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The missing link. A role for nuclear techniques

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The missing link. A role for nuclear techniques

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Acronyms and abbreviations

AM	Antimicrobials
AMA	Antimicrobial agent
AMR	Antimicrobial resistance
ARG	Antibiotic-resistant genes
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
APLI	Atmospheric pressure laser ionization
CSIA	Compound stable isotope analysis
DNA	Deoxyribonucleic acid
FAO/IAEA	Food and Agriculture Organization of the United Nations/International Atomic Energy Agency
GAP	Global action plan
HPLC	High performance liquid chromatography
HILIC	Hydrophilic interaction chromatography
OIE	World Organization for Animal Health
WGS	Whole genome sequencing (WGS)
WHA	World Health Assembly
WWTPs	Waste water treatment plants

Executive summary

Antimicrobials (AM) play a critical role in the treatment of human and animal (aquatic and terrestrial) diseases, which has led to their widespread application and use. Antimicrobial resistance (AMR) is the ability of microorganisms (e.g. bacteria, viruses and some parasites) to stop an antibiotic, such as an antimicrobial, antiviral or antimalarial, from working against them. Globally, about 700 000 deaths per year (33 000 in Europe[1]) arise from resistant infections as a result of the fact that antimicrobial drugs have become less effective at killing resistant pathogens. Antimicrobial chemicals that are present in environmental compartments can trigger the development of AMR. These chemicals can also cause antibiotic-resistant bacteria (ARB) to further spread antibiotic resistance genes (ARG) because they may have an evolutionary advantage over non-resistant bacteria.

For some time now, AMR has been approached mainly from the human and animal health angles, however little is known about the impacts that AMR in the environment may have on health. The Food and Agriculture Organization of the United Nations (FAO), the World Health Organization (WHO) and the World Organization for Animal Health (OIE) have a long-standing partnership to combat health threats associated with interactions between humans and animals. In a recent Memorandum of Understanding, this tripartite alliance agreed to strengthen its joint work with a focus on tackling AMR.

Global efforts on AMR must recognize the extent to which antibiotics, and resistant bacteria spread through manufacturing run-off and human and animal waste. Inviting UN Environment to collaborate with FAO, WHO and OIE on the environmental aspects of AMR is fully in line with the directives in Resolution EA.3/Res.4[2], which was adopted by the UN Environment Assembly in 2017. This resolution notes that human, animal and plant health and the environment are interconnected and requests the UN Environment to prepare, by the fifth session of the United Nations Environment Assembly in 2021, a report on the environmental impacts of AMR. Furthermore, a preliminary report on the Joint FAO/WHO Expert Meeting in collaboration with OIE on *Foodborne Antimicrobial Resistance: Role of the Environment, Crops and Biocides* (November 2018) highlighted the importance of AMR and antibiotic-resistant genes (ARGS) in the immediate plant production environment. The report indicated that conventional and organically grown vegetables could be vehicles for the dissemination of AMR bacteria and their resistance genes to humans if consumed raw, and that soil, organic fertilizers and irrigation water are sources of microbial residues and contamination. Thus, AMR bacteria are present in agricultural soil and may spread into the food.

Potentially high levels of antibiotics and, correspondingly, high levels of ARB and ARG can be found in the effluents of waste water treatment plants. The sewage of industrial plants that have produced antibiotics might contain more ARB and ARG than does sewage from hospitals or from urban areas. In general, waste water can act as a potential reservoir. The spread of AMR in the environment is generally not controlled by national or international legal regulations. The European Union (EU)

[1] https://www.thelancet.com/action/showPdf?pii=S1473-3099%2818%2930605-4

[2] https://papersmart.unon.org/resolution/uploads/k1800154.english.pdf

Water Framework Directive contains a provision on ensuring high quality of fresh water according to agreed standards. However, the phenomenon of AMR is not referenced. The other transmission route of ARG and ARB – over manure into soil that is connected to surface water or groundwater – is even more complicated to control. One bottleneck is the lack of reporting and definitions of ARG and ARB in the environmental context. In contrast, clinical strains are much better described.

This paper will provide alternative screening methods useful for environmental samples and surveillance approaches in planning such screening efforts. We consider a range of methods for studying environmental horizontal gene transfer. A better understanding of how antimicrobial resistance moves from agricultural areas to the environment through soil and water is important if we are to develop guidance to managing it cost effectively. Based on case studies, this paper aims to summarize the current understanding of the occurrence of ARG in the environment, and the antimicrobial movement from agricultural areas to the environment.

Current convectional chemical methods used to analyze antibiotics and study ARB with metagenomics provide comprehensive surveys on high concentrations of contaminants in the environment, but fail to elucidate sources and sinks, or to reveal how the two parameters influence each other. Nuclear techniques could help determine the sources of antibiotics and where they are eliminated through isotopic fingerprinting of sources and may detect the degradation of antibiotics by transformation-induced isotopic effects.

The cost-benefit and cost-effectivity of analyses are not discussed in this technical paper. Neither does the paper touch on risk management approaches, such as hazard identification, exposure assessment, toxicological assessments, and overall risk assessment. Instead, the focus is on the methodologies that can be used to detect and trace the source and transport of antibiotics through soil and water, and, more importantly, the nuclear techniques (multi-element stable isotopes fingerprinting) that can determine the origin, production process and transport of AM, using Compound-Specific Stable Isotope Analysis (CSIA). We offer recommendations on how to handle samples gathered in the field, including sampling strategies and protocols, the transport and preservation of samples, and methods for analyzing them. The paper concludes with our thoughts on the way forward, including knowledge gaps on AMR that warrant investigation using nuclear and microbiological methods.

This technical paper was developed by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture (AGE), the Animal Health Service (AGAH) and the Land and Water Division (CBL), Climate, Biodiversity, Land and Water Department of the Food and Agriculture Organization of the United Nations (FAO), with inputs from accredited colleagues in the field of nuclear techniques in agriculture and the environment.

Introduction

Antimicrobials (AM) have been vitally important to human and veterinary medicine since the discovery of an antimicrobial agent against syphilis in 1909 (Gensini, 2007) and the discovery of the first antibiotic – penicillin – in 1928, which gave rise to a revolution in medicine. With their applicability to the treatment of various microbial infections, the amount of antimicrobials used in human medicine is on a continual rise around the world. The downside of this extensive use, and the resulting release of antibiotics into the environment, is an increasing spread of antimicrobial resistance (AMR), including bacteria that are resistant to antibiotics. For the most part, the use of antibiotics in animals is not for therapeutic purposes. Instead, significant amounts are either used prophylactically on healthy animals to stop the development of an infection, or to promote growth and speed up the pace at which animals gain weight.

As long as there have been antimicrobials, there has been AMR, but its rise in recent decades has caused significant problems for the treatment of infections. Until now, AMR has mostly been approached from the human and animal health perspectives. However, global efforts to manage AMR must also consider how antibiotics and resistant bacteria spread through manufacturing run-off and human and animal waste. This means expanding current efforts to include the plant and environmental sectors, a move that is fully in line with Resolution EA.3/Res.4, adopted by the UN Environment Assembly in 2017. The resolution notes that human, animal and plant health, and the environment are all interconnected and requests UN Environment to prepare – by the fifth session of the United Nations Environment Assembly in 2021 – a report on the environmental impacts of antimicrobial resistance that includes an assessment of the gaps in knowledge around the impact and causes of AMR in our environment.

There are currently no definitive methods for monitoring AMR in environmental compartments. We need to better understand the persistence dynamics of antimicrobial-resistant bacteria, antimicrobial residues, antimicrobial resistance genes and potential for the exchange of genes in wastes and wastewater (and how these factors vary with treatment) in order to precisely define the target indicators associated with environmental sources such as water and soil. Environmental target organisms have not yet been identified, which prevents efforts to provide guidance on effective AMR management. Targets and indicators will most likely be region-specific depending on climate and type of production system.

In 2015, a global action plan (GAP) on AMR was adopted by the World Health Assembly (WHA). The plan committed all member states to developing their own national action plans for tackling AMR. In addition, the Food and Agriculture Organization of the United Nations (FAO) has developed an action plan on AMR, specifically to support the food and agriculture sectors in implementing the GAP. Other global initiatives to control AMR include the Interagency Coordination Group on Antimicrobial Resistance (IACG), established by the Political Declaration of the High-Level Meeting on Antimicrobial Resistance (UN Resolution A/RES/71/3). The IACG's mandate is to provide practical guidance on approaches needed to ensure sustained effective global action to address antimicrobial resistance. The IACG will report its recommendations to the UN Secretary-General in 2019.

The presence of antibiotic chemicals in environmental compartments creates a selection pressure that can lead to the development of AMR. These chemicals may also cause the further spread of antibiotic-resistant bacteria (ARB) because they often have an evolutionary advantage over non-resistant bacteria. ARB carry antibiotic resistance genes (ARGs), which code for enzymes or changes in the cell that can counter the effect of the specific antibiotics. Antibiotic resistance genes may be passed from one organism to the other in a process known as horizontal gene transfer. Hence, is it not only the chemicals, but also the ARGs that must be regarded as environmental contaminants. Not all ARBs are equally harmful to humans. Resistant pathogenic bacteria are of greater concern than nonpathogenic bacteria. It is therefore also important to know the exact bacterial species that is carrying the ARG. To sum up, when looking at the environmental consequences of antimicrobial use, three different types of contaminants emerge:

1. the original antibiotics released into the environment;
2. antibiotic resistance and the corresponding ARGs;
3. ARBs, especially pathogenic bacteria harboring antibiotic resistance.

