water

MDPI

*Perspective*

# Reusing Treated Wastewater: Consideration of the Safety Aspects Associated with Antibiotic-Resistant Bacteria and Antibiotic Resistance Genes

**Pei-Ying Hong [1,*], Timothy R. Julian [2,3,4], Marie-Laure Pype [5], Sunny C. Jiang [6], Kara L. Nelson [7], David Graham [8], Amy Pruden [9] and Célia M. Manaia [10,*]**

1 Water Desalination and Reuse Center, Division of Biological and Environmental Science and Engineering, King Abdullah University of Science and Technology, Thuwal 23955-6900, Saudi Arabia
2 Eawag, Swiss Federal Institute of Aquatic Science and Technology, CH-8600 Dübendorf, Switzerland; tim.julian@eawag.ch
3 Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, P.O. Box, CH-4002 Basel, Switzerland
4 University of Basel, P.O. Box, CH-4003 Basel, Switzerland
5 Advanced Water Management Centre, The University of Queensland, Brisbane, QLD 4072, Australia; m.pype@awmc.uq.edu.au
6 Department of Civil and Environmental Engineering, University of California, Irvine, CA 92697, USA; sjiang@uci.edu
7 Engineering Research Center for Re-Inventing the Nation's Urban Water Infrastructure (ReNUWIt), Department of Civil and Environmental Engineering, University of California, Berkeley, CA 94720, USA; karanelson@berkeley.edu
8 School of Engineering, Newcastle University, Newcastle upon Tyne NE1 7RU, UK; david.graham@newcastle.ac.uk
9 Department of Civil and Environmental Engineering, Virginia Tech, Blacksburg, VA 24061, USA; apruden@vt.edu
10 CBQF—Centro de Biotecnologia e Química Fina—Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Arquiteto Lobão Vital, Apartado 2511, 4202-401 Porto, Portugal
* Correspondence: peiying.hong@kaust.edu.sa (P.-Y.H.); cmanaia@porto.ucp.pt (C.M.M.)

Received: 13 February 2018; Accepted: 24 February 2018; Published: 27 February 2018

**Abstract:** As more countries engage in water reuse, either intended or de facto, there is an urgent need to more comprehensively evaluate resulting environmental and public health concerns. While antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are increasingly coming under the spotlight, as emerging contaminants, existing water reuse regulations and guidelines do not adequately address these concerns. This perspectives paper seeks to frame the various challenges that need to be resolved to identify meaningful and realistic target types and levels of antibiotic resistance benchmarks for water reuse. First, there is the need for standardized and agreed-upon methodologies to identify and quantify ARB and ARGs. Second, even if methodologies are available, identifying which ARB and ARGs to monitor that would best relate to the occurrence of disease burden remains unknown. Third, a framework tailored to assessing the risks associated with ARB and ARGs during reuse is urgently needed. Fourth, similar to protecting drinking water sources, strategies to prevent dissemination of ARB and ARGs via wastewater treatment and reuse are required to ensure that appropriate barriers are emplaced. Finally, current wastewater treatment technologies could benefit from modification or retrofit to more effectively remove ARB and ARGs while also producing a high quality product for water and resource recovery. This perspectives paper highlights the need to consider ARB and ARGs when evaluating the overall safety aspects of water reuse and ways by which this may be accomplished.

**Keywords:** water reuse; wastewater treatment; source prevention; monitoring and surveillance; risk assessment

## 1. Introduction: Benefits of Water Reuse

At least 45 countries worldwide face moderate to severe water scarcity issues [1]. Water stresses are further compounded by the need to utilize freshwater for food production. Currently, about 70% of the global freshwater supplies are withdrawn for agricultural irrigation [2]. Food production is water-thirsty and with the projected increase in global human population from the current 7 billion to 9.7 billion in 2050, the amount of water that is to be channeled into food production is expected to increase. Intensive mining of groundwater supplies is unsustainable and alternative water resources must be considered.

Worldwide, ~60% of all irrigated croplands fall within 20 km of an urban area, making irrigation with municipal wastewater a potential option [3]. Municipal wastewater provides several advantages over other alternative sources of irrigation water—It is a reliable water source for farmers, hence expanding the growing season and the variety of crops that can be grown [4]. Furthermore, depending on the degree of treatment, wastewater generally contains higher nutrient content as compared to groundwater. Such nutrients can be assimilated by crops, which can increase crop yields and the decrease reliance on chemical fertilizers. The intentional reuse of treated wastewater for agricultural irrigation is already practiced throughout the world—about 5000 $km^2$ are irrigated with treated wastewater worldwide [1]. On the other hand, the majority of the world's wastewater is not treated and is discharged directly into the environment, contaminating surface waters. An estimated $2.9 \times 10^7$ $km^2$ of croplands (14% of all irrigated croplands) are dependent on surface waters that are likely to contain a moderate to high fraction of inadequately treated wastewater [5].

The intentional reuse of treated wastewater for other non-potable purposes (e.g., landscape irrigation, cooling towers, toilet flushing) also occurs throughout the world; in some cases, wastewater is even being treated to permit potable reuse [6]. The majority of the wastewater treatment plants (WWTP) worldwide utilize some form of settling and biological treatment, often with disinfection as the final step. Activated sludge is one common type of biological treatment, and was first developed more than one hundred years ago by Ardern and Lockett [7], with the main intention of reducing organics through biodegradation and metabolic processes carried out by dense and highly active microbial populations. Public health has improved drastically over the last century, with a marked decrease in waterborne disease outbreak, as WWTPs became a widely implemented foundation of urban infrastructure. Various benchmarks for assessing WWTP performance and resulting water quality have evolved over the years, with most existing regulations requiring utilities to assess basic parameters, such as total suspended solids, chemical oxygen demand, biochemical oxygen demand, ammonia, nitrate, total phosphorus, and fecal coliforms or *Escherichia coli*.

In recent decades, "emerging" contaminants have been identified that may present "new" environmental and public health concerns (designated as contaminants of emerging concern, CECs) that are not necessarily represented by traditional means of assessing water quality. Substantial effort has been placed on improving the activated sludge process and developing new and advanced treatment technologies for removing pharmaceuticals and personal care products (PCPPs) [8,9]. However, most recent concerns regarding the need for water reuse to provide a safeguard against the spread of antibiotic resistance remain to be addressed by the available treatment technologies and existing regulations.

Given that the threat from antimicrobial resistance "has reached alarming levels in many parts of the world and that in some settings, few, if any, of the available treatments options remain effective for common infections" [10], urgent action is needed in all sectors, including water reuse. Successive warnings that antibiotic resistance is one of the most serious human health threats have

arisen in parallel with the growing evidence of rising levels of antibiotic-resistant bacteria (ARB) and the antibiotic resistance genes (ARGs) that they carry in the aquatic environment associated with anthropogenic inputs [10–12]. ARGs enable bacteria to survive in the presence of antibiotics and belong to various classes corresponding to their mechanism of action and the class of antibiotic to which they encode resistance. A general pattern has been observed, in which ARB and ARGs emerge not long after new antibiotics are introduced to the market [13]. Correspondingly, ARGs associated with particularly life-threatening infections, encoding resistance to a wide range of antibiotics, have been detected in WWTPs or in the environment soon after the first reports of clinical cases [14,15]. Phenomena of proliferation, bioaccumulation, and transport have been observed for ARB and ARGs in human-impacted environments, bringing to light the potential for exposure to humans and the need to delineate possible health risks [11,16,17].

The fact that WWTPs receive wastewater that is laden with ARB and ARGs; along with residual antibiotics, metals, and other potential selective agents, cannot be ignored, and it is thus critical to establish fundamental understanding of the factors that contribute to their proliferation and dissipation in the treatment process and to identify effective barriers to their dissemination during subsequent reuse. Notably, the highly dense and active microbial populations within a WWTP are potentially an ideal setting for selection of ARB and ARGs in treated effluents as well as a site for horizontal gene transfer (HGT), or sharing, of ARGs among the bacterial populations. For example, bacteriophages are found at extremely high concentrations in secondary sewage effluent, in the range of $10^7$ to $10^8$ virus-like particles (VLP) per milliliter [18], where HGT is possible as they mix with and infect the dense bacterial populations at a high infection rate [19,20]. The active growing bacteria encourage rapid lytic viral replication and large viral burst size [21], conditions that are well-suited for generalized gene transfer by transduction, in which bacteriophages mistakenly pick up and transfer bacterial DNA fragments, including ARGs [22]. Such concerns arising from ARB and ARGs may thus impede subsequent efforts to reuse the treated wastewaters.

With this context in mind, there is a need to include a more comprehensive assessment of the role of wastewater treatment and reuse in the propagation and dissemination of antibiotic resistance. This will build up the needed scientific knowledge that is required to inform effective mitigation strategies for ARB and ARGs and minimize the potential risks that are associated with water reuse. Through this perspectives paper, the aim is to highlight the need to consider ARB and ARGs as emerging contaminants in wastewater, to understand the risks that are incurred from ARB and ARGs in water reuse contexts, and to lay out various intervention strategies that can be utilized to mitigate the risks that are associated with these emerging contaminants. To achieve this aim, the following outline topics are discussed in the context of antibiotic resistance and water reuse:

(i) Current regulations pertaining to microbiological water quality
(ii) Monitoring and surveillance associated with water reuse
(iii) Risk assessment of ARB and ARGs
(iv) Mitigation approaches to safeguard water quality for reuse

## 2. Current Regulations Pertaining to Microbiological Water Quality

Using the European Union (EU) as an example, in spite of the abundance of freshwater in most European regions, the Wastewater Directive recommends that treated wastewater should be reused whenever appropriate [23]. Although this recommendation was made as early as 1991, a common European legislation on water reuse is still lacking to this date. Instead, some countries have developed their own recommendations regarding the reuse of treated wastewater. Recommended parameters for monitoring the quality of recycled water include microbiological parameters, in particular bacteria of enteric origin and indicators of possible fecal contamination (see Table 1 and those that are listed by Becerra-Castro et al. [24]).

**Table 1.** Type of parameters and treatment objectives in different sectors of the urban water cycle according to European legislation.

| Type of Water | Type of Parameters | References |
| --- | --- | --- |
| **Wastewater** | Chemical oxygen demand, COD<br>Biochemical oxygen demand, BOD—(can be replaced by total organic carbon, TOC, or total oxygen demand, TOD)<br>(BOD5 at 20 °C)<br>Total suspended solids<br>Total phosphorus<br>Total nitrogen | [23,25] |
| **Water for reuse** | pH<br>Electrical conductivity, EC<br>Sodium absorption rate, SAR<br>Total suspended solids, TSS<br>Biological oxygen demand, BOD<br>Chemical oxygen demand, COD<br>Total nitrogen, TN or Nitrate nitrogen, or N–$NO_3$<br>Phosphate<br>Sulphate<br>**Faecal coliforms** or *Escherichia coli*<br>Nematode eggs | See [24] for examples of guidelines for Italy, Spain, Portugal, France. |
| **Surface water** | 45 priority substances or groups of substances, 21 of which classified as priority hazardous substance.<br>Includes plant protection products, biocides, metals and other groups like Polyaromatic Hydrocarbons (PAH) that are mainly incineration by-products and Polybrominated Biphenylethers (PBDE) that are used as flame retardants | [26,27] |
| **Drinking water** | A total of 48 microbiological, chemical and indicator parameters must be monitored and tested regularly<br>Microbiological:<br>*Escherichia coli*<br>**Enterococci**<br>***Pseudomonas aeruginosa***<br>***Clostridium perfringens* including spores**<br>**Other heterotrophic bacteria** | [28] |

Note: Microbial contaminants are in bold.

