



EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

17 February 2021
EMA/CVMP/ERA/632109/2014
Committee for Medicinal Products for Veterinary Use (CVMP)

Reflection paper on antimicrobial resistance in the environment: considerations for current and future risk assessment of veterinary medicinal products

| | |
|---|------------------|
| Draft agreed by the Environmental Risk Assessment Working Party (ERAWP) | 30 April 2018 |
| Draft agreed by the Antimicrobials Working Party (AWP) | 30 May 2018 |
| Adopted by CVMP for release for consultation | 8 November 2018 |
| Start of public consultation | 16 November 2018 |
| End of consultation (deadline for comments) | 31 August 2019 |
| Draft agreed by Environmental Risk Assessment Working Party (ERAWP) | 6 January 2021 |
| Draft agreed by the Antimicrobials Working Party (AWP) | 6 January 2021 |
| Adoption by CVMP | 17 February 2021 |

| | |
|----------|--|
| Keywords | antibiotic, environmental fate, human health, animal health, risk assessment, antimicrobial resistance bacteria (ARB), antimicrobial resistance genes (ARGs) |
|----------|--|

Official address Domenico Scarlattilaan 6 • 1083 HS Amsterdam • The Netherlands

Address for visits and deliveries Refer to www.ema.europa.eu/how-to-find-us

Send us a question Go to www.ema.europa.eu/contact **Telephone** +31 (0)88 781 6000

An agency of the European Union



Reflection paper on antimicrobial resistance in the environment: considerations for current and future risk assessment of veterinary medicinal products

Table of contents

| | |
|--|-----------|
| 1. Executive summary | 4 |
| 2. Aims of the reflection paper | 5 |
| 3. Background | 6 |
| 4. Mechanisms of development of antimicrobial resistance | 7 |
| 5. Consumption of veterinary antibiotics | 9 |
| 6. Emissions and fate of VMPs as sources of antimicrobial substances to and within the environment | 10 |
| 6.1. Emissions | 10 |
| 6.2. Fate and behaviour of veterinary antimicrobials in the environment..... | 11 |
| 6.2.1. Terrestrial environment | 12 |
| 6.2.2. Aquatic environment..... | 14 |
| 6.2.3. Further considerations and recommendations on environmental fate..... | 16 |
| 7. Emissions and fate of ARB and ARGs to and within the environment..... | 16 |
| 7.1. Excretion of ARB and ARGs from treated animals..... | 16 |
| 7.2. Selection of ARGs in environmental bacteria exposed to antimicrobial residues and ARGs from VMPs | 17 |
| 7.3. Exchange of ARGs between environmental bacteria and animal/human bacteria in the environment | 19 |
| 8. Risks to human and animal health from AMR and ARGs in the environment..... | 20 |
| 8.1. Environmental transfer of ARGs and ARB and potential route of transfer to humans and animals from the use of VMPs | 20 |
| 8.1.1. Food from crops | 20 |
| 8.1.2. Food from aquaculture | 20 |
| 8.1.3. Contaminated drinking water | 21 |
| 8.1.4. Contaminated recreational places | 21 |
| 8.2. Consequences for the risk assessment of human and animal health | 21 |
| 9. Evaluation of the current risk assessment process for VMPs: consideration of AMR – knowledge gaps and research needs | 22 |
| 9.1. Current risk assessment approach for VMPs..... | 22 |
| 9.2. Knowledge gaps and research needs..... | 24 |
| 10. Mitigation of AMR in the environment | 27 |
| 11. Conclusions | 29 |
| Annex I | 31 |
| Mechanisms of AMR | 31 |
| Annex II..... | 34 |
| UK Department of Health 'AMR Systems Map' (Dobra et al., 2014)..... | 34 |

12. Glossary 35
13. References 37

1. Executive summary

This reflection paper considers the impact(s) on ecosystems, animal and human health from the presence of antimicrobial residues (ARs) and/or antimicrobial resistance genes (ARGs; a DNA region within the microbial genome that encodes for reduced sensitivity against specific antimicrobials) in the environment resulting from the use of veterinary medicinal products (VMPs). At the outset, we define antimicrobial resistance (AMR) as the ability of microorganisms such as bacteria to become increasingly resistant to an antimicrobial to which they were previously susceptible.

It is recognised and acknowledged by the Committee for Medicinal Products for Veterinary Use (CVMP) that current guidelines on the environmental risk assessment (ERA) of VMPs for use in the European Union do not address how to assess the impact of antimicrobials, as veterinary pharmaceuticals, on the prevalence of AMR in the receiving environments e.g. soil, surface water, groundwater.

To produce this paper, an interdisciplinary team working across the Antimicrobials Working Party (AWP) and the Environmental Risk Assessment Working Party (ERAWP) of the CVMP has reviewed the current available data on antimicrobials in the environment and their role in the transmission of ARGs that may have clinical consequence for both human and animal health. This paper is unique in its remit and timely, given the level of discussion within and across organisations such as the World Organisation for Animal Health (OIE), the World Health Organisation (WHO) and the Food and Agriculture Organisation (FAO). It focuses specifically on information pertaining to veterinary medicines, particularly antimicrobials, the sources of potential resistance genes and their pathways in the environment, and the effects other pressures such as co-factors or contaminants have on the persistence of AMR. However, it is acknowledged that VMPs that are antimicrobial in nature act similarly to their human medicine counterparts and that there are other pressures driving the development of environmental AMR by natural selection. Therefore, it has been taken into consideration that antimicrobial use in veterinary medicine is not the only contributor to presence of AMR genes in the environment.

With a focus on veterinary medicines, this paper has identified the major exposure pathways and release scenarios and, subsequently, considered the likely extent of the accumulation and mobility of ARs and ARGs excreted from treated animals against a background of naturally occurring levels of ARGs. It has also considered the potential consequences of AMR in the environment on animal and human health.

There is evidence that ARGs are transported through the environment. Further, the environment acts as a bridge to different compartments; e.g. animal to manure/biosolids to soil to water to sediment, whilst simultaneously the environment acts like a reservoir or sink for the mixing of mobile genetic elements (MGEs) that interact and disperse to other compartments or to human and animal hosts. There is evidence that some anti-microbial resistant pathogenic bacteria have developed through these pathways and have impacted on human and animal wellbeing (Singer et al., 2016).

This paper reflects on what is required to address the data gaps and to better understand the factors that influence resistance emergence in the environment such as, movement of ARs and ARGs between different environmental compartments.

However, one of the main conclusions of this paper is that there are significant gaps in our knowledge around the specific mechanisms and pathways of AMR. Further, there is little information on the potential impacts that ARs and ARGs, resulting from VMP use, can have on the functioning of the ecosystem and its key species. While several publications have indicated that bacteria in the environment may be the source of antibiotic resistance in human clinical pathogens (Finley et al., 2013; Forsberg et al., 2012; Wellington et al., 2013), the extent to which VMP use contributes to the

emergence and spread of AMR in bacteria of clinical relevance for humans or animals (some of which have the ability to survive and grow in the environment) is unknown (ECDC/EFSA/EMA/SCENIHR, 2009; FAO/WHO/OIE, 2008).

Possible risk mitigation measures to reduce the incidence of AMR in the environment are identified. These measures tend to involve the implementation of best practices on disposal of manure and setting up systems to correctly dispose medicines as well as best practices on enhancing animal welfare where it reduces the risk of microbial infections and the implementation of education and training programmes for farmers and practitioners. Considering the correct disposal of medicines, this is now included in the new veterinary regulation (Regulation [EU] 2019/6¹). Implementation of best practices and guidelines on disposal of manure and medicines as well as on reducing the risk of microbial infections through increased animal welfare may help limit the emergence, spread or development of AMR at the farm level.

This reflection paper has also considered whether the risk assessment of VMPs, in EU member states, should be amended to include or address the risk from AMR in the environment arising from the use of VMPs containing antimicrobials. In response to this fundamental question, this paper concludes that the current ERA for VMPs cannot yet be amended to consider the risks posed by the accumulation of ARs and ARGs in the environment from the use of VMPs. In particular, it is noted that: the relative contribution from the use of veterinary medicines to the overall burden of AMR in the environment is not known. Uncertainty remains as to whether or not the presence of ARs and ARGs in the environment, resulting from veterinary medicinal use, is likely to result in a significant problem for the ecosystem and/or for animal/human health and it is not currently possible to provide clear advice on what data/studies would be required to quantify and address the issue of AMR in the environment, from the use of veterinary medicines, and how regulatory bodies could interpret such data.

In conclusion, while it is accepted that there is evidence that the environment is likely to play a role in the spread and/or persistence of AMR, the extent to which VMP use contributes to ARs and/or ARGs in the environment with consequential impact(s) on ecosystems, animal and/or human health is unknown. To evaluate the risks of AMR development appropriately, alternative tools (e.g. minimal selective concentration [MSC] assays) and models to understand the environment from the microbiological perspective are needed. The EMA/CVMP will continue to monitor scientific developments in this area and, as the science evolves and improved or alternative assessment methodologies are developed, the need to revise the current approach to environmental risk assessment for VMPs containing antimicrobials will be further considered.

2. Aims of the reflection paper

This reflection paper aims to review the potential impact(s) on ecosystems, animal and human health from the possible presence of ARs and/or ARGs in the environment arising from the use of VMPs. This paper will differentiate the key exposure pathways and, subsequently, consider the likely extent of accumulation and mobility in the environment of ARs and ARGs excreted from animals treated with VMPs. In addition, the potential effects on the functioning of bacterial communities and the overall impacts on ecosystems, as a consequence of either AMR or by changing the microbial diversity without selecting for acquired antibiotic resistance, are considered. Moreover, an evaluation and understanding of the degree to which the environment is altered by VMP use, how it may contribute to the cycling of resistance genes between different ecosystem compartments (e.g. soil, water, animals and/or humans), and the effect or consequences of this on animal and human health is performed. Furthermore, as VMPs are not the only source of antimicrobials that enter the environment, the

¹ Regulation (EU) 2019/6 of the European Parliament and of the Council of 11 December 2018 on veterinary medicinal products and repealing Directive 2001/82/EC (OJ L 4, 7.1.2019, p. 43–167)

consideration of any potential impacts from VMP use needs to be done within the context of the global use of antimicrobials giving consideration to the 'One Health' approach.

This reflection paper also considers whether the current ERA for VMPs should or could be further developed to appropriately assess the potential risks posed by veterinary medicines with antimicrobial properties to drive environmental AMR.

3. Background

The European Commission has recognised in its 2017 Action Plan against AMR (European Commission, 2017) that the problem of AMR cannot be successfully tackled by isolated, sectoral efforts. A holistic approach is needed, which takes into consideration the different sectors committed to addressing AMR, in-line with the globally recognized 'One Health' approach (the concept of 'One Health' is used in the political declaration on AMR adopted during the high-level meeting of the UN General Assembly in 2016 (United Nations, 2016)), defined as "the integrative effort of multiple disciplines working locally, nationally and globally to attain optimal health for people, animals and the environment". To support the EU actions on combatting AMR, in the European Union Strategic Approach to Pharmaceuticals in the Environment from 2019 is also included to "identify actions to be taken or further investigated to address the potential risks from pharmaceutical residues in the environment" (European Commission, 2019). More specifically, the term is used to describe a principle which acknowledges that human and animal health and the environment are interconnected, that diseases are transmitted from humans to animals and *vice versa*. Therefore, AMR requires a systematic approach that considers humans, animals and the environment.

The CVMP strategy on antimicrobials 2016-2020 (EMA/CVMP, 2016a) considers the interaction between humans, animals and the environment as sources of antimicrobial resistance genes in a 'One Health' context, and states that: "The importance of the environment as a reservoir for antimicrobial resistance genes is now widely recognised. Use of antimicrobials in humans, animals (including in aquaculture) and plants leads to contamination of the environment both with antimicrobials and resistant bacteria. The presence of antimicrobials in the environment exerts a selective pressure for resistance genes in bacteria in a variety of ecosystems including animals, humans and plants. The cycling of these resistance genes between the different ecosystems is extremely complex and requires further research. The CVMP acknowledges that further consideration should be given to the contribution of veterinary antimicrobial use to the environmental resistome²". In the draft of the CVMP strategy for 2021–2025 the action that the "CVMP will explore the development of improved or alternative methodologies to assess if improvements can be made to the environmental risk assessment for antimicrobial VMPs" is included (EMA/CVMP, 2020).

Although the majority of AMR action plans and monitoring programmes currently focus on human and livestock activities, there has been growing concern that the natural environment may play a substantial role in the evolution, persistence and spread of AMR and thus may impact our ability to control and treat AMR-associated infections in both animals and humans. A review of the scientific literature on this issue has shown that the origin of many ARGs of clinical relevance can be traced back to bacteria that occur in the wider environment (Wright, 2007), hence indicating the environment to be an important reservoir of AMR. Yet, there is little information on the potential impacts that ARs and ARGs, resulting from VMP use, can have on the functioning of the ecosystem and its key species. There are also indications that shed of AMR (ARB and ARGs) occurs at similar levels from cattle raised without using antibiotics (Vikram et al., 2017). Furthermore, it is still unknown whether putative changes induced in communities of bacteria naturally present in the environment may affect the

² The resistome is considered to be the pool of antimicrobial resistance genes within the natural environment (see section 4 for details)

emergence and spread of AMR in bacteria of clinical relevance for humans or animals (some of which have the ability to survive and grow in the environment) (ECDC/EFSA/EMA/SCENIHR, 2009; FAO/WHO/OIE, 2008).

Knowledge gaps exist concerning the interplay between antimicrobial use in food-producing species, resistance in the environment, potential adverse impacts on human and animal health as well as other environmental side effects. Currently, it is not possible to analyse trends in AMR from environmental sources over time due to the absence of standardised or routine monitoring systems, safe thresholds for antimicrobials in the environment (in terms of impact on AMR) and standardised requirements as well as methods for susceptibility testing of bacteria from soil samples. An independent review on AMR (Review on Antimicrobial Resistance, 2016) recommended that a coordinated effort should be taken to establish a global surveillance system to monitor the emergence and spread of drug-resistant infections. This review highlights the need to reduce unnecessary antimicrobial use in animals (uses not related to animal welfare or food security, e.g. use for infection prevention) to mitigate any effects that could occur on animal health, ecosystems and public health from animal waste. Although out of the direct scope of the present reflection paper, it is noted that the above-mentioned review also recommends pharmaceutical companies to establish a systematic monitoring of waste products from their antibiotic manufacturing processes and to support the installation of effective waste processing facilities to reduce or eliminate active pharmaceutical ingredients (APIs) from being discharged into the environment. Related to this, targets have been developed by industry for antibiotics in receiving waters from pharmaceutical manufacturing operations (Tell et al., 2019). This line of action could, for example, be considered within the regulatory framework for good manufacturing practice (GMP) to include compulsory environmental criteria.

In response to the rising threat from AMR, it is necessary for the CVMP, as part of the 'One Health' approach, to reflect on the current state of knowledge. There is a need to consider any interventions that could reduce the environmental drivers that enable the development of AMR, following use of antimicrobials in animal health, while maintaining the efficacy of the products. Such possible intervention measures under the remit of the CVMP include:

- promoting prudent use of antimicrobials, leading to a reduction of consumption of antimicrobials,
- any improvements in the risk assessment for VMPs containing antimicrobial agents, and
- the identification of practical and effective risk mitigation measures for the registration of new VMPs and maintenance of the longevity of existing VMPs.

Between the drafting of this paper and its publication, the new EU Regulation on veterinary medicinal products (Regulation [EU] 2019/6) entered into force. In this regulation is stated that, together with an application for authorisation of an antimicrobial veterinary medicinal product, documentation on the direct or indirect risks to public or animal health or to the environment and information about risk mitigation measures to limit antimicrobial resistance development should be submitted (Article 8[2][a] and [b]). In addition to this, the European Medicines Agency (EMA) has advised on amending the section in Annex II to this regulation relating to the requirements for the technical documentation in such a way that resistance in the environment shall be addressed.

4. Mechanisms of development of antimicrobial resistance

Antimicrobial resistance (AMR) is the ability of a microorganism to survive and multiply in the presence of a compound with antimicrobial properties that would normally inhibit or kill this microorganism. AMR is one of the adaptive traits that bacterial subpopulations may possess or acquire, enabling them to survive and overcome host strategies aimed against them. AMR is a natural phenomenon that pre-

dates the modern selective pressure of clinical antimicrobial use (D'Costa et al., 2011) because natural antimicrobials (antibiotics) are ubiquitously present in microbial and fungal communities. Several different mechanisms are involved in the development of resistance to antimicrobials (for more detail on the specific mechanisms see Annex I). The pool of ARGs within the environment, the so-called environmental resistome, is now widely recognised as a complex and diversified reservoir of resistance genes that can be transferred into and between environmental and clinically relevant bacteria (Cantas et al., 2013; Wellington et al., 2013). The recruitment of MGEs such as plasmids, transposons, insertion sequences, and integrative conjugative elements, including the genes they carry, will also occur. These MGEs enable the movement of DNA within and between genomes of prokaryotic species and the total collection of MGEs in a genome is known as the mobilome (Gillings, 2013).

Generalised concerns exist that antimicrobial use selects for resistant bacteria (i.e. increased abundance of a sub-population of a resistant bacterial species relative to the total population of the species) which may have a deleterious impact either as a pathogen itself or by horizontal gene transfer (HGT) of ARG(s) to a potential bacterial pathogen. Concerning AMR, a distinction should be made between intrinsic resistance and acquired resistance (Holzbauer and Chiller, 2006). Intrinsic resistance occurs as a result of a structural or functional trait which allows tolerance to a particular substance or antimicrobial class by all members of a bacterial taxon. Acquired resistance results from a genetic change in the genome of formerly susceptible bacteria, which can be the consequence of a mutation (endogenous resistance) or following HGT of foreign genetic information (exogenous resistance) (Alekshun and Levy, 2007; Davies and Davies, 2010). The selection of bacteria with intrinsic or acquired resistance could result in a threat to human and/or animal health. For example, intrinsically resistant bacteria for many classes of antimicrobials, for instance *Clostridioides difficile*, can be selected during antimicrobial therapy and thereby cause harm, including casualties in both human and veterinary medicine (Moono et al., 2016).

The environment receives inputs of ARs and ARGs as result of different anthropogenic activities such as pharmaceutical manufacturing or the use of antimicrobials in human and veterinary medicines (Bengtsson-Palme et al., 2018). It is suggested that these activities increase environmental selection pressure and therefore the environmental resistome, notably by increasing the recruitment of MGEs and the genes they carry (Jechalke et al., 2014).

For a risk assessment on AMR, especially in the context of the environment where different bacterial populations may be exposed to different substances simultaneously, cross-resistance (a single resistance mechanism confers resistance to almost a whole antimicrobial class and potentially to other classes) and co-resistance (resistance to more than one class of antimicrobial in the same bacterial strain, as might occur when different resistance genes are found on the same plasmid) to antimicrobials and other substances should also be regarded carefully. Due to cross- and co-resistance, bacteria resistant to a certain antimicrobial substance can be selected by exposure to another antimicrobial or even another substance with antimicrobial properties. For example, biocides and heavy metals are known to have the potential to select for resistance to antimicrobial agents because the genes encoding resistance to various molecules often coexist on the same genetic elements (Cavaco et al., 2010; Singer et al., 2016; Soumet et al., 2012; Wales and Davies, 2015). This adds further complication to the already complex issue of resistance in the environment.