CLASSES OF ANTIBIOTICS, MODES OF ACTION AND RESISTANCE STRATEGIES

To better understand antimicrobial effects and the development of resistance, it is useful to review how different antibiotics work.

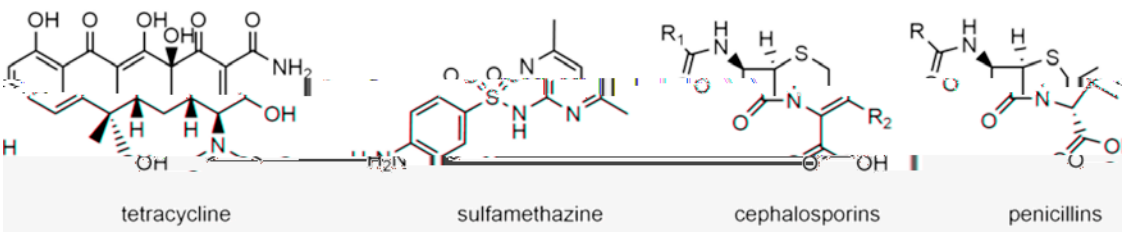
Mode of action of different antibiotics.

Among the most widely used antibiotics in human medicine are penicillins, which belong to the class of β-lactam antibiotics, together with e.g. cephalosporins, carbapenems and monobactams. Their common chemical structure is the characteristic β-lactam ring (see Figure 1). This four-membered ring is an easy target for hydrolysis, which can be mediated by enzymes. Hydrolysis renders antibiotics ineffective and is responsible for their rather short half-life in the environment. β-lactam antibiotics attach to penicillin-binding proteins and are bactericidal, meaning that they kill bacteria through inhibiting the synthesis of the bacterial cell wall by inducing a futile cycle of peptidoglycan synthesis and degradation, which depletes the cells of resources (Cho, 2014).

Tetracyclines are another class of antibiotics that are also used both in human and veterinary medicine and are, as penicillins, of natural origin. Over time, research has led to the discovery of various natural or semi-synthetic tetracyclines. They work by inhibiting the binding of aminoacyl-t-RNA to the mRNA-ribosome complex. Because of their octahydrotetracene-2-carboxamide skeleton, degradation is not so easily achieved, and this causes these compounds to persist longer in the environment (see Figure 1) (Nguyen, 2014).

Sulfonamides, another antibiotic class, were the first fully synthetic antimicrobials and the first to be used systematically. They are bacteriostatic, meaning that they inhibit the cell division of bacteria through competitive inhibition of dihydropteroate synthetase (DHPS), which is a crucial enzyme in the synthesis of folate. Due to their structure, they are not readily degradable and, therefore an understanding of their environmental fate is particularly important (see Figure 1) (Sköld, 2000). All of these antibiotic classes are used both in human and veterinary medicine.

FIGURE 1
Chemical structures of tetracycline, sulfamethazine, cephalosporins and penicillins



Different resistance strategies.

Antibiotic resistance can originate from several mechanisms: 1) the transformation ('breakdown') of antibiotics; 2) changes in the bacterial target site; 3) active efflux or decreased permeability; and 4) general cell adaption (see Figure 2). Often, bacteria develop several strategies in parallel in order to circumvent antibiotic action.

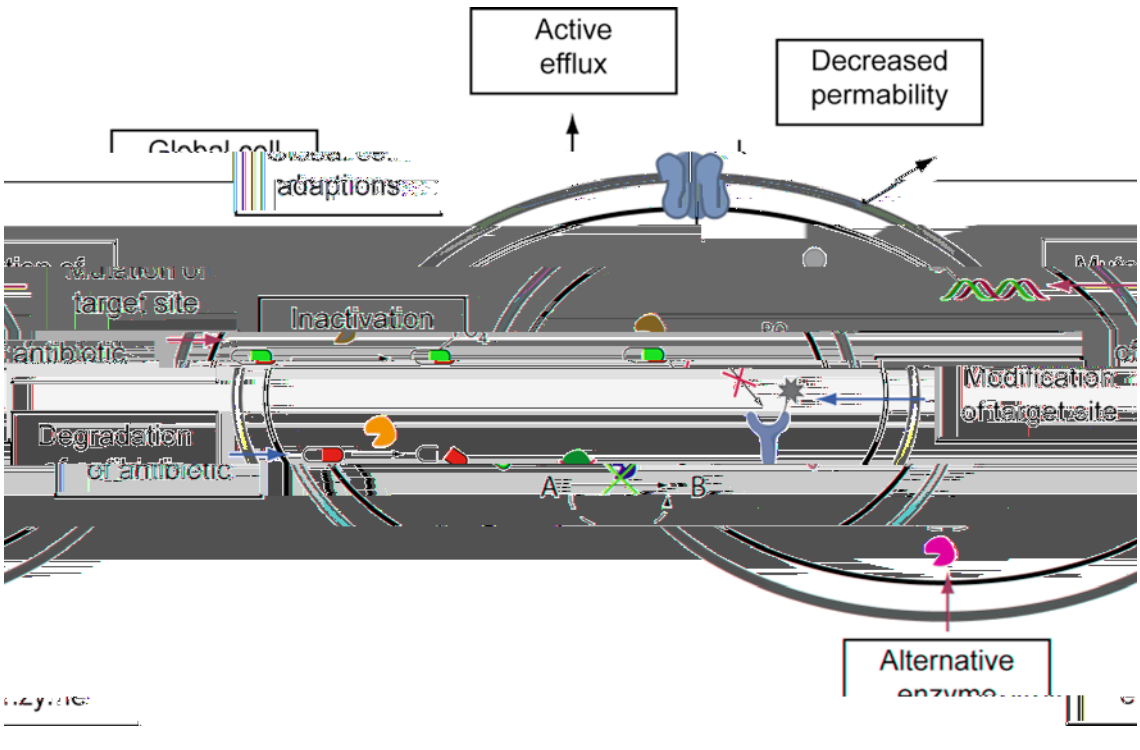
The transformation of antibiotics includes different degradation mechanisms. One example is the hydrolysis of β-lactams through β-lactamases, which releases CO_2, rendering the process irreversible. Extended spectrum β-lactamases (ESBL) are one of the enzyme classes that can hydrolyze penicillins and cephalosporins, thus leading to multi-resistance (Poole, 2004). The addition of functional groups and the resulting change in chemical structure can also cause the ineffectiveness of the antibiotic, for example through acetylation, phosphorylation, and adenylation. Steric hindrance is one of the main reasons for this kind of resistance.

Changes in target sites represent another major group of resistance mechanisms. Target protection is, among other things, a mechanism for creating tetracycline resistance when specific enzymes dislodge the tetracycline from its binding site after interacting with the ribosome (Nguyen, 2014). One of the most prevalent resistance mechanisms is caused by modifications to the target site, either by point mutations in gene coding the target, enzymatic alteration of the binding site, or replacing or bypassing the original target. This mechanism is most prominently represented in sulfonamide resistance. Resistant bacteria express DHPS enzymes, which can distinguish sharply between the substrate and the antibiotic (Sköld, 2000). Penicillin-binding proteins have also been altered in different bacteria to complicate binding β-lactam antibiotics and making the bacteria resistant (Poole, 2004).

Active efflux is another mechanism that is extremely important for tetracycline resistance (Nguyen, 2014). In this case, efflux pumps use proton transfer to transport the antibiotic out of the bacteria. Decreased permeability, in turn, conveys resistance by reducing the amount of antibiotic that can enter the cell, a mechanism especially important for hydrophilic antibiotics such as β-lactams and tetracyclines. These need to be channeled across the membrane through porins (water-filled diffusion channels) or active transport systems, which can be altered, or their expression level within the cell can be changed, to minimize antibiotic intake.

Finally, in recent years it has been observed that the entire bacterial cell adapts to the presence of antibiotics, making it possible for the bacteria to survive (Munita and Arias, 2016). While resistance against some antibiotics (e.g. penicillins, tetracyclines) has developed in bacteria naturally over a long period of time, resistance against synthetically-produced sulfonamides has not evolved over the same time frame. These

FIGURE 2
Overview of different resistance strategies of microorganisms



resistances should therefore not be readily detected in areas without agricultural or anthropogenic influences. Understanding the occurrence of antibiotics, associated resistance genes and pathogens harbouring these resistances is, therefore, particularly relevant for sulfonamide structures.

ENVIRONMENTAL OCCURRENCE

Occurrence of antimicrobial chemicals in the environment.

Tetracyclines and sulfonamides – among other antibiotics – are found in different concentrations in diverse environmental compartments. Tetracyclines and sulfonamides have been detected in the range of ng/L in both surface and ground waters in various regions and countries (Lindsey, 2001; Kemper, 2008). In addition, these antibiotics adsorb to soil and a release is possible if changes appear in the pH or ionic strength of the soil or water passing through the soil (dissolution) (Baquero *et al.*, 2008). The concentration of sulfonamides in surface waters from Germany is shown in Table 1.