Rationale for the assessment of water quality for reuse purposes is largely derived from minimum quality requirements established for drinking water, in which the presence of bacteria of enteric origin above a certain threshold is considered a criterion of non-compliance (Table 1). In the Drinking Water Directive, target thresholds for various water quality parameters have been selected based on a combination of scientific information and the precautionary principle. Generally, quality requirements are established, assuming that water intended for human consumption must protect consumer health on a life-long basis. For example, the World Health Organization (WHO) recommends establishing water quality guidelines for different practices using a uniform risk-based approach based on Disability-Adjusted Life Years (DALYs). This approach allows for risk to be quantified and compared for all types of disease, including acute and chronic, infectious and non-infectious; where the conversion from illness to DALYs is based on the severity of the disease [29]. A commonly used acceptable burden of disease is $10^{-6}$ DALYs per person per year (pppy). This risk level corresponds to maintaining genotoxins to levels that limit lifetime cancer risk to less than 1 in 100,000 per person [30]. This risk threshold is also recommended for establishing water quality targets for reuse in agriculture. The corresponding tolerable risk for diarrheal pathogens is approximately 1 in 1000 annual risk per person [31]. While this is a useful framework that could also be applied to assessing risk from ARB, it is not straightforward, based on current data, to define equivalent risk endpoints.

Although fecal coliforms are commonly considered in guidelines of reclaimed water intended for crop irrigation as indicators of fecal pathogens that cause diarrheal illness, ARB and ARGs are not currently considered. There are various barriers to more meaningful and intentional guidelines to protect against the spread of antibiotic resistance via water reuse. In particular, (i) the recognition of the importance of ARB and ARGs as contaminants is relatively new; (ii) threshold values have not been established and it will be difficult to do so; (iii) mechanisms that influence their selection and

fate in water and wastewater treatment systems are still poorly understood; (iv) there are logistical and economical challenges to putting in place cost-effective and sensitive monitoring and surveillance methodologies; and (v) no consensus among the relevant stakeholders on the relative risk posed by water reuse compared to other pathways that may contribute to the spread of antibiotic resistance.

Such challenges have motivated discussion centered on defining maximum admissible values of ARB and ARGs in treated wastewater [11,32]. Ideally, the maximum admissible value should correspond to the minimal risk of transmission and infection to humans. Such a value is still very difficult to estimate in the face of limited knowledge about the most prominent sources, paths of dissemination, and modes of transmission/colonization/infection in humans [17]. However, as for other environmental contaminants, the application of the precautionary principle to the dissemination of ARB and ARGs is logical.

Several questions arise in moving towards proposing maximum admissible values for ARB and ARGs. First, even if a given threshold value is proposed and is possible to achieve through wastewater treatment, it is not possible to guarantee that ARB and ARGs will not recover and proliferate subsequently when the effluent is released to the environment. This is not unlikely, given the complex microbial ecology and interplay among bacteria inhabiting sewer lines, water distribution system biofilms, river sediments, groundwater, and other receiving environments. Thus, monitoring efforts should ideally encompass such receiving environments, with selection of threshold targets and values taking into consideration potential for survival, regrowth, and HGT between ARB and ARGs with native bacteria residing in such environments. These phenomena are still not fully understood and are difficult to predict.

To resolve this uncertainty, one can propose that a threshold of maximum admissible ARB or ARGs in treated wastewater should be "low enough" to reduce the risk of subsequent proliferation to very low levels (acceptable risk threshold). However, again there are some obstacles. One is that it is difficult to define the "low enough" value; the other is that to propose such a threshold, it would be necessary to guarantee that wastewater treatment and disinfection strategies could reach such a low value in an affordable manner [33,34]. This may not be realistic, especially for smaller wastewater treatment facilities and for low and middle-income regions. A second obstacle is that routine monitoring methods to measure ARB and ARGs would need to be able to target such "low levels", meaning that very low quantification limits are required from these methods while maintaining the important characteristics of low cost, ease of use, and rapid turnaround time for analysis.

## 3. Monitoring and Surveillance of Antibiotic Resistance

In clinical settings, the current procedure that is established to determine if bacteria isolated from an infected host are resistant to antibiotics include cultivation, determination of phenotypic traits, serotyping, and antimicrobial susceptibility tests [35]. However, such procedures are often not suitable for use in environmental monitoring and surveillance programs given the cost and time requirements, nor do they always provide quantitative information. Also, culture-based methods do not provide a comprehensive picture of the broader ARG presence in the microbial community beyond the cultured target and the potential for HGT unless deeper reconnaissance and sequencing of isolates is performed, which is often impractical. However, knowing the abundance of a range of ARB along with ARGs they carry in wastewater provides valuable information for assessing the potential risks to human health through incorporation of wastewater unit process treatment efficiencies with risk models. Similarly, knowing the abundance of ARB and ARGs in the final effluent of WWTP allows for one to refine the assessment of treatment effectiveness and to determine the associated contaminant load discharged into the environment.

To address the needs for quantitative measurements, one can utilize various approaches, such as (i) enumeration of bacteria on antibiotic-supplemented culture media, (ii) quantitative polymerase chain reaction (qPCR) targeting specific ARB or ARG per volume or mass of sample, and (iii) high-throughput

DNA sequencing combined with measurement of total cell counts or total DNA, to allow determination of the absolute abundance of cells or genes.

The recent advancement of metagenomics-based approaches have provided a powerful means to give a snapshot of the diversity of ARGs and mobile genetic elements (MGEs) that facilitate HGT (e.g., plasmids, integron gene cassettes) in wastewater and environmental samples [36,37]. Metagenomics also makes it possible to assess the roles of other microbes besides bacteria, such as phages, in propagating resistance in WWTPs, as elucidated in a recent review paper [38].

To its credit, qPCR allows for the quantitation of gene targets with higher precision and lower detection limits when compared with conventional endpoint PCR. For example, up to $10^4$ gene copies (GC) of beta-lactamase $bla_{\text{TEM}}$ gene, $10^2$ GC of beta-lactamase $bla_{\text{CTX-M}}$ gene, and $10^2$ GC of *mec*A gene were found to be present in phages isolated from sewage [39]. Recent review papers have collated abundance of ARGs determined by qPCR in various wastewater effluent streams [40,41], while others discussed the use of metagenomics to track and perhaps to assign risks that are associated with ARGs [42–44]. An extensive elaboration of the various studies has demonstrated the use of these tools, and therefore will not be reiterated in this paper. However, depending on the ARB and ARG targets, the detection limits provided by qPCR and metagenomics may still not be low enough to allow protection of human health from these contaminants. Still, various sample concentration techniques can be applied if this truly is a limitation.

In moving towards selecting targets for environmental monitoring, it is important to recognize that all tools have limitations. Molecular methods like qPCR cannot directly differentiate between live or dead hosts, nor whether the DNA was extracellular, bacteria-associated, or phage-associated. Such distinctions are relevant to fully assessing human and environmental impacts. qPCR is also limited in that it is necessary to select one gene at a time, though arrays are now available that can target well over 280 genes [45]. Current high-throughput DNA sequencing platforms generate short read lengths of up to ~300 bp, which are random in terms of which gene segments are or are not captured given the "shot-gun" nature of the approach. Various bioinformatics approaches are under development to identify and classify ARGs that are obtained from shot-gun metagenomics sequencing, but are limited both by the length and quality of the read as well as the quality of available databases for identifying the sequences. Currently, several databases and pipelines are available to identify ARGs, MGEs, and other genes of interest [46–48], but there is need for improved curation and standardization for comparability purposes. New sequencing platforms, like PacBio, that provide long read lengths are now available and provide richer information for gene identification and also advance confidence in assembly. Assembly is the process by which overlapping portions of reads are identified to produce "contigs" and "scaffolds", enabling the prediction of genomes and moving towards identifying which ARB carry which ARGs.

There is no doubt that advances in DNA sequencing technologies will play a significant role in facilitating future surveillance of ARB and ARGs in the environment. However, sequencing methods are not inherently quantitative and provide only relative abundance of genes normalized to total reads. Hence, depending on the sequencing coverage, relative abundance obtained from sequencing approaches may vary substantially and not be conducive for comparisons across different studies/sample sets. If combined with a measure of total cell counts or total DNA (e.g., from qPCR of a universal target, flow cytometry, or direct microscopy), relative abundance of genes can be converted to total abundance, providing quantitative results. However, this is not yet a common approach and may not be accurate enough for risk assessments. Improvements are also needed in terms of detection limits, precision, and accuracy, especially if critical ARB or ARG dose thresholds are found to be very low or cannot be usefully correlated with genetic markers. Nevertheless, one particular advantage of metagenomics data is that it can be assembled in order to identify presence of ARBs on an MGE or within a pathogenic host. Reduction in cost can greatly facilitate the application of DNA-sequencing based tools to become standard protocols for monitoring ARB and ARGs.

The next point to consider is which ARB and ARG targets to monitor. Defining which ARB or ARG are likely to contribute to infection and disease burden has not been straightforward and is often fraught with uncertainty. Furthermore, ARB are known to be ubiquitously present in nature, even in "pristine" environments that have not been exposed to antibiotics [49,50]. Yet, bacterial populations were found to possess ARGs, either intrinsically present in their genome or acquired through HGT of MGEs. Thus, there is a key need to distinguish ARB and ARGs that are truly representative of risk relative to the background. Furthermore, as HGT itself is considered as a risk, monitoring a MGE, such as the clinical integron 1 gene cassette [51], which has been proposed as a comprehensive indicator of genetic recombination events and antibiotic resistance potential of anthropogenic origin [52], would be useful. There is, however, some debate about the utility of this marker. This is because most studies rely on detecting only the integrase gene alone without the detection of the recombinases and promoter sequence. Hence, the mere detection of integrase gene may not necessarily mean that the downstream ARGs contained within the gene cassette would undergo HGT (recombination) or be expressed.

Until suitable methods and targets are agreed upon, surveying a wide suite of ARB, ARGs, and MGEs is advisable, enabling the scientific community to move towards eventually identifying which targets most meaningfully represent increased disease risks and burdens. It may also be beneficial to align a portion of environmental surveillance efforts to match the priorities listed in the WHO's Combat Plan against Antimicrobial Resistance. Specifically, WHO has listed several pathogens as priority targets. This list includes carbapenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacteriaceae* (including *Klebsiella*, *E. coli*, *Serratia* and *Proteus*) [53].

## 4. Advancing Risk Assessment Frameworks for ARB and ARGs in Recycled Water

Quantitative microbial risk assessment (QMRA) framework provides a useful starting place for evaluating the risks that are associated with antibiotic resistance [29,54]. The classical QMRA typically consists of: (i) problem formulation or hazard identification; (ii) exposure assessment; (iii) dose-response relationships; and (iv) risk characterization and management (Figure 1). However, as illustrated here, this framework will require substantial adaptation to understand the risks from ARB and ARGs [16,55]. In particular, hazards should include both pathogenic and non-pathogenic ARB and ARGs due to the potential for HGT in the environment and within human hosts; the exposure assessment should account for HGT of newly acquired ARB in the environment; the dose response models should account for impacts of ARGs, history of antibiotic use, and HGT within the host. Finally, risk characterization should include impacts of ARB on infection, treatment delays and/or failures, and increased risks of both morbidity and mortality.

