+B. In contrast, B excess alone had no significant impact on leaf P concentration comparatively to controls. Vermicompost tea decreased leaf P concentration in response to As or to B excess but significantly increased it in response to the mixed treatments (As+B). In the absence of vermicompost tea, root P concentration significantly increased in plants exposed to B excess and remained unaffected in the other treatments. Exposure to vermicompost tea significantly decreased P concentration in all cases.

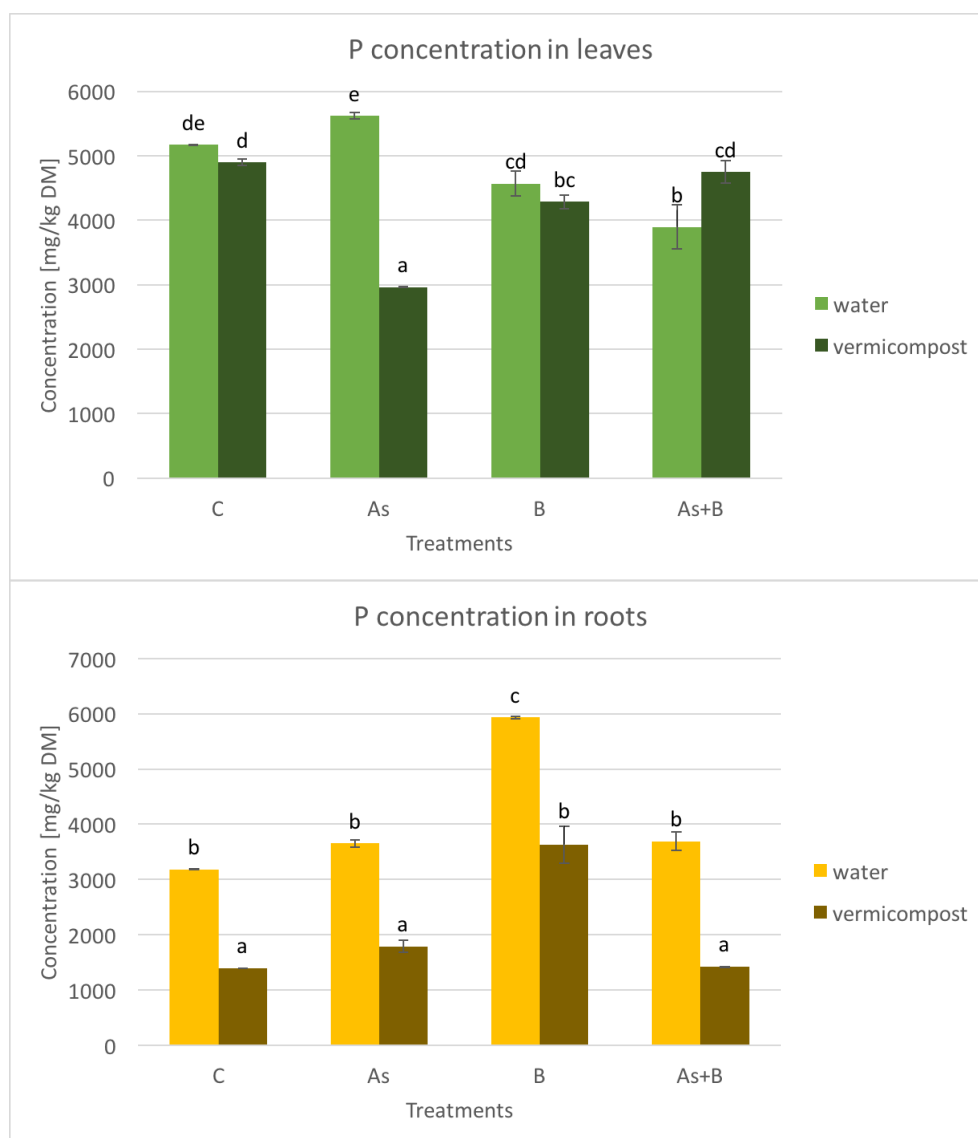


Figure 29 Phosphorous content in leaves and roots of *Atriplex atacamensis* after two weeks of treatment (control, As ($500 \mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As ($500 \mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different ($p\text{-value} < 0.05$).

As shown on Figure 30, in the absence of vermicompost tea, the leaf S concentration increased in response to As and decreased in response to B excess while it remained unaffected in response to the mixed treatment. Vermicompost tea treatment significantly increased leaf S concentration in all cases, except in the mixed treatment where a significant decrease was recorded. The root S content also obviously increased in As-treated plants while it decreased, in the absence of vermicompost tea, in plants exposed to B excess and to the mixed treatment. Vermicompost tea does not have significant impact on S content in roots excepted for plants under As treatment where it significantly increases the S concentration.

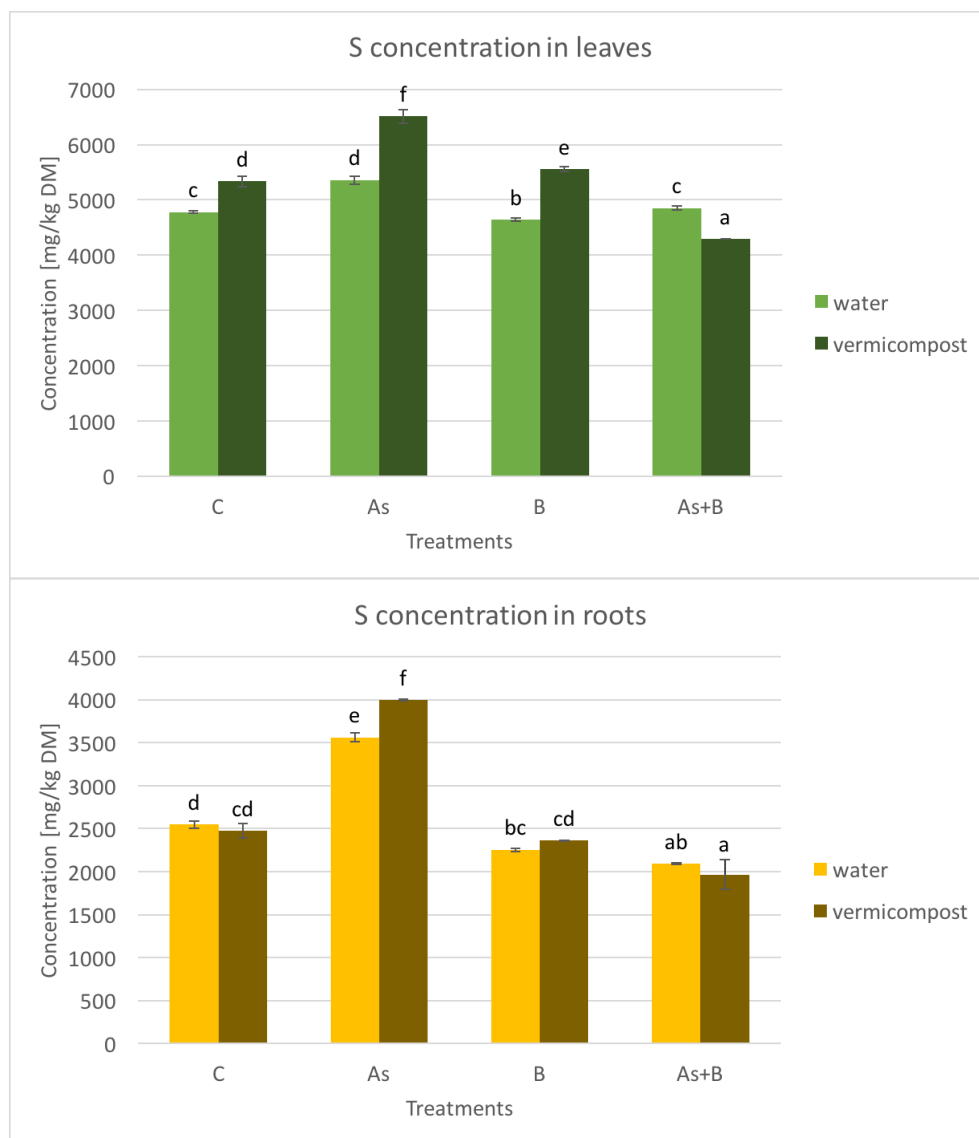


Figure 30 Sulphur content in leaves and roots of *Atriplex atacamensis* after two weeks of treatment (control, As ($500 \mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As ($500 \mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different ($p\text{-value} < 0.05$).

3.2.5 Proline

Arsenic toxicity or B excess did not affect leaf proline concentration (Figure 31) comparatively to control, although proline concentration was higher in plants exposed to B excess than in those exposed to As toxicity. It is noteworthy that the mixed treatment strongly increased the leaf proline concentration. Vermicompost tea decreased proline accumulation in plants exposed to As or to B excess. Analysis was not performed on vermicompost tea impact on As+B treatment because of a lack of plant material. The root proline concentration was unaffected by the treatments but it clearly decreased in response to vermicompost tea exposure.

