**Mitigating antimicrobial resistance risks when using reclaimed municipal wastewater for
agriculture**

Pei-Ying Hong^{1#}, Changzhi Wang¹, David Mantilla-Calderon¹

1. King Abdullah University of Science and Technology (KAUST), Water Desalination and
Reuse Center, Division of Biological and Environmental Science and Engineering,
Thuwal 239455-6900, Saudi Arabia

Corresponding author

Pei-Ying Hong

Email: peiyong.hong@kaust.edu.sa

Tel: +966 12 808 2218

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24 **Abstract**

25 The global need for food is posing a serious threat to water security. Treated wastewater can be
26 used as an alternative water supply to mitigate our reliance on non-renewable waters (defined
27 as water that cannot be replenished within our life span). However, concerns related to
28 emerging contaminants such as antibiotic resistant bacteria (ARB) and antibiotic resistance
29 genes (ARGs) can impede efforts to push for widespread use of treated wastewater in
30 agricultural irrigation. This chapter aims to provide a better understanding of the potential
31 concerns by first using case studies in two countries that have already practiced water reuse.
32 Second, we collate and analyze data that suggests that wastewater treatment plants able to
33 achieve at least 8-log reduction in microbiological contaminants may suffice as appropriate
34 intervention barriers for ARB dissemination to the environment. This chapter also recognizes
35 that extracellular DNA carrying ARGs may not be effectively removed even with membrane-
36 based treatment. There is therefore a need to assess whether extracellular DNA may
37 accumulate in agricultural soils due to repeated use of treated wastewater, and to determine the
38 concentrations of extracellular DNA needed to significantly increase horizontal gene transfer
39 (HGT) in the natural environment. Given the large knowledge gaps that hinder an accurate
40 assessment of the associated risks, it would be prudent to adopt the precautionary principle and
41 to implement appropriate intervention strategies and best management practices that minimize
42 the impacts and concerns arising from the reuse of treated wastewater in agriculture.

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Introduction

Almost 70% of our global freshwater is used to produce food to feed the 7 billion people worldwide ¹. The global population is expected to increase to 9.8 billion by 2050 ², meaning even more freshwater is needed to produce sufficient food to feed us all. If no alternative sources of water are made available, the long-term consequence will be a depleted water supply.

Clearly, innovative solutions have to be considered to replenish the rapidly depleting fossil fresh waters we rely on (e.g. through aquifer recharge) or to substitute a portion of the non-renewable water supplies with recycled or desalinated waters. Desalinated seawater, a process whereby saline waters (either coastal seawaters or inland brackish waters) are converted to freshwaters either by means of thermal or membrane-based processes, can be used as an alternative water source. The advantage of doing so is that high quality water is produced. However, the energy costs associated with desalination typically range from 3 to 4 kWh/m³ ³. Considering the amount of water that is used per day for food production (estimated at 57.1 million m³ for Saudi Arabia) ⁴, relying on desalinated waters alone to minimize our reliance on freshwater is going to amount to huge economic burden, even in a country such as Saudi Arabia which is the world's largest producer of fossil fuels (and hence has lower energy costs than non-producing countries).

In contrast, treating wastewater by converting it from dirty water to a level clean enough for reuse takes less energy than desalination. The conventional wastewater treatment process requires about 0.3 to 1 kWh/m³ ⁵⁻⁷, which is three to four times lower than the energy required by desalination. The downside is that this practice is commonly associated with a "yuck" factor. Moreover, municipal wastewaters contain a diverse suite of contaminants that are not completely removed by conventional treatment processes ^{8,9}. The problem is further compounded when the list of contaminants that are found in our wastewaters becomes

increasingly more complex too. Consumers are becoming more aware of health impacts arising from bacteria and viruses in our daily environment and in our food. This increased awareness in turn drives up demand for antimicrobial products. Retail companies respond to this demand by offering new products that promise antimicrobial effects through the addition of silver nanoparticles, triclosan, quaternary ammonium salts and so on, just to list a few. A modern individual is also consuming more pharmaceutical compounds with every passing year ¹⁰. Unmetabolized residues of these pharmaceutical compounds as well as metabolites and transformation products of the parent compounds will find their way down the toilet to our sewage. We do not and cannot know the full extent of detrimental impacts on public health during reuse events.

