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Review

Anaerobic Membrane Bioreactor Effluent Reuse: A Review of Microbial Safety Concerns

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Abstract: Broad and increasing interest in sustainable wastewater treatment has led a paradigm shift towards more efficient means of treatment system operation. A key aspect of improving overall sustainability is the potential for direct wastewater effluent reuse. Anaerobic membrane bioreactors (AnMBRs) have been identified as an attractive option for producing high quality and nutrient-rich effluents during the treatment of municipal wastewaters. The introduction of direct effluent reuse does, however, raise several safety concerns related to its application. Among those concerns are the microbial threats associated with pathogenic bacteria as well as the emerging issues associated with antibiotic-resistant bacteria and the potential for proliferation of antibiotic resistance genes. Although there is substantial research evaluating these topics from the perspectives of anaerobic digestion and membrane bioreactors separately, little is known regarding how AnMBR systems can contribute to pathogen and antibiotic resistance removal and propagation in wastewater effluents. The aim of this review is to provide a current assessment of existing literature on anaerobic and membrane-based treatment systems as they relate to these microbial safety issues and utilize this assessment to identify areas of potential future research to evaluate the suitability of AnMBRs for direct effluent reuse.

Keywords: antibiotics; pathogens; antibiotic resistance; anaerobic digestion; membrane bioreactors (MBR)

1. Shifting Paradigms in Wastewater Treatment

1.1. The Anaerobic MBR as an Alternative to Conventional Wastewater Treatment

The anaerobic membrane bioreactor (AnMBR) is a subclass of MBRs that has great potential for improving wastewater treatment process efficiency and sustainability. The primary advantages to wastewater treatment by AnMBRs are the inherent aspects of the bioprocess that allow for low energy expenditure and potential for energy harvesting. The use of an anaerobic reactor eliminates the requirement of aeration for treatment while also introducing the potential for recovery of methane generated by anaerobic digestion [1]. Additional advantages to the application of AnMBRs are their low solid waste production and a small reactor footprint due to higher anaerobic degradation rates while still maintaining high quality effluent by the use of membrane filtration [2].

AnMBRs also serve to consolidate and/or eliminate many of the steps in conventional wastewater treatment, including activated sludge aeration, secondary clarification, and sludge digestion (Figure 1). Because of these advantages, research involving their use as decentralized and/or mainline wastewater treatment systems has garnered significant interest over the past several years. Recent advances in the understanding of critical operational issues associated with AnMBRs, such as reduction of methane loss and minimizing biofouling mechanisms, have further improved prospects of the technology for becoming a preferred treatment method [3,4].

AnMBRs alone cannot achieve significant nitrogen and phosphorus nutrient removal that would allow for direct discharge of the effluent without tertiary treatment [5]. They do however allow treated effluent to be reused for agricultural irrigation due to these high residual nutrient concentrations. Although this is potentially a major advantage for wastewater treatment sustainability, introducing the idea of AnMBR effluent recycling also brings about additional concerns regarding the impacts of emerging contaminants (i.e., organic micropollutants, antibiotic-resistant pathogens, antibiotic resistance genes etc.) on the environments exposed to irrigation [6–8].

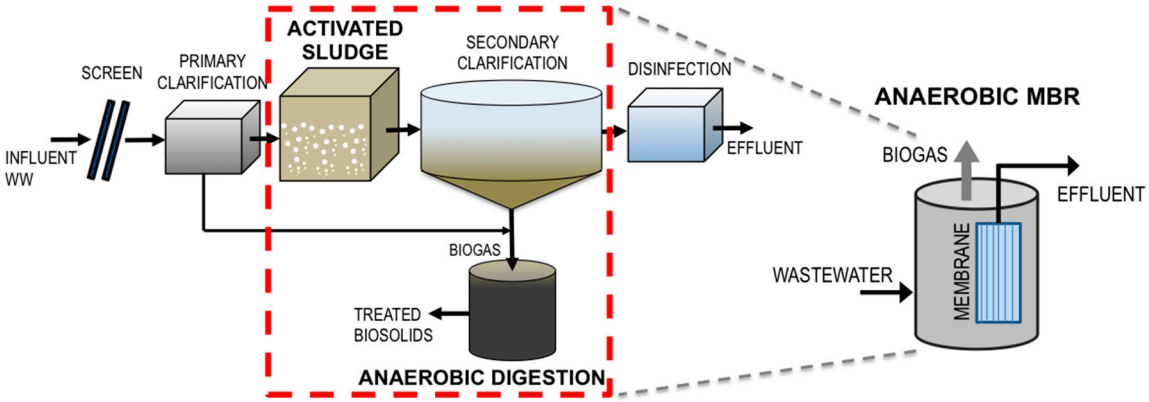

Figure 1. Schematic diagram of anaerobic MBR (AnMBR) potential to reduce central wastewater treatment plant processes and footprint. AnMBR can be used to replace activated sludge, secondary clarification and anaerobic digestion (i.e., processes enclosed within the red dashed line), hence reducing the footprint of a wastewater treatment plant.

1.2. The Need to Consider Emerging Contaminants in Wastewater

1.2.1. Non-Microbial Contaminants

When exploring the use of treated wastewater for irrigation or other types of reuse, there are several contributing factors that need to be considered. Among the primary concerns for domestic and agricultural wastewaters is the presence of bacteria and viruses that are harmful to human or plant health. These microbial pathogens can be effectively reduced to acceptable levels through means of disinfection that include chlorination, ozonation and ultraviolet (UV) inactivation [9,10]. Nonetheless, chlorination remains the dominant method used in wastewater treatment applications. This poses a problem for water reuse applications due to the potential for toxic or carcinogenic disinfection byproduct (DBP) formation through chlorine interaction with organic compounds in the wastewater effluents [11,12]. One possible approach to circumvent this issue is direct effluent reuse without any chemical disinfection. In order to evaluate this as a possibility for AnMBRs specifically, subsequent content of this review will examine microbial risks reported in the existing literature on a pre-disinfection basis.

In addition to DBPs, another emerging threat associated with wastewater effluent reusability is that of organic micropollutant (OMP) fate and persistence [13]. OMPs are generally present in domestic wastewater at trace levels (typically in the low μg/L range) due to the household use of pharmaceuticals and personal care products, as well as combined waste streams that include hospitals and other facilities producing high OMP concentration effluents. These trace organic compounds are known to be persistent in the environment, which can pose a serious issue for direct effluent reuse. One specific subclass of OMPs that is particularly relevant to wastewater microbial-associated risks is that of antibiotics [14]. Both OMPs generally and antibiotics specifically have been extensively studied for their removal rates and mechanisms in various wastewater treatment environments, including conventional wastewater treatment, membrane bioreactors, and anaerobic digestion [15–18].

Furthermore, the potential impact of antibiotic-type OMPs is conceivably far greater reaching than other OMP subclasses due to their influence on the presence and abundance of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) [19].

In aerobic wastewater treatment processes, such as conventional activated sludge and aerobic MBRs, sorption onto biosolids accounts for a significant fraction of treatment systems' overall OMP removal capacity [20]. This phenomena, however, still leaves the issue of OMP accumulation in the wasted activated sludge and its impact during subsequent disposal. Nonetheless, several previous studies have shown that membrane-based wastewater treatment more effectively reduces OMP and antibiotic concentrations than conventional processes [21–24]. Alternatively, AnMBRs require little to no sludge wasting as part of the anaerobic digestion process, effectively eliminating the possibility of long-term OMP removal by biosolids sorption. As a result, AnMBRs have been observed to rely on biodegradation as opposed to sorption as the primary mechanism for OMP removal from wastewaters [25,26]. This difference in removal mechanisms can be attributed to the overall better biodegradability of OMPs, and specifically antibiotics, under anaerobic digestion conditions as compared to aerobic [27,28].

1.2.2. Microbial-Associated Contaminants

Widespread and increasing usage of antibiotics in both domestic and agricultural environments has resulted in higher antibiotic loading rates on wastewater treatment plants (WWTPs) across the world. The general lack of biodegradability of antibiotics combined with the fact that wastewater treatment systems are not generally designed for their removal has led to their persistence within treatment plants and their affected environments [29]. This has further resulted in an increase in antibiotic resistance within the different stages of wastewater treatment due to the high density of bacteria in the influent and activated sludge that are exposed to these antibiotics, ultimately leading to WWTPs serving as a hotbed for antibiotic resistant bacteria (ARB) emergence and horizontal gene transfer [30]. This concept is illustrated in Figure 2. Studies assessing antibiotic resistance in WWTPs using metagenomics have shown that a wide range of ARGs are still present in plant effluents as well as in activated sludge and that the ARG subtypes can vary significantly between wastewater effluents and activated sludge samples [31,32].

