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Antimicrobial Resistance and Water Reuse

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The potential impacts of antimicrobial resistance (AMR) in the environment is a subject of increasing attention. Wastewater and wastewater treatment are of particular concern, as they may have comparatively high concentrations of both antibiotics and bacteria. This creates ideal conditions for formation of AMR and transfer of this resistance between organisms. The prevalence of such AMR is beginning to be investigated. The potential transfer of AMR in reused waters and its impact in downstream applications is only beginning to be understood. The development of next-generation DNA sequencing techniques provides the potential for intensive analysis of microbial genomes in environmental samples, allowing identification of known and novel antimicrobial resistance genes. These approaches will be valuable for understanding the risks of AMR transmission associated with water reuse and may provide a tool for monitoring water treatment in the future.

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Antimicrobial resistance and water reuse

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Acronyms and Abbreviations

AMR: Antimicrobial resistance

NGS: Next-generation (DNA) sequencing

PCR: Polymerase chain reaction

qPCR: Quantitative polymerase chain reaction

Executive Summary

The potential impacts of antimicrobial resistance (AMR) in the environment is a subject of increasing attention. Wastewater and wastewater treatment are of particular concern, as they may have comparatively high concentrations of both antibiotics and bacteria. This creates ideal conditions for formation of AMR and transfer of this resistance between organisms. The prevalence of such AMR is beginning to be investigated. The potential transfer of AMR in reused waters and its impact in downstream applications is only beginning to be understood. The development of next-generation DNA sequencing techniques provides the potential for intensive analysis of microbial genomes in environmental samples, allowing identification of known and novel antimicrobial resistance genes. These approaches will be valuable for understanding the risks of AMR transmission associated with water reuse and may provide a tool for monitoring water treatment in the future. This report reviews the state of knowledge on antimicrobial resistance, the role of wastewater treatment the development and transmission of AMR, and the use of molecular biological tools available to identify AMR genes.

Contents

Antibiotics and Antimicrobial Resistance

Antimicrobials, commonly referred to as antibiotics, are agents that kill or stop the growth of microorganisms like bacteria. First discovered in 1928 by Alexander Fleming, antibiotics are a crucial facet of modern medicine and have changed the prognosis of infection drastically. However, since the invention of antibiotics and theoretically even before, bacteria have developed mechanisms of resistance that present growing challenging for medical methodology.

For an antibiotic to be effective, three things must occur. First, there must be targets susceptible to the antibiotic present in the cell (Dzidic et al. 2008). Second, the antibiotic must reach the target in sufficient quantities. Finally, the antibiotic must be in a functional state, not modified or inactivated. The three main categories of antibiotic resistance – target modification, efflux pumps and outer membrane permeability, and antibiotic inactivation – work by preventing one of these three conditions from being met.

The first class of antibiotic resistance mechanisms is those that modify the target site for the antibiotic to reduce binding (Blair et al. 2015). As most target sites are key to cell vitality, the mechanisms that have developed change the site enough to significantly reduce binding while still allowing normal cellular functions. In some cases, cells compensate for altered functioning of the target site with additional changes. Within this mechanism, there are both mutations to the genes encoding for target structures and post-translational modifications that do not change the genetic sequence. One of the molecules most commonly targeted by antibiotics is peptidoglycan, an essential component of the cell wall needed for growth and stability. Resistance has evolved as both mutations in the domain of the peptidoglycan bound by antibiotics, and in genes encoding for a distinct kind of peptidoglycan called PBP2a. Other mechanisms that do not function on genetic change include methylation of target ribosomes and encoding of proteins to coat and protect enzymes and DNA.

The second class of antibiotic resistance is mechanisms that reduce the concentration of the antibiotic in the cell. Many antibiotics are hydrophilic, so cannot diffuse through the hydrophobic lipopolysaccharides that fill the outer membrane of Gram-negative bacteria, moving instead through non-specific channels called porins. Down regulation of porin number, size, and selectivity reduces the amount of antibiotics that enter the cell. Efflux pumps are another membrane protein, but one that actively transport antibiotics out of the cell. Although some are specialized, many efflux pumps function on a diverse range of antibiotics, so can factor towards multi-drug resistance (MDR). Many organisms have intrinsic antibiotic resistance from innate inducible multidrug efflux pumps, but mutations to the regulatory elements can upregulate efflux activity and increase resistance further.