In contrast, penicillins and other β-lactam antibiotics have rarely been found in samples taken from water sources (Kemper, 2008). This is due to the high hydrolysis rate of these antibiotics. Other antibiotics have been detected in high concentrations near facilities where they are produced for the global bulk drug market. Concentrations up to the mg/L range, three orders of magnitude higher than found in unaffected surface waters, have been detected in surface waters and concentrations up to the low µg/L range in wells close to production facilities. The study identified two factors as responsible for the high concentrations: the massive amount of antibiotics produced by the facility and insufficient treatment of waste water (Fick *et al.*, 2009). Waste water treatment plants (WWTPs) are not equipped to degrade persistent antibiotics in an efficient way, despite the fact that production facilities are found to release antibiotics and ARB into

the environment through waste water effluent (Rodriguez-Mozaz *et al*., 2015). Disinfection (e.g. ozonation and chlorination) is rarely performed as a standard operation, while biodegradation (e.g. activated sludge treatment) is relatively common. As a result, WWTPs have been found to be hot spots for ARB (Michael *et al*., 2013, Rizzo *et al*., 2013). In order to assess the impact of the continuous release of antibiotics on ecosystems and human health, it is crucial to understand degradation and the long-term environmental fate of these chemicals.

TABLE 1
Measured sulfonamide concentrations in different surface waters in Germany

Sulfonamide	Surface water in Germany (µg/L)
Sulfamethoxazol	0.023 – 1.13[a] 0.005 – 0.09[b] 0.008 – 0.17[c] 0.023 – 0.46[d]
Sulfadimidin (Sulfamethazin)	0.009 – 0.84[a] 0.0015[d]
Sulfadiazin	0.017 – 0.23[a] 0.003[d]
Sulfamerazin	0.01[a]
Sulfathiazol	0.01[a]

Note a: maximum measurement value of a monitoring series; b: minimal measurement value of a monitoring series; c: median of a monitoring series; d: mean of a monitoring series.

Source: MEC database, German Environment Agency

Occurrence of ARGs in the environment.

In addition to direct cultivation-based evidence, ARB can be indirectly revealed by detecting the presence of ARGs on plasmids or genomic DNA. ARGs code for resistance mechanisms such as degradation enzymes or changes in target sites. These genes have been detected in a range of environmental compartments, including soil, sediment, ocean, surface water, groundwater, and even in arctic and Antarctic locations (Li *et al*., 2014; Nesme *et al*., 2014, Conte *et al*., 2017). They also can be observed in WWTP influent and effluent, indicating the presence of ARB throughout the natural and urban environments (Rizzo *et al*., 2013). Different ARGs can code for resistance against a single antibiotic so it is crucial to identify the ARGs and their respective targets. The reverse is also true, as certain enzymes coded by a single ARG can create resistance to several different antibiotic classes thus making the bacteria multi-resistant. Resistance through production of ESBL can be conveyed by different genes and by enzymes, such as the β-lactamase genes $bla_{CTX\text{-}M}$, bla_{TEM}, bla_{SHV} and bla_{OXA}, which present different occurrence patterns depending on location (Canton and Coque, 2006; Bush, 2010; Kittinger *et al*., 2016). Tetracycline resistance is mainly coded by tet genes, such as *tetA*, *tetW* and *tetX*, which use different mechanisms (active efflux, ribosomal protection and degradation) and have been detected in clinical isolates as well as in environmental samples (Nguyen *et al*., 2014). Sulfonamide resistance is mainly due to sul genes, particularly *sul1* and *sul2*, which code for insensitive DHPS enzymes in the bacteria (Skold, 2000). They are also prevalent in soil, WWTPs, and water samples.

Occurrence of genes (ARG) in different bacteria (ARB)

It is also important to consider the distribution of ARGs among different bacterial species. While ARGs are typically considered harmful irrespective of their bacterial host, it is crucial to differentiate between pathogenic bacteria, which can cause disease, and non-pathogenic bacteria, which are generally considered harmless or even beneficial. On the one hand, ARGs harbored in pathogens are a reason for the widespread ineffectiveness of certain antibiotics, independent of the resistance mechanism coded for by these genes. On the other hand, coding by ARGs for degradation enzymes in non-pathogenic bacteria can help to reduce the amount on antibiotics present. Bacterial species and genetic determinants, e.g. ARGs and integrons that have been suggested as indicators, are listed in Table 2

There are currently no commonly-agreed protocols for monitoring AMR in environmental sources. Thus, a better understanding of the persistence dynamics of

antimicrobial-resistant bacteria, antimicrobial residues, antimicrobial resistance genes and the potential for exchange of genes in wastes and waste water (and how these factors vary with treatment) is needed to allow a more precise definition of the target indicators associated with environmental sources such as water and soil.

Overall, AMR presents a major threat to human and animal health: many treatment methods are now ineffective and nosocomial infections, in particular, are becoming more difficult to treat. The direct and visible health impacts of AMR are accompanied by less obvious but no less damaging impacts on the environment. The presence of antimicrobials in the environment can prompt changes in biodiversity, since resistant organisms may have evolutionary advantages. AMR could also contaminate the food chain in various ways. For example, resistance genes could be present in microorganisms used for food production (e.g. starter cultures, bio-conserving microorganisms); ARB could be present on food due to contamination by antibiotics; and cross-contamination with ARB could occur during processing (Verraes *et al*., 2013).

TABLE 2
Suggested possible indicators for antibiotic-resistant bacteria and antibiotic resistance genes in environmental settings

Bacterial groups	Antibiotic resistance genes (and the proteins they encode)
Escherichia coli Klebsiella pneumonia Aeromonas spp. Pseudomonas aeruginosa Enterococcus faecalis Enterococcus faecium	*intI1* (integrase gene of class 1 integrons, a genetic platform for ARG capture)
	sul1 and *sul2* (sulfonamide-resistant dihydropteroate synthase)
	$bla_{CTX\text{-}M}$ and *blaTEM* (β-lactamases, frequently identified in *Enterobacteriaceae*)
	$bla_{NDM\text{-}1}$ (New Delhi metallo-β-lactamase)
	bla_{VIM} (carbapenemase, frequently found in clinical *Pseudomonas aeruginosa* in certain areas)
	bla_{KPC} (*Klebsiella pneumoniae carbapenemase*)
	qnrS (quinolone pentapeptide repeat family)
	aac-(6)-Ib-cr (aminoglycoside acetyltransferase)
	vanA (vancomycin resistance operon gene)
	mecA (penicillin binding protein)
	ermB and *ermF* (rRNA adenine N-6-methyltransferase, associated with macrolide resistance)
	tetM (ribosomal protection protein, associated with tetracycline resistance)
	aph (aminoglycoside phosphotransferase)

Source: Berendonk et al., (2015); FAO/WHO, (2018): https://www.who.int/foodsafety/areas_work/antimicrobial-resistance/FAO_WHO_AMR_Summary_Report_June2018.pdf

Antimicrobial movement from agricultural areas to the environment

Antimicrobials and, more specifically, antibiotics reach the environment through different pathways and spread further into various compartments. The same is true for antibiotic resistance and for bacteria that are resistant to antibiotics. Antibiotics can enter the environment during the production process when they are discharged into waste water or, in some cases, directly into surface water, or as a result of their use in human and veterinary medicine or in plant production. While water is the main vector for moving compounds from human medicine and antimicrobial production (via waste water and release into surface water), soil is the most important vector when antibiotics are used as pesticides, or when manure and slurry used as fertilizers contain antibiotics from veterinary medicine and agricultural use. (Singer *et al.*, 2016).

Antibiotic resistance is particularly notable in places where antibiotics are present in elevated concentrations and where antibiotic-resistant organisms have ideal growing conditions, such as WWTPs (Rizzo *et al.*, 2013), hospitals and slurry storage tanks, which provide excellent environments for bacterial growth. Nevertheless, their dissemination pathway throughout different environmental compartments is unclear. For example, there has been little systematic effort to routinely monitor and evaluate the burden of antibiotics, antibiotic resistance, and antibiotic-resistant pathogens on irrigation water. Hence, this could well be an entry path of undetected contamination, especially in developed countries, whereas drinking water and animal products are monitored more closely[3]. An overview of the different input pathways is given in the left-hand column of Figure 3.