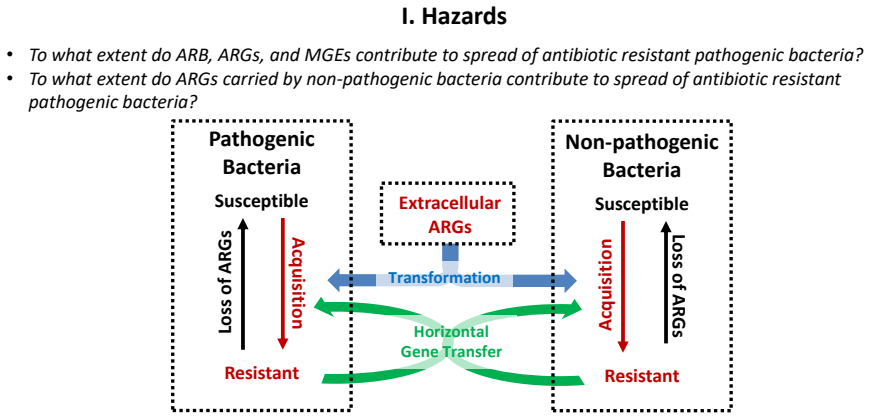


**Figure 1.** *Cont.*

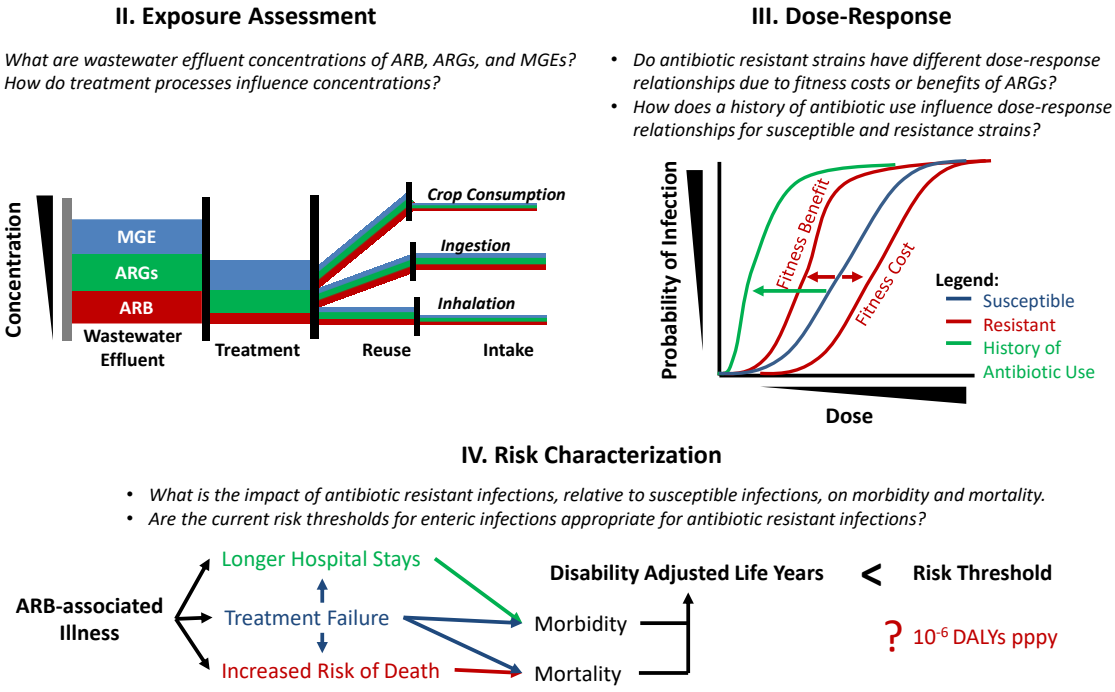


**Figure 1.** An overview on how the classical quantitative microbial risk assessment framework can be applied to understand and assess the risks from antibiotic resistant bacteria and genes.

### *4.1. Hazards*

Determining the burdens from antimicrobial resistance exposure requires consideration of the following contaminants:

#### 4.1.1. *Bacteria*

Pathogenic ARB are of most direct concern, given that they can lead directly to adverse health outcomes in terms of increasing duration and failure rates of medical treatment and increasing risk of death [56]. However, non-pathogenic ARB are also a concern as a reservoir with the potential to transfer ARGs to non-antibiotic resistant pathogens (see Dose-Response, and Ashbolt et al. [16]). Moreover, ARB can be more resistant to water treatment than their non-resistant counterparts, which prolongs their survival in the environment. For example, a recent study revealed that an antibiotic-resistant virulent *E. coli* strain persisted longer than the less resistant non-virulent *E. coli* strain upon solar radiation, despite both being of the same genus and species [57]. In addition to fecal bacteria, which tend to be the focus of monitoring, non-fecal-bacteria are also of concern. Most opportunistic pathogenic bacteria are not of fecal origin, but are now the primary source of waterborne disease in industrialized countries [58].

#### 4.1.2. *Genes*

ARGs are subject to mobilization across hosts, via MGEs, which amplifies the risk. They are considered as "emerging contaminants" that are independent of the ARB host. ARGs are transported through wastewater treatment processes in multiple states, including as functional genes in bacteria, on plasmids, encapsulated within phages, and exogenously as extracellular DNA [39,59,60]. HGT subsequently can occur via conjugation, transduction, and/or transformation. The rate of HGT by each mechanism has not been extensively quantified in the WWTP, where it would be useful to identify which mechanisms play the most dominant role. However, inferences can be made from other ecosystems. To illustrate, transduction rates that are reported for aquatic environments suggest substantial contribution of bacteriophages to the emergence of bacterial strains with new traits and

potential antibiotic resistant pathogens. The reported frequencies of HGT vary widely; ranging from $10^{-2}$ to $10^{-10}$ transductants per recipient cell, the most common values ranging from $10^{-5}$ to $10^{-9}$, depending on the environmental matrix, the physiological state of the recipient bacteria and the bacterium–bacteriophage pair itself [61–63]. The significance of transduction in the environment has been presented in several recent reviews [38,64,65] and will not be elaborated upon here. When compared to transduction, similar data on conjugation and transformation in environmental systems are comparatively limited.

The fate of ARGs after exiting the WWTP still merits consideration, given their potential to remain functional and to be transformed into and replicate within downstream bacterial hosts. Notable characteristics of wastewater effluent (namely high nutrient, calcium chloride levels, and presence of lytic phages, termed as "phage superspreaders") have been shown to increase cell competency and transformation frequency [66,67]. However, it is unclear whether or not these factors, along with other unknown factors, would facilitate exogenous ARGs from wastewater effluent to be taken up via transformation and at what rate. Although the risks of possible downstream ARG amplification are greatly reduced after wastewater treatment, where designs typically achieve 3–4 log removal of bacteria and virus [68,69], the fate and health relevance of exogenous DNA remains largely unknown. Clearly more information on the fate, transport, and health impacts of ARGs in the scope of water reuse is needed.

### *4.2. Exposures*

Exposure assessments integrate data with respect to contaminant concentrations, impact of control processes, and specific exposure volumes for each exposure pathway (e.g., consumption of food crops, ingestion of water, inhalation) in order to estimate the magnitude and frequency of exposures (Figure 1). Data for QMRA is most useful if it is quantitative with estimates of associated uncertainty and variability. For antibiotic resistance exposure assessments, quantitative data on concentrations in wastewater before and after treatment is needed for ARB, ARGs, and MGEs (see Mitigation Strategies). Risks are influenced by state of ARGs (i.e., extracellular, bacteria-associated, phage-associated), so data on the state of ARGs is also important. The methods that were used to collect the relevant data, including associated challenges and opportunities, are discussed above in Monitoring and Surveillance.

Exposure assessments also require estimates of exposure volumes and intakes. Prior work established for enteric and opportunistic pathogens is relevant, especially in the context of both potable and non-potable reuse. For example, exposures are estimated for multiple water reuse scenarios within the World Health Organization Guidelines for the Safe Use of Wastewater, Excreta and Greywater [31] and World Health Organization Quantitative Microbial Risk Assessment: Application for Water Safety Management [29].

### *4.3. Dose-Response Relationships*

Existing dose-response relationships used in QMRA are largely based on human and animal challenge studies for pathogens without consideration of antibiotic resistance. Antibiotic resistance may influence bacterial fitness, i.e., relative advantage for survival and proliferation. Therefore, existing dose-response relationships may not adequately reflect infectivity of antibiotic resistant strains. Specifically, antibacterial resistance carries a fitness cost that most often reduces growth rate and/or virulence [70,71]. However, there are some resistance mechanisms with little-to-no impact on growth rate and/or virulence [70], possibly because they are encoded in plasmids that share long evolutionary history and hence are highly stable in a particular bacterial host [72]. Occasionally, resistance even confers a fitness advantage [70,73]. The impact of ARGs on pathogen infectivity is therefore difficult to predict and is based on the combination of the bacterial species and the resistance mechanisms, including resistance location (chromosome or plasmid) [70,71]. Dose-response relationships also do not account for the impact of recent antibiotic use on pathogen infectivity. A recent history of antibiotic use may allow for resistant strains to proliferate and dominate colonization sites [74].

New dose-response models are necessary for antibiotic resistant pathogens, especially accounting for the increased likelihood of infection following antibiotic treatments.

An additional complication with existing dose-response relationships is that risks are modeled for exposures to single pathogenic strains. Treated wastewater, however, contains complex microbial communities, inclusive of antibiotic-susceptible, antibiotic-resistant, pathogenic, and non-pathogenic bacteria. The current dose-response relationships may be insufficient to model risks from exposures to mixed communities. Exposures to mixed communities may increase the likelihood of pathogens acquiring ARGs or virulence within the human body through, for example, HGT. Not much is known about the HGT of ARGs from MGEs or non-pathogenic strains to pathogens inside the human body. Studies indicate that ingestion of naked DNA containing ARGs is not a direct concern, as transformation into the commensal mammalian gut microbiota is undetectable [75–78]. This may be due, in part, to the harsh environment of the mammalian gut and its impact on survival of extracellular DNA. Furthermore, human-associated phages in gut microflora and fecal wastes do not or rarely carry ARGs [79]. The potential for gene transfer of ARGs to nose, ear, throat, or skin microbiota has yet to be studied. Thus, transformation and transduction likely present more risk in the environment, rather than in the human gut, in terms of potential for propagation via transformation.

Improved dose-response and other experimental data based on a combination of epidemiological data, animal and human challenge studies, and mathematical modeling are necessary to accurately characterize risks from exposures to ARB and ARGs during wastewater reuse.

