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REVIEWS

Review

Antibiotic-Resistance Genes in Waste Water

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Waste water and waste water treatment plants can act as reservoirs and environmental suppliers of antibiotic resistance. They have also been proposed to be hotspots for horizontal gene transfer, enabling the spread of antibiotic resistance genes between different bacterial species. Waste water contains antibiotics, disinfectants, and metals which can form a selection pressure for antibiotic resistance, even in low concentrations. Our knowledge of antibiotic resistance in waste water has increased tremendously in the past few years with advances in the molecular methods available. However, there are still some gaps in our knowledge on the subject, such as how active is horizontal gene transfer in waste water and what is the role of the waste water treatment plant in the environmental resistome? The purpose of this review is to briefly describe some of the main methods for studying antibiotic resistance in waste waters and the latest research and main knowledge gaps on the issue. In addition, some future research directions are proposed.

Trends

Waste water and waste water treatment plants are potential hot spots of selection of antibiotic resistance and horizontal gene transfer.

Hospitals are only a small proportion of the sources of antibiotics, antibiotic-resistant bacteria (ARB), and antibiotic-resistance genes (ARGs); municipal wastes are also vital sources.

Mobile genetic elements are likely to play a role in the dissemination of antibiotic resistance in waste water.

The detection of an ARG does not mean that this is conferring resistance in the host. Thus, molecular methods are needed that can distinguish between ARG carriage in the host chromosome and ARG which confers resistance or a risk to the treatment of pathogens.

Waste waters contain traces of antibiotics and other compounds which can cause a selection pressure for antibiotic resistance, and even low concentrations are able to cause selection pressure.

Waste Water Is a Meeting Place for Antibiotics, Antibiotic-Resistance Genes, and Bacteria from Different Sources

There is a global concern about the spread of antibiotic resistance, and the problem is not restricted to the clinic, even though the consequences are clinical. Most of the antibiotics given to humans are used in the household and eventually end up in the sewage. Therefore urban waste water treatment plants (WWTPs) are among the main sources of both antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) released into the environment [1,2]. WWTPs receive sewage from various sources, and bacteria from different environments, making it possible for the bacteria to interact and exchange genes horizontally. WWTPs can act as reservoirs and environmental suppliers of antibiotic resistance and have been proposed to be hotspots for horizontal gene transfer (HGT), enabling even broader dissemination of ARGs [3,4]. However, clear evidence showing the evolution of resistance and the spread ARGs in WWTPs is still not widely available. The high bacterial densities, biofilms, and stress caused by pollutant compounds, such as antibiotics, biocides, pharmaceuticals, and heavy metals, can promote horizontal gene transfer in waste waters [5]. In fact, WWTPs are a unique interface between human society and the environment as sewage from households and hospitals contain antibiotics and bacteria of human origin, potentially providing a selective pressure for ARB and ARGs prior to their release into the environment [4]. The concentrations of different compounds that can select for antibiotic resistance are below therapeutic concentrations used in clinical settings [6]. Even sub-MIC (minimal inhibitory concentration) levels have been shown to select for resistance phenotypes, but the studies have normally used simplified communities (reviewed in [7]), so the effects on complex communities are still largely unknown, although some work has been done [8]. Also, the bioavailability of the compounds and their fate during the treatment process vary depending on the compound. This unique environment,

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 https://doi.org/10.1016/j.tim.2017.09.005

containing a mixture of compounds, may pose a serious threat of spreading resistance, possibly to pathogenic bacteria, and this should be studied further. This review focuses on the methods used to study antibiotic resistance in waste waters and the current view and knowledge gaps related to the subject.