5. Consumption of veterinary antibiotics³

The European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) project, set up by the EMA following a request from the European Commission, publishes an annual report on sales of veterinary antibiotic active ingredients in EU/EEA countries and Switzerland (31 countries reported data for 2018, 25 of which provided data for the full period that the report covered from 2011 to 2018 (EMA/ESVAC, 2020). The most recent report, published in October 2020, showed that sales of antibiotics for use in animals in Europe fell by 34.6% between 2011 and 2018 (EMA/ESVAC, 2020). It is noted that these sales data do not cover other antimicrobials such as antifungals and parasiticides. In addition, these sales data do not take into account wastage, imports or exports of veterinary antibiotics, but are considered the best currently available approximation of the quantity of antibiotics used in animals. Many EU/EEA countries have developed, or are developing, more robust systems to collect and collate data on antibiotic use by animal species. Additionally, the European Centre for Disease Prevention and Control (ECDC) records human use of antibiotics based on population-normalised daily doses per year (ESAC-Net, 2020). However, it is noted that several countries have only recently set up monitoring systems to record these data, and these data are aggregated at a high level.

Significant issues are raised when considering the merit of using these sales/consumption data in isolation to give an accurate picture of the exposure of the environment and the prediction of likely AMR hotspots (which would be correlated with veterinary or human health concerns). It is overly simplistic to suggest that the likely excretion of ARs, antibiotic resistant bacteria (ARBs) and ARGs from treated animals into the environment is expressly related to the levels of antibiotics sold. For instance, several antibiotics (e.g. β -lactams, streptomycins and aminoglycosides) are produced by environmental bacteria, thus contributing to the natural background level of antibiotics in the environment. Further, besides consumption, production and manufacturing can also be important sources of antibiotics to the environment. Also, since antibiotic substances and AMR genes have different rates of depletion/degradation in the body of the treated animal and the environment, the AMR hotspots may not be those compartments where antibiotic substance consumption is the highest. Therefore, it is important to consider the physicochemical and environmental fate properties of antibiotics, especially in terms of their stability, sorption and persistence characteristics as well as partitioning to soil or water compartments. Therefore, sales data alone should not be used to predict the extent of the occurrence and spread of AMR in the environment. The recommendations section of this reflection paper, together with the emissions and fate sections, consider additional data that may be useful in determining the extent of exposure and persistence of ARs and ARGs in the environment.

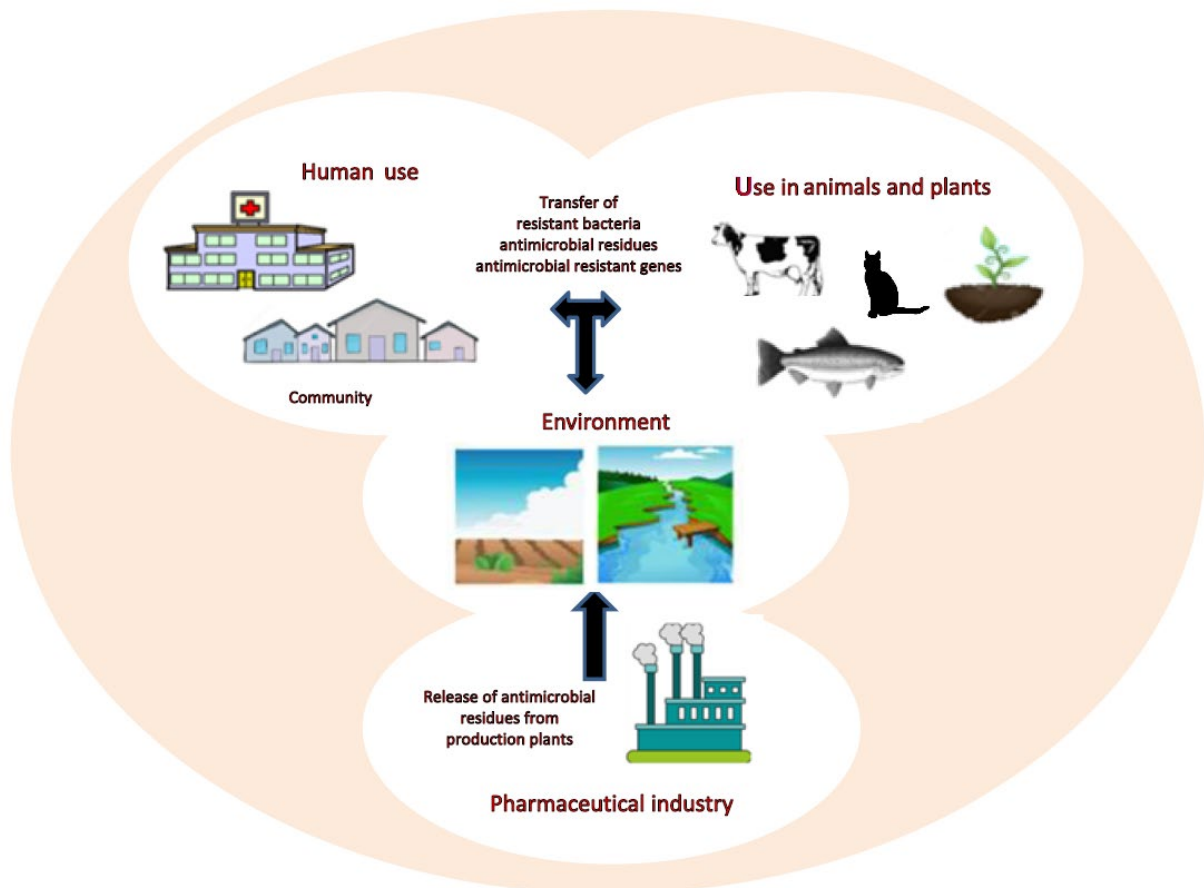
³ Note the distinction in terminology. The term 'antibiotics' is synonymous with antibacterials whereas, the term 'antimicrobials' is a general term for any compound with a direct action on microorganisms used for treatment or prevention of infections. Antimicrobials are inclusive of antibacterials, antivirals, antifungals and antiprotozoals. Therefore, in this case, reference is made to sales of VMPs that contain an active substance which is considered as an antibacterial.

6. Emissions and fate of VMPs as sources of antimicrobial substances to and within the environment

6.1. Emissions

Figure 1 provides a simple representation of how antibiotics are cycled between different environmental compartments, for instance from medical sources (e.g. hospitals), agricultural settings, aquaculture, the pharmaceutical industry and the wider environment.

Figure 1. A simple schematic of the pathways for dispersion of AMR



In terms of the emission pathways of antimicrobials from animals treated with VMPs, a large fraction of the antimicrobials can be released into the environment in an active form, via excretion in urine and faeces (and other materials such as discarded milk and blood). As the activity of antimicrobial substances does not necessarily end when a bacterial infection has been treated in the animal, a selective pressure on bacteria in the environment may be imposed. This in turn may lead to the selection of resistant strains, which are also capable of moving between different environments, thereby creating the potential for the movement of ARGs and associated MGEs (further covered in chapter 7 on the emissions and fate of ARB and their AMR genes to and within the environment).

Excretion rates of ARs depend on a number of factors, including the antimicrobial itself, its mode of application, the animal (e.g., species and age) and the time elapsed since administration. Data on absorption, distribution, metabolism and excretion (ADME) are available in regulatory submissions relating to both maximum residue limits (MRLs) and marketing authorisation applications (MAAs). Such information, together with the exposure assessment as carried out for the ERA, can provide useful

information on the potential extent of (a) microbiologically active substance(s) passing into the environment. Exact figures of the rates of excretion of an antimicrobial are not always available, but for some highly consumed antibiotic classes such as β -lactams, tetracyclines, phenicols and trimethoprim, excretion generally exceeds 50% of the administered dose (Alavi et al., 2015; Elizalde-Velázquez et al., 2016; EMA, 2015), depending on the route of administration. For sulfonamides, excretion is more variable, and for macrolides, the excreted fraction is generally lower. Amoxicillin is relatively inert in the body and will be excreted mainly as parental form (degradation rate of 10–20%), whilst sulfamethoxazole is extensively degraded (up to 85%) (Hirsch et al., 1999). For some ARs like sulfadiazine and trimethoprim, excretion might be low when they are dosed at low concentrations in the feed (comparable to concentrations caused by cross-contamination), but this is not the case for tetracyclines (Peeters et al., 2016). Additionally, metabolites formed in the treated animal and subsequently excreted may retain their antibiotic activity. Therefore, a range of rates of excretion and degradation as well as possible transformation events are seen which are dependent on the individual active substance. It is noted that for VMPs containing antimicrobials where an extended (Phase II) environmental risk assessment is required, information on excretion, degradation, and transformation may already be available in the submission dossiers. Although relatively simplistic, assimilation of such data could be used to highlight those substances that can potentially enter the environment and their persistence in the environment.

In addition to their indirect discharge, antimicrobials are also used in aquaculture where they are generally used as in-feed preparations. Ultimately, antimicrobials can reach various external environmental compartments such as rivers, lakes and soils (Kümmerer, 2009; Martínez, 2009; Sukul and Spittler, 2007) where they can continue to exert their effects.

Based on the pattern of use of VMPs and the handling of the waste from treated animals, the main pathways by which antimicrobials used in veterinary medicine and their metabolites reach soils and water systems are considered to be the:

- direct excretion onto the land by pasture-reared animals,
- application of animal manure(s) or slurry to areas of agricultural use as fertilisers, and
- discharge of effluents from animal production units (husbandry and slaughterhouses) to surface waters and soils, including aquaculture.

Antimicrobials partition into different environmental compartments according to their physicochemical properties and may further be transformed by abiotic or biological processes. Additional information on fate and behaviour in terrestrial and aquatic compartments is covered in section 6.2 of this reflection paper below.

6.2. Fate and behaviour of veterinary antimicrobials in the environment

As mentioned in section 5, accurate consumption data for animals treated with antimicrobials does not give a representative picture of the environmental exposure to antimicrobials. Furthermore, although antibiotics from almost all substance classes have been detected in liquid manure at relevant concentrations (from μg to mg per kg , as discussed in section 6.2.1), there is presently no systematic monitoring of antibiotic compounds in environmental matrices such as water, soil, sediment or sewage sludge, and manure or residues from anaerobic digesters. Therefore, to assess the role of veterinary use of antimicrobials in the complex biological phenomena of the environmental resistome and mobilome, it is particularly important to understand and accurately model the fate and behaviour of veterinary antimicrobials in the environment as well as the waste matrix, which includes manure and

slurry. In these, the biological component (e.g. biodegradation of the ARs) is an important factor. Therefore, further research on fate and behaviour is required.

The initial distribution and fate of antimicrobials in the environment is largely dependent on the pattern of use, the metabolism and transformation occurring within the animal and the excretion potential of such compounds from the animal. However, once released from the animal into the environment, the fate of antibiotics will depend on their physicochemical properties (e.g. molecular structure, size, shape, solubility and hydrophobicity) and a variety of environmental factors (e.g. climatic conditions, soil types and hydrological effects). In addition, sorption properties of these antimicrobials together with transformation potential by abiotic or biological processes will also determine how they partition into different environmental compartments. In particular, antimicrobials released into the environment are likely to be naturally degraded by biodegradation processes (by bacteria and fungi) and non-biotic elimination processes such as hydrolysis, oxidation and reduction. Degradation processes are influenced (amongst others) by temperature, moisture, pH and ionic strength of the environment and the composition of the local microbiota. It appears reasonable to suggest that factors that prolong the persistence of a compound in the environment could also enhance the potential of a substance to select for resistance in the environmental microbiome.

6.2.1. Terrestrial environment

For the terrestrial compartment, one of the main sources of antimicrobials comes from the spreading of manure from animals treated with antimicrobials (Hamscher et al., 2005). The spreading of unprocessed manure is recognised in the European legislation (Article 13[f] of Regulation [EC] 1069/2009⁴) as a way of fertilising the soil and disposing of manure, and is practiced if the manure does not represent a risk of spreading serious transmissible diseases (such as Newcastle disease or, in the case of unprocessed poultry manure, avian influenza). The risk from AMR is not explicitly covered under this legislation.

Approximately 96 million tonnes of farm manures (both solid manures and slurries) are applied to agricultural land in the United Kingdom alone (DEFRA, 2010). Taking into consideration application rates, it is estimated that, as a result of application of manure to land, antimicrobials are being released into the environment in the region of kilograms per hectare and per year (Kemper, 2008). This represents an immense potential for environmental contamination from antibiotics used in livestock.

Knowledge of the concentrations of ARs in manure is important as it can give an indication as to the maintenance of bacterial resistance in the environment, as all bacteria replicating in the manure are still subject to selection processes taking place there. A European study (Hölzel et al., 2010) investigating the association between ARs (of 24 antibiotics used in animal and/or human medicine) and bacterial AMR of *Escherichia coli*, *Enterococcus faecalis* and *Enterococcus faecium* in liquid pig manure used as fertiliser reported a range of antibiotic concentrations in manure from residual levels to commonly 1–10 mg/kg or mg/l, but also concentrations of more than 50 mg/kg (see Table 1).

⁴ Regulation (EC) No 1069/2009 of the European Parliament and of the Council of 21 October 2009 laying down health rules as regards animal by-products and derived products not intended for human consumption and repealing Regulation (EC) No 1774/2002 (OJ L 300, 14.11.2009, p. 1–33)

Table 1. Detected antibiotics in pig manure (n = 305, adapted from Hölzel et al. (2010))

| Antibiotic | Positive findings* | | Concentration (mg/kg) | |
|-------------------|--------------------|-----|-----------------------|-----------------|
| | (n) | (%) | Median** | Range (min-max) |
| Chlortetracycline | 113 | 37 | 0.33 | 0.1-50.8 |
| Tetracycline | 93 | 31 | 0.71 | 0.1-46.0 |
| Oxytetracycline | 18 | 6 | 0.14 | 0.1-0.9 |
| Doxycycline | 4 | 1 | 0.29 | 0.1-0.7 |
| Σ TETs | 166 | 54 | 0.73 | 0.1-52.6 |
| Σ SULs | 154 | 51 | 0.15 | 0.05-38.4 |

SULs: sulfonamides; TETs: tetracyclines

*TETs: > 0.1 mg/kg; SULs: >0.05 mg/kg

**Positive findings

The levels of antibiotics found in manure might seem generally low, but European pigs and cows are reported to jointly produce 1.27 billion tonnes of manure per year. Data on antibiotic consumption for those two species are not available, but consolidated data from 31 EU/EEA countries shows that more than 6,500 tonnes of active ingredients were sold for use in animals in 2018 (EMA/ESVAC, 2020).

Further data from a global perspective (Massé et al., 2014) also depict a wide range of reported values of antibiotic concentrations in manure. A summary of the findings is presented in Table 2.

Table 2. Example of concentration of antibiotics in manure from global sources (adapted from Massé et al. (2014))

| Antibiotic | Matrix | Concentration | Reference |
|-------------------|-----------------------------|---------------|--------------------------------------|
| Chlortetracycline | Beef manure stockpile | 6.6 mg/kg | Dolliver and Gupta (2008) |
| Chlortetracycline | Swine manure | 764.4 mg/l | Pan et al. (2011) |
| Chlortetracycline | Swine manure | 139 mg/l | Chen et al. (2012) |
| Chlortetracycline | Swine manure storage lagoon | 1 mg/l | Campagnolo et al. (2002) |
| Doxycycline | Swine manure | 37 mg/l | Chen et al. (2012) |
| Monensin | Beef manure stockpile | 120 mg/kg | Dolliver and Gupta (2008) |
| Oxytetracycline | Manure | 136 mg/l | Martínez-Carballo et al. (2007) |
| Oxytetracycline | Cow manure | 0.5-200 mg/l | Ince et al. (2013) |
| Oxytetracycline | Fresh calf manure | 10 mg/kg | De Liguoro et al. (2003) |
| Oxytetracycline | Swine manure | 354 mg/l | Chen et al. (2012) |
| Sulfadiazine | Swine manure | 7.1 mg/l | Chen et al. (2012) |
| Sulfonamides | Swine manure | 2 mg/kg DM | Jacobsen and Halling-Sørensen (2006) |
| Tetracycline | Swine manure | 98 mg/l | Chen et al. (2012) |
| Tylosin | Fresh calf manure | 0.11 mg/kg | Jacobsen and Halling-Sørensen (2006) |
| Tylosin | Beef manure stockpile | 8.1 mg/kg | Dolliver and Gupta (2008) |

The variation in antibiotic concentrations in manure can be attributed to differences in the total usage of the compounds, but also to differences in the antibiotic's metabolism, degradation processes or to the methods used for sampling and quantification of antibiotic concentrations.

Massé et al. (2014) highlighted that, regarding the studies cited in their publication to determine concentrations of antibiotics in manure, the majority did not provide sufficient description of manure handling and management conditions before sampling. Furthermore, there is a need for more reliable and standardised methods of quantification in complex matrices such as soil and biological sludge. Without improvements in these areas, dependency on the results from such studies or the ability to make inter- and even intra-study comparisons is problematic.

Once in the environment, some antibiotics bind strongly to soil and sediments, which contributes to their persistence as they become inaccessible to degradation (these 'trapped' compounds can persist in soil for many years). There is a significant amount of debate as to whether or not these non-extractable residues are bioavailable. Some studies suggest that sorption and fixation can reduce, but not necessarily eliminate their antimicrobial activity (Sengeløv et al., 2003). For example, tetracycline and tylosin remain active even when tightly adsorbed by clay particles (Chander et al., 2005). These sorbed compounds might represent a reservoir of pollutants that can be mobilised to further contaminate other compartments (Pedersen et al., 2003). Persistence and accumulation of tetracycline in the environment has been reported (Hamscher et al., 2002). It is clear that additional research is needed to better understand the kinetics of biodegradation and the potencies of degraded products of various antibiotics in manures and the receiving soils.

Following application of manure to land, antibiotics may become mobile as a result of water flow through the soil and subsequent leaching. This could result in a flow of the substance in question (or mobile resistant genes) from soil into surrounding surface water and groundwater. The extent to which this will occur is dependent on the properties of the antibiotic, soil, and hydrological effects. Further research is needed to gain a better understanding of the mobility and transport of antibiotics in the environment. From the available studies, it is possible to conclude that there are considerable differences regarding the environmental behaviour of the various antibiotics. For example, oxytetracycline is not transported into deeper soil segments as it is strongly adsorbed to soil, whilst olaquinox is only weakly adsorbed (Rabølle and Spliid, 2000). Distribution coefficients vary in different environments. In the case of oxytetracyclines and tylosin, the distribution coefficient is lower in manure than in soils (Loke et al., 2002).

It is acknowledged that the above considers the spreading of manure whereas another similar route of environmental exposure is that from recently antimicrobial-treated pasture-reared animals defecating directly onto land. It is assumed that this route will likely result in less extensive exposure when compared to exposure from the spreading of manure. Nevertheless, this route of exposure could still produce localised impacts resulting in an increased selection pressure for AMR development.

6.2.2. Aquatic environment

As mentioned in section 6.2.1, following application of manure to land, some of the antimicrobials may leach/transport into aquatic compartments, including surrounding surface water and groundwater. Further, pasture animals that have been recently treated with an antimicrobial could, in theory, directly excrete (via faeces or urine) higher concentrations of antimicrobials than those expected via the spreading of manure. Little information is available on the relative contribution from the direct excretion exposure route.

In general, antibiotic concentrations reported in aquatic environments are less than 10 µg/l (sewage treatment plant effluents, receiving surface waters, groundwater) (Kümmerer, 2009). The relative

contribution of the use of antibiotics in veterinary medicine to the levels observed in environmental compartments is largely unknown, particularly considering that many of these antibiotics are also used in human medicine.

