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Improving the agronomic quality of a residual red gypsum landfill through phytomanagement.

José Zapata Carbonell

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Université de Bourgogne Franche-Comté
École Doctorale Environnement Santé
Laboratoire Chrono-Environnement -- UMR 6249 CNRS

THÈSE

Présentée en vue de l'obtention du titre de

Docteur de l'Université de Bourgogne Franche-Comté
Spécialité << Sciences Agronomiques >>

**Améliorer la qualité agronomique d'un dépôt
de gypse rouge résiduel par phytomanagement**

Présentée et soutenue par

José Gonzalo ZAPATA CARBONELL

Le 15 mai 2020 à Besançon

Membres du jury:

Engracia María MADEJÓN RODRÍGUEZ (Directrice de recherche, IRNAS, CSIC)

Michel MENCH (Directeur de recherche, INRAE, U-Bordeaux)

Olivier FAURE (Maître de conférences, PEG, EMSE)

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Rapporteuse

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Examineur

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Abstract

The industrial development may bring along significant environmental concerns regarding the storage of residues. The residual red gypsum (RRG), which is the neutralization product of the TiO₂ extraction effluent, is an alkaline substrate rich in Ca, Fe, S, Mg, Mn, among others. Such characteristics complicate the proper natural reclamation, therefore requiring adequate management for meeting the state regulations. This PhD dissertation focused on the study of the RRG landfill found at the Ochsenfeld site in Alsace, France. **The general objective of this doctorate was to provide assistance on the plant reclamation of the Ochsenfeld site by following two virtual approaches or axes: the phytostabilization and the phytoextraction of Mn.** The first specific objective was to characterize the study site. Then the second and third specific objectives were to evaluate the use of organic amendments and mycorrhizae as assistance for the plant development. The last objective was to evaluate a specialized plant species capable of developing in RRG with minimum assistance. In this work, it was found that some species of the *Betulaceae* and *Salicaceae* families established spontaneously in the site, whose characteristic was the resistance to poor agronomic quality and tolerance to trace elements. The application of crushed pine bark chips not only decreased significantly the pH of RRG for *Betula pendula* plants to accumulate in leaves up to five-fold the natural concentrations of Mn, but also induced some drawbacks in the plants. The application of raw digestate to RRG increased the growth and biomass production of *Betula pendula* over the short-term, whereas its inoculation with mycorrhizal fungi induced a similar outcome only with visible effects over the medium- to long-term. Finally, the trials using *Lupinus albus* indicated that few was the assistance needed in order to allow its growth and development. Furthermore, the plant's mechanism that allowed the Mn phytoextraction provided some insight on the potential use of this species for further field-scale applications in seasonal co-cultures next to other Mn accumulating species in order to recover Mn from the RRG. **From an applied point of view, this PhD research allowed to determine the limiting factors that prevented the natural installation of vegetation in the RRG landfill. Furthermore, it allowed the generation of pertinent information on agronomic solutions to the encountered problematic. Treatment recommendations are made in order to meet the needs required by the French state, which would avoid possible impacts to the ecosystem and public health. Additionally, the management hereby mentioned may also provide insights for an alternative revalorization method for the RRG with an economically interesting perspective.**

Résumé

Le développement industriel peut entraîner d'importantes préoccupations environnementales concernant le stockage des résidus. Le gypse rouge résiduel (RRG), qui est le produit de neutralisation de l'extraction des effluents de TiO_2 , est un substrat alcalin riche en Ca, Fe, S, Mg, Mn, entre autres. De telles caractéristiques compliquent la remise en état naturelle appropriée, nécessitant donc une gestion adéquate pour répondre aux réglementations de l'État français. Cette thèse portait sur l'étude de la décharge de RRG localisée sur le site de l'Ochsenfeld en Alsace, France. **L'objectif général de cette thèse était d'apporter une assistance à la remise en état du site d'Ochsenfeld en suivant deux approches ou axes virtuels: la phytostabilisation et la phytoextraction du Mn.** Le premier objectif spécifique était de caractériser le site d'étude. Ensuite, les deuxièmes et troisièmes objectifs spécifiques étaient d'évaluer l'utilisation d'amendements organiques et de mycorhizes comme aide au développement de la plante. Le dernier objectif était d'évaluer une espèce végétale spécialisée capable de se développer en RRG avec un minimum d'assistance. Dans ce travail, il a été constaté que certaines espèces des familles *Betulaceae* et *Salicaceae* s'établissaient spontanément sur le site. Leurs caractéristiques étaient la résistance à une mauvaise qualité agronomique et la tolérance aux oligo-éléments. L'application de copeaux d'écorce de pin broyé a non seulement diminué de manière significative le pH du RRG permettant aux plantes de *Betula pendula* d'accumuler dans les feuilles jusqu'à cinq fois les concentrations naturelles de Mn, mais a également induit certains dysfonctionnements dans les plantes. L'application de digestat brut au RRG a augmenté la croissance et la production de biomasse de *Betula pendula* à court terme, tandis que son inoculation avec des champignons mycorhiziens a induit un effet similaire visible seulement à moyen et à long terme. Enfin, les tests utilisant *Lupinus albus* ont indiqué que l'assistance testée était insuffisante pour permettre sa croissance et son développement. De plus, le mécanisme de la plante qui a permis la phytoextraction de Mn a fourni un aperçu de l'utilisation potentielle de cette espèce pour d'autres applications à l'échelle du terrain dans des co-cultures saisonnières à côté d'autres espèces accumulant du Mn **D'un point de vue appliqué, cette recherche doctorale a permis de déterminer les facteurs limitants qui ont empêchés l'installation naturelle de végétation dans la décharge du RRG. De plus, elle a permis de générer des informations pertinentes sur les solutions agronomiques à la problématique rencontrée. Des recommandations de traitement ont été faites afin de répondre aux besoins requis par l'Etat français, ce qui éviterait des impacts possibles sur l'écosystème et la santé publique. De plus, la gestion proposée par ce travail peut fournir un aperçu d'une autre méthode de revalorisation pour le RRG avec une perspective économiquement intéressante.**

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Abbreviations

ACC: 1-aminocyclopropane-1-carboxylate

ADEME: The French Environmental and Energy Agency (Agence de l'Environnement et de la Maîtrise de l'Énergie)

AM: Arbuscular mycorrhizae / endomycorrhizae

ANR: National Research Agency (Agence Nationale de la Recherche)

BASOL: Data base of polluted sites and soils (Base de données sur les sites et sols pollués)

CEC: Cation exchange capacity

DM: Dry matter

DW: Dry weight

EC: Ectomycorrhizae

ER: Ericoid/orchid mycorrhizae

ES: Ecosystem services

GRO: Gentle remediation options

HC: Hydrocarbons

HM: Heavy metals

MNR: Monitoring natural recovery

MTES: Ministry of the Ecology and Solidarity Transition (Ministère de la Transition Écologique et Solidaire)

PAH: Polycyclic aromatic hydrocarbons

PCA: Principal components analysis

PGPB: Plant growth-promoting bacteria

PM: Particulate matter

PTE: Potentially toxic elements

REE: Rare earth elements

ROS: Reactive oxygen species

RRG: Residual red gypsum

TE: Trace elements

Scientific Valorization

Publications to peer-review journals

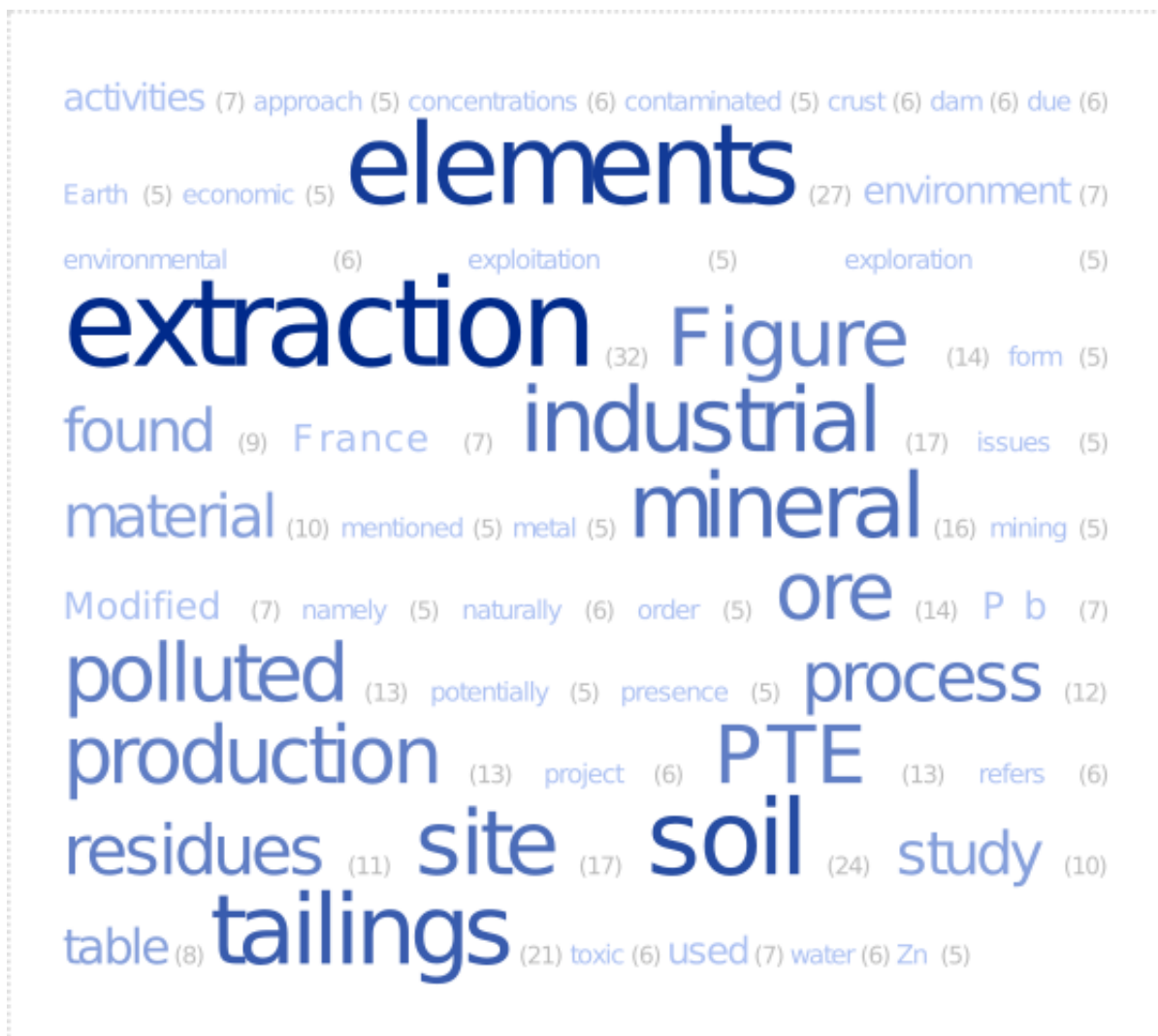
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Scientific Dissemination

Colloquim	References
FJC UBFC Besançon	Zapata-Carbonell, J. , Chalot, M., Tatin-Froux, F. Waste Revalorization: The enhancement of Industrial Soil Conditions. (June 14 -15, 2018).
15th IPC IPS Novi-Sad, Serbia	Zapata-Carbonell, J. , Bégeot, C., Carry, N., Choulet, F., Gillet, F., Girardclos, O., Mouly, A., Chalot, M. Natural Revegetation of a Red Gypsum Landfill. (October 1-5, 2018).
4th RNRSSP ADEME Paris	Zapata-Carbonell, J. , Maurice, N., Ciadamidaro, L., Pfendler, S., Tatin-Froux, T., Parelle, J., Chalot, M. Revitalising a Residual Red Gypsum Technosol with Indigenous Plant Species. (November 26-27, 2019, Poster).

Chapter 1

General introduction



Evolution allowed human beings to use reason for problem solving. This need led human to build tools to aid in some basic tasks such as hunting spears and arrow tips, knives and even soil exploiting tools for agriculture, another humankind's great discovery. For the construction process of their *utensils*, the human beings discovered that all the materials needed were found in the ground under their feet. Therefore, exploration and extraction of naturally occurring and precious materials in all its forms, i.e. solid, liquid or gas, have been part of the heritage of early civilizations passing from the Stone Age, before 4000 B. C, through the Bronze, Iron and Steel and the Nuclear Age, from 1945 (Hartman, 1987).

1.1 Definitions and concepts

1.1.1 The extractive industry and the production of hazardous material

The continuous application and changing of such innovating techniques led to modern practices, so common and important that soon they became industries. Nowadays, mining is the name of the industry dedicated to find, retrieve valuable elements either metalliferous minerals or non-metals, i.e. ore, from the Earth's crust (Moran et al., 2014). These elements may include not only raw material for manufacturing tools and building infrastructure, but also the extraction of precious rocks and minerals such as gold, silver and diamonds, which have been used as currency, probably due to their rarity. In the same fashion, extraction of coal, oil, since the industrial revolution and some actinide elements are now used as fuel to power other industries and for energy production, which are of great public and economic concern and therefore, have a great and increasing value in modern times (Figure 1). Some of the most economically important minerals may be sphalerite, galena, chalcopyrite, tetrahedrite-friebergite-tennantite, bornite, cassiterite, pyrite, quartz, chlorite, sericite, carbonate minerals, amphiboles, pyroxenes, feldspars, barite, arsenopyrite, monazite, titanite and zircon (Petruk, 2000).

The extraction from the Earth's crust can be classified in two types: aboveground and underground exploitation. The former refers to the use of material that is available for exploitation from the shallowest soil horizons, this extractive industry may comprise excavation, and transportation of the ore and disposal of the remaining overburden (Sobek et al., 1978); whereas the latter refers to the exploitation of ores that are found lower into the crust that may require complex underground exploration and installation of infrastructure for transportation of extractive equipment, this generally comprehends to send personnel underground to identify the mineral seam and install the required machinery (Frosch et al., 1980). In order to extract the existing ore, the basic steps to follow are exploration,

development, and production (Hustrulid et al., 2013). Once the ore is mined out of the ground, it is taken to the extraction facility for a cleaner extraction and concentration of the ore: the production.

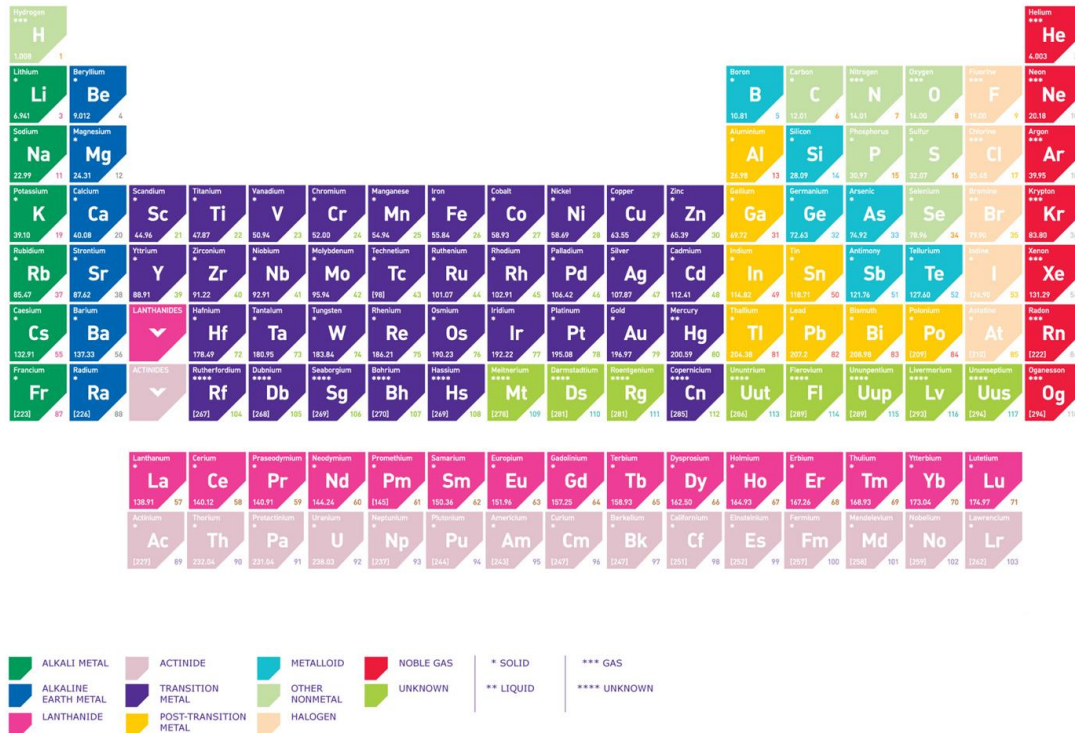


Figure 1. Mendeleev's periodic table of elements (Modified from <https://www.sigmaaldrich.com/>).

Prior to any process, the study of the area is necessary in order to characterize the mineral and material form, to determine the smelter products and related materials and solve any possible problem that may be encountered: the exploration. This is assessed by the applied mineralogy (Petruk, 2000). This discipline is therefore responsible of exploration of the site, the processing of the minerals, the disposal and treatment of the tailings and the refining. Once the elements are found and retrieved from the ground, a series of different processes are carried out in order to process this material (Figure 2). These processes give place to different by-products of environmental concern; namely slags, overburden, heaps and tailings.

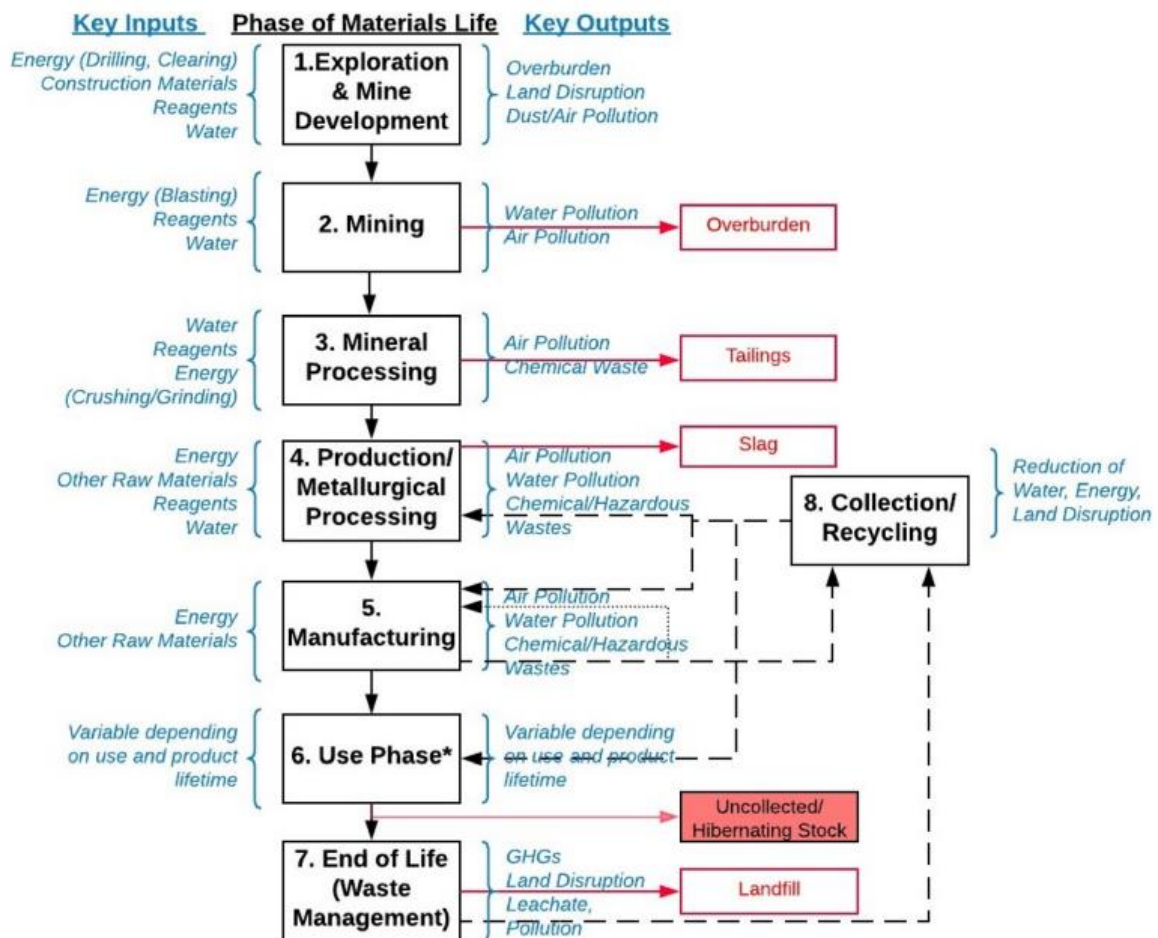


Figure 2. Life Cycle Assessment relative to overall mineral extractive industry (Modified from Gorman and Dzombak, 2018).

1.1.2 The residues of the extractive industry and risks

Tailing is the term given to the residues of the mineral extraction: both the fine grounded rock from 1 to 600 μm , and the used water of the process (Edraki et al., 2014). Tailings are often landfilled as open-air dumps and ponds or slag heaps (Johansson, 2016; Wang et al., 2017; Park et al., 2019). The study of tailings is of great relevance given the magnitude of the metallic ore extraction occurred in recent times (Figure 3), these may cause diverse environmental issues such as aerial dispersion of dusty particles, instability of tailings storage and the presence of sulfides and trace elements that might lead to leaching and groundwater pollution (Sun et al., 2018).

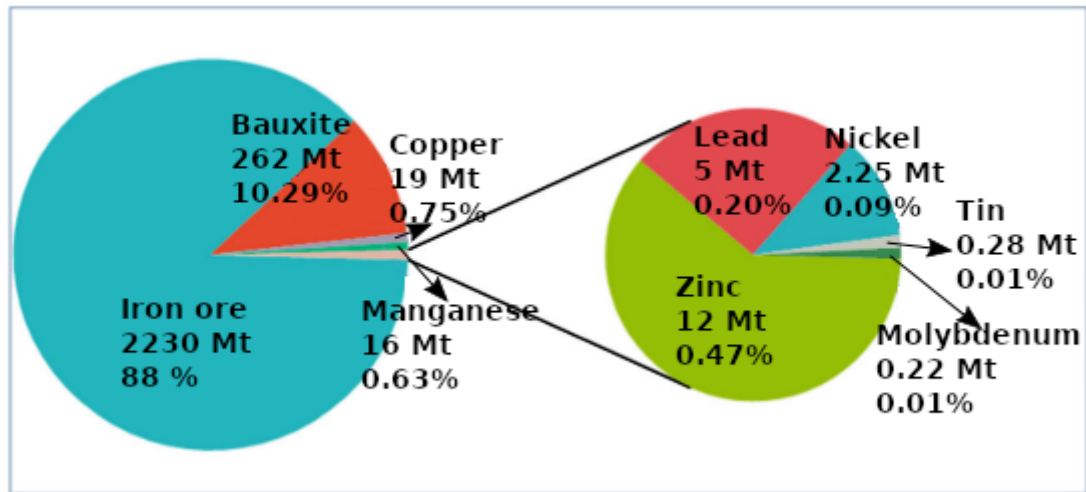


Figure 3: Worldwide production of metal ore in 2016 (Modified from Sun et al., 2018).

In addition, the location of the tailings influences a lot on the risk assessment of the site. For instance, the Panasqueira tin-tungsten mine in Portugal has produced over 8 million m³ of tailing piles and over 2 million m³ of mud dams, that are located nearby few small villages and the Zêzere River that feeds one of the dams and is also Lisbon's main water supply source (Grangeia et al., 2011). Notwithstanding the closeness and the quantities of tailings, the real danger comes from the composition of the tailings and its dispersion in the area, where over 220, 200, 163 and 320 ppm of As, Ba, Cu and Zn have been found in the rhizosphere of soils of nearby rural areas (Candeias et al., 2014) (Figure 4).

The characteristics of the tailings depend on the ore used and the reagents used during the extraction processes. Once the valuable mineral has been extracted, the composition of the tailings may be characterized by metals such as As, Cu, Mn, Pb or Zn (Mendez and Maier, 2008). These residues have been also known as anthroposols (Paterson, 1996) or more recently named Technosols by the Reference Group (Baxter, 2015).

Various are the case studies found in the literature of extractive activities. For instance, in Thailand, Sn is extracted in the form of cassiterite (SnO₂) from their western granite formation and also from the weathered rock, either *in-situ* or naturally retrieved by rain or streams (from alluvial deposits). The tailings resulting from this extraction process may contain Sn, Nb and Ta (Saisinchai et al., 2016). Although there is no data confirming the toxicity of any of the mentioned elements, the industry of SnO₂ extraction is an example of how the exploitation of valuable minerals can expose some embedded elements to the environment.

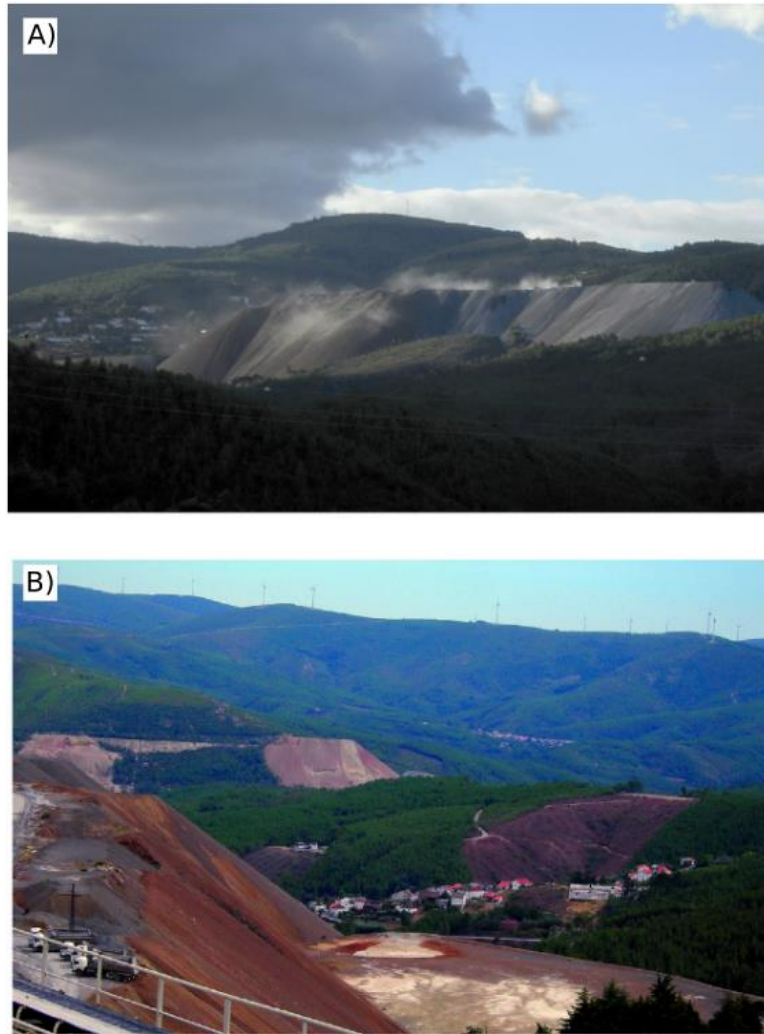


Figure 4. A) Views of deposit or in Barroca Grande, Portugal. A) Dust deposition by the wind of fine tailings. B) Machinery unloading coarse material. From Candeias et al., 2014.

Conversely, the extraction of alumina (Al_2O_3) from the bauxite ore requires a more complex process of extraction, namely the Bayer process (Jones and Haynes, 2011). The extraction method comprehends submitting the ores to elevated temperatures (106 to 240°C) and the addition of NaOH. Because the bauxite naturally contains a variety of trace elements, the bauxite residue, namely red-mud (RM), may contain elements such as Al, As, Cd, Cr, Cu, Fe, Hg, Mn, Mo, Na, Ni, V and Zn in concentrations significantly higher than soil baselines (Di et al., 2019). Additionally, the red-mud tailings have a solid and liquid phase, which involves the use of dams of contention that in turn require cautious and continuous supervision to avoid incidents as it has been seen in the past.

The sudden release or dam bursts of this type of residue may expose the environment and nearby populations to alkaline floods of sediment charged with trace elements, which could affect the productivity of agricultural fields and threaten the well-being on any organism that enters in contact with it (Aragon and Rud, 2012) (Figure 5)



Figure 5. Sediment of the dam burst accident in Ajka, Hungary in 2010. (Modified from Gelencsér et al., 2011).

The occurrence of dam bursts in the past has demanded the interest of the industrial sector and the authorities to react, which has led to the creation of the Red Mud Project[®]. The objective of this project is to encourage research on how to improve the management of the residue and the study of its properties and valorization (Red Mud Project, 2015). This approach can be thus extrapolated to tailings of other nature to meet the following needs:

- to understand the possible outcomes should a leak or contamination occurred from the tailings dump upon the ecosystem, either urban or wild.
- to seek for the benefit and utility of the tailings based on its content, in order to reduce its storage and accumulation.
- to optimize the purification of the element of interest, in order to obtain the maximum economic profit.

As mentioned before, the mineral extraction is not complete, therefore either the focal element of the industry or other valuable elements may still remain in the tailings. The interest

of the industrial partner might depend on the element to recover. A clear example is the Au extraction. The extraction method includes oxidation of the ores through cyanidation leach; however, depending on the ore sizes, the extraction may be as efficient as 90% (Brooy et al., 1994). This leaves a 10% of residual Au in the tailings. Added to this, the environmental impact of producing and dumping tailings sometimes means that the soil surface is occupied and left unused and uncovered by vegetation, which in turn entails a source of pollution and a potential health hazard for humans and the environment (Zhang et al., 2006) due to the presence of potentially toxic elements and cyanides within it (Salas-Luévano et al., 2017).

1.1.3 The potentially toxic elements (PTEs)

In the Earth's crust we may find a great number of elements of the Mendeleev's periodic table (Rudnick and Gao, 2003) (Table 1). The distribution of such elements over the surface of the Earth is random and ubiquitous, giving place to diverse habitats, and exceptional soil characteristics. It is through the factors mentioned at first that new habitats can be created, sometimes originating harsh and poor habitats (Zhao et al., 2006). As mentioned above, the mineral extraction from ore can lead to the exposure of the environment and living organisms to potentially toxic elements (PTEs) (Alloway, 1995) that may entail public health issues or impact ecosystems at a small and large scale (Förstner, 1987).

The term PTEs refers to a substance with a toxicological importance that can have adverse health effects when living organisms are exposed to it in excessive concentrations. Some PTEs are naturally present in the rock parent material and can be released gradually onto other environmental compartments thanks to lithogenic or pedogenetic processes, i.e. weathering, earthquakes, and volcanism (Veldkamp, 2005; Kosheleva et al., 2018). Nevertheless, it is through anthropogenic activities that such elements have become more abundant and thus more regulated (EEC, 2015).

PTEs is a term that has been found in the literature many times since 1988. This term refers indifferently to the inorganic elements that are found in either compartment in the environment: soil, water, air (Burton et al., 1988; Teixeira et al., 2018). Notably, a term often seen in the literature is heavy metal (HM), but this term may vary depending on the context: it could refer to those elements with a rather important density, the atomic number or chemical behavior (Nieboer and Richardson, 1980). Due to some discrepancies, the term used from now on will be PTEs. The trace elements (TE) also form part of the PTEs. This group is formed by 68 mineral elements naturally present in the Earth's crust with an abundance lower than 0.1% (Baize, 2000).

Table 1. Estimated composition of the upper continental crust. Modified from Rudnick and Gao, 2003.

Elements	Units	Upper crust	%	Elements	Units	Upper crust	%
SiO ₂		66.6	2	Ag	ng g ⁻¹	53	5
TiO ₂		0.64	13	Cd	μg g ⁻¹	0.09	15
Al ₂ O ₃		15.4	5	In		0.056	14
FeO _T		5.04	10	Sn		2.1	26
MnO	wt %	0.10	13	Sb		0.4	28
MgO		2.48	14	I		1.4	50
CaO		3.59	6	Cs		4.9	31
Na ₂ O		3.27	15	Ba		628	13
K ₂ O		2.80	8	La		31	9
P ₂ O ₅		0.15	15	Ce		63	6
Li		24	21	Pr		7.1	
Be		2.1	41	Nd		27	8
B		17	50	Sm		4.7	6
N		83		Eu		1.0	14
F		557	10	Gd		4.0	7
S		62	53	Tb		0.7	21
Cl		370	103	Dy		3.9	
Sc		14.0	6	Ho		0.83	
V		97	11	Er		2.3	
Cr		92	19	Tm		0.30	
Co		17.3	3	Yb		1.96	18
Ni		47	24	Lu		0.31	17
Cu	μg g ⁻¹	28	14	Hf		5.3	14
Zn		67	9	Ta		0.9	13
Ga		17.5	4	W		1.9	54
Ge		1.4	9	Re	ng g ⁻¹	0.198	
As		4.8	10	Os		0.031	29
Se		0.09	54	Ir		0.022	32
Br		1.6		Pt		0.5	95
Rb		84	20	Au		1.5	26
Sr		320	14	Hg		0.05	76
Y		21	11	Tl		0.9	57
Zr		193	14	Pb		17	3
Nb		12	12	Bi	μg g ⁻¹	0.16	38
Mo		1.1	28	Th		10.5	10
Ru	ng g ⁻¹	0.34	6	U		2.7	21
Pd		0.52	3				

Although studying the PTEs concentrations and behavior in the environment would be the outcome of this reflection, the real problematic would actually be linked to study whether the speciation of a PTEs is elevated or not (Rieuwertts et al., 1998). A clear example of this is the case studied by Turpeinen et al (2000) in the old abandoned and reforested shooting range

of Hollola, south Finland. More than 4 decades of using ammunition has left the humic soil charged with over 9800 mg kg⁻¹ of Pb determined in topsoil, that was rich in acidic organic matter, without removing the shots off the soil; however, due to decomposition, the Pb leaches down to the mineral soil where it may continuing its path to the groundwater (Table 2). The understanding of this situation involved analyzing soil samples through CaCl₂ extractions for determining the soluble fraction of Pb in the soil, that would be equivalent to understand the fraction of Pb that plants can absorb (Houba et al., 2000), that filters into the mineral soil.

Table 2. Soil characteristics of Pb-contaminated and control samples of the Hollola forest, Finland. (Modified from Turpeinen et al., 2000).

Soil depth cm	pH	Water content %	Organic matter %	Pb	Sb	As	Cr	Cu	Ni	Zn
				mg kg ⁻¹						
Contaminated										
0-5	3.5± 0.4	17.0± 3.3	30.3±4.3	9804± 1599	5.7± 2.1	6.5± 3.2	8.1± 2.9	17.8± 3.6	28.3± 13.3	64.7± 14.9
5-20	4.2± 0.2	8.3±1. 4	7.1±2.2	325.3 ±96.5	0.6± 0.2	4.8± 1.1	19.5 ±4.5	12.6± 3.9	26.9± 7.4	75.8± 22.6
Control										
0-5	3.6± 0.3	16.2± 1.9	36.7±3.9	32.7± 5.7	0.3± 0.1	1.2± 0.6	2.1± 0.9	25.1± 7.9	23.3± 11.9	26.9± 8.9
5-20	4.3± 0.2	6.9±2. 4	8.4±3.1	17.6± 6.3	0.2± 0.1	0.8± 0.2	2.9± 1.6	29.2± 5.5	19.2± 8.8	18.9± 12.3

As mentioned, the presence of PTEs can induce health issues, especially those that enter the trophic chains. For instance, in urban gardens the PTEs may enter the plant system by aerial deposition (Assad et al., 2019) or by the use of contaminated soils (Rinklebe et al., 2019) or even irrigation of contaminated water (Kiskun et al., 2000). A case study of the Kastor site near Cologne, Germany, assessed the presence and phytoavailability of As, Cu, Mn, Hg, Pb and Zn in soils used for growing vegetables. The site has been utilized for the mining of galena (PbS) and sphalerite (ZnS) since the 12th century. Antoniadis et al. (2017) found that the presence of these elements surpassed not only the permissible concentrations in soils and vegetables but also the toxicity thresholds calculated for beans, carrots and lettuce grown in four gardens at the vicinities of the Kastor site (Table 3).

Table 3. Total aqua regia concentrations and ammonium nitrate extractions of PTEs (mg kg^{-1}) of soils in gardens nearby the Kastor site, and permissible limits and background values. (Modified from Antoniadis et al., 2017).

	As	Cu	Fe	Mn	Pb	Zn	Hg
Aqua regia							
Garden-1	15.51	33.99	12.165	520.3	294.2	206.8	0.69
Garden-2	267.2	420.9	15.059	3562	13789	7426	36.79
Garden-3	25.63	33.09	15.063	3547	687.6	596.0	1.35
Garden-4	22.66	42.39	15.064	2473	661.1	508.0	1.36
Regulation limits	50	40	N.A.	N.A.	400	150	5
Background	3	16	N.A.	540	49	102	0.14
Ammonium nitrate							
Garden-1	N.D.	0.05	N.D.	2.39	N.D.	0.074	N.D.
Garden-2	N.D.	0.71	0.13	14.56	16.27	69.41	N.D.
Garden-3	N.D.	0.02	N.D.	25.63	N.D.	1.53	N.D.
Garden-4	N.D.	0.03	N.D.	22.44	N.D.	1.53	N.D.
Regulation limits	0.4	1.0	N.A.	N.A.	0.1	2.0	N.A.

1.1.4 The soil pollution situation in France

In Europe, approximately 2.5 million of contaminated sites may exist in the territory, from which, about 73% of the contaminated sites belong in the *Industrial and commercial activities* category that comprises commercial activities, mining residues, oil production and extraction and electricity production plants only for France (Eurostat, 2016).

In France, the available information concerning polluted sites, produced soil and water pollutants can be found in the BASOL and GEORISQUES databases from the Ministry of Ecology and Solidarity Transition or MTES (Ministère de la Transition Écologique et Solidaire). According to the MTES, about 6800 sites have been identified in early 2018 that are polluted or potentially polluted by either, industrial activities, gas emissions, spillage during waste management or confinement of toxic products (Lesueur et al., 2019). These identified sites are under surveillance and some are already being treated (Figure 6).

Pollutants in France may vary from metal and metalloids, polycyclic aromatic hydrocarbons, chlorinated hydrocarbons (HAPs), pesticides, radioactive substances, cyanides, etc. These elements not only may be a risk due to wind erosion and weathering but also due to leaching and mixing with the underground waters. Hence, their proportions have been determined (Figure 7) (Lesueur et al., 2019).

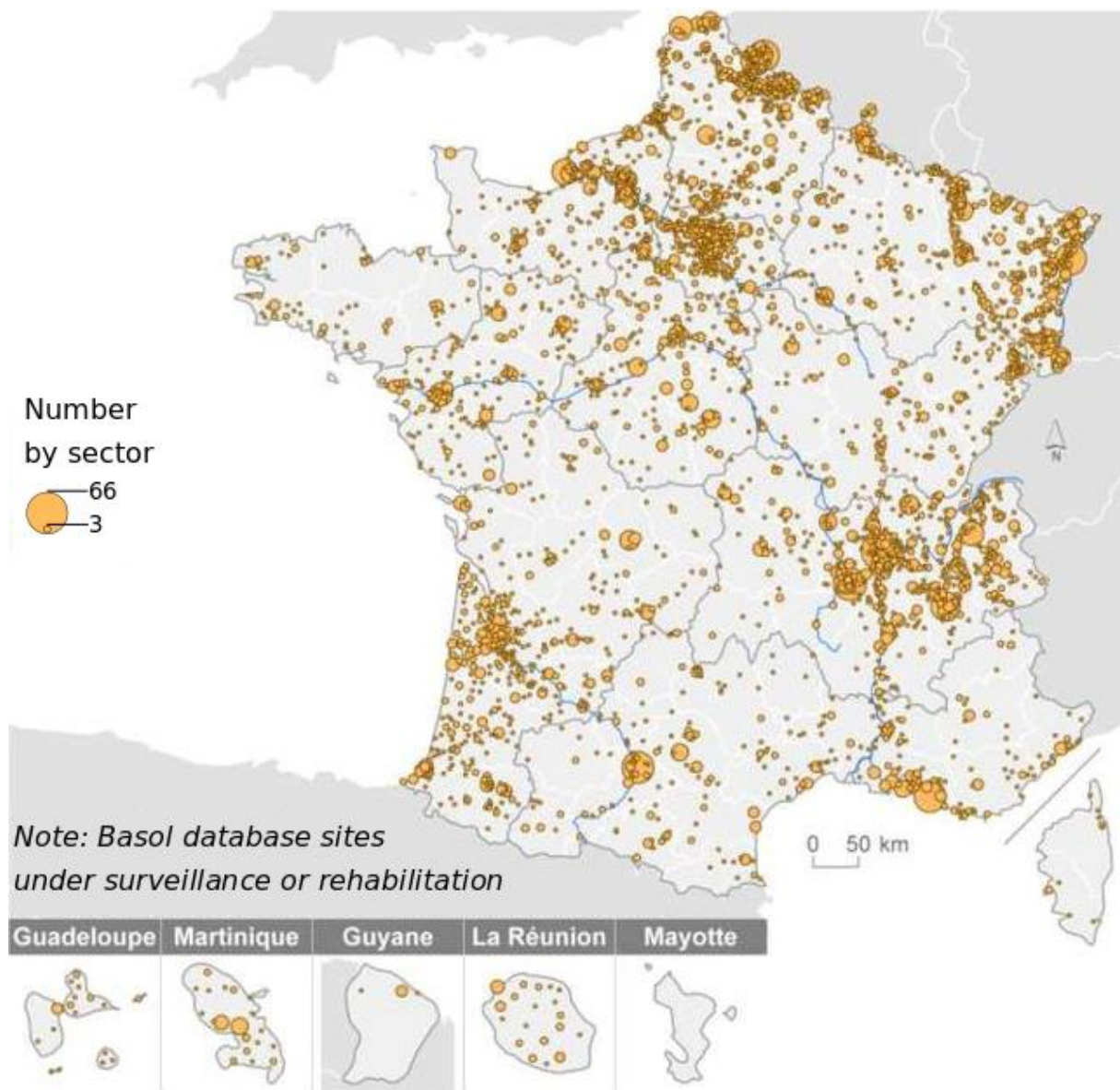


Figure 6. Soils and polluted sites with undergoing rehabilitation and monitoring in early 2018 (Lesueur et al., 2019).

Thanks to the green-borne energetic transition law of august 17th of 2015, France is engaged to follow a circular economic approach rather than the traditional linear economic approach that comprises extraction, production, consumption and final disposition. For fulfilling the objectives of the circular approach, it is necessary to take advantage of the residues produced and find an added value to them. The French Environmental and Energy Agency (Agence de l'Environnement et de la Maîtrise de l'Énergie, ADEME) is in charge of aiding through the provision of funds for the development of projects and application of policies aiming to the correct management of residues. An example is the remediation implementation from 1996 to 2006 at La-Combe-du-Saut in Southern France (Drouhot et al., 2014).

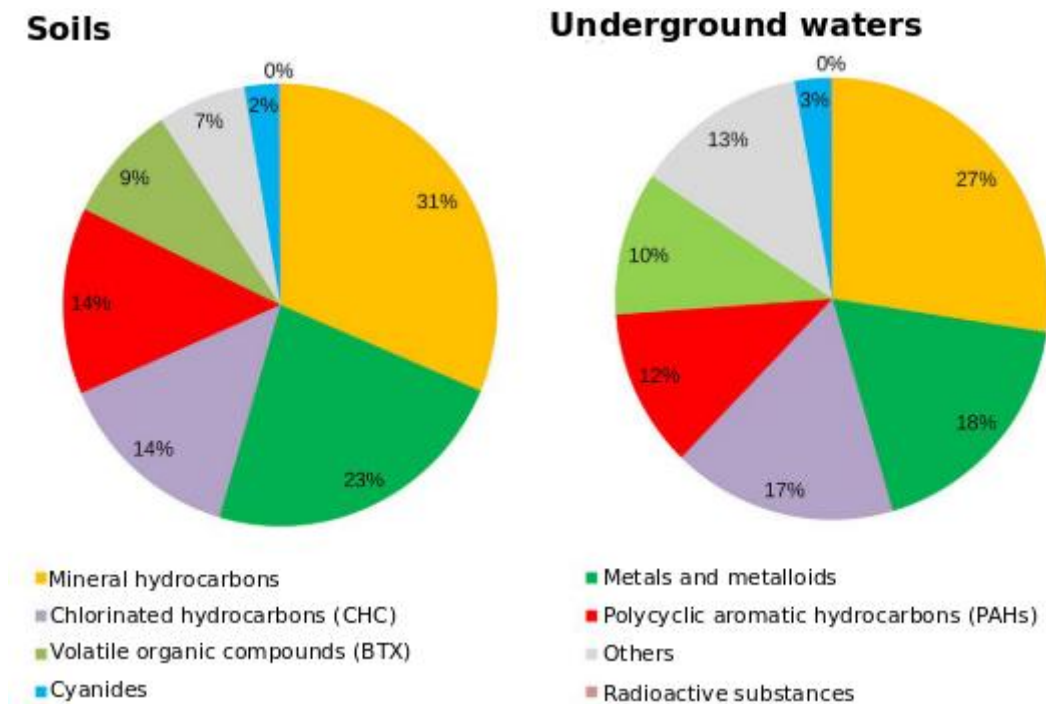


Figure 7. Abundance of identified pollutants in soils (on the left) and groundwater (on the right) in polluted soils and sites in France.

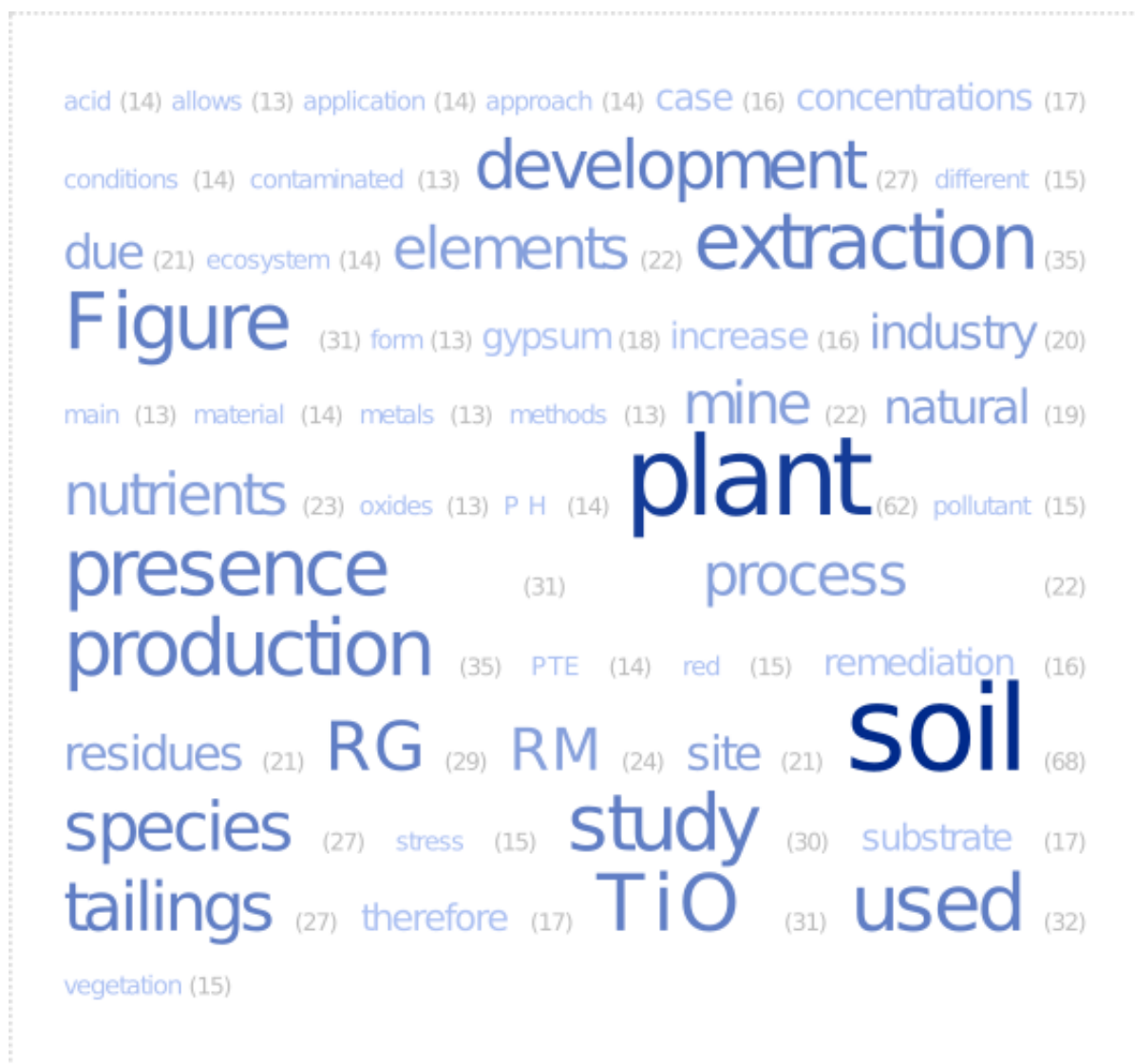
1.2 PhD context, justification and funding

The work developed for this PhD work intends to study PTEs present in soils issued from anthropogenic activities and plants adapted to live in polluted and nutrient-poor environments. The research endeavor urges to better understand whether it is possible to establish a vegetation cover upon the substrate itself and to re-valorize this so-called Technosol.