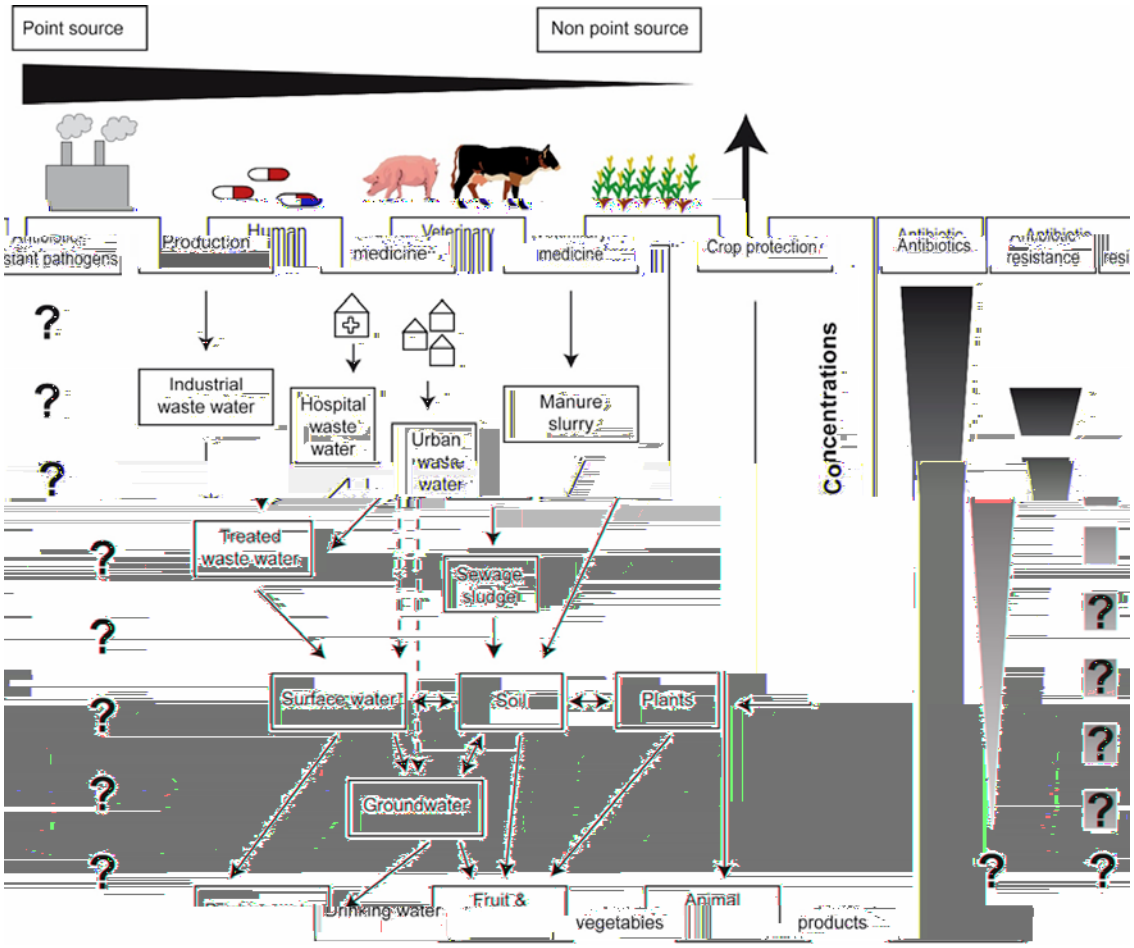
Antibiotics are discharged at particular points as a result of human activity. Over time, these chemical or pharmaceutical compounds may be degraded, sorbed, or chemically modified thus reducing the overall concentration of active substance down the dissemination pathway. Many pharmaceuticals are regarded as emerging substances in surveys. However, with respect to their behavior in the environment, these chemicals may be regarded as 'traditional' contaminants.

Assessing the persistence of antibiotic resistance transferred through ARGs is more challenging. ARGs can also be classified as contaminants because they did not originally occur in large quantities in the environment and are the result of human activity. Unlike antibiotics, however, they may self-replicate, so that they are not only degraded but can also increase in concentration through replication, horizontal gene transfer etc. Consequently, it is crucial to monitor these 'non-traditional' contaminants in all different potential dissemination steps. At sites where conditions for bacteria are ideal (i.e. WWTPs, slurry storage tanks), the concentration of ARGs and antibiotic-resistant pathogens may increase, because antibiotic resistance presents an advantage for bacteria when antibiotics are present (Allen *et al.*, 2010).

As can be seen in Figure 3, there are important knowledge gaps regarding the interdependency of antibiotics concentrations, ARGs concentrations and the abundance of pathogenic antibiotic-resistant bacteria. For antibiotics, we need to better

[3] https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32013D0652

FIGURE 3
Input pathways for antibiotics, antibiotic resistance and antibiotic-resistant pathogens into the environment and the food chain (left) and current knowledge gaps about their concentration behavior throughout the process (right).



understand how these chemicals spread from different sources in the environment and the environmental compartments in which they are degraded, so that elimination can be better predicted, and management efforts better targeted. With regard to ARGs, we need to understand how AR gene abundance is affected by decreasing antibiotic concentrations and whether ARG abundances may even increase by self-replication. There are no current efforts to monitor ARGs in environmental samples so that our knowledge is based on 'snapshots' from a limited number of surveys. The processes that cause or influence the spread of ARGs throughout the environment are not fully understood. Finally, it is not well-known how antibiotic-resistant pathogens behave in the environment, where their concentrations change, and what influences or triggers these changes. Governments are encouraged to explore targeted, effective and cost-efficient research and monitoring efforts to learn more about the factors that determine the amplification and longevity of ARG occurrence

Existing conventional methods for monitoring

MONITORING ANTIBIOTICS IN THE ENVIRONMENT

Liquid chromatography. The chemical analysis of antibiotics in environmental surveys is typically conducted by chromatographic separation followed by mass spectrometric detection. The first step separates target compounds from other substances and environmental matrix interferences. Mass spectrometric (MS) analysis subsequently facilitates the identification and quantification of the compounds from their mass spectrum and fragmentation pattern. Since most antibiotics are non-volatile, high performance liquid chromatography (HPLC), rather than gas chromatography, is commonly used. Separation relies on reversed phase HPLC (polar solvent, non-polar stationary phase), but may also include ion exchange separation and hydrophilic interaction chromatography (HILIC) for particularly polar and water-soluble antibiotics and their derivatives. Compounds are transferred from the liquid phase to the high vacuum of the mass spectrometer in the ion source. There, an electrospray converts the eluent into charged droplets, which are dried until the target compounds take up the charge and enter the high vacuum of the mass spectrometer in ionized form (electrospray ionization, ESI). Less frequent alternatives for ionization are atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI) or atmospheric pressure laser ionization (APLI).

Mass spectrometry. Once the target molecules have been ionized, mass spectra are recorded. This involves guiding the ions through a quadrupole in which opposing magnets have the same polarity. Superimposed modulation with a direct current/alternating current make it possible to discard all of the ions except those with one specific m/z. By scanning through m/z, either full mass spectra can be obtained, or specific ions can be selected for further analysis. Subsequent fragmentation in a collision cell of the mass spectrometer can produce fragment ion spectra, which are analyzed in a second quadrupole mass spectrometer (MS/MS). Alternatively, ions can be measured at high mass resolution in a time of flight MS (TOF-MS) or an Orbitrap mass spectrometer. The selective fragmentation of molecular structures in the collision cell of MS/MS leads to specific daughter ions that allows the identification of target compounds, even in the presence of complex environmental matrices. This facilitates *target analysis* to identify specific antibiotics in trace amounts in environmental samples. In turn, the high mass accuracy of TOF-MS or Orbitrap-MS instruments makes it possible to obtain exact elemental compositions. With this source of information, even the emergence of unknown analytes can be detected, because these compounds may be characterized more closely according to their unique mass, their retention time and their fragmentation pattern *(non-target analysis)* (Krauss *et al.*, 2010).

'Conventional' chemical analysis based on chromatography and mass spectrometry has the advantage that these methods can analyze a broad range of target compounds, are very sensitive and allow precise quantification over a large linear range of concentrations. A disadvantage is that elaborate instrumentation is required, which does not allow for rapid field testing. In this case, bioanalytical quantification offers an attractive alternative (see below). Finally, while LC-MS identifies compound structures

and determines concentrations, conventional mass spectrometry cannot distinguish the same chemical from different sources or tell whether a compound in a sample may have undergone environmental transformation. There is a need for isotope-based ('nuclear') methods that can provide such unique complementary information (see Section 5).

Bioanalytical quantification of antibiotics. Bioanalytical methods for rapidly screening of antibiotics use enzyme-linked receptors or antibodies. Screening methods should be simple, quick, inexpensive and specific, with low detection limits and high sample throughput (Gaudin, 2017). Screening antibiotic residues in food samples is done with binding tests using dipsticks or microtiter plates as analysis platform and colorimetry or chemiluminescence as a detection principle. Antibiotic binding proteins selectively react with a certain class of antibiotics like penicillins or tetracylines. Antibodies can be designed for any antibiotic by immunizing animals like rabbits or mice. Antibodies are more selective than receptor proteins and thus, in many cases, they are even able to differentiate between antibiotics of the same antibiotic class (e.g. sulfamethazine and sulfadiazine). A variety of antibodies has been developed over the past 30 years to quantify antibiotics from different classes, like sulfonamides, aminoglycosides, β-lactams, polyketides, fluoroquinolones, macrolides, or tetracyclines.

The analysis principle is usually based on indirect, competitive immunoassays where antibiotics in the sample and the immobilized equivalents of these antibiotics compete for the binding sites of added antibodies. The higher the analyte concentration in the sample, the fewer antibodies bind to the immobilized antibiotics. The antibodies that have bound are detected by incubation with a secondary enzyme-labelled (e.g. horseradish peroxidase) antibody. A multi-analyte immunoassay is available for the quantification of multiple antibiotics in raw milk by a regenerable antibiotic microarray (Kloth *et al*., 2009b). By internal calibration and the use of regeneration cycles, samples can be analyzed without changing the microarray chip during a full working day. The analysis is carried out on the microarray analysis platform MCR 3, which is designed for the automated processing of flow-based chemiluminescence microarray chips in laboratories or in the field (Kloth *et al*., 2009a). The antibiotic microarray chip has already been shown to quantify 80% of the antibiotics administered to cows (Kloth *et al*., 2009b); it allows the quantification of antibiotics, even in honey (Wutz *et al*., 2011), and can easily be adapted to natural water samples. Standard operation procedures exist for validating multiplex antibiotic immunoassays (Meyer *et al*., 2017).

The advantages of antibiotic microarray immunoassays are their rapidity, cost-effectiveness and simplicity. Disadvantages lie in the fact that that compounds can only be analyzed if their antibodies are available, and that the effect of antibiotics on microorganisms cannot be detected. In the analysis of antibiotic residue in foods, LC-MS is thus routinely used to confirm contamination and microbial inhibition tests are used to prove antibiotic activity. The transfer to environmental applications is constrained at the moment because of the sensitivity of the tests – in the range of 1 – 100 µg/L – whereas reported concentrations in environmental samples range from 1 – 1000 ng/L. Therefore, as in most organic trace analysis procedures, the enrichment of antibiotics by solid phase extraction or other concentration methods is important as a first step of sample pretreatment.

