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Microbial community analysis of membrane bioreactor incorporated with biofilm carriers and activated carbon for nitrification of urine

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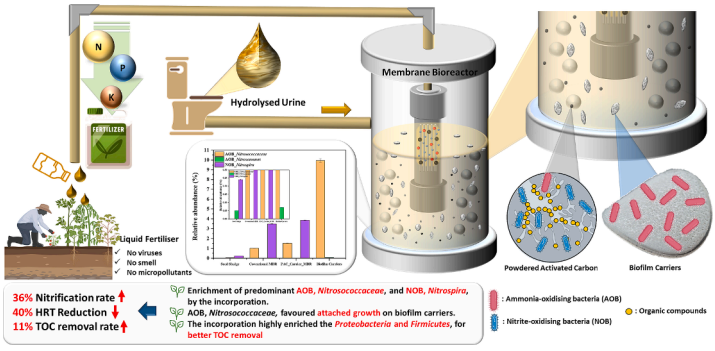
HIGHLIGHTS

- PAC and biofilm carrier incorporated MBR showed higher nitrification and lower HRT.
- Microbial diversity and richness reduced by PAC and biofilm carrier addition.
- Source-separated urine feeding significantly shifted microbial community dynamics.
- The dominant AOB and NOB were *Nitrosococcaceae* and *Nitrospira*, respectively.
- The addition of biofilm carrier highly favoured the enrichment of *Nitrosococcaceae*.

GRAPHICAL ABSTRACT



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ABSTRACT

The integration of powdered activated carbon and biofilm carriers in a membrane bioreactor (MBR) presents a promising approach to address the challenge of long hydraulic retention time (HRT) for nitrification of hydrolysed urine. This study investigated the effect of the incorporation in the MBR on microbial dynamics, focusing on dominant nitrifying bacteria. The results showed that significant shifts in microbial compositions were observed with the feed transition to full-strength urine and across different sludge growth forms. Remarkably, the nitrite-oxidizing bacteria *Nitrospira* were highly enriched in the suspended sludge. Simultaneously, ammonia-oxidizing bacteria, *Nitrosococcaceae* thrived in the attached biomass, showing a significant seven-fold increase in relative abundance compared to its suspended counterpart. Consequently, the incorporated MBR displayed 36% higher nitrification rate and 40% HRT reduction compared to the conventional MBR. This study provides valuable insights on the potential development of household or building scale on-site nutrient recovery from urine to fertiliser.

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1. Introduction

With the exponential growth of the global population, the demand for food production has escalated substantially. This heightened requirement has led to an increased reliance on synthetic fertilisers, thereby resulting in the accumulation of excessive nutrients in domestic waste (FAO, 2021). Source-separation of urine as a nutrient-rich waste stream presents a unique opportunity for implementing a circular economy approach (Kabir et al., 2023; Zheng et al., 2023). By recovering nutrients from urine and recycling them as fertilizers, the dependence on virgin synthetic fertilizers can be reduced, thereby mitigating their environmental impact since synthetic fertilizer production is highly energy-intensive process. Several methods encompassing physical, chemical, and electro-chemical treatments such as stripping, precipitation, and adsorption, have been investigated for nutrient recovery from source-separated urine (Sohn et al., 2023a; Tang et al., 2023; Zhang et al., 2021).

Among these methods, biological nitrification in a membrane bioreactors (MBRs) has emerged as a highly promising option for nutrient recovery. Through the use of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), nitrification can convert ammonia to nitrate, leading to a decrease in pH without the need for chemical additives (Wu et al., 2022). At the same time, heterotrophic bacteria play a vital role in the removal of organic matter. Subsequently, the effluent from the MBR is nitrified urine, primarily in the form of ammonium nitrate, thereby recovering other major nutrients such as phosphorous and potassium (Ren et al., 2021; Volpin et al., 2020). Common AOB species in wastewater treatment plants (e.g., *Nitrosomonas, Nitrosospira, Nitrosococcus*) oxidize ammonia to nitrite, while the globally distributed genus *Nitrospira* is a diverse group of NOB responsible for further oxidizing nitrite to nitrate (Chen et al., 2023; Daims et al., 2015; Derwis et al., 2023; Olsson, 2017; Zhang et al., 2023).

The composition of AOB and NOB populations can vary depending on specific environmental factors such as substrate availability and salinity (Fumasoli et al., 2016; Lin et al., 2023). Achieving a balanced oxidation rate between these two bacterial groups is crucial for maintaining stable nitrification. However, this can be challenging due to the influence of parameters such as temperature, dissolved oxygen, and pH (Jiang et al., 2021). The nitrite concentration can be accumulated if the NOB activity fails to keep up with that of AOB, which can be detrimental to both AOB and NOB (Peng et al., 2023; Zuo et al., 2023; Zuo et al., 2022). Therefore, a significant amount of time is required for the acclimation and enrichment of these slow-growing nitrifying microbes during the start-up phase when transitioning from diluted to undiluted urine (Sohn et al., 2023b).

In addition to the challenges associated with biological nitrification, the high salinity and ammonia concentrations (up to 30–50 times, respectively) in urine compared to municipal wastewater present another bottleneck, resulting in longer hydraulic retention times (HRT) in MBRs (Jiang et al., 2023; Liu et al., 2023). This longer HRT leads to larger system footprint, increased capital and operating costs, and poses operational challenges (Sohn et al., 2023a; Yang et al., 2020). The incorporation of additives such as biofilm carriers or activated carbon in MBRs can effectively improve nitrification performance by modifying the properties of the sludge, including the composition of microbial communities (Song et al., 2023). This enhancement is attributed to the attachment of microorganisms onto the porous surfaces of these additives, which provides a conducive environment for their growth, resulting in stable proliferation of AOB and NOB. Furthermore, this attachment facilitates increased resilience against shock loading rates and promotes efficient organics degradation (Mahendran et al., 2012).