Justified by the increasing industrial activity and the seemingly equal residues production, this PhD work seeks to provide options to treat this problematic by similarly implementing less environmentally impacting practices. The sample analysis costs along with the experimental site would have not been possible without the sustenance of the PHYTOCHEM project, funded by the ANR (2013-2017). PHYTOCHEM promoted the application of phytotechnologies for the management of industrial polluted sites with the aims of using biomass grown in such substrates for chemical re-valorization and transformation into higher value products. The issued biomasses would be produced following two opposed approaches: the phytoextraction and phytostabilization. This project provided the partnership with our industrial associate Cristal (now renamed TRONOX) that allowed us the access to the study site (The Ochsenfeld site, Thann).

Chapter 2

Literature review



The following section provides the literature background and theoretical principles that were required to base the methodology and comprehension of this thesis. Hereby, we also have covered the recent studies that have been undertaken and are considered a state-of-the-art in the subject of titanium dioxide's extraction, residual red gypsum's production and revalorization, vegetation's adaptation strategies, and land reconditioning and remediation.

2.1 The Ti industry

Titanium is a metallic element whose name is derived from the *Titans* of the Greek mythology. First records of Titanium go back as early as the late 18th century, when the element was discovered but extracted with impurities, it was until the beginning of the 20th century, where its extraction became more elaborated and thus its purity. Some of its initial interests were notably as a construction material as metallic titanium, due to its strength similar to that of steel and lighter weight. Then research led to the improvement of its properties through the creation of alloys, notably for the aerospace industry (Donachie, 2000).

Later on, some of its properties have allowed it to be used as the raw material for the manufacture of surgical and dental implants and prosthesis, due to its capacity to be accepted by bone tissue, namely osseointegration (Sáenz de Viteri and Fuentes, 2013). Some other capabilities of Ti were also explored given its chemical properties of absorbing and reflecting ultraviolet and visible light, respectively, that therefore allowed it to be used as a photocatalyst: in the form of titanium dioxide or titania (TiO₂). In its purest form, titanium dioxide is the chemical form of the whitest of all pigments. These characteristics made it the most preferable pigment to use in the painting, cosmetic, paper, pharmaceutical and even the food industries (TDMA, 2013).

The exploitation of TiO₂ starts with the extraction of the ores then taken to appropriate facilities for extraction. The most common ores found in nature and used for the extraction are **ilmenite and rutile**; the difference between them being the purity of the TiO₂ itself. The former may contain from 43 to 65% of TiO₂, whereas the latter may reach up to 95% (Duyvesteyn et al., 2002). Sometimes, TiO₂ from lower grade ores might be purified into titaniferous slag, which would allow from 70 up to 80% of TiO₂ prior to the main extraction (Gázquez et al., 2009). Moreover, two main extraction methods dominate the industry, both of which use strong oxidants: chloride and sulfate processes (Figure 8).

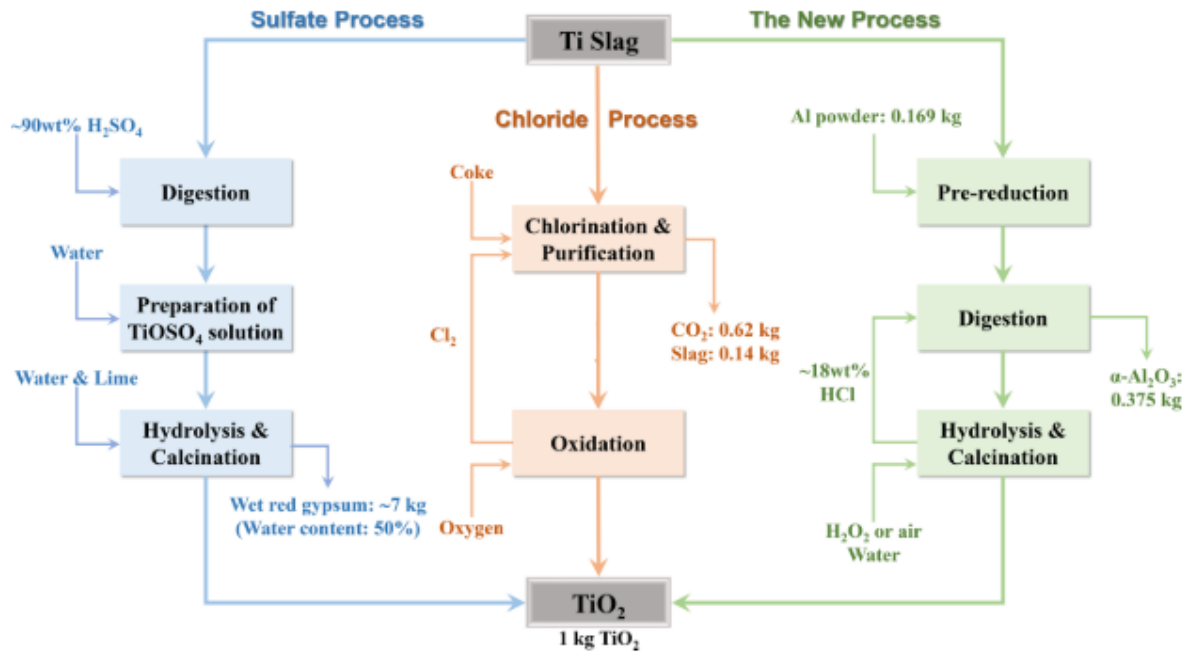
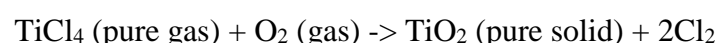
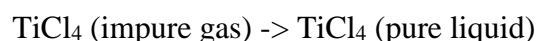


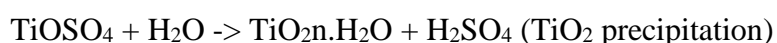
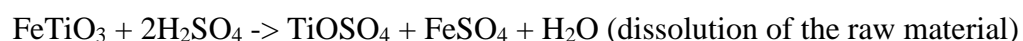
Figure 8. Existing extraction procedures of TiO_2 (blue and red): experimental extraction method. From Zhu et al., 2019.

As explained by Gázquez et al. (2014), the process consists of mixing the raw materials with chlorine gas at hot temperature, this mixture of gas will produce an impure mixture of titanium tetrachloride (TiCl_4) and carbon oxides. Then, the hot impure gas is exposed to already cool down TiCl_4 gas, which in turn allows to condensate and therefore eliminate contamination by zirconium and silica. Once the gas is pure, it is condensate, to later be finally heated up to approximately 1500°C and then oxidized to become TiO_2 . This process allows the recycling of the reagents, which in turn makes this process more efficient not only energetically but also environmentally, due to the reduction of residues produced (Braun et al., 1992). The chloride process follows the path shown below:



On the other hand, the sulfate process utilizes ilmenite or titaniferous slag or a mix of both to be digested with sulfuric acid, giving place to titanyl sulfate (TiOSO_4), iron sulfate

(FeSO₄). The titanium resulting liquor is then hydrolyzed, then the ferrous sulfate is separated by differences of densities from the hydrated titanium dioxide, which is in turn concentrated into a slurry. Then, the slurry is calcinated, allowing TiO₂ crystals to grow. The resulting by products are water and sulfuric acid (Gázquez et al., 2014). The process is summarized with the equations below:



It exists an organism that coordinates the production of titanium dioxide worldwide: the titanium dioxide manufacturer association (TDMA). The TDMA explains how TiO₂ is used in different ways. Among its main properties is its color, which is used to the manufacture, opacity and whitening of paints, coatings and plastics. Additionally, it may also be used as a *food* grade pigment to give whiteness or opacity. In the food industry, this pigment is known as the *colorant E171*; in the UK market this pigment is commercialized at around £14 per kilogram. The global production of only TiO₂ pigments reached approximately 4 million tons per year (Ortlieb, 2010), becoming a seriously important industry.

Skin care products are also manufactured using the properties and advantages of the TiO₂. Such is the example of sunscreen and face creams, due to its effectiveness to absorb UV light and scatter visible light (Weir et al., 2012). It may also be found in deodorants, lip balms, toothpaste and shaving cream (Rompelberg et al., 2016; Calle et al., 2017).

Additionally, the catalyst properties of TiO₂ nanoparticles are used in order to aid in the oxidation reactions of organic molecules, as pollutants in aqueous form, since the product of the oxidation of any organic molecule may result in CO₂ and H₂O (Kondo and Jardim, 1991). The quantities of this product go upwards, and thus it does the consumption of raw materials, reagents and the generation of residues. During the 70's in Spain, the acid residues of the TiO₂ sulfate extraction were dumped 40 miles into the sea in the Gulf of Cadiz (Contreras et al., 2016). Nowadays, this practice is forbidden, nevertheless other processes are undertaken.

The TiO₂ is produced all over the world by different groups. Even though some small producers exist over the world that do not belong to the TDMA, some of those registered are:

- CINKANA Metallurgical and Chemical Industry Celije, Inc. This is a Slovenian group that reaches over 150 million euros per year on revenues and has been producing TiO₂ since 1968 (<https://www.cinkarna.si/en/>).
- Kronos Worldwide Inc., a USA company, has been producing TiO₂ since 1916. It operates in 5 countries over Europe and Northamerica, their sole production process is based on the chloride method and have a yearly capacity of TiO₂ of 555,000 tons (<https://kronostio2.com/en/>).
- The Lomon Billions Group Co. Ltd, is a Chinese company with the 3rd largest production of TiO₂ in the world and the 1 producer in Asia. They produce TiO₂ since the 1990 and they use the chloride and sulfate methods (<https://www.lomonbillions.global/>).
- Tronox Holdings plc, a USA multinational whose full 2019 revenue was somewhere between 2650 to 2700 million dollars. They offer over 16 different TiO₂ based products and own more than 21 manufacturing plants. They are the virtual leader in TiO₂ production today (<https://www.tronox.com/>).

2.1.1 The production of red gypsum

One of the residues of the sulfuric extraction of TiO₂ is the **red gypsum (RG)**. The RG receives its name due to the color it shows, which is given by the presence of iron oxides from the ilmenite (FeTiO₃). It is notably formed by a mixture of gypsum (CaSO₄·2H₂O) and ferric hydroxide (Fe(OH)₃) (Zhang et al., 2019).

The RG is originated after using concentrated sulfuric acid (>90%) for the extraction of TiO₂, because the resulting recycled sulfuric acid has a concentration of approximately 20%, therefore the most cost-effective method is the neutralization using lime and disposal of it, hence the mixture of CaO and H₂SO₄. For producing one ton of TiO₂ it is necessary to use between 8 to 10 tons of H₂SO₄, at this rate, the RG production may reach up to approximately 7 kg·kg⁻¹ of extracted TiO₂ (Zhu et al., 2019). It is worth-mentioning that RG is not the only by-product of the extraction of TiO₂. The initial neutralization at a lower pH produces white gypsum, but it is at a higher pH that the remaining sulfates and iron oxides precipitate, hence giving place to a red gypsum (Peacock and Rimmer, 2000).



Figure 9. A) Tailings dump of residual red gypsum at the Ochsenfeld site, Eastern France; B) Red mud tailings pond at the Aluminium-Oxide Stade GmbH plant in Stade, Germany.

The final disposal of RG is generally by landfilling the tailings in special ponds, usually isolated through a membrane or protective dykes, with the purpose of separating and then accumulating the sediments until full, then the process is repeated in new sites (Figure 9); liquid phases can be air-dried or extracted prior to treating them in an appropriate facility (Peacock and Rimmel, 2000). The RG contain several elements, the literature indicates the presence of major elements among which we would probably find Fe, Ca and S; on the other hand, RG can also contain trace (Table 4). Furthermore, ilmenite ore is a natural occurring radioactive material and thus, the presence of radionuclides such as radium (Ra), thorium (Th) or uranium (U) in such industrial processes is relatively common, nonetheless, the presence of radionuclides in RG is negligible, since most of them remain in the sludge (Contreras et al., 2016).

Table 4. Presence of trace elements in residual red gypsum, expressed as % or mg kg⁻¹ dry weight. Modified from Peacock and Rimmel, 2000.

Component	Content
	%
CaSO ₄ ·H ₂ O	58.5-59.3
Fe ₂ O ₃ ·H ₂ O	32.9-36.6
Ti	1.0-1.3
Al	0.1-0.8
Mg	0.5-0.6
Mn	0.2-0.5
Si	0.3-0.5
Cl	0.02-0.2
	mg kg ⁻¹
Cr	500-800
Zn	200-400
Sr	100-300
Ni	50-60
Co	20-30
Ba	1-3
Pb	1-2

2.1.2 The application and valorization of RG tailings

Landfill mining is a used technique that allows the exploitation of landfills for purposes of extracting aggregated value to already considered *residues* (Johanssen, 2016). This practice allows meets the postulates of *Green Chemistry* proposed by Anastas and Wang (1998), in which they call for the reuse of industrial waste material for the development of new chemical components that may act as raw materials for other industries. Given its composition and the quantities in which it is produced around the globe RG may have the potential to be given an economic value in addition to the environmental importance of reducing the possible pollution sources. It is therefore why several studies have been conducted overtime giving place to a great quantity of published information about different applications for RG. Consequently, a synthesis is proposed below.

One of the most found uses in the literature is as soil amendment for different purposes. For instance: RG has been used as a soil amendment in Malaysian soils prone to flocculation, as a mean to avoid soil erosion and loss. At this point it was suggested that its composition could also improve the Ca and S content in the soil, therefore increasing its fertility (Fauziah et al., 1996).

Other studies showed that it is possible to use RG as a stock of S to be released slowly over time in sulfur-poor soils. Additionally, the same study suggested that, due to the affinity to iron oxides, RG could be used for the immobilization of Pb in some agricultural soils (Peacock and Rimmer, 2000). In acid agricultural soils of Spain, RG has been tested for its capacity to immobilize PTEs. Garrido et al. (2005) reported that by using RG the pH increased by up to 1 unit, this had the effect of immobilizing Pb and Cu from 380 and 486 mg·kg⁻¹ to 151 and 405 mg·kg⁻¹, respectively. A similar case was shown for Se (Park et al., 2011), for Al (Vázquez et al., 2017), Cd (Illera et al., 2004) and for As (Gadepalle et al., 2007). The main reason of this usage is due to the effect of an alkaline pH on nutrients.

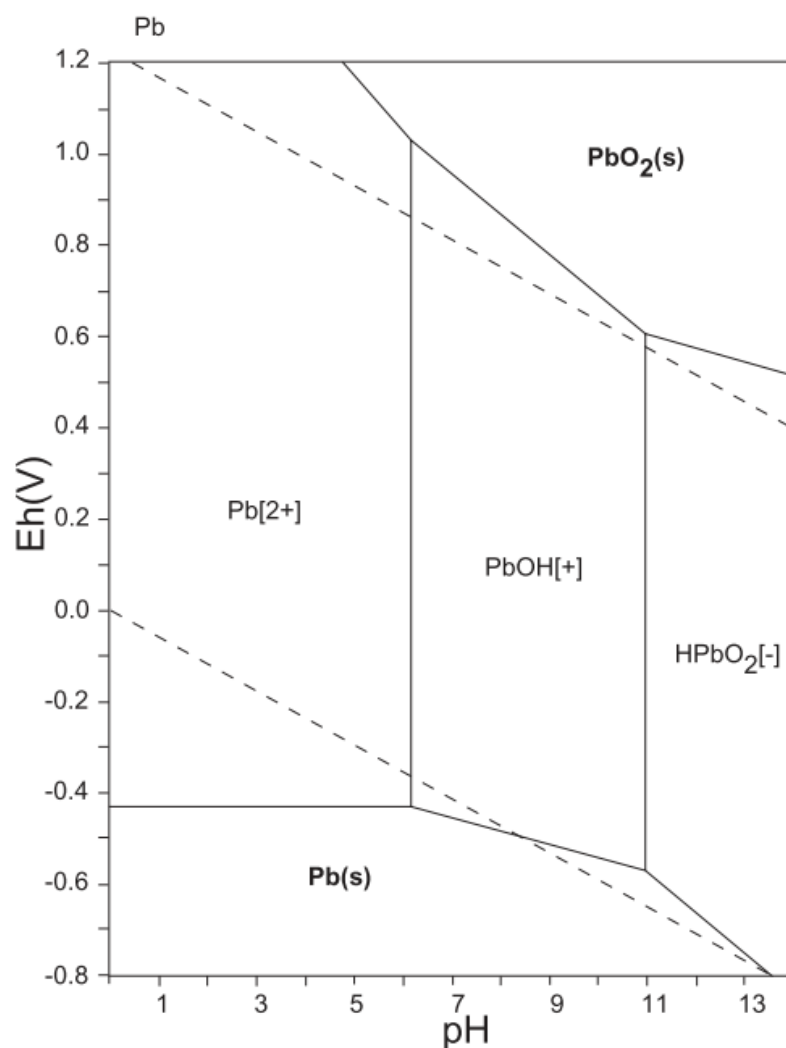


Figure 10. Eh-pH diagram of lead (Pb) in solid form. Modified from Takeno, 2005.

For instance, Pb may be in its most simple and soluble form at a pH of 6 or lower, but an increase of pH will turn it into a less soluble form (Takeno, 2005) (Figure 10). Not only has

this application been tested over time in unproductive soil, also the effects of this substrate have been looked at in the production of edible vegetables (Assad et al., 2017) to be used in productive soils.

Some authors explain how to take advantage of the physical properties of gypsum itself for construction and manufacture of gypsum-based products. For instance, Kamarudin and Zakaria (2007) concluded that it is possible to use the RG to produce ceramic glazes. In their work, the authors proposed to replace the CaCO_3 in the ZnO process for CaO, giving place to different types of glazes that could be used for low-pressure-prone applications, such as wall or table glazes. In the context of construction and manufacture the substrate has been studied in the past to be used in subsoil stabilization as a binder agent to replace concrete, to be used in zones with water saturated or soft soils (Hughes et al., 2011). The literature highlighted that its slower process of strength development could be improved by the inclusion of additives to the mixture. Furthermore, (Pérez-Moreno et al., 2013) tested out and proved that RG and tioxide (another TiO_2 extraction by-product) can be used in different mixtures for the manufacture of fire protective plates to be used in building construction. They determined that a plate conceived of 75% RG and 25% tioxide might even be stronger than commercial plates for the same purpose.

Red gypsum has been studied as a reactor for carbon capture and storage, through mineral carbonation, which is the transformation of atmospheric CO_2 into a solid and stable carbonate by bonding atmospheric CO_2 oxides (such as calcium, magnesium or iron) (Azdarpour et al., 2014). This study resulted in a better carbon sequestration through calcium than iron, although temperature and particle size of the oxides ought to be regulated in order to have a maximum efficiency. This utility has been studied several times by different authors due to the climate change crisis endured in recent times (Rahmani, 2018). Although the use of RG for the construction has constraints, since ferric sulfate is abundant in RG, which may produce stains when in humid conditions (Azdarpour et al., 2014).

Finally, should some of the elements contained and forming RG be extracted, the tailings could become the feedstock for other industrial processes. For instance, TiO_2 has been extracted from the RG using EDTA (Figure 11), giving place to a 95% purity TiO_2 with nanoparticle size between 30 and 50 nm (Borhan and Nee, 2015). This example implies an economic gain for the extraction industry, which would exploit to a maximum level their residues. On the other hand, some studies have shown that the reuse of RG could be beneficial not only for the RG extraction industry but also for third party industries, due to the existence of other potential markets. Such is the case of the use of RG as a natural source of sulfur to be

used as a catalyst that would aid in the conversion of glycerol waste to glycerol carbonate, an important feedstock for the production of solvents (Zuhaimi et al., 2015). Similarly, the use of RG can also be the feedstock for the production of iron oxide pigments, this process was patented by Auer et al. (2003).

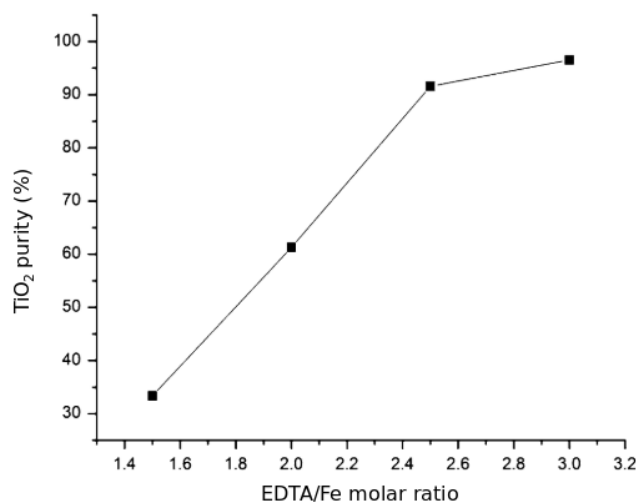


Figure 11. Effect on extraction purity of TiO₂ using EDTA/Fe.

2.2 Vegetation functions in Technosols

The presence of vegetation in highly anthropized areas is important, due to the great number of ecological functions and ecosystem services provided, which are the advantages provided by these organisms for the human beings (Chapin et al., 2011). Some of these perturbations could be due to either anthropogenic or natural factors such as deforestation, grazing, slash-and-burn practices, or floods, wild fires, landslides, among others (Lal, 1993).

Among the functions and services, we will find the most important of all: the oxygen production, but more importantly in modern times, due to global warming, is the CO₂ consumption. In the same trend, the food production is a significant service provided by plants. On the other hand, plants could also be used as raw materials for the energy production, for the industrial manufacture of paper, the preparation of medicines, and the source of some natural fibers, among others. Despite the obvious and direct services often supplied, the presence of plants can procure the ecosystem, and the human included, other indirect services.

For instance, the presence of vegetation in wet ecosystems can work as a humidity regulator of the soil's water content through evapotranspiration. In the same manner, in dry

ecosystems the presence of trees can create microclimates that can help in the establishment and development of other species among them (Jones, 2014). Additionally, the presence of plants and more specifically root systems can aid in the soil stabilization and hence avoid soil loss and erosion (Tisdall and Oades, 1979). It can also provide the soil biota with the appropriate conditions to develop and aid in the biogeochemical cycles (Hobbie, 1992; Chapin and Körner, 1995). In agricultural systems, the use of polycultures, notably perennial vegetation, can increase the number of ecosystem services (ES); such is the case of the Corn Belt in the Midwestern US (Asbjorsen et al., 2013). The modelling in this area showed a significant increase in some ES when increasing the presence of perennial species in the system. The mentioned ES may be hydrologic control and water purification, climate control, biological regulation, nutrient recycling (Figure 12).

A Technosol is by definition any natural soil that is affected by a significant quantity of artifacts that in turn are any hard material of anthropic origins. These artifacts can be of organic or inorganic nature (Hicham et al., 2016). This classification of soils includes mine spoils, tailings, landfills, combustion residues, sludge, among others (Baxter et al., 2015). Given this definition, the presence of PTEs in Technosols could be expected to exceed natural background values (Alloway, 1995). In addition to the presence of PTEs, the Technosols often times lack ideal agronomic characteristics (Table 5).

In the previous section it was mentioned that the purer the raw material (i.e. ilmenite) the purer the residual acid liquor will result (i.e. during the TiO_2 extraction), however contaminants may come from the use of uncontrolled limestone for neutralization (Peacock and Rimmer, 2000). This presence may not only affect the production difficulty but also the quality of the residues. The presence of PTEs in tailings poses a risk to the near environment, but the risk of leaving an open landfill comes from the loss of tailings charged with of PTEs that may cause harm to either human populations, water bodies or complete ecosystems, including the plants present in these by producing stress.

2.2.1 Plant stressing factors

The re-emergence of vegetation after an ecosystem perturbation, also known as colonization, is triggered as a function of the new characteristics of the site and it is specific for each species in order for them to survive to the new soil conditions (Taiz and Zeiger, 2006). Among the stressful conditions that plants might encounter when colonizing, it may be found drought stress, heat stress, cold stress, salinity stress, oxidative stress or hypoxia stress (Taiz and Zeiger, 2006).

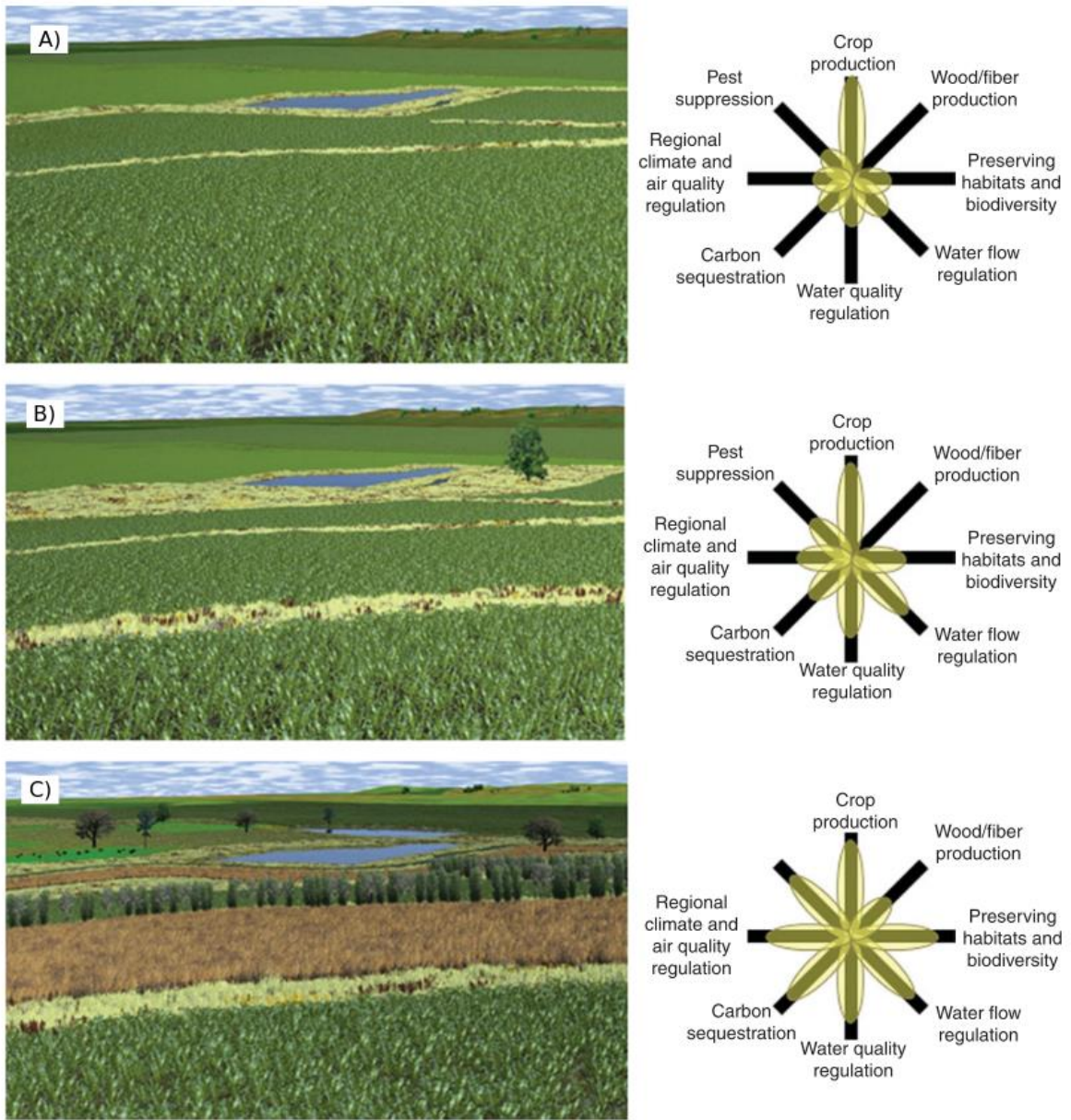


Figure 12. Comparison of agricultural landscape systems. Left: models with different proportions of presence of perennial vegetation. Right: Provided ecosystem services by the corresponding system. Proportions: A) 4%, B) 16%, C) 64%.

Table 5. Tissue levels of elements that may be required by plants From Taiz and Zeiger, 2006.

Element	Chemical Symbol	Concentration in dry matter (% or ppm*)
Hydrogen	H	6
Carbon	C	45
Oxygen	O	45
Macronutrients		
Nitrogen	N	1.5
Potassium	K	1.0
Calcium	Ca	0.5
Magnesium	Mg	0.2
Phosphorus	P	0.2
Sulfur	S	0.1
Silicon	Si	0.1
Micronutrients		
Chlorine	Cl	100
Iron	Fe	100
Boron	B	20
Manganese	Mn	50
Sodium	Na	10
Zinc	Zn	20
Copper	Cu	6
Nickel	Ni	0.1
Molybdenum	Mo	0.1

*Only micronutrients are given in ppm, the rest is given in %.

As it was mentioned in the previous section, few are the nutrients that are essential for plants. Weathering, is an influencing factor on the availability of most plant nutrients, except for N. The lack of N in soils may also play a role on the stress reactions of plant species (Landeweert et al., 2001). The growth of a plant in a substrate that lacks an essential nutrient could lead to a nutrient deficiency in other tissues which consequently could break the balance of nutrient absorption and lead to detrimental effects. Conversely, if a plant is exposed to a substrate with a higher available concentration of a micronutrient, even when essential for the development it may provoke a toxic effect (Taiz and Zeiger, 2006; Marschner, 2012; Emenike et al., 2018). Oxidative stress is the imbalance of reactive oxygen and antioxidant elements, due to the excessive production of reactive oxygen species (ROS) (Syta et al., 2019), and may be induced by the exposure to toxic levels of TE (Gaetke and Kuang, 2003; Halliwell and Gutteridge, 2015).

For instance, gypsum ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$) is a pretty common mineral that can influence the soil quality drastically. Even when a soil contains essential nutrients the abundance in the ionic

form of gypsum can regulate the availability of the nutrients for plants. Moreover, the presence of gypsum is often times associated with high salinity, therefore exposing plants to toxic levels of Na (Elrashidi et al., 2010). Similarly, the exposure of birch seedlings to PTEs such as Cd may affect the root development and their functions. In assay exposing birch seedlings to a solution of $2\mu\text{M CdCl}_2$, significant decrease in the development of root growth after only 2 days was registered. The indirect effect of this reaction can impede the water absorption and the root accessibility to nutrients (Gussarsson, 1994). Similarly, in *Betula platyphylla var japonica*, the presence of Mn in concentrations higher than 1 mg l^{-1} in liquid medium showed signs of toxicity that started by brown speckles and turned into leaf chlorosis when increasing the concentration (Kitao et al., 2001). Aluminum could be considered a toxic element under acidic conditions and it is considered as one of the main stress inducing elements in agricultural lands. It has been well studied over time and therefore its consequences can go from root growth inhibition, stomatal opening, to leaf chlorosis and necrosis (Ali et al., 2008). For colonization to happen, plants must have the capacity to adapt to specific stressing factors and harshness of the conditions present in the new site (Sytar et al., 2019).

2.2.2 Plant adaptations

Some plant nutrition strategies to compensate for lack of nutrients and excess of competition comprise the development of particular behaviors such as carnivorism, and parasitism (Brewer, 2003). In other instances, structural changes such as the development of cluster roots, and highly developed hydrophytes allow the extraction of nutrients from unavailable soils (Tomasi et al., 2009). Associations with other organisms, i.e. mycorrhization and nodule development, have allowed to adapt to environments with nutrient shortages (Brundrett, 2009). Changes and adaptations to harsh soils may sometimes be regulated by the production of phytohormones that will stop or allow the access to the root cells of a certain toxic element present in the substrate (Sytar et al., 2019).



Figure 13. Ultramafic and calcareous vegetation in Dun Mountain, New Zealand. The *Nothofagus* forest in the left and the metallophyte vegetation in the right (*Chionochloa pallens*, *Hebe odora*, *Cassinia vauvilliersii*, *Dracophyllum uniflorum*, etc). From Robinson, 1997.

One thing in common that Technosols, ultramafic or serpentine soils and calamine soils have is the presence of metals, this is why there are considered as *metalliferous* soils (Wojcik et al., 2017). In such metal-abundant substrates few are the specialized plant species that may develop, due to the abundance of toxic concentrations of some elements and the lack of other essential nutrients (Shallari et al., 1998). These notable species are well specialized to live in environments heavily charged with metals that even plants of the nearest ecosystem that accustomed to the weather characteristics would avoid colonization (Figure 13).

The species that can accumulate and tolerate great concentrations of metal(loid)s, i.e. usually up to 0.1% of its dry weight for Ni (Table 6), are known as hyperaccumulators (Brooks et al., 1977). These plants are usually endemic of metalliferous soils and since the last century, the identification of *metallophyte* plant species has become a usual activity for these types of substrates, given their applicability in polluted sites, i.e. phytoextraction (Baker and Whiting, 2002). A particularity of hyperaccumulating species is the greater concentrations of some element when compared to common ranges (Van der Ent et al., 2013).

Table 6. Hyperaccumulator criteria of trace elements. From Verbruggen et al., 2009.

Trace element	Concentration criterion (% in leaf dry matter)	Number of taxa	Number of families represented
Sb	≥ 0.1	2	2
Cd	≥ 0.01	4	2
Co	≥ 0.1	(26)	11
Cu	≥ 0.1	(35)	15
Pb	≥ 0.1	14	7
Mn	≥ 1.0	10	6
Ni	≥ 0.1	390	42
Tl	≥ 0.1	1	1
Zn	≥ 1.0	14	6

The soils of New Zealand and New Caledonia have hosted a great number of publications regarding the description of hyperaccumulating plant species thanks to the presence of ultramafic or ultrabasic rocks (Jaffre et al., 1976; Brooks et al., 1998; Becquer et al., 2003; Demenois et al., 2017). Although more recently, the research and application of hyperaccumulators has expanded all over the world, aiming to take advantage of this particular trait for phytoextraction of different elements. An example is the case of Ni. This element has been studied and extracted from different soil types now by *Alyssum* spp. (Brooks et al., 1979), *Amaranthus dubius* (Mellem et al., 2012), whereas *Thlaspi caerulescens* (now *Noccaea caerulescens*) has been studied for its hyperaccumulating characteristics for Zn and Cd (Brown et al., 1995; Assunção et al., 2003) as well as *Arabidopsis halleri* (Marquès et al., 2004). Similarly, Mn hyperaccumulation through *Gossia fragrantissima* (Fernando et al., 2013) and *Phytolacca Americana* (Min et al., 2007).

On the other hand, when the agronomical characteristics of a soil are insufficient to satisfy the plant needs, the plant can develop survival strategies, this type of substrate is called an oligotrophic soil (Bakker et al., 2002) and the plants adapted to this type of habitat are called oligotrophs (Raven et al., 2005). The oligotrophic soils may be characterized by either the lack of nutrients or the lack of availability of these nutrients.

A plant species that is well adapted to live in oligotrophic soils, such as the arid wastelands of Ganqika in Northern China where switchgrass (*Panicum virgatum* L.). This species is intended to be introduced for serving as a multiple ecosystem services provider, i.e. soil stability, perennial growth, and carbon storage and bioenergy feedstock. This species has a

rather efficient nutrient use and drought resistance and whose only limitation is the nitrogen availability (Ameen et al., 2019).

A peculiar trait of plants accustomed to live in environments with inadequate nutrient presence is the development a rather significant underground system, which promotes the association with microorganisms: i.e. mycorrhizae and bacteria (Hobbie, 1992).

2.2.2.1 Mycorrhizal symbiosis

Mycorrhiza is a symbiotic association of plants and fungi with interests on trading water; photosynthetic carbohydrates and available nutrients, i.e. N, K and especially P, (Landeweert et al., 2001; Strullu-Derrien and Strullu, 2007; Courty et al., 2017). Mycorrhization may occur in all type of environments, however it may be limited by the excess of dryness; salinity, nutrients, also in sites with severe soil disturbances, floods, and soils with excessive lack of nutrients (Marschner, 2012). The presence of any type of mycorrhizae will vary depending on the compatibility between the fungi with the plant species. The fungi that form the mycorrhiza develop a large radicular system named hyphae whose function is to access beyond the pore space in the soil matrix (Drew et al., 2003). Even is mycorrhizal fungi presence being almost ubiquitous in terrestrial ecosystems, the mycorrhizal colonization has been improved in some cases, i.e. the addition of biochar in agricultural soils, thanks to the increase of colonization sites (Atkinson et al., 2010).

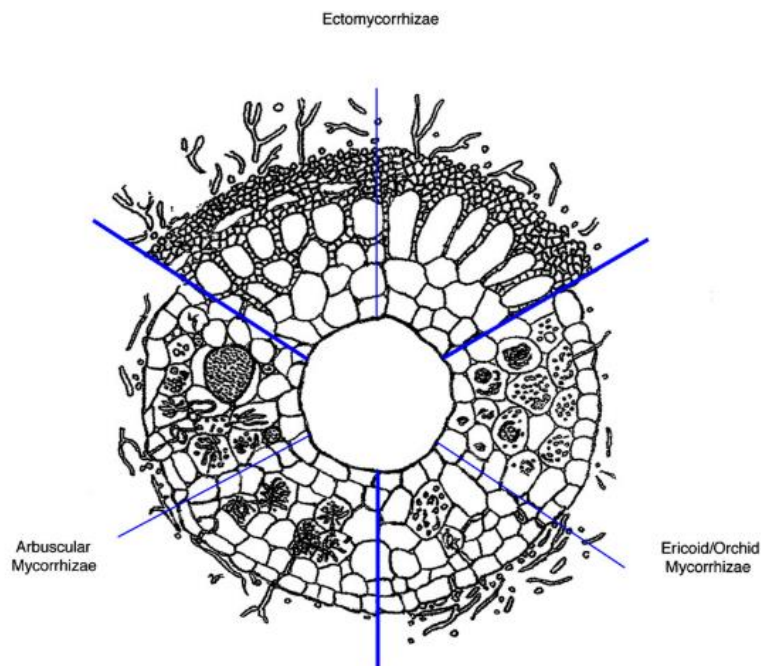


Figure 14. Types of mycorrhization. From Strullu-Derren and Strullu, 2007.

These associations are divided in arbuscular mycorrhizae or endomycorrhizae (AM) ectomycorrhizae (EC) (Marschner, 2012) and ericoid/orchid mycorrhizae (ER) (Webster, 2008) (Figure 14). The AM are characterized for the production of arbuscules, which are branch-like structures that enter the plant cell walls and allow the solute exchange between the plant and the fungus (Marschner, 2012). This is the most common type of all and is dominated by the Glomeromycota phylum (Schüßler et al., 2001) (Figure 15A). The EC are fungi that develop among the plant root's cell walls creating a mantle-like shape from which hyphae grow and extends towards the soil (Landeweert et al., 2001) (Figure 15B). Lastly, ER mycorrhization refers to the specific mycorrhizal fungi that can colonize the Ericaceae family. This is the most recently determined mycorrhizal type and it is found in environments with high acidity, abundance of recalcitrant phenolic compounds and non-decomposed organic matter, as it happens in peatlands (Martino et al., 2018).

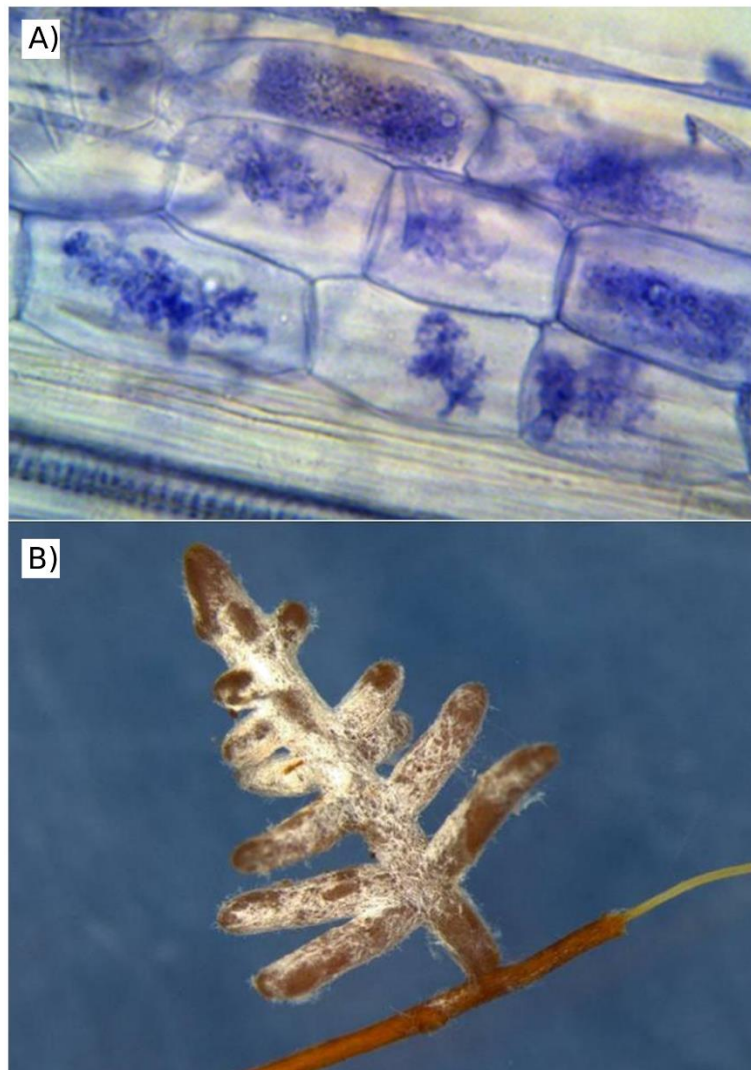


Figure 15. A) Endomycorrhizal infection on *Helianthus annuus* (Modified from Turrini et al., 2016). B) Ectomycorrhizal infection. Photo from Yong-long, Wang.

2.2.2.2 Bacterial activity

The presence of bacteria is of incomparable importance in the soil ecosystem due to the functions and services provided (Figure 16). As it has been stated before the nitrogen cycle is the only biogeochemical cycle that is independent from the pedogenic processes. Therefore, thanks to bacterial role as main actors of the N cycle, i.e. *Rhizobium spp.* in creating nodules in roots of legumes (García-de-los-Santos et al., 1996), the bacterial activity is of extreme importance (Doran et al., 1998; Jetten, 2008). The trade of C from root exudates for nutrients extracted by rhizospheric bacteria are the base of this association (Tinker, 1984; Clarholm, 1985).



Figure 16. Root section and rhizospheric bacteria. Photo from the National Science Foundation (NFS).

In remediation, the presence of plant growth-promoting bacteria (PGPB) and specialized plant species can enhance the remediation success on contaminated sites with hydrocarbons (HC) (Khan et al., 2013). The PGPB may also regulate plant homeostasis under abiotic stressing conditions, i.e. drought, by the degradation of the plant ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) through bacterial secretions of ACC deaminase, relieving plant stress (Yang et al., 2008). All these functions in addition to the already mentioned nitrogen contribution.

2.3 Land remediation methods and tailings perspectives

In previous sections it was mentioned that the industrial development brought the production of hard to treat, sometimes intractable, substrates. In this section, the known ways of restoring impacted soils and tailings will be discussed. The traditional methods applied for remediation of sites with polluted soils and particularly tailings may vary depending on the pollutant to be treated, the magnitude and abundance of it and the location of the site to treat. Additionally, such methods have advanced and sometimes even improved over the years. In general, they are divided in three categories: physical or engineering, chemical and biological approaches (Tordoff et al., 2000).

Table 7. Summary of remediation physico-chemical techniques on soil. Modified from Hamby, 1996.

Techniques	Method
Actinide Chelators	Chemical Treatments
Chemical immobilization	
Fluid extraction	
Oxidation	
Peroxidation remediation	
Photodegradation	
Capping	Physical techniques
Cementitious waste forms	
Electrokinetics	
Incineration	
In-situ grouting	
In-situ vitrification	
Soil washing	
Sorting	
Stabilization/solidification	
Thermal desorption	
Vapor stripping	

In a literature review by Hamby (1996) he registered the known remediation techniques at the end of the 20th century, in order to facilitate the future search of existing approaches and make them available for the private sector and other users. One of the main classification parameters he used was, other than the type of approach used, the medium itself: either soil or groundwater. Over seventeen physical and chemical methods were reviewed and classified in Table 7, at that time only for soil and surface layers.

In synthesis, the mentioned methods help in either the displacement, the immobilization or the precipitation of the contaminants from the substrate, whether these were organic or

inorganic components. As mentioned before, our main concern are the PTEs contained in the tailings, therefore this research is focused in the study of remediation techniques of inorganic pollutants. Some of the main limiting or determining factors depend on the nature of the pollutant and the cost of the operation (Cappuyns, 2013). Added to this, the environmental cost of the chemical and the engineering approaches may prompt to habitat destruction, lead to leaching of some other metals, moreover some chelators used could even be toxic (Lin and Lin, 2005). Therefore, the application of such technique drifts away from the concept of habitat or substrate *restoration*.

On the other hand, in the initially cited review, not much information about the biological approaches was mentioned. This particular approach aims to changing the chemical form of the compounds to render them either less toxic or more available for the vegetation or microorganisms to degrade them, metabolize them, or simply to trap them within the substrate for avoiding losses to other environments through run-off, percolation or leaching, or erosion (Gadd, 2000; Malik, 2004). The main advantage, besides the cost-effectiveness, is the low environmental impact. As it has been pointed out using life cycle assessments, the use of greener and less invasive remediation methods is preferable to traditional approaches because it impacts less on the ecosystems (Cappuyns, 2013). Bioremediation methods are used not only for soil but also for liquid mediums; it comprises the use of bacteria, fungus or plants to biotransformate or biodegrade the pollutant and transform it into a harmless or at least less harmful compound (Fulekar, 2010).

According to Shishir et al. (2019) bioremediation can be divided in three types: Monitoring Natural Recovery (MNR), biostimulation and bioaugmentation. The first one consists in letting the pollutants *in-situ* attract the organisms capable of tolerate and metabolize it, hence rendering it less hazardous. The only human intervention being for status updates on the process condition. Secondly, biostimulation refers to the use of either synthetic or natural surfactants in order to render contaminant molecules more available for the microorganisms to decompose. Finally, bioaugmentation refers to the introduction of specialized microorganisms into the polluted system in order to assist the indigenous biota to degrade the contaminant compounds. For inorganic pollutants such as metals, the bioremediation processes consist mainly of the absorption and the toxicity reduction of the pollutant, notably through phytoremediation (Sun et al., 2018).

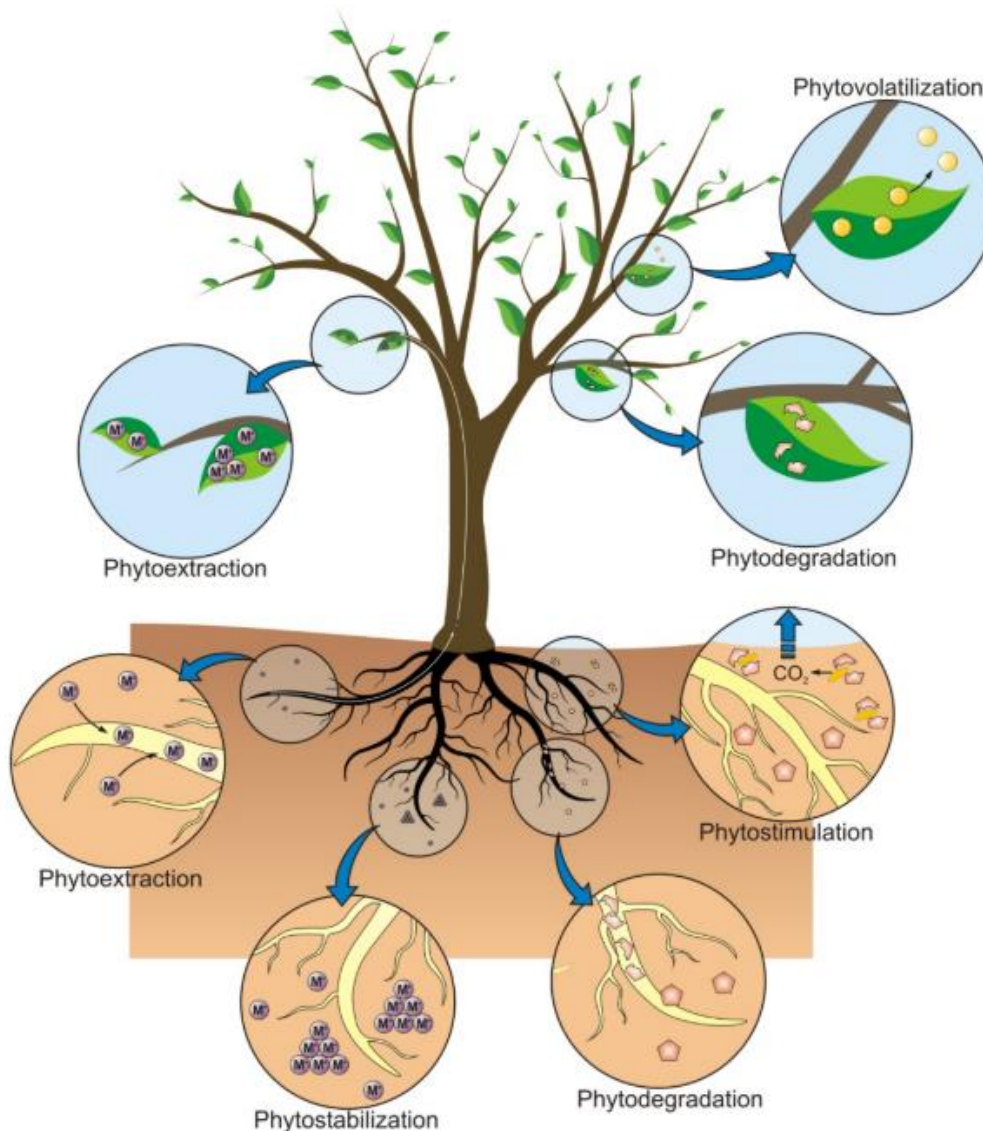


Figure 17. Schematic representation of phytoremediation strategies. From Favas et al., 2014.

