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A survey of sub-inhibitory concentrations of antibiotics in the environment

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ABSTRACT

Antibiotics are poorly metabolized, and can enter the environment via human waste streams, agricultural run-off and pharmaceutical effluent. We consequently expect to see a concentration gradient of antibiotic compounds radiating from areas of human population. Such antibiotics should be thought of as pollutants, as they can accumulate, and have biological effects. These antibiotic pollutants can increase rates of mutation and lateral transfer events, and continue to exert selection pressure even at sub-inhibitory concentrations. Here, we conducted a literature survey on environmental concentrations of antibiotics. We collated 887 data points from 40 peer-reviewed papers. We then determined whether these concentrations were biologically relevant by comparing them to their minimum selective concentrations, usually defined as between 1/4 and 1/230 of the minimum inhibitory concentration. Environmental concentrations of antibiotics surveyed often fall into this range. In general, the antibiotic concentrations recorded in aquatic and sediment samples were similar. These findings indicate that environmental concentrations of antibiotics are likely to be influencing microbial ecology, and to be driving the selection of antibiotic resistant bacteria.

Introduction

Antibiotic resistance is one of the greatest threats to human health in the 21st century. Approximately 70% of nosocomial infections are resistant to at least one type of antibiotic (Zhang et al., 2011) and resistance is expected to increase as bacteria acquire genes that confer increasingly higher levels of resistance to diverse classes of antimicrobials. It is estimated that by 2050, antibiotic resistant infections might account for 10 million deaths annually, increasing from 700,000 deaths currently (O'Neill, 2014). Furthermore, antibiotic resistance leads to longer hospital stays and an overall economic burden that is most felt in low-income nations. On its current trajectory, the effect of antimicrobial resistant infections could damage the global economy to an extent level similar to the Global Financial Crisis in 2008, and could see a further 28.3 million people enter into extreme poverty (Adeyi et al., 2017).

Following the World Health Organisation's Global Action Plan on antimicrobial resistance, there has been an increase in research on antibiotic resistance and increased implementation of monitoring and surveillance strategies (Adeyi et al., 2017; Prestinaci et al., 2015). Until recently, the method often used to manage antibiotic resistant infections was to replace an antibiotic with either a higher concentration of the same antibiotic, or more commonly, a different antibiotic class. This is not a sustainable solution, as little development of new antibiotics has occurred in in recent years, largely due to the marginal commercial benefit arising from antibiotic development (Bartlett et al., 2013).

Furthermore, current management and control strategies are not working. Between 2000 and 2015 antibiotic usage increased by 65%, as measured by defined daily doses (DDD), with a concerning increase in last-line of defense antibiotics.

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Based on this trajectory, antibiotic usage is expected to increase 200% by 2030 (Klein et al., 2018). While many countries have implemented national antibiotic usage surveillance plans to monitor and control use of antibiotics, a unified global response is needed to adequately address the growing problem of antibiotic resistance.

Bacteria may be intrinsically resistant to one or more antibiotics, or they may acquire resistance. Antibiotics are often naturally occurring molecules produced by bacteria or fungi and are present in all natural environments (Allen et al., 2010). While it is not known exactly what the original function of these antimicrobials might have been, it follows that some bacteria exhibit resistance to naturally occurring antibiotics. This is known as intrinsic resistance, which is likely the origin of many resistance genes that we see today (Davies and Davies, 2010). However, use of antibiotic compounds at unnaturally high concentrations places significant selective pressure on bacteria, killing most cells and fixing just those cells that can acquire resistance.

Bacteria acquire resistance in two ways, via mutations or horizontal gene transfer. Huge selective pressures are placed on microbial communities as a result of antibiotic use in human medicine, agriculture, aquaculture, and veterinary medicine. Selection takes place at the treatment site, where selection pressure is high enough to make acquisition of resistance necessary for survival. The mechanisms involved in acquisition or generation of resistance under these circumstances are well understood (Blair et al., 2015; Blair et al., 2014; Giedraitienė et al., 2011; Levy and Marshall, 2004).

