Journal of Environmental Management 394 (2025) 127692

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Research article

Effective strategies for pathogen reduction in decentralized wastewater treatment systems

Marta Lois [a], Matías Rivadulla [b], Ravi K. Chhetri [c], Sonia Suárez [b], Henrik R. Andersen [c], Francisco Omil [b], Jesús L. Romalde [a,*]

[a] CRETUS, Department of Microbiology and Parasitology, CIBUS-Faculty of Biology, Universidade de Santiago de Compostela, 15782, Santiago de Compostela, Spain
[b] CRETUS, Department of Chemical Engineering, Universidade de Santiago de Compostela, Santiago de Compostela, 15782, Galicia, Spain
[c] Department of Environmental Engineering, Technical University of Denmark, Miljøvej 113, 2800, Kgs. Lyngby, Denmark

ARTICLE INFO

Keywords:
Decentralized treatment
Membrane bioreactor
Enteric viruses
Enterococcus
Klebsiella

ABSTRACT

Decentralized wastewater treatment using membrane bioreactors (MBRs) is a suitable alternative for the removal of pathogens, including bacteria and viruses, thus reducing the risk of infectious disease outbreaks. In this research, the effectiveness of different MBRs in removing pathogenic bacteria with clinical relevance (*Klebsiella* spp. and *Enterococcus* spp.) and enteric viruses (Norovirus – NoV – genogroups GI and GII, Sapovirus – SaV – and Hepatitis E Virus –HEV–) was evaluated in two decentralized collection systems: Demosite 1 (urban wastewater separated in black and grey fractions) and Demosite 2 (hospital effluents). We also evaluated the applicability of pepper mild mottle virus (PMMoV) as general fecal contamination biomarker in water samples and its potential as indicator of viral removal in the two decentralized systems. Our data demonstrated that decentralized treatment through anaerobic and combined anoxic/aerobic treatment methods at both demosites efficiently eliminated pathogenic bacteria and enteric viruses. Log Removal Values (LRVs) at Demosite 1 and Demosite 2 reached up to >4.98 and > 4.95 for bacteria, respectively, and >7.53 and > >6.78 for enteric viruses, respectively. *Enterococcus* spp. such as *E. faecalis* and *E. hirae*, and NoV (GII and a lesser extent GI) were the most recalcitrant pathogens in the systems. We also demonstrated the potential of PMMoV as an indicator of enteric viral reduction during decentralized treatment process. This work highlights the reliability of decentralized treatment systems in reducing pathogenic microorganisms, offering a practical solution for improving public health and environmental safety.

1. Introduction

Wastewater treatment is an essential process for protecting public health and preserving environmental quality. In this way, decentralized wastewater treatment systems offer a promising solution for managing wastewater in areas where centralized treatment infrastructure is limited or impractical. These systems provide scalable, cost-effective and flexile solutions tailored to local requirements, minimizing infrastructure, transportation, and energy demands while ensuring compliance with environmental regulations (Al-Hazmi et al., 2022; Sharma et al., 2022; Ventura et al., 2024). However, challenges such as operational efficiency, maintenance, and long-term scalability must be carefully considered when implementing these systems.

One of the critical concerns in decentralized wastewater treatment is the effective removal of pathogenic microorganisms, which pose significant health risks to human populations. Special concern has been raised in the last years about the multidrug resistant bacteria which could lead to serious public health, economic and social implications (Denissen et al., 2022). Enteric bacteria and viruses are primarily transmitted through the fecal-oral route, representing a major public health concern due to their ability to cause widespread gastrointestinal disease outbreaks (Cai and Zhang, 2013; Hai et al., 2014). The major enteric bacteria and viruses in wastewater include *Escherichia* spp., *Shigella* spp., *Klebsiella* spp., *Salmonella* spp., *Enterococcus* spp., Norovirus, Sapovirus, Rotavirus, Adenovirus, or Hepatitis A and E viruses (Cai and Zhang, 2013; Hai et al., 2014; Park et al., 2024). These pathogenic microorganisms are highly resilient and possess a remarkable ability to persist in various environments, posing considerable challenges for effective wastewater management (Hai et al., 2014). Targeting these pathogens in wastewater treatment requires a multifaceted

* Corresponding author.
E-mail address: jesus.romalde@usc.es (J.L. Romalde).

https://doi.org/10.1016/j.jenvman.2025.127692
Received 15 June 2025; Received in revised form 9 September 2025; Accepted 16 October 2025
Available online 21 October 2025

approach that combines biological, chemical, and physical processes (Al-Hazmi et al., 2022).

Membrane bioreactors (MBRs) have become a suitable technology for decentralized wastewater treatment due to their compact design, high efficiency, and ability to produce high-quality effluent suitable for reuse. Additionally, MBRs are highly effective in removing various micropollutants and pathogens from aquatic environments, making them a reliable solution for ensuring safe water quality in decentralized systems (Ottoson et al., 2006; Zhang and Farahbakhsh, 2007; Hai et al., 2014; Zuo et al., 2021; Al-Hazmi et al., 2022; Nasir et al., 2022; Tang et al., 2024). The main difference between MBRs and conventional biological treatments is the use of a membrane with a specific pore size (0.1–10 μm for microfiltration and 5–100 nm for ultrafiltration) that acts as a physical barrier, successfully retaining most pathogenic microorganisms through the size exclusion principle (Hai et al., 2014; Eloffy et al., 2022). Besides the membrane pore size, several factors influence pathogen removal in MBR systems, including membrane fouling, pre-treatment quality, and operational conditions such as sludge retention time (SRT) and hydraulic retention time (HRT) (Al-Hazmi et al., 2022; Eloffy et al., 2022). These operational variables are critical in optimizing treatment performance and ensuring compliance with health standards.

Several studies have demonstrated that decentralized systems employing MBRs effectively achieve high levels of pathogen removal, with reported efficiencies often surpassing 99.9 % (Chaudhry et al., 2015; Purnell et al., 2016; Harb and Hong, 2017; Gurung et al., 2017; Miura et al., 2018; Ji et al., 2019; Wang et al., 2020; Zuo et al., 2021; Al-Hazmi et al., 2022; Nasir et al., 2022; Rivadulla et al., 2024; Tang et al., 2024).

This study investigates the efficiency of different decentralized wastewater treatment systems in removing pathogenic bacteria and enteric viruses under different operational conditions. For this purpose, different MBRs were tested for their effectiveness in reducing bacterial counts and viral concentrations at two pilot-scale wastewater treatment plants: Demosite 1 (Vigo, Spain) for segregated urban wastewater and Demosite 2 (Copenhagen, Denmark) for hospital wastewater treatment. Given their high prevalence in hospital-acquired infections and the growing concerns over antibiotic resistance, the pathogenic bacteria *Enterococcus* spp. and *Klebsiella* spp. were selected for this study, focusing specifically on drug-resistant strains. In addition, Norovirus Genogroups I (NoV GI) and II (NoV GII), Sapovirus (SaV), and Hepatitis E Virus (HEV) were selected for this study due to their high transmission rates and capacity to cause gastrointestinal illnesses, which can lead to outbreaks, especially in regions with inadequate sanitation.

2. Materials & methods

2.1. Description of demosites

2.1.1. Demosite 1

The experimental setup was used to treat the wastewaters produced in three office buildings in Galicia, NW Spain, which are segregated into two different fractions: on one side the streams collected from the flush of the toilets (Black Water -BW-) and on the other side, those collected from the drainage of the sinks (Grey Water -GW-). An Anaerobic Membrane Bioreactor (AnMBR) treating BW and a Hybrid preanoxic Membrane Bioreactor (H-MBR) treating a mixture of GW and AnMBR effluent in two different proportions (75:25 and 25:75, v/v), were operated in an integrated treatment train strategy. Two different stages were defined: Period I (March 2022–June 2023) with a ratio of 25:75 among BW effluent:GW was applied; and Period II (July 2023–March 2024) with ratio of 75:25 among BW effluent:GW (Fig. 1A). Lastly, a short-term operation (April 2024) with the addition of powdered activated carbon PAC (500 mg L^{-1}) in the H-MBR (Period III), maintaining the same operational conditions as Period II (AnMBR permeate:GW ratio of 75:25) (Fig. 1B).

The 3.4 m^3 AnMBR, is divided in a 2.4 m^3 anaerobic digester and a 1 m^3 filtration chamber containing a 6.25 m^2 ultrafiltration flat sheet membrane (Fig. 1), with a pore size of 0.035 μm (Martins System, Berlin, Germany). The HRT was fixed at 2.36 ± 0.19 d. The system was operated as a fed-batch with level control in which the membrane operated in cycles of 6.5 min, of which 5 min of filtration and 1.5 min of relaxation. The biogas produced in the digester was bubbled through the bottom of the filtration chamber using a blower, to prevent membrane

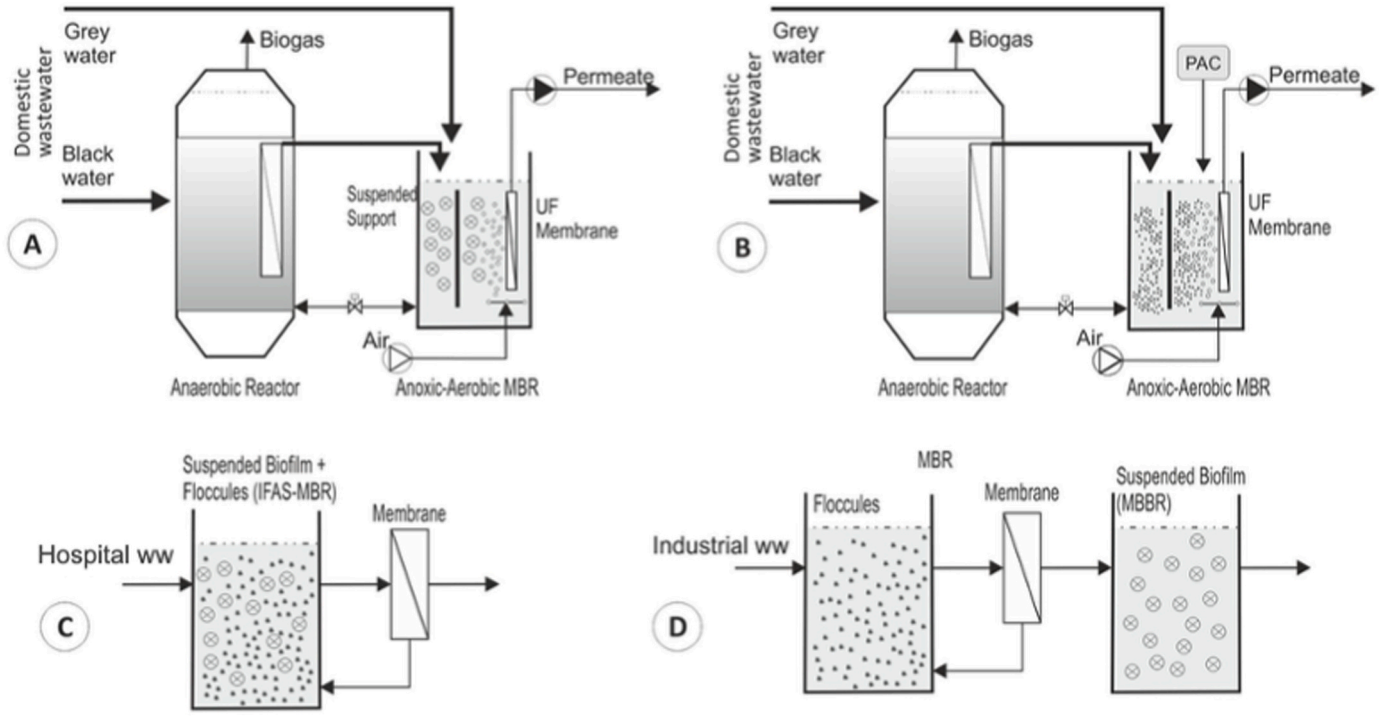

Fig. 1. Treatment configurations applied in Demosite 1 (A, B) and 2 (C, D).

fouling. Stirring and homogenization was achieved by mechanical stirring in the digester and pneumatic stirring thanks to the bubbled biogas in the filtration chamber. This plant was inoculated with anaerobic sludge collected from a full-scale WWTP nearby and operated during 3 years at the same location prior to this study.

