N° ordre : 42370

UNIVERSITE LILLE 1, Sciences et Technologies
Ecole doctorale SMRE- Science de la Matière, du Rayonnement et de l'Environnement
Laboratoire de Génie Civil et géoEnvironnement-LGCgE

Thèse

présentée par

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En vue de l'obtention du grade de
Docteur de l'Université des Sciences et Technologies de Lille 1
Discipline : Géosciences, Ecologie, Paléontologie et Océanographie

Interactions Sol-Plante dans un contexte de phytomanagement de sols pollués par des métaux : Application à *Miscanthus x giganteus*

Date de soutenance publique : 5 mai 2017

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Remerciements

En écrivant ces mots, je suis à quelques étapes près de finir une autre phase inoubliable de ma vie, «**la thèse** » et habilitée avec toutes sortes d'enthousiasme et de détermination dans le but de commencer un nouveau chapitre qui est « la vie professionnelle ». Mais avant de tourner cette page, quelques mots de gratitude devrait être dit à certaines personnes qui se tenaient à mes côtés et ont participé à l'écriture des lignes et lignes de travail acharné, à la diffusion de la joie et de rires, même à des moments difficiles.

Tout d'abord, je tiens à exprimer ma sincère gratitude à **l'Association de spécialisation et l'orientation scientifique**, pour leur bourse, sans laquelle il aurait été impossible de faire cette thèse. Également je voudrais remercier ISA Lille et les gens la bas pour leur gentillesse, leur amabilité et leur hospitalité.

Aussi, Je remercie les membres de jury de ma thèse, Madame Valérie Bert et Messieurs Thierry Lebeau, Eric Pinelli et Jean Louis Hilbert, pour avoir évaluer mon travail et m'avoir donné l'autorisation de le défendre.

Passer au laboratoire, à l'endroit où j'ai passé les trois dernières années, à partir du 7 janvier 2014, le jour de mon arrivée à Lille. Pour être honnête, quand j'avais quitté le Liban, je n'avais aucune idée de ce qui m'attendait, en tenant compte que je n'avais pas l'habitude de parler en Français. Pour cela, j'avais un peu de peur au début, mais, j'avais tellement de chance de rencontrer et de se joindre à un groupe de gens assez modestes, accueillants et aimables, avec qui j'ai eu beaucoup de magnifiques souvenirs inoubliables.

Je suis excessivement reconnaissant à mon directeur de thèse Francis Douay et mon co-encadrant Bertrand Pourrut, qui m'ont accepté au début d'être un membre de l'équipe et m'ont aidé tout au long du chemin à atteindre et à achever mes objectifs. Nous avons partagé beaucoup de moments que je n'oublierai jamais. Peut-être que je n'ai pas eu de la chance de vous le dire avant, c'est grâce à vous que j'ai beaucoup appris et j'ai acquis assez de connaissances et des leçons qui m'accompagneront pour toujours. Merci beaucoup... J'espère que vous me souviendrez pour si longtemps, et que j'ai laissé un bon impact en vous deux...

Un grand merci à Sébastien, Aurélie, Geraldine, Arnaud et Christelle pour être autant aimable et respectueux. Si bien que nous n'avons pas travaillé ensemble, mais les groupes de discussion et de rire dont vous faisiez partie, que soit dans les pauses café ou soit dans les pots, resteront graver dans ma mémoire à jamais. Je suis également reconnaissant envers, Karin qui était toujours là, à chaque fois que je lui avais besoin pour un conseil statistique ou une explication et David, qui était toujours gentil, doux et serviable tout au long de ces années. Christophe, l'homme qui nous accueillerait toujours avec un grand sourire sur son visage.... Je te remercie pour ta touche personnelle dont tu as faites ainsi que pour ton aide et spécialement durant les derniers jours avec spectro. Je n'oublierai pas le sens d'humour

d'Hervé et son aide dans l'échantillonnage du sol et aussi bien la gentillesse de Pierre-François ... Une vive appréciation va à Brice qui m'avait appris beaucoup, et grâce à lui, ma première partie de la thèse et donc le premier article ne seraient pas pu être atteints sans son aide, ses conseils et son coopération. Notre sortie de groupe ont été aussi amusant et j'ai eu de la chance de te plus connaître et de découvrir ta gentillesse et ton bon sens d'humour. Adeline, ma collègue que j'ai passé avec elle la plus longue période, nous avons partagé le même bureau, j'ai eu de la chance et suis ravi de te reconnaître... Je n'oublierai pas, notre pause de fumer à 15:00, notre sortie ensemble avec la team, nos innombrables bons moments et ceux qui étaient mauvais ... Ils resteront pour toujours et à jamais... Bonne chance mon amie dans ton travail puisque tu mérites tout le meilleur. Les Dransart (Julie et Mathieu), merci beaucoup mes copains, puisque j'ai eu de la chance d'assister à cette belle occasion « un mariage Français » et mieux connaitre vos traditions... J'étais tellement heureux de partager ces beaux moments avec vous, aussi bien que d'autres moments qui étaient pleins de joie et de bonheur…. Xuan et Marie, les nouveaux doctorants, bien que nous nous sommes rencontrés il y a peu de temps, mais on a passé aussi des moments amusants et cool et nous avons déjà fait quelques bons souvenirs ensemble. Julien... Je me souviens bien des jours d'échantillonnage, je peux te dire que t'étais l'un de mes premiers tuteurs de français et c'est pour cette raison que je te remercie forcement. Je n'oublierai jamais nos sorties, les jeux de football et les quelques semaines qu'on a passé ensemble au gymnase lorsque tu étais mon coach... Tu es vraiment un ami dont les gens peuvent compter sur... Ta connaissance est un grand honneur pour moi. Finalement, Sarah, j'ai beaucoup de choses à te dire, mais parfois les mots ne peuvent pas exprimer les idées, mais j'essayerai quand même. Permettez-moi te dire que ta connaissance est l'une des meilleures choses qui m'est arrivée durant toute cette période ... Merci pour nos nombreuses discussions et tout ce que tu m'avais fait, ton aide, être là pour moi et me soutenir quand j'ai due besoin, … Nous avons partagé beaucoup de souvenirs mémorables, des sorties bien que d'autres moments ... J'espère que nous garderons cet esprit pour toujours ...

Je vais essayer ne pas d'oublier aucun de mes amis qui m'avaient soutenue durant toute cette période et avec lesquels nous avons vécu de beaux souvenirs inoubliables ... Je vais commencer avec Zohour et Sarah, les plus belles, les plus gentilles et les plus compatissantes filles que j'ai connu. Je vous remercie du fond de mon cœur d'avoir été toujours là pour moi... Je n'oublierai jamais tous les moments qu'on a passé ensemble que ce soit agréables ou tristes. Je suis très chanceux d'être votre ami. Rami et Manon, je vous remercie beaucoup pour ce que vous m'avez fait, votre amitié, vous avez été si aimable et serviable dès le moment où je me suis arrivé... Je n'oublierai jamais votre aide au début et grâce à vous, j'ai pu trouver mon garant Ali, à qui je suis tellement reconnaissant... Aussi, nous avons partagé quelques moments sportifs (basket) ensemble, des longues discussions politiques intéressantes et également les mercredi soir pour regarder les films avec d'autres amis. Mahmoud, Roba et Sabet, bien que nous nous sommes rencontrés il y a peu de temps, mais je suis très reconnaissant pour être des vrais amis dont je peux compter sur. Zeinab, même si nous n'avons pas eu assez d'occasion de se rencontrer pendant ces trois années, mais je

n’oublierai jamais le fait que nous parlions presque tous les jours ainsi que tu as été là pour me soutenir et m’encourager. Je tiens à te remercier et j’espère de te revoir prochainement.

Enfin, sans le support de ma belle et précieuse famille, je n'aurais pas été en mesure d’être à la place où je me suis maintenant. Nour et Rami, vous ne pouvez pas s’imaginer ma joie le moment que j’ai su que vous allez me partager la journée de ma défense et se tenir à mes côtés. Je vous remercie tellement mes chers, votre présence signifie beaucoup pour moi. Wassim, mon petit frère, je te remercie pour le soutien que tu m’as donné, nos discussions sur les sujets de sport et autres ... Je te souhaite une bonne chance dans ton avenir. Maman et papa... l’amour inconditionnel, la lumière de mes yeux, mon âme, tout ce que je suis tellement grâce à vous. Les sacrifices que vous faites, les nuits et les moments difficiles que vous aviez passé afin de nous soutenir… dire merci et tous les mots sont très peu pour vous s’exprimer combien je vous suis énormément reconnaissant. À tous ces moments, à vos larmes, à votre amour infinie, je dédie ce travail...

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General introduction

Anthropogenic activities (mining, industrial activities, combustion of fossil fuels, waste disposal, agriculture, etc.) are the main sources of soil metal pollution (Gomez-Sagasti et al., 2012; Panagos et al., 2013). The corresponding pollution is considered as a global critical environmental issue, which draws the attention of various social groups (local authorities, researchers, environmentalists, and ordinary people), due to the diverse detrimental effects it creates on plants, animals, soil microorganisms and even human health (Singh and Kalamdhad, 2011). It is noteworthy to mention that the areas in the vicinity of the pollutant sources are more exposed to contamination of air, water and/or soil (De Bartolomeo et al., 2004; Miro et al., 2004).

The agricultural lands in the proximity of the former lead smelter "Metaleurop Nord" (Northern France), in function for more than a century and which had emitted large quantities of metals mainly Cd, Pb and Zn to atmosphere, are an explicit example about the negative impacts of pollution sources. In details, a vast area around the corresponding smelter (120 km^2) was subjected to considerable levels of metal contamination as a reflection of the smelter atmospheric emissions and deposition of dust particles (ADEME, 2011). Consequently, the agricultural topsoils had been heavily contaminated mainly by Cd, Pb and Zn. Most of the food crops do not meet the European threshold values for Cd and Pb (Douay et al., 2013), and several human diseases and dysfunctions were documented due to the chronic exposure to the corresponding metals (ADEME, 2011). Therefore, the remediation of such degraded area is a great challenge, taking into consideration that the corresponding pollutants are miscellaneous, persistent, and widespread.

Due to the inconvenience of the conventional physico-chemical remediation methods to cleanup this large area (high costs, environmental-unfriendly, negative impacts on the ecosystem, and mainly the surrounding environment), the choice went towards the development of innovative i*n situ* cost-effective and environmental-friendly methods that might ameliorate the ecological state of the contaminated area and reducing the corresponding metal hazards (Mench et al., 2010). One of these techniques is the *in situ* phytostabilization, based on the metal-tolerant plants able to precipitate metals and/or induce metal complex formation in rhizosphere and/or accumulate them in their root tissues, reducing their translocation to aerial organs, consequently reducing their mobility, leaching, bioavailability and land erosion possibilities in such large areas (Mench et al., 2010).

In the scope of finding the most suitable technique for reclaiming the large contaminated area, several plants species had been investigated. Among which, the herbaceous ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) that exhibited phytostabilization potentials via limiting the metal transfer to their aerial parts over time and relatively tolerating the stress arisen (Bidar et al., 2007). Another approach was the aided-phytostabilization, combining the use of five woody species (*Robinia pseudoacacia*, *Alnus glutinosa*, *Quercus robur*, *Acer pseudoplatanus*, and *Salix alba*) and fly ash amendments. It

demonstrated successful afforestation of the site, along with metal availability reductions, especially in the amended plots (Lopareva-Pohu et al., 2011; Pourrut et al., 2011). However, the economic interest of these crops were rather limited, which restraint the phytomanagement development in a contaminated area as large as the Metaleurop area.

Therefore, depending on the previous outcome, the cultivation of the bio-energy crop *Miscanthus x giganteus* seemed to be a suitable choice for the phytostabilization in this context (Nsanganwimana, 2014). The C4 perennial, rhizomatous lignocellulosic noninvasive grass demonstrated high tolerance to the wide spectrum of metals and mainly accumulated them in its underground parts, thus reduced their mobility, transfer and bioavailability thereby alleviating the human and environmental hazards. In the frame of the Phytener project and the PhD work of Nsanganwimana (2014), miscanthus showed high potentials to growing well and proliferating in the highly contaminated agricultural plots surrounding Metaleurop smelter and thereby yielding high biomass which might be invested economically (energy and biofuel production, building and construction materials and diverse agricultural uses).

Previous works on the miscanthus plants have demonstrated its positive impacts on limiting the metal mobility and transfer via the high capacity of accumulation in the belowground parts (Nsanganwimana et al., 2014, 2015). Miscanthus plants played a significant role in decreasing the extractability of the metals from the soil to the aerial organs (Nsanganwimana, 2014). Its contribution in increasing the soil organic carbon pool has been also proved in several papers (Hromádko et al., 2010; Técher et al., 2012). Finally, miscanthus plants have played a significant role in the metal redistribution in the different soil fractions which eventually decreased the human oral bioaccessibility (Pelfrêne et al., 2015). However, the impacts of the plant on restoring the degraded soil functionality and biological parameters have never been investigated.

Moreover, it is noteworthy to mention that the choice of late miscanthus harvest was taken in the contaminated agricultural of Metaleurop. However, little is known about the impact of accumulation of the leaf litter debris aver the topsoils on the soil parameters, nutrient cycle and mainly the speciation and mobility of the metals. In addition, the health impacts and the metal accumulations in the crops supposed to succeed the miscanthus culture after 15 or 20 years of cultivation represent great concern especially in the context of a highly metal contaminated area such as Metaleurop.

Finally, as mentioned before, the efficacy of miscanthus plants in phytostabilization of metal contaminated soils has been demonstrated. It has shown good potentials in tolerating the high soil metal concentrations, yielding considerable biomass in the contaminated fields and mainly accumulating less metal quantities in its aboveground organs in comparison to the underground ones (Nsanganwimana et al., 2015, 2016). However, the impact of the corresponding contamination on the plant health and resistance mechanisms was not investigated, or if investigated it was in hydroponic or soil metal spiked media, which might

not reflect the real field conditions, or for a period not exceeding 2 to 3 months or one growing cycle as a maximum.

Therefore, the current thesis is considered as a continuity of the previous works launched in the Metaleurop site. However, it is to go a step forward in the scope of research in an attempt to investigate *Miscanthus x giganteus* impacts on restoring the degraded contaminated soil functionality via studying certain physico-chemical and biological parameters in an *ex situ* pot experiment. Moreover, the impact of the late harvest and miscanthus leaf fall was extrapolated by (i) performing an *ex situ* artificial aging process under controlled laboratory conditions, (ii) studying the modifications occurring in soil physico-chemical, biological parameters as well as the metal availability, and (iii) evaluating the health of the succeeding crop that is supposed to replace the miscanthus culture after certain period of time. Finally, the response of the miscanthus plants to the soil gradient metal contamination was also investigated in an *ex situ* experiment during the first (5 months) and the second growing cycle (18 months), via studying plant health using several biomarkers.

In order to facilitate its reading, this manuscript is divided into six parts. The first part (Part A) introduces the contamination issue in the Metaleurop Nord megasite. It highlights the complexity to manage this site, and summarizes the phytoremediation experiments that have been led on site. The second part (Part B) presents the materials and methods, in which we describe in details the experimental *ex situ* set up, modes of soil and plant sampling, as well as the different laboratorial analysis.

The part C discusses the impacts of miscanthus on the soil agronomic parameters and comprises two chapters. Chapter C-1 sheds the light on the impacts of the miscanthus root exudates on restoring the contaminated soil functionality, via studying the evolution of different soil parameters (physico-chemical parameters, metals availability, microbial biomass and certain biological activities) after one year of cultivation in the *ex situ* experimentation. The second chapter (chapter C-2) investigates the impacts of the late harvest, over a long period, on the soil agronomic parameters and metal behavior taking into consideration the considerable amounts of organic debris of miscanthus leaves accumulated during 20 years of miscanthus cultivation. The impacts on the health of the crop that is supposed to replace the miscanthus crops on long term are investigated as well.

The part D of this work studies the influence of the soil metal contamination on the *Miscanthus x giganteus* health, via studying metal accumulation in leaves, plant growth and certain stress biomarkers after a first growing cycle (chapter D-1) and a second growing cycle (chapter D-2).

The part E refers to the general discussion based on the outcome of the work done, and elaborates certain remarkable points demonstrated in the previous chapters. Finally, the part F presents the conclusions inferred from the entire work and proposes certain

perspectives of work that might be beneficial to investigate further *Miscanthus x giganteus* potential to phytomanage contaminated areas.

Part A- General context, study site and thesis objectives

A.1- General context

Polluted sites by current or former human activities present a real or potential risk to the environment. In France, there are 4142 polluted site, of which 571 present in the department of Nord-Pas de Calais representing 13.8 % of the total (CGDD, 2013). Of these 571 sites, the "Metaleurop Nord" site is located between Lens and Douai, in the heart of the former coal-mining area (Figure 1), and is considered as one of the most contaminated site in Europe.

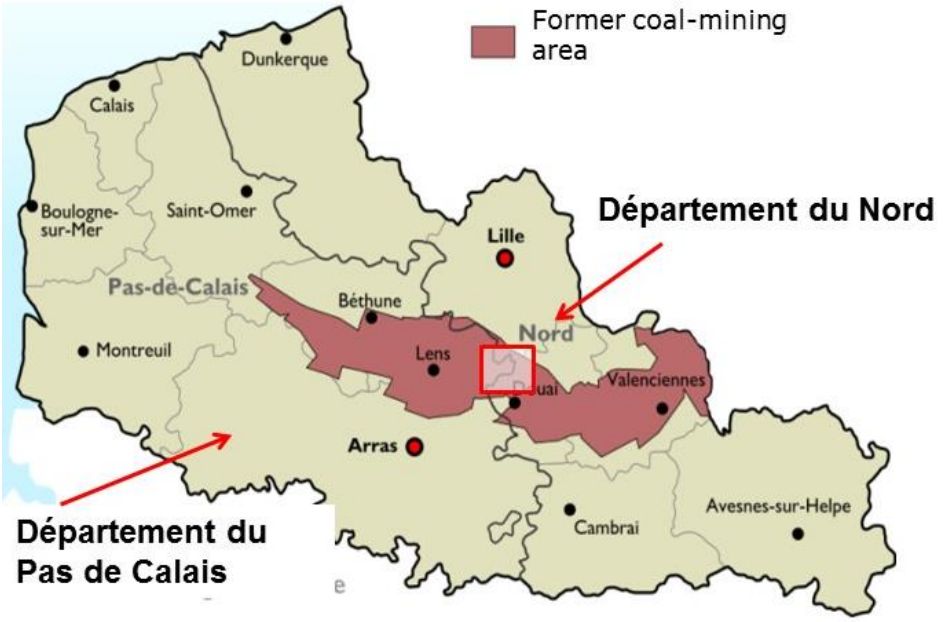

Figure 1: Location of the Metaleurop megasite (red square) in the former Nord-Pas-de-Calais French Region

For more than a century, the area around Metaleurop Nord has been suffering from atmospheric metallic emissions and deposition of the former lead (Pb) smelter Metaleurop Nord in Noyelle-Godault and the zinc (Zn) foundry Nyrstar, formerly known as Umicore, in Auby. The productions of the Metaleurop Nord reached their highest values during the 70s (150000 t yr^{-1} Pb and 100000 t yr^{-1} Zn, representing 2/3 and 1/3 of the national production). In addition, the Noyelles-Godault smelter became the world leading producer of germanium (Ge, 20 t yr^{-1}) and indium (In, 50 to 70 t yr^{-1}), in addition to the production of cadmium (Cd, 400 t yr^{-1}), silver (Ag, 210 t yr^{-1}), arsenic (As), antimony (Sb) and sulfuric acid (H_2SO_4, 245,000 t yr^{-1}) (Mazade, 2010).

The consequences of the industrial activity were the massive release of contaminated dusts from the site by both channeled dust emissions (Figure 2), and diffuse emissions. The first was the result of dust evacuation from the chimneys of the plant and had represented 75% of metal emissions from the site (Mattielli et al., 2009). The second resulted from the suspension of particles by wind and the movement of the handling equipment.

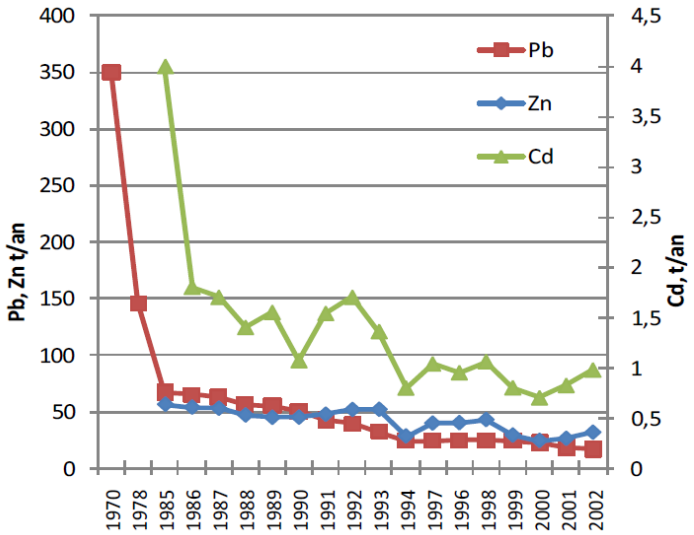

Figure 2: Evolution of the atmospheric canalized emissions from Metaleurop Nord between 1970 and 2002 (DRIRE Nord - Pas-de-Calais, 2003)

After this period, under the pressure exerted by the public and the local authorities, the two plants were forced to undergo certain operational modifications to reduce the quantities of metal emissions. Gas filters were replaced or modernized in the Metaleurop Nord smelter (Souto, 1996) and the pyrometallurgical process at Nyrstar was replaced by an electrolytic process (hydrometallurgy) which considerably limited the metal emissions into the atmosphere (DRIRE Nord-Pas de Calais, 2002). It is noteworthy to mention that the Nyrstar plant is still active until nowadays. However, Metaleurop Nord was forced to close in 2003 due to the economic crisis and lack of competitivity with other producers (mainly in China) (Mazade, 2010). Despite the closedown, the surrounding area is still suffering from the negative impacts originated by the one hundred years of metallic emissions.

A.2- Complexity of Metaleurop Nord megasite: specific case of agricultural lands

According to The Megasite Management Guideline (http://www.ufz.de/mmt-guideline-en/), Metaleurop Nord contaminated area can be considered as a megasite, due to the complexity to manage the site: very large contaminated area; complex contaminations in soils; varying metal concentrations; multiple stakeholders with various interests and goals to be involved in the management; economic and planning at all relevant (regional and local) levels must be considered; long-term management needed.

A.2.1- A large contaminated area

Early in the 1980s, the first mapping of the soil contamination around the two foundries showed the high topsoil metallic contamination (Luttringer and de Cormis, 1979; Sarcia and Talbot, 1984). Later on, intensive research work was launched in relation with a multidisciplinary program initiated by the Region of Nord-Pas de Calais, covering and investigating different themes: impacts of metallic pollutants on environment, population health, and economic consequences.

The soils at the Metaleurop megasite have been showed as highly metal contaminated, acquiring Cd, Pb and Zn concentrations 20-50 times higher in the ploughed soil horizons (0-25 cm) than the regional backgrounds (Sterckeman et al., 2002). However, the degree of contamination is not homogeneous all over the site and depends on the distance from the source, in which the metal contamination decreases as the distance from the smelter increase (Douay et al., 2011). The total surface affected by the emissions from the two sites, over a period of more than one century, is estimated over 120 km² (Figure 3).

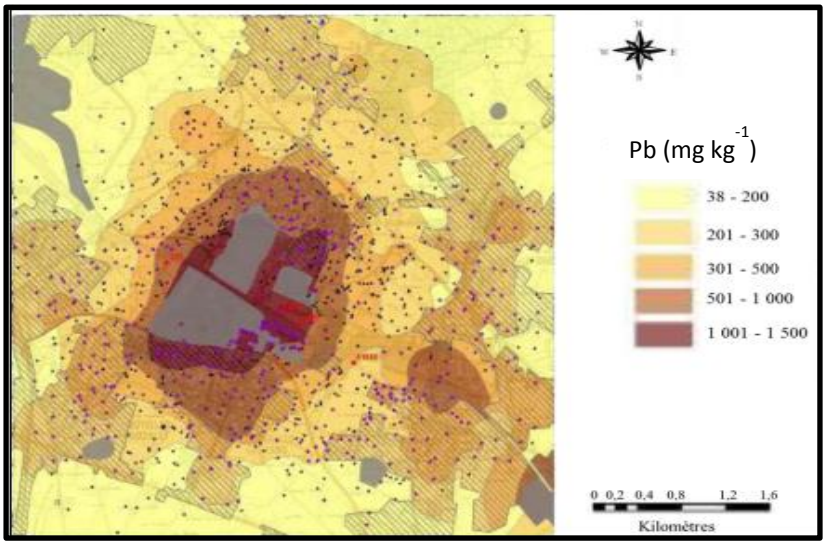

Figure 3: Map of Pb isoconcentration in soils of the Metaleurop site (Douay et al., 2011)

The surface of agricultural soils contaminated by more than 200 mg kg^{-1} Pb in the ploughed horizon is estimated at about 750 hectares, among which certain area near the old smelter with levels exceeding 1000 mg kg^{-1} Pb (Figure 3).

A.2.2- A complex geo-pedo-morphology

The management complexity of the Metaleurop Nord megasite is not only based on soil contamination extend, but also on the strong differences in soil types encountered in this area. Actually, the contaminated area covers over three natural regions well defined by their physical characteristics (Figure 4):

- the North, constituting a plain with geological substratum consisting of sandy and clayey tertiary formations;
- the central part, with the alluvial valley drained by the Deûle canal and extended to the East by a vast colluvium plain, the Plain of the Scarpe;
- the South, comprising a large plain with cretaceous calcareous substrate covered by quaternary silts whose thickness often reaches several meters.

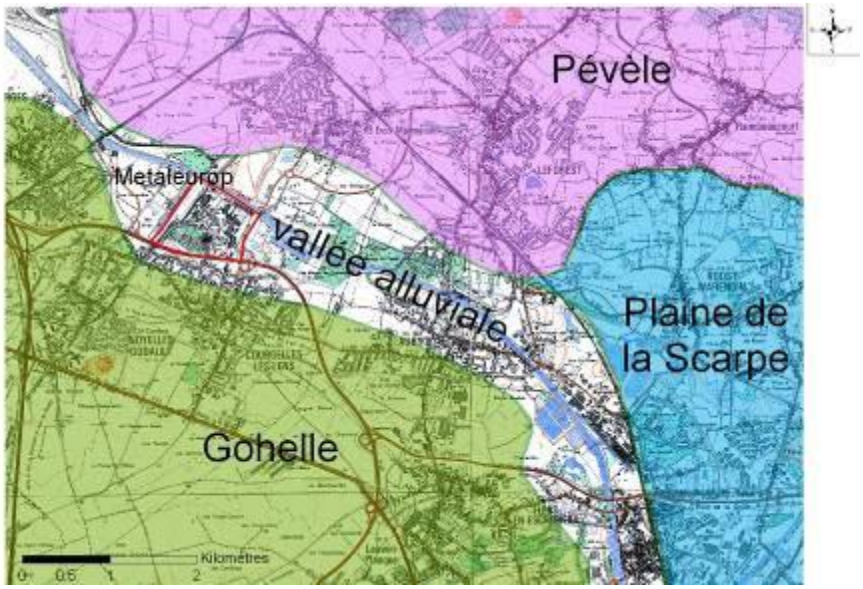

Figure 4: Geomorphologic units of the site

A.2.3- Various land uses and numerous stakeholders

The contaminated area is characterized by intensive periurban agriculture along with former coal-mining activities as well as present or former industrial activities, a very dense infrastructure network (canals, rails, roads, and highways), and urban areas (Figure 5). This complex land use can be classified into four main areas: residential, agricultural, leisure (park, sportive facilities…), and artisanal and tertiary sector. The agricultural activities represent a bit less than 45 % of the area studied, comprising essentially ploughed plots.

Figure 5: Metaleurop Nord megasite territory

The area of interest is also characterized by a strong urbanization counting around 55,000 inhabitants, half of which live in the municipalities of Noyelles-Godault, Courcelles-lès-Lens, Evin-Malmaison, and Leforest that are in close vicinity to the former smelter where the soil contamination is in the upper limits (Figure 6). However, five other cities also surround the former smelter and are affected as well.

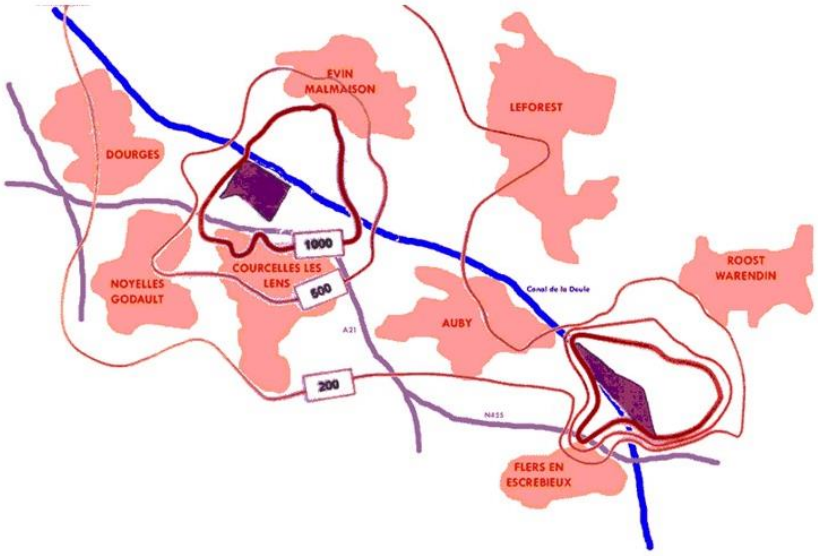

Figure 6: Location of the height cities included in the Metaleurop megasite. Red lines represent the Pb isoconcentration curves (200, 500 and 1000 mg kg^{-1})

In addition, it is worth noting that the administrative border between the Département du Nord and the Département du Pas-de-Calais crosses the contaminated site (Figure 1), which adds another constraint when stakeholder involvement is needed. This very important number of private (inhabitants, companies, associations…) or public (Region, Départements, cities) stakeholders, with different goals and interests makes it difficult for decision makers to implement certain contamination control techniques.

A.2.4- Human and environmental risks on agricultural soils

A.2.4.1- Food crop contamination

Numerous studies have been led to investigate the level of crop contamination before Metaleurop closedown in 2003 (Des Ligneris et al., 1999; Douay et al., 2011, 2002a, 2002b; Douay and Pruvot, 2006; Luttringer and de Cormis, 1979; Souto, 1996). They demonstrated that most of the food production was contaminated by Cd and Pb, and their contaminations exceeded French and European threshold values. However, since then, research about the soil metal contamination impact on agricultural crop quality has been scarce.

After the closure of the smelter in 2003, some investigations were launched to assess the impact of the cessation of the smelter activities on Cd and Pb concentrations in wheat and grain in 15 plots surrounding the smelter (Douay et al., 2008). Statistical analysis of the results showed that the Cd concentrations of the straws decreased significantly, whereas those of the grains did not evolve. For Pb, grain and straw concentrations were significantly lower than in 2003. Among the 15 grain samples, 13 had Pb concentrations below the limit of detection (0.2 mg kg^{-1} DW). However, with respect to Cd and Pb concentrations, 80% of the wheat grains were not suitable for human consumption. This rate was 90% during the activity of Metaleurop Nord (Douay et al., 2011).

Another study was established in 2010, 7 years after the closure of the smelter, to determine the agricultural crop contamination for a better assessment of the population exposure to metals (Douay et al., 2013). The investigated crops were wheat (grain and straw), barley (grain and straw), maize (grain and forage), horse bean, potato and sugar beet. The results showed that highest metal concentrations were detected in wheat straw and foraged maize, and to a lesser extent in wheat grain, horse bean, barely straw and sugar beet, while the lowest concentrations were recorded in barley and maize grains as well as potato. However, most of the Cd and Pb concentrations were beyond the threshold values stated by the European commission for human consumption (European Commission, 2006).

A.2.4.2- Human risks linked with soil exposure and dust accumulation

Food crop contamination is not the only problem to tackle on the contaminated agricultural area of Metaleurop. Direct exposure with contaminated soils (and especially soil ingestion

via hand-to-mouth contact by children) and dust emissions are major issues that need to be taken into consideration for the future management of the site. Indeed, investigations on Pb concentrations into child blood revealed that, despite Metaleurop closedown, the concentration was still considered relatively high (40 µg L^{-1}) in comparison with the national standards (28 µg L^{-1}) (Chatelot et al., 2008). Moreover, 1.4% of children of this area possessed a high blood lead concentration of more than 100 µg L^{-1} of Pb in the blood (Declercq and Ladrière, 2004).

This problem can be explained by several factors including soil ingestion or dust inhalation. Indeed, using the Unified Barge Method to mimic oral exposure pathway and the effects of the human digestion process, the works of (Pelfrene et al., 2012, 2011) showed that Cd and Pb were moderately (during the gastrointestinal phase) to highly (during the gastric phase) bioaccessible, conversely to Zn. Moreover, despite Metaleurop closedown, dust particles collected in the surrounding area are still contaminated by considerable concentrations of metals and present an important hazard to human health (Pelfrêne, 2016). Oral and pulmonary bioaccessibility tests were established to evaluate the associated human risks. Results showed that the bioaccessible Cd and Pb portions are still high (respectively 74% and 69%) in the dust particles in the Metaleurop megasite (Pelfrêne, 2016).

A.2.4.4- Environmental impact

Along with the soil metal contamination, soil dysfunction was observed in the most contaminated agricultural soils, expressed by the highly elevated C/N ratio. This slowing down of organic matter degradation has been correlated to the decrease in fauna wealth as well as the microbial activities (Pruvot et al., 2002). Moreover, different investigations were launched to determine the impacts of the soil metal contamination in the Metaleurop site on *Eisenia fetida*. Results showed that the concentrations of the Cd, Pb and Zn strongly increased in *E. fetida* upon their exposure to the high metal concentrations which caused a great modification at the level of gene expression in *Eisenia fetida* (Bernard et al., 2010; Brulle et al., 2011, 2008). Another study, performed on the site 10 years after the smelter closure, showed that the earthworm communities were composed of few species with moderate abundance in comparison with communities found in similar habitats outside the metal-contaminated area (Grumiaux et al., 2015).

The impact of the soil metal contamination in the megasite was also assessed on terrestrial fauna. The natural impregnation of Zn, Pb and Cd of various organisms belonging to different classes was assessed by sampling at 21 sites polluted with varying degrees of metal concentrations (Pruvot et al., 2009). The results showed elevated metal concentrations measured in the organisms collected primarily near the former Metaleurop site. Field experiments conducted by Fritsch et al. (2011) showed that the metal bioaccumulation in snail populations (*Cantareus aspersus* and *Cepaea nemoralis*) increased with the increase in the soil metal concentration. The contaminated snails possessed heavier shells upon metal

exposure in comparison to uncontaminated controls. The indirect effects of the soil metal contamination on woodlice (*Porcellio scaber*) were also assessed. Results clearly demonstrate the negative effects of the contaminated poplar litter collected from the woody habitats in the area on the growth and metal accumulation of this terrestrial isopod (Godet et al., 2012, 2011).

Several studies were applied on animals as well and demonstrated the negative impacts of contamination on their health. For example, Coeurdassier et al. (2012) showed that the European blackbirds (*Turdus merula*) coming from the Metaleurop site exhibited high residues of Cd and Pb in their blood, and the highest proportions (73 and 99% respectively) were associated to erythrocytes. Moreover, Fritsch et al. (2010) showed that the wild small mammals in Metaleurop accumulated metals in both liver and kidneys. These authors also demonstrated that the internal concentrations of non-essential metals used to increase with the increase in the soil metal concentrations for the wood mice, bank vole and the common shrew. Finally, Tête et al. (2014) demonstrated an increase in the Cd and Pb concentrations in liver and kidneys of wood mice present in the vicinity of the former smelter accompanied by histological alterations.

Plant health was negatively affected as well because of the metal contamination prevailing in the Metaleurop area. An *ex situ* and *in situ* experiments were carried out on two herbaceous models by cultivating a grass (*Lolium perenne*) and a legume (*Trifolium repens*) in contaminated agricultural soils (25 mg Cd, 1 200 mg Pb and 1 250 mg Zn kg^{-1}) originating from the polluted area (Bidar, 2007). The obtained results showed an increase in the plants metal concentrations beyond the background values, an augmentation in malondialdehyde (MDA) production as well and significant decrease and modification in the fatty acids contents upon the metal exposure (Bidar et al., 2009, 2008).

A.3– Management of agricultural fields in the Metaleurop Nord megasite

The Metaleurop megasite poses a threat to soil resources, causes considerable environmental and health risks as well as economic and social (ADEME, 2011). Its efficient and sustainable management requires innovative investigation and remediation technologies as well as integrated assessment approaches in order to optimize reuse visions for a viable and sustainable local and regional development. The critical situation in the Metaleurop area united the state services, farmers, local authorities, industrials, and researchers, to find satisfactory solutions to limit the environmental and health hazards of the corresponding pollution, while conserving and enhancing agricultural productions in this degraded area. Taking in consideration the vast polluted surface, the conventional methods are not applicable. Therefore, the approach was to implement techniques able (i) to decrease the mobility and bioavailability of metals, (ii) to reduce contained dust emissions, (iii) to limit the access to contaminated agricultural soils by maintaining an activity, and (iv)

to be socially and economically acceptable. For almost twenty years, the use of plants associated or not with soil amendments has been considered as a suitable choice to manage this area.

A.3.1- Principle of phytomanagement

In the context of management of contaminated sites, phytotechnologies can be chosen and evaluated taking into account not only their remediation efficiency but also integrating a socio-economic value (Figure 7). Indeed, sustainable solutions need to meet societal and economic expectations.

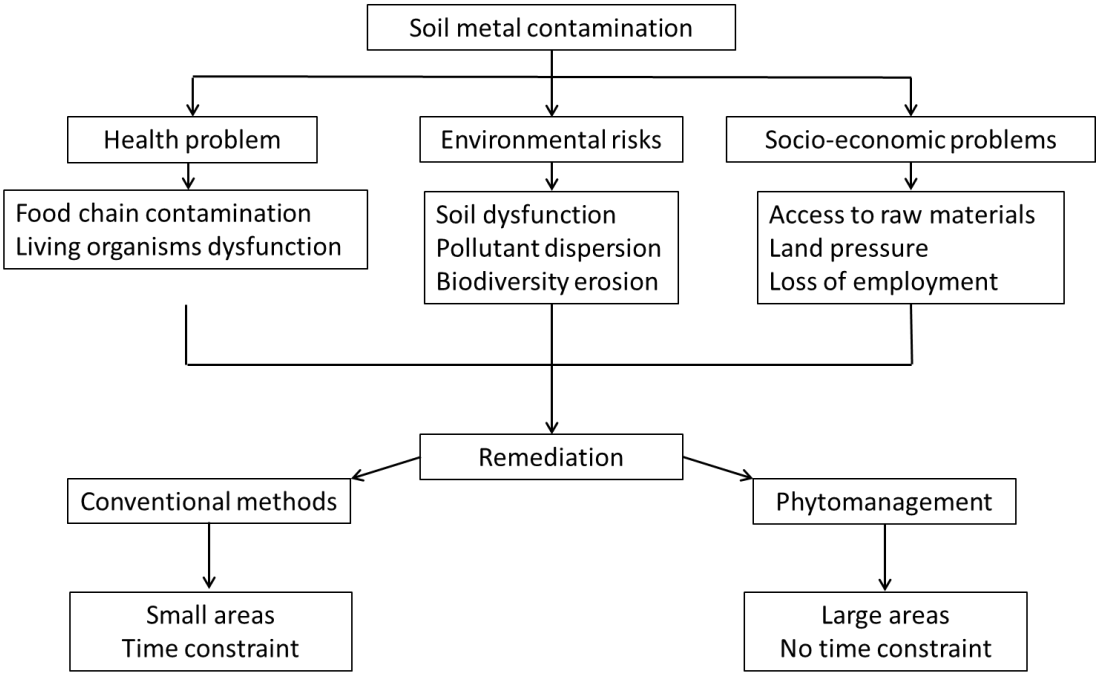

Figure 7: Conceptual framework of phytomanagement (adapted from Nsanganwimana, 2014)

The primary objectives of phytomanagement are (1) to clean up contaminated matrices including water, effluent, soil, sediment, mining waste and air (Nsanganwimana, 2014), (2) to improve food safety (Singh et al., 2011), (3) to restore ecosystem services, and (4) to meet economic needs with the production of renewable raw materials for industrial use and low impact on greenhouse gas emissions (Conesa et al., 2012; Evangelou et al., 2012). The biomass from contaminated lands can be implemented in many fields including the production of renewable energies (biofuels) and various eco-materials. Moreover, the production of high biomass bio-energetic plants on marginal lands, where food crop could not be cultivated (or badly), increases the biomass crop surfaces without creating controversy (food crop vs biomass crop). For these reasons, phytomanagement might be considered as environmentally friendly and well perceived by the populations because it

takes into account both environmental, societal and economic expectations and can constitute a sustainable management mode integrating an ecological and economic viability.

A.3.2- Main phytomanagement trials applied in Metaleurop site

Phytomanagement incorporates a range of phytoremediation techniques which differ according to the process by which plants remove, immobilize, or degrade contaminants (Pilon-Smits and Freeman, 2006). In the approach for phytomanaging the Metaleurop site, two different phytoremediation techniques were trialed: the phytoextraction and phytostabilization processes.

A.3.2.1- Phytoextraction

Phytoextraction involves the use of plants for the purpose of extracting metals from soils and concentrating them in aerial parts which are then harvested (McGrath and Zhao, 2003). The corresponding metal accumulation in plants depends on the nature and speciation of metals, the soil physico-chemical parameters and the species and varieties of the plants (Tangahu et al., 2011). Until recently, studies on phytoextraction focused mainly on the use of hyperaccumulating plants. However, hyperaccumulating plants are often plants that have slow growth and therefore low biomass production (McGrath et al., 2002). These reasons explain today interest in non-hyperaccumulative species capable of producing significant biomass on contaminated soils. To be used in phytoextraction, an ideal species must possess the following characteristics: high biomass production, high metal bioaccumulation and rapid implantation and propagation as well as tolerance to variations in climatic conditions (Tangahu et al., 2011).

a) Herbaceous plants

Arrhenatherum elatius, known as "French ryegrass", is a cosmopolitan and ubiquitous perennial species that has all the characteristics to be a potentially ideal species for phytoextraction (Deram et al., 2000). In Northern France, this species grows on unpolluted sites as well as on polluted sites, which is characteristic of plants called pseudo-metallophytes. This species has been shown to be effective in phytoremediation operations on cobalt, copper and nickel polluted soils or on Pb-rich soils (Deram et al., 2000). Following these first encouraging results, Deram et al. (2006) investigated the differences between and within several *A. elatius* populations in terms of Cd and Zn accumulation, in order to judge the potential for improvement of this species for phytoextraction in the areas around Nyrstar and the slag heap of the former Metaleurop smelter. To this end, the Cd and Zn accumulation capacities of nine populations of *A. elatius* were investigated. The accumulation of Zn in the aerial parts of the *A. elatius* was statistically different depending on the origin of the population. It has been shown that the accumulation of Zn in the aerial parts of *A. elatius* ranged from 150 to 2 100 mg kg^{-1} DM. However, these concentrations

remained well below those of a plant described as Zn hyperaccumulator (> 10 000 mg Zn kg^{-1} DM). The same remark can be said concerning the Cd accumulation, in which it varied between 83 ± 75 and 123 ± 41 mg Cd kg^{-1} DM. Overall, these results combined with others from a study on seasonal variation in metal accumulation (Deram et al., 2006) were quite deceiving and did not support this plant as a suitable candidate for the phytoextraction of Metaleurop area.

b) Woody plants

Since the 95's, in order to exclude agricultural production on the most contaminated soils, Metaleurop Nord began the creation of a green belt around the smelter. Each year, 5 to 10 hectares were undergoing a reconversion with the installation of herbaceous vegetation and trees (sycamore maple, oak and red oak, beech, cherry, alder, and poplar), without questioning the most appropriate species or the future uses of the biomass. Since the closure of the foundry, some plantations have been carried out by ADEME, which is also in charge of the management of the green belt plots.

Thirteen years later, three sites were selected from this green belt to compare the accumulation of metals in 25 different woody species[1] (Migeon, 2009). In addition, naturally occurring trees and shrubs were also sampled[2]. The results showed that among the studied species, the poplar and willow presented the best potential for metal phytoextraction at the site (strong accumulation for Cd and Zn), for they seemed capable of rapidly accumulating the metals present in the soil in their stems and leaves. In details, the Zn levels in the leaves were between 30 to 150 mg kg^{-1} DM for most of the investigated woody species. However, the poplar and willow accumulated between 2 and 6 times more Zn than the other plants. These species displayed bioconcentration factor (BF: ratio between the metal concentrations in the leaves and in those the soil) of 0.6 to 1.2, which suggests that they are Zn accumulators. Similarly, the highest Cd contents in leaves were found in the poplar and willow species as well (> 13 mg kg^{-1}), with bioconcentration factors (BF = 2.26) even more important than Zn. The other investigated species possessed much lower BF (Migeon, 2009).

Despite these promising results, this phytoextraction option was not considered as suitable for the Metaleurop context (ADEME, 2011) for several reasons:

[1] The species collected were *Acer campestre* L. (Field maple), *Acer pseudoplatanus* L. (Sycamore maple), *Alnus glutinosa* (L.) Gaertn. (Common Alder), *Betula pendula* Roth (White Birch), *Crataegus monogyna* Jacq. (Common hawthorn), *Prunus avium* L. (Wild Cherry), *Quercus robur* L. (Pedunculate oak), *Quercus rubra* L. (Red oak), *Robinia pseudoacacia* L. (black locust), *Salix alba* L. (White willow), *Salix caprea* L. (Goat willow), *Salix purpurea* L. (Purple willow) and the following poplar hybrids: *Populus trichocarpa x Populus deltoides* (Poplar cultivar Beaupré), *P.deltoides x Populus nigra* (Black poplar hybrid), *P. tremula x Populus alba* (Grey willow), and *P. tremula × Populus tremuloides* (Hybrid Aspen).

[2] *Cornus sanguinea* L. (Common Dogwood), *Corylus avellana* L. (Common hazel), Crataegus monogyna Jacq. (Common hawthorn), *Genista tinctoria* L. (Dyer's greenweed), *Ligustrum vulgare* L. (Wild privet), *M. sylvestris* Mill. (European crab apple), *Sambucus nigra* L. (Black Elderberry) and *Ulmus minor* Mill. (Field elm).

- metal extraction rates were not satisfying regarding the massive and multiple contamination of the megasite. Moreover, metal such as Pb were poorly extracted. Thus, phytoextraction process would have to last several decades to centuries to reach satisfying less hazardous soil metal concentration;

- fate of contaminated biomass: one of the main limitation of phytoextraction is the unknown use of contaminated biomass;

- metal recycling into soils: the choice to massively grow trees on the contaminated agricultural area of Metaleurop might create an important issue due to contaminated leaf fall in autumn and thus reintroduction of the metals into the soil. No investigation has been lead to understand the influence of tree leaves' fall on metal recycling and to anticipate potential risk for human and environment.

A.3.2.2- Phytostabilization

On the contrary to phytoextraction, phytostabilisation is not a technology aiming at cleaning-up contaminated soils, but it is a management strategy for stabilizing contaminants which are potentially toxic (Vangronsveld et al., 2009). The main purpose is to establish a metal non-accumulating vegetation cover, which is tolerant to high soil metal contaminations and effective in immobilizing contaminants in the soil via root accumulation and precipitation within the rhizosphere and thus limit their transfer to the aerial parts (Bolan et al., 2011; Seregin and Ivanov, 2001). In addition, the plant cover could also improve the physico-chemical and biological parameters of the contaminated soils by increasing the organic matter, nutrient contents, cation exchange capacity, and biological activities (Arienzo et al., 2004). Plants may also help in providing the necessary surface stability to prevent wind-blow of contaminated particulates, and in reducing water pollution by interception of a substantial proportion of incident precipitation (Tordoff et al., 2000).

Two different approaches of metal stabilization (phytostabilization assisted or not) were implicated in the Metaleurop site, on the same experimental site. The experimental site (50°26'N, 3°01'E) was set up in 1999 on a former agricultural field which had been cultivated with maize and wheat. The approximately 10000 m^2 site is located at Evin-Malmaison, 600 m north and downwind of the former smelter. In spring 2000, the site was divided into three plots of about 3000 m^2 each. The first one was not amended and used as a reference plot (R). The other two plots were amended with silico-aluminous fly ash FA1 (plot F1) and the third with sulfo-calcic fly ash FA2 (plot F2) at a rate of 23.3 kg m^{-2} then ploughed up to a 25- to 30-cm soil depth (Figure 8).

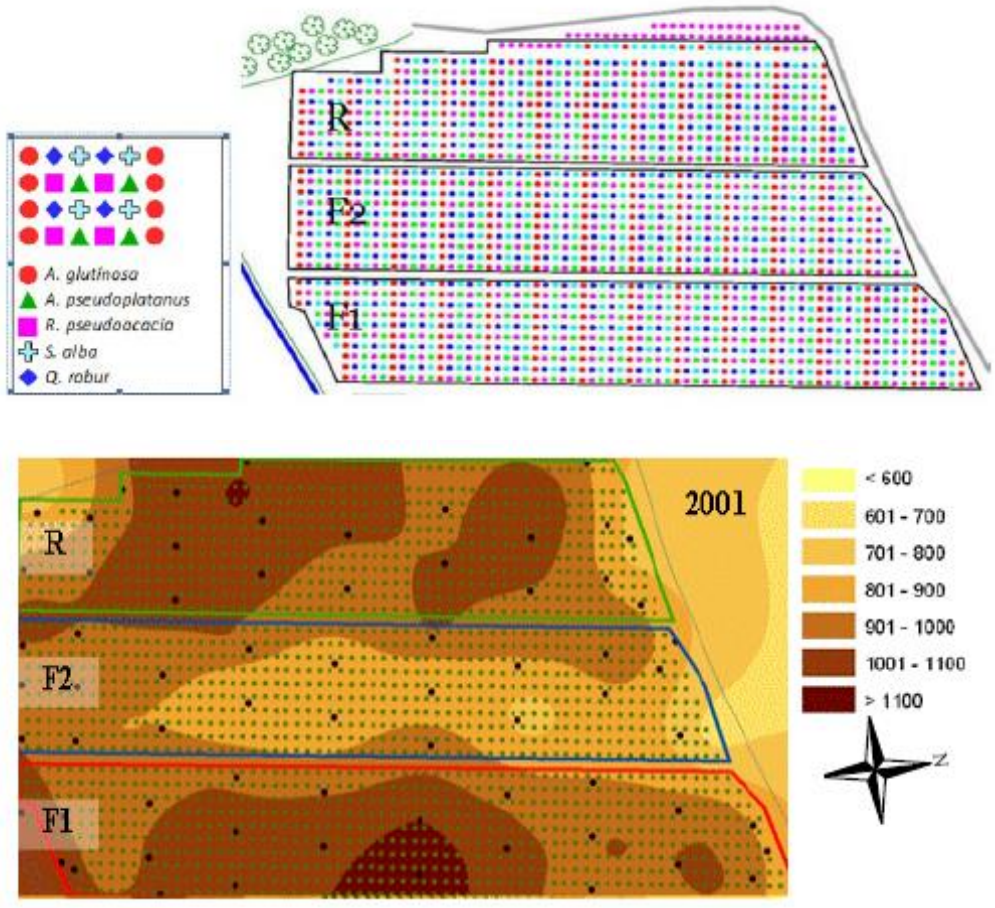

Figure 8: Details of the woody experimental setup close to the former Metaleurop Nord smelter: Tree plantation scheme (top figure) and soil Pb concentrations (bottom figure) (Lopareva-Pohu, 2011)

A herbaceous mixture of *Festuca ovina L., Lolium perenne L., Bromus catharticus Vahl* and *Trifolium repens L.* was sown (10 kg of each species). In winter 2000, the whole site was planted with a tree mix: black locust (*Robinia pseudoacacia* L.), black alder (*Alnus glutinosa* L.), pedunculate oak (*Quercus robur* L.), sycamore maple (*Acer pseudoplatanus* L.) and white willow (*Salix alba* L.). About 1800 trees were planted altogether according to a regular scheme alternating the species. The distances between the trees were 2 m within the rows and 2.50 m between the rows within each plot. The distance between the plots was 4 m. All plant species (herbaceous and trees) are usually used by the Etablissement Public Foncier Nord-Pas de Calais for restoring brownfield lands. As Salix species are known to accumulate Cd and Zn in above-ground parts, it is not suitable for soil phytostabilisation. However, this species was planted in order to follow regional practices. Planted trees originated from the local nursery (situated in an uncontaminated area) and were about 70 cm in height.

a) Phytostabilization

In the frame of her PhD work, Bidar (2007) studied the interest of English ryegrass (*Lolium perenne* L.) and dwarf white clover (*Trifolium repens* L.) for phytoremediation of the highly

metal contaminated agricultural soils. The first is one of the most frequently studied species, defined by some authors as an optional metallophyte plant with high biomass production (Bidar et al., 2007). Conversely, at this time, *T. repens* had little attention on its potential use in phytoremediation and more specifically in phytostabilization (Bidar et al., 2007). The comparative study about the interest of these two species in phytoremediation was carried out on two experimental sites (the R plot of the above-mentioned experimental site, and a non-contaminated site located 20 km far from the former smelter). The concentrations of Cd, Pb, and Zn in the soil surrounding the root system of the two species were 0.60, 35 and 62 mg kg^{-1} respectively for the control site, and 25, 1200 and 1300 mg kg^{-1} for the contaminated site. Concentrations of Cd, Pb, and Zn exported to both the roots and the shoots in both species collected from the contaminated soils were significantly higher than in the control (Table 1).

	T. repens				*L. perenne*			
	Roots		Shoots		Roots		Shoots	
	Control	Exposed	Control	Exposed	Control	Exposed	Control	Exposed
Cd	1.55 ± 0.44^{d}	126.89 ± 21.36	2.10^{-7} (*)	9.01 ± 1.57	1.39 ± 0.29^{d}	131.84 ± 8.54	2.10^{-7} (*)	12 ± 1.6
Pb	1.65 ± 0.31^{d}	166.98 ± 66.98	10^{-6} (*)	35.80 ± 7.32	3.13 ± 0.54^{d}	269.98 ± 64.59	0.27 ± 0.10^{d}	45.65 ± 4.03
Zn	83.04 ± 18.95^{d}	1563.3 ± 294.4	27.19 ± 5.43^{d}	96.70 ± 15.62	86.94 ± 20.31^{d}	1511.18 ± 92.70	15.52 ± 4.71^{d}	218.15 ± 20.87

Table 1: Metal concentrations (mg kg^{-1} DM) in control and exposed *T. repens* and *L. perenne* roots and shoots (Bidar et al., 2007)

Bioaccumulation and transfer factors confirmed that metals were preferentially accumulated in the roots as follows: Cd>Zn>Pb, and their transfer to shoots was limited (Bidar et al., 2009, 2007). Results also showed that there were seasonal and annual variations of metal accumulation in the two studied plant species. With regard to this storage, the plants seemed to limit the metal transfer to their aerial parts over the time, thereby indicating their availability for metal phytostabilization. Aerial deposition was another source of plant exposure to nonferrous metals. Despite the occurrence of metal-induced oxidative alterations in plant organs (increase in SOD activities, MDA and 8-OHdG concentrations), both plant species seemed to tolerate a high metal concentration in soils (Bidar et al., 2009, 2007). These results confirmed the interest of these two species (*T. repens* and *L. perenne*) for phytostabilization of soils contaminated by Cd, Pb, and Zn.

b) Assisted phytostabilization

In order to increase the efficiency of phytostabilisation, organic and/or mineral, amendments can be associated with the vegetation cover (aided phytostabilisation). They could help to reduce pollutants mobility and bioavailability, and/or to promote the planting and development of a vegetation cover (Lopareva-Pohu et al., 2011b). As a follow-up to the PhD work of Bidar (2007), Lopareva-Pohu et al. (2011a) evaluated the benefits of aided

phytostabilisation of metals for the same experimental site, but this time investigating the three plots (R, F1 and F2).

First, Lopareva-Pohu studied the influence of fly ashes on ryegrass and clover abilities to stabilize metals. Her results confirmed the results of Bidar (2007) on their suitability to reduce the mobility and the availability of Cd, Pb and Zn. Moreover, FA exhibited significant positive effects by enhancing both plant growth and reducing plant sensitivity to metal-induced physiological stress, as studied through photosynthetic pigment contents and oxidative damages. These results supported the usefulness of aided phytostabilisation of metal contaminated soils.