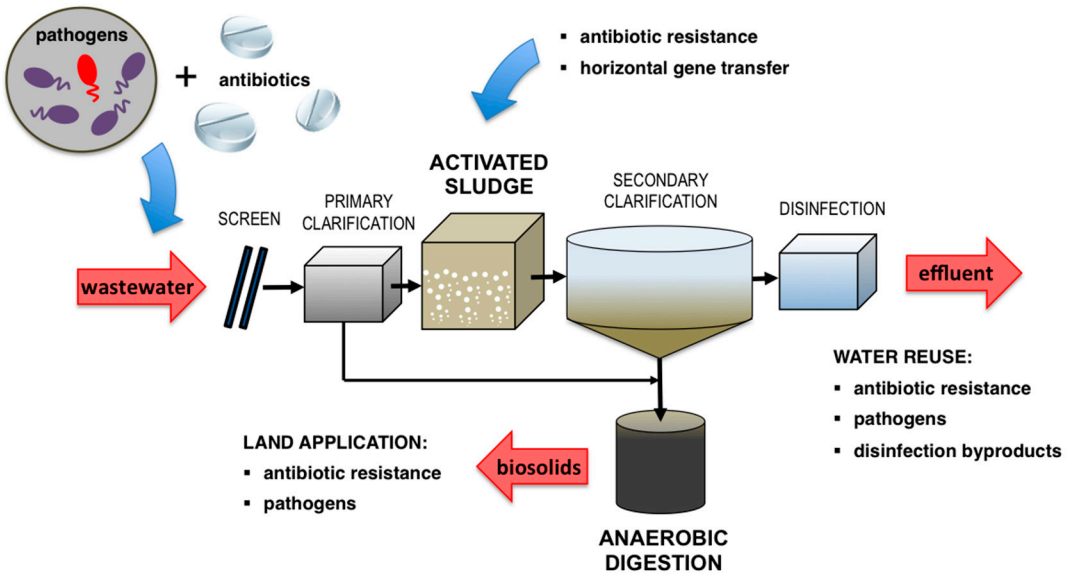

Figure 2. Schematic diagram of microbial antibiotic-associated threats in conventional wastewater treatment systems.

Given the ultimate release into the environment of WWTP effluents either through discharge or irrigation, it is important to understand the implications of these ARG profile variations. Recent studies have shown that although post-treatment disinfection can effectively reduce concentrations of ARB, disinfection methods such as chlorination and UV inactivation have little to no effect on ARG presence [33]. This leaves the possibility of further ARG persistence and gene transfer once wastewater effluents are discharged. Furthermore, recent studies have shown that ARGs (as compared to ARB) are particularly persistent in soils irrigated with wastewater effluents [34,35]. Both of these factors give rise to the possibility of increased antibiotic resistance among bacteria that are harmful to human health through horizontal gene transfer.

Besides ARB and ARG, the presence of pathogenic bacteria in wastewater is also a direct threat to effluent safety during reuse. This problem is compounded by their potential for regrowth in reclaimed water storage facilities [36]. The microbial consortium of stored wastewater effluents is largely dependent on the water quality parameters and nutrient content of those effluents, which can either diminish or enrich pathogenic microbial communities even after disinfection [37]. Furthermore, many pathogenic bacteria are also known to be antibiotic-resistant. This resistance has recently been shown to coincide with heightened persistence levels. For instance, although solar irradiation is able to effectively inactivate pathogens in wastewater effluents, hence reducing their potential for regrowth [38], a recent study showed that an *E. coli* strain exhibiting a stronger spectrum of antibiotic resistance required higher solar irradiation intensity to achieve the same log reduction as compared to another *E. coli* strain with fewer antibiotic resistance traits [39].

Hence, it seems that current disinfection strategies when coupled with conventional wastewater treatment are not sufficiently capable of containing these microbial-associated contaminants, particularly those related to antimicrobial resistance. Membrane-based wastewater treatment processes have proven to be a significant improvement from conventional treatment systems in reducing biotic contaminants, and thus making progress towards reuse without disinfection and DBPs [40]. Anaerobic digestion has also been shown to be an effective way at reducing the burden of pathogens in waste treatment [41]. Furthermore, both of the aforementioned treatment methods have been identified as potential means for ARB and ARG abundance reduction in WWTP effluents and biosolids, respectively [19]. AnMBR technology combines these two processes and reduces the potential outlets of environmental impact from two (WWTP biosolids and effluent) to one (effluent only) due to the lack of sludge production from AnMBR systems [42]. This, in combination with the technology's promise for improving wastewater treatment's sustainability, merits a thorough evaluation of AnMBRs for their possible future role in the reduction of both pathogenic threats and antibiotic resistance proliferation.

Despite this, literature on the subject has not been thoroughly reviewed nor evaluated from this perspective. Additionally, research that evaluates AnMBRs for pathogenic presence and antibiotic resistance dissemination has been limited to a handful of studies. As such, the remainder of this review is dedicated to assessing how both MBRs and anaerobic digestion have been independently shown to contribute to the reduction of these microbial-associated risks. The overall intent of the review is to determine if AnMBR technology can provide improved microbial safety during wastewater treatment as compared to conventional and aerobic MBR systems.

To achieve this, existing literature from peer-reviewed journals available on PubMed and Scopus was systematically reviewed based on its relevance to the aforementioned topic. Specific studies were included and subsequently grouped based on their relevance to either anaerobic digestion or membrane bioreactor (MBR) systems that were investigated for one or more of the following microbial contaminants: (1) antibiotic resistant bacteria, (2) antibiotic resistance genes, (3) potentially pathogenic bacteria, and (4) microbial indicator organisms. Given the potential significance of antibiotics in regards to these biotic contaminants, studies addressing the impact of antibiotics on reactor microbial communities in general were also examined. Any existing works that have focused on these issues in AnMBRs specifically were given particular attention and used as a basis for determining potential areas to be expanded on in future research.

2. Antibiotic-Associated Microbial Risks and Impact

2.1. The Effect of Anaerobic Digestion on ARB

The effect of antibiotics during anaerobic digestion has long been a topic of interest, primarily due to the use of digesters for the treatment of animal wastes [43]. Despite the widespread ban of the use of antibiotics as growth promoters in the European Union, this method is still commonly used in the United States and throughout the world. As such, the issue of antibiotic resistance of bacteria arising from this practice has also been studied for quite some time [44]. A recent study of the anaerobic digestion of cattle manure found that multidrug resistant bacteria were abundant in the effluents of these digesters, with specific resistance to penicillin, levofloxacin, ampicillin, and chloramphenicol [45]. This is a particular issue of concern due to the widespread land application of digested cattle manure in agricultural environments. The increasing use of both Class A and Class B WWTP biosolids as fertilizers has proven to be a cause for similar concern due to their potential for harboring both ARB and ARGs [19]. It has also been determined that pathogenic species in WWTP activated sludge are likely to have higher resistance occurrence rates, further compounding this concern [33].

One proposed method for the reduction of multidrug resistant ARB during anaerobic digestion is the use of thermophilic digesters (>50 °C) in the place of mesophilic (~35 °C). From a sustainability perspective, there is the obvious additional cost of heating associated with thermophilic digestion to be considered. Nonetheless, biogas production rates from those digesters have also been observed as significantly higher [46], potentially offsetting some of the required energy expenditure. Studies by Han et al. and Beneragama et al. have shown that although after mesophilic digestion several multidrug resistant ARB, including pathogenic species, were still detected in swine and dairy manure, respectively, thermophilic anaerobic digestion resulted in 100% destruction of both pathogenic and multidrug resistant bacteria [46,47]. A similar study by Miller et al. instead evaluated ARB survival during anaerobic digestion of wastewater sludge at both mesophilic and thermophilic conditions [48]. This work found that a known tetracycline-resistant isolate was fully inactivated in the thermophilic digester, but not its mesophilic counterpart. Furthermore, the study showed that the survival of this isolate was also responsible for higher levels of tetracycline resistance genes. In general, the findings of the previously discussed studies have shown that ARB in anaerobically digested effluents remain a significant concern in both agricultural and domestic wastewater treatment. The ARB-associated threat to microbial safety is further compounded by the potential for these bacteria to be of pathogenic nature (further discussed in Section 3.2).