A prior investigation compared conventional MBR with powdered activated carbon (PAC)-added MBR for source-separated urine treatment, reporting a 5 % increase in the nitrification rate, with a primary focus on micropollutant removal and fouling mitigation (Jiang, 2022). In the subsequent study, the additional incorporation of biofilm carriers demonstrated a noteworthy enhancement in nitrification performance, resilience, and a reduction in the start-up period (Sohn et al., 2023b). These findings underscored the significance of integrating PAC and biofilm carriers to enhance the rapid and efficient production of safe liquid fertiliser and these were the main motivation behind this microbial study. Building on this research, microbial analysis becomes crucial for optimizing urine MBR processes and advancing sustainable nutrient recovery strategies. To the best of current knowledge, the impact of this incorporation on microbial dynamics in the urine MBR process has not been investigated to date, despite its close relationship to nitrification and HRT. Therefore, this study aims to investigate, for the first time, the effect of incorporating biofilm carriers and PAC in an MBR treating source-separated urine on the operational performance as well as microbial community dynamics. Specifically, this study explored the changes in microbial diversity and community structure, focusing on nitrifying bacteria, in both suspended and attached sludge in comparison to a conventional MBR system for urine treatment. This study provides valuable insights for the development of compact and efficient MBR systems tailored for source-separated urine treatment, contributing to the achievements of a circular economy in nutrients.

2. Materials and methods

2.1. Feed urine collection and seed sludge

Building 11 at the University of Technology Sydney (UTS) has urine separation pipes from male toilets using waterless urinals installed on each floor. The collected urine is stored in a large water tanks located in the basement. For the operation of lab-scale MBR, a 20L tank of hydrolysed urine was collected. Upon collection, nutrient concentrations, pH, and total organic carbon (TOC) were promptly measured. The inoculative sludge used in this study was sourced from a decentralised wastewater treatment plant located in Central Park Sydney, Ultimo, NSW Australia. The inoculum was systematically acclimated to urine treatment, beginning with ten-fold diluted urine with around 400 mg/L of total ammonia nitrogen and progressively transitioning to normal urine concentration.

2.2. Experimental set-up and operational conditions

Two parallel aerobic MBR systems with a working volume of 15 L each were operated continuously. Both MBRs were fed with hydrolysed source-separated urine after the gradual start-up period with diluted urine feeding to allow time for microorganisms to acclimate. The experimental setup of the MBR with the addition of biofilm carriers and PAC is illustrated in Fig. 1. The control system was composed of a conventional MBR with an identical setup but without any carriers and PAC. Round and paraboloid-shaped polyethylene biochips (Mutag Biochip 30TM, Germany) with a diameter of 30 mm and thickness of approximately 1.1 mm were chosen for their large surface area (5,500 m^2/m^3) and light weight (165 g/L). While the shape of biofilm carriers can significantly influence the reactor performance, the round and paraboloid-shaped configuration facilitated the continuous removal of excess biomass, subjected to shear stress while rotating in the reactor, preventing biofilm clogging. Such clogging could otherwise lead to the formation of an anoxic layer, negatively impacting the nitrification process (Raj Deena et al., 2022). These biofilm carriers were introduced to the MBR system at a filling ratio of 4.75 % w/v, resulting in a total active surface area of 4.125 m^2. The virgin biofilm carriers were added from the initial day of operation and reached saturation during the start-up phase. Additionally, 2 g/L of PAC (Darco KB-B, Norit, US) having 500–1000 m^2/g of surface area and particle size within the range of 100–325 mesh was included after the start-up period. The PAC was rinsed at least three times with deionized (DI) water and dried at 105 °C before use. The images of biofilm carrier and PAC were provided (see supplementary material).