Second, Lopareva-Pohu investigated the influence of afforestation and fly ash amendments to reduce metal mobility. After eight years, some soil physico-chemical parameters, including cadmium (Cd), lead (Pb) and zinc (Zn) extractability were modified. In particular, pH decreased on the whole site while organic carbon content increased (Lopareva-Pohu et al., 2011a). The alteration of these parameters influencing trace element mobility is explained by afforestation. Over time, concentrations of $CaCl_2$-extractable metals increased and were correlated with the soil pH decrease. In the amended soils, extractable Cd, Pb and Zn concentrations were lower than in the reference soil. The results indicated that the two fly ashes buffered natural soil acidification due to vegetation development and limited trace element mobility and thus could limit their bioavailability.

In parallel, other results showed that low metal concentrations were measured (Table 2) in aerial part (leaves and twigs) of A. *glutinosa, A. pseudoplatanus* and *R. pseudoacacia* (Pourrut et al., 2011a). Moreover, these trees exhibit a very low bioconcentration factor (BCF<< 1), suggesting them as suitable for phytostabilisation of highly metal-contaminated soils, unlike *S. alba* and *Q. robur*.

	Alnus glutinosa			*Acer pseudoplatanus*			*Robinia pseudoacacia*		
	Cd	Pb	Zn	Cd	Pb	Zn	Cd	Pb	Zn
Leaves									
R	0.48 ± 0.04^{a}	6.2 ± 0.5^{a}	224 ± 18^{a}	0.83 ± 0.2^{a}	3.1 ± 0.5^{a}	109.0 ± 8.7^{a}	0.73 ± 0.10^{a}	2.2 ± 0.2^{a}	79.4 ± 4.9^{a}
F1	0.34 ± 0.04^{ab}	6.2 ± 0.6^{a}	174 ± 16^{ab}	0.76 ± 0.1^{a}	3.9 ± 0.3^{a}	83.6 ± 3.2^{b}	0.56 ± 0.05^{a}	1.6 ± 0.2^{a}	42.5 ± 2.1^{b}
F2	0.31 ± 0.03^{b}	5.8 ± 0.4^{a}	160 ± 10^{b}	0.87 ± 0.1^{a}	3.5 ± 0.4^{a}	62.9 ± 3.0^{c}	0.47 ± 0.04^{a}	1.0 ± 0.1^{b}	32.7 ± 2.3^{b}
Twigs									
R	$1.84 \pm 0.15^{a*}$	$58.1 \pm 7.9^{a*}$	$159 \pm 13^{a*}$	$2.07 \pm 0.26^{a*}$	$24.9 \pm 3.3^{a*}$	$47.8 \pm 3.5^{a*}$	$2.81 \pm 0.28^{a*}$	$28.8 \pm 1.6^{a*}$	$116.8 \pm 9.9^{a*}$
F1	$0.79 \pm 0.08^{b*}$	$37.4 \pm 3.0^{a*}$	$128 \pm 10^{ab*}$	0.95 ± 0.16^{b}	$16.4 \pm 2.0^{b*}$	$38.2 \pm 2.0^{ab*}$	$1.49 \pm 0.22^{b*}$	$14.2 \pm 1.0^{b*}$	$87.9 \pm 4.9^{ab*}$
F2	$0.76 \pm 0.05^{b*}$	$22.9 \pm 1.3^{b*}$	$102 \pm 6^{b*}$	1.18 ± 0.16^{ab}	$13.4 \pm 2.0^{b*}$	$34.4 \pm 1.5^{b*}$	$0.99 \pm 0.11^{b*}$	$13.7 \pm 1.4^{b*}$	$62.9 \pm 4.4^{b*}$

Table 2: Mean concentrations of Cd, Pb and Zn in the leaves and twigs of *A. glutinosa*, *A. pseudoplatanus* and *R. pseudoacacia* trees in 2008 (mg kg^{-1} DM) (Pourrut et al., 2011a).

Fly ash amendments strongly decreased TE availability *to A. glutinosa, A. pseudoplatanus* and *R. pseudoacacia*, and metal translocation to aboveground organs. These decreases fit well together with the FA amendment-induced $CaCl_2$ extractability depletion (Lopareva-Pohu

et al., 2011b). Although both FAs were useful to decrease Cd, Pb and Zn concentrations in aboveground parts of trees, the sulfo-calcic ash (FA2) were more efficient.

A.3.3- Phytomanagement with *Miscanthus x giganteus*

The previous phytostabilization experiments on the Metaleurop megasite showed promising results. However, none of the plant production (trees or ryegrass and clover) has met the economic interest which could encourage their cultivation by farmers in the vast surfaces of Metaleurop site (ADEME, 2011). Thus, there was a need to find a crop able to reduce metal risks and to restore soil functions on metal contaminated soils, while bringing socio-economic benefits. Among all the potential candidates, *Miscanthus x giganteus* was selected and its suitability to phytomanage the agricultural soils of Metaleurop megasite was investigated in the frame of the Phytener multidisciplinary project (2009-2014).

A.3.3.1- Interest of miscanthus for phytomanagement of contaminated sites

a) Miscanthus x giganteus characterization

With an average height of 2 to 3 meters, *Miscanthus* x *giganteus* is a *Poaceae* sterile plant, none invasive and triploid clone (n = 57), resulting from the hybridation of *Miscanthus sinensis* (diploid, n = 36) and *Miscanthus sacchariflorus* (tetraploid, n = 76) (Figure 9).

Figure 9: Miscanthus plant, A: aerial part and B: rhizomes

It is a perennial (> 15 years without rotation) C4 plant originating from eastern Asia and known as a promising biomass crop (Heaton et al., 2010; Nsanganwimana et al., 2014).The main advantage of this perennial crop is its capacity to produce huge biomass yield rich in lingo-cellulose starting from the second or third year of cultivation (25 t ha^{-1}) (Karp and Shield, 2008). Excluding the first years of cultivation, fertilization and other agricultural practices are not needed as most of the key nutrients are translocated to the rhizome at the end of the growing season in order to support the following year growth cycle.

The growth cycle starts by the plants sprouting early in the spring. It is during this period that the planting of rhizomes or seedlings (plantings pre-started) is carried out. After that, tillers

start to form especially starting from the second year, resulting in the production of multiple stems per plant and dense clump formation. Generally, the number of shoots increases rapidly during the months of May, June and July. The months of August, September and even October correspond to the growth and therefore to the progressive production of aerial biomass. The end of growth coincides with a drop in temperature. The senescence of the leaves is progressive beginning with the most elderly ones. During the senescence, certain nutrients are remobilized from the aerial parts to the rhizomes. Complete senescence of the aerial parts occurs from the first frosts, in which the leaves dry very quickly and detach themselves from the stems, thus constituting mulch that completely covers the ground. Later on, the stems gradually dry during the winter (Nsanganwimana, 2014) (Figure 10).

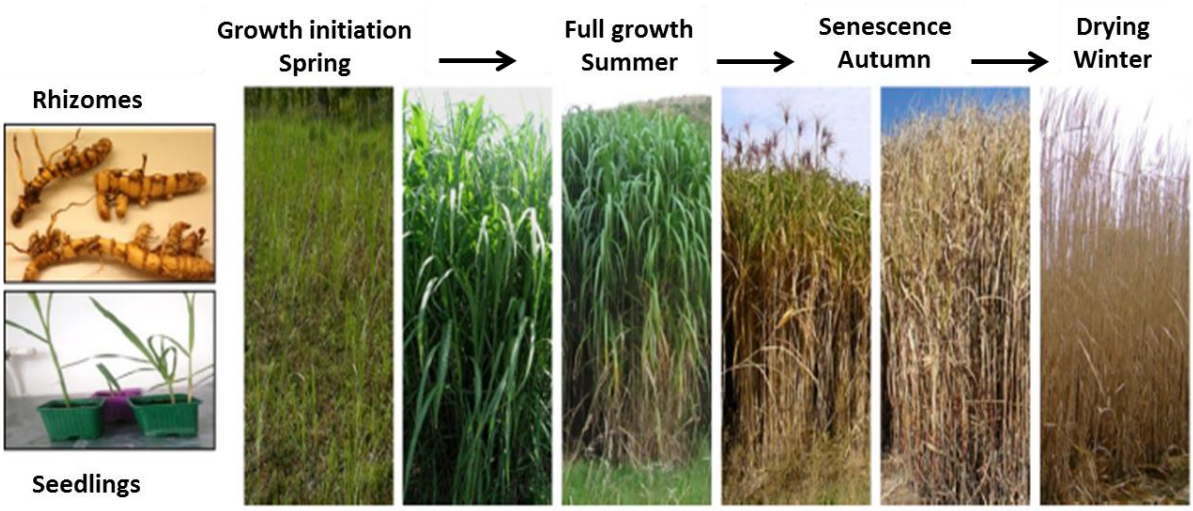

Figure 10: Miscanthus x giganteus growing cycle (adapted from Nsanganwimana, 2014)

In view of this annual growth cycle, the miscanthus aerial biomass can be harvested at two intervals. Harvesting can take place either at the end of growth (mid-October to mid-December) or during the winter. The first harvest period, also known as "green harvest" or "early harvest", whereas the second is called “dry harvest” or “late harvest”. In the Metaleurop site, the late harvest choice was depended, as the biomass content in mineral and water is low, a positive factor in order to improve the biomass combustion. Another advantages of miscanthus late harvest is obtaining a drier biomass which eventually lowers the costs by limiting the need of a drying storage place and special equipment and at the same time yield better results for most of thermochemical biorefining technologies (pyrolysis, gasification) (Nsanganwimana et al., 2014; Roncucci et al., 2015). However, it is noteworthy to mention that the biomass yield associated with an early harvest can be approximately 30% more than that associated with the late harvest.

Moreover, miscanthus is well perceived by the local population through economic and environmental opportunities or impacts proven and expected, which are associated with

multiple biomass use: heating, composting, refinery, litter animal (horse, poultry), mulch, biochar and soil amendments (Nsanganwimana et al., 2014).

b) Interest of *M. x giganteus* to manage contamination

Miscanthus plants are well known for their high ability to tolerate different types of soil contamination. Investigations have proved that *Miscanthus* x *giganteus* have the capability to grow up and well resist different kinds of soil pollution (metals, organic compounds, pesticides…) (Pandey et al., 2016). For instance, Pidlisnyuk et al. (2014) and Techer et al. (2011) showed their crucial role in the bacterial degradation of the polycyclic aromatic hydrocarbons, petroleum and pesticides via its root exudates.

They have also demonstrated high potentials to withstand high soil metal concentrations, while accumulating them in their underground parts (mainly the roots), with low levels in the aboveground organs (stems and leaves) (Nsanganwimana et al., 2014). Not only do they tolerate high soil metal concentrations, but also they can grow well and proliferate in the contaminated soils. For instance, Kocoń and Matyka (2012) established a three year experiment in Pb and Zn contaminated soils and demonstrated that the corresponding plant biomass yield was increasing progressively and recording its highest values during the third year of culture (average 7 times higher than the first year). Moreover, Pidlisnyuk et al. (2016) launched an experiment by cultivating miscanthus plants in a former military site in Kamenetz-Podilsky (Ukraine), contaminated particularly by Fe, Mn, Ti, and Zr, and to a lesser extent As, Cu, Pb, and Zn. The results showed that despite the high concentrations of metal in the soil, the growth of *Miscanthus x giganteus* was not affected and the plant height was comparable to regular plantation growing at the clean agricultural land with similar climates.

Moreover, the miscanthus plants also play an important role in decreasing the corresponding soil metal availability, via contributing in the changes of the soil physico-chemical parameters and modifying the metal distribution between the exchangeable and less exchangeable soil fractions (Yang et al., 2010). For instance, the 0.01 M $CaCl_2$ extractable As and Pb concentrations decreased by 19 and 52% respectively after 3 months of miscanthus cultivation in acid technosoils (Ollivier et al., 2012).

In addition, due to their corresponding considerable biomass, miscanthus plants are well known for their high capacity to sequestrate carbon, produce organic compounds and translocate them into the soil, thereby increasing the organic carbon levels and prospering the soil biological activities and biodiversity (Anderson et al., 2009; Técher et al., 2012).

Several reasons might be standing behind the upper mentioned resistive properties of the miscanthus plants against the elevated soil metal contamination, which are basically related to their responses. To begin with, the avoidance of the local metal accumulation and distribution might constitute a homeostasis mechanism to avoid the severe toxic effects. For example, in *M. sinensis*, excessive metal ions as Al, Cr or Zn are removed from the growing

root tip tissues and are stored in mature ones or distributed to shoots (Ezaki et al., 2008). Another possible reaction undertaken by the metal to mitigate the metal toxic effects might be the formation of inert metal-organic acid (citrate, malate, and oxalate) complexes in the apoplast and vacuoles (Lyubenova et al., 2013). Moreover, the activation of the antioxidative defence system comprising the enzymatic activities as well as the non-enzymatic pathway. Indeed, as a result of stressful environments expressed by the soil metal contamination, the production of a large amount of reactive oxygen species and the resulting generation of oxidative stress are among the best-known and earliest aspects of metal toxicity counteracting in plants (Pourrut et al., 2011b; Shahid et al., 2014). For example, the activation of the catalase, ascorbate peroxidase and superoxide dismutase was observed more rapidly in roots than in leaves of Cd-treated (0-6.6 mM in hydroponic culture) *M. x giganteus* (Scebba et al., 2006). Moreover, Firmin et al. (2015) stated that the soil metal contamination (12, 746 and 953 mg kg^{-1} corresponding to Cd, Pb, and Zn, respectively) in an agricultural field surrounding the Metaleurop smelter cultivated by *Miscanthus x giganteus* affected mostly the plant leaves in spite the fact that the metals accumulated way more in the roots. In details, the authors recorded an oxidative stress in the miscanthus leaves expressed by a strong decrease of fatty acid contents, an increase of the lipid peroxidation biomarker malondialdehyde (MDA), a decrease of the GSSG/GSH ratio in the leaves of exposed plants, and finally an increase in the peroxidase (PO) and superoxide dismutase (SOD) activities. Moreover, the hydroponic experimentations of Guo et al. (2016) demonstrated the progressive increase of MDA, H_2O_2 contents and the activities of superoxide dismutase, peroxidase, ascorbate peroxidase, and glutathione reductase activities with the increase in the Cd concentration in three miscanthus species (*M. sinensis*, *M. sacchariflorus* and *M. floridulus*). Zhang et al. (2015) also obtained similar results while cultivating the *M. sacchariflorus* in soils spiked by increasing concentrations of Cd concerning the MDA concentrations and the activities of SOD, POD, and CAT. However, the hydroponic solutions and metal spiked soils experiments might not reflect the real field observations on the plant.

c) Interest of miscanthus for phytomanagement of metaleurop megasite

Nsanganwimana (2014) in his thesis has proved the suitability of *Miscanthus x giganteus* to be implemented in the Metaleurop Nord contaminated area. Briefly, several agricultural plots with gradient metal (Cd, Pb, and Zn) concentrations were cultivated by miscanthus. The plant showed high tolerance capacities in all the studied fields expressed by the progressive increase in its corresponding biomass yield with the passage of the years of cultivation (Nsanganwimana et al., 2015). It was also demonstrated that the plant mainly accumulated metals in its roots. Moreover, he also indicated that mycorrhization increased metal (mainly Cd and Zn) accumulation in the different organs and in the shoot yields (Nsanganwimana et al., 2015). In his *ex situ* pot experiments, Nsanganwimana (2014) demonstrated the positive effects of miscanthus on decreasing the extractability of the metals from soil to the aerial organs, due to their corresponding accumulation in the roots as well as the increase of the

soil organic carbon derived from the plant root exudation. Iqbal et al. (2013) as well demonstrated the decrease in the Cu and Pb extractable contents in an agricultural plot in the Metaleurop site cultivated by *Miscanthus x giganteus* in comparison to an adjacent field cultivated by annual crop. The reasons behind this corresponding decline in the metal availability in soils cultivated by miscanthus might be the increase in cation exchange capacity, and water retention capacities as a result of miscanthus culture (Lewandowski et al., 2000), and the increase in the soil organic matter derived from the plant litter and root exudates (Dondini et al., 2009; Iqbal et al., 2013). Finally, Pelfrêne et al. (2015) also shed the light on the substantial impacts of the plant on the metal redistribution in soils.

However, the impacts of the corresponding plant on restoring soil functionality and biological parameters were never studied, as well as the impacts of their corresponding late harvest with the leaf fall and accumulation over the surface of the soil with the concern of decomposition and the mobility of the metals. Moreover, little is known about the impacts of the field soil metal contamination on the plant health and its corresponding mode of reaction against the stress induced by the metals, and the present information is based on experimentations implicating metal spiked soils or metal contaminated hydroponic solutions.

A.4- Objectives of the thesis

The Metaleurop Nord megasite has been extensively contaminated by metal emissions (mainly Cd, Pb and Zn) for more than one century. The level of metal contamination poses high risks for human and environment, and this is particularly true for agricultural areas. However, the complexity of the site (size, different degrees of contamination, complex land uses, many stakeholders at different levels…) make it very difficult for the decision makers to find a sustainable management. Numerous studies have investigated potential management solutions of the contaminated soils for almost 20 years. If several of them exhibited promising results, especially on agricultural areas, none of them enables a sustainable management.

In the frame of the Phytener project (2009-2014) and the PhD work of Florien Nsanganwimana (2011-2014), the potential of *Miscanthus x giganteus* to phytomanage the contaminated agricultural soils had been investigated. These studies showed that soil contamination does not affect *M. × giganteus* growth and shoot yields. Cadmium, Pb and Zn are mainly accumulated in roots and *M. × giganteus* reduces their transfer to the aboveground organs. This plant presents therefore a great potential for metal phytostabilisation, and in a more comprehensive way, for phytomanagement.

However, given the perennial nature of the plant, several questions were raised by Nsanganwimana (2014). In particular, he highlighted the fact that the effects of metal-

induced stress on the plant health need to be assessed as well as the fate of contaminants in relation to the accumulation of soil organic matter in miscanthus plantations. Moreover, the influence of the plant on soil biological parameters has to be investigated. Thus, the present PhD work is a continuation of the work of Nsanganwimana (2014). It aims at further analyzing the mode of interaction between *Miscanthus x giganteus* and the degraded soil ecosystem in the area of Metaleurop by:

1) Studying the impacts of the miscanthus cultivation on restoring the soil functionality via studying certain soil physico-chemical and biological indicators of soil health;
2) Determining the impacts of the late harvest on the mobility and the bioavailability of metals following the leaf decomposition and the corresponding consequences on the subsequent culture;
3) Investigating the impacts of the soil metal contamination on the corresponding plant health and its tolerance via studying certain biomarkers of stress.

Part B- Materials and methods

B.1- Choice of the experimental plots and miscanthus plantations

At the Metaleurop site, agricultural soils are contaminated to varying degrees depending on the distance to the sources of contamination (Sterckeman et al., 2002). The experimental plots integrated in this thesis represent the contamination gradient that characterizes the site and they represent the base of the Phytener project (2009-2014)

The work of the thesis is based on *ex situ* experimentations that are based initially on the *in situ* experimental sites of LGCgE. However, the soils implicated were collected from the agricultural plots surrounding the Metaleurop smelter, and represent the gradient concentration of metals. The miscanthus agricultural fields were established in 2007 in the scope of demonstrating their feasibility to phytomanagement in the Metaleurop site.

B.1.1- Location of miscanthus cultivated plots

The experimental system consisted of four agricultural plots (named M200, M500, and M700 based on their approximate Pb concentration in soil), located in the peri-urban areas around the former lead smelter (Figure 11). The forth agricultural plot (MC) is located in the village of Linzeux.

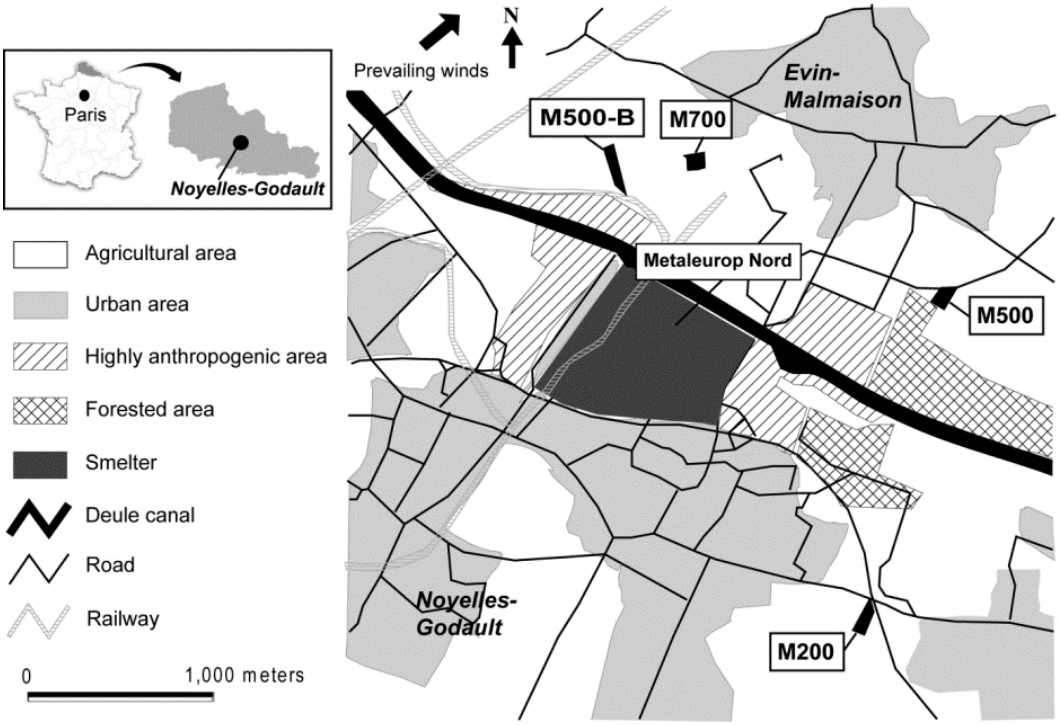

Figure 11: Agricultural experimental site

The M200 plot (1.4 ha) is at the most distant contaminated plot from the former smelter (about 1.8 km away), and also the least contaminated. It is located in the town of Courcelles-

lès-Lens. The M500 plot (0.8 ha) is at a distance of about 1.4 km from the former plant, in the commune of Evin-Malmaison. It is located in the alluvial valley of the Deûle. The M700 (0.8 ha) plot is located within the closest perimeter of the former metallurgical plant, about 1 km north of the former industrial site.

The plot (MC) covers an area of 1.3 ha and is about 75 km far from the site. It was introduced to the plots experimental design to serve as the control uncontaminated site, with metals concentrations slightly lower than the regional background levels (Sterckeman et al., 2002).

B.1.2- Soil characteristics of the studied plots

The studied plots are characterized by certain soil parameters which distinguish one from the other. For instance, the fine and coarse silt concentrations of MC soil (690 g kg^{-1}) were higher than those of the other soils studied (498, 522, and 530 g kg^{-1} for M200, M500, and M700 soils, respectively). The sand fraction was highest in M200 and M700 soils (289 and 274 g kg^{-1}). The soil of the M500 plot is more clayey (311 g kg^{-1}) than those of the other plots with clay content of about 200 g kg^{-1}.

The soil texture in the M200 plot soil is derived from well-drained clay-sandy silt strata resting around 1.10 m on chalk (g: 30 - 50 cm). The M500 soil is derived from limestone-clayey alluvial deposits (g: 30 cm). The soil of the M700 plot consists of silt and clay loam resting on argilo-silty clay-sandy materials (g: 30 - 50 cm). Finally, the MC soil is developed on loess silts over 1.20 m thick (g > 1.20 cm).

B.1.3- Implementation of miscanthus plantations

At that time of initiation in 2007, the miscanthus (Mis-B) rhizomes from Bical France (currently NovaBiom) were planted in MC, M200 and M500 plots using a potato planter with a density of 20 000 plants ha^{-1}. There was no mineral fertilization of these plots. This management mode reflects the agricultural practices implemented in the region. However, in the M700 plot, the plantations took place in 2010. Three different miscanthus origins were cultivated. The Mis-B (designated as B), is derived from the uncontaminated MC plot, Mis-I (designated as I), supplied by a farm located in the region of Paris and Mis-A (designated as A), which was supplied by Rhizosphere.

B.2- *Ex situ* experimentations

B.2.1- Pot experiment

For the sake of studying the impacts of the *Miscanthus x giganteus* on restoring the metal-contaminated soil functionalities and assess their capacities to stabilize the metals on one side, and the impacts of this contamination of the health on the plant health on the other side, an *ex situ* pot experiment was established in an area away from the roads in the campus of the Lille 1 University.

B.2.1.1- Soil sampling and preparation from the site

Soil samples were collected between the period of in March 2014 from the ploughed horizon (0-25 cm) of each of the upper mentioned agricultural plots (MC, M200, M500, and M700), to sustain the gradient metal contamination. Moreover, the metal distribution in the M700 plot is not homogeneous according to the distance from the smelter (Nsanganwimana, 2014), in which there are locations where the Pb concentration exceeds 700 mg kg^{-1} (Figure 12). In order to obtain a wider range of gradient metal concentration, we found it interesting to collect two different samples from the plot. The first represents an approximate concentration of 750 mg kg^{-1} of Pb thereby termed M750 collected from the north side of the plot, and the other with a Pb concentration of 900 mg kg^{-1} and designated as M900.

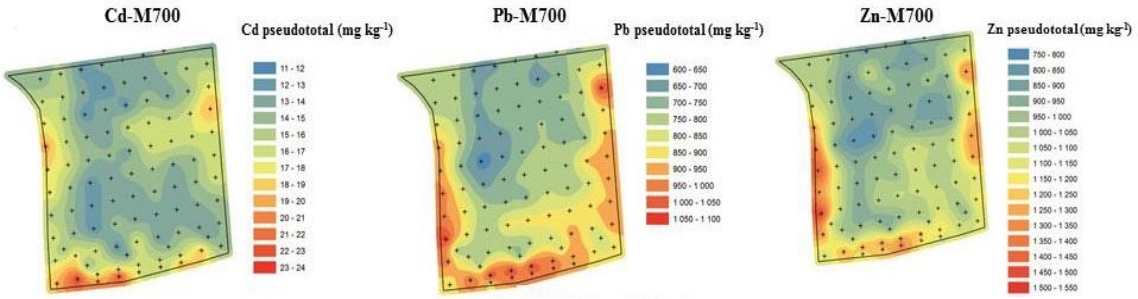

Figure 12: Cd, Pb and Zn pseudototal concentration distribution in M700 plot

Upon collection, the samples were homogenized, air dried and sieved to pass a 10 mm mesh prior to cultivation.

B.2.1.2- Miscanthus origins and preparation

Three miscanthus cultivars were used in this experiment. Besides the B and A cultivars previously mentioned, a new cultivar was introduced originating from Iowa university in the USA was introduced and designated as U. Their corresponding rhizomes (5-7 cm, 2-3 buds) were set to grow in polyethylene pots (9 x 9 x 9 cm) filled with potting compost that was constantly watered. The rhizomes were kept in the pots until the miscanthus plantlets reached 20–25 cm in height. At that moment the *ex situ* experimentation was launched.

B.2.1.3- Experimental setup

For a period of 18 months (May 2014 to October 2015), an *ex situ* pot experiment (Figure 13) was established in an area away from the roads in the campus of the Lille 1 University

(50.6090° N, 3.1381° E, Villeneuve d'Ascq. Approximately 100 kg of each of the homogenized sampled soils were equally distributed in five grey-colored pots (to avoid the temperature elevation occurrence). Thereafter, the miscanthus plantlets (B, U, and A) were transferred from the small pots to the 20-kg pots. In order to infer the effects of the plant on restoring the soil functionality and assess it capacity in stabilizing metals two other modalities were introduced. Of which, uncultivated covered pots "C" (to prevent any type of spontaneous vegetation to develop) and uncovered "UC" control pots (n = 5).

Figure 13: *Ex situ* experiment at Lille 1 University

Briefly, the experiment was comprised of 75 cultivated pots (three different miscanthus cultivars x 5 soils x 5 replicates) presented in (Figure 14), and 50 unplanted (C and UC x 5 soils x 5 replicates). The pots were disposed over woody rafters to avoid contact with the ground of the experimental area, and were randomly distributed to avoid point and borderline effects. The soil humidity in the pots was maintained by regular watering throughout the entire duration of the experiment, as was as the rain water. Finally, the weeds were manually removed and kept on the surface of the soils to avoid a possible metal exportation.

MC	MC	MC	MC	MC	
M500	M750	M200	MC	M750	
M750	M900	M500	M500	M200	
M200	M200	M750	M900	M500	B
M900	M500	M900	M200	M750	U
M500	M900	M200	M750	M900	A
MC	M750	M500	M900	M200	
M200	MC	M900	M750	M500	
M750	M500	M200	MC	M900	
M900	M200	M750	M500	MC	
M750	MC	M500	M900	M200	
M200	M500	MC	M750	M900	
M500	M900	M200	MC	M750	
M900	M200	M750	M500	MC	
MC	M750	M900	M200	M500	

Figure 14: *Ex situ* pot experimental design including three miscanthus cultivars (B, U, and A)

B.2.1.4- Soil sampling from the pots and preparations

Soil samples were collected twice from the pots, at the beginning of the experiment which was considered as T0 (June 2014), and one year after during the same period (T+1). The sample collection was done with an auger (composite samples were prepared via collecting five soil samples from each pot). Back to the laboratory, after homogenization, a part was sieved manually to 2 mm, packed in plastic bags and conserved at 4°C for the characterization of microbial activities (Abellan et al., 2011). The other part was oven dried at 40°C then sieved to 2 mm according to the recommendations of the standard NF ISO 11464 (1994). A third part was crushed using a shredder ultra-centrifuge (ZM 200, Retsch) to pass through a 250 µm mesh. It is noteworthy to mention that the analysis of the physico-chemical parameters, metal concentrations as well as the biological parameters in the investigated soils were performed according to standardized protocols.

B.2.1.5- Plant growth parameters, leaf sampling and preparations

For the sake of evaluating the impact of the soil metal contamination on the corresponding miscanthus cultivars, by the end of the first and second growing seasons (October 2014 and 2105), plant growth parameters such as the stem height, diameter and the number of tillers per pot were determined. The total aerial biomass was not harvested and measured in order not to prevent the nutrient translocation towards the rhizomes, and thus not compromising the second year of the experiment.
In order to specify the plant health via certain biomarkers of stress, three leaves (4th, 5th and 6th foliar stage) were harvested from each one of the 75 pots and immediately flash frozen into liquid nitrogen. Samples were then stored at – 80°C prior to the biomarkers analysis.

The other leaves were also harvested to measure metal concentrations, put into plastic bags and kept in a cool-box. Back at the laboratory, leaves were washed 3 times with osmosed water to remove dust particles. They were then oven-dried at 40°C for 48 h, afterwards ground into fine powder using a knife mill (GM200, Retsch) before metal analysis.

B2.2- Aging experiment

To extrapolate the effect of *M. x giganteus* after 18-20 years of cultivation and their corresponding late harvest, with what it results in accumulation of organic residues (leaf litter) in the cultivated area, and thus its following consequences on the cycle of elements in the soil as well as on the metal speciation and extractability. Finally, the post miscanthus impact was determined as well by studying the impacts of the late harvest on the succeeding

crop culture. For this sake, we simulated in the laboratory an artificial aging process under controlled conditions trying to answer the presented questions.

B2.2.1- Soil and leaf litter origins, sampling and preparations

The soils from the 0-25 cm ploughed horizon implicated in the experiment were sampled in February 2015 from the agricultural plots (MC, M200 and M500) cultivated with miscanthus since 2007. The collected samples were homogenized, oven-dried at 40°C and sieved through a 2 mm mesh before launching the experiment.
From each plot, samples of the leaf litter of *Miscanthus x giganteus* were collected as well, from a specific area (1 m^2). The leaf litter was weighed and stored in plastic bags. In the laboratory, the leaf samples oven dried at 40°C for 48 h, sieved and ground to 1-2 mm.

B2.2.2- Soil artificial aging and experimental setup

500 g of soils sieved to pass a 2 mm mesh of the three different origins (MC, M200, and M500) were attentively mixed with 1 g of the sieved leaf samples (1-2 mm), corresponding to the estimated amount of leaf litter that might be present after 18-20 years in the agricultural field. Thereafter, the corresponding mixture (soil and incorporated leaf fragments was transferred to small polyethylene pots (9 x 9 x 9 cm; n = 6). Concomitantly, another plastic pots (n = 6) were filled with MC, M200, and M500 soils yet without the leaf powder.
The aging experiments were launched in the laboratory at different time intervals (1, 3, and 6 months) at 25°C. Deionized water was added to the pots to maintain soil moisture at 60 % of their water holding capacities (WHC). At the end of the incubation, 3 pots of each treatment were sacrificed for determining certain physico-chemical and biological parameters, whereas on the other three were cultivated with ryegrass.

B.2.2.3- Ryegrass cultivation

The other portion of the aged soils was planted with 1 g of ryegrass seeds (*Lolium perenne*). The cultivation period lasted for 2 months in controlled environment (20°C, 16 h photoperiod). Deionized water was added to soil samples to maintain moisture at 60% WHC. At the end of the cultivation period, the ryegrass shoots were harvested by cutting 1 cm above the soil with scissors. A small portion was immediately flash frozen into liquid nitrogen directly after sampling and stored at – 80 °C prior to the biomarkers analysis. The remaining portion was then washed 3 times with osmosed water, to remove any dust particles. The samples were oven-dried at 40 °C for 48 h and weighed to determine their corresponding dry biomass. Finally, the samples were ground into fine powder using a knife mill (GM 200, Retsch) for metal concentration determination.

B.3- Soil analysis

B.3.1- Soil physico-chemical parameters and metal concentrations

B.3.1.1- Particle size distribution

Particle size distribution was determined by sedimentation and sieving after destruction of organic matter by H_2O_2 according to the French NFX 31-107 standard.

B.3.1.2- Soil organic carbon (SOC)

Soil organic carbon levels were measured through a colorimetric dosage following a method of sulfochromic oxidation (NF X 31-109, 1993). The organic carbon is oxidized following the dichromate method causing the reduction of Cr VI to Cr III and a change in color from orange to green. The intensity of the green color is proportional to the concentration of the Cr III which is related to the carbon content of soils.
Under a hood, 5 mL of potassium dichromate ($K_2Cr_2O_7$, 80 g L^{-1}), then 7.5 mL of sulfuric acid (H_2S0_4, 95%) were added to a glass tube containing 400 mg of soil sample crushed to 250 µm. The tube was stirred gently and then placed in a heating block (DK Heating Digester (Velp Scientifica®) at 140°C for 30 min. Once cooled, the solution was poured in a 100 mL graduated flask, and then the level was adjusted with osmosed water. After stirring, an aliquot of 20 mL was taken and centrifuge for 10 min (4500 rpm; 2000 g). A calibration curve was made with a solution of potassium dichromate ($K_2Cr_2O_7$, 80 g L^{-1}) and glucose (1 g). The samples were then placed in a microplate and their absorbance were read at the wavelength λ = 585 nm using a spectrophotometer (spectrophotometer, Multiskan® GO). Organic carbon (g kg^{-1}) released in the sample was obtained for each of the samples after calculating the absorbance of a blank and referring to the calibration curve. For quality assurance, replicas were used, white and certified equipment (BCR® 129) IRMM, Belgium) was included in the analyses.

B.3.1.3- Available phosphorus

The quantification of the available phosphorus in the soil samples was held according to Joret-Hébert method (NF X 31-161, 1993) (Joret and Hébert, 1955). One gram of dried, crushed 2 mm soil was deposited in a test tube to which 25 mL of extraction solution (0.1 M ammonium oxalate solution) was added. The solution was set to agitate at 20°C for 2 h. Thereafter, the solution was centrifuged for 15 min at 1200 rpm, and then filtered. A coloring reagent was added to color the solution. After preparing a standard curve from orthophosphate, solutions were measured spectrophotometrically (UV - 1800 Shimadzu) at wavelength λ = 825 nm. The available phosphorus (mg kg^{-1}) released in the sample was obtained for each of the samples after calculating the absorbance of a blank and referring to the calibration curve.

B.3.1.4-Total $CaCO_3$

The total $CaCO_3$ content of soil (dried and ground up to 250 µm) was determined through the quantification of the release of CO_2 following an acid reaction. For this purpose, a calcimeter was used. The test portion varies according to the carbonate content. The results expressed in dry weight, resulting from the difference between two volumes before and after the acid reaction. The blank (B) and the calibration curve (C) were taken into account while calculation:

$$W\ CaCO_3 = \frac{1000\ x\ m2\ x\ (Vsample - B)}{m1\ x\ (C - B)}\ x\ \frac{100 + w(H20)}{100}$$

where:
W $CaCO_3$ = content of carbonate (g kg^{-1}) of soil dry
m1 = mass of the sample in g
m2 = average mass of the standards of calcium carbonate in g
V sample = the volume of CO_2 produced by the reaction of the sample in mL
C = average volume of CO_2 produced by the reaction of carbonate standard in mL
B = the change in volume in response to the blank sample (might be negative)
W H_2O = water content expressed in the form of % dry mass

B.3.1.5- Cationic exchange capacity

The method used to determine the Cationic Exchange Capacity (CEC) in the soil was the extraction with ammonium acetate (NF X 31-130, 1999). It was composed of three successive steps: 1) percolation (exchange) with acetate ammonium allowing the substitution of cations from soil with the NH_4^+ ions; 2) extraction with NaCl in order to extract the NH_4^+ ions fixed to the soil; 3) dosage of NH_4^+ ions extracted by spectrophotometry according to ISO-7150/1 (1984) (Figure 15).

Initially, 2.5 g of soil dried and sieved to 2 mm was deposited in a specific percolation tube. This tube was then filled with a 1 M ammonium acetate solution and left to percolate. The operation was repeated five times. Five ethanol rinses were carried out. The soil was left to dry out at room temperature for 24 h. For the extraction procedure, the soil as well as the cotton of the extension socket was transferred to a centrifugation tube, to which 50 mL of 1 M NaCl was added then started to agitate for 1 h. The solution was then filtered (0.45 µm). Two solutions of reactions (Color Reagent and sodium dichloroisocyanurate DIC) were added to color the solution. Finally, the NH_4^+ ions were assayed by colorimetry. A standard curve was realized with a solution of NH_4^+, color reagent and DIC. The absorbance of the solutions was then measured spectrophotometrically (UV/VIS spectrophotometer, Multiskan® GO) at the wavelength λ = 655 nm. The CEC value for each sample ($cmol^+\ kg^{-1}$) was obtained after calculating the absorbance of a blank and referring to the calibration curve.

Figure 15: Different steps of CEC measurement in soil

B.3.1.6- pH $_{water}$

Soil samples (dry and sieved to 2 mm) were used for pH measurement (ISO 11464, 1994). Briefly, a soil suspension prepared in a volume representing five times its corresponding volume (5 mL of soil to 25 mL of osmosed water). The corresponding suspension was mixed via magnetic agitator (750 turn min^{-1}) for 1 h. After 2 hours of decanting, pH measurement was performed using a glass electrode (Knick Portamess® electrode).

B.3.1.7- Residual humidity

The residual humidity of a sample (soil or plant) corresponds to the difference in mass before and after drying. It allows expressing the concentrations obtained in dry matter.
After weighing a glass cup (*m0)*, approximately 10 g of soil or plants were used (*m1*). After 24 h in an oven at 105°C, the cups were taken out and weighed (*m2*) after cooling. The residual moisture in g kg^{-1} of sample, was calculated as following: humidity = [(*m1* - *m2*) / (*m2* - *m0)]*) x 1000.

B.3.1.8- Nitrogen content

The determination of nitrogen content in the soil samples was based on a colorimetric method described by Saha et al. (2012). This method is modified version of Kjedhal method: titanium dioxide (TiO_2) is used as a catalyst in place of selenium (Se) for sanitary concerns (NF ISO 11261, 1995).

Under a hood and in one glass tube, 3.5 g of a catalyst composed of a mixture of 3.35 g of potassium sulfate (K_2SO_4), 0.1 g of copper sulfate ($CuSO_4.5H_2O$) and 0.1 g of titanium dioxide (TiO_2) was added and well mixed to 400 mg soil sample crushed to 250 µm. Later on, carefully, 10 mL H_2SO_4 (98%) was poured and then in threefold, 10 mL (3 x 3.35) of hydrogen peroxide (H_20_2) was added. The solution was mixed using a vortex. The tube was then placed in a heating block (DK Heating Digester, Velp Scientifica®) at 200°C for 20 min, and then at 380°C for 45 min. Mineralization of samples resulted in a light green color. Once cooled for 5 min at room temperature, 40 mL ultrapure water was added. The solution was diluted by the addition of ultrapure water in a 100 mL graduated flasks (final solution).

The color measurement is adapted from O'dell (1993). In a test tube, 1 mL of the final solution and 1 mL of ultrapure water were introduced, then in the order:

- 7.5 mL of O'Dell buffer solution (Na_2HPO_4, $NaKC_4H_4O_6.4H_2O$ and NaOH);
- 2 mL of sodium salicylate reagent ($C_7H_5NaO_3$ 15% and Na_2 [Fe $(CN)_5NO$] 3%);
- 1 mL of sodium hypochlorite (6 %).

The tube was closed with a parafilm and then agitated in order to mix the contents. To ensure a clean development of color, it was then placed for 1 h in the dark. The range of calibration was performed from ammonium chloride (NH_4Cl). The intensity of the blue-green color is directly proportional to the concentration of N - NH_4 in the sample. The samples were then placed in a microplate to read their absorbance spectrophotometrically (UV/VIS spectrophotometer, Multiskan® GO) at λ = 660 nm. The content of nitrogen (g kg^{-1}) released in the sample was obtained for each of the samples after calculating the absorbance of a blank and referring to the calibration curve. For quality assurance, replicates have been used, blanks and certified equipment (BCR® 129) IRMM, Belgium) were included in the analyses.

B.3.1.9- Pseudo-total metal concentrations in the soils

The determination of metal concentrations in soil required a step of mineralization. For this sake, 300 mg of sample ground to 250 µm was mineralized with a solution of hydrochloric acid (4.5 mL HCl) and concentrated nitric acid (HNO_3, 70%, 1.5 mL) heated to 120°C on a plate (Hotblock Environmental Express, USA) for 1 h 30 min. Once cooled, the QSP (25 mL) was done using osmosed water. The solution was filtered out then. The Cd, Pb, and Zn concentrations were measured through atomic absorption spectrometry (AAS) (Shimadzu AA - 6800 Tokyo, Japan) (Waterlot et al., 2013). A correction factor of the analytical values was made by integrating the residual moisture values. The quality control of the extraction and analysis was provided by the introduction of two internal reference samples and a certified soil reference (CRM 141, IRMM, Belgium).

B.3.1.10- 0.01 M $CaCl_2$ extractable metal concentrations

The 0.01 M $CaCl_2$ extraction of Cd, Pb and Zn was carried out on the soil samples as well. 3 g of dry soil ground to 250 µm were deposited in a centrifuge tube, then 30 mL of $CaCl_2$ (0.01 M) solution was added. After being agitated for 2 h, the tubes were placed in the centrifuge for 20 min to 4 500 g. The solution was filtered (0.45 µm) then acidified (92 µL HNO_3 65 % for 30 mL) to allow better conservation. The Cd, Pb, and Zn in extracts were determined via AAS (Shimadzu AA-6800 Tokyo, Japan) coupled with a (Shimadzu, Tokyo, Japan) ASC-6100 autosampler (Waterlot and Douay, 2012).

B.3.2- Soil biological parameters

The follow-up of the microbial activities was conducted through different analyses: 1) determination of the microbial biomass carbon (MBC) via chloroform fumigation experiment, 2) quantification of the soil basal respiration (overall activity of the microbial biomass) and hydrolysis of the FDA (overall microbial activity potential), 3) monitoring the fungal activities (laccases) and 4) evaluation of the enzymatic activities related to certain elements cycles (ureases and acid phosphatases).

B.3.2.1- Microbial biomass carbon

The determination of the microbial biomass carbon (MBC) was based on the chloroform-fumigation extraction method at atmospheric pressure according to the method stated by Vance et al. (1987) and Wenhao et al. (2013), in which each soil sample is divided into two portions, one to be fumigated and the other left non-fumigated. Later on, the carbon in the two solution extracts is oxidized using the dichromate method causing the reduction of Cr VI to Cr III and a change in color from orange to green. The intensity of the green color is proportional to the concentration of the Cr III. The difference in the absorbance between the fumigated and the non-fumigated samples represent the MBC expressed in mg kg^{-1}.

For the entire chloroform fumigation extraction process, 40 g of fresh soil samples were needed. The first 20 g were weighed into 250-mL bottles with screw caps, and were extracted immediately with 80 mL of 0.5 M K_2SO_4 solution for 60 min using a rotary shaker at 35 rev min^{-1} for they were not supposed to undergo the chloroform fumigation. Upon finishing, the bottles are centrifuged for 10 min at 5000 rpm, and thereafter filtered using 0.45 µm filters. The other 20 g of the fresh soil samples were fumigated with ethanol-free chloroform. Ethanol was removed by washing 100 mL chloroform with 100 mL of 5 % H_2SO_4 using a separating funnel. Thereafter, the chloroform was washed 3 times with 100 mL deionized water. For fumigation, multiple soil samples were fumigated at the same time, where the soils were spread in small glass dishes and allowed to fumigate in a desiccator, with 50 mL of ethanol-free chloroform in the dark at 25 °C for 24 h to expose the maximum surface area to chloroform vapor. After incubation, the chloroform was allowed to evaporate under a fume hood before the soils were extracted with 80 mL of 0.5 M K_2SO_4 solution as described above.

Extractable C (EC) in soil extracts was determined using a colorimetric dosage following a method of sulfochromic oxidation. The organic carbon in both the fumigated and non-fumigated is oxidized in the dichromate method causing the reduction of Cr VI to Cr III and a change in color from orange to green. Soil extracts (4 mL) were pipetted into glass digestion tubes and treated with 1 mL $K_2Cr_2O_7$ (2.5 g L^{-1}) and 5 mL H_2SO_4 (98 %). Samples were carefully mixed by vortex. In addition, a standard curve was realized with the solution of $K_2Cr_2O_7$ (2.5 g L^{-1}) and glucose (20 g L^{-1}). After some time, the samples were placed in a

microplate to determine their corresponding absorbance spectrophotometrically at wavelength λ = 350 nm (UV/VIS spectrophotometer, Multiskan® GO).

The microbial biomass C (expressed in mg kg^{-1}) was calculated as the difference in the absorbance between the fumigated and non-fumigated soils and after calculating the absorbance of a blank and referring to the calibration curve.

B.3.2.2- Soil basal respiration

The determination of soil microbial respiration depended on the calculation of the production of CO_2 without the addition of any substrate (basal respiration) (ISO 16072, 2002). The method used relies on a colorful indicator (cresol red), which shifted from red/pink to yellow based on a decrease in pH due to CO_2 liberation as a result of the microbial activities (Rowell, 1995).

The cresol red solution was prepared by mixing cresol red (12.5 µg mL^{-1}), potassium chloride (150 mM), sodium bicarbonate (2.5 mM) and deionized water. In a tube, 10 mL of the solution of cresol red were added and on the other hand 10 g of fresh soil sieved to 2 mm were placed in a second tube. Both were then placed gently in a larger jar which was then closed with a lid and placed in the dark for five days at 20°C (Figure 16).

Figure 16: Schematic representation of the soil basal respiration measurement protocol

Later on, 200 µL of the cresol red solution were deposited in a well of a microplate. A range of calibration was carried out using a solution of sodium bicarbonate. The absorbance of the samples was then read spectrophotometrically (UV/VIS spectrophotometer, Multiskan® GO) at wavelength λ = 590 nm. The CO_2 (mg C kg^{-1} h^{-1}) level released in the sample was determined for each of the samples after calculating the absorbance of a blank and referring to the calibration curve.

B.3.2.3- Fluorescein diacetate hydrolytic activities

Soil proteases and lipolytic enzymes (such as lipases and esterases) released by microorganisms play an important role in the turnover of proteins and lipids in the soil system. Their activities can be measured following the hydrolysis of fluorescein. The measurement of the fluorescein diacetate hydrolytic activities (FDHA) was based on the

quantification of fluorescein liberated after incubating soil in a sodium phosphate buffer according to Green et al. (2006).

In a centrifuge tube, 1 g of fresh soil sieved to 2 mm was introduced. Afterwards 45 mL of sodium phosphate buffer (60 mM; pH 7.6) and 0.5 mL of substrate (FDA solution 4.9 mM) were added. After swirling for a few seconds to mix the contents, the tubes were incubated at 37°C for 3 h. In order to stop the hydrolysis, 2 mL of acetone were added and mixed with the solution using a vortex. The solution was centrifuged for 5 min at 4500 rpm and filtered (0.45 µm). A range of calibration was performed using a solution of a standard of fluorescein (Sigma Aldrich) in the same matrix as samples. Then, the absorbance of each of the sample was measured spectrophotometrically at the wavelength λ = 490 nm (UV/VIS spectrophotometer, Multiskan® GO). The fluorescein content (mg fluo g^{-1}) released in the sample was obtained for each of the samples after calculating the absorbance of a blank and referring to the calibration curve.

Acetone can release visible compounds at 490 nm; controls were made for each sample to determine that the coloring did not come from the hydrolysis of the FDA. For the controls, the procedure described above was also used, but instead of the addition of the 0.5 mL of the substrate (FDA solution), 0.5 mL of acetone was added. A blank (samples without soil, only reagents), was also taken into account and the value was subtracted from the results achieved.

B.3.2.4- Laccases activity

Laccases play a central role in organic matter recycling as they are involved in lignin degradation and humic substances formation. Different protocols exist to determine laccase activities using several substrates as DOPA (L-3, 4-dihydroxyphenylalanine), DMP (2, 6-dimethoxyphenol), catechol or syringaldazine (Eichlerova et al., 2012). However, the most widely used substrate is the 2,2-azinobis-(-3-ethylbenzthiazoline-6-sulfonate), known as ABTS (Floch et al., 2007; Terron et al., 2004) . Therefore, the measurement of the activities was done by quantifying the rate of oxidation of ABTS to $ABTS^+$ in the supernatant.

In a centrifugation tube, 1 g of fresh soil sieved to 2 mm was mixed with 10 mL of modified universal buffer (MUB) at pH 2 and 200 µL ABTS (0.01 M). The tube was incubated at 30°C for 5 minutes. It was then cooled (2-3 min) in crushed ice in order to stop the reaction before being centrifuged at 4500 rpm for 4 min. The solution was then filtered (0.45 µm) before being transferred to a microplate. A range of calibration was carried out using a solution of $ABTS^+$ (0.01 M), made the day before by adding commercial laccases (Trametes versicolor; Sigma Aldrich) (Floch et al., 2007). Then the absorbance of the sample was measured spectrophotometrically at the wavelength λ = 420 (UV/VIS spectrophotometer, Multiskan® GO). The $ABTS^+$ (µmol g^{-1}) content released in the sample was obtained for each of the samples after calculating the absorbance of a blank and referring to the calibration curve.

B.3.2.5- Ureases

Ureases participate actively to nitrogen cycle by releasing inorganic N into soils. Their activity can be evaluated after a phase of extraction performed according to Kandeler and Gerber (1988). The measurement of the urase activity is based on their ability to release N, and is done through the colorimetric determination of ammonium (NH_4^+) release (O'dell, 1993; Saha et al., 2012) (Figure 17).

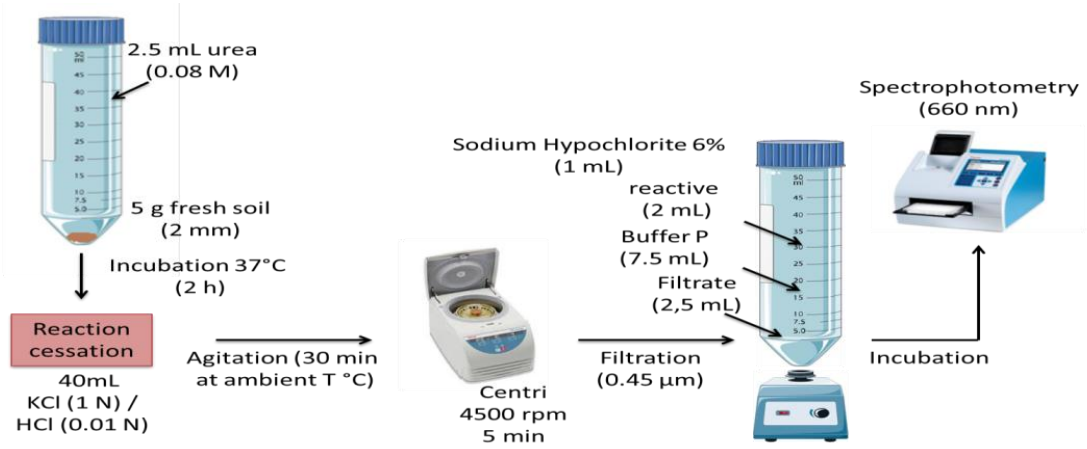

Figure 17: Schematic representation of the of soil urease activities measurement protocol

In centrifugation tube, 2.5 mL of urea solution (0.08 M) was added to 5 g of fresh soil sieved to 2 mm. It was incubated for 2 h at 37°C. In order to stop the reaction, 40 mL of potassium chloride (1 M KCl) and hydrochloric acid (0.01 M HCl) solution were poured in the tube that was then set to agitate for 30 min at room temperature. The solution was filtered (0.45 µm) and centrifuged for 5 min at 4500 rpm. A control was made by replacing the solution of urea by deionized water. Later on, 2.5 mL of the filtered and centrifuged solution was poured to another centrifugation tube and then 7.5 mL of O'Dell buffer solution (O'dell, 1993) was added along with 2 mL of sodium salicylate and sodium nitroprusside reagent and 1 mL of a solution of sodium hypochlorite (6 %). After a quick vortex, the solution was incubated for 1 h in the dark. A range of calibration was performed with a solution of ammonium chloride in the same matrix as samples. Then the absorbance of the sample was measured spectrophotometrically at the wavelength λ = 660 nm (UV/VIS spectrophotometer, Multiskan® GO). The content of NH_4^+ (µg N g^{-1}) released in the sample was obtained for each of the samples after calculating the absorbance of a blank and referring to the calibration curve.

B.3.2.6 Acid phosphatases

Similarly, to ureases for N, phosphatases are actively involved in P cycle. In particular, they mineralize organic phosphorous in soil. Their activity can be measured by several methods. Considered as the most sensitive and accurate, the protocol of Tabatabai and Bremner (1969) is based on the colorimetric estimation of the p-nitrophenol released by the acid

phosphatases activity upon soil incubation with buffer solution and the p-nitrophenyl phosphate (Eivazi and Tabatabai, 1977) (Figure 18).

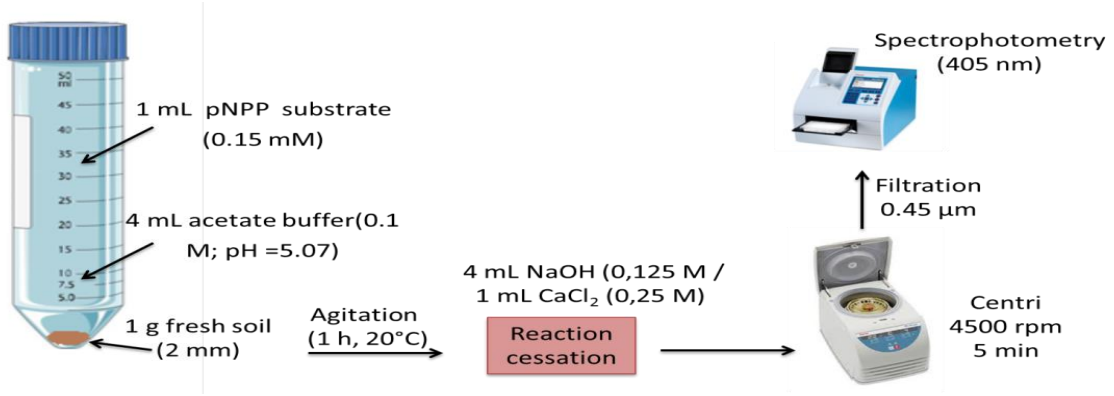

Figure 18: Schematic representation of the soil acid phosphatase activities measurement protocol

In a centrifugation tube, 4 mL of sodium acetate buffer (pH= 5) and 1 mL of para-nitrophenyl phosphate 0.15 mM (p-NPP; colorless) were added to 1 g of fresh soil sieved to 2 mm. The phosphatase hydrolysis of p-NPP released a monophosphate ion and the para-nitrophenol, (yellow-brown in color). After incubation at 37°C for one hour on a rotary Shaker, the mixture was completed with 1 mL of $CaCl_2$ (0.25 M) and 4 mL of NaOH (0.125 M) in order to stop the enzymatic reaction. Centrifugation of the sample was performed for 5 min at 4500 rpm. A blank was made by mixing 4 mL of sodium acetate, 1 mL of p-NPP, 1 mL of $CaCl_2$, and 4 mL of NaOH. This blank was used to figure out the absorbance of p-NPP in soil samples. A calibration range was performed from a preparation of para-nitrophenol (also called 4-nitrophenol or pNP; 0.15 mM). Then the absorbance of the sample was measured spectrophotometrically at the wavelength λ = 405 nm (UV/VIS spectrophotometer, Multiskan® GO). The quantity of p-nitrophenol (µg Np g^{-1}) released in the sample was obtained for each of the samples after calculating the absorbance of the blank and referring to the calibration curve.

