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ANTIBIOTICS IN AGROECOSYSTEMS: STATE OF THE SCIENCE

Antibiotics in Agroecosystems: Introduction to the Special Section

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Abstract

The presence of antibiotic drug residues, antibiotic resistant bacteria, and antibiotic resistance genes in agroecosystems has become a significant area of research in recent years and is a growing public health concern. While antibiotics are used in both human medicine and agricultural practices, the majority of their use occurs in animal production where historically they have been used for growth promotion, in addition to the prevention and treatment of disease. The widespread use of antibiotics and the application of animal wastes to agricultural lands play major roles in the introduction of antibiotic-related contamination into the environment. Overt toxicity in organisms directly exposed to antibiotics in agroecosystems is typically not a major concern because environmental concentrations are generally lower than therapeutic doses. However, the impacts of introducing antibiotic contaminants into the environment are unknown, and concerns have been raised about the health of humans, animals, and ecosystems. Despite increased research focused on the occurrence and fate of antibiotics and antibiotic resistance over the past decade, standard methods and practices for analyzing environmental samples are limited and future research needs are becoming evident. To highlight and address these issues in detail, this special collection of papers was developed with a framework of five core review papers that address the (i) overall state of science of antibiotics and antibiotic resistance in agroecosystems using a causal model, (ii) chemical analysis of antibiotics found in the environment, (iii) need for background and baseline data for studies of antibiotic resistance in agroecosystems with a decision-making tool to assist in designing research studies, as well as (iv) culture- and (v) molecular-based methods for analyzing antibiotic resistance in the environment. With a focus on the core review papers, this introduction summarizes the current state of science for analyzing antibiotics and antibiotic resistance in agroecosystems, discusses current knowledge gaps, and develops future research priorities. This introduction also contains a glossary of terms used in the core reivew papers of this special section. The purpose of the glossary is to provide a common terminology that clearly characterizes the concepts shared throughout the narratives of each review paper.

Core Ideas

- Antibiotic resistant bacteria are an emerging threat to human, animal, and ecological health.
- Agroecosystems often contain elevated levels of antibiotics and antibiotic resistance.
- The impact of antibiotics at low concentrations in the environment is not fully known.
- Research is needed to understand the spread of antibiotic resistance within and beyond agroecosystems.
- Standardized approaches will help bring a consensus among scientific community datasets.

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Since the discovery of penicillin in 1928 by Alexander Fleming and the inception of the "antibiotic era," the use of antibiotics in medicinal and agricultural practices has significantly advanced public health and food production (Knapp et al., 2010). Antibiotics became widely available for use in human and veterinary medicine in the 1940s and have been used as feed additives for growth promotion in livestock since 1950 (Halling-Sørensen et al., 1998; Kumar et al., 2005; Kummerer, 2009). Current worldwide consumption of antibiotic compounds is approximately 100,000 to 200,000 Mg per year (Hollis and Ahmed, 2013; Van Boeckel et al., 2014). The use of large quantities of antibiotics raises concerns and questions about the release of these drugs and the increasing prevalence of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in the environment. If antibiotic resistance continues to rise, effective treatments for a large number of infectious diseases in human and animal health may be jeopardized (CDC, 2013). Furthermore, ecological health, including nutrient cycling, may be altered by shifts in indigenous aquatic and terrestrial microbial communities that are affected by the enrichment and/or release of antibiotics, ARB, and ARGs into the environment.

While the use of antibiotic drugs is believed to selectively enrich ARB and ARGs, large knowledge gaps remain when examining the relationship between antibiotic drugs, ARB, and ARGs in diverse agricultural and environmental systems. In these complex systems, discerning direct links between the presence or absence of antibiotic drugs and the occurrence of antibiotic resistance is challenging due to many factors. Foremost among these challenges is the natural phenomenon of ARGs

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Abbreviations: ARB, antibiotic resistant bacteria; ARG, antibiotic resistance gene; ELISA, enzyme-linked immunosorbent assay; FISH, fluorescent in situ hybridization; HGT, horizontal gene transfer; LC/MS/MS, liquid chromatography and tandem mass spectrometry; MIC, minimum inhibitory concentration; MGE, mobile genetic element; PCR, polymerase chain reaction; qPCR, real-time quantitative polymerase chain reaction.

being present within microorganisms due to intrinsic resistance. Bacteria can acquire resistance during horizontal gene transfer (HGT) of mobile genetic elements (MGEs) that contain not only ARGs but also other functional genes. The spread of resistance can occur quickly due to HGT and the rapid growth of microorganisms, both of which facilitate the passage of advantageous mutations and genetic elements (Normark and Normark, 2002). These uncertainties create a number of challenges related to determining the environmental fate, bioavailability, and effects of antibiotics, ARB, and ARGs in agroecosystems.

Recently, the World Health Organization released a special report (WHO, 2014) that suggests that antibiotic failure is already a global reality and that investigation of antibiotic drugs, ARB, and ARGs in the environment is a critical area for future research. Of special interest are those agroecosystems in which antibiotic use is nontherapeutic and mitigation efforts to prevent antibiotics, biologically active degradation compounds (i.e., metabolites), ARB, and ARGs from reaching the environment are limited or nonexistent. Unlike human biosolids and wastewaters that undergo treatment before land application, it is common for untreated animal waste to be applied to land. As a result, coordinated full-scale investigations concerning the impact of agroecosystems on the spread of antibiotic resistance in the wider environment are necessary to elucidate the potential influence of these systems on the development, movement, and survival or persistence of ARB and ARGs.

Critical Research Areas and Priorities

Many hurdles exist in the identification and quantification of antibiotic drugs, metabolites, ARB, and ARGs in agroecosystems and in determining the specific human and agricultural practices that may be sources and/or facilitate the spread of antibiotics and antibiotic resistance. First, antibiotic use in humans and animals is not the only cause of enrichment of ARB and ARGs. Resistance can be an innate characteristic of certain bacterial species (Cox and Wright, 2013). For example, *Pseudomonas aeruginosa* has a high intrinsic resistance to numerous antibiotics without prior exposure to those compounds (Hancock and Speert, 2000). Moreover, many bacteria and fungi in the environment can produce compounds that are structurally similar to antibiotics and serve both communication and antagonistic functions (Linares et al., 2006). These low molecular weight compounds are sometimes produced at concentrations that are high enough to exert an effect similar to antibiotics, enriching ARB and ARGs in soil and water environments (Aminov, 2010). In these instances, the presence of antibiotics, ARB, and ARGs does not equate to an anthropogenic impact.

Because the study of anthropogenic sources of antibiotics and antibiotic resistance in the environment is relatively new, levels that existed before the extensive use of antibiotics in human medicine and agroecosystems are not well characterized. Defining the amount of antibiotics and ARB that would occur naturally in the environment is challenging (Durso et al., 2012). Environmental systems that have already been affected due to anthropogenic inputs of antibiotic-related contamination cannot be analyzed to determine what the levels were in the system before current or past antibiotic inputs. Furthermore, attempting to define and use pristine systems is difficult since environmental systems are extremely variable and heterogeneous, especially with regard to microbial community composition. Direct comparisons cannot be made easily between pristine environments and those affected by antibiotic resistance because land-use and past histories are not equivalent (Franklin and Mills, 2009); however, pristine environments allow us to see that ARB and ARGs exist naturally.

The ways in which antibiotics and antibiotic resistance are measured play an important role in our ability to make comparisons between published research studies. The types of antibiotic compounds, ARB, and/or ARGs that are analyzed need to be carefully considered and taken into account when drawing conclusions about how levels of antibiotics and antibiotic resistance within a particular environment may have changed as a result of agroecosystem practices (Levy, 2002). If the types and quantities of ARB and ARGs in an environment are being altered, then the impact on human, animal, and ecological health may be significant. Antibiotic compounds, ARB, and ARGs have the potential to move throughout an ecosystem or between ecosystems, and the possible effects on environmental health are intimately connected with the health of humans and animals, a concept known as "One Health" (Papadopoulos and Wilmer, 2011).