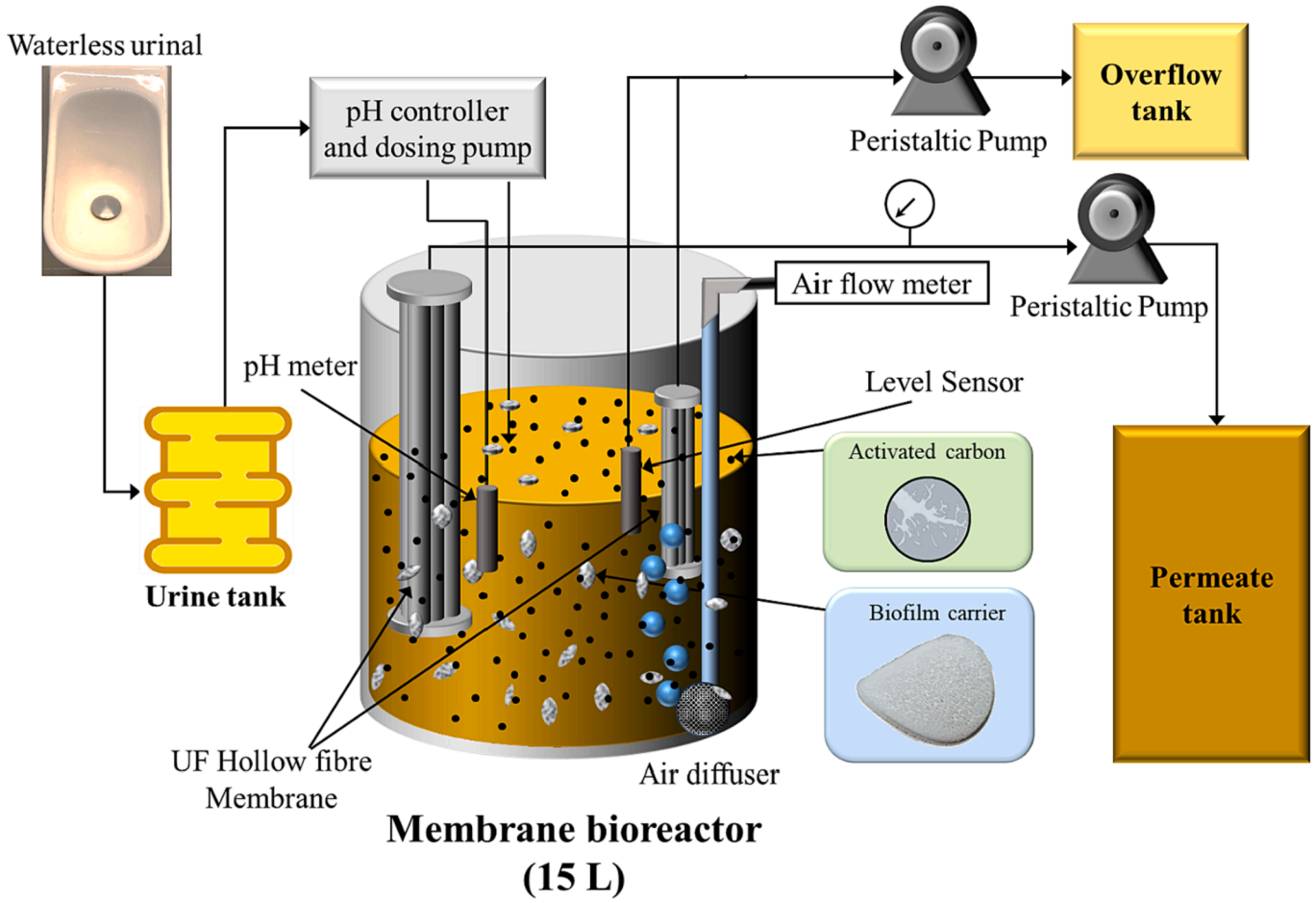

Fig. 1. Schematic diagram of experimental set up of a membrane bioreactor incorporated with PAC and biofilm carriers.

Hollow fibre ultrafiltration (UF) membranes (PVDF, 0.02 μm pore size, OriginWater, China) with inner and outer diameters of 0.55 mm and 1.65 mm, individually, and a surface area of 0.02 m^2 were utilised in both MBRs. The membrane module was operated under low trans-membrane pressure conditions, maintaining a flux at 3 $L/m^2 \cdot h$, which contributed to minimal membrane fouling. The pH values in both reactors were continuously detected and maintained using a pH controller with a dosing pump (BL7916-1, Hanna Instruments, Australia) by connecting a pH meter (HI6100405, Hanna Instruments, Australia). Whenever the pH value decreases owing to the alkalinity consumption during nitrification, the dosing pump automatically introduced pH 9.2 of urine to adjust the pH level at 6.2 ± 0.1. In the context of MBR operations, pH levels below neutrality is preferred to prevent ammonia loss through volatilization as certain amount of nitrogen is still present in the form of volatile ammonia. Earlier study has shown that, operating at neutral pH led to higher accumulation of NO_2^- which undermines the microbial activity and hence process efficiency as NO_2^- is toxic to microorganisms. Hence, the pH was adjusted to 6.2 ± 0.1 to minimize the risk of NO_2^- accumulation (Volpin et al., 2020). Consequently, the HRT was determined depending on the dosed urine to maintain a steady pH level, while the water level in the reactor was maintained using a water level controller connected to another UF module. The sludge retention time (SRT) was set to an infinite value, with 5 mL of sludge samples collected 1–2 times per week throughout the entire operational time. Air diffusers were employed in both reactors to maintain a dissolved oxygen (DO) level of 4.5 ± 0.1 mg/L, facilitating fully mixed conditions in the presence of biofilm carriers and PAC. The average concentrations of Mixed Liquor Suspended Solids (MLSS) and Mixed Liquor Volatile Suspended Solids (MLVSS) during the operational period were 5.5 ± 0.1 g/L and 4.8 ± 0.1 g/L, respectively, in the control system. Conversely, in the incorporated MBR with 2 g/L of PAC, these concentrations were 7.4 ± 0.3 g/L and 6.4 ± 0.4 g/L, correspondingly. The MLVSS/MLSS ratio in both reactors were maintained above 0.8. The amount of immobilised biomass attached on the biofilm carriers was maintained at 2.9 g, after the carriers were saturated with attached biomass during the start-up phase.

2.3. Analytical methods

2.3.1. Nutrient, total organic carbon and sludge properties analysis

The MLSS and MLVSS concentrations were assessed periodically 1–2 times per week, according to the standard methods. Quantification of immobilized biomass on the carrier was conducted by periodically replacing 3 carriers, which accounted for less than 1 % of the total number of carriers employed in the system. This minimal replacement had negligible impact on the overall results. The saturated carriers with biomass were subjected to 24-hour drying process at 80 °C and subsequently weighed. To remove the biomass, the carriers were soaked in a 5 % NaOH solution for 12 h, followed by rinsing with DI water. Afterward, the carriers were dried again at 80 °C, and they were weighed to determine the mass of the immobilised biomass on the carriers. By considering the known total number of carriers added to the reactor (310), the total immobilized biomass was calculated by multiplying the weight of the biomass in the collected samples.

For the analysis of nutrient concentrations, including ammonium nitrogen (NH_4-N), nitrate nitrogen (NO_3-N), and nitrite nitrogen (NO_2-N), a standard test kit (Merck Millipore, Burlington, USA) in conjunction with a spectrophotometer (Spectroquant NOVA 60, Merck, Germany)

was employed. The samples from the influent and effluent were collected on the same day and TOC concentration was determined using a TOC analyser (Analytik Jena Multi N/C 2000, Germany) to calculate the daily removal rates. Prior to analysis, each sample underwent filtration using a 0.45 μm cellulose esters filter, and subsequent dilution at a 100-fold ratio was applied.

2.3.2. Amplicon sequencing and bioinformatics analysis

The samples of suspended sludge from both reactors and attached biomass were collected at the end of the operation, while seed sludge was sampled prior to seeding into each reactor. The attached biomass was carefully scraped off and dissolved in DI water for sample preparation. The samples were then sent to the UTS Next Generation Sequencing Facility, Sydney, Australia, for DNA extraction and amplicon sequencing. While the sample from the inoculative sludge can be referred as 'Seed sludge', the suspended sludges sampled from each reactor are defined as 'Conventional MBR' and 'PAC_Carriers_MBR'. In addition, 'Biofilm carriers' represents the attached biomass from the biofilm carriers in this study.

The bacterial and archaeal 16S rRNA V3-V4 regions, were targeted using the universal primer set Pro341F (5′-CCTAYGGGRBGCASCAG-3′) and Pro806R (5′-GGACTACNNGGGTATCTAAT-3′), to capture a comprehensive representation of the microbial community (Sohn et al., 2021). Paired-end amplicon sequencing (2 × 300 bp) was performed on the Illumina MiSeq platform at the UTS sequencing facility. The initial processing of raw sequence data was carried out using the Illumina bcl2fastq pipeline. The raw sequencing data were submitted to the National Center for Biotechnology Information (NCBI) with accession No. PRJNA1067394.