B.4- Plant health evaluation

B.4.1- Plant metal concentrations

The concentrations of Cd, Pb, and Zn were measured on plant samples crushed and sieved to 250 µm. A sample of 0.3 g was mineralized with HNO_3 (70%, 5 mL) at 95°C in a mineralization plate (Hotblock Environmental Express, USA) for 75 minutes. Once cooled, 5 mL of hydrogen peroxide were added (H_2O_2, 30%), and the solution was heated for 3 h at 95°C. The determination of the metal concentrations has been achieved by means of a AAS (Shimadzu AA - 6800 Tokyo, Japan) coupled with a (Shimadzu, Tokyo, Japan) ASC-6100 autosampler (Waterlot et al., 2013). The residual moisture was measured according to standard NF ISO

11465. For quality assurance, replicates were used, blanks and clean laboratory reference material were included as well in the analyses.

B.4.2- Stress biomarkers

The responses of the plant against the metal stress in the different soils were determined by measuring quantitative biological parameters: oxidative stress, photosynthetic pigments, and secondary metabolism.

B.4.2.1- Antioxidative enzymatic activities determination

The production of reactive oxygen species (ROS) is one the most well-known effect of metal-induced stress. Plant cells possess very efficient antioxidative systems (both enzymatic and non-enzymatic) to detoxify ROS. The evaluation of antioxidative enzymatic activities provides an indirect information about metal-induced oxidative stress and direct information about plant ability to cope with this oxidative stress. In this work, we investigated the three main enzymes involve in metal-induced oxidative stress (): superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR).

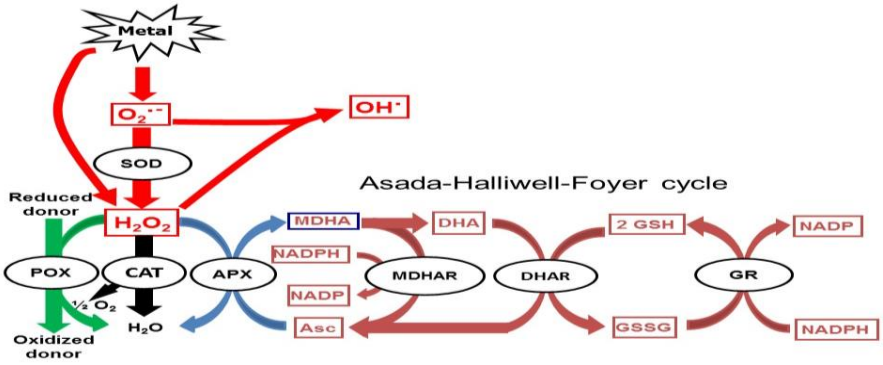

Figure 19: Enzymatic detoxification pathway of metal-induced ROS in plant cell

APX: Ascorbate peroxidase; CAT: Catalase; DHAR: Dehydroascorbate reductaseGR: Glutathione reductase; MDHAR: Monodehydroascorbate reductase; POX: Peroxidase; SOD: Superoxide dismutase.

Antioxidative enzymatic activity assays were evaluated spectrophotometrically according to the high-throughput protocols developed by Liné et al. (in preparation) using a plate reader (Thermo Scientific Multiskan™ GO). Briefly, five foliar discs (0.5 cm in diameter) per plant sample were collected from frozen leaves using a manual punch. Then they were put into in 96-deepwell plates (2 mL) with one 4-mm-diameter glass bead. Samples were then ground under frozen conditions using a Mixer Mill MM 400 (Retsch), twice for 1.5 min at 30 Hz, after addition of 1 mL of ice-cold Tris extraction buffer pH 7.0 containing 0.01 M EDTA, 0.4 M PVP, 0.05 ascorbate, 11.44 mM β-mercaptoethanol, and protease cocktail inhibitor. Samples were homogenated for 2 min at 15 Hz with the MM400 grinder. Plates were then centrifuged at

5000 *g* for 15 min at 4°C. Supernatants were collected and protein content was determined according to Bradford (1976), using bovine serum albumin (BSA, Sigma) as standard.

B.4.2.1.1- Superoxide dismutase (SOD)

SOD is considered the first line of against ROS, dismutating $O_2^{\cdot -}$ to oxygen molecule and H_2O_2 according to the following reaction:

$$2\ O_2^{\cdot -} + 2\ H^+ \xrightarrow[SOD]{} 2\ H_2O_2 + O_2$$

In the laboratory, the SOD determination was determined by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT), according to the method of Giannopolitis and Ries (1977). The method is based on the presence of a photosensitive molecule (riboflavine) which releases an electron when lit by intense light. This electron reduces dioxygen to produce the anion superoxide. Then, this anion superoxide is able to reduce Nitro blue tetrazolium (NBT; colorless) into formazan blue (blue):

$$\text{Riboflavine} + h\nu + O_2 \rightarrow \text{riboflavine}^{\cdot} + O2^{\cdot -}$$
$$O_2^{\cdot -} + \text{NBT} \rightarrow \text{formazan blue} + O_2$$

The reaction mixture contained 0.47 mM NBT (Sigma), 3.85 µM riboflavin (Sigma), 19.23 mM methionine (Sigma), 36.54 mM phosphate buffer (pH 7.8), and 20 µL enzyme extract. The test tubes containing the mixture were placed 30 cm below a light source (30 W fluorescent lamps). The reaction was started by switching on the light and was allowed to run for 10 min. The reaction was stopped by switching off the light with the absorbance at 560 nm. An unirradiated reaction mixture that did not develop color served as the control, and its absorbance was subtracted from that of the test tube. One unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of NBT reduction.

B.4.2.1.2- Ascorbate peroxidase (APX)

APX is one of the most important antioxidative enzymes in plants. Moreover, unlike catalase, its activity is not negatively affected by metal contamination. This enzyme has a high affinity for H_2O_2 and reduces it to H_2O in chloroplasts, cytosol, mitochondria, and peroxisomes, as well as in the apoplastic space, utilizing ascorbate as specific electron donor (Sofo et al., 2015) according to the following reaction:

$$H_2O_2 + \text{Ascorbate} \xrightarrow[APX]{} H_2O + \text{Monodehydroascorbate (MDA)}$$

Therefore, the APX activity was determined by its ability to oxidize its co-substrate, the ascorbate (yellow) into monodehydroascorbate (colorless) according to Nakano and Asada (1981).

The reaction mixture contained 434 mM phosphate buffer (pH 7.0), 3.77 mM H_2O_2, 0.56 mM ascorbic acid, and 10 µL enzyme extract. One enzyme unit was defined as 1 µmol of ascorbic

acid oxidized per min at 290 nm using an ascorbate standard curve. The enzyme activity was expressed as µmoles of ascorbate oxidized min^{-1} mg^{-1} protein.

B.4.2.1.3- Glutathione reductase (GR)

GR plays a vital role in maintaining the regulation of the cytosolic redox environment which is important for the cell endurance. It converts the oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH as co-substrate (Gill et al., 2013), according to the following reaction:

$$GSSG + NADPH + H^+ \xrightarrow[GR]{} 2\ GSH + NADP^+$$

Moreover, GR is a key enzyme in metal detoxification as GSH and phytochelatines (oligomers of GSH) play a fundamental role in metal chelation.
GR was assayed as the decrease in absorbance at 340 nm caused by the oxidation of NADPH (Dringen and Gutterer, 2002). This assay is based on the reduction of oxidized glutathione (GSSG) by NADPH in the presence of GR. The reaction mixture contained 0.1 M Tris buffer (pH 7.5), 1 mM GSSG (Sigma), 0.1 mM NADPH (Sigma), and 20 µL enzyme extract. The amount of NADPH oxidized was calculated from the extinction coefficient of 3.732×10^{-3} mL $nmol^{-1}$ of NADPH. The enzyme activity was expressed as nmol of NAPDH oxidized min^{-1} mg^{-1} protein.

B.4.2.2- Photosynthetic pigments and secondary metabolites determination

The photosynthetic pigments (chlorophyll a, b, and carotenoids) are necessary for the photosynthesis process. Carotenoids, as well, are plant pigments functioning as non-enzymatic antioxidants. They play an influential role in protecting chlorophyll pigments and cell membranes under stress conditions either by dissipating excess excitation energy as heat or by scavenging ROS and suppressing lipid peroxidation (Baek et al., 2012; Gill and Tuteja, 2010). The concentration of the corresponding pigments is considered as sensitive biomarker of metal stress and may predict subsequent events at the organism level (MacFarlane and Burchett, 2001). The absorbance properties of the pigments facilitate their qualitative and quantitative analysis.

The non-enzymatic antioxidants represent the other half of the antioxidative machinery, comprising several compounds. Among which, the secondary metabolites (phenolic compounds, tannins, flavonoids, and anthocyanins) which play an important role in not only protecting the different components of the cell from damage, but also in plant growth and development by tweaking cellular process like mitosis, elongation, senescence and cell death (Das and Roychoudhury, 2014). Moreover, they also play a role in metal tolerance and detoxification.

The photosynthetic pigments and the secondary metabolites were evaluated spectrophotometrically according to Liné et al. (in preparation) using a plate reader (Thermo

Scientific Multiskan™ GO). Briefly, two foliar discs (0.5 cm in diameter) per plant sample were collected from frozen leaves using a manual punch and weighed. Then they were put into a 96-deepwell plate (2 mL) with one 4-mm-diameter glass bead. Samples were then ground under frozen conditions using a Mixer Mill MM 400 (Retsch) twice for 1.5 min at 30 Hz. After the addition of 1.5 mL of ice-cold 95% methanol in each well, samples were homogenated for 2 min at 15 Hz with the MM400 grinder. Plates were left in the dark for 24 h and 48 h of incubation.

After 24 h of incubation, leaf extracts were homogenated for 2 min at 15 Hz with the Mixer Mill MM 400. A total of 100 µL was collected for photosynthetic pigment analysis. The absorbance was measured at 470, 652, and 666 nm. Concentrations of total carotenoids, chlorophyll a and b and were calculated according to the extinction coefficients and equations reported by Lichtenthaler (1987). Finally, data were averaged and the mean concentrations obtained were expressed as mg g^{-1} FW of leaf.

After 48 h of incubation, plates were centrifuged at 5000 *g* for 5 min, prior to secondary metabolism molecule extraction. The total phenolic compound was determined based on Folin Ciocalteu assay. Briefly, the 200-µL reaction mixture contained 20 µL of supernatant, 40 µL of Folin reagents (10% v/v), and 0.098 mM of Na_2CO_3. The mixture was allowed to stand 2 h at room temperature for color development and then absorbance was measured at 510 nm. Concentrations of phenolic compounds were calculated using a standard curve of gallic acid. The results were expressed as mM of gallic acid equivalent (GE) per gram of fresh weight of leaf. The flavonoid content was determined using the aluminum chloride method using catechin as the reference compound. Briefly, the reaction mixture contained 25 µL of methanolic extract, 0.00724 mM $NaNO_2$, 0.01125 mM $AlCl_3$, and 0.05 mM NaOH. The mixture was homogenated for 1 min and absorbance was measured at 595 nm. Flavonoid concentrations were calculated using a standard curve of catechin. The results were expressed as mg catechin equivalent (CE) per gram of fresh weight of leaf. For tannins, the reaction mixture contained 50 µL of methanolic extract and 100 µL of vanillin solution 1%. The mixture was let in the dark for 15 min and absorbance was measured at 500 nm. The tannin concentration was calculated using a standard curve of catechin. The results were expressed as mg L^{-1} catechin equivalent (CE) per gram of fresh weight of leaf. Anthocyanins were measured using the differential pH method based on the property of anthocyanin pigments to change color with pH. Two dilutions of the same sample were prepared, the first one in potassium chloride buffer (0.2 M, pH 1.0) and the second in sodium acetate buffer (0.4 M, pH 4.5). After equilibration at room temperature for 15 min, the absorbance was read at 510 and 700 nm. The results were expressed as mg cyaniding 3-glucoside equivalent per gram of fresh weight of leaf.

B.5- Statistical analysis

Analysis of variance was done to compare modalities in every experiment. Fisher test was considered for significance ($p \leq 0.05$). If statistically significant differences were found, the Tukey HSD test was used for pair-wise comparisons. Principal component analysis was performed to study the relationship between the measured soil parameters in C-1 (studying the impact of the miscanthus plants on the soil parameters). All statistical analyses were performed using XLSTAT software.

Part C- Impacts of the *Miscanthus x giganteus* cultivation on the soil agronomic parameters

C.1- Assessment of *Miscanthus x giganteus* capacity to restore the functionality of metal-contaminated soils: *ex situ* experiment

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Foreword

Miscanthus x giganteus has demonstrated high potentials to adapt and to grow particularly well in the metal contaminated area surrounding the former lead smelter "Metaleurop nord" (Nsanganwimana et al., 2014). The corresponding metal concentrations were mostly accumulated in the underground parts and to a lesser extent in the aboveground ones (Nsanganwimana et al., 2014, 2015, 2016). Thus, miscanthus was proposed as a good candidate to phytomanage this area by reducing metal mobility, transfer and bioavailability. Beyond the above-mentioned information, the information about the impact of the miscanthus plants on soil biological parameters are scares in general, and has never been investigated in the case of the degraded soil in the Metaleurop area. Indeed, when the *in situ* experimental site has been set up between 2008 and 2010, these parameters were not taken into consideration, and no analyses were performed before the establishment of miscanthus culture. Thus, it is impossible for us to investigate the influence of miscanthus on soil system *in situ.*

Therefore, the work presented in this chapter aimed to determine the influence of miscanthus on restoring the degraded soil functionality. An *ex situ* experiment was established by cultivating three miscanthus cultivars in 20-kg pots filled with soil collected from different agricultural plots surrounding Metaleurop, representing a gradient metal contamination (M200, M500, M750 and M900, the number corresponding to their approximate concentration in lead), and another uncontaminated soil taken as a reference (MC). The corresponding experiment started in May 2014 and lasted for one year. In order to study specifically the role of miscanthus roots on restoring soil functionality, all the fallen leaves were removed from the soil surfaces during the experiment.

Several analyses were applied on the collected soil samples in an attempt to study the impacts of three miscanthus cultivars on:

- physico-chemical parameters of the contaminated soils,
- Cd, Pb and Zn extractability,
- soil biological parameters (microbial biomass carbon, basal respiration and certain enzymatic activities involved in C, N and P cycles).

Publication

Al Souki, K. S., Louvel, B., Douay, F., Pourrut, B., 2017. Assessment of *Miscanthus x giganteus* capacity to restore the functionality of metal-contaminated soils: *ex situ* experiment. Accepted in Applied Soil Ecology.

C.1- Assessment of *Miscanthus x giganteus* capacity to restore the functionality of metal-contaminated soils: *ex situ* experiment

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Abstract: Phytostabilisation can be an appropriate choice for managing soils contaminated by diverse metals. *Miscanthus x giganteus* was selected to phytostabilise polluted soils surrounding the former lead smelter Metaleurop Nord, in Northern France. The aim of the current study was to determine the impact of miscanthus plants on restoring the corresponding soil functionality. *Ex situ* experiments revealed that soil biological activities (basal respiration, fluorescein diacetate hydrolytic activity, acid phosphatases, laccases and ureases) as well as microbial biomass carbon increased in contaminated soils cultivated with miscanthus, compared to unplanted soils. These results verify the capacity of miscanthus to restore polluted soil functionalities, indicating the positive effect of phytostabilisation on soil biological activities. In conclusion, revegetating polluted soils with miscanthus plants can restore their capacity to perform biochemically and biologically.

Keywords: *Miscanthus x giganteus*, phytostabilisation, metal contamination, soil biological indicators, soil enzymatic activities, phytomanagement

C.1.1- Introduction

Anthropogenic activities (mining, industrial activities, combustion of fossil fuels, waste disposal, agriculture, etc) are the main causes behind soil metal pollution (Gomez-Sagasti et al., 2012; Panagos et al., 2013). Areas in the vicinity of pollutant sources are more exposed to contamination of air, water and/or soil (De Bartolomeo et al., 2004; Miro et al., 2004). Such exposure might pose not only severe health threats to humans, but also to plants, animals and microorganisms.

Remediating polluted areas represent a great challenge because of the persistence and/or the existence of multiple pollutants at the site and/or the extension of pollution. Conventional soil remediation commonly involves physical methods such as excavation and removal of soil to landfills (Dermont et al., 2008). Taking into consideration the fact that such methods are expensive, environmentally unfriendly and unsuitable for large areas, it is not surprising that there is a growing interest to overcome these drawbacks and meet regulatory guidelines, by developing *in situ* environmentally friendly as well as cost-effective methods capable of ameliorating the ecological state of the contaminated area (Conesa et al., 2012).
The last two decades have witnessed a remarkable interest in phytoremediation, comprising numerous techniques that use tolerant plants to mitigate pollutant diffusion and risks (Nsanganwimana et al., 2014) including phytostabilization, which aims to manage large scale and/or poly-contaminated areas. It is based on metal-tolerant plants able to precipitate

metals and/or induce metal complex formation in the rhizosphere and/or accumulate them in their root tissues, reducing their translocation to aerial organs, consequently reducing their mobility and bioavailability (Gomez-Sagasti et al., 2012; Wong, 2003).

Phytostabilisation can also play a crucial role in restoring soil functionality that could be disturbed by metal contamination. The plant root system stabilizes the soil by preventing erosion and leaching by reducing water percolation through the soil (Chibuike and Obiora, 2014). In addition, plant roots ameliorate the physical, chemical and biological conditions of the rhizosphere by enriching it with organic substances of plant and microbial origin including organic acids, sugars, proteins, enzymes, carbohydrates and other compounds (Hinsinger et al., 2005). For instance, Dary et al. (2010) enlightened the positive effects of *Lupinus luteus* for *in situ* phytostabilisation and reclamation of metal-polluted soils. Epelde et al. (2009a) demonstrated the impact of phytostabilisation with *Lolium perenne* on soil biological properties, by increasing the biomass, activity and functional diversity of the soil microbial community. Moreover, Burges et al. (2016) showed the capacity of *Festuca rubra* to grow well in metal-contaminated mine soils, to reduce the bioavailability of Cd and Zn, in addition to enhancing several soil microbial parameters.

Among plants used for phytostabilisation, the C4 perennial rhizomatous grass *Miscanthus x giganteus* has been shown to be a good candidate. *In situ* and *ex situ* experiments of Nsanganwimana et al. (2014) demonstrated its high capacity to grow well on metal-contaminated soils, reduce their potential mobility, mainly accumulate them in roots and limit their transfer into aerial organs. Moreover, its high biomass could be invested in energy and biofuel production, building and construction materials and diverse agricultural uses, making the plant valuable for the phytomanagement of metal-contaminated areas (Nsanganwimana et al., 2014). However, the effect of this species on soil biological parameters has not yet been investigated.

The main objective of the current study was to determine the impact of *Miscanthus x giganteus* on restoring the functionality of polluted soils with a gradient concentration of metals. For this purpose, we studied the effects of three different miscanthus cultivars on: i) the physico-chemical parameters of soils with gradient concentration of Cd, Pb and Zn, ii) the corresponding metals' extractability and iii) the soil biological parameters (microbial biomass carbon, basal respiration and certain enzymatic activities in the corresponding soils).

C.1.2- Materials and Methods

C.1.2.1- Soil and plant origins and preparations

Soil samples (plowed horizon, 0–30 cm) were collected from different metal-contaminated (Cd, Pb and Zn) agricultural fields, located around the former Pb smelter Metaleurop Nord (Northern France) (Lopareva-Pohu et al., 2011). These soils were designated M200, M500, M750 and M900, corresponding to their approximate Pb concentrations (mg kg^{-1} soil), which

increase as the distance to the smelter decreases (Douay et al., 2013; Sterckeman et al., 2002). The M200 (50°24’52”N, 3°01’51”E, Courcelles-les-Lens) and M500 (50°25’49”N, 3°02’13”E, Evin-Malmaison) plots are located plots are 1.8 km Southeast and 1.4 km Northeast to the former smelter (50°25’42”N 3°00’55”E, Noyelles-Godault), whereas the third plot where M750 and M900 soil samples were collected from is at approximately 1 km distance and located at Evin-Malmaison (50°26'15.0"N 3°01'05.7"E).

Another uncontaminated sample taken as control “MC” was also collected from an agricultural field established 75 km from the smelter (50°20’46”N 2°12’15”E) at the village of Linzeux. Thereafter, samples were homogenized, dried and sieved through a 10 mm mesh prior to cultivation.

Three different cultivars of *M. x giganteus,* referred to as B, U and A, originating from England, USA and Austria respectively, were used throughout the experiment. Rhizomes were grown in polyethylene pots (9 x 9 x 9 cm) filled with potting compost. The compost was kept wet by watering. Rhizomes were maintained in the pots, until miscanthus plantlets reached 20–25 cm in height.

C.1.2.2- Experimental design and plant growth

Ex situ experiment was conducted over a 1-year (May 2014 to June 2015). About 100 kg of each of the above mentioned homogenized soils (MC, M200, M500, M750 and M900) was equally distributed in five pots (light grey in color in order to avoid temperature elevation possibilities). The *M. x giganteus* plantlets (B, U and A) were displaced from the small pots to 20-kg soil pots (two plantlets in each pot). Moreover, to clearly understand the effects of the plant, and to assess its capacity to stabilize metals and restore soil functionality, two other modalities were established: uncultivated covered pots “C” (aimed to prevent any type of spontaneous vegetation to develop) and an uncovered “UC” control pots (n = 5).

To sum up, 75 planted pots were used (three different miscanthus cultivars x 5 soils x 5 replicates), in addition to another 50 unplanted pot (C and UC x 5 soils x 5 replicates). The experiment was established in an area away from roads on the University campus, by which the pots were disposed over wooden rafters to avoid contact with the ground of the experimental area. The pots were randomly distributed over the location to avoid point and borderline effects. In addition to the rain water, soil humidity was maintained by regular watering during the entire experiment. Weeds were manually removed and kept on the surface of the soils to avoid a possible metal exportation.

C.1.2.3- Soil physico-chemical parameters and metal concentrations

Physico-chemical parameters and metal concentrations were measured at the beginning of the experiment (T0) and 1 year after (T+1) during the same season according to standardized protocols, where fresh soil samples were collected from the pot using an auger (composite samples were prepared via collecting 5 soil samples from each pot). Afterwards in the laboratory, the soil samples were dried at 40 °C for 24 h, ground and sieved to 2 mm and 250 µm.

Particle size distribution was determined by sedimentation and sieving after destruction of organic matter by H_2O_2 according to the French NFX 31-107 standard. pH (H_2O) was measured after stirring a mixture of soil and deionized water (1:5, v/v) according to the ISO 10390 standard. Total carbonate content was determined measuring the CO_2 formed after HCl (4 M) addition according to the NF ISO 10693 standard. Soil organic carbon (SOC) content was extracted and measured according to the ISO 14235 standard. Total nitrogen (TN) content was determined by the dry combustion method according to ISO 13878. Available phosphorus (P_2O_5) concentration was measured according to the French NFX 31-161 standard and Joret and Hébert (1955) via extraction in ammonium oxalate solution ($(NH_4)_2$ C_2O_4, 0.1M, pH = 7). Cationic exchange capacity (CEC) was determined after percolation of CH3COONH4 (1M, pH = 7) solution into soil samples followed by an extraction of ammonium ions ($NH4^+$) with sodium chloride (NaCl, 1 M) according to the French NF X31-130 standards.

For the determination of Cd, Pb and Zn pseudototal concentrations, soil acid digestion was performed using a digestion plate (HotBlock (TM) Environmental Express, USA). The aqua regia solution (HCl + HNO3, 3:1, 6 mL) was added and the aliquot heated at 120 °C for 120 min. The quality control of the extraction and analysis was provided by the introduction of two internal reference samples and a certified soil reference (CRM 141, IRMM, Belgium). The Cd, Pb, and Zn in extracts were determined by atomic absorption spectrophotometry (AA-6800, Shimadzu).

For the determination of available Cd and Zn concentrations, soils were mixed with a 0.01 M calcium chloride ($CaCl_2$) solution at a ratio of 1:10, m/v according to Waterlot and Douay (2012). The Cd, Pb, and Zn in extracts were determined by atomic absorption spectrophotometry (AA- 6800, Shimadzu).

C.1.2.4- Soil biological parameters

Fresh soil samples were collected, sieved to 2 mm, and conserved at 4°C, until the beginning of the experiments.

Soil microbial biomass carbon (MBC) was quantified via the chloroform fumigation extraction method stated by Vance et al. (1987) and Wenhao et al. (2013). Soil basal respiration (BR) was determined according to Rowell (1995). Fluorescein diacetate hydrolytic

activities (FDHA) were determined according to the optimized method adopted by Green et al. (2006). Laccase, acid phosphatase and urease activities were measured according to Eichlerova et al (2012), Eivazi and Tabatabai (1977), Kandeler and Gerber (1988), respectively. All enzyme activities were determined at optimum conditions of pH, temperature and substrate concentration to assess their maximum potential activities in the soils.

C1.2.5- Statistical analysis

Analysis of variance was done to compare modalities. Fisher test was considered for significance ($p \leq 0.05$). If statistically significant differences were found, the Tukey HSD test was used for pair-wise comparisons. Principal component analysis was performed to study the relationship between the measured soil parameters. All statistical analyses were performed using XLSTAT software.

C.1.3- Results

C.1.3.1- Soil initial physico-chemical parameters, particle size distribution and metal concentrations

The initial physico-chemical parameters, particle size distribution and metal concentrations were determined for the studied soils (Table 1). As explained above, the contamination degree of the soils collected from different agricultural plots varied with their distance from the former smelter. Metal concentrations were between 20 and 50 times higher than regional background values (0.42, 38 and 74 mg kg^{-1} corresponding to Cd, Pb and Zn, respectively). On the other hand, the Cd, Pb and Zn concentrations of MC soils met the regional background values. pH values in the different soils ranged from slightly acidic in MC (6.4) to slightly alkaline in M500 (7.6). Moreover, M500 soils contained the highest $CaCO_3$ content (14.8 g kg^{-1}), whereas MC had the least (0.4 g kg^{-1}). Concerning the particle size distribution, M500 soils were more clayey than the others. However, the soil grain size of the five studied soils was dominated by silt (49.7 to 69.5).

	MC	M200	M500	M750	M900
pH	6.4	7.1	7.6	7.3	7.1
$CaCO_3$ (g kg^{-1})	0.4	0.9	14.8	5.4	7.6
Clay (%)	20.8	17.8	30.6	19.2	16.4
Silt (%)	69.5	57.3	49.7	56	59.2
Sand (%)	9.7	24.9	19.7	24.8	24.4
Cd (mg kg^{-1})	0.3 ± 0.0	3.8 ± 0.2	9.0 ± 0.2	13.5 ± 0.3	16.0 ± 0.3
Pb (mg kg^{-1})	37.3 ± 1.3	260.3 ± 2.0	528.6 ± 5.3	747.1 ± 16.9	898.6 ± 16.3
Zn (mg kg^{-1})	54.6 ± 3.1	388.0 ± 14.8	537.0 ± 10.9	906.0 ± 16.8	1116.0 ± 18.7

Table 1: Initial physico-chemical parameters, particle size distribution and metal (Cd, Pb and Zn) concentrations of studied soils (mean ± standard deviation)

C.1.3.2- Changes in Soil physico-chemical parameters

Table 2 presents in the form of ratios the changes of the physico-chemical parameters in the studied soils after one year of experiment (T+1). Values superior to 1 indicate that the corresponding parameter had increased in comparison to T0, whereas values inferior to 1 imply the opposite.

		MC	M200	M500	M750	M900
B	pH	0.95 ± 0.01 f	1.00 ± 0.01 e	1.03 ± 0.01 d	1.03 ± 0.01 d	1.04 ± 0.01 bcd
	P_2O_5 (mg kg^{-1})	0.56 ± 0.13 cde	0.80 ± 0.09 abc	0.54 ± 0.19 de	0.83 ± 0.05 ab	0.81 ± 0.05 abc
	CEC (cmol kg^{-1})	1.47 ± 0.04 a	1.31 ± 0.03 bcde	1.21 ± 0.10 ef	1.34 ± 0.06 abcd	1.27 ± 0.04 cde
	SOC (g kg^{-1})	1.61 ± 0.03 bcd	1.53 ± 0.10 cde	1.41 ± 0.05 cdefg	1.96 ± 0.09 a	1.80 ± 0.19 ab
	TN (g kg^{-1})	0.62 ± 0.02 hi	0.64 ± 0.06 hi	0.76 ± 0.02 cdefg	0.70 ± 0.03 gh	0.76 ± 0.03 cdefg
U	pH	0.95 ± 0.03 f	0.99 ± 0.01 e	1.03 ± 0.01 d	1.02 ± 0.01 d	1.03 ± 0.01 d
	P_2O_5 (mg kg^{-1})	0.57 ± 0.06 bcde	0.78 ± 0.05 abc	0.40 ± 0.11 e	0.86 ± 0.02 a	0.79 ± 0.05 abc
	CEC (cmol kg^{-1})	1.33 ± 0.06 abcde	1.34 ± 0.04 abcd	1.37 ± 0.03 abc	1.36 ± 0.05 abcd	1.31 ± 0.03 bcde
	SOC (g kg^{-1})	1.41 ± 0.04 cdefg	1.33 ± 0.12 defgh	1.42 ± 0.09 cdefg	1.62 ± 0.15 bc	1.47 ± 0.09 cdef
	TN (g kg^{-1})	0.60 ± 0.01 hi	0.67 ± 0.01 ghi	0.74 ± 0.01 efg	0.72 ± 0.03 fgh	0.75 ± 0.06 efg
A	pH	0.95 ± 0.02 fg	0.99 ± 0.01 e	1.03 ± 0.01 d	1.03 ± 0.01 d	1.04 ± 0.01 cd
	P_2O_5 (mg kg^{-1})	0.56 ± 0.10 cde	0.73 ± 0.16 abcd	0.42 ± 0.21 e	0.69 ± 0.03 abcd	0.70 ± 0.03 abcd
	CEC (cmol kg^{-1})	1.40 ± 0.08 abc	1.43 ± 0.06 ab	1.23 ± 0.04 de	1.25 ± 0.06 cde	1.20 ± 0.05 ef
	SOC (g kg^{-1})	1.58 ± 0.04 bcde	1.37 ± 0.13 cdefgh	1.47 ± 0.08 cdef	1.91 ± 0.17 a	1.85 ± 0.20 ab
	TN (g kg^{-1})	0.58 ± 0.00 i	0.69 ± 0.10 ghi	0.75 ± 0.04 defg	0.69 ± 0.03 ghi	0.75 ± 0.05 defg
C	pH	0.86 ± 0.01 h	1.03 ± 0.01 d	1.06 ± 0.01 abc	1.07 ± 0.01 a	1.06 ± 0.01 a
	P_2O_5 (mg kg^{-1})	0.87 ± 0.02 a	0.90 ± 0.09 a	0.88 ± 0.06 a	0.92 ± 0.01 a	0.91 ± 0.03 a
	CEC (cmol kg^{-1})	0.81 ± 0.09 ij	0.73 ± 0.10 ijk	1.07 ± 0.06 fg	0.63 ± 0.05 k	0.61 ± 0.06 k
	SOC (g kg^{-1})	1.10 ± 0.01 hi	1.01 ± 0.06 i	1.07 ± 0.03 hi	1.19 ± 0.05 fghi	1.16 ± 0.08 ghi
	TN (g kg^{-1})	0.81 ± 0.01 abcdef	0.89 ± 0.05 ab	0.92 ± 0.01 a	0.87 ± 0.07 abc	0.91 ± 0.06 a
UC	pH	0.92 ± 0.02 g	1.02 ± 0.01 d	1.06 ± 0.01 ab	1.06 ± 0.01 abc	1.06 ± 0.01 a
	P_2O_5 (mg kg^{-1})	0.81 ± 0.07 abc	0.89 ± 0.08 a	0.81 ± 0.02 abc	0.91 ± 0.09 a	0.95 ± 0.03 a
	CEC (cmol kg^{-1})	0.77 ± 0.03 ijk	0.68 ± 0.01 jk	1.03 ± 0.04 gh	0.87 ± 0.03 hi	0.64 ± 0.01 jk
	SOC (g kg^{-1})	1.26 ± 0.05 efghi	1.17 ± 0.05 fghi	1.34 ± 0.03 cdefgh	1.45 ± 0.02 cdefg	1.26 ± 0.15 efghi
	TN (g kg^{-1})	0.73 ± 0.02 efgh	0.76 ± 0.02 cdefg	0.86 ± 0.02 abcd	0.77 ± 0.03 bcdefg	0.85 ± 0.04 abcde

Table 2: Ratio (T+1/T0) of physico-chemical parameters of soils (MC, M200, M500, M750, and M900) planted with three different miscanthus cultivars (B, U and A), and unplanted covered (C) and uncovered (UC) soils, ± standard deviation. CEC: cationic exchange capacity, SOC: soil organic carbon,

TN: total nitrogen (For every parameter, different letters in lines refer to significant differences between soils) (Tukey HSD test, $n = 5$, $p \leq 0.05$)

In MC soils, the pH at T0 was 6.1, 6.0, 6.0, 6.1 and 6.1 corresponding to B, U, A, C, and UC respectively, whereas it fluctuated between neutral in the M200 soils and slightly alkaline in M500, M750 and M900 soils respectively (supplementary data Table 1). The pH evolution reveals slight changes throughout the duration of the experiment. However, two different trends were detected. The MC soil became more acidic, whereas the other soils turned out to be more basic. Yet, the shifts in both sides were less in the miscanthus cultivated pots, than in the unplanted ones. The (T+1/T0) ratio in MC soils was 0.95 in the pots with miscanthus, 0.92 in the uncovered pots and 0.86 in the covered soils. The same trend appeared in the contaminated soils, where pH significantly increased compared to the miscanthus pots.

At T0, the lowest available P_2O_5 concentrations were detected in the MC soils (0.16, 0.13, 0.14, 0.10 and 0.08 mg kg^{-1} corresponding to B, A, U, UC and C respectively), while the highest values were found in M900 soils (0.60, 0.65, 0.58, 0.48 and 0.62 mg kg^{-1} corresponding to B, A, U, UC and C respectively) (supplementary data Table 1). The corresponding available concentrations decreased in all soil modalities. Yet, in MC and M500 soils, the difference was significant between planted and unplanted pots, by which the decline was greater in the former (T+1/T0 in MC soils = 0.56, 0.56, 0.57, 0.81 and 0.87 corresponding to B, A, U, UC and C respectively).

The highest CEC at T0 were detected in the M500 soils (25.9, 23.7, 26.5, 24.8 and 24.6 cmol kg^{-1} corresponding to B, A, U, UC and C respectively) in comparison with the other soils where it was fluctuating between 10.2 and 12.4 cmol kg^{-1}(supplementary data Table 1). The CEC increased in miscanthus-cultivated soils and decreased in C and UC soils. However, in M500 soils, the CEC increased in all modalities, yet it was significantly higher in the planted pots (T+1/T0 = 1.21, 1.37, 1.23, 1.07 and 1.03 corresponding to B, U, A, C, and UC respectively).

SOC concentrations in the M500 soils at T0 were higher than the other soils modalities (29.5, 26.0, 27.4, 26.7 and 24.4 g kg^{-1} corresponding to B, U, A, C, and UC respectively), while the lowest values were detected in the M750 and M900 with approximately similar SOC concentrations (supplementary data Table 1). However, the corresponding SOC concentrations increased in all soils, especially those cultivated (a significant difference existed between the cultivated and uncultivated pots). The highest increase in SOC was always observed in the M750 soils (T+1/T0 = 1.96, 1.62, 1.91, 1.19 and 1.45 corresponding to B, U, A, C and UC respectively).

Finally, total nitrogen (TN) contents at T0 where the most elevated in M500 soils (2.4 g kg^{-1} in B, U and A cultivated soils and 2.3 g kg^{-1} in the C and UC soils respectively). On the other hand the TN lowest values were displayed in the M900 soils (0.9, 0.8, 0.8, 0.9 and 0.9 g kg^{-1}

in B, U, A, C and UC soils respectively) (supplementary data Table 1). The corresponding TN concentrations decreased in all soils with a significant difference between miscanthus cultivated pots and the unplanted soils. In addition, MC soils showed the highest decrease mainly in miscanthus containing pots (T+1/T0 = 0.62, 0.60, 0.58, 0.81 and 0.73 corresponding to B, U, A, C and UC respectively).

C.1.3.3- $CaCl_2$ extractable metal concentrations

The Cd and Zn extractable concentrations by 0.01 M $CaCl_2$ at T0 of the different studied soils were presented (supplementary data Table 2). It should be mentioned that the $CaCl_2$ extractable Pb concentration was measured, yet the values were below the limit of detection (0.0636 mg/L) due its low mobility and strong adsorption to Fe/Mn oxides and organic matter (Cerqueira et al., 2011). Results reveal that the lowest extractable Cd concentration was recognized in the MC soils (0.08, 0.08, 0.07, 0.06 and 0.06 mg kg^{-1} corresponding to B, U, and A, C and UC respectively), whereas the highest extractable values were witnessed in the M900 soils (0.25, 0.24, 0.24, 0.23 and 0.23 mg kg^{-1} corresponding to B, U, and A, C and UC respectively). The lowest Zn extractable portion were in the M500 soils (0.19, 0.16, 0.14, 0.16 and 0.16 mg kg^{-1} corresponding to B, U, and A, C and UC respectively), whereas the highest values were recorded in the M200 soils (0.79, 0.78, 0.86, 0.81, and 0.80 mg kg^{-1} corresponding to B, U, and A, C, and UC, respectively).

The changes in Cd and Zn extractable concentrations by the 0.01 M $CaCl_2$ displayed in Table 3 demonstrate that the extractable Cd concentration decreased in both planted and unplanted pots, yet this was more evident in miscanthus-cultivated pots. For the soils planted, (T+1/T0) ratio ranged from 0.57 in MC and M500 soils with miscanthus B and 0.73 in M900 soil with miscanthus A. No significant differences were observed among the planted soils. In the non-planted modalities, the ratio varied between 0.80 in certain uncovered pots and 0.93 in some covered ones. No significant differences existed in the unplanted pots.

The $CaCl_2$ extractable Zn concentration decreased more in the planted pots (significant differences exist between the planted and unplanted soils). Among the cultivated soils, the highest decrease took place in the M500 soil (T+1/T0 = 0.25, 0.37 and 0.24 corresponding to B, U, and A respectively), whereas the M200 soil showed the smallest decrease in the Zn $CaCl_2$ extractable concentration (T+1/T0 = 0.81, 0.79 and 0.78 corresponding to B, U, and A respectively). At T+1, the change in the unplanted soil extractable concentrations ranged from 0.73 in the uncovered M500 soil to 1.12 in the covered M200 soil.

		MC	M200	M500	M750	M900
B	Cd (mg kg^{-1})	0.57 ± 0.04 e	0.68 ± 0.06 cde	0.57 ± 0.06 e	0.65 ± 0.03 cde	0.65 ± 0.03 cde
	Pb (mg kg^{-1})	ND	ND	ND	ND	ND
	Zn (mg kg^{-1})	0.59 ± 0.05 hij	0.81 ± 0.05 cdef	0.25 ± 0.02 l	0.59 ± 0.04 hij	0.70 ± 0.08 fghi
U	Cd (mg kg^{-1})	0.61 ± 0.04 de	0.68 ± 0.06 cde	0.61 ± 0.04 de	0.67 ± 0.07 cde	0.70 ± 0.04 bcd
	Pb (mg kg^{-1})	ND	ND	ND	ND	ND
	Zn (mg kg^{-1})	0.50 ± 0.06 jk	0.79 ± 0.02 cdef	0.37 ± 0.06 kl	0.53 ± 0.05 j	0.72 ± 0.10 fgh
A	Cd (mg kg^{-1})	0.61 ± 0.01 de	0.68 ± 0.10 cde	0.63 ± 0.12 de	0.68 ± 0.03 cde	0.73 ± 0.03 bcd
	Pb (mg kg^{-1})	ND	ND	ND	ND	ND
	Zn (mg kg^{-1})	0.56 ± 0.06 ij	0.78 ± 0.05 cdef	0.24 ± 0.02 l	0.57 ± 0.09 ij	0.63 ± 0.05 ghij
C	Cd (mg kg^{-1})	0.91 ± 0.01 a	0.90 ± 0.02 a	0.93 ± 0.03 a	0.93 ± 0.03 a	0.92 ± 0.05 a
	Pb (mg kg^{-1})	ND	ND	ND	ND	ND
	Zn (mg kg^{-1})	0.90 ± 0.00 bcd	1.12 ± 0.03 a	0.89 ± 0.04 bcde	0.80 ± 0.01 cdef	0.98 ± 0.01 ab
UC	Cd (mg kg^{-1})	0.80 ± 0.02 abc	0.85 ± 0.07 ab	0.86 ± 0.04 ab	0.80 ± 0.02 abc	0.80 ± 0.04 abc
	Pb (mg kg^{-1})	ND	ND	ND	ND	ND
	Zn (mg kg^{-1})	0.75 ± 0.04 defg	0.93 ± 0.02 bc	0.73 ± 0.07 efgh	0.77 ± 0.04 cdefg	0.90 ± 0.03 bcd

Table 3: Ratio (T+1/T0) of $CaCl_2$ extractable Cd, Pb and Zn concentrations in soils, planted with three different miscanthus cultivars (B, U and A), and unplanted covered (C) and uncovered (UC) soils, ± standard deviations (For every parameter, different letters in the lines refer to significant differences between soils) (Tukey HSD test, $n = 5$, $p \leq 0.05$). ND: not detected

C.1.3.4- Changes in Soil biological parameters

Microbial biomass carbon

The results at T0 (supplementary data Table 3) show that the highest biomass was observed in M500 soils (308.9, 314.3, 313.3, 320.5 and 312.7 mg kg^{-1} dry soil, corresponding to B, U, A, C, and UC respectively), whereas M750 and M900 soils contained the lowest. As for the changes in MBC present in Figure 1, significant differences existed between the ratio of microbial biomass in planted and unplanted soils. However, the changes were approximately the same in all soil modalities independently of the metal concentration. MBC in the planted soils ranged from 1.4 to 1.5xT0, whereas it evolved very little in the unplanted soils.

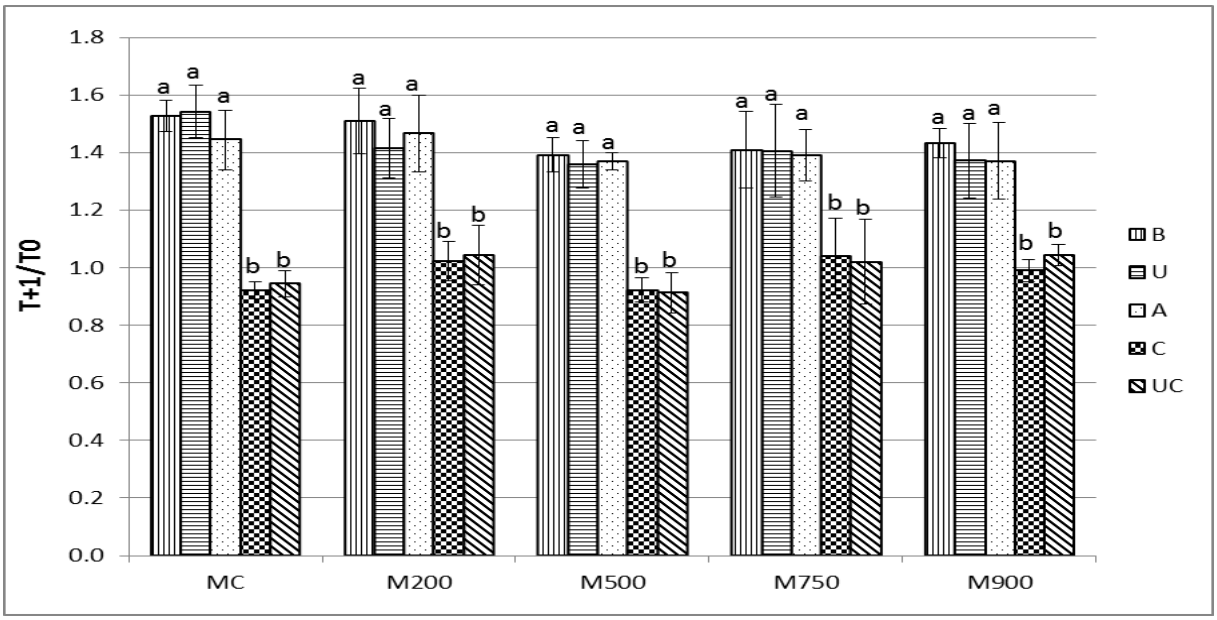

Fig. 1: Microbial biomass carbon (MBC) ratio (T+1/T0) in soils (MC, M200, M500, M750, M900), planted with three different miscanthus cultivars (B, U and A), and unplanted covered (C) and uncovered (UC) soils. Values are presented as means ± SD. Different letters refer to significant differences between soils (Tukey HSD test, $n = 5$, $p \leq 0.05$)

Basal respiration

The basal respiration (BR) activity at T0 (supplementary data Table 3) was always the highest in MC soils (0.6 mg kg^{-1} C dry soil h^{-1} corresponding in B, U, A, C and UC respectively), and decreased as the metal concentration increased; M900 (0.2 mg kg^{-1} C dry soil h^{-1} corresponding to B, U, A, C and UC respectively). The basal respiration rose in all soils (Figure 2). Yet the ratios of contaminated soils with miscanthus were higher than the unplanted soils. They fluctuated between 2.4 in the M900 soil with miscanthus B (the lowest increase) and 3.4 in the M200 soil with miscanthus A. On the other hand, unplanted soils did not obtain the same manner of activity augmentation, where they approached 1.6 in the UC modality of the M900 soil. As for the uncontaminated soils, no significant differences were figured between planted and unplanted soils, even though miscanthus-cultivated soils demonstrated higher respiration than the others (1.6, 1.7, 1.4, 1.1 and 1.2 corresponding to miscanthus B, U and A, in addition to C and UC respectively).

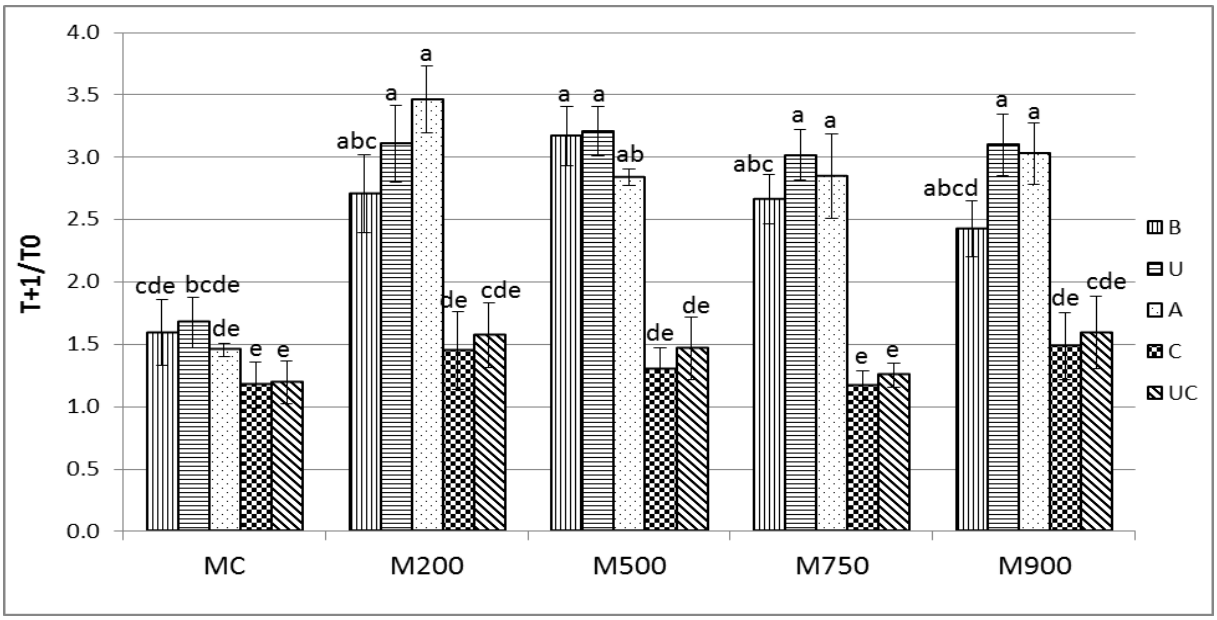

Fig. 2: Basal respiration ratio (T+1/T0) in soils (MC, M200, M500, M750, M900), planted with three different miscanthus cultivars (B, U and A), and unplanted covered (C) and uncovered (UC) soils. Values are presented as means ± SD. Different letters refer to significant differences between soils (Tukey HSD test, $n = 5$, $p \leq 0.05$)

Fluorescein diacetate hydrolytic activity

The fluorescein diacetate hydrolytic activity (FDHA) at T0 was always the highest in MC soils (0.1 mg fluo g^{-1} soil h^{-1} corresponding to B, U, A, C and UC respectively), and decreased as the contamination degree increased (supplementary data Table 3). The results presented in Figure 3 showed that FDHA strongly increased in soils with miscanthus in comparison with the unplanted soils, where the mass of fluorescein released in soil, was multiplied by 4 in miscanthus B- and A- cultivated soils, and approximately by 5 in the miscanthus U-cultivated soils (4.1, 4.9, and 4.7 x T0 corresponding to B, U and A respectively), compared to 1.4 and 1.2 x T0 in UC and C soils.

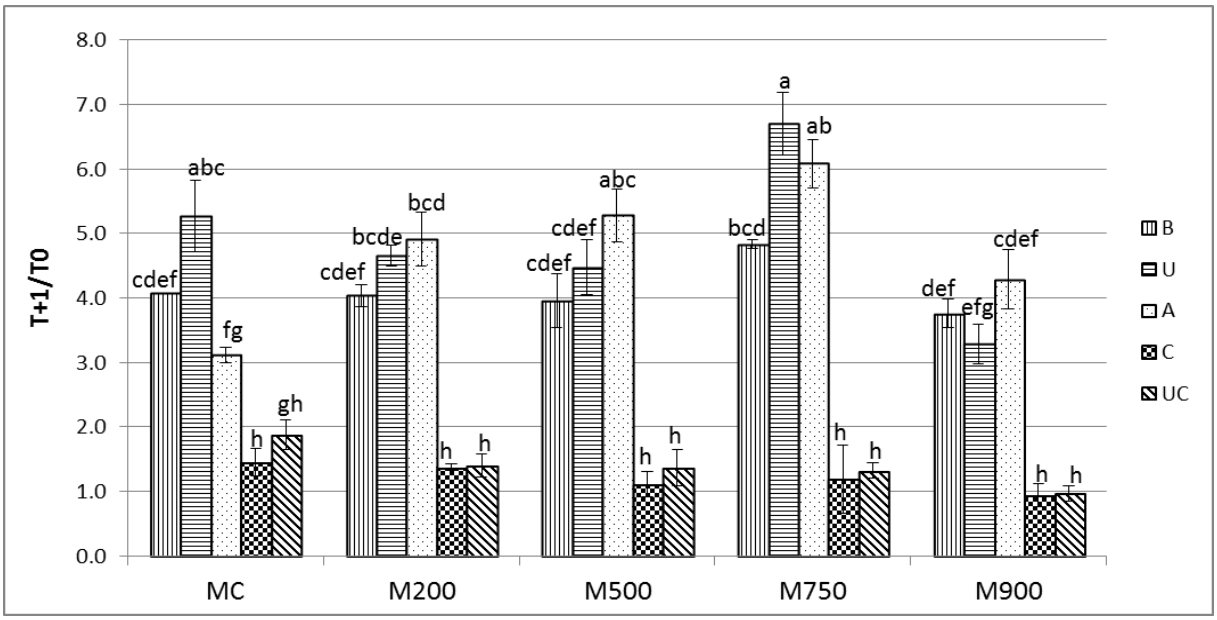

Fig. 3: Ratio (T+1/T0) of the fluorescein diacetate hydrolytic activity in soils (MC, M200, M500, M750, M900), planted with three different miscanthus cultivars (B, U and A), and unplanted covered (C) and uncovered (UC) soils. Values are presented as means ± SD. Different letters refer to significant differences between soils (Tukey HSD test, $n = 5$, $p \leq 0.05$)

Laccase activity

At T0, the laccase activity was always the highest in the M200 soils (63.9, 61.6, 65.6, 67.3 and 66.3 µmol $ABTS^{+}$ g^{-1} dry soil corresponding to B, U, A, C and UC respectively), whereas the lowest was in the M500 soils (36.9, 41.1, 41.2, 37.6 and 35.1 µmol $ABTS^{+}$ g^{-1} dry soil corresponding to B, U, A, C and UC respectively) (supplementary data Table 3). The activities' changes present in the Figure 4, showed that they had increased 2.6 x T0 for miscanthus A and B, and 2.5 x T0 for miscanthus U, whereas it was lower in C and UC (1.37 and 1.5 x T0 respectively). In addition, the gradient of metal contamination did not affect the changes, where the corresponding values in contaminated soils were approximately the same as in the uncontaminated soils.

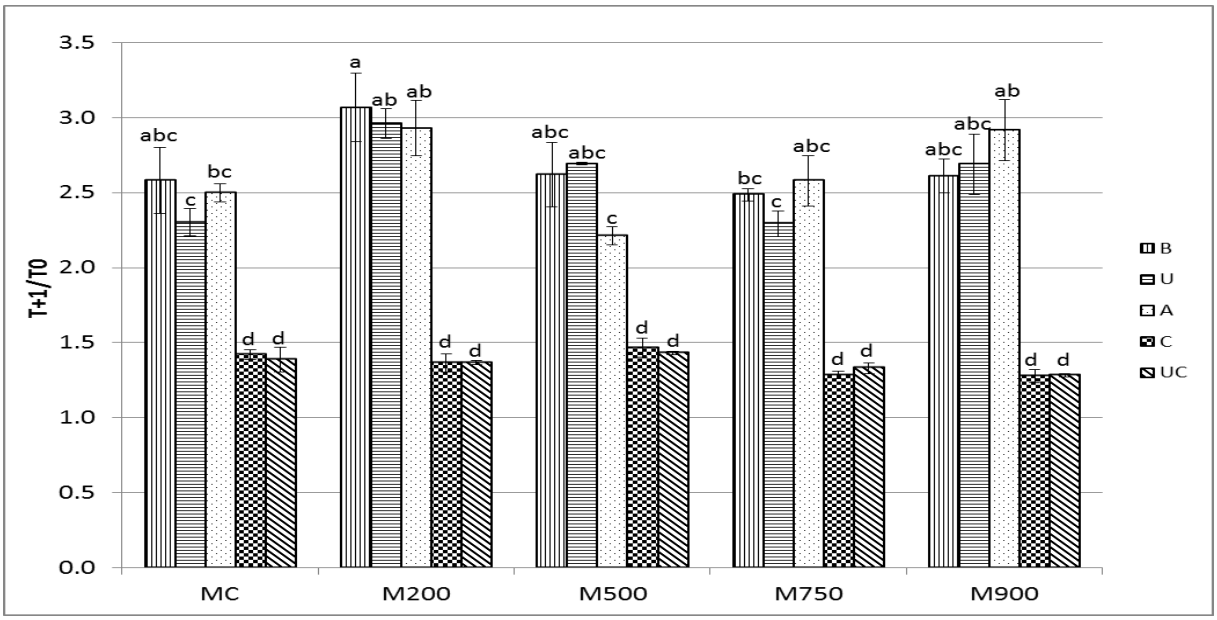

Fig. 4: Laccase activity ratio (T+1/T0) in soils (MC, M200, M500, M750, M900), planted with three different miscanthus cultivars (B, U and A), and unplanted covered (C) and uncovered (UC) soils. Values are presented as means ± SD. Different letters refer to significant differences between soils (Tukey HSD test, $n = 5$, $p \leq 0.05$)

Acid phosphatase activity

At T0, the acid phosphatase activity in MC soils was always the highest in all the modalities (0.02 µg Np g^{-1} dry soil corresponding to B, U, A, C and UC respectively) and it decreased as contamination increased, with the lowest values remarked in the M900 soils (0.004 µg Np g^{-1} dry soil corresponding to B and U and 0.003 µg Np g^{-1} dry soil corresponding to A, C and UC respectively) (supplementary data Table 3). Figure 5 shows significant differences among cultivated and uncultivated pots. The cultivated soils demonstrated an increase in the corresponding activity ranging from 1.6 x T0 in the MC soil where the increase was the lowest in comparison to the contaminated soils, to 2.2 x T0 in the M500 soil planted with miscanthus B. On the other hand, the increase of the activities in the unplanted pots did not exceed 1.3xT0, which was present in the M500 and M750 uncovered pots.