2.2. The Effect of Anaerobic Digestion on ARGs

In addition to ARBs, another antibiotic-associated microbial threat is that of antibiotic resistance genes (ARGs). In some sense, they are more of a direct representation of the potential for pathogenic bacteria to acquire resistance by horizontal gene transfer than are ARB [19]. Furthermore, ARG have been shown to persist in wastewater treatment systems even when associated ARB can be removed [49]. This is largely due to their significantly smaller size as compared to ARB and the ineffectiveness of post-treatment processes such as conventional disinfection in their removal [50]. The presence of ARGs in WWTP biosolids has led to extensive research investigating their fate during anaerobic digestion. A summary of studies investigating the dynamics of ARGs in anaerobic-associated treatment processes is presented in Table 1, along with the specific gene types and their reported abundances. The majority of these studies targeted ARGs known to promote resistance against sulfonamides and tetracycline, although genes associated with erythromycin, trimethoprim, and multidrug resistance were also examined in some of the works. It is important to point out, however, that sulfonamides and tetracycline are among the earliest developed antibiotics and that there is a broad spectrum of emerging gene classes that confer resistance to newer antibiotics which have yet to be studied in wastewater-affected environments [51].

A number of the aforementioned studies also sought to evaluate thermophilic digestion as a means for ARG abundance reduction from WWTP biosolids. The general findings of these studies were that thermophilic processes are usually more effective at reducing ARG concentrations than mesophilic, although in all instances targeted ARGs remained at detectable levels. For example, Ghosh et al. determined that *tet* genes, which confer resistance to tetracycline by ribosome alteration and inactivation enzymes [52], were effectively reduced by the thermophilic pretreatment in a thermophilic-mesophilic treatment train [53]. However, it was also observed that abundances often rebounded during the subsequent mesophilic step. Likewise, a study by Diehl et al. found that *tet* genes were most effectively removed by anaerobic digestion at 55 °C as compared to at 37 °C and 47 °C, or in an aerobic digester where almost no reduction was seen [54]. Other studies evaluating *sul* genes, which confer resistance to sulfonamides by encoding for variant dihydropteroate synthase (DHPS) enzymes [55], along with *tet* gene concentrations also reached similar conclusions [48,56]. Conversely, a study by Ma et al. found that a mesophilic digester more effectively reduced *tetC*, *tetG*, *tetX*, and *intl1* gene abundances. However, *tetO* and *tetW*, as well as genes known to promote sulfonamide and erythromycin resistance, were still better removed by thermophilic digestion [57]. These findings suggest that increased temperature of anaerobic digestion could have variable effects on gene abundances, even within the same ARG class. This is likely attributable to the association of specific ARGs with different groups of microorganisms within the anaerobic digesters in combination with the observed microbial community clustering based on digester temperature.

Mesophilic anaerobic digestion was also studied independently in several scenarios. For example, a long-term project by Resende et al. found that ARGs including *ermB*, *aphA2*, and $bla_{\text{TEM-1}}$, which confer resistance to macrolides, aminoglycoside, and beta-lactams, respectively, had abundances that were significantly lower in summer months as compared to the winter months during ambient temperature anaerobic digestion in Brazil [58]. Additionally, several studies have investigated the effects of specific antibiotics on their associated ARG abundances in anaerobic digestion systems. Results have generally exhibited a positive correlation between antibiotics at high concentrations and their relevant ARGs [59,60]. Other studies have examined the impact of antimicrobial agents such as triclosan and triclocarban on ARG abundances. Results from these works have shown variable results. For example, *mexB* genes, which confer multidrug efflux pump-based resistance, increased in mesophilic anaerobic digesters at high triclosan concentrations, while *intl1* abundances decreased [61]. Triclocarban, a related antimicrobial, also increased *mexB* gene levels under similar conditions, while simultaneously reducing *ermF* genes [62]. In this study, *intl1* gene abundances were not significantly affected; unlike when anaerobic sludge was exposed to triclosan. The investigation of *intl1* gene dynamics is particularly relevant to ARG horizontal gene transfer potential in AD systems, as it encodes for the class 1 integron which is commonly associated with cassettes containing ARGs [63]. For example, its observed correlation with *sulI* gene abundances implies *sulI*'s possible presence on mobile genetic elements and its implied higher potential for horizontal gene transfer [48].

Recent literature has also focused on evaluating antibiotic resistance using a metagenomic approach. Utilizing metagenomics for ARG evaluation offers particular advantages as compared to conventional quantitative PCR. A metagenomics based approach includes comprehensive gene evaluation by blasting against existing ARG databases, lack of reliance on existing individual ARG primers, and the potential for direct correlative analysis with their microbial community dynamics. This method has also been utilized to evaluate ARG abundances in anaerobic digesters treating both primary and secondary WWTP sludge [64]. Results of this evaluation showed that a wide range of ARGs were present in digesters with the highest abundances coming from tetracycline, aminoglycoside, beta-lactam, and sulfonamide resistance genes. Furthermore, this study revealed that several of the dominant ARGs exhibited strong correlations (Pearson's coefficient > 0.80) with several pathogenic bacteria that include *Streptococcus* and *Eubacterium* species. In a separate study, the effect of temperature on ARG abundances was also studied during anaerobic digestion of sewage sludge by utilizing metagenomics [65]. Results of this work showed that although up to 13 different ARG subtypes were

removed by over 90% in the anaerobic digesters, total ARG abundances and diversity level were the same in both thermophilic and mesophilic treatment. These findings reinforce the observations of the previously mentioned study that utilized qPCR to show that particular ARGs were better removed at thermophilic temperatures while others were better removed under mesophilic conditions [57]. Yet another study employing metagenomics sought to evaluate anaerobic treatment of wastewater as compared to both aerobic and low-energy anaerobic-aerobic treatment [66]. Results revealed that although all three of the methods greatly reduced total ARG abundance, low-energy anaerobic-aerobic treatment showed the most promising results. A classification of ARGs by their resistance mechanism further revealed that efflux pump activation was the most dominant form of resistance in all systems, although in anaerobic treatment target inactivation was also a dominant mechanism. Overall, these studies have shown that the utilization of metagenomics for ARG assessment can provide new and significant insights into their dynamics in waste treatment systems.

2.3. Impact of Antibiotics on The Anaerobic Digestion Process

Although anaerobic digestion has demonstrated potential in reducing ARB and ARG, the impact that antibiotics can have on the microbial community dynamics of anaerobic digesters must also be considered. It has been shown, for example, that antibiotic concentrations of above 1 mg/L in anaerobic digestion systems can break down acetate, butyrate and propionate degradation pathways and lead to an accumulation of volatile fatty acids (VFAs) in digesters [60,67]. Furthermore, a similar study by Aydin et al. found that concentrations of combined sulfamethoxazole, tetracycline and erythromycin antibiotic mixtures of above 10 mg/L also inhibited chemical oxygen demand (COD) removal and biogas generation rates [68]. A different study of the same anaerobic digesters used quantitative PCR to quantify formate-tetrahydrofolate ligase (FTHFS), acetyl-coenzyme A synthetase (ACAS), and methyl coenzyme M reductase (*mcrA*) genes and reveal that this antibiotic mixture inhibited reactor performance by disrupting acetogenesis, acetoclastic methanogenesis, and hydrogenotrophic methanogenesis pathways, respectively [69]. Likewise, the use of DGGE analysis showed that acetoclastic methanogens were most directly impacted by increasing antibiotic concentrations [70]. A recent study by Xiong et al. has further confirmed the previously discussed results by determining through high-throughput sequencing that both syntrophic VFA utilizing bacteria and methanogenic populations changed significantly at high concentrations of tetracycline in anaerobic reactors [60]. The results of these studies generally confirm that antibiotics at concentrations in the range of 1 mg/L and above can significantly impact the keystone microbial communities responsible for maintaining stable anaerobic digestion processes. This range of antibiotic concentrations is generally representative of those found in agricultural biosolid wastes (4–40 mg/L) [71], and implies that their inhibitory effect should be taken into consideration during digester operation. Although municipal biosolids generally contain specific antibiotic concentrations in a much lower range than can affect the anaerobic process (0.2–10 μg/L) [72], they are also known to contain a much broader range of antibiotic types simultaneously, which can potentially have a long term effect on microbial community dynamics. Therefore, when considering anaerobic digestion in combination with membrane separation as a means for removal of ARB and ARGs from municipal wastewaters, the potential inhibitory effect of antibiotics must also be considered.