As shown in Fig. 1, the 47.5 L H-MBR was divided in three different chambers, comprising a mechanically stirred anoxic (17.8 L), an aerobic (20.2 L) and a filtration (9.5 L) chamber. The latter contained a 0.47 m^2 Puron® ultrafiltration hollow fiber membrane (Koch membrane systems), with a pore size of 0.03 μm, which was continuously aerated to prevent fouling. The membrane operated in cycles of 3.5 min, of which 3 min corresponded to filtration and 0.5 min to backwashing. The HRT was maintained at 4.71 ± 0.03 h in the H-MBR. An internal recirculation stream allowed the transport of suspended biomass and nitrate/nitrite from the (aerated) filtration to the anoxic chamber. This recirculation ratio (R) was fixed at a value of 3. Both anoxic and aerobic chambers contained 20 % volume of Mutag BioChip carrier elements, with a specific area of 4000 m^2 m^{-3}, which were confined in their respective chambers. The biomass in this system was inoculated from a previous aerobic MBR treating GW in the same facility.

2.1.2. Demosite 2

The setup was composed of two parallel treatment lines which were established as on-site pilot plants treating hospital wastewater (HWW), inside the Herlev Hospital Wastewater Treatment Plant (WWTP) in Copenhagen, Denmark. Each line was composed of two reactors in series as shown in Fig. 1C and D. Series 1 consisted of a 500L hybrid Integrated Fixed Film Activated Sludge (IFAS) and MBR reactor (IFASMBR), followed by a 200L polishing Moving Bed Biofilm Reactor (MBBR). Series 2 consisted of a 500L conventional MBR followed by a 200L polishing MBBR. Biofilm carriers were K5 supplied by AnoxKaldnesTM and were added at a filling ratio of 20 % in the IFAS and of 50 % in both polishing MBBR tanks. The membrane modules were provided by Alpha Laval, Denmark with Polyvinylidene fluoride flat sheet 0.2 μm pore size membranes, with a surface area of 3.65 m^2. Mixing was performed by bottom aeration in all tanks. The transmembrane pressure (TMP) of the membrane system inside both the IFAS and the MBR was monitored by pressure gauges between the membrane outlet/permeate circuit and was surveyed across the operational time. The inlet and permeate pumps were controlled by level sensors installed inside the respective tanks to maintain the flow.

Three distinctive operational conditions were implemented with SRT 30 days, HRT 4 days; operation duration 206 days (phase 1), SRT 15 days, HRT 0.75–1.3 days; operation duration 114 days (phase 2) and SRT 8 days, HRT 0.27 days; operation duration 223 days (phase 3) with daily sludge wastage. During all phases the two systems were monitored and optimized as for their continuous loading and treatment efficiencies. Prior to SRT control the system operated for 15 days for stabilization and optimization.

2.2. Sample collection

Samples from Demosite 1 (n = 52) were collected from mid-October 2022 to April 2024 at different compartments, including influent, effluent, biomass from the two membrane bioreactor systems, and biofilm attached to carriers. The assessment of pathogenic bacteria and viruses in the influents and effluents of black and grey water was conducted through two sampling campaigns along Period I (March 2023–June 2023), five sampling campaigns during Period II (July 2023–March 2024) and one sampling campaign along Period III (April 2024). Samples were collected in the early morning (8–9 am), transported to the laboratory, refrigerated at 4 °C, and concentrated within 24 h of arrival.

Samples from Demosite 2 (n = 9) were collected from September 2023 to May 2024 from untreated hospital wastewater, outlet from MBR and outlet from IFASMBR.

Samples from biofilm and sludge at Demosite 1 were obtained only during periods II and III. For biofilm, two samplings were obtained during period II (November 2023 and March 2024) and one during period III (April 2024) for bacterial analysis. For viral analysis, three samplings were carried out during period II (July 2023, February 2024, and March 2024) and one during period III (April 2024). Sludge was sampled once during period II (March 2024) and once during period III (April 2024) for bacterial analysis. For viral analysis, five sludge samplings were carried out during period II (July 2023, October 2023, November 2023, February 2024, and March 2024) and one during period III (April 2024).

2.3. Bacterial isolation and identification

Samples from the different compartments were serially diluted, and 100 mL of appropriate dilutions were filtered through 0.45 μm pore size nitrocellulose sterile membrane filters. Filters were placed on Enterococcus Agar (m-EA) and Klebsiella Chromogenic Agar Base media (KChA) plates (both in triplicate) supplemented separately with ciprofloxacin (CPX; 1 μg mL^{-1}), trimethoprim (TMP; 100 μg mL^{-1}) and sulfamethoxazole (SMX; 7 μg mL^{-1}) and incubated for 24–48 h at 37 °C. Concentrations of chemotherapeutic agents were selected on the basis of EUCAST data (https://www.eucast.org/clinical_breakpoints?utm_source=chatgpt.com). Inoculated plates from Demosite 2 were shipped within 24–48 h to the USC laboratory to further analyses.

Counts of resistant bacteria to each antibiotic (expressed in colony-forming units per 100 mL; CFU/100 mL) were determined and putative *Klebsiella* spp. and *Enterococcus* spp. were isolated for further characterization. Selected isolates were subjected to phenotypic characterization and 16S rRNA gene sequencing as previously described (Gerpe et al., 2017; Romalde et al., 1990).

2.4. Viral concentration and nucleic acid extraction

BW samples (200 mL), sludge (20 mL) and biofilm samples (50 mL) were concentrated using the aluminum hydroxide adsorption-precipitation method (Carcereny et al., 2021), and concentrates were resuspended in 1–2 mL of phosphate buffered saline (PBS). GW and HWW samples (2 L) were primary concentrated by ultrafiltration using Rexeed-25A-filters (Asahi Kasei Medical America Inc) and then subjected to the aluminum hydroxide adsorption-precipitation method as above. All samples were spiked with 10 μL (final concentration 10^5 genomic copies (GC)/mL) of Mengovirus (MgV) as viral process control (Costafreda et al., 2006).

Viral RNA from concentrates was extracted using the Nucleospin® RNA/DNA Virus Kit (Macherey-Nagel GmbH & Co., Düren, Germany) following the manufacturer's instructions. 150 μL of the concentrated sample was mixed with 25 μL of Plant RNA Isolation Aid (Thermo Fisher Scientific, Vilnius, Lithuania) and 600 μL of lysis buffer from the NucleoSpin Virus kit. Viral RNA was eluted in 50 μL of RNAse free dH_2O and analyzed by RT-qPCR in the same day. Each extraction included a negative control and a positive control used to estimate the virus recovery efficiency. Samples with a virus recovery ≥1 % were considered as acceptable.

2.5. RT-qPCR assays

Viral RNA was detected by RT-qPCR on a Mx3000P qPCR system (Stratagene; USA) instrument. PrimeScript™ One Step RT-PCR Kit (Takara Bio, USA) was used in a 10 μl total volume, using 2.5 μl of extracted RNA. The primer/probe sets employed and amplification conditions for Mengovirus, Norovirus genogroups I and II, SaV, HEV and PMMoV were as follows: MgV (Pintó et al., 2009), NoV GI and NoV GII (Polo et al., 2015), SaV (Varela et al., 2015), HEV (Santos-Ferreira et al., 2020) and PMMoV (Haramoto et al., 2013), respectively. The thermal cycling conditions for the PMMoV RT-qPCR assay consisted of an initial

RT at 50 °C for 30 min, followed by denaturation at 95 °C for 30 s and 45 cycles of amplification at 95 °C for 5 s and 60 °C for 1 min.

Each RNA was tested in duplicate and as pure (undiluted RNA) and ten-fold diluted to detect possible presence of inhibitors. Every RT-qPCR assay included negative controls containing no nucleic acid and 1 well with each corresponding synthetic virus as positive control at 10^4 copies/μL. Calibration curves for all viruses were constructed using a minimum of five 10-fold dilutions and 3 wells for each dilution using the different synthetic virus controls. Results were expressed as number of viral genome copies per liter of wastewater (GC/L), following the guidelines of the ISO 15216-1 (ISO, 2017).

For each specific enteric virus target, Cq values ≤ 40 were converted into GC/L using the corresponding standard curve and volumes tested. Occurrence of inhibition was estimated by comparing average viral titers obtained from duplicate wells tested for the undiluted RNA with duplicate wells tested on ten-fold diluted RNA (Carcereny et al., 2021). Inhibition was ascertained when difference in average viral titers was higher than 0.5 log10 and, in these cases, viral titers were inferred from the ten-fold RNA dilution.

2.6. Interpretation and statistical analysis

All statistical analysis and data plotting were performed with R Studio (version 4.3.1). Based on the normality of the data, Welch's two-sample *t*-test or the Mann-Whitney test were used to compare bacterial counts and viral loads between the influent and effluent in each period at Demosite 1, after the AnMBR and H-MBR processes, and at Demosite 2, after the MBR and IFAS processes. Bacterial and viral LRVs from the AnMBR and H-MBR bioreactors across the three periods were compared to assess differences using the parametric one-way ANOVA test. The difference of means between groups was resolved via confidence intervals using Tukey's test. Bacterial and viral LRVs from the MBR and IFAS bioreactors were compared by using the Welch's two-sample *t*-test or the Mann-Whitney test. Spearman's rank correlation analysis was employed to correlate the concentrations of PMMoV with the concentrations of each enteric virus. Differences were considered statistically significant with p-values <0.05.

3. Results

3.1. Bacterial removal efficiency

3.1.1. Demosite 1

In most sampling events, BW influents (BWI) exhibited high bacterial counts on both m-EA and KChA plates compared to GW influents (GWI) (Table 1). Generally, bacteria grown on m-EA and KChA plates supplemented with SMX showed higher counts in both BWI and GWI compared to those grown on m-EA and KChA plates supplemented with TMP and CPX (Table 1). Bacteria grown on m-EA and KChA plates supplemented with TMP and CPX showed great reduction in BW effluents (BWE) and GW effluents (GWE), often reaching 0 CFU/100 mL (Table 1). In contrast, bacteria grown on m-EA and KChA plates supplemented with SMX were more persistent, with one case showing levels of 7.47×10^4 CFU/100 mL in GWE (Table 1).

In period I, bacterial counts in BWE were reduced by 2–3 log units on m-EA and KChA supplemented with the different antibiotics, obtaining in almost cases counts lower than 100 CFU/100 mL (Table 1). On the m-EA supplemented with CPX, no growth was observed for the BWE (Table 1). In GWE, bacterial counts varied depending on the type of bacteria and the antibiotic, with reductions of 2 or nearly 3 log units observed for *Klebsiella* grown on TMP and CPX (Table 1). In period II, bacterial counts in BWE were reduced to 2 to nearly 5 log units on m-EA and KChA supplemented with SMX, TMP and CPX (Table 1). *Klebsiella* resistant to TMP, as well as enterococci resistant to SMX, reached 0 CFU/100 mL in all samplings, whereas *Klebsiella* resistant to CPX was present in only one sampling at 127 CFU/100 mL (Table 1). Enterococci

Table 1
Pathogenic bacteria (CFU-Colony Forming Units-/100 mL) and Log Removal Value (LRV) during different samplings in all effluent samples from BW and GW from Demosite 1. SMX: Sulfamethoxazole; TMP: Trimethoprim; CPX: Ciprofloxacin.