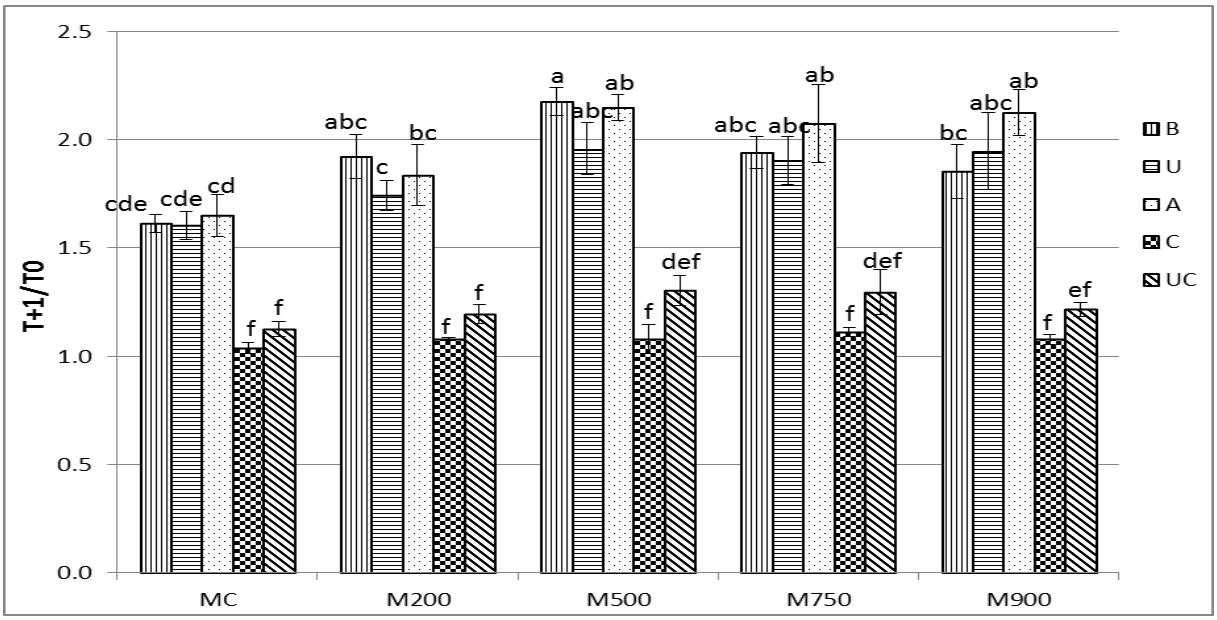

Fig. 5: Acid phosphatase activity ratio (T+1/T0) in soils (MC, M200, M500, M750, M900), planted with three different miscanthus cultivars (B, U and A), and unplanted covered (C) and uncovered (UC) soils. Values are presented as means ± SD. Different letters refer to significant differences between soils (Tukey HSD test, $n = 5$, $p \leq 0.05$)

Urease activity

Urease activity in the soils at T0 was always the highest in the M500 soil (95.0, 90.4, 96.4, 106.0 and 103.7µg N g^{-1} dry soil corresponding to B, U, A, C and UC respectively), and the lowest in those of M900 (60.8, 55.2, 59.2, 65.4 and 61.9 µg N g^{-1} dry soil corresponding to B, U, A, C and UC respectively) (supplementary data table 3). Figure 6 illustrates that urease activity was nearly the same in cultivated soils, yet slightly lower in the M750 soil (1.6–1.7 x T0) compared to the others (2–2.2 x T0). However, unplanted soils demonstrated less change in the corresponding activity, where the highest activities recorded were in the M900 UC soil yielding a ratio of 1.5 x T0.

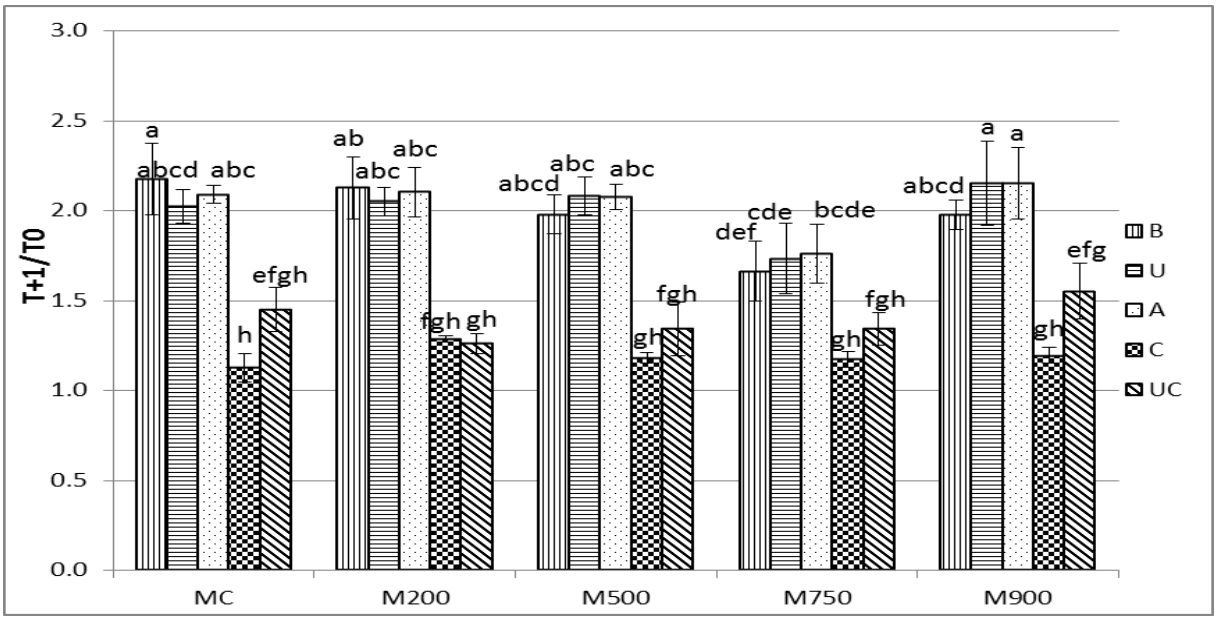

Fig. 6: Urease activity ratio (T+1/T0) in soils (MC, M200, M500, M750, M900), planted with three different miscanthus cultivars (B, U and A), and unplanted covered (C) and uncovered (UC) ones. Values are presented as means ± SD. Different letters refer to significant differences between soils (Tukey HSD test, $n = 5$, $p \leq 0.05$)

C.1.4- Discussion

The capacity of miscanthus plants to influence the soil's physico-chemical parameters as well as the Cd and Zn availability and some biological parameters was assessed throughout the experiment. It is noteworthy to indicate that significant differences do not exist between the soils planted with B, U and A cultivars, by which the results were very similar regarding their corresponding soil parameters, which might be referred to the relative short duration of the experiment (12 months). Certain changes regarding the soil properties took place in the non-planted pots, revealing the existence of interactions in the corresponding media. However, the results obtained showed that the soil modifications that came together in the planted pots exceeded those in the non-planted ones (significant differences between the cultivated and uncultivated soils), and thereby exposing the net effect and efficacy of the miscanthus plants in the process of phytostabilisation.

C.1.4.1- Effects on soil physico-chemical parameters and available Cd and Zn concentrations over the duration of the experiment

Regarding pH values, the distinguishing remark was the resistance to variations demonstrated in miscanthus cultivated soils in comparison with the unplanted soils. MC soils with miscanthus showed a lower tendency to acidification than unplanted soils. The same held true for the other modalities, yet the resistance was to alkalization. To obtain optimal growth conditions for survival and nutrient uptake, plants might induce pH variations via

root exudate composition and ionic forms, which induces acidification to increase elements' mobility or alkalization as a part of the plant's defense mechanism to alleviate toxicity caused in acidified media (Hinsinger et al., 2003).

The SOC concentrations as well as the CEC showed a remarkable increase in the miscanthus-planted soils. The results are in accordance with those reported by Hromadko et al. (2010) and Techer et al. (2011). These authors attributed the corresponding augmentation to the miscanthus root exudates, which comprise carbohydrates, proteins and amino acids, and the enlargement of rhizosphere biota including free living fungi, bacteria and soil microfauna augmenting the SOC, which in turn affects the corresponding CEC.

The decrease in the available P_2O_5 and TN concentrations was more evident in the miscanthus planted soils than in the uncultivated soils. These results corroborate those reported by Nsangawimana et al. (2016), who investigated the accumulation of metals and nutrients during the growth cycle of miscanthus plants cultivated in metal-contaminated soils. Their work demonstrated the plant's need to uptake certain nutrients that are necessary to fulfill its growth, including nitrogen and phosphorous, which cause their corresponding concentrations in the soil to decrease (Nsanganwimana et al., 2016; Schachtman et al., 1998).

Cd and Zn bioavailability is influenced by physico-chemical parameters in soil, such as soil pH, redox conditions, metal concentrations, organic matter and clay contents, as well as contact time with the soil matrix (Kim et al., 2015). In agreement with Nsanganwimana et al. (2014), the significant decrease in metal availability in planted soils could stem from the miscanthus capacity to accumulate metals mainly in their rhizosphere and thus reduce their mobility and availability. In addition, a negative correlation existed between the SOC and the $CaCl_2$-extractable Cd and Zn concentrations (- 0.88 and - 0.67, respectively) as well as with CEC (- 0.51 and - 0.59, respectively). In conformity with Epelde et al. (2009a), the increase in the SOC and concomitantly its corresponding CEC might have enhanced the metal association and binding in soil forming insoluble metal–organic complexes with humic acids and thereby decreasing the risk of toxicity to plants and microorganisms and creating a self-sustaining ecosystem (Epelde et al., 2009a; Rieuwerts et al., 1998).

C.1.4.2- Effects on soil biological activities

Miscanthus cultivation also played a crucial role in restoring soil functionality that was disturbed by the metal contamination. The various biological parameters tested (MBC, BR and enzymatic activities) were demonstrated as good indicators of soil health and hence quality for their rapid response, sensitivity and capacity to provide information that integrates many environmental factors (Garbisu et al., 2011; Mijangos et al., 2006).

As for the present study, it was clearly demonstrated that all the biological parameters investigated (MBC, BR, FDHA, laccase, urease and acid phosphatase activities) showed higher

values in the miscanthus cultivated soils than the unplanted soils. Consequently, C, N and P cycles in soils were accelerated, and concomitantly functions had been re-established (e.g. nutrient supply, recycle materials, establish a niche for microorganisms) (Moreno-Jimenez et al., 2012). The results are in total agreement with various studies related to phytoremediation where it has been proved that presence of phytoremediators (excluders or extractors) remarkably boosted the enzymatic activities of polluted soils compared to unplanted controls (Hernandez Allica et al., 2006; Jiang et al., 2010; Moreno-Jimenez et al., 2012).

In general, biological parameters exhibited lower values in metal-polluted soils than unpolluted soils. It has been proved that metals exert a negative influence on soil functionality affecting growth, morphology and metabolism of soil microorganisms through functional disturbance, alteration of protein and cell membrane integrity (Epelde et al., 2009b; Leita et al., 1995). Current results of BR, FDHA, and acid phosphatases are in agreement with the previous studies (Liao and Xie, 2007; Papa et al., 2010; Renella et al., 2005; Renella et al., 2003; Wang et al., 2007; Zhang et al., 2010; Zhang et al., 2008), where a negative correlation existed between these parameters and Pb concentration in soil (r^2 = - 0.58, - 0.45 and - 0.79 respectively). Normally, plant effects are related besides to its nature (extractor or excluder), to the secretion of the easily metabolizable root exudates (sugars, amino compounds, organic acids, fatty acids, growth factors and nucleotides) and surfaces for microbial colonization, where it is considered a vital factor in controlling microbial biomass and activities rendering the soil a favorable environment for microbes to thrive (Bais et al., 2006; Epelde et al., 2009b; Wenhao et al., 2013).

Another factor affecting enzymatic activities is the metal bioavailability in soil. Since the mechanisms that prevent soil enzyme inhibition by metals are likely to be the same mechanisms limiting metal uptake by plants and soil organisms (Epelde et al., 2009b; Speir and Ross, 2002), then, the increase in activity of basal respiration, FDHA, acid phosphatases, ureases in addition to the microbial biomass C (r^2 =-0.60, -0.48, -0.75, -0.55 and -0.69 respectively) observed may well be due to the decrease in available Cd concentration found mainly in the cultivated pots at T+1, demonstrating that toxicity to soil microorganisms is linked directly to metal availability (Wang et al., 2007; Zhang et al., 2010), and therefore microbial biomass (C, N, and P) might be used as a sensitive indicator of soil pollution by metals.

Concerning SOC, the results revealed that there is a positive correlation between this parameter and all the other biological parameters except for laccase (r^2 = 0.53, 0.87, 0.47, 0.58 and 0.75 corresponding to BR, MBC, FDHA, acid phosphatases, and ureases, respectively), by which the increase in the SOC at T+1 led to the prosperity of the parameters mentioned. This phenomenon can be explained by the fact that the increase in the quantity of the organic carbon in soils had stimulated the enzymatic activities by supplying them with materials that activate enzyme synthesis and simultaneously enhance

microbial development (Pardo et al., 2014). Another example that demonstrates the positive effect of SOC on MBC is the work of Cheng et al. (2013) who showed that the accumulation of SOC over time increased the microbial biomass in Wilson spruce forest.

As for pH, a negative correlation exists between this factor and certain enzymatic activities, notably BR, FDHA and acid phosphatase (r^2 = - 0.75, - 0.6 and - 0.88 respectively), and another positive correlation with Cd, Pb and Zn concentrations (r^2 = 0.83, 0.84 and 0.80 respectively). Higher phosphatase activity was shown in the soils with the lowest pH values, confirming the concept and results of Sarapatka et al. (2004). As for the positive correlations between pH value and metal concentrations, the rule states that as pH increases, the solubility and thus the availability of the corresponding metals decreases (Venditti et al., 2000), but this is not the case here. This finding can be explained by the high concentrations of metals in soils which greatly augment their availability and toxicity (Baran et al., 2014; Favas et al., 2011). Thus the negative relation presented between pH and BR and FDHA is basically the effect of the metal pollution on the activity of the corresponding parameters.

It was shown that a positive correlation exists between TN and the MBC (r^2 = 0.62), agreeing with Yang et al. (2010), who had similar results and explained them by the fact that the variations of soil microbial biomass reflect the degree of immobilization–mineralization of soil carbon and nitrogen, by which a decrease in soil microbial biomass can result in mineralization of nutrients, whereas an increase in microbial biomass may lead to immobilization of nutrients. Another negative correlation does exist between TN and laccase activities (r^2 = -0.57). These results were confined with Eggert et al. (1996) who demonstrated that nitrogen-deficient medium caused the production of laccase in *Pycnoporus sanguineus. More precisely, Hernandez et al. (2015) concluded that* laccase activity is inhibited by urea being used as a source of nitrogen. In the present work, urease activity increased from T0 to T+1, especially in miscanthus-cultivated soils, and a positive correlation existed between urease and laccase (r^2 = 0.45). Thus the decrease in the amount of urea in soils due to urease activities caused the laccase activities to prosper.

C.1.5- Conclusion and perspectives

It has been proved that *Miscanthus x giganteus* is a great candidate for phytostabilization for its capacity to reduce Cd and Zn mobility and availability, as well as its contribution to augmenting the soil organic carbon concentrations. In addition, miscanthus plants played a crucial role in the studied soil's biological parameters such as the microbial biomass carbon, basal respiration and other enzymatic activities. Nevertheless, variations were not recorded concerning the parameters among the soils cultivated with different miscanthus cultivars. Therefore a prolongation of the experiment might be an interesting project for specifying the impact of the cultivars on their corresponding soil characteristics. Even though a considerable quantity of soil was used in the pots, the experiment was conducted under optimal conditions that allowed the occurrence of maximum interactions between the

different soil parameters. Therefore, further *in situ* experimentations should take place to validate the current work's results. Moreover, the influence of heavy metal-contamination on the miscanthus plants should be investigated. Determining the stress biomarkers in the corresponding plants would clarify the mode of interaction and responses of the plants when exposed to detrimental conditions.

Acknowledgments

The authors are grateful to the Association for Specialization and Scientific Guidance for the PhD scholarship offered to Karim Al Souki. They also wish to thank ADEME (French Agency for the Environment and Energy Management) for the financial support of the Phytener program.

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Supplementary data

		MC	M200	M500	M750	M900
B	pH	6.05 ± 0.04	7.11 ± 0.01	7.48 ± 0.04	7.50 ± 0.06	7.50 ± 0.06
	P_2O_5 (mg kg^{-1})	0.16 ± 0.01	0.44 ± 0.05	0.19 ± 0.05	0.43 ± 0.01	0.60 ± 0.05
	CEC (cmol kg^{-1})	10.17 ± 1.13	12.18 ± 0.92	25.90 ± 1.15	11.49 ± 1.30	11.80 ± 1.34
	SOC (g kg^{-1})	20.55 ± 1.27	14.04 ± 0.77	29.46 ± 1.30	9.13 ± 1.17	9.74 ± 0.79
	TN (g kg^{-1})	1.50 ± 0.09	1.19 ± 0.06	2.39 ± 0.07	1.20 ± 0.05	0.85 ± 0.06
U	pH	6.02 ± 0.09	7.11 ± 0.52	7.56 ± 0.06	7.58 ± 0.04	7.54 ± 0.03
	P_2O_5 (mg kg^{-1})	0.13 ± 0.01	0.50 ± 0.02	0.21 ± 0.02	0.46 ± 0.04	0.65 ± 0.02
	CEC (cmol kg^{-1})	11.43 ± 0.54	12.26 ± 1.16	23.72 ± 1.78	11.21 ± 0.89	11.70 ± 1.26
	SOC (g kg^{-1})	20.26 ± 0.89	15.27 ± 1.10	25.97 ± 1.88	8.87 ± 0.85	9.01 ± 0.76
	TN (g kg^{-1})	1.43 ± 0.06	1.09 ± 0.02	2.38 ± 0.04	1.12 ± 0.03	0.83 ± 0.06
A	pH	6.02 ± 0.09	7.09 ± 0.02	7.50 ± 0.02	7.53 ± 0.07	7.53 ± 0.03
	P_2O_5 (mg kg^{-1})	0.14 ± 0.02	0.38 ± 0.03	0.20 ± 0.04	0.42 ± 0.02	0.58 ± 0.02
	CEC (cmol kg^{-1})	10.91 ± 0.53	11.63 ± 1.43	26.46 ± 1.51	12.07 ± 1.03	12.36 ± 0.83
	SOC (g kg^{-1})	20.49 ± 1.19	16.32 ± 1.02	27.43 ± 1.10	8.88 ± 0.79	9.12 ± 0.84
	TN (g kg^{-1})	1.58 ± 0.07	1.10 ± 0.09	2.37 ± 0.07	1.17 ± 0.03	0.84 ± 0.05
C	pH	6.12 ± 0.02	7.02 ± 0.02	7.46 ± 0.02	7.51 ± 0.04	7.50 ± 0.02
	P_2O_5 (mg kg^{-1})	0.10 ± 0.01	0.43 ± 0.07	0.21 ± 0.02	0.35 ± 0.08	0.48 ± 0.05
	CEC (cmol kg^{-1})	10.37 ± 0.34	11.75 ± 1.07	24.84 ± 1.00	11.68 ± 1.25	11.77 ± 0.89
	SOC (g kg^{-1})	20.86 ± 1.52	14.14 ± 0.87	26.71 ± 1.11	9.14 ± 0.71	8.98 ± 0.78
	TN (g kg^{-1})	1.83 ± 0.02	1.02 ± 0.04	2.34 ± 0.09	1.16 ± 0.04	0.89 ± 0.03
UC	pH	6.11 ± 0.02	7.14 ± 0.02	7.55 ± 0.03	7.54 ± 0.05	7.50 ± 0.03
	P_2O_5 (mg kg^{-1})	0.08 ± 0.01	0.36 ± 0.04	0.24 ± 0.01	0.38 ± 0.07	0.62 ± 0.03
	CEC (cmol kg^{-1})	11.38 ± 0.18	12.35 ± 1.28	24.60 ± 0.92	10.34 ± 1.16	11.57 ± 1.28
	SOC (g kg^{-1})	20.34 ± 1.70	14.15 ± 0.69	24.40 ± 0.96	8.82 ± 1.25	9.49 ± 1.10
	TN (g kg^{-1})	1.69 ± 0.08	1.09 ± 0.07	2.31 ± 0.09	1.18 ± 0.06	0.87 ± 0.02

Table 1: Physico-chemical parameters of soils (MC, M200, M500, M750, and M900) planted with

three different miscanthus cultivars (B, U and A), and unplanted covered (C) and uncovered (UC) soils at T0 (mean ± standard deviation). CEC: cationic exchange capacity, SOC: soil organic carbon, TN: total nitrogen

		MC	M200	M500	M750	M900
B	Cd (mg kg^{-1})	0.08 ± 0.01	0.16 ± 0.01	0.11 ± 0.01	0.24 ± 0.02	0.25 ± 0.01
	Pb (mg kg^{-1})	< LD	< LD	< LD	< LD	< LD
	Zn (mg kg^{-1})	0.30 ± 0.02	0.79 ± 0.06	0.19 ± 0.01	0.49 ± 0.03	0.63 ± 0.05
U	Cd (mg kg^{-1})	0.08 ± 0.00	0.17 ± 0.01	0.10 ± 0.01	0.24 ± 0.01	0.24 ± 0.01
	Pb (mg kg^{-1})	< LD	< LD	< LD	< LD	< LD
	Zn (mg kg^{-1})	0.35 ± 0.03	0.78 ± 0.07	0.16 ± 0.02	0.53 ± 0.07	0.71 ± 0.03
A	Cd (mg kg^{-1})	0.07 ± 0.00	0.16 ± 0.01	0.09 ± 0.00	0.25 ± 0.01	0.24 ± 0.02
	Pb (mg kg^{-1})	< LD	< LD	< LD	< LD	< LD
	Zn (mg kg^{-1})	0.30 ±0 .05	0.86 ± 0.04	0.14 ± 0.01	0.39 ± 0.03	0.58 ± 0.04
C	Cd (mg kg^{-1})	0.06 ± 0.00	0.14 ± 0.01	0.07 ± 0.00	0.20 ± 0.01	0.23 ± 0.01
	Pb (mg kg^{-1})	< LD	< LD	< LD	< LD	< LD
	Zn (mg kg^{-1})	0.31 ±0 .02	0.81 ± 0.03	0.16 ± 0.01	0.36 ± 0.04	0.58 ± 0.03
UC	Cd (mg kg^{-1})	0.06 ± 0.00	0.14 ± 0.02	0.06 ± 0.00	0.21 ± 0.01	0.23 ± 0.01
	Pb (mg kg^{-1})	< LD	< LD	< LD	< LD	< LD
	Zn (mg kg^{-1})	0.30 ± 0.02	0.80 ± 0.03	0.16 ± 0.02	0.43 ± 0.02	0.58 ± 0.03

Table 2: 0.01 M $CaCl_2$ extractable Cd, Pb and Zn concentrations of soils (MC, M200, M500, M750, and M900) planted with three different miscanthus cultivars (B, U and A), and unplanted covered (C) and uncovered (UC) soils at T0 (mean ± standard deviation). < LD: below limits of detection

		MC	M200	M500	M750	M900
B	MBC (mg kg^{-1})	210.48 ± 9.87	237.38 ± 27.81	308.87 ± 19.19	164.12 ± 18.86	143.33 ± 12.33
	BR (mg C kg^{-1} dry soil hr^{-1})	0.63 ± 0.05	0.23 ± 0.03	0.18 ± 0.01	0.18 ± 0.02	0.18 ± 0.02
	FDHA (mg fluo g^{-1} fry soil hr^{-1})	0.11 ± 0.00	0.07 ± 0.00	0.06 ± 0.00	0.05 ± 0.00	0.06 ± 0.00
	Laccase (µ mol $ABTS^{+}$ g^{-1} dry soil)	44.78 ± 3.65	63.86 ± 5.34	36.85 ± 2.80	58.89 ± 2.39	56.19 ± 4.80
	Acid phosphatase (µg Np g^{-1} dry	0.019 ± 0.001	0.008 ± 0.000	0.008 ± 0.000	0.004 ± 0.000	0.004 ± 0.001
	Urease (µg N g^{-1} dry soil)	74.21 ± 3.50	79.83 ±6.15	95.02 ± 5.84	77.28 ± 3.58	60.75 ± 4.58
U	MBC (mg kg^{-1})	207.32 ± 10.46	249.46 ± 18.90	314.30 ± 27.60	163.00 ± 18.07	154.79 ± 9.56
	BR (mg C kg^{-1} dry soil hr^{-1})	0.59 ± 0.06	0.21 ± 0.02	0.19 ± 0.01	0.19 ± 0.02	0.17 ± 0.02
	FDHA (mg fluo g^{-1} fry soil hr^{-1})	0.09 ± 0.00	0.07 ± 0.00	0.06 ± 0.01	0.04 ± 0.00	0.06 ± 0.01
	Laccase (µ mol $ABTS^{+}$ g^{-1} dry soil)	51.83 ± 6.45	61.62 ± 3.42	41.11 ± 4.08	59.67 ± 3.70	56.07 ± 4.21
	Acid phosphatase (µg Np g^{-1} dry	0.019 ± 0.001	0.009 ± 0.000	0.008 ± 0.001	0.004 ± 0.000	0.004 ± 0.000
	Urease (µg N g^{-1} dry soil)	78.58 ± 6.16	80.07 ± 4.05	90.36 ± 6.89	76.34 ± 2.39	55.24 ± 4.12
A	MBC (mg kg^{-1})	216.80 ± 15.61	233.59 ± 19.04	313.31 ± 12.03	163 ± 14.63	146.60 ± 6.67
	BR (mg C kg^{-1} dry soil hr^{-1})	0.64 ± 0.03	0.24 ± 0.03	0.21 ± 0.03	0.22 ± 0.03	0.17 ± 0.02
	FDHA (mg fluo g^{-1} fry soil hr^{-1})	0.14 ± 0.01	0.06 ± 0.00	0.05 ± 0.00	0.04 ± 0.01	0.05 ± 0.01
	Laccase (µ mol $ABTS^{+}$ g^{-1} dry soil)	45.09 ± 2.04	65.59 ± 3.97	41.21 ± 3.59	51.78 ± 3.37	56.88 ± 2.42
	Acid phosphatase (µg Np g^{-1} dry	0.019 ± 0.001	0.008 ± 0.001	0.007 ± 0.000	0.004 ± 0.000	0.003 ± 0.000
	Urease (µg N g^{-1} dry soil)	76.89 ± 2.12	84.02 ± 3.46	96.42 ± 3.92	74.71 ± 5.80	59.22 ± 2.92
C	MBC (mg kg^{-1})	199.38 ± 7.56	22.47 ± 6.89	320.54 ± 36.99	161.38 ± 13.39	164.62 ± 11.81
	BR (mg C kg^{-1} dry soil hr^{-1})	0.55 ± 0.03	0.22 ± 0.02	0.22 ± 0.02	0.21 ± 0.01	0.15 ± 0.02
	FDHA (mg fluo g^{-1} fry soil hr^{-1})	0.11 ± 0.01	0.07 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	0.05 ± 0.00
	Laccase (µ mol $ABTS^{+}$ g^{-1} dry soil)	39.47 ± 2.53	67.32 ± 4.93	37.56 ± 4.11	52.45 ± 4.77	57.01 ± 2.43
	Acid phosphatase (µg Np g^{-1} dry	0.020 ± 0.001	0.007 ± 0.000	0.007 ± 0.000	0.004 ± 0.000	0.003 ± 0.000
	Urease (µg N g^{-1} dry soil)	71.46 ± 5.69	87.51 ± 3.07	105.97 ± 3.88	76.76 ± 6.63	65.42 ± 3.26
UC	MBC (mg kg^{-1})	207.06 ± 13.83	214.71 ± 6.00	312.69 ± 16.57	168.42 ± 14.49	151.54 ± 9.52
	BR (mg C kg^{-1} dry soil hr^{-1})	0.57 ± 0.07	0.22 ± 0.01	0.21 ± 0.03	0.22 ± 0.02	0.16 ± 0.02
	FDHA (mg fluo g^{-1} fry soil hr^{-1})	0.11 ± 0.01	0.08 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
	Laccase (µ mol $ABTS^{+}$ g^{-1} dry soil)	41.32 ± 4.37	66.27 ± 4.09	35.09 ± 2.54	50.51 ± 2.63	54.96 ± 3.52
	Acid phosphatase (µg Np g^{-1} dry	0.019 ± 0.001	0.008 ± 0.000	0.007 ± 0.000	0.004 ± 0.001	0.003 ± 0.000
	Urease (µg N g^{-1} dry soil)	72.26 ± 4.14	96.19 ± 7.87	103.67 ± 5.32	80.57 ± 3.62	61.94 ± 3.74

Table 3: Biological parameters of soils (MC, M200, M500, M750, and M900) planted with three different miscanthus cultivars (B, U and A), and unplanted covered (C) and uncovered (UC) soils at T0 (mean ± standard deviation). MBC: microbial biomass carbon, BR: basal respiration, FDHA: fluorescein diacetate hydrolytic activity

C.2- *Miscanthus x giganteus* culture on soils highly contaminated by metals: Impact of late harvest on the soil–plant system

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Foreword

As the miscanthus plantations reached their tenth year of cultivation on field, corresponding to the mid-term range of their higher biomass production potential (around 20-25 years), speculations about the impact of late harvests and of the resulting metal-contaminated leaves incorporation into soils are arisen. Indeed, the PhD work of Nsanganwimana (2014) demonstrated that even if *M x giganteus* is a metal-excluder and mainly accumulates metals in roots and rhizomes, a non-negligible quantity of metals can be translocated to leaves,

especially on the most contaminated soils (M750 and M900). The second question was about the influence of this long-term metal-recycling on metal phytoavailability and on the health of the future crop that is supposed to succeed the miscanthus culture in the field after a certain period.

The first step of this study was to estimate the leaf input into field over a period of 20 years. In spring 2015, we sampled ten 1 m^2 quadrats on each of the three oldest miscanthus fields of our experimental site (MC, M200 and M500). The leaf litter that has been accumulated for 7 years was weighted and the total leaf input over a period of 20 years was estimated. The collected leaves were dried, grinded and sieved to 2 mm to facilitate their incorporation into soils. Then, the soils of the three plots were mixed with their corresponding leaf powder. The second step of this experiment was to apply an artificial aging process on each soil (amended and non-amended soils) for 1, 3 and 6 months according to the experimentations of Cui et al. (2011) and Du et al. (2008). The artificial aging process was launched in small pots (500 g pots) due to material constraints. Indeed, the incubator was fully used during 6 months for this experiment, and it cannot contain bigger pots. Incubation was performed under controlled conditions in the laboratory (constant humidity and temperature). At the end of the aging process, we investigated the influence of leaf incorporation and artificial aging on (i) certain soil physico-chemical parameters, (ii) pseudototal and $CaCl_2$-extractable metal concentrations, and (iii) soil microbial biomass carbon and basal respiration.

Moreover, at the end of the aging process, the third step of this study was to select a crop that could represent the future culture succeeding miscanthus. Due to the limited volume of soils available (500 g), it was not possible to cultivate the food crops that could potentially replace miscanthus in this area (corn, potatoes, beet…). Therefore, it was decided to cultivate ryegrass (*Lolium perenne*) that can be used as fodder, has a fast growth and have the ability to stabilize metals (Bidar et al., 2007). Moreover, this plant is commonly used in the laboratory as model plant for ecotoxicology studies. Ryegrass was grown into greenhouse on the aged soils for two months. At the end of the growth, the influence of leaf incorporation and soil aging on metal phytoavailability was evaluated. The impact of the different soil treatments (leaf amendment and aging duration) on ryegrass was then assessed using biomarkers (photosynthetic pigments and antioxidative enzymatic activities).

Publication

Al Souki, K. S., Louvel, B., Liné, C., Waterlot, C., Douay, F., Pourrut, B., Submitted to Ecotoxicology and Environmental Safety. *Miscanthus x giganteus* culture on soils highly contaminated by metals: Impact of late harvest on the soil–plant system

C.2- *Miscanthus x giganteus* culture on soils highly contaminated by metals: Impact of late harvest on the soil–plant system

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Abstract: *Miscanthus x giganteus* has been proved a suitable candidate for phytostabilization of metal-polluted soils. Its late harvest in winter leaves large amounts of leaf litter on the soil surface. However, little is known about the mobility and the bioavailability of metals following leaf decomposition and the corresponding consequences on the succeeding culture. *Ex situ* artificial aging for 1, 3, and 6 months was conducted with miscanthus leaf fragments incorporated into three agricultural soils displaying a gradient concentration in Cd, Pb, and Zn to simulate the leaf litter input over 20 years of miscanthus culture. We investigated the impacts on physicochemical and biological soil parameters, $CaCl_2$-extractable metal, and their subsequent ryegrass shoot concentrations, and hence on ryegrass health. The results showed that the amended soils had higher pH, greater available phosphorous and higher soil organic carbon values. The respiratory activity and microbial biomass carbon in the amended soils increased mainly after 1 month of aging, and decreased afterwards. Despite the higher $CaCl_2$ extractability measured for Pb and Zn in the amended soils, the phytoavailability slightly increased only on the most contaminated soils. Moreover, leaf incorporation did not affect the ryegrass biomass, photosynthetic pigment contents, nor the antioxidative enzyme activities. Conclusively, leaf incorporation induced slight variations in the soil's physicochemical and biological parameters, as well as metal extractability, but not to an extent that might cause a considerable threat to the subsequent culture.

Keywords: *Miscanthus x giganteus*, organic matter restitution, metal cycle, metal behavior, ryegrass, stress biomarkers

C.2.1- Introduction

Soil metal pollution is a worldwide environmental issue, caused by detrimental human activities such as smelting and mining operations, industrial production, and intensive agriculture (Tchounwou et al., 2012). Consequently, human health and the ecosystem are threatened with alteration (Kim et al., 2015).

Elevated costs of traditional remediation techniques have urged development of innovative cost-effective biological methods of soil treatments (Hernandez-Allica et al., 2007). These include phytoremediation comprising several techniques that depend on plants and associated microorganisms, which aims to remove pollutants from soil and/or reduce their mobility, thereby rendering them harmless by decreasing potential health and environmental risks (Ali et al., 2013; Tangahu et al., 2011). Among the various methods, phytostabilization is a process whereby plants are established and function primarily to accumulate metals in root tissue and/or to aid in their precipitation in the rhizosphere. This process is mainly adopted to revegetate degraded soils and decrease metal bioavailability (Epelde et al., 2009).

A promising plant for phytostabilization is the C4 perennial, rhizomatous lignocellulosic grass *Miscanthus x giganteus*, well known for its high capacity to withstand a wide spectrum of metals and mainly accumulate them in its underground parts (Nsanganwimana et al., 2015; Nsanganwimana et al., 2016). Miscanthus species are also well known for their ability to sequestrate inorganic contaminants and promote bacterial degradation of persistent organic pollutants (Técher et al., 2012). Evidence of the efficiency of this plant in phytomanagement is the highly metal-polluted (mainly by Cd, Pb, and Zn) area surrounding the former lead smelter Metaleurop Nord (northern France). Nsanganwimana et al. (2016) demonstrated that *Miscanthus x giganteus* mainly accumulated metals in their underground parts, and Pelfrêne et al. (2015) shed light on its ability to reduce human risks by decreasing metal oral bioaccessibility. Moreover, Al Souki et al. (2017b) recently demonstrated miscanthus positive effects on improving the contaminated soils' physicochemical and biological parameters. However, the Cd, Pb, and specifically Zn concentrations in the aerial parts were not negligible and accumulated in leaves more than in stems (Nsanganwimana et al., 2016). This raises the question of metal cycles after several years of leaf fall.

After the establishment phase of 3–5 years, miscanthus biomass remains stable for at least two decades (Dufossé et al., 2014; Lewandowski et al., 2000). Early harvest of miscanthus could be organized at the end of the growing period (September to October), to obtain the highest yield by collecting both stems and leaves. On the other hand, late harvest (December to February) reduces harvesting yield but allows obtaining a drier biomass than the early harvest, which finally lowers the costs by limiting the need of a drying storage place and special equipment (Nsanganwimana et al., 2014; Roncucci et al., 2015). Leaf fall reduces soil nutrient losses and prevents weed growth due to the cover formed by the fallen leaves. In addition, late harvest allows a complete nutrient translocation from the miscanthus above-ground organs to the rhizome (Nsanganwimana et al., 2014; Roncucci et al., 2015). In the case of miscanthus plants established on contaminated areas, late harvest should be examined cautiously considering the potential risk posed by contaminated leaf incorporation into soils.

Some researchers claimed that after a 15- to 20-year period of establishment, the crop yield tends to decrease (Arundale et al., 2014; Clifton-Brown et al., 2007; Dufossé et al., 2014). Thus, economic reasons might lead to establishing another crop after the removal of the miscanthus stand. This crop change needs to be planned, especially with crop productions on contaminated areas. Indeed, the biodegradation of plant residues is considered a crucial step in the cycling of the potential polluting elements in soil (Boucher et al., 2005; Cui et al., 2011). This process would modify the cycle of elements such as carbon, nitrogen, phosphorus, and metals. Consequently, metal speciation and extractability, in addition to soil organism abundance and activities, are affected (Cui et al., 2011; Zhou et al., 2014). Concerning the *Miscanthus x giganteus* culture, the effects of the corresponding litter incorporation on metal extractability as well as on the crops that succeed leaf litter incorporation are totally unknown and therefore require investigation.

In the present work, *ex situ* experiments in controlled conditions (in the laboratory) were launched simulating the input of 20 years of miscanthus leaf litter to three different soils. For this reason, we artificially aged soils mixed with an estimated input of 20 years of leaf litter and i) monitored changes in certain soil physicochemical parameters, microbial biomass carbon, and basal respiration, ii) measured the pseudototal and $CaCl_2$-extractable metal concentrations, and iii) evaluated the phytoavailable metals in the succeeding culture (*Lolium perenne*) and their effects on plant health using biomarkers (photosynthetic pigments and antioxidative enzymatic activities).

C.2.2- Materials and Methods

C.2.2.1- Soil and leaf litter origin and preparations

Soils (plowed horizon, 0–25 cm) used throughout the experiment were sampled (February 2015) from contaminated agricultural fields cultivated with miscanthus plants since 2007, located around the former Metaleurop Nord Pb smelter (northern France) and designated as M200 and M500 according to their approximate Pb concentrations in the topsoil (in mg kg^{-1}). The M200 plot (50°24’52”N, 3°01’51”E, Courcelles-les-Lens) is located 1.8 km from the former smelter and characterized by its sandy clay loam soil. The M500 plot (50°25’49”N, 3°02’13”E, Evin-Malmaison) is approximately 1.4 km from Metaleurop Nord and has a clay loam soil. A third uncontaminated plot, MC (50°20’46”N 2°12’15”E), was taken as the control and is located 75 km from the smelter; a silt portion dominates. Soil samples were homogenized, oven-dried at 40°C and sieved through a 2-mm mesh before launching the experiment.

From each field, samples of *Miscanthus x giganteus* leaf litter were collected from several 1-m^2 spots, weighed and then oven dried at 40°C in the laboratory, sieved, and ground to 1–2 mm.

C.2.2.2- Determination of metal concentration in miscanthus leaves

Metals in the miscanthus leaves were determined according to the method described by Waterlot et al. (2013), in which 300 mg of each sample were acid-digested with nitric acid (HNO_3, 70%) and heated at 95°C for 75 min. Following the addition of hydrogen peroxide (H_2O_2, 30%), the solution was heated for further 180 min prior to adding osmosed water. The Cd, Pb, and Zn concentrations in the extracts were determined by atomic absorption spectrophotometry (AA 6800, Shimadzu, Kyoto, Japan). Quality control for chemical extraction and digestion was performed by including blanks, internal, and certified (Polish Virginia tobacco leaves, INCTPVTL-6, Poland) reference materials.

C.2.2.3- Soil aging experiment

To each soil (500 g, 2 mm), 1 g of sieved leaf samples (1–2 mm) was introduced, based on the leaf mass collected from each 1-m^2 spot on miscanthus fields and the estimation of total input of leaf litter over 20 years of miscanthus cultivation. Later on, the soils and leaves were mixed attentively with a powder mixer. The mixture was transferred to small plastic pots (9 × 9 × 9 cm; n = 6). Simultaneously, other plastic pots (n = 6) were filled with MC, M200, and M500 soils without leaf powder.

The aging experiments were conducted at different time intervals (1, 3, and 6 months, named I1, I3, and I6, respectively) at 25°C according to the experiments conducted by Cui et al. (2011) and Du et al. (2008). Deionized water was added to the pots to maintain soil moisture at 60% of their water-holding capacity (WHC). At the end of the incubation, three pots of each treatment were sacrificed for analysis, whereas the other three were cultivated with ryegrass to simulate a post-miscanthus culture.

C.2.2.4- Soil analysis

Soil samples underwent several investigations, before and after the different aging periods. A portion was kept fresh and sieved to 2 mm for measurement of biological activities. The remaining portion was oven-dried at 40 °C, sieved, and ground to pass a 2-mm and 250-µm mesh for the physicochemical analysis, pseudototal and $CaCl_2$-extractable metal concentrations.

C.2.2.4.1- Pseudototal metal concentrations and physicochemical parameters of soils

To determine the pseudototal Cd, Pb, and Zn concentrations, soil acid digestion was applied utilizing a digestion plate (HotBlock™ Environmental Express, USA). Aqua regia solution (HCl + HNO_3, 3:1, 6 mL) was added and the aliquot heated at 120°C for 90 min. Quality control of the extraction and analysis was provided by the introduction of two internal reference samples and a certified soil reference (CRM 141, IRMM, Belgium). The Cd, Pb, and Zn concentrations were determined by atomic absorption spectrophotometry (AA 6800, Shimadzu) (Waterlot et al., 2012).

Soil pH (H_2O) was measured after agitating a mixture of soil and deionized water (1:5, v/v) according to the ISO 10390 standard. The available phosphorus (P_2O_5) concentration was measured according to Joret and Heber (1955) via extraction in ammonium oxalate solution ($(NH_4)_2\ C_2O_4$, 0.1 M, pH = 7) and afterwards quantified spectrophotometrically upon the addition of a coloring reagent. Soil organic carbon (SOC) content was measured through a colorimetric dosage following extraction by potassium dichromate ($K_2Cr_20_7$, 80 g L^{-1}) according to the ISO 14235 standard.

C.2.2.4.2- Soil $CaCl_2$-extractable metal concentrations

The $CaCl_2$-extractable Cd, Pb, and Zn concentrations in the different soil modalities were investigated as well. The soils were mixed with a 0.01 M calcium chloride ($CaCl_2$) solution at a ratio of 1:10, m/v (Waterlot and Douay, 2012). The Cd, Pb, and Zn concentrations in extracts were determined by atomic absorption spectrophotometry (AA 6800, Shimadzu). The quality of analytical data was verified by including the certified reference material CRM BCR®-701.

C.2.2.4.3- Soil biological parameters

Soil basal microbiological respiration (BR) and microbial biomass carbon (MBC) were determined based on the quantification of the CO_2 liberated in the soil and via the chloroform fumigation–extraction method, respectively, according to Al Souki et al. (2017b).

C.2.2.5- Ryegrass cultivation in the aged soil and analysis

The remaining three aged soils were cultivated with the 1 g of ryegrass seeds (*Lolium perenne*). The cultivation period lasted 2 months in a controlled environment (20 °C, 16 h photoperiod). Deionized water was added to soil samples to maintain moisture at 60% WHC. At the end of the cultivation period, the ryegrass shoots were harvested by cutting 1 cm above the soil with scissors. Part of the shoots were immediately flash frozen in liquid nitrogen directly after sampling and stored at –80 °C until biomarker analysis. The remaining part was then washed three times with osmosed water to remove any dust particles. The samples were oven-dried at 40°C for 48 h and weighed to determine their corresponding dry biomass. Finally, the samples were ground into fine powder using a knife mill (GM 200, Retsch) to determine metal concentration.

C.2.2.5.1- Metal concentrations in the ryegrass shoots

The Cd, Pb, and Zn concentrations in the ryegrass shoots were determined by atomic absorption spectrophotometry (AA 6800, Shimadzu) following the same protocol described in section C.2.2.2-.

C.2.2.5.2- Quantification of ryegrass photosynthetic pigment

Photosynthetic pigments were evaluated spectrophotometrically according to Liné et al. (in preparation) using a plate reader (Thermo Scientific Multiskan™ GO). Briefly, two foliar discs (0.5 cm in diameter) per plant sample were collected from frozen leaves using a manual punch and weighed. Then they were put into in 96-deepwell plate (2 mL) with one 4-mm-diameter glass bead. Samples were then ground under frozen conditions using a Mixer Mill MM 400 (Retsch), twice for 1.5 min at 30 Hz. After the addition of 1.5 mL of ice cold 95% methanol in each well, samples were homogenated for 2 min at 15 Hz with the MM400 grinder. Plates were left in the dark for 24 h of incubation.

After 24 h of incubation, leaf extracts were homogenated for 2 min at 15 Hz with the Mixer Mill MM 400. A total of 100 µL was collected for photosynthetic pigment analysis. The absorbance was measured at 470, 652, and 666 nm. Concentrations of total carotenoids (Car), chlorophyll a (Chl a) and b (Chl b), respectively, were calculated according to the extinction coefficients and equations reported by Lichtenthaler (1987). Finally, data were averaged and the mean concentration obtained was expressed as mg g^{-1} FW of leaf.

C.2.2.5.3- Quantification of ryegrass antioxidative enzymatic activity

Antioxidative enzymatic activity assays were evaluated spectrophotometrically according to Liné et al. (in preparation) using a plate reader (Thermo Scientific Multiskan™ GO). Briefly, five foliar discs (0.5 cm in diameter) per plant sample were collected from frozen leaves using a manual punch. Then they were put into a 96-deepwell plate (2 mL) with one 4-mm-diameter glass bead per well. Samples were then ground under frozen conditions using a Mixer Mill MM 400 (Retsch), twice for 1.5 min at 30 Hz. After addition of 1 mL of ice cold Tris extraction buffer pH 7.0 containing 0.01 M EDTA, 0.4 M PVP, 0.05 ascorbate, 11.44 mM β-mercaptoethanol, and protease cocktail inhibitor, samples were homogenated for 2 min at 15 Hz with the MM400 grinder. Plates were then centrifuged at 5000 *g* for 15 min at 4°C. Supernatants were collected and protein content was determined according to Bradford (1976), using bovine serum albumin (BSA, Sigma) as standard.

The total activity of superoxide dismutase (SOD) was determined by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) according to the method reported in Giannopolitis and Ries (1977). The reaction mixture contained 0.47 mM NBT (Sigma), 3.85 µM riboflavin (Sigma), 19.23 mM methionine (Sigma), 36.54 mM phosphate buffer (pH 7.8), and 20 µL of enzyme extract. The test tubes containing the mixture were placed 30 cm below a light source (30 W fluorescent lamps). The reaction was started by switching on the light and was allowed to run for 10 min. The reaction was stopped by switching off the light with the absorbance at 560 nm. An unirradiated reaction mixture that did not develop color served as the control, and its absorbance was subtracted from the test

tube's absorbance. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction.

Ascorbate peroxidase (APX) activity was evaluated by the decrease of absorbance at 290 nm due to ascorbate oxidation (Nakano and Asada, 1981). The reaction mixture contained 434 mM phosphate buffer (pH 7.0), 3.77 mM H_2O_2, 0.56 mM ascorbic acid, and 10 µL enzyme extract. One enzyme unit was defined as 1 µmol of ascorbic acid oxidized per minute at 290 nm using an ascorbate standard curve. The enzyme activity was expressed in µmoles of ascorbate oxidized min^{-1} mg^{-1} protein.

C.2.2.6- Statistical analysis

Analysis of variance was calculated to compare modalities. The Fisher test was considered for significance ($p \leq 0.05$). If statistically significant differences were found, the Tukey HSD test was used for pair-wise comparisons. All statistical analyses were performed using XLSTAT software.

C.2.3- Results

C.2.3.1- Metal concentrations of the miscanthus leaves

The concentrations of Cd, Pb, and Zn in the miscanthus leaves collected on each miscanthus plot are presented in Table 1.

	MC	M200	M500
Cd (mg kg^{-1})	< LD	0.6 ± 0.0 b	1.5 ± 0.1 a
Pb (mg kg^{-1})	7.7 ± 0.8 b	10.8 ± 1.7 b	19.5 ± 1.8 a
Zn (mg kg^{-1})	32.8 ± 3.0 c	73.1 ± 4.6 b	106.3 ± 8.1 a

Table 1: Cd, Pb, and Zn concentration in the miscanthus leaves collected on MC, M200 and M500 soils. Values represent means ± standard deviations. Different letters in rows refer to significant differences between plots (Tukey HSD test, $n = 3$, $p \leq 0.05$). LD: Limit of detection (Cd: 0.0049 mg L^{-1}; i.e. 0.4 mg kg^{-1})

The leaves sampled from the uncontaminated MC plot displayed the lowest metal concentrations (< LD: 0.4, 7.7, and 32.8 mg kg^{-1} corresponding to the Cd, Pb, and Zn concentrations, respectively). The metal concentrations increased in the leaves as soil contamination increased: the leaves sampled from the M500 plot displayed, respectively, 3.7, 2.5, and 3.2 times greater Cd, Pb, and Zn concentrations than those in the MC soil.

C.2.3.2- Pseudototal metal concentrations and physicochemical parameters of soils

Table 2 presents the pseudototal metal concentrations, pH, available P_2O_5, and soil organic carbon (SOC) of the control, unamended, and amended soils throughout the different aging periods (1, 3, and 6 months).

		Control	Non-amended soil			Amended soil		
			I1 NL	I3 NL	I6 NL	I1 L	I3 L	I6 L
Cd (mg kg^{-1})	MC	0.6 + 0.1 c	0.5 + 0.0 c	0.5 + 0.1 c	0.6 + 0.1 c	0.6 + 0.0 c	0.6 + 0.0 C	0.6 + 0.1 c
	M200	3.1 ± 0.2 b	3.0 ± 0.2 b	3.1 ± 0.3 b	2.9 ± 0.3 b	3.5 ± 0.2 b	3.5 ± 0.3 b	3.5 ± 0.1 b
	M500	7.9 ± 0.2 a	8.0 ± 0.8 a	7.9 ± 0.5 a	7.9 ± 0.6 a	8.3 ± 0.6 a	8.3 ± 0.2 a	8.4 ± 0.2 a
Pb (mg kg^{-1})	MC	32.0 ± 6.7 c	29.0 ±1.8 c	28.9 ± 4.0 c	29.1 ± 5.2 c	36.8 ± 3.5 c	33.9 ± 3.0 c	38.0 ± 5.1 c
	M200	194.6 ± 21.1 b	181.7 ± 13.1 b	185.9 ± 8.0 b	181.3 ± 3.3 b	201.3 ± 6.5 b	202.9 ± 10.9 b	198.9 ± 10.0 b
	M500	468.6 ± 6.8 a	458.4 ± 30.6 a	461.0 ± 12.0 a	475.0 ± 10.8 a	478.7 ± 11.3 a	477.9 ± 15.7 a	475.0 ± 10.8 a
Zn (mg kg^{-1})	MC	48.4 ± 6.4 d	50.3 ± 1.3 d	51.7 ± 1.6 d	50.8 ± 1.6 d	54.5 ± 3.0 d	54.9 ± 3.5 d	53.5 ± 5.2 d
	M200	276.3 ± 7.1 c	276.9 ± 9.6 c	274.8 ± 12.6 c	275.0 ± 7.8 c	287.6 ± 6.7 c	287.5 ± 6.0 c	288.0 ± 7.9 c
	M500	490.2 ± 4.1 ab	490.0 ± 10.3 ab	479.5 ± 18.6 b	488.6 ± 15.0 ab	510.4 ± 11.3 a	500.6 ± 9.7 ab	505.8 ± 6.9 ab
pH	MC	6.1 + 0.1 i	6.1 + 0.0 i	6.0 + 0.3 i	6.0 + 0.0 i	7.0 + 0.1 fgh	6.9 + 0.1 gh	6.8 + 0.0 h
	M200	7.3 + 0.1 bcde	7.3 + 0.0 def	7.2 +0.0 ef	7.2 + 0.1 efg	7.6 +0.1 ab	7.5 +0.0 abcd	7.4 + 0.1 abcde
	M500	7.4 + 0.1 abcde	7.4 + 0.1 abcde	7.4 + 0.0 bcde	7.3 + 0.0 cde	7.7 +0.1 a	7.6 + 0.1 a	7.6 + 0.1 abc
P_2O_5 (mg kg^{-1})	MC	0.2 + 0.0 e	0.2 + 0.0 e	0.2 + 0.0 e	0.2 + 0.0 e	0.3 + 0.0 de	0.3 + 0.0 de	0.3 + 0.0 d
	M200	0.4 + 0.0 c	0.4 + 0.0 c	0.5 + 0.0 c	0.4 + 0.0 c	0.5 + 0.0 b	0.6 + 0.0 ab	0.6 + 0.0 a
	M500	0.2 + 0.0 e	0.2 + 0.0 e	0.2 + 0.0 e	0.2 + 0.0 e	0.3 + 0.0 de	0.3 + 0.0 de	0.3 + 0.0 de
SOC (g kg^{-1})	MC	20.1 + 1.5 de	19.5 + 1.4 e	19.8 + 1.2 de	19.1 + 1.7 ef	21.7 + 1.9 cde	22.9 + 1.3 cd	23.4 + 1.8 c
	M200	13.2 + 1.4 g	13.2 + 1.7 g	13.0 + 1.4 g	13.1 + 1.2 g	15.2 + 1.9 g	15.5 + 1.2 g	16.0 + 1.3 fg
	M500	27.8 + 1.9 b	27.7 + 1.6 b	27.3 + 2.3 b	27.3 + 1.6 b	31.7 + 1.4 a	31.8 + 1.9 a	31.8 + 1.8 a

Table 2: Evolution of pseudototal metal concentrations, pH, P_2O_5 content and Soil organic carbon (SOC) of the control, non-amended (NL) and amended (L) MC, M200, and M500 soils after 1, 3, and 6 months of artificial aging referred to as I1, I3, and I6 respectively. Values represent means ± standard deviations. Different letters in rows refer to significant differences between plots (Tukey HSD test, $n = 3$, $p \leq 0.05$).

To begin with, the control MC soil displayed the lowest metal concentrations (0.6, 32.0, and 48.4 mg kg^{-1} corresponding to the concentrations of Cd, Pb, and Zn, respectively), followed by the M200 and M500 soils. Naturally, incubation did not influence the pseudototal metal concentrations in unamended soils (I1 NL, I3 NL, and I6 NL). Interestingly, the addition of the miscanthus leaves did not modify the corresponding metal concentrations in the three amended soils (no significant differences were detected between the different soil modalities).

In general, the unamended soils did not undergo any significant variation in comparison with their corresponding controls throughout the entire incubation process regarding the physicochemical parameters investigated (pH, available P_2O_5, and SOC). However, the

amended soils underwent certain variations compared to their corresponding control unamended soils. The pH of the MC soil was slightly acidic (pH = 6.1) in comparison to the M200 and M500 soils, which tended to be slightly alkaline (pH = 7.3 and 7.4, respectively). The leaf incorporation increased only the pH of the MC soil. After 1 month of incubation, the pH augmentation in the amended MC soil reached 12.9 % in comparison to the control pots. No significant differences were observed on M200 and M500 soils.

The available P_2O_5 content in the M200 control soil was higher than the MC and M500 control soils (0.4, 0.2, and 0.2 mg kg^{-1} in the M200, MC, and M500 control soils, respectively). The corresponding concentration increased progressively in the MC and M200 amended soils, where they reached their highest levels at I6, constituting a rise of 23.2 and 27.8% relative to their controls. The M500 amended soils did not undergo significant variations in comparison with its corresponding control.

The highest SOC concentrations were detected in the M500 control soil, followed by the MC and M200 controls (20.1, 13.2, and 27.8 g kg^{-1} corresponding to the MC, M200, and M500 control soils, respectively; significant differences existed between the three soils). The corresponding concentrations increased progressively in the amended MC and M500 soils throughout the incubation period, in which the highest levels were recorded at I6, an increase of 14.2 and 15%, respectively. The M500 amended soils did not undergo significant variations in comparison with their corresponding control.

C.2.3.3- Soil $CaCl_2$-extractable metal concentrations

The 0.01 M $CaCl_2$-extractable Cd, Pb, and Zn concentrations of the control, unamended, and amended MC, M200, and M500 soils after 1, 3, and 6 months of incubation are presented in Table 3. Globally, $CaCl_2$-extractable concentrations of the three metals increased as soil metal contamination increased (MC < M200 < M500).