The majority of research and public concern about antibiotics and antibiotic resistance in the environment has centered on the potential risks to humans and animals that consume and utilize antibiotic drugs for the prevention and treatment of disease (Snary et al., 2004; Ashbolt et al., 2013). Even when antibiotics entering the environment are below clinically determined minimum inhibitory concentrations (MICs), research has shown that some antibiotics can lead to increased abundance of ARGs within susceptible organisms and shifts in microbial community structure (Liu et al., 2011). In particular, alterations in environmental microbial populations can have negative impacts on critical processes, especially in soil systems where microorganisms perform important biological transformations of carbon and nutrients (Ding and He, 2010), thereby affecting plant growth and ecosystem functions (Van der Heijden et al., 2008; Lau and Lennon, 2011; Wagg et al., 2011). Consequently, in addition to human and animal health, ecological health could be jeopardized by the release of antibiotics and development of antibiotic resistance in agroecosystems, and the health of ecosystems should be an additional area of future research.

Contents of the Special Section

This special section contains five core review papers, 19 technical, review, and issues papers (Table 1), and a glossary of commonly used terms. The topics include the occurrence (Durso et al., 2016; McCall et al., 2016), detection (Wallace and Aga, 2016), dissemination (Hafner et al., 2016; Ruuskanen et al., 2016; Sura et al., 2016), fate (Amarakoon et al., 2016; Kulesza et al., 2016; Liu et al., 2016; Xu et al., 2016; Youngquist et al., 2016), plant uptake (Franklin et al., 2016; Kumar and Gupta, 2016), microbiology (Nordenholt et al., 2016; Roberts and Schwarz, 2016; Rothrock et al., 2016a; Whitehead and Cotta, 2016; Zwonitzer et al., 2016), and ecological risk (Subbiah et al., 2016) of antibiotics and/or antibiotic resistance in agroecosystems and surrounding natural areas. The antibiotic drugs, ARB, and ARGs discussed in this special section are predominantly associated with animal production but also include fruit and vegetable production, as well as

those associated with the application of biosolids to agricultural lands. The core review papers, the main focus of this introduction, examine critical research priorities and directions including (i) causal model of antibiotics and antibiotic resistance pathways in agroecosystems (Williams-Nguyen et al., 2016), (ii) detection, measurement, and risk assessment of antibiotics (Aga et al., 2016), (iii) baseline and background levels of antibiotic resistance with a decision-making tool (Rothrock et al., 2016b), and (iv) culture- (McLain et al., 2016) and (v) molecular-based (Luby et al., 2016) methods of antibiotic resistance detection. These reviews provide a synthesis of available information on past and current research as well as current needs and ways of improving research strategies so that knowledge of antibiotics and antibiotic resistance in agroecosystems can be improved.

Summary of Core Review Papers

Antibiotics and Antibiotic Resistance in Agroecosystems: State of the Science

As noted by Williams-Nguyen et al. (2016), large quantities of antibiotics are routinely introduced to agroecosystems, yet the pathways by which humans, animals, and all other biota may be exposed to antibiotics and antibiotic resistance are complex and poorly understood. The potential effects of environmental exposures on human, animal, and ecosystem health are unclear. In recent years, causal modeling diagrams have been used in an attempt to illustrate and visualize ordered relationships between factors in complex systems, typically as it relates to epidemiology. Causal modeling helps to minimize bias and makes assumptions explicit when assessing causal effects from data (Shrier and Platt, 2008; Joffe et al., 2012). To help illustrate pathways by which exposure to antibiotic drugs, ARB, and ARGs may occur and relate to expected effects, a causal model was proposed for agroecosystems (Williams-Nguyen et al., 2016). The causal model takes a One Health approach and describes the key interactions between antibiotics, ARB, and ARGs as well as their resulting interactions within the agroecosystem and on three specific endpoints: (i) human health, (ii) ecosystem function, and (iii) agricultural system productivity. This review evaluates the current state of understanding of these interactions using available literature so that key knowledge gaps can be identified.

Table 1. List of papers in the special section Antibiotics in Agroecosystems: State of the Science.

Reference	Title	Category
Williams-Nguyen et al. (2016)	Antibiotics and antibiotic resistance in agroecosystems: State of the science	Core review
Aga et al. (2016)	Challenges in the measurement of antibiotics and in evaluating their impacts in agroecosystems: A critical review	Core review
Rothrock et al. (2016b)	How should we be determining background and baseline antibiotic resistance levels in agroecosystem research?	Core review
McLain et al. (2016)	Culture-based methods for detection of antibiotic resistance in agroecosystems: Advantages, challenges, and gaps in Knowledge	Core review
Luby et al. (2016)	Molecular methods for assessment of antibiotic resistance in agricultural ecosystems: Prospects and challenges	Core review
Amarakoon et al. (2016)	Dissipation of antimicrobials in feedlot manure compost after oral administration versus fortification after excretion	Technical
Durso et al. (2016)	Assessment of selected antibiotic resistances in ungrazed native Nebraska prairie soils	Technical
Franklin et al. (2016)	Uptake of three antibiotics and an antiepileptic drug by wheat crops spray irrigated with wastewater treatment plant effluent	Technical
Hafner et al. (2016)	Evaluation of monensin transport to shallow groundwater after irrigation with dairy lagoon water	Technical
Kulesza et al. (2016)	Manure injection affects the fate of pirlimycin in surface runoff and soil	Technical
Kumar and Gupta (2016)	A framework to predict uptake of trace organic compounds by plants	Technical
Liu et al. (2016)	Sorption of lincomycin by manure-derived biochars from water	Technical
McCall et al. (2016)	Metagenomic comparison of antibiotic resistance genes associated with liquid and dewatered biosolids	Technical
Nordenholt et al. (2016)	Veterinary antibiotic effects on atrazine degradation and soil microorganisms	Technical
Roberts and Schwarz (2016)	Tetracycline and phenicol resistance genes and mechanisms: Importance for agriculture, the environment, and humans	Review
Rothrock et al. (2016a)	Antibiotic resistance patterns of major zoonotic pathogens from all-natural, antibiotic-free, pasture-raised broiler flocks in the southeastern United States	Technical
Ruuskanen et al. (2016)	Fertilizing with animal manure disseminates antibiotic resistance genes to the farm environment	Technical
Subbiah et al. (2016)	Not all antibiotic use practices in food-animal agriculture afford the same risk	Issues
Sura et al. (2016)	Transport of three antimicrobials in runoff from windrows of composting beef cattle manure	Technical
Wallace and Aga (2016)	Enhancing extraction and detection of veterinary antibiotics in solid and liquid fractions of manure	Technical
Whitehead and Cotta (2016)	Examination of the aerobic microflora of swine feces and stored swine manure	Technical
Xu et al. (2016)	Dissipation of antimicrobial resistance determinants in composted and stockpiled beef cattle manure	Technical
Youngquist et al. (2016)	Fate of antibiotics and antibiotic resistance during digestion and composting: A review	Review
Zwonitzer et al. (2016)	Quantifying attachment and antibiotic resistance of *Escherichia coli* from conventional and organic swine manure	Technical

Increased use of antibiotics globally, coupled with advancements in analytical technology, has resulted in more frequent detection of antibiotic compounds and, to a lesser extent, their metabolites, in a variety of agroecosystem compartments, including soil, water, sediment, and biota (Kolpin et al., 2002; Aga et al., 2005; Batt et al., 2006; Pruden et al., 2012; Zhang et al., 2013). Despite advances in detection methods, limited data are available on the occurrence and fate of antibiotics in agroecosystems, as well as their spatial and temporal distribution. Recently, predictive modeling has been proposed as an alternative to large-scale and high-cost monitoring programs to assist in estimating expected concentrations of antibiotics at the landscape-scale (Boxall et al., 2014). These models rely on accurate antibiotic usage data as well as mechanistic knowledge of the metabolism, fate and transport, and landscape and hydrologic processes; however, usage data is not universally available, and data for a number of medically important antibiotic classes are lacking.

A number of pathways have been identified for the introduction of antibiotics into agroecosystems, and their effect on the abundance and proliferation of ARB and ARGs may depend on the specific pathway. Land application of manure solids and wastewater is a common route for antibiotics to enter the environment. Application of manure, with or without antibiotics, is a common practice and is known to increase both ARB and ARGs (Pruden et al., 2006; Zhou et al., 2010; Udikovic-Kolic et al., 2014), but available data are limited, with inconsistent results on both short- and long-term environmental impacts (Auerbach et al., 2007; Munir and Xagoraraki, 2011; Negreanu et al., 2012; McLain and Williams, 2014), highlighting the need for further research.