Table 1. Average abundance of antibiotic resistance genes (ARGs) in anaerobic digester (AD) and membrane bioreactor (MBR) effluents and their sources.

Treatment Method	Source	Associated Antibiotic	Gene Class	Average Gene Abundance from Source	Average Observed Gene Abundance Post-Treatment	Comments	Ref.
Thermophilic-mesophilic AD	WWTP biosolids	tetracycline	*tetA*	8×10^{-5}–4×10^{-4} (/16S)	4×10^{-5}–6×10^{-4} (/16S)	Thermophilic-mesophilic more effective than mesophilic alone.	[53]
			tetO	4×10^{-6}–3×10^{-5}	1×10^{-6}–2×10^{-5}		
			tetX	2×10^{-3}–1×10^{-2}	5×10^{-5}–3×10^{-4}		
		class 1 integron	*intl1*	4×10^{-4}–2×10^{-3}	1×10^{-4}–2×10^{-4}		
Thermophilic AD	WWTP biosolids	Tetracycline	*tetA*	6.0×10^{4} (µL)	5.0×10^{3} (µL)	Gene reduction by anaerobic digestion effective at 37, 47, and 55 °C while aerobic digestion was ineffective.	[54]
			tetL	4.2×10^{5}	1.0×10^{4}		
			tetO	1.8×10^{5}	2.0×10^{4}		
			tetW	8.4×10^{1}	1.0×10^{1}		
			tetX	3.3×10^{4}	1.0×10^{3}		
		class 1 integron	*intl1*	1.0×10^{6}	5.0×10^{3}		
Mesophilic or thermophilic AD	WWTP biosolids	Sulfonamide	*sulI*	2×10^{-2}–2×10^{-1} (/16S)	5×10^{-3}–4×10^{-2} (/16S)	Thermophilic more effective than mesophilic. *sulI* correlated with *intl1*.	[48]
		tetracycline	*tetO*	5×10^{-4}–3×10^{-3}	1×10^{-4}–4×10^{-4}		
			tetW	5×10^{-4}–4×10^{-3}	1×10^{-4}–1×10^{-3}		
		class 1 integron	*intl1*	1×10^{-3}–5×10^{-2}	1×10^{-3}–5×10^{-3}		
Mesophilic or thermophilic AD	WWTP biosolids	Sulfonamide	*sulI*	1×10^{10} (g)	8×10^{8} (thermophilic)	*Sul* and *erm* genes more effectively reduced by thermophilic treatment while mesophilic treatment more effectively reduced three *tet* resistance genes and *intl1*.	[57]
			sulII	6×10^{9}	7×10^{8} (thermophilic)		
		macrolide	*ermB*	1×10^{9}	2×10^{8} (thermophilic)		
			ermF	1×10^{9}	7×10^{7} (thermophilic)		
		tetracycline	*tetO*	1×10^{9}	4×10^{8} (thermophilic)		
			tetW	2×10^{9}	3×10^{8} (thermophilic)		
			tetC	4×10^{9}	5×10^{8} (mesophilic)		
			tetG	1×10^{10}	1×10^{9} (mesophilic)		
			tetX	2×10^{8}	1×10^{7} (mesophilic)		
		class 1 integron	*intl1*	6×10^{9}	5×10^{8} (mesophilic)		
Thermophilic AD	WWTP biosolids	Sulfonamide	*sulI*	1×10^{7} (µL)	1×10^{6} (µL)	Silver nanoparticles/sulfamethoxazole had no effect on ARG abundance. *Tet* abundances were higher in mesophilic.	[56]
			sulII	1×10^{6}	1×10^{5}		
		tetracycline	*tetO*	1×10^{5}	1×10^{4}		
			tetW	1×10^{5}	3×10^{4}		
		class 1 integron	*intl1*	4×10^{6}	2×10^{5}		
Mesophilic AD	cattle manure	Macrolide	*ermB*	10^{6} (g)	10^{5} (g)	ARG decreases were greater during summer month ambient AD.	[58]
		aminoglycoside	*aphA2*	10^{4}	10^{2}–10^{3}		
		beta-lactam	$bla_{\text{TEM-1}}$	10^{7}	10^{4}–10^{6}		

Table 1. *Cont.*

Treatment Method	Source	Associated Antibiotic	Gene Class	Average Gene Abundance from Source	Average Observed Gene Abundance Post-Treatment	Comments	Ref.
Mesophilic AD	synthetic mixture	Multidrug	*mexB*	NR	10^5–10^6 (g)	*mexB* increased with high levels of triclosan and *intl1* decreased.	[61]
		class 1 integron	*intl1*	NR	10^{-6}–10^{-2}/16S		
Mesophilic AD	synthetic mixture	Erythromycin	*ermA, B, F* *ereA*	NR	10^1–10^3 (mL)	Increasing conc. of erythromycin and tetracycline lead to increases in their associated ARGs.	[59]
		tetracycline	*tetA, B, C, D, E, M, S, Q*	NR	10^2–10^4		
Mesophilic AD	synthetic sludge	Erythromycin	*ermF*	NR	10^{-5}–10^{-2} (per 16S)	Triclocarban conc. Increases *mexB* and *tetL* genes while reducing *ermF* genes.	[62]
		tetracycline	*tetL*	NR	10^{-6}–10^{-2}		
		multidrug	*mexB*	NR	10^{-7}–10^{-4}		
		class 1 integron	*intl1*	NR	10^{-5}–10^{-4}		
Mesophilic AD	synthetic mixture	Tetracycline	*tetW*	NR	4×10^5–2×10^6 (mg)	Increasing propionate conc. with high tetracycline increased gene abundance.	[60]
			tetQ	NR	2×10^2–8×10^4		
Anaerobic EGSB-aerobic MBR	pharmac-eutical WW	Sulfonamide	*sulI*	10^5 (mL)	10^6 (EGSB) 10^7 (MBR)	EGSB and anaerobic sludge had the lowest ARG concentrations while aerobic activated sludge had higher abundances.	[73]
			sulII	10^4	10^6 (mL) 10^7 (mL)		
		tetracycline	*tetM*	10^6	10^7 10^8		
			tetO	$<10^3$	10^4 10^5		
			tetQ	10^4	10^7 10^7		
			tetW	10^4	10^6 10^7		
		erythromycin	*ermB*	10^5	10^6 10^7		
		class 1 integron	*intl1*	10^7	10^7 10^8		
AnMBR AeMBR	synthetic mixture	Sulfonamide	*sulI*	NR	10^{-4} (Ana.) 10^{-2} (Aer.)	Lower ARG conc. than aerobic MBR. *sulI* correlated with *intl1* abundance.	[74]
			sulII	NR	10^{-4} (/16S) 10^{-2} (/16S)		
		trimethoprim	*dfrA*	NR	10^{-3} 10^{-2}		
		class 1 integron	*intl1*	NR	10^{-4} 10^{-3}		
Anoxic-oxic-MBR	Synthetic mixture	Sulfonamide	*sulI*	NR	3×10^7–2×10^8 (mL)	*tet* genes incr. at lower SRTs, *sulI* incr. then decr. and *sulII* decr. with lower SRT.	[75]
			sulII	NR	1×10^7–1×10^9		
		tetracycline	*tetC*	NR	3×10^6–1×10^7		
			tetE	NR	6×10^7–1×10^8		
Aerobic MBR	domestic WW	Sulfonamide	*sulI*	10^8 (100 mL)	$10^{5.5}$ (100 mL)	MBR had higher LRV of *tet* genes than other treatment processes.	[76]
		tetracycline	*tetO*	10^9	10^2		
			tetW	10^9	10^3		

2.4. The Role of Membrane-Based Treatment on Antibiotic Resistance Reduction

Membrane bioreactors (MBRs) have been shown in previous studies to significantly improve specific removal rates of antibiotics from influent wastewaters as compared to conventional processes [23,77,78]. Additionally, new membrane-based treatment processes, such as an MBR with nanofiltration-based effluent recycling, have also shown promise in further reducing antibiotic concentrations and impact [79,80]. Despite this, the role of membrane-based wastewater treatment in the reduction of ARB and ARG abundances has been much less studied, specifically in the case of membrane-coupled anaerobic digestion. Nonetheless, there has been some insight provided by several recent papers investigating the fate of ARGs in membrane-based wastewater treatment. These studies are summarized in Table 1.