However, there is increasing interest in the potential for sub-inhibitory levels of antibiotics to promote resistance (Andersson and Hughes, 2012; Davies et al., 2006). Antibiotics used in medicine and animal husbandry are often poorly metabolized, with the result that up to 90% of the therapeutic dose can be excreted unchanged (Berge et al., 2005; Kümmerer and Henninger, 2003). Antibiotics are not removed by standard waste management processes, but are released into the environment via human waste effluent, or via manuring of crops with animal waste (Chee-Sanford et al., 2009; Heuer et al., 2011). Antibiotics also enter the environment directly via crop spraying (McManus, 2014), landfill leachate (Chung et al., 2018), and pharmaceutical factory run off (Larsson, 2014; Tahrani et al., 2016). Consequently, antibiotics are increasingly being viewed as an emerging environmental contaminant (Martinez, 2009; Milic et al., 2013).

It is clear that sub-clinical levels of antibiotics still have significant biological effects. In particular, they affect the very processes involved in the acquisition or generation of resistance, including mutation, recombination and lateral gene transfer (Andersson and Hughes, 2012; Chow et al., 2015; Martinez, 2009). But what concentrations of antibiotics are needed to stimulate these effects, and are these concentrations found in environmental compartments? Here we gather the available information on environmental levels of antibiotics, and determine whether these concentrations are biologically relevant.

1. Summary of current antibiotic use

Between 2000 and 2015, antibiotic use increased 65%, with the increase mainly seen in low income countries (Klein et al., 2018). While high income countries still have higher overall use, it is expected that rising average income will boost antibiotic use in low income countries, to equal or exceed current use in high income countries. The defined daily dose (DDD) of antibiotics used per 1000 inhabitants per day varies significantly between countries, with less than 10 DDD in Central America to over 40 in Turkey and Tunisia (Klein et al., 2018).

Of all antibiotics manufactured globally, approximately 70%-80% are used in agriculture (Rushton et al., 2014). In animal husbandry, the Population-Corrected Unit (PCU) is defined as milligrams of total antibiotic used per kilogram of meat production. This varies significantly between socially and economically comparable countries, with countries such as Iceland, Estonia, Latvia and Slovenia being well below 500 PCU and other countries such as France, UK and Spain being above 6000 PCU (Klein et al., 2018).

This variation in antibiotic consumption suggests that antibiotic usage could be reduced dramatically without negative health consequences for either human medicine or animal husbandry (Kummerer and Henninger, 2003). This is also an example of the importance of antibiotic monitoring systems, since they allow comparisons of antibiotic use. Unfortunately, surveillance systems for antibiotic use and production are not uniform between countries. The availability of antibiotics without prescription, and the widespread lack of regulation for antibiotic use in animal husbandry makes accurate estimates difficult. There are also differences in use patterns, for instance streptomycin is widely used for fruit spraying in the USA, while this use is banned in much of Europe (Wise, 2002).

In human medicine, the β-lactams, including penicillin, are most widely used, accounting for 50%-70% of antibiotic consumption (Kummerer and Henninger, 2003; Monteiro and Boxall, 2010). Tetracyclines and fluoroquinolones are the main classes of antibiotics used in animal husbandry, however, this varies significantly, since some classes of antibiotics are banned from use in livestock in some countries (Karcı and Balcıoğlu, 2009; Rushton et al. 2014). Generally, heavy use of antibiotics is inevitably followed by the emergence of resistant bacterial strains. For example, use of colistin in Chinese agriculture has been associated with the appearance of plasmid mediated colistin resistance (Liu et al., 2016). The global consumption of last resort antibiotics, such as carbapenems and colistin, has increased (Klein et al., 2018). As use increases, there will be an increasing influx of antibiotic pollution into the environment.

2. Concentrations in the environment

Theoretically, it is possible to predict environmental levels of antibiotics by examining consumption, excretion, and effluent volume (Bound and Voulvoulis, 2004; Kummerer and Henninger, 2003). However, little is known about rates of dissemination and degradation for different antibiotic classes, or how different environmental conditions might affect these rates. Antibiotic concentrations in environmental samples can be measured directly using analytical methods such as high-performance liquid chromatography (HPLC). Antibiotics can be detected in aquatic and sediment samples, allowing detection of antibiotics that are soluble in water, and those which exhibit sorption to soil.