Although ARB and ARGs are present in manure, biosolids, and wastewater effluent, separating the effects of these compounds from preexisting resistance found in populations of native bacteria adds another level of complexity to modeling efforts. Separating resistance of the pristine soils from that induced by the release of ARB, ARGs, and trace levels of antibiotics would help confirm the relationship between antibiotic use with antibiotics and antibiotic resistance present in agroecosystems. Another confounding factor when investigating antibiotic resistance is the intrinsic relationship between ARB and ARGs and the risks associated with each. Antibiotic resistant bacteria pose a direct risk to the three-agroecosystem health endpoints based on the extensive studies of pathogenic bacteria. On the other hand, ARGs pose an indirect risk with impacts linked to HGT, yet little information is known about the transport and fate of extracellular DNA in the environment.

Currently, the health effects in humans who are exposed to low levels of antibiotics and ARB from environmental sources are unknown. For antibiotics, various pathways exist for human exposure, including ingestion of contaminated food and water and inhalation of contaminated dust particles. Antibiotic residues have been measured in food crops, water sources, and animal-based food products, but often at levels several orders of magnitude lower than the acceptable daily intake values in developed countries (Holmes et al., 2007). Although the effects of long-term chronic exposures to low levels of antibiotics in humans have yet to be investigated, data suggest that low levels of antibiotics may select for ARB and/or ARGs (Lin et al., 2014). Off-target effects of antibiotics have shown that at environmentally relevant concentrations, cyanobacterial species are affected (Guo et al., 2015). While studies have also shown a toxic response to antibiotics in invertebrates and fish, once again the concentrations at which adverse effects occur are orders of magnitude higher than what is typically considered as environmentally relevant.

Research investigating the impacts of antibiotics on ecosystem function primarily focuses on soil microorganisms. Microcosm studies have shown that antibiotics in the environment have the potential to alter the microbial biomass, affect community structure, and modify functional endpoints such as substrate-induced respiration, iron reduction, ammonification, N-mineralization, and nitrification (Schmitt et al., 2004; Hammesfahr et al., 2008; Gutiérrez et al., 2010; Kleineidam et al., 2010; Solis et al., 2011; Toth et al., 2011). These alterations in soil microbial function may in turn affect higher-level organisms and ecosystem processes. Little is known about the effects of ARB or ARGs on ecosystem function. Various hypotheses have been proposed for alterations in microbial diversity, function, and composition (Martinez, 2009), as well as effects on wildlife health (Gillings, 2014), but more evidence is necessary for their thorough evaluation.

While effects of single antibiotic compounds in agroecosystems have been selectively characterized, toxicological impacts of mixtures are not well understood. Limited research on antibiotic mixtures has shown that combinations of compounds can often result in synergistic, antagonistic and additive effects, depending on the compounds present (Yang et al., 2008; Liu et al., 2014). In addition, not only will mixtures of antibiotics be present, but so will their metabolites and other environmental toxins, such as metals, which have been shown to affect mixture toxicity and further enrich for ARB (Majewsky et al., 2014; Yu et al., 2015). Predicting the biological effects of these mixtures is challenging because changes in the composition of compounds can change mixture toxicity from synergistic to antagonistic due to alteration in relative contribution of each compound (Liu et al., 2014). Given the complex mixtures of contaminants found in agroecosystems, future environmental risk assessment of antibiotics must also evaluate the effects of these mixtures.

Finally, the expected link between the occurrence of ARB in the environment and agricultural systems has yet to be determined. The ARB from environmental sources are likely to spread to agricultural systems given the documented links between wildlife (e.g., birds) and common foodborne pathogens in agricultural products (Greig et al., 2015), indicating evidence of transfer to animals and crops within an agroecosystem.

Challenges in the Measurement of Antibiotics and in Evaluating Their Impacts in Agroecosystems

Detection and measurement of antibiotic residues are essential for understanding their potential to adversely affect human health, ecosystem function, and agricultural systems, including animal health. While the importance of accurate measurements is clear, prioritizing which antibiotics to measure is difficult. The potential for an antibiotic to have adverse impacts in agroecosystems is directly related to its original use, in vivo, and its environmental persistence and inherent biological activity. Not only is the wide variety of antibiotic compounds of concern, but some

of their transformation products and metabolites may also affect biological activity and therefore need to be considered when conducting environmental studies. Aga et al. (2016) examine the state of the science for detection, quantification, and risk assessment of antibiotics and their transformation products in the agroecosystem.

The concentration of antibiotics and their metabolites in different environmental compartments vary greatly, but residues have been detected up to levels of milligram per kilogram in animal manure for persistent compounds, such as tetracyclines and sulfonamides (Haller et al., 2002; Aga et al., 2005). Depending on environmental conditions, physicochemical characteristics, and routes of entry, environmental concentrations of the antibiotics and/or their metabolites typically decrease over time due to irreversible sorption to particulate matter, dispersion, and/or degradation. In most environmental compartments, the concentrations eventually fall below the limits of detection by most analytical methods (Homem and Santos, 2011). However, even subinhibitory and nonlethal concentrations of antibiotics and/or their metabolites have been shown to act as signaling molecules between microorganisms and may contribute to the evolution of antibiotic resistance (Aminov, 2010).

The development of sensitive analytical methods is needed to measure environmentally relevant concentrations of antibiotics in complex environmental samples. While instrumentation has improved greatly in recent years with detection limits in the picogram per gram or parts per trillion range, difficulties in separating antibiotics and their degradation products from complex matrices (e.g., soils, manures, and wastewaters) still limits the ability to accurately and reproducibly measure them (Wilga et al., 2008). An even greater challenge is the determination of the ecological implications and significance of the biologically available (bioavailable) fractions of antibiotics at their predicted environmental concentrations. The definition of *bioavailability* often varies considerably, mainly due to lack of standard methods to measure this fraction in the environment. In addition, bioavailability is dependent on the chemical analysis of extracted compounds, which in turn depends on the efficiencies of the extraction method. Unfortunately, absolute recovery of multiple residues from environmental matrices is typically not possible, and even with improved analytical techniques, the fraction recovered from soil or other matrices may not necessarily correspond with the fraction that plants or microbes are exposed to in the environment (Naidu, 2008).

Quantitative analysis often requires elaborate extraction and clean-up procedures to minimize interferences. The extraction and clean-up technique of choice for aqueous samples is solid phase extraction (SPE) because of improved selectivity, specificity and reproducibility, minimal organic solvent consumption, shorter sample preparation time, ease of operation, and the potential for automation (Poole, 2003). Preparation of solid and semisolid samples, such as manure or soil, is extremely challenging due to high concentrations of natural organic matter. Instrumental analysis using high performance liquid chromatography and tandem mass spectrometry (LC/MS/MS) has become the primary analytical tool for quantification of antibiotics. High-resolution instruments such as quadrupole time-of-flight and Orbitrap MS (Thermo Scientific) are best suited for identification of unknowns, whereas triple quadrupole provides high selectivity for detecting antibiotics (Johnson et al., 1990). Ion trap mass spectrometry can help identify transformation products, which is critical, as many transformation products retain antimicrobial properties (Diaz-Cruz and Barcelo, 2007).

Currently, standard methods do not exist for detection of antibiotics in environmental samples, although some laboratories have used some variation of USEPA Method 1694 (USEPA, 2007). Because methods are not yet standardized, well-described procedures, including details of validation, are necessary to help make comparisons between studies. In addition, methods and procedures for determining limits of detection vary between laboratories and have been the subject of environmental literature for decades (Keith et al., 1983). Without regulations to monitor the occurrence antibiotics in the environment, however, other means to stimulate development of standard analytical methods are needed.

Costs for quantifying antibiotics can be prohibitive in some instances, leading to the development of screening tools to quickly detect and measure antibiotics and to estimate bioavailability. Enzyme-linked immunosorbent assay (ELISA) is often used as a screening tool and a semiquantitative method for determining total analyte concentrations within a class of antibiotics (Aga et al., 2005). The value of this approach is that the ELISA has the ability to estimate bioavailability regardless of a compound's structure, while targeted analysis using LC/MS/MS would not detect an unknown transformation product. Bioreporters, genetically engineered cells capable of producing detectable signals in the presence of a target compound, may also be a useful alternative to chemical analysis (Meighen, 1991). These tools have been used in aqueous and solid samples, and matrix effects are corrected using a control strain that constitutively produces bioluminescence. Bioreporters have already been developed for the detection of macrolides (Möhrle et al., 2007) and tetracyclines (Korpela et al., 1998).