The raw reads underwent microbial community analysis using Quantitative Insights into Microbial Ecology (QIIME) 2 (version 2020.11.1). To ensure data quality, a series of pre-processing steps were performed, including quality filtering, denoising (primer and read trimming), merging of paired-end reads, dereplication, and chimera filtering, employing the q2-dada2 denoise-paired plugin. The resulting high-quality reads were then taxonomically assigned with GraftM, utilizing hmmsearch (HMMER), against the SILVA database (release v132). Specifically, the identified sequences were placed into a pre-constructed phylogenetic tree for quick phylogenetic annotations. To assess the diversity within the microbial community, various alpha-diversity metrics such as the Shannon index and rarefaction curves were calculated using in-house Python scripts, where all samples were rarefied to a standardized sequencing depth of 110,000 sequences per sample. Additionally, to investigate differential abundances between groups, log2foldchange results were calculated. These approaches provided a more comprehensive and detailed analysis, yielding valuable insights into the microbial community structure and dynamics.

3. Results and discussion

3.1. Performance of membrane bioreactors

Two parallel MBRs were initiated by acclimating the inoculative sludge initially using diluted hydrolysed urine. Following an appropriate start-up period, both MBRs were gradually fed with increasing urine concentrations until acclimation with full strength real hydrolysed urine as shown in our previous study (Sohn et al., 2023b). Fig. 2 illustrates the nitrification performance of the control MBR and the PAC incorporated MBR with carriers, focusing on ammonium, nitrite, and nitrate concentrations, and ammonia to nitrate conversion rates. After the acclimation phase, the conventional MBR was operated with full strength real hydrolysed urine for 45 days, while the other MBR with saturated biofilm carriers and PAC was operated for 57 days. The NH_4^+ to NO_3^- conversion rates were 46 % and 48 % on average for the control and incorporated MBR, respectively, indicating higher stability in the latter compared to the slightly fluctuating control MBR. There was a slight

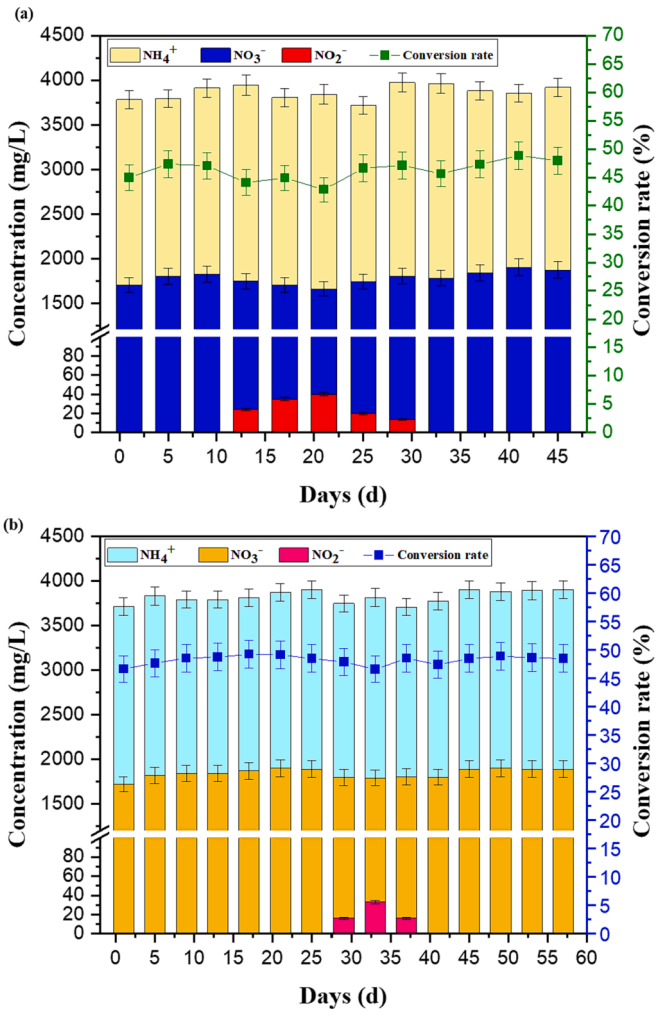

Fig. 2. Nitrification performance of (a) the conventional MBR; and (b) PAC_Carriers_MBR. Samples were taken every 4 days throughout the entire operational period and triplicated for analysis.

nitrite accumulation up to 40 mg/L and 33 mg/L in the conventional system and the incorporated system, individually, owing to the imbalanced activity of AOB and NOB (Liu & Wang, 2014). As a response, urine feeding was temporarily stopped to facilitate restoration and stabilisation of the system (Jiang et al., 2021; Volpin et al., 2020). The control system returned to a stable and steady state after 20 days following the NO_2^- accumulation event, whereas the incorporated MBR restored within a shorter period of 12 days. This faster recovery in the incorporated MBR might be attributed to the presence of carriers, which exhibit stronger survivability and resilience compared to the suspended microorganisms

Table 1
Performance results from the conventional MBR and the incorporated MBR.

Component in effluent	Conventional MBR	Incorporated MBR[b]
Nitrification rate (mgN/L·d)	194 ± 60	304 ± 73
HRT[a] (days)	10 ± 3	6 ± 2
NH_4^+-N ($mg_N.L^{-1}$)	2084 ± 86	1977 ± 54
NO_3-N ($mg_N.L^{-1}$)	1769 ± 85	1843 ± 60
NO_2-N ($mg_N.L^{-1}$)	< 1	< 1
TOC[a] removal rate (%)	85 ± 1	96 ± 1

*The result indicates the average value of all the samples collected during the operation period. Samples were taken every 4 days and triplicated for analysis.
[a] HRT, hydraulic retention time; TOC, total organic carbon.
[b] Incorporated MBR indicates the membrane bioreactor with powdered activated carbon and biofilm carrier addition.

(Dan & Le Luu, 2021; Liu et al., 2022).