It should be noted that the treatment of water, sewage and other contaminated residues can reduce concentrations of certain classes of antibiotics but, invariably, a fraction of antibiotics will remain in effluents after treatment (Watkinson et al., 2007). Water chlorination helps to degrade antibiotics such as β -lactams and trimethoprim (Dodd and Huang, 2007; Li et al., 2008). Traditional methods for wastewater treatment can eliminate up to 80% of fluoroquinolones and tetracyclines but they are less efficient in the removal of macrolides (Gulkowska et al., 2008; Shellie et al., 2002; Sukul and Spittler, 2007).

An increase in the prevalence of ARB, including enterococci, *E. coli* and *Acinetobacter spp.*, after wastewater treatment has been observed by several authors, despite a reduction of bacterial load in treated wastewater compared to raw wastewater (Ferreira da Silva et al., 2007; Łuczkiwicz et al., 2010; Zhang et al., 2009b). Concerning ARGs, the situation is more complex given that they are not 'degradable pollutants' but auto-replicative elements.

Antibiotics can also enter the aquatic environment directly from pharmaceutical production facilities. Emissions from industrial sites can be considerable, especially in developing countries (Larsson, 2014). Antibiotics are also used in culture medium for the production of biological pharmaceuticals. This exposure route is, however, not within the scope of the present reflection paper.

6.2.2.1. Aquaculture

Antimicrobials have the propensity to reach the aquatic compartments directly from the faeces of fish treated with VMPs containing antimicrobial active substances as in-feed preparations. An additional waste stream of antimicrobials could result from uneaten treated feed.

According to an aquaculture sustainability briefing (European Commission, 2015), most VMPs used to manage finfish diseases have been judged to have minimal negative environmental impacts if used correctly (IUCN, 2007), and VMP use is closely regulated and inspected in all EU Member States. Problems such as risks to non-target species do occur where VMPs are used inappropriately. The use of antimicrobials is of particular concern in open marine aquaculture where they enter the surrounding marine environment via fish faeces and can persist for extended periods in sediments. In Europe, antibiotics are typically administered via medicated feed, but only a percentage is absorbed by the fish. Rigos et al. (2004) estimated that 60–73% of oxytetracycline administered to sea bass in Greek farms is released to the environment. Little is known regarding the significant impacts of antimicrobials on the marine environment. However, available studies indicate potential ecological risks. High concentrations of oxytetracycline and florfenicol, both active against *furunculosis* in salmon, have been shown to inhibit growth of algal species. This also highlights the need to understand the effects of 'real-world' chronic, low-level exposure of wild species to antimicrobials (Pittenger et al., 2007).

In the past, antimicrobials were used much more liberally in aquaculture. In response to growing awareness and stricter regulations, they are now generally used as a last resort. Improvements in farming practices have led to improved animal health and have reduced the need for the use of antimicrobials (European Commission, 2015). Moreover, the development and use of vaccines is also a key factor in reducing antibiotic use. Nevertheless, aquaculture has the potential to contribute to the widespread pool of resistant bacteria in the environment. Taylor et al. (2011) suggest research is needed to understand its impacts in comparison to far more dominant sources of resistant bacteria, particularly from other animal sources such as manures.

6.2.3. Further considerations and recommendations on environmental fate

Regarding the fate of antimicrobials in the environment, there is a dynamic relationship between relevant compartments (wastewater, manure, soil, surface water and groundwater). It is acknowledged that the existing MAA process for a VMP requires provision of certain data (including information on metabolism and excretion of a compound in the treated animal as well as on physicochemical properties, persistence and adsorption data in sediments and soils) that could, in some instances, be used to determine the significance of some of the key exposure and fate mechanisms in a simplistic manner. However, in order to produce a more robust evaluation of AMR in the environment, a better understanding of excretion, transformation and sorption of antimicrobial compounds would be required to quantify the environmental fate of antimicrobials.

Ciprofloxacin, a transformation product of enrofloxacin, which is used in veterinary medicine and is a commonly prescribed fluoroquinolone in human medicine, can be used as an example to illustrate the complex fate of antimicrobials in the environment: while waste-water treatment removes up to 90% of ciprofloxacin by sorption to sewage sludge, biological degradation is poor. As a result, ciprofloxacin accumulates in human sewage sludge and, if the sludge is used as fertiliser and subsequently applied to land, it can be found in the soil in up to 30 cm depth in concentrations in the low mg per kg range (Golet et al., 2002; Martínez-Carballo et al., 2007). In soil, ciprofloxacin persists for more than 90 days with only minimal transformation (Girardi et al., 2011). Although the strong adsorption to soil might reduce its bioavailability, it could still elicit effects on soil microorganisms as reported by Girardi et al. (2011), who found indications that the resistance gene *qnrS* was present in soil treated with that compound.

In conclusion, a better understanding of excretion, transformation and sorption of antimicrobial compounds is required to accurately predict the fate of these substances and, subsequently, to elucidate the role of the environment in the potential transfer of relevant AMR to bacteria with associated risks to human and animal health.

7. Emissions and fate of ARB and ARGs to and within the environment

7.1. Excretion of ARB and ARGs from treated animals

Antimicrobial treatment is usually indicated against specific pathogenic bacteria responsible for the infection (EMA/CVMP, 2018). As the use of antimicrobials creates a selection pressure, any use of antimicrobials to treat diseased animals may have the potential to select or disseminate AMR within the pathogenic bacteria against which the antimicrobial is used (Aarestrup, 2005; Holzbauer and Chiller, 2006; Toutain et al., 2016). The potential to select for resistance is mainly correlated to the antimicrobial substance, the administered dose and the corresponding concentration that is reached in the target tissue (Toutain et al., 2016). In addition, antimicrobials (or corresponding microbiologically active metabolites) administered to treat a specific pathogen exert a collateral selection pressure on the commensal microbiota (Baron et al., 2016; Beyer et al., 2015; Toutain et al., 2016). The importance of this 'non-desired exposure' is dependent on the pharmacokinetic profile of the drugs and may also be driven by the route of administration of the VMPs (Bibbal et al., 2007; Bibbal et al., 2009; De Smet et al., 2017; Holman and Chénier, 2015). Exposure of the gastrointestinal tract (GIT) microbial population could lead to major modifications in the microbial equilibrium and to some extent contribute, as demonstrated in scientific literature, to increase the reservoir of resistance genes in the colon (Baron et al., 2016; Beyer et al., 2015; D'Costa et al., 2011; Martínez et al., 2015; Toutain et al., 2016).

The high microbial load and the diversity of the bacterial population in the GIT, especially in the distal portion where the greatest population of established resident bacteria occurs, serves as a hotspot for AMR development (Toutain et al., 2016). It is suggested that, even if commensal bacteria are not considered clinically relevant, they will harbour a range of resistance genes which may subsequently be directly excreted into the environment via the faeces of the treated animal (Bibbal et al., 2007; Fleury et al., 2015; Thames et al., 2012). Apart from the GIT, other reservoirs are also possible, including the skin, the upper respiratory and the urinary tract (e.g. methicillin-resistant *Staphylococcus* species, multidrug-resistant [MDR] *E. coli*) (Antunes-Lopes et al., 2020; EMA/CVMP, 2015).

Product characteristics such as the dosage regimen and the route of administration will influence the pharmacokinetic profile, in particular the extent of exposure of the GIT to antimicrobials and their metabolites (Bibbal et al., 2007; Bibbal et al., 2009; Lees and Toutain, 2012; Zhang et al., 2013). For example, tetracyclines (tetracycline, chlortetracycline, oxytetracycline, and doxycycline) are the antimicrobial class most commonly administered by the oral route in food-producing animals (EMA/ESVAC, 2020). They have a very low oral bioavailability in pigs, with values typically ranging between 5–15% of the total dose (Nielsen and Gyrd-Hansen, 1996; Toutain and Bousquet-Mélou, 2004). The unabsorbed fraction (85–95%) remains in the GIT, exposing the dense microbial environment for a duration that could exceed the treatment period, and with subsequently unabsorbed fractions being released into the environment in the faeces of treated animals. Furthermore, tetracyclines can also trigger an enterohepatic cycle, meaning that microbial communities in the GIT might undergo consecutive selection pressures in the frame of only one treatment (Toutain et al., 2016). Therefore, in terms of risk assessment, the following characteristics increase the risk to drive the selection and/or excretion of resistance determinants into the environment:

- antimicrobials administered orally that are poorly systemically bioavailable,
- antimicrobials administered parentally that are excreted into the GIT,
- and those above that are, additionally, associated with a collective treatment (herd/group treatment).

Indeed, in addition to ARs, waste from treated food-producing animals may contain many pathogenic and commensal bacteria, with some harbouring ARGs. For example, it is recognised that the spread of manure leads to a temporary increase in the occurrence of AMR in manure-fertilised soil (Bengtsson-Palme et al., 2018; Kumar et al., 2018; Topp et al., 2018; Wall et al., 2016; Yu et al., 2017). However, it should be noted that untreated animals may also harbour ARBs or ARGs even without selection pressure, as shown in several scientific reports (Beyer et al., 2015; Nilsson et al., 2019). Thus, the use of waste from animals for manure spreading may contribute to the environmental resistome, although further research is still needed to understand the environmental impact of these observations (Heuer et al., 2011; Jensen et al., 2002; Sengeløv et al., 2003).

7.2. Selection of ARGs in environmental bacteria exposed to antimicrobial residues and ARGs from VMPs

Sources of antimicrobials contaminating different environmental compartments include food-producing animals excreting active compounds (as parent form or as metabolites) in faeces and/or urine. Those residues have the potential for exerting a selective pressure on the bacterial populations in animal waste, sludge or manure and, thereafter, in environmental compartments (Heuer et al., 2011). Once in the environment, several antimicrobials remain stable for several weeks or even months (Halling-Sørensen et al., 2003).

For non-environmental bacteria, survival seems more critical than growth in the environment. For those bacteria, which use the environment for dispersal only, the advantage of harbouring resistance genes even in the presence of antimicrobials is likely to be small (Bengtsson-Palme et al., 2018; Heuer et al., 2011). However, there is evidence that HGT still continues in the absence of growth. Considering that even enteric bacteria can grow in the environment under some circumstances, the relationship is not clear cut. Nonetheless, for bacteria that can grow outside the animal and use the environment as an alternative or main habitat, antimicrobial exposure would be more likely to contribute to the selection of resistant determinants during environmental dissemination. Bacteria from the latter include opportunistic and emerging pathogens such as *Aeromonas* spp; *Acinetobacter* spp; *Pseudomonas putida*, *Burkholderia cepacia*, *Stenotrophomonas maltophilia* and *Bacillus cereus* (D'Costa et al., 2006; Denet et al., 2017; Forsberg et al., 2016; Forsberg et al., 2012; Goñi-Urriza et al., 2000; Holmes et al., 1998; Raphael and Riley, 2017).

The selection pressure exerted by the concentration(s) of antimicrobial(s) in contact with environmental bacteria might have a different impact on the development and spread of AMR. As a function of the exposure, the bacterial response could differ and result in different levels of genotypic and phenotypic adaptations (Andersson and Hughes, 2011; Gullberg et al., 2014; Gullberg et al., 2011; Rodríguez-Rojas et al., 2013). Results from *in vitro* experiments suggest that, if a bacterial population is challenged by high concentrations of (an) antimicrobial(s), for instance concentrations higher than the minimum inhibitory concentration (MIC), the pre-existing resistant or less susceptible strains will be selected, eventually establishing a highly resistant bacterial population. However, if a bacterial population encounters antimicrobial concentrations below the MICs, different mechanisms could increase their genetic variability. This could include an increase in the mutation rates, in genetic recombination and also in HGT, and finally lead to a greater heterogeneity in the resistance profile within the exposed bacterial community (Aminov, 2009; Andersson and Hughes, 2014; Gullberg et al., 2014; Gullberg et al., 2011; Jolivet-Gougeon et al., 2011; Rodríguez-Rojas et al., 2013; Sandegren, 2014). Nevertheless, at concentrations even well below the MIC of susceptible strains, resistant strains can maintain a selective advantage. This represents the concept of minimal selective concentration (MSC) as described by Gullberg et al. (2011), whereby clonal expansion (the relative increase in growth of ARB) is possible even at low concentrations. For example, Gullberg et al. (2011) demonstrated resistance at levels as low as ng/l for ciprofloxacin. Such *in vitro* studies indicate, in theory, that even at the lowest concentrations of an antimicrobial (sub-MIC concentrations) present in the environment, selection for some ARB/ARGs is still possible. All these observations would need to be confirmed under real environmental conditions before direct extrapolation from *in vitro* to *in vivo* could be performed.

The consequences for the environment itself are currently difficult to estimate without specific knowledge on the direct impact on resident microbial communities and ecological process functions. Nevertheless, all selection processes might occur and may increase, at least, the environmental resistome and subsequently also the potential risk of transfer of resistant bacteria or resistance genes to clinically relevant bacteria that could have an impact on public or animal health. However, it is acknowledged that the significance of this risk is yet to be elucidated. The fact that ARs and ARGs are introduced to soils together means that disentangling the selective effects of antibiotic residues from changes in ARB/ARG diversity and abundance associated with introduction of manure-borne bacteria is challenging. However, one study by Cleary et al. (2016) on soil experimentally exposed to VMPs at levels close to the maximum recorded in animal faeces (i.e. 10 mg/kg of tylosin, sulfamethazine and chlortetracycline that are commonly used in commercial pig production) showed selection for AMR and changes in bacterial community structure, including decreased relative abundance of key proteobacteria taxa such as *Rhizobium* sp.

Further research would be needed to understand the environmental conditions that could positively or negatively influence the spatio-temporal dynamics of selection and dissemination of resistant determinants.

7.3. Exchange of ARGs between environmental bacteria and animal/human bacteria in the environment

The HGT of DNA plays a profound role in the evolution of prokaryotes. Acquisition of genes from other organisms provides an efficient way to acquire new traits and adapt to new or changing environments (Bobay and Ochman, 2017; Gillings, 2017). In the context of AMR, HGT contributes to the rapid dissemination of ARGs among commensal and/or pathogenic microbiota during a short period (Hiltunen et al., 2017). This dissemination of ARGs from antimicrobial-producing organisms to clinically relevant species has occurred within the antibiotic era and is mediated by diverse MGEs (e.g. plasmids, transposons, genomic islands) and integrons (Perry and Wright, 2013).

Three main mechanisms of HGT exist and are well described in the scientific literature: conjugation, transduction and transformation. Most of the demonstrations of transfer of DNA were realised *in vitro*, but the heterogeneity of bacterial communities *in vivo* might facilitate the spread of ARGs (Cooper et al., 2017). The ability, for example, of soil bacteria to transfer ARGs by conjugation has been demonstrated, especially in the rhizosphere (Schwaner and Kroer, 2001; Sengeløv et al., 2000; van Elsas et al., 1998). Furthermore, the presence of similar MGEs in pathogenic and environmental bacteria harbouring ARGs implies that exchange between those reservoirs has occurred and probably still takes place (Huijbers et al., 2015; Nesme and Simonet, 2015).

HGT might occur in all environments but has been most studied in soil, where particular physical properties (e.g. temperature, pH, concentration of nutrients and oxygen) and a huge microbial diversity create favourable conditions for this process (Aminov, 2011; Forsberg et al., 2015; Perry and Wright, 2013; van Elsas and Bailey, 2002). Currently, there is some evidence suggesting that aminoglycoside and vancomycin resistance enzymes, the extended-spectrum β -lactamase CTX-M as well as the quinolone resistance gene *qnr* originated in environmental bacteria (Beyer et al., 2015; Nilsson et al., 2019; Nilsson et al., 2009). Different examples of transfer of ARGs between environmental matrices and clinical isolates have been described in the literature (Baquero et al., 2008; Benveniste and Davies, 1973; Forsberg et al., 2012; Humeniuk et al., 2002). For example, Poirel et al. (2005) screened a collection of 48 Gram-negative clinical and environmental bacterial species belonging to *Enterobacteriaceae*, *Aeromonadaceae*, *Pseudomonadaceae*, *Xanthomonadaceae*, *Moraxellaceae* and *Shewanellaceae* and identified that the *qnrA* gene originated from the chromosome of *Shewanella* algae.

Therefore, a key consideration is the possibility that pathogenic or commensal bacteria that have acquired a resistance determinant from environmental bacteria can make it back to their human or animal host. Vice versa, transfer of ARGs to environmental bacteria is possible and has already been described. This enables human or animal-associated bacteria to use strains of the environmental bacterial population as recipients for resistance genes that can later return to the human or animal-associated resistome.

8. Risks to human and animal health from AMR and ARGs in the environment

8.1. Environmental transfer of ARGs and ARB and potential route of transfer to humans and animals from the use of VMPs

Environmental dissemination of ARBs is increasingly identified as a potential route for the spread of AMR (Marshall and Levy, 2011). Different environmental compartments might contribute to the dissemination of resistant pathogens and commensal bacteria associated with humans and animals alike. Opportunistic pathogens such as *Acinetobacter spp.* and *Pseudomonas spp.* are located within different environmental compartments. Thus, sewage, wastewater treatment plants, agricultural and veterinary hospital effluents (Zhang et al., 2009a), drinking water (consumed either by humans or domesticated animals in close contact with humans), recreational water, air-borne aerosols, dust, wildlife fauna and contaminated food from agriculture or aquaculture are all vectors enabling the potential transmission of bacteria and ARGs between hosts through the environment (Singer et al., 2016).

The next section provides illustrative evidence from European studies to depict several of the many different routes that allow the transmission of ARGs and ARBs to humans and/or animals through the environment.

8.1.1. Food from crops

During cultivation in soil to which animal manure is applied, crops and irrigation water may be contaminated with resistant bacteria, for example Extended Spectrum Beta-Lactamases (ESBL) and AmpC Beta-Lactamases (AmpC)-producing bacteria. A number of studies have investigated the prevalence of AMR bacteria in or on vegetables and fruits. Since fresh produce is often consumed raw, consumption may result in the ingestion of resistant bacteria that, depending on the bacterial species, are able to colonise the gut or pass through the intestine, thus posing a potential public health risk (Wall et al., 2016). For example, a study from the Netherlands revealed that 3rd-generation cephalosporin (3GC)-resistant faecal *Enterobacteriaceae* were isolated from 2.7%, 1.3% and 1.1% of supermarket vegetables, iceberg lettuce from farms and agricultural soil, respectively. Comparison of fresh produce and its agricultural environment indicates that the *Enterobacteriaceae* population on fresh produce reflects that of the soil in which it is grown (Blaak et al., 2014).

8.1.2. Food from aquaculture

The occurrence and spread of ARB and ARGs in areas designated for fish farming (marine and freshwater) has exponentially increased during recent decades (Elbashir et al., 2018; Topp et al., 2018). The application of antimicrobials to the aquatic environment may select for ARGs not only in fish pathogens, but also in environmental bacteria (Muziasari et al., 2016). Resistance has been most frequently reported against oxytetracycline, tetracycline, ampicillin and florfenicol (Caruso, 2016), but some ARGs coding for resistance to quinolones and β -lactams can be found in fish pathogens, human pathogens and aquatic bacteria (Cabello et al., 2013). Furthermore, Cabello and colleagues suggested that the use of antimicrobials in aquaculture, notably the use of colistin in Asian aquaculture, could be correlated with the emergence of the plasmids encoded mobile colistin resistance (MCR) determinants (Cabello et al., 2017). That said, it is noted that colistin is not authorised as a VMP for use in aquaculture in the EU.