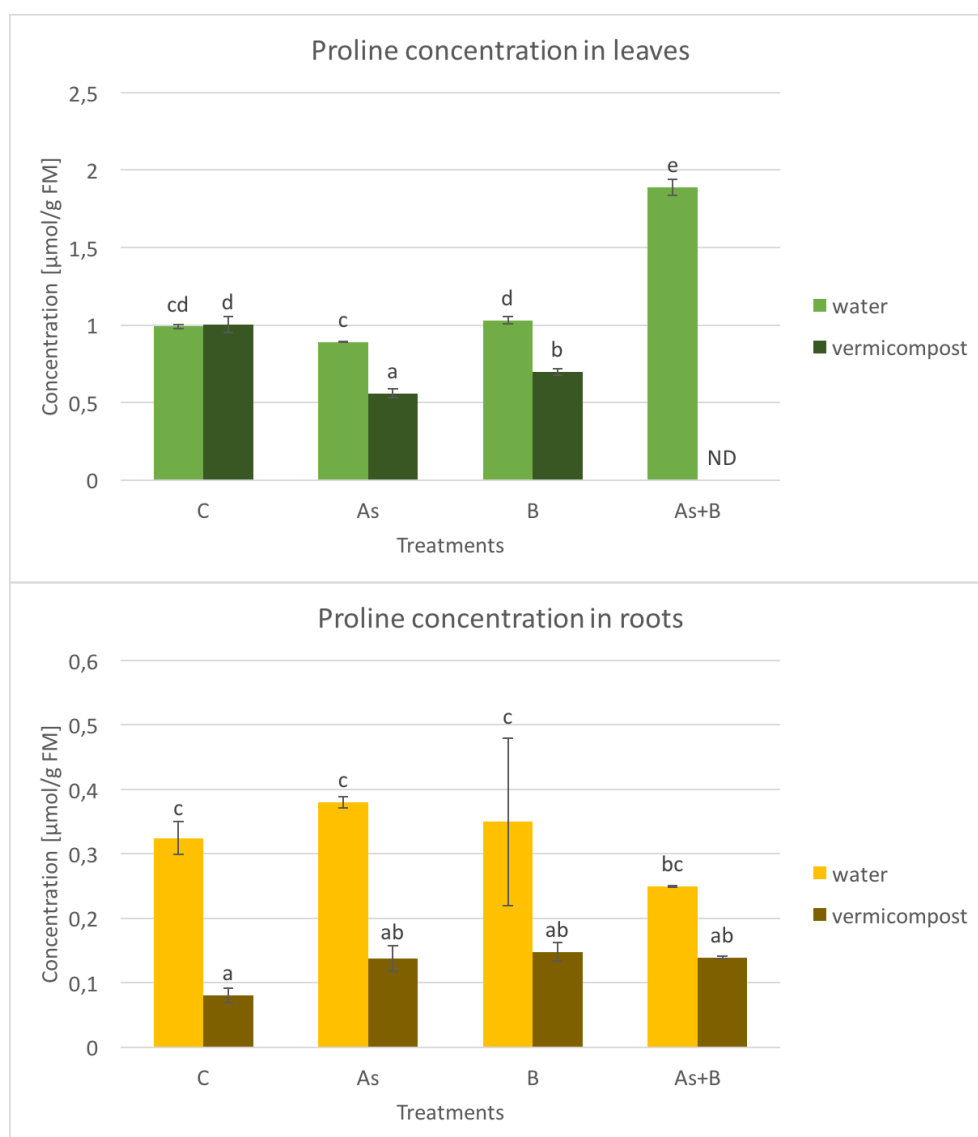


Figure 31 Proline concentration in leaves and roots of *Atriplex atacamensis* after two weeks of treatment (control, As ($500 \mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As ($500 \mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different ($p\text{-value} < 0.05$), ND corresponds to no data.

3.2.6 Glycinebetaine

The leaf glycinebetaine concentration increased in response to B excess and to a higher extent in mixed treatment than in plants exposed to B excess alone. Vermicompost tea slightly increased glycinebetaine concentration in control plants only. These results are presented on Figure 32. Glycinebetaine was not determined in the leaves of plants exposed to vermicompost tea and As+B because of a lack of material. Glycinebetaine was undetected in the roots of plants, whatever the treatment.

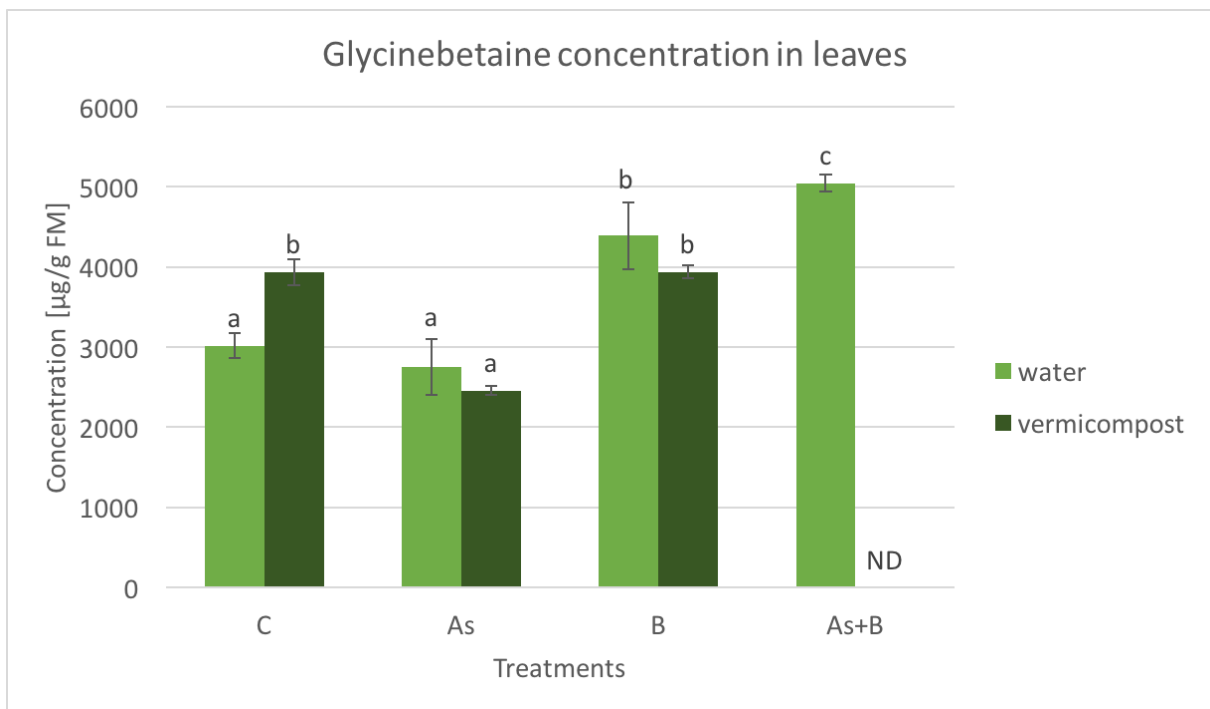


Figure 32 Glycinebetaine concentration in leaves and roots of *Atriplex atacamensis* after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different ($p\text{-value} < 0.05$), ND corresponds to no data.

3.2.7 Malondialdehyde

As shown on Figure 33, leaf MDA increased in B-treated plants and exhibited the highest value in plants exposed to As+B. In contrast, As alone had no impact on leaf MDA content. Vermicompost tea significantly reduced leaf MDA concentration in response to B and As+B treatments. The root MDA was decreased in As-treated plants comparatively to controls, while B excess had no significant impact on root MDA in the presence or absence of vermicompost tea. Root MDA also decreased in plants exposed to the mixed treatment and vermicompost tea increased the concentration.

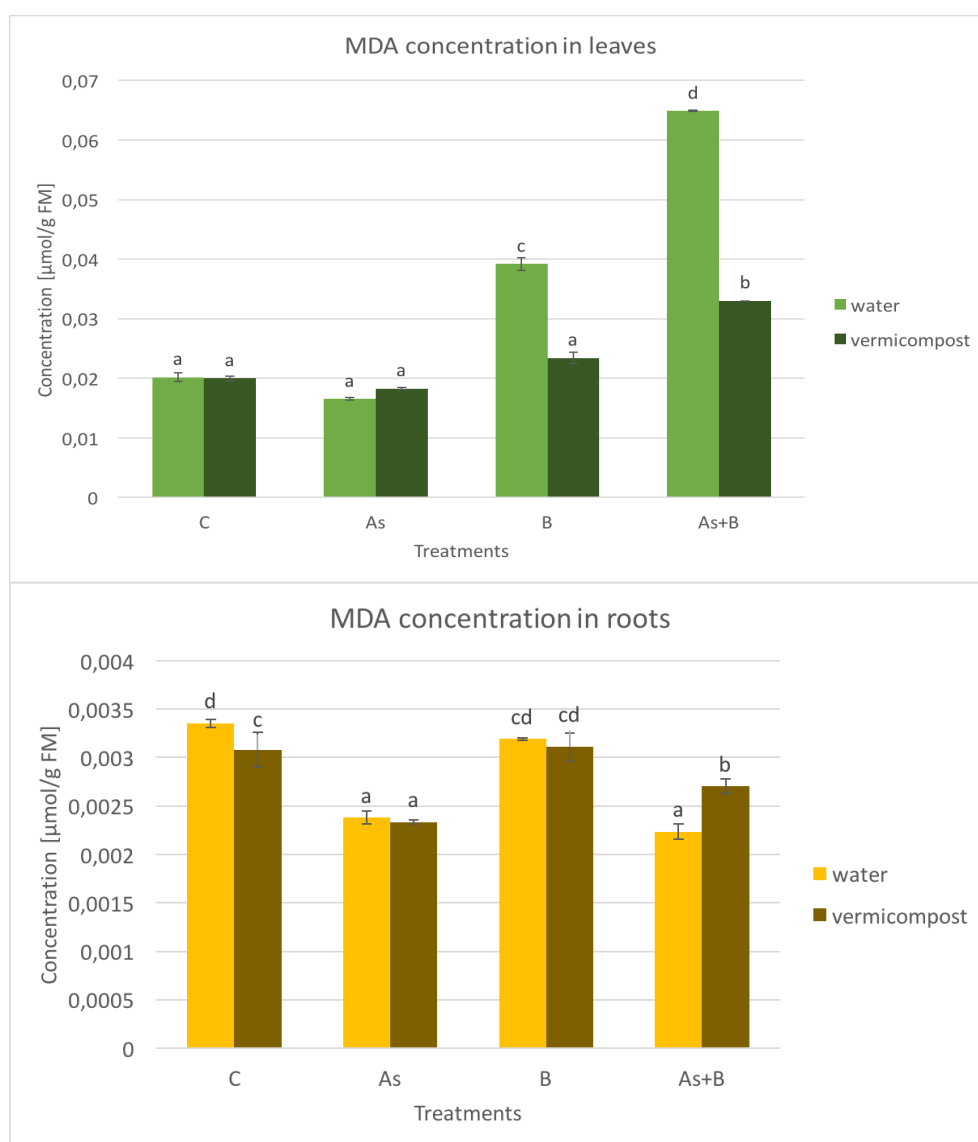


Figure 33 Malondialdehyde concentration in leaves and roots of *Atriplex atacamensis* after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different ($p\text{-value} < 0.05$).