Pulford and Watson (2003) interpreted phytoremediation as the exclusive use of plants for the extraction or retrieval of a certain pollutant. Additionally, they classified such process in five main axes (Table 8). Although in more recent publications other strategies are taken in consideration when describing phytoremediation such as phytostimulation or rhizodegradation (Figure 17), which consists on the stimulation of the microorganisms communities living in the rhizosphere by the plant, notably through the production of exudates that provide carbon and energy for the developing microcosm (Favas et al., 2014). The advantage of phytoextraction is the removal of the contaminant from the polluted site through the production of metal-enriched biomass. This biomass in turn is incinerated, which reduces the volume and allows the search of appropriate storage for the ashes (Kabata-Pendias and Pendias, 2011).

Table 8 Subgroups of phytoremediation strategies Modified from Pulford and Watson 2003.

Name	Description
Phytoextraction	The plant takes up elements from the substrate and stock them in its harvestable parts.
Phytodegradation	The microorganisms associated to the roots degrade the pollutants
Rhizofiltration	In liquid medium, the plant's roots absorb the pollutants
Phytostabilization	The plant immobilizes and reduces the bioavailability of the pollutants through secretion of pH modifying substances
Phytovolatilization	The plants allow the volatilization of the pollutants into the atmosphere

Even when quicker and more efficient solutions have been developed over the years, the use of passive, greener, easier to manage and less expensive solutions for remediation are preferred by the decision makers to keep the PTEs from entering other compartments of the environment (Salas-Luévano et al., 2017). Since tailings themselves considered as a pollutant when they are landfilled due to the large quantities of production, the most suitable management for this substrate would be the MNR.

Later on, this gave place to the concept of phytotechnologies, which refers to the use of science based on plants and microorganisms for providing solutions to contaminated sites or substrate (Mench et al., 2009). A list of the application of phytotechnologies has been reviewed by Mench et al (2010), in which they list successful cases of phytostabilization, phytoextraction, phyto- and rhizodegradation, and rhizofiltration, and the plant species used. More recently, the literature indicated advances on the field of phytotechnologies. The application of known hyperaccumulating species for the recovery of metals and metalloids, i.e. Zn, Cd, As; and high biomass-producing crops with low metal extraction rate, form part of such advances (Burges et al., 2018). The latter highlighted the use of tree species for the continuous recovery of metals from the soil, similarly increasing the substrate stabilization. Likewise, the gentle remediation options (GROs) were defined as measures to treat contaminated sites that required low maintenance aiming to minimize the soil functions disturbances. These comprised the use of plants and soil microorganisms (Cundy et al., 2016). Kumpiene et al (2014) aimed to compare two GROs: phytoextraction and phytostabilization. Authors concluded that total TE concentrations were more significantly reduced when more than one approach was aimed for

in comparison to only phytoextraction. Therefore, the application of mixed approaches is desired in phytomanagement cases.

2.3.1 Phytomanagement in Technosols

Phytomanagement is the association of plant-based biological remediation methods such as phytoextraction, phytostabilization and biofortification with biomass valorization. For instance, phytomanagement may be used to provide a change on land use, i.e. transforming ancient agricultural lands into forest through the use of stands of pioneer species *Betula pendula* Roth. in Southeastern Estonia (Uri et al., 2007). The association of phytomanagement techniques is notably focused on the mitigation of not only deficient essential TE but also elevated and potentially hazardous TE concentrations; alternatively, the role of plants is increasing the value of the soil that is being treated (Robinson et al., 2009).

The urgent increase of Technosols from the landfilling of extractive and chemical industries, and abandonment of mining sites plus the already existent acknowledgement of *metallophytes* that can survive in ore deposits has created a new field of application: the phytomanagement of Technosols (Kumar et al., 1995). The focus of phytomanagement in Technosols will depend on the needs of the site itself. Often times, the site has only the potential to be phytostabilized, whereas other times the use of some native plants would permit the phytoextraction.

2.3.1.1 Phytomanagement of tailings

Before any site treatment it is essential to perform a screening of the properties of the substrate to work with and the existent indigenous flora of the site. In the literature, various cases have been identified (Shallari et al., 1998; Shu et al., 2005; Yang et al., 2013; Randelović et al., 2014; Ciadamidaro et al., 2019). The case of the Cartagena-La Union mining district in Spain is an example of why the selection of phytomanagement over other techniques is suitable. The surface to treat and thus the cost tend unrealistic to propose neither engineering nor chemical remediation approaches, therefore phytomanagement is preferred (Parraga-Aguado et al., 2013).

In general, the production of rather acidic tailings brings its own concerns, for instance, the acid mine/rock drainage (AMD), that is the leaching and loss of PTEs found in the tailings due to the acidity and presence of sulfide residues (Park et al., 2019). This is one of the reasons why extraction tailings are generally alkaline. Nevertheless, some stored muds are rather acidic, such is the case of the Aznalcóllar pyrite mining residue. A spill of pyritic slurry occurred in

Andalucía, Spain in early 1998 that covered around 40 km² of valuable agricultural land releasing enormous concentrations of Pb, As, Cu and Zn (Simón et al., 1999). In this site, natural colonization occurred by *Chenopodium album* L. and thus it was studied as a pilot plant for revegetation (Walker et al., 2004). Studies identified the use of pH-increasing amendments, i.e. cow manure, for increasing plant growth, reducing leaching and plant accumulation of PTEs (Figure 18). For substrate of the same site, Madejón et al (2006) used biosolid compost, sugar beet lime and a mixture of sugar beet lime with leonardite to reduce the PTEs solubility. The use of such amendments assisted in the phytostabilization of the area by improving the establishment of spontaneous colonization.

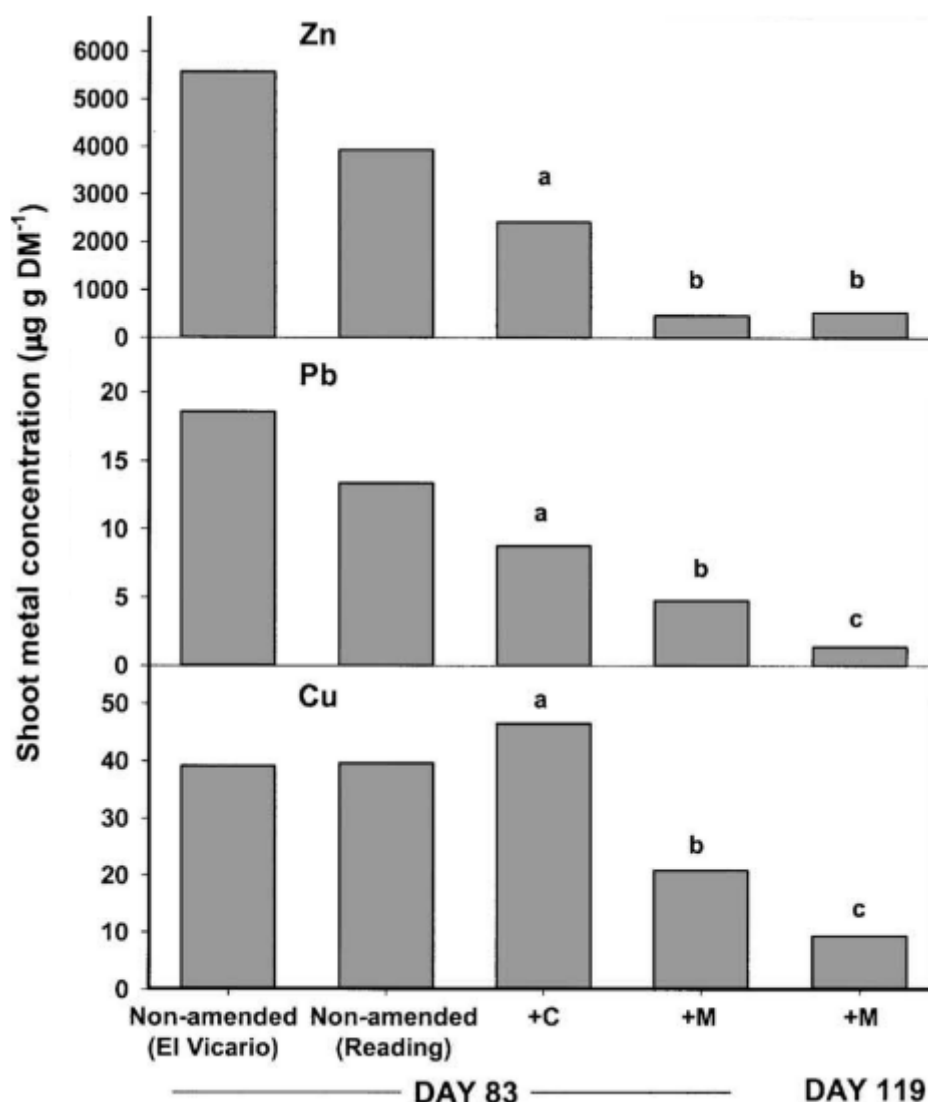


Figure 18. Metal concentrations in shoots DM of *Chenopodium album* after 83 and 119 days of growing in soil contaminated pyritic slurry, without and with amendments: compost (C); cow

manure (M). Non-amended soils were obtained from El Vicario and Reading communes. From Walker et al., 2004.

The tailings of the already closed Gunnar gold mine in Canada have been pumped down to a valley where they occupy over 11 hectares, from which about a half is under water. The tailings are mainly composed by quartz, calcite, pyrite, Fe-oxides and galena (Young et al., 2015). Phytostabilization of a non-vegetated area of tailings was the approach undertaken in this site using native plant species (*Picea mariana*, *Medicago sativa*, *Agropyron trachycaulum*, and *Festuca rubra*), inorganic fertilizers and some organic amendments. The latter allowed to increase the soil structure and fertility for the plant development (Young et al., 2015).

Similarly, a tailings deposit in Quebec, Canada, the Sigma-Lamarc gold mine has been used to test the performance of the indigenous white spruce (*Picea glauca*) in a greenhouse when using indigenous and allochthonous mycorrhizal and bacterial inoculum. The findings indicated that the inoculation of *Pseudomonas putida* did not necessarily improved the uptake of NPK from the soil but rather enhanced the balance of Mg and Ca. The concentration of the latter was already identified as a limiting growing factor. Similarly, the case of the mycorrhizal inoculum *Cadophora finlandia* regulated the uptake of Ca and Fe (Nadeau et al., 2018). Clemente et al. (2012) used waste products from the olive oil extraction and pig slurry in order to improve the overall conditions of a contaminated and arid soil by using *A. halimus*. The aim was to phytostabilize some PTEs present (Cd, Pb, Cu and Zn). The pot assay resulted in improvement of the major soil physical conditions as well as plant development and rhizospheric microbial community growth. However, this measure was somewhat limited due to the elevated metal concentrations in leaves and fruits, which suggests that this plantation could disseminate the PTEs through the loss of dry biomass.

2.3.1.2 Phytomanagement and reclamation of abandoned mines

Mining is motivated by the existence of valuable ore within the soil. After extraction, all that remains is the sterile and unstructured soil, called spoil or heaps. When this land is abandoned, it becomes nobody's responsibility (Fields, 2003). The natural process of abandoned mining sites is therefore natural colonization and spontaneous succession over a long period of time (Allen et al., 1997). The spontaneous natural succession is an approach that has been seen upon abandoned post-Communist coal-mine areas. For instance, the mining district in Czech Republic, old mines have been left to either revegetate naturally or have been reclaimed through technical afforestation (Hodačová and Prach, 2003). The study suggested that natural

succession, even though it is a slower approach, provides a more complete vegetation cover with indigenous plant species, whereas afforestation may introduce exotic, possibly invasive, species (Figure 19).

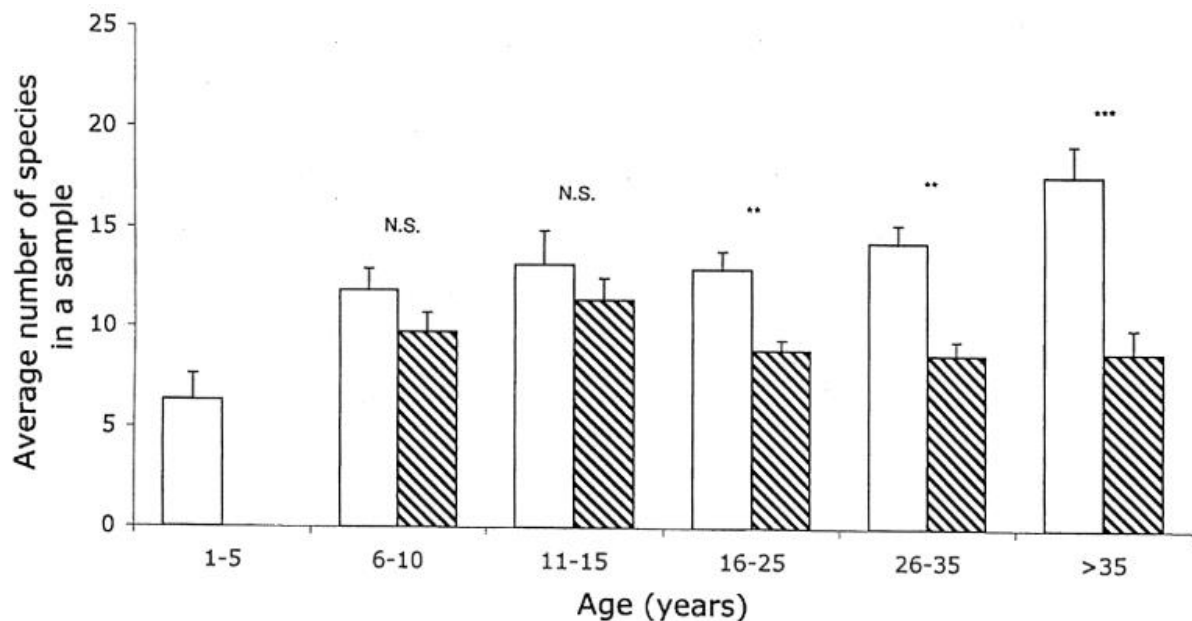


Figure 19. Plant species abundance in abandoned brown-coal mine heaps in Czech Republic. Hollow bars: Naturally (unassisted) afforested area, hatched bars: assisted afforested area. From Hodačová and Prach, 2003.

In the USA, the application of biosolids cakes was used for the reclamation of abandoned mines. This approach allowed to increase the nitrogen content of the soil and also contribute to the organic matter content when mixed or laid upon mine spoils (Walker, 1994). The origin of these biosolid cakes was basically from sewage sludge that has been neutralized, composted and pathogen-free. Some of the most remarkable characteristics of biosolids cakes were alkaline pH, high organic N content, and contained carbon material from composted mulch (Haering and Daniels, 2000). The drawbacks of using biosolids cakes on mine spoils were the sudden leaching of organic N and P from the soil profile into the groundwater (Figure 20). This could lead to eutrophication and loss of water supply sources (Stehouwer et al., 2006).

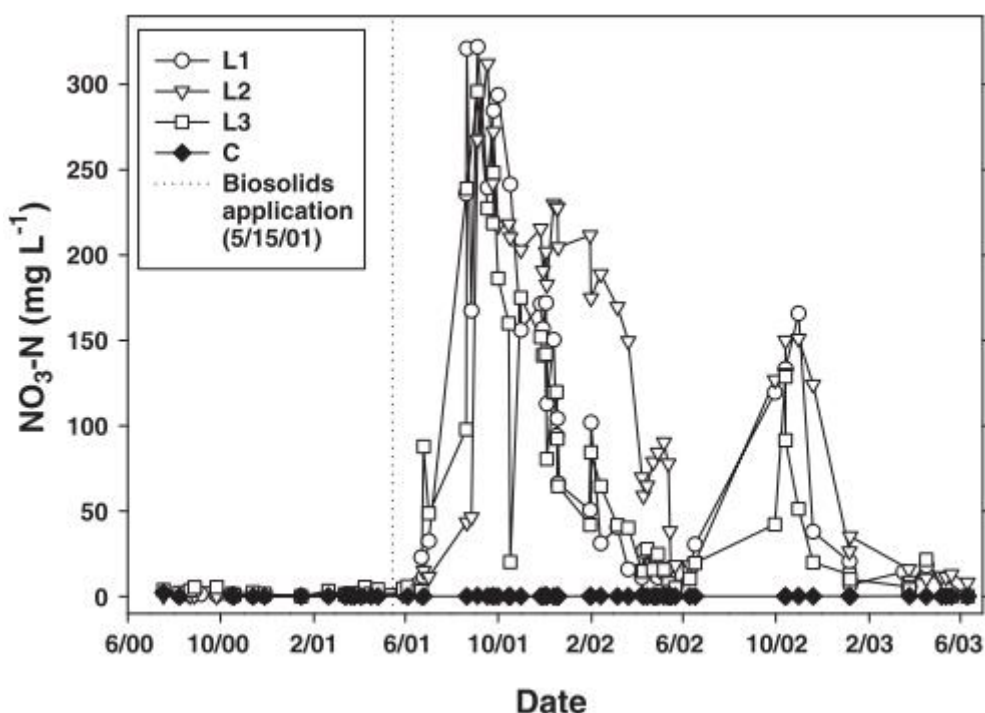


Figure 20. Quantification of $\text{NO}_3\text{-N}$ in samples from percolated water through zero-tension lysimeters over time. L1, L2 and L3 in the zone with biosolids application and C is untreated area.

The spontaneous revegetation of abandoned mines must therefore comprise the use of PTEs-tolerating vegetation. Some of the comprised aspects for such task have been compiled by Wong (2003). In his text, the author mentioned that pioneer plant species must be used in order to allow ecological succession of the site. Finally, the use of hyperaccumulating species is suggested for the cleaning up of soils, therefore recovery of PTEs.

Phytomining is the use of metal-accumulating or hyperaccumulating plants for the production of crops charged with valuable metals from soils ore-based or with too low metal concentrations for conventional mining extraction (Brooks et al., 1998). A classic application has occurred in abandoned gold mines. The discovery of gold hyperaccumulation plant species initiated the investigations of ways for increasing the accumulation. With this aim, some chemicals have been determined to increase the solubilization and induce a sort of *gold hyperaccumulation* (Wilson-Corral et al., 2012). Similarly, the recovery of Rare Earth Elements (REE) through phytomining has made its emergence due to the recent relevance of its uses in high-tech devices and the rareness of ore deposits. A phytoscreening profile showed that grasses had a better affinity for accumulation of Ge in shoots when compared to herbs. Concentrations of lanthanide elements were greater in *Brassica napus* with 250, 247, 95 and 56 ng g⁻¹ of La, Nd, Gd and Er, respectively; whereas Ge was found in concentrations of up to 358 ng g⁻¹ DM

in *Zea mays* (Wiche and Heilmeyer, 2016). Based on a collection of 49 hardy fern species, a recent study highlighted the preferential transfer of light REE transfer to the fronds over heavy REE (Grosjean et al., 2019).

The phytomining application has been considered by some as the new tendency to revalorize waste, notably abandoned mines or industrial residues. For the concept of phytomining to work, three main conditions must be met: 1) the identification of hyperaccumulating species, which has been repeatedly explained before; 2) the development of agronomic practices to ensure the cost-effectiveness of the operation; and finally, 3) the development of methodology that allows the exploitation of the phytomined element from the plant biomass (Chaney et al., 2018). As a response to the latter conditions, studies such as that proposed by Grison et al. (2015) support the reuse of actual waste and residues may be the key to adapt to the imminent changes in our society (reduction in the access to natural resources and reduced quality of the remaining available resources). This concept indicates the use of *bio-inspired* remediation approaches (Figure 21). The elements contained in plants grown in contaminated substrates would therefore be used for the production of special catalysts that would contribute not only economically to the industrial partners but also at an environmental level to the ecosystem. These catalysts are now known as eco-catalysts (Escande et al., 2017). The eco-catalysts production is now underdevelopment and covers a variety of elements that can be extracted: Mn (Escande et al., 2015), Cu (Clavé et al., 2016). Over time, various are the cases that have inquired the need for remediation techniques development. Such is the case of red mud (RM) and red gypsum (RG).

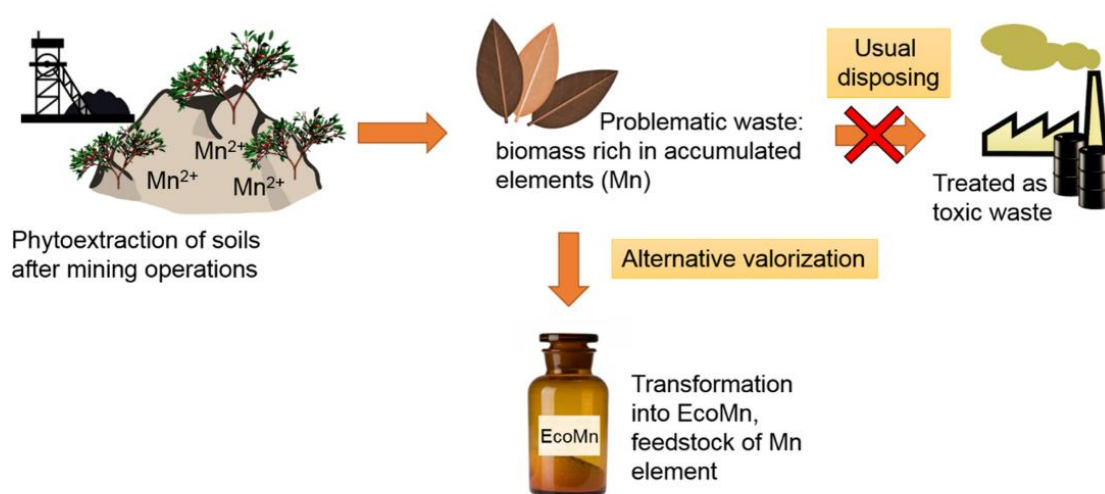


Figure 21. The process of Eco-catalyst production using Mn-enriched biomass grown in metal-enriched tailings. From Escande et al., 2017.

2.3.1.3 Case study 1: The revegetation of a red mud landfill

The red mud, as mentioned in earlier sections of this thesis, is the residue of the Bayer® extraction process. The recurrent spillage or dam bursts of tailing ponds containing RM have inquired international efforts for treating impacted areas. Therefore, the following subsection will provide some of the developments and knowledge acquired regarding RM.

Studies of RM dust resuspension by the wind (PM_{10}) have been undertaken after the spillage in Ajka, Hungary. Toxicological assessments of the particulate matter were carried out but found that no potential risk was taken by inhaling RM dust. However, the authors exhorted that the comparison values they used were considered for environmental health standards and not human health standards (Gelencsér et al., 2011). From an ecotoxicological point of view, the RM of the Ajka spill was analyzed from different perspectives. One case was the microcosm's study of RM's mid-term possible consequences and use perspectives of Ujaczki et al (2015). The outcomes of this study indicated a maximum dose of 5% of RM may be used without affecting soil biota nor surpassing Hungarian guidelines. Other studies focused in the consequences of the RM in plants. Ruyters et al (2011) studied the long-term effects of the exposition of RM to plants and their potential uptake of PTEs. The results indicated that no uptake of TE, due to the elevated pH, was detected and the only serious issues for the RM revegetation were the excessive salinity and possibly slow leaching, however, gypsum amendment was recommended to balance leaching and control salinity overtime. This measure was by the way adopted as a countermeasure right after the Ajka accident to prevent the salinity issues down the Torna Creek, Marcal and Rába Rivers. The neutralization of RM produces $CaCO_3$, from atmospheric carbon (CO_2), therefore, carbon sequestration was considered as positive outcome of the accident applied remediation measures. Some 13 to 26 Mt of CO_2 were estimated for the Ajka RM spill, whereas a potential 572 Mt of CO_2 were estimated for the global alumina industry (Renforth et al., 2012).

As explained before, the RM incidents compelled to RM producers to develop a strategy for revalorization of this material. One case of this underdevelopment research was the use of RM and coal fly ash as absorbents for the removal of dye (methylene blue) from wastewaters in Australia. This seemed promising, although the results showed that coal fly ash was a better absorbent material than RM for methylene blue from wastewaters (Wang et al., 2005). Similarly, the capabilities of RM were assessed against those of beringite and lime as an amendment for immobilization and bioavailability reduction of PTEs in soils from sewage sludge irrigated soils charged with Zn, Cd, Ni and Cu from France and the UK (Lombi et al.,

2002). The mesocosm study confirmed the hypothesis that RM may be used for metal immobilization from contaminated soils, especially those of the UK thanks to the pH influences (Figure 22).

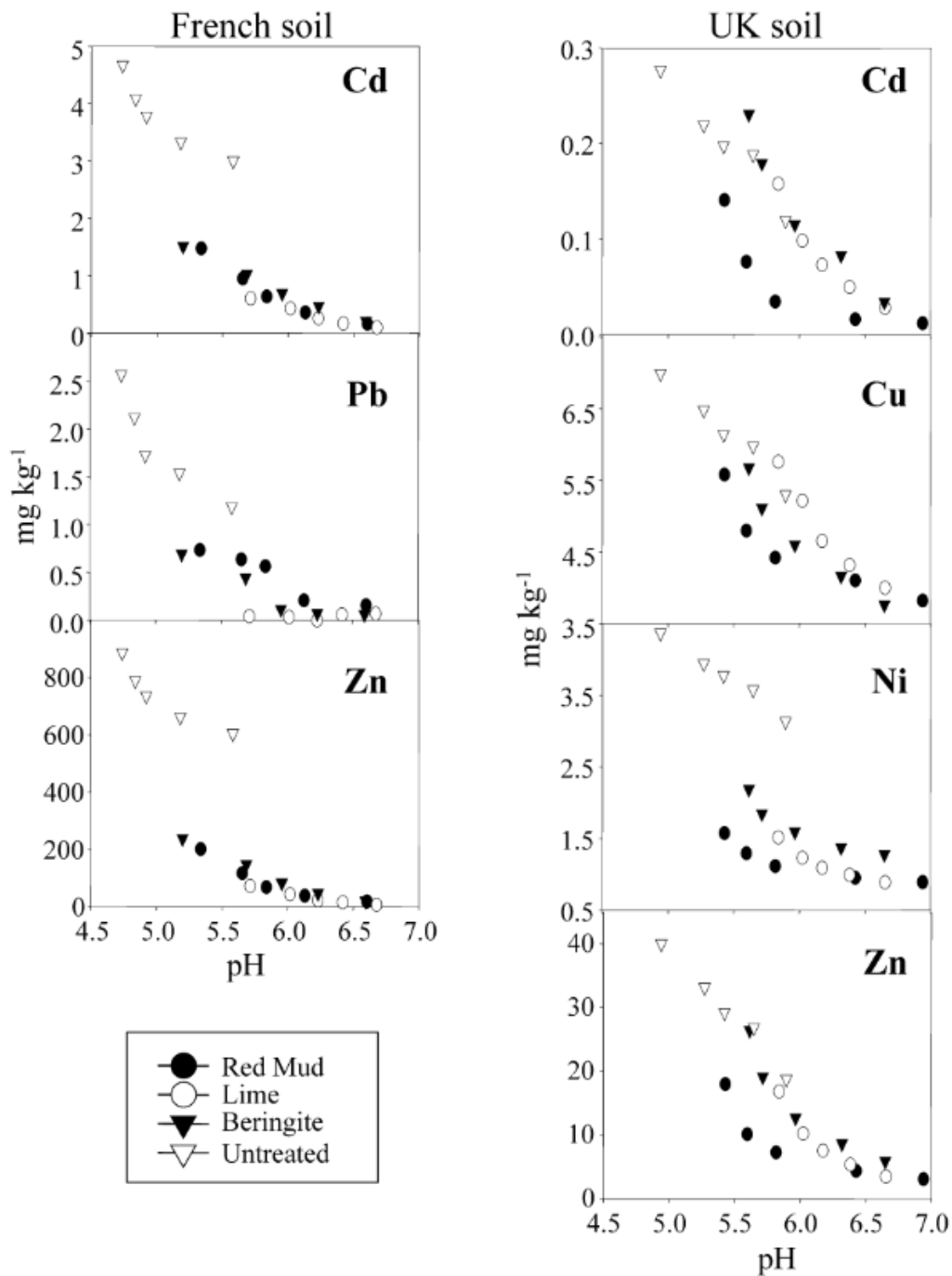


Figure 22. Effects of pH changes on the immobilization of Cd, Cu, Ni and Zn in French and UK soils. From Lombi et al., 2002.

Regardless of the accidents in the RM context, the study of the stabilization of RM tailings existed beforehand. At the Aughinish Alumina Limited Bayer Plant in Southwestern

Ireland, the concerns about an imminent closure of the tailing ponds led them to experiment with remediation strategies. The assessment of growth of *Lolium perenne* and *Holcus lanatus* was carried out in RM plots treated with gypsum and thermally dried sewage sludge. The treatments had already been tested in Western Australia in the past (Wong and Ho, 1994). The results indicated that indeed RM itself is a quite inadequate in agronomical quality. The application of essential nutrient amendments is imperative for grasses after only one year. Although the presence of Na presented no concern after treatments (Courtney and Timpson, 2005). Finally, a successful case of revegetation was described by Wehr et al (2006). The Alcan Gove bauxite mine and refinery in Australia deposited over 80 ha of RM were treated with seawater, to neutralize the Na presence, dried, capped with clayey soil and ripped prior to planting alkali- and salt- tolerant species i.e. *Chloris gayana*, *Cynodon dactylon*, *Acacia holosericea*, *Eucalyptus alba*, *Casuarina equisetifolia* L., etc., and mineral fertilization. The approach showed after 20 years that pH has been ameliorated (from 9.8 to 6.8) by capping and inducing vegetation development. Furthermore, the sustainable sufficiency was reached by the inclusion of legumes due to the atmospheric N fixation.

2.3.1.4 Case study 2: studies at a residual red gypsum landfill

The importance of the **residual red gypsum (RRG)** lays in the increasing demand for TiO₂. The main difference between this and the previous residue is notably the pH and the liquid phase presence. Despite that, the RRG is still a rather special substrate with quite interesting properties. Some characterization studies of RRG have been carried out over time (Fauziah et al., 1996; Peacock and Rimmer, 2000; Gázquez et al., 2009; Mahazam and Azmi, 2016) all of which coincided with slightly alkaline pH (~8), pretty low cation exchange capacity (CEC) and the presence of majoritarian elements such as Ca, S, Fe and Al. These conditions represent a significant drawback for the revegetation of such tailings. Few works have been performed in the past regarding RRG, but most of them consider the forms of valorization of RRG, these have been commented before. Regardless, the low CEC and alkaline pH have turned RRG into a recurrent soil amendment for treating PTEs contaminated soils. Garrido et al (2005) compared the use of RRG, dolomitic residue, phosphogypsum and sugar foam for the immobilization of Pb, Cu and Cd in acidic agricultural soils from Spain. The results showed a correct Pb immobilization and recommended using mixtures of the amendments for a more accurate immobilization of the other metals (Illera et al., 2004). Only recently has research focused on the rehabilitation and reclamation of RRG landfills. Works such as that by Assad et al (2017), describing the distribution of PTEs taken up by vegetables grown in mineral-fertilized RRG;

Zappelini et al (2018), describing the rhizospheric microbiota of a RRG landfill; and by Ciadamidaro et al (2019) , identifying potential tree species for phytomanagement in a RRG landfill, have been found. This represents a wide opportunity for the search of new knowledge and therefore prompts the fulfillment of this thesis.

Chapter 3

Aims and objectives



The literature state of the art showed that very few studies involving red gypsum have been carried out in the past and more importantly, that no study was found on re-vegetation or reestablishment of a vegetation cover upon a residual red gypsum landfill for its reclamation. Since the occurrence of these landfills is parallel to the demand of TiO_2 , this investigation is relevant for a future where TiO_2 production is on the rise. The knowledge produced hereby on how to stabilize such substrate when the time comes, therefore avoiding possible environmental crisis and political issues with the TiO_2 producers will be cherished. The central question of this work was, therefore, whether the reclamation of a red gypsum landfill was possible. In order to reply to this central nuisance, some more specific questions were made:

- What is the actual state of the site?
- Is there any vegetation developing in the site?
- What are the limiting factors that keep plants from developing in an optimal way?
- Is there a plant species capable of being used as a pilot plant to carry out experiments and be used to establish a vegetation cover, which is the main aim of this work?
- Is there a way to improve the plant development at the RRG landfill in order to aid the re-vegetation?
- Is there a way to re-valorize the biomass issued from a RRG landfill?

The common factor that binds these queries altogether gave place to the global aim of this PhD dissertation that was to provide assistance on the reclamation of a red gypsum landfill in a sustainable fashion in order to minimize the environmental impact. The pursuit of this aim was focused in two main tasks that became the axes of focus: the site stabilization and the revalorization of the substrate through the use of vegetation through phytomanagement technology.

Several options exist to achieve this, but in this case the path of application of either organic or biological amendments on specialized vegetation was chosen.

Derived from the central aim, four specific objectives or tasks were formulated in order to answer to the questions asked beforehand.

- Achieve the characterization of RG and vegetation present *in-situ* and their spatial distribution.
- Assess the effectiveness of organic pH-decreasing amendments to improve the development of a vegetation cover upon the experimental site.
- Assess the effects on the application of organic and biological soil amendments the one that will aid to improve the Mn solubilization.
- Assess the development of the specialized species *Lupinus albus* for the phytoextraction of Mn from the experimental site.

In order to explore these objectives and provide an answer to the main query, the results are divided in four parts, one for each objective. The next chapter will introduce the site, presenting the known and available information in the literature about the experimental site and its history.

Chapter 4

Experimental residual red gypsum site



The site of study is located in the Alsace region (Eastern France) in the department of Haut-Rhin, namely the Ochsenfeld plain. This is located at the south of the Vosges ridge where the Thur River is born at 1304m of altitude, then 25 km downstream it traverses the villages of Thann, old Thann, and Cernay and descends to the Alsace plains (Martin et al., 2015).



Figure 23. The location of the millenium factory (blue) and the Ochsenfeld landfill (red) at the outskirts of Thann and Cernay in Eastern France. (From <https://www.google.com/maps/>).

In this area, the Thann & Mulhouse factory was founded in 1808 by Philippe-Charles Kestner and it has dedicated its operations to the production of sulfuric acid, chlorine, potassium hydroxide and, after 1922, it became the first plant in the world to produce TiO_2 . Thann & Mulhouse was divided in 1993 into Albermarle PPC and the other part was sold to Millenium Chemicals in 1998, which in turn was acquired by the Saudi group Cristal Global in 2007 (Stoskopf, 2014), in red in Figure 23. The ownership and administration of these old companies has varied over time. Nowadays, the chemical industries, a 15-ha complex within Thann are the titanium and derivates production recently acquired by the North American group Tronox and the potassium derivates industry by the European group Vynova, naming the site Vynova PPC.

The Cristal group is one of the largest manufacturers of titanium chemicals in the world, the largest producer of merchant titanium chemicals, a leading manufacturer of specialty

titanium products and a fast-growing producer of mineral sands and titanium metal powder. Added to this, the group's titanium portfolio is comprised mainly by titanium dioxide (TiO_2), titanium tetrachloride (TiCl_4) and ultrafine TiO_2 . The product of the extraction is in the form of anatase (Cristal).



Figure 24. Evolution of the Ochsenfeld landfill over time. This landfill received residues from the production of the potash and other elements coming from PPC; later on, the production of TiO_2 started (in 1922), then the site started receiving RRG. (From <https://www.geoportail.gouv.fr>).

The Ochsenfeld landfill has been the storage area of the chloride electrolysis residues and iron sulfate since 1930 and 1990, respectively and in more recent years it has been covered with the residual red gypsum from the sulfuric extraction for covering the ancient residues (Basol, 2017b). This storage facility is comprised by an 80-ha surface whose photographic records go as early as 1935 (Figure 24). The site has witnessed changes due to the nature of the elements deposited inside. Due to the presence of Hg , SO_x and Cl^- in underground water, an excavation took place in 2004 to build an impermeable barrier in order to avoid more percolation and runoff of these elements given the low pH conditions of the substrate. Consequently, the company acted by covering the site with residual red gypsum (Basol, 2017a).

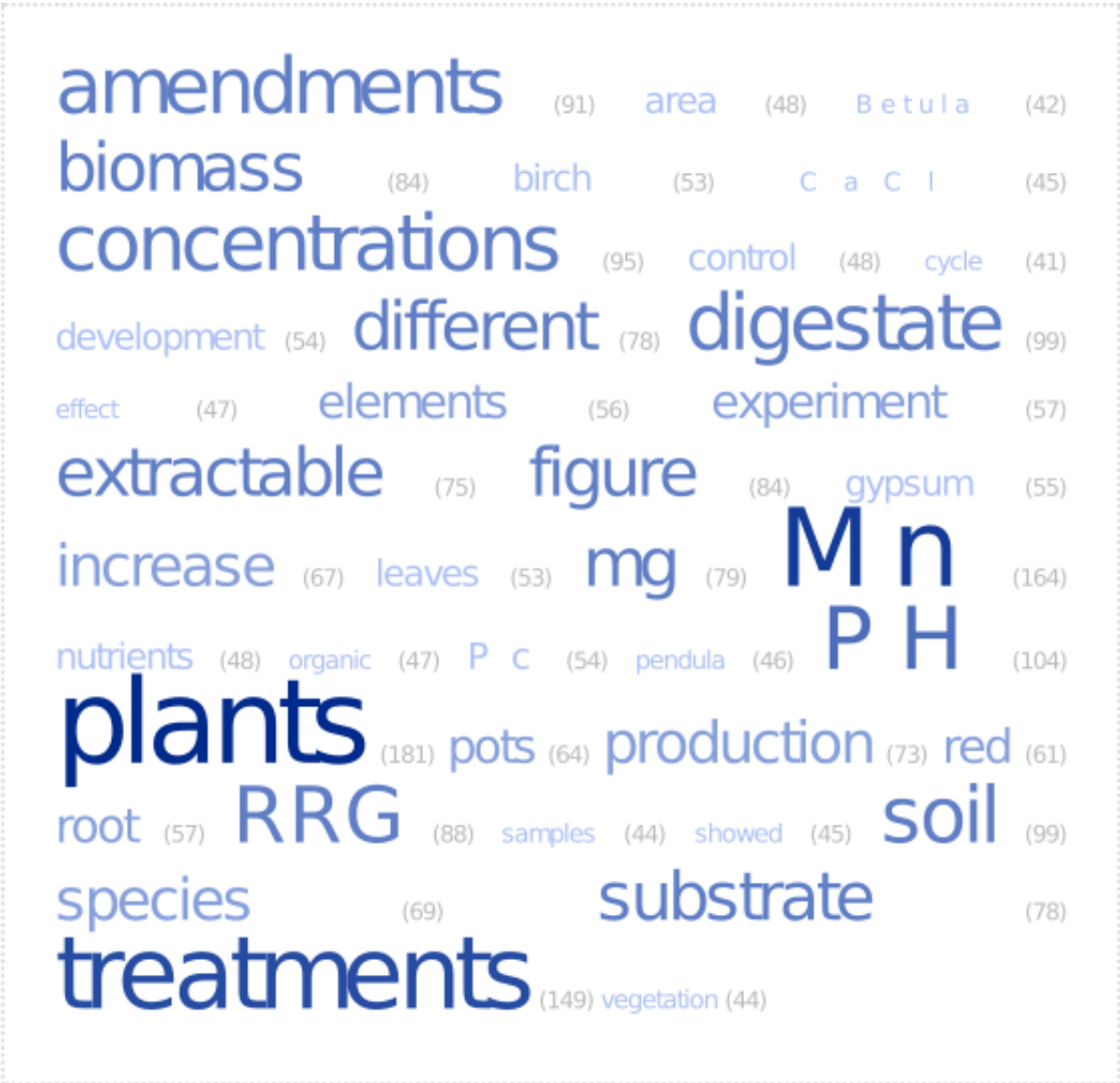
The overall works of this thesis were carried out within and with substrate sourced from the same plot, namely the D1 plot in yellow (Figure 25). The D1 plot is a nearly 3 ha area that has been inactive for approximately 10 years and is over 20 m depth.



Figure 25. The Ochsenfeld landfill and the D1 plot (yellow). (From <https://www.google.com/maps/>).

Chapter 5

Results



Part I

5.1 Spontaneous ecological recovery of vegetation in a red gypsum landfill: *Betula pendula* dominates after 10 years of inactivity

5.1.1 Context and publication

The literature on the chemical composition of the red gypsum was indeed scarce. The only bits of information that could be found related to red gypsum were about its valorization as a construction additive. Few were the works found that only referred to the influence of red gypsum on the dynamics of some PTEs, let alone the influence on the development of plants. Therefore, this section covers the first specific objective of this dissertation, which is the characterization of the vegetation and physico-chemical properties of the RRG landfill at the Ochsenfeld site and the analysis of the spatial distribution of plants and trace elements.



In consistency with the scope of the PHYTOCHEM project, we took advantage of the access we had to the Ochsenfeld site at the outskirts of Thann to assess the relations between the natural plant occurrence and the substrate's characteristics *in-situ* and use it as a first re-vegetation of an RRG landfill case study. It is worth mentioning that the approach mentioned in this section could be repeated over time in order to assess the evolution and changes of the vegetation *in-situ*. The use of statistical multivariate tools was essential to understand for the interpretation of this first approach. The development of this first case study gave place to the following publication in *Ecological Engineering* from early 2019.



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Spontaneous ecological recovery of vegetation in a red gypsum landfill: *Betula pendula* dominates after 10 years of inactivity.

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1. Abstract

Red gypsum is the product of the neutralization of titanium dioxide (TiO₂) extraction residue from ilmenite and anatase. The disposal of red gypsum creates heterogeneous plots with layers that may include Fe, Ca, Al, Mg, Mn, S, and other elements and an alkaline pH that makes revegetation complicated and slow. The vertical and horizontal dispersion of the sediment particles are the main concern. Therefore, the establishment of precise vegetation cover is needed to address this issue. One of the aims of this study was (1) to explore the distribution of the spontaneous vegetation found along a red gypsum-formed landfill located at the Ochsenfeld site in eastern France. Additionally, (2) some pedological parameters were also studied to determine the most significant red gypsum chemical drivers influencing the occurrence and abundance of the vegetation within the site. The *Braun-Blanquet* scale was used to rate the species presence in the field contained in the spontaneous vegetation dataset. The vegetation survey revealed the presence of 59 species from 23 families. The most abundant species was *Betula pendula*, and a further cluster analysis enabled the differentiation of areas with this species. The CaCl₂ extractable concentrations of the nutrients and trace elements, as well as the pH and the organic matter (OM) present in the sampled substrate, were used to form the pedological parameters dataset. A multiple factor analysis (MFA) was performed to link the large datasets together and revealed 3 groups of plants. Group 1 was composed of pH-tolerant species such as *B. pendula* and *S. caprea*. Group 2 was formed by Cr-Zn-tolerant species, including *Echium vulgare* and *R. pseudoacacia*. Finally, group 3 was characterized by species such as *Clematis vitalba* and *Artemisia vulgaris* that tolerate the presence of Na. The MFA revealed a correlation between the *Betula pendula* distribution and the pH, CaCl₂ extractable P concentration and organic matter. On the other hand, a lack of relation of the CaCl₂ extractable concentrations of Fe, Mn, Na, Si and K were also found. This study aided in the selection of an adapted candidate for the implementation of the revegetation strategy of a red gypsum landfill in eastern France. Further tests were performed at the site using white birch for Mn accumulation and topsoil stabilization *in situ*.

Keywords - Red gypsum, *Betula pendula*, trace elements, spontaneous vegetation

2. Introduction

The increase in the global population has led to an increase in natural resource demands. Consequently, industries consume increasing amounts of feedstock for producing enough goods to satisfy demands; therefore, the byproduct yield is expected to be equally elevated. The different extraction and transformation processes are countless, but only ore processing and tailing production have been classified among the ten worst polluting sources since 2012 (Mills-Knapp et al., 2012; Ericson et al., 2014). Approximately 7 Gt of tailings are generated globally each year by mineral processing and extraction activities of mine ores (Edraki et al., 2014). Among only the 28 EU member states, up to 300 million tons of industrial waste had been produced by 2014 (European Commission, 2009). Mine tailings consist of fine-grained rock that remains after the extraction of economic minerals and is associated with the solution used to process the ore. The latter contains dissolved metals and ore processing reagents (Edraki et al., 2014). The tailings are classified as one subset of the Technosol Reference Soil Group (Baxter, 2015) that comprises soils formed from waste materials produced during mineral and energy extraction and refining activities, which means that depending on their origin, ore processing residues are likely to contain one or more potentially toxic elements (PTEs) (Kosheleva et al., 2018). According to Driussi and Jansz (2006), tailings represent a potential source of environmental pollution due to the risks of sediment displacement through runoff or leaching, spills, dust scattering and the instability of the substrate. The risk of scattering by the wind has been widely studied either from industrial or traffic sources for being related to the rise of cases of health disorders of exposed individuals (Siegel et al., 2012). Moreover, the containment of the extraction residues could also result in dam bursts. These bursts have been witnessed throughout time, and some of the most frequently mentioned have occurred with the Bayer bauxite extraction residue: the red mud (RM), stored in basins as a liquid. The WISE Uranium Project (1994) has listed and updated every accident, including tailings dam failures. These incidents gave rise to research that enabled the understanding of the impacts and the possible ways for amending them. For instance, after the Ajka incident in Hungary in 2010, the acid effluents affected hundreds of hectares of crops where all kinds of organisms were exposed to PTEs and elevated levels of pH and salinity present in the mixture (Gelencsér et al., 2011; Ruyters et al., 2011). Moreover, in other sites, *in vitro* risk assessments have been carried out to evaluate the toxicity of potentially hazardous elements (PHEs) produced by the bauxite production industry and scattered by the wind (Reis et al., 2014). Later, in 2015, the Red Mud

Project was founded as a network for the reutilization and revalorization of red mud (Red Mud Project, 2015).

Similar to RM, red gypsum (RG) is the byproduct of titanium refining, mainly for the TiO₂ pigment industry. This byproduct is mainly composed of Fe oxides and hydroxides, Mn oxides, Ti dioxide, and calcium carbonate/aluminates and therefore has an alkaline pH (Fauziah et al., 1996). The demand for the pigment was estimated to be approximately 5.5 million tons annually in 2008, with a 3% growth every year (Vincentz Network, 2008). From the Huelva factory in Spain alone, 142,000 tons of raw material are used and some 30,000 tons of RG are produced annually (Gázquez et al., 2014). Recent market forecasts have noted that an increase in the demand for TiO₂ pigments can be expected in future years (ICIS News, 2018); therefore, an increase in RG production is also expected. RG has been studied and proposed to be used as an amendment for salinity excess (Renforth et al., 2012) and soluble metals excess (Garrido et al., 2005) and is used as a natural gypsum substitute in the cement industry (Gázquez et al., 2014). These proposed valorization options are based on the chemical properties of RG. However, for the same reason, RG landfills could be inhospitable environments, though they are rarely mentioned in the literature. In general, RG disposal is slurry-like, but since the moisture is drained, the tailings are exposed to the wind, which is the main threat. If tailings were to leave the landfill heaps, the PTEs contained within could cause health issues (Assad et al., 2016). Therefore, the best solution to this problem is topsoil stabilization. Leaving aside traditional *ex situ* remediation techniques (Jadia and Fulekar, 2009), a more recent review described an ideal management of tailings that aims at improving the environmental, social and economic outcomes (Edraki et al., 2014). For instance, they suggested the design of more thoughtful emplacements for the landfill and the establishment of a stable top layer made out of thickened sediments. However, these techniques involve some constraints linked to costs, pretreatment and maintenance to keep the properties of the top layer. The authors suggested reuse and recycle tactics to address this problem.