### *4.4. Risk Characterization and Management*

Risk characterization refers to estimating the number of people impacted by the identified hazard, while management refers to active measures to maintain that level below an acceptable burden. In the case of antibiotic resistance, the impact of infections will vary depending on whether the outcome of interest is colonization, infection, treatment failure, illness, or death. Adaptation of the QMRA framework to antimicrobial resistance will require updating the relationships between infection, illness, and DALYs necessary to estimate disease burden. For enteric diseases, this conversion is based on a combination of the: (i) probability of illness given infection; (ii) the probability of various outcomes given illness (e.g., mild diarrhea, severe diarrhea, or death); and (iii) the severity rating of each outcome (representative of the impact of disease on one's well-being). For example, a single rotavirus infection is equivalent to 0.013 DALYs, based on the assumptions that rotavirus illness lasts seven days, there is a 97.5% likelihood of mild diarrhea with corresponding severity rating of 0.1, a 2.5% likelihood of severe diarrhea with a severity rating of 0.23, and a 0.015% likelihood of death [30]. Similar conversion factors for antimicrobial resistant infections do not yet exist, but are expected to be higher than those for infections that can be cured by antibiotics. This is because antibiotic-resistant infections are usually more serious, last longer, and are more likely to lead to death than susceptible infections [56]. Therefore, adaptation of QMRA for antimicrobial resistant infections will require expanding conversion factors to account for longer disease duration and greater severity. Expanding conversion factors will require consideration of country-level differences, as conversion is based on disease surveillance data that are regionally-bounded, and therefore may not be representative of the population from different countries [80].

Risk characterization for antimicrobial resistant infections will also require reconsidering the acceptable disease burden. The WHO acceptable disease burden is defined by DALYs ($<10^{-6}$ DALYs pppy). If resistant infections have a higher infection-to-DALY conversion factor than treatable infections, as expected, risk characterization will have to account for the probability that an infection is resistant. In contrast, the U.S. EPA defines acceptable disease burden in terms of infections (specifically $< 10^{-4}$ infections pppy). Thus, whether or not an infection is resistant will not influence whether or not the tolerable disease burden is exceeded. Generally, the U.S. EPA and WHO disease burdens align for enteric diseases (like rotavirus) because the infection-to-DALY conversion factor is at

or below 0.01 [30]. However, the increased duration and severity of antimicrobial resistant infections will shift this relationship.

Integrating unique considerations with respect to antibiotic resistance as it relates to the four core components of the QMRA framework provides a useful conceptual model to understand and mitigate risks of antibiotic resistance from water reuse. However, substantial modification and better defined knowledge is required to fully utilize this conceptual model for assessing risks of ARB and ARGs.

## 5. Mitigation Strategies for Antimicrobial Resistance in Recycled Water

Mitigation and risk assessment ideally go hand in hand, with the latter informing the former with respect to target endpoints. However, such target endpoints have yet to be defined as risk models tailored to antimicrobial resistance are still in their infancy. Given the gravity of concern for the spread of antimicrobial resistance, precautionary action towards adoption and implementation of appropriate mitigation strategies is warranted. Here, the holistic perspectives of both the "One Health" [81] and "One Water" [82] paradigms, and their convergence, provide useful guidance. Ideally, mitigation practices should be in harmony with other environmental and health benefits and tailored to local conditions and constraints. In terms of endpoints, mitigation and monitoring should complement each other. Given that antibiotic resistance exists in the background, a reasonable endpoint target is a level and profile of both ARB, ARGs, and HGT potential that would be comparable to the background arising from the use of conventional water source. To exemplify, a comparison between treated-wastewater-irrigated and freshwater-irrigated soils found that ARB and ARG levels were on the whole identical or sometimes even lower in treated-wastewater irrigated soils [83]. Another study irrigated soil microcosms with chlorinated or dechlorinated effluents, and did not observe any significant changes in the ARG levels when compared to microcosms that are irrigated with deionized water [84]. Still, another study comparing several soils irrigated with treated wastewater versus a background soil in urban parks of Victoria, Australia found clear elevation of several ARGs in those soils exposed to treated wastewater [85]. Increased monitoring of multiple endpoints can provide feedback towards better informing both selection of monitoring targets and optimization of risk models.

Here, we focus on two general aspects of mitigation: Source prevention and treatment technologies.

### *5.1. Source Prevention as a Barrier to Downstream Proliferation of ARB and ARGs*

Given the existence of antibiotic resistance in the natural background, the concept of source control is correspondingly focused on human activities that either directly augment this background or result in conditions that stimulate the elevation and mobilization of antibiotic resistance. An example of the latter would be dumping of antibiotic-laden waste or heavy metals into waste streams or aquatic environments and correspondingly imposing pressure on microbial communities both towards selection of strains carrying ARGs [86]. Thus, continued education towards appropriate antibiotic use, limited to cases of need in humans and animals remains as a top priority across the board for combating antibiotic resistance.

In the context of water reuse, it is important to consider the upstream influences on the WWTP. In particular, several studies have noted that hospital sewage is worthy of special handling considerations. Hospital sewage is known to contain higher levels of antibiotics, as well as corresponding ARB and ARGs, and markers of hospital-derived ARGs have been ultimately noted in water bodies receiving hospital-influenced WWTP effluent [87–89]. Thus, a reasonable consideration is the requirement of on-site pre-treatment of hospital sewage before discharging to municipal sewers [90,91]. It is important to also recognize that pharmaceutical manufacturing wastes are often laden with extremely high levels of antibiotic mixtures and poses a special risk. Especially for pharmaceutical plants operating in countries with limited or ineffective environmental regulation and high population, there is special concern [92]. However, in certain instances, it is important to consider that antibiotics are also taken in domestic households. In a study by Schwartz [93],

the abundance of ARGs encoding resistance to ampicillin was actually higher in a housing area than in corresponding clinical wastewater. Thus, even with focused source control, mitigation within the wastewater and recycled treatment processes makes sense.

Depending on the intended water reuse application, the target endpoints may differ. For example, potable reuse should be held to a higher standard than non-potable reuse. Given the limited resources and the need to think holistically regarding the goals of water reuse, more costly treatments are less justifiable for the latter. Still, exposures associated with non-potable reuse are often overlooked. For example, aerosols can carry opportunistic pathogens, such as *Legionella pneumophila* and *Pseudomonas aeruginosa*, which can cause pneumonia or colonize the skin and cause infections later when the dermal layer is compromised [94]. Thus, it is important in identifying mitigation strategies to also manage the safety of the water if it is piped to a different location for reuse. In particular, there is also a need to consider preventing regrowth in the distribution system. Currently, there is little to no guidance specifically for managing water reuse distribution systems, with consideration needed for whether the water is used to irrigate crops, irrigate recreational fields, or other activities. Basic questions such as the ideal disinfectants, water storage conditions and water age management need to be also addressed.

Regardless of the intended reuse purpose or local constraints, the need and benefits of basic sanitation and hygiene are crystal clear. This is well-illustrated by work documenting the elevation of the multi-antibiotic resistance-associated genetic element encoding New Delhi metallo beta-lactamase NDM-1, in surface waters during pilgrimage to remote sites in India without sanitation facilities [95] and in the recovery of NDM-positive *E. coli* pathogen in untreated wastewater in Saudi Arabia during the Hajj pilgrimage month [96]. Thus, an important message is that WWTPs are not to blame. Instead, they are a key aspect of the solution.

### *5.2. Treatment Technologies to Remove ARB and ARGs*

WWTPs typically employ several steps, including physical, biological and chemical processes. A recent surge in research is beginning to provide insight into the relative effects of these treatments on ARBs and ARGs. In terms of general bacterial removal, it is estimated that the concentration of bacteria in wastewater influent and final effluent are about $10^9$–$10^{12}$ colony forming unit (CFU)/day and $10^4$–$10^6$ CFU per day per inhabitant equivalent, respectively [97]. Within a typical WWTP, ARB, and ARGs specifically may be removed via several processes, but there is also potential to increase if the conditions preferentially select for ARB [68,98] or promote the potential for HGT [99].

Initial screening and settling of primary sewage influent produces primary sludge, which may be subject to further treatment via sludge digestion and the residuals reused as a soil amendment. While the physical processes that are applied to primary sludge will reduce the load of bacteria, including ARB, to subsequent stages of treatment, it is important to also consider the effects to agroecosystems when residuals are land applied. The effects of residuals application to soil and crops varied depending on the region and do not always elevate ARB and ARGs, but the potential of residual antibiotics and metals to select and influence horizontal transfer of ARGs in soils [94–96] and subsequently to crops is a legitimate concern. Because of such concerns and other unknowns, land application has been avoided in some countries. Instead, the incineration of antibiotic-laden sludge generated by conventional WWTPs have been opted by some countries to be a better solution in reducing antibiotics, ARB, and ARGs than land application [100].

Following primary treatment, biological treatment is usually employed as an economical means of removing organic matter, nutrients, and other pollutants in the water. Specific biological treatment processes are highly variable, from suspended floc growth in the case of standard activated sludge treatment to attached biofilm processes, and mixed aerobic and anaerobic stages for biological nutrient removal. Following primary treatment, the water still contains some raw-sewage associated bacteria and several field studies have been conducted tracking ARB and ARGs through various biological treatment processes [68,101,102]. General log removal value (LRV) of bacteria following biological

treatment is typically around 1-log, while the specific effects on ARB and ARGs are relatively more varied, which may be reflective of the nature of the sewage, process performance, climatic and unknown factors.

A general concern is that because biological treatment by nature depends upon highly concentrated and active bacteria that are interacting with influent pathogens and ARGs, along with residual metals and antibiotics, WWTPs could be a "hot spot" for the transfer of ARGs [60,103–107]. One recent study showed a positive correlation between the solids retention time and numbers of Gram-positive ARB [108], demonstrating that nutritional status of the bacteria and the time of exposure to antibiotics and other selective agents in the aeration basin can play a role in promoting antibiotic resistance. On the other hand, attempts to estimate HGT rates in activated sludge suggest that it is not an overly common phenomenon in WWTPs relative to background [109], although more work is needed to confirm this observation. In any case, while biological treatment surely will and should remain as an important pillar to wastewater treatment, it is doubtful that it can be relied upon fully for the reduction of ARB and ARGs. Therefore, other downstream barriers should be explored, especially when intending the water for reuse.

Tertiary treatments of WWTP effluents for the purpose of non-potable reuse are usually comprised of filtration (typically granular media filtration or membrane filtration, such as micro- or ultra-filtration), and disinfection. Chlorination is one of the most used disinfection systems to decrease the total amount of bacteria in wastewater effluents. However, questions remain with respect to the ability of impaired bacteria to survive and regrow, especially in water reuse distribution systems [94]. According to the WaterVal chlorine disinfection validation protocol [110], based on the U.S. EPA disinfection profiling and benchmarking guidance manual [111], a treatment plant could validate for a 2-log LRV of bacteria by using a typical CT (i.e., concentration of disinfectant multiplied by contact time) value of 5 mg·min/L at 20 °C, pH 7–7.5 and turbidity $\leq$5 NTU. However, several studies showed an increase of the proportion of ARB over a typical dose range (0.5–5 mg/L) and CT value used in WWTPs [112–115]. In contrast, higher dose and CT values (i.e., lethal doses) result in extensive fragmentation and precipitation of DNA-protein complexes [116], which may signify a good way to reduce ARB and ARGs. At extremely high doses (e.g., 80 mg Cl·min/L), Guo and coworkers demonstrated that ARG transfer was greatly suppressed [112], but such a high dose is not practical and could have other consequences. Besides the formation of toxic, carcinogenic disinfection byproducts (DBPs), which would significantly increase the chemical risks that are associated with water reuse, work has also shown that the DBPs produced by chlorination could also select for ARGs [99]. Thus, the ultimate impact of chlorine on ARB and ARGs is still debatable and it is not wise to rely on chlorination as the sole barrier.