3.2.8 Glutathione

The leaf total glutathione content was not affected by the treatments (Figure 34) and exogenous vermicompost tea had a slight and non-significant impact on this parameter. The root total glutathione decreased in response to As toxicity and exhibited the lowest value in plants exposed to B and to As+B treatments. Vermicompost tea increased root total glutathione in control and B-treated plants and slightly decreased it in As-treated ones.

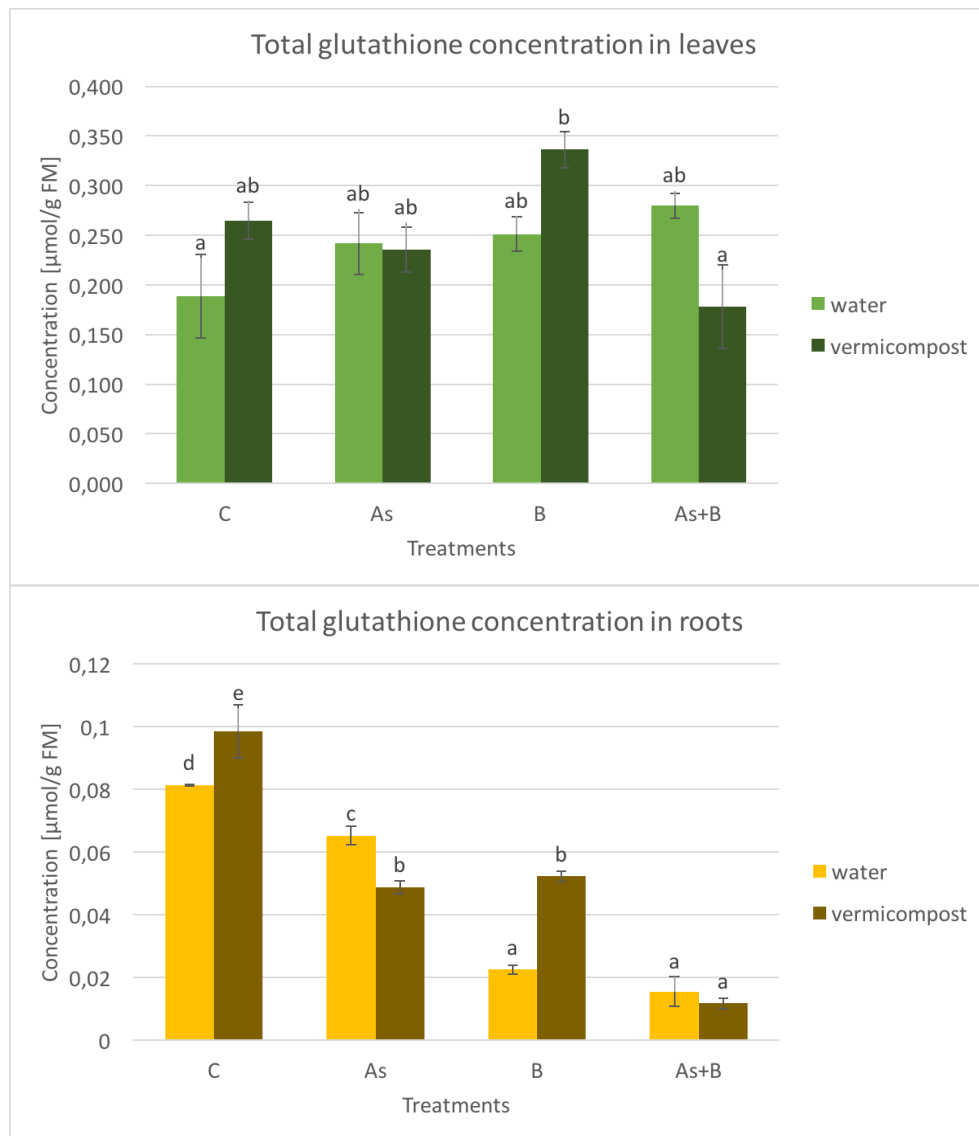


Figure 34 Total glutathione concentration in leaves and roots of *Atriplex atacamensis* after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different ($p\text{-value} < 0.05$).

The leaf GSSG to GSH concentrations ratio (Figure 35) was not affected by the treatments in the absence of vermicompost tea. The very high standard errors suggested that an important variability was recorded. Vermicompost tea tended to decrease this ratio, but its impact was significant in As+B-treated plants only. The root GSSG and GSH concentrations ratio increased in plants exposed to B excess or to As+B, and vermicompost had no significant impact on the recorded values.

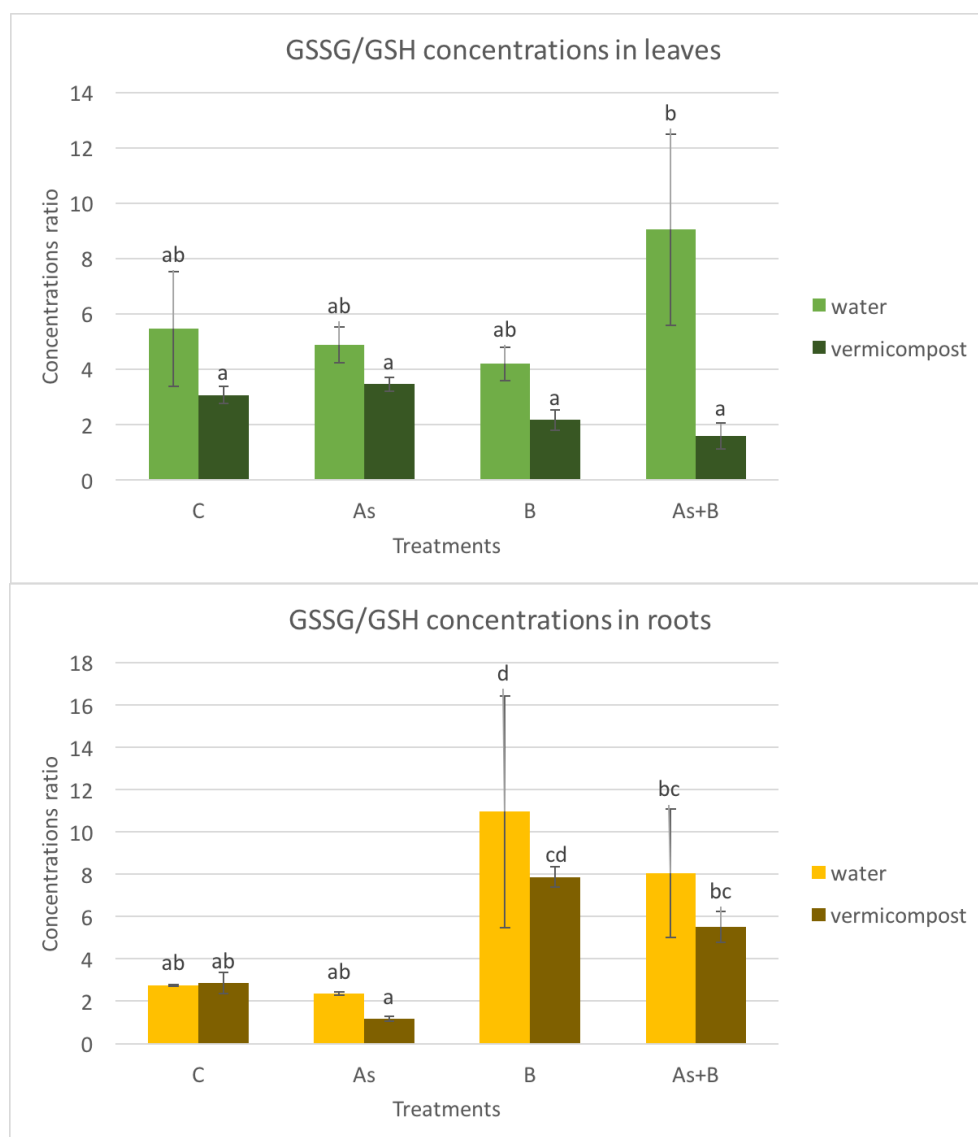


Figure 35 GSSG and GSH concentrations ratio in leaves and roots of *Atriplex atacamensis* after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different ($p\text{-value} < 0.05$).

3.2.9 Phytochelatin

The leaf phytochelatin concentration increased in response to As toxicity but not in response to B excess or mixed treatment (Figure 36). Vermicompost tea abolished the As-induced increase in phytochelatin content. Arsenic also strongly increased the root phytochelatin concentration and such increase was also mitigated by vermicompost tea. No significant increase in root phytochelatin was recorded in plants exposed to B excess or mixed treatment.