		Control	Non-amended soil			Amended soil		
			I1 NL	I3 NL	I6 NL	I1 L	I3 L	I6 L
Cd-$CaCl_2$ (mg kg^{-1})	MC	0.01 + 0.00 f	0.01 + 0.00 f	0.01 + 0.00 f	0.01 + 0.00 f	0.01 + 0.00 ef	0.01 + 0.00 f	0.01 + 0.00 f
	M200	0.02 + 0.0 cd	0.02 + 0.00 cde	0.02 + 0.00 cd	0.02 + 0.00 de	0.03 + 0.00 c	0.02 + 0.00 cd	0.02 + 0.00 cd
	M500	0.04 + 0.0 ab	0.04 + 0.00 ab	0.04 + 0.00 ab	0.04 + 0.00 b	0.05 + 0.00 a	0.04 + 0.00 a	0.04 + 0.00 ab
Pb-$CaCl_2$ (mg kg^{-1})	MC	0.21 + 0.03 gh	0.21 + 0.02 gh	0.19 + 0.0 1 h	0.19 + 0.02 h	0.28 + 0.03 cdef	0.25 + 0.02 fgh	0.25 + 0.02 efgh
	M200	0.26 + 0.02 efgh	0.27 + 0.01 defg	0.27 + 0.02 defg	0.26 + 0.02 efgh	0.36 + 0.01 ab	0.35 + 0.02 b	0.35 + 0.02 bc
	M500	0.33 + 0.01 bcd	0.33 + 0.03 bcde	0.32 + 0.03 bcde	0.32 + 0.04 bcde	0.43 + 0.03 a	0.38 + 0.02 ab	0.37 + 0.02 ab
Zn-$CaCl_2$ (mg kg^{-1})	MC	0.08 + 0.01 ijk	0.06 + 0.00 jk	0.07 + 0.01 jk	0.06 + 0.01 k	0.12 + 0.01 fg	0.11 + 0.01 gh	0.10 + 0.00 h
	M200	0.09 + 0.01 hij	0.07 + 0.01 ijk	0.08 + 0.01 ijk	0.08 + 0.01 ijk	0.18 + 0.01 d	0.15 + 0.01 e	0.14 + 0.01 ef
	M500	0.14 + 0.00 ef	0.14 + 0.00 ef	0.14 + 0.01 ef	0.14 + 0.01 ef	0.28 + 0.01 a	0.25 + 0.01 b	0.23 + 0.00 c

Table 3: Cd, Pb, and Zn $CaCl_2$ extractable fractions of the control, non-amended (NL) and amended (L) MC, M200, and M500 soils after 1, 3, and 6 months of artificial aging referred to as I1, I3, and I6 respectively. Values represent means ± standard deviations. Different letters in rows refer to significant differences between plots (Tukey HSD test, $n = 3$, $p \leq 0.05$).

The $CaCl_2$-extractable Cd, Pb, and Zn concentrations in the unamended soils were quite similar to their corresponding concentrations in the control soils throughout the entire incubation period (no significant differences were detected).

The leaf amendment did not impact Cd $CaCl_2$-extractable concentrations in the three soils, in which no significant differences were detected between the different soil modalities with the same metal concentration. On the other hand, leaf incorporation raised the Pb $CaCl_2$-extractable concentrations throughout the entire incubation period in the M200 soil only after the 1st month of incubation in the MC and M500 soils. In I1 L pots, in comparison with the control pots, the corresponding Pb $CaCl_2$ concentrations increased by 26.2, 27.8, and 23.0% in the MC, M200, and M500 amended soils, respectively.

Incorporating leaves increased the Zn $CaCl_2$-extractable concentrations in the three amended soils, with values at I1 displaying the highest augmentations (36.2, 52.8, and 49.9%, corresponding to Zn-$CaCl_2$ concentrations of the amended MC, M200, and M500 soils, respectively). However, the values tended to decrease with time throughout the incubation phase, remaining higher than their corresponding controls and unamended soils (significant differences were still detected).

C.2.3.4- Soil microbial carbon

The influence of soil aging and leaf incorporation was studied on soil microbial carbon (Figure 1).

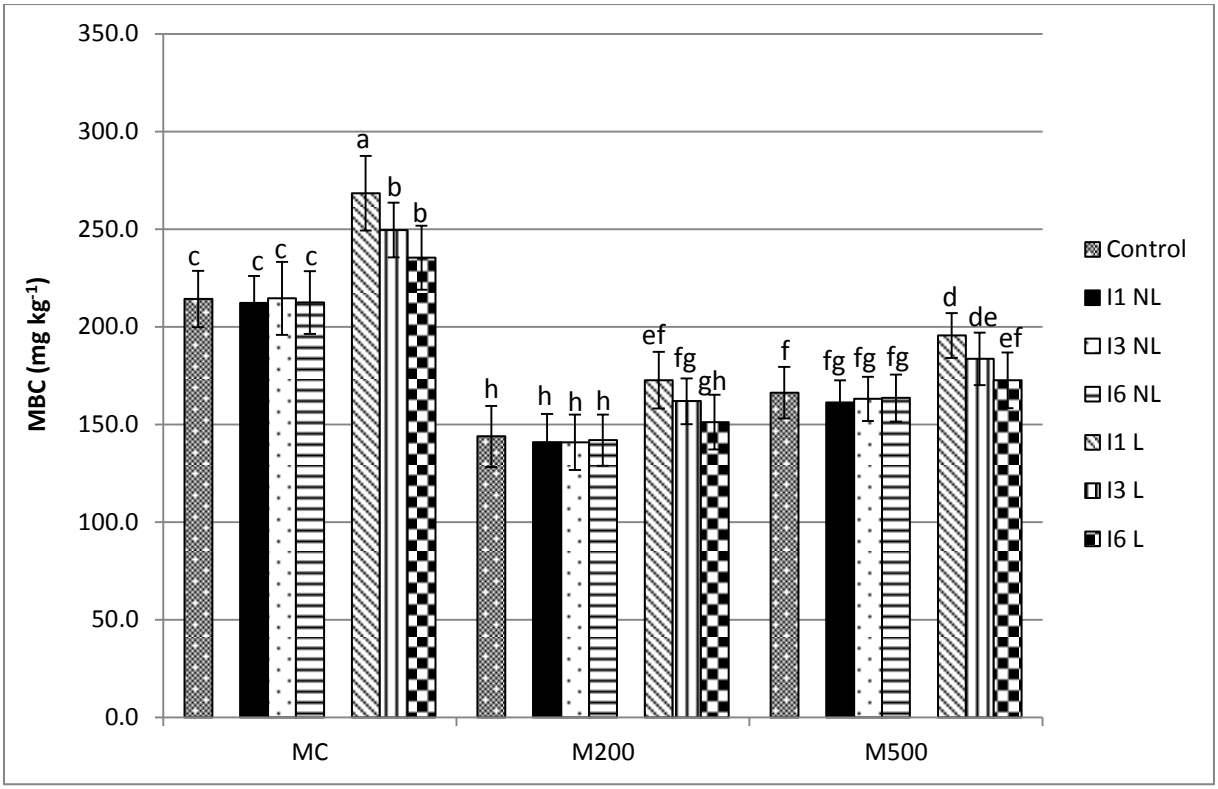

Fig 1: Microbial biomass carbon (MBC) of the control, non-amended (NL) and amended (L) MC, M200 and M500 soils after 1, 3 and 6 months of artificial aging referred to as I1, I3 and I6 respectively. Bars represent means ± standard deviations. Different letters refer to significant differences between plots (Tukey HSD test, $n = 3$, $p \leq 0.05$).

The highest MBC was measured in the MC control soil, followed by the MBC levels of the M500 and M200 controls (significant differences were noted among the soils). No significant variations were recorded between the unamended soils and their corresponding controls.

On the other hand, leaf incorporation boosted the biomass in the MC soil mainly at I1 with a 20.2% increase with respect to its corresponding control. Values tended to decrease with time yet remained significantly higher than their corresponding unamended and control soils.

The MBC in the amended M200 and M500 soils were significantly higher than their corresponding controls and unamended soils only after the 1st and 3rd month of incubation, with the largest biomass detected at I1 s, representing a rise of 16.7 and 15.0%, respectively, relative to their corresponding controls.

C.2.3.5- Soil basal respiration

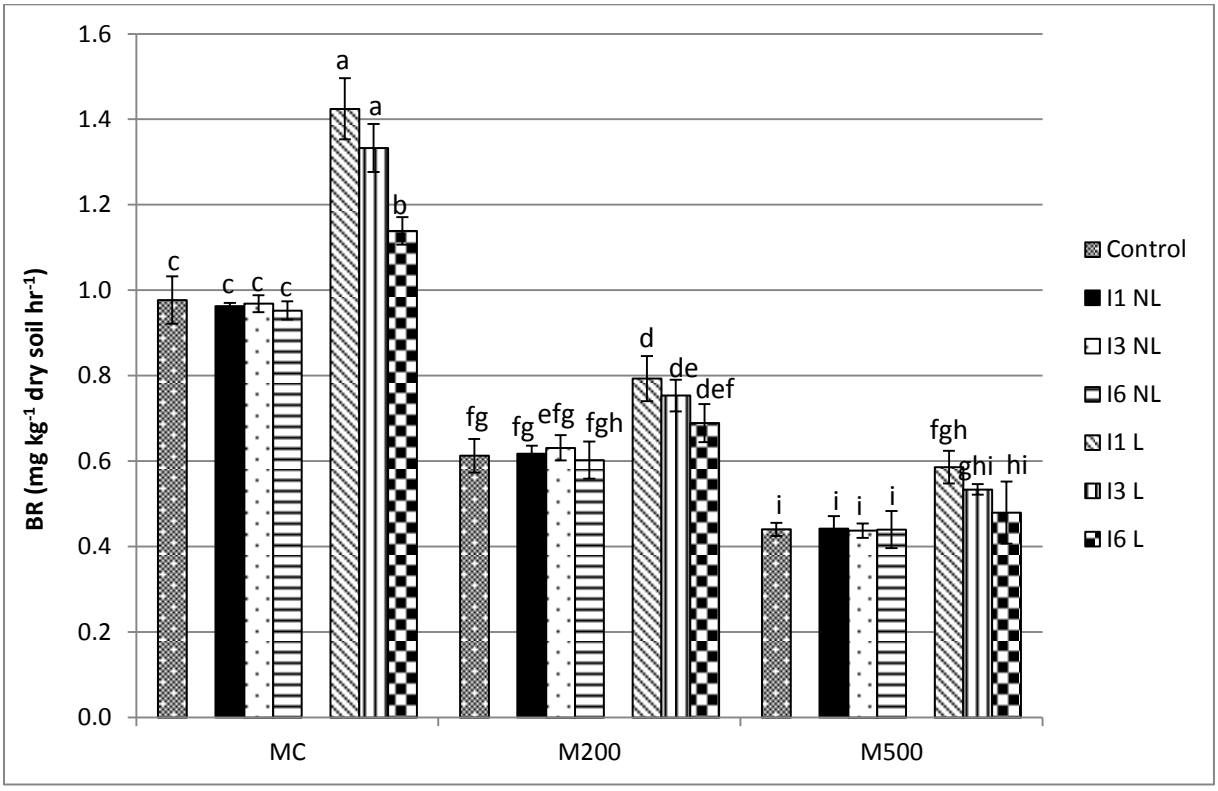

Fig 2: Basal respiration (BR) of the control, non-amended (NL) and amended (L) MC, M200 and M500 soils after 1, 3 and 6 months of artificial aging referred to as I1, I3 and I6 respectively. Bars represent means ± standard deviations. Different letters refer to significant differences between plots (Tukey HSD test, $n = 3$, $p \leq 0.05$).

The basal respiration of MC, M200, and M500 soils showed that the respiratory activities decreased in the control soils as the contamination increased (Figure 2). The MC soil control displayed the greatest activity, followed by the M200 and M500 soil controls, respectively (significant differences were determined among the soils). No significant variations were detected between the unamended soils and their corresponding controls.

However, the leaf incorporation led to a significant increase in the activity in the MC soil during the entire incubation period, up to 31.4% at I1 in comparison with the activity in the control soil. On the other hand, the leaf incorporation improved the respiratory activity only after the 1st month of incubation in the M200 and M500 soils, a 22.7 and 24.9% rise, respectively, compared with their corresponding controls. Afterwards, no significant differences were detected in the amended M200 and M500 soils with their corresponding controls and unamended soils after the 3rd and 6th month of incubation.

C.2.3.6- Ryegrass shoot dry biomass

The ryegrass shoot dry biomass did not vary between those cultivated in the amended and unamended soils (no significant differences were detected) whatever the metal contamination (Figure 3).

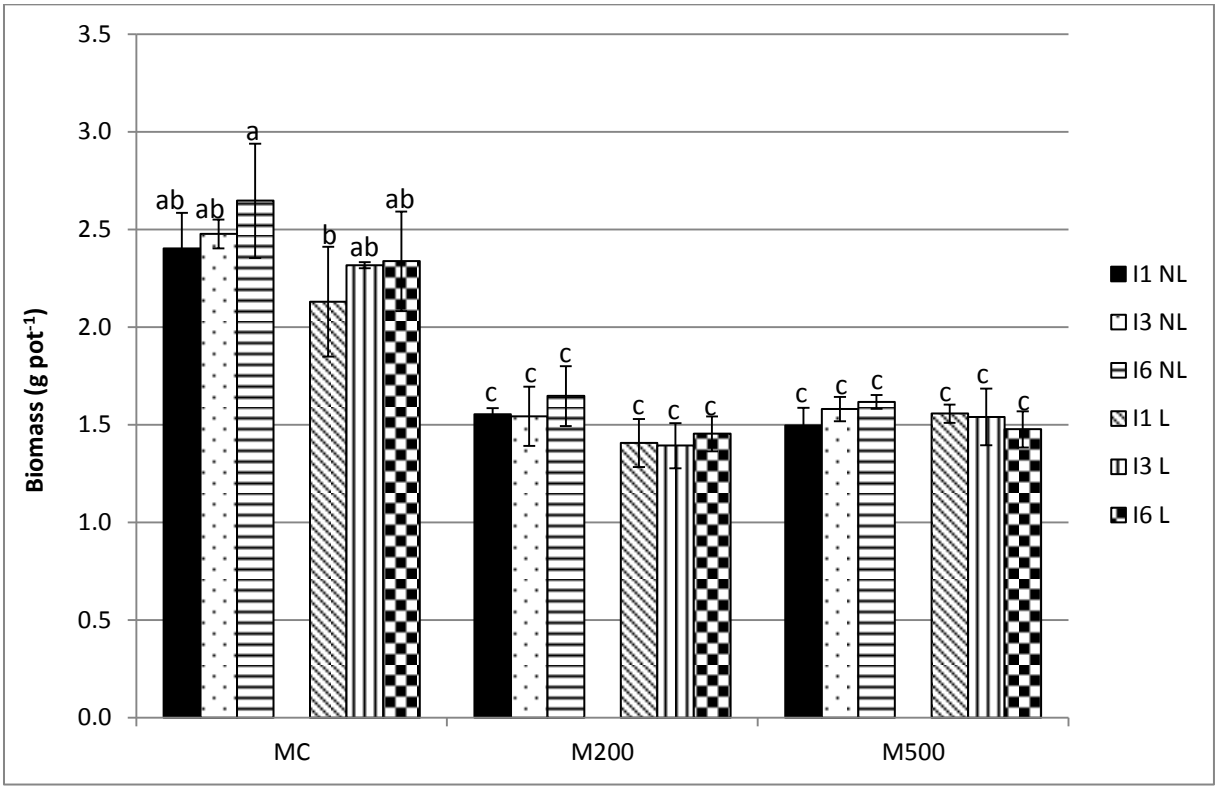

Fig 3: Shoot dry biomass of the ryegrass cultivated in the non-amended (NL) and amended (L) MC, M200 and M500 soils after 1, 3 and 6 months of artificial aging referred to as I1, I3 and I6 respectively. Bars represent means ± standard deviations. Different letters refer to significant differences between plots (Tukey HSD test, $n = 3$, $p \leq 0.05$).

The ryegrass samples planted in the contaminated soils displayed a decline in their corresponding biomass, with significant differences between the corresponding dry biomass of the ryegrass shoots planted in the MC soil and those cultivated in the M200 and M500 soils (Figure 3). The average shoot dry biomass of the ryegrass cultivated in the MC unamended soil was 2.4 g pot^{-1}, and decreased by 37.1 and 35.2 % in the shoots cultivated in the M200 and M500 soils, respectively. However, no significant variations were recorded between the results obtained in the M200 and M500 soils.

C.2.3.7- Metal concentrations in ryegrass shoots

The Cd and Zn concentrations in the ryegrass shoots cultivated in both the amended and unamended soils after incubation are presented in Table 3. The Pb concentrations are not shown because they were below the detection limit (0.052 mg L^{-1}; i.e., 4.3 mg kg^{-1}) in all the samples studied.

		Non-amended soil			Amended soil		
		I1 NL	I3 NL	I6 NL	I1 L	I3 L	I6 L
Cd (mg kg^{-1})	MC	< LD	< LD	< LD	< LD	< LD	< LD
	M200	0.4 + 0.0 d	0.4 + 0.0 d	0.5 + 0.0 d	0.6 + 0.0 d	0.5 + 0.0 d	0.5 + 0.0 d
	M500	1.3 + 0.1 c	1.4 + 0.2 bc	1.4 + 0.2 bc	1.9 + 0.1 a	1.7 + 0.3 ab	1.5 + 0.2 bc
Zn (mg kg^{-1})	MC	15.6 + 1.2 d	15.9 + 2.0 d	14.1 + 1.1 d	21.0 + 3.0 d	17.3 + 2.1 d	17.4 + 1.0 d
	M200	40.3 + 3.7 c	41.7 +3.8 c	41.7 + 3.9 c	42.8 + 2.9 c	42.3 + 3.2 c	41.9 + 2.2 c
	M500	67.8 + 4.2 b	64.4 + 2.9 b	68.3 + 5.2 b	77.9 + 3.9 a	77.8 + 3.0 a	76.6 + 4.3 a

Table 3: Cd and Zn concentrations in the ryegrass shoots cultivated in the non-amended (NL) and amended (L) MC, M200, and M500 soils after 1, 3, and 6 months of artificial aging referred to as I1, I3, and I6 respectively. Values represent means ± standard deviations. Different letters in rows refer to significant differences between plots (Tukey HSD test, $n = 3$, $p \leq 0.05$).
LD: Limit of detection (Cd: 0.4 mg kg^{-1})

The Cd concentration in the ryegrass shoots increased progressively with the soil metal concentration. It was below the detection limit (0.4 mg kg^{-1}) in the shoots of the MC soil. The Cd concentration fluctuated between 0.4 and 0.6 mg kg^{-1} in the ryegrass shoots cultivated in the M200 soil with no significant differences detected between plants cultivated in the different soil modalities. The ryegrass in the M500 soil displayed the highest Cd concentration, yet no significant variations were detected in the shoots except for those cultivated in the I1 L soils (1.9 mg kg^{-1}).

The Zn concentrations in the ryegrass shoots increased progressively with the increase in soil contamination. No significant differences were detected in the shoot metal concentrations of the plants cultivated in the amended and unamended MC and M200 soils. The ryegrass cultivated in the M500 soil exhibited the highest Zn concentration in their shoots, with a slight but significant increase recorded in the shoots cultivated in the leaf-amended soils, in which the Zn in the shoots increased by an average of 13.7% in comparison with the plants in the unamended soils.

C.2.3.8- Ryegrass photosynthetic pigments

The influence of soil modalities on photosynthetic pigments (Chl a, Chl b, and Car) contents are shown in Figures 4, 5, and 6, respectively.

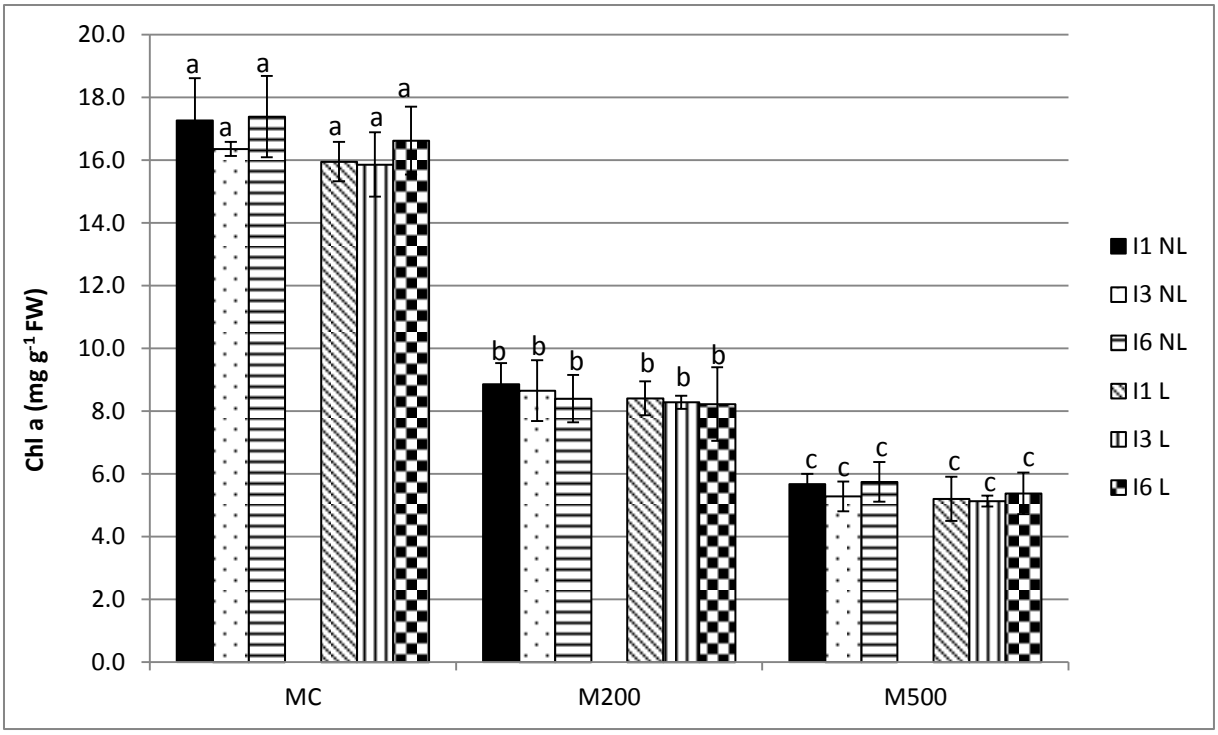

Fig 4: Chl a contents in the ryegrass shoots cultivated in the non-amended (NL) and amended (L) MC, M200, and M500 soils after 1, 3 and 6 months of artificial aging referred to as I1, I3, and I6 respectively. Bars represent means ± standard deviations. Different letters refer to significant differences between plots (Tukey HSD test, n = 3, $p \leq 0.05$).

For these three biomarkers, no significant differences were observed between ryegrass plants cultivated in the amended or unamended soils. The Chl a mean concentration in the ryegrass shoots cultivated in the MC soil was 16.6 mg g^{-1} FW (Figure 4). The corresponding concentration decreased progressively in the ryegrass shoots cultivated in the M200 and M500 soils by 48.9 and 67.4%, respectively.

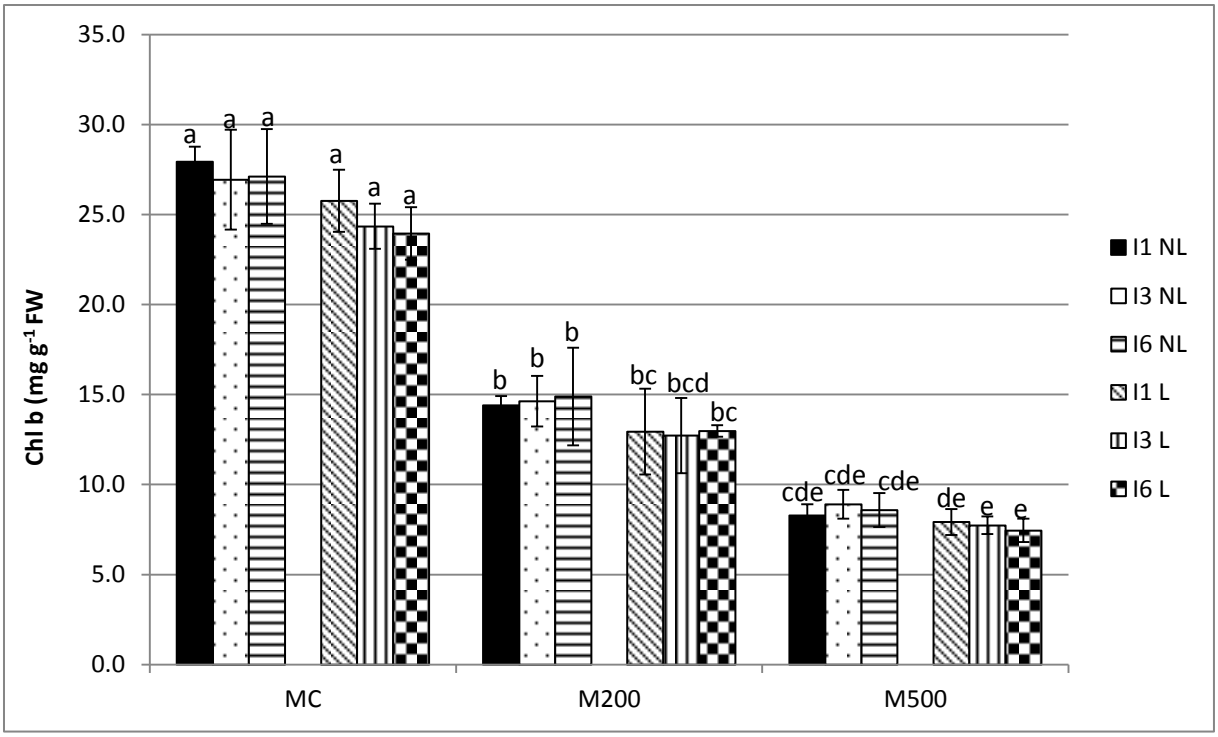

Fig 5: Chl b contents in the ryegrass shoots cultivated in the non-amended (NL) and amended (L) MC, M200 and M500 soils after 1, 3 and 6 months of artificial aging referred to as I1, I3 and I6 respectively. Bars represent means ± standard deviations. Different letters refer to significant differences between plots (Tukey HSD test, n = 3, $p \leq 0.05$).

The same trends were observed for Chl b (Figure 5). The average Chl b concentration in the ryegrass shoots cultivated in the MC soil was 26.0 mg g^{-1} FW, and decreased by 47.1 and 68.7% in those cultivated in the M200 and M500 soils, respectively.

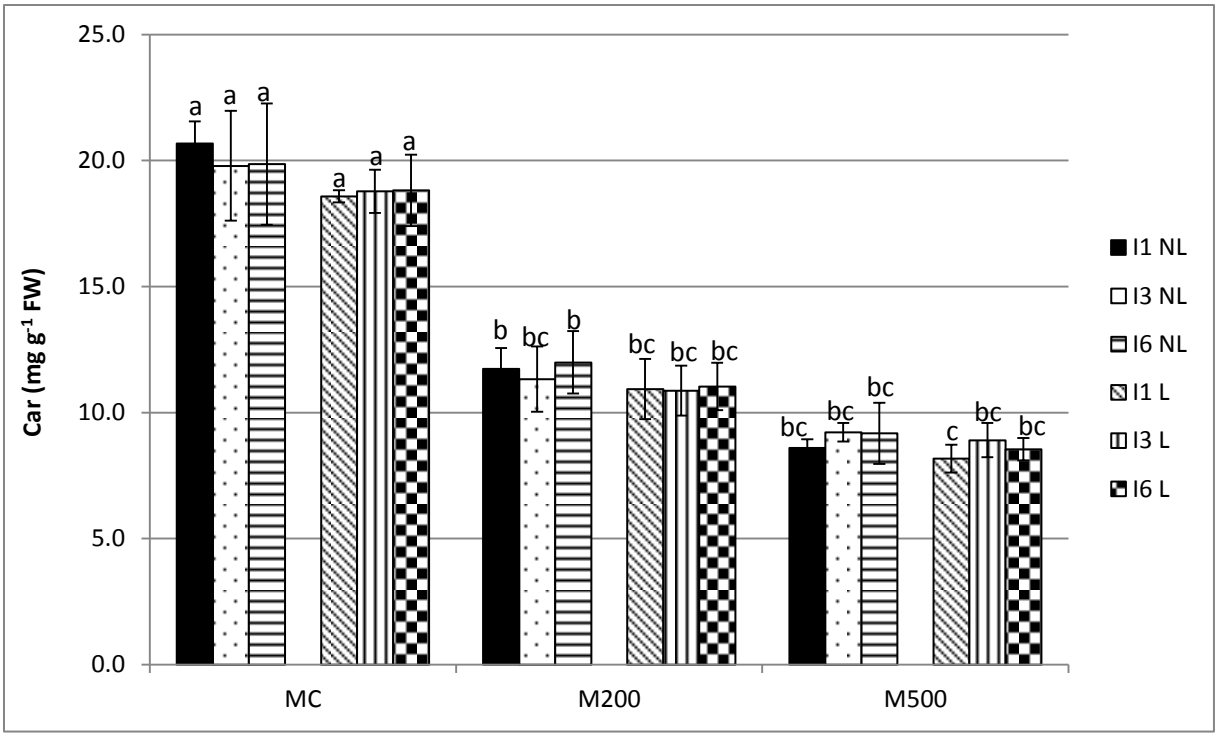

Fig 6: Car contents in the ryegrass shoots cultivated in the non-amended (NL) and amended (L) MC, M200 and M500 soils after 1, 3 and 6 months of artificial aging referred to as I1, I3 and I6 respectively. Bars represent means ± standard deviations. Different letters refer to significant differences between plots (Tukey HSD test, $n = 3$, $p \leq 0.05$).

The Car content was also strongly affected by soil contamination (Figure 6). The average Car level in ryegrass plants grown in the MC soil was 19.4 mg g^{-1} FW, and decreased by 41.7 and 54.8% in the ryegrass shoots cultivated in the M200 and M500 soils, respectively. However, unlike Chl a and b, no significant differences were observed between plants cultivated in the M200 and M500 soils.

C.2.3.9- Ryegrass antioxidative enzymatic activity assay

The antioxidative activities of SOD and APX are displayed in Figures 7 and 8, respectively.

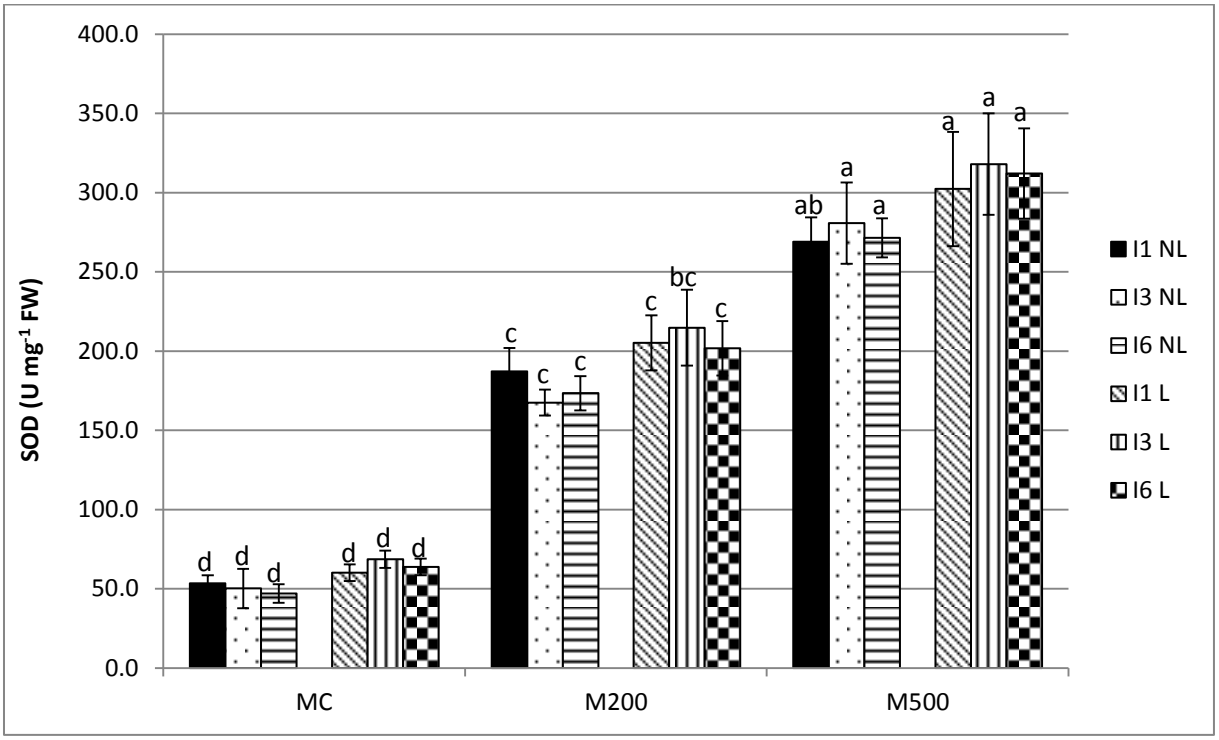

Fig 7: SOD activities in the ryegrass shoots cultivated in the non-amended (NL) and amended (L) MC, M200, and M500 soils after 1, 3, and 6 months of artificial aging referred to as I1, I3 and I6 respectively. Bars represent means ± standard deviations. Different letters refer to significant differences between plots (Tukey HSD test, $n = 3$, $p \leq 0.05$).

The SOD activities in the ryegrass samples significantly increased as the soil gradient contamination rose (Figure 7). The plants cultivated in the M200 and M500 soils displayed activities 3.3 and 5.1 times higher than those recorded in the plants grown in the MC soil (basal average value, 57.3 U mg^{-1} FW).

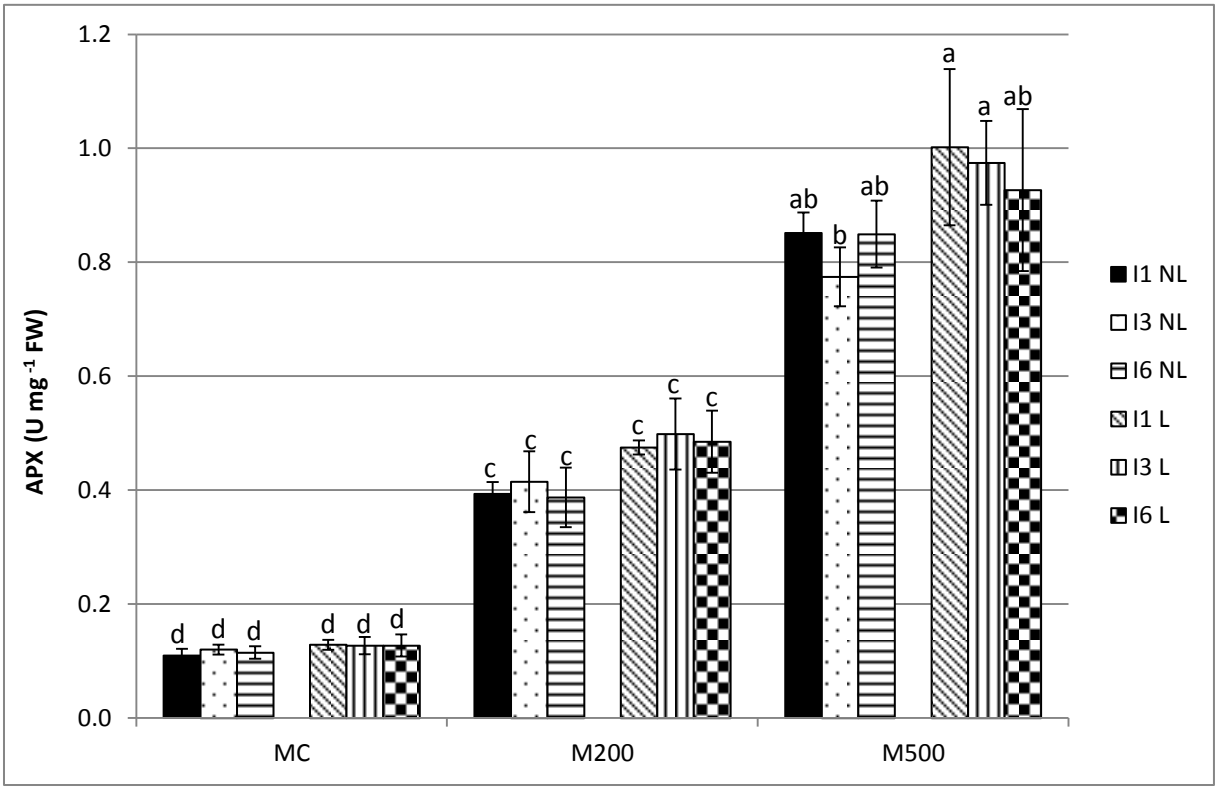

Fig 8: APX activities in the ryegrass shoots cultivated in the non-amended (NL) and amended (L) MC, M200 and M500 soils after 1, 3 and 6 months of artificial aging referred to as I1, I3 and I6 respectively. Bars represent means ± standard deviations. Different letters refer to significant differences between plots (Tukey HSD test, $n = 3$, $p \leq 0.05$).

The same trends were observed for APX (Figure 8). APX increased progressively in the plants cultivated in the M200 and M500 soils, respectively, recording values 3.6 and 7.4 times higher than the basal values obtained on the MC soil (0.1 U mg^{-1} FW). No significant variations were recorded between the SOD and APX activities in the ryegrass shoots cultivated in the amended and unamended soils of the same metal concentrations.

C.2.4- Discussion

C.2.4.1- Influence of leaf incorporation on soil parameters

Miscanthus has been proved to be a suitable candidate for phytomanaging metal-polluted soils due to its capacity to accumulate high metal concentrations in its underground parts, and to a lesser extent in the aerial organs, as well as producing high biomass when cultivated in contaminated areas (Nsanganwimana et al., 2015; Nsanganwimana et al., 2016). Moreover, it displayed a positive influence on improving the physicochemical and biological parameters of the contaminated soils as well as decreasing the corresponding human health risks (Al Souki et al., 2017b; Pelfrêne et al., 2015). However, the present study focuses on the impacts of leaf residue decomposition throughout the period of miscanthus culture on different soil parameters as well as one potential crop that could replace the miscanthus

plants after their removal. For this reason, a controlled artificial soil aging process incorporating powder of the miscanthus leaves (estimated quantity, 20 years of leaf fall) into different uncontaminated and contaminated soils was launched to determine the consequences.

To begin with, leaf incorporation did not impact soil's pseudototal metal concentrations (Table 2). Indeed, the quantity of leaves incorporated represented 2/1000 of the soil mass. Moreover, their corresponding metal concentrations (in milligrams per kilogram of leaves) account for 18.9–19.3% for Cd, 4.1–24% for Pb, and 21.7–67.6% for Zn of the total soil metal concentrations in the MC, M200, and M500 plots from which they were collected. Thus, soil contamination was even slightly diluted. However, the main concern regarding leaf incorporation was not its influence on the pseudototal metal concentration but on their available fractions.

This study demonstrates the influence of miscanthus leaf incorporation on $CaCl_2$ metal extractability in the amended soils studied (Table 3). It increased the Pb and Zn $CaCl_2$-extractable metal concentrations in the amended soils mainly at I1 in comparison with the control and unamended soils. However, no impacts were detected on the Cd $CaCl_2$-extractable concentrations throughout the incubation process. This increase in metal $CaCl_2$ extractability cannot be explained by the decrease of pH or organic carbon content, which are considered as two of the main parameters influencing metal extractability. Indeed, although pH values were relatively high during the experiment, the amendment with miscanthus leaves caused a slight pH increase in the soil, mainly the MC amended soil (Table 2). Boucher et al. (2005) and Cui et al. (2011) demonstrated that *Arabidopsis halleri* and *Brassica juncea* leaf incorporation sharply increased soil pH, and the maximum level was obtained mainly after the 1st few days of incubation. Afterwards, a trend showing a slight decline followed by consistency prevailed. Wang et al. (2015) recorded the same outcome by incorporating Cd-contaminated rice straw into soils. The fast rise in soil pH may be due to the adsorption of H^+ ions by organic materials or the rapid release of excess cations from the residues reacting with H^+ in the soil (Tang et al., 1999). Another factor affecting the soil pH might be the plant material being incorporated: cations may be released from plant residues during microbial degradation, thus increasing the corresponding soil pH (Cui et al., 2011; Vazquez et al., 2008; Xu et al., 2016).

Leaf incorporation increased the available P_2O_5 and soil organic carbon (SOC) concentrations, compared to the control and unamended soils. These results are in agreement with Xu et al. (2016), who demonstrated the augmentation of SOC concentrations in the soils amended with rice straw, and Wei et al. (2015) who stated that wheat straw incorporation resulted in higher available phosphorous and SOC concentrations in the amended soil. Many studies (Dufossé et al., 2014; Medina et al., 2015; Zhang et al., 2016) have shown the significant effects of crop residue addition in improving the soil's organic matter dynamics and nutrient cycling in the soil, and thus replenishing the soil's organic matter content and supplying

essential nutrients after mineralization (N, P, etc.). Moreover, factors such as suitable moisture levels and temperature might have promoted the subsequent decomposition of the plant residues incorporated and thus the increase of organic carbon and essential nutrient concentrations such as P in the amended soils (Surekha et al., 2003; Wei et al., 2015). The reason behind the increase in Pb and Zn $CaCl_2$ extractability might be the increase in the amount of the metals in the exchangeable fractions of soil due to their corresponding influx from the incorporated leaves (Du et al., 2008), as well as their corresponding mobility, which will be discussed in the following paragraph.

The present study also demonstrated an influence of leaf incorporation on soil microbial biomass carbon (Figure 1) and basal respiration (Figure 2). Globally, these biological parameters exhibited higher values in the uncontaminated MC soil and decreased with the soil's metal concentration. According to Al Souki et al. (2017b), metal contamination negatively impacts soil functionality and affects the growth, morphology, and metabolism of soil microorganisms through functional disturbance and alteration of protein and cell membrane integrity. The BR and MBC varied as well between the amended and the unamended soils. After 1 month of incubation (I1), both the respiratory activities and MBC increased to reach their maximum values in all amended soils (significant differences were detected between the amended soils and their corresponding controls). On the other hand, the second phase (I3 and I6) represented the declining phase, where both parameters followed a descending pattern to reach their minimum levels by the end of the incubation period. The increase in values at the first stage could stem from the utilization of easily available organic compounds, most of which are water-extractable from the incorporated plant leaves, which caused the increase in BR and MBC (Shi and Marschner, 2014). Thus, the subsequent decline in activity and biomass to return to control levels might be due to the depletion of carbon substrates and easily available compounds, in which the utilization of the more recalcitrant compounds required energy for enzyme synthesis and therefore left less energy for growth (Pan et al., 2016; Quinn et al., 2011; Shi and Marschner, 2014).

C.2.4.2- Impacts of leaf incorporation on the ryegrass crop

Ryegrass is an important, widespread perennial cool-season turf grass. It was selected because it was a valuable forage crop that can be used for plant cover as well as for phytoremediation due to its capacity to accumulate toxic substances in its biomass (Bai et al., 2015a; Bidar et al., 2009; Houben et al., 2013). The results reported herein show that ryegrass accumulated more Cd and Zn in its aboveground parts depending on the contamination gradient (Table 3). However, only the ryegrass plants cultivated in the amended M500 soils exhibited higher Zn concentrations in their shoots, in comparison with those planted in the unamended soils. These results are quite consistent with our data obtained on $CaCl_2$ metal extractability (except for Pb), even if the effect of leaf incorporation on metal accumulation in shoots is only notable on the M500 soil. However, the low

accumulation of Pb in ryegrass shots is due to its very low mobility into plants and its poor translocation from roots to shoots (Pourrut et al., 2013; Pourrut et al., 2011).

Our results on Cd and Zn are in agreement with those obtained by Perronnet et al. (2000) and Wang et al. (2015), who conducted similar experiments, in which soils were amended with Cd- and Zn-contaminated *Thalspi caerulescens* leaves and Cd-contaminated rice straws, respectively. These authors demonstrated that the incorporated metals exhibited high mobility and were transferred in large amounts to the subsequent crop, proving the idea that the effects of plant leaf addition on metal speciation and phytoavailability in soils depend not only on soil pH and organic carbon, but also on the plant material and the amounts of metal within.

Du et al. (2008) also investigated the impact of amending soils with arsenic (As)-rich Chinese brake fern (*Pteris vittata* L.). After 1, 3, and 6 months of soil aging, germinated wheat was planted in the soils, and the results showed that the As concentration in the shoots significantly increased with the increasing amount of As in the leaves. In addition, the shoots planted in the soils incubated for 1 month exhibited the highest concentrations. The relative increase in the shoot metal concentration in the subsequent ryegrass might be attributed to the high mobility of the metals contained in the miscanthus leaves incorporated, which were mainly transferred to the subsequent crop (Perronnet et al., 2000; Wang et al., 2015).

Even if the miscanthus leaf incorporation into soils slightly increased metal accumulation in ryegrass shoots on the most contaminated soils (Table 3), it did not affect the plant's health (Figures 3–8). Yet, there were significant variations between the ryegrass samples cultivated in the uncontaminated MC soils and those cultivated in the contaminated soils. The biomass results (Figure 3) showed a significant 37.1 and 35.2% decrease in the plants cultivated in M200 and M500 soils, respectively, relative to those in the MC. The corresponding impact of the soil gradient contamination was more evident in the photosynthetic pigment concentrations (Figures 4–6), in which Chl a decreased by 48.9 and 67.4% in the shoots cultivated in the M200 and M500 soils, respectively. Similar trends were demonstrated for Chl b and Car levels. Finally, The SOD ad APX activities in the plants sampled from the M200 and M500 soils were three to seven times higher than those recorded in the MC soil plants. Overall, the results obtained in the present study indicate that the ryegrass species suffered from stressful conditions in the contaminated amended and unamended soils.

These results are consonant with the study reported by Wang et al. (2013), who revealed the significant negative effects on the Chl a, Chl b, and Car contents as well as the biomass of ryegrass seedlings upon exposure to Cd. Bai et al. (2015b) also demonstrated the negative impact Pb had on the ryegrass biomass, the net photosynthetic rate, in addition to the Chl and Car contents of their corresponding leaves. The decrease in the ryegrass biomass could be attributed to the negative influence exerted by the metals over the main metabolic processes including photosynthesis, respiration, as well as nutrient and water uptake (Chibuike and Obiora, 2014; Pourrut et al., 2013; Pourrut et al., 2011). Moreover, the metal

concentration-dependent increase of SOD and APX activities in the present study agrees with several other experiments (Bonnet et al., 2000; Cao et al., 2016; Lou et al., 2015; Luo et al., 2011). In addition, the gene expression of SOD and APX were boosted in the ryegrass that was subjected to Pb stress in the experiment of Li et al. (2012). Finally, Al Souki et al. (2017a) demonstrated the negative impacts of the gradient metal concentration in soils on the corresponding photosynthetic pigments and antioxidative SOD and APX activities in the miscanthus leaves.

Interestingly, in the current work, we demonstrated that ryegrass plants underwent substantial oxidative stress, while photosynthetic pigments were impacted to a lesser extent. The impact of metal contamination was less notable on plant biomass and no significant differences were observed between plants grown on the M200 and M500 soils. This different response could stem from the short cultivation period (2 months). Significant variations might appear in the corresponding shoot biomass in the contaminated soils if the duration was prolonged. The results obtained were in accordance with Li et al. (2012), who showed that the Chl contents of ryegrass were not affected by the increasing Cd concentrations in a 1-week experiment, contrary to the activities of the antioxidative enzymes, which responded immediately to the increasing metal stress. Luo et al. (2011) made similar observations while studying the impact of increasing levels of Cd on ryegrass, in which the corresponding Chl a and b levels in the plants treated with 0.2 and 0.5 mM Cd were the same. Overall, the results obtained indicate that the antioxidative enzymes responsible for the early stage of defense against metal-induced oxidative stress are the most severely affected.

C.2.5- Conclusions and perspectives

The main objective of the present study was to assess the impacts of incorporating leaf litter into the soil on several soil parameters, metal availability, as well as on the health of the crop succeeding the miscanthus culture (ryegrass). Nevertheless, miscanthus leaf incorporation induced slight variations in the soil physicochemical parameters studied, mainly after the 1st month of aging. Litter incorporation did not influence the pseudototal metal concentrations in soils but significantly increased Pb and Zn $CaCl_2$-extractable concentrations. Leaf incorporation increased the available P_2O_5 and soil organic carbon concentrations compared to control and unamended soils. Meanwhile, the soil respiratory activities and microbial biomass carbon rose mainly after the 1st month of incubation as a result of leaf amendment. However, no significant effects of leaf incorporation were observed on the subsequent ryegrass plant health. In conclusion, the incorporating miscanthus leaves did not have significant and persistent impacts on the soil–plant system studied, which might be considered as a very positive point when contaminated areas are harvested late. However, the results obtained might be used with caution given that they were obtained *ex situ*, in controlled conditions, using artificial aging. They must be

considered as preliminary data that need to be confirmed by long-term *in situ* experiments, which are ongoing on the Metaleurop Nord experimental sites.

Acknowledgements

The authors are grateful to the Association for Specialization and Scientific Guidance for the PhD scholarship offered to Karim Al Souki. They also wish to thank ADEME (French Agency for the Environment and Energy Management) for the financial support of the Phytener program.

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Part D- Influence of the soil metal contamination on the Miscanthus x giganteus health

D.1- Response of three *Miscanthus x giganteus* cultivars to metal stress. Part 1: Metal accumulation and plant health

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Foreword

The transition from laboratory experiments to field experiments could often provide deceiving results in the field of phytoremediation (Vangronsveld et al., 2009). This can be explained by two main factors:

- artificial conditions vs field conditions: the majority of the experiments to determine plant potential for phytoremediation *ex situ* are based on hydroponic cultures or spiked soils with no or limited period for chemical stabilization in soils. These conditions poorly mimic *in situ* situation and overestimate plants ability to uptake and translocate metals. Moreover, in the field, there are stressors that affect phytoremediation that are not encountered at laboratory or greenhouse scale: variations in temperature, nutrients, precipitation and moisture, plant pathogens and herbivory, uneven distribution of contaminants, soil type… Based on these biased data obtained in greenhouse experiments, and unrealistic extrapolations, too enthusiastic interpretations and promises have been made concerning the possibilities of metal phytoextraction (Vangronsveld et al., 2009).
- no or poor evaluation of plant health (only based on growth parameters): phytoremediation technologies are usually set up *in situ* for several years to several decades. Thus, taking in consideration the impact of pollutants on plant health is crucial to estimate the long-term ability of the plant potential to survive or to settle on the contaminated area.

Same remarks can be done on miscanthus studies. Most of the researches dealing with miscanthus ability to cope with metal contamination were based on hydroponic or spiked soil studies. Moreover, authors often used environmental unrealistic metal concentrations. In the other hand, our previous studies (Phytener program, Nsanganwimana PhD work (2014)) clearly demonstrated the ability of miscanthus to stabilize metals and reduce their

availabilities *in situ*, in Metaleurop Nord area. However, we have not investigated the effect of metals on miscanthus health.

In 2016, a parallel work, but complementary to this study, has been done in our laboratory to develop a high-throughput biomarker set able to deeply investigate stress (biotic, abiotic) influences on plant health (Liné et al., in preparation). This biomarker set aims at monitoring oxidative stress (antioxidative enzyme, lipid and protein peroxidation), redox status (glutathione and ascorbate contents), photosynthesis (photosynthetic pigments content, RUBISCO activity), secondary metabolism (phenolic compounds, tannins, flavonoids, and anthocyanins contents), sugar metabolism (Sucrose Phosphate Synthase, Sucrose Synthase…) and DNA degradation (comet assay). Using a preliminary set of biomarkers that were totally validated and calibrated, we aimed to evaluate the metal-induced stress on miscanthus plants grown on Metaleurop Nord contaminated soils.

For this sake, the same *ex situ* experiment established to investigate the impacts of the plant on restoring the soil functionality was used (C1). The impact of the soil gradient metal contamination on the plant health after the first growing cycle was investigated by monitoring:

- Cd, Pb and Zn accumulation into leaves,
- plant growth parameters,
- photosynthetic pigment and secondary metabolite contents in the leaves,
- activities of the antioxidative enzymes in the leaves.

Publication

Al Souki, K. S., Liné, C., Gruyer, N., Dorais, M., Douay, F., Pourrut, B., Submitted to Journal of Hazardous Materials. Response of three *Miscanthus x giganteus* cultivars to metal stress. Part 1: Metal accumulation and plant health

D.1- Response of three *Miscanthus x giganteus* cultivars to metal stress. Part 1: Metal accumulation and plant health

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Abstract: *Miscanthus x giganteus* is considered a suitable candidate for phytostabilizing vast territories contaminated by metals. However, information on their tolerance to elevated metal concentrations is scarce. To examine plant responses to stressful metal conditions, an *ex situ* pot experiment was launched using three different miscanthus cultivars (named B, U, and A) grown in soils with a gradient of Cd, Pb, and Zn contamination. Control plants were also cultivated on an uncontaminated soil. The results showed that the number of tillers per plant and diameter were negatively affected by soil contamination, as was the leaf photosynthetic pigment (chlorophyll a and b, carotenoids). On the other hand, phenolic compound, flavonoid, tannin, and anthocyanin contents in addition to the antioxidative enzymatic activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase increased in the plants grown on contaminated soils, unlike the results obtained in the control plants. Altogether, these data demonstrate that miscanthus is impacted by metal contamination but tolerates high levels of soil contamination well. These results may help understand miscanthus strategy allowing it to tolerate high contamination levels and its corresponding efficiency in phytoremediation.

Keywords: *Miscanthus x giganteus*, metal contamination, photosynthetic pigments, phenolic compounds, antioxidative enzymes

D.1.1- Introduction

Anthropogenic activities (mining, industrial activities, combustion of fossil fuels, waste disposal, agriculture, etc.) are the main sources of metal enrichment in soils [1, 2]. This pollution could represent a critical environmental problem because of its diverse toxic effects on human health, as well as on plants, animals, and microorganisms.

Remediating polluted areas is considered a great challenge mainly when the corresponding pollutants are miscellaneous, persistent, and widespread. Conventional physicochemical techniques are expensive, environmentally unfriendly, and unsuitable in large areas. Therefore, an interest of *in situ,* environmentally friendly, and cost-effective biotechnologies that are capable of enhancing the ecological quality of the polluted area has arisen [3, 4].

Phytoremediation comprises numerous techniques that use plants to reclaim polluted areas by reducing pollutant diffusion and hazards or rendering them harmless [3, 5]. Within these techniques comes *in situ* phytostabilization, a process whereby metal-tolerant plants are established to accumulate metals in root tissues and/or stabilize metal in the rhizosphere [4]. As a result, metal translocation to aerial organs, mobility, leaching, bioavailability, and land erosion possibilities are reduced [1, 3].

Among the several plants used for phytoremediation, the promising bio-energy crop *Miscanthus x giganteus* has been increasingly chosen, due to its capacity to diminish human and environmental risks because it accumulates metals more in the roots, limiting transfer of these metals to the shoots and thus reducing their potential mobility and bioavailability [5]. Moreover, the plant has shown potential to stimulate PAH degradation [6, 7], which can be useful in managing multi-contaminated sites. These capacities combined with a high biomass yield and a remarkable adaptability to different environments make this C4 perennial, lignocellulosic, rhizomatous, and noninvasive grass a great candidate for phytomanagement of various contaminated areas.

In northern France, around the former lead smelter Metaleurop Nord, the miscanthus plants grown on highly metal-contaminated agricultural plots exhibited good development and low metal accumulation in its aboveground parts [8, 9]. Pelfrêne et al. [10] displayed the substantial positive impacts of miscanthus culture on metal redistribution in soils by decreasing their proportions in the exchangeable fractions of soils, and thus decreasing their corresponding oral accessibility to humans. Finally, Al Souki et al. [11] highlighted the positive impact of *Miscanthus x giganteus* in restoring metal-contaminated soil functionality via enhancing the soil's biological activities (basal respiration, fluorescein diacetate hydrolytic activity, acid phosphatases, etc.), in addition to improving soil microbial biomass carbon as well as several soil physicochemical parameters.

However, investigations regarding the pollutant's effects on miscanthus and its ability to tolerate the resulting stress are scarce, and papers dealing with the topic are rare. These include Arduini et al. [12], who investigated the response of the *Miscanthus x giganteus* to increasing Cd concentrations in hydroponic solutions, Zhang et al. [13], who studied the impact of Cd-spiked soils on the *Miscanthus sacchariflorus* in a pot experiment, and finally Guo et al. [14], who launched an experiment to determine the impact of increasing Cd concentrations on three different miscanthus species (*M. sinensis, M. floridulus*, *and M. sacchariflorus*) in hydroponic cultures.

