Universitat Autònoma de Barcelona

The occurrence of contaminants in crops grown under organic soil amendments and peri-urban soils: phytotoxicity and human health implications

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PhD programme in Environmental Science and Technology

DOCTORAL THESIS

The occurrence of contaminants in crops grown under organic soil amendments and peri-urban soils: phytotoxicity and human health implications

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Barcelona, November 2020

WE DECLARE:

That the thesis titled "The occurrence of contaminants in crops grown under organic soil amendments and peri-urban soils: phytotoxicity and human health implications" presented by Rui You to obtain a doctoral degree, has been completed under our supervision and meets the requirements to opt for a Doctoral degree for the UAB

For all intents and purposes, we hereby sign this document.

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This thesis was financially supported by Spanish Ministry of Economy, Industry, and Competitiveness through Project AGL2017-89518-R and China Scholarship Council through a scholarship.

Acknowledgements

First and Foremost, I would like to give special thanks to my supervisor, Sergi Díez, for giving me the opportunity to do research in his laboratory, for excellent support and guidance in the past four years. His conscientious academic spirit, open-minded personality and positive attitude toward life inspire me both in academic study and daily life. Also, I would like to express my sincere gratitude to my tutor, Dr. Montse, who gives me much help and advice during the whole period of my Ph.D study, which has made my accomplishments possible.

My sincere gratitude to dream group in IDAEA, in which people come and go, but it maintains unity and enthusiasm. I would like to thank all these people I met here. To Dr. Josep Maria Bayona and Dr. Victor Matamoros, for all the contributions, advice and help they have offered over the years. To Dr. Carmen, for your patience, you always offer timely help when I need something. To Yolanda, for your support and company, without you, the experiment will be boring and tough. To Sandra, for your persistence in improving my Spanish, and trust me can do better. To Marta, for your generously offered help with the experiment, it is you who guide me to be familiar with the various equipment in the laboratory. To Siday, for your concern and support， you have the superpower to warm others. To Anna, for your selfless and computer knowledge. Whenever I encounter a problem with computer technology, you are always the first person I ask for help. To Đorđe, for your humor, you always make people around you feel relaxed and happy. To Carles, for your friendliness and kindness Although we have only been gotten along for a year, I always remember your smile.

I also cannot forget all members from the DAMA project. I want to thank Dr. Núria Cañameras, Dr. Núria Carazo , Dr. Benjamin, Claudia, Marta Casado. Without their help , experimental design, sampling, and analysis would be impossible.

Thanks to China Scholarship Council, who financed the expenses of my Ph.D. study here.

Last but not least, I am very grateful to my family, especially my parents for their unconditional support. their care and trust motivate me to move on and make me want to be a better person.

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List of acronyms

ARB	antibiotic resistant bacteria
ARG	antibiotic resistant gene
ATP	adenosine triphosphate
AZM	azithromycin
CECs	contaminants of emerging concern
CF	chemical fertilizer
CIP	ciprofloxacin
DW	dry weight
EC	European Commission
EU	European Union
FW	fresh weight
HQ	hazard quotient
HGT	horizontal gene transfer
Kd	soil-water partition coefficient
Kow	octanol-water partition coefficient
LIN	lincomycin
MGE	mobile genetic elements
OECD	organization for Economic Cooperation and Development
OFMSW	organic fraction of municipal solid waste
OTC	oxytetracycline
PAHs	polycyclic aromatic hydrocarbons
PCA	principal component analysis
PM	particulate matter
POPs	persistent organic pollutants
PPCPs	pharmaceutical and personal care products
RfD	reference dose
SM	swine manure
SMZ	sulfamethoxazole
SS	sewage sludge
TEs	trace elements
THQ	total hazard quotient
UPA	peri-urban agriculture
WHO	World Health Organization
WWTPs	wastewater treatment plants

DEET	N,N-Diethyl-meta-toluamide
TCEP	Tris(2-chloroethyl) phosphate
BPF	Bisphenol F
MPB	Metylparaben
OHBT	1-Hydroxybenzotriazole
TCPP	Tris (chloroisopropyl) phophate

Abstract

Modern agriculture has been continually searching for effective methods to meet the exponentially increasing food demand. Amending soil with fertilizers has been widely adopted, as it could efficiently and fast supply nutrients to vegetables.

Since 2015, the European Commission has proposed a circular economy plan which encourages the soil amendment with biosolids. Nevertheless, the presence of a wide range of contaminants, such as trace elements (TEs), organic pollutants, and emerging pollutants such as antibiotics (ABs) and antibiotic resistance genes (ARGs), has been widely reported in many organic fertilizers. Vegetables can absorb the contaminants from the amended soil, and further threaten human health. For this reason, risk assessment of organic fertilizer applications is necessary.

The most commonly used organic fertilizers are animal-based waste (manure), compost (plant sources or food waste), and urban waste (sewage sludge and household waste). Currently, no study evaluates the plant uptake of TEs and ABs under these three organic fertilizers, nor research evaluates the impact of repeated organic fertilization. Additionally, multiple application of sewage sludge might result in the accumulation of Zn and Cu in amended soil, and their presence would influence uptake of other contaminants.

Therefore, in this doctoral thesis, three aspects related to the contaminants in vegetable under different agriculture activities are addressed: (1) amending soils with different doses of different organic fertilizers, which aims to assess the impact of organic fertilizers on the occurrence of TEs and ABs in vegetables, (2) repeated amending soil with organic fertilizers and monitoring the variation of TEs concentrations in vegetables of different productive cycles, which aims to assess the impact of long-term organic fertilization, and (3) amending soils with sludge and different amounts of Zn and Cu, which aims to assess the effect of Zn and Cu on the accumulation of TEs, ABs, and ARGs in vegetables. Furthermore, in every case the risk to human health associated with the consumption of vegetables was evaluated.

In peri-urban area, the agricultural soil may receive the potential pollution from fertilizer, but also from potential contaminants due to urban activities. Those pollutants would influence the growth and development of vegetables. In this thesis, a simple and rapid method to assess soil pollution was also developed. Here, we use two plant growth indexes (seed germination rate and root elongation at the initial stage) for three vegetable seeds to assess soil chemical contamination on proximity agriculture.

Resumen

La agricultura moderna ha estado buscando continuamente métodos efectivos para satisfacer la demanda de alimentos que aumenta exponencialmente a nivel mundial. La aplicación de residuos orgánicos como fertilizante o enmienda de suelo es una medida ampliamente aceptada, ya que suministra nutrientes de manera eficiente y rápida a los cultivos. De hecho, desde 2015, la Comisión Europea ha propuesto un plan de economía circular que fomenta la enmienda del suelo con biosólidos. No obstante, la presencia de una amplia gama de contaminantes como elementos traza (ET), contaminantes orgánicos y contaminantes emergentes como antibióticos (AB) y genes de resistencia a antibióticos (ARG), ha sido reportada en algunos fertilizantes orgánicos. Las verduras pueden incorporar los contaminantes procedentes de suelos fertilizados con residuos orgánicos, amenazando la salud humana. Por esta razón, es necesaria la evaluación de los riesgos que puede provocar la aplicación de estos residuos orgánicos.

Los fertilizantes orgánicos más utilizados son los desechos de origen animal (estiércol), el compost (fuentes vegetales o desechos de alimentos) y los desechos urbanos (lodos de depuradora y residuos domésticos). Actualmente, no existe ningún estudio que evalúe la incorporación de ET y AB por parte de las plantas con estos tres tipos de fertilizantes orgánicos, ni tampoco el impacto de la aplicación repetida de fertilizantes orgánicos sobre las mismas parcelas. Además, esta aplicación repetida de lodos de depuradora resultaría en la acumulación de Zn y Cu en el suelo modificado, y su presencia puede influir en la incorporación de otros contaminantes.

Por tanto, en esta tesis doctoral se abordan tres aspectos relacionados con los contaminantes en hortalizas en diferentes actividades agrícolas: (1) aplicación de diferentes dosis de distintos residuos orgánicos a suelos de cultivo, que tiene como objetivo evaluar el efecto de esta fertilización en la incorporación de ET y AB en la hortaliza, (2) aplicación reiterada de residuos orgánicos en suelos de cultivo y seguimiento de la concentración de ET en hortalizas durante diferentes ciclos productivos, cuyo objetivo es evaluar el impacto de la fertilización orgánica a largo plazo, y (3) aplicación de lodos de depuradora y diferentes cantidades de Zn y Cu al

suelo, con el objetivo de evaluar el efecto de estos metales sobre la acumulación de otros contaminantes como ET, AB y ARG en las hortalizas. Además, en todos los casos se evaluó el riesgo para la salud humana asociado al consumo de hortalizas.

En áreas periurbanas, el suelo agrícola puede recibir la contaminación potencial de contaminantes procedentes de suelos fertilizados con residuos orgánicos, pero también los contaminantes potenciales derivados de las actividades urbanas. Estos contaminantes pueden influir en el crecimiento y desarrollo de los vegetales. En esta tesis, también se desarrolló un método simple y rápido para evaluar la contaminación del suelo. Para ello se utilizaron dos índices de crecimiento de plantas (tasa de germinación de semillas y alargamiento de raíces en la etapa inicial) en tres semillas de hortalizas para evaluar la contaminación química del suelo en agricultura de proximidad.

Resum

L'agricultura moderna ha estat buscant contínuament mètodes efectius per satisfer la demanda d'aliments que augmenta exponencialment a nivell mundial. L'aplicació de residus orgànics com a fertilitzants o esmenes de sòl és una mesura molt utilitzada, ja que subministra nutrients de manera eficient i ràpida als cultius. De fet, des de 2015, la Comissió Europea ha proposat un pla d'economia circular que fomenta la utilització de biosòlids. No obstant això, la presència d'una àmplia gamma de contaminants com elements traça (ET), contaminants orgànics i contaminants emergents com antibiòtics (AB) i gens de resistència a antibiòtics (ARG), ha estat descrita en alguns fertilitzants orgànics. Les verdures poden incorporar els contaminants procedents de sòls fertilitzats amb residus orgànics, suposant així una amenaça per a la salut humana. Per aquesta raó, és necessària l'avaluació dels riscos que pot provocar la utilització d'aquests residus orgànics.

Els fertilitzants orgànics més emprats són les deixalles d'origen animal (fems), el compost (fonts vegetals o deixalles d'aliments) i les deixalles urbanes (llots de depuradora i residus domèstics). Actualment, no existeix cap estudi que avaluï la incorporació dels ET i AB per part de les plantes amb aquests tres tipus de fertilitzants orgànics, ni tampoc l'impacte de l'aplicació repetida de fertilitzants orgànics sobre les mateixes parcel·les. A més, aquesta aplicació repetida de llots de depuradora podria resultar en l'acumulació de Zn i Cu als sòls agrícoles, i la seva presència influiria en la incorporació d'altres contaminants.

Així doncs, en aquesta tesi doctoral s'aborden tres aspectes relacionats amb els contaminants en hortalisses en diferents activitats agrícoles: (1) aplicació de diferents dosis de diferents residus orgànics en sòls de cultiu, que té com a objectiu avaluar l'efecte d'aquesta fertilització en la incorporació de ET i AB en l'hortalissa, (2) aplicació reiterada de residus orgànics en sòls de cultiu i seguiment de la concentració de ET en hortalisses durant diferents cicles productius, amb l'objectiu d’avaluar l'impacte de la fertilització orgànica a llarg termini, i (3) aplicació de fangs de depuradora i diferents quantitats de Zn i Cu a sòls, amb l'objectiu d'avaluar l'efecte d'aquests metalls sobre

l'acumulació d'altres contaminants com ET, AB i ARG en les hortalisses. A més d’això, en tots els casos s’ha avaluat el risc per a la salut humana associat al consum d’hortalisses.

En àrees periurbanes, el sòl agrícola pot rebre la contaminació potencial de contaminants procedents de sòls fertilitzats amb residus orgànics, però també els contaminants potencials derivats de les activitats urbanes. Aquests contaminants poden influir en el creixement i desenvolupament dels vegetals. En aquesta tesi, també s’ha desenvolupat un mètode simple i ràpid per tal d’avaluar la contaminació de sòls. Per a això, es van utilitzar dos índexs de creixement de plantes (taxa de germinació de llavors i allargament d'arrels en l'etapa inicial) en tres llavors de verdures per avaluar la contaminació química dels sòls en agricultura de proximitat.

Structure of the thesis

This thesis is divided into 5 chapters. Chapter 1 gives an overview of the subject and presents the hypotheses and objectives of the PhD project. Chapter 2 describes materials and general methods implemented in this work. Chapter 3 presents results obtained within this framework, and includes three publications and another one that has been submitted to a peer review journal following four topics:

Topic 1: Assessment of the occurrence of trace elements and antibiotics in lettuce grown with different organic fertilizers and evaluation of the human health risk

Article 1: **Occurrence and human health risk assessment of antibiotics and trace elements in *Lactuca sativa* amended with different organic fertilizers**

Environmental Research, 190 (2020) 109946

https://doi.org/10.1016/j.envres.2020.109946

Topic 2: Assessment of the effect of sewage-sludge soil with different Cu and Zn content on the uptake of TEs, ABs, and ARGs in the edible part of lettuce (leaf) and radish (root), and evaluation of the phytotoxicity and human health implications.

Article 2: **Dose effect of Zn and Cu in sludge-amended soils on vegetable uptake of trace elements, antibiotics, and antibiotic resistance genes: Human health implications**

Environmental Research, 191 (2020) 109879

https://doi.org/10.1016/j.envres.2020.109879

Topic 3: Evaluation of the variation of trace elements in vegetable edible tissue after cycling organic fertilization, and assessent of the long-term impact of organic soil amendments.

Article 3: **Health risk assessment of trace elements exposure through the soil-vegetables-human pathway under repeated applications of organic amendments**

Submitted to a peer review journal

Topic 4: Evaluation of the chemical characterization of peri-urban soil surrounding Barcelona and phytotoxicity assessment using two growth indexes (seed germination rate and root elongation) on three vegetables.

Article 4: **Chemical characterization and phytotoxicity assessment of peri-urban soils using seed germination and root elongation tests**

Environmental Science and Pollution Research, 26 (33), 34401-34411.

https://doi.org/10.1007/s11356-019-06574-0

Finally, Chapter 4 presents the general discussion and suggestions for future research, whereas the main conclusions of the thesis are summarized in Chapter 5.

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Chapter 1: General Introduction and Objectives

Chapter 1 overviews the main contaminant sources facing peri-urban agriculture, as well as the fate and influence of chemicals in plants and human body. The hypothesis and objectives are also present in this chapter.

1.1 Overview challenges of peri-urban agriculture

1.1.1 Peri-urban agriculture

At least 55% of the world's population already lives in urban areas and that number is expected to up to 66% by 2050 (FAO, 2009). UN-HABITAT (2006) suggests that the rate of population growth will lead to an increase of urban slum areas, due to high levels of unemployment, food insecurity and malnutrition. Such rapid urbanization and the harsh reality urgently require strategies to ensure adequate food supply and distribution systems to address escalating levels of urban food insecurity together with its adverse consequences.

Since 1999, peri-urban agriculture (UPA) has been considered as an essential part of agricultural production systems, as feeding the cities, creating employment and generating income for the urban poor. There is still no consensus on the precise definition of UPA. One of the most widely accepted definition was established by the Organization for Economic Cooperation and Development (OECD) in 1979 which defines UPA as the cultivation area for crops and rearing of animals surrounding the cities. This reagin is in a radius of 20 km from the urban nucleus with more than 200.000 inhabitants and 10 km of radius from the cities between 50.000 and 100.000 inhabitants.

There are many advantages related to UPA. For instance, it minimizes the carbon dioxide footprint in terms of food transportation, offers fresher food products to large cities, and furthermore, helps deal with some parts of urban waste (Ayambire et al. 2019). However, disadvantages for UPA, like its benefits, arise from its proximity to densely built urban areas. The contaminants released large infrastructure (e.g. solid waste incineration, airports, highways, harbors) of the large cities exerts an environmental pressure that can become pollution sources of UPA. Additionally, large-scale vegetable production brings intensive use of fertilizers, pesticides, and reclaimed water, which also contributes to an important source of pollution.

1.1.2 Sources of contaminants

1.1.2.1 Air

Earth atmosphere mainly consists of oxygen (O_2), nitrogen (N_2) and carbon dioxide (CO_2). However, since the industrial revolution, rapid economic development, transportation, and urbanization during the last 3-4 decades have deteriorated the atmosphere quality by emitting various contaminants. Crop yield and safety are highly dependent on environmental conditions among which air quality can play an important role.

Gaseous air pollutants can be divided into two types. (1) Primary pollutants, such as nitric oxide (NO_X) and sulphur dioxide (SO_2), are emitted directly into the atmosphere. The concentration of primary pollutants is highly associated with release source. (2) Secondary pollutants, such as photochemical ozone and acid rain, which are formed by subsequent chemical reaction in the atmosphere. Secondary pollutants have a wider impact range, as it could be transported to far areas with the atmospheric flow, and influence ecosystem thousands of kilometers from emission sources. The major gaseous air pollutants of concern associated with agriculture are listed in Table 1.1, which also presents some sensitive vegetable species and the damage concentration.

Table 1.1 Summary of major gaseous air pollutants (Ashmore, 1991)

Pollutant	Major sources	Threshold for vegetables		
		Sensitive crops	Visible injury ($\mu g/m^3$)	Yield reduction ($\mu g/m^3$)
Sulphur dioxide (SO_2)	Combustion plants, smelters	Barley,clover, lucerne	250	20
Ozone (O_3)	Secondary pollutant; primarily motor vehicles	Tobacco,bean	80	40
Nitric oxide (NO_X)	Combustion plants, motor vehicles	Bean,pea	1000	100

Hydrogen fluoride (HF)	Brickworks, smelters, coal burning	Barley,maize	100	0.1
Ammonia (NH_3)	Fertilizer works, agriculture	Bean,sunflower	5000	100

Besides gaseous pollutants, some suspended particles (solid and liquid) in the air termed as particulate matter (PM) are also regarded as serious environmental threats. For example, fine particulate matter could cover plant's leave and reduce light penetration, blocking the opening of stomata (Gao et al., 2020) . Most fine particles originate from combustion processes in traffic, power plants, industry, household energy use, biomass burning, and from agriculture and natural sources. PM could be transported and deposit up to several kilometers away from their sources, and cause adverse impacts even at very low concentrations (Fig 1.1).

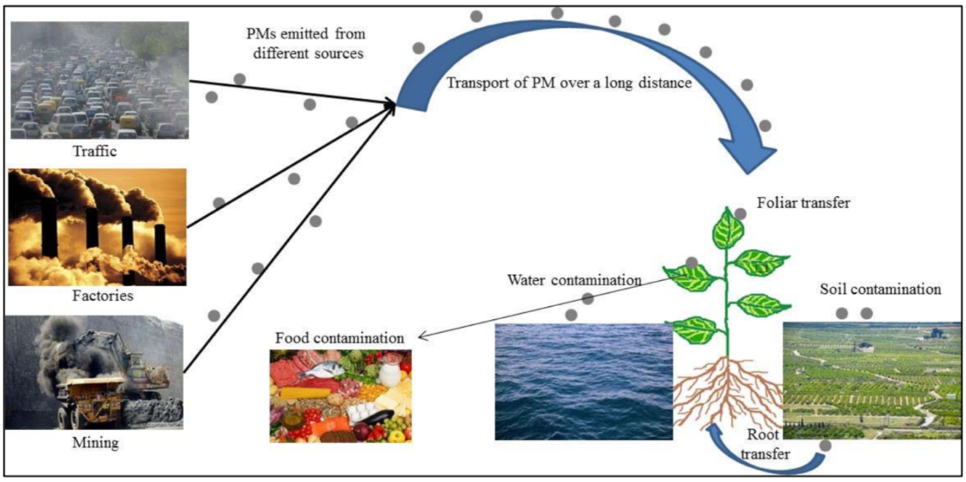


Fig. 1.1 The fate of PM (Shahid et al., 2017)

PM with a diameter of 2.5 microns or less ($\leq PM_{2.5}$) can penetrate the lung barrier and enter the blood system. Chronic exposure to $PM_{2.5}$ increases the risk of developing cardiovascular and respiratory diseases, as well as of lung cancer (Burnettet al., 2018). Currently, the European Union legislation poses a limit for annual mean $PM_{2.5}$ concentrations at 25 μg/m^3 (EC, 2020). The corresponding limit imposed by the U.S. Environmental Protection Agency is 12 μg/m^3 (EPA, 2012), while the World Health Organization (WHO) ambient air quality guidelines suggest an annual mean $PM_{2.5}$

concentration threshold of 10 μg/m^3 (WHO, 2018). The concentration of PM has decreased sharply in North America and Europe in the last decades, but it is still high in many major cities and industrial areas.

Apart from size and concentration, the chemical properties are important for PM hazardous influence. Goix et al (2014) proposed global threat scores to prioritize the harmfulness of anthropogenic fine and ultrafine metallic particles (FMP) emitted into the atmosphere at the global scale. This study evaluated ecotoxicity, human bioaccessibility, cytotoxicity, and oxidative potential of trace elements emitted into the atmosphere and reported following order of the hazard classification: $CdCl_2$ ~ CdO_4 > CuO_4 > PbO_4 > ZnO_4 > $PbSO_4$ > Sb_2O_3. Industrial emitted metals and metalloids such as As, Cr, Pb, Ni, Zn, Cd and V are carcinogenic. In particular, As, Cd, Pb, Cr, and Hg are among the most toxic elements with respect to their potential toxicity and exposure to living organisms. Atmospheric pollution by trace elements is caused primarily by stationary or mobile sources, such as waste incineration, domestic oil burning, power generation plants, industrial units, vehicular traffic, and the resuspension/remediation of contaminated sites (Manno et al., 2006). Among these, trace element emission from industrial and traffic activities represent the most important sources of atmospheric pollution. Smelters and industrial plants that involve burning of ore such as refuse and waste incinerators, coal-fired units can emit comparatively high quantities of metals and metalloids into the atmosphere (Uzu et al., 2011). Emissions of trace elements to the air by industrial processes occur via crushing, fusion, reduction, refining and processing (Table 1.2).

Table 1.2 Trace elements (tons) from different sources into the air (De Lurdes Dinis and Fiúza, 2011)

Industrial activity	Cd	Hg	Pb	As
Energy sector	5.72	19.8	80.9	2.069
Mineral oil and gas refineries	1.09	1.04	2.14	1.63
Thermal power stations and other combustion facilities	3.72	18.50	61.1	205
Coke ovens	1.01	0.28	17.4	-
Production and processing metals	9.66	4.77	398.3	-
Mineral industry	1.79	4.00	60.9	1
Chemical industry	0.72	6.14	2.34	-

Waste and waste water treatment	0.24	1.22	5.41	0.3
Paper and wood production processing	0.56	0.22	3.17	9
Animal and vegetable products from the food and beverage sector	0.05	-	-	-
Other activities	0.03	0.01	-	-
Total	25	56	632	219

Trace elements levels in atmosphere vary greatly between urban and rural areas, as well as with distance from emission sources. For example, WHO (2000) reported that mean levels of As in ambient air in the United States range from <1 to 3 ng/m^3 in remote areas and from 20 to 30 ng/m^3 in urban areas. Trace elements levels in air vary in different countries (Table 1.3), which mainly depend on the intensity of industrialization and urbanization.

Table 1.3 Trace elements levels in air reported in different countries (ng/m^3)

	Poland	Pakistan	Spain	Algeria	Iran	India	UK	Nigeria
Pb	23.6	16.24	9.24	299	120.92	-	10.22	0.832
Cd	0.806	31.66	0.25	21.2	0.33	0.02	0.2	-
Zn	66.5	0.85	354	-	164.58	7.13	-	1.712
Ni	2.15	65.78	3.38	42.4	5.33	0.29	1.74	0.478
As	0.534	-	0.55	-	7.77	-	0.91	-
Fe	-	-	-	639.8	652.41	20.81	-	1.081
Co	0.271	12.69	-	37.7	5.13	-	-	-
Al	0.058	3.02	-	-	241.51	13.89	-	-

Sources: Krzemińska-Flowers et al., 2006; Fernández-Olmo et al., 2016; Oucher et al., 2015; Hassanvand et al., 2015; Vandana et al., 2012; Mafuyai et al., 2014.

1.1.2.2 Soil

Soil pollution refers to the presence of a chemical or substance in the soil at a higher than background concentration that has adverse effects on any non-targeted organism (FAO and ITPS, 2015). The diversity of contaminants is keeping evolving with agrochemicals and industrial developments. So, identifying soil contaminants is both difficult and expensive.

Soil contaminants have natural and anthropogenic origin. Natural events, such as volcanic eruptions or forest fires, can release huge number of toxic elements into the environment. Despite the contribution of natural resources, the major pressure of pollution on soil ecosystem usually originates from anthropogenic processes.

As shown in Fig 1.2, the main anthropogenic sources of soil pollution are the chemicals used in or produced as byproducts of industrial activities, domestic, livestock and municipal wastes (including wastewater), agrochemicals, and petroleum-derived products (FAO, 2018). These chemicals are released to the environment accidentally, like oil spills or leaching from landfills, or intentionally, as is the case with the use of fertilizers and pesticides, irrigation with untreated wastewater, or land application of biosoilds.

Fig. 1.2 Potential interrelated pathways for soil–subsurface chemical contamination (FAO, 2018)

Soil pollution is a concern problem that has been identified as the third most important threat to soil functions in Europe and Eurasia (FAO and ITPS, 2015). There are approximately 3 million potentially polluted sites in the European Economic Area and cooperating countries in the West Balkans (EEA-39) (EEA, 2014). The most common soil pollutants affecting agricultural areas are (1) trace elements, (2) nitrogen and phosphorus, (3) pesticides, (4) polycyclic aromatic hydrocarbons, (5) persistent organic pollutants, (6) radionuclides, (7) contaminants of emerging concern, (8) antibiotic resistance bacteria and genes.

(1) Trace elements

The term "trace elements" has never been defined precisely. It has been used in geochemistry for chemical elements that occur in the Earth's crust in amounts less than 0.1% (1000 mg/kg). The term has commonly been defined based on their abundance and includes elements of various chemical properties: metals and metalloids. Trace elements (TEs) is like a double-edged sword for organism, on the one hand, many of them are essential micronutrients for plants, animals and humans. Lacking TEs would cause irreversible damage to organisms (Keskin et al., 2018). On the other hand, TEs would also induce phytotoxicity and harm human health at high concentrations (Shahid et al., 2020). Nowadays, the threaten of TEs is getting worse, since more and more TEs are released to the environment with intensive human activities. Unlike other contaminants, TEs appear to be virtually permanent. Metals accumulated in soils are able to be depleted by leaching, plant uptake, erosion, or deflation. However, the processes are pretty slow. The first half-life of trace metals, as calculated by Iimura et al (1977) for soils in lysimetric conditions, varies greatly (in years): Zn, 70–510; Cd, 13–1100; Cu, 310–1500; and Pb, 740–5900. From the compilation of data given by Bowen (1979), the following residence time of trace elements in soils of temperate climate can be estimated (in years): for Cd, 75–380; for Hg, 500–1000; and for Ag, Cu, Ni, Pb, Se, and Zn, 1000–3000. In soils of tropical rainforests, the rate of leaching of some elements is much shorter and is calculated at about 40 years. All similar estimations have indicated that the complete removal of metallic contaminants from soils is nearly impossible. After entering food-chain, TEs would cause higher risk to higher animals. Because unlike most organic compounds, TEs are not subject to metabolic breakdown, and would accumulate in the tissues of living organisms.

(2) Nitrogen and phosphorus

Nitrogen (N) is an essential component of all living structures such as proteins, DNA, RNA, hormones, enzymes and vitamins. It has both organic and inorganic forms. Plants need inorganic forms, such as ammonium (NH_4^+) and nitrate (NO_3^-), while animals require organic forms, such as amino acids and nucleic acids (Englander et al., 1983). Phosphorus (P) is one of the main macronutrients for all living organisms. It forms part of biological molecules, such as DNA and RNA, and it is used to transport cellular energy via adenosine triphosphate (ATP). In order to increase vegetable productivity and supply the nutrient needs of the many deficient soils around the world, applying of synthetic fertilizers including abundant N, P and potassium (K) were widely adopted through the twentieth century. As shown in Fig 1.3, The fertilizer demand has increased globally, with an average annual growth of 1.9 percent, and it is expected to over 200 million tonnes by 2022 (FAO, 2019).

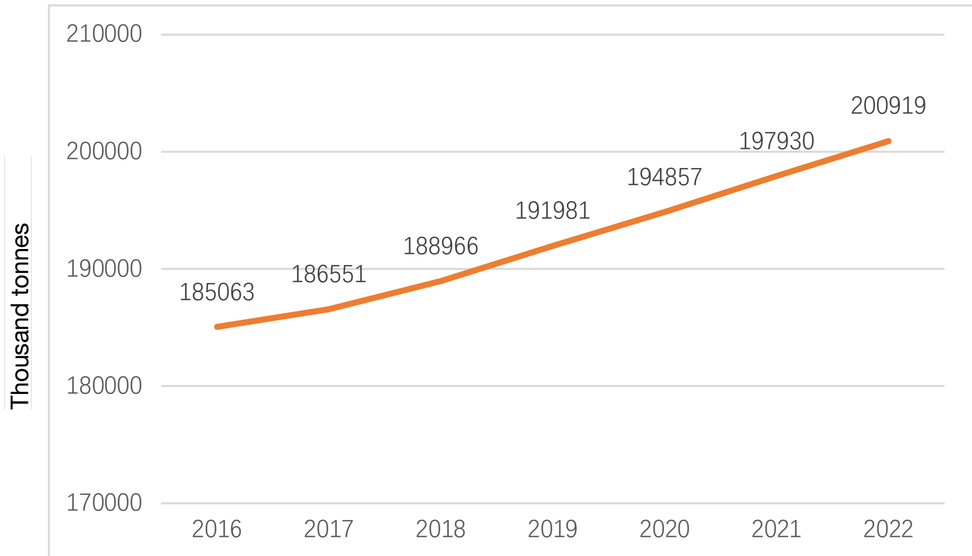


Fig. 1.3 Global synthetic fertilizers consumption (FAO, 2019)

However, it is not a linear correlation between an increase in the amount of fertilizer applied and an increase in crop production. On the contrary, increases may result in low nutrient use efficiency and in turn lower crop yields (Hossain et al., 2005; Zhu et al., 2005). Excessive nutrient would disrupt plant metabolism and alter soil microbial community. Additionally, the surplus nutrient may cause acute environmental problems (Good and Beatty, 2011; Vitousek et al., 2009; Withers et al., 2014). For example, the nutrient may leach into the groundwater or be transported to surface water bodies by runoff, causing eutrophication. Many TEs have also been documented in phosphate and nitrate fertilizers including As, Cd, Cr, Hg, Pb, and Zn (Brevik et al., 2015).

(3) Pesticides

Pesticides are applied to reduce crop losses due to insect pests, weeds and pathogens, and thus to guarantee global food supplies (FAO and ITPS, 2017). Once again, the problem arises when a misuse of pesticides occurs. For example, overuse pesticide would upset the activities of soil microbes and therefore affect the nutritional quality of soils.

(4) Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a large group of mutagenic and carcinogenic compounds with two or more fused aromatic rings, which have attracted attention due to their high toxicity, mutagenicity, carcinogenicity, and widespread presence in the environment. The composition of PAH mixtures in soil are dominated by two major source patterns: natural PAHs and anthropogenic emissions. Fig 1.4 shows the contribution of sorts of sources, where fossil fuel combustion occupied the majority portion.

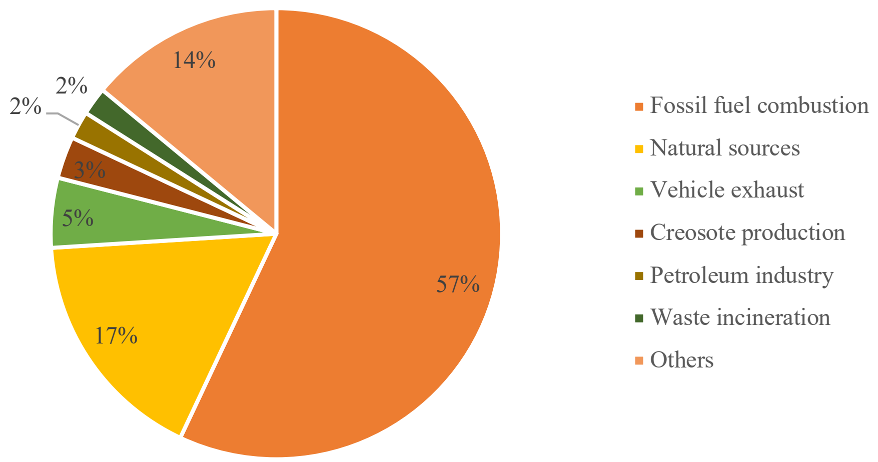


Fig. 1.4 PAHs global emission sources (Kuppusamy, et al., 2017)

(5) Persistent organic pollutants

Persistent organic pollutants (POPs) are chemical substances that persist in the environment, bioaccumulate through the food chain, and have adverse effects on human health and the environment (UNEP, 2001). Soils are the main environmental sink for POPs which form stable bonds with soil organic matter, where they remain in a non-extractable form. Changes in the soil environment, however, can change partitioning rates of POPs in the soil, leading them to become readily extractable. For example, an increase of 1 °C in air temperature induces an increase in the volatilization of POPs from soils by 8% (Komprda et al., 2013).

(6) Radionuclides

The most common natural and anthropogenic radionuclides found in soils are ^{40}K, ^{238}U, ^{232}Th, ^{90}Sr and ^{137}Cs (Wallova et al., 2012). Long-term monitoring on agricultural products has been conducted strictly after the Fukushima accident, indicating that radionuclides remained bioavailable in soils long after initial contamination (Absalom et al., 1999; Falciglia et al., 2014; Yablokov et al., 2009).

(7) Contaminants of emerging concern

Contaminants of emerging concern (CECs) refers to a large number of synthetic or naturally occurring chemicals that have recently appeared in the environment and are not commonly regulated (Geissen et al., 2015). CECs encompass chemicals such as pharmaceuticals, endocrine disruptors, hormones and toxins, among others, and biological pollutants, such as micropollutants in soils, which include bacteria and viruses. They may enter the environment and result in known or suspected adverse effect. For instance, the presence of pharmaceutical and personal care products (PPCPs) has been linked to the development of antibiotic resistant bacteria, feminization of male fish, and genotoxicity in aquatic organisms (Daughton and Ternes, 1999).

(8) Antibiotic resistance bacteria and genes

Bacteria are very adaptable genetically when confronted with antibiotics. Mutational changes would occur in normally sensitive bacteria, allowing it to survive and further proliferate as antibiotic resistant bacteria (ARB) that carry antibiotic resistant genes (ARGs) (Amarasiri, 2020). On the other hand, horizontal gene transfer (HGT) favors the transfer of ARGs among bacteria though mobile genetic elements (MGE), including plasmids, integrons, and prophages.

The main concern about antibiotic resistance bacteria (ARB) and genes (ARGs) is related to the declined effect of antibiotics on therapeutic potential against human and animal pathogens. The estimate number of deaths caused annually by ARB in the USA and in the EU are calculated as 23,000 and 25,000, respectively. (https://www.cdc.gov/drugresistance/) (https://ec.europa.eu/health/ amr/antimicrobial-resistance_en)

1.1.2.3 Water

Water is widely regarded as one of the most essential natural resources, however, freshwater systems are threatened by human activities. For instance, natural water ecosystems are transformed by widespread land cover change, urbanization, industrialization and engineering schemes like reservoirs and irrigation to maximize human benefit (Falkenmark et al., 2003). The advantage of water supply to economic development are always accompanied the impairment to ecosystems and biodiversity, with potentially serious but unquantified cost (Vörösmarty et al., 2010).

Water quality issues are a major challenge that humanity is facing in the twenty-first century. These problems are going to be more aggravated in the future by climate change, resulting in higher water temperatures, melting of glaciers, and an intensification of the water cycle, with potentially more floods and droughts (Huntington, 2006; Oki and Kanae, 2006).

More than one-third of Earth's accessible renewable freshwater is consumptively used for agricultural, industrial, and domestic purposes (Schwarzenbach et al., 2006). As most of these activities lead to water contamination with diverse synthetic and geogenic natural chemicals, it comes as no surprise that chemical pollution of natural water has become a major public concern in almost all parts of the world.

Chemical water pollutants can be divided into two categories: macropollutants and micropollutants (Schwarzenbach et al., 2010). Macropollutants are the relatively small number of mostly inorganic pollutants occurring at mg/L level and include nutrients (e.g. N and P) as well as natural organic constituents (Gruber and Galloway, 2008; Filippelli, 2008). The sources and impacts of these common contaminants are reasonably well understood, but designing sustainable treatment strategies remains a scientific challenge (Larsen et al., 2007). Micropollutants are the thousands of inorganic and organic trace pollutants occurring at ng/L level. Many of these micropollutants may exert toxic effects even at such low concentrations, particularly when present as mixtures (Schwarzenbach et al., 2006).

Table 1.4 illustrates various aspects of global water pollution, including major contaminantion sources as well as different temporal and spatial problems, ranging from long-term global persistent organic pollutants (POPs) to long-term regional (mining) to long-term local (hazardous waste sites) to short-term regional (agriculture) to local (wastewater) pollutants.

Table 1.4 The discussion of water pollution issues (Schwarzenbach et al., 2010)

Pollutant sources	Source type	Pollutant types addressed	Illustrative examples	Main water quality problems	Major challenges
Multiple (waste sites, spills, agriculture, combustion, and others)	Globally distributed point and diffuse	Persistent organic pollutants (POPs)	PCBs, PBDEs, DDT, PAHs, PCDDs, PCDFs	Biomagnification in food chain, diverse health effects	Phase out existing POPs, confine existing sources, prevent use of new POPs
Agriculture	Diffuse	Pesticides	Triazines, chloraceanilides, DDT, lindane	Contamination of ground and surface water with biologically active chemicals; accidental poisoning (particularly in developing countries)	Control of pesticide runoff from agricultural land, pesticide misuse
Natural contaminants Geogenic contaminants,	Diffuse	Inorganic contaminants, cyanotoxins,	As, F, Se, U, microcystins, geosmin	Cancer, fluorosis, human health, aesthetics (taste and odor)	Development of effective household treatment systems, control,

Biogenic contaminants		taste and odor compounds			eutrophication, consumer acceptance
Mining	Mostly point	Acids, leaching agents, heavy metals	Sulfluric acid, cyanide, mercury, copper	Metal remobilization, acute toxicity, chronic neurotoxicity	Acid neutralization, metal removal, introducing effective nontoxic reagents
Hazardous waste	Point	Diverse	U, technetium, chromium, chlorinated solvents, nitroaromatic explosives	Long-term contamination of drinking water resources	Containment of pollutants, monitoring of mitigation processes including natural attenuation
Urban wastewater in industrialized countries	Point	Pharmaceuticals, hormones	Pharmaceuticals, hormones	Pharmaceuticals, hormones	Reduction of micropollutant loads from wastewater by polishing treatment
Urban wastewater in developing and emerging countries	Point	Microorganisms and viruses	Cholera, typhoid fever, diarrhea, hepatitis A and B, schistosomiasis, dengue	Human health, child mortality, malnutrition	Improving sanitation and hygiene, safe drinking water, cheap adequate drinking water disinfection techniques

For inorganic pollutants, including metals (e.g., Cr, Ni, Cu, Zn, Cd, Pb, Hg) and metalloids (e.g., As), the main challenge in evaluating their environmental risks is related to their contrasting solubility behavior under different redox conditions. For example, under oxic conditions, Fe and Mg would form finely dispersed oxide particles which strongly adsorb metals and metalloids. When oxygen is depleted, these oxide particles undergo reductive dissolution and release their adsorbed elements.

When dealing with organic pollutants, the major challenge is the large number and the great variety of chemicals with a large range of physical-chemical properties and reactivities. Fig 1.5 shows the differences in partitioning behavior between water and air or water and an organic phase of some organic micropollutants. The huge difference would result in different environmental effects. For instance, the K_{aw} and the K_{ow} of hydrophobic polychlorinated biphenyls (PCBs) are much higher than sulfonamide antibiotics. This different partitioning behavior suggests that PCBs have higher bioaccumulative ability.

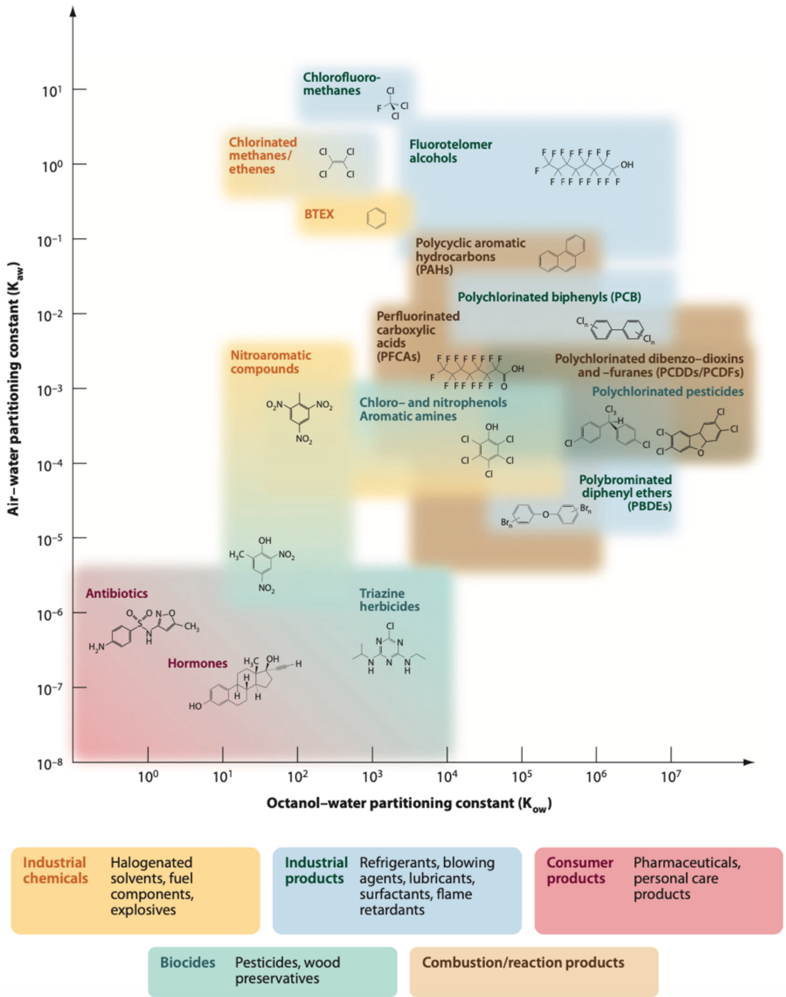


Fig. 1.5 Air-water (K_{aw}) versus octanol-water partitioning constants (K_{ow}) of different organic water pollutants (BTEX stands for benzene, toluene, ethylbenzenes, and xylenes, i.e. fuel constituents). Colored areas indicate the approximate range of the compound properties as well as the origin/usage of the contaminants (i.e., industrial chemicals and products, consumer products, biocides, or combustion/reaction products). (Schwarzenbach et al., 2010)

Besides of the chemical pollution, another risk of water safety is viruses, bacteria, and protozoa, especially, in developing and transition countries. In high-income countries, outbreaks caused by pathogenic *E. coli* and cryptosporidiosis are often reported. According to WHO records of infectious disease outbreaks in 132 countries (from 1998 to 2001), outbreaks of water- borne diseases are at the top of the list, with cholera as the next most frequent disease, followed by acute diarrhea, legionellosis, and typhoid fever (WHO, 2002).

1.2 The metabolic fate of contaminants in plants

The metabolic fate of contaminants in plants can be characterized in relation to some basic processes such as:

- Uptake (absorption) from growth media
- Transport to other tissue within a plant (translocation)
- Detoxification

1.2.1 Uptake

Contaminants enter plants via two major pathways: root uptake and foliar uptake (Fig 1.6). For most contaminants, root uptake is predominant; the concentration of pollutants in plants depends in principle on growth media, the physiochemical properties of contaminants, and the particular plant species.

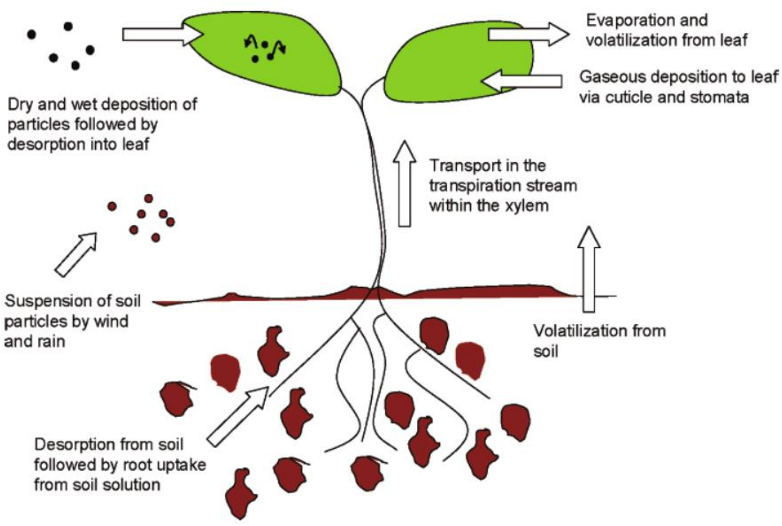


Fig. 1.6 Principal plant uptake pathways of chemical contaminants by plants (Collins, 2006)

1.2.1.1 Root uptake

The first structure to emerge from the germinating seed is the root, enabling the developing seedling to become anchored in the soil and to absorb water. This reflects the two primary functions of roots: anchorage and absorption (Evert, 2013). Fig 1.7 shows the structure of root. In young roots, the epidermis is specialized as an absorbing tissue. The uptake of water and substrate by the root is facilitated by root hairs- tubular extensions of epidermal cells- which greatly increase the absorptive surface of the root. The epidermis lacks a cuticle and offers little resistance to the passage of substrate into the root.