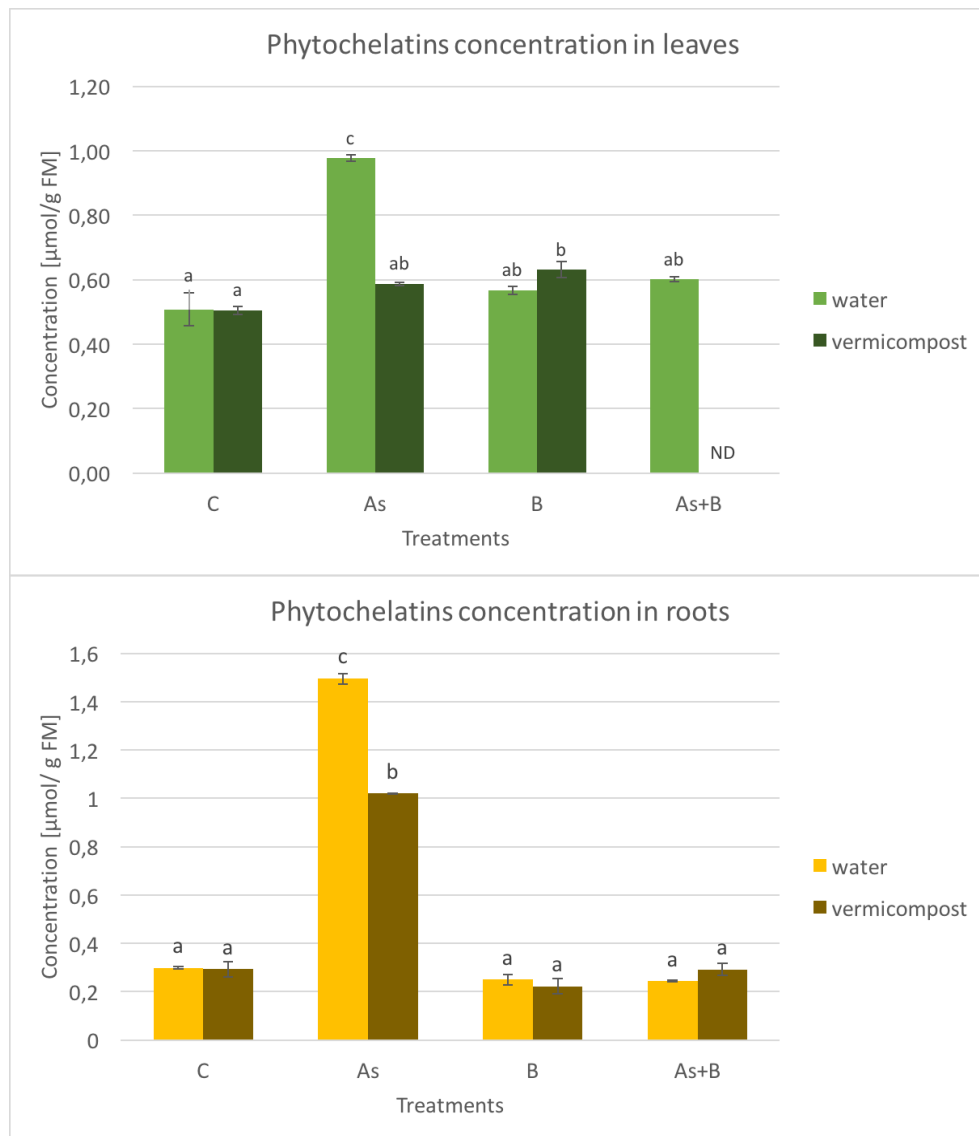


Figure 36 Phytochelatin concentrations in leaves and roots of *Atriplex atacamensis* after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different ($p\text{-value} < 0.05$), ND corresponds to no data.

3.2.10 Total phenolics and flavonoids

As shown on the Figure 37, the total phenolics concentration was higher in the leaves than in the roots. In the absence of vermicompost tea, the leaf total phenolics concentration did not increase in As-treated plants but increased in plants exposed to B excess or As+B treatments. Vermicompost tea slightly increased leaf total phenolics in controls but decreased them in As and in B-treated plants. The root total phenolics increased in response to As treatment and decreased in response to As+B exposure. Vermicompost tea increased the root total phenolics in response to this mixed treatment.

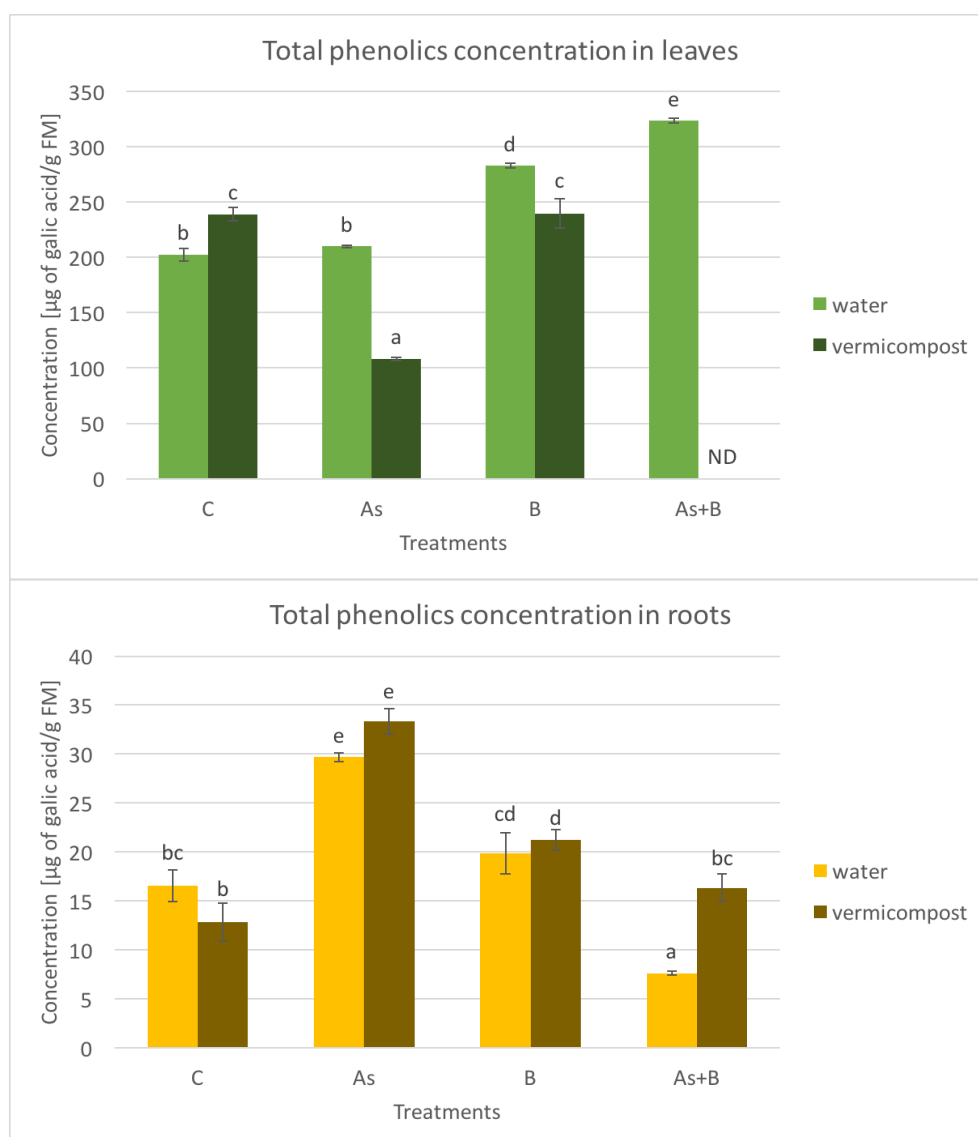


Figure 37 Total phenolics concentrations in leaves and roots of *Atriplex atacamensis* after two weeks of treatment (control, As ($500 \mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As ($500 \mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different ($p\text{-value} < 0.05$), ND corresponds to no data.

Chapter 3: Results analysis

The leaf flavonoids (Figure 38) remained unaffected in response to As toxicity and increased in B and As+B-treated plants. As previously noticed for total phenolics, vermicompost tea decreased the leaf flavonoids in B-treated and As-treated plants and slightly increased it in controls. The root flavonoids increased in response to As toxicity but decreased in response to B excess or to mixed treatments. In all cases, vermicompost tea increased the root flavonoid content.