Whereas numerous studies have examined the occurrence of antibiotics in manure, soil, water and other matrices, less work has focused on ecological effects and risk. To accurately assess risk, toxicity data on antibiotics, transformation products, and contaminant mixtures are essential, but currently lacking. Due to high costs of regional and national monitoring programs, predictive models have become necessary to evaluate exposure and ecological risks of antibiotics in agroecosystems. Various proposed models have been useful in representing toxicity data based on the type and concentration of contaminants (Loewe and Muischnek, 1926; Bliss, 1939; Gonzalez-Pleiter et al., 2013). Continued development of sensitive and robust analytical methods will permit improved measurement of bioavailable fractions of these compounds and improve risk analysis. Large-scale efforts involving multiple agencies and university research groups would be valuable in attempting to unify information and approaches to improve fate and risk assessment of antibiotics in agroecosystems.

How Should We Be Determining Background and Baseline Antibiotic Resistance Levels in Agroecosystem Research?

While research in isolated and pristine environments indicates that antibiotic resistance is an ancient phenomenon (Bhullar et

al., 2012), the use of anthropogenic antibiotics also influences the presence of antibiotic compounds, ARB, and ARGs in an environment. Consequently, Rothrock et al. (2016b) emphasize that the determination of background and baseline levels of antibiotic resistance is crucial for an accurate assessment of the impacts of anthropogenic inputs in agroecosystems. Universally accepted definitions of background and baseline levels are not found in the literature; therefore, for this review article, *background* is defined as the concentration in an environment not influenced by local human activity, and *baseline* as the numerical average and/or range of antibiotic drugs, ARB, and/or ARGs levels present at the beginning of a study (Rothrock et al., 2016b). Without knowledge of background and/or baseline levels at the beginning of a study, it is difficult to draw conclusions regarding the impact of human activities in applied animal production systems (Durso and Cook, 2014). Normalization of antibiotic resistance found in agroecosystems against background and baseline levels will (i) allow evaluation of significant alterations in the occurrence of ARB and/or ARGs within a study, (ii) improve the ability to compare results between studies, and (iii) identify links between agricultural or environmental activities and treatments.

Research questions and experimental designs should be properly framed so that background and baselines levels are established and the data collected accurately assess impact. In addition, bacterial communities associated with antibiotic resistance need to be considered during the experimental design phase. Native ARB are those that are ubiquitous in the environment before any anthropogenic influences; selected ARB are the subset of the native community that are enriched in affected environments following the application of manure or wastewater or release of antibiotic compounds; and adapted ARB are the gastrointestinal tract–associated bacteria that enter the environment through manure application and are incorporated into the soil flora.

Animal manures are a major source of antibiotics, ARB, and ARGs that can potentially reach the environment. While animals being fed antibiotics appear to have increased levels of ARB and ARGs in their manure (Durso et al., 2012; Zhang et al., 2013), the effects of manure application in soil are less clear, with variable responses in ARB and ARG levels that do not directly correlate with animals receiving antibiotic treatments (Udikovic-Kolic et al., 2014). Likewise, ARB and ARGs are present in biosolids with HGT being demonstrated, yet data about biosolid application and the effects on ARB and ARGs in agroecosystems are contradictory (D'Costa et al., 2006; Brooks et al., 2007; Munir and Xagoraraki, 2011).

The degree to which the resistome in modern soils has been influenced by human antibiotic use since the beginning of the antibiotic era is not clear. While analyzing the resistomes of background soils would help gauge the impact of agroecosystems on antibiotic resistance, few studies have focused on background resistomes, and even fewer have included appropriate background soils when analyzing impacted agricultural soils. Based on research to date, soils appear to harbor distinct ARGs compared with human-associated microbial communities (Gibson et al., 2015), and the large diversity of ARGs in soil may favor preexisting genotypes rather than selecting for new ARGs (Udikovic-Kolic et al., 2014).

The release of antibiotics, ARB, and/or ARGs into surface and groundwaters is often associated with urban and agricultural sources that are widespread, which creates difficulty in acquiring background data (Chee-Sanford et al., 2009; Munir et al., 2011; Garder et al., 2014). However, waters downstream of point sources of antibiotic-related contamination (e.g., wastewater treatment plant, animal feedlot) have consistently contained elevated levels of ARGs compared with upstream (i.e., background) samples (Sapkota et al., 2007; Storteboom et al., 2010). Similar challenges in obtaining background and baseline data have been observed in other agroecosystems, including aquaculture (Schmidt et al., 2000; Sobecky and Hazen, 2009; McDaniel et al., 2010; Seyfried et al., 2010; Tamminen et al., 2011) and horticulture (Duffy et al., 2011; Walsh et al., 2011; Popowska et al., 2012). Given these inconsistent results and lack of background data, more information about antibiotic resistance in agroecosystems is necessary to understand the links between environmental, human, and animal systems.

Despite some knowledge gaps in surveillance programs, successful antibiotic resistance surveillance programs exist globally (DANMAP, 2014; NethMap, 2014; CDC, 2015; EUCAST, 2015; Public Health Agency of Canada, 2015). However, for datasets to be successfully correlated and compared, surveillance programs would need to use standardized testing methods for antibiotic resistance monitoring (Wray and Gnanou, 2000). While attempts have been made to prioritize which antibiotic drugs from human and animal medicine should be examined in antibiotic resistance research (Boxall et al., 2003; FDA, 2003; WHO, 2011), determining which drugs that may enter agroecosystems pose the greatest risk to human, animal, and/or ecological health is difficult. Therefore, with the purpose of aiding scientists working in agroecosystem research, an antibiotic resistance decision-making tool (AR-DMT) was created to assist in selection of the most important and relevant antibiotics to evaluate given particular research goals/criteria, as well as to guide the experimental design process (Rothrock et al., 2016b). Antibiotics are rated using three main criteria: (i) use within agroecosystems, (ii) ranking within major scientific databases and surveillance programs, and (iii) target bacteria or ARB for treatment. In short, once the user has provided the data of interest, the tool will provide the rankings of all of the World Health Organization critically important antibiotics for those specific search criteria (allowing the user to further investigate the most appropriate class- or drug-specific ARG targets based on research design or goals).

Given the expansive diversity of antibiotic resistance–related targets, the agroecosystem antibiotic resistance research community is encouraged to begin a standardization of (i) definitions of background and baseline antibiotic resistance levels, (ii) assessments of within and between study normalization, and (iii) determination of the most appropriate antibiotic resistance–related targets in each agroecosystem. Adoption of these criteria when conducting antibiotic resistance–related research in agroecosystems would assist in accurately assessing the impacts of any treatment or management regime. In addition, the inclusion of these data in publications would unify the scientific literature, allowing for a broader and more accurate understanding of the direct and indirect effects that agriculture has on antibiotic resistance in the environment.

Culture-based Methods for Detection of Antibiotic Resistance in Agroecosystems: Advantages, Challenges, and Gaps in Knowledge

The review by McLain et al. (2016) addresses the current knowledge of culture-based techniques for the assessment of antibiotic resistance in agroecosystems, including the wide-range of methods, bacterial groups, and antibiotics commonly targeted for resistance studies, data interpretation, and confounding factors. Numerous culture-based methods exist for analyzing antibiotic resistance in environmental bacteria with target bacteria isolated on either general or selective media. While culture techniques are time consuming, they have distinct advantages, including direct identification and analysis of antibiotic resistance in individual bacterial isolates. Culture-based methods provide opportunities to link phenotypic and genotypic characteristics and assess ARG transfer potential, allowing for greater understanding of overall resistance patterns, as well as identification of multiple-antibiotic resistance within single organisms.

Standard clinical classification protocols exist that categorize a bacterial isolate as resistant, susceptible, or intermediate to an antibiotic based on the bacterium's growth at defined antibiotic concentrations, known as breakpoints (Silley, 2012). In clinical settings, these breakpoints, measured as MICs, are used to determine specific dosage formulations for antibiotic treatment. The MIC is the lowest concentration that will inhibit microbial growth following overnight incubation for rapidly grown bacteria (Andrews, 2001). These clinical breakpoint concentrations can alter over time and vary between the United States and Europe, with standards published by the Clinical and Laboratory Standards Institute (CLSI, 2015) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015).