Therefore, alternative revegetation studies on harsh environments have been carried out. Natural succession processes suggested that unaided restoration can be achieved through nature-based solutions (Bradshaw, 1997), leading to functional soils. These approaches have identified the main drivers for revegetation success. In general, the physical and chemical properties of the deposited substrate, such as fine texture, high mechanical impedance, extreme pH conditions, hypersalinity, and elevated concentrations of metals and metalloids, are considered deleterious for plant and rhizosphere development (Huang et al 2012; Edraki et al., 2014).

Moreover, plant residues and the diaspore carriage strategy also play an important role by influencing the next generation of species that will develop (Martínez-Ruiz et al, 2005). Even though knowledge on the limiting drivers exists, every site is different in its chemistry, biological richness and weather, and thus, each case should be studied separately. Time determines the stage of succession and hence, the state of revegetation. There are many different studies of natural colonization and ecological succession carried out in different time spans. For instance, in southern China, natural colonization on five lead/zinc (Pb/Zn) mine tailing heaps was studied (Shu et al., 2005). The natural colonization of plants on these tailings was limited even after years of inactivity, with only some small patches distributed, mainly on the edge of the tailing ponds and even fewer patches at the center of the ponds. Poaceae and Asteraceae were major families present in the flora at these sites. Li and Liber (2018) performed a study in eastern China at a coal gob pile to compare the development of seven pioneer plant communities 9 years after planting. They found that the best-adapted association of leguminous and nonleguminous species was by *Medicago sativa*, *Amorpha fruticosa*, *Salix babylonica* and *Populus tomentosa*. A similar substrate, a coalmine in Spain, was assessed after 40 years with and without a topsoil treatment, showing a large difference in the speed of succession between the treatments. Alday et al. (2011) confirmed that the establishment of resilient vegetation in a topsoil treatment will speed up ecological succession. However, no related approach was found in the literature for RG tailings.

The present study aims to establish a flora inventory of a red gypsum-formed Technosol located at the Ochsenfeld site in order to determine the main chemical drivers of the native vegetation that occurred after 10 years of inactivity. We assessed the plant diversity of the area through the determination of the absolute cover values of the plant species and we determined the bulk mineral composition of the substrate and key soil parameters (pH, OM). We further applied a Multiple Factor Analysis (MFA) ordination method to different subsets of variables, to determine whether there is a link between the vegetation and the pedological datasets.

3. Materials and Methods

3.1 Study Site

The Ochsenfeld site (47.79686 N, 7.132775 E) is an 80-ha complex (Figure 26 left) that uses limestone for neutralization and stockpiles the sulfur-rich effluents produced by the TiO₂ production plant 3 km away, located within the Thann village in eastern France. The site belongs to the world's largest TiO₂ producer, Cristal. The disposal surface of the Ochsenfeld

site was prepared to protect the watershed from the leaching of industrial pollutants through an impermeable barrier. Normally, the effluents are deposited in lagoons to dry out and layer up until the maximum capacity of each lagoon is reached. The present study was performed on a 3 ha 10-year inactive lagoon defined as D1 area (Figure 26). The sediments are colored red due to the presence of iron oxides (Assad et al., 2016), and even though 10 years have passed since the last pour, D1 area shows little to nonexistent vegetation presence. In addition, the presence of cracks where water infiltrates is visible over the surface.

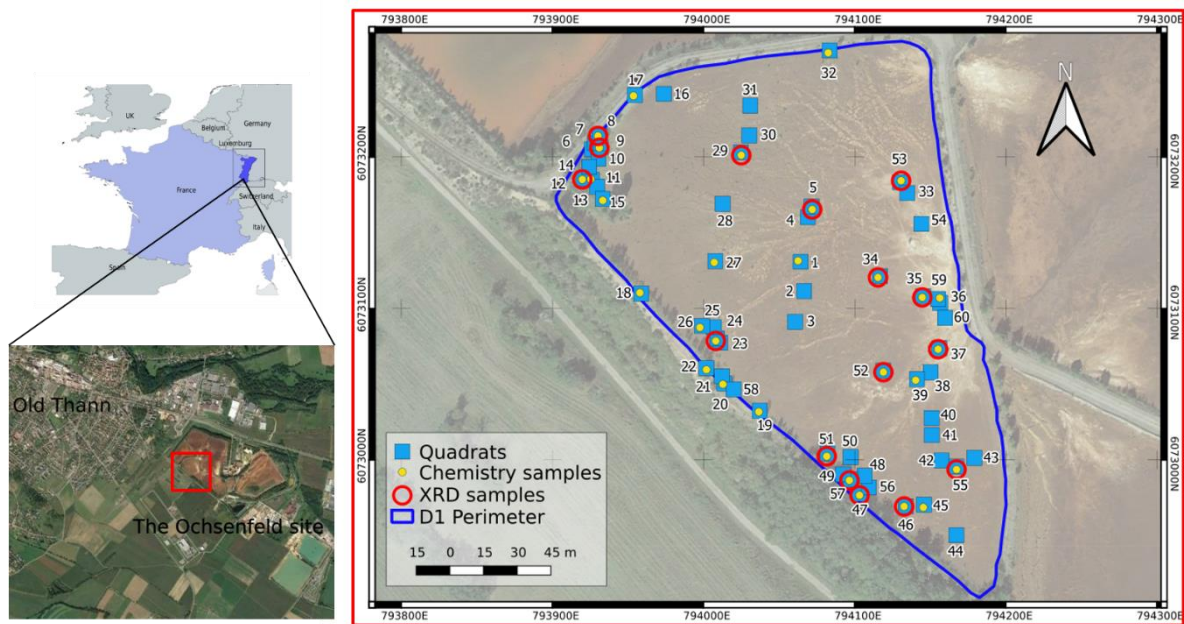


Figure 26. The Cristal neutralization plant. Geographical location of the red gypsum landfill at the Ochsenfeld site (left); View of the D1 area showing the location of the 60 sampling quadrats as well as the quadrats used for further chemical ($n=30$) and XRD ($n=16$) analysis.

3.2 Plant Surveying

In spring 2016, the plant diversity of the D1 area was assessed through the determination of the absolute cover values of the plant species, which are represented through the 7-level Braun-Blanquet scale (r, +, 1, 2, 3, 4, 5, for 0.5, 2.5, 15, 37.5, 62, 87.5 and 100%, respectively) (Wikum and Shanholtzer, 1978). Following substantial prospection on plant density, sixty 1-m² quadrats were scattered throughout the D1 area, as illustrated in Figure 26, in six different zones that were established to be as representative as possible of the whole D1 area. There were thus 10 quadrats per zone, randomly allocated.

3.3 Substrate Characterization

The available information about the substrate indicates a slightly alkaline pH and approximately 19% and 9% of the major elements Ca and Fe, respectively (Fauziah et al., 1996). We further analyzed the D1 study area more precisely by collecting five auger-extracted topsoil cores (0-20 cm) from each of the six zones (representing five randomly selected quadrats per zone) for a total of 30 cores, which were further oven-dried (40 °C for 72 h), ground and sieved at 2 mm.

The total element concentrations of the 30 cores were measured using inductively coupled plasma atomic emission spectrometry (ICP-AES, Thermo Fischer Scientific, Inc., Pittsburg, USA) after the acid digestion of 500 mg of sample (DigiPREP system, SCP Sciences, Courtaboeuf, France) using a mixture of 2 mL of 67% nitric acid, 6 mL of 34% hydrochloric acid and 2 mL of 48% hydrofluoric acid. Another 2.5 g of dried sample were extracted with 0.01 M of CaCl₂ solution in a 1:10 proportion under constant agitation at 140 rpm for 3 h. Then, the sample was filtered and quantified by ICP-AES (Ciadamidaro et al. 2014) to determine the CaCl₂ extractable fraction of the trace elements (TE) and nutrients, which is related to nutrient availability (Houba et al., 1996). The total concentration / CaCl₂ extractable concentration ratios were calculated.

The organic matter (OM) content of the same 30 topsoil samples was estimated by comparing the mass loss before and after calcination using 1 g of dried soil at 550 °C for 5 h. The pH was measured with a Hach HQ40d pH meter after shaking 1 g of dry soil in 2.5 mL of 1 M KCl for 1 h (Ciadamidaro et al. 2014).

Finally, the bulk mineral composition of the soil samples was assessed through X-ray diffractometry (XRD, Bruker, United States) measurements in order to estimate the possible external inputs, which may be responsible for the plant presence in the D1 area. Out of the 30 previously selected samples, approximately 1 g of oven-dried crushed (< 100 µm) substrate of 16 representative samples was analyzed using a D8 Advance Bruker X-ray diffractometer equipped with a LinxEye detector Parallel beam geometry and Cu-K α radiation at 1.54184 Å. XRD patterns were acquired from 5 to 60°2 θ and further processed using the EVA software package.

3.4 Statistical Analyses

The analyses were performed in *R* (Ver. 3.4.2) using the packages *ade4* (Dray and Dufour, 2007), *vegan* (Oksanen et al., 2018), *MASS* (Venables and Ripley, 2002), *FactoMineR* (Lê et al., 2008) and *Factoclass* (Lebart et al., 1995) and graphed using *ggplot2* (Wickam,

2017). We applied a cluster analysis to the Jaccard distance matrix from the presence-absence plant data to define the typology of the 60 quadrats (with 3 groups). We performed a principal component analysis (PCA) on the vegetation data from the 60 quadrats using the Hellinger transformed dataset to identify the main gradients in the composition of the plant communities within the D1 area. The Hellinger transformation is available in *vegan*. The quadrats 2, 3, 25, 27, 28 and 42 were removed from the analysis because they contained no vegetation, which was required for using the Hellinger transformation. We further performed a PCA on the soil variables (pH, OM and the CaCl₂ extractable fraction of elements) to explore their correlations and the chemical gradients throughout the D1 area. We further used a Multiple Factor Analysis (MFA), which is a symmetrical canonical ordination method applied to different subsets of variables (Borcard et al., 2011), to determine whether there is a link between the vegetation and the pedological datasets. The subsets were formed by 3 sets of data: the chemical properties (pH and OM), the vegetation (the Hellinger transformed plant abundances) and the extractable nutrients (the soil CaCl₂ extractable fractions) of the same 30 sampled quadrats. It is worth mentioning that the CaCl₂ extractable dataset was chosen over the total concentration dataset due to the connection with plant nutrient uptake.

4. Results and Discussion

4.1 Plant distribution on the landfill

The survey revealed a species richness of 59 comprising 23 families (Table 9) that naturally recolonized the 54 m² sampled surface, among which *Asteraceae*, *Poaceae* and *Fabaceae* were the best represented among herbaceous plants while *Salicaceae* and *Betulaceae* were the most represented woody species groups. A similar trend was found in reforested mine tailings in Mexico, where the vegetation spectra suggest that these families are more tolerant to heavy metals in soil (Salas-Luévano et al, 2017). Most of the species present on the D1 area were native to the region and are commonly encountered in ruderal environments. The plant communities were dominated by perennials, either herbaceous (60%) or pioneer trees (20%), in addition to 20% by annuals. A previous study on the revegetation of an industrial wasteland contaminated by polycyclic aromatic hydrocarbons (PAHs) and PTEs from eastern France showed that the pioneering community, essentially made of annuals and bi-annuals during the first year, was gradually colonized by perennials, which dominated the second successional year (Dazy et al., 2008). In our site, the dominance of perennials is justified due to 10 years of inactivity. It is worth mentioning that the substantial community changes could also be due to

invasions of non-native organisms (Prach and Walker, 2011). In this regard, 2 herbaceous species that may be considered invasive, *Conyza canadensis* and *Erigeron annuus*, were found in a few quadrats, as well as *Robinia pseudoacacia* for the tree species. The presence of the latter may be due to the presence of this species in the embankments surrounding the D1 area.

Table 9. Taxonomic levels of the surveyed species present in the D1 area of the red gypsum landfill.

Family	Genus	Species	ID	Family	Genus	Species	ID
Adoxaceae	<i>Sambucus</i>	<i>nigra</i>	<i>S.nig</i>	Onagraceae	<i>Epilobium</i>	<i>angusifolium</i>	<i>E.aun</i>
Amaranthaceae	<i>Beta</i>	<i>sp</i>	<i>B.sp</i>	Onagraceae	<i>Epilobium</i>	<i>parviflorum</i>	<i>E.par</i>
Amaranthaceae	<i>Chenopodium</i>	<i>album</i>	<i>C.alb</i>	Papaveraceae	<i>Papaver</i>	<i>rhoeas</i>	<i>P.rho</i>
Apiaceae	<i>Daucus</i>	<i>carotta</i>	<i>D.car</i>	Plantaginaceae	<i>Chaenorrhinum</i>	<i>minus</i>	<i>C.min</i>
Asteraceae	<i>Artemisia</i>	<i>vulgaris</i>	<i>A.vul</i>	Plantaginaceae	<i>Plantago</i>	<i>lanceolata</i>	<i>P.lan</i>
Asteraceae	<i>Cirsium</i>	<i>arvense</i>	<i>C.arv</i>	Plantaginaceae	<i>Plantago</i>	<i>major</i>	<i>P.maj</i>
Asteraceae	<i>Cirsium</i>	<i>vulgare</i>	<i>C.vul</i>	Poaceae	<i>Bromus</i>	<i>hordaceus</i>	<i>B.hor</i>
Asteraceae	<i>Conyza</i>	<i>canadensis</i>	<i>C.can</i>	Poaceae	<i>Bromus</i>	<i>sterilis</i>	<i>B.ste</i>
Asteraceae	<i>Erigeron</i>	<i>annuus</i>	<i>E.ann</i>	Poaceae	<i>Festuca</i>	<i>pratensis</i>	<i>F.pra</i>
Asteraceae	<i>Hieracium</i>	<i>sp</i>	<i>H.sp</i>	Poaceae	<i>Festuca</i>	<i>rubra</i>	<i>F.rub</i>
Asteraceae	<i>Leontodon</i>	<i>hispidus</i>	<i>L.his</i>	Poaceae	<i>Poa</i>	<i>annua</i>	<i>P.ann</i>
Asteraceae	<i>Matricaria</i>	<i>chamomilla</i>	<i>M.cha</i>	Poaceae	<i>Poa</i>	<i>trivialis</i>	<i>P.tri</i>
Asteraceae	<i>Picris</i>	<i>hieracioides</i>	<i>P.hie</i>	Poaceae	<i>Vulpia</i>	<i>myuros</i>	<i>V.myu</i>
Asteraceae	<i>Pilosella</i>	<i>officinalis</i>	<i>P.off</i>	Primulaceae	<i>Lysimachia</i>	<i>nemorum</i>	<i>L.nem</i>
Asteraceae	<i>Scorzonera</i>	<i>humilis</i>	<i>S.hum</i>	Ranunculaceae	<i>Clematis</i>	<i>vitalba</i>	<i>C.vit</i>
Asteraceae	<i>Sonchus</i>	<i>asper</i>	<i>S.asp</i>	Resedaceae	<i>Reseda</i>	<i>lutea</i>	<i>R.lut</i>
Asteraceae	<i>Taraxacum</i>	<i>officinale</i>	<i>T.off</i>	Rosaceae	<i>Crataegus</i>	<i>laevigata</i>	<i>C.lae</i>
Betulaceae	<i>Betula</i>	<i>pendula</i>	<i>B.pen</i>	Rosaceae	<i>Potentilla</i>	<i>argentea</i>	<i>P.arg</i>
Boraginaceae	<i>Cynoglossum</i>	<i>officinale</i>	<i>C.off</i>	Rosaceae	<i>Prunus</i>	<i>avium</i>	<i>P.avi</i>
Boraginaceae	<i>Echium</i>	<i>vulgare</i>	<i>E.vul</i>	Rosaceae	<i>Rosa</i>	<i>arvensis</i>	<i>R.arv</i>
Caryophyllaceae	<i>Arenaria</i>	<i>serpyllifolia</i>	<i>A.ser</i>	Rosaceae	<i>Rubus</i>	<i>fruticosus</i>	<i>R.fru</i>
Caryophyllaceae	<i>Cerastium</i>	<i>fontanum</i>	<i>C.fon</i>	Rubiaceae	<i>Galium</i>	<i>mollugo</i>	<i>G.mol</i>
Euphorbiaceae	<i>Euphorbia</i>	<i>cyparissias</i>	<i>E.cyp</i>	Salicaceae	<i>Populus</i>	<i>nigrum</i>	<i>P.nig</i>
Fabaceae	<i>Lotus</i>	<i>corniculatus</i>	<i>L.cor</i>	Salicaceae	<i>Populus</i>	<i>tremula</i>	<i>P.tre</i>
Fabaceae	<i>Medicago</i>	<i>indica</i>	<i>M.ind</i>	Salicaceae	<i>Salix</i>	<i>caprea</i>	<i>S.cap</i>
Fabaceae	<i>Medicago</i>	<i>lupulina</i>	<i>M.lup</i>	Salicaceae	<i>Salix</i>	<i>purpurea</i>	<i>S.pur</i>
Fabaceae	<i>Robinia</i>	<i>pseudoacacia</i>	<i>R.Pse</i>	Sapindaceae	<i>Acer</i>	<i>pseudoplatanus</i>	<i>A.pse</i>
Fabaceae	<i>Trifolium</i>	<i>pratense</i>	<i>T.Pra</i>	Scrophulariaceae	<i>Verbascum</i>	<i>blattaria</i>	<i>V.bla</i>
Fabaceae	<i>Vicia</i>	<i>sativa</i>	<i>V.Sat</i>	Verbenaceae	<i>Verbena</i>	<i>officinalis</i>	<i>V.off</i>
Hypericaceae	<i>Hypericum</i>	<i>perforatum</i>	<i>H.per</i>				

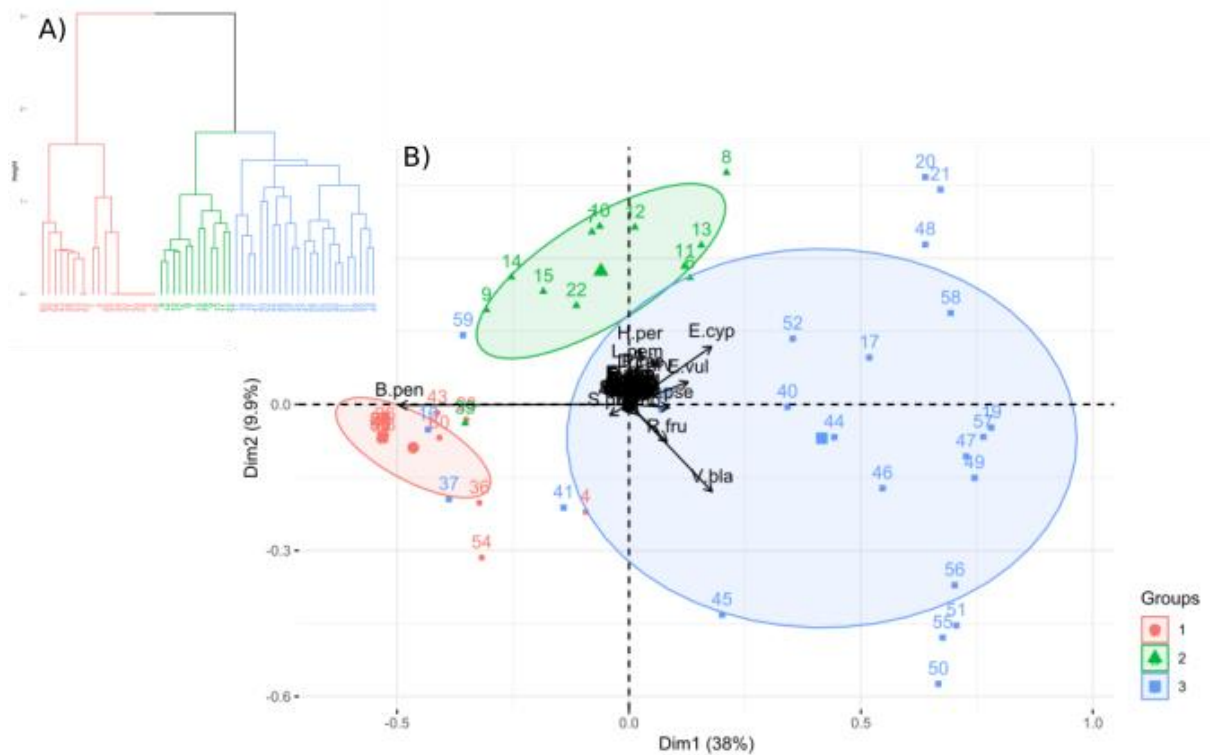


Figure 27. Distribution of the plant species within the D1 area. A) Cluster formation using the Jaccard dissimilarity index through the Ward D2 method (Murtagh and Legendre, 2014); B) PCA of the vegetation dataset ($n=54$) highlighting the clusters formed within the D1 area. The numbers refer to the quadrats shown in Figure 26.

Prach et al. (2014) considered *Robinia pseudoacacia* as an alien species in dry and warm regions that forms dense, species-poor stands dominated by nitrophilous species in the herb layer, which is not acceptable from the restoration point of view. The surface of the D1 area was remarkably heterogeneous in terms of vegetation cover. The ward cluster analysis derived from the Jaccard transformed presence-absence scale of species revealed the formation of three groups (Figure 27A). The three clusters were thus defined: cluster 1 included the quadrats where *Betula pendula* (birch trees) dominated; cluster 2 contained mixed vegetation with birch present and showed the most diverse vegetation; finally, cluster 3 included the quadrats with less diverse vegetation, where birch remained at a low frequency. Indeed, the data ordination of the PCA (Figure 27B) allowed for the describing of the distribution throughout the D1 area based on the *Braun-Blanquet* ranking dataset (Figure 31).

The first cluster, which was dominated by birch trees, did not share the space with any other species (i.e., quadrats 29 to 32). A mechanism to avoid competition through releasing allelopathic substances may be used by some *Salix* species, though this may not be the strategy for *Betula pendula*. Only non-important phenolic compound production has been registered for

the substrate beneath *Betula pendula* (Suominen et al., 2003). Thus, its dominance may be linked to its prolific seed production and dispersion strategy, as well as to its fast growing potential at the juvenile stage (Fischer et al., 2002). These characteristics allow this pioneer tree species to occupy afforested abandoned areas quite readily (Frouz et al., 2008). In a non-reclaimed post-mining chronosequence (Mudrak et al., 2016), *B. pendula*, along with *Salix caprea* and *Populus tremula*, had the highest cover value that increased with succession age.

The quadrats with the highest plant richness (cluster 2) were found in the edge of the D1 area (i.e., quadrats 8, 9 and 10), especially at the NW corner (Figure 26), which included species such as *Hypericum perforatum*, *Cirsium arvense*, *Daucus carota*, and *Epilobium angustifolium*, among others. The substrate of this corner showed a visual difference with the rest of the lagoon; it seemed to be composed similarly to the embankments surrounding the basin. The edge effect is a well-known ecological phenomenon observed in various environments where the vegetation is different between the edge and the internal area of the study site (Murcia, 1995). There is a clear species assortment close to the embankments of the D1 area, where at least twice the number of species were seen but not registered in this study of the D1 area. For instance, this was the case with the pioneering species *Tussilago farfara* or *Scleranthus annuus*. This result was mostly due to the composition of the road surrounding the basin, which created a different habitat for the vegetation. The effect here was mostly positive, as it led to greater plant cover on the edge, especially for quadrats 36, 54 and 60. Finally, the third cluster was mostly located south of the lagoon and filled in the gaps between the first two groups. This last cluster included species such as *Echium vulgare*, *Euphorbia cyparissias*, *Rubus fruticosus* and *Verbascum blattaria*, among others.

Based on the frequency values, the most abundant species found were *Betula pendula*, *Echium vulgare*, *Euphorbia cyparissias*, *Hypericum perforatum*, *Robinia pseudoacacia*, *Rubus fruticosus* and *Verbascum blattaria*, with presence recorded in quadrats 20, 15, 8, 9, 10, 11 and 9. The presence of different vegetation closer to the edges was not surprising, since this has been observed elsewhere (Popma et al., 1988; Shu et al., 2005). Quadrats 35, 36, 37, 39, 53, 59 and 60 may have also served as a reservoir for some dumped CaCO₃, as shown in Figure 26. The pipeline access was commonly set at this central point of the D1 area. The edge area may be readily colonized by tolerant species, especially weedy species of native plants, which induced changes in the environment at the expense of the landfill interior. Species such as *Echium vulgare* have been documented as developing well in poor environments, thriving in well-drained infertile soils, and tending to be excluded from very dense vegetation (Klemow et al., 2002). Interestingly, higher frequency of genetic mutations in response to environmental

stress was found to allow *Echium vulgare* to improve their ability to colonize unfavourable environments (Dresler et al., 2015). *Verbascum blattaria* was found to develop a higher biomass per plant in the soils with a low nutrient content, indicating its adaptability to infertile soils (Bretzel et al., 2009). *Euphorbia cyparissias* was also found to grow spontaneously on heaps left by historical mining for Zn-Pb ores (Stefanowicz et al., 2016).

4.2 Red gypsum characterization

The collected RG samples revealed an average pH of 8.3 ± 1.1 with values ranging from 7.7 up to 12.5 (Figure 28 and Table 10). It is worth mentioning that only 3 of the 30 quadrats analyzed (39, 53 and 59) showed pH values well above the average (Figure 28). The CaCl_2 extractable concentrations of some elements, such as Fe and Mn, were also remarkably heterogeneous over the D1 area. This heterogeneity probably plays a significant role in the availability of nutrients for native plants (Hallock, 1988). However, the averaged pH values at our site are within the range observed at other RG dumps, since neutralization of the effluent is required during the industrial process at this site (Gázquez et al., 2009). Additionally, the OM content presented a normal distribution, varying from 8.1 to 18.1% and averaging $13.1 \pm 2.4\%$. Surely, OM is not formed during the industrial production of TiO_2 ; hence, OM presence must be due to droppings of the local fauna and inputs of dead plants within and surrounding the site.

The total concentrations of the major macroelements, such as Ca, S and Mg (Table 11), were consistent with the data from Fauziah et al. (1996), who described a similar process of extraction and residual percentages of RG. Similarly, micronutrients, such as Fe, Mn, Cu and Zn, were also found in elevated total concentrations when compared to agricultural soil values (Rudnick and Gao, 2003 and Kabata-Pendias, 2011). The concentration of total Mn for instance in agricultural soils is estimated at the range 1500–3000 mg kg^{-1} (Kabata-Pendias, 2011), whereas at the Thann site, concentrations of total Mn reached 11400 mg kg^{-1} at quadrat 47. Similarly, the concentration of Fe in the RG substrate reached 9.5 % (Table 11), whereas the global abundance of Fe is calculated to be around 4.5% (Kabata-Pendias, 2011). Manganese is a member of the iron family of elements and is closely associated with Fe in geochemical processes (Kabata-Pendias, 2011). Thus, the Mn cycle follows the Fe cycle in various geochemical environments, probably also at the Ochsenfeld site. The high concentrations of some elements highlights the potential nutrient richness of the RG substrate. However, the analysis of the CaCl_2 -extractable fraction of the RG substrate revealed the remarkably low availability of some nutrients (Fe, Mn, P, and Zn) with a ratio (CaCl_2 extractable concentrations / total concentrations) $< 0.1\%$ (Table 10 and Table 11). These ratios are estimated at the ranges

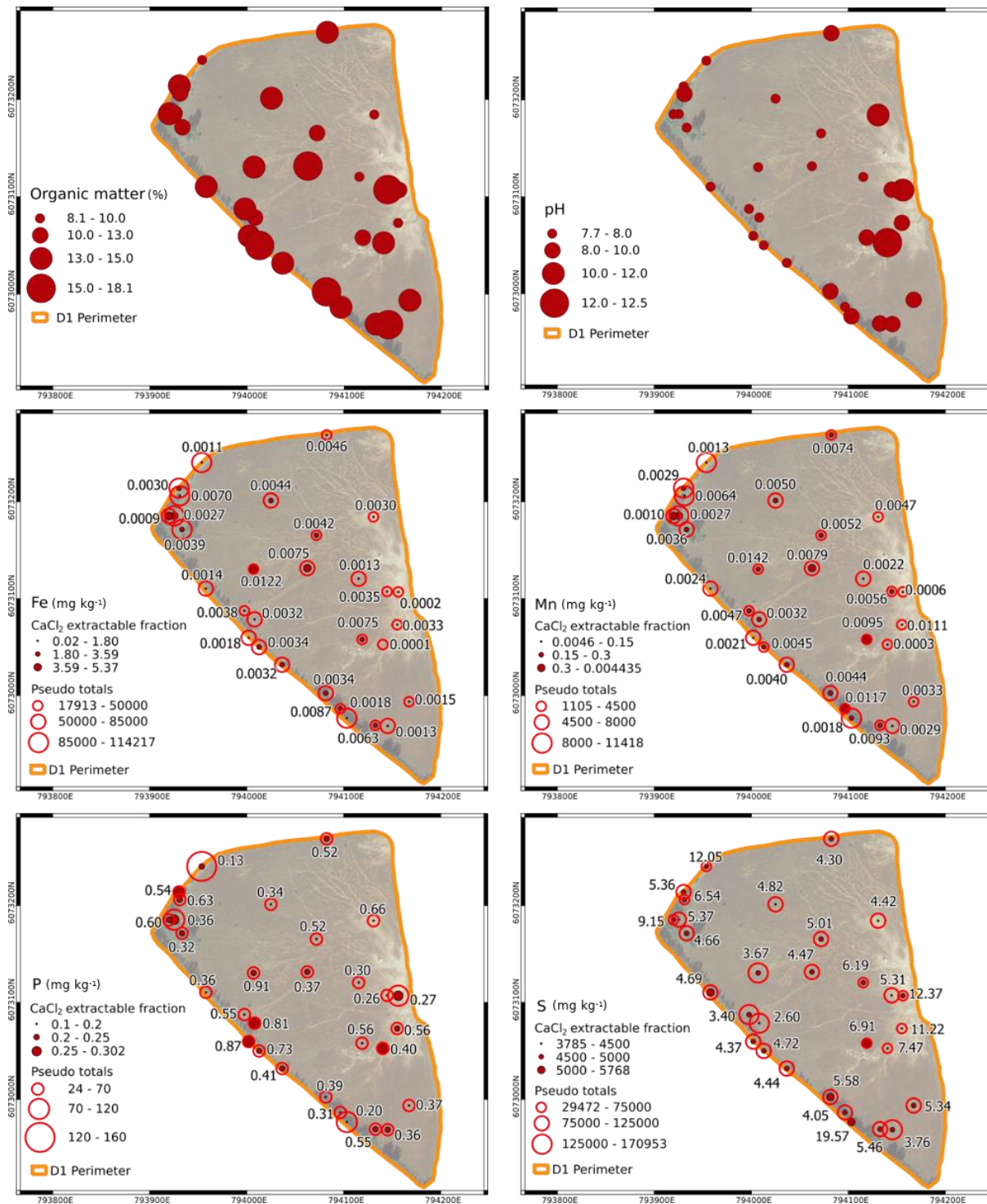


Figure 28. Spatial distribution of OM, pH, Fe, Mn, P and S properties within D1 area in the red gypsum landfill. The ratios of extractable CaCl₂ over the total concentrations are displayed as percentages on the map.

0.0002-0.01 for Fe, 0.00006-0.01 for Mn, 0.13-0.9 for P and 0.001-0.13 for Zn (Figure 28). On the other hand, some elements such as Mg, S (see Figure 28) and K showed ratios > 2%. Most importantly, the total N content of the RG samples was very low, often below the detection limit (Zappellini et al., 2018). These harsh conditions (elevated pH combined with

low N) often suppress the root growth of plants and cause leaf chlorosis and lower biomass production (Wang et al., 2017). The large amount of Fe and Mn, mostly in oxide forms, may also limit the availability of other nutrients in this area due to adsorption occurring during the precipitation of Fe and Mn oxides (Alloway et al., 1995).

We further performed a PCA to explore the distribution of the elements over the D1 area, which highlighted the correlations between the various soil factors (Figure 32). We included the pH, OM and the total concentrations of the macro- and micronutrients. The first two principal components (PC) represented 44.3% of the variation in the dataset, with 26.8% and 17.5% for PC 1 and PC 2, respectively. The variables driving $(-0.6 > x < 0.6)$ principal component 1 were pH, B, S, Mn, Mg and Fe, and Fe, Mn and S drove principal component 2. The biplot showed that the higher the pH, the lower the CaCl_2 extractable concentration of Fe, Mn, B, Cr, Zn, S, Mg and Na. This has been documented several times (Bewley, 2007, Cooling et al., 1970, Takeno, 2005). On the other hand, pH was positively correlated with P, K and Si. The quadrats 39, 53 and 59 seemed to share similarities, especially an elevated pH, as explained before and highlighted in Figure 26. The correlation between Fe and Mn may be linked to their coprecipitation in oxic conditions (Alloway et al., 1995). The PCA at the NW corner showed that the richness of this zone was due to the relatively lower pH than the rest of the site, as seen in Figure 28. This pH change could therefore allow for a higher nutrient availability.

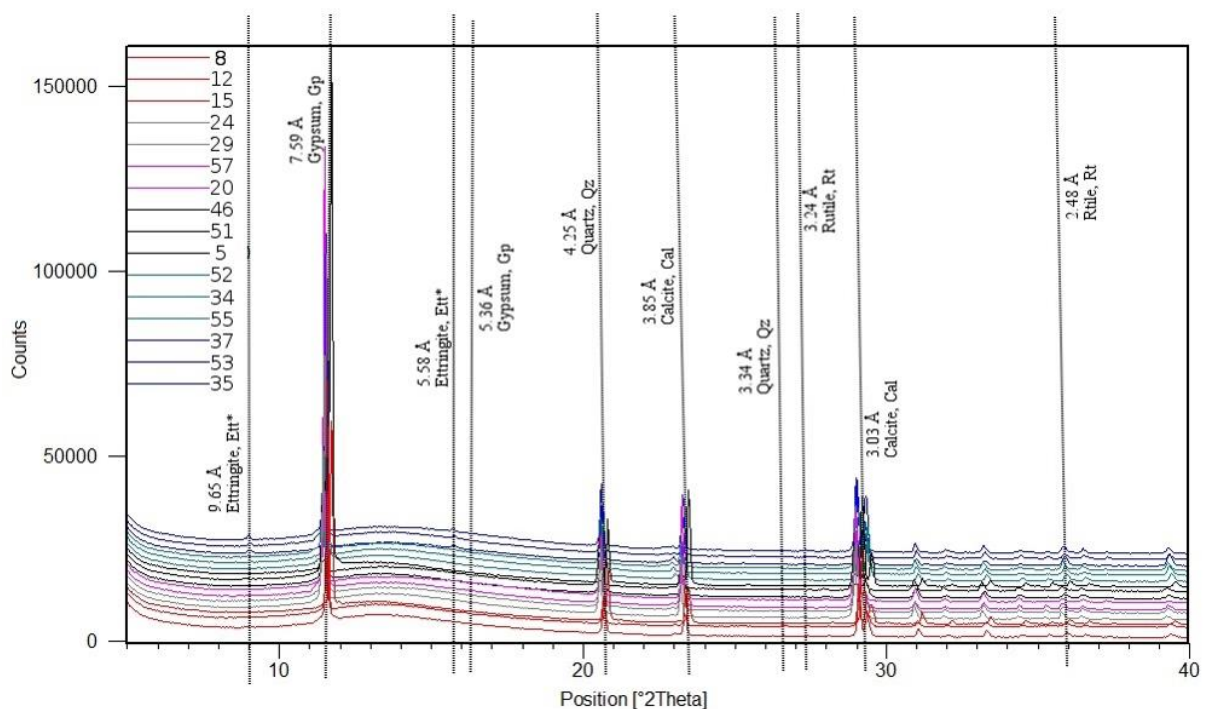


Figure 29. Bulk mineral phase identification of the red gypsum substrate by XRD. Cal, calcite; Gp, gypsum; Rt, rutile; Qz, quartz; Ett, ettringite (Abbreviations according to Whitney and Evans, 2010). *not validated abbreviation.

Further mineral characterization of the RG by XRD analysis was performed on 16 representative out of the 60 collected samples (Figure 29). In the whole dataset, 5 different crystal phases were identified: gypsum (Gp) and calcite (Cal) were found in 100% of the sampled sites (n=16), which is consistent with some Cristal group reports, since they form part of the residues. Rutile (Rt), a titanium dioxide, appeared in 6.2% of the samples (n=1) and is one of the main chemical forms of the products of the Cristal group, and it is thus commonly found in the residues (Gázquez et al., 2009). Finally, quartz (Qz), was found in 31.2% of the sampled sites and represented an expected outcome of the process, as seen by Gázquez et al. (2009). None of the latter minerals was unfamiliar to the TiO₂ extraction process except for ettringite (Ett), which was identified in 3 samples. Ett is a hydrated sulfate mineral formed in clayey soils with a pH between 10 and 14, and it contains Ca and Al (Ouhadi and Yong, 2008). These elements are present in the D1 area. This mineral is frequently reported in construction sites in cements and concretes as a secondary phase. Within the D1 area, Ett was found only in quadrats 39, 53 and 59, which share the same location as the highest pH quadrats. In terms of life support, the presence of Ett may represent an increase in the water retention capacity of the soil (Ouhadi and Yong, 2008). This observation could reflect the modifications that affected the substrate to become a soil. Moreover, fluctuation in the quantities was observed; for instance, quadrat 5 presented a noticeable Gp crystallization compared to the other samples. However, for quadrats 58, 16 and 37, Gp appeared to be barely crystallized. In addition, samples 52, 16, 41, 37, 49 and 54 contained more calcite than the other samples.

4.3 Parameters influencing the native plant abundance

The model obtained by the MFA presented in Figure 5 explained 41.2% of the variance using the first two dimensions. The correlation circle (Figure 30A) allowed us to detail the correlation among the quantitative variables and the first two dimensions. The variables influencing the first dimension were *Verbascum blattaria*, *Echium vulgare*, *Euphorbia cyparissias*, *Cirsium arvense*, *Robinia pseudoacacia* and elements such as Mg, B, S, Mn, Fe, Zn, Na and Cr. On the opposite side we found *Betula pendula*, *Chaenorrhinum minus*, *Taraxacum officinale*, *Salix purpurea*, *Populus tremula* and *Salix caprea*, along with pH, P and Si. The arrangement of variables in this dimension explained 26.8% of the variance. The second dimension explained another 15.4% of the variance. In this axis, organic matter was the variable that contributed the most. This axis showed no correlation with the rest of the variables. A hierarchical clustering of the quadrats was performed using the ordination proposed by the MFA, which suggested three groups (Figure 30B). The first one included quadrats 1, 5, 15, 24, 26, 29, 32 and 35, which were characterized by their organic matter content. The second group (quadrats 8, 9, 11, 12,

17, 18, 19, 20, 22, 45, 46, 47, 51, 52, 55 and 57) were characterized by the presence of Mg, Mn, S, Zn, Cr, Mn, Fe, Na; and the species *Verbascum blattaria*, *Echium vulgare*, *Euphorbia cyparissias*, *Cirsium arvense*, *Picris hieracioides*. Finally, the third group was formed by quadrats 34, 37, 39, 53 and 59 that were characterized by the presence of *Betula pendula*, *Salix purpurea*, *Salix caprea*, *Populus tremula*, *Chaenorrhinum minus*, *Taraxacum officinale*, high pH, P and Si.

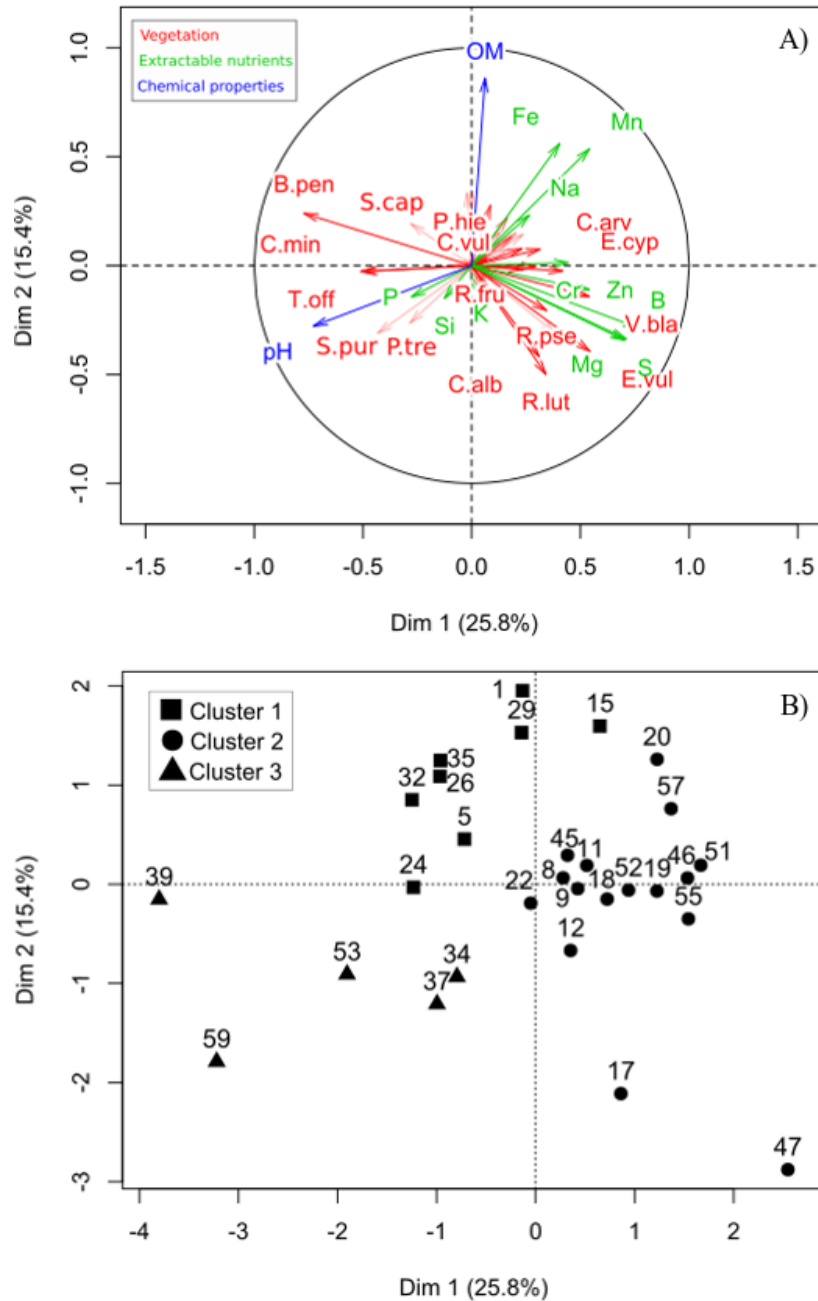


Figure 30. Multiple factor analysis (MFA) of the vegetation dataset at the D1 area of the Cristal plant. (A) Correlation circle displaying the distribution of the quantitative variables of the 3 datasets: chemical properties (pH and OM), vegetation (the Hellinger transformed plant abundances) and extractable nutrients (the soil CaCl₂ extractable fractions) of the 30 samples collected on the red gypsum landfill. (B) MFA-based clustering of the quadrats formed 3 groups correlating to the principal components.

In dimension one we noticed that extractable concentrations of Mg, B, S and Zn were inversely correlated to *Betula pendula* and *Salix caprea* abundances. Given the pedological and physico-chemical constraints of tailings, revegetation success may be clearly improved by using native well-adapted plant species, since sometimes these species can bear the harshest conditions (Alday et al., 2011). The species sharing pioneer traits are fast growing and have low nutrient requirements, a high tolerance to toxic elements and could grow anywhere, leaving the foundations for future generations (Eltrop et al., 1991; Jones, 1998; Hynynen et al., 2009). In Europe, birch, willow, poplar, and alder represent deciduous pioneer tree species, which produce large quantities of seeds, although alder, poplar and *Salix* generally produce much lower seed numbers (< 1 million seeds per tree) than birch (up to 10 million seeds per tree) (see Tiebel et al., 2018 for review). This review of 33 publications indeed revealed that birch is the only pioneer tree species of temperate forests with longer-lived seeds, persisting in the soil for 1-5 years in deeper soil layers. These characteristics are regarded as an advantage for pioneer tree species with regard to their strategy of fast colonization of disturbed areas. This might partly explain the success of birch at the red gypsum landfill. Furthermore, species such as *Salix purpurea*, *Populus tremula*, *Chaenorrhinum minus* and *Taraxacum officinale* followed a similar trend and were correlated to pH, P and Si. A similar structure of the woody chronosequence was found at non-reclaimed post-mining sites in the Czech Republic (Frouz et al., 2008). The preference for alkaline substrates has been observed in other studies of natural revegetation on mine tailings; those species were considered native disturbances adapted (Alday et al., 2011).

Conversely, species such as *Echium vulgare*, *Verbascum blattaria*, *Reseda lutea*, *Chenopodium album*, *Rubus fruticosus* and *Euphorbia cyparissias* seemed to be more likely to establish in quadrats with high extractable concentrations of Mg, B, S, Cr and Zn. The tolerance to high levels of Cr, Cu and Zn in soils has been documented for *Verbascum blattaria* (Shallari et al., 1998) in a relatively short time span, which suggests its potential use for phytostabilization (Morina et al., 2016). Some of these plants are documented to exist in metalliferous substrates (Klemow et al., 2002), sandy loam and bare soil areas (Gross, 1980). Despite their importance for optimal plant development, the restricted availability of P and K, as discussed in section 3.2, does not seem to affect the species found at the site, as observed by Dogan (2001), which may mean tolerance by some plant species of the site for restricted availability of these elements.

5. Conclusions

The present study described some important characteristics of a red gypsum landfill in which natural revegetation has occurred. A spontaneous tree succession has been established, mostly formed by *Betula pendula* in the central area, whereas additional tree species (*Salix caprea*, *Populus nigra*, *Populus tremula*, and *Robinia pseudoacacia*) have colonized the edges. Other herbaceous species include *Echium vulgare*, *Verbascum blattaria*, and *Reseda lutea*. We can conclude that in spite of the great abundance of certain nutrients essential for appropriate plant development, such as Ca, S, Mg, P and K, conditions of the substrate, such as pH, restrict the access of plants growing there to the nutrients they need. The abundance of species was classified into 3 groups based on their apparent nutrient preferences and trace element intolerances. The identified constraints for plants to develop at the D1 area were the poor availability of some nutrients, as well as the lack of nitrogen. Therefore, future phytostabilization approaches at the D1 area will have to include processes to improve the agronomic status of the area. Current laboratory and field trials include the use of organic (digestate) and biological (bacterial- and fungal-based) amendments.