MONITORING ANTIBIOTIC RESISTANCE IN THE ENVIRONMENT

Detection of ARB and ARGs in the environment. Antimicrobial resistance in the environment is either identified by the cultivation of bacteria or by molecular biological methods. ARBs are isolated after cultivation on selective media, which

contain the antibiotic of interest. The ARBs are picked depending on the morphology of the colonies on agar plates or their colour. If possible, isolated bacteria should be specified by MALDI-TOF-MS (Schumacher *et al*., 2018).

The quantification, detection, typing and characterization of ARGs in environmental samples are possible using molecular biological methods. These methods are based on nucleic acid amplification tests (NAATs), DNA sequencing, or DNA hybridization (Zhang *et al*., 2009) and are applied either after cultivation or directly after filtration of water samples. Filtration is used mainly for concentrating bacteria on a membrane filter that can be used directly for cultivation on agar plates or for harvesting bacterial cells with a cell lysis buffer. The first important step in obtaining the target DNA of bacteria is to extract and purify DNA after cell lysis. Afterwards, the total DNA, which contains ARGs in plasmids or chromosomes, is further processed for the analysis of ARGs.

Functional metagenomics is possible without cultivation by cloning ARGs into a library (Allen *et al*., 2010). Such a library is introduced into Escherichia coli or other established host cells. The effect of antibiotic resistance can be screened on selective media containing a certain antibiotic. Whole-genome sequencing of isolated bacteria is a powerful modern technology, which is able to screen for all ARGs using databases for antibiotic resistance genes (Berendonk *et al*., 2015; Gomi *et al*., 2018). Bacteria with genes encoding for several antibiotic resistances can be identified easily in environmental samples. Dynamic studies on the movement of antibiotic resistance genes by horizontal gene transfer – even in the absence of selection or movement by chemical or physical stress – can be undertaken in the future (Allen *et al*., 2010).

NAAT uses enzymes to amplify extracted target DNA. Polymerase chain reaction (PCR) is the most common DNA amplification method. After cultivation, PCR was used to find antibiotic-resistant bacteria and their resistance genes in waste water, surface water, and drinking water biofilms (Schwartz *et al*., 2003). Currently, the detection of amplified genes mostly involves fluorescence, using instrumentations for quantitative PCR. Multi-resistant Gram-negative bacteria were isolated from waste water and surface waters, and they were shown to harbor genes for carbapenemase and extended spectrum β-lactamase identified by qPCR (Kittinger *et al*., 2016; Müller *et al*., 2018). Distribution studies of ARG were conducted by cultivation-independent qPCR using environmental samples such as soil (Wang *et al*., 2014), surface water (Stoll *et al*., 2012), and waste water treatment plants (Laht *et al*., 2014).

Isothermal NAATs are alternative methods that are able to amplify DNA at a certain temperature (Zhao *et al*., 2015). Examples are recombinase polymerase amplification (RPA), helicase-depending amplification (HDA) or loop-mediated isothermal amplification assays (LAMP). Multiplexed analysis of genes is possible using a heterogeneous asymmetric RPA performed with chemiluminescence DNA microarrays (Kunze *et al*., 2015). Treatment with propidium or ethidium monoazide and subsequent photolinkage of the DNA hybridization agents by blue LEDs before DNA extraction can be used to inhibit the amplification of free DNA in the sample (Nocker *et al*., 2006). This is an option for identifying or quantifying antibiotic-resistant genes in viable bacteria cells. The principle of PMA-haRPA for the quantification of Legionella spp. has shown the potential for investigating water samples (Kober *et al*., 2018). Miniaturization and multiplexing should be further developed to enable the analysis of the diversity of ARB and ARG in the field. DNA hybridization uses the selective interaction between target DNA and oligonucleotide probes for the detection of ARGs. Magnetic nanoparticles were modified with DNA probes to capture

target DNA. A detection probe was used to identify hybridized ARGs on magnetic nanoparticles by forming a DNA sandwich. With this construct, antibiotic resistance genes could be quantified in 96-well microtiter plates by fluorescence spectrometry (Son *et al*., 2010). DNA microarrays for the detection of 90 antibiotic resistance genes of Gram-positive bacteria were established in another approach (Perreten *et al*. 2005). The target DNA was labelled with biotin by a randomly-primed nucleic acid amplification reaction and imaged after staining.

The cultivation of bacteria is the common way to identify ARG or ARB. However, this method is time consuming, and sometimes viable but non-culturable (VBNC) bacteria are excluded. The identification of certain genes without cultivation by molecular biological methods can be performed directly. Nevertheless, one has to keep in mind that the total DNA of all bacteria concentrated by filtration is extracted, and therefore differentiation between ARB is not possible because ARGs could be found in plasmids of various bacteria. Another issue is the limited sensitivity of the cultivation-independent methods, which makes it necessary to enrich bacteria by a mechanical treatment such as filtration. Besides the bacteria of interest, suspended matter, humic acids, or other matrix components can be co-enriched by filtration, which may disturb subsequent molecular detection methods. The adherence of bacterial cells to tubing or other surfaces of technical sampling systems could damage the cells as well as compromising the capacity of molecular methods to correctly estimate the extent of antibiotic-resistant genes. In addition to standard membrane filtration methods, researchers have studied the use of cross-flow microfiltration (Peskoller *et al*., 2009) and monolithic adsorption filtration (Wunderlich *et al*., 2016) to collect larger sample volumes. Molecular methods are able to detect a greater variety of bacteria among highly concentrated intact bacteria, not only the bacteria that are present in high concentration. This is a general 'iceberg problem' in environmental microbiology. The effects of horizontal gene transfer in microbial communities or in antibiotic-resistant pathogens may be overlooked because of their presence in low concentrations.

Nuclear techniques (multi-element stable isotopes fingerprinting) and tools for determining the source and transport of AM

It is crucial for governments to choose the most appropriate tools for determining the source and transport of AM and to have monitoring regimes, and cost-benefit and cost-effective approaches in place to assess the economic aspects of nuclear versus conventional methods. Nuclear techniques trace the antibiotic medicine – the chemical, not the antimicrobial resistance which is the pathogen in question. Once a selection pressure is imposed, antimicrobial resistance genes may potentially originate, amplify and distribute in a dynamic of their own. To confine the spread of AMGs, the question is therefore not so much about the original sources of AM, but rather about the processes leading to their spread and amplification. In contrast, when AM are concerned, the question about sources is extremely relevant because the release of the chemical agents imposes the selection pressure. Here, conventional monitoring tools (LC-MS, bioanalytical tools, see above) can detect the existence of a chemical, but they are unable to assess its origin, or whether it has been further degraded.

Compound-specific stable isotope analysis (CSIA) is a powerful tool that can provide answers when existing monitoring methods fall short (see Section 4). On the one hand, *isotopic source fingerprinting* may reveal different sources of an identical chemical; on the other hand, degradation-induced changes in isotope ratios (*isotope fractionation*) may detect when a given chemical has been transformed or degraded. Successful applications have focused on the sources and fates of many common groundwater contaminants such as chlorinated solvents and BTEX (benzene, toluene, ethylbenzene, and xylene) compounds (Badin *et al.* 2014, Fischer *et al.*, 2007, Fisher *et al.*, 2016). While a decrease in concentration of a contaminant can result from transformation (Barber *et al.*, 2009), dilution or sorption, pronounced changes in the stable isotope composition of a contaminant are the hallmark of kinetic isotope effects and are thus a reliable indicator that (bio)transformation has occurred (Elsner *et al.*, 2005). The observed isotope fractionation *in situ* can be compared with laboratory observations, determining a pathway-specific enrichment factor. Thus, CSIA can be used to qualify and quantify *in situ* transformations. The latest methodological advances even allow the analysis of several elements (H, C, Cl, N) within a molecule. This multi-element isotope information can be used to elucidate *in situ* transformation pathways and underlying reaction mechanisms. In addition, an analysis of stable isotope patterns can be used to determine the source of a contamination, because the ground stocks and synthesis pathway used during production can leave a typical 'stable isotope fingerprint' (Nijenhuis *et al.*, 2016).

CSIA may also help to determine the source and fate of antibiotics in the environment, with some restrictions. Routinely, researchers use gas chromatography (GC) coupled with isotope ratio mass spectrometry (IRMS); however, polar substances such as antibiotics require liquid chromatography (LC) approaches (Rodriguez-Mozaz *et al.*, 2015). LC-IRMS, thus far, only allows the analysis of carbon-stable isotope

composition; the analysis of other elements (e.g. H or N) requires prior derivatization to make the substance amenable to GC. Further challenges are associated with low environmental concentrations of antibiotics. CSIA using GC- or LC-IRMS requires relatively high amounts of a compound injected on-column, and corresponding low-background (interfering substances) in a sample, since all substances exiting the GC or LC column are converted, during combustion or pyrolysis, to a simple analysis gas (e.g. CO_2, N_2 or H_2). As a result, extensive pre-concentration and clean-up procedures are necessary. A further complicating factor is that kinetic isotope effects occur only in a reacting bond, whereas CSIA measures the 'bulk' changes in the molecular average. Hence, the general high molecular mass of antibiotic tends to 'dilute' degradation-induced isotope effects in CSIA.

The following paragraphs describe the general concepts behind the use of CSIA to investigate sources and degradation pathways, in laboratory and field studies, including examples of antibiotics, pharmaceuticals or common groundwater contaminants, as available.

