Suitability of marginal sites contaminated by trace elements for the production of non-food biomass: lessons from lysimeter experiments

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Résumé

Dans le contexte de post-révolution industrielle, les techniques de phytomanagement pour la revalorisation de sites pollués sont considérées comme des nouvelles techniques prometteuses mais elles présentent encore quelques difficultés au niveau de leur mise en application. Le risque de transfert des polluants doit ainsi pouvoir être limité. Le présent travail avait comme but général d'évaluer l'aptitude des friches industrielles à la production de biomasse par la mise en place de deux groupes d'expérimentations en lysimètres. La première expérimentation visait à évaluer l'impact de trois types de fertilisants appliqués à différentes doses sur la production de biomasse ainsi que sur la lixiviation des éléments traces et de l'azote total. La deuxième expérimentation faisait partie du projet ECOSOL et impliquait plusieurs variétés de colza ainsi que des herbacées. L'objectif général de ce projet était d'évaluer l'impact de la croissance des plantes sur la solubilité des éléments traces.

La première expérience a montré que l'augmentation de l'application d'azote sur le sol permet d'augmenter la production de biomasse, quelle que soit la fertilisation utilisée. Cependant, compte tenu de la grande quantité d'azote lessivé et de l'augmentation possible du pH induite dans le cas de l'azote minéral, la fertilisation organique a été envisagée pour les conseils de fertilisation, étant donné que les amendements organiques ont semblé se démarquer positivement. En particulier, les boues ont parfois permis de réduire la biodisponibilité de certains éléments traces tout en permettant un apport régulier grâce à la minéralisation de l'azote organique. L'augmentation de la biomasse sur le sol non pollué était extrêmement marquée, par rapport au sol pollué, et les quantités d'éléments-traces phyto-extraits étaient plus élevées dans le sol non pollué pour le Cd et le Zn et dans le sol pollué pour le Cu et le Pb. Comme de très faibles quantités d'élements traces ont été détectées dans le lixiviat, aucune tendance n'a été identifiée concernant leur évolution.

En ce qui concerne la deuxième expérimentation, aucun résultat n'a été considéré comme étant de bonne qualité en ce qui concerne l'évolution temporelle du lixiviat. Ce manque de données a toutefois permis d'apporter des perspectives et de mettre en évidence la possibilité de sélectionner les variétés selon des critères de biodiversité pour de futures expériences.

Abstract

In the context of the post-industrial revolution, phytomanagement techniques for the rehabilitation of polluted sites are considered promising new techniques but still present some difficulties in their application. The risk of pollutant transfer must therefore be limited. The general aim of this work was to assess the suitability of brownfield sites for biomass production by setting up two groups of experiments using lysimeters. The first experiment aimed to assess the impact of three types of fertilisers applied at different doses on biomass production and on the leaching of trace elements and total nitrogen. The second experiment was part of the ECOSOL project and involved several varieties of rapeseed as well as herbaceous plants. The overall objective of this project was to assess the impact of plant growth on trace element solubility.

The first experience has shown that increasing nitrogen application to the soil increases biomass production, regardless of the fertilisation used. However, given the large amount of leached nitrogen and the possible increase in pH induced in the case of mineral nitrogen, organic fertilisation was considered for fertilisation advice, as organic amendments seemed to stand out positively. In particular, sludge sometimes reduced the bioavailability of certain trace elements while allowing a regular supply thanks to the mineralisation of organic nitrogen. The increase in biomass on unpolluted soil was extremely marked compared to polluted soil, and the quantities of phyto-extracted trace elements were higher in unpolluted soil for Cd and Zn and in polluted soil for Cu and Pb. As very small quantities of trace elements were detected in the leachate, no trend was identified regarding their evolution.

For the second experiment, no results were considered to be of good quality regarding the temporal evolution of the leachate. This lack of data did, however, make it possible to provide perspectives and to highlight the possibility of selecting varieties according to biodiversity criteria for future experiments.

Abbreviation list

Abbreviation	Meaning
ANOVA	Analysis of variance
glmer	Generalized Linear Mixed Model
ICP	Inductively coupled plasma mass spectrometry
lmer	Linear mixted-effect models
ROS	Reactive oxygen species
SSV	Soil screening value
TE	Trace elements
OM	Organic Matter

Contents

List of Figures

List of Tables

1 Introduction

1.1 Context

After having made Walloon region prosperous for a century, the industrial sector has left polluted soils in its downfall, unsuitable for agronomic or land use, commonly called brownfields or marginal contaminated sites (Parmentier, 2008; Evlard and Gossiaux, 2018). In the European Union, the soil of approximately three millions sites are suspected of being contaminated and 250 000 contaminated sites are known to require cleanup (Payá Pérez and Rodríguez Eugenio, 2018). In Wallonia, 6 000 sites have been identified as potentially polluted (Colinet et al., 2009), covering an area of 49 100 hectares (ValBiom, 2014).

Currently, the revaluation of brownfield sites are at the centre of concerns, for several reasons. Exposure to contaminants generated by industrial activity causes environmental issues, as well as a serious risk to public health (Su et al., 2014). The limited availability of land makes it necessary to clean up these sites while still being able to develop them economically. In this way, especially in the case of trace elements (TE) contamination, phytoremediation appears as a non-intrusive and inexpensive new approach for remediating contaminated soil, and combining it on marginal land while supporting bioenergy or phyto-products production is an interesting mean for landowner to add economical value to their land without compromising food security (Van Ginneken et al., 2007). Other possibilities for valorization can also be explored, such as the use of specific molecules from these plants in the industrial or pharmaceutical field (Masarovičová and Kráľová, 2012).

Despite proven success of phytoremediation in laboratories, its field applicability is still facing difficulties (Saxena et al., 2019), one of them being in association with risks associated with the potential leaching and spreading of trace elements enhanced by soil acidification. In this way, attention is paid to pH regulation, as it is considered to be the main driver regulating the mobility of trace elements in the soil (White and Broadley, 2009).

1.2 Objectives

It is in this context that this master thesis takes place, with the general objective of assessing the suitability of brownfield sites for the production of non-food biomass. The main focus of the study was put on the issue of leaching of contaminants during the exploitation of the site. It is hoped to progress in the knowledge which govern the mobility of trace elements in marginal contaminated soil by setting up two series of lysimeter experiments, each with their own specific objectives.

1.2.1 Effect of nitrogen fertilisation on willow growth and on the leaching of trace elements as well as total nitrogen in marginal soil

The first experiment was launched in April 2019 and aimed to compare the growth of willows on two types of soil and using several types of amendments. The polluted soil studied came from the former iron and steel site of Carsid (Duferco), which was compared with an unpolluted soil from Gembloux. The fertilisation regimes used were digestate, sewage sludge and mineral fertilizer and these were tested on both types of soil at different doses.
The first part of this work is a continuation of this project and aims to answer the following research questions:

1. How much biomass is produced in the second year and at what growth rate?
2. Which fertilisation regime gives the highest yields?
3. How much total nitrogen and TE are leached during the second year?

1.2.2 ECOSOL project

The second series of experiments launched is intrinsically part of the ECOSOL project which started in July 2016 and will end in September 2022. It is a multidisciplinary project that involves five laboratories of the University of Liège specialised in agronomy, plant biology and genomics, chemistry, pedology and pharmacy. These units work together with the common objective of enabling the reconversion and greening of the industrial wastelands of the former Auvelais chemical factory. The production of biomass and ecosystem services and the valorisation of molecules with high added value would be the main economic strategies favoured. This work investigates the ability of selected non-food plants to grow when planted on the polluted soil of the former Auvelais chemical plant. Species are selected according to their potential for phytoremediation use and/or pharmaceutical valorisation.

1.2.2.1 One-meter high lysimeters

The first lysimetric set-up measure 1m high and received in 2020 *Alliaria petiolata* (Bieb.) Cavara and Grande., *Lolium perenne* L., *Tanacetum vulgare* L. and the spring rapeseed axana, cleopatra, mosaïk and theia varieties. The research questions related to these experiences are as follows:

1. Can rapeseed grow and produce seeds on TE contaminated soil?
2. Are TE likely to migrate into the soil-plant water system?
3. Is the solubility of TE affected by plant growth, depending on the depth considered?

1.2.2.2 Fifteen-centimeter high lysimeters

In the second fifteen-centimeter high lysimetric set-up, the same four spring rape species were sown in 2020 for two months. After harvest, eight herbaceous species were implanted. These experiences aim to answer the following questions:

1. Are the selected species suitable for growth in heavily contaminated soil?
2. Can they improve soil properties in the long term?
3. Is TE solubility affected by plant growth?

2 State of art

2.1 Marginal lands

2.1.1 An attempted definition

Currently, the definition for marginal lands differs across discipline and is not scientifically agreed (Liu et al., 2011). However, according to the report of Nuffield Council on Bioethics (2011), "it has been commonly used to refer either to land that is unsuitable for food agriculture or land that has a low carbon stock". From the fertility point of view, FAO (2011) defines marginal land as an unsuitable land who produces less than 40 percent of potentially attainable yields, whereas cultivable lands are supposed to produce 80 percent. They can be unsuitable for food crops because of either poor soil quality, nutrient depletion, steep land, inorganic or organic contamination (Nsanganwimana et al., 2014).

Within the framework of this master thesis and given the lack of consensus on this term, a definition adapted to the realities of Wallonia's soils was selected. In the guidebook written by Evlard and Gossiaux (2018), a marginal use site is defined as "a site that cannot be used for food or land (real estate) purposes and/or shows signs of abandonment (abandoned site, no visible site maintenance or no site maintenance in the near future). This site may or may not present soil alterations (through pollution and/or erosion and/or loss of organic matter)". This definition therefore includes brownfield as well as unused land for biomass production. In Wallonia, wasteland of this type covers 49 100 hectares (ValBiom, 2014). From here on, the continuation of this literature review focuses mainly on marginal contaminated lands such as brownfield sites.

2.1.2 Phytomanagement practices and valorisation tracks

In view of the risks related to the spread of soil and groundwater contamination caused by brownfield sites (Beames et al., 2015), the first step to consider would be to remediate them. However, the cost for cleaning up are extremely high (Megharaj and Naidu, 2017), although it depends on various factors, such as the type of pollutant, the soil characteristics and the remediation technology used. On top of that, conventional remediation methods cause greenhouse gas emissions (Witters et al., 2012) and even if they have a rapid effect, they tend to destroy soil biological activities (McGrath et al., 2001) .

Over the last few years, a lot of research has been done on one technique in particular, phytoremediation, which is defined as "the use of plants to remove pollutants from the environment or to render them harmless" (Raskin et al., 1997). It appears to be the least destructive, most eco-friendly and cost efficient remediation technique (Khalid et al., 2017), and has proved to be particularly suitable for TE-contaminated soil (Pandey et al., 2019). Phytoremediation is generally classified into phytostabilisation, phytodegradation, phytovolatilisation and phytoextraction, depending on the mechanism involved (Figure 1).

Phytoextraction	The use of pollutant-accumulating plants to remove metals or organics from soil by concentrating them in the harvestable parts
Phytodegradation	The use of plants and associated microorganisms to degrade organic pollutants
Phytostabilisation	The use of plants to reduce the bioavailability of pollutants in the environment
Phytovolatilisation	The use of plants to volatilise pollutants

Figure 1: Definitions of the different areas of phytoremediation, according to Salt et al. (1998)

Since phytoremediation without biomass valorisation is not economically sustainable in the long term (Vigil et al., 2015), the current trend is to go towards integrating phytoremediation objectives with sustainable and cost-effective soil management, which is called phytomanagement. The economic aspect is often favoured over the remediation aspect, as long as care is taken not to provoke the dispersion of contaminants in the environment (Robinson et al., 2009). Moreover, many benefits can be obtained from the vegetalisation of contaminated site, the main one being the restoration of some ecosystem services, such as erosion control, nutrient cycling, carbon storage (Burges et al., 2018) and biodiversity support (Jan and Parry, 2016).

The industrial use of biomass grown on polluted soils depends on the plants' response to pollutants. Plants that are specifically adapted to survive in TE-rich soil, known as metallophytes, are divided into three categories (Figure 2). Hyperaccumulators are usually used for phytoextraction and excluders may be efficient for phytostabilisation purposes (Ali et al., 2013; McGrath et al., 2000). Even if many authors tend to classify metallophytes, caution should be exercised with regard to this classification in view of the wide genetic inter- and intra-specific variation (Pollard et al., 2002).

Excluders	Plant species that restrict the transport and entry of TE into aerial part
Hyperaccumulators	Plant species capable to accumulate trace elements in their shoot tissues to levels far above those present in the soil or in non-accumulating plant species, without exhibiting any toxicity symptoms.
Indicators	Plant species having a TE content in their shoot tissues proportional to the TE concentration in the substrate

Figure 2: Categories of metallophytes, as defined by McGrath et al. (2000) and Ali et al. (2013)

The sustainable processing of transforming metallophytes into a spectrum of marketable products - e.g. chemicals, composite materials - and energy is called “biorefinery” (Sotenko et al., 2016).

Figure 3 gives an overview of the wide number of industries involved as well as some possibilities of by-products generated from metals-enriched biomass.

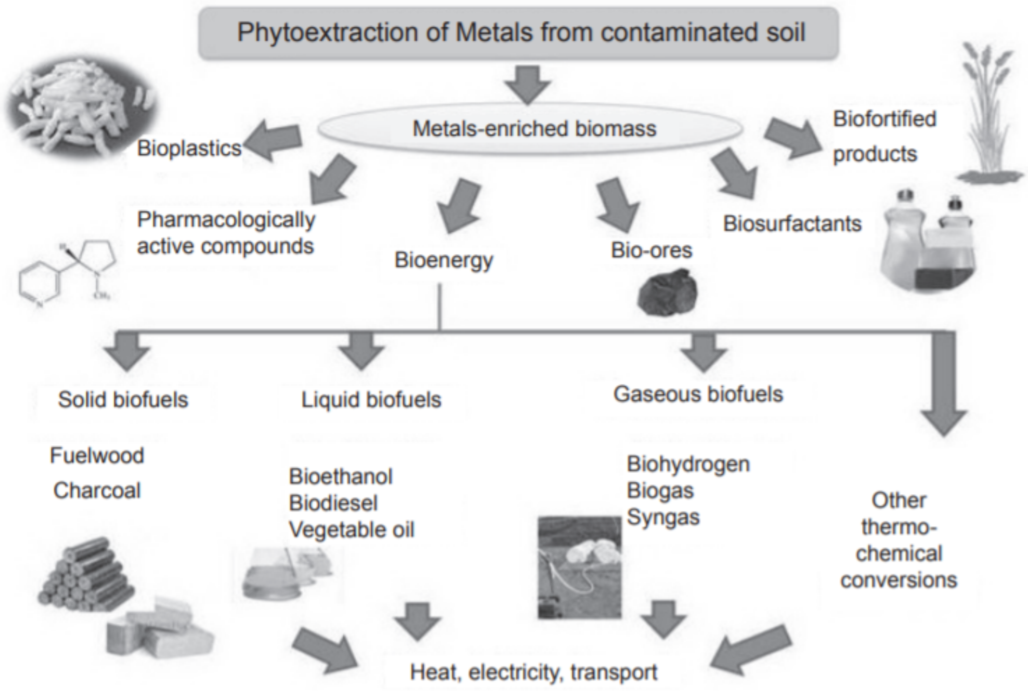


Figure 3: Valorisation tracks for metal enriched biomass (Donati, 2018)

2.1.2.1 Bioenergy industry

Wood energy has been used since the dawn of time as the very first source of renewable energy and today it is still providing about 6% of the global total primary energy supply (FAO, 2020). The term “bioenergy” is used to design all types of energy stem from biofuels, which are fuels derived from matter of a biological origin, or biomass (FAO, 2004). FAO classify biofuels in three group according to the source of biomass used: woodfuels, agrofuels and municipal by-products. Each of these groups is divided into solid, liquid and gaseous forms of fuels that can be used for heat or power generation (Mabee and Saddler, 2007).

At first, the use of biofuels as an alternative to fossil fuels was seen as a green solution to cover energy needs (Timilsina and Shrestha, 2011) but soon, controversy over the use of agronomic land for bioenergy production surfaced (Mabee and Saddler, 2007). Problems of land-use competition, implications for food prices (Headey and Fan, 2008; McMichael, 2010; Ciaian and Kancs, 2011; Rezitis and Sassi, 2013) and increased incentives for deforestation (Deepak, 1983; Danielsen et al., 2009) were quickly identified. Considering crossed environmental and socio-economic externalities implied by the use of bioenergy (Brose et al., 2010), a commonly accepted solution is to limit energy crop production to marginal productivity sites. It is indeed an interesting mean for landowner to add economical value to their land without inducing land-use change or compromising food security (Van Ginneken et al., 2007).

2.1.2.2 Other industries

In addition to the utilisation for energy purposes, a large number of industries are involved in the valorisation of biomass produced from the phytoextraction process. A process called phytomining allows to extract bio-ores from hyperaccumulators (Brooks et al., 1998). Apart from ores, some elements are necessary for humans in small quantities and can be used as supplement in case of deficiency, as in the case of selenium (Bañuelos, 2006). In addition, some studies have shown that trace elements, when present in certain medicinal plants, can act as remedies (Lichtfouse, 2016). Metal-based drugs, known as "bhasmas", are commonly used in the traditional Indian Ayurveda to propel metabolic (metallomic) processes (Prasad, 2008). Trace element contaminated soil can also be suitable for the cultivation of medicinal plants (Augustina and Adriana, 2014) and aromatic crops (Pandey et al., 2019), which can be used widely, for example in cosmetics, soaps, perfumery industry, as insect repellents and aromaterapy (Gupta et al., 2013). In the construction industry, insulating materials or biocomposites can also been made from TE-rich plant fibres such as hemp fibres (Linger et al., 2002).

2.1.2.3 Limitations and concerns

Despite proven success of phytoremediation in laboratories, its field applicability is restricted by several technical difficulties, which need to be caught up. For example, phytoextraction is limited by low metal bioavailability, slow plant growth rate and biomass, reduced metal accumulation and tolerance (Saxena et al., 2019). In reality, it is a process that takes a long time before having a depolluting or stabilising effect and is only effective on shallow pollutants, located around the root zone (Shackira and Puthur, 2019). New phytoremediation assisted technologies such as chelating agents (EDTA) were created to increase TE bioavailability and speed remediation time. However, they are far for miraculous and can lead to undesirable environmental consequences such as disruption of physicochemical properties of soils (Saxena et al., 2019).

Furthermore, some concerns remain about the use of biomass contaminated with TE. Indeed, metal-contaminated plant biomass would still require treatment prior to disposal, or at least precautions during treatment. Existing pre-treatment steps for volume reduction are composting and compaction, with leachate-collection. Pyrolisis seems directly suitable, but combustion must take place in controlled conditions, with filters adapted to retain dust containing metal (Ghosh and Singh, 2005; Prasad, 2003) .

2.2 Biochemical behaviour and physiological functions of some elements in plants and soil

2.2.1 Classification of elements in plants

Elements important for plant growth are commonly classified according both to their relative concentrations within the plant and to their biochemical and physiological functions (Figure 4). The abundance of elements in the tissues allows to distinguish two distinct groups of nutrients. The elements H, C, N, O, K, Ca, Mg, P and S are refered as macronutrients because they are present at a minimum of 0,1% of dry mass (Welch, 1995), whereas micronutrients refer to other nutrients such as Cl, Fe, B, Mn, Zn, Cu, Ni, Mo, that are equally important but present in much lower concentrations. Macro- and micronutrients are considered essential because a plant cannot complete its normal life cycle without them, they are irreplaceable and directly included in the metabolism (Arnon and Stout, 1939).

Group number and elements included inside it		General role of the group	Element	Critical leaf concentration (mg/g)	
				Sufficiency	Toxicity
1	C, H, O, N, S	Major constituents, enzymatic processes and redox reactions	N	15 - 40	N.D
			S	1,0 - 5,0	N.D
2	P, B, Si	Structural integrity, energy transfer reactions	P	2 - 5	> 10
			B	5 - 100 x 10^{-3}	0,1 - 1,0
3	K, Na, Ca, Mg, Mn, Cl	Osmotic potential, enzyme activation, membrane permeability and potential	K	5 - 40	> 50
			Ca	0,5 - 10	> 100
			Mg	1,5 - 3,5	> 15
			Mn	10 - 20 x 10^{-3}	0,2 - 5,3
			Cl	0,1 - 6,0	4,0 - 7,0
4	Fe, Cu, Zn, Mo	Chelated form in enzymes, electron transport	Fe	50 - 150 x 10^{-3}	> 0,5
			Cu	1 - 5 x 10^{-3}	15 - 30 x 10^{-3}
			Zn	15 - 30 x 10^{-3}	100 - 300 x 10^{-3}
			Mo	0,1 - 1,0 x 10^{-3}	1

Figure 4: Classification of most plant nutrients and critical leaf concentrations for sufficiency and toxicity in non-tolerant plants, adapted from Marschner (2012); Taiz et al. (2014) and White and Brown (2010)

The next point develops one macronutrient in particular, nitrogen, as it is the evolution of this element that is studied in one part of this work.

2.2.2 Nitrogen cycle

Seventy-eight percent of the atmosphere is made up of nitrogen in the N_2 form, yet this is the most frequently deficient nutrient in non-legumes plants. Apart from the application of N fertilisers, N_2 can be immobilised in soil through biological fixation by rhizobium (in legumes) or by other free-living microorganisms, or directly through the formation of N oxides by atmospheric electrical discharge (during thunderstorm for example) (Havlin et al., 2014; Kumar Chakravorti et al., 2018). The N applied as fertiliser or fixed is not entirely collected by plants, a huge part of it is incorporated into the soil organic matter, released in the atmosphere as N_2O, N_2 or NO, or leached as nitrite or nitrate (figure 5). The last 50 years of applying N-rich fertilisers have led to an increase in nitrate contamination in surface and ground water, posing health and environmental problems. Presence of nitrate in drinking water is harmful for human and livestock health and causes diseases like methemoglobinemia. It was also reported that it would causes abortions in cattle. Apart from that, excessive nitrate quantity in surface water causes eutrophication problems, which result in algal bloom and fish poisoning (Di and Cameron, 2002). Following the World Health Organization (2011), the maximum nitrate concentration that is considered harmless for short-term exposure in drinking water would be 11mg NO^{3-}N L^{-1} or 50mg/l as nitrate ion[1]. Although nitrite ions are not present in high concentrations in drinking water due to their lower oxidation stability compared to nitrate ions, a limit concentration has also been defined. The acceptable limit for nitrite ion is 3mg/l, or 0,9mg as nitrite-nitrogen[2]. In order to comply with these guidelines and for all the reasons mentioned above, estimating the ideal amount of fertiliser to be applied is essential in order to ensure productivity, while reducing the negative impacts of N inputs in the environment (Quemada et al., 2013).

The forms of nitrogen that can be taken up by plants are NO_3^- and NH_4^+. Nitrates cannot be used directly by the plant and have to be reduced to ammonium first. This process is carried out thanks to the enzymes nitrate reductase and nitrite reductase, and requires the use of two NADH, which consumes energy (Maathuis, 2009). Although ammonium is the prefered N-source (Havlin et al., 2014), it has proved to be toxic when applied alone (Babourina et al., 2007) and in any case, more difficult to maintain in soil than nitrate. Therefore, for most plants, growth is improved when nourished with both NO_3^- and NH_4^+ (Havlin et al., 2014), because nitrate can alleviate ammonium toxicity (Babourina et al., 2007). Nitrogen nutrition affects both organic acid metabolism and the element composition of plant tissues (Marschner, 2012). In this way, N deficiency induces accelerated leaf senescence and chlorosis: the lack of N protein in the chloroplasts causes yellowing of the oldest leaves, followed by browning and death in the most severe cases (Havlin et al., 2014).

[1] 1 mg/l as nitrate = 0.226 mg/l as nitrate-nitrogen
[2] 1 mg/l as nitrite= 0,304 mg/l as nitrite-nitrogen

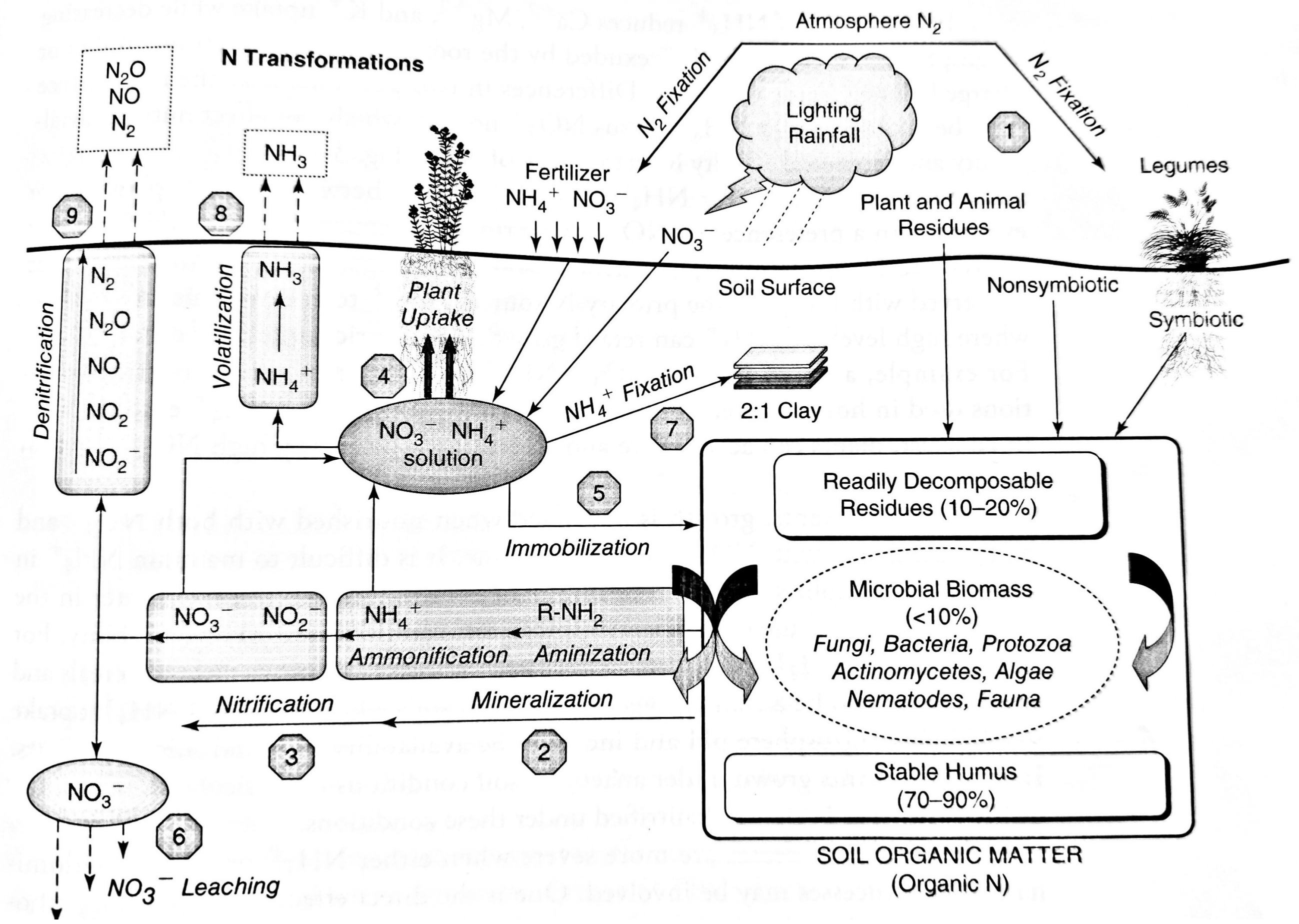


Figure 5: Nitrogen cycle (Havlin et al., 2014)

2.2.3 Trace elements

2.2.3.1 Definition and sources

Trace elements (TE) are geochemically defined as chemical elements present in the earth's crust in quantities of less than 0,1% (1000mg/kg) (Baize, 1997). This definition indicates only their abundance and not their physiological characteristics, even though some elements have been recognised as essential for plants. At present, 17 trace elements (Al, B, Br, Cl, Co, Cu, F, Fe, I, Mn, Mo, Ni, Rb, Si, Ti, V and Zn) have been recognised as essential for plants, although not all the mechanisms in which they are involved are known to date (Kabata-Pendias, 2011). "Heavy metals" is another term widely use in the literature to refer to trace elements. However, TE include elements of various chemical properties and not all of them have metallic properties (Stengel and Gelin, 2003). In this work, the term "heavy metal" will not be used in order to avoid any confusion.

Initially, TE are naturally present in the soil from the pedogenic processes and weathering of parent materials (Kabata-Pendias, 2011). However, some anthropogenic activities may have released TE or disrupt their geochemical cycle. Thus, the TE content in soil can change over time and a soil may accumulate TE at a concentration above the threshold considered to be at risk for organisms and other media (Wuana and Okieimen, 2011).

2.2.3.2 Physiology of TE in plants

Whether essential or not, any mineral element present in too high concentration in the soil solution becomes toxic and can therefore inhibit plant growth and reduce yield (White and Brown, 2010). Upon exposure to TE, plants produce reactive oxygen species (ROS) such as superoxide O_2^- and hydrogen peroxide H_2O_2. A simultaneous increase in these ROS causes the production of hydroxyl radicals (OH●), which are the most reactive and short-lived (1 ns) ROS (Sharma and Dietz, 2009). ROS lead to disturbance of cellular ionic homeostasis and cellular damage such as membrane dismantling, DNA-strand cleavage and macromolecule deterioration, to the point of inducing programmed cell death (Carrasco-Gil et al., 2012; Shahid et al., 2014; Sharma and Dietz, 2009; Parent et al., 2008).

Globally, TE-content inside the cell affect photosynthesis, respiration, mineral nutrition, enzymatic reactions and many other physiological factors (He et al., 2011; Van Assche and Clijsters, 1990). Besides the concentration, the TE toxicity effects also depend on the metal in question, the duration of exposure, the stage of plant development, the severity of plant stress, the particular organs studied and the plant species (Shahid et al., 2014; Sharma and Dietz, 2009). In order to cope with the negative consequences of heavy-metal toxicity, plants have developed two main strategies: avoidance and tolerance (Dalvi and Bhalerao, 2013).

A. Avoidance

The first defence mechanism is mainly extracellular and consists of limiting the uptake of TE and prohibits their entry in plant tissues through root cells. It involves:

- Immobilisation by mycorrhizal associations: ectomycorrhizas (ECM) and arbuscular mycorrhizas (AM) act as an exclusion barrier to metal uptake (Jentschke and Godbold, 2000; Leyval et al., 1997)
- Complexation by root exudates: root cells release a large number of secretions (e.g. ectoenzymes and polymeric carbohydrates), excretions (e.g. protons, carbon dioxide, bicarbonates) and diffusates or exsudates (e.g. amino or organic acids, ions, sugars, flavonoid-type phenolics etc), mucilage and mucigel (polysaccharides) (Kidd et al., 2009; Marschner, 2012) . Some of them can diminish the bioavailability of TE by making stable complexes or by increasing the pH of the rhizosphere, which induce TE-precipitation (Dalvi and Bhalerao, 2013).

B. Tolerance

The second defence line mainly focuses on intra-cellular TE-detoxification. This involves accumulation, storage and immobilisation of TE by binding them with amino acids, proteins or peptides. More specifically, the processes involved are:

- Immobilisation by TE-binding to cell wall (Dalvi and Bhalerao, 2013; Lal, 2010)
- Active efflux pumping at plasma membrane permits to lower the intracellular TE-concentration to subtoxic levels (Reichman, 2002)
- Complexation of TE with organic acids within cell (Reichman, 2002)
- Inactivation of toxic metals: metal-phytochelatins/ metallothioneins complex are formed and then actively transported from the cytosol across the tonoplast into the vacuole where it is stored (Tong et al., 2004).
- Oxidative stress defense mechanisms:
    1. Proline or phenol accumulation and/or release of stress related proteins like heat shock proteins (Pál et al., 2006; Singh et al., 2016; Hall, 2002)
    2. Release of hormones such as salicilic acid (Metwally et al., 2003) jasmonic acid (Xiang and Oliver, 1998), ethylene (Keunen et al., 2016; Lynch and Brown, 1997) and gibberellic acid (Mansour and Kamel, 2005)
    3. Antioxydant defence mechanisms: superoxide dismutase detoxifies O_2^- and ascorbate peroxidase (APX), peroxiredoxins (PRXes) and catalase (CAT) decompose H_2O_2 (Sharma and Dietz, 2009; Gwóźdź et al., 1997)

A summary of all these mechanisms can be seen in the figure 6.

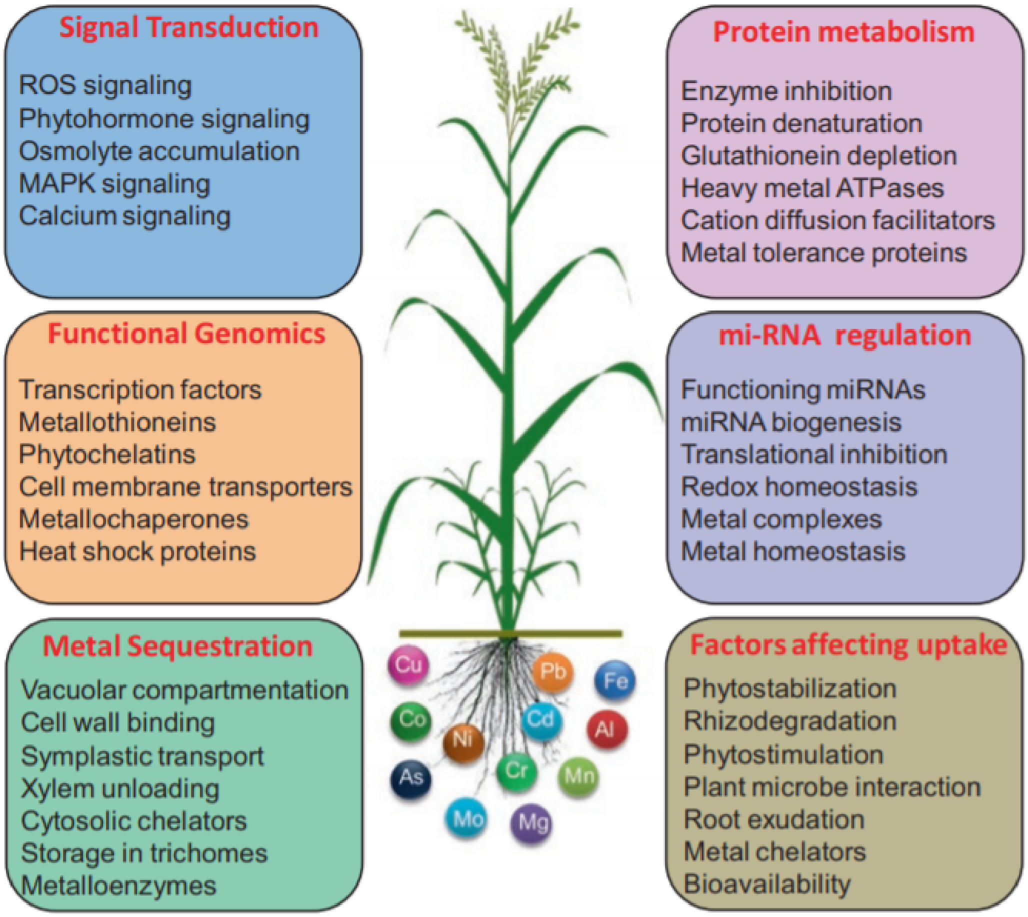


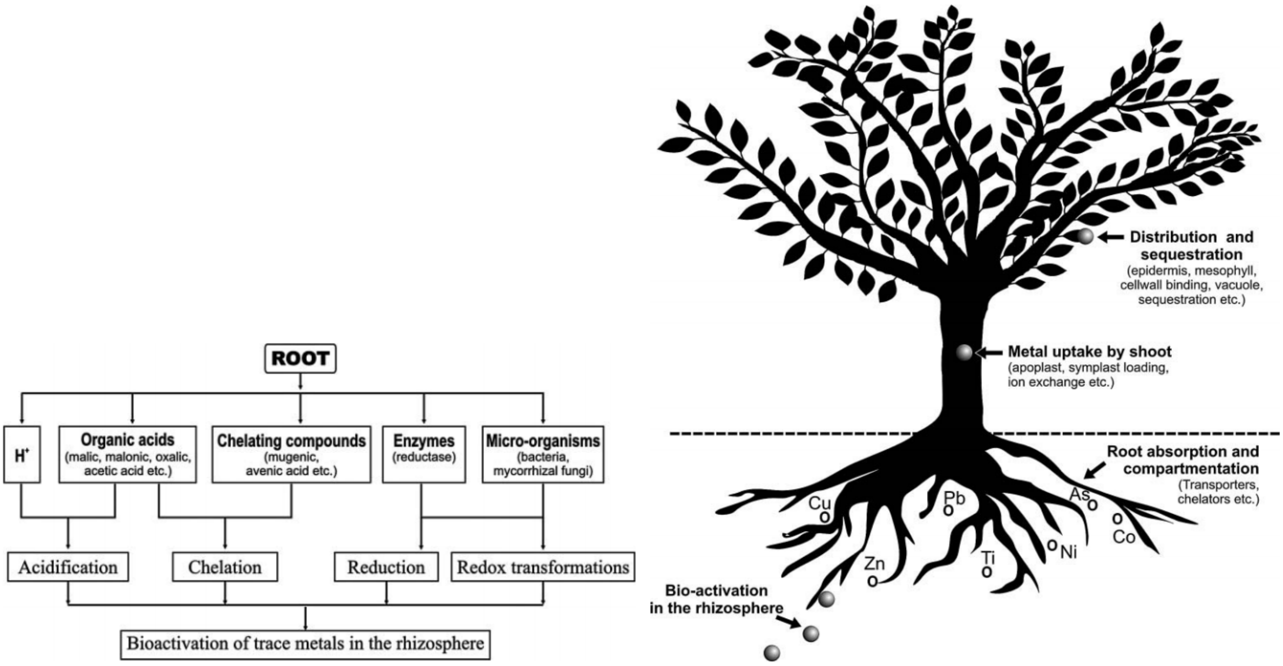
Figure 6: Cellular response mechanisms of plants to TE (Joshi et al., 2019)

C. Relations with plant behaviour

These cellular defence mechanisms are related to the behaviours developed in the section 2.1.2. Excluders are more apt to restrict transport from root to shoot than entirely regulate metal uptake (Mehes-Smith et al., 2013).

Phytostabilisation mecanisms include microorganisms present in the rhizosphere, root exudates, cell wall binding of metal ions, chelation of metal ions by metal-binding molecules and, eventually, sequestration in vacuole (Shackira and Puthur, 2019).

The hyperaccumulation of trace elements takes place in several stages: bioactivation in rhizome, uptake then translocation in shoots and sequestration in leaves (figure 7), where TE first have to cross the physiological barriers that stand in their way. There are two initial barriers for metal translocation: metal ions can be adsorbed on the extracellular negatively charged sites (COO^-) of the root cell walls and once they enter, impermeable suberin layers also limit transport of metals from root apoplast to root xylem. After that, an efficient translocation from root to shoot is possible thanks to TE chelation with ligand, before being unload and stored in vacuole of leaves cell (Dalvi and Bhalerao, 2013).



(a) (b)

Figure 7: a) Processes possibly involved in TE mobilization in the rhizosphere (Yang et al., 2005) b) Major processes involved in TE hyperaccumulation (Sheoran et al., 2011)

2.2.3.3 Factors influencing TE mobility and soil-plant transfer

The fraction of TE that the plant might be able to take up is determined by the fraction of TE in soil that is soluble, mobile, and bio-available (Carrillo-González et al., 2006). Indeed, the pool of metal ions present in the soil solution is the most easily extractable and available part for root absorption, but is also the most easily leachable (Wuana and Okieimen, 2011). The behaviour and bioavailability of TE is mainly regulated by the transfer between soil phases and therefore cannot be considered independently of the intrinsic properties of the soil (Kabata-Pendias, 2004).

TE retention in soil increases proportionally to cation exchange capacity (CEC), as it determines the total capacity of a soil to hold exchangeable cations by adsorbing them thanks to the negative charges located on the surface of the ion exchange sites (Chapman, 1965). In principle, CEC rises with the amount of organic matter and clay in the soil, even if CEC values vary from one type of clay to another according to the following sequence: montmorillonite > imogolite > vermiculite > illite, chlorite > halloysite > kaolinite (Kabata-Pendias, 2011). While CEC only account for ion exchange sites, another adsorption process, specific adsorption, involves the exchange of metal cations with surface ligands to form partly covalent bonds with charged mineral surfaces (Rieuwerts et al., 1998).

Usually, the majority of TE in soil is associated with the solid phase, where they can be bound to the surface of the soil's minerals, (co-)precipitated or bound to organic molecules. The binding capacity of the soil components differs according to the TE considered (Blume and Brümmer, 1991). Only a small portion of TE can be dissolved in the soil solution, where they can be found in the form of free ions or complexes with affinity ligand. Figure 15 summarises the main processes influencing the mobility of TE in soils and the factors impacting the processes. These factors are pH, redox conditions and temperature, and the following paragraphs will help to explain the mechanisms in which they come into play.

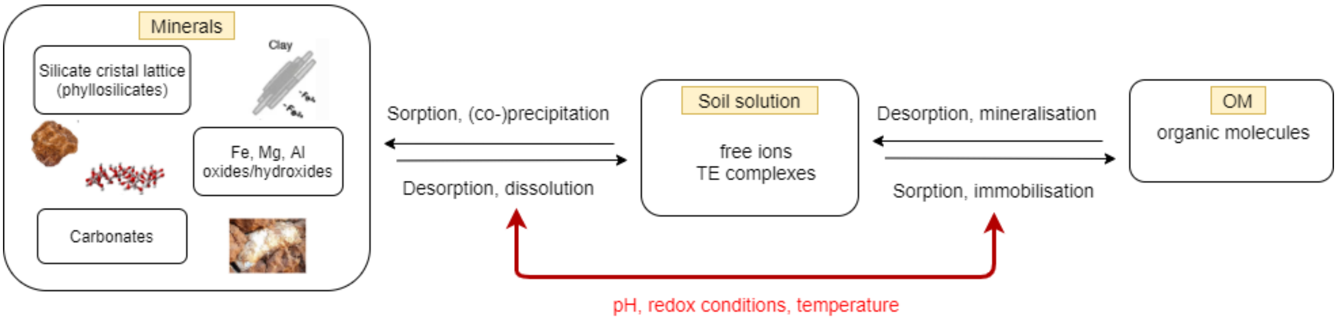


Figure 8: Synthesis of the main processes influencing the mobility of trace elements in soils, adapted from Carrillo-González et al. (2006)

A. pH

The scientific community has commonly accepted that pH is the most influencing factor on metal bioavailability in soils, solubility and pH usually showing an inverse relationship (Rieuwerts et al., 1998; White and Broadley, 2009). Indeed, in alkaline soil, TE tend to precipitate and have therefore a very low availability (De Matos et al., 2001) whereas in acid soils, the increase of protons in the soil solution can cause their substitution with metals on the exchange complex or can lead to the desorption of metal-ligand complexes (Rieuwerts et al., 1998). This is the case for all TE except V and Cr(VI), which tend to desorb from iron oxides at higher pH (Adriano, 2001; Wuana and Okieimen, 2011).

B. Redox conditions

Changes in redox conditions affect elements that have several oxidation stages. This includes Hg, As, Se, Cr, Fe, Mn but also their associated oxides-hydroxides (Qu et al., 2019; Kabata-Pendias, 2004). Sometimes the transition of a trace element from one oxidation state to another has a strong influence on its mobility and behavior, as it is the case for the redox couple Cr(III) - Cr(VI) (Adriano, 2001)

C. Climatic parameters

Climatic factors such as humidity and temperature have an impact on the development of plants and the activity of microorganisms. According to Li et al. (2012), the increase in temperature would affect soil dynamics and availability of some TE and enhance their potential for transfer from soil to plant.

E. Other factors

- **Temporality**
  The physico-chemical parameters influencing the transfer of trace elements into the soil solution are likely to change considerably over time (Kabata-Pendias, 2011). It implies therefore to take into account this dynamic behavior when studying processes governing TE mobility.

- **Organisms**
  As seen in section 2.2.3.2 root exudates cover a large number of substances, which may influence the mobility of trace elements (Marschner, 2012). Depending on the substance secreted, root exudates may either enhance or reduce the availability of TE, by directly affecting pH (acidification or alcalinisation), chelation, precipitation and redox reactions, or indirectly, through their effects on microbial activities, physical and chemical properties of the rhizosphere and root growth system (Kidd et al., 2009). For example, Ali et al. (2013) reported that some root exudates can lower the rhizosphere soil pH generally by one or two units over that in the bulk soil. The influence of other organisms on the mobility of trace elements has also been stated. According to Qu et al. (2019), microorganisms play a role as important as clays in the binding of TE and Wen et al. (2004) reported that earthworm activities would increase the mobility and bioavailability of TE in soils.

Remarks and variability
Even if some generalities can be drawn regarding the behaviour of trace elements in soils, the bioavailability of each element still differs according to its own chemical properties. In that respect, TE can be classified into 3 categories according to their bioavailability (Prasad, 2003):

- readily bioavailable (Cd, Ni, Zn, As, Se, Cu)
- moderately bioavailable (Co, Mn, Fe)
- least bioavailable (Pb, Cr, U)

2.2.3.4 Guidance

A. Legislation
In the context of a remediation project, the soil screening value (SSV) is commonly adopted in Europe as the limit concentration of pollutant content in soil safe for human use (Chernova and Beketskaya, 2011). In Wallonia, threshold values to be reached are defined for selected TE (As, Cd, Cr, Cu, Hg, Ni, Pb, Zn) in the soil decree (Service Public de Wallonie, 2018). The effects of these TE on human health are shown in figure 9.

Pollutants	Major sources	Effects on human health	Treshold values	
			in water (µg/l)	in soil (mg/kgDM)
Arsenic (As)	Pesticides, fungicides, metal smelters	Bronchitis, dermatitis, poisoning	10	30 - 65
Cadmium (Cd)	Welding, electroplating, pesticide fertilizer, Cd and Ni batteries, nuclear fission plant	Renal, lung and kidney damages or diseases, bone defects, increased blood pressure , gastrointestinal disorder, cancer	5	1,8 - 20
Lead (Pb)	Paint, pesticide, smoking, automobile emission, mining, burning of coal	Children diseases (mental retardation, developmental delay, fatal encephalopathy), congenital paralysis, acute or chronic damage to the nervous system (sensor neural, epilepticus), liver, kidney and gastrointestinal damage	10	120 - 1840
Mercury (Hg)	Pesticides, batteries, paper industry	Tremors, gingivitis, minor psychological changes, acrodynia, spontaneous abortion, damage to nervous system, protoplasm poisoning	1	1,1 - 5
Zinc (Zn)	Refineries, brass manufacture, metal Plating, plumbing	Zinc fumes have corrosive effects on skin, cause damage to nervous membrane	200	196 - 3000
Chromium (Cr)	Mines, mineral sources	Damage to the nervous system, fatigue, irritability	50	57 - 288
Copper (Cu)	Mining, pesticide production, chemical industry, metal piping	Anemia, liver and kidney damage, stomach and intestinal irritation	100	53 - 600
Manganese (Mn)	Welding, fuel addition, ferromanganese production	Damage to central nervous system after inhalation or contact	N.D.	N.D.

Figure 9: Anthropogenic sources and types of TE and their effect on human health with their permissible limits, adapted from Singh et al. (2011) and Service Public de Wallonie (2018)

In Wallonia, theses regulations therefore only apply to the total TE content analysed, even if the threshold values do not inform us of their real dangerousness and potential for transfer to living organisms (plants and humans) (Adamo et al., 2002). Moreover, when the clay and OM contents in the soil increase, the total concentration of TE also increases ((Dobrovol'skii, 1998), cited by Chernova and Beketskaya (2011)), but they can be immobilised and remain in non-exchangeable forms (Usman et al., 2004). For example, Bashkin (2003) has showed that the toxicity of Cu does not necessarily correlate with the total content of this metal or even total soluble content.

The majority of European countries does not take soil properties into account when determining the SSV, but a few of them still consider (Chernova and Beketskaya, 2011):

- the contents of fractions <0.002 mm and OM % (in Slovakia, Netherlands and Flanders (Belgium))
- the pH and organic carbon % (in Great Britain)
- the degree of base saturation (in Poland)

B. Indicators for a phytoremediation project

Among the indicators that exist within the framework of trace element contamination, some of them have been created in order to determine the aptitude of plants to integrate a phytoremediation project and categorise them (Mishra and Pandey, 2019; Wuana and Okieimen, 2011):

- Plant ability to uptake TE from soil is evaluated by the bioconcentration factor (BCF)[3]

  $\text{BCF} = \frac{[\text{Element (plant part)}]}{[\text{Element (soil)}]}$

- Translocation factor (TF) determines in which part of the plant TE are preferentially stored

  $\text{TF} = \frac{[\text{Element (plant shoot)}]}{[\text{Element (plant root)}]}$

- the amount of metal extracted (M) is calculated according to:

  $M(\text{mg}) = \text{Metal concentration in plant tissue (mg/kg)} \times \text{Biomass (kg)}$

In general, plants with a BCF $>$ 1 and a TF $>$ 1 are suitable for phytoextraction, whereas plants with a BCF $>$ 1 and a TF $<$ 1 may have the potential for phytostabilisation (Jutsz and Gnida, 2015).

2.3 Willow in short-rotation coppice (SRC)

2.3.1 Crop Characteristics

Willow (*Salix* genus) belongs to the Salicaceae family and contains 330-500 species, which shapes in a wide diversity of morphology. Even today, identifying and classifying them remains very difficult due to their constant inter-specific hybridisation and considerable variation in ploidy, resulting in a remarkable phenotype plasticity (Karp et al., 2011).

Willow is considered as a promising biomass crop and can be used for combustion, pyrolysis and gasification. It spreads easily and quickly in SRC, where cut stools constantly re-sprout to provide new shoots for many years (Karp et al., 2011). Some species, such as *Salix Viminalis* are more adapted to survive on polluted soil and still produce high biomass, although an excessive increase in TE concentrations can *in fine* decrease biomass production (Evlard, 2013). The phytoextraction characteristics of *Salix Viminalis* confirm its remediating potential (Mleczek et al., 2018): Tőzsér et al. (2018) showed that *Salix Viminalis* accumulates Cu, Fe, Mn, and Zn in root, and Cd and Zn in leaves. However, its extraction efficiency may decline over the years, as indicated by the decrease in TE concentration in shoots with time (Hammer et al., 2003). The woody biomass produced is of better quality when it does not contain too many trace elements as it requires reduced pre-treatment steps (see section 2.1.2.3). Some fertilisers, such as sewage sludge and ashes, do contain

[3] At present, there is no consensus about the definition of this indicator (Egendorf et al., 2020) and it may vary in name (Transfer factor, Biological absorption coefficient, Bioaccumulation factor) and definition in the literature (Kabata-Pendias, 2011; Mirecki et al., 2015; Wuana and Okieimen, 2011). Here, the bioconcentration factor defined by (Mishra and Pandey, 2019) has been chosen.

TE which, depending on the environmental conditions, could potentially be available to plant and accumulate in the harvestable shoot biomass (Adler et al., 2008), reducing the quality of the wood fuel.

2.3.2 Fertilisation requirement

A coppice farmer could decide to apply a certain dose of fertiliser only if this procedure can increase the profitability of his system, i.e. if the net revenue from fertilisation balance fertilisation costs (Aronsson et al., 2014). However, in real condition, SRC re-distribute nutrients and require only minimal nitrogen fertiliser for growth (Karp et al., 2011). The literature is divided on this subject and no consensus has been reached on the amount of fertiliser to be applied for SRC willow (Fabio and Smart, 2018), as the application of fertiliser to willow coppice does not always lead to an increase in biomass production (James and McDonald, 1989; Sevel et al., 2014). Sevel et al. (2014) have a balanced opinion on the subject and recommend to consider the nutrient status of the soil first before making decisions regarding fertiliser dosages. They noticed that increasing nitrogen doses rises sagging shoots frequency, which is problematic for harvest.

2.3.3 Non-destructive biomass estimation

Estimating the standing biomass of a forest stand is a research topic in silviculture. Generally, when a one or two-year rotation is considered, or to measure biomass at the end of a rotation period, destructive methods are used. This is done by cutting the entire crop stems, drying and then weighing them to obtain the total dry plant mass. However, when the biomass to be measured is large, applying such a method is too laborious and another estimation technique should be used (Hytönen et al., 1987).

Allometric equations

The measurements of individuals of an identical tree species living in the same conditions increase in the same proportion. The search for the nature of this statistical relationship is the basic principle of allometry. In that case, regression equations can relate biomass and/or volume to diameter and/or height (Picard et al., 2012). The most commonly used forms of equations are exponential or logarithmic equations (Schumacher and Hall, 1933; Kershaw et al., 2017), which are fitted from biomass data obtained by destructive methods (Shi and Liu, 2017).

A huge number of studies have investigated biomass production of SRC willow clones but there is no real consensus on how to obtain allometric equations. The height at which the diameter is measured varies according to studies and a very strong influence of both genotype and environment has been pointed out (Rönnberg-Wästljung and Thorsén, 1988; Nordh and Verwijst, 2004; Linkevičius et al., 2019). GlobAllomeTree (2020) database gathers some allometric equations. Each of them were constructed using diameter at breast height (DBH), which is diameter taken at 1m30. Twenty-seven equation are available on this database for "Salix Viminalis" keyword.

3 Material and methods

3.1 Effect of nitrogen fertilisation on willow growth and on the leaching of trace elements as well as total nitrogen in marginal soil

The first experiment was launched in April 2019 in the experimental garden of Gembloux Agro-Bio Tech within the framework of Camille Soetaert's master's thesis (Soetaert, 2019). This consists in comparing the effect of several dosages of three types of amendment (mineral, digestate and sludge) on the growth of willows (*Salix Viminalis* L.) and on the fate of nitrogen and TE leaching in marginal soil.

3.1.1 Experimental design

Soil characteristics

The first soil used was a polluted soil from the former Carsid steel site (Duferco, Wallonia) and the second one was a healthy soil collected in Gembloux Agro-Bio Tech. These two soils were analysed and their characteristics are described in table 1. Both soil types were referenced on the lysimeters using the acronyms P for polluted and NP for unpolluted.

Table 1: Initial characteristics of the two soils placed in the lysimeters (Soetaert, 2019)

Parameters	Carsid soil	Faculty soil
Pseudo-totals TE (Aqua regia)	[Cu] = 70 mg/kg [Zn] = 1416 mg/kg [Pb] = 252 mg/kg	[Cu] = 22,8 mg/kg [Zn] = 322 mg/kg [Pb] = 10,2 mg/kg
Availables TE (Lakanen-Ervio)	[Cu] = 44 mg/kg [Zn] = 179 mg/kg [Pb] = 242 mg/kg	[Cu] = 15 mg/kg [Zn] = 53 mg/kg [Pb] = 52 mg/kg
Solubles TE ($CaCl_2$ 0,01M)	[Cu] < 0,3 mg/kg [Zn] = 0,24 mg/kg [Pb] < 1 mg/kg	[Cu] < 0,3 mg/Kg [Zn] = 0,32 mg/Kg [Pb] < 1 mg/Kg
pH	8,09	6,97
Granulometry	Fraction < 2 mm = 63 % Fraction > 2 mm = 37 %	Fraction < 2 µm = 13,8 % Fraction <50 µm = 58,6 % Fraction < 2 mm = 27,6 %
Total organic carbon	[TOC] = 24,5 g/kg	[TOC] = 49,9 g/K
Total nitrogen	[N_{tot}] = 0,5432 g/100g	[N_{tot}] = 0,245 g/100g
Nitrates	[NO_{3-}] = 3,21 mg/kg	[NO_{3-}] = 6,12 mg/kg

Amendments used

Several doses of three types of amendments have been applied on both soil type, as shown in table 2. A mineral fertilizer (Ammonitrate 27%) was tested in single, double or quadruple dose , sludge and digestate were tested in single or double nitrogen dose. All amendments were applied with a maximum mineral nitrogen rate of 200kg N/ha. The gross quantity in lysimeters differs due to the different amount of mineral nitrogen available per ton in the amendments. As the amount of available mineral nitrogen was not initially known when the experiment began, it was estimated at 5kg/T for the sludge and 2.65kg/T for the digestate. These values were selected by Soetaert (2019) on the basis of the literature and the suppliers' data sheets.

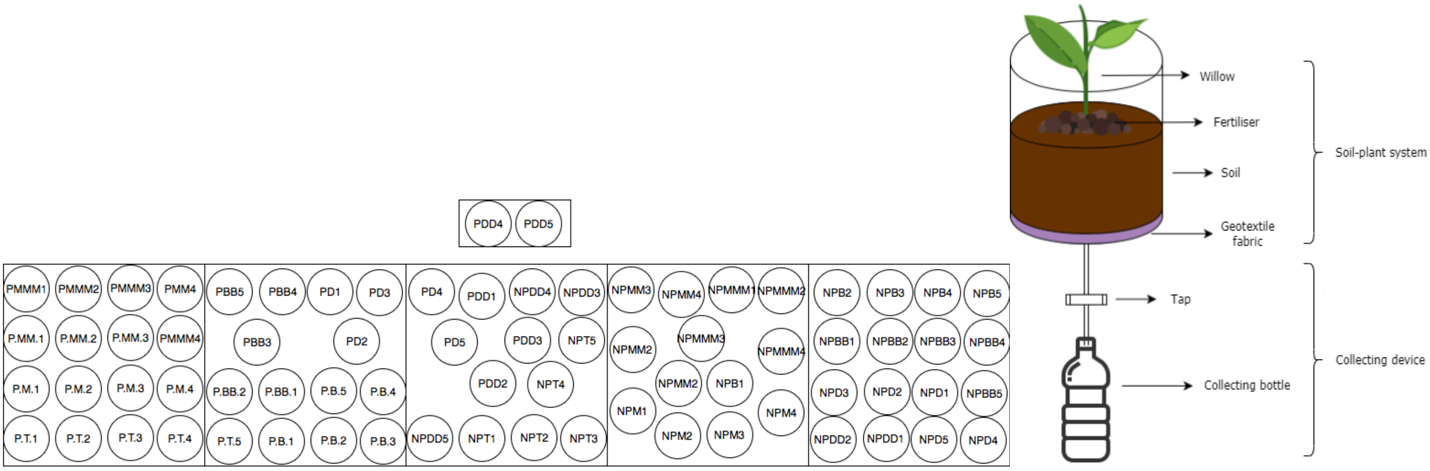
Table 2: Amount of fertiliser placed in the lysimeters and corresponding mineral nitrogen dose

Amendment types	Acronym	Quantity [kg N min/ha]	Quantity in lysimeters [g]
Mineral	M	50	1
Mineral	MM	100	2
Mineral	MMM	200	4
Sewage sludge	B	100	100
Sewage sludge	BB	200	200
Digestate	D	100	185
Digestate	DD	200	370
None (control)	T	0	0

As the nitrate concentration in the leachate was measured below the quantification limit (NANOCOLOR® Nitrate 1-65 test) during the second year of experimentation, a second fertilisation was carried out using the same quantities of fertiliser as Soetaert (2019).

Setup

Each modality were repeated four or five times to build the experimental set-up, giving a total of 74 lysimeters. They were placed on raised wooden pallets according to the configuration shown in figure 10 a). Between the 17th and 22nd April 2019, willows of the *Salix Viminalis* L. variety were implanted in lysimeters. Each lysimeter forms the experimental unit and is itself separated into two parts: the soil-plant system and the collecting device (figure 10 b)).



(a) (b)

Figure 10: a) Experimental design b) Experimental unit

3.1.2 Monitoring and task timeline

Figure 11 shows the chronology of the tasks and measures taken during the experiment, which was worth the time equivalent of two master's thesis. The rotation lasted one year and a half, since willows were planted in April 2019 and cut down in October 2020. Throughout the duration of the experiment, regular and even watering was brought to the lysimeters as well as pest and weed management. More particularly, a detergent-based treatment was applied to eliminate aphids whereas weeds and caterpillars were removed by hand.

3.1.2.1 Leachate harvesting

The effect of fertilisation on the leaching of nitrogen and trace elements was determined using leachate harvesting campaigns, carried out after each fertilisation (April 2019 and July 2020). For each leachate harvest, the soil of each lysimeter was previously moistened with 1 litre of distilled water, either the same morning or the preceding afternoon, so that the micropores in the soil were filled with water. After a waiting period (from a few hours to one night), each soil was subsequently watered with 1 litre of distilled water, so that the percolated water could be collected using a bottle preliminarily attached to the lysimeter's collecting tube.

Figure 11: Task timeline

3.1.2.2 Biomass estimation

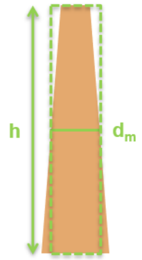
In order to see if the growth rate differs between modalities, willow branches were measured monthly after the second fertilisation (three times). Initially, the use of allometric equations had been considered due to the limited data required for its use: only a single pair of measurements is needed to estimate the biomass of a main stem. However, even for the same variety of willow, obtaining a single equation seems difficult because of the myriad sources of variation that can make the equation vary, such as the type of clone and the environment (climate, soil type) (see section 2.3.3). Furthermore, the diameter commonly used to build the equation is the diameter measured at 1m30, a height rarely reached by the individuals in this experiment. In addition, two distinct morphological features can be observed, some trees showing a large number of adjacent stems per main stem while others show few[4](figure 12). Thus, using the same equation for visibly heterogeneous individuals could be an additional source of error, since an allometric equation is already a model subject to a certain degree of inaccuracy[5]. For all these reasons, the use of such an equation was considered inadequate for this experiment.

(a) (b)

Figure 12: a) Individuals which do not develop many adjacent branches per main stem b) Individuals with many secondary branches per main stem

[4]This can be explained by the higher mortality of apical meristems in the group with a lot of adjacent branches. This mortality removes the dominance of the apical meristem, allowing the growth of secondary meristems (Stafstrom, 1995).

[5]Last year, Soetaert (2019) had actually tested the equation that she had built to predict the biomass of some principal stems of the willows of the experiment but the error was too large between the estimation and the real biomass.



Another method was therefore used, which allows to approximate the volume of a stem (main or adjacent), based on the assumption that a tree stem is formed from a composite of geometric solids. Among the many formulas that exist, Huber's formula (1) was selected because only one diameter (d_{m}) (taken at mid-height) and one height (h) is needed to obtain the volume (v) (de León and Uranga-Valencia, 2013; Rondeux, 1999).

$$v = \frac{\pi h}{4} d_m^2 \tag{1}$$

As the measurement was already rather time-consuming, the diameter at mid-height and the height were measured for all branches $\geq$ 20cm height. Estimated differences in volume between two dates can then be used to determine whether the growth differs between modalities.

3.1.3 Laboratory analyses

3.1.3.1 Leachate

pH and TE

After each harvest, the pH of the leachate was measured with a pH-meter before they were filtered through 602 $H^{1/2}$ filters. Part of the liquid was then frozen for further nitrogen analysis, while the other part was acidified with nitric acid at a concentration of 100µl HNO_3 per 100ml. In the latter, Cd, Pb, Cu and Zn were quantified using an Atomic Absorption Spectrometer (SpectrAA 220).

N analysis

Concerning nitrogen analysis, it was initially planned to use the NANOCOLOR® Nitrate 1-65 test, as performed by Soetaert (2019). Indeed, the most soluble form of nitrogen is the nitric form (nitrite and nitrate) and, as nitrates are more stable than nitrites, it is mainly nitrates that are leached in greater quantities and are more often the subject of standards. However, after preliminary nitrite quantification with QUANTOFIX® Nitrate/Nitrite strips, it was observed that the quantity of nitrite present in the leachate was too high and could cause interference with NANOCOLOR® Nitrate 1-65 test (Hartley and Asai, 1963). Instead, Total Nitrogen was quantified in the leachate using the NANOCOLOR® Total Nitrogen 0-83 test.

3.1.3.2 Willows

On 5 October 2020, each willow was cut, tied in bundles and put in the oven at 50°C for drying. Once dry, the plants were ground and mineralised according to the protocol of the "Water - Soil - Plant" axis of Gembloux Agro-Bio Tech. After mineralisation, Cd, Pb, Cu and Zn were quantified using an Atomic Absorption Spectrometer (SpectrAA 220). The total amount of phyto-exported

TE in the dry biomass (mg) can be calculated by multiplying the dry biomass (kg) by the amount of trace elements in the dry biomass (mg/kg).

3.1.3.3 Soils

An analysis of the nitrates present in the soils at the end of the experiment was carried out by the team of the "Water - Soil - Plant" axis.

3.2 ECOSOL project

The second series of experiments launched is intrinsically part of the ECOSOL project and focuses on the selection of species in the context of the regreening of the polluted site of the former Auvelais chemical plant. The common goal of these experiments is to evaluate the effect of the growth of these species on the trace element content of the soil solution, when planted on the polluted soil of the former Auvelais chemical plant.

3.2.1 One-meter high lysimeters

The first experimental set-up consisted of 48 one-metre high lysimeters. Six replicates of four varieties of rapeseed and three other herbaceous species were planted on 18 March 2020 in random order, as well as six lysimeters without plants (control) (Figure 13). The varieties of rapeseed tested were axana, theia, cleopatra and mosaic and the herbaceous species were *Lolium perenne* L., *Tanacetum vulgare* L. and *Alliaria petiolata* (Bieb.) Cavara and Grande. Regular and even watering was given to the lysimeters throughout the experiment. In each lysimeter, suction cups were placed at a depth of 10 and 35 cm, thanks to which capillary water was collected every 15 days from 29 April to 16 July 2020.

Figure 13: One-meter high lysimeters: rapeseed varieties and herbaceous species

3.2.2 Fifteen-centimeter high lysimeters

The second experimental device consisted of 24 fifteen-centimeter high lysimeters where six replicates of the same four varieties of rapeseed were planted (mosaïk, theia, axana and cleopatra) in late March (Figure 14). The above-ground biomass was cut down on 27 May 2020.

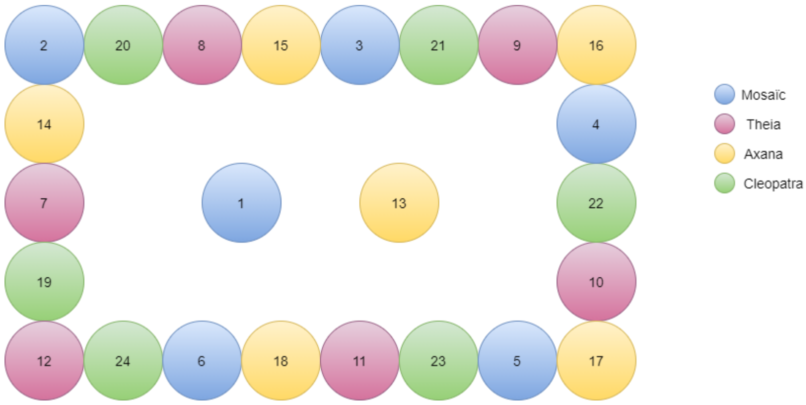


Figure 14: Experimental set-up of the first experiment in 15cm-high lysimeters

On 8 June 2020, 3 replicates of 8 herbaceous plants were then planted in the same lysimeters as rapeseed varieties. The herbaceous species were: *Lolium perenne* L., *Echium vulgare* L., *Verbascum thapsus* L., *Matricaria recutita* L., *Hypericum perforatum* L., *Achillea millefolium* L., *Valeriana repens* Host. and *Stachys officinalis* (L.) Trev. The above-ground biomass was cut down on 20 October 2020.

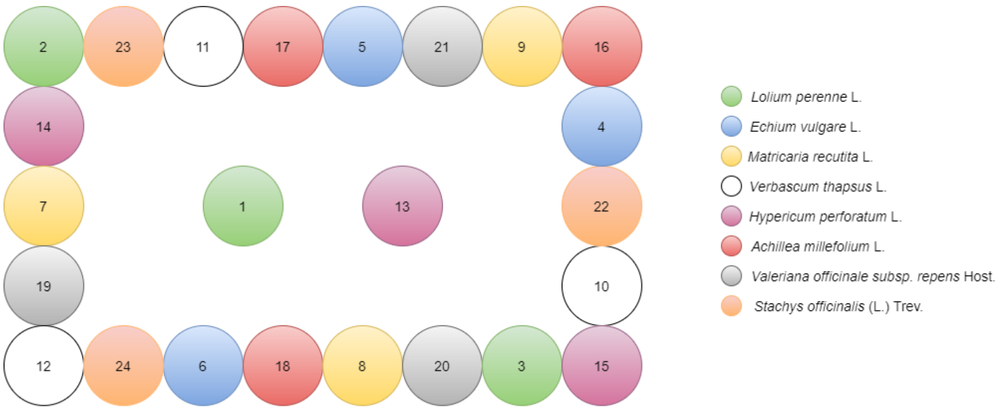


Figure 15: Experimental set-up of the second experiment in the 15-cm high lysimeters

For both experiments in 15cm-high lysimeters, regular and even watering was given to the lysimeters throughout the experiment. The temporal monitoring of the soil solution was carried out every week, thanks to suction cups placed at mid-depth of lysimeters. After being harvested, the biomass was dried in an oven at 50°C, weighted and mineralised. Cd, Pb, Zn and Cu content was then quantified using SpecrAA 220.

3.2.3 Laboratory analysis

For each soil solution collected with suction cups, pH was measured with a pH-meter and 9 trace elements (As, Cd, Cu, Mo, Zn, Ni, Pb, Mn, Cr) were quantified using ICP analysis.

3.3 Statistical tests

Statistical analyses for all experiments were achieved using R software. Before performing any statistical test, NA-values and outliers detected by Cook's distance were first eliminated from the dataset. The conditions of application necessary to carry out analyses of variance (ANOVA) were verified using the Shapiro-Wilk test to check normality of populations and residues and the Levene test to verify homoscedasticity. The samples were assumed to be random, simple and independent. If these conditions were not met, even after data transformation (logarithmic or square root), non-parametric tests were performed. In particular, Kruskal-Wallis tests were used instead of one-way ANOVA and permutation ANOVA instead of two or three-way ANOVA. In case of significant interaction between several factors, the dataset was decomposed according to each factor before doing new ANOVA on these dataset subdivisions.

In the case of repeated measurements over time (non-independent samples), where data distribution is not known a priori, a repeated measures ANOVA was performed using the linear mixed models (lmer) of the lme4 package. The normality of the residues has also been verified by the Shapiro-Wilk test. For repeated measurement when data distribution is known (binomial), generalized linear mixed model (glmer) are used.

In order to highlight the differences between groups, the following posthoc tests of mean comparison were used: Student-Newman-Keuls (SNK) for two or three ways ANOVA and permutation ANOVA, TukeyHSD for one way ANOVA, Dunn test for Kruskal-Wallis and Tukey contrasts for lmer.

4 Results

4.1 Effect of nitrogen fertilisation on willow growth and on the leaching of trace elements as well as total nitrogen in marginal soil

4.1.1 Leachate evolution

4.1.1.1 Impact of fertilisation on pH

Bi-directional ANOVA carried out on subdivided data sets revealed that, before the second fertilisation, pH differed significantly according to soil and fertilisation types, showing a significantly higher pH in polluted soils (Appendices 2a and 2b). On the polluted soil, the fertilised modalities indicated a lower pH than the control, while on the unpolluted soil, sludge-type amendment contributed to a significant increase in pH compared to digestate. Control and mineral fertiliser did not differ from sludge or digestate.

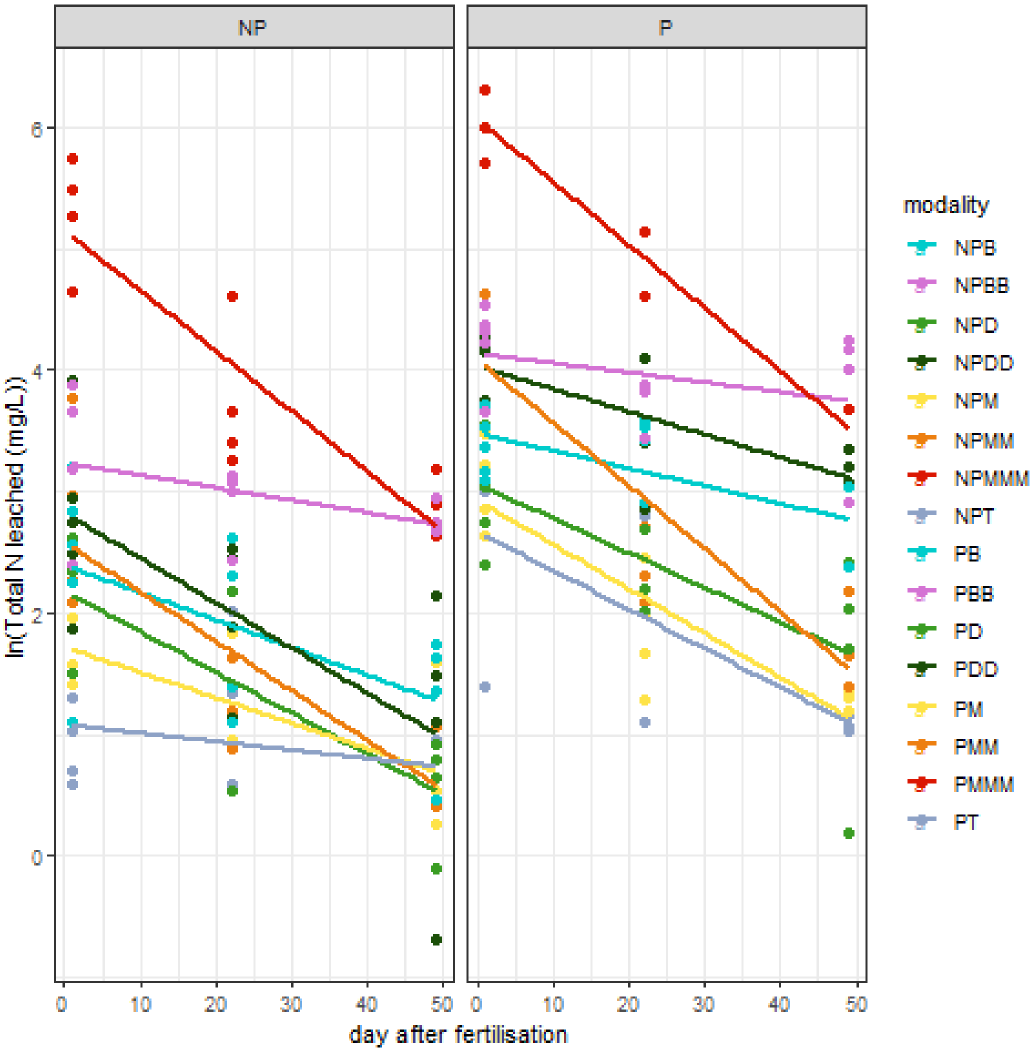
The direct impact of fertilisation was assessed by a repeated measures ANOVA between the pH before and after the second fertilisation (Appendix 2c). On the polluted soil, sludge significantly increased pH, as well as the 50 and 100 doses of mineral fertiliser and the 100 dose of digestate. 200 dose of digestate and mineral fertiliser did not significantly impacted pH right after fertilisation. On unpolluted soil, sludge significantly acidified pH as well as dose 200 of mineral fertiliser. The other doses of mineral fertiliser did not significantly impacted pH. Digestate, on the other hand, basified pH on unpolluted soil.

A repeated measures ANOVA was also carried out on the values of pH of the leachates after fertilisation (Appendix 2d). Globally, the factor day was very highly significant for all fertilisers on both soils, except for modalities NPMMM and NPB. The classes formed by the Tukey contrasts are variable, but still show that the pH value for the 2nd leachate harvest tends to be lower than the other two sampling, except for the NPB modality. The scatter plot of pH values can be seen in the appendix 2e.

4.1.1.2 Impact of fertiliser type and dose on total nitrogen leached

A repeated measures ANOVA was carried out on the logarithmic transformation of nitrogen measurements. It revealed, for the same dose examined, a very highly significant interaction between fertiliser and day factors (Appendix 3), which traduces the fact that fertilisers evolve very differently over time. This trend is illustrated on figure 16, where linear regressions of the neperian logarithmic transformation of nitrogen measurement were applied to each modalities. Even if the regression coefficients obtained are far from optimal for each modality, they are nevertheless considered acceptable in this case in view of the low number of repetitions. The comparison of slopes and intercepts of regression lines shows that for the same dose of nitrogen applied (100: MM, B and

D; 200:MMM, BB and DD), total nitrogen is the most strongly leached for modalities amended with mineral fertiliser, followed by digestate then sludge.



	Unpolluted (NP)		Polluted (P)	
Fertilisation	Equation	R^2	Equation	R^2
T	$y = 1,1 - 0,007x$	0,079	$y = 2,7 - 0,032x$	0,54
M	$y = 1,7 - 0,021x$	0,52	$y = 2,9 - 0,0037x$	0,79
MM	$y = 2,6 - 0,041x$	0,69	$y = 4,1 - 0,052x$	0,86
MMM	$y = 5,2 - 0,05x$	0,79	$y = 6,1 - 0,052x$	0,92
D	$y = 2,2 - 0,034x$	0,66	$y = 3,1 - 0,025x$	0,42
DD	$y = 2,8 - 0,037x$	0,47	$y = 4 - 0,019x$	0,59
B	$y = 2,4 - 0,023x$	0,34	$y = 3,5 - 0,015x$	0,49
BB	$y = 3,2 - 0,01x$	0,23	$y = 4,1 - 0,008x$	0,14

Figure 16: Graph of linear regressions applied to the neperian logarithm of total nitrogen leached over time according to the fertilisation modalities.

4.1.1.3 Impact of fertiliser type and dose on trace elements leached

Cd and Pb were measured below the quantification limit of the spectrometer (0,1mg/L for Pb and 0,02mg/L for Cd) in all leachates. The Cu and Zn content of the leachates was also either very close to or below the quantification limits (0,01mg/L for Zn and 0,03mg/L for Cu). A repeated measures ANOVA was performed using a binomial (detected/undetected) distribution for Cu and Zn content in the leachates. However, as the glmer model presented convergence problems, this result is not shown here. Appendix 4 contains raw data on Zn and Cu content of the leachate.

4.1.2 Effect of fertilisation on biomass production and TE content in willow stems

4.1.2.1 Estimated evolution of the increase in volume following the second fertilisation

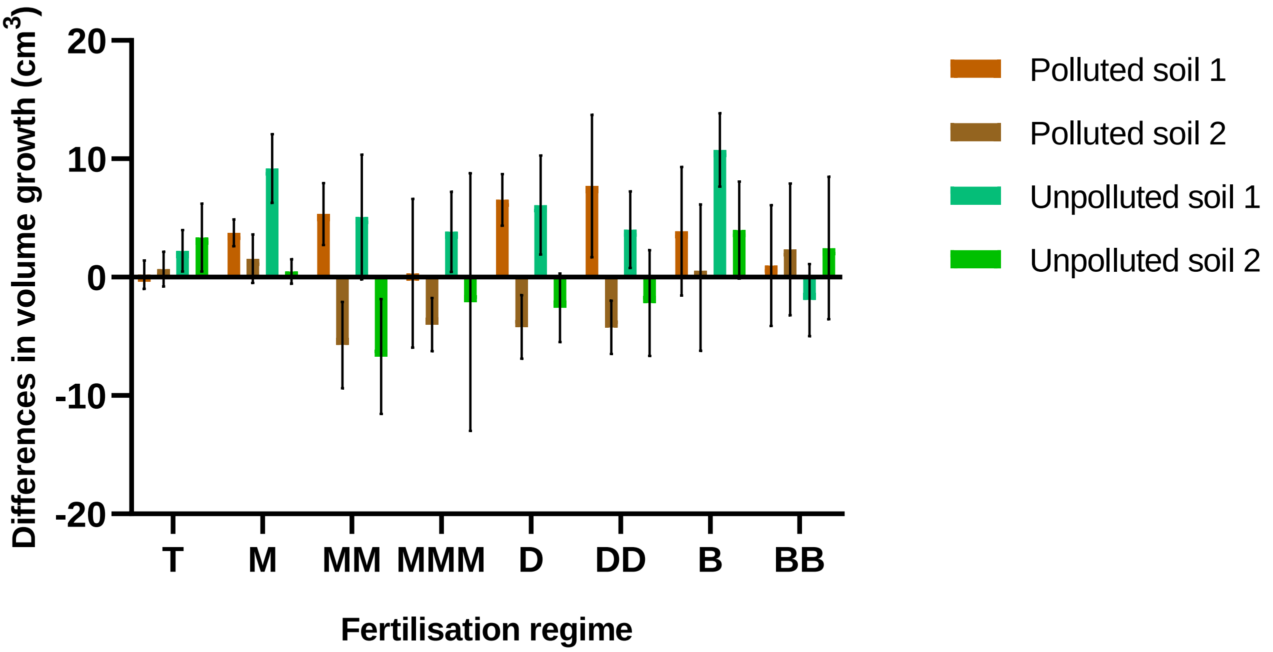


Figure 17: Means and standard deviations of the estimated differences in volume increase, 1: between the first and the second measurements, 2: between the second and third measurements

Figure 17 shows means and standard deviations of the estimated differences in volume increase, relative to the branches measurement campaign. Two separated ANOVAs were carried out on them (Appendices 5c and 5d). For the first estimated difference in volume, the dose was significant for the unpolluted soil fertilised with the sludge modality, showing a greater difference in volume for the 100 dose than for the 200 dose. The type of fertiliser was also significant, when considering an N dose of 200, as the sludge produced significantly less biomass than the digestate. The fertiliser and dose factors were significant for the second difference in volume. The control and the sludge allowed a greater increase in volume than the mineral fertiliser and the digestate. The SNK test did not detect differences between means for the dose factor. Raw data are available in the appendices 5a and 5b.

4.1.2.2 Total dry biomass production

Figure 18 illustrates the total dry above-ground willow biomass produced according to fertilisation regimes. For all the ANOVAs carried out (Appendix 6b), biomass production was significantly higher on unpolluted soil than on polluted soil. On both soil types, all fertilisers (mineral, sludge and digestate) significantly improved biomass production compared to the control, in significant proportion to the dose of nitrogen applied. Means comparison showed that the biomass produced at nitrogen dose 200 was significantly higher than at doses 100 and 50, which in turn was above dose 0. Mean and standard deviation of willow biomass produced as well as the contents of trace elements contained in biomass are available in the appendix 6a.

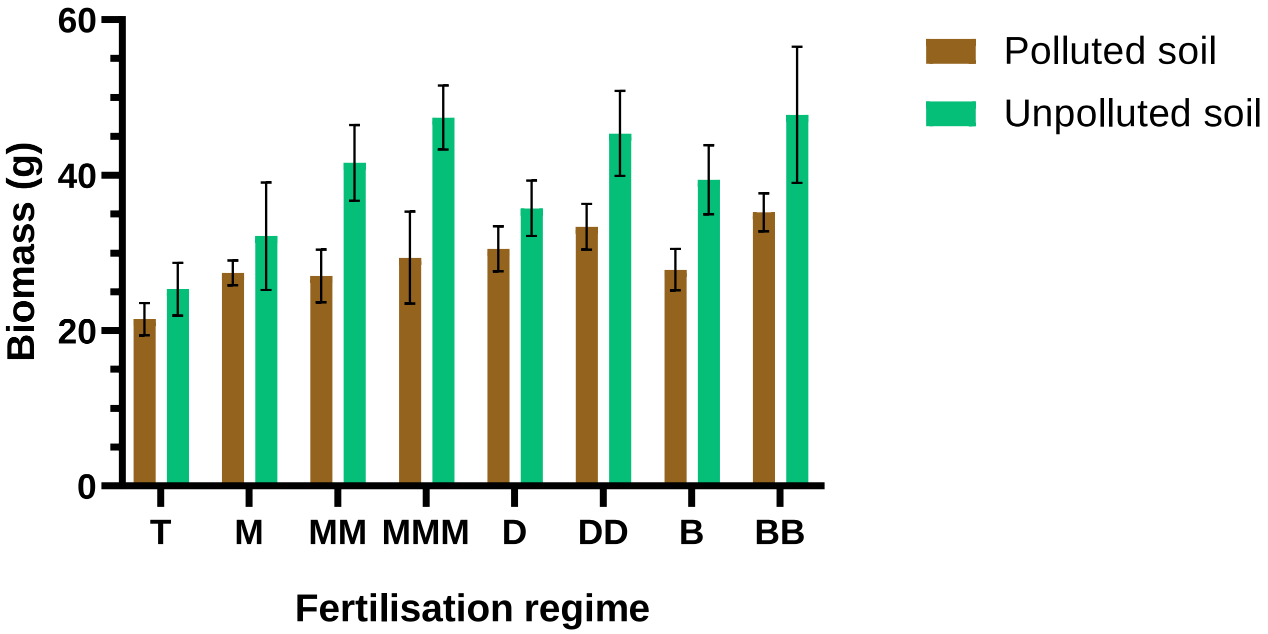


Figure 18: Dry biomass produced according to fertilisation regimes and soil

4.1.2.3 Trace element content in dry biomass

Figure 19 illustrates the amount of TE contained in the dry aerial biomass of willows. The soil factor was significant for all the ANOVAs carried out for each TE (Appendices 6c to 6f). The amount of copper and lead accumulated was significantly higher for polluted soil while the accumulation of cadmium and zinc was higher for unpolluted soil.

Cadmium (Cd)

A summary table of the ANOVAs carried out for cadmium content is available in appendix 6c.

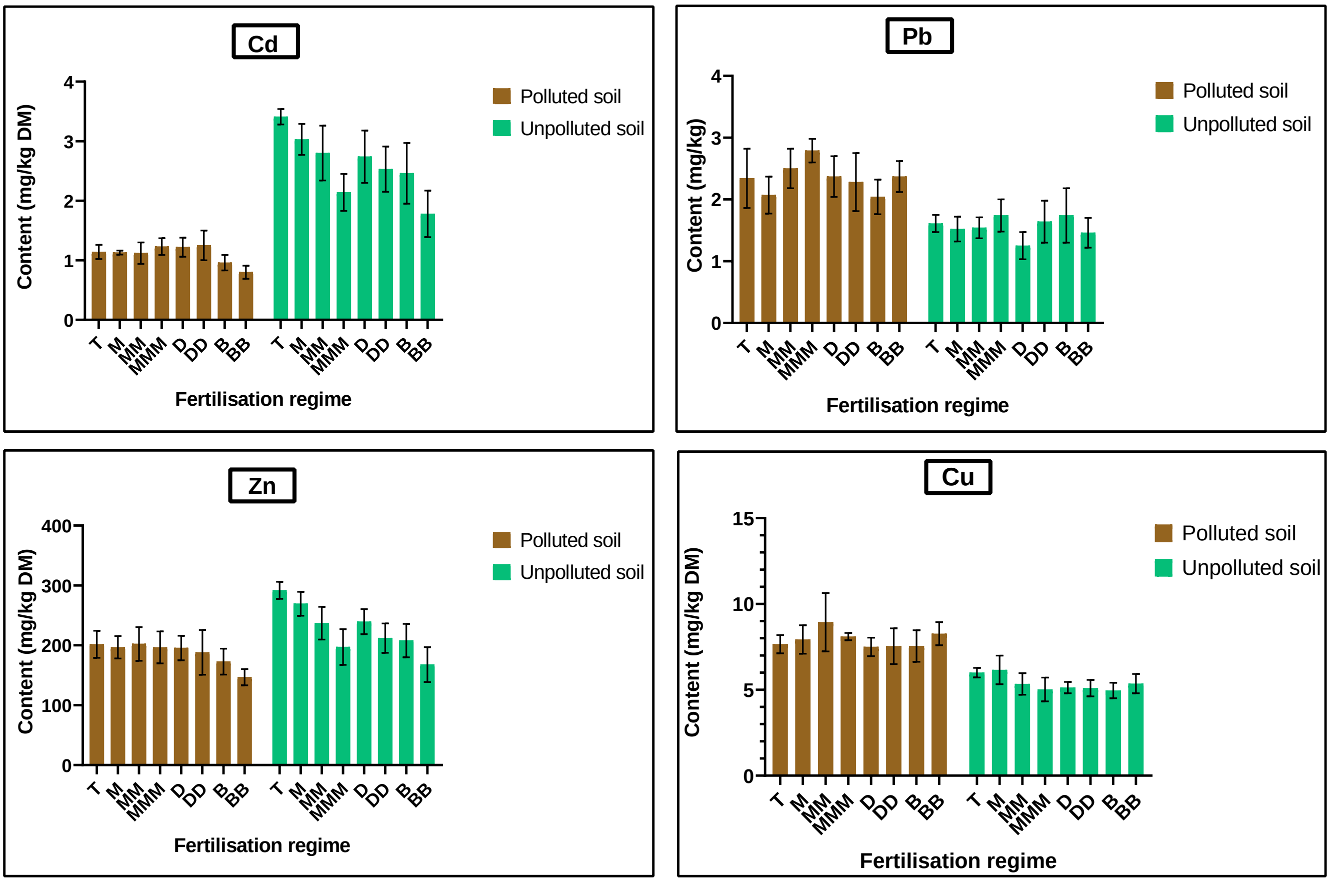


Figure 19: Trace elements content (mg/kg dry matter) in willow dry branches and twigs

Fertilisation was significant for the polluted soil, where the sludge-based fertiliser resulted in a lower cadmium content in the willows. It was not the case for the unpolluted soil, where the fertiliser factor was not significant but the dose factor was. However, the dose factor was significant for this soil, where the SNK test showed that increasing the amount of nitrogen in the soil significantly reduced the cadmium content in the willows.

Lead (Pb)

Concerning lead content, soils fertilised with the 200 dose of the mineral fertiliser caused a significant increase in the lead content in the willow biomass, compared to the 50 dose (Appendix 6d).

Copper (Cu)

The fertiliser and dose factors did not cause a significant change in the copper content of the willow biomass (Appendix 6f).

Zinc (Zn)

The fertiliser factor was significant for both soil types, and the dose factor was significant for the unpolluted soil. On the latter, the 200 dose produced a decrease in the amount of zinc compared to the 100 dose, which is itself lower than the 0 and 50 dose. On the unpolluted soil, all fertilisers were able to reduce the amount of zinc in the willows, but the sludge-type fertiliser was able to cause a greater reduction than the mineral and digestate fertiliser. On polluted soil, the sludge type amendment also seems to stand out significantly from mineral fertiliser, digestate and control, causing a significant decrease in the zinc content in the willows (Appendix 6e).

4.1.2.4 Total amount of trace element extracted according to the type of soil

Figure 20 shows the mean total amount of TE extracted in willow biomass, according to the type of soil.

Total amount of TE extracted in willow biomass (mg)	Unpolluted soil	Polluted soil
Cu	0,207 ± 0,048	0,226 ± 0,046
Zn	8,63 ± 1,57	5,31 ± 1,176
Cd	0,099 ± 0,021	0,031 ±0,008
Pb	0,061 ± 0,020	0,067 ± 0,018

Figure 20: Means and standard deviations of the total quantity in TE phyto-extracted (mg), according to the type of soil

4.1.3 Nitrate content in soils at the end of the experiment

The quantity of nitrates dosed in soils at the end of the experiment was significantly higher for lysimeters amended with sludge compared to the control for both soil types. Soil that received mineral fertiliser and digestate did not differ significantly from either the control or the sludge. In polluted soil, the dose factor was also significant. Soils fertilised with a nitrogen dose of 200 contained a quantity of nitrates at the end of the experiment that was significantly different from the 50 dose and the 0 dose (Appendix 7).

4.2 ECOSOL project

4.2.1 One-meter high lysimeters

Figure 21 shows the levels of significance for each repeated measure ANOVAs carried out on the monitoring of solutions over time in one-meter high lysimeters. The exact p-values are available in appendix 8. Figures 22 and 23 illustrate the groups identified by the Tukey's contrast posthoc test. Repeated measures ANOVAs were directly subdivided according to depth and logarithmic transformations of variables were applied as this allowed the normalisation of the residuals. The note about logarithmic transformation also applies to sections 4.2.2.1 and 4.2.2.2.

Herbaceous species	Variety	Depth	Ln(pH)	Ln(As) (ppb)	Ln(Cd) (ppb)	Ln(Cr) (ppb)	Ln(Cu) (ppb)	Ln(Mn) (ppb)	Ln(Mo) (ppb)	Ln(Ni) (ppb)	Ln(Pb) (ppb)	Ln(Zn) (ppb)
Control (no plant)		10	*	n.s	***	x	n.s	n.s	n.s	x	**	n.s
		35	n.s	n.s	***	x	n.s	***	n.s	x	x	*
Lolium perenne L.		10	n.s	***	n.s	x	*	n.s	***	x	n.s	n.s
		35	**	n.s	***	x	***	n.s	n.s	x	n.s	*
Alliaria petiolata (Bieb.) Cavara and Grande		10	n.s	**	*	x	n.s	*	n.s	x	***	n.s
		35	**	*	*	x	***	***	***	x	***	n.s
Tanacetum vulgare L.		10	*	n.s	***	x	***	n.s	**	x	***	**
		35	**	n.s	***	x	x	***	n.s	x	***	n.s
Brassica napus L.	Axana	10	n.s	n.s	n.s	x	***	***	n.s	x	x	**
		35	***	n.s	***	x	***	n.s	n.s	x	x	***
	Cleopatra	10	n.s	n.s	n.s	x	n.s	n.s	*	x	***	***
		35	***	***	**	x	x	n.s	n.s	x	n.s	*
	Mosaik	10	***	n.s	**	x	***	n.s	n.s	x	n.s	***
		35	***	n.s	n.s	x	*	***	n.s	x	***	n.s
	Theia	10	*	n.s	n.s	x	n.s	n.s	*	x	n.s	*
		35	x	x	x	x	x	x	x	x	x	x

*Codes: n.s: non significant, *: significant (p-value < 0,05) , **: highly significant (p-value < 0,01) , ***: very highly significant (p-value < 0,001) , x: not enough replicate to fit any lmer (production of NA or non-compliance with application conditions).*

Figure 21: Levels of significance for repeated measures ANOVAs carried out on solutions collected in one-meter high lysimeters

Species	Control (no plant)																			
Day ↓ / Depth →	ln(pH)		ln(As)		ln(Cd)		ln(Cr)		ln(Cu)		ln(Mn)		ln(Mo)		ln(Ni)		ln(Pb)		ln(Zn)	
	10	35	10	35	10	35	10	35	10	35	10	35	10	35	10	35	10	35	10	35
42	ab				ab	ab						b					a			a
56	a				ab	a						b					b			a
69	ab	n.s	n.s	n.s	c	bc	x	x	n.s	n.s	n.s	ab	n.s	n.s	x	x		x	n.s	a
92	ab				ac	c						a					b			a
105	ab				b	ac						ab								a
120	b				bc	a						ab								a

Species	*Lolium perenne* L.																			
Day ↓ / Depth →	ln(pH)		ln(As)		ln(Cd)		ln(Cr)		ln(Cu)		ln(Mn)		ln(Mo)		ln(Ni)		ln(Pb)		ln(Zn)	
	10	35	10	35	10	35	10	35	10	35	10	35	10	35	10	35	10	35	10	35
42		a	d			ac							e							b
56		b	b			ab							b							ab
69	n.s	ab		n.s	n.s	c	x	x	b		n.s	n.s	d	n.s	x	x	n.s	n.s	n.s	ab
92		ab	c			bc				a			a							a
105		ab				ab			ab	b			b							a
120		b	a			a			a	b			c							ab

Species	*Alliaria petiolata* (Bieb.) Cavara and Grande.																			
Day ↓ / Depth →	ln(pH)		ln(As)		ln(Cd)		ln(Cr)		ln(Cu)		ln(Mn)		ln(Mo)		ln(Ni)		ln(Pb)		ln(Zn)	
	10	35	10	35	10	35	10	35	10	35	10	35	10	35	10	35	10	35	10	35
42		a	a		a	a					b	bc		b			ab	d		
56		b	ab	a	b	a					b	c		ab			b	c		
69	n.s	b	b	a	ab	a	x	x	n.s		a	ac	n.s	b	x	x	a	b	n.s	n.s
92		ab	b	ab	ab	a					ab	a		a			b	bd		
105			ab	b	ab	a				b	ab	ac		ab			a	a		
120		ab	ab	a	ab	a				a	ab	ab								

Species	*Tanacetum vulgare* L.																			
Day ↓ / Depth →	ln(pH)		ln(As)		ln(Cd)		ln(Cr)		ln(Cu)		ln(Mn)		ln(Mo)		ln(Ni)		ln(Pb)		ln(Zn)	
	10	35	10	35	10	35	10	35	10	35	10	35	10	35	10	35	10	35	10	35
42	ab	ab			ab	bc						b	b				b		a	
56	a	b			a	a						b	ab				b	ab	a	
69	ab	ab	n.s	n.s	b	b	x	x		x	n.s	b	ab	n.s	x	x	a		a	n.s
92	b	a			b	b						a	a				b	b	a	
105	b	ab			b	b			a				a						a	
120	ab	b			ab	ac			b			b	ab					a	a	

Species	*Brassica napus* L., axana variety																			
Day ↓ / Depth →	ln(pH)		ln(As)		ln(Cd)		ln(Cr)		ln(Cu)		ln(Mn)		ln(Mo)		ln(Ni)		ln(Pb)		ln(Zn)	
	10	35	10	35	10	35	10	35	10	35	10	35	10	35	10	35	10	35	10	35
42		a				b					b								b	b
56		c				a			a		b								ab	c
69	n.s	a	n.s	n.s	n.s	c	x	x		a	b	n.s	n.s	n.s	x	x	x	x	ab	c
92						bc					b								a	abc
105									c		a								a	
120		b				a			b	b	ab								ab	a

Species	*Brassica napus* L., cleopatra variety																			
Day ↓ / Depth →	ln(pH)		ln(As)		ln(Cd)		ln(Cr)		ln(Cu)		ln(Mn)		ln(Mo)		ln(Ni)		ln(Pb)		ln(Zn)	
	10	35	10	35	10	35	10	35	10	35	10	35	10	35	10	35	10	35	10	35
42		ab		a		ab							a				b		bc	ab
56		d		ab		ab							a				b		c	ab
69	n.s	cd	n.s	c	n.s	b	x	x	n.s	x	n.s	n.s	a	n.s	x	x	a	n.s	ac	a
92		bc		c		ab							a						ab	b
105		a		b		ab							a				b		a	ab
120		ab				a							a						ab	ab

Note: The letters in the table represent the groups detected by Tukey's contrast mean comparison method, which are ranked in ascending order (the group with the letter "b" has a significantly higher mean than group "a"). Shaded cells indicate too little or no data at these dates, making it impossible to compare means.

Figure 22: Groups detected using the Tukey's contrast means comparison method

<table>
<tr><td colspan="2">Species</td><td colspan="20">Brassica napus L., mosaïk variety</td></tr>
<tr><td rowspan="2">Day ↓</td><td rowspan="2">Depth →</td><td colspan="2">ln(pH)</td><td colspan="2">ln(As)</td><td colspan="2">ln(Cd)</td><td colspan="2">ln(Cr)</td><td colspan="2">ln(Cu)</td><td colspan="2">ln(Mn)</td><td colspan="2">ln(Mo)</td><td colspan="2">ln(Ni)</td><td colspan="2">ln(Pb)</td><td colspan="2">ln(Zn)</td></tr>
<tr><td>10</td><td>35</td><td>10</td><td>35</td><td>10</td><td>35</td><td>10</td><td>35</td><td>10</td><td>35</td><td>10</td><td>35</td><td>10</td><td>35</td><td>10</td><td>35</td><td>10</td><td>35</td><td>10</td><td>35</td></tr>
<tr><td colspan="2">42</td><td>ab</td><td>a</td><td rowspan="6">n.s</td><td rowspan="6">n.s</td><td>ab</td><td rowspan="6">n.s</td><td rowspan="6">x</td><td rowspan="6">x</td><td></td><td></td><td rowspan="5">n.s</td><td>ab</td><td rowspan="6">n.s</td><td rowspan="6">n.s</td><td rowspan="6">x</td><td rowspan="6">x</td><td rowspan="6">n.s</td><td>b</td><td>c</td><td rowspan="6">n.s</td></tr>
<tr><td colspan="2">56</td><td>a</td><td>ab</td><td>a</td><td></td><td></td><td>a</td><td>c</td><td>ac</td></tr>
<tr><td colspan="2">69</td><td>ab</td><td>a</td><td>b</td><td></td><td>a</td><td>c</td><td>d</td><td>bc</td></tr>
<tr><td colspan="2">92</td><td>ab</td><td>ab</td><td>ab</td><td></td><td></td><td>ac</td><td>a</td><td>ab</td></tr>
<tr><td colspan="2">105</td><td>a</td><td></td><td>a</td><td>b</td><td></td><td>bc</td><td></td><td>a</td></tr>
<tr><td colspan="2">120</td><td>b</td><td>b</td><td>a</td><td>a</td><td>b</td><td></td><td>a</td><td></td><td>ac</td></tr>
</table>

<table>
<tr><td colspan="2">Species</td><td colspan="20">Brassica napus L., theia variety</td></tr>
<tr><td rowspan="2">Day ↓</td><td rowspan="2">Depth →</td><td colspan="2">ln(pH)</td><td colspan="2">ln(As)</td><td colspan="2">ln(Cd)</td><td colspan="2">ln(Cr)</td><td colspan="2">ln(Cu)</td><td colspan="2">ln(Mn)</td><td colspan="2">ln(Mo)</td><td colspan="2">ln(Ni)</td><td colspan="2">ln(Pb)</td><td colspan="2">ln(Zn)</td></tr>
<tr><td>10</td><td>35</td><td>10</td><td>35</td><td>10</td><td>35</td><td>10</td><td>35</td><td>10</td><td>35</td><td>10</td><td>35</td><td>10</td><td>35</td><td>10</td><td>35</td><td>10</td><td>35</td><td>10</td><td>35</td></tr>
<tr><td colspan="2">42</td><td>c</td><td rowspan="6">x</td><td rowspan="6">n.s</td><td rowspan="6">x</td><td rowspan="6">n.s</td><td rowspan="6">x</td><td rowspan="6">x</td><td rowspan="6">x</td><td rowspan="6">n.s</td><td rowspan="6">x</td><td rowspan="6">n.s</td><td rowspan="6">x</td><td>ab</td><td rowspan="6">x</td><td rowspan="6">x</td><td rowspan="6">x</td><td rowspan="6">n.s</td><td rowspan="6">x</td><td>a</td><td rowspan="6">x</td></tr>
<tr><td colspan="2">56</td><td>ab</td><td>b</td><td>a</td></tr>
<tr><td colspan="2">69</td><td>ac</td><td>ab</td><td>a</td></tr>
<tr><td colspan="2">92</td><td>c</td><td>a</td><td>a</td></tr>
<tr><td colspan="2">105</td><td>a</td><td></td><td>a</td></tr>
<tr><td colspan="2">120</td><td>bc</td><td>ab</td><td>a</td></tr>
</table>

Note: The letters in the table represent the groups detected by Tukey's contrast mean comparison method, which are ranked in ascending order (the group with the letter "b" has a significantly higher mean than group "a"). Shaded cells indicate too little or no data at these dates, making it impossible to compare means.

Figure 23: Groups detected using the Tukey's contrast means comparison method

4.2.2 Fifteen-centimeter high lysimeters

4.2.2.1 Rapeseed Varieties

Temporal evolution of the pH and TE content in the solutions collected with suction cups

The levels of significance of the repeatedly measured ANOVAs carried out on the temporal monitoring of the solutions before the rapeseed harvest are available on the figure 24, exact p-values can be seen in appendix 9a. Figure 25 illustrates the groups identified by the Tukey's contrast means comparison test, performed after the ANOVA.

Brassica napus L. varieties	Ln(pH)	Ln(As) (ppb)	Ln(Cd) (ppb)	Ln(Cr) (ppb)	Ln(Cu) (ppb)	Ln(Mn) (ppb)	Ln(Mo) (ppb)	Ln(Ni) (ppb)	Ln(Pb) (ppb)	Ln(Zn) (ppb)
Mosaïk	***	***	*	***	***	n.s	***	***	n.s	***
Theia	n.s	***	**	***	n.s	n.s	***	***	***	n.s
Axana	***	***	n.s	***	**	n.s	***	n.s	***	n.s
Cleopatra	*	***	***	***	**	n.s	***	***	n.s	*

*Codes: n.s: non significant, *: significant (p-value < 0.05) , **: highly significant (p-value < 0.01) , ***: very highly significant (p-value < 0.001)*

Figure 24: Levels of significance for repeated measures ANOVAs carried out on solutions collected in one-meter high lysimeters

<table>
<tr><td>Species</td><td colspan="20">Brassica napus L.</td></tr>
<tr><td>Variety</td><td colspan="10">Mosaik</td><td colspan="10">Theia</td></tr>
<tr><td>Day</td><td>pH</td><td>As</td><td>Cd</td><td>Cr</td><td>Cu</td><td>Mn</td><td>Mo</td><td>Ni</td><td>Pb</td><td>Zn</td><td>pH</td><td>As</td><td>Cd</td><td>Cr</td><td>Cu</td><td>Mn</td><td>Mo</td><td>Ni</td><td>Pb</td><td>Zn</td></tr>
<tr><td>1</td><td></td><td>a</td><td>ab</td><td>a</td><td>a</td><td rowspan="6">n.s</td><td>a</td><td>a</td><td rowspan="6">n.s</td><td>b</td><td rowspan="6">n.s</td><td>a</td><td>ab</td><td>a</td><td rowspan="6">n.s</td><td rowspan="6">n.s</td><td>a</td><td>a</td><td>b</td><td rowspan="6">n.s</td></tr>
<tr><td>7</td><td>bc</td><td>b</td><td>ab</td><td>a</td><td>a</td><td>a</td><td>a</td><td>bc</td><td>a</td><td>ab</td><td>a</td><td>a</td><td>ab</td><td>a</td></tr>
<tr><td>14</td><td>b</td><td>b</td><td>b</td><td>a</td><td>a</td><td>a</td><td>a</td><td>bc</td><td>a</td><td>ab</td><td>a</td><td>a</td><td>bc</td><td>a</td></tr>
<tr><td>21</td><td>c</td><td>c</td><td>a</td><td></td><td>a</td><td>b</td><td>b</td><td>a</td><td>b</td><td>a</td><td>b</td><td>b</td><td>c</td><td></td></tr>
<tr><td>27</td><td>a</td><td>b</td><td>ab</td><td>b</td><td>b</td><td>b</td><td>b</td><td>ac</td><td>a</td><td>b</td><td>c</td><td>a</td><td>bc</td><td>a</td></tr>
<tr><td>34</td><td>ab</td><td>bc</td><td>ab</td><td>c</td><td></td><td>ab</td><td></td><td>ab</td><td>ab</td><td>ab</td><td></td><td></td><td>ac</td><td></td></tr>
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<table>
<tr><td>Species</td><td colspan="20">Brassica napus L.</td></tr>
<tr><td>Variety</td><td colspan="10">Axana</td><td colspan="10">Cleopatra</td></tr>
<tr><td>Day</td><td>pH</td><td>As</td><td>Cd</td><td>Cr</td><td>Cu</td><td>Mn</td><td>Mo</td><td>Ni</td><td>Pb</td><td>Zn</td><td>pH</td><td>As</td><td>Cd</td><td>Cr</td><td>Cu</td><td>Mn</td><td>Mo</td><td>Ni</td><td>Pb</td><td>Zn</td></tr>
<tr><td>1</td><td></td><td>a</td><td rowspan="6">n.s</td><td>a</td><td>a</td><td rowspan="6">n.s</td><td>a</td><td rowspan="6">n.s</td><td>b</td><td rowspan="6">n.s</td><td></td><td>bc</td><td>ab</td><td></td><td>a</td><td rowspan="6">n.s</td><td>a</td><td>a</td><td rowspan="6">n.s</td><td>ab</td></tr>
<tr><td>7</td><td>c</td><td>ab</td><td>a</td><td>a</td><td>a</td><td>a</td><td>b</td><td>a</td><td>c</td><td>a</td><td>a</td><td>ab</td><td>ab</td><td>b</td></tr>
<tr><td>14</td><td>a</td><td>bc</td><td>ab</td><td>b</td><td>c</td><td>ab</td><td>ab</td><td>bc</td><td>bc</td><td></td><td>b</td><td>c</td><td>b</td><td>ab</td></tr>
<tr><td>21</td><td>b</td><td>c</td><td>b</td><td></td><td>b</td><td>b</td><td>ab</td><td>c</td><td>a</td><td></td><td></td><td>bc</td><td>c</td><td>a</td></tr>
<tr><td>27</td><td>a</td><td>ab</td><td>c</td><td>a</td><td>a</td><td>b</td><td>a</td><td>ab</td><td>c</td><td>b</td><td>ab</td><td>a</td><td>ab</td><td>ab</td></tr>
<tr><td>34</td><td>c</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
</table>

Note: The letters in the table represent the groups detected by Tukey's contrast mean comparison method, which are ranked in ascending order (the group with the letter "b" has a significantly higher mean than group "a"). Shaded cells indicate too little or no data at these dates, making it impossible to compare means.

Figure 25: Groups detected using the Tukey's contrast means comparison method

Biomass production and TE content in biomass according to rapeseed varieties

Boxplots and statistical tests carried out on biomass and TE content in biomass can be seen on figures 26 and 27.

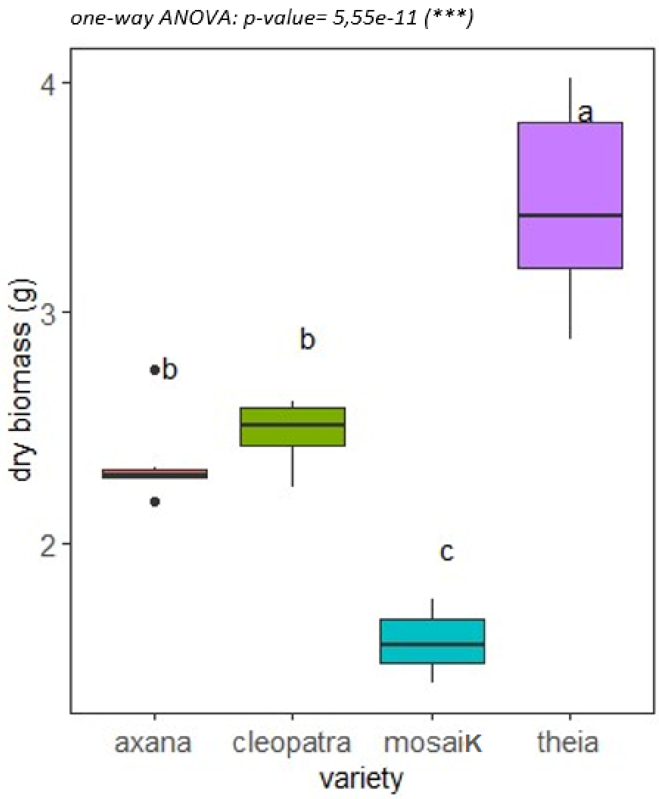

Figure 26: Boxplot, ANOVA and groups identified by TukeyHSD on dry biomass according to rapeseed varieties

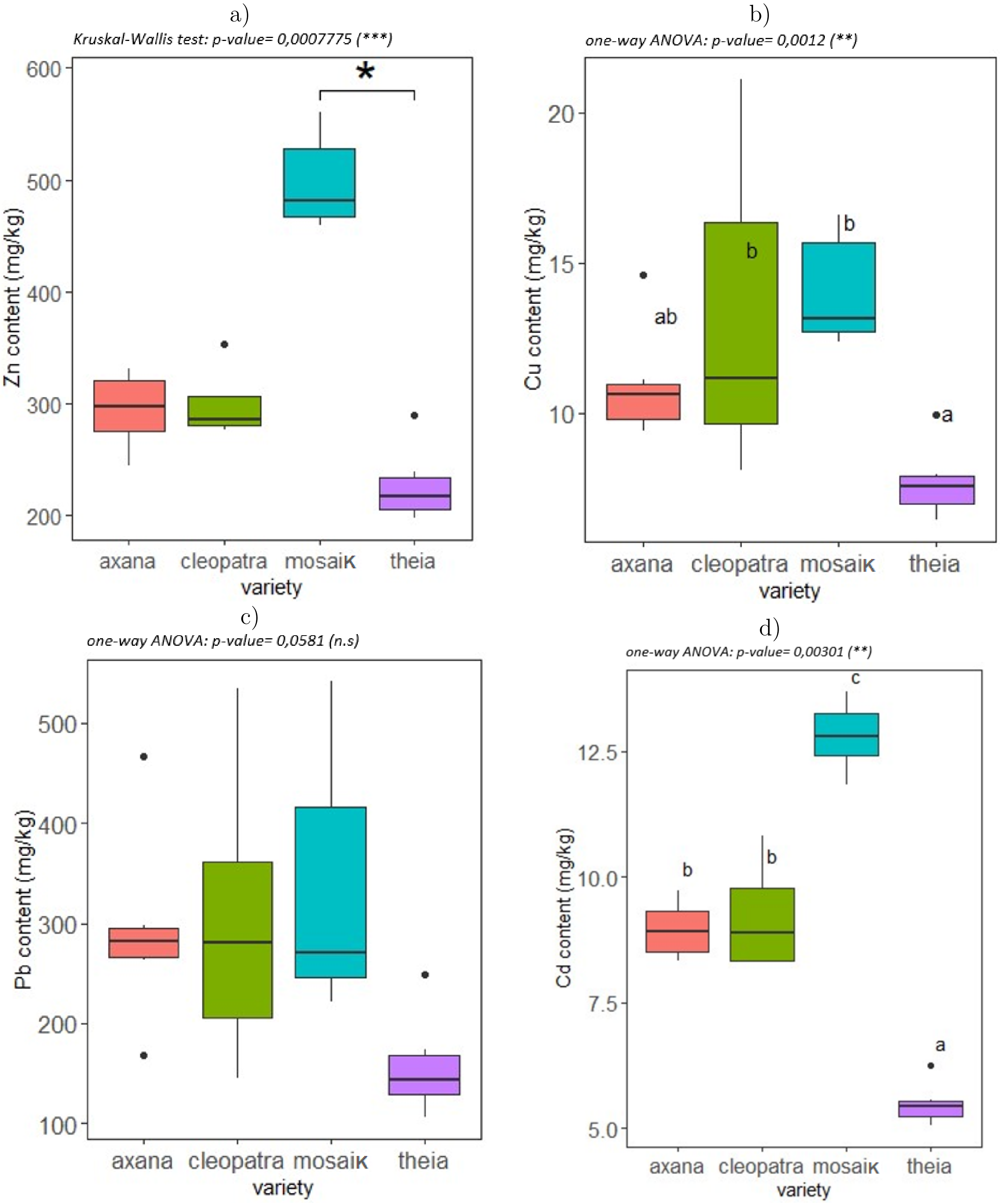

Figure 27: Boxplots and statistical tests for each TE content (mg/kg dry matter) in rapeseed dry biomass according to varieties. a) Kruskal-Wallis followed by Dunn test; b), c) and d): One-way ANOVAs followed by TukeyHSD posthoc test

4.2.2.2 Herbaceous Species

Temporal evolution of leachate

The levels of significance of the repeated measures ANOVAs carried out on solutions collected throughout the growth of herbaceous plants are available on the figure 28, exact p-values can be seen in appendix 9b. Figures 29 and 30 illustrates the groups identified by the Tukey's contrast means comparison test, performed after ANOVAs.

Herbaceous species	Ln(pH)	Ln(As) (ppb)	Ln(Cd) (ppb)	Ln(Cr) (ppb)	Ln(Cu) (ppb)	Ln(Mn) (ppb)	Ln(Mo) (ppb)	Ln(Ni) (ppb)	Ln(Pb) (ppb)	Ln(Zn) (ppb)
Lolium perenne L.	n.s	n.s	**	***	n.s	***	**	n.s	n.s	***
Echium vulgare L.	*	*	*	***	***	***	***	n.s	n.s	***
Matricaria recutita L.	***	***	n.s	x	***	**	***	n.s	**	***
Verbascum thapsus L.	***	n.s	n.s	**	***	***	***	n.s	x	***
Hypericum perforatum L.	***	***	***	**	***	n.s	n.s	x	n.s	n.s
Achillea millefolium L.	n.s	n.s	***	n.s	***	n.s	***	n.s	x	***
Valeriana repens Host	***	**	***	***	***	*	***	n.s	x	***
Stachys officinalis (L.) Trev	x	x	x	x	x	x	x	x	x	x

*Codes: n.s: non significant, *: significant (p-value < 0,05) , **: highly significant (p-value < 0,01) , ***: very highly significant (p-value < 0,001) , x: not enough replicate to fit any lmer (production of NA or non-compliance with application conditions).*

Figure 28: Levels of significance obtained for repeated measures ANOVAs applied to pH and TE content for each herbaceous species

Species	*Lolium perenne* L.										*Echium vulgare* L.										*Matricaria recutita* L.									
Day	pH	As	Cd	Cr	Cu	Mn	Mo	Ni	Pb	Zn	pH	As	Cd	Cr	Cu	Mn	Mo	Ni	Pb	Zn	pH	As	Cd	Cr	Cu	Mn	Mo	Ni	Pb	Zn
1			a	b		ab	a			ab	ab	ab	ab	ab		abc	ad			bcdf	a	bc				b	ac			bcd
6			a	b		a	a			a	ab	ab	ab	c	b	bd	ab			cdf	a	ac				ab	a			bcd
14			a	b		c	a			ab	ab	ab	ab	b	a	cd	d			ad	ab	ac			a	ab	ac		a	ab
20			a	a		ac	a			ab	ab	a	ab			a	bcd			ac	ab	ab				ab	ac			ac
27			a				a				ab	ab	ab	b	c	ab	bcd			ac	ab	a			a	ab	ac			ac
34			a			ab	a			a	ab	ab	a		cd	abc	a			a	ab	ac				ab	ac		ab	ac
41	n.s	n.s	a		n.s	ab	a	n.s	n.s	ab	ab	ab	ab			d		n.s	n.s	bcde	a	ac	n.s	x		ab	ac	n.s		ac
48			a			c	a			a	b	ab	ab		c	abc	bcd			ab	ab	bc			c	b	c		b	a
57			a			bc	a			ab	a	ab	ab		d	bc	cd			cdf	a	c			c	b	bc			d
68			a			ab	a			ab	ab	ab	ab		c	abc	ad			ef	ab	ac			bc	ab	ac			cd
77			a			a	a			a	b	ab	ab		c	abc	ac			bcde	a	bc			c	ab	ac			bc
85			a			ab	a			a	ab	b	ab		c	abc	a			bcdf	a	c			ab	ab	ab			ac
90			a	b		a	a			ab	b	ab	b	ab		bc	ad			cf	ab	c				ab	ac		ab	bc
106			a	b		ab	a			b	b	ab	b	bc	ab		bcd			f	b					a	a		b	cd

Species	*Verbascum thapsus* L.										*Hypericum perforatum* L.										*Achillea millefolium* L.									
Day	pH	As	Cd	Cr	Cu	Mn	Mo	Ni	Pb	Zn	pH	As	Cd	Cr	Cu	Mn	Mo	Ni	Pb	Zn	pH	As	Cd	Cr	Cu	Mn	Mo	Ni	Pb	Zn
1	a			b		bc	ab			c													ab				a			ac
6				b	a	ab	ab			bc													a		ab		df			a
14	ab			ab	bd	ab	ab			ab	a	a	ab			b							ab				f			a
20	ab			b	ab	a	ab			ab	ab	ab	ab	b	a	ab							ab				cde			ab
27	e			b		c	ab			ab	ad	ab	ab	ab	ab								ab		bc		ab			ab
34					b		a			a	ad	ab	ab			ab							ab				ab			ac
41	a	n.s	n.s		cde		a	n.s	x	ab	ab	ab	a		ac	ab	n.s	x	n.s	n.s	n.s	n.s	b	n.s	bd	n.s		n.s	x	ac
48	bcd				e	a	b			a	ad	b	b		cd	ab							ab				f			ab
57	ac				de	ab	b			ab	ab	b	ab		d	ab							ab		d		ef			ac
68	ab				e	a	ab			bc	abc	ab	ab		bcd	ab							ab		bc		cf			ac
77	bd				bc	a	a			ab	bd	ab	ab		bcd	ab							ab		cd		bc			ac
85	bd			b	de	a	ab			ab	ad	ab	ab	a	cd	ab							ab		a		ab			ac
90	bd			b		a	ab			ab	d	ab	b	b		ab							ab				bcd			bc
106	d			a		a	ab			ab	cd	ab	ab			a							ab				bc			c

Note: The letters in the table represent the groups detected by Tukey's contrast mean comparison method, which are ranked in ascending order (the group with the letter "b" has a significantly higher mean than group "a"). Shaded cells indicate too little or no data at these dates, making it impossible to compare means.