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Methods Used for Analyzing ARGs

For the past 70 years, research in antibiotic resistance has focused mainly on pathogens. Isolating pure cultures has been, and still is, the most important method in clinical microbiology. Antibiotic susceptibility testing of bacteria is relatively inexpensive and gives important data on resistance patterns that are needed for the clinical treatment of patients. Databases of clinical breakpoints (such as EUCAST, www.eucast.org) help in monitoring antibiotic resistance worldwide. However, clinical breakpoints cannot be applied to bacteria in waste water. In addition to clinical breakpoints, EUCAST has established common epidemiological cut-off values for resistance (ECOFFs), which can, in principle, be applied to waste waters; however, the drawback of ECOFF is that it requires the analysis of a large number of independent isolates which reduces its use in waste waters [9]. In general, culturing and susceptibility testing have their limits with environmental bacteria [10], as only a fraction of environmental bacteria can be grown under laboratory conditions. However, when combined with molecular biology tools, data from susceptibility testing can be used to find previously unknown resistance determinants, either intrinsic or acquired through mutations or horizontal gene transfer. Sequencing of whole microbial genomes gives insight about the genetic environment of the ARGs. Genes located on mobile genetic elements, capable of horizontal transfer, pose a bigger risk for the spread of resistance [9,11–13].

Quantitative PCR

PCR and quantitative PCR (qPCR) methods can be used in the analysis of genes from environmental DNA without the need for culturing. The need for prior knowledge of primer design limits their use to known genes or to genes with high homology to known ones. High-throughput qPCR arrays can address the throughput limitations associated with traditional qPCR [14–16]. With the qPCR array, the simultaneous quantification of hundreds of ARGs and other genes of interest is possible as parallel assays in just one run. This creates an opportunity for quantification of many relevant ARGs, sequences related to mobile genetic elements, and genes specific to certain bacterial species in WWTPs or related environments.

Metagenomics

Metagenomics, the sequencing of the whole-community DNA, can overcome the need for prior knowledge of resistance genes. Metagenomics has been used to detect antibiotic resistance in diverse environments [17–23] and is not restricted to few *a priori* chosen genes but, through sequencing the total community DNA, can capture the whole resistome. However, the annotation of ARGs still relies on known genes in public antibiotic-resistance gene databases [24–29]. The most reliable are those that contain only experimentally verified ARGs [30]. Also, the HMM (Hidden Markov Model)-based database, Resfams [24], and updated version of the CARD database [28], contain sets of verified genes.

In most environments, ARGs are rare in number in comparison to other functional genes, and therefore deep sequencing is needed to capture the whole diversity [21,31]. Most metagenomic sequencing platforms produce short reads that, as such, give only limited information about the sequenced genes. Assembling short reads to longer overlapping DNA segments (contigs) can give information about the phylogeny and genetic location of the genes. Partial or even complete genomes can be reconstructed from metagenome data [32,33]. This knowledge is important in ranking the risks of ARGs in the environment.

Functional Metagenomics

Functional metagenomics, the cloning and expression of environmental DNA in a laboratory host, can overcome the limits of PCR and metagenomic sequencing in detecting mostly known resistance genes. In functional metagenomics, environmental DNA is cloned in large fragments (10–200 kb) in a laboratory host, for example, *Escherichia coli*, and the susceptibility of the host to different antibiotics is tested. Clones with a resistance phenotype are screened for the antibiotic-resistance determinant by subcloning, mutagenesis, or *in silico* analysis, which can be laborious and time consuming. Cloning and expressing the genes in the host can be difficult and are the main disadvantages of functional metagenomics, although they can be solved to some extent by using hosts other than *E. coli*. Proteomics combined with functional metagenomics is a promising new way to overcome the tedious screening of potential clones containing each of the segments of DNA. Using proteomic tools in combination with functional metagenomics, the expressed proteins can be identified in a high-throughput manner and by comparing to a strain without the cloned DNA and the putative new resistance determinants identified [34].

Emerging Methods

The development of new methods constantly brings new possibilities for the analysis of ARGs in the microbial community. There is an urgent need for a method that could resolve the host of an ARG without culturing and in a high-throughput format. Recently published epicPCR [35], is one such promising tool. It is a generic method for linking two genes, originating from one cell, to one amplicon, which can be sequenced. If one gene partner is a 16S RNA gene, the method can be used to find out the host of an ARG. Also, new high-throughput single-cell genome sequencing techniques, where >50 000 cells can be analyzed at once, might be promising in analyzing WWTP bacterial communities [36].