8.1.3. Contaminated drinking water

The prevalence and resistance patterns of various bacteria isolated from drinking water distribution systems have been recently reported. For example, in Romania, multiple AMR *E. coli* strains isolated from drinking water were found to harbour ARGs encoding resistance to aminoglycosides, β -lactams, tetracyclines and trimethoprim/sulfamethoxazole (Cernat et al., 2007). In France, a strain of *E. coli* carrying the *bla*_{CTX-M-1} IncI1/ST3 plasmid was isolated from drinking water. The plasmid was identical to those found in animals and humans (Madec et al., 2016). In another study performed in Germany, the *vanA* and *ampC* ARGs were detected in drinking water biofilms (Schwartz et al., 2003).

8.1.4. Contaminated recreational places

ARBs have been detected in natural aquatic environments and direct ingestion of water from recreational locations (e.g. seawater, lakes) is a route by which the population could be directly exposed (Leonard et al., 2015). In England, Leonard and colleagues showed that 0.12% of *E. coli* isolated from surface waters were resistant to 3GCs and could represent a human exposure risk for water users (Leonard et al., 2015). Leonard et al. also used a targeted metagenomic approach to estimate exposure to *E. coli* in UK coastal bathing waters carrying all known ARGs, concluding that all exposure events result in ingestion of at least one *E. coli*-associated ARG (> 100 million events per year) and that 2.5 million events per year involving the ingestion of 100 *E. coli* borne ARGs are likely to occur (Leonard et al., 2018a). The relationship between exposure, colonisation and infection with AMR-opportunistic pathogens is uncertain and there are no dose-response data available for colonisation, infection or HGT of ARGs from ingested bacteria to those of the microbiome. A cross-sectional study of surfers and non-surfers in the UK found that surfers were 3 times as likely to be colonised by 3GC-resistant *E. coli* and > 4 times as likely to be colonised by *bla*_{CTX-M} *E. coli*, suggesting an association between coastal bathing water exposure and gut colonisation by ARB (Leonard et al., 2018b).

In the Netherlands, ESBL-producing *E. coli* were detected in four different recreational waters nearby wastewater treatment plants (three recreational waters appointed under European Bathing Water Directive 2006/7/EC and one not appointed). *E. coli* were detected in all four recreational waters, with an average concentration of 1.3 colony-forming units/100 ml and in 62% of all samples (Blaak et al., 2014).

This section considered the sources and pathways of AMR transmission into humans and animals. The next section will elaborate the potential consequences these pathways may have on health.

8.2. Consequences for the risk assessment of human and animal health

The contribution of VMPs to the environmental resistome and the subsequent impact on human and animal health is difficult to quantify at present. Antimicrobials, ARBs and ARGs may originate from various sources. In addition, the same classes of antimicrobials are used in both human and veterinary medicine and there is also the potential for co-selection. Consequently, the presence of ARBs/ARGs in the environment cannot be clearly attributed to the use of antimicrobials in humans or to their use in animals, and it is indeed problematic to attempt to associate any consequences of the risk assessment to the use of VMPs only. However, the risk for human and animal health associated with the dissemination of resistance through the environment involves: the possibility of ARGs being transferred from non-pathogenic environmental bacteria to pathogenic or commensal bacteria (e.g. the environmental origin of *qnr* genes, as described above); the potential transfer of antimicrobial-resistant pathogens directly from animal to human with the environment acting as a 'vehicle'; and the possibility of infection by resistant bacterial pathogens originating from environmental compartments

(e.g. those represented by the ESKAPE family: *Enterococcus*, *Staphylococcus*, *Klebsiella*, *Acinetobacter*, *Pseudomonas*, *Escherichia*). Evidence of environmental transfer of AMR to humans or animals causing direct adverse health events is scarce.

A well-described consequence for human health is the worldwide spread of the New Delhi Metallo-beta-lactamase (NDM) carbapenemase. As carbapenems are not known to be authorised in veterinary medicine and off-label use is only possible in non-food producing animals, it is more likely that the development and spread of NDM carbapenemases can be traced back to the use of antimicrobials in humans rather than in animals. After contact with a contaminated environment, the resistance determinant can be maintained within the gut microbiota for several months, leading to dramatic health consequences when patients are hospitalised.

AMR compromises our ability to deliver high quality medical care in the community and in the hospital environment. Effective antibiotic therapy is essential for many advanced medical procedures (e.g. heart transplants, hip replacements or any procedure associated with a high risk of bacterial infection) as well as for the treatment of patients with a suppressed immune system.

Resistance to antibiotics among human and veterinary pathogens increases the risks of treatment failure, increases mortality by increasing the time from an initial diagnosis to an effective therapy, and can also lead to morbidity by increasing the use of more toxic antibiotics as replacements for those rendered ineffective due to resistance. This issue also imposes an additional healthcare cost and productivity loss that, in the EU, was estimated to be at least €1.5 billion in 2007 with regards to the most frequently isolated bacteria from blood cultures (ECDC/EMEA, 2009).

In 2016, a review commissioned by the UK government entitled 'Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations' (Review on Antimicrobial Resistance, 2016) suggested that, if left unchecked, AMR will, in the future, impose a financial burden on society in addition to increasing morbidity and mortality in humans and animals. The CVMP also recognises this and continues to build upon its efforts to gain a better understanding of the role of veterinary use of antimicrobials on the development of AMR, in this instance, relating to the reservoir in the environment. Importantly, the CVMP promotes responsible use of antimicrobials in animals, since eliminating unnecessary use in animals and humans is expected to have a beneficial impact on the occurrence of AMR (ECDC/EFSA/EMA, 2017).

9. Evaluation of the current risk assessment process for VMPs: consideration of AMR – knowledge gaps and research needs

9.1. Current risk assessment approach for VMPs

Commission Directive 92/18/EEC⁵ introduced the requirement for the assessment of environmental safety as part of the submission for a MA of a new VMP. The ERA is performed in two phases (as described in VICH guidelines GL6 (CVMP/VICH, 2000) and GL38 (CVMP/VICH, 2004b) and in the supporting CVMP guideline (EMA/CVMP, 2016b)) and aims to evaluate the potential harmful effects caused to the environment through the use of VMPs and to identify the risk from such effects. From the perspective of antimicrobials, soil bacteria and cyanobacteria are considered the most sensitive organisms that require assessment. However, certain antimicrobials have also elicited adverse effects in higher organisms tested (especially plants). It is noted that for human medicines an additional study

⁵ Commission Directive 92/18/EEC of 20 March 1992 modifying the Annex to Council Directive 81/852/EEC on the approximation of the laws of Member States relating to analytical, pharmacotoxicological and clinical standards and protocols in respect of the texturing of veterinary medicinal products (OJ L 97, 10.4.1992, p. 1–23)

on inhibition of respiration activity in activated sludge also needs to be determined (OECD, 2010). Therefore, in terms of the effects part of the assessment, few data are of significant relevance to AMR. With regard to the data requirements on the physicochemical and fate properties of compounds in soil (sometimes in manure) and aquatic compartments, greater relevance is seen. In particular, the findings from the existing studies on biodegradation and sorption of VMPs containing antibiotics could, in a simplistic manner, be used to evaluate the likely stability and persistence of an antimicrobial in the environment and, in combination with knowledge to be obtained on AMR development in microorganisms, its subsequent potential selection pressure for AMR could be assessed. It is also noted that the current phase II ERA process allows for the refinement of the exposure of a compound in the environment through the provision of data on metabolism and excretion. Such studies on metabolism and excretion may already be available as part of an MRL application or the safety part of a MA dossier and could provide useful information on the potential extent of excretion of microbiologically active substance(s) into the environment.

The effects of antibiotics on environmental bacteria can range from simple parameters such as a decrease in biomass, respiration rate or denitrification rate, to more complex parameters like community shifts and the survival of new genetic information. The findings from the few studies available on the effects of some antimicrobials on nitrification and decomposition in soil indicated effects in standard studies at very high concentrations, compared to expected field concentrations (Jensen, 2001; Thiele-Bruhn, 2003). However, the guidelines mentioned above do not currently take into consideration any aspects of AMR. Consequently, the trigger value of 100 µg/kg soil in the phase I assessment allows for high soil concentrations, and the safe concentrations based on traditional ecotoxicological endpoints (e.g. respiration and nitrification rate) fall short in avoiding minimal 'resistance-selective' concentrations (Berendonk et al., 2015; Montforts, 2005). Contamination with ARGs may change the genotype of autochthonous bacteria (Pruden et al., 2006; Schwartz et al., 2003). Acquiring resistance to antimicrobial substances, however, is part of the natural evolution of microbial species and may not, by definition, result in a deleterious effect. It is evident that alternative tools and models are needed to understand the environment from the microbiological perspective (e.g. applying tools from landscape ecology (Singer et al., 2006)), and that different tools are needed to assess the risk of ARGs in the environment (Midtvedt, 2004). For example, Hughes Martiny et al. (2006) chose to describe the biotic communities at the taxon level, not at the species level. The question as to whether taxa in communities or at biocenoses as a whole should be considered is yet to be agreed. Studies that focus on the effects of ARGs on complete (autochthonous) microbial communities are currently not available. The ecological relevance of the introduction of ARGs and the associated shifts in community composition have not been determined to date (McVey and Montforts, 2012; Mensink and Montforts, 2007).

In accordance with VICH GL27 (CVMP/VICH, 2004a), in the pre-approval information for registration of new antimicrobial veterinary medicines for use in food-producing animals, an applicant must provide data addressing the potential for such products to select for resistant bacteria in the treated animal that might be of human health concern (zoonotic pathogens and commensals). Once in the environment, such ARBs and ARGs could equally be a risk to animal health. The required data, where available, may include information on the concentration of microbiologically active substance(s) within the animal's gastrointestinal tract, which could be used to inform on the possibility of selection of resistance in the organisms of concern. There is no specific requirement for studies to investigate directly the excretion of ARGs from treated animals, despite this hazard being well documented (section 8.1).

The CVMP's draft risk assessment guideline for VMPs containing antimicrobials (EMA/CVMP/AWP, 2018) identifies general environmental contamination as a route of human exposure to ARGs resulting from the use of VMPs, but this route is not within scope of this guidance.

In summary, although the ERA process for VMPs does not currently take into consideration any aspects of AMR, there are data available from the existing process that could be useful in evaluating the significance of the fate mechanisms for certain antimicrobials in terms of AMR. Of particular note is the current data on physicochemical and fate properties as well as information on metabolism and excretion of antimicrobials used as VMPs. In terms of the effects assessment for the current ERA process, none of the current data requirements are considered relevant to AMR. It is clear that, in order to evaluate AMR in the environment appropriately, alternative tools (e.g. MSC assays) and models to understand the environment from the microbiological perspective would be required. Finally, any evaluation of AMR in the environment would have to consider the effects from the introduction of ARGs resulting from antimicrobial use as VMPs. Finally, it is important to note that the AMR/ERA assessment is hampered by the fact that, even if appropriate models were available to predict the magnitude of an AMR shift in an environmental compartment from VMP use, the relevance of such information and its subsequent consideration within the risk-benefit evaluation would be problematic at best. In order to rectify this, it is of critical importance to know how and to what extent human/animal health and ecosystems will be impacted by certain environmental AMR 'levels' or shifts and how this relates to certain quantities of certain antimicrobial substances in the different environmental compartments. Therefore, the above highlights fundamental issues that prevent a straightforward amendment of the current ERA to account for AMR in the environment. In particular, these are:

- 'Safe levels' for antimicrobials (that will not adversely impact human or animal health, or the environment) have not been established for AMR.
- The current ERA is based on the active substance and its major transformation products. An assessment for AMR would need to evaluate these as well as the potential environmental contaminants of ARB and the ARGs.
- Absence of appropriate validated assays to model and quantify AMR.
- The extent to which human/animal health and ecosystems will be impacted by certain environmental AMR 'levels' or shifts need elucidation.

9.2. Knowledge gaps and research needs

Assessing the risks of AMR in the environment due to the use of antimicrobials in veterinary medicine towards human and animal health is challenging given the complexity of the problem and the paucity of knowledge regarding the mechanisms and pathways involved at the genetic, cellular and population levels. In addition, the lack of understanding regarding the role that the receiving environment has on the fate of ARs, ARBs and ARGs is a significant knowledge gap as well.

To properly assess the risks of AMR in the environment, it would be beneficial if the differential contribution of the environment as compared to the contribution from other sources could be quantified in relation to the problem of AMR. It is clear that ARGs have the potential to move from environmental bacteria to human and animal pathogens and *vice versa*, as transfer of genes between bacteria can, in theory, occur anywhere (Bengtsson-Palme et al., 2018) and is more likely to occur between phylogenetically closely related bacteria (Philippot et al., 2010). However, for the transfer of resistance to occur, the host and receiving bacteria need to share the same ecological niche, at least temporarily (Wiedenbeck and Cohan, 2011). Following this rationale, it is reasonable to suggest that the frequency of transfer of resistance would be higher between animal-to-animal, and human-to-human-associated bacteria (Porse et al., 2017; Salyers et al., 2004). The transfer of ARGs to animal and human bacteria from environmental bacteria, which are often less phylogenetically related, would therefore likely be less common, but not necessarily insignificant, as environmental stressors may induce HGT to and from (opportunistic) human pathogens in environmental settings (Bengtsson-Palme

et al., 2018). The potential exposure pathways for environmental bacteria (whether only transient or not) to humans have already been discussed above, for instance recreational activities, interaction with farmed/wild animals or eating/drinking contaminated food/water (Allen, 2014; Allen et al., 2010; Baquero et al., 2008; Ghaly et al., 2017; Lupo et al., 2012; Rolain, 2013). In theory, all of these could be linked to VMP use. However, the actual significance of any of these exposure scenarios remains uncertain due to the lack of knowledge of the factors triggering transfer of ARGs in environmental bacteria and subsequent persistence/continued viability of the ARG and/or host bacteria once transferred under these scenarios. Furthermore, it is almost impossible to quantify the potential for opportunistic pathogens, which have been shown to thrive in soil (Johnning et al., 2013), to act as intermediary hosts of ARGs which they then transfer to human and veterinary pathogens at a later time. It is also recognised that VMPs select for AMR and that livestock production is associated with elevated environmental reservoirs of AMR (Magouras et al., 2017). Furthermore, environmental concentrations of several antibiotics used as VMPs are above the MSCs and it is possible to quantify the environmental exposure routes.

Recently, research papers have addressed the possibilities of conducting quantified assessments of exposure pathways for AMR in the environment (Ashbolt et al., 2013; Schmitt et al., 2017). Information on how to conduct such risk quantification is considered currently as lacking, given the number of knowledge gaps that need to be addressed to enable a proper risk assessment of AMR (Bengtsson-Palme, 2016). Some progress has, however, been made in addressing recognised knowledge gaps, as outlined in Table 3.

Table 3. Selected knowledge gaps hindering the assessment of risks associated with environmental AMR from VMP use (Adapted from Bengtsson-Palme (2016))

| OPEN QUESTION | SOME SUGGESTIONS |
|--|--|
| Where do horizontally transferrable resistance determinants emerge? | Polluted environments, sewage treatment plants, aquaculture, agriculture (Ashbolt et al., 2013; Berendonk et al., 2015). The spreading of manure is considered as most significant for VMPs. |
| What concentrations of antibiotics used in animals and other toxicants are selective for resistance? | Determination and predictions of minimal selective concentrations for antibiotics (Gullberg et al., 2014; Gullberg et al., 2011; Tello et al., 2012). |
| Which environments have the potential to drive resistance selection in bacterial communities? | Likely: animals given antibiotics, aquaculture, and application of manure to land. Possible: discharges from slaughter houses, sewage, sewage treatment plants, waste disposal, wastes from animal housing (Ashbolt et al., 2013; Larsson, 2014). |
| What roles do MGEs play in resistance development? | Transfer of resistance between bacteria, mobilisation of chromosomal resistance genes, rearrangement of existing resistance determinants (Stokes and Gillings, 2011). |
| What concentrations of antibiotics and other toxicants induce HGT? | Sub-inhibitory concentrations of antibiotics (Beaber et al., 2004; Prudhomme et al., 2006), few minimal concentrations determined (Jutkina et al., 2016). |
| What are the dissemination routes for resistance | Water bodies (Lupo et al., 2012; Pruden et al., |

| OPEN QUESTION | SOME SUGGESTIONS |
|---|--|
| genes to human and animal pathogens? | 2013), agriculture and food trade (EFSA/ECDC, 2013; Rolain, 2013). |
| Which dissemination routes from selective environments connect to environments with human and animal pathogens? | Water bodies and agriculture have large potential. |

The EU, international organisations and several national research councils are currently engaged in funding a range of activities to address the knowledge gaps on AMR in the environment. In particular, OIE, WHO, UK Natural Environment Research Council (NERC), the Biotechnology and Biological Sciences Research Council (BBSRC) and the Medical Research Council (MRC), as a cross-council initiative, have recently awarded large research grants and pump priming grants under the theme of 'understanding real world interactions'. This theme aims to address the need for a greater understanding of the role of the bacterial environment in influencing the evolution, acquisition and spread of AMR, and as a reservoir for resistance. The grants that have been awarded through this cross-council initiative include projects entitled 'Is AMR in the environment driven by dissemination of antibiotics or antibiotic resistance genes?' and 'Evaluating the threat of antimicrobial resistance in agricultural manures and slurry' (NERC, 2018). A previous NERC 'Environmental Microbiology and Human Health' call also funded a project on AMR ('Using next-generation sequencing to reveal human impact on aquatic reservoirs of antibiotic resistant bacteria at the catchment scale').

In addition to national research grants, the 'Joint Programming Initiative on Antimicrobial Resistance' (JPIAMR) is coordinating a partnership approach at EU level with the aim of pooling national research efforts to tackle the challenge of AMR more effectively. There are a number of grants that have been awarded that encompass the environment, examples of which include PREPARE ('Predicting the Persistence of Resistance Across Environments'), STARCS ('Selection and Transmission of Antimicrobial Resistance in Complex Systems') and three separate projects concerning AMR in wastewater (JPIAMR, 2018). As well as these commissioned projects, the JPIAMR has also provided funding to establish working groups to enhance alignment and maximise existing and future research efforts. One of these working groups is named 'Bridging the gap between exposure to AMR in the environment and impact to human health' and one of the outputs from this group will be to publish a defined toolbox of existing approaches, best practices for study protocols, and to identify research gaps.

Some authors have proposed the use of the concept of MSC as a quantitative indicator of the level of antibiotics necessary for the development of resistance. MSC is defined as the concentration at which the fitness cost of resistance is balanced by the antibiotic-conferred selection of the mutant and, according to *in vitro* data, usually corresponds to 1/4 to 1/230 of the MIC of the susceptible strains (depending on the antibiotic and type of mutation considered) (Gullberg et al., 2011). However, there are concerns regarding the ability to extrapolate results from *in vitro* competition experiments to the complexity of microbial communities in the environment. In a recent study, Bengtsson-Palme and Larsson (2016) used a theoretical method for the assignment of MSCs for 111 antibiotics based on observed lowest MIC values for target organisms available on the EUCAST ('European Committee on Antimicrobial Susceptibility Testing') database (EUCAST, 2018). This framework relies on the assumption that an antibiotic concentration that inhibits growth of some bacteria will have selective effects at the community level, and the values obtained correspond to the upper boundaries of the MSC for each compound. Subsequently, the predicted no effect concentrations (PNECs) for resistance selection in microbial communities were estimated by applying a flat assessment factor of 10 to each (to account for the difference between inhibitory concentration and selective concentration of antibiotics) for each calculated value of MSC. According to the authors, such values could be

considered as analogous to the lowest observed effect concentrations (LOECs) used in environmental risk assessment for different chemicals. Rico et al. (2017) proposed an alternative method to derive resistance thresholds based on theoretical MSC distributions derived from MIC data in a similar way as proposed by Bengtsson-Palme and Larsson (2016). The method provided by Rico et al. (2017) is based on probabilistic risk assessment theory and can be used to calculate the probability that antibiotic exposure concentrations result in antibiotic resistance at the community level. The method can be used to perform preliminary risk assessments in environmental compartments with measured or modelled exposure concentration distributions.