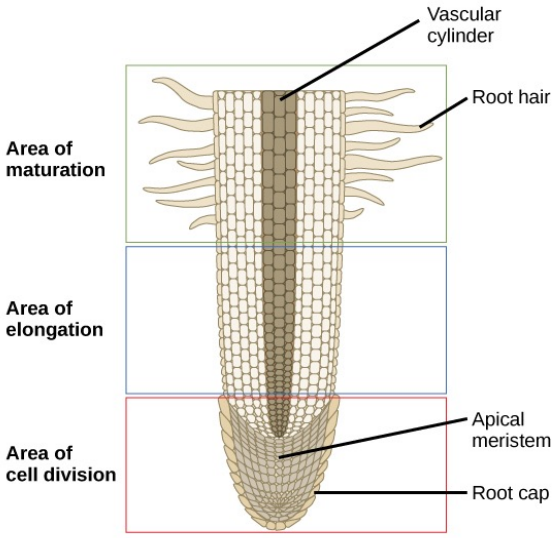


Fig. 1.7 A longitudinal view of the root reveals the zones of cell division, elongation, and maturation. Cell division occurs in the apical meristem. (Clark et al, 2018)

Water and solutes can move from soil pore water to the xylem or phloem (and consequently be transported to above-ground tissues) via three pathways (Fig 1.8). The first one is symplastic, where substances move in the between cells through interconnecting plasmodesmata. The second pathway is the transmembrane route, where substrates move between cells through cell walls and membranes. Another route is the apoplastic route, where substances are transported along cell walls through the intercellular spaces. Compounds taken up solely by the apoplastic route would be stopped unless cross Casparian strip. This strip acts as a hydrophobic barrier between the apoplast (extracellular space in the epidermis) and the vascular tissue (Schreiber, 2010). The cell membranes are semipermeable, and they are able to control over which molecules can or cannot pass through them. It is important because there would be selective passage of solutes, especially when they might bring harm to the plant.

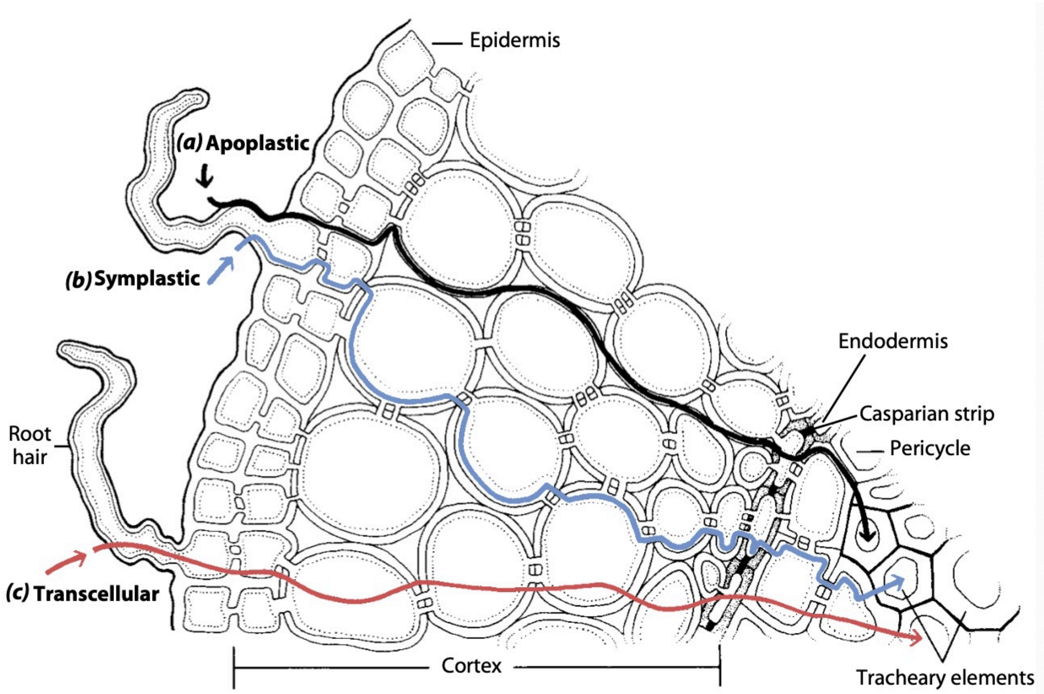


Fig. 1.8 Possible pathways for the movement of substrate from the soil, across the epidermis and cortex, and into the tracheary elements, or water-conducting elements, of the root. (a) Apoplastic movement (black line); (b) symplastic movement (blue line); and (c) transcellular movement (red line) (Raven et al., 2005)

There are two major ways that molecules can across a membrane: passive and active transport mechanisms (Fig 1.9). Passive transport is the movement of molecules or solute from a region with a higher concentration to a region with lower concentration. Process of passive transport does not need energy which include: (a) simple diffusion, small nonpolar molecules and small uncharged polar molecules, such as water, pass directly through the lipid bilayer following their concentration gradient; (b) facilitated diffusion occurs via either carrier proteins or channel proteins. Carrier proteins bind the specific solute and undergo conformational changes as the solute molecule is transported. Channel proteins allow selected solutes- commonly ions such as Na^+ and K^+- to pass directly through water-filled pores. Channel proteins are gated. When the gates are open, solutes pass through, but when they are closed, solute flow is blocked. On the other hand, active transport is the movement of molecules across a cell membrane from a region of lower concentration to a region of higher concentration against the concentration gradient. Therefore, active transport requires cellular energy to achieve this movement, usually supplied by the ATP.

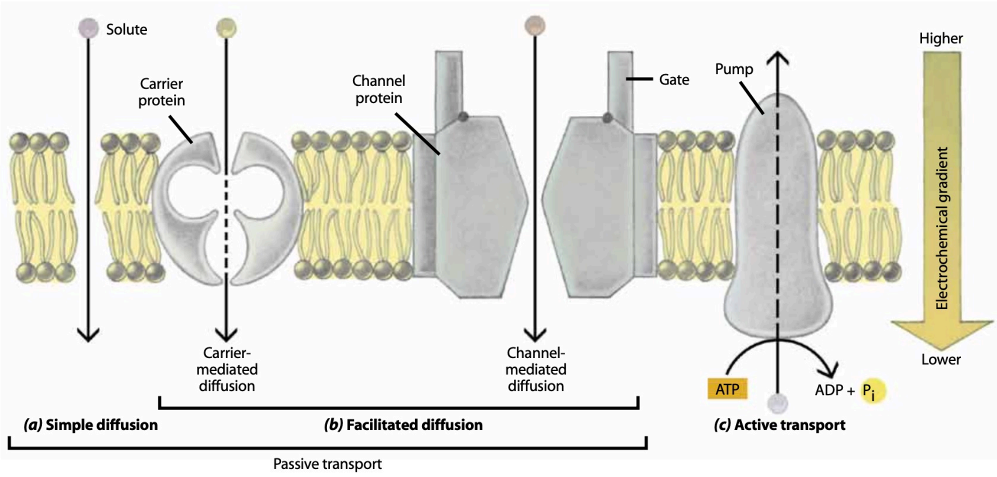


Fig. 1.9 Modes of transport through the plasma membrane (Raven et al., 2005).

(1) Inorganic contaminants

For trace element, passive and active transport are both involved during the root uptake process. Morel (1996) reported that at low external cation concentration in the soil solution (<0.5 μM), active absorption predominates, whereas at higher concentrations (>0.1 mM) the absorption is dominated by passive processes. For certain elements, plants have different preference. For instance, Pb and Ni are preferably absorbed passively, while Cu, Mo, and Zn are preferably absorbed actively (Kabata-Pendias, 2011). However, when concentrations of elements over the threshold value for a physiological barrier, all elements would be taken up passively.

(2) Organic contaminants

For organic contaminants, like CECs and antibiotics, the absorption process is mainly by passive diffusion or advection. Because they are usually xenobiotic to the plants, there are no specific transporters for these compounds in the plant cell membranes (Pilon-Smits, 2005). The lipophilicity of organic contaminants strongly affects their ability to passively cross membranes. Higher lipophilicity favor more rapid diffusion.

1.2.1.2 Foliar uptake

The potential of plant foliar parts to absorb nutrients, water and contaminants was documented about three hundred years ago. Unlike root uptake, which has been largely studied, little is known about uptake by plant leaves from the atmosphere. The absorb process depends on a number of factors, such as (1) the physicochemical properties of the compound and cuticle, (2)the morphology and surface area of the plant leaf, (3) environmental conditions (Beckett et al., 2000).

(1) Inorganic contaminants

According to the pollution context, the foliar uptake of TEs can be neglected, or in contrast appears as the main pathway of pollution, particularly when ultra-fine particles are involved in the process (Calderón-Preciado et al., 2013; Schreck et al., 2014). Plant canopy can efficiently adsorb PM in the atmosphere by capturing the airborne PM on their foliar parts. In fact, vegetables growing near smelters show high foliar levels of metals (Uhlig and Junttila, 2001), while other studies also indicated enhanced levels of metal in foliar plant organs near roadside or industrial areas in Bahrain (9–420 lg/g Pb) (Madany et al., 1990) and Canada (100–3000 lg/g Pb) (Cannon and Bowles, 1962).

Uptake of TEs by foliar surfaces occurs through stomata, cuticular cracks, lenticels, ectodesmata and aqueous pores (Fig 1.10). In fact, absorption of foliar deposited TEs takes place mainly through ectodesmata, which are non-plasmatic channels positioned mainly between subsidiary cells and guard cells in the cuticular membrane or epidermal cell wall (Shahid et al., 2017). Moreover, the cuticle present above the guard cell is comparatively more permeable compared to epidermal cells. Uzu et al. (2010) showed that PM adsorbed on plant leaves is mainly retained by trichomes and cuticular waxes, but some of metals linked to PM can enter inside plant leaf tissues. Overall, foliar uptake of metals is considered a surface phenomenon (Bondada et al., 2004).

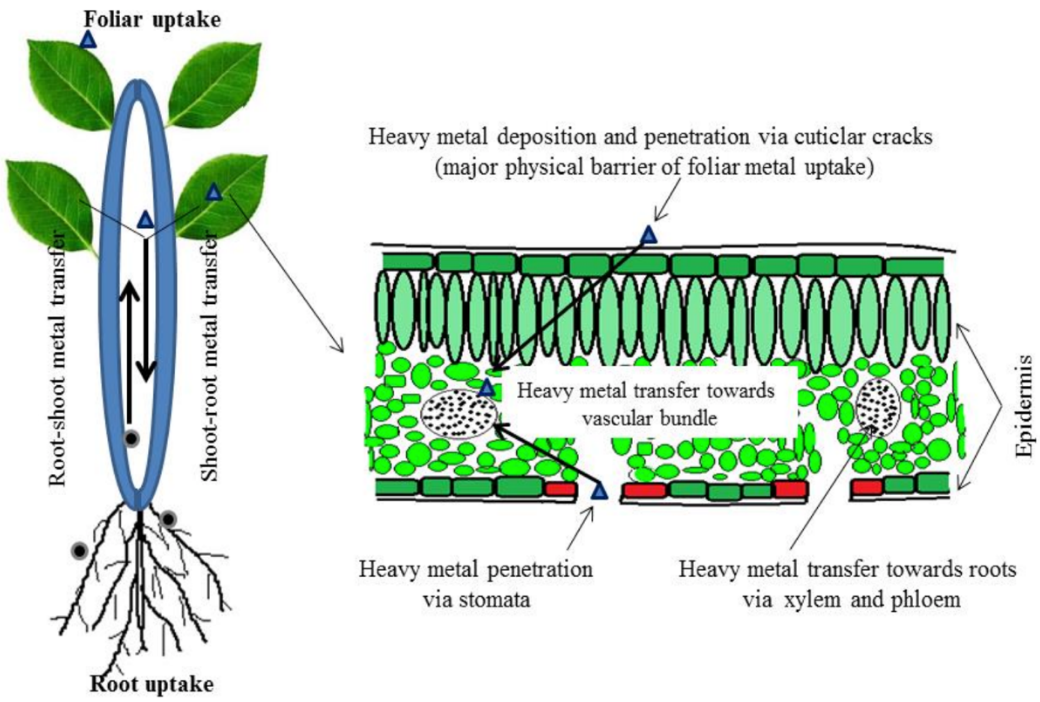


Fig. 1.10 Foliar pathways of heavy metal entrance to plants (Shahid et al., 2017)

According to Chamel et al. (1991), metal penetration via leaf cuticle mainly involves four steps:

(i) adherence to the cuticle;

(ii) penetration through the cuticle (possibly via endocytosis);

(iii) desorption in the apoplast;

(iv) absorption by the subjacent cells.

Like root uptake, foliar uptake of TEs may also occur in a dose dependent manner. For example, Bondada et al. (2004) reported positive linear relationship between As contents in the leaves and foliar sprays amount. Morphology of the surface of leaves is an important factor governing foliar uptake of trace elements. Some plants (e.g., lichens, mosses, mushrooms) are especially susceptible to absorb elements and some compounds from aerial sources. In contrast, cereal tops show a relative sensitivity to aerial pollution (especially Pb and Ni) (Kabata-Pendias, 2011).

(2) Organic contaminants

All organic contaminants except the polar and non-volatile contaminants will preferably be taken up from air and the concentration in soil does not have much impact on its concentration in leaves, unless it is far above chemical equilibrium (Trapp and Legind, 2011). Kipopoulou et al (1999) demonstrated that the mixture of PAHs in inner vegetable tissue is very similar to that in air vapor, therefore suggesting that gaseous deposition is the principal pathway for the accumulation of PAHs.

Environmental factors such as relative humidity can improve the cuticular penetration of hydrated ions by reducing the hydrophobic properties of the cuticle surface, causing cuticle swelling, delaying droplet drying, maintaining deposits in hydrated form, and/or redissolving salt deposits. Chemicals in contact with foliage can partition onto the cuticle and be translocated to other plant compartments or diffuse into the plant through stomata, although the relative importance of the latter pathway is still a matter of controversary (Calderón-Preciado et al., 2013).

1.2.2 Translocation

The concentration of contaminants in different tissues of plants are determined largely by the amount entered in the plants, sequestration within vacuoles, translocation in the xylem and phloem, and dilution within the tissues through growth. To prevent contaminants accumulation in shoot tissues, plants have evolved various mechanisms to restrict the entry to the xylem, which include (i) the production of chelates in the cytoplasm of root cells and the sequestration of chelates in the vacuole to restrict contaminants delivery to the xylem from the symplast, and (ii) the development of physical barriers to the extracellular movement of contaminants to the xylem to restrict the delivery to the xylem from the apoplasm.

1.2.2.1 Inorganic contaminants

For TEs, the concentrations are often (but not always) higher in the root than in the shoot. The chelating ligands are most important in the control of translocation of TEs in plants. However, numerous other factors such as pH, the redox state, competing cations, hydrolysis, polymerization, and formation of insoluble salts (e.g., phosphate, oxalate, etc.) also govern mobility within plant tissues. Tiffin (1972) reported that long-distance transport of TEs in higher plants depends on the vascular tissues (xylem and phloem) and is partly related to the transpiration intensity. The distribution and accumulation patterns of TE vary considerably for each element, kind of plant, and growth season. Table 1.5 shows the translocation factors (leaves/root) of several elements in the different plant species, indicating that translocation across internal tissues is mainly species-specific.

Table 1.5 Values of the leaf/root translocation factor per element in wetland plants (Bonanno et al., 2018)

Name	As	Cd	Cr	Cu	Hg	Mn	Ni	Pb	Zn
Alisma plantago-aquatica	0.44	0.56	0.78	0.81	0.10	0.46	0.58	0.30	0.45
Apium nodiflorum	0.48	0.90	0.59	0.77	0.50	0.79	0.53	0.44	0.65
Arundo donax	0.33	0.30	0.61	0.48	0.31	0.52	0.28	0.16	0.43
Bolboschoenus maritimus	0.41	0.77	0.53	0.68	0.81	0.58	0.61	0.44	0.66
Carex cuprina	0.68	0.77	0.71	0.75	0.70	0.75	0.77	0.73	0.59
Cyperus longus	0.12	0.69	0.63	0.59	0.71	0.78	0.74	0.70	0.58
Eichhornia crassipes	0.53	0.79	0.83	0.81	0.79	0.74	0.70	0.62	0.78
Epilobium hirsutum	0.61	0.68	0.66	0.73	0.70	0.74	0.63	0.61	0.82
Equisetum arvense	0.57	0.64	0.47	0.60	0.53	0.56	0.39	0.36	0.58
Juncus acutus	0.45	0.63	0.56	0.52	0.75	0.62	0.41	0.63	0.61
Juncus maritimus	0.67	0.64	0.68	0.54	1.37	1.49	1.38	1.40	1.33
Lemna minor	1.13	1.21	1.17	1.10	1.08	1.22	1.31	1.20	1.11
Lemna gibba	1.32	1.20	1.29	1.29	1.09	1.19	1.26	1.10	0.84
Nasturtium officinale	0.38	0.56	0.63	0.78	0.67	0.51	0.53	0.67	0.64
Paspalum paspaloides	0.48	0.88	0.57	0.55	0.60	0.62	0.35	0.42	0.53
Phragmites australis	0.15	0.75	0.65	0.57	0.79	0.85	0.67	0.24	0.49
Typha angustifolia	0.16	0.37	0.35	0.28	0.38	0.18	0.33	0.14	0.64
Typha domingensis	0.14	0.28	0.31	0.41	0.58	0.19	0.22	0.13	0.66
Typha latifolia	0.17	0.20	0.30	0.49	0.53	0.16	0.38	0.16	0.43
Veronica anagallis-aquatica	0.57	0.62	0.68	0.88	0.51	0.45	0.53	0.48	0.53

1.2.2.2 Organic contaminants

Polar organic contaminants are preferably translocated from soil and accumulate in leaves and fruits. Lipophilic contaminants will be retained in roots and the lower stem and will not reach the leaves or fruits in significant amounts. Thus, polar and non-volatile contaminants, i.e. contaminants with low K_{ow} and K_{aw}, have the highest accumulation potential from soil into plants (Trapp and Legind, 2011). This is in conflict with the usual concept of bioaccumulation, where high bioaccumulation is assumed to occur for highly lipophilic contaminants (Mackay and Fraser 2000). Tanoue et al (2012) reported that intermediate hydrophobic compounds ($logK_{ow}$ from 0.5 to 3) could be translocated easier than highly hydrophobic ones.

1.2.3 Detoxification

Plants have developed several biochemical mechanisms to adapt and detoxify environmental contaminants during their evolution and course of life (ontogeny and phylogeny).

1.2.3.1 Inorganic contaminants

Gonzalez-Mendoza (2008) overviewed the cellular mechanisms involved in the detoxification to potentially toxic elements. These mechanisms include: (1) mycorrhizal association, (2) metal binding to cell wall, (3) precipitation by extracellular exudates, (4) reduction in uptake at the plasma membrane, (5) chelation by various peptides, and (6) compartmentation of metals in the vacuole.

1.2.3.2 Organic contaminants

Plants are primary producer in the nature, they do not need any organic compounds as a source of carbon, energy or nitrogen. Therefore, all organic chemicals are useless to plants, once they enter tissue, plants would convert the xenobotic pollutant into less phytotoxity products by three phases (Fig 1.11).

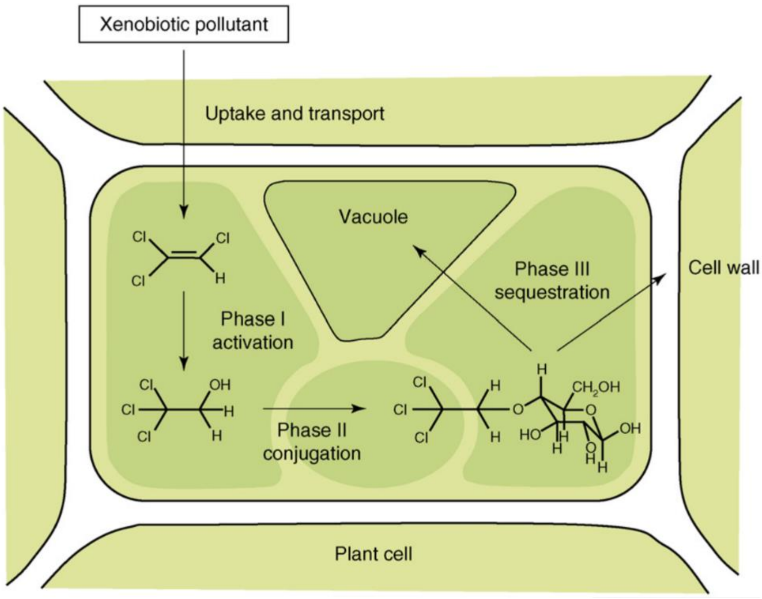


Fig. 1.11 Detoxicity processes of xenobiotic pollutant (Van Aken, 2008)

Phase I (activation) is focus on the transformation of the compound by oxidation, reduction or hydrolysis, which aims to make the molecules more polar, chemically active and more soluble in water (Komives and Gullner, 2005). Conjugation is usually the next step after transformation (phase II). In plants, the major conjugates are glucosides, glutathione conjugates and amino acid. Conjugates produced in phase II are commonly highly water soluble and less toxic than their derivatives of phase I, and cannot usually be excreted by plants. In phase III (sequestration) the xenobiotics are stored in the vacuole (soluble conjugates) or incorporated in the cell walls (insoluble conjugates) to avoid further harm.

1.3 Impacts of chemicals on plants

1.3.1 Essentiality and deficiency

Some of trace elements, unlike organic compounds, are important for the health and developments of plant. To now, there are 17 TEs (Al, B, Br, Cl, Co, Cu, F, Fe, I, Mn, Mo, Ni, Rb, Si, Ti, V, and Zn) considered to be essential for all plants, several are proved necessary for a few species only, and others are known to have stimulating effects on plant growth, but their functions are not yet recognized. Table 1.6 lists several functions of TEs (Kabata-Pendias, 2011).

Table 1.6 Principal functions of trace elements (Kabata-Pendias, 2011)

Element	Constituent of	Involved in
Al	-	Controlling colloidal properties in the cell, possible activation of some dehydrogenases and oxidases
As	Phospholipid (in algae)	Metabolism of carbohydrates in algae and fungi
B	Phosphogluconates	Metabolism and transport of carbohydrates, flavonoid synthesis, nucleic acid synthesis, phosphate utilization, and polyphenol production
Br	Bromophenols (in algae)	-

Co	Cobamide coenzyme	Symbiotic N_2 fixation, possibly also in non-nodulating plants, and valence changes stimulation synthesis of chlorophyll and proteins
Cu	Various oxidases, plastocyanins, and ceniloplasmin	Oxidation, photosynthesis, protein and carbohydrate metabolism, possibly involved in symbiotic N_2 fixation, and valence changes, cell wall metabolism
F	Fluoracetate (in a few species)	Citrate conversions
Fe	Hemo-proteins and nonheme iron proteins, dehydrogenases, and ferrodoxins	Photosynthesis, N_2 fixation, and valence changes
I	Tyrosine and its derivatives (in angiosperms and algae)	-
Li	-	Metabolism in halophytes
Mn	Many enzyme systems	Photoproduction of oxygen in chloroplasts and, indirectly, NO_3^- in reduction
Mo	Nitrate reductase, nitrogenase, oxidases, and molybdoferredoxin	N_2 fixation, NO_3^- reduction, and valence changes
Ni	Enzyme urease (in *Canavalia* seeds)	Possibly in action of hydrogenase and translocation of N
Rb	-	Function similar to that of K in some plants
Se	Glycene reductase (in *Clostridium* cells) combined with cysteine and methionine	Can replace S in some plants
Si	Structural components	-
Sr	-	Function similar to that of Ca in some plants
Ti	-	Possibly photosynthesis and N_2 fixation
V	Porphyrins, hemoproteins	Lipid metabolism, photosynthesis (in green algae), and, possibly, in N_2 fixation Porphyrins, hemoproteins
Zn	Anhydrases, dehydrogenases, proteinases, and peptidases	Carbohydrate, nucleic acid, and lipid metabolism

If essential TEs were not supplied adequately, the growth of plants would be abnormal, especially disorder metabolic cycles. Table 1.7 sums most frequent deficiency symptom of several elements (Kabata-Pendias, 2011).

Table 1.7 Symptoms of micronutrient deficiency in some common cultivars (Kabata-Pendias, 2011)

Element	Symptoms	Sensitive Crop
B	Chlorosis and browning of young leaves; killed growing points; distorted blossom development; lesions in pith and roots, and multiplication of cell division	Legumes, *Brassica* (cabbage and relatives), beets, celery, grapes, and fruit trees (apples and pears)
Cu	Wilting, melanism, white twisted tips, reduction in panicle formation, and disturbance of lignification and of development and fertility of pollen	Cereals (oats), sunflower, spinach, and lucerne (alfalfa)
Fe	Interveinal chlorosis of young organs	Fruit trees (citrus), grapes, and several calcifuge species
Mn	Chlorotic spots and necrosis of young leaves and reduced turgor	Cereals (oats), legumes, and fruit trees (apples, cherries, and citrus)
Mo	Chlorosis of leaf margins, "whiptail" of leaves and distorted curding of cauliflower, "fired" margin and deformation of leaves due to NO_3 excess, and destruction of embryonic tissues	*Brassica* (cabbage and relatives) and legumes
Zn	Interveinal chlorosis (mainly of monocots), stunted growth, "little leaf" rosette of trees, and violet-red points on leaves	Cereals (corn), legumes, grasses, hops, flax, grapes, and fruit trees (citrus)

1.3.2 Toxicity and tolerance

Metabolic disorders are affected not only by deficiency, but also by excess. In general, plants are much more resistant to an increased concentration than to an insufficient content.

1.3.2.1 Inorganic contaminants

A number of various plants grow well in the soil contaminated by trace elements, therefore some of them are used for amending the environmental pollution. For example, hyperaccumulators, which are able to accumulate several times higher TEs than the growth media without any symptoms of toxicity. Mesjasz-Przybyłowicz et al. (2004) reported that the concentration of Ni in the healthy leaves of *Berkheya coddii* exceed 10 times its content of soil. Although plants adapt rather readily to chemical stress, they also may be very sensitive to an excess of a particular TEs. Toxic concentrations of these TEs in plant tissues are very difficult to establish. Visible harmful symptom could be an index for environmental pollution. Table 1.8 lists most common and nonspecific symptoms of phytotoxicity.

Table 1.8 General effects of trace element toxicity on common cultivars

Element	Symptoms	Sensitive crop
Al	Overall stunting, dark green leaves, purpling of stems, death of leaf tips, and coralloid and damaged root system	Cereals
As	Red-brown necrotic spots on old leaves, yellowing or browning of roots, depressed tillering, wilting of new leaves	Legumes, onion, spinach, cucumbers, broomgrass, apricots, and peaches
B	Margin or leaf tip chlorosis, browning of leaf points, decaying growing points, and wilting and dying-off of older leaves In severely affected pine trees, necrosis occurs on needles near the ends of shoots and in upper half on the tree	Cereals, potatoes, tomatoes, cucumbers, sunflowers, and mustard, apple, apricots, citrus, and walnut
Be	Inhibition of seed germination and reduced growth, degradation of protein enzymes	-
Cd	Brown margin of leaves, chlorosis, reddish veins and petioles, curled leaves, and brown stunted roots Severe reduction in growth of roots, tops, and number of tillers (in rice) Reduced conductivity of stem, caused by deterioration of xylem tissues. Reduction of chlorophyll and carotenoids	Legumes (bean, soybean), spinach, radish, carrots, and oats
Co	Interveinal chlorosis in new leaves followed by induced Fe chlorosis and white leaf margins and tips, and damaged root tips	-
Cr	Chlorosis of new leaves, necrotic spots and purpling tissues, injured root growth	-
Cu	Dark green leaves followed by induced Fe chlorosis, thick, short, or barbed-wire roots, depressed tillering. Changes in lipid content and losses of polypeptides involved in photochemical activities	Cereals and legumes, spinach, citrus seedlings, and gladiolus

F	Margin and leaf tip necrosis, and chlorotic and red-brown points of Leaves	Gladiolus, grapes, fruit trees, and pine tree
Fe	Dark green foliage, stunted growth of tops and roots, dark brown to purple leaves of some plants (e.g., “bronzing” disease of rice)	Rice and tobacco
Hg	Severe stunting of seedlings and roots, leaf chlorosis and browning of leaf points	Sugar beets, maize, and roses
Li	Chlorotic and necrotic spots on leaves and injured root growth	Citrus
Mn	Chlorosis and necrotic lesions on old leaves, blackish-brown or red necrotic spots, accumulation of MnO_2 particles in epidermal cells, drying tips of leaves, and stunted roots and plant growth	Cereals, legumes, potatoes, and cabbage
Mo	Yellowing or browning of leaves, depressed root growth, depressed tillering	Cereals
Ni	Interveinal chlorosis (caused by Fe-induced deficiency) in new leaves, gray-green leaves, and brown and stunted roots and plant growth	Cereals
Pb	Dark green leaves, wilting of older leaves, stunted foliage, and brown short roots	-
Rb	Dark green leaves, stunted foliage, and increasing amount of shoots	-
Se	Interveinal chlorosis or black spots at Se content at about 4 mg/kg, and complete bleaching or yellowing of younger leaves at higher Se content, pinkish spots on roots	-
Ti	Chlorosis and necrosis of leaves, stunted growth	Beans
Tl	Impairment of chlorophyll synthesis, mild chlorosis and slight cupping of leaves, reduced germination of seeds and growth of plants	Tobacco and cereals
Zn	Chlorotic and necrotic leaf tips, interveinal chlorosis in new leaves, retarded growth of entire plant, and injured roots resemble barbed wire	Cereals and spinach

Source: Bergmann and Cumakov (1977); Kitagishi and Yamane (1981); Mengel and Kirkby (1978); Singh et. al., 1980; Gettier et. al., 1987.

A common characteristic of plants is their ability to prolong survival under harsh conditions. Lower plants are believed to be more tolerant of pollutant. For example, mosses, liverworts, and lichens show an extremely high level of adaptation to toxic concentrations of certain trace elements, because of quite rapid evolution process

(Basile et al., 2017). Although higher plants show less tolerant to pollution, they are also widely known to accumulate these elements.

1.3.2.2 Organic contaminants

Although the effect of organic contaminants on vegetable health is not fully understood, there are some studies reported the potential risk to plants. Hurtado et al. (2017) studied the effects of the presence of 11organic chemicals (pharmaceuticals, personal care products, anticorrosive agents and surfactants) on lettuce morphology and physiology. Fig 1.12 shows part of these results which indicates significant metabolic alteration and morphological damage in vegetables grown under organic chemicals contaminated condition.

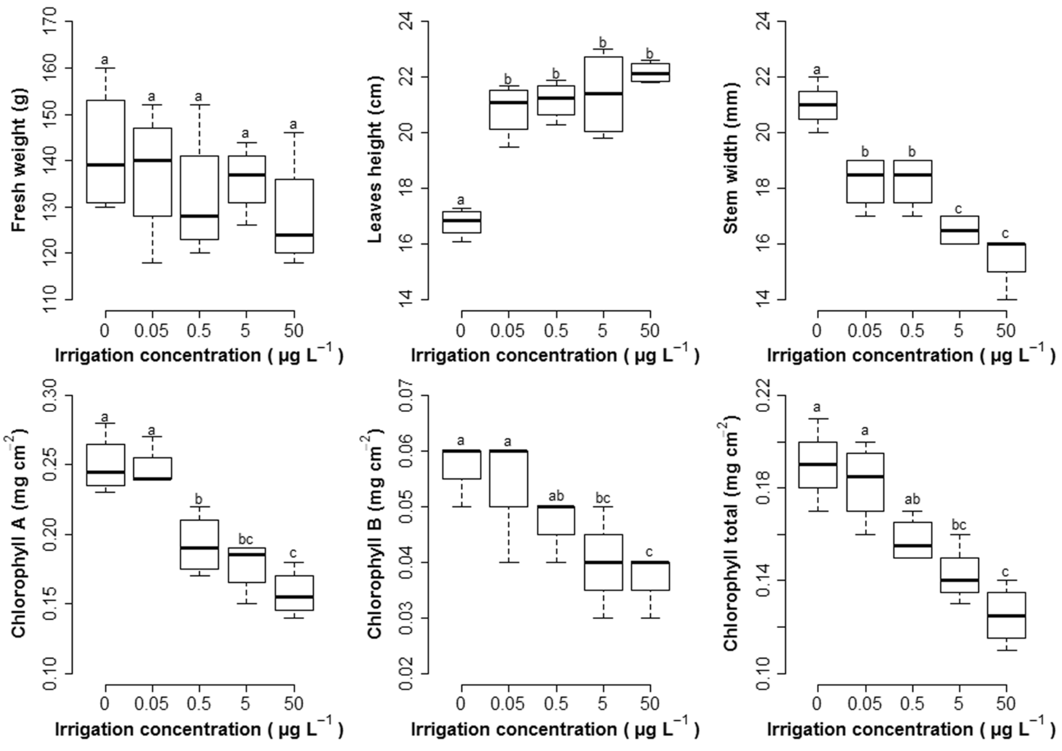


Fig. 1.12 Boxplot of the studied agronomic parameters for the different irrigation concentrations (0, 0.05, 0.5, 5 and 50 μg L^{-1}). Properties with the same letters are not significantly different (*p* < 0.05) (Hurtado et al., 2017)

Bellino et al. (2018) tested the impact of four antibiotics on seed germination and root development of tomato (Solanum *lycopersicum* L.), and reported that antibiotics impair root elongation and cell division in root apical meristem, but do not affect seed germination. Nevertheless, the concentrations tested (ppm) were much higher than the concentrations that are generally found in the environment. Acute phytotoxic effect of antibiotics is rare in the environment, but the presence of low concentrations of such pollutants does not exclude the possibility of chronic effects, nor the presence of acute effects related to the additive or synergistic interactions of different antibiotics (Margenat et al., 2017).

1.4 Human health risk

1.4.1 Significance

Human exposure through consumption of vegetables should be investigated since chemicals from soil can be taken up, subsequently bioaccumulate in vegetables, and threaten human health (Fig 1.13). This pattern includes the following three steps:

(1) The absorption of compounds from one of the mobile phases of the soil (pore water or soil gas) into vegetables.

(2) The vegetables intake by human beings.

(3) The transportation of digested chemicals to target organs via blood stream and the excretion of the remaining part of the chemicals.

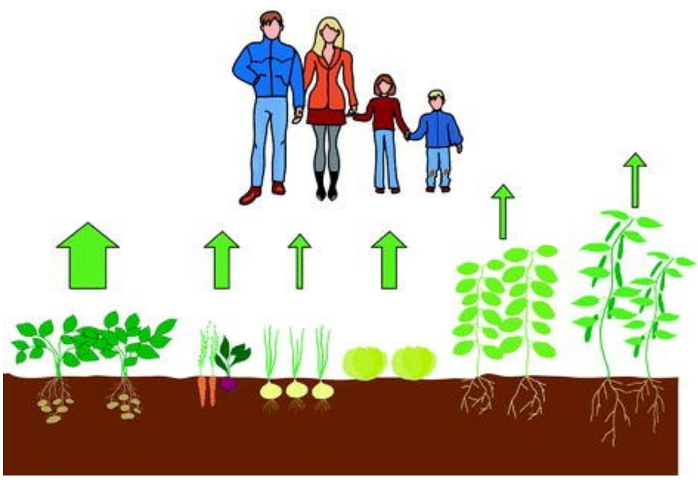


Fig. 1.13 Schematic representation of uptake of chemicals in vegetables and exposure of humans through consumption of these vegetables (Elert et al. 2011)

Vegetable consumption is important intake pathway for most trace elements. For example, eating vegetable contribute 90% of total Cd intake, while the percentage vary between 80% and 90% of Cu, Hg, Mo and Zn (Elert et al. 2011). Organic contaminants exposure through vegetable consumption also contributes a large fraction to total intake. Over 80% dioxins is absorb by human via vegetable pathway, and the value up to 90% for several aromatic contaminants (phenols, catechol, resorcinol and hydroquinone) (Lijzen et al. 2001).

1.4.2 Essentiality in human health

TEs are essential components of biological structures. Currently, according to the report of WHO (1996), the following elements are considered essential for human nutrition: I, Zn, Cu, Mo and Se. In biological systems, these TEs are mostly conjugated or bound to proteins forming metalloproteins, or to smaller molecules, such as phosphates, phytates, polyphenols and other chelating compounds. In enzymes, TEs participate in catalytic processes as: (1) constituents of enzyme active sites; (2) stabilizers of enzyme tertiary or quaternary structure; or (3) associates in forming weak- bonding complexes with the substrate that can contribute to orienting the substrate for reactions, or stabilizing charged transition states. Table 1.9 lists main functions, vegetable sources and presence for I, Zn, Cu, Mo and Se.

Table 1.9 Functions, vegetable sources, presence of I, Zn, Cu, Mo and Se (Wikimedia Commons)

	Functions	Sources	Presence
I	I is essential element to make thyroid hormones	wholegrains, green beans, courgettes, kale	Different enzymes
Zn	Zn is involved in the activity of about 100 enzymes	nuts, seeds, and whole grains.	There are 2–3 g of Zn present in the human body (second to Fe in body content)
Cu	Cu is necessary for the development of connective tissue, nerve coverings, and bone.	nuts, seeds, and whole grains.	There is about 80 mg of Cu in the adult body (highest

			concentrations in liver and brain)
Mo	Mo activates enzymes that help break down harmful sulfites and prevent toxins from building up in the body.	Lentils, dried peas, bean	Different enzymes
Se	Se is incorporated into proteins to make selenoproteins, which are important antioxidant enzymes.	nuts, seeds, and whole grains.	Se is found in glutathione peroxidase, thioredoxins, and selenoprotein P

1.4.3 Toxicity in human health

It is generally accepted that TEs can become toxic at high concentration. One very important feature when considering adverse health effects of TEs is their bioaccumulation. In fact, acute effects of TEs are rarely reported, whereas chronic exposure to low dose which leads to buildup of higher concentration and onset of disease is commonplace.

The toxicity of organic contaminants is depended on the the exposure duration and route, and the individual health status are relevant to assess the possible health effect, also organic contaminants may be metabolized, excreted, stored, or bioaccumulated in the body fat (Pirsaheb et al., 2015).

When chemicals enter human body, they could accumulate in and damage many organs such as liver, heart, kidney, and brain disturbing normal biological functioning. Table 1.10 shows principal harmful effects of some contaminants on human beings.

Table 1.10 Harmful effects of the chemicals on human health

chemical	Harmful effects
As	As (V) (as arsenate) is an analogue of phosphate and thus interferes with metabolic processes such as ATP synthesis and oxidative phosphorylation
	Hyperpigmentation, keratosis and possible vascular complications
B	Most ingested boron is absorbed and leaves the body within 4 days.
	Decreased fetal weight (developmental)
Ba	Barium is a competitive potassium channel antagonist that block the passive efflux of intracellular potassium, results in a decrease of K in the blood plasma.

	Hypokalemia, which can result in ventricular tachycardia, hypertension and/or hypotension, muscle weakness, and paralysis.
Cd	Carcinogenic, mutagenic, and teratogenic; endocrine disruptor; interferes with calcium regulation in biological systems; causes renal failure and chronic anemia
Co	Has both beneficial and harmful effects on human health. It is a part of the vitamin B12, has been used for the treatment of anemia because it causes red blood cells.
Cr	Chromium is a human carcinogen mainly by inhalation exposure in occupational sceneries. Hair loss
Cu	Elevated levels have been found to cause brain and kidney damage, liver cirrhosis and chronic anemia, stomach and intestinal irritation
Hg	Anxiety, autoimmune diseases, depression, difficulty with balance, drowsiness, fatigue, hair loss, insomnia, irritability, memory loss, recurrent infections, restlessness, vision disturbances, tremors, temper outbursts, ulcers and damage to brain, kidney and lungs
Li	A single large dose may result in vomiting and diarrhea.
Mn	Central nervous system effects
Ni	Allergic dermatitis known as nickel itch; inhalation can cause cancer of the lungs, nose, and sinuses; cancers of the throat and stomach have also been attributed to its inhalation; hepatotoxic, immunotoxin, neurotoxic, genotoxic, reproductive toxic, pulmonary toxic, nephrotoxic, and hepatotoxic; causes hair loss
Pb	Its poisoning causes problems in children such as impaired development, reduced intelligence, loss of short-term memory, learning disabilities and coordination problems; causes renal failure
Sb	Affects longevity, blood glucose, and cholesterol
Zn	Over dosage can cause dizziness and fatigue.
Amide pesticides	Abdominal cramps, anemia, ataxia, dark urine, cyanosis, hypothermia, collapse, convulsions, diarrhea,
Bipyridyl herbicides	The main effects are dehydration (resulted from vomiting), their high oxidative stress causes necrosis in the gastrointestinal tract, kidney tubules, liver, and lung; in the latter case, respiratory failure and pulmonary fibrosis may take place.
Carbamate pesticides	They poorly penetrate the blood-brain barrier. Their main symptoms of carbamates intoxication are miosis, salivation, sweating, tearing, rhinorrhoea, behavioural change, abdominal pain, vomiting, diarrhea, urinary incontinence, bronchospasm, dyspnea
Triazine herbicides	Human exposure has been associated with carcinogenicity and endocrine disruption, but these effects are still debatable.
Dithiocarbamate pesticides	Low acute oral and dermal toxicity due to their slow absorption.
Organophosphate pesticides	The skin, conjunctiva, gastrointestinal tract, and lungs rapidly absorb most these compounds and their metabolites arise 12 to 48 h..

Triazole, diazole pesticides	Propiconazole was classified as a possible human carcinogen by EPA and its ingestion of can irritate the gastric mucosa.

Sources: Ali et al., 2013; ATSDR, 2018; EPA, 2018; Fenner et al., 2013b; Pereira et al., 2015

1.4.4 Conceptual Model

The calculation of exposure through the consumption of vegetables is performed in two stages:

1. the calculation of contaminant concentrations in the edible parts of vegetables;
2. the calculation of human exposure through consumption of contaminated vegetables.

Obviously, exposure through consumption of contaminated vegetables depends strongly on the vegetable type. Some plants that can absorb and accumulate large amounts of TEs, while others are not effective in the uptake. Generally, fast growing leafy vegetables, like lettucea, cabbage, and leek show high metal uptake and accumulation rates (Li et al., 2018). In addition, different cultivars of the same species can exhibit substantial differences in uptake and accumulation rates. Wang et al. (2007) made an investigation of inter- and intraspecific variations of Cd concentration in 13 species including 39 cultivars of leafy vegetables and found that cultivar was a more representative taxon level for selecting pollutant-safe leafy vegetables than species.

1.4.5 Mathematical Equations

In a general form, The estimated daily intakes (EDIs) of contaminants through vegetable consumption is calculated as follows:

$$\mathrm{EDI} = \frac{\mathrm{DI} \times C_P}{\mathrm{BW}} \qquad (1)$$

Where DI is the daily intake of vegetables; C_p is the concentration of each pollutant in the crop; BW is body weight.

To evaluate the human health risk, the hazard quotient (HQ) was calculated.

The HQ is the ratio between the EDI and the reference oral dose (RfD), as shown in Equation 2.

$$\mathrm{HQ} = \frac{\mathrm{EDI}}{\mathrm{RfD}} \qquad (2)$$

Where RfD is the maximum tolerable daily intake (μg/kg/day) of a specific element that does not result in carcinogenic effects for human beings, obtained from IRIS (2020). An HQ＞1 implies a potential risk to the population; otherwise, the consumer is safe.

Finally, the total hazard quotient (THQ), used to assess the total risk of all chemicals to which an individual might be exposed, was calculated as the sum of the HQ of all the elements.

1.5 Hypothesis and Objectives

Based on the facts that vegetables could absorb contaminants from soil and fertilizer, and sometimes show visible symptoms, the following hypothesizes were proposed:

1. Application of organic fertilization would affect the occurrence of pollutants in vegetables.
2. The repeated application of organic fertilizers would increase the concentration of contaminants in vegetables.
3. Increasing amounts Zn and Cu in sludge-amended soils, would exert extra accumulation of pollutants in vegetables.
4. Peri-urban soil of Barcelona has more burden of contaminants compared with the pristine site.
5. Bio-monitoring indexes such as seed germination rate and root elongation can reflect the peri-urban soil pollution.

To address these hypotheses, the following objectives were established.

The overall aim of this thesis is to evaluate the concentration of contaminants in crops grown with organic fertilizer in the peri-urban soil of Barcelona and assess their effects on phytotoxicity and human health.

Therefore, in order to accomplish the general objective, this thesis comprises the following specific objectives:

1. Monitor the concentration of TEs and ABs in lettuces following the application of three different organic fertilizers at greenhouse scale.
2. Assess the dose effect of Zn and Cu in sludge-amended soils on the occurrence and accumulation of TEs, ABs, ARGs in vegetables in greenhouse studies.
3. Compare the concentration of TEs in vegetables after several organic fertilizer amendment applications in the field.
4. Evaluate the potential human health risk in greenhouse and field studies.
5. Detect the content of selected chemical contaminants (TEs, PAHs, CECs) in peri-urban soil.
6. Assess the effect of peri-urban soils quality on seed germination and root elongation to develop a bio-monitoring index to reflect soil pollution.

Chapter 2: Materials and general methods

Chapter 2 includes the materials and general methods implemented to carry out this work and which are not completely described through the research articles content. It is devised into three parts, the first one concerned experimental layout, the second part described sample analytical procedures, the third part is devoted to data analysis.

2.1 Experimental layout

2.1.1 Experimental site

The experiments performed for topic 1, 2 were conducted in a glass greenhouse facility located at the Agròpolis-UPC agriculture experimental station (41°17′18″N, 2°02′43″E) in Viladecans (Barcelona, Spain), while topic 3 was field experiments done at same experimental station. Finally, the experiment of topic 4 was conducted in lab of IDAEA-CSIC.

2.1.2 Experimental subject

2.1.2.1 Soil

The soil used in topic 1, 2 and 3 was collected from an agricultural area located in the Llobregat River Delta (Fig. 2.1) where had never been applied intentionally antibiotic products before this work.

Fig. 2.1 Sampling site picture

The soil used for topic 4 was sampled from one pristine site (S1) and two peri-urban sites (S2, S3) (sampling map shown in the chapter 3: Fig 3.4.1). These three sites were chosen based on the gradient of the impact of industrial, urban, and agricultural activities. Site 1 (S1) is an organic farming area irrigated by a drip system, manure-amended, relatively far away from Barcelona, and protected from urban pollution, while site 2 (S2) and site 3 (S3) are located in the peri-urban area and furrow irrigated with treated wastewater from the Llobregat River. Compared with S2, which has car traffic pollution, S3 is mainly influenced by the adjacent airport.