Just on the topic of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in treated wastewater alone, there has been an ongoing debate about whether their presence can potentially impact public health during reuse events. In the absence of robust antimicrobial risk assessment, there is currently no consensus among the various stakeholders (e.g. researchers, regulators, utilities) about whether there is a need to mandate new regulations or invest in state-of-the-art treatment technologies (e.g. membrane bioreactors, advanced oxidation processes) to enhance removal efficiencies of ARB and ARGs from wastewater prior to reuse for non-potable purposes. The reluctance to implement new treatment technologies like membrane bioreactors to existing WWTPs is in part due to the energy and operational costs associated with running membrane bioreactors. An aerobic membrane bioreactor requires about 2 kWh/m³ ¹¹, which is ca. 2 times higher than the energy costs needed to operate a conventional treatment process. Furthermore, fouled membranes have to be routinely cleaned or replaced. Similarly, advanced oxidation processes like UV/H₂O₂ or UV/ozone require additional energy for the operation of UV lamps, and incur chemical costs that may be more expensive than the conventional chlorine. Under any scenario, both the

operational and capital costs associated with either new or upgraded treatment technologies to enhance removal of ARB and ARGs would be significantly higher than before.

The question therefore arises: does the benefit of excessively treating our wastewaters outweigh the costs involved? This is a valid question considering that antimicrobial resistance threats are especially more acute in many developing countries with a lack of available funds to revamp or upgrade existing treatment infrastructure. There has to be a point where investment is made to an optimal level, and not beyond that needed, so that the invested technology safeguards the risk at the acceptable level without overburdening the financial system.

In this chapter, we note that there has been no strong evidence to suggest a significant risk related to antimicrobial resistance threats in countries that have already actively reused good quality treated wastewater for both potable and nonpotable purposes. Treatment technologies are assessed to determine what level of log removal values (LRVs) are needed to investigate whether remnant levels of ARB and ARGs are likely to pose concerns during agricultural irrigation. These include concerns such as the potential for horizontal gene transfer arising from the presence of extracellular DNA. The objective of this chapter is to provide a pragmatic outlook on how to facilitate safe and sustainable reuse of treated wastewater, thereby reducing our reliance on non-renewable water supplies.

No direct evidence of increased antimicrobial resistance threats in Singapore

The greatest safety and public health concerns arise in relation to drinking treated wastewater. However, it is important to recognize that multi-barrier technologies are put in place by countries (e.g. Singapore) or cities (e.g. Orange County) opting for indirect potable reuse. To exemplify, Singapore has implemented indirect potable reuse of treated wastewater (referred to as NEWater) since 2003. The country treats wastewater through a membrane bioreactor

(MBR)-based wastewater treatment plant (WWTP) prior to reverse osmosis (RO)-based filtration at a water reclamation plant. The treated product is termed NEWater and is subsequently blended with surface water stored in catchment reservoirs, and retained in the reservoirs for a certain duration that varies depending on local water usage rates. Eventually, this blended water (comprising up to 40% NEWater) ¹² then goes through the conventional drinking water treatment, which includes chemical coagulation, filtration and disinfection, before being distributed to individual households.

Given the relatively high percentage of NEWater in its drinking water supplies, one can in theory track the population over time to determine if consumption of highly treated recycled wastewater is likely to cause an increase in antimicrobial resistance rates among the local community pre- and post-introduction of NEWater, particularly since Singapore is a small country with full nationwide coverage of piped potable waters provided for only by Singapore Public Utilities Board (the main agency in charge of all water-related matters in Singapore). It is unknown if any epidemiological studies were commissioned since the start of NEWater implementation with this research question in mind. However, in response to the 2017 National Strategic Action Plan on Antimicrobial Resistance, Singapore Public Utilities Board funded a number of projects that include monitoring for ARB and ARGs in the local reservoirs. In Singapore, local surface reservoirs usually contain rainwater, storm water, surface water from Malaysia as well as reclaimed water (NEWater). Despite local reservoirs receiving reclaimed water, the abundance of most ARG-like sequencing reads detected was comparatively lower than that detected in hospital wastewaters or untreated wastewater¹³. Using quinolone resistance gene (i.e., *qnrS2*, which confers resistance through protection of sites targeted by quinolone) as an example, the relative abundance of reads (per Gb of sequencing data) increased more than 5 times, from an average 1973 reads per Gb of sequencing data in the local reservoir surface waters to approximately 12000 reads/Gb of sequencing data derived

from untreated wastewaters. Instead, the levels in the local reservoir approximate to that sampled from treated effluents (383 to 637 reads/Gb), suggesting that those relative abundances detected in the local reservoirs were most likely baseline environmental concentrations¹³. This meant that even though ARGs are detected in local reservoirs (which is used as a source of drinking water), these positive detections may not be directly due to the mixing of treated wastewater, but instead due to background environmental exposure.

These findings were also in agreement with another independent study conducted by Yi et al. Over a two year period, the authors performed fluorescence in-situ hybridization on water samples collected throughout Singapore, and noted that the percentage of macrolide-lincosamide-streptogramin B (MSLB) resistant bacteria in total bacterial community of reservoirs were consistently lower than that detected in surface waters collected from urban parks, commercial, residential and industrial zones of Singapore¹⁴. Their observations, along with the earlier study¹³, reiterate that the multi-barrier approaches put in place by Singapore water utilities are able to effectively minimize the dissemination of ARBs and ARGs through indirect potable reuse.