6. Acknowledgments

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7. Supplemental data

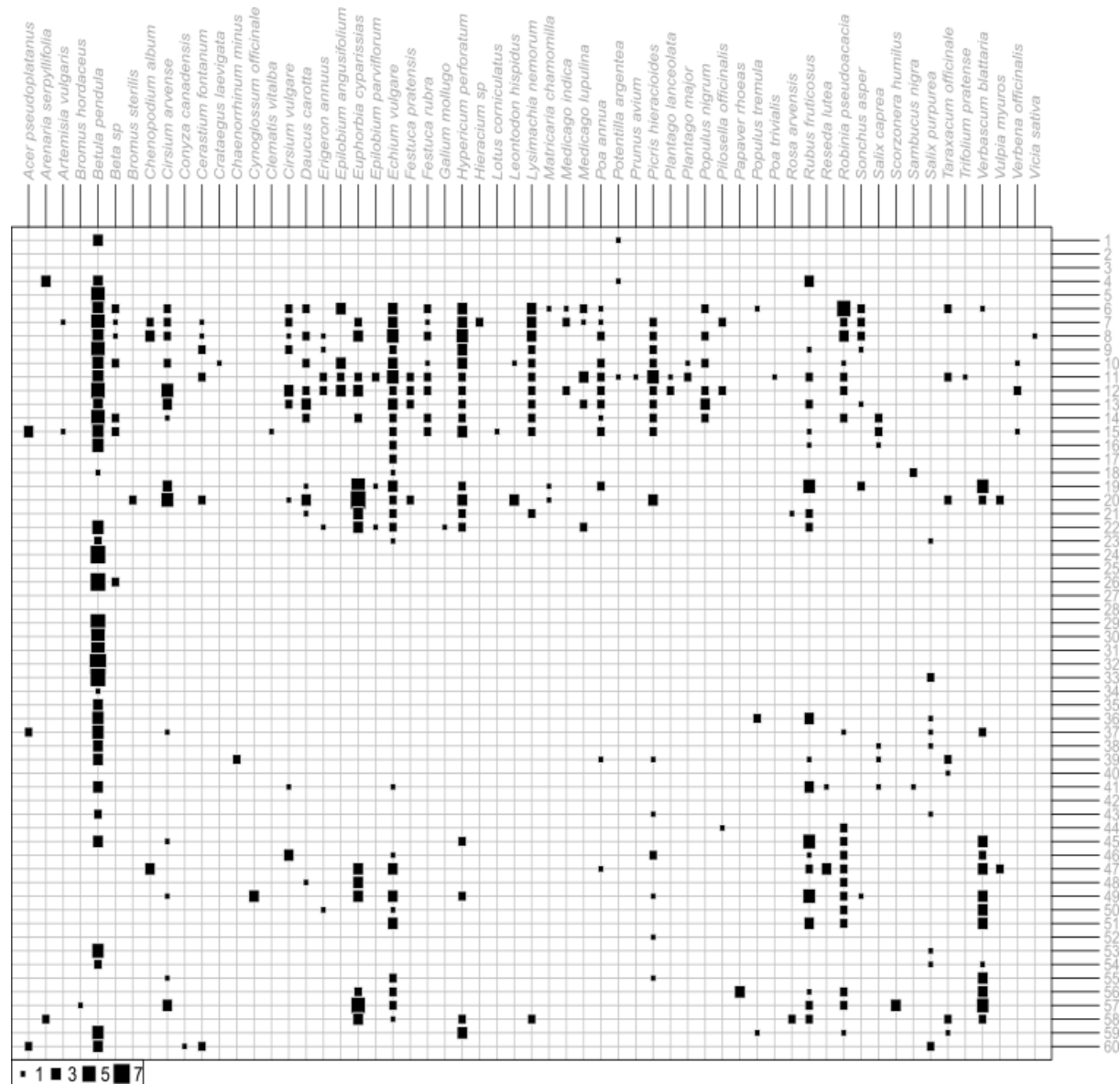


Figure 31. Plant species abundances present at the D1 area of the Ochsenfeld site.

Table 10. Chemical characteristics pH, OM and CaCl₂-extractable element concentrations of the 30 samples collected on the D1 area of the red gypsum landfill.

ID	pH	OM	B	Cr	Cu	Fe	K	Mg	Mn	Na	P	S	Si	Ti	Zn
		%		mg/kg D Wt											
1	7.8	15.2	0.33	4.28	0.006	4.85	7.03	92.6	0.44	6.343	0.22	4520.6	7.69	0.99	0.052
5	7.8	12.9	0.19	4.36	0.006	1.83	5.49	72.6	0.19	6.177	0.18	4728.8	9.77	0.54	0.04
8	7.8	13.1	0.75	3.59	0.019	2.78	18.14	422.7	0.24	11.75	0.3	4651.6	8.49	0.49	0.003
9	7.7	12.3	0.95	7.73	0.006	2.58	13.16	795.7	0.21	10.083	0.21	4931	5.77	0.8	0.003
11	7.8	12.6	0.98	4.95	0.017	3.93	87.4	495	0.3	12.157	0.28	4133.6	6.76	0.99	0.027
12	8.4	12.5	0.83	7.47	0.006	0.99	22.19	784.2	0.1	12.542	0.25	4936.3	6.14	0.23	0.056
15	7.9	14.5	0.93	5.9	0.06	5.37	15.48	356.8	0.41	305.473	0.23	4631.1	7.52	1.46	0.001
17	7.8	8.2	0.95	5.49	0.006	0.94	17.87	310	0.13	19.564	0.21	4823.1	6.94	0.05	0.031
18	7.9	13.7	0.9	7.03	0.006	0.96	10.7	434.5	0.14	11.122	0.15	5007.2	7.05	0.23	0.048
19	7.9	13.5	0.58	6.73	0.02	2	17.01	372.3	0.23	9.912	0.24	4869.1	8.25	0.45	0.046
20	7.9	18.1	0.53	5.41	0.019	1.83	41.49	639.2	0.2	10.964	0.23	4877.8	6.52	0.29	0.068
22	7.9	13.4	0.58	3.81	0.006	0.89	17.2	634.5	0.1	8.719	0.28	4859.6	6.79	0.17	0.054
24	7.7	11.3	0.31	1.78	0.019	1.65	11.54	54.4	0.15	5.766	0.28	4438.2	6.17	0.27	0.001
26	7.9	14.9	0.26	3.53	0.006	1.75	6.38	154.1	0.15	7.938	0.2	4548.3	5.58	0.41	0.003
27	7.9	14.8	1.16	3.01	0.065	3.67	10.8	66.9	0.26	364.74	0.25	4758.3	8.83	1.29	0.037
29	7.8	14.9	1	5.29	0.072	2.74	10.72	93	0.27	308.742	0.13	4333.4	7.72	0.68	0.014
32	8.1	14.5	0.2	1.99	0.006	1.3	6.97	103.1	0.17	8.26	0.23	4561.4	8.62	0.62	0.001
34	7.8	9.5	0.66	5.02	0.006	0.71	8.03	216.1	0.1	5.021	0.19	4528.9	8.12	0.07	0.031
35	8.7	16.6	0.52	4.96	0.006	1.27	12.48	159.7	0.16	8.036	0.14	4480	10.94	0.18	0.041
37	8.6	9.7	0.76	4.31	0.019	0.58	23.89	51.9	0.12	8.65	0.22	4414.8	13.9	0.11	0.065
39	12.5	14.9	0.04	1.06	0.094	0.02	18.14	1.5	0.001	8.55	0.27	3784.6	4.02	0.02	0.008
45	8.2	15.4	0.38	6.11	0.006	0.68	8.76	464.3	0.13	9.367	0.2	4962.1	12.31	0.24	0.069
46	8.1	13.5	1.3	6.66	0.042	2.5	10.32	409.9	0.28	324.526	0.24	4881.2	11.7	0.6	0.049
47	8.0	8.1	1.87	2.62	0.018	1.73	15.62	1685.7	0.21	6.531	0.19	5768.1	7.74	0.05	0.059
51	8.3	15.5	0.65	8.1	0.006	1.87	11.87	930.2	0.22	9.682	0.15	5077.1	4.54	0.42	0.049
52	8.1	11.8	0.49	4.62	0.006	3.16	7.76	510.1	0.35	8.177	0.18	5069.8	7.12	0.67	0.026
53	10.1	9.9	0.19	8.71	0.006	1.02	7.63	36.2	0.13	10.214	0.16	4445.2	4.39	0.41	0.001
55	8.2	13.2	0.93	9.31	0.053	0.6	14.62	305.3	0.14	360.441	0.17	4560.1	8.11	0.17	0.108
57	7.9	14.7	1	2.11	0.019	3.32	13.93	411.4	0.34	8.241	0.14	4579.8	7.6	0.52	0.036
59	11.5	10.3	0.04	2.11	0.176	0.06	38.87	4.2	0.01	14.013	0.28	4545.9	15.16	0.02	0.001

Table 11. Total element concentrations (mg kg⁻¹ DWt) of the 30 samples collected on the D1 area of the red gypsum landfill.

ID	Al	B	Ca	Cd	Co	Cr	Cu	Fe	K	Mg	Mn	Na	Ni	P	Pb	S	Si	Ti	Zn
mg/kg DWt																			
1	3946.5	5.4	217667.9	0.3	24	217.8	49.3	64532.6	137.6	1833	5595.3	121.8	31.7	58.9	23.8	101186.9	250.1	6532.9	103.1
5	3021.3	0.4	218913.9	0.0	21.5	144.5	40.8	44183	210.7	1746.4	3637.4	85.2	19.3	35.1	21.3	94306.3	128.6	8468.9	79.8
8	6703.2	6.2	176130.6	0.0	41.1	423.9	79.5	93425.9	251.4	3829.2	8113.5	121.1	40.6	55.5	41.6	86790.4	86.9	14247.3	166.9
9	6639.9	12.1	172303.1	0.6	47.7	475.9	92	96475.4	242.3	6066.4	7781.3	131.1	58.9	57	54.3	91836.6	226.4	16618.1	171.9
11	7725.3	11.5	163739.8	0.7	49.7	494.1	95	101594.3	382.3	4707	8491.3	123.6	59.6	88.6	56.5	88700.8	231.6	15736.4	197.6
12	8176.1	6.9	147756.3	0.0	49.6	544.4	105.4	114216.6	377.3	6102.6	9528	100	66.6	41.6	66.4	53934.2	69.8	15976.3	201.9
15	4702.6	9.5	155607.6	0.0	29.6	343.7	62.1	76548.8	179.6	2583.8	6336.6	93.7	31.7	35.9	35	70824.7	114.1	10721.1	121.7
17	7638.3	14.2	219647.4	0.8	42.3	156.9	68.5	88198.9	377.1	3914.3	10504.5	280	56.1	160.4	21.1	40021.7	201.7	3816.9	163.9
18	4465.5	6.8	203307.7	0.5	26	323.2	58.1	68022.2	178.4	3387.5	5923.9	126.6	33.4	40.7	32.2	106709.4	234.6	9657.5	102.8
19	4436.5	6.7	209734.1	0.9	25.8	257.3	56.1	62740.1	236.5	3273.8	5837.8	104.8	34.5	57.9	28.3	109582.6	223.9	8481.5	114.1
20	2861	0.4	164661.6	0.0	16	225	37.7	53392.6	111.1	1673.9	4416.3	80.3	18.7	32	21.6	103240.3	100.4	6441.3	68.7
22	3481	6.2	211367.4	0.6	16.8	163.3	41.5	50879.2	143.8	4665.8	4710.8	97.2	25.7	31.9	20.8	111258.4	158.2	3490.4	95.2
24	3217.5	2.5	209157.1	0.0	19.6	192.3	38.3	52387.9	138.4	1405.7	4720.1	126.4	25.9	35	18.6	170953.1	121.2	6590.8	92.4
26	2876	3.4	207251.7	0.7	16.7	183.6	36.2	46054.3	153	1764.5	3282	78.6	22.2	35.8	20.5	133645.2	402.8	5517.1	87.3
27	1835.8	2	201603.9	0.2	11.4	134	24.9	30067.2	101.7	810.9	1837.9	85.1	14.6	27.5	17.2	129498.2	314.1	433.4	57.5
29	3626	0.4	189858.6	0.1	21.9	244	45.7	61678.8	174.4	1322.1	5384.2	91.7	27.6	38.9	25.4	89934.8	138.2	9049	95.3
32	2017.2	6.2	240130	0.3	14.1	87.4	23.7	28322	172.5	1659.8	2275.6	83.1	10.7	44.3	17.7	106116.7	359.6	5406.4	67
34	3640.5	4.9	257908.7	0.2	19.4	127.9	36.2	53108.4	201.2	2343.2	4514.4	85.5	26.2	63.9	16.6	73196.1	91.2	4968.6	95.3
35	2987.4	6.4	210169	0.1	12.9	150.5	27	36452.3	200.8	7017.2	2922.1	67.8	17.2	54	17	84372.8	123.8	5164.2	72.7
37	1862.9	9.2	234603.6	0.1	7.1	64.5	15	17912.6	157.9	4786.2	1104.9	58.4	9.6	39.2	10.2	39357	77.7	2599.4	49.5
39	2034.4	9.1	286266	0.4	7.6	46.8	15	19697.9	125.5	5028.8	1649.4	82.4	11	67.1	8.2	50677.7	266.9	1746.9	59.3
45	3915.1	5.4	203563.6	0.3	20.7	229.4	49.3	53916.7	274	7103.4	4524.2	101.3	25.6	56	29.9	132138.2	347.7	7834.2	93.1
46	2543.9	8.3	175662.1	0.0	15.5	141.2	31.3	39632.4	170.2	7389.5	3042.9	63.3	19.5	42.9	19.4	89442.7	157.5	6043.1	68.6
47	6318.2	25	227494.3	1.1	50.4	64.1	72.3	95940.2	5.2	20478.2	11418.3	107.4	65.3	94.6	15.4	29471.6	242.8	2670.9	195.8
51	3814.9	4.3	211056.2	0.1	19.3	245	40.8	55800.4	264.9	8232.7	4917.2	86.5	23.6	39.3	22.4	90934.1	121.4	7468.1	94.2
52	3422.8	3.8	242667.3	0.2	18.6	142.1	32.3	42022.6	352.8	8682.4	3628.6	75.4	20.8	31.1	16.6	73394.2	138.5	8012.7	91.9
53	2072.8	4.4	177052	0.0	13.2	140.5	24	34595	86.9	3918.1	2737.5	71.6	15.2	24	15.9	100582.4	88.1	5698.9	55.2
55	3544	5	221976.3	0.1	18.3	155.5	31.9	41247	282.2	9952.6	4118.4	68.6	21.9	45.2	18	85409.8	100.8	6271.5	87.1
57	2591.7	3.5	208683.7	0.0	15.5	125.5	31.6	38263.3	185.3	4139	2920.1	74.9	18.6	45.8	20.6	112947	127	6542.2	68.6
59	3624	6.6	273852	0.4	12.4	117.8	22.7	32069.3	234.7	4488.7	2277.9	101.1	17.7	103.8	14.2	36756.5	223.2	4813.7	75.6

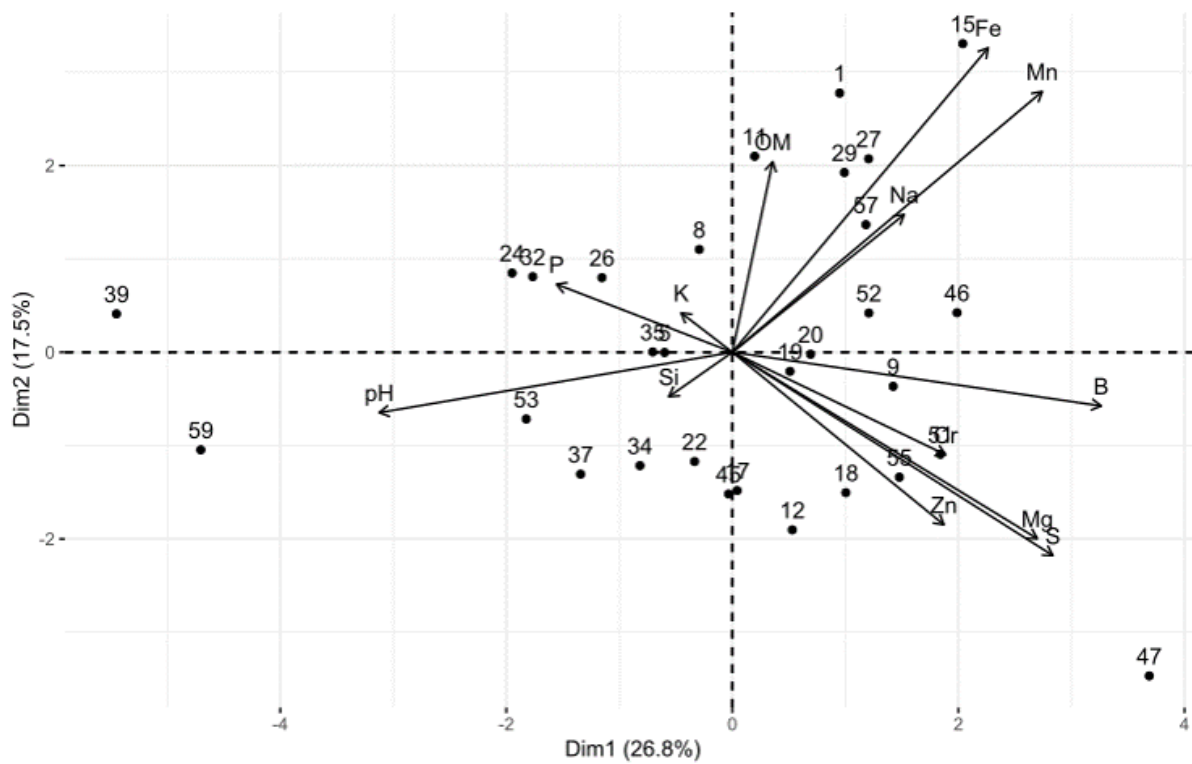


Figure 32. PCA ordination of pH, OM and CaCl₂ extractable concentrations of B, Cr, Cu, Fe, K, Mg, Mn, Na, P, S, Zn in RG over the sampled plots of the D1 area on the red gypsum landfill.

Part II

5.2 Improving silver birch (*Betula pendula*) growth and Mn accumulation in residual red gypsum using organic amendments.

5.2.1 Context and publication

The previous section established the baseline for the subsequent experiments by providing the main characteristics of the site's vegetation and substrate. Consequently, the dominance of the pioneer species *Betula pendula* was determined in the landfill, as well as the abundance of manganese in the substrate. Since we found some tolerance of this species to the presence of PTEs and accumulation of Mn, we therefore intended to use this species for covering the phytostabilization and phytoextraction axes of this PhD dissertation.



This part meets the second objective of this dissertation that was to use organic amendments to decrease the pH. The experiment ran under the assumption that by increasing the availability of essential nutrients present in the RRG, the *Betula pendula* seedlings would have a better development and therefore grow faster. With this, we intended to cover the phytostabilization axis. In parallel, we intended to cover the phytoextraction axis by phytoextracting Mn through the acidification of the RRG.



Improving silver birch (*Betula pendula*) growth and Mn accumulation in residual red gypsum using organic amendments.

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1. Abstract

The increasing production of wastes that are landfilled might contribute to sources of potentially toxic elements; this is the case of residual red gypsum tailings, a by-product of titanium dioxide extraction. Revegetation of such a site is essential, and Mn phytoextraction may render the operations economically profitable. This study aimed to apply phytomanagement techniques for increasing the plant development, tailings revegetation and an optimal Mn phytoextraction using silver birch, the most abundant plant species on this site. To enhance the nutrient availability from the tailings, amendments that reduce the pH, i.e. pine bark chips, *Miscanthus* straw, white peat, and ericaceous compost, were mixed with residual red gypsum and birches were allowed to grow for 3 months. The pine bark chips and ericaceous compost led to a maximum decrease in pH, allowing the accumulation of up to 1400 mg Mn kg⁻¹ dry matter in the leaves of silver birch. However, some nutrient competition was found in the pine bark treatment, which halved biomass production as compared to control. Further amendment addition may be needed to take advantage of the pine bark capabilities as a soil conditioner and Mn solubilizing treatment in residual red gypsum.

Keywords: *Betula pendula*, organic amendments, Potentially toxic elements, Phytomanagement, Red gypsum,

2. Introduction

The global population surpassed 7 billion inhabitants by January 2016, increasing concerns about food security, climate change, public health, resource extraction and waste recycling (Abdulkadyrova et al., 2016). Waste generation and its management is currently of major importance (EEA, 2015). Total waste production includes waste produced in manufacturing, mineral extraction, and households, with some of these main waste sources in Europe reaching up to 2500 million tons (Eurostat, 2016) and becoming landfill waste. The number of potentially contaminated sites in Europe is as high as 3 million (EEA, 2007), and this number could eventually increase with the increase in the population and industry. Industrial landfills may contain mine slags and tailings that generally contain potentially toxic elements (PTEs) that affect organisms differently (Kurt-Karakus, 2012). The urbanization near landfill areas may increase population exposure to PTEs by dust inhalation, ingestion of home-grown vegetables (Assad et al., 2019), and dermal contact with contaminated substrates (Jiang et al., 2019). Additionally, tree development in mining soils with high rhizosphere pH, *i.e.* from the bauxite extraction or coal mining industries, may be affected by a nutrient deficiency, rather than by soil metal(loid)s in excess (Zhang and Zwiazek, 2016).

Management and remediation of industrial residues usually imply engineering technologies, although these are often disregarded due to the unappealing cost-effectiveness of operations and further environmental impact (Ciadamidaro et al., 2019). Alternatively, phytomanagement could be employed, through the combination of chemical, biological or the use of organic amendments, for the reclamation of contaminated sites or extraction of certain PTEs (Robinson et al., 2009). A previous study (Hueso-González et al., 2017) used straw and pine chips for mulching and mixing with the soils to improve water retention and therefore increase plant survival rate in dry environments. These organic residues were selected due to their low additional cost for reforestation and for the services they offered to the ecosystem (e.g., improvement of aggregate stability, aeration and hydraulic conductivity) (Hueso-Gonzalez et al., 2018). Peat mineral mix was also used as a soil cover for tailings and mine overburden to improve the availability of micronutrients for *Pinus contorta* and *Picea glauca* (Manimel Wadu and Chang, 2017). In a field experiment carried out in a gold mining tailings in Southeastern Manitoba, Canada, papermill sludge and woodchips were used to improve substrate aggregation and organic content. The use of these substrates in low quantities (<5.6 t ha⁻¹) improved directly the physico-chemical soil conditions and consequently the biological activity without altering the pH (Young et al., 2015).

Previous studies on the residual red gypsum (RRG) of the Ochsenfeld site, in Eastern France, showed that the harshness and nutrient deficiencies of the substrate somehow restrained plant colonization (Zappellini et al., 2018). The absence of a vegetation cover on such mine tailings may represent a health concern for the villagers and settlements surrounding the site. Managing the Ochsenfeld site by following the Green Chemistry principles would allow this RRG site to be returned to a productive area, since RRG has abundant presence of some metals of commercial interest (Zapata-Carbonell et al., 2019), where the raw material can be exploited for secondary uses. In this regard, the production of commercial glazes for ceramic production (Kamadurin and Zakaria, 2007) and the production of cement as a binder (Hughes et al., 2011; Gázquez et al., 2014) are some of the main uses for RRG. Moreover, given recent chemical studies, RRG might also be used as raw material for the synthesis of other compounds, such as catalysts (Grison et al., 2015; Deng et al., 2016).

Silver birch (*Betula pendula*), which is naturally occurring and dominates the vegetation cover at the RRG Ochsenfeld site (Zapata-Carbonell et al., 2019), showed tolerance and Mn accumulation rates that are close to those found in other *Betula* species (Ciadamidaro et al., 2019). This colonist would be a suitable species for the phytomanagement of Mn-contaminated sites. As it is well known, the chemical speciation and solubility of nutrients and PTEs is mainly driven by the pH, and thus, we hypothesized that a reduction in pH of the RRG, currently at 7.8, would render the nutrients more readily available, which will improve the plant development and hence revegetation. This work aims to demonstrate the hypothesis that amending the RRG with commercial organic amendments known for lowering soil pH, i.e. ericaceous compost, white peat, pine bark chips, and *Miscanthus* straw, will: (1) increase growth of the silver birch by allowing the revegetation of this particular contaminated site and (2) enhance the amount of Mn taken up by the tree roots and, hence, its accumulation in harvestable plant parts for possible valorization.

3. Materials and methods

3.1 Nature of substrate and origins

The substrate used was RRG, the product of the neutralization of the sulfuric TiO₂ extraction effluent, and the landfill is located at the Ochsenfeld site (47.79686 N, 7.132775 E), near Thann in Eastern France. The RRG has been *in-situ* for over a decade and contains abundant concentrations of Ca, S, Fe, Mn and Mg (approximately 200000, 110000, 650000, 5000 and 3500 mg kg⁻¹ DW, respectively) (Assad et al., 2017, Zapata-Carbonell et al., 2019). We also found other essential elements such as Co, Cu, K, Na, P, S, Si and Zn, or other PTEs such as

Cr, Cd, or Ni. However, for most of the PTEs, we did not register higher accumulation of these elements in the edible parts of crops or in poplar leaves grown on red gypsum compared with the control soil in a previous experiment (Assad et al., 2017), except for Cr for which we indeed found higher amounts in poplar leaves.

3.2 Tree production

Betula pendula seeds were collected from the Thann site in 2017 and used in all subsequent experiments. Seeds were sown on peat (Brill Typical, Germany), and seedlings were grown for 3 months in a culture chamber with a 16 h, 23°C and 8 h, 21°C day/night regime, with an average moisture of 70% and a daylight intensity of approximately 600 $\mu\text{mol photons photosynthetically actives m}^{-2} \text{ s}^{-1}$.

3.3 Experimental setup

The RRG was collected at the Ochsenfeld site on a fully characterized area (Zapata-Carbonell et al., 2019) in February 2017. The amendments selected for lowering the pH were ericaceous compost (Ec) (Sorexto, France), white peat (Wp) (Hawita Baltic, Latvia), pine bark chips (Pc) (Eden Garden, France) and *Miscanthus* straw (Ms). Mixtures of substrates 1:1 (v/v) were homogenized and set up in 0.5 L pots ($n=12$) using RRG, some 320 g DW of substrate, and each of the above amendments were applied in their crushed form. We selected the amendment doses, following recommendations found in previous studies where incorporation of feedstocks in similar proportions into a mine soil (Tandy et al. 2009) or a contaminated sediment (Mattei et al., 2017) were found to be efficient in promoting plant growth. The four treatments were as follows (1) ericaceous compost + red gypsum (Ec), (2) white peat + red gypsum (Wp), (3) pine bark + red gypsum (Pc), and (4) *Miscanthus* straw + red gypsum (Ms). Untreated RRG was used as a control treatment (Unt). Pots were placed on one plate per treatment to avoid leakage exchange among the treatments. One seedling of silver birch was planted per pot immediately after mixing the amendments in the pots and kept in the culture chamber mentioned above. Pots were watered from the bottom every four days in order to maintain a 23% of humidity as found *in-situ*. The leaves and roots were harvested after 3 months, washed and rinsed with distilled water to avoid contamination, and then oven-dried at 70°C and weighed.

3.4 Monitoring and sampling

The initial and final element concentrations (Al, Ca, Cr, Fe, K, Mn, Na, P and S) were determined in substrates and leaf samples. Substrate samples were taken monthly in the shape of thin cores (5-10 mm in diameter) along the edges of the pots oven-dried at 40°C, ground and sieved through a 2 mm sieve to analyze and monitor changes in the pH and CaCl_2 extractable

fractions of the elements cited above. Similarly, the physiological response was assessed by measuring the relative chlorophyll content index (CCI) through a CCM-200 plus chlorophyll meter (Opti-Sciences, Hudson, USA) using the third most developed leaf each time this parameter was measured in every plant for consistency.

3.5 Elemental analysis

The substrate pH was determined using a Hach HQ40d pH meter (Colorado, USA) after 1:2.5 (w/v) parts of substrate in 1 M KCl had been shaken for 1 h (Hesse, 1971). Analysis of element concentrations in substrate extracts was performed using induced coupled plasma atomic emission spectrometry (ICP-AES, Thermo Fisher Scientific, Inc., Pittsburg, USA). To determine the CaCl₂ extractable fraction, 2.5 g of substrate were mixed with 25 mL of 0.01 M CaCl₂ solution and incubated at room temperature for 3 h under agitation (140 rpm). This solution was finally filter through a Whatman 2 filter paper (Houba et al., 2000).

Initial total concentrations in the substrates and the ionome in the birch leaves were determined in wet-digested samples using either 0.5 g of substrate + 2 mL HNO₃ + 5 mL HCl or 0.125 g of leaf DM + 1.75 mL HNO₃ + 0.5 mL H₂O₂, respectively, in a digestion block (DigiPREP, SCP Sciences, Courtaboeuf, France) prior to the ICP-AES analysis (Assad et al., 2017). The calculated recovery percentages of the studied elements ranged from 89 to 156% for the substrates, and 60 to 101% for the leaves. The reference materials used were loamy clay soil (CRM052, LGC Promochem, Molsheim, France) and oriental basma tobacco leaves (INCT-OBTL-5, LGC Promochem, Molsheim, France) for the substrate and leaves, respectively.

For a better understanding of the soil-plant interaction and nutrient exchanges, bioconcentration factor (BCF), which integrates both soil and plant concentrations was computed for each element (Chopin and Alloway, 2007; Kabata-Pendias and Pendias, 2001). The bioconcentration factors were calculated with the foliar element concentration over the substrate element concentration, as explained by Takarina and Pin (2017). The phytoextracted Mn was calculated taking into account the Mn concentration accumulated in leaves (mg kg⁻¹ DM) times the biomass produced (kg DM).

3.6 Statistical analysis

Data obtained was processed with *R* (Ver. 3.4.2) packages *FactoMineR* (Lê et al., 2008), *factoextra* (Kassambra and Mundt, 2017), and *agricolae* (Mendiburu, 2014), and the graphs were made with *ggplot2* (Wickam, 2017). All tests were considered significant when $p < 0.05$. Analysis included two-way analysis of variance (ANOVA), with the Tukey-Kramer test for

differences in the means used for group classification. For the CaCl_2 extractable concentrations of Mn and Cr, data were log-transformed relative to the ANOVA postulates. Principal component analysis (PCA) were performed using the *FactoMineR* package to explain the distribution of the elements determined in the leaves and in the substrates among the treatments. Ellipses were drawn at the 95% confidence interval of the barycenter of each treatment. The results reported in the tables are presented in means \pm standard deviation (SD).

4. Results

4.1 Amendment influence on the substrate properties

Compared to the untreated substrate, the amended substrates displayed changes in physico-chemical parameters. First, changes in pH were found at the initial time (T_0), with a decrease of up to one unit for the Pc soil. Few to no significant differences were determined for Wp, Ms and Ec soils. The differences remained similar during the experiment, with the highest pH observed for the Unt soil, followed by the Wp, Ec and Ms soils, and the lowest pH being in the Pc soil. Throughout the experiment, the amendments tended to initially decrease the pH which was increased over time, including the Unt soil (Figure 33).

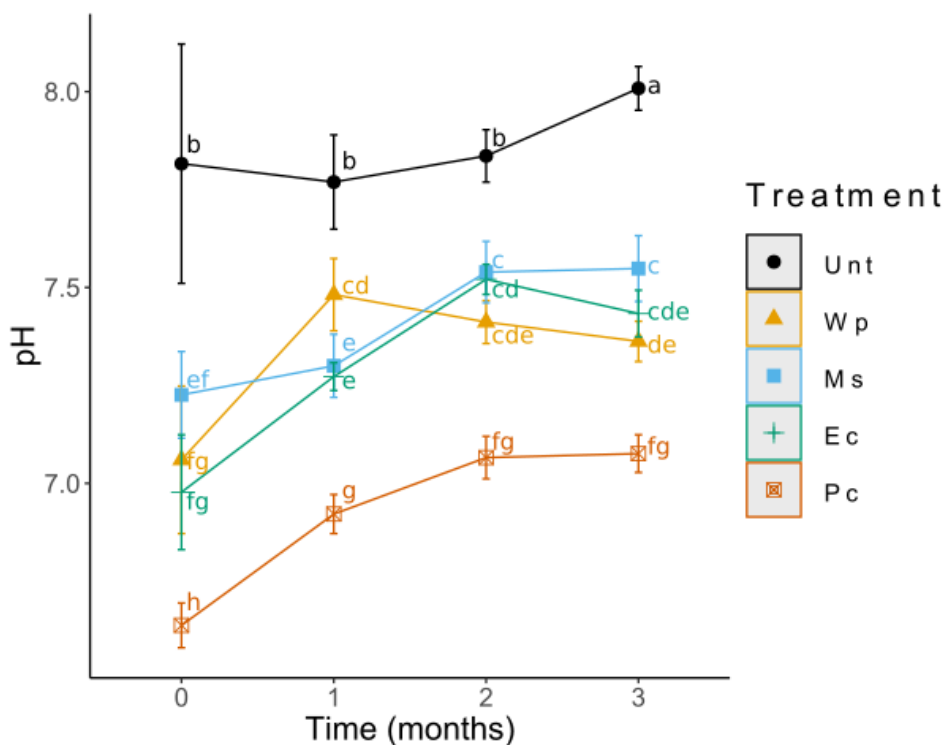


Figure 33. Changes in soil pH for each treatment during a 3-month growth period. Values shown (means \pm SD) represent the results of 12 replicates.

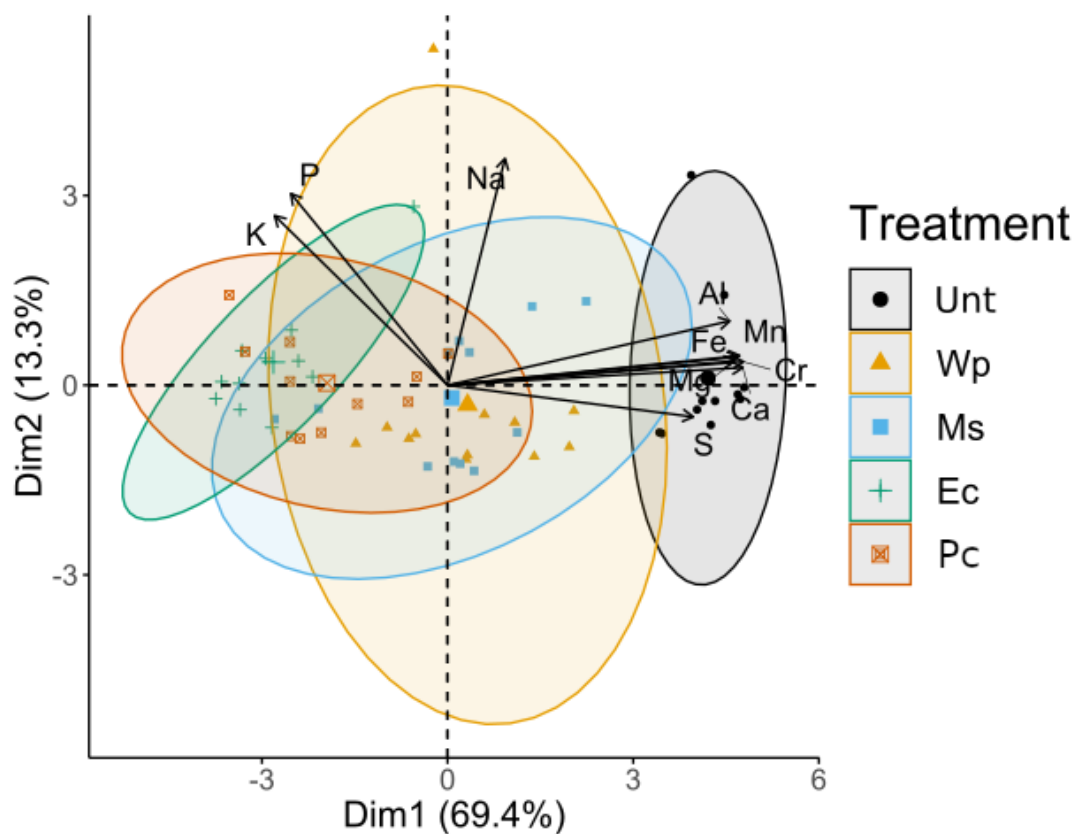


Figure 34. Principal component analysis (PCA) of total element concentrations in the soils. The largest symbols in the graphic represent the barycenter of each treatment. Ellipses represent the confidence intervals at 95%.

A PCA was performed using only the soil total concentrations of elements (Figure 34). This analysis shows the distribution of the elements among the treatments. The first component explained 69.4% of the variance, as most of the arrows, representing the elements determined by ICP-AES, were oriented in this axis towards the Unt soil (Al, Ca, Cr, Fe, Mg, Mn, and S) and on the opposite side towards Ec and Pc soils (P and K). On the other hand, the second component explained 13.3% of the variance, the variable that had more influence on this component was Na. Further changes noticed in the amended soils included changes in the solubility (CaCl_2 extractable fraction) of some elements (Table 12), notably Mn and Cr. The incorporation of ericaceous compost, white peat and pine bark chips decreased significantly the CaCl_2 extractable Cr concentration and likely root exposure from T_0 as compared to the Unt soil (Figure 35). The use of *Miscanthus* straw induced a more progressive change in Cr extractability; however, equally different from the Unt soil. In contrast, Mn solubility significantly increased in the Pc, Ec, and Wp soils. Additionally, the highest Mn solubility occurred in the Pc soil at T_0 , reaching up to 16 mg Mn kg^{-1} soil DW (Figure 36).

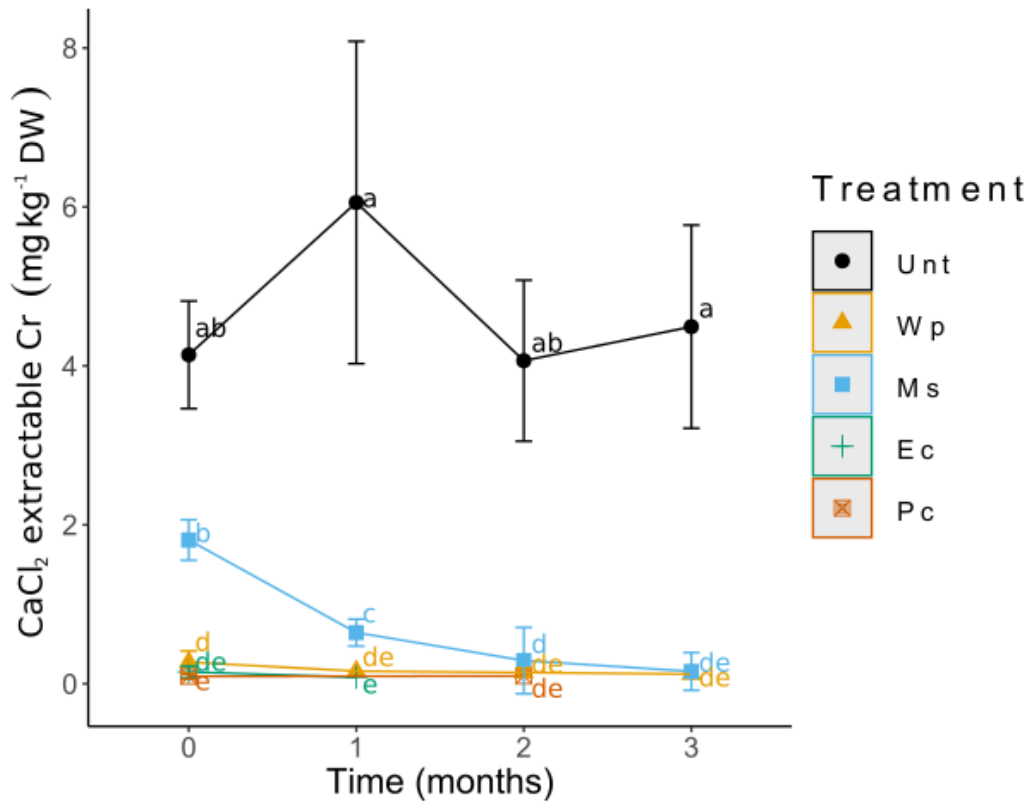


Figure 35. Changes in the CaCl₂ extractable soil fractions of Cr. Values shown represent (means ± SD) the results of 12 replicates.

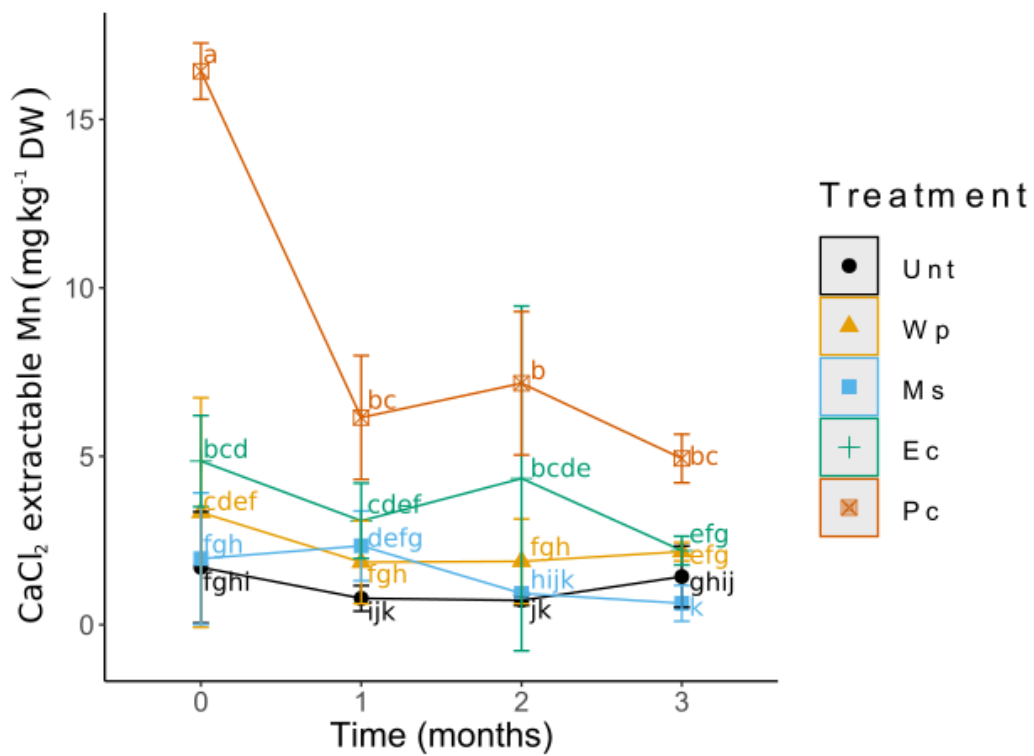


Figure 36. Changes in the CaCl₂ extractable soil fractions of Mn. Values shown represent (means ± SD) the results of 12 replicates.

4.2 Birch growth

The plant answers somewhat differed across the treatments. At harvest, the mortality rate for plants growing in the Ec and Ms treatments was one specimen in each treatment (8.3%), whereas no plants died in the Unt and Wp treatments. Finally, for Pc, the mortality rate was 25% (3 specimens).

The amendment effects on the biomass production at harvest are shown in Figure 37. Unexpectedly, the root biomass production was similar for the Unt, Wp and Ec treatments, whereas the Pc plants significantly displayed a lower root biomass than the Unt plants ($p < 0.01$). The root biomass of the Ms plants did not differ from the other ones. As for the roots, leaf biomass production was similar for the Unt, Wp, and Ec plants. In contrast, the leaf biomass of the Pc and Ms plants were lower than that of the Unt plants.

The chlorophyll content index showed no significant differences across the treatments after one month; however, after 3 month, the Unt, Wp and Ms birches showed similar chlorophyll values, whereas the Pc and Ec plants had significant lower chlorophyll values (Figure 38).

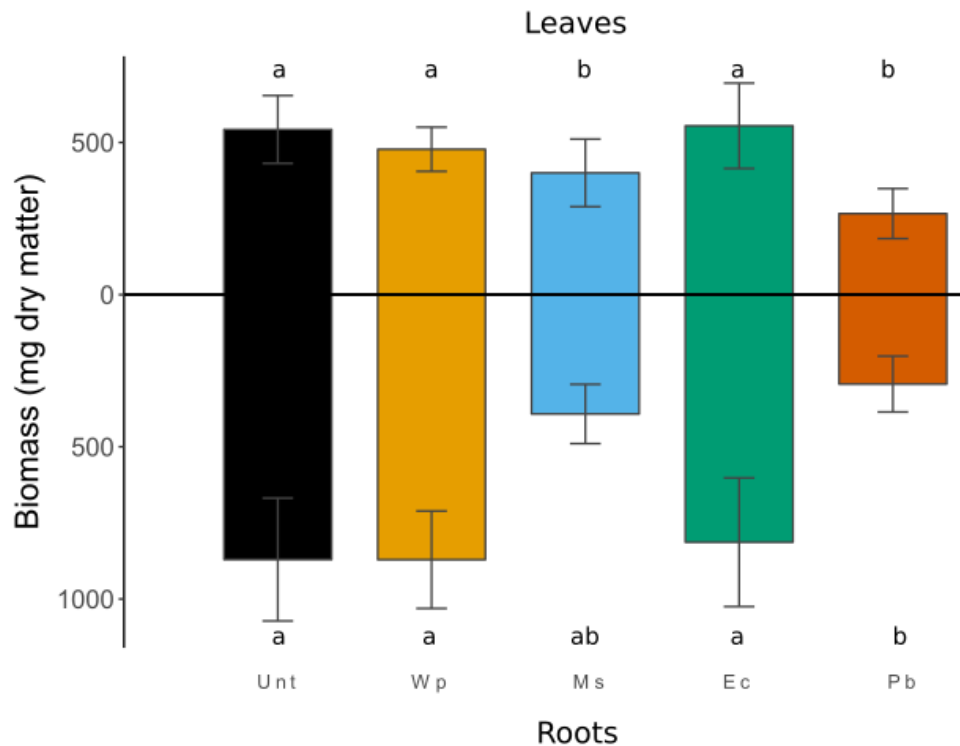


Figure 37. Root and leaf DM yields of birch plants (mg plant^{-1}) harvested after a 3-month growth period. Values shown represent (means \pm SD) the results of 12 replicates. Identical letters indicate that no significant differences ($p < 0.05$) occurred in the birch biomass across the various treatments.

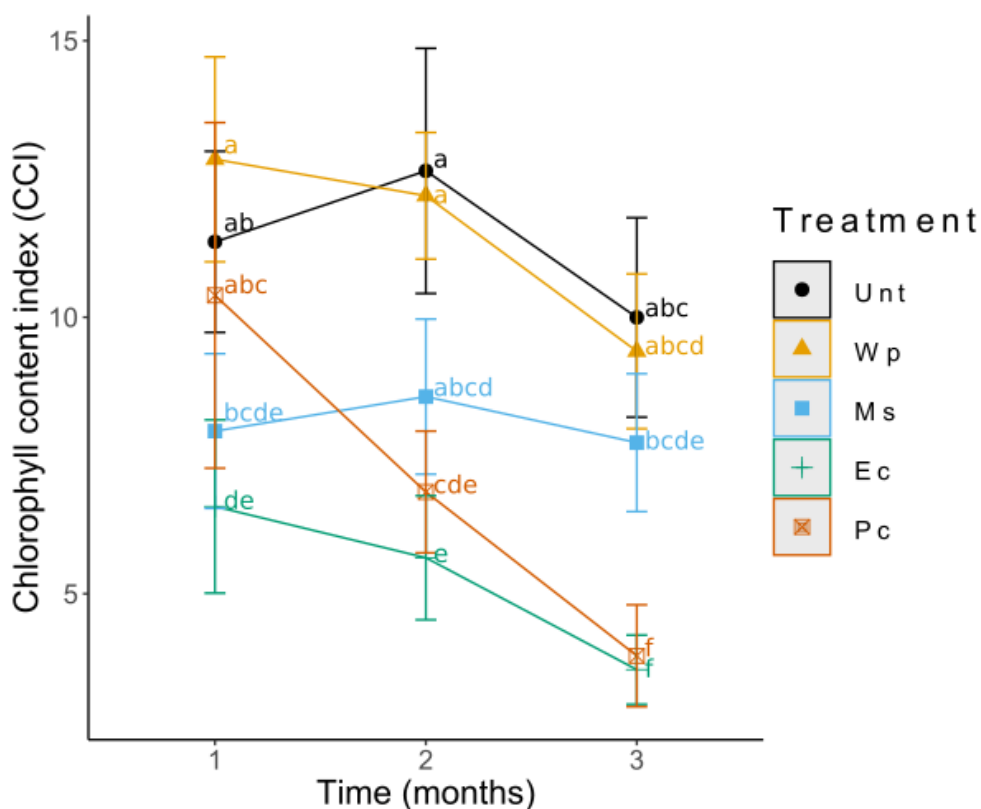


Figure 38. Changes in the chlorophyll index of birch leaves during the 3-month growth period. Analyses were performed using the 3rd most developed leaf of each treatment and measured by a CCM-200 plus chlorophyll meter. Values shown represent (means \pm SD) the results of 12 replicates.

4.3 Foliar ionome of birch plants

The elements determined in the leaves and the substrates are summarized in

Table 13. The PCA based on the foliar ionomes and soil concentrations explained 43% of the variance and 12% in its first and second component, respectively (Figure 39). The barycenter of untreated RRG, Ec and Pc were aligned with the first component, where total concentrations of S, Mg, Cr, Ca, Al, Mn and Fe in soils, Cr and Mg in leaves were more abundant in the Unt direction and Mn, P in leaves and total concentrations of K and P in soil were most abundant in Ec and Pc. The second component was mostly influenced by Fe, Al, Na in leaves and on the opposite end K in leaves. Again, some leaf samples of Pc and Ms were characterized by high concentrations of the former group of elements, whereas some samples of Wp were characterized by the latter group.

Some elements in foliar ionomes were influenced by the treatments. The foliar Cr concentration of plants from amended soils was 10-fold lower than that of the Unt plants. Foliar Mn concentration for the Wp and Ec plants exceeded that the Unt plants by 2-fold, whereas in

the Pc leaves, the Mn concentration was 3-fold that of the Unt leaves. Foliar Mg concentrations for the Wp, Ms, Ec and Pc plants were 0.3, 0.5, 0.5 and 0.6 times lower than that for the Unt plants, respectively. For foliar Na concentrations, Unt and Wp values were similar, Ms and Ec values were 0.5 times lower than the Unt ones whereas the Pc value increased 6 times as compared to the Unt one. Foliar P concentration for the Ec plants was 2-fold higher than for the Unt ones.