Ultraviolet (UV) disinfection is an alternative to chlorine since it does not produce toxic DBPs, prompting many WWTPs to switch from chlorine to UV. According to the U.S. EPA ultraviolet disinfection guidance manual [117], the required dose to achieve 2-log inactivation for *Cryptosporidium*, *Giardia* and virus is 5.8, 5.2 and 100 mJ/cm$^2$, respectively. In addition, the guidelines recommend that the highest UV dose would be 186 mJ/cm$^2$ to achieve 4-log inactivation of viruses. This is generally thought to be sufficient to kill most co-occurring bacteria, since viruses are generally harder to inactivate than bacteria. However, an earlier study has found that, while this recommended dose would be sufficient to inactivate ARB (10 to 20 mJ/cm$^2$ to achieve 5-log inactivation), it would result in varying efficacies in removing different types of ARGs. For instance, *tet*A and *amp*C genes (which confer resistance against tetracycline and ampicillin, respectively) would only be reduced by 1 to 2-log, while *mec*A and *van*A (which confer resistance against methicillin and vancomycin, respectively) would be reduced by 3 to 4-log [34]. This large variation in inactivation efficiencies suggests a selective enrichment of certain types of ARGs that may be more resistant against UV treatment in the final treated water. Prior work demonstrated that the nature of the DNA sequence (e.g., number of adjacent thymine bases) dictates the susceptibility of ARGs to UV disinfection [34]. Collectively, these findings seem to suggest that tertiary treatment processes demonstrate limited effectiveness at removing ARB

and ARGs. However, it remains unknown if the corresponding risks levels arising from the remnant ARB and ARGs would exceed that of the acceptable level for non-potable reuse.

Beyond tertiary treatment, advanced treatment, including ozone, advanced oxidation processes (AOPs, which generally involve in-situ production of reactive hydroxyl radicals in the presence of primary oxidant and/or energy source or catalysts), and nanofiltration or reverse osmosis can be used as an additional treatment for non-potable or potable reuse. Ozone ($O_3$) is a disinfectant that is becoming increasingly popular in water reuse treatment trains. Ozone is a strong oxidizing agent that can fragment contaminants and other organics, thus making these contaminants more amenable to biodegradation. In the case of antibiotic resistance, ozonation and other advanced oxidation processes are promising because they can move past pathogen destruction and actually destroy DNA and potentially block the ability of downstream bacteria to acquire antibiotic resistance via transformation [55]. However, as is the case for the other disinfection processes that are cited here, studies focused on effects of ozone are not fully conclusive and ultimate use of ozonation would be very costly. Lüddeke et al. [118] studied the removal of total bacteria and ARB in pilot-scale advanced wastewater treatment by ozone in combination with different filtering techniques (i.e., sand filtration and/or granulated activated carbon). Ozone was able to improve bacterial removal by about 1 LRV with a contact time of 20 min in the presence of 0.73 mg $O_3$/mg dissolved organic carbon. However, the study shows that the total level of resistant *E. coli* and *Staphylococcus* spp. isolates increased. According to Sigmon et al. [119], ozone is able to remove around 2 LRV of *E. coli* with a CT of 0.65 mg·min/L at pH 7.6, a temperature of 16 °C and a total organic carbon (TOC) concentration of 17.1 mg/L. This reported disinfection strategy achieved approximately the same CT value as that in the study of Luddeke et al. [118]. However, pH and temperature of the experiment in that study was not measured. These findings demonstrate that environmental or operating conditions can have a significant influence on the final LRV achieved.

On the other hand, combining chlorine (i.e., an oxidant) with UV (i.e., energy source) was shown to achieve higher log reduction for ARGs like *sul*1, *tet*X, *tet*G, and MGEs *intI*1 than using either disinfection strategy alone [120]. The maximum reported LRV for *tet*X was approximately 2.2-log with 249.5 mJ/cm$^2$ of UV irradiation in combination with 30 mg/L chlorine. This is in contrast to approximately 0.6 LRV from the same UV fluence alone and 1.5 LRV from the same concentration of chlorine [120]. It is important to note that the amount of UV irradiation and chlorine to be added to the wastewater effluent to achieve this reported LRV is exceedingly high and may be costly for long-term operations. Another study demonstrated that the combination of UV and $H_2O_2$ was able to reduce the UV fluence from ca. 65 mJ/cm$^2$ to 40 mJ/cm$^2$ for the same LRV of 3 when extracellular ampicillin resistance genes were suspended in phosphate saline buffer. However, when the same extracellular ampicillin resistance genes were suspended in wastewater effluent, the combination of $H_2O_2$ and UV result in neither a significant reduction in the UV fluence that is required to achieve the same log reduction, nor improvement in overall log reduction of ARGs [121]. These findings demonstrate that while AOP can potentially be useful for inactivating ARGs in wastewater matrices, the additional costs associated with using an oxidant in tandem with an energy source may not be entirely justified.

Among all of the available treatments that are commonly applied for water reuse, membrane treatments are most likely to be effective. Membrane treatment options ranked from the loosest to tightest size removal criteria include: microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO). MF, UF, and NF would suffice if the treated water is intended for non-potable reuse purpose while an additional RO step is typically used to achieve a final product suitable for potable reuse. MF, UF, and NF membranes are commonly applied in tandem with activated sludge treatment as an alternative to a settling tank. LRV of bacterial contaminants reported by MBRs are collated in a recent review paper [41]. When comparing the reported influent and effluent abundances of bacteria, it is apparent that membranes are a well-established barrier to remove bacteria at high LRV, but whether these reported LRVs can be translated to ARB would remain unknown. This is because antimicrobial molecules are known for inducing alterations in the bacterial envelope and in its

mechanical properties [122,123]. Variances in cell rigidity may apply to ARB, which can consequently trigger passage of ARB through membranes at a different rate than non-antibiotic bacterial cells. Besides size exclusion, other factors, such as adsorption onto the membrane, can also aid in removing ARB and ARGs from wastewater. In fact, adsorption may play a more important role in removing ARGs than size exclusion alone. This is because studies have indicated that naked DNA containing ARGs could be effectively removed by fouled UF [124] and MF membranes [125]. These are promising results since UF- and MF-based MBRs are typically operated at lower energy requirement than NF or RO, and thus incur lower costs.

Membrane separation processes can also be applied together with anaerobic fermentation process to further lower energy and costs that are required to operate MBRs. Life cycle analysis revealed that anaerobic membrane bioreactors (anMBRs) hold promise in providing a sustainable way to treat municipal wastewater due to its capacity to produce methane that can be harvested to become an energy source and its low solid waste production rate [126]. This is in contrast to the aerobic MBRs, which require energy for aeration and generate large volumes of antibiotics-laden sludge requiring disposal. Recent studies also suggest that anMBR may provide treated effluent that is safer than that produced by aerobic MBR. This is exemplified from the higher removal rates of the majority of the detected bacterial contaminants than aerobic MBR [127], and a lower occurrence of ARGs in anMBR than aerobic MBR when exposed to similar concentrations of antibiotics [128]. However, anaerobic processes alone do not remove ammonia and phosphorus from municipal wastewaters, and can result in environmental concerns (e.g. eutrophication in surface waters) or breaches in the potable water quality when used upstream of a water reclamation plant. Hence, combining aerobic and anaerobic processes by using sequential redox conditions to select/remove different types of ARB and ARGs with lower energy use and nutrient removal may be the ultimate solution [129].

Overall, it is reasonable to conclude that the advanced treatment processes applied in potable reuse treatment trains are likely to reduce ARB and ARGs to levels that are negligible compared to background concentrations [130].

## 6. Concluding Statement

There is an urgent need to more comprehensively evaluate resulting environmental and public health concerns that may arise from water reuse. A growing volume of research has brought to light the ubiquitous presence of ARB and ARGs in wastewater and has demonstrated that conventional WWTPs do not fully remove these contaminants from the final treated effluent. Despite mounting scientific evidence, existing regulations and policies have yet to determine permissible levels of ARB and ARGs in final treated water. A "One Water" framework in which multiple barriers are emplaced to protect public health is advisable. Addressing various knowledge gaps that were highlighted in this perspectives paper, including the need to identify suitable targets and inadequate risk assessment models, can help to facilitate effective recycled water management to ensure utmost protection of public health. In the meantime, it is important to consider both source prevention and treatment technologies to minimize the potential detrimental impact arising from ARB and ARGs in wastewaters.

**Acknowledgments:** This paper was conceived at the KAUST Research Conference "Changing paradigms of wastewater treatment—from waste to resource" held on 27–29 March 2017. The conference was sponsored by KAUST Office of Sponsored Research (OSR) and was organized by Water Desalination and Reuse Center (WDRC).

**Author Contributions:** This paper is a collaborative work among all authors. P.-Y.H. and C.M.M. conceived the outline of this paper; C.M.M. and P.-Y.H. contributed to Section 2; A.P., C.M.M. and P.-Y.H. contributed to Section 3; T.R.J., S.C.J. and K.L.N. contributed to Section 4; A.P., P.-Y.H., K.L.N., M.-L.P. and D.G. contributed to Section 5; P.-Y.H. collated and synthesized the contributions from all authors. All authors contributed to the overall framing and revisions of the manuscript at multiple stages.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References and Notes