Pathogenic bacteria	Period (Sampling)	Black Waters						LRV AnMBR			Grey Waters						LRV H-MBR		
		Influent			Effluent						Influent			Effluent					
		SMZ	TMP	CPX	SMZ	TMP	CPX	SMZ	TMP	CPX	SMZ	TMP	CPX	SMZ	TMP	CPX	SMZ	TMP	CPX
Klebsiella spp.	I (1)	97,211	44,000	2900	80	30	160	3.08	3.17	1.26	248	1177	12	478	1	1	−0.39	2.95	1.69
	I (2)	6620	4370	2020	50	30	0	2.12	2.16	>3.31	400	710	280	10	0	1	1.49	>2.73	2.32
	II (1)	3353	330	783	77	0	127	1.64	>2.52	0.79	1443	843	770	0	0	0	>2.62	>2.32	>2.46
	II (2)	69,930	39,960	95,830	410	0	0	2.23	>4.60	>4.98	2057	733	100	667	0	0	0.09	>2.26	>1.40
	II (3)	68,200	2467	1167	100	0	0	2.83	>3.39	>3.97	149,850	977	7357	74,740	0	33	−0.30	>2.39	1.75
	III (1)	24,567	767	100	0	0	0	>4.39	>2.88	>2.00	10,630	1980	3437	0	0	0	>3.42	>2.69	>2.93
Enterococcus spp.	I (1)	85,574	9600	755	65	17	0	3.12	2.75	>2.88	63	17	0	25	41	0	0.40	−0.38	–
	I (2)	2512	911	341	42	14	0	1.78	1.81	>2.53	390	90	0	15	27	1	1.31	0.42	–
	II (1)	583	300	166	1	1	0	2.77	2.48	>2.22	2727	1357	517	0	0	0	>2.83	>2.53	>2.11
	II (2)	2795	922	217	1	1	0	3.45	2.96	>2.34	1533	447	33	76	33	0	0.70	0.53	>0.92
	II (3)	55,500	2667	333	0	3	0	>4.74	2.95	>2.52	4593	7870	9943	0	0	0	>3.06	>3.29	>3.40
	III (1)	16,400	16,433	333	0	0	0	>4.21	>4.22	>2.52	17,073	7377	7157	0	0	0	>3.63	>3.27	>3.25

resistant to SMX and TMP reached 1 CFU/100 mL in most samplings, while *Klebsiella* resistant to SMX exhibited the highest counts (Table 1). In GWE, bacterial counts were reduced by 2 to over 3 log units in most samplings on m-EA and KChA supplemented with the three antibiotics, with no *Klebsiella* resistant to TMP or *Enterococcus* resistant to CPX detected in any of the samplings (Table 1). As in BWE, *Klebsiella* resistant to SMX showed the highest bacterial counts, reaching 667 and 7.47 × 10^4 CFU/100 mL (Table 1). *Klebsiella* resistant to CPX were detected only in sampling 3 with a count of 33 CFU/100 mL, and enterococci resistant to SMX and TMP were detected only in sampling 2, with counts of 76 and 33 CFU/100 mL, respectively (Table 1). In period III, complete removal of resistant enterococci and *Klebsiella* was achieved in both BWE and GWE (Table 1).

Overall, decentralized treatment at Demosite 1 was highly effective in eliminating bacteria across all three periods. Removal rates ranged from 94.5 to 100 % for AnMBR (LRVs ranging from 1.3 to >3.3) and to 60.6–100 % (LRV ranging from 0.4 to 2.9) for H-MBR in period I, and from 83.8 to 100 % for AnMBR (LRVs ranging from 0.8 to >5.0) and from 18.8 to 100 % (LRV ranging from 0.1 to >3.4) for H-MBR in period II (Table 1). In period III, complete removal (100.00 %) of bacteria grown on both m-EA and KChA plates was achieved by the AnMBR (LRVs ranging from to >2.0 to >4.4) and H-MBR (LRVs ranging from to >2.7 to >3.6) bioreactors, when PAC was added to the H-MBR treatment (Table 1). Resistant enterococci and *Klebsiella* were significantly reduced ($p < 0.05$) in all periods analyzed by both the AnMBR and H-MBR processes.

Regarding bacterial LRVs after the AnMBR process, no statistically significant differences were found between the periods in the one-way ANOVA analysis. However, a slight performance improvement was observed for Periods II and III. Although a minor improvement in bacterial removal was also noted in period II after H-MBR process, no significant differences were observed between the two periods (I and II). Nevertheless, bacterial LRVs in period III showed significant differences ($p < 0.05$) compared to periods I and II. Therefore, H-MBR was more effective in eliminating bacteria in Period III, when PAC was added before H-MBR treatment.

3.1.2. Demosite 2

Inlets from Demosite 2 also exhibited higher bacterial counts on both m-EA and KChA plates, compared to the MBR and IFASMBR effluents (Table 2). Bacteria grown on m-EA and KChA plates supplemented with SMX showed higher counts in both IFASMBR and MBR compared to those grown on m-EA and KChA plates supplemented with TMP and CPX (Table 2). Bacteria grown on m-EA and KChA plates supplemented with SMX were more persistent after MBR and IFASMBR processes (Table 2).

Generally, bacteria grown on m-EA and KChA plates supplemented with the three antibiotics showed reductions of more than 1 to nearly 5 log units in both bioreactors (Table 2). *Klebsiella* and enterococci resistant to TMP reached 0 CFU/100 mL in most samplings after MBR treatment, while higher counts were observed after IFASMBR treatment (Table 2). Enterococci resistant to CPX were only detected in sampling 1 after MBR and in sampling 2 after IFASMBR, with counts of 333 and 73 CFU/100 mL, respectively (Table 2).

Overall, decentralized treatment at Demosite 2 efficiently eliminated bacteria, achieving high removal rates ranging from 91.4 to 100 % (LRVs ranging from 1.1 to >4.9) for MBR and from 96.7 to 100 % (LRVs ranging from 1.5 to >4.2) for IFASMBR (Table 2). Resistant enterococci and *Klebsiella* were significantly reduced ($p < 0.05$) after the MBR and IFASMBR processes, according to Mann-Whitney test. No significant differences in bacterial LRVs were observed between the two bioreactors. Therefore, both MBR and IFASMBR systems appear to be equally effective in removing bacteria.

3.2. Bacterial identification through 16S rRNA gene sequencing

3.2.1. Demosite 1

In Period I, a total of 69 isolates were selected from the different m-EA (37 isolates) and KChA (32 isolates) plates for further characterization using 16S rRNA gene sequencing: 25 from BWI, 18 from BWE, 12 from GWI, and 14 from GWE (Supplementary Table S1). In BWI, the *Enterococcus* and *Klebsiella* species detected were *E. faecalis* and *E. lactis*, *K. quasipneumoniae* subsp. *similipneumoniae*, *K. michiganensis* and *K. quasipneumoniae* subsp. *similipneumoniae*. In BWE, the identified species included *E. faecalis*, *E. hirae*, *E. lactis*, *E. durans* and *Klebsiella pneumoniae* subsp. *ozaenae*. In GWI, the species detected were *E. lactis*, *E. durans*, *E. faecalis*, *E. faecium*. In GWE, only *E. lactis* was identified. It seems that *Klebsiella* spp. are completely removed after H-MBR process in this period, but certain *Enterococcus* spp. manage to pass through the bioreactor membranes.

In Period II, a total of 79 isolates were selected from the different m-EA (31 isolates) and KChA (48 isolates) plates for further characterization using 16S rRNA gene sequencing: 40 from BWI, 11 from BWE, 22 from GWI, and 6 from GWE (Supplementary Table S1). The *Enterococcus* and *Klebsiella* species detected in BWI were: *E. innesii*, *E. casseliflavus*, *E. faecalis*, *E. faecium*, *E. lactis*, *E. durans*, *E. hirae*, *E. hulanensis*, *K. michiganensis*, *K. quasivariicola*, *K. quasipneumoniae* subsp. *similipneumoniae*, *K. variicola* subsp. *tropica*, and *K. variicola* subsp. *variicola*. In BWE, only *E. faecalis* and *K. michiganensis* were identified. In GWI, the species identified were *E. faecium*, *E. casseliflavus*, *E. faecalis*, and *K. michiganensis*. No *Enterococcus* or *Klebsiella* species were detected in GWE. These results suggest that the H-MBR process in this period worked efficiently in removing *Enterococcus* and *Klebsiella* species.

In Period III, 7 isolates were selected from the different m-EA (2 isolates) and KChA (5 isolates) plates for further characterization using 16S rRNA gene sequencing: 5 from BWI, and 2 from GWI (Supplementary Table S1). In BWI, only *E. hirae* was detected, while in GWI, only *K. michiganensis* was identified. *E. hirae* was detected in AnMBR, and *E. lactis* and *E. hulanensis* were detected in H-MBR. In the aerobic biofilm, only *E. hirae* was detected, while in anaerobic biofilm, the only species detected was *E. casseliflavus*. Since no bacteria were detected after the AnMBR and H-MBR processes during this period, both bioreactors were effective in removing *Enterococcus* spp. and *Klebsiella* spp.

3.2.2. Demosite 2

A total of 69 isolates were selected from the different m-EA (36

Table 2
Pathogenic bacteria (CFU-Colony Forming Units-/100 mL) and Log Removal Value (LRV) during different samplings in all effluent samples from Demosite 2. SMX: Sulfamethoxazole; TMP: Trimethoprim; CPX: Ciprofloxacin.

Pathogenic bacteria	Sampling	Inlet			MBR outlet			IFASMBR outlet			LRV MBR			LRV IFASMBR		
		SMZ	TMP	CPX	SMZ	TMP	CPX	SMZ	TMP	CPX	SMZ	TMP	CPX	SMZ	TMP	CPX
Klebsiella spp.	1	87,712	88,813	91,575	2077	0	3	93	413	333	1.63	>4.95	4.48	2.97	2.33	2.44
	2	48,840	32,370	48,663	2400	100	2567	1287	37	1620	1.31	2.51	1.28	1.58	2.94	1.48
	3	45,510	33,300	26,640	0	0	3	3	3	10	>4.66	>4.52	3.95	4.18	4.05	3.43
Enterococcus spp.	1	101,349	7037	3883	3	0	333	1167	0	0	4.53	>3.85	1.07	1.94	>3.85	>3.59
	2	95,830	71,040	34,377	3967	667	0	367	73	73	1.38	2.03	>4.54	2.42	2.99	2.67
	3	113,643	22,940	9413	7	0	0	10	3	0	4.21	>4.36	>3.97	4.06	3.88	>3.97

isolates) and KChA (33 isolates) plates for further characterization using 16S rRNA gene sequencing: 40 strains from the inlet, 12 strains from the MBR effluent and 17 strains from the IFASMBR effluent (Supplementary Table 2). In the inlets, the *Enterococcus* and *Klebsiella* species detected were *E. faecalis*, *E. lactis*, *E. faecium*, *E. hirae*, *E. saccharolyticus* subsp. *taiwanensis*, *K. michiganensis*, *K. pneumonie* subsp. *pneumoniae*, *K. variicola* subsp. *variicola*, *K. quasipneumoniae* subsp. *quasipneumoniae*, and *K. pneumoniae* subsp. *ozaenae*. After MBR, we detected *E. lactis*, *E. casseliflavus*, *E. faecalis*, *E. thailandicus*, *E. hulanensis*, and *E. hirae*. After IFASMBR process, the species detected were: *E. hirae*, *E. faecalis*, *E. hulanensis*, and *E. faecium*.

All these results suggest that both MBR and IFASMBR bioreactors are highly effective in removing pathogenic *Klebsiella* spp., although some *Enterococcus* species were able to pass through the membranes more easily.

3.3. Virus removal efficiency

3.3.1. Recovery efficiency of the MgV process control

The recoveries of MgV, used as viral process control, ranged between 1.1 and 77.5 % (mean 25.2 ± 24.1 SD) for Demosite 1, and 17.7 and 59.0 % (mean 33.6 ± 16.0 SD) for Demosite 2. The limits of detection (LoD) and quantification (LoQ) as well as calibration curves for each RT-qPCR assay are described in Supplementary Table 3.

3.3.2. Demosite 1

In general, BWI exhibited higher viral loads compared to GWI (Table 3). SaV was the enteric virus with the highest levels of detection in BWI across all sample periods, followed by NoV GI and NoV GII (Table 3). In GWI, all enteric viruses, when detected, were present at similar concentrations (Table 3).