Another interesting feature of an ARG, and one which has relevance in terms of phenotype, is its genetic environment. If an ARG is situated in a mobile genetic element (MGE) it is evaluated to possess an increased risk in the proposed risk analysis [9]. A combination of inverse-PCR with a long-read sequencing platform has recently been shown to be useful in the determination of the genetic environment of tetracycline- and sulfonamide-resistance genes [37]. When compared with metagenomics, the developed method was superior in detecting the ARGs in sediment under a fish farm since the ARGs were present in low frequency in the metagenomes. The metagenomic analysis of low-frequency genes can potentially be facilitated by a gene-capture approach in which the ARGs are captured from the isolated DNA before sequencing [38].

There exists a wide variety of different methods for studying antibiotic resistance in waste waters and, depending on the research question, the researchers need to choose the best one to answer the question. The reduction in sequencing costs will probably advance the study of antibiotic resistance in WWTPs in the near future. Even though new methods are developed, and seem tempting, all methods are still relevant and are needed to study antibiotic resistance in all its dimensions.

Transfer of Antibiotic-Resistance Genes

The high density of bacteria in WWTPs could provide an optimum environment for HGT among environmental bacteria and human pathogens [39]. The ARG-related MGEs have been most frequently identified in the cultured indicator bacteria *Enterococcus* and coliforms. The location of ARGs on MGEs, such as plasmids, transposons, and integrons, makes the transfer of resistance possible and easy to achieve among bacteria with the same or different origins [40]. The transfer of resistance plasmids of *Enterococcus faecalis* in the activated sludge of two WWTPs in Germany was examined [41]. The transfer rates between different strains of *E. faecalis* resistance plasmids that have a broad host range for Gram-positive bacteria in

the activated sludge conditions were at least 10 times lower than they were under laboratory conditions. The ARGs were located in multidrug-resistance (MDR) plasmids which could have been transferred into an *E. coli* recipient strain, indicating a high possibility of HGT among bacteria in the waste water environment [42].

Recent work has also demonstrated that HGT is promoted in subinhibitory concentrations of antibiotics [43] and that positive selection might even inhibit HGT by eliminating possible recipients in the environment [44]. However, the analysis of ARG transfer in WWTPs is seriously hindered by the lack of suitable methods for high-throughput assessment of ARG transfer under real WWTP conditions, so it is not surprising that there is a knowledge gap in our understanding of the transfer of ARGs in WWTPs [45,46].

Selection of ARGs in Waste Water

Selection pressure is a key issue in the presence and dissemination of ARGs in waste water (Figure 1, Key Figure). It is now well established that even low concentrations of antibiotics can result in the selection of ARGs [47], which makes it very difficult to establish a safe concentration of an antibiotic compound in the waste water. It must be noted that these experiments have been performed in simple communities, and the concentrations selective in diverse communities found in waste water are still to be assessed. Moreover, it is difficult to assess the bioavailable concentrations of antibiotics in waste water for different bacterial species in real conditions; this leaves us with the possibility of either an over- or underestimation of the selection pressure.

One MGE can, and often does, contain resistance genes for more than one antibiotic compound, meaning that a resistance gene can be selected by a wide range of antibiotics. Furthermore, the same mobile element can also contain a resistance gene for a disinfectant or a metal, which leads to the situation where antibiotic resistance is selected by those compounds [48].

Waste Water Treatment and Antibiotic Resistance

Once ARB successfully enter WWTPs, they can spread their resistance determinants among bacteria of the endogenous microbial community and those transiting through the WWTP. ARB have been found in WWTPs and in their effluent, indicating that WWTPs are not fully effective at removing these bacteria [1,49–51]. However, the overall levels of resistance are reduced due to the treatment as the bacterial loads are reduced 10–100-fold, and the ARGs are also reduced, but not eliminated [31,52]. The resistance profiles of such bacteria comprise resistance to all clinically important antibiotics. Classical microbiology methods, such as cultivation and antibiotic susceptibility testing, as well as culture-independent methods, have been used to detect ARB and genes [31,39,52–58]. While hospitals contribute to the problems of ARB and ARGs, and they are considered as hotspots for the dissemination of ARB and ARGs, the actual evidence for their role is not strong [59]. Generally, hospital effluents contribute less than 1% of the total amount of municipal sewage, so hospital waste water is diluted extensively in WWTPs, suggesting that the municipal waste water also contains a high amount of ARB [60].