The microorganisms that are commonly targeted in culture-based studies to evaluate antibiotic resistance in agroecosystems are microbial groups that are clinically relevant and easy to culture. Frequently, these target microorganisms are also indicators of water quality. Generally, the most common microbes targeted for environmental analysis are *Escherichia coli, Enterococcus* spp., *Salmonella* spp., and *Staphylococcus* spp., and recent research has suggested the addition of *Aeromonas* spp., *Klebsiella pneumonia,* and *Pseudomonas aeruginosa* (Berendonk et al., 2015). *Salmonella* spp. account for 38% of foodborne illnesses in the United States (CDC, 2013). *Enterococcus* spp. and *E. coli* are currently used as water quality indicators by the USEPA (USEPA, 2012), and *Klebsiella pneumonia* has been suggested as a model organism based on its high persistence in the environment and animal guts (Tzouvelekis et al., 2012). The antibiotics selected for these studies are typically those used in agriculture, as well as those prescribed for human use. Other considerations for antibiotic selection include mechanism of action and the extent to which they are used for prophylaxis, growth promotion, or treatment of disease in animals.

Before antibiotic resistance testing, identification of bacterial isolates is essential. Once target organisms have been successfully isolated and identified, antibiotic resistance testing can be performed via three common methods: broth and agar dilution, agar disk diffusion, and E-tests. These three methods for the analysis of antibiotic resistance are well standardized and reproducible. The results for these culture-based techniques have been found to be reliable and comparable in clinical settings. The choice of method predominantly depends on the scope of research, but other considerations include laboratory limitations and whether qualitative or quantitative results are desired (Baker et al., 1991; Joyce et al., 1992). While agar disk diffusion studies report numbers of isolates that are susceptible and resistant, broth microdilution methods are more quantitative and produce MIC_{50} values that represent the concentration at which ≥50% of the isolates in a population are inhibited. Given the quantitative nature of this method, researchers are encouraged to not overemphasize MIC_{50} values in small test populations (10–30 isolates), when a few strains with high MIC values may skew the MIC_{50}. Questions remain, however, regarding how many isolates are necessary per sample and how many samples within an agroecosystem must be analyzed to produce a representative dataset for accurate analysis of antibiotic resistance (Persoons et al., 2011).

Culture-based methods have certain limitations, including inherent culture bias. Most of the bacterial species in soil and water are not able to be cultivated; therefore, culture-based approaches apply only to a small subset of the microbial species and do not provide the full spectrum of diversity present in environmental samples. When ARB are identified using both culture- and molecular-based techniques, the results have been found to be different (Garcia-Armisen et al., 2013). Another notable limitation is that culture methods do not identify bacteria that are in the viable but nonculturable state. This state has important implications with regard to antibiotic resistance, since bacteria become resistant to antibiotics, yet have the potential to eventually return to being metabolically active and pathogenic (Ehrlich et al., 2002). Another potential culture bias with regard to antibiotic resistance is the presence of persister cells that are dormant variants of regular cells and highly tolerant to antibiotics (Lewis, 2010).

Even with their limitations, culture-based methods are the basis of international surveillance efforts to monitor antibiotic resistance, and standardized molecular methods are presently not available to replace them. While direct polymerase chain reaction (PCR)-based and metagenomic techniques show great promise in helping to characterize ARG diversity and abundance in complex environments, these methods do not enable functional validation of identified resistance mechanisms and generally cannot correlate between bacterial phyla and specific ARGs. Multiple studies have compared the effectiveness of culture-based and phenotypic characterization with culture-independent methods that generally target specific antibiotic resistant genes instead of bacteria, but no single method or group of methods has been identified as providing more accurate results (Jorgensen and Ferraro, 2009; Campbell et al., 2011; Nordmann et al., 2012). Future assessment of antibiotic resistance in the environment will depend on standardized methods and techniques that incorporate culture- and molecular-based procedures.

Molecular Methods for Assessment of Antibiotic Resistance in Agricultural Ecosystems: Prospects and Challenges

Luby et al. (2016) discuss existing molecular techniques for identifying and tracking antibiotic resistance in agricultural ecosystems. Molecular methods offer the distinct advantage of providing direct information about the extractable pool of DNA,

RNA, and/or proteins within a sample. The isolated DNA, RNA, or protein(s) can be sequenced and directly compared against publicly available databases. Utilization of molecular methods also helps to avoid biases associated with culture-based methods. For the analysis of antibiotic resistance in agroecosystems, molecular methods offer a means of tracking the fate of various antibiotic resistance indicators in and between systems. Utilization of molecular methods as a measure of antibiotic resistance analysis does require certain knowledge, including familiarity with common molecular techniques, properly framed research questions based on specific molecular targets, and awareness of advantages and disadvantages of various methods for the correct interpretation of data.

Antibiotic resistant genes are the most common molecular targets of interest when assessing antibiotic resistance in environmental samples. These genes encode various functions that allow bacteria to survive and grow in the presence of antibiotic concentrations that are inhibitory to susceptible cells. Following the extraction of DNA from a sample, ARGs are normally identified by PCR-based methods and, more recently, by metagenomic techniques. However, the identification of an ARG in a sample only indicates the potential for resistance since the gene may not be expressed, may be in a nonfunctional form (mutated or incomplete), or may be present in a dead cell or as extracellular DNA.

Other common targets for antibiotic resistance analysis include RNA and proteins, which can be targeted to specifically track expression of antibiotic resistance mechanisms. However, RNA- and protein-based methods are challenging techniques, and, as a result, DNA-based methods are generally preferred for tracking ARGs. Horizontal gene transfer allows bacteria to share ARGs through MGEs, such as plasmids, integrons, and transposons. Several studies have incorporated the analysis of markers associated with MGEs when analyzing ARGs in soil and manure (Nandi et al., 2004; Binh et al., 2008; Popowska et al., 2012; Klümper et al., 2015), which provides a line of evidence that gene transfer may be a factor in the proliferation of ARGs.

Traditional PCR is one of the most popular methods of detecting known ARGs in environmental samples since it is highly sensitive, provides relatively rapid results in 2 to 3 h, and produces direct information about the DNA sequence of interest. Polymerase chain reaction is an enzyme-dependent reaction that utilizes highly specific primers that recognize sections of a target gene and amplify it. However, challenges and limitations exist for applying PCR to samples from agroecosystems. One of the most significant challenges is that PCR is dependent on the extraction of DNA, which should be optimized for the matrix of interest to capture clean DNA from as many different kinds of bacteria as possible and applied consistently across samples intended for comparison. When working with environmental samples, sequencing a subset of the PCR products obtained during analysis is advisable to verify that PCR is amplifying the intended product.

Real-time quantitative PCR (qPCR) provides the same benefits as PCR, while yielding additional information about the copy number (or abundance) of a particular ARG. For qPCR, use of a probe that fluoresces when bound to the target DNA or dyes, like SYBR Green (Thermo Fisher Scientific), that bind to double-stranded DNA allows detection of the amplification of target DNA during the PCR reaction. As a quantitative method, determination and reporting of limits of quantification are critical. In addition, normalization to 16S rRNA genes is believed to aid in accounting for minor variations in extraction efficiency as well as providing information about the proportion of total bacteria carrying ARGs in the sample (Pruden et al., 2006; Knapp et al., 2010; Heuer et al., 2011). Quantification of ARGs with qPCR methods has been successfully conducted on samples from diverse agroecosystems, including swine lagoons (Koike et al., 2007), groundwater (Koike et al., 2007), river sediments (Pei et al., 2006), and manure and soil (Heuer and Smalla, 2007). The development of qPCR arrays is a promising way to analyze multiple targets; however, it may be best used as a screening tool since the limit of detection is higher than traditional qPCR. One major drawback of PCR-based methods is that sequences for the genes of interest must be known and selected ahead of time, which may bias the results and overlook key genetic elements associated with antibiotic resistance.

Horizontal gene transfer is a key process to characterize since it is the means by which antibiotic resistance actually spreads among bacteria. Documentation of HGT occurrence and potential can occur through PCR-based analysis of specific marker genes associated with MGEs (Nandi et al., 2004), retrospective genome or metagenome analysis (Nesme et al., 2014; Nesme and Simonet, 2015), and direct assays of transfer including conjugation, transduction, and transformation (Coque et al., 2008; Musovic et al., 2010; Seitz and Blokesch, 2013). Direct assays are useful for determining mechanisms of action, host ranges, and transfer rates of ARGs on mobile elements as well as identifying whether ARGs are functional. However, these analyses require that the recipient cells be culturable, which limits their application, especially in agroecosystem research. The use of a reporter gene, such as green fluorescent protein, could reduce the need for culturing and selection steps while still confirming that the genes of interest are actually being expressed under the conditions of the study (Klümper et al., 2015).