No direct evidence of public health outbreak caused by reclaimed water

The above case study in Singapore suggests that properly treated wastewater, particularly that which has gone through a multi-barrier treatment train, may not significantly elevate risks associated with ARB and ARGs if reuse is coupled with a proper management and governance system. In the US, nurseries and vegetables are irrigated with treated wastewater¹⁵. California is the leading state in reusing 46% of reclaimed water for agricultural irrigation¹⁶. The state stipulates at least tertiary treatment and disinfection of treated wastewater before irrigation¹⁵. The guideline further recommends that not more than one sample in any 30 day

period should have > 23 most-probable-number (MPN) of total coliforms per 100 mL of reclaimed water applied onto food crops that is consumed raw ¹⁷. Alternatively, if the level of treatment falls below tertiary treatment, more restrictive agronomic practices are required. For example, if only secondary treatment is available, drip irrigation (where water drips slowly from the soil surface/subsurface to roots of plants) instead of spray irrigation must be used, and only for orchards or animal feed production, not for the production of vegetables ¹⁸. In such instances, the guideline still requires no more than 240 CFU per 100 mL of reclaimed water collected within any 30 day period ¹⁷.

Based on the abovementioned guideline, to date there have not been any reports of public health outbreaks tied to food irrigated with treated wastewaters in California. Instead, non-point contamination of irrigation waters and unsanitary practices have been more of a concern. Using the April-June 2018 outbreak of *E. coli* O157:H7 in romaine lettuce as a case study, 210 people from 36 states in US were infected with the outbreak strain. Among them, 27 people developed kidney failure and 5 deaths were reported ¹⁹. Following this outbreak, US Food and Drug Administration (FDA) performed a traceback analysis to determine a common point of contamination in the distribution chain. Records collected from restaurants and stores where sick people ate or shopped were reviewed. This led to the identification of 23 farms in the Yuma growing region as the common point source supplying the contaminated romaine lettuce across states. Environmental assessments were then carried out at these farms. Specifically, canal waters at these farms were positive for *E. coli* O157:H7, and through whole genomic sequencing, identified to be of the same strain causing the outbreak ¹⁹. Since the canal water was used to irrigate the romaine lettuce, it was believed that the *E. coli* strain came into contact with the romaine lettuce either during germination or at various times during growing season, including after a freeze event that may have led to damage of some portion of the crop and facilitated internalization of the pathogenic strain. When trying to account for how this outbreak strain was introduced into the irrigation canal, the environmental assessment team noted that a

large concentrated animal feeding operation (CAFO) is located adjacent to the irrigation canal. However, they were unable to identify an obvious route for contamination from the CAFO to canal since the same outbreak strain was not identified at the CAFO by whole genome sequencing ²⁰.

In October to December 2018, a new outbreak involving *E. coli* O157:H7, also in romaine lettuce, from Central Coastal growing regions of northern and central California was reported ²¹. There were in total 62 reported illnesses, with 25 hospitalizations and two cases of kidney failure. Using the same approach as illustrated earlier, sediment samples from an on-farm water reservoir tested positive for the outbreak strain in this instance, and were likely transferred to the irrigation water when conveyor pumps were activated and perturbed the sediments. However, the reservoir was not connected to other water sources or distribution systems in the growing region and hence no apparent source of contamination could be identified. In addition, the farm did not have an optimal disinfection system in place for the agricultural water ²².

These two recent outbreaks suggest that (i) open-air surface waters are particularly vulnerable to contamination, (ii) it is not easy to pinpoint the exact source of contamination and implement remediation measures, (iii) farmers may not be well-trained to carry out appropriate treatment and disinfection of irrigation waters on-site, and (iv) both outbreaks were not associated with reusing treated wastewater. This suggests that a safe reuse of treated wastewater is feasible, and perhaps safer than the current practice of using traditional sources of irrigation waters, as long as the appropriate intervention strategies and policies are put in place.

Wastewater treatment processes are important barriers

A recent multivariate regression analysis using datasets obtained from 73 countries reported that better sanitation and governance were associated with lower antimicrobial resistance indices ²³. This reiterates the absolute need for proper treatment of wastewater prior to reusing the water in an open environment.