The third class of antibiotic resistance is mechanisms that directly inactivate the antibiotic molecules through hydrolysis, group transfer, or redox. Hydrolysis occurs when hydrolytically susceptible bonds in the antibiotic are targeted and cleaved by specific enzymes. Many bacteria excrete these enzymes, so antibiotics are inactivated before even reaching the cell. Group transfer inactivates antibiotics by chemical substitution, adding adenylyl, phosphoryl, or acetyl group to prevent the antibiotic from being able to successful bind to the target site. The catalyzing transferase enzyme in these reactions requires a co-substrate, an energy-providing

molecule, so their activity is limited to the cytoplasm. Redox processes, while not common, function when the bacteria oxidizes or reduces a critical molecule of the antibiotic to a weaker one.

Underlying all these mechanisms of resistance are antimicrobial resistance genes (ARGs), which represent changes to the genetic code that are directly responsible for bacteria being capable of surviving in the presence of specific antimicrobial compounds that would normally be lethal. AMR, and the ARGs underlying it, can be innate or acquired, and arises both spontaneously and from selective pressures. Acquired resistance arises in two ways, through mutation or through horizontal gene transfer.

Mutations occur both spontaneously and under selective pressure and are the original source of all antibiotic resistance. Each mutation, regardless of origin, can also be classified as positive, negative, or neutral based on their impact on the fitness of the organism, with the presence of positive mutations increasing each sequential generation. Spontaneous mutations confer resistance prior to or in the absence of selective pressure, arising from errors in DNA replication or incorrect repair of DNA. Many spontaneous mutations negatively impact the fitness of the organism, as maintaining genetic information is critical for the endurance of the species, but positive spontaneous mutations are the source of evolution. Some environments provide such great selective pressure for change that the risk of negative mutation is outweighed by the potential gain of positive mutation. In these circumstances, some cells enter a state of an elevated mutation rate and are known as hypermutators. Caused by a reduction of the mechanisms that ensure DNA fidelity, these changes increase the possibility of acquiring antibiotic resistance through mutation. Non-lethal selective pressure can also lead to the rise of adaptive mutagenesis, when mutations occur in non-dividing or slowly dividing cells instead of only during cell division as originally theorized.

Once a mutation that confers antibiotic resistance has occurred and permeated a population, it can potentially be transferred to other species through horizontal gene transfer. Genetic material can be shared or exchanged through mechanisms of conjugation, transformation, and transduction, utilizing functions that occur naturally in many bacteria as well as viruses and mobile genetic elements to transfer resistance genes. In recent studies, hundreds of antibiotic resistance-containing mobile elements called plasmids have been identified in a single species of bacteria. In addition, resistance genes are often clustered together, so are more likely to be moved together and confer multi-drug resistance.

Antibiotic resistance may be the result of hundreds of different mutations that target a wide range of antibiotic mechanisms. The breath of the field makes identifying and countering resistance an extensive task, the magnitude of which will only continue to grow as selective pressure and horizontal gene transfer confer greater resistance

Antimicrobial resistance in wastewater & water reuse

Awareness of antimicrobial resistance has traditionally been focused on clinical settings, where pathogenic bacteria with antimicrobial resistance present a significant challenge for the treatment

of patients. This issue is a growing problem with the increasing prevalence of multi-drug resistant strains that are becoming increasingly difficult to combat. The resultant decrease in the efficacy of antibiotics and their growing inability to combat many infections has become a global issue for human health.

Recently there has been growing interest in movement of antibiotics through waste streams, and the distribution and emergence of antimicrobial resistance in the environment. Antibiotics may enter the environment through a variety of routes, including wastewater, improper disposal of unused prescription medicines, or agricultural runoff. From the standpoint of wastewater treatment and water reuse, a major contributor is antibiotic medicines that have been ingested but not fully metabolized. A large percentage of antibiotics may be excreted in active forms that can contribute to the development of AMR genes. AMR bacteria and the ARGs they possess may also be released into wastewater, where the AMR bacteria may proliferate and ARGs may spread to other bacteria.

Although modern wastewater treatment has contributed significantly to improvements in human health, commonly used treatment techniques present particular challenges with regards to AMR bacteria and ARGs. Most current wastewater treatment systems rely heavily on microbial activity to treat input wastewater. Bacterial growth is encouraged in parts of the treatment process to reduce pollutants through their metabolism or sequestration. Many conventional wastewater treatments use both aerobic and anaerobic systems to encourage the growth of physiologically distinct populations of bacteria and other microbes, thereby accomplishing different steps in the treatment process (Harb and Hong 2017).