Figure 29: Groups detected using the Tukey's contrast means comparison method, realised after repeated measures ANOVAs

Species	*Valeriana officinale subsp. repens* Host									
Day	pH	As	Cd	Cr	Cu	Mn	Mo	Ni	Pb	Zn
1	a	ab	bc	c		a	ac			ad
6	a	ab	a	b		a	d			ab
14	a	a	ac	cd		a	bcd			ab
20	a	ab	ac	d		a	bcd			a
27	a	ab	ab	a	a	a	ac			a
34	a	ab	ab			a	ab			a
41	a	ab	ac			a	ac	n.s	x	ab
48	a	b	bc		ab	a	bcd			ac
57	a	b	c		b	a	cd			bcd
68	a	ab	ac		a	a	a			ad
77	a	ab	ac		a	a	ad			ad
85	a	ab	ac	c	a	a	bcd			ad
90	a	ab	bc	cd		a	bcd			bd
106	a	ab	ac			a	bcd			d

Note: The letters in the table represent the groups detected by Tukey's contrast mean comparison method, which are ranked in ascending order (the group with the letter "b" has a significantly higher mean than group "a"). Shaded cells indicate too little or no data at these dates, making it impossible to compare means.

Figure 30: Groups detected using the Tukey's contrast means comparison method, realised after repeated measures ANOVAs

Biomass production and TE content in biomass according to herbaceous varieties

The dry biomass collected and TE content for each herbaceous species planted is displayed on figure 31. No statistical tests were carried out on these results as it was difficult to clean the plants properly due to the limited amount of biomass collected. These results should therefore be taken with "a grain of salt" and can only give an overview.

Herbaceous species	Number of lysimeter	Dry biomass (g)		Cd (ppm)		Pb (ppm)		Zn (ppm)		Cu (ppm)	
		Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd
Lolium perenne L.	3	2,61	1,04	2,53	0,19	180,60	45,26	296,32	39,56	7,21	0,79
Echium vulgare L.	1*	0,45		12,21		520,83		413,14		16,93	
Matricaria recutita L.	3	0,54	0,26	55,17	10,49	477,54	194,97	593,35	60,79	26,48	0,92
Verbascum thapsus L.	3	1,78	0,23	4,92	1,81	647,3	327,76	483,07	166,21	18,91	3,65
Hypericum perforatum L.	3	0,85	0,20	6,88	3,81	379,50	319,63	381,31	184,79	16,63	6,61
Achillea millefolium L.	3	2,63	0,14	20,58	1,95	518,94	172,34	448,33	34,02	18,68	2,08
Valeriana repens Host	0*										
Stachys officinalis (L.) Trev	3	0,81	0,12	6,72	4,57	703,29	194,48	573,10	154,69	18,68	4,67

**Echium vulgare* L.: only 1 plant grew in only 1 of the 3 lysimeters. *Valeriana repens* Host.: no plants were found in any of the 3 lysimeters that were sown.*

Figure 31: Means and standard deviations of dry biomass produced per lysimeter and TE content in biomass

5 Discussion and prospects

5.1 Effect of nitrogen fertilisation on willow growth and on the leaching of trace elements and total nitrogen in marginal soil

5.1.1 Effect of the second nitrogen fertilisation on willow growth rates

In view of the discrepancy between the three measurement campaigns, the analysis of the difference in volume increases does not enable to deduce which fertilisation regime produced a higher growth rate from the second fertilisation. It can be noticed that some differences in volume increase between two dates were negative, which is due to several reasons. Firstly, measurements are subject to the accuracy of the instruments themselves and secondly, it was observed in the field that the willows had suffered badly from the heat and drought between the second and third measurements. Thus, the drought caused an increase in branch mortality as well as water loss within the biomass (Savage and Cavender-Bares, 2011), causing a smaller measured diameter and thus a negative volume increase. However, this lack of revealing results does not necessarily mean that the second fertilisation had no effect on biomass production. Indeed, according to Dušek and Květ (2006), the seasonal growth rate of *Salix caprea* starts to slow down in September. However, given the high branch mortality caused by the drought, it is reasonable to assume that willow growth-rate was no longer tremendous from the second branch measurements (1 August 2020).

5.1.2 Effect of the second nitrogen fertilisation on pH as well as total nitrogen and trace elements leached

Total nitrogen leaching from the second fertilisation was considerably higher for mineral fertilisers, followed by digestate then sludge. These characteristics are directly linked to the amount of mineral and nitric nitrogen compared to the amount of organic nitrogen in fertilisers. Nitrogen in nitric and ammoniacal forms are the two forms of nitrogen that can be leached, with a preponderance for nitrate ions which are more abundant than nitrite and cannot be fixed to clay particles, in contrast to ammoniacal nitrogen (Havlin et al., 2014). As the mineral fertiliser used (27% Ammonitrate) is composed of 13,5% nitric nitrogen and 13,5% of nitrogen ammonia, it is therefore logical that mineral fertiliser leachs more quickly than fertilisers containing organic nitrogen, which must first be mineralised before nitrates can be leached (Hernández et al., 2002). Nitrates quantification in soils at the end of the experiment also showed that soils amended with sludge-type fertiliser had a significantly higher nitrate content than the control, which was expected, given that the amount of organic nitrogen in the sludge is being mineralised continuously (Zare and Ronaghi, 2019).

Drawing conclusions on the evolution of the leaching of Zn, Cu, Cd and Pb seems difficult for this experiment, as they could rarely be quantified by flame spectrometer. However, given the inverse relationship between pH and the bioavailability of trace elements (White and Broadley, 2009), some hypotheses can be made by looking at the evolution of pH in these same leachates. The long-term impact of fertilisation on the pH of the leachates was assessed using an ANOVA on the pH of the leachate collected one year after the first fertilisation and before the second fertilisation. Fertilisation impact then differed within and, as at the beginning of the experiment, between soil types. Fertilisation had increased acidity on polluted soil, whereas on unpolluted soil, the sludge fertiliser seemed to have risen pH compared to digestate. Right after the second fertilisation, the acidification caused by the application of the mineral fertiliser was only visible in the unpolluted soil, for an application of a mineral nitrogen dose of 200. The buffering capacity of polluted soil might therefore be higher than unpolluted soil. Acidification caused by the application of fertilisers should be considered with care in the future, as it may cause an increase in the bioavailability of trace elements (White and Broadley, 2009). Buffering capacity of soil varies considerably depending on their composition. In general, the buffering capacity is low in sandy soils or soil with low organic matter content (low CEC) but it increases in calcareous soils (Hinsinger et al., 2003). In this case, higher buffering capacity in polluted soil is not due to the content of organic carbon or clays because they are higher on the unpolluted soil, but can possibly be caused by calcareous molecules or other molecules that neutralise H_+.

5.1.3 Fertilisation efficiency with regard to total biomass production and trace elements content in biomass

The results showed that the increase in biomass production is more pronounced on unpolluted soil than on polluted soil, which can easily be explained by the fact that willows growing on unpolluted soil are not subject to the same level of abiotic stress compared to polluted soil, where TE can be toxic and limit growth. The nitrogen supply has, not surprisingly, led to an increase in the production of biomass, regardless of the fertiliser type used. However, even if this effect can be noticed in both soil types, it still needs to be put into perspective. Indeed, while on unpolluted soil, the application of a nitrogen dose of 200 N_{min}/ha has led to a mean rise in dry biomass from 25,35 $\pm$ 3,8 g (control) to 46,73 $\pm$ 6,71 g, on polluted soil it has only increased from 21,48 $\pm$ 2,31 g to 31,77 $\pm$ 6,19 g. The mean increase in biomass produced by nitrogen fertilisation is therefore double for unpolluted soil compared to polluted soil. This raises the question of whether the application of such a high dose of fertiliser in real phytomanagement conditions is really beneficial to the system and whether the costs of fertilisation will be offset by the increase in biomass produced by the fertilisation.

Copper and lead contents in dry biomass were significantly higher for willows planted on polluted soil, as opposed to cadmium and zinc, which were higher in the biomass planted on unpolluted soil (more than twice the amount in the case of cadmium). These results may be surprising at first, as unpolluted soil is not supposed to contain a high level of TE available for plant uptake. However, the cadmium content had not been measured before the experiment and this result could therefore reveal the existence of cadmium pollution in the supposedly unpolluted Gembloux soil. Another way to explain this difference may be by highlighting the significant difference in pH between the two types of soil. Even if trace elements are not supposed to be bioavailable at such a neutral pH (around 7 for unpolluted soil), polluted soil is still more basic as it oscillates around 8. This higher pH for polluted soil could imply a decrease in the bioavailability of zinc and cadmium (De Matos et al., 2001). The trace elements extraction by *Salix Viminalis* despite their theoretically low bioavailability can be explained by reading a recent study written by Nworie et al. (2019). They suggest that the uptake of trace elements by plant roots is not controlled by the soil pH since they did not obtain any relationship between the concentration of root-borne trace elements and pH. They therefore hypothesised that the low-molecular-weight organic acids released from the roots possibly played a more important role in mobilising the soil-borne trace elements in the rhizosphere than pH, making TE bioavailable for the plant uptake.

The amount of TE phytoextracted was also impacted by fertilisation type and nitrogen dosage, and this effect varied depending on the trace element considered. Concerning Zn content, sludge-based fertiliser led to a diminution in concentration on both soils, whereas sludge diminished Cd content only on polluted soil. The reduced bioavailability produced by the sludge-type amendment may be due to its very basic pH as it has been limed, which can cause a increase in pH in the fertilised soil. On the other hand, increasing doses of nitrogen contributed to reducing the quantities of Zn and Cd, but it is only marked on unpolluted soil. This effect was also observed by Jacobs et al. (2018) for Cd and Zn, where the increase in biomass production induced by N supply led to a diminution of TE concentration compared to control. As mentioned before, the fact that biomass production is more strongly marked with the increase in nitrogen for unpolluted soil could explain why this dose effect is not significant in the case of polluted soil. Finally, the willow ability to grow despite high concentrations of Cd and Zn without showing signs of physiological stress confirm the fact that the willow has a hyperaccumulative behaviour for these two elements (Tőzsér et al., 2018). Concerning Pb content, an opposite behaviour was observed, where triple mineral nitrogen dose increased lead content in biomass for both soil. This can be explained by the fact that mineral nitrogen fertilisation generally produces acidification, which was enough to increase lead bioavailability of Pb in biomass. Finally, Cu-content in biomass did not seemed impacted by fertilisation type or nitrogen doses.

5.1.4 Manuring advice and phytoextraction efficiency

All these results combined make it possible to draw some conclusions concerning the fertilisation advice that can be applied in the context of a willow SRC phytomanagement project. The significant but still limited increase in biomass in the case of polluted soil raises the question of whether the cost of buying fertiliser will be compensated by the increase in biomass that fertilisation will allow. It therefore seems more judicious, in the case of a real phytomanagement project, to first consider the nutrient status of the soil before making any decision regarding fertilisation (Sevel et al., 2014), especially since in real conditions, *Salix* tend to redistribute nutrients efficiently (Karp et al., 2011).

If the decision to apply a nitrogen-based fertiliser is kept, the type of fertiliser does not seem to impact biomass production compared to the dose of nitrogen that is applied. However, when a large amount of mineral nitrogen is applied, the use of nitrogen by the plant is not optimal as nitrogen is too quickly leached to be used efficiently (Quemada et al., 2013). This practice therefore seems to be rather deleterious and can cause groundwater contamination and eutrophication problems (Di and Cameron, 2002). Thus, a fertiliser containing a good proportion of organic nitrogen should be favoured, as it would make it possible to release a smaller dose of mineral nitrogen to the plant over time and limit leaching issues. If the selection of such a fertiliser is not possible, smaller doses of mineral fertiliser should be applied over time in order to limit leaching, but this could add burden to the coppice farmer. Care should also be taken to supply a quantity of water adapted to the crop needs. Moreover, particular attention must also be paid to the possible acidification of the soil after the application of mineral fertilisers, which could increase the bioavailability of trace elements or potentially allow their leaching. During this experiment, the sludge organic soil fertiliser stood out from other fertilisers by making it possible to reduce the bioavailability of Zn and Cd thanks to its lime content. This effect is corroborated in the literature, where it is also mentioned that organic fertilisers could improve the physical, chemical and biological properties of the soil and increase soil organic carbon (Hernández et al., 2002). However, sewage sludge is not a miracle fertiliser either and its application should be carefully considered as it may contain microorganisms and trace elements.

The quantities of total phytoextracted trace elements were all found to be higher for willows growing on unpolluted soil than for willows growing on polluted soil, which is directly caused by the higher production of biomass on unpolluted soil. As the amount of TE phytoextracted can only be counted as a few mg, it is expected that many more years of phytoremediation will be required before a noticeable effect on the amount of TE in the soil can be deduced.

5.2 ECOSOL project

5.2.1 Preliminary note

It is important to note that the capricious behaviour of suction cups has led to the impossibility to obtain a minimum number of three repetitions per collecting day for both the fifteen-centimeter high lysimeters and the one-meter high lysimeters. This resulted in singularities problems (overfitting) for nearly all fitted lmer models. Thus, the quality of the data and of the temporal comparison can be seriously questioned and the results can only give an idea of the reality.

5.2.2 Impact of plant growth on the leaching of trace elements on both lysimeter devices

The results obtained for the three experiments confirm the doubts expressed about the quality of the results. The non-homogeneous lack of data, sometimes due to suction cups and sometimes due to ICP, made it impossible to switch to multivariate statistic analysis in order to obtain a general overview. Thus, this large amount of data (around 200 ANOVAs) made interpretation difficult, especially as no general trend emerges, neither for pH nor for TE.

The presence of significance for pH and TE content evolution of the herbaceous plants that grew in the fifteen-centimeter high lysimeters reinforced the doubt about the quality of the data. For all fifteen-centimeter high lysimeters, the biomass obtained from the herbaceous planted were not considerable (Appendix 10a and 10b), which was probably due either to the late sowing date, either to a lack of nutrients possibly pumped up by the previous rapeseed. As chlorosis were noticed on all herbaceous plants, the lack of nutrient hypothesis is then confirmed. Moreover, two herbaceous, *Echium vulgare* L. and *Valeriana repens* Host., globally failed to germinate in the lysimeters, as only one plant was found for *Echium vulgare* L. and none for *Valeriana repens* Host. Since Dresler et al. (2017) stated that secondary metabolites concentration were enhanced and organic acid were accumulated in *Echium vulgare* as a reaction to Zn and Pb stress. Thus, the trace elements levels in the soil of the former Auvelais chemical plant could be too high for these two species and cause cell death. Thus, in view of the low vegetation cover formed by herbaceous species, a significant influence of plant growth on pH is therefore very unlikely in this case, unless the significant variation in pH occurs naturally and independently of plant growth over time. On both lysimeter devices, the Tukey's contrast test even identified a significant increase in soil pH, which was not really expected. Root-mediated pH changes are due to several processes, and even if some of them, as redox reaction or release of carbonic acid, can increase pH, this is far from being the general rule (Hinsinger et al., 2003). Durand et al. (2001) found out that the net flux of charge released by the plants was strongly impacted by the buffering capacity of the soil solution.vThis buffering capacity is not known a priori for the soil of the Auvelais chemical plant, which limits the possibilities of predicting the behaviour of the root zone. Moreover, environmental stress such as nutrient-constraint also play a role on root-mediated pH changes, sometimes causing localised release of H^+ in subapical zones of the roots as a response to environmental stresses (Hinsinger et al., 2003).

In view of all the doubts expressed, it seems that no clear answer could be submitted regarding the influence of plant growth on the leaching of trace elements, for both lysimeters setups. During the continuation of the experiments, particular care must be taken to sample the solutions and obtain enough repetitions, otherwise the results would be difficult to interpret or simply unusable.

5.2.2.1 Rapeseed varieties

Concerning the rapeseed varieties that were harvested in small lysimeters, the statistical tests carried out on the biomass and TE content demonstrated that the theia and mosaïk varieties stood out from the two other varieties. Biomass production of the theia variety has proved to be significantly higher compared to the other three varieties, while its TE content tended to be lower (p-value was significant for all TE except for Pb). The mosaïc variety showed the opposite behaviour, tending to contain higher levels of TE in the biomass, while producing significantly less biomass than the other varieties. This inverse relationship between biomass and TE content has been described in the litterature, as in Pinto et al. (2014). During the harvest, this premature growth for the theia variety was also noticed, given that the individuals of the theia variety were the only ones who had developed buds after two months of growths. Despite this more important growth rate, the mass of seeds collected in one-meter high lysimeters for this variety was the lowest (result not shown). Comparaison between varieties is thought difficult as very few data are available for the theia variety.

5.2.3 Prospects brought to herbaceous species experiment

Figures 32, 33 and 34 present main characteristics of plants used this year in the lysimeter experiments. *Echium vulgare* L. and *Valeriana repens* Host are not shown here because they seem not to be adapted to thrive on the polluted soil of Auvelais. With the need to carry out more experiments to study the evolution of the leachability of heavy metals with plant growth, a selection from among these varieties could be made on the basis of biodiversity criteria. If this way, the species to be selected must be those that are both the most attractive to a wide variety of pollinators, and containing the highest quantity of pollen or nectar. Nectar being the principal energy source for pollinators, it should be prefered to the quantity of pollen. Thus, the last in regard to this classification is *lolium perenne*, which is wind-pollinated and therefore does not bring biodiversity of pollinators. The two species in the top are *Verbascum thapsus* and *Stachys officinalis*, with a preference for *Verbascum thapsus* in view of its innumerable valorisation potential in the medical field compared to *Stachys officinalis*.

Crop characteristics		*Matricaria recutita* L.		*Hypericum perforatum* L.		*Achillea millefolium* L.	
Phytoremediation characteristics	Cd	Cd accumulation in shoots [1]		Cd accumulation in shoots [9]		Low ability to contain and accumulate TE in biomass [19]	
	Zn	TE tolerant, elements more likely to be restricted to roots. Pb is the least mobile element within the plant [2]		Variable behavior according to the growing media [10]			
	Cu						
	Pb			N.D.			
	Ni			Potential Ni accumulation in aerial parts [11]			
	Co	N.D.		Limited translocation of Cr and Co, tolerance to Cr [12]			
	Cr						
	Hg			N.D.		Hg accumulation [20]	
Economic aspect	Yield (t ha^{-1})	2,3-4 [3]		9 [13]		N.D.	
	Profitability (€ ha^{-1})	1500 (in Serbia) [4]		6300 [14]		N.D.	
	Main uses	Medicinal and cosmetic industry (extracts and essential oils), in foodstuffs (extracts and teas) [5]		Medicinal industry (antiviral, anticancer, antibacterial effects, antidepressive agent), for essential oil production [15]		Medicinal industry (healing properties: antiseptic, antispasmodic,...), in decoction [21]	
Field-oriented side	Benefits	Adapted to grow on many environmental conditions, TE content does not affect essential oil yield and composition [6]		High versatility crop, Cr content in soil does not affect essential oils quality [16]		Drought tolerant, efficient settlement (rhizomes) and high seed dynamic [22]	
	Disadvantages	The productivity and chemical composition of essential oils is affected by the local agroclimatic conditions, lead toxicity affects pollen grains [7]		Can sometimes be difficult to grow, usually not cultivated more than 3 years due to susceptibility to fungal disease, can be invasive in some countries. Active compound content (hypericin) changes according to the season. [17]		Can compete with food crop production (in New Zealand) [23]	
Biodiversity aspects	Favoured pollinating insects	Bees, bumblebees, wasps, hoverflies, bombilids	[8]	Short-tongue wild bees, hoverflies, flies, beetles	[18]	Bees, butterflies, bumblebees, hoverflies	[24]
	Pollination type	Entomogamous		Autogamous / Entomogamous (Apomixis / Heterogamy)		Entomogamous	
	Amount of pollen	N.D.		large quantity		N.D.	
	Nectar quantity	N.D.		N.D.		N.D.	

Figure 32: Main characteristics of *Matricaria recutita* L., *Hypericum perforatum* L. and *Achillea Millefolium* L.