Treated wastewater is currently reused in three main ways, namely managed aquifer recharge, agricultural irrigation, and indirect potable reuse. At the time of writing, there has been no full-scale practice of direct potable reuse although this may happen in the future. Existing regulations pertaining to the treated water quality do not explicitly list the permissible level of ARB or ARGs for each type of reuse activity. Instead, recommendations are based on coliform counts, biochemical oxygen demand (BOD)/chemical oxygen demand (COD) reduction, total nitrogen and total phosphate concentrations, and other parameters related to the chemical constituents of these waters ²⁴⁻²⁶. Table 1 lists some of the reuse water quality limits recommended by the Ministry of Water, Environment and Agriculture in Saudi Arabia, US EPA and International Organization for Standardization (ISO). The list is not exhaustive and readers can refer to the references for a more complete list.

Given that the permissible concentrations of contaminants can vary depending on the type of reuse purpose, an appropriate treatment train therefore needs to be devised accordingly. This is otherwise termed as fit-for-purpose. In most instances, it is recommended that wastewater goes through at least secondary treatment if it is intended for aquifer recharge and/or agricultural irrigation ^{27, 28}. In addition, ISO guidelines also suggest that in order to achieve the restricted irrigation (defined as irrigation in places where public access is controlled or restricted by physical or institutional barriers), a potential corresponding treatment should include activated secondary wastewater treatment process, sand filtration and disinfection ²⁶. An earlier study reported that a conventional secondary treatment process in Saudi Arabia, which includes a primary clarifier, an activated sludge tank, a secondary clarifier and a final

disinfection (Figure 1a), was able to achieve ≤ 1000 CFU/100 mL of fecal coliforms and hence can be used for restricted irrigation ²⁹.

Collating results from other studies, the anticipated log removal rates for total bacteria or total suspended solids are ca. 1-log for both clarifiers (i.e., 70% removal for primary and secondary clarifier, respectively) ³⁰, 1 to 2-log by the activated sludge tanks depending on the operating conditions ³¹⁻³⁴, about 2-log removal (i.e., 99% removal) for slow sand filtration ³⁵, and an additional > 3 -log (i.e., 99.9% removal) if chlorination and ozonation, the two most commonly used disinfection processes, are effectively carried out ^{29, 32, 36} (Figure 1a). If membrane filtration, typically a microfiltration or ultrafiltration membrane, is also retrofitted to the wastewater treatment plant, ca. average 5 log removal of microbiological contaminants are routinely reported ^{37, 38} (Figure 1b). However, because different WWTPs use different treatment trains and different operational parameters that may affect the overall efficiency of each individual treatment unit, log removal values can potentially vary over a wide spectrum ³¹.

Table 1. Recommended contaminant limits in reclaimed water intended for different reuse purposes. * restricted irrigation refers to irrigation where public access is controlled or restricted by physical or institutional barriers. ** unrestricted irrigation refers to irrigation in settings where public access is not restricted.

Reference	Regulatory agency	Parameter	Restricted irrigation*	Unrestricted irrigation**	
25	Saudi Arabia Ministry of Water, Environment and Agriculture	Fecal coliforms (CFU/100 mL)	≤ 1000	≤ 2.2	
		Biochemical oxygen demand, BOD (mg/L)	40	10	
		Total suspended solids, TSS (mg/L)	40	10	
		Turbidity (NTU)	5	5	
			Landscape irrigation, toilet flushing	Surface or spray irrigation of any food crops	Surface irrigation of orchards and vineyards
24	US-EPA	Fecal coliforms	No detectable per 100 mL	No detectable per 100 mL	≤ 200
		Biochemical oxygen demand, BOD (mg/L)	≤ 10	≤ 10	≤ 30
		Total suspended solids, TSS (mg/L)	Not specified	Not specified	≤ 30
		Turbidity (NTU)	≤ 2	≤ 2	Not specified
			Restricted irrigation and agricultural irrigation of processed food crops	Unrestricted irrigation and agricultural irrigation of food crops consumed raw	Agricultural irrigation of non-food crops
26	ISO	Fecal coliforms (CFU/100 mL)	≤ 200	≤ 10	≤ 1000
		Biochemical oxygen demand, BOD (mg/L)	≤ 10	≤ 5	≤ 20
		Total suspended solids, TSS (mg/L)	≤ 10	≤ 5	≤ 30
		Turbidity (NTU)	Not specified	≤ 2	Not specified

Figure 1. An illustration of the wastewater treatment processes and the approximate log removal values (LRV) of total microbiological contaminants reported at each stage. Treatment process can include the use of **(a)** primary clarifier, activated sludge, secondary clarifier and disinfection, or **(b)** membrane bioreactor prior to disinfection.