Using these techniques, antibiotics can be detected at ng/L concentrations in a variety of environments close to human influence. These low antibiotic concentrations do not provide the same selection pressure as clinical levels of antibiotics, but can still significantly promote the acquisition of antibiotic resistance genes (Andersson and Hughes, 2012). Because waste streams can contain both antibiotics and the resistance genes under selection, diverse resistance determinants can be readily acquired by an array of bacterial species.

There is a gradient of antibiotic concentration radiating from areas of dense human population and around agricultural operations (Campagnolo et al., 2002; Kümmerer, 2009).

The key questions that remain unanswered are: What are the concentrations of various antibiotic classes in environmental samples; and Are these concentrations above the threshold predicted to exert selective pressure?

To address these questions, we collated data from literature that reports measurements of antibiotic concentrations in diverse environments. Literature was collected in late 2018 from scholarly databases (Google Scholar ®, PubMed) using a combination of the following search terms; "antibiotic concentration", "HPLC", "antibiotics in the environment", "distribution of antibiotics in the environment", "levels of antibiotics in the environment", "release of antibiotics" and "antibiotic pollution". Papers were also retrieved by examining previous review papers. All papers were original research, peer-reviewed scientific literature. From the search results, research papers reporting concentrations of antibiotics in the environment were retained and used as data sources. Forty papers were used as sources of primary data, covering the period from 1999 to 2018.

Reported concentrations of antibiotics were recorded, and where possible, the minimum, maximum, mean or median concentration was recorded. The sample number, frequency of detection, location, environment type, detection method and reference were recorded alongside the concentration (Appendix A Table S1). Eight hundred and eighty-seven environmental antibiotic concentrations were recorded of which 212 were from sediment and 675 from aquatic environments, encompassing Europe, Asia and North America.

Environmental concentrations of antibiotics were compared with their MIC distributions for wild-type bacteria, these data being directly obtained from EUCAST (http://www.eucast.org/). The distributions of MIC measurements were based on collated data from 1,892,215 MIC measurements. The range of MICs for each antibiotic, for all available organisms, was collated (Antibiotic concentrations and pooled MIC are available as a CSV file File S1 & File S2 in Appendix A).

3. Summary of data

Environmental concentration data were collected for 39 different antibiotics belonging to 9 different antibiotic classes. Absolute concentrations ranged from 10^6 ng/L to 10^{-2} ng/L. MIC values recorded in the EUCAST database were retrieved for as many antibiotic types as possible. These data covered 24 of the antibiotics for which environmental concentrations were available. Antibiotic concentration data were plotted by antibiotic class and type as scatterplots (Fig. 1). These were overlain with box and whisker plots of the reported MIC data extracted from EUCAST.

Examining these plots, approximately 2% of antibiotic concentrations in environmental samples have measured antibiotic concentrations that overlap the range of MICs observed for a diverse range of organisms. At these concentrations, the growth of a significant number of environmental bacteria is likely to be inhibited, and cells will be under strong selection for antibiotic resistance.

A significant number of measured environmental concentrations fall within values thought to be above the minimum selective concentration (MSC), usually estimated to lie between 1/4 and 1/230 of the MIC (Bengtsson-Palme and Larsson, 2016; Gullberg et al., 2011). We can expect that these environmental antibiotic concentrations have significant biological effects, including effects on transcription (Davies et al., 2006) and on rates of recombination, mutation and lateral gene transfer events (Andersson and Hughes, 2014; Mesak et al., 2008).

Generation of *de novo* resistance need only arise once under such selective pressure to fix in a bacterial lineage, and then rapidly spread to other species and locations (Liu et al., 2016b; Skov and Monnet, 2016; Zhi et al., 2016; Zhu et al., 2017). Many records in the dataset are concentrations that are sufficient to select for *de novo* resistance. The majority of measured antibiotic concentrations do fall below the MSC, into the range of the predicted no effect concentration (PNEC), generically defined as concentrations below the lower range of the MSC (<1/230 MIC) (Bengtsson-Palme and Larsson, 2016).