The predominant bacterial species analyzed to date from WWTPs belong to the common indicators of faecal contamination: *E. coli*, total coliforms, and enterococci. However, in addition to these bacteria, a wide variety of clinically important ARB have been detected, including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus* spp., and Gram-negative bacteria (e.g., *Enterobacteria*, *Pseudomonads*, and *Acinetobacter*). These bacteria were resistant to fluoroquinolones and carbapenems, and were producers of extended-spectrum β-lactamases [61–66]. The number of bacteria, including the total number of resistant bacteria, significantly decreases after the waste water treatment process [67,68].

Key Figure

Selection and Transfer of Antibiotic Resistance in Waste Water

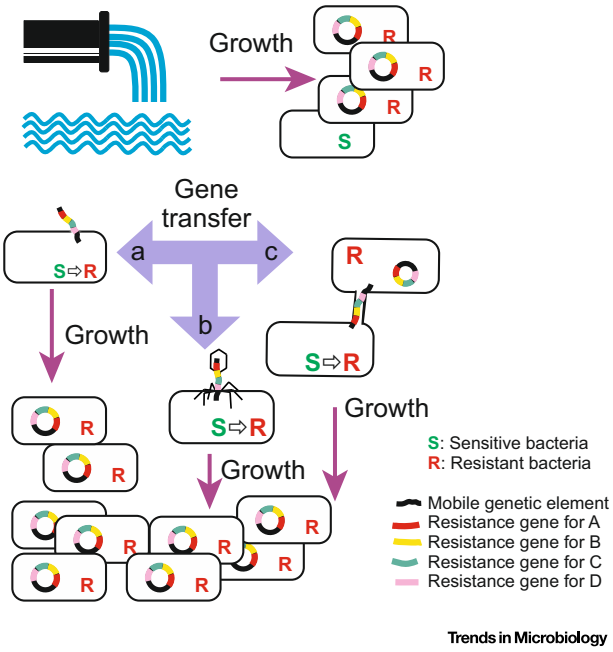

Figure 1. When there is selection pressure for antibiotic-resistant bacteria (ARB) (R), they overgrow the sensitive bacteria (S). The sensitive bacteria can become resistant by acquiring a resistance gene by transformation (a), transduction (b), or conjugation (c). Selection pressure can be caused by antibiotics, metals, or biocides present in the waste water. Selection pressure against one resistance gene can select other resistance genes also by coselection, as indicated by different resistance genes.

Guo *et al*. [69] identified a reduction in the proportion of heterotrophic bacteria resistant to erythromycin, cephalexin, gentamicin, and ciprofloxacin, while the proportion of bacteria resistant to sulfadiazine, vancomycin, rifampicin, and tetracycline increased after UV treatment in WWTPs. The operating conditions of the treatment system in WWTPs have different effects on the fates of ARB. For instance, in the study of Munir *et al*. [70], the concentrations of bacteria resistant to tetracycline and sulphonamides decreased several orders of magnitude in the treated water in comparison with the raw influent water, but the concentration of ARB remained very similar in pre- and post-disinfected effluents. However, the conditions in WWTPs may be favourable for the selection of ARB which, in turn, can transfer the resistance determinants to susceptible bacteria [71–74].

In addition to resistant bacteria detected with culture-dependent methods, culture-independent methods have been able to detect genes conferring resistance on all classes of antibiotics in WWTPs all over the world, and these genes can also be found in the WWTP effluent [31,52,54,70,75–78]. Activated sludge may be a reservoir of ARGs and a hotspot for ARG transfer between resident bacteria and those transiting the WWTPs. Thirty ARGs encoding resistance to tetracycline, sulphonamides, quinolones, or macrolides were identified by qPCR from the activated sludge of two WWTPs [79]. WWTPs can definitely be considered a hotspot for ARB and ARGs but the picture of the dynamics of antibiotic resistance in WWTPs is far from complete.