Potential impacts of treated wastewater irrigation on the indigenous soil microbial community

In the context of this chapter, we argue that most of the treated wastewater should be reused for agricultural irrigation since this is the sector that uses the most water. Hence, emphasis was made to evaluate if ARB in treated wastewater would complicate public health concerns during agricultural irrigation. A similar assessment has been done by Pepper et al., in which they evaluated the abundance of ARB that were reported by past studies, and compared against that reported in the baseline soil microorganisms³⁹. The authors noted that applying conventionally treated effluent (i.e., with no MBR) on agricultural lands during a single application event resulted in ca. 0.001% increment in ARB compared to the baseline ARB concentrations in soils. This increment is lower than the percentage increase arising from single

application of biosolids (0.1%) and manure (0.05%) application³⁹. A further comparison between treated wastewater-irrigated and freshwater-irrigated soils revealed identical levels in both ARB levels after irrigation⁴⁰, and support the calculations made by Pepper et al³⁹. It is therefore likely that effluent or manure-associated ARB would not be able to compete successfully against the resident soil bacteria^{40, 41}, and would attenuate to baseline levels in between irrigation events.

To further investigate the potential for the accumulation of total bacterial cells in irrigated soils due to long term irrigation, we took the average cell counts (ca. 10^{11} cells per L) sampled after the primary clarifier and determined by flow cytometry³⁸, and the known LRVs reported by each stage of the WWTP discussed earlier, and estimated that the total cells found in the final reclaimed water would range between 10^4 to 10^6 cells per L, depending on the treatment process (Figure 1). We assume that the WWTP provides 4000 m³ of treated wastewater per day, therefore discharging in a worst case scenario 4×10^{12} cells/d into the agricultural soils if all of the reclaimed water were to be used for irrigation. Assuming a gross irrigation depth of 70 mm over 0.01 km² irrigated land, this volume of wastewater is sufficient to irrigate over 0.06 km² land mass. Using an average soil bacterial cell density of 10^9 cells per g³⁹, this would equate to 753.9 cells per mm² of land⁴², and therefore 4.5×10^{13} cells would be exposed to the treated wastewater (4×10^{12} cells/d) (Figure 2). This abundance of indigenous soil bacterial counts is 1-log higher than that from the treated wastewater, and a single irrigation of the treated wastewater would therefore unlikely to result in a significant increment in cell mass balance.

While a single irrigation event with treated wastewater would be unlikely to result in a significant increment in cell counts, in practice, irrigation is routinely carried out throughout the growing season, with specific irrigation schedules varying depending on the type of crop, stage of crop growth, weather, and soil characteristics. Assuming that irrigation is done once every 10 days in Saudi Arabia for crops like lettuce, cucumbers and peppers, and with an assumed

bacterial decay of 5-log due to solar irradiation ^{43, 44} in between consecutive irrigation events, the contributed amount of bacteria by conventionally treated wastewater would still be approximately on the same magnitude of abundance than that in the indigenous soil microbiota after 1 month of using treated wastewater (Figure 2). However, the contributed portion may be comprised of mainly ARB since an earlier study found that the fraction of ARB normalized to the total culturable bacteria increased after the WWTP than in the untreated wastewater ²⁹, and that a pathogenic and antibiotic-resistant strain of *E. coli* survived harsh environmental conditions better than a commensal *E. coli* strain ⁴⁴.

In contrast, the amount of bacteria in MBR-treated effluent is 3-log lower than that in the baseline soil. Assuming the same irrigation frequencies and decay rates, it would not be possible to accumulate to levels higher than the indigenous bacteria already present in the soil within the entire growing season (Figure 2). These calculations assume that an agricultural site uses undiluted stream of treated wastewater on site. However, in most agricultural sites, the demand for irrigation water is substantially higher than the volume provided for by treated wastewater alone. For example, as already mentioned, an estimated 57.1 million m³ of water per day is needed for agricultural irrigation in Saudi Arabia ⁴. A full capture of all wastewater in the kingdom would generate only 8.7 million m³ (based on an average 260 L of water used per day per capita in 2017 that will be discharged back into sewers as waste by the current 33.4 million population ⁴⁵) of treated municipal wastewater, which is not sufficient to meet the demand for agricultural irrigation. Hence, dilution of treated wastewater with groundwater or desalinated water is most likely needed, and this dilution would further lessen the contribution of bacteria from treated wastewater into the natural soils.

