The potential implications of reclaimed wastewater reuse for irrigation on the agricultural environment: the knowns and unknowns of the fate of antibiotics and antibiotic resistant bacteria and resistance genes – A review

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Running title: Fate of antibiotics and related resistant elements in wastewater-irrigated agricultural systems

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ABSTRACT

The use of reclaimed wastewater (RWW) for the irrigation of crops may result in the continuous exposure of the agricultural environment to antibiotics, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs). In recent years, certain evidence indicate that antibiotics and resistance genes may become disseminated in agricultural soils as a result of the amendment with manure and biosolids and irrigation with RWW. Antibiotic residues and other contaminants may undergo sorption/desorption and transformation processes (both biotic and abiotic), and have the potential to affect the soil microbiota. Antibiotics found in the soil pore water (bioavailable fraction) as a result of RWW irrigation may be taken up by crop plants, bioaccumulate within plant tissues and subsequently enter the food webs; potentially resulting in detrimental public health implications. It can be also hypothesized that ARGs can spread among soil and plant-associated bacteria, a fact that may have serious human health implications. The majority of studies dealing with these environmental and social challenges related with the use of RWW for irrigation were conducted under laboratory or using, somehow, controlled conditions. This critical review discusses the state of the art on the fate of antibiotics, ARB and ARGs in agricultural environment where RWW is applied for irrigation. The implications associated with the uptake of antibiotics by plants (uptake mechanisms) and the potential risks to public health are highlighted. Additionally, knowledge gaps as well as challenges and opportunities are addressed, with the aim of boosting future research towards an enhanced understanding of the fate and implications of these contaminants of emerging concern in the agricultural environment. These are key issues in a world where the increasing water scarcity and the continuous appeal of circular economy demand answers for a long-term safe use of RWW for irrigation.

Keywords: antibiotics; accumulation; human health risks; antibiotic-resistance genes; uptake; reclaimed wastewater irrigation

Abbreviations: APCI, atmospheric pressure chemical ionization; ARB, antibiotic resistant bacteria; ARGs, antibiotic resistance genes; CytcOx, cytochrome c oxidase; DDA, data-dependent acquisition; DOM, dissolved organic matter; D_{ow}, pH-dependent speciation of ionic compounds; ESI, electrospray ionization; GSTs, glutathione S-transferases; H^+-ATPase, proton pump; HGT, horizontal gene transfer; HILIC, Hydrophilic interaction liquid chromatography; K_{oc}, organic carbon-normalized sorption coefficient; LC HRMS, liquid chromatography high-resolution mass analyzers; LC, liquid chromatography; MAE, microwave-assisted extraction; MGEs, mobile genetic elements; NER, non-extractable residues; PCR, polymerase chain reaction; PLE, pressurized liquid extraction; QqQ, triple quadrupole; QqQ-LIT, hybrid triple quadrupole-linear ion trap; QuEChERS, quick, easy, cheap, effective, rugged and safe; ROS, reactive oxygen species; RWW, reclaimed wastewater; SLE, solid liquid extraction; SOM, soil organic matter; SRM, selected reaction monitoring; TPs, transformation products; TTC, threshold of toxicological concern; UHPLC, ultra-high performance liquid chromatography; USE, ultrasound-assisted solvent extraction; WWTPs, wastewater treatment plants;

Contents

1. Introduction

1.1. Why should antibiotics and antibiotic resistance be considered as contaminants of emerging concern?

Since their introduction into medicine in the 1940s, antibiotics have been central to modern healthcare (Center for Disease Dynamics and Economics & Policy, 2015; Nesme and Simonet, 2015). This has involved their extended usage for the treatment of serious infections related to human health and welfare and for the promotion of growth and disease prevention in livestock and other food animals. Together with population growth, increasing prosperity and inappropriate use, have stimulated the production of thousands of tons of antibiotics, with projections for further increased production in the forthcoming years (Van Boeckel et al., 2014; Van Boeckel et al., 2015). Over the last decades, an increasing body of evidence has shown that antibiotics entering the environment, subsequently pose potential adverse effects on non-target organisms and humans (Boxall, 2004; Runnalls et al., 2010; Vasquez et al., 2014; Brandt et al., 2015). Antibiotics are introduced into the environment via various human activities, including direct disposal of unused or expired medication, release from pharmaceutical manufacturing plants and hospitals, and veterinary drug use (Grossberger et al., 2014). Moreover, most antibiotics are poorly absorbed and not completely metabolized in human and animal bodies. Hence, a high percentage of the intake dosage (30-90%) of most antibiotics is excreted via urine and faeces within hours after application either as the parent compound or as metabolites (Liu et al., 2010; Zhang et al., 2014). As a result, antibiotics may directly enter the environment through the application of manure to soil and excretion by grazing livestock (Pan and Chu, 2016a). The use of RWW for irrigation and the use of biosolids as soil amendments constitute additional significant pathways for the introduction of antibiotics in the agricultural environment, as conventional wastewater treatment processes are only moderately effective at removing antibiotics from the RWW (Michael et al., 2012; Petrie et al., 2015). It should be noted that the removal efficiency of antibiotics during wastewater treatment processes varies and is mainly dependent on a combination of antibiotics' physicochemical properties and the operating conditions of the treatment systems (reported concentration of antibiotics in RWW range from low ng L^{-1} to low μg L^{-1} depending on the type of antibiotic, the treatment technology applied in WWTPs and the season of the year) (Michael et al., 2012). The most commonly applied biological treatments (i.e. conventional activated sludge, membrane bioreactor, moving bed biofilm bioreactor) are usually unable to efficiently remove antibiotics from RWW (low removal efficiency especially for polar antibiotics); as a result the application of costly advanced treatment processes downstream of conventional biological process (i.e. membrane filtration such as reverse osmosis, activated carbon adsorption, activated oxidation processes such as ozonation, fenton oxidation or sonolysis) and disinfection (i.e. ultraviolet irradiation) should be applied for the significant improvement of antibiotics removal efficacy (up to 100% in many cases) (Michael et al., 2012; Luo et al., 2014). Consequently, antibiotics are routinely detected in

RWW and biosolids, and in RWW-irrigated agricultural soils and runoff from such sites, biosolids- and manure-amended soils, and surface and groundwater systems and sediments receiving RWW (Kolpin et al., 2002; Pedersen et al., 2003; Kinney et al., 2006; Fatta-Kassinos et al., 2011a; Gottschall et al., 2012; Luo et al., 2014; Meffe and de Bustamante, 2014). The reported uptake of antibiotics by crop plants and aquatic organisms and their subsequent entry into the human food web warrants special concern due to possible public health effects (Rand-Weaver et al., 2013; Malchi et al., 2014; Pan et al., 2014; Prosser and Sibley, 2015).

Along with antibiotics, RWW, biosolids and manure may carry significant loads of antibiotic resistant bacteria (ARB) and resistance genes (ARGs) (Szczepanowski et al., 2009; Munir and Xagoraraki, 2011; Rizzo et al., 2013; Manaia et al., 2016). Rizzo et al. (2013) reviewed the spread of ARB and ARGs from WWTPs and concluded that conventional WWTPs are important hotspots for the spread and selection of ARB as well as ARG transfer, and that advanced treatment technologies (i.e. ozonation, fenton oxidation and sonolysis) and disinfection processes are regarded as possible tools to control the spread of ARB into the environment. While antibiotic residues may exert selective pressure on exogenous or on soil resident bacteria, the spread of ARB and ARG to terrestrial and aquatic and, eventually other environments, may be enhanced (Bondarczuk et al., 2016). WWTPs could be regarded as “genetic reactors” that assemble bacteria from a myriad of human and environmental sources and offer conditions that may favor the exchange of genetic material, ARB selection, and hence their rapid evolution (Ju et al., 2016; Manaia et al., 2016). Simultaneously, the environmental matrices receiving RWW and biosolids constitute additional genetic reactors, where bacteria originated from the abovementioned sources may be mixed and counteract with environmental organisms (Baquero et al., 2008). Thus, the continuous disposal of RWW, biosolids and manure in the environment contribute to the enrichment of soil with ARB and ARGs, with soil being already considered as one of the largest environmental reservoir of antibiotic resistance (Nesme et al., 2014; Nesme and Simonet, 2015). ARGs may persist in the environment and, even worse, they can be transferred to other bacteria including human commensals or pathogens of clinical relevance, through horizontal gene transfer (HGT) of mobile genetic elements (MGEs). The implications of the widespread distribution of this type of contaminants can be substantial at both the public health and economic levels (Han et al., 2016; Johnson et al., 2016). Reaching alarming levels in many parts of the world (World Health Organization, 2014), the increasing emergence and propagation of ARGs are threatening modern medicine and posing major risks to human health and ecological sustainability in the 21st century (Bush et al., 2011; Berendonk et al., 2015; Price et al., 2015).

Diminishing availability of good quality freshwater due to the growing demand of an increasing world population and climate change-driven frequent and prolonged dry periods, render RWW as a valuable alternative water source in arid and semi-arid regions worldwide (Bixio et al., 2006). Currently, RWW is commonly used to irrigate agricultural land and urban greeneries, and replenish surface and groundwater resources (Hamilton et al., 2007; Pan et al., 2014).

This review collates recent knowledge on antibiotics and ARB and ARGs in the agricultural environment as a result or the use of RWW for irrigation. Among others, the effects of biotic and abiotic factors on the fate of these contaminants of emerging concern in the agricultural environment receiving RWW irrigation, as well as implications for plant uptake and potential negative effects on public health, will be discussed and highlighted.

2. Fate of antibiotics in reclaimed wastewater-irrigated agricultural soil

A great number and variety of antibiotics and related elements are present in RWW, constituting mixtures which may continuously vary (intra- and inter-daily, seasonally and inter-annually) in composition and concentrations (Diwan et al., 2013; Petrie et al., 2015). Therefore, RWW irrigation may result in the continuous exposure of the agricultural environment to a variety of antibiotics and ARB and ARGs (Wang et al., 2014). RWW-irrigated soils have been found to accumulate antibiotics in concentrations that are several folds higher than the ones found in the irrigation water (Kinney et al., 2006; Calderón-Preciado et al., 2011). Kinney et al. (2006) detected erythromycin in RWW-irrigated soils in Colorado State, USA, at concentrations of 0.02-15 μg kg^{-1}. Furthermore, Wang et al. (2014) explored the effects of long-term RWW irrigation of six public parks in Beijing, China, on the concentration of five tetracyclines (tetracycline, oxytetracycline, chlortetracycline, methacycline, and doxycycline) and 9 of their degradation products, four sulfonamides (sulfadimethoxine, sulfamerazine, sulfamethizole, sulfamethoxazole), and six fluoroquinolones (ofloxacin, enrofloxacin, sarafloxacin, danofloxacin, ciprofloxacin, norfloxacin) in the rhizosphere soil. The total concentration of tetracyclines was in the range of 12.7-145.2 μg kg^{-1}, with the parent compound being found in higher concentrations compared to their degradation products. Fluoroquinolones were randomly detected in sampled soils with their highest total concentration being 79.2 μg kg^{-1}, whereas none of the four sulfonamides examined were found in all soil samples (Wang et al., 2014). Grossberger et al. (2014) found sulfamethoxazole in soil in concentrations ranging from 0.12-0.28 μg kg^{-1} (depending on the soil type) following the irrigation of carrot crop with RWW for a single growing period.

Antibiotics are ionizable molecules and can occur as neutral and/or charged species (zwitterionic, negative or positive) in the RWW used for irrigation and in the receiving soil (Wu et al., 2015). The chemical form of antibiotics in soil pore water (bioavailable/bioaccessible fraction), along with the properties of soil and the surrounding water, shape the fate of antibiotics in agricultural soils. Once introduced into soil, antibiotics are subjected to sorption/desorption and transformation processes (both biotic and abiotic), which influence the concentrations available for biodegradation, transport into soil (runoff and leaching) and plant uptake, ultimately specifying the potential of accumulation of antibiotics in soil (Grossberger et al., 2014). The chemical properties of antibiotics that significantly impact and shape their environmental fate are polarity, hydrophobicity and water solubility. The polarity of organic compounds is determined by the presence of ionizable functional groups, such as carboxyl, phenolic hydroxyl and amine moieties within the molecules, which may be protonated and deprotonated depending on soil pore water pH, thus acquiring a positive or negative charge, respectively. As a result, polar and ionizable antibiotics engage in interactions with the soil organic matter (SOM), the mineral surfaces and the dissolved organic matter (DOM). Such interactions include hydrophobic partitioning, electron donor-acceptor interactions (e.g., hydrogen bonding), cation-anion exchange, protonation, water binding, cation binding and surface complexation (Thiele-Bruhn et al., 2004; Vasudevan et al., 2009). Therefore, the physicochemical properties of antibiotics as well as the chemistry of soil pore-water (i.e. pH, mineral concentration, cation exchange capacity, dissolved organic matter), and the soil organic matter (SOM) content and structure (i.e. clay composition) are critical factors controlling the retention of antibiotics in soil (Vasudevan et al., 2009; Wu et al., 2013a; Miller et al., 2016; Park and Huwe, 2016) (Table 1).

In the following sections, studies concerning the main processes affecting the environmental fate of antibiotics in soils receiving RWW for irrigation (i.e. sorption, transformation and transport) are presented. These studies are the ones performed under real-scale applications or under controlled condition simulating RWW irrigation. Other controlled studies (performed either in the lab or in greenhouse) that corroborate the limited findings regarding the environmental fate of antibiotics in the agricultural environment receiving RWW for irrigation, are also discussed.

2.1. Sorption

Polar and ionizable antibiotics tend to remain soluble in soil pore water rather than be retained in soil organic matter and mineral surfaces, whereas, conversely, nonpolar and moderately polar neutral antibiotic compounds are instantaneously absorbed by SOM, while also spontaneously get desorbed to soil water till an equilibrium is established (Thiele-Bruhn et al., 2004; Wegst-Uhrich et al., 2014)(Table 1). Partitioning of antibiotics between SOM and soil pore water is typically described using an organic carbon-normalized sorption coefficient, K_{oc} (compounds with log K_{oc} values <2 are considered to be capable for only weak sorption) but equilibrium partitioning isotherms in many cases do not fit with linear models (Chefetz et al., 2008; Xu et al., 2009; Revitt et al., 2015; Park and Huwe, 2016). To this effect, the Freundlich isotherms has been revealed to be the most successful ones to describe the sorption of antibiotics to soil (Chefetz et al., 2008; Xu et al., 2009; Revitt et al., 2015; Park and Huwe, 2016) Interestingly, Tulp et al. (2008) reported a reduction of polar, multifunctional compounds partitioning behaviour over a large range of environmental matrices by a factor of 7-60, compared with nonpolar and moderately polar neutral compounds. Currently, the ability to predict sorption for ionized organic compounds to SOM from solute descriptors is limited, although sorption of organic anions to SOM is generally lower than that of the corresponding neutral species (Miller et al., 2016). Moreover, the extent of association of polar ionizable antibiotics with soil particles and SOM is strongly determined by the chemistry of soil pore water (i.e. pH, ionic strength, concentration of competing ions) (Gu et al., 2007; Vasudevan et al., 2009; Kodešová et al., 2015). Kurwadkar et al. (2007) have observed that sulfathiazole and sulfamethazine demonstrated a strong pH dependency for sorption in three soils having distinguished texture (i.e. loamy sand, sandy loam and loam), showing higher sorption capacity at soil pore water pH values lower than 7.5, where these antibiotics exist primarily in their neutral/cationic form. Weakly acidic antibiotics (i.e. sulfamethoxazole), which regularly carry a negative charge in RWW-irrigated soils, may be poorly retained by soil particles and SOM due to repulsion forces between the deprotonated radicals and the negatively charged soil particles and SOM, consequently being prompted to leaching; thus are regularly being detected in aquifers (Chefetz et al., 2008; Borgman and Chefetz, 2013). Moreover, compounds that display higher hydrophobicity are adsorbed to a lower extent in organic soils with high clay content (Durán-Álvarez and Jiménez-Cisneros, 2014), showing significant rate of desorption and higher potential for reaching the aquifer during rainfall events or continuous RWW irrigation (Chefetz et al., 2008). Conversely, positively charged antibiotics, such as tetracyclines, may be retained onto soil particles by cation exchange, while simultaneously desorption at low rates may occur as a result of competition with metal and organic cations (Gu et al., 2007). Antibiotics that carry both positive and negative charge due to the existence of several functional groups in their complex structure (i.e. ciprofloxacin) can undergo both sorption and desorption from soil mineral surfaces and soil organic matter (Wu et al., 2013a). Importantly, these processes are strongly pH-dependent and vary in the presence of metal cations, probably due to surface complexation with Al^{3+}, Na^{+} and Ca^{2+} (Vasudevan et al., 2009; Wu et al., 2013a). Overall, the soil pore water pH can be considered as a dominant factor controlling the sorption of polar and ionizable antibiotics in soil. Ionizable antibiotics regularly show lower sorption rates in alkaline soils, whereas lower rates of sorption may be registered in acidic soils receiving RWW irrigation, as RWW regularly has pH values greater than 7 (7 to 8), which may result in elevated pH values of the soil pore water (Borgman and Chefetz, 2013; Christou et al., 2017).

The effects of rhizosphere in antibiotics speciation and bioavailability should also be taken into account, as the root exudates, which may contain organic acids such as citric, oxalic and malonic acids, can reduce the soil pore water pH up to two units as far as 2-3 mm from the root surface compared with bulk soil (Hinsinger et al., 2003). Such alteration in the rhizosphere pH values may influence the properties of soil, the microbial mineralization of SOM (Keiluweit et al., 2015), and therefore the speciation and sorption of neutral and ionizable antibiotics (Hurtado et al., 2016).

2.2. Transport in soil

Antibiotics may be transported to soil if dissolved in soil pore water, either vertically leading to their presence in deeper soil depths and the aquifer, or horizontally, causing contamination of unpolluted sites and adjacent water bodies (Alder et al., 2001; Davis et al., 2006). The migration of antibiotics in soil is closely related to their sorption capacity onto the soil matrix, influenced by their physicochemical properties, the properties of soil and the chemistry of soil pore water (Chefetz et al., 2008; Zhang et al., 2014; Park and Huwe, 2016) (Table 1). Transport studies can be performed using different approaches in the laboratory, either by using packed soil columns or undisturbed soil columns tests. The presence of expansive clays in soil results in the disappearance of preferential path in the porous network of soil once clay becomes wet, which in turn provokes the decay in transport of organic contaminants contained in soil pore water

(Durán-Álvarez and Jiménez-Cisneros, 2014). Moreover, increases in SOM, due to manure, compost or biosolids soil amendment, may also result in decreased mobility of antibiotics in RWW-irrigated soils as a result of enhanced sorption (Borgman and Chefetz, 2013). In addition, DOM inputs due to RWW irrigation can affect transport and sorption of antibiotics in agricultural soils. DOM can increase the antibiotics' apparent solubility and therefore enhance their mobility (facilitated by co-transport), or conversely, reduce their mobility due to co-sorption and cumulative sorption to the soil's solid phases (Chefetz et al., 2008; Haham et al., 2012) (Table 1). These contradictory effects may be attributed to the fact that the processes affecting the mobility of antibiotics and DOM, as well as of their complex, are controlled by the binding affinity of antibiotics to the DOM , the DOM-antibiotics complexation kinetics as well as by the hydrophobicity of DOM and the DOM-antibiotics complex, which in turn determine their binding affinity to the soil organic and inorganic matrices (Chefetz et al., 2008). Therefore, the mobility of the antibiotics may be increased or decreased if the binding affinity of the DOM and DOM-antibiotics complex to the soil matirces is low or high, respectively.

Kinney et al. (2006) reported that the differences in concentrations of erythromycin and sulfamethoxazole in 5-cm increments of a soil profile (0-30 cm soil layer) in RWW-irrigated fields might indicate interactions of these antibiotics with soil components during leaching through the vadose zone. Indicatively, sulfamethoxazole has been detected at a mean concentration of 0.11 μg L^{-1} in groundwater beneath soils subjected to long-term irrigation (45 years) with RWW (secondary treated, activated sludge) in Germany (Ternes et al., 2007). The application of biosolids was found to increase the retardation of antibiotics in soils, thus mitigating their leaching potential, whereas RWW irrigation may increase the mobility of weakly acidic antibiotics, as the elevation of soil pore water pH due to the neutral-basic nature of RWW results in the predominant presence of the anionic form of these antibiotics in soil (the repulsion of antibiotics from the negatively charged soil surfaces enhance the leaching potential) (Borgman and Chefetz 2013). Bondarenko et al. (2012) evaluated the ability of turfgrass systems in attenuating trimethoprim and sulfamethoxazole antibiotics during RWW irrigation by monitoring the leachate water at the 90-cm depth, and revealed the higher leaching potential of trimethoprim compared with sulfamethoxazole, as the former displayed higher concentrations and frequency of detection in the leachate over the latter. Horizontal transport of antibiotics may result from runoff, or the deposition of RWW aerosol close to irrigation channels, the deposition of soil material derived from RWW-irrigated sites by wind erosion, or the transport of soil material between fields with farm machinery (Dalkmann et al., 2012).

2.3. Transformation in soil

The bioavailable/bioaccessible concentrations of antibiotics in wastewater-irrigated soils may be altered by abiotic and biotic (microbial) transformation processes. Such processes may result in the mineralization of these organic molecules, or the formation of biologically active transformation products that may be taken up by plants (Jechalke et al., 2014; Miller et al., 2016). Abiotic transformation processes that may take place in agricultural soils include photolysis, hydrolysis and redox reactions (Table 1). Although these transformation processes of antibiotics are well documented in wastewater treatment plants and receiving water sources (Fatta-Kassinos et al., 2011b; Homem and Santos, 2011; Ganiyu et al., 2015), only limited information is available regarding the abiotic transformation processes of antibiotics occurring in soil. Direct photolysis of antibiotics in soils is considered trivial due to light attenuation (Hebert and Miller, 1990), since the SOM and DOM can act as quenchers of UV irradiation decreasing the photodegradation kinetics of antibiotics compared with clean water (Fatta-

Kassinos et al., 2011b). While no evidence exists regarding the hydrolysis of antibiotics in RWW-irrigated soils, β-lactam antibiotics were found to be rapidly hydrolyzed in manure-amended soils (Jechalke et al., 2014). In addition, Solliec et al. (2016) found tetracycline antibiotics in the range of $\mu g\,kg^{-1}$ in soils following swine manure amendment, while degradation products resulting from oxidation, hydrolysis and biodegradation processes were sometimes found at higher concentrations compared with the ones of the parent compounds.

Microbial biodegradation and biotransformation are considered to be dominant biotic processes that greatly shape the fate of antibiotics in soil (Lin and Gan, 2011; Ding et al., 2016; Pan and Chu, 2016a). The biotic transformation of antibiotics in soil was found to be influenced by their initial concentrations, microbial activities, oxygen status in the soil, soil type and environment (moisture, temperature, salinity, pH), the presence of SOM and clay content, and the physicochemical properties of the antibiotic (Table 1) (Lin and Gan, 2011; Grossberger et al., 2014; Pan and Chu, 2016a). The biodegradation of antibiotics in soil is also influenced by their sorption capacity to soil matrices, which in turn determines their bioavailable fraction in soil pore water. Therefore, soil characteristics such as the SOM content, soil texture and soil pH greatly shape the rates of antibiotics degradation in RWW-irrigated soils (Lin and Gan, 2011; Wu et al., 2012; Durán-Álvarez and Jiménez-Cisneros, 2014). High SOM and clay content often correlate with decreased biodegradation rates, probably due to the reduced bioavailability of antibiotics because of the increased sorption to SOM (mainly humic acids) (Xu et al., 2009; Wu et al., 2012) and the formation of non-extractable residues (NER) (Yang et al., 2009; Müller et al., 2013). Biosolids or manure amendment may either reduce biodegradation due to increase sorption or increase biodegradation due to enhanced microbial activity (Walters et al., 2010). Generally, the biodegradation of antibiotics in soil is faster and more complete under aerobic (topsoil) as compared to anaerobic conditions (deeper layers of vadose zone) (Liu et al., 2010; Wu et al., 2012; Pan and Chu, 2016a). However, it is widely accepted that both aerobic and anaerobic processes are needed for the overall biodegradation of antibiotics in RWW-irrigated soils. Aerobic biodegradation may result in the fast initial enzymatic attack and the decomposition of alkyl side-chains and other easily degradable factional croups (e.g. carboxyl groups), whereas anaerobic biodegradation may include various enzymatic processes that biodegrade more complex and stable functional groups and structural moieties, such as aromatic groups, naphthalene rings and sulfonamides (Ghattas et al., 2017). Low concentration of antibiotics in soils (in the range of low ng kg^{-1}) in fields irrigated with RWW for the short time may result in limited biodegradation rates, indicating that higher concentrations (i.e. due to prolonged RWW irrigation or irrigation with RWW containing high concentrations of antibiotics) are necessary to induce changes in the soil microbial community structure or to promote the expression of biodegradation enzymes (Grossberger et al., 2014; Miller et al., 2016). Moreover, the degradation rates of macrolide antibiotics were found to be accelerated in soils that were previously exposed to antibiotics as a result of RWW irrigation or biosolids amendment (Topp et al., 2016). Nevertheless, Grossberger et al. (2014) reported contradictive results, as pre-exposure of soils to sulfamethoxazole via RWW irrigation did not enhance its biodegradation rate. Wang et al. (2014) reported the presence of nine biodegradation products of tetracycline antibiotics in the rhizosphere soil of RWW-irrigated parks in Beijing, China, with 4-epianhydrochlortetracycline being detected in all soil samples in the range of 1.3-5.1 μg kg^{-1}. Biotic transformation of antibiotics in the rhizosphere may be increased compared with that in bulk soil (Kopmann et al., 2013), as plants and rhizosphere–associated microorganisms secrete enzymes, such as laccases and peroxidases, that can effectively biodegrade antibiotics; laccase oxidation was reported as an efficient mechanism for the removal of sulfonamide antibiotics from soil (Ding et al., 2016). The limited information with regard to the biotransformation of antibiotics in soils irrigated with RWW is due to the small number of studies conducted under real field conditions. Therefore, there is a need for further studies, since the transformation products (TPs) may exert biological effects or be taken up by crop plants.

3. Detection and quantification of antibiotics, ARB and ARGs in soils and crops

The detection and quantification of antibiotics and/or ARB and ARGs in soil and crop samples are laborious and challenging tasks, although possible nowadays, thanks to developments in analytical instrumentation and techniques. The wide range of extraction protocols and the chromatographic techniques, along with the challenges raised, for the detection and quantification of antibiotics are discussed in detail. Cultivation based methods for the characterization of antibiotic resistance, and molecular biology methods applied for revealing the diversity and the abundance of ARB and ARG in RWW and in soil samples have been reviewed by different authors (Rizzo et al., 2013; Luby et al., 2016; Manaia et al., 2016) and hence are summarized in Fig. 2.

3.1. Extraction and analysis of antibiotics in soils and crops

The determination of antibiotics in the agricultural environment including soils and crops is necessary for getting a better understanding of their fate, impact and human exposure assessment. This objective has been achieved only lately, attributed to the developments in analytical instrumentation and techniques that have enabled researchers to detect and quantify such organic micro-pollutants in these environmental matrices. However, because of the relatively recent interest in such studies, there is still a lack of validated and standardized protocols of analysis that guarantee the quality and fit to purpose of the results obtained. The main difficulties in the analysis of antibiotics in agricultural soils and crops are associated to the low concentration expected of these compounds, especially in the vegetal material, and the complexity of these matrices, which contain large amounts of endogenous components, like organic matter, pigments and fatty or waxy materials, which can interfere in the determination.

To evaluate the uptake and distribution of antibiotics in plants, different parts of crops, plant leaves, stems, roots and fruits, must be analyzed separately. If we add to this the large variety of soils and crops that can be studied, we can get an idea of the complexity of the analytical problem, since validated methods must be developed for specific matrices presenting differences in matrix effects. Other difficulties arise from differences in the structure and physicochemical properties of the antibiotics, which not only affect the behavior of these compounds in the agricultural environment as it is discussed in this paper, but also the extraction efficiency and analysis. For example, the strong interaction of tetracyclines with organic matter and clay components in soils results in their poor extraction, as well as in lowering the reproducibility of the measurements (Kulshrestha et al., 2004), while the pH values greatly influence the extraction of tetracyclines and fluoroquinolones from soil and vegetables (Hu et al., 2014). Consequently, reported methods usually include a limited number of target analytes, and a method for multi-residue determination of a broad range of antibiotics in soils and plants at environmentally relevant levels is demanded.

Finally, many antibiotics are metabolized or degraded after use, resulting in the formation of transformation products (TPs), which can be present in the irrigation water or can be generated once the antibiotics reach the agricultural media. These TPs represent a risk still not evaluated since scarce information about type and behavior of TPs present in the soil-plant system is available and effort has to be paid in developing analytical strategies for their identification.

3.1.1. Extraction methodologies

Previous to the analysis, a sampling strategy must be carefully designed in order to obtain representative samples, which provide reliable results. The type of soil and crop and agricultural practices in real conditions are crucial in defining the sampling protocol. Once the sample is obtained, sample preparation, including pretreatment and extraction, is critical to assure maximum recovery together with efficient removal of potential interferences. Pretreatment is usually focused to sample homogenization, and includes cutting, grinding, blending, sieving and lyophilization steps. As a common practice, plant materials are rinsed with deionized water to discriminate between contaminant deposition and uptake, whereas soils are dried at room temperature or lyophilized, before extraction (Matamoros et al., 2012).

With regard to the extraction, several techniques have been tested (Agüera and Lambropoulou, 2016). Those more commonly reported include solid liquid extraction (SLE) (Hawker et al., 2013), ultrasound-assisted solvent extraction (USE) (Solliec et al., 2016; Koba et al., 2017), pressurized liquid extraction (PLE) (Jacobsen et al., 2004; Franklin et al., 2016; Azanu, et a., 2016) or more recently QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction (Hu et al., 2014; Salvia et al. 2012). Table 2 includes, as an example, information of some of the reported methods. In most of the cases water, methanol, acetone, ethyl acetate and acetonitrile are the solvents of choice, in combination with buffer solutions (McIlvaine buffer, citrate buffer) and chelating agents (EDTA) that are being used to avoid the formation of chelate complexes between some antibiotics (tetracyclines) and metal ions present in soils (Li et al., 2011a). PLE and USE have been extensively used to extract various classes of antibiotics from soil (Jacobsen et al., 2004; O'Connor and Aga, 2007) and recently also from plant material, though a post-extraction "cleanup" is often required to remove co-extracts. SPE is the most widely used clean-up procedure and thus the extract is directly percolated or diluted in water (<5% organic content) and then percolated through the cartridge. Polymeric materials are the sorbents of choice because of its ability to retain compounds in a wide range of polarities.

Recently, the well-known QuEChERS extraction method, extensively applied in pesticide multi-residue analysis in crops, has extended its application to other environmentally relevant analytes and matrices (Bruzzoniti et al., 2014). In most of the cases, an appropriate method optimization is required and modified versions of the original procedure have been reported. Hu et al. (2014) propose the application of a modified QuEChERS method for determination of 26 veterinary antibiotics in vegetables. In this case, acetonitrile:methanol (85:15, v/v) was selected as the extraction solvent and the buffer composition was modified to increase the acidity of the extraction system in order to improve fluoroquinolones and tetracycline extraction. Recoveries higher than 60% were obtained for 23 of the 26 antibiotics tested. The use of UAE (Ferhi et al., 2016) and changes in the clean-up step (Salvia et al., 2012) have been also introduced for improving the recovery of antibiotics in soils.

3.1.2. Instrumental analysis

At present, liquid chromatography (LC) in combination with mass spectrometry (MS) is the primary technology used to analyze multiple antibiotics in wastewater as well as in soil and plant tissue extracts. Innovation in the theory and development of novel chromatographic columns has made reversed phase (RP) LC an accurate and reliable method for the determination of antibiotic residues (Seifrtová et al., 2009). The extra resolution provided by Ultra-High Performance Liquid Chromatography (UHPLC) systems gives greater information and reduces the risk of not detecting potentially important co-eluting analytes. In the last years, the use of sub-2 µm, or sub-2 µm core-shell columns steadily increased (Salvia et al., 2012; Solliec et al., 2016). Typically, C_{18}, modified or not with more polar functional groups (Hawker et al., 2013; Huang et al., 2015; Franklin et al., 2016) is by far the most widely used stationary phase; However, other phases (i.e phenyl, C_8 etc.) that offer different retention characteristics are also used (Hu et al., 2014; Azanu et al., 2016). As regards the mobile phase, in multi-residue studies, gradient elution of mixtures of water/acetonitrile or water/methanol is regularly reported. Volatile additives (e.g. formic acid, acetic acid, and ammonium acetate) at different concentration are used to modify mobile phase in order to improve the ionization of analytes and the MS detection sensitivity in the analysis of antibiotics, as well as to control pH (Hawker et al., 2013; Azanu et al., 2016).

With respect to mass spectrometers, tandem MS involving atmospheric pressure ionization, is nowadays state of the art, since these kind of analyzers combine two or more mass-to-charge ratio separation devices of the same or different types. Triple quadrupole (QqQ) and hybrid triple quadrupole-linear ion trap (QqQ-LIT) are among the most common and well established analyzers for target multi-residue methods (Azanu et al., 2016; Pan and Chu, 2016a). Selected Reaction Monitoring (SRM) is the preferred choice as acquisition mode. In this mode, selection of at least two specific transitions (precursor ion/product ion) is needed to fulfill requirements for a reliable quantification and confirmation of the analytes in the sample. Electrospray ionization (ESI), either positive (PI) or negative (NI), is usually the ionization mode of choice as it is more efficient for polar and ionizable compounds. In general, acidic groups are more compatible with NI whereas the presence of amine groups provides better performance in PI. Nevertheless, if wide scope methods are designed, this behavior is hardly predictable when multiple groups are present and both modes should be used. In multi-analyte methods, polarity-switching during the same run (available in modern instruments) between PI and NI may be necessary to cover the range of compound classes (Seifrtová et al., 2009; Farouk et al., 2015). Both, PI and NI modes were evaluated for the analysis of different classes of antibiotics (tetracyclines, macrolides, sulfonamides). Tetracyclines and macrolide antibiotics can be easily protonated and analyzed in PI mode. Sulfonamides can be readily detected in both NI and PI modes.

Signal suppression from the sample matrix and isobaric spectral interferences are among the major drawbacks of ESI mode that affect the sensitivity and should be always taken into consideration (Solliec et al., 2016; Koba et al., 2017). For this reason, the atmospheric pressure chemical ionization (APCI) mode was proposed as an alternative approach (Schlüsener et al., 2003). To overcome some of the ESI difficulties related to the matrix effects, matrix-matched calibration methodology is also applied (Koba et al., 2017).

Despite the tremendous technological advances of tandem triple quadrupole mass spectrometry in antibiotic target analysis, its use in non-target screening is still challenging. The desirable goal is the development of quantitative multiclass methods fully replacing the screening/confirmation traditional scheme, by expanding the ability to monitor a wider range of analytes, at the same time. In this context, the gradual introduction of high-resolution mass analyzers (LC HRMS), such as time-of-flight (TOF), Orbitrap, and hybrid mass spectrometer of quadrupole-time of-flight (Q-TOF) or Q-Orbitrap), has changed the mass spectrometry landscape for the determination of antibiotic residues, with non-target analysis. Among the different HRMS instruments, TOF is the least applied probably because it cannot operate as tandem MS spectrometer. On the other hand, hybrid QqTOF and Orbitrap with a linear ion trap (LTQ-Orbitrap) or a quadrupole (Q-Orbitrap) are recently applied for target and non-target screening (Laganà and Cavaliere, 2015; Senyuva et al., 2015; Tong et al., 2016). More specifically, in a very recent study, LC-Q-Orbitrap MS was applied for selective quantification and identification of veterinary antibiotics and their various transformation products in soils (Solliec et al., 2016). The use of hybrid quadrupole Orbitrap operated in a combination of full scan and fragmentation events named data-dependent acquisition (DDA) has previously been employed for the research of unknowns. The proposed extraction procedure (liquid solid extraction and solid-phase extraction were used for sample pre-concentration and purification) and the detection technique showed to be adequate, with LOD values ranging from 1.0 to 7.4 μg kg^{-1} for soils. Similarly, Koba et al. (2017), demonstrated the successful use of LC-Q-Orbitrap MS for the accurate determination of three antibiotics (clindamycin, sulfamethoxazole, and trimethoprim) and five of their metabolites in different soils matrices.

Overall, most of the published methods for the measurement of antibiotics in soil and plant matrices are designed to analyze several compounds belonging to the same family. Methods covering several families of antibiotics are still scarce. The current strategy is focused towards multi-residue and multi-class methods for the simultaneous determination of antibiotic compounds having different physicochemical properties. To this effect, HRMS and especially orbitrap-HRMS is the promising technology within this area and is expected to be also the clear leader in the antibiotic crop uptake studies.

3.2. Detection and quantification of ARB and ARGs in RWW, soil and crop samples

The occurrence and abundance of ARB and ARG in RWW and soil samples may be determined either based on culture-dependent methods or on the direct analyses of total DNA (see Fig. 2). Wastewater samples need to be concentrated prior to analyses and hence the membrane filtration method is frequently used, either for bacterial cultivation or total DNA extraction. In contrast, soil samples may be suspended in water to desorb bacteria prior to cultivation, or be used directly for DNA extraction (Fig. 2). Given the fact that culture-dependent methods are more laborious, time-consuming and generally regarded as less informative, than culture-independent approaches, they have been successively replaced by metagenomics and quantitative PCR methods (Szczepanowski et al., 2009; Nesme et al., 2014). However, culture dependent methods, in particular targeting enterococci and *Escherichia coli*, are still relevant approaches to target indicator bacteria. These methods, besides allowing a direct comparison with routine monitoring analyses, if adequately adapted for instance with the supplementation of antibiotics, may lead to the detection of rare resistance phenotypes and genotypes (da Costa et al., 2006; Varela et al., 2013; Varela et al., 2015). Bacterial isolates may be characterized for the antibiotic resistance profiles and the mobile genetic elements or ARGs, supporting a comprehensive survey of the multidrug resistance phenotypes or capability to spread resistance by HGT. Culture independent methods are nowadays simpler to perform and have the important advantage of allowing the detection of ARGs in non-culturable bacteria. These approaches can target specific ARGs or related genes, and are being used as quantitative methods (quantitative PCR, qPCR) or as qualitative analyses (metagenomics). The method of qPCR, if adequately calibrated, allows the measurement of the abundance of a specific gene per volume of wastewater or gram of soil (and plant tissue as well), or of the prevalence of that same gene, expressed as the ratio between the gene under analysis and a housekeeping gene, normally 16S rRNA. In metagenomics analyses the relative abundance of a given gene is normally expressed as a ratio between the number of sequence reads of the analysed gene vs the total number the reads or in relation to the number of reads of the 16S rRNA gene. Next generation sequencing analyses offer the possibility to have a broad view of ARGs, MGEs and bacterial populations, allowing simultaneously a good insight of the bacterial phylogenetic diversity in RWW or soil (Nesme et al., 2014). Not much is known about the occurrence of endophytic ARB in crops and this is in part due to technical challenges, referring mainly to low abundance of bacteria in such habitats.

4. Effects of antibiotics on soil biota (microbiome and soil fauna)

Antibiotics are regarded as persistent or 'pseudo-persistent' environmental contaminants of emerging concern. The ecotoxicological effects of antibiotics have been extensively studied, mainly with regard to the aquatic environment and the analysis of microorganisms; however, evidence on ecotoxicological effects on soil biota is still scant (Puckowski et al., 2016). Due to their persistence and known mode of action, antibiotics entering the soil are likely to disturb the complex regulatory networks in the soil microbiome and soil fauna, which are closely associated with soil quality and ecological function (Wardle et al., 2004; Becerra-Castro et al., 2015; Lopes et al., 2015). The effects of antibiotics on soil biota depend essentially on their bioavailability and, therefore, on soil properties, as well as on the availability of nutrients and the presence of root exudates (Halling-Sørensen et al., 2003; Bernier et al., 2011). Assays targeting the impact of antibiotics on soil microbial community function include microbial growth, respiration and enzyme activity, as well as functional diversity based on the community-level physiological profiling approach (culture independent methods) (Becerra-Castro et al., 2015; Brandt et al., 2015). The effects of antibiotics on soil bacteria, due to the application of antibiotic-contaminated manure or the artificial contamination of soil, are well documented using controlled pot experiments (Reichel et al.; Schmitt et al., 2005; Liu et al., 2009; Schauss et al., 2009; Lin et al., 2016). For example, Baguer et al. (2000) reported that tylosin and oxytetracycline antibiotics spiked in soil at environmentally relevant concentrations had no effect on earthworms, springtails and enchytraeids, following 21 days of incubation (the lowest observed effect concentration was 3000 mg kg^{-1}). Yu et al. (2011) showed that the observed behavior and growth defects (body bending frequency, reversal movement, omega turns and body length) of nematodes (*Caenorhabditis elegans*) following their exposure to sulfamethoxazole at environmentally relevant concentrations (1 ng L^{-1} - 100 mg L^{-1}) for 96 h transferred to the progeny. In addition, by using molecular assays, Pike and Kingcombe (2009) showed that the bacterial endosymbiont *Wolbachia*, which causes a variety of reproductive peculiarities, were successfully eliminated from the diplodiploid collembolan *Folsomia candida* through the continuous exposure of the populations (over two generations and several weeks) to rifampicin administered as 2.7% dry weight of their yeast food source, leading to the total sterility of all individuals of *Folsomia candida*, despite the continuation of normal egg production. Collectively, direct evidence indicating the impact of antibiotics on the microbial function and community structure in the agricultural environment as a result of RWW irrigation is extremely scarce. More precisely, only Ma et al. (2016) verified the effects of antibiotics applied through irrigation on soils microbial community, by conducting a controlled pot experiment using topsoil (0-20 cm) from a vegetable field following long-term RWW irrigation in northeast China. The studied soil was sprayed daily (for 120 days) with aqueous oxytetracycline solution in order to add 0.03 mg kg^{-1} day^{-1} of the antibiotic, aiming to mimic concentrations relevant in RWW irrigation. The daily oxytetracycline treatments promoted microbial carbon biomass (up to 2.64 times) and increased the McIntosh index of diversity ($p < 0.05$) between 60 and 90 days as calculated using Biolog data and compared with the zero oxytetracycline control, indicating a slight recovery in the soil microbial community and function (Ma et al., 2016).

Overall, RWW irrigation may introduce multiple changes to the soil biota, though it is extremely difficult to attribute such effects solely to antibiotics, as irrigation itself, or the introduction of other contaminants of emerging concern, or DOM or nutrients through RWW, may be responsible for such changes. Therefore, the effects of antibiotics on soil fauna due to RWW can be verified only through the performance of controlled experiments, where antibiotics may be applied either individually (see Ma et al., 2016) or in mixtures.

5. Antibiotic resistant bacteria and resistance genes

Irrigation with RWW may result in the continuous release of ARB and ARGs to natural and agricultural environments (Fatta-Kassinos et al., 2011a; Negreanu et al., 2012; Gatica and Cytryn, 2013; Rizzo et al., 2013), which in turn can potentially cause risks to human health, as human-associated susceptible pathogenic bacteria can become resistant by acquiring resistance genes or other organism that are already resistant in the soil environment (Berendonk et al., 2015). In the last years, a plethora of studies revealed the presence of ARB and ARGs in the RWW of WWTPs worldwide (da Costa et al., 2006; Szczepanowski et al., 2009; Munir et al., 2011; Gao et al., 2012). Advanced wastewater treatment processes (i.e. membrane biological reactors) are proved to significantly reduce the amount of these resistant elements in the RWW (Munir et al., 2011). RWW was reported to contain tetracycline and sulfonamide resistant bacteria among others, as well as few dozens of clinically relevant ARGs, including genes conferring resistance to aminoglycosides, β-lactams, chloramphenicol, fluoroquinolones, macrolides, rifampicin, tetracycline, trimethoprim, and sulfonamide antibiotics and other multidrug resistance genes (Szczepanowski et al. 2009). Moreover, the class 1 integron genes (*int*I1), often reported as a proxy of antibiotic resistance, was found in RWW (LaPara et al., 2011). The presence of ARB and ARGs in the agricultural environment receiving RWW and the potential implications that this phenomenon may pose to public health has recently attracted the attention of the scientific community (Holvoet et al., 2013; Broszat et al., 2014; Jechalke et al., 2015; Ben Said et al., 2016). Fahrenfeld et al. (2013) monitored the distribution of ARGs in the point of use of three RWW distribution systems in the western US and found that a broad spectrum of ARGs was present in the RWW passed through the distribution system, highlighting the importance of bacterial re-growth. The presence of L*mip* and *gad*AB genes at the point of use of RWW distribution system also revealed the presence of the waterborne pathogens *Legionella pneumophila* (L*mip*) and *Escherichia coli* (*gad*AB) in RWW. In addition, batch microcosm experiments revealed the presence of ARGs corresponding to resistance to sulfonamides (*sul1*, *sul2*) in soil following repeated irrigation with secondary-treated effluent (Fahrenfeld et al., 2013). With regard to the presence of ARB and ARGs in RWW-irrigated agricultural fields, results are controversial. Gatica and Cytryn (2013) reviewed recent studies that assessed the impact of RWW irrigation on antibiotic resistance in agricultural soils and concluded that RWW irrigation does not seem to impact antibiotic resistance levels in the soil microbiome. In addition, Negreanu et al. (2012) found identical or even lower levels of ARB and ARGs in agricultural soils irrigated with secondary-treated effluent for a prolonged period (6-18 years) in Israel compared with freshwater-irrigated soils. These findings suggest that antibiotic resistant elements released in RWW-irrigated soils are not able to compete or survive in the soil environment and that they do not significantly contribute ARGs to soil bacteria, corroborating to already reported existence of native AR in soil microbiome (D'Costa et al., 2006). Worth noting, tetracycline and ciprofloxacin resistant bacteria were absent from the freshwater samples, whereas their abundance in the RWW applied for irrigation ranged between 50 and 450 and between 700 and 1100 CFU mL^{-1} for tetracycline and ciprofloxacin, respectively (Negreanu et al., 2012). In contrast, higher diversity and increased abundance of various ARGs in soils of urban parks irrigated with RWW compared with freshwater irrigation or pristine soil were recently reported (Wang et al., 2014; Han et al., 2016). RWW irrigation of urban parks in Beijing, China, resulted in the increased abundance of *tet*G, *tet*W , *sul*1, and *sul*2 ARGs (Wang et al., 2014). The integrase gene (*int*I1) was also detected in high abundance and had significant positive correlation with *tet*G, *sul*1, and *sul*2 genes. Additionally, bacteria hosting *sul*2 and *int*I1 genes were related with bacteria, such as *Klebsiella oxytoca*, *Acinetobacter baumannii*, *Shigella flexneri*, whose potential to get in contact with humans may raise public health concerns (Wang et al., 2014). Han et al. (2016) reported that the ARGs detected in urban parks in Victoria, Australia, were significantly more abundant in RWW irrigated areas. Although the abundance of the genes *int*I1 and the transposase tnpA were not significantly higher in RWW-irrigated urban parks compared with the non RWW-irrigated ones, the patterns of ARGs in both types of area were different, demonstrating that the impact of RWW irrigation was noticeable (Han et al., 2016). The overview of the studies described above, indicates that ARB and ARG dynamics along the RWW-soil-crop continuum are highly complex and that the persistence of ARB and the horizontal transfer of ARGs across these environmental barriers undoubtedly depend on a myriad of biotic and abiotic factors. The apparently controversial findings about the impacts in soil due to RWW irrigation may result, at least in part, to some practical and methodological limitations that can be illustrated based on some simple assumptions. One refers to the fact that soil contains a high abundance of bacteria, which means that even if RWW-derived bacteria accumulate in the soil, it may take several decades to produce noticeable effects. One gram of bulk soil can contain 10^8 bacterial cells and more than 10^4 species (Raynaud and Nunan, 2014), while one mL of RWW may contain less than 10^6 bacterial cells, of which, in average, less than 10^3 host an acquired antibiotic resistance gene (Laht et al., 2014; Manaia et al., 2016). Assuming a soil water concentration of 10% (w/w), it could be estimated that the prevalence of acquired ARGs in soil would be of 0.0001%. Considering the unlikely scenario that due to aggregation or bacterial growth, the prevalence of that ARG increased 100 times, it would be 0.01%. This practical aspect stumbles on the second type of limitation, the methodological constraints. When DNA extracts, used for ARGs quantification, are prepared, normally from an amount equivalent to 0.25-1 g of soil, the ARGs that are being recovered are most probably close or below the quantification limit of most quantitative polymerase chain reaction (PCR) protocols commonly used and that ranges $1:10^3$-10^6 (ARGs:16S rRNA gene, referring to total bacteria) (Laht et al., 2014; Narciso-da-Rocha and Manaia, 2017). Moreover, since in the environment bacteria live mainly as aggregates, the quantification of ARGs may be also affected by random events. Hence, the heterogeneity of samples, not always overcome by technical replicates, and the aforementioned scarcity of ARGs, may lead to contradictory findings, as those reported above. Another approach that can be used to assess impacts of ARGs dissemination via RWW irrigation is based on metagenomic surveys. In metagenomics, as in quantitative PCR, the results are frequently expressed as a ratio between the gene of interest and a bacterial housekeeping gene, usually the 16S rRNA or, in alternative, to the total number of sequence reads (Graham et al., 2011; Christgen et al., 2015; Munck et al., 2015). Again, these values express a prevalence, which for the reasons evoked above may be too low to give an expressive result. It should be also noted that DNA may persist in soil for long periods of time and therefore targeting of ARGs using qPCR-based methods may in essence be targeting relic DNA bound to clay particles and/or organic matter and not viable bacteria (Becerra-Castro et al., 2015; Carini et al., 2016).

However, the apparent inconsistency of the studies that aim to assess the potential impacts of the use of RWW for irrigation, should not be perceived as the absence of risk. A given ARB and ARG even at very low prevalence in a given environment may represent a high risk for the spreading of antibiotic resistance or for human health. It will contribute for the spreading of antibiotic resistance, if the ARB has the capacity to proliferate in the environment, is ubiquitous and harbors mobile genetic elements that can be transferred by horizontal gene transfer. In addition, an ARB and ARG will represent a threat for human health, if humans have a high exposure to places where the ARB is present (e.g. food crops cultivated in RWW-irrigated fields), if the ARB is able to colonize humans and, in the worst case, if the ARB contains also virulence factors (Manaia, 2017). In this aspect, it is worth mentioning that numerous wastewater ARB and ARGs are also potential human pathogens (Vaz-Moreira et al., 2014). For example, members of the families *Pseudomonadaceae*, *Burkholderiaceae* or *Moraxellaceae* that include opportunistic pathogens such as *Pseudomonas aeruginosa*, *Burkholderia cepacia* or *Acinetobacter baumannii*, are frequently detected in RWW and are also able to survive in soil and even to have an endophytic lifestyle in different crops (Vaz-Moreira et al., 2014; Becerra-Castro et al, 2015).

6. Uptake of antibiotics by reclaimed wastewater-irrigated crop plants in real and simulated field conditions

Several classes of antibiotics have been proven to be taken up through roots and translocated to the aerial parts of crop plants grown under hydroponic or greenhouse control conditions, as well as in manure- and biosolids-amended and RWW-irrigated soils, in real agricultural systems (Boxall et al., 2012; Tanoue et al., 2012; Goldstein et al., 2014; Wu et al., 2015; Miller et al., 2016; Christou et al., 2017). Among them, chloramphenicol, sulfonamides, fluoroquinolones, and lincosamides are the ones with the highest bioconcentration factors (Pan et al., 2014). However, despite the relatively large number of predominantly descriptive studies undertaken in order to investigate root uptake of antibiotics, the mechanistic understanding of antibiotics uptake by crop plants remains rather limited (Miller et al., 2016). It has been previously shown that the uptake of antibiotics by crop plants is largely dependent on their bioavailability/bioaccessibility in soil pore water near the rhizosphere (sorption to soil constituents and transformation by soil organisms reduce bioavailability), and thus on their physicochemical properties and the properties of the soil environment (see Fig. 1) (Goldstein et al., 2014). Once taken up, the transport of antibiotics within the plant vascular translocation system (xylem and phloem) largely depends on their physicochemical properties (i.e. lipophilicity and electrical charge), as well as on the plant's physiology and transpiration rate (Goldstein et al., 2014; Dodgen et al., 2015) and environmental conditions (i.e. drought stress) (Zhang et al., 2016). Several antibiotics enter the root through the epidermis of growing root tips and subsequently pass through the cortex and the endodermis to reach the vascular tissues, where they can then be transported via the xylem to aboveground tissues. The movement of antibiotics from the soil pore water to the vascular tissues of plants may be distinguished to transmembrane, symplastic and apoplastic, depending on the ability of antibiotics to cross the membranes of plant cells (Miller et al., 2016). The presence of the Casparian strip in the endodermis which acts as a hydrophobic barrier between the apoplast and the vascular tissue, suggests that antibiotics must at least once follow the symplastic route, which is constituted of selective binding sites and channels (Kong et al., 2007; Tanoue et al., 2012; Malchi et al., 2014). As a result, the lipophilicity and speciation of antibiotics strongly affects their root uptake by and translocation within the plants. The octanol-water partition coefficient (K_{ow}) has been suggested as a predictor of uptake behavior of non ionizable organic compounds (Hsu et al., 1990). However, the movement of polar and ionizable antibiotics (the majority of antibiotics fall into this category) through plant cell membranes may be impeded by interactions with the negative surface potential of the cytoplasmic membrane (Trapp, 2004), by ion trapping, which is common for sulfonamides (Goldstein et al., 2014; Christou et al., 2016) and by sorption to plant cell walls (Trapp, 2004), making K_{ow} an inappropriate indicator for the estimation of ionizable antibiotics movement within and through plant cells. The pH-dependent speciation of ionic compounds (D_{ow}) is considered to be a more appropriate descriptor for the ability of ionizable antibiotics to cross cell membranes and translocated within the plant than K_{ow} (Wu et al., 2013b; Hyland et al., 2015).

The uptake and translocation of antibiotics within RWW-irrigated crop plants grown in real agricultural systems, where a cocktail of antibiotics occurs in RWW and the complexity of soil-plant-environment interactions prevails, has not been widely studied. Only few studies followed experimental setups where real RWW was applied for the irrigation of crop plants in field, representing actual farming practices, or genuine soil, or ecological conditions typical for commercial agriculture farming, simultaneously allowing for the assessment of the actual potential uptake of antibiotics by crops and its integration into a database for risk assessment (Malchi et al., 2014; Prosser and Sibley, 2015) (see Table 3). Wu et al. (2014) did not detect sulfamethoxazole and trimethoprim in plant tissues (root, leaf, stems, fruits) of vegetables growing in field and irrigated with both tertiary-treated effluent or tertiary-treated effluent spiked with the two antibiotics and 17 other pharmaceuticals and personal care products at a concentration of 250 ng L^{-1}, each. Lincomycin and ofloxacin antibiotics where detected in the leaves (0.12 and 0.10 μg kg^{-1} wet weight, respectively) of *Eruca sativa* L. plants grown in soil in pots under greenhouse conditions and irrigated with water spiked with these antibiotics, based on the mean concentration of these antibiotics found in RWW and rivers in Italy (0.25 and 0.15 μg L^{-1}, respectively) (Marsoni et al., 2014). However, these antibiotics were not detected in the grains of *Zea mays* L. grown under similar experimental set up and conditions (Marsoni et al., 2014). Moreover, sulfamethoxazole and sulfapyridine was not detected in tomato and cucumber fruits from plants grown in different types of soils (sand, aeolian, alluvial) under greenhouse conditions and irrigated with RWW (the mean concentrations of sulfamethoxazole and sulfapyridine was 0.28 and 0.17 μg L^{-1}, respectively), whereas sulfamethoxazole was found in the leaves of tomato plants grown in all three soil types and in the leaves of cucumber grown in sand, suggesting that sulfamethoxazole is preferentially transported in the xylem rather than in the phloem of tomatoes and cucumber plants (Goldstein et al., 2014). Sulfamethoxazole was also found in the roots (edible parts) of carrots and sweet potatoes (0.05-0.24 μg kg^{-1} wet weight) grown in soil in lysimeters and irrigated with RWW provided by a conventional activated-sludge wastewater treatment facility (the mean concentration of sulfamethoxazole in RWW was 0.05 μg L^{-1}) (Malchi et al., 2014). Riemenschneider et al. (2016) detected ciprofloxacin in the edible tissues of cabbage and carrot (~5 and ~10 μg kg^{-1} dry weight) grown in field and irrigated with water abstracted from the Zarqa River (the mean concentration of ciprofloxacin was 0.3 μg L^{-1}), which is constituted of RWW from the largest WWTP in Jordan (As Samra WWTP, activated sludge and chlorination) as the main component and spring and runoff water, in Jordan.

By conducting a field study, Christou et al. (2017) explored the long-term (three years) effects of two distinctly tertiary-treated effluents (effluent from WWTP applying activated sludge, slow sand filtration and chlorination, and effluent derived from an MBR treatment) applied for the irrigation of tomato plants under commercial agricultural farming on the fate of sulfamethoxazole and trimethoprim in soil and their uptake and bioaccumulation in tomato fruits. The concentration of these antibiotics was determined in fruits harvested at the end of the harvesting period (last harvest) for the first two years of the study, while at the third year of the study antibiotics' concentrations were determined at fruits harvested at the beginning (first harvest), middle (fourth harvest) and the end of the harvesting period (seventh harvest) (seven to eight harvests took place in each year of the study). Results revealed that the concentration of these antibiotics in both the soil and tomato fruits varied depending on the qualitative characteristics of the RWW applied for irrigation and the duration of irrigation. The concentration of both antibiotics in fruits increased with the increasing duration of RWW irrigation, reaching the highest concentration values during the last harvest of the third year of the study (5.26 μg kg^{-1} for sulfamethoxazole and 3.40 μg kg^{-1} for trimethoprim; in dry weight basis); the bioconcentration factor of sulfamethoxazole and trimethoprim reached its highest values during the last harvest of the third year of the study (5.42 and 6.44, respectively) (Christou et al., 2017).

The uptake of sulfamethoxazole, trimethoprim and ofloxacin by wheat plants (*Triticum aestivum* L.) grown in field and spray irrigated with RWW (effluent from WWTP where activated sludge and trickling filters were applied for treatment and disinfection) was evaluated at harvest, as well as three weeks before harvest, by Franklin et al. (2016). Straw and grain samples were rinsed with methanol prior to the extraction and analysis of antibiotics in order to remove chemical compounds adhering to the outer surfaces, simultaneously allowing for the estimation of antibiotics within these tissues, as well as on their surfaces. Residues of each compound were present on most plant surfaces. Ofloxacin was found throughout the plant, with higher concentrations in the straw (10.2±7.05 μg kg^{-1}) and lower concentrations in the grain (2.28±0.89 μg kg^{-1}). Trimethoprim was found only on the surfaces of grain (5.15±2.79 μg kg^{-1}) and straw (1.1±0.54 μg kg^{-1}), whereas sulfamethoxazole was concentrated within the grain (0.64 ± 0.37 μg kg^{-1}) (Franklin et al., 2016).

The above low volume of literature indicates that antibiotic uptake, translocation and accumulation in the edible parts of crop plants irrigated with RWW under real agricultural systems is feasible and likely dependent on crop species, soil type and soil pore water chemistry, the physicochemical properties of antibiotics, the concentration of antibiotics in RWW applied for irrigation and the duration that RWW irrigation is being practiced. Nonetheless, plenty of knowledge gaps still exist, requiring further studies utilizing RWW irrigation under field conditions. Such studies should incorporate a wider spectrum of plant species, while the concentration of antibiotics in RWW applied for irrigation, the soil and the edible parts of plants should be quantified, allowing for more accurate estimations of the bioconcentration factors and the estimation of potential public health risk associated with the consumption of such produce. The metabolites of antibiotics in plant tissues should also be quantified in studies evaluating the uptake of antibiotics by RWW-irrigated plants, since metabolites may occur in concentrations similar or even higher compared with the ones of parent compounds, while also being more toxic (Malchi et al., 2014; Miller et al., 2016; Paltiel et al., 2016). The potential uptake of ARB and ARGs by RWW-irrigated crop plants under real agricultural systems and their subsequent entry into the food web with serious human health implications is not yet systematically evaluated, despite the fact that these antibiotic related contaminants are continuously released in agricultural soils due to the use of RWW for irrigation (Munir et al., 2011; Fahrenfeld et al., 2013). At the same time, studies revealed the potential internalization of ARB and ARGs by plants irrigated with RWW or grown in antibiotic polluted soil under controlled greenhouse conditions (Ye et al., 2016; Zhang et al., 2016).

7. Phytotoxic effects

Exposure to antibiotics has been shown to exert significant effects on plant development and physiology, such as lower rates of germination, inhibition of growth, tissues deformation, reduced photosynthetic rate and chlorophyll content, disturbances in redox homeostasis and other stress-related phenomena (Michelini et al., 2013; Bártíková et al., 2016; Christou et al., 2016) (see Fig. 3). With regard to model organisms, a study with *Arabidopsis* indicated that chlortetracycline interfered with plant calcium homeostasis, thereby causing severe stress symptoms in both roots and shoots (O'Connor and Aga, 2007). Using hairy root cultures of *Helianthus annuus*, the direct involvement of stress mediated reactive oxygen species (ROS) in oxytetracyclin degradation could be proven (Bruzzoniti et al., 2014). However, the majority of studies uncovering the adverse effects of antibiotic exposure to plants were conducted under hydroponic experimental set up in laboratory conditions using unrealistic antibiotic exposure concentrations (Migliore et al., 2003; Kong et al., 2007; Farkas et al., 2009; Xie et al., 2010; Hillis et al., 2011; Li et al., 2011b; Michelini et al., 2013; Pan and Chu, 2016b). Fewer studies have been conducted in soil under control greenhouse or field conditions by using slurries and manures (Migliore et al., 2010), or by spiking the soil with the studied antibiotics (Liu et al., 2009). Marsoni et al. (2014) found no effects of lincomycin and ofloxacin on *Eruca sativa* L. and *Zea mays* L. grown in soil in greenhouse when applied through irrigation at concentrations of 1x, 10x and 100x of these antibiotics found in Italian RWW and rivers (0.15 and 0.25 μg L^{-1}, respectively). Christou et al. (2016) monitored the phytotoxic effects of sulfamethoxazole and trimethoprim in alfalfa plants grown in sand and irrigated for 50 days with nutrient solution spiked with the targeted antibiotics at environmentally relevant concentrations (10 μg L^{-1}) and found that stress-related effects, manifested via membrane lipid peroxidation and oxidative burst, were local and confined to the roots rather than systemically to shoots and leaves, and exacerbated when the tested antibiotics were applied in mixture. Moreover, Christou et al. (2016), uncovered the role of both H_2O_2 and NO in signal transduction for the orchestration of the detoxification mechanisms (induced antioxidant armory, induced expression of glutathione S-transferases; GSTs) in the leaves of alfalfa plants exposed to sulfamethoxazole and trimethoprim, as well as the involvement of proton pumps (H^+-ATPase) and cytochrome c oxidase (CytcOx) towards the detoxification of these antibiotics (Fig. 3). Overall, phytotoxicity is greatly dependent on factors including the compounds' properties and concentration in soil pore water, sorption kinetics, soil organic matter and pH, compound biodegradation rate, and the presence of other compounds in the soil.

8. Public health implications / Risk assessment

The antibiotic-mediated risks to human health associated with the consumption of agricultural produce from RWW-irrigated crops may be assessed either by estimating the daily or annual exposure of humans to antibiotics and converting it to medical dose equivalent (Marsoni et al., 2014; Pan et al., 2014; Wu et al., 2014), or by following the threshold of toxicological concern

(TTC) (Malchi et al., 2014), or the hazard quotient approach (Prosser and Sibley, 2015). The risks to human health due to the intake of antibiotics with the consumption of RWW-irrigated vegetables were revealed to be negligible by using the medical equivalent dose, as the daily intake due to the entrance of antibiotics in the food chain was 10-200 folds lower than the medical dose (Marsoni et al., 2014; Wu et al., 2014). By applying the TTC and hazard quotient approaches, Christou et al. (2017) assessed the effects of the intake of sulfamethoxazole and trimethoprim to both adults and toddlers due to the consumption of tomato fruits harvested from plants irrigated with tertiary-treated effluents during three consecutive growing periods in field, and found that the consumption of these fruits does not pose a health threat, since the daily consumption of tomato fruits by an adult in order to reach the TTC with regard to sulfamethoxazole and trimethoprim were 363.3 and 596.6 kg, whereas the respective values for toddler were 62.3 and 102.3 kg, respectively (the values of hazard quotient were equal or lower than 0.015). Marsoni et al. (2014) stated that the potential adverse effects of antibiotics along the food chain should not be neglected. Special attention should be given to antibiotics with structural alert for potential genotoxicity and carcinogenicity (i.e. sulfapyridine, sulfamethoxazole and ciprofloxacin) when the TTC approach is used, as the TTC value to be used for assessing the associated risks to human health is low (2.5 ng kg body weight^{-1} day^{-1}). In a recently published review article, Prosser and Sibley (2015) assessed the human health risks of antibiotics and other pharmaceuticals in plant tissues due to RWW irrigation (as well as biosolids and manure amendments) and found that the majority of individual pharmaceuticals in the edible tissue of plants due to RWW irrigation represent a *de minimis* risk to human health, although when assuming additivity, the mixture of pharmaceuticals could potentially present a hazard. This is not only true for the parent compounds as such, but also for the metabolites, especially in mixtures.

It is obvious from the above that further in-field studies need to be performed in order to obtain more solid information on the safety of RWW use for irrigation. Such studies should examine the effects of the mixture of antibiotics present in the treated flows used for irrigation, as well as the potential additivity or synergies of the mixture of antibiotics and heavy metals towards toxicity, as well as the toxicity of metabolites, some of which may be accumulated in greater concentrations and exert higher toxic effects compared with the parent compounds (Prosser and Sibley, 2015; Christou et al., 2017). Another type of risks and public health impacts associated with RWW-irrigation are the potential uptake of ARB and ARGs by plants via soil and their entry into the food chain. These are issues poorly understood, but the current knowledge cannot exclude the possibility that ARB thriving in the environment can be transmitted to humans (Ashbolt et al., 2013; Manaia, 2017). Even if at very low abundance these bacteria may be transmitted to humans, eventually in an asymptomatic long-term colonization, noticed only when for some reason the general health condition is compromised (Manaia, 2017). The assessment of these risks is still difficult to achieve, due to different types of limitation, for example: i) the technical shortcomings on the detection and quantification of ARB and ARG in environmental matrices described above; ii) the ignorance about the number of ARB that may be required to start a successful colonization in the human body; or iii) the scant information on the paths of dissemination and transmission from the environment to humans. All these are important limitations to establish adequate recommendations about maximum admissible threshold values or to define critical control points or critical sources for ARB dissemination.

9. Concluding remarks and recommendations for future research

The number of in-field studies aiming to examine the fate of antibiotics, ARB and ARGs in the agricultural environment as a result of RWW use for irrigation is currently limited. Moreover, comparison of the results among studies for reaching a more solid conclusion is rendered difficult due to the variations in plant growth conditions, analytical methods, RWW variability, species/cultivars studied, and data reporting methods (e.g. fresh vs dry weight), or due to insufficient information given (plant, environmental, and soil properties, irrigation regime, antibiotics concentration in RWW and soil, etc.) (Miller et al., 2016). Based on the knowledge gaps identified, we attempt here to provide recommendations for future research and suggest future directions:

- Studies should preferably be conducted under field conditions with genuine fully characterized soil, real RWW flows, and by following common agricultural practices. Importantly, the data regarding the antibiotics concentration in RWW applied for irrigation and in the soil, should be reported. Moreover, other data regarding (a) soil properties (e.g. the historical data of the field, soil pH, texture, CEC, electrical conductivity, organic matter content, nutrient concentration), (b) environmental conditions (e.g. temperature, humidity, abiotic stresses that may prevail during the experimental period), (c) irrigation regime and (d) agricultural practices undertaken, should also be reported. To this effect, apart from the extensive depiction of RWW-associated treatments, appropriate control treatments should be applied and fully described, as well. Control treatments may refer to the irrigation of plants with the same irrigation system (i.e. sprinkler, drip, sub-irrigation) and the same volumes and frequency as RWW-irrigated plants, with tubewell water or tap water in which the absence of antibiotics is verified before their use.
- The transformation products of antibiotics in RWW and in soil should be monitored. The potential uptake of metabolites and in general of the TPs present in agricultural soils as a

result of biotic and abiotic transformation by plants warrants further investigation. Metabolites in plant tissues should also be monitored, since sometimes they may exceed the concentration of the parent compounds and exert more acute toxicity.

- Comprehensive field-scale and microcosm studies should be conducted using a combination of culture-based and culture-independent analyses in order to measure the impacts in terms of ARGs and MGEs abundance and patterns, generating a body of information that support the assessment of potential risks of resistance propagation through the path wastewater-agricultural soil-crops-humans.
- Further in-field studies need to be performed in order to obtain more solid information on the possible public health risks of RWW reuse for irrigation. Such studies should examine the effects of the mixture of antibiotics present in the RWW used for irrigation, as well as the potential additivity or synergies of the mixture of antibiotics and heavy metals towards toxicity, as well as the toxicity of metabolites, some of which may be accumulated in greater concentrations and exert higher toxic effects compared with the parent compounds (Prosser and Sibley, 2015; Christou et al., 2017).
- Phytotoxic and other stress-related phenomena induced in crop plants under field conditions due to their exposure to antibiotics as a result of RWW irrigation, as well as the detoxification and overall defense mechanisms induced in response to such exposure, merit further investigation. Studies should be conducted in soils using RWW (mixture of pharmaceuticals), where additive or synergistic effects may prevail towards phytotoxicity.
- An important question still seeking answer is the potential effects of antibiotics uptake and accumulation in crop plant tissues on crop yield and fruit quality characteristics (marketability, taste, antioxidant activity, etc.).

- The effects of processes taking place in rhizosphere (root exudates and rhizosphere microbiota) on antibiotic uptake by plants merit further investigation. The uptake mechanisms of ionizable antibiotics (the majority of antibiotics) should be further explored in order to unravel passive and active (energy dependent via channels and transporters) uptake, as well as the translocation and reallocation of antibiotics within plant tissues (leaves, fruits) through the vascular tissues.
- The lack of validated and standardized protocols of analysis for the detection and precise quantification of antibiotics in all environmental matrices that can guarantee the quality of the results obtained, along with the complexity of these matrices and the reported metabolism and transformation of antibiotics within this matrices (formation of TPs), highlight the need for the development of validated and standardized methods for specific environmental matrices.

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Figure captions:

Figure 1. Main sources and fates of antibiotics in the agricultural environment receiving RWW for irrigation. The reuse of RWW for irrigation constitutes a significant pathway for the introduction of antibiotics to the agricultural environment, as conventional wastewater treatment processes are only moderately effective at removing antibiotics from the RWW. Consequently, antibiotics are routinely detected in RWW, and in RWW-irrigated agricultural soils and runoff from such sites, and surface and groundwater systems and sediments receiving RWW. Antibiotics are taken up by, and bioaccumulate in the edible tissues of RWW-irrigated crop plants, thus entering the food web with potential negative implications to public health.

Figure 2. Examples of objectives and applications of approaches commonly used to detect and quantify ARB and ARG in wastewater and in soil.

Figure 3. Following their uptake, translocation and bioaccumulation within crop plants, antibiotics may exert phytotoxic and stress related phenomena in an organ-specific manner. Plants employ sophisticated defense and detoxification mechanisms to overcome these adverse effects, with the enhancement of the antioxidant defense system and the induction of glutathione S-transferases (GSTs) and cytochrome P450 at the enzymatic and transcript level to be of high significance. Antibiotics are also metabolized within the plant cells through oxidation, reduction and hydrolysis, and through their conjugation with sugars and other macromolecules, which in turn facilitate their

sequestration in the vacuole or their exclusion to the apoplast. Figure is modified from Christou et al. (2016).

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