Recent work has shown that aerobic MBRs have the potential to reduce ARG abundances in WWTP effluents more effectively than conventional treatment processes. For example, a study by Munir et al. investigating ARB and ARGs in five full-scale WWTP effluents and biosolids found that both tetracycline resistant and sulfonamide resistant bacteria showed higher log removal values (LRVs) before disinfection in an MBR plant when compared to four other conventional WWTPs [76]. This work further showed that tetracycline-associated ARGs (*tetO* and *tetW*) were removed by up to 6–7 log in the MBR plant while *sulI* gene LRVs were in the range of 2–3. Tetracycline-associated resistance gene LRVs were also significantly higher than in other treatment plants. A similar study by Wang et al. that investigated ARG abundance in pharmaceutical wastewater treatment systems further determined that membrane separation of aerobic sludge removed up to 99.8% of ARGs and was more effective than secondary clarification [73]. Both MBRs in the aforementioned studies employed membranes with pore sizes in the ultrafiltration (UF) range, which are generally larger than the average plasmid that would represent extracellular ARGs. However, given that a previous analysis of the mechanisms affecting ARG removal by membrane filtration revealed a positive correlation between colloid presence in UF membrane retentates and ARG removal rates [81], it is likely that this phenomenon is also responsible for the higher ARG LRVs in the MBR systems studied. Furthermore, the development of membrane biofilms has been shown to improve water quality parameters of MBR effluents such as COD removal [82], which can potentially serve as an additional mechanism for the removal of ARGs from wastewater effluents.

The previously discussed study by Wang et al. additionally showed that, for the same influent wastewater, aerobic sludge ARG concentrations were significantly higher than those of both an anaerobic digester and an expanded granular sludge bed (EGSB) reactor (anaerobic), implying that there may be potential advantages to combining anaerobic processes with membrane-based treatment [73]. This concept was specifically investigated in a recent study by Harb et al., which compared ARG abundance reduction by anaerobic versus aerobic sludge-based MBR treatment systems [74]. The results of this work showed that when normalized against 16S rRNA gene copies, the concentrations of sulfonamide and trimethoprim resistance genes were 1–2 log lower in an AnMBR system than in its aerobic counterpart. These observations, along with those of Wang et al. [73], reinforce the concept that ARG abundances can be effectively reduced by anoxic or anaerobic conditions [83]. Furthermore, the work of Harb et al. [74] is an example of how the combination of anaerobic digestion can be complimented by the advantages of MBR-based treatment for overall improved ARG removal capacity. Another possibility for combining biological treatment with membrane separation is the inclusion of multiple biological steps, as is often done during conventional wastewater treatment. A study by Xia et al., for example, examined ARG abundances an anoxic-oxic MBR system and showed that operational parameters such as solids retention time (SRT) can also impact ARG presence in MBR sludge [75].

3. Pathogen-Associated Microbial Risks

3.1. Pathogens in Wastewater Treatment

Wastewater effluent reuse potential is directly dependent on the risks associated with the presence of microbial pathogens. As such, a comprehensive understanding of the effectiveness and implications of different wastewater treatment technologies for their removal is necessary. Changes in the microbial communities of natural environments by antibiotics can have a consequential effect on pathogen persistence [84]. These dynamic circumstances associated with the threat of pathogens in the environment call for a heightened level of scrutiny of the microbial assessment of wastewater effluents and biosolids.

The traditional methods for evaluating microbial safety of wastewater effluents were developed over 100 years ago and are still used as standards today [85]. These methods include the culture-based detection of total coliforms, fecal coliforms, *E. coli* and *Enterococci*. These microbial indicators, although being historically effective tools for the assessment of water quality and safety, are generally nonspecific and limited in their ability to determine specific pathogenic risk. This is largely due to the high variability of non-cultivable pathogenic bacteria that remain unaccounted for by these methods [86]. Furthermore, for the indicator bacteria that are currently used (e.g., *E. coli*), not all of the strains associated with each species actually exhibit pathogenic traits.

The introduction of molecular techniques has offered new insight into the presence and removal capacity of pathogenic bacteria by wastewater treatment systems [87]. Molecular-based detection has its own limitation, especially in its capacity to distinguish between viable cells and DNA from damaged or non-viable bacteria. However, recent advances have offered potential solutions to these limitations [88,89]. These solutions have included monoazide-based staining of DNA to differentiate cells with intact cell walls against those with compromised ones. Furthermore, the merits of molecular detection of pathogenic bacteria are well established and can offer specific insight into various wastewater treatment process strengths for increasing microbial safety [90–94]. These merits include the quantification of non-cultivable pathogens by quantitative PCR and semi-comprehensive comparative analysis of potentially pathogenic bacterial abundance by high-throughput sequencing. Despite this, the technology has yet to be employed as a primary detection method by most regulatory and monitoring agencies.

3.2. The Role of Anaerobic Digestion in Pathogen and Indicator Microorganism Removal

Land application of treated sewage sludge for agricultural use accounts for over half of the WWTP produced biosolids in the United States and Western Europe, while in developing countries the percentage is often even higher [95]. Class B biosolids in the United States differ from Class A biosolids in that they contain detectable levels of human pathogens and are restricted by specific preventative practices during the process of land application. Pathogen levels in Class B biosolids can be highly variable due to the lack of stringent regulations regarding indicator bacteria concentrations (fecal coliform $< 2 \times 10^6$ CFU per gram), resulting in potentially high short-term microbial risks [96]. Anaerobic digestion of both agricultural and municipal sludge has long been established as an effective method for reducing pathogenic risk arising from sludge disposal and land application [97,98]. Furthermore, increasing practices involving land application of municipal biosolids for agricultural fertilization has ultimately led to the establishment of mesophilic anaerobic digestion as a preferred sludge stabilization method for Class B biosolids due to its efficient reduction of organic matter, retention of nutrients necessary for fertilization, and its relatively predictable destruction capabilities of indicator microorganisms [3,99].

A recent review by Avery et al. summarized the current studies of pathogenic and indicator bacteria in anaerobic digesters and provided an overview of their LRVs [41]. The studies assessed in the review utilized a range of different sludge types. A source-based comparison of their findings revealed that, compared to municipal sludge and swine slurry (LRV of 1–2), the anaerobic

digestion of cattle manure resulted in much higher removal rates (LRV of approx. 4). The relevant potential pathogens detected for in previous studies included total coliform, fecal coliform, *E. coli*, Enterobacteriaceae, and species of *Salmonella*, *Shigella*, *Enterococcus*, *Mycobacterium*, *Staphylococcus*, *Listeria*, *Campylobacter*, *Yersinia, Vibrio*, and *Clostridium*. Overall, results in the literature showed that among the most commonly targeted groups, *E. coli* and *Salmonella* exhibited the highest average LRVs (approx. 3) while *Enterococcus*, *Clostridium* and *Mycobacterium* were among the lowest (below 1.5). The bacterial groups that showed these lower LRVs during the anaerobic process are generally known to exhibit characteristics that promote persistence in strenuous conditions such as spore formation (*Clostridium*), resistance to extreme temperature and pH (*Enterococcus*), and resistant cell wall structure (*Mycobacterium*). The review also reinforced the conclusion that temperature of anaerobic digestion greatly affected LRVs. However, an analysis of existing literature showed that psychrophilic digestion provided overall better removal (3–4 log) of indicator organisms than did mesophilic digestion (approx. 2 log). This was generally attributed to the optimal growth conditions of most fecal-associated microorganisms being in the mesophilic range.