1. Jimenez, B.; Asano, T. *Water Reuse: An International Survey of Current Practice, Issues and Needs (Scientific and Technical Report)*; IWA Publishing: London, UK, 2008.
2. AQUASTAT. FAO's Global Water Information System. Available online: http://www.fao.org/nr/water/aquastat/infographics/Infographics_all_eng.pdf (accessed on 5 October 2017).
3. Thebo, A.L.; Drechsel, P.; Lambin, E.F. Global assessment of urban and peri-urban agriculture: Irrigated and rainfed croplands. *Environ. Res. Lett.* **2014**, *9*, 114002. [CrossRef]
4. Pescod, M.B. *Wastewater Treatment and Use in Agriculture. Fao Irrigation and Drainage Paper 47*; FAO of the United Nations: Rome, Italy, 1992; p. 125.
5. Thebo, A.L.; Drechsel, P.; Lambin, E.F.; Nelson, K.L. A global, spatially-explicit assessment of irrigated croplands influenced by urban wastewater flows. *Environ. Res. Lett.* **2017**, *12*, 074008. [CrossRef]
6. Nuclear Regulatory Commission (NRC). Water Reuse: Potential for Expanding the Nation's Water Supply through Reuse of Municipal Wastewater. Available online: https://www.nap.edu/catalog/13303/water-reuse-potential-for-expanding-the-nations-water-supply-through (accessed on 9 November 2017).
7. International Water Association (IWA). History of Activated Sludge. Available online: http://www.iwa100as.org/history.php (accessed on 5 October 2017).
8. Luo, Y.; Guo, W.; Ngo, H.H.; Nghiem, L.D.; Hai, F.I.; Zhang, J.; Liang, S.; Wang, X.C. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. *Sci. Total Environ.* **2014**, *473–474*, 619–641. [CrossRef] [PubMed]
9. Barbosa, M.O.; Moreira, N.F.; Ribeiro, A.R.; Pereira, M.F.; Silva, A.M. Occurrence and removal of organic micropollutants: An overview of the watch list of eu decision 2015/495. *Water Res.* **2016**, *94*, 257–279. [CrossRef] [PubMed]
10. World Health Organization (WHO). *Antimicrobial Resistance: Global Report on Surveillance*; WHO: Geneva, Switzerland, 2014.
11. Berendonk, T.U.; Manaia, C.M.; Merlin, C.; Fatta-Kassinos, D.; Cytryn, E.; Walsh, F.; Burgmann, H.; Sorum, H.; Norstrom, M.; Pons, M.N.; et al. Tackling antibiotic resistance: The environmental framework. *Nat. Rev. Microbiol.* **2015**, *13*, 310–317. [CrossRef] [PubMed]
12. Bush, K.; Courvalin, P.; Dantas, G.; Davies, J.; Eisenstein, B.; Huovinen, P.; Jacoby, G.A.; Kishony, R.; Kreiswirth, B.N.; Kutter, E.; et al. Tackling antibiotic resistance. *Nat. Rev. Microbiol.* **2011**, *9*, 894–896. [CrossRef] [PubMed]
13. Clatworthy, A.E.; Pierson, E.; Hung, D.T. Targeting virulence: A new paradigm for antimicrobial therapy. *Nat. Chem. Biol.* **2007**, *3*, 541–548. [CrossRef] [PubMed]
14. Kieffer, N.; Poirel, L.; Bessa, L.J.; Barbosa-Vasconcelos, A.; da Costa, P.M.; Nordmann, P. VIM-1, VIM-34, and IMP-8 carbapenemase-producing *escherichia coli* strains recovered from a Portuguese river. *Antimicrob. Agents Chemother.* **2016**, *60*, 2585–2586. [CrossRef] [PubMed]
15. Mahon, B.M.; Brehony, C.; McGrath, E.; Killeen, J.; Cormican, M.; Hickey, P.; Keane, S.; Hanahoe, B.; Dolan, A.; Morris, D. Indistinguishable NDM-producing *escherichia coli* isolated from recreational waters, sewage, and a clinical specimen in Ireland, 2016 to 2017. *Eurosurveillance* **2017**, *22*, 30513. [CrossRef] [PubMed]
16. Ashbolt, N.J.; Amezquita, A.; Backhaus, T.; Borriello, P.; Brandt, K.K.; Collignon, P.; Coors, A.; Finley, R.; Gaze, W.H.; Heberer, T.; et al. Human health risk assessment (HHRA) for environmental development and transfer of antibiotic resistance. *Environ. Health Perspect.* **2013**, *121*, 993–1001. [CrossRef] [PubMed]
17. Manaia, C.M. Assessing the risk of antibiotic resistance transmission from the environment to humans: Non-direct proportionality between abundance and risk. *Trends Microbiol.* **2017**, *25*, 173–181. [CrossRef] [PubMed]
18. Huang, X.; Zhao, Z.; Hernandez, D.; Jiang, S. Near real-time flow cytometry monitoring of bacterial and viral removal efficiencies during water reclamation processes. *Water* **2016**, *8*, 464. [CrossRef]
19. Kokjohn, T.A.; Sayler, G.S. Attachment and replication of *pseudomonas aeruginosa* bacteriophages under conditions simulating aquatic environments. *J. Gen. Microbiol.* **1991**, *137*, 661–666. [CrossRef]
20. Muniesa, M.; Jofre, J. Factors influencing the replication of somatic coliphages in the water environment. *Antonie Van Leeuwenhoek* **2004**, *86*, 65–76. [CrossRef] [PubMed]
21. Fuhrman, J.A. Marine viruses and their biogeochemical and ecological effects. *Nature* **1999**, *399*, 541–548. [CrossRef] [PubMed]

22. Miller, R.V.; Ripp, S.; Replicon, J.; Ogunseitan, O.A.; Kokjohn, T.A. Virus-mediated gene transfer in freshwater environments. In *Gene Transfers and Environment: Proceedings of the Third European Meeting on Bacterial Genetics and Ecology (bageco-3), Villefranche-sur-mer, France, 20–22 November 1991*; Gauthier, M.J., Ed.; Springer: Berlin/Heidelberg, Germany, 1992; pp. 51–62.
23. European Economic Community (EEC). Concerning Urban Wastewater Treatment, Council Directive 91/271/EEC, 1991.
24. Becerra-Castro, C.; Lopes, A.R.; Vaz-Moreira, I.; Silva, E.F.; Manaia, C.M.; Nunes, O.C. Wastewater reuse in irrigation: A microbiological perspective on implications in soil fertility and human and environmental health. *Environ. Int.* **2015**, *75*, 117–135. [CrossRef] [PubMed]
25. European Economic Community (EEC). Amending council directive 91/271/EEC, Commission directive 98/15/EC, 1998.
26. European Economic Community (EEC). Establishing a framework for community action in the field of water policy–EU water framework, Directive (2000/60/EC), 2000.
27. European Economic Community (EEC). Amending directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy, Directive 2013/39/EU, 2013.
28. European Economic Community (EEC). On the quality of water intended for human consumption, Council directive 98/83/EC, 1998.
29. World Health Organization (WHO). *Quantitative Microbial Risk Assessment: Application for Water Safety Management*; WHO: Geneva, Switzerland, 2016.
30. World Health Organization (WHO). Guidelines for Drinking Water Quality: Fourth Edition Incorporating the First Addendum. Available online: http://www.who.int/water_sanitation_health/publications/drinking-water-quality-guidelines-4-including-1st-addendum/en/ (accessed on 17 December 2017).
31. World Health Organization (WHO). *Who Guidelines for the Safe Use of Wastewater, Excreta and Greywater*; WHO: Geneva, Switzerland, 2006.
32. Manaia, C.M.; Macedo, G.; Fatta-Kassinos, D.; Nunes, O.C. Antibiotic resistance in urban aquatic environments: Can it be controlled? *Appl. Microbiol. Biotechnol.* **2016**, *100*, 1543–1557. [CrossRef] [PubMed]
33. Di Cesare, A.; Fontaneto, D.; Doppelbauer, J.; Corno, G. Fitness and recovery of bacterial communities and antibiotic resistance genes in urban wastewaters exposed to classical disinfection treatments. *Environ. Sci. Technol.* **2016**, *50*, 10153–10161. [CrossRef] [PubMed]
34. McKinney, C.W.; Pruden, A. Ultraviolet disinfection of antibiotic resistant bacteria and their antibiotic resistance genes in water and wastewater. *Environ. Sci. Technol.* **2012**, *46*, 13393–13400. [CrossRef] [PubMed]
35. Washington, J.A. Principles of diagnosis. In *Medical Microbiology*, 4th ed.; Baron, S., Ed.; University of Texas Medical Branch at Galveston: Galveston, TX, USA, 1996.
36. Garner, E.; Wallace, J.S.; Argoty, G.A.; Wilkinson, C.; Fahrenfeld, N.; Heath, L.S.; Zhang, L.; Arabi, M.; Aga, D.S.; Pruden, A. Metagenomic profiling of historic Colorado front range flood impact on distribution of riverine antibiotic resistance genes. *Sci. Rep.* **2016**, *6*, 38432. [CrossRef] [PubMed]
37. Gatica, J.; Tripathi, V.; Green, S.; Manaia, C.M.; Berendonk, T.; Cacace, D.; Merlin, C.; Kreuzinger, N.; Schwartz, T.; Fatta-Kassinos, D.; et al. High throughput analysis of integron gene cassettes in wastewater environments. *Environ. Sci. Technol.* **2016**, *50*, 11825–11836. [CrossRef] [PubMed]
38. Balcazar, J.L. Bacteriophages as vehicles for antibiotic resistance genes in the environment. *PLoS Pathog.* **2014**, *10*, e1004219. [CrossRef] [PubMed]
39. Colomer-Lluch, M.; Jofre, J.; Muniesa, M. Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. *PLoS ONE* **2011**, *6*, e17549. [CrossRef] [PubMed]
40. Hong, P.Y.; Al-Jassim, N.; Ansari, M.I.; Mackie, R.I. Environmental and public health implications of water reuse: Antibiotics, antibiotic resistant bacteria, and antibiotic resistance genes. *Antibiotics* **2013**, *2*, 367–399. [CrossRef] [PubMed]
41. Harb, M.; Hong, P.-Y. Anaerobic membrane bioreactor effluent reuse: A review of microbial safety concerns. *Fermentation* **2017**, *3*, 39. [CrossRef]
42. Garmendia, L.; Hernandez, A.; Sanchez, M.B.; Martinez, J.L. Metagenomics and antibiotics. *Clin. Microbiol. Infect.* **2012**, *18 Suppl 4*, 27–31. [CrossRef]
43. Martinez, J.L.; Coque, T.M.; Baquero, F. Prioritizing risks of antibiotic resistance genes in all metagenomes. *Nat. Rev. Microbiol.* **2015**, *13*, 396. [CrossRef] [PubMed]