In general, the antibiotic concentrations recorded in aquatic and sediment samples were similar (Appendix A Fig. S1). This might be expected, given that antibiotics are most likely to be transported into the environment via water, and some of these antibiotics are then sequestered into sediment. The dynamics of how antibiotics bind to sediment are not completely known, and the standing concentration in sediment should be an interaction between absorption from the surrounding water and degradation within the sediment. Consequently, we need to know the rate at which antibiotics are being shed into the environment, the absorption rate of antibiotics into sediment, and the half-life of antibiotics in both water and sediment. If the rate of degradation of antibiotic is slower than the rate that antibiotics are being released into the environment, we expect to see accumulation of antibiotics within environmental compartments.

4. Limitations of the data

This meta-analysis provides an overview of clinically relevant antibiotics in aquatic environments and sediment. In some cases, antibiotics with high usage rates do not appear in the Table. For example, penicillin, is a commonly used antibiotic that might be expected to be found at high concentrations. However, it is not represented in the data, because it is rapidly degraded in the environment (Monteiro and Boxall, 2010). This demonstrates that direct analysis of environmental samples is important, since it detects antibiotic concentrations that result from the dynamic interaction between rate of release and environmental half-life of particular classes of antibiotics.

For every analytical tool, there are limits of detection. For some of the antibiotics, the minimum concentration required for detection is higher than the concentration where biological effects are predicted to occur (1/4 -1/230 the MIC) (Bengtsson-Palme and Larsson, 2016; Gullberg et al., 2011). This means that these antibiotics could be present in the environment at undetectable, but biologically relevant concentrations (Armbruster and Pry, 2008). For example, the MIC of ciprofloxacin for 82 bacterial species falls between 0.002 and 4 mg/L, and for amoxicillin it falls between 0.002 and 16 mg/L for 30 bacterial species (The European Committee on Antimicrobial Susceptibility Testing). However, the limit of detection (LOD) for both these antibiotics is 0.005 mg/L by high-performance liquid chromatography (HPLC-MS/MS) (Kemper, 2008). This value is sometimes higher than the lowest measured MIC, and is often significantly higher than sub-MIC concentrations that have been suggested to select for resistance (1/4 - 1/230 the MIC). Consequently, low, but relevant concentrations of antibiotics present in the environment might not be detected via commonly used analytical techniques. Furthermore, some antibiotics may not be detected in water samples because they bind strongly to sludge or sediment, for example tetracyclines and fluoroquinolones (Tolls, 2001). Therefore, sampling of both aquatic and sediment samples is necessary.

There is a need for accurate and accessible testing methods that can detect low levels of antibiotics in liquid and sediment