Forensics: determining the origin of antibiotic contamination. The stable isotope composition of a chemical can provide information on its origin and the production process used for synthesis. For example, the carbon-stable isotope signature of chlorinated aliphatic compounds at a contaminated site in Ferrara, Italy, allowed researchers to distinguish two major sources: 1) a source from recent production, with C >-40‰, reflecting its production from petroleum compounds; and 2) a source derived from historic production lines using depleted methane for synthesis with C <-60‰ (Nijenhuis *et al*., 2013). Similarly, carbon-stable isotope signatures could be used to distinguish naturally-produced from industrially-produced chloroform (Hunkeler *et al*., 2012). For pharmaceuticals, only a few studies are available to date. One such study presented a multi-element (C, H, O) isotope analysis of ibuprofen to identify its origin of production (Gilevska *et al*., 2015). Five main sources could be distinguished for ibuprofen, with typical isotope patterns that reflect the raw materials and synthetic pathways used, including the potential isotope fractionation during synthesis. Another study reported the low variability in carbon and nitrogen isotope composition of diclofenac, allowing for an easier application of CSIA to characterize natural attenuation *in situ*, but a limitation for source apportionment (Maier *et al*., 2014).

Comprehensive studies of other pharmaceuticals are limited (Krummen *et al*., 2004; Wokovich *et al*., 2005; Deconinck *et al*., 2008) and studies of antibiotics are lacking. However, the analysis of natural-abundance stable isotope composition of C, N, O, S and H of pharmaceuticals has become an approach used in process patent protection (Sabatelli *et al*., 2017).

There is a clear need for a database that contains the multi-element stable isotope compositions of the antibiotic products of important producers, in order to identify, on the one hand, sources of antibiotics and, on the other, allow for investigations *in situ* (Barber *et al*., 2009).

CSIA for analysis of degradation pathways – lab studies. To detect *in situ* degradation pathways, laboratory studies are needed that determine changes in stable isotope effects in reference experiments. By relating the relative change in isotope composition to a corresponding change in concentration, we can derive the underlying pathway-specific stable isotope enrichment factors. Multi-element isotope analysis, in particular, can provide additional information for distinguishing degradation pathways. Triple-

element (C/Cl) CSIA has already proven valuable for distinguishing different reaction pathways for microbial 1,2-dichloroethane biotransformation, with specific patterns observed for dihalo elimination and oxidative degradation (Palau *et al*., 2014; Franke *et al*., 2017; Palau *et al*., 2017a; Palau *et al*., 2017b).

In the case of pharmaceuticals, diclofenac N and C analysis has shown specific isotope fractionation patterns for oxidative (low C and high N isotope fractionation) and reductive transformation (high C and low N isotope fractionation) (Maier *et al*., 2014). For the antibiotic sulfamethaxozole (SMX), only carbon stable isotope effects have been reported thus far. Beside the aerobic biodegradation of SMX by Microbacterium sp. strain BR1, enrichment factors were reported for abiotic reactions, including photolysis, and reaction with ozone or chlorine dioxide (see Table 3). Based on the carbon enrichment factor alone, the detection of a pathway would not be possible and isotope effects for other elements, such as S, N or H, and detailed metabolite analysis will be necessary (see Section 6).

Detection of degradation pathways – field studies. While CSIA is now routinely applied for groundwater contaminants, such as chlorinated solvents and BTEX (see, for example, Hunkeler *et al*., 2008), its application for pharmaceuticals are still rare. Schurner *et al*. (2016) investigated the fate of selected pesticides and pharmaceuticals in a mesoscale aquifer model system and were able to perform CSIA on carbon and nitrogen. Combined with a tracer test to characterize the water flow, the CSIA provided information on both degradation and sorption in the system. Neither the pesticide metabolite 2,6-dichlorobenzamide and the pharmaceutical ibuprofen experienced degradation or sorption; the pesticide bentazone was transformed but did not sorb significantly while the pharmaceutical diclofenac was subject to both degradation and sorption.

Another study evaluated the natural attenuation of the antibiotic sulphanilamide in a contaminated chalk aquifer (Bennett *et al*., 2017). Here, an investigation of the carbon stable isotope signature *in situ* was combined with an ex situ laboratory microcosm study involving C-labelled sulphanilamide14. Both a decrease in concentration and carbon stable isotope enrichment could be observed *in situ*, suggesting biotransformation in the contaminated groundwater. The ex situ microcosm studies provided information on the potential for biodegradation in the groundwater microbial community and investigated the conditions that would enhance such degradation. In this case, it was suggested that sulphanilamide functions as a carbon and putative nitrogen and sulphur source, with preferential transformation under iron-reducing conditions.

While these studies provide interesting preliminary insights, the application of CSIA to analyse the fate of antibiotics in the environment will be limited. Due to low *in situ* concentrations, which require high concentration-factors and extensive clean-up procedures to produce sufficient quantities of antibiotics necessary for analysis via IRMS, major efforts should be made to develop preparatory steps for extraction, concentration and clean-up. Most importantly, these preparatory steps as well as subsequent (optional) derivatization to make compounds GC-amenable, should be ideally isotope effect-free. Thus, uniform, validated protocols with associated quality control procedures are essential before CSIA can be routinely applied to assess the fate of antibiotics in the environment.

TABLE 3
Summary of isotope enrichment factors for pharmaceuticals

Compound	System	εC	εN	other elements	Reference
Diclofenac	Oxidative	n.s.	-7.1±0.4‰		(Maier *et al*., 2014)
--	Reductive dechlorination	-2.0±0.1‰	n.s.		(Maier *et al*., 2014)
--	MnO_2	-1.5±0.3‰	-7.3±0.6‰		(Maier *et al*., 2016)
--	Photolysis	-1.7±0.3‰	+1.9±0.1‰		(Maier *et al*., 2016)
--	Ozonation	n.s.	+1.5±0.2‰		(Maier *et al*., 2016)
Sulfamethoxazole	*Microbacterium* sp. strain BR1	-0.6±0.1‰			(Birkigt *et al*., 2015)
--	Direct photolysis pH 5 (ddH_2O)	-3.0±0.2‰			(Birkigt *et al*., 2015)
--	Direct photolysis pH 7.4 (MMO)	-2.0±0.1‰			(Birkigt *et al*., 2015)
--	O_3 (pH 8)	n.s.			(Willach *et al*., 2017)
--	O_3 (pH 3)	-1.2±0.1‰			(Willach *et al*., 2017)
--	O_3+DMSO (pH 8)	n.s.			(Willach *et al*., 2017)
--	O_3+DMSO (pH 3)	-2.2±0.1‰			(Willach *et al*., 2017)
--	ClO_2 (pH 8)	-1.3±0.1‰			(Willach *et al*., 2017)
--	ClO_2 (pH 3)	-0.8±0.1‰			(Willach *et al*., 2017)
--	Direct photolysis (LP 254 nm) pH 3	-0.8±0.1‰			(Willach *et al*., 2017)
--	Direct photolysis (LP 254 nm) pH 8	n.s.			(Willach *et al*., 2017)
--	Direct photolysis (MP 200-600 nm) pH 3	n.s.			(Willach *et al*., 2017)
--	Direct photolysis (MP 200-600 nm) pH 8	n.s.			(Willach *et al*., 2017)
--	Direct photolysis (MP 310-600 nm) pH 3	-4.8±0.1‰			(Willach *et al*., 2017)
--	Direct photolysis (MP 310-600 nm) pH 8	-3.9±0.1‰			(Willach *et al*., 2017)
--	Direct photolysis (HP 220-500 nm) pH 3	-1.9±0.1‰			(Willach *et al*., 2017)
--	Direct photolysis (HP 220-500 nm) pH 8	-2.2±0.2‰			(Willach *et al*., 2017)

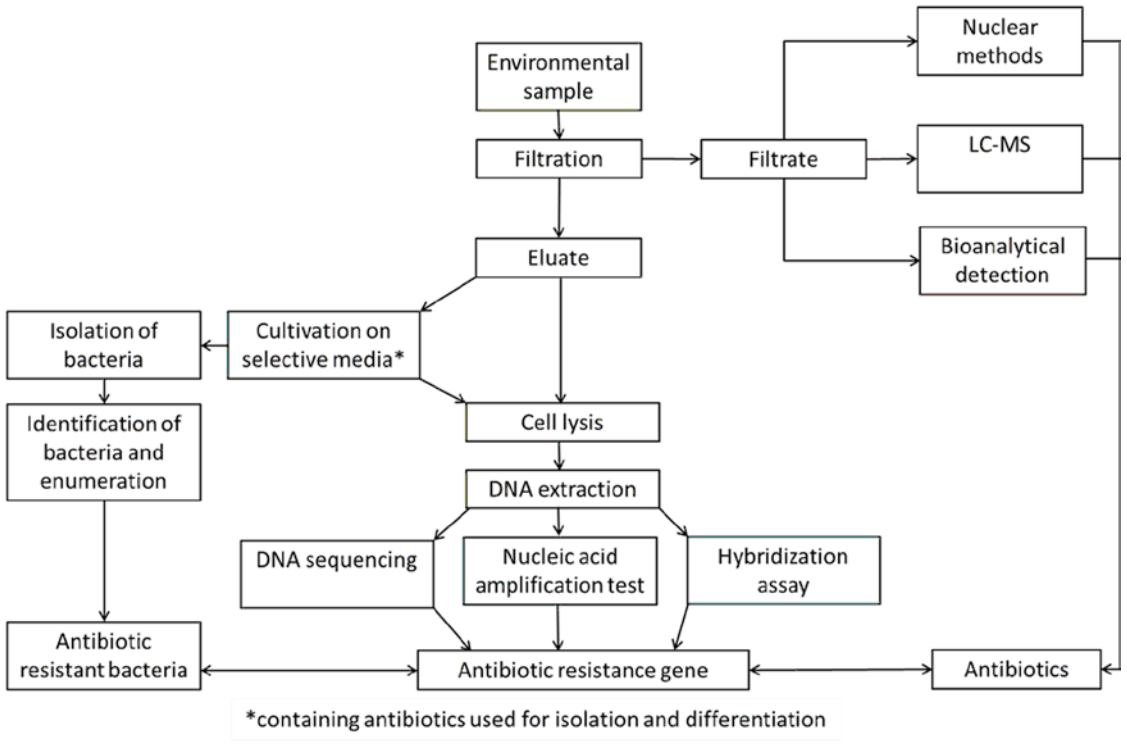
n.s.: not significant

Current methodology for detecting and tracing the source and transport of antibiotics through soil and water

A clear methodology or standard operating procedure for tracing antibiotic resistance through soil and water does not yet exist. However, any future protocol should include the detection of: 1) antibiotics and their transformation products; 2) antibiotic-resistant bacteria; and 3) antibiotic resistance genes, with the option to include nuclear techniques (i.e., compound-specific isotope analysis). Figure 4 shows an overview of current methodologies for all three types of analytes.