Table 1 summarises the concentration of nitrified urine from the two MBRs as well as the nitrification rate, HRT and TOC removal rate during the operation. The PAC-carriers-incorporated MBR exhibited an overall nitrification rate of 304 ± 73 mg_N/L·d, whereas the control MBR showed a lower rate of 194 ± 60 mg_N/L·d. This significantly higher nitrification rate (by 36 %) can be primarily attributed to the thriving nitrifying microorganisms on the porous surface of the biofilm carriers (Bassin et al., 2016). Moreover, the addition of PAC likely contributed to the enhanced nitrification by enriching the bacterial community through further biofilm attachment (Asif et al., 2020; Du et al., 2017). The enhanced nitrification rate resulted in a reduced HRT for the incorporated MBR, making it more compact, with an average HRT of 6 ± 2 days, representing an approximate 40 % reduction.

The TOC removal rate, which reflects the efficiency of organic matter removal, remained steady at around 85 % throughout the entire operational period in the conventional MBR. The initial TOC concentration of the source-separated urine stood at 828 ± 37 mg/L, which subsequently decreased to 116 ± 10 mg/L in the effluent. Nevertheless, the introduction of PAC to the carrier-incorporated MBR led to 11 % increase in the TOC removal rate, reaching 96 %. This enhancement can be attributed to the substantial adsorption capacity of PAC and the augmented growth of attached heterotrophic microorganisms dedicated to organics removal (Kim & Nerenberg, 2022; Sohn et al., 2021). This increased removal efficiency is reflected in the reduced TOC concentration in the effluent, measuring 33 ± 7 mg/L. The resulting effluent quality from the MBRs distinctly emphasizes the potential for efficient stabilization of source-separated urine through biological nitrification.

3.2. Microbial diversity and richness

As an overview, 859,117 denoised sequences in total were clustered to a total of 4,543 operational taxonomic units (OTUs), which had the minimum and maximum numbers of sequences per denoised samples were 175,068 and 231,069, respectively. Table 2 summarised the sequencing depth and observed OTUs, along with the diversity estimator as Shannon index for each sample. The Shannon index serves as an indicator of microbial community diversity, with higher values signifying increased richness in diversity. Rarefaction curves were generated based on the observed OTUs to examine the difference in microbial richness (Fig. 3). The curves indicated that all samples reached a saturation plateau at a sequencing depth of approximately 110,000, confirming that the sequencing depth utilised in this study was sufficient. On the basis of Shannon index and rarefaction curves, the inoculative sludge exhibited the highest diversity level (Shannon index of 7.6) as well as the community richness. Transition to the source-separated urine as a feed water in the conventional MBR resulted in a significant reduction in both diversity and richness. However, in the MBR with the incorporation of PAC and biofilm carriers, even lower diversity and richness were observed. This result indicates that the addition of PAC and biofilm carriers was able to create a selective force that enriched certain species, making them predominant microbes in the system (Sohn et al., 2021). When comparing the biodiversity between the carrier-attached biomass and the suspended biomass, the latter exhibited slightly lower levels of biodiversity and richness. This finding aligns with results from other studies that have analysed diversity in biocake and mixed liquor samples (Wolff et al., 2021).

Table 2
Microbial diversity of each sample.

Samples[a]	Sequences	OTUs[b]	Shannon index
Seed sludge	227,868 ± 6,836	1,347 ± 40	7.6 ± 0.2
Conventional MBR	225,112 ± 6,753	1,108 ± 33	6.2 ± 0.2
PAC_Carrier_MBR	231,069 ± 6,932	1,026 ± 31	5.9 ± 0.2
Biofilm carriers	175,068 ± 5,252	1,062 ± 32	6.1 ± 0.2

[a] Seed sludge, inoculum; Conventional MBR, suspended sludge from the control MBR; PAC_Carrier_MBR, suspended sludge from the incorporated MBR; Biofilm carriers, attached sludge from the incorporated MBR.
[b] OTUs, operational taxonomic units.

3.3. Microbial composition

The functional bacterial communities were characterized by assigning the sequence reads to known phyla levels, resulting in the detection of 10 major phyla, while those with a relative abundance of less than 1 % were grouped under 'others' in Fig. 4a. In the seed sludge, the dominant sequence of four major phyla was as follows: *Chloroflexi* (22.7 %) > *Proteobacteria* (20.5 %) > *Planctomycetes* (17.2 %) > *Actinobacteria* (14.8 %). However, the introduction of source-separated urine as a feed significantly altered the dominant sequence order. In the suspended sludge of the conventional MBR and PAC incorporated MBR with carriers, *Proteobacteria* became the predominant phylum, accounting for 36.1 % and 34.2 % individually. This was followed by the subdominant phyla *Bacteroidetes* > *Actinobacteria* > *Chloroflexi*, which were present at 17.4 %, 15.0 %, and 13.9 % in the conventional MBR sludge and 18.8 %, 17.8 %, and 13.1 % in the PAC_Carriers_MBR sludge, respectively. The introduction of PAC did not change the order of dominance among microbial phyla, however it resulted in a slight increase in their abundance by 1.4–2.8 %, except for *Chloroflexi*. Conversely, the attached biomass on the biofilm carriers was largely dominated by *Proteobacteria* (48.9 %), followed by *Actinobacteria* (14.3 %) > *Firmicutes* (14.2 %) > *Bacteroidetes* (13.3 %). The presence of aforementioned dominant phyla identified in each sludge sample is in line with their ubiquity in biological reactors for wastewater treatment (Asif et al., 2020).