A recent study that investigated the occurrence of indicator organisms in mesophilic anaerobically digested Class B biosolids at 18 different locations across the United states revealed that fecal coliform levels were between 1×10^4 and 6×10^5 CFU per g at a 95% confidence interval [100], which is reasonably below the maximum allowable level of 2×10^6 CFU/g. This range of digested sludge concentrations however is considerably broader than what was observed in associated undigested sludge (1.5×10^8 to 4.5×10^8 CFU/g), implying that there are other factors also contributing to the variability of mesophilic digester effluents than their respective influent concentrations. In other studies temperature has been determined to be the most significant factor controlling rate of pathogen destruction in anaerobic digesters [101], while hydraulic retention time (HRT) and mixing rates have also been established as directly influential [102].

Although it has been shown that mesophilic digestion may be sufficiently capable of producing Class B biosolids, the production of Class A biosolids would require more advanced treatment [103]. Thermophilic anaerobic digestion has been proposed as a potentially suitable technology to achieve this level of biosolids production. The previously discussed review by Avery et al. [41] did not include literature addressing thermophilic anaerobic digestion, as it was mainly focused on operation at ambient temperatures in warm climates. In several recent studies that have investigated running digesters under thermophilic conditions, results generally showed vastly more effective indicator bacterial removal rates. For example, a study of *E. coli* inactivation in dairy manure showed that the time necessary for a 1 log removal was 7–8 days at 37 °C and <1 day at 52.5 °C [104]. Another work reiterated these findings, showing a similar difference (8 fold) in *E. coli* inactivation rates [105]. The study also discovered that *E. coli* was significantly more sensitive to heat increases than were *Enterococcus faecalis* and *Clostridium perfringens*. Furthermore, a recent work addressing the anaerobic digestion of municipal sewage sludge revealed that LRVs for *E. coli* and *E. faecalis* were both in the range of 1 log and 3 log for digestion at 37 and 55 °C, respectively [106]. The study also proposed possibility of high-heat short-term pretreatment of sewage sludge for mesophilic anaerobic digestion, which resulted in LRVs in the range of 17–20. Overall, the potential of thermophilic anaerobic digestion to produce high quality biosolids is well established, but the intended use of the treated waste would ultimately determine whether treatment at above 50 °C and its associated energy expenditure should be considered.

The use of quantitative PCR (qPCR) for pathogenic detection in anaerobically treated biosolids has been considered a possible alternative to culture-based enumeration [107]. qPCR-based detection offers the specific advantage of determining quantities of non-cultivable pathogenic species that non-molecular methods inherently overlook. Despite this, it has not yet been widely adopted for the regulation of Class A and Class B biosolids in practice. However, the evaluation of anaerobic digestion using molecular-based methods has recently provided useful insight into the suitability of existing culture-based methods for indicating microbial risks. For example, it has been determined

that, for the same indicator microorganisms, culture-based quantification during anaerobic digestion can potentially lead to an overestimation of LRVs as compared to those determined by qPCR, with differences in LRV of up to 2 log [108]. Another recent study that compared qPCR to culture-based enumeration determined that *Enterococci* were accurately quantified at similar concentrations using both detection techniques in Class B biosolids, but varied significantly between methods when used for Class A biosolids [109]. The same study also illustrated the merit of qPCR for the evaluation of the non-cultivable *Lactococcus lactis* in both types of biosolids. A similar work also utilized qPCR to quantify non-cultivable *Legionella*, *Clostridium*, and *Staphylococcus* species, revealing mesophilic anaerobic digester effluent concentrations of between 10^3 and 10^4 genomic units per g of biosolids for associated known pathogenic species [110].

Furthermore, a study by Ju et al. employing high-throughput sequencing revealed that although the overall richness of potentially pathogenic species was reduced by full-scale anaerobic digestion (from 74 to 10–14 species), six pathogenic species actually increased in total relative abundance [64]. Those species were associated with the genera *Collinsella*, *Streptococcus*, *Mycobacterium*, *Eggerthella*, *Gordonia*, and *Propionibacterium*. Species associated with at least 3 of these genera showed strong and significant correlations with ARG abundance levels, implying that antibiotic resistance by these bacteria could have contributed to their persistence within the anaerobic digestion process. Overall, the results of the study by Ju et al. [64] exemplify the advantages of high-throughput sequencing in determining the vastly different removal rates of pathogenic species by anaerobic digestion as well as identifying probable influencing factors contributing to these differences (e.g., antibiotic resistance of potential pathogens). A similar work by Bibby et al. that used 16S rRNA gene pyrosequencing to target bacterial pathogens reiterated the aforementioned advantages of high-throughput methods for the comprehensive analysis of pathogen diversity in biosolids [111]. However, the study also illustrated the key limitations presently associated with high-throughput sequencing, namely those of insufficient sequencing depth for accurate quantification and uncertainty in pathogen identification using the 16S rRNA gene [111].

3.3. Pathogen-Associated Risk Reduction by Membrane Bioreactors

MBRs have proven to be a significant improvement from conventional treatment systems in their ability to reduce both total bacterial presence and pathogenic threats in wastewater effluents [40]. There has been extensive research done to evaluate MBRs using culture-based methods in this regard (summarized in Table 2). Although these studies have shown the merits of membrane-based technologies, all of the systems that have been tested using microfiltration (MF) and ultrafiltration (UF) membranes thus far still contain detectable levels of indicator bacteria at varying levels of log reduction values (LRVs). This subsection will review the findings of existing research that addresses different MBR systems for indicator bacteria removal rates as well as focus on the specific works that have employed molecular-based detection and examine the particular insights gained from those studies.

The majority of studies addressing the removal of potentially pathogenic bacteria by MBR systems have utilized culture-based enumeration of indicator bacteria. Among the most commonly detected indicators are total coliform, fecal coliform, *E. coli*, and *Enterococci*. For the studies assessed in this review, the concentrations in influent wastewater of these indicators were in the log range of 7.0 to 8.2, 5.3 to 7.1, 5.1 to 7.0, and 4.2 to 7.9 per 100 mL, respectively. Total coliform LRVs were in the range of 4.7 to 7.0 for the aerobic MBR systems studied while the only anaerobic MBR targeting total coliform showed an LRV of 6.9. Likewise, fecal coliform LRVs were generally in similar range of 5.4 to 6.9 for aerobic MBRs, 6.6 for an anaerobic MBR, and 6.0 for an anaerobic-anoxic-oxic (AAO) MBR system. Studies utilizing culture-based plate counts of *E. coli* showed LRVs ranging from 3.5 to 6.8 in aerobic MBR systems, 6.8 in an anaerobic MBR, and 6.1 in an AAO MBR. Studies targeting *Enterococci* showed a slightly lower range of LRVs for aerobic MBRs (3.0 to 6.3), while the study addressing an anaerobic MBR using culture-based *Enterococci* enumeration revealed an LRV of 7.0. In addition to these four indicator bacteria, the class Clostridia was also targeted in four separate studies and showed LRVs

in the range of 2.8 to 4.9 (including 4.8 in an AAO MBR). Based on these results, the anaerobic MBR systems studied using culture-based methods showed removal rates of the four primary indicator bacteria that were at the high end of the range determined for studies involving aerobic MBRs and the AAO MBR [112,113]. These differences in LRVs, however, cannot be deemed significant based on this information, as only two studies of anaerobic MBRs for these comparable indicator bacteria have been done. The different ranges of indicator bacteria LRVs within the same MBR type (e.g., aerobic) may also be attributable to differences in operational conditions of each system. However, due to the variability in parameter information provided in the studies reviewed, an overall analysis of these parameters was not performed in this review. An additional study of an AAO MBR reported LRVs of indicator bacteria in the range of 0.1 to 0.7 [114], however these results represent a significant outlier as compared to the rest of existing literature. With the exception of the aforementioned, all of the MBR systems studied using culture-based methods have shown improved microbial indicator removal rates as compared to conventional WWTP processes [115–117].

These improved removal rates by membrane-based systems have important implications for effluent reuse potential, as they introduce the possibility of reuse without disinfection. Whether this would be feasible in practice is largely contingent on the level of indicator microbial quality required. Present reuse restrictions vary widely based on national and local guidelines for maximum indicator bacteria allowable [118]. In the United States, Environmental Protection Agency guidelines indicate that fecal coliform must be below detection limits for unrestricted agricultural reuse and below 200 CFU per 100 mL for restricted reuse [119], along with containing a minimum of 1 mg/L chlorine residual. In Germany, for example, restricted reuse guidelines are less stringent, requiring under 10,000 and 1000 CFU per 100 mL for total coliform and fecal coliform, respectively [120]. Based on the range of influent wastewater concentrations and LRVs in the reviewed literature (Table 2), a majority of the studied MBR effluents would be below or close to the fecal coliform levels necessary for restricted reuse.