It is important to emphasize that sampling is the main source of potential errors in deriving quantitative and qualitative analytical results. Sampling microorganisms is even more error-prone than it is for antibiotics, because additional biological effects like proliferation, inactivation, or agglomeration may come into play. Sampling in the environment is always a challenge making experience and planning critical to success. And comparable results in environmental analyses of antibiotics, ARGs and pathogens can only be achieved if the sample and sampling conditions are described thoroughly.

FIGURE 4
Scheme for the detection of antibiotics, antibiotic-resistant bacteria and antibiotic resistance genes in environmental samples



The following recommendations provide basic guidelines for collecting, preserving and storing environmental samples selected in the field.

a.) General plan for sampling

- Protocols for sampling should be well organized.
- All equipment should be set up and tested for functionality.
- Pay close attention to the weather conditions in the days before and during sampling.
- Long-time planning may be needed, especially if seasonal effects can influence research results.
- Sampling points should be identified based on to the quality of the expected samples and their information value.
- The sampling points should be explored to determine accessibility, and the quality of water sources.

b.) Sampling points

- Sampling should always be done at the same place.
- The sampling points must be clearly marked on the site and on a map.
- At least three samples are needed for reliable analytical results.
- The sampling volume should be larger than the volume used for analysis.
- The origin of a sample should be clearly identified.
- Any changes to the sampling procedure during the sampling process should be noted and explained.

c.) Sampling vessels and type of sampling

- Sterilized glass containers should be used for sampling. Antibiotics adsorbed on plastic container or tubing may reduce the collected concentration of antibiotics.
- Sampling devices should be sterilized to avoid bacterial contamination.
- Temperature, pH and turbidity of the sample should be measured for the general characterization of water samples.
- Use filtration to separate bacteria and antibiotics and to mechanically enrich bacteria.
- Use sterile membrane filters (0,22 µm or 0,45 µm) for water samples with low turbidity and low sample volume (1 – 100 mL) and crossflow ultrafiltration modules or monolithic adsorption filtration devices for larger sample volumes (> 100 mL) to separate bacteria and antibiotics by filtration.
- Ideally, filtration should be performed at the sampling point is preferable to ensure that qualitative and quantitative statements can be made concerning the content of ARG, pathogens and antibiotics. Peristaltic pumps with an electricity supply are needed for this process. Otherwise, filtration by gravitational forces or by manual sample injection should be applied.
- The type of sampling procedure used should be well documented.

d.) Sampling protocol

The sampling protocol should contain at least the following elements:

- Name of the person performing the sampling;
- Location of the sampling point, including the full address and description of the sampling point (including photos if possible);
- Exact name of sampling point;
- Date and time of sampling;
- Type of sample taken (groundwater, waste water effluent, waste water influent, drinking water, surface water from river, lake, or other);
- Description of the sampling technique (scooped sample or pumped water, type of disinfection);
- Temperature and pH of the sample;
- Abnormalities in sampling that could affect the outcome.

e.) Transportation and sample preservation

- The time between sampling and analysis in the laboratory should be kept as short as possible.
- Samples should preferably be prepared for analysis within 24 hours of sampling and no longer than 48 hours after sampling.
- If transport to the lab takes less than eight hours, the samples can be transported at ambient temperature. However, the samples must be protected from light and strong temperature effects (e.g. in cool boxes).
- For longer transport times (more than 8 hours), the samples must be cooled - ideally to (5 ± 3) °C and protected from light (e.g. in cool boxes with rechargeable batteries). Care must be taken that the samples are not frozen. The temperature must be monitored and recorded.
- The transport conditions must be documented.
- The samples must be stored at (5 ± 3) °C in the laboratory.
- Preservation should be avoided for culture methods, molecular biological methods or immunoassays because this may result in false negative results.
- Samples bound for chemical analysis and nuclear methods should be preserved by adding 36% hydrochloric acid diluted 1:1 in water to produce a pH < 2 in the sample, or using another preservative (NaOH) that protects the integrity and stability of the analyte.
- Samples can be extracted onsite using a large-volume solid phase extraction protocol. Polystyrene-divinylbenzene and hyper-crosslinked polystyrene-divinylbenzene-ethylvinylbenzene polymers such as a mixture of StrataX (Phenomenex) and Bakerbond SDB-1 (J.T. Baker) are useful options.
- Extraction recoveries and the integrity of compound-specific isotope values of the target analytes after extraction must be validated before an extraction protocol can be implemented for large-scale sample analysis.

f.) Consideration for analysis methods

Immunoassays:

- Immunoassays for antibiotics can be used as a screening or quantitative method.
- In screening, a cut-off value must be defined to allow the identification of antibiotics in samples.
- Quantitative immunoassays need calibration with spiked antibiotics in environmental samples. Matrix effects may influence the interaction between antibodies and analytes, resulting in over- or underestimations.
- Blank measurements should be repeated 20 times.
- Extreme conditions in samples, such as low pH (< pH 4) or large pH (> pH 9), storage at high (<10°C) or low temperature (>30°C), storage time (>1 day), high turbidity, etc., should be tested with spiked samples at concentrations representing the test midpoint of a calibration curve.

Molecular biological methods

- Laboratories need high quality standards to avoid cross-contamination.
- DNA controls should be imposed to control the efficiency of DNA extraction and nucleic acid amplification.
- Calibration with cultivated microorganisms for NAATs is important to evaluate all sample preparation steps (filtration, elution, cell lysis, DNA extraction, nucleic acid amplification).
- Biosafety level 1 is sufficient for the cultivation of bacteria from environmental samples.
- Experiments with genetically-modified microorganisms need to be done in accordance with the relevant regulation on genetic engineering.

Cultivation methods

- Laboratories need high quality standards so that cross-contamination is avoided.
- Laboratories needs biosafety level 2 or higher for the cultivation of pathogenic microorganisms.
- Biosafety level 1 is sufficient for cultivating bacteria from environmental samples

Chemical analysis/nuclear methods

- Analyses should be performed using appropriate international references, compound-specific working standards and reference gases.
- All sample preparation steps should be validated and controlled for lack of isotope effects by standardized quality assurance/quality control procedures, using a compound-specific working standard with a known stable isotope composition.
- The ratio of target-analyte to background concentrations (dissolved organic matter) should be monitored in the extract, and additional clean-up steps such as preparative HPLC, are likely necessary if this ratio greatly exceeds 100 (Bakkour *et al*., 2018).

Outlook: the way forward

Tracing antibiotics and antibiotic resistance is extremely complex because it requires us to consider three different contaminants at the same time: antibiotics, resistance genes and pathogenic bacteria. To make matters worse, the conditions under which antibiotics are eliminated are not necessarily those under which resistance genes and pathogenic bacteria are eliminated. This raises three major questions relating to knowledge gaps that require investigation with nuclear and microbiological methods:

- What are the sources of antibiotics and where are the antibiotics eliminated?
- How is AR gene abundance influenced by antibiotic concentrations and environmental drivers
- In which host organisms are antibiotic resistances located?

KNOWLEDGE GAP 1: WHAT ARE THE SOURCES OF ANTIBIOTICS AND WHERE ARE THE ANTIBIOTICS ELIMINATED? THE CASE FOR NUCLEAR METHODS

Preventing the release and spread of antibiotics in the environment requires a fundamental understanding of the sources and locations, zones and environmental conditions where antibiotics are eliminated. Sources, such as insufficient wastewater treatment, spillage at production sites, from plant residues, or from diffuse pollution (i.e. agricultural land receiving organic fertilization through manure application) may initially be identified by concentration analysis, i.e. high concentrations imply a major source of antibiotic contamination. However, concentration analysis alone cannot identify a source or its relative contribution to local contamination if multiple sources of antibiotics are present. Multi-element stable isotope analysis may provide a basis for a mass balance approach and relative contributions, using producer/source-specific multi-element stable isotope fingerprints. For a full-scale analysis, the multi-element isotope composition of antibiotics from key producers should be evaluated for their differences and similarities. Ideally, all elements in the molecule of interest should be analysed. This is currently routinely available for H, C, N in GC-amenable compounds and, in the future, taking advantage of current methodological advances, may also include O, Cl, Br and S.