The overview of knowledge gaps presented above, together with the UK Department of Health 'AMR systems map' presented in Annex II, highlight that there is a conceptual understanding of the role of the environment with respect to AMR. The systems map details the numerous different inputs and transmission pathways that are likely contributing to the potentially ever-growing burden of AMR in clinical, animal and environmental settings. In addition, the AMR systems map comprehensively demonstrates how AMR is a highly complex problem that spans multiple sectors. Furthermore, as discussed in section 3.6 of the 2017 European Commission action plan against the rising threat from AMR (European Commission, 2017), there is a specific need to support research, develop tools and explore risk assessment methodologies to successfully understand and combat AMR.

10. Mitigation of AMR in the environment

Risk mitigation is an essential part of the evaluation of VMPs. Risk mitigation can be used to restrict the risks associated with a product to an acceptable level or even to completely remove such a risk. Further research is needed in order to estimate the exposures and risks associated with environmental pathways of antibiotics as VMPs that drive AMR in the environment. Nonetheless, certain management options might contribute to the reduction of these risks, acting synergistically with existing policies and goals. For example, Muurinen et al. (2017) studied the influence of manure application on the environmental resistome under Finnish agricultural practice with restricted antibiotic use. The publication reports that many genes spread from animals to the soil through manure application, but that these genes do not appear to persist beyond 12 months in the soil environment. This and similar studies suggest that practices that minimise or control the frequency of repeat spreading of manure from treated animals, as followed in Finland, may lead to lower levels of clinically relevant ARGs in agricultural soils.

It is worth noting that an EU-wide ban on the use of antibiotics as growth promoters in animal feed was introduced in 2006 (European Commission, 2005); Regulation [EC] No 1831/2003⁶), which has impacted on the overall use of antibiotics in the EU. Furthermore, the EMA has produced recommendations regarding the use of critically important antibiotics for human medicine in animals (e.g., fluoroquinolones, colistin (EMA/AMEG, 2016) and 3rd- and 4th-generation cephalosporins (EMA/CVMP/SAGAM, 2009)).

In this context, mitigation measures should aim to reduce the input of antibiotics into environmental compartments. For VMPs, this can be done by:

- reducing the quantities of antimicrobials prescribed/used (e.g. prudent use and enhancing animal welfare),
- reducing the release of antimicrobials to the environment by establishing effective barriers (e.g. avoid the release of urine and faeces from antibiotic-treated animals into aquatic environments for a determined timespan) and by

⁶ Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition (OJ L 268, 18.10.2003, p. 29–43)

- setting up systems and best practice guidelines to correctly dispose medicines in line with Article 117 of Regulation (EU) 2019/6.

Considering the first bullet point, the RONAFA paper, developed by the European Food Safety Authority (EFSA) and CVMP (EMA/EFSA, 2017), already describes different strategies in relation with the reduction of use of antimicrobials. The application of manure as a fertiliser to agricultural soils is expected to be a relevant vehicle for the dissemination of antibiotics and ARB into the environment and could, therefore, be a target for intervention. Manure is often stored prior to its application to land. Degradation of residues occurs for some antibiotics (to different extents according to the bacterial species), but not others. Further work is needed to determine the best methods of storing manure such that the efficiency of manure treatment to reduce the levels of antibiotics and ARGs is maximised. This is particularly relevant as certain antibiotics have been shown to have long elimination half-lives in manure, for example 100 days in the case of tetracyclines (Chee-Sanford et al., 2009). So, if the rate of application of contaminated manure exceeds the degradation of the antibiotics, then net accumulation is expected. One possibility could be to optimise the process of anaerobic digestion of liquid manure with a focus on eliminating ARs, a process already used for waste management with associated methane/biogas production. It should, however, be investigated if the digestion tanks themselves provide a novel source of AMR, since the conditions in the tanks are likely to support AMR development. Methods that improve nutrient reuse such as phosphate recycling from sewage sludge and other manure treatment systems like biogas production or nitrogen removal (van der Meersche et al., 2019) could, after optimisation, also contribute to lessen the amount of antibiotics spread onto agricultural land.

Finally, there is clear scope to couple environmental management of manure to show the positive impact that interventions have already delivered in terms of reducing release of antimicrobials into the environment (Pruden et al., 2013). This is evidenced in Table 4 for optimising antibiotic use and minimising impacts from antimicrobials present in animal manures.

Table 4. Management options for reducing the release of antibiotics and ARGs from manure to the environment (based on recommendations by Pruden et al. (2013))

| Optimising antibiotic use | Maintaining good animal health | Alternatives to antibiotics |
|--|---|--|
| Limiting the use of antimicrobials (especially critically important compounds) Banning the use of antibiotics as growth promoters* | Optimising management practices by: <ul style="list-style-type: none"> - reducing animal density - improving nutritional status | Developing better vaccines and vaccination programmes |
| Management of manure containing antibiotics | Biological treatment of ARGs in manure | Containment of ARGs in manure |
| Composting can eliminate 50–70% of certain antibiotics Watering, aeration and turning of compost can accelerate decay of some antibiotics Fermentation is more effective at removing other antibiotics | Response to biological treatments varies greatly | Prevention of lagoon spills and seepage Control of surface runoff Improved manure collection Long-term manure storage Manure separation Limiting sediment erosion and |

| Optimising antibiotic use | Maintaining good animal health | Alternatives to antibiotics |
|---------------------------|--------------------------------|-----------------------------|
| | | transport from animal farms |

* Growth promoters are banned in the EU since 2006 on the grounds that such use could potentially lead to the unnecessary development of AMR.

11. Conclusions

This reflection paper aimed to consider how the presence of ARs and ARGs in the environment might impact ecosystems as well as animal and human health.

Assessing the risks of AMR in the environment due to the use of antimicrobials in veterinary medicine towards human and animal health is challenging given the complexity of the problem and the paucity of knowledge regarding the mechanisms and pathways involved at the genetic, cellular and population levels. In addition, the lack of understanding regarding the role that the receiving environment has on the fate of ARs, ARBs and ARGs is a significant knowledge gap. Nevertheless, on reflection, there is a growing body of work demonstrating that the use of antibiotics in veterinary (and human) medicine contributes to environmental reservoirs of ARBs or ARGs, which facilitates the transfer of MGE either directly or indirectly to humans and animals. In this context, the difficulty in identifying the specific source of ARs or ARGs is important to note, as analogues of compounds exist between human and veterinary medicines. Therefore, it is problematic to attribute a single source as the major origin of the contamination (e.g. wastewater versus manure discharge) and, in particular, to disentangle the input from human versus veterinary antimicrobials. Consequently, the contribution of VMPs to the environmental resistome and the subsequent impact on human and animal health is difficult to quantify at present.

In conclusion, there is currently limited information to propose a methodology to modify the current ERA for VMPs to consider also the risks posed by the accumulation of ARs and ARGs in the environment from the use of these VMPs. However, the CVMP will continue to explore the development of improved or alternative risk assessment methodologies, with the support of other national and EU scientific agencies and bodies, to assess if improvements can be made. In particular, some areas for consideration are noted below:

A 'One Health' approach should be taken to minimise environmental contamination with ARs and ARBs/ARGs

In the EU, a greater volume of antimicrobials is sold to treat diseases in animal husbandry than for use in human medicine (ECDC/EFSA/EMA, 2017). Although some of those will be naturally degraded or transformed, there is a growing body of work demonstrating that the use of antibiotics in veterinary (and human) medicine contributes to environmental reservoirs of ARGs that can directly or indirectly drive the transference of MGEs to humans and animals.

The WHO global action plan on AMR (WHO, 2015) included the environment in the 'One Health' approach. The JPIAMR takes the starting point that the holistic assessment of the contributions of pollution on the environment with antibiotics, ARs and resistant bacteria is a necessity.

The development of strategies to minimise environmental contamination by antimicrobials and resistant bacteria is one of the priorities of the 'One Health' approach. Therefore, any measure(s) taken should follow this approach, in which the reduction of the exposure of the environment to substances with antimicrobial properties is not limited to one regulatory arena only, but all relevant sources of antimicrobial contamination are to be considered and reduced as much as possible.

Assessment of the risk for the environment from the authorisation of antimicrobial VMPs

Consideration of AMR in the environment, in the context of an MAA for a VMP, might have an impact on ecosystems as well as animal and human health. Due to the interdisciplinary nature of the problem, the CVMP acknowledges that any future changes to the risk assessment, if needed, would likely span parts 3 and 4 of the dossier.

The CVMP will continue to monitor for new data/approaches/technologies which could be used to improve the current risk assessment process, especially regarding the identification of potential hazards, risks and risk management measures. Specifically, there is a need to monitor scientific developments and gain a better understanding of:

- The emerging area of using MSCs to determine risks posed by ARs. Additional data are due to be published on determining MSCs in complex microbial communities.
- The level and duration of excretion of ARBs and ARGs from treated and untreated animals as well as transfer of ARGs between animals via the environment.
- The fate of ARGs in manure, together with the fate of antibiotics. This gap in our knowledge concerns whether there is an increase or decrease of ARGs during manure storage, and the main factors affecting this. Risk mitigation measure(s) (e.g. relating to storage of manure) could subsequently be developed.
- The impact of environmental AMR on ecosystems and human/animal health.
- The identification of relevant resistance determinants (e.g. on MGEs) and determination of antimicrobials concentrations that select for them. It is noted that the generation of such data is already a requirement for new active substances undergoing the application process for a new MA.
- The identification of environmental properties and environmental exposure profiles both for the antimicrobials and the ARGs.

As a result, it is acknowledged that there is a need to build expertise in regulatory agencies on approaches to evaluate the risks to the environment emanating from AMR and the ensuing risks to animal and public health.

Risk management measures to be applied in general to limit environmental contamination by ARs and ARGs

From a public and animal health perspective, it is a priority to use antimicrobials in an optimal way, to treat a disease effectively, but also to reduce any unnecessary consumption of antimicrobials and their subsequent release into the environment. Measures taken and proposed that promote the prudent use of antibiotics will also reduce the amount of ARs entering the environment. The detailed EMA/EFSA recommendations on how to reduce the need to use antimicrobials in food-producing animals (RONAFA) (EMA/EFSA, 2017) should be implemented as far as possible to reduce the use of antimicrobials in animals, and, as a result, exposure of the environment to those antimicrobials.

In conclusion, the field of environmental AMR is a rapidly evolving scientific discipline and new insights and findings are published almost weekly. The EMA/CVMP will continue to monitor scientific developments in this area and, as the science evolves and improved or alternative assessment methodologies are developed, the need to revise the current approach to environmental risk assessment for VMPs containing antimicrobials will be further considered.

Annex I

Mechanisms of AMR

Development of AMR

Some bacteria are innately resistant to certain types of antibiotics. However, bacteria may also develop antimicrobial resistance in two ways: either by a genetic mutation or by the acquisition or resistance from another bacterium (which could be from the same or a different species). Most antimicrobial substances have an environmental origin and are produced by microorganisms that protect themselves from threats by other organisms. In time, some of these prokaryotic organisms (certain types of bacteria) have developed a resistance against these antimicrobial substances through mutation of their genes. Resistance can appear spontaneously as a consequence of random mutations, more commonly following gradual build-up over time or due to the presence of antimicrobials (Geenen et al., 2011). Thus, the presence of ARGs causes the bacteria to be resistant to antimicrobial substances. The transfer of these AMR genes may take place from parent to offspring bacteria but may also take place from one bacterial species to another different species. When AMR genes are transferred from one bacterium to another bacterium that causes illness in humans or animals, the presence of these AMR genes may cause treatment failure in the human or animal patient or animal.

Selection and spread of AMR

Exposure of bacteria to antimicrobial substances is a known driver of AMR and encourages selection of antimicrobial resistance genes. Antimicrobial substances include antibiotics used in human and veterinary health care as well as disinfectants used in cosmetics (e.g. triclosan) and in biocides (hand and surface disinfection). Other compounds, like metals (e.g. copper, zinc and silver) are also known to elicit co-selection for AMR genes and thus are attributed to play a role in the development and spread of AMR.

Figure A1. Possible effects of antimicrobials on antimicrobial resistance in the environment (Schmitt et al., 2017)

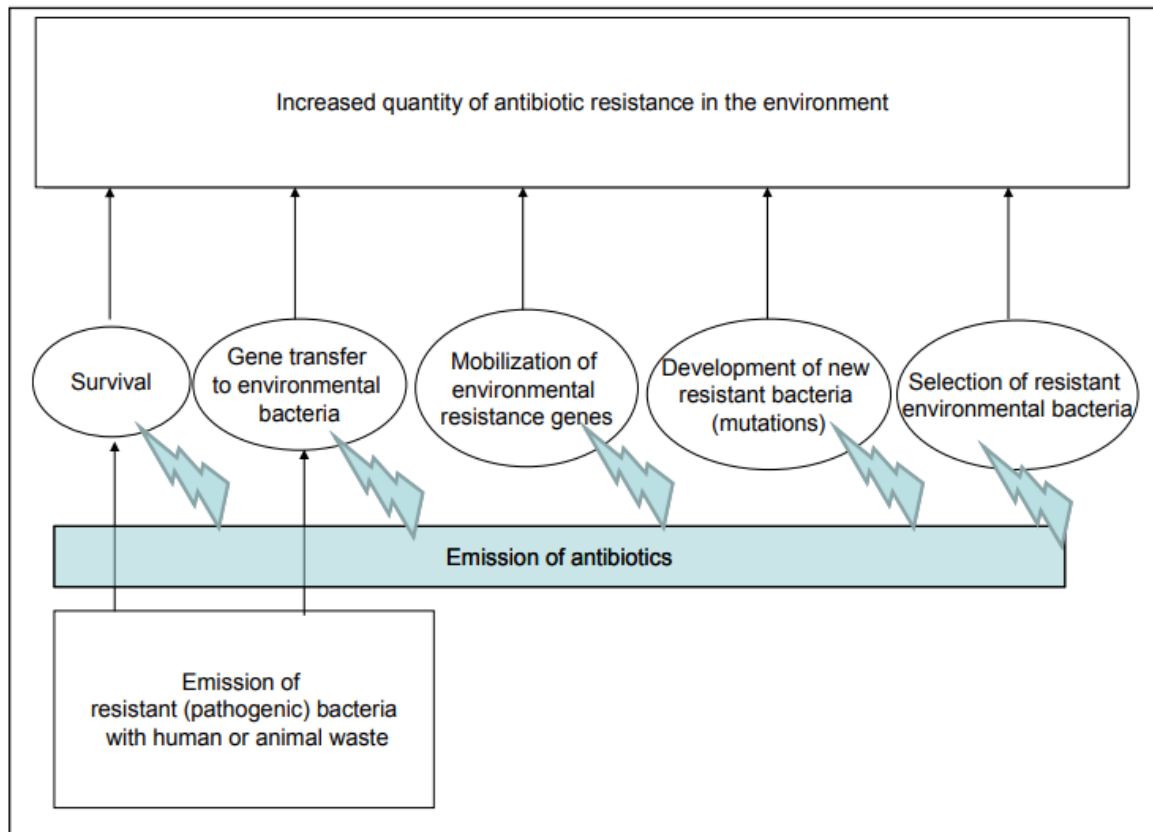


Figure A1 depicts the possible spread of resistant microorganisms following the release of ARBs in the presence of an antimicrobial-selective pressure. Potential releases may originate from manure or wastewater treatment plants (WWTPs). Irrespective of the type of release, the presence of antimicrobials in the environment in biologically relevant concentrations can select for resistant microorganisms. Although there is a relatively clear picture of how AMR develops and spreads in hospitals, the pathways that act via environmental matrices are not well understood (Berkner et al., 2014).

Berkner et al. (2014) suggested that several environmental hotspots with the potential for the development and spread of AMR have been identified so far, for instance biofilms, certain sediments, treated effluents and sewage sludge (human medicines), pharmaceutical production sites, aquaculture facilities, liquid manure tanks and soil repeatedly fertilised with manure. In particular, it was proposed that intestinal bacteria from livestock treated with antibiotics might survive in manure storage facilities only to be directly transmitted onto land. A further example involves locations such as biogas production units, where large numbers of microorganisms under favourable nutrient conditions are exposed to antibiotic concentrations that can select for resistance. In addition, manure contains metal ions from animal feed and biocides from the disinfection of livestock housing that are implicated in co-selecting for resistance or that could enhance mutation frequencies that may lead to the development of resistance.

The influence of metals on the selection and spread of AMR

The environmental conditions in which a bacterium resides can have a significant effect on its potential to develop or acquire ARGs. The multitude of unique factors and stressors at play in the wider environment (soils, sediments, water) create a complex arena in which AMR can develop and persist.

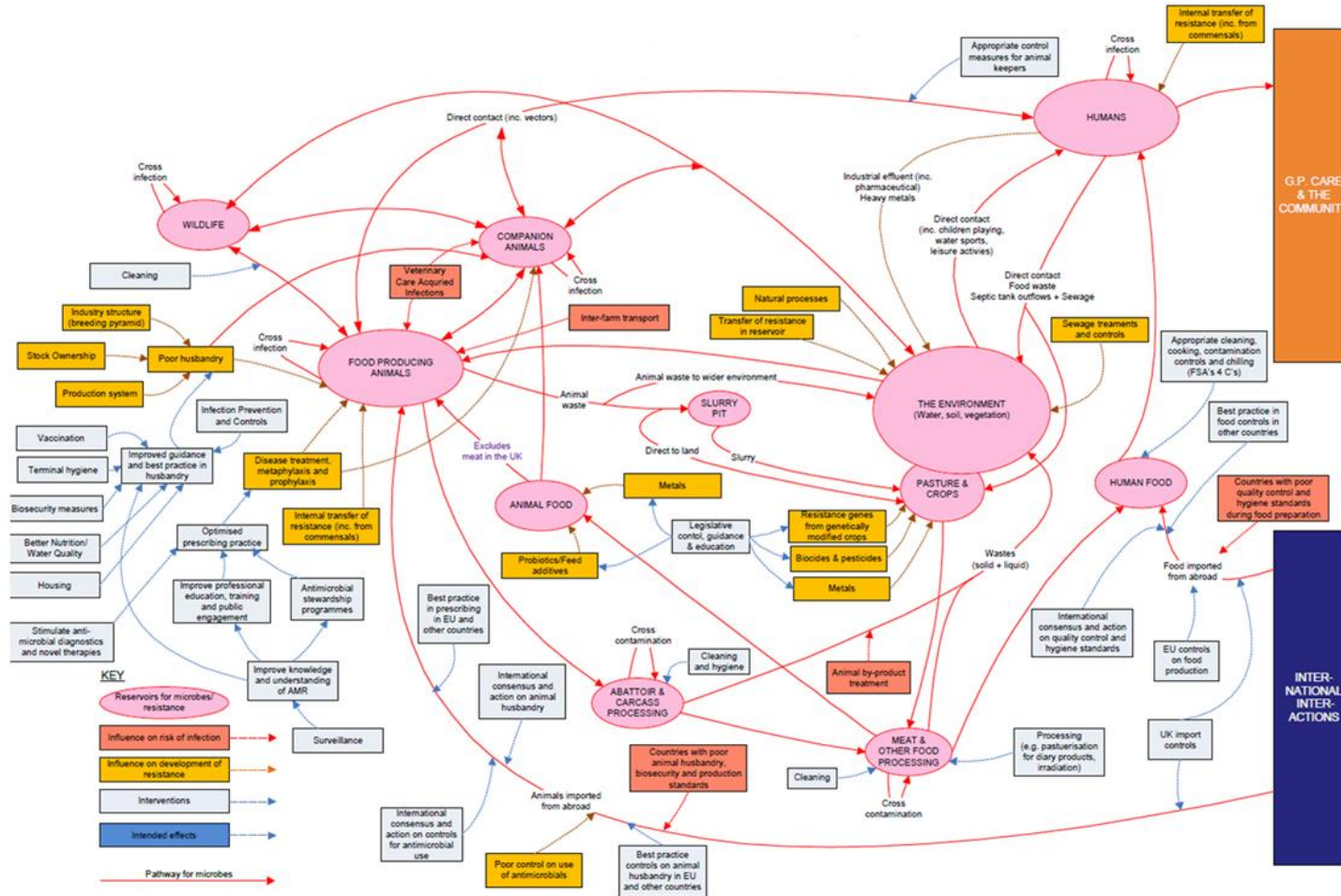
Heavy metals are one such set of stressors that are commonly found in the environment and have long been implicated in the development, persistence, and spread of AMR. This is because of their ability to co-select for ARGs (Poole, 2017).