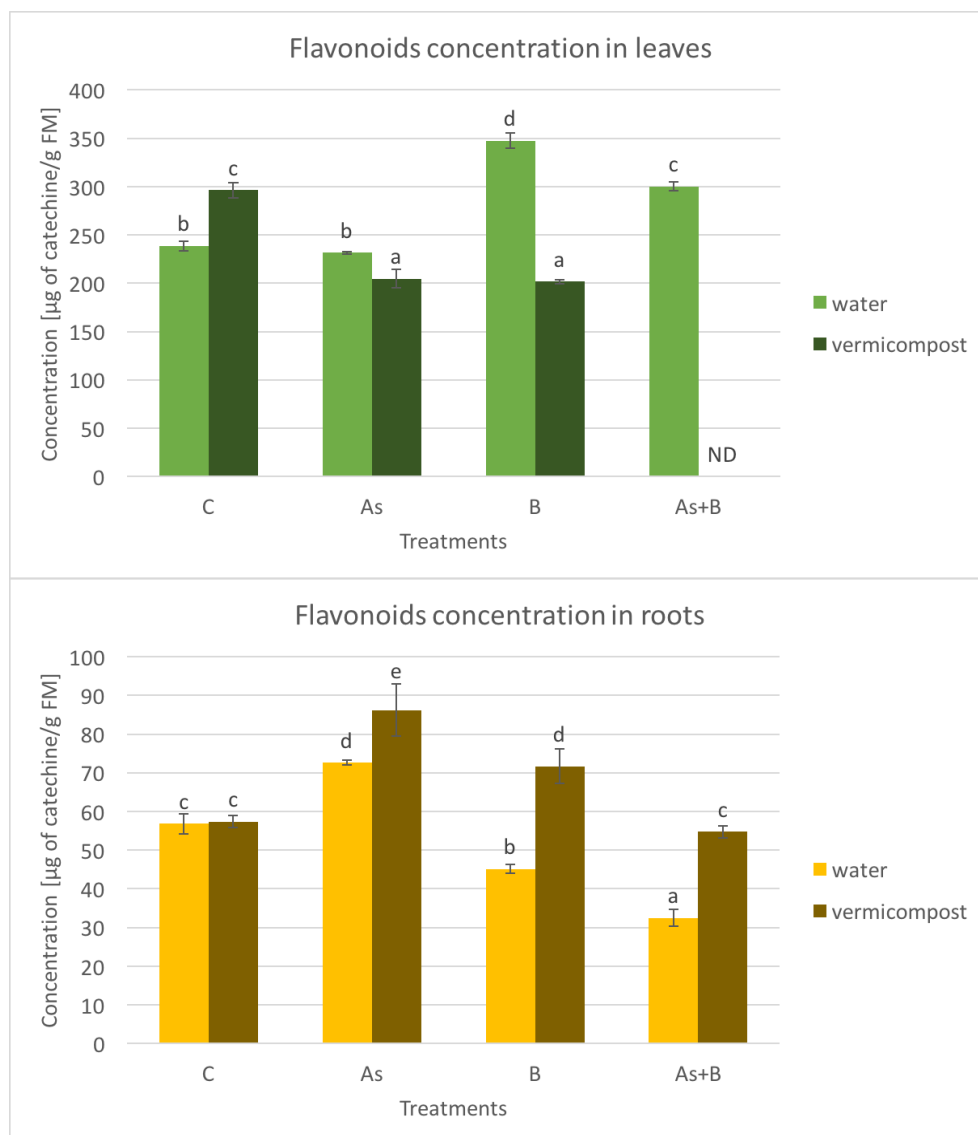


Figure 38 Flavonoids concentrations in leaves and roots of *Atriplex atacamensis* after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates and vertical bars are S.E.. The values with different letters are significantly different ($p\text{-value} < 0.05$), ND corresponds to no data.

Chapter 4: Discussion

Atriplex Atacamensis is an endemic plant species from the Antofagasta region in Chile and is known to grow in the presence of high As concentrations in its direct environment. Therefore, although the applied As concentration of 500 μM seems high regarding classical studies performed with other plant species (see Farooq et al., 2016 for review), *A. atacamensis* was able to cope with it. Indeed, Vromman et al. (2011) already carried out an experiment on *A. atacamensis* and showed that 75% of plants exposed to 1000 μM in hydroponic culture during 28 days survived until the end of the treatment. In its natural environment (Quillagua oasis, Atacama Desert) the As concentration in the soil reached $0.22 \pm 0.02 \text{ mg.kg}^{-1}$. This region is also characterized by a high B concentration in the soil that reaches $1.02 \pm 0.05 \text{ mg.kg}^{-1}$ (Vromman et al., 2016b). Therefore, *A. atacamensis* is simultaneously subject to high As and B concentrations (Tapia et al., 2013; Vromman et al., 2011). It is the reason why it is relevant to study the combined effect of these metals on the plant behaviour.

The results show that *A. atacamensis* could actually deal with the applied As concentration because the mortality percentage was 0%. In contrast, a B concentration of 500 mg.L^{-1} seems to be too high and induces toxic effects on plants. The mortality percentage of plants under this treatment exceeded 50%. Plants exposed to the mixed treatment showed the highest mortality percentage. However, some plants resisted to the B excess. This can probably be explained by the substantial natural variability of non-domesticated plants grown from field collected seeds like *A. atacamensis*. Most species of the *Atriplex* genus are indeed characterized by a high level of intraspecific variability (Bouda et al., 2008; Ruas et al., 2001; Walker et al., 2014). Although the resistance of *A. atacamensis* to As has been recently studied (Vromman, 2018; Vromman et al., 2017, 2016b, 2016b, 2011), the B excess resistance of *A. atacamensis* was never analyzed to the best of our knowledge.

4.1 The impact of boron excess on the arsenic absorption by *atriplex atacamensis* phil.

In order to identify the specific effect of As and/or B on the physiological behaviour of *A. atacamensis*, the present discussion is based on the comparison of metal treatments performed on plants grown first in the absence of VCT.

4.1.1 Plant water status and plant growth

Leaf water content was not affected by the treatments and it slightly decreased in roots under the mixed treatment compared to As only. This low impact of metal stress on water content was expected for a xerohalophyte plant species well adapted to extremely arid conditions (Tapia et al., 2013).

Beside stomatal closure allowing to drastically reduce the loss of water through transpiration, osmotic adjustment has been demonstrated to play a key role in regulation of the plant water status in *Atriplex* plant species (Ben Hassine et al., 2008; Ben Hassine and Lutts, 2010; Martínez et al., 2003; Martínez et al., 2005; Martínez et al., 2004). Unfortunately, for practical reasons, neither transpiration nor osmotic adjustment were determined in the present work, but proline and GB, acting in osmotic adjustment in *Atriplex* genus were quantified. Proline and GB are an osmolyte that accumulated in order to lower osmotic potential of plant allowing the maintenance of a favorable water balance. Besides their major role in plant adaptation to water stress, they also acts as endogenous antioxidant (Kaur and Asthir, 2015).

According to Camacho-Cristóbal et al. (2008) and Hasanuzzaman et al. (2015), As and B induce water and osmotic stress and as a response, increase the accumulation of proline and GB by the plant. However, roots proline concentration remained unaffected by metal treatments and GB was not detected in this organ. Since GB is produced from choline in the chloroplast compartment, its absence in non-photosynthetic organs could be explained, although some authors previously reported that GB could be translocated to the root by phloem sap or that proplast present in the roots are fully able to synthesize it (Chen and Murata, 2008; Mansour and Ali, 2017). The results also show that As did not induce water stress and the plants did not accumulate proline or GB suggesting that As-treated plants did not encountered an internal constraint which could justify the synthesis of protective compounds. This indicates that *A. atacamensis* was able to deal with such As concentration. Vromman et al. (2011) reported that leaves and roots water contents did not vary between 0, 100 and 1000 μM of As applied during two weeks. This confirms the relevance of using *A. atacamensis* for phytoremediation applications in arid regions. On the contrary, results show that B induced this specific response

in leaves (enhanced by As) which then efficiently synthesize proline and GB allowing an effective adaptive response since no decrease in WC was recorded during the experiment.

Concerning the plant growth, no significant result was recorded. However, As tended to slightly increase the leaves and roots fresh masses while B tended to decrease it. The mixed treatment shows that As mitigated the B effect. This may not be due to difference in the water content since it was not significantly different as previously mentioned. According to Camacho-Cristóbal et al. (2008), Nable et al. (1997) and Macho-Rivero et al. (2017) B excess may have a deleterious impact on chlorophyll content, stability of PSII and hence net photosynthesis. It is therefore not surprising that plant growth was affected by B excess in our experiments. It has however to be mentioned that growth-related parameters were recorded on living plants only and that a selection process occurred during the experiment which lead us to underestimate the impact of B on plant growth.

4.1.2 Mineral nutrition

Arsenic was mainly accumulated in the roots of *A. atacamensis* where its concentration was about ten times higher than in leaves. The poor translocation of As from root to shoot in most plant species was already reported by Zhao et al. (2009) and was mentioned in *A. atacamensis* by Vromman et al. (2011). A similar process was reported for most if not all toxic elements and could be related to an avoidance strategy and an attempt to protect stress-sensitive photosynthetic tissues from pollutants accumulation. Only hyperaccumulating plants display a preferential accumulation of toxic elements in the aerial parts (Verbruggen et al., 2009).

It is therefore noteworthy that B behaved in a very contrasting way: B was indeed well transported and even under B excess, root sequestration appeared inefficient and the concentration in leaves was two times higher than in roots which corroborates previous data published by Camacho-Cristóbal et al. (2008), Matoh (1997) and Uluisik et al. (2018).

In leaves and roots, the mixed treatment increased the B content compare to plants only treated with B. In contrast, mixed treatment significantly decreased As concentration in roots as well as the As translocation to leaves.

To explain this mutual inverse effect on the ions concentrations, the transport mechanisms of both can be look closer. They can be transported across the lipid bilayer by passive diffusion as well as by facilitated transport through aquaporins (Garbinski et al., 2019; Tanaka and Fujiwara, 2008; Zhao et al., 2010). In terms of concentration in the nutrient solution, B was by far at higher concentration than As in the mixed treatment.

Nodulin 26-like intrinsic protein (NIP) are involved in the transport of neutral molecules such as arsenite and boric acid. These transporters allow the uptake but also the efflux detoxification mechanism of plants to tolerate metal stress (Camacho-Cristóbal et al., 2008; Reid and Fitzpatrick, 2009; Uluisik et al., 2018; Zhao et al., 2010). The two metal species may be absorbed by two similar aquaporins: the NIP5;1 and the NIP3;1 (Kumari et al., 2018; Tanaka and Fujiwara, 2008; Wang et al., 2016). Wang et al. (2016) reported that genes encoding for NIP aquaporins are stimulated under B excess in order to reduce its accumulation in the plant tissues by efflux or to increase the vacuolar compartmentation of the metal, depending on the precise identity of stimulated gene. This means that under B excess, the efflux mechanisms by roots could be enhanced and since aquaporins may be involved in both B and As transport, As mainly stored in roots was more extruded in the concomitant presence of B excess than when alone. This hypothesis could explain why As concentration in roots decreased under mixed treatment.

Sun et al. (2018) recently showed that the overexpression of OsNIP1;1 and OsNIP3;1 aquaporins genes did not affect the As absorption but markedly decreased the root-to-shoot translocation of arsenite and thus its concentration in aerial parts. On the other hand, the aquaporin OsNIP3;1 is the closest homolog of AtNIP5;1 and is also involved in the B concentration regulation (Tanaka and Fujiwara, 2008). Hence B excess could have reduced the translocation of As from root to shoot which could explain the lower concentration of As in leaves under mixed treatment.

To summarize, the combination of the As translocation reduction and the efflux mechanism increase induced by the B excess could explain the As content reduction in the plant under mixed treatment.