In period I, quantification levels for BWI were 1.2×10^6 and 3.5×10^6 GC/L for NoV GI, 1.5×10^6 and 2.0×10^6 GC/L for NoV GII, and 5.5×10^7 and 3.8×10^8 GC/L for SaV (Table 3). NoV GI and NoV GIII were detected only in BWE in sampling 1, with a concentration of 7.0×10^4 and 8.9×10^4 GC/L, respectively (Table 1). SaV was detected in both samplings, with concentrations of 2.7×10^5 and 9.3×10^4 (Table 3). For GWI and GWE, NoV GI and NoV GII were detected below the LoQ in one sampling, while SaV was not detected (Table 3). HEV was not detected in BWI or BWE but was detected below the LoQ in GWI and GWE in sampling 2 (Table 3). In period II, quantification levels for BWI ranged between ≤ LoQ and 6.3×10^5 GC/L for NoV GI, between 1.2×10^5 and 6.8×10^5 GC/L for NoV GII, and between 5.0×10^6 and 3.4×10^7 GC/L for SaV (Table 3). HEV was detected only in BWI from sampling 4 with a concentration of 1.06×10^5 GC/L (Table 3). NoV GI, SaV and HEV were not detected in any BWE sampling, and NoV GII was detected in only sampling 3, below the LoQ (Table 3). In GWI, NoV GI and NoV GII were detected in sampling 1 below the LoQ, and NoV GII was also detected in sampling 2 with a concentration of 1.0×10^5 GC/L (Table 3). SaV was detected only in sampling 5, with a concentration of 7.3×10^4 GC/L, while HEV was not detected in any GWI samples (Table 3). None of the viruses were detected in GWE (Table 3). In period III, none of the viruses were detected in BWE, GWI, or GWE (Table 3). NoV GI, NoV GII and SaV were detected only in BWI, with concentrations of 1.7×10^5, 2.5×10^5 and 5.4×10^6 GC/L, respectively, while HEV was not detected (Table 3). Generally, decentralized treatment at Demosite 1 proved to be highly effective in removing enteric viruses throughout all three periods.

After AnMBR process, levels in the effluents of BW from period I showed reductions of 98.0–100 % for NoV GI (LRVs ranging from 1.7 to >6.0), 95.7–100 % for NoV GII (LRVs ranging from 1.4 to >6.2), and 99.8–99.9 % for SaV (LRVs ranging from 2.8 to >3.2) (Table 3). After H-MBR process, the removal rate for NoV GI was 93.8 % (LRV = 1.2)

Table 3
Enteric virus concentration (genome copies/liter, GC/L) and Log Removal Value (LRV) during different samplings in all effluent samples from BW and GW, bioreactors and biofilms from Demosite 1. Each value represents the average of RT-qPCRs technical duplicates of a single concentrated sample. <LoQ: below the limit of quantification.

Enteric virus	Period (Sampling)	Black Waters		LRV AnMBR	Grey Waters		LRV H-MBR
		Influent	Effluent		Influent	Effluent	
NoV GI	I (1)	3.54E+06	7,05E+04	1.70	<LoQ	<LoQ	1.21
	I (2)	1.25E+06	ND	>6.01	ND	ND	–
	II (1)	8.14E+05	ND	>5.91	<LoQ	ND	>1.12
	II (2)	<LoQ	ND	>3.96	ND	ND	–
	II (3)	6.34E+05	ND	>5.80	ND	ND	–
	II (4)	1.75E+05	ND	>5.24	ND	ND	–
	II (5)	1.31E+04	ND	>4.12	ND	ND	–
	III (1)	1.70E+05	ND	>5.23	ND	ND	–
NoV GII	I (1)	2.05E+06	8,88E+04	1.36	ND	<LoQ	1.79
	I (2)	1.47E+06	ND	>6.17	<LoQ	ND	>3.73
	II (1)	6.81E+05	ND	>5.83	<LoQ	ND	>1.84
	II (2)	1.24E+05	ND	>5.09	9,96E+04	ND	>4.40
	II (3)	4.12E+05	<LoQ	1.92	ND	ND	>3.57
	II (4)	1.43E+05	ND	>5.16	ND	ND	–
	II (5)	1.79E+05	ND	>5.25	ND	ND	–
	III (1)	2.55E+05	ND	>5.41	ND	ND	–
SaV	I (1)	3.76E+08	2,66E+05	3.16	ND	ND	>4.82
	I (2)	5.51E+07	9,34E+04	2.78	ND	ND	>4.37
	II (1)	3.39E+07	ND	>7.53	ND	ND	–
	II (2)	4.99E+06	ND	>6.70	ND	ND	–
	II (3)	1.24E+07	ND	>7.09	ND	ND	–
	II (4)	5.06E+06	ND	>6.70	ND	ND	–
	II (5)	1.14E+07	ND	>7.06	7,28E+04	ND	>4.26
	III (1)	5.39E+06	ND	>6.73	ND	ND	–
HEV	I (1)	ND	ND	–	ND	ND	–
	I (2)	ND	ND	–	<LoQ	<LoQ	0.43
	II (1)	ND	ND	–	ND	ND	–
	II (2)	ND	ND	–	ND	ND	–
	II (3)	ND	ND	–	ND	ND	–
	II (4)	1.06E+05	ND	>5.03	ND	ND	–
	II (5)	ND	ND	–	ND	ND	–
	III (1)	ND	ND	–	ND	ND	–

(Table 3). For NoV GII, removal rates ranged between 98.4 and 100 % (LRVs ranging from 1.8 to >3.7) (Table 3). SaV was completely removed (100 %) by the H-MBR (LRVs reached up to >4.8) (Table 3). HEV was only detected in sampling 2, showing a reduction of 63.0 % (LRV = 0.4) by the hybrid MBR (Table 4). In period II, NoV GI and SaV were completely removed by the AnMBR in all samplings (LRVs reached up > 5.9 for NoV GI and >7.5 for SaV) (Table 3). For NoV GII, removal rates ranging from 98.8 to 100 % (LRVs ranging from 1.9 to >5.8) (Table 3). HEV was only detected in BWI in sampling 4, being completely removed (100 %; LRV >5.0) (Table 3). NoV GI was detected only in GWI in sampling 1, being completely removed after H-MBR process (LRV = >1.1) (Table 3). NoV GII was also completely removed when detected, with LRVs ranging from >1.8 to >4.4 (Table 3). SaV was detected only in GWI in sampling 5, being completely removed (LRV = >4.3) (Table 3). In period III, all enteric viruses were removed after AnMBR process, with LRVs of >5.2, >5.4 and > 6.7 for NoV GI, NoV GII and SaV, respectively (Table 3). None of the four enteric viruses analyzed were detected in either GWI or GWE (Table 3). Enteric viruses were significantly reduced ($p < 0.05$) by the AnMBR and H-MBR processes in all periods analyzed.

Regarding viral LRVs after the AnMBR process and considering all analyzed viruses together, statistically significant differences ($p < 0.05$) were found between period I and II in the one-way ANOVA analysis. Therefore, AnMBR was more effective in eliminating enteric viruses in Period II than in Period I. No significant differences were observed between Periods II and III. Although a minor improvement in viral removal was observed in period II after the H-MBR process, no significant differences in viral LRVs were found between periods I and II. Therefore, H-MBR appears to be equally effective in eliminating enteric viruses, regardless of the period.

3.3.3. Demosite 2

NoV GI, NoV GII and SaV were detected in all inlet samples, with concentrations ranging between 1.9×10^3 and 2.1×10^4 GC/L for NoV GI, 4.7×10^4 and 6.1×10^6 GC/L for NoV GII, and $2{,}5 \times 10^5$ and $1{,}3 \times 10^6$ GC/L for SaV (Table 4). HEV was not detected throughout the study (Table 4). SaV was not detected in any MBR outlet or IFASMBR outlet samples (Table 4). NoV GI was and NoV GII were detected in two MBR outlet samples below the LOQ (Table 4). NoV GII was detected below the LOQ in one IFAS sample, while NoV GI was not detected in any IFASMBR samples (Table 3).

SaV was completely removed after treatment using MBR and IFASMBR, with LRVs reaching >6.1. For NoV GI, MBR LRVs ranged from 1.9 to >3.3 (removal efficiencies ranging from 98.7 to 100 %), and for NoV GII LRVs ranged from 2.8 to >6.8 (removal efficiencies ranging from 99.8 to 100 %) (able 9). NoV GI was completely removed using IFASMBR, with LRVs ranging from >3.3 to >4.3. For NoV GII, LRVs ranged from 3.2 to >6.8 after IFASMBR treatment (removal efficiencies ranging from 99.9 to 100 %) (able 9).

All enteric viruses were significantly reduced ($p < 0.05$) by the MBR and IFAS processes. No significant differences were observed between the LRVs of the MBR and IFASMBR bioreactors, indicating that both are equally effective in removing enteric viruses.

Table 4
Enteric viruses (genome copies/liter, GC/L) and Log Removal Value (LRV) during different samplings in all effluent samples from Demosite 2. Each value represents the average (GC/L) of RT-qPCRs technical duplicates of a single concentrated sample. <LoQ: below the limit of quantification.

Enteric virus	Sampling	Inlet	MBR outlet	IFASMBR outlet	LRV MBR	LRV IFASMBR
NoV GI	1	1.87E+03	ND	ND	>3.27	>3.27
	2	6.21E+03	<LoQ	ND	1.91	>3.79
	3	2.07E+04	<LoQ	ND	1.89	>4.32
NoV GII	1	4.74E+05	<LoQ	<LoQ	3.52	3.23
	2	5.22E+05	<LoQ	ND	2.76	>5.72
	3	6.08E+06	ND	ND	>6.78	>6.78
SaV	1	4.74E+05	ND	ND	>5.68	>5.68
	2	1.31E+06	ND	ND	>6.12	>6.12
	3	2.53E+05	ND	ND	>5.40	>5.40
HEV	1	ND	ND	ND	–	–
	2	ND	ND	ND	–	–
	3	ND	ND	ND	–	–

3.4. PMMoV as an indicator of viral reduction

PMMoV was detected in all samples from Demosite 1 across all periods (Supplementary Table S4). Generally, BWI exhibited higher viral loads compared to GWI. PMMoV LRVs ranged from 0.9 to 3.2 for the AnMBR, and 0.1 to 2.7 for the H-MBR (Supplementary Table S4). PMMoV concentrations were significantly reduced ($p < 0.05$) following AnMBR and H-MBR treatments at Demosite 1. To determine whether the concentrations of the fecal indicator virus (PMMoV) were correlated with the concentrations of NoV GI, NoV GII and SaV, the nonparametric Spearman's rank correlation coefficient was calculated. Significant strong positive correlations were observed between NoV GI and PMMoV ($\rho = 0.8566185$), NoV GII and PMMoV ($\rho = 0.8652076$) and SaV and PMMoV ($\rho = 0.8606777$) in black waters. In grey waters, we observed a moderate negative correlation between NoV GI and PMMoV ($\rho = -0.4134388$), and weak positive correlations between NoV GII and PMMoV ($\rho = 0.1712178$) and SaV and PMMoV ($\rho = 0.1984677$). Therefore, this fecal indicator seems to function as an indicator of enteric virus reduction following AnMBR treatment at Demosite 1, but not after H-MBR treatment.

In Demosite 2, PMMoV was also detected in all samples (Supplementary Table S4). The MBR showed LRVs ranging from 1.9 to 2.7, while the IFASMBR showed LRVs ranging from 2.1 to 2.9 (Supplementary Table S5). PMMoV concentrations were reduced after the MBR and IFASMBR processes, although no significant differences were observed. Additionally, no significant differences were found between the LRVs of the MBR and IFASMBR bioreactors, indicating that both processes are equally effective in reducing PMMoV loads. We also observed significant strong positive correlations between NoV GII and PMMoV ($\rho = 0.864531$) and SaV and PMMoV ($\rho = 0.8218225$) at Demosite 2. A moderate positive correlation between NoV GI and PMMoV concentrations ($\rho = 0.6005679$) was also noted. Thus, PMMoV also served as an indicator of, at least, the reduction of NoV GII and SaV following MBR and IFASMBR treatments.