A recent review conducted by Poole (2017) summarises the knowledge regarding the influence of zinc (Zn) and copper (Cu) on AMR development and spread:

- Metals provide a selective pressure for metal resistance which can, in turn, co-select for AMR due to physical genetic linkages between genes.
- Concentrations of Cu in the environment have been shown to correlate with an increased occurrence of ARGs and MGEs in environmental bacteria.
- The use of Zn and Cu in veterinary medicine has been linked to the development and persistence of resistant organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA) and MDR *E. coli* and *Salmonella* spp.
- Cu and Zn can promote biofilm formation in certain organisms and also promote the induction of dormant persistence states in a number of Gram-negative bacteria.
- Cu and Zn have been shown to inhibit as well as synergistically enhance a number of antibiotic compounds.

Annex II

UK Department of Health 'AMR Systems Map' (Dobra et al., 2014)



This systems map shows the influences on the development of AMR in humans, animals and the environment.

12. Glossary

| | |
|-------|--|
| 3GCs | 3 rd -generation cephalosporins |
| ADME | Absorption, distribution, metabolism and excretion |
| AMR | Antimicrobial resistance |
| API | Active pharmaceutical ingredient |
| ARBs | Antibiotic resistant bacteria |
| ARGs | Antimicrobial resistance genes |
| ARs | Antimicrobial residues |
| AWP | Antimicrobials Working Party |
| CVMP | Committee for Medicinal Products for Veterinary Use |
| ECDC | European Centre for Disease Prevention and Control |
| EFSA | European Food Safety Authority |
| EMA | European Medicines Agency |
| ERA | Environmental risk assessment |
| ERAWP | Environmental Risk Assessment Working Party |
| ESBL | Extended Spectrum Beta-Lactamases |
| ESVAC | European Surveillance of Veterinary Antimicrobial Consumption |
| FAO | Food and Agriculture Organisation |
| GIT | Gastro-intestinal tract |
| GL | Guideline |
| HGT | Horizontal gene transfer |
| LOECs | Lowest observed effect concentrations |
| MA | Marketing authorisation |
| MAAs | Marketing authorisation applications |
| MGEs | Mobile genetic elements |
| MRL | Maximum residue limit |
| MRSA | Methicillin-resistant <i>Staphylococcus aureus</i> |
| MSC | Minimal selective concentration assays |
| NDM | New Delhi metallo-beta-lactamase |
| OIE | World Organisation for Animal Health |
| PNECs | Predicted no effect concentrations |
| VICH | International Cooperation on Harmonisation of Technical Requirements for |

Registration of Veterinary Medicinal Products

| | |
|-------|-------------------------------|
| VMPs | Veterinary medicinal products |
| WHO | World Health Organisation |
| WWTPs | Wastewater treatment plants |

13. References

- Aarestrup, F.M., 'Veterinary drug usage and antimicrobial resistance in bacteria of animal origin', *Basic & Clinical Pharmacology & Toxicology*, Vol. 96 (4), 2005, pp. 271–281.
- Alavi, N., A.A. Babaei, M. Shirmardi, A. Naimabadi and G. Goudarzi, 'Assessment of oxytetracycline and tetracycline antibiotics in manure samples in different cities of Khuzestan Province, Iran', *Environmental Science and Pollution Research*, Vol. 22 (22), 2015, pp. 17948–17954.
- Alekshun, M.N. and S.B. Levy, 'Molecular mechanisms of antibacterial multidrug resistance', *Cell*, Vol. 128 (6), 2007, pp. 1037–1050.
- Allen, H.K., 'Antibiotic resistance gene discovery in food-producing animals', *Current Opinion in Microbiology*, Vol. 19 2014, pp. 25–29.
- Allen, H.K., J. Donato, H.H. Wang, K.A. Cloud-Hansen, J. Davies and J. Handelsman, 'Call of the wild: antibiotic resistance genes in natural environments', *Nature Reviews Microbiology*, Vol. 8 (4), 2010, pp. 251–259.
- Aminov, R.I., 'The role of antibiotics and antibiotic resistance in nature', *Environmental Microbiology*, Vol. 11 (12), 2009, pp. 2970–2988.
- Aminov, R.I., 'Horizontal gene exchange in environmental microbiota', *Frontiers in Microbiology*, Vol. 2 (158), 2011, pp. 1–19.
- Andersson, D.I. and D. Hughes, 'Persistence of antibiotic resistance in bacterial populations', *FEMS Microbiology Reviews*, Vol. 35 (5), 2011, pp. 901–911.
- Andersson, D.I. and D. Hughes, 'Microbiological effects of sublethal levels of antibiotics', *Nature Reviews Microbiology*, Vol. 12 (7), 2014, pp. 465–478.
- Antunes-Lopes, T., L. Vale, A.M. Coelho, C. Silva, M. Rieken, B. Geavlete, T. Rashid, S.M. Rahnama'i, J.N. Cornu, T. Marcelissen and E.A.U. Young Academic Urologists (YAU) Functional Urology Working Group, 'The role of urinary microbiota in lower urinary tract dysfunction: a systematic review', *European Urology Focus*, Vol. 6 (2), 2020, pp. 361–369.
- Ashbolt, N.J., A. Amézquita, T. Backhaus, P. Borriello, K.K. Brandt, P. Collignon, A. Coors, R. Finley, W.H. Gaze and T. Heberer, 'Human health risk assessment (HHRA) for environmental development and transfer of antibiotic resistance', *Environmental Health Perspectives (Online)*, Vol. 121 (9), 2013, pp. 993–1001.
- Baquero, F., J.-L. Martínez and R. Cantón, 'Antibiotics and antibiotic resistance in water environments', *Current Opinion in Biotechnology*, Vol. 19 (3), 2008, pp. 260–265.
- Baron, S., E. Jouy, F. Touzain, S. Bougeard, E. Larvor, C. de Boisseson, M. Amelot, A. Keita and I. Kempf, 'Impact of the administration of a third-generation cephalosporin (3GC) to one-day-old chicks on the persistence of 3GC-resistant *Escherichia coli* in intestinal flora: an in vivo experiment', *Veterinary Microbiology*, Vol. 185 2016, pp. 29–33.
- Beaber, J.W., B. Hochhut and M.K. Waldor, 'SOS response promotes horizontal dissemination of antibiotic resistance genes', *Nature*, Vol. 427 (6969), 2004, pp. 72–74.
- Bengtsson-Palme, J., 'Antibiotic resistance in the environment: a contribution from metagenomic studies', *Sahlgrenska Academy, University of Gothenburg, Gothenburg (Sweden)*, 2016.
<http://hdl.handle.net/2077/41843>.
- Bengtsson-Palme, J., E. Kristiansson and D.G.J. Larsson, 'Environmental factors influencing the development and spread of antibiotic resistance', *FEMS Microbiology Reviews*, Vol. 42 (1), 2018, pp. 68–80.

Bengtsson-Palme, J. and D.G.J. Larsson, 'Concentrations of antibiotics predicted to select for resistant bacteria: proposed limits for environmental regulation', *Environment International*, Vol. 86 2016, pp. 140–149.

Benveniste, R. and J. Davies, 'Mechanisms of antibiotic resistance in bacteria', *Annual Review of Biochemistry*, Vol. 42 1973, pp. 471–506.

Berendonk, T.U., C.M. Manaia, C. Merlin, D. Fatta-Kassinos, E. Cytryn, F. Walsh, H. Bürgmann, H. Sørum, M. Norström and M.-N. Pons, 'Tackling antibiotic resistance: the environmental framework', *Nature Reviews Microbiology*, Vol. 13 (5), 2015, pp. 310–317.

Berkner, S., S. Konradi and J. Schönfeld, 'Antibiotic resistance and the environment—There and back again', *EMBO Reports*, Vol. 15 (7), 2014, pp. 740–744.

Beyer, A., S. Baumann, G. Scherz, J. Stahl, M. von Bergen, A. Friese, U. Rösler, M. Kietzmann and H. W., 'Effects of ceftiofur treatment on the susceptibility of commensal porcine *E. coli*—Comparison between treated and untreated animals housed in the same stable', *BMC veterinary research*, Vol. 11 (265), 2015, pp. 1–13.

Bibbal, D., V. Dupouy, J.-P. Ferré, P.-L. Toutain, O. Fayet, M.-F. Prère and A. Bousquet-Mélou, 'Impact of three ampicillin dosage regimens on selection of ampicillin resistance in *Enterobacteriaceae* and excretion of blaTEM genes in swine feces', *Applied and Environmental Microbiology*, Vol. 73 (15), 2007, pp. 4785–4790.

Bibbal, D., V. Dupouy, M.-F. Prère, P.-L. Toutain and A. Bousquet-Mélou, 'Relatedness of *Escherichia coli* strains with different susceptibility phenotypes isolated from swine feces during ampicillin treatment', *Applied and Environmental Microbiology*, Vol. 75 (10), 2009, pp. 2999–3006.

Blaak, H., A.H.A.M. van Hoek, C. Veenman, A.E.D. van Leeuwen, G. Lynch, W.M. van Overbeek and A.M. de Roda Husman, 'Extended spectrum β -lactamase-and constitutively AmpC-producing *Enterobacteriaceae* on fresh produce and in the agricultural environment', *International Journal of Food Microbiology*, Vol. 168–169 2014, pp. 8–16.

Bobay, L.-M. and H. Ochman, 'The evolution of bacterial genome architecture', *Frontiers in Genetics*, Vol. 8 2017, pp. 72.

Cabello, F.C., H.P. Godfrey, A. Tomova, L. Ivanova, H. Dölz, A. Millanao and A.H. Buschmann, 'Antimicrobial use in aquaculture re-examined: its relevance to antimicrobial resistance and to animal and human health', *Environmental microbiology*, Vol. 15 (7), 2013, pp. 1917–1942.

Cabello, F.C., A. Tomova, L. Ivanova and H.P. Godfrey, 'Aquaculture and mcr colistin resistance determinants', *mBio*, Vol. 8 (5), 2017, pp. e01229–01217.

Campagnolo, E.R., K.R. Johnson, A. Karpati, C.S. Rubin, D.W. Kolpin, M.T. Meyer, J.E. Esteban, R.W. Currier, K. Smith and K.M. Thu, 'Antimicrobial residues in animal waste and water resources proximal to large-scale swine and poultry feeding operations', *Science of the Total Environment*, Vol. 299 (1), 2002, pp. 89–95.

Cantas, L., S. Shah, L. Cavaco, C. Manaia, F. Walsh, M. Popowska, H. Garelick, H. Bürgmann and H. Sørum, 'A brief multi-disciplinary review on antimicrobial resistance in medicine and its linkage to the global environmental microbiota', *Frontiers in Microbiology*, Vol. 4 2013, pp. 1–14.

Caruso, G., 'Antibiotic resistance in fish farming environments: a global concern', *Journal of Fisheries Sciences.com*, Vol. 10 (4), 2016, pp. 9–13.

Cavaco, L.M., H. Hasman, M. Stegger, P.S. Andersen, R. Skov, A.C. Fluit, T. Ito and A. F.M., 'Cloning and occurrence of *czrC*, a gene conferring cadmium and zinc resistance in methicillin-resistant *Staphylococcus aureus* CC398 isolates', *Antimicrobial Agents and Chemotherapy*, Vol. 54 (9), 2010, pp. 3605–3608.

Cernat, R., C. Balotescu, D. Ivanescu, D. Nedelcu, V. Lazar, M. Bucur, D. Valeanu, R. Tudorache, M. Mitache and M. Dragoescu, 'P1024 Mechanisms of resistance in multiple-antibiotic-resistant *Escherichia*

coli strains isolated from drinking and recreational, salmaster waters', International Journal of Antimicrobial Agents, Vol. 29 (Supplement 2), 2007, pp. S274.

Chander, Y., K. Kumar, S.M. Goyal and S.C. Gupta, 'Antibacterial activity of soil-bound antibiotics', Journal of Environmental Quality, Vol. 34 (6), 2005, pp. 1952–1957.

Chee-Sanford, J.C., R.I. Mackie, S. Koike, I.G. Krapac, Y.-F. Lin, A.C. Yannarell, S. Maxwell and R.I. Aminov, 'Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste', Journal of Environmental Quality, Vol. 38 (3), 2009, pp. 1086–1108.

Chen, Y.S., H.B. Zhang, Y.M. Luo and J. Song, 'Occurrence and assessment of veterinary antibiotics in swine manures: a case study in East China', Chinese Science Bulletin, Vol. 57 (6), 2012, pp. 606–614.

Cleary, D.W., A.H. Bishop, L. Zhang, E. Topp, E.M.H. Wellington and W.H. Gaze, 'Long-term antibiotic exposure in soil is associated with changes in microbial community structure and prevalence of class 1 integrons', FEMS Microbiology Ecology, Vol. 92 (10), 2016, pp. fiw159.

Cooper, R.M., L. Tsimring and J. Hasty, 'Inter-species population dynamics enhance microbial horizontal gene transfer and spread of antibiotic resistance', eLife, Vol. 6 2017, pp. e25950.

CVMP/VICH, 'VICH GL6: Environmental impact assessment (EIAS) for veterinary medicinal products – Phase I' (CVMP/VICH/592/98-FINAL), European Medicines Evaluation Agency (EMA), London (UK), 2000.

http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/10/WC500004394.pdf.

CVMP/VICH, 'VICH GL27: Guidance on pre-approval information for registration of new veterinary medicinal products for food producing animals with respect to antimicrobial resistance ' (CVMP/VICH/644/01-FINAL), European Medicines Evaluation Agency (EMA), London (UK), 2004a.

http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/10/WC500004308.pdf.

CVMP/VICH, 'VICH GL38: Environmental impact assessments for veterinary medicinal products (VMPs) – Phase II' (CVMP/VICH/790/03-FINAL), European Medicines Agency (EMA), London (UK), 2004b.

http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/10/WC500004393.pdf.

D'Costa, V.M., C.E. King, L. Kalan, M. Morar, W.W.L. Sung, C. Schwarz, D. Froese, G. Zazula, F. Calmels, R. Debruyne, G.B. Golding, H.N. Poinar and G.D. Wright, 'Antibiotic resistance is ancient', Nature, Vol. 477 (7365), 2011, pp. 457–461.

D'Costa, V.M., K.M. McGrann, D.W. Hughes and G.D. Wright, 'Sampling the antibiotic resistome', Science, Vol. 311 (5759), 2006, pp. 374–377.

Davies, J. and D. Davies, 'Origins and evolution of antibiotic resistance', Microbiology and Molecular Biology Reviews, Vol. 74 (3), 2010, pp. 417–433.

De Liguoro, M., V. Cibir, F. Capolongo, B. Halling-Sørensen and C. Montesissa, 'Use of oxytetracycline and tylosin in intensive calf farming: evaluation of transfer to manure and soil', Chemosphere, Vol. 52 (1), 2003, pp. 203–212.

De Smet, J., S. Croubels, P. De Backer and M. Devreese, 'Effect of administration route and dose alteration on sulfadiazine-trimethoprim plasma and intestinal concentrations in pigs', International Journal of Antimicrobial Agents, Vol. 50 (6), 2017, pp. 707–714.

DEFRA, 'Fertiliser manual (RB209), 8th edition', Department for Environment, Food and Rural Affairs (DEFRA), The Stationery Office (TSO), Norwich (UK), 2010.

Denet, E., B. Coupat-Goutaland, S. Nazaret, M. Pélandakis and S. Favre-Bonté, 'Diversity of free-living amoebae in soils and their associated human opportunistic bacteria', Parasitology Research, Vol. 116 (11), 2017, pp. 3151–3162.

Dobra, S., P. Johnston, M. Baugh, C. Barfoot, P. Bennett, S. Eckford and G. Chappelle, 'Antimicrobial resistance (AMR) systems map. Overview of the factors influencing the development of AMR and the interactions between them', Department of Health (DH), Public Health England (PHE), Department for Environment Food & Rural Affairs (DEFRA), Veterinary Medicines Directorate (VMD), London (UK), 2014.

https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/387746/Microbial_Maps.pdf.

Dodd, M.C. and C.-H. Huang, 'Aqueous chlorination of the antibacterial agent trimethoprim: reaction kinetics and pathways', *Water Research*, Vol. 41 (3), 2007, pp. 647–655.

Dolliver, H.A.S. and S.C. Gupta, 'Antibiotic losses from unprotected manure stockpiles', *Journal of Environmental Quality*, Vol. 37 (3), 2008, pp. 1238–1244.

ECDC/EFSA/EMA, 'ECDC/EFSA/EMA second joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals. Joint interagency antimicrobial consumption and resistance analysis (JIACRA) report', *EFSA Journal*, Vol. 15 (7), 2017, pp. 4872.

ECDC/EFSA/EMA/SCENIHR, 'Joint opinion on antimicrobial resistance (AMR) focused on zoonotic infections', *EFSA Journal*, Vol. 7 (11), 2009, pp. 1372.

ECDC/EMA, 'ECDC/EMA joint technical report. The bacterial challenge: time to react. A call to narrow the gap between multidrug-resistant bacteria in the EU and the development of new antibacterial agents', European Centre of Disease Prevention and Control (ECDC), European Medicines Agency (EMA), Stockholm (Sweden), 2009.

http://www.ecdc.europa.eu/en/publications/Publications/0909_TER_The_Bacterial_Challenge_Time_to_React.pdf.

EFSA/ECDC, 'The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2011', *EFSA Journal*, Vol. 11 (5), 2013, pp. 3196.

Elbashir, S., S. Parveen, J. Schwarz, T. Rippen, M. Jahncke and A. DePaola, 'Seafood pathogens and information on antimicrobial resistance: a review', *Food Microbiology*, Vol. 70 2018, pp. 85–93.

Elizalde-Velázquez, A., L.M. Gómez-Oliván, M. Galar-Martínez, H. Islas-Flores, O. Dublán-García and N. SanJuan-Reyes, 'Amoxicillin in the aquatic environment, its fate and environmental risk'. In: *Environmental Health Risk: Hazardous Factors to Living Species*, M.L. Larramendy and S. Soloneski (eds.). InTech, London (UK), 2016, pp.