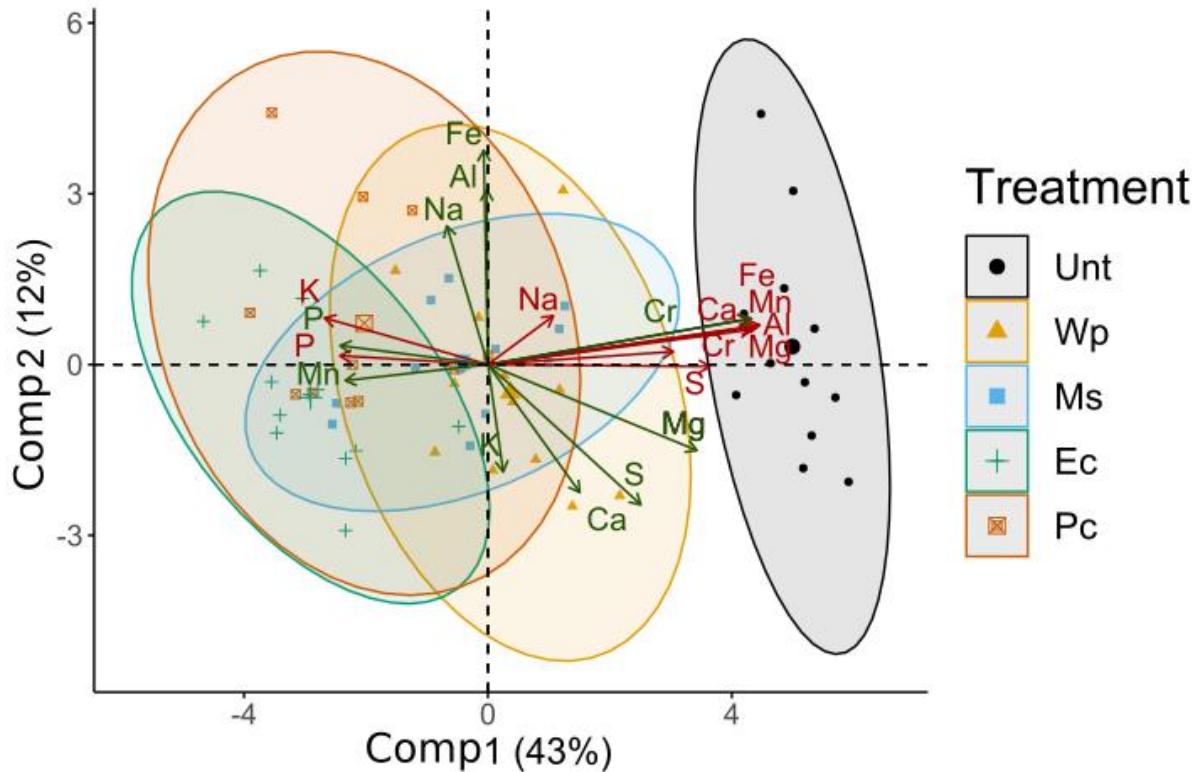


Figure 39. Principal component analysis (PCA) of the concentrations of elements determined in leaves (green) and the total concentrations of elements determined in the substrates at T0 (red). The largest symbols in the graphic represent the barycenter of each treatment. Ellipses represent the confidence intervals at 95%.

The foliar Mn removal per plant (foliar Mn concentration x leaf biomass), and the analysis of variance indicated that Unt and Ms plants had similar foliar Mn removal, and the lowest values across the treatments. The highest foliar Mn removal was obtained in Ec. An intermediate group was formed by Pc and Wp (Figure 40).

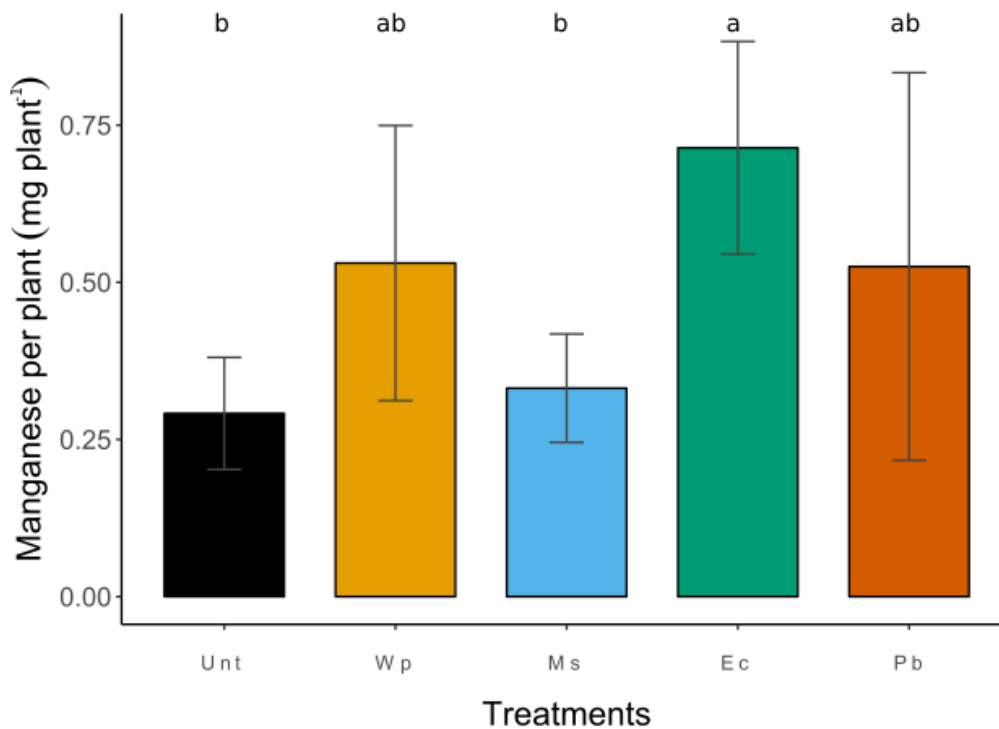


Figure 40. Mn recovered by birch biomass by soil treatments. Values shown represent (means \pm SD) the results of 12 replicates. Identical letters indicate no significant differences ($p < 0.05$) across the treatments.

5. Discussion

The pH decrease occurring into the soils was expected, as all amendments were commercially known for their pH-decreasing properties, and on their own, the used amendments have a much lower pH themselves (Jackson et al., 2009). The progressive increase in soil pH found over the duration of the experiment was probably due to the presence of other salts in the RRG and the quality of the water used for irrigation, as reported by Al-Busaidi and Cookson (2003) in their alkaline substrates given the presence of sodium carbonates. In trials aiming at improving geranium and marigold growth on horticultural substrates, pine bark chips alone allowed to reach a pH of 4.5 (Jackson et al., 2009). Mixing peat with field soil in a strawberry-peat bioassay allowed for reaching a soil pH within the 4.5 - 4.8 range (Tender et al., 2016). The pH values measured throughout the experiment would be acceptable for the plants in terms of tolerance since the pH for all treatments stabilized between 6.5 and 7.5.

Some changes occurred for the element solubility in the amended soils. The Mn solubility, for instance, may be affected by both pH and redox potential at a 6.5-7.5 pH, whereas at values of 5 or lower, the speciation will be mainly driven by pH (Gotoh and Patrick, 1972).

For Cr, all the amendments tested reduced significantly its solubility, a theoretically normal effect of acid pH (Takeno, 2005). This might be due to the binding properties of the dissolved organic matter and the presence of Fe contained in the added amendments, rendering it undetectable (Fendorf, 1995).

In the case of Mn, oxidation states from II to VII are all possible, some bound to carbonates, silica and other forms of oxides and oxyhydroxides, although the soluble Mn^{2+} is the preferred form for plants (Alloway, 1995). In general, Mn speciation is regulated by the redox potential and pH, making Mn available to plants (II) under reducing conditions and acid pH or unavailable (>II) under oxidizing conditions and rather alkaline pH (Kabata-Pendias and Pendias, 2011). At pH values of 5.5 and higher, available Mn begins to decrease in the soil solution (Van Winkle and Pullman, 2003). Furthermore, Mn may be absorbed either from the soil solution directly by the roots or can be solubilized after reduction in the rhizosphere (Reuter et al., 1988).

The determination of chlorophyll content index is a commonly used parameter to assess, among essential physiological parameters, nitrogen uptake by plants (Lebourgeois et al., 2012). Chlorophyll measurements in seedlings from the Pc and Ms treatments showed a drop from the start. Since nitrogen is a macronutrient essential for plant growth, a lack of this element, reflected by the chlorophyll content index, may be related in the low birch biomass produced compared to the other treatments. As reference, in uncontrolled conditions in the field, 4-month-old birch plants produced up to 17 g DM of leaves while using mineral fertilization and planted in a mixture of peat and sand (Kasurinen et al., 2012). At a similar age, the trees in our experiment growing in RRG barely reached 600 mg DM of leaves, demonstrating the presence of harsh conditions that the plants must endure. We have indeed evaluated that certain 5-year-old birches on the site measured less than 1 m in height. This also indicates the need for soil management to render nutrients more available to plants. The poor growth of plants in the Pc treatment could be related to the absence of N provided in the RRG (Zappellini et al., 2018), to the competition with microbes or by the possible but untested presence of allelopathic compounds that often characterize *Pinus* species (Prévosto et al., 2008).

Moreover, the excess or lack of some elements in the leaves may involve health issues for plants. Mn is a micronutrient essential for various reactions in the cells (Millaleo et al., 2010); nevertheless, problems in plant development may occur with deficiency (10-20 mg kg⁻¹ DM, Marschner, 2012) or excess (200-5300 mg kg⁻¹ DM, Ducic and Polle, 2005). The tolerance to Mn in plants shall depend on adaptations and genotypes. Kitao et al. (2001) determined that, even when exposed to highly available concentrations, *Betula platyphylla* var.

japonica reached up to 6300 mg Mn kg⁻¹ DM in its leaves without showing signs of toxicity, thus our study only showing 1400 mg Mn kg⁻¹ DM suggests that Mn toxicity levels were not reached. This may be further proved by measuring oxidative stress indicators such as some key reactive oxygen species.

In the same trend, the difference in foliar Mg concentration might indeed be a cause of the low leaf biomass production found in Pc, since Mg is a macronutrient present in the chlorophyll molecule and therefore related to plant growth (Marschner, 2012). In addition, some competition between the Mg, Mn and Ca might exist given the involvement of these elements in some reactions because of their similar atomic radii; this could explain why a drop was reported in the birch leaf Mg when no differences were observed in the CaCl₂ extractable Mg among the treatments (Table 12). Chen et al. (2010) noted that the Mg deficiency in *Betula alnoides* seedlings produced an impaired root to shoot ratio, along with reductions in the stem width, height and leaf area. The decrease in Mg leaf concentration directly affects the chlorophyll concentration (Farhat et al., 2016), which is seen in the chlorophyll content index drop in Figure 38. Additionally, due to this Mn-Mg-Ca relation, a decline in leaf Ca was also observed in birch from the Ec and Pc treatments (Figure 40) (White and Broadley, 2003). A similar behavior was seen in *Beta vulgaris* (Hermans et al., 2005). Ericsson and Kahr (1995) also reported low values of Mg in *B. pendula* Roth., between 1400 and 1800 mg kg⁻¹ DM, although their experiment used younger seedlings than the ones used in our study.

Chromium is a non-essential element for plants that can be detrimental for growth (Shanker et al., 2005). Concentrations in birches (Figure 40) were within the 0.006-18 mg kg⁻¹ DM range for common foliar concentrations (Shanker et al., 2005). However, all the organic treatments induced a strong decrease of Cr in birch leaves, as compared to the untreated RRG (Figure 34). Our findings indicate that treating Cr-contaminated soils with organic amendments might be beneficial for both plant and human health (Kumpiene et al., 2008).

The foliar Na concentration of Pc reached up to 6-fold the control concentration (some 930 mg kg⁻¹ DM) nonetheless Modrzewska et al. (2016) reported foliar Na concentrations up to 264 mg kg⁻¹ DM for *B. pendula* growing alongside roads with heavy traffic exposed to salt for winter. Leaves of Pc plants accumulated the highest amount of Mn, P and Na, whereas they accumulated lower amounts of Cr, Mg, and Ca, confirming what was stated above. Differences on foliar Mn removal may be attributed to the variation in the nutrients supplied by the amendments; for instance, the ericaceous compost is undeniably more highly charged than Ms and even Pc with macronutrients (P, K, Mg, and Ca) and organic matter (Chevoleau et al., 2005 from Barbier, 2010). Even when the pH affected virtually only the solubility of Mn and Cr, the

balance of Ca and Mg was affected, also inducing deficiencies, which are one of the most common problems in plant development (Zhang and Zwiazek, 2016). Despite the high Mn concentration in leaves induced by Pc, the foliar Mn removal was still lower than that of Ec because of the difference in biomass production. Finally, in the control treatment the BFC of Mn had a notably low factor (BCF = 0.08); however, when amendments were present values increased notoriously: 0.15 for Ms, 0.22 for Wp, 0.31 for Ec and 0.36 for Pc (Table 13 and Figure 40). The BCF analysis indicated that even the use of a weak pH-modifying amendment such as *Miscanthus* straw increased the Mn bioconcentration factor by 2-fold the BCF of RRG. Using ericaceous compost and pine bark amendments showed similar BCF values for Mn with around 4-fold the values of RRG; however, the ericaceous compost allowed the highest Mn accumulation per plant, and this result should be considered when amendments are chosen.

6. Conclusions

The residual red gypsum tailings of the Ochsenfeld site is a potential micronutrient sink, i.e. Mn, Mg, and Ca. Such nutrients would be useful for the site revegetation but phytomanagement practices ought to be applied to increase the plant development and nutrient extraction. Additionally, the revalorization of such substrate as a phytoextracted raw material (*i.e.*, for the production of ecocatalysts) would also be possible, providing that a cost benefit analysis is achieved. Out of the four amendments the ericaceous compost allowed a decent plant development and biomass production, plus changes in substrate pH leading to a higher leaf Mn removal per plant. The use of pH-decreasing amendments resulted in Mn solubilization, notably when amending RRG with ericaceous compost and pine bark chips. In contrast, the addition of amendments reduced the Cr solubility preventing phytotoxicity issues in all treatments. Finally, the revegetation of the Ochsenfeld site is possible but the substrate may require a long-term source of macronutrients, e.g. nitrogen, for ensuring a relevant development of *B. pendula*.

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8. Author contributions statements

LC, FTF, JP and MC contributed to the conception and design of the experiment; JZC, LC, JP and FTF contributed to the acquisition of the data; JZC and JP contributed to the data analysis; JZC wrote the first draft of the manuscript; LC, JP, MC and FTF provided critical revision and suggestions to the first draft. All authors contributed to manuscript revision, read and approved the submitted version.

9. Supplemental data

Table 12. Abundance of CaCl₂ extractable elements in substrates under the five treatments. Values shown represent (means ± SD) the results of 12 replicates. Unt: Untreated residual red gypsum; Wp: white peat + residual red gypsum; Ms: Miscanthus straw + residual red gypsum; Ec: ericaceous compost + residual red gypsum + Pc: pine bark + residual red gypsum.

Treatment	Time (months)	Al (mg kg ⁻¹ dry soil)	Ca (mg kg ⁻¹ dry soil)	Cr (mg kg ⁻¹ dry soil)	Cu (mg kg ⁻¹ dry soil)	Fe (mg kg ⁻¹ dry soil)	K (mg kg ⁻¹ dry soil)	Mg (mg kg ⁻¹ dry soil)	Mn (mg kg ⁻¹ dry soil)	Na (mg kg ⁻¹ dry soil)	Ni (mg kg ⁻¹ dry soil)	P (mg kg ⁻¹ dry soil)	S (mg kg ⁻¹ dry soil)	Zn (mg kg ⁻¹ dry soil)
Unt	0	1.450 ± 0.467	8254.430 ± 367.703	4.140 ± 0.272	0.113 ± NA	18.730 ± 7.309	24.780 ± 2.12	766.280 ± 67.261	1.700 ± 0.665	273.880 ± 26.891	0.038 ± NA	NA	4559.190 ± 298.165	0.083 ± 0.030
	1	0.750 ± 0.097	7688 ± 396.69	6.060 ± 0.816	NA	8.340 ± 1.839	48.130 ± 3.897	1736.600 ± 271.643	0.780 ± 0.152	404.680 ± 35.473	NA	NA	5030.520 ± 303.274	0.071 ± 0.007
	2	0.610 ± 0.116	6668.270 ± 120.036	4.070 ± 0.408	0.057 ± NA	7.410 ± 0.551	40.050 ± 11.39	1312.300 ± 294.828	0.720 ± 0.061	197.110 ± 46.352	NA	0.100 ± 0.007	4988.240 ± 266.056	NA
Wp	3	1.130 ± 0.228	6356.800 ± 123.102	4.490 ± 0.514	0.048 ± 0.005	15.820 ± 4.501	63.530 ± 12.374	1442.600 ± 396.636	1.430 ± 0.366	332.640 ± 83.869	NA	NA	4950.790 ± 372.677	0.028 ± 0.030
	0	1.280 ± 0.815	8302.230 ± 253.404	0.270 ± 0.057	NA	16.940 ± 12.679	71.100 ± 5.372	683.100 ± 57.515	3.330 ± 1.371	370.990 ± 46.241	0.042 ± NA	0.200 ± 0.018	4832.940 ± 382.051	0.076 ± 0.018
	1	1.030 ± 0.250	7483.730 ± 408.620	NA	NA	12.110 ± 4.601	90.490 ± 8.488	1385.300 ± 128.066	1.850 ± 0.495	554.180 ± 43.117	0.040 ± NA	0.180 ± 0.015	5705.150 ± 358.756	0.076 ± 0.009
Ms	2	0.940 ± 0.292	6624.170 ± 97.489	0.140 ± 0.023	0.070 ± 0.011	14.110 ± 5.282	79.130 ± 9.458	1301.900 ± 277.810	1.880 ± 0.507	412.070 ± 151.537	0.044 ± 0.009	0.220 ± 0.026	4830.680 ± 264.547	0.035 ± 0.013
	3	1.190 ± 0.088	6480.330 ± 10.247	NA	0.055 ± 0.000	19.670 ± 1.408	137.440 ± 3.602	1428.300 ± 209.652	2.170 ± 0.111	640.740 ± 105.972	0.044 ± 0.001	0.140 ± 0.046	4911.030 ± 218.138	0.047 ± 0.026
	0	1.290 ± 0.496	8018.530 ± 347.091	1.810 ± 0.102	0.169 ± 0.033	18.380 ± 9.633	602.480 ± 23.422	714.120 ± 38.508	1.960 ± 0.786	383.570 ± 58.077	0.053 ± 0.008	0.240 ± 0.026	5090.830 ± 300.858	0.082 ± 0.026
Ec	1	1.510 ± 0.165	7708.500 ± 91.188	0.640 ± 0.068	0.148 ± 0.013	20.890 ± 3.798	711.180 ± 227.710	1019.740 ± 207.127	2.340 ± 0.416	425.050 ± 33.796	0.066 ± 0.005	0.260 ± 0.027	5432.230 ± 707.823	0.091 ± 0.003
	2	0.540 ± 0.019	7001.200 ± 248.188	0.290 ± 0.168	0.069 ± 0.022	748.130 ± 471.669	471.669 ± 6.700	912.530 ± 551.418	0.930 ± 0.016	296.970 ± 177.817	0.059 ± 0.025	0.280 ± 0.116	4896.630 ± 622.110	NA
	3	0.470 ± 0.117	6602 ± 190.873	0.160 ± 0.026	0.077 ± 0.011	3.820 ± 1.353	236.588 ± 923.710	1182.190 ± 341.780	0.640 ± 0.214	181.418 ± 364.200	0.056 ± 0.011	0.260 ± 0.046	4935.590 ± 299.532	0.032 ± 0.022
Pc	0	0.970 ± 0.161	7950.970 ± 248.936	0.150 ± 0.029	NA	11.910 ± 2.652	295.500 ± 16.920	673.190 ± 11.714	4.860 ± 0.544	364.200 ± 23.277	NA	0.340 ± 0.031	4932.520 ± 463.934	0.059 ± 0.006
	1	0.880 ± 0.037	7478.330 ± 121.138	NA	0.130 ± 0.017	10.140 ± 0.743	490 ± 28.391	1389.130 ± 135.172	3.080 ± 0.449	515.890 ± 38.034	0.042 ± NA	0.270 ± 0.029	5867.620 ± 611.275	0.077 ± 0.007
	2	0.770 ± 0.008	6654.570 ± 120.885	NA	0.079 ± 0.008	10.400 ± 0.417	673.230 ± 82.487	1932.600 ± 98.503	4.340 ± 2.060	547.260 ± 35.735	0.043 ± 0.000	0.320 ± 0.017	5862.920 ± 94.685	NA
3	0.960 ± 0.067	6657.500 ± 107.027	NA	0.065 ± 0.004	15.510 ± 0.583	556.640 ± 90.728	1550.400 ± 117.847	2.200 ± 0.172	609.120 ± 96.555	0.038 ± 0.002	0.290 ± 0.047	5266.080 ± 89.283	0.085 ± 0.104	

Treatment	Time (months)	Al	Ca	Cr	Cu	Fe	K	Mg	Mn	Na	Ni	P	S	Zn
Pc	0	1.830 ± 0.223	8046.230 ± 43.466	0.090 ± 0.013	NA	11.680 ± 2.584	324.720 ± 14.858	757.010 ± 30.647	16.430 ± 0.336	391.080 ± 29.312	0.053 ± 0.011	0.200 ± 0.023	4604.300 ± 62.693	0.078 ± 0.015
	1	1.210 ± 0.104	7539.700 ± 200.325	NA	NA	6.950 ± 1.236	825.540 ± 426.051	1573.130 ± 632.449	6.150 ± 0.740	604.620 ± 130.565	NA	0.170 ± 0.075	5555.750 ± 760.529	0.066 ± 0.002
	2	0.860 ± 0.141	7114.200 ± 72.774	NA	NA	7.270 ± 2.361	469.250 ± 126.381	910.340 ± 164.327	7.170 ± 0.857	279.180 ± 73.105	NA	0.180 ± 0.012	4828.810 ± 187.445	NA
	3	1.140 ± 0.235	6760.730 ± 36.715	NA	NA	12.830 ± 4.531	534.070 ± 16.126	1075.770 ± 37.550	4.930 ± 0.289	442.990 ± 13.422	NA	0.150 ± 0.026	4784.900 ± 62.227	0.045 ± 0.030

Table 13. Concentrations of elements in the RRG substrates and in leaves of birch seedlings under the five RRG treatments after a 3-month growth period. Bioconcentration factors (BCF) are provided for each element. Values shown represent (means \pm SD) the results of 12 replicates.

Element	Parameter	Unt	Wp	Ms	Ec	Pc
Al	Leaf (mg kg ⁻¹ dry matter)	17.93 \pm 8.37	15.43 \pm 7.23	8.42 \pm 3.07	15.91 \pm 8.88	20.42 \pm 10.4
	Substrate (mg kg ⁻¹ dry weight)	4302.03 \pm 141.24	3352.18 \pm 233.59	3363.88 \pm 253.81	3060.57 \pm 266.99	3005.84 \pm 181.49
	BCF	0.0042	0.0046	0.0025	0.0052	0.0068
Ca	Leaf (g kg ⁻¹ dry matter)	19.12 \pm 2.41	19.31 \pm 4.05	12.62 \pm 1.37	17.94 \pm 3.34	11.97 \pm 4.94
	Substrate (g kg ⁻¹ dry weight)	206.51 \pm 6.85	167.48 \pm 11.52	168.3 \pm 17.36	138.1 \pm 9.37	142.82 \pm 11.88
	BCF	0.0926	0.1153	0.075	0.13	0.0838
Cr	Leaf (mg kg ⁻¹ dry matter)	10 \pm 3.12	1.68 \pm 0.88	1.68 \pm 0.51	1.15 \pm 0.09	1.95 \pm 0.97
	Substrate (mg kg ⁻¹ dry weight)	270.89 \pm 5.03	213.22 \pm 15.01	212.69 \pm 22.51	174.42 \pm 10.78	186.3 \pm 14.41
	BCF	0.0369	0.0079	0.0079	0.0066	0.0105
Fe	Leaf (g kg ⁻¹ dry matter)	0.19 \pm 0.12	0.15 \pm 0.07	0.12 \pm 0.07	0.14 \pm 0.09	0.3 \pm 0.13
	Substrate (g kg ⁻¹ dry weight)	67.61 \pm 2.24	56.56 \pm 3.38	58.73 \pm 6	47.01 \pm 2.88	49.85 \pm 3.42
	BCF	0.0028	0.0027	0.0021	0.0031	0.006
K	Leaf (mg kg ⁻¹ dry matter)	8164.15 \pm 2522.78	8355.4 \pm 2485.14	9064.22 \pm 2899.74	8104.15 \pm 1886.99	8503.72 \pm 1666.72
	Substrate (mg kg ⁻¹ dry weight)	162.37 \pm 75.76	164.71 \pm 82.06	342.41 \pm 189.21	408.9 \pm 31.74	425.26 \pm 44.66
	BCF	50.281	50.7289	26.4721	19.8194	19.9964
Mg	Leaf (g kg ⁻¹ dry matter)	9.46 \pm 1.22	6.16 \pm 1.29	4.16 \pm 0.7	5.89 \pm 0.85	3.95 \pm 1.32
	Substrate (g kg ⁻¹ dry weight)	3.34 \pm 0.16	2.71 \pm 0.2	2.72 \pm 0.2	2.3 \pm 0.2	2.52 \pm 0.19
	BCF	2.8295	2.2704	1.5318	2.5578	1.5673
Mn	Leaf (mg kg ⁻¹ dry matter)	509.04 \pm 148.68	1030 \pm 432.4	735.14 \pm 132.66	1192.45 \pm 375.23	1408.74 \pm 492.21
	Substrate (mg kg ⁻¹ dry weight)	5739.57 \pm 202.56	4663.15 \pm 310.34	4772.99 \pm 570.39	3789.24 \pm 265.09	3964.65 \pm 283.29
	BCF	0.0887	0.2209	0.154	0.3147	0.3553
Na	Leaf (mg kg ⁻¹ dry matter)	128.63 \pm 112.33	124.84 \pm 171.34	60.59 \pm 59.03	51 \pm 23.08	931.56 \pm 1204.56
	Substrate (mg kg ⁻¹ dry weight)	180.15 \pm 22.76	170.9 \pm 42.31	135.43 \pm 4.89	167.36 \pm 28.68	163.5 \pm 21.82
	BCF	0.714	0.7305	0.4474	0.3047	5.6977
P	Leaf (mg kg ⁻¹ dry matter)	442.98 \pm 141.98	447.36 \pm 109.57	597.99 \pm 105.73	811.38 \pm 304.83	642.87 \pm 156.76
	Substrate (mg kg ⁻¹ dry weight)	52.13 \pm 22.76	83.44 \pm 34.63	98.89 \pm 15.5	103.48 \pm 11.24	84.06 \pm 40.22
	BCF	8.4976	5.3613	6.047	7.8412	7.648
S	Leaf (g kg ⁻¹ dry matter)	14.77 \pm 3.88	13.45 \pm 4.82	8.15 \pm 2.61	10.26 \pm 3.25	9.15 \pm 2.44
	Substrate (g kg ⁻¹ dry weight)	110.49 \pm 6.14	95.01 \pm 24.39	95.74 \pm 8.54	70.88 \pm 4.74	83.34 \pm 10.88
	BCF	0.1337	0.1416	0.0852	0.1449	0.1098

Part III

5.3 Digestate improves the development of *Betula pendula* grown on residual red gypsum.

5.3.1 Context and publication

In the previous part, we highlighted the potential of using pine bark chips as a pH-decreasing amendment. This allowed a significant decline on the substrate's pH, which induced a higher Mn phytoextraction.



In the third section, we focused on meeting the third specific objective while also aiming to maintain the parallel the two axes of focus. Similarly, we introduced another organic amendment in order to improve the agronomic quality of the RRG: raw digestate, which is the product of the anaerobic digestion of organic matter. Hereby, the synergistic effect of a mycorrhizal inoculum, pine bark chips and organic the product of raw digestate, were put to test in setups under control and uncontrolled conditions. Furthermore, a field run using a biological amendment over a medium- to long-term period was put to test. The selection of such treatments was motivated by the need of a self-sufficient setup that could be repeatable at a bigger scale.

Digestate improves the development of Betula pendula grown on residual red gypsum.

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1. Introduction

The presence of vegetation on any soil provide many functions and services to the ecosystem such as soil stabilization to avoid soil loss and erosion, as an interface for soil moisture control through evapotranspiration, as influences for local temperature changes, CO₂ consumers and O₂ producers and as the primary food producers on trophic chains (Chapin et al., 2011). Land changes, including vegetation loss, are linked to about 60 % to anthropogenic causes directly and some 40 % to indirect changes such as climate change (Song et al., 2018). In general, plants require macronutrients, some 1 to 25 g kg⁻¹ of dry matter (DM) and micronutrients between 0.1 to 500 mg kg⁻¹ DM due to their importance in cell structure, osmoregulation, electrochemical balance and enzymatic activity (Taiz and Zeiger, 2006; West, 2014). Essential macro and micronutrients are found in the soil minerals, but their abundances depend on the nature of the parent material and the age of the soil (Kabata-Pendias, 2011). The lack of essential nutrients in mine waste sites is one of the principal constraints for revegetation (Festin et al., 2019). For instance, at the effluent basin of the now inactive Camaqua copper mine in south Brazil levels of P, S and Na up to 100-fold lower than the reference values are found that lays particular conditions for specialized species to survive, such is the case of the shrub *Solanum viarum* (Afonso et al., 2019). In Northern France at the Homécourt experimental site, former coke factory, the C/N ratios were found lower by 2-fold in samples of polluted soil when compared to a control unpolluted soil. In the same trend, TE were 10-fold higher in the polluted than the unpolluted samples. Additionally, 16 polycyclic aromatic hydrocarbons (PAHs) were determined in rather elevated concentrations in the polluted samples (Dazy et al., 2008). In the latter study, only 15 plant species were determined growing within the site including *Chenopodium album*, *Artemisia vulgaris*, and *Festuca rubra*, among others, the abundance and richness of such vegetation match with the typical values found in sites at early stages of succession.

Finally, N values were below detection levels, along with at least 100-fold higher concentrations of Fe, Mn, Mg and S, in residual red gypsum (RRG) issued from the TiO₂ neutralization at the Ochsenfeld site, in Eastern France (Zappelini et al., 2018; Zapata-Carbonell et al., 2019). The vegetation in the previous study was dominated by the pioneer species *Betula pendula*. In order to avoid the soil loss, the use of a vegetation cover is a common practice (Hueso-Gonzalez et al., 2017). Given the shortages of essential nutrients, Bradshaw (1997) determined that management applied for mitigation could comprise the selection of plant species for revegetation and the appropriate techniques when planting. Therefore, the strategy commonly adopted is the use of fertilizers to increase the mineral content available for

the plants. However, the use of such fertilizers ought to be justified economically, meaning that costs of fertilizers should be lower than the income of the yield of grains or biomass produced (West, 2014). Particularly for revegetation in sites with acidity or alkalinity problems and presence of potentially toxic elements (PTEs) it has been recommended to apply tolerant vegetation and apply the needed fertilization before seeding (Tordoff et al., 2000). Mulching is a common agricultural practice used for the conservation of hydric resources of plantations. For instance, in a semi-arid Mediterranean forest, Hueso-Gonzalez et al (2017) validated the use of straw and pine needles of *Pinus hallepensis* Mill for the conservation of humidity during the Mediterranean summer in the rhizosphere of a plantation. In a semi-arid environment where semi-desert shrub communities are installed in Colorado, USA, wood chips of *Pinus* sp. were mixed with the soil and this improved water holding capacity and aggregate stability, lowered the bulk density and increased the organic matter content, which in turn can aid improving microbial activity (Eldridge et al., 2007). Moreover, the increase of organic matter into a system is intended as a binder of the mineral particles in the soil composition. The existence of this binder may increase the water holding capacity. One common fertilizer that agrees with economic feasibility is the digestate. The digestate is one of the by-products of the anaerobic digestion of raw organic residues of agriculture, the other one being the biogas production (Do and Scherer, 2012; Rojas et al., 2012). Digestate can either be in solid (10-20%) or liquid phase (80-90%), and is used for being rich in nutrients such as P, K, Ca, Mg and a high NH_4^+/N ratio (Losak et al., 2012); which are important nutrients for plant development. More information about the properties of digestate and application examples have been compiled in reviews by Makadi et al. (2012); Möller and Müller (2012). Some studies on polluted sites have included digestate as a nutrient contributor to microbial communities for the remediation of contaminated soils with total petroleum hydrocarbons (Gielnik et al., 2019). Digestate has also been used as a covering layer for neutralizing and geochemical stabilization of flooded sulfidic tailings from the Boliden mine in Northern Sweden, this reduced the metal precipitation of the tailings and kept the conditions for bio-solid degradation (Jia et al., 2015). More recently, the use of digestate has been investigated for other factors. For instance, microalgae as were used for consumption of nutrients present in liquid digestate in order to avoid potential drawbacks of using it in agricultural lands, which may be legislated (Stiles et al., 2018): NH_3 volatilization, soil contamination (biologically, chemically or physically) and *ex-situ* eutrophication through nutrient lixiviation (Xia and Murphy, 2015). The latter paper lays out the importance of determining the correct dosage and management of digestate as a soil amendment. A recent report also pointed out the risks associated with contaminants in digestate (hereafter referred to as C/D) used as a fertilizer and soil improver (EC, 2019). As an alternative to chemical

amendments, the use of mycorrhizae has been widely studied in the past given their symbiotic relationship. Such exchange consists on trading some photosynthetates (carbon rich) for nutrients unavailable for the plant roots (N, P, S, K, etc.) (Courty et al., 2017). The use of mycorrhizae has been tested in several derelict environments to aid the plant development. For instance, at the Sigma-Lamaque gold mine, the tailings are a harsh environment for plant development; however, seedlings of *Picea glauca* were found *in-situ* with different fungal communities than those present in the forest surrounding the site. Additionally, an experiment in controlled conditions showed the synergistic interactions of mycorrhizal consortia and bacteria (Nadeau et al., 2018). In another unused gold mine tailing landfill in Baberton, South Africa, mycorrhizae were identified naturally in spontaneous shrub and grass vegetation of *Andropogon eucomus*, *Imperata cylindrical* and *Dodonaea viscosa*, suggesting that mycorrhization is an important strategy for ecological succession and land reclamation (Orlowska et al., 2011). Despite the presence of colonizing plant species at the mentioned Ochsenfeld site, in Eastern France, the development of a vegetation cover may take several years, therefore we hypothesized that through the use of organic and biological amendments (pine bark chips, digestate and mycorrhizal inoculum INOQ) we could increase the development of *Betula pendula*, present at the Ochsenfeld site. The aims of this work therefore were to i) determine the minimum digestate dose needed for obtaining a substantial increase in the plant biomass production and its development. Similarly, ii) to evaluate the chosen dose of digestate in combination with pine bark chips and the biological amendment INOQ in pot and field experiments in order to determine the best mix of treatment that would allow a better vegetation development for the revegetation and phytostabilization of the RRG tailings of the Ochsenfeld site.

2. Materials and methods

2.1 Substrates preparation and planting conditioning

The substrate used for the pot experiments below was RRG collected at the D1 plot of the Ochsenfeld site at the sampling point 32 and hence the initial substrate characteristics are fully detailed in Zapata-Carbonell et al. (2019). At the D1 plot, the sampled RRG had a moisture content of about 27% and contains abundant concentrations of Ca, S, Fe, Mn and Mg (approximately 200000, 110000, 650000, 5000 and 3500 mg kg⁻¹ DW, respectively). The raw digestate was acquired from Agrivalor (<https://www.agrivalor.eu/>), which processes residues obtained from the food industry in the French region of Alsace. The seedlings used originated from *Betula pendula* seeds that were collected from the Ochsenfeld site in 2017 and kept at 5°C, these were used in all subsequent experiments. Seeds were sown, germinated on peat (Hawita Baltic, Latvia), and pre-grown for three months in a culture chamber with a 16-h, 23°C/8-h, 21°C day/night regime, with an average moisture of 70% and a daylight intensity of approximately 600 μmol m⁻² s⁻¹. The mycorrhizal consortium INOQ Forest[®] was used as a biological amendment as recommended by the provider (<https://inoq.de/>).

2.2 Part I: Optimizing the digestate dose

In order to determine the best minimum digestate concentration required for plant development, four different concentrations were assessed in 1 L pots containing 650 g of RRG in a preliminary experiment. The concentrations were based on the quantity (in g) of nitrogen (N) per litre of raw digestate (0.025, 0.05, 0.075 and 0.1%). Raw digestate contained about 0.8% of total N, according to data recovered from the producer, Agrivalor (see values provided in Table 14), therefore 40, 80, 120 and 160 mL of raw digestate were the equivalent quantities to the percentages mentioned above. An extra additional treatment was added as control using untreated RRG.

Pre-grown birch seedlings were then transplanted to the pots containing RRG and the corresponding digestate dose (referred to as control, 0.025, 0.05, 0.075 and 0.1), and kept in cultured chamber for six weeks with the same under the growth conditions mentioned above. Each treatment was comprised by 10 replicates; one tray was used for three pots in order to avoid contamination or infections, then all trays were kept randomly separately within the culture chamber to avoid biased effects should light variations appear. The trays were for six weeks, plant physiological parameters were measured each week. Finally, above and underground biomasses were harvested, washed with distilled water prior to deionized water, oven-dried at 70°C then weighed and stored.

2.3 Part II: Biological and organic amendments assays on *Betula pendula*

A pot experiment was performed using three months old *Betula pendula* seedlings transplanted into 1 L pots with 650 g of RRG using 1:3 v/v of <2cm pine bark chips (Eden Garden, France) and the selected digestate dose as organic amendments. An additional treatment consisted in 20 mL of the mycorrhizal consortium INOQ Forest[®] was used as a biological amendment. Eight treatments consisted in mixing RRG (residual red gypsum) with pine bark chips (Pc), digestate (Dg) and a mycorrhizal inoculum (In) alone or in combinations. An untreated treatment was also included to be used as control (Ct). Each treatment was comprised by 12 replicates; one tray was used for three pots in order to avoid contamination or infections, then all trays were kept randomly separately to avoid biased effects should light variations appear, for a period of three months before harvesting. Growth conditions were similar to those detailed above. Plant physiological parameters were also sampled in a weekly basis.

The field experiment was undertaken at the Ochsenfeld site in a 12 m² surface in June 2018, kept in a fence to protect the plants from herbivores (Figure 46). The plants and treatments used were the same that were used in the pot experiment described above, this time with 25 plant replicates and 0.5 m² per treatment randomly separated from each other by a 15 cm wide corridor. The plants were kept for 12 weeks. At harvest, birch heights were recorded. After harvesting, leaf and root biomass were washed with tap water, in order to remove excess of dust, then deionized water, oven-dried at 70°C then weighed and stored. A portion of the root biomass was kept fresh and dissected to determine the mycorrhizal-colonized proportion by counting the fungal mantles present in 300 counts of root apices (Nagati et al., 2019).

2.4 Part III: Testing the mycorrhizal inoculum in field

In June of 2017, a field trial was undertaken using *Betula pendula*, the same conditions of the previous experiments for pre-growing the seedlings were met. Some 40 three-months-old *B. pendula* seedlings were planted per set at the D1 plot of the Ochsenfeld site. Three sets were planted, within each set, 2 subsets of plants were established: inoculated (I) with 20 mL of INOQ Forest[®] consortium, and untreated (C). The substrate was worked similarly for all subsets: tillage with a rototiller for 2 minutes. The plants were harvested after 18 months, keeping the underground and aboveground biomass, which was washed with distilled water, oven-dried and weighed. The fresh roots were also analyzed for mycorrhizal apex counting.

2.5 Physiological parameters

The plant physiological parameters measured for assessing the effectiveness of digestate were relative chlorophyll content index that was estimated through a CCM-200 plus chlorophyll-

meter (Opti-Sciences, Hudson, USA) using the third most developed leaf at each measurement for consistency. The photosystem II (PSII) efficiency was recorded after 4 hours of obscurity through a MINI PAM II (Walz, Effeltrich, Germany). Additionally, final leaf and root biomass were measured and initial and final height were measured in order to calculate de relative growth rate (RGR): final height – initial height / number of weeks. The pH of substrate samples taken at T₀ to T_f that were oven-dried at 40°C for three days was measured through a Hach HQ40d pH meter (Colorado, USA) after 1:2.5 w:v parts of substrate in 1 mol L⁻¹ KCl had been shaken for 1 h (Hesse, 1971).

2.6 Statistical analysis

Data obtained was processed with *R* (Ver. 3.4.2) packages *FactoMineR* (Lê et al., 2008), *factoextra* (Kassambara and Mundt, 2017), and *agricolae* (Mendiburu, 2009), and the graphs were made with *ggplot2* (Wickam, 2017). All tests were considered significant when $p < 0.05$. Analysis included two-way analysis of variance (ANOVA), with the Tukey-Kramer test for differences in the means used for group classification. Some data was log-transformed relative to the ANOVA postulates. The results reported in the tables are presented in means \pm standard deviation (SD).

Table 14. Measured parameters of digestate. Determined by Agrivalor[®].

Date	pH	Organic C % DW	Total N % DW	NH ₄ % DW	OM % DW	P ₂ O ₅ % DW
20/03/2017	8.70	2.73	0.85	0.22	4.84	0.20
20/03/2017	8.40	3.25	0.81	0.33	5.57	0.29
08/04/2017	8.20	2.36	0.79	0.47	4.73	0.28
30/05/2017	8.60	3.30	0.79	0.29	5.68	0.27
12/07/2017	8.40	2.97	0.83	0.27	5.19	0.25
26/07/2017	8.40	3.10	0.85	0.30	5.37	0.30
10/10/2017	8.30	2.45	0.80	0.31	4.27	0.21
Mean	8.43	2.88	0.82	0.31	5.09	0.26

3. Results

3.1 Optimization of digestate doses for *Betula pendula* growth improvement

We first performed a preliminary experiment to determine the optimal dose of digestate to bring to *Betula pendula*. The differences in plant development between *Betula pendula* with the different digestate doses and the control plants were significant. The mortality rates were only affected by the two highest concentrations. Starting at a pH of 7.9, the RRG suffered very few pH changes, notably reducing it (Table 15). The first effect recorded was the chlorosis of some leaves at first week after the transplantation for the overall treatments, including control. From this point, the plants with digestate presented a faster leaf development and growth than those in the control.

Table 15. Effects of various doses of digestate on birch survival rates, biomasses and heights registered at harvest (6 weeks). KCl-pH of the various substrates were measured at T_0 and T_f .

		Control	0.025	Digestate (% of N)		
				0.05	0.075	0.1
pH	T_0	7.94 ± 0.01 ^a	7.96 ± 0.05 ^a	7.93 ± 0.02 ^a	8.00 ± 0.03	7.99 ± 0.01 ^a
	T_f	7.83 ± 0.06 ^a	7.78 ± 0.02 ^b	7.77 ± 0.04 ^b	7.80 ± 0.02 ^b	7.81 ± 0.03 ^b
Survival (%)	T_f	100.00	100.00	100.00	80.00	60.00
Biomass (g)	Leaves	0.80 ± 0.12 ^{ab}	1.27 ± 0.45 ^a	1.32 ± 0.52 ^a	1.33 ± 0.82 ^a	0.49 ± 0.37 ^b
	Roots	1.60 ± 0.46 ^{bc}	2.73 ± 0.65 ^a	2.19 ± 1.19 ^{ab}	1.71 ± 1.21 ^{abc}	0.69 ± 0.37 ^c
RGR (cm week⁻¹)	-	0.15 ± 0.09 ^b	0.75 ± 0.41 ^a	0.45 ± 0.33 ^{ab}	0.65 ± 0.65 ^a	0.01 ± 0.05 ^b

Differences were determined by ANOVA and post-hoc analysis Tukey-Kramer test. *: p -value < 0.05. Groups formed indicated by letters. Values represent mean ± the standard deviation of the mean.

After 6 weeks, the plants in most treatments grew notably faster than the control, except for those amended with at 0.1% of N. Similarly in the biomass, the treatments with 0.025, 0.05 and 0.075% N developed almost a 1.6-fold more leaves than control, and 4-fold than those in 0.1% N (Table 15) ($p < 0.05$). The biomass produced after six weeks showed a slight increase in the leaf compartment for digestate treatments with 0.025, 0.05 and 0.075 % N over the control treatment. However, the 0.1 % N treatment showed the lowest leaf biomass production overall. A similar trend was found for root biomass production, found in treatments with 0.025 and 0.05 % N having the highest production, being 1.6-fold higher than the control. The 0.1% N treatment showed the lowest root biomass production, being 0.5-fold lower than the control. (Table 15). Additionally, the chlorophyll content index indicated significant differences ($p < 0.05$) among treatments at T_2 that were consistent until T_f for treatments 0.05 and 0.075% N (Figure 41).

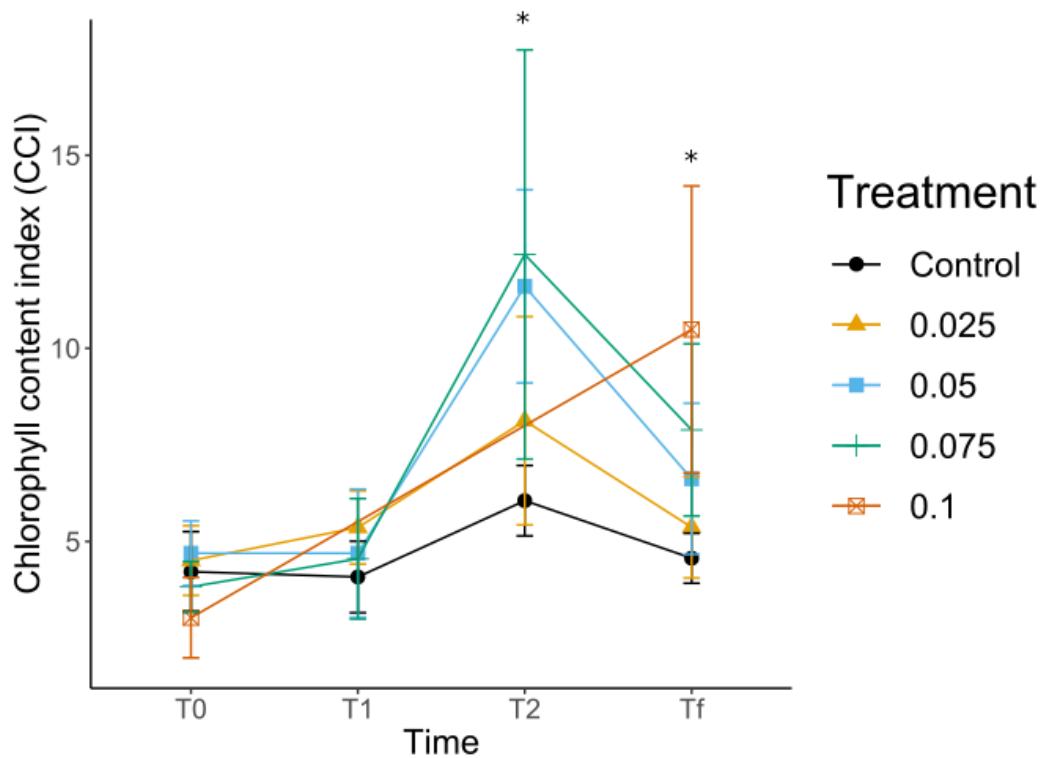


Figure 41. Effects of various doses of digestate on chlorophyll content index of birch leaves. Digestate was mixed at various doses with residual red gypsum (RRG). *Significant difference from control at p -value <0.05 . Values represent mean and error bars the standard deviation of the mean.

Overall, the digestate concentrations that provided the best results were 0.025, 0.05 and 0.75% N, based on the best leaf and root biomass production. Additionally, the consideration of the mortality rate aided to narrow the options to 0.025 and 0.05% N. Based on the chlorophyll values, the chosen concentration for the subsequent assays was 0.05% N.

Effects of *Betula pendula* when grown with organic and biological amendments in controlled conditions. The subsequent experiment in controlled conditions revealed notable and significant differences regarding the physiological plant responses among the treatments that included digestate from those who did not.