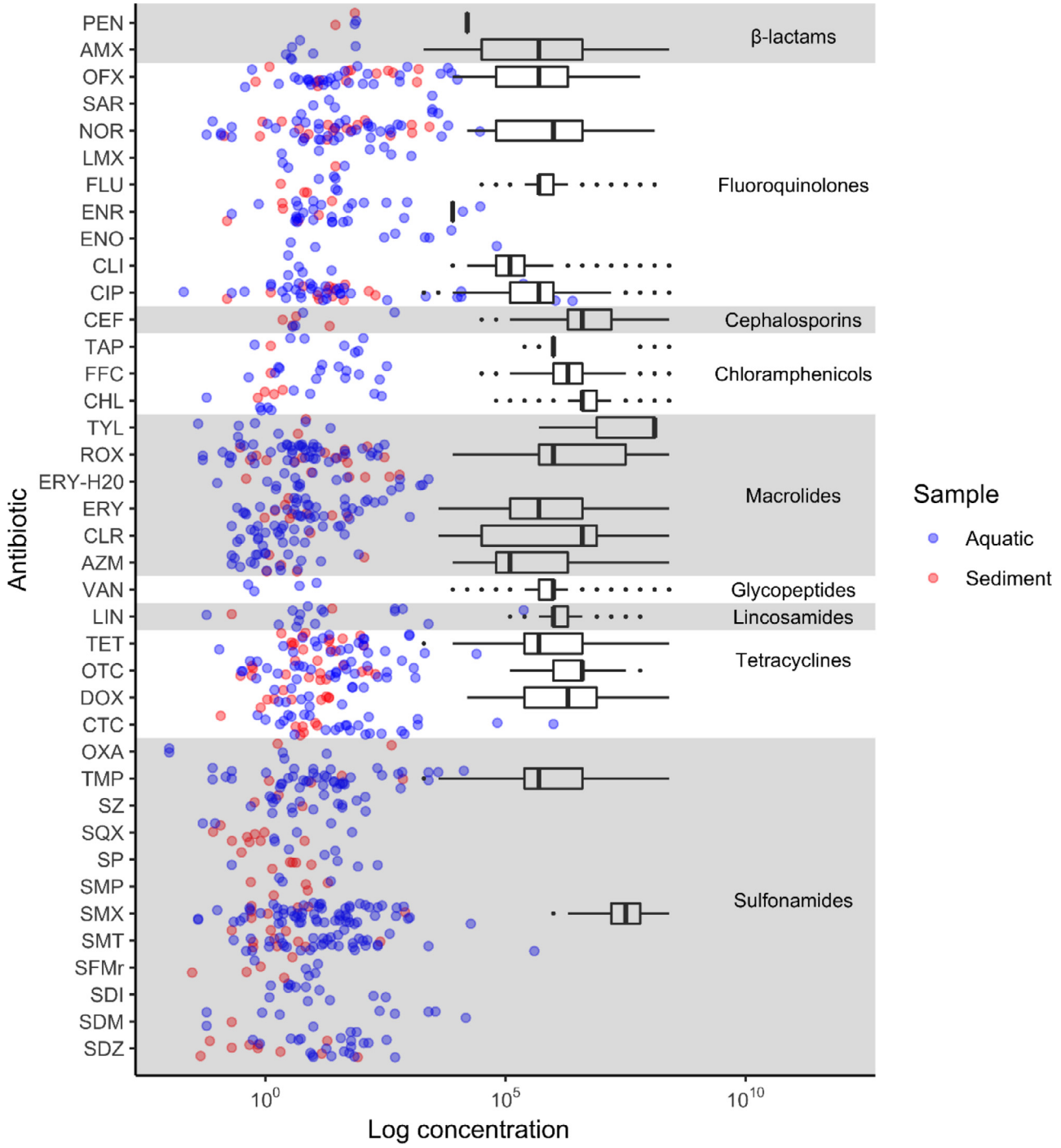

Figure 1 – Concentrations of antibiotics detected in the environment, aquatic concentrations are reported in ng/L and sediment concentrations are ng/kg. Measurements were derived from 675 aquatic (blue) and 212 sediment (red) samples. Box and whisker plots indicate MIC distributions (n = 1,892,215) for all wild type bacteria available from EUCAST. MIC concentrations are reported in ng/L Antibiotics are grouped according to class. For standard antibiotic abbreviations, see Appendix A Table S1.

samples. HPLC is an accurate tool for measuring antibiotics in the laboratory, but there is an increasing demand for in-field measurement equipment. This would increase accessibility of environmental antibiotic measurement (Parthasarathy et al., 2018). The question arises, how can we best determine the concentration of antibiotics in the environment? It would be possible to predict environmental levels of antibiotics by taking into account consumption, metabolic rates, efflux and half-life (Kummerer and Henninger, 2003). However, none of these values are known with any certainty.

5. Predicted effects of environmental concentrations of antibiotics

In the environment, sub-inhibitory concentrations of antibiotics can upregulate the rate of mutation and gene transfer, ultimately increasing the prevalence of antibiotic resistance (Gillings and Stokes, 2012). The microbiomes of humans and livestock contain diverse resistance genes (Salyers et al., 2004; Zhu et al., 2013), therefore, humans and livestock should be thought of as both a source of antibiotics (excreted during treatment) and of antibiotic resistance genes (from the endemic resistome and carriage of clinically relevant resistance genes). Indeed, resistance genes are now being regarded as a new form of pollutant: one that has the ability to replicate (Gillings et al., 2018).