The development of next-generation DNA sequencing methods has led to a new era of molecular characterization of environmental ecosystems. These technologies circumvent the need for PCR and provide a broad snapshot of the ARGs, MGEs, virulence genes, and various other functional genes in the samples of interest. Application of metagenomic approaches to agroecosystems has revealed a wide range of ARGs and MGEs (Kristiansson et al., 2011; Bengtsson-Palme et al., 2014). It also provides broad contextual information beyond identification of ARGs and other targets of interest. Identification of HGT elements can provide information about how ARGs may pass from one environment to another (Nesme and Simonet, 2015). Identification of genes of interest from metagenomic datasets is facilitated by publicly available databases and tools; however, numerous challenges are associated with data analysis, and further development of approaches and consensus in the scientific community for standardized analysis would be beneficial.

Combining molecular- and culture-based methods presents some advantages and can assist in linking genotype with phenotype. However, most culture-based assays require a great deal of time and only recover a small subset of the total bacterial community. A summary of the major pros and cons associated with using molecular- and culture-based methods is found in Table

2. Recent work has focused on expanding molecular-based techniques into single, rapid assays that would provide information about antibiotic resistance phenotypes. These molecular phenotype methods include membrane hybridization (Jindal et al., 2006) and fluorescent in situ hybridization (FISH) methods (Zhou et al., 2009) and have been applied to manure and soil samples. While membrane hybridization scales up more easily, FISH has the capability of identifying resistant microorganisms when used in combination with phylogenetic probes (Zhou et al., 2009).

Regardless of the method or combination of methods selected, experimental design is paramount and must be carefully planned to address the research question(s) of interest. Antibiotic resistance in agroecosystems is multifaceted not only because of the diverse environments within these systems but also because of the complexity of the origins of antibiotic resistance. A successful research design involves (i) inclusion of appropriate controls and accounting for background and/or baseline antibiotic resistance, (ii) obtaining representative samples and statistical resolution in systems that may be spatially heterogeneous and temporally variable, (iii) accurately capturing the factors that may play critical roles in the field (e.g., application of manure, temperature, precipitation), (iv) garnering insight into ARG hosts and viability, and (v) combining methods to support multiple lines of evidence to support conclusions.

Knowledge Gaps and Future Research Directions

Currently, the pathways that allow antibiotic compounds, ARB, and ARGs to move through the environment are not fully understood. The causal model presented by Williams-Nguyen et al. (2016) helps identify the environmental sectors or reservoirs where these antibiotic contaminants may be found and outlines the main pathways by which they may move through agroecosystems. Yet this information is not complete, and additional research is necessary to fully elucidate current reservoirs and pathways of antibiotic-related contaminants in the environment, while also identifying those that are not known. The need for risk assessment of antibiotics and antibiotic resistance in the environment is also critical but hindered by the lack of knowledge about the quantities and types of antibiotic drugs, ARB, and ARGs that are present and where within the agroecosystem they are located. Lastly, although selection of targets is normally driven by human and animal health, ecological health should be another consideration.

Well-developed standard methods for accurate analysis of antibiotics, ARB, and ARGs from environmental samples are rare. While methods have been developed for analysis of antibiotics and antibiotic resistance in clinical settings, these methods cannot readily be applied in environmental settings. The matrices found in agroecosystems are complex and routinely contain compounds that interfere with subsequent analysis. Because standard methods have not been developed for antibiotic research in the environment, most laboratories must develop their own methods. This severely limits the ability to make comparisons between samples analyzed in different laboratories and hinders risk assessment analysis. The development of standard methods for the detection and quantification of antibiotics, ARB, and ARGs in agroecosystems is a critical research need.

Surveillance programs for monitoring antibiotics and antibiotic resistance in the environment are lacking to date. The development and implementation of these types of programs at local, national and international levels would provide long term, comprehensive information about how and where antibiotics and antibiotic resistance are affecting agroecosystems. These programs would provide information about the overall impacts within agroecosystems to assist in determining areas that require additional research focus. Surveillance data would also assist in identifying environmental reservoirs of antibiotics, ARB, and ARGs; routes that allow these contaminants into and out of agroecosystems; and pathways that pose potential health risks to humans, animals, and other biota by allowing contact with contaminants.

Table 2. Pros and cons associated with the use of culture- and molecular-based methods to evaluate antibiotic resistance is agroecosystems.

Culture techniques	
Pros	**Cons**
• Direct identification and analysis of antibiotic resistance in individual isolates	• Time consuming; results can take days
• Opportunity to link results with phenotypic and genotypic characteristics	• There is an inherent cultivation bias; easily cultivated microbes are generally targeted most often
• Ability to assess antibiotic resistance gene transfer potential	• Not all microogansims are culturable; cannot identify bacteria that are viable but nonculturable
• Do not require complex instrumentation and can be performed at a relatively low cost	
• Can be used to determine clinical breakpoint concentrations	
Molecular techniques	
Pros	**Cons**
• Direct detection of target nucleic acid without cultivation	• Entire DNA pool cannot be extracted from environmental samples
• High specificity and sensitivity	• Inability to distinguish between nonviable and viable microorganisms and extracellular DNA
• Results obtained from traditional polymerase chain reaction (PCR) and real-time quantitative polymerase chain reaction (qPCR) within a few hours	• Detection of antibiotic resistance gene only indicates a potential for resistance
• Next-generation sequencing circumvents need for PCR and provides a broader snapshot of genes	• While useful for determining gene expression, it is difficult to analyze RNA and proteins

Conclusions

The analysis of antibiotics and antibiotic resistance in agroecosystems is an important area of research that requires a One Health approach to fully understand the health implications of antibiotic drugs, ARB, and ARGs in the environment. Since the use of antibiotics is not diminishing and incidences of antibiotic resistance are on the rise in human and animal populations, a greater understanding of the transport and fate of antibiotics, ARB, and ARGs in the environment is critical to determine the possible risks and impacts on human, animal, and ecological health. While food production systems and biosolid applications are recognized as significant input sources of antibiotic-related contaminants, the direct and indirect impacts in agroecosystems are not known. Development of standard methods and practices among the scientific community is necessary for accurate identification and quantification of antibiotics, ARB, and ARGs in soil, water, manure and other environmental matrices. Additional focus on standard research methods and practices is a critical first step in obtaining the reliable data necessary to provide a comprehensive evaluation of antibiotics and antibiotic resistance in agroecosystems and begin to determine the potential risks to human, animal, and ecological health.

Glossary

The accurate analysis and discussion of antibiotic resistance in agroecosystems requires a precise and standardized vocabulary, in addition to the use of adequate experimental controls. Many terms used when describing antibiotic resistance research have meanings that vary across disciplines or do not have clearly established definitions. For example, the terms *antimicrobial* and *antibiotic* are often used interchangeably; however, in this review, *antimicrobial* is defined as a natural, semisynthetic or synthetic chemical that kills or inhibits the growth of microorganisms, and *antibiotics* are described as the subset of antibacterial compounds that target bacteria (Fig. 1).

Absolute recovery. The ratio of the instrument response (e.g., area) of the analyte spiked into the sample before extraction to the response of the analyte spiked in a pure solvent (standard solution), defined at a particular concentration. This can be expressed in percentage by multiplying the ratio by 100. Absolute recovery does not take into account any matrix suppression or enhancement in the detection system.

Acquired resistance. Antibiotic or antimicrobial resistance coded by genes obtained by transformation, transduction, or conjugation. The term *acquired resistance* is typically used in contrast to *intrinsic* or *inherent resistance*, in that the organism exhibiting acquired resistance was previously susceptible to an antibiotic or antimicrobial.

Agroecosystem. Region of agricultural production functionally defined as an ecosystem: land or water areas used for agricultural purposes (poultry houses, feedlots, aquaculture, crop production fields and pastures, greenhouses, and adjacent areas including surface water, soil, and groundwater). Includes living and nonliving components and agricultural inputs and outputs such as feed, manure, fertilizers, and biosolids.

Antibacterial. Any natural, semisynthetic, or synthetic compound that results in bacterial cell death or inhibition of bacterial growth. Disinfectants and antiseptics with antibacterial activity are considered antibacterial, as are ionophores (see Fig. 1).