Figure 32 is based on the following sources: [1,9] Masarovičová and Katarína (2007), [2] Geneva et al. (2014), [3] Masarovičová et al. (2010), [4] Pljevljakušić and Brkić (2020), [5] Mežaka et al. (2020), [6] Geneva et al. (2014), [7] Mežaka et al. (2020); Albooghobaish and Zarinkamar (2011), [8,18,24] e-FLORA-sys (2009), [10-12] Bonari et al. (2019),[13,14] Kazemi et al. (2013), [15,16] Barner et al. (2001); Bonari et al. (2019), [17] Poutaraud and Girardin (2005); Rahnavrd (2017), [19] Murtic et al. (2019), [20] Wang et al. (2012), [21] Panda (2004), [22,23] Bourdôt and Field (1988)

Crop characteristics		***Lolium perenne* L.**		***Verbascum thapsus* L.**		***Stachys officinalis* (L.) Trev**	
Phytoremediation characteristics	Cd	TE preferentially accumulated in roots than in shoots, as follow: Cd > Zn > Pb. Species commonly used in assisted phytoremediation technologies [25]		Cd hyperaccumulator [29]		N.D	
	Zn			N.D.			
	Cu						
	Pb						
	Ni	N.D.				medium content of Ni [34]	
	Co	N.D				N.D.	
	Cr	N.D					
	Hg	N.D.					
Economic aspect	Yield (t ha^{-1})	N.D.		flowers: 0,84-1,68; leaves: 3,46-5,68 (United States) [30]		N.D. (Note: in Serbia, Egypt and Montenegro: oil yield is 0,04% v/dry weight) [35]	
	Profitability (€ ha^{-1})	N.D.		N.D		N.D.	
	Main uses	forage grass [26]		medicinal (pulmonary treatments, antioxidant, antiseptic, cardiovascular disease, antitumor activity, and many others), essential oil [31]		medicinal (antioxidant properties, phenolic compounds), foodstuffs (decoctions), essential oil [36]	
Field-oriented side	Benefits	quick germination, fast settlement [27]		prolifically producing seeds and efficient germination , but rarely becomes aggressively invasive, not competitive [32]		N.D.	
	Disadvantages	N.D.		N.D.		yield of essential oil of Stachys species is lower than other Lamiaceae family members [37]	
Biodiversity aspects	Favoured pollinating insects	N.D. (Note: poor pollination index compared to other dicotyledons)	[28]	Short-tongue wild bees, hoverflies, flies, beetles	[33]	Hymenoptera	[38]
	Pollination type	Anemogram		Autogamous / Entomogamous		Autogamous / Entomogamous	
	Amount of pollen	N.D.		N.D.		N.D.	
	Nectar quantity	N.D		large quantity		large quantity	

Figure 33: Main characteristics of *Lolium perenne* L., *Verbascum thapsus* L. and *Stachys officinalis* L. Trev

The following sources were used to build the table on figure 33: [25] Bidar et al. (2007); li Li et al. (2020), [26,27] Sampoux et al. (2011), [28] Ricou et al. (2014), [29] Čudić et al. (2016), [30] Kleitz et al. (2003), [31] Panchal et al. (2010), [32] Turker and Gurel (2005), [33,38] e-FLORA-sys (2009), [34] Bani et al. (2013), [35] Gören (2014) , [36] Šliumpaite et al. (2013), [37] Vundac et al. (2006)

Crop characteristics		*Alliaria petiolata* (Bieb.) Cavara and Grande		*Tanacetum vulgare* L.		*Brassica napus* L. (rapeseed)	
Phytoremediation characteristics	Cu	Low ability to accumulate TE [39]		Can accumulate high Cd, Cr, Ni and Pb content in leaves. Co, Cu, Fe, Hg, Mn and Pb are accumulated mostly in the roots [44]		The entire plant is tolerant to Pb, Cd and Zn. In seeds: low concentration of TE except Zn, with concentration affected by TE translocation from shoots. [48]	
	Zn						
	Ni						
	Pb						
	Cd						
	Cr	N.D.					
	Co	N.D.					
	Hg	N.D.					
Economic aspect	Yield (t ha^{-1})	N.D.		2,92-4,96 [45]		2-5 [49]	
	Profitability (€ ha^{-1})	N.D.		N.D		N.D.	
	Main uses	Depurative, diuretic, young leaves are edible [40]		Insect repellent, essential oil, antioxidant extracts, secondary metabolites [46]		Energy source (oil), therapeutic properties (diuretic, anti-scurvy, anti-inflammatory) [50]	
Field-oriented side	Benefits	Stage-structured, short-lived, high fertility (also a disadvantage as it can behave like an invasive species) [41]		N.D.		More than half the amount of TE is left in residues after extraction process [51]	
	Disadvantages	invasive (in Canada) [42]		N.D.		N.D.	
Biodiversity aspects	Favoured pollinating insects	Bees, hoverflies	[43]	Bees, bumblebees, wasps, hoverflies, bombilids	[47]	Bees, bumblebees, solitary bees, hoverflies	[52]
	Pollination type	Autogamous/Entomogamous		Entomogamous		Entomogamous	
	Amount of pollen	N.D.		N.D.		N.D.	
	Nectar quantity	medium quantity		medium quantity		N.D.	

Figure 34: Main characteristics of *Alliaria petiolata* (Bieb.) Cavara and Grande, *Tanacetum vulgare* L. and *Brassica napur* L.

Figure 34 is based on the following sources: [39] Drozdova et al. (2019), [40]e-FLORA-sys (1986) [41,42] Pardini et al. (2009), [43,47,52] e-FLORA-sys (2009) [44] Adamcová et al. (2017); Konieczny and Ślezak (2020), [45] Dragland et al. (2005), [46] Stevović et al. (2010), [48] Angelova et al. (2017), [49] Harker et al. (2015), [50] Soodabeh Saeidnia (2012), [51] Park et al. (2012)

6 Conclusions

The present work aimed at assessing the suitability of brownfield sites to biomass production by setting up two groups of lysimeter experiments, the first involving willow crop and the second implying rapeseed and some other herbaceous plants having a medicinal value. The principal objective for willow crop in SRC was to assess the impact of different types of fertilisation on the production of biomass as well as on the leaching of both total nitrogen and trace elements. The main objective of the second lysimeter experiment was to determine if the solubility of trace elements was affected by plant growth.

The first experiment has shown that the increase in nitrogen application on soil makes it possible to increase the production of biomass, regardless of the fertilisation used. However, in view of the large amount of leached nitrogen and possible increase in pH induced in the case of mineral nitrogen, organic fertilisation was considered for the fertilisation advice, the organic amendments seemed to stand out positively. In particular, sludge sometimes allowed to reduce bioavailability of some trace elements as well as allowing continuous release of mineral nitrogen. The increase in biomass on unpolluted soil was extremely marked, compared to polluted soil and quantities of trace elements phyto-extracted were higher in unpolluted soil for Cd and Zn and on polluted soil for Cu and Pb. As very small amounts of trace elements were detected in the leachate, no trend were identified concerning their evolution.

Concerning the second part of this master thesis, no results were considered to be of good quality with regard to the evolution of leachate over time. This lack of data is not without its rewards, however, as it has made it possible to bring perspectives and highlight the selection of varieties according to biodiversity criteria for future experiments.

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Appendices

Appendix 1: Characteristics of the digestate and sludge used for the second fertilisation

Results on raw matter	**Digestate (Cinergie)**	**Sewadge Sludge (Roselies)**
Dry matter at 60° C	6,48	35,22
Total Nitrogen (%)	0,43	1,208
Organic Nitrogen (%)	0,262	0,858
Mineral Nitrogen (NH_4^+) (%)	0,167	0,349
pH	8	13,2

It is important to mention that analyses were carried out by the B.E.A.G.X. 5 months after keeping the samples in cold storage. However, these values seem plausible, given that the total nitrogen values correspond to the values of the measurement campaign carried out in 2019. The amount of Total Nitrogen (%) in the sludge ranged between 1.185 and 1.353 and in the digestate between 0.423 and 0.617.

Appendix 2a: Summary table of the statistical analyses made on pH before fertilisation

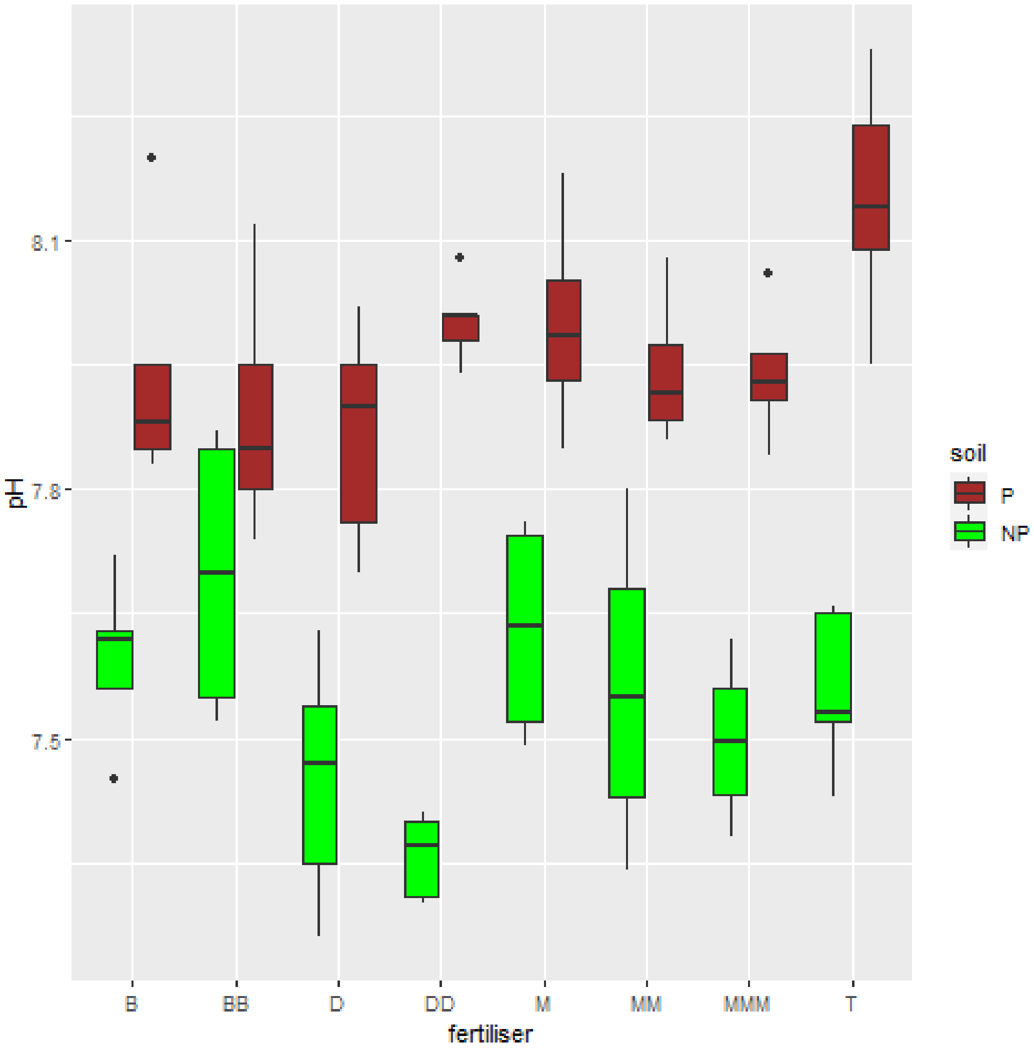
Statistical test	Factors	p-value
ANOVA	soil	I.**
	fertiliser	
	dose	

Statistical test	Dataset subdivision		Factors	p-value		SNK groups a	SNK groups ab	SNK groups b
	Fertiliser					a	ab	b
ANOVA	M		soil	9,18e-07	***	P		NP
			dose	0,365	n.s			
	B		soil	0,000639	***	P		NP
			dose	0,689260	n.s			
	D		soil	I.*				
			dose					
	Fertiliser	Dose						
	D	100	soil	0,00157	**	P		NP
		200	soil	4,02e-08	***	P		NP
	Soil			p-value				
	P		fertiliser	0,0124	*	T		M, D, B
			dose	0,6096	n.s			
	NP		fertiliser	0,00261	**	B	M,T	D
			dose	0,44058	n.s			

*Codes: n.s: non significant, *: significant (p-value < 0,05) , **: highly significant (p-value < 0,01) , ***: very highly significant (p-value < 0,001), I.: significant interaction between factors.*

Note: The groups defined by the SNK method of mean comparison are identified by letters in decreasing order (the group with the letter "a" has a significantly higher mean than group "b"). Since it makes no sense to consider the factors separately when there is interaction, the individual p-values of the factors are not shown.

Appendix 2b: Boxplot of the pH according to the modalities



Appendix 2c: Direct impact on pH before vs after fertilisation

Statistical test	Factors	p-value
Repeated measures ANOVA	soil, fertiliser, day, dose	I.**

Statistical test	Dataset subdivision: Soil	Fertiliser	dose	Factors	p-value		Tukey contrast groups: a	b
Repeated measures ANOVA	P			dose				
	P			fertiliser, day	I.*			
	NP			fertiliser, dose, day	I.***			
	P	M		dose, day	I.*			
	P	B		dose	0,7698	n.s		
	P	B		day	2,148e-06	***	before	after
	P	D		dose, day	I.*			
	NP	M		dose, day	I.***			
	NP	B		dose	0,33645	n.s		
	NP	B		day	0,02757	*	after	before
	NP	D		dose	0,98292	n.s		
	NP	D		day	2,641e-05	***	before	after
	P	M	50	day	0,02556	*	before	after
	P	M	100	day	0,006608	**	before	after
	P	M	200	day	0,2691	n.s		
	P	D	100	day	1,851e-06	***	before	after
	P	D	200	day	0,1606	n.s		
	NP	M	50	day	0,3946	n.s		
	NP	M	100	day	0,5502	n.s		
	NP	M	200	day	1,677e-06	***	after	before

*Codes: n.s: non significant, *: significant (p-value < 0,05) , **: highly significant (p-value < 0,01) , ***: very highly significant (p-value < 0,001), I.: significant interaction between factors.*
Note: The groups defined by the Tukey contrast method of mean comparison are identified by letters in increasing order (the group with the letter "b" has a significantly higher mean than group "a"). Since it makes no sense to consider the factors separately when there is interaction, the individual p-values of the factors are not shown.

Appendix 2d: Repeated measures ANOVAs on pH after fertilisation

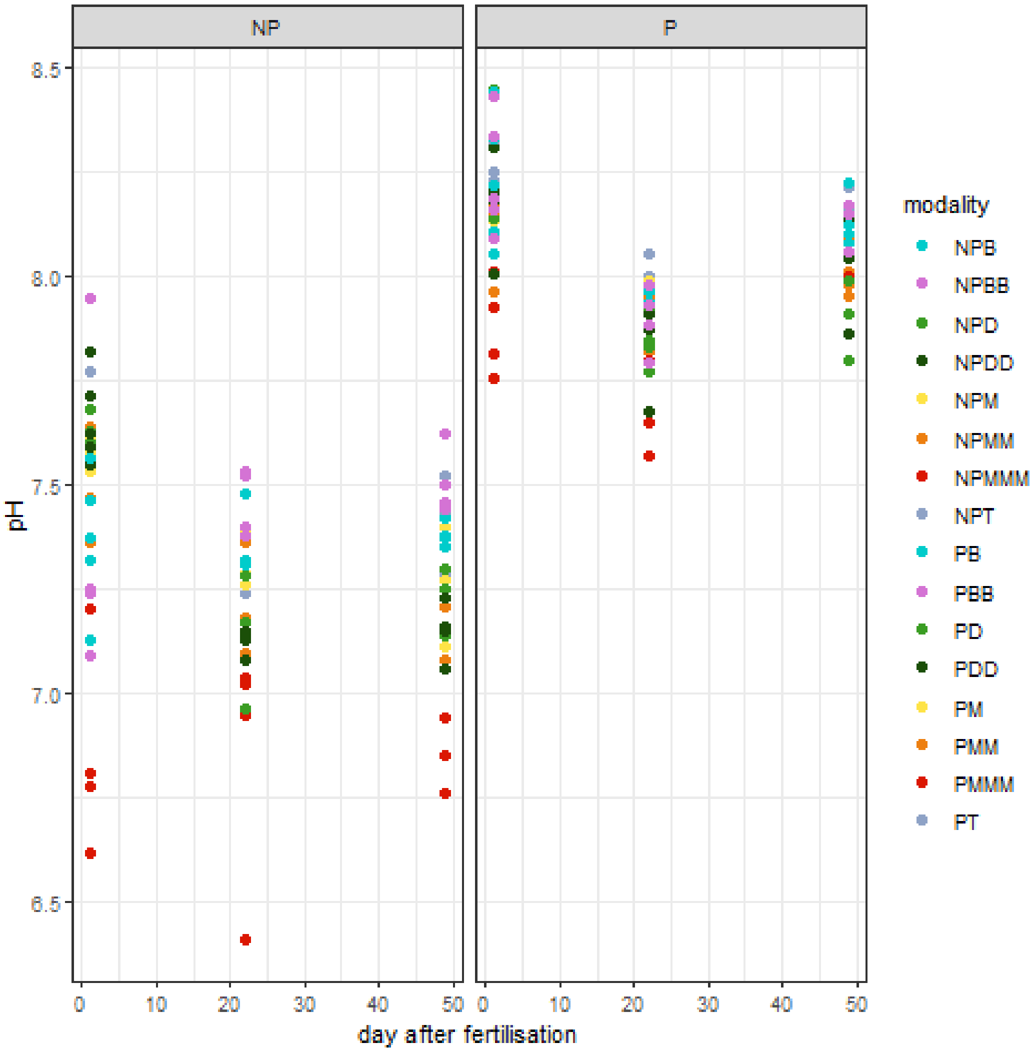
Statistical test	Factors	p-value	
Repeated measures ANOVA	soil	I.***	
	fertiliser		
	day		
	dose		

Statistical test	Dataset subdivision			Factors	p-value		Tukey contrast groups		
	Soil						a	b	c
Repeated measures ANOVA	P			fertiliser	I.*				
				dose					
				day					
	NP			fertiliser	I.***				
				dose					
				day					
	Soil	dose							
	P	100		fertiliser	I.**				
				day					
		200		fertiliser	I.**				
				day					
	NP	100		fertiliser	I.***				
				day					
		200		fertiliser	I.***				
				day					
	Soil	Fertiliser							
	P	M		dose	I.*				
				day					
		B		dose	0,6615				
				day	2,784e-14	***	22	1, 49	
		D		dose	I.***				
				day					
	NP	M		dose	I.***				
				day					
		B		dose	I.**				
				day					
		D		dose	0,95258				
				day	<2e-16	***	22, 49	1	
	Soil	Fertiliser	dose						
	P	M	50	day	<2,2e-16	***	22	49	1
			100	day	2,189e-05	***	22	1,49	
			200	day	2,708e-05	***	22	1,49	
		D	100	day	<2,2e-16	***	22,49	1	
			200	day	<2,2e-16	***	22	49	1
	NP	M	50	day	1,785e-11	***	22,49	1	
			100	day	<2,2e-16	***	22,49	1	
			200	day	0,3226	n.s			
		B	100	day	0,9899	n.s			
			200	day	1,088e-06	***	1	22,49	

*Codes: n.s: non significant, *: significant (p-value < 0,05) , **: highly significant (p-value < 0,01) , ***: very highly significant (p-value < 0,001), I.: significant interaction between factors.*

Note: The groups defined by the Tukey contrast method of mean comparison are identified by letters in increasing order (the group with the letter "b" has a significantly higher mean than group "a"). Since it makes no sense to consider the factors separately when there is interaction, the individual p-values of the factors are not shown.

Appendix 2e: pH evolution after fertilisation



Appendix 3: Evolution of total nitrogen leached

Statistical test	Factors	p-value	
Repeated measures ANOVA	soil		
	fertiliser	I.***	
	day		I.**
	dose		

Statistical test	Dataset subdivision	Factors	p-value			Tukey contrast groups		
	Dose					a	b	c
Repeated measures ANOVA	200	soil						
		fertiliser	I.***					
		day						
	100	soil						
		fertiliser	I.***					
		day						
	Fertiliser							
	M	soil	I.*					
		day		I.***				
		dose						
	B	soil						
		dose	I.**					
		day						
	D	soil	1,558e-10	***		NP	P	
		dose	1,618e-05	***		100, 200		
		day	1,568e-13	***		49	22	1

*Codes: n.s: non significant, *: significant (p-value < 0,05) , **: highly significant (p-value < 0,01) , ***: very highly significant (p-value < 0,001), I.: significant interaction between factors.*

Note: The groups defined by the Tukey contrast method of mean comparison are identified by letters in increasing order (the group with the letter "b" has a significantly higher mean than group "a"). Since it makes no sense to consider the factors separately when there is interaction, the individual p-values of the factors are not shown.

Appendix 4: Raw data of trace elements leached

name	May 19 2020 (before fertilisation)		July 13 2020		August 4 2020		September 22 2020	
	Cu	Zn	Cu	Zn	Cu	Zn	Cu	Zn
PT1	<QL	<QL	0,051	<QL	0,041	<QL	<QL	0,012
PT2	<QL	<QL	0,038	<QL	0,035	<QL	0,0306	0,012
PT3	<QL	<QL	0,034	<QL	<QL	<QL	<QL	<QL
PT4	<QL	<QL	0,037	<QL	0,032	<QL	<QL	<QL
PT5	<QL	<QL	0,042	<QL	<QL	<QL	<QL	<QL
PM1	<QL	<QL	0,033	<QL	0,035	<QL	<QL	<QL
PM2	<QL	<QL	0,036	<QL	0,034	<QL	<QL	<QL
PM3	<QL	<QL	<QL	<QL	<QL	<QL	<QL	<QL
PM4	<QL	<QL	0,034	<QL	0,032	0,012	0,032	<QL
PMM1	<QL	<QL	<QL	<QL	<QL	<QL	<QL	<QL
PMM2	<QL	<QL	0,030	<QL	<QL	<QL	<QL	<QL
PMM3	<QL	<QL	0,030	<QL	0,030	0,106	<QL	<QL
PMM4	<QL	<QL	<QL	<QL	<QL	<QL	<QL	<QL
PMMM1	<QL	<QL	<QL	0,013	<QL	<QL	<QL	<QL
PMMM2	<QL	<QL	<QL	0,014	<QL	0,011	<QL	<QL
PMMM3	<QL	<QL	<QL	0,022	<QL	<QL	<QL	<QL
PMMM4	<QL	<QL	<QL	0,011	<QL	0,013	<QL	<QL
PD1	<QL	<QL	0,056	<QL	<QL	<QL	<QL	<QL
PD2	<QL	<QL	0,036	<QL	0,032	<QL	0,0315	<QL
PD3	<QL	<QL	0,055	<QL	0,041	<QL	0,0387	<QL
PD4	<QL	<QL	0,049	<QL	0,033	<QL	<QL	<QL
PD5	<QL	<QL	0,047	0,013	<QL	<QL	<QL	<QL
PDD1	<QL	<QL	0,060	0,013	0,044	0,011	0,037	<QL
PDD2	<QL	<QL	0,074	0,025	0,058	0,010	0,045	<QL
PDD3	<QL	<QL	0,044	0,013	0,044	0,013	0,040	<QL
PDD4	<QL	<QL	0,060	0,014	0,033	0,010	<QL	<QL
PDD5	<QL	<QL	0,061	0,018	0,040	0,013	0,030	<QL
PB1	<QL	<QL	0,103	<QL	0,088	0,019	0,057	<QL
PB2	<QL	<QL	0,108	0,010	0,087	0,019	0,070	<QL
PB3	<QL	<QL	0,097	<QL	0,105	0,017	0,069	0,011
PB4	<QL	<QL	0,103	<QL	0,089	<QL	0,068	<QL
PB5	<QL	<QL	0,100	0,011	0,069	0,012	0,052	<QL
PBB1	0,040	<QL	0,120	0,013	0,099	0,013	0,070	<QL
PBB2	<QL	<QL	0,191	0,014	0,109	0,017	0,071	<QL
PBB3	<QL	<QL	0,170	0,017	0,100	0,015	0,071	<QL
PBB4	0,033	<QL	0,194	0,024	0,092	0,013	0,058	<QL
PBB5	<QL	<QL	0,145	0,014	0,088	0,011	0,064	0,011

name	May 19 2020 (before fertilisation)		July 13 2020		August 4 2020		September 22 2020	
	Cu	Zn	Cu	Zn	Cu	Zn	Cu	Zn
NPT1	<QL	<QL	<QL	0,011	<QL	0,026	<QL	<QL
NPT2	<QL	<QL	<QL	0,018	<QL	0,021	<QL	0,013
NPT3	<QL	<QL	<QL	<QL	<QL	0,024	<QL	0,012
NPT4	<QL	<QL	<QL	0,014	<QL	0,031	<QL	0,010
NPT5	<QL	<QL	<QL	<QL	<QL	0,025	<QL	0,013
NPM1	<QL	<QL	<QL	<QL	<QL	0,025	<QL	<QL
NPM2	<QL	<QL	<QL	<QL	<QL	0,016	<QL	0,011
NPM3	<QL	0,01	<QL	0,011	<QL	0,038	<QL	0,012
NPM4	<QL	<QL	<QL	0,018	<QL	0,038	<QL	0,016
NPMM1	<QL	<QL	<QL	<QL	<QL	0,015	<QL	<QL
NPMM2	<QL	<QL	<QL	0,011	<QL	0,039	<QL	0,011
NPMM3	<QL	<QL	<QL	0,029	<QL	0,045	<QL	0,019
NPMM4	<QL	<QL	<QL	<QL	<QL	0,028	<QL	0,011
NPMMM1	<QL	<QL	<QL	0,100	<QL	0,047	<QL	0,034
NPMMM2	<QL	<QL	<QL	<QL	<QL	<QL	<QL	0,014
NPMMM3	<QL	<QL	<QL	0,053	<QL	0,013	<QL	0,012
NPMMM4	<QL	<QL	<QL	1,095	<QL	0,013	<QL	0,011
NPD1	<QL	<QL	<QL	0,052	<QL	0,035	<QL	<QL
NPD2	<QL	<QL	<QL	0,032	<QL	0,014	<QL	<QL
NPD3	<QL	<QL	<QL	<QL	<QL	0,021	<QL	<QL
NPD4	<QL	0,012	<QL	0,012	<QL	0,025	<QL	<QL
NPD5	<QL	<QL	<QL	<QL	<QL	<QL	<QL	<QL
NPDD1	<QL	<QL	<QL	<QL	<QL	0,012	<QL	<QL
NPDD2	<QL	<QL	<QL	0,012	<QL	0,018	<QL	<QL
NPDD3	<QL	<QL	<QL	0,025	<QL	0,029	<QL	<QL
NPDD4	<QL	<QL	<QL	0,015	<QL	0,018	<QL	<QL
NPDD5	<QL	<QL	<QL	0,011	<QL	0,014	<QL	0,012
NPB1	<QL	<QL	0,055	0,016	<QL	0,024	<QL	<QL
NPB2	<QL	<QL	<QL	<QL	<QL	0,026	<QL	<QL
NPB3	<QL	<QL	<QL	<QL	<QL	0,020	<QL	0,011
NPB4	<QL	<QL	0,034	0,017	<QL	0,015	<QL	0,015
NPB5	<QL	<QL	0,047	0,012	<QL	0,017	<QL	0,014
NPBB1	<QL	<QL	0,051	0,020	<QL	0,025	<QL	0,016
NPBB2	<QL	<QL	0,030	0,016	<QL	0,022	<QL	0,014
NPBB3	<QL	<QL	0,076	0,015	0,034	0,028	<QL	0,011
NPBB4	<QL	<QL	0,056	0,014	<QL	<QL	<QL	<QL
NPBB5	<QL	<QL	<QL	0,012	0,03	0,029	<QL	0,014