3.5. Bacterial and viral quantification in biofilms and sludge from Demosite 1

Pathogenic bacteria and viruses were also analyzed in biofilms from the anoxic (Bfan-HMBR) and aerobic (Bfaer-HMBR) chambers of the H-MBR at periods II and III, as well as in sludge from AnMBR (Sl-AnMBR) and H-MBR (Sl-HMBR) reactors.

During period I, *Klebsiella* resistant to the three antibiotics were more abundant in Bfan-HMBR than in Bfaer-HMBR, while enterococci resistant to SMX and TMP showed higher abundance in Bfaer-HMBR (Fig. 2A). No CPX-resistant enterococci were detected in Bfaer-HMBR, whereas their count in Bfan-HMBR was limited to 7 CFU/100 mL (Fig. 2A). During periods II and III, *Klebsiella* resistant to SMX were more abundant in Bfan-HMBR than in Bfaer-HMBR, while no *Klebsiella* resistant to TMP or CPX were detected in any biofilm (Fig. 2C). Also, resistant enterococci were more abundant in Bfan-HMBR. In period II, enterococci resistant to TMP were detected in higher numbers than those resistant to other drugs, whereas in period III number of enterococci resistant to SMX was higher and TMP-resistant enterococci were only found in Bfan-HMBR at just 3 CFU/100 mL (Fig. 2C).

Regarding sludge during period II, *Klebsiella* and enterococci resistant to the different antibiotics were more abundant in Sl-AnMBR than in Sl-HMBR (Fig. 3A). The abundance of SMX-resistant *Klebsiella* and enterococci, as well as TMP-resistant enterococci, was higher compared

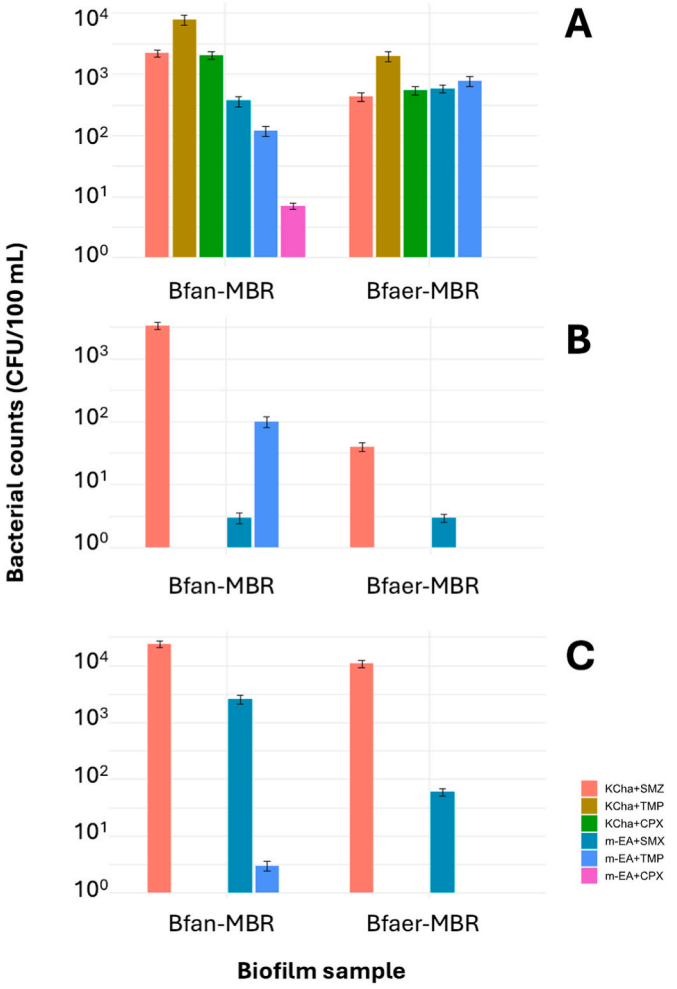

Fig. 2. Pathogenic bacteria (CFU/100 mL) in biofilms from anaerobic (Bfan-MBR) and aerobic (Bfaer-MBR) chambers of H-MBR reactor at Demosite 1 durting period I (A), II (B) and III (C). m-EA: m-Enterococcus Agar; KChA: Klebsiella Chromogenic Agar Base media; SMX: Sulfamethoxazole; TMP: Trimethoprim; CPX: Ciprofloxacin.

to TMP- and CPX-resistant *Klebsiella* and CPX-resistant enterococci in Sl-AnMBR (Fig. 3A). In H-MBR, the number of *Klebsiella* resistant to SMX was high compared to *Klebsiella* resistant to TMP and CPX (Fig. 3A). Similarly, the number of enterococci resistant to TMP was higher than enterococci resistant to SMX, which were detected at only 7 CFU/100 mL, while no CPX-resistant enterococci were detected (Fig. 3A). During period III, resistant *Klebsiella* and enterococci were more abundant in Sl-AnMBR than in Sl-HMBR except for enterococci resistant to CPX (Fig. 3B). In both Sl-AnMBR and Sl-HMBR, SMX-resistant *Klebsiella* and enterococci were more abundant than bacteria resistant to the other antibiotics, with no enterococci resistant to CPX detected (Fig. 3B). In Sl-HMBR, the number of SMX and CPX-resistant *Klebsiella* were 2–3 log unit higher than TMP-resistant *Klebsiella* (Fig. 3B). The numbers of enterococci resistant to SMX, TMP and CPX were low in Sl-HMBR, with counts of 37, 10 and 13 CFU/100 mL, respectively (Fig. 3B).

In period II, a total of 58 isolates were selected from the different m-EA (33 isolates) and KChA (25 isolates) plates for further characterization using 16S rRNA gene sequencing (Supplementary Table S1): 14 from Bfaer-MBR, and 18 from Bfan-MBR, 17 from Sl-AnMBR, and 11 from Sl-HMBR. No *Klebsiella* spp. were identified among these isolates. Regarding enterococci, *E. lactis*, *E. durans*, *E. faecium*, and *E. hirae* were identified in Bfan-MBR, *E. thailandicus* and E. hirae in Bfaer-MBR, *E. hirae*, *E. faecalis*, *E. casseliflavus* and *E. thailandicus* in Sl-AnMBR, and *E. hirae*, *E. faecium, E. lactis* and *E. hulanensis* and *E. thailandicus* in Sl-HMBR.

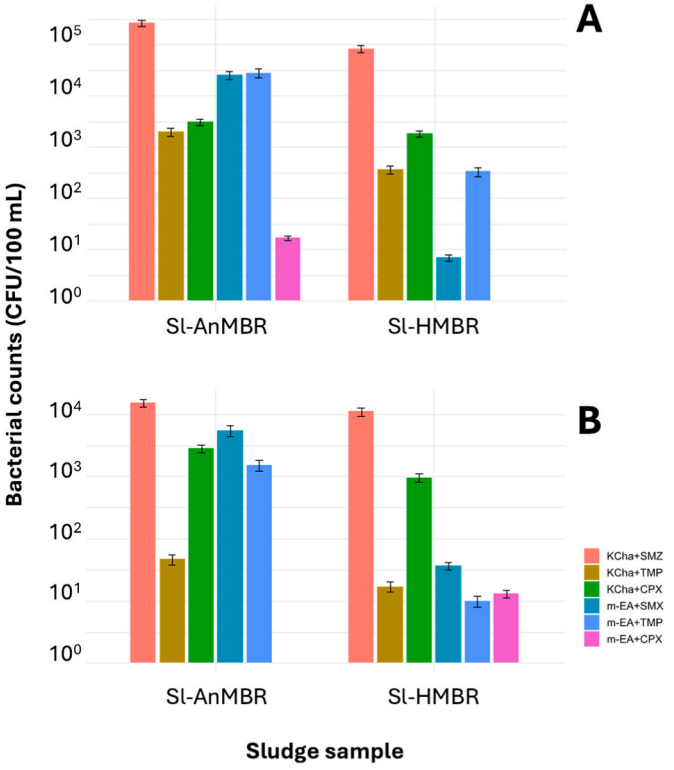

Fig. 3. Pathogenic bacteria (CFU/100 mL) in sludge from anaerobic (Sl-AnMBR) and aerobic (Sl-HMBR) reactors during periods II (A) and III (B) at Demosite 1. m-EA: m-Enterococcus Agar; KChA: Klebsiella Chromogenic Agar Base media; SMX: Sulfamethoxazole; TMP: Trimethoprim; CPX: Ciprofloxacin.

Regarding viral RNA concentrations in biofilms (Fig. 4; Supplementary Table 6), in period II, NoV GII was detected at similar concentrations (~1-3 x 10^4 GC/L) in all Bfan-MBR samples, while in Bfaer-MBR it was detected in only one sampling below the LoQ. NoV GI was detected in two samplings in both Bfan-MBR and Bfaer-MBR, with the concentration in Bfan-MBR higher than in AER. SaV and HEV were detected in only one sampling in Bfan-MBR with concentrations of 2.68 $\times$ 10^5 and 8.10 $\times$ 10^5 GC/L, respectively. In addition, SaV was also detected in one sample of Bfaer-MBR at a concentration 2.15 $\times$ 10^5 GC/L. In period III, none of the viruses were detected in biofilms, except for SaV, which was detected in only one Bfan-MBR sample, slightly below the LoQ.

SaV was the most frequently detected enteric virus in sludge during both periods in AnMBR and H-MBR, showing also the highest concentration levels (ranging from 1.51 $\times$ 10^5 GC/L to 4.09 $\times$ 10^7 GC/L), followed by NoV GII and NoV GI (Fig. 5; Supplementary Table 6). Generally, the RNA concentration levels of the three enteric viruses were higher in Sl-AnMBR than in Sl-HMBR at least by 1 log unit (Fig. 5; Supplementary Table 6). HEV was not detected in any samplings from either type of sludge.

Finally, PMMoV was also analyzed in both biofilms and sludge during periods II and III. PMMoV was detected in all biofilms and sludge samples in both periods at concentration levels around 10^8 GC/L (Supplementary Fig. S1). In sludge, the levels of PMMoV were always slightly higher in Sl-AnMBR.

4. Discussion

The reuse of treated wastewater is an increasingly vital but challenging solution in the context of global warming and rising water scarcity. Hence, decentralized wastewater treatment systems offer a

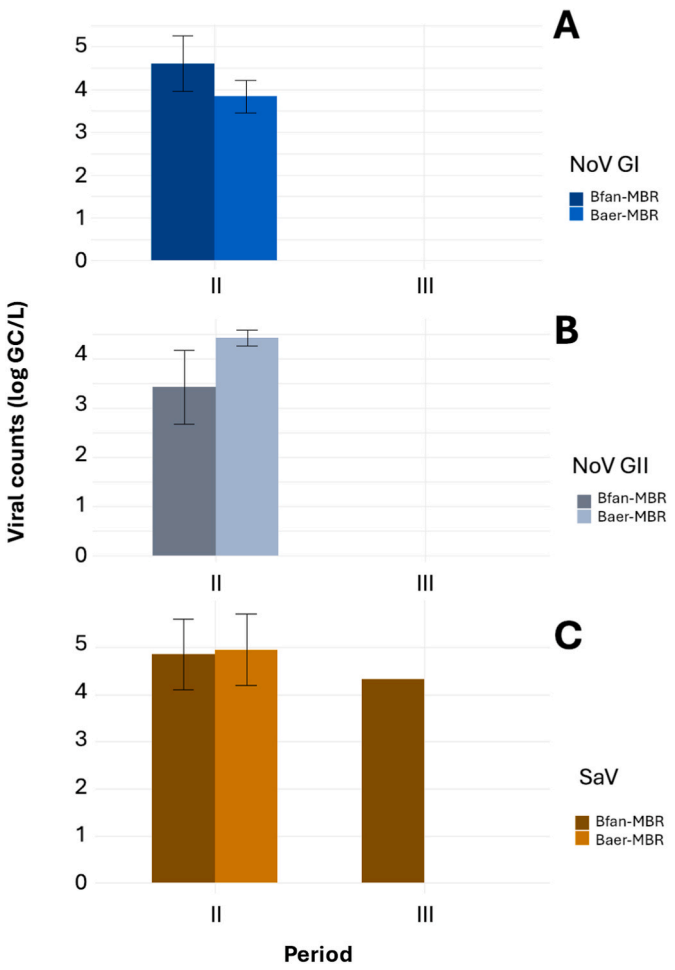

Fig. 4. Enteric virus concentration (genome copies/liter, GC/L) in biofilms from anaerobic (Bfan-MBR) and aerobic (Bfaer-MBR) chambers of H-MBR reactor during periods II and III at Demosite 1. A) NoV GI; B) NoV GII; C) SaV. Data represent the mean ± SD (standard deviation) based on three samplings in period II.