44. Martinez, J.L.; Coque, T.M.; Baquero, F. What is a resistance gene? Ranking risk in resistomes. *Nat. Rev. Microbiol.* **2015**, *13*, 116–123. [PubMed]
45. Wang, F.H.; Qiao, M.; Su, J.Q.; Chen, Z.; Zhou, X.; Zhu, Y.G. High throughput profiling of antibiotic resistance genes in urban park soils with reclaimed water irrigation. *Environ. Sci. Technol.* **2014**, *48*, 9079–9085. [CrossRef] [PubMed]
46. Arango-Argoty, G.; Singh, G.; Heath, L.S.; Pruden, A.; Xiao, W.; Zhang, L. Metastorm: A public resource for customizable metagenomics annotation. *PLoS ONE* **2016**, *11*, e0162442. [CrossRef] [PubMed]
47. Jia, B.; Raphenya, A.R.; Alcock, B.; Waglechner, N.; Guo, P.; Tsang, K.K.; Lago, B.A.; Dave, B.M.; Pereira, S.; Sharma, A.N.; et al. Card 2017: Expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res.* **2017**, *45*, D566–D573. [CrossRef] [PubMed]
48. Yang, Y.; Jiang, X.; Chai, B.; Ma, L.; Li, B.; Zhang, A.; Cole, J.R.; Tiedje, J.M.; Zhang, T. ARGS-OAP: Online analysis pipeline for antibiotic resistance genes detection from metagenomic data using an integrated structured arg-database. *Bioinformatics* **2016**, *32*, 2346–2351. [CrossRef] [PubMed]
49. Bhullar, K.; Waglechner, N.; Pawlowski, A.; Koteva, K.; Banks, E.D.; Johnston, M.D.; Barton, H.A.; Wright, G.D. Antibiotic resistance is prevalent in an isolated cave microbiome. *PLoS ONE* **2012**, *7*, e34953. [CrossRef] [PubMed]
50. Brown, M.G.; Balkwill, D.L. Antibiotic resistance in bacteria isolated from the deep terrestrial subsurface. *Microb. Ecol.* **2009**, *57*, 484–493. [CrossRef] [PubMed]
51. Gillings, M.; Boucher, Y.; Labbate, M.; Holmes, A.; Krishnan, S.; Holley, M.; Stokes, H.W. The evolution of class 1 integrons and the rise of antibiotic resistance. *J. Bacteriol.* **2008**, *190*, 5095–5100. [CrossRef] [PubMed]
52. Gillings, M.R.; Gaze, W.H.; Pruden, A.; Smalla, K.; Tiedje, J.M.; Zhu, Y.G. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *ISME J.* **2015**, *9*, 1269–1279. [CrossRef] [PubMed]
53. World Health Organization (WHO). Who Publishes List of Bacteria for Which New Antibiotics Are Urgently Needed. Available online: http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/ (accessed on 5 October 2017).
54. Haas, C.N.; Rose, J.B.; Gerba, C.P. *Quantitative Microbial Risk Assessment*, 2nd ed.; World Health Organization (WHO): Geneva, Switzerland, 2014; p. 440.
55. Pruden, A. Balancing water sustainability and public health goals in the face of growing concerns about antibiotic resistance. *Environ. Sci. Technol.* **2014**, *48*, 5–14. [CrossRef] [PubMed]
56. ECDC/EMEA. *The Bacterial Challenge: Time to React*; ECDC/EMEA: Solna, Sweden, 2009; ISBN 9789291931934.
57. Al-Jassim, N.; Mantilla-Calderon, D.; Wang, T.; Hong, P.Y. Inactivation and gene expression of a virulent wastewater *escherichia coli* strain and the nonvirulent commensal *escherichia coli* DSM1103 strain upon solar irradiation. *Environ. Sci. Technol.* **2017**, *51*, 3649–3659. [CrossRef] [PubMed]
58. Brunkard, J.M.; Ailes, E.; Roberts, V.A.; Hill, V.; Hilborn, E.D.; Craun, G.F.; Rajasingham, A.; Kahler, A.; Garrison, L.; Hicks, L.; et al. Surveillance for waterborne disease outbreaks associated with drinking water—United States, 2007–2008. *MMWR Surveill. Summ.* **2011**, *60*, 38–68. [PubMed]
59. Parsley, L.C.; Consuegra, E.J.; Kakirde, K.S.; Land, A.M.; Harper, W.F., Jr.; Liles, M.R. Identification of diverse antimicrobial resistance determinants carried on bacterial, plasmid, or viral metagenomes from an activated sludge microbial assemblage. *Appl. Environ. Microbiol.* **2010**, *76*, 3753–3757. [CrossRef] [PubMed]
60. Rizzo, L.; Manaia, C.; Merlin, C.; Schwartz, T.; Dagot, C.; Ploy, M.C.; Michael, I.; Fatta-Kassinos, D. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. *Sci. Total Environ.* **2013**, *447*, 345–360. [CrossRef] [PubMed]
61. Imamovic, L.; Jofre, J.; Schmidt, H.; Serra-Moreno, R.; Muniesa, M. Phage-mediated Shiga toxin 2 gene transfer in food and water. *Appl. Environ. Microbiol.* **2009**, *75*, 1764–1768. [CrossRef] [PubMed]
62. Jiang, S.C.; Paul, J.H. Gene transfer by transduction in the marine environment. *Appl. Environ. Microbiol.* **1998**, *64*, 2780–2787. [PubMed]
63. Ogunseitan, O.A. Genetic transduction in freshwater ecosystems. *Freshwater Biol.* **2008**, *53*, 1228–1239. [CrossRef]
64. Brown-Jaque, M.; Calero-Caceres, W.; Muniesa, M. Transfer of antibiotic-resistance genes via phage-related mobile elements. *Plasmid* **2015**, *79*, 1–7. [CrossRef] [PubMed]
65. Penades, J.R.; Chen, J.; Quiles-Puchalt, N.; Carpena, N.; Novick, R.P. Bacteriophage-mediated spread of bacterial virulence genes. *Curr. Opin. Microbiol.* **2015**, *23*, 171–178. [CrossRef] [PubMed]

66. Davison, J. Genetic exchange between bacteria in the environment. *Plasmid* **1999**, *91*, 73–91. [CrossRef] [PubMed]
67. Keen, E.C.; Bliskovsky, V.V.; Malagon, F.; Baker, J.D.; Prince, J.S.; Klaus, J.S.; Adhya, S.L. Novel "superspreader" bacteriophages promote horizontal gene transfer by transformation. *MBio* **2017**, *8*, e02115–e02116. [CrossRef] [PubMed]
68. Al-Jassim, N.; Ansari, M.I.; Harb, M.; Hong, P.Y. Removal of bacterial contaminants and antibiotic resistance genes by conventional wastewater treatment processes in Saudi Arabia: Is the treated wastewater safe to reuse for agricultural irrigation? *Water Res.* **2015**, *73*, 277–290. [CrossRef] [PubMed]
69. Jumat, M.R.; Hasan, N.A.; Subramanian, P.; Heberling, C.; Colwell, R.R.; Hong, P.Y. Membrane bioreactor-based wastewater treatment plant in Saudi Arabia: Reduction of viral diversity, load, and infectious capacity. *Water* **2017**, *9*, 534. [CrossRef]
70. Melnyk, A.H.; Wong, A.; Kassen, R. The fitness costs of antibiotic resistance mutations. *Evol. Appl.* **2015**, *8*, 273–283. [CrossRef] [PubMed]
71. Vogwill, T.; MacLean, R.C. The genetic basis of the fitness costs of antimicrobial resistance: A meta-analysis approach. *Evol. Appl.* **2015**, *8*, 284–295. [CrossRef] [PubMed]
72. Mantilla-Calderon, D.; Hong, P.Y. Fate and persistence of a pathogenic ndm-1-positive *escherichia coli* strain in anaerobic and aerobic sludge microcosms. *Appl. Environ. Microbiol.* **2017**, *83*, e00640-17. [CrossRef] [PubMed]
73. Roux, D.; Danilchanka, O.; Guillard, T.; Cattoir, V.; Aschard, H.; Fu, Y.; Angoulvant, F.; Messika, J.; Ricard, J.D.; Mekalanos, J.J.; et al. Fitness cost of antibiotic susceptibility during bacterial infection. *Sci. Transl. Med.* **2015**, *7*, 297ra114. [CrossRef] [PubMed]
74. Donskey, C.J. Antibiotic regimens and intestinal colonization with antibiotic-resistant gram-negative bacilli. *Clin. Infect Dis.* **2006**, *43* (Suppl. 2), S62–S69. [CrossRef]
75. Nordgard, L.; Brusetti, L.; Raddadi, N.; Traavik, T.; Averhoff, B.; Nielsen, K.M. An investigation of horizontal transfer of feed introduced DNA to the aerobic microbiota of the gastrointestinal tract of rats. *BMC Res. Notes* **2012**, *5*, 170. [CrossRef] [PubMed]
76. Nordgard, L.; Nguyen, T.; Midtvedt, T.; Benno, Y.; Traavik, T.; Nielsen, K.M. Lack of detectable DNA uptake by bacterial gut isolates grown in vitro and by *Acinetobacter baylyi* colonizing rodents in vivo. *Environ. Biosafety Res.* **2007**, *6*, 149–160. [CrossRef] [PubMed]
77. Rizzi, A.; Brusetti, L.; Arioli, S.; Nielsen, K.M.; Tamagnini, I.; Tamburini, A.; Sorlini, C.; Daffonchio, D. Detection of feed-derived maize DNA in goat milk and evaluation of the potential of horizontal transfer to bacteria. *Eur. Food Res. Technol.* **2008**, *227*, 1699–1709. [CrossRef]
78. Wilcks, A.; Jacobsen, B.B. Lack of detectable DNA uptake by transformation of selected recipients in mono-associated rats. *BMC Res. Notes* **2010**, *3*, 49. [CrossRef] [PubMed]
79. Enault, F.; Briet, A.; Bouteille, L.; Roux, S.; Sullivan, M.B.; Petit, M.A. Phages rarely encode antibiotic resistance genes: A cautionary tale for virome analyses. *ISME J.* **2017**, *11*, 237–247. [CrossRef] [PubMed]
80. Lim, K.Y.; Hamilton, A.J.; Jiang, S.C. Assessment of public health risk associated with viral contamination in harvested urban stormwater for domestic applications. *Sci. Total Environ.* **2015**, *523*, 95–108. [CrossRef] [PubMed]
81. CDC. One Health. Available online: https://www.cdc.gov/onehealth/index.html (accessed on 9 November 2017).
82. USWaterAlliance. One Water Roadmap: The Sustainable Management of Life's Most Essential Resource. Available online: http://uswateralliance.org//one-water/roadmap (accessed on 9 November 2017).
83. Negreanu, Y.; Pasternak, Z.; Jurkevitch, E.; Cytryn, E. Impact of treated wastewater irrigation on antibiotic resistance in agricultural soils. *Environ. Sci. Technol.* **2012**, *46*, 4800–4808. [CrossRef] [PubMed]
84. Fahrenfeld, N.; Ma, Y.; O'Brien, M.; Pruden, A. Reclaimed water as a reservoir of antibiotic resistance genes: Distribution system and irrigation implications. *Front. Microbiol.* **2013**, *4*, 130. [CrossRef] [PubMed]
85. Han, X.M.; Hu, H.W.; Shi, X.Z.; Wang, J.T.; Han, L.L.; Chen, D.; He, J.Z. Impacts of reclaimed water irrigation on soil antibiotic resistome in urban parks of victoria, australia. *Environ. Pollut.* **2016**, *211*, 48–57. [CrossRef] [PubMed]
86. Bengtsson-Palme, J.; Larsson, D.G.J. Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. *Environ. Int.* **2016**, *86*, 140–149. [CrossRef] [PubMed]

87. Czekalski, N.; Berthold, T.; Caucci, S.; Egli, A.; Burgmann, H. Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into Lake Geneva, Switzerland. *Front. Microbiol.* **2012**, *3*, 106. [CrossRef] [PubMed]
88. Iweriebor, B.C.; Gaqavu, S.; Obi, L.C.; Nwodo, U.U.; Okoh, A.I. Antibiotic susceptibilities of enterococcus species isolated from hospital and domestic wastewater effluents in alice, eastern cape province of South Africa. *Int. J. Environ. Res. Public Health* **2015**, *12*, 4231–4246. [CrossRef] [PubMed]
89. Kotlarska, E.; Luczkiewicz, A.; Pisowacka, M.; Burzynski, A. Antibiotic resistance and prevalence of class 1 and 2 integrons in *escherichia col*i isolated from two wastewater treatment plants, and their receiving waters (Gulf of Gdansk, Baltic Sea, Poland). *Environ. Sci. Pollut. Res. Int.* **2015**, *22*, 2018–2030. [CrossRef] [PubMed]
90. Narciso-da-Rocha, C.; Varela, A.R.; Schwartz, T.; Nunes, O.C.; Manaia, C.M. BlaTEM and vanA as indicator genes of antibiotic resistance contamination in a hospital–urban wastewater treatment plant system. *J. Glob. Antimicrob. Resist.* **2014**, *2*, 309–315. [CrossRef] [PubMed]
91. Timraz, K.; Xiong, Y.H.; Al Qarni, H.; Hong, P.Y. Removal of bacterial cells, antibiotic resistance genes and integrase genes by on-site hospital wastewater treatment plants: Surveillance of treated hospital effluent quality. *Environ. Sci.-Wat. Res.* **2017**, *3*, 293–303. [CrossRef]
92. Bengtsson-Palme, J.; Boulund, F.; Fick, J.; Kristiansson, E.; Larsson, D.G. Shotgun metagenomics reveals a wide array of antibiotic resistance genes and mobile elements in a polluted lake in India. *Front. Microbiol.* **2014**, *5*, 648. [CrossRef] [PubMed]
93. Schwartz, T. Strategies to assess and minimize the biological risk of antibiotic resistance in the environment. In *Antimicrobial Resistance in the Environment*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2011; pp. 251–264.
94. Garner, E.; Zhu, N.; Strom, L.; Edwards, M.; Pruden, A. A human exposome framework for guiding risk management and holistic assessment of recycled water quality. *Environ. Sci. Water Res. Technol.* **2016**, *2*, 580–598. [CrossRef]
95. Ahammad, Z.S.; Sreekrishnan, T.R.; Hands, C.L.; Knapp, C.W.; Graham, D.W. Increased waterborne blaNDM-1 resistance gene abundances associated with seasonal human pilgrimages to the upper ganges river. *Environ. Sci. Technol.* **2014**, *48*, 3014–3020. [CrossRef] [PubMed]
96. Mantilla-Calderon, D.; Jumat, M.R.; Wang, T.; Ganesan, P.; Al-Jassim, N.; Hong, P.Y. Isolation and characterization of NDM-positive *escherichia coli* from municipal wastewater in Jeddah, Saudi Arabia. *Antimicrob. Agents Chemother.* **2016**, *60*, 5223–5231. [CrossRef] [PubMed]
97. Novo, A.; Manaia, C.M. Factors influencing antibiotic resistance burden in municipal wastewater treatment plants. *Appl. Microbiol. Biotechnol.* **2010**, *87*, 1157–1166. [CrossRef] [PubMed]
98. Li, D.; Zeng, S.; He, M.; Gu, A.Z. Water disinfection byproducts induce antibiotic resistance-role of environmental pollutants in resistance phenomena. *Environ. Sci. Technol.* **2016**, *50*, 3193–3201. [CrossRef] [PubMed]
99. Zhang, Y.; Gu, A.Z.; He, M.; Li, D.; Chen, J. Subinhibitory concentrations of disinfectants promote the horizontal transfer of multidrug resistance genes within and across genera. *Environ. Sci. Technol.* **2017**, *51*, 570–580. [CrossRef] [PubMed]
100. Pruden, A.; Larsson, D.G.; Amezquita, A.; Collignon, P.; Brandt, K.K.; Graham, D.W.; Lazorchak, J.M.; Suzuki, S.; Silley, P.; Snape, J.R.; et al. Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. *Environ. Health Perspect.* **2013**, *121*, 878–885. [CrossRef] [PubMed]
101. Mao, D.Q.; Yu, S.; Rysz, M.; Luo, Y.; Yang, F.X.; Li, F.X.; Hou, J.; Mu, Q.H.; Alvarez, P.J.J. Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants. *Water Res.* **2015**, *85*, 458–466. [CrossRef] [PubMed]
102. Yuan, Q.B.; Guo, M.T.; Wei, W.J.; Yang, J. Reductions of bacterial antibiotic resistance through five biological treatment processes treated municipal wastewater. *Environ. Sci. Pollut. Res.* **2016**, *23*, 19495–19503. [CrossRef] [PubMed]
103. Marcinek, H.; Wirth, R.; Muscholl-Silberhorn, A.; Gauer, M. *Enterococcus faecalis* gene transfer under natural conditions in municipal sewage water treatment plants. *Appl. Environ. Microbiol.* **1998**, *64*, 626–632. [PubMed]
104. Ferreira da Silva, M.; Tiago, I.; Verissimo, A.; Boaventura, R.A.; Nunes, O.C.; Manaia, C.M. Antibiotic resistance of enterococci and related bacteria in an urban wastewater treatment plant. *FEMS Microbiol. Ecol.* **2006**, *55*, 322–329. [CrossRef] [PubMed]

105. Auerbach, E.A.; Seyfried, E.E.; McMahon, K.D. Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Res.* **2007**, *41*, 1143–1151. [CrossRef] [PubMed]
106. Kim, S.; Jensen, J.N.; Aga, D.S.; Weber, A.S. Tetracycline as a selector for resistant bacteria in activated sludge. *Chemosphere* **2007**, *66*, 1643–1651. [CrossRef] [PubMed]
107. Łuczkiewicz, A.; Jankowska, K.; Fudala-Książek, S.; Olańczuk-Neyman, K. Antimicrobial resistance of fecal indicators in municipal wastewater treatment plant. *Water Res.* **2010**, *44*, 5089–5097. [CrossRef] [PubMed]
108. Neyestani, M.; Dickenson, E.; McLain, J.; Robleto, E.; Rock, C.; Gerrity, D. Impacts of solids retention time on trace organic compound attenuation and bacterial resistance to trimethoprim and sulfamethoxazole. *Chemosphere* **2017**, *182*, 149–158. [CrossRef] [PubMed]
109. Munck, C.; Albertsen, M.; Telke, A.; Ellabaan, M.; Nielsen, P.H.; Sommer, M.O. Limited dissemination of the wastewater treatment plant core resistome. *Nat. Commun.* **2015**, *6*, 8452. [CrossRef] [PubMed]
110. WaterSecure. Chlorine disinfection. In *WaterVal Validation Protocol*; Australian WaterSecure Innovations Ltd.: Brisbane, Australia, 2017.
111. USEPA. *Disinfection Profiling and Benchmarking Guidance Manual*; Agency, U.S.E.P., Ed.; U.S.EPA: Washington, DC, USA, 1999.
112. Guo, M.-T.; Yuan, Q.-B.; Yang, J. Distinguishing effects of ultraviolet exposure and chlorination on the horizontal transfer of antibiotic resistance genes in municipal wastewater. *Environ. Sci. Technol.* **2015**, *49*, 5771–5778. [CrossRef] [PubMed]
113. Munir, M.; Wong, K.; Xagoraraki, I. Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Res.* **2011**, *45*, 681–693. [CrossRef] [PubMed]
114. Murray, G.E.; Tobin, R.S.; Junkins, B.; Kushner, D.J. Effect of chlorination on antibiotic resistance profiles of sewage related bacteria. *Appl. Environ. Microbiol.* **1984**, *48*, 73–77. [PubMed]
115. Oncu, N.B.; Menceloglu, Y.Z.; Balcioglu, I.A. Comparison of the effectiveness of chlorine, ozone, and photocatalytic disinfection in reducing the risk of antibiotic resistance pollution. *J. Adv. Oxid. Technol.* **2011**, *14*, 196–203.
116. Suquet, C.; Warren, J.J.; Seth, N.; Hurst, J.K. Comparative study of HOCl-inflicted damage to bacterial DNA ex vivo and within cells. *Arch. Biochem. Biophys.* **2010**, *493*, 135–142. [CrossRef] [PubMed]
117. USEPA. Ultraviolet disinfection guidance manual for the final long term 2 enhanced surface water treatment rule. In *EPA 815-R-06-007*; United States Environmental Protection Agency: Washington, DC, USA, 2006; p. 436.
118. Luddeke, F.; Hess, S.; Gallert, C.; Winter, J.; Gude, H.; Loffler, H. Removal of total and antibiotic resistant bacteria in advanced wastewater treatment by ozonation in combination with different filtering techniques. *Water Res.* **2015**, *69*, 243–251. [CrossRef] [PubMed]
119. Sigmon, C.; Shin, G.A.; Mieog, J.; Linden, K.G. Establishing surrogate-virus relationships for ozone disinfection of wastewater. *Environ. Eng. Sci.* **2015**, *32*, 451–460. [CrossRef]
120. Zhang, Y.; Zhuang, Y.; Geng, J.; Ren, H.; Zhang, Y.; Ding, L.; Xu, K. Inactivation of antibiotic resistance genes in municipal wastewater effluent by chlorination and sequential UV/chlorination disinfection. *Sci. Total Environ.* **2015**, *512–513*, 125–132. [CrossRef] [PubMed]
121. Yoon, Y.; Chung, H.J.; Wen Di, D.Y.; Dodd, M.C.; Hur, H.G.; Lee, Y. Inactivation efficiency of plasmid-encoded antibiotic resistance genes during water treatment with chlorine, UV, and UV/H2O2. *Water Res.* **2017**, *123*, 783–793. [CrossRef] [PubMed]
122. Formosa, C.; Grare, M.; Duval, R.E.; Dague, E. Nanoscale effects of antibiotics on *p. aeruginosa*. *Nanomed. Nanotechnol. Biol. Med.* **2012**, *8*, 12–16. [CrossRef] [PubMed]
123. Formosa, C.; Grare, M.; Jauvert, E.; Coutable, A.; Regnouf-de-Vains, J.B.; Mourer, M.; Duval, R.E.; Dague, E. Nanoscale analysis of the effects of antibiotics and CX1 on a *pseudomonas aeruginosa* multidrug-resistant strain. *Sci. Rep.* **2012**, *2*, 575. [CrossRef] [PubMed]
124. Breazeal, M.V.; Novak, J.T.; Vikesland, P.J.; Pruden, A. Effect of wastewater colloids on membrane removal of antibiotic resistance genes. *Water Res.* **2013**, *47*, 130–140. [CrossRef] [PubMed]
125. Cheng, H.; Hong, P.Y. Removal of antibiotic-resistant bacteria and antibiotic resistance genes affected by varying degrees of fouling on anaerobic microfiltration membranes. *Environ. Sci. Technol.* **2017**, *51*, 12200–12209. [CrossRef] [PubMed]

126. Shoener, B.D.; Zhong, C.; Greiner, A.D.; O. Khunjar, W.; Hong, P.-Y.; Guest, J.S. Design of anaerobic membrane bioreactors for the valorization of dilute organic carbon waste streams. *Energy Environ. Sci.* **2016**, *9*, 1102–1112. [CrossRef]
127. Harb, M.; Hong, P.Y. Molecular-based detection of potentially pathogenic bacteria in membrane bioreactor (MBR) systems treating municipal wastewater: A case study. *Environ. Sci. Pollut. Res. Int.* **2017**, *24*, 5370–5380. [CrossRef] [PubMed]
128. Harb, M.; Wei, C.H.; Wang, N.; Amy, G.; Hong, P.Y. Organic micropollutants in aerobic and anaerobic membrane bioreactors: Changes in microbial communities and gene expression. *Bioresour. Technol.* **2016**, *218*, 882–891. [CrossRef] [PubMed]
129. Christgen, B.; Yang, Y.; Ahammad, S.Z.; Li, B.; Rodriquez, D.C.; Zhang, T.; Graham, D.W. Metagenomics shows that low-energy anaerobic-aerobic treatment reactors reduce antibiotic resistance gene levels from domestic wastewater. *Environ. Sci. Technol.* **2015**, *49*, 2577–2584. [CrossRef] [PubMed]
130. Olivieri, A.W.; Crook, J.; Anderson, M.A.; Bull, R.J.; Drewes, J.E.; Haas, C.N.; Jakubowski, W.; McCarty, P.L.; Nelson, K.L.; Rose, J.B.; et al. *Expert Panel Final Report: Evaluation of the Feasibility of Developing Uniform Water Recycling Criteria for Direct Potable Reuse*; National Water Research Institute for the State Water Resources Control Board: Sacramento, CA, USA, 2016.