2.1.2.2 Fertilizers

Three types of organic fertilizers were evaluated in this work: solid fraction of swine manure (SM) (Cooperativa Agraria, Torelló, Osona,Spain), digested sewage sludge (SS) from a wastewater treatment plant (WWTP) surrounded by industries in the nearby city of Barcelona (Gavà-Viladecans, Spain), and organic fraction of municipal solid waste (OFMSW) from a composting plant (Torrelles del Llobregat, Spain). This OFMSW was obtained from a mixture of pruning waste from the nearby area and organic waste from the Hospital Universitari Vall d'Hebrón kitchen. Fig. 2.2 present samples of three organic fertilizers. A chemical fertilizer (CF) - as a control - was obtained from reagent grade chemicals (NH_4NO_3, P_2O_5, and K_2O), and no TEs impurities are expected.

Fig. 2.2 Picture of three organic fertilizers (from left to right is SS, SM,and OFMSW, respectively)

2.1.2.3 Vegetables

In this work, vegetables representing four different crop types (leafy, inflorescence, berry and root plant) were studied:

- lettuce *(Lactuca sativa* L. cv. Quattro Stagioni and *Lactuca sativa* L. cv. Maravilla de Verano)
- cauliflower (*Brassica cretica* L. cv. Maravilha)
- tomato (*Solanum lycopersicum* L. cv. Marmande)
- radish (*Raphanus sativus* cv. Redondo Rojo Vermell).

2.1.3 Experimental set-up

2.1.3.1 Topic 1: Greenhouse - lettuce - 4 amendements

A total of 60 units were placed individually in 2.5 L amber glass pots (15 cm diameter, 20 cm high) with an inverted bottle shape fitted with a bottom outlet connected to drainage tubing (0.5 cm diameter) and filled with 2.3 kg of soil sieved to 2 mm (Fig 2.3). The experimental set-up consisted of 4 treatments at 3 fertilization doses of SS (primary and secondary sludge with anaerobic digestion), OFMSW (composted municipal organic food waste composted with wood waste), SM (solid fraction), and chemical fertilization (CF) as a control. This resulted in 12 treatments with 5 repetitions each, for a total of 60 randomly distributed containers planted with lettuce. The estimated nutrients to be added (N, P, K) were based on previously reported studies for lettuce crops (80–100 kg of N per ha; 30–50 kg of P_2O_5 per ha; and 160–210 kg K_2O per ha) (García-Serrano Jiménez et al., 2009). According to the chemical composition of the soil, nitrogen was selected as the limiting nutrient. The amount of organic fertilizer added per pot was calculated to ensure the same quantity of total nitrogen in all treatments (100 kg of N per ha). Based on this value, 3 doses were tested: dose 1 (half the optimal N dose), dose 2 (optimal N dose) as the reference nitrogen dose, and dose 3 (twice the optimal N dose). The total duration of greenhouse cultivation was 57 days, from October 8 until harvesting on December 4, 2018.

Fig. 2.3 Greenhouse experiment facility

Drip irrigation was applied through a connection to a reservoir containing mainly rainwater combined with groundwater. The physicochemical parameters of irrigation water are described in Table 2.1.

Table 2.1 Main characteristics of the irrigation water

Parameter	Irrigation water
pH	7.9
Conductivity	1.724 dS/m
Total suspended solids (TSS)	1.103 mg/L
Ca^{2+}	2.014 meq/L
Mg^{2+}	2.534 meq/L
Na^{+}	10.414 meq/L
HCO_3^-	4.672 meq/L
Cl^-	9.790 meq/L
$N\text{-}NO3^-$	0.016 meq/L
$N\text{-}NH4^+$	0.069 meq/L
SO_4^{2-}	2.399 meq/L
PO_4^-	0.001 meq/L
K^+	0.466 meq/L
B	0.34 mg/L

2.1.3.2 Topic 2: Greenhouse - lettuce and radish - sewage sludge with Cu & Zn

In the second experiment, the basic facilities were same as topic one. While radish was added into this experiment to further assess the risk facing root vegetable. Radish prefers sandy soil. To reach the best growth condition, the pots added a certain amount of sand. The exact amount was 1652 g fresh soil, 878 g sand, and 70 g wet sewage sludge. To evaluate the effect of Cu and Zn content in sewage sludge on the uptake of contaminants in vegetables grown in amended soil, the following treatments were established: (i) control: unspiked sewage sludge containing 240 mg/kg Cu and 700 mg/kg Zn (dw); (ii) treatment 1 (T1): sewage sludge spiked with $CuSO_4 \cdot 5H_2O$ and

$ZnSO_4 \cdot 7H_2O$ to two-fold the background value of Cu and Zn, i.e. to 480 mg/kg and 1400 mg/kg (dw); and (iii) treatment 2 (T2): sewage sludge spiked with more $CuSO_4 \cdot 5H_2O$ and $ZnSO_4 \cdot 7H_2O$ to four-fold the original Cu and Zn concentration, i.e. to 960 mg/kg and 2800 mg/kg (dw). All treatments were replicated five times. One lettuce or radish seedling was planted in each experimental unit on March 4, 2019, and harvested until they reached the commercial size (35 productive days for radish, 56 for lettuce).

2.1.3.2 Topic 3: Field experiment – lettuce and radish- cycle fertilization

The filed trials were carried out on in same agricultural experimental station (Agrópolis) from 2019 to 2020. The experimental site covers 340 m^2. One part of the field was used to grow lettuce (*Lactuca sativa L*. cv. Maravilla de Verano), including 12 plots of 3x2 m each (with 1 m margin between plots). The other part used for radish (*Raphanus sativus*. cv. Redondo rojo) was also divided into 12 experimental plots of 2.4 x 1 m each. The plots are randomized block design with 3 treatments and 4 replicates for both vegetables (Fig. 2.4). Treatments consisted of a control (chemical fertilizer), and two different organic fertilizers (SS and OFMSW). The sources of fertilzers for field experiment are as same as previous greenhouse experiment. The required amount of fertilizers was calculated according to the optimal N content for crops (100 kg per ha) (Pomares and Ramos, 2010). The applied amount for each plot was shown in Table 2.2.

Fig. 2.4 Field experiment picture

Table 2.2. Dose of fertilizers applied to the soil.

	Lettuce	Radish
Sewage sludge (fresh)	18 kg	5.5 kg
Compost (dry)	26 kg	9 kg
Chemical fertilizer	353 g	113 g

The first experiment was conducted in 04/2019. After fertilizers were incorporated and homogeneously distributed in soil, both crops were implanted. The lettuce was transplanted on 04/03/2019 and the radish experiment began on 04/08/2019. Vegetables were drip inrrigated, with flow rate of 1.14 L / h. Irrigation system was provided by the scientific-technical unit of the Polytechnic University of Catalonia (UPC). Plants were harvested until they reached the commercial size (37 productive days for radish, 56 for lettuce).

The second trial was conducted on 06/11/2019. The experimental process and the amount of applied fertilizer are same as first cycle. Since the growth period of second experiment is winter, crops took longer timeto reach commercial size (74 days for radish, 120 days for lettuce). Fig. 2.5 presents the average temperature and participation during trials.

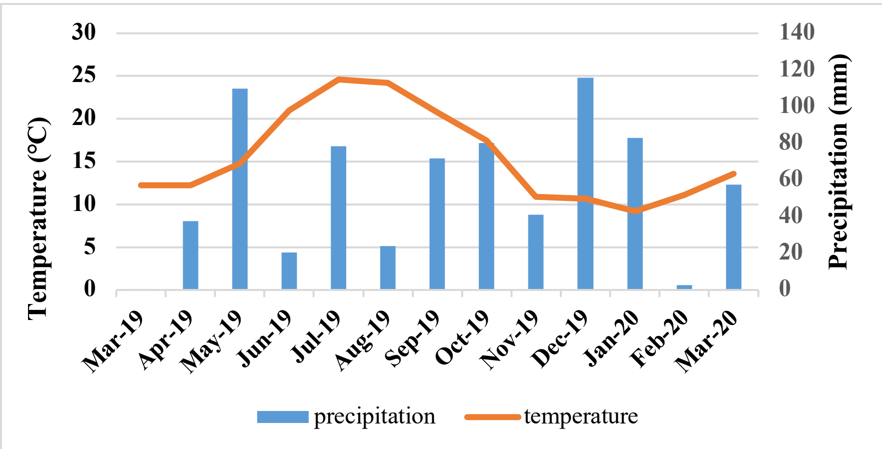


Fig. 2.5 Average temperatures and monthly accumulated precipitation during 2019/03 -2020/03. Source: Estación meteorológica Viladecans (Meteocat).

2.1.3.3 Topic 4: Laboratory experiment-lettuce, tomato, and cauliflower-phytotoxicity experiment

The phytotoxicity experiment was designed based on the guideline issued by Environmental Protection Agency (EPA 712-C-96-154) about seed germination/root elongation toxicity test and ISO 11269-1 root growth test. Seeds were purchased from a local garden material store. Before being tested for bioassays, their germination potential was examined under 24 ± 2 °C in the dark for 2 days. The germination rate for all tested seeds was over 90% which guaranteed the viability of the seed.

Soil was air-dried, mixed, and grounded to fine powder (< 2 mm). Then, 30 g of soil per site was weighed into a 100 × 15mm Petri dish and wetted with deionized water to reach 80% of its total water-holding capacity.

As for seeds, at first, they were sterilized with 2.5% sodium hypochlorite for 15 min and carefully washed with distilled water. Ten clean seeds were evenly put, at least 0.5 inches from the edge, on the surface of the soil per dish. Then, cover the dish to avoid moisture evaporation. Subsequently, all dishes were randomly placed on the lab workbench at 24 ± 2 °C in darkness for germination. According to the EPA guideline, germination means the resumption of active growth by an embryo. Moreover, as defined by Finkelstein et al. (2008), germination is the initial emergence of the radicle from the seed coat. The primary root should attain a length of 5 mm for the seed to be counted as having germinated. When at least 65% of seeds of control have germinated and developed roots that are at least 20 mm long, germination experiment concludes, and germination rate could be calculated. In our case, although tomato, lettuce, and brassica vary in their germination time in our control soil (30 to 45 h), all of them germinated within 48 h. Then, these seedlings were put under fluorescent lamp for 16:8 h light to dark cycles for the next 72 h for root to continue growing. Five replicates of each treatment were tested. Fig. 2.6 shows the image of seedlings under the lamp.

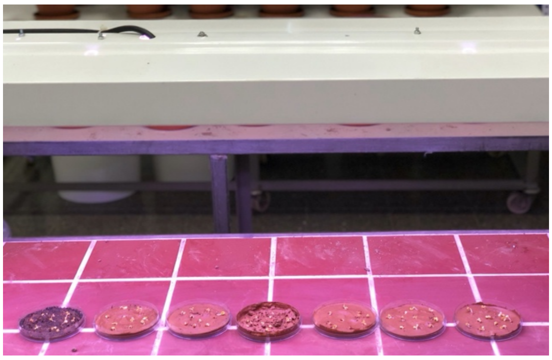


Fig. 2.6 Lab experiment facility

After the initial 48 h, in each dish, the number of acceptable seedlings was counted and divided by the total number of seeds added (10) to calculate the germination rate. At the end of the experiment (3 days later), all seedlings were pulled out and the root elongation was measured which is defined as the length from the tip to radicle.

2.2 Sample analytical procedures

2.2.1 Reagents and standards

2.2.1.1 Trace elements

The reagents for extraction were prepared using nitric acid (69%) (Panreac), 37% hydrochloric acid (Merck, Darmstadt, Germany), and 31% hydrogen peroxide (Merck). All solutions were diluted with doubly deionized water obtained from Millipore water purification systems (Elix&Rios) (18.2 MΩ/cm resistivity and TOC, Total Organic Carbon <30 μg/L). CRM 1570a, supplied by the National Institute of Standards and Technology (USA), has certified values for As, Cd, Pb, Cr, Cu, Hg and Zn. Digestion reagents were also used as a blank matrix. For quality control purposes, in each sample digestion series, CRM and laboratory reagent blank were added. A limit of detection (LOD) of in the solution analyzed was determined from three times the standard deviation obtained from the analysis of ten runs of blank samples on the same day as the determinations. Similarly, the limit of quantification (LOQ) was calculated by multiplying the standard deviation by ten times.

2.2.1.2 Antibiotics

Antibiotic standards of sulfonamides (sulfacetamide, sulfadiazine, sulfamethazine, sulfamethizole, sulfamethoxazole, sulfapyridine and sulfathiazole) were obtained from Sigma-Aldrich (Bornem, Belgium). Fluoroquinolones (Ciprofloxacin, enrofloxacin and ofloxacin), tetracyclines (chlortetracycline hydrochloride, oxytetracycline hydrochloride and tetracycline hydrochloride), azithromycin di-hydrate and lincomycin hydrochloride monohydrate were supplied by Fluka (Buchs, Switzerland). Surrogate standard used, enrofloxacin d5 hydrochloride, ofloxacin d3 hydrochloride, clindamycin d3 hydrochloride and sulfamethoxazole d4, were purchased from Sigma-Aldrich. Strata-X solid-phase extraction (SPE) cartridges (200 mg/ 6 mL) were purchased from Phenomenex (Torrance, CA, USA) and 0.70 µm of glass-fiber filters 47 mm in diameter were obtained from Whatman (Maidstone, UK).

In order to enable equilibration, 100 ng/g (dw) ofloxacin-d3, clindamycin-d3, enrofloxacin-d3, and sulfamethoxazole-d4 were spiked one hour prior to extraction into the samples (1 g) as surrogate standards. LOD and LOQ were calculated as the mean background noise in a blank triplicate plus three and ten times.

2.2.1.3 Antibiotic resistance gene

Four plasmid vectors were used as standards for absolute quantification of qPCR amplicons. For 16S rDNA gene, *bla*TEM, $bla_{\text{CTX-M-32}}$, *sul*1, *qnr*S1, *intl*1, purified DNA samples from pNORM1 plasmid were used as quantification standards. For *tet*M, $bla_{\text{OXA-58}}$ and *mec*A, purified DNA samples from three different pUC19 plasmids were used as standards. Standard curves (Ct per log copy number) for quantification of all genes were obtained for each run, using 10- fold serial dilutions of the corresponding standards. Three technical replicates were run for the standard curves; complete reaction mixes plus nuclease-free water, but no template, were used as negative control qPCR reactions. The limit of quantification (LOQ) was defined as the lowest point on the linear part of the standard curve: 100 gene copies per reaction for ARGs and intl1 and 1000 gene copies per reaction for 16S rDNA. To ensure the absence of bacteria on the surface of vegetable, the last rinsing water was plated in LB agar media for 72 hours at 28 °C.

2.2.1.4 Polycyclic Aromatic Hydrocarbons (PAHs)

Gas chromatography (GC) grade (Suprasolv) hexane, acetone and ethyl acetate were obtained from Merck (Darmstadt, Germany). Triphenylamine was purchased from Sigma–Aldrich (Steinheim, Germany). The LODs and LOQs were calculated for each analyte as three and ten times the signal from the baseline noise (S/N ratio), respectively.

2.2.1.5 Contaminants of Emerging Concern (CECs)

Analytical grade flame retardants (tris(2-chloroethyl) phosphate (TCEP) and tris (1-chloro-2-propyl phosphate (TCPP), benzotriazoles and benzothiazoles (i.e., 1,3-benzothiazole, 2-mercaptobenzothiazole, benzotriazole, 5-methyl-2H-benzotriazole and 1-hydroxybenzotriazole), parabens (methylparaben, ethylparaben, butylparaben and propylparaben), antioxidant (i.e., butylated hydroxyanisole (BHA)), plastifiers (bisphenol A, bisphenol F and 4-tert-octylphenol), surfactant (2,4,7,9-tetramethyl-5-decyne-4,7-diol (surfynol 104)), some pharmaceuticals (i.e., carbamazepine, diazepam, lamotrigine, lorazepam, primidone, oxazepam) and some pesticides (i.e., azoxystrobin, dimethomorph, pyraclostrobin, chlorpyrifos, diazinon, pymetrozin, indoxacarb, DEET) were purchased from Sigma-Aldrich (Bornem, Belgium). Other standard compounds (i.e., carbamazepine-10,11-epoxide, carbendazim, atrazine and simazine) were supplied by Fluka (Buchs, Switzerland). Surrogate standards were bisphenol A-d16, carbamazepine-13C6, diazepam-d5, 5,6-dimethyl-1H-benzotriazole (XbTri), ethylparaben-13C and lamotrigine-13C15N4 purchased from Sigma-Aldrich and caffeine-13C3 obtained from Fluka. Internal standard triphenylamine (TPhA, 98%) was purchased from Sigma-Aldrich and the derivatization agent trimethylsulfonium hydroxide (TMSH) was obtained from Fluka. Suprasolv® grade methanol, hexane, ethyl acetate and LiChrosolv® were purchased from Merck (Darmstadt, Germany). Reagent water was deionized using the ultrapure water system Arium 611 from Sartorius (Aubagne, France), N,N-dimethylformamide was obtained from Merck. Strata-X solid-phase extraction (SPE) cartridges (200 mg/ 6 mL) were purchased from Phenomenex (Torrance, CA, USA) and 0.70 µm of glass-fiber filters 47 mm in diameter were obtained from Whatman (Maidstone, UK). The LODs and LOQs were calculated for each analyte as three and ten times the signal from the baseline noise (S/N ratio), respectively.

2.2.2 General parameters

2.2.2.1 Soils and fertilizers

Measurement of pH in the suspension with a soil/fertilizer to water ratio of 1:2.5 was performed using a pH meter, and electrical conductivity was measured in the saturation paste extract. Cation exchange capacity was determined by saturation with sodium acetate solution, replacement of the absorbed sodium with ammonium, and determination of displaced sodium by flame atomic absorption spectrometry. The concentration of $NaHCO_3$-extractable P was measured by the procedures developed by Olsen as described in Page et al. (1982). The total N was determined by Kjeldahl method, and the concentration of NO_3-N was measured by Hach-Lange spectrophotometer (DR 1900 Portable Spectrophotometer), while NH_4-N in the soil/fertilizer was quantitatively displaced solutions of K salts and measured colorimetrically after reaction with phenol(s) and hypochlorite. The NH_4Ac (CH_3COONH_4)-extractable speciation of K, Ca, Mg, and Na was determined by inductively coupled plasma optical emission spectrometry (Thermo Scientific, iCAP 6500 ICP-OES).

2.2.2.2 Vegetables

The length and number of leaves of both vegetables of topic 3 were measured weekly until the end of the experiment. The chlorophyll content in the leaves and weight of the edible part of both vegetables were measured in situ. Chlorophyll was gauged using a chlorophyll meter (Opti-Sciences, Hudson, NH, USA). Each measurement was performed on three leaves per crop. A calibration curve was obtained to correlate the chlorophyll content with the previously measured absorbance. Round leaf samples (4 cm diameter) were then extracted with 5 mL of *N*,*N*-dimethylformamide (DMF) and kept in the dark at 4 °C for 48 h before the spectrophotometric determination. The extracts were measured at two wavelengths, 647 and 664.5 nm, so that the chlorophylls could be calculated using Inskeep and Bloom's co- efficients (Inskeep and Bloom, 1985; Porra, 2002). Lipid extraction was carried out by adding 15 mL of ethanol/hexane (1/1, v/v) to a glass tube with 3 g of fresh sample (Margenat et al., 2018). The sample was then sonicated for 15 min and centrifuged at 2500 rpm for 15 min. It was further filtered through a 0.22 μm nylon filter (Scharlab, Barcelona, Spain). After the solvent was removed by purging and drying the sample with nitrogen gas, the sample remaining in the tube was weighed and operationally defined as lipid content.

2.2.3 Trace elements

2.2.3.1 Soils and fertilizers

The TEs in soils and fertilizers were measured according to the method published by EPA (1994). 1 g dried and homogenized sample was digested with 4 mL HNO_3 (concentrated HNO_3 to ASTM type I water ratio of 1:1, v:v) and 10 mL HCl (concentrated HCl to ASTM type I water ratio of 1:4, v:v) on a hot plate at 85 °C around 30 min, then transferred the mixture to 100 mL volumetric flask, and diluted to volume with reagent water. After centrifuging at 3756 × g for 20 min, 10 mL supernatant was diluted 5 times again, and the solution was measured using inductively coupled plasma mass spectrometry (Thermo Scientific, xSeries 2 ICP-MS). The mercury concentration was measured with an AMA-254 (Altec, Prague, Czech Republic).

2.2.3.2 Vegetables

The edible part of the vegetables (the leaf of the lettuce and root of the radish) was freeze-dried and digested with a microwave oven (Milestone Ethos). 0.1 g of powdered sample was weighed into the PTFE vessels, and 8 mL of HNO_3 and 2 mL of 31% H_2O_2 were added. The digestion program carried out was as follows: 15 min from room temperature to 90 ℃; 10 min at 90 ℃; 20 min from 90 ℃ to120 ℃; 15min from 120 ℃ to 190 ℃; 20 min at 190 ℃. After cooling to room temperature, the digested sample was analyzed by ICP-MS. The mercury concentration was measured with an AMA-254 (Altec, Prague, Czech Republic).

2.2.4 Antibiotics

2.2.4.1 Soils and fertilizers

A 0.5 g portion of freeze-dried soil or fertilizer matrix was weighed in a 50 mL glass tube with 25 µL of a mix of surrogates at 2 ppm and left to equilibrate for 1 h. Four mL of McIlvain-EDTA buffer and 1 mL of ACN were added to the sample, which was then vortexed and centrifuged at 2500 rpm for 15 min. Two mL of lead acetate (200 mg/L) was added to the sample to remove proteins, and it was centrifuged again for 15 min. The supernatant was transferred to a clean vial and diluted by adding 13 mL of 0.2 M EDTA solution. A polymeric reverse-phase Strata-X (200 mg/6 mL) SPE cartridge was conditioned with 5 mL MeOH and 5 mL water. Following the percolation of the crude extract, the cartridge was washed with 5 mL of water and dried. The cleaned extract was eluted with 5 mL MeOH followed by evaporation of the solvent under a gentle N_2 stream and dissolved in 200 μL of mobile phase (H_2O/ACN, 95/5, v/v) before analysis in the UPLC-MS/MS. The final extracts were filtered through a 0.22 μm pore nylon filter and injected into a UPLC-MS/MS.

2.2.4.2 Vegetables

25 μL of a surrogate standard (4 μg/mL) was added to a 5 g wet weight sample and left to equilibrate for 1 h. A vegetable sample was then extracted with 10 mL of methanol in an ultrasonic bath for 15 min. The extracts were centrifuged for 15 min at 3000 g. Two extraction cycles were required. The supernatants were decanted and combined in a glass vial and evaporated under a gentle nitrogen stream at a temperature of 40 °C until they had been reduced to 1 mL. The concentrated extracts were then diluted with 10 mL of water to perform the SPE clean-up step. The SPE cartridges (polymeric reverse phase Strata-X cartridge, 100 mg/6 mL) were first preconditioned with 6 mL of methanol and 6 mL of water. After sample loading, the polymeric cartridge was washed with 1 mL of water with 5% methanol. The cartridges were then dried under a nitrogen stream, followed by elution with 2 mL of a mixture of methanol and ethyl acetate (1:1, v:v). The eluted fraction was evaporated to dryness and reconstituted in 1 mL of mobile phase (H_2O/ACN, 95/5, v/v). The final extracts were filtered through a 0.22 μm pore nylon filter and injected into a UPLC-MS/MS.

2.2.5 Antibiotic resistance gene

2.2.5.1 Soils and fertilizers

DNA was extracted from the soil and sewage-sludge samples (250 mg) was extracted using a DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol, to a final elution volume of 100 μL. The concentration and quality of DNA were measured using a NanoDrop Spectrophotometer 8000 (Thermo Fisher Scientific).

Dynamo ColorFlash SYBR Green (Thermo Scientific, Inc.) chemisry was used for *mec*A, *tet*M, and $bla_{\mathrm{OXA\text{-}58}}$ qPCR quantifications; all the other ARGs were quantified with LightCycler 480 SYBR Green I Master (A F. Hoffmann–La Roche AG, Inc). Two thermal cycling conditions were employed. For *sul*1, *intl*1, bla_{TEM}, $bla_{\mathrm{CTX\text{-}M\text{-}32}}$, *qnr*S1, and 16S rDNA amplification: 95 °C for 10 min as activation step, 45 extension cycles (15 s at 95 °C, 1 min at the selected annealing temperature, Supplementary). For *tet*M, *mec*A, and $bla_{\mathrm{OXA\text{-}58}}$ amplification: 7 min at 95 °C as activation step, 40 amplification cycles (10 s at 95 °C, 30 s at the selected annealing temperature). All qPCR reactions were performed in a Lightcycler 480 II (A F. Hoffmann–La Roche AG, Inc). Melting curves were obtained to confirm amplification specificity. Reactions were conducted in 20 μL volumes on 96-well plates. Optimal primer concentrations were 0.15 μM for *tet*M, *mec*A, and bla_{OXA} primers and 0.3 μM for *sul*1, intl1, bla_{TEM}, $bla_{\mathrm{CTX\text{-}M\text{-}32}}$, *qnr*S1, 16S rDNA primers. All qPCR assays were run as technical duplicates. A fixed dilution of raw DNA extract was used. The quality criteria used was an $R^2 > 0.99$, the slope of the standard curve should be between -3.1 and -3.4, the accepted efficiency of the reactions was ranging from 90% to 110%.

2.2.5.2 Vegetables

The edible part of the vegetable (90–100 g) was processed in a grinder (Grindomix GM200, Retsch, Inc). The crushed vegetable matter was transferred to a beaker along with 50 mL of sterile phosphate-buffered saline (PBS), mixed gently, and then filtered through a sterile gauze to remove the pulp. The resulting filtrate was transferred to 50 mL tubes through a 100 μm nylon mesh cell strainer (Corning® Cell Strainer) and centrifuged at 4000 rpm for 15 min. DNA extraction was carried out from the pellets and absolute quantification of ARGs (*sul*1*, qnr*S1*, bla*$_{\mathrm{TEM}}$*, tet*M*, bla*$_{\mathrm{CTX\text{-}M\text{-}32}}$*, bla*$_{\mathrm{OXA\text{-}58}}$, and *mec*A), *intI*1, and the 16S genes was performed as indicated in the soil and sludge section. The 16S values were subsequently corrected as determined by the 16S sequencing to remove plastidial (chloroplasts, leucoplasts, and mitochondria) 16S sequences.

2.2.6 PAHs

The extraction of PAHs in soil was performed on a PSE-One (Applied Instruments, USA) by duplicate. Briefly, 3 g of freeze-dry soil was extracted with n-hexane/acetone 1:1. The extraction conditions were 110 °C, 140 bar, and 3 cycles with solvent mixture of 15 min each. The cleanup of the extracts was carried by adsorption chromatography on a glass column packed with 5 g of neutral alumina (activated at 400 °C, deactivated with 3% water) and anhydrous sodium sulfate (top). Then, different fractions were eluted: FI (10 mL n-hexane), FII (10 mL n-hexane/ ethyl acetate (9/1, v/v)), and FIII (10 mL n-hexane/ethyl acetate (1/1)). For PAH determination, FII was analyzed in a Bruker Scion 436GC SQ apparatus (Bruker Daltonics Inc., Billerica, MA, USA) using the following: splitless injection (hexane, 290 °C; the purge valve was activated 50 s after the injection); helium carrier gas (0.6 mL/min); a 20 m × 0.18 mm i.d. column with a 0.18μm coating of TRB-5MS stationary phase (from Teknokroma, Barcelona, Spain); a column temperature program consisting of 1.20 min at 60 °C, a 14 °C/min ramp up to 200 °C, a 7.5 °C/min ramp up to 300 °C, and 10 min at 300 °C; transfer line and ion source temperatures of 280 °C and 290 °C, respectively; data acquisition in full-scan mode from 50 to 500 amu, with 6.2 min of solvent delay.

2.2.7 CECs

5 g of soil (fw), homogenized and sieved through 2 mm mesh, were placed in a glass tube. It was fortified with 31.25 ng of a mixture of 6 surrogates and left to equilibrate for 30 min. The extraction was performed by sonication for 15 min three times with 5 mL of acetone/ethyl acetate (1:1, v/v). The extract was then centrifuged at 3100 rpm for 10 min, and decanted the supernatant. The soil was extracted three additional times with 4 mL, 5 mL, and 4 mL of solvent. The supernatants were

combined and were nitrogen-evaporated in a water bath at 40 °C to about 1 mL. Then, the concentrated solution was re-dissolved in 500 mL de-ionized water, and the pH was adjusted to 3 by concentrated sulfuric acid. Then, the extraction was loaded onto previously conditioned SPE cartridges (STRATA X, 100 mg, 6 mL). The cartridge was eluted with 10 mL of ethyl acetate and was dried by nitrogen for 20 min to 250 μL. Besides, 37.25 ng of triphenylamine (TPhA) was added as an internal standard. Finally, a 50-μL aliquot was analyzed by GC–MS/ MS without derivatization, and another 50 μL aliquot was analyzed derivatized with 10 μL of MTBSTFA.

2.3 Data analysis

The experimental results were statistically evaluated using the SPSS package (Chicago, IL, US). All data sets were checked for normal distribution using the Kolmogorov–Smirnov test. If data is normal, ANOVA/ t-test, Pearson's correlation analysis were performed for comparisons or for analyzing interactive effects between different factors. Otherwise, a non-parametric Mann-Whitney U test was performed, and Spearman's correlation coefficient was calculated. Principal component analysis (PCA) was conducted and varimax rotation was applied because orthogonal rotation minimizes the number of variables with a high loading on each component and facilitates the interpretation of results. Statistical significance was defined as $p < 0.05$.

Chapter 3 Results and discussion

The present thesis assesses the occurrence of several contaminants in crops grown under different soil fertilization schemes and in peri-urban soils and furthermore evaluates the impact on plant growth and human health implication. The results obtained within this framework are divided into 4 topics

Topic 1: stands on being the first study where the plant uptake and human health implications of TEs and ABs were monitored in lettuces following the application of three organic fertilizers (manure, sewage sludge, and organic fraction of municipal solid waste) under real agricultural practices.

Topic 2: assesses the effect of different Cu and Zn content in sewage-sludge soil amendment on the uptake of TEs, ABs, and ARGs in the edible part of lettuce (leaf) and radish (root), as well as evaluate the phytotoxicity and human health implications.

Topic 3: compares the concentration of trace elements in vegetables grown on soils amended once and twice with fertilizer, and evaluates the potential risk of repeated organic fertilization for human health.

Topic 4: monitors the chemical characterization of soil surroundings on peri-urban agriculture in the Baix Llobregat Agrarian Park (BLAP) in the city of Barcelona, and uses two bio-monitoring indexes to assess soil pollution.

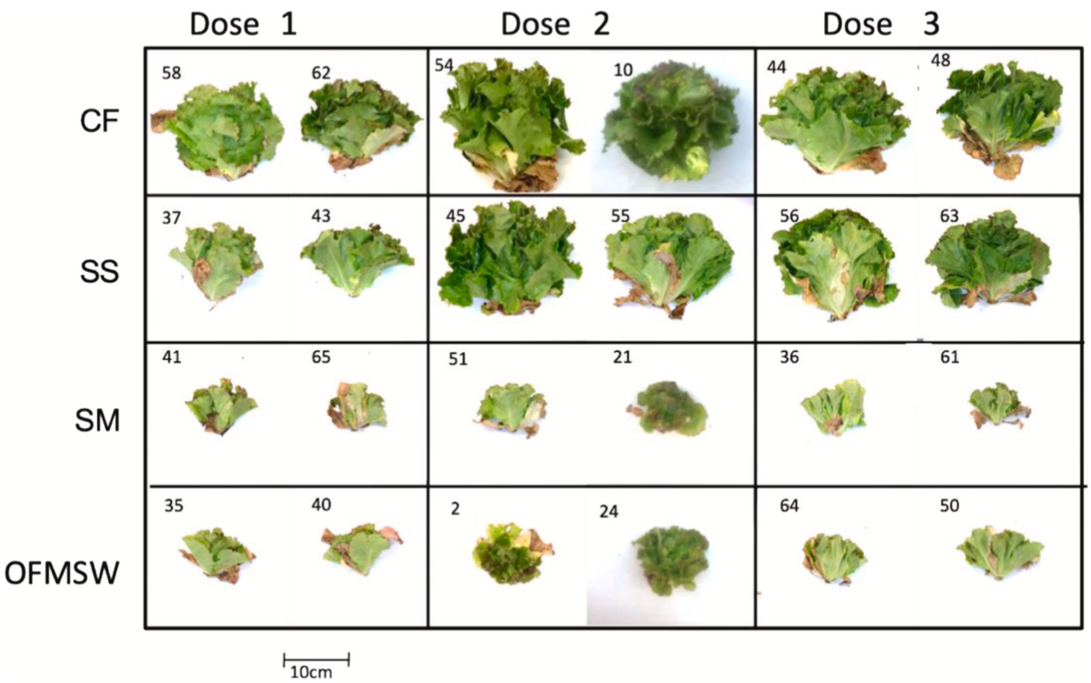
3.1 Topic 1: Plant uptake and human health implications of TEs and ABs under different fertilization

This topic assessed potential effects of organic fertilizers on the vegetal productivity, physiology, and the accumulation of contaminants, as well as evaluated human health risk.

Based on the article:

Occurrence and human health risk assessment of antibiotics and trace elements in *Lactuca sativa* amended with different organic fertilizers.

Environmental Research 190, 109946



Occurrence and human health risk assessment of antibiotics and trace elements in *Lactuca sativa* amended with different organic fertilizers.

Abstract

Soil amendment with organic fertilizers is an effective approach to improve soil fertility. However, organic fertilizers may contain pollutants such as trace elements (TEs) and antibiotics (ABs), which, once deployed in arable soil, can be taken up by vegetables and have adverse effects on crops and human health. This study assesses the presence of 15 TEs and 16 ABs in lettuce grown in a greenhouse facility and amended with 3 different organic fertilizers (sewage sludge (SS), organic fraction of municipal solid waste (OFMSW), and swine manure (SM)) at 3 different fertilization doses. The results show that lettuces amended with SS resulted in the lowest content of TEs. Although 11 ABs were detected in the SM and SS fertilizers, only 3 ABs were detected in lettuce leaves. The concentrations of detected ABs in lettuce ranged from 0.67 ng/g fw (lincomycin) to 14.2 ng/g fw (ciprofloxacin) in SS. The organic fertilization dose did not affect the lettuce uptake of TEs or ABs. Moreover, the use of SS resulted in the highest lettuce yield of the organic amendments. The total hazard quotients (THQs) obtained for TEs and ABs were less than 1 for all the studied fertilization treatments. The highest THQs for TEs were observed in lettuce amended with SM (0.11–0.16), whereas the highest THQs for ABs were observed in SS treatments (0.06–0.09). The results thus suggest that consumption of lettuces amended with organic fertilizers would not pose a risk to human health due to the presence of studied TEs or ABs, but potentially harmful combined effects cannot be ruled out.

Keywords: Organic fertilizers, Trace elements ,Antibiotics, Uptake, Yield, Human health Risk assessment

3.1.1 Introduction

Over the past century, the world population has quadrupled, and it is estimated that it will grow by more than a third in the period between 2017 and 2050 (Elferink and Schierhorn, 2016). This scenario would require an increase in food production of around 70% by 2050 (FAO, 2011). Around 90% of this growth in crop production would be accounted for by higher yields, greater cropping intensity, and agricultural land expansion (FAO, 2009). As agriculture becomes more dependent on plant nutrients, fertilization must be used to replenish the soil's depleted stores of them (mainly N, P, and K). Between 30 and 50% of yield growth may be attributable in one way or another to commercial fertilizers (Blanco, 2011).

Indeed, organic amendments such as manure, organic waste, compost, and biochar could be a reliable and effective approach to enhance soil fertility in a circular economy/sustainable agriculture framework. The organic fertilizers most often used in the context of the circular economy are animal-based waste (manure), compost (plant sources or food waste), and urban waste (sewage sludge and household waste) (Chew et al., 2019). Manure has been used on farms across the planet for centuries. The organic fraction of municipal solid waste (OFMSW) refers to a mixture of treated (digested or composted) waste from parks, gardens, and kitchens (Campuzano and González-Martínez, 2016). It can be converted into valuable organic matter through the activity of microorganisms during the composting or digestion pro- cesses, which can naturally decompose the waste and transform it into usable compost. The OFMSW is also rich in organic components such as carbohydrates, proteins, lipids, and organic acids, making it a potential source of fertilization (Paritosh et al., 2017). Sewage sludge (SS) is a form of organic waste with a high concentration of phosphorus. Nevertheless, these amendments may pose a potential human health risk if they are applied in farming activities, since they contain biological and chemical contaminants. The presence of a wide range of contaminants, including trace elements (TEs), organic pollutants, potential human pathogens, and emerging pollutants such as antibiotics (ABs) and antibiotic resistance genes (ARGs), has already been reported in some of these organic fertilizers (EMA, 2009; Mao et al., 2015; Wohde et al., 2016; Xie et al., 2018).

The application of fertilizers may affect the presence of TEs and ABs in food crops. Morera et al. (2002) found a positive correlation between the sludge application rate and the overall mean percentage of metals extracted in non-residual fractions of soils. Other studies have reported that the presence of TEs and ABs could lead to changes in plant morphology and physiology. Reis et al. (2014) compared the effects of inorganic and compost fertilization on Lactuca sativa L. grown in a greenhouse and found greater growth (plant height, stem diameter, and chlorophyll content) to be related to the presence of compost. Boxall et al. (2006) reported a conceptual model for veterinary pharmaceutical uptake in vegetables (i.e., lettuce and carrot roots) grown in manure-amended soils. Moreover, several reports demonstrate that a variety of crops can uptake ABs, including sulfonamides, fluoroquinolones, tetracyclines, and trimethoprim, from manure-amended soil (Dolliver et al., 2007; Kumar et al., 2005; Mullen et al., 2019; Wang et al., 2016). Vertical transport of veterinary antibiotics has been evaluated in manure-amended soils, but they accumulate on the soil subsurface (Gros et al., 2019). In a review of 169 compounds in SS and 450 wastewater treatment trains, Verlicchi and Zambello (2015) reported that, following anaerobic digestion, some ABs (fluoroquinolones and macrolides) are the predominant class of pharmaceuticals in SS. Therefore, the application of biosolids in soils can cause phytotoxicity and can contaminate human and animal food chains (Sidhu et al., 2019). However, an integrated assessment including both TEs and ABs, as one of the most important classes of contaminants of emerging concern, and their impact on agronomical and human health has not yet been carried out.

The novelty of this manuscript stands on being the first study where the plant uptake and human health implications of TEs and ABs were monitored in lettuces following the application of three organic fertilizers under real agricultural practices. Accordingly, the assessment of the occurrence and accumulation of 15 TEs and 16 ABs (fluoroquinolones, sulfonamides, tetracyclines, a lincosamide, and a macrolide) in lettuce crops grown with different organic amendments (SS, OFMSW, and SM) compared with chemical fertilization (CF) under real agriculture conditions. Moreover, the potential effects on the crop yield and morphological response of plants grown with and without organic fertilization will be assessed. Finally, the human health risk associated with the consumption of lettuces amended with the aforementioned organic fertilizers will also be assessed.

3.1.2 Material and methods

3.1.2.1 Experimental design

This experiment was conducted in a glass greenhouse facility located at the Agròpolis-UPC agriculture experimental station (41°17′18″N, 2°02′43″E) in Viladecans (Barcelona, Spain). Lactuca sativa L. was selected as the horticultural vegetable, and a total of 60 units were placed individually in 2.5 L amber glass pots (15 cm diameter, 20 cm high) with an inverted bottle shape fitted with a bottom outlet connected to drainage tubing (0.5 cm diameter) and filled with 2.3 kg of soil sieved to 2 mm (Hurtado et al., 2016). The soil used was collected from the agricultural area located in the Llobregat River Delta (41°170′ N, 2°02′E). The soil had a loam-clay texture (40% sand, 35% silt, 25% clay), a pH of 8.5, and an electrical conductivity of 0.24 dS m^{-1}. The total organic carbon content was 1.27% and the nitrogen content (Kjeldahl) was 0.09% of the soil dry weight. The Olsen phosphorous concentration was 33 mg/kg, whereas the K^+ , Ca^{2+} , Mg^{2+}, and Na^+ cations were 344, 7014, 362, and 91 mg/kg of the soil dry weight, respectively (Table S1.1).

Three types of organic fertilizers were evaluated: solid fraction of swine manure (SM) (Cooperativa Agraria, Torelló, Osona, Spain), digested SS from a wastewater treatment plant (WWTP) surrounded by industries in the nearby city of Barcelona (Gavà-Viladecans, Spain), and OFMSW from a composting plant (Torrelles del Llobregat, Spain). This OFMSW was obtained from a mixture of pruning waste from the nearby area and organic waste from the Hospital Universitari Vall d'Hebrón kitchen. The organic fertilizer parameters are shown in Table S1.2. Chemical fertilizer was obtained from reagent grade chemicals (NH_4NO_3, P_2O_5, and K_2O), so no TE impurities such as heavy metals are expected.

The experimental set-up consisted of 4 treatments at 3 fertilization doses of SS (primary and secondary sludge with anaerobic digestion), OFMSW (composted municipal organic food waste composted with wood waste), SM (solid fraction), and chemical fertilization (CF) as a control. This resulted in 12 treatments with 5 repetitions each, for a total of 60 randomly distributed containers planted with lettuce. The estimated nutrients to be added (N, P, K) were based on previously reported studies for lettuce crops (80–100 kg of N per ha; 30–50 kg of P_2O_5 per ha; and 160–210 kg K_2O per ha) (García-Serrano Jiménez et al., 2009). According to the chemical composition of the soil, nitrogen was selected as the limiting nutrient. The amount of organic fertilizer added per pot was calculated to ensure the same quantity of total nitrogen in all treatments (100 kg of N per ha). Based on this value, 3 doses were tested: dose 1 (half the optimal N dose), dose 2 (optimal N dose) as the reference nitrogen dose, and dose 3 (twice the optimal N dose).

Batavia lettuce (*Lactuca sativa* L. cv. Maravilla de Verano) was planted in the pots. The total duration of greenhouse cultivation was 57 days, from October 8 until harvesting on December 4, 2018. Finally, drip irrigation was applied through a connection to a reservoir containing mainly rainwater combined with groundwater.

3.1.2.2 Analytical procedures

(1) TE extraction

TE extraction from the soil was adapted from Llorente-Mirandes et al. (2010). A 0.1 g dried powdered sample was weighed in the PTEE microwave digestion vessels, and 8 mL HNO_3 (1:1) and 2 mL of 31% H_2O_2 were added to perform the microwave digestion (Milestone, Sorisole, BG, Italy). The following digestion program was run: 15 min from room temperature to 90 °C, 10 min maintained at 90 °C, 20 min from 90 °C to 120 °C, 15 min from 120 °C to 190 °C, and 20 min maintained at 190 °C. After cooling to room temperature, the digested samples were kept at 4 °C until analysis. Digestion blanks were also prepared in each sample digestion series.

(2) AB extraction from soil and organic fertilizers

The ABs were selected based on their occurrence in organic fertilizers and wastewater. Table S1.3 shows the physicochemical properties of these ABs. The reagents used are detailed in Surporting information. ABs were extracted from the soil matrix following the extraction procedure described earlier (Berendsen et al., 2015). A 0.5 g portion of freeze-dried soil or fertilizer matrix was weighed in a 50 mL glass tube with 25 µL of a mix of surrogates at 2 ppm and left to equilibrate for 1 h. Four mL of McIlvain-EDTA buffer and 1 mL of ACN were added to the sample, which was then vortexed and centrifuged at 2500 rpm for 15 min. Two mL of lead acetate (200 mg/L) was added to the sample to remove proteins, and it was centrifuged again for 15 min. The supernatant was transferred to a clean vial and diluted by adding 13 mL of 0.2 M EDTA solution. A polymeric reverse-phase Strata-X (200 mg/6 mL) SPE cartridge was conditioned with 5 mL MeOH and 5 mL water. Following the percolation of the crude extract, the cartridge was washed with 5 mL of water and dried. The cleaned extract was eluted with 5 mL MeOH followed by evaporation of the solvent under a gentle N_2 stream and dissolved in 200 µL of mobile phase (H_2O/ACN, 95/5, v/v) before analysis in the UPLC-MS/MS. The final extracts were filtered through a 0.22 µm pore nylon filter and injected into a UPLC-MS/MS. The UPLC-MS/MS methodology is described elsewhere (Tadić et al., 2019).

(3) AB extraction from lettuce

25 µL of a surrogate standard (4 µg/mL) was added to a 5 g wet weight sample and left to equilibrate for 1 h. A vegetable sample was then extracted with 10 mL of methanol in an ultrasonic bath for 15 min. The extracts were centrifuged for 15 min at 3000 g following a previously described methodology (Tadić et al., 2019). Two extraction cycles were required. The supernatants were decanted and combined in a glass vial and evaporated under a gentle nitrogen stream at a temperature of 40 °C until they had been reduced to 1 mL. The concentrated extracts were then diluted with 10 mL of water to perform the SPE clean-up step. The SPE cartridges (polymeric reverse phase Strata-X cartridge, 100 mg/6 mL) were first preconditioned with 6 mL of methanol and 6 mL of water. After sample loading, the polymeric cartridge was washed with 1 mL of water with 5% methanol. The cartridges were then dried under a nitrogen

stream, followed by elution with 2 mL of a mixture of methanol and ethyl acetate (1:1, v:v). The eluted fraction was evaporated to dryness and reconstituted in 1 mL of mobile phase (H_2O/ACN, 95/5, v/v). The final extracts were filtered through a 0.22 µm pore nylon filter and injected into a UPLC-MS/MS. The analytical quality parameters used for the determination of ABs in lettuce tissues are shown in Table S1.4 and Table S1.5.

3.1.2.3 Morphological and physiological parameters

The length and number of lettuce leaves were measured at the end of the experiment for each mesocosm. Chlorophyll content in leaves and weight were measured in situ. Chlorophyll was gauged by a chlorophyll meter (Opti-Sciences, Hudson, NH, USA). Lipid extraction was carried out in the laboratory by adding 15 mL of ethanol/hexane (1:1, v/v) to a glass tube with 3 g of fresh sample. The sample was then sonicated for 15 min and centrifuged at 2500 rpm for 15 min. It was further filtered by a 0.22 µm nylon filter (Scharlab, Barcelona, Spain). The solvent was removed by purging and drying with nitrogen gas, and the sample remaining in the tube was weighed and operationally defined as lipid content.

