UNIVERSITAT POLITÈCNICA DE CATALUNYA BARCELONATECH

Fate and effects of waterborne contaminants of emerging concern in the soil-plant System : impact of biochar soil amendment to mitigate their plant uptake

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Fate and effects of waterborne contaminants of emerging concern in the soil-plant system. Impact of biochar soil amendment to mitigate their plant uptake

Presentada per

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Maig 2017

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A Manuel y Pascual,
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Abstract

Water scarcity is an issue of global concern due to the increase of the population and the climate change, which both increase the water demand. Many arid and semiarid countries are facing high water stress and the use of reclaimed water becomes a valuable resource. Many countries' economy is based on the agro-food sector, with amounts ca. the 70% of water demand. For this reason, reclaimed water represents an important component of wise water management.

Wastewater treatment plants (WWTP) are designed to remove efficiently some biodegradable compounds, however, they are not able to remove a number of recalcitrant organic contaminants known as chemical oxygen demand (COD). There are many sources of water pollution, and contaminants of emerging concern (CECs) including many compounds that they are not legislated and recently some effects to the environment have been observed. For example, pharmaceuticals, personal care products, flame retardants, microplastics, etc.

Consequently, plants are exposed to a huge number of chemical contaminants that are present not only in water, but also in air or soil. For this reason, it is important to understand the dynamics involved in the plant uptake of these CECs and more specifically in crops.

In this Thesis, the uptake of some CECs, chosen by their occurrence in the environment and their physical-chemical properties, has been assessed. To elucidate the factors that are involved in the uptake of these contaminants, three different experiments were performed in a greenhouse. Therefore, this Thesis is divided in five sections. In two of them (Chapter 3 and Chapter 4) the plant uptake of some CECs with a perlite:sand mixture and its modeling were assessed. Moreover, a mass balance was performed to evaluate the persistence of the CECs in the substrate. Then, by inverse modeling, the half-lives of CECs in the soil-plant system were estimated.

In the experiment conducted in Chapter 3, most of the CECs that were added in the irrigation water were taken up by lettuce. Hence, in Chapter 5, biochar, which is a soil improver, was assessed as a soil amendment to mitigate the uptake of these CECs in lettuce.

Since the first three sections we demonstrated that CECs can be uptaken by crops and translocated to edible parts, and it well known that plants can metabolize xenobiotics

through transformation, conjugation and sequestration steps, in Chapter 6, an enzymatic digestion was performed to determine the conjugated CECs fraction. Interestingly, the conjugated fraction accounted up to more than 80%, which should be taken into account in risk assessment studies.

Finally, in Chapter 7 the effects of CECs to lettuce were elucidated. Visual differences between non-exposed and exposed lettuce were observed. For this reason, a metabolomic approach was applied to correlate the presence of CECs with the changes in the metabolome and the changes in chlorophyll content and plant morphology.

Resum

L'escassetat d'aigua és una preocupació global degut a l'augment de la població i al canvi climàtic, ja que ambdós augmenten la demanda d'aigua. Molts països àrids i semiàrids estan fent front a un estrès d'aigua molt elevat i l'ús d'aigua regenerada esdevé un recurs molt preat. L'economia de molts països està basada en el sector agroalimentari, el qual comptabilitza a nivell global el 70% de la demanda d'aigua. Per aquest motiu, l'aigua regenerada representa un important component en la gestió eficient de l'aigua.

Les estacions depuradores d'aigües residuals (EDARs) estan dissenyades per eliminar compostos biodegradables, però aquestes no són capaces d'eliminar un elevat nombre de contaminants orgànics recalcitrants, fracció coneguda com la demanda química d'oxigen (DQO). Hi ha diverses fonts de contaminació aquàtica, i els contaminants emergents (CECs), que inclouen un elevat nombre de contaminants, no estan legislats per les autoritats. A més, recentment, s'han observat efectes sobre el medi ambient. Entre d'altres CECs, podem trobar fàrmacs, productes de cura personal, retardants de flama, microplàstics, etc.

Conseqüentment, les plantes es troben exposades a un gran nombre de contaminants químics que estan presents, no només a l'aigua, sinó també en l'aire o el sòl. Per aquest motiu, és important entendre la dinàmica involucrada en la incorporació d'aquests CECs a les plantes, i més específicament, en conreus.

En aquesta Tesi, s'ha avaluat la incorporació d'alguns CECs, que han estat escollits en funció de la seva presència en el medi ambient i les seves propietats físico-químiques. Per dilucidar els factors implicats en la incorporació d'aquests CECs, tres experiments s'han dut a terme en un hivernacle. Per tant, aquesta Tesi es troba dividida en cinc capítols. En els dos primers (Capítol 3 i 4), s'ha estudiat la incorporació d'alguns CECs a enciam, emprant una mescla de sorra i perlita com a substrat. A més, es va dur a terme un balanç de masses per avaluar la persistència dels CECs en el substrat. Llavors, mitjançant modelització inversa, s'han estimat els temps de vida semi-mitjos dels contaminants en el sistema sòl-planta.

En l'experiment del Capítol 3 es va observar que la majoria de CECs que es van afegir a l'aigua de reg van ser incorporats als enciams. Per això, en el Capítol 5 es va emprar el carbó vegetal (biochar) com a modificador del sòl per mitigar la incorporació d'aquests contaminants en enciams.

Com que en les tres primeres seccions es va poder observar que aquests contaminants poden ser incorporats pels enciams, arribant a les parts comestibles d'aquest conreu, i és sabut que les plantes poden metabolitzar xenobiòtics a través de processos de transformació, conjugació i segrest, en el Capítol 6 es va dur a terme una digestió enzimàtica per determinar la fracció dels CECs que pot ser conjugada amb la glucosa. Curiosament, aquesta fracció representava fins a més del 80% respecte el contaminant parental. El que demostra que aquesta fracció hauria d'estar inclosa en els estudis de valoració del risc.

Finalment, en l'experiment dut a terme en el Capítol 7, es van observar diferències visuals entre enciams que van ser exposats a CECs i els que no. Per aquest motiu, amb un enfocament metabolòmic, es va correlacionar la presència dels CECs amb els canvis en el metaboloma de l'enciam i els efectes observats (morfologia i contingut de clorofil·les).

Resumen

La escasez de agua es una preocupación global debido al aumento de la población y el cambio climático, ya que ambos aumentan la demanda de agua. Muchos países con clima árido o semiárido están haciendo frente a un estrés de agua muy elevado y el uso de agua regenerada se convierte en un recurso muy valioso. La economía de muchos países está basada en el sector agroalimentario, el cual contabiliza globalmente el 70% de la demanda de agua. Por este motivo, el agua regenerada representa un importante componente en la gestión eficiente del agua.

Las estaciones depuradoras de aguas residuales (EDARs) están diseñadas para eliminar compuestos biodegradables, pero éstas no son capaces de eliminar un elevado número de contaminantes orgánicos recalcitrantes, cuya fracción es conocida como la demanda química de oxígeno (DQO). Hay varias fuentes de contaminación acuática, y los contaminantes emergentes (CECs), que incluyen un elevado número de estos contaminantes, no están legislados por las autoridades. Además, recientemente, se han observado efectos sobre el medio ambiente. Dentro de los CECs, podemos encontrar fármacos, productos de cuidado personal, retardantes de llama, microplásticos, etc.

Consecuentemente, las plantas se encuentran expuestas a un gran número de contaminantes químicos que están presentes no sólo en el agua, sino también en el aire o el suelo. Por este motivo, es importante entender la dinámica involucrada en la incorporación de estos CECs en plantas, especialmente, en cultivos.

En esta Tesis, se ha evaluado la incorporación de algunos CECs, elegidos en función de su presencia en el medio ambiente y sus propiedades físico-químicas. Para dilucidar los factores implicados en la incorporación de estos CECs, tres experimentos se han llevado a cabo en un invernadero. Por lo tanto, esta Tesis se encuentra dividida en cinco capítulos. En los dos primeros (Capítulo 3 y 4), se ha estudiado la incorporación de algunos CECs en lechuga, utilizando una mezcla de arena y perlita como sustrato. Además, se llevó a cabo un balance de masas para evaluar la persistencia de los CECs en el sustrato. Entonces, mediante modelización inversa, se han estimado los tiempos de vida semi-medios de los contaminantes en el sistema suelo-planta.

En el experimento del Capítulo 3 se observó que la mayoría de CECs que se añadieron al agua de riego, fueron incorporados por las lechugas. Por ello, en el Capítulo 5 se empleó carbón vegetal (biochar) como modificador del suelo para mitigar la incorporación de estos contaminantes en lechugas.

Como en las tres primeras secciones se pudo observar que estos contaminantes pueden ser incorporados por las lechugas, llegando a partes comestibles de este cultivo, y es sabido que las plantas pueden metabolizar xenobióticos a través de procesos de transformación, conjugación y secuestro, en el Capítulo 6, se llevó a cabo una digestión enzimática para determinar la fracción de estos CECs que puede ser conjugada con la glucosa. Curiosamente, esta fracción representaba hasta más del 80% respecto al contaminante parental. Lo que demuestra que esta fracción debería estar incluida en los estudios de valoración del riesgo.

Finalmente, en el experimento llevado a cabo en el Capítulo 7, se observaron diferencias visuales entre lechugas que fueron expuestas a CECs y las que no. Por este motivo, con un enfoque metabolómico, se correlacionaron la presencia de los CECs con los cambios en el metaboloma de la lechuga y los efectos observados (morfología y contenido de clorofilas).

Index

List of tables

List of supplementary tables

List of figures

List of supplementary figures

List of acronyms

AC	Active carbon
ALS	Alternating least squares
ARB	Antibiotic resistant bacteria
ARG	Antibiotic resistant gene
ATP	Adenosine triphosphate
BC	Biochar
BCF	Bioconcentration factor
BHT	Butylated hydroxytoluene
BPA	Bisphenol A
BZP	Benzophenone
C_A	Concentration in air
CAF	Caffeine
CBZ	Carbamazepine
CECs	Contaminants of emerging concern
CFA	Clofibric acid
C_O	Concentration in octanol
COD	Chemical oxygen demand
C_W	Concentration in water
DMF	Dimethylformamide
D_{ow}	Octanol-water distribution ratio
EC	European Commission
ECHA	European Chemicals Agency
EDA	Electron donor-acceptor
EDC	Endocrine disruptor compound
EF	Enantiomeric factor
EU	European Union
FDA	Food and Drug Administration
f_i	Ionic fraction
f_n	Neutral fraction
FUR	Furosemide
GHG	Greenhouse gas
GSH	Glutathione
GST	Glutathione S-transferase
H	Henry law's constant
IBI	International biochar initiative
IBU	Ibuprofen
K_{AW}	Air-water partition coefficient
K_d	Soil-water distribution coefficient
K_{OA}	Octanol-air partitioning coefficient
K_{OC}	Soil adsorption coefficient

K_{ow}	Octanol-water partitioning coefficient
LCF	Leaf translocation factor
MDHJ	Methyl dihydrojasmonate
MeBT	5-Methyl-1H-benzotriazole
MePB	Methyl paraben
NER	Non-extractable residues
OPL	4-Octylphenol
PAH	Polycyclic aromatic hydrocarbon
PBs	Parabens
PCB	Polychlorinated biphenyls
PLS	Partial least squares
PROP	Propranolol
ϱ_s	Saturation vapor pressure
PVC	Polyvinyl chloride
PZE	Phenazone
R	Universal gas constant
RCF	Root concentration factor
RD	Royal Decree
S	Solubility
SMZ	Sulfamethazine
SSA	Specific surface area
T	Temperature
TCA	Tricarboxylic acid
TCP	Tris(2-chloroethyl) phosphate
TCS	Triclosan
TF	Translocation factor
TON	Tonalide
TSCF	Transpiration concentration factor
TWW	Treated wastewater
UN	United Nations
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
VIP	Variable importance of projection
VOC	volatile organic compounds
WHC	water holding capacity
WHO	World Health Organization
WWTP	Waste water treatment plant

Chapter 1. Introduction

1.1 Reclaimed water

Water comprises over 70% of Earth's surface; however, fresh surface-water represents only about a 0.007% of the total water. Moreover, the constantly increasing of population in Earth (in 2017 population has already reached 7.5 billion inhabitants), is reducing the per capita water resources available (United Nations, 2014). The World Economic Forum considers water crises, the biggest threat facing the planet today (World Economic Forum, 2016) and the climate change will further increase the pressure on the availability of water resources (Intergovernmental Panel on Climate Change, 2014).

In arid and semiarid countries, climate change impacts add additional pressure to already difficult water management challenges. Rain is expected to reduce in frequency but increase in intensity, resulting in frequent droughts and floods (Trenberth, 2011). The consumption of groundwater is likely to become unsustainable and the need of an effective water reuse is demanded. This complicates the supply of water for domestic, industrial, and agricultural uses. By 2025, 1,800 million people are expected to be living in countries or regions with water scarcity (UN-Water, 2007). In many arid areas, water stress causes deterioration of fresh water resources in terms of quantity and quality, because the demand for water exceeds the available amount during a certain period of the year (Gassert et al., 2014). As it can be seen in Figure 1.1, many countries have still high exposure to water-related risks, and with the existing climate change scenarios, almost half the world's population will be living in areas of high water stress by 2030 (UN-Water, 2007).

In the southern Europe (EU), the agricultural sector represents approximately ca. 70% of the total water withdrawal; and, together with the agro-food industry, it is one of the most important economic activities. For many developing countries and transitional economies, agriculture sector accounts for more than 90% of water withdrawals (FAO-AQUASTAT, 2012).

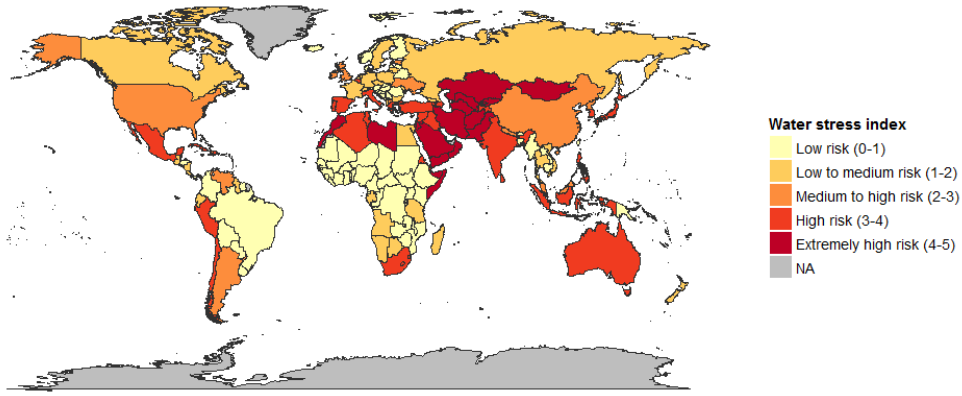


Figure 1.1: Overall water stress index from the different world countries. Data obtained from Aqueduct Water Risk Atlas (Gassert et al. 2014).

In EU, reclaimed water (RW) is used for non-potable reuse, such as agriculture, industrial cooling water, toilet flushing, aquifer's recharge, and to increase river flows and streams. In this context, the reuse of treated wastewater can be considered a water supply for many essential human activities such as agriculture. While Israel is the leading country in water reuse with more than 70% of its sewage, Spain has the largest waste reclamation program in Europe with about 17% of its sewage (FAO-AQUASTAT, 2012).

Some Member States of the EU, especially the ones experiencing water scarcity, have issued their own legislative framework or guidelines for water reuse applications. In Spain, the Royal Decree (RD/1620/2007) was published and introduced the concept of reclaimed water. In this RD, the possible uses of reclaimed water are described and it establishes the quality that the water must comply depending on the different uses. Five kinds of uses are described in the RD: urban, agricultural, industrial, recreational and environmental. In all uses, the parameters to be controlled are nematodes, *Escherichia coli*, suspended solids and turbidity. Moreover, only the organic contaminants listed in the Annex IV of the RD/907/2007 are targeted to ensure the quality of water.

1.2 Contaminants of emerging concern

Contaminants of emerging concern (CECs) are substances that are introduced in the water cycle and can affect the environment and/or human health but they are still not regulated or in the process of regulation. CECs may be new substances, or maybe they

have been in the environment for a long time, but recently some negative effects for humans and the environment have been recently discovered.

Early environmental surveys were focused on chemical contaminants like priority pollutants (e.g. toxic pesticides or persistent organic pollutants) (Maliszewska-Kordybach, 1999). However, pharmaceuticals were targeted as possible contaminants at the end of 1990s (Daughton and Ternes, 1999). In fact, pharmaceuticals are bioactive molecules *per se* and can exert deleterious effects to non-targeted organisms. In the last years, the development of new analytical techniques has allowed to detect CECs in different environmental matrices, where CECs usually occur at trace levels (very low ng L^{-1} to µg L^{-1}).

Several hundred CECs have been detected and they can be categorized based on their physical-chemical properties or their impact on the environment. For example, common CECs are pharmaceuticals, personal care products, flame retardants, pesticides, plasticizers, disinfestation-by-products, nanomaterials, microplastics, etc. (Bhandari et al., 2015).

Several effects have been related to the presence of CECs in the environment; however, the high number of different CECs detected in the different environmental compartments makes it very difficult to elucidate the cause-effects to the living organisms.

Among the deleterious effects, one of the highest concern is the endocrine disruption to human or wildlife. In fact, some CECs can interfere with the functioning of body's endocrine system, regulated by the endogenous hormones. This alteration may lead adverse health effects in the targeted organism (e.g. cancer, birth defects or development disorders). Due to similar structural properties as hormones or other natural compounds, some CECs can mimic a natural hormone, and this makes the body to respond the stimulus like a natural hormone (Le et al., 2008). They can alter the growth of hormones or even inhibit part of the endocrine system (Schug et al., 2016).

Another important environmental and human health concern is the spread of antibiotic resistant bacteria (ARB) and microbe antibiotic resistant genes (ARG). In fact, the WHO stated that antibacterial resistance is a serious global problem (World Health Organization, 2014). For instance, antibiotics and antimicrobials are widely used among population and farming. Their use are mainly to treat or prevent animal diseases or even used illicitly as growth promoters. However, high amount of antibiotics are excreted by

the body without being metabolized (Qiu et al., 2016). This is causing that microbes and other living microorganisms adapt and resist the effects of medication used to treat them (Agersø et al., 2006).

To improve the water quality in the EU water bodies, the EU created the Water Framework Directive (WFD, 2000/60/CE). Briefly, it sets out the strategies against contamination of water and outlines the steps to be taken. For this purpose, a list of priority substances was issued. These substances present significant hazard to or via aquatic environment and usually can be persistent or prone to bioaccumulate in biota. In this regard, Environmental Quality Standards (EQS) set the annual average and the maximum allowable concentration for the priority substances and other pollutants, with the aim of achieving good surface water chemical status. In the 2008/105/EC Directive, the limits on concentrations of the priority substances in surface water of the listed pollutants were established. Moreover, the EQS established the list of 33 priority substances in Annex II as Annex X of the WFD.

The priority list is periodically being revised and in 2013, three pharmaceuticals were added for the first time (2013/39/CE) in the Watch List namely, diclofenac, and the hormones 17α-ethinylestradiol (EE2) and 17β-estradiol (E2) which can disrupt the endocrine system in humans and harm fish reproduction (Stewart et al., 2014; Zenker et al., 2014). Due to the new research and the need of reusing wastewater, CECs regulation is expected in the forthcoming years (Lapworth et al., 2012). In 2015, the list was revised adding several contaminants as candidates of priority hazardous substances (e.g. erythromycin, azithromycin, methiocarb, etc.), and in 2017, a new revision is expected.

1.2.1 Sources of CECs

Because CECs can be found in many of the daily used products by humans, the release to the environment is continuous. The main sources of CECs entrance in the water system are summarized in Figure 1.2.

CECs enter the water cycle mostly due to anthropogenic activities. For example, pharmaceuticals are consumed by humans or used in animal or fish farming, and usually their metabolization rate in the body is not complete (5 to 95%). Then, the active ingredients are excreted, being introduced to the water cycle. Similarly, personal care products are found in a myriad of products such as soaps, fragrances, cosmetics, etc. Consequently, several CECs are released to the environment and they have been yet

detected in water bodies. Industrial sector and hospitals also contribute to the discharge of CECs directly to the water cycle. For example, pharmaceuticals, dyes, plasticizers, solvents, and surfactants are released to the environment, and end up in the sewerage system with or without preliminary treatment.

Intensive agricultural practices use large amounts of pesticides and herbicides and release these contaminants directly to the environment. Furthermore, a common agricultural practice is the application of biosolids and manure as soil amendment. These biosolids are usually composed by sewage sludge that can be used as a soil conditioner. As it will be mentioned later, many CECs are not removed efficiently from the sewage treatment plants and they can be another entrance of CECs to the aquatic system. Moreover, depending on the properties, several CECs can be accumulated in the soil or leach to the groundwater and pollute the aquifers.

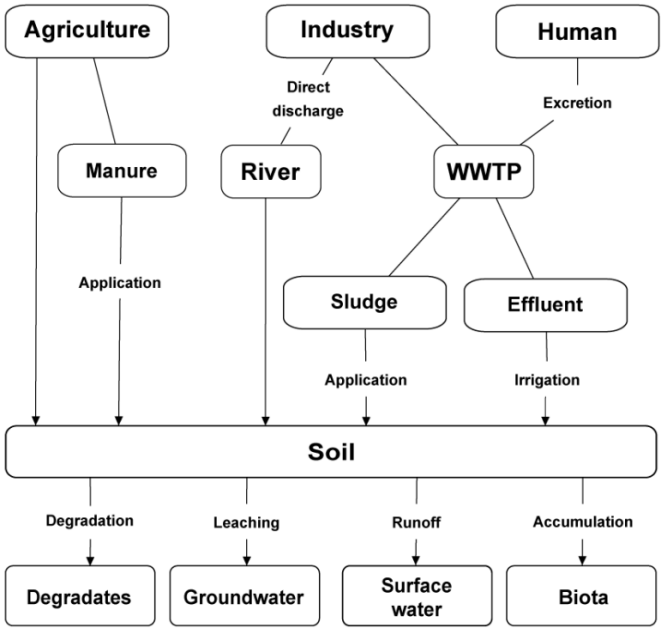


Figure 1.2: Routes of sources and fate of CECs in soil system (Boxall, 2011).

Usually, tertiary treatment wastewater plants, including disinfection as the last step, are used for water to reclamation that can be used for the applications mentioned before. However, WWTP are not designed to remove specific compounds (like CECs) but to meet quality parameters required for the authorities. Conventional WWTP consists of a combination of physical, chemical, and biological treatments to remove solids, reduce

biochemical oxygen demand and some nutrients. Although the treatment of wastewater may remove a fraction of CECs, there is a significant potential that CECs are released in treated effluent into the rivers or other aquatic systems (Daughton and Jones-Lepp, 2001).

1.2.2 Physical-chemical parameters of CECs

A myriad of CECs has been identified in different environmental matrices like water, soil or biota. The fate of these contaminants can be partially explained with their physical-chemical properties. Some of the main properties are described here.

Solubility

The solubility (*S*) is a chemical property that specifies the ability for a substance (solute) to dissolve in a solvent. Normally, it is referred as the maximum amount of solute dissolved in a solvent at equilibrium (in mol m^{-3}). Solubility is related to the hydrophobicity of the compounds. CECs with high *S* tend to be transported through water, while CECs with low *S* tend to precipitate or bioconcentrate easier (Verschueren, 1983; van Leeuwen and Vermeire, 2007).

Air-water partitioning coefficient

The air-to-water partitioning coefficient (K_{AW}), or so-called non-dimensional Henry's Law Constant) can be described as the partitioning of a contaminant between air and water. The Henry's Law constant (H) can be calculated from solubility in water (*S*) and the saturation vapor pressure (ρ_S):

$$H = \frac{\rho_S}{S} \tag{1.1}$$

Then, K_{AW} can be calculated as:

$$K_{AW} = \frac{H}{RT} = \frac{C_A}{C_W} \tag{1.2}$$

Where, *R* is the universal gas constant (8.314 J mol^{-1} K^{-1}), *T* is temperature (K), C_A and C_W are the equilibrium concentration of a contaminant in both air and water phase. When a CEC has a high K_{AW} value, then tends to be in air, and therefore, the CEC is

tagged as volatile. On the other hand, low K_{AW} values refer to non-volatile contaminants that tend to be in the water.

Octanol-water partitioning coefficient

The n-octanol-water partitioning coefficient (K_{ow}) describes the equilibrium partitioning between a hydrophobic phase and water and it can be calculated as:

$$K_{ow} = \frac{C_O}{C_W} \tag{1.3}$$

Where C_O and C_W are the equilibrium concentrations of a contaminant in both octanol and water phases. It is used as a predictor for the environmental partitioning between lipid and water phases. CECs with high log K_{ow} values (> 4) are hydrophobic, while low log K_{ow} (< 1) values are hydrophilic substances.

Several CECs have in their structure functional groups (such as hydroxyl or amino) that make the contaminant ionizable depending on the pH that they are found. Since the neutral and ionic exhibit different polarities, K_{ow} value should be corrected based on the pH and the pK_a values of the different CECs. The neutral and ionic fractions can be calculated as:

$$f_n = \frac{1}{1+10^{i(pH-pK_a)}} \quad \text{and} \quad f_i = f_n - 1 \tag{1.4 and 1.5}$$

Then, an octanol-water distribution ratio (D_{ow}) can be calculated taking into account the octanol-water partitioning coefficient (K_{ow}) and the neutral fraction at a given pH:

$$\log D_{ow} = \log K_{ow} + \log f_n \tag{1.6}$$

Soil adsorption coefficient

The soil adsorption coefficient (K_{oc}) provides a measure of the ability of a chemical to sorb to the organic portion of soil. Therefore, K_{oc} indicates the potential for the CEC to leach through soil and be introduced into groundwater, or be sorbed strongly to the soil, reducing its mobility.

K_{oc} is calculated following the following equation:

$$K_{oc} = \frac{K_d}{OC} \times 100 \quad (1.7)$$

Where K_d is the distribution coefficient between the soil and water phases at equilibrium; and OC is the percentage of organic content in the soil.

1.2.3 Overview of the CECs studied in this work

As it has been mentioned in the previous section, several hundred CECs have been detected in the environment. To understand the fate of some different CEC families, the different experiments conducted in this thesis were carried out with more than 20 CECs. Their selection was mainly based on: (i) physical-chemical properties and (ii) occurrence in the environment.

In Table 1.1, the main physical-chemical properties mentioned in section 1.2.2 are listed for the studied CECs in this work. Moreover, a short description of each selected CEC is provided depending on their use.

Industry

Plasticizers are used in plastic manufacture to improve its properties like resistance or stability. There are many different additives, for example, bisphenols or phthalates. Moreover, other compounds can be precursors or degradation products of surfactants such as alkylphenols.

One of the CECs that received increasing attention over the last decade, it is **bisphenol A** (BPA), which is a synthetic compound widely used for epoxy resins and polymer monomer (polycarbonate) production. For this reason, BPA is found in many daily products like food containers, drinking bottles, electronics or even medical devices (Fung et al., 2000; Kubwabo et al., 2009; Choi et al., 2012). As BPA can leach from food containers or can lining, it can be absorbed by food (Takao et al., 2002). Consequently, BPA has been detected in over 90% of tested humans in the US (Calafat et al., 2005). BPA has similar chemical structure as the potent estrogen receptor agonist, diethylstilbestrol and, because its estrogenic activity, BPA is considered to be an endocrine disruptor compound (EDC) (Rubin, 2011). Hence, several studies pointed out that BPA could have affect metabolic diseases such as diabetes; liver malfunction or other cardiovascular diseases (Wang et al., 2011; Muhamad et al., 2016). In 2011, the EU restricted the amount of BPA in baby's bottles to 600 µg kg^{-1} (European Comission,

2011/8/EU) and the limit of BPA migration in toys to 100 µg L^{-1} (European Comission, 2014/81/EU).

Finally, alkylphenols, which are named based on the chain size (e.g. ethyl-, propyl-, octyl-, and nonyl-) are precursors of the alkylphenol ethoxylates that are used as surfactants. Alkylphenols are considered to be EDCs (Bonefeld-Jørgensen et al., 2007). In 2003, EU regulated only certain uses of nonylphenol (European Comission, 2003/53/EC). Later, nonylphenol and **octylphenol** (OPL) were added as priority substances in the list of the WFD (2008/105/EC). OPL is used in the production of *p-tert*-octylphenol based resins, which are used as in tyre manufacture. OPL is persistent in the environment (Staniszewska et al., 2014) and several studies pointed out that OPL has a xenoestrogenic activity, even it differs chemically from the natural hormones; it has estrogenic activity in the organism (Markey et al., 2001). Moreover, OPL possesses potential risks to freshwater and marine aquatic and terrestrial compartments (DEFRA, UK, 2008).

Several substances can be added to plastic, textiles or furniture to reduce their flammability. Flame retardants inhibit or delay the spread of fire by suppressing the chemical reactions in the flame or by the formation of a protective layer on the surface of a material. One example of flame retardant is **tris(2-chloroethyl) phosphate** (TCP). Besides as a flame retardant, it can be used also as plasticizer and viscosity regulator in different polymers. TCP has been found in many environmental matrices and due to its suspected reproductive toxicity, it was listed by the European Chemical Agency (ECHA) as a substance of very high concern (European Comission, ED/6/2009).

In the agro-food industry, antioxidants are needed to reduce the oxidation of food. For example, **butylated hydroxytoluene** (BHT) is one of these additives, also known as E-321. Although it is forbidden in some countries (Japan, Sweden or Australia), it is still used in the US and in EU. It is added to many food products and also in cosmetics or pharmaceuticals. Some authors pointed out that BHT could possess endocrine disruptor activity preventing expression of male sex hormones (Schrader and Cooke, 2000) and in 2015 the EU added BHT to the Watch List (European Comission, ED/2015/495).

Pharmaceuticals

Due to human consumption, many pharmaceuticals are present in the environment. For example, **carbamazepine** (CBZ) is a drug used for the treatment of epilepsy or bipolar affective disorder. It is considered a recalcitrant compound in the environment (Maeng

et al., 2011), and it can be accumulated from year to year in soil (Kinney et al., 2006). CBZ exhibits limited removal efficiency in WWTP (Kot-Wasik et al., 2016; Yuan et al., 2017) and therefore, it is one of the most frequently detected pharmaceuticals in European natural waters, at concentrations up to μg L^{-1} (Barra Caracciolo et al., 2015). Due to its solubility and lipophilicity and its present in water, it can be easily taken up by plants (Shenker et al., 2011).

Another important class of pharmaceuticals is anti-inflammatory drugs that provide analgesic, antipyretic and anti-inflammatory effects. Among these, ibuprofen and phenazone were chosen in this work. In Spain, **ibuprofen** (IBU) is in the top 10 of the most prescribed drugs (Ministerio de Sanidad, 2015). IBU has been found in many types of waters reaching levels up to μg L^{-1}, although it has a high removal rate in WWTP (Gómez et al., 2007; Kosma et al., 2010; Martínez-Bueno et al., 2012).

Phenazone or antipyrine (PZE) is often detected in WWTP effluents because its elimination is not very effective and it has been found in groundwater (Reddersen et al., 2002).

Clofibric acid (CFA) is an active metabolite of some blood lipid regulators (e.g. clofibrate, etofibrate or theofibrate) used to reduce the concentration of lipoproteins in blood which are responsible for controlling the cholesterol and triglycerides (Spellman, 2014). CFA has been reported to be persistent in the environment and to hold endocrine disruption activity through interference with cholesterol synthesis (Pfluger and Dietrich, 2001).

Furosemide (FUR) is a diuretic drug that prevents the body from absorbing too much salt, passing in the urine. FUR is used to treat edema (fluid retention) in people with kidney disorders, liver diseases or heart failure and it is also used to treat hypertension. The human excretion rates for FUR range from 60 to 90% (Nowicki et al., 1995) and because its low removal rate in TWWP, it is commonly detected in water in the μg L^{-1} level (Jelic et al., 2011).

Propranolol (PROP) is a β-blocker drug used for the treatment of cardiovascular disorders such as high blood pressure. It has been found in many WWTP effluents, in drinking water and in river water (Bendz et al., 2005; Roberts and Thomas, 2006; de Jesus-Gaffney et al., 2015). Moreover, some studies have considered PROP as toxic to aquatic organisms (Cleuvers, 2005).

Antibiotics and antimicrobials

Antibiotics or antimicrobials are medicines used to prevent and treat several diseases by killing or inhibiting the growth of bacteria. For many years, they have been administrated to humans and animals (Kümmerer, 2009a, b). Furthermore, antibiotic consumption is expected to grow in the oncoming years, and recently, one of the biggest concerns by the World Health Organization (WHO) is the antibiotic/antimicrobial resistance. Microorganisms (such as fungi, bacteria, and parasites) have developed to reduce the efficacy of these compounds. This reduction of effectiveness may be a global human health issue.

In this work, **sulfamethazine** (SMZ) was selected among many antibiotics. SMZ is mainly used in veterinary farming. Although, its excretion rate has been found to be very high, reaching the 90% (Kim et al., 2011). SMZ is relative persistent in the environment (Pérez et al., 2005; Carstens et al., 2013), and due to its properties, SMZ is expected to be highly mobile in soil and potentially leachable to groundwater (Thiele-Bruhn, 2003).

Triclosan (TCS) is an antibacterial and antifungal compound widely used as an antibacterial, bactericide, and disinfectant agent. TCS was first registered in 1969 as a pesticide by the United States Environmental Protection Agency (USEPA). In 1972, TCS was used in hospitals and health care settings for its antibacterial properties. Today it is still registered as a pesticide, although it has been used in many personal care products (such as soaps, deodorants or toothpastes) to slow or stop the growth of bacteria or fungi. TCS is fat- soluble compound that can penetrate the skin and enter to the bloodstream (Moss et al., 2000). A study conducted by Calafat et al. (2008) exhibited that TCS was detected in human urine in 74.6% of the samples examined. Laboratory studies suggested that TCS has reproductive endocrine-disrupting effects (Wang and Tian, 2015). Therefore, in 2015 the European Chemicals Agency (ECA) restricted the use of TCS and in 2016, the US Food and Drug Administration (FDA) also banned from soaps, although it can still be used in toothpaste and other personal care products with a maximum concentration of 0.3% w/w (FDA, 2016).

Parabens (esters of 4-hydroxybenzoic acid, PBs) are also used in personal care products as preservatives for their bactericidal and fungicidal properties to prevent the growth of harmful bacteria and mold. PBs can be found in cosmetic products (e.g. makeup, hair and shaving products) or in food (as food additive). The most common PBs are methyl,

ethyl, and propyl paraben. They have been found in a huge amount of food products such as beverages, meat or fruit (Liao et al., 2013). **Methyl paraben** (MePB, E218) and ethyl paraben were the most detected PBs in food samples. Despite that MePB is rapidly degraded in human body (Moos et al., 2016), it has been found in the free form in 75% of human urine samples in a study from the USA (Calafat et al., 2010). MePB concentration ranged from 1.0 to 17,300 μg L^{-1}. Several studies pointed out that PBs could have adverse human health effects such as estrogenic properties (Chen et al., 2007; Darbre and Harvey, 2008), sperm DNA damage (Meeker et al., 2011), and reproductive tract disorders (Fernandez et al., 2016); the EU has banned some of them and limited the maximum concentration of parabens in 0.4% for single ester and 0.8% for mixtures of esters (European Comission, ED/1004/2014).

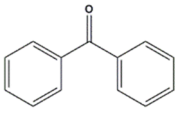
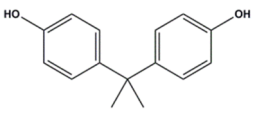
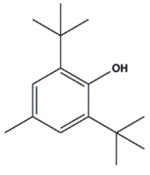
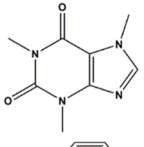
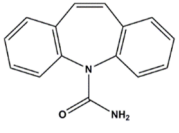
Personal Care Products

Benzophenone (BZP) is used as ultraviolet (UV) filter in many personal care products and packaging because it prevents photo-degradation from ultraviolet (UV) light. There are many BPZ derivatives which have functional groups in the aromatic structure and are used as ingredients in sunscreen. Derivatives of BPZ are common ingredients in sunscreen. Benzophenone is persistent, bioaccumulative and toxic (Kim and Choi, 2014). BPZ and BPZ-derivatives are linked to cancer, endocrine disruption, and organ system toxicity (European Food Safety, 2009).

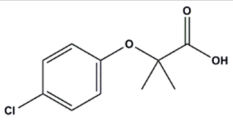
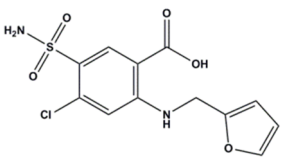
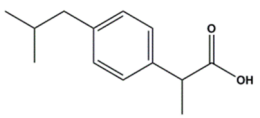
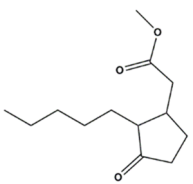
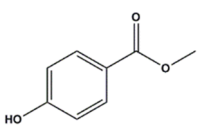
Many personal care products contain fragrances that can be obtained from natural products or can be synthetized. Two examples of fragrances in this work are tonalide and methyl dihydrojasmonate. **Tonalide** (TON) is a polycyclic musk that has been found in rivers and in WWTP effluents (Ortiz de García et al., 2013; Lange et al., 2015) even it is a volatile compound. Similar to TON, **methyl dihydrojasmonate** (MDHJ) is a fragrance with a smell similar to jasmine. It is commonly found in WWTP effluents and its removal rates are around 50-60% (Matamoros et al., 2017).

Finally, perhaps one of the most world-consumed substances is **caffeine** (CAF) which is found in several beverages, food, medicaments, etc. (Chen et al., 2002). Because CAF is mainly used by humans, it is commonly used as urban tracer, and large amounts of CAF have been detected in all kind of waters, reaching high μg L^{-1}.

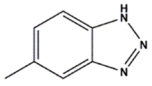
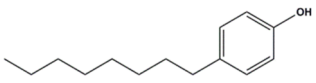
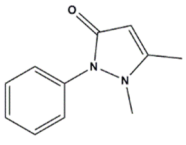
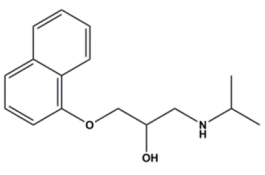
Table 1.1: Physical-chemical properties of the studied CECs in this work.

CEC	Chemical structure	Molecular weight (g mol^{-1})	pKa	Speciation (z)	Solubility (mg mL^{-1})	Log K_{AW} (m^3 m^{-3})	Neutral log K_{ow} (m^3 m^{-3})	Log D_{ow} pH = 7 (m^3 m^{-3})
Benzophenone (BZP)		182.22	NA	0	0.17	-4.3	2.98	2.98
Bisphenol A (BPA)		228.29	9.7/10.5	0/-1/-2	0.13	-7.3	3.46	3.46
Butylated hydroxytoluene (BHT)		220.35	12.1	0/-1	0.0046	-1.8	5.06	5.06
Caffeine (CAF)		194.19	NA	0	21.5	-9.7	0.11	0.11
Carbamazepine (CBZ)		236.27	NA	0	0.084	-7.1	2.23	2.23

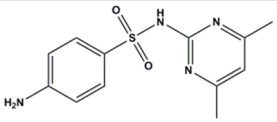
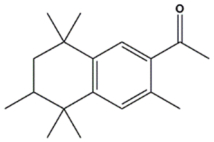
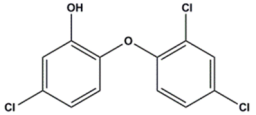
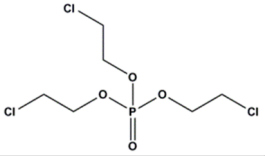
(continuation) Table 1.1: Physical-chemical properties of the studied CECs in this work.

CEC	Chemical structure	Molecular weight (g mol^{-1})	pKa	Speciation (z)	Solubility (mg mL^{-1})	Log K_{AW} (m^3 m^{-3})	Neutral log K_{ow} (m^3 m^{-3})	Log D_{ow} pH = 7 (m^3 m^{-3})
Clofibric acid (CFA)		214.64	4.0	0/-1	0.55	-5.7	2.68	-0.34
Furosemide (FUR)		330.74	3.5/9.0	0/-1/-2	0.1	-10.7	2.42	-0.99
Ibuprofen (IBU)		206.28	4.3	0/-1	0.12	-4.9	3.63	0.95
Methyl dihydrojasmonate (MDHJ)		226.31	NA	0	0.2	-4.4	3.6	3.6
Methyl paraben (MePB)		152.15	8.5	0/-1	1.3	-4.6	1.97	1.95

(continuation) Table 1.1: Physical-chemical properties of the studied CECs in this work.

CEC	Chemical structure	Molecular weight (g mol^{-1})	pKa	Speciation (z)	Solubility (mg mL^{-1})	Log K_{AW} (m^3 m^{-3})	Neutral log K_{ow} (m^3 m^{-3})	Log D_{ow} pH = 7 (m^3 m^{-3})
5-Methyl-1H-benzotriazole (MeBT)		133.15	1.6 / 8.5	+1/0/-1	3.7	-5.4	1.57	3.6
4-Octylphenol (OPL)		206.32	10	0/-1	0.0026	-3.0	5.64	1.95
Phenazone (PZE)		188.23	1.8	+1/0	75.8	-7.3	0.85	1.56
Propranolol (PROP)		259.34	9.5/13.9	+1/0/-2	1.2	-9.5	2.92	0.53

(continuation) Table 1.1: Physical-chemical properties of the studied CECs in this work.

CEC	Chemical structure	Molecular weight (g mol^{-1})	pKa	Speciation (z)	Solubility (mg mL^{-1})	Log K_{AW} (m^3 m^{-3})	Neutral log K_{ow} (m^3 m^{-3})	Log D_{ow} pH = 7 (m^3 m^{-3})
Sulfamethazine (SMZ)		278.33	1.3/3.1/7.2	+2/+1/0/-1	0.51	-11.5	0.31	0.1
Tonalide (TON)		258.4	NA	0	0.0019	-3.7	5.71	5.71
Triclosan (TCS)		258.4	8.8	0/-1	0.0046	-4.0	5.21	5.21
Tris(2-chloroethyl) phosphate (TCP)		285.49	NA	0	5	-6.5	1.72	1.72

NA: Not applicable; z is charge number (valency). All values were estimated by using the ACD Advanced Chemistry Development (2010), ACD/i-lab 2.0. Toronto, 2010.

In the last years, several authors have reported the occurrence of several CECs in all kind of waters. As the occurrence of CECs is mainly because of anthropogenic activities, their concentrations depend on the region, the seasonality or other human activities.

Table 1.2 shows the concentration of some of the studied CECs in WWTP influents and effluents, in surface water and in drinking water.

Table 1.2: Occurrence of CECs in the aquatic environment.

CEC	WWTP influent (ng L^{-1})	WWTP effluent (ng L^{-1})	Surface water (ng L^{-1})	Drinking water (ng L^{-1})	Reference
BZP	453-1,548	339-1,450	1-68	1-790	11, 20, 25, 26, 35, 41, 58, 64
BPA	13-5,620	30-1,100	0.5-2,970	0.5-2,550	11, 13, 16, 28, 36, 37, 39, 42, 47, 48, 57, 61, 65
BHT	213-2,420	48-262	49-620	62-455	5, 6, 33, 56
CAF	220-7,370	3-2,440	62-1,048	291-526	4, 7, 8, 14, 18, 34, 55, 67
CBZ	27-3,780	5-4,600	20-595	1-601	8, 27, 31, 43, 48, 49
CFA	12-420	5-2,102	11-21	5.3-630	12, 32, 36, 60, 61
FUR	50-2,000	60-1,100	1.72-3,200	ND[a]	9, 24, 52
IBU	4-115,000	13-10,160	14-414	0.5-5850	12, 21, 30, 35, 67
MDHJ	320-10,000	10-234	22-529	ND[a]	10, 29, 38
MePB	0.3-79,600	0.5-3,830	0.3-2875	0.3-86	27, 35, 45, 46
MeBT	67-6758	85-2800	2-702	2-21.4	11, 17, 34, 50, 62
OPL	160-13,000	450-1,200	50-6,300	1.6-22	23, 43, 61
PZE	300-920	160-410	1.96-450	2-80	1, 60, 69
PROP	2-168	13-290	2-40	54-270	22
SMZ	4,010-9,720	173	0.1-100	23-84	8, 14,
TON	86-2,590	13-1,200	100-226	ND[a]	2, 10, 15, 46, 63
TCS	52-86,200	40-5,370	3-478	0.6-734	3, 10, 31, 35, 52
TCP	200-1010	100-1,730	3-640	12-200	19, 32, 34, 35, 44, 51, 53

[a]N.D. Not detected; 1. Andreozzi et al. (2003); 2. Artola-Garicano et al. (2003); 3. Bedoux et al. (2012); 4. Behera et al. (2011); 5. Bendz et al. (2005); 6. Benotti et al. (2009); 7. Boleda et al. (2011); 8. Cabeza et al. (2012); 9. Calamari et al. (2003); 10. Calderón-Preciado et al. (2011b); 11. Careghini et al. (2015); 12. Carmona et al. (2014); 13. Céspedes et al. (2008); 14. Choi et al. (2008); 15. Clara et al. (2011); 16. Felix-Canedo et al. (2013); 17. Focazio et al. (2008); 18. Fram and Belitz (2011); 19. Fries and Puttmann (2001); 20. Gago-Ferrero et al. (2013); 21. Gracia-Lor et al. (2012); 22. Huerta-Fontela et al. (2011); 23. Janex-Habibi et al. (2009); 24. Jelic et al. (2011); 25. Jeon et al. (2006); 26. Kameda et al. (2011); 27. Kasprzyk-Hordern et al. (2008); 28. Kasprzyk-Hordern et al. (2009); 29. Klaschka et al. (2013); 30. Kleywegt et al. (2011); 31. Kumar et al. (2010); 32. Li et al. (2014); 33. Liu et al. (2015); 34. Loos et al. (2013); 35. Loraine and Pettigrove (2006); 36. Luo et al. (2014); 37. Martin et al. (2012); 38. Matamoros et al. (2012); 39. Nie et al. (2012); 40. Patrolecco et al. (2015); 41. Pojana et al. (2007); 42. Pothitou and Voutsa (2008); 43. Rajasärkkä et al. (2016); 44. Reemtsma et al. (2008); 45. Regueiro et al. (2009); 46. Reiner et al. (2007); 47. Rosal et al. (2010); 48. Santhi et al. (2012); 49. Santos et al. (2009); 50. Scheurer et al. (2011); 51. Singer et al. (2002); 52. Singer et al. (2010); 53. Stackelberg et al. (2004); 54. Stamatis et al. (2010); 55. Stasinakis et al. (2008); 56. Teijon et al. (2010); 57. Terzić et al. (2008); 58. Tsui et al. (2014); 59. Valcárcel et al. (2011); 60. Versteegh and Dijkman (2007); 61. Vethaak et al. (2005); 62. Wang et al. (2016c); 63. Yang and Metcalfe (2006); 64. Yoon et al.

(2010); 65. Yu and Chu (2009); 66. Zhou et al. (2010); 67. Zorita et al. (2009); 68. Zuccato et al. (2000); 69. Zuehlke et al. (2006).

1.2.4 Dynamics of CECs in soil

Once the chemicals reach the soil, CECs are subjected to different biotic or abiotic processes such as plant bioaccumulation, sorption or desorption, and degradation processes which influence the bioavailable concentration.

Sorption is the physical-chemical process that includes the possible interactions that determine the retention of a chemical in the soil. It governs the fate, mobility and the effects that can have the contaminants in the environment. The sorption process corresponds to the interchange of substance (sorbates) between a liquid or gas phase and a solid phase (sorbent) at equilibrium. A CEC can interact with the sites in the surface of the sorbent by physical or chemical processes. Soil contains several functional groups (phenols, hydroxy or carboxy) in the organic matter and in the mineral phase where CECs can interact with. Depending on the solubility and hydrophobicity, the partition between soil and CEC can change and the fate of the different CECs may differ.

Moreover, as it has been mentioned before, CECs can occur as neutral or ionic form (e.g. anionic, cationic or zwitterionic), depending on the soil pH. Consequently, the behavior in the soil depends on the compound and the pH that they are found and different interactions can take place. Neutral species exhibit hydrophobic interactions, while ionic species can have other interactions such as ionic interchange.

Sorption is usually described by K_d (see section 1.2.2) and the higher K_d values, the stronger CECs are sorbed to the soil and less bioavailable are. On the other hand, CECs with low K_d values (e.g. sulfonamides) are less sorbed in the soil and they are more mobile and can leach to groundwater or be taken up by plants.