Proteobacteria, a gram-negative bacterial phylum, stood out as the most abundant among all the samples from the MBRs. This phylum includes diverse range of functionally important bacteria that play essential roles in the biodegradation of pollutants and the removal of organic matter (Ding et al., 2019). While *alpha* (α), *gamma* (γ), and *delta* (δ) *Proteobacteria* were detected, the majority of the *Proteobacteria* identified belonged to the γ-*Proteobacteria*, known for being pioneers in surface colonization (Deb et al., 2022). Given their high abundance in the attached sludge, this finding is consistent with their role as key players in biofilm formation and surface attachment. It was reported that *Bacteroidetes* and *Chloroflexi* are filamentous bacteria proceeding the surface colonisation and proliferation as well as degrading the cell decay materials and soluble microbial products (Feng et al., 2022). The switch to urine feeding resulted in a 2-fold increase in the abundance of *Bacteroidetes*, while the abundance of *Chloroflexi* was reduced by half. *Actinobacteria* were almost evenly abundant in all sludge samples, however the suspended sludge from the PAC-added MBR showed a higher abundance by around 3 % compared to the other samples, which may explain the better TOC removal rate in the PAC_Carriers_MBR. *Firmicutes* was the third predominant phylum in the attached biomass with 14.2 %, which was almost 12 % higher abundance than other samples. This phylum is known for high resistance against extreme environmental conditions, which is in line with the performance of PAC-Carriers_MBR showing high resistance against ammonia shock loadings (ElNaker et al., 2018).

Further taxonomic analysis down to genus level was conducted for more thorough and detailed information on the bacterial communities. Fig. 4b illustrates the top 37 bacterial genera, with individual representation limited to those with a relative abundance above 1 % in at least one sample, while the remaining genera are grouped under the category 'Others'. Following the transition to source-separated urine feeding, there were significant shifts in the dominant genera of *Proteobacteria* and *Bacteroidetes* compared to the seed sludge. In the *Proteobacteria* phylum of the seed sludge, the prevailing genera were *Hyphomicrobium* and *Candidatus_Alysiosphaera*, which belong to the class of *α-Proteobacteria*. However, in the suspended sludge from the two MBRs, *Burkholderiaceae*

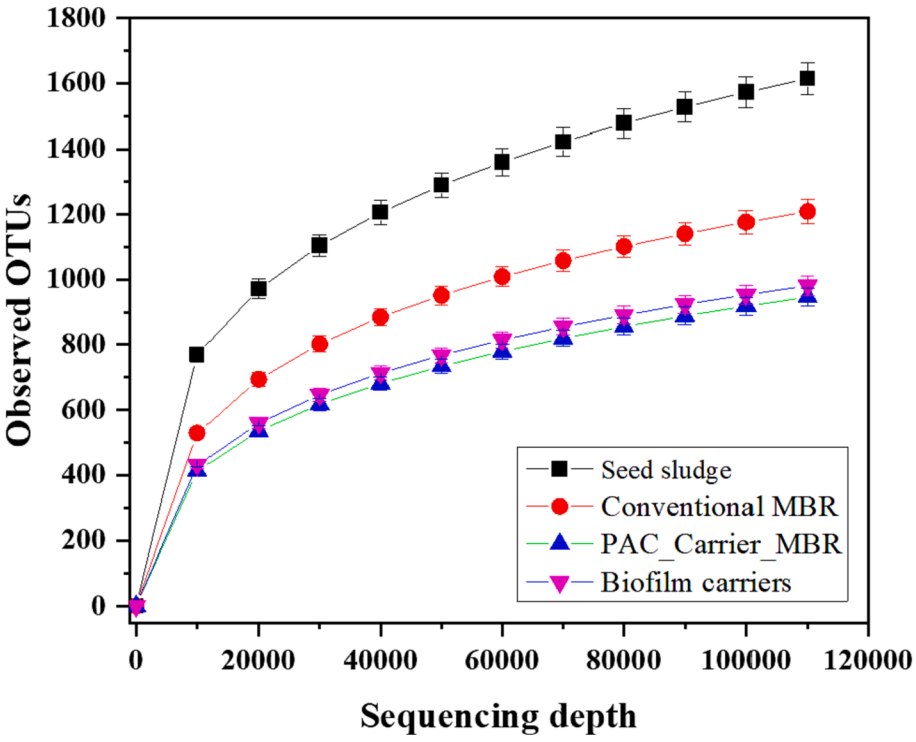

Fig. 3. Rarefaction curves of seed sludge, conventional MBR, PAC_Carrier_MBR, and biofilm carriers ("Seed sludge", inoculum; "Conventional MBR", suspended sludge from the control MBR; "PAC_Carrier_MBR", suspended sludge from the incorporated MBR; "Biofilm Carriers", attached sludge from the incorporated MBR).

and *Rhodanobacteraceae_uncultured_graftm_285* emerged as the top two abundant family, both belonging to the γ-*Proteobacteria* class. Regarding the *Bacteroidetes* phylum, the highest relative abundance in the inoculum was observed for *Ignavibacteria_SJA_28* and *Sphingobacteriales_AKYH767*, with 4.1 % and 1.4 % respectively. However, this dominance in the *Bacteroidetes* phylum shifted to *Chitinophagaceae* at 7.5 % and *Ornithobacterium* at 4.7 % in the PAC-incorporated sludge, which showed slightly higher abundance than the control MBR (6.9 % and 3.7 %, individually). Consequently, the addition of PAC to the suspended sludge did not significantly alter the relative abundance of genera compared to the one without PAC. However, a noteworthy increase in abundance was observed in the genus *Mycobacterium* from the phylum *Actinobacteria*, which was 2.2 % higher than the conventional MBR.

Similarly, in the immobilised biomass, *Mycobacterium* accounted for the highest abundance among all genera, with a relative abundance of 10.1 %. This result aligns with previous studies showing *Mycobacterium* as the most abundant genus in attached biomass, primarily associated with biofilm formation (De Sotto et al., 2018). Given the highest abundance of the phylum *Proteobacteria* in the immobilised biomass, the following four abundant genera, which are *Nitrosococcaceae* (9.9 %), *Comamonas* (8.2 %), *Ottowia* (4.2 %), and *Castellaniella* (3.9 %), belong to the phylum *Proteobacteria*. *Comamonas* has been reported as one of the heterotrophic nitrifiers, indicating its significance in environments with high ammonium strength wastewater and the degradation of recalcitrant pollutants (Asif et al., 2020; Nguyen et al., 2020). While other sludge samples showed a low abundance of around 0.2 %, the attached biomass exhibited a significantly higher abundance of *Bacillaceae*, *Dolosicoccus*, and *Clostridiales_Family_XI_W5053* by 2.5–3.6 %, all of which belong to the phylum *Firmicutes*.