A comprehensive database of multi-element stable isotope compositions of internationally used antibiotic products is still lacking. Such a repository will be needed to provide a basis for identifying and quantifying the contribution of respective sources in case significant variabilities are observed. However, in instances where the isotope patterns of commercial products are relatively uniform, source identification is precluded, but would allow a more straightforward identification of degradation-induced changes of these isotope ratios to pinpoint transformation (Barber *et al.*, 2009).

The elimination of antibiotics can similarly be assessed by determining concentration gradients, where a decrease in concentration would imply a removal from the environment. However, a decrease in concentration is the sum of transformation (Barber *et al.*, 2009) and other processes such as dilution and sorption. Field surveys of the main, presumed sources and downstream locations are necessary to address

the variability of stable isotope compositions of antibiotics in the environment. By comparing those environmental signals to source isotope fingerprints, a first indication of biotransformation may be obtained if isotope shifts are observed for certain elements. Subsequent analysis to correlate isotope shifts with local hydrochemistry and microbial communities, zones and conditions supporting biotransformation can be used to develop management approaches. However, such field surveys are almost completely lacking at the moment, representing an important knowledge gap.

Dominant transformation pathways that lead to the elimination of antibiotics can be identified by observing product formation (Barber *et al*., 2009). However, transformation products can be intermediates in the pathway and further degraded. Additionally, these products may be formed from multiple pathways and different original antibiotic compounds. Thus, complementary, multiple lines of evidence, are needed, e.g. approaches using multi-element stable isotope fractionation analysis to provide insights into dominant transformation pathways *in situ*. Furthermore, the quantification of the extent of transformation becomes possible by comparing such observations to laboratory reference experiments. Again, such studies are still limited at present and only a few microbial strains capable of antibiotic transformation have been described thus far. Overall, stable isotope analysis of antibiotics is challenging due to their polar nature, making them difficult to extract, which precludes their immediate analysis with state-of-the-art GC-approaches, and their low concentrations *in situ*, which requires extensive concentration and clean-up procedures. Thus, future efforts should both focus on the development of analytical approaches and sample preparation as well as obtaining a fundamental understanding of potential transformation reactions and pathways (Barber *et al*., 2009).

KNOWLEDGE GAP 2: HOW IS AR GENE ABUNDANCE INFLUENCED BY ANTIBIOTICS CONCENTRATIONS AND ENVIRONMENTAL DRIVERS? THE NEED FOR QUANTITATIVE AND TIME-RESOLVED DATA

Pollution from ARGs increases the chance that human pathogens will acquire antibiotic resistance. This fact poses a growing threat to global public health. Waste water and manure slurry are the main paths by which ARG, pathogens, and antibiotics enter into the environment. All three contaminants can be identified in any water compartment. Once ARGs are in the environment, they can spread among different bacterial species and habitats and, unlike antibiotics, ARBs are capable of replication. Therefore, contamination by antibiotics and ARG have to be considered together within a space and time context. In particular, quantitative and time-resolved data on the abundance of resistance genes in soil and water compartments are necessary for risk assessment. Extreme weather events like heavy rainfall, floods or drought also should be closely monitored to understand the fluctuations of ARGs and pathogens in the environment. In addition, technical water treatment systems should be analyzed in more detail regarding the presence of antibiotics and ARGs. Early studies at the fourth purification stage in WWTPs have shown that ARGs increase in relation to the general reduction of bacteria and antibiotic concentrations. However, the reasons for this effect still need to be explored in detail. In addition, the links between human pathogenic bacteria and the resistance genes they harbor will be important for risk assessment. Because of the natural abundance of ARGs in apathogenic bacteria, it is important to identify the organisms in which the ARGs were originally located. This is only possible if quantification of ARGs in pathogens is possible.

The direct correlation between ARB and ARG in environmental samples can only be made once bacteria has been cultivated in selective media, containing the antibiotics of

interest. Culture-based methods take a long time and are only able to detect culturable bacteria. Nor are viable-but non-culturable bacteria accessible for environmental monitoring studies of ARGs. In conclusion, it is not possible to determine dynamic effects in environmental samples by cultivation alone. Rapid cultivation-independent methods are the alternative. When detecting antibiotic resistance from environmental samples by molecular biological methods, current methods can only detect the presence of ARGs in a whole sample (by qPCR, DNA hybridization, or whole genome sequencing) because once the DNA is extracted, no link between the ARG and its host can be made.

To overcome current constraints to accessing quantitative and time-resolved data around ARG, antibiotics and pathogens in water, the following research is needed:

- Molecular biological tools and methods are needed for quantitative and time-resolved monitoring of antibiotic resistance in the environment, with focus pathogens and multi-resistance. Microarray-based analysis in combination with live/death differentiation and selective cell sorting are promising tools for multiplexed study of pathogenic ARB. A selective separation of bacteria cells before detection by molecular biological methods would be useful. This can be performed by magnetic cell-sorting with antibody-labelled magnetic nanoparticles or segmented monolithic affinity filtration systems.
- Molecular biological methods must be standardized. Protocols for sampling, DNA extraction, nucleic acid amplification and DNA quantification (qPCR, DNA microarrays) and DNA sequencing need to be harmonized.
- Quantitative and time-resolved data on antibiotics in the environment are needed. Microarray immunoassays for the rapid and parallel quantification of antibiotics are important tools that can analyse at least 13 different antibiotics in about ten minutes.
- Molecular biological methods and microarray immunoassays have to be combined to gain time-dependent and quantitative data on antibiotics, ARGs and pathogens in parallel

KNOWLEDGE GAP 3: IN WHICH HOST ORGANISMS ARE ANTIBIOTIC RESISTANCES LOCATED?

Understanding dynamic effects like horizontal gene transfer or the loss of antibiotic resistance is important for developing approaches to managing potentially contaminated water and soils. Studying of dynamic effects at the single cell level would be the most convincing approach but is also the most challenging (Blainey, 2013). Single-cell methods need to be developed and implemented. Separation of single bacteria cells can be accomplished by Raman-activated cell sorting using stable isotope approaches (Berry *et al*., 2015; Taubert *et al*., 2018). Single-cell sequencing can identify more than one ARG present in a cell. After a whole genome amplification, the genetic profile of a single bacterial cell is analysed independently of the ability to replicate, and therefore ARGs in plasmids or on chromosomes can be assigned to a particular organism. Early successful approaches have been tested, including microfluidic digital PCR (Ottesen *et al*., 2006) or single-cell genomic sequencing of bacteria (Lasken, 2007).

Conclusions

Antibiotics kill bacteria, healing infections that cause diseases and death. Unfortunately, bacteria have learned how to quickly evolve and resist the effect of antibiotics. The antibiotics are either destroyed or transported out of the bacterial cell, or the bacteria change their targets to escape the antibiotic effect. The antibiotic resistance genes coding for these adaptations may be transferred to pathogens which can lead to multi-resistances causing millions of deaths each year. Hence, an urgent need exists to understand the sources and environmental fate of antibiotics, antibiotic resistance genes and the pathogens carrying the resistance genes. In contrast to 'traditional' contaminations, the problem therefore requires the study of three contaminants at the same time.

Current methods use conventional chemical methods to analyse antibiotics and study ARBs with metagenomics. Both approaches provide comprehensive surveys about high concentrations of contaminants in the environment, but fail to elucidate sources and sinks, or reveal how the two parameters influence each other. In the past, resistant bacteria have been studied using cultivation-based approaches, which are slow-moving and fail to visualize the detectable, but not culturable fraction. However, next generation sequencing methods, including whole genome sequencing (WGS), are evolving rapidly, with potentially transformational food safety applications.

This paper identifies three major knowledge gaps and suggests how to close them using a combination of advances in nuclear and microbiological analysis. To answer the question "Where are the sources of antibiotics and where are the antibiotics eliminated?" we propose that further advances in compound-specific isotope analysis could contribute to isotopic fingerprinting of sources and may detect the degradation of antibiotics by transformation-induced isotope effects. To answer the question "How is AR gene abundance influenced by antibiotic concentrations and environmental drivers?" we argue that quantitative and time-resolved data are needed. To this end, we propose microarray-based bioanalytics for targeted analysis of ARGs and ARBs as a rapid, cultivation-independent approach. Finally, to answer the question "In which host organism are resistances located?" we argue that further advances – based on sorting and/or single-cell analysis – will be necessary to link resistance to infectivity, and to characterize the spread of antibiotic resistance based on antibiotic concentrations and environmental factors. To pave the path for future developments in this direction, and to lay a basis for comparison between studies, we suggest sampling guidelines that may be instrumental in harmonizing efforts in different countries and laboratories.

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LAND AND WATER DISCUSSION PAPER

Antimicrobial movement from agricultural areas to the environment:

The missing link. A role for nuclear techniques

Antimicrobials (AM) play a critical role in the treatment of human and animal (aquatic and terrestrial) diseases, which has led to their widespread application and use. Antimicrobial resistance (AMR) is the ability of microorganisms (e.g. bacteria, viruses and some parasites) to stop an antibiotic, such as an antimicrobial, antiviral or antimalarial, from working against them. Globally, about 700 000 deaths per year arise from resistant infections as a result of the fact that antimicrobial drugs have become less effective at killing resistant pathogens. Antimicrobial chemicals that are present in environmental compartments can trigger the development of AMR. These chemicals can also cause antibiotic-resistant bacteria (ARB) to further spread antibiotic resistance genes (ARG) because they may have an evolutionary advantage over non-resistant bacteria. This paper will provide alternative screening methods useful for environmental samples and surveillance approaches in planning such screening efforts. Based on case studies, this paper aims to summarize the current understanding of the occurrence of ARG in the environment, and the antimicrobial movement from agricultural areas to the environment.

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