Another important soil process is the formation of non-extractable residues (NER), which are chemical species (active ingredient, metabolites and fragments) that are not extracted by methods which do not significantly change the chemical nature of these residues but which remain in the soil. Non-extractable CECs residues involve various physical and/or chemical interactions between the compound and the soil structure, depending on the xenobiotic in question. Types of interactions that may be involved in non-extractable residue formation are van

der Waals forces, ligand exchange, charge-transfer complexes, hydrophobic partitioning, covalent bonding and sequestration (Mordaunt et al., 2005).

Several CECs can suffer from other abiotic processes such as photolysis or hydrolysis. Hydrolysis is a chemical transformation process of CECs in the environment and it is mainly governed by pH and temperature (Maszkowska et al., 2014a; Yin et al., 2017). Photolysis, or also called photo-dissociation, is usually a chemical oxidation reaction activated by photons. Several researches are focused on photo-degradation of CECs in water, because ultraviolet light (UV) can be used instead of chlorine, iodine or bromine in WWTP. UV radiation causes damage to the genetic structure of bacteria, viruses, and other pathogens leading to their inactivation and it has been shown that in combination with H_2O_2, the degradation rates of CECs in the WWTP increased (Kim et al., 2009; Chu et al., 2016). The main problem is that this tertiary treatment is expensive and can form reactive disinfection by-products that can impair human health (Benitez et al., 2013; Chu et al., 2014).

Finally, there can be other biotic processes that reduce the bioavailability of CECs. In this regard, bioaccumulation and biotransformation play an important role. For example, in water or in soil, there are many organisms that can be in contact with CECs and absorb them. Once the contaminants are taken up, depending on its recalcitrance, they can be accumulated or transformed to be removed from the organism's body. In this present work, the fate of CECs in the soil-plant system has been studied.

1.3 Uptake of CECs by plants

Plants are exposed to many CECs that are present in the environment, especially in the soil. This exposure may lead to a bioaccumulation of CECs in plants such as crops, which may possess a human risk if they are consumed.

Depending on environmental factors and plant physiology, crops require large amounts of water to develop. Water flows through plants in interconnected system of pipes called xylem that are closed at both ends, the roots and the leaves, by cell walls. The roots are organs taking up the water from the soil, and for this purpose, they have very high surface area. Water and solutes move from soil to the interior of roots and at the endodermis, this movement is stopped by the

Casparian strip. Both water and solutes must pass one cell to enter the symplast. 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. The xylem is the conductive system for the transpiration stream. Water and solutes are transported upwards to the leaves, where photosynthesis takes place. Then, the produced assimilates there are transported to the sinks through the phloem, the other conductive system that leads to all parts of the plant.

There are two major ways that molecules can be moved across a membrane: passive mechanisms like diffusion or osmosis that use no energy or active transport that requires energy. Active transport refers to a movement of a molecule or a solute from a region with lower concentration of ions to a region with a higher concentration of ions. Conversely, passive transport is the movement of molecules or solute from a region with a higher concentration of ions to a region with lower concentration of ions.

Amino acids, sugars, and lipids need to enter the cell by protein pumps, which require active transport powered by energy. This energy is known as adenosine triphosphate (ATP). On the other hand, CECs are taken up by plants mainly by passive diffusion or advection and their transport does not require energy. In fact, CECs reach the plants predominantly from the use of reclaimed wastewater for irrigation, the manure for fertilization, and the application of biosolids. The main passive transport and uptake processes are: (i) uptake with transpiration water, (ii) diffusion from soil into roots, (iii) attachment of soil particles followed by diffusion into plant tissue, (iv) diffusive exchange with air, and (v) particle deposition from air on plant surfaces followed by diffusion into plant tissue (Trapp, 2004).

Once the CECs enter to the roots, they can go through three different pathways. The first one is the apoplastic route, where CECs are transported along cell walls through the intercellular spaces. Another route is the simplastic one, where CECs move in the between cells through interconnecting plasmodesmata (Figure 1.3). Finally, the third pathway is the transmembrane route, where CECs move between cells through cell walls and membranes (Raven et al., 2005). The contaminants will undergo in the different routes depending on the ability of

them to cross membranes into cells, however CECs taken up only by the apoplastic route cannot cross the Casparian strip. CECs need to cross at least one lipid bilayer to enter the xylem and be transported to aerial parts (Miller et al., 2016).

The ionization form and the lipophilicity play an important role in root uptake. Neutral compounds can easier cross membranes than ionic compounds. For this reason, uptake of neutral CECs is usually expected to be higher than ionic CECs and their translocation is also higher because they can move easily from xylem to phloem transported with the water stream. On the contrary, ionic compounds can suffer from electrostatic interactions that may reduce their uptake (Trapp, 2009).

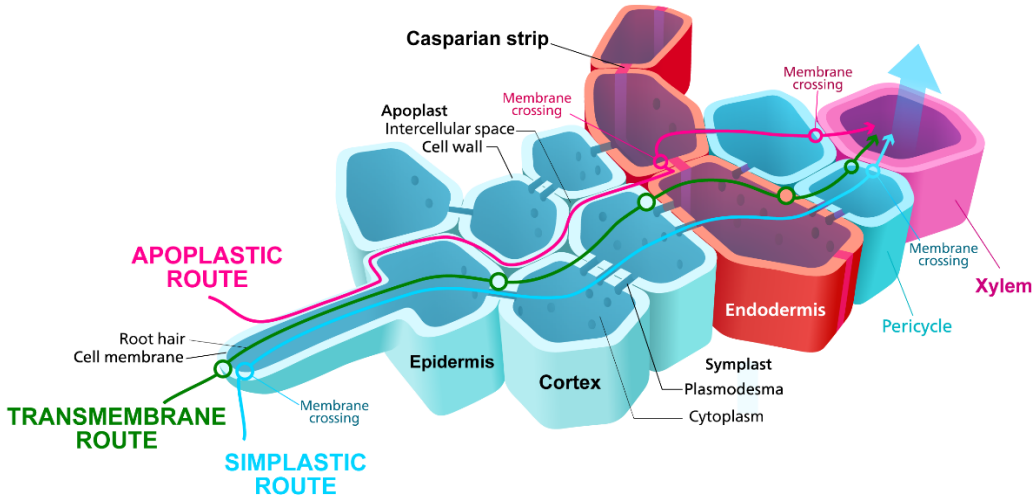


Figure 1.3: Transversal cut of root cells showing the apoplastic (red), simplastic (blue), and transmembrane routes. Original image was obtained from Plant cell cycle, Wikimedia Commons.

Hydrophilic CECs are highly soluble in water and they are able to cross easily cell membranes. For this reason, hydrophilic CECs move with water transpiration stream reaching the aerial parts. Oppositely, hydrophobic CECs, and therefore high lipophilic compounds, may be predominantly retained by root tissue due the interactions with cell walls and lipids.

Bioconcentration factors (BCFs) are used to express uptake of a contaminant. It is usually the ratio between a contaminant in plant tissue and in soil or solution,

if working in hydroponics. As plants have specific tissues (i.e. roots, leaves, stems, and fruits), specific BCFs can be described.

For example, the root concentration factor (RCF) is a ratio between the concentrations of a CEC in the roots and in soil or the irrigation concentration (Equation 1.8).

$$RCF = \frac{C_{root}}{C_{soil}} \tag{1.8}$$

Briggs et al. (1982) studied the root uptake of several organic neutral compounds and it was the first study that reported a relationship between RCF and the hydrophobicity of the compounds using the log K_{ow} (Figure 1.4).

For the non-ionic studied compounds, the RCF increased with log K_{ow} and the fit curve between them is as follows:

$$RCF = 0.82 + 0.03\,K_{ow}^{0.77} \tag{1.9}$$

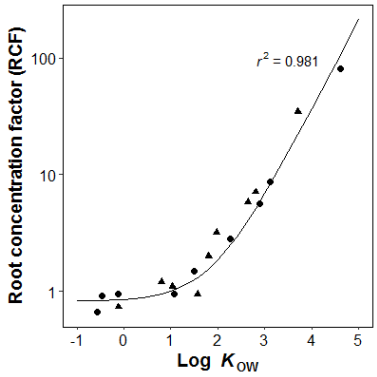


Figure 1.4: Relationship between root concentration factors (RCF) and log K_{ow} from Briggs et al. (1982)

Neutral lipophilic CECs (log K_{ow} > 4) can sorb to lipids found in the root membranes, exhibiting a higher accumulation. Conversely, lipophobic CECs are more water soluble and their sorption and accumulation in roots is expected to be much lower.

Depending on the media pH, ionizable CECs may be found in both neutral and ionic species in soil, solution or cell system. Interestingly, neutral and ionic species have different chemical behaviors. For instance, the neutral form can be sorbed to soil, dissolved in the soil solution or in the cell lipids, and it can even cross cell membranes faster than ionic form. Ionic fraction exhibits lower lipophilic sorption than the neutral form due to the permeability of cell membranes (Trapp, 2004). Because of the negative charge in the protoplast, cationic form accumulates usually higher than anionic form due to attraction, while in anionic may undergo with repulsions (Goldstein et al., 2014).

Furthermore, ionizable CECs may be subjected to ion trap effect (Figure 1.5). For example, an ionizable CEC found predominantly in anionic form in soil solution, may become uncharged in the rhizosphere, where the pH is lower than in the soil solution (Azcón-Bieto and Talón Cubillo, 2000), and be taken up easier. Inside the cell, cytosol has a neutral pH (7 – 7.4); however, the neutral form may become negatively charged in the vacuole (pH 4 – 5.5), which is the largest fraction of an adult cell, and this may limit its further transport, becoming trapped in the vacuole (Trapp, 2004).

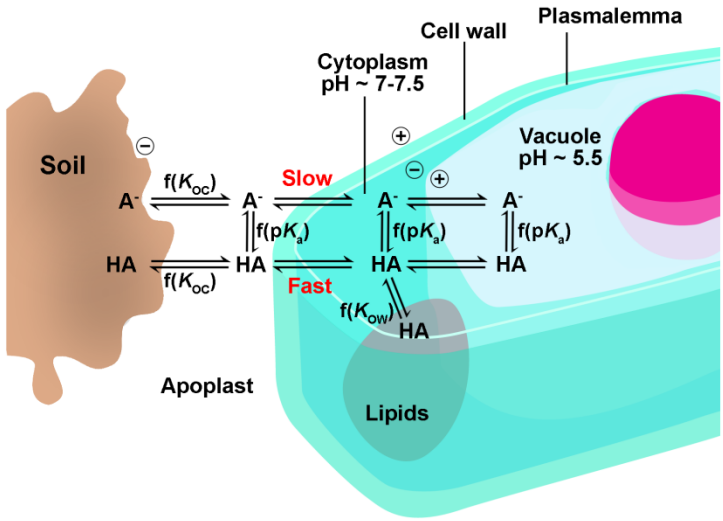


Figure 1.5: Molecule species involved in the ion trap effect of a weak acid (HA) and the dissociated anion (A^-) based on Trapp (2000).

Once the CECs are taken up by the roots, their translocation is mostly carried out by the transpiration stream of water up to the leaves. Therefore, the transpiration stream concentration factor (TSCF) is defined as the concentration ratio between xylem sap and soil or solution. Although it is very useful for removal calculations, it is difficult to measure experimentally and it is time dependent. Chemicals that are taken up actively, have TSCF values higher than 1 (N, P or K), while those that move with the water by transpiration stream have values less than one.

In the same experiment, Briggs et al. (1982) found also a Gaussian-like relationship between the TSCF and the log K_{ow}:

$$TSCF = 0.784 \exp \frac{-(\log K_{ow} - 1.78)^2}{2.44} \tag{1.10}$$

Few years later, Hsu et al. (1990) reported a similar relationship for neutral oxabicycloalkanes:

$$TSCF = 0.7 \exp \frac{-(\log K_{ow} - 3.07)^2}{2.78} \tag{1.11}$$

Recently, Dettenmaier et al. (2009) measured the TSCF of 25 neutral organic compounds in two different plants and found out that compounds with low values of log K_{ow} were also translocated, and reported the relationship as follows:

$$TSCF = \frac{11}{11 + 2.6^{\log K_{ow}}} \tag{1.12}$$

The three different relationships are shown in Figure 1.6.

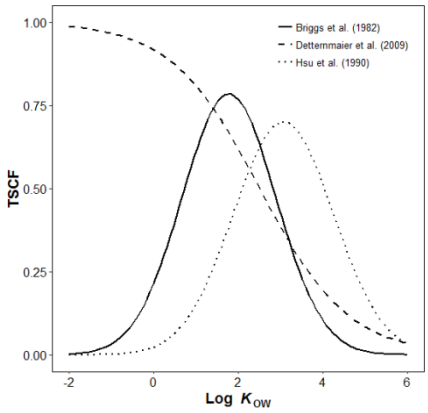


Figure 1.6: TSCF versus log K_{ow} of the three selected regressions.

While the first two authors reported low translocation for highly hydrophilic compounds, Dettenmayer et al. (2009) reported that neutral compounds (such as sulfolane or caffeine) with log K_{ow} ranging from -2 up to 3 can be easily translocated. In fact, hydrophilic CECs with log $K_{ow} < 0$ are mobile in both xylem and phloem, intermediate lipophilic CECs ($0 < \log K_{ow} < 3$) are mobile mainly in xylem, while hydrophobic CECs tend to be sorbed to the cell membranes, and for this reason, their TSCF decrease (Trapp, 2009).

Nevertheless, ionic CECs do no follow none of these relationships, even if log D_{ow} is used instead of log K_{ow} probably due ion trap effects (Miller et al., 2016).

Another important uptake pathway of CECs is through air. Leaves hang in the air, and they can be exposed to the presence of CECs in the atmosphere. Therefore, CECs accumulation in aerial tissue can occur from dry or wet deposition from air on plant surfaces followed by diffusion or by diffusive exchange with air. Volatile CECs such as fragrances may volatilize from soil to the atmosphere and diffuse into the leaves via the cuticle or the stomata, which control the gas exchange. Air enters to the plant through stomata by gaseous diffusion, and is used in the photosynthesis and respiration. The stomata provide an entry to CO_2 and an exit route for water vapor, but the stomatal entry route can only be significant for CECs present in air in the gaseous phase (Smith and Jones, 2000). These CECs can diffuse into intercellular air spaces and partition to the aqueous and lipophilic phases of adjacent plant tissues (Collins et al., 2006).

Compounds such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and other volatile compounds have been reported to be taken up by plants mainly by gaseous uptake (Simonich and Hites, 1994; Böhme et al., 1999; Meneses et al., 2002). Different studies have related the gaseous uptake to the octanol-air partition coefficient (K_{OA}) for many organic chemicals (Paterson et al., 1991; Tolls and McLachlan, 1994; Kömp and McLachlan, 1997). K_{OA} is related to Kow and H and can be estimated with the following equation:

$$K_{OA} = \frac{K_{ow} RT}{H} \tag{1.13}$$

where R is the ideal gas constant and T is the absolute temperature. H/RT is the unitless Henry's law constant, which can be quantified by the experimental K_{AW} (Meylan and Howard, 1991; Meylan and Howard, 2005). Then, the equation can be rewritten as:

$$K_{OA} = \frac{K_{ow}}{K_{AW}} \tag{1.14}$$

Taking into account the physical-chemical properties, the dominant uptake pathways can be estimated. In roots, the dominant uptake processes are advection in with soil pore water for chemicals with log $K_{ow} > 1$ and phloem mediated uptake from the leaf compartment for chemicals with log $K_{ow} < 1$ and log $K_{AW} < -6$. In air, for instance, CECs with log $K_{OA} < 11$ and $K_{AW} > -3$ are mainly taken up in lettuce leaves by gaseous deposition to the leaf surface, due to the high volatility of CECs. For hydrophilic chemicals of low log K_{ow} (< 4) and low log K_{AW} (< −5), uptake occurs mainly via the roots. For these CECs, the uptake in the leaf will be controlled by the soil concentration and the transpiration stream flow. For very lipophilic chemicals (log $K_{OA} > 11$ and log $K_{ow} > 6$), such as fragrances, partitioning to airborne particles and their deposition on the leaf surface is the dominant uptake route to the lettuce leaf (Undeman et al., 2009).

1.3.1 Other factors affecting the plant uptake

Finally, several parameters in the soil-water-plant system may affect the uptake and translocation of CECs. In soil, the texture, organic matter content, and pH will be determinant in the bioavailability of CECs, and they can reduce the plant uptake.

Neutral compounds exhibit higher polar interactions with the organic matter in soil than ionic compounds. For example, Shenker et al. (2011) studied the uptake of CBZ by cucumbers with 3 types of soil and concluded that the amount of CBZ taken up by cucumbers was lower when soil had higher amount of organic content. Similar results were obtained by Goldstein et al. (2014) for neutral CECs, observing an increase in plant uptake when the soils had lower organic content. For ionizable CECs, pK_a is not only the determinant parameter, but also the soil pH, which can alter the ionizable bioavailable fraction.

Another parameter that has been shown to have a great determination is water quality. There are many parameters in water that can have influence on the bioavailability of CECs. Similar to organic matter in soil, the dissolved organic carbon and the pH can affect significantly the uptake of CECs. Goldstein et al. (2014) and Malchi et al. (2014) studied the influence of differences between fresh water and reclaimed water in PPCPs plant uptake. They observed that for neutral PPCPs, there were no differences; however, for weak acids, the uptake in reclaimed water was lower compared to the fresh water, probably due to polar interactions between the weakly acidic PPCPs and the dissolved organic matter in water, reducing the bioavailability (Maoz and Chefetz, 2010).

Table 1.3: Literature bioaccumulation factor (BCF) values of the studied CECs in this work in lettuce.

CEC	Experiment type	Tissue	BCF	Ref
BPA	Hydroponic	Leaf	7.8 L kg^{-1} dw	1
		Root	9519 L kg^{-1} dw	1
CAF	Hydroponic	Leaf	0.14 - 6.4 L kg^{-1} dw	6
		Root	0.06 - 3.0 L kg^{-1} dw	6
BZ	Hydroponic	Leaf	50 - 58 L kg^{-1} dw	6
		Root	20 - 26 L kg^{-1} dw	6
	Soil	Leaf	126.88 L kg^{-1} dw	5
		Root	15.71 L kg^{-1} dw	5
		Leaf	60 L kg^{-1} dw	3
IBU	Hydroponic	Leaf	0 L kg^{-1} dw	6
		Root	0.6 - 0.7 L kg^{-1} dw	6
PMD	Hydroponic	Leaf	12 – 17 L kg^{-1} dw	6
		Root	5.4 - 7.2 L kg^{-1} dw	6
SMT	Soil	Leaf	0.064 - 0.0124 kg kg^{-1} dw	4
		Leaf	0.01 - 0.02 L kg^{-1} dw	2
TCP	Soil	Leaf	65 L kg^{-1} dw	3
TCS	Hydroponic	Leaf	0 L kg^{-1} dw	6
		Root	112 – 138 L kg^{-1} dw	6

1. Dodgen et al. (2013); 2. Dolliver et al. (2007); 3. Hyland et al. (2015); 4. Rajapaksha et al. (2014); 5. Riemenschneider et al. (2016); 6. Wu et al. (2013).

There are many plant parameters that can affect the uptake of contaminants. For example, there are many types of plants or crops, which have different plant morphology. Depending on the plant species, parameters such as water and lipid content, and growth rate, the concentration of CECs in different parts of the plant can be completely different. One of the most important parameter in plant uptake is transpiration, because most CECs are taken up and translocated with the transpiration stream. Transpiration depends on the plant species, temperature, humidity, and availability of water.

Table 1.4: Carbamazepine bioconcentration factor (BCF) values found in the literature for different crops.

Plant tissue	BCF	Ref
Alfalfa		
Leaf	0.2 L kg^{-1} dw	1
Apple tree		
Leaf	0.3 L kg^{-1} dw	1
Cabbage		
Fruit	5.8 L kg^{-1} dw	5
Leaf	46.5 L kg^{-1} dw	5
Root	36.1 L kg^{-1} dw	5
Carrot		
Leaf	2.5 - 7.3 kg kg^{-1} dw	4
	36.0 L kg^{-1} dw	5
Root	1.0 - 3.0 kg kg^{-1} dw	4
	8.2 L kg^{-1} dw	5
Cucumber		
Fruit	4.1 - 41.5 L kg^{-1} dw	3
Leaf	60 - 406 L kg^{-1} dw	3
	6.8 - 16.1 L kg^{-1} fw	6
Root	0.67 - 3.04 L kg^{-1} fw	6
Stem	0.37 - 1.36 L kg^{-1} fw	6
Eggplant		
Fruit	18.9 L kg^{-1} dw	5
Leaf	45.6 L kg^{-1} dw	5
Root	113.3 L kg^{-1} dw	5
Stem	8.2 L kg^{-1} dw	5
Lettuce		
Leaf	126.9 L kg^{-1} dw	5
Root	15.7 L kg^{-1} dw	5
Parsley		
Leaf	53.3 L kg^{-1} dw	5
Root	24.0 L kg^{-1} dw	5
Pea		
Leaf	1.4 - 4.6 L kg^{-1} fw	7
Root	0.18 - 0.44 L kg^{-1} fw	7
Stem	0.67 - 0.99 L kg^{-1} fw	7
Pepper		
Fruit	4.9 L kg^{-1} dw	5
Root	23.5 L kg^{-1} dw	5
Stem	17.8 L kg^{-1} dw	5
Potato		
Leaf	101.8 L kg^{-1} dw	5
Root	45.1 L kg^{-1} dw	5
Stem	35.1 L kg^{-1} dw	5
Sweet potato		
Leaf	0.8 - 1.5 kg kg^{-1} dw	4
Root	0.2 - 0.25 kg kg^{-1} dw	4
Radish		
Leaf	60.6 kg k^{-1} dw	2
Root	8.3 kg k^{-1} dw	2
Rucola		
Leaf	35.7 L kg^{-1} dw	5
Root	22.1 L kg^{-1} dw	5
Stem	4.4 L kg^{-1} dw	5
Ryegrass		
Leaf	65.3 kg k^{-1} dw	2
Soybean		
Leaf	2.5 - 3.2 L kg^{-1} dw	8
Root	0.24 - 0.33 L kg^{-1} dw	8
Stem	0.25 - 0.42 L kg^{-1} dw	8
Tomato		
Fruit	0.46 - 2.9 L kg^{-1} dw	3, 5
Leaf	61.5 -321 L kg^{-1} dw	3
Root	15.7 L kg^{-1} dw	5
Stem	24.0 L kg^{-1} dw	5
Zucchini		
Fruit	4.0 L kg^{-1} dw	5
Leaf	24.6 L kg^{-1} dw	5
Root	40.6 L kg^{-1} dw	5
Stem	5.5 L kg^{-1} dw	5

1. Calderón-Preciado et al. (2011b); 2. Carter et al. (2014); 3. Goldstein et al. (2014); 4. Malchi et al. (2014); 5. Riemenschneider et al. (2016); 6. Shenker et al. (2011); 7. Tanoue et al. (2012); 8. Wu et al. (2010)

1.3.2 Plant metabolism

All biological organisms have self-defense mechanisms to protect them from xenobiotic compounds. In animals, the main site of xenobiotic transformation is the liver, where nonpolar contaminants are metabolized to more soluble forms that are typically excreted in urine. Although plants do not have effective excretion pathways, the term “green-liver” was firstly adopted by Sandermann (1994), to describe it similar to human detoxification system. The model is based

on three steps or phases: (i) transformation, (ii) conjugation, (iii) and elimination or storage. Therefore, CECs accumulated in plants may become metabolized, thus reducing their concentration within the plant's tissues by increasing CEC hydrophilicity and storage them as non-toxic compounds.

Transformation

The first step is the transformation or functionalization of the parent compounds to predispose them to Phase II reactions. It includes several reactions such as hydroxylation, hydrolysis or other oxidation reactions producing compounds with higher polarity (Phase I metabolism). One of the most prevalent transformations has been identified as hydroxylation, which is the process of adding an OH^- group (Burken, 2004). The addition of the OH functional group results in a suitable site for conjugation to occur. Oxidation reactions involve the loss of electrons and most of these reactions are catalyzed by cytochrome P-450 monooxygenases enzymatic system. The enzyme introduces one atom of molecular oxygen into the xenobiotic substrate and reduces the second atom of oxygen into water using a reductant, usually $NADPH_2$ o $NADH_2$. Cytochrome P-450 monooxygenases are usually membrane bound and xenobiotic metabolism occurs in the endoplasmic reticulum. Other enzymes such as peroxygenases and carboxylesterase play also important role in contaminant transformation. Although oxidations are the main reactions, some processes like reduction, ester hydrolysis or amide hydrolysis have been identified (Shang et al., 2004; Thompson et al., 2004). For instance, the most common reduction reaction in plants is aryl nitroreduction where a nitro group on a phenyl ring is reduced to an amino group. These reactions are catalyzed by aryl nitroreductases, which require a reductant such as NADPH.

Conjugation

Conjugation is usually the next step after transformation; nevertheless, in some cases, CECS are conjugated without being transformed. In mammals, sulfate glucuronic acid, and glutathione conjugates usually predominate; however, in plants major conjugates are glucosides, glutathione conjugates and amino acid conjugates. The conjugates produced in this phase are highly water-soluble and the toxicity is lower than the parent compound. Examples of enzymes that

catalyze the conjugation of compounds are the glutathione *S*-transferases (GSTs) or the glucosyltransferases (Brazier-Hicks and Edwards, 2005).

Glutathione conjugation involves the attachment of glutathione (GSH), a small tripeptide of the three amino acids glutamate, cysteine and glycine, to xenobiotic that containing usually halogen, alkyl sulfoxide or phenolate groups. Then, these conjugates are further transported into plant vacuole to remove the conjugate from the cytoplasm.

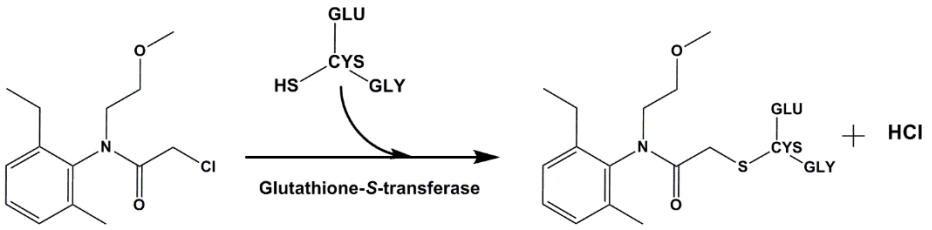


Figure 1.7: Example of a glutathione conjugation of a xenobiotic (methachlor).

In addition to inactivation of contaminants, GSTs are important in the management of plant hormones and secondary plant metabolites or the response to oxidative stress (Burken, 2004).

Glucosidation is another process of conjugation where usually, on the functional groups obtained in Phase I or other functional groups from the parent compounds, a glucoside is added. Glucosidation results in soluble glucosides that can be stored as soluble conjugates in the vacuoles or incorporated into lignin (Phase III). These reactions are catalyzed by enzymes such as UDP-glucosyl-transferase and require UDP-glucose (UDPG) as a substrate. The glucose can be attached to phenol (*O*-glucosylation) or N-arylamine (*N*-glucosylation) groups via a ß-glucoside linkage.

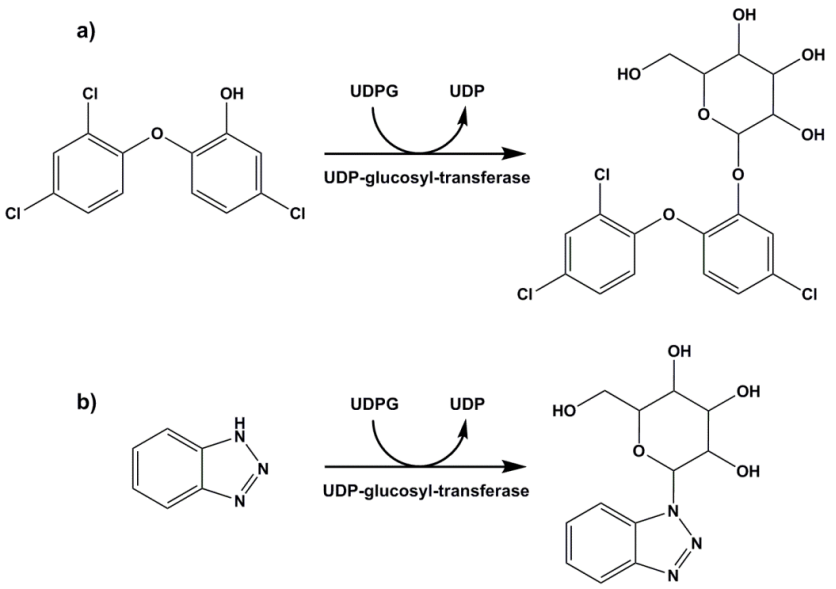


Figure 1.8: Examples of glucosilation of two different xenobiotics: (a) O-glucosylayion of triclosan and (b) N-glucosylation of benzotriazole

Sequestration

Finally, the third step in the green liver model is the sequestration of the conjugated CECs. As plants are not able to excrete conjugates via the urine, they storage the soluble conjugates in the vacuole and insoluble conjugates in the cell walls (Komossa et al., 1995). However, before the sequestration a secondary conjugation may occur to enhance the vacuolar sequestration. The most common reaction is the malonyl CoA conjugation, where a malonic acid residue is attached by an ester linkage to a Phase II xenobiotic conjugate. Then, the sequestration may lead to bound residues that are not extractable in common laboratory solvents and are not accessible to standard residue analysis. Some studies have shown that this fraction could be higher than the parent compound (Sandermann, 2004).

Recently, some studies have been focused on the detection of Phase I and II metabolites in plants. For instance, among the studied CECs in this work, some metabolites of BPA, CBZ and TCS have been detected in different crops such as lettuce, potato or tobacco (Malchi et al., 2014; Riemenschneider et al., 2016).

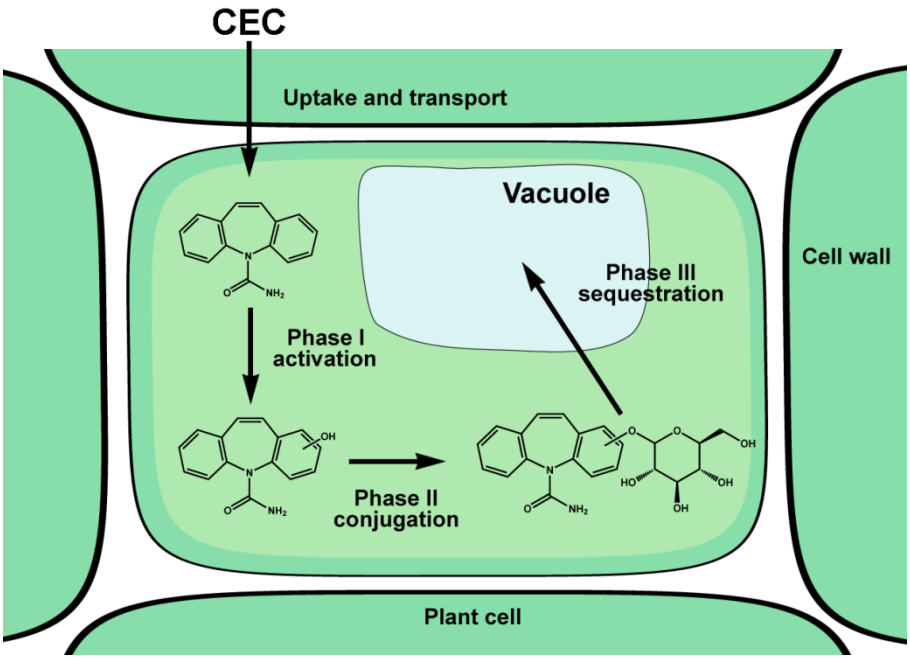


Figure 1.9: Representation of the three phases of the green liver model. Hypothetical example of CBZ transformation, conjugation and sequestration in plant cells (adapted from Van Aken (2008)).

Although green liver model could explain the metabolism in plants, there can be other CEC degradation processes. For example, photolytic degradation on plant surfaces has been demonstrated to be a significant loss mechanism (Niu et al., 2004). This degradation process is enhanced by the leaves movement to maximize the interception of sunlight. Moreover, plant transpiration stream can move CECs to foliar tissues from which CECs can be volatized. This pathway was found to be significant for volatile organic compounds like trichloroethylene or benzene (Collins et al., 2000; Ma and Burken, 2003).

Many microorganisms such as bacteria and fungi live in the soil-plant system that can metabolize these contaminants. Endophytic bacteria live inside the plants, in roots and leaves, and it has been shown that these bacteria could metabolize some contaminants (Lodewyckx et al., 2002). For this reason, it is difficult to discern where exactly CEC metabolization takes place in plants (Sauvetre and Schröder, 2015).

Plants respond to fluctuations in their environment. For example, changes in temperature, humidity or other environmental stressors may affect the growth or productivity. Biotic and abiotic stress conditions produce reactive oxygen species in plants causing oxidative stress damage (Krishnamurthy and Rathinasabapathi, 2013). Furthermore, CECs could act as stressors for plants and some adverse effects have been yet reported. Some antibiotics like enrofloxacin induced toxic effects and hormesis in cucumber, lettuce, and radish by modifying the length of roots and leaves at concentrations between 0.05 – 50 mg L^{-1} (Migliore et al., 2003). CBZ effects were observed at much higher concentration (10 mg L^{-1}) than environmental levels with a biomass reduction of 50% and reduced root length and the number of leaves (Shenker et al., 2011).

The effects of most CECs at relevant environmental concentrations to plants are still unknown. To study them, there are two main strategies: measuring the enzymatic activity or studying the differences in plant metabolites, proteins or genes, when plants are exposed to contaminants. Metabolomics aims to study the plant system at the molecular level to provide a characterization of the total metabolome of a plant and includes the analysis of carbohydrates, amino acids and other organic compounds. Thus, measuring all these compounds is still an analytical challenge due to the complexity of the matrix samples (Allwood et al., 2011).

As it has been discussed before, surface and groundwater are polluted with many contaminants. Removing these contaminants from irrigation water is very expensive. In the agricultural sector, a common practice is the addition of amendments to the soil to improve its fertility and crop productivity. For this reason, inorganic and organic substances are added to improve the soil structure and water holding capacity, support the nutrient cycle and to add nutrients to the soil. Among organic amendments, waste disposal substances such as manure, compost or biosolids and leachates are commonly applied because they are economically affordable. Despite these amendments, biochar has received the attention of not only farmers but also among the scientific community, because its interesting high sorbent capacity, that could be able to reduce the concentration of contaminants in water.

1.4 Biochar

Biochar (BC) is a type of black carbon described as a "solid material obtained from the carbonization of biomass which can be added to the soil to improve its fertility and reduce greenhouse gas (GHG) emissions" by the International Biochar Initiative (IBI). Biochar has a high content of carbon, produced during biomass pyrolysis under limited amount of oxygen, and temperatures ranging from 300 up to 900°C (Zhang et al., 2015b). It is produced by thermal decomposition of many different organic materials (e.g. leaves, straw, wood, manure or other organic waste biomass such as sewage sludge) (Briens et al., 2008; Lehmann and Joseph, 2009). Therefore, depending on the feedstock and the pyrolysis conditions, the biochar properties can change (Demirbas, 2004; Zhang et al., 2015c).

Biomass pyrolysis produces gaseous, liquid and solid products, and biochar is usually a product of the pyrolysis technology used to produce syngas ad bio-oil (Lehmann, 2007). It can be produced from minutes up to hours or days, depending on the proportion of syngas, bio-oil and biochar desired. For instance, a fast pyrolysis usually is performed to produce higher amount of bio-oil, and as a consequence, the aromaticity of the biochar produced is generally higher (Kim et al., 2012).

During BC pyrolysis, the organic matter thermally decomposes and not only water evaporates but also lignin, cellulose structures change to a polycyclic aromatic structure. For this reason, BC surface usually is highly aromatic and similar to activated carbon surface. Nevertheless, BC surface contain several functional groups (e.g. carboxylic, phenolic, lactones, etc.) that can provide BC acid-basic or hydrophobic-hydrophilic behaviors (Figure 1.10).

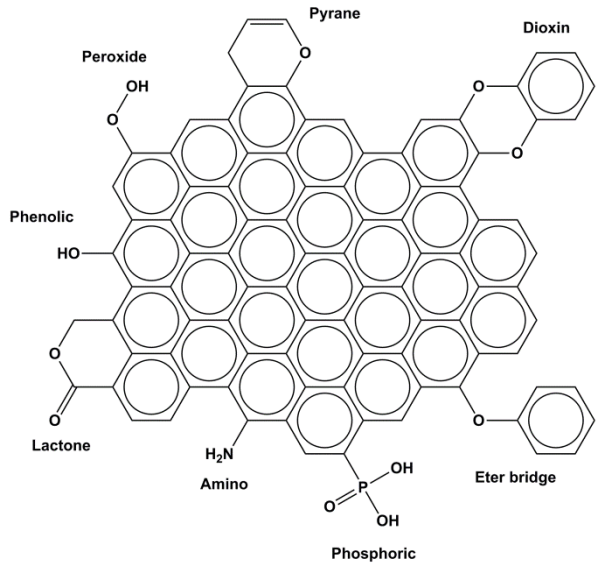


Figure 1.10: Example of functional groups in biochar surface (adapted from Lehmann and Joseph, 2009)

Several interactions between BC surface and CECs can occur due to these functional groups. For example, carboxylic or phenolic groups provide a negative charged surface, while amino groups may provide a positive charged surface. Both the graphene-like surface and these functional groups can interact with CECs mostly by non-covalent interactions: e.g. electrostatic attractions, hydrogen bonds, van der Waals forces, electron-donor-acceptor (EDA) interactions (Radovic et al., 2001; Zhu et al., 2005). The molecular structure of the CEC and the biochar surface will determine the interactions that can take place and the strength of them (Ahmad et al., 2014).

Moreover, BC can have a great specific surface area (SSA) and microporous. Both properties play an important role in the adsorption capacity of BC (Mohan et al., 2014). As mentioned before, depending on the feedstock and pyrolysis conditions, SSA can vary from few to several hundred $m^2\ g^{-1}$ (Figure 1.11).

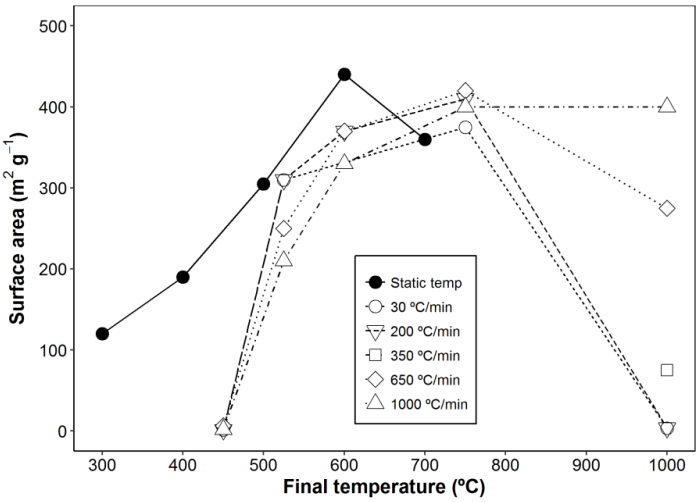


Figure 1.11: Surface area of different BCs produced at different ramp temperatures (adapted from Kookana et al. 2011)

1.4.1 Agronomic potential of BC in the environment

Over the last decade, BC has demonstrated to be an inert material that can temporary storage elemental carbon and then sequestered to CO_2 and in addition it can improve soil fertility. BC can reduce the emissions of GHG such as methane (CH_4) and nitrogen oxides (NOx), which contribute at higher magnitude than CO_2 to the greenhouse effect (Forster et al., 2007).

Moreover, BC amendment in soils has many benefits like increasing the water holding capacity (WHC) of soils (Karhu et al., 2011; Mangrich et al., 2015). It can also help to improve soil fertility, which can increase crop productivity (Kloss et al., 2014; Sänger et al., 2017). Besides, BC can increase soil cation exchange capacity, which prevents nutrient leaching.

In the last years, many other applications of BC have been developed. For instance, it has been used in animal farming (as feed additive, litter additive, manure composting) (Schmidt, 2012), in building sector (because it can help the air decontamination and humidity regulation) (Schmidt, 2008) and in the textile sector (Lin and Chang, 2008).

Finally, another BC application is the adsorption of contaminants from water and soil such as metals and organic contaminants. This application started with the

adsorption of herbicides, pesticides and PAHs (Yang et al., 2006; Yu et al., 2006, 2009; Beesley et al., 2010). Nevertheless, it has been demonstrated that BC can adsorb many other organic contaminants such as pharmaceuticals, antibiotics, hormones, etc. For this reason, in the last years, several studies have been carried out to try to reduce the mobility of organic contaminants in the soil. A recent study has evaluated the use of BC in tertiary wastewater treatment for removing micropollutants from water and concluded that, although BC is not as efficient in micropollutant removal as activated carbon (AC), it is cheaper and more sustainable. Therefore, BC could be optimized for micropollutants removal and substitute the expensive AC (<250 \$ ton^{-1} BC vs 1500 \$ ton^{-1} AC) (Thompson et al., 2016).

The sorption of CECs to BC occurs mainly through physi-sorption processes (non-covalent bonds) and in the BC pores (micro, meso and macro). Chemisorption processes may take also place and sorb CECs in their charged form to BC, however, it involves surface reactions with bond processes with organic compounds (Schwarzenbach et al., 2003). The most important interactions between CECs and BC are hydrophobic interactions, pore filling and EDA.

Although in BC surface there are micropores (< 2 nm), mesopores (2 – 50 nm) and macroporus (> 50 nm), most of BC surface is comprised by micro and mesopores. The relative small size of CECs benefits the process of pore-filling (Nguyen et al., 2007; Hao et al., 2013).

BC has an aromatic carbon surface with many functional groups, such as protonated aromatic amines or N-heteroaromatic rings, which is capable of accepting π-electrons (Hunter and Sanders, 1990; Hunter et al., 2001). On the other hand, some CECs such as BPA have electron-rich arenes (π-donors). Then, π-πEDA processes can take place between BC and some CECs. This donor capacity is correlated with polarizability and the number of substituents that have electron-donor properties (Zhu et al., 2004).

In plants, the first experiments that used BC amendments to soil to reduce the plant uptake were focused on pesticides and metals (Yu et al., 2009; Yang et al., 2010; Park et al., 2011). Recently, some authors have studied the plant uptake

reduction of some CECs (i.e. antibiotics or CBZ) into plants (Kumar et al., 2005; Williams et al., 2015).

Nevertheless, it has to be pointed out that BC can also have negative environmental impacts. Firstly, due to the process of pyrolysis, potential addition of PAHs, dioxins or other volatile organic compounds (VOCs) could be added to the environment. Moreover, the stability of BC has not been proved to be up to several hundred years (Chen et al., 2013). In fact, aging processes can reduce BC stability and its adsorption ability (Ghaffar et al., 2015; Wang et al., 2016a; Rechberger et al., 2017). Finally, as BC can increase soil WHC, some authors pointed out that BC could change soil microbiota (Thies et al., 2015; Tammeorg et al., 2016).

Chapter 2. Objectives

The overall aim of this Thesis is to study the dynamics involved in the uptake of CECs in lettuce crops. Therefore, this general aim will be met by achieving the following specific objectives:

- Evaluate the transference of CECs from irrigation water to lettuce under controlled conditions, to understand the main parameters involved in the uptake and translocation of CECs.
- Estimate degradation rate constants in the substrate and in roots and leaves of lettuces exposed to CECs using an inverse modeling approach.
- Elucidate the effect of BC amendment to soil in the plant uptake of a mixture of CECs.
- Develop a method based on an enzymatic digestion to determine the glycosylated fraction of CECs.
- Determine the effects of the exposure of CECs in lettuces and correlate them with the metabolic pathways.

Chapter 3. Study of the uptake of CECs under controlled conditions

This chapter is based on the article:

Hurtado, C., Domínguez, C., Pérez-Babace, L., Cañameras, N., Comas, J., Bayona, J.M., (2016). Estimate of uptake and translocation of emerging organic contaminants from irrigation water concentration in lettuce grown under controlled conditions. Journal of Hazardous Materials 305, 139-148.

The widespread distribution of contaminants of emerging concern (CECs) in the water cycle can lead to their incorporation in irrigated crops, posing a potential risk for human consumption. To gain further insight into the processes controlling the uptake of organic microcontaminants, Batavia lettuce (*Lactuca sativa* L) grown under controlled conditions was watered with CECs (e.g., non-steroidal anti-inflammatories, sulfonamides, β–blockers, phenolic estrogens, anticonvulsants, stimulants, polycyclic musks, biocides) at different concentrations (0 - 40 μg L^{-1}). Linear correlations were obtained between the CEC concentrations in the roots and leaves and the watering concentrations for most of the contaminants investigated. However, large differences were found in the root concentration factors (RCF = 0.27 – 733) and translocation factors (TF = 0 – 3) depending on the persistence of the target contaminants in the rhizosphere and their specific physicochemical properties. With the obtained dataset, a simple predictive model based on a linear regression and the root bioconcentration and translocation factors can be used to estimate the concentration of the target CECs in leaves based on the dose supplied in the irrigation water or the soil concentration. Finally, enantiomeric fractionation of racemic ibuprofen from the initial spiking mixture suggests that biodegradation mainly occurs in the rhizosphere.

3.1 Introduction

In a context of climate change and a burgeoning world population (Gerland et al., 2014), the pressure on water resources will grow, particularly in arid and semiarid regions. Agriculture is the sector that consumes the most water at the global level, accounting for approximately 70% of total consumption (Frenken and Gillet, 2012).

However, contaminants of emerging concern (CECs) such as pharmaceuticals and personal care products (PPCPs) have been detected in surface water used for irrigation in agriculture (Kümmerer, 2009b; Calderón-Preciado et al., 2011b). It is thus necessary to assess the behavior, fate, and health risks these compounds pose.

Some studies have already shown the potential uptake of pesticides, veterinary medicines, and other CECs by crops in different experimental setups, e.g. in *in vitro* experiments from the nutrient solution (Herklotz et al., 2010; Calderón-Preciado et al., 2012), in greenhouse conditions with treated wastewater (TWW) or soil amended with biosolids, plants can uptake several CECs (Wu et al., 2010; Macherius et al., 2012b; Calderón-Preciado et al., 2013; Zhang et al.), and finally, in field trials from TWW spiked with PPCPs and from reclaimed water (Calderón-Preciado et al., 2011a; Macherius et al., 2012b; Goldstein et al., 2014; Malchi et al., 2014; Wu et al., 2014).

Several empirical and process-based models have been developed to try to predict the concentration of CECs in plants (Trapp and Eggen, 2013; Terzaghi et al., 2015). However, while useful for building a theoretical framework for risk assessment, some of these models (Legind et al., 2011; Rein et al., 2011) are too data intensive to assess the uptake of emerging contaminants in practice (Trapp, 2015).

Many studies have reported the use of rhizobacteria to promote plant growth and in phytoremediation. Among them, endophytic bacteria (Lodewyckx et al., 2002; Barac et al., 2004) were recently proposed for the biodegradation of organic pollutants (Afzal et al., 2014; Sauvetre and Schröder, 2015). Moreover, it is well established that biotic processes are enantioselective, affecting one of the enantiomers of chiral contaminants (e.g., ibuprofen). Therefore, the enantiomeric

fractionation of chiral contaminants can be used to assess the occurrence of biotic processes in environmental compartments.

This study aimed to evaluate the uptake of eight CECs with a broad range of physicochemical properties supplied at four concentrations to lettuce (*Lactuca sativa* L) through soil with a low colloidal fraction. The CECs were selected based on their high detection and occurrence in all types of waters and their effects in humans and try to be representative compounds of some CECs families. For example, compounds with an endocrine disruptor activity such as bisphenol A (BPA) (Benachour and Aris, 2009; Careghini et al., 2015), persistent and highly bioaccumulable compounds such as carbamazepine (CBZ) (Bahlmann et al., 2014; Jurado et al., 2014), propranolol (PROP) (Maszkowska et al., 2014a; Maszkowska et al., 2014b) and tonalide (TON) (Wang et al., 2013; Parolini et al., 2015). Moreover, compounds which main concern is the bacterial resistance like the veterinary antibiotic sulfamethazine (SMT) (Wegst-Uhrich et al., 2014; Ou et al., 2015) and the biocide triclosan (TCS) present in many care products (Bedoux et al., 2012; Giuliano and Rybak, 2015). Finally, the biological active compound caffeine (CAF) which is also recognized as a contaminant of freshwater and urban aquatic environment (Thomas et al., 2010; Letić et al., 2015) and ibuprofen (IBU) which is one of the most used analgesics and it has been detected also in most of the aquatic system (Tixier et al., 2003; Petrie et al., 2015).

The concentration of the supplied CECs in the soil close to the roots, in the roots themselves and in the leaves was determined in order to quantify the incorporation and translocation of the different CECs to develop a simplified model using the data for all four concentrations to predict the concentration of a given CEC in the leaves for a specific initial treatment, which could be useful for risk assessment.