Activated Sludge

The activated sludge process is the usual method used to remove nutrients from waste water. In the process, microbes, produced in aerobic conditions, oxidize carbonaceous biological matter and nitrogenous matter and clump together to form the sludge – which can be

separated from the liquid phase, forming biosolids. Part of the sludge is recycled back to the process, but part of it is removed as excess sludge or sewage sludge. The excess sludge goes through anaerobic digestion and composting before it is used as, for example, land fill. It has been shown that, compared to sewage sludge, effluent contains different bacteria and antibiotics [31,52]; therefore, this part of the WWTP output should also be studied when analyzing the environmental effects of WWTPs. Karkman *et al*. [54] showed that *erm*(F) from macrolide, lincosamide, and streptogramins B (MLSB) resistance genes, and the tetracycline resistance genes, *tetP*(A) and *tetP*(B), were the most enriched genes in the digested and dried sludge. Similar results were found for genes encoding resistance to polymyxin, tetracycline, vancomycin, and MLSB class antibiotics in the WWTP sludge [52]. WWTP sludge was recognized as the main source of tetracycline- and sulfonamide-resistant bacteria and genes discharged into the water environment [70]. WWTP sludge can play an important role in the selection and spread of ARGs. Normally, the sludge collected from the process is anaerobically digested and further composted before application to the land. Still, some ARGs, such as genes conferring resistance to sulphonamides, tetracyclines, β-lactams, and vancomycin, have been shown to be enriched during digestion [31,52,54] and composting [80]. The growing demand to reuse the sewage sludge for important nutrients poses a risk of further dissemination of antibiotic resistance in the environment.

Effluent

The prevalence of ARB and ARGs in the rivers receiving WWTP effluent may increase downstream of the WWTP [81,82]. In the study of waste water samples in Germany, 123 clinically relevant ARGs were detected in the effluents, including aminoglycoside-, β-lactam-, chloramphenicol-, fluoroquinolone-, macrolide-, rifampicin-, tetracycline-, trimethoprim-, and sulphonamide-resistance genes, as well as genes encoding multidrug efflux pumps capable of conferring resistance to wide variety of compounds [57].

The analysis of the flow of 30 ARGs (20 *tet*, four *sul*, four *qnr,* and two *erm* genes) through each unit of the WWTPs in Northern China showed the proliferation and release of ARGs [79]. In the final effluent, there was a significant enrichment of the ten ARGs [*tet*(B), *tet*(G), *tet*(H), *tet*(S), *tet*(T), *tet*(X), *sul1*, *sul2*, *qnrB*, and *erm*(C)] in comparison to the 16S rRNA genes ($P < 0.05$). The ARB were also more resistant to chlorination than the susceptible bacteria. It was shown that there was a reduction in the abundance of ARGs from the raw influent to the effluent; however, 12 ARGs [*tet*(A), *tet*(B), *tet*(E), *tet*(G), *tet*(H), *tet*(S), *tet*(T), *tet*(X), *sul1*, *sul2*, *qnrB*, and *erm*(C)] were discharged from WWTPs at higher rates than were found in the influent [79]. ARG enrichment ratios ranged from 8 ± 1 [*tet*(G)] to 268 ± 248 [tet(T)], while the 16S rRNA resulted in 5 ± 2 [79]. The analysis of WWTPs in Hong Kong by metagenomic sequencing showed the seasonal change of few ARG types, and the decrease of genes in the WWTP effluent [21,52]. Yang *et al*. [52] indicated that most of the ARGs were removed from WWTP influent after the waste water treatment. Indeed, by using metagenomic sequencing, more than a 98% reduction of ARGs in the effluent, in comparison with the raw influent, was observed. The reduction in ARGs, after the treatment process, was also reported in some other studies [31,54,76]. Other studies showed that there was no change in the relative number of ARGs or that the number increased. By contrast, the enrichment of some ARGs was observed in the effluent community [75,76,83]. The selective conditions in WWTPs may provide a selective advantage for the ARGs and ARB, or for HGT among the bacterial community. The ARB may also be enriched due to other nonantibiotic selective pressures, such as metals or biocides [48,84].