Literature addressing pathogenic bacteria using molecular-based methods so far has been limited. One such study, however, utilized qPCR to target *E. coli*, *Salmonella*, and *Shigella* in an AAO MBR and showed LRVs of 4.4, 3.3 and 2.5, respectively [121]. These results indicate a high level of variability between various potentially pathogenic bacteria in AAO MBR systems. Furthermore, the lower LRV for *E. coli* seen in this study as compared to a similar AAO MBR, which was examined using culture-based methods [122], illustrates the potential for disparities in results obtained by different detection techniques. Another recent work also employed quantitative digital PCR for the detection of pathogenic bacteria in both a full-scale aerobic MBR and a lab-scale anaerobic MBR treating the same municipal wastewater [123]. Results of the same study targeting total bacteria, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* showed LRVs of 4.1, 2.7, 1.0, and 3.1, respectively, in the aerobic MBR system and 3.1, 4.2, 1.2, and 3.9, respectively, in the anaerobic MBR [123]. In addition to reiterating the potential for variability among pathogenic species, these results also accentuate the potential for improved pathogen removal rates by anaerobic MBRs. Although the reason for observed higher LRVs by anaerobic MBRs is not clear due to the limited number of studies addressing this issue, one potential explanation could be associated with differences in membrane fouling mechanisms observed in aerobic and anaerobic MBRs [124]. These differences are potentially significant given recent observations that membrane biofilms can improve bacterial LRVs and effluent water quality in general [125].

Further insight into the removal and dynamics of potentially pathogenic bacteria can be drawn from the utilization of high-throughput sequencing of MBR system effluents. In one such study, pyrosequencing was utilized to characterize influent, sludge, and effluent microbial characteristics of a full-scale aerobic MBR [126]. Results of this study showed that influent samples were highly diverse and contained up to 39% relative abundance of potentially pathogenic bacteria. Furthermore, several potentially pathogenic genera such as *Aeromonas*, *Enterobacter*, *Enterococcus*, and *Pseudomonas* were undetected in the MBR effluents, while the genera of *Legionella, Clostridium*, and *Mycobacterium* were

the most dominant of potential pathogens. Overall, these recent studies of MBRs using molecular methods accentuate the variability of pathogen abundances within the same treatment system as well as across different membrane-based treatment system types. This implies the necessity of further systematic study of various MBR types using both molecular- and culture-based methods in order to better characterize the threats arising from pathogenic bacteria in their effluents.

Table 2. Log removal rates of various indicator bacteria and bacterial pathogens in membrane bioreactor systems and their operating conditions. N.A. denotes information not provided. N.D. denotes not detected.

System Type	Membrane Nominal Pore Size	Wastewater Type	Detection Method	Indicator/Pathogen	Abundance in Influent	LRV	Ref.
Aerobic MBR	0.4 μm	Municipal wastewater	Culture-based plate count	*E. coli* *Enterococci* *C. perfringens*	N.A. N.A. N.A.	5.0 4.5 3.0	[116]
Aerobic MBR	0.4 μm	Municipal wastewater	Culture-based plate count	Total coliform Fecal coliform *E. coli* *Enterococci*	~10^{8} CFU/100 mL ~10^{7} CFU/100 mL ~$10^{6.9}$ CFU/100 mL ~$10^{5.9}$ CFU/100 mL	6.0 6.9 6.7 5.7	[127]
Aerobic MBR	0.4 μm	Municipal wastewater	Culture-based plate count	Fecal coliform *E. coli* *Enterococci*	~$10^{7.8}$ CFU/100 mL ~$10^{7.4}$ CFU/100 mL ~$10^{7.5}$ CFU/100 mL	>6.7 >6.1 >6.3	[115]
Aerobic MBR	0.4 μm	Municipal wastewater	Culture-based plate count	*E. coli* *Enterococci*	~$10^{6.9}$ CFU/100 mL ~$10^{5.9}$ CFU/100 mL	6.8 5.7	[128]
Aerobic MBR	0.4 μm	Municipal wastewater	Digital PCR	Total bacteria *A. Baumannii* *P. aeruginosa* *K. pneumoniae*	~$10^{8.5}$ copies/L ~$10^{6.5}$ copies/L ~$10^{3.8}$ copies/L ~10^{6} copies/L	4.1 2.7 1.0 3.1	[123]
Aerobic MBR	0.4 μm	Hospital wastewater	Culture-based plate count	Total coliform Fecal coliform *Enterococci*	~$10^{8.1}$ CFU/100 mL ~$10^{7.3}$ CFU/100 mL ~$10^{6.2}$ CFU/100 mL	3.2 3.6 3.1	[117]
Aerobic MBR	0.2 μm	Hospital wastewater	Culture-based plate count	*E. coli* *Enterococci*	~10^{5} CFU/100 mL ~10^{6} CFU/100 mL	3.8 4.5	[129]
Aerobic MBR	0.2 μm	Municipal wastewater	Culture-based plate count	Total coliform *E. coli* *Enterococci* Clostridia	~$10^{8.2}$ CFU/100 mL ~$10^{6.8}$ CFU/100 mL ~$10^{6.0}$ CFU/100 mL ~$10^{5.9}$ CFU/100 mL	5.3 5.1 5.0 4.6	[130]
Aerobic MBR	0.1–0.2 μm	Municipal wastewater	Culture-based plate count	Total coliform *E. coli* Clostridia	~10^{8} CFU/100 mL ~$10^{6.5}$ CFU/100 mL ~$10^{5.5}$ CFU/100 mL	5.9 5.1 4.9	[125]

Table 2. *Cont.*

System Type	Membrane Nominal Pore Size	Wastewater Type	Detection Method	Indicator/Pathogen	Abundance in Influent	LRV	Ref.
Aerobic MBR	0.05 μm	High-strength greywater	Culture-based plate count	Total coliform *E. coli* *Enterococci* Clostridia *P. aeruginosa*	~$10^{7.4}$ CFU/100 mL ~$10^{3.8}$ CFU/100 mL ~$10^{2.7}$ CFU/100 mL ~$10^{2.9}$ CFU/100 mL ~10^{7} CFU/100 mL	>7 N.A. 2.6 2.8 6.5	[118]
Aerobic MBR	0.04 μm	Municipal wastewater	Culture-based plate count	Total coliform Fecal coliform	N.A. ~10^{7} CFU/100 mL	5.5 6.5	[131]
Aerobic MBR	0.04 μm	Municipal wastewater	Culture-based plate count	Total coliform Fecal coliform	~10^{7} CFU/100 mL ~10^{4} CFU/100 mL	5.5 2.6	[132]
Aerobic MBR	0.04 μm	Municipal wastewater	Culture-based plate count	Total coliform *E. coli*	~10^{6} to 10^{8} CFU/100 mL N.A.	4.7–5.1 N.D.	[133]
Aerobic MBR	0.04 μm	Synthetic wastewater	Culture-based plate count	Total coliform *E. coli* *C. perfringens*	N.A. N.A. N.A.	6.5 5.5 5.0	[134]
Aerobic MBR	0.04 μm	Municipal wastewater	Culture-based plate count	Fecal coliform *Enterococci*	~10^{7} CFU/100 mL ~$10^{6.4}$ CFU/100 mL	6.9 6.1	[135]
Aerobic MBR	0.03–0.1 μm	Municipal wastewater	Culture-based plate count	Total coliform Fecal coliform	~10^{7} CFU/100 mL ~10^{6} CFU/100 mL	5.8–6.9 5.4–6.0	[136]
Aerobic MBR	0.03–0.2 μm	Municipal wastewater	Culture-based plate count	Total coliform Fecal coliform	~10^{6} to 10^{8} CFU/100 mL ~10^{6} to 10^{7} CFU/100 mL	5.5–6.7 5.3–6.5	[137]
AAO MBR	N.A.	Combined blackwater, greywater and kitchen wastewater	Culture-based plate count Quantitative PCR	Fecal coliform *E. coli* *Salmonella* *Shigella*	~$10^{4.8}$ CFU/100 mL ~$10^{3.5}$ CFU/100 mL ~$10^{1.5}$ CFU/100 mL ~$10^{0.8}$ CFU/100 mL	6.2 4.7 2.3 2.6	[121]
AAO MBR	0.45 μm	Greywater	Culture-based plate count	Fecal coliform *E. coli* *Staphylococcus* *Salmonella*	~10^{4} CFU/100 mL ~10^{4} CFU/100 mL ~10^{4} CFU/100 mL ~10^{4} CFU/100 mL	0.5 0.7 0.1 0.1	[114]
AAO MBR	0.4 μm	Municipal wastewater	Culture-based plate count	*E. coli* Clostridia	~10^{6} CFU/100 mL ~$10^{5.6}$ CFU/100 mL	6.1 4.8	[122]