3.2 Materials and methods

3.2.1 Experimental layout

The experiment was carried out in a glass greenhouse located at the Agròpolis-UPC agriculture experimental station (41º 17' 18" N, 2º 02' 43" E) in Viladecans (Barcelona, Spain). The experimental units consisted of 2.5 L cylindrical amber glass pots (Ø = 15 cm and 20 cm high) fitted with a tubing outlet at the bottom (Ø = 3 cm). In order to minimize potential interactions between CECs and soil

colloids, the experimental units were filled with 2 L of a mixture of perlite and sand (2:1, v/v, average dry weight 1.2 kg). One Batavia lettuce (*Lactuca sativa*, cv. Arena) seedling was planted in each experimental unit and watered with the Hoagland and Arnon solution prepared with harvested rainwater (pH = 5.5). A nutrient solution was supplied through an on-line drip irrigation system. A dose of 50 mL of irrigation water was applied to each experimental unit per day. The number of daily irrigations was regulated to keep water in the soil at field capacity, thereby preventing leachate production.

Treatments consisted of the direct application of 14, 35, 70 and 140 μg of eight CECs per experimental unit. This procedure made it possible to avoid possible adsorption of the applied products by the irrigation tubing and associated biofilm. Taking into account the irrigation water supplied, this corresponds to an average CEC concentration in the irrigation water (C_{IW}) of 4, 10, 20 and 40 μg L^{-1}, and taking into account the soil mass in each experimental unit, it corresponds to an average concentration in the soil (C_S) of 11.7, 29.2, 58.3 and 116.7 μg kg^{-1}. The control consisted of planted experimental units to which no CECs were applied.

Treatments were distributed among eight applications over the course of four weeks, starting six weeks after planting. The experiment had a total duration of 10 weeks. The treatments and control were replicated four times. The selected CECs were as follows: bisphenol A (BPA, 99%), caffeine (CAF, 99%), carbamazepine (CBZ, 99%), ibuprofen (IBU, 98%), propranolol (PROP, 99%), sulfamethazine (SMT, 99%), triclosan (TCS, 97%), and tonalide (TON, 97%). The BPA, CAF, CBZ, IBU, PROP, SMT, and TCS were purchased from Sigma-Aldrich (St. Louis, MO, USA), and the TON from Ventós (Sant Just Desvern, Spain). Table 3.1 shows their structure and physicochemical properties.

Table 3.1: Physical-chemical properties of the selected contaminants of emerging concern (CECs) in this study.

CEC	pKa[1]	Solubility (mg L^{-1})	Log K_{ow}[2]	Log K_{OA}[2]	Log K_{AW}[2]	f_n[3]
Bisphenol A (BPA)	8.7[0/-]	173	3.32	12.75	-9.43	0.995
Caffeine (CAF)	0.8[+/0]	2632	-0.07	8.77	-8.83	0.999
Carbamazepine (CBZ)	2.45[+/0]	17.7	2.45	10.81	-7.20	0.999
Ibuprofen (IBU)	4.3[0/-]	41.1	3.97	9.18	-5.21	0.008
Propranolol (PROP)	9.5[+/0]	228	3.48	13.97	-10.49	0.001
Sulfamethazine (SMT)	2.7[+/0] 7.4[0/-]	2846	0.89	8.29	-8.10	0.797
Tonalide (TON)	NA[4]	0.29	5.70	7.95	-2.04	1.000
Triclosan (TCS)	7.9[0/-]	4.62	4.76	11.45	-4.08	0.967

[1]Dissociation reaction, [0]: neutral; [+]: cationic; [-]: anionic.
[2]Log K_{ow}, log K_{OA} and log K_{AW} from database provided by Episuite v4.11 (http://www.epa.gov/opptintr/exposure/pubs/episuite.htm)
[3]The neutral fraction f_n was calculated from Trapp et al.(Trapp, 2009) at substrate pH 6.42 .
[4]Not applicable

3.2.2 Analysis of vegetable tissues and substrate

Upon conclusion of the experiment (at 10 weeks), the plants were harvested and the substrate close to roots, the roots themselves, and the entire aerial part of the plant (mostly leaves) were analyzed.

After the sampling was performed, the roots were watered with deionized water to remove the adhered perlite-sand mixture. The roots and leaves were comminuted with liquid nitrogen and stored at -20ºC until analysis. The extraction of vegetable tissue was performed as reported elsewhere and a brief description is found in the supplementary information (see section 3.7.2) (Calderón-Preciado et al., 2009). Aliquots of samples were fortified with a mixture of surrogates (see section 3.7.1). A matrix solid phase was performed followed by a pressurized solvent extraction. Then, a liquid-liquid extraction was performed and after a

cleanup, all samples were injected in both GC-MS/MS and LC-MS/MS (see sections 3.7.3 and 3.7.4).

Extraction of the CECs in the soil close to the roots was performed as follows: a 1 g aliquot spiked with the same mixture of surrogates was mixed with 0.5 g of sodium sulfate anhydrous, equilibrated for 1 h, and extracted twice with 10 mL of an acetone:hexane (3:1, v/v) mixture for 15 min by sonication. A third extraction with 10 mL of methanol was performed. The resulting extracts were combined, evaporated to 2 mL, and dried by percolation through an anhydrous sodium sulfate column. The extraction solvent mixture was replaced with ethyl acetate prior to the samples' injection in the GC system. Qualitative and quantitative analysis was performed based on retention time and the selected reaction monitoring (SRM) mode of two product ions, and the ratio between the product ions was determined by confirmation (Table S3.2). The limits of detection and quantification for all the targeted analytes and matrices are given in Table S3, and the recoveries are given in Table S4.

Finally, sample extract aliquots were subjected to chiral derivatization of IBU as described by Hashim and Khan (2011) The full procedure is described in the supplementary information (see section 3.7.5).

3.2.3 Data analysis

Standardized concentrations

The standardized concentrations of the tested CECs in the soil close to the roots (SC_{SR}) were calculated as follows:

$$SC_{SR} = \frac{1}{4}\sum_{T_i}^{4}\frac{C_{SR_i}}{C_{S_i}} \tag{3.1}$$

where T_i stands for the treatment applied (1 to 4), C_{SR} is the concentration of a given CEC in the soil close to the roots, and C_S is the initial soil concentration (11.7, 29.2, 58.3 or 117 µg kg^{-1}). The standardized concentrations in the roots (SC_R) and leaves (SC_L) were calculated similarly.

Enantiomeric fraction (EF)

Many CECs are produced synthetically as racemic mixtures. Hence, 50% of the compound is the *R* form, and 50% the *S* form. The enantiomeric fraction is a descriptor of enantiomeric (chiral) mixtures. Although in the natural environment many physical processes are not enantioselective (e.g., hydrolysis, photolysis), microbial degradation and biological metabolism can be (Harner et al., 2000; Qi et al., 2014). The EF was calculated as described in Equation 3.2.

$$EF = \frac{S}{S+R} \tag{3.2}$$

Bioconcentration and translocation factors

The concentration factor for soil close to the roots in treatment *i* (SCF_i) was calculated as follows:

$$SCF_i = \frac{SR_{C_i}}{S_{C_i}} \tag{3.3}$$

where SR_{Ci} and C_{Si} are the concentration of a given CEC in the soil close to the roots and the average concentration of CEC for the soil mass in treatment *i*, respectively.

Likewise, the root concentration factor in treatment *i* (RCF_i) was calculated as follows:

$$RCF = \frac{C_R}{C_S} \tag{3.4}$$

where C_R is the concentration in the roots.

For each CEC, the linear regression coefficient (assuming an intercept of zero) of C_R over C_S was also calculated ($b_{CR/CS}$).

The translocation factor (TF) was calculated as follows:

$$TF = \frac{C_L}{C_R} \tag{3.5}$$

where C_L is the concentration in the leaves in the different treatments.

Finally, the percentage of degradation among the compartments was calculated as follows:

$$\%\ Degradation = \frac{m_{ini} - m_{soil} - m_{root} - m_{leaf}}{m_{ini}} \times 100 \qquad (3.6)$$

Where m_{ini} is the initial added mass of each CEC in the pot, m_{soil}, m_{root} and m_{leaf} are the masses of CEC in soil, roots and leaves respectively, taking into account the mass and the concentration in each compartment. This overall degradation would include degradation in soil, non-extractable residues and biotransformation in plant.

Modeling of concentration in leaves

The predicted concentration of a given CEC in the leaves (C'_L) was calculated by means of the following equation:

$$C'_L = TF_{mean} \times b_{C_R / C_S} \times C_S \qquad (3.7)$$

where TF_{mean} is the average translocation factor, and $b_{CR/CS}$ is the linear regression coefficient of C_R over C_S over the course of the different treatments.

C'_L could also be expressed relative to the average concentration of a particular CEC in the irrigation water (C_{IW}) in a given treatment *i* as follows:

$$C'_{L_i} = TF_{mean} \times b_{C_R / C_{IW}} \times C_{IW_i} \qquad (3.8)$$

where, $b_{CR/CIW}$ is the linear regression coefficient of C_R over C_{IW} over the course of the different treatments *i*. This model has been validated for soil with very low CEC and no leachates.

The regressions, analysis of variance (ANOVA), and subsequent mean separation (LSD) were conducted in R (R Development Core Team, 2015).

3.3 Results

3.3.1 Occurrence of CECs in the different compartments

Concentration in the soil close to the roots

The concentrations of the tested CECs in the soil close to the roots (C_{SR}) were lower than in the roots themselves (C_R) or in the leaves (C_L). They ranged from 0.3 to 167 ng g^{-1} dw depending on the product and dose applied (Table 3.2). Overall, TCS was the CEC to exhibit the highest concentration, while SMT had the lowest; their standardized concentrations (Equation 3.1) were 1.47 ± 0.45 and 0.03 ±0.01, respectively (Figure 3.1A).

Concentrations in the roots

Generally, the average concentration in the roots (C_R) was between 2.6 and 150 times higher than in the soil close to the roots (C_{SR}). In absolute terms, C_R varied widely, from below the LOQ to 1630 ng g^{-1} dw, again depending on the CEC and treatment (Table 3.2). Overall, CBZ had the highest concentrations and IBU the lowest; their standardized concentrations were 9.67 ± 1.99 and 0.90 ± 0.78, respectively (Figure 3.1B).

Concentration in the leaves

Overall, CEC concentration in the leaves (C_L) averaged between 0.5 and 110 times lower than in the roots. The concentration varied, depending on the CEC tested and the treatment used; however, the concentration of CBZ in the leaves was much higher than that of the other products (Figure 3.1C).

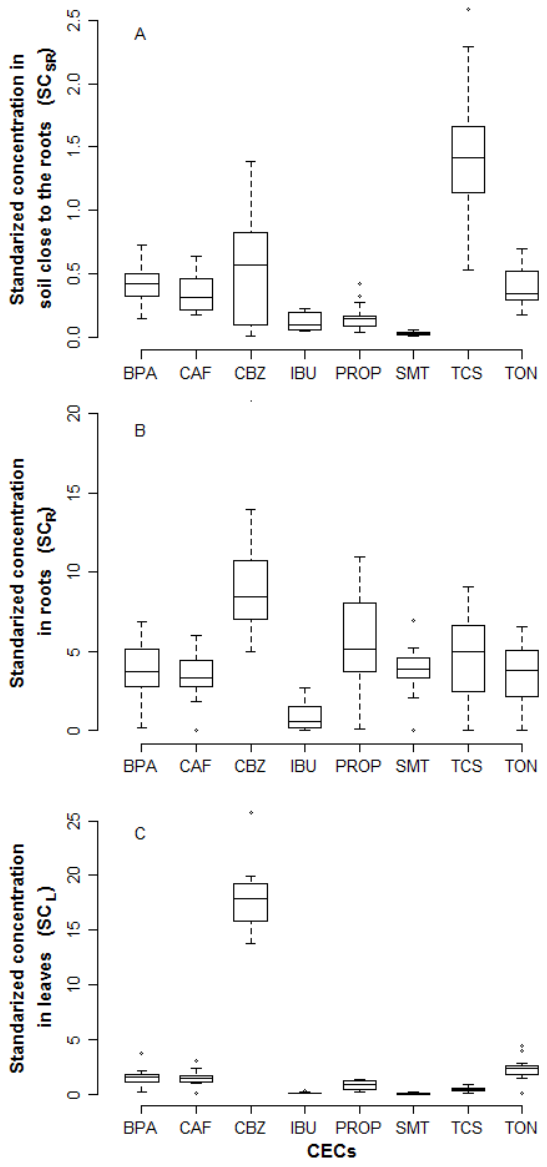


Figure 3.1: Boxplots of standardized concentration of tested CECs in the three different analyzed compartments (A) in the soil close to the roots (SC_{SR}), (B) in the roots (SC_R) and (C) in the leaves (SC_L).

Table 3.2: Mean concentration (N = 4. ± sd; soil in ng g^{-1}; root in ng g^{-1} dw; leaf: ng g^{-1} dw) of the emerging organic contaminants in the different compartments at the end of the exposure experiment (70 d) and the estimated degradation (%) using a mass balance

CEC	Compartment	Treatment (µg L^{-1})				
		0	4	10	20	40
BPA	**Soil**	< LOD	5.1 ± 3.5	11 ± 3	25 ± 3	55 ± 16
	Root	< LOD	73 ± 11	124 ± 18	212 ± 71	325 ± 69
	Leaf	< LOD	33 ± 17	54 ± 8	83 ± 19	158 ± 53
	Deg (%)	NA	53 ± 31	59 ± 8	54 ± 5	51 ± 13
CAF	**Soil**	1.5 ± 0.9	4.2 ± 1.0	5.8 ± 0.6	18 ± 6	64 ± 10
	Root	< LOD	32 ± 9	126 ± 50	255 ± 61	398 ± 105
	Leaf	< LOD	32 ± 6	53 ± 11	77 ± 8	147 ± 20
	Deg (%)	NA	62 ± 10	76 ± 9	65 ± 10	43 ± 8
CBZ	**Soil**	< LOD	0.85 ± 0.91	10.4 ± 10	37 ± 4	117 ± 30
	Root	< LOD	142 ± 88	234 ± 98	473 ± 116	1214 ± 314
	Leaf	< LOD	233 ± 47	461 ± 48	1031 ± 149	2054 ± 315
	Deg (%)	NA	64 ± 16	44 ± 34	20 ±10	<5 ± 15
IBU	**Soil**	< LOD	0.73 ± 0.22	2.1 ± 0.81	8.7 ± 3.4	24 ± 3
	Root	< LOD	< LOD	13 ± 5	69 ± 32	223 ± 68
	Leaf	< LOD	0.93 ± 0.32	2.4 ± 1	4.9 ± 1.1	24 ± 7
	Deg (%)	NA	90 ± 2	92 ± 3	86 ± 6	80 ± 3
PROP	**Soil**	< LOD	1.5 ± 0.2	3.8 ± 1.7	9.7 ± 6.9	27 ± 18
	Root	< LOD	113 ± 14	195 ± 60	313 ± 49	393 ± 47
	Leaf	< LOD	< LOD	29 ± 8	67 ± 11	119 ± 26
	Deg (%)	NA	83 ± 7	81 ± 6	78 ± 11	75 ± 15
SMT	**Soil**	< LOD	0.30 ± 0.11	0.82 ± 0.44	2.4 ± 0.9	4.7 ± 1.9
	Root	< LOD	60 ± 18	92 ± 22	243 ± 54	495 ± 64
	Leaf	< LOD	< LOD	< LOD	< LOD	< LOD
	Deg (%)	NA	94 ± 3	93 ± 5	93 ± 7	93 ± 9
TON	**Soil**	1.5 ± 1.1	5.3 ± 1.8	13 ± 3	21 ± 14	39 ± 12
	Root	< LOD	9.4 ± 4.3	117 ± 27	270 ± 69	587 ± 122
	Leaf	< LOD	26 ± 14	73 ± 6	105 ± 19	321 ± 99
	Deg (%)	NA	54 ± 16	52 ± 9	60 ± 18	61 ± 19
TCS	**Soil**	< LOD	10 ± 4	56 ± 18	97 ± 25	167 ± 32
	Root	< LOD	21 ± 18	147 ± 92	353 ± 95	772 ± 206
	Leaf	< LOD	13 ± 2	17 ± 1	25 ± 3	32 ± 3
	Deg (%)	NA	15 ± 36	<5 ± 38	<5 ± 21	<5 ± 27

NA: not available; <LOD: lower than limit of detection.

Enantiomeric fractionation of IBU

IBU is sold as a racemic mixture; however, in the soil close to the roots, the S enantiomer predominated over the *R*-enantiomer (EF = 0.74 ± 0.02), which means that the *R*-form was degraded more easily than the *S* form. In the roots, the *S*-enantiomer was still predominant, although less so than in the C_S, as the EF decreased (0.68 ± 0.09). Finally, in the leaves, a complete racemization (EF = 0.50 ± 0.03) was observed (Figure 3.2).

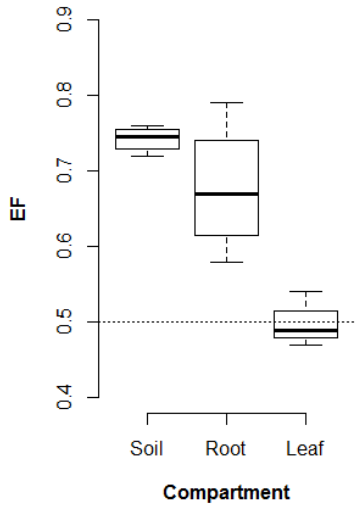


Figure 3.2: Boxplots of the enantiomeric factors (EF) of IBU in the soil close to the roots, in the roots and in the leaves. The horizontal line was the value of the commercial racemic mixture of IBU (EF = 0.50).

Bioconcentration processes

TCS was the only tested CEC to have a concentration factor in the soil near the roots (SCF) greater than 1; its average was 3.5. The SCF of the remaining CECs was significantly lower, averaging between 0.1 and 0.4 (Figure 3.3A). The root concentration factor (RCF) values were much greater than the SCF. Their values ranged from 0.43 to 11.7. The CEC with the highest RCF was CBZ (average of 9.3), followed by PROP (average of 6.0) (Figure 3.3B).

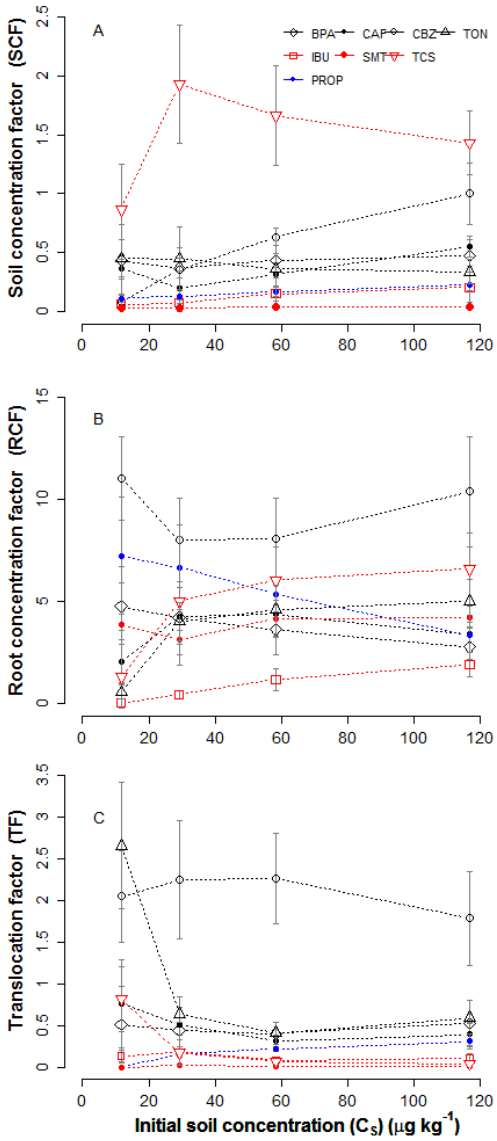


Figure 3.3: Mean of the concentration factors (A) in the soil close to the roots (SCF), (B) in the roots (RCF) and (C) and leaf translocation factor (TF) along the initial applied concentration in soil (C_S).

The other CECs tested exhibited much lower values. For IBU, the RCF clearly increased as larger and larger doses were applied; the opposite was true of PROP. For TON, at the lower application rate, the value of RCF was very low. It then stabilized at a greater value as the application rates increased. For the remaining

products, the values of RCF were relatively independent of the application rates (Figure 3.3B).

It is noteworthy that the leaf translocation concentration factor (TF) for CBZ is much higher (average of 3.4) than that of the remaining products, which, on average, are lower than 1. The TF values are also slightly dependent on the concentration, declining at the highest concentrations (Figure 3.3C).

Modeling the uptake of CEC

The concentration of the tested CECs in the roots showed a strong linear relationship with the application rate expressed as the average concentration in the soil (C_S) or in the irrigation water (C_{IW}) (Figure 3.4). The coefficients of determination (R^2) always take values higher than 90%. This strong linear relationship is held even for IBU, PROP, and TON, for which the R_{CF} clearly depends on the rate of application. The high values of the slopes indicate the ease with which most products are taken up by the roots. Moreover, translocation from roots to leaves remained relatively stable regardless of the treatments applied, as shown by the values of the translocation concentration factors (Figure 3.3C).

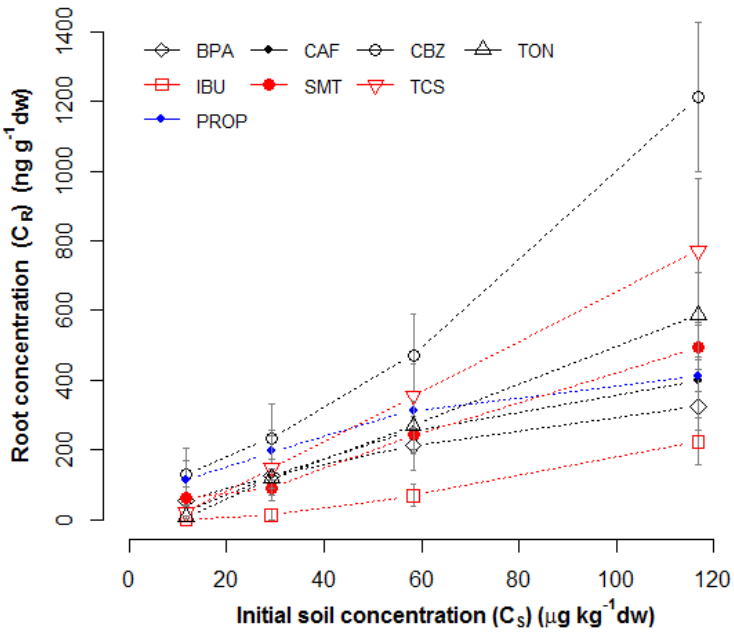


Figure 3.4: Concentration of tested CECs in the roots (C_R, ng g^{-1} dw) over application rate expressed as the average concentration in the soil (C_S, µg kg^{-1} dw).

The above considerations make it possible to build a simplified model to predict the concentration of a given CEC in the leaves (C'_L) for a specific treatment i, multiplying the average leaf translocation concentration factor (TF_{mean}), the slope of the linear relationship of the root concentration ($b_{CR/CS}$) over the mean soil concentration of the given CEC, and the mean concentration of the CEC in the soil in a given treatment i (C_S) (Equation 3.7)

Figure 3.5 shows that there is strong agreement between the predicted concentration values in the leaves using Equation 3.7 and the observed values (R^2 = 0.9985). Depicted values are located very close to the bisecting line, even for CECs like BPA, IBU, and TCS, for which the linear relationship between the concentration in leaves and the initial applied concentration is less strong (Figure 3.4).

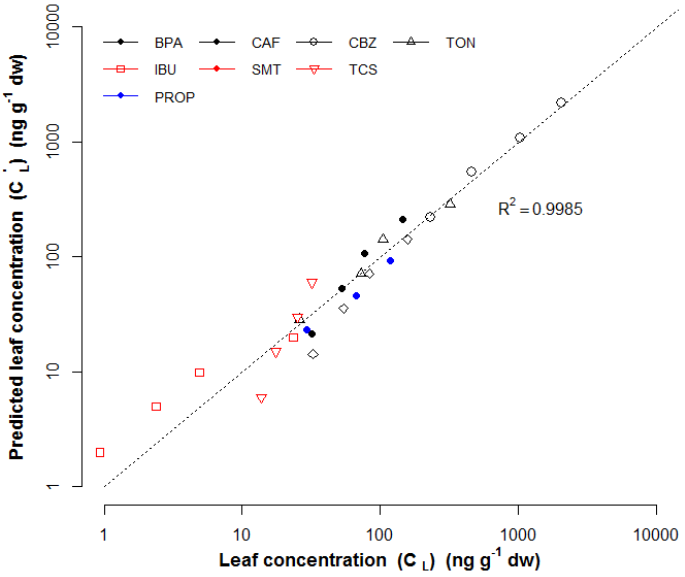


Figure 3.5: Values of tested CEC in the leaves compared with the values obtained from the product of the concentration of supplied CEC in the soil (C_S), the concentration factor of the roots (RCF) and leaf translocation concentration factor (TF) (Equation 3.2).

3.4 Discussion

Although the uptake of pharmaceuticals by plants from irrigation water and biosolids has been widely documented (Wu et al., 2010; Calderón-Preciado et al., 2011a; Calderón-Preciado et al., 2011b; Shenker et al., 2011; Wu et al., 2012; Goldstein et al., 2014; Malchi et al., 2014; Wu et al., 2014), the exact soil-root-plant system processes involved in this uptake are not yet well understood. This paper looked at the uptake of several CECs by lettuce when the plants are grown in a soil with a very low cation exchange capacity and the dose of irrigation is adjusted to prevent leaching. The experiment was carried out in a greenhouse and based on the overall water added to the substrate and the final water in soil, evapotranspiration was expected to be lower than other studies (Shenker et al., 2011; Malchi et al., 2014; Prosser et al., 2014a). As most of these compounds are described to be taken up by plants by water transpiration, this is supposed to be the main uptake process. Dodgen et al. (2015) demonstrated that

evapotranspiration was correlated to the uptake of organic chemicals to the roots and leaves for both neutral and ionizable compounds. Therefore, the permanence of a CEC in the soil should depend on its recalcitrance and the ease with which it is taken up by roots. Volatilization from the soil can also be a significant transport pathway for semivolatile CECs such as TON (log K_{AW} = -2.04, log K_{OA}= 7.95, Table 3.1). According to the non-steady state model for both hydrophilic and hydrophobic neutral organic chemicals described by Undeman et al. (2009), volatilization becomes a potential source of leaf contamination through the soil–air pathway (Collins and Finnegan, 2010).

3.4.1 Root uptake and translocation

The chemical speciation of the CECs can be anticipated from their pKa and the pH of the soil (6.42). Moreover, in this experiment neutral and ionizable compounds were selected (Table 3.1).

For neutral compounds, such as CAF, CBZ and TON, the accumulation to roots is usually related to lipophilicity (Briggs et al., 1982). Wu et al. (2014) found a positive correlation between the RCF and log K_{ow} only for neutral compounds in different plants. As neutral compounds can cross the cell membranes more easily than ionic compounds (Hsu et al., 1990; Malchi et al., 2014), these can be easily translocated to leaves by the water transpiration stream and accumulate there. This translocation is also dependent on the lipophilicity. Dettenmaier et al. (2009) showed that in pressurized chambers, hydrophilic compounds can also be translocated to leaves, that decreases along the log K_{ow}. Tanoue et al. (2012) reported that intermediate hydrophobic compounds (log K_{ow} from 0.5 to 3) could be translocated easier than highly hydrophobic ones. Hence, in our study CBZ was the product found in the highest concentrations in the roots. This can be explained by its neutrality and medium hydrophobicity (log D_{ow} = 2.25). In addition, they exhibit a low interaction with the soil's organic colloids (Briggs et al., 1982). CAF is more hydrophilic and water soluble so it has more difficulties to cross the lipophilic cell membranes and that could explain the concentration in roots and leaves lower than CBZ (Goldstein et al., 2014). TON, although is neutral and has a high log K_{ow}, was found in leaves at high concentration, however, TON follows another uptake route and its transport to the leaves was not related to water movement but volatilization (Undeman et al., 2009).

For ionic compounds, the correlation between RCF or TF and log D_{ow} was not found by Wu et al. because other mechanisms such as ion trap (Trapp, 2004) take place and may affect to the root concentration. In this experiment, BPA, IBU, PROP, SMT and TCS are ionizable compounds. Although BPA and TCS were found mostly in neutral forms (99.5 and 96.7% respectively) at substrate pH, IBU was found anionic, PROP was found cationic and finally SMT was around 80% neutral however, it has a zwitterionic equilibrium. Accordingly, the low concentration of IBU in the roots (Table 3.2) could be explained by its electronegativity, as long as the root membranes have a negatively charged potential (Trapp, 2004), which would hinder the absorption of negatively charged ions. Instead, PROP occurred in the root at a higher concentration than IBU, but at a lower concentration than most of the neutral products (Figure 1B). However, since the soil we used had a low CEC, more hydrophobic products (log K_{OW} > 4.66) could also be easily sorbed by roots. This is the case of TCS and TON.

The high concentration of CBZ found in roots and leaves was consistent with other studies in the literature. For example, in soybean plants irrigated with 10 µg L^{-1} of CBZ and TCS, Wu et al. (2010) reported that TCS was found mostly in the roots (16.9 ± 2.6 ng g^{-1} dw). In this study, TCS likewise exhibited a higher concentration in the roots (147 ± 92 ng g^{-1} dw), while CBZ was found mostly in the leaves (216 ± 75 ng g^{-1} dw). Shenker et al. (2011) irrigated cucumbers with fresh and reclaimed water spiked with 1 µg L^{-1} of CBZ and it was found in the roots was between 2 and 4.5 µg g^{-1} in fw, while the concentration in the leaves ranged from 19 to 39 µg g^{-1}. Wu et al. (2014) reported that several PPCPs were detected in edible parts of common vegetables that had been watered with PPCP-spiked treated wastewater. CBZ concentrations between 0.1 and 2.5 ng g^{-1}, depending on the plant species, were detected. Interestingly, like most of the compounds examined here, the PPCPs were found at higher concentrations in the roots than in the leaves. Goldstein et al. (2014) reported CBZ levels between 50 and 500 ng g^{-1} dw in cucumbers and tomatoes. In the same experiment, CAF was detected at concentrations from 1 to 9 ng g^{-1} dw in the same plants. In this study, CAF was also found in leaves at lower concentration than CBZ. BPA was studied in hydroponics by Dodgen et al. (2014) and was taken up by lettuces and collards (200 – 442 ng g^{-1} dw in roots) and there was low translocation to leaves (0.2 – 3.5 ng g^{-1} dw in leaves). Although the experimental performance was different, BPA accumulated higher in roots than in leaves with this perlite:sand mixture. SMT has been also studied and most authors point out that it can be

accumulated in the roots but its translocation is not very high (Pan et al., 2014; Rajapaksha et al., 2014). For example, Pan et al. (2014) found that SMZ accumulated only in roots of radish and white cabbage. In our experiment, SMT was not detectable in leaves, despite that; it was found in roots, similarly to these studies. TCS has been widely studied in many crops grown in soil and most of these studies accumulated mainly in the roots and just in few studies were detected in leaves with ryegrass grown in soil (Carter et al., 2014; Prosser et al., 2014a; Wu et al., 2014), and Macherius et al. (2012b) studied the uptake of TON in barley and meadow fescue and found TON in both roots and leaves and suggested that volatilization can be a pathway since TON was detected in control leaves of barley. Hence, although this experiment used a simplified set-up and a low CEC soil, the findings are comparable to those of other studies performed with real soil.

3.4.2 Biodegradability

Biodegradation can occur both in soil and in plant. However, biodegradation of CECs in the rhizosphere is considered to be the most significant removal mechanism for CECs that are not readily absorbed by the roots. Indeed, as much as 40% of a plant's photosynthate can be released into the soil as sugars, organic acids, and larger organic compounds such as root exudates (Leigh et al., 2002). These exudates are used as carbon and energy sources by soil microbial biomass, leading to a significant enrichment compared with soil that is uninfluenced by roots (Chaudhry et al., 2005). Several studies have addressed the dissipation of pharmaceuticals in soil, but the interaction between soil and the rhizosphere effect has been neglected (Grossberger et al., 2014). This notwithstanding, it is widely accepted that, in phytoremediation, the rhizosphere plays a role in removing organic contaminants from soil through a synergistic interaction of many factors (Gerhardt et al., 2009). The results of this study underscore the importance of the relative persistence of CECs in the rhizosphere as a key primary parameter for assessing plants' exposure to them. The final concentration measured in the soil near the roots was for almost all the compounds much lower than the applied. In fact, TCS is the only tested CEC with a positive concentration factor in the soil near the roots (SCF). This accumulation is consistent with the recalcitrance resulting from its biocidal activity; nevertheless, several studies have reported half-lives of TCS from 18 to 187 d (Ying et al., 2007; Walters et al., 2010); hence, TCS was persistent in soils. In plants, Macherius et al. (2014) found eight

phase II metabolites of TCS in carrots. Other CECs have been studied in degradation assays with soils and some of them in plants. For example, Li et al. (2013) reported the degradation of CBZ in three soils and they found some metabolites and that less than 2% of CBZ was mineralized and non-extractable residues (NERs) were around 4%. High DT50 were found for the three soils (46 - 273 d) suggesting that CBZ was a persistent CEC in soil. In plant, two CBZ metabolites have been detected by Goldstein et al. (2014); Malchi et al. (2014) BPA half-lives in soils ranged from 1 to 7 d and 3 metabolites of BPA were detected in soil. Dodgen et al. (2013) investigated the uptake of BPA from hydroponics to collards and lettuces, and they found out that in the nutrient solution BPA tend to degrade and degradation was faster when the nutrient solution was exposed with plants. CAF is rapidly biodegraded in soil (DT50 from 1 to 3 d) and metabolites have been detected in soil (Topp et al., 2006) and in microorganisms (Dash and Gummadi, 2006). Recently, some metabolites of IBU have been detected in an aquatic plant (Pietrini et al., 2015).

Interestingly, IBU was supplied as a racemic mixture; however, an EF of 0.74 was found in the substrate. Furthermore, an EF of 0.69 was observed in the roots. This could indicate biotic degradation in both the rhizosphere and the roots as biotic degradation could be enantioselective (Matamoros et al., 2009). However, the EF was 0.50 in the leaves; therefore, racemization was taking place inside the plant. This could be explained by different detoxification processes that occur in plants. Plants have their own detoxification system with many enzymes that can metabolize organic contaminants (e.g., cytochrome P450, monooxygenases, peroxidases, glutathione S-transferases) and endophytic bacteria can live inside plants and have a potentially large impact on their metabolism (Brader et al., 2014; Sauvetre and Schröder, 2015). *R*-IBU has been studied to be transformed by microorganisms in biofilm reactors (Hussain et al., 2015). Chen and Rosazza (1994) reported that the reduction rate of *R*-IBU was twice faster than *S*-IBU in *Nocardia*. Moreover, Hashim et al. (2010) and Hashim and Khan (2011) found out that some fungi and some bacteria can induce metabolic chiral inversion. Finally, there are some membranes that can be enantioselective and this could affect to the EF (Tsuchiya and Mizogami, 2012); nevertheless as racemization was observed in plants, it does not seem that this process is the predominant. Deeper research in the field of degradation routes in soil and plants is needed.

3.4.3 Modeling plant uptake

The relationship between CEC concentration in soil and plant uptake has seldom been studied. Usually, root concentration factors (RCF) are calculated based on their nominal concentrations; however, as demonstrated in the previous section, their behavior in the rhizosphere is largely dependent on the compound. One of the few existing studies used a simplified two-compartment model (Cropp et al., 2010) to assess the plant concentration and found a linear relationship between soil-water concentration and plant concentration. However, that model was only validated for norfloxacin. Kumar et al. (2005) observed an increase of chlortetracycline in onions and cabbage related to the dose of manure applied to the soil.

However, to the best of our knowledge, this is the first study to report a linear relationship between root and leaf concentrations for a wide range of CECs supplied in irrigation water. Moreover, the fact that the leaf translocation concentration factors (TF) remain fairly stable regardless of the dose of CEC applied (Figure 3.3C) makes it possible to predict fairly accurately the content of the tested CEC based on the dose supplied and the calculated average soil concentration (Figure 3.5).

Although the experimental setup used in this study was rather simple (low CEC, no leachates produced), the approach could be particularly useful in risk assessment studies for estimating CEC concentrations in crops in the worst-case scenario, in which the soil-contaminant interaction is negligible.

3.5 Conclusions

Although previous studies in real scenarios have shown that several organic pollutants can be taken up by plants, it is difficult, if not impossible to reproduce the experimental set-up elsewhere. In this study, a mesocosm characterized by a low CEC exhibited similar behavior with regard to the evaluated CECs as in previous studies. Degradation, uptake and translocation processes were all highly dependent on the specific CEC evaluated and the compartment.

Linear relationships observed between the root concentration and the application dose, along with the stability of the leaf translocation concentration factors,

makes it possible to predict the leaf concentrations of tested CECs fairly accurately.

Enantiomeric IBU degradation was detected in the soil, and a racemization trend was observed in the plants, from the roots to the leaves. This would seem to suggest that mixed biotic degradation pathways might occur in the plant either through endophytic bacteria or the plant's own detoxification system, leading to complete racemization in the leaves. Further research is required to address the complexity of the biotic degradation pathways for CECs in plants.

3.6 Annex

Definition of symbols used in this chapter

$b_{CR/CS}$	Linear regression coefficient of C_R over C_S over the course of the different treatments
C_{IW}	Calculated average irrigation water concentration of a given CEC
C_L	Leaf concentration of a given CEC
C'_L	Predicted leaf concentration of a given (Eq. 3.5)
SC_L	Standardized leaf concentration of a given CEC (Eq. 3.1)
TCF	Leaf translocation factor of a given (Eq. 3.5)
TCF_{mean}	Mean leaf translocation factor of a given CEC
C_R	Root concentration of a given CEC
RCF	Root concentration factor of a given CEC (Eq. 3.4)
SC_R	Standardized root concentration of a given CEC (Eq. 3.1)
C_S	Calculated average soil concentration of a given CEC
C_{SR}	Concentration in the soil close to the roots of a given CEC
SC_{SR}	Standardized concentration in the soil close to the roots of a given CEC (Eq. 3.1)
SCF	Concentration factor in the soil close to the roots of a given CEC (Eq. 3.3)

3.7 Supporting information

3.7.1 Materials and reagents

Internal standard triphenylamine (TPhA, 98 %) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Trimethylsulfonium hydroxide (TMSH) was obtained from Fluka (Buchs, Switzerland). 10,11-Dihydrocarbamazepine (DHCBZ, 99 %), 2,2'-dinitrobiphenyl (DNBP, 97 %), 2-(2,4,5-trichlorophenoxy)propionic acid (FEN, Pestanal) and sulfamethoxazole (SMX,

99 %) were purchased from Sigma-Aldrich; tonalide-d3 (TON-D3) was purchased from Dr. Ehrenstorfer (Ausburg, Germany).

Florisil was purchased from Merck (Darmstadt, Germany). Sodium sulfate anhydrous and sodium chloride were purchased from Fluka (Buchs, Switzerland). Disodium hydrogen citrate sesquihydrate and trisodium citrate dihydrate were obtained from Sigma-Aldrich. Suprasolv® grade acetone, methanol, hexane, ethyl acetate and LiChrosolv® grade acetonitrile were purchased from Merck. Hydrochloric acid (37% v/v) and potassium carbonate (98 %) were purchased from Panreac (Barcelona, Spain). The Na_2SO_4 was baked for 5 hours at 450 ºC in a muffle furnace before using. Reagent water was deionized in the laboratory using the ultrapure water system Arium 611 from Sartorius (Aubagne, France).

(*R*)-(+)-α-methylbencylamine for chiral derivatization (*R*-1-PEA, ≥ 99%), triethylamine (TEA, ≥ 99%) and ethyl chloroformate (ECF, 97%) were purchased from Sigma-Aldrich. Strata-X, Polymeric HLB-Phase, solid phase extraction (SPE) cartridges (30 mg / 3 mL) were purchased from Phenomenex (Torrance, CA, USA).

3.7.2 Vegetal extraction

Briefly, a 0.5 g aliquot of plant tissue (root or leaf) was spiked at 25 ng g^{-1} with a mixture of surrogate. The sample was then blended with florisil, Na_2SO_4, Na_3-citrate dihydrate, NaCl, Na_2H-citrate sesquihydrate, and Hydromatrix using a pestle. The mixture was extracted with acetone:hexane (1:1, v/v) using a pressurized solvent extraction (PSE) apparatus (Applied Separations (Allentown, PA, USA). Samples were extracted with two 14-minute cycles at 104 ºC and 110 bar. Neutral-basic and acid fractions were obtained by solvent partitioning at neutral and acid pH respectively.

The aliquots of the sample extracts were analyzed first using an EI GC-MS/MS Bruker 450-GC gas chromatograph coupled to a Bruker 320-MS triple-stage quadrupole mass spectrometer (Bruker Daltonics Inc., Billerica, MA, USA). Qualitative and quantitative analyses were performed based on retention time and the selected reaction monitoring (SRM) mode of two product ions, as well as the ratio between the product ions (Table S3.1).

Another sample extract aliquot was evaporated and reconstituted with methanol:water (20:80, v/v). SMT and PROP were analyzed by LC-MS/MS using a TSQ Quantum triple-stage quadrupole mass spectrometer equipped with an ESI source (Thermo Fischer Scientific, San Jose, CA, USA).

Table S3.1. Monitoring ions in GC-MS/MS

Segment	Compound	RT (min)	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
1	**IBU**	11.02	161*	91	23
			220	161	11
2	**FEN**	13.68	196*	132	20
			284	198	15
3	**CAF**	14.79	194*	109	14
			194	55	20
3	**TON**	14.96	258*	243	10
			243	187	13
3	**TON-d3**	14.97	261*	246	10
			246	190	13
4	**CBZ**	16.81	193*	191	23
			193	167	18
4	**DHCBZ**	17.58	195*	152	30
			195	180	18
4	**TPhA**	17.16	245*	167	30
			245	141	21
5	**BPA**	17.74	241*	133	15
			241	211	17
5	**DNBP**	17.95	198*	168	15
			198	138	25
6	**TCS**	18.25	302*	252	19
			302	189	37

* Transition used for quantification

3.7.3 GC-MS/MS determination

BPA, CAF, CBZ, IBU, TON and TCS were analyzed by GC-MS/MS. Methylation of the acidic carboxyl group for both vegetal tissue and soils extracts was performed in a programmed temperature vaporizing (PTV) injector of the gas chromatograph by adding 10 µL TMSH to a 50 µL sample aliquot before

injection. A volume of 5 µL was injected into a Bruker 450-GC gas chromatograph coupled to a Bruker 320-MS triple quadrupole mass spectrometer (Bruker Daltonics, Billerica, MA, USA) fitted with a 20 m × 0.18 mm ID, 0.18 µm film thickness Sapiens X5-MS capillary column coated with 5 % diphenyl 95 % dimethyl polysiloxane from Teknokroma (Sant Cugat del Vallès, Spain). The PTV was set at 60 ºC for 0.5 min and rapidly heated to 300 ºC at 200 ºC min^{-1}, and hold for 7 min. Then the injector was cooled to initial 60 ºC at 200 ºC min^{-1}. The oven temperature was held at 60 ºC for 3.5 min and then the temperature was programmed at 30 ºC min^{-1} to a 150 ºC and finally at 8 ºC min^{-1} to 320 ºC, holding the final temperature for 6 minutes. Gas flow rate was set at 0.6 mL min^{-1}. Ion source temperature and the transfer line both were held at 250ºC. A solvent delay of 8 minutes was applied. Argon gas was used for CID at a pressure of 1.8 mTorr, and the optimum collision energy (CE) was selected for each transition.

Qualitative and quantitative analysis was performed based on retention time and selected reaction monitoring (SRM) mode of two product ions, and the ratio between the product ions (Table S3.1). The limit of detection (LOD) and the limit of quantitation (LOQ) for both vegetal tissue and soil were defined as the mean background noise in a blank triplicate plus three and ten times, respectively, the standard deviation of the background noise from three blanks. LODs and LOQs were compound dependent and for leaves and roots ranged from 0.8 to 5 ng g^{-1} dry weight (dw) and for soil ranged from 0.5 to 1 ng g^{-1} dw (Table S3.2). The recoveries of the surrogates added can be seen in Table S3.4.

3.7.4 LC-MS/MS determination

Extract aliquots were evaporated to dryness and reconstituted with methanol:water (20:80, v/v) for SMT and PROP determination by LC-MS/MS. A TSQ Quantum triple-stage quadrupole mass spectrometer equipped with and ESI source (Thermo Fischer Scientific, San Jose, CA, USA), a Finnigan Surveyor MS Pump Plus and an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) were used for LC-MS/MS determination.

The chromatographic separation was performed on a Kinetex® C18 Phenomenex® (50 × 2.1 mm, 2.6 µm). The mobile phase consist of water (A) and methanol (B) both solvents with 0.1 % formic acid and is set at 350 µL min^{-1}. The elution started at 20 % B for 1 min and was then linearly ramped up to 99

% B in 14 min, where it was held for 1 min before returning to the initial conditions in 1 min. The injection volume was 5 μL, and the column was maintained at 35 °C. The MS/MS determination was carried out in ESI positive ion mode with the spray voltage at 5.0 kV and the optimum tube lens voltage (TL) were optimized for each m/z. The ion transfer temperature was set at 250 °C. Nitrogen (purity, >99.999 %) was used as a sheath gas, ion sweep gas, and auxiliary gas at 70 psi. Data were acquired in the selected reaction monitoring (SRM) mode. Argon gas was used for CID at a pressure of 1.3 mTorr, and the optimum collision energy (CE) was selected for each transition (Table S3.3).

Qualitative and quantitative analysis was performed based on retention time and SRM mode of two product ions, and the ratio between the product ions as confirmation. The limit of detection (LOD) and the limit of quantitation (LOQ) for both vegetal tissue and soil were calculated as the mean background noise in a blank triplicate plus three and ten times, respectively, the standard deviation of the background noise from three blanks. LODs and LOQs were compound dependent and for leaves and roots ranged from 2.1 to 3.2 ng g^{-1} dry weight (dw) and for soil ranged from 0.05 to 0.10 ng g^{-1} dw respectively. LODs and LOQs for each compound in the different compartments are presented in Table S3.3.The recoveries of the spiked surrogates can be seen in Table S3.4.

Table S3.2. Monitoring ions in LC-MS/MS

Segment	Compound	RT (min)	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
1	**SMT**	3.51	279*	149	17
			279	186	18
2	**SMX**	3.82	254*	183	17
			254	155	25
3	**PROP**	4.75	260*	156	16
			260	92	29

* Transition used for quantification

Table S3.3. Limits of detection (LOD) and quantification (LOQ) of the selected CECs in the three compartments studied.

Compound	Compartment	LOD (ng g^{-1} dw)	LOQ (ng g^{-1} dw)
BPA	**Soil**	0.91	0.98
	Root	3.9	4.3
	Leaf	4.5	5.3
CAF	**Soil**	0.52	0.58
	Root	1.2	1.3
	Leaf	1.5	1.6
CBZ	**Soil**	0.5	0.6
	Root	1.1	1.2
	Leaf	1.3	1.5
IBU	**Soil**	0.6	0.7
	Root	1.1	1.2
	Leaf	0.8	0.9
PROP	**Soil**	0.8	0.9
	Root	2.3	2.9
	Leaf	5.3	6.0
SMT	**Soil**	0.5	0.6
	Root	0.8	0.9
	Leaf	3.9	4.3
TCS	**Soil**	0.41	0.44
	Root	0.85	0.91
	Leaf	0.93	1.2
TON	**Soil**	0.53	0.60
	Root	1.1	1.3
	Leaf	1.1	1.4

Table S3.4. Recoveries of the surrogates added in each compartment (N = 20).

Compound	Compartment	Recovery (%)
DHCBZ	**Soil**	52 ± 5
	Root	77 ± 6
	Leaf	81 ± 7
DNBP	**Soil**	68 ± 5
	Root	65 ± 5
	Leaf	70 ± 8
FEN	**Soil**	41 ± 4
	Root	77 ± 6
	Leaf	71 ± 7
SMX	**Soil**	61 ± 12
	Root	38 ± 10
	Leaf	35 ± 7
TON-d3	**Soil**	59 ± 7
	Root	73 ± 12
	Leaf	68 ± 14

3.7.5 Chiral derivatization of IBU

The derivatization procedure was described by Hashim and Khan (2011). The extracts were subjected to chiral derivatization by adding 30 µL of TEA (50mM in acetonitrile) and 40 µL of ECF (60mM in acetonitrile). This mixture was sonicated for 2 min and 10 µL of *R*-1-PEA (0.5 M in acetonitrile) were added. Then, the mixture was again sonicated for 2 min. Sulfuric acid 0.1 M and ultrapure water were added to stop the reaction, lower the pH and prepare the sample for further extraction of the diastereomeric derivatives.

The SPE cartridges were initially conditioned with 1.5 mL of ethyl acetate, 1.5 mL of methanol and 1.5 mL of ultra-pure water adjusted to pH 9.5. The aqueous solutions were passed through the cartridges under gravity and the cartridges were rinsed twice with 1.5 mL of ultra-pure water adjusted to pH 9.5. The cartridges were then dried under vacuum for 10 min. Finally, the amide derivatives were eluted with ethyl acetate (1 mL) to 2 mL GC vials.

The ibuprofen derivatives analysis was performed on a Trace GC-MS 2000 gas chromatograph – mass spectrometer (GC-MS) equipped with a 20 m × 0.18 mm

ID, 0.14 μm film thickness TRB-50 column coated with (50%) diphenyl-(50%) dimethyl polysiloxane from Teknokroma. The carrier gas flow rate was 0.6 mL min^{-1}. 1 μL samples were injected in splitless mode and the injector temperature was set at 280 ºC. The oven temperature was held at 65 ºC for 2 min and then the temperature was programmed at 15 ºC min^{-1} to 120 ºC, at 6 ºC min^{-1} to 220 ºC and 12 ºC min^{-1} to 310 ºC, holding the final temperature for 10 min. Mass spectrometric ionization was undertaken in electron impact (EI) mode (70 eV) and the GC interface temperature was held at 270 ºC. Acquisition was performed in single-ion monitoring (SIM) mode with dwell times ranging from 0.300 to 0.190 s depending on the time segment, to achieve a minimum of 7 points per GC peak. The ions 161/119/105 (25 - 30 min) were monitored for ibuprofen derivatives and 245 (16 - 25 min) for internal standard tryphenylamine.

Table S3.5. Linear regression coefficients between the applied dose of CEC and the concentration found in each compartment.

Soil	Compound	Slope	R^2	p-value
	BPA	0.461	0.939	1.04E-10
	CAF	0.488	0.923	5.60E-10
	CBZ	0.894	0.899	4.63E-09
	IBU	0.188	0.937	1.30E-10
	PROP	0.211	0.710	1.32E-05
	SMT	0.040	0.879	1.70E-08
	TON	0.348	0.882	1.42E-08
	TCS	1.495	0.954	1.24E-11
Roots				
	BPA	3.030	0.926	4.21E-10
	CAF	3.625	0.931	2.54E-10
	CBZ	9.862	0.935	1.56E-10
	IBU	1.693	0.865	3.91E-08
	PROP	3.931	0.903	3.30E-09
	SMT	4.170	0.975	1.14E-12
	TON	4.891	0.958	1.59E-10
	TCS	6.396	0.931	2.60E-10
Leaves				
	BPA	1.394	0.917	1.05E-09
	CAF	1.296	0.968	7.30E-13
	CBZ	17.55	0.982	1.27E-14
	IBU	0.173	0.855	6.82E-08
	PROP	1.040	0.958	5.72E-12
	SMT	NA	NA	NA
	TON	2.533	0.911	1.73E-09
	TCS	0.320	0.910	1.90E-09

Chapter 4. Inverse modeling to estimate degradation

This chapter is based on the article:

Hurtado, C., Trapp, S., Bayona, J.M., (2016). Inverse modeling of the biodegradation of emerging organic contaminants in the soil-plant system. Chemosphere 156, 236-244.

Understanding the processes involved in the uptake and accumulation of organic contaminants into plants is very important to assess the possible human risk associated with. Biodegradation of contaminants of emerging concern (CECs) in plants has been observed, but kinetical studies are rare. In this study, we analyze experimental data on the uptake of CECs into lettuce derived in a greenhouse experiment. Measured soil, root and leaf concentrations from four contaminants were selected within the applicability domain of a steady-state two-compartment standard plant uptake model: bisphenol A (BPA), carbamazepine (CBZ), triclosan (TCS) and caffeine (CAF). The model overestimated concentrations in most cases, when no degradation rates in plants were entered. Subsequently, biodegradation rates were fitted so that the measured concentrations were met.

Obtained degradation kinetics are in the order, BPA < CAF ≈ TCS < CBZ in roots, and BPA ≈ TCS < CBZ << CAF in leaves. Kinetics determined by inverse modeling are, despite the inherent uncertainty, indicative of the dissipation rates. The advantage of the procedure that is additional knowledge can be gained from existing experimental data. Dissipation kinetics found via inverse modeling is not a conclusive proof for biodegradation and confirmation by experimental studies is needed.

4.1 Introduction

Pharmaceuticals, biocides and drugs as well as other chemicals from human use, reach sewer systems and are partially removed during conventional wastewater treatment processes (Halling-Sørensen et al., 1998). By irrigation with reclaimed water, or sewage sludge amendment, these chemical residues may reach agricultural soils. Uptake into crops can lead to human exposure to such chemicals (Hospido et al., 2010). In the European Union, the environmental risk from pharmaceutical products is assessed only for veterinary drugs (EMA European Medicines Agency, 2011), and only few pharmaceuticals and drugs are regularly monitored according with the Watch List of the Water Framework Directive (European Comission, 2008). Then, human exposure to contaminants of emerging concern (CECs) relies partly on scientific studies, and an increasing number of studies on their uptake into vegetables is reported (Wu et al., 2015; Miller et al., 2016).

Prosser and Sibley (2015) found no human health hazards from the plant uptake of the "majority of pharmaceuticals and personal care products". However, Malchi et al. (2015) stated that "current data are insufficient to support a comprehensive human health risk assessment" of pharmaceuticals and personal care products in plant tissue due to biosolids and manure amendments, or reclaimed water irrigation. Due to the high number of compounds potentially present in reclaimed water (Calderón-Preciado et al., 2011a; Loos et al., 2013; Luo et al., 2014), prediction tools for pre-screening of chemicals and priority setting for safety assessments are of high value (Polesel et al., 2015). Prosser et al. (2014b) examined the ability of two prediction models to estimate the uptake of pharmaceuticals and personal care products (PPCPs) into plants from sludge-amended soils. Predictions of plant uptake of PPCPs within one order of magnitude near the experimental results were achieved for some of the investigated compounds. Polesel et al. (2015) developed and tested a simulation tool for fate prediction from human pharmaceuticals down the drain through a sewage treatment plant and via sludge amendment and irrigation to agricultural fields and crops. However, simulations were performed disregarding degradation in plants. To reduce discrepancies between model predictions and measurements, the authors stressed the need for more measured input parameters (e.g., K_d) and kinetics of biotransformation in plant tissues.

For polar compounds, efficient translocation in xylem of plants can be expected (Trapp, 2007; Dettenmaier et al., 2009), leading to accumulation in leaves, if no losses occur. Biodegradation has been identified as among the most relevant dissipation processes of chemicals from plants (Fantke et al., 2012; Jacobsen et al., 2015), but is often unknown or uncertain and depends on a number of factors, such as species and temperature (Fantke and Juraske, 2013; Fantke et al., 2014; Jacobsen et al., 2015). Methods to measure metabolism in soil and plants have been developed early, typically employing the use of ^{14}C-labeled compounds to close the mass balance (Trapp et al., 1990; Kästner et al., 2014). There are also OECD guidelines for pesticide metabolism in crops to elucidate the degradation pathway available (i.e. OECD Tests Nr. 501, 502). The drawback is that studies with hot labels are expensive, and safety issues arise. These safety issues can be solved by using stable isotopes (^{13}C and ^{15}N), but require IRM-MS equipment, if isotopically labeled compounds are available at all.

An alternative method to assess biodegradation that has rarely been attempted is the use of inverse modeling. Hereby, predictable loss due to physical-chemical processes (volatilization, translocation, dilution) is contrasted with measured dissipation. The difference is contributed to biodegradation. This method cannot prove degradation but can help to quantify loss processes (Jacobsen et al., 2015).

The kinetics of biodegradation affects the relation between concentrations in plants and soil. First-order degradation kinetics, either in soil or in plants, will change the slope of the trend line (lower for degradation in plants, higher for degradation in soil), but the relation will remain linear. In a study with lettuce grown under controlled conditions and irrigated with water containing eight contaminants of emerging concern (CECs), Hurtado et al. (2016) obtained mostly linear correlations between watering concentrations and concentrations measured in roots and leaves. Besides hydrophobicity (log D_{ow}) of chemicals, their persistence was identified as a key determinant for plant uptake and accumulation of the CECs.

In this study, we supplemented a standard plant uptake model (Rein et al., 2011) with different degradation kinetics for soil and plant. The model was parameterized to simulate the uptake experiments of emerging organic contaminants into lettuce performed by Hurtado et al. (2016). Degradation rate constants in soil were derived from the measured concentrations, while rates in

leaves and roots were fitted, based on the difference between the model prediction (without degradation) and the measured data. The resulting rates were compared to data from literature.

4.2 Materials and methods

4.2.1 Experimental section

Experiments were conducted in a glass greenhouse located in Viladecans (Barcelona, Spain) as described in Hurtado et al. (2016). Briefly, lettuce (*Lactuca sativa*) was planted in pots in a mixture of perlite and sand (2:1 v/v, approx. 1.2 kg) and watered with Hoagland nutrient solution (Hoagland and Arnon, 1950) diluted 1:1 with rain water. A dose of 50 mL of irrigation water was applied to each experimental unit per day. The number of daily irrigations was regulated to keep water in the soil below field capacity, thereby preventing leachate production.

After 40 days, CECs were added to soil. Five treatments consisted of direct application of 0, 14, 35, 70 and 140 μg of eight CECs per experimental unit in eight applications during 28 days. Taking into account the soil substrate mass in each experimental unit, this corresponds to an average nominal initial concentration in the substrate of 0, 11.7, 29.2, 58.3 and 116.7 μg kg^{-1} dw. After 28 days, substrate, roots and leaves of lettuces were separated and analyzed. The data used in this study can be found in the SI and are also reported in Hurtado et al. (2016).

The CECs measured in the experimental study were bisphenol A, caffeine, carbamazepine, ibuprofen, propranolol, sulfamethazine, triclosan and tonalide. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), except tonalide from Ventós (Sant Just Desvern, Spain). The extraction of CECs from vegetal tissue and substrate and the analytical parameters are listed in Hurtado et al. (2016). The properties of the compounds are listed in Table 4.1.

Table 4.1: Properties of the compounds added in the experiment by Hurtado et al. (2016). All values were obtained using ACD Advanced Chemistry Development (2010), ACD / i-lab 2.0, Toronto, 2010.

CEC	Molar mass (g mol^{-1})	p*K*a values	Speciation (z)	Neutral log K_{ow}	log D_{ow} at pH 6.4	log K_{AW}	log K_{HSA}
Bisphenol A (BPA)	228.29	9.7, 10.5	0/-1/-2	3.46	3.46	-9.43	3.57
Caffeine (CAF)	194.19	NA	0	0.11	0.11	-8.83	2.53
Carbamazepine (CBZ)	236.27	NA	0	2.23	2.23	-7.20	3.74
Ibuprofen (IBU)	206.28	4.3	0/-1	3.63	1.53	-5.21	4.42
Propranolol (PROP)	259.34	9.5	1/0	2.69	0.13	-10.49	3.54
Sulfamethazine (SMT)	278.33	3.1, 7.2	1/0/-1	0.31	0.25	-11.33	4.1
Tonalide (TON)	258.40	NA	0	5.71	5.71	-2.04	4.71
Triclosan (TCS)	289.54	8.8	0/-1	5.21	5.21	-4.08	4.81

NA: Not applicable; z is charge number (valence); K_{ow} (L L^{-1}) is the partition coefficient octanol to water for the neutral molecule; D_{ow} (L L^{-1}) is the apparent partition coefficient of the neutral and ionic molecules at pH 6.4 (soil pH); K_{AW} (L L^{-1}) is the partition coefficient air to water for neutral molecules (known as dimensionless Henry's Law constant) and K_{HSA} (L mol^{-1}) is the adsorption to human serum albumin (as predictor for the adsorption to proteins).

4.2.2 Model section

The plant uptake model is based on the commonly used "standard model" for plant uptake (Legind and Trapp, 2009; Legind et al., 2011; Rein et al., 2011; Trapp, 2015). Modifications were introduced to consider different degradation kinetics. This version of the model is primarily designed for neutral compounds. As long as the fraction of ionic molecules is small, ionization only slightly affects the outcome when measured K_d-values are used. PROP, IBU and SMT were not included in the plant uptake simulations because the ionization prohibits the use of this model version. TON was excluded because of its high volatility. In a separate approach, *Michaelis-Menten* degradation kinetics in roots and leaves were calculated, but for mathematical reasons with initial (constant) concentration in soil.

The underlying differential equation for the change of concentration in roots (C_R, mg kg^{-1}) with time t (d) is:

+ inflow from soil - translocation upwards - dilution by growth – degradation

$$\frac{dC_R}{dt} = \frac{Q}{M_R} \times \frac{C_S}{K_d} - \frac{Q}{M_R \times K_{RW}} \times C_R - k_{growth} \times C_R - degrad. \quad (4.1)$$

where R is index for roots, Q is the transpiration (L d^{-1}), M is the plant mass (kg), C_S is the concentration of chemical in soil (mg kg^{-1}), K_d is the distribution coefficient between substrate and pore water (L kg^{-1}), K_{RW} is partition coefficient roots to water (and xylem sap) (L kg^{-1}), and $k_{growth,R}$ is the growth rate of roots (d^{-1}).

The differential equation for the change of concentration in leaves (C_L, mg kg^{-1}) with time, neglecting uptake of chemical from air, is

+ translocation from roots - loss to air - dilution by growth – degradation

$$\frac{dC_L}{dt} = \frac{Q}{M_L \times K_{RW}} \times C_R - \frac{A_L \times g \times 1000}{K_{LA} \times M_L} \times C_L - k_{growth,L} \times C_L - degrad. \quad (4.2)$$

where L is index for leaves, A is area (m^2), g is conductance (m d^{-1}) and K_{LA} is partition coefficient between leaves and air (L kg^{-1}).

a) Coupled dynamic differential equation system with first-order degradation

The concentrations in soil, roots and shoots are calculated from a system of coupled ordinary differential equations that form a triangular matrix and are solved analytically.

The concentration in soil is considered time-dependent, with

$$C_S(t) = C_S(0) \times e^{-k_1 t} \quad (4.3)$$

The loss rate from soil k_1 (matrix element 1) was calculated from the measured initial and final concentrations at time t, $C_S(t)$, assuming first-order loss due to degradation, plant uptake and volatilization:

$$k_1 = \frac{\ln \frac{C_S(0)}{C_S(t)}}{t} \tag{4.4}$$

The transfer rate from soil to roots is

$$k_{12} = \frac{Q}{M_R \times K_d} \tag{4.5}$$

The rate k_2 is the sum of all loss terms (to shoots, dilution, degradation) from roots (d^{-1})

$$k_2 = \frac{Q}{M_R \times K_{RW}} + k_{growth,R} + k_R \tag{4.6}$$

k_R is the 1st order degradation rate that is to be fitted.

The rate k_3 (d^{-1}) is the sum of all loss terms (to air, dilution, degradation) from leaves

$$k_3 = \frac{A_L \times g \times 1000\ L\,m^{-3}}{K_{LA} \times M_L} + k_{growth,L} + k_L \tag{4.7}$$

k_L is the 1st order degradation rate that is to be fitted.

The transfer rate from roots to leaves is

$$k_{23} = \frac{Q}{M_L \times K_{RW}} \tag{4.8}$$

The analytical solution for the concentration in roots (matrix element 2) is

$$C_R(t) = \frac{k_{12} \times C_S(0)}{k_2 - k_1} \times \left(e^{-k_1 \times t} - e^{-k_2 \times t}\right) \tag{4.9}$$

and for leaves (matrix element 3) is

$$C_L(t) = k_{12} k_{23} C_S(0) \left\{ \frac{e^{-k_1 t}}{(k_2 - k_1)(k_3 - k_1)} + \frac{e^{-k_2 t}}{(k_1 - k_2)(k_3 - k_2)} + \frac{e^{-k_3 t}}{(k_1 - k_3)(k_2 - k_3)} \right\} \tag{4.10}$$

This model resembles the cascade model presented and tested by Rein et al. (2011) and applied by Legind et al. (2011) and Prosser et al. (2014b).

b) Michaelis-Menten degradation kinetics in roots and leaves

Enzymatic reactions often follow the Michaelis-Menten kinetics

$$degradation = \frac{v_{max} \times C}{K_M + C} \tag{4.11}$$

where v_{max} is the maximal enzymatic removal in roots or leaves (mg d^{-1}) and K_M (mg kg^{-1}) is the concentration at which removal is half v_{max}. With *Michaelis-Menten* type kinetics, the shape of the trendline between concentrations in soil and plant is no longer linear. This kinetic has been observed for the degradation of cyanide by plants (Larsen et al., 2004; Yu et al., 2004) and for the exclusion of salt NaCl and NaF from roots (Trapp et al., 2008; Clausen et al., 2015). The assumption of steady-state was made to allow for a closed analytical solution, and requires constant concentration in soil $C_S(0)$. The steady-state leads to a quadratic equation which was solved using Vieta's formulas (Larsen et al., 2004; Trapp et al., 2008).

The comparison of experimental values from different studies with different concentration levels is done by calculation of root concentration factor (RCF) and leaf concentration factor (LCF) which are defined as

$$RCF = \frac{C_R(t_2)}{C_S(t_1)} \tag{4.12}$$

$$LCF = \frac{C_L(t_2)}{C_S(t_1)} \tag{4.13}$$

Here, t_1 and t_2 stand for the time when the concentrations were measured. While t_2 (the time when the concentration in root and leaf is determined) typically refers to the time of harvest, there is no standard for t_1, and initial, nominal or final (at harvest) concentrations have been used for the calculation of RCF and LCF.

Model input data

Where available, input data for the plant properties were taken from the experiment. As shown recently, plant properties can significantly affect the

outcome of the model simulations (Trapp, 2015). The experiment was carried out in a greenhouse in Spain, but in the winter period. Growth was moderate (growth rate 0.05 d^{-1}), and the ratio of transpiration to plant mass was relatively low (Table 4.2).

Table 4.2: Input data for the simulation of the uptake experiment with lettuce. Data shown are for an individual pot. Displayed is the data set for an experiment with carbamazepine (experimental unit number 17). Data source Hurtado et al. (2016).

Input data	Symbol	value	unit	Comment
distribution coefficient	K_d	0.72	L kg^{-1}	measured
total loss rate	k_1	0.0895	d^{-1}	calculated from measurements
water content roots	W_R	0.898	L kg^{-1}	measured
lipid content roots	L_R	0.025	kg kg^{-1}	default
mass of roots	M_R	0.0833	kg	measured
transpiration	Q	0.053	L d^{-1}	calculated from added water
growth rate root	$k_{growth,R}$	0.05	d^{-1}	calculated from measurements
shoot mass	M_L	0.2227	kg	measured
leaf area	A	1	m^2	default
conductance	g	0.001	m s^{-1}	default
lipid content leaves	L_L	0.02	g g^{-1}	default
water content leaves	W_L	0.954	g g^{-1}	measured
time between dosing and harvest	t	28	d	measured
growth rate shoots	$k_{growth,L}$	0.05	d^{-1}	calculated from measurements

4.3 Results

Dry weight and water content of substrate, root and leaf for the different experimental units can be found in Table S4.1. Final concentrations in the three compartments can be found in Tables S4.2, S4.3 and S4.4.

Bioconcentration factors derived from experimental data with lettuce

Root concentration factor (RCF, kg kg^{-1} dw) and leaf concentration factor (LCF, kg kg^{-1} dw) were calculated as the slopes of the linear regression of the concentration in plant versus either the initial (nominal) or the final concentration in the soil substrate. Figure 4.1 shows the RCFs and LCFs of CBZ and IBU. RCFs

obtained for CBZ were rather similar when initial or final concentrations of CBZ in soil were used to establish the regression. The slope, interpreted as RCF, was 10.4 kg kg^{-1} dw with the initial and 9.6 kg kg^{-1} dw with the final substrate concentration, however, the y-intercept went from -45 μg kg^{-1} dw to +92 μg kg^{-1} dw. Also, the LCF of CBZ changed very little, from 17.6 to 15.1 kg kg^{-1} dw (but with high Y-intercept of 268 μg kg^{-1} dw). Conversely, for IBU the slope of the RCF regression changed from 2.0 to 9.4 kg kg^{-1} dw and from 0.20 to 0.94 kg kg^{-1} dw for the LCF with initial or final soil concentrations, and Y-intercepts were negative. The slopes for the other compounds can be seen in Figure S4.1 (RCF) and S4.2 (LCF). All compounds showed good uptake into roots with RCF > 1 kg kg^{-1} dw. Translocation to leaves was highest for CBZ and lowest for TCS. Most slopes increased when the final substrate concentration was used for the regression.

Modeling

The data obtained in the experiments (Table 4.2), such as dry weights at harvest and transpiration, were used to simulate plant uptake with the standard plant uptake model with degradation as described above (Equations. 4.1-4.9). The loss rates from soil (k_1) were calculated from the nominal initial concentration and the final measured concentration assuming exponential (1st order) decay (Table 4.3). Except one case (CAF, lowest applied amount), the loss rate from soil of all studied CECs decreased when the initial concentration increased. For example, for CBZ the loss rates from soil were 0.090, 0.037, 0.015 and 0.006 d^{-1} for the four treatments (11.7, 29.2, 58.3 and 116.7 μg kg^{-1} dw). For IBU, the rates were 0.100, 0.094, 0.067 and 0.055 d^{-1} for the same treatments.

Degradation rates in roots and leaves were determined by fitting simulated concentrations in plants to the measured ones. Figure 4.2 shows an example for the simulations with fit. The simulation of BPA succeeds without added degradation in roots or leaves, but only when dissipation from soil is considered. For CAF, on the other hand, the simulation improves when a fast degradation rate in leaves is assumed. The results of the linear model appear curved due to the changing degradation rates in soil. Judged from the correlation between calculated and average measured concentrations, the *Michaelis-Menten* kinetics in this case is closer to the measured result. In all cases, however, omitting degradation in soil (labeled as 00 in Figure 4.2) leads to drastic overestimation.

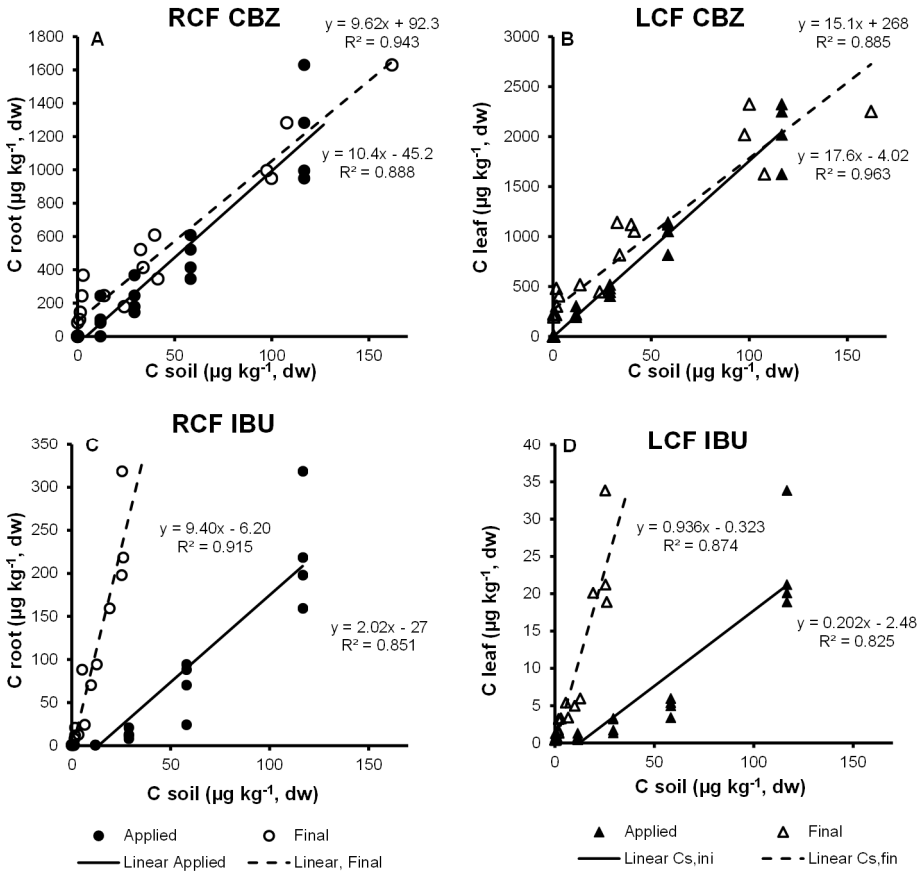


Figure 4.1: Root and leaf bioconcentration factors (RCFs and LCFs) of carbamazepine (CBZ) and ibuprofen (IBU). The solid and dashed lines represent the linear regression of the root and leaf concentration on the applied initial and the final soil concentration.

Fitted first-order dissipation rates of selected CECs are shown in Table 4.3b. The fastest first-order dissipation rate from roots was fitted for CBZ (0.35 d^{-1}). Dissipation from roots also affects leaves, but nonetheless a rapid dissipation rate of CAF from leaves was required to meet the measured data. The adjusted parameters for the *Michaelis-Menten* kinetics in roots and leaves can be found in Table 4.3c. Fitted v_{max} was higher in roots than in leaves, except for CAF. The values have to be taken with care because the two parameters cannot be verified independently, but also because the fitted degradation must replace partly the missing dissipation from soil which, for mathematical reasons, could not be considered in the simulation with *Michaelis-Menten* kinetics.

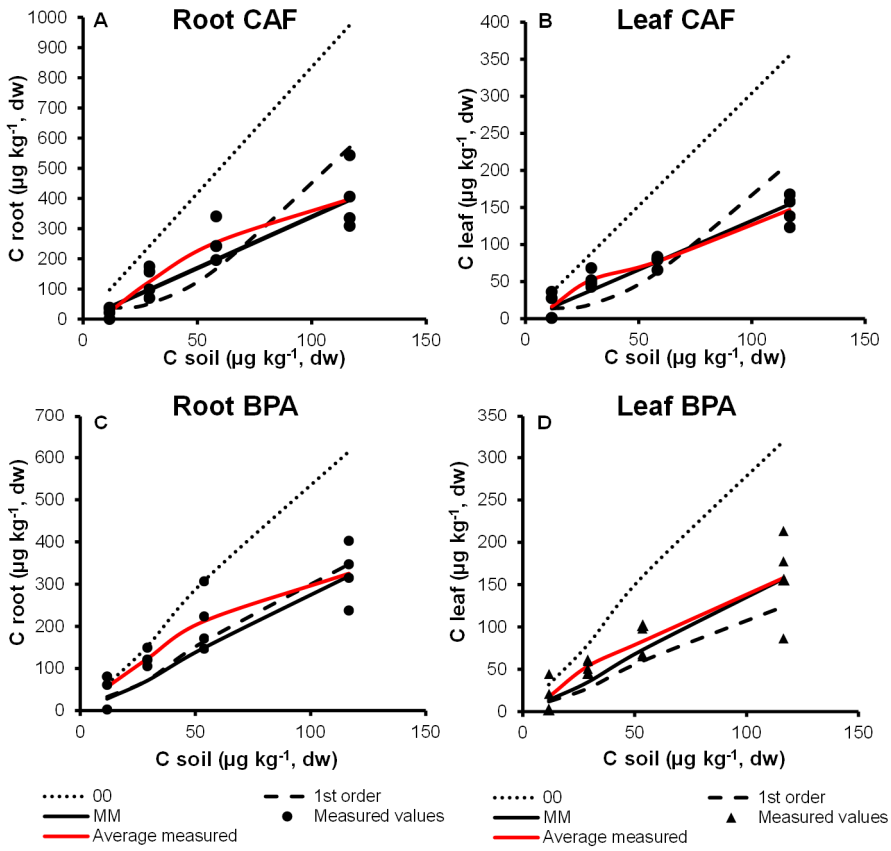


Figure 4.2: Simulated and measured concentrations in a) top left: CAF roots, b) top right: CAF leaves, c) bottom left: BPA roots; d) bottom right: BPA leaves. Solid points represent measured values; 00: no degradation in soil or plant; 1st: first-order degradation in soil, roots and leaves; MM: *Michaelis-Menten* degradation in roots and leaves.

Table 4.3: Calculated first order degradation rates in soil, k_{soil}, (d^{-1}) for the four different treatments of selected CECs (in brackets: standard deviation, N = 4).

Treatment (µg kg^{-1} dw)	BPA	CAF	CBZ	IBU	PROP	TCS
11.7	0.039 (0.030)	0.038 (0.008)	0.109 (0.040)	0.104 (0.010)	0.088 (0.024)	0.008 (0.010)
29.2	0.038 (0.008)	0.060 (0.004)	0.058 (0.048)	0.099 (0.013)	0.080 (0.023)	0.001 (0.012)
58.3	0.031 (0.005)	0.044 (0.012)	0.017 (0.004)	0.073 (0.015)	0.076 (0.034)	0.001 (0.020)
116.7	0.029 (0.011)	0.022 (0.006)	0.001 (0.009)	0.059 (0.005)	0.063 (0.032)	0.001 (0.008)

Table 4.3b: Fitted first-order degradation rates (d^{-1}) in roots (k_{root}) and leaves (k_{leaf}) of selected CECs.

	BPA	CAF	CBZ	TCS
k_{root} (d^{-1})	0.00	0.05	0.35	0.10
k_{leaf} (d^{-1})	0.00	1.50	0.05	0.00

Table 4.3c: Adjusted *Michaelis-Menten* parameters v_{max} (mg d^{-1}) and K_M (mg kg^{-1}) for enzymatic degradation in kinetics equation of selected CECs.

	BPA	CAF	CBZ	TCS
v_{max} root	0.01	6.0	0.20	0.20
K_M root	0.10	5.0	0.45	1.00
v_{max} leaf	0.0003	7.0	0.07	0.00
K_M leaf	0.1	5.0	0.0001	none

4.4 Discussion

4.4.1 Bioconcentration factors

Bioconcentration factors (BCF) are defined as concentration ratio between organism and surrounding medium. However, the calculations can be done in various ways. Some authors calculate BCFs from the concentration in the irrigation water, others from the concentration in soil and some from the concentration in the soil solution. Moreover, in some studies the nominal concentration is used while others use the final concentration in soil at harvest to calculate the BCFs. In this study, BCFs were derived as slope of the regression line so measurements at all concentrations contributed simultaneously, without contribution of background (Y-intercept), and with both initial (nominal) and final concentration (Figures S4.1, S4.2). For CBZ there were no big differences in the BCF when it was calculated with initial or final substrate concentration (Figure 4.1). On the other hand, for IBU the difference was almost 5 times (2.0 to 9.4 g g^{-1} dw). Thus, it is important to consider that dissipation from soil or substrate will affect the BCF.

For most of the studied CECs, experimental BCFs can be found in the literature (Table 4.4). The reported values show large variance and are generally far higher in hydroponics. In comparison to BCFs derived from experiments with soil, our values are at the higher end, probably due to the lower adsorption capacity of perlite and sand, compared to soil organic matter.

The half-lives (DT50) in the perlite and sand mixture are slower than those derived in soil. The perlite and sand mixture was chosen as a substrate because in laboratory studies we observed that there were lower interactions than when using soil. The uptake simulations were done with measured K_d-values, thus, the difference in adsorption does not affect the simulations. But the substrate for this experiment lacked of organic matter, which can be used as main substrate by bacteria degrading co-metabolically CEC. This may explain why most loss rates from our substrate were lower than rates found in soil (Fent et al., 2003; Langdon et al., 2012; Matsumura et al., 2015). For example, DT50 of bisphenol A in soil have been determined from 0.5 to 7 days (Cousins et al., 2002; Ying and Kookana, 2005; Xu et al., 2009), while we found half-lives from 17.8 to 23.9 days. BPA dissipation is related to bacteria in soil and it dissipates faster in more aerobic environments (Fent et al., 2003).

Table 4.4: Experimental values of bioconcentration factors (BCF) and dissipation time in soils (DT50) reported and obtained in this study.

CEC	Experiment ype	Plant	Tissue	BCF literature	BCF in this study (kg kg^{-1} dw)	DT50 (d) in literature	DT50 (d) this study
BPA	Hydroponics	Dracaena[a]	Stem	170 L kg^{-1} dw	Leaf 1.3 – 2.7	0.5 – 7[i,j,k]	18 – 24
			Root	110 L kg^{-1} dw	Root 2.6 – 4.5		
		Lettuce, collard[b]	Leaf	7 - 66 L kg^{-1} dw			
			Root	4339 - 9587 L kg^{-1}			
CAF	Soil	Cucumber, tomato, sweet potato, carrot[c,d]	Leaf	0.4 – 17 L kg^{-1} dw	Leaf 1.2 – 0.94	1.5 – 3[l,m]	12 – 32
		Cucumber, tomato[c]	Fruit	0.4 – 5.3 L kg^{-1} dw	Root 3.5 – 5.3		
		Sweet potato, carrot[d]	Root	0.1 – 0.8 kg kg^{-1} dw			
CBZ	Soil	Soybean, radish, cucumber, tomato, sweet potato[c,d,e,f]	Leaf	0.6 - 425 kg kg^{-1} dw	Leaf 15 – 17.6	60 – 533[n,o]	6.4 – >40
					Root 9.9 – 10.4		
		Soybean, radish, sweet potato, carrot[d,e,f]	Root	0.1 – 8.3 kg kg^{-1} dw			
		Cucumber, tomato[c]	Fruit	0.4 - 27 kg kg^{-1} dw			
IBU	Hydroponics	Typha, phragmites, iris, juncus[g]	Root	7 – 201 L kg^{-1}	Leaf 0.2 – 0.94	1 – 6[i,p]	6.7 – 11.7
					Root 2.0 – 9.4		
PROP	Soil	Radish, ryegrass[f]	Leaf	– 11.9 kg kg^{-1} dw	Leaf 1.1 – 2.9	> 40[f]	7.9 – 11
			Root	1.2 kg kg^{-1} dw	Root 3.2 – 6.7		
			Root	1.2 kg kg^{-1} dw			
TCS	Soil	Radish, ryegrass[f]	Leaf	0.1 - 38 kg kg^{-1} dw	Leaf 0.15 – 0.24	18 – 187[q,r,s]	>40
			Root	0.12 kg kg^{-1} dw	Root 4.4 – 6.9		
TON	Soil	Carrot	Leaf	0.18 kg kg^{-1} dw	Leaf 2.7 – 5.9dw	50 – 133[s]	
		Carrot, barley, meadow	Root	0.50 – 2.74 kg kg^{-1} dw	Root 5.3 – 12 kg kg^{-1} dw		

[a]Saiyood et al. (2010), [b]Dodgen et al. (2013), [c]Goldstein et al. (2014), [d]Malchi et al. (2014), [e]Wu et al. (2010), [f]Carter et al. (2014), [g]Zhang et al. (2015d), [h]Macherius et al. (2012b), [i]Xu et al. (2009), [j]Cousins et al. (2002), [k]Ying and Kookana (2005), [l]Lin et al. (2010), [m]Topp et al. (2006), [n]Monteiro and Boxall (2009), [o]Walters et al. (2010), [p]Löffler et al. (2005), [q]YYing et al. (2007), [r]Walters et al. (2010), [s]Chen et al. (2014).

4.4.2 Degradation

Dissipation rates from soil in Table 4.3a were calculated from initial nominal and final measured substance concentrations in the substrate. Rates decreased with increasing concentrations. Such kinetics, i.e. decreasing (pseudo) first-order rates with increasing substrate concentrations, can occur by enzymatic degradation when the half-saturation concentration K_M of the reaction is within the range of occurring concentrations and when at the same time the amount of enzymes is constant. Thus, co-metabolic (non-growth) degradation by microbes living in the substrate or near and in the roots, but also degradation by roots itself can lead to this kinetics. At higher soil concentrations, PROP, IBU, BPA and CAF had the highest dissipation rates, while CBZ and TCS showed the lowest dissipation. As it can be seen in Table 4, several studies suggest that CBZ is relative persistent with half-lives in soil (DT50) over 60 days. Moreover, TCS and its metabolites were found in soil still four years after the application with biosolids Macherius et al. (2014). The other compounds are more labile to degradation. For example, metabolites of BPA have been found in soil such as 4-hydroxyacetophenone, 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid Dodgen et al. (2014). Hurtado et al. (2016) determined the concentrations of test chemicals both in the bulk soil, and in the vicinity of roots. Only TCS showed an enrichment in the soil around roots, underlining its persistence. Low concentrations in the soil near roots were found for PROP and IBU, which also had the highest loss rates from soil Table 4.3a). This makes it likely that degradation is enhanced by roots. Furthermore, enantiomeric fractionation of IBU was observed, with an enrichment of the *S*-enantiomer.

Inverse modeling

Inverse modeling can be a powerful tool to determine missing processes or rate constants and is often used for model calibration. Jacobsen et al. (2015) used the technique to find missing in-planta-degradation of pesticides, and the principle applied here is similar: model predictions are compared to measured concentrations, and the difference is contributed to dissipation by degradation. This method is of course highly uncertain because both model simulation and experiment have their own uncertainties, and unknown dissipation processes can lead to reduced uptake. It is therefore a positive sign that the model in no case underestimated the experimental concentrations, and that often only a rather small dissipation rate was sufficient (Table 4.3).

Two degradation kinetics were evaluated: first-order kinetics and Michaelis-Menten. It is noteworthy to mention that the first-order kinetic has only one parameter to adjust (k) while Michaelis-Menten has two (v_{max} and K_M). Moreover, mathematical constraints did not allow considering the (known) dissipation from soil. The first-order fit is therefore preferable in our case.

Measured root and leaf concentrations of BPA were very close to those predicted, thus, no degradation rates were fitted (Table 4.3b). We did not find degradation studies of BPA in plants for comparison. A large difference between predicted CAF concentrations in leaves and measured values required to fit the k_{leaf} to 1.50 d^{-1}. Dettenmaier et al. (2009) derived the transpiration stream concentration factor (TSCF) with a pressure chamber experiment of several organic compounds and reported that polar neutral compounds such as CAF should be taken up rapidly by roots and translocated to the leaves. On the other hand, Goldstein et al. (2014) and Wu et al. (2014) reported that CAF was translocated to leaves less than CBZ due to polar interactions. In the experiments of Hurtado et al. (2016), similar behavior was observed, and the concentrations predicted for leaves were far above the measured ones. Both phenomenon reduced translocation or rapid transformation of CAF in leaves can lead to this discrepancy. In the literature, no degradation rates have been reported for CAF in plants. Regarding CBZ, transformation products (TPs) have been reported both for soil and plants, such as 10,11-epoxy carbamazepine, 10,11-dihydroxycarbamazepine or 10,11-dihydro-10,11-dihydroxy-carbamazepine (Goldstein et al., 2014; Malchi et al., 2014). Malchi et al. (2014) reported that in soil, CBZ parent compound was dominant (90%) and in leaves it depended on the species (potato or carrot). Goldstein et al. (2014) reported similar TPs and similar percentages in cucumbers and tomatoes. Metabolites formed in soil can also be taken up by plants, which makes it difficult to differentiate where exactly the degradation occurred.

Recently, Pietrini et al. (2015) detected 11 TPs of IBU in Lemna gibba L. plant extracts when plants were exposed to 1 mg L^{-1} of IBU. In microalgae reactors, IBU and CAF were rapidly biodegraded (Matamoros et al., 2016), while CBZ appeared to be recalcitrant. In this case, the *S*-enantiomer of IBU was degraded faster. TCS was metabolized in carrot and horseradish to conjugates, and the final amount of conjugates was five times higher than that of TCS (Macherius et al., 2012a). With the fitted degradation rate in roots of 0.1 d^{-1}, such a ratio would be reached after 18 days, it is thus reasonable. In horseradish, 33 metabolites of TCS were detected, hereof 23 identified (Macherius et al., 2014).

4.5 Conclusions

Biodegradation of emerging contaminants in plants has been observed in many cases but kinetics data are rare. Inverse modeling may provide a way to obtain this missing information. Rates determined by inverse modeling are, despite the inherent uncertainty, indicative of the dissipation rates. In the present study, degradation kinetics was in the order BPA < CAF ≈ TCS < CBZ for roots, and BPA = TCS < CBZ << CAF for leaves. There are indications that the high rate for CAF could also compensate for less translocation than predicted.

In soil, decreasing first-order dissipation rates with increasing concentration were observed. Co-metabolic degradation can explain this kinetics. The dissipation rates were in the order TCS < BPA ≈ CAF < PROP ≈ IBU ≈ CBZ for the lowest initial concentration, and TCS ≈ CBZ < CAF ≈ BPA < IBU ≈ PROP at the highest applied dose.

The shape of the BCF-curve (the ratio of concentration in plant to soil) and the Y-intercept gives information on the type of degradation kinetics. A negative Y-intercept can be obtained from (rapid) enzymatic degradation in plants. Finally, the use of inverse modeling provides additional knowledge in the biodegradation of chemicals that can be taken up and further translocated in plants. This can be very helpful to assess where biodegradation takes places and this method can be used to study further metabolism in plant.

Dissipation kinetics found via inverse modeling is not a conclusive proof for biodegradation and confirmation by experimental studies (for example, by determination of metabolites or by studies with labeled compounds) is needed.

4.6 Supporting information

Table S4.1. Fresh and dry weight (in g) of the roots and leaves of the different experimental units.

Unit number	Root fw (g)	dw (g)	Leaf fw (g)	dw (g)
1	127.1	12.4	277.4	15.1
2	139.6	13.6	256.3	16.0
3	104.1	10.1	244.1	13.0
5	115.0	11.2	261.7	13.1
6	109.3	10.6	268.6	11.5
7	110.5	10.7	262.1	12.8
8	100.0	9.7	229.0	10.5
9	113.0	11.0	241.3	13.0
10	149.6	14.5	295.2	18.6
11	129.5	12.6	250.4	16.6
12	106.9	10.4	231.6	11.2
13	83.3	8.1	222.7	10.2
14	104.9	10.2	207.1	13.5
15	98.4	9.6	228.8	13.3
16	84.2	8.2	210.2	9.0
17	97.3	9.5	199.5	9.7
18	103.3	10.0	220.9	9.8
19	127.1	12.4	204.1	12.1
20	102.8	10.0	211.5	10.2

Table S4.2. Final soil concentration in µg kg^{-1} dw of the selected CECs in each experimental unit.

Unit number	Applied concentration	BPA	CAF	CBZ	IBU	PROP	TCS
1	0	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
2	0	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
3	0	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
5	11.7	1.76	3.33	< LOD	0.62	0.41	16.4
6	11.7	8.55	4.11	1.19	1.04	1.40	10.4
7	11.7	2.40	3.81	< LOD	0.68	1.33	6.25
8	11.7	7.64	5.63	1.98	0.55	1.76	7.64
9	29.2	10.2	6.64	1.49	1.45	1.34	36.0
10	29.2	9.76	5.24	2.73	1.81	4.80	75.6
11	29.2	14.4	5.77	13.6	1.87	4.29	48.6
12	29.2	8.81	5.48	23.8	3.28	4.78	65.4
13	58.3	29.9	11.8	33.9	6.65	2.14	82.7
14	58.3	24.5	17.3	39.8	9.90	7.77	90.1
15	58.3	21.6	18.3	32.5	5.31	10.1	81.2
16	58.3	25.3	26.0	41.5	12.9	18.8	133
17	117	37.9	52.8	108	19.3	6.58	165
18	117	57.2	70.1	100	26.0	48.83	187
19	117	49.3	59.2	98	25.3	32.00	123
20	117	76.6	74.9	162	25.5	19.37	194

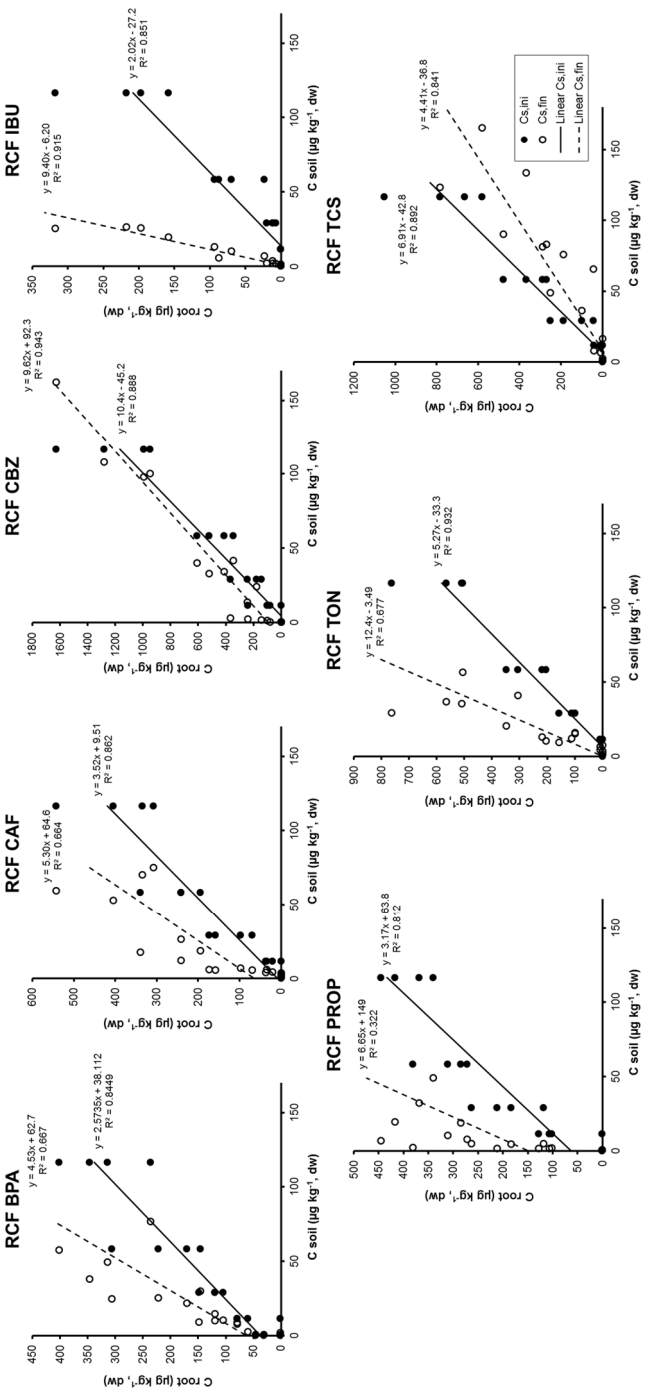
Table S4.3. Final root concentration in µg kg^{-1} dw of the selected CECs in each experimental unit.

Unit number	BPA	CAF	CBZ	IBU	PROP	TCS
1	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
2	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
3	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
5	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
6	80.1	21.6	101	< LOD	107	9.28
7	60.9	38.1	82.0	< LOD	128	10.3
8	80.1	35.7	244	< LOD	103	42.2
9	105	98.1	145	7.9	212	100
10	120	159	367	11.6	264	191
11	120	175	245	20.7	184	252
12	149	70.3	179	12.7	119	45.6
13	147	242	414	24.0	382	272
14	307	341	609	70.0	273	480
15	171	195	522	88.0	311	290
16	223	242	346	94.0	285	370
17	347	406	1282	159	446	583
18	402	335	949	218	341	667
19	314	543	995	318	369	784
20	237	309	1630	198	417	1055

Table S4.4. Final leaf concentration in µg kg^{-1} dw of the selected CECs in each experimental unit.

Unit number	BPA	CAF	CBZ	IBU	PROP	TCS
1	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
2	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
3	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
5	< LOD	< LOD	220	< LOD	< LOD	< LOD
6	2.25	< LOD	217	0.83	< LOD	14.3
7	20.8	27.7	194	0.66	< LOD	14.6
8	44.3	36.2	301	1.29	< LOD	12.2
9	50.4	51.6	479	1.70	25.9	15.3
10	45.3	42.8	403	1.34	39.4	15.9
11	61.2	47.6	515	3.20	30.2	17.1
12	59.7	68.0	445	3.28	21.2	18.0
13	65.2	65.6	815	3.38	76.7	20.7
14	101.9	83.4	1119	4.95	68.0	27.1
15	67.7	79.4	1140	5.38	72.9	26.3
16	97.9	80.4	1052	5.95	52.3	26.8
17	86.4	158	1624	20.1	94.1	27.1
18	156	138	2323	18.9	114	33.7
19	177	123	2017	21.2	156	35.0
20	213	168	2250	33.8	113	32.0

Figure S4.1 Plot and regression between concentrations in root and nominal and final soil concentration of selected CECs. Slope is interpreted as RCF (kg kg^{-1} dw); N = 16, R^2crit = 0.247 (α = 5%).



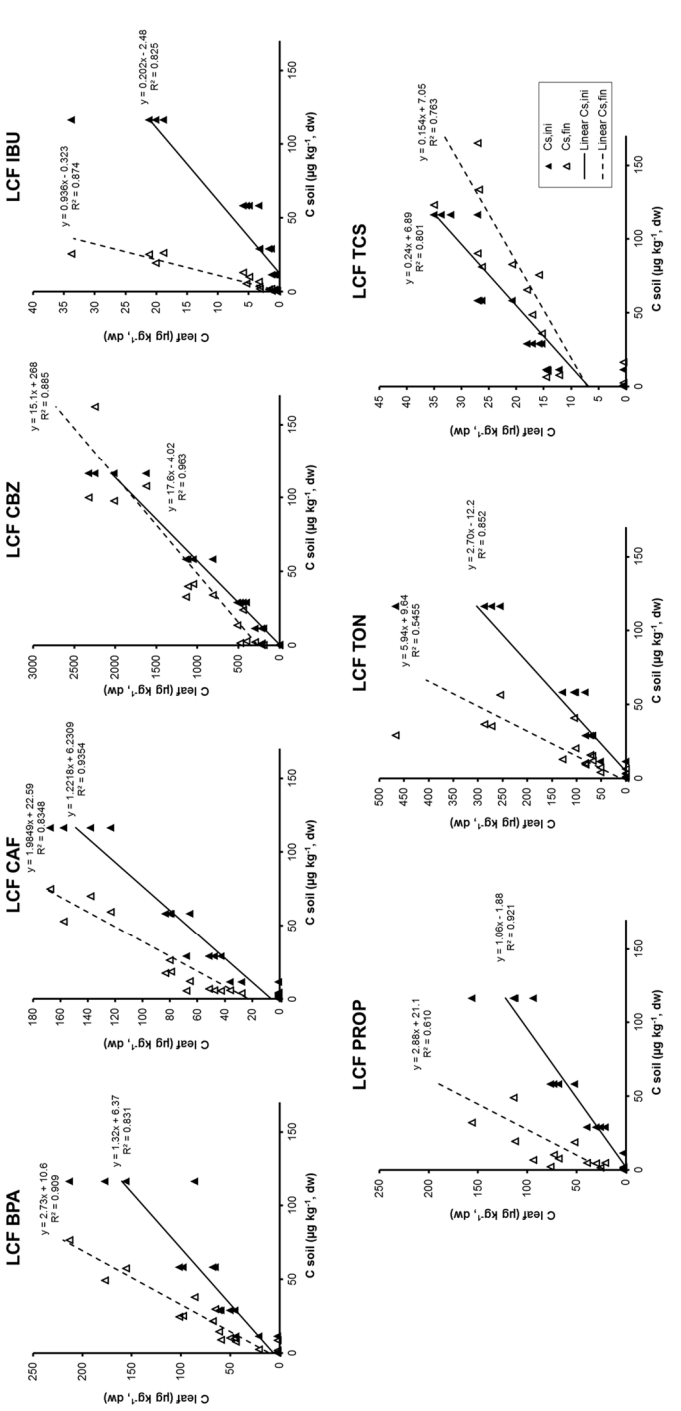


Figure S4.2. Plot and regression between concentrations in leaves and nominal and final soil concentration of selected CECs. Slope is interpreted as LCF (kg kg^{-1} dw); N = 16, R^2crit = 0.247 (α = 5%).

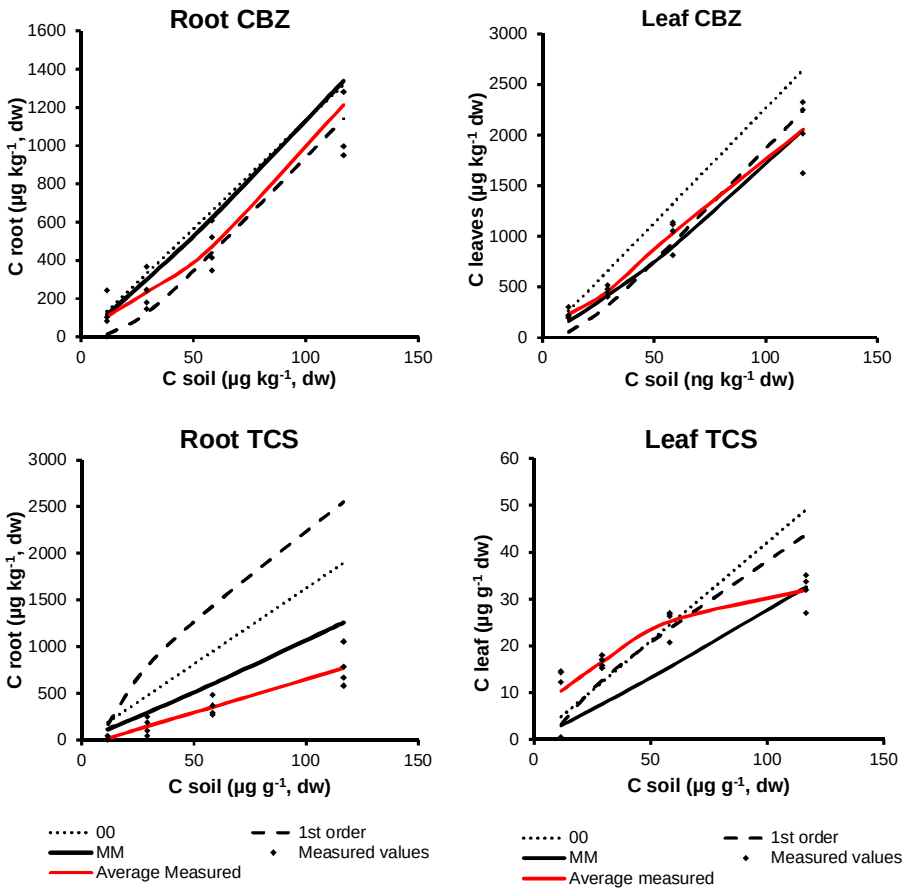


Figure S4.3 Simulated and measured concentrations in a) top left: CBZ roots, b) top right: CBZ leaves, c) bottom left: TCS roots; d) bottom right: TCS leaves. Solid points represent measured values; 00: no degradation in soil or plant; 1st: first-order degradation in soil, roots, and leaves; MM: *Michaelis-Menten* degradation in roots and leaves.

Chapter 5. Biochar amendment to reduce CEC plant uptake

This chapter is based on the article:

Hurtado, C., Cañameras, N., Domínguez, C., Price, G.W., Comas, J., Bayona, J.M., (2017). Effect of soil biochar concentration on the mitigation of emerging organic contaminant uptake in lettuce. J. Hazard. Mater. 323, Part A, 386-393.

Although crop uptake of emerging organic contaminants (CEC) from irrigation water and soils has been previously reported, successful mitigation strategies have not yet been established. In this study, soil was amended with a wood-based biochar (BC) at two rates (0, 2.5 and 5% w/w) to evaluate the effect on mitigation of CEC uptake (i.e. bisphenol A, caffeine, carbamazepine, clofibric acid, furosemide, ibuprofen, methyl dihydrojasmonate, tris(2-chloroethyl) phosphate, triclosan, and tonalide) in lettuce (*Lactuca sativa L*). After 28 days of irrigation with water containing CECs at 15 μg L^{-1}, the average CEC concentration in roots and leaves decreased by 20 to 76% in biochar amended soil relative to non BC-amended soil. In addition, the enantiomeric fractions (EF) of ibuprofen (IBU) in biochar amended soils (EF = 0.58) and unamended soils (EF = 0.76) suggest that the IBU sorbed fraction in BC is more recalcitrant to its biodegradation.

5.1 Introduction

Biochar (BC) is a solid carbonaceous rich material produced from slow pyrolysis using different feedstocks under a low oxygen atmosphere and at temperatures ranging from 350 to 900ºC. Biochar is applied to agricultural soils to aid in carbon dioxide sequestration, to increase soil water holding capacity and reduce nutrient leaching (J. Lehmann, 2009; Yao et al., 2012). In addition, BC-amended soils have been shown to increase sorption capacity to pesticides (Cabrera et al., 2014), polycyclic aromatic hydrocarbons (PAHs) (Anyika et al., 2015), halogenated phenols (Oh and Seo, 2015), veterinary antibiotics such as sulfamethazine (Teixidó et al., 2013) and PPCPs (Zhang et al., 2013). Consequently, mitigation in plant uptake of organic pollutants in BC-amended soils have been reported for PAH contaminated soils (Khan et al., 2013), pesticides at 50 mg kg^{-1} applied directly to soil (e.g. pyrimethanil, carbofuran and chlorpyrifos) (Yu et al., 2009; Yu et al., 2010) and, recently, carbamazepine at 1 mg kg^{-1} (Williams et al., 2015).

Irrigation water reclaimed from municipal wastewater treatment may contain variable concentrations of emerging organic contaminants (CECs) ranging from ng L^{-1} up to several µg L^{-1} (Calderón-Preciado et al., 2011a; Lapworth et al., 2012; Li, 2014). Among them, pharmaceutical and personal care products (PPCPs), biocides, fragrances, veterinary products, artificial sweeteners and disinfection byproducts (DBPs) have been detected (Richardson and Ternes, 2011). Accordingly, some CECs occurring in reclaimed water used for irrigation can be taken up by crops (Goldstein et al., 2014; Malchi et al., 2014; Wu et al., 2014). Therefore, strategies to mitigate crop uptake of CECs have become of great interest from both a food safety and human health perspective.

Biochar has been reported to possess high affinity to some CECs (BPA, CBZ and IBU). Limited information exists on the absorption – adsorption dynamics and competitive displacement of BC sorbates in the soil-root system, where soil organic matter and BC compete for the CECs occurring in the irrigation waters (Ulrich et al., 2015). Proposed sorption mechanisms for bisphenol A (BPA) and ibuprofen (IBU) are through π–π electron donor-acceptor interactions and through hydrophobic adsorption for CBZ (Sun et al., 2011; Jung et al., 2013). In contrast, no information exists for other contaminants commonly found in sewage and effluents subjected to different treatment degree including caffeine (CAF), clofibric acid (CFA), furosemide (FUR), methyl dihydrojasmonate (MDHJ), tris(2-chloroethyl) phosphate (TCP) and tonalide (TON).

We hypothesize that amending soils with BC, particularly soils depleted in mineral or organic colloids, will reduce the bioaccessibility of CECs by plants. Therefore, the objective of this study is to assess the attenuation capacity of BC in soils to mitigate the uptake and translocation by lettuce of 10 CECs commonly found in reclaimed irrigation water. The study consists of using CECs with different physical-chemical properties in a soil amended with two rates of BC, 2.5 and 5% (w/w). In addition, the enantiomeric fraction of IBU was calculated in order to assess the impact of BC on CEC biodegradation in the soil.

5.2 Materials and methods

5.2.1 Experimental layout

The experiment was conducted in a research greenhouse belonging to the Universitat Politècnica de Catalunya, located in Agròpolis (Viladecans, Barcelona, Spain). Experimental units consisted of 2.5 L cylindrical amber glass pots (Ø = 15 cm and 20 cm high) fitted with a bottom outlet connected to drainage tubing (Ø = 3 cm) and filled with 2.3 kg of air-dried soil sieved to 2 mm as reported elsewhere (Comas et al., 2014). To evaluate the effect of amending the soil with BC on the uptake of a mixture of ten CECs, the following treatments were established: (i) unamended unspiked soil (control), (ii) BC- amended soil at 2.5% and 5% (w/w) with 70 μg of each CEC dissolved in water at 15 μg L^{-1} (30.4 μg kg^{-1} soil + BC dw) and supplied directly into the pot, to avoid adsorption to the irrigation tubing, over eight applications in a four week period beginning twenty-seven days after planting, and (iii) unamended soil and CECs (30.4 μg kg^{-1} soil dw) supplied as described above. Treatments were replicated five times. In Table 5.1, the physical-chemical properties of the selected CECs are listed. One seedling of Batavia lettuce (*Lactuca Sativa* L, cv. Arena) was planted in each experimental unit and watered with a Hoagland and Arnon solution (Hoagland and Arnon, 1950) using a time programmed drip irrigation system (V = 100 mL d^{-1}).

The soil used was collected from the surface horizon of a typical Xerorthent soil from the Llobregat River Delta's agricultural area (longitude 2°03'E, latitude 41°17'N) and sieved between 0.12 and 2 mm. The soil had a sandy texture (90% sand, 8% silt, and 2% clay) with a pH of 7.42 ± 0.03 (soil-to-water ratio 1:5) and soil electrical conductivity of 3.8 dS m^{-1} (soil-to-water ratio 1:5). Total organic carbon and total organic nitrogen content was 5 g kg^{-1} and 0.7 g kg^{-1}, respectively. The cation exchange capacity (CEC) was 3.8 meq 100 g^{-1} and exchangeable Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} were 2.82, 0.64, 0.25, and 0.15 meq 100 g^{-1} soil, respectively.

Biochar was produced by Bodegas Torres (Vilafranca del Penedès, Barcelona, Spain) from vineyard wood feedstock and pyrolyzed at 650 ºC. Biochar was crushed and sieved to particle sizes between 0.12 and 2 mm. Morphological properties of BC such as surface area (SA), pores or molar H/C are usually the most important factors in sorption of compounds (Ahmad et al., 2014). The N_2-B.E.T SA was 387 m^2 g^{-1}, pore volume was 0.0679 cm^3 g^{-1}, and pore size was 3.26 nm for the BC. Ultimate analysis of BC resulted in C, H, N and S contents of 62.8, 1.1, 0.3 and less than 0.1%, respectively, and a molar H/C ratio of 0.21. Biochar pH was 9.82 ± 0.04 (1:10 solid:solution ratio with deionized water), conductivity was 2158 ± 46 µS cm^{-1} and specific weight was 1.72 ± 0.05. An FTIR spectrum was obtained with KBr pellets and it can be found in the supplementary information (Figure S5.1). Biochar amended soil pH increased to 7.48 ± 0.04 and 7.56 ± 0.03 with 2.5 and 5% BC amendment, respectively.

5.2.2 Chemical analysis

At the end of the experiment, soil, plant roots and aboveground biomass, i.e. leaves, from each experimental unit were analyzed. Prior to analysis, roots were soaked in deionized water to remove adhered soil. Roots and leaves were comminuted separately with liquid nitrogen and stored at -20ºC until analysis.

Plant tissue

The extraction of CECs from plant tissues has been reported elsewhere (Calderón-Preciado et al., 2009). Briefly, a matrix solid-phase dispersion method was applied to the plant root and leaf tissue spiked with a mixture of surrogates (see section 3.7.2). The plant material was extracted with a mixture of acetone:hexane (1:1, v/v) using a pressurized solvent extraction (PSE) system (Applied Separations, Allentown, PA, USA). Neutral-basic and acid fractions were obtained by solvent partitioning at neutral and acid pH, respectively. The final extracts were analyzed by GC coupled to electron impact tandem mass spectrometry (EI-MS/MS) in a Bruker 450-GC gas chromatograph coupled to a Bruker 320-MS triple stage quadrupole mass spectrometer (Bruker Daltonics Inc., Billerica, MA, USA). Qualitative and quantitative analysis was performed based on retention time and selected reaction monitoring (SRM) mode of two product ions, and the ratio between the product ions was used for confirmation. Monitoring ions (Table S5.1), LODs and LOQs (Table S5.2) and recoveries (Table S5.3) can be found in the supplementary information.

Table 5.1: Physical-chemical properties of the selected contaminants of emerging concern (CECs) in this study.

Name	pKa[a]	Solubility (mg L^{-1})	Log K_{ow}[b]	Log K_{oc}[b]	DT50 (d)	f_n[c]
Bisphenol A (BPA)	9.7[0/-] 10.5[-/2-]	173	3.32	3.2	7[1]	0.995
Caffeine (CAF)	0.8[+/0]	2632	-0.07	1.3	1.5[2]	1.000
Carbamazepine (CBZ)	0.1[+/0]	17.7	2.45	2.8	60[3]	1.000
Clofibric acid (CFA)	4.0[0/-]	583	2.84	2.9	4.5 – 18.5[4]	0.001
Furosemide (FUR)	3.5[0/-] 9.0[-/2-]	149	2.03	3.1	ND[e]	0.001
Ibuprofen (IBU)	4.3[0/-]	41.1	3.97	3.4	0.91 – 6.09[4]	0.001
Methyl dihydrojasmonate (MDHJ)	NA[d]	92	2.98	2.7	ND	1.000
Tris(2-chloroethyl) phosphate (TCP)	NA[d]	878	1.63	1.6	167[5,6]	1.000
Tonalide (TON)	NA[d]	0.29	5.70	4.8	50[7]	1.000
Triclosan (TCS)	8.8[0/-]	4.62	4.76	4.2	187[8]	0.962

[a]Dissociation reaction, [0]: neutral; [+]: cationic; [-]: anionic. [b]Log K_{OW} from database provided by ACD/iLab (https://ilab.acdlabs.com/iLab2/index.php). [c]Neutral fraction at soil pH (7.4) was calculated from (Trapp, 2009); [d]Not applicable. [e]Not in database (ND). [1] Ying et al. (2003), [2] Lin et al. (2010), [3]Monteiro and Boxall (2009), [4]Xu et al. (2009), [5]Römbke J (1995), [6]EU European Comission (2009b), [7]Chen et al. (2014) and [8]Walters et al. (2010).

Soil

For the extraction of CECs in soil, 1 g aliquot was spiked with a mixture of surrogates (details in SI), and mixed with 0.5 g of sodium sulfate anhydrous, equilibrated for 1 h at 6 ºC and extracted twice with 10 mL of a mixture acetone:hexane (3:1, v/v) for 15 min by ultrasonication. A final extraction of the soil in 10 mL of methanol was subsequently performed. After methanol evaporation, the extract residue was reconstituted in acetone:hexane and combined, evaporated to 2 mL and dried by percolation through an anhydrous sodium sulfate column. The dried extract was reconstituted in ethyl acetate

prior to injection in the GC system. Aliquots of the sample extracts were also analyzed by EI GC-MS/MS as detailed above.

5.2.3 Data and statistical analysis

Root concentration factors (RCF) were calculated for individual CECs in each experimental unit at harvest as follows:

$$RCF = \frac{C_R}{C_S} \quad (5.1)$$

where C_R is the concentration in the root and C_S the concentration applied to the soil.

Translocation factors (TF) were calculated as follows:

$$TF = \frac{C_L}{C_R} \quad (5.2)$$

where C_L is the concentration of CECs in leaves.

The enantiomeric fraction (EF) of IBU was calculated as described in Equation 5.3 below:

$$EF = \frac{S}{S+R} \quad (5.3)$$

where *S* and *R* are the concentrations of (*S*)- and (*R*)-ibuprofen enantiomers, respectively.

The recovered mass of each CEC in the soil, roots and leaves were calculated as the product of the dry weight (Table S5.4) by the CEC concentration measured (Tables S5.5 – S5.7). The difference in mass of CEC initially spiked into soil and the masses recovered in the soil, roots, and leaves was considered to be an Unrecovered Fraction (UF) lost through biodegradation, volatilization, and/or irreversible binding to mineral surfaces. A two-way analysis of variance (ANOVA) was performed to determine if the UF depended on the treatments and the contaminants.

Finally, a one-way ANOVA and subsequent mean separation (LSD), alpha = 0.05, was performed to study the significant differences between CEC concentrations in the three

compartments and differences in bioconcentration factors for the BC treatments. All analyses were conducted in R (R Development Core Team, 2015).

5.3 Results

5.3.1 Dynamics of CECs in unamended soils

Concentration in soil, lettuce roots and leaves

In unamended CEC spiked soil, mean concentrations of the tested CECs were lower than in the lettuce roots and leaves. Mean concentration in the soil was 8.1 ± 3.3 ng g^{-1} dw, where the lowest concentration corresponded to IBU (5.6 ± 1.8 ng g^{-1} dw) and the highest concentration to TCS (15 ± 1 ng g^{-1} dw) (Table 5.2). The average concentration of individual CEC in the lettuce roots was 174 ± 75 ng g^{-1} dw and ranged from 102 ± 26 ng g^{-1} dw for IBU to 322 ± 102 ng g^{-1} dw for CBZ (Table 5.2). The average RCF was 5.7 ± 2.5, while the lowest value of the *RCF* corresponded to CAF, 3.4 ± 0.8 g g^{-1} dw, and the highest value to TCS, 11 ± 3 g g^{-1} dw (Table 5.2).

Generally, CEC concentration in the lettuce leaves was much lower than in the roots. The average concentration of individual CEC in the leaves was 71 ± 82 ng g^{-1} dw. IBU was found at the lowest concentration in the leaves while CBZ exhibited the highest concentration (4.9 ± 15 and 246 ±44 ng g^{-1} dw, respectively). FUR was the only CEC not detected in the leaves. The average *TF* was 0.5 ± 0.6 g g^{-1} dw but ranged from 0.03 ± 0.01 to 1.7 ± 0.2 g g^{-1} dw for TCS and CBZ, respectively.

Unrecovered fraction (UF)

Despite CEC concentrations measured in the lettuce root and leaf samples being greater than the soil (Table 5.2), the small overall mass contribution of the plant material relative to the soil resulted in only a small proportion residing in either roots or leaves (Table 5.3). The average amount recovered in lettuce plants, i.e. roots plus leaves was 0.9 ± 0.8%, although there were significant differences between the various CECs in the mixture. For example, CBZ exhibited the highest plant recovery in lettuce (2.6 ± 0.4%) followed by TCP, MDHJ, and TON (between 1.3 and 1.7%). The IBU amount recovered in plant was the lowest at 0.2 ± 0.1%. In the unamended spiked soil, the average amount recovered, across all CECs, was 27 ± 11% but significant differences in CEC recoveries were observed. The highest recovery was with TCS (50 ± 2%) followed by TCP, BPA, and CFA (29 to 34%). On the other hand, CBZ, CAF and IBU were the CECs with lowest recoveries (between 18 and 20%) in soil.

On average, the UF in the unamended soil was 72 ± 11%, ranging from 49 ± 2 to 82 ± 4%, depending on the CEC considered (Table 5.3). The highest UF were in IBU and CAF, at 82 ± 4% and 81 ± 6%, respectively, while the lowest UF were measured in TCS, TCP and BPA, 49 ± 2%, 64 ± 8% and 69 ± 4%, respectively.

5.3.2 Effect of biochar on CEC dynamics

Concentration in soil, lettuce roots and leaves

The concentration of CECs in the soil increased with each added level of BC, with 5% BC retaining significantly more than the 2.5% or control in most instances. When soils were amended with BC at 5%, the average concentration of CEC in soil increased by 65 ± 45% relative to unamended soils. The CECs that exhibited the highest increase were IBU, TON, BPA, CBZ, MDHJ and FUR (65 to 10 5%), while CAF and CFA had the lowest increase (8 to 20%). Moreover, significant decreases in the soil IBU's enantiomeric factors (EF) with 0.76 ± 0.12 (N = 5) measured in unamended soil and 0.58 ± 0.03 (N = 5) in soil amended with BC at 5% (Figure 5.1). Soil concentrations of CAF or CFA were not significantly different between any of the BC treatments or control. Significant differences between CEC recovered in the soil were also detected with the highest recoveries in TCS, BPA, TECP, and FUR over all treatments.

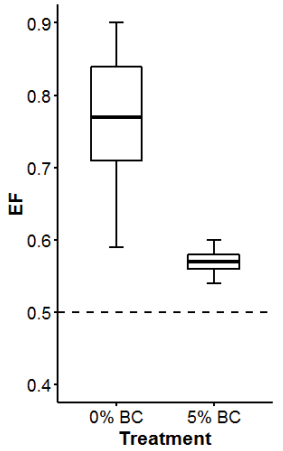


Figure 5.1: Boxplots of the enantiomeric fraction (EF) of IBU in soil spiked with a racemic mixture of IBU and amended with biochar at 5% (5% BC) or without BC (0%). The horizontal line was the value of the commercial racemic mixture of IBU (EF = 0.50).

Table 5.2: Mean CEC concentrations in ng g^{-1} dw, root concentration factor (RCF) g g^{-1}, dw, and translocation factor (TF), g g^{-1}, dw with standard deviations (N = 5), in the soil, lettuce root and lettuce leaf after amendment with different rates of biochar (BC).

0% BC	Soil	Root	Leaf	RCF	TF
BPA	9.2 ± 1.1^{bc}	174 ±26^{bc}	20 ± 5.4^{de}	5.7 ± 0.8^{bc}	0.11 ± 0.03^{e}
CAF	5.6 ± 1.8^{e}	102 ± 25^{d}	30 ± 3.6^{d}	3.4 ± 0.8^{d}	0.32 ± 0.12^{d}
CBZ	5.8 ± 1.3^{e}	145 ± 23^{bcd}	246 ± 44^{a}	4.8 ± 0.8^{bcd}	1.7 ± 0.2^{a}
CFA	9.0 ± 1.4^{bc}	200 ± 49^{b}	8.7 ± 2.8^{de}	6.6 ± 1.6^{b}	0.04 ± 0.01^{e}
FUR	8.0 ± 1.9^{cd}	203 ± 57^{b}	<LOD	6.7 ± 1.9^{b}	NA
IBU	5.6 ± 1.3^{e}	112 ± 27^{cd}	4.9 ± 1.5^{de}	3.7 ± 0.9^{cd}	0.04 ± 0.02^{e}
MDHJ	6.3 ± 1.5^{de}	189 ± 65^{b}	126 ± 31^{c}	6.2 ± 2.1^{b}	0.72 ± 0.3^{c}
TCP	10 ± 2^{b}	148 ± 23^{bcd}	152 ± 18^{b}	4.9 ± 0.8^{bcd}	1.05 ± 0.2^{b}
TCS	15 ± 1^{a}	322 ± 102^{a}	9.2 ± 2.0^{de}	11 ± 3^{a}	0.03 ± 0.01^{e}
TON	6.2 ± 1.9^{de}	146 ± 52^{bcd}	115 ± 25^{c}	4.8 ± 1.7^{bcd}	0.83 ± 0.22^{c}

2.5% BC	Soil	Root	Leaf	RCF	TF
BPA	12 ± 2^{bc}	105 ± 34^{cd}	18 ± 6^{c}	3.5 ± 1.1	0.17 ± 0.05^{e}
CAF	5.9 ± 1.7^{f}	59 ± 21^{d}	29 ± 6^{c}	1.9 ± 0.7	0.56 ± 0.26^{d}
CBZ	8.3 ± 1.4^{de}	93 ± 18^{cd}	148 ± 36^{a}	3.1 ± 0.6	1.6 ± 0.19^{a}
CFA	8.3 ± 0.9^{de}	115 ± 48^{bcd}	3.2 ± 2.4^{d}	3.8 ± 1.6	0.03 ± 0.02^{e}
FUR	11 ± 3^{cd}	142 ± 44^{abc}	<LOD	4.7 ±1.4	NA
IBU	7.0 ± 1.6^{ef}	64 ± 17^{d}	4.3 ± 1.4^{d}	2.1 ± 0.6	0.07 ± 0.04^{e}
MDHJ	8.7 ± 1.7^{de}	162 ± 63^{ab}	94 ± 10^{b}	5.3 ± 2.1	0.68 ± 0.32^{cd}
TCP	13 ± 3^{b}	105 ± 25^{cd}	111 ± 36^{b}	3.5 ± 0.8	1.0 ± 0.2^{b}
TCS	18 ± 2^{a}	175 ± 86^{a}	9.0 ± 4.8^{cd}	5.8 ± 2.8	0.07 ± 0.06^{e}
TON	9.0 ± 1.7^{de}	114 ± 34^{bcd}	88 ± 28^{b}	3.7 ± 1.1	0.77 ± 0.09^{c}

5% BC	Soil	Root	Leaf	RCF	TF
BPA	18 ± 3^{b}	52 ± 24^{cd}	6.7 ± 2.4^{d}	1.7 ± 0.8^{cd}	0.14 ± 0.07^{e}
CAF	6.8 ± 1.6^{f}	62 ± 15^{bcd}	24 ± 4^{c}	2.0 ± 0.5^{bcd}	0.40 ± 0.14^{cd}
CBZ	11 ± 3de	91 ± 28^{abc}	94 ± 20^{a}	3.0 ± 0.9^{abc}	1.1 ± 0.3^{a}
CFA	9.7 ± 1^{ef}	62 ± 15^{bcd}	4.0 ± 1.4^{d}	2.0 ± 0.5^{bcd}	0.07 ± 0.04^{e}
FUR	13 ± 2^{cd}	121 ± 13^{a}	<LOD	4.0 ± 0.4^{a}	NA
IBU	11 ± 2de	40 ± 11^{d}	1.8 ± 1.3^{d}	1.3 ± 0.4^{d}	0.05 ± 0.02^{e}
MDHJ	11 ± 3de	120 ± 48^{a}	58 ± 16^{b}	3.9 ± 1.6^{a}	0.54 ± 0.23^{bc}
TCP	16 ± 4^{bc}	100 ± 28^{ab}	69 ± 13^{b}	3.3 ± 0.9^{ab}	0.72 ± 0.14^{b}
TCS	23 ± 4^{a}	131 ± 58^{a}	4.6 ± 3.2^{d}	4.3 ± 1.9^{a}	0.04 ± 0.04^{e}
TON	12 ± 3de	99 ± 45^{ab}	27 ± 8^{c}	3.3 ± 1.5^{ab}	0.34 ± 0.22^{d}

Values with the same letter within a column are not significantly different ($\alpha \geq 0.05$). NA: Not available

In the roots and leaves, CEC concentrations decreased with increasing rates of added BC, although no significant differences between treatments were detected for CAF and TCS in the leaves or for MDHJ and TON in the roots. The overall decrease in concentration of CECs in the roots and leaves in the 5% BC treatment was consistent in both plant compartments. Over all CECs measured, root concentration decreased on average by 34 ± 22% when soils were amended with BC at 2.5% and by 48 ± 22% with BC amended to soil at 5%. In the 5% BC treatment, CECs exhibiting the least decrease in concentration were TCP and TON (32 ± 31% and 32 ± 19%, respectively) while the CECs exhibiting the greatest decrease were BPA, CFA and IBU (70 ± 14%, 69 ± 8%, 64 ± 10%, respectively). Indeed, the average RCF in unamended spiked soil was 5.7 ± 2.5, while the average RCF in soil amended with BC at 2.5% and 5% was 3.7 ± 1.8, and 2.9 ± 1.4, respectively (Table S5.8). Not all the CECs behaved similarly, the RCF of CAF, TCP and CBZ were not significantly different between BC treatments, while the other compounds had significant reductions in RCF (Figure 5.2). Uptake of CECs in lettuce leaves followed a similar pattern to the roots. Overall, no significant decreases in TF between BC treatments and soil control were observed for most CECs (Table 5.2). The exceptions were in CBZ, TCP, and TON which had between 30 and 59% lower TF in the 5% BC treatment than either the 2.5% BC treatment or soil control.

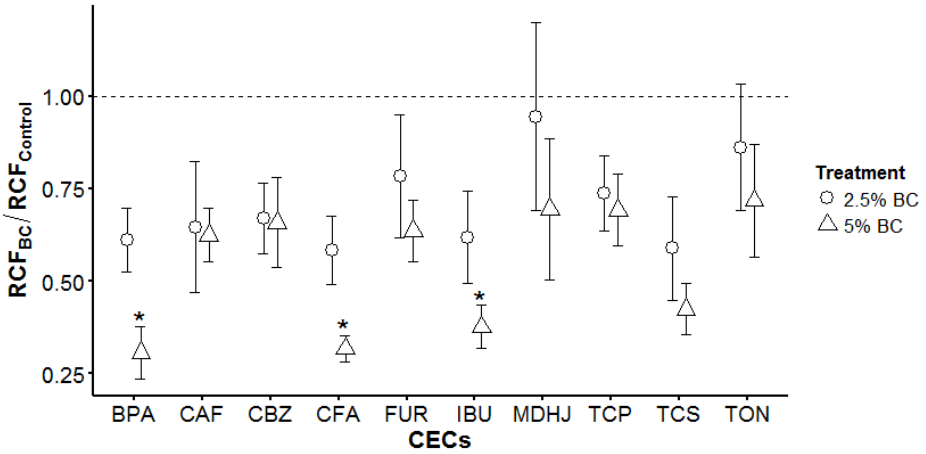


Figure 5.2: Mean and standard error of the RCFs of soils amended with biochar (BC) at 2.5 and 5% w/w relative to the unamended soil of the selected CECs. CECs with an asterisk between biochar treatments are significantly different at $p < 0.05$.

Effect of BC on the Unrecovered Fractions (UF)

When the soil was amended with BC at 2.5%, the average UF was 66 ± 13% over all the CECs (Table 5.3), but TCS and CAF had UF ranging from 40 to 80%. In the soil amended with 5% BC, the average UF was 57 ± 16%, and again, CAF was the CEC that exhibited the highest UF (78 ± 5%) and TCS the lowest UF (25 ± 12%). The trend for most CECs was for a decrease in the UF with increasing BC treatments although this observed effect was not always significant.

Table 5.3: Proportion (%) of recovered fraction of CECs (mass basis), in the soil, lettuce root, lettuce leaf, and unrecovered fraction (UF) in biochar (BC) amended soil at 0, 2.5 and 5% (w/w).

	Soil			Root		
	0% BC	**2.5% BC**	**5% BC**	**0% BC**	**2.5% BC**	**5% BC**
BPA	30 ± 4^{bc}	40 ± 6^{bc}	57 ± 10^{b}	0.23 ± 0.08^{bcd}	0.13 ± 0.06^{bc}	0.05 ± 0.03^{cd}
CAF	18 ± 6^{e}	19 ± 6^{f}	22 ± 5^{f}	0.13 ± 0.05^{d}	0.07 ± 0.03^{c}	0.06 ± 0.02^{bcd}
CBZ	19 ± 4^{e}	27 ± 5de	36 ± 10de	0.18 ± 0.05^{bcd}	0.11 ± 0.04^{bc}	0.09 ± 0.04^{abc}
CFA	29 ± 5^{bc}	27 ± 3de	32 ± 3^{ef}	0.26 ± 0.09^{b}	0.14 ± 0.05^{bc}	0.06 ± 0.03^{bcd}
FUR	26 ± 6^{cd}	34 ± 8^{cd}	44 ± 5^{cd}	0.26 ± 0.09^{b}	0.18 ± 0.07^{ab}	0.12 ± 0.03^{ab}
IBU	18 ± 4^{e}	23 ± 5^{ef}	37 ± 8de	0.14 ± 0.05^{cd}	0.08 ± 0.02^{c}	0.04 ± 0.01^{d}
MDHJ	21 ± 5de	28 ± 6de	35 ± 9de	0.25 ± 0.14^{bc}	0.19 ± 0.07^{ab}	0.13 ± 0.07^{a}
TCP	34 ± 8^{b}	44 ± 9^{b}	53 ± 12^{bc}	0.19 ± 0.06^{bcd}	0.13 ± 0.03^{bc}	0.10 ± 0.05^{abc}
TCS	50 ± 2^{a}	59 ± 6^{a}	74 ± 12^{a}	0.40 ± 0.14^{a}	0.22 ± 0.15^{a}	0.14 ± 0.09^{a}
TON	20 ± 6de	29 ± 6de	39 ± 9de	0.18 ± 0.04^{bcd}	0.13 ± 0.03^{bc}	0.10 ± 0.06^{abc}
	Leaf			**Unrecovered Fraction**		
	0% BC	**2.5% BC**	**5% BC**	**0% BC**	**2.5% BC**	**5% BC**
BPA	0.20 ± 0.05^{d}	0.18 ± 0.08^{c}	0.07 ± 0.03^{d}	69 ± 4^{cd}	60 ± 6de	43 ± 10^{e}
CAF	0.29 ± 0.04^{d}	0.30 ± 0.08^{c}	0.26 ± 0.11^{c}	81 ± 6^{a}	80 ± 6^{a}	78 ± 5^{a}
CBZ	2.4 ± 0.4^{a}	1.5 ± 0.5^{a}	0.98 ± 0.20^{a}	78 ± 4^{ac}	71 ± 5^{bc}	63 ± 10^{bc}
CFA	0.08 ± 0.02^{d}	0.03 ± 0.03^{c}	0.04 ± 0.01^{d}	70 ± 5^{cd}	73 ± 3^{abc}	68 ± 3^{ab}
FUR	NA	NA	NA	73 ± 6^{bc}	65 ± 8^{cd}	56 ± 5^{cd}
IBU	0.04 ± 0.01^{d}	0.04 ± 0.01^{c}	0.02 ± 0.01^{d}	82 ± 4^{a}	77 ± 5^{ab}	63 ± 8^{bc}
MDHJ	1.3 ± 0.4^{bc}	1.0 ± 0.2^{b}	0.60 ± 0.15^{b}	78 ± 5^{ab}	71 ± 6^{bc}	64 ± 9^{bc}
TCP	1.5 ± 0.2^{b}	1.1 ± 0.3^{b}	0.73 ± 0.18^{b}	64 ± 8^{d}	55 ± 9^{e}	46 ± 12de
TCS	0.09 ± 0.01^{d}	0.09 ± 0.05^{c}	0.05 ± 0.03^{d}	49 ± 2^{e}	40 ± 6^{f}	25 ± 12^{f}
TON	1.1 ± 0.3^{c}	0.87 ± 0.25^{b}	0.28 ± 0.08^{c}	79 ± 6^{ab}	70 ± 5^{bc}	60 ± 9^{bc}

NA: not applicable.

Means with the same letter within a column are not significantly different ($\alpha \geq 0.05$)

5.4 Discussion

5.4.1 Effect of BC on CEC bioavailability

Concentrations of CECs in plant leaves depend on the concentration in irrigation water and on the RCF and TF. We have previously reported that the final CEC concentration in lettuce leaves was directly proportional to the concentration of CECs in the irrigation water, i.e. RCF and TF remained stable, even when soil CEC concentrations were increased (Hurtado et al., 2016). The decrease in lettuce leaf concentrations of CECs observed in our study is partly attributed to a lower entry through the roots, rather than to a decrease of the translocation factors from roots to the leaves.

Although significant differences in the TF between CECs studied were observed, BC content in the soil had little effect on most of them (Table 5.2). In fact, observed differences in the TF of CECs can be explained by the physical-chemical properties of the compounds (Table 5.1). Neutral compounds are able to be translocated to leaves through transpiration stream where they can accumulate (Malchi et al., 2014). For instance, CAF, CBZ, TCP, MDHJ and TON exhibited higher TF than the other compounds. CBZ, MDHJ and TCP have more intermediate log K_{ow} (1.6 – 2.5) than CAF (-0.07) or TON (5.70) which explains differences in TF between these neutral compounds (Goldstein et al., 2014). Other compounds, such as BPA, CFA, IBU, FUR, and TCS, are ionizable and their neutral fraction is pH dependent. In our study, at a soil pH (from pH 7.42 to 7.56), BPA and TCS are found mostly in a neutral form but the pH range in a plant can vary from 5 to 10, meaning that different ionic species of BPA and TCS may occur inside the plant (Dodgen et al., 2013).

This neutral fraction is more susceptible to be taken up by roots than CFA, FUR and IBU, which are found mostly in an anionic form (pK_a 3.5 to 4.3) in soil. Plant uptake and translocation can be further reduced through charge interactions between the CECs and soil or BC surfaces (Trapp, 2009).

In contrast, no differences between neutral and ionizable compounds were observed in the lettuce roots. Interestingly, TCS, which exhibited the highest uptake by roots, did not translocate to the leaves because of its high log K_{ow}.

Biochar additions changed the soil-plant system dynamics with certain CECs. Biochar has strong sorbent properties and can interact with CECs through direct surface interactions (Ahmad et al., 2014). In our study, soil concentrations increased

significantly with addition of the BC treatments in most of the studied CECs. Specifically, CECs with higher log $K_{oc,}$ such as BPA and TCS, increased soil concentration especially in response to higher BC amendments to the soil. Conversely, CAF, with a log K_{oc} of 1.3, did not respond to BC amendments in soil. Overall, a trend between CEC soil concentration and log K_{oc} was observed for the compounds studied but the correlations were generally very poor, with R^2 ranging from 0.01 to 0.20.

The RCFs decreased when the soil was amended with BC for a number of CECs (Figure 5.2). For all the CECs, except MDHJ, the RCFs when soils were amended with BC decreased compared to the unamended soil (Table 5.2). The relative RCFs ($RCF_{BC}/RCF_{control}$) decreased in all CECs (< 1) when BC was added. The relative RCF of BPA, CFA, and IBU were significantly lower at the 5% BC rate than the 2.5% (Figure 5.2). On the other hand, for most other CECs, the relative RCFs did not significantly decrease when more BC was supplied to the soil (CAF, CBZ and TCP), although a trend for lower relative RCFs was observed in TON, TCS, MDHJ, and FUR. The decrease in the relative RCF was lowest with neutral compounds while the CECs that responded to the higher rate of BC were ionizable hydrophobic compounds with some hydroxyl and carboxylic groups and some conjugated aromatic (TCS, BPA, CFA and IBU). Hence, differences in the interactions between CECs and BC can be explained by the structure and properties of the CECs and surface properties of the BC. These ionizable compounds with benzene rings interact more strongly with the aromatic surface of BC (e.g. hydrophobic interactions and π-π interactions) than neutral compounds without these functional groups (Mukherjee et al., 2011). These interactions could take place as the FTIR spectrum (Figure S5.1) showed C6 aromatic rings, C=C, C=O and –COOH bands. However, further studies need to be done to assess the different interactions between CEC mixtures and BC and the stability of BC. In this experiment a high temperature biochar was used because of its high SA, but also because these BC are more recalcitrant in soils due to more stable C forms (Zhang et al., 2015b).

There is limited information on the interactions between the CECs selected in our study and BC amendments to soil. In fact, most studies are conducted with individual compounds and not in mixtures, whereby compound behavior may differ in a mixture than when present as an individual compound. In sorption experiments, Sun et al. (Sun et al., 2011) reported that BPA was strongly bound to BC surfaces through π-π electron donor acceptor (EDA) interactions, mainly as a result of the two benzene rings with a phenol group attached providing the donor electrons and through hydrophobic interactions. Similar interactions were reported by Jung et al. (Jung et al., 2013) for IBU,

although weak binding with CBZ was observed attributable mainly to hydrophobic interactions. In our study, BPA concentrations decreased in plant when more BC was added. Therefore, BC amendments likely reduce the soil pore water concentration of some CECs, thus limiting bioavailability of compounds to the plant.

Translocation of CECs to lettuce leaves was significantly reduced when BC was amended, particularly as the BC rate increased from 2.5% to 5%. Similar effects have been reported for CBZ, with a 17 to 42% decrease in leaves when soil was amended with different BCs (Williams et al., 2015). In our study, CBZ decreased by 40 to 62% in leaves although the rate of BC amended was five to ten times higher than what was reported by Williams et al. (Williams et al., 2015). It is noteworthy to mention that CBZ is one of the most studied compounds in plant uptake studies due to its high translocation and potential to accumulate in leaves (Shenker et al., 2011; Goldstein et al., 2014; Malchi et al., 2014). Additions of BC reduced CBZ leaf concentrations by half in our study, which is a significant finding for the potential of BC to mitigate movement of some CECs. Furthermore, a novel feature of our study was the spiking of CECs in a mixture, which may have resulted in a range of interactions with binding sites. In some cases, it is possible that competitive or selective binding with BC reduced the effectiveness of CEC sorption. Overall, TFs for CECs in our study did not vary widely such that once entering into the plant; translocation was more dependent on water movement and other specific properties of the compounds.

5.4.2 Effect of BC on Unrecovered Fraction (UF)

The same initial concentration of CECs was spiked into the irrigation water but the recovered and unrecovered fractions (UF) were always different, independent of the BC amendments. The mass balance results for CECs in the soil, roots, and leaves, suggest that overall recoveries, and losses, were CEC dependent. Differences in the calculated UF can occur through a number of possible pathways, including biodegradation, volatilization, or photodegradation. In our study, leachate was not considered to be a significant loss pathway. A proportion of the UF may have formed non-extractable residues (NERs) during biodegradation such that either the parent compound or metabolites are irreversibly bound to soil solids (Bhandari et al., 1997). In the unamended spiked soil, the range of the UF was from 49 to 82% while for BC amended soils, at 2.5 and 5%, the range of the UF was 25 to 80%. The addition of BC to the soil increased the fraction recovered in soil in the range of 19 to 74%. Therefore, BC sorption of the CECs reduced these loss pathways in the short-term but most

importantly reduced plant availability for uptake. As the UF was greatest in the control than in BC treatments, the risk of formation of metabolites of some CECs can be also higher.

Half-life values (DT50) are widely used to estimate the persistence of organic microcontaminants in soils. In the literature, DT50 values have been found for many of the selected CECs. In this study, however, no data was found for MDHJ or FUR. A negative correlation (R^2 = 0.64) was observed between the average UF of each CECs and the DT50 reported in the literature (Figure S5.2). Hence, compounds such as TCS or TCP that are more persistent in soil (higher DT50 values) had higher recoveries than more labile compounds such as CAF and IBU. These results suggest that soil biodegradation is one of the most significant pathways for most of the CEC evaluated although some irreversible binding to soil cannot be ruled out. Moreover, these results are further supported by the enantioselective degradation of IBU. Although IBU is usually sold as a racemic mixture (EF = 0.5), it has been reported that in natural systems where biological processes occur, the EF varies (Hashim et al., 2010; Hashim and Khan, 2011). The soil rhizosphere around plants contains a rich diversity of bacterial communities that can selectively degrade enantiomers. In this regard, the EF of IBU in the unamended spiked soil was 0.76, while the EF of IBU in BC amended soil at BC 5%, declined to 0.58 (Figure 5.1). This result indicates that the rate of enantiomeric IBU biodegradation was reduced after BC was amended to soil. Thus, BC reduced the fraction of IBU in pore space available to bacteria, and as such, the EF is a good indicator supporting biotic degradation of this CEC.

5.5 Conclusions

Results of our study demonstrate that many CECs can be taken up by lettuce plants over the course of its growth cycle. Of particular relevance was the fact that these CECs were translocated to the leaves, which are the edible portions of the plant. Biochar amendments to soil were shown to be an effective sorbent for most of the CECs evaluated in this study. In soil, BC amendment decreased the biodegradation of CECs as supported by the reduced EF for IBU in the presence of BC. Interestingly, this decrease in biodegradation of the CECs did not lead to higher concentrations in lettuce roots or leaves. In fact, concentrations in roots and leaves were also reduced when BC was amended to soil (averaging 34 to 48% in roots and 23 to 55% in leaves at 2.5 and 5% of biochar amendments). Therefore, bioavailabilities of these CECS through BC amendments were reduced for both microbial and plant access. Our study supports the

need of further research into the long-term impacts of BC in soils to mitigate CEC plant uptake and persistence, particularly in the context of CEC dynamics when present as mixtures.

5.6 Supplementary information

5.6.1 Materials and reagents

The information of most of reagents and CECs are described in section 3.7.1. Surrogates used were caffeine-$^{13}C_3$ (CAF-$^{13}C_3$, 99%), carbamazepine-$^{13}C_6$ (CBZ-$^{13}C_6$, 99%), 2,2'-dinitrobiphenyl (DNBP, 97%) and ibuprofen-d_3 (IBU-d_3, 98%),purchased from Sigma-Aldrich; and mecoprop-d_3 (MEC-d3, 98%) and tonalide-d3 (TON-d_3), purchased from Dr. Ehrenstorfer (Ausburg, Germany).

(*R*)-(+)-α-methylbenzylamine for chiral derivatization (*R*-1-PEA, ≥ 99%), triethylamine (TEA, ≥ 99%) and ethyl chloroformate (ECF, 97%) were purchased from Sigma-Aldrich. Strata-X, Polymeric HLB-Phase, solid-phase extraction (SPE) cartridges (30 mg / 3 mL) were purchased from Phenomenex (Torrance, CA).

5.6.2 GC-MS/MS determination

All the CECs were analyzed by GC-MS/MS. Methylation of the acidic carboxyl group and the hydroxyls group of BPA for both plant tissue and soil extracts was performed in a programmed temperature vaporizing (PTV) injector of the gas chromatograph by adding 10 µL TMSH to a 50 µL sample aliquot before injection. The GC-MS/MS conditions are described in section 3.7.3.

Qualitative and quantitative analysis was performed based on retention time and SRM mode of two product ions, and the ratio between the product ions (Table S5.1). The LODs and LOQs for both plant tissue and soil were defined as the mean background noise in a blank triplicate plus three and ten times, respectively, the standard deviation of the background noise from three blanks. LODs and LOQs were compound dependent and for leaves and roots ranged from 0.2 to 5 ng g^{-1} dry weight (dw) and for soil ranged from 0.2 to 2.4 ng g^{-1} dw (Table S5.2). The recoveries of the surrogates added can be seen in Table S3.

5.6.3 Chiral derivatization of IBU

The derivatization procedure was described by Hashim and Khan (2011) and are described in section 3.7.5.

Table S5.1. Monitoring ions in GC-MS/MS

Segment	Compound	RT (min)	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
1	**CFA**	8.59	128*	65	25
			128	100	15
2	**IBU**	11.23	161*	91	23
			220	161	11
2	**IBU-d_3**	11.19	164*	122	16
			223	164	13
2	**MEC-d_3**	11.34	231*	172	14
			172	128	19
3	**MDHJ**	12.19	153*	83	20
			156	125	15
4	**TCP**	13.54	249*	125	15
			249	125	30
5	**CAF**	14.74	194*	109	14
			194	55	20
5	**CAF-$^{13}C_3$**	14.78	197*	110	12
			110	82	17
5	**TON**	15.03	258*	243	10
			243	187	13
5	**TON-d_3**	14.99	261*	246	10
			246	190	13
6	**CBZ**	17.09	193*	191	23
			193	167	18
6	**CBZ-$^{13}C_6$**	17.14	199*	173	25
			199	197	20
6	**TPhA**	17.47	245*	167	30
			245	141	21
7	**BPA**	17.81	241*	133	15
			241	211	17
7	**DNBP**	17.99	198*	168	15
			198	138	25
8	**TCS**	18.25	302*	252	19
			302	189	37
9	**FUR**	25.32	372*	81	20
			372	339	15

*Transition used for quantification

Table S5.2. Limits of detection (LOD) and quantification (LOQ) of the selected CECs in the three studied compartments.

Compound	Compartment	LOD (ng g^{-1} dw)	LOQ (ng g^{-1} dw)
BPA	**Soil**	1.7	2.0
	Root	2.4	2.7
	Leaf	0.6	1.2
CAF	**Soil**	1.0	1.4
	Root	1.4	1.9
	Leaf	0.3	0.6
CBZ	**Soil**	1.6	1.9
	Root	1.5	2.0
	Leaf	1.0	1.4
CFA	**Soil**	1.3	1.8
	Root	2.4	3.0
	Leaf	0.3	1.0
FUR	**Soil**	1.8	2.4
	Root	4.3	5.0
	Leaf	4.0	4.6
IBU	**Soil**	0.4	0.7
	Root	0.8	1.3
	Leaf	0.2	0.5
MDHJ	**Soil**	0.6	1.2
	Root	2.8	3.5
	Leaf	0.3	0.9
TCP	**Soil**	1.3	1.7
	Root	2.4	2.9
	Leaf	0.9	1.3
TCS	**Soil**	0.8	1.5
	Root	1.5	1.9
	Leaf	0.5	1.0
TON	**Soil**	1.3	1.6
	Root	1.0	1.4
	Leaf	0.4	0.7

Table S5.3. Average recovery of the surrogates added in each compartment and the standard deviation (N = 20).

Compound	Compartment	Recovery (%)
CAF-$^{13}C_3$	**Soil**	67 ± 4
	Root	64 ± 6
	Leaf	60 ± 4
CBZ-$^{13}C_6$	**Soil**	78 ± 5
	Root	70 ± 7
	Leaf	63 ± 6
DNBP	**Soil**	52 ± 3
	Root	71 ± 4
	Leaf	73 ± 6
IBU-d_3	**Soil**	69 ± 7
	Root	56 ± 8
	Leaf	63 ± 7
MEC-d_3	**Soil**	73 ± 5
	Root	58 ± 6
	Leaf	61 ± 8
TON-d_3	**Soil**	65 ± 4
	Root	60 ± 7
	Leaf	62 ± 6

Table S5.4. Dry weight (g) of non-spiked soil (control), and spiked soils amended with 0, 2.5, and 5% BC, roots and leaves. of each experimental unit.

Experimental Unit	Soil	Root	Leaf
Control 1	2255	1.1	12.3
Control 2	2263	1.5	10.7
Control 3	2296	1.6	9.9
Control 4	2322	1.2	8.3
Control 5	2279	1.1	9.5
0% BC 1	2283	1.3	6.2
0% BC 2	2321	1.0	7.1
0% BC 3	2336	1.2	7.4
0% BC 4	2286	0.8	7.0
0% BC 5	2243	0.9	6.9
2.5% BC 1	2314	0.8	6.9
2.5% BC 2	2278	0.7	6.8
2.5% BC 3	2283	0.7	6.9
2.5% BC 4	2292	1.0	7.1
2.5% BC 5	2261	1.0	8.2
5% BC 1	2290	0.4	7.3
5% BC 2	2260	0.8	6.8
5% BC 3	2300	0.9	6.8
5% BC 4	2294	0.6	9.0
5% BC 5	2274	0.8	7.0

Table S5.5. Fraction of RCF in soils amended with 2.5% or 5% BC relative to the soil without BC with standard deviation (N = 5).

CEC	$RCF_{2.5/0\%}$	$RCF_{5/0\%}$
BPA	0.61 ± 0.19[a]	0.31 ± 0.16[c,*]
CAF	0.65 ± 0.39[a]	0.62 ± 0.16[ab]
CBZ	0.67 ± 0.21[a]	0.66 ± 0.27[ab]
CFA	0.58 ± 0.20[a]	0.32 ± 0.08[c,*]
FUR	0.78 ± 0.37[a]	0.64 ± 0.19[ab]
IBU	0.62 ± 0.28[a]	0.38 ± 0.13[c,*]
MDHJ	0.95 ± 0.57[a]	0.69 ± 0.43[a]
TCP	0.74 ± 0.23[a]	0.69 ± 0.22[a]
TCS	0.59 ± 0.31[a]	0.42 ± 0.15[bc]
TON	0.86 ± 0.38[a]	0.72 ± 0.34[a]

Means with the same letter in the same column are not significantly different (α = 0.05) and means with asterisk (*) are CECs that exhibited significant differences between treatments.

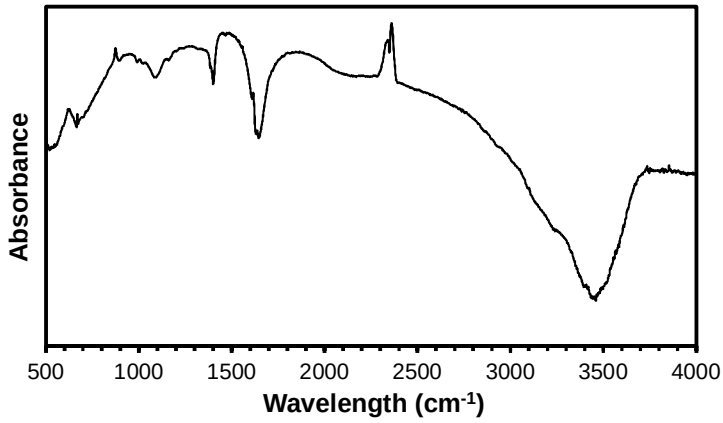


Figure S5.1 FTIR spectra of the studied biochar produced from vineyard wood pyrolyzed at 650 ºC obtained with a Thermo Nicolet Avatar 360 FTIR and KBr pellets.

The spectrum showed bands in the region of 3400 to 3500 cm^{-1}, which can be related to the stretching of hydroxyl groups. In the region of 1600 to 1700 cm^{-1} bands of double bonds between carbons but also between aromatic carbon and oxygen. (e.g. –COOH). Bands around 1350-1450 cm^{-1} can be related to C6 ring modes, while bands around 1100 cm^{-1} represent C-O-C groups (Komnitsas et al.; 2015).

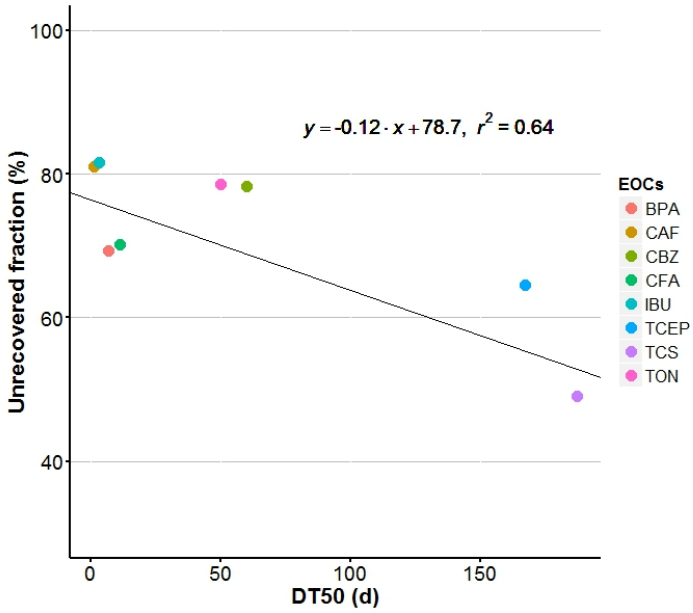


Figure S5.2 Regression between the DT50 values found in the literature of the selected CECs and the unrecovered fraction (p = 0.017).

Chapter 6. Determination of the β-glycosylate fraction of CECs in lettuce

The uptake of a large variety of CECs by crops has already been reported, and the occurrence of Phase II metabolites or conjugates has only been detected in plant cell cultures. However, the extent of their formation under cropping conditions is largely unknown. In this study, an analytical strategy to assess the conjugation of 11 CECs in lettuce (*Lactuca sativa* L) irrigated with different concentrations (0, 0.05, 0.5, 5, and 50 μg L^{-1}) of CECs was developed. The methodology involved enzymatic digestion with β-glucosidase to obtain the total fraction (free form + conjugates) of CECs. The conjugation fraction was then obtained based on the difference. The highest extent of conjugation (i.e., 27 to 83%) was found with the most hydrophobic compounds, such as bisphenol A, carbamazepine, methyl paraben, and triclosan. So, the CEC conjugate fraction cannot be neglected in the human daily intake assessments.

6.1 Introduction

The uptake of a large variety of CECs in different crops has been reported under different controlled conditions and in field studies (Malchi et al., 2014; Wu et al., 2014; Riemenschneider et al., 2016). Once the CECs are taken up by plants, several transformation processes can occur. Plants are known to have their own detoxification system, leading to the formation of transformation intermediates that do not result in mineralization (Burken, 2004). First, the uptaken contaminants are transformed into more polar and soluble compounds by oxidation, hydrolysis, or hydroxylation (Phase I). Functional groups (e.g., -OH, -SH, -COOH, or -NH_2) may be added to the molecule. Alternatively or subsequently, the uptaken compound or Phase I metabolite is conjugated with glucose, malonic acid, glutathione, or other amino acids (Phase II). Usually, these intermediates are more water-soluble and less phytotoxic. Finally, these conjugated compounds can be sequestered from the plant's metabolism to the cell vacuole or in the cell wall (Phase III) (Coleman et al., 1997).

Recently, some studies have focused on identifying Phase I transformation products in cell cultures (Macherius et al., 2014; LeFevre et al., 2015; Wu et al., 2016). These transformation products have also been reported in some vegetables (Goldstein et al., 2014; Malchi et al., 2014).

However, to estimate the human daily intake of contaminants occurring in crops, it is important to determine the fraction of the contaminant that has been conjugated since the parent compounds may be released in the human digestive tract (Day et al., 1998). In this study, the plant uptake and the fraction of β-glycosylate conjugates of eleven CECs were determined for the first time. The selection of CECs was based on their occurrence and their physical-chemical properties. The chosen CECs were mostly neutral or very weak acids with functional groups prone to conjugation. The study was carried out under controlled conditions by using spiked water at different concentrations. Although many conjugated contaminants can be formed, this study focused on glycosylated conjugates, because humans can break the glycosidic bond, releasing the corresponding aglycone inside the body (Saitoh et al., 2004). It could thus be formed to a greater extent than others conjugates (LeFevre et al., 2016).

6.2 Materials and methods

6.2.1 Experimental conditions

The experiment was conducted at an agricultural experimental station (Agròpolis) belonging to the Universitat Politècnica de Catalunya (Viladecans, Spain) in winter (January 29 to March 21, 2016). The average temperature inside the greenhouse was 21 ºC, and the relative humidity was 56%. The soil used was collected from the surface horizon of a typical Xerorthent soil from an agricultural area located at the Llobregat River Delta (longitude 2°03'E, latitude 41°17'N). The soil had a sandy texture (90% sand, 8% silt, and 2% clay) with a pH of 7.4 (soil-to-water ratio 1:5) and electrical conductivity of 3.8 dS m^{-1}. Total organic carbon and total organic nitrogen content was 5 g kg^{-1} and 0.7 g kg^{-1}, respectively. The cation exchange capacity (CEC) was 3.8 meq 100 g^{-1} and exchangeable Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} were 2.82, 0.64, 0.25, and 0.15 meq 100 g^{-1} soil, respectively.

Lettuce (*Lactuca sativa* L, cv. Arena) was planted in pots and watered with Hoagland nutrient solution (Hoagland and Arnon, 1950) diluted 1:1 with rainwater. About 100 mL of irrigation water was applied to each experimental unit per day. The number of daily irrigations was regulated to keep water in the soil below field capacity, thereby preventing leachate production. Eighteen days after the seedling implant, CECs were added to the soil. Five treatments were used, consisting of the direct application of 0, 0.05, 0.5, 5, and 50 $\mu g\ L^{-1}$ to the irrigation water in four replicates per each treatment (20 experimental units in total).. After 34 days, the lettuce was harvested and the soil, roots, and lettuce leaves were separated. The leaves and roots were comminuted separately with liquid nitrogen and stored at -20 °C until analysis.

6.2.2 Reagents and materials

The CECs measured in the experimental study are listed in Table 6.1 with their properties.

A 0.1 N acetate:acetic acid buffer was prepared and adjusted to a final pH of 5.5 with a solution of 0.1 N NaOH. Finally, ethanol was added to reach 3% ethanol in the buffer. See the section 6.5.1 for detailed information on the reagents.

Table 6.1: Physical-chemical properties of the contaminants of emerging concern (CECs) investigated in this study.

Compound	Molar mass (g mol^{-1})	p*K*a	Speciation (z)	Neutral log K_{ow}	Solubility (mg mL^{-1})
Benzophenone (BZP)	182.22	NA	0	2.98	0.17
Bisphenol A (BPA)	228.29	9.7 10.5	0/-1/-2	3.46	0.13
Butylated hydroxytoluene (BHT)	220.35	12.1	0/-1	5.06	0.0046
Caffeine (CAF)	194.19	NA	0	0.11	21.5
Carbamazepine (CBZ)	236.27	NA	0	2.23	0.084
Methyl paraben (MePB)	152.15	8.5	0/-1	1.97	1.3
5-Methyl-1H-benzotriazole (MeBT)	133.15	1.6 8.5	+1/0/-1	1.57	3.7
4-Octylphenol (OPL)	206.32	10.0	0/-1	5.64	0.0026
Phenazone (PZE)	188.23	1.8	+1/0	0.85	75.8
Triclosan (TCS)	258.40	8.8	0/-1	5.21	0.0046
Tris(2-chlorethyl) phosphate (TCP)	285.49	NA	0	1.72	5

NA: Not applicable; z is charge number (valency); K_{OW} (L/L) is the partition coefficient octanol to water for the neutral molecule and K_{AW} (L/L) is the partition coefficient air to water for the neutral molecule (known as dimensionless Henry's Law constant). *All values were estimated by using the ACD Advanced Chemistry Development (2010), ACD/i-lab 2.0. Toronto, 2010.

6.2.3 Extraction and enzymatic reaction

To determine the amount of conjugated contaminants, a first-step extraction was performed, followed by an enzymatic reaction. One gram of fresh-weight leaf tissue was extracted three times with 2.5 mL acetate buffer pH of 5.5 by means of 15 minutes of sonication. It was then centrifuged at 3500 rpm for 10 minutes. Two parallel subsamples were extracted per replicate: in one subsample, the enzyme was added to obtain the total (free form + conjugate); the other subsample was used to calculate the concentration of the parent compound extracted with the aqueous buffer (free form).

In order to assess the amount of enzyme needed, three enzyme doses were evaluated, namely, 5, 10 and 20 enzyme units to the final extract volume (0.25, 0.5, and 1.0 mg enzyme). The enzyme was added directly to the extracts and kept under orbital agitation at room temperature. The enzymatic reaction time was also optimized by monitoring the enzymatic reaction yield at 2, 6, 24, 48, and 72 h (see section 6.3.1).

Following the enzymatic reaction, the extracts were spiked with a mixture of surrogates (see section 6.5) and percolated through 30 mg / 3 mL Strata-X SPE cartridges (Phenomenex, Torrance, CA) previously conditioned with 3 mL of ethyl acetate, 3 mL of methanol, and 3 mL of buffer. After being dried under vacuum, the cartridges were eluted with 6 mL of ethyl acetate and concentrated to a final volume of 300 µL under a nitrogen stream. Triphenylamine was added as an internal standard, and samples were derivatized with 10 µL of TMSH prior to injection into the GC-MS/MS. The final extracts were analyzed by GC coupled with electron impact ionization tandem mass spectrometry (EI-MS/MS) in a Bruker 450-GC gas chromatograph coupled to a Bruker 320-MS triple-quadrupole mass spectrometer (Bruker Daltonics Inc., Billerica, MA). Qualitative and quantitative analyses were performed based on the retention time and selected reaction monitoring (SRM) mode of two product ions, and the ratio between the product ions was used for confirmation. Monitoring ions, LODs and LOQs, and recoveries can be found in the supplementary information (Table S6.1-S6.4).

6.2.4 Data calculation

Statistical analysis (non-parametric Wilcoxon signed-rank test) was performed using STATA 14 (StataCorp LP, College Station, TX).

6.3 Results and discussion

6.3.1 Optimization of the deglycosylation reaction

Amount of enzyme

Four enzyme doses of β-glucosidase (0, 5, 10, and 20 units) were added to the leaf extracts of lettuces exposed to the highest concentration. The concentrations of the parent compounds are shown in Figure 6.1.

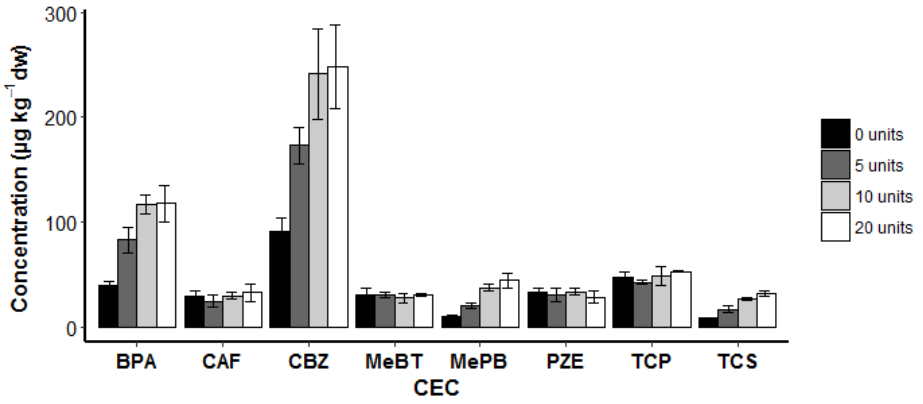


Figure 6.1: Average concentration and standard deviation (N = 3) of parent contaminant (in µg kg^{-1} dw) in lettuces exposed to the highest concentration (50 µg L^{-1}) when different amounts of enzyme (0, 5, 10, and 20 units) were added after the extraction.

For the samples not subjected to enzymatic digestion, the parent compounds were usually detected. For some compounds (BPA, CBZ, MePB, and TCS), the concentration of extracted CECs was significantly higher ($p < 0.05$) when enzyme was added. The amount of enzyme selected was 10 units because concentrations with 20 units were not significant higher.

In contrast, CAF, MeBT, PZE, and TCP concentrations did not increase after the addition of enzyme, suggesting that β-glycosylate conjugates were not formed under the cropping conditions used.

Time of enzymatic digestion

After the extraction with the aqueous buffer, 10 units of enzyme were added to the extracts, and they were soft-stirred for 2, 6, 24, 48, and 72 h. The concentration of the parent compound according to the enzymatic reaction time is shown in Figure 6.2. The selected time was 24 h because, for most CECs, no significant increase in concentration was obtained beyond that time.

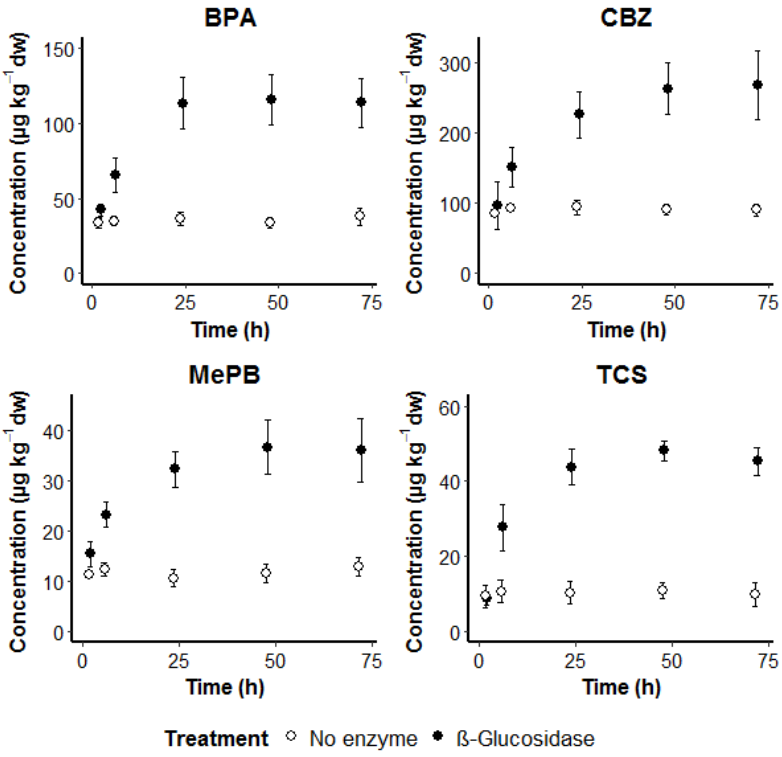


Figure 6.2: Average concentration and standard deviation (N = 3) of parent contaminant (in µg kg^{-1} dw) in lettuces exposed to the highest concentration (50 µg L^{-1}) at different reaction time (2, 6, 24, 48, and 72 h) without enzyme and with 10 units of β-glucosidase.

6.3.2 Determination of the conjugated fraction

After optimizing the amount of enzyme and the reaction time, all samples were incubated with 10 units of enzyme for 24 h. Control samples (the same experimental units without enzyme for 24 h) were also extracted to subtract the amount of parental compound extracted with the buffer. The differences between the control samples and the samples with enzyme are shown in Figure 6.3 for selected contaminants (9 out of 11).

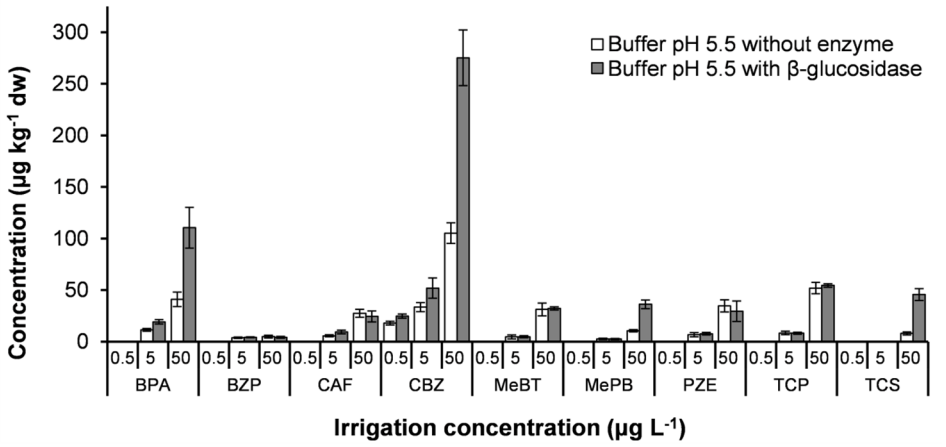


Figure 6.3: Average concentration and standard deviation (N = 4) of parent contaminant (in µg kg^{-1} dw) in different exposure concentrations (0.5, 5, and 50 µg L^{-1}) for samples without enzyme and with 10 units of β-glucosidase for 24 h.

For many CECs in different treatments, the concentration of non-conjugated parent compound in the plant was below the LOD. In the lettuce samples without spiking and the samples exposed to the lowest level, CECs were not detected in the leaves. Differences between enzymatic treatments became evident at higher spiking concentrations. BHT and OP were not detected in any treatment, which is consistent with a high hydrophobicity (log K_{ow} > 5) and, thus, low availability through the radicular pathway (Hurtado et al., 2016).

BPA was detected and quantified at the two highest treatments (5 and 50 µg L^{-1}). The average conjugated concentration ranged from 7.8 to 70 µg kg^{-1}, and it accounted for a conjugated fraction of between 40 and 63% for both treatments. In a previous study, BPA glucopyranoside conjugation was detected in tobacco seedlings exposed at 10 mg L^{-1} by using ^{14}C-labeled BPA (Nakajima et al., 2002). These authors suggested that BPA was metabolized to its β-glucoside metabolite and translocated to the leaves. BPA is highly hydrophobic and its translocation to aerial parts should be low, as has been reported (Lu et al., 2015; Hurtado et al., 2016). However, these studies do not take the conjugated fraction into account. The present study shows that an important fraction of BPA can be conjugated and translocated to the leaves.

CBZ was detected at three exposure levels (0.5, 5, and 50 µg L^{-1}). The conjugated fraction increased with the exposure level from 27 to 62% of the CBZ. Several authors have studied CBZ degradation, mainly Phase I products. Malchi et al. (2014) detected

two metabolites (10,11-epoxycarbamazepine and 10,11-dihydroxycarbamazepine) in the roots and leaves of sweet potatoes and carrots watered with treated wastewater. However, to date no Phase II metabolization metabolites have been reported.

Although no differences were observed for MePB at exposure level of 5 μg L^{-1}, the conjugated fraction accounted for 70% of the total MePB at 50 μg L^{-1}. No studies of MePB conjugation in plants have been previously reported; however, it should be noted that its conjugation can be significant at high exposure levels.

TCS was only detected at the highest treatment (50 μg L^{-1}). Interestingly, its conjugated form accounted for 83% of the total. As TCS has a very high K_{ow} (5.2), its translocation is not expected to be high (Trapp, 2009). However, TCS could be passively taken up in the roots and then conjugated and translocated to aerial parts of the lettuce. Macherius et al. (2012a) identified several glucopyranosyl forms in horseradish cell cultures and in carrot plants in pots exposed at 3 mg kg^{-1} of TCS. In their experiment, the total amount of TCS conjugates exceeded the free form of TCS in carrots, which is similar to the results of the present study.

Other CECs, such as CAF, PZE and 5-MeBT, exhibited no significant differences with the addition of the enzyme. This may be due to the fact that these CECs may undergo a transformation in Phase I, but it is uncertain whether they can be conjugated to Phase II (Luckner, 2013). Huber et al. (2009) suggested that plants have two independent pathways for the metabolization of acetaminophen. The free form of acetaminophen was conjugated directly with glucose without prior transformation, while diclofenac was hydroxylated prior to conjugation (Huber et al., 2012). Unfortunately, the methodology developed in the present study does not enable the quantification of CECs that have undergone Phase I and II metabolization.

Although hydrophobicity is one of the molecular descriptors for molecular conjugation, other molecular factors (e.g. steric hindrance) might affect the extent of the conjugation. Thus, it is important to elucidate whether these hydrophobic contaminants could be conjugated and translocated to the aerial parts of the plant. These contaminants may be being underestimated, as most assessments focus on the target compound. Moreover, this could explain why some hydrophobic contaminants, such as estrogenic hormones, are unexpectedly uptaken by plants; since they are excreted as conjugates, these forms could be taken up by plants through the roots (Sabourin et al., 2012; Malchi et al., 2015).

6.4 Conclusion

This study has demonstrated that once CECs are taken up by plants, glucose conjugates may be formed for some of them. The conjugated fraction accounted for between 27 and 83% of the free parent compound and usually increased in accordance with the hydrophobicity and concentration of the contaminant in question. Therefore, not only Phase I transformation products but also Phase II conjugates should be taken into account in risk assessments based on daily intake (Malchi et al., 2015; Prosser and Sibley, 2015), since the non-conjugated forms can be released during digestion. Additional conjugates could be formed with other molecules such as amino acids. Future research should seek to identify and quantify other Phase II metabolites.

6.5 Supporting information

6.5.1 Reagents and materials

Benzophenone (BZP, 99%), bisphenol A (BPA, 99%), butylated hydroxytoluene (BHT, 98%) caffeine (CAF, 99%), carbamazepine (CBZ, 99%), methyl paraben (MePB, 99%), 5-methyl-1*H*-benzotriazole (MeBT, 98%), 4-octylphenol (OPL, 99%), phenazone (PZE, 98%), triclosan (TCS, 97%) and tris(2-chloroethyl) phosphate (TCP, 97 %) and all were purchased from Sigma-Aldrich (St. Louis, MO). Ethyl acetate, ethanol and methanol SupraSolv®-quality were purchased to Merck (Darmstadt, Germany). Glacial acetic acid was purchased to Panreac (Castellar del Vallès, Spain), sodium hydroxide (>97%) and the enzyme β-glucosidase (10-30 units/mg) from almonds were purchased to Sigma-Aldrich.

The GC-MS/MS determination is described in section 3.7.3. Qualitative and quantitative analysis was performed based on retention time and SRM mode of two product ions, and the ratio between the product ions (Table S6.1). The LODs and the LOQ for leaf were defined as the mean background noise in a blank triplicate plus three and ten times, respectively, the standard deviation of the background noise from three blanks. LODs and LOQs were compound dependent and ranged from 2.1 to 10 μg kg^{-1} (Table S6.2). The recoveries of the surrogates added can be seen in Table S6.3. The relative recoveries of the studied CECs in the enzymatic procedure can be seen in Table S6.4.

Table S6.1. Monitoring ions in GC-MS/MS

Compound	RT (min)	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
MePB	9.13	135	77	18
		166*	135	13
BHA	9.50	179*	149	20
		194	179	16
EPB	9.92	135*	77	18
		180	152	11
MeBT	10.46	118	77	17
		147*	118	14
BHT	10.50	205	177	12
		220*	205	17
OPL	11.72	135*	77	18
		149	121	15
BzP	12.13	105	77	14
		182*	105	17
XTTri	12.31	132	91	16
		161*	132	16
TCP	13.57	249	99	30
		249*	125	15
CAF	14.76	194	55	20
		194*	109	14
CAF-$^{13}C_3$	14.77	110	82	12
		197*	110	17
PZE	15.31	188	96	15
		188*	159	11
CBZ	16.52	193	167	18
		193*	191	23
CBZ-$^{13}C_6$	16.55	199	171	20
		199*	197	22
TPhA	16.91	245	141	32
		245*	167	30
BPA-d_6	17.22	252*	139	20
		270	252	14
BPA	17.30	241*	133	15
		256	241	13
TCS	17.99	302	189	32
		302*	252	19

*Transition used for quantification

Table S6.2. Limits of detection (LOD) and quantification (LOQ) in µg kg^{-1} in dw of the studied CECs with the buffer extraction and enzymatic reaction.

Compound	LOD (µg kg^{-1})	LOQ (µg kg^{-1})
BHT	ND	ND
BPA	7.3	9.8
BzP	4.6	6.2
CAF	2.6	4.3
CBZ	7.7	10
MeBT	2.1	3.1
MePB	1.5	1.9
OPL	ND	ND
PZE	2.7	4.1
TCP	4.3	5.5
TCS	4.0	5.5

ND: Not detected

Table S6.3. Average recoveries (%) of the surrogates added before the SPE concentration and the standard deviation of all samples (N = 19).

Compound	Recovery (%)
BHA	87 ± 3
BPA-d_6	90 ± 4
CAF-$^{13}C_3$	92 ± 3
CBZ-$^{13}C_6$	91 ± 6
EPB	84 ± 5
XTTri	90 ± 4

Table S6.4. Average absolute and relative recoveries (%) of the CECs added with the buffer extraction and enzymatic reaction.

Compound	Absolute recovery (%)	Relative recovery (%)
BHT	17 ± 2	22 ± 3
BPA	18 ± 3	28 ± 3
BZP	13 ± 2	25 ± 3
CAF	35 ± 2	42 ± 2
CBZ	21 ± 2	37 ± 3
MeBT	35 ± 2	39 ± 2
MePB	32 ± 2	44 ± 3
OPL	ND	ND
PZE	22 ± 4	24 ± 4
TCP	25 ± 3	28 ± 3
TCS	20 ± 1	22 ± 2

Chapter 7. Effects of CECs to lettuce

The occurrence of contaminants of emerging concern (CECs) in irrigation waters (up to low μg L^{-1}) and irrigated crops (ng g^{-1} in dry weight) has been reported, but the linkage between plant morphological changes and plant metabolomic response has not yet been addressed. In this study, a non-targeted metabolomic analysis was performed on lettuce (*Lactuca sativa* L) exposed to 11 CECs (pharmaceuticals, personal care products, anticorrosive agents and surfactants) by irrigation. The plants were watered with different CEC concentrations (0-50 μg L^{-1}) for 34 days under controlled conditions and then harvested, extracted, derivatized and analyzed by comprehensive two-dimensional gas chromatography coupled to a time-of-flight mass spectrometer (GC×GC-TOFMS). The resulting raw data were analyzed using multivariate curve resolution (MCR) and partial least squares (PLS) methods. The metabolic response indicates that exposure to CECs at environmentally relevant concentrations (0.05 μg L^{-1}) can cause significant metabolic alterations in plants (carbohydrate metabolism, the citric acid cycle, pentose phosphate pathway and glutathione pathway) linked to changes in morphological parameters (leaf height, stem width) and chlorophyll content.

7.1 Introduction

Contaminants of emerging concern (CECs), including compounds such as pharmaceuticals and personal care products, are increasingly detected in agricultural irrigation waters as a consequence of multiple inputs throughout the water cycle and partial removal during water reclamation and potabilization (Calderón-Preciado et al., 2011b; Cabeza et al., 2012; Riemenschneider et al., 2016). Both plant uptake of CECs and changes in plant morphological (e.g. biomass production and shoot growth) and physiological (e.g. phytohormones and chlorophyll content) parameters have been reported under different experimental conditions. Plants can transform the uptaken CECs through plant detoxification mechanisms involving enzymatic (phase I) and conjugation (phase II) processes (Macherius et al., 2014; Wu et al., 2016). For instance, the presence of CECs can increase antioxidant enzymatic activities due to detoxification processes (Christou et al., 2016). Plants are known to be able to biosynthesize specialized or secondary metabolites to adapt to biotic or abiotic environmental stressors, such as drought, salt, low soil oxygen, metals, temperature, light and oxidative stress (Jorge et al., 2015; Nakabayashi and Saito, 2015). Nevertheless, the application of metabolomics to study plant response to CEC exposure in agricultural irrigation waters has not yet been investigated.

Metabolomics is the comprehensive analysis of all of an organism's metabolites to understand the complexity of molecular interactions in biological systems (Fiehn, 2002; Bino et al., 2004). Plant metabolomics aims to study the plant system at the molecular level to provide a non-biased characterization of the total metabolite pool (metabolome) of a plant's tissue in response to its environment (Jorge et al., 2015). Plant metabolomics includes the analysis of a wide range of chemical compounds, from ionic inorganic compounds to biochemically derived hydrophilic carbohydrates, organic and amino acids, and a range of hydrophobic lipid-related compounds. Analytical methodologies based on gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), or nuclear magnetic resonance (NMR) are commonly used (Farag et al., 2014; Simmler et al., 2014). However, measurement of all these compounds is still an analytical challenge due to the complexity of the matrix samples (Allwood et al., 2011). In this regard, the use of hyphenated chromatographic techniques, such as comprehensive two-dimensional gas chromatography-mass spectrometry (GC×GC-MS), has emerged as a powerful separation technique to help solve this issue (Koek et al., 2008).

The three main benefits of GC×GC compared to one-dimensional (1D) GC are: (i) increased chromatographic resolution; (ii) improved analyte detectability due to the cryofocusing in the thermal modulator; and (iii) chemical class ordering in the contour plots (Dallüge et al., 2003). The coupling of two-dimensional (2D) GC×GC to a fast detector such as time-of-flight (TOF) working at acquisition rates up to 500 MHz and combined with a proper spectral deconvolution helps to fully resolve peak metabolites and enables the detection of up to several thousand peaks in a single GC×GC run (Ramos and Brinkman, 2009; Reichenbach, 2009). However, one problematic feature of metabolomic data is the complexity and large volume of data provided by GC×GC–TOFMS and the difficulty of analyzing highly polar or thermal labile metabolites, which require complex derivatization reactions. Despite the technique's increased resolution and the significant separation improvement over 1D GC – the 2D separation space provides large peak capacity – there is still some overlap (Mohler et al., 2006; Mohler et al., 2007; Beckstrom et al., 2011).

In the last decade, the generalized rank annihilation method (GRAM) (Fraga et al., 2001), parallel factor analysis (PARAFAC) (Hoggard et al., 2009), PARAFAC2 (Skov et al., 2009) and the partial least squares (PLS) (Johnson et al., 2004; Radović et al., 2014) multivariate resolution method have been proposed to overcome fundamental challenges occurring during GC×GC analyses. The most effective way to handle both elution time shifts and peak shape changes is to use methods that do not require the fulfilment of the trilinear model, such as the PARAFAC2 method (Amigo et al., 2008; Skov et al., 2009) or, in more general cases, multivariate curve resolution-alternating least squares (MCR-ALS) (Parastar et al., 2011; Parastar et al., 2012; Parastar et al., 2013; Parastar and Tauler, 2014). As the MCR–ALS method is based only on fulfilment of the bilinear model's assumption, three-way and four-way GC×GC–TOFMS data should be arranged in a column-wise super-augmented data matrix in which mass-to-charge ratios (m/z) are allocated in the columns and the elution times in the second and first chromatographic columns are listed in the rows (Tauler, 1995; Tauler et al., 1995). Since m/z values are common to all measured spectra in all second-column modulations, unavoidable chromatographic challenges, such as shifts in retention time within and between GC×GC–TOFMS chromatographic runs, are properly handled in the column-wise augmented mode. In addition, baseline/background contributions can be modelled by adding extra components to the MCR–ALS model. Another outstanding aspect of MCR–ALS analysis is its extreme flexibility to consider all samples (standard, unknown and replicates) in a single super-augmented data matrix, enabling joint analysis (Parastar

and Tauler, 2014), even in cases where the individual data matrices have different numbers of rows (retention times).

As mentioned above, the metabolic response of plants to various stresses of abiotic origin (e.g. metals, pesticides) is receiving increasing attention (Wang et al., 2015; Pidatala et al., 2016; Zhao et al., 2016). For instance, the occurrence of Cd and Pb have been shown to affect carbohydrate metabolism and glutathione metabolism, whilst crop exposure to herbicides has been shown to decrease antioxidant levels and disturb the tricarboxylic acid (TCA) cycle and other amino acid-related pathways (Wang et al., 2015; Zhao et al., 2016). However, the effect of the presence of CECs in irrigation waters on plant metabolomics has not yet been considered. The aim of the present study was to conduct, for the first time, a non-targeted metabolomic analysis of lettuce (*Lactuca sativa* L) exposed to 11 CECs (benzophenone (BZP), bisphenol A (BPA), butylated hydroxytoluene (BHT), caffeine (CAF), carbamazepine (CBZ), methylparaben (MePB), 5-methyl-1H-benzotriazole (MeBT), 4-octylphenol (OPL), phenazone (PZE), triclosan (TCS) and tris(2-chloroethyl) phosphate (TCP)) supplied in irrigation water in order to determine the affected metabolic pathways. Extracted leaf samples were derivatized and analyzed by GC×GC–TOFMS combined with MCR and PLS to identify the endpoints affected by the exposure to the contaminants (Figure 7.1).

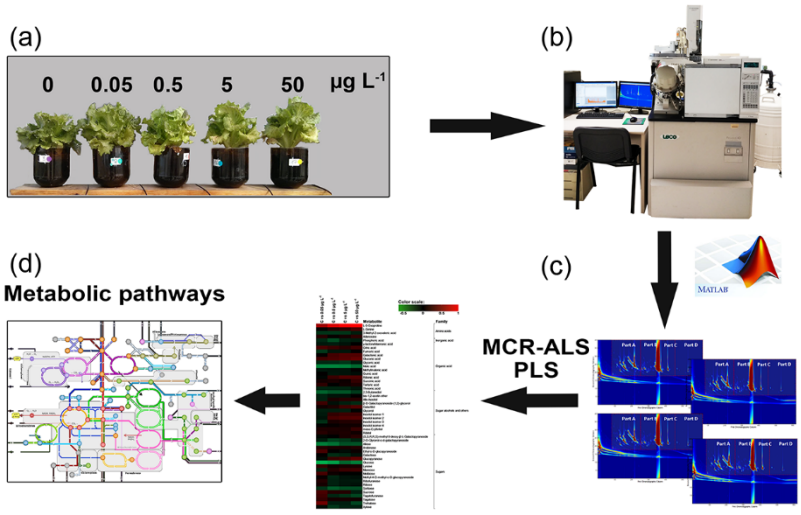


Figure 7.1: General framework used in this study: from samples, data acquisition, data transformation, and modelling.

7.2 Methods

7.2.1 Experimental setup

The experiment was conducted in an agricultural experimental station (Agròpolis) belonging to the Universitat Politècnica de Catalunya (Viladecans, Spain) in winter period (January 29 to March 21, 2016). Further details are explained in section 6.2.1. Lettuce (*Lactuca sativa* L) was chosen because it is an economically important vegetable crop that alone accounts for 14% of all leafy vegetable sales in the US (Fantke et al., 2011).

CEC extraction from the lettuce leaves

The extraction procedure for the determination of the CECs from the leaves is described elsewhere (Calderón-Preciado et al., 2009). For each experimental unit, a 0.5 g portion of the comminuted leaves was transferred to a porcelain mortar and spiked with a mixture of surrogates (see section 7.6). Briefly, a matrix solid-phase dispersion (MSPD) method followed by a pressurized solvent extraction using a mixture of acetone:hexane (1:1, v/v) is performed. The final extracts were analyzed by GC coupled to electron impact tandem mass spectrometry (EI-MS/MS) in a Bruker 450-GC gas chromatograph coupled to a Bruker 320-MS triple stage quadrupole mass spectrometer (Bruker Daltonics Inc., Billerica, MA, USA). Qualitative and quantitative analysis were performed based on retention time and selected reaction monitoring (SRM) mode of two product ions, and the ratio between the product ions was used for confirmation. Monitoring ions, limits of detection (LODs) and limits of quantification (LOQs) and recoveries can be found in the section 7.6 (Tables S7.1-S3). The mean concentration and standard deviation of each CEC in the different treatments were then calculated with the four replicates of each treatment.

Metabolite extraction from the lettuce leaves

The extraction procedure was adapted from the described elsewhere (Garreta-Lara et al., 2016). Briefly, for the extraction of polar plant compounds, 60 mg of plant material was transferred into an Eppendorf tube. Then, 400 µL methanol, 30 µL of a 50 µg mL^{-1} D-glucose (U-13C6, 99%) solution and 30 µL of a 50 µg mL^{-1} salicylic acid-d_6 solution were added to the tube. The samples were vortexed and sonicated in an ultrasonic bath for 15 min. 200 µL of chloroform was then added and the samples were vortexed for 1 min and sonicated for 15 min. Next, 400 µL of water was then added and the samples

were again vortexed for 1 min and sonicated for 15 min. The tubes were then centrifuged at 10,000×*g* for 15 min in order to separate the aqueous and lipid phases. Finally, 700 µL of the aqueous phase was transferred to a 4 mL glass vial. The extracts were vacuum-dried with a Speedvac (Thermo Scientific) at 40 ºC for 4 h. The samples were stored at -80 ºC until analysis. 200 µL of 20 mg mL-1 methoxyamine (MeOX) in pyridine was added to the dry residue. The mixture was vortexed for 1 min and then incubated at 40 ºC for 90 min. Thereafter, 30 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) was added and the mixture was vortexed for 1 min and incubated at 40 ºC for 45 min. Finally, the extracts were filtered through a 0.22 µm filter (Ultrafree®-MC, Millipore) and then transferred to a chromatographic vial. Triphenylamine (TPhA) was added as an instrumental standard (25 µL) and samples were injected into the GC×GC-TOFMS.

Instrumental analysis

The GC×GC-TOFMS system consisted of an HP 6890N (Agilent Technologies, Palo Alto, CA) gas chromatograph equipped with a split/splitless injector, a secondary oven to fit the secondary column, and a ZX1 (Zoex, Houston, TX) two-stage thermal modulator operating at 4 s per modulation with 0.5 s hot pulse duration and a 30 ºC modulator temperature offset. Liquid nitrogen was used to cool down the nitrogen gas for cold pulses and was automatically filled into a dewar using a liquid leveller, which accessed a 60 L liquid nitrogen storage tank. For the first dimension, a 20 m × 0.18 mm I.D., 0.36 µm film thickness Sapiens-X5.MS coated with 5% diphenyl 95% dimethyl polysiloxane from Teknokroma (Sant Cugat del Vallès, Spain) was used. For the second dimension, a 2 m × 0.10 mm I.D., 0.10 µm film thickness TRB-50HT from Teknokroma was used. The oven temperature was held at 75 ºC for 2 min and then programmed to rise at 7 ºC min^{-1} to 100 ºC, then at 5 ºC min^{-1} to 260 ºC, and finally at 10 ºC min^{-1} to 310 ºC, with the final temperature being held for 5 minutes. The secondary oven was kept 5 ºC above the first-dimension temperature throughout the chromatographic run. Helium was used as the carrier gas at a constant flow of 0.6 mL min^{-1}. In addition, 1 µL of each sample was injected via the autosampler in splitless injection mode. The MS system was a low resolution Pegasus 4D TOF system (LECO, St. Joseph, MI) operating in the electron impact ionization mode. The applied electron energy was 70 eV, and the transfer line and ion source were set at 250ºC and 200 ºC, respectively. Scanning was performed from 60 to 700 m/z at 100 spectra s^{-1} with unit mass resolution and a detector voltage of 1800 V. The data was pre-processed in Chroma-TOF 3.32 software using the peak find and peak and spectra deconvolution software routines for the

identification and annotation of detected peaks. A signal-to-noise ratio of 100 was used for this study.

7.2.2 Data processing and statistical analysis

Agronomic parameters

Morphological (fresh weight, leaf height and stem width) and physiological (chlorophyll A and B) parameters were measured after 34 days. For the determination of chlorophyll A and B, three circles of inner, middle and outer leaf were cut using a circular cutting press (4 cm diameter per circle, 12.6 cm2 foliar area) and individually extracted with 5 mL N,N-dimethylformamide (DMF) (Porra, 2002). Tubes were kept from the light and stored at 4 ºC for 48 h. UV absorbance at 647 and 664.5 nm was then measured using a Varian Cary 400 spectrophotometer (Agilent Technologies, CA, USA). Chlorophyll concentration was calculated based on the absorbance values and the foliar area as described in the section 7.6 (Equations. S7.1-S7.3). Mean values for the three circles of each experimental unit were used to calculate the average chlorophyll content of each treatment.

Data arrangement, compression and MCR-ALS analysis

Data sets were in .CDF format and were imported into MATLAB using MATLAB's Bioinformatics toolbox. Data from GC×GC-TOFMS analysis can be arranged in a three-way data cube or array with two retention time axes and one m/z values axis. If more than one sample is analyzed, the data will be a four-way data array with two retention time axes, one m/z values axis and one sample axis.

Due to huge the huge amount of data collected in the GC×GC-TOFMS data sets for the 20 lettuce samples, a data segmentation and compression strategy based on the use of wavelets (Walczak and Massart, 1997; Shao et al., 1999) especially in the time direction was proposed to make their chemometric analysis more feasible and reduce computer storage requirements. To this end, GC×GC-TOFMS data for the 20 samples were segmented into four parts (A-D) by visual inspection of the chromatograms (Figure S7.1). The bleeding part of the chromatogram was excluded from the data.

Since the same m/z range (e.g., 60-700 amu with 640 m/z points) was selected for all the chromatographic runs, GC×GC-TOFMS data for segments A-D for 20 lettuce samples were arranged in a column-wise super-augmented matrix with their m/z values

in the common column mode. In this column-wise matrix augmented arrangement, the same number of m/z values was observed for all modulations, whereas the number of elution times considered in each data modulation could be different. Thus, components common to different modulations can be described by elution profiles (peaks) with different shapes and retention times, even if they belong to the same compound in different modulations. This cannot generally be done with methods such as PARAFAC or PARAFAC2 (Parastar et al., 2011; Bortolato and Olivieri, 2014; Parastar and Tauler, 2014; Ahmadvand et al., 2017).

Since GC×GC-TOFMS produces a series of modulated peaks for each component, summation of the modulated peaks for each component can be used for quantitative analysis. MCR-ALS can resolve pure modulated peaks (second-dimension elution profiles) in the presence of baseline/background contribution and other chemical constituents. Relative quantitative information for one target compound can then be directly derived from the comparison of MCR-ALS-resolved second-dimension elution profiles for different samples. After resolving the GC×GC-TOFMS data for the 20 samples and obtaining the resolved elution profiles in two chromatographic columns along with their mass spectra, the lettuce metabolites were quantified and identified. These pure spectra can be used to identify the resolved components by comparing them with those of standard compounds in the National Institute of Standards and Technology (NIST) MS and Golm Metabolome databases. A reverse match factor (RMF) based on the correlation coefficient between the MCR-ALS-resolved and experimental mass spectra reported by the NIST software was used to select the best identified compound for the MCR-ALS-resolved mass spectra. This match factor is reported between 0 (no match) and 1000 (perfect match). As a general guide, a value of 900 or greater was considered to be a very good match; between 800 and 900, a good match; between 700 and 800, a fair match; and less than 600 a poor or very poor match.

PLS modelling

The relative concentrations of the identified metabolites were obtained using simple peak integration of resolved elution profiles in the second chromatographic dimension for each component. These concentrations were used to build a new matrix containing relative concentrations of all the resolved metabolites for the 20 lettuce samples (control and exposed). This new data matrix was then correlated to agronomic parameters obtained for the 20 samples including leaf height, stem width, chlorophyll A concentration, total chlorophyll concentration and soil pH by the PLS2 multivariate

regression method to find the most discriminant metabolites for each agronomic parameter. For a straightforward interpretation of the PLS2 model, variable importance in projection (*VIP*) (Chong and Jun, 2005) scores were used instead of the commonly used regression coefficients vectors. *VIP* scores give information about how the variables combine to form the quantitative relation between **X** and **Y**, thereby providing a better assessment of their relative importance in the model. Hence, these *VIP* scores are useful for understanding which **X**-variables are important (numerically large *VIP* score values), and which **X**-variables provide the same information (similar profiles of *VIP*-score values). A large *VIP*-score value in a chromatographic region indicates that the compounds eluted in that retention-time region will have a large impact on the prediction model, whilst a low value indicates that the compounds are less influential.

Heat map

To study the change in metabolites between the non-exposed lettuces (control samples) and the lettuces exposed to the four different concentrations, the ratio of the areas between the exposed and non-exposed lettuces was calculated. The logarithms of the means for each treatment were then used to plot a heat map (Table S7.4) for the different metabolites. Log ratio values above 0 indicate an up-regulation of metabolites in the exposed samples compared to the control (non-exposed) samples. Conversely, log10 ratio values below 0 indicate a down-regulation of metabolites in exposed units compared to the control ones.

$$\log\ ratio\ = \log\ \frac{\bar{A}_i}{\bar{A}_c} \qquad (7.1)$$

Where $\bar{A_i}$ is the average abundance of a selected metabolite in lettuce samples exposed to different CECs and $\bar{A_c}$ is the average abundance of a selected metabolite in the control samples.

Metabolite and pathways identification

After the metabolites were identified using the NIST database, they were characterized with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The same database was used to investigate the possible metabolic pathways (Kanehisa and Goto, 2000; 2017).

The KEGG pathway analysis tool was used with the *Arabidopsis thaliana* database was used to construct an incidence table in which any given pair of metabolites was

considered related if they appeared in at least one common pathway (Table S7.5). Identified pathways with at least two hits were included in a network analysis, using the reshape2 and igraph packages in R (R Development Core Team, 2015). Only pathways with at least two identified metabolites were included in the analysis. The general pathways ath01100 (Metabolic pathways) and ath02010 (ABC transporters) were excluded from the analysis.

Software

GC×GC-MS data were acquired using ChromaTOF version 3.3.2 (LECO, St. Joseph, MI, USA) converted to CSV format in the 60-700 m/z range and imported into MATLAB by using Bioinformatics Toolbox (The Mathworks, Inc., Natick, MA, USA). NIST MS Search version 2.2 (National Institute of Standards and Technology, USA) and the GOLM metabolome database (GMD) of derivatized compounds were used for metabolite identifications. MATLAB's Wavelet Toolbox was used for data compression. The PLS Toolbox (http://www.eigenvector.com/) and MCR-ALS Toolbox were used for chemometric analysis (Jaumot et al., 2005).

7.3 Results

Occurrence and effect of CECs on plant morphology and physiology

Figure 7.2 shows the concentration of the different CECs in the lettuce leaves of all the experimental units following an exposure period of 34 days. No CECs were detected in the non-exposed lettuce. At the lowest treatment concentration (i.e. 0.05 µg L^{-1}), only CBZ was detected (2 ng g^{-1} dw). At higher irrigation concentrations (i.e. 0.5 µg L^{-1}), BPA, CAF, 5-MeBT, MePB and TCP were also detected (1 – 24 ng g^{-1} dw). Finally, at the two highest treatment concentrations (i.e. 5 and 50 µg L^{-1}), all the CECs except OPL were detected (1 – 724 ng g^{-1} dw). CEC concentrations increased over the course of the treatments in all cases. CBZ was the CEC to exhibit the highest accumulation in leaves, followed by TCP and CAF. BHT and TCS exhibited the lowest leaf accumulation. Linear correlation (Pearson correlation coefficient > 0.90; $p < 0.05$, N = 16) between leaf concentrations and irrigation concentration of CBZ was observed (Figure S7.2).

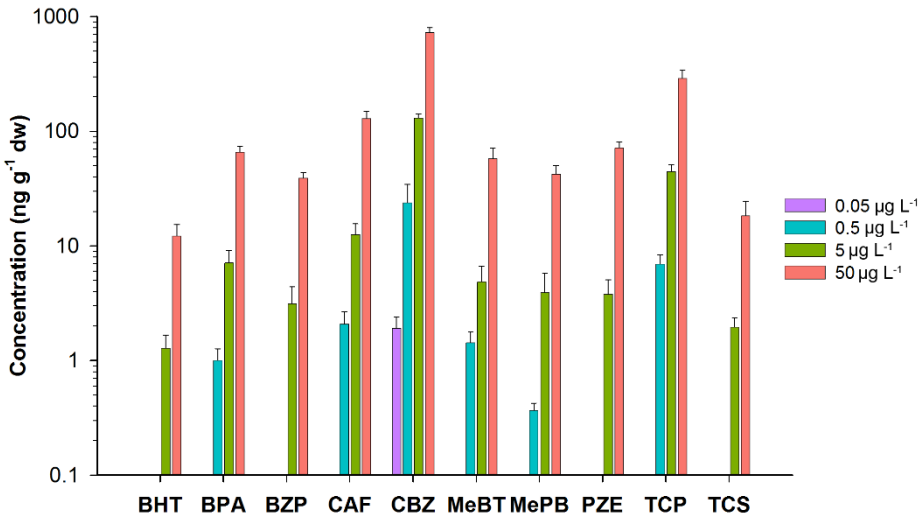


Figure 7.2: Mean concentration and standard deviation (N = 4) of the studied CECs in leaves (ng g^{-1} dw) for the four different irrigation concentrations (0.05, 0.5, 5, and 50 µg L^{-1}) in log scale.

Figure 7.3 shows the effect of the presence of these CECs on plant morphology (fresh weight, leaf height and stem width) and physiology (chlorophylls A and B and total chlorophyll). Although there were visual differences in the lettuce exposed to different CEC concentrations, no significant differences ($p > 0.05$) were observed in the fresh weight. In contrast, differences in height were apparent between the exposed and non-exposed samples, although not between samples exposed to different concentrations. Differences in stem width were significant between exposed and non-exposed units and between different treatment levels. In addition, a significant reduction (between 15 and 37%) was observed in chlorophyll A and B content compared to non-exposed samples at different treatment levels (Figure 7.3).

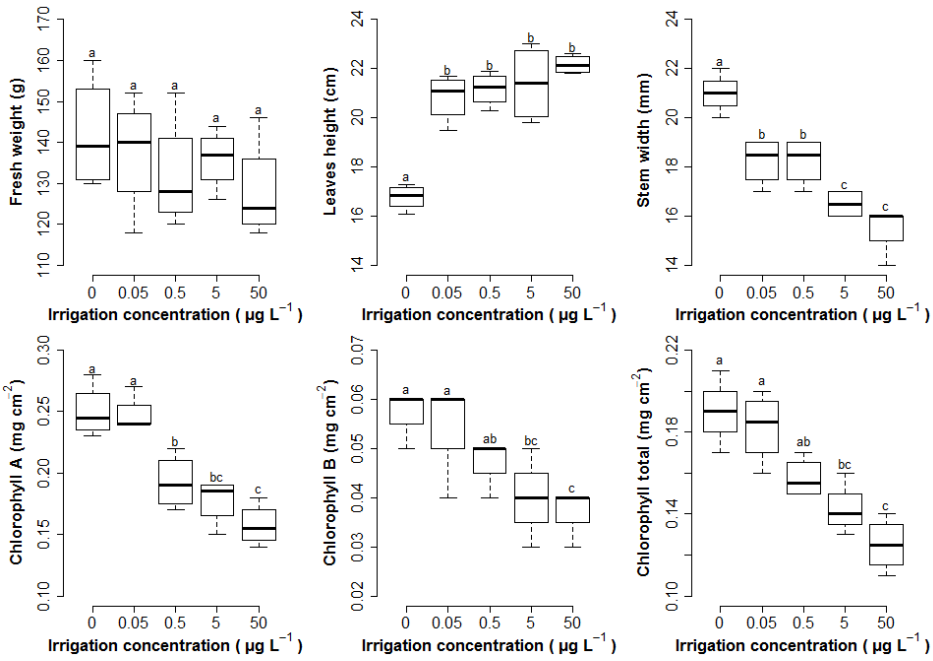


Figure 7.3: Boxplot of the studied agronomic parameters for the different irrigation concentrations (0, 0.05, 0.5, 5, and 50 µg L^{-1}). Properties with the same letters are not significant different ($p < 0.05$).

GC×GC–TOFMS combined with MCR-ALS-PLS: a powerful tool for elucidating metabolic profiling

Data pre-processing with ChromaTOF software (LECO) revealed more than 1000 peaks (S/N ratio ≥ 100) in the lettuce extracts (Figure S7.3). Several metabolites were identified, including sugars (e.g. glucose, galactose, ribose), alcohols (arabinitol and ribitol), and some organic acids (malic acid and gluconic acid). The highly overloaded peak at the 300-400 time point of the contour plot was assigned to fructose, the monosaccharide occurring at the highest concentration in lettuce (0.43% w/w) (Nutrient Data Laboratory et al., 2001).

Despite the unsurpassed GC×GC peak capacity, many plant components exhibited a strong coelution. Therefore, the application of chemometric tools was advisable to improve the resolution of the metabolite chromatographic profiles and their associated mass spectra for accurate metabolite identification. Raw GC×GC–TOFMS data from the 20 lettuce sample extracts were column-wise augmented and analyzed by MCR-ALS applying the proper constraints (see section 7.6, Figure S7.4 for further information and

previous work) (Tauler, 1995; Tauler et al., 1995; Parastar et al., 2011; Parastar et al., 2012; Parastar et al., 2013; Parastar and Tauler, 2014). With 75 MCR-ALS components selected, R^2 values (see Equation S7.5) were higher than 99.8% and LOF values (see Equation S.6) were below 3.6%. Of these 75 MCR-ALS-resolved profiles, 50 were unambiguously assigned to characteristic lettuce metabolites by library search. These 50 MCR-ALS-resolved components were further considered for identification and linked to the lettuce metabolome (Table S7.6).

Following the resolution and identification of the 50 lettuce metabolites, the peak areas of the same MCR-ALS component profiles in every sample and for all components and treatments were arranged in a data table. This data table with the 50 metabolite peak areas for the 20 lettuce samples (**X** data block) was then correlated to the 5 morphological and physiological plant parameter responses in the 20 lettuce samples (**Y** data block) using PLS2 (see section 7.2). The **X** and **Y** data blocks were auto-scaled before PLS2. Cross-validation (CV) was used to test the number of significant latent variables (LVs) needed in the PLSR correlation model in a practical and reliable way. Ultimately, 4 LVs were considered in the model. The cumulative captured variances were 85.5% for the X block and 79.8% for the Y block. Table S7.7 shows the explained variance by LV.

The *VIP* scores represent the influence of each variable on the PLS model (Equation S7.7). In other words, the *VIP* score for each variable was computed to quantify its importance using the PLS weights associated with each LV. The *VIP* score values were very useful in determining which of the metabolites detected in the lettuce extracts were most influential. Therefore, examination of the *VIP* scores is a reliable and simple technique for determining the effective metabolites in the best final PLS predictive model. Table 7.1 shows the *VIP* scores for the three morphological and physiological plant parameters of leaf height, stem width and total chlorophyll for 50 metabolites. In this study, the "greater than one rule" was generally used as a criterion for variable selection (Chong and Jun, 2005). Therefore, **X**-variables with a *VIP* greater than one were important. With the aid of *VIP* scores, it was possible to determine the most influential variables amongst a large number of variables in the **X** block. In this regard, 21 metabolites were found to significantly affect the four morphological and physiological parameters studied.

Table 7.1: *VIP* scores in the concentration of metabolites in response to CECs stress exposure in lettuce crops to the three morphological-physiological parameters measured: leaves height, stem width and total content of chlorophylls.

Metabolite	Leaves height	Stem width	Total chlorophyll
L-5-Oxoproline	2.42	3.39	3.20
L-Serine	1.40	1.84	1.75
3-Methyl-2-oxovaleric acid	1.35	1.29	1.31
Adenosine	0.30	0.34	0.33
Phosphoric acid	0.51	0.54	0.53
γ-lactoneMannonic acid	0.20	0.03	0.05
Citric acid	0.92	0.39	0.49
Fumaric acid	0.64	0.12	0.24
Galactonic acid	2.52	3.46	3.27
Gluconic acid	2.70	3.35	3.26
Glyceric acid	0.85	0.90	0.90
Malic acid	2.17	2.47	2.46
Methylmalonic acid	0.32	0.04	0.09
Quinic acid	0.24	0.29	0.25
Ribonic acid	2.03	2.94	2.76
Succinic acid	1.53	1.44	1.42
Tartaric acid	0.64	0.13	0.24
Threonic acid	0.97	1.00	0.97
2,3-Butanediol	1.89	1.65	1.75
bis-1,2-acetin ether	0.68	0.09	0.21
Allo-Inositol	1.77	1.83	1.74
β-D-Galactopyranoside-(1,2)-glycerol	0.50	0.41	0.45
Galactitol	1.99	2.26	2.19
Glycerol	0.53	0.16	0.24
Inositol isomer 1	2.23	2.74	2.59

(continuation) Table 7.1: *VIP* scores in the concentration of metabolites in response to CECs stress exposure in lettuce crops to the three morphological-physiological parameters measured: leaves height, stem width and total content of chlorophylls.

Metabolite	Leaves height	Stem width	Total chlorophyll
Inositol isomer 2	2.24	3.15	2.97
Inositol isomer 3	1.96	1.82	1.76
meso-Erythritol	1.87	1.67	1.67
Inositol isomer 4	0.58	0.50	0.46
Ribitol	0.43	0.12	0.19
(S,S,R,R,S)-methyl 6-deoxy-β-L-Galactopyranoside	0.91	1.34	1.25
2-O-Glycerol-α-d-galactopyranoside	0.87	1.04	0.99
Allose	1.19	0.99	1.05
Arabinose	0.81	0.43	0.55
Ethyl α-D-glucopyranoside	0.35	0.05	0.13
Galactose	0.46	0.60	0.57
Glucopyranose	2.11	3.09	2.90
Glucose	1.85	1.60	1.67
Lyxose	0.43	0.05	0.15
Mannose	1.55	2.19	2.06
Melibiose	0.31	0.05	0.13
Methyl-4-O-methyl-α-D-glucopyranoside	0.39	0.39	0.40
Ribofuranose	0.62	0.42	0.49
Ribose	0.42	0.33	0.36
Sorbose	1.79	2.32	2.17
Sucrose	0.46	0.09	0.15
Tagatofuranose	0.53	0.40	0.44
Tagatose	0.51	0.03	0.16
Trehalose	0.55	0.23	0.33
Xylose	0.61	0.75	0.68

Metabolic changes in response to the presence of CECs

Of the more than 1000 peaks detected by GC×GC–TOFMS (LECO's ChromaTOF software) the mass of only 50 increased differently in lettuce leaves in response to the occurrence of CECs in irrigation waters (Figure S7.3). The metabolites identified included amino acids, organic acids and sugars (Table S7.6).

The heat map showed up- and down-regulation of metabolites when exposed and non-exposed lettuces were compared (Fig. 7.4). One of the strongest effects was found for

L-5-oxoproline, whose relative abundance increased in CEC-exposed lettuces in a dose-response manner (up-regulation). Differences in other amino acids, such as L-serine and adenosine, were observed between the control and exposed lettuces; however, the effects were lower than for L-5-oxoproline. More than 10 organic acids exhibited up- and down-regulation between treatments compared to the control samples. Malic acid was the organic acid to exhibit the greatest down-regulation when treated and control samples were compared. Threonic and glyceric acids were also found to be down-regulated when exposed and non-exposed samples were compared. In contrast, organic acids such as gluconic acid or galactonic acid, were up-regulated (log ratio > 0.2). Many carbohydrates (e.g. sorbose or allose) exhibited down-regulation. Nevertheless, some sugars were up-regulated by the presence of CECs, such as mannose (up to 0.23) or glucopyranose (up to 35). In fact, there is evidence that plants respond to environmental factors through sugar-sensing mechanisms (Rosa et al., 2009). Finally, many alcohols and other compounds, such as inositol isomers and 2,3-butanediol, also exhibited significant up or down-regulation respectively.

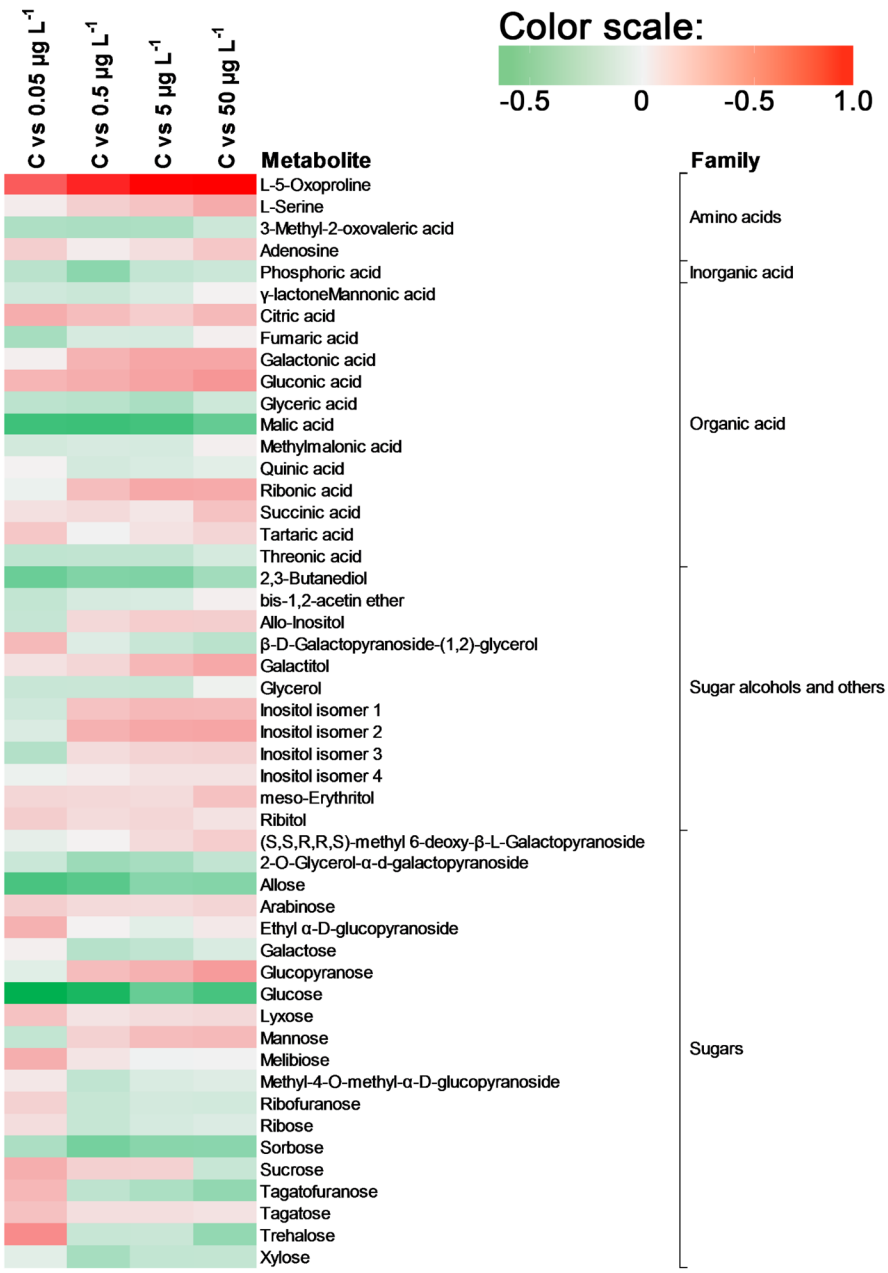


Figure 7.4: Heat map of changes in the levels of 50 metabolites in response to the CEC exposure concentration compared to unexposed lettuces (C). The log10 fold change in abundance ratios (see Equation 7.1) are shown in Table S7.4. Red indicates an up-regulation effect, whilst green indicates a down-regulation effect.

Morphological changes and metabolic pathways impaired by the presence of CECs in irrigation waters

KEGG pathway analysis of the analyzed metabolites using the *A. thaliana* pathway data set identified 29 functional modules that included at least two metabolites (Table S7.5). Pathways related to secondary metabolism (ath01110, ath01200, ath00630, ath00020) and sugar metabolism (ath0052, ath00040, ath00030, ath00520) were especially well-represented, probably indicating an effect of the treatment on these two functions (Table S7.5). These two functional groups can be observed in the bipartite plot shown in Fig. 4. The graph also shows the coordinate reduction of several saccharides (glucose, galactose, ribose, trehalose, allose, sorbose, and xylose, labelled in green in Figure 7.5) and the increase in some of their metabolites (gluconate, ribitol, galactitol, and galactonate, labelled in red in Figure 7.5), which may indicate an increase in the catabolism of non-structural carbohydrates. The graph likewise shows an increase in the inositol pathway. Arrows in the plot indicate metabolites that show a differential response at the lowest treatment concentration (see also the heat map in Figure 7.4); it is revealing that strong and mild treatments had virtually opposite effects on several sugars and on the inositol pathway.

The secondary metabolism cluster is centered on four TCA cycle metabolites (citrate, succinate, malate and fumarate), although it is unclear whether or not these changes can affect the plant's energy metabolism capacity, as none of the other related functional modules appeared to be affected. The connection of these metabolites with other pathways related to secondary metabolites (2,3-butanediol, tartrate, glycerate) may suggest an effect on the plants' secondary metabolism, possibly related to their defense mechanisms, as occurs with embryos exposed to BPA (Pelayo et al., 2012; Chen and Reese, 2013; Rochester, 2013; LaKind et al., 2014; Porreca et al., 2017).

A comparison of the metabolite clusters shown in Figure 7.5 and the calculated PLSR model and VIP scores revealed that 21 metabolites significantly impaired the four plant morphological and physiological parameters studied. These metabolites included those involved in galactose metabolism (e.g. mannose, galactitol and inositol), three of the metabolites involved in the TCA cycle (succinate, malate and fumarate), and other components of sucrose metabolism, glycerolipid metabolism, the pentose phosphate pathway and the amino acid pathways (Figure 7.6).

7.4 Discussion

The plant uptake of CECs by the lettuce crops was consistent with previous findings in which neutral compounds, such as carbamazepine, have been shown to be prone to uptake in crops (Wu et al., 2010; Goldstein et al., 2014; Hurtado et al., 2016). The relationship between the CEC concentrations in leaves and irrigation water was only fairly linear for CBZ, which could be detected at the four assessed concentrations. Therefore, bioaccumulation factors (concentration ratio in leaves in dry weight vs irrigation concentration) were CEC dependent. CBZ and TCP exhibited the highest values (14 and 5.7 L kg^{-1} respectively), whilst BHT and TCS exhibited the lowest values (0.24 and 0.36 L kg^{-1} respectively). These values were similar to the values reported in the literature for different experimental setups (Wu et al., 2010; Calderón-Preciado et al., 2013; Wu et al., 2013; Goldstein et al., 2014; Malchi et al., 2014). Ionic and hydrophobic (BPA, TCS) CECs exhibited lower translocation than non-ionic and polar compounds (CBZ and TCP), in keeping with previously reported findings (Dettenmaier et al., 2009; Trapp, 2009).

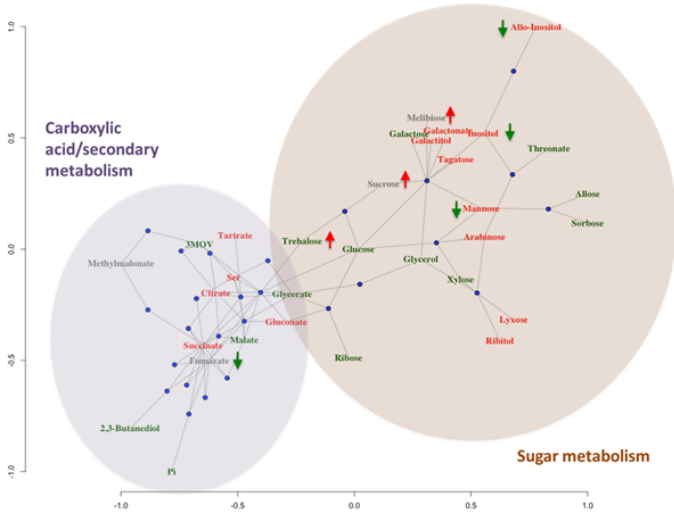


Figure 7.5: KEGG pathway analysis of the analyzed metabolites, using *A. thaliana* pathway dataset (Table S7.5). Two functional groups can be observed at the bipartite plot (carboxyclic acid / secondary metabolism and sugar metabolism).

Metabolomic studies require powerful analytical tools. In the present study, the use of GC×GC–TOFMS proved to have a high separation capacity for complex samples, resulting in the detection of over 1,000 peaks with a S/N ration > 100. The main limitations are the thermal stability of analytes during GC analysis and the large size of the database when TOFMS raw data are processed. The present study demonstrated that chemometric tools such as MCR-ALS followed by PLS could be used to compare the metabolic profiles of different samples.

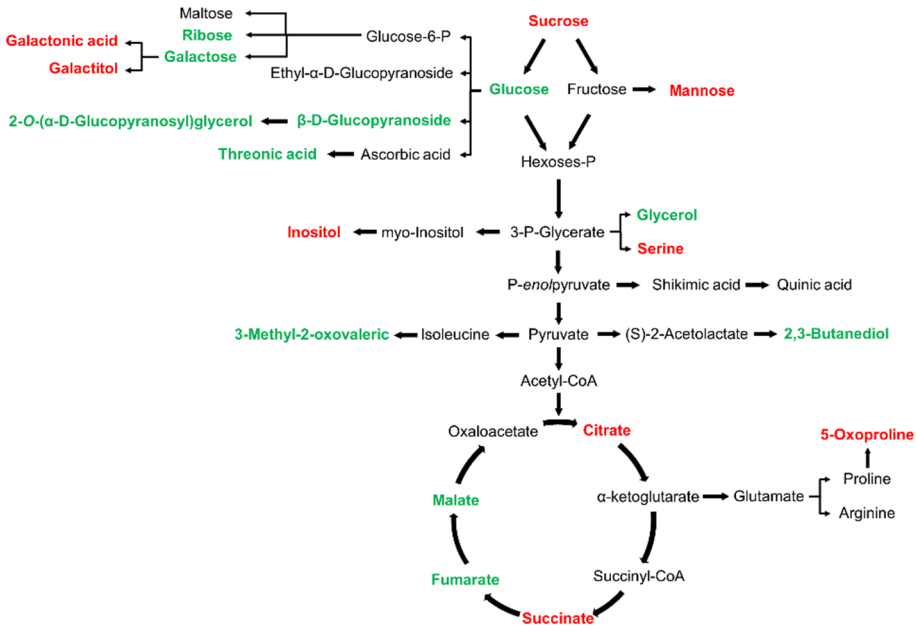


Figure 7.6: Metabolic changes involved in the primary pathways of lettuce exposed to CECs. The significant up and down regulated metabolites are indicated in red and green respectively.

With regard to the effect of CECs on plant physiology and morphology, recent findings have proved that the presence of CECs produces changes in plant hormone concentrations (e.g. auxins, cytokinins, abscisic acid and jasmonates), the nutrient composition of crop leaves (Carter et al., 2015), biomass production, chlorophyll content and plants' shoot growth (Liu et al., 2009; Zhang et al., 2012; Pan et al., 2013; Qiu et al., 2013). However, these studies were carried out exposing plants at several orders of magnitude (i.e. mg L^{-1} levels) higher than the relevant environmental concentrations. The results of the present study expand on those findings, confirming that crop exposure to CECs affected morphological and physiological parameters, such

as leaf height, steam width and chlorophyll A and B content of lettuce at the relatively low concentration of 0.05 μg L^{-1}. This is the first time that plant exposure to relevant environmental concentrations of CECs has been shown to affect plant morphology and physiology.

Primary metabolism plays an essential role in plant metabolism and is vital for plants' survival and development (Wang et al., 2016b). The present study explored the metabolic response of lettuce crops exposed to different CEC concentrations (Figure 7.4). Carbon (C) and nitrogen (N) metabolism are closely coordinated in the fundamental processes permitting plant growth, e.g. photosynthesis and N uptake (Kusano et al., 2011). Figure 7.4 shows that CEC concentrations play an important role in plants' metabolic responses. The response is therefore normally greater (either up- or down-regulated) at higher concentrations (e.g. 5-oxoproline and gluconic acid). However, for most of the metabolites (e.g. mannose, glucose, malic acid, and allose) the dose-response relationship was non-lineal (Figure 7.4). This is in keeping with the fact that at low doses organic pollutants such as herbicides (e.g. glyphosate and simazine) stimulate plant growth or protein content, whereas at higher doses they produce phytotoxicity; indeed, such hormetic responses are not unusual in (eco)toxicology (Duke et al., 2006).

The metabolites can be classified as upregulated (5-oxoproline, serine, adenosine, citric acid, galactonic acid, gluconic acid, ribonic acid, succinic acid, glucopyranose, mannose, inositols, galactitol) and downregulated (allose, glucose, ribose, tagatofuranose, trehalose, xylose, malic acid, and butanediol). The amino acid 5-oxoproline showed the highest up-regulation response to CEC exposure (Figure 7.4). This is consistent with the fact that this metabolite is an intermediate of the metabolism of glutathione, one of the main plant defense substances (Ohkama-Ohtsu et al., 2008). It is involved in many detoxifying mechanisms, such as the reduction of active oxygen species, and also regulates cell defense systems, including the detoxification of toxic xenobiotics, such as herbicides (Katerova and Miteva, 2010). Other metabolites up-regulated by the presence of CECs include amino acids (serine and adenosine), organic acids (citric acid, galactonic acid, gluconic acid, ribonic acid and succinic acid), sugars (glucopyranose and mannose), sugar alcohols and others (inositols and galactitol). The amino acid serine is also involved in the glutathione response through the stabilization of the glutathione thiolate anion (Cummins et al., 2011). Citric acid is associated with oxidative stress tolerance to heavy metals (Hassan et al., 2016). Similarly, a concentration increase in gluconic acid has been reported in plants exposed to different metal elements (i.e. Cd and Pb) (Kavita

et al., 2008; Wang et al., 2015).Wang et al. (2015) reported that under the stimulus of Cd stress, carbon flow in radish roots mainly concentrated in gluconic acid, suggesting that it was being used to alleviate the Cd toxicity. Moreover, myo-inositol has been observed to be very strongly stimulated by glyphosate at doses above 10 or 40 μM, depending on the plant (Piotrowicz-Cieślak et al., 2010). Recent studies in *Arabidopsis* suggest that nuclear pools of myo-inositol may play a specific role in programmed cell death. In fact, the regulation of myo-inositol levels is critical to maintain levels of ascorbic acid (Meng et al., 2009), phosphatidylinositol, and ceramides, which regulate growth, development and cell death (Donahue et al., 2010). In contrast, sugars (e.g. allose, glucose, ribose, tagatofuranose, trehalose and xylose), malic acid and butanediol were the metabolites to show the highest down-regulation response to CEC exposure. The sharp decline in glucose after plant exposure to CECs indicates a shift from C accumulation to C assimilation (Rosa et al., 2009). Non-structural sugars play many roles in plant physiology, not only as energy reserves, but also as protection against osmotic pressure and other forms of stress (Martínez-Vilalta et al., 2016). Glucose concentration has been observed to decline in plants exposed to pesticides including glufosinate, sulcotrione, AE 944 [N2-(1-ethyl-3-phenylpropyl)-6-(1-fluoro-1-methylethyl)-1,3,5-triazine-2,4-diamine], foramsulfuron, benfuresate and glyphosate (Trenkamp et al., 2009). Additionally, the lower amounts of central C metabolism intermediates (glycolysis and TCA cycle) observed in lettuce crops exposed to CECs is likely associated with increases in energy consumption (Bowne et al., 2012; Zhao et al., 2015) (Figure 7.6). These results are also consistent with a previous study showing down-regulation of malic acid during Pb stress in radish (Wang et al., 2015). Finally, butanediol has been described as a signalling molecule involved in plant/bacterium interactions and, notably, able to induce plant systemic resistance (Effantin et al., 2011). The decrease in this metabolite under the occurrence of CECs may suggest a reduction of plant resistance to bacterial infection.

Changes in the metabolic profile could be linked with morphological parameters, such as plant elongation or chlorophyll content. In this study, we have observed that the increase in the level of 5-oxoproline and sugar metabolites (i.e. gluconic acid, glucopyranose, inositol isoforms, ribonic acid, galactitol and galactonic acid) was associated with an increase in leaf height and a decrease in stem width and total chlorophyll content (Table 7.1, *VIP* scores > 2). Therefore, the occurrence of CECs may be associated with an increase in 5-oxoproline from the TCA cycle and sugar metabolism, which is related with plant cell elongation. For example, Zhao et al. (2015) observed that an increase in 5-oxoproline, amongst other metabolites, was associated

with cell elongation. Proline biosynthesis deficiency leads to abnormal plants and cell wall defects, suggesting that it plays a role in structural proteins, some of which modulate stem elongation and shoot growth (Kavi Kishor et al., 2015). In contrast, a decrease in malic acid level was associated with an increase in leaf height, but a decrease in stem width and chlorophyll content. This is in keeping with the fact that foliar application of malic acid significantly increases chlorophyll content in *Lilium* sp. plants (Darandeh and Hadavi, 2011) and that malic acid concentration has been observed to increase during rapid cell elongation of cotton plants (Naoumkina et al., 2015). The malate valve uses malic acid to transport nicotinamide adenine dinucleotide phosphate (NADPH) generated by photosynthesis between cell compartments (chloroplast, cytosol and mitochondria) (Hebbelmann et al., 2011; Heyno et al., 2014). Therefore, the decrease in chlorophyll content observed in the present study suggests an association with a reduction in photosynthesis and, consequently, a reduction in the malic acid needed to transport NADPH. Whilst malic acid could not be utilised, the oxidative pentose phosphate pathway (PPP) may serve as a major non-photosynthetic source of NADPH production, which is needed for the reduction of oxidized glutathione (Crecelius et al., 2003; Prosser and Sibley, 2015) due to CEC exposure. Accordingly, the observed increase in gluconic acid (Figure 7.4), an intermediate metabolite of PPP, could be the response to the CEC occurrence.

7.5 Conclusion

Morphological and metabolic analysis of lettuce crops irrigated with CECs demonstrated that the presence of these compounds in irrigation waters disrupted carbohydrate metabolism, including of glucose, inositol, sorbose, mannose and allose, the TCA cycle (e.g. malic acid, fumarate and citric acid), the pentose phosphate pathway (gluconic acid), and the glutathione pathway (5-oxoproline and serine) at all levels (Figure 7.5). Proline metabolism is apparently the most disrupted pathway, which may be explained by an enhancement of the glutathione detoxification pathway. These metabolic changes were associated with morphological (leaf height, stem width) and physiological (chlorophyll content) changes. According to the therapeutic doses, the concentration at which CECs have been found in lettuce crops do not pose a human health risk (Prosser and Sibley, 2015). Nevertheless, the present results show that vegetable quality is affected by, amongst other things: secondary metabolites (sugars, amino acids and carboxylic acids) and morphological-physiological parameters (leaf height, stem width and total chlorophyll content). The profile alteration of these metabolites may result in CEC-exposure-induced changes in flavour and nutritional

supply. Further studies at genomic, transcriptomic and proteomic levels need to be performed to understand the mechanisms involved in the plant response to CECs.

7.6 Supplementary information

The CECs used for this experiment can be seen in section 6.5.1. D-glucose (U-13C6, 99%) was supplied by Cambridge Isotope Laboratories, Inc. (Andover, MA, USA), salicylic acid-d6 (98%D), triphenylamine (TPhA) and trimethylsulfonium hydroxide (TMSH) were purchased from Sigma Aldrich. Pyridine (anhydrous, 99.8%), chlorotrimethylsilane (TMCS), methoxyamine hydrochloride (98%) (MeOX) and N-methyl-N-trimethylsilyl trifluoroacetamide (>98.5%) (MSTFA), used as derivatizating agents, were also obtained from Sigma-Aldrich. Hexane, methanol and chloroform were analytical reagent grade, and sodium chloride (NaCl) salt was supplied by Merck (Darmstadt, Germany). N,N-dimehtylformamide (DMF) was purchased to Sigma-Aldrich.

The extraction of CECs from the lettuce leaves is explained in section 3.7.2. Qualitative and quantitative analysis was performed based on retention time and selected reaction monitoring (SRM) mode of two product ions, and the ratio between the product ions (Table S7.1). The limit of detection (LOD) and the limit of quantitation (LOQ) for plant tissue were defined as the mean background noise in a blank triplicate plus three and ten times, respectively, the standard deviation of the background noise from three blanks. LODs and LOQs were compound dependent and ranged from 0.3 to 4.5 ng g^{-1} dry weight (Table S7.2). The recoveries of the surrogates added can be seen in Table S7.3.

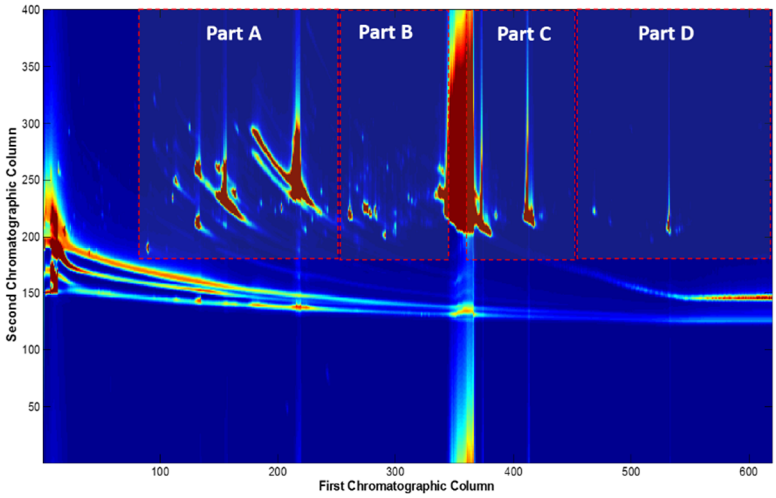


Figure S7.1 Total ion chromatogram (TIC) of GC×GC-MS data of one of the control samples. Four different chromatographic segments are shown in this figure.

Chlorophyll determination

Chlorophyll concentration was calculated with the following equations:

$$\text{Chlorophyll A} = (12.70*Abs^{664.5}) - (2.79*Abs^{647}) \quad \text{(S7.1)}$$

$$\text{Chlorophyll B} = (20.70*Abs^{647}) - (4.62*Abs^{664.5}) \quad \text{(S7.2)}$$

$$\text{Total chlorophylls} = (17.90*Abs^{647}) - (8.08*Abs^{664.5}) \quad \text{(S7.3)}$$

where Abs is the absorbance at the specified wavelength (647 and 664.5 nm), and dividing it for the foliar surface.

Finally, soil pH was measured at a soil-water ratio of 1:5 $CaCl_2$ 0.01 M with a Crison GLP 22 pH meter equipped with a gel filled pH electrode IntelliCAL™ PHC101 (Hach Lange, CO, USA).

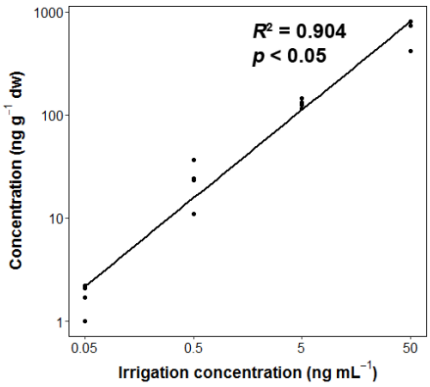


Figure S7.2 Linear correlation between the leaf concentration (ng g^{-1} dw) and the irrigation concentration (ng mL^{-1}) of CBZ.

Data arrangement, compression and MCR-ALS analysis

Due to huge size of data sets collected in GC×GC-MS data sets of 20 lettuce samples, a data segmentation strategy was used. In this regard, GC×GC-TOFMS data for 20 samples segmented into four parts (A-D) by visual inspection of the chromatograms. Figure S7.1 shows these chromatographic segments as an example on GC×GC-TIC of one of the control sample.

Wavelet decomposition and compression is applied on every column (m/z) of X_{aug} independently. Compression reduces the size of data 2^{nd} times, which, n is the compression level (Walczak and Massart, 1997; Shao et al., 1999). The compressed matrix (X_{compr}) contains the same information as X_{aug}, but needs much lower computer storage. For the datasets under study in this paper, level-3 wavelet compression was sufficient without loss of relevant information in the elution time direction, and spectral domain remained unchanged.

Before starting MCR-ALS analysis, some prior knowledge is required. One of the main difficulties in MCR analysis is determination of the number of chemical components exist in data matrix. In this work, the size of singular values and changes in lack of fit (LOF) of MCR-ALS solutions by adding or removing components into the model were used as criteria to estimate the number of significant components.

To start ALS optimization, simple-to-use interactive self-modeling mixture analysis (SIMPLISMA) (Windig and Guilment, 1991) was used to estimate the initial values of mass spectral profiles. In addition, proper constraints involving non-negativity

(concentration and spectral modes), unimodality (concentration mode), spectra normalization (to unit length) and component correspondence (if applicable) were applied during ALS optimization to obtain reliable results with minimum rotational ambiguity (Tauler, 2001).

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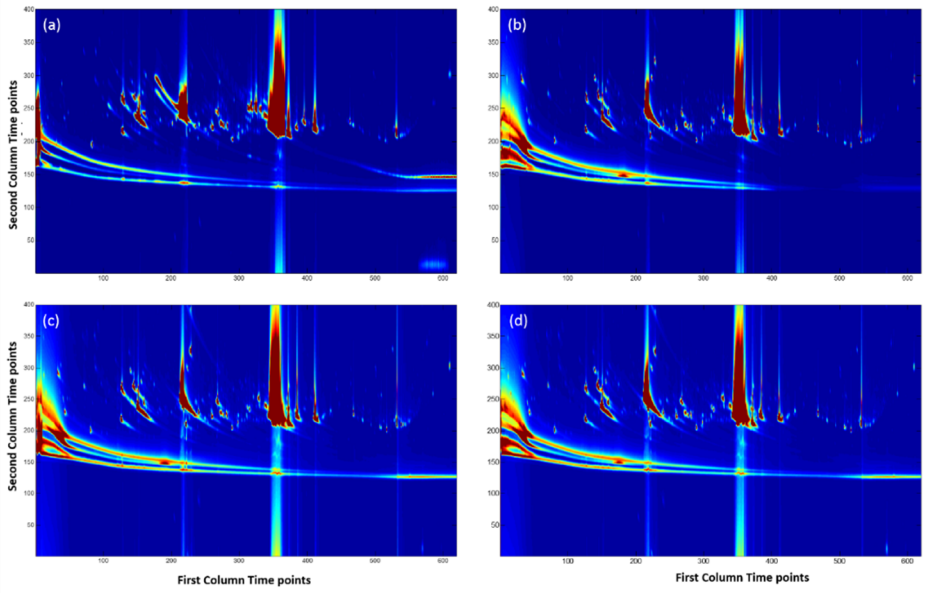

Figure S7.3 Contour plots of lettuce extracts analyzed by GC×GC–TOFMS. Lettuces exposed at (a) 0 µg L^{-1}, (b) 0.05 µg L^{-1}, (c) 0.5 µg L^{-1}, and (d) 50 µg L^{-1} of 11 CECs.

The four column-wise super-augmented data matrix for four segments (A-D) in 20 samples were then analyzed using MCR-ALS. The MCR bilinear model for a data matrix such as the one taken from a modulation of the first column in GC×GC-TOFMS is based on the description of the variation of the measurements as a linear mixture of the contributions of their pure components (Parastar and Tauler, 2014). The MCR bilinear model can be straightforwardly extended to higher order data, i.e., to the GC×GC-TOFMS data sets obtained in the analysis of different samples and arranged in a super-augmented data matrix. In linear algebra notation, the general MCR bilinear model applied to a super-augmented GC×GC-TOFMS data set obtained in the simultaneous analysis of multiple samples is as follow:

$$X_{aug} = C_{aug} S^T + E_{aug} \quad \text{(S7.4)}$$

where $X_{aug}(KLI,J)$ is column-wise super-augmented GC×GC-TOFMS data matrix with *K* data modulations taken from the first column with *I* rows (second column elution time points) and *J* columns (m/z values) for L samples. The C_{aug} (KLI×N) is the super-augmented matrix containing resolved second dimension elution profiles for the different modulations. Note that the profiles of the same component in the different

modulations may be different, both in shape and in peak position. The $S^T(N \times J)$ is the matrix of common (invariant) pure mass spectra, and E_{aug} (KLI×J) is the residual matrix with the data variance unexplained by the bilinear model $C_{aug}S^T$. In addition, N is the number of chemical components considered in the factor matrices. MCR-ALS solves Eq. S7.4 for C and S^T, using an iterative algorithm based on two constrained linear least-squares steps (Tauler, 1995; Tauler et al., 1995). The values of coefficient of determination (R^2) and LOF were used for evaluation of MCR-ALS model and they can be defined as follows:

$$R^2(\%) = \sqrt{\frac{\sum_{i=1}^{I}\sum_{j=1}^{J}\sum_{k=1}^{K}(\hat{x}_{aug,i,j,k})^2}{\sum_{i=1}^{I}\sum_{j=1}^{J}\sum_{k=1}^{K}x_{aug,i,j,k}^2}} \times 100 \qquad \text{(S7.5)}$$

$$LOF(\%) = \sqrt{\frac{\sum_{i=1}^{I}\sum_{j=1}^{J}\sum_{k=1}^{K}(x_{aug,i,j,k} - \hat{x}_{aug,i,j,k})^2}{\sum_{i=1}^{I}\sum_{j=1}^{J}\sum_{k=1}^{K}x_{aug,i,j,k}^2}} \times 100 \qquad \text{(S7.6)}$$

where $x_{aug,i,j,k}$ is the element of the original data matrix and $\hat{x}_{aug,i,j,k}$ is the recovered value using MCR-ALS method.

Metabolite detection and NIST identification

MCR-ALS resolved profiles (S^T) were assigned to metabolites and identified by comparing the mass fragmentation patterns associated to the MCR-ALS resolved mass spectra profiles using the standard mass spectral database of the National Institute of Standards and Technology (NIST) and GOLM. For each mass spectrum, 100 hits were retrieved by the NIST Mass Spectral Search 2.2 software distributed with the NIST 2014 library. A reverse match factor (RMF) based on the correlation coefficient between the MCR-AS resolved and experimental mass spectra reported by NIST software was used for selection of the best identified compound for MCR-ALS resolved mass spectra. This match factor is reported between 0 (no match) and 1000 (perfect match). As a general guide, a value of 900 or greater was considered to be a very good matching; between 800 and 900, a good match; between 700 and 800, a fair match; and less than 600 a poor or very poor match.

PLS modeling

Root mean squares error in leave-one-out cross-validation (RMSECV-LOO) has been used for choosing the significant number of latent variables (LV) in PLS model (Wold et al., 2001). Root mean squares error in prediction (RMSEP) and relative error in prediction (REP) were the quantitative measures of prediction validity. The knowledge of the presence of some individual samples and/or variables which are mainly influential for a given model is very important. For a straightforward interpretation of the PLS2 model, *VIP* (Chong and Jun, 2005) was used instead of commonly used weights and regression coefficients vectors. This method is based on the obtained PLS2 loading weights for variables. The *VIP* scores for each variable (j) is equal to its accumulated weights from all the selected LVs. This value is calculated as:

$$VIP_j = \sqrt{\frac{J\sum_{f=1}^{F} w_{jf}^2 SS_f}{SS_{y,F}}} \qquad \text{(S7.7)}$$

where SS_f and SS_y are the sum of squares of the explained variance for the f^{th} LV and total sum of squares of response matrix, respectively. Also, w_{jf} is the weight of the variable (j) on the f^{th} LV and J and F are total number of the variables and LVs, respectively. Since the average of squared *VIP* scores equals 1, "greater than one rule" is generally used as a criterion for variable selection. Therefore, X-variables that have a *VIP* larger than one are important. With the aid of *VIP* scores, it is possible to determine the most influential variables among a huge number of variables in X-block.

The *VIP* gives information about how the variables combine to form the quantitative relation between X and Y, thus providing an interpretation of the scores. Hence, these *VIP* scores are essential for the understanding of which X-variables are important (numerically large *VIP* values), and which X-variables that provide the same information (similar profiles of *VIP* values). A large *VIP* value in a chromatographic region indicates that the compounds eluted in that retention time region have a large impact on the prediction model, while, on the contrary, a low value indicates less influential components.

Example the MCR-ALS analysis (control and exposed samples)

Figures S7.4a and S7.4b show the resolved elution profiles in first and second chromatographic dimensions, respectively. Also, Figure S7.4c depicts the resolved mass

spectra for 20 components. In Figure S7.4d, second column elution profiles for one of the resolved metabolite in 20 samples are demonstrated. As it can be seen, this metabolite has a very low concentration in control sample. However, its concentration increases by increasing the concentration of contaminant exposed to the lettuce samples. Using the MCR-ALS resolved mass spectrum for this metabolite and by comparing with NIST MS database and GOLM Metabolome database this metabolite was identified as L-5-Oxoproline (2TMS) (RMF = 949). Other metabolites were also identified by comparing their MCR-ALS resolved mass spectra with NIST and GOLM databases

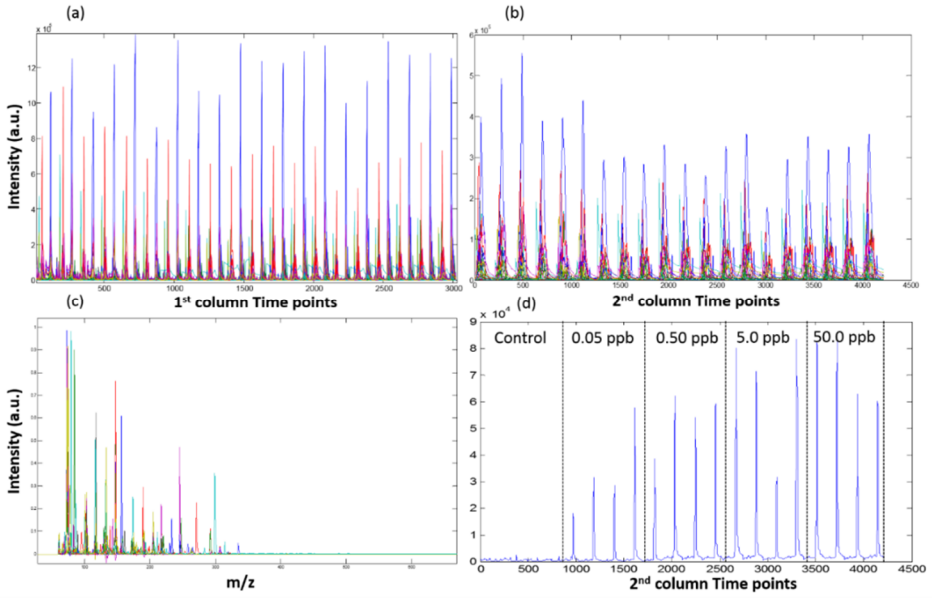

Figure S7.4 MCR-ALS analysis of segment 1 of 4 segments in 20 samples (control and exposed samples). The number of components was 20 in this case which confirmed using singular value decomposition (SVD). The value of lack of fit (LOF) and R^2 for the developed model were respectively 4.6% and 99.8%, which were acceptable according to the noise level of data.

Table S7.1. Monitoring ions in GC-MS/MS

Segment	Compound	RT (min)	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
1	**MePB**	9.06	166*	135	13
			135	77	18
1	**BHA**	9.41	194*	179	14
			179	149	14
2	**EPB**	9.83	180*	152	11
			135	77	18
3	**BHT**	10.40	220*	205	17
			205	177	12
3	**MeBT**	10.36	147*	118	14
			118	77	17
4	**OPL**	10.91	149*	121	15
			135	77	18
5	**BZP**	12.04	182*	105	17
			105	77	14
5	**XTTri**	12.23	161*	132	16
			132	91	18
6	**TCP**	12.65	249*	125	15
			249	99	30
7	**CAF**	14.65	194*	109	14
			194	55	20
7	**CAF-$^{13}C_3$**	14.61	197*	110	12
			110	82	17
7	**PZE**	15.21	188*	159	11
			188	96	15
8	**CBZ**	16.44	193*	191	23
			193	167	18
8	**CBZ-$^{13}C_6$**	16.40	199*	173	25
			199	197	20
8	**TPhA**	16.78	245*	167	30
			245	141	21
9	**BPA**	17.11	241*	133	15
			241	211	17
9	**BPA-d6**	17.09	270*	252	14
			252	139	20
10	**TCS**	17.86	302*	252	19
			302	189	37

*Transition used for quantification

Table S7.2. Limits of detection (LOD) and quantification (LOQ) of the selected CECs in the lettuce leaves.

Compound	LOD (ng g^{-1} dw)	LOQ (ng g^{-1} dw)
BHT	0.9	1.1
BPA	0.7	0.9
BZP	1.8	2.3
CAF	1.7	1.9
CBZ	1.0	1.5
MeBT	1.1	1.3
MePB	0.3	0.4
OPL	2.9	4.5
PZE	2.4	3.1
TCP	0.7	1.0
TCS	1.4	1.7

Table S7.3. Average recoveries of the surrogates added in each compartment and the SD of all the samples (N = 20).

Compound	Recovery (%)
BHA	67 ± 4
BPA-$^{13}C_6$	78 ± 5
CAF-$^{13}C_3$	52 ± 3
CBZ-$^{13}C_6$	69 ± 7
EPB	73 ± 5
XTTri	65 ± 4

Table S7.4. Log fold ratios of control in front of the four CECs exposure concentrations 0.05, 0.5, 5 and 50 µg L^{-1}.

Metabolite	Control vs 0.05 µg L^{-1}	Control vs 0.5 µg L^{-1}	Control vs 5 µg L^{-1}	Control vs 50 µg L^{-1}
L-5-Oxoproline	0.61	0.82	0.96	0.97
L-Serine	0.03	0.14	0.19	0.29
3-Methyl-2-oxovaleric acid	-0.19	-0.20	-0.19	-0.11
Adenosine	0.14	0.03	0.08	0.17
Phosphoric acid	-0.16	-0.29	-0.13	-0.11
γ-lactoneMannonic acid	-0.10	-0.11	-0.07	0.00
Citric acid	0.28	0.21	0.15	0.23
Fumaric acid	-0.21	-0.08	-0.08	0.02
Galactonic acid	0.02	0.25	0.31	0.31
Gluconic acid	0.25	0.28	0.32	0.37
Glyceric acid	-0.15	-0.16	-0.20	-0.10
Malic acid	-0.50	-0.51	-0.49	-0.40
Methylmalonic acid	-0.09	-0.07	-0.08	0.02
Quinic acid	0.01	-0.08	-0.07	-0.04
Ribonic acid	-0.02	0.22	0.30	0.29
Succinic acid	0.07	0.10	0.05	0.19
Tartaric acid	0.17	0.00	0.07	0.12
Threonic acid	-0.14	-0.14	-0.14	-0.08
2,3-Butanediol	-0.38	-0.32	-0.32	-0.22
bis-1,2-acetin ether	-0.13	-0.08	-0.07	0.02
Allo-Inositol	-0.12	0.11	0.15	0.15
beta-D-Galactopyranoside-(1,2)-glycerol	0.23	-0.06	-0.12	-0.16
Galactitol	0.07	0.11	0.24	0.30
Glycerol	-0.12	-0.11	-0.12	-0.01
Inositol isomer 1	-0.10	0.19	0.24	0.23
Inositol isomer 2	-0.07	0.26	0.30	0.31
Inositol isomer 3	-0.18	0.09	0.13	0.13
meso-Erythritol	0.11	0.11	0.10	0.20
Inositol isomer 4	-0.02	0.03	0.06	0.07
Ribitol	0.15	0.09	0.11	0.06
(S,S,R,R,S)-methyl 6-deoxy-β-L-Galactopyranoside	-0.03	0.01	0.10	0.15
2-O-Glycerol-α-d-galactopyranoside	-0.11	-0.24	-0.21	-0.13
Allose	-0.47	-0.43	-0.30	-0.31

(continuation) Table S7.4. Log fold ratios of control in front of the four CECs exposure concentrations 0.05, 0.5, 5 and 50 µg L^{-1}.

Metabolite	Control vs 0.05 µg L^{-1}	Control vs 0.5 µg L^{-1}	Control vs 5 µg L^{-1}	Control vs 50 µg L^{-1}
Arabinose	0.15	0.10	0.09	0.12
Ethyl α-D-glucopyranoside	0.26	0.01	-0.04	0.04
Galactose	0.02	-0.17	-0.14	-0.07
Glucopyranose	-0.05	0.22	0.26	0.35
Glucose	-0.68	-0.61	-0.39	-0.48
Lyxose	0.19	0.06	0.09	0.10
Mannose	-0.13	0.13	0.22	0.23
Melibiose	0.27	0.06	-0.01	0.00
Methyl-4-O-methyl-α-D-glucopyranoside	0.05	-0.14	-0.07	-0.05
Ribofuranose	0.13	-0.12	-0.09	-0.09
Ribose	0.09	-0.12	-0.08	-0.06
Sorbose	-0.20	-0.35	-0.29	-0.29
Sucrose; alpha-D-Glc-(1,2)-beta-D-Fru]	0.28	0.14	0.13	-0.12
Tagatofuranose	0.24	-0.15	-0.20	-0.27
Tagatose	0.20	0.08	0.08	0.07
Trehalose	0.42	-0.12	-0.11	-0.27
Xylose	-0.04	-0.21	-0.13	-0.13

Table S7.5. Pathway Search Results from KEGG (1).

ath0110 Metabolic pathways - (22)	
C00009	Orthophosphate
C00031	D-Glucose
C00042	Succinate
C00065	L-Serine
C00089	Sucrose
C00116	Glycerol
C00122	Fumarate
C00124	D-Galactose
C00137	myo-Inositol
C00149	(S)-Malate
C00158	Citrate
C00159	D-Mannose
C00181	D-Xylose
C00212	Adenosine
C00257	D-Gluconic acid
C00258	D-Glycerate
C00259	L-Arabinose
C00474	Ribitol
C00671	3-Methyl-2-oxopentanoic acid
C00880	D-Galactonate
C01083	Trehalose
C01697	Galactitol
ath02010 ABC transporters - (14)	
C00009	Orthophosphate
C00031	D-Glucose
C00065	L-Serine
C00089	Sucrose
C00116	Glycerol
C00121	D-Ribose
C00137	myo-Inositol
C00159	D-Mannose
C00181	D-Xylose
C00259	L-Arabinose
C00503	Erythritol
C01083	Trehalose
C01487	D-Allose
C05402	Melibiose
ath01110 Biosynthesis of secondary metabolites - (10)	
C00031	D-Glucose
C00042	Succinate
C00065	L-Serine
C00122	Fumarate
C00149	(S)-Malate
C00158	Citrate
C00257	D-Gluconic acid
C00258	D-Glycerate
C00671	3-Methyl-2-oxopentanoic acid
C01083	Trehalose

ath00030 Pentose phosphate pathway - (4)	
C00031	D-Glucose
C00121	D-Ribose
C00257	D-Gluconic acid
C00258	D-Glycerate
ath00520 Amino sugar and nucleotide sugar metabolism - (4)	
C00031	D-Glucose
C00159	D-Mannose
C00181	D-Xylose
C00259	L-Arabinose
ath00020 Citrate cycle (TCA cycle) - (4)	
C00042	Succinate
C00122	Fumarate
C00149	(S)-Malate
C00158	Citrate
ath01230 Biosynthesis of amino acids - (3)	
C00065	L-Serine
C00158	Citrate
C00671	3-Methyl-2-oxopentanoic acid
ath00620 Pyruvate metabolism - (3)	
C00042	Succinate
C00122	Fumarate
C00149	(S)-Malate
ath00500 Starch and sucrose metabolism - (3)	
C00031	D-Glucose
C00089	Sucrose
C01083	Trehalose
ath00250 Alanine, aspartate and glutamate metabolism - (3)	
C00042	Succinate
C00122	Fumarate
C00158	Citrate
ath00053 Ascorbate and aldarate metabolism - (3)	
C00137	myo-Inositol
C00259	L-Arabinose
C01620	Threonate
ath00190 Oxidative phosphorylation - (3)	
C00009	Orthophosphate
C00042	Succinate
C00122	Fumarate
ath00650 Butanoate metabolism - (3)	
C00042	Succinate
C00122	Fumarate
C03044	(R,R)-Butane-2,3-diol
ath00051 Fructose and mannose metabolism - (3)	
C00159	D-Mannose
C00247	L-Sorbose
C01487	D-Allose
ath00350 Tyrosine metabolism - (2)	
C00042	Succinate
C00122	Fumarate

(continuation) Table S7.5. Pathway Search Results from KEGG (1).

ath00052 Galactose metabolism - (10)	
C00031	D-Glucose
C00089	Sucrose
C00116	Glycerol
C00124	D-Galactose
C00137	myo-Inositol
C00159	D-Mannose
C00795	D-Tagatose
C00880	D-Galactonate
C01697	Galactitol
C05402	Melibiose
ath01200 Carbon metabolism - (7)	
C00042	Succinate
C00065	L-Serine
C00122	Fumarate
C00149	(S)-Malate
C00158	Citrate
C00257	D-Gluconic acid
C00258	D-Glycerate
ath00630 Glyoxylate and dicarboxylate metabolism (6)	
C00042	Succinate
C00065	L-Serine
C00149	(S)-Malate
C00158	Citrate
C00258	D-Glycerate
C00552	meso-Tartaric acid
ath00561 Glycerolipid metabolism - (2)	
C00116	Glycerol
C00258	D-Glycerate

ath00040 Pentose and glucuronate interconversions - (5)	
C00116	Glycerol
C00181	D-Xylose
C00259	L-Arabinose
C00474	Ribitol
C00476	D-Lyxose
ath00562 Inositol phosphate metabolism - (2)	
C00137	myo-Inositol
C19891	1D-chiro-Inositol
ath00260 Glycine, serine and threonine metabolism - (2)	
C00065	L-Serine
C00258	D-Glycerate
ath00360 Phenylalanine metabolism - (2)	
C00042	Succinate
C00122	Fumarate
ath00760 Nicotinate and nicotinamide metabolism - (2)	
C00042	Succinate
C00122	Fumarate
ath00280 Valine, leucine and isoleucine degradation - (2)	
C00671	3-Methyl-2-oxopentanoic acid
C02170	Methylmalonate
ath01210 2-Oxocarboxylic acid metabolism - (2)	
C00158	Citrate
C00671	3-Methyl-2-oxopentanoic acid
ath00640 Propanoate metabolism - (2)	
C00042	Succinate
C02170	Methylmalonate
ath00920 Sulfur metabolism - (2)	
C00042	Succinate
C00065	L-Serine

1) Pathways with less than two metabolites detected are not included. Pathway dataset from *Arabidopsis thaliana*.

Table S7.6. Number of finally resolved peaks using MCR-ALS model related to lettuce metabolome and their corresponding information including chemical name, derivatization order, empirical formula and RMF.

No	Metabolite	Derivatization	Formula	RMF
1	L-5-Oxoproline	2TMS	C11H23NO3Si2	949
2	Succinic acid	2TMS	C10H22O4Si2	935
3	Glyceric acid	3TMS	C12H30O4Si3	965
4	Phosphoric acid	3TMS	C9H27O4PSi3	858
5	Fumaric acid	2TMS	C10H20O4Si2	915
6	Glycerol	3TMS	C12H32O3Si3	939
7	Malic acid	2TMS	C10H22O5Si2	880
8	meso-Erythritol	4TMS	C16H42O4Si4	886
9	2,3-Butanediol	2TMS	C10H26O2Si2	817
10	Threonic acid	4TMS	C16H40O5Si4	880
11	Methylmalonic acid	2TMS	C10H22O4Si2	862
12	L-Serine	2TMS	C9H23NO3Si2	774
13	3-Methyl-2-oxovaleric acid	1TMS	C9H18O3Si	796
14	bis-1,2-acetin ether	2TMS	C11H26O4Si2	672
15	Tartaric acid	4TMS	C16H38O6Si4	887
16	Ribofuranose	4TMS	C17H42O5Si4	874
17	Citric acid	4TMS	C18H40O7Si4	836
18	Xylose	4TMS	C20H52O5Si5	749
19	Quinic acid	5TMS	C22H52O6Si5	791
20	Ribitol	5TMS	C20H52O5Si5	795
21	Galactose	4TMS	C19H47NO5Si4	849
22	Tagatose	5TMS	C22H55NO6Si5	745
23	Sorbose	5TMS	C22H55NO6Si5	753
24	Arabinose	4TMS	C19H47NO5Si4	767
25	Methyl-4-O-methyl-α-D-glucopyranoside	3TMS	C17H40O6Si3	823
26	Ribose	4TMS	C17H42O5Si4	823
27	Lyxose	4TMS	C18H45NO5Si4	748
28	Glucose	5TMS	C22H55NO6Si5	920
29	Inositol isomer 4	6TMS	C24H60O6Si6	972
30	2-O-Glycerol-α-d-galactopyranoside	6TMS	C27H66O8Si6	857
31	Gluconic acid	6TMS	C24H60O7Si6	907
32	Glucopyranose	5TMS	C21H52O6Si5	870
33	D-Allose	5TMS	C22H55NO6Si5	843
34	Inositol isomer 1	6TMS	C24H60O6Si6	822

(continuation) Table S7.6. Number of finally resolved peaks using MCR-ALS model related to lettuce metabolome and their corresponding information including chemical name, derivatization order, empirical formula and RMF.

No	Metabolite	Derivatization	Formula	RMF
35	Ribonic acid	5TMS	C20H50O6Si5	800
36	Inositol isomer 2	6TMS	C24H60O6Si6	841
37	Inositol isomer 3	6TMS	C24H60O6Si6	889
38	Galactitol	6TMS	C24H62O6Si6	742
39	Mannonic acid, γ-lactone	4TMS	C18H42O6Si4	762
40	Galactonic acid	6TMS	C18H42O6Si4	801
41	(S,S,R,R,S)- methyl 6-deoxy-β-L-Galactopyranoside	3TMS	C16H38O5Si3	802
42	Mannose	5TMS	C22H55NO6Si5	817
43	Allo-Inositol	6TMS	C24H60O6Si6	842
44	beta-D-Galactopyranoside-(1,2)-glycerol	6TMS	C27H66O8Si6	859
45	Trehalose	8TMS	C36H86O11Si8	839
46	Adenosine	3TMS	C19H37N5O4Si3	731
47	Ethyl α-D-glucopyranoside	4TMS	C20H48O6Si4	771
48	Tagatofuranose	5TMS	C21H52O6Si5	771
49	Melibiose	8TMS	C36H86O11Si8	746
50	Sucrose	8TMS	C35H84O11Si8	842

Table S7.7. Percent variance captured by PLS model.

	X-block		Y-block	
LV	Individual	Total	Individual	Total
1	23.09	23.09	64.35	64.35
2	23.90	46.99	10.25	74.61
3	31.84	78.83	1.41	76.02
4	6.63	85.46	3.78	79.80

Chapter 8. General discussion

Although water was once considered an abundant resource, climate change and population growth are straining our finite freshwater supplies. By 2050, the world will increase 55%demand in water and 70% in energy (UN-Water, 2017). For this reason, wastewater reuse can be a sustainable way to recycle water. Nevertheless, and mainly due to anthropogenic activities, CECs are present in all kind of waters, including reclaimed water, which can be used for agricultural purposes.

Therefore, crops are exposed to CECs by water and/or air. In this work, the uptake and translocation to edible parts of several CECs has been studied in controlled conditions, to further understand the fate of these contaminants in the water-soil-plant system.

A large number studies (ca. 74) have been published about plant uptake of emerging organic contaminants during the current century, comprising more than a hundred of plant species and organic contaminants. However, most of these experiments have been performed in different experimental set up including greenhouse or field conditions; or using soil or in hydroponics. Thus, it is generally difficult to compare results due to many factors that can affect to the experimental results. In this regard, a simple experimental setup with a mixture of perlite and sand was conducted in Chapter 3, to study the different uptake of some CECs. The substrate mixture was selected because it had no organic matter, where CECs could be sorbed or be degraded. Moreover, it can be easily reproduced, as it is not soil dependent. Those contaminants were chosen by its occurrence in water and their physical-chemical properties. Although the irrigation concentrations (0 – 40 µg L^{-1}) were higher than environmental values (from ng L^{-1} to low µg L^{-1}), the relevance of the experiment was to study the different behaviors among CECs in the soil-plant environment.

Then concentration of CECs in the substrate was measured at the end of experiment (see section 3.3.1). Different final substrate concentrations were found for the different CECs. Together with the mass balance performed (Table 3.2), it indicated that, although

it was a mixture of sand and perlite, there were differences in the degradability of these contaminants. For example, in Figure 8.1, the initial substrate concentration was plotted together with the final concentrations of some CECs (BPA, CBZ and IBU). It can be seen that while CBZ final concentration was near the initial concentration. On the other hand, IBU exhibited a high degradation in the substrate.

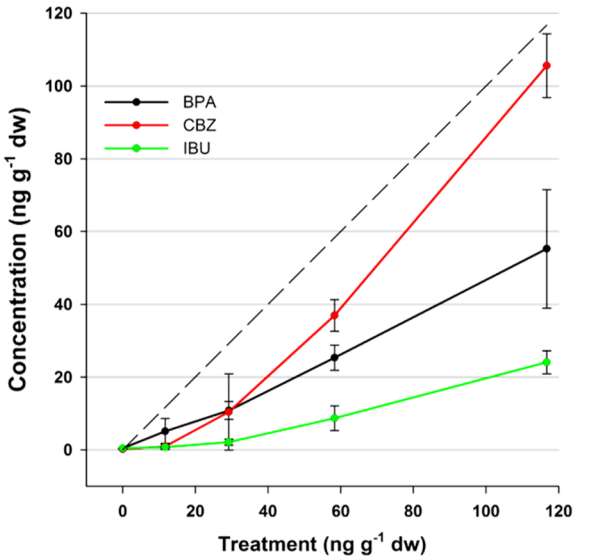

Figure 8.1: Substrate concentration of three different CECs (black: bisphenol A, red: carbamazepine, and green: ibuprofen) along the applied substrate concentration. The dashed line represents the initial applied substrate concentration.

After an exposure time of 28 days, all CECs were detected in roots, and all CECs except SMZ were detected in leaves. This indicated that most of the CECs could be easily taken up plants, although their translocation is somehow restricted. Several authors have tried to correlate between the uptake and specific physical-chemical properties (see section 1.3); although to make these correlations, authors used similar families of compounds with different substituents. In contrast, in this work, CECs from different families have been evaluated, which differ widely from their chemical structure. The different CECs were divided in ionic and neutral compounds. In the experiment, the neutral CECs (CBZ and CAF) exhibited higher translocation than ionic compounds. This behavior was also observed in the other experiments conducted in Chapter 5 and Chapter 7, where neutral compounds, generally were taken up and translocated at higher extent than ionic CECs. However, as it can be seen in Chapter 7, hydrophobic neutral CECs like BHT or BZP exhibited lower translocation than the hydrophilic neutral CECs (CAF, CBZ, and TCP).

In the last years, several authors have focused their research on the degradation of CECs inside plants, mostly by determining transformation products. To study deeply the degradation in plant, the use of labeled compounds such as ^{14}C is usually preferred. However, as it has been discussed previously, these studies are not typically performed because of the lack of these compounds together with their safety issues and high costs. In this regard, in Chapter 4, an inverse model was used to estimate the degradation of CECs in the soil-plant system.

Different plant uptake predictive models are able to estimate the concentration of a compound in roots and leaves, depending on a large number of specific parameters. These predictive models are used by many regulatory agencies: RAIDAR (Risk Assessment IDentification And Ranking, Environment Canada), EUSES (European Union System for the Evaluation of Substances), CSoil (An exposure model for human risk assessment of soil contamination, RIVM, Netherlands), CLEA (Contaminated Land Exposure Assessment, Environment Agency, UK) and CalTOX (CALifornia TOXic Substances control, USA).

The output of these predictive models depend on the input of specific data to the model, and generally, a direct relationship between data and accurate results can be obtained (Trapp, 2015). In addition, these models can be classified depending on whether the concentration of contaminant, which is exposed to the plant, is in steady-state conditions (no change with time) or dynamic (variable with time) conditions. Moreover, there are models based on the activity of the contaminants, which drives the diffusion and thermodynamic equations describe the exchange between environmental compartments (Trapp, 2009). This approach can be used for neutral and ionizable contaminants, however, large biased results upon the predicted and the reported values have been observed for ionics (Hawker et al., 2013). For ionizable CECs, the partition processes between soil solution and plant cells can no longer be described with the log K_{ow}. Instead, dissociation and speciation (dependent on pH), different membrane permeabilities of the occurring molecule species (leading to ion trap effects), and electrical attraction by charged, living cells need to be considered.

These predictive models have also limitations, for example, the one used in this Thesis (Chapter 4), the *Standard Plant Uptake* model based on the assumption that all processes (if not mentioned otherwise) are passive and the occurring processes are based on advection or on diffusion (Trapp, 2004). Despite that, is only applicable for neutral

compounds, for exponentially growing plants, and it assumes the steady state conditions.

The controlled experiment conducted in Chapter 3 generated a large dataset that was used to estimate the degradation in the substrate and in the lettuce roots and leaves. The Standard Plant Uptake model was slightly modified to add two degradation kinetics in it to be able to estimate the degradation rates. For the first time, estimated degradation rates in lettuce of some CECs were reported (Table 4.3).

Two degradation kinetics were studied: first-order and *Michaelis-Menten* kinetics. First-order kinetics is the most widely used, because it refers to a process that is directly proportional to the contaminant concentration involved in the degradation process. However, biotic processes can also occur and in these reactions, enzymes are usually involved. Therefore, *Michaelis-Menten* kinetics appears to be reasonable and was evaluated in this Thesis. Although the latter model is based on a kinetic equation containing two parameters to be adjusted instead of the one used in the first-order kinetics, interestingly, *Michaelis-Menten* did not improve the fitting with experimental results in comparison to first-order kinetics. Nevertheless, the good fit of CECs degradation to a *Michaelis-Menten* kinetics suggested that biodegradation processes in both soil and plant are predominant, which is in agreement with the fact that many authors found Phase I and II metabolites, which are related to reactions with cytochrome P450 and other enzymes.

As most of CECs were taken up by lettuces in the experiment conducted in Chapter 3, a mitigation strategy in Chapter 5 was proposed to diminish the bioavailability and the translocation of CECs to edible part of lettuces. In this regard, BC was selected because it is a soil amendment used in agriculture. Although there is a concern regarding the lack of long-term experiments with BC soil amendment, it has been proved that in arid areas, BC can help to improve soil fertility (Van Zwieten et al., 2010; Yue et al., 2017).

In the experiment conducted in Chapter 5, the uptake and translocation of several CECs was performed using BC as soil amendment. In contrast with the first experiment, soil was used instead of the perlite:sand mixture, to evaluate the effect of BC in a more realistic scenario. In this regard, a soil from the Llobregat River Delta was collected, and could be representative as an agricultural soil for this experiment. Two BC rates were evaluated: 2.5 and 5% (w/w). Based on the density of the soil and the percentages added of BC, it could be estimate an application of 35 to 70 t ha^{-1} at 10 cm depth, although

the optimum rate for soil application depends greatly on the type of the soil due to differences in bulk densities and reactive particles (Biederman and Harpole, 2013). In this regard, the International Biochar Initiative suggests that applications between 5 to 50 t ha^{-1} have been used successfully in different studies. As the soil used in the experiment is a sandy soil and has a low organic matter content, higher doses of BC could be applied to increase the organic C and to improve water holding capacity of a sandy soil. Additionally, from an agronomic point of view, BC is usually recommended for acidic and neutral soils rather than basic ones because its application leads to an increase of the soil pH (Jeffery et al., 2011; Liu et al., 2013).

Even with the soil amendments with BC, a fraction of CECs can be translocated to edible parts such as leaves, however, their uptake and translocation decreased significantly, suggesting that BC can be an effective sorbent for most of the evaluated CECs.

Interestingly, the concentration of CECs in the BC amended soils was higher than the unamended soil. This, together with the reduced EF for IBU in the presence of BC (Figure 5.1), indicates that BC amendment decreased the biodegradation of CECs in most cases attributable to a decrease in the bioavailability of CEC sorbed on BC.

Most biological molecules are present in one of several enantiomeric forms, for instance, the amino acids are found in the *L*-enantiomer (levorotatory) and natural sugars are mostly found in the *D*-form (dextrorotatory). However, many other substances are found in both L and *D*-form. In this regard, more than half of the drugs currently in use are chiral products and most of them are sold as racemic mixture (Nguyen et al., 2006). Despite the chemical structure, they can exhibit different biological activities between enantiomers (i.e. metabolism or pharmacology), while some physical processes are not enantioselective (e.g. hydrolysis or photolysis).

In this Thesis, the racemization of IBU has been studied in two chapters (Chapters 3 and 5). IBU is usually sold as a racemic mixture, which means that has a 50% of the *S*-form and 50% of the *R*-form. However, only the *S*-enantiomer has the desired pharmacological activity while the *R*-enantiomer is pharmacologically inactive (McCullagh, 2008). A derivatization reaction with a chiral molecule ((*R*)-(+)-α-methylbencylamine) was performed in order to transform the chiral molecules in diastereomers and it showed that while it was added as a racemic mixture, different EF was obtained in the soil, roots and leaves. In fact, *R*-form degraded in the substrate

much faster than *S* form, thus increasing the EF (Equation 3.2). This is in agreement with observed IBU's EF in lakes, rivers, and in WWTP influents where high enantiomeric excess of the pharmacologically active *S* enantiomer is found (Buser et al., 1999; Hashim and Khan, 2011). Buser et al. (1999) found that while in the WWTP influent the EF ranged from 0.8 to 0.9, in the treated effluent IBU's EF decreased to 0.5 – 0.7, similar to the values obtained by Matamoros et al. (2009) in a WWTP in Spain (0.9 to 0.6). However, in the experiment conducted in Chapter 3, IBU's EF decreased up to an almost racemic mixture in leaves.

As it is reported by Khan et al. (2014), the changes in EF have generally assumed to the consequence of a rapid degradation of one enantiomer relative to the other (enantioselective degradation); however, a chiral inversion of enantiomer to the other may also take place. In humans, the chiral inversion of IBU has been demonstrated through enzymatic action (Wsol et al., 2004). Moreover it has been shown that some bacteria (*Nocordia diaphanozonaria*) can produce enzymes that invert the chirality of 2-arylpropionic acid derivatives from the *S*-form to the *R*-form.

For this reason, IBU's EF can be a good indicator of biodegradability. In fact, the degradation of IBU was one of the highest in the experiments conducted in Chapter 3 and Chapter 5. Interestingly, IBU's recovery when the agronomic soil was not amended with BC was just 18%, while with 5% BC amendment, the recovery increased up to a 37%. Together with the fact that IBU's EFs were 0.76 and 0.58 for unamended and 5% BC amended soil, indicates that BC reduced the bioavailability of IBU.

Recently, the study of CEC transformation products in plants has become a hot topic by the scientific community, because some of these products can be more harmful than the parent compound. For example, transformation products of BPA, CBZ, and TCS have been reported in plant cell culture. The research has been focused mostly on Phase I metabolites, where parent compounds are functionalized to be later conjugated in Phase II. For example, Malchi et al. (2014) reported that after exposing root vegetables to CBZ, in soil, roots, and leaves, the 10,11-epoxyCBZ metabolite was detected. Interestingly, in soil, the metabolite accounted just a 10%, which supports the low biodegrability in soil of CBZ, observed also in Chapter 3 and Chapter 5. In roots, the metabolite fraction accounted also only a 10%, however, in leaves the metabolite fraction accounted more than 50%. Therefore, CBZ can be metabolized by plants, which is in agreement of the degradation values obtained in Chapter 4.

Other authors studied the Phase II metabolites firstly in cell cultures (Macherius et al., 2014; Wu et al., 2016), and then in real crops in hydroponics (Macherius et al., 2012a; LeFevre et al., 2015). The reported Phase II metabolites are mainly saccharides or sulfosaccharides, and also disulfosaccharide and amino acid based metabolites were identified. These results showed that some CECs may be metabolized and this fraction should be accounted for risk assessment. However, this fraction has never been quantified due to the lack of standards and the variety of conjugates that can be originated.

As most of these studies reported a higher abundance of glycosylated metabolites compared to the other conjugation products, in Chapter 6, an enzymatic digestion was proposed to determine the conjugated fraction. Hence, an extraction of both conjugated and non-conjugated fraction was performed. Then, an enzyme (β-glucosidase) was added to break the glycosidic bond between the CEC and the sugar. By difference, the conjugated fraction can be calculated. The results in this experiment exhibited that the conjugated fraction accounted up to 83% to the non-conjugated CEC. Interestingly, the most hydrophobic CECs (BPA and TCS) were the ones that exhibited the highest conjugated fraction. As it has been discussed in the introduction (see section 1.3.2), the hydrophobic xenobiotics are conjugated to become more soluble, then, plants excrete or sequester them into the vacuoles. Despite that, CBZ exhibited a significant conjugated fraction, which is interesting because many studies are focused only on Phase I metabolites, and as CBZ exhibits a high uptake, this fraction should be accounted for risk assessment studies, where only the target contaminant is considered.

In the greenhouse were the experiments were conducted, all the experimental units were at the same temperature and humidity, received the same sunlight, and irrigated with the same water quality. In the third experiment (Chapter 6 and Chapter 7), lettuces were exposed to a cocktail with 11 CECs at 4 different concentrations. The first two concentrations (0.05 and 0.5 µg L^{-1}) are environmental relevant conditions, while the two higher concentrations (5 and 50 µg L^{-1}) are one and two orders of magnitude higher, although in some countries, these high concentrations have been reported in TWW effluents (Larsson et al., 2007; Kostich et al., 2014). Following the exposure time (28 d), visual morphological differences in lettuces among treatments were observed. While lettuces irrigated with the non-spiked were smaller and thicker, the ones irrigated with spiked water were taller and thinner (Figure 7.1). Moreover, the pigments were also slightly different and the content of chlorophyll content was measured for all

experimental units. Statistical differences were observed for many of these parameters when lettuces were exposed to CECs, suggesting that they could affect to crops.

In the literature, most of these phytotoxic effects have been reported for exposure with a single compound at concentrations well above the environmental levels. In lettuce, phytotoxicity has been observed mostly from antibiotics. In this regard, Migliore et al. (2003) reported an alteration in the post-germinative development of the lettuces and hormesis. D'Abrosca et al. (2008) reported a decrement of the photosynthetic pigments when lettuces were exposed to Atorvastatin. Moreover, Hillis et al. (2011) observed difference in root length when lettuces were exposed to several antibiotics. Boxall et al. (2006) reported a reduction in growth of plants exposed to phenylbutazone, oxytetracycline and enrofloxacin.

In different plants, some CECs of this work have exhibited toxic effects. For instance, González-Naranjo et al. (2015) observed a decrease efficiency of photosystem II in *Sorghum bicolor* plants exposed in soil at 83 mg kg^{-1} dw with IBU. TCS exposure to algae affected to their biomass at very low concentrations (0.012 – 1.2 µg L^{-1}) (Wilson et al., 2003). Ferrari et al. (2003) exposed algae with CBZ and CFA and reported a growth reduction at relative high exposure concentration (5 – 75 mg L^{-1}).

Therefore, many studies suggest that some CECs have toxic effects in plants. In this regard, in Chapter 7, a metabolomic analysis was performed to elucidate whether lettuce metabolites were altered. This metabolomic approach in plants has been reported only for metals (i.e. Pb and Cd) and some pesticide application (e.g. copper nanoparticles and mancozeb) (Wang et al., 2015; Pidatala et al., 2016; Zhao et al., 2016). There are evidences that plants respond to environmental stress factors like drought, salinity and other abiotic factors (Ahuja et al., 2010; Khan et al., 2015). To perform this study, metabolites were extracted from the leaves of both lettuce exposed and non-exposed to CECs. Following a derivatization step,, they were analyzed with a powerful separation technique such as comprehensive two-dimensional GC coupled to time of flight mass spectrometry (GCxGC-TOF) and the use of chemometric tools to treat the data generated was needed. It is noteworthy, that the intention of the study was not to elucidate the different effects that each CEC may exert on specific metabolites, but to have an overview of the alterations in the lettuce's metabolome. For this reason, several CECs were selected based on the occurrence in water, and used them as a mixture to simulate environmental contamination in irrigation water.

The results obtained in Chapter 7 showed that lettuce metabolome was altered when plants were exposed to the CEC mixture. Differences in abundance were observed in sugars, amino acids, alcohols, and other plant metabolites. Hence, many of these metabolites are involved in many important pathways. For example, the citric acid cycle was affected: citric acid was down-regulated, while malate and fumarate were up-regulated. The galactose metabolism was also affected with the changes in mannose, galactitol or myo-inositol. In the firsts Chapters, the uptake and translocation of CECs was demonstrated. Moreover, the formation of Phase I and II metabolites have been discussed along this Thesis. In this regard, plants can metabolize some CECs by transformation, conjugation, and sequestration steps (Burken, 2004). Despite that, using a non-target metabolomics, the morphological effects of CECs to lettuce were elucidated and related to the changes in the metabolites.

8.1 Risk assessment

In this present work, the uptake and translocation to aerial parts of several CECs has been studied. It has been shown that CECs such as CBZ or TCP can be easily translocated and accumulated to edible parts, which may pose human risk. Because the experiments of this Thesis have been performed in pots in a greenhouse with spiked water, no risk assessment has been performed with the data generated. However, the human risk associated with eating crop irrigated with TWW is discussed here.

There are different ways to estimate the possible human risk, which typically are based on the threshold of a critical toxicological effect, usually derived from animal experiments. For example, the Threshold of Toxicological Concern (TTC) is useful to establish a level of exposure for chemicals below this value, no appreciable risk to human health is expected. This TTC approach is useful to set Estimated Daily Intakes (EDIs) for chemicals with known toxicological profiles or similar structure. The TTC is often used to estimate the safety of exposure to chemicals found at very low concentration in food or water (Larsen, 2006; Houeto et al., 2012).

In most cases of toxicological assessment, there is no information and/or toxicological data or it is only limited to *in vitro* studies. Then, a TTC approach is performed based on the relation to structural of chemicals. The most used approach for structuring chemicals on order to make a TTC estimation is the Cramer classification tree (Cramer et al., 1976). This TTC approach has been widely used, for example, by the US FDA or

the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 1999; Food and Drug Administration, 2007). Substances are classified into one of three classes:

Class I: substances of simple chemical structure with known metabolic pathways and innocuous end products which suggest a low order of oral toxicity

Class II: substances that are intermediate. They possess structures that are less innocuous than those in Class I but they do not contain structural features that are suggestive of toxicity like those in Class III.

Class III: substances with a chemical structure that permit no strong initial impression of safety and may even suggest a significant toxicity.

The thresholds used in the TTC approach are intakes, expressed in μg per person per day, below which a given compound of known structure is not expected to present a toxicological concern (Kroes et al., 2004). Many authors classifies compounds using the Cramer decision tree, includes also a Class IV, which refers to compounds that have evidence to be genotoxic (Munro et al., 1996). The TTC concept is a risk-assessment tool that was developed to establish human-exposure threshold values for chemicals occurring at very low concentrations and lacking specific toxicological data. The TTC values are based on the calculated 5th percentile values of "No Observed Adverse Effect Levels" (NOAELs) divided by an uncertainty factor of 100 to derive an acceptable daily intake.

As some CECs are pharmaceutically active compounds, they are easy labeled as Class III compounds. In this work, most of the studied CECs are Class III compounds. The Class III compounds have a TTC value of 1500 ng kg^{-1} of body weight per day, while potentially genotoxic compounds have a TTC value of 2.5 ng kg^{-1} of body weight per day (Kroes et al., 2004) Consumption of contaminants above these TTC values indicates a possible risk of exposure and suggests that further toxicity data is required to assess the human health risk.

In the literature, there are different studies that used different methods to assess the human risk. For example, Prosser and Sibley (2015) reviewed the literature values of CECs in edible plants and concluded that the majority of individual CECs present a *de minimis* risk to human health. Their approach derives values by estimating for example a NOAEL from an observed dose-response curve and applying factors for possible differences between human populations. For pharmaceuticals, an uncertainty factor of 1000 was applied to all minimum therapeutic doses (MTD) to determine acceptable daily

intakes (ADI) values. However, this approach is not used for genotoxic carcinogens, where it is assumed there is no threshold (Hanlon et al., 2016).

In the study of Malchi et al. (2014), they watered root vegetables (sweet potato and carrot) with TWW from Israel. As they detected CBZ, some of its metabolites, and lamotrigine in roots and leaves, they performed a risk assessment based on a TTC approach to estimate the required daily consumption of those vegetables. CBZ is a Class III compound and for this reason, to reach the TTC level (1500 ng per kg body weight per day), the EDI of carrots should be more than 100 kg. However, as 10,11-epoxyCBZ and lamotrigine are considered as genotoxic compounds, an adult could reach the TTC level of lamotrigine (2.5 ng per kg body weight per day) by eating 180 g of carrot per day (2 carrots), while for a child (25 kg) by consuming 60 g carrot per day (half-a carrot).

Riemenschneider et al. (2016) studied the uptake of more than 20 CECs in 10 vegetable species irrigated with TWW and conducted a risk assessment study with the values obtained in their study. They concluded that the human exposure via consumption of food crops irrigated with TWW ranged from 0.003 – 15 ng kg^{-1} body weight per day of the compounds investigated. That means that the human risk is low, and these values are in agreement with other studies (Pan et al., 2014; Wu et al., 2014). Interestingly, for CBZ, the estimated annual doses were about 0.001% of the minimum daily doses (10 – 200 mg d^{-1} for a 70 kg person). To reach the TTC level of CBZ, at least 9 kg of vegetables per day should be consumed. In contrast, for a potentially genotoxic compound (10,11-epoxyCBZ or ciprofloxacin), a consumption 100 g of potato or half an eggplant (177 g) per day would exceed the TTC values for a 70 kg person. It is noteworthy that consumption above the TTC value should not be presumed to be toxic. It should, however, indicate a demand for specific toxicity analysis of the CEC.

Nevertheless, these studies take only into account the single effects of each contaminant. Plant are exposed to multiple contaminants, resulting in mixtures of CECs being present in edible tissue of plants. Although different CECs may have totally independent actions, in many cases two or more CECs may act in the same site in ways they can be additive or non-additive (Sexton and Linder, 2011). Moreover, synergic (greater than the sum of either effect alone) or antagonistic (lower than the sum of either effect alone) effects can also occur in the environment (Carpenter et al., 2002). Although it is difficult to elucidate these effects, these should be accounted in the risk assessment (Monosson, 2005).

In addition, these studies have considered only the parent compound or the main Phase I metabolites. In Chapter 6, it has been demonstrated that a large fraction (27-83% respect to the parental compound) of glycoside metabolites can be formed in plants. As it has been discussed previously, in the digestive system, humans can break these glycosidic bonds, releasing the parental contaminant to the body, and thus, this fraction should be accounted in risk assessment studies. Likewise, the formation of other Phase II conjugates has been reported (LeFevre et al., 2015), and the effects in human body should be studied.

8.2 Recommendations for future research

The use of RW becomes increasingly necessary in many arid and semiarid countries, where water scarcity is a fact. As it has been discussed along the Thesis, CECs can be taken up by crops and translocated to edible parts. Some of these CECs have been shown to alter plant's metabolism. Moreover, plants are able to transform and metabolize some of the studied CECs. It is noteworthy that the conducted experiments in this Thesis have been performed in a greenhouse. For this reason, further studies should be done at a real field scale to confirm the CECs behavior and their fate observed in this work.

Furthermore, in this work, using an enzymatic digestion, a glycosylation fraction has been determined. However, other conjugation processes such as sulfo-saccharides or other amino acid-based conjugates can also take place. Therefore, the formation of the whole conjugated fraction should be studied.

Similar to the uptake, the transformation would be time-dependent, hence, the use of *in vivo* techniques could be an interesting option to analyze them. For example, the low invasive technique *in vivo* solid-phase microextraction (SPME) could be proposed as a sampler to analyze the uptake and production of transformation products. The SPME consists of insertion of a fiber directly into the living system of a plant (e.g., leaf, stem or bulb) (Bojko et al., 2011). It reduces the amount of solvent to use, there are different commercial coatings, and it can be easy to analyze. However, to be quantitative, a calibration step is required (Bojko and Pawliszyn, 2014).

Furthermore, in Chapter 7, some metabolic effects were observed when lettuces were exposed to CECs. Alterations in the lettuce metabolism were reported and should be deeper investigated. As rivers or TWW effluents can be contaminated, a myriad of CECs

may reach crops, which may affect them. For this reason, it is important to assess the impact of the contaminants in a real field scenario.

As it has been reported by Riemenschneider et al. (2016), Prosser and Sibley (2015), and Malchi et al. (2014), the human risk via food consumption is still unclear; however, many Phase I metabolites can be formed in plants. The use of non-target analysis can provide a good knowledge to study the main formation routes; however, high resolution mass spectrometry techniques needed, are expensive and experiments at a dose higher than the environmental ones are required.

BC was proposed as a mitigation strategy of the uptake of CECs to lettuce. However, long-term experiments should be performed, because it has been demonstrated that aging effects may lower the sorption capacity of BC. Besides, further studies are needed to characterize the desorption hysteresis effects. Moreover, other mitigation to reduce the bioavailability of CECs should be taken into account.

Chapter 9. Conclusions

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

- Most of the CECs studied in this Thesis were taken up by lettuce and some of them were translocated to aerial parts. Their bioaccumulation factors in roots and leaves ranged from 0.27 to 733 and from 0 to 3.
- The main parameters that could affect to the uptake and translocation of CECs are the biodegradability, the chemical speciation and the hydrophobicity (log K_{ow} or log D_{ow}).
- The uptake of CECs in lettuce was fairly lineal with the irrigation concentration, which makes it possible to predict the leaf concentration of tested CECs fairly accurately.
- The IBU EF suggested that mixed biotic degradation pathways might occur in the plant either through endophytic bacteria or the plant's own detoxification system, leading to complete racemization in the leaves.
- Degradation rates in the soil-plant system were determined by inverse modeling for the first time. In soil, IBU and PROP were the CECs that exhibited the highest degradation rates. On the other hand, CBZ and TCS were the CECs with lowest degradation rates.
- In plant, BPA was the CEC with the lowest degradation rate, while CAF and CBZ were the CECs with highest degradation rates.
- The addition of 2.5 and 5% BC to the soil decreased the concentration of CECs from 34 to 48% in roots and 23 to 55% in leaves at 2.5 and 5% respectively of biochar amendments.
- Moreover, BC amendment decreased the biodegradation of CECs as supported by the reduced EF for IBU in the presence of BC.

- For some CECs (BPA, CBZ, and TCS) the glycosylated fraction accounted for between 27 and 83% of the free parent compound, increasing with the hydrophobicity. Therefore, not only Phase I transformation products but also Phase II conjugates should be taken into account in risk assessments based on daily intake.
- Lettuces exposed to CECs exhibited morphological and agronomical differences compared to the non-exposed lettuces. Moreover, the presence of CECs in irrigation waters disrupted several metabolic pathways such as TCA and sugar metabolism.

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