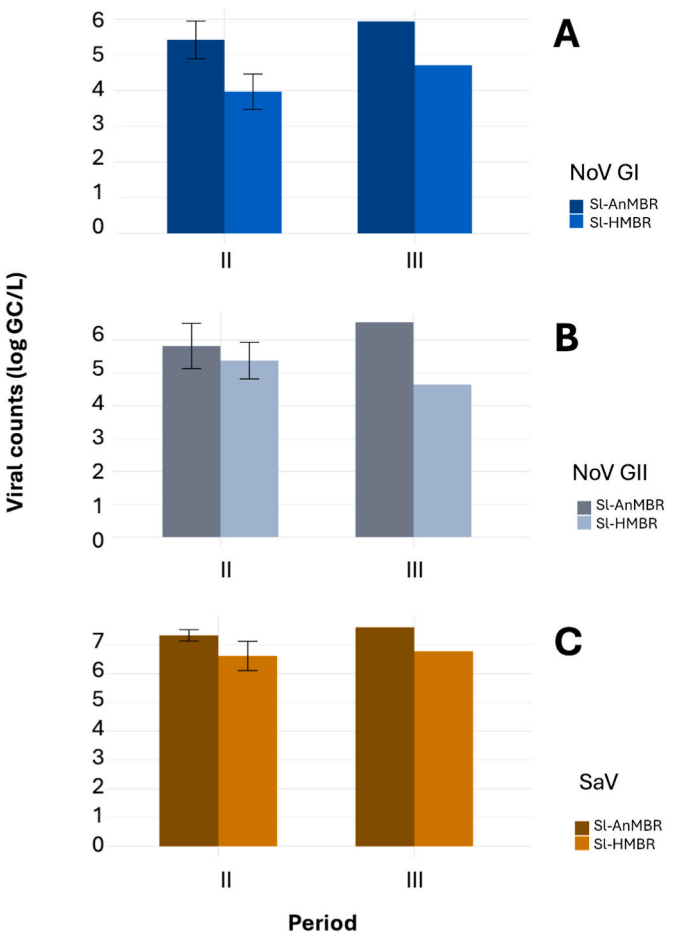

Fig. 5. Enteric virus concentration (genome copies/liter, GC/L) in sludge from anaerobic (Sl-AnMBR) and aerobic (Sl-HMBR) reactors during periods II and III at Demosite 1. A) NoV GI; B) NoV GII; C) SaV. Data represent the mean ± SD (standard deviation) based on five samplings in period II.

promising approach by improving public health and environmental sustainability while reducing pathogen risks associated with untreated wastewater (Al-Hazmi et al., 2022; Sharma et al., 2022; Ventura et al., 2024). Decentralized systems using advanced treatment technologies like MBRs address growing water demands especially in areas without centralized infrastructure by treating wastewater locally and eliminating the need for extensive transport networks. These compact systems also feature low sludge production and can handle varying influent loads, making them ideal for decentralized applications (Eloffy et al., 2022; Ventura et al., 2024). In addition, MBRs are well-known for their greater effectiveness in removing pathogenic microorganisms compared to conventional secondary treatments (Ottoson et al., 2006; Zhang and Farahbakhsh, 2007; Francy et al., 2012; Hai et al., 2014; Chaudhry et al., 2015; Harb and Hong, 2017; Miura et al., 2018; Tang et al., 2024). While the biological treatment process in MBRs is based on the same principles as conventional activated sludge, these systems separate solids from the effluent by integrating membranes that vary in pore size, typically microfiltration or ultrafiltration (Chaudhry et al., 2015; Miura et al., 2018; Zhao et al., 2022; Tang et al., 2024), although nanofiltration and reverse osmosis membranes may also be employed (Al-Hazmi et al., 2022; Eloffy et al., 2022).

Based on the use of aeration in the bioreactor, MBRs can be classified as aerobic MBRs (AeMBRs) and anaerobic MBRs (AnMBRs). Since AnMBRs have high organic load capacity, reduced energy consumption, and the ability to recover energy as biogas from wastewater, they are commonly employed in decentralized wastewater treatment systems (Harb and Hong, 2017; Zhao et al., 2022; Ventura et al., 2024). Unlike conventional MBRs, where biological treatment and membrane filtration occur in the same tank, hybrid MBRs (H-MBRs) integrate both aerobic and anaerobic processes in a two-stage system. The first stage involves biological treatment, followed by membrane filtration to remove solids, pathogens, and other contaminants, resulting in high-quality effluents (Harb and Hong, 2017; Ji et al., 2019; Zhao et al., 2022).

In this study, different types of MBRs demonstrated their effectiveness in removing enteric bacteria and viruses at two Demosites (1 and 2). At Demosite 1, black water was initially treated in an AnMBR with an ultrafiltration membrane, and the resulting effluent was subsequently mixed with grey water for further treatment in a H-MBR also incorporating an ultrafiltration membrane. At Demosite 2, two separate MBRs with a microfiltration membrane (MBR and IFAS) were employed to treat hospital influents.

Bacterial counts in influents and effluents were variable for each sampling, but removal efficiencies were generally close to 99.9 % at both Demosites. Both enterococci and *Klebsiella* were significantly reduced in water effluents using the AnMBR and H-MBR bioreactors across all periods (I, II, and III). Similarly, significant reductions were observed with the bioreactors at Demosite 2 (IFASMBR, MBR and polishing MBBR). At Demosite 1, the AnMBR consistently demonstrated high LRVs across all periods (I, II, and III), without significant differences among them. In contrast, the LRVs of the H-MBR varied depending on the period, with period III being significantly different from periods I

and II, suggesting that the addition of PAC improved the H-MBR's ability to eliminate bacteria, even under the same operational conditions as period II. It has been documented previously that the addition of PAC alleviates the blockage of MBR membrane pores, thereby improving the removal of bacteria and viruses (Ravindran et al., 2009; Sun et al., 2024). At Demosite 2, both the MBR and the IFASMBR exhibited high LRVs, with no differences between them, indicating that both were equally effective in removing pathogenic bacteria.

Wastewater, especially hospital wastewater, often contains a high burden of antibiotic-resistant bacteria, along with antibiotic-resistant genes and antibiotics such as sulfamethoxazole, trimethoprim, and ciprofloxacin (Wang et al., 2020; Sharma et al., 2022; Zhao et al., 2022; Godinho et al., 20204). In this way, the removal of *Enterococcus and Klebsiella* can be challenging due to their persistence in adverse environmental conditions and their potential to spread antibiotic resistance genes, particularly when antibiotics such as sulfamethoxazole, trimethoprim, and ciprofloxacin are present in water systems (Godinho et al., 2024; Park et al., 2024). Additionally, stress factors within decentralized treatment systems can promote other bacterial resistance mechanisms (Park et al., 2024). Bacterial LRVs observed at both Demosites are in line with the removal rates reported for microbial indicator bacteria (e.g., *Escherichia coli*, enterococci) in other studies using MBRs (Ottoson et al., 2006; Zhang and Farahbakhsh, 2007; Zanetti et al., 2010; Francy et al., 2012; Cheng and Hong, 2017; Wang et al., 2020). For example, Francy et al. (2012) reported LRVs between 4.82 and 7.49 for enterococci after MBR process, and in the studies of Ottoson et al. (2006) and Zanetti et al. (2010), LRVs for enterococci ranged between 4.6 and 6.2 after MBR treatment. In the study of Harb and Hong (2017), LRVs ranged from 2.7 to 5.6. Wang et al. (2020) reported removal rates of nearly 94 % for various antibiotic-resistant bacteria (including *Klebsiella* spp.), with the removal rate for ciprofloxacin-resistant bacteria reaching nearly 85 %. Complete removal of enterococci was observed in the studies of Ottoson et al. (2006), Zhang and Farahbakhsh (2007) and Purnell et al. (2016) using MBRs.

We detected several different species of *Enterococcus* and *Klebsiella* in the influents of Demosite 1 (urban wastewater) and 2 (hospital effluents), in accordance to other studies (Park et al., 2024; Godinho et al., 2024). Moreover, certain *Enterococcus* spp. were also detected in the final effluents at Demosite 2. The presence of bacterial pathogens in effluents, including *Enterococcus* and *Klebsiella* species, has been observed in many studies after the MBR process (Harb and Hong, 2017; Wang et al., 2020; Godinho et al., 2024; Park et al., 2024). Godinho et al. (2024) detected *Enterococcus* and *Klebsiella* species resistant to CPX. As in the study of Park et al. (2024), *E. faecium*, *E. faecalis*, *E. hirae, E. casseliflavus* and *E. thailandicus* were detected at the effluents in Demosite 2. In the study of Park et al. (2024), *E. faecalis*, *E. faecium* and *E. hirae* exhibited high resistance rates to antibiotics, with *E. faecium* showing a particularly greater ability to resist the effects of ciprofloxacin. They also detected low levels of CPX (0.133 μg/L), SMX (0.03 μg/L), and TMP (0.018 μg/L) in the effluents, which could be one factor contributing to the detection of antibiotic-resistant bacteria in our study. Thus, while the decentralized treatment appears to be efficient in eliminating *Klebsiella* spp. at both Demosites, some *Enterococcus* spp. seem to manage to pass through the reactor membranes at Demosite 2. Besides *Enterococcus* spp. and *Klebsiella* spp., the target organisms in this study, 22 and 11 different pathogen-associated genera were identified in Demosite 1 and 2, respectively (Supplementary Tables S1 and S2). Some of them were detected in the final effluents of Demosite 1 during periods I and II, as well as in the MBR and IFAS effluents, predominantly Gram-negative bacteria, including *Aeromonas* spp., *Pseudomonas* spp., *Citrobacter* spp., *Aerococcus viridans*, *Raoultella ornithinolytica*, *Enterobacter sichuanensis*, *Staphylococcus edaphicus*, *Phytobacter dIzotrophicus*, *Brevundimonas diminuta* and *Chryseobacterium rhizoplanae*. Godinho et al. (2024) also detected many of these pathogenic bacteria, which were resistant to CPX, after MBR treatment.

Due to the small pore size of the membranes, bacteria should be completely removed in MBR systems. However, other factors can influence bacterial removal by the MBRs, such as SRT or fouling (Cheng and Hong, 2017; Wang et al., 2020). Membrane fouling leads to an increase in hydrophobicity and a decrease in the membrane's electrical charge, which facilitates bacterial removal through adsorption (Cheng and Hong, 2017). The interaction of bacteria with biofilms is also noteworthy in their removal, as demonstrated in Demosite 1, where bacteria were a high number of bacteria were retained in both aerobic and anaerobic biofilms. Bacteria may experience alterations such as decreased rigidity in their cell walls or physical deformation due to filtration pressures, which enable them to pass through the membranes (Harb and Hong, 2017). Additionally, the membrane pore size can have defects or widenings, which could also help explain the passage of bacteria in the effluents at Demosite 2. MBRs have registered high LRV values for a wide variety of viruses, including NoV GI, NoV GII and SaV (Ottoson et al., 2006; Sima et al., 2011; Simmons et al., 2011; Francy et al., 2012; Chaudhry et al., 2015; Qiu et al., 2015; Gurung et al., 2017; Miura et al., 2018; Ji et al., 2019; Purnell et al., 2016; Tang et al., 2024).

In this work, we also demonstrated the effectiveness of MBRs in reducing NoV GI, NoV GII and SaV RNA levels at Demosites 1 and 2. In Demosite 1, SAV was the virus with the highest levels of detection in black influents, followed by NoV GI, NoV GII and HEV. In Demosite 2, NoV GII was the virus with highest levels of detection in the inlets, followed by SaV and NoV GI. Viral RNA concentrations were significantly reduced in water effluents from Demosite 1 using AnMBR and H-MBR bioreactors across all periods, as well as with MBR and IFAS bioreactors at Demosite 2. Complete removal of enteric viruses by both bioreactors was achieved in Demosite 1 during periods II and III. We observed that AnMBR was more effective in eliminating enteric viruses in periods II and III. However, H-MBR consistently maintained its virus-eliminating efficiency across all periods, suggesting that the addition of PAC had no effect on H-MBR′s ability to eliminate enteric viruses. SaV and NoV were completely removed in the study of Qiu et al. (2015) after MBR treatment, and Ji et al. (2019) observed completely removal with H-MBR of NoV GI, NoV GII and RV. At Demosite 2, both the MBR and the IFASMBR series exhibited high LRVs, with no differences between them, indicating that both were equally effective in removing enteric viruses. Although SaV was completely removed after MBR and IFAS processes, NoV GI and NoV GII continued to be detected below the LoQ at effluents of both bioreactors. Gurung et al. (2017) detected NoV GI and NoV GII in the MBR permeate, similar to the findings at Demosite 2 in our study.

Viruses generally exhibit higher resistance and stability to wastewater treatment than bacteria, although their resistance varies depending on the virus type and treatment process. Since the pore sizes of ultrafiltration membranes and virus particle sizes are comparable, mechanisms other than size exclusion, such as adsorption and electrical charge repulsion, are involved in virus removal by MBRs (Hai et al., 2014; Chaudhry et al., 2015; Zuo et al., 2021; Eloffy et al., 2022; Tang et al., 2024). Virus adsorption to solid surfaces differs among viral species due to variations in their surface characteristics, with both surface charge and hydrophobicity playing a role in this process, and these variations are closely linked to differences in virus removal efficiencies (Miura et al., 2018; Nasir et al., 2022; Tang et al., 2024). When the virus particles and the membrane surfaces have the same charge, they repel each other, preventing viruses from passing through the membrane (Hai et al., 2014; Zou et al., 2021). The hydrophobic capsid proteins of viruses can differ between viral species, which can also influence viral adsorption (Chaudhry et al., 2015; Miura et al., 2018). Viruses can be removed by MBRs as they attach to mixed liquor suspended solids (MLSS), composed of bacteria and organic compounds larger than membrane pores, preventing their passage through the membrane (Ottoson et al., 2006; Simmons et al., 2011; Chaudhry et al., 2015; Gurung et al., 2017; Miura et al., 2018; Zuo et al., 2021). Surface charge and variations in hydrophobic interactions between viruses (or their capsid proteins) and MLSS influence the efficiency of viral adsorption. For example, Miura et al. (2018) observed that the adsorption of NoV GI

to MLSS improved its LRV.

Besides adsorption and electrical charge repulsion, other mechanisms influencing virus removal by MBRs include retention of viruses by the cake layer formed on the membrane surface (Ottoson et al., 2006; Hai et al., 2014; Tang et al., 2024) and inactivation of viruses through interactions with other organisms or enzymes (e.g., proteases), particularly in AnMBRs (Chaudhry et al., 2015). The viral removal efficiency in MBRs could also be influenced by design and operating conditions, including pH, isoelectric point, temperature, dissolved oxygen, SRT, and HRT (Eloffy et al., 2022; Nasir et al., 2022). For example, low pH or extended HRT can enhance viral adsorption to solid particles, thereby improving virus removal (Miura et al., 2018; Nasir et al., 2022). In the study of Miura et al. (2018), an acidic pH (pH = 4) resulted in higher LRVs for NoV GI, NoV GII and SaV compared to a neutral pH. Membrane fouling can also influence virus removal (Hai et al., 2014; Chaudhry et al., 2015; Zhao et al., 2022; Nasir et al., 2022). However, maintaining a balance between moderate fouling and occasional membrane cleaning (e.g., backwashing or chemical cleaning) is necessary for the efficient operation of MBRs (Hai et al., 2014; Chaudhry et al., 2015; Eloffy et al., 2022; Nasir et al., 2022; Zhao et al., 2022). All these factors could contribute to the presence of lower viral loads of NoV GI and NoV GII in Demosite 2. Regarding membrane size exclusion mechanism, the occurrence of abnormal membrane pores or other membrane failures who may lead to virus passage or decreased filtration performance (Zuo et al., 2021) could also help explain the passage of NoV GI and NoV GII in the effluents at Demosite 2.

Viruses attached to the sludge are retained within the bioreactor or separated by the membrane, preventing them from passing into the effluent and thereby contributing to higher removal efficiency in MBR treatment (Al-Hazmi et al., 2022; Tang et al., 2024). Additionally, the adhesion of viruses to the biofilm on the membrane surface further enhances MBR removal efficiency (Hai et al., 2014; Chaudhry et al., 2015; Qiu et al., 2015; Zou et al., 2021; Nasir et al., 2022). We observed the accumulation of NoV GI, NoV GII and SaV in the biofilms and sludge of AnMBR and H-MBR bioreactors, contrary to the study of Miura et al. (2018), who found that those viruses were efficiently adsorbed to MLSS in the MBR. Sima et al. (2011) detected NoV GI and NoV GII in sludge samples, and Simmons et al. (2011) showed that NoV GII predominantly accumulated in primary sludge particles. The presence of higher RNA loads of NoV GI, NoV GII and SaV in sludge and biofilms demonstrated their accumulation and retention by the MBR processes in Demosite 1.

At Demosite 2, SaV was removed more efficiently than NoV GI and NoV GII, as observed in the study of Sima et al. (2011). In contrast, Miura et al. (2018) and Qiu et al. (2015) observed a more efficient removal of NoV GI and NoV GII compared to SaV. Moreover, SaV exhibited the highest LRVs in Demosite 1 compared to NoV GI and NoV GII, although all viruses were completely removed in period III. The varying concentrations of viruses in the influents, along with their detection limits, can lead to higher or lower removal values (Qiu et al., 2015; Gurung et al., 2017) which explains the higher LRVs of SaV compared to NoV GI and NoV GII in Demosite 1. Despite evidence from several studies indicating that removal efficiency is unrelated to virus morphology (Sima et al., 2011; Gurung et al., 2017), the complete removal of SaV in Demosite 2, in contrast to the incomplete removal of NoV GI and NoV GII, could still be attributed to differences in viral particle characteristics (Tang et al., 2024).

The determination of virus removal by MBRs depends not only on the type of virus but also, if it is the case, on the specific indicator microorganism monitored for such purpose (Hai et al., 2014; Chaudhry et al., 2015; Qiu et al., 2015; Purnell et al., 2016). Several studies have utilized indicator microorganisms to represent the efficiency of enteric virus removal (Ottoson et al., 2006; Francy et al., 2012; Tang et al., 2024). However, in some cases, the removal of enteric viruses in effluents was not correlated with the presence of indicator microorganism (Ottoson et al., 2006). In our study, PMMoV removal correlated with the removal of NoV GI, NoV GII and SaV by the AnMBR at Demosite 1, and by the MBR and IFAS at Demosite 2. Moreover, AnMBR and HMBR in Demosite 1 and MBR and IFAS in Demosite 2 removed efficiently PMMoV, with LRVs higher than 2 log units. Our results align with those of Tang et al. (2024), who demonstrated higher LRVs of PMMoV following the MBR process. Moreover, we detected PMMoV in all effluent samples from the MBRs at both Demosites, with higher concentration levels than those of the enteric viruses analyzed. However, the concentrations of PMMoV were reduced in these samples when the enteric viruses were also reduced or not detected. These findings are consistent with those of Tang et al. (2024). Therefore, PMMoV could serve as a reliable indicator of wastewater treatment efficiency.

The wastewater treatment employed in period III (combination 75:25 BW effluent:GW with the adsorbent PAC) at Demosite 1 successfully eliminates all the enteric bacteria and viruses. However, at Demosite 2, some *Enterococcus* spp. and Gram-negative bacteria (but not multiresistant *Klebsiella* spp.) manage to pass through the MBR and IFASMBR bioreactors. It was also noted that IFAS removed all enteric viruses, whereas MBR completely removed only SaV, with low concentrations of NoV GI and NoV GII still detected in the effluents. It should be indicated that the RT-qPCR approach is in fact detecting viral genomic material and not infective particles, so further studies are needed to determine the viability of the virus detected, especially at the effluents.

Our work reinforces the importance of monitoring bacteria and viruses within decentralized systems, as these pathogens might enter in contact with humans and animals representing a public health risk. Overall, the two pilot decentralized treatment plants could be effective solutions for reducing pathogenic bacteria and enteric viruses that cause gastrointestinal diseases.

CRediT authorship contribution statement

Marta Lois: Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Matías Rivadulla:** Writing – review & editing, Investigation. **Ravi K. Chhetri:** Writing – review & editing, Investigation. **Sonia Suárez:** Writing – review & editing. **Henrik R. Andersen:** Writing – review & editing. **Francisco Omil:** Writing – review & editing, Funding acquisition. **Jesús L. Romalde:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Funding

This research was supported by the projects PRESAGE (PCI2021-121990) financed under the ERA-NET AquaticPollutants Joint Transnational Call (GA N○ 869,178), and ARES (PID2022-141203OB-I00) funded by MCIN/AEI/10.13039/501,100,011,033/FEDER, UE. The ERA-NET AquaticPollutants is an integral part of the activities developed by the Water, Oceans and AMR Joint Programming Initiatives,".

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Jesus L. Romalde reports financial support was provided by ERA-NET AquaticPollutants Joint Transnational Call. Francisco Omil reports financial support was provided by State Agency of Research. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors want to acknowledge the company FCC Aqualia for the assistance in the operation and control of the decentralized system. The authors also want to acknowledge the company Veolia Denmark and

Alfa Laval Denmark for various assistance with constructing the pilot system for Demosite 2.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2025.127692.

Data availability

Data will be made available on request.

References

Al-Hazmi, H.E., Shokrani, H., Shokrani, A., Jabbour, K., Abida, O., Mousavi Khadem, S. S., Habibzadeh, S., Sonawane, S.H., Saeb, M.R., Bonilla-Petriciolet, A., Badawi, M., 2022. Recent advances in aqueous virus removal technologies. Chemosphere 305, 135441. https://doi.org/10.1016/j.chemosphere.2022.135441.

Cai, L., Zhang, T., 2013. Detecting human bacterial pathogens in wastewater treatment plants by a high-throughput shotgun sequencing technique. Environ. Sci. Technol. 47, 5433–5441. https://doi.org/10.1021/es400275r.

Carcereny, A., Martínez-Velázquez, A., Bosch, A., Allende, A., Truchado, P., Cascales, J., Romalde, J.L., Lois, M., Polo, D., Sánchez, G., Pérez-Cataluña, A., Díaz-Reolid, A., Antón, A., Gregori, J., Garcia-Cehic, D., Quer, J., Palau, M., González Ruano, C., Pintó, R.M., Guix, S., 2021. Monitoring emergence of the SARS-CoV-2 B.1.1.7 variant through the Spanish National SARS-CoV-2 Wastewater Surveillance System (VATar COVID-19). Environ. Sci. Technol. 55, 11756–11766. https://doi.org/10.1021/acs.est.1c03589.

Chaudhry, R.M., Nelson, K.L., Drewes, J.E., 2015. Mechanisms of pathogenic virus removal in a full-scale membrane bioreactor. Environ. Sci. Technol. 49, 2815–2822. https://doi.org/10.1021/es505332n.