Overall, the observed shifts in dominant genera following the introduction of source-separated urine reveal a significant influence on the microbial community within the MBR system, particularly in terms of the abundance and distribution of key functional genera related to biodegradation and pollutant removal. Moreover, the presence and prevalence of specific genera in the attached sludge emphasize their potential importance in improving the overall performance and efficiency of urine treatment in MBR. These findings highlight the importance of understanding the microbial dynamics in both the suspended and attached sludge and leveraging this knowledge to optimize urine MBR processes and enhance sustainable nutrients recovery strategies.

3.4. Enrichment of nitrifying bacteria

Taking a closer look at the enriched nitrifying bacteria present in both suspended and attached growth within the urine treating MBR is crucial for gaining insights to the relationship between these two growth forms and their potential impact on nitrification performance, which is a fundamental objective of this MBR system. As such, this section focused on investigating the dynamics of nitrifying bacteria and their enrichments following the source-separated urine feeding, while considering the influence of different growth types on the nitrification process. As depicted in Fig. 4a, the relative abundance of the *Nitrospirae* phylum significantly increased from the inoculative sludge (0.2 %) to the suspended sludge in both the conventional MBR (4.2 %) and PAC-incorporated MBR (4.6 %). The dominant genus within the phylum *Nitrospirae* was *Nitrospira*, with relative abundance of 3.5 % and 3.9 % in the conventional and PAC-added sludge, respectively, showing a slight increase of 0.4 % with PAC addition (Du et al., 2017). The prevalence of *Nitrospira* facilitated efficient nitrite oxidation to nitrate in both MBRs. In contrast, *Nitrobacter*, another well-known NOB genus belonging to the *Proteobacteria*, was rarely detected in both MBRs. This scarcity might be attributed not only to its lower substrate affinity compared to *Nitrospira* but also to the unfavourable conditions for its enrichment, likely due to low nitrite concentrations during stable MBR operation (Gu et al., 2022). Regarding AOB, *Nitrosococcaceae* from *Proteobacteria* was the dominant functional family in both MBRs, with relative abundances of 1.0 % and 1.5 %, respectively. Although *Nitrosomonas* is commonly known as the main AOB in bioreactors, it was not detected in the suspended sludge acclimated with urine, whereas a small abundance of 0.1 % was observed in the seed sludge. The relative abundance of nitrifying

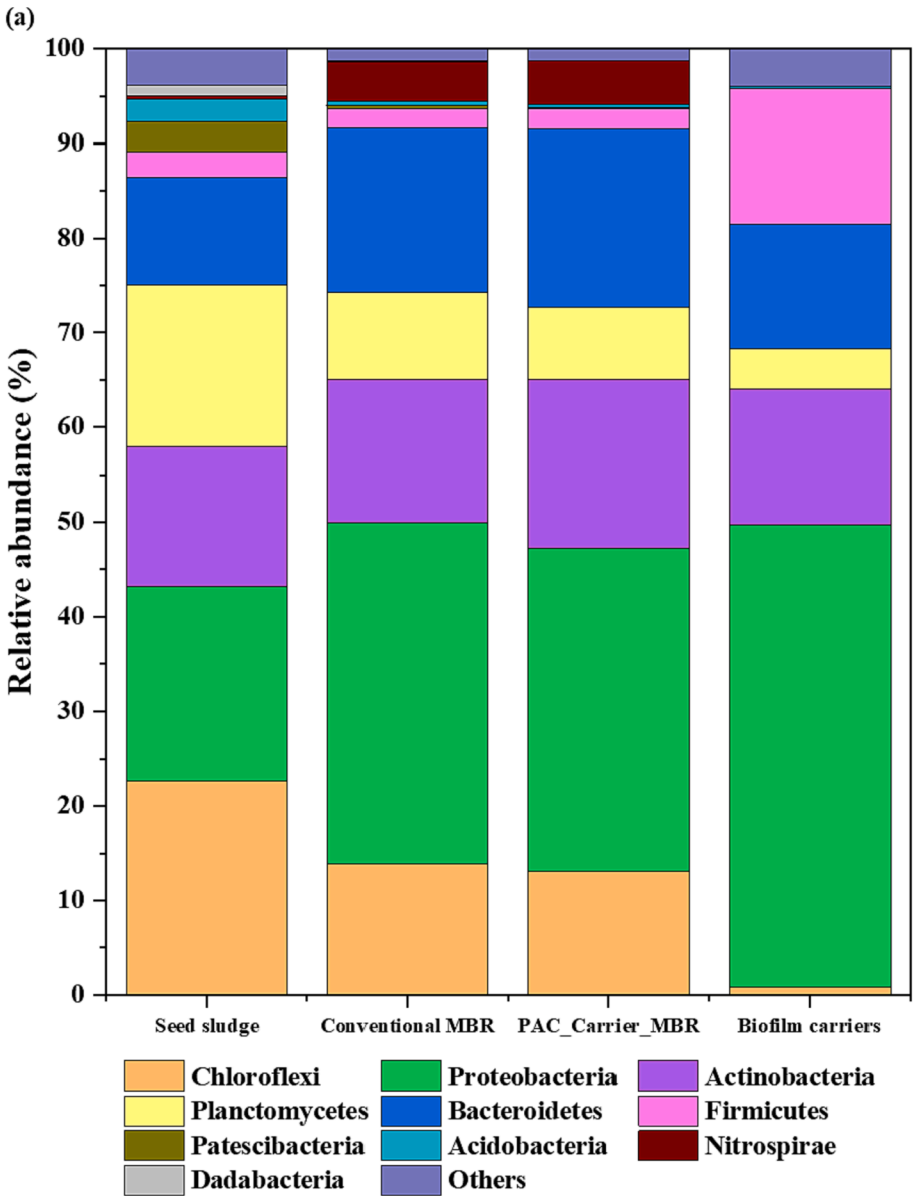

Fig. 4. Microbial composition at (a) phyla level and (b) genera level in each sample. Only phyla and genera with relative abundance >1 % in at least one sample are shown individually, the rest are grouped into "Others".

bacterial genera is illustrated in Fig. 5a.

The incorporation of biofilm carriers resulted in a significant shift in the relative abundances of nitrifying bacterial genera. In the attached sludge, the most dominant AOB family was *Nitrosococcaceae*, with a relative abundance of 10.0 %, while 0.1 % of the community was observed to be *Nitrosomonas*. Interestingly, no *Nitrospira* was detected on the biocarriers, whereas *Nitrobacter* was rarely presented, making it the only NOB genus detected. This phenomenon could be attributed to the depth of the biofilms which closely correlates with the growth of AOB and NOB. In this context, AOB tend to dominate the upper layer of biofilms, while NOB are more prevalent in the deeper layers. The carriers used in this study, as per prior research, formed biofilms exclusively in small cavities of the porous phase, maintaining the thickness at around 0.8–1.2 mm when saturated (Al-Amshawee & Yunus, 2021). Considering the amount of immobilised biomass attached on the saturated biofilm carriers, in this study, remained at around 2.9 g throughout the operation, indicating effective thickness maintenance. This thin biofilm layer has facilitated the proliferation of AOB, potentially suppressing the growth of NOB due to their relatively less protection by the AOB-rich upper layer (Piculell et al., 2016). The fact that the biofilm carriers effectively enriched the *Nitrosococcaceae* belonging to the γ-*Proteobacteria*, is likely due to their filamentous properties, which facilitated their thriving on the carriers' surface. Additionally, it implies that the biofilm carriers could provide a favourable environment for slow-growing autotrophic AOB for their enrichment. While AOB belonging to *Nitrosococcaceae*, for example, *Nitrosococcus* and *Ca.* Nitrosoglobus, is rarely found in conventional WWTPs, it has been frequently reported as a predominant AOB in saline aquaculture systems (Brescia et al., 2023; Li et al., 2022; Lo et al., 2022). This can be attributed to its prominent role as a primary AOB in this research, considering that source-separated urine exhibits salinity levels 5 to 30 times higher than those in typical municipal wastewater. Consequently, the enrichment of the specific AOB, *Nitrosococcaceae* on the carriers played a crucial role in promoting the MBR performance. Overall, *Nitrospira* emerged as the predominant NOB genus in the suspended sludge, showing an almost 20-fold higher relative abundance

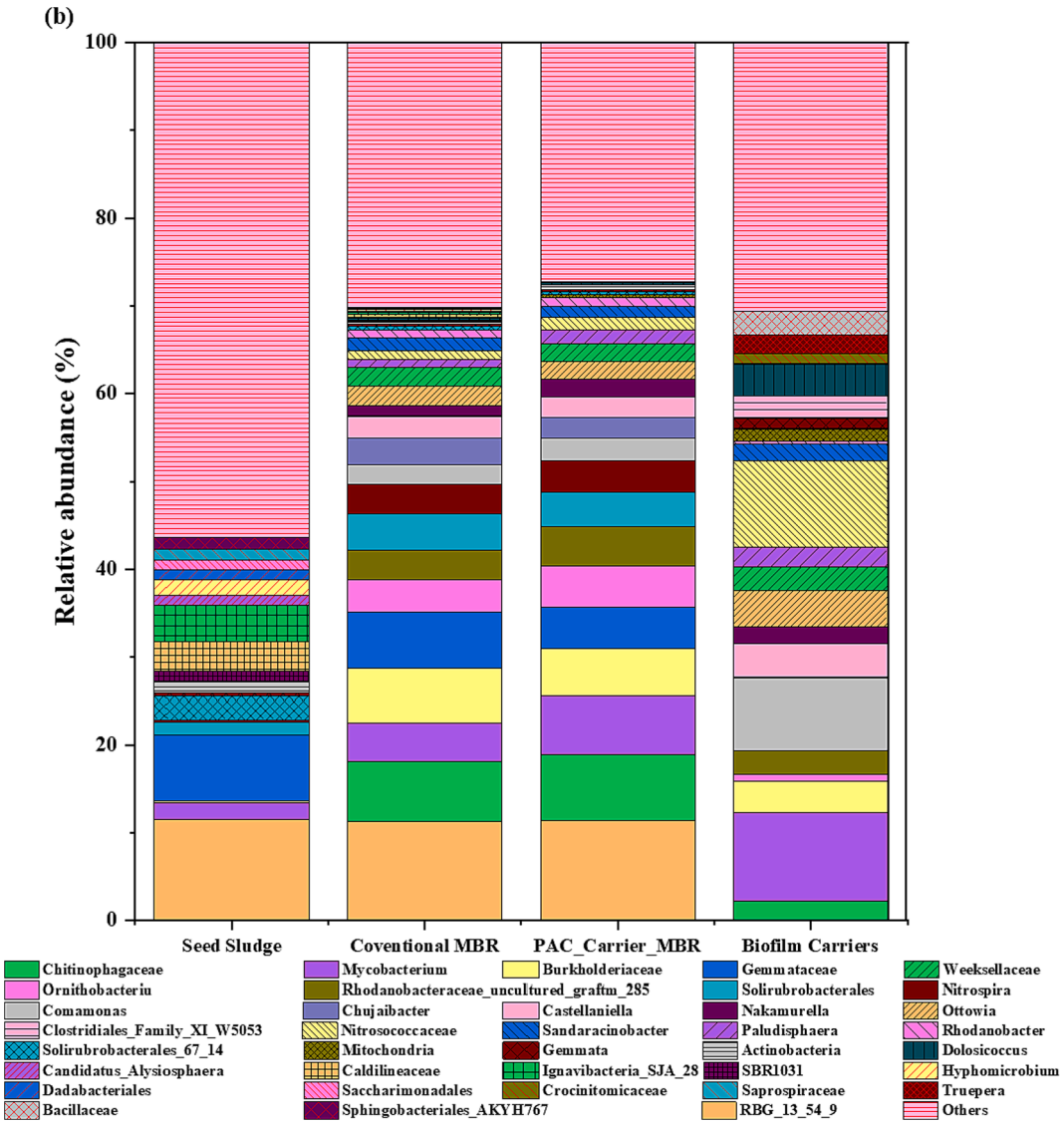

Fig. 4. (*continued*).

than that of the inoculative sludge. On the other hand, the AOB genus *Nitrosