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ANTIBIOTICS IN AGROECOSYSTEMS: STATE OF THE SCIENCE

Challenges in the Measurement of Antibiotics and in Evaluating Their Impacts in Agroecosystems: A Critical Review

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Abstract

Large quantities of antibiotics are used in agricultural production, resulting in their release to agroecosystems through numerous pathways, including land application of contaminated manure, runoff from manure-fertilized fields, and wastewater irrigation of croplands. Antibiotics and their transformation products (TPs) exhibit a wide range of physico-chemical and biological properties and thus present substantive analytical challenges. Advances in the measurement of these compounds in various environmental compartments (plants, manure, soil, sediment, and water) have uncovered a previously unrealized landscape of antibiotic residues. These advanced multiresidue methods, designed to measure sub-ng g^{-1} concentrations in complex mixtures, remain limited by the inherent intricacy of the sample matrices and the difficultly in eliminating interferences that affect antibiotic detection. While efficient extraction methods combined with high sensitivity analysis by liquid chromatography/mass spectrometry can provide accurate quantification of antibiotics and their TPs, measured concentrations do not necessarily reflect their bioavailable fractions and effects in the environment. Consequently, there is a need to complement chemical analysis with biological assays that can provide information on bioavailability, biological activity, and effects of mixtures. Enzyme-linked immunosorbent assays (ELISA), often used as screening tools for antibiotic residues, may be useful for detecting the presence of structurally related antibiotic mixtures but not their effects. Other tools, including bioreporter assays, hold promise in measuring bioavailable antibiotics and could provide insights on their biological activity. Improved assessment of the ecological and human health risks associated with antibiotics in agroecosystems requires continued advances in analytical accuracy and sensitivity through improvements in sample preparation, instrumentation, and screening technologies.

Core Ideas

- Analysis of antibiotics in the agroecosystem is challenged by matrix effects.
- Bioreporters may be useful in measuring bioavailability of antibiotics.
- Identification of transformation products and information on their toxicity are needed.
- Knowledge on synergistic and antagonistic effects of antibiotic mixtures is lacking.
- Measured and predicted environmental concentrations differ significantly.

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Measurement of antibiotic residues in the environment provides critical information on their potential to cause undesirable effects to ecosystem function and to animal and human health. Prioritization of which antibiotics should be included in the analytical methods for detection in agroecosystems has traditionally focused on heavily used antibiotics, especially those frequently used in treating or preventing infections or in improving feed conversion efficiency. Although there are large variations in the quantities of individual antibiotics sold for food-producing animals (Table 1), there are equally large variations in the accuracy of measurements and reported occurrences of antibiotics in various environmental compartments (manure, soil, and water) (Hernández et al., 2007; Kummerer, 2009; Vanderford et al., 2014). Because the potential for antibiotics to cause undesirable effects in agroecosystems is linked to their use, persistence (in vivo and environmental), and inherent biological activity (USEPA, 2013), prioritization of which antibiotics or class of antibiotics should be measured is not a straightforward task.

Residues of persistent and frequently administered antibiotics, such as tetracyclines and sulfonamides, are typically detected at μg kg^{-1} to mg kg^{-1} levels in animal manure (Hamscher et al., 2005; Ho et al., 2013; Hu et al., 2010; Kumar et al., 2005) and agricultural runoff (Campagnolo et al., 2002). Previous studies (Aga et al., 2003; Aga et al., 2005) have also shown that tetracyclines introduced into the soil through manure application are transformed into persistent by-products, some of which may still be biologically active and are potentially more toxic than the parent antibiotic. Many veterinary antibiotics that have been linked to the development of antibiotic resistance in human pathogens, such as fluoroquinolones, are now used less frequently in agriculture because of food and human safety concerns (Lipsitch et al., 2002; Nelson et al., 2007; Luby et al., 2016). Depending on environmental conditions, physicochemical characteristics, and manure handling practices, antibiotic

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; LC-MS/MS, liquid chromatography–tandem mass spectrometry; NOM, natural organic matter; PEC, predicted environmental concentration; PLE, pressurized liquid extraction; QqQ, triple quadrupole; SPE, solid-phase extraction; ToF, time-of-flight; TP, transformation product.

concentrations in the agroecosystem will decrease over time due to irreversible sorption, dispersion, and/or degradation. Dissipation through multiple mechanisms results in concentrations in most compartments falling below the detection limits of existing analytical methods in the absence of further manure application (Homem and Santos, 2011). Nevertheless, evidence suggests that even subinhibitory concentrations of antibiotics have the ability to function as signaling molecules and can accelerate the evolution of antibiotic resistance in exposed bacteria (Andersson and Hughes, 2014). Recent evidence suggests that the occurrence of antibiotic resistance is highly favored in environmental compartments located near point sources for antibiotic residues, such as feed lots that are constantly receiving antibiotics from animal excretions (Sura et al., 2015). It has been suspected that the occurrence of antibiotic resistance in these hotspots that are subjected to pollution by antibiotic residues from manure may be due in large part to the presence of more persistent and biologically active transformation products (TPs) of antibiotics.

In recent years, modern instrumentation has advanced to achieve ultra–trace level detection limits (sub-pg kg^{-1} or parts per quadrillion) for some antibiotics. Nevertheless, difficulties in separating antibiotics from complex sample matrices still limit the ability to accurately and reproducibly measure antibiotics and their TPs. Even more challenging to assess are the ecological implications of biologically available (or bioavailable) antibiotic residues at their predicted environmental concentrations (PECs) in soil and water. This paper aims to summarize and examine the state of the science for detection and quantification of antibiotics and their TPs in the agroecosystem. Although the analytical methods described in this review are not unique to antibiotics, the discussion in this paper will focus on specific analytical challenges posed by manure, soil, and other relevant matrices affecting trace analysis of antibiotics in agroecosystems. The challenges and advances in the accurate measurement of antibiotics and their TPs in complex environmental matrices (e.g., soil, manure, plants) are highlighted, along with the current limitations of published studies on the ecological impacts of antibiotics. The need for faster, more cost-effective analytical approaches for on-site monitoring of antibiotic residues is discussed. Finally, research gaps critical to advancing our understanding of the human and ecological risks associated with antibiotic residues resulting from agricultural use are identified.

Determining Occurrence, Fate, and Biological Effects of Antibiotics in the Environment

Antibiotics are administered in animals to inhibit the reproduction of pathogenic bacteria through either the bacteriostatic (growth inhibition) or the bactericidal (killing bacterial cells) mode of action. In 2013, approximately 14.8 million kg of antibiotics and antimicrobials were sold in the United States for use in food-producing animals (USFDA, 2015); of this amount, tetracyclines accounted for 44%, and ionophores accounted for 30%. Table 1 presents a list of the major classes of veterinary antibiotics used in animal agriculture in the United States, classified based on their mechanisms of action. The table also provides a sample antibiotic from each class and reports important parameters relevant to their environmental fate and behavior. Depending on the animal species and the antibiotic class, up to 90% of the drug dosage can be eliminated in nonmetabolized form in feces or urine (Kumar et al., 2005; Sukul et al., 2009). Consequently, cropland application of manure from treated animals is a major pathway for antibiotics to enter the environment.

Residues of veterinary antibiotics have been reported in multiple compartments of the agroecosystem, from animal feeding operations to soils and receiving waters. Reported concentrations of antibiotic residues in animal manure are wide ranging, depending on the use patterns (e.g., growth promotion, therapeutic, or subtherapeutic) and the type of livestock operation. In general, dairy facilities in the United States with no young calves contain relatively low antibiotic concentrations due to restricted use for therapeutic purposes (Watanabe et al., 2010), with typical concentrations ranging from 40 to 180 μg kg^{-1} for

Table 1. Antibiotics used in animal agriculture in the United States.

Drug class	Average quantity sold in the United States for veterinary use 2013†	Example compound	Water solubility‡	Octanol/water partition coefficient‡	Elimination half-life§	Primary metabolism reaction§	Fraction unmetabolized§
	kg		mg L^{-1}		h		%
Aminoglycosides	270,342	neomycin	10,000–500,000	−8.1 to −0.8	2–3	–	80–90
Cephalosporins	28,337	ceftiofur	22–10,100	0.9 to 2.7	0.5–6	deacetylation	<10
Ionophores	4,434,657	monensin	<0.003¶	5.4 to 8.5	2–3	demethylation	50–80
Lincosamides	236,450	lincomycin	>500,000#	0.2 to 2.6#	3–4	demethylation	10–50
Macrolides	563,251	tylosin	0.45–15	1.6 to 3.1	1–5	demethyation	10–80
Pencillins	828,721	penicillin G	22–10,100	0.9 to 2.7	0.5–3	hydrolysis	80
Sulfonamides	384,371	sulfadimethoxine	7.5–1,500	−0.1 to 1.7	3–10	oxidation, deactylation	20–50
Tetracyclines	6,514,779	chlortetracycline	230–52,000	−1.3 to 0.05	6–13	epimerization	60–80
Others††	1,527,646	–	–	–	–	–	–

† USFDA (2015).

‡ Thiele-Bruhn (2003).

§ Merck manual (Merck, 2015).

¶ Ionophore solubility may be much higher depending on presence of sodium.

Lincosamide properties estimated.

†† "Other" includes aminocoumarins, amphenicols, diaminopyrimidines, fluoroquinolones, glycolipids, pleuromutilins, polypeptides, quinoxalines, and streptogramins.

sulfonamides and tetracyclines in lagoon sediments. Other large-scale livestock feeding operations that administer subtheraputic levels of antibiotics often exhibit greater antibiotic concentrations that can reach as high as mg kg^{-1} dry weight but that often range from a few μg kg^{-1} to tens of mg kg^{-1} for fluoroquinolones, sulfonamides, and tetracyclines in poultry and swine (Hu et al., 2010; Martinez-Carballo et al., 2007; Zhao et al., 2010).

Antibiotic residues have been measured in other compartments after manure application on agricultural fields, resulting in detectable quantities of antibiotics in soils and surface water (Campagnolo et al., 2002; Martinez-Carballo et al., 2007; Sura et al., 2015; Wei et al., 2011). Levels ranging from below detection limits to mg kg^{-1} quantities have been observed in soil after manure application (Hu et al., 2010; Kemper, 2008). Corresponding antibiotic concentrations in runoff and surface waters have been reported at concentrations higher than 200 μg kg^{-1} (Watanabe et al., 2010; Wei et al., 2011); however, concentrations in the aqueous fraction are strongly governed by the sorption partition coefficient of the antibiotics (Kummerer, 2009).

Transfer from manure to soils and subsequent uptake into crops have been reported for a number of antibiotics. Notably, some crops, such as barley and rice, may concentrate antibiotics in their root systems. For example, ciprofloxacin and narasin have been detected in the root compartments of barley (Eggen et al., 2011). Hawker et al. (2013) reported the bioconcentration of zwtterionic antibiotics, including oxytetracycline, chlortetracycline, and norfloxacin, in rice roots. Oxytetracycline has additionally been shown to bioconcentrate in several aquatic plants used for human consumption after fertilization with contaminated swine manure (Boonsaner and Hawker, 2015).

Plant uptake of antibiotics from soil has been shown to be both compound specific and plant dependent, with differences reported at the subspecies level (Sallach et al., 2015). Uptake into plants is thought to be dependent on partitioning in soil pore water (Carter et al., 2014), and translocation within the plant has been shown to correlate with ion speciation, both inside and outside of the plant (Dodgen et al., 2013; Trapp and Mc Farlane, 1995), as well as with the lipophilicity of individual compounds (Briggs et al., 1982; Hawker et al., 2013). In addition, evidence suggests that antibiotic compounds are transformed inside the plant (Goldstein et al., 2014). However, few studies monitor the uptake and accumulation of transformation products in the soil–plant system. Generally, studies investigating the uptake of antibiotics into plants irrigated with recycled wastewater have found plant concentrations to be low, rarely exceeding the limits of detection (Goldstein et al., 2014; Wu et al., 2014). Plant uptake of pharmaceuticals originating from municipal source (wastewater recycling and biosolid soil amendments) has been recently reviewed elsewhere (Wu et al., 2015).

Certain widely used antibiotics have additionally been shown to bioconcentrate in aquatic organisms after prolonged exposure. Antibiotics belonging to the fluoroquinolone, lincosamide, macrolide, sulfonamide, and tetracycline classes have been shown to bioconcentrate in the bile, plasma, liver, or muscle of nine fish species endemic to rivers near established point sources (Zhao et al., 2015b). Erythromycin was detected in more than 80% of all samples and at concentrations of up to 2390 μg kg^{-1} (wet weight) in liver samples, whereas other antibiotics were detected in varying quantities at a lesser frequency; for instance, tetracyclines were detected in the liver in only 2% of the sampled fish. The use of erythromycin in aquaculture as a feed additive has widely resulted in its detection at ng g^{-1} to μg g^{-1} quantities in both cultivated (Chen et al., 2015) and wild (Zhang et al., 2015; Zhao et al., 2015b) aquatic organisms. Similarly, sulfonamide antibiotics, such as sulfadiazine and sulfamethoxazole, have been shown to bioconcentrate in noncultivated fish species, including the common carp (Zhao et al., 2015a).

Because the biological activity of antibiotics is governed by their physicochemical properties, compounds within a class that are structurally very similar can be expected to have similar environmental fate and behavior (Figueroa-Diva et al., 2010). Unlike neutral organic pollutants, the mobility of antibiotics in soil is not directly related to their Log octanol-water partition coefficient or water solubility values (Table 1) because many antibiotics have ionic properties that govern their sorption behavior in soil. For instance, antibiotics that are organic bases (e.g., aminoglycosides) can adsorb to cation exchange sites of clays at pH lower than their acid dissociation constant (p*K*a) values via cation bridging (McBride, 1994; Thiele-Bruhn, 2003). On the other hand, antibiotics that are organic acids (e.g., cephalosporins, penicillins) are minimally bound to soil surfaces, except in rare cases when soil surfaces are positively charged (Aksu and Tunç, 2005; McBride, 1994). Some antibiotics are sorbed in soil by more than one mechanism. For example, tetracyclines have three different p*K*a values; they exist as cationic, zwitterionic, or anionic species and are sorbed in soil by various mechanisms, depending on the pH of the soil (Kulshrestha et al., 2004; Sassman and Lee, 2005). Other polyfunctional ionogenic antibiotics, such as amphenicols and fluoroquinolones, can interact with the soil in multiple ways, and therefore their environmental behavior is difficult to predict based on electronic and hydrophobic effects alone (MacKay and Vasudevan, 2012).

The physicochemical properties of antibiotics determine to a large degree if antibiotics are bioavailable in soil, which in turn dictates whether they will exhibit biological effects or if they will biodegrade into nonbiologically active compounds. The term "bioavailability" has many definitions, depending on the field of science. Some researchers (e.g., Semple et al., 2004) define the bioavailable fraction of a contaminant to be the portion that can freely cross the cellular membrane of an organism at any given time and environmental condition. On the other hand, the "bioaccessible fraction" is the portion that can freely cross the cellular membrane only when the organism has access to it. According to these definitions, both bioavailable and bioaccessible fractions may be transferred from the environment and penetrate a cell, potentially causing biological effects. Ideally, each fraction should be measured to assess the ecological impacts of antibiotics present in the environment; however, measuring bioavailability of antibiotics presents unique challenges.

A common procedure for measuring the bioavailability of contaminants involves extracting the contaminant in question from the matrix (e.g., soil) using organic solvents and then quantifying the concentrations in the extract. Unfortunately, there is a risk that a given extraction procedure will change the form of these compounds (e.g., degradation), leading to their nondetection or to a false-negative detection (Wegst-Uhrich et al., 2014). Therefore, in practice the bioavailable fraction of an antibiotic is difficult to determine because its recovery in soil or other

complex matrices does not necessarily correspond to the fraction that plants or microbes can assimilate in nature (Naidu, 2008).

Several attempts have been made to examine the bioavailable fraction of organic compounds in soil and sediment using nonexhaustive, mild extraction techniques. Kelsey et al. (1997) demonstrated a time-dependent reduction in the bioavailability of anthropogenic contaminants to earthworm and bacteria and showed that it is possible to approximate uptake over time by means of mild solvent extraction. In similar work, β-cyclodextrin–based extractions have been shown to extract labile hydrophobic materials, such as polycyclic aromatic hydrocarbons, from soils and sediments with acceptable correlation to biotic uptake (Reid et al., 2000). Such methods have additionally proven successful for contaminants in dissimilar soils (Swindell and Reid, 2006). Other methods using selective supercritical fluid extraction (Björklund et al., 2000) have also been reported for the determination of bioavailable compounds.

Because bioavailability is strongly dependent on the chemical species, a suitable extraction method that does not change the chemical structure of the antibiotics is important (e.g., no redox reaction, no dehydration reaction occurs during extraction). Moreover, it is more useful to examine the bioavailable fraction of antibiotics in the natural environment and relate this to the fraction that can induce selection pressure for antibiotic resistance. Progressing from detection of antibiotic residues to the evaluation of ecological effects is critical in linking the detected traces of antibiotics to their biological effects and should receive substantive attention in future research activities. Because bioavailability depends on existing environmental conditions (e.g., pH, organic carbon content, soil type) and the organisms in that environment (Semple et al., 2004), a gap likely occurs between what is measured in the laboratory and what is truly bioavailable. The potential use of bioreporters in assessing bioavailable antibiotics in soil is discussed in a later section of this review.

Extraction of Antibiotics from Complex Matrices

Aqueous Samples

Analysis of antibiotics in various environmental samples typically requires a complex set of extraction and clean-up strategies to eliminate or minimize interferences while ensuring quantitative recovery between widely variable sample matrices. Extraction is also performed to concentrate the target compounds to improve detection limits for antibiotics. Extraction of antibiotics from water samples is relatively straightforward compared with extraction from solid samples. Solid-phase extraction (SPE) has become the method of choice for aqueous samples because it offers several advantages over liquid–liquid extraction, such as improved selectivity, specificity and reproducibility, minimal organic solvent consumption, shorter sample preparation time, ease of operation, and the potential for automation (Poole, 2003). Preconcentration, isolation of antibiotics, and removal of sample matrix can be achieved simultaneously with a properly designed SPE procedure. Varieties of commercial sorbents are available with different surface functionalities that allow customized preconcentration and purification of antibiotics. Reversed-phase polymers, sometimes in combination with anion exchangers, can be used to selectively retain target antibiotics and allow interferences to pass through. The adsorbed antibiotics are then quantitatively recovered through elution with a properly selected organic solvent to overcome the affinity of the compounds to the sorbent while minimizing elution of unwanted matrix components.

Among current commercial SPE cartridges used for the extraction of antibiotics from environmental samples are polymer blend sorbents with mixed polarity, which have found wide applications in the simultaneous extraction of multiple classes of antibiotics (Diaz et al., 2013; Tong et al., 2009; Wei et al., 2011). Highly specific sorbents, such as molecularly imprinted polymers, have also been used to improve selectivity (Chen et al., 2010; He et al., 2007). Unfortunately, commercially available molecularly imprinted polymers for antibiotics are very limited and are often prohibitively expensive; most of them also have limited sample capacity and have not proven useful for practical applications in antibiotic analysis in real environmental samples. Although matrix minimization is often achieved using SPE, matrix effects can remain problematic in samples with high concentrations of natural organic matter (NOM), such as wastewater, manure, and soil extracts. Natural organic matter, typically characterized as polyphenols and long-chain carboxylic acids, can inhibit antibiotic–sorbent interactions in SPE by either competing with the sorption sites or by complexing with the target antibiotics. Often, the matrix interferes with antibiotic recovery in SPE, reducing the number of free sites available for retention (Turiel et al., 2003). In addition, coextracted matrix components eluted with antibiotics during solvent elution can result in signal suppression during analysis by liquid chromatography/mass spectrometry (LC/MS), as discussed below.

Solids or Semisolids

Extraction of antibiotics from soil, manure, and other solid samples (e.g., fish, meat, vegetables, etc.) is even more challenging than extraction from aqueous samples due to the abundance of potential interferences. For example, extraction of tetracyclines from soil is often poorly reproducible due to variable concentrations of NOM combined with the occurrence of multivalent cations that complexes with tetracyclines (Lindsey et al., 2001). The strong interaction of tetracyclines with NOM and with clay components in soil can lead to poor extraction efficiencies and reproducibility (Kulshrestha et al., 2004).

Pressurized liquid extraction (PLE) is a relatively simple, yet exhaustive, extraction technique designed to reduce overall extraction time through the use of elevated temperatures and pressure to rapidly extract analytes using minimal volumes of organic solvents (Tobiszewski et al., 2009). The use of subcritical solvents for extraction results in dramatically increased analyte desorption rates and improved solubility above the solvent's ambient boiling point, allowing for substantially shortened extraction times without a decrease in recoveries relative to other common extraction methods (Tobiszewski et al., 2009). The use of an inert solid support in the extraction cell also provides an added filtration (Nieto et al., 2010b). Pressurized liquid extraction has been used to improve recovery of tetracyclines from soils (Jacobsen et al., 2004; O'Connor et al., 2007) and has been used to extract other classes of antibiotics from soil and sewage sludge (Golet et al., 2002; Schlusener et al., 2003). However, the high extraction efficiency offered by PLE is a "double-edged sword" because it also leads to coextraction of NOM, which interfere

in the detection (Jacobsen et al., 2004; O'Connor et al., 2007). Postextraction "cleanup" or separation of interferences is often implemented when analyzing extracts from highly complex matrices, such as manure and soil.

Methods using PLE have been published in recent years to extract multiple classes of antibiotics and pharmaceuticals from sludge and manure (Barron et al., 2008; Chen et al., 2013; Diaz-Cruz et al., 2006; Ding et al., 2011; Jelic et al., 2009; Lillenberg et al., 2009; Nieto et al., 2010a; Okuda et al., 2009; Pamreddy et al., 2013; Radjenovic et al., 2009) as well as vegetative matrices (Wu et al., 2012). These studies demonstrate the difficulty in the development and application of multiresidue methods for solid samples. For example, recoveries for β-lactams were reported to be less than 1%, whereas sulfonamides exhibited a large range of recoveries from near 1 to 104% (Diaz-Cruz et al., 2006). Recovery of antibiotics can be optimized for a particular application based on a subset of compounds and sample matrix. However, individual recoveries for each antibiotic will vary depending on the physicochemical properties of target antibiotics and the nature of the sample matrix.

Despite its limitations, PLE may be optimized by individually tuning the principal extraction parameters, including solvent or solvent composition and pH (Barron et al., 2008; Chen et al., 2013; Diaz-Cruz et al., 2006; Göbel et al., 2005; Golet et al., 2002), temperature (Diaz-Cruz et al., 2006; Golet et al., 2002), extraction pressure and static extraction time (Golet et al., 2002; Pamreddy et al., 2013), number of extraction times and cycles (Diaz-Cruz et al., 2006; Radjenovic et al., 2009), and sample mass (Barron et al., 2008). Each variable may have a significant effect on recovery (Nieto et al., 2010b). Pressurized liquid extraction remains advantageous over newer techniques, such as microwave-assisted extraction, because it is not limited to microwave-active solvents (Nieto et al., 2010b); however, PLE remains limited in its "tenability" toward specific analytes during extraction and minimization of coextraction of matrix interferences.

Detection of Antibiotics in Environmental Samples

High-Performance Liquid Chromatography with Mass Spectrometric Detection

High-performance liquid chromatography with tandem mass spectrometry (LC/MS/MS) has emerged as the primary analytical tool for quantifying antibiotics in environmental samples, either with a triple quadrupole (QqQ), a quadrupole time-of-flight (Q-ToF) MS, or an Orbitrap MS. A QqQ MS detector can provide high-selectivity detection of antibiotics through "in-space" tandem mass spectrometry (Johnson et al., 1990). An ion trap MS also provides "in-time" tandem MS capabilities (March, 1997) but may be subject to space charge effects or "fragile ions" (Snow et al., 2003). High-resolution (ToF, Q-ToF, and Orbitrap) MS instruments are best suited to identification, using accurate mass determination of molecular and fragment ions. Although high-resolution MS instruments are more expensive than QqQ MS, they are becoming more widely available in many environmental laboratories because of the increased interest in nontarget analysis, where unknown contaminants are detected and identified based on the elemental composition predicted by accurate mass measurements and mass spectral fragmentation pattern. Screening methods for antibiotics have been performed by Q-ToF MS in waste and surface waters (Ibanez et al., 2009) and in animal hair and feed (van der Heeft et al., 2009).

Advances in analytical instrumentation have allowed detection of low concentrations of antibiotics in complex environmental matrices due to the high sensitivity and selectivity of mass spectrometers. However, the complex nature of soil and manure, combined with the low concentrations of antibiotics typically found in these samples, pose special analytical challenges. For instance, LC/MS/MS with electrospray ionization source is prone to signal suppression, particularly in the presence of high amounts of NOM. As shown in Fig. 1, the signal of 25 ng mL^{-1} minocycline (a tetracycline antibiotic commonly used as an internal standard) spiked into a manure extract is more than 50% suppressed relative to the signal of the same concentration spiked in the mobile phase used in the LC/MS/MS method. Signal suppression results in poor detection limits and variability in recovery. Signal suppression encountered in LC/MS/MS analysis of antibiotics may be alleviated with extensive sample clean-up steps, but these steps could result in increased cost and greater sample loss and could potentially introduce errors in the analysis.

Analysis of antibiotics in complex matrices also results in variable chromatographic retention times, which could lead to either false-negative or false-positive detection if peaks are not carefully integrated. One study reported retention time drift up to 3 min when analyzing manure samples for tetracyclines compared with the standard mixture (Aga et al., 2003). Although quantification by isotope dilution or standard addition methods can correct for potential errors in the identification and quantification due to retention time drifts and signal suppression (Vanderford et al., 2003), labeled isotopes will add additional cost to the analysis and are sometimes not readily available.

Differences in the chemical properties between multiple parent antibiotics and their metabolites or TPs can be quite substantial. Because chemical properties, sample complexities, and study objectives often dictate what compounds are measured and where, analytical methods are developed and tested using representative matrices. Multiresidue methods afforded by LC/MS/MS are preferred for studies focused on assessing antibiotic

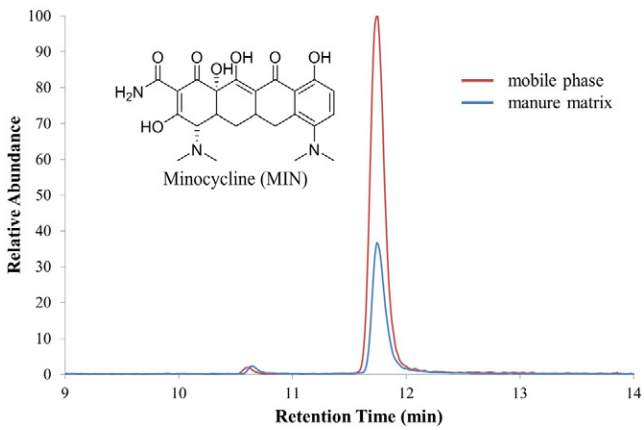

Fig. 1. Liquid chromatography–tandem mass spectrometry chromatograms of a 25 ng mL^{-1} minocycline internal standard spiked in 500 mg dried, raw manure extract (blue line) and 95/5 water/methanol diluent (red line), illustrating more than 50% signal suppression in manure matrix.

occurrence in the environment and are necessary for evaluating degradation, partitioning, and ultimate fate of these compounds in the environment (Gros et al., 2006). However, there are always trade-offs in sensitivity and accuracy when developing multiresidue analytical methods because "one method does not always fit all." Although some analytes may have excellent recoveries and have signals that are not affected by matrix, others may suffer from poor recoveries or significant matrix effects. Therefore, although it is an added expense, it is very important to use isotope-labeled analogs as surrogate standards in LC/MS/MS analysis whenever possible.

Isotope Dilution Mass Spectrometry

Isotope dilution uses a labeled surrogate standard to account for losses due to sample handling and variable analyte response arising from matrix effects. In isotope dilution, a known amount of analyte labeled with a stable isotope (e.g., deuterium, ^{13}C, ^{15}N) is added to the sample before any sample preparation or extraction to correct for matrix effects, losses during extraction, and instrumental variations for the unlabeled target analytes. Ideally, an isotopically labeled analog of each analyte is added for every target compound. However, this approach can be cost prohibitive and is sometimes not possible because of the lack of available labeled analogs.

Although structurally related compounds from the same class of antibiotics can be used as an internal standard in lieu of isotopically labeled analogs, accurate quantification is not always guaranteed. For example, demeclocycline, which is not used in animals and is a structural analog to the approved tetracycline antibiotics (Fig. 2), may be used as an internal standard in analysis of tetracyclines because there are no isotopically labeled tetracyclines available. However, demeclocycline does not have the same chemical properties or retention times as chlortetracycline, oxytetracycline, or tetracycline antibiotics. Thus, the ionization efficiency of demeclocycline may be affected slightly differently compared with the other tetracycline compounds. In addition, demeclocycline has been shown to epimerize in the presence of some complex matrices that may increase variability in peak integration (Sallach et al., 2015). Selection of internal standards in LC/MS/MS must consider chromatographic retention times, relative recoveries, and susceptibility to matrix effects.

Identification of Transformation Products

Many antibiotics are metabolized or degraded after use, resulting in the formation of transformation products (TPs). Many TPs retain some antimicrobial activity and continue to exert selection pressure on microbiota after undergoing structural changes, whereas other TPs are converted back into the active ingredient (Díaz-Cruz and Barceló, 2007). Transformation products often go unidentified but may exist in an environmental compartment long after the parent antibiotic, and it is becoming increasingly important to make efforts to identify and quantify TPs in environmental studies. Ion trap MS can be used to identify TPs through successive fragmentation. For example, Eichhorn and Aga (2004) demonstrated the utility of both ion trap MS and high-resolution MS in identifying transformation products of chlortetracycline in hog manures. Similarly, Hoff et al. (2014) isolated and identified several previously unknown, species-specific metabolites of sulfaquinoxaline in the tissues of

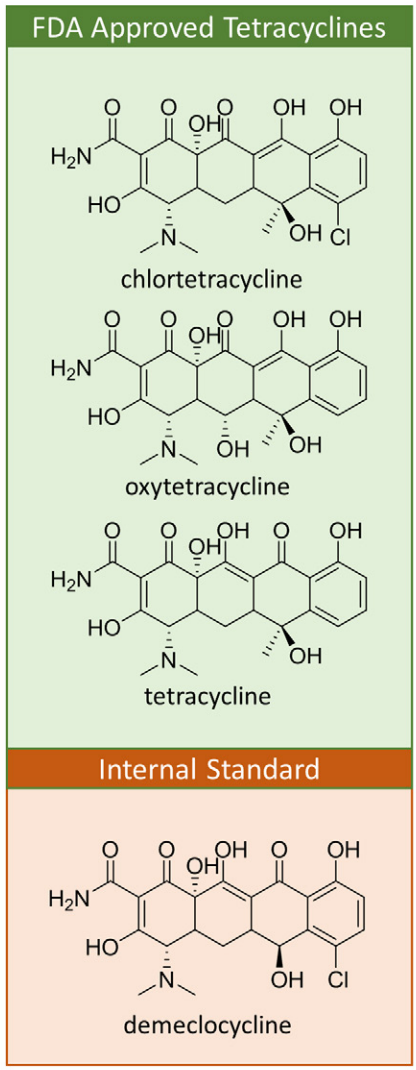

Fig. 2. Structures of target tetracycline antibiotics and demeclocycline as an appropriate internal standard.

poultry, bovine, ovine, and porcine tissues using Orbitrap MS. Hybrid Q-ToF instruments have additionally been used to identify TPs of commonly used antibiotics (e.g., amoxicillin) in waste and surface waters (Perez-Parada et al., 2011). Because reference standards often are not available commercially, identification of these TPs may be tentative. Identification and quantitation of TPs of a wide variety of contaminants is becoming more assessable as tools such as computer modeling combined with MS (Kern et al., 2009), laboratory bioreactors (Helbling et al., 2010), and mass spectral databases for LC/MS/MS (Gómez-Ramos et al., 2011) become more widely available. Mass spectrometry combined with these and other tools such as bioassays will ultimately help to determine the occurrence and biological effects of parent antibiotics and TPs. Even with these tools, there will always be a need to efficiently and quantitatively extract parent antibiotics and TPs from a complex matrix to accurately detect these compounds and evaluate their biological effects.

Units and Detection Limits

Antibiotic concentrations in literature studies are typically reported as mass per unit volume in aqueous samples and mass

per unit mass for solids and semisolids. Because of the wide variety of matrices, ranging from relatively uncontaminated groundwater, to fresh animal feces and urine, to soil and sludge, there can be inconsistency in how units are expressed. Additionally, due to the variations in matrices, no standard methods of analysis exist for antibiotics, with most methods developed in-house or adapted from USEPA Method 1694 for the analysis of pharmaceuticals in environmental matrices (USEPA, 2007). Inconsistency and variations in sample preparation are especially problematic for solids and semisolids with highly variable moisture content. To make comparisons between studies, ideally solid and semisolid concentrations are measured and expressed in both a "wet weight" and "dry weight" basis. Manure, a heterogeneous mixture of feces, urine, bedding, and soil, is often collected and analyzed for low levels of antibiotics, but the process for homogenizing this mixture may or may not be well described. Given that the distribution of antibiotics within these sample types is likely to be uneven, homogenization will help minimize variability in analytical results.

Evaluation of method limits of detection is another area where there is variability in procedures followed by different laboratories. The variation in the procedures for estimating limits of detection is not unique to antibiotic analysis and has been a subject of environmental literature for many decades (Keith et al., 1983). Issues in reporting analyte concentrations near or below detection limits are especially problematic in analytical methods where the measured concentrations are used to determine compliance with federal regulations (Kimbrough and Wakakuwa, 1993). Because there are no "standard" analytical methods for monitoring antibiotics in the environment, the variability in the reported concentrations determined from the same samples analyzed by different laboratories could be highly significant, as illustrated in a recent interlaboratory study of analytical methods for emerging contaminants (Vanderford et al., 2014). Unless the environmental occurrence of antibiotics leads to regulatory actions, the development of standard analytical methods for their measurement is unlikely.

Although there have been efforts to standardize detection limit terminology and validation approaches (Taverniers et al., 2004), because data are created for different purposes it is likely that inconsistency will remain a problem. Usually, the purpose of any analytical method is to deliver quantitative information with an acceptable well-defined level of uncertainty. Thus, the purpose of validation is to measure and report analytical uncertainty for any method (Taverniers et al., 2004). Ideally, method details are presented either in a methods article or in supporting information to the extent that a reader can evaluate the total uncertainty of the measurements. Method performance characteristics, such as precision, selectivity, linearity and range, recovery, and a variety of detection limit estimates are critical in evaluating the suitability of the procedures in producing the data presented (Taverniers et al., 2004). Of these measures, a summary of recovery and a set of well-defined detection limits are probably the most useful. Ultimately, a systematic comparison of measured antibiotics in different studies using various methods requires sufficient descriptive detail to permit any reader to evaluate the analytical uncertainty and method performance across a variety of samples likely to be collected from any compartment in an agroecosystem.

Biological Assays

Because of the high cost of instrumental methods and the need for rapid assays that can also measure the effects of antibiotic residues, biological assays are used in the assessment of antibiotics in agroecosystems. These methods generally use some biological property to separate and selectively quantify a chemical. Two of the most useful methods are enzyme-linked immunosorbent assay (ELISA) and bioreporter assays, which can also be used for measuring the bioavailable fraction of antibiotics.

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay is an alternative technique for measuring antibiotics, which provides a high-throughput and low-cost method relative to LC/MS/MS analysis (Aga et al., 2003; Zhang et al., 2013). Enzyme-linked immunosorbent assay offers utility as both a screening tool and a quantitative method for total analyte concentration (Aga et al., 2005). Although generally developed for a single target analyte, the antibodies involved in analysis by ELISA are generally class specific, exhibiting a high degree of cross-reactivity with structurally similar compounds (Aga et al., 2005; Huet et al., 2006; Wang et al., 2007). Where slight modifications in the structure of the analyte may prevent detection by LC/MS/MS, broad cross-reactivity ELISA is advantageous in screening processes because the assay can also detect TPs; cross-reactivity could be considered a strength in measuring bioavailability of structurally similar TPs that are important to detect, especially if they have potential biological activities.

Previous studies have used ELISA to examine antibiotics in environmental systems such as manure and soil (Aga et al., 2003), groundwater (Barber et al., 2009; Bradley et al., 2014), surface water (Kumar et al., 2004), wastewater (Černoch et al., 2012), or milk (Adrian et al., 2009). The results indicate that ELISA can be useful for low-cost screening of cephalosporins (Chen et al., 2009), monensin (Dolliver et al., 2008), tetracyclines (Aga et al., 2003; Jeon et al., 2008), and tylosin (Kumar et al., 2004) and its TPs (Hu et al., 2008) in agroecosytems. Furthermore, ELISA procedures are rapid, portable, and easily adaptable for testing of multiple samples simultaneously. Although ELISA is not a replacement for quantitative instrumental methods, there are clear advantages to using screening methods to reduce costs and assess the presence of structurally similar compounds resulting from the transformations of an antibiotic in the environment.

Detection with Bioreporters

One limitation of measuring the concentrations of antibiotics by instrumental methods is that concentrations obtained may not represent the true biological activity of the antibiotics in the natural environment. Biological effects of antibiotic mixtures can sometimes be synergistic or antagonistic. Therefore, the ecological impacts of antibiotic residues may be underestimated or overestimated if the biological activity is inferred directly from the concentrations determined by specific instrumental methods (Backhaus et al., 2000). Because many antibiotics are adsorbed on soil particles, the sample matrix will also affect bioavailability and potential effects. For example, Subbiah et al. (2011) found that a mixture of ciprofloxacin, neomycin, and tetracycline was partially neutralized due to adsorption in soil and caused minimal effects to *Escherichia coli*. However, β-lactams and florfenicol

in the same mixture remained active in supernatant extracted from soil. In contrast, Chander et al. (2005) reported that tetracycline and tylosin were biologically available to *E. coli* and *Salmonella*, even though the majority of the compounds were likely bound to soil particles.

As with ELISA, the use of bioreporters may be an alternative to chemical analysis. Bioreporters (Fig. 3) are genetically engineered living cells capable of producing detectable signals when sensing the target chemical. Bioreporters can be constructed using common gene technology methods (Ivask et al., 2001); for example, firefly or bacterial luciferase genes (Meighen, 1991) that encode reporter proteins can be used to produce a signal that is easy to detect with a luminometer. In addition to *lux* and *luc* genes, *gfp* and *lacZ* genes are also used for encoding the reporter protein (e.g., Hansen and Sørensen, 2000).

A bioreporter typically has a genetically engineered plasmid that contains two genetic elements: (i) gene(s) for one or more proteins specific to the chemical(s) of interest and (ii) the promoter/operator site of the original operon located upstream of the genes that encode a reporter protein for signal production (Fig. 3B). The protein specific to the chemical of interest (e.g., tetracycline) regulates the expression of reporter genes (i.e., the production of the signal), and therefore the signal is produced only in the presence of the chemical of interest.

Construction of bioreporters for the detection of antibiotic residues is achieved with a recombinant DNA method by replacing the resistance gene with a gene that encodes a signal-producing protein (Fig. 3). Currently, bioreporters have been developed for the detection of macrolides (Möhrle et al., 2007) and tetracyclines (Korpela et al., 1998). Because bioreporters use resistance operons occurring in antibiotic-resistant bacteria, they hold promise in measuring the selection pressure caused by trace antibiotic residues in agroecosystems. In another words, with bioreporters the bioavailable fraction of antibiotics could be measured from environmental samples without the need for harsh extraction conditions.

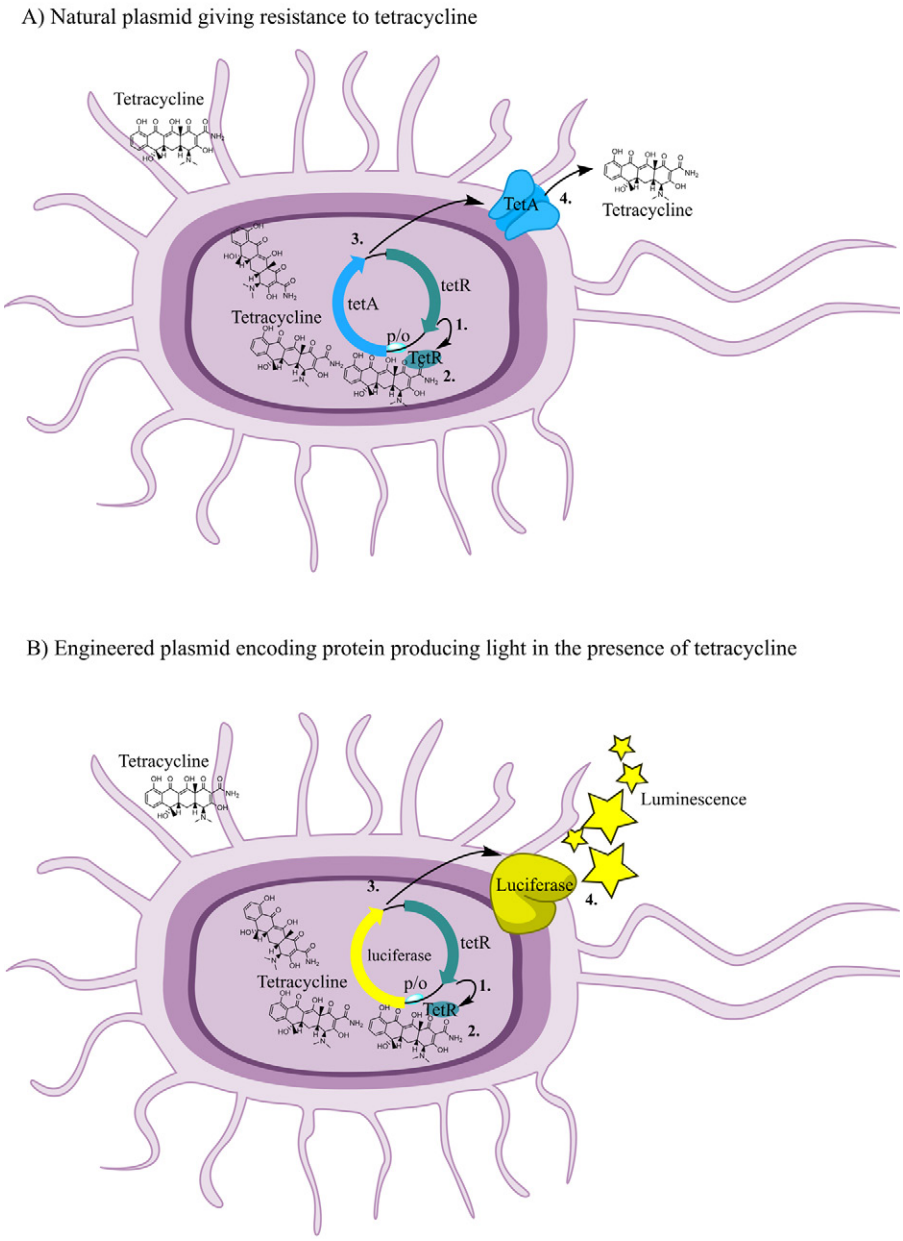

Fig. 3. (A) A bacterium with naturally occurring plasmid that gives resistance to tetracycline. (B) A whole cell bacterial bioreporter with an engineered plasmid. 1: Gene *tetR* encodes a repressor protein TetR. 2: Tetracycline binds to TetR causing a conformational change and releasing the TetR from promoter/operator region (p/o). 3(A): This allows the transcription of the resistance gene, *tetA*, and synthesis of the tetracycline efflux pump, TetA. 3(B): This allows the transcription of reporter gene and synthesis of reporter protein, Luciferase. 4(A): Protein TetA pumps tetracycline molecules from the cell, and this way the resistance against tetracycline is achieved. 4(B): Reporter protein Luciferase produces luminescence (light signal).

As discussed by Semple et al. (2004), the bioavailability of chemicals in the environment is dependent on physical and chemical conditions, the compound, and the organism. The "target" organisms of antibiotics are bacteria, and therefore use of whole-cell bacterial bioreporter assays to measure the bioavailable fraction of antibiotics would be ideal. To our knowledge, few studies have used bioreporters to determine bioavailable fractions of antibiotics. Hansen et al. (2002) used a bacterial whole cell biosensor (bioreporter) to evaluate the fraction of bioavailable chlortetracycline from pig feces. They also demonstrated the persistence of the tetracycline resistance phenotype among the coliforms long after treatment with chlortetracycline ended, which could not be explained by the selection pressure and would need further studies. The same bioreporter strain (Hansen and Sørensen, 2000) has been used in studies to assess the bioavailability of tetracyclines in different environmental conditions (Zhang et al., 2014). Tamminen et al. (2011) used a bioreporter assay to measure the concentrations of the tetracycline traces from sediments in aquaculture farms; however, in

their study the total concentrations of tetracyclines were below the detection limits of the bioreporter assay.

Although only two bioreporter strains have been used for the detection of bioavailable tetracyclines, there have been several studies where bacterial bioreporters have been used for the detection of bioavailable heavy metals or organic contaminants (Bondarenko et al., 2008; Ivask et al., 2001; Leedjarv et al., 2006). Because knowledge on genetic elements and regulation systems in antibiotic resistance operons is increasing, opportunities may exist for the construction of additional bioreporter strains capable of measuring the selection pressure caused by antibiotic contaminants in the environment.

Bioreporters may also be used for detection of antibiotics in solid samples when the measurement is performed in the soil–water suspension. Quenching by the matrix can be corrected by using a control strain that produces bioluminescence constitutively. Peltola et al. (2005) and Ivask et al. (2009) have successfully measured heavy metals from soil with this type of bioreporter. Finally, a promising design by Ivask et al. (2007) that immobilizes cells onto optical fibers for measuring heavy metals in soils offers potentially reusable and portable bioreporters, which may be adapted for use in antibiotic screening methods.

Effects of Mixtures

Antibiotic residues in the agroecosystem rarely occur as a single compound; rather, they occur as a "cocktail" of veterinary antibiotics and TPs. Although toxicological data are available for many individual antibiotics, understanding how these compounds behave as mixtures is key to predicting and identifying risks. Compounds of a particular class, such as sulfonamides, combined with other antibiotics from another class but with similar mode of action, can have additive toxic effects (Backhaus et al., 2000; Bona et al., 2014; Faust et al., 2000). Mixtures of antibiotics from different classes, such as macrolides, tetracyclines, and fluoroquinolones, suggest that both synergistic and antagonistic effects are possible (Gonzalez-Pleiter et al., 2013; Yang et al., 2008). Simple binary mixtures of antibiotic classes have been predicted to increase environmental risk, reported as toxicity, by as much as 50 to 200% over the individual parent compounds alone (Marx et al., 2015). The ecological effect of antibiotic mixtures is challenging to predict because any change in the ratio of compounds or in the antibiotic composition in the mixture could exhibit a reversal in the effect of the mixture, from synergistic to antagonistic, as a result of the changes in the relative contribution of each antibiotic to the overall biological effect (Liu et al., 2014).

Although bioactivity has been demonstrated for a number of TPs (Dimitrakopoulou et al., 2012; Girardi et al., 2011; Wan et al., 2013), evidence suggests that some TPs can have increased toxicity relative to the parent compound (Lewis et al., 2012; Majewsky et al., 2014); some may even have a completely different mode of action than the parent compound (Halling-Sørensen et al., 2002). Furthermore, the presence of other common environmental contaminants (e.g., metals) contributes to the overall toxicity of antibiotic mixtures such that predicting toxicity based on the toxicity of each single compound becomes a futile exercise (Yu et al., 2015).

Regional Monitoring and Modeling in Agroecosystems

Large-scale monitoring programs present many challenges, not the least of which is the cost. Programs such as those conducted by the US Geological Survey (Kolpin et al., 2002) and the USEPA (McClellan and Halden, 2010) provide critical information on antibiotic concentrations in the environment. Such programs are only possible with substantial funding and logistical coordination between different research units. Smaller-scale wastewater treatment plant monitoring programs (Benotti et al., 2009; Karthikeyan and Meyer, 2006; Michael et al., 2013) that help predict environmental loadings of antibiotics and other pharmaceutical compounds from municipal sources have been limited to the urban contributions to agroecosystems.

The high cost of regional and national monitoring programs have made it necessary to use predictive measures in analyzing the environmental exposure and ecological risks of antibiotics. Such studies are often based on sales data because information on actual animal use is difficult to obtain and largely unavailable. For example, the quantities of antibiotics listed in Table 1 are based on the average masses sold in the United States for use in food-producing animals during the year 2013. Connecting exposure data with hydraulic and geologic databases and Geographic Information Systems has been shown to be a powerful, low-cost alternative to monitoring programs for identifying and prioritizing risks associated with antibiotics in the environment. These modeling strategies have been used to identify at-risk soils (de la Torre et al., 2012; Iatrou et al., 2014; Wajsman and Ruden, 2006) and water bodies (Schowanek and Webb, 2002; Wajsman and Ruden, 2006; Williams et al., 2009) on a landscape scale for antibiotics and other biologically active emerging contaminants. Predictive models have recently been successfully used to predict environmental occurrence of antibiotics with acceptable accuracy based on regional consumption and excretion, with added methodologies for linking the potential for concurrent antibiotic resistance (Zhang et al., 2015).

Where usage data are unreliable or unavailable, strategies of reverse modeling that take advantage of previous monitoring data have also shown to be effective (Boxall et al., 2014). However, validation of environmental fate and transport models continues to be a significant challenge. System boundaries, such as using consumption of antibiotics or wastewater effluent concentration for the loading parameter, have a large effect on the reliability of PECs (Schwab et al., 2005). Differences between measured environmental concentrations and PECs can be significant and illustrate the complexity of fate and transport modeling, especially when temporal variations are considered (Celle-Jeanton et al., 2014). An important contributor to the differences between measured environmental concentrations and PECs that should not be ignored is the accuracy of the analytical methods used in measuring antibiotic concentrations in various compartments of the agroecosystem.

Different models have been proposed to facilitate prediction of the environmental toxicity and risks associated with mixtures of contaminants. Historically, the predictive assessment of mixture toxicity has followed either concentration addition (Loewe and Muischnek, 1926) or independent addition (Bliss, 1939), both representing additive behavior. The concept of concentration addition assumes that each compound in the mixture elicits

a response through the same mode of action. A weighted average depends on the potency and concentration of each compound present in a mixture. Independent addition describes the cumulative toxicity of a mixture in which the effect of each compound is assumed to behave independently of the other compounds in the mixture. Perhaps a more appropriate method is the combination index, which normalizes the effects of a mixture with the effects of each individual contaminant. A combination index value greater than one represents a synergistic interaction, whereas a value less than one indicates an antagonistic interaction. Further, compounds that act additively would be represented by a combination index of one. All three models have been shown to be valuable for representing toxicity data depending on concentration and specific compound interactions (Gonzalez-Pleiter et al., 2013). Regardless, future environmental risk assessment of antibiotics should attempt to incorporate mixture toxicity and, when available, should account for toxicity contributions from TPs as well as other forms of contamination.

Conclusions

Although there have been a number of studies on the occurrence of antibiotics in manure, soil, water, and other matrices in the agroecosystem, the ecological effects of antibiotics and their role in the development and spread of antibiotic resistance have not been clearly defined. The challenges in achieving accurate risk assessment remain formidable due to various factors, ranging from the accuracy and variability in analytical techniques to measuring bioavailability and toxicity of antibiotic mixtures and their TPs. Although advances in LC/MS/MS instrumentations have facilitated the detection of trace levels of antibiotics in complex environmental samples, care must be taken in validating methods for specific matrices and applications because the severity of matrix effects can vary substantially between the types of samples being analyzed (e.g., soil vs. manure). To better understand the impact of antibiotics in agroecosystems and accurately predict their contribution in the development of antibiotic resistance in bacteria, methods that allow the measurement of the bioavailable fraction of antibiotics are extremely valuable. Finally, large-scale surveys coupled with accurate environmental models will be key in predicting the fate, transport, and risks of antibiotics and their TPs in the environment. The latter information will be important in developing mitigation strategies for minimizing emergence, proliferation, and spread of antibiotic resistance in the agroecosystem.

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