Advanced Treatment Technologies

Advanced treatment technologies are methods that are targeted to remove emerging contaminants from waste water treated by the active sludge process. The main categories of emerging contaminants include pharmaceuticals, personal care products, endocrine-

disrupting compounds, surfactants, pesticides, and flame retardants. The advanced treatment technologies, such as photocatalysis, membrane filtration, activated carbon adsorption, and advanced oxidation processes (AOPs), have been shown to be efficient in removing emerging contaminants from waste water [85]. However, the ARGs are not necessarily removed by advanced treatment technologies. Moreover, at least some of the technologies create conditions that induce the SOS response in bacteria. The SOS response increases the mutation rate in bacteria by increasing the expression of error-prone DNA polymerases [86] and HGT of ARGs [87]. The possible contribution of advanced treatment technologies to the dissemination of ARGs should be evaluated before these processes are implemented in large scale.

Concluding Remarks and Future Perspectives

Waste water and WWTPs are considered as potential hot spots for the dissemination of antibiotic resistance and the transfer of resistance genes. Indeed, resistance genes are found universally in municipal, hospital, and relevant industrial waste waters. However, it is currently difficult to tell how much is too much by using quantitative analysis, that is, to assess if a difference in the quantity of ARGs in a particular environment is relevant. There is also an urgent need to obtain expression data for ARGs in different environments (see Outstanding Questions). Different WWTPs seem to give different results for the removal of ARGs, but we do not really know if that is because of how they have been constructed and managed or their microbial contents, or other factors. Comparison of WWTPs is also hindered by the lack of standards or generally adopted methods and protocols. There are justified concerns that the advanced treatment step may promote antibiotic resistance by inducing bacterial stress, and those concerns should be resolved rather quickly before large-scale investments in the waste water treatment plants are made globally. The risk analysis should be developed further before we can draw conclusions on the actual risk of the ARGs in waste waters. In particular, we should find out effective ways to proceed beyond the quantity and/or sequence of the resistance genes, and there are at least candidate methods to do that. Only after understanding which organisms carry ARGs, and how mobile these genes are, we can make evidence-based conclusions on the risk caused by antibiotic resistance in waste waters and the possible mitigation of those risks.

Outstanding Questions

How to develop methods to go beyond the quantity/sequence of a gene without cultivation? Who has the genes, and how mobile are the genes, should be answered in order to measure the relevance of the genes in context.

How to assess if a difference in the quantities of AMR genes is relevant? How much is a risk? What should be used for comparison? Which of these genes are expressed, and to what differing levels are they expressed in waste water and WWTPs?

Different WWTPs seem to give different results; is this due to their construction and management? The data that would enable comparison should be produced. These data should include the selective concentrations in the WWTP and possible coselection patterns.

Which ARB and ARGs are the WWTPs (classified according to levels of treatment, e.g., primary, secondary, tertiary) capable of removing? Which do they never remove, and for which are the WWTP bacterial communities sources of antibiotic resistance?

Which ARB and ARGs can survive in the environment after they are released from UWTPs (e.g., surviving to 10 km downstream)?

Which ARB and ARGs can survive in animals or people that drink the water?

What novel ARB and ARGs are present in our WWTPs currently, and what levels of risk do they pose to human and animal health?

How much money are we willing to invest to ensure that the risks from waste water and WWTPs are minimised?

Acknowledgments

M.V. was supported by Academy of Finland (Grant Nos. 256811, 268643, and 288897) and the Water JPI (Pilot Call project StARE). F.W. and T.T.D. were supported by the Water JPI (Pilot call project StARE) funded national by the Environmental Protection Agency. F.W. was supported by the Department of Agriculture, Fisheries and the Marine and has received funding from Alltech.

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