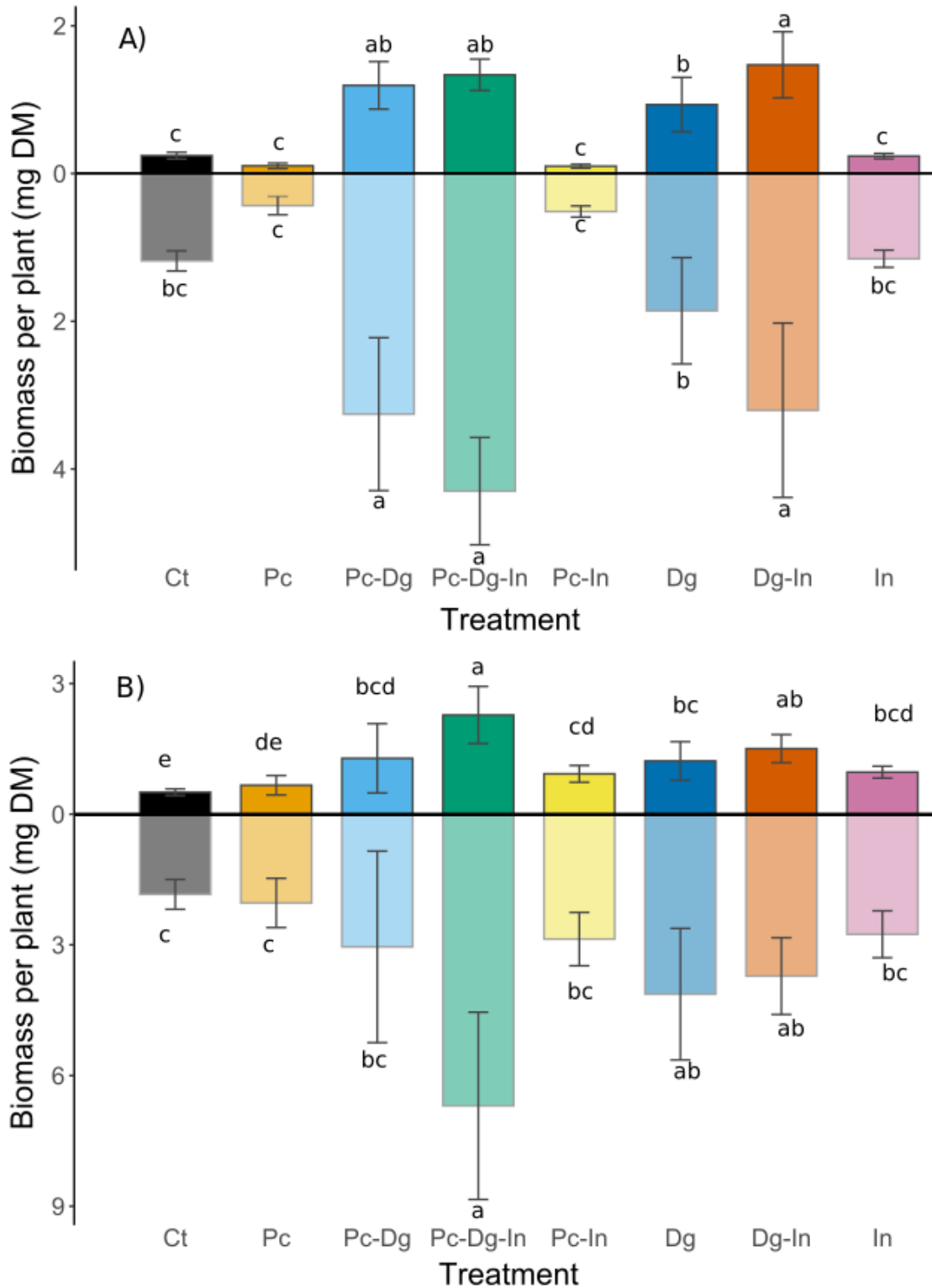


Figure 42. Effects of organic and biological amendments on birch biomass production in A) pot, and B) field experiments. Organic and biological amendments consisted in mixing residual red gypsum (RRG) with pine bark chips (Pc), digestate (Dg) and a mycorrhizal inoculum (In) alone or in combinations. Values represent mean and error bars the standard deviation of the mean.

In pots, the leaf biomass production of the treatments without digestate showed no significant differences between one another, whereas the combinations that included digestate showed significant differences among each other being Dg-In higher than Dg by almost two-fold. The same modalities showed no significant difference when pine bark was added (Pc-Dg and Pc-Dg-In). A difference of almost three-fold was found between Dg and Ct treatments (Figure 42A).

Similarly, the root biomass production of treatments without digestate was significantly lower than that of treatments with digestate; it was notably more reduced in Pc and Pc-In when compared to Ct and In for 2-fold. For the treatments including digestate, no significant difference was found for the overall treatments except for Dg that was two-fold lower than the rest. Overall, plants growth treated with digestate produced almost three-fold root biomass than that of Ct (Figure 42A). The chlorophyll content index also exhibited a significant difference among treatments with and without digestate from the first week of application and it became more evident toward the end of the experiment (Figure 43).

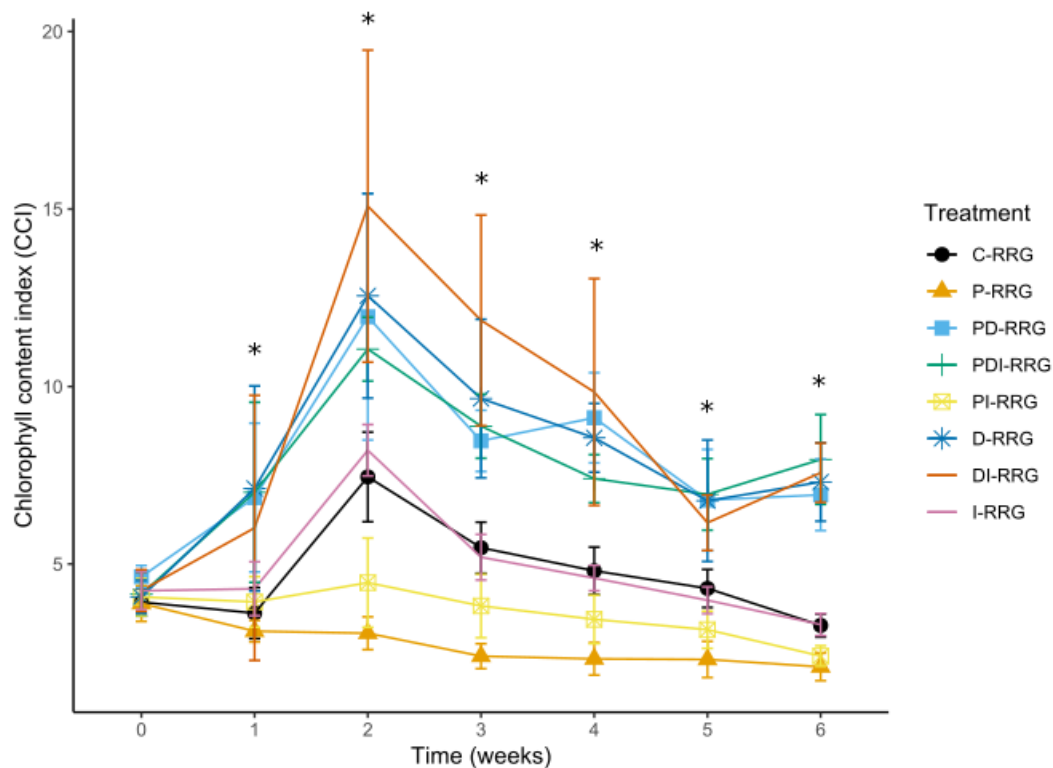


Figure 43. Effects of organic and biological amendments on chlorophyll content index of birch leaves. Details of the treatments are provided in legend to figure 2. *Significant difference from control at p -value < 0.05 . Values represent mean and error bars the standard deviation of the mean.

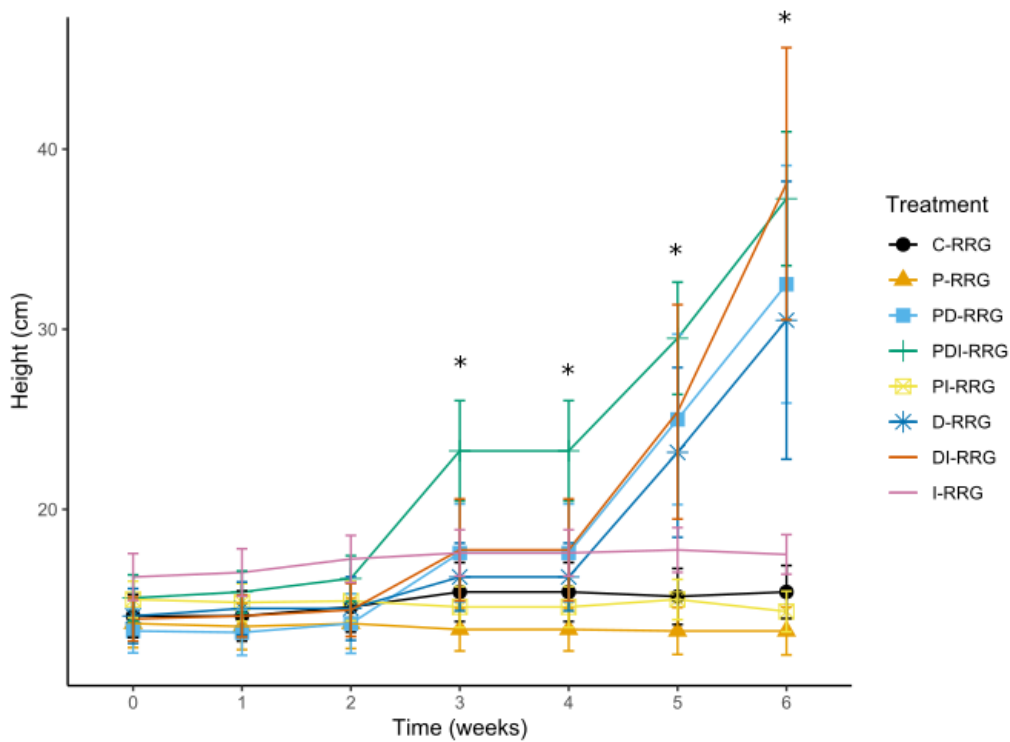


Figure 44. Effects of organic and biological amendments on birch heights. Details of the treatments are provided in legend to figure 2. *Significant difference from control at p -value < 0.05 . Values represent mean and error bars the standard deviation of the mean.

The height record showed that significant differences were recorded for the digestate treatments after three weeks, and reached up to three-fold the values of the treatments with no digestate (Figure 44). A faster growth was identified for specimens growing in the Pc-Dg-In treatment starting between the 2nd and 3rd week.

3.2 Effects of *Betula pendula* when grown with organic and biological amendments in field conditions

In the field experiment, results of leaf biomass showed significant differences among all treatments with respect to the control treatment, except for the Pc treatment. The highest and most significant leaf biomass produced was in the Pc-Dg-In treatment that produced over two-fold more than Ct (Figure 42B). Conversely, the root biomass production showed that all treatments had a higher root biomass production than Ct with significant differences except for Pc. The highest production was found in Pc-Dg-In. The latter was almost four-fold higher than that found on Ct (Figure 42B). Given the accessibility to the site, the height of this approach was only registered at T_f . The plants developed in Pc-In were significantly higher than those developed in Dg-In and Dg-Pc. No differentiation was found between treatments with and without Dg. Plants in these treatments were not significantly different from Ct; however, plants

growing on Pc-Dg, Dg and Dg-In presented the lowest heights of the overall treatments (Table 16).

Finally, the colonized root apices counts showed the highest proportion in the inoculated treatments in the pot experiment when compared to non-inoculated treatments; however, no significant difference was found between the former and Ct. On the other hand, the count on the field experiment showed a significant 0.5-fold increase for Pc-In, when compared to Pc and Dg-Pc (Table 16).

Table 16. Effects of organic and biological amendments on birch survival and mycorrhizal colonization of birch root apices at final time in pot and field experiments among treatments..

Treatments	Field survival (%)	Colonized Apex counts (%)	
	T _f	Pot	Field
Ct	93.33	42.0 ± 9.5 ^a	42 ± 13.7 ^{abc}
Pc	80.00	34.7 ± 9.1 ^{ab}	35 ± 7.5 ^{bc}
In	88.00	57.1 ± 19.2 ^a	56.7 ± 15.9 ^{ab}
Pc-In	100.00	46.7 ± 13.9 ^a	58.9 ± 9.4 ^a
Dg	52.00	22.3 ± 14.5 ^b	34.2 ± 6.5 ^{bc}
Dg-Pc	36.00	38.9 ± 9.5 ^{ab}	33.8 ± 11.6 ^c
Dg-In	56.00	54.1 ± 10.8 ^a	54.1 ± 5.5 ^{ab}
Dg-Pc-In	44.00	44.3 ± 9.6 ^a	52.6 ± 9.7 ^{abc}

Differences were determined by ANOVA and post-hoc analysis Tukey-Kramer test. Significant difference at p-value < 0.05. Groups formed indicated by letters. Values represent mean ± the standard deviation of the mean.

3.3 Long-term effects of inoculation with INOQ Forest[®]: field trials

The field plantation set up in June of 2017 was harvested after two years of development. The survival rate was of 56.6 and 66.6% of plants growing in untreated (Ct) and inoculated (In) substrates, respectively. The statistical analysis on the measures of height, leaf and root biomass and presence of fungal mantle in the root apices of inoculated with the mycorrhizal consortium were significantly higher than that of those growing in untreated substrate (Figure 45).

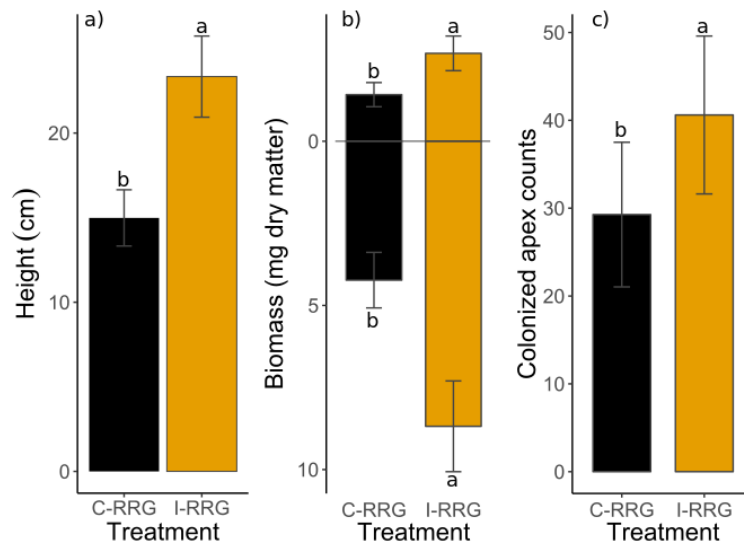


Figure 45. a) Height, b) biomass production and c) colonized root apices counted between inoculated and untreated treatments.

4. Discussion

The lack of nutrients (especially N) or their poor biological availability of the RRG renders these type of landfills highly unfavorable to plant growth (Zapata-Carbonell et al., 2019). Here we showed that birch biomass production could be efficiently improved using digestate. The mere presence of essential nutrients in digestate can explain the differences in biomass production and an optimal digestate dose corresponding to 0.05 % of N seemed to be optimal for birch growth. The addition of digestate doses with 0.075 to 0.1% N might be producing phytotoxic effects on *B. pendula*. The effect is reflected on the birch mortality rates in these two treatments and the reduction on biomass for the 0.1% treatment. The main influencing factor could be the presence of ammonium (Möller and Müller, 2012), which can damage chloroplast structure, disrupt photosynthesis, cause oxidative stress and deplete carbon supply (Bittsanszky et al., 2015), the latter can be also justified given the low C/N ratio of digestate (Table 14). Additionally, the presence of volatile fatty acids in the digestate might also play an important role on plant phytotoxicity (Drennan and Distefano, 2010).

The chlorophyll changes further helped us to narrow the selection of the digestate dose. The CCI for treatments 0.05 and 0.075 registered the highest values, but it is worth mentioning that the size and chlorosis of leaves were influencing factors that hampered the collection of data, especially for the treatment 0.01% N, that provided no data. Our CCI values indicated that plants may be suffering from stress, when compared to those values of Richardson et al (2002). They determined that CCI values of *Betula papyrifera* could fluctuate from 0 up to 25

for stressed to optimum plant conditions, respectively. Since the digestate used is in liquid form, the loss by lixiviation is also a possibility; therefore, the use of a more recalcitrant dose is recommended. The use of mulching amendment that allows the retention of moisture is highly recommended.

The other organic or biological amendments did not improve birch growth in the pot experiment when used alone. However, in the pot experiment that used combinations of organic and biological amendments, noticeable birch growth and fitness improvements were observed when digestate was included in the treatments, notably Pc-Dg, Pc-Dg-In, Dg-In, with respect to Dg or to control. This indicates a synergistic positive effect given the individual characteristics of the amendments. Pine bark chips have been used in the past in acidic mine soils at a pH of 3 to sorb and immobilize PTEs such as Cu, Zn, Ni, Pb, and Cd (Cutillas-Barreiro et al., 2014; Fernández-Calviño et al., 2017). Even though, PTEs are also present at the RRG of the Ochsenfeld site, these are rather unavailable given the alkaline pH of the RRG (Zapata-Carbonell et al., 2019). Thus, the addition of pine bark chips together with digestate amendments could encourage moisture retention (Pérez-Esteban et al., 2012), aggregate stability and most importantly to increase the organic carbon content in the substrate (Young et al., 2015). The latter may play an important role by increasing the C/N ratio that is lowered when adding digestate and therefore $\text{NH}_3\text{-N}$ to the substrate (Bittsanszky et al., 2015). The addition of pine bark chips together to digestate may help to synergistically improve the C/N ratio of the RRG substrate. As suggested in the literature, the presence of the relevant fungal inoculum is necessary for an efficient establishment of vegetation. However, given the harsh conditions and the sorbing properties of the RRG, natural mycorrhizal colonization at the RRG site is probably impaired, and the introduction of the commercial inoculum INOQ could be an ideal compromise (Orłowska et al., 2011). In our pot experiment, the addition of a commercial inoculum slightly increased mycorrhizal colonization of birch roots, although not significantly, that had however no positive effect on birch growth. On the other hand, the addition of digestate to the RRG substrate significantly reduced the number of mycorrhizal root tips on birch roots. A similar negative effect of liquid digestate on AMF colonization of triticale has been observed in a previous experiment (Caruso et al., 2018). However, the results dataset suggested that the presence addition of a commercial the mycorrhizal inoculum together with digestate increased significantly the number of mycorrhizal apices on birch roots when comparing the digestate treatment alone with the digestate combined with the mycorrhizal inoculum. This combination may have increased the nutrient availability in the rhizosphere that would explain the higher biomass production in roots and leaves (Vierheilig et al., 2005; Nadeau et al., 2018). However,

in treatments not treated with no digestate the differences between control and inoculated treatments showed no difference. The digestate may therefore have had an adverse effect on birch root colonization that was alleviated when a mycorrhizal inoculum was added.

The field experiment showed similar results than those in the pot experiment in respect to the use of digestate. However, a less recalcitrant effect of digestate was recorded regarding the biomass production. Only the treatment that included digestate, pine bark and inoculum achieved the highest biomass production. The leaf and root biomass produced in the field was slightly higher than that produced in pots, probably due to restrictions in the pots, judging by the size of the root systems (Figure 47). The height did not necessarily varied among treatments at T_f in the field experiment, but when compared to the overall height of plants in the pot experiment, the latter developed almost 2.5-fold higher plants than the field experiment. An influencing factor of such difference might be the scarce watering plants had in the field (Li et al., 1996). Since no inoculated treatment showed significant effect of amelioration of biomass production, height or fungal apex colonization when compared to a non-inoculated treatment it is expected to have reacted similar to the pot experiment.

Finally, the field longer term plantation resulted in notable differences on inoculated *B. pendula* over untreated, which differs from the previous field experiment using the same inoculum. The variant parameter is the time of development and exposition to the inoculum. After two years, the inoculum has probably had enough time to build its way towards the root cells and therefore building a mycelial network in order to improve the nutrient reach of plant and fungus association (Nadeau et al., 2018).

A dose of digestate of 0.05% N (80 ml kg⁻¹ substrate) was selected and used for the subsequent experiments due to its elevated content of inorganic nitrogen. This dose induced significant improvements on the plant development while avoiding phytotoxic effects of ammonium in the leaves and root development of *Betula pendula*. Furthermore, this treatment had lower mortality rate compared to 0.075 and 0.1% N treatments. The combination of digestate and pine bark chips might have influenced notably the plant development in the pot experiment by increasing the bioavailable nutrients in the substrate but also the aggregate stability, along with the organic matter content that could influence the development of microbial activity. In the field, however, the influence of other detrimental factors such as water deficit may play an important role to be taken into consideration when implementing revegetation strategies. The inoculation with mycorrhizal consortium INOQ Forest[®] may have a positive effect on birch development over the long-term through the development of a plant-fungi network. The addition of all three treatments (digestate, pine bark chips and mycorrhizal

inoculum) may be a viable amendment combination used for revegetation of an RRG plot whose effects would cover from the short to the long-term.

5. Acknowledgements

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6. Supplemental data

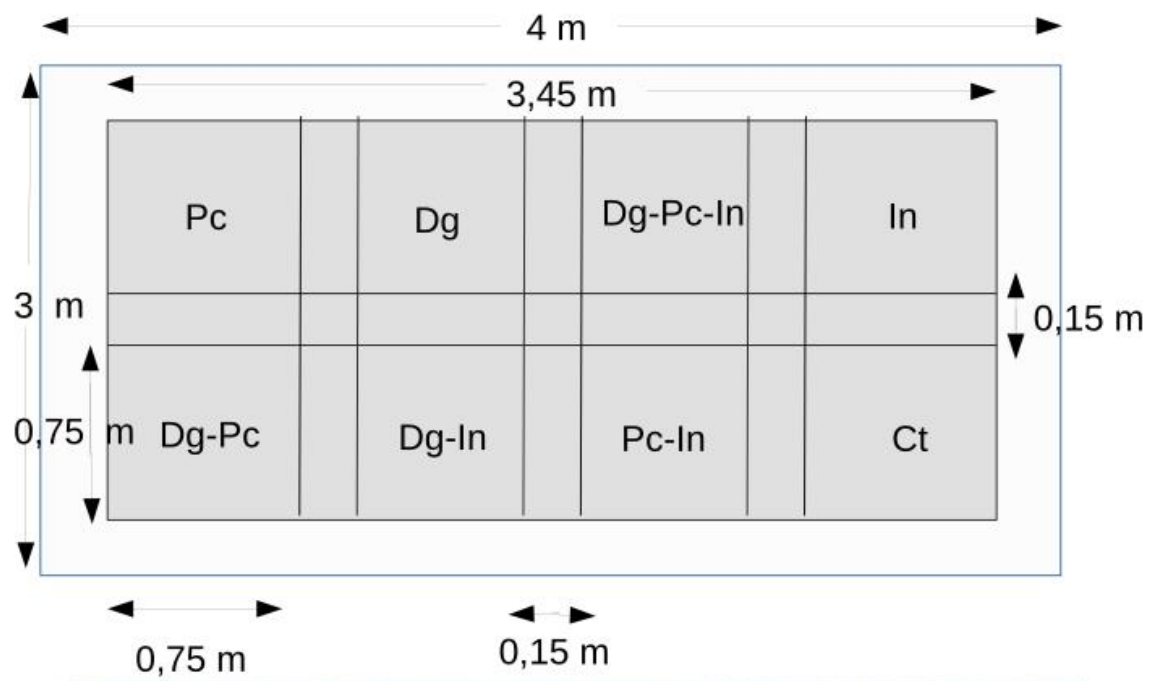


Figure 46. Planning and field setup.



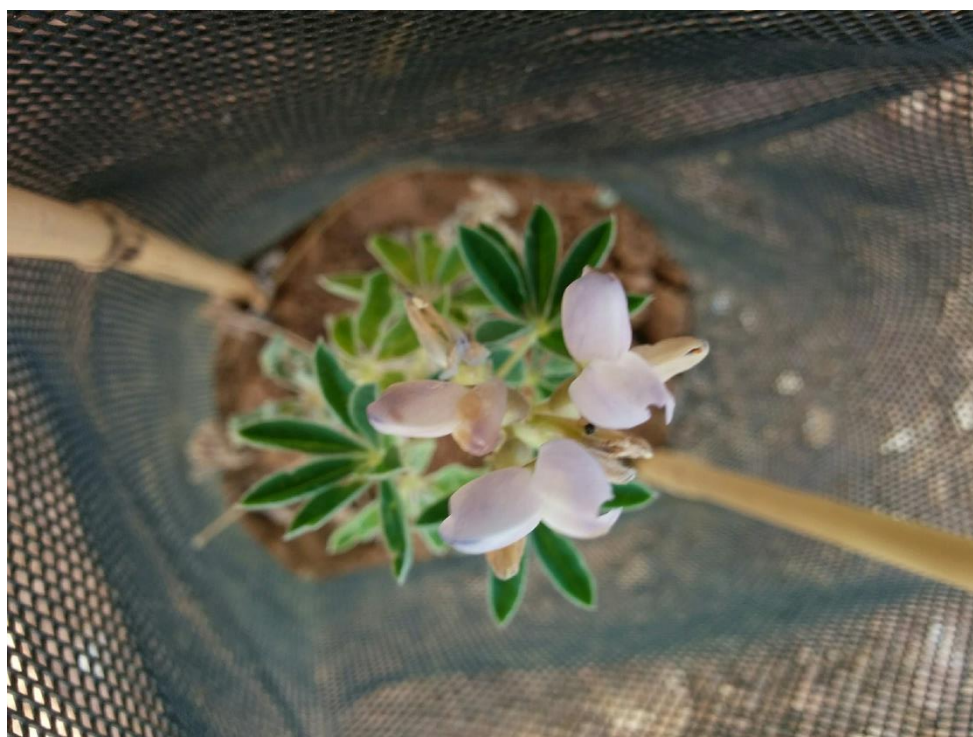
Figure 47. Root system on Betula pendula in A) field experiment, and B) pot experiment.

Part IV

5.4 Phytoextraction of Mn from a residual red gypsum landfill for revalorization through the development of *Lupinus albus*.

5.4.1 Context and publication

The results of the last section validated the hypothesis of the potential of raw digestate for increasing the development of *Betula pendula* in RRG thanks to the addition of nitrogen, which covers the axis of phytostabilization. Unfortunately, the axis of Mn phytoextraction still remained uncovered by the use of *Betula pendula*.



The literature comprising Mn-hyperaccumulators and other Mn accumulating species mentioned some species that could be compatible with the RRG landfill at the Ochsenfeld site, as the case of *Phytolacca acinosa*. Unfortunately, preliminary assay in pots using this species and RRG indicated low Mn phytoextraction and poor development, even when adding digestate. Due to this, a more specialized plant species was tested and proved to be effective and almost self-sufficient for developing in RRG and phytoextracting Mn. This part covers the last specific objective focused on the Mn phytoextraction through *Lupinus albus*. Furthermore, the use of the already organic amendments was assessed.

Phytoextraction of Mn from a residual red gypsum landfill for revalorization through the development of *Lupinus albus*.

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1. Introduction

The many centuries of mineral and ore extraction through mining have left abundant residues that are considered sterile (Babel et al., 2016). According to Adiansyah et al. (2015), some mining operations have an extraction rate of valuable elements of approximately 1 to 3%, meaning that the remainder is considered waste and is discarded. The Green Chemistry framework promotes the reuse of industrial waste to be exploited as raw material for other industries (Anastas and Warner, 1998). Likewise, the Red Mud Project[®] (2015) was developed after the recurrent dam bursts of bauxite residues from the Alumina Bayer process. This project encourages fellow enterprises to use proper management and promote revalorization of their produced tailings. The mentioned approaches claim similar values: the exploitation of what industry may call residues, with the aims of potential revalorization. Residual red gypsum (RRG) is the neutralization product of the sulfuric acid extraction of TiO₂, which in turn is the white pigment most used in the paint and food industry (Fauziah et al., 1996; Gázquez et al., 2014). The formation of gypsum (CaSO₄·2H₂O) gives RRG the potential to be used as raw material for the production of plaster and plasterboard (LeBel, 1980). Other applications that take advantage of its physico-chemical characteristics have been studied in the past (Kamarudin and Zakaria, 2007; Hughes et al., 2011; Pérez-Moreno et al., 2013). Another study (Azdarpour et al., 2017) took advantage of the carbonation properties of gypsum. In the mentioned study, the authors obtained solid and stable carbonates by using atmospheric CO₂, therefore inducing carbon sequestration that would be relevant for climate change regulation. Recent publications on the synthesis of organometallic molecules by Grison et al. (2015) have opened the possibility of profiting from the enriched biomass of metallophytes from contaminated sites or industrial residues, especially those rich in Ni, Zn, Pd and Mn. The Ochsenfeld site, near Thann, Eastern France, is the landfill for these effluents produced after TiO₂ extraction known as RRG. The RRG at the Ochsenfeld site has been accumulating in this area for almost a century now and covers a surface of 80 ha (Cristal, 2009). The rather elevated concentrations of Fe, Ca, Al, S, Mg and Mn, a pH of 8.2, a cation exchange capacity of 26.8 meq kg⁻¹, and a silty texture that predominate in the substrate make the RRG of the Ochsenfeld site rich in profitable TE but also a harsh environment for the establishment of vegetation. Some essential nutrients such as the N-total values and plant available P (CaCl₂ extractions) in the RRG are below the detection levels and 0.14 mg kg⁻¹, respectively (Zappellini et al., 2018). The phytomanagement of RRG with the aims of phytoextraction of Mn at the Ochsenfeld site could allow the use of Mn-rich biomass for the synthesis of Eco-Mn[®], the Mn eco-catalyst (Escande et al., 2015). In a previous analysis of the flora present at the D1 plot of the

Ochsenfeld site, 59 species were identified; however, none of those were identified as a Mn hyperaccumulator ($>10000 \text{ mg kg}^{-1}$ of Mn) (Baker and Brooks, 1989; Zapata et al., 2019). The fabaceous *Lupinus albus* (white lupin), in addition to its symbiotic capacity that allows fixation of atmospheric N, has a particular mechanism for P absorption when it is not available in the soil (Dinkelaker et al., 1989). Lupins have the capacity to develop cluster roots, namely, proteoid roots that produce citric and malic acids and protons that can induce soil acidification and therefore the solubilization of P (Gardner et al., 1983; Dinkelaker et al., 1989; Massonneau et al., 2001; Tomasi et al., 2009). Interestingly, the root exudation of malic acid allows the solubilization of rhizospheric Fe and notably Mn (Jauregui and Reisenauer, 1982); therefore, *Lupinus albus* might be used for the phytoextraction and accumulation of Mn from the RRG. In the published literature, *Lupinus* is a genus commonly mentioned in the phytomanagement of contaminated sites, i.e., phytostabilization of bauxite extraction residue (Dary et al., 2010). Furthermore, *Lupinus* has also been used for phytoextraction of Cd (Zornoza et al., 2010; Trejo et al., 2016), As (Franchi et al., 2017), U (Henner et al., 2018), chemically assisted extraction of Hg (Rodríguez et al., 2016), and multi-elemental assisted phytoextraction (Koopmans et al., 2007; Peñalosa et al., 2007). Regarding Mn, Martínez-Alcalá et al. (2009) recorded accumulated concentrations of up to 4969 mg kg^{-1} in the leaves of lupins. The review of Haynes (1983) concluded that the successive application of legumes in a pasture system has cumulative effects on the acidification of soils and therefore on the increase in solubility of nutrients in the soil when compared to pastures without legumes, including, notably, an increase in available N. Based on these principles, we hypothesized that *Lupinus albus* may be used for the phytoextraction of Mn from the RRG. To maximize the production of Mn-rich biomass of *Lupinus albus*, two strategies were proposed: to increase the phytoextracted Mn concentration and to increase the biomass production. The biomass may be increased by the addition of the NPK-rich amendments raw digestate and compost (Losak et al., 2012; Carlile et al., 2015). On the other hand, the Mn concentrations may be increased by decreasing the pH through the use of organic mulching amendments such as pine bark chips and ericaceous compost, since this has been achieved in *Betula pendula* growing in RRG (Zapata-Carbonell et al., 2020), a successive culture and the use of specialized organic amendments. The combination of the mentioned amendments with a successive legume setup may be beneficial in large-scale field applications. Taking this into account, this study aimed to maximize the production of Mn-enriched biomass of *Lupinus albus* through Mn solubilization and biomass production stimulation, so that production could be applied at a large scale in the field. To do so, two experiments were utilized. First, the Mn solubilization from the RRG was tested in a successive pot experiment using pine bark and ericaceous compost as acidifying amendments. The second

experiment focused on biomass stimulation in a pot experiment by adding digestate and compost to the RRG.

2. Materials and methods

2.1. Preparation of *Lupinus albus* and growth chamber conditions

Lupinus albus seeds were purchased from Semences du Puy (France). The seeds were planted directly in the soils at 0.5 cm depth and kept in the growth chamber with a 16-h at 23°C and 8-h at 21°C day/night regime, an average moisture of 70% and a daylight intensity of approximately 600 $\mu\text{mol m}^{-2}\text{s}^{-1}$.

2.2. Stage A: pH-decreasing amendments in a successive culture system

2.2.1. Experimental Setup

The consecutive culture system consisted of 4 cycles (T_1 to T_4), each consisting of 4 weeks. One cycle consisted of planting 4 white lupin seeds per 1-L pot, letting them grow for 4 weeks, then harvesting the 10 pots and repeating the operation. At the end of each cycle, 10 pots per treatment were collected.

Three treatments were tested: untreated RRG from the Ochsenfeld site was used as a control treatment (RRG). The two other treatments were a mixture of RRG and <2 cm pine bark chips (Pc) (Natura'lis, Longvic, France) and RRG and ericaceous compost (Ec) (Sorexto, St-Victor-de-Morestel, France). These amendments were homogeneously mixed with RRG at a 1:1 v/v ratio to ensure the desired pH-decreasing effect (Zapata-Carbonell et al., 2020). The mixtures were placed in 1-L pots. As a control measure, we used 5 pots per treatment per cycle but this time without planting white lupin sprouts. The pots were placed in two plates per treatment to avoid contamination by leaching. Then, they were randomly distributed within the plates and were also randomly distributed in the culture chamber. All pots were watered from the plates until saturation, which ensured the original RRG moisture content of 23%.

2.2.2. Sampled parameters

The physiological parameters were measured every week during the overall cycles. These parameters comprised the chlorophyll content index (CCI), which was estimated with a CCM-200 plus chlorophyll meter (Opti-Science, Hudson, USA) through the ratio of transmittance at 660 and 940 nm (Markwell, 1995), and the photosystem II (PSII) efficiency after 4 hours of obscurity, which was measured with a MINI PAM II (Walz, Effeltrich, Germany). The latter,

which was maintained at 0.8, was used to corroborate the correct functioning of the plants throughout the four cycles and the three treatments.

At the end of each cycle, a 2-cm-diameter core sample of the rhizosphere soil was taken from each pot, oven-dried at 40°C for three days, ground and sieved through a 2 mm mesh. The substrate pH was measured with a Hach HQ40d pH meter (Colorado, USA) in a 1:2.5 (w/v) mixture of the sampled substrate in 1 M KCl that had been shaken for 1 h (Hesse, 1971). The CaCl₂-extractable fraction of Mn in the substrates was extracted using 2.5 g DW of substrate mixed with 25 mL of 0.01 M CaCl₂ solution and incubated at room temperature for 3 h under agitation (140 rpm) to finally obtain the liquid phase through Whatman 2 filter paper (Houba et al., 2000). This extract was then analysed by flame atomic absorption spectroscopy (FAAS) (Model AA-240 FS, Varian, Palo Alto, USA). The white solutions were prepared following the same protocol but excluding the sample.

Additionally, at the end of each cycle, both the aboveground and underground biomass of the white lupins were harvested, thoroughly washed first with tap water and then with deionized water, oven-dried at 70°C for 3 days and weighed. The total Mn concentration in the leaves was also analysed in FAAS after extracting 0.125 g of leaf dry matter (DM) through 1.75 mL HNO₃ and 0.5 mL H₂O₂ in a digestion block (DigiPREP, SCP Sciences, Courtaboeuf, France). The white solutions were prepared following the same protocol, but instead of sample, we used oriental basma tobacco leaves (INCT-OBTL-5, LGC Promochem, Molsheim, France) with a known concentration of 170 ± 12 mg/kg DM of Mn. The phytoextracted foliar Mn was calculated using the Mn concentration in the leaves (mg/kg DM) times the biomass produced (kg DM).

2.3. Stage B: Growth promoting amendments

2.3.1. Dosage of amendments

The growth-promoting amendments used were digestate from the anaerobic digestion of agricultural organic matter waste for the production of methane and compost from agricultural organic matter waste, both obtained from the group Agrivalor (France). To determine which growth-promoting amendment was the best and at what dose, we decided to base the doses on the total N contained in each amendment. According to previous analyses provided by Agrivalor (Table 14 and Table 19), the digestate and compost each had 0.8 and 1.1% DW of total N, respectively. We used 1-L pots for this experiment; thus, the concentrations that were added at the top of the RRG contained 0.025, 0.05, 0.075 and 0.1% N for raw digestate (20, 40, 60 and 80 mL per pot). For the compost, 30 and 90 g were added per pot (labelled as low

and high concentrations). Compost was dosed in such quantities as to achieve 0.66 and 1.33% of total N per pot. Each dose of the amendments was considered a treatment, which was composed of 10 replicates each. As a control treatment, we used 10 pots with untreated RRG. Each pot of each treatment had a consistent weight of 650 g, considering the original RRG moisture of 23%.

2.3.2. Experimental setup

White lupins were pregerminated in wet cotton in the growth chamber and kept for seven days until cotyledons were visible. Then, sprouts were ready to be planted directly in the pots. In each pot, we planted two sprouts that were kept under the same controlled conditions. Pots were placed in two plates per treatment to avoid contamination by leaching. Then, they were randomly distributed within the plates and were also randomly distributed in the culture chamber. All pots were watered from the plates until saturation to ensure the original RRG moisture content. The setup was maintained for four weeks, when both the aboveground and underground biomass were harvested, thoroughly washed with deionized water, oven-dried at 70°C for 3 days and weighed.

2.3.3. Sampled parameters

For this assay, CCI and PSII fluorescence were also determined but only three times in 4 weeks: once at start of the experiment, then two weeks into the experiment and at harvest time. For height, initial and final heights were measured to calculate the relative growth rate (final height-initial height/total number of days). Finally, the foliage-recovered Mn was calculated from the biomass and the phytoextracted Mn concentrations in leaves, excluding the stems, and was determined by FAAS, as explained previously.

2.4. Statistical analysis

The data obtained were processed with the *R* (Ver. 3.6.2) packages *FactoMineR* (Lê et al., 2008), *factoextra* (Kassambara and Mundt, 2016), and *agricolae* (Mendiburu, 2009), and the graphs were made with *ggplot2* (Wickam, 2017). All tests were considered significant when $p < 0.05$. Analysis included two-way analysis of variance (ANOVA), and the Tukey-Kramer test was used to determine differences in the means used for group classification. For the CaCl₂ extractable concentrations of Mn, data were log-transformed relative to the ANOVA postulates. The results reported in the tables are presented as the means \pm standard deviation (SD).

3. Results

3.1. Effects of a pH declining on *Lupinus albus* in successive culture

The consecutive culture induced a significant aboveground biomass reduction after 1 cycle of culture (Figure 48), which then stabilized for the following cycles. Likewise, for the underground biomass, a similar decrease was observed, and the lowest quantified values were observed at the end of the 4th cycle (Figure 48). The aboveground biomass produced at the end of the first cycle was twice the aboveground biomass of the other cycles. Similarly, we observed a global decrease in the chlorophyll content index throughout the four cycles for all of the tested treatments (Figure 49).

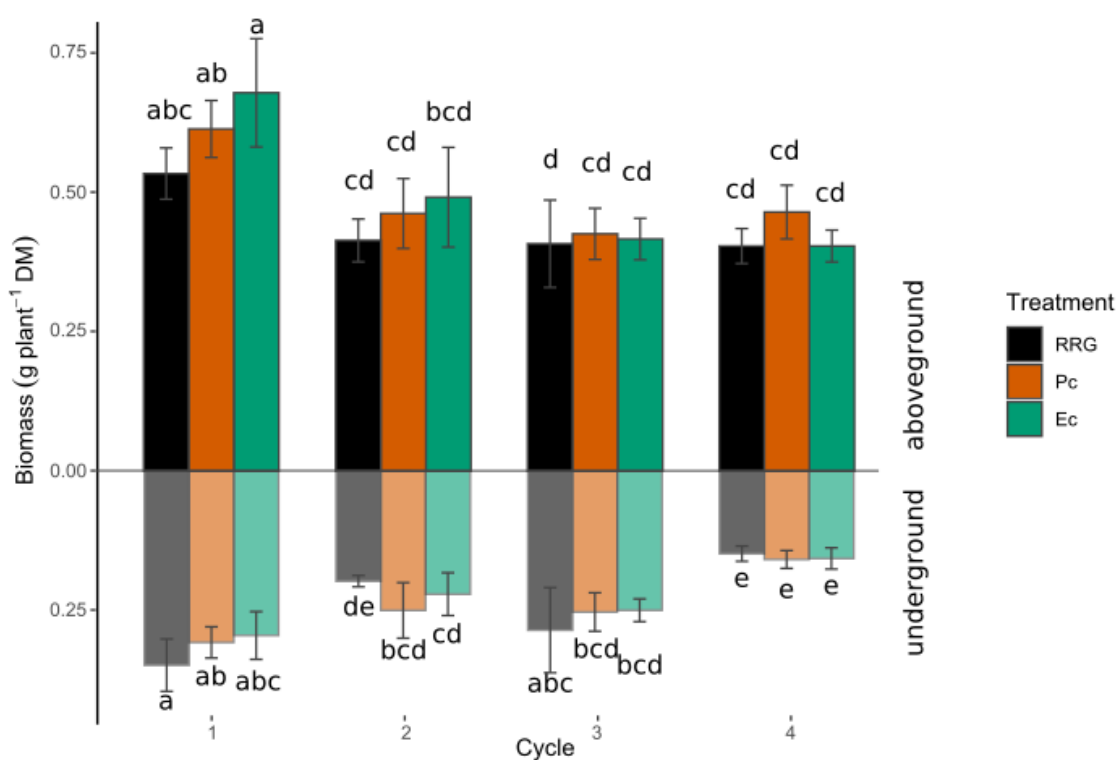


Figure 48. Average above- and underground biomass production (g plant^{-1}) of *Lupinus albus* per treatment ($n=10$) at the end of each cycle. Mean \pm SD. Different letters: $p\text{-value} < 0.05$.

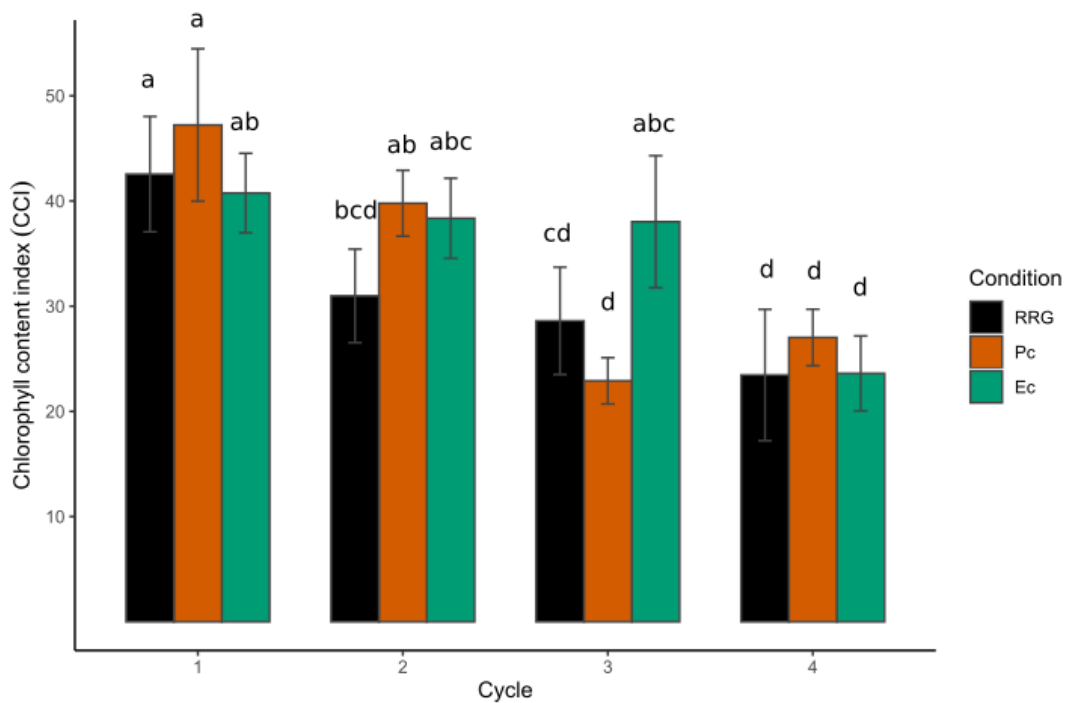


Figure 49. Average foliar chlorophyll content index per treatment ($n=10$) determined by chlorophyll meter at the end of each cycle. Mean \pm SD. Different letters: p -value < 0.05 .

Overall, the pH values measured in Pc and Ec were significantly lower than those of the control. In the pots without plants, regardless of the treatment, when the pH values were higher than 7.7, the CaCl_2 extractable Mn remained below 1.58 mg kg^{-1} , which was the maximum value of RRG and was reached by cycle 4. In Pc, an increase in the CaCl_2 extractable Mn occurred by cycle 4, reaching up to 9.53 mg kg^{-1} . For Ec, the maximum CaCl_2 extractable Mn concentration of 5.4 mg kg^{-1} was reached at cycle 1 (Figure 54).

In pots with plants, the CaCl_2 extractable Mn concentrations were the lowest at pH 7.6 and higher. Again, this was the case for RRG. Below this pH, the soluble Mn increased from 0.07 to 11.9 mg kg^{-1} . In Pc, values reached the maximum Mn solubility of 11.9 mg kg^{-1} in cycle 4, whereas for Ec, the maximum value was 10.9 mg kg^{-1} , which was reached at cycle 3 (Figure 50a). At pH values higher than 7.6, the phytoextracted Mn in leaves reached 1000 mg kg^{-1} or lower (Figure 50b). Finally, values of phytoextracted Mn higher than the threshold of 1000 mg kg^{-1} were only seen when the CaCl_2 extractable Mn values were lower than 2.8 mg kg^{-1} (Figure 50c). All values and their deviations were summarized in Table 17.

Table 17. Average values and standard deviations of pH, substrate CaCl₂ extractable Mn (mg kg⁻¹ DW) and foliar Mn concentration (mg kg⁻¹ DM) per treatment (n=3) measured from the consecutive pot experiment at the end of each cycle (n=4) in the presence of *Lupinus albus*. Groups are based on a mixed effect linear model with 95% confidence.

Parameter	Cycle	Treatments		
		RRG	Pc	Ec
pH	1	7.60±0.06 ^b	7.19±0.07 ^{ef}	7.08±0.06 ^f
	2	7.62±0.07 ^b	7.44±0.05 ^{bc}	7.26±0.08 ^{def}
	3	7.82±0.36 ^a	7.28±0.06 ^{cde}	7.15±0.10 ^{ef}
	4	7.93±0.08 ^a	7.15±0.09 ^{ef}	7.40±0.10 ^{cd}
CaCl ₂ extractable Mn (mg kg ⁻¹ DW)	1	0.14±0.04 ^g	0.60±0.24 ^{cde}	0.75±0.44 ^{cd}
	2	0.23±0.13 ^{fg}	0.72±0.32 ^{cd}	1.18±0.55 ^c
	3	0.32±0.11 ^{ef}	0.91±0.22 ^c	7.44±2.52 ^a
	4	0.41±0.16 ^{d^{ef}}	4.76±2.83 ^{ab}	2.57±0.51 ^b
Mn in leaves (mg kg ⁻¹ DM)	1	1451.18±190.07 ^b	2374.43±512.07 ^a	1493.92±291.05 ^b
	2	1289.94±271.77 ^{bc}	1277.46±249.11 ^{bc}	1001.46±373.92 ^{cde}
	3	988.20±203.14 ^{cd}	828.00±170.73 ^{de}	818.89±207.50 ^{de}
	4	699.92±204.32 ^e	845.41±118.42 ^{de}	480.28±130.48 ^f

RRG: Residual red gypsum, used as control treatment. Pc: RRG and pine bark chips. Ec: RRG and ericaceous compost.

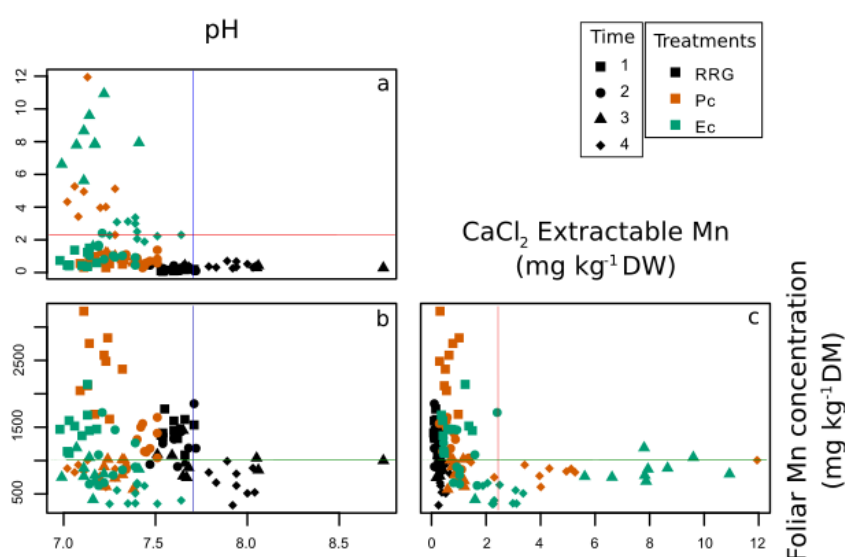


Figure 50. Scatterplot matrix of a) CaCl₂ extractable Mn (mg kg⁻¹ DW) as a function of pH; b) the foliar Mn concentration as a function of pH and c) the foliar Mn concentration (mg kg⁻¹ DM) as a function of CaCl₂ extractable Mn. Thresholds: red at 2.8 mg kg⁻¹ DW; blue at pH 7.77; green at 1000 mg kg⁻¹ DM.

The results of the calculated foliage-recovered Mn per plant indicated that the highest significant values were determined for cycle 1. In Pc, the foliage-recovered Mn reached twice the value of the untreated treatment. On the other hand, Ec showed intermediate values, reaching 25% more than the untreated treatment. For the other cycles, no significant differences were found, but overall, the foliar recovered Mn was almost three times higher at cycle 1 than at the other cycles (Figure 51).

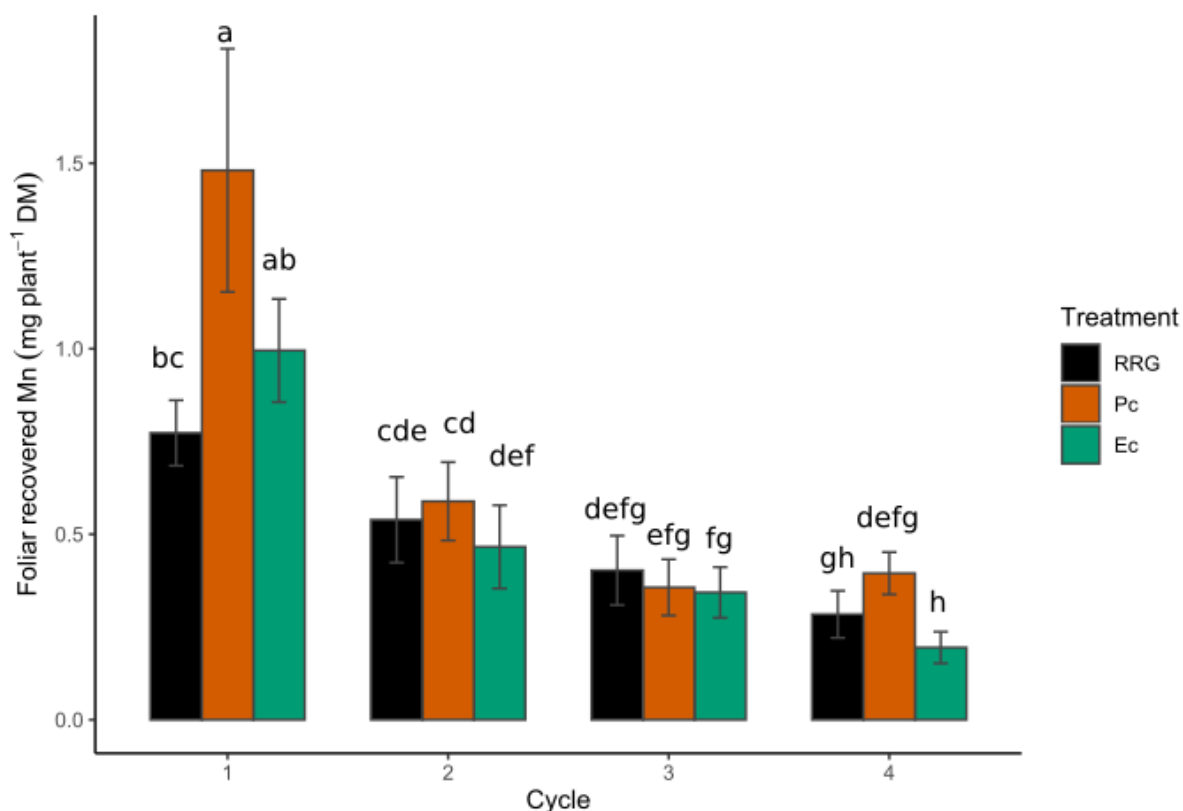


Figure 51. Foliage-recovered Mn per plant (mg DM) by treatment ($n=10$) at the end of each cycle. Mean \pm SD. Different letters: p -value < 0.05 .

3.2. Effects on *Lupinus albus* when adding growth-promoting amendments

After 4 weeks, the white lupins showed some visible differences, especially in terms of their physiological responses like, for instance, the survival rate. The survival rate was reduced by half in the plants growing in the 0.1% N treatment, whereas it was unchanged in the rest of the treatments, except for the 0.075% N treatment (Table 18).

The above- and underground biomasses showed significant differences (p -value < 0.05). In leaves, the treatments containing digestate showed no significant difference from the control, even in the 0.1% N treatment, the plants produced barely half of the leaf biomass produced in the control. On the other hand, the plants growing in treatments including compost

showed an increase of up to 40%. The same behaviour was observed for root production, but the decrease in root biomass in the 0.1% N treatment was 4-fold lower than that in the control, whereas in the compost treatment, the increase reached up to 50%. In addition, the relative growth rate calculated indicated that plants in all treatments showed significant growth increases when compared to the untreated plants. White lupins growing in compost showed a similar growth rate to those growing in digestate with doses of 0.05 and 0.075% N. The same 0.025% N treatment showed a significant increase of nearly 33% compared to the control. On the other hand, the 0.1% N treatment resulted in similar growth to that resulting from the untreated soil (Table 18).

Table 18. Synthesis of survival rate, leaf and root biomass and relative growth and CCI changes over time among treatments in Lupinus albus registered at harvest time of the pot experiment

Treatment	Dose	Survival (%)	Biomass		Relative growth	Chlorophyll content index		
			Above-ground	Under-ground		Day 1	Day 15	Day 30
Control	-	100.00	0.59±0.18 ^{bc}	0.28±0.15 ^b	0.22 ^c	40.62±18.21 ^a	23.54±6.72 ^{ab}	13.18±6.25 ^{bcd}
	0.025	100.00	0.57±0.21 ^{bc}	0.27±0.08 ^b	0.32 ^a	22.80±14.58 ^{ab} _{cd}	27.29±6.89 ^{ab}	17.54±5.57 ^{bcd}
Digestate	0.05	100.00	0.52±0.15 ^c	0.21±0.07 ^{bc}	0.27 ^{abc}	33.53±14.30 ^{ab}	26.63±12.40 ^{ab}	20.06±7.51 ^{abc} _d
	0.075	70.00	0.52±0.15 ^{bc}	0.17±0.09 ^{bc}	0.26 ^{abc}	18.75±0.00 ^{abc} _d	23.38±13.56 ^{ab} _{cd}	19.90±4.30 ^{abc} _d
	0.1	50.00	0.28±0.11 ^c	0.07±0.01 ^c	0.23 ^{bc}	NA	14.00 ^{bc} _d	15.73±10.12 ^{bc} _d
Compost	Low	100.00	0.81±0.23 ^{ab}	0.45±0.13 ^a	0.27 ^{ab}	36.55±10.26 ^a	21.43±4.95 ^{abc}	10.96±3.66 ^{cd}
	High	100.00	0.91±0.23 ^a	0.50±0.14 ^a	0.26 ^{bc}	37.26±8.26 ^a	22.11±6.99 ^{abc}	10.08±4.99 ^d

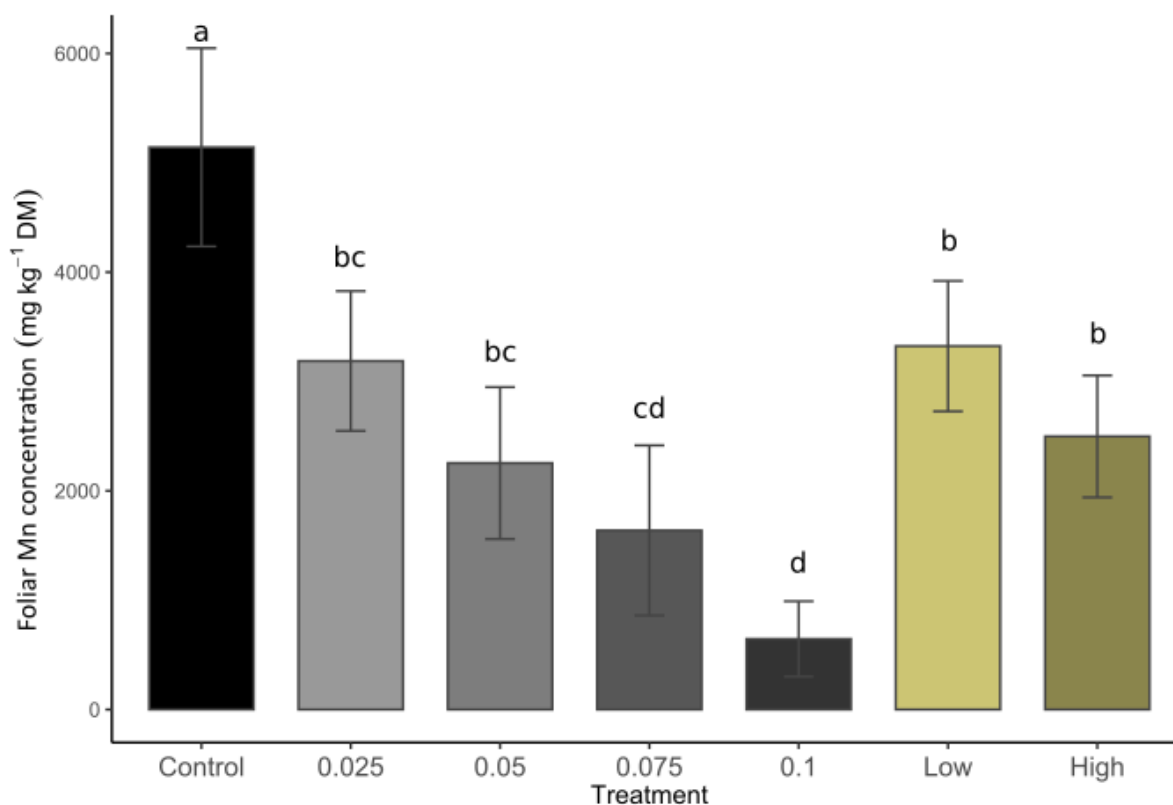


Figure 52. Total Mn concentration (mg kg^{-1}) determined in leaves of *Lupinus albus* per treatment of different doses of digestate and compost after 4 weeks. Mean \pm SD. Different letters: p -value < 0.05 .

Furthermore, the PSII fluorescence was verified, and the values were approximately 0.8 for all treatments, showing no variation among them. Conversely, the chlorophyll content index was not significantly different among treatments with high variability. At day 1, values obtained in plants growing in both compost treatments were similar to those of the control, whereas the rest were virtually lower. Throughout time, the CCI tended to decrease. At day 30, the CCI in most treatments had decreased dramatically, although it decreased significantly for both compost doses (Table 18).

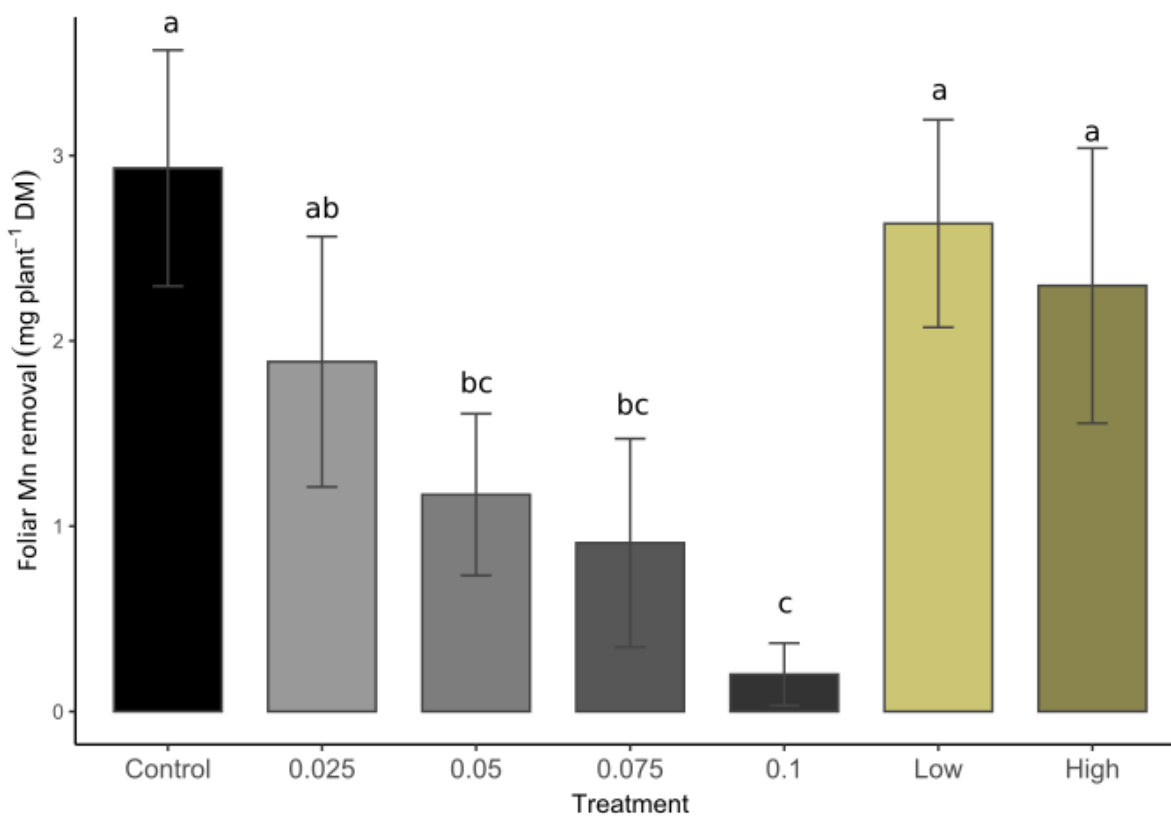


Figure 53. Foliage-recovered Mn per plant (mg DM) of *Lupinus albus* per treatment of different doses of digestate and compost after 4 weeks. Mean \pm SD. Different letters: p -value < 0.05.

Another parameter that was determined was the Mn concentration accumulated in the leaves. The Mn concentrations found in leaves under all doses of all treatments were significantly lower than those of the control. Although all treatments were statistically similar, except for the Mn determined in the 0.1% N treatment, which had roughly 20% of the Mn determined in the control, some differences were visible among them. Both of the compost doses resulted in Mn concentrations lower than those of the control by 30%, whereas the leaves of the digestate treatment groups at doses of 0.025, 0.05 and 0.075% N had up to a 30, 50, and 75% reduction, respectively, in the Mn concentrations in the leaves compared to those of plants grown in the control treatment (Figure 52).

A similar trend was registered when analysing the foliage-recovered Mn per plant per treatment. This parameter was the product of the Mn concentrations in leaves and the foliar biomass per plant (mg kg⁻¹ DM * kg DM). The control treatment resulted in the most Mn per plant; however, no significant difference was found when compared to both doses of compost. The different doses of digestate were significantly different from the control treatment by 33, 66 and 80% but not significantly different from each other (Figure 53).

4. Discussion

Overall, the results of the pH decreasing amendments highlighted two main outcomes: that a significant improvement in pH, CaCl₂ extractable Mn and phytoextracted Mn occurred when amendments were used when compared to the untreated RRG and that the highest significant values in all measured parameters were found at cycle 1.

At first glance, the significant difference between cycle 1 and the other cycles could be due to some sort of toxicity effect that might be causing stress (Matsui et al., 2019). However, the consistent occurrence of near 0.8 values in the PSII efficiency among the cycles indicated that photosynthetic activity was working correctly; therefore, plants were probably not suffering stress (Björkman and Demmig, 1987; Kromkamp et al., 1998). Other clues suggested that the clustering and accumulation of old roots in the pots could have caused space restriction and therefore decreases in the production of aboveground biomass (LaRoche, 1980). Nevertheless, we assumed, based on the chlorophyll content index that N deficiency occurred after the first cycle. This proxy has been used confidently in different plants in the past because of the linear correlation between it and the real chlorophyll content (Ghasemi et al., 2011). By adding Pc and Ec, we may have partially improved the N content for the white lupins, but the effect only lasted until cycles 3 and 2 for Ec and Pc, respectively. Ma et al. (1998) indicated that even when adding N-rich amendments, the absence of N₂ in the rhizospheric soil system of *Lupinus angustifolius* L. would significantly decrease biomass and seed production. Therefore, we can only assume that the decrease in biomass production might be linked to the depletion of N in the RRG or even the depletion of rhizobia (Rennie and Dubetz, 1984). Nonetheless, these latter parameters were not measured. It is worth mentioning that the presence of P from RRG of the Ochsenfeld site is rather low in both its total and CaCl₂ extractable form at a maximum of 160 and 0.3 mg kg⁻¹, respectively (Zapata-Carbonell et al., 2019).

The intended effect of mixing the pine bark and ericaceous compost into the RRG was to decrease the soil pH. This effect was clearly achieved without the influence of plants. The pH values determined for Ec in pots without plants reached pH 7, which was consistent with past experiences using Ec in RRG (Zapata-Carbonell et al., 2020). On the other hand, we expected the use of Pc to decrease the pH even more than Ec, as was seen in the previous work where amendment with pine bark chips resulted in a pH of 6.4. Nevertheless, the lowest average value we observed was approximately 7.1. This difference in pH gain induced by Pc might be related to the texture of the added amendment. The Pc used by Zapata-Carbonell et al. (2020)

was crushed, whereas the Pc used in our experiment was only sieved through a 2 cm mesh. The pH increase in the untreated soil may be due to the presence of carbonates in the tap water used for irrigation (Guo and Sims, 2000). This effect was not noticed in the pots with amendments. Furthermore, the presence of white lupins decreased the bulk soil pH by 0.1 units in addition to the pH increase from Pc and Ec. These results were consistent with the explanation by Dinkelaker et al. (1989), in which they describe that near the proteoid roots, the pH decrease may be lower than in the bulk soil.

The Mn solubilization by the addition of amendments was clearly improved as expected. In planted and non-planted pots, the CaCl₂ extractable Mn was inversely proportional to the pH, resulting in the highest soluble Mn in the Pc pots, followed by the Ec pots and finally the control. The presence of CaCl₂ extractable Mn only decreased considerably when plants were present due to root nutrient extraction (Marschner, 1995). The phytoextracted Mn decreased throughout time in the plants growing in the untreated pots, which suggests that it may be related to the already mentioned lack of N that would have considerably decreased the size of the plants. This can also be supported by the significant reduction in root biomass at cycle 4 in all treatments, which is when the Mn accumulation in leaves decreased dramatically. The decrease in the phytoextracted Mn in Ec and Pc at cycles 3 and 4, respectively, could be explained by the increase in the Mn CaCl₂ extractable fraction. The more plants absorbed and accumulated Mn, the less soluble Mn was left to detect. Finally, even though the foliage-recovered Mn was only increased in the amended treatments, notably in Pc at cycle 1, the Mn recovery decreased over time. This was most likely related to the nutrient shortage that considerably reduced root production, affecting the root reach and finally reflecting Mn accumulation in leaves.

N-rich growth-promoting amendments were used to increase the biomass of the plants and thus increase Mn recovery. Contrary to the most common effect found in the literature for most plants, the use of digestate in *Lupinus albus* did not increase biomass production. Digestate even induced a drop in biomass production, notably root production at the 0.075 and 0.1% N doses. The low biomass production and survival rate when compared to the untreated soil suggests that a phytotoxic effect occurred at these doses. Indeed, the presence of ammonium in the digestate was elevated in comparison to that in the compost treatment, with some 37% and 0.01% of the total N in the digestate and compost, respectively. Leguminous plants might be especially sensitive to excess ammonium (Britto and Kronzucker, 2002). Similar phytotoxic effects of ammonium were described in the past in crops and horticultural plants following the use of pig slurry by Lencioni et al. (2016). The mentioned work indicated

low root elongation and even germination reduction in pea seeds as effects. Applying compost mildly but significantly increased the overall biomass production of plants. This effect was previously observed by Castaldi et al. (2005) when adding compost to Pb-, Cd- and Zn-rich mining soils of southwestern Sardinia, allowing a biomass increase of 3-fold in the shoots and 6-fold in roots of *Lupinus albus* L. The use of compost can contribute to plant development by increasing the organic matter content and microbial activity as well as improving aggregate stability and texture (Walker et al., 2004).

Neither of the amendments induced nearly as much leaf Mn accumulation as the control treatment. The foliage-recovered Mn was therefore affected by this low accumulation in addition to the low biomass production. Most likely, the reason for the low Mn phytoextraction is that through the increase in nutrients in the digestate and compost, we also increased the P concentrations, given that the digestate and compost contained 0.26 and 0.44% DW of P₂O₅, respectively. Should this be true, proteoid root system production would be reduced, which in turn would mean less carboxylic acid production and therefore less solubilization (Lambers et al., 2012). The P concentrations in soil and leaves were not measured; however, this effect was seen by Abdolzadeh et al. (2010), where the deliberate increase in P in aqueous solution significantly decreased the production of proteoid root clusters.

Together, both of the stages in this work allowed us to assess both objectives: the augmentation of phytoextracted Mn and the augmentation of biomass production. The Mn phytoextraction was notably increased by using Pc; however, the effect was not sustained for more than 1 culture cycle. This strategy should probably be changed, and the time of decomposition between cycles should be increased to eliminate possible overcrowding in the pots. The use of growth-promoting amendments resulted in improvement in the biomass by using compost in either of the doses studied. However, the phytoextracted Mn concentration was affected by the nutrients input through the amendments, which betrays the whole purpose of this experiment, which was to increase the foliar Mn recovery for its use as a raw material for eco-catalysts. The combination of both approaches could perhaps provide a solution to the N deficiency found in successive cultures using pH-increasing amendments. The application of successive coculture with *Lupinus albus* could be interesting for field applications since it would aid in increasing N fixation and decreasing the pH in winter to prepare the RRG for phytoextraction of Mn by other species.

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6. Supplemental data

Table 19. Measured parameters of compost. Determined by Agrivalor[®].

Date	pH	Organic C % DW	Total N % DW	NH ₄ % DW	OM % DW	P ₂ O ₅ % DW
19/04/2017	8.20	14.27	1.21	0.02	23.52	0.52
20/04/2017	-	14.76	1.33	0.03	24.22	-
29/06/2017	9.10	17.93	1.45	0.02	29.68	0.56
18/12/2017	9.00	14.39	0.85	0.00	23.21	0.32
18/12/2017	8.50	15.70	0.90	0.00	27.62	0.35
Mean	8.70	15.41	1.15	0.01	25.65	0.44

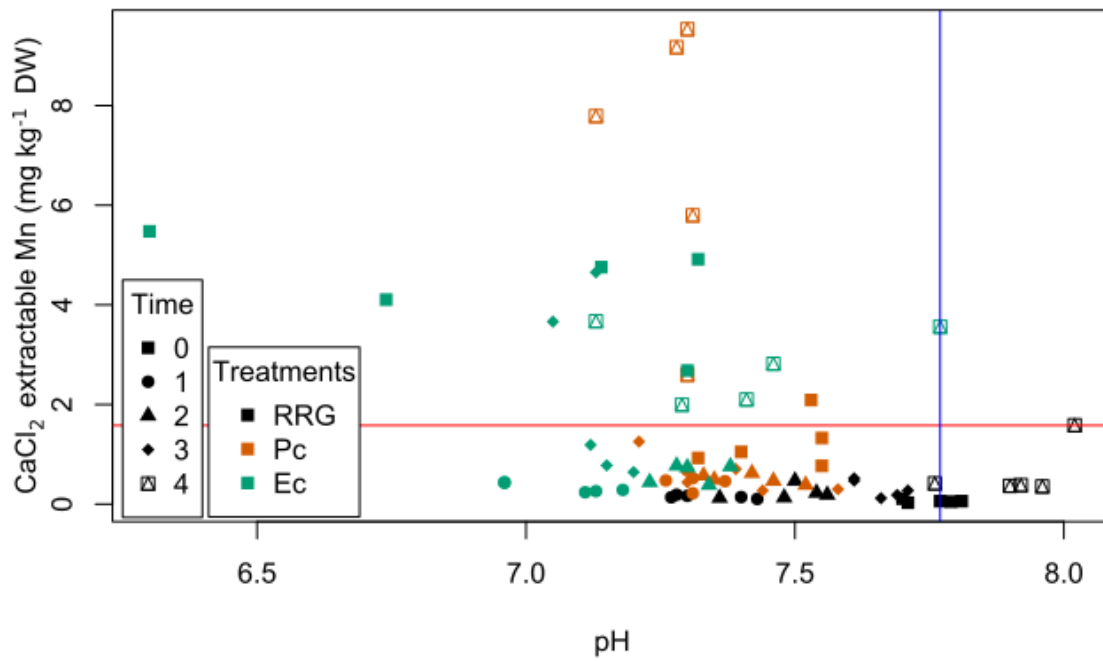


Figure 54. Scatterplot of the CaCl₂ extractable Mn (mg kg⁻¹ DW) variations as a function of pH per treatments from the consecutive pot experiment at the end of each cycle (n=5) in the control pots without the presence of *Lupinus albus*. Thresholds: red at 1.58 mg kg⁻¹ DW; blue at pH 7.77.

Chapter 6

General discussion

addition (6) albus (10) analysis (6) application (11) bark (10)
Betula (18) biomass (14) birch (6) concentration (6)
considered (7) develop (8) digestate (11) effects (11)
experiment (14) extract (6) field (10) increasing (11)
indicated (8) inoculum (6) introduce (6) lack (7) landfill (11) leaching (9)
leaves (6) lupins (9) **Lupinus** (11) mg (7) **Mn** (34) nutrients (7)
Ochsenfeld (11) order (10) **pendula** (18) per (8)
phytoextraction (11) pine (10) **plants** (26)
pots (10) presence (11) **results** (16) **RG** (20) root (8)
RRG (7) **soil** (9) solubilization (7) substrate (8) sustainable (6) system (9)
tests (6) used (10) **white** (12)

6.1 Initial objectives

This work was developed motivated by the lack of knowledge on the establishment of a plant cover upon red gypsum landfills issued from the neutralization of the TiO₂ extraction effluents. In particular, the Ochsenfeld site was used to search for answers to the original questions:

- What is the actual state of the site?
- Is there any vegetation developing in the site?
- What are the limiting factors that keep plants from developing in an optimal way?
- Is there a plant species capable of being used as a pilot plant to carry out experiments and be used to establish a vegetation cover, which is the main aim of this work?
- Is there a way to improve the plant development at the RRG landfill in order to aid the re-vegetation?
- Is there a way to re-valorize the biomass issued from a RRG landfill?

In synthesis, the phytomanagement approach gives an integral alternative for the treatment of the RRG landfill of the Ochsenfeld site, where one of the needs was to provide an answer to the potential environmental and public health issue of having an uncovered landfill of industrial tailings. As found and stated by a literature state of the art, the stabilization of the topsoil is needed to avoid the loss of material and the establishment of a plant cover is the most long-term and cost-effective way of achieving this. However, the development of such project requires planning and previous knowledge that is lacking in the literature for RRG so far. In this chapter, the overlapping of the two main axis, i.e. RG phytostabilization and Mn phytoextraction will be explored; the reasoning behind the decision-making during the experiments will be explained as well as how the experiments are linked together. Finally, a summary with the proposals for the Ochsenfeld site will be proposed.

6.2 Main findings

The first part of the results chapter allowed for investigating and understanding the chemistry and the ecological organization of the plant species present at the site. Indeed, the Ochsenfeld site was already colonized by pioneer plant species, prior to the start of this PhD work. These are specialized plants since their presence in other abandoned industrial/mining sites is rather common in the literature. Among these, some tree species are highlighted, *Poplar*, *Salix*,

Betula, *Robinia*. The physico-chemical analysis indicates that the site is rather rich in Mn, Mg, Fe, Al, Ca, and S. Furthermore, the presence of K and P may indicate that RG could be used for growing plants correctly. Nevertheless, given the presence of CaCO₃, the predominant alkaline pH of the substrate does not allow an acceptable nutrient availability, which is evidenced by the low CaCl₂ extractability of all elements and the low CEC determined in the past (Zappelini et al., 2018). Additionally, the lack of N suggests that the site requires some kind of N input for plants to develop faster. Previous studies indicated that *Betula pendula*, the most abundant species in the site, is the tree species most capable of accumulating Mn from those found in the Ochsenfeld site (Ciadamidaro et al., 2019). This turns out interesting in the context of Mn-rich biomass production for the synthesis of Eco-catalysts, where a content of over 1% Mn of dry matter is needed to provide good results (Grison, 2015). The results of the multivariate analysis in the site indicated that most species are distributed following the abundance of nutrients such as P and K. On the other hand, some species, in order to avoid competition with other plants, have colonized zones with higher concentrations of PTEs and with less abundance of P and K. This study evidenced the species that may be specialized for tolerating these elements, which is interesting from the point of view of our two main axes. For instance, *Betula pendula* may be used for re-vegetation of the site. The reason may not only be its pioneer traits: tolerance to toxic metals, high potential for Mn accumulation and tolerance to lack of nutrients, *B pendula* is also interesting due to its broad-spreading root system. This might be taken advantage for increasing the soil transpiration, therefore reducing the soil moisture content and hence lowering the ground water volumes that might be contaminated with leached metals. The latter provides an important economic service to the managers of the site, since they ought to treat all leaching contained within the membranes of the ponds by law. Furthermore, this study showed other spontaneous herbaceous species that may be used for increasing topsoil stabilization, especially from the Fabaceous family. In this regard, evidence of the presence of black locust (*Robinia pseudoacacia*) brought insight of its potential use for the phytostabilization of RG (Batzli et al., 1992). The Fabaceous family has the potential to host *Rhizobium* bacteria that could fix atmospheric N in the RG, which would complement the lack of N in RG at the Ochsenfeld site in a sustainable manner. After this first *in-situ* investigation, some more questions were raised: could we aid in the Ochsenfeld site reclamation by the introduction of some autochthonous plant species? What are the needs for meeting the proposed axis of RG phytostabilization and Mn phytoextraction?

As a response to both questions asked above, the second part of the results chapter evaluated the influence of the application of pH-decreasing amendments with short supply

chain and that were otherwise considered as residues (pine bark chips and Miscanthus straw), or that were used as conventional horticultural substrates (Baltic peat and ericaceous compost), on RG in controlled experiments. In these conditions, *Betula pendula* seedlings grown from seeds collected at the site were planted in pots. Even though the use of ericaceous compost and Baltic peat were not as sustainable as the use of pine bark chips and Miscanthus straw, the former were chosen as to compare their already confirmed influence with the latter. The pH reduction was reached through the use of pine bark, Baltic peat and ericaceous compost. These amendments also achieved the mobilization of elements that were contained in the substrate, notably Mn up to 1400 mg kg⁻¹. However, the effects were only reached temporarily despite the great volume and pre-treatment of the amendments (crushed pine bark chips). This reduces the chances of reproduction of this effect in a field application for land reclamation. Considering the estimated density of *Betula pendula* trees is 1600 trees per hectare (Hytönen et al., 2014), the 1:1 v/v ratio application and the 3 hectares of the D1 plot, some 2400 L of crushed pine bark chips would be required for reproducing the same effect upon the site. Moreover, the solubilization of RG elements caused imbalances in the nutrient absorption by the plants, causing deleterious physiological effects in white birch (stress, chlorosis and biomass reduction). Through this approach, we also noticed that plants reacted negatively to the lack of N, which was undetectable in previous substrate analysis. This parameter was not analyzed; however, it was thought to be the main obstacle to overcome in order to reach land reclamation of the site, based on the chlorophyll content index results. Initially, we hypothesized that by increasing the available Mn in the substrate, the plants would instinctively react by uptaking it, based on the results seen by Kitao et al (2001). They indicated that, in a hydroponic setup, by increasing the available Mn the passive transport could increase significantly the Mn phytoextraction of *Betula platyphylla* var. *japonica* reaching up to 18 mg g⁻¹ DM. However, the results obtained in our assay indicated that even though Mn is being solubilized, the available Mn²⁺ is being hindered from accessing the plants and stayed trapped or leached from the RG, raising some more inquiries: how could we manipulate the substrate in order to allow access to the Mn²⁺ in the RG? Could we increase the plant growth by using biological or other organic amendments? Can the active transport of nutrients be increased in the root-soil interphase in order to extract the available Mn more thoroughly?

The latter questions led to designing the experiment reported in the third part of the results chapter, which comprised the dosage of digestate, to use in addition to pine bark and a commercial mycorrhizal inoculum in pots and field conditions. Within the findings, the balance between the mortality rate and the boosting on the biomass production of *Betula pendula*

determined that the addition of digestate at a concentration of 0.05% N per pot, which was equivalent to 80 ml of digestate per litter of soil, was the best application quantity. The field effects on the biomass production were considerably lower than those found in the pots. This difference might have been due to the leaching of digestate, although this last part was not corroborated through analysis in controlled conditions, it is still something to develop. The analysis of samples where mycorrhizae was introduced showed significant growing effects, especially in the root system, (Figure 55).



Figure 55. Root system of *Betula pendula* inoculated with a commercial inoculum (INOQ, DE). Root apices in presence of mycorrhizal mantle (red) and with no signs of presence of mantle (blue).

This was not seen in the pot experiments, although the count of colonized apices was indeed significant where the inoculum was introduced in both field and pot experiments. The effects of mycorrhizae are more visible over middle- to long-term exposure (Figure 45). Over the short-term, the immediate induced effects in aboveground biomass production were most likely linked to the presence of nutrients in the inoculum substrate. Furthermore, the addition of uncrushed pine bark did not succeed at solubilizing Mn when added to RG but its presence complemented in the addition of digestate and the inoculum showing a significant increase in the aboveground biomass production of *Betula pendula*. These differences were hypothetically attributed to the moisture retention caused by mulching, which helped avoiding the leaching of digestate. Although, the latter is only an assumption, since it was not confirmed through experimentation. The difference between the controlled and uncontrolled condition tests was rather astonishing. In this case, we might have neglected the effect of digestate leaching when

it was added in the field. This could be alternatively solved by undertaking another pot experiment with deeper pots in order to avoid the root systems to have access to the leaching solution in the plates (Mench et al., 2010). In the field, the use of mulch may represent an alternative to counteract the loss of nutrients by leaching as consequence of the soil texture and the lack of organic matter. Moreover, the outcomes of the field tests using mycorrhizal inoculum with *Betula pendula* suggested that the use of mycorrhizae could be pursued for the biomass increase. This raises some curiosity about the capacities of microorganisms to reduce Mn in the rhizosphere. In parallel to these experiments, we carried out a mushroom sampling campaign at the Ochsenfeld site with the aims of recovering spores of RG-adapted fungi. Nevertheless, the collected spores could not be exploited nor tested.

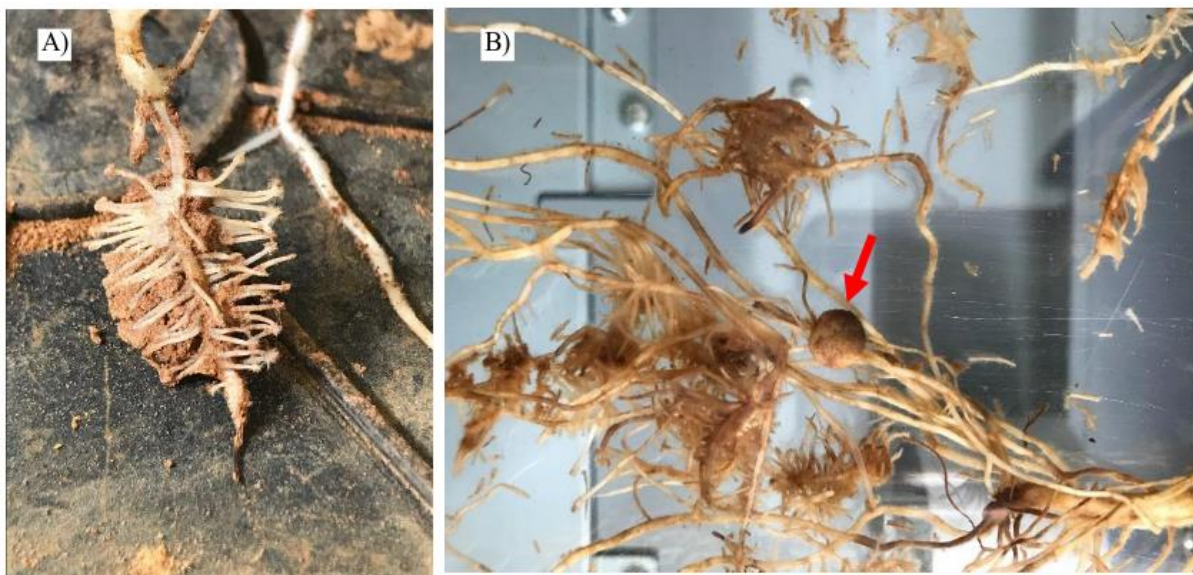


Figure 56. Different structures in root system of *Lupinus albus*: A) proteoid cluster root, B) nodule hosting *Rhizobium* bacteria. Photo from Léa Mounier.

The final part of the results chapter was based under the assumption that other species could be considered for the Mn recovery from RG, as in the case of the Mn hyperaccumulator *Phytolacca acinosa*. Preliminary tests using this species in untreated RG and RG acidified through crushed pine bark resulted in decreased biomass production and Mn phytoextraction only reaching 200 mg kg⁻¹ DM in either treatment (Moyen, unpublished data). Additionally, this species is not considered as local, therefore its use was not followed up. Conversely, the Fabaceous species *Lupinus albus* was considered given the services that its presence could provide in field conditions. The literature results showed that this plant species was able to accumulate Mn in the leaves, up to 4900 mg kg⁻¹ DM (Martinez-Alcala et al., 2009). Also, due to their capacity to produce carboxylic acids in the rhizospheric soil; its presence could be

exploited to perform a more sustainable acidification of the substrate in order to solubilize Mn (Figure 56). Finally, their capacity to function as symbionts of rhizobia may also be exploited as a more permanent input of N for the enrichment of the agronomical characteristics of the RG. The results of the fourth experiment indicated that indeed the presence of white lupins could extract up to 8000 mg of Mn per kg of RG DW, although this is mostly concentrated in the leaves, notably in the outside of the leaves of *Lupinus albus* (Page et al., 2002). This means that at a larger scale, more industrialized setup, the stems of white lupins recollected must be retrieved before processing, otherwise a dilution effect in total Mn concentration in the biomass may be induced.

The application of digestate as amendment in the experiment did not succeed at increasing the biomass of *Lupinus albus*. On the contrary, its use reduced the plant growth, probably due to the addition of stress-inducing substances such as ammonium. When using compost, even though the biomass was somewhat stimulated, the Mn phytoextraction potential of white lupins case decreased due to the addition of P, which in turn probably led to the reduction of proteoid root systems. The Mn phytoextraction from the RG was considerably increased by the use of uncrushed pine bark mulch in the RG. Pine bark mulching could therefore be considered for further field applications over long-term, although the duration of its effect in the Mn accumulation must be further investigated. The applicability of successive cultures, which ran under the assumption of sustainable acidification through *Lupinus albus*, could not be confirmed after 4 successive culture cycles, however, a trend of pH decrease was found in RG pots that contained plants, compared to unplanted pots. It would be worth exploring the application of this setup over a longer period of time, as well as the Mn solubilization and the N fixation in the soil. The latter was not looked at during this experiment, but empiric agricultural results confirmed that Fabaceous species are used in successive mixed cropping for their capabilities on N fixation.

Parallel to the last experiment, a test using co-culture of *Lupinus albus*-*Betula pendula* showed interesting results in the influence of the acidifying capacity of white lupin over the Mn phytoextraction of white birch. Analysis were made on white birch leaves that where white lupins were not present, then white lupins were introduced and leaves were analyzed. The results indicated concentrations going from 300 up to 6000 mg kg⁻¹ DM on birch leaves growing next white lupins after 1 month (Figure 57).

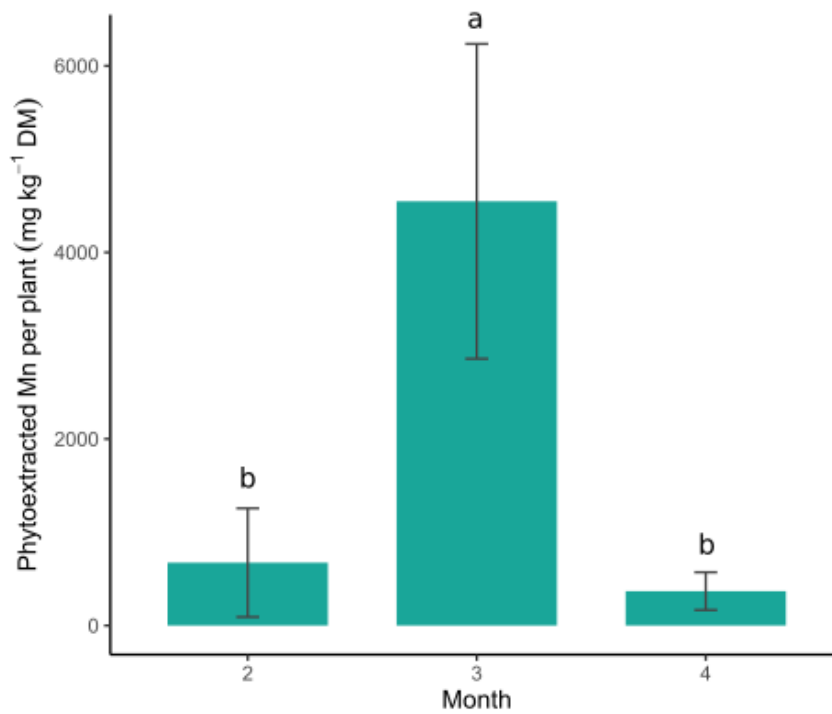


Figure 57. Phytoextracted concentration of Mn per plant of *Betula pendula* when growing after 2 to 4 months with *Lupinus albus*.

This opens the possibility of setting up white lupins along white birch with the aims of Mn solubilization by lupins that could be release into the soil and further taken up by birch. This should be further explored since the influence of the first in the absorption of Mn has not yet been determined. The question raised is, can *Lupinus albus* allow *Betula pendula* to extract most of the Mn solubilized? Only after those tests can the co-culture system between the two species be validated. The validation of this model may involve a sustainable Mn-phytoextractive system for the RG landfill. Another inquiry would be whether other Fabaceous could be used for placing a successive and co-culture systems alongside *Betula pendula*, i.e. *Robinia pseudoacacia*, which was spotted in the site in the vegetation survey of the first experiment?

6.3 Perspectives

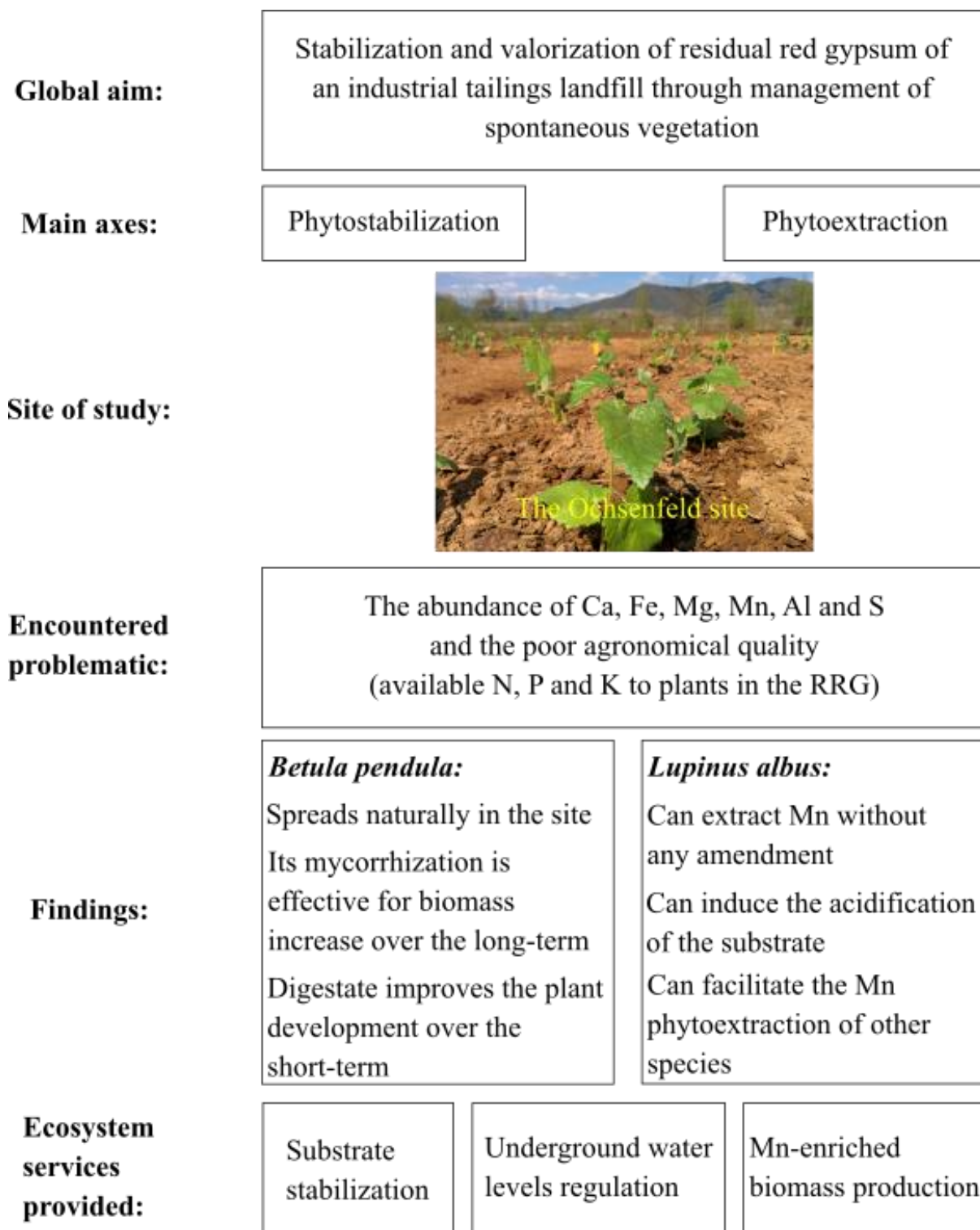


Figure 58. Research findings overview.

The findings gathered during this study form now part of the relevant literature concerning the revegetation of a red gypsum landfill and the possibilities for Mn-enrichment of biomass

through phytoextraction for its valorization as Eco-catalysts (Figure 58). The original questions were answered but some others regarding the field applications arose. The answers of such form now part of the bases for the reclamation of the RRG landfill at the Ochsenfeld site.

The results provided in this dissertation may now be exploited for the management of the residual red gypsum landfill at the Ochsenfeld site. The recommendations for the management of the RRG site may include:

- To establish a systematic collection and culture of *Betula pendula* seeds from plants present *in-situ* in order to create enough supply for an afforestation strategy, considering the recommended density of 1600 stands per hectare.
- To develop a methodology for the application of raw digestate at a maximum dose of 80 mL per L of soil (some 80 000 L per hectare), that may include spreading and soil ploughing, for a short-term stimulation and starting boost of the afforested plants.
- To introduce some mulching prior to the digestate application at each stand placement to ensure the retention of moisture and avoid leaching of digestate.
- To introduce at each *Betula pendula* stand a mycorrhizal inoculum to establish a symbiotic relationship that may ensure the improvement of the agronomical quality of the RRG in a sustainable and self-sufficient fashion over the long-term.
- To introduce winter successive cultures of *Lupinus albus* next to the *Betula pendula* seedlings in order to stimulate the rhizospheric acidification and therefore the Mn phytoextraction in *Betula pendula*.
- To dedicate a small area for the Mn phytoextraction through *Lupinus albus* that would require minimum assistance, but would require supervision and protection from herbivores in order to avoid biomass loss. The use of strengthened plastic nets in small surfaces was found useful.

The suggestions made in this PhD dissertation may not only allow a correct management of the residual red gypsum landfill at the Ochsenfeld site through *Betula pendula* and *Lupinus albus*, but also could be extrapolated in landfills of other TiO₂ extraction plants around the globe.

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