Aerobic steps such as activated sludge treatment are of particular concern because by design they contain high concentrations of diverse and active bacterial populations (Barancheshme and Munir 2018, 2019). Such systems create conditions well suited to the development of AMR in response to antibiotics present in the influent wastewater. In addition, the high concentrations of bacteria also facilitate the movement of ARGs between organisms through horizontal gene transfer. This movement of ARGs through the community is likely exacerbated by high viral loads in aerobic sludge digesters, with viruses transferring genetic material, including ARGs, from one host to another in the system.

Although conventional wastewater treatment systems are effective in the removal of many contaminants, they have generally not been designed with AMR in mind and may not be well suited to limiting the release of ARGs. Indeed, while many wastewater treatment systems include physical and/or chemical disinfection processes (such as chlorination and UV irradiation) before final release of effluent, antibiotics, ARGs, and pathogens may be refractory to these treatments, leading to their release into the environment. Recent studies have shown high proportions of AMR in wastewater effluent, and possible stabilization of AMR in receiving waters (Ju et al. 2019, Corno et al. 2019).

The propagation of AMR though wastewater treatment is becoming a subject of increasing concern as there is increased reliance on indirect and direct potable reuse of effluent from wastewater treatment. With the advent of water reuse there has been increased adoption of advanced water treatments, such as micro- or nanofiltration, and reverse osmosis, and ozonation and oxidation, which provide additional removal of contaminants. Initial research suggests that

these treatments are effective in increasing removal or ARGs in the treated water, but considerable work still needs to be done on this subject.

As water reuse increases, so to does the relevance of monitoring for AMR, ARGs, and pathogenic organisms, to understand how effective wastewater treatment and advanced water treatments are at facilitating removal of these contaminants, and to facilitate risk assessment. The techniques for identifying these contaminants are rapidly evolving and are reviewed below.

Detection of AMR, ARGs, and pathogens

Culturing

Prior to the advent of modern molecular biology techniques for DNA analysis, identification of AMR bacteria relied on culturing samples on selective growth media containing antibiotics. Bacteria capable of growing on such media could then be identified as having resistance to the antibiotic in question. Although such techniques have been, and remain widely used in clinical settings, they are time and labor intensive, and have not been widely adopted in analysis of wastewater treatment. In addition, because many bacteria are not culturable on commonly used growth media, culturing assays are likely to have high instances of false negative results.

PCR and qPCR

The polymerase chain reaction (PCR) is an enzymatic method that allows exponential amplification of a fragment of DNA flanked by know sequences. With respect to identification of AMR, this technique allows identification of an ARG of known sequence from a sample. PCR assays have been developed for a wide variety of ARGs.

A more recent advancement of this approach is quantitative PCR (qPCR), also referred to as real-time PCR. While conventional PCR relies on an endpoint analysis where the final product is tested to determine whether the DNA fragment of interest is present in the tested sample, qPCR monitors the amplification of PCR product throughout the course of the reaction using fluorescent signals that are generated when the new product is replicated. In qPCR there is initially lag phase where no amplification is apparent, followed by a logarithmic phase of amplification where product becomes detectible above background levels of fluorescence. The number of amplification cycles required to reach this logarithmic phase is proportional to the amount of template DNA in the reaction, and so the amount of starting material in the sample can be calculated based on appropriate calibrations with known standards. Using this technique, the quantity of a specific ARG in a water sample can thus be determined.

Specific assays for many ARGs have been designed and validated for both conventional PCR and qPCR. Required instrumentation is not cost prohibitive and is available in most labs set up for molecular biology work. In addition, the cost per reaction is relatively low, and assays can be run quickly, allowing for rapid sample analysis and generation of results. These techniques are limited by the fact that generally only one ARG can be tested per reaction, and so cost and effort can increase if a whole panel of ARG are to be tested for. In addition, these assays cannot identify the source organisms for the tested fragment, and therefore it cannot be determined is

identified ARGs are from pathogenic organisms that would represent an increased risk to human health.

Next-generation sequencing

Next-generation sequencing (NGS) DNA sequencing encompasses a broad range of technologies that have emerged over the last decade, including platforms from Illumina, PacBio, and Oxford Nanopore, among others. While the methodologies underlying these different sequencing technologies vary greatly, they all are characterized by the ability to read thousands or millions of individual sequences in parallel. This is in dramatic contrast to the Sanger method of DNA sequencing, in use since its invention in the 1970s, in which only one DNA sequence at a time could be determined. This massive increase in the ability to acquire DNA sequences, along with precipitous decreases in the per base cost of such sequencing, has revolutionized biological research in the last few years.

For detection of AMR and ARGs these technologies allow the entire population bacteria and genes present in an environmental sample (such as one taken from a wastewater treatment system or its effluent) to be analyzed in a parallel. Regardless of the platform used, there are two approaches to sequencing acquisition that are particularly relevant, metabarcoding and metagenomics.

Metabarcoding of bacterial populations is used to characterize what bacteria are present in a sample. All bacteria share certain genes that are required for basic biological functions such as DNA replication, RNA transcription, and protein synthesis. One of these genes, the 16S ribosomal RNA (16S rRNA) gene has proven particularly useful for identification of a wide range of bacteria. Although metabarcoding cannot directly identify which bacteria in a community possess ARGs and can provide important information on community composition. Another important caveat is that this approach is that the data obtained may not identify bacteria to the "species" level, but rather only as being part of a broader phylogenetic clade, or closely related group of bacteria. This is particularly relevant with respect to pathogenic bacteria, some of which have closely related relatives that are not pathogenic. Currently widely used metagenomic approaches may not provide sufficient resolution to distinguish between pathogenic and non-pathogenic members of a group. This limitation can be overcome with additional sequencing, but this also entails additional effort and cost.

Metagenomic analysis produces DNA sequence data from across the genomes of the bacteria and other organisms present in a sample. After DNA from a sample is isolated, it is fragmented into smaller pieces, and randomly labelled for sequencing. Millions or fragments are then sequenced in parallel. Using computational methods, the resultant DNA sequence fragments can be matched to one another to reconstitute longer stretches of DNA through a process called assembly. The contigs resulting from this assembly can be compared to databases of now sequences to characterize the genes in the sampled population. Though this method ARGs in the population can be identified. However, these ARGs generally cannot be matched to the bacteria they were derived from, and so it cannot be determined with certainty whether the ARGs reside in pathogenic bacteria that pose a direct risk to human health. However, there are emerging technologies that may facilitate the matching of ARGs and the bacteria that possess them.

Although all NGS technologies produce large numbers of DNA sequences in parallel, that are important technical differences that are important to consider. Illumina sequencing is currently the most widely used NGS platform. Illumina sequencing produces sequences with relatively short read lengths, 150 basepairs (bp) being the most common. This means that for metagenomics any individual sample is not likely to provide sufficient information for gene characterization. These reads are always subject to assembly prior to any downstream characterization. Because millions of sequences are produced, and the error rate of the reads is very low, high quality contigs can be produced. But the assembly process is computationally intensive, and conducting these analyses currently require considerable expertise. In addition, the high capital costs for instrumentation and intensive process of sample preparation mean that Illumina sequencing is almost always performed by a commercial laboratory, rather than onsite. This coupled with the several days being required for samples preparation and sequence generation mean that this technology will likely be valuable for characterizing wastewater treatment and water reuse systems but may not be suitable for regular monitoring where rapid analysis of data is required for effective corrective actions to be taking.

Recently, there has be interest in nanopore based NGS DNA sequencing technology, particularly the MiniION and related instruments from Oxford Nanopore. This technology has several enticing attributes. The instruments can very small, allowing portability and use on site. Sample prep and sequencing are rapid, providing the potential for near real-time data analysis. Also, the length of sequence reads is not constrained as it is for Illumina, so longer reads can be acquired to aid with species identification and metagenomic assembly. A major downside of this platform is that per base accuracy is not nearly as high as with Illumina platforms. This means that even with derivation of consensus sequences from overlapping fragments included in assemblies, the potential exists for sequencing errors that could confound the results of downstream analyses. How this may affect applications such as identification of ARGs from environmental samples is currently being investigated.

Future prospects

As development of water reuse increases, so to will the need to evaluate risks associated with transmission of AMR and ARGs. Analysis of these issues is going significant attention, and the technologies available to study and monitor these phenomena are developing rapidly. At the present the computational expertise required for analysis of NGS data still represents a barrier to entry for application of these techniques by most for the water industry. However, it is expected that in the near future best practices will be established for use of NGS technologies in monitoring AMR and ARGs, and with this, more streamlined and accessible workflows for data analysis will become available.

Note

A reference list of publications cited in this report is presented below. A more comprehensive bibliography of publications relating to the topics addressed in this report is presented in Appendix A.

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