On the other hand, vacuolar sequestration mechanisms are extremely important for plants exposed to As toxicity (Farooq et al., 2016; Zhao et al., 2009). This is especially the case for *A. atacamensis* since it is known to accumulate As in its root cells and thereby considered as a potential candidate for phytostabilisation applications (Benzarti et al., 2013; Lutts and Lefèvre, 2015; Vromman, 2018). Some aquaporins may also be involved in the vacuolar sequestration of As and B (Camacho-Cristóbal et al., 2008; Finnegan and Chen, 2012; Reid and Fitzpatrick, 2009; Uluisik et al., 2018; Zhao et al., 2010, 2009). Therefore, the addition of As to B might have increased the vacuolar accumulation of the later in leaves and roots: since B was in much higher concentration than As in plants, the vacuolar sequestration induced by As could have been completely overwhelmed by the B.

However, it is important to notice that B and As forms used are $\text{Na}_2\text{B}_4\text{O}_7$ and HAsNa_2O_4 which are not boric acid and arsenite. Vromman et al. (2011) highlighted that a poor fraction of

arsenate is reduced in arsenite in the roots of *A. atacamensis* but this fraction increases while the stress increases. The fact that additional oxygen was not provided to the plants may also impact the redox potential, leading to an increase in the proportion of As(III). Arsenate reduction is also accepted as the first detoxification step of As within the plants (Farooq et al., 2016; Zhao et al., 2010, 2009). Concerning B, the initial form may be transformed in B(OH)₃ according to the following equations:

Ionic dissociation in water: $\text{Na}_2\text{B}_4\text{O}_7 \rightarrow 2 \text{Na}^+ + \text{B}_4\text{O}_7^{2-}$

Acid/base reaction: $\text{B}_4\text{O}_7^{2-} + 5 \text{H}_2\text{O} + 2 \text{H}^+ \rightarrow 4 \text{B(OH)}_3$

Since the Yoshida solution pH used is equal to 5.7 (Yoshida et al., 1976), the acid condition allows the reaction to occur and the resulting form of B could be absorbed by the plant and might have induced the previously described mechanisms.

Vromman et al. (2018, 2017, 2016, 2011) used similar nutrient solution under similar environmental conditions. The use of speciation programs (VisualMinteq and Geochem) clearly indicated that an important fraction of As remains as arsenate in the solution. Similarly, these authors provided clear evidence that As(V) reduction in *A. atacamensis* is not operating at full efficiency and that some portion of As remain within the plants as As(V). Arsenate is thought to be absorbed by phosphate transporters (DiTusa et al., 2016; Puckett et al., 2012). In the present work, As alone did not reduce P concentration, suggesting that in our nutrient solution, P was fully available to ensure normal plant nutrition. The situation may be somewhat different in soils exhibiting low fertility levels, such as those present in the Atacama desert and Vromman et al. (2017) reported that P deficiency may increase As absorption, probably through an increase in the expression of genes coding for P transporters.

Complexation of As with non-protein thiols rich compounds, especially PCs is also an important strategy of As resistance and has been reported to operate especially in the roots of *A. atacamensis* (Vromman et al., 2016b, 2011). Synthesis of these protecting compounds requires an important S absorption and Reid et al. (2013) demonstrated that As may increase the expression of genes coding for S transporters. It is noteworthy that S concentration in the roots (and to a lower extent in the leaves) increased in *A. atacamensis* exposed to As toxicity. This may be related to the drastic increased in PCs concentration. It has to be mentioned that As-induced PCs accumulation in roots was completely abolished by B excess which also decreased the S concentration in the roots. To the best of our knowledge, no exhaustive data are available regarding B impact on S nutrition in halophyte plant species but it still may be hypothesized that a decrease in S absorption may, to some extent, affect PCs synthesis in plants exposed to the mixed treatment.

4.1.3 Oxidative stress and plant response strategy

Malondialdehyde quantification was performed as an indicator of lipid peroxidation due to oxidative stress. The results indicated that an oxidative stress occurred in leaves as a result of B excess. Arsenic toxicity and B excess are known to generate ROS that cause oxidative stress and lipid peroxidation (Camacho-Cristóbal et al., 2008; Farooq et al., 2016). In the present case, however, Arsenic had no impact on MDA content in the leaves, which confirms that, beside root sequestration, tolerance mechanisms to As may also act in the shoot part. It also confirms the high level of resistance of *A. atacamensis* to accumulated As (up to 70 mg.kg⁻¹ DW!). Arsenic, however, clearly exacerbated the negative effect of B in mixed treatment.

In roots B did not induce a specific oxidative stress and As mitigated the MDA concentration even in comparison to control. This may be explained by the difference of concentrations and could be a further indication of As tolerance of *A. atacamensis*.

The MDA concentration was ten times higher in leaves than in roots. Again, this is probably because the B concentration was the highest in this organ. Another reason could be that chloroplasts are major sites of ROS synthesis occurring as a result of stress-induced disruption of thylakoid membranes and that oxidative stress is commonly higher in photosynthetic tissues (Miller et al., 2010).

Plants dispose of different mechanisms to deal with oxidative stress. The common strategy against As toxicity is the increase of endogenous antioxidant, such as ascorbate or glutathione. Ascorbate was not quantified in the present work, although it was reported to increase in *A. atacamensis* exposed to As toxicity (Vromman, 2018). Glutathione is also a well-known antioxidant. In the specific case of As toxicity, it however has a dual impact since it also acts as a precursor of the PCs involved in As vacuolar sequestration (Farooq et al., 2016; Jozefczak et al., 2012; Zhao et al., 2010). In the present case, As applied alone reduced the root GSH content, which may be explained by the strong recorded increase in PCs. This however implies that GSH is less available for antioxidative purposes and thus could not explain the lower MDA concentration. Other compounds may assume antioxidative properties among which anthocyanins and carotenes that were previously reported to increase in As-treated plants of *A. atacamensis* (Vromman et al., 2016b). These compounds, however, are high in the leaves but not in the roots where an As-induced decrease in MDA was recorded here.

Regarding B treatments, GSSG and GSH ratio increased in roots. This indicates that GSH was mainly used as an antioxidant in roots. The GSht concentration and the S fraction used in it decreased (see Annex A). Furthermore, the PCs concentration was as low as in control which indicates that B complexation with this molecule is probably not the strategy adopted by the

plant. From these results, it seems that B and As does not share the same detoxification pathway. Indeed, one of the B excess response is the complexation with organic molecules containing hydroxyl groups (Roessner et al., 2006).

In leaves, no significant effect of B was observed concerning the GSSG/GSH ratio and the GSht concentration. The total S content tended to decrease under B treatment but under mixed treatment, it was mitigated by As addition. The same trend was observed concerning the fraction of S used in GSH. However, the total S, the total GSH and the PCs concentrations were much higher in leaves than in roots.

The concentration of antioxidants (total phenolics and flavonoids) was also higher in leaves than in roots and it increased under B treatments. These results seem logical since the oxidative stress was much higher in this organ. On the contrary, the total phenolics and flavonoids roots concentrations increased under As and decreased under B treatments. Some osmocompatible solutes such as proline (Kaur and Asthir, 2015) and GB (Chen and Murata, 2008) were also shown to act as antioxidant. Glycinebetaine mainly accumulated in the leaves under the B excess conditions while proline accumulated under the mixed treatment.

Based on these results, it might be hypothesized that the production site of GSH and antioxidants is located in leaves tissues of *A. atacamensis*. Total phenolics and flavonoids could be preferably used by the plant under high oxidative stress and thereby a poor fraction would remain available to be translocated to the roots. On the contrary, GSH would not especially be used in leaves since the results obtained does not change from the control but it could be used in roots mainly as an “emergency” antioxidant.

4.2 The impact of vermicompost tea on the arsenic absorption by *Atriplex atacamensis* PHIL.

In order to identify the specific effect of VCT on the physiological behaviour of *A. atacamensis* under metal stress, the present discussion is based on the comparison of plants treated with and without VCT.

4.2.1 Plant growth and water status

The present work suggests that the impact of VCT remained limited under control conditions. Numerous studies mentioned that plant growth could be improved by vermicompost even in the absence of stress (Chinsamy et al., 2014; Haghighi et al., 2016; Mengistu et al., 2017). Vermicompost and VCL have been recommended to increase crop yield (Arancon et al. 2007; Atiyeh et al. 2000; Kiyasudeen et al. 2016; Sharma et al. 2005). It has however to be mentioned that our plants were cultivated in hydroponic culture with an optimal nutrient composition while the positive impact of vermicompost may be due to an improvement of the soil structure (Azarmi et al., 2010; Zucco et al., 2015). Moreover, the duration of VCT treatment remained limited and any beneficial advantage of it may require longer exposure. A slight, although non-significant, increase in root growth was recorded in control conditions. Some studies reported that such improvement may be related to auxin properties of humic acids (Canellas et al., 2002) and the analyzed VCT obtained from PUR VER contains bioactive IAA which could hasten root growth.

As far as stressed plants are concerned, results demonstrates that VCT improved the plant resistance to B excess since it significantly reduced the mortality percentage of treated plants. Water content of the considered organs remained unaffected by VCT. This parameter was already unaffected by metal stress, and such observation is relevant to the fact that *A. atacamensis*, as mentioned before, is quite well adapted to it (Vromman 2018).

It is noteworthy that proline content was significantly lower in plants exposed to stress in the presence of VCT. This is at first sight a surprising result, since proline has been detected in VCT. Similar findings were however reported by Chinsamy et al. (2014) and by Benazzouk et al. (2018) on tomato exposed to water and salt stress, respectively. Despite the decrease in proline content, WC remained unaffected in plants simultaneously exposed to ion toxicity and VCT. It may be hypothesized that proline accumulation is triggered by signaling pathways induce by an internal metabolic constraint. In plants exposed to VCT, the internal constraint was lower than in plants exposed to stress in its absence, thus explaining a lower proline accumulation. Although this hypothesis could be valid for the leaves, a decrease of proline in the roots of control plants however remains puzzling.