EMA, 'European public MRL assessment report for tulathromycin', European Medicines Agency (EMA), London (UK), 2015. https://www.ema.europa.eu/documents/mrl-report/tulathromycin-modification-microbiological-adi-mrls-bovine-porcine-species-after-provisional-maximum_en.pdf.

EMA/AMEG, 'Updated advice on the use of colistin products in animals within the European Union: development of resistance and possible impact on human and animal health', European Medicines Agency (EMA), London (UK), 2016.

http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2016/05/WC500207233.pdf.

EMA/CVMP, 'Reflection paper on the risk of antimicrobial resistance transfer from companion animals', European Medicines Agency (EMA), London (UK), 2015.

http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2015/01/WC500181642.pdf.

EMA/CVMP, 'CVMP strategy on antimicrobials 2016–2020', European Medicines Agency (EMA), London (UK), 2016a.

http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2016/10/WC500214901.pdf.

EMA/CVMP, 'Guideline on environmental impact assessment for veterinary medicinal products in support of the VICH guidelines GL6 and GL38' (EMA/CVMP/ERA/418282/2005-Rev.1- Corr.), European

Medicines Agency (EMA), London (UK), 2016b.

http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/10/WC500004386.pdf.

EMA/CVMP, 'Guideline on the summary of product characteristics (SPC) for veterinary medicinal products containing antimicrobial substances. Draft' (EMA/CVMP/383441/2005-Rev.1), European Medicines Agency (EMA), London (UK), 2018. https://www.ema.europa.eu/en/documents/regulatory-procedural-guideline/draft-guideline-summary-product-characteristics-spc-veterinary-medicinal-products-containing_en.pdf.

EMA/CVMP, 'CVMP strategy on antimicrobials 2021–2025. Draft', European Medicines Agency (EMA), Amsterdam (The Netherlands), 2020. https://www.ema.europa.eu/en/documents/scientific-guideline/cvmp-strategy-antimicrobials-2021-2025_en.pdf.

EMA/CVMP/AWP, 'Guideline on the assessment of the risk to public health from antimicrobial resistance due to the use of an antimicrobial veterinary medicinal product in food-producing animals. Draft 2' (EMA/CVMP/AWP/706442/2013), European Medicines Agency (EMA), London (UK), 2018. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2015/03/WC500183774.pdf.

EMA/CVMP/SAGAM, 'Revised reflection paper on the use of 3rd and 4th generation cephalosporins in food producing animals in the European Union: development of resistance and impact on human and animal health', European Medicines Agency (EMA), London (UK), 2009. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/10/WC500004307.pdf.

EMA/EFSA, 'EMA and EFSA joint scientific opinion on measures to reduce the need to use antimicrobial agents in animal husbandry in the European Union, and the resulting impacts on food safety (RONAFA)', EFSA Journal, Vol. 15 (1), 2017, pp. 4666.

EMA/ESVAC, 'Sales of veterinary antimicrobial agents in 31 European countries in 2018. Trends from 2010 to 2018. Tenth ESVAC report.', European Medicines Agency (EMA), Amsterdam (The Netherlands), 2020. https://www.ema.europa.eu/en/documents/report/sales-veterinary-antimicrobial-agents-31-european-countries-2018-trends-2010-2018-tenth-esvac-report_en.pdf.

ESAC-Net, 'Antimicrobial consumption interactive database', European Centre for Disease Prevention and Control (ECDC), Stockholm (Sweden), 2020. <http://ecdc.europa.eu/en/healthtopics/antimicrobial-resistance-and-consumption/antimicrobial-consumption/esac-net-database/Pages/database.aspx> (accessed: 2020).

EUCAST, 'Clinical breakpoints', European Society of Clinical Microbiology and Infectious Diseases (ESCMID), Basel (Switzerland), 2018. http://www.eucast.org/clinical_breakpoints/ (accessed: 2018).

European Commission, 'Ban on antibiotics as growth promoters in animal feed enters into effect', European Commission, Brussels (Belgium), 2005. http://europa.eu/rapid/press-release_IP-05-1687_en.htm.

European Commission, 'Future brief: sustainable aquaculture', European Commission, Brussels (Belgium), 2015. http://ec.europa.eu/environment/integration/research/newsalert/pdf/sustainable_aquaculture_FB11_en.pdf.

European Commission, 'A European one health action plan against antimicrobial resistance (AMR)', European Commission, Brussels (Belgium), 2017. https://ec.europa.eu/health/sites/health/files/antimicrobial_resistance/docs/amr_2017_action-plan.pdf.

European Commission, 'Communication from the Commission to the European Parliament, the Council and the European Economic and Social Committee – European Union strategic approach to pharmaceuticals in the environment', European Commission, Brussels (Belgium), 2019.

FAO/WHO/OIE, 'Joint FAO/WHO/OIE expert meeting on critically important antimicrobials. Report of the FAO/WHO/OIE expert meeting. FAO headquarters, Rome, 26–30 November 2007', Food and Agriculture Organization of the United Nations (FAO), World Health Organization (WHO), World Organisation for Animal Health (OIE), Rome (Italy), 2008. <http://www.fao.org/3/a-i0204e.pdf>.

Ferreira da Silva, M., I. Vaz-Moreira, M. Gonzalez-Pajuelo, O.C. Nunes and C.M. Manaia, 'Antimicrobial resistance patterns in *Enterobacteriaceae* isolated from an urban wastewater treatment plant', *FEMS Microbiology Ecology*, Vol. 60 (1), 2007, pp. 166–176.

Finley, R.L., P. Collignon, D.G.J. Larsson, S.A. Mcewen, X.-Z. Li, W.H. Gaze, R. Reid-Smith, M. Timinouni, D.W. Graham and E. Topp, 'The scourge of antibiotic resistance: the important role of the environment', *Clinical Infectious Diseases*, Vol. 57 (5), 2013, pp. 704–710.

Fleury, M., G. Mourand, E. Jouy, F. Touzain, L. Le Devendec, C. de Boisseson, F. Eono, R. Cariolet, A. Guérin and O. Le Goff, 'Impact of ceftiofur injection on gut microbiota and *Escherichia coli* resistance in pigs', *Antimicrobial Agents and Chemotherapy*, Vol. 59 (9), 2015, pp. 5171–5180.

Forsberg, K.J., S. Patel, T.A. Wencewicz and G. Dantas, 'The tetracycline destructases: a novel family of tetracycline-inactivating enzymes', *Chemistry & Biology*, Vol. 22 (7), 2015, pp. 888–897.

Forsberg, K.J., S. Patel, E. Witt, B. Wang, T.D. Ellison and G. Dantas, 'Identification of genes conferring tolerance to lignocellulose-derived inhibitors by functional selections in soil metagenomes', *Applied and Environmental Microbiology*, Vol. 82 2016, pp. 528–537.

Forsberg, K.J., A. Reyes, B. Wang, E.M. Selleck, M.O.A. Sommer and G. Dantas, 'The shared antibiotic resistome of soil bacteria and human pathogens', *Science*, Vol. 337 (6098), 2012, pp. 1107–1111.

Geenen, P.L., M.G.J. Koene, H. Blaak, A.H. Havelaar and A.W. Van de Giessen, 'Risk profile on antimicrobial resistance transmissible from food animals to humans', National Institute for Public Health and the Environment (RIVM), Bilthoven (The Netherlands), 2011. <https://www.rivm.nl/bibliotheek/rapporten/330334001.pdf>.

Ghaly, T.M., L. Chow, A.J. Asher, L.S. Waldron and M.R. Gillings, 'Evolution of class 1 integrons: mobilization and dispersal via food-borne bacteria', *PLoS ONE*, Vol. 12 (6), 2017, pp. e0179169.

Gillings, M.R., 'Evolutionary consequences of antibiotic use for the resistome, mobilome and microbial pangenome', *Frontiers in Microbiology*, Vol. 4 (4), 2013, pp. DOI: 10.3389/fmicb.2013.00004.

Gillings, M.R., 'Lateral gene transfer, bacterial genome evolution, and the Anthropocene', *Annals of the New York Academy of Sciences*, Vol. 1389 (1), 2017, pp. 20–36.

Girardi, C., J. Greve, M. Lamshöft, I. Fetzer, A. Miltner, A. Schäffer and M. Kästner, 'Biodegradation of ciprofloxacin in water and soil and its effects on the microbial communities', *Journal of Hazardous Materials*, Vol. 198 2011, pp. 22–30.

Golet, E.M., A. Strehler, A.C. Alder and W. Giger, 'Determination of fluoroquinolone antibacterial agents in sewage sludge and sludge-treated soil using accelerated solvent extraction followed by solid-phase extraction', *Analytical Chemistry*, Vol. 74 (21), 2002, pp. 5455–5462.

Goñi-Urriza, M., M. Capdepuy, C. Arpin, N. Raymond, P. Caumette and C. Quentin, 'Impact of an urban effluent on antibiotic resistance of riverine *Enterobacteriaceae* and *Aeromonas* spp. ', *Applied and Environmental Microbiology*, Vol. 66 (1), 2000, pp. 125–132.

Gulkowska, A., H.W. Leung, M.K. So, S. Taniyasu, N. Yamashita, L.W. Yeung, B.J. Richardson, A. Lei, J.P. Giesy and P.K. Lam, 'Removal of antibiotics from wastewater by sewage treatment facilities in Hong Kong and Shenzhen, China', *Water Research*, Vol. 42 (1–2), 2008, pp. 395–403.

Gullberg, E., L.M. Albrecht, C. Karlsson, L. Sandegren and D.I. Andersson, 'Selection of a multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals', *mBio*, Vol. 5 (5), 2014, pp. 1–9.

Gullberg, E., S. Cao, O.G. Berg, C. Ilbäck, L. Sandegren, D. Hughes and D.I. Andersson, 'Selection of resistant bacteria at very low antibiotic concentrations', *PLoS Pathogens*, Vol. 7 (7), 2011, pp. e1002158.

Halling-Sørensen, B., G. Sengeløv, F. Ingerslev and L.B. Jensen, 'Reduced antimicrobial potencies of oxytetracycline, tylosin, sulfadiazin, streptomycin, ciprofloxacin, and olaquinox due to environmental processes', *Archives of Environmental Contamination and Toxicology*, Vol. 44 (1), 2003, pp. 7–16.

Hamscher, G., H.T. Pawelzick, H. Höper and H. Nau, 'Different behavior of tetracyclines and sulfonamides in sandy soils after repeated fertilization with liquid manure', *Environmental Toxicology and Chemistry*, Vol. 24 (4), 2005, pp. 861–868.

Hamscher, G., S. Sczesny, H. Höper and H. Nau, 'Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry', *Analytical Chemistry*, Vol. 74 (7), 2002, pp. 1509–1518.

Heuer, H., H. Schmitt and K. Smalla, 'Antibiotic resistance gene spread due to manure application on agricultural fields', *Current Opinion in Microbiology*, Vol. 14 (3), 2011, pp. 236–243.

Hiltunen, T., M. Virta and A.-L. Laine, 'Antibiotic resistance in the wild: an eco-evolutionary perspective', *Philosophical Transactions of the Royal Society B*, Vol. 372 (1712), 2017, pp. 20160039.

Hirsch, R., T. Ternes, K. Haberer and K.-L. Kratz, 'Occurrence of antibiotics in the aquatic environment', *Science of the Total Environment*, Vol. 225 (1), 1999, pp. 109–118.

Holman, D.B. and M.R. Chénier, 'Antimicrobial use in swine production and its effect on the swine gut microbiota and antimicrobial resistance', *Canadian Journal of Microbiology*, Vol. 61 (11), 2015, pp. 785–798.

Holmes, A., J. Govan and R. Goldstein, 'Agricultural use of *Burkholderia (Pseudomonas) cepacia*: a threat to human health?', *Emerging Infectious Diseases*, Vol. 4 (2), 1998, pp. 221–227.

Holzbauer, S. and T.M. Chiller, 'Antimicrobial resistance in bacteria of animal origin', *Emerging Infectious Diseases*, Vol. 12 (7), 2006, pp. 1180–1181.

Hölzel, C.S., K.S. Harms, H. Küchenhoff, A. Kunz, C. Müller, K. Meyer, K. Schwaiger and J. Bauer, 'Phenotypic and genotypic bacterial antimicrobial resistance in liquid pig manure is variously associated with contents of tetracyclines and sulfonamides', *Journal of Applied Microbiology*, Vol. 108 (5), 2010, pp. 1642–1656.

Hughes Martiny, J.B., B.J.M. Bohannan, J.H. Brown, R.K. Colwell, J.A. Fuhrman, J.L. Green, M.C. Horner-Devine, M. Kane, J.A. Krumins and C.R. Kuske, 'Microbial biogeography: putting microorganisms on the map', *Nature Reviews Microbiology*, Vol. 4 (2), 2006, pp. 102–112.

Huijbers, P.M.C., H. Blaak, M.C.M. de Jong, E.A.M. Graat, C.M.J.E. Vandenbroucke-Grauls and A.M. de Roda Husman, 'Role of the environment in the transmission of antimicrobial resistance to humans: a review', *Environmental Science & Technology*, Vol. 49 (20), 2015, pp. 11993–12004.

Humeniuk, C., G. Arlet, V. Gautier, P. Grimont, R. Labia and A. Philippon, ' β -Lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types', *Antimicrobial Agents and Chemotherapy*, Vol. 46 (9), 2002, pp. 3045–3049.

Ince, B., H. Coban, G. Turker, E. Ertekin and O. Ince, 'Effect of oxytetracycline on biogas production and active microbial populations during batch anaerobic digestion of cow manure', *Bioprocess and Biosystems Engineering*, Vol. 36 (5), 2013, pp. 541–546.

IUCN, 'Guide for the sustainable development of mediterranean aquaculture. Interaction between aquaculture and the environment', International Union for Conservation of Nature and Natural Resources (IUCN), Spanish Ministry of Agriculture, Fisheries and Food, European Federation of Aquaculture Producers (FEAP), Gland (Switzerland), Malaga (Spain), 2007.
<https://www.iucn.org/content/guide-sustainable-development-mediterranean-aquaculture-interaction-between-aquaculture-and-environment>

https://www.iucn.org/sites/dev/files/import/downloads/acua_en_final.pdf.

Jacobsen, A.M. and B. Halling-Sørensen, 'Multi-component analysis of tetracyclines, sulfonamides and tylosin in swine manure by liquid chromatography-tandem mass spectrometry', *Analytical and Bioanalytical Chemistry*, Vol. 384 (5), 2006, pp. 1164–1174.

Jechalke, S., H. Heuer, J. Siemens, W. Amelung and K. Smalla, 'Fate and effects of veterinary antibiotics in soil', *Trends in Microbiology*, Vol. 22 (9), 2014, pp. 536–545.

Jensen, J., 'Veterinary medicines and soil quality: the Danish situation as an example'. In: *Pharmaceuticals and Personal Care Products in the Environment: Scientific and Regulatory Issues*, C.G. Daughton and T.L. Jones-Lepp (eds.). American Chemical Society, Washington, D.C. (USA), 2001, pp. 282–302.

Jensen, L.B., Y. Agersø and G. Sengeløv, 'Presence of erm genes among macrolide-resistant Gram-positive bacteria isolated from Danish farm soil', *Environment International*, Vol. 28 (6), 2002, pp. 487–491.

Johnning, A., E.R.B. Moore, L. Svensson-Stadler, Y.S. Shouche, D.G.J. Larsson and E. Kristiansson, 'The acquired genetic mechanisms of a multi-resistant bacterium isolated from a treatment plant receiving wastewater from antibiotic production', *Applied and Environmental Microbiology*, Vol. 79 (23), 2013, pp. 7256–7263.

Jolivet-Gougeon, A., B. Kovacs, S. Le Gall-David, H. Le Bars, L. Bousarghin, M. Bonnaure-Mallet, B. Lobel, F. Guillé, C.-J. Soussy and P. Tenke, 'Bacterial hypermutation: clinical implications', *Journal of Medical Microbiology*, Vol. 60 (Part 5), 2011, pp. 563–573.

JPIAMR, 'The third JPIAMR joint call "Transmission Dynamics"', Joint Programming Initiative on Antimicrobial Resistance (JPIAMR), Stockholm (Sweden), 2018.
<https://www.jpiamr.eu/supportedprojects/third-joint-callresult/> (accessed: 2018).

Jutkina, J., C. Rutgersson, C.-F. Flach and D.G.J. Larsson, 'An assay for determining minimal concentrations of antibiotics that drive horizontal transfer of resistance', *Science of the Total Environment*, Vol. 548–549 2016, pp. 131–138.

Kemper, N., 'Veterinary antibiotics in the aquatic and terrestrial environment', *Ecological Indicators*, Vol. 8 (1), 2008, pp. 1–13.

Kumar, D., S. Pornsukarom, G.K. Sivaraman and S. Thakur, 'Environmental dissemination of multidrug methicillin-resistant *Staphylococcus sciuri* after application of manure from commercial swine production systems', *Foodborne Pathogens and Disease*, Vol. 15 (4), 2018, pp. 210–217.

Kümmerer, K., 'Antibiotics in the aquatic environment—A review—Part I', *Chemosphere*, Vol. 75 (4), 2009, pp. 417–434.

Larsson, D.G.J., 'Pollution from drug manufacturing: review and perspectives', *Philosophical Transactions of the Royal Society B*, Vol. 369 (1656), 2014, pp. 20130571.

Lees, P. and P.-L. Toutain, 'The role of pharmacokinetics in veterinary drug residues', *Drug Testing and Analysis*, Vol. 4 (Supplement 1), 2012, pp. 34–39.

Leonard, A.F.C., X.L. Yin, T. Zhang, M.C. Hui and W.H. Gaze, 'A coliform-targeted metagenomic method facilitating human exposure estimates to *Escherichia coli*-borne antibiotic resistance genes', *FEMS Microbiology Ecology*, Vol. 94 (3), 2018a, pp. fiy024.

Leonard, A.F.C., L. Zhang, A.J. Balfour, R. Garside and W.H. Gaze, 'Human recreational exposure to antibiotic resistant bacteria in coastal bathing waters', *Environment International*, Vol. 82 2015, pp. 92–100.

Leonard, A.F.C., L. Zhang, A.J. Balfour, R. Garside, P.M. Hawkey, A.K. Murray, O.C. Ukoumunne and W.H. Gaze, 'Exposure to and colonisation by antibiotic-resistant *E. coli* in UK coastal water users:

environmental surveillance, exposure assessment, and epidemiological study (Beach Bum Survey)', *Environment International*, Vol. 114 2018b, pp. 326–333.

Li, D., M. Yang, J. Hu, Y. Zhang, H. Chang and F. Jin, 'Determination of penicillin G and its degradation products in a penicillin production wastewater treatment plant and the receiving river', *Water Research*, Vol. 42 (1), 2008, pp. 307–317.

Loke, M.-L., J. Tjørnelund and B. Halling-Sørensen, 'Determination of the distribution coefficient (logK_d) of oxytetracycline, tylosin A, olaquinox and metronidazole in manure', *Chemosphere*, Vol. 48 (3), 2002, pp. 351–361.

Łuczkiwicz, A., K. Jankowska, S. Fudala-Książek and K. Ołańczuk-Neyman, 'Antimicrobial resistance of fecal indicators in municipal wastewater treatment plant', *Water Research*, Vol. 44 (17), 2010, pp. 5089–5097.

Lupo, A., S. Coyne and T.U. Berendonk, 'Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies', *Frontiers in Microbiology*, Vol. 3 2012, pp. 18.

Madec, J.-Y., M. Haenni, C. Ponsin, N. Kieffer, E. Rion and B. Gassilloud, 'Sequence type 48 *Escherichia coli* carrying the bla_{CTX-M-1} IncI1/ST3 plasmid in drinking water in France', *Antimicrobial Agents and Chemotherapy*, Vol. 60 (10), 2016, pp. 6430–6432.

Magouras, I., L.P. Carmo, K.D.C. Stärk and G. Schüpbach-Regula, 'Antimicrobial usage and resistance in livestock: where should we focus?', *Frontiers in Veterinary Science*, Vol. 4 2017, pp. 148.

Marshall, B.M. and S.B. Levy, 'Food animals and antimicrobials: impacts on human health', *Clinical Microbiology Reviews*, Vol. 24 (4), 2011, pp. 718–733.

Martínez-Carballo, E., C. González-Barreiro, S. Scharf and O. Gans, 'Environmental monitoring study of selected veterinary antibiotics in animal manure and soils in Austria', *Environmental Pollution*, Vol. 148 (2), 2007, pp. 570–579.

Martínez, J.L., 'Environmental pollution by antibiotics and by antibiotic resistance determinants', *Environmental Pollution*, Vol. 157 (11), 2009, pp. 2893–2902.

Martínez, J.L., T.M. Coque and F. Baquero, 'What is a resistance gene? Ranking risk in resistomes', *Nature Reviews Microbiology*, Vol. 13 2015, pp. 116–123.

Massé, D.I., N.M.C. Saady and Y. Gilbert, 'Potential of biological processes to eliminate antibiotics in livestock manure: an overview', *Animals*, Vol. 4 (2), 2014, pp. 146–163.

McVey, E.A. and M.H.M.M. Montforts, 'Regulatory research on antimicrobial resistance in the environment'. In: *Antimicrobial Resistance in the Environment*, P.L. Keen and M.H.M.M. Montforts (eds.). John Wiley & Sons, Hoboken, N.J. (USA), 2012, pp. 549–567.

Mensink, B.J.W.G. and M.H.M.M. Montforts, 'The ecological risks of antibiotic resistance in surface waters: a literature review', Dutch National Institute for Public Health and the Environment (RIVM), Bilthoven (The Netherlands), 2007.

Midtvedt, T., 'The ECO-SHADOW concept—A new way of following environmental impacts of antimicrobials'. In: *Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks*, K. Kümmerer (eds.). Springer, Berlin (Germany), 2004, pp. 311–316.

Montforts, M.H.M.M., 'The trigger values in the environmental risk assessment for (veterinary) medicines in the European Union: a critical appraisal', Dutch National Institute for Public Health and the Environment (RIVM), Bilthoven (The Netherlands), 2005.

Moono, P., N.F. Foster, D.J. Hampson, D.R. Knight, L.E. Bloomfield and T.V. Riley, '*Clostridium difficile* infection in production animals and avian species: a review', *Foodborne Pathogens and Disease*, Vol. 13 (12), 2016, pp. 647–655.

Muurinen, J., R. Stedtfeld, A. Karkman, K. Pärnänen, J. Tiedje and M. Virta, 'Influence of manure application on the environmental resistome under Finnish agricultural practice with restricted antibiotic use', *Environmental Science & Technology*, Vol. 51 (11), 2017, pp. 5989–5999.

Muziasari, W.I., K. Pärnänen, T.A. Johnson, C. Lyra, A. Karkman, R.D. Stedtfeld, M. Tamminen, J.M. Tiedje and M. Virta, 'Aquaculture changes the profile of antibiotic resistance and mobile genetic element associated genes in Baltic Sea sediments', *FEMS Microbiology Ecology*, Vol. 92 (4), 2016, pp. fiw052.

NERC, 'List of awards', Natural Environment Research Council (NERC), Swindon (UK), 2018. http://gotw.nerc.ac.uk/list_them.asp?them=AMR (accessed: 2018).

Nesme, J. and P. Simonet, 'The soil resistome: a critical review on antibiotic resistance origins, ecology and dissemination potential in telluric bacteria', *Environmental Microbiology*, Vol. 17 (4), 2015, pp. 913–930.

Nielsen, P. and N. Gyrd-Hansen, 'Bioavailability of oxytetracycline, tetracycline and chlortetracycline after oral administration to fed and fasted pigs', *Journal of Veterinary Pharmacology and Therapeutics*, Vol. 19 (4), 1996, pp. 305–311.

Nilsson, O., E. Alm, C. Greko and B. Bengtsson, 'The rise and fall of a vancomycin-resistant clone of *Enterococcus faecium* among broilers in Sweden', *Journal of Global Antimicrobial Resistance*, Vol. 17 2019, pp. 233–235.

Nilsson, O., C. Greko, J. Top, A. Franklin and B. Bengtsson, 'Spread without known selective pressure of a vancomycin-resistant clone of *Enterococcus faecium* among broilers', *Journal of Antimicrobial Chemotherapy*, Vol. 63 (5), 2009, pp. 868–872.

OECD, 'Test guideline no. 209: activated sludge, respiration inhibition test (carbon and ammonium oxidation)', Organisation for Economic Co-operation and Development (OECD), Paris (France), 2010. <https://doi.org/10.1787/9789264070080-en>.

Pan, X., Z. Qiang, W. Ben and M. Chen, 'Residual veterinary antibiotics in swine manure from concentrated animal feeding operations in Shandong Province, China', *Chemosphere*, Vol. 84 (5), 2011, pp. 695–700.

Pedersen, J.A., M.A. Yeager and I.H. Suffet, 'Xenobiotic organic compounds in runoff from fields irrigated with treated wastewater', *Journal of Agricultural and Food Chemistry*, Vol. 51 (5), 2003, pp. 1360–1372.

Peeters, L.E.J., E. Daeseleire, M. Devreese, G. Rasschaert, A. Smet, J. Dewulf, M. Heyndrickx, H. Imberechts, F. Haesebrouck, P. Butaye and S. Croubels, 'Residues of chlortetracycline, doxycycline and sulfadiazine-trimethoprim in intestinal content and feces of pigs due to crosscontamination of feed', *BMC Veterinary Research*, Vol. 12 (209), 2016, pp. 1–9.

Perry, J. and G.D. Wright, 'The antibiotic resistance 'mobilome': searching for the link between environment and clinic', *Frontiers in Microbiology*, Vol. 4 2013, pp. 138.

Philippot, L., S.G.E. Andersson, T.J. Battin, J.I. Prosser, J.P. Schimel, W.B. Whitman and S. Hallin, 'The ecological coherence of high bacterial taxonomic ranks', *Nature Reviews Microbiology*, Vol. 8 (7), 2010, pp. 523–529.

Pittenger, R., B. Anderson, D.D. Benetti, B. Dewey, R. Goldberg, A. Rieser, B. Sher and A. Sturgulewski, 'Sustainable marine aquaculture: fulfilling the promise; managing the risks. Report of the marine aquaculture task force', Marine Aquaculture Task Force, Takoma Park, MD (USA), 2007. <http://www.pewtrusts.org/en/research-and-analysis/reports/2007/01/09/sustainable-marine-aquaculture-fulfilling-the-promise-managing-the-risks>.

Poirel, L., J.-M. Rodriguez-Martinez, H. Mammeri, A. Liard and P. Nordmann, 'Origin of plasmid-mediated quinolone resistance determinant QnrA', *Antimicrobial Agents and Chemotherapy*, Vol. 49 (8), 2005, pp. 3523–3525.

Poole, K., 'At the nexus of antibiotics and metals: the impact of Cu and Zn on antibiotic activity and resistance', *Trends in Microbiology*, Vol. 25 (10), 2017, pp. 820–832.

Porse, A., H. Gumpert, J.Z. Kubicek-Sutherland, N. Karami, I. Adlerberth, A.E. Wold, D.I. Andersson and M.O.A. Sommer, 'Genome dynamics of *Escherichia coli* during antibiotic treatment: transfer, loss, and persistence of genetic elements in situ of the infant gut', *Frontiers in Cellular and Infection Microbiology*, Vol. 7 2017, pp. 126.

Pruden, A., D.G.J. Larsson, A. Amézquita, P. Collignon, K.K. Brandt, D.W. Graham, J.M. Lazorchak, S. Suzuki, P. Silley and J.R. Snape, 'Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment', *Environmental Health Perspectives (Online)*, Vol. 121 (8), 2013, pp. 878–885.

Pruden, A., R. Pei, H. Storteboom and K.H. Carlson, 'Antibiotic resistance genes as emerging contaminants: studies in Northern Colorado', *Environmental Science & Technology*, Vol. 40 (23), 2006, pp. 7445–7450.

Prudhomme, M., L. Attaiech, G. Sanchez, B. Martin and J.-P. Claverys, 'Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*', *Science*, Vol. 313 (5783), 2006, pp. 89–92.

Rabølle, M. and N.H. Spliid, 'Sorption and mobility of metronidazole, olaquinox, oxytetracycline and tylosin in soil', *Chemosphere*, Vol. 40 (7), 2000, pp. 715–722.

Raphael, E. and L.W. Riley, 'Infections caused by antimicrobial drug-resistant saprophytic Gram-negative bacteria in the environment', *Frontiers in Medicine*, Vol. 4 2017, pp. 183.

Review on Antimicrobial Resistance, 'Tackling drug-resistant infections globally: final report and recommendations', Wellcome Trust, UK Government, London (UK), 2016. http://amr-review.org/sites/default/files/160525_Final%20paper_with%20cover.pdf.

Rico, A., R. Jacobs, P.J. van den Brink and A. Tello, 'A probabilistic approach to assess antibiotic resistance development risks in environmental compartments and its application to an intensive aquaculture production scenario', *Environmental Pollution*, Vol. 231 (Part 1), 2017, pp. 918–928.

Rigos, G., I. Nengas, M. Alexis and G.M. Troisi, 'Potential drug (oxytetracycline and oxolinic acid) pollution from Mediterranean sparid fish farms', *Aquatic Toxicology*, Vol. 69 (3), 2004, pp. 281–288.

Rodríguez-Rojas, A., J. Rodríguez-Beltrán, A. Couce and J. Blázquez, 'Antibiotics and antibiotic resistance: a bitter fight against evolution', *International Journal of Medical Microbiology*, Vol. 303 (6–7), 2013, pp. 293–297.

Rolain, J.-M., 'Food and human gut as reservoirs of transferable antibiotic resistance encoding genes', *Frontiers in Microbiology*, Vol. 4 2013, pp. 173.

Salyers, A.A., A. Gupta and Y. Wang, 'Human intestinal bacteria as reservoirs for antibiotic resistance genes', *Trends in Microbiology*, Vol. 12 (9), 2004, pp. 412–416.

Sandegren, L., 'Selection of antibiotic resistance at very low antibiotic concentrations', *Upsala Journal of Medical Sciences*, Vol. 119 (2), 2014, pp. 103–107.

Schmitt, H., T. ter Laak and K. Duis, 'Development and dissemination of antibiotic resistance in the environment under environmentally relevant concentrations of antibiotics and its risk assessment', German Federal Environment Agency (UBA), Dessau-Roßlau (Germany), 2017.

Schwanner, N. and N. Kroer, 'Effect of plant species on the kinetics of conjugal transfer in the rhizosphere and relation to bacterial metabolic activity', *Microbial Ecology*, Vol. 42 2001, pp. 458–465.

Schwartz, T., W. Kohnen, B. Jansen and U. Obst, 'Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms', *FEMS Microbiology Ecology*, Vol. 43 (3), 2003, pp. 325–335.

- Sengeløv, G., Y. Agersø, B. Halling-Sørensen, S.B. Baloda, J.S. Andersen and L.B. Jensen, 'Bacterial antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry', *Environment International*, Vol. 28 (7), 2003, pp. 587–595.
- Sengeløv, G., G.A. Kowalchuk and S.J. Sørensen, 'Influence of fungal-bacterial interactions on bacterial conjugation in the residuesphere', *FEMS Microbiology Ecology*, Vol. 31 (1), 2000, pp. 39–45.
- Shellie, R.A., L.-L. Xie and P.J. Marriott, 'Retention time reproducibility in comprehensive two-dimensional gas chromatography using cryogenic modulation: an intralaboratory study', *Journal of Chromatography A*, Vol. 968 (1–2), 2002, pp. 161–170.
- Singer, A.C., H. Shaw, V. Rhodes and A. Hart, 'Review of antimicrobial resistance in the environment and its relevance to environmental regulators', *Frontiers in Microbiology*, Vol. 7 2016, pp. 1728.
- Singer, R.S., M.P. Ward and G. Maldonado, 'Can landscape ecology untangle the complexity of antibiotic resistance?', *Nature Reviews Microbiology*, Vol. 4 (12), 2006, pp. 943–952.
- Soumet, C., E. Fourreau, P. Legrandois and P. Maris, 'Resistance to phenicol compounds following adaptation to quaternary ammonium compounds in *Escherichia coli*', *Veterinary Microbiology*, Vol. 158 (1–2), 2012, pp. 147–152.
- Stokes, H.W. and M.R. Gillings, 'Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens', *FEMS Microbiology Reviews*, Vol. 35 (5), 2011, pp. 790–819.
- Sukul, P. and M. Spiteller, 'Fluoroquinolone antibiotics in the environment'. In: *Reviews of Environmental Contamination and Toxicology*, G.W. Ware and D.M. Whitacre (eds.). Springer, New York, N.Y. (USA), 2007, pp. 131–162.
- Taylor, N.G.H., D.W. Verner-Jeffreys and C. Baker-Austin, 'Aquatic systems: maintaining, mixing and mobilising antimicrobial resistance?', *Trends in Ecology & Evolution*, Vol. 26 (6), 2011, pp. 278–284.
- Tell, J., D.J. Caldwell, A. Häner, J. Hellstern, B. Hoeger, R. Journal, F. Mastrocco, J.J. Ryan, J. Snape, J.O. Straub and J. Vestel, 'Science-based targets for antibiotics in receiving waters from pharmaceutical manufacturing operations', *Integrated Environmental Assessment and Management*, Vol. 15 (3), 2019, pp. 312–319.
- Tello, A., B. Austin and T.C. Telfer, 'Selective pressure of antibiotic pollution on bacteria of importance to public health', *Environmental Health Perspectives (Online)*, Vol. 120 (8), 2012, pp. 1100–1106.
- Thames, C., A. Pruden, R. James, P. Ray and K. Knowlton, 'Excretion of antibiotic resistance genes by dairy calves fed milk replacers with varying doses of antibiotics', *Frontiers in Microbiology*, Vol. 3 2012, pp. 139.
- Thiele-Bruhn, S., 'Pharmaceutical antibiotic compounds in soils—A review', *Journal of Plant Nutrition and Soil Science*, Vol. 166 (2), 2003, pp. 145–167.
- Topp, E., D.G.J. Larsson, D.N. Miller, C. Van den Eede and M.P.J. Virta, 'Antimicrobial resistance and the environment: assessment of advances, gaps and recommendations for agriculture, aquaculture and pharmaceutical manufacturing', *FEMS Microbiology Ecology*, Vol. 94 (3), 2018, pp. fix185.
- Toutain, P.-L. and A. Bousquet-Mélou, 'Bioavailability and its assessment', *Journal of Veterinary Pharmacology and Therapeutics*, Vol. 27 (6), 2004, pp. 455–466.
- Toutain, P.-L., A.A. Ferran, A. Bousquet-Melou, L. Pelligand and P. Lees, 'Veterinary medicine needs new green antimicrobial drugs', *Frontiers in Microbiology*, Vol. 7 2016, pp. 1196.
- United Nations, 'Political declaration of the high-level meeting of the General Assembly on antimicrobial resistance', United Nations, New York, N.Y. (USA), 2016.
http://digitallibrary.un.org/record/842813/files/A_71_L-2-EN.pdf.

van der Meersche, T., G. Rasschaert, F. Haesebrouck, E. van Coille, L. Herman, S. van Weyenberg, E. Daeseleire and M. Heyndrickx, 'Presence and fate of antibiotic residues, antibiotic resistance genes and zoonotic bacteria during biological swine manure treatment', *Ecotoxicology and Environmental Safety*, Vol. 175 2019, pp. 29–38.

van Elsas, J.D. and M.J. Bailey, 'The ecology of transfer of mobile genetic elements', *FEMS Microbiology Ecology*, Vol. 42 (2), 2002, pp. 187–197.

van Elsas, J.D., B.B.M. Gardener, A.C. Wolters and E. Smit, 'Isolation, characterization, and transfer of cryptic gene-mobilizing plasmids in the wheat rhizosphere', *Applied and Environmental Microbiology*, Vol. 64 (3), 1998, pp. 880–889.

Vikram, A., P. Rovira, G.E. Agga, T.M. Arthur, J.M. Bosilevac, T.L. Wheeler, P.S. Morley, K.E. Belk and J.W. Schmidt, 'Impact of 'raised without antibiotics' beef cattle production practices on occurrences of antimicrobial resistance', *Applied and Environmental Microbiology*, Vol. 83 (22), 2017, pp. e01682-01617.

Wales, A.D. and R.H. Davies, 'Co-selection of resistance to antibiotics, biocides and heavy metals, and its relevance to foodborne pathogens', *Antibiotics*, Vol. 4 (4), 2015, pp. 567–604.

Wall, B.A., A. Mateus, L. Marshall and D.U. Pfeiffer, 'Drivers, dynamics and epidemiology of antimicrobial resistance in animal production', *Food and agriculture organization of the United Nations (FAO)*, Rome (Italy), 2016. <http://www.fao.org/3/a-i6209e.pdf>.

Watkinson, A.J., E.J. Murby and S.D. Costanzo, 'Removal of antibiotics in conventional and advanced wastewater treatment: implications for environmental discharge and wastewater recycling', *Water Research*, Vol. 41 (18), 2007, pp. 4164–4176.

Wellington, E.M., A.B. Boxall, P. Cross, E.J. Feil, W.H. Gaze, P.M. Hawkey, A.S. Johnson-Rollings, D.L. Jones, N.M. Lee and W. Otten, 'The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria', *The Lancet Infectious Diseases*, Vol. 13 (2), 2013, pp. 155–165.

WHO, 'Global action plan on antimicrobial resistance', *World Health Organisation (WHO)*, Geneva (Switzerland), 2015. http://www.who.int/drugresistance/global_action_plan/en/.

Wiedenbeck, J. and F.M. Cohan, 'Origins of bacterial diversity through horizontal genetic transfer and adaptation to new ecological niches', *FEMS Microbiology Reviews*, Vol. 35 (5), 2011, pp. 957–976.

Wright, G.D., 'The antibiotic resistome: the nexus of chemical and genetic diversity', *Nature Reviews Microbiology*, Vol. 5 (3), 2007, pp. 175–186.

Yu, Z., L. Gunn, P. Wall and S. Fanning, 'Antimicrobial resistance and its association with tolerance to heavy metals in agriculture production', *Food Microbiology*, Vol. 64 2017, pp. 23–32.

Zhang, L., Y. Huang, Y. Zhou, T. Buckley and H.H. Wang, 'Antibiotic administration routes significantly influence the levels of antibiotic resistance in gut microbiota', *Antimicrobial Agents and Chemotherapy*, Vol. 57 (8), 2013, pp. 3659–3666.

Zhang, X.-X., T. Zhang and H.H.P. Fang, 'Antibiotic resistance genes in water environment', *Applied Microbiology and Biotechnology*, Vol. 82 (3), 2009a, pp. 397–414.

Zhang, Y., C.F. Marrs, C. Simon and C. Xi, 'Wastewater treatment contributes to selective increase of antibiotic resistance among *Acinetobacter* spp. ', *Science of the Total Environment*, Vol. 407 (12), 2009b, pp. 3702–3706.