Antibiotic. A chemical used to treat infectious bacterial diseases in humans, animals, or plants that results in bacterial cell death or inhibition of bacterial growth. This includes natural, semisynthetic, and synthetic compounds. Antibiotics are a subset of antibacterials (see Fig. 1).

Antibiotic class. A group of chemically related antibiotics having a similar mode of action on susceptible bacteria.

Antibiotic resistance. The ability of a microorganism to survive and/or grow in the presence of an antibiotic at a concentration that would normally prevent its growth or reproduction.

Antibiotic resistance gene (ARG). A gene conferring resistance to one or more antibiotics or different antibiotic classes. Genes involved in the transfer or expression

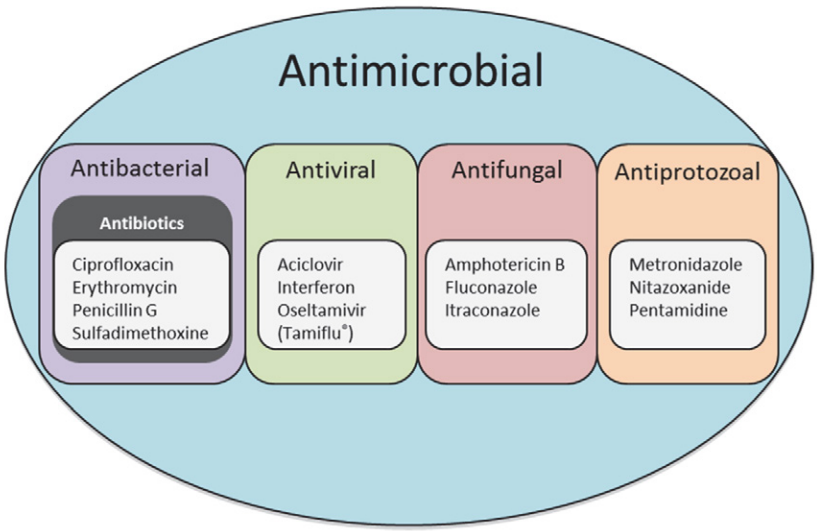

Fig. 1. Concept diagram of antimicrobial chemicals, which may be natural, semisynthetic, or synthetic and are used to kill or inhibit the growth of microorganisms. Antibiotics, a subset of antibacterials, are a type of antimicrobial used in the treatment and prevention of bacterial infections.

of resistance genes are not included in this definition since they are considered mobile genetic elements, not ARGs.

Antibiotic resistant bacteria (ARB). Bacteria able to grow in the presence of an antibiotic at a particular concentration. The specific concentration is either determined empirically by clinical standards that correlate phenotypic isolate measurements with treatment failure, or epidemiologically by determining the concentration of the target drug that inhibits the growth of the majority of strains in a species.

Antibiotic resistant determinant (ARD). An older term that is equivalent to ARG. It is generally no longer used and has been replaced by the term *antibiotic resistance gene* (ARG).

Antimicrobial. Any chemical compound (natural or synthetic) that inhibits growth or kills microorganisms. Antimicrobials include antibacterial, antiviral, antiprotozoal, and antifungal compounds. Disinfectants, antiseptics, and ionophores are also considered antimicrobial agents. This is a general term for agents used against all microbes, not just bacteria (see Fig. 1).

Antimicrobial resistance. The ability of a microorganism to grow and survive the toxic effects of exposure to an antimicrobial agent.

Background. The concentration of antimicrobial drugs, antibiotic resistant bacteria, or antibiotic resistance genes that would exist without a local anthropogenic source or stressor being present. Background can be represented by a range rather than an absolute value.

Baseline. Concentration of antibiotic drug, resistant bacteria, or resistance genes representing the present state of the sampled environment and used to provide information against which any changes can be measured.

Broad host range plasmid (BHP). A plasmid that can be transferred and maintained in phylogenetically diverse bacteria, which represent multiple genera.

Clinical Laboratory Standards Institute (CLSI). A US-based not-for-profit organization established with the objective of developing clinical and laboratory practices and promoting their use worldwide. Among other things, the CLSI develops MIC (see below) guidelines for specific antibiotics against various human-associated commensals and pathogens.

Clonal. In reference to bacteria, this term refers to bacterial cells arising via the process of binary fission from a single source and are assumed to be more closely related to each other than isolates from other clones.

Colony-forming unit (CFU). A unit used to evaluate the number of viable bacteria or fungal cells in a sample. The process of calculating colony forming units includes serial dilutions of the sample, plating on agar medium, and counting the resulting colonies. The intention is to separate cells, so that each individual cell grows into a bacterial colony. However, since the potential exists for two or more cells to stick to each other, land in the same place on the agar, and result in only a single colony instead of more, it is customary to refer to the plate counts in terms of number of colonies, not number of cells.

Concentrated animal feeding operation (CAFO). Regulated animal agriculture enterprise that utilizes high-density livestock production requiring feed delivery to the animals, as opposed to grazing, where designation is given on the basis of the number of animal units (e.g., 1000 or more cattle in the United States) or in the collection and discharge of livestock waste.

Conjugation. Cell-to-cell mediated gene transfer of a mobilizable genetic element, plasmid, or transposon. It requires that a live donor and live recipient have physical contact with each other and be actively growing.

Epidemiological cutoff value (ECOFF). The normal distribution of MIC breakpoints (see below) in a given bacterial species.

European Committee on Antimicrobial Susceptibility Testing (EUCAST). A standing European committee aimed at developing MIC breakpoints (see below) for selected antibiotics toward specific bacteria. Can be considered the European counterpart of the Clinical Laboratory Standards Institute.

Extraction recovery. The ratio of the instrument response (e.g., area) of the analyte spiked into the sample before extraction to the response of the analyte spiked in a sample after extraction (sample matrix), defined at a particular concentration. This can be expressed in percentage by multiplying the ratio by 100. Extraction efficiency accounts for matrix suppression or enhancement in the detection system because the analyte response is relative to the signal of the spiked standard in the sample matrix.

Feed additive. A food supplement for livestock production, including vitamins, amino acids, fatty acids, minerals, steroid hormones, and antimicrobials.

Horizontal gene transfer (HGT). Transfer of genes and/or mobile elements between bacteria in a manner other than traditional meiosis. The three most studied mechanisms of HGT in bacteria are conjugation, transformation, and transduction, although other mechanisms exist. *Lateral gene transfer* is occasionally used as a synonym.

Integrons. A mobile genetic element found on bacterial chromosomes and/or plasmids composed of an integrase-encoding gene and an integration site where gene cassettes can be inserted via site-specific recombination. Integrons often harbor antibiotic resistance genes. They can collect multiple gene cassettes and are therefore often associated with multidrug resistance. Not unique to prokaryotes.

Internal standard. A known amount of compound that is added to samples, blanks, and calibration solutions that has a very similar structure and behavior with the analyte, yet

different enough to have a separate and distinguishable signal from the analyte. Inefficiencies in sample preparation, matrix effects, and drift in instrument performance should have similar effects on the signals of both the analyte and the internal standard; thus, using the ratio of the two signals in calculating analyte concentrations reduces variability and improves accuracy of the analytical method.

Intrinsic resistance. Antibiotic or antimicrobial resistance that results from the structural or functional properties inherent to a particular bacterial species. These inherent properties predate the antibiotic era and are chromosomally encoded or bacteria lack a pathway/target site. These traits are transmitted vertically from mother to daughter cells. For example, Gram-negative bacteria are intrinsically resistant to vancomycin due to the inability of the drug to cross the outer membrane, and anaerobic bacteria are intrinsically resistant to aminoglycosides because uptake of the drug is linked to electron transport, which is not present in anaerobic bacteria. The term is used in contrast to *acquired resistance*. The term *innate resistance* is sometimes used as a synonym for *intrinsic resistance*. For example, if the genus, such as mycoplasma, does not make a cell wall, they have always been intrinsically resistant to all β-lactam antibiotics.

Ionophores. A chemical compound that facilitates the transport of ions across a cell membrane, either by binding with the ion or by creating a channel through the membrane. Ionophores disrupt membrane potentials by conducting ions through a lipid membrane in the absence of a protein pore and thus exhibit cytotoxic properties. Used as antimicrobial agents in food animal production, ionophores alter rumen fermentation by increasing the amount of food that is digested by the animal. Ionophores are commonly classified as antibiotics; however, they are not used in human medicine.