4.2.2 Vermicompost tea interesting compounds

Some compounds present in VCT may help the plant to cope with abiotic stress and this is especially the case for phytohormones. Vermicompost tea contains significant amounts of ABA, which is a well-known « stress hormone » involved in stress resistance (Khadri et al., 2007). Absciscic acid induce stomatal closure. Although this parameter was not recorded in the present study, a lower stomatal conductance may explain the maintenance of water content despite a decrease in proline concentration (Kudoyarova et al., 2010). Besides its important role in stomatal closure, ABA is also inducing the expression of several genes coding for protective chaperones proteins and involved in the stabilization of intracellular structures (Ton et al., 2009). In the present work, we also detect the presence of CK, especially of *trans*-zeatin CK types. These compounds also assume protective functions and mainly act as antisenescent hormones. Cytokinins are clearly involved in plant resistance to ions toxicities (Aremu et al., 2014; Ghanem et al., 2008). We do not have experimental proofs, at this stage, that CK present in VCT was absorbed by *A. atacamensis*. Nevertheless, Benazzouk et al. (2019b) recently reported that VCL drastically modifies CK endogenous content of salt-treated tomato which could, at least partly, explain the beneficial impact of VCL on salinity resistance. In this latter case, however, CK were mainly present in the *cis*-zeatin type, which is considered to be quite less active than the *trans*-zeatin type recorded in the PURE VER product. We also reported the presence and *trans*-zeatin riboside which is a stable form mainly used by the plant for long distance translocation.

Salicylic acid was detected at high concentration and may also assume protective functions of the stressed tissues. It may induce the synthesis of protective heat shock proteins and reduce the impact of water stress (Bezrukova et al., 2001). It contributes to maintain efficient photosynthesis through stabilization of PSII and avoidance of stress-induced decrease in chlorophyll content. It is also interesting to mention that SA also plays a key role in biotic stress signalling and could thus be involved in numerous disease resistance processes (Moreau et al., 2012). Shahba et al. (2010) demonstrated that SA decreases proline concentration in tomato and this corresponds to the present results obtained on *Atriplex atacamensis*.

4.2.3 Mineral nutrition

The beneficial impact of VCT on As-exposed plants was evident from a decrease in As concentration in the roots; such a decrease was recorded for both plants exposed to As alone but also for plants exposed to the mixed treatment. A vermicompost-induced decrease in As content in the roots was not leading to an increase in the shoot, confirming that VCT reduced the absorption process with no impact on the translocation step. It still remains to be determined if exogenous vermicompost act on the transporters involved on As absorption or if the VCT modifies the As speciation within the solution, leading to less bioavailable As forms.

One hypothesis could be that VCT improved the selectivity of phosphate transporters allowing them to more efficiently discriminate between phosphate and arsenate. This, probably, is not valid since P content in the roots of vermicompost-treated plants also decreased comparatively to plants that were not exposed to VCT. Such a decrease is surprising since Bhattacharya and Chattopadhyay (2002) reported that earthworms increased the bioavailability of P present in fly-ashes.

Vermicompost tea contains relatively high concentrations of S, GSH and proteins. The latest can be constituted by some cysteine amino acids. These compounds contains thiols functions known to form complexes with As species (Serves et al., 1995; Shen et al., 2013). Thereby, it may be possible that As-thiols complexes could not be absorbed by the plant. Pati and Makhopadhyay (2009) also report a reduction of the As uptake by the rice species *Oryza sativa* L. treated with vermicompost or farmyard manure. In our work, VCT slightly, although significantly, increased S content in both leaves and roots of As-treated plants. However, this additional S absorbed at the root was probably not use for non-protein thiol synthesis since PCs content decreased in this organ as a natural consequence of a decrease in accumulated As.

In contrast to As, B accumulation was increased in the leaves of plants exposed to VCT, while accumulation in the roots strongly decreased. This suggests that VCT is acting at the translocation step and may perhaps influence the activities of transporters involved in the xylem loading. Some compounds present in VCT may directly influence the regulation of membrane transporters, including transporters involved in the efflux of elements from the parenchyma cells to the xylem vessels. Polyamines are small aliphatic amine interacting with a wide range of transporters and Benazzouk et al (2019b) hypothesized that spermine and spermidine present in VCL may help to reduce Na^+ absorption by salt-treated tomato. However, in the present case, polyamines were not detected in VCT. Because polyamines may be synthesized by the plant in response to a wide range of phytohormones, the possibility that VCT indirectly stimulate endogenous polyamine synthesis independently of its own content in these products could not be ruled out.

4.2.4 Effect of vermicompost tea on oxidative stress management

The results of MDA quantification indicate that VCT had globally a beneficial effect on the oxidative status of the plants, mainly in leaves of plants exposed to B toxicity and to mixed treatment. Indeed, leaf MDA strongly increased in response to B but VCT strongly reduced the oxidative stress in these plants. In the case of B-treated plants, it is important to notice that VCT decreased oxidative stress, although it increased B accumulation in the tissue, which implies that VCT significantly improved tolerance to endogenous B and not only acts through a decrease in B accumulation.

The protective effects of VCT in the leaves of B-treated plants could hardly be explained by an increase in GSH content since VCT had no significant impact on this parameter, which is surprising considering that VCT contained high amounts of GSH ($480 \mu\text{mol.L}^{-1}$). Vermicompost however increased GSH in the roots of B-treated plants. Ascorbate was also present in VCT but at lower amounts than GSH and it was not quantified in the tissues.

Phytochelatin concentrations in leaves and roots were not affected by VCT addition, except under As treatment: in this case, the significant PCs concentration decrease may be linked to the reduced As absorption.

The total phenolics and flavonoids roots concentrations were not affected or increased under VCT treatment. These compounds are frequently reported to act as antioxidant and were detected in the PUR VER VCT. Leaves however, presented a different behaviour and both total phenolics and flavonoids decreased in response to VCT in As and in B-treated plants but increased in control plants.

Naguib et al. (2012) reported that total phenolic and flavonoid contents were slightly higher in broccoli cultivars as a result of organic fertilizer treatment. Kheiry et al. (2016) also observed a significant effect of organic fertilizers on the flavonoid content of calendula plants. Anari Anaraki et al. (2016) tested foliar and soil application of humic acids on pomegranate fruit and reported an increase of phenolics and flavonoids concentrations as well. Finally, Gholami et al. (2018) tested the effect of vermicompost and humic acid on the antioxidant activity of chicory. They also reported an increase in total phenolics, flavonoids and others antioxidants concentrations. These authors suggest a hormonal change that may increase the activity of enzymes involved in antioxidant synthesis or the presence of compounds inducing the production of more shikimic acid, precursor of antioxidants. The ratio of carbon and nitrogen may also have an influence on the antioxidant compounds production.

Beside antioxidant compounds, some antioxidant enzymes also play a crucial role in plant resistance to environmental constraints. Most of them are encoded by multigene family and some of those genes may be induced by specific phytohormones: some genes coding for superoxide dismutase (EC. 1.15.1.1), catalase (EC. 1.11.1.6), ascorbate peroxidase (EC. 1.11.1.11) and glutathione reductase (EC. 1.8.1.7) may, to some extent, be regulated by phytohormones (Miller et al., 2010). Cytokinins, SA and ABA play key roles in this respect (Aremu et al., 2014). It could thus not be excluded that vermicompost acts on antioxidative properties through the stimulation of antioxidative enzymes rather than on synthesis of endogenous antioxidant compounds.

These results confirm the reduction of oxidative stress as well as the reduction of the use of GSH as an antioxidant. It seems evident that VCT, especially humic acids and/or

phytohormones that it contains, increases the antioxidant activity of the plant and thereby help it to resist metal stress. This could be due to an external simulation of their synthesis or because of the absorption of antioxidants present in the VCT. The decrease of phenolics and flavonoids leaves concentrations under metal stress may be explain by the involvement of other antioxidants more effective in abiotic stress induced by metals. Indeed, VCT contains also anthocyanins, ascorbate and maybe others that might have mitigated the oxidative stress.

Conclusion and perspectives

Atriplex atacamensis is able to cope with high As concentration (500 μM) since the mortality percentage was 0%. On the contrary, the applied B concentration (500 mg.L^{-1}) induced a mortality percentage above 50% and was probably too high. Arsenic treatment did not affect the leaf WC and the absence of synthesis of protective compounds (proline and GB) suggests the absence of internal constraint and thus confirms the relevance of using it for phytoremediation applications in arid conditions. On the contrary, results show that B induced the synthesis of proline and GB, but this allows an effective adaptive response since no decrease in WC was recorded. Furthermore, we observed that mixed treatment exacerbated the synthesis of these osmoprotectants.

Arsenic was mainly accumulated in roots unlike B which mainly accumulated in leaves. In both organs, the mixed treatment increased the B content compare to plants only treated with B but decreased As concentration in roots as well as the As translocation to leaves. This result might be explained by the shared transport mechanism of both elements by similar aquaporins. B could have increased the ion efflux mechanisms and therefore simultaneously increased the efflux of As stored in roots. Furthermore, simulation of specific aquaporins by B could have induced a decrease of As translocation to aerial part. On the other hand, As enhances vacuolar sequestration mechanism. Therefore, the addition of As to B might have increased the vacuolar accumulation of the later in leaves and roots.

MDA quantification indicates that an oxidative stress occurred in leaves as a result of B excess. Arsenic toxicity alone did not increase the MDA concentration in leaves, which confirms high level of resistance of *A. atacamensis* to accumulated As. However, it clearly exacerbated the negative effect of B in mixed treatment.

Glutathione is a well-known antioxidant but also a precursor of the PCs involved in As vacuolar sequestration. The recorded results confirm the importance of this detoxification mechanism. They also confirm that B does not share the same detoxification pathway and suggest that total phenolics and flavonoids are preferably used under high oxidative stress while GSH appears to be an “emergency” antioxidant.