3.1.2.4 Human health risk assessment

(1) Hazard quotient for TEs

The potential risk to human health associated with the consumption of TEs in vegetables was assessed using the hazard quotient (HQ) approach, as described in Margenat et al. (2019):

$$\mathrm{HQ} = \frac{\mathrm{EDI}}{\mathrm{RfD}}$$

Where RfD is the reference dose, i.e., the maximum tolerable daily intake (µg/kg bw/day) of a specific metal without appreciable risk, and EDI is the estimated daily intake (µg/kg bw/day), calculated as follows:

$$\mathrm{EDI} = \frac{\mathrm{DI} \times C_M}{\mathrm{BW}}$$

where DI represents the daily intake in g fw per day. The DI for fresh vegetables in Spain used in the calculations was taken from the EFSA's Comprehensive Food Consumption Database (87 g of fresh lettuce per day for adults and 38 g for children). The average body weights of a Catalan male adult (20–65 years old) and child (4–9 years old) were used, i.e. 70 and 24 kg, respectively. C_M is the 95th percentile value for the concentration of each TE in lettuce (µg/g fresh weight (fw)), and BW is the body weight (kg) of the target individual.

The total hazard quotient (THQ), calculated as the sum of all the HQs for all the chemicals to which an individual might be exposed, was also evaluated. The risk assessment for Cr was conducted using the RfD of the Cr^{3+} form, due to its predominance in lettuce (Asfaw et al., 2017). Oral RfDs were used to evaluate the human health risk, except for Co, Rb, and Hg, for which this value was not available. Therefore, the risk assessment was evaluated for 12 out of the 16 TEs.

(2) Human health risk of ABs

The EDI (µg/kg/day) was calculated according to the aforementioned equation for TEs, where DI (g/day) and BW (kg) are the daily intake of the edible part of vegetables and body weight, respectively. In this case C_M (µg/g) indicates the 95th percentile value for the concentration of each AB in vegetables.

The potential risk was estimated using the hazard quotient (HQ) approach (SPN, 2003; WHO, 1997). The HQ is the ratio between the EDI and threshold levels considered acceptable daily intakes (ADIs). The ADI (µg/kg/day) values used were taken from a list based on microbiological and toxicological endpoints previously compiled by Wang et al. (2017). The ADIs for the ABs detected in the lettuce samples were as follows: 0.15 µg/kg/day for ciprofloxacin, 25 µg/kg/day for lincomycin, and 1.7 µg/kg/day for azithromycin. The THQ is estimated as the sum of the individual HQs. If the THQ is less than 1, the risk is generally deemed to be acceptable.

3.1.2.5 Data analysis

The experimental results were statistically evaluated using the SPSS v. 26 package (Chicago, IL, US). All data sets were checked for normal distribution using the Kolmogorov–Smirnov test to ensure that para- metric statistics were applicable. The overall comparison of the occurrence of chemical contaminants between fertilizer products was performed with a paired-sample t-test (dependent samples), whereas the comparison of the concentration of each chemical contaminant between plots was analyzed with an independent-samples t-test. Statistical significance was defined as p

< 0.05. Principal component analysis (PCA) was performed on the concentration of TEs and ABs in the lettuce samples, as well as on the lettuce morphological and physiological parameters through the use of a correlation matrix.

3.1.3 Results and discussion

3.1.3.1 Occurrence of TEs and ABs in soil and organic fertilizers

Table S1.6 shows the TE content for each type of fertilizer used. The total average TE concentration was greatest in the SS (2043 mg/kg dw), followed by the agricultural soil (1221 mg/kg dw), the SM (1058 mg/kg dw), and, finally, the OFSMW (838 mg/kg dw). The most abundant TEs in the soil and organic fertilizers were Cu, Zn, B, Sr, Mn, and Ba. The TE levels were compliant with Catalan Law 5/2017 for agricultural soil. Likewise, the use of the different organic fertilizers was compliant with the limits set by the European Commission under Directive 86/278/EEC and with Spanish Royal Decree 1310/1990 for agricultural use.

According to the requirements of Spanish law for fertilizers of animal and vegetable origin (Royal Decree 506/2016), SM is Class C and subject to a maximum yearly dosage. The high concentration of Cu and Zn found in this fertilizer is consistent with the fact that these TEs are often added to livestock diets as supplements or growth factors, but barely assimilated by the animals. Therefore, up to 90% of the ingested Cu and Zn is excreted (Berenguer et al., 2008). Nevertheless, SS showed greater concentration of Cu and Zn than SM (Table S1.4). The high concentration of Cu may be explained by the impact related to industrial activities in the surrounding area of the WWTP.

The soil used in this study was quite rich in TEs and showed greater concentrations of Co, Mn, As, and Pb than the organic fertilizers assessed for soil amending. Only the SS fertilizer exceeded the concentration of most of the TEs analyzed in the soil, and it was the only case in which soil concentrations of TEs increased when the fertilizer was applied. This is consistent with previous studies carried out in the area, which have shown that soil from the Llobregat Delta is very heterogeneous. In some cases, as in this study, autochthonous sand soil is used for construction and backfilled with building demolition debris from the nearby city of Barcelona (Custodio, 2012).

Regarding the soil's physicochemical properties (Table S1.1), the pH was in the same range in the soil and fertilizers, so it was not expected to cause differences between treatments. However, fertilization enhances the organic matter content in soil and may influence the uptake of TE by plants. TEs are expected to be more strongly linked to soil organic matter and, thus, in the short term, their incorporation into the plant can be diminished (Rieuwerts et al., 1998). The organic matter content was up to 92 times higher in the SM than the soil (1%, dw), but also greater than in the OFMSW (42%, dw) and SS (41%, dw). However, in the medium term, once the organic matter begins to degrade, TEs can be released and uptaken by plants (Smith, 2009).

Five families of antibiotics were analyzed including fluoroquinolones, sulfonamides, tetracyclines, macrolides, and lincosamides. The concentrations of the 16 ABs ranged from undetected to 9831 ± 2200 ng/g dw in the organic fertilizers (Table S1.7). Nine ABs were identified in the SM, 6 in the SS, and only 1 in the OFMSW. ABs were not identified in the unamended soil. Ciprofloxacin was the most abundant AB in the SS (9317 ng/g dw), whereas the other detected ABs were below 250 ng/g dw. Conversely, 2 ABs (lincomycin and azithromycin) and 1 degradation intermediate (8-(hydroxyquinoline)) were detected in the SM above 3000 ng/g dw. These results are consistent with other published studies on SM, where fluoroquinolones, tetracyclines, and sulfonamides were detected at levels between 400 and 46000 ng/g dw (Xie et al., 2018) and with the average values reported by Wohde et al. (2016). With regard to the SS composition, McClellan and Halden (2010) performed a survey study in 94 U.S. WWTPs and found that of the 72 pharmaceuticals and personal care products monitored, ciprofloxacin was the most abundant AB with an average concentration in SS of 6800 ± 2300 ng/g dw. No information is available on the occurrence of ABs in OFMSW, which was the organic amendment with the lowest AB concentration (Table S1.7).

3.1.3.2 Effect of organic fertilizers and dose on the occurrence of TEs in vegetables

The sum of the average concentrations of the TEs analyzed in lettuce leaves from dose 2 ranged from 13 mg/kg fw in the CF to 29 mg/kg fw in the lettuce amended with

SM (Table 3.1.1). The concentration of Cu and Zn in lettuce amended with organic fertilizers was statistically greater than in those grown with the CF. This is surprising since the concentration of Cu in soil was greater than in the SM and OFMSW amendments (Table S1.6). This suggests that Cu plant bioavailability in these organic fertilizers is greater than in soil. On the other hand, the use of the SM and OFMSW fertilizers resulted in a significantly greater concentration of Sr, Ba, Cr, and Mo in lettuce leaves than the use of CF and SS. Despite the greater concentration of these TEs found in the SS amendment in comparison with the other organic fertilizers (Table S1.6), the concentrations of these TEs were greater in lettuce amended with the SM and OFMSW fertilizers. This may indicate that the organic matter composition of the SS had a greater interaction with these elements and, therefore, lower plant bioavailability (Zeng et al., 2011). The concentrations of individual TEs in lettuce leaves were similar to those found in other studies, where SS or organic manure were used (Zubillaga and Lavado, 2002), as well as to those reported in previous field-scale studies in lettuce irrigated with reclaimed water (Margenat et al., 2018). It is worth noting that the Pb and Cd concentration values found in lettuce were always compliant with European Commission regulation No. 181/2006 for leaf vegetables (0.30 mg/kg fw for Pb and 0.20 mg/kg fw for Cd).

Table 3.1.1 Minimum, maximum and average concentration (mg/kg, fw) of the selected TEs in lettuce samples (n = 5). Dose 1 (half of the optimal N dose), dose 2 (optimal N dose) acting as a referential nitrogen dose and dose 3 (twice the N optimal dose).

Sample	Cu	Zn	B	Co	Sr	Mn	Cd	Ba	Cr	Mo	Hg	As	Ni	Pb
CF dose 1	(0.6–0.8)	(2.2–4.1)	(2.2–3.5)	(0.03–0.05)	(2.5–3.7)	(3.2–6.0)	(0.01–0.01)	(0.2–0.7)	(0.05–0.07)	(0.02–0.05)	(0.001–0.001)	(0.003–0.005)	(0.02–0.07)	(0.003–0.014)
	0.7	2.8	2.9	0.04	3.3	4.8	0.01	0.6	0.06	0.04	0.001	0.004	0.05	0.007
CF dose 2	(0.6–0.7)	(1.6–2.4)	(2.3–2.8)	(0.002–0.004)	(2.9–3.7)	(2.5–3.2)	(0.0–0.01)	(0.5–0.6)	(0.05–0.07)	(0.01–0.03)	(0.001–0.001)	(0.002	(0.04–0.05)	(0.003–0.018)
	0.6	1.9	2.5	0.003	3.4	2.9	0.01	0.6	0.06	0.02	0.001	0.005)	0.04	0.010
												0.004		
CF dose 3	(0.6–0.8)	(2.3–2.9)	2.2–3.2)	(0.003–0.003)	(2.3–3.4)	(2.5–3.4)	(0.01–0.01)	(0.3–0.6)	(0.04–0.07)	(0.02–0.03)	(0.001–0.001)	(0.003–0.004)	(0.02–0.04)	(0.004–0.009)
	0.7	2.5	2.7	0.003	2.9	3.1	0.01	0.4	0.05	0.02	0.001	0.003	0.03	0.006
SS dose 1	(0.7–1.0)	(3.2–4.4)	(2.5–4.8)	(0.004–0.007)	(2.8–4.7)	(3.7–6.7)	(0.01–0.02)	(0.5–0.8)	(0.04–0.17)	(0.03–0.06)	(0.001–0.001)	(0.005–0.011)	(0.04–0.07)	(0.004–0.010)
	0.9	3.6	3.8	0.006	3.9	5.8	0.01	0.6	0.10	0.04	0.001	0.007	0.05	0.007
SS dose 2	(0.8–1.0)	(3.0–3.7)	(2.7–4.1)	(0.003–0.005)	(2.5–3.4)	(3.0–4.6)	(0.01–0.01)	(0.3–0.4)	(0.04–0.06)	v0.03–0.03v	(0.001–0.001)	(0.003–0.005)	(0.03–0.06)	(0.002–0.011)
	0.9	3.4	3.2	0.004	2.9	3.9	0.01	0.3	0.05	0.03	0.001	0.004	0.04	0.006
SS dose 3	(0.6–1.2)	(2.6–4.3)	(2.0–3.6)	(0.004–0.006)	(2.4–4.6)	(2.6–3.4)	(0.01–0.01)	(0.2–0.4)	(0.03–0.07)	(0.02–0.05)	(0.001–0.001)	(0.004–0.008)	(0.05–0.11)	(0.007–0.023)
	0.8	3.5	2.7	0.004	3.0	3.1	0.01	0.3	0.05	0.03	0.001	0.005	0.08	0.013
SM dose 1	(0.9–1.5)	(3.2–7.7)	(3.6–5.7)	(0.007–0.017)	(5.6–8.5)	(5.8–15.6)	(0.01–0.03)	(1.3–1.9)	(0.06–0.17)	(0.09–0.17)	(0.002–0.002)	(0.006–0.014)	(0.06–0.28)	(0.014–0.035)
	1.1	5.5	4.5	0.011	6.9	9.4	0.02	1.6	0.10	0.12	0.002	0.010	0.16	0.024
SM dose 2	(1.0–1.8)	(4.5–7.0)	(2.9–5.0)	(0.009–0.013)	(4.5–6.0)	(6.3–7.9)	(0.01–0.02)	(1.0–1.5)	(0.13–0.22)	(0.07–0.15)	(0.002–0.002)	(0.006–0.009)	(0.07–0.11)	(0.014–0.031)
	1.3	5.4	3.9	0.011	5.4	7.0	0.01	1.3	0.16	0.10	0.002	0.008	0.09	0.022
SM dose 3	(1.1–1.4)	(4.3–6.0)	(4.0–5.8)	(0.007–0.010)	(5.1–7.4)	(6.4–13)	(0.01–0.02)	(1.2–1.6)	(0.06–0.12)	(0.12–0.16)	(0.002–0.002)	(0.006–0.014)	(0.07–0.14)	(0.012–0.026)
	1.2	4.9	4.6	0.009	6.1	8.6	0.02	1.5	0.08	0.13	0.002	0.010	0.10	0.018
OFMSW dose 1	(1.0–1.4)	(4.3–4.8)	(4.0–4.8)	(0.007–0.009)	(5.6–7.7)	(3.9–5.2)	(0.01–0.03)	(1.7–2.2)	(0.13–0.24)	(0.09–0.13)	(0.001–0.002)	(0.009–0.015	(0.07–0.28)	(0.018–0.029)
	1.2	4.6	4.5	0.008	6.8	4.5	0.02	2.0	0.19	0.10	0.001	0.013	0.14	0.022
OFMSW dose 2	(1.2–2.0)	(3.7–5.9)	(4.4–6.8)	(0.007–0.011)	(6.0–7.9)	(5.9–8.5)	(0.02–0.03)	(1.6–2.2)	(0.11–0.18)	(0.09–0.15)	(0.002–0.002)	(0.010–0.014)	(0.12–0.29)	(0.015–0.021)
	1.5	5.1	5.4	0.009	6.7	7.5	0.02	1.8	0.15	0.12	0.002	0.012	0.20	0.018
OFMSW dose 3	(0.7–1.0)	(3.1–4.4)	(3.1–4.4)	(0.005–0.009)	(3.9–5.2)	(4.5–5.8)	(0.01–0.01)	(1.1–1.5)	(0.07–0.24)	(0.07–0.10)	(0.001–0.002)	(0.004–0.006)	(0.09–0.17)	(0.003–0.007)
	0.9	3.9	3.5	0.007	4.4	5.0	0.01	1.3	0.15	0.08	0.001	0.005	0.13	0.005

Although there were differences between the TE composition of the organic amendments, no statistical differences were found between fertilization doses. In other words, a greater dosage of any of the fertilizers applied did not imply a greater accumulation of TEs in the cultivated lettuce. This may be due to the fact that the control soil had a high concentration of TEs, making differences between doses difficult to clearly discern. Similarly, Kidd et al. (2007) found that repetitive applications of SS in crops did not lead to an increase in TEs (Cu, Zn, and Mn) in plant shoot (Alyssum serpyllifolium, Cistus ladanifer, Zea mays) concentrations and concluded that metal leaching losses are greater than the transfer of metals into plant tissue and/or the food chain in the case of crops. The present results are also consistent with those of Castro et al. (2009), who observed that in lettuce leaves, the continued addition of organic fertilizers (SS and OFSMW) did not have an accumulative effect of TEs compared with inorganic fertilizers. Therefore, the application of fertilizers should also be considered to entail an increase in organic matter, which may reduce plant uptake of some elements as previously described by Rieuwerts et al. (1998). Additionally, it is worth noting that the soil used in this study was being exposed to the organic fertilizers for the first time, and, thus, the interaction between TEs from the organic fertilizers and the soil may have been stronger and somewhat reduced the plant bioavailability of the TEs. Further organic amendment cycles should be studied to elucidate this effect.

3.1.3.3 Effect of organic fertilizers and dose on the occurrence of ABs in vegetables

Although several antibiotics were identified in the organic fertilizer products, only 3 were detected above the LOQ in lettuce leaves (lincomycin, ciprofloxacin, and azithromycin), and only in lettuces amended with the SM and SS fertilizers (Table 3.1.2). No ABs were detected in lettuce grown with the CF or OFSMW fertilizers. The average AB concentrations detected in lettuce grown with SM and SS ranged from 0.67 to 1.10 ng/g fw for lincomycin and from 11.8 to 14.2 ng/g fw for ciprofloxacin in the SS. These results are consistent with the fact that ciprofloxacin was the most abundant AB found in the SS amendment (Table S1.7), but it also has to do with its physicochemical structure and biodegradability. Among the studied ABs, those that are present in their neutral form at the soil pH (Table S1.1and Table S1.3) had greater uptake. Overall, the incorporation of non-charged compounds would be greater than for ionic species, since they are repelled by cell membranes with electrical potential, while cationic species are attracted to the cell membranes, restricting their incorporation into the plant (Chuang et al., 2019). Nevertheless, these concentrations are one thousand times lower than those found in organic fertilizers (Table S1.7) and similar to those found in lettuces irrigated with reclaimed water or amended with SM (Azanu et al., 2018; Kang et al., 2013; Tadić et al., 2019). In fact, Kang et al. (2013) showed that the use of manure in 11 vegetable crops resulted in similar concentrations of ABs (chlortetracycline, monensin, sulfamethazine, tylosin, and virginiamycin) in vegetables to those observed in crops grown with CF (in all cases <10 ng/g fw).

With regard to the effect of the fertilization dose, the present results show that the concentration of lincomycin and ciprofloxacin slightly increased depending on the amount of SS added, but these differences were not statistically significant ($p>0.05$). Nevertheless, although the abundance of ABs was low, further studies should include the presence of transformation products since these by-products or metabolites have been observed to be present at greater concentrations in lettuce than parent compounds (Tadić et al., 2019).

Table 3.1.2 Minimum, maximum, and average concentration (µg/kg, fw) of ABs in lettuce samples (n = 5). Only ABs above the LOQ are shown.

Compound	SM dose 1 (n =5)	SM dose 2 (n =5)	SM dose 3 (n =5)	SS dose 1 (n =5)	SS dose 2 (n =5)	SS dose 3 (n =5)
Lincomycin	(0.83–1.2)0.93	(0.68–1.3)1.10	(0.82–1.2)1.1	(0.60–1.1)0.93	(0.68–2.6)1.1	(0.77–3.4)1.1
Ciprofloxaci	-	-	-	(9.3–34)12	(8.0–18)14	(7.8–29)16
Azithromycin	-	-	-	(0.55–2.7)1.7	(1.7–7.6)3.0	(1.6–10)4.4

3.1.3.4 Effect of organic fertilizers on crop morphology and yield

As with the TE and AB content in the lettuce samples, differences were also observed in crop morphology and yield between treatments (Fig. 3.1.1). For instance, leaf length ranged from 16.7 to 30.7 cm, the number of leaves ranged from 19 to 48, and the fresh weight showed considerable variability, ranging from 18 to 290 g/unit (Table 3.1.3). Lettuce yields and morphological values were similar to those found in other greenhouse conditions (Hurtado et al., 2017). These results show that lettuces amended with SS had similar morphological parameters (i. e., fresh weight, leaf height, number of leaves) to those amended with CF, while the other organic fertilizers (OFMSW and SM) resulted in lower morphological values (Table 3.1.3, $p<0.05$). The chlorophyll content was similar in the CF and SS treatments, but lower in the other treatments. No differences were observed in the carbohydrate content, but the lipid content followed the same trend as the changes in the morphological parameters. These results thus indicate that the SS fertilization had a similar agronomic yield to the application of the CF, while the other treatments exhibited lower values.

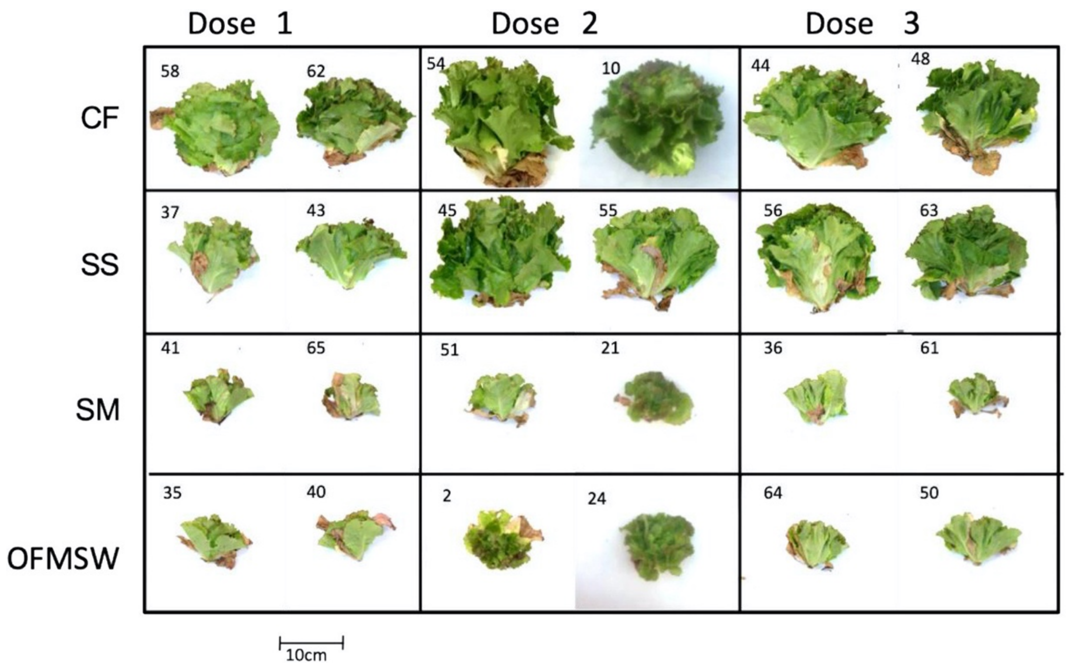


Fig 3.1.1 Images of lettuces growing under the different fertilization treatments (chemical fertilization (CF), sewage sludge (SS), swine manure (SM) solid fraction, and organic fraction of municipal solid waste (OFMSW). Dose 1 (half the optimal N dose), dose 2 (optimal N dose) as the reference nitrogen dose, and dose 3 (twice the optimal N dose). The number showed in each picture is the random location of the lettuce plant in the experimental set-up.

Table 3.1.3 Minimum, maximum and average levels of different lettuce quality parameters studied (n = 5).

Treatment	Fresh weight (g)	Leaves length (cm)	Number of leaves	Lipids (%)	Carbohydrates (%)	Total chlorophyll content (mg/cm^2)

CF dose 1	(104–147)^{a} 121	(19.6–21.5) 20.4^{a}	(32–37) 33^{a}	(0.18–0.20) 0.20^{a}	(0.57–0.59) 0.59^{a}	(0.014–0.022) 0.016^{a}
CF dose 2	(184–228)^{b} 219	(21.4–26.2) 25.2^{b}	(33–43) 37^{a}	(0.25–0.28) 0.26^{b}	(0.23–0.49) 0.33^{b}	(0.015–0.024) 0.021^{a}
CF dose 3	(257–285) 268^{c}	(23.4–24.7) 24.0^{b}	(35–41) 39^{a}	(0.21–0.29) 0.29^{b}	(0.38–0.70) 0.44^{a,b}	(0.015–0.019) 0.018^{a}
SS dose 1	(113–134) 119^{a}	(20.3–26.3) 22.3^{a}	(31–38) 36^{a}	(0.17–0.21) 0.19^{a}	(0.17–0.28) 0.23^{a}	(0.014–0.017) 0.014^{a}
SS dose 2	(176–203) 183^{b}	(23.0–28.4) 25.2^{a}	(35–44) 40^{a}	(0.14–0.25) 0.22^{a}	(0.26–0.37) 0.31^{b}	(0.019–0.021) 0.020^{b}
SS dose 3	(228–290) 244^{c}	(27.4-30.7) 29.5^{a}	(37–48) 42^{a}	(0.32–0.36) 0.35^{b}	(0.42–0.68) 0.52^{c}	(0.016–0.022) 0.022^{b}
SM dose 1	(24.0–30.0) 28.1^{a}	(16.7–17.6) 17.4^{a}	(21–22) 21^{a}	(0.03–0.30) 0.12^{a}	(0.37–0.71) 0.53^{a}	(0.012–0.016) 0.014^{a}
SM dose 2	(28.3–32.6) 30.5^{a}	(16.9–17.5) 17.2^{a}	(19–24) 21^{a}	(0.13–0.15) 0.14^{a}	(0.30–0.68) 0.54^{a}	(0.013–0.016) 0.014^{a}
SM dose 3	(31.5–36.3) 32.8^{a}	(17.1–18.2) 17.3^{a}	(21–24) 22^{a}	(0.12–0.14) 0.14^{a}	(0.31–0.59) 0.43^{a}	(0.013–0.015) 0.014^{a}
OFMSW dose 1	(29.4–37.1) 32.0^{a}	(17.2–17.9) 17.6^{a}	(21–28) 23^{a}	(0.12–0.17) 0.16^{a}	(0.23–0.51) 0.46^{a}	(0.014–0.015) 0.014^{a}
OFMSW dose 2	(34.1–43.7) 37.2^{a}	(17.3–18.7) 17.9^{a}	(24–28) 25^{a}	(0.17–0.19) 0.18^{a}	(0.19–0.59) 0.42^{a}	(0.012–0.016) 0.014^{a}
OFMSW dose 3	(45.4–52.3) 51.8^{b}	(18.1–19.9) 18.4^{a}	(24–26) 25^{a}	(0.16–0.21) 0.18^{a}	(0.20–0.48) 0.31^{a}	(0.011–0.013) 0.013^{a}

Different letters show statistical differences between doses ($p<0.05$).

Since the pollutant content in lettuce leaves was similar between the treatments, the main explanation for the agronomic difference between the SS and other treatments may be nitrogen availability (Kiba and Krapp, 2016). In fact, a lack of synchronization between nitrogen mineralization and nitrogen demand has been described as a challenge in culture strategies using organic fertilization (Pang and Letey, 2000). Therefore, although the organic fertilizers were applied ensuring a similar amount of Kjeldahl nitrogen (ranging from 2.3 to 2.8% dw), only ammoniacal nitrogen is easily available to plants. This is consistent with the fact that the ammoniacal-N content was greater in the CF and SS treatments (Table S1.2), whereas organic nitrogen was the predominant nitrogen form in the OFMSW and SM fertilizers (Urquiaga and Zapata, 2000). This fact, coupled with the short production cycle of lettuce and a low mineralization rate, prevented the organic nitrogen from decomposing and becoming available to the culture.

The fertilizer doses greatly affected lettuce yield. Whereas increased SS and CF doses resulted in an increase in the fresh weight, the increased SM and OFMSW doses did not statistically affect lettuce yield. The other studied parameters (morphology and lipid, carbohydrate, and chlorophyll content) were not affected by the dose of fertilizer added, except for the carbohydrate content in the SS and CF treatments, which increased in keeping with the dose. These changes can be explained by the amount of ammoniacal nitrogen present in each organic fertilizer, as mentioned above. Therefore, from the point of view of crop yield and morphology, the SS fertilizer was the most suitable amendment with similar results to those for the CF.

3.1.3.5 Sources and distribution of TEs and ABs

Principal component analysis (PCA) was performed on the whole data set (excluding soil due to the low sample size) to gain further insight into the TE and AB sources and the distribution behavior of the different assessed parameters in the lettuce samples. The PCA reduced the 26 measured variables to 4 principal components, which explained 78% of the total variability observed. Nevertheless, only the first two principal components, which accounted for 61% of total variability, were studied. The first component, which explained 51% of the variability, had high positive values (>0.8) for most of the studied TEs (Fig S1.1) and negative loadings for lettuce yield, morphological parameters, and carbohydrate content. This indicates that the lettuces with high yields were also the ones showing the lowest TE content. This was the case of the lettuces amended with CF and SS. The second component explained 10% of the variability and had high positive values (>0.6) for ABs (Fig S1.1). This indicates that these lettuces had a high accumulation of these compounds, which was the case with the SS and SM treatments.

Fig. 3.1.2 shows the score plot for PC1 vs. PC2, since it was the only plot that grouped samples. The plot separated the lettuce samples into three distinct groups.

Group I includes lettuces amended with all 3 doses of the CF, which had the lowest PC1 and PC2 values, indicating their lower concentration of pollutants compared with the other amendment treatments. Group II includes lettuce samples with a low TE impact and high yield, but a high presence of ABs (all 3 studied doses of the SS treatment), and Group III includes the samples with the greatest TE impact and a low yield (all 3 doses of the SM and OFMSW treatments). These results are consistent with the low uptake of TEs in SS and the high yield obtained in previous studies (Castro et al., 2009; Kidd et al., 2007) and suggest that the use of SS in agriculture is suitable, despite the presence of ABs.

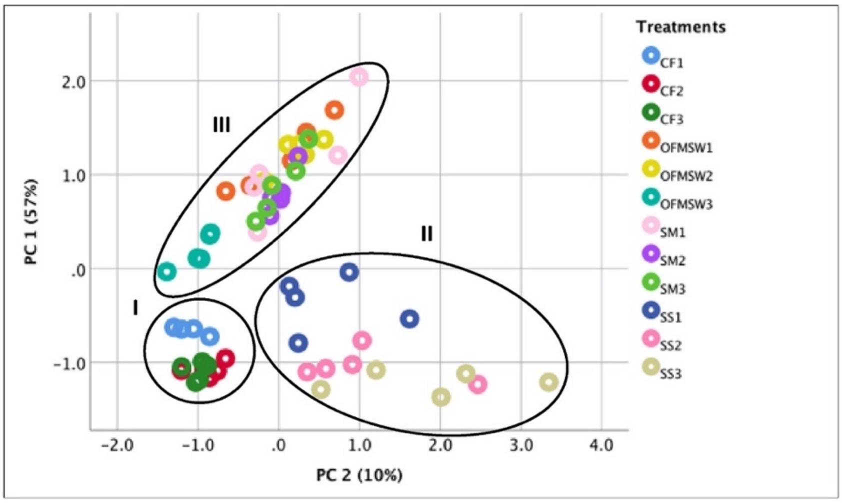


Fig 3.1.2 Score plot of the principal components PC 1 and PC 2 analysis obtained from 6 quality parameters, 15 TEs and 16 ABs in lettuce grown under the four treatments namely, chemical fertilization (CF), sewage sludge (SS), swine manure (SM) and organic fraction of solid municipal waste (OFMSW). The numeral 1, 2 and 3 denotes the dose of fertilizer applied in every treatment.

3.1.3.6 Human health implications

As mentioned earlier, both the TE and the AB concentrations in the lettuce were always compliant with European Commission regulation No. 181/2006 for leafy vegetables and EU Directive No. 37/2010 for foodstuff of animal origin. Nevertheless, not all pollutants are always included in these lists of contaminants and the risks should be assessed using HQ approaches.

Tables 3.1.4 and 3.1.5 show the estimated HQ and THQ for the consumption of these lettuce samples by a human adult and a child. HQs ranged from $2.15x10^{-5}$ to 0.05 in adults for Cr in CF dose 3 and Mn in SS dose1, respectively. Of these 12 TEs, on average, Mn posed the greatest health risk to adults and children, followed by As, B, Mo, Cd, Zn, Ni, Sr, Ba, Pb, Cu, and Cr in decreasing order. These findings are consistent with those of other studies carried out in Europe, China, and Ethiopia (Chang et al., 2014; Margenat et al., 2019; Woldetsadik et al., 2017). The THQ values obtained were less than 1 for all the studied fertilization treatments and apparently lower in the application of CF (0.05–0.07) and SS (0.06–0.09) than in the OFMSW (0.08–0.13) and SM (0.11–0.16) treatments. These results are consistent with the greater concentration of TEs found in lettuces amended with OFMSW and SM fertilizers (Table 3.1.1). Nevertheless, no statistically significant differences ($p > 0.05$) between treatment doses were observed. These results are likewise consistent with those from previous studies in which lettuces were amended with manure or irrigated with reclaimed water (Margenat et al., 2019).

Table 3.1.4 Values of hazard quotients (HQ and THQ) for lettuce considering the different treatments for adults.

Sample	B	Cr	Mn	Ni	Cu	Zn	Mo	Cd	Sr	Ba	Pb	As	THQ
CF (dose 1)	0.009	2.31 E-05	0.024	0.0017	0.0011	0.0064	0.0052	0.0070	0.0036	0.0018	0.0019	0.0084	0.07
CF (dose 2)	0.007	2.47 E-05	0.012	0.0012	0.0009	0.0040	0.0029	0.065	0.0032	0.0016	0.0026	0.0088	0.05
CF (dose 3)	0.008	2.15 E-05	0.012	0.0011	0.0010	0.0049	0.0027	0.0057	0.0028	0.0014	0.0012	0.0061	0.05
SS (dose 1)	0.012	5.37 E-05	0.024	0.0018	0.0012	0.0072	0.0054	0.0136	0.0040	0.0019	0.0023	0.0177	0.09
SS (dose 2)	0.010	2.16 E-05	0.017	0.0014	0.0013	0.0063	0.0034	0.0053	0.0029	0.0011	0.0015	0.0082	0.06
SS (dose 3)	0.009	2.38 E-05	0.012	0.0027	0.0014	0.0072	0.0044	0.0069	0.0036	0.0010	0.0031	0.0129	0.06
SM (dose 1)	0.014	5.40 E-05	0.053	0.0071	0.0018	0.0131	0.0170	0.0138	0.0071	0.0049	0.0049	0.0226	0.16
SM (dose 2)	0.012	7.17 E-05	0.029	0.0028	0.0022	0.0115	0.0146	0.0078	0.0051	0.0037	0.0043	0.0152	0.11
SM (dose 3)	0.015	4.22 E-05	0.045	0.0036	0.0018	0.0098	0.0174	0.0105	0.0062	0.0042	0.0038	0.0224	0.14
OFMSW (dose 1)	0.012	8.05 E-05	0.019	0.0067	0.0018	0.0144	0.0099	0.0127	0.0066	0.0056	0.0042	0.0264	0.12

OFMSW (dose 2)	0.017	6.20 E-05	0.031	0.0073	0.0025	0.0100	0.0412	0.0137	0.0066	0.0054	0.0030	0.0237	0.13
OFMSW (dose 3)	0.011	8.15 E-05	0.021	0.0043	0.0013	0.0075	0.0278	0.0067	0.0044	0.0038	0.0011	0.0114	0.08

Table 3.1.5 Values of hazard quotients (HQ and THQ) for lettuce considering the different treatments for children.

Sample	B	Cr	Mn	Ni	Cu	Zn	Mo	Cd	Sr	Ba	Pb	As	THQ
CF (dose 1)	0.025	6.48E-05	0.0002	0.0048	0.0030	0.0180	0.0145	0.0197	0.0102	0.0051	0.0054	0.0236	0.13
CF (dose 2)	0.020	6.91E-05	0.0001	0.0033	0.0026	0.0111	0.0081	0.0183	0.0089	0.0045	0.0073	0.0247	0.11
CF (dose 3)	0.023	6.01E-05	0.0001	0.0030	0.0028	0.0138	0.0074	0.0160	0.0080	0.0040	0.0033	0.0172	0.10
SS (dose 1)	0.033	1.50E-04	0.0003	0.0050	0.0034	0.0203	0.0150	0.0381	0.0112	0.0053	0.0066	0.0494	0.19
SS (dose 2)	0.028	6.05E-05	0.0002	0.0040	0.0035	0.0177	0.0095	0.0148	0.0080	0.0030	0.0043	0.0229	0.12
SS (dose 3)	0.025	6.65E-05	0.0001	0.0075	0.0040	0.0202	0.0124	0.0195	0.0102	0.0028	0.0087	0.0360	0.15
SM (dose 1)	0.040	1.51E-04	0.0005	0.0198	0.0049	0.0367	0.0476	0.0388	0.0198	0.0136	0.0138	0.0632	0.30
SM (dose 2)	0.035	2.01E-04	0.0003	0.0078	0.0063	0.0322	0.0409	0.0218	0.0142	0.0104	0.0120	0.0425	0.22
SM (dose 3)	0.041	1.18E-04	0.0005	0.0102	0.0050	0.0276	0.0488	0.0295	0.0173	0.0117	0.0107	0.0627	0.26
OFMSW (dose 1)	0.034	2.25E-04	0.0002	0.0186	0.0050	0.0404	0.0352	0.0355	0.0184	0.0157	0.0117	0.0739	0.29
OFMSW (dose 2)	0.047	1.74E-04	0.0003	0.0204	0.0069	0.0280	0.0412	0.084	0.0185	0.0151	0.0085	0.0663	0.29
OFMSW (dose 3)	0.031	2.28E-04	0.0002	0.0120	0.0037	0.0211	0.0278	0.0187	0.0122	0.0107	0.0031	0.0319	0.17

The EDI for ABs is dependent on both the AB concentration in a vegetable and the amount of that vegetable consumed. Since ABs were only detected in lettuces grown with SM and SS amendments, the EDI values were only calculated for these lettuces. The EDI for ABs ranged from 1 to 20 ng/kg bw/day for adults and from 1 to 25 ng/kg bw/day for children (Tables S1.8 and S1.9). Lettuces amended with SS showed greater total EDI values (18 - 22 ng/kg bw/day) than those amended with SM (1 ng/kg bw/day). This is mainly due to the high occurrence of ciprofloxacin in SS, which accounted for more than 80% of the total EDI. These values are similar to those observed for lettuces irrigated with reclaimed water (Azanu et al., 2018). Similarly, the HQ for ciprofloxacin in the SS treatment accounted for more than 95% of the total HQ in lettuces due to the presence of ABs (Table 3.1.6). The THQs in lettuces amended with SM (0.00005 - 0.00007) were much lower than those found for SS (0.10 - 0.17) for both adults and children. In all cases, the THQs at all amending doses were always much lower than 1, indicating no potential human health risk. These results are consistent with the majority of previous studies in which the use of biosolids, manure, and wastewater resulted in HQs lower than 0.1 for different vegetables (Prosser and Sibley, 2015).

Table 3.1.6 Values for hazard quotients (HQ and THQ) for ABs in lettuces. Only treatments in which ABs were detected above the LOQ has been included. First value shown in table is for adults and the second one for children.

	SM			SS		
	Dose 1	Dose 2	Dose 3	Dose 1	Dose 2	Dose 3
Lincomycin	0.00005/ 0.00006	0.00005/ 0.00007	0.00005/ 0.00007	0.00003/ 0.00004	0.00005/ 0.00007	0.00005/ 0.00007
Ciprofloxacin	-	-		0.0978/ 0.1246	0.1177/ 0.1499	0.1301/ 0.1657
Azithromycin	-	-		0.0012/ 0.0016	0.0022/ 0.0028	0.0032/ 0.0041
THQ	0.00005/ 0.00006	0.00005/ 0.00007	0.00005/ 0.00007	0.0991/ 0.1262	0.1199/ 0.1527	0.1336/ 0.1699

The present results suggest the consumption of lettuces amended with organic fertilizers would not pose a risk to human health due to the presence of TEs or ABs. The EDI of ABs is much lower than the human therapeutic dose and the minimum inhibitory concentration for ABs. Nevertheless, bacteria are frequently exposed to nonlethal (that is, subinhibitory) concentrations of ABs, and recent evidence suggests that this is likely to play an important role in the evolution of antibiotic resistance (Andersson and Hughes, 2014). In fact, the presence of ABs in lettuces has recently demonstrated to result in horizontal transfer of resistance genes from crops to mouse gut microbiome (Maeusli et al., 2020). In addition, the combination of different ABs, TEs, and other pollutants has been shown to promote antibiotic resistance through co-selection (Pal et al., 2017). Results from Gullbert et al. (2014) indicate that very low AB and heavy metal levels found in polluted environments and in treated humans and animals might be sufficiently high to maintain multiresistance plasmids. Song et al.(2017) observed that tetracycline resistance increased significantly in soils containing environmentally relevant levels of Cu and Zn, but not in soil spiked with high levels of tetracycline. In fact, metal (Cu and Zn) and antibiotic resistance genes co-occur in animal isolates of multidrug-resistant Salmonella and methicillin-resistant Staphylococcus aureus (MRSA)(Poole, 2017). Nevertheless, other studies pointed out evidence for co-selection of antibiotic resistance genes and mobile genetic elements in metal polluted urban soils (Zhao et al., 2019). Therefore, further studies should be done to understand the interaction between TEs and ABs present in vegetables in relation to the human health risk.

3.1.4 Conclusions

The results show that soil amendment with organic fertilizers (sewage sludge, swine manure, and the organic fraction of municipal solid waste) resulted in changes in the abundance of TEs and ABs in lettuce leaves, as well as differences in plant morphology. The concentration of Cu and Zn in lettuces amended with organic fertilizers was statistically greater than in those grown with chemical fertilizer. ABs were only detected in SM and SS amended lettuce leaves. Nevertheless, the dose of organic fertilizers did not affect the plant uptake of TEs and ABs in any of the studied organic fertilizers. The results show that the use of SS resulted in the highest lettuce yield compared with the other organic amendments, with values similar to those for chemical fertilization. The THQs for TEs and ABs were far below 1 in all cases, suggesting that there is no human health risk associated with the consumption of these food crops. Nevertheless, the co-selection of antibiotic resistance genes through the presence of TEs and ABs cannot be ruled out. The present results indicate that the use of SS resulted in the highest lettuce yield and lowest presence of TEs of any of the analyzed organic fertilizers, it also resulted in the greatest presence of ABs. These results should be taken with care as they were obtained under greenhouse conditions in only one cultivation cycle, that is considering that repetitive applications of these fertilizers in soil could increase crop exposure to pollutants due to soil accumulation.

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3.1.6 Supporting information

3.1.6.1 Chemicals and reagents

Antibiotic standards of sulfonamides (sulfacetamide, sulfadiazine, sulfamethazine, sulfamethizole, sulfamethoxazole, sulfapyridine and sulfathiazole) were obtained from Sigma-Aldrich (Bornem, Belgium). Fluoroquinolones (Ciprofloxacin, enrofloxacin and ofloxacin), tetracyclines (chlortetracycline hydrochloride, oxytetracycline hydrochloride and tetracycline hydrochloride), azithromycin di-hydrate and lincomycin hydrochloride monohydrate were supplied by Fluka (Buchs, Switzerland). Surrogate standard used, enrofloxacin d5 hydrochloride, ofloxacin d3 hydrochloride, clindamycin d3 hydrochloride and sulfamethoxazole d4, were purchased from Sigma-Aldrich. Strata-X solid-phase extraction (SPE) cartridges (200 mg/ 6 mL) were purchased from Phenomenex (Torrance, CA, USA) and 0.70 µm of glass-fiber filters 47 mm in diameter were obtained from Whatman (Maidstone, UK).

3.1.6.2 Supplementary tables

Table S1.1 Soil physicochemical characteristics

Parameter	Soil
Texture	Loamy
pH	8.48
Electrical conductivity (EC) (dS/m)	0.240
Humidity (%)	1.33
Organic matter (%, dw)	1.27
Total Kjeldahl N (%, dw)	0.089
Olsen P (mg/kg, dw)	33
Equivalent calcium carbonate (%, dw)	28
Ca (mg/kg, dw)	7014
Mg (mg/kg, dw)	362

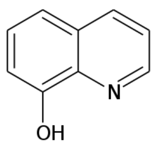
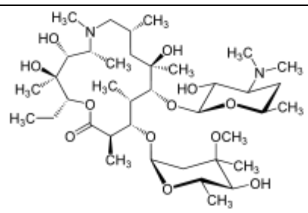
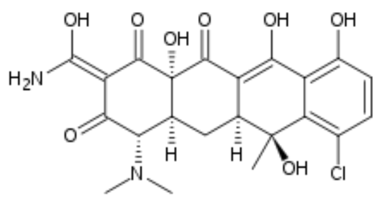
Na (mg/kg, dw)	91
K (mg/kg, dw)	344

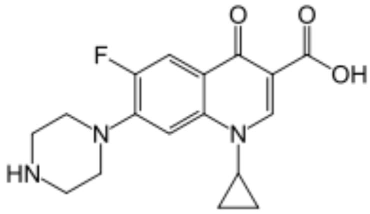
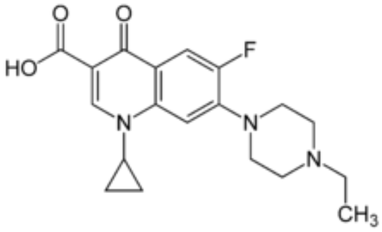
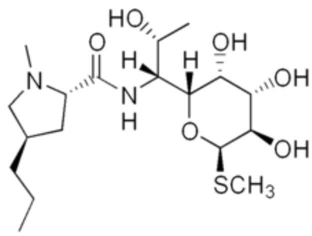
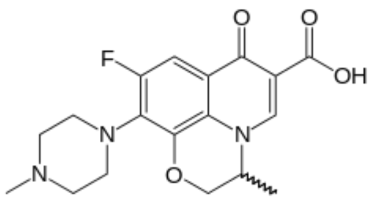
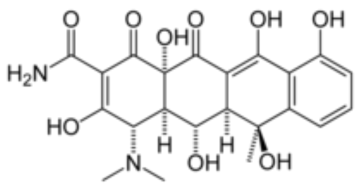
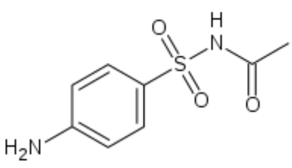
Table S1.2 Characteristics of organic fertilizers used in this experiment on dry weight basis

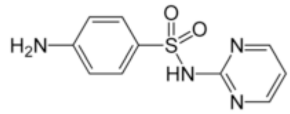
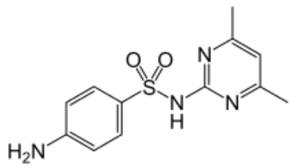
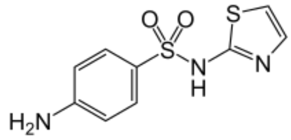
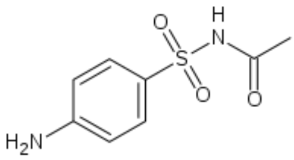
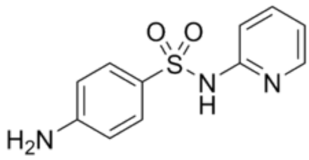
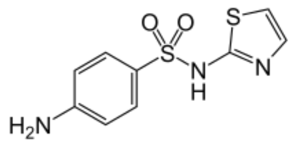
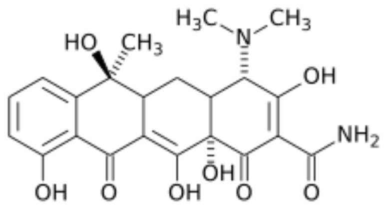
Parameter	Swine Manure	OFSMW	Sewage Sludge
pH	8.26	8.25	8.40
Humidity (%)	40	20	87
Organic matter (%)	92.0	53.8	51.5
N-ammoniacal (%)	0.56	0.29	1.34
N Kjeldahl (%)	2.29	2.61	2.77
K (%)	0.82	2.42	0.207
Olsen-P (%)	0.61	0.55	1.55

*Composted solid fraction **Anaerobically digested

Table S1.3 Physicochemical properties of the selected antibiotics.