Table 2. *Cont.*

System Type	Membrane Nominal Pore Size	Wastewater Type	Detection Method	Indicator/Pathogen	Abundance in Influent	LRV	Ref.
Anaerobic MBR	0.3 µm	Municipal wastewater	Digital PCR	Total bacteria	~$10^{8.5}$ copies/L	3.1	[123]
				A. baumannii	~$10^{6.5}$ copies/L	4.2	
				P. aeruginosa	~$10^{3.8}$ copies/L	1.2	
				K. pneumoniae	~10^{6} copies/L	3.9	
Anaerobic MBR	0.03 µm	Dairy wastewater	Culture-based plate count	*E. coli*	~10^{7} CFU/100 mL	6.7	[113]
				Enterococci	~$10^{7.9}$ CFU/100 mL	7.4	
				C. perfringens	~$10^{6.5}$ CFU/100 mL	N.D.	
Anaerobic MBR	100 kDa MWCO	Municipal wastewater	Culture-based plate count	Total coliform	~$10^{6.9}$ CFU/100 mL	N.D. (in 1 mL)	[112]
				Fecal coliform	~$10^{6.6}$ CFU/100 mL	N.D.	
				Streptococci	~$10^{5.7}$ CFU/100 mL	N.D.	

4. Wastewater Treatment Sustainability: The Role of AnMBRs in Reducing Effluent Reuse Risk

The recent rise in interest associated with treated wastewater effluents for their reuse can be attributed to a worldwide move towards improved water process sustainability. However, the current state of conventional wastewater treatment does not sufficiently address the concerns arising from emerging contaminants in agricultural and municipal wastewaters to allow for direct reuse in most instances [7]. Furthermore, microbial safety is at the forefront of the emerging concerns associated with wastewater reuse and is directly linked to non-microbial issues such as antibiotic presence and formation of DBPs [138,139].

To gain insight into the prospect of AnMBRs for improving effluent quality associated with these contaminants, literature addressing both anaerobic digestion and MBR-based treatment were investigated in this review. In general, studies of anaerobic digestion systems have shown that mesophilic treatment is an effective method for the reduction of pathogenic risk by 1–2 log and the subsequent production of biosolids suitable for restricted land application (i.e., Class B). Studies assessing thermophilic anaerobic treatment have shown their capability in further reducing pathogenic risk as well as shortening necessary retention times. Existing literature on potentially pathogenic bacteria in MBRs (predominantly aerobic) has shown that their LRVs are a significant improvement from conventional wastewater treatment. Nonetheless, disinfection of effluents remains necessary for the purposes of discharge and restricted reuse based on current US guidelines. As related to antibiotic resistance, both anaerobic digesters and MBR have been shown to harbor lower levels of both ARB and ARGs when compared to aerobic biological sludge treatment and conventional WWTPs, respectively.

These concepts have been positively reinforced by the findings of the few studies that have addressed AnMBR systems specifically. The evaluation of indicator microorganisms in AnMBRs has shown that they are capable of producing LRVs in the range of the highest observed for aerobic MBRs. Furthermore, the detection of specific pathogenic species using quantitative PCR in both aerobic and anaerobic MBR systems has also revealed higher LRVs for AnMBRs, with effluent pathogen levels close to those that would be acceptable for disinfection-free reuse based on QMRA. In addition to these possible advantages, the notion that anaerobic digesters harbor lower levels of ARGs as compared to aerobic systems was further reiterated in a study evaluating this in respective MBR systems, suggesting a possible 1–2 log difference in ARG abundances. Although the results of these studies accentuate the potential of AnMBRs for improving microbial safety, the lack of existing literature on the subject renders these implications inconclusive.

Another limitation of the existing literature in its ability to address true pathogenic risks in both anaerobic digesters and MBR systems is that the vast majority of studies performed so far have relied on culture-based enumeration of indicator bacteria. This is inherently restrictive given the lack of specificity associated with traditional microbial indicators, especially considering the vast differences in wastewater reuse guidelines presently used in different regions worldwide [118]. Furthermore, existing works have shown a high level of variability of LRVs for the indicator microorganisms detected, even within the same treatment system. This variability is likely representative of the same for actual pathogenic bacteria, especially in the case of MBRs [126]. Both quantitative and high-throughput molecular-based analyses of MBR systems have provided valuable insight into this concept, revealing that different MBR system types produce vastly diverse effluent microbial communities [123]. High-throughput microbial analysis specifically has shown the effect that non-microbial emerging contaminants (e.g., antibiotics) can have on reactor microbial communities [74] and has also provided insight into the potential link between ARG levels in anaerobic digesters and associated pathogenic bacterial abundances [64]. Based on these observations and the lack of consensus regarding what level of treatment qualifies as sufficient for safe reuse, future research on the subject would benefit from combining a systematic molecular-based approach with QMRA analysis for defining and standardizing pathogenic risk. Determining acceptable level of risk associated with antibiotic resistance is even more challenging to define, as no water quality standards addressing ARB and ARG in wastewater effluents are presently available from regulatory agencies. Molecular-based

methods are a powerful tool that can be used to deepen understanding of both of these microbial risks and establish a framework for the relationship between antibiotic resistance and pathogenic bacterial persistence. As such, it would be useful in future studies to utilize a combination of culture-based and molecular-based methods to study the effects of antibiotics on AnMBR systems' microbial communities, the pathogenic potency of their effluents, and their impact on antibiotic resistance.

Additional studies examining the effects of operational conditions on anaerobic membrane-based wastewater treatment systems can further be used to optimize their effluent quality and minimize microbial risks for the purpose of direct reuse. For example, given the higher pathogen and ARG removal rates observed for anaerobic digesters operating at thermophilic conditions, investigating the idea of operating AnMBRs at higher temperatures may be an appealing option from a microbial effluent quality perspective, particularly considering the higher methane production associated with the process [140]. These higher biogas production rates, however, are limited when digesters are operated at the organic loading rates associated with AnMBR treatment of low to medium strength wastewaters. Furthermore, as a means to improve process sustainability, the prospect of operating AnMBRs at psychrophilic and low mesophilic temperatures has been gaining attention in recent studies [141,142]. This practice could well be advantageous to the removal of pathogens from MBR systems, especially given the observations associated with higher LRVs of pathogens in anaerobic digesters at psychrophilic conditions as compared to mesophilic [41]. Nonetheless, it remains unknown whether this advantage can be extrapolated to AnMBRs specifically. Furthermore, the effect of psychrophilic AnMBR systems on antibiotic resistance proliferation is yet to be determined at any capacity and would be a valuable area of investigation for future research.

Another area of research that is intimately connected with AnMBRs and membrane-based wastewater treatment in general is that of membrane biofilm development. Given that recent studies have elucidated the advantages of membrane biofilms in improving membrane rejection capabilities of both organic compounds and microbial indicators, future research should focus on evaluating the potential contribution of this phenomenon towards removal of known bacterial pathogens, antibiotics, and ARGs in MBRs and AnMBR systems specifically. These contributions, if determined substantial, will have to be weighed against the practical drawbacks arising from non-conventional system parameters such as membrane biofilm development and psychrophilic reactor operation in AnMBRs. Overall, any design improvements resulting in higher removal of microbial-based contaminants will also need to take into consideration their impact on chemical risks as well. Ultimately, the incorporation of all three of these issues (i.e., process sustainability, chemical risks, and microbial risks) into the life cycle analysis of wastewater management by AnMBRs will facilitate safe and responsible practices for their effluents' reuse.

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