Cheng, H., Hong, P.Y., 2017. Removal of antibiotic-resistant bacteria and antibiotic resistance genes affected by varying degrees of fouling on anaerobic microfiltration membranes. Environ. Sci. Technol. 51, 12200–12209. https://doi.org/10.1021/acs.est.7b03798.

Costafreda, M.I., Bosch, A., Pintó, R.M., 2006. Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. Appl. Environ. Microbiol. 72, 3846–3855. https://doi.org/10.1128/AEM.02660-05.

Denissen, J., Reyneke, B., Waso-Reyneke, M., Havenga, B., Barnard, T., Khan, S., Khan, W., 2022. Prevalence of ESKAPE pathogens in the environment: antibiotic resistance status, community-acquired infection and risk to human health. Int. J. Hyg Environ. Health 244, 114006. https://doi.org/10.1016/j.ijheh.2022.114006.

Eloffy, M.G., El-Sherif, D.M., Abouzid, M., Elkodous, M.A., El-nakhas, H.S., Sadek, R.F., Ghorab, M.A., Al-Anazi, A., El-Sayyad, G.S., 2022. Proposed approaches for coronaviruses elimination from wastewater: membrane techniques and nanotechnology solutions. Nanotechnology Rev 11, 1–25. https://doi.org/10.1515/ntrev-2022-0001.

Francy, D.S., Stelzer, E.A., Bushon, R.N., Brady, A.M., Williston, A.G., Riddell, K.R., Borchardt, M.A., Spencer, S.K., Gellner, T.M., 2012. Comparative effectiveness of membrane bioreactors, conventional secondary treatment, and chlorine and UV disinfection to remove microorganisms from municipal wastewaters. Water Res. 46, 4164–4178. https://doi.org/10.1016/j.watres.2012.04.044.

Gerpe, D., Buján, N., Diéguez, A.L., Lasa, A., Romalde, J.L., 2017. *Kiloniella majae* sp. Nov., isolated from spider crab (*Maja brachydactyla*) and pullet carpet shell clam (*Venerupis pullastra*). Syst. Appl. Microbiol. 40, 274–279. https://doi.org/10.1016/j.syapm.2017.05.002.

Godinho, O., Lage, O.M., Quinteira, S., 2024. Antibiotic-resistant bacteria across a wastewater treatment plant. Appl. Microbiol. 4, 364–375. https://doi.org/10.3390/applmicrobiol4010025.

Gurung, K., Ncibi, M.C., Sillanpää, M., 2017. Assessing membrane fouling and the performance of pilot-scale membrane bioreactor (MBR) to treat real municipal wastewater during winter season in Nordic regions. Sci. Total Environ. 579, 1289–1297. https://doi.org/10.1016/j.scitotenv.2016.11.122.

Hai, F.I., Riley, T., Shawkat, S., Magram, S.F., Yamamoto, K., 2014. Removal of pathogens by membrane bioreactors: a review of the mechanisms, influencing factors and reduction in chemical disinfectant dosing. Water 6, 3603–3630. https://doi.org/10.3390/w6123603.

Haramoto, E., Kitajima, M., Kishida, N., Konno, Y., Katayama, H., Asami, M., Akiba, M., 2013. Occurrence of pepper mild mottle virus in drinking water sources in Japan. Appl. Environ. Microbiol. 79, 7413–7418. https://doi.org/10.1128/AEM.02354-13.

Harb, M., Hong, P.Y., 2017. Molecular-based detection of potentially pathogenic bacteria in membrane bioreactor (MBR) systems treating municipal wastewater: a case study. Environ. Sci. Pollut. Res. 24, 5370–5380. https://doi.org/10.1007/s11356-016-8211-y.

International Organization for Standardization (ISO), 2017. Microbiology of Food Chain—Horizontal Method for Determination of Hepatitis A Virus and Norovirus Using Real-Time RT-PCR—Part 1: Method for Quantification; ISO 15216-1:2017. International Organization for Standardization, Geneva, Switzerland, 2017.

Ji, Z., Wang, X.C., Xu, L., Zhang, C., Rong, C., Rachmadi, A.T., Amarasiri, M., Okabe, S., Funamizu, N., Sano, D., 2019. Fecal source tracking in a wastewater treatment and reclamation system using multiple waterborne gastroenteritis viruses. Pathogens 8, 170. https://doi.org/10.3390/pathogens8040170.

Miura, T., Schaeffer, J., Le Saux, J.-C., Le Mehaute, P., Le Guyader, F.S., 2018. Virus type-specific removal in a full-scale membrane bioreactor treatment process. Food Environ. Virol. 10, 176–186. https://doi.org/10.1007/s12560-017-9330-4.

Nasir, A.M., Adam, M.R., Mohamad Kamal, S.N.E.A., Jaafar, J., Othman, M.H.D., Ismail, A.F., Aziz, F., Yusof, N., Bilad, M.R., Mohamud, R., A Rahman, M., Wan Salleh, W.N., 2022. A review of the potential of conventional and advanced membrane technology in the removal of pathogens from wastewater. Sep. Purif. Technol. 286, 120454. https://doi.org/10.1016/j.seppur.2022.120454.

Ottoson, J., Hansen, A., Björlenius, B., Norder, H., Stenström, T.A., 2006. Removal of viruses, parasitic protozoa and microbial indicator in conventional and membrane processes in a wastewater pilot plant. Water Res. 40, 1449–1457. https://doi.org/10.1016/j.watres.2006.01.039.

Park, J.-H., Bae, K.-S., Kang, J., Park, E.-R., Yoon, J.-K., 2024. Comprehensive study of antibiotic resistance in *Enterococcus* spp.: comparison of influents and effluents of wastewater treatment plants. Antibiotics 13, 1072. https://doi.org/10.3390/antibiotics13111072.

Pintó, R.M., Costafreda, M.I., Bosch, A., 2009. Risk assessment in shellfish-borne outbreaks of hepatitis A. Applied Environ. Microbiol. 75, 7350–7355. https://doi.org/10.1128/AEM.01177-09.

Polo, D., Varela, M.F., Romalde, J.L., 2015. Detection and quantification of hepatitis A virus and norovirus in Spanish authorized shellfish harvesting areas. International J. Food Microbiolo. 193, 43–50. https://doi.org/10.1016/j.ijfoodmicro.2014.10.007.

Purnell, S., Ebdon, J., Buck, A., Tupper, M., Taylor, H., 2016. Removal of phages and viral pathogens in a full-scale MBR: implications for wastewater reuse and potable water. Water Res. 100, 20–27. https://doi.org/10.1016/j.watres.2016.05.013.

Qiu, Y., Lee, B.E., Neumann, N., Ashbolt, N., Craik, S., Maal-Bared, R., Pang, X.L., 2015. Assessment of human virus removal during municipal wastewater treatment in Edmonton, Canada. J. Appl. Microbiol. 119, 1729–1739. https://doi.org/10.1111/jam.12971.

Ravindran, V., Tsai, H.-H., Williams, M.D., Pirbazari, M., 2009. Hybrid membrane bioreactor technology for small water treatment utilities: process evaluation and primordial considerations. J. Membr. Sci. 344, 39–54. https://doi.org/10.1016/j.memsci.2009.07.032.

Rivadulla, M., Lois, M., Elena, A.X., Balboa, S., Suarez, S., Berendonk, T.U., Romalde, J. L., Garrido, J.M., Omil, F., 2024. Occurrence and fate of CECs (OMPs, ARGs and pathogens) during decentralised treatment of black water and grey water. Sci. Total Environ. 915, 169863. https://doi.org/10.1016/j.scitotenv.2023.169863.

Romalde, J.L., Toranzo, A.E., Barja, J.L., 1990. Changes in bacterial populations during red tides caused by *Mesodinium rubrum* and *Gymnodinium catenatum* in North West Coast of Spain. J. Appl. Bacteriol. 68, 123–132. https://doi.org/10.1111/j.1365-2672.1990.tb02556.x.

Santos-Ferreira, N., Mesquita, J.R., Rivadulla, E., Inácio, A.S., da Costa, P.M., Romalde, J. L., Nascimento, M.S.J., 2020. Hepatitis E virus genotype 3 in echinoderms: first report of sea urchin (*Paracentrotus lividus*) contamination. Food Microbiol. 89, 103415. https://doi.org/10.1016/j.fm.2020.103415.

Sharma, M., Yadav, A., Dubey, K.K., Tipple, J., Das, D.B., 2022. Decentralized systems for the treatment of antimicrobial compounds released from hospital aquatic wastes. Sci. Total Environ. 840, 156569. https://doi.org/10.1016/j.scitotenv.2022.156569.

Sima, L.C., Schaeffer, J., Le Saux, J.C., Parnaudeau, S., Elimelech, M., Le Guyader, F.S., 2011. Calicivirus removal in a membrane bioreactor wastewater treatment plant. Appl. Environ. Microbiol. 77, 5170–5177. https://doi.org/10.1128/AEM.00583-11.

Simmons, F.J., Kuo, D.H., Xagoraraki, I., 2011. Removal of human enteric viruses by a full-scale membrane bioreactor during municipal wastewater processing. Water Res. 45, 2739–2750. https://doi.org/10.1016/j.watres.2011.02.001.

Sun, Y., Guo, Q., Rao, W., Li, N., Bai, Y., Zhang, J., Liang, S., 2024. Effect of powder activated carbon (PAC) addition on the performance of anaerobic dynamic membrane bioreactor (AnDMBR): stratification analysis of dynamic membrane characteristics. Chem. Eng. J. 499, 155889. https://doi.org/10.1016/j.cej.2024.155889.

Tang, Y., Sasaki, K., Ihara, M., Sugita, D., Yamashita, N., Takeuchi, H., Tanaka, H., 2024. Evaluation of virus removal in membrane bioreactor (MBR) and conventional activated sludge (CAS) processes based on long-term monitoring at two wastewater treatment plants. Water Res. 253, 121197. https://doi.org/10.1016/j.watres.2024.121197.

Varela, M.F., Polo, D., Romalde, J.L., 2015. Prevalence and genetic diversity of human sapoviruses in shellfish from commercial production areas in Galicia, Spain. Appl. Environ. Microbiol. 82, 1167–1172. https://doi.org/10.1128/AEM.02578-15.

Ventura, J.R.S., Tulipan, J.U., Banawa, A., Umali, K.D.C., Villanueva, J.A.L., 2024. Advancements and challenges in decentralized wastewater treatment: a comprehensive review. Desalin. Water Treat. 320, 100830. https://doi.org/10.1016/j.dwt.2024.100830.

Wang, S., Ma, X., Liu, Y., Yi, X., Du, G., Li, J., 2020. Fate of antibiotics, antibiotic-resistant bacteria, and cell-free antibiotic-resistant genes in full-scale membrane bioreactor wastewater treatment plants. Bioresour. Technol. 302, 122825. https://doi.org/10.1016/j.biortech.2020.122825.

Zanetti, F., De Luca, G., Sacchetti, R., 2010. Performance of a full-scale membrane bioreactor system in treating municipal wastewater for reuse purposes. Bioresour. Technol. 101, 3768–3771. https://doi.org/10.1016/j.biortech.2009.12.091.

Zhang, K., Farahbakhsh, K., 2007. Removal of native coliphages and coliform bacteria from municipal wastewater by various wastewater treatment processes: implications

to water reuse. Water Res. 41, 2816–2824. https://doi.org/10.1016/j.watres.2007.03.010.

Zhao, Y., Qiu, Y., Mamrol, N., Ren, L., Li, X., Shao, J., Yang, X., van der Bruggen, B., 2022. Membrane bioreactors for hospital wastewater treatment: recent advancements in membranes and processes. Front. Chem. Sci. Eng. 16, 634–660. https://doi.org/10.1007/s11705-021-2107-1.

Zuo, K., Wang, K., DuChanois, R. M., Fang, Q., Deemer, E. M., Huang, X., Xin, R., Said, I. A., He, Z., Feng, Y., Shane Walker, W., Lou, J., Elimelech, M., Huang, X., & Li, Q. 2021. Selective membranes in water and wastewater treatment: Role of advanced materials, Mater. Today 50, 516-532. doi : 10.1016/j.mattod.2021.06.013.