Although MBR-based treatment can lower the contribution of bacterial load from treated wastewater into natural soils, one still needs to consider the possibility of conjugation between the remnant bacteria in the reclaimed water and the indigenous soil microorganisms. This

328 concern stems from a number of recent studies that showed *in-vitro*, chemical pollutants
329 prevalent in the wastewater stream (e.g. carbamazepine, triclosan, copper nanoparticles or
330 copper ions) can trigger intracellular reactive oxygen species (ROS) production and enhance
331 cell membrane permeability ⁴⁶⁻⁴⁸. This in turn resulted in a higher conjugation rates between *E.*
332 *coli* strains or between *E. coli* and species of other genera (e.g. *Pseudomonas putida* and
333 *Salmonella enterica* serovar Typhimurium) compared to the baseline spontaneous conjugation
334 rates of 10^{-5} (Table 2a). In addition, sub-lethal concentrations of chlorine and hydrogen
335 peroxide, both of which are disinfectants used in conventional WWTPs, were also able to
336 independently promote conjugation within and across bacterial genera at sublethal
337 concentrations ⁴⁹. However, these conjugation experiments were performed in oligotrophic
338 buffer solution matrix and with a ratio of 1 to 1 of donor to recipient bacterial cells, each in cell
339 density of 10^8 CFU/mL (Table 2a), and are not conditions simulating that in the natural
340 environments. Therefore, further studies should look into replicating the same experiments in
341 conditions more similar to that in the natural environment so as to determine whether enhanced
342 conjugation would indeed happen between the microorganisms in soil and reclaimed water.

343 **Table 2.** Collation of studies performed to assess **(a)** conjugation frequencies, and **(b)** transformation frequencies in the absence and
344 presence of stressors.

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a)	Conjugation studies ^[Ref]	Tested donor	Tested recipient	Donor: recipient ratio (cell density)	Test conditions	Detected frequency
	Zhang et al. (2016) ⁴⁹	<i>E. coli</i> S17-1	<i>E. coli</i> K12	1.5: 1 (10 ⁸ CFU/mL)	No stressor (baseline) PBS 4 h reaction 25 °C	10 ⁻⁵
					Free chlorine (0.1 to 1 mg/L) PBS 4 h reaction 25 °C	Concentration-dependent increase (3 to 6-fold higher than baseline)
					H ₂ O ₂ (0.24 to 3 mg/L) PBS 4 h reaction 25 °C	Concentration-dependent increase (2 to 6-fold higher than baseline)
	Wang et al. (2018) ⁴⁷	<i>E. coli</i> K12 LE392	<i>E. coli</i> K12 MG1655 mutant	1: 1 (10 ⁸ CFU/mL)	No stressor (baseline) PBS 8 h 25 °C	6.85 x 10 ⁻⁵
					Carbamazepine (0.05 to 50 mg/L)	Concentration-dependent increase (4 to 12-fold higher than baseline)
		<i>E. coli</i> K12 LE392	<i>Pseudomonas putida</i> KT2440	1: 1 (10 ⁸ CFU/mL)	No stressor (baseline) PBS 8 h 25 °C	2.67 x 10 ⁻⁶
					Carbamazepine (0.05 to 50 mg/L)	Concentration-dependent increase (5 to 8-fold higher than baseline)
	Lu et al. (2018) ⁴⁶	<i>E. coli</i> K12 LE392	<i>E. coli</i> K12 MG1655 mutant	1: 1 (10 ⁸ CFU/mL)	No stressor (baseline) PBS 8 h 25 °C	3.13 x 10 ⁻⁵

					Triclosan (0.02 to 2 µg/L)	Concentration-dependent increase (2 to 6-fold higher than baseline)
		<i>E. coli</i> K12 LE392	<i>Pseudomonas putida</i> KT2440	1: 1 (10 ⁸ CFU/mL)	No stressor (baseline) PBS 8 h 25 °C	4.91 x 10 ⁻⁶
					Triclosan (0.02 to 2 µg/L)	Concentration-dependent increase (1.8 to 2.2-fold higher than baseline)
	Zhang et al. (2019) ⁴⁸	<i>E. coli</i> K12 LE392	<i>Pseudomonas putida</i> KT2440	1: 1 (10 ⁸ CFU/mL)	No stressor (baseline) PBS 24 h 25 °C	1.8 x 10 ⁻⁶
					Copper (0.5 to 1 µmol/L)	Concentration-dependent increase (2 to 2.2-fold higher than baseline)
b)	Transformation studies ^[Ref]	Tested recipient		Cell: DNA ratio (CFU: µg)	Test conditions	Detected frequency
	Mantilla-Calderon et al. (2019) ⁵⁰	<i>Acinetobacter baylyi</i> ADP1		2 x 10 ⁸ : 2	No stressor (baseline) 24 h 37 °C	1.9 x 10 ⁻⁶
					Bromoacetic acid (100 to 600 µM)	Concentration-dependent increase (1.1 to 1.8-fold higher than baseline)
					Disinfection byproducts cocktail containing trichloromethane (1.4 µM), bromoacetic acid (1.9 µM), formaldehyde (3.8 µM), N-nitrosodimethylamine (0.7 nM), tribromoacetamide (0.03 µM)	1.7-fold higher than baseline
	Augsburger et al. (2019) ⁵¹				No stressor (baseline) 24 h 37 °C	3.4 x 10 ⁻⁶
					Sunlight (70 to 150 mJ/cm ²)	Concentration-dependent increase (1.5 to 2-fold higher than baseline)

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349 **Figure 2.** An estimation of the amount of bacterial cells and extracellular DNA (eDNA) that
 350 would be contributed by the effluent treated by either activated sludge (AS) processes or by
 351 membrane bioreactor (MBR). The estimation is made given an assumed irrigation scenario, soil
 352 characteristics and decay rates.