Isotope dilution. A method of standard addition by which a known amount of a stable isotope-labeled analyte is added to a sample before extraction. The concentration of the unknown analyte (native) is then determined based on its signal relative to the signal of the known isotope-labeled analog and a previously determined response factor. The response factor is the ratio of the detector response of the same amounts of the native analyte and isotope-labeled analyte. Quantification by isotope dilution provides an automatic correction for sample losses and matrix effects in the target analyte concentration because the isotope-labeled analyte is subjected to the same conditions and procedures as the unknown native analyte.

LC/MS/MS. An analytical method that involves separation of multiple analytes using high performance liquid chromatography (LC) and detection by tandem mass spectrometry (MS/MS). Trace analysis of organic compounds such as antibiotics using LC/MS/MS is typically performed using multiple reaction monitoring in a triple quadrupole MS but may also be performed in an ion-trap MS. LC/MS/MS is more selective and provides higher signal-to-noise ratios than selected ion monitoring that is performed in a single quadrupole LC/MS.

Limit of detection (LOD). In analytical chemistry, LOD is the lowest amount of analyte that gives a signal that is distinguishable from the background signal of the sample matrix in the absence of that analyte. The LOD is typically calculated as the analyte concentration corresponding to three times the standard deviation of the blank signal ($n = 7$). In microbiology, LOD is the lowest number of target cells or genes that can be detected and measured per unit of mass or volume using a specific assay.

Limit of quantification (LOQ). The lowest concentration of a compound that can be determined with both precision and accuracy, under a stated level of confidence (e.g., 95% confidence level). The LOQ is typically calculated as the analyte concentration corresponding to 10 times the standard deviation of the blank signal ($n = 7$).

Matrix effects. The combined effects on an analytical signal from components of a sample other than the analyte resulting in reduced accuracy, reproducibility, and sensitivity of a method. Matrix effects can cause signal suppression or enhancement in analysis by gas or liquid chromatography with mass spectrometric detection. Percentage matrix effects can be evaluated by determining the ratio of the analyte response recorded for the analyte spiked in a sample after extraction (sample matrix) to the response of the same amount of analyte spiked in a pure solvent (standard solution).

Metabolites. Reaction products formed during the biological degradation of chemical compounds through enzyme-catalyzed reactions that leads to conjugation, bond cleavage, isomerization, and/or other chemical modifications on the parent compound.

Minimum inhibitory concentration (MIC). A measurement equal to the lowest concentration of the target drug that is able to inhibit the visible growth of a bacterium after a specified period of time, most commonly an overnight incubation using a standardized method.

Mobile genetic element (MGE). Genes involved in the transfer or expression of resistance genes and DNA that move within cells or between genomes, including integrons, plasmids, insertion sequences, transposons, conjugative transposons, and bacteriophage. Antibiotic resistance genes are normally associated with MGEs; however, a MGE does not have to be associated with an ARG.

Multi-drug resistance (MDR). In general, the state whereby a microbe is classified as resistant to more than two or three antibiotic classes; however, a specific functional definition varies widely. In human and veterinary treatment settings, the term refers to a demonstrated resistance of isolates. However, the term is also widely used when describing the carriage of ARGs, which may or may not have the capacity to be expressed. One common definition is that an organism carries more than two or three different resistance genes or mutations in different targets that confer resistance

to different classes of antibiotics. When used in this way, the term does not apply when a single ARG confers resistance to multiple classes of antibiotics such as the *erm* genes conferring resistance to macrolides, lincosamides, and streptogramin B. Due to the lack of consensus on the definition of this term, it is recommended that it be clearly defined in the materials and methods sections when reporting in the scientific literature.

National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS). A public health surveillance system that tracks changes in the antimicrobial susceptibility of certain enteric (intestinal) bacteria found in ill people, retail meats, and food animals in the United States.

Narrow-host-range plasmids (NHP). Plasmids that are only shared within genera or between isolates within a single species. The *Haemophilus* plasmids are an example of NHP.

One Health. The concept that human, veterinary, and environmental health are not separate entities, but are interconnected. It is the collaborative effort of multiple disciplines—working locally, nationally, and globally—to attain optimal health for people, animals, and the environment.

Plasmid. Small, heritable, double-stranded DNA distinct from the chromosome. It can replicate independently or integrate into bacterial chromosome and often carry nonessential host genes, including ARGs.

Polymerase chain reaction (PCR). A technology in molecular biology used to amplify a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region, along with a DNA polymerase, the enzyme catalyzing DNA replication, are key components to enable selective and repeated amplification.

Pressurized liquid extraction (PLE). A method of sample extraction that incorporates the use of liquid solvents at increased temperature and pressure, sometimes approaching the supercritical region. Increased temperature results in higher rates of diffusion and increased solubility while the increased pressure keeps the solvent from reaching its boiling point. The combination allows for efficient extraction while limiting solvent consumption.

Proto-resistance. A state whereby a sample contains genes with no current activity against antibiotics but that have the potential to gain this function, i.e., genes that confer subminimum inhibitory concentration (MIC) levels of resistance that combined with subsequent mutations or acquisition of additional genes can generate full MICs.

Quality assurance/quality control (QA/QC). The complete set of procedures used to measure and document the quality of data produced from an analytical process to ensure the integrity of results and that specific criteria are met. QA/QC typically includes a number of different techniques used to validate the results of analytical measurements, including the preparation and analysis of fortified matrix and laboratory blanks, replicate sample analysis, or inclusion of a surrogate for monitoring recovery between samples.

Resistome. The resistance gene reservoir; all existing antibiotic resistance genes (ARGs) in both pathogenic and nonpathogenic bacteria, usually defined within a given site, e.g., "the human gut resistome," or "the soil resistome."

Silent/cryptic resistance. Phenotypic susceptibility to the target antibiotic concurrent with carriage of genes that code for resistance to the target but are not expressed. This type of resistance may become clinically important if expression is restored by mutation or mobilization.

Solid phase extraction (SPE). A sample preparation technique that combines clean-up and concentration of analytes into one procedure by passing the liquid sample through a solid stationary phase to separate the target analytes from the rest of the sample matrix. Separation of analytes can be achieved by selectively adsorbing them in the solid stationary phase and letting the rest of the sample components pass through, or vice versa. In practice, many other compounds in the sample, other than the target analytes, are coextracted and concentrated with the analytes, potentially leading to significant matrix effects.

Standard addition (SA). A quantification method whereby a known amount of analyte is added to the sample, before or after extraction. If added before extraction, losses during sample extraction can be taken into account; this approach can be time consuming and costly and may only be possible if enough sample is available for extraction of spiked and unspiked samples. If added after extraction, sample processing is shortened but losses during extraction are not corrected for in the quantification of the analyte. One-point standard addition, whereby only one concentration of analyte is added to a sample, can be performed if the concentration of the analyte and the added sample are within the linear range. If the unknown concentrations in the samples are expected to be widely variable, a series of increasing concentrations of standards are added into various samples; the total additive signal from the analyte and the standard added are plotted against the concentration added. A linear regression of these responses, extrapolated to zero, is used to calculate the concentration of the analyte in the original sample.

Surrogate. A compound that is chemically similar to the analyte of interest and is added in known amounts to samples. Surrogates are used to determine extraction efficiencies and matrix interferences and, therefore, should behave similarly to the analytes in the experimental samples.

Transduction. Viral-mediated transfer of bacterial DNA from one host cell to another; primarily among closely related strains.

Transformation product. In chemistry: stable, or relatively stable, intermediary compounds formed by the incomplete mineralization of a parent compound. In biology: a mosaic gene that has some new nonhost DNA sequences mixed with host DNA, often conferring new properties such as increased resistance to an antibiotic.

Transposons and conjugative transposons. Transposons are small self-contained segments of DNA that can insert themselves into a chromosome or plasmids within a host bacterial cell. Conjugative transposons have the same ability to move within a host among different DNA regions but also carry additional genes that allow transfer from one host bacterial cell to another.

Vertical gene transfer. In bacteria, the transfer of genetic material from mother to daughter cells through asexual reproduction. The term *clonal*, or *clonal spread*, is a synonym.

Wastewater. Spent or used water that has been adversely affected by anthropogenic processes (agricultural, industrial, and municipal), typically resulting in diminished or impaired quality.

Wastewater treatment plant (WWTP). A facility that receives wastewaters from domestic and/or industrial sources and that through a combination of physical, chemical, and biological processes, treats the wastewater to reduce concentrations of regulated contaminants.

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