Regarding the effect of VCT, no significant effect on the plant growth was recorded as well as on the WC. However, a significant decrease of proline concentration in leaves and roots of stressed plants suggests that VCT is a valuable tool to reduce the internal constraint inducing the proline accumulation. This protective effect of VCT is confirmed by the VCT-induced decrease in MDA.

Conclusion and perspectives

The PUR VER[®] vermicompost tea contains several phytohormones that may help the plant to cope with abiotic stress. Abscissic acid, a well-known “stress hormone”, induces the stomatal closure that may explain the maintenance of WC despite a decrease in proline concentration. Cytokinins were also detected and are involved in plant resistance to ions toxicities. Salicylic acid was recorded as well at high concentration and may also assume protective functions of the stressed tissues. VCT also contains minerals nutrients as well as different antioxidants.

The results confirm that VCT reduced the absorption of As. This may be due to modification of the As speciation within the culture solution because of the presence of thiol functions in VCT complexing with it, leading to less bioavailable As forms. In contrast to As, B accumulation was increased in the leaves of plants exposed to VCT while accumulation in the roots strongly decreased. Despite the B content increase, leaf MDA decreased under the action of VCT which implies that it significantly improved tolerance to endogenous B. Indeed, results show that VCT, especially humic acids and/or phytohormones that it contains, increase the antioxidant activity of the plant and thereby help it to resist metal stress.

According to the obtained results, the use of *A. atacamensis* for phytostabilisation purpose stay relevant. However, B excess clearly reduces its ability of As absorption and sequestration. At this concentration, B also induces a strong oxidative stress exacerbated by the mixed treatment that cause the dead of more than 50% of the plants. It would be interesting to quantify the ions concentrations in dead plants as well as evaluate the impact of lower B concentration. The potential shared transport mechanism should also be look closer.

Further work could be interesting to deepen our knowledge on As and B impact on *A. atacamensis*, especially in the presence of VCT. From a physiological point of view, the influence of VCT on stomatal conductance, transpiration rate and net photosynthesis may provide interesting information on its influence of ion absorption and translocation. The precise localization of elements in the tissues through adapted technics (laser ablation micro-pixe) could also be of special interest. Speciation of accumulated As may help us to understand the detoxification mechanisms used by the plants against this element. Analysing by RT-PCR the expression of genes coding for aquaporins may also provide information to support or invalidate our hypothesis concerning the efflux process in plants exposed to mixed treatment. It could also be quite interesting to establish the phytohormonal profile inside the plant tissues of plants exposed to the various treatments and to compare it with the phytohormonal profile established here for VCT.

Finally, it could also be important to apply the treatment for longer periods and at different growth stages and to lower the B doses in order to more adequately match to field conditions.

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Annex

Annex A: S fraction used in GSht

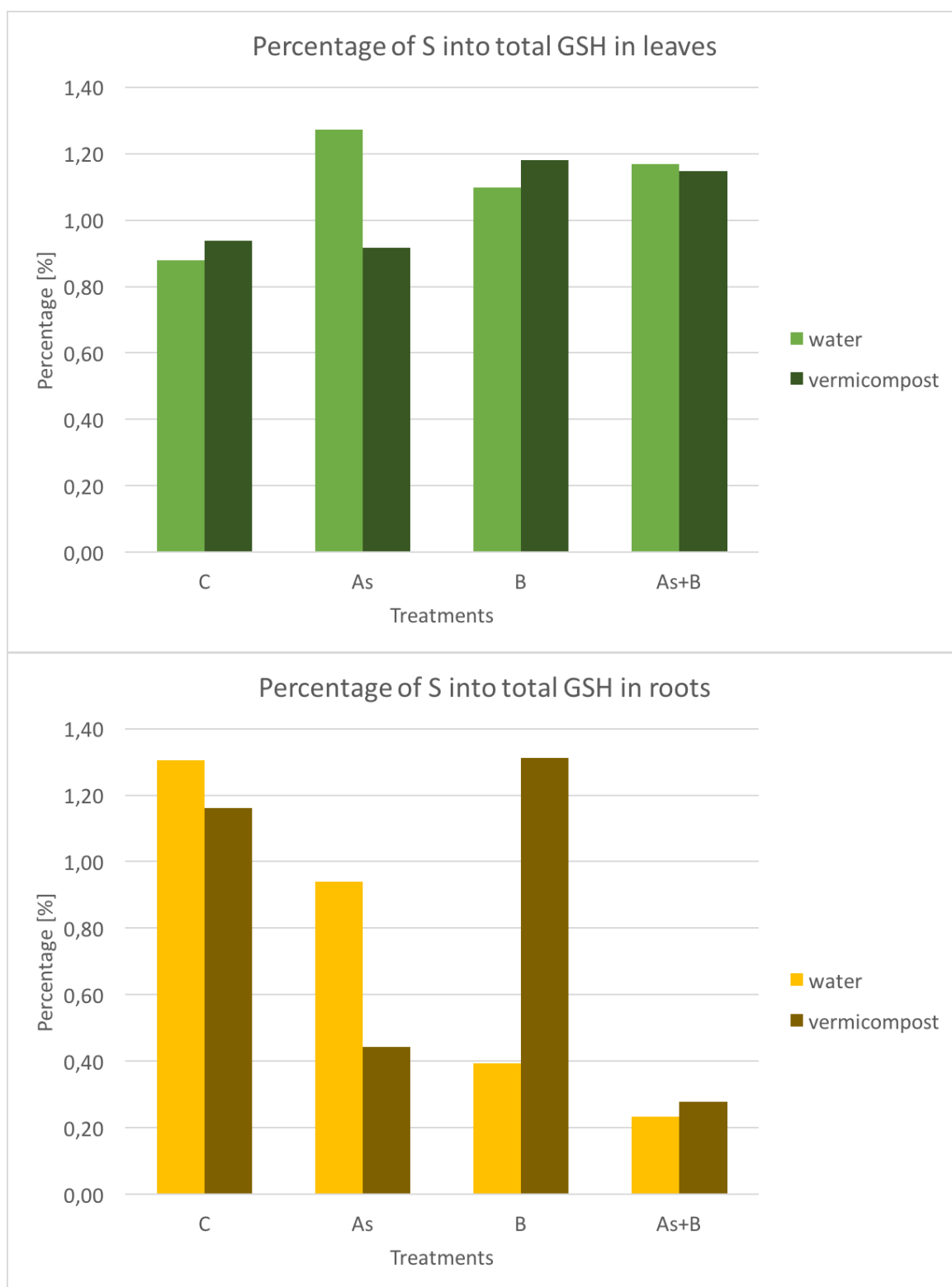


Figure 39 Fraction of S presents into GSht in leaves and roots of *Atriplex atacamensis* after two weeks of treatment (control, As (500 $\mu\text{mol.L}^{-1}$), B (500 mg.L^{-1}), As (500 $\mu\text{mol.L}^{-1}$) and B (500 mg.L^{-1})) in hydroponic culture with or without vermicompost tea addition in the Yoshida solution (10%) (vermicompost or water respectively). Each value is the mean of 3 replicates. No statistics were performed on these data since it is calculated from mean values of different measurement

The impact of boron excess and vermicompost tea on the arsenic absorption by *Atriplex atacamensis* PHIL.

Ségolène Marcelis

The Atacama Desert, in Northern Chile, is one of the most arid place in the world. Some regions like the Quillagua oasis are highly contaminated by As and B. In addition to natural reasons, mining activities are the cause of the increasing contamination of the unique source of water, the Rio Loa river

A. atacamenis is a xerohalophyte plant species endemic of this region. This plant is almost the only species able to survive in these very harsh conditions. Previous researches have mentioned its potential for phytostabilisation applications of As-contaminated soils since the plant is able to accumulate As in its roots. The plant can also deal with an excess of B but the behaviour of the plant simultaneously exposed to As toxicity and B excess has not yet been explored.

On the other hand, vermicompost is subject to growing curiosity in the scientific community. This consists in the digestion product of organic matter by earthworms. It contains interesting chemicals and is known to act as a biostimulant. Its beneficial effects on plant growth, biotic and abiotic stresses have already been observed. However, the effect of vermicompost on metal stress is poorly documented.

The present study aims to highlight these gaps; its first objective is to provide the global composition of the vermicompost tea (VCT) used, the second to identify the effect of B excess and VCT on As absorption by *A. atacamensis*. To carry out this experiment, *A. atacamensis* were grown in hydroponic culture. Half of plants were treated with 10% in volume of VCT, kindly provided by the company PUR VER®. As (500 µM; in the form of arsenate) and B (500 mg.L⁻¹) were applied separately or simultaneously. The eight treatments (Control, As, B and As+B, with and without VCT) lasted two weeks. VCT composition was analysed in detail. Leaves and roots masses and WC were measured as well as ions content. Two osmoprotectants, proline and GB, were quantified. MDA, GSH, PCs, total phenolics and flavonoids concentrations were also quantified in leaves and roots.

Results confirm the resistance of the plant to such As concentration but the B concentration applied induced a high level of plant mortality. Surviving plants, however, efficiently produced osmoprotectants and were able to maintain the WC. B significantly decreased the As content while As increased B content in the plant. B induced a high oxidative stress and plants mitigated it by producing antioxidants while As was mainly complexed with PCs.

VCT did not have significant impact on plant growth and WC but improve plant tolerance to accumulated ions. It drastically decreased the proline concentration probably by having an effect on the internal signalling pathway. VCT contains numerous phytohormones (ABA, CK, SA,...) putatively involved in plant resistance against abiotic stress. It increased B translocation to aerial parts without affecting the MDA concentration. VCT increased the antioxidant activity of the plant and thereby help it to resist metal stress. VCT thiols rich compounds may modify the As speciation decreasing its bioavailability. It is concluded that *Atriplex atacamensis* is a promising species for phytomanagement of B and As-contaminated areas and that VCT is a useful tool to improve its level of stress tolerance.