The main objective of the current study was to determine the effects of cultivating *Miscanthus x giganteus* in the polluted soils surrounding the former Pb smelter Metaleurop Nord, with a gradient concentration of Cd, Pb, and Zn. For this purpose, we studied the impacts on three different miscanthus cultivars via: i) the plant growth parameters and the concentrations of Cd, Pb, and Zn in the leaves, ii) the photosynthetic pigment (chlorophyll a, chlorophyll b, and carotenoids) and secondary metabolite contents (phenolic compounds, tannins, flavonoids, and anthocyanins), and iii) the activities of the antioxidant enzymes in the leaves (superoxide dismutase [SOD], ascorbate peroxidase [APX], and glutathione reductase [GR]).

D.1.2- Materials and Methods

D.1.2.1- Soil and plant origins and preparations

The area surrounding the former lead smelter Metaleurop Nord is heavily contaminated by Cd, Pb, and Zn [15]. Soil samples (plowed horizon, 0–25 cm) were collected from agricultural plots called M200, M500, M750, and M900, corresponding to their approximate Pb concentrations (in mg kg^{-1} of soil), which decrease as the distance from the smelter increases [16, 17]. The M200 (50°24’52”N, 3°01’51”E, Courcelles-les-Lens) and M500 (50°25’49”N, 3°02’13”E, Evin-Malmaison) plots are located 1.8 km southeast and 1.4 km northeast of the former smelter (50°25’42”N 3°00’55”E, Noyelles-Godault), respectively, whereas the third plot where M750 and M900 soil samples were collected approximately 1 km away is located at Evin-Malmaison (50°26'15.0"N 3°01'05.7"E). In addition, to have a clear idea of the impact of contamination, another uncontaminated soil sample was also collected from an agricultural field established 75 km from the smelter (50°20’46”N 2°12’15”E), used as the control plot and named MC. These soil samples were homogenized, dried, and sieved through a 10-mm mesh.

Three different cultivars of *M. x giganteus*, referred to as B, U, and A (originating from England, the USA, and Austria, respectively) were used throughout the experiment. Their rhizomes were cut into small pieces, one fragment (5–7 cm long, 2–3 buds) of which was grown in polyethylene pots (9 × 9 × 9 cm) filled with potting compost, until obtaining miscanthus plantlets (20–25 cm in height). The planted compost was kept wet by constant watering.

D.1.2.2- Experimental design

For a period of 18 months (May 2014 to October 2015), an *ex situ* experiment was conducted in an area away from roads on the Lille 1 University campus (50.6090° N, 3.1381° E). About 100 kg of each of the above-mentioned homogenized soils (MC, M200, M500, M750, and M900) were equally distributed in five pots (light grey in color to avoid temperature elevation). The *M. x giganteus* plantlets (B, U, and A) were displaced from the small pots to the 20-kg soil pots (two plantlets in each pot).

A total of 75 planted pots were used (three different miscanthus cultivars × 5 soils × 5 replicates). The pots were disposed over wooden rafters to avoid contact with the ground of the experimental area. The pots were randomly distributed over the location to avoid point and borderline effects. In addition to rain water, soil humidity was maintained by regular watering during the entire experiment. Weeds were manually removed and left on the surface of the soils to avoid metal exportation

D.1.2.3- Soil sampling and analysis

Once the experimental site was set up, fresh composite soil samples were collected from the pots using an auger, dried at 40°C for 24 h, ground and sieved to 2 mm and 250 µm. All the soil analyses were conducted following standardized protocols.

Particle-size distribution was determined by sedimentation and sieving after destruction of organic matter by H_2O_2 according to the French NFX 31-107 standard. pH (H_2O) was measured after stirring a mixture of soil and deionized water (1:5, v/v) according to the ISO 10390 standard.

For the determination of Cd, Pb, and Zn pseudototal concentrations, soil acid digestion was performed according to Waterlot et al. [18] using a digestion plate (HotBlock™ Environmental Express, USA). The aqua regia solution (HCl + HNO_3, 3:1, 6 mL) was added and the aliquot heated at 120°C for 120 min. The quality control of the extraction and analysis was provided by the introduction of two internal reference samples and a certified soil reference (CRM 141, IRMM, Belgium). The Cd, Pb, and Zn concentrations in the extracts were determined by atomic absorption spectrophotometry (AA-6800, Shimadzu, Kyoto, Japan).

D.1.2.4- Plant sampling and analysis

D.1.2.4.1- Plant growth parameter measurements, leaf sampling and preparation

By the end of the first growing season (October 2014; 5 months of cultivation), plant growth parameters such stem height and diameter were measured. The number of tillers per plant was determined as well. The total aerial biomass was not harvested and measured, in order not to prevent nutrient translocation towards the rhizomes, and thus not compromise the 2nd year of the experiment.

To evaluate plant health, three leaves (4^{th}, 5^{th}, and 6^{th} foliar stage) were harvested from each of the 75 pots and immediately flash frozen in liquid nitrogen. Samples were then stored at –80°C until biomarker analysis.

The other leaves were also harvested to measure metal concentrations, put into plastic bags, and kept in a coolbox. In the laboratory, leaves were washed three times with osmosed water to remove dust particles. They were then oven-dried at 40°C for 48 h and afterwards ground into fine powder using a knife mill (GM200, Retsch) before metal analysis.

D.1.2.4.2- Metal concentrations in leaves

To determine the miscanthus leaf metal concentrations, 300 mg of ground leaf powder was acid digested with nitric acid (HNO_3, 70%) and heated at 95°C for 75 min, followed by hydrogen peroxide addition (H_2O_2, 30%) and another 180 min heating prior to adding osmosed water. The Cd, Pb, and Zn concentrations in extracts were determined by atomic absorption spectrophotometry (AA-6800, Shimadzu). Quality control for chemical extraction and digestion was performed by including blanks, internal, and certified (Polish Virginia tobacco leaves, INCTPVTL-6, Poland) reference materials [19].

D.1.2.4.3- Antioxidative enzymatic activity assays

Antioxidative enzymatic activity assays were evaluated spectrophotometrically according to Liné et al. [20] using a plate reader (Thermo Scientific Multiskan™ GO). Briefly, five foliar discs (0.5 cm in diameter) per plant sample were collected from frozen leaves using a manual punch. Then they were put into in 96-deepwell plates (2 mL) with one 4-mm-diameter glass bead. Samples were then ground under frozen conditions using a Mixer Mill MM 400 (Retsch), twice for 1.5 min at 30 Hz, after addition of 1 mL of ice-cold Tris extraction buffer pH 7.0 containing 0.01 M EDTA, 0.4 M PVP, 0.05 ascorbate, 11.44 mM β-mercaptoethanol, and protease cocktail inhibitor. Samples were homogenated for 2 min at 15 Hz with the MM400 grinder. Plates were then centrifuged at 5000 *g* for 15 min at 4°C. Supernatants were collected and protein content was determined according to Bradford [21], using bovine serum albumin (BSA, Sigma) as standard.

The total SOD activity was determined by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) according to the method reported by Giannopolitis and Ries [22]. The reaction mixture contained 0.47 mM NBT (Sigma), 3.85 µM riboflavin (Sigma), 19.23 mM methionine (Sigma), 36.54 mM phosphate buffer (pH 7.8), and 20 µL enzyme extract. The test tubes containing the mixture were placed 30 cm below a light source (30 W fluorescent lamps). The reaction was started by switching on the light and was allowed to run for 10 min. The reaction was stopped by switching off the light with the absorbance at 560 nm. An unirradiated reaction mixture that did not develop color served as the control, and its absorbance was subtracted from that of the test tube. One unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of NBT reduction.

APX activity was evaluated by the decrease of absorbance at 290 nm due to ascorbate oxidation [23]. The reaction mixture contained 434 mM phosphate buffer (pH 7.0), 3.77 mM H_2O_2, 0.56 mM ascorbic acid, and 10 µL enzyme extract. One enzyme unit was defined as 1 µmol of ascorbic acid oxidized per min at 290 nm using an ascorbate standard curve. The enzyme activity was expressed as µmoles of ascorbate oxidized min^{-1} mg^{-1} protein.

GR was assayed as the decrease in absorbance at 340 nm caused by the oxidation of NADPH [24]. This assay is based on the reduction of oxidized glutathione (GSSG) by NADPH in the presence of GR. The reaction mixture contained 0.1 M Tris buffer (pH 7.5), 1 mM GSSG (Sigma), 0.1 mM NADPH (Sigma), and 20 µL enzyme extract. The amount of NADPH oxidized was calculated from the extinction coefficient of 3.732×10^{-3} mL $nmol^{-1}$ of NADPH. The enzyme activity was expressed as nmol of NAPDH oxidized min^{-1} mg^{-1} protein.

D.1.2.4.4- Secondary metabolism molecule and photosynthetic pigment quantification

Photosynthetic pigments, phenolic compounds, tannins, flavonoids, and anthocyanins were evaluated spectrophotometrically according to Liné et al. [20] using a plate reader (Thermo Scientific Multiskan™ GO). Briefly, two foliar discs (0.5 cm in diameter) per plant sample were collected from frozen leaves using a manual punch and weighed. Then they were put into a 96-deepwell plate (2 mL) with one 4-mm-diameter glass bead. Samples were then ground under frozen conditions using a Mixer Mill MM 400 (Retsch) twice for 1.5 min at 30 Hz. After the addition of 1.5 mL of ice-cold 95% methanol in each well, samples were homogenated for 2 min at 15 Hz with the MM400 grinder. Plates were left in the dark for 24 h and 48 h of incubation.

After 24 h of incubation, leaf extracts were homogenated for 2 min at 15 Hz with the Mixer Mill MM 400. A total of 100 µL was collected for photosynthetic pigment analysis. The absorbance was measured at 470, 652, and 666 nm. Concentrations of total carotenoids, chlorophyll a and b and were calculated according to the extinction coefficients and equations reported by Lichtenthaler [25]. Finally, data were averaged and the mean concentrations obtained were expressed as mg g^{-1} FW of leaf.

After 48 h of incubation, plates were centrifuged at 5000 *g* for 5 min, prior to secondary metabolism molecule extraction. The total phenolic compound was determined based on Folin Ciocalteu assay. Briefly, the 200-µL reaction mixture contained 20 µL of supernatant, 40 µL of Folin reagents (10% v/v), and 0.098 mM of Na_2CO_3. The mixture was allowed to stand 2 h at room temperature for color development and then absorbance was measured at 510 nm. Concentrations of phenolic compounds were calculated using a standard curve of gallic acid. The results were expressed as mM of gallic acid equivalent (GE) per gram of fresh weight of leaf. The flavonoid content was determined using the aluminum chloride method using catechin as the reference compound. Briefly, the reaction mixture contained 25 µL of methanolic extract, 0.00724 mM $NaNO_2$, 0.01125 mM $AlCl_3$, and 0.05 mM NaOH. The mixture was homogenated for 1 min and absorbance was measured at 595 nm. Flavonoid concentrations were calculated using a standard curve of catechin. The results were expressed as mg catechin equivalent (CE) per gram of fresh weight of leaf. For tannins, the reaction mixture contained 50 µL of methanolic extract and 100 µL of vanillin solution 1%. The mixture was let in the dark for 15 min and absorbance was measured at 500 nm. The tannin concentration was calculated using a standard curve of catechin. The results were

expressed as mg L^{-1} catechin equivalent (CE) per gram of fresh weight of leaf. Anthocyanins were measured using the differential pH method based on the property of anthocyanin pigments to change color with pH. Two dilutions of the same sample were prepared, the first one in potassium chloride buffer (0.2 M, pH 1.0) and the second in sodium acetate buffer (0.4 M, pH 4.5). After equilibration at room temperature for 15 min, the absorbance was read at 510 and 700 nm. The results were expressed as mg cyaniding 3-glucoside equivalent per gram of fresh weight of leaf.

D.1.2.5- Statistical analysis

Analysis of variance was done to compare modalities. The Fisher test was considered for significance ($p \leq 0.05$). If statistically significant differences were found, the Tukey HSD test was used for pair-wise comparisons. All statistical analyses were performed using XLSTAT software.

D.1.3- Results

D.1.3.1- Granulometry, pH, and pseudototal metal concentrations of soils

The different soils used in the experiment were analyzed for their corresponding particle size distribution, pH, and pseudototal metal concentrations. The results presented in the Table 1 demonstrate that the silt portion was the most dominant in all the soils studied ranging from 49.7% in the M500 soil to 69.5% in the MC soil.

	MC	M200	M500	M750	M900
Clay (%)	20.8	17.8	30.6	19.2	16.4
Silt (%)	69.5	57.3	49.7	56	59.2
Sand (%)	9.7	24.9	19.7	24.8	24.4
pH	6.4	7.1	7.6	7.3	7.1
Cd (mg kg^{-1})	0.3 ± 0.0	3.8 ± 0.2	9.0 ± 0.2	13.5 ± 0.3	16.0 ± 0.3
Pb (mg kg^{-1})	37.3 ± 1.3	260.3 ± 2.0	528.6 ± 5.3	747.1 ± 16.9	898.6 ± 16.3
Zn (mg kg^{-1})	54.6 ± 3.1	388.0 ± 14.8	537.0 ± 10.9	906.0 ± 16.8	1116.0 ± 1.7

Table 1: Particle size distribution, pH and metal (Cd, Pb and Zn) concentrations of the studied soils (mean ± standard deviation)

The M500 soil was more clayey (30.6%) in comparison to the other soils. The pH values in the soils investigated fluctuated between slightly acidic in the MC (6.4) and slightly basic in the M500 soils (7.6). The degree of metal contamination in the soils studied validated the previous data, ranging from 20 to 50 times more than the regional agricultural background values (0.42, 38, and 74 mg kg-1, corresponding to Cd, Pb, and Zn, respectively). Finally, the metal concentrations in the MC soil coincided with the regional background values.

D.1.3.2- Plant growth parameters and metal concentration in leaves

Table 2 presents the accumulation of Cd, Pb, and Zn in the miscanthus leaves as well as plant stem diameter and the number of tillers at the end of the first growing season. Tiller heights were also measured in each pot. However, during the first growing season, the emergence of tillers was strongly heterogeneous among pots with the same metal concentrations, resulting in very high standard deviation values. Therefore, the corresponding results are not displayed in Table 2.

The metal concentrations in leaves increased according to the concentrations in the soils where the plants were cultivated. No differences in metal accumulation were observed between the three miscanthus cultivars. In the MC soil, the Cd concentration in the leaves of the three cultivars was below the limit of detection (0.4 mg kg^{-1}). The highest Cd leaf concentrations (2.2–2.3 mg kg^{-1}) were detected in the plants cultivated in the most polluted soil (M900). The same was noted concerning Zn, whose corresponding leaf concentrations increased from 32.2, 21.6, and 39.0 mg kg^{-1} (corresponding to the B, U, and A cultivars, respectively) in the MC soil to 74.0, 68.8, and 71.2 mg kg^{-1} in the M900 soil. However, the Pb concentrations in the leaves were always below the limits of detection (4.3 mg kg^{-1}) in all the samples investigated.

Significant differences were also observed in plant growth (Table 2). The number of tillers was the highest in plants cultivated in the MC soil (8, 14, and 8 tillers per plant, corresponding to the B, U, and A cultivars, respectively). Despite the decrease in the number of tillers of the B and U cultivars grown in the contaminated soils (approximately four tillers per pot), the results did not display significant differences between the modalities. However, the U cultivar exhibited significant differences between the uncontaminated and contaminated soils, where the number of tillers decreased from 14 tillers in the former to five tillers in the M900 soil, a 62.9% decrease.

The stems of the B and A cultivars planted in the uncontaminated MC soil were thicker than those present in the contaminated pots (8.2 and 7.4 mm corresponding to the stem diameter of the B and A cultivars, respectively, planted in the MC soil). However, significant differences were noted only in the B plants cultivated in the contaminated soils. No significant differences were present for the U cultivar, and the stems displayed approximately the same thickness in the plants obtained in the contaminated and uncontaminated soils (6.1 mm in the MC soil and 6.2 mm in the M900 soil).

		Cd (mg kg^{-1})	Pb (mg kg^{-1})	Zn (mg kg^{-1})	Number of tillers	diameter (mm)
B	MC	< LD*	< LD*	32.2 + 2.8 fg	8.0 + 1.0 b	8.2 + 0.6 a
	M200	0.5 + 0.0 e	< LD*	60.5 + 2.6 bcd	4.0 + 1.6 bc	7.1 + 0.8 ab
	M500	1.0 + 0.1 d	< LD*	59.7 + 7.1 cd	5.0 + 1.6 bc	5.1 + 0.7 c
	M750	1.8 + 0.3 c	< LD*	72.9 + 8.5 abc	4.0 + 0.9 bc	5.3 + 0.6 bc
	M900	2.2 + 0.1 ab	< LD*	74.0 + 8.9 a	4.0 + 0.4 bc	4.9 + 0.8 c
U	MC	< LD*	< LD*	21.6 + 2.1 g	14.0 + 1.6 a	6.1 + 0.4 abc
	M200	0.5 + 0.1 e	< LD*	54.7 + 5.7 d	7.0 + 1.2 b	6.2 + 0.9 abc
	M500	0.9 + 0.1 d	< LD*	52.1 + 7.2 de	8.0 + 1.3 b	5.7 + 0.6 bc
	M750	2.1 + 0.3 abc	< LD*	64.8 + 5.5 abcd	5.0 + 0.8 bc	6.1 + 1.0 abc
	M900	2.3 + 0.2 a	< LD*	68.8 + 5.7 abc	5.0 + 1.6 bc	6.2 + 0.3 abc
A	MC	< LD*	< LD*	39.0 + 1.7 ef	8.0 + 1.7 b	7.4 + 1.2 ab
	M200	0.5 + 0.0 e	< LD*	60.0 + 5.8 cd	4.0 + 1.1 bc	5.9 + 0.7 abc
	M500	1.1 + 0.1 d	< LD*	62.6 + 3.3 abcd	4.0 + 1.0 bc	7.1 + 0.3 ab
	M750	1.9 + 0.1 bc	< LD*	71.2 + 5.7 abc	4.0 + 1.0 bc	5.7 + 0.5 bc
	M900	2.2 + 0.1 ab	< LD*	73.5 + 6.6 ab	4.0 + 0.7 bc	5.2 + 0.7 bc

Table 2: Leaf metal concentrations, number of tillers per pot, and stem diameter of the cultivars (B, U, and A) in soils with a gradient metal concentration (MC, M200, M500, M750, and M900) at the end of the first growing period. Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$).
*: Limit of detection (Cd: 0.4 mg kg^{-1}, Pb: 4.3 mg kg^{-1})

D.1.3.3- Antioxidative enzymatic activity assays

The activities of the different antioxidative enzymes were significantly boosted in the leaves of the miscanthus cultivars in response to the augmentation of the soil contamination.

The lowest SOD activities (Figure 1) were observed in the plant leaves cultivated in the MC soil (69.7, 67.2, and 75.5 U mg^{-1} FW corresponding to the B, U, and A cultivars, respectively) and their highest values in those planted in the M900 soil for the B cultivar (158.8 U mg^{-1} FW) and the M750 soil for the U and A cultivars (153.0 and 160.2 mg^{-1} FW, respectively). Compared to the control leaves, the increase ranged from 96.5, 87.7, and 76.8% in the leaves of the B, U, and A cultivars, respectively, cultivated in the M200 soil, to 127.7% in the leaves from the B cultivar planted in the M900 soil and 127.5 and 112.1% in the U and A cultivars of the M750 soil. Significant differences were recorded between the plants cultivated in the contaminated and uncontaminated soils. However, they were not detected among those obtained in the contaminated pots.

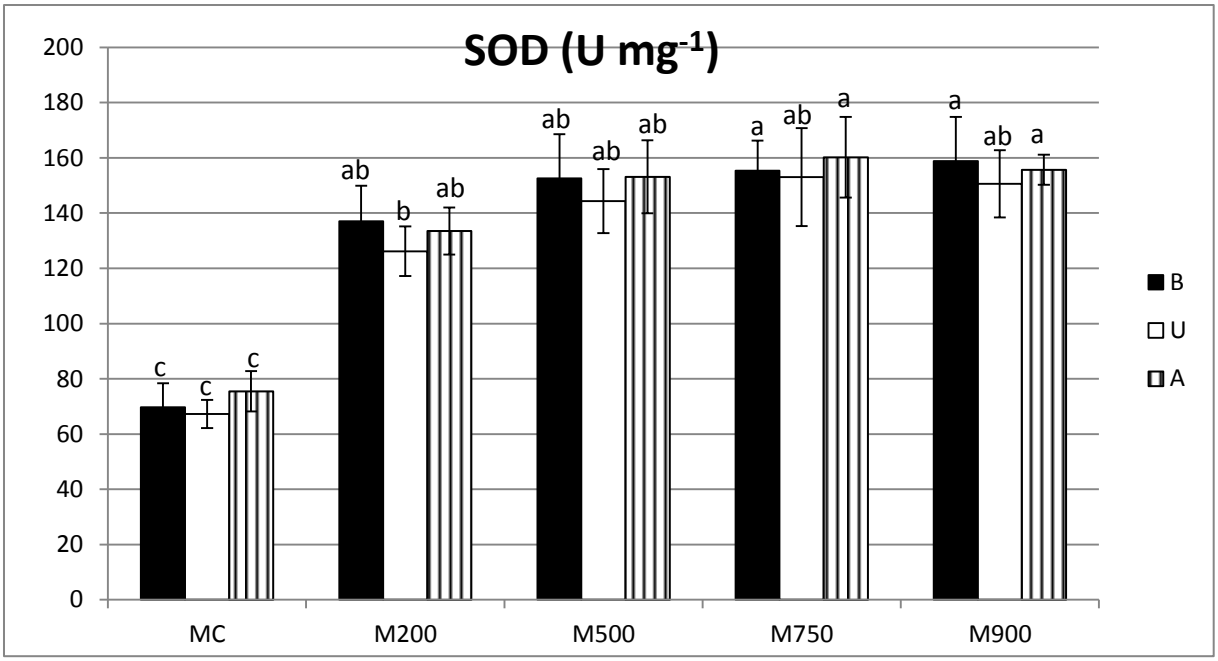

Figure 1: SOD activities in the leaves of three different miscanthus cultivars (B, U, and A) cultivated in soils with gradient concentrations of metals (MC, M200, M500, M750, and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$)

Figure 2 shows that the APXs also exhibited their lowest activity values in the leaves of the plants cultivated in the MC soil (0.1 U mg^{-1} FW corresponding to the B, U, and A cultivars, respectively) and the highest values in those planted in the M750 soil (0.6 U mg^{-1} FW for the B, U, and A cultivars). The APX activities in the leaves of the plants cultivated in the contaminated soils increased from 212.2, 237.9, and 279.0% in the B, U, and A cultivars, respectively, cultivated in the M200 soil, to 376.4, 456.9, and 402.9% in those in the B, U, and A cultivars, respectively, cultivated in the M750 soil, in comparison with the APX activities in the control plants. Significant differences were found between the cultivars of the uncontaminated and contaminated soils. However, there were no significant differences in the results of APX activities in the three cultivars planted in the M500 soil.

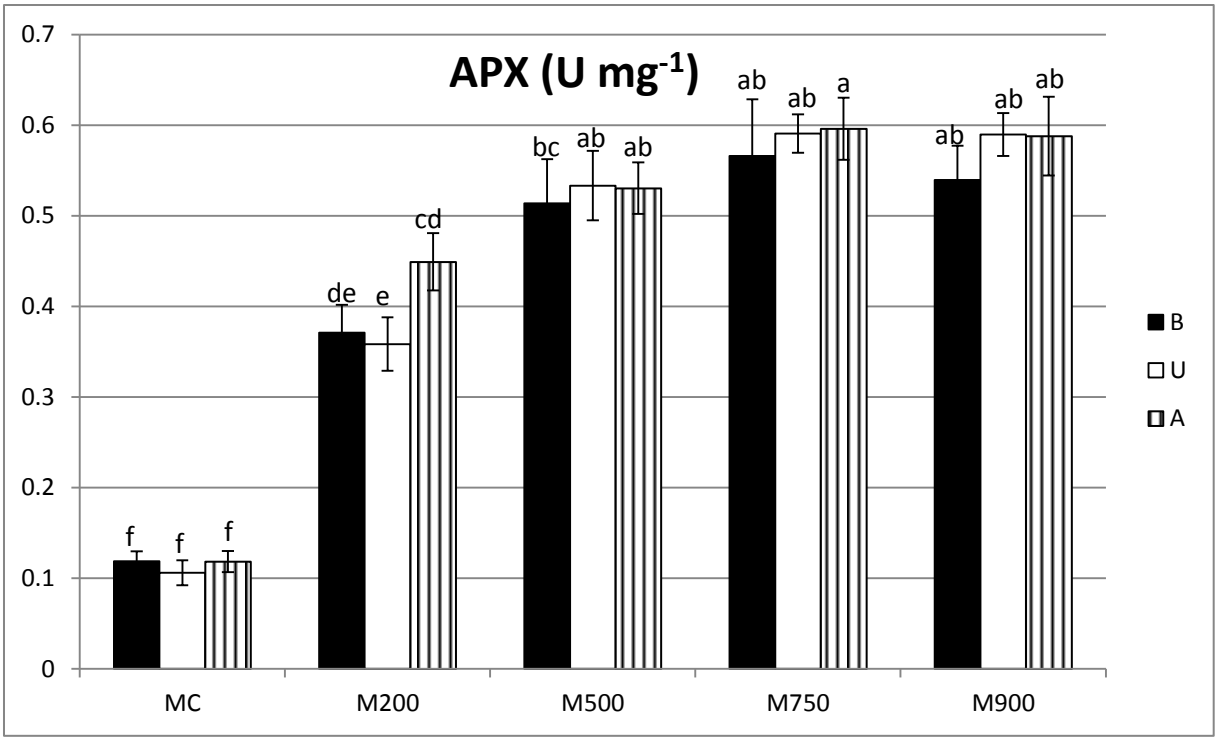

Fig. 2: APX activities in the leaves of three different miscanthus cultivars (B, U, and A) cultivated in soils with gradient concentrations of metals (MC, M200, M500, M750, and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$)

The lowest GR activities (Figure 3) were demonstrated in the leaves of the plants cultivated in the MC soil (0.2, 0.2, and 0.3 U mg^{-1} FW corresponding to the B, U, and A cultivars, respectively) and the most elevated levels were present in those planted in the M750 soil for the B and A cultivars (0.6 and 0.7 U mg^{-1} FW, respectively) and in the M500 soil for the U cultivar (0.7 mg^{-1} FW). The activities in the leaves of the plants cultivated in the contaminated soils increased from 197.7 and 111.8% in the U and A plants, respectively, cultivated in the M200 soil and 181.2% in the B cultivar plant of the M500 soil to 201.9 and 123.9% in those of the B and A cultivars, respectively, cultivated in the M750 soil and 207.1% in the U cultivar of the M500 soil. Significant differences were found between the plants cultivated in the uncontaminated and contaminated soils, but not in those present in the contaminated soils.

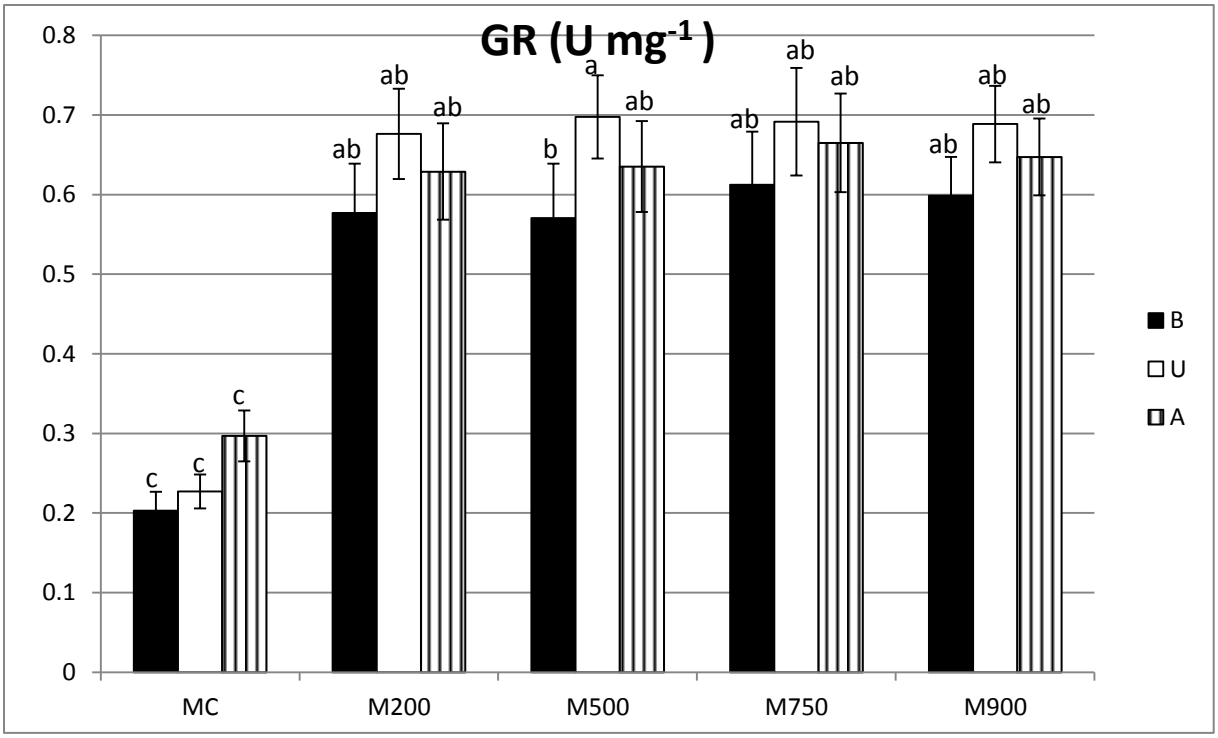

Fig. 3: GR activities in the leaves of three different miscanthus cultivars (B, U, and A) planted in soils with gradient concentrations of metals (MC, M200, M500, M750, and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, n = 5, $p \leq 0.05$)

D.1.3.4- Secondary metabolite quantification

The miscanthus plants cultivated in contaminated soils exhibited increasing phenolic compound, tannin, flavonoid, and anthocyanin concentrations in their leaves in comparison with those grown in the uncontaminated soils (Figures 4–7).

The phenolic compound concentrations in the control plants were 98.6, 92.7, and 96.3 mg gallic acid g^{-1} FW corresponding to the B, U, and A cultivars, respectively, representing the lowest quantities among the modalities (Figure 4). On the other hand, the highest phenolic compound concentrations were recorded in the M900 soil for the B and U cultivars (135.2 and 134.8 mg gallic acid g^{-1} FW, respectively) and in the M500 soil for the A cultivar (134.6 mg gallic acid g^{-1} FW). The minimal augmentation was detected in the B and U plants cultivated in the M500 soil (17.7 and 13.7% respectively) and the A cultivar in the M200 soil (15.9%), whereas the maximum concentration increase was detected in the leaves of the B and U plants cultivated in the M900 soil (37.1 and 45.4%, respectively), and the A plants cultivated in the M500 soil (39.7%). Nevertheless, the significant differences in the corresponding concentrations of the phenolic compounds started to appear in the plants cultivated in the severely contaminated pots (M750 and M900 for the B and U cultivars and M500 for cultivar A).

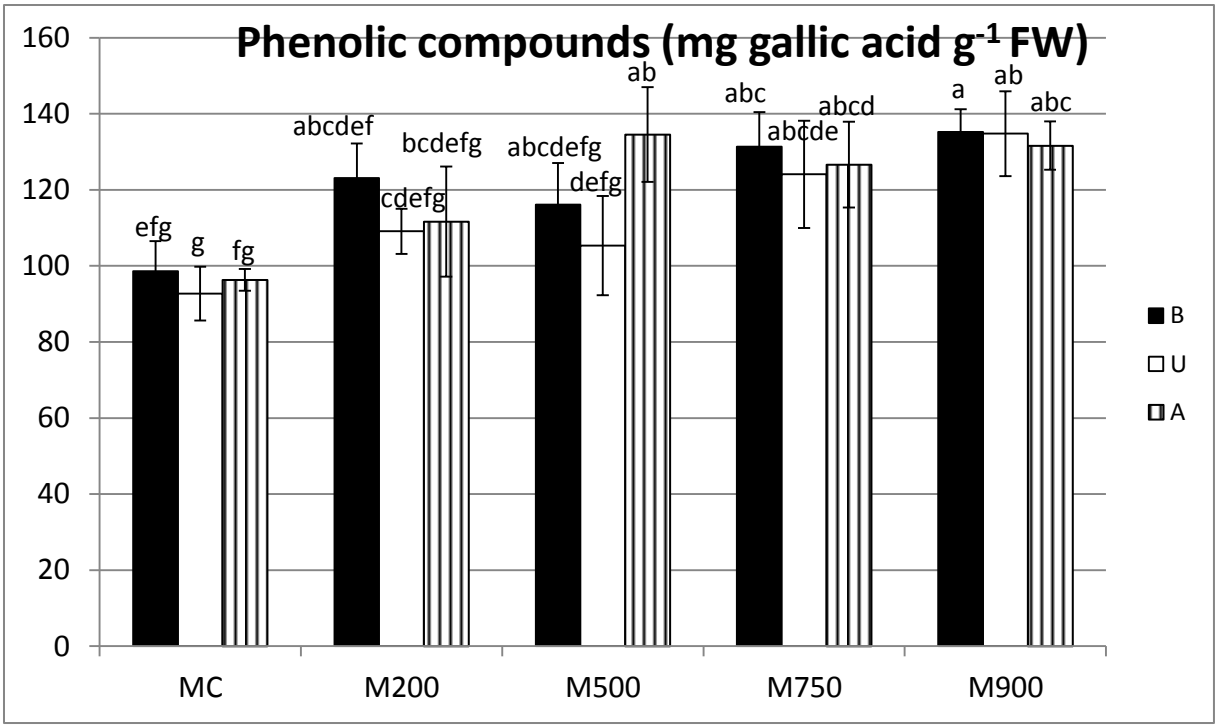

Fig. 4: Phenolic compound concentrations in the leaves of three different miscanthus cultivars (B, U, and A) cultivated in soils with gradient metal concentration (MC, M200, M500, M750, and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$)

The lowest tannin concentrations (Figure 5) were recorded in the leaves of the miscanthus sampled from the MC soil (2018.7, 2144.0, and 2168.1 mg L^{-1} catechin g^{-1} FW corresponding to the B, U, and A cultivars, respectively), whereas the highest values were observed in the U and A plants of the M900 soil (3023.6 and 2970.5 mg L^{-1} catechin g^{-1} FW, respectively) and in the M750 soil for the B plants (3150.2 mg L^{-1} catechin g^{-1} FW). The corresponding concentrations were increasing by 29.7, 22.8, and 12.9% in the B, U, and A cultivars, respectively, grown in the M200 soil, reaching 56.1% in the B plants grown in M750 soils and 41.0 and 37.0% in U and A cultivars, respectively, cultivated in the M900 soil. Significant differences in the results in the different modalities were detected starting from the M500 soil.

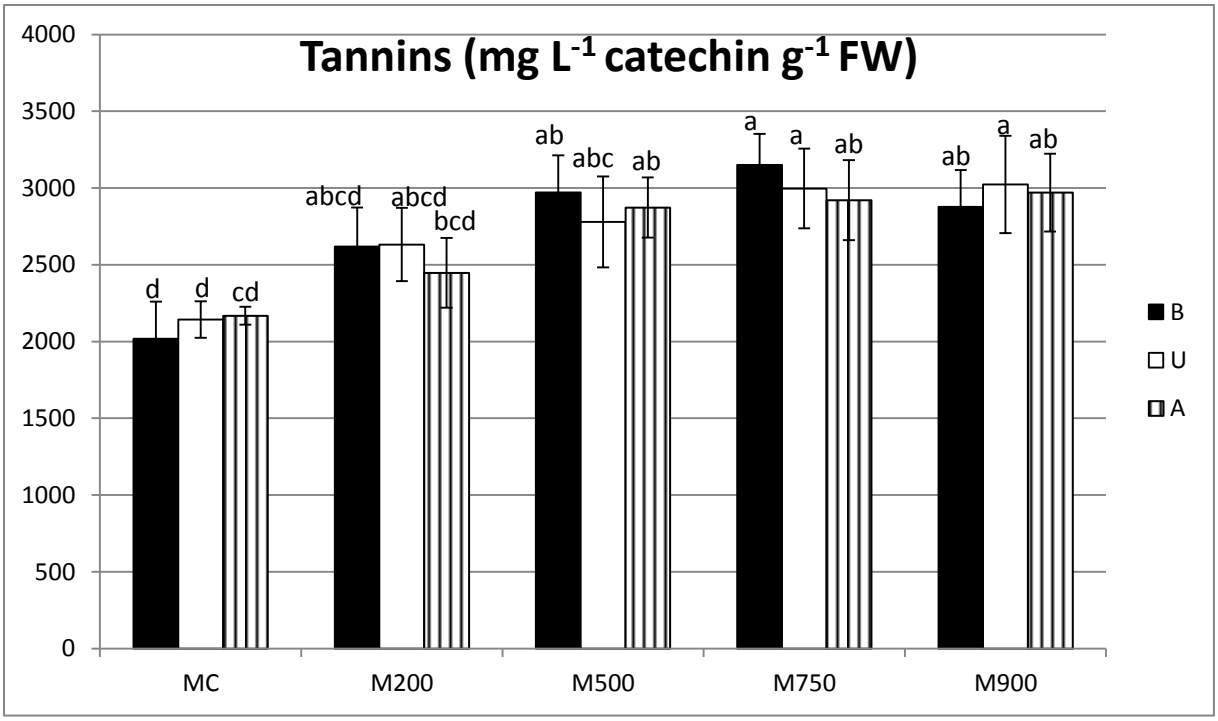

Fig. 5: Tannin concentrations in the leaves of three different miscanthus cultivars (B, U, and A) cultivated in soils with gradient metal concentration (MC, M200, M500, M750, and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$)

The flavonoid results displayed in Figure 6 show that the lowest concentrations were detected in the plants cultivated in the MC soil (2347.5, 2235.5, and 2044.8 mg catechin L^{-1} g^{-1} FW in the B, U, and A cultivars, respectively), whereas the highest concentrations were detected in the M900 soil for the B cultivar, M500 for the U cultivar, and M200 for the A cultivar (3631.8, 3831.9, and 3997.1 mg catechin L^{-1} g^{-1} FW in the B, U, and A cultivars, respectively). The lowest induction increases were detected in the B plants cultivated in the M200 soil (32.1%), the U plants cultivated in the M750 soil (55.1%), and the A plants cultivated in the M900 soil (62.9%), whereas the highest increases were displayed by the B plants of the M900 soil (54.7%), the U plants of the M500 soil (71.4%), and the A plants of the M200 soil (95.5%). However, significant differences were detected between the results displayed in the leaves of the plants sampled from the contaminated and uncontaminated soils, but not among those collected from the contaminated soils, except the B cultivar where the differences started to appear in the M500 soil.

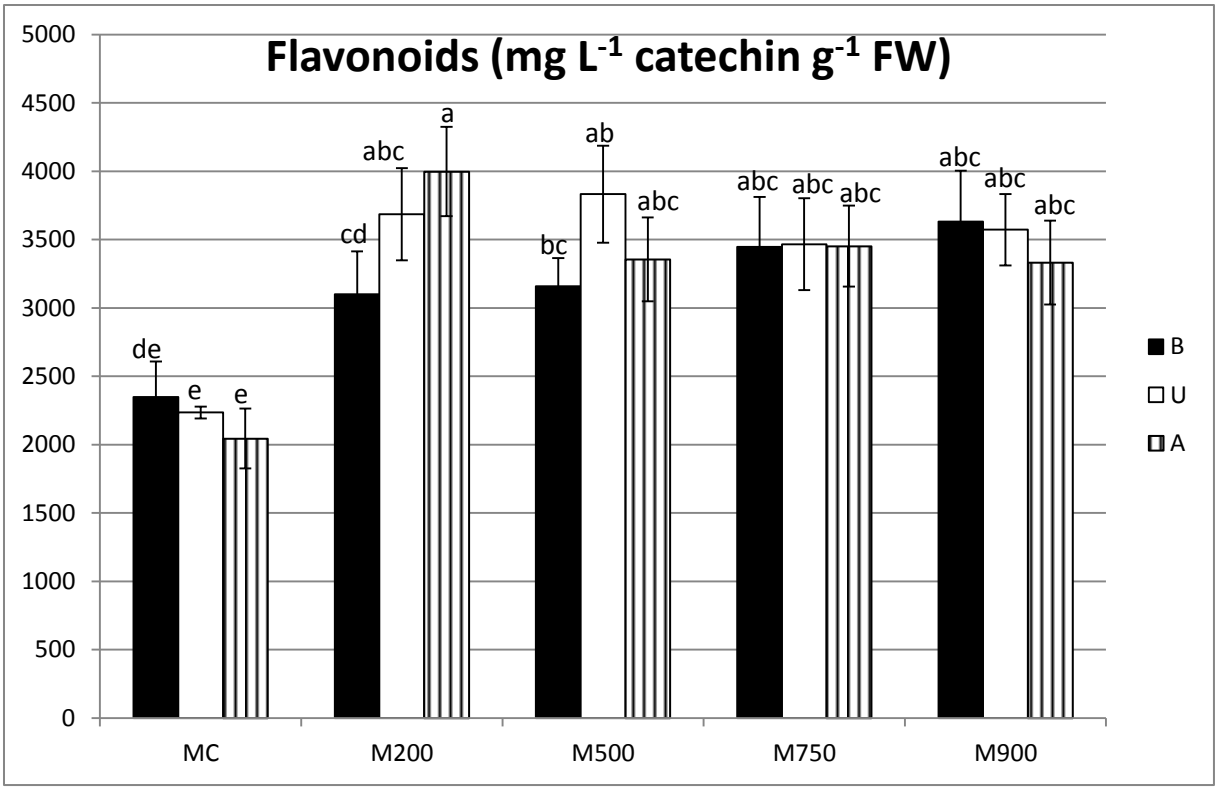

Fig. 6: Flavonoid concentrations in the leaves of three different miscanthus cultivars (B, U, and A) cultivated in soils with gradient metal concentration (MC, M200, M500, M750, and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$)

The anthocyanin concentrations (Figure 7) indicate that the lowest concentrations were measured in the leaves of the plants in the MC soil (0.9, 1.0, and 0.8 mg cyanidin g^{-1} FW corresponding to the B, U, and A cultivars, respectively), whereas the B and A plants in the M900 soil recorded the highest values (2.6 and 3.0 mg cyanidin g^{-1} FW, respectively). The U plants displayed their highest anthocyanin concentrations in the M750 soil (2.4 mg cyanidin g^{-1} FW). Relative to the control plants, the anthocyanin concentrations increased by 134.0 and 129.3% in the B and A cultivars, respectively cultivated in the M200 soil, as well as 114.8% in the U cultivar planted in the M500 soil, and reached 207.3 and 266.1% in the B and A cultivars cultivated in the M900 soil and 154.0% in the U cultivar of the M750 soil. Significant differences were detected concerning the results of the plants cultivated in the uncontaminated and contaminated soils; nevertheless, these differences existed among the cultivars planted in the contaminated soils as well (the B plants cultivated in the M900 soil and the U cultivar grown in M500, M750, and M900 soils).

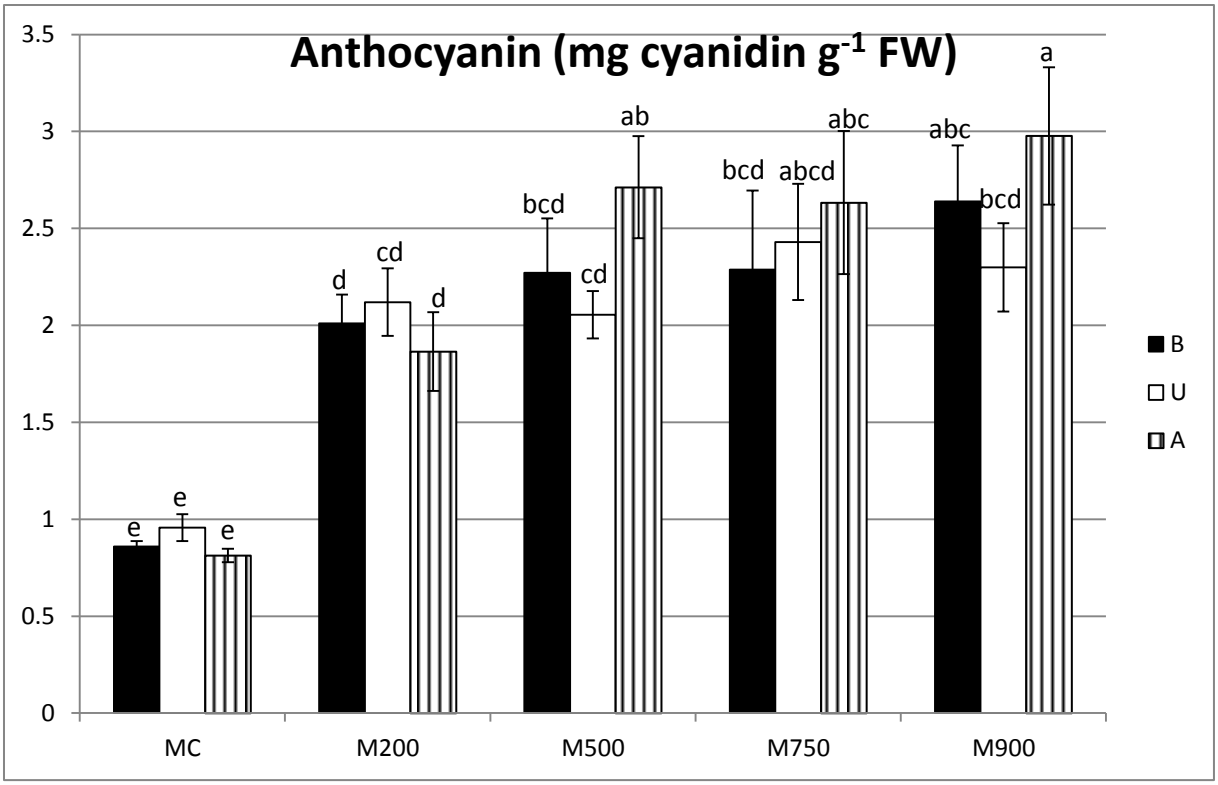

Fig. 7: Anthocyanin concentrations in the leaves of three different miscanthus cultivars (B, U, and A) cultivated in soils with gradient metal concentration (MC, M200, M500, M750, and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$)

D.1.3.5- Photosynthetic pigment quantification

The photosynthetic pigment contents displayed a significant decrease for the three miscanthus cultivar plants grown in contaminated soils compared to control plant cultivars (Figures 8–10).

The chlorophyll a contents in the leaves of the miscanthus plants presented in Figure 8 significantly decreased from 12.2, 12.5, and 12.1 mg g^{-1} FW corresponding to the miscanthus B, U, and A cultivars, respectively, cultivated in the MC soil, to 8.6, 7.1, and 7.3 mg g^{-1} FW in miscanthus cultivated in the M900 soil. These declines varied from 3.4, 27.1, and 15.4% in the leaves of the B, U, and A cultivars, respectively, cultivated in the M200 soil, to 29.1, 43.2 and 39.2% in those cultivated in the M900 soil. Within cultivars and in comparison with their corresponding controls, significant variations started to appear in the B plants cultivated in the M750 soil, the U plants grown in the M200 soil, and the A plants cultivated in the M500 soil.

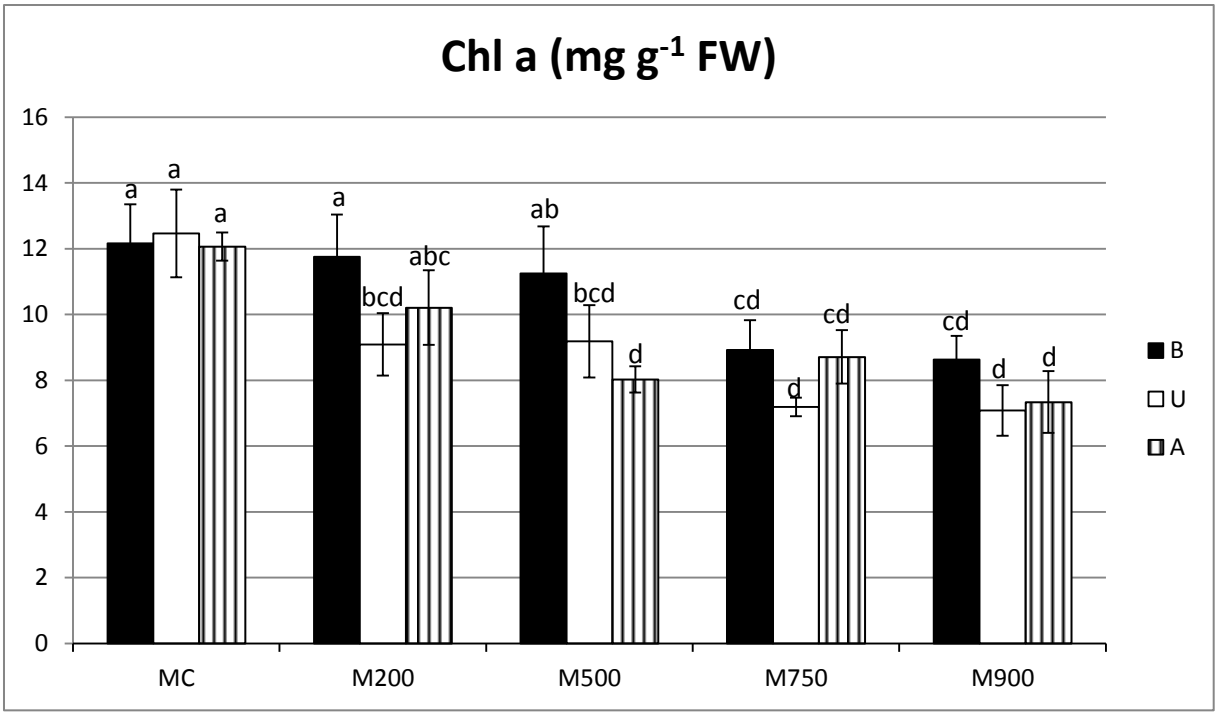

Fig. 8: Chlorophyll a contents in the leaves of three different miscanthus cultivars (B, U, and A) cultivated in soils with gradient metal concentration (MC, M200, M500, M750, and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$)

The highest chlorophyll b concentrations (Figure 9) were measured in the plants cultivated in the MC soil (17.4, 17.4, and 17.0 mg g^{-1} FW corresponding to the B, U, and A cultivars, respectively), whereas the lowest concentrations were observed in the plants cultivated in the M900 soil (13.2, 11.7, and 13.6 mg g^{-1} FW corresponding to the B, U, and A cultivars, respectively). The corresponding decrease in the B cultivar chlorophyll concentrations was between 7.1 and 24.3 % in the plants cultivated in the M500 and M750 soils, respectively. In the U and A cultivars, this value ranged from 9.3 to 11.6% in the plants grown in the M200 soil and 32.4 and 20.0% in the plants grown in the M900 soil. Significant differences started appearing in the plants cultivated in the M750 soil for the three cultivars.

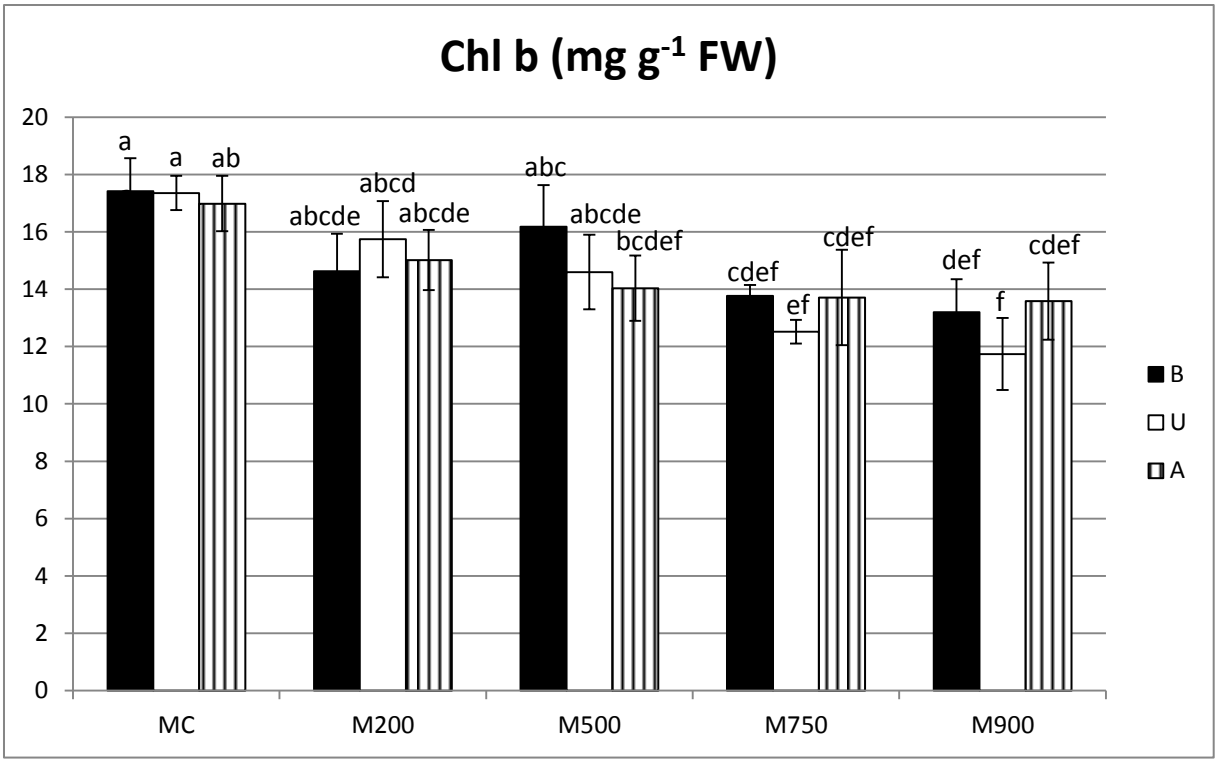

Fig. 9: Chlorophyll b contents in the leaves of three different miscanthus cultivars (B, U, and A) cultivated in soils with gradient metal concentration (MC, M200, M500, M750, and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$)

The highest carotenoid (car) contents were recorded in the leaves of the plants cultivated in the MC soil (15.9, 15.1, and 15.6 mg g^{-1} FW corresponding to the B, U, and A cultivars, respectively), whereas the lowest values were obtained in those of the M900 soil (10.5, 10.0, and 11.0 mg g^{-1} FW corresponding to the B, U, and A cultivars, respectively) (Figure 10). Compared to the car contents in the control leaves, the drop ranged between 5.2% in the leaves of the B cultivars cultivated in the M500 soil, and 17.2 and 4.3% in the leaves of the U and A cultivars, respectively, grown in the M200 soil, to 34.3, 33.8, and 29.1% in those of the B, U, and A cultivars, respectively, cultivated in the M900 soil. Referring to their corresponding controls cultivated in the uncontaminated soil, the car concentrations in the leaves of the B and U cultivars began to display significant differences in the plants cultivated in the M750 soil. The A cultivars displayed significant differences in comparison to the controls in the plants cultivated in the M500 and M900 soils.

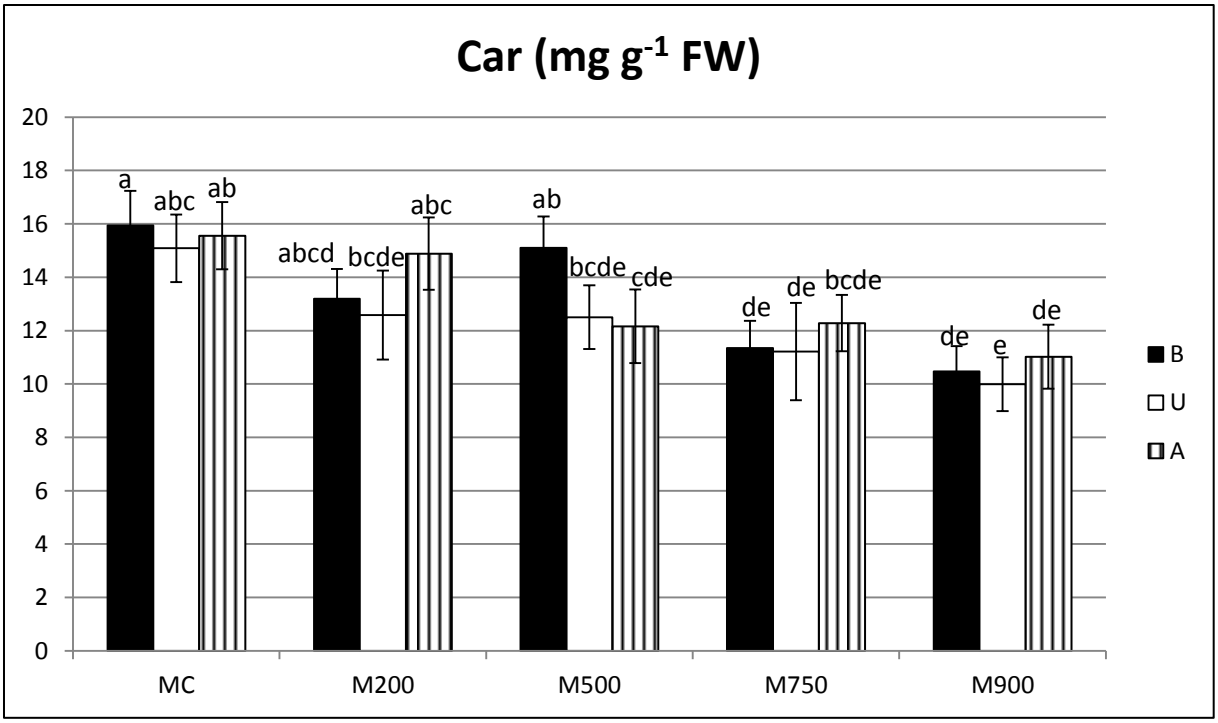

Fig. 10: Carotenoids contents in the leaves of three different miscanthus cultivars (B, U, and A) cultivated in soils with gradient metal concentration (MC, M200, M500, M750, and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$)

D.1.4- Discussion

Miscanthus x giganteus has demonstrated capacities in phytostabilization and management of different kinds of polluted areas, by accumulating the metals mainly in their underground parts and decreasing their corresponding oral bioaccessibility [8-10] as well as contributing to restoring soil functionality and biological activities [11]. However, the distinctive attribute of miscanthus is its ability to thrive and develop well in such degraded areas. Nevertheless, acclimation to the stressful conditions prevailing in these areas demands certain adaptations, which result in strong physiological, enzymatic, and molecular alterations.

In the present study, the metal concentrations increased in the corresponding leaves of the three cultivars with the augmentation of the soil metal concentrations (Table 2). These data are in agreement with other results obtained *in situ* on several contaminated plots surrounding Metaleurop Nord, with gradient soil metal concentrations [9]. In addition to the experiments in Metaleurop Nord, the current results are consonant with the outcome in phytotron experiments using highly metal-polluted soils [26] and in greenhouse experiments on Cd-spiked soils cultivated with *M. sacchariflorus* [13]. Guo et al. [14] also observed the same trend on three different miscanthus species (*M. sinensis*, *M. floridulus*, and *M. sacchariflorus*) grown in a hydroponic solution with gradient Cd concentrations that might be considered environmentally unrealistic. However, the results demonstrated in Table 2 conform with the above-mentioned studies, which showed a significant increase in the leaf metal concentrations with the increase in metal concentrations in both the soil and the

nutrient solution. For example, Guo et al. [14] demonstrated that under 200 µM Cd treatment (hydroponic solution), the Cd concentrations in the leaves of *M. sinensis*, *M. floridulus*, and *M. sacchariflorus* were 146, 210, and 71 µg g^{-1} dry weight, respectively. Zhang et al. [13] showed that the Cd concentration in the aboveground parts of *M. sacchariflorus* increased proportionally along with the spiked-soil Cd concentrations augmentations, reaching 18.4 mg kg^{-1} in the most contaminated soil (100 mg kg^{-1} of Cd). The corresponding leaf Cd concentrations are much higher than the results obtained in the present experiment, in which the maximum Cd leaf concentrations did not exceed 2.3 mg kg^{-1} in the plants cultivated in the M900 soil. However, the rise in the leaf metal concentrations in the current study might stem from the limited quantity and volume of available soil, which might increase the corresponding metal exposure and thus uptake and translocation by the plant [13, 14].

The reaction of the miscanthus plants to stress from various metals in the present study was expressed in the significant reduction in the number of tillers per plant in the U cultivar (14 tillers in the MC soil to five tillers in the M900 soil), as well as the stem diameter in the B cultivar (8.2 mm thick in the plants cultivated in the MC soil to 4.9 mm in those planted in the M900 soil) (Table 2). The results were confined with the work of Fernando and Oliveira [27], who demonstrated the negative effects of metal-contaminated soils on the number of tillers per *M. giganteus* plant. In the present study, the decline in plant growth may be an outcome of the metal impacts on the main metabolic processes such as photosynthetic activities, respiration, as well as nutrient and water uptake [28-30].

Generally, oxidative stress is one of the main consequences of exposure to high metal concentrations in higher plants. Metals induce the production of reactive oxygen species (ROSs) including superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2), which are strong oxidative agents that might cause irreparable damage to biomolecules such as DNA, proteins, and lipids, thereby affecting plant growth and development, which might eventually lead to cell death [31-33]. However, plants possess a series of antioxidative enzymatic systems involving SODs, peroxidases (including APs), GRs, and others, as well as nonenzymatic constituents including ascorbate, glutathione (GSH), and secondary metabolites that can counteract the negative influence of ROSs, to restore redox homeostasis and normal metabolism [34-36].

In our study, the activities of the antioxidative enzymes studied (Figures 1, 2, and 3 corresponding to SOD, APX, and GR, respectively) in the leaves of the three miscanthus cultivars (B, U, and A) displayed a significant accretion in the contaminated soils compared to the control soils (between 100 and 400% in the most severely contaminated soils, M750 and M900). The data obtained are in total agreement with the results of Guo et al. [14] in *M. sinensis*, *M. floridulus*, and *M. sacchariflorus* exposed to Cd in hydroponic conditions. These authors showed that SOD, APX, as well as GR increased in all three species, yet the corresponding increase in the enzymatic activities mentioned is more acute in the results

reported by Guo et al. [14] in which the increases in the activities at the highest Cd concentration exceeded those obtained in the controls by more than 3 or 4 folds.

Secondary metabolites are also important in plant metal tolerance and ROS detoxification [37]. It should be specified that no one has investigated the impact of metal pollution on the secondary metabolite secretion in miscanthus plants before the current study. Our results (Figures 4, 5, 6, and 7) clearly demonstrated the increase in phenolic compound, tannin, flavonoid, and anthocyanin concentrations, respectively, in the three cultivars, mainly those cultivated in the highly contaminated soils (M500, M750, and M900). Various groups of phenolic compounds exist in plants, discriminated by their number of constitutive carbon atoms associated with the structure of the basic phenolic skeleton. These include the simple phenolic compounds, tannins, flavonoids, and anthocyanins [38]. The phenolic compounds possess a high capacity to chelate metals because of the hydroxyl and carboxyl groups existing in their structure [38]. Analogous to all other phenolic compounds, tannins are reported to exhibit antioxidative properties by forming variable affinity complexes with metals and are thus involved in their detoxification [39, 40]. Flavonoids are considered to be plant metabolites and acquire their antioxidative capacity from their molecular structure, comprising conjugated double bonds in addition to the functional groups in the rings. The main role of flavonoids is lowering the production of and quenching ROSs [41]. Anthocyanins can also be released as a reaction against metal stresses and are believed to increase the antioxidative response of plants in order to uphold their regular physiological status against both biotic and abiotic stresses [42]. Gill and Tuteja [34] also discussed accretion in the flavonoid concentration following biotic and abiotic stresses, such as wounding, drought, metal toxicity, and nutrient deprivation. Finally, the accumulation of the phenolic compounds in leaf tissues was also observed in various species exposed to metals and in plants grown in multi-polluted soils [43-45]. Altogether, the increase in phenolic compound concentrations might be considered a clear indication of their corresponding implication in miscanthus protection against metal stress via the effective addition of defensive responses to the plant repertoire [42, 46].

Another significant indicator of metal-induced stress is the modification of photosynthetic pigment contents in leaves. Chlorophyll loss is one of the common symptoms of metal exposure [32, 45]. Meanwhile, carotenoid production is enhanced or reduced depending on the types of metals present [47]. Carotenoids are also plant pigments functioning as nonenzymatic antioxidants. They play an influential role in protecting chlorophyll pigments and plant organs under stress conditions by either dissipating excess excitation energy such as heat or scavenging ROSs and suppressing lipid peroxidation [34, 42]. In the present study, the results (Figures 8–10) demonstrate that metal exposure reduced chlorophyll a at a maximum 29.1, 43.2, and 39.2% in the B, U, and A cultivars, respectively. Chlorophyll b concentrations were affected as well: a maximum 24.3, 32.4, and 20.0%, corresponding to the maximum reduction in the B, U, and A cultivars, respectively). Finally the carotenoid contents showed significant reductions in the three miscanthus cultivars with the metal

concentration increase in the soil (maximum 34.3, 33.8, and 29.1% in the B, U, and A cultivars, respectively). These results agree with Guo et al. [14], who demonstrated a decrease in the above-mentioned pigments in all three miscanthus species (*M. sinensis*, *M. floridulus*, and *M. sacchariflorus*) as the Cd contamination increased, reaching a maximum of more than 50% at the highest Cd concentration, which might be considered as the most intense response in comparison with the results obtained in the present study. Zhang et al. [13] also recorded more than a 30% decrease in the corresponding photosynthetic pigment contents of the leaves of *M. sacchariflorus* with as the Cd concentrations increased in the soils. The decrease in the chlorophyll pigments might be attributed to the direct effects of metal accumulation on photosynthesis by impairing chlorophyll biosynthesis or the increased metal-induced activity of chlorophyllase [32, 48]. According to Hattab et al. [49], chloroplasts have a complex system of membranes rich in polyunsaturated fatty acids, which are potential targets for peroxidation. This could also partially explain the deleterious effects of the metals on the chlorophyll contents and other photosynthetic parameters. Nevertheless, it is noteworthy that sometimes the reduction of the chlorophyll content might be an adaptive defensive response in the leaves of plants grown under contaminated environments, which permits them to protect the photosynthetic apparatus from photo-inhibition and photo-oxidation and thus enduring stressful conditions [50, 51]. As for the carotenoids, the decline in the contents of the corresponding photosynthetic pigment may be an outcome of reduced synthesis and/or their enhanced oxidative degradation by the oxidative stress imposed [52]. In addition, according to Baek et al. [42], carotenoids play an important role in protecting against mild metal stress conditions, but with the increasing concentrations of metals anthocyanins participate more actively in protecting the plant. This might explain the results, where it was evident that the carotenoid levels decreased in the miscanthus plants as the metal concentrations increased, while anthocyanin inductions increased with rising contamination.

Altogether, the collective work of the antioxidative enzymatic network and secondary metabolites might lead to alleviating damage to the cells by means of decreasing ROS levels and oxidative damage to lipids [51]. This is considered a good indicator of the efficiency of the antioxidative defense system of the miscanthus plants, which could scavenge ROSs, maintain integrity of the chloroplast, and restore redox homeostasis and normal metabolism under stress [14, 34], thus confirming their high tolerance to elevated concentrations of metals [53].

D.1.5- Conclusion and perspectives

The present study sheds light on the response of the three miscanthus cultivars to the increasing concentrations of metals (Cd, Pb, and Zn). The results showed that the number of tillers and stem diameter were slightly affected by soil metal contamination. The inhibitory effects on the photosynthetic pigments were evident when comparing the contaminated

and the uncontaminated plants. In addition, the resistance of the cultivars to metals was enforced by the rising levels of phenolic compounds as well as the activities of the antioxidative enzymes, which proportionally increased as contamination rose. However, slight variations were noted between the three cultivars investigated in all the parameters studied, in which differences were mainly observed between the plants cultivated in the contaminated and uncontaminated soils for the B, U, and A cultivars. The various laboratory analyses were performed on the samples collected during the first growing season. However, it might be useful to pursue the changes in cultivar reactions in response to metal contamination during further growing seasons. Moreover, the corresponding study was carried out in pots with a specific quantity of soil, which might be considered a forcing environment. Therefore, further *in situ* experiments might be useful to validate the results obtained and specify the impact of metal contamination on miscanthus plants.

Acknowledgements

The authors are grateful to the Association for Specialization and Scientific Guidance for the PhD scholarship offered to Karim Al Souki. They also wish to thank ADEME (French Agency for the Environment and Energy Management) for the financial support of the Phytener program.

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D2- Response of three *Miscanthus x giganteus* cultivars to metal stress. Part 2: Comparison between two growing seasons

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Foreword

All the experiments dealing with miscanthus and metals are until nowadays established in either hydroponic solutions or metal-spiked soils and for a short period that does not exceed couple of months or maximum one growing cycle. Thus, this chapter is considered as a continuity of the previous one (D.1-) and aims at studying the effect of metals on miscanthus plants after two growing season (T2). Moreover, at the end of the first growing cycle, no significant differences were observed between cultivar. These second objective of the present study was to investigate if the same results would be obtained at T2.

The essence of the corresponding work was not so far from that of the previous chapter (D.1-). The same biomarkers were used to evaluate miscanthus health (antioxidant enzymes, photosynthetic pigments, secondary metabolites), and were compared with the corresponding leaf metal accumulation, in order to compare the difference of responses between the two growing cycles. However, a special interest in the leaf metal concentrations was triggered. The relevance of "long-term" (2 years) pot experiments, hydroponic culture or spiked soils experiments to evaluate plant suitability for phytoremediation was especially discussed.

Publication

Al Souki, K. S., Liné, C., Gruyer, N., Dorais, M., Douay, F., Pourrut, B., Submitted to Journal of Hazardous Materials. Response of three *Miscanthus x giganteus* cultivars to metal stress: Part 2: Comparison between two growing seasons.

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Abstract: The positive impact on restoring soil functionality, decreasing the human metal bioaccessibility and enhancing the physico-chemical and biological activities in soil made the *Miscanthus x giganteus* convenient species to phytomanaging wide metal contaminated areas. However, the information about the plant's mode of reaction against elevated soil multi-metal concentrations is still rare. For the sake of investigating the miscanthus response to stressful metal concentrations, *ex situ* pot experiment was initiated for a period of 18 months, with three miscanthus cultivars referred to as B, U and A planted in soils with gradient Cd, Pb and Zn concentrations. Another non-contaminated soil was added and considered as control, and plants were cultivated within. Results revealed that the long exposure of increasing soil metal concentration caused the number of tillers per plant to decline and the metal concentrations in the leaves to increase progressively with the soil contamination. Moreover, the photosynthetic pigments (chlorophyll a, b, and carotenoids) were negatively affected as well. However, the phenolic compounds, flavonoids, tannins, and anthocyanins in addition to the antioxidative enzymatic activities of superoxide dismutase, ascorbate peroxidase and glutathione reductase elevated progressively with the metal concentration and exposure duration. Conclusively, miscanthus plants demonstrated an intensified and synchronized antioxidative response against the corresponding metal concentration.

Keywords: *Miscanthus x giganteus*, metal contamination, pot experiment, antioxidative response

D.2.1- Introduction

Considerable areas of agricultural lands in the proximity of mining and smelting sites are vulnerable to contamination by multiple metals as a result of the atmospheric emissions and deposition of dust particles [1]. Metal accumulation in the agricultural soils is of concern due to food safety issues and potential health risks as well as detrimental effects on soil ecosystems [2]. The areas surrounding the former Pb smelter Metaleurop Nord in Northern France are an explicit example about the complexities that might arise as a result of the intense land metal pollution [3, 4]. The former smelter was in activity for more than a century and generated great quantities of dust. Consequently, the agricultural topsoils around the smelter are extremely contaminated by metals (mainly Cd, Pb and Zn), and the agricultural food crops might not meet the European standards [3]. In addition, several human diseases dysfunctions were documented between the inhabitants as result of their chronic exposure to the corresponding metals [1, 3, 5]. Therefore, sustainable management of these soils is crucial.

The conventional physico-chemical remediation methods are not convenient for treating vast areas, for their high costs and negative impacts on the ecological system. Therefore, the environmentally friendly and cost effective biotechnologies capable of reducing risks while enhancing the ecological quality of the polluted areas are under investigation [6, 7]. The most appropriate alternative technology for the vast multi-contaminated area surrounding the former Metaleurop Nord smelter is the *in situ* phytostabilization. Upon the application of phytostabilization, metal leaching through polluted soil is reduced as well as their corresponding availability [5]. Moreover, the metals are sequestered in the roots and their translocation and accumulation in the aboveground parts of the plant is limited [5]. In the scope to find the most appropriate technique for reclaiming the large contaminated area, several plants species were trialed. The herbaceous ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) were first selected for their ability to form a dense plant cover on soil, thus limiting soil erosion and dust emissions [8, 9]. These plants were able to limit the metal transfer to their aerial parts over time and relatively tolerated the stress arisen, making them suitable for phytostabilization. Meanwhile the efficiency of aided-phytostabilization combining the use of five woody species (*Robinia pseudoacacia*, *Alnus glutinosa*, *Quercus robur*, *Acer pseudoplatanus*, and *Salix alba*) and fly ash amendment was also evaluated [10-12]. The results demonstrated a successful afforestation of the site, combined with the reduction of metal availability, especially on the amended plots. However, the economic interest of these crops is rather limited, which restraint their interest for the phytomanagement of a contaminated area as large as the Metaleurop area.

In this specific context, the most promising phytomanagement option appeared to be the cultivation of *Miscanthus x giganteus* [13, 14]. This plant is a C4 perennial herbaceous, lignocellulosic, rhizomatous and noninvasive grass, characterized by its high yield, remarkable adaptability to the high metal conditions prevailing in the targeted area, as well as its high capacity to accumulate metals mainly in the roots and hence reduce their potential mobility and bioavailability, thereby alleviating human and environmental risks

[13]. Several papers have been released reporting the positive impacts of the miscanthus plants on the degraded area surrounding Metaleurop Nord. Of which, a study demonstrating the high capacity of accumulating low metal and nutrient concentrations in the aboveground parts and well proliferating in the highly contaminated studied agricultural plots acquiring a considerable dry biomass which could be invested economically [14, 15]. Pelfrêne et al. [5] demonstrated their substantial impacts on the metal redistribution in soils as well as on decreasing the human metal oral bioaccessibility. Finally, Al Souki et al. [16] displayed the positive impacts of the *Miscanthus x giganteus* in restoring metal contaminated soil functionality via enhancing the soil biological activities, in addition to the microbial biomass carbon as well as certain physico-chemical parameters such as the soil organic carbon content and cationic exchange capacity. Nevertheless, information is scarce concerning metal exposure effects on the plant health and resistance mechanisms [17-19]. Moreover, the few publications that came out discuss the impact of the metals on the miscanthus for a period between three to four months or maximum during one growing cycle. Thus, there is a need to understand the metal tolerance and resistance mechanisms of miscanthus plants.

The current work is part of a larger study aiming at understanding the influence of *Miscanthus x giganteus* on soil parameters [16] and its tolerance to metals during two growing seasons (Part D-). In this study, we investigated the response of *Miscanthus x giganteus* plants cultivated in pots filled with soils from Metaleurop area with a gradient concentration of Cd, Pb and Zn, after 18 months of cultivation (two growing periods). We studied the effects of soil metal contamination on three different miscanthus cultivars i) on plant growth parameters and accumulation of Cd, Pb and Zn in leaves, ii) on oxidative stress induction in leaves following antioxidant enzyme activities, iii) and on secondary metabolism (phenolic compounds, tannins, flavonoids and anthocyanins) and photosynthetic pigments (chlorophyll a, b and carotenoids).

D.2.2- Materials and Methods

D.2.2.1- Soil and miscanthus origins and preparations for pot experiments

As previously mentioned, the agricultural land surrounding the former Pb smelter Metaleurop Nord is massively contaminated by several metals, including Cd, Pb, and Zn [10]. Soil samples (plowed horizon, 0-25 cm) were collected from different agricultural plots in the territory surrounding the former smelter, and characterized by their gradient concentration of metals which increased as the distance to the smelter decreased [3, 20]. The samples were thereby designated based on their corresponding Pb concentration (in mg kg^{-1} in soil), in which the M200 (50°24’52”N, 3°01’51”E, Courcelles-les-Lens, 1.8 km from the smelter, 1.4 ha), M500 (50°25’49”N, 3°02’13”E, Evin-Malmaison, 1.4 km from the smelter, 0.8 ha), M750 and M900 (50°26'15.0"N 3°01'05.7"E, Evin-Malmaison, 1 km from the smelter, 0.8 ha) samples contained approximately 200, 500, 750 and 900 mg kg^{-1} Pb in soil. For the sake of

comparing the impact of contamination on the plants, soil samples were collected from a non-contaminated agricultural plot located 75 km from the smelter (50°20'46"N 2°12'15"E, Linzeux, 1.3 ha) and considered as controls. Thereafter, samples were homogenized, dried and sieved through a 10 mm mesh prior to cultivation.

Three different miscanthus cultivars (B, U, and A) from different origins were implicated for the pot experiment. Small 2-3 budded rhizomes (5-7 cm) of the corresponding plants were grown in pots (9 x 9 x 9 cm) filled with potting compost that was kept wet via constant watering.

D.2.2.2- Pot experimental design

When the miscanthus plantlets reached 20-25 cm, *ex situ* experiments were launched for a period of 18 months (May 2014 to October 2015) in an area distant from roads in the Lille 1 University campus (50.6090° N, 3.1381° E). Approximately 100 kg of the upper mentioned homogenized and dry soils (MC, M200, M500, M750, and M900) were equally distributed in five 20-kg capacity pots (light grey colored in order to limit temperature elevation possibilities) and the *M. x giganteus* plantlets (B, U, and A) were thereafter displaced from the small pots to the 20-kg soil pots (two plantlets in each pot).

	MC	M200	M500	M750	M900
Cd (mg kg^{-1})	0.3 ± 0.0	3.8 ± 0.2	9.0 ± 0.2	13.5 ± 0.3	16.0 ± 0.3
Pb (mg kg^{-1})	37.3 ± 1.3	260.3 ± 2.0	528.6 ± 5.3	747.1 ± 16.9	898.6 ± 16.3
Zn (mg kg^{-1})	54.6 ± 3.1	388.0 ± 14.8	537.0 ± 10.9	906.0 ± 16.8	1116.0 ± 1.7

Table 1: Cd, Pb and Zn pseudototal concentrations of studied soils (mean ± standard deviation)

As previously shown in Al Souki et al. [16] the metal concentrations in MC were in agreement with the regional background values (0.42, 38 and 74 mg kg^{-1} corresponding to Cd, Pb, and Zn, respectively). Whereas, the other soils possessed pseudototal concentrations 20 to 50 times more than the regional agricultural background values.

Briefly, 75 planted pots were used (3 different miscanthus cultivars x 5 soils x 5 replicates). The pots were displaced over wooden rafters to avoid direct contact with the ground of the experimental area. Random distribution of the pots took place in an attempt to avoid the point and borderline effects. Moreover, the pots were regularly watered during the entire experiment for the sake of conserving the soils humidity, in addition to the rain water as well. Weeds were manually removed and kept on the surface of the soils to avoid a possible metal exportation.

D.2.2.3- Miscanthus sampling and sample preparation

By the end of the second growing season (October 2015), plant growth parameters such as the stem height, diameters as well as the number of tillers per pot were determined for the second time.

For the sake of investigating the effects of contamination on plants health, three leaves (4^{th}, 5^{th} and 6^{th} foliar stage) were collected from every plant. The samples were instantly flash frozen into liquid nitrogen, and then conserved at – 80 °C in the laboratory prior to biomarker analysis.

The rest of the leaves within each pot were also collected to determine the corresponding metal concentrations within. Samples were placed in a plastic bag and kept in a cool box. When in the laboratory, leaves were washed 3 times with osmosed water in order to eliminate the dust particles. Later on, samples were oven-dried at 40 °C for 48 h and ground into fine powder using a knife mill (GM200, Retsch) before metal analysis.

D.2.2.3.1- Metal concentrations in the collected leaves

The Cd, Pb and Zn concentrations in miscanthus leaves were determined via acid digestion. 300 mg of the ground leaf powder was digest with nitric oxide (HNO_3 70 %) and heated. Afterwards, hydrogen peroxide (H_2O_2 30 %) was added and the sample was reheated for another 180 min prior to osmosed water addition. The metals in the extracts were determined by atomic absorption spectrophotometry (AA- 6800, Shimadzu). Quality control for chemical extraction and digestion was performed by including blanks, internal and certified (Polish Virginia tobacco leaves, INCTPVTL- 6, Poland) reference materials [21].

D.2.2.3.2- Antioxidative enzymatic activities assays

Antioxidative enzymatic activity assays were evaluated spectrophotometrically according to Liné et al. [22] using a plate reader (Thermo Scientific Multiskan™ GO). Briefly, five foliar discs (0.5 cm diameter) per plant sample were collected from frozen leaves using a manual punch. Then, they were put into in 96-deepwell plate (2 mL) with one 4 mm diameter glass bead. Samples were then ground under frozen conditions using a Mixer Mill MM 400 (Retsch) for 2 times (1.5 min at 30 Hz). After addition of 1 mL of ice cold Tris extraction buffer pH 7.0 containing 0.01 M EDTA, 0.4 M PVP, 0.05 ascorbate, 11.44 mM β-mercaptoehtanol and proteases cocktail inhibitor. Samples were homogenated for 2 min at 15 Hz with the MM400 grinder. Plates were then centrifuged at 5000 g for 15 min at 4°C. Supernatant were collected and protein content was determined according to Bradford [23], using bovine serum albumin (BSA, Sigma) as standard.

The total activity of superoxide dismutase (SOD) was determined by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) according to the method

of Giannopolitis and Ries [24]. The reaction mixture contained 0.47 mM NBT (Sigma), 3.85 µM riboflavin (Sigma), 19.23 mM methionine (Sigma), 36.54 mM phosphate buffer (pH 7.8), and 20 µL enzyme extract. The test tubes containing the mixture were placed 30 cm below a light source (30 W fluorescent lamps). The reaction was started by switching on the light and was allowed to run for 10 min. The reaction was stopped by switching off the light and the absorbance at 560 nm. An unirradiated reaction mixture that did not develop color served as the control, and its absorbance was subtracted from that of the test tube. One unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of NBT reduction.

Ascorbate peroxidase (APX) activity was evaluated by the decrease of absorbance at 290 nm due to ascorbate oxidation [25]. The reaction mixture contained 434 mM phosphate buffer (pH 7.0), 3.77 mM H_2O_2, 0.56 mM ascorbic acid and 10 µL enzyme extract. One enzyme unit was defined as 1 µmole of ascorbic acid oxidized per min at 290 nm using a standard curve of ascorbate. The enzyme activity was expressed as µmoles of ascorbate oxidized min^{-1} mg^{-1} protein.

Glutathione reductase (GR) was assayed as the decrease in absorbance at 340 nm caused by the oxidation of NADPH [26]. This assay is based on the reduction of oxidized glutathione (GSSG) by NADPH in the presence of GR. The reaction mixture contained 0.1 M Tris buffer (pH 7.5), 1 mM GSSG (Sigma), 0.1 mM NADPH (Sigma) and 20 µL of enzyme extract. The amount of NADPH oxidized was calculated from the extinction coefficient of 3.732×10^{-3} mL $nmole^{-1}$ of NADPH. The enzyme activity was expressed as nmoles of NAPDH oxidized min^{-1} mg^{-1} protein.

D.2.2.3.3- Photosynthetic pigments and secondary metabolism molecule quantification

Photosynthetic pigments, phenolic compounds, tannins, flavonoids, and anthocyanins were evaluated spectrophotometrically according to Liné et al. [22] using a plate reader (Thermo Scientific Multiskan™ GO). Briefly, two foliar discs (0.5 cm diameter) per plant sample were collected from frozen leaves using a manual punch, and weighed. Then, they were put into in 96-deepwell plate (2 mL) with one 4 mm diameter glass bead. Samples were then ground under frozen conditions using a Mixer Mill MM 400 (Retsch) for 2 times 1.5 min at 30 Hz. After the addition of 1.5 mL of ice cold 95 % methanol in each well, samples were homogenated for 2 min at 15 Hz with the MM400 grinder. Plates were let in the dark for 24 h and 48 h of incubation.

After 24 h of incubation, leaf extracts were homogenated for 2 min at 15 Hz with the Mixer Mill MM 400. 100 µL was collected for photosynthetic pigment analysis. The absorbance was measured at 470, 652, and 666 nm. Concentrations of chlorophyll a, b, and total carotenoids were calculated according to extinction coefficients and equations reported by Lichtenthaler

[27]. Finally, data were averaged and the obtained mean concentrations were expressed as mg g^{-1} FW of leaf.

After 48 h of incubation, plates were centrifuged at 5000 g for 5 min, prior to secondary metabolism molecule extraction. The total phenolic compound was determined based on Folin Ciocalteu assay. Briefly reaction mixture of 200 µL contained 20 µL of supernatant, 40 µL of Folin reageants (10 % v/v) and 0.098 mM of Na_2CO_3. The mixture was allowed to stand 2 h at room temperature for color development. And then absorbance was measured at 510 nm. Concentrations of phenolic compounds were calculated using a standard curve of gallic acid. Results were expressed as mM of gallic acid equivalent (GE) per gram of fresh weight of leaf. The flavonoid content was determined by aluminum chloride method using catechine as a reference compound. Briefly reaction mixture contained 25 µL of methanolic extract, 0.00724 mM $NaNO_2$, 0.01125 mM $AlCl_3$ and 0.05 mM NaOH. The mixture was homogenated during one minute and absorbance was measured at 595 nm. Concentrations of flavonoids were calculated using a standard curve of catechin. Results were expressed as mg catechin equivalent (CE) per gram of fresh weight of leaf. For tannins, reaction mixture contained 50 µL of methanolic extract and 100 µL of vanilline solution 1 %. Mixture was let in the dark during 15 min and absorbance was measured at 500 nm. Tannin concentration was calculated using a standard curve of catechin. Results were expressed as mg L^{-1} catechin equivalent (CE) per gram of fresh weight of leaf. Anthocyanins were measured using the differential pH method based on the property of anthocyanin pigments to change the color with pH. Two dilutions of the same sample were prepared, the first one in potassium chloride buffer (0.2 M, pH 1.0) and the second in sodium acetate buffer (0.4 M, pH 4.5). After equilibration at room temperature during 15 min, the absorbance was read at 510 and 700 nm. Results were expressed as mg cyaniding 3-glucoside equivalent per gram of fresh weight of leaf.

D.2.2.4- Statistical analysis

Analysis of variance was done to compare modalities. The Fisher test was considered for significance ($p \leq 0.05$). If statistically significant differences were found, the Tukey HSD test was used for pair-wise comparisons. All statistical analyses were performed using XLSTAT software.

D.2.3- Results

D.2.3.1- Miscanthus leaf metal concentrations at the end of the second growing season

The metal concentrations in the leaves increased progressively with their corresponding concentrations in the soils where the plants were cultivated (Table 2).

		Cd (mg kg^{-1})	Pb (mg kg^{-1})	Zn (mg kg^{-1})
B	MC	0.4 ± 0.0 h	6.9 ± 0.8 f	37.2 ± 3.1 h
	M200	0.7 ± 0.1g	12.6 ± 1.4 e	76.1 ± 5.3 f
	M500	1.4 ± 0.1 e	19.8 ± 1.7 cd	90.9 ± 4.8 de
	M750	2.4 ± 0.1 c	25.9 ± 3.8 b	114.7 ± 4.5 b
	M900	3.1 ± 0.1 a	31.0 ± 2.1 a	138.2 ± 4.1 a
U	MC	0.4 ± 0.0 h	5.9 ± 0.4 f	29.0 ± 3.7 h
	M200	0.7 ± 0.1 g	11.2 ± 2.0 ef	66.2 ± 4.4 g
	M500	1.1 ± 0.1 f	18.0 ± 1.7 d	82.2 ± 4.6 ef
	M750	2.1 ± 0.1 d	23.1 ± 1.9 bc	104.7 ± 4.0 c
	M900	2.7 ± 0.1 b	27.4 ± 3.0 ab	116.3 ± 3.3 b
A	MC	0.4 ± 0.0 h	7.0 ± 0.9 f	41.5 ± 6.0 h
	M200	0.7 ± 0.0 g	12.8 ± 1.1 e	77.1 ± 5.2 f
	M500	1.2 ± 0.1 e	19.5 ± 1.4 cd	93.5 ± 2.1 d
	M750	2.3 ± 0.2 c	25.6 ± 2.6 b	120.4 ± 4.3 b
	M900	3.1 ± 0.1 a	31.7 ± 3.9 a	140.7 ± 4.9 a

Table 2: Leaf metal concentrations, number of tillers per pot, height and stem diameter of the cultivars (B, U and A) in soils with a gradient metal concentration (MC, M200, M500, M750, and M900) at the end of the second growing period. Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$).

The Cd concentration in the leaves of the three miscanthus cultivars cultivated in the MC soil was 0.4 mg kg^{-1} and increased with the concentration of metal in soil where it was 7.8, 6.8 and 7.8 times more in B, U, and A plants cultivated in the M900 soil.

Concomitantly the Pb concentration in the leaves of the three cultivars of the M900 was 4.5-fold its corresponding concentration in the plants of the MC soil (6.9, 5.9, and 7.0 1 mg kg^{-1} corresponding to B, U, and A).

Concerning Zn, the concentrations in the leaves increased with the soil metal contamination as well. The values in the leaves of the plants cultivated in the M900 soils were 3.7, 4.0 and 3.4 times more than their corresponding plants in the MC soil (37.2, 29.0, and 41.5 mg kg^{-1} corresponding to B, U, and A cultivars, respectively).

D.2.3.2- Stem height, diameter and number of tillers per plant at the end of the second growing season

The Table 3 presents stem height, diameter and number of tillers of the 3 miscanthus cultivars at the end of the second growing season in each pot of the studied soils.

		Number of tillers	Height (cm)	Diameter (mm)
B	MC	16.3 + 1.5 bc	109.3 ± 9.1 a	9.3 ± 1.8 a
	M200	10.0 + 2.9 def	99.2 ± 4.3 ab	8.7 ± 1.2 a
	M500	9.4 + 2.3 def	97.6 ± 7.1 abc	9.0 ± 1.4 a
	M750	6.0 + 1.7 f	96.0 ± 5.9 abc	8.4 ±1.6 a
	M900	6.0 + 1.9 f	94.8 ± 4.1 abc	8.7 ± 1. a
U	MC	27.3 + 4.2 a	92.7 ± 5.9 abc	7.3 ± 0.7 a
	M200	12.0 + 1.2 bcd	87.6 ± 7.2 bc	8.2 ± 0.7 a
	M500	11.2 + 2.2 bcde	86.2 ± 4.1 bc	8.1 ± 0.6 a
	M750	9.0 + 2.8 def	86.0 ± 7.9 bc	7.8 ± 1.0 a
	M900	7.4 + 2.7 def	84.4 ± 5.4 c	8.3 ± 0.4 a
A	MC	17.0 + 2.0 b	106.3 ± 6.8 a	9.2 ± 0.3 a
	M200	10.8 + 2.6 cdef	100.2 ± 5.9 ab	8.7 ±0.8 a
	M500	10.0 + 2.3 def	98.8 ± 6.9 ab	8.7 ± 0.7 a
	M750	6.4 + 1.6 ef	97.2 ± 9.3 abc	8.4 ± 1.4 a
	M900	6.0 + 0.9 f	95.2 ± 9.2 abc	8.8 ± 1.1 a

Table 3: Number of tillers per pot, height and stem diameter of the cultivars (B, U, and A) in soils with a gradient metal concentration (MC, M200, M500, M750, and M900) at the end of the second growing period. Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$).

The highest number of tillers was observed in the three miscanthus cultivars that were planted in the MC soil (16.3, 27.3, and 17.0 tillers per plant corresponding to B, U, and A cultivars, respectively). Significant differences occurred within the plants cultivated in the non-contaminated MC soil, in which the U cultivar exhibited more tillers in the pots than B and A plants. The number of tillers significantly decreased in the contaminated soils averaging between 38.8 and 72.9% compared to the MC soil. However, no significant differences were displayed among the three cultivars present in the contaminated soils.

There were no significant differences detected concerning the stem height and diameter of the three miscanthus cultivars in all the studied soils. The shoots in the MC soil were slightly higher (109.3, 92.7, and 106.3 cm corresponding to miscanthus B, U, and A, respectively). It is noteworthy to mention that the tallest U plant was shorter than the shortest plant in the other two cultivars. The thinnest stem was detected in the U plant cultivated in the MC soil (7.3 mm), whereas the thickest stem belonged to the B cultivar presented in the MC soil as well (9.3 mm).

D.2.3.3- SOD, APX and GR activity determination

Globally, the activities of the three investigated antioxidative enzymes (SOD, APX and GR in Figures 1, 2, and 3, respectively) exhibited the same response patterns to metal contamination. The enzymes were strongly activated in plants grown on the M200 soil compared to the MC soil. Then, their activities slowly increased but in a concentration-dependent manner. For the three enzymes, no significant differences were observed among the miscanthus cultivars.

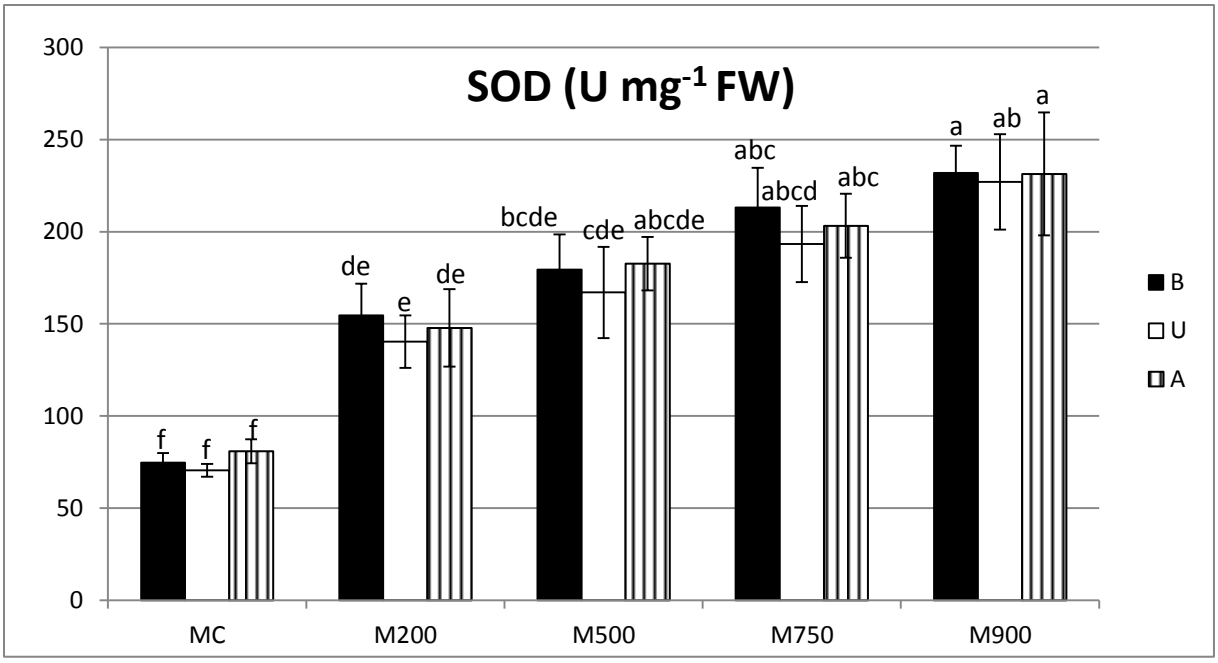

Fig. 1: SOD activities in the leaves of three different miscanthus cultivars (B, U, and A) cultivated in soils with gradient concentrations of metals (MC, M200, M500, M750, and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$)

The least SOD activities were obtained in the leaves of the plants cultivated in the MC soil (74.6, 70.5, and 80.9 U mg^{-1} FW corresponding to B, U, and A, respectively), and progressively increased with the soil metal concentration, in which the highest values were recorded in the plants present in the M900 soil scoring 210.8, 222.0, and 186.1% accretion compared to background values.

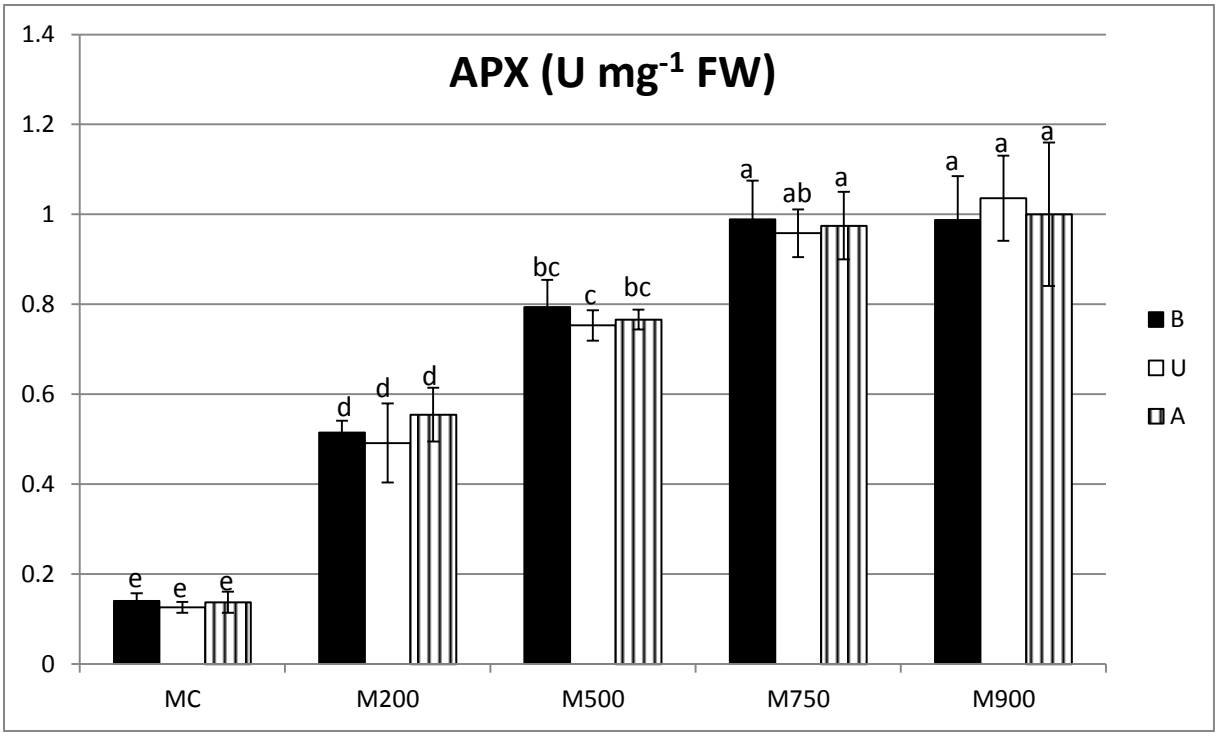

Fig. 2: APX activities in the leaves of three different miscanthus cultivars (B, U, and A) cultivated in soils with gradient concentrations of metals (MC, M200, M500, M750, and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$)

APX minimal activities as well were recorded in the leaves of the plants present in the MC soil (0.1 U mg^{-1} FW in the leaves of B, U, and A, respectively), and continued augmenting until possessing their maximal levels in the leaves of the plants cultivated in the M900 soil with 602.7, 720.9, and 627.2% augmentation compared to background values.

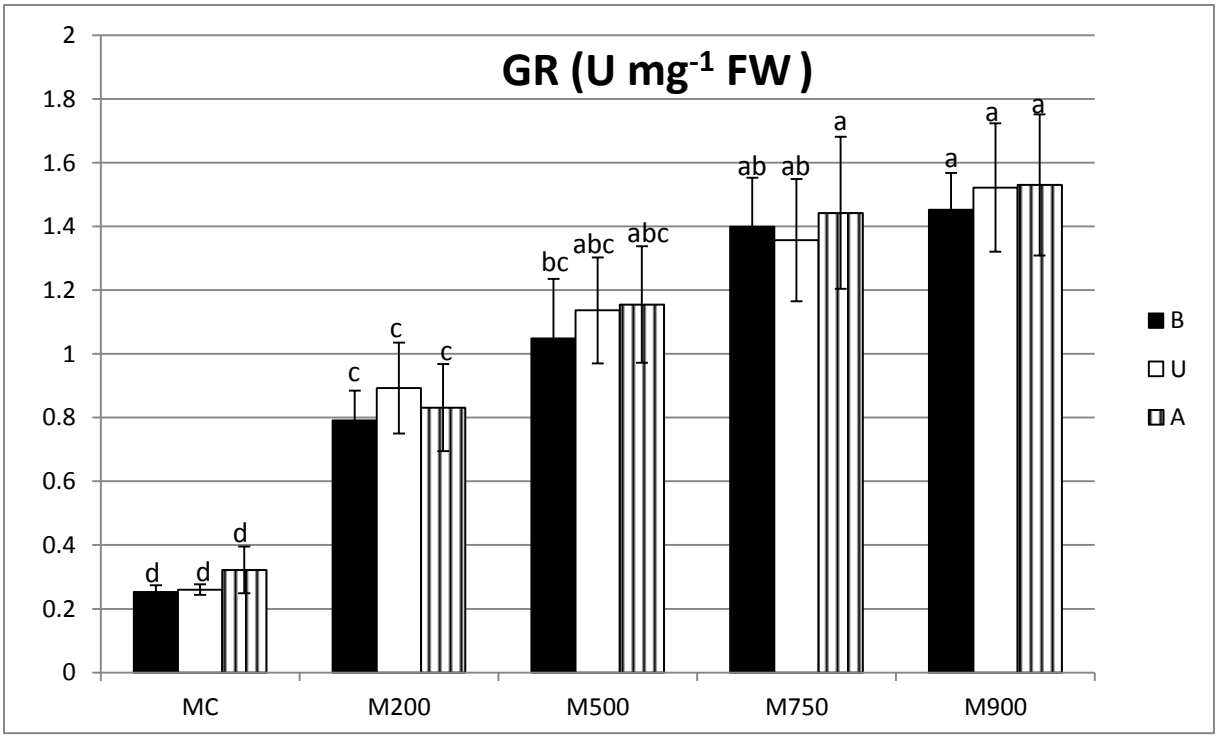

Fig. 3: GR activities in the leaves of three different miscanthus cultivars (B, U, and A) cultivated in soils with gradient concentrations of metals (MC, M200, M500, M750, and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$)

The GR recorded their lowest activities in the leaves of the plants cultivated in the MC soil (0.3 U mg^{-1} FW corresponding to B, U, and A, respectively), and increased with soil metal concentration recording their maximal values in the plants cultivated in the M900 soil with a raise of 475.7, 485.3, and 374.9% compared to background values in those of B, U, and A cultivars, respectively.

D.2.3.4- Photosynthetic pigment quantification

The photosynthetic pigments (chl a, b and car) concentrations in miscanthus leaf tissues exhibited an inverse response patterns compared to antioxidative enzymes activities (Figures 4, 5, and 6). Globally, their concentrations were negatively affected by metal contamination. However, the same two-level response was observed. Actually, the concentrations of the investigated photosynthetic pigments strongly decreased in plants grown on the M200 soil compared to the MC soil. Then, their contents in leaves slowly decayed in a concentration-dependent manner. For the corresponding pigments, no significant differences were observed among the miscanthus cultivars.

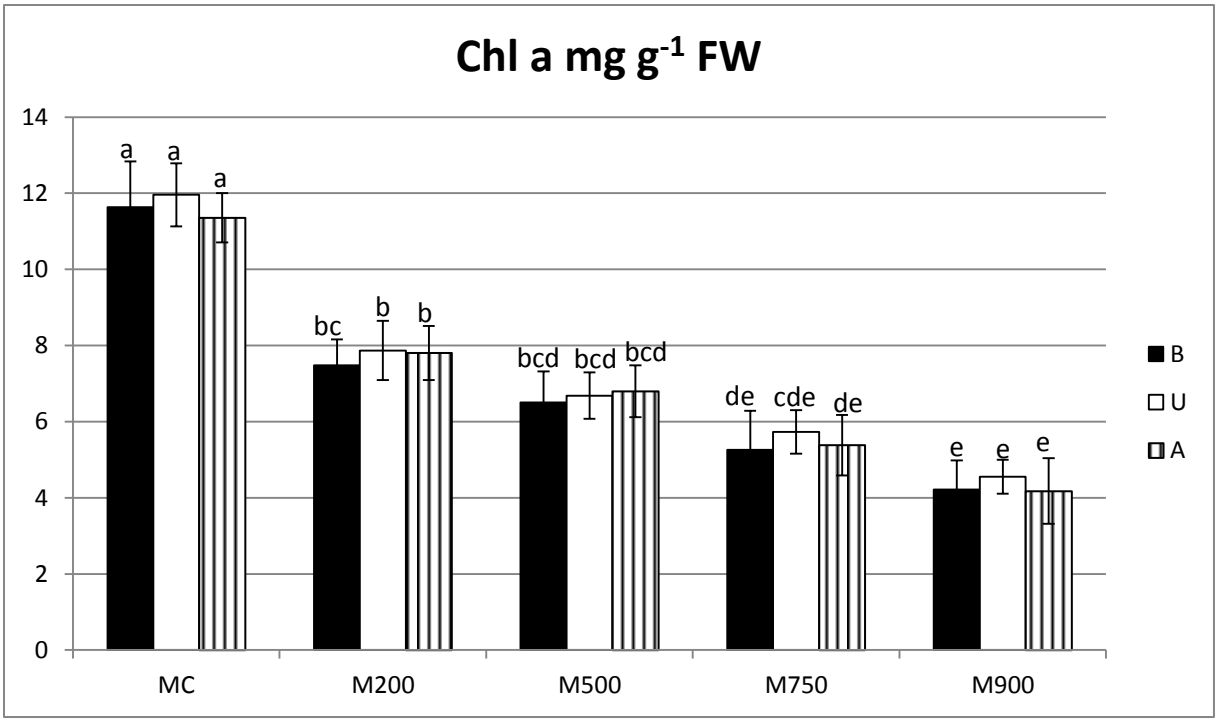

Fig. 4: Chlorophyll a contents in the leaves of three different miscanthus cultivars (B, U, and A) cultivated in soils with gradient metal concentration (MC, M200, M500, M750, and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$)

Chl a concentration significantly declined in the leaves of the plants as the contamination of soil increased (Figure 4). The highest concentrations were observed in the leaves of the miscanthus that were cultivated in the MC soil (11.63, 11.96 and 11.36 mg g^{-1} FW corresponding to miscanthus B, U, and A, respectively). The corresponding depression recorded 63.8, 61.9, and 63.2% in B, U, and A shoots cultivated in the M900 soil.

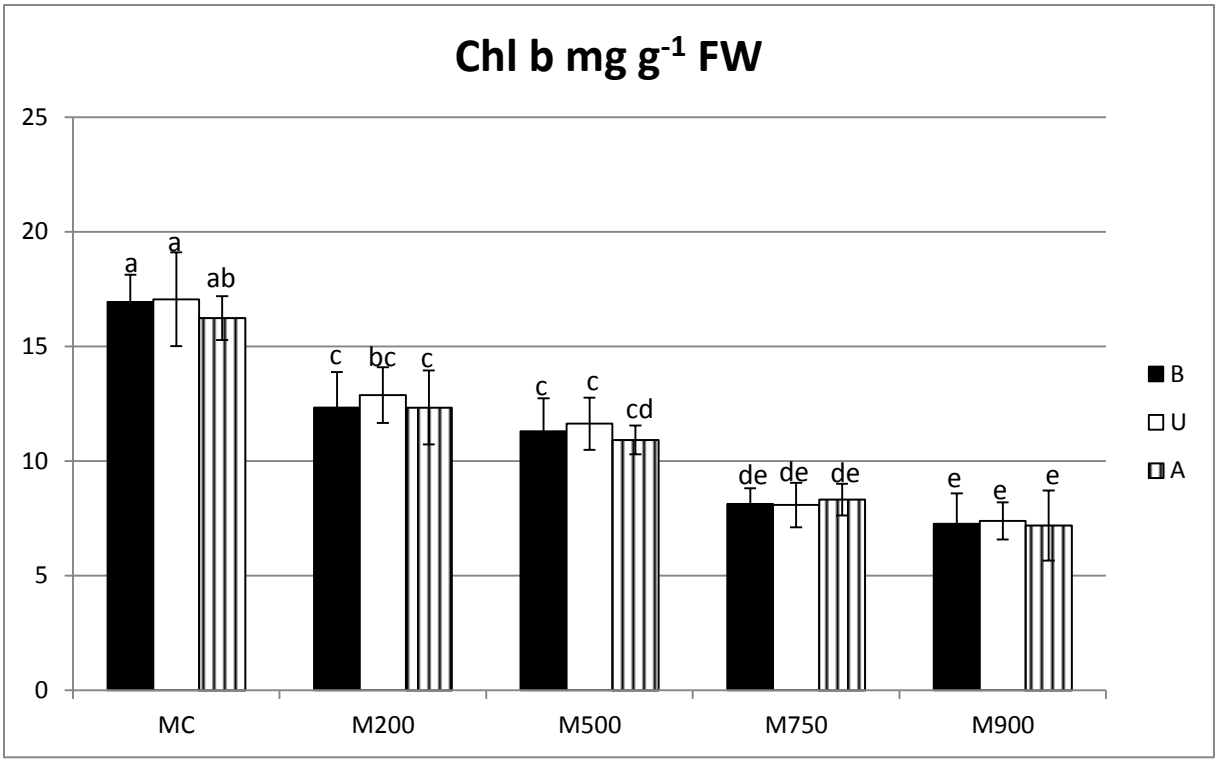

Fig. 5: Chlorophyll b contents in the leaves of three different miscanthus cultivars (B, U, and A) cultivated in soils with gradient metal concentration (MC, M200, M500, M750, and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$)

Chl b levels recorded 16.9, 17.1 and 16.2 mg g^{-1} FW corresponding to miscanthus B, U, and A respectively cultivated in the MC soil, and decreased by 57.1, 56.7, and 55.7 % in those cultivated in the M900 soil.

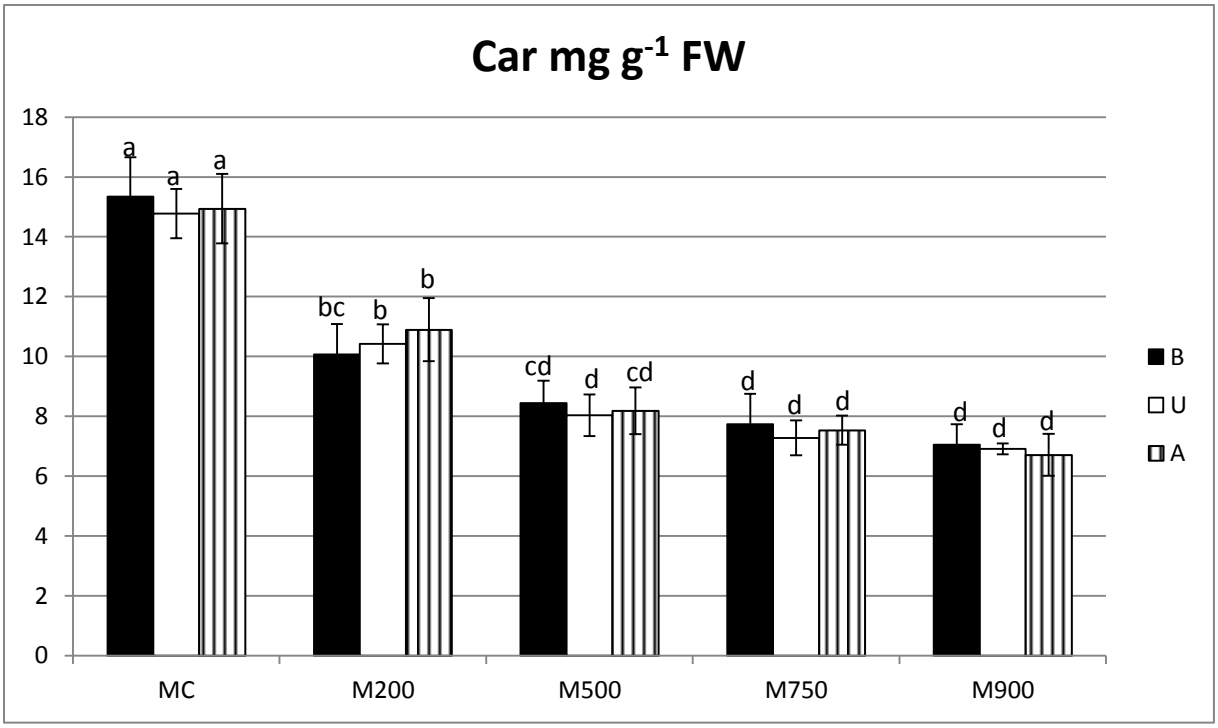

Fig. 6: Carotenoid contents in the leaves of three different miscanthus cultivars (B, U, and A) cultivated in soils with gradient metal concentration (MC, M200, M500, M750, and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$)

Maximum car concentrations were detected in the leaves of the plants that were cultivated in the MC soil (15.3, 14.8, and 14.9 mg g^{-1} FW corresponding to miscanthus B, U, and A, respectively). The minimum levels in the M900 soil were reduced by 54.0, 53.2, and 55.1% in the B, U and A cultivars respectively.

D.2.3.5- Secondary metabolite quantification

The secondary metabolite accumulations in miscanthus leaf tissues show the same response patterns to soil metal concentration increase as antioxidative enzymes (Figures 7 to 10). Globally, the concentrations of the investigated secondary metabolites (phenolic compounds, tannins, flavonoids, anthocyanins) strongly increased in plants grown on the M200 soil compared to the MC soils. Then, their accumulation in leaves slowly continues in a concentration-dependent manner. For the four metabolites, no significant differences were observed among the three miscanthus cultivars.