Compound	Molecular structure	Molecular weight (g/mol)	pK_a	$logK_{OW}$	Water solubility (mg/L)	$logK_{OA}$
8-hydroxyquinoline		145.16	5.02[e] [+/0] 9.81[e] [0/-]	1.66	7965	6.483
Azithromycin		749.00	7.34[b] [+/0]	3.24	0.06204	30.684
Chlortetracycline		478.89	3.95[b] [+/0] 5.59[b] [0/-]	-0.72	742.8	23.771

			7.94[b] [-/2-]			
Ciprofloxacin		331.35	6.32[b] [+/0] 8.73[b] [0/-]	0.28	1.148e+4	16.962
Enrofloxacin		359.40	6.68[b] [+/0] 7.87[b] [0/-]	0.70	3397	16.918
Lincomycin		406.54	7.60[d] [+/0]	0.29	92.19	21.111
Ofloxacin		361.38	6.67[b] [+/0] 7.92[b] [0/-]	-0.39	2.826e+4	17.491
Oxytetracycline		460.44	3.79[b] [+/0] 5.41[b] [0/-] 8.46[b] [-/2-]	-1.72	1.433E+5	22.994
Sulfacetamide		214.24	1.95[b] [+/0] 5.30[b] [0/-]	-0.60	1.194E+4	9.744

Sulfadiazine		250.28	2.10[b] [+/0] 6.28[b] [0/-]	-0.34	2.814E+4	8.100
Sulfamethazine		278.33	2.37[b] [+/0] 7.49[b] [0/-]	0.76	1.127E+4	8.293
Sulfamethizole		270.33	1.86[b] [+/0] 5.29[b] [0/-]	0.41	6292	12.509
Sulfamethoxazole		253.28	1.85[b] [+/0] 5.60[b] [0/-]	0.48	3942	11.298
Sulfapyridine		249.29	4.25[c] [+/0] 8.43[c] [0/-]	0.53	1.199E4	11.705
Sulfathiazole		255.31	2.01[b] [+/0] 7.11[b] [0/-]	0.72	2.003E+4	11.671
Tetracycline		444.44	3.32[b] [+/0] 7.78[b] [0/-] 9.58[b] [-/2-]	-0.018	8780	23.098

[a] Dissociation reaction, [0]: neutral; [+]: cationic; [−]: anionic

[b] *Babić, S., Horvat, A.J.M., Mutavdžić Pavlović, D., Kaštelan-Macan, M., 2007. Determination of pKa values of active pharmaceutical ingredients. TrAC - Trends Anal. Chem. 26, 1043–1061.*

[c] *Yang, H., Li, G., An, T., Gao, Y., Fu, J., 2010. Photocatalytic degradation kinetics and mechanism of environmental pharmaceuticals in aqueous suspension of TiO2: A case of sulfa drugs. Catal. Today 153, 200–207.*

[d] *O'Neil, M.J. (ed.). The Merck Index - An Encyclopedia of Chemicals, Drugs, and Biologicals. Whitehouse Station, NJ: Merck and Co., Inc., 2006., p. 953*

[e] *Perrin DD; Dissociation constants of organic bases in aqueous solution. IUPAC Chem Data Ser: Suppl 1972. But tersworth, London. (1972)*

Log K_{OW}, Log K_{OA}, Henry LC and Log K_{AW} from database provided by Episuite v4.11 (http://www.epa.gov/opptintr/exposure/pubs/episuite.htm)

Note: Log K_{OW}, Log K_{OA}, Henry LC and Log K_{AW} are estimated values.

Table S1.4 MRM method used in the UPLC-MS/MS instrument.

Compound	RT[a] (min)	CV[b] (V)	Precursor ion	Quantification (CE[c])	Qualifica
8-hydroxyquinoline	0.92	35	146	128 (25)	118 (25)
Lincomycin	0.99	35	407	126 (30)	359 (20)
Sulfacetamide	1.72	30	215	92 (20)	156 (15)
Sulfadiazine	1.79	30	251	156 (15)	108 (20)
Sulfapyridine	2.01	30	250	156 (15)	184 (20)
Oxytetracycline	2.19	30	461	426 (20)	443 (15)
Sulfathiazole	2.28	30	256	156 (15)	108 (20)
Tetracycline	2.55	30	448	410 (20)	427 (15)
Sulfamethazine	2.86	20	279	156 (20)	124 (20)
Ofloxacin	3.11	30	362	318 (25)	261 (15)
Ofloxacin-d3	3.11	40	365	261 (30)	221 (20)
Ciprofloxacin	3.39	30	332	288 (20)	245 (25)
Enrofloxacin-d3	3.48	30	365	245 (25)	347 (25)
Enrofloxacin	3.50	30	360	316 (20)	342 (20)
Sulfamethizole	3.67	30	271	156 (15)	108 (20)
Azithromycin	3.67	50	749	158 (40)	591 (25)
Clindamycin-d3	3.78	50	428	129 (30)	380 (20)
Chlortetracycline	3.78	30	479	444 (25)	462 (20)
Sulfamethoxazole-d4	4.32	30	258	160 (15)	112 (25)
Sulfamethoxazole	4.35	30	254	92 (25)	156 (20)

[a]RT: retention time,[b]CV: cone voltatge, [c]CE: collision energy

Table S1.5 Analytical quality parameters (relative recovery, standard deviation LOD, and LOQ) for ABs in lettuce samples (n=3).

Compound	R% relative ± RSD	LOD (ng/g, fw)	LOQ (ng/g, fw)
8-hydroxyquinoline	118±21	0.45	0.47
Lincomycin	88±20	0.22	0.23
Sulfacetamide	134±14	0.14	0.17
Sulfapyridine	111±6	0.34	0.36
Sulfadiazine	129±16	0.29	0.31
Sulfathiazole	83±5	0.07	0.11
Tetracycline	122±24	0.02	0.06
Oxytetracycline	128±31	0.04	0.08
Sulfamethizole	77±10	0.39	0.41
Sulfamethazine	133±27	0.61	0.69
Ciprofloxacin	43±12	0.76	0.80
Enrofloxacin	24±6	0.004	0.010
Ofloxacin	23±7	0.03	0.09
Azithromycin	55±17	0.02	0.06
Chlortetracycline	43±15	1.13	1.52
Sulfamethoxazole	124±24	0.27	0.29

Table S1.6 Concentrations (mg/kg, dw) of selected TEs in soil and organic fertilizers (n=1).

	Cu	Zn	B	Co	Sr	Mn	Cd	Ba	Cr	Mo	Hg	As	Ni	Pb	ΣTEs
Soil	92	74	97	10	216	470	<0.5	125	21	2.0	0.03	20	24	58	1221
SM	72	535	220	0.50	76	115	<0.5	24	5.5	2.2	0.01	0.50	3.2	2.7	1058
OFMSW	60	160	160	2.4	172	135	<0.5	90	15	0.63	0.08	1.7	9.4	29	838
SS	244	700	120	3.5	450	102	0.51	270	57	3.6	0.25	7.7	53	30	2043

Table S1.7 Concentrations (ng/g, dw) of selected antibiotics detected in fertilizer samples and soil (n=3)

Compound	SM	SS	OFMSW	Soil
8-hydroxyquinoline *	3397±604	149±27	-	-
lincomycin	9831±2200	25±6	-	-
sulfacetamide	3.9±0.9	-	-	-
sulfapyridine	-	-	-	-
sulfadiazine	11±2	-	-	-
sulfathiazole	-	203±12	-	-
tetracycline	5.9±1.2	169±33	-	-
oxytetracycline	918±222	-	-	-
sulfamethizole	-	-	-	-
sulfamethazine	-	-	-	-
ciprofloxacin	-	9317±2600	-	-
enrofloxacin	-	-	-	-
ofloxacin	12±2	-	-	-
chlortetracycline	2.9±0.6	-	-	-
azithromycin	6912±2100	165±51	1.1±0.3	-
sulfamethoxazole	-	-	-	-

-:*Non detected. *Intermediate in the degradation of several ABs.*

Table S1.8 EDI values (ng/kg/bw/day) calculated for ABs and adults in lettuces amended with SM and SS.

	SM			SS		
	Dose 1	**Dose 2**	**Dose 3**	**Dose 1**	**Dose 2**	**Dose 3**
Lincomycin	1.2	1.4	1.3	0.8	0.9	1.0
Ciprofloxacin	-	-	-	14.7	17.6	19.5
Azithromycin	-	-	-	2.1	3.7	2.0
Total EDI	1.2	1.4	1.3	17.6	22.3	22.4

Table S1.9 EDI values (ng/kg/bw/day) calculated for ABs and children in lettuces amended with SM and SS.

	SM			SS		
	Dose 1	**Dose 2**	**Dose 3**	**Dose 1**	**Dose 2**	**Dose 3**
Lincomycin	1.6	1.7	1.7	1.1	1.1	1.2
Ciprofloxacin	-	-	-	18.7	22.5	24.9
Azithromycin	-	-	-	2.7	4.8	2.5
Total EDI	1.6	1.7	1.7	22.4	28.4	28.6

3.1.6.3 Supplementary figure

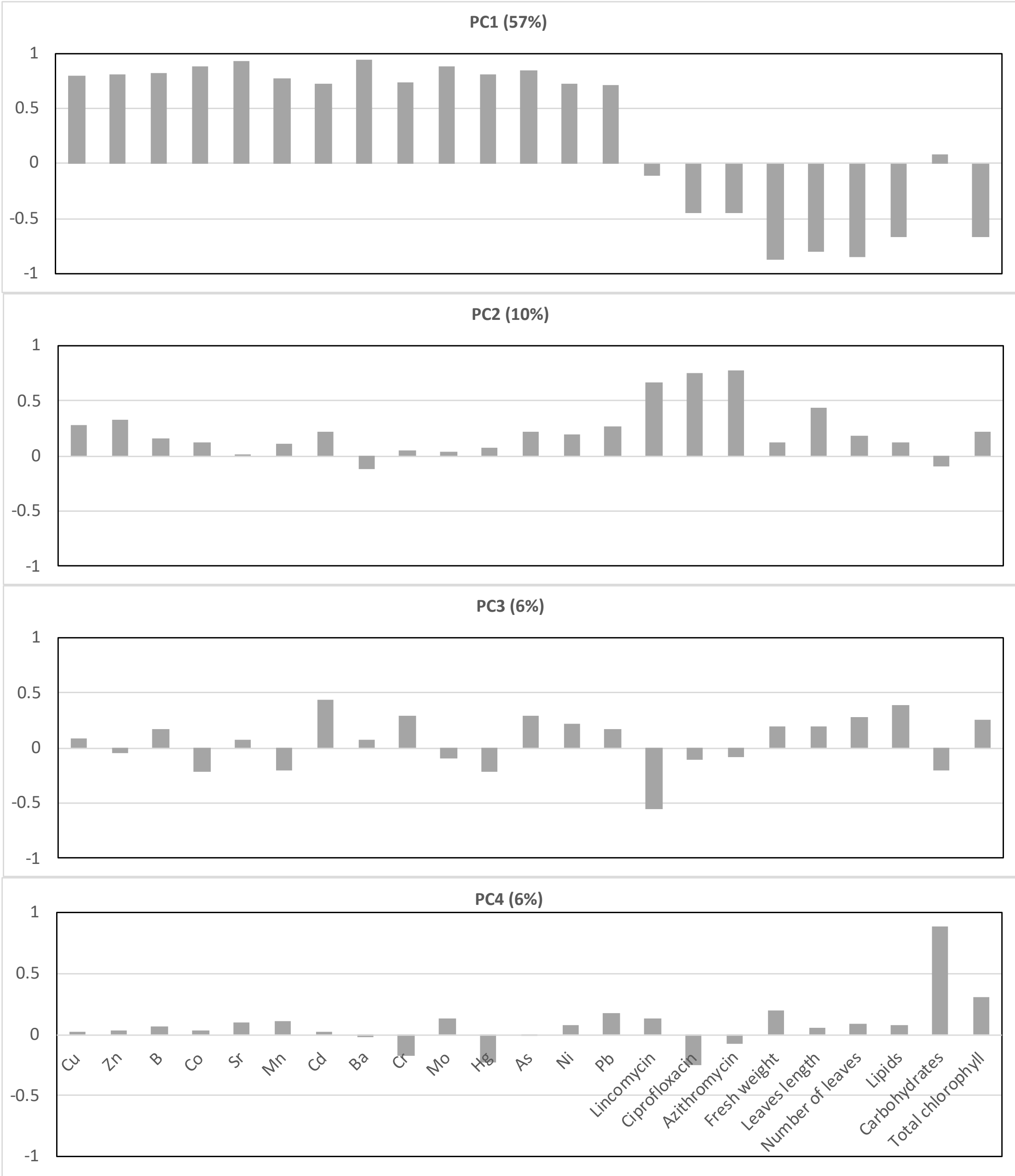


Fig S1.1 Loading for the first 4 principal components and total variance explained (in percentage).

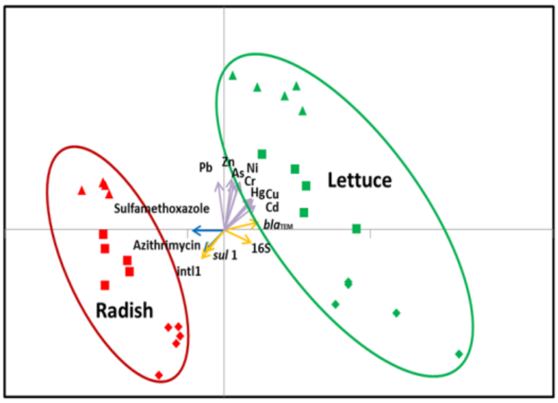
3.2 Topic 2: Effect of different Cu and Zn content in sewage-sludge-amended soils on plant uptake and human health risk

This topic assesses the effect of different Cu and Zn content in sewage-sludge soil amendment on the uptake of TEs, ABs, and ARGs in the edible part of lettuce (leaf) and radish (root), as well as evaluate the phytotoxicity and human health implications.

Based on the article:

Dose effect of Zn and Cu in sludge-amended soils on vegetable uptake of trace elements, antibiotics, and antibiotic resistance genes: Human health implications.

Environmental Research 191, 109879



Dose effect of Zn and Cu in sludge-amended soils on vegetable uptake of trace elements, antibiotics, and antibiotic resistance genes: Human health implications.

Abstract

The application of sewage sludge to agricultural fields reduces the need for mineral fertilizers by increasing soil organic matter, but may also increase soil pollution. Previous studies indicate that zinc and copper, as the most abundant elements in sewage sludge, affect plant uptake of other contaminants. This paper aims to investigate and compare the effect of increasing amounts of Zn and Cu in sludge-amended soils on the accumulation of trace elements (TEs), antibiotics (ABs), and antibiotic resistance genes (ARGs) in lettuce and radish. The vegetables were grown under controlled conditions, and the influence on plant physiology and human health were also evaluated. The results show that the addition of Zn and Cu significantly increased the concentration of TEs in the edible tissue of both vegetables. According to the hazard quotient (HQ) of the TEs, the human health risk increased 2 to 3 times and was 3 - 4 times greater in lettuce than in radish. In contrast to the TEs, the occurrence of ABs and most of the ARGs was higher in radish roots than lettuce leaves. ABs were not detected in lettuce leaves, and the amount of all ARGs except *bla*$_{TEM}$ was 10 times lower than in radish roots. On the other hand, the addition of Zn and Cu had no significant effect on the occurrence of ABs and ARGs in the edible part of the vegetables, and no damage was found to plant productivity or physiology. The results show that the consumption of lettuce and radish grown in sewage-sludge-amended soils under tested doses of Cu and Zn does not pose an adverse human health effect, as the total HQ value was always less than 1, and the presence of ABs and ARGs was not found to have any potential impact. Nevertheless, further studies are needed to estimate the long-term effect on human health of crops grown under frequent application of biosolids in arable soil.

Keywords: Lettuce, Radish, Metals, Antibiotics, ARG, Sewage sludge

3.2.1 Introduction

The implementation of the Urban Waste Water Treatment Directive (91/271/EC) (EC, 1991) in the European Union led to an increase in the quantity of sewage sludge, a by-product of the wastewater treatment process. According to a forecast by Milieu Ltd. (2010), the amount of sludge generated in the EU would exceed 13 million tons by 2020. Furthermore, the safe disposal of sewage sludge accounts for up to 50% of the operating costs of wastewater treatment plants. The European Commission thus proposed an action plan related to the circular economy (EC, 2015), which encourages the application of treated sewage sludge to agricultural soil due to its high content of nutrients and organic matter, provided it complies with Directive 86/278/EEC (EC, 1986) in terms of chemical (trace elements) and biological (pathogens) pollutants.

However, soil amendment with sewage sludge remains a challenging task because of the presence of organic and inorganic contaminants. For example, Iglesias et al. (2018) found a significant increase in the amount of Pb, Hg, Zn, and Ag in sludge-amended soils. Cheng et al. (2014) detected that the concentrations of fluoroquinolones (FQs), tetracyclines (TCs), and sulfonamides (SAs) in 58 sewage sludge samples ranged from 1569 to 23,825 μg/kg, 592 to 37,895 μg/kg, and 20.1 to 117 μg/kg dry weight (dw), respectively. Therefore, concerns have emerged about the chemical contamination of crops cultivated in sewage-sludge-amended soil, as plants can take up TEs and ABs from the substrate (Guoqing et al., 2019).

In addition to these contaminants, the antibiotic resistome, which results from the application of sewage sludge, has gotten more and more attention. As reported in many studies, sewage sludge is a hotspot for bacteria carrying antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) (He et al., 2019; Zielínki et al., 2019). Owing to the abundant carriers derived from sludge, ARGs could be disseminated in soil by horizontal gene transfer (HGT) after application (Zhou et al., 2019). Furthermore, the presence of TEs and ABs in sewage sludge can exert long-term selective pressure on soil microorganisms and increase the over-expression of ARGs (Urra et al., 2019). Some bacteria carrying ARGs might colonize plants as endophytes or adhere to plant surfaces and manage to survive and persist throughout the vegetable growth stage (Pu et al., 2019). Thus, there will be a notable human health risk of exposure to ARGs through the consumption of contaminated vegetables (Zhao et al., 2019). Yang et al. (2018) detected numerous ARGs (*catB*8, *php*, *van*B, and *str*) in lettuce grown in amended soil and discovered that the application of sewage sludge boosts the evolution and dissemination of ARGs in the soil-plant system. Murray et al. (2019) compared the effect of sewage sludge on the occurrence of ARGs in several vegetables and reported that root vegetables carry more abundant ARGs than leafy vegetables.

As the most plentiful elements in sewage sludge, copper and zinc could be crucial for the occurrence and long-term accumulation of other contaminants in crops as a result of three effects or mechanisms. The first is the antagonist effect, which reduces the concentration of other TEs in the crop through competition for the same binding sites on the root. The second is the complexation effect, which affects the fate of ABs as a result of complexing with a large number of amino groups, carboxyl groups, hydroxyl groups, and heterocycles in ABs. One common consequence, a reduction in antibiotic potency, has long been known, with Zn inactivation of penicillin first having been reported in 1946 (Eisner and Porzecansri, 1946) and later having been shown to result from Zn binding to and promoting the hydrolysis of this β-lactam (Gensmantel et al., 1980). Nevertheless, Sayen et al. (2019) revealed that the presence of Cu favored the plant uptake of enrofloxacin (Enro), a fluoroquinolone antibiotic, which could be taken up as both free (mainly in zwitterionic form) and Cu-Enro complexes (as positively charged complexes). Finally, the third is the co-selection mechanism, which triggers the proliferation of ARGs (Liu et al., 2019). ARGs and metal resistance genes (MRGs) are frequently located together on the same genetic elements, such as a plasmid, transposon, or integron. This physical linkage results in an increase in the expression of ARGs in bacteria under the pressure of Cu and Zn.

Many studies have separately examined the levels of TEs, ABs, and ARGs in vegetables grown in soil amended with sewage sludge (Shamsollahi et al., 2019; Wei et al., 2020). However, information on the accumulation and interaction of these contaminants is still limited, especially under the selective pressure of Cu and Zn. Therefore, the present paper aims to assess the effect of different Cu and Zn content in sewage-sludge soil amendment on the uptake of TEs, ABs, and ARGs in the edible part of lettuce (leaf) and radish (root), as well as evaluate the phytotoxicity and human health implications.

3.2.2 Materials and method

3.2.2.1 Experimental layout

The experiment was conducted in a greenhouse located at the Agròpolis-UPC agricultural experiment station (41° 17′ 18″ N, 2° 02′ 43″ E) in Viladecans (Barcelona, Spain) with an average temperature of 21°C and a relative humidity of 56%. In accordance with the optimum N concentration needed for plant growth (Pomares and Ramos, 2010), experimental units consisted of 2.5 L cylindrical amber glass pots (Ø = 15 cm, 20 cm high) filled with 1652 g fresh soil, 878 g sand, and 70 g wet sewage sludge with a humidity of 79.1%. Soil samples were collected from the adjacent area (<20 cm) to which no antibiotic products hadintentionally been applied before this project. Sewage sludge was ob- tained from the Gavà-Viladecans wastewater treatment plant (Parc del Baix Llobregat, Barcelona). $ZnSO_4 \cdot 7H_2O$ and $CuSO_4 \cdot 5H_2O$ were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) with a purity > 99%. Lettuce (*Lactuca sativa* L. cv. Maravilla de Verano) and radish (*Raphanus sativus* cv. Redondo Rojo Vermell) were chosen for the study, because they are some of the most widely consumed vegetables in the Mediterranean region and could represent two different crop types (leafy and root plant). To evaluate the effect of Cu and Zn content in sewage sludge on the uptake of contaminants in vegetables grown in amended soil, the following treatments were established: (i) control: unspiked sewage sludge containing 240 mg/kg Cu and 700 mg/kg Zn (dw); (ii) treatment 1 (T1): sewage sludge spiked with $CuSO_4 \cdot 5H_2O$ and $ZnSO_4 \cdot 7H_2O$ to two-fold the background value of Cu and Zn, i.e. to 480 mg/kg and 1400 mg/kg (dw); and (iii) treatment 2 (T2): sewage sludge spiked with more $CuSO_4 \cdot 5H_2O$ and $ZnSO_4 \cdot 7H_2O$ to four-fold the original Cu and Zn concentration, i.e. to 960 mg/kg and 2800 mg/kg (dw). All treatments were replicated five times. One lettuce or radish seedling was planted in each experimental unit on March 4, 2019, and irrigated with harvested rainwater (50 mL/pot/day) through a drip irrigation system. Plants were harvested until they reached the commercial size (35 productive days for radish, 56 for lettuce), and the lettuce leaf and the radish root were stored at − 20 °C until analysis.

3.2.2.2 Analytical procedures

(1) Reagents and standards

This information is included in the Supplementary Material.

(2) Analyses of soil and sewage-sludge samples

Physical and chemical parameters: Humidity, pH, electrical conductivity (EC), organic matter content (OM), total nitrogen, and available nitrogen, phosphorus, and potassium (NPK) were determined using standard methods described by Sparks (1996).

Trace elements: Soils and sewage sludge were digested in concentrated HCl–HNO_3 at 95 °C for 30 min. After cooling and filtering (<0.45 μm), samples were analyzed by ICP-MS (Thermo Scientific) (Martin et al., 1994). The mercury concentration was measured using an AMA-254 (Altec, Prague, Czech Republic).

Antibiotics: No products containing antibiotics had ever been intentionally applied to the soil used in the experiment. The extraction method for the sewage sludge sample was as described by Berendsen et al. (2015). Briefly, 500 mg sludge was weighed into a 50 mL centrifuge tube and 4 mL of McIlvain–EDTA buffer and 1 mL ACN were added to the sample. The sample was vortexed and centrifuged at 2500 rpm for 15 min. Then 2 mL of lead acetate solution was added and the sample was centrifuged again (2500 rpm, 15 min). After centrifugation, the supernatant was transferred to a clean vial and diluted with 13 mL 0.2 M EDTA solution. A Phenomenex (Torrance, CA, USA) Strata-X RP 200 mg/6 mL reversed-phase solid phase extraction (SPE) cartridge was conditioned with 5 mL MeOH and 5 mL water. The complete extract was applied to the SPE cartridge, which was subsequently washed with 5 mL of H_2O and dried. The antibiotics were eluted from the cartridge using 5 mL MeOH followed by evaporation to dryness. The residue was dissolved in 200 μL of mobile phase (H_2O/ACN, 95/5, v/v) before being transferred into an UPLC–MS/MS sample vial.

Antibiotic resistance genes: DNA was extracted from the soil and sewage-sludge samples using a DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. DNA concentration was measured using a NanoDrop Spectrophotometer 8000 (Thermo Fisher Scientific). Seven ARGs (*sul1, qnrS1,* bla_{TEM}, *tetM,* $bla_{\mathrm{CTX\text{-}M\text{-}3}}2$, $bla_{\mathrm{OXA\text{-}58}}$, and *mecA*), the integron *intl1*, and the 16S ribosomal DNA gene were quantified by qPCR following the protocol described by Cerqueira et al. (2019b).

(3) Analyses of vegetables

Plant phytotoxicity: The length and number of leaves of both vegetables were measured weekly until the end of the experiment. The chlorophyll content in the leaves and weight of the edible part of both vegetables were measured in situ. Chlorophyll was gauged using a chlorophyll meter (Opti-Sciences, Hudson, NH, USA). Each measurement was performed on three leaves per crop. A calibration curve was obtained to correlate the chlorophyll content with the previously measured absorbance. Round leaf samples (4 cm diameter) were then extracted with 5 mL of N,N-dimethylformamide (DMF) and kept in the dark at 4 °C for 48 h before the spectrophotometric determination. The extracts were measured at two wavelengths, 647 and 664.5 nm, so that the chlorophylls could be calculated using Inskeep and Bloom's coefficients (Inskeep and Bloom, 1985; Porra, 2002). Lipid extraction was carried out by adding 15 mL of ethanol/hexane (1/1, v/v) to a glass tube with 3 g of fresh sample (Margenat et al., 2018). The sample was then sonicated for 15 min and centrifuged at 2500 rpm for 15 min. It was further filtered through a 0.22 μm nylon filter (Scharlab, Barcelona, Spain). After the solvent was removed by purging and drying the sample with nitrogen gas, the sample remaining in the tube was weighed and operationally defined as lipid content.

Trace Elements: The edible part of the vegetables (the leaf of the lettuce and root of the radish) was freeze-dried and digested with a microwave oven (Milestone Ethos), as described by Llorente-Mirandes et al. (2010). Briefly, 0.1 g of powdered sample was weighed into the PTFE vessels, and 8 mL of HNO_3 and 2 mL of 31% H_2O_2 were added. The digestion program carried out was as follows: 15 min from room temperature to 90 °C;10 min at 90 °C;20 min from 90°C to 120 °C;15 min from 120 °C to 190 °C; 20 min at 190 °C. After cooling to room temperature, the digested sample was analyzed by ICP-MS. The mercury concentration was measured with an AMA-254 (Altec, Prague, Czech Republic).

Antibiotics: 5 g of homogenized vegetable sample was extracted with 10 mL of methanol in an ultrasonic bath for 15 min. The extracts were centrifuged for 15 min at 3000 g following a previously described methodology (Tadić et al., 2019). Two extraction cycles were required. The supernatants were decanted and combined in a glass vial and evaporated under a gentle nitrogen stream at a temperature of 40 °C until they had been reduced to 1 mL. The concentrated extracts were then diluted with 10 mL of water to perform the SPE clean-up step. The SPE cartridges (polymeric reverse phase Strata-X cartridge, 100 mg/6 mL) were first preconditioned with 6 mL of methanol and 6 mL of water. After sample loading, the polymeric cartridge was washed with 1 mL of water with 5% methanol. The cartridges were then dried under a N_2 stream, followed by elution with 2 mL of a mixture of methanol and ethyl acetate (1/1, v/v). The eluted fraction was evaporated to dryness and reconstituted in 1 mL of mobile phase (H_2O/ACN, 95/5, v/v). The final extracts were filtered through a 0.22 μm pore nylon filter and injected into a UPLC-MS/MS.

Antibiotic resistance genes: The preliminary procedure for DNA extraction from vegetables was performed as described by Cerqueira et al. (2019a). Briefly, the edible part of the vegetable (90–100 g) was processed in a grinder (Grindomix GM200, Retsch, Inc). The crushed vegetable matter was transferred to a beaker along with 50 mL of sterile phosphate-buffered saline (PBS), mixed gently, and then filtered through a sterile gauze to remove the pulp. The resulting filtrate was transferred to 50 mL tubes through a 100 μm nylon mesh cell strainer (Corning® Cell Strainer) and centrifuged at 4000 rpm for 15 min. DNA extraction was carried out from the pellets and absolute quantification of ARGs (*sul*1, *qnr*S1, bla_{TEM}, *tet*M, $bla_{CTX\text{-}M\text{-}32}$, $bla_{OXA\text{-}58}$, and *mec*A), intl1, and the 16S genes was performed as indicated in the soil and sludge section. The 16S values were subsequently corrected as determined by the 16S sequencing (Cerqueira et al., 2019b) to remove plastidial (chloroplasts, leucoplasts, and mitochondria) 16S sequences.

3.2.2.3 Estimated daily intake

The estimated daily intake (EDI) of TEs and ABs were determined based on both the content in the vegetables and the consumption amount of the respective crop. The EDI for a Spanish adult was calculated as follows (Equation (1)):

$$\text{EDI} = \frac{\text{DI} \times C_P \times \text{F}}{\text{BW}} \qquad (1)$$

Where DI is the daily intake of vegetables (according to the EFSA's Comprehensive Food Consumption Database, the average weights of lettuce and radish consumed by a Spanish adult are 0.045 and 0.022 kg/day (wet mass), respectively); C_p is the concentration of each pollutant in the crop (mg/kg dw); and F is a factor (0.091) to convert fresh weight (fw) to dry weight. BW is body weight, which is assumed to be 70 kg.

3.2.2.4 Human health risk assessment

To evaluate the human health risk, the hazard quotient (HQ) was calculated.

The HQ of TEs (HQ_{TE}) is the ratio between the EDI and the reference oral dose (RfD), as shown in Equation 2.

$$HQ_{TE} = \frac{\text{EDI}}{\text{RfD}} \qquad (2)$$

Where RfD is the maximum tolerable daily intake (μg/kg/day) of a specific element that does not result in carcinogenic effects for human beings, obtained from IRIS (2020). An HQ＞1 implies a potential risk to the population; otherwise, the consumer is safe.

Finally, the total hazard quotient (THQ_{TE}), used to assess the total risk of all chemicals to which an individual might be exposed, was calculated as the sum of the HQ_{TE} of all the elements.

The HQ of ABs (HQ_{AB}) was calculated using the acceptable daily intake (ADI), as shown in Equation 3.

$$HQ_{AB} = \frac{EDI}{ADI} \quad (3)$$

Two antibiotic ADIs were calculated based on different endpoints. For therapeutic purposes, ADI1 was calculated by dividing the lowest daily therapeutic dosage for an adult (mg/day) by a safety index (1000) and body weight (70 kg) (Prosser and Sibley, 2015), while ADI2 was adopted from provisional values established in the literature or derived using toxicological, microbiological, or therapeutic approaches (Wang et al., 2017). Consumption of vegetables contaminated with antibiotics is only one pathway of human exposure. Therefore $HQ_{AB}>1$ indicates a potential hazard.

3.2.2.5 Data analysis

A non-parametric Mann-Whitney U test was performed and Spearman's correlation coefficient was calculated for multiple comparisons or to analyze interactive effects between different factors. Principle component analysis (PCA) was conducted on the concentrations of TEs, ABs, and ARGs. Varimax rotation was applied because orthogonal rotation minimizes the number of variables with a high loading on each component and facilitates the interpretation of results. Statistical significance was defined as $p < 0.05$. The data analysis was performed with the SPSS v25 package (Chicago, IL, US).

3.2.3 Results and discussion

3.2.3.1 Characterization of soil and sewage sludge

Some physical and chemical properties of soil and sewage sludge are shown in Supplementary Table S2.2. Due to its significantly higher content of NPK and organic matter, sewage sludge has proven to be a useful source of nutrients for vegetables. However, the higher electrical conductivity of sludge also poses a potential threat, namely, possible salt toxicity to plants and soil organisms (Zhou et al., 2005).

The TEs were selected according to the applicable regulation of the European Parliament and European Council (PE-CONS 76/18, 2019). A total of 8 TEs need to be tested for in sewage sludge used for fertilizer. Supplementary Table S2.3 shows their concentrations in the soil and sewage-sludge samples, as well as the corresponding maximum admissible limits for agricultural soil and biosolids fertilizer. In the present soil sample, TE concentrations (dw) ranged from 0.025 mg/kg (Hg) to 92 mg/kg (Cu), and all content was below the generic reference value for contaminated soil in Catalonia. In the sewage sludge, the most abundant element was Zn (700 mg/kg), followed by Cu, Cr, Ni, Pb, As, Hg, and Cd. The TE contents were similar to the abundance of TEs reported in sludge from other sewage treatment plants in Barcelona, Spain (0.12 - 1270 mg/kg) (Husillos Rodríguez et al., 2013). Only the level of Ni was found to be slightly higher than allowed under fertilizer regulations (see Table S2.3).

The concentration of ABs (dw) in the sewage sludge ranged from undetected to 5790 μg/kg (ciprofloxacin), which is 45 times higher than the other detected ABs. Supplementary Table S2.4 shows that 6 of the 16 ABs analyzed had values over the limit of detection in sewage sludge. According to previous studies, AB concentrations in sewage sludge vary from ng to mg per kg dw depending on the treatment techniques, operational conditions, and sources (Li et al., 2013). In the present sludge, the concentration of ciprofloxacin was similar to that in sludge from 11 Swedish sewage treatment plants (1600–11000 μg/kg dw) (Östman et al., 2017).

Quantification of *mec*A in both soil and sewage sludge samples was not possible since it was found to be under detection limits. The rest of the targeted genetic elements were all detected, but $bla_{\text{CTX-M-32}}$ in sewage sludge samples and qnrS1, bla_{TEM}, $bla_{\text{CTX-M-32}}$, and $bla_{\text{OXA-58}}$ in soil samples were found to be below the quantification limits (Supplementary Fig. S1). Since ARGs are more abundant in sewage sludge than soil, this indicates a potential risk of increased antibiotic resistance in soil after amendment. In addition, the high values of intl1 in sewage sludge imply that the application of sludge may accelerate the dissemination of ARGs in the matrix through horizontal gene transfer. Among the ARGs, *sul*1 was detected at levels around 100 times higher than the others, except for *tet*M. This is consistent with the report that the sulfonamide-resistant gene is prevalent in sewage sludge, due to the very low removal of the sul gene during sewage treatment plant processes (Xu et al., 2015).

3.2.3.2 Effect of Zn and Cu addition to sludge on plant uptake of trace elements

The addition of Zn and Cu to sludge resulted in significant changes in most of the TEs in the edible part of both vegetables (Fig. 3.2.1). The difference became clear at higher spiking levels, except for Cd (in lettuce and radish) and Hg (in radish). Zn - Cd interactions are controversial, since both antagonism and synergism between the two elements have been reported. One field experiment showed that the interaction mechanism of these two metals was synergistic, such that increased Cd and Zn content in soils could increase the bioaccumulation of Zn or Cd in crops (Nan et al., 2002). Later findings, however, found antagonism between Zn and Cd in the uptake - transport process (Wu et al., 2003). In the present study, the changes observed in Cd with increasing Zn were not steady. This may be caused by the interaction of these two mechanisms. Hg has comparatively high electronegativity values and easily forms bonds with other elements, especially with S anions. Hg compounds are unaffected by hydrolysis and hardly used by plants. Therefore, the addition of Zn and Cu neither favored nor inhibited Hg uptake by radish. Unsurprisingly, the quantity of Zn grew notably in plant tissue when extra Zn was added to the substrate. Specifically, Zn increased from 28.75 mg/kg to 46.54 mg/kg in lettuce, and from 17.50 mg/kg to 36.42 mg/kg in radish. In contrast, Cu tends to be more strongly absorbed in soil, and plants regulate its uptake more effectively than Zn (Kabata-Pendias, 2011). In the present study, Cu only increased from 4.02 mg/kg to 4.45 mg/kg in lettuce, and from 2.17 mg/kg to 2.69 mg/kg in radish. Thus, plant tissue concentrations and availability of Cu are usually much lower and less sensitive to soil inputs of this element as a component of sludge compared with more mobile elements such as Zn. On the other hand, a Zn–Cu antagonistic interaction has been reported, due to the involvement of the same carrier sites in their absorption mechanisms (Kabata-Pendias, 2011). The concentrations of other TEs increased to different extents. For lettuce, the increase of TEs was as follows: Pb (344%) > As (289%) > Ni (243%) > Cr (235%) > Zn (162%) > Hg (137%) > Cu (111%) > Cd (103%). In the radish root, the sequence was Pb (332%) >

Ni (288%) > As (266%) > Zn (208%) > Cr (187%) > Cu (124%) > Cd (105%) > Hg (97%).

As shown in Fig. 3.2.1, the accumulation of TEs was significantly ($p < 0.05$) greater in the edible part of lettuce than in radishes. The con- centration in lettuce leaves ranged from 0.012 to 46.54 mg/kg vs. 0.005 - 36.42 mg/kg in radish root. The relatively longer growth period of lettuce compared to radish (2 months vs. 1 month) makes it easier for lettuce to absorb more TEs. In addition, the higher transpiration and translocation rates of lettuce facilitate element transport to aerial parts (Gupta et al., 2019). The sequential increase in the abundance of TEs in both crops was equivalent (Zn > Cu > Cr > Ni > Cd > As > Pb > Hg), indicating that although the absolute amount of TEs in vegetable tissue differs between plants, the relative abundance of TEs is more dependent on the type of trace elements. In this sense, Zn and Cu, the essential metals in plants, are constituents of several key enzymes and also play important functions in physiological processes. In contrast, there is no evidence to date that the rest of the TEs play an essential role in plant metabolism, or that they can even be considered phytotoxic elements for plants (As, Pb, and Hg). Accordingly, their uptake was limited.

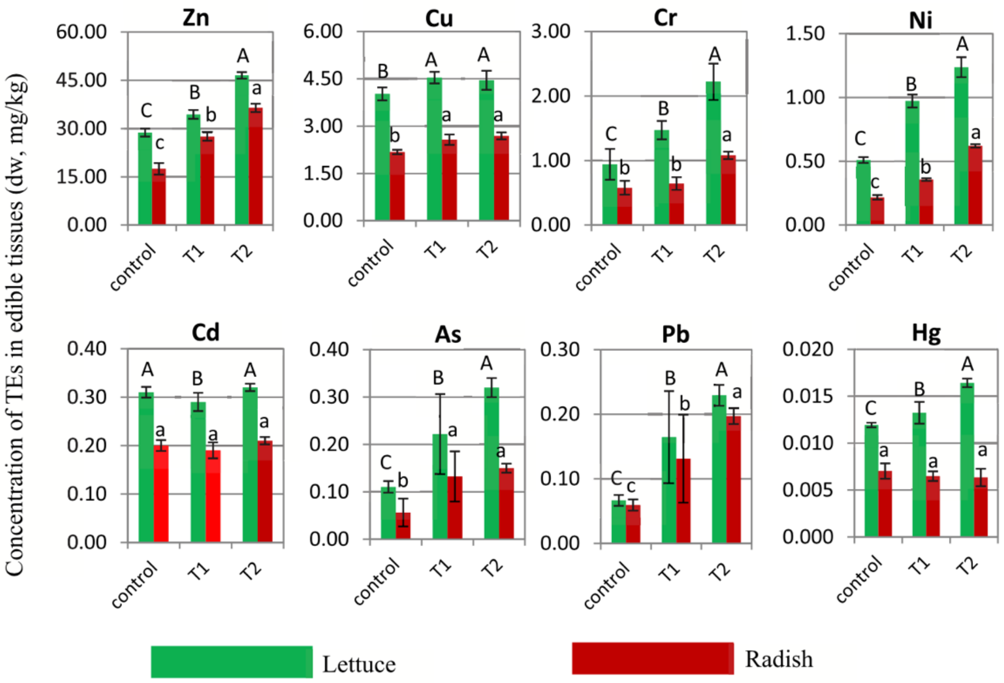


Fig. 3.2.1 Effect of Cu and Zn on the uptake of TEs by leaves of lettuce and root of radish (Mean ± SD, N = 5). Uppercase and lowercase letters refer to significance ($p < 0.05$) for lettuce and radish, respectively. Control:

unspiked sewage sludge containing 240 mg/kg Cu and 700 mg/kg Zn (dw); T1: sewage sludge spiked with 480 mg/ kg Cu and 1400 mg/kg Zn (dw); T2: sewage sludge spiked with 960 mg/kg Cu and 2800 mg/kg Zn (dw).

3.2.3.3 Effect of Zn and Cu addition to sludge on plant uptake of antibiotics

Table 3.2.1 shows the occurrence of ABs in plant samples grown under different concentrations of Zn and Cu. Target ABs were below the LOD in lettuce samples, and 2 (azithromycin and sulfamethoxazole) out of 16 ABs were close to or slightly above the LOQ in radish samples.

Table 3.2.1. Concentration of detected ABs (μg/kg, fw) in vegetable tissue.

	Lettuce					Radish				
	Control	T1	T2	LOD	LOQ	Control	T1	T2	LOD	LOQ
AZM	＜LOD	＜LOD	＜LOD	0.029	0.054	0.02± 0.01[a]	0.02± 0.01[a]	0.01± 0.01[a]	0.010	0.019
SMZ	＜LOD	＜LOD	＜LOD	0.018	0.036	0.03 ± 0.01[a]	0.05±0.01[a]	0.06±0.02[a]	0.030	0.059

Azithromycin (AZM) and sulfamethoxazole (SMZ). Different letter indicates significant difference ($P < 0.05$).

It was somewhat surprising to find sulfamethoxazole in the radish samples, as its value in the sludge was lower than the LOD. There are two possible explanations for this. The first might be related to the possibility that the sulfamethoxazole in the sewage sludge is in its conjugate form and, consequently, was not detected. Once in the soil, enzyme activity could release the parental product, which could be accumulated in the radish. The second possible explanation is that sulfamethoxazole simply has a very high bioconcentration factor, which would explain its accumulation in radish even though its concentration was below the LOD in the sludge (Table S2.4). In this sense, a recent study found sulfamethoxazole in all the vegetables tested, with the highest contamination levels being found in lettuce leaves compared to tomato fruits, cauliflower, and broad bean seeds (Tadić et al., 2019). This is also consistent with a previous study that found that sulfamethoxazole has high leaching potentials from soil and sludge due to the low K_d value (1.7 L/kg) (Höltge and Kreuzig, 2007).

The AB concentrations varied slightly across the treatments ($p > 0.05$), which may be due to their low abundance levels in vegetables. In fact, most were below the LOQ, suggesting they may not be able to express a precise fluctuation. The sulfamethoxazole increased along the gradient of Cu and Zn concentrations by around two-fold; however, the difference was not significant. Liu et al. (2017) reported that the presence of Cu^{2+} inhibited the sorption of sulfamethoxazole by competing in the hydrophobic adsorption region in soils. Desorbed sulfamethoxazole could be taken up by plants along with pore water. In the present study, AB concentrations in plant tissues were fairly low. This is probably due to three factors: (a) the background AB value in the substrate was much lower than in other studies (Qian et al., 2016; Ye et al., 2016); (b) the poor mobility of ABs such as ciprofloxacin, which is considered non-mobile (Tolls, 2001) and has a high affinity to soil particles (K_d 430 L/kg), hinders their uptake by vegetables from soil, despite their relatively high abundance in the substrate; and (c) the plant's detoxification mechanism, which may lead to undetectable AB accumulation in plant tissue (Farkas et al., 2007).

3.2.3.4 Effect of Zn and Cu addition to sludge on plant uptake of antibiotic resistance genes

Absolute values of 16S rDNA sequences in lettuce and radish (0.7- 10.2 × 10^8 copies/g and 0.9 - 4.4 × 10^8 copies/g, respectively; Fig. 3.2.2) were slightly higher than in other vegetables (e.g., in broad beans, the range is from 140 to 5.2 × 10^7 copies/g) treated with chemical fertilizers in peri-urban plots of Barcelona (Cerqueira et al., 2019c). These results suggest that the application of sewage sludge may favor the proliferation of endophytic bacteria in crops. In contrast, *intI*1 was found at lower levels than in other comparable studies (10^4 for lettuce, 10^5 for radish in Lau et al., 2017), which suggests a relatively low potential for horizontal gene transfer linked to group I integrons, especially for lettuce.

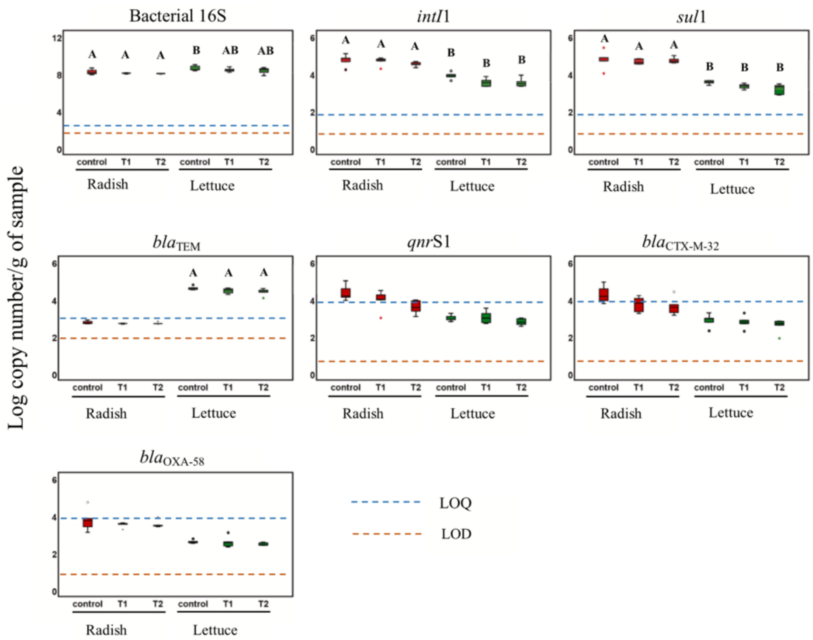


Fig. 3.2.2 Relative abundances of the different genetic elements in vegetable samples. Data are expressed as copies of each sequence per g of tissue (log10 values), and code colored by vegetable type: green for lettuce and red for radish. Different uppercase letters indicate statistically difference ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article). Control: unspiked sewage sludge containing 240 mg/kg Cu and 700 mg/kg Zn (dw); T1: sewage sludge spiked with 480 mg/kg Cu and 1400 mg/kg Zn (dw); T2: sewage sludge spiked with 960 mg/kg Cu and 2800 mg/kg Zn (dw).