Multi-barrier intervention strategies for low resource countries

Based on the literature gathered for this study, we are leaning towards the opinion that the risks arising from ARB in reclaimed water used for agricultural irrigation may be sufficiently mitigated if (i) the effluent is derived from a treatment process that is cleaning municipal wastewaters containing approximately 10^{12} cells/L and provides 8-log or more reduction in total bacterial cell count, or (ii) appropriate agronomic practices are put in place (e.g. dilution of treated wastewater, drip irrigation and non-leafy crops).

Although the general scientific evidence supports the conclusion that MBRs generate treated effluent that would result in low risks during agricultural irrigation, it is important to remember that countries that rely heavily on agriculture for their gross domestic product also include low resource countries that have less monetary capacity to invest in their sanitary infrastructure ⁵². Therefore, instead of trying to push for advanced state-of-art technologies (e.g. MBR or RO) which incur high operational and capital investments and are out of reach for these countries, the question to ask is whether low cost treatment strategies, possibly through a multi-barrier approach would be equally effective to mitigate risks associated with reuse.

Examples of low cost multi-barrier approach could be coagulation, flocculation and sedimentation (typically achieves 1 log reduction ⁵³), followed by retention of supernatant in evaporation ponds or wetlands (typically achieves 1 to 2 log reduction ^{54, 55}), and then surface spreading for infiltration of the water through sand gravel layers prior to storage in underground aquifers (typically achieves 1 log reduction for every 8.5 m of infiltration ^{53, 56}). The stored water can then be pumped up from the aquifer and disinfected (typically achieves 3 log reduction) before using for agricultural irrigation. In such instances, the foremost criteria would be abundance of land and long retention time for natural attenuation to occur effectively. The aim would be to achieve a comparable 8-log reduction in total with this multi-barrier approach using resources that are abundant, low cost or free.

Feasibility assessment of low-cost multi-barrier approaches would need to be performed – first, by determining the LRV at each stage of the implemented barrier; and second, by evaluating the energy and operational costs associated with the entire treatment train. For successful implementation, different stakeholders (e.g. engineers, regulators, researchers, utility operators, end users) should work together to form a better picture of the best estimated extent of risks involved in using treated wastewater. For example, researchers can lead with regular monitoring of their treatment technologies, and in evaluating water for new emerging contaminants. They could also work with engineers to design or implement improved versions of treatment technologies in instances where the existing treatment technologies do not provide sufficient barriers. Relevant utilities can then make use of the data derived by scientists and engineers to design best management practices for farmers and to protect public health.

ARGs: living in the cloud of uncertainties

Although the LRVs of bacteria achieved by each individual stage along the WWTP is generally well studied, LRV of extracellular DNA by the same treatment process is not as well characterized. In an earlier study, a lab-scale MBR-based reactor only achieved ca. 2 to 4-log reduction of extracellular DNA⁵⁷, suggesting that the final reclaimed water still contains these potential contaminants. Indeed, the presence of extracellular DNA is not unique to treated wastewater; it has been reported to be present in a median concentration of about 0.07 ng/L in Singapore's drinking water⁵⁸.

Since there are currently no similar studies performed in estimating the amount of extracellular DNA present in the final reclaimed water, we assume all reclaimed water should have gone through a treatment that would achieve 0.07 ng extracellular DNA per liter of water since it approximates to that present in clean drinking water in Singapore⁵⁸. Subsequently, we

used this value to estimate the amount of extracellular DNA that can be disseminated into the environment if the reclaimed water were to be reused. Based on the same parameters used in earlier calculations for ARB, we calculated that approximately 0.3 g of extracellular DNA is disseminated into the environment each day through reuse of reclaimed water based on the assumed irrigation frequencies, land area etc. stated in Figure 2.

Earlier studies have found that extracellular DNA can persist in soil environments for longer period of time than newly introduced bacterial cells. For example, DNA introduced by agricultural irrigation and/or manure application could still be detected by PCR-based methods after as many as 16 months⁵⁹⁻⁶¹. The degradation rates of DNA in the soil are well studied but multiple factors are known to affect the decay rates⁶². A general degradation rate of DNA in soils cannot easily be determined due to contradictory results reported by various studies. Therefore, assuming 50% of extracellular DNA decays by the time of next irrigation event, ca. 1.2 g of DNA would be accumulated by end of 1 month and 2.8 g of DNA by end of 2 months (Figure 2). Concerns may arise if this extracellular DNA is taken up by naturally competent bacteria cells present in the recipient environment, and subsequently integrated into the genome to be expressed into problematic gene products (e.g. virulence traits, antibiotic resistance). However, it is also difficult to know how much of these DNA would contain such problematic gene products, reiterating that the true extent of risks from extracellular DNA is difficult to ascertain.