Appendix 5a: Main descriptive statistics of volume increase estimation between first and second measurements

Modality	Mean	Sd	1st Qu.	3rd Qu.	Min	Max
PT	0,23	1,34	0,17	0,81	-2,00	1,56
PM	3,74	1,30	3,22	4,07	2,36	5,50
PMM	5,35	3,02	2,93	7,75	2,40	8,30
PMMM	0,33	7,24	-2,97	15,34	-5,94	10,78
PD	6,53	2,44	5,19	8,17	2,86	8,44
PDD	7,71	6,72	2,70	11,99	-0,70	15,79
PB	3,90	6,07	1,04	4,34	-2,60	13,71
PBB	0,97	5,70	2,73	3,65	-6,22	8,54
NPT	2,22	1,95	1,38	2,99	0,15	5,21
NPM	9,17	3,35	6,73	10,64	6,55	13,74
NPMM	5,08	6,09	2,24	9,59	-3,11	9,95
NPMMM	3,84	3,91	2,63	5,92	-1,61	7,55
NPD	6,09	4,67	2,34	9,41	1,52	12,38
NPDD	4,02	3,62	1,72	6,48	0,60	9,13
NPB	10,75	3,48	9,76	10,25	7,27	16,60
NPBB	-1,93	3,42	-3,73	1,10	-6,65	1,57

Appendix 5b: Main descriptive statistics of volume increase estimation between second and third measurements

Modality	Mean	Sd	1st Qu.	3rd Qu.	Min	Max
PT	0.70	1.64	0.18	1.78	-1.47	2.79
PM	1.56	2.37	1.02	2.60	-1.76	3.85
PMM	-5.72	4.21	-7.54	-5.01	-9.52	0.31
PMMM	-4.00	2.61	-5.48	-3.24	-6.30	-0.30
PD	-4,21	3.00	-5,96	-3,44	-7,13	0,59
PDD	-4.23	2.50	-5.29	-2.83	-7.59	-0.98
PB	-0,04	6.91	-4,00	1,97	-6,69	10,97
PBB	2,35	6.22	1,87	5,57	-8,16	7,51
NPT	3,34	3.20	2,7	3,11	-0,42	8,46
NPM	0,47	1.19	-0,22	1,17	-0,93	1,84
NPMM	-6,71	5.60	-10,57	-4,57	-11,11	1,11
NPMMM	-2,11	12.57	-11,71	5,44	-13,48	13,37
NPD	-2,58	3.25	-4,75	-0,27	-6,07	1,79
NPDD	-2,18	5.00	-5,75	2,44	-7,14	3,88
NPB	3,98	4.58	2,71	5,24	-2,82	9,74
NPBB	2,46	6.73	0,4	5,50	-7,95	9,9

Appendix 5c: ANOVAs on difference in volume increase estimation between first and second measurements

Statistical test			factors	p-value		SNK groups		
ANOVA			soil					
			fertiliser	I.*				
			dose					
Statistical test	**Dataset subdivision**		**factors**	**p-value**		**SNK groups**		
	Fertiliser					a	ab	b
ANOVA	M		soil	0,141	n.s			
			dose	0,175	n.s			
	D		soil	0,335	n.s			
			dose	0,832	n.s			
	B		soil	I.*				
			dose					
	Fertiliser	Soil						
	B	P	dose	0,455	n.s			
		NP	dose	0,000399	***	100		200
	Dose							
	100		soil	0,206	n.s			
			fertiliser	0,620	n.s			
	200		soil	0,5060	n.s			
			fertiliser	0,0428	*	D	M	B

*Codes: n.s: non significant, *: significant (p-value < 0,05) , **: highly significant (p-value < 0,01) , ***: very highly significant (p-value < 0,001), I.: significant interaction between factors.*
Note The groups defined by the SNK method of mean comparison are identified by letters in decreasing order (the group with the letter "a" has a significantly higher mean than group "b"). Since it makes no sense to consider the factors separately when there is interaction, the individual p-values of the factors are not shown.

Appendix 5d: ANOVAs on difference in volume increase estimation between second and third measurements

Statistical test	factors	p-value		SNK groups	
				a	b
permutation ANOVA	soil	0,80392	n.s		
	fertiliser	0,02	*	T, B	M, D
	dose	0,02399	*	0,50,100,200	

*Codes: n.s: non significant, *: significant (p-value < 0,05) , **: highly significant (p-value < 0,01) , ***: very highly significant (p-value < 0,001).*
Note: The groups defined by the SNK method of mean comparison are identified by letters in decreasing order (the group with the letter "a" has a significantly higher mean than group "b")

Appendix 6a: Raw data on total dry willow biomass production and trace element content in dry biomass

Modality	biomass (g)		Cu (mg/kg DM)		Zn (mg/kg DM)		Pb(mg/kg DM)		Cd (mg/kg DM)	
	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd
PT	21,48	2,31	7,66	0,59	201,64	25,25	2,34	0,54	1,14	0,14
PM	27,42	1,85	7,94	0,96	196,73	21,62	2,07	0,34	1,14	0,04
PMM	27,02	3,93	8,95	1,97	202,26	32,49	2,50	0,37	1,12	0,21
PMMM	29,38	6,82	8,10	0,25	196,55	30,78	2,79	0,22	1,24	0,16
PD	30,54	3,23	7,50	0,61	195,51	22,92	2,36	0,37	1,22	0,18
PDD	30,22	7,64	7,54	1,16	188,22	41,88	2,28	0,52	1,25	0,28
PB	27,84	2,98	7,56	1,03	172,77	24,20	2,04	0,31	0,96	0,15
PBB	35,23	2,74	8,27	0,75	146,70	15,13	2,37	0,27	0,80	0,12
NPT	25,35	3,80	6,00	0,32	291,89	15,92	1,61	0,15	3,41	0,14
NPM	32,16	7,97	6,17	0,96	269,38	23,11	1,53	0,23	3,03	0,30
NPMM	41,59	5,63	5,34	0,73	237,00	31,59	1,54	0,20	2,80	0,53
NPMMM	47,41	4,76	5,03	0,80	197,20	34,51	1,74	0,30	2,14	0,36
NPD	35,73	4,00	5,13	0,37	239,50	23,40	1,25	0,25	2,74	0,49
NPDD	45,36	6,12	5,10	0,54	211,98	27,42	1,64	0,37	2,53	0,42
NPB	39,41	4,97	4,96	0,50	207,98	31,16	1,74	0,49	2,46	0,57
NPBB	47,76	10,12	5,36	0,65	167,77	33,67	1,46	0,27	1,78	0,46

Appendix 6b: ANOVA carried out on biomass production

Statistical test	Factors	p-value	
ANOVA	dose	I.**	
	soil		I.**
	fertiliser		

Statistical test	Dataset subdivision				SNK groups		
	Fertiliser	factors	p-value		a	b	c
ANOVA	M	soil	1,15e-16	***	NP	P	
		dose	0,0514	n.s			
	B	soil	3,64e-06	***	NP	P	
		dose	6,35e-05	***	200	100	
	D	soil	0,000599	***	NP	P	
		dose	0,006220	**	200	100	
	Dose	factors	p-value				
	100	soil	1,74e-06	***	NP	P	
		fertiliser	0,8402	n.s			
	200	soil	2,82e-07	***	NP	P	
		fertiliser	0,0878	n.s			
	Soil	factors	p-value				
	P	fertiliser	5,29e-05	***	M, D, B	T	
		dose	0,0128	*	200	100, 50	0
	NP	fertiliser	7,72e-07	***	M, D, B	T	
		dose	2,16e-05	***	200	100, 50	0

*Codes: n.s: non significant, *: significant (p-value < 0,05) , **: highly significant (p-value < 0,01) , ***: very highly significant (p-value < 0,001), I.: significant interaction between factors.*

Note The groups defined by the SNK method of mean comparison are identified by letters in decreasing order (the group with the letter "a" has a significantly higher mean than group "b"). Since it makes no sense to consider the factors separately when there is interaction, the individual p-values of the factors are not shown.

Appendix 6c: ANOVA carried out on Cd content in biomass

Statistical test	Factors	p-value	
permutation ANOVA	dose	I.**	
	soil		I.**
	fertiliser		

Statistical test	Dataset subdivision		Factors	p-value		SNK groups				
	Fertiliser					a	ab	b	bc	c
ANOVA	D		soil	2,14e-07	***	NP		P		
			dose	0,588	n.s					
	B		soil	2,05e-06	***	NP		P		
			dose	0,0333	*	100		200		
	M		soil	I.**						
			dose							
	Fertiliser	dose								
	M	50	soil	1,53e-05	***	NP		P		
		100	soil	0,00105	**	NP		P		
		200	soil	0,00358	**	NP		P		
	Fertiliser	soil								
	M	P	dose	0,301	n.s					
		NP	dose	0,00733	**	100		200		
	Soil									
	P		fertiliser	0,000181	***	D, M, T		B		
			dose	0,923607	n.s					
	NP		fertiliser	0,0505	n.s					
			dose	4,55e-05	***	0	50		100	200
	Dose									
	100		soil	6,04e-10	***	NP		P		
			fertiliser	0,269	n.s					
	200		soil	1,62e-08	***	NP		P		
			fertiliser	0,00227	**	D, M		B		

*Codes: n.s: non significant, *: significant (p-value < 0,05) , **: highly significant (p-value < 0,01) , ***: very highly significant (p-value < 0,001), I.: significant interaction between factors.*

Note: The groups defined by the SNK method of means comparison are identified by letters in decreasing order (the group with the letter "a" has a significantly higher mean than group "b"). Since it makes no sense to consider the factors separately when there is interaction, the individual p-values of the factors are not shown.

Appendix 6d: ANOVA carried out on Pb content in biomass

Statistical test	Factors	p-value
ANOVA	dose	I.*
	soil	
	fertiliser	

Statistical test	Dataset subdivision		Factors	p-value		SNK groups a	ab	b
	Fertiliser					a	ab	b
ANOVA	M		soil	8,26e-07	***	P		NP
			dose	0,014	*	200	100	50
	B		soil	0,00262	**	P		NP
			dose	0,81015	n.s			
	D		soil	0,000116	***	P		NP
			dose	0,395008	n.s			
	Dose							
	200		soil	3,4e-06	***	P		NP
			fertiliser	0,107	n.s			
	100		soil	I.*				
			fertiliser					
	Dose	Fertiliser						
	100	M	soil	0,00387	***	P		NP
		B	soil	0,287	n.s			
		D	soil	0,000489	***	P		NP
	Dose	Soil						
	100	P	fertiliser	0,166	n.s			
		NP	fertiliser	0,127	n.s			
	Soil							
	P		fertiliser	0,5258	n.s			
			dose	0,0401	*	0,50,100, 200		
	NP		fertiliser	0,5591	n.s			
			dose	0,5449	n.s			

*Codes: n.s: non significant, *: significant (p-value < 0,05) , **: highly significant (p-value < 0,01) , ***: very highly significant (p-value < 0,001), I.: significant interaction between factors.*

Note: The groups defined by the SNK method of mean comparison are identified by letters in decreasing order (the group with the letter "a" has a significantly higher mean than group "b"). Since it makes no sense to consider the factors separately when there is interaction, the individual p-values of the factors are not shown.

Appendix 6e: ANOVA carried out on Zn content in biomass

Statistical test	factors	p-value	
ANOVA	dose	I.*	
	soil		I.*
	fertiliser		

Statistical test	Dataset subdivision	factors	p-value		SNK groups		
	Fertiliser				a	b	c
ANOVA	M	soil	0,00771	**	NP	P	
		dose	0,07016	n.s			
	B	soil	0,0248	*	NP	P	
		dose	0,0171	*	100	200	
	D	soil	0,0222	*	NP	P	
		dose	0,2118	n.s			
	Dose						
	100	soil	0,00134	**	NP	P	
		fertiliser	0,05353	n.s			
	200	soil	0,1507	n.s			
		fertiliser	0,0138	*	D, M	B	
	Soil						
	P	fertiliser	0,0107	*	T, M, D	B	
		dose	0,4394	n.s			
	NP	fertiliser	6,55e-06	***	T	M, D	B
		dose	0,000487	***	0, 50	100	200

*Codes: n.s: non significant, *: significant (p-value < 0,05) , **: highly significant (p-value < 0,01) , ***: very highly significant (p-value < 0,001), I.: significant interaction between factors.*
Note: The groups defined by the SNK method of mean comparison are identified by letters in decreasing order (the group with the letter "a" has a significantly higher mean than group "b"). Since it makes no sense to consider the factors separately when there is interaction, the individual p-values of the factors are not shown.

Appendix 6f: ANOVA carried out on Cu content in biomass

Statistical test	factors	p-value		SNK groups	
				a	b
permutation ANOVA	soil	<2e-16	***	P	NP
	fertiliser	0,07731	n.s		
	dose	0,82353	n.s		

*Codes: n.s: non significant, *: significant (p-value < 0,05) , **: highly significant (p-value < 0,01) , ***: very highly significant (p-value < 0,001), I.: significant interaction between factors.*
Note: The groups defined by the SNK method of mean comparison are identified by letters in decreasing order (the group with the letter "a" has a significantly higher mean than group "b"). Since it makes no sense to consider the factors separately when there is interaction, the individual p-values of the factors are not shown.

Appendix 7: ANOVA carried out on nitrates contained in soils at the end of the experiment

Statistical test	Factors	p-value
permutation ANOVA	soil	
	fertiliser	I.*
	dose	

Statistical test	Dataset subdivision		Factors	p-value		SNK groups		
	Soil					a	ab	b
permutation ANOVA	P		fertiliser	0,0212	*	B	D,M	T
			dose	0,0318	*	200	100	50,0
	NP		fertiliser	0,0032	**	B	D,M	T
			dose	0,1861	n.s			
	Dose							
	100		soil	0,575	n.s			
			fertiliser	0,389	n.s			
ANOVA	200		soil	0,383	n.s			
			fertiliser	0,040	*	B	D	M
	Fertiliser							
	M		soil	I.*				
			dose					
	D		soil	0,3977	n.s			
			dose	0,0706	n.s			
	B		soil	0,7420	n.s			
			dose	0,0508	n.s			
	Fertiliser	soil						
	M	P	dose	0,0301	*	50, 100, 200		
		NP	dose	0,265	n.s			

*Codes: n.s: non significant, *: significant (p-value < 0,05) , **: highly significant (p-value < 0,01) , ***: very highly significant (p-value < 0,001), I.: significant interaction between factors.*
Note: The groups defined by the SNK method of means comparison are identified by letters in decreasing order (the group with the letter "a" has a significantly higher mean than group "b"). Since it makes no sense to consider the factors separately when there is interaction, the individual p-values of the factors are not shown.

Appendix 8: Exact p-values obtained for all repeated measures ANOVA realised for one-meter high lysimeters

Herbaceous species	Variety	Depth	Ln(pH)	Ln(As) (ppb)	Ln(Cd) (ppb)	Ln(Cr) (ppb)	Ln(Cu) (ppb)	Ln(Mn) (ppb)	Ln(Mo) (ppb)	Ln(Ni) (ppb)	Ln(Pb) (ppb)	Ln(Zn) (ppb)
Control (no plant)		10	0,04217	0,3528	0,00097	x	0,8122	0,1175	0,3245	x	0,004262	0,2936
		35	0,4995	0,5471	6,953e-06	x	0,4224	0,000227	0,1731	x	x	0,03465
Lolium perenne L.		10	0,8133	<2,2e-16	0,9815	x	0,0139	0,2662	<2,2e-16	x	0,8761	0,6369
		35	0,001803	0,9045	0,0001161	x	<2,2e-16	0,2408	0,9193	x	0,1008	0,01285
Alliaria petiolata (Bieb.) Cavara and Grande		10	0,08471	0,002196	0,02443	x	0,4121	0,0316	0,8498	x	0,0002544	0,3183
		35	0,005112	0,03519	0,04259	x	<2,2e-16	0,0007376	2,063e-05	x	<2,2e-16	0,7681
Tanacetum vulgare L.		10	0,02647	0,7265	1,854e-05	x	9,697e-05	0,09993	0,001292	x	<2,2-e16	0,00803
		35	0,002391	0,9975	2,929e-07	x	x	2,27e-08	0,7295	x	0,0008393	0,76
Brassica napus L.	Axana	10	0,4995	0,1322	0,2535	x	3,634e-09	1,648e-05	0,83	x	x	0,006738
		35	<2,2e-16	0,6736	<2,2e-16	x	<2,2e-16	0,1601	0,08737	x	x	3,187e-08
	Cleopatra	10	0,3513	0,132	0,05464	x	0,08278	0,07851	0,0379	x	1,824e-09	1,457e-06
		35	<2,2e-16	<2,2e-16	0,00445	x	x	0,5035	0,9509	x	0,7936	0,03958
	Mosaik	10	0,0007785	0,06033	0,005038	x	1,414e-07	0,09977	0,4096	x	0,5855	0,0006486
		35	3,154e-05	0,606	0,0831	x	0,01701	2,979e-07	0,0859	x	<2,2e-16	0,4405
	Theia	10	7,105e-06	0,6239	0,07223	x	0,1934	0,2499	0,02213	x	0,308	0,03314
		35	x	x	x	x	x	x	x	x	x	x

Codes: x: not enough replicate to fit any lmer (production of NA or non-compliance with application conditions). The numbers in the table are p-values obtained after repeated measures ANOVA (type II Wald Chisquare test)

Appendix 9a: Exact p-values obtained for all repeated measures ANOVA realised on herbaceous grew in fifteen-centimeter high lysimeters

Herbaceous species	Ln(pH)	Ln(As) (ppb)	Ln(Cd) (ppb)	Ln(Cr) (ppb)	Ln(Cu) (ppb)	Ln(Mn) (ppb)	Ln(Mo) (ppb)	Ln(Ni) (ppb)	Ln(Pb) (ppb)	Ln(Zn) (ppb)
Lolium perenne L.	0,3889	0,8039	0,002671	8,029e-11	0,4515	5,163e-16	0,004629	0,06875	0,4922	4 ,104e-07
Echium vulgare L.	0,01096	0,0263	0,02667	2,406e-05	<2,2e-16	2,364e-11	1,012e-10	0,6356	0,4259	3,12e-16
Matricaria recutita L.	8,589e-05	0,0002949	0,2046	x	4,544e-14	0,00219	5,376e-06	0,9006	0,001456	<2,2e-16
Verbascum thapsus L.	<2,2e-16	0,6044	0,1466	0,001924	<2,2e-16	4,318e-15	0,0001182	0,9381	x	4,411e-08
Hypericum perforatum L.	1,496e-07	0,000255	0,0002606	0,007242	5,252e-09	0,0532	0,08115	x	0,3763	0,3863
Achillea millefolium L.	0,214	0,09352	0,0001478	0,08305	2,505e-11	0,5651	<2,2e-16	0,3125	x	6,519e-06
Valeriana repens Host	0,02298	0,001051	8,146e-06	<2.2e-16	2,088e-06	0,03361	0,1349	0,1513	x	6,379e-10
Stachys officinalis (L.) Trev	x	x	x	x	x	x	x	x	x	x

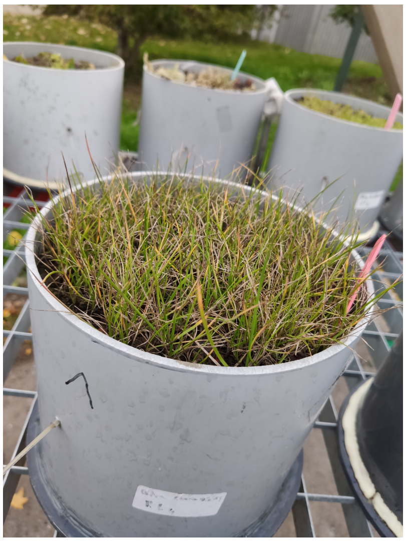
Codes: x: not enough replicate to fit any lmer (production of NA or non-compliance with application conditions), numbers in the table are p-values obtained after repeated measures ANOVA (type II Wald Chisquare test)

Appendix 9b: Exact p-values obtained for all repeated measures ANOVA realised on colza varieties grew in fifteen-centimeter high lysimeters

Brassica napus L. varieties	Ln(pH)	Ln(As) (ppb)	Ln(Cd) (ppb)	Ln(Cr) (ppb)	Ln(Cu) (ppb)	Ln(Mn) (ppb)	Ln(Mo) (ppb)	Ln(Ni) (ppb)	Ln(Pb) (ppb)	Ln(Zn) (ppb)
Mosaïk	4,116e-14	1,876e-13	0,01261	<2,2e-16	4,909e-15	0,07037	1,054e-10	7,443e-07	0,1721	3,589e-06
Theia	0,0882	1,65e-05	0,001691	6,562e-16	0,3477	0,05876	<2,2e-16	1,778e-16	2,686e-09	0,3477
Axana	1,216e-15	0,0003844	0,4056	<2,2e-16	0,001882	0,1773	<2,2e-16	0,3828	3,428e-05	0,05845
Cleopatra	0,03787	4,002e-07	2,573e-08	<2,2e-16	0,00451	0,2216	6,557e-07	3,364e-08	0,634	0,02959

Codes: numbers in the table are p-values obtained after repeated measures ANOVA (type II Wald Chisquare test)

Appendix 10a: 1st serie of pictures of the herbaceous plants that grew in the small lysimeters



(a)

(b)

(c)

(d)

On pictures: a) *Lolium perenne* L., b) *Echium vulgare* L., c) *Matricaria recutita* L., d) *Verbascum thapsus* L.

Appendix 10b: 2nd serie of pictures of the herbaceous plants that grew in the small lysimeters

(a)

(b)

(c)

(d)

On pictures: a) *Hypericum perforatum* L., b) *Achillea millefolium* L., c) *Valeriana officinale subsp. repens* Host., d) *Stachys officinalis* L. Trev