Among the ARGs, abundance levels varied by several orders of magnitude, with *sul*1 being the most abundant ARG in radish and *bla*$_{TEM}$ in lettuce. Quantification of *mec*A and *tet*M was not possible since they were found to be under detection limits. On the other hand, *bla*$_{TEM}$, in the case of radish samples, and *qnr*S1, *bla*$_{CTX\text{-}M\text{-}32}$, and *bla*$_{OXA\text{-}58}$, for both radish and lettuce, were found below quantification limits. The distribution of quantified genetic elements abundance in vegetable samples was evaluated using the Mann-Whitney *U* test. The analysis shows that ARG abundance was more influenced by crop type than additional Cu and Zn. Radish may pose a higher ARG risk, except in the case of *bla*$_{TEM}$. This finding is consistent with prior studies showing radishes had a greater load of ARGs than lettuce (Guron et al., 2019; Tien et al., 2017). Both growth time and vegetable species could affect the occurrence of ARGs in vegetable tissues. For instance, the *tet*AP gene was detected in the endive phyllosphere at 30 d, but not at 60 d (Wang et al., 2015). Crops could shape the overall rhizosphere and phyllosphere microbiota through the secretion of various proteins, amino acid, phenols, etc. (Berg and Smalla, 2009; Bulgarelli et al., 2013). Another study (Fogler et al., 2019) found differences in the lettuce and radish resistomes and suggested that the extent of soil contact should be considered. Furthermore, studies carried out in our group have established that 0.01 - 1% of the ARG loads present in the soil can be found in the edible parts of the plant (Cerqueira et al., 2019a, 2019b; 2019c).

In the present study, no significant differences in ARGs in plant tissue were observed with the additional dose of Zn and Cu. Metal cations have a broad ability to complex with antibiotics to decrease their bioavailability (Uivarosi, 2013). Hence, following amendment with extra Zn and Cu, soil bacteria may face less pressure from antibiotics, thereby preventing the spread of ARGs in the matrix microbial community. Owing to the low abundance of intl1 in plant tissue, the possibility for ARGs to be transferred from soil bacteria to endogenous bacteria in plants is relatively rare. On the other hand, bacteria have developed a variety of resistance mechanisms to counteract TE stress, including altered gene expression (increasing metal resistance genes, which may induce the expression of ARGs) and changes in the physiological state (Teitzel and Parsek, 2003).

Supplementary Fig. S2.2 shows the relationship among quantified genetic elements in the edible tissue of both vegetables. In radish samples, *sul*1 was significantly related to bacterial 16S and *intl*1. This indicates that the occurrence of *sul*1 in radish was strongly dependent on the number of endophytic bacteria and positively related to the ability of horizontal transfer of bacterial genes, whereas this relationship is weak in lettuce. ARGs in air particulates have been reported to be diverse and abundant (Li et al., 2018) and may be deposited on the surface of plant leaves and invade them via leaf stomata. The airborne ARGs may impair the association of ARGs-intl1-bacterial abundance in microbiomes in the aerial part of the plant.

3.2.3.5 Distribution and relationship of contaminants

The correlations between the TE, AB, and ARG variables were further analyzed by PCA, as shown in the biplot (Fig. 3.2.3). Two principal components explain 79% of the variation in the data. The first principal component (PC1), which accounted for 61% of the variance, clearly separated the samples into two distinct groups, lettuce and radish. This trend indicates that vegetable type is the key factor for the accumulation of contaminants in edible tissue. Cd and *bla*$_{TEM}$ have high positive loading values (>0.80) in PC1, while sulfamethoxazole has a high negative loading value (− 0.873) (Table S2.5). This result indicates that the occurrence of these three contaminants is significantly different between vegetables and that sulfamethoxazole may inhibit the absorption of Cd and the expression of *bla*$_{TEM}$.

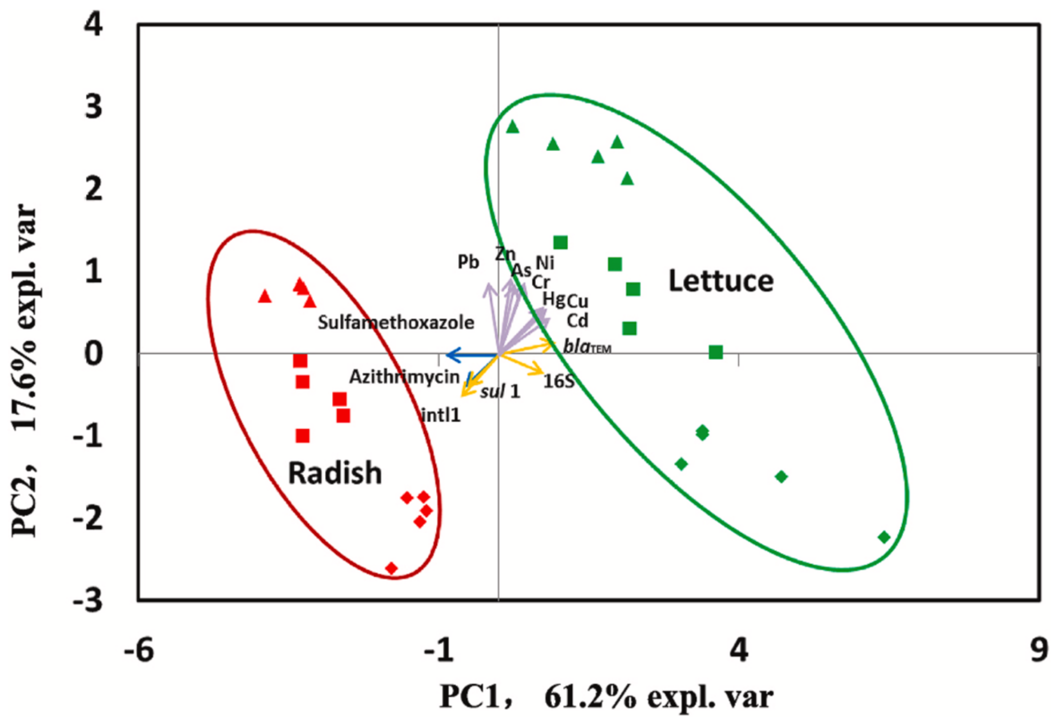


Fig. 3.2.3 The PCA biplot of lettuce and radish data showing the loading of each variable (arrows) and the scores of each treatment (symbols). Treatments are expressed as rhombus (control), square (T1), triangles (T2). Green and red symbols correspond to lettuce (L) and radish (R). TEs, ABs and genetic elements loading are represent by violet, blue and yellow arrow. The length of the arrows approximates the variance of the variables, whereas their angles among them estimate their correlations. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Additionally, PC2 accounted for 18% of the total variance. As can be seen in Fig. 3.2.3, this second component grossly separated the control and the treatments applied to vegetables. Negative values correspond to the control and higher positive values are related to the highest concentrations of Cu and Zn, in treatment T2. The results for PC2 also indicate some associations between TEs and suggest that the addition of Cu and Zn favored the accumulation of other TEs. More specifically, high positive loadings (> 0.80) were found for As, Zn, Pb, Cr, and Ni, indicating the same trend as the observed variables. In this sense, adding Zn significantly favored the accumulation of most TEs in vegetables. In general, the main factor affecting the uptake and distribution of TEs was their speciation. The increased TEs in the vegetable tissue may originate from the substrate-bound compounds. When the Zn and Cu are added, they compete for the binding sites in the soil and sludge with other elements, resulting in many more TEs being released into pore water as free ions, which are easily absorbed by plants (Norvell et al., 2000).

Finally, the individual scores for each vegetable in the biplot showed a clearly separate pattern for lettuce and radish, as well as for each treatment. A first cluster made up of the lettuce treatments can be observed (lower and upper right), with a pattern distributed along the PC2 axis for the TEs and bla_{TEM}. In a second cluster, radish scores (lower and upper left) were scattered toward positive and negative values along the PC2 axis. This is consistent with the lower TE values, but higher AB and genetic elements values here than in lettuce.

3.2.3.6 Effect on phytotoxicity

Supplementary Fig. S2.3 shows the change in the length and number of leaves according to the different studied conditions. No significant differences were found between the two growth indices in T1 and T2 and the control, indicating that extra Zn and Cu did not hinder the development of either vegetable. This finding is contrary to that reported by Wolf et al. (2017), who found that equivalent Zn and Cu hampered lettuce growth. This was due to the strong complexing ability of the sewage sludge in the present case, which prevented plants from absorbing overdoses of Zn and Cu.

At the end of the experiment, the total chlorophyll content, lipids, and carbohydrates in the leaves, the fresh weight of the edible potion, and the height of the aerial part of both vegetables were analyzed to evaluate the effect of contaminants on productivity (Table S2.6). No significant differences were observed among the different treatments in either vegetable, indicating that added Zn and Cu have no influence on crop productivity.

3.2.3.7 Potential human health risk

(1) Risk assessment for trace elements

The estimated daily intake (EDI) of elements was calculated according to the concentration in edible tissues and daily consumption amount, which is shown in Fig. 3.2.4. The EDI value was dependent on the element, treatment, and vegetable type. Generally, consuming vegetables grown under treatment T2 led to the highest intake of TEs (3.1×10^{-3} mg/kg/bw/day for lettuce and 1.1×10^{-3} mg/kg/bw/day for radish). As for quantity, Zn accounted for the majority in both crop tissues (2.8×10^{-3} mg/kg/bw/day for lettuce and 0.9×10^{-3} mg/kg/ bw/day for radish). According to Commission Regulation (EC) No 1881/2006, the maximum accepted levels of Pb and Cd in lettuce are 0.3 and 0.2 mg/kg, respectively. In radish, this level is 0.1 mg/kg for both metals. Therefore, according to Equation (1), the maximum acceptable EDIvalueforlettuceis1.9×10^{-4}mg/kg/bw/dayforPband1.3×10^{-4} mg/kg/bw/day for Cd. For radish, the maximum acceptable EDI value is 3.1×10^{-5} mg/kg/bw/day for both metals. In the present study, the highest EDI values for these two elements were both lower than the limit.

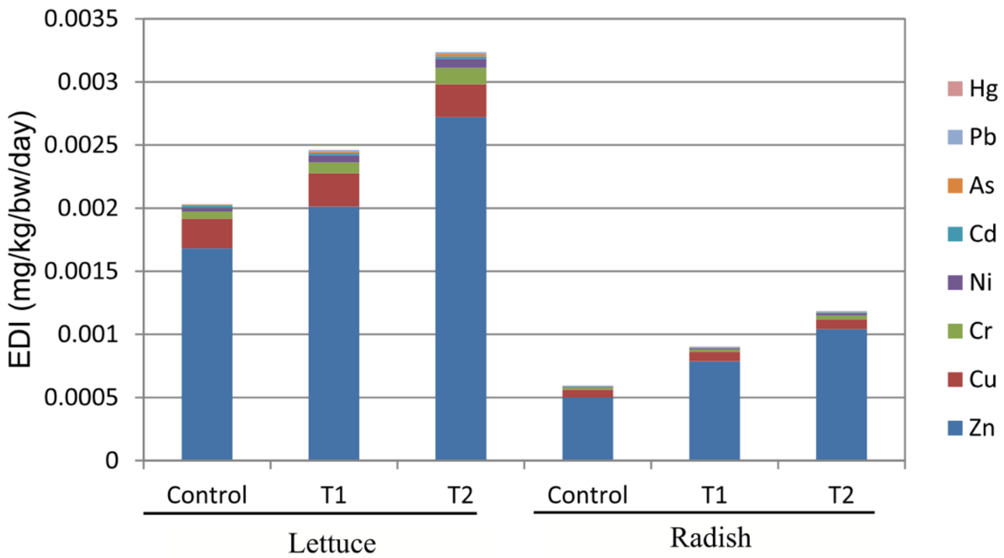


Fig. 3.2.4. Estimated daily intake of trace elements.

HQ_{TE} was calculated based on the EDI value and the oral reference dose for non-carcinogenic effects (Table 3.2.2). Arsenic had the highest HQ_{TE} value in the edible parts of the vegetables and thus posed the highest health risk to humans. This finding is consistent with that reported in Shamsollahi et al. (2019), in which the risk of As was around 20 times higher than Pb and Cd in lettuce samples grown under sewage sludge amendments. In the present study, the level was around 10 times lower than in previous work, due to different background values of TEs in the sewage sludge. The additional pressure of Zn and Cu influenced the uptake of TEs, but it did not change the order of health risks posed by the elements, which was As > Cd > Zn > Pb > Cu > Hg > Cr.

The THQ_{TE} was <1 for all the samples. The THQ_{TE} values were around 3–4 times higher in lettuce than in radish. This is due not only to the higher TE values in lettuce, but also to its higher consumption in Spain. Vegetables grown with the extra pressure of Cu and Zn showed a higher risk, although, since the values were still <1, no risk should be assumed. Nevertheless, the risk was 2–3 times higher in T1 and T2, respectively, compared to the control.

Table 3.2.2 Hazard quotient of TE (HQ_{TE}) and total hazard quotient (THQ_{TE}) in vegetable samples

	Lettuce			Radish			RfD (mg/kg bw/day)
	Control	T1	T2	Control	T1	T2	
As	0.0218	0.0437	0.0631	0.0063	0.0129	0.0165	3×10^{-4}
Cu	0.0006	0.0007	0.0007	0.0002	0.0002	0.0003	0.4
Ni	0.0015	0.0028	0.0036	0.0003	0.0005	0.0009	0.02
Zn	0.0057	0.0068	0.0090	0.0017	0.0027	0.003	0.3
Pb	0.0011	0.0028	0.0039	0.0005	0.0011	0.0011	3.5×10^{-3}
Cd	0.0078	0.0076	0.0090	0.0021	0.0021	0.0033	1×10^{-3}
Cr	3.7×10^{-5}	5.8×10^{-5}	8.7×10^{-5}	1.0×10^{-5}	1.2×10^{-5}	2.5×10^{-5}	1.5
Hg	0.0004	0.0004	0.0005	0.0001	0.0001	0.0002	2×10^{-3}
THQ	0.0390	0.0648	0.0900	0.0112	0.0195	0.0251	

(2) Risk assessment for antibiotics

The consumption of vegetables containing ABs is one pathway of human exposure. Generally, the daily intake of ABs varies considerably between reports and depends on the AB and the vegetable growing conditions. Table 3.2.3 lists the EDI values of ABs detected in the vegetables in the present study and other similar studies. The daily intake value of azithromycin in radish varied from 3.1×10^{-6} to 6.3×10^{-6} μg/kg/day, whereas the value of sulfamethoxazole was relatively higher, varying from 9.4×10^{-6} to 1.9×10^{-5} μg/kg/day. As can be seen, compared with other reports, the EDI of antibiotics associated with the consumption of organic vegetables was significantly lower, probably due to the significant difference in substrates.

Table 3.2.3 Estimated daily intake of detected antibiotics of vegetables in several reports

Crop species	Treatment	Antibiotics	EDI (μg/kg/day)	Reference
Radish	Control	AZM	6.3×10^{-6}	Our study
Radish	T1	AZM	6.3×10^{-6}	Our study
Radish	T2	AZM	3.1×10^{-6}	Our study
Radish	Control	SMZ	9.4×10^{-6}	Our study
Radish	T1	SMZ	1.6×10^{-5}	Our study
Radish	T2	SMZ	1.9×10^{-5}	Our study
Lettuce	Manure-amended	SMZ	5.1×10^{-3}	Dolliver et al., 2007
Radish	Manure-amended	OTC	2.4×10^{-4}	Hu et al., 2010
Radish	Manure-amended	LIN	8.9×10^{-5}	Hu et al., 2010
Lettuce	Sewage sludge-amended	CIP	1.3×10^{-2}	Lillenberg et al., 2010
Lettuce	Sewage sludge-amended	AZM	4.7×10^{-5}	Sidhu et al., 2019

AZM: azithromycin; SMZ sulfamethoxazole; LIN: Lincomycin; OTC oxytetracycline; CIP: ciprofloxacin

Table 3.2.4 lists the HQ value of the detected antibiotics (azithromycin and sulfamethoxazole) in radish, as well as their acceptable daily intake (ADI) values based on two evaluation methods. Although the risk of both antibiotics varied according to different targets, the HQ_{AB} values of both ABs were significantly lower than the HQ of ≥ 1, identified as a potential hazard to human health for the current assessment. Therefore, consumption of radish and lettuces grown in soil amended with sewage sludge with high concentration levels of Zn and Cu has no hazardous effect in terms of antibiotics. However, further research is required, as many other exposure pathways for human beings to antibiotics, e.g. farm fish, drinking water, or inhaled particles, are plausible.

Table 3.2.4 Acceptable daily intake (ADI) and hazard quotient of ABs (HQ_{AB}) of radish based on different effect endpoints

	[a]ADI1 (μg/kg/day)	HQ_{ADI1}			[b]ADI2 (μg/kg/day)	HQ_{ADI2}		
		Control	T1	T2		Control	T1	T2
Azithromycin	7.1[c]	8.9×10^{-7}	8.9×10^{-7}	4.4×10^{-7}	1.7[e]	3.7×10^{-6}	3.7×10^{-6}	1.8×10^{-6}
Sulfamethoxazole	5.7[d]	1.6×10^{-6}	2.8×10^{-6}	3.3×10^{-6}	130[e]	7.2×10^{-8}	1.2×10^{-7}	1.5×10^{-7}

[a] Based on therapeutic purpose

[b] Based on microbiological and toxicological effect

[c] For ADI_1 calculations therapeutic dosage for azithromycin is 500 mg/day (https://www.healthline.com/health/azithromycin-oral-tablet#dosage)

[d] For ADI_1 calculations therapeutic dosage for sulfamethoxazole is 400 mg/day (Prosser and Sibley, 2015)

[e] Wang et al., 2017

(3) Risk assessment of antibiotic resistant genes

The human health concern posed by foodborne antibiotic resistance is derived from the fact that pathogenic bacteria may acquire antibiotic resistance genes from strains in ready-to-eat food, thereby jeopardizing the future of antibiotic therapy (e.g., due to the loss of the option of clinical antibiotic use) and increasing the severity of infection as manifested in the prolonged duration of disease.

The mechanism of resistance of bla_{TEM} is the production of β-lacta- mases, which are able to hydrolyze the four-membered β-lactam ring present in some antibiotics, such as penicillin and cephalosporin. bla_{TEM} is the predominant gene in lettuce leaves but at levels slightly above the quantification limit. Even at these low values, the presence of this gene could be a cause of concern regarding lettuce leaf consumption, although the real importance of this potential risk has yet to be determined.

On the other hand, *sul*1 accounted for the largest share of the target ARGs in radish samples. This gene codes for resistance to sulfonamide antibiotics, which can be related to the detected presence of sulfamethoxazole in radish. In this regard, it is worth considering the possibility that the actual antibiotic selective pressure may occur in the soil, rather than in the root, as the plant resistome is highly influenced by the soil (Cerqueira, 2019b). The similar trends observed in *sul* 1 and *intl*1 in both matrices is probably linked to the fact that *sul*1 is a group I integron-associated gene (Ma et al., 2020). This further supports the hypothesis that some selective pressure for sulfonamide resistance exists in either soil or the plant.

This study thus demonstrates that foodborne antibiotic resistance depends on the type of vegetable consumed and that Cu and Zn accumulation in the soil does not affect its spread. However, the question of the extent to which foodborne ARGs could affect human health remains. Appropriate models for translating foodborne antibiotic resistance to measures of human health risk are needed. The present study provides data that could be useful for making such a determination in the future, as they suggest that the target antibiotic resistance differs among different vegetables.

3.2.4 Conclusions

Increased doses of Zn and Cu in sludge-amended soils influence TE accumulation in vegetables; however, crop type, rather than increased Zn and Cu concentrations, seems to be the key factor. This is especially evident for the ABs and ARGs slightly detected in vegetables, at least, at the Zn and Cu spiking doses used in this study. Although the addition of Zn and Cu increased the uptake of TEs in the vegetables, ABs and ARGs did not significantly change. In this sense, TE uptake and the health risk were greater (3 - 4 times) in lettuce than in radish, probably because of the high consumption rate among the Spanish population. However, there was no health risk for the consumer. In contrast, ABs were not detected in lettuce and the abundance of ARGs was 10 times higher in radish. On the other hand, only two ABs, sulfamethoxazole and azithromycin, were quantified and efficiently accumulated in radish root, which had a greater number of gene copies of endophytic bacteria and ARGs than lettuce leaves, except for *bla*$_{\mathrm{TEM}}$. Finally, the health risk posed by ABs in the edible tissue of vegetables in this study was relatively lower than reported elsewhere, and the main risk of antibiotic resistance depended on vegetable type.

Although the present study showed that lettuces and radish grown under sewage-sludge-amended soil with different accumulated levels of Zn and Cu did not significantly affect the accumulation of ABs and ARGs in the edible part of vegetables, and that increased TEs likewise had no hazardous effect on human health, long-term monitoring is necessary. Frequent amendments with sewage sludge inevitably result in the accumulation of other TEs and ABs in the soil, and increasing environ- mental pressure could trigger an outbreak of ARGs in vegetables, especially the selection pressure of non-biodegradable TEs.

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3.2.6 Supporting information

3.2.6.1 Reagents and standards

Trace elements. The reagents for extraction were prepared using nitric acid (69%) (Panreac), 37% hydrochloric acid (Merck, Darmstadt, Germany), and 31% hydrogen peroxide (Merck). All solutions were diluted with doubly deionized water obtained from Millipore water purification systems (Elix&Rios) (18.2 MΩ/cm resistivity and TOC, Total Organic Carbon <30 μg/L). CRM 1570a, supplied by the National Institute of Standards and Technology (USA), has certified values for As, Cd, Pb, Cr, Cu, Hg and Zn. Digestion reagents were also used as a blank matrix. For quality control purposes, in each sample digestion series, CRM and laboratory reagent blank were added. A limit of detection (LOD) of in the solution analyzed was determined from three times the standard deviation obtained from the analysis of ten runs of blank samples on the same day as the determinations. Similarly, the limit of quantification (LOQ) was calculated by multiplying the standard deviation by ten times.

Antibiotics: Sulfathiazole, sulfamethizole, sulfadiazine, sulfamethazine, sulfamethoxazole, ofloxacin, enrofloxacin, ofloxacin-d3, enrofloxacin-d3 hydrochloride, lincomycin, tetracycline, ciprofloxacin, and azithromycin were purchased from Sigma-Aldrich whereas ofloxacin methyl ester, and ofloxacin ethyl ester were purchased from LGC Standards S.L.U. (Barcelona, Spain). Clindamycin-d3 hydrochloride was purchased from Toronto Research Chemicals (Toronto, Canada), and sulfamethoxazole-d4 was purchased from Analytical Standard Solutions – A2S

(Saint Jean d'Illac, France), respectively. All standards were high purity (95% or higher). Methanol and water (both LC-MS grade), ethyl acetate (GC-ECD/FID grade), and formic acid (98–100%, pro-analysis) were obtained from Merck, whereas acetonitrile (LC-MS grade) was obtained from Fisher Scientific UK (Loughborough, UK). SPE Strata-X cartridges (100 mg/6 mL) were obtained from Phenomenex (Torrance, CA, USA), and 0.22-μm pore nylon filters were purchased from Sigma-Aldrich. In order to enable equilibration, 100 ng/g (dw) ofloxacin-d3, clindamycin-d3, enrofloxacin-d3, and sulfamethoxazole-d4 were spiked one hour prior to extraction into the samples (1 g) as surrogate standards. LOD and LOQ were calculated as the mean background noise in a blank triplicate plus three and ten times.

Antibiotic resistance gene: Primers for sul1, tetM, qnrS1, mecA, blaTEM, blaCTX-M-32, blaOXA-58, and bacterial 16S rDNA sequences are listed in supplementary Table S1. To ensure the absence of bacteria on the surface of vegetable, the last rinsing water was plated in LB agar media for 72 hours at 28 °C. For blank control of qPCR reactions, complete reaction mixes plus nuclease free water, but no template, were used. PNORM1 plasmid and pUC19 plasmids were used as quantification standards for ARGs. The limit of quantification (LOQ) was defined as the lowest point on the linear part of the standard curve: 100 gene copies per reaction for *sul*1, $bla_{\text{CTX-M-32}}$ and *intI1* and 1000 gene copies per reaction for 16s rDNA, *tet*M, bla_{TEM}, $bla_{\text{OXA-58}}$ and *mec*A .

3.2.6.2 Supplementary tables

Table S2.1 Primers utilized for detection and quantifications of antibiotic resistance genes in soil, sewage sludge and plant parts

Target gene	Primers	Sequence (5'-> 3')	Amplicon size (bp)	Tm(°C)	Reference
intl1	intI1LC5	GATCGGTCGAATGCGTGT	196	60	Barraud et al, 2010
	intI1LC1	GCCTTGATGTTACCCGAGAG			
16s rDNA	331F	TCCTACGGGAGGCAGCAGT	195	60	Bräuer et al., 2011
	518R	ATTACCGCGGCTGCTGG			
blaTEM	blaTEM-F	TTCCTGTTTTTGCTCACCCAG	113	60	Di Cesare et al., 2016

	blaTEM-R	CTCAAGGATCTTACCGCTGTTG			
blactx-m-32	ctx-m-32-FW	CGTCACGCTGTTGTTAGGAA	156	60	Hembach et al., 2017
	ctx-m-32-R	CGCTCATCAGCACGATAAAG			
blaOXA-58	OXA58F	GCAATTGCCTTTTAAACCTGA	152	63	Laht et al., 2014
	OXA58R	CTGCCTTTTCAACAAAACCC			
mecA	mecAF	AAAAAGATGGCAAAGATATTCAA	185	63	Szczepanowski et al., 2009
	mecAR	TTCTTCGTTACTCATGCCATACA			
qnrS	qnrSrtF11	GACGTGCTAACTTGCGTGAT	118	60	Marti & Balcázar, 2013
	qnrSrtR11	TGGCATTGTTGGAAACTTG			
sul1	sul1-FW	CGCACCGGAAACATCGCTGCAC	162	60	Pei et al., 2006
	sul1-RV	TGAAGTTCCGCCGCAAGGCTCG			
tetM	tetMF	GCAATTCTACTGATTTCTGC	186	60	Tamminen & Karkman, 2011
	tetMR	CTGTTTGATTACAATTTCCGC			

Table S2.2 Physical and chemical parameters of soil and sewage sludge sample

Parameters	Soil	Sewage sludge
Humidity (%)	1.33	79.1
pH	8.48	8.40
EC (dS/m)	0.24	2.56
OM (%)	1.27	51.5
Total N (mg/kg)	890	27700
Available N (mg/kg)	298	2800
Available P (mg/kg)	33	15500
Available K (mg/kg)	344	2070

Table S2.3 Content of TEs in soil and sewage sludge (dw, mg/kg). The generic reference levels of these elements for contaminated soil in Catalonia and legislation for fertilizer

Element	Soil	Standard for soil	Sewage sludge	Legislation for sludge
Cu	92.45 ± 1.78	-	240.12 ±2.14	300 [b]

Zn	74.32 ± 2.17	170 [a]	700.78 ± 1.14	800 [b]
Pb	58.12 ± 0.99	60 [a]	30.85 ± 1.09	120 [b]
Ni	33.01 ± 0.32	45 [a]	53.01 ± 1.08	50 [b]
Cr	21.01 ± 0.12	400 [a]	57.96 ± 0.21	100 [c]
As	19.61 ±1.01	30 [a]	7.71 ± 0.01	40 [b]
Cd	<0.5	2.5 [a]	0.51 ± 0.01	1.5 [b]
Hg	0.0252 ± 0.0001	2 [a]	0.67 ± 0.09	1 [b]

[a] Generalitat de Catalunya (2017); [b] PE-CONS 76/18 (2019); [c] Saveyn and Eder. (2014).

Mean ± SD (N = 3).

Table S2.4 Concentration of ABs in sewage sludge

Compound	LOD (dw, µg/kg)	LOQ (dw, µg/kg)	Concentration (dw, µg/kg)	Molecular weight	Log K_{ow}	Solubilty (mg/L)	pKa	K_d (L/kg)
ciprofloxacin	1.29	4.35	5789.47	331.3	0.28	30000	6.09	430 [a]
doxycycline	1.39	4.69	130.57	444.4	0.63	50	3.09	na
sulfathiazole	1.39	4.69	126.32	255.3	0.05	373	7.2	4.9 [b]
tetracycline	1.20	4.02	104.78	444.4	-1.37	231	3.3	1140 [c]
azithromycin	0.72	2.49	102.39	749	4.02	2.37	8.74	na
lincomycin	0.29	1.05	15.87	406.5	0.20	927	7.6	66 [d]
sulfamethoxazole	0.77	2.49	<LOD	253.28	0.89	610	1.6	1.7 [e]

[a] Nowara et al., (1997); [b] Tolls, (2001); [c] Sithole and Guy, (1987); [d] Li et al., (2019); [e] Höltge and Kreuzig, (2007)
na: not available.

Table S2.5 Loadings for PCA with relatively high scores in bold typeface

	Component	
	1	2
Cd	**.856**	.444
As	.254	**.851**

Cu	.792	.552
Cr	.400	**.852**
Zn	.203	**.930**
Pb	-.186	**.877**
Hg	.751	.577
Ni	.420	**.880**
Sulfamethoxazole	**-.873**	-.119
Azithromycin	-.577	-.412
16S	.746	-.234
blatem	**.945**	.141
intl1	-.640	-.525
sul1	-.539	-.401

Table S2.6 Vegetable quality parameters (n=5)

	LC	LT1	LT2	RC	RT1	RT2
Chl_T ($\mu g/cm^2$)	0.19 ± 0.06	0.20± 0.06	0.20± 0.06	0.31± 0.05	0.26± 0.06	0.31± 0.07
Lipids (%)	0.27 ± 0.03	0.26 ± 0.04	0.34 ± 0.14	0.07 ± 0.01	0.11 ± 0.08	0.07 ± 0.01
Carbohydrates (%)	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.01	0.06± 0.01	0.06± 0.02	0.07± 0.02
Fresh weight (g)	215.8 ± 51.4	223.7 ± 22.9	247.5 ± 48.0	50.3± 18.2	35.7± 35.7	55.5 ± 14.6
Height (cm)	13.8 ± 0.3	14.4 ± 0.4	13.9 ± 1.2	15.90 ± 1.0	16.7 ± 1.0	16.7 ± 1.3

LC= lettuce in control; LT1= lettuce in T1; LT2= lettuce in T2; RC= radish in control;RT1= radish in T1; RT2= radish in T2

3.2.6.3 Supplementary figures

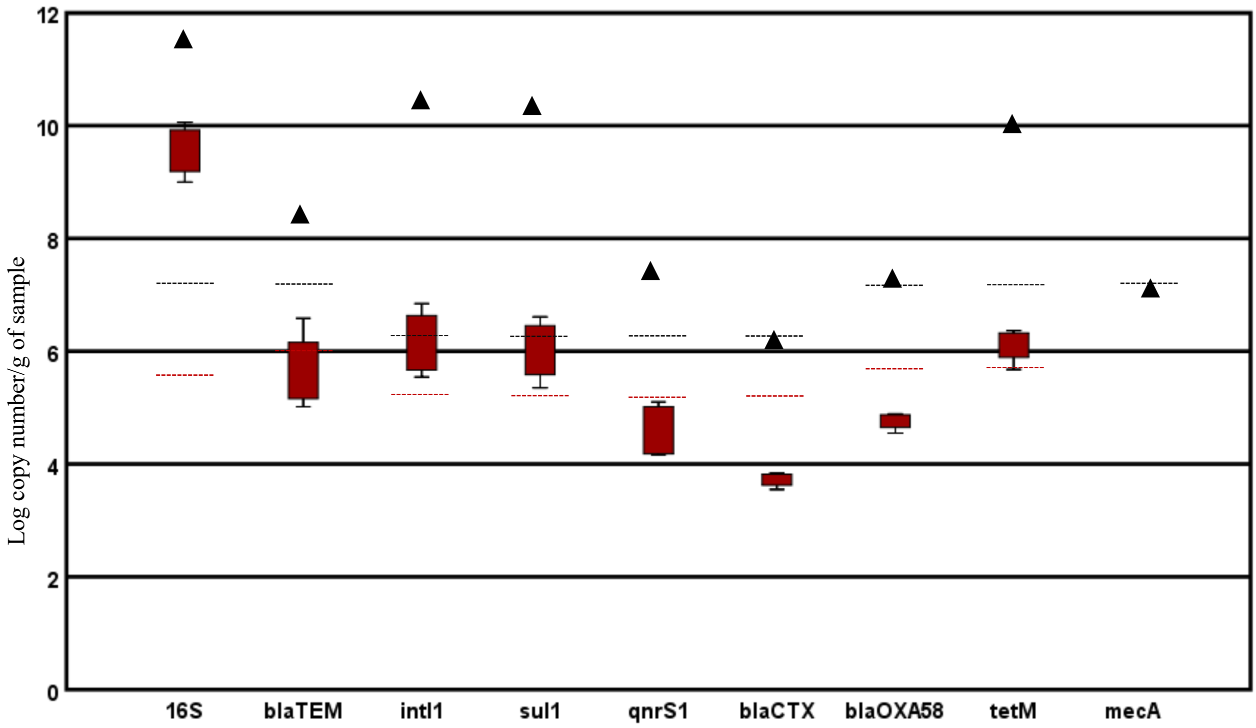

Fig S2.1 Abundance of genetic elements in fresh soil and sewage sludge. Data are expressed as copies of each sequence per g of sample (log 10 values). Box represents soil samples (n=4), while triangle is the abundance in sewage sludge (n=1). Dotted line is LOQ of genetic element (black for sludge, red for soil).

	*bla*TEM	Intl 1	Sul 1	16S rDNA
16S rDNA	-	**	**	
Sul 1	-	**		**
Intl 1	-		**	**
*bla*TEM	-	-	-	-

(Radish)

	*bla*TEM	Intl 1	Sul 1	16S rDNA
16S rDNA	**			
Sul 1	**			
Intl 1	*			
*bla*TEM		*	**	**

(Lettuce)

Fig S2.2 Correlation maps between the different genetic elements. Asterisks indicate significant Pearson correlations (*, p<0.01, **, p<0.05), while white corresponds to no correlation.

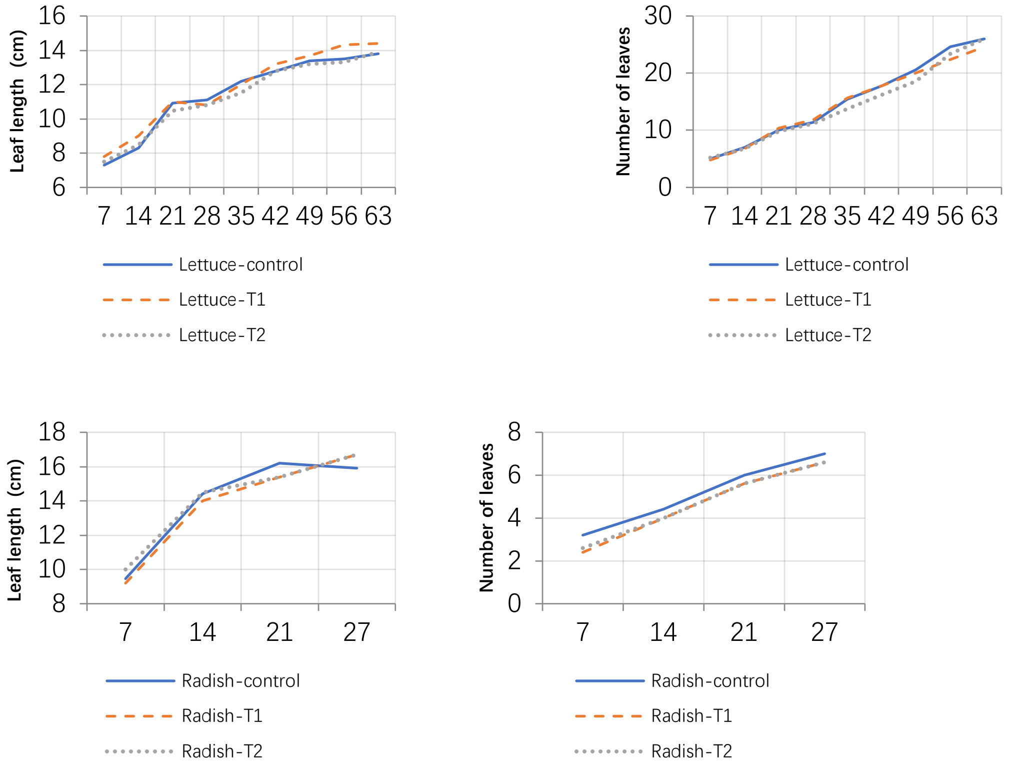

Fig. S2.3 The change of growth indexes of vegetables over time

3.2.6.4 References

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3.3 Topic 3: Influence of repeated organic fertilization on the accumulation of trace elements in vegetables

This topic compares the concentration of trace elements in vegetables grown soils amended once and twice with fertilizer, and evaluates the potential risk of repeated organic fertilization for human health.

Based on the article:

Health risk assessment of trace elements exposure through the soil-vegetables-human pathway under repeated applications of organic amendments

Submitted to a peer review journal

Health risk assessment of trace elements exposure through the soil-vegetables-human pathway under repeated applications of organic amendments

Abstract

Amendment soil with organic fertilizers provides a valuable source of plant nutrients and organic matter. Nevertheless, there is still a concern for human health safety due to the presence of trace elements in organic fertilizers. Long term organic soil fertilization would result in the accumulation of trace elements in soils that can be uptaken by plants and threaten human health. Therefore, we studied representative trace elements (TEs) in soil-plant-human contamination pathway of two production cycles, for evaluating the effect of repeated organic fertilizer application on the vegetable production and contaminantion (i.e. lettuce and radish). The results showed that the use of sewage sludge (SS) and chemical fertilizer (CF) resulted in higher vegetable yield than organic fraction of municipal solid waste (OFMSW). Soil amendment had limited/no impact on the total TE concentration in soil. However, the TEs concentration in vegetable grown with organic fertilizers was higher than those grown with chemical fertilizer. After repeated fertilization, the TE concentrations in the edible tissue of vegetables was significantly increased. According to the hazard quotient (HQ), lettuce had higher TEs risk than radish, and the highest THQs for TEs were observed in vegetable amended with OFMW. Total HQ value increased 1.2 to 2-fold in the second production cycle. Nevertheless, no consumption of lettuce and radish grown in amended soil pose an adverse human health effect, as the total HQ value was always less than 1.

Keyword: trace elements, repeated organic fertilization, lettuce, radish, hazard quotient

3.3.1. Introduction

Accelerated industrialization and urban development have resulted in generation of huge amounts of diverse organic solid waste (OSW) worldwide. According to a World Bank report, global OSW generation level is approximately 1.3 billion tonnes per year, and the number is expected to increase to approximately 2.2 billion by 2025 (Hoornweg and Bhada-Tata, 2012). How to proper ecofriendly disposal of such a huge amount of OSW is still a global issue (Collivignarelli et al., 2019).

The abundance of plant beneficial components in OSW, such as organic matter and plant nutrients, especially N and P, makes it a good soil amendment. Compost (plant sources and food waste) and sewage sludge (SS) are two mostly used municipal organic waste for agricultural fertilization (Chew et al., 2019). Approximately, half of solid faction of compost and sewage sludge is constituted of organic matter which has a significant effect on altering soil physicochemical properties. Therefore, soil trace elements bioavailability by transforming labile metal to metallo-organic complexes is reduced (Sharma et al., 2017). Apart from the beneficial components of biosolids, they are characterized by the presence of toxic substances, such as pesticides, pharmaceuticals, personal care products, and various trace elements (TEs) (Sidhu et al., 2019; Nkinahamira et sl., 2019). European Directive 86/278/EEC (Council of European Communities, 1986) restricts the concentration of trace elements in biosolids applied to agricultural soil. In the United States, EPA (1993) also limits the maximum TEs loading in soil-amendment biosolids in "The Standard for the Use or Disposal of Sewage Sludge". However, all these regulations were established based on the risk assessment of a single dose fertilization, none of them take the potential risk of long-term application into account. In this sense, biosolids would evolve during weathering processes, as for example, the mineralization of organic fertilizer-derived organic matter. During this process, TEs complexed with organic matter would be released and transformed to more soluble forms that are easily absorbed by vegetables. Therefore, Change et al (1997) proposed ‘‘the sewage sludge time bomb hypothesis’’ which regards the organic amendments as a potential threat in long-term.

The negative effects derived from the organic amendments, largely depend on their quality as well as their application frequency. Most of studies focused their attention whether or not a single time application of organic fertilizer may affect crop productivity and safety (Zandvakili et al., 2019; Huang et al., 2016). Only a few available studies compared the effects of frequent applications of organic amendments on the occurrence of trace elements and human health implication. However, most of these previous studies were conducted under controlled condition in laboratory or greenhouse (Chen et., 2014; Morra., 2010). Consequently, it is difficult to translate the achieved knowledge about repeated organic fertilizer application influence into effective and practically feasible guidelines for agricultural systems. Therefore, the aim of this study was to evaluate the behaviour of trace elements (TEs) in soil-plant-human contamination pathway during two production cycles, evaluating their accumulation after repeated organic fertilizer application on vegetables (i.e. lettuce and radish).

3.3.2. Material and methods

3.3.2.1. Experiment layout

Field trials were carried out at the Agròpolis-UPC agriculture experimental station (41°17′18″N, 2°02′43″E) in Viladecans (Barcelona, Spain). The experimental site covers 340 m^2. One part of the field was used to cultivate lettuce (*Lactuca sativa L.* cv. Maravilla de Verano), including 12 plots of 3x2 m each (with 1 m margin between plots). The other part was used to farm radish (*Raphanus sativus.* cv. Redondo rojo) and was also divided into 12 experimental plots of 2.4 x 1 m each. The plots were randomized block design with 3 treatments and 4 replicates for both vegetables. Treatments consisted of a control (chemical fertilizer), and two different organic fertilizers (sewage sludge and compost). Chemical fertilizer was obtained from reagent grade chemicals (NH_4NO_3, P_2O_5, and K_2O). Anaerobically digested sewage sludge, which was a mixture of by-products collected in primary and secondary wastewater treatment process, was obtained from Gavà-Viladecans wastewater treatment plant (Parc del Baix Llobregat, Barcelona). The organic fraction of municipal solid waste

(OFMSW) was obtained from a composting plant (Torrelles del Llobregat, Spain). It is a mixture of pruning waste from the nearby area and organic waste from the Hospital Universitari Vall d'Hebron kitchen. The applied amount of fertilizers was calculated according to the optimal N content for crops (100 kg per ha) (Pomares and Ramos, 2010).

The first experiment was conducted in April 2019, while the second round was carried out in November 2019. Before each experiment, three kinds of fertilizers were homogeneously mixed with soil, the amended soil was collected for analyzing soil TEs amount. On the same day, crops were implanted in plots and irrigated with harvested rainwater and groundwater though irrigation tape (dropper flow rate of 1.14 L/h). Vegetables were harvested until they reached the commercial size. In the first trial, 35 productive days for radish and 56 days for lettuce were necessary. The second experiment was conducted in winter, where crops took longer time to mature (74 days for radish, 120 days for lettuce). Once collected, lettuce leaf and the radish root were stored at − 20 °C until analysis.

3.3.2.2 Sample analysis

Soil and organic fertilizers: Samples were digested with 4 mL HNO_3 (concentrated HNO_3 to ASTM type I water ratio of 1:1, v:v) and 10 mL HCl (concentrated HCl to ASTM type I water ratio of 1:4, v:v) on a hot plate at 85 °C for 30 min. After cooling and filtering (<0.45 μm), samples were analyzed by ICP-MS (Thermo Scientific) (Martin et al., 1994)..

Vegetables: The leaf of the lettuce and root of the radish were freeze-dried and digested with a microwave oven (Milestone Ethos). A 0.1 g of powdered sample was digested with 8 mL of HNO_3 and 2 mL of H_2O_2 in a PTFE vessel, and digested following the program: 15 min from room temperature to 90 °C; 10 min at 90 °C; 20 min from 90 °C to 120 °C, 15 min from 120 °C to 190 °C, 20min at 190 °C. After cooling to room temperature, the digested samples were analyzed by ICP-MS.

3.3.2.3 Contamination indices

(1) Enrichment factor

The Enrichment factor (EF) is an indicator used to differentiate soil trace elements sources derived from anthropogenic or natural activities. This index calculated as the double ratio of the concentration of target element to the reference element in study soil and the background value, as shown in Eq. (1). Due to a more meaningful basis and for better estimation, the TEs value of a pristine site of Barcelona (which is barely influenced by human activities) was chosen as background value rather than the average earth's crust values. EF value smaller than 2 suggests low enrichment and TEs may come entirely from natural processes. The increase of EF value points out that sources of TEs are more likely related to anthropogenic activities. The EF value between 2 and 5 indicates moderate enrichment, while EF higher than 5 shows a significant enrichment.

$$EF = \frac{\left(\frac{C_{El}}{C_R}\right)soil}{\left(\frac{C_{El}}{C_R}\right)Background} \qquad (1)$$

where C_{El} and C_R are the concentrations studied TEs and the reference element, respectively. Typical reference elements used in many studies are Al, Fe, Ti, Mn, Li, Sc and Zr (Reimann and de Caritat, 2005). In our study, Ti was chosen as reference element.

(2) Geoaccumulation index

Geoaccumulation index (I_{geo}) is another indicator to assess the intensity of anthropogenic contaminants deposition on surface soil (Antoniadis et al., 2017), which is expressed as follows:

$$I_{geo} = log_2\left(\frac{C_{El}}{1.5C_B}\right) \qquad (2)$$

where C_{El} is the measured concentration of the TE in soil and C_B is the geochemical background value. The constant 1.5 allows us to analyze natural fluctuations in the content of a given substance in the environment and to detect very small anthropogenic influence. Geoaccumulation index is defined in Table 3.3.1 from Class 0 (I_{geo}=0, unpolluted) to Class 6 (I_{geo}>5, extremely polluted) (Barbieri, 2016).