The problem of antibiotic resistance is compounded by the ability of bacteria to acquire genes from their environment and from other bacteria, regardless of species, via horizontal gene transfer. The majority of known antibiotic resistance genes are carried by mobile genetic elements, such as transposons, integrative-conjugative elements and plasmids (Allen et al., 2010; Gillings, 2014). This means that a resistance gene could arise in a single bacterium but be rapidly disseminated around the globe (Ghaly et al., 2017). One well documented example of this is the colistin resistance gene, *mcr-1*, which spread globally following a single *de novo* mutation event. It is likely that this occurred in the Shandong province in China, driven by the heavy agricultural use of colistin in swine farms. Following the initial mobilization event, the *mcr-1* gene was rapidly distributed and has now been detected in five continents in both humans and in livestock (Liu et al., 2016b; Skov and Monnet, 2016; Zhi et al., 2016).

Sub-inhibitory concentrations of antibiotics increase rates of mutation and conjugation via the SOS response (Andersson and Hughes, 2014). The SOS response is a general response to DNA damage, such as the damage inflicted by some antibiotics. There are approximately 40 genes involved in the SOS response, several of which are translesion DNA polymerases which allow the replication machinery to bypass damaged regions of DNA. This maintains chromosomal integrity but also significantly increases the likelihood of base substitutions (Baharoglu and Mazel, 2014; Mesak et al., 2008; Cirz et al., 2006). Sub-inhibitory concentrations of several classes of antibiotics (such as aminoglycosides, fluoroquinolones and β-lactams) are well documented to activate the SOS response (Andersson and Hughes, 2014; Mesak et al., 2008b).

Long term exposure to sub-clinical levels of antibiotics could be a major factor in the generation and transfer of resistance genes (Kümmerer, 2004; Uslu et al., 2008). Subclinical concentrations of antibiotics are continuously discharged through sewage effluent, and sludge or manure application, providing continual selective pressure. It has been documented that resistance that evolves in response to clinical levels of antibiotics will be high cost (Andersson and Hughes, 2014), whereas *de novo* resistance that is generated at sub-clinical levels is less likely to have a significant cost on bacterial fitness, and can allow these bacteria to out-compete strains in which mutations are costly (Andersson and Hughes, 2014). Consequently, *de novo* resistance may be maintained on chromosomes for longer when generated in environmental settings. This in turn provides a greater opportunity for newly formed resistance genes to be captured by a mobile element via an insertion or recombination event. Once mobilized, however, the cost of a resistance gene to its bacterial host does not necessarily affect its capacity to persist (Ghaly and Gillings, 2018; Lopatkin et al., 2017; Stevenson et al., 2017).

6. Degradation of antibiotics in the environment

It is clear that some antibiotics persist in the environment, but the length of time an antibiotic can persist in the environment varies depending on the type of antibiotic and the environmental conditions. For many antibiotics, the degradation products are still effective antimicrobials and therefore still have an impact on microbial function (García-Galán et al., 2008; Kümmerer, 2009). There is huge variation in stability of antibiotics, for example, some antibiotics have high sorption into soil, making them able to persist for significant lengths of time, while some antibiotics rapidly degrade under ultraviolet light and this would be a significant factor in the degradation of antibiotics in aquatic environments. Although sorption of antibiotics into sediment removes them from water sources, they are still active in sediment, and here they may be protected from oxidization and UV degradation (Alder et al., 2004; Girardi et al., 2011). While sorption of antibiotics to soil reduces surface and ground water contamination, it increases the exposure of soil-dwelling microorganisms to antibiotics.

In general, the half-life of antibiotics in manure is estimated to lie between 2-100 days (Boxall et al., 2004), allowing ample time for them be applied, mix with the soil and be transported via run-off. Biodegradability of antibiotics in aquatic samples can be measured *in vitro* using a Closed Bottle Test. This has been done for several antibiotics, and none were found to be readily biodegradable, defined as greater than 60% degradation within 28 days (Alexy et al., 2004; Kummerer et al., 2000).