Recent studies using *Acinetobacter baylyi* ADP1 as model bacterium demonstrated a baseline transformation frequency of about 10^{-6} when extracellular DNA was introduced to the competent bacterium in DNA to cell ratio of 1 μ g to 10^8 cells^{50, 51} (Table 2b). Furthermore, it was observed that in lab studies, when these extracellular DNA are present along with sub-lethal concentrations of disinfection byproducts (DBPs) and solar irradiation, transformation frequencies are increased in a concentration dependent manner by up to 2-fold^{50, 51}. The

increase in transformation frequencies was found to be due to the mutagenic but non-lethal effect caused by DBPs and sunlight. In both instances, there was an upregulation of *recA* gene expression while the upregulation of competency-related genes were only observed in the presence of sunlight. In bacteria, *recA* plays a shared role in both the repair of DNA double-strand breaks⁶³ and the chromosomal integration of foreign DNA during natural transformation⁶⁴. The increase in transformation frequencies observed in both DBPs and sunlight was therefore likely to be due to increased integration of extracellular DNA into *A. baylyi* ADP1 as the bacteria seeks to repair damage induced by both stressors.

Although there was a concentration-dependent increment in transformation frequencies in the presence of stressors introduced during the treatment or in the natural environment, the fold increment observed in these studies were at most 2-fold^{50, 51}. This would mean that the transformation frequency remains in the same magnitude as that of baseline – 10^{-6} . It remains unknown if multiple stressors, for example combinations of DBPs and sunlight, would impose an additive or antagonistic effect on transformation frequencies, and if so, whether it would significantly increase or decrease the hazards arising from horizontal gene transfer. There remains a knowledge gap as to whether this extracellular DNA is truly of concern and should constitute a new class of emerging contaminants, e.g. when disseminated into the environment through water reuse.

More questions to be addressed

This review only focuses on antibiotic resistant bacteria and antibiotic resistance genes, and argues that the risks arising from them would be low if sufficient intervention measures are put in place. However, these are not the only contaminants present in the treated wastewater matrix. Currently, we can never presume to have tested all of the individual contaminants that

may potentially be of concern. However, what we do know now is that pharmaceutical compounds and other chemical contaminants (e.g. triclosan, heavy metals) are also present alongside microbiological contaminants in the reclaimed water⁶⁵⁻⁶⁷. As such, many burning questions remain. Have we evaluated the treatment technologies to determine the log removal values of microbiological and chemical contaminants that are of concern? Are there potential additive or synergistic effects when multiple contaminants of emerging concerns and environmental factors come into confluence, such that the associated risks would be significantly elevated? Or are the interactions going to be antagonistic such that risks would be minimally low? To further compound the problem, climate change is predicted to make it even more challenging to ensure consistently good water quality for potable and reuse purposes. For example, changing rainfall patterns may mean variability in water resources⁶⁸ – drought in certain places or extreme rainfall events in other places would mean that existing wastewater treatment plants that are typically designed within a certain safety factor might have to operate under sub-optimal conditions. This can potentially lead to partially treated wastewater being released for reuse. How can we then ensure a continuous delivery of good water quality?

Answering these questions will not be straightforward considering that it is technically and logistically challenging to monitor every single contaminant present in the wastewater. Rapid, high-throughput, non-target based monitoring methods will be needed. Some of these methods include metagenomics for identifying the microbial and functional gene diversity, high-throughput toxicity screening to determine whether treatment processes are able to reduce toxicity levels, and high resolution mass spectrometry to identify various compounds in wastewater simultaneously. Besides expanding our monitoring capacity, detecting mere presence and/or absence of contaminants will not be beneficial without quantifying the risks. However, the risk models related to antibiotic resistant bacteria and resistance genes or many of the emerging contaminants are not yet established⁶⁹, and gathering information related to

dose response or probability of transmission will require animal models to start with. Clearly, a multidisciplinary approach involving chemists, biologists, toxicologists, microbiologists and expertise in other related fields will be needed to address these pressing issues.

In the meantime, it is not practical to steer away entirely from reusing treated wastewater just to avoid any potentially unfounded detrimental consequences, especially when water scarcity is a real and growing global concern. Instead, we recommend adopting the precautionary principle and implementing appropriate intervention strategies and best management practices to minimize the impacts and concerns arising from the reuse of treated wastewater in agriculture.

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