Table 3.3.1 The degree of metal pollution in terms of seven enrichment classes

Class	Value	Soil quality
0	$I_{geo} \leq 0$	Uncontaminated
1	$0 < I_{geo} < 1$	Uncontaminated to moderately contaminated
2	$1 < I_{geo} < 2$	Moderately contaminated
3	$2 < I_{geo} < 3$	Moderately contaminated to heavily contaminated
4	$3 < I_{geo} < 4$	Heavily contaminated
5	$4 < I_{geo} < 5$	Heavily to extremely contaminated
6	$I_{geo} \geq 5$	Extremely contaminated

(3) Bioconcentration factor

Bioconcentration factor (BCF) is the soil-to plant element accumulation index calculated as follows:

$$BCF = C_P/C_S \qquad (3)$$

where C_P and C_S are element concentration in plant and soil (dw), respectively.

3.3.2.4 Human health risk assessment

The estimated daily intake (EDI) of TEs were determined based on both the content in the vegetables and the average consumption amount of the respective crop. The EDI was calculated as follows

$$EDI = \frac{\mathrm{DI} \times C_P \times \mathrm{F}}{\mathrm{BW}} \qquad (4)$$

where DI is the daily intake of vegetables, F is a factor (0.091) to convert fresh weight (fw) to dry weight (dw), C_P is the concentration of each pollutant in the crop (mg/kg, dw), and BW is body weight. According to the EFSA's Comprehensive Food Consumption Database, in Spain, the average consumption of lettuce and radish for adults are 0.045 and 0.022 kg/day fw, and for children are 0.027 and 0.009 kg/day fw, respectively). The average BW of Catalan male adult (20–65 years old) and children (4–9 years old) are 70 and 24 kg, respectively.

To evaluate the human health risk, the hazard quotient (HQ) was calculated as follows

$$HQ = \frac{EDI}{RfD} \quad (5)$$

where RfD is the maximum tolerable daily intake (μg/kg/day) of a specific element that does not result in carcinogenic effects for human beings, obtained from IRIS (2020). An $HQ > 1$ implies a potential risk to the population; otherwise, the consumer is safe.

Finally, the total hazard quotient (THQ) was calculated to assess the total risk of all chemicals to which an individual might be exposed, as the sum of the HQ of all the elements.

3.3.2.5 Data analysis

The experimental results were statistically evaluated using the SPSS v25 package (Chicago, IL, US). The comparison of the concentration of TEs between treatments was analyzed with an independent-samples t-test. Statistical significance was defined as $p < 0.05$.

3.3.3 Results and discussion

3.3.3.1 Soil and organic fertilizers

(1) Physicochemical properties

The physical and chemical parameters of soil and organic fertilizers are shown in Table 3.3.2. The soil sample used was collected before the study and constitutes 40% sand, 35% silt, 25% clay. The pH values of soil and organic fertilizers were in the same range (alkaline) which indicates that, after fertilization, pH would not result in the variation of speciation of TEs in soil. Nevertheless, the huge amount of organic matter in fertilizers was expected to combine liable TEs in the soil. In the short term, their incorporation would inhibit plant uptake of TEs from the substrate. However, in the long term, once the organic matter was decomposed, the absorbed TEs would be released into the soil solution again. Electrical conductivity (EC) values of organic fertilizers were significantly higher than soil which would affect soil microbial activities. USDA-NRCS (2014) reported that for substrate EC higher than 1 ds/m, the salinity would inhibit the growth of crop.

Table 3.3.2 Physical and chemical parameters of soil and organic fertilizer samples

	Soil	SS	OFMSW
pH	8.48	8.3	8.8
Electrical conductivity (dS/m)	0.240	2.56	7.47
Humidity (%)	1.33	72	39.9
Organic matter (%, dw)	1.27	55	43.9
Total Kjeldahl N (%, dw)	0.089	4.8	2.68
Ammoniacal-N (%, dw)	-	1.5	0.25
Olsen P (dw)	33 mg/kg	1.75%	1.01%
Ca (dw)	7014 mg/kg	5.7%	7.7%
Mg (dw)	362 mg/kg	0.73%	0.86%
K (dw)	344 mg/kg	0.52%	2.05 %

(2) Occurrence of trace elements

Table 3.3.3 summarizes the information of TEs in soil and organic fertilizers. The TEs level of studied soil was compliant with Catalan law 5/2017. According to EF results (i.e. EF$<$2), most soil TEs come from natural resources, excepted for Pb and Cu. The possible anthropogenic sources of Pb and Cu in studied area are industrial activities of surrounding plants and traffic activities (Margenat et al., 2020). The I_{geo} value of Ni, Pb and Cu was higher than 0 ($0 < I_{geo}$ (Ni)$< I_{geo}$ (Cu)$< 1 < I_{geo}$ (Pb)<2), indicating a gentle to moderate soil contamination caused by these elements.

In SS sample, the TE concentrations ranged from 0.51 mg/kg (Cd) to 978 mg/kg (Zn). While in OFMSW, the concentration ranged from 0.57 mg/kg (Cd) to 240 mg/kg (Zn). It should be noted that the concentration of Zn, Cr, Ni in SS were above the suggested value which may result in a damage to vegetables. Although TE concentration (dw) is high in SS, in our study applied SS containing a huge amount of water which would reduce the burden.

Table 3.3.3 The concentration and maximum acceptable amount of TE of soil and organic fertilizers (mg/kg, dw), as well as EF, I_{geo} value of studied soil.

	Zn	Pb	Cr	Cd	Ni	As	Cu
Soil	74.32 ±2.17	58.12 ±0.99	21.01±0.12	＜0.5	33.01±0.32	19.61±1.01	92.45±1.78 [a]
BV [a]	132	16	26	0.5	17	16	34
EF [b]	0.54	3.49	0.78	-	1.86	1.14	2.61
I_{geo} [c]	-1.5	1.27	-0.89	-	0.37	-0.34	0.85
RSV-soil [d]	170	60	400	2.5	45	30	-
SS	978.21±4.31	70.11±2.72	106.01±3.21	0.51±0.17	88.08±3.21	7.71±1.01	370.44±7.22
OFMSW	240.01±5.77	34.21±4.21	15.71±1.99	0.57±0.21	11.41±2.55	8.72±0.99	90.01±4.21
RSV-fertilizer [e]	800	120	100	1.5	50	40	300

[a] BV: Soil TEs background value of Barcelona (You et al., 2019).

[b] EF: Enrichment factor of soil.

[c] I_{geo}: Geoaccumulation Index of soil.

[d] RSV: Risk screening value for soil contamination in agricultural soil in Catalonia (Generalitat de Catalunya, 2017).

[e] RSV-fertilizer: Risk screening value of TEs in organic fertilizer for agriculture (PE-CONS 76/18, 2019; Saveyn and Eder, 2014).

(3) The effect of organic fertilization on soil trace elements

The amended soil samples were collected before each productive cycle, and the TE concentration is shown in Table 3.3.4. Compared with original soil, only Zn content steadily increased in the amended soil (original soil < 1st cycle soil < 2nd cycle soil) ($p < 0.05$). This may be caused by the high Zn concentration gap between soil and fertilizers. No significant difference was observed for other elements, which indicates that amending soil with SS and OFMSW did not increase TE amount, at least in terms of total concentration.

Table 3.3.4 Concentration of trace elements in original soil, fertilizer-amended soil in two consecutive productive cycles (mg/kg, dw).

	Original soil	Soil +SS 1st cycle	Soil +OFMSW 1st cycle	Soil +SS 2nd cycle	Soil +OFMSW 2nd cycle
Zn	74.32 ±2.17 [a]	80.28±3.17 [b]	81.78±1.17 [b]	89.21±1.71 [c]	90.21±2.01 [c]
Pb	58.12 ±0.99 [a]	57.99±1.21 [a]	57.22±2.01 [a]	57.88±2.01 [a]	58.01±1.91 [a]

Cr	21.01±0.12 ^{a}	21.01±0.91 ^{a}	21.21±2.01 ^{a}	21.00±0.37 ^{a}	20.19±0.77 ^{a}
Cd	＜0.5 ^{a}	＜0.5 ^{a}	＜0.5 ^{a}	＜0.5 ^{a}	＜0.5 ^{a}
Cu	92.45±1.78 ^{a}	91.77±3.77 ^{a}	92.11±2.17 ^{a}	91.74±3.22 ^{a}	91.99±2.91 ^{a}
Ni	33.01±0.32 ^{a}	34.01±2.01 ^{a}	33.78±2.11 ^{a}	33.21±3.22 ^{a}	32.01±2.79 ^{a}
As	19.61±1.01 ^{a}	19.77±2.11 ^{a}	19.09±2.11 ^{a}	19.88±2.78 ^{a}	19.41±2.77 ^{a}

3.3.3.2 Vegetable samples

(1) Vegetable growth parameters

Vegetable morphology and yield showed significant differences between different treatments (Table 3.3.5). For instance, lettuce carbohydrates ranged from 0.32% to 1.25%, and radish ranged from 7.27 to 31.66 g fw. Overall, fertilization with SS and CF results in similar vegetable morphology and crop production, however, were different in contrast with OFMSW treatment. One explanation for the agronomic difference between OFMSW versus other treatments may be N availability (Kiba and Krapp, 2016). Despite we supplied the same amount of Kjeldahl nitrogen to each plot, only inorganic N can be used by vegetables. Thus, the organic N, which is the predominant N form in OFMSW, needs 3-5 year to convert to plant-available form (Pang (2000). Scow et al. (1994) also reported a lag period after a transition from conventional to organic farming, where there were lower yields under organic farming treatments. This could be confirmed by the decreased yield gap between OFMSW and other treatments in the second cycle. Lettuce yield did not show a significant difference between first and second production cycle, while radish production is significantly reduced in the second cycle. During the second production cycle, the experimental area experienced several heavy rains, and radishes were completely submerged in rainwater, which may be an important reason for the observed yield reduction.

Table 3.3.5 Vegetables quality parameters. For same vegetables, different letters indicate a significant difference).

	Fresh weight (g)	Lipids (%)	Carbohydrates (%)	Total chlorophyll content (mg/cm^2)
Lettuce -CF 1st cycle	465.9 ± 195.1^{a}	0.063 ± 0.027^{a}	0.322 ± 0.186^{a}	0.018 ± 0.002^{a}

Lettuce-OFMSW 1st cycle	224.9 ±107.7 ^{b}	0.029 ± 0.023 ^{a}	1.254 ± 0.302^{b}	0.013 ± 0.001 ^{b}
Lettuce-SS 1st cycle	424.9 ± 107.7 ^{a}	0.064 ± 0.036 ^{a}	0.309± 0.206 ^{a}	0.020 ± 0.002 ^{c}
Lettuce-CF 2nd cycle	425.9 ± 121.1 ^{a}	0.151 ± 0.055 ^{b}	0.477± 0.428^{a}	0.018 ± 0.002 ^{a}
Lettuce-OFMSW 2nd cycle	326.3 ± 139.0 ^{c}	0.183 ± 0.062 ^{b}	1.088± 0.808^{b}	0.017 ± 0.002 ^{a}
Lettuce-SS 2nd cycle	461.0± 81.5 ^{a}	0.126 ± 0.031 ^{b}	0.415± 0.461^{a}	0.017 ± 0.002 ^{a}
Radish -CF 1st cycle	27.42 ±11.75^{a}	0.124± 0.089 ^{a}	1.308± 0.267 ^{a}	0.027 ± 0.003 ^{a}
Radish -OFMSW 1st cycle	17.17 ±12.37 ^{b}	0.026± 0.006 ^{a}	0.130± 0.114 ^{b}	0.024 ± 0.003 ^{b}
Radish -SS 1st cycle	31.66 ± 15.56 ^{c}	0.065 ± 0.048 ^{a}	0.373± 0.267 ^{b}	0.025 ± 0.003 ^{b}
Radish -CF 2nd cycle	10.02 ± 3.56 ^{d}	0.125± 0.053 ^{b}	1.428± 0.367 ^{a}	0.021± 0.002 ^{c}
Radish -OFMSW 2nd cycle	7.27 ± 4.56 ^{e}	0.178± 0.091 ^{b}	0.179± 0.114 ^{b}	0.019± 0.003 ^{d}
Radish -SS 2nd cycle	10.27± 2.96 ^{d}	0.191± 0.148 ^{b}	0.361± 0.313 ^{b}	0.022± 0.001 ^{c}

(2) Occurrence of trace elements

It is surprising that soil amendment with organic fertilizers significantly increased TE concentration in the edible part of both vegetables (Fig. 3.3.1), since no difference was shown in the total TE concentrations of the original soil and amended soil. This may suggest that the speciation of TEs were different between soil and fertilizer, and the concentration of bioavailable TEs was greater in fertilizers than in soil. For single fertilization, the treatment with OFMWS results in a highest TE accumulation in vegetables, followed by SS and CF. After repeated fertilization, the TEs concentration in vegetable grown with SS increased a lot, especially for Zn and As. In the second productive cycle, concentrations of Zn in lettuce and As in radish using SS fertilizer were higher than those using OFMWS. Compared to the results of two productive cycles, it is obvious that TE concentrations on the second productive cycle were higher than on the first one. This may result from (1) different planting timing: the second productive cycle was carried out in winter and the first one was in spring. In winter, more fuel is burned for city heating, which induces the release of large amounts of contaminants. Besides, winter is the rainy season in Barcelona, and wet deposition of air pollutants increase their concentrations in soil and plants; (2) relatively longer growth period: during this winter's second cycle, vegetables needed more time to reach commercial size (lettuce: 37 days in first cycle vs. 74 days in second; radish: 56 days in first vs. 120 days in second). Therefore, longer growth time means longer exposure to contaminants; and (3) the organic matter in SS and OFMSW from first experiment could be decomposed, and if undergo this process, the organic-bounded elements would be released and transformed to other species (e.g. water-soluble speciation) increasing the amount of bioavailable TEs in the second application period. This can be confirmed by the fact that the increase rate under chemical fertilizer treatment is less than the one under organic treatment.

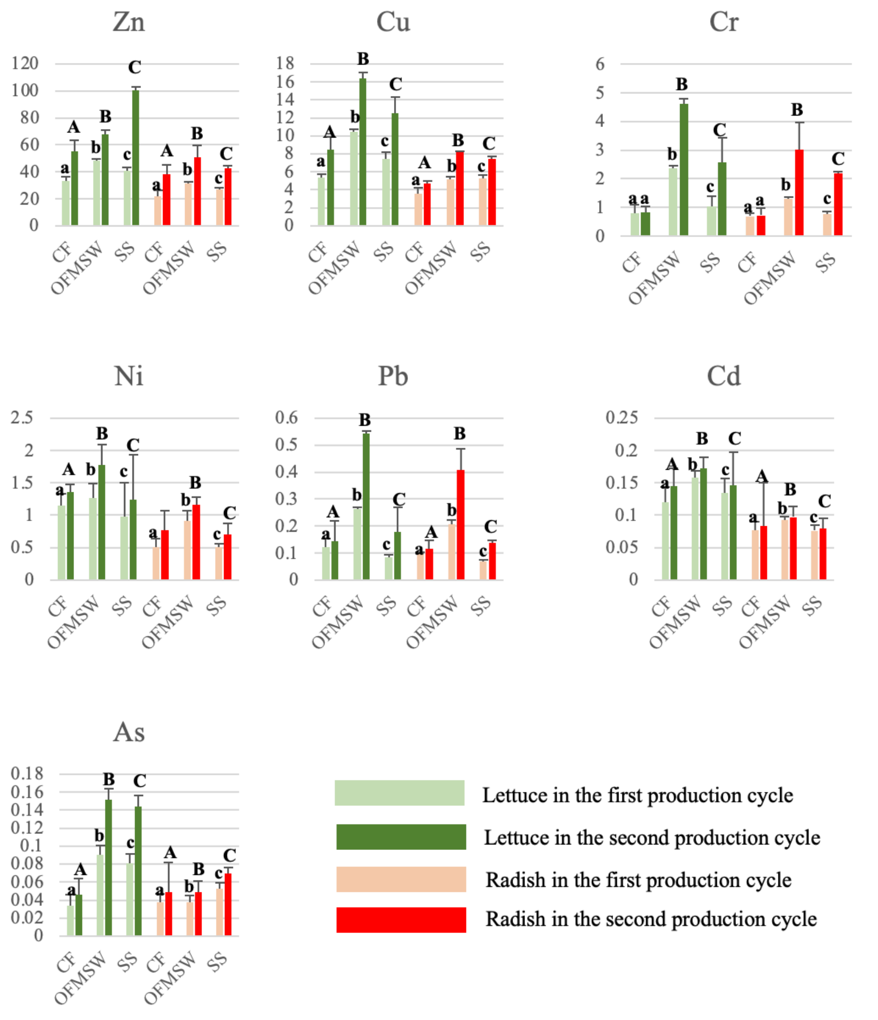


Fig. 3.3.1 The concentration of TEs in the edible part of vegetables under different amendments (dw, mg/kg). Green bars represent lettuce, and red bars represent radish. While the light color represents 1st productive cycle, dark color represents 2nd productive cycle. For the same vegetable, different letters indicate a significant difference ($p < 0.05$).

(3) Bioaccumulation factor of trace elements

The Bioaccumulation factor (BCF) values of TEs of lettuce and radish under different treatments are shown in Table 3.3.6. BCF of lettuce was always higher than radish for the same condition. The higher transpiration rates of lettuce facilitate element transport from soil to vegetable (Gupta et al., 2019). The highest BCF was found for Cd, however, this value is not extremely accurate, because in the substrate, the Cd concentration was below the LOQ. During calculations, 1/2 LOQ was used to represent the concentration in the soil. Nevertheless, there is no doubt that BCF of Cd was high. The high BCF value for Zn means that vegetables had a high capacity to absorb Zn from the matrix. Cd and Zn not only can easily be absorbed by lettuce and radish but also by other plants, such as maize (Cao et al., 2020) and peanuts (Gu et al., 2019). Compared with these two studies, the BCF value was higher in our study. This may result from a relatively long translocate routine for TEs from soil to the fruit of maize and peanut. During the transportation process, TEs could be bound to the cell wall or/and compartmented in the vacuole of other tissues (e.g. stem). Pb and As had the lowest BCF value (0.01-0.09 and 0.002-0.008) which indicates that, to some extent, lettuce and radish have a strong defence mechanism inhibitating the accumulation of Pb and As. The lower mobility of these two elements in the soil (Zhuang et al., 2013) could also explain this behaviour. Additionally, the insoluble compounds ($Pb(OH)_2$, $PbCO_3$, and $PbSO_4$) are the main Pb forms in soil (Cao, 2020), and so therefore it is difficult for Pb to be absorbed from soil and accumulated in vegetable tissue.

Table 3.3.6 Bioaccumulation factor (BCF) of trace elements

		Zn	Pb	Cr	Cd	Ni	As	Cu
CF-1st	Lettuce	0.446	0.002	0.038	0.483	0.035	0.002	0.059
	Radish	0.294	0.002	0.033	0.310	0.016	0.002	0.039
OFMSW-1st	Lettuce	0.593	0.005	0.113	0.631	0.038	0.005	0.113
	Radish	0.388	0.004	0.062	0.369	0.028	0.002	0.056
SS-1st	Lettuce	0.506	0.001	0.049	0.536	0.030	0.004	0.080
	Radish	0.300	0.001	0.037	0.306	0.015	0.003	0.057
CF- 2nd	Lettuce	0.293	0.002	0.039	0.579	0.041	0.002	0.092
	Radish	0.519	0.002	0.035	0.337	0.024	0.003	0.051

OFMSW- 2nd	Lettuce	0.388	0.009	0.220	0.687	0.054	0.008	0.178
	Radish	0.630	0.007	0.144	0.389	0.035	0.003	0.089
SS- 2nd	Lettuce	0.300	0.003	0.123	0.584	0.038	0.007	0.136
	Radish	0.477	0.002	0.105	0.319	0.021	0.004	0.081

3.3.3.3 Health risk assessment

The estimated daily intake (EDI) of TEs for adult and children were calculated according to the concentration in edible tissues and daily consumption. Fig. 3.3.2 shows the EDI of radish samples. Radish grown with OFMSW always trigger the highest TEs intake. During the first production cycle, EDI of OFMSW were 0.0011 and 0.0013 mg/kg/bw/day for adult and children, respectively. In the second production cycle, EDI of OFMSW have increased with values of 0.0018 and 0.0022 mg/kg/bw/day for adult and children, respectively.

Figure 3.3.3 shows the results for lettuce. After repeated amending soil with SS, the EDI of lettuce increased rapidly, the increased value was higher than OFMSW. In the second productive cycle, the value was twice higher than the first one. This was due to the significantly increasing amount of Zn in lettuce. According to Commission Regulation (EC) No 1881/2006, the maximum accepted levels of Pb and Cd in lettuce are 0.3 and 0.2 mg/kg (fw), respectively, whereas the value is 0.1 mg/kg (fw) for both metals in radish. Therefore, according to equation (4), for daily lettuce consumption, the maximum acceptable EDI value (mg/kg/bw/day) for Pb is 1.9×10^{-4} and 3.3×10^{-4} for adult and children, respectively; and the EDI value (mg/kg/bw/day) for Cd is 1.3×10^{-4} and 2.2×10^{-4} for adult and children, respectively. For radish, the maximum acceptable EDI of both elements are 3.1×10^{-5} and 3.7×10^{-5} mg/kg/bw/day for adult and child. In the present study, the highest EDI values for these two elements were both lower than the threshold.

Fig. 3.3.2 Estimated daily intake of radish samples

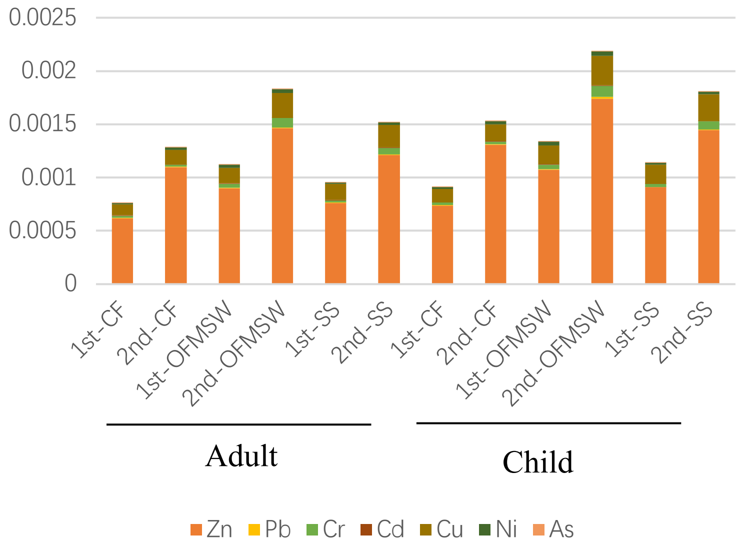
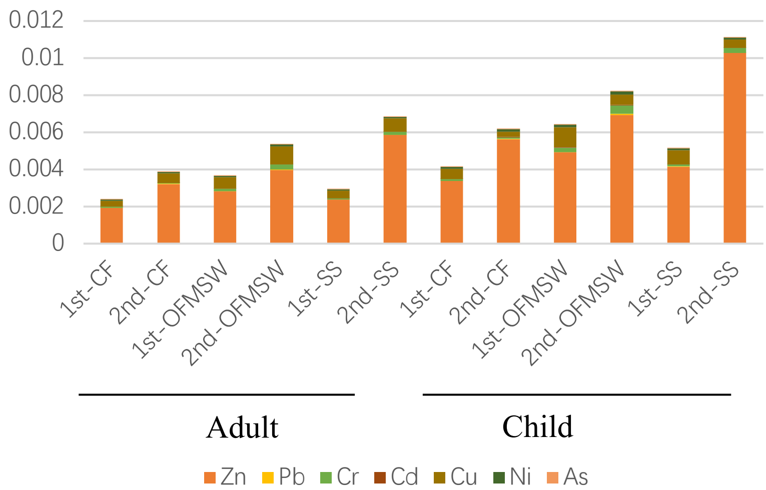


Fig. 3.3.3 Estimated daily intake of lettuce samples



HQ was calculated based on the EDI value and the oral reference dose for non-carcinogenic effects. Table 3.3.7 presents the health risk values for adults. HQ ranged from 1.22 x 10^{-5} (Cr in the first cycle of radish) to 2.95 x 10^{-2} (As in the second cycle of lettuce). For adult, vegetable safety was threatented mainly by Zn, Cd, As. Repeated soil amendment with CF, OFMSW and SS resulted in the THQ value increased 1.35, 1.52, 1.75-fold in lettuce leaves, and the values increased 1.42, 1.43, 1.37-fold in radish root. Table 8 summarizes the health risk values for children. Similarly to adults, HQ ranged from 1.22 x 10^{-5} (Cr in first cycle of radish) to 5.16 x 10^{-2} (As in second cycle of lettuce). According to the results, when the human risk is assessed, Pb should to be taken into account due to their high HQ values. Repeated soil amendment with CF, OFMSW and SS resulted in the THQ value increased 1.24, 1.67, 1.97-fold in lettuce leaves, whereas the value increased 1.37, 1.43, 1.37-fold in radish root. However, the THQ value was <1 for all the samples, indicating that no risk should be assumed.

Table 3.3.7 Values of hazard quotients (HQ and THQ) for vegetables considering the different treatments for adults

	Lettuce						Radish					
	CF (1st)	OFMSW (1st)	SS (1st)	CF (2nd)	OFMSW (2nd)	SS (2nd)	CF (1st)	OFMSW (1st)	SS (1st)	CF (2nd)	OFMSW (2nd)	SS (2nd)
Zn	6.44×10^{-3}	9.36×10^{-3}	7.90×10^{-3}	1.07×10^{-2}	1.32×10^{-2}	1.96×10^{-2}	2.07×10^{-3}	2.99×10^{-3}	2.54×10^{-3}	3.66×10^{-3}	4.87×10^{-3}	4.04×10^{-3}
Pb	2.02×10^{-3}	4.39×10^{-3}	1.41×10^{-3}	2.42×10^{-3}	9.06×10^{-3}	2.99×10^{-3}	7.90×10^{-4}	1.70×10^{-3}	5.58×10^{-4}	9.57×10^{-4}	3.33×10^{-3}	1.13×10^{-3}
Cr	3.07×10^{-5}	9.29×10^{-5}	4.01×10^{-5}	3.22×10^{-5}	1.80×10^{-4}	1.01×10^{-4}	1.22×10^{-5}	2.50×10^{-5}	1.46×10^{-5}	1.39×10^{-5}	5.76×10^{-5}	4.19×10^{-5}
Cd	7.07×10^{-3}	9.23×10^{-3}	7.84×10^{-3}	8.46×10^{-3}	1.01×10^{-2}	8.54×10^{-3}	2.22×10^{-3}	2.64×10^{-3}	2.19×10^{-3}	2.41×10^{-3}	2.78×10^{-3}	2.28×10^{-3}
Cu	7.87×10^{-4}	1.52×10^{-3}	1.08×10^{-3}	1.24×10^{-3}	2.39×10^{-3}	1.83×10^{-3}	2.58×10^{-4}	3.71×10^{-4}	3.74×10^{-4}	3.37×10^{-4}	5.84×10^{-4}	5.33×10^{-4}
Ni	3.37×10^{-3}	3.71×10^{-3}	2.88×10^{-3}	3.98×10^{-3}	5.20×10^{-3}	3.64×10^{-3}	7.35×10^{-4}	1.30×10^{-3}	7.23×10^{-4}	1.11×10^{-3}	1.66×10^{-3}	1.01×10^{-3}
As	6.63×10^{-3}	1.75×10^{-2}	1.58×10^{-2}	9.06×10^{-3}	2.95×10^{-2}	2.80×10^{-2}	3.15×10^{-3}	3.55×10^{-3}	5.06×10^{-3}	4.19×10^{-3}	4.69×10^{-3}	6.63×10^{-3}
THQ	2.63×10^{-2}	4.58×10^{-2}	3.69×10^{-2}	3.59×10^{-2}	6.96×10^{-2}	6.47×10^{-2}	9.63×10^{-3}	1.26×10^{-2}	1.15×10^{-2}	1.32×10^{-2}	1.80×10^{-2}	1.57×10^{-2}

Table 3.3.8 Values of hazard quotients (HQ and THQ) for vegetables considering the different treatments for children.

	Lettuce						Radish					
	CF (1st)	OFM SW (1st)	SS (1st)	CF (2nd)	OFM SW (2nd)	SS (2nd)	CF (1st)	OFM SW (1st)	SS (1st)	CF (2nd)	OFM SW (2nd)	SS (2nd)
Zn	1.13×10^{-2}	1.64×10^{-2}	1.38×10^{-2}	1.88×10^{-2}	2.31×10^{-2}	3.42×10^{-2}	2.46×10^{-3}	3.57×10^{-3}	3.04×10^{-3}	4.37×10^{-3}	5.81×10^{-3}	4.83×10^{-3}
Pb	2.30×10^{-2}	6.97×10^{-2}	3.01×10^{-2}	2.42×10^{-2}	1.35×10^{-2}	7.57×10^{-2}	9.42×10^{-3}	2.03×10^{-3}	6.66×10^{-4}	1.14×10^{-3}	3.98×10^{-3}	1.35×10^{-3}
Cr	5.38×10^{-5}	1.63×10^{-4}	7.01×10^{-5}	5.64×10^{-5}	3.15×10^{-4}	1.77×10^{-4}	1.59×10^{-5}	2.98×10^{-5}	1.75×10^{-5}	1.65×10^{-5}	6.88×10^{-5}	5.00×10^{-5}
Cd	1.24×10^{-2}	1.61×10^{-2}	1.37×10^{-2}	1.48×10^{-2}	1.76×10^{-2}	1.49×10^{-2}	2.65×10^{-3}	3.15×10^{-3}	2.61×10^{-3}	2.87×10^{-3}	3.32×10^{-3}	2.72×10^{-3}
Cu	1.38×10^{-3}	2.66×10^{-3}	1.89×10^{-3}	7.25×10^{-4}	1.40×10^{-3}	1.07×10^{-3}	3.08×10^{-4}	4.43×10^{-4}	4.46×10^{-4}	4.03×10^{-4}	6.97×10^{-4}	6.36×10^{-4}
Ni	5.91×10^{-3}	6.49×10^{-3}	5.03×10^{-3}	6.96×10^{-3}	9.10×10^{-3}	6.36×10^{-3}	8.77×10^{-4}	1.55×10^{-3}	8.62×10^{-4}	1.32×10^{-3}	1.98×10^{-3}	1.20×10^{-3}
As	1.16×10^{-2}	3.07×10^{-2}	2.76×10^{-2}	1.59×10^{-3}	5.16×10^{-2}	4.91×10^{-2}	4.11×10^{-3}	4.23×10^{-3}	6.03×10^{-3}	4.13×10^{-3}	4.23×10^{-3}	6.03×10^{-3}
THQ	6.56×10^{-2}	1.42×10^{-1}	9.22×10^{-2}	8.14×10^{-2}	2.38×10^{-1}	1.82×10^{-1}	1.15×10^{-2}	1.50×10^{-2}	1.37×10^{-2}	1.44×10^{-2}	2.01×10^{-2}	1.68×10^{-2}

3.3.4 Conclusion

In our study area, soil was lightly contaminated with Pb and Cu. Amending soil with organic fertilizers did not significantly influence the total TEs concentration in soil. However, TEs accumulation in the edible part of vegetables increased with the application of organic fertilizers, and this trend would be enhance after succesive fertilization cycles. The vegetable safety was threatened mainly by As, Cd and Zn. Child were more sensitive than adult to the non-carciofenic risk of vegetable comsuption, but no adverse effect was observed.

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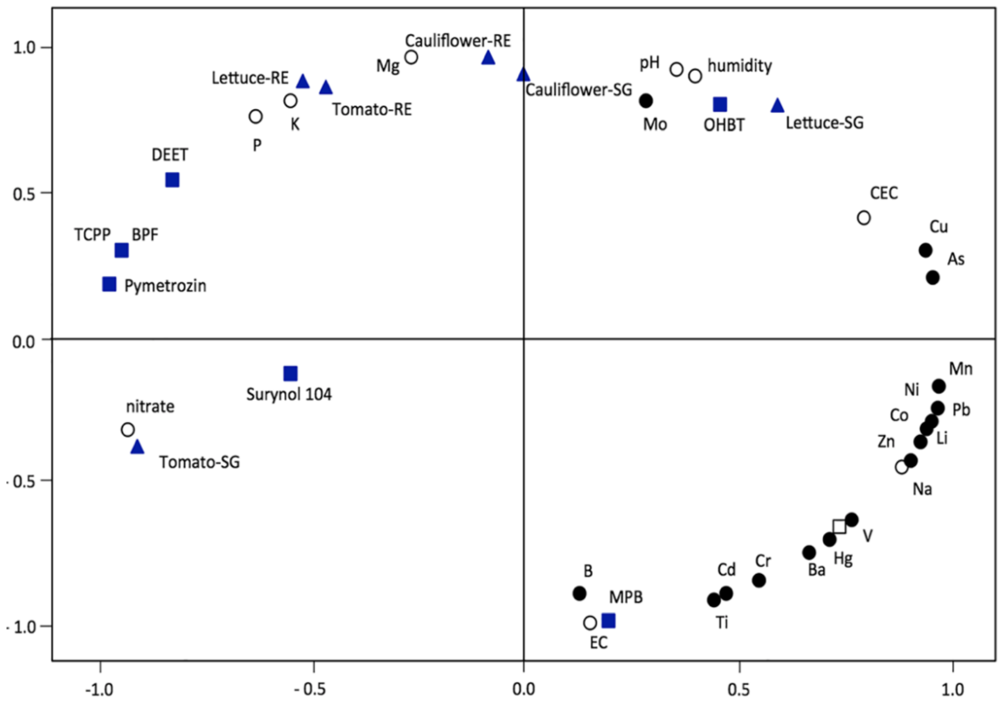
3.4 Topic 4: Soil contamination in peri-urban area of Barcelona and phytotoxicity assessment

This topic monitors the chemical characterization of soil surroundings on peri-urban agriculture in the Baix Llobregat Agrarian Park (BLAP) in the city of Barcelona, and uses two bio-monitoring indexes to assess soil pollution.

ased on the article:

Chemical characterization and phytotoxicity assessment of peri-urban soils using seed germination and root elongation tests.

Environmental Science and Pollution Research, 26 (33), 34401-34411.



Chemical characterization and phytotoxicity assessment of peri-urban soils using seed germination and root elongation tests

Abstract

The peri-urban soil is exposed to pollutants because of its proximity to the city, which may influence the quality of agricultural products. In this study, the occurrence of 16 trace elements (TEs), 16 polycyclic aromatic hydrocarbons (PAHs), and 33 contaminants of emerging concern (CECs) was analyzed in two soil sites of the peri-urban area of Barcelona (Spain) (S2 and S3) and a pristine site (S1). Levels of Pb (S2 164 and S3 150 mg/kg) are around 2.5 times higher than the guideline values. Values for Cu (178 mg/kg) in S2 are 1.8-fold higher, whereas for Zn, levels are slightly above the threshold in S2 (208 mg/kg) and S3 (217 mg/kg). The total concentrations of PAHs are significantly below the limits: 24 ng/g dw (S1), 38 ng/g dw (S2), 49 ng/g dw (S3), whereas only some CECs are detected with low concentrations. We also developed a simple and rapid method to assess soil pollution. Here, we use two plant growth indexes (seed germination rate and root elongation at the initial stage) of three seeds (lettuce, tomato, and cauliflower) to assess soil chemical contamination on agriculture. In the peri-urban soil, the concentration of Pb was 2.5 times higher than the guideline values, whereas for Cu and Zn, values were slightly above their limits, while only few PAHs and CECs were detected. Results for principal component analysis suggest that root elongation is a more sensitive measurement endpoint than germination rate, especially for lettuce. The germination rate of tomato relied on the nitrate in the soil and decreased sharply in the site with pollution of Cu and As. Under the specific conditions of this study, cauliflower should not be recommended to assess environmental pollution due to its low sensitivity to pollutants. In conclusion, this is a low-cost, simple, and rapid method for evaluating the effects of chemical pollution of agriculture soils on seed growth.

Keywords: Peri-urban soil · Trace elements · Polycyclic aromatic hydrocarbons · Contaminants of emerging concern · Seed germination rate · Root elongation

3.4.1 Introduction

The expansion of large cities has increased the demand for food (Ferreira et al. 2018; Tan et al. 2005). Among lots of solutions, peri-urban agriculture seems to be a favorable choice, since it minimizes the carbon dioxide footprint in terms of food transportation, helps recycle urban reclaimed water and biosolids, and furthermore offers fresher food products to the adjacent city (Duvernoy et al. 2018; Zasada 2011). However, compared with traditional rural agriculture, the peri-urban ecosystem inevitably faces numerous additional pressures and tensions for two interacting reasons. Firstly, a number of contaminations from large infrastructures (e.g., solid waste incineration, airports, highways, industrial emissions) are discharged into the peri-urban area. Secondly, large-scale vegetable production brings intensive use of fertilizers, pesticides, and reclaimed water, which also contributes to an important source of pollution (Margenat et al. 2017, 2018).

Soil is an important medium for plant growth; however, once it is polluted, some contaminants can be uptaken by crops and furthermore threaten human health. Therefore, when promoting peri-urban agriculture, it is quite necessary to consider the extent of soil contamination.

Among numerous classes of pollutants posed to the peri-urban soil (inorganic and organic), trace elements (TEs) have been monitored over decades years, since they are closely related to urban development. For example, Kabata-Pendias (2011) observed that from 1977 to 1997, in Warsaw (Poland), the concentrations of Li, Ni, Zn, Cr, Pb, Ba, Sr, and Fe in soil have increased sharply due to the extensive use of vehicles. Huang et al. (2018) reported that compared with background levels, the concentrations of many TEs in peri-urban soil increased significantly, especially Pb, with levels even higher than the Chinese legislative limits. In addition, Hu et al. (2018) revealed that surrounding land use and agricultural activities have a significant influence on TEs accumulation in peri-urban soil.

Polycyclic aromatic hydrocarbons (PAHs) are a large group of mutagenic and carcinogenic compounds with two or more fused aromatic rings. It was proved that marked increases of PAHs in soil following industrial revolution are mainly influenced by human activities, especially the incomplete combustion of organic materials in the urban area (Kim et al. 2018; Wilcke 2007), such as industrial production, residential heating, power generation, and vehicular emissions (Hussain and Hoque 2015; Moore et al. 2015; Riaz et al. 2019). Peng et al. (2016) reported that the mean concentrations of total 16 PAHs in suburban and rural soils of Beijing were 322 and 219 ng/g respectively, and the major sources of PAHs in these soils were coal and biomass combustion.

Besides traditional pollutants, contaminants of emerging concern (CECs), a diverse group of largely unregulated chemicals, have become another major concern for the scientific community and regulatory agencies (Du et al. 2014; Fairbairn et al. 2016). Because some of CECs exhibit toxic or endocrine disruption potential to biota at the relatively low concentration, such as di(2-ethylhexyl)phthalate, triclosan and propylparaben (Herrero et al. 2012), and others persist, could accumulate and eventually biomagnify in higher trophic level species (e.g., nonyphenol and fire retardants). Wu et al. (2014) reported that pharmaceutical and personal care products (PPCPs) were detected in 8 vegetables irrigated by reclaimed water without and with a fortification of PPCPs at 250 ng/L, and the concentrations in edible tissues were in the range of 0.01–3.87 and 0.15–7.3 ng/g (dry weight), respectively. CECs are continuously discharged into the environment from domestic and industrial sewage systems and affected by the level of urbanization and industrial point sources, even the concentration showed a clearly increasing pattern along the rural-suburban-urban gradient (Peng et al. 2016).

To date, the effects of TEs, PAHs, and CECs on peri-urban agriculture have been researched separately (Da Silva et al. 2017; Pan et al. 2014), but the knowledge about the co- influence of these compounds is scarce (Margenat et al. 2018; Marquès et al.

2017). When identifying this influence along with city activities on peri-urban soil, it is infeasible to test the toxicity of every chemical and their mixtures to all species. Thus, bio-monitoring strategies are becoming more used (Bagur-González et al. 2011; Li et al. 2017). As part of the battery of bioassays, seed germination and root elongation have been well developed and recommended by many regulatory agencies, because of the short experimental cycle and cost savings (Luo et al. 2018; Zaccheo et al. 2009). A previous study shows that germination was higher in seeds watered with irrigation waters than with distilled water, probably because the higher concentration of nutrients in the irrigation waters that would help break dormancy to facilitate seed germination (Margenat et al. 2017).

Given the facts mentioned above, this study aimed to develop a simple and rapid method to assess the effect of soil surroundings on peri-urban agriculture in the Baix Llobregat Agrarian Park (BLAP) in the city of Barcelona. To this end, we evaluated two plant growth indexes (seed germination rate and root elongation) under different soil environments (from peri-urban area and a pristine site). Here we present the different responses of three seeds: lettuce (*Lactuca sativa* L. cv. Quattro Stagioni), cauliflower (*Brassica cretica* L. cv. Maravilha), and tomato (*Solanum lycopersicum* L. cv. Marmande). They were selected because they represent three types of vegetables: leafy crop, inflorescence, and berry, which have been recommended by the US Food and Drug Administration and the Organization for Economic Cooperation and Development (OECD 2006) for bioassays and phytotoxicity tests. In addition, these three vegetables are popular in the Mediterranean diet.

3.4.2 Methods

3.4.2.1 Sampling site description

The study area (Fig. 3.4.1) is located in the delta and low valley of the Llobregat River (NE Spain) where adjoins Barcelona. In this region, based on the gradient of the impact of industrial, urban, and agricultural activities, three sites were chosen. Site 1 (S1) is an organic farming area irrigated by a drip system, manure-amended, relatively far away from Barcelona, and protected from urban pollution, while site 2 (S2) and site 3 (S3) are located in the peri-urban area and furrow irrigated with treated wastewater from the Llobregat River. Compared with S2, which has car traffic pollution, S3 is mainly influ- enced by the adjacent airport.

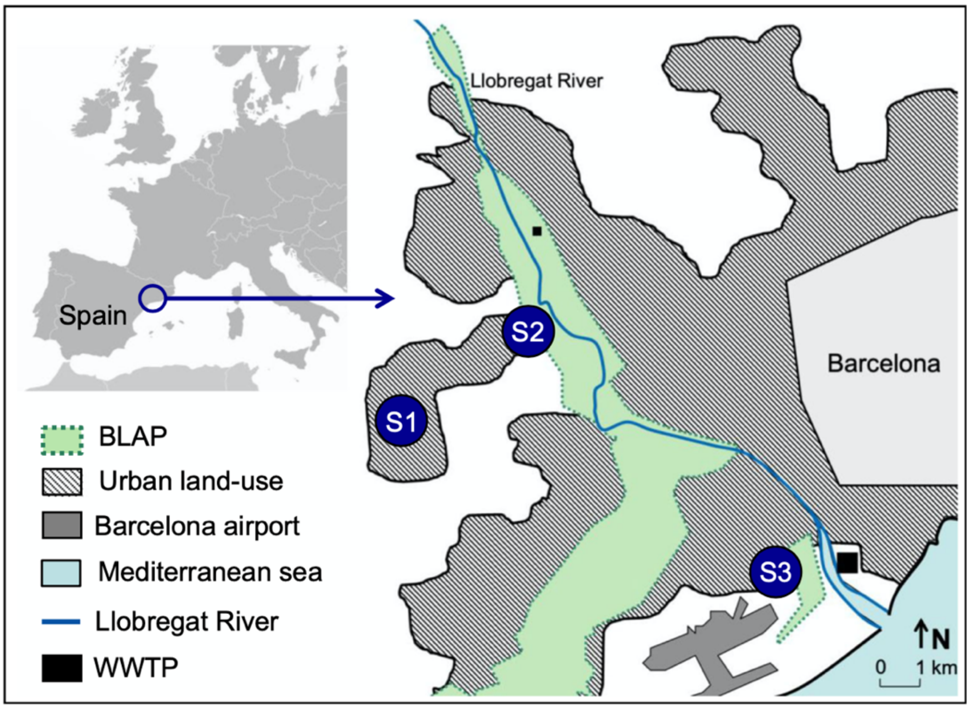


Fig. 3.4.1 Map of the agricultural area with sampling points. S1 is an organic farming area irrigated by a drip system; S2 and S3 are furrow irrigated with different treated wastewater from the Llobregat River

3.4.2.2 Sampling strategy

All soil samples were collected from the surface (0 - 20 cm depth) of three different study sites. A composite soil sample was obtained from five subsamples in each farm plot. Soil samples were sieved through a 2.0-mm mesh and stored at - 20 °C. According to the USDA (1987) classification, soil samples from farm plots S1 and S2 were loamy sand, while the soils from S3 was sandy.

3.4.2.3 Analysis procedure

(1) Chemical analysis

Soil pH was determined in the suspension with a soil to water ratio of 1:2.5 using a pH meter, and electrical conductivity was measured in the saturation paste extract. Cation exchange capacity was determined by saturation with sodium acetate solution, replacement of the absorbed sodium with ammonium, and determination of displaced sodium by flame atomic absorption spectrometry. The concentration of $NaHCO_3^-$ extractable P was measured by the procedures developed by Olsen as described in Page et al. (1982). The soil samples were analyzed for nitrate as nitrate nitrogen (NO_3-N) by Hach-Lange spectrophotometer (DR 1900 Portable Spectrophotometer), and the NH_4Ac (CH_3COONH_4)-extractable speciation of K, Ca, Mg, and Na was determined by inductively coupled plasma optical emission spectrometry (Thermo Scientific, iCAP 6500 ICP-OES).

To determine the concentration of total trace elements, soils were analyzed by the method published by EPA (1994). Briefly, 1 g dried and homogenized soil was digested with 4 mL HNO_3 (concentrated HNO_3 to ASTM type I water ratio of 1:1, v:v) and 10 mL HCl (concentrated HCl to ASTM type I water ratio of 1:4, v:v) on a hot plate at 85 °C around 30 min, then transferred the mixture to 100 mL volumetric flask, and diluted to volume with reagent water. After centrifuging at 3756 × g for 20 min, 10 mL supernatant was diluted 5 times again, and the solution was measured using inductively coupled plasma mass spectrometry (Thermo Scientific, xSeries 2 ICP-MS).

According to the method published by Chamorro et al. (2013), the extraction of PAHs in soil was performed on a PSE-One (Applied Instruments, USA) by duplicate. Briefly, 3 g of freeze-dry sediment was extracted with n-hexane/acetone 1:1. The extraction conditions were 110 °C, 140 bar, and 3 cycles with solvent mixture of 15 min each. The cleanup of the extracts was carried by adsorption chromatography on a glass column packed with 5 g of neutral alumina (activated at 400 °C, deactivated with 3% water) and anhydrous sodium sulfate (top). Then, different fractions were eluted: FI (10 mL n-hexane), FII (10 mL n-hexane/ ethyl acetate (9/1, v/v)), and FIII (10 mL n-hexane/ethyl acetate (1/1)). For PAH determination, FII was analyzed in a Bruker Scion 436GC SQ apparatus (Bruker Daltonics Inc., Billerica, MA, USA) using the following: splitless injection (hexane, 290 °C; the purge valve was activated 50 s after the injection); helium carrier gas (0.6 mL/min); a 20 m × 0.18 mm i.d. column with a

0.18μm coating of TRB-5MS stationary phase (from Teknokroma, Barcelona, Spain); a column temperature program consisting of 1.20 min at 60 °C, a 14 °C/min ramp up to 200 °C, a 7.5 °C/min ramp up to 300 °C, and 10 min at 300 °C; transfer line and ion source temperatures of 280 °C and 290 °C, respectively; data acquisition in full-scan mode from 50 to 500 amu, with 6.2 min of solvent delay; and data processing by Workstation 8 software.

Soil CEC extraction was adapted from a slightly modified method (Xu et al. 2008). Briefly, 5 g soil, homogenized and sieved through 2.0 mm mesh, was mixed with 5 mL acetone/ ethyl acetate (1:1, v:v) solvent in 50 mL of screw Teflon lined cap centrifuge tube, sonicated at 42 kHz for 15 min, centrifuged at 8452 × g for 10 min, and decanted the supernatant. The soil was extracted three additional times with 4 mL, 5 mL, and 4 mL of solvent. The supernatants were combined and were nitrogen-evaporated in a water bath at 40 °C to about 1 mL. Then, the concentrated solution was re-dissolved in 500 mL de-ionized water, and the pH was adjusted to 3 by concentrated sulfuric acid. Then, the extraction was loaded onto previously conditioned SPE cartridges (STRATA X, 100 mg, 6 mL). The cartridge was eluted with 10 mL of ethyl acetate and was dried by nitrogen for 20 min to 250 μL. Besides, 37.25 ng of triphenylamine (TPhA) was added as an internal standard. Finally, a 50-μL aliquot was analyzed by GC–MS/ MS without derivatization, and another 50 μL aliquot was analyzed derivatized with 10 μL of MTBSTFA.

The LODs and LOQs were calculated for each analyte as three and ten times the signal from the baseline noise (S/N ratio), respectively, and are provided in the tables. For calculation, levels below the LOQ and LOD were replaced by 1⁄2 LOQ and 1⁄2 LOD, respectively.

(2) Seed germination and root elongation tests

The experiment was designed based on the guideline issued by Environmental Protection Agency (EPA 712-C-96-154) about seed germination/root elongation toxicity test and ISO 11269-1 root growth test. The seed species assayed were let- tuce (*Lactuca sativa* L. cv. Quattro Stagioni), cauliflower (*Brassica cretica.* cv. Maravilha), and tomato (*Solanum lycoperscum*. cv. Marmande). Seeds for the experiments were purchased from a local garden material store. Before being tested for bioassays, their germination potential was examined under 24 ± 2 °C in the dark for 2 days. The germination rate for all tested seeds was over 90% which guaranteed the viability of the seed.

Soil was air-dried, mixed, and grounded to fine powder (< 2 mm). Then, 30 g of soil per site was weighed into a 100 × 15mm Petri dish and wetted with deionized water to reach 80% of its total water-holding capacity.

As for seeds, at first, they were sterilized with 2.5% sodium hypochlorite for 15 min and carefully washed with distilled water. Ten clean seeds were evenly put, at least 0.5 inches from the edge, on the surface of the soil per dish. Then, cover the dish to avoid moisture evaporation. Subsequently, all dishes were randomly placed on the lab workbench at 24 ± 2 °C in darkness for germination. According to the EPA guide- line, germination means the resumption of active growth by an embryo. Moreover, as defined by Finkelstein et al. (2008), germination is the initial emergence of the radicle from the seed coat. The primary root should attain a length of 5 mm for the seed to be counted as having germinated. When at least 65% of seeds of control have germinated and developed roots that are at least 20 mm long, germination experiment concludes, and germination rate could be calculated. In our case, although tomato, lettuce, and brassica vary in their germination time in our control soil (30 to 45 h), all of them germinated within 48 h. Then, these seedlings were put under fluorescent lamp for 16:8 h light to dark cycles for the next 72 h for root to continue growing. Five replicates of each treatment were tested.

After the initial 48 h, in each dish, the number of acceptable seedlings was counted and divided by the total number of seeds added (10) to calculate the germination rate. At the end of the experiment (3 days later), all seedlings were pulled out and the root elongation was measured which is defined as the length from the tip to radicle.

3.4.2.4 Statistical analysis

The experimental data were statistically evaluated by SPSS v. 24 package (Chicago, IL, USA). One- or multi-way analysis of variance (ANOVA) and Pearson's correlation analysis were performed for multiple comparisons or for analyzing interactive effects between different factors. Principal component analysis (PCA) was conducted on the concentrations of TEs, PAHs, CECs, and agronomical indexes. Varimax rotation was applied because orthogonal rotation minimizes the number of variables with a high loading on each component and facilitates the interpretation of results. Statistical significance was defined as $p < 0.05$.

3.4.3 Result and discussion

3.4.3.1 Conventional soil quality parameters

Table 3.4.1 shows the soil characteristics from the different sites. The driest soil is from S3, which is significantly different from soils from S1 and S2 ($p < 0.05$), probably due to different soil textures. According to the USDA (1987) classification, the soil in S3 is sandy, while soils in S1 and S2 are loamy sand. It is well known that sandy soil with unstable structure has less capacity for holding soil water and fertilizer than sandy loam. Besides, high electrical conductive values were detected in all soil sites. Margenat et al. (2017) reported that in this area, treated wastewater has high conductivity, which may bring the high CE level in soil irrigated by it (S2, S3). As for S1, owing to long-term manure fertilization, the CE in soil inevitably increases. This is also consistent with the high values of N, P , and K.

Table 3.4.1 General quality parameters in the studied soils. Mean ± SD (N = 3). Different letters indicate significant differences among sites

	S1	S2	S3
Humidity 105°C (%)	$46±1^{a}$	$64±3^{b}$	$32±2^{c}$
pH	$7.61 ± 0.01^{a}$	$7.74 ± 0.07^{b}$	$7.50 ± 0.40^{c}$
Electrical conductivity (µS/cm)	$2331 ± 17^{a}$	$2198 ± 23^{b}$	$5010 ± 41^{c}$
Nitrate (mg/kg)	$9.0 ± 0.7^{a}$	$2.0 ± 0.3^{b}$	$10.0 ± 1.1^{c}$
Phosphorous (mg/kg)	$64±4^{a}$	$35±2^{b}$	$7±3^{c}$
Potassium (mg/kg)	$375 ± 21^{a}$	$346 ± 19^{a}$	$297±9^{b}$
Calcium (mg/kg)	$2984 ± 23^{a}$	$6561 ± 21^{b}$	$6422 ± 43^{c}$
Magnesium (mg/kg)	$379 ± 22^{a}$	$360 ± 27^{a}$	$135 ± 13^{b}$

Sodium (mg/kg)	32±2^{a}	136±3^{b}	152±7^{c}
Cation exchange capacity (ms/cm)	7.5 ± 0.2^{a}	9.7 ± 0.7^{b}	8.0 ± 1.2^{ab}
Texture	Sandy loam	Sandy loam	Sandy

3.4.3.2 Occurrence of trace elements in soils

The concentration of TEs (16 out of the 58 elements analyzed) in the soils and the maximum acceptable values in agricultural soil established by the Catalan Law 5/2017 in accordance with the Spanish Royal Decree 9/2005 are listed in Table 3.4.2. The TEs detected at the highest value in all soils were Mn (361 - 494 mg/kg), Zn (132 - 217 mg/kg), and Ti (184 - 458 mg/kg). Mn and Zn as essential micronutrients participate in the structure of photosynthetic proteins and enzymes in plants and have a stimulating effect on auxin promoting coleoptile growth. However, once their concentrations in soil exceed the specific thresholds, they would also damage plants like other pollutants. In the peri-urban area, due to the high intensity of industrial activities, soil often contains high levels of Mn and Zn. In our studies, Mn and Zn levels in S2 and S3 soil are close or even above the regulated level values, and the result is consistent with the abundance of Mn (103 - 13,584 mg/kg) and Zn (23 - 3770 mg/kg) reported in soil from other Baix Llobregat sites (Zimakowska-Gnoińska et al. 2000). Therefore, it is imperative to control the sources of these two elements to avoid the negative effect, whereas the high abundance of Ti is consistent with geogenic origin. The content mainly depends on the parent rock and the degree of differentiation. S3 soil was mainly derived from shallow sea sediments that generally contain more Ti than the other two soil sites developed from granite (Boström et al. 1973).

Table 3.4.2 Concentrations of TEs in three sites and the limit value according to the Catalan soil law (mg/kg, dw). Mean ± SD (N = 3). Different letters indicate significant differences among sites. Number in bold indicates that the value exceeds the limit

	S1	S2	S3	Threshold value
Ti	184 ± 15^{a}	260 ± 22^{b}	**458 ± 21^{c}**	390
V	46±7^{a}	61±5^{a}	71 ± 2^{b}	100
Cr	26±2^{a}	66±4^{b}	117 ± 12^{c}	400

Mn	361 ± 9^{a}	494 ± 18^{b}	471 ± 15^{b}	500
Co	7±4^{a}	10±5^{a}	10 ± 3^{a}	25
Ni	17±3^{a}	34±5^{b}	33 ± 5^{b}	45
Cu	34±8^{a}	**179 ± 21^{b}**	91 ± 12^{c}	100
Zn	132±7^{a}	**208 ± 11^{b}**	**217 ± 12^{b}**	200
As	16±1^{a}	**38±2^{b}**	26 ± 1^{c}	30
Ba	86±3^{a}	305±3^{b}	**510 ± 7^{c}**	500
Pb	16±1^{a}	**164±5^{b}**	**150 ± 8^{b}**	60
Cd	0.5 ± 0.1^{a}	0.7 ± 0.1^{b}	1.2 ± 0.1^{b}	2.5
B	92±7 ^{a}	81±8 ^{a}	101 ± 3 b	-
Li	11±2 ^{a}	28±1 ^{b}	28 ± 3 ^{b}	-
Mo	2.0 ± 0.2^{a}	2.0 ± 0.7^{a}	1.9 ± 0.4^{a}	3.5
Hg	0.01 ± 0.01^{a}	0.37 ± 0.01 ^{b}	0.60 ± 0.01^{c}	2

The total average concentration of TEs per site was as follows: 143 mg/kg (S3), 121 mg/kg (S2), and 64 mg/kg (S1). ANOVA test showed that soil from S1 was less contaminated by many trace elements (i.e., Cu, Pb, Zn) than either of soil in peri-urban (S2, S3, $p < 0.05$). In fact, according to the regional decree (Generalitat de Catalunya, 2017), the levels of all trace elements in S1 were lower than the threshold. In the S2 soil, the concentrations of Cu, Zn, As, and Pb exceed the standard guideline, while in the S3 soil, Zn, Pb, and Ti concentrations are higher than the norm. This fact may be explained by the different fertilization method, traffic pressure, and industrial inputs in each site. In S1, an organic amendment was used for soil fertilization, whereas in S2 and S3, inorganic fertilizer is commonly employed. Alloway (2012) reported that the application of phosphate fertilizer generally gives rise to the high concentration of most metal(loid)s such as As, Cd, and Zn. In addition, a large concentration of Pb was found in the S2 and S3 partly derived from legacy leaded automobile exhaust emissions in the urban area, and partly result from the irrigation water. Cabeza et al. (2012) reported that the value of Pb in irrigation water of studied area exceeded the maximum allowable concentration. Moreover, Nziguheba and Smolders (2008) found that phosphate fertilizers are the main source of increased Pb levels in cropped soils. High Zn levels in

S2 and S3 are associated with traffic density since Zn sources include, among others, brake linings and rubber tires (Councell et al. 2004). Moreover, Zn has been used as a tire-wear particle tracer (Amato et al. 2011; Harrison et al. 2012).

3.4.3.3 Occurrence of PAHs in soils

Table 3.4.3 shows the 16 PAHs in soil samples and the maximum concentration of PAHs allowed by the Catalan legislation (Busquet 1997). Apparently, all of them are significantly be- low the limit values. The total concentrations of PAHs in detected soil were as follows: 24 ng/g dw (S1), 38.4 ng/g dw (S2), and 49 ng/g dw (S3), and they were in the same range as those in an uncontaminated area reported by Nadal (2004) in Catalonia. Based on these results, in Barcelona, the agriculture soil is not obviously polluted by PAHs. However, the contamination of PAHs is still positively related to the nearby urbanization level and traffic pressure (S3 > S2 > S1).

Table 3.4.3 PAHs concentrations (ng/g, dw) in three sites and the maximum concentrations allowed (data from the Catalan legislation for soil)

	S1	S2	S3	LOD	LOQ	Maximum value
Naphthalene	<LOD	<LOD	<LOQ	0.4	1.0	5000
Acenaphthene	<LOQ	<LOQ	<LOQ	0.1	0.1	-
Acenaphtnylene	<LOD	<LOQ	<LOQ	0.1	0.1	-
Fluorene	<LOD	<LOD	<LOQ	0.4	0.6	-
Phenanthrene	<LOQ	<LOD	<LOQ	1.1	2.3	5000
Anthracene	0.5	0.8	0.6	0.4	0.5	100000
Fluoranthene	2.0	< LOQ	2.3	1.1	1.7	15000
Pyrene	1.9	1.5	1.9	0.9	2.1	-
Benzo[a]anthracene	1.6	1.9	2.4	1.0	1.4	10000
Chrysene	2.3	2.1	2.4	0.9	1.4	-
Benzo[b]fluoranthene	2.9	4.2	4.8	1.0	1.6	-
Benzo[k]fluoranthene	< LOQ	4.1	5.0	1.5	3.6	50000
Benzo[a]pyrene	1.9	3.4	4.3	1.7	3.4	80
Dibenz[a,h]anthracene	1.1	1.9	02.0	0.9	0.9	-
Benzo[g,h,i]perylene	2.1	4.2	5.4	0.9	1.1	-

Indeno[1,2,3- c,d]pyrene	4.2	12.3	16.8	1.2	2.0	50000
∑16 PAHs	24	38.4	49			
∑ HMW PAHs	19.8	35.6	45			

PAHs could be divided into two main classes: low and high molecular weight PAHs (LMW and HMW, respectively). The LMW PAHs (2 - 3 ring PAHs) such as naphthalene, fluorene, phenanthrene, and anthracene are shown to have significantly less toxicity compared to the HMW PAHs of 4 - 7 rings (from pyrene to indeno [1,2,3-c,d]pyrene in Table 3.4.3) which are recalcitrant and carcinogenic to humans (Duan et al. 2015; Kuppusamy et al. 2015). In our study, the concentrations of LMW were almost all below the limit of quantification. This may result from the high irradiation rate (mean 15 MJ/ m^2, up to 25 MJ/m^2 in July) in Barcelona (Meteocat 2019). It is well known that PAHs suffer degradation in the atmosphere by photo-oxidation (Balducci et al. 2017; Chao et al. 2019), especially for 2- and 3 - ring PAHs which possess the low molecular weight, high vapor pressure, and high fugacity ratio (Mackay et al. 2000). In 1990, Park monitored that volatilization accounted for approximately 30 and 20% loss of naphthalene and 1-methylnaphthalene, respectively; but for the remaining compounds, volatilization was negligible. Kuppusamy et al. (2017) also reported that LMW PAHs would be lost rapidly and become more mobile and degradable once entering the soil, while HMW PAHs are more persistent and resistant to degradation (Kuppusamy et al. 2015). In addition, for HMW PAHs, due to relatively higher molecular weight, they tend to fall down to the soil near the emitted point by dry and wet deposition. Therefore, the concentrations of HMW PAHs in peri-urban soil (S2, S3) almost double that in the pristine site (S1). The total concentrations of HMW PAHs are low, but representative; therefore, only the total contamination of HMW PAHs will be involved in subsequent evaluations about phytotoxicity assessment, instead of individual PAH and all PAHs.

3.4.3.4 Occurrence of contaminants of emerging concern

A total of 33 CECs was analyzed; Table 3.4.4 shows only 11 of them which were detected above LOQs in at least one site. For different soils, the fluctuation of CEC concentration is different. S1 ranged from non-detectable to 397 ng/g (TCPP), S2 ranged from non-detectable to 6.68 ng/g (chlorpyrifos), and S3 ranged from non-detectable to 30 ng/g (MPB), separately. It is interesting to note that the concentration of TCPP and BPF in S1 is obviously higher than S2 and S3. This phenomenon could be due to S1 unique irrigation system (dripping by plastic tubing) and organic fertilization method (manure). TCPP is an organic flame retardant used as a raw material in the manufacture of polyester, rubber, binder, and resins. In 2000, European production of TCPP reached 36,000 tonnes/year (Föllmann and Wober 2006), while Bisphenol F (BPF) is used in the production of epoxy resins and polycarbonate polymers for lining large food containers, water pipes, and mulch plastic film. Due to their chemical characteristics (TCPP: log K_{ow} = 2.98, water solubility = 1200 mg/L; BPF: log K_{ow} = 3.06, water solubility = 360 mg/L), both TCPP and BPF would release into the environment through the plastic tubing during irrigation. In addition, Fromme (2002) detected BPF in liquid manure (2.2–62.6 µg/kg dw). Overall, the concentrations of CECs in our studied soils were lower than other peri-urban areas (Mac Loughlin et al. 2017)

Table 3.4.4 Concentrations of CECs (ng/g, dw) in the different soils evaluated

	S1	S2	S3	LOD	LOQ
Azoxystrobin	nd	3.82	nd	0.36	0.37
Chlorpyrifos	nd	6.68	nd	0.04	0.06
N,N-Diethyl-meta-toluamide (DEET)	1.40	0.48	< LOQ	0.19	0.22
Tris(2-chloroethyl) phosphate (TCEP)	1.10	nd	nd	0.17	0.18
Bisphenol F (BPF)	199	< LOD	< LOQ	9.0	10.1
Carbamazepin	< 0.12	0.14	< 0.12	0.12	0.14
Metylparaben (MPB)	< LOD	< LOD	30	6.18	6.92
1-Hydroxybenzotriazole (OHBT)	5.60	5.80	5.50	10.8	11.0

Pymetrozine	2.00	1.30	1.40	0.88	0.89
Carbamazepine-10,11-epoxide	nd	< LOD	< LOD	0.21	0.40
Tris (chloroisopropyl) phosphate (TCPP)	397	< LOD	< LOD	20.9	21.4

Concentration values have been corrected by the recoveries
nd not detected

3.4.3.5 Sensitivity of plant species

(1) Effect of different soils on plant growth indexes

There were two endpoints measured, namely seed germination rate and root elongation (Table 3.4.5). The germination rates of lettuce and tomato could reflect the difference between three soil sites ($p < 0.05$), while cauliflower germination rate is relatively insensitive, only could separate S3 from S1 and S2. Furthermore, unlike lettuce and cauliflower of which the germination rate was as follows: S2 > S1 > S3, tomato germination rate in S2 (67%) is extremely lower than S1 (91%) and S3 (83%).

As for root elongation, lettuce also is a good bioindicator specie which grew significantly differently in different soils, S1 (36) > S2 (30) > S3 (21), whereas, for tomato and cauliflower, the lengths of root are not significantly different between S1 and S2 soil.

Table 3.4.5 Comparison of seed germination and root elongation for three kinds of vegetable in three studied sites

Site	Plants	Germination rate (%)	Root elongation (mm)
S1	Lettuce	94.0 ± 0.6^{a}	35.8 ± 3.6^{a}
	Tomato	91.4 ± 2.3^{a}	37.3 ± 9.4^{a}
	Cauliflower	89.4 ± 2.7^{a}	48.3 ± 7.9^{a}
S2	Lettuce	99.0 ± 0.9^{b}	30.2 ± 4.4^{b}
	Tomato	67.0 ± 4.3^{b}	38.3 ± 7.5^{a}
	Cauliflower	90.4 ± 1.2^{a}	50.6 ± 9.5^{a}
S3	Lettuce	91.0 ± 1.7^{c}	21.4 ± 3.2^{c}
	Tomato	83.0 ± 2.2^{c}	32.2 ± 4.7^{b}
	Cauliflower	80.2 ± 2.5^{b}	42.7 ± 8.1^{b}

For the same plant species, different letters indicate significant differences between soils ($p < 0.05$)

(2) Relationships between soil parameters and plant growth indexes

Table 3.4.6 shows Pearson's correlation coefficients among soil parameters, seed germination rate, and the length of roots. It was found that for lettuce and cauliflower, their germination rates were significantly correlated with humidity and pH, while other factors were less significant (i.e., Mg, CEC, Mo) or had no correlation with them (i.e., Hg, Na, DEET), indicating that their seed germination is more sensitive to the moisture and pH rather than environmental pollution. Similar results were also reported by Rezvani et al. (2014). This may be explained by the following reasons. First, seed germination is simply an appearance of cell elongation instead of cell division (Haber and Luippold 1960), and it depends on the water and the reserves in the seed itself instead of surrounding condition; therefore, the response to environmental pollution is not sensitive. Second, it is necessary to consider that the effect of contaminants on seed germination relies on their ability to reach embryo tissues across the physiological barriers, mainly, the seed coating. The contaminants occurring in the soil may be absorbed by the seed coating, thus would not affect the growth of the embryonic root.

Table 3.4.6 Pearson correlation coefficients between the soil parameters and plant index

	Germination rate			Root elongation		
	Lettuce	Tomato	Cauliflower	Lettuce	Tomato	Cauliflower
Humidity	0.971	-	0.919	-	-	-
pH	0.959	-	0.930	-	-	-
EC	-	-	-	-0.920	-0.934	-0.960
NO_3	-	0.988	-	0.991	0.978	-
K	-	-	-	1.000	0.988	-
Mg	-	-	-	0.958	0.966	0.946
Ti	-	-	-	-0.996	-0.990	-
V	-	-	-	-0.947	-	-
Cr	-	-	-	-1.000	0.986	-
Cu	-	-0.977	-	-	-	-
As	-	-0.981	-	-	-	-

Ba	-	-	-	-0.983	-0.960	-
Cd	-	-	-	-0.998	-0.991	-
Hg	-	-	-	-0.962	-0.939	-
MPB	-	-	-	-0.936	-0.947	-0.956
HWM PAHs	-	-	-	-0.958	-0.936	-

All correlations shown are significant at the 0.01 level (2-tailed)

The seed germination of tomato is obviously different from lettuce and cauliflower which has a significant negative relation with Cu and As and positively correlated with nitrate. It is consistent with the phenomenon reported by Ashagre et al. (2013) that increasing Cu concentrations to 100 mg/kg decreased significantly the tomato germination rate. This may be caused by (1) the key role of peroxidases which are Cu stress-related enzymes is regarded as stiffening cell wall. Peroxidase-catalyzed lignification decreases the cell wall plasticity and therefore reduces cell elongation (Sánchez et al. 1995). (2) Cu toxicity causes oxidative damage for seedling; Mazhoudi et al. (1997) observed the accumulation of lipid peroxidation products when the concentration of sur- rounding Cu increased. In addition, compared with lettuce and cauliflower, the germination of tomato seed seems to be also more sensitive to the excessive As and NO_3^- in S2. In this sense, recently, Seifi et al. (2019) studied the effect of nitrate- reducing bacteria on the growth of tomato plants, since these bacteria have an important role in the biological removal of harmful nitrogen compounds. Some isolates increased significantly the growth parameters of tomato as well as seed germination, hypocotyl, and epicotyl length compared with control. Likewise, the existence of nitrate-reducing isolates in high nitrate surrounding may be the reason that tomato germination rate is higher in high NO_3^- soil in our study.

On the other hand, the root elongation may be a better index to reflect soil condition due to the high correlation coefficients with more factors, especially with TEs and HMW PAHs. We will discuss it later based on PCA results.

3.4.3.6 Determination of the contributions of soil parameters to plant endpoints by PCA

Two principal components (PCs) extracted by PCA explained 63.1% and 32.3% of data variation (Fig. 3.4.2). The highest positive loading of PC1 was focused on TEs (Mn, Co, Ni, Cu, Zn, As, Pb, Li), indicating PC1 is mainly influenced by TEs. And the negative loadings of root length suggested that root elongation, especially lettuce root elongation, is a good index for the contamination of TEs, which is consistent with the results of Pearson's correlation analysis. Although the germination rate of tomato also has a high negative loading on PC1, it is not a perfect indicator for soil TEs, because as discussed before, it is only significantly related to pollution of Cu and As.

Except for tomato germination rate, all plant endpoints have high positive loading on PC2. For PC2, the highest positive loadings were humidity and pH which have been proved to be essential for the growth of seeds, and the highest negative loadings were Ti, Cd, B, EC, and OHBT, indicating these elements hinder the growth.

Figure 2 shows the loadings of PC1 versus PC2. The relationship between soil parameters and plant indexes is clearly observed. It was found that most TEs and PAHs were located in an adjacent area, focused on the fourth quadrant, especially concentrated on PC1, indicating that their variation may follow similar trends. All plant indexes in the second quadrant could reflect contamination, but their sensitivities are different. Among these indexes, cauliflower is less sensitive to pollution, due to the low loading on PC1. Excepted pollution, root length can suggest the soil fertility, since this index and nutrients (P, K, Mg) are located in the proximity area. Unexpectedly, CECs show a positive relationship with plant indexes. This may be because, in our study area, their concentrations are relatively low which may inhibit the growth of pathogens in the soil.

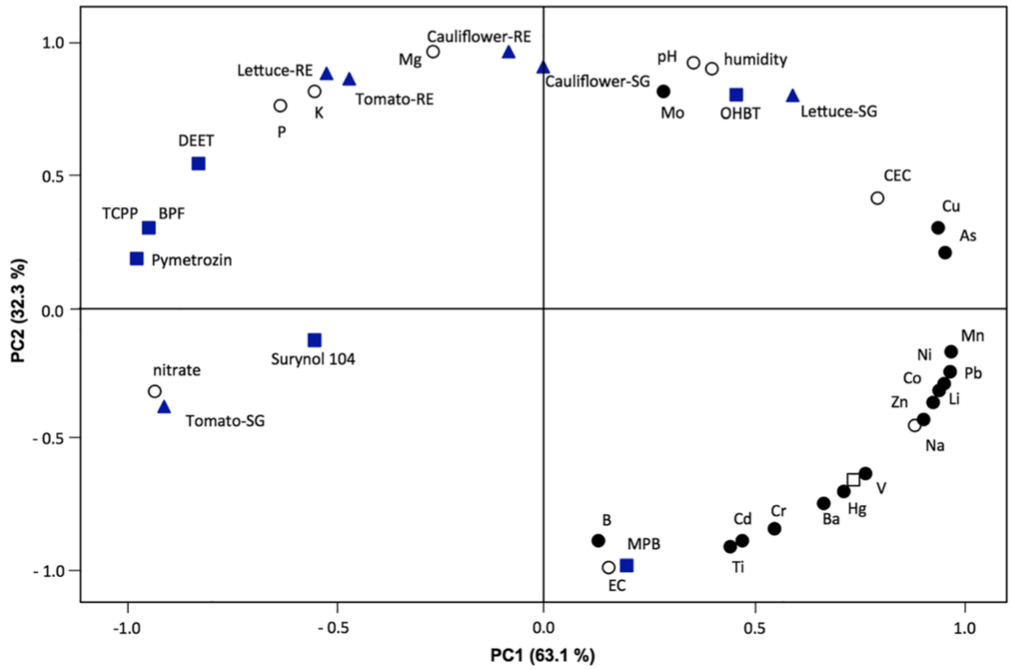


Fig 3.4.2 Principal component analysis results, loading plot PC1 vs PC2. Trace elements: black circles; polycyclic aromatic hydrocarbons (PAHs): white square; contaminants of emerging concern: blue squares; conventional soil quality parameters and nutrients: open circles. Root elongation and seed germination: open triangles. RE: root elongation; SG: seed germination

3.4.4 Conclusions

The results of this study show that risks posed to peri-urban agriculture ecosystem come from not only urban activities, but also from modern agricultural practices, so choosing proper plant can increase seed survival and improve yield. On the other hand, the feedback on the growth of seeds also could be an indicator of soil surrounding. In the present study, seed germination rate and root elongation were suitable for evaluating the chemical pollution of peri-urban agriculture soil of Barcelona. Along with the advantage of sensitivity of plant indexes, short experiment period (5 days), and low cost, the described method is useful when assessing peri-urban agriculture soils. Several key conclusions can be drawn:

1) The concentrations of TEs and PAHs in the soil from peri- urban agriculture area are below the guidelines' threshold, except for Cu, Zn, and Pb that exceed the limits.

2) Seed germination mostly depends on the humidity and pH. So compared with it, seed elongation is a better index which could reflect more aspects of the surrounding.

3) Low-dose CECs promote the growth of seedling.

4) Seed elongation could obviously show the pollution of TEs and PAHs in soil. Lettuce is the best indicator for assessing soil, due to the high seed germination and the significant decrease facing contamination.

5) It seems that tomato plants are not suitable for planting in the area with the highest Cu concentration, since it would significantly affect the seed germination rate, whereas cauliflower is less sensitive to pollution, therefore could be planted in the relative contaminated area.

3.4.5 References

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Chapter 4: General Discussion

This chapter presents the general discussion drawn from this dissertation and some suggestions for future research.

4.1 Occurrence of contaminants in vegetables and soil.

By 2050, the world's population will increase by 34%, and the percentage of the population living in urban areas is excepted to increase to 66% (FAO, 2009). Therefore, it is imperative to increase food production to feed the increasing urban population. Amending soil with fertilizers is the most common way to increase crop yield, as between 30 and 50% of yield growth is attributable to commercial fertilizers (Blanco, 2011). Since 2015, The European Commission proposed an action plan related to the circular economy which encourages farmers to amend soil with organic fertilizer instead of chemical fertilizer. Because biosolids fertilization not only beneficially disposes waste but also reduces cost. The most often used biosolids fertilizer are animal-based waste (manure), compost (plant sources or food waste), and urban waste (sewage sludge and household waste) (Chew et al., 2019). However, the presence of a wide range of contaminants, including trace elements (TEs), organic pollutants, potential human pathogens, and emerging pollutants such as antibiotics (ABs) and antibiotic resistance genes (ARGs), has already been reported in some of these organic fertilizers (Mao et al., 2015; Wohde et al., 2016; Xie et al., 2018). As highlighted throughout this thesis, the application of organic fertilizers may affect the presence of contaminants in food crops, and trigger adverse human health risk.

To explore the occurrence and accumulation of TEs and ABs in lettuce crops grown due to the application of different doses of different organic amendments, in topic 1, sewage sludge (SS), swine manure (SM), and municipal organic food waste composted with wood waste (OFMSW) were compared with chemical fertilization (CF). The amount of organic fertilizer added per pot was designed based on the optimal N dose (100 kg of N per ha): dose 1 (half the optimal N dose), dose 2 (optimal N dose), and dose 3 (twice the optimal N dose). Despite the greater concentration of TEs found in the SS in comparison with the other organic fertilizers, the TEs concentrations were greater in lettuce amended with the SM and OFMSW fertilizers (Table 3.1.1). This may indicate that the organic matter composition of the SS had a stronger interaction with these elements and, therefore, lower plant bioavailability. On the other hand, only 3 antibiotics were detected above the LOQ in lettuce leaves (lincomycin, ciprofloxacin, and azithromycin), and only in lettuces amended with the SM and SS fertilizers. No ABs were detected in lettuce grown with the CF or OFSMW fertilizers. Although there were differences caused by the type of fertilizers, no statistical differences were found between fertilization doses ($p>0.05$) (Table 3.1.2).

According to these results of topic 1, we found that, among three organic fertilizers, SS resulted in the lowest TEs accumulation in lettuce. However, the concentration of Cu and Zn in lettuce amended with SS was statistically greater than in those grown with chemical fertilizer. This migth be originated by the high content of Zn and Cu in sewage sludge. Long-term SS application inevitably causes the continuous accumulation of Zn and Cu in the matrix, and previous studies proved that these two elements affect plant uptake of other contaminants (Sayen et al., 2019; Liu et al., 2019). Therefore, in topic 2, we designed an experiment to assess the dose-effect of Zn and Cu in sludge-amended soils on the occurrence of contaminants (TEs, ABs, and ARGs) in vegetables, and hypothesized that increasing Cu and Zn amount would induce an increase accumulation of contaminants in the edible part of lettuce (leaf) and radish (root). Results showed that the addition of Zn and Cu significantly increased the concentration of TEs in both vegetables (Fig. 3.2.1). Nevertheless, the influence on the occurence of ABs and ARGs in plant tissue can be ingored. Only 2 (azithromycin and sulfamethoxazole) out of 16 ABs were close to or slightly above the LOQ in radish samples, and no target ABs were found in lettuce samples (Table 3.2.1). This result is different from topic 1 where 3 ABs (lincomycin, ciprofloxacin, and azithromycin) were detected in lettuce grown using sewage sludge (Table 3.1.2) . This disagreement could be caused by (1) the difference between sludges: despite the sludges were collected from the same wastewater plant, the background value of ABs differs from time to time; (2) different planting time: experiment described in topic 1 was conducted from October to December, while experiment explained in topic 2 was from March to June. It is possible that different temperatures and sun intensity will cause different degradation rates of antibiotics; (3)

growth dilution: the best growing seasons for lettuce are spring and fall. Although the growth period for two experiments is similar (57 and 56 days), in topic 1 lettuces grown in fall and winter were significantly smaller than lettuces grown in topic 2 (spring) (average fresh weight:183 vs 215 g). For similar contaminant amount, the concentration is lower in larger vegetables; and finally (4) irrigation water: the irrigation water for both experiments was from the reservoir of the experimental base, the origins are rainwater and groundwater, temporal dynamics of the compontsof irrigation water may influence the presence of antibiotics in plants. Like other contaminants, the occurence of ARGs shows a clear interspecies difference. Most of ARGs in radish roots was 10 times higher than lettuce leaves, except bla_{TEM} (Fig. 3.2.2). This finding is consistent with prior studies showing that radishes had a greater load of ARGs than lettuce (Guron et al., 2019; Tien et al., 2017). Crops could shape the overall rhizosphere and phyllosphere microbiota through the secretion of various proteins, amino acid, phenols, etc. (Berg and Smalla, 2009; Bulgarelli et al., 2013). The extent of soil contact also needs to be considered when explaining the difference between lettuce and radish resistomes (Fogler et al., 2019).

Zn and Cu are the most abundant elements in our studied organic fertilizers, however, repeated fertilization not only brings more Zn and Cu but also introduces more other TEs and organic matter. For this reason, we conducted two runs of field experiments in order to evaluate the effect of frequent applications of fertilizers on the occurrence of trace elements in vegetables (topic 3). Results show that the application of organic fertilizers had no impact on soil total TE concentration (Table 3.3.4). Nevertheless, the TEs concentration in vegetable grown with organic fertilizers was higher than in vegetable grown with chemical fertilizer (Fig. 3.3.1). This is consistant with results from the greenhouse experiment. In the field experiment, soil was applied with the same amount fertilizers as the treatment of dose 2 in topic 1. For some elements, the concentration in lettuce is similar between experiments (e. g. Cu: 6-15 mg/kg (dw) in topic 1; 5-13 mg/kg in topic 3). For some other elements, the concentration is higher in topic 3 (e.g. Pb：0.07-0.18 mg/kg in topic 1; 0.09-0.26 mg/kg in topic 3). Topic 1 was conducted under controlled condition in greenhouse which excludes the influence of many natural factors, such as rainfall which deposits air pollution into soil and plant. Additionally, the TEs concentration of vegetable grown in the mixture of sewage sludge and the chemical compound of Zn and Cu (in topic 2, Fig. 3.2.1) is lower than vegetable grown in repeat sewage sludge amendment (in topic 3, Fig 3.3.1), even controlling the same background value of Cu and Zn, which indicates that the consequence of long-term sewage sludge fertilization is more than an increase of the soil element content. Decomposed organic matter from organic fertilizer released TEs into pore water which is easily absorbed by plants.

Besides the pollution coming from fertilizers, peri-urban soil has to face multiple contaminants released from large infrastructures (i.e. port and airport) and city traffic. Soil pollution level is highly related to the urbanization and industrialization level of nearby cities. To date, there are no studies of TEs, CECs and PAHs pollution in the peri-urban agricultural area of Barcelona. In this regard, topic 4 developed the experimental design performed in two peri-urban sites and one pristine site of Barcelona, and assessed the occurrence of 16 TEs, 33 CECs and 16 PAHs in the soil. In the peri-urban area, the most abundant elements are Mn and Zn, with values close or even above the maximum acceptable values of agricultural soil established by the Catalan Law 5/2017 (Table 3.4.2). Thus, it is imperative to control the sources of these two elements to avoid their negative effects. However, the most hazardous element in peri-urban soil is Pb, whose value is around 2.5 times higher than the guideline values. The large concentration of Pb was found in peri-urban soil partly derived from legacy leaded automobile exhaust emissions in the urban area, and partly resulting from the irrigation water. These values agree with those reported for Pb in irrigation water of the studied area that exceeded the maximum allowable concentration (Cabeza et al. 2012). Our findings also agree with Nziguheba and Smolders (2008) that found that phosphate fertilizers are the main source of increased Pb levels in cropped soils. Unlike for TEs, the levels of 16 PAHs analyzed are significantly below the Catalan legislation limit values (Table 3.4.3). The total concentrations of PAHs in soil were in the same range as those in an uncontaminated area reported by Nadal (2004) in Catalonia. Based on these results, in Barcelona, the agriculture soil is not noticeably polluted by PAHs. However, the contamination of PAHs is still positively related to the nearby urbanization level and traffic pressure since pristine site showed lower values than peri-urban sites. Some CECs were detected at low concentrations, and their occurrence is more related to agricultural activities instead of urbanization (Table 3.4.4). Pristine site has much higher TCPP and BPF than peri-urban soil. These contaminants may originate from the dripping tube and organic fertilizer. Overall, the concentrations of CECs in our studied soils (rang from not detected to 199 ng/g) were lower than in other peri-urban areas such as Gran La Plata, Argentina (Mac Loughlin et al. 2017) (rang from not detected to 649 ng/g)

4.2 Risk assessment

4.2.1 Phytotoxicity

Amending soil with organic fertilizer is an effective method to supply soil nutrients, which aims to improve crop yield. However, the contaminants in organic fertilizer may offset the benefit, and even damage plant growth. Different organic fertilizers result in different crop morphology and yield, that is reflected by both experiments of topic 1 and 3. Vegetables grown with SS had a similar result to vegetables grown with chemical fertilizer, while the other organic fertilizers (OFMSW and SM) brought lower morphological values and vegetable production (Fig 3.1.1 and Table 3.3.5). The main explanation for the agronomic differences may be nitrogen availability. SS and CF have more ammoniacal-N content which can be directly used by plant (Table 3.3.2), while the abundant organic N in other fertilizers need time to mineralize before being absorbed by vegetables. This conjecture got confirmed by experiment of topic 3 in which the yield gap between OFMSW and CF decreased on the second production cycle. On the other hand, the fertilizer doses greatly affected lettuce yield. The fresh weight of lettuce increased by increasing doses of SS and CF, while did not statistically change with SM and OFMSW (Fig 3.1.1). Thus, from the point of view of crop yield and morphology, the SS fertilizer was the most suitable amendment with similar results to CF.

Zn and Cu are essential micronutrients, which participate in the structure of photosynthetic proteins and enzymes in plants and have a stimulating effect on auxin promoting. Nevertheless, once their concentrations exceed the specific thresholds, they would also hinder plant development. Topic 2 assess the phytotoxicity of lettuce and radish grown in sludge-amended soils with increasing Zn and Cu doses. No significant difference was observed in crop yield or morphology (Fig. S2.3). This finding is contrary to that reported by Wolf et al. (2017), who found that equivalent Zn and Cu hampered lettuce growth. This was due to the strong complexing ability of the sewage sludge amended soil in the present case, which prevented plants from absorbing overdoses of Zn and Cu.

Phytotoxicity is an important index to assess soil pollution, therefore, in topic 4, two bio-monitoring indexes (seed germination rate and root elongation) were aseessed in peri-urban soil and pristine soil. The results show that the germination rate is more correlated with soil humidity and pH, while root elongation was highly influenced by soil pollution, especially TEs and PAHs (Table 3.4.6).

4.1.2 Human health implication

As discussed before, many adopted agricultural activities (organic fertilization, the addition of Zn and Cu, and repeated soil amendments) significantly increased the concentration of TEs in vegetables. Although the amounts of TEs were always compliant with European Commission Regulation No. 181/2006 for leafy and root vegetables, not all elements are included in the regulation. Therefore, human health risk was further assessed by calculating the hazard quotient. The THQ obtained from topic 1, 2 and 3 show that the consumption of vegetables grown with organic fertilizers would not pose a risk to human health, as values of all treatments were less than 1. Nevertheless, compared with chemical fertilizer, organic fertilization brought a higher health risk. The potential threat increased with the repeated application (increased 1.2-2-fold) (Table 3.3.7 and Table 3.3.8). Besides, the addition of Zn and Cu to sewage sludge supplied also resulted in higher human health risk, as THQ value increased 2-3 times (Table 3.2.2).

Nowadays, no regulation is established for antibiotic in terms of vegetable consumption. Therefore, we calculated the hazard quotient of detected ABs, based on microbiological and toxicological endpoints. In topic 1, the highest HQ was observed in ciprofloxacin under SS treatments (0.16) (Table 3.1.6). While, in the experiment of topic 2 which was also amended by SS, the highest HQ was found in sulfamethoxazole at 3.7×10^{-6} (Table 3.2.4). This huge difference between HQ values might be caused by several factors. In the first place, the difference in ABs occurrence: in topic 1, the value of the highest AB (ciprofloxacin) reaches 16 µg/kg (fw) (Table 3.1.2), whereas in topic 2, the highest AB is sulfamethoxazole (0.06 µg/kg, fw) (Table 3.2.1). Secondly, different acceptable daily intakes (ADIs) values: according to the study of Wang (2017), ADI of ciprofloxacin is 0.15 µg/kg/day, and ADI of sulfamethoxazole is 130 µg/kg/day. Overall, all HQ values suggest that not risk to human health due to the presence of ABs, but potentially harmful combined effects cannot be ruled out. There are many other exposure pathways for human beings to antibiotics, e.g. farm fish, drinking water, or inhaled particles.

The risk of antibiotic resistant gene is its ability to help bacteria to resist the effects of an antibiotic which they were previously sensitive (Amarasiri et al., 2020). Antibiotic resistant bacterial (ARB) infections have higher mortality and morbidity rates and result in longer hospital stays (Cosgrove, 2006). The most abundant ARG in lettuce sample of topic 2 is *bla*$_{TEM}$ (Fig. 3.2.2). The resistance mechanism of *bla*$_{TEM}$ is the production of β-lactamases, which are able to hydrolyze the four-membered β-lactam ring present in some antibiotics, such as penicillin and cephalosporin. The predominant gene in radish root was *sul*1. This gene codes for resistance to sulfonamide antibiotics. The difference between lettuce and radish samples indicates that the foodborne antibiotic resistance highly depends on the type of vegetable consumed. However, still remains the question of the extent to which foodborne ARGs could affect human health.

4.3 Future research needs

The following recommendations are proposed for future work that could be undertaken and could give more knowledge about the dissertation:

(1) Co-occurrence of contaminants (trace element, antibiotics, antibiotic resistant genes) in edible parts of food crops have been studied in this thesis. It is noteworthy that the experiments concerning topic 1 and 2 were performed in a greenhouse. For this reason, further studies should be done at a real field-scale to confirm contaminant behavior and their fate observed in this work.

(2) The positive relation between trace elements and ARGs has been reported in many studies (He et al., 2014; Chen et al., 2020). Nevertheless, this phenomenon was not observed in our experiment devoted to assess the effect of Zn and Cu on the occurrence of ARGs. Since there are agricultural areas in some parts of the world highly contaminated by trace elements (including Cu and Zn), new experiments could be performed increasing the amount of both trace elements to see their effect at high loadings.

(3) Metabolites of antibiotics in plant tissues ought to be monitored, because they may surpass the concentration of the parent compounds and reveal more acute toxicity in some cases (Tadić et al., 2021)

Chapter 5: Conclusions

This chapter summarizes important conclusions of this dissertation.

The general main conclusions extracted from the research conducted in this thesis are summarized as follows:

- Soil amendment with different fertilizers resulted in significant changes in the abundance of TEs and ABs in lettuce leaves, as well as differences in plant morphology.
- TEs concentration in vegetables grown in soil amended with organic fertilizers was statistically greater than in those grown in soil amended with the chemical fertilizer. This difference increased with repeated application.
- ABs were only detected in lettuce leaves grown in manure and sewage sludge amended soils.
- The use of sewage sludge resulted in the highest lettuce yield compared with the other organic amendments, with values similar to those for chemical fertilization.
- The dose of organic fertilizers did not affect the plant uptake of TEs and ABs in any of the studied organic fertilizers.
- The concentration of Zn and Cu in sludge-amended soils has a positive relation with the amount of TEs in vegetable tissue, while this relation is negligible with the occurrence of ABs and ARGs.
- Vegetable type is a key factor for the accumulation of TEs, ABs and ARGs. In contrast to the TEs, the occurrence of ABs and most of the ARGs was higher in radish roots than in lettuce leaves.
- The consumption of vegetables grown in organic fertilizer-amended soils does not pose an adverse human health effect, no matter the kind of organic fertilizer and the application times.
- The concentrations of majority TEs in peri-urban soil of Barcelona are below the guidelines' threshold, except for Cu, Zn, As, and Pb at some sampling sites.
- Compared with pristine site, the relative high level of TEs originates from traffic and industrial activities.

- The concentration of PAHs and CECs in Barcelona peri-urban soil are pretty low, and therefore their pollution is negligible.
- Compared with seed germination rate, root elongation is a better index to assess soil pollution, because of its sensitivity.
- Lettuce is the best indicator for assessing soil, due to the high seed germination and its significant decrease facing contamination.

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