7. Solutions and future research

Currently there are no regulations or environmental limits on antibiotic pollution, in contrast to many other pollutants such as chlorine, oil and grease, heavy metals, sulfates and nitrogen. All of these can be monitored, have reference standards, and if necessary, treatment protocols (Carraro et al., 2016). Since there are no global guidelines for antibiotic reference standards and treatment of sewage effluent, there is a significant difference between how countries monitor and treat their sewage effluent. Hospital effluent is well documented to have high concentrations of antibiotics (Kümmerer, 2001), however, in the majority of countries, hospital effluent is classed as “domestic” waste and enters the municipal sewage system. Only a few countries treat this effluent separately before it enters the municipal sewage system (Carraro et al., 2016). It has been suggested that the minimum selective concentration (MSC) would be more useful than the MIC when proposing acceptable limits of antibiotics in the environment. The minimum selective concentration is the minimum concentration of an antibiotic that provides resistant strains a growth advantage over susceptible strains. The MSC varies between 1/4 and 1/230 of the MIC depending on the antibiotic (Bengtsson-Palme and Larsson, 2016; Gullberg et al., 2011). For example, the MSC of ciprofloxacin is between 8.6×10^{-6} and 1 mg/L. Whist this is a large range, it gives an indication of what the upper limits of environmental concentrations of ciprofloxacin should be and shows that the levels highlighted in this review are biologically relevant. We should better regulate antibiotic pollution and maintain environmental antibiotic levels below the range of MSC. Antibiotics need to be recognized by governing bodies as pollutants and need to have regulatory status. There should be global guidelines for the reference standards and treatment protocols for antibiotic pollution in human waste streams.

Treatment of human sewage and livestock waste is necessary to prevent antibiotics from entering the environment in the quantities we see here. Generally, antibiotics have long half-lives, and if soluble, are highly mobile and exhibit strong bacteriostatic qualities. Chemical treatment of waste to remove antibiotics is uncommon, as this risks contamination of water with the treatment chemical. Furthermore, the metabolites of antibiotics are generally still active antimicrobials. Removal of antibiotics would be ideally done via physical methods such as reverse osmosis membranes which can remove approximately 90% of antibiotics (Li et al., 2018). The use of sorbents to remove antibiotics is also a viable option as many antibiotics strongly bind with sediment, as long as the sediment is then disposed of in such a way that it does not enter the environment, for example, disposal in lined landfills prevents environmental contamination (Li et al., 2018). Photodegradation of antibiotics is one of the most common and effective ways to remove antibiotics, however, this process takes time and requires space (Sturini et al., 2012). Once antibiotics have regulatory status, it would be easier to enforce treatment of waste and more research into effective removal methods would follow.

We should not only look to better usage and treatment of antibiotics and waste but also to conditions that can reduce the need for antibiotic treatment. Practices that reduce antibiotic consumption, such as vaccines or hygiene systems, particularly in low-income countries, can be highly effective. For example, when clean water and basic sanitation are available, diarrheal diseases decrease (Nandi et al., 2017), and effective use of vaccines can reduce future antibiotic needs (Lee et al., 2014). New antimicrobials will be ineffective in solving the resistance problem in the long term if these novel drugs are then used in the same way that antibiotics have been used previously. Likewise, antibiotics that are important in human health must be preserved and not used in agriculture. The World Health Organization publishes a list of antibiotics essential to human health. We would argue that there should be a global ban on use of these antimicrobials in agriculture in order to conserve their effectiveness in treating human diseases.

Antibiotics enter the environment, where they can persist at biologically relevant concentrations for significant periods of time. When exposed to these levels of antibiotics, there is an upregulation of mutation and DNA transfer which can lead to bacteria acquiring antibiotic resistance genes. This poses significant threat to human health. We acknowledge that antibiotic usage is ingrained into every step of modern medicine, and that mass food production might not be possible without prophylactic usage of antibiotics. However, there needs to be a shift in antibiotic monitoring, usage and control at every level. The true extent of antibiotic use must be known in order to form workable solutions. Current antibiotic usage is unsustainable and will set the conditions for loss of human life, decreased livestock production and huge economic costs.

The fact that antibiotics are losing their effectiveness after decades of misuse cannot be ignored. Common infectious diseases like tuberculosis, pneumonia, sexually transmitted bacterial infections and diarrhea are becoming untreatable due to the rise of drug resistant strains. The spread of antibiotic resistance is a global phenomenon. Although resistance genes may arise in one location they can rapidly spread to all parts of the globe. Addressing the problem of antibiotic resistance requires a rapid, and unified global response.

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Appendix A. Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2020.05.030.

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