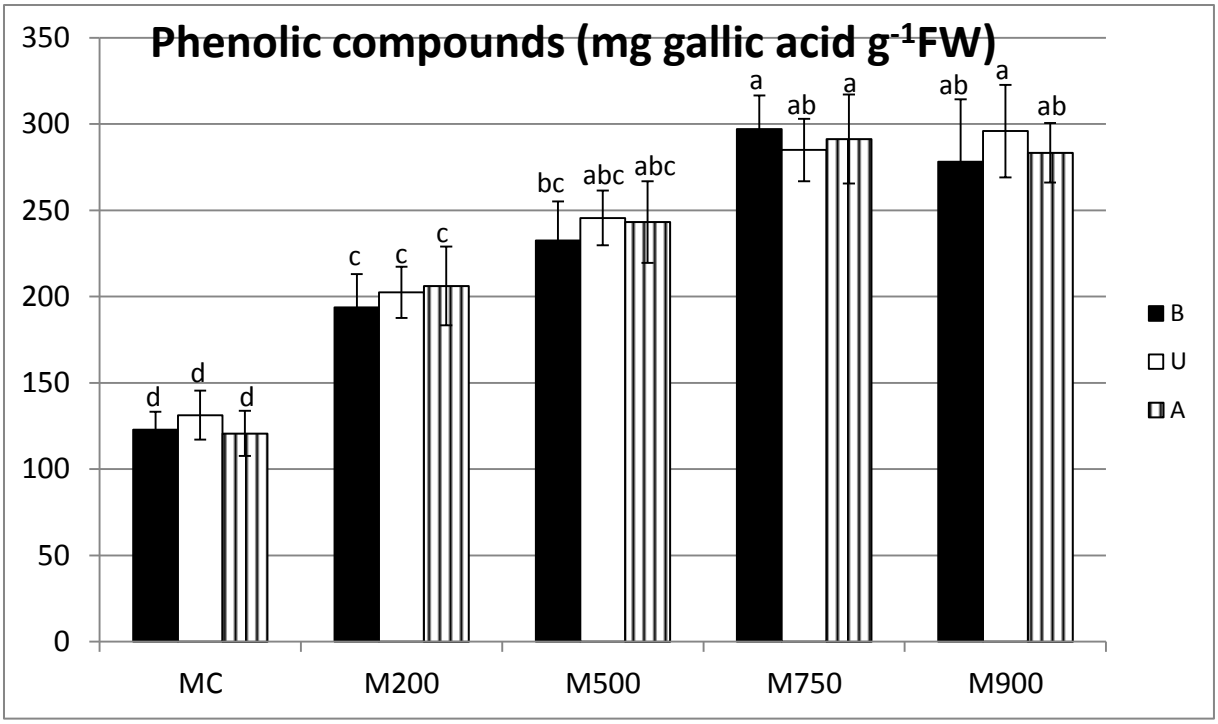

Fig. 7: Phenolic compound concentrations in the leaves of three different miscanthus cultivars (B, U and A) cultivated in soils with gradient metal concentration (MC, M200, M500, M750, and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$)

The levels of phenolic compounds gradually increased from 122.8, 131.3, and 120.8 mg gallic acid g^{-1} FW corresponding in the leaves B, U, and A plants cultivated in the MC soil, to 297.0, 295.9, and 291.3 3 mg gallic acid g^{-1} FW corresponding to B, U, and A cultivated in the M750 (Figure 7). The M900 and M750 soils respectively recording 141.8, 125.4, and 141.3% accretion compared to background values.

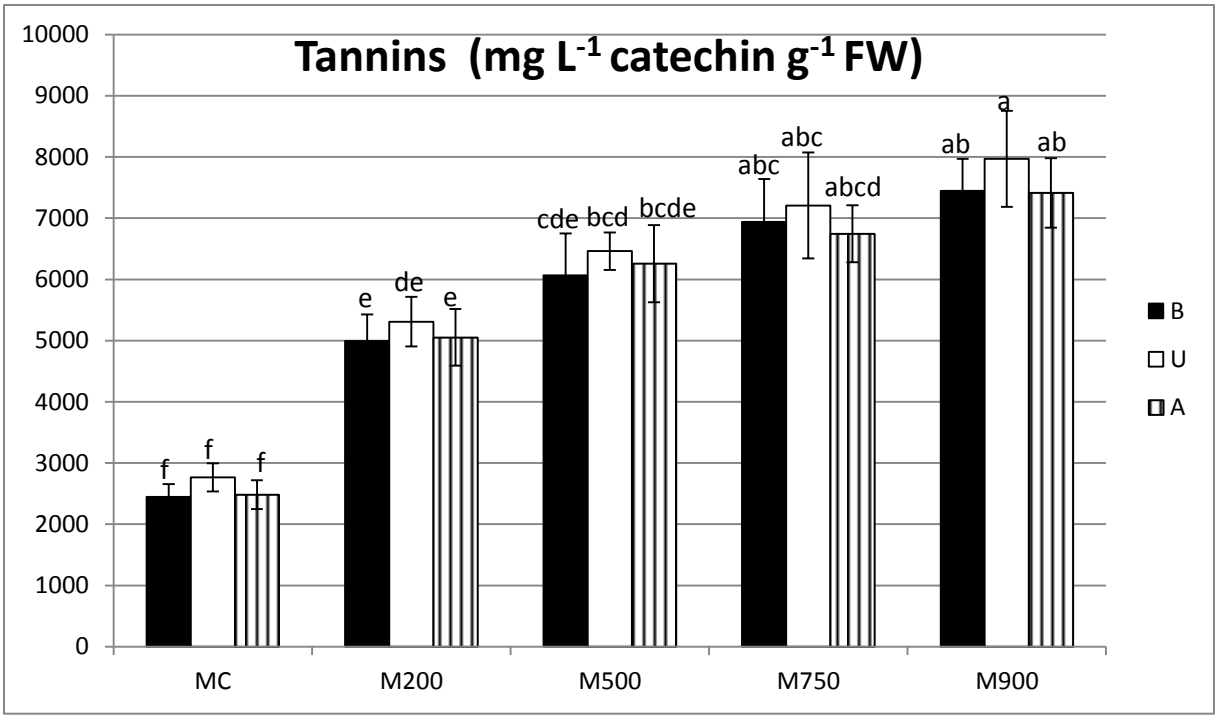

Fig. 8: Tannin concentrations in the leaves of three different miscanthus cultivars (B, U, and A) cultivated in soils with gradient metal concentration (MC, M200, M500, M750, and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$)

The tannin concentration increased in the miscanthus leaves as well with the increase in the soil contamination (Figure 8). Values recorded 2444.6, 2765.3, and 2483.4 mg L^{-1} catechin g^{-1} FW in the B, U, and A cultivars of the MC soil, and increased by 204.3, 188.2, and 198.5% compared to background values in the plants of the M900 soil.

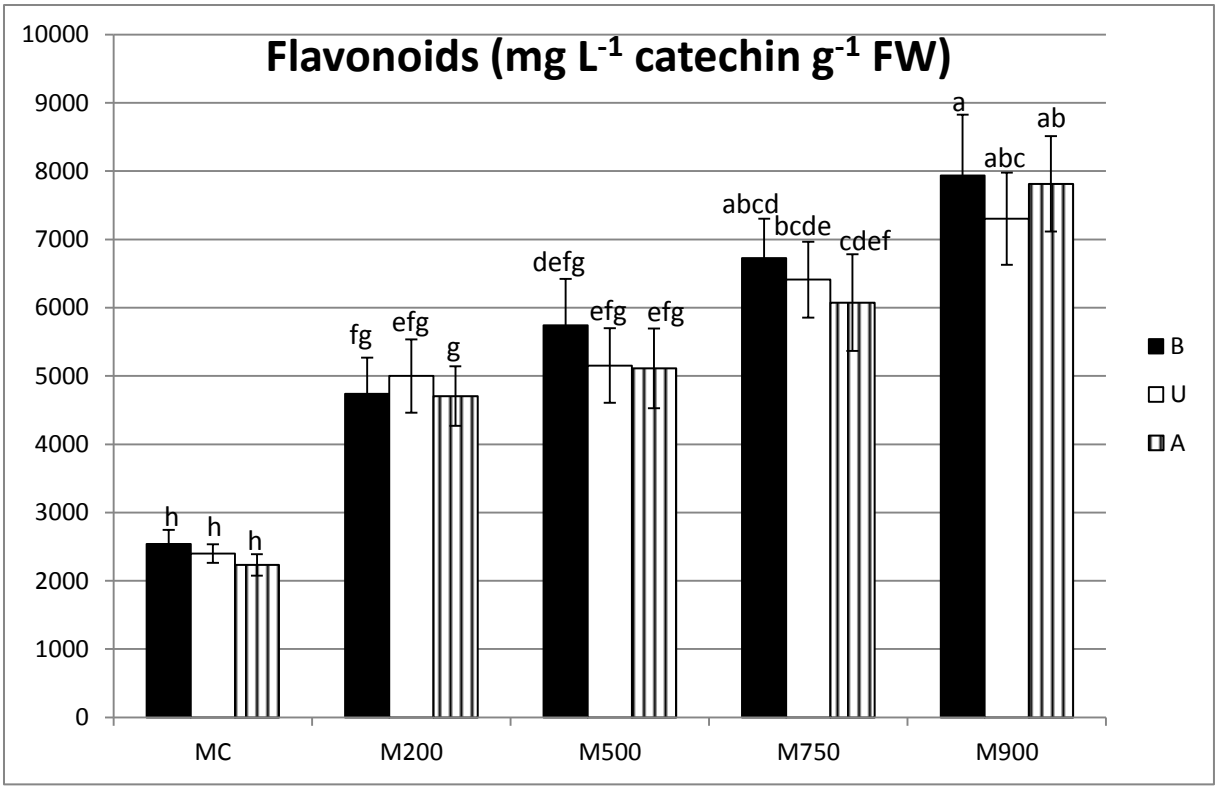

Fig. 9: Flavonoids concentrations in the leaves of three different miscanthus cultivars (B, U and A) cultivated in soils with gradient metal concentration (MC, M200, M500, M750 and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$)

The flavonoid quantity increased in the miscanthus leaves with the soil contamination (Figure 9). Minimal inductions were detected in the leaves of the miscanthus planted in the MC soil (2541.1, 2400,1 and 2232.8 8 mg catechin L^{-1} g^{-1} FW in B, U, and A cultivars, respectively), and raised progressively yielding 212.3, 204.3, and 249.9% augmentation compared to background values in those cultivated in the M900 soil.

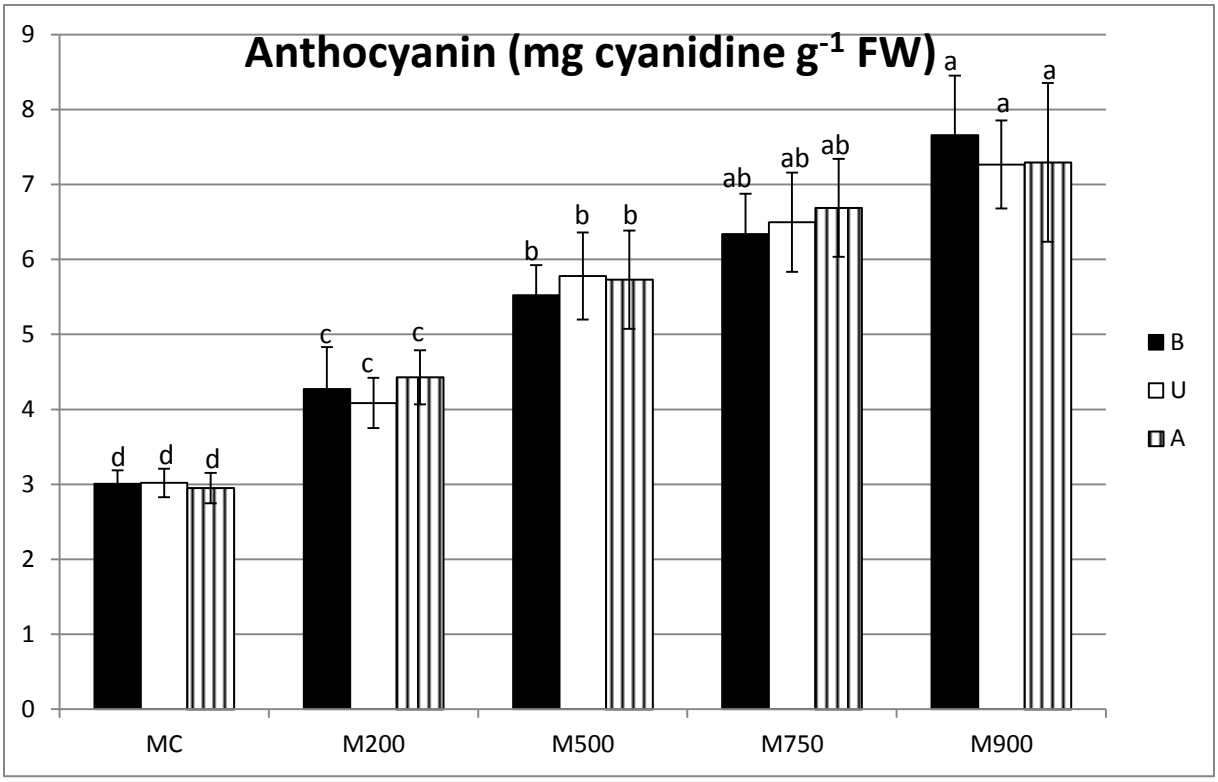

Fig. 10: Anthocyanin concentrations in the leaves of three different miscanthus cultivars (B, U, and A) cultivated in soils with gradient metal concentration (MC, M200, M500, M750, and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$)

The anthocyanin results in the leaves of the 3 cultivars showed that the plants cultivated in the MC soil recorded the lowest concentrations (3.0 mg cyanidin g^{-1} FW in the leaves of the 3 cultivars), and boosted by 154.8, 140.7, and 147.3% compared to background values in B, U, and A plants respectively cultivated in the M900 soil (Figure 10).

D.2.4- Discussion

The ability of *Miscanthus x giganteus* to thrive in contaminated areas was discussed in several papers, and it was nominated as a good candidate for phytostabilization and phytomanagement of severely contaminated areas [13, 14]. Its corresponding positive impacts on decreasing metal bioaccessibility, and restoring soil functionality via enhancing the soil biological parameters were discussed in some previous works as well [5, 16]. However, the experiments that evaluate metal impacts on miscanthus health are scarce and have never been lead for more than three months. In the previous work (D.1-), after a full growing cycle on metal-contaminated soils (T1), we demonstrated that miscanthus plants exhibited a metal-induced stress (increase in antioxidative enzyme activities, decrease of photosynthetic pigment content, and enhancement of secondary metabolite accumulation). However, results also demonstrated that miscanthus plants were quite tolerant to metals as no significant differences were observed between plants growing on different soils with an

increasing gradient in metal concentrations. The aim of this discussion is not to examine the influence of metals on *M. x giganteus* physiology which has been already discussed in the first part of this work (D.1-), but to consider its metal tolerance between the two growing seasons, and to compare it with other miscanthus species' ones.

In the present study, at the end of second growing cycle (T2), miscanthus exhibited significant difference in response to the exposure to multiple metal concentrations. The decline in the number of tillers in the contaminated soils in comparison to the non-contaminated ones expressed well the stressful cultivation environment (Table 2). Our data showed a clear decline in the tillers numbers, up to 60% in the highly contaminated in comparison to the MC soil. In comparison, during the first growing season, a decrease was observed only on the more contaminated soil (M900) and only with U cultivar (D.1-). Fernando and Oliveira [28] also demonstrated the negative impacts of soil contamination on the miscanthus growth. However, on two soils multi-contaminated with metals, this impact was less intense with a 33.3% decrease of number of tillers per plant compared to control plants. On the other hand, in the present work, no significant differences were recorded concerning the height of the plants, unlike Fernando and Olivera [28] who showed a 21.6% decrease in the plants cultivated in contaminated soil. Guo et al. (a) [18] as well showed that the height of three miscanthus species (*M. sinensis*, *M. floridulus* and *M. sacchariflorus)* decreased with the increase of Cd concentration (respectively 18.3, 17.1, and 12% shorter than their corresponding controls). Zhang et al. [19] as well observed up to 47.4 % decrease in the height of the *M. sacchariflorus* cultivated in Cd-spiked soil (100 mg kg^{-1}).

The observed negative impact of metal contamination on plant growth can be explained by several deleterious effects induced by metals at physiological and molecular levels [29, 30]. Our results on antioxidant activities clearly suggest that miscanthus is facing an intense oxidative stress (Figures 1 to 3). For each antioxidative enzymes (SOD, APX, GR), a strong activity increase was observed on the M200 soil compared to the control soil (MC), followed by a dose-dependent enhancement. These results contrast with the first growing season (T1), when no significant difference were observed between plants grown on the contaminated soils (D.1-). In addition, the measured activities at T2 were in average 27.7% higher than at T1. However, our T2 results are in accordance with those of Guo et al. [18]. These authors showed a dose-dependent increase of the SOD, APX, and GR activities as well as catalase and peroxidases activities. Nevertheless, the metal-induced increases were 2 to 3 times higher in hydroponic conditions. Conversely, a dose-dependent increase in SOD, APX, and peroxidase activities, followed by a significant decrease at the higher concentrations was observed in *M. x giganteus* plants grown in hydroponic conditions [31] and in *M. sacchariflorus* grown on spiked soils with Cd [19].

In our experiment, this metal-induced oxidative stress was along with a significant decrease in the photosynthetic pigments (Chl a, Chl b, and Car) in the 3 miscanthus cultivars. The response shows the same two-level pattern (Figures 4 to 6). A strong decrease in pigment

concentrations was observed on the M200 soil compared to the MC soil, followed by a slower but concentration-dependent decline. This concentration-dependent pigment concentration reduction was also demonstrated in *M. sinensis*, *M. floridulus* and *M. sacchariflorus* exposed to Cd in hydroponic conditions [18] or in *M. sacchariflorus* grown on Cd-spiked soils [19], but are in contradiction with our T1 results (D.1-). Moreover, in comparison with first growing season results (D.1-), the pigment concentration decays were higher at the end of the second growing season (in average, on the M900 soil , up to 62.9% in T2 vs 37.2% in T1 for Chl a; 56.5% vs 26.1% for Chl b; 54.1% vs 32.4% for Car). These results are in accordance with the results of Guo et al. [18] which show a total chlorophyll loss up to 57.7, 56.8, and 36.6 % in *M. sinensis*, *M. floridulus* and *M. sacchariflorus* plants cultivated in the most Cd-contaminated solution (200 µM), concomitant with, respectively, a 48.6, 44.8, and 20.8% decrease in carotenoids content. In the metal tolerant *M. sacchariflorus* grown on Cd-spiked soils, the decrease in Chlo a, Chlo b and Car contents was up to 34.9, 26.9, and 18.4%, respectively [16].

Secondary metabolism plays an important role in plant metal tolerance and defense against oxidative stress [32]. However, no study has investigated the effect of metals on miscanthus secondary metabolism yet. In our experimental conditions, after one growing season, results showed that all the investigated secondary metabolite (phenolic compounds, tannins, flavonoids, anthocyanins) concentrations increased in response to metal stress (D.1-). However, no significant differences were observed between plants growing on the different contaminated soils. Conversely, at the end of the second growing season, our data demonstrate a clear concentration-dependent response to the soil metal contamination (Figures 7 to 10). The same two-level patterns, as already observed for antioxidative enzymes and photosynthetic pigments responses, were noticed for phenolic compounds, tannins, and flavonoids. Their concentrations strongly increased in plants grown on the M200 soil compared to the MC soil, and then slightly rose in concentration-dependent manner. Anthocyanin accumulation in response to metal contamination did not exhibit this two-level pattern and was totally concentration-dependent (Figure 10). Moreover, as already observed for the other biomarkers, the response intensity was significantly higher but for anthocyanin (on the soil M900 up to 128.8% at T2 vs 39.7% at T1, for phenolic compounds; 197.0% vs 40.2 % for tannins; 222.1% vs 59.1% for flavonoids: 147.6% vs 204.6% for anthocyanins).

Altogether, our data clearly demonstrated that our miscanthus plants were subjected to an important metal-induced stress. During the first growing season, the plants were able to tolerate the increasing concentrations of metals. However, at the end of the second growing season (T2), the evaluated stress was concentration-dependent, and in average twice higher compared to T1. Moreover, this stress was comparable with the level of stress observed in experiments growing several miscanthus species in hydroponic solutions or in spiked-soils, which usually poorly reflect the field reality. These significant differences between T1, T2 and other experiments could be due to the differences in metal uptake. Indeed, at T2, Cd,

Pb, and Zn concentrations in the plants cultivated in contaminated soils were up to, respectively, 2.2, 7.2 and 1.9 times higher than at T1. The T2 Cd concentrations were also comparable to Cd concentrations found in miscanthus grown in Cd-spiked soils [16] or in hydroponic solution enriched with Cd [31, 33]. Despite the fact of using extremely high concentrations of Cd in hydroponic solutions, and of observing a very important stress in *M. sinensis, M. floridulus* and *M. sacchariflorus* leaves, Guo et al. [18] measured lower Cd concentrations in the corresponding leaves. This could be explained by the very short experimentation duration (16 days).

However, these observed metal-induced stresses and impacts on miscanthus biomass are quite surprising regarding the fact that miscanthus is supposed to be a stress-tolerant plant [13]. Moreover, these results are not in agreement with field experiments. Actually, no significant effects of metal contamination on *M. x giganteus* biomass have been demonstrated on our *in situ* experimental plots in Metaleurop area, for almost ten years (unpublished data). Despite the fact that we used 20 kg of soils per pot (which is a big quantity of soils compared to other experiments which do not use more than 8 kg of soils), these results suggest that we over-estimated the metal-induced stress and impacts after two growing season. Although *Miscanthus x giganteus* plants do not transfer intensively metals to their corresponding biomass, yet the prolonged time of metal exposure (18 months) in a precise soil volume and quantity might suggest stronger metal absorption and transport from the roots to the aboveground parts and hence result in increased leaf metal concentrations [18, 19, 34]. The outcome obtained is in conformity with Pourrut et al. [29] who stated that the intensity of the contamination effects on plants depend on the metal concentrations as well as their duration of exposure.

Same conclusion can be drawn for hydroponic and spiked soil experiments. Indeed, several works have criticized these types of experimentations and shed the light precisely on the corresponding metal availability, translocation and accumulation in the plants in culture [35, 36]. For example, Hamels et al. [37] showed that the metal availability and toxicity to barley seedlings grown on field-contaminated soils was up to 30 times lower than that on corresponding spiked soils. Moreover, Sinnett et al. [36] demonstrated that the Cd leaf concentration in a poplar variety grown in hydroponic solution was 30 mg kg^{-1} which was crucially higher than the normal field ranges (0.2-0.8 mg kg^{-1}).

D.2.5- Conclusions and Perspectives

The current work is a part of series of studies that aim to evaluate the efficiency of the *Miscanthus x giganteus* plants in remediating highly metal contaminated soils. After proving the positive impacts of cultivating *M. giganteus* in highly contaminated soils via decreasing metal bioaccessibility and thus alleviating the risks on human, restoring soil functionality and improving its corresponding physico-chemical and biological parameters, the results

obtained in the present study show that the miscanthus number of tillers was negatively affected by the soil metal contamination. Moreover, the corresponding response to the increase soil metal concentration was expressed by declining in the photosynthetic pigment (Chl a, Chl b, and car) levels from one hand, and from the other hand the progressive inductions of the phenolic compounds, tannins, flavonoids and anthocyanin. In addition, the antioxidative enzymatic activities of SOD, APX, and GR were also elevating with the metal concentrations in the soil. It was also recognized that in comparison with the results of the first growing season the response of the plant was intensified as a result of the longer metal exposure duration and the limited volume and quantity of soils presented in the pots. This outcome might pose an important question about the relevance of long term pot experiments in simulating the actual field conditions. Therefore, further *in situ* investigations are recommended to legitimize the obtained results concerning the impact of the metals on the miscanthus plants.

Acknowledgements

The authors are grateful to the Association for Specialization and Scientific Guidance for the PhD scholarship offered to Karim Al Souki. They also wish to thank ADEME (French Agency for the Environment and Energy Management) for the financial support of the Phytener program.

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Part E- General discussion

The former Pb smelter Metaleurop Nord (Northern France) that was functioning for more than a century (1894-2003) had strongly contaminated the soil in the vicinity due to its atmospheric emissions containing high quantity of metals, and particularly Cd, Pb and Zn. The affected area might be considered as a megasite (approximately 120 km^2), showing different degrees of contamination, depending on the distance from the source (Douay, 2014). The impacts on soil functionality, agriculture and human health are quite remarkable and alerting (Douay et al., 2013). Conventional soil remediation techniques are not appropriate to manage this contaminated area considering their high costs and the fact they are environmentally unfriendly (Dermont et al., 2008). However, phytostabilization meets the environmental, economic and social expectations. Therefore, the relevance of two management modes based on the non-food biomass production has been investigated under the Phytener program (2009-2014). The first one was the cultivation of woody plants, and the other was the herbaceous vegetation expressed by the promising C4, perennial and non-invasive *Miscanthus x giganteus* (Douay, 2014).

Several works in our lab demonstrated the suitability of *Miscanthus x giganteus* in the phytostabilization process of the Metaleurop contaminated area for (i) its ability to produce high yield biomass, (ii) minimizing the human and environmental risks via accumulating the metals mainly in the roots, (iii) limiting their transfer to the shoots and thus reducing their potential mobility and bioavailability (Nsanganwimana et al., 2015, 2016; Pelfrêne et al., 2015). However, the impacts of the plant on the contaminated soil functionality and restoration have been rarely assessed. Similarly, the contamination influence on plant health has been poorly studied, and only in artificial conditions (spiked soils, hydroponic cultivations) which generate results that could differ strongly from results obtained on field. Therefore, the current work is complementary to the PhD work of Florien Nsanganwimana. It aimed at assessing the *Miscanthus x giganteus* capacity to restore the soil functionality in the metal-contaminated areas surrounding the former Pb smelter Metaleurop, as well as investigating the impact of the elevated metal soil concentrations on plant health and thus infer its tolerance to stressful environments. For this sake *ex situ* pot experiments were set up for a period of 18 months.

Due to several advantages (the uncollected fallen leaves reduces soil nutrient losses and prevents weed growth; drier biomass does not require drying storage or special equipment (Nsanganwimana et al., 2014; Roncucci et al., 2015), late harvest of miscanthus plots was favored on Metaleurop area. As the miscanthus plants approached their tenth consecutive year of cultivation, it was quite interesting to consider the long-term effects of their corresponding leaf incorporation into the soil. However, the collected samples represented the mid-term effects, and for this reason, it was decided to mimic, under laboratory-controlled conditions, the artificial aging process of the soil. The aim was to extrapolate the long-term effects of plant residue decomposition on the corresponding metal speciation and bioavailability in soils, on the soil biological activities as well as the subsequent future crop

that might replace the miscanthus plants in the field. For practical purposes, the ryegrass was chosen.

E.1- Impacts of soil metal contamination on the miscanthus plant health: critical evaluation

To investigate the impacts of the elevated metal concentrations in the agricultural plots surrounding the former Pb smelter Metaleurop on miscanthus health and thus infer its tolerance to stressful environments, two sampling campaigns were held (T1: October 2014 and T2: October 2015) from the *ex situ* experiment. The results obtained at the end of the first and second growing seasons clearly demonstrated a metal-induced stress: oxidative stress, decay of photosynthetic pigments, and increase of secondary metabolites concentrations. At the plant scale, the number of tillers per pot and stem height decreased in the plants growing in the heavily contaminated soils in comparison with those in the uncontaminated soil, inferring a decrease in the biomass. However, our data show significant differences between the two growing seasons. At T1, soil metal contamination induced stress in miscanthus plants, but no differences were observed between little and high contaminated soils (D.1). In addition, only slight effects were observed at macroscopic level. This suggests miscanthus plants are able to cope with high level of metal contamination and quite resistant to metal-induced stress. In the other hand, at T2, a stronger and concentration-dependent impact was measured, combined with significant negative effects on plant growth (D.2). These last data are totally in agreement with the few articles dealing with metal-induced stress on miscanthus plants (Guo et al., 2016; Scebba et al., 2006; Zhang et al., 2015).

All together, these results are surprising as they are in contradiction with the field experiment results obtained by Nsanganwimana (2014) during his PhD work. He did not observe any negative impact of soil metal contamination on the corresponding plant biomass (Figure 20). Miscanthus harvest yield was even higher on contaminated plots than on the non-contaminated one. Actually, Nsanganwimana's work (2014) demonstrated that miscanthus growth is more influenced by soil agronomic parameters than metal contamination. Preliminary results obtained using biomarkers on miscanthus grown on field also confirm this conclusion (Pourrut et al., unpublished results).

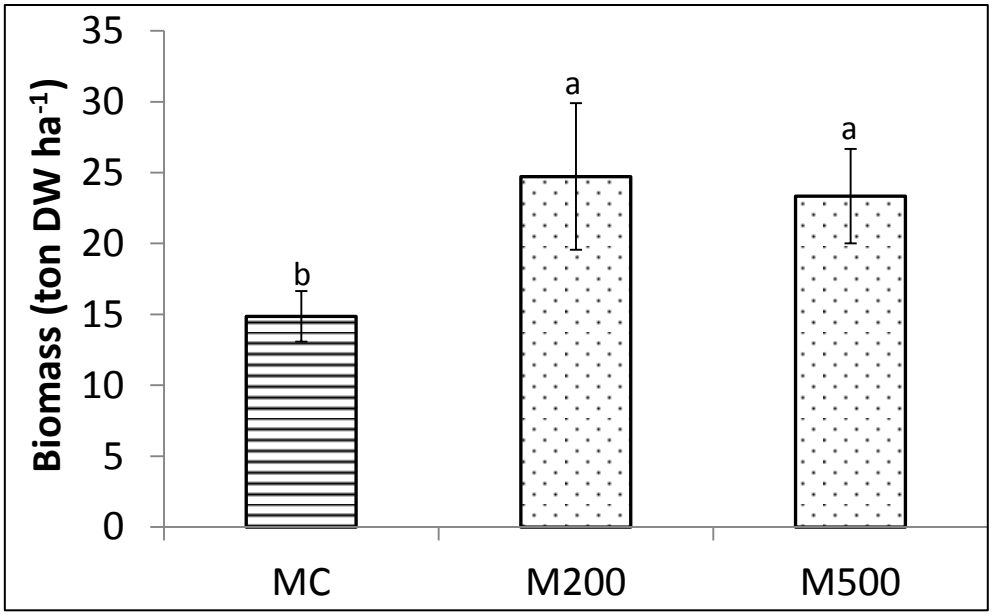

Figure 20: 6-year old Miscanthus biomass (ton DW ha^{-1}) in MC, M200 and M500 agricultural fields. Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, n = 5, $p \leq 0.05$) (Nsanganwimana, 2014).

We drew the hypothesis that the main reason behind the difference in the intensity of response might be the significant differences in the corresponding plant leaf metal accumulation. We probably over-estimated metal-induced stress at T2, due to a metal over-accumulation. Indeed, at T2, the metal accumulation in leaves of miscanthus grown on contaminated soils were up to 2 times higher for Cd and Zn, and 7 times higher for Pb than at T1. Moreover, this metal accumulation at T2 was similar to the ones measured on spiked soils (Zhang et al., 2015) or hydroponic cultivations (Arduini et al., 2006; Scebba et al., 2006).

The comparison of metal uptake and translocation to aboveground part between miscanthus plants grown on field in Metaleurop area and in pots (results from the current study) also supports our hypothesis (Table 3). Two field sampling campaigns were organized in 2011 and 2014. The results obtained show that the corresponding leaf metal concentrations in the plants from the field were approximately identical and no significant variations were determined among them. Moreover, the corresponding pot results in 2014 were quite similar and comparable despite a significant increase in Cd accumulation in the highly contaminated soils. However, the leaf metal concentrations in the T2 plants (grown in pots) were significantly higher than the ones from plants grown on field: Cd, Pb, and Zn concentrations were respectively up to 4.3, 2.1 and 2.1 times higher on M900 soil.

		Cd (mg kg^{-1})	Pb (mg kg^{-1})	Zn (mg kg^{-1})
MC	Field 2011	0.43 + 0.03 h	12.46 + 0.99 de	27.56 + 2.13 k
	Field 2014	0.42 + 0.02 h	10.09 + 0.69 ef	31.41 + 2.29 k
	Pot 2014	< LD*	< LD*	32.18 + 2.8 jk
	Pot 2015	0.38 + 0.02 h	6.86 + 0.82 f	37.20 + 3.14 ijk
M200	Field 2011	0.42 + 0.02 h	10.26 + 1.09 ef	46.66 + 2.92 hij
	Field 2014	0.43 + 0.03 h	9.64 + 2.29 ef	44.73 + 2.62 ij
	Pot 2014	0.49 + 0.04 gh	< LD*	60.53 + 2.6 fgh
	Pot 2015	0.72 + 0.14 fg	12.56 + 1.41 de	76.06 + 5.27 d
M500	Field 2011	0.46 + 0.03 h	9.79 + 0.58 ef	51.66 + 4.60 gh
	Field 2014	0.46 + 0.04 h	11.87 + 1.48 de	55.80 + 5.13 gh
	Pot 2014	1.04 + 0.13 e	< LD*	59.71 + 7.1 fgh
	Pot 2015	1.40 + 0.13 d	19.77 + 1.74 c	90.95 + 4.83 c
M750	Field 2011	0.83 + 0.04 ef	13.70 + 1.24 de	61.86 + 4.43 efgh
	Field 2014	0.69 + 0.04 fg	12.85 + 2.16 de	64.60 + 5.69 defg
	Pot 2014	1.76 + 0.33 c	< LD*	72.89 + 8.5 de
	Pot 2015	2.37 + 0.14 b	25.92 +3.79 b	114.72 + 4.55 b
M900	Field 2011	0.69 + 0.03 fg	14.95 + 1.03 d	62.02 + 6.13 efgh
	Field 2014	0.77 + 0.06 f	14.50 + 1.33 d	68.66 + 5.58 def
	Pot 2014	2.20 + 0.12 b	< LD*	73.99 + 8.9 de
	Pot 2015	3.14 + 0.10 a	30.99 + 2.13 a	138.19 + 4.08 a

Table 3: Leaf metal concentrations of the miscanthus B plants cultivated in the field for the years 2011 and 2014 and in the pots for the years 2014 and 2015 in soils with a gradient metal concentration (MC, M200, M500, M750, and M900). Values are presented as means ± SD. Different letters refer to significant differences between plants (Turkey HSD test, $n = 5$, $p \leq 0.05$).*: Limit of detection (Cd: 0.4 mg kg^{-1}, Pb: 4.3 mg kg^{-1})

Despite the fact that we cultivated our miscanthus plants in 20 kg-pot, which could be considered as a large quantity of soil in comparison with other pot experiments found in the literature (where miscanthus is grown in pots of less than 8 kg), our second growing cycle does not reflect the reality of what happens on field, and over-evaluated the impact of contamination on miscanthus plant health. In general, plants grown in pot experiments may contain higher concentrations of metals than those grown in the field (Zhivotovsky et al., 2011). The differences in metal uptake between field and controlled pot conditions may be attributed to the different physiological state of the plant and/or to some modifications of soil parameters in the pot conditions. To begin with, the soils used in the pots were dried before launching the experiment. Soil drying modifies the distribution of the metal fractions resulting in an increase in the water soluble and exchangeable fractions (Nowack et al., 2004; Wang et al., 2002). Moreover, soils were sieved and coarse fragments were broken up.

The homogeneous mixture resulting from this preparation may have increased the contact between roots, soil water and the soil matrix (Conesa et al., 2007).

On the other hand, miscanthus plants in the field had a higher biomass and were older than the ones in the pot experiment. Plants may be more vulnerable to metals in the early stages of their life cycle (Cheng, 2003). Lower metal concentrations in older plants can also be explained by the phytodilution process due to the higher biomass present in the field than in the pots (Robinson et al., 1998). In addition, the differences in root exudates or other rhizosphere processes may have contributed to the lower accumulation in the field. Root exudates can modify the soil parameters around the roots (Wang et al., 2002). Adult plants in the field do not only have a more developed root system and have more time to modify the rhizosphere, they can also invest more into exudation than the younger plants (Wang et al., 2002). Finally, in the field, miscanthus roots can progressively extend downwards to the deeper soil layer that is less contaminated, resulting in a decrease in metal uptake with time unlike the situation in the pots (Hu et al., 2013). This fact was confirmed by Sterckeman et al. (2000) who demonstrated that the soil contamination in the agricultural plots in the Metaleurop site is limited to the ploughed horizon.

Same statements could draw on spiked soils and hydroponic experiments, which have been published. Indeed, several studies have investigated the reliability of hydroponic and metal spiked soil experimentations on the phytoavailability of the corresponding metals. The outcome was not so promising, in which it was evident that the metal concentrations in the plants were largely exceeding the field experimental results. For instance, the work of Migeon (2009) demonstrated that the metal concentrations of the poplar species grown in hydroponic solutions were way more than the corresponding field concentrations. Sinnett et al. (2006) as well developed simple models of metal uptake by one variety of poplar, which were developed based on metal spiked hydroponic trials and compared them with results from pot and field experiments. The results showed that the Cd leaf concentrations were approximately 30 mg kg^{-1} and considered substantially higher than the normal range of 0.2-0.8 mg kg^{-1}. The corresponding high variation between the hydroponic and field trials might be attributed to the high availability of the metals in the solution in comparison to the field soils. It could also be due to the absence of interactions between the roots and the soil particles and microorganisms which are absent in the hydroponic culture, which crucially affects the absorption and translocation of the corresponding metals (Migeon, 2009; Sinnett et al., 2006).

The metal availability in the spiked soils also does not reflect the actual field values. For instance, Hamels et al. (2014) showed that the metal toxicity to barley seedlings grown on field-contaminated soils was up to 30 times lower than that on corresponding spiked soils. In their work, McBride et al. (2009) stated that the efficiency of Cu and Zn extraction from field-contaminated soils was much lower than that from the laboratory-spiked soils. For Mehlich 3 and DTPA tests, Cu and Zn in field-contaminated soils were less extractable by a

factor of about 2 compared with the spiked soils. With less aggressive tests (i.e. $CaCl_2$ extraction), the difference in extractability was even greater. These finding suggest that field-contaminated soils have a small and highly variable metal exchangeable metal fractions depending on their corresponding speciation (Hamels et al., 2014).

E.2- Impacts of soil metal contamination on the miscanthus plant health: 1st growing cycle

As stated before, the metal accumulation in the miscanthus plants after the first growing season was comparable with the results obtained in the agricultural field experiments (Table 3). Thus, to evaluate metal-induced stress on miscanthus plant, we consider only the first year of cultivation. In our experiment, the leaf Cd and Zn concentrations were increasing with the soil gradient concentration, unlike Pb concentrations which were always below the limits of detection (D.1-). However, as consequences, only slight influences of metal contamination were observed on plant growth. Interestingly, no significant differences were found between plants grown on contaminated soils, despite the increasing metal concentrations. These results are totally in agreement with those of Nsanganwimana (2014). During a 3 month greenhouse experiment, this author cultivated miscanthus plants (Bical cultivar) on MC, M200, M500 and M750 soils, and observed the same trends. The results confirmed an augmenting accumulation of Cd and Zn in leaves and stems, as well as no variation for Pb concentrations. Meanwhile, Nsanganwimana also measured a negative effect of metal contamination on leaf number, stem height, stem diameter and shoot dry weight, but no significant differences were observable between plants cultivated in the different soils.

To understand better the effects of soil metal contamination on miscanthus plants, we evaluated their health using a set of biomarkers to monitor oxidative stress, photosynthetic pigments, and secondary metabolism (D.1-). Certain response variations were triggered in the plants cultivated in the contaminated soils, indicating that they were under stressful conditions. There were two types of responses comprising the results of the plants of the uncontaminated soils and the plants of the contaminated soils. Significant variations were rarely detected among the plants of the contaminated soils. In other words, the metal-induced physiological stress was not dose-dependent and these results confirm the ones observed at macroscopic level (plant biomass, number and diameter of stems…). Altogether, our data show that miscanthus is slightly affected by metal contamination and can easily cope with extreme level of pollution.

This outcome contrasts with the results of a parallel experimentation established in the laboratory greenhouse. In this experiment, miscanthus plants but also ryegrass (*Lolium perenne*) and clover (*Trifolium repens*) plants were grown on the same soils with the same gradient of contamination (Pourrut et al. in preparation). These two plants are commonly

used in phytoremediation experiments (Bidar et al., 2009). However, preliminary results (Figure 21) clearly demonstrated that these plants suffered from severe stress with the increase in the metal concentration: intense oxidative stress (increase in antioxidant enzymes activities, and lipid peroxidation), significant photosynthetic pigment concentration decrease, severe DNA degradation… In comparison, miscanthus plants tolerated very well these stressful conditions. The results on photosynthetic pigments (Figure 21) and antioxidant enzymes (data not shown) are totally in agreement with our pot experiment data.

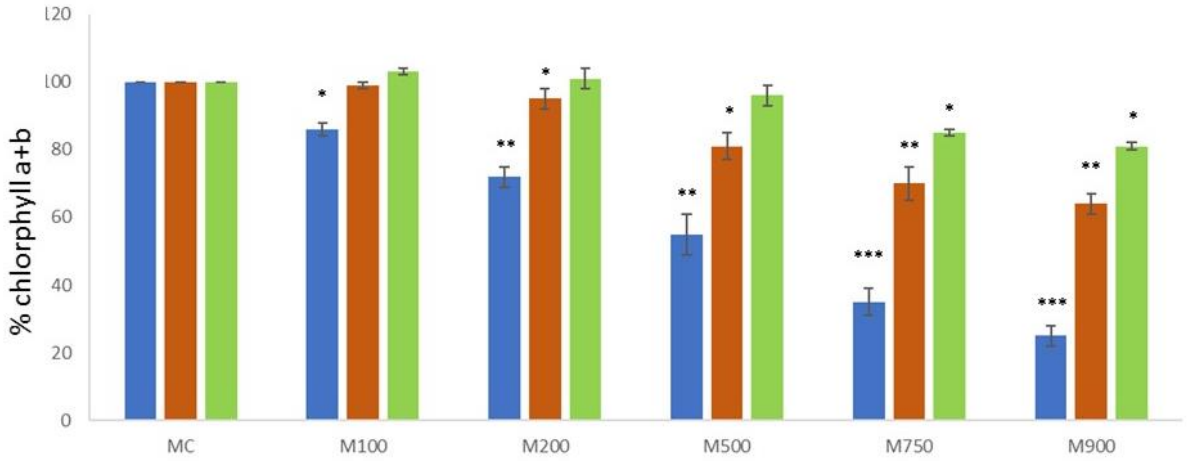

Figure 21: Influence of metal contamination on chlorophyll a+b contents (expressed in percentage compared to control plants) in ryegrass (*Lolium perenne*; blue bars), white clover (*Trifolium repens*; red bars) and miscanthus (*Miscanthus x giganteus* cultivar; green bars) plants grown for 3 months on MC, M100, M200, M500, M750, and M900 soils, in pots experiments in greenhouse. Values are presented as means ± SD. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (Anova test, $n = 6$). From Pourrut et al. (in preparation)

Interestingly, miscanthus plants exhibited almost no lipid peroxidation, and no DNA degradation was noticed even at the highest concentrations. In the other hands, all these biomarkers analysis were performed on plants grown in pots (outside or in greenhouse) which could induce a bias as already discussed in (D.1-). Considering together the miscanthus yield on field (Figure 20) and the slight influence of metal in pot experiments, our data strongly confirm the high level of tolerance for metal of miscanthus, and its suitability for remediation of metal contaminated area.

E.3- Positive impacts of the miscanthus on the soil

E.3.1- Root influence on soil system

The *ex situ* pot experiment showed the positive impacts of the miscanthus plant on increasing the overall SOC by 35.7% (C.1-). Similar results were observed on non-contaminated soils (Hromádko et al., 2010; Kaňová et al., 2010) and on fly ashes from a

lignite power-plant landfill (Techer et al., 2012a). The positive aspects of increasing soil organic carbon (SOC) are numerous, such as soil stabilization (Conant et al., 2004), soil aggregate formation (Jastrow et al., 2007), increase of fertility and soil water capacity (Lal, 2004). Moreover, the increase in the corresponding SOC, due to miscanthus cultivation, decreased the metal availability from the soils (decrease of up to 40% in metal $CaCl_2$-extractability; C.1-), via enhancing the metal association and binding in the soil forming insoluble metal-organic complexes (Epelde et al., 2009; Rieuwerts et al., 1998), and thus reduces the human health risks (Pelfrêne et al., 2015).

Our experiments also showed the positive impacts of the miscanthus plant on restoring the contaminated soil functionality via enhancing the different bacterial activities and biomass (C.1-). Similar results were observed on non-contaminated soils (Hromádko et al., 2010), PAH-contaminated soils (Techer et al., 2012b), and on fly ashes from a lignite power-plant landfill (Techer et al., 2012a). In our work, the fallen leaves were removed constantly and excessively from the soil surface and the shoots were harvest. Therefore, the obtained enhancement in soil bacterial activities and biomass only reflects the influence of miscanthus roots on the soil system.

Metal-contaminated soil functionality restoration could be explained by root rhizodeposition, providing a suitable environment for the growth and development of microorganisms (Stępień et al., 2014). Indeed, this phenomenon is the major source of substrates for microbial activity in the rhizosphere (Lynch and Whipps, 1990). Rhizodeposition compounds comprise exudates, lysates mucilage and secretions, that microbial populations colonizing the soil might have a great access to and induce the corresponding increase in their biomass upon utilization. Hromádko et al. (2010) demonstrated that *M. x giganteus* roots have a very high rate of root exudation compared to other plants such as rice (*Oryza sativa*), sorghum Sudangrass (*Sorghum × drummondii*) or crested wheatgrass (*Agropyron cristatum*). These exudates are composed of carbohydrates (mainly glucose and saccharose; Kaňová et al. (2010), organic acids (succinic > propionic > citric > tartaric > malic > oxalic > ascorbic > acetic > fumaric; Kaňová et al. (2010)) and amino acids (mainly aspartic acid, arginine, alanine and glutamic acid; Hromádko et al. (2010)). Otherwise, in an *in vitro* study, Techer et al. (2011) observed a considerable promotion in the bacterial growth and PAH-degradation activity in microplates enriched with miscanthus root exudates. These exudates contained a broad range of flavonoid-derived compounds might have played a role in the cometabolic processes. More precisely, their work suggested a partial involvement of quercetin and rutin in bacterial biostimulation process. All these organic compounds released by miscanthus roots play a role in metals availability in soil, their uptake by plant root and translocation to aboveground part (Harter and Naidu, 1995; Shahid et al., 2012). However, their exact role is very complex and depends on several factors (type of metal, soil physico-chemical parameters…).

E.3.2- Influence of late harvest on soil properties

Normally, the best period for the harvest of miscanthus plants is considered between mid-December to the end of February which is termed late harvest (Bilandzija et al., 2014). There are several advantages for late harvest, among which guaranteeing more dryer shoots with less moisture contents, less ash, chlorine, nitrogen, and sulfur which assure better combustion properties (Bilandzija et al., 2014). It also allows the translocation of nutrients from the aboveground organs to the rhizome, and thus complementing their corresponding loss and consumption throughout the growing cycle and creating a reserve for further usage in the subsequent year of plant growth (Nsanganwimana, 2014; Roncucci et al., 2015). Moreover, delaying the harvesting time saves storage place and drying equipment. However, in the case of miscanthus grown on metal-contaminated area (case of Metaleurop site), the main concern is the quantity of metals in the leaves, which will fall and accumulate on the soil surface. This leaf fall creates a possibility to metal re-introduction in soils and their mobilization and could modify their speciation and thus influence bioavailability in the soil due to the decomposition and degradation of the organic matter over long period of time (not less than 20 years), given that the average life span of the plant is at least 20 years.

Upon documenting the impact of the root exudates on the different soil parameters via the pot experiment (leaf influence was omitted due to their corresponding removal from the soil surface in the pots), the impact of the miscanthus leaves was investigated through the artificial aging experiment established in the laboratory under controlled conditions (C.2-). Reversely, the root exudation impact was not considered in this experiment. To begin with, the incorporation of the leaf fragments induced slight positive alterations in the physico-chemical parameters of the soil. In details, the pH increased mainly in the MC soil in comparison with the non-amended ones especially at the beginning of the experiment. The available phosphorous concentration progressively increased as well throughout the entire incubation process. More importantly, we were able to answer the main concern and objective of the corresponding experiment, concerning the influence of the leaf litter incorporation on the metal extractability and availability in the soil. If a slight increase in the metal $CaCl_2$-extractability was observed due to the organic matter incorporation, no influence on metal phytoavailability was noticed. Moreover, ryegrass health was not affected by this organic incorporation, in which no modification on the antioxidative plant response nor on photosynthetic pigments was measured.

Otherwise, the leaf litter incorporation contributed in the increase of the organic carbon by approximately 13.2 % in the amended soils, as well as in the increase of the soil biological parameters. Soil basal respiration and microbial biomass carbon increased upon the leaf addition due to the input of the organic compounds that were used by the soil microorganisms to proliferate. However, SOC increase linked with 20 years of litter incorporation into soil could be considered as negligible in comparison with the 35.7 % increase of SOC due to root rhizodeposition after a single growing season (Hromádko et al., 2010; Kaňová et al., 2010). Moreover, Amougou et al. (2011) demonstrated that the accumulation of carbon, in field, in miscanthus rhizomes and roots was 7.5 to 10 t C ha^{-1}

after 3 years of cultivation compared to 1.5 t C ha^{-1} per year in the senescent leaves. Beuch et al. (2000) as well observed similar outcome based on a field study of 4-8 year old stand of *Miscanthus x giganteus*. These authors stated that on long-term bases the potential supply to soil organic carbon was 3.1 t C ha^{-1} annually by the leaf litter and 9.1 t C ha^{-1} by the rhizomes and roots.

Altogether, late harvesting in Metaleurop area can be considered a positive strategy from both an economic and environmental point of view. Moreover, Ruf and Emmerling (2017) recently demonstrated the adverse effects of early harvest (autumn) on soil microbial parameters, as well as earthworm community, in comparison with the late harvest.

Part F- Conclusions and perspectives

The main objective of this PhD work was to investigate further the suitability of miscanthus to manage metal contaminated soils and particularly in Metaleurop Nord area. First, the miscanthus plants have been proved as great candidate for phytostabilization of metal-polluted sites for its fundamental impacts on (i) decreasing metal mobility and availability, (ii) enhancing the soil organic carbon pool, and (iii) restoring the degraded soil functionality via improving the biological parameters.

Second, based on the *ex situ* soil artificial aging experimentation, we can predict that the late harvest of miscanthus is a good and an interesting strategy. Indeed, the incorporation of the miscanthus leaf litter in the soil had certain positive impacts on augmenting the soil available nutrient concentrations (carbon, phosphorous…) and enhancing the soil biological parameters as well (basal respiration, microbial biomass carbon). In addition and most importantly, the impacts of the corresponding incorporation on soil total and bioavailable metal concentrations, as well as on the subsequent culture (ryegrass in our case) health were negligible.

If this PhD work provided a significant number of interesting results, it raises many questions, and open new research paths to investigate the soil-miscanthus system:

- Effect of miscanthus on soil functionality: *in situ* experimentations must be lead to confirm our *ex situ* results. Moreover, we investigated only some global biological soil parameters (respiration, biomass, specific activities). In order to complete our understanding on miscanthus influence on soil carbon, nitrogen, and phosphorus cycles, it could be interesting to study other soil enzymes that play an important role in these cycles such as β-glucosidases or proteases.
  In addition, the determination of the rhizosphere microbial diversity sounds an interesting idea. It can be measured by various techniques such as traditional plate counting and direct counts as well as the fatty acid analysis or the newer molecular-based procedures (metabarcoding, metagenomics). A more detailed analysis of microbial gene expression in the plant rhizosphere could be also considered to understand better plant effects.

- Mechanisms of miscanthus influence on soil: as already performed by Techer et al. (2012a) on PAH-contaminated soils, the composition determination of root exudates on metal-contaminated soils could help us to understand the influence of miscanthus on the soil system. Several techniques of root exudate collection exist (Vranova et al., 2013), but most of them are based on hydroponic cultivation or on solid medium with nutrient solution (quartz sand, perlite, vermiculite…). Beside the already discussed bias induced by these types of experimentations at the beginning of the discussion, hydroponic sampling of root exudates may lead to underestimation of exudation rates after 24h of assay (Oburger et al., 2013). However, certain techniques for root exudate collection (such as

the Rhizobox) that allow non-destructive and repetitive sampling might enhance our knowledge of fundamental rhizosphere processes.

- Soil carbon pool: soil organic carbon changes associated with land conversion to energy crops are central to the debate on bioenergy and their potential carbon neutrality. A simple comparison between the cultivation of annual crops and the perennial C4 bioenergy crops, demonstrates that the annual crop cultivation leads to the production of carbon debts due to high CO_2 emissions from soil as a result of SOM mineralization (Fargione et al., 2008). However cultivation with perennial bioenergy crops might transform the soil from carbon source to carbon sink (Fargione et al., 2008; Zatta, 2013). Since the miscanthus plants has been cultivated for 10 years on MC, M200, and M500 plots, it might be interesting to determine if their culture have affected the soil carbon pool. This could be done by comparing miscanthus plots with adjacent annual crop plots.

- Miscanthus plant health *in situ*: our results clearly demonstrated that pot experiments tend to force the soil-plant system and over-estimate the impact of metals on miscanthus plant health. Therefore, it was initially planned in this PhD work to compare *ex situ* and in *situ* results obtained on miscanthus experimental plots around Metaleurop, since the plant will be growing in an unrestricted area with unlimited soil quantity. Unfortunately, due to time constraints, it was impossible to include them in this PhD thesis. However, the analysis of the same plant health biomarkers (oxidative stress, photosynthetic pigments, and secondary metabolism) is currently ongoing in the laboratory. The plant samples were collected during the 2011 (PhD work of Florien Nsanganwimana) and 2014 sampling campaigns (current PhD work). The comparative analysis of these data will help us to understand the real impact of metal contamination on miscanthus plants and their corresponding responses.

- Miscanthus tolerance to metals: in order to understand better miscanthus tolerance to metals, further investigation using different biomarkers could be performed ex situ and in situ. It could be interesting to analyze plant defenses against metal such as concentrations in chelating molecule (glutathione, phytochelatins…) or to investigate histological localization of metal into leaves. Moreover, it would be interesting to investigate further the metal impacts on plant physiology, and especially on redox status (glutathione, ascorbate…), energy status (ATP/NAD/NADP) or sugar metabolism. The development of high-throughput methods to analyze these new biomarkers is currently ongoing in the laboratory.

- Miscanthus cultivar selection: our study is based on three different cultivars. Two of them has been used by the laboratory for several years (B and A). In this study, it was decided to integrate a new cultivar (U) provided by the University of Iowa, which exhibits a very

different morphotype (shorter stems, more tillers). However, our results as the ones of Nsanganwimana (2014) did not show any significant differences between miscanthus cultivar responses. It would be interesting to trial new miscanthus cultivars to select highly productive and metal tolerant cultivar, which could also accumulate less in their aboveground organs. Some contact has been taken with the FP7 project OPTIMISCS which aims at trialing elite germplasm types to optimize production of bioenergy and bioproducts from miscanthus.

- Suitability of miscanthus to phytomanage other types of soils: the research works conducted during the last years by LGCgE (including the current work) have clearly demonstrated the suitability of *Miscanthus x giganteus* to phytomanage the contaminated soils around the former lead smelter Metaleurop Nord. It would be interesting to investigate its influence on different types of soils (acid, sandy, technosoils…) and pollutions (organic pollutants).

- Influence of miscanthus cultivation of succeeding culture: our experiment of leaf litter incorporation suggested that the input of organic matter derived from the fallen leaves slightly influences soil physico-chemical and biological parameters. However, these results are based on an artificial aging experiment. Leading experiment on field, by destroying a section of the MC, M200 and M500 plots, which have been cultivated with miscanthus for 10 years, could provide data about the influence of 10 years of miscanthus culture. Moreover, incorporating the corresponding cultivar leaves in soils, permitting the aging of the soil for certain time and thereafter cultivating another culture in the field, would be considered a good idea for the sake of validating the obtained results in the current work.

Summing up, the world is heading more and more towards utilizing phytotechnologies for energy production as well as remediating the different contaminated areas. The promising *Miscanthus x giganteus* crop is being invested on both sides as it is suitable for bioenergy production and phytostabilization of metal and organic contaminated sites. However, further investigations need to be established combining *in* situ and *ex* situ experimentations for the sake of investigating deeper the potentiality of *Miscanthus x giganteus* in phytomanaging marginal lands, technosoils, soils contaminated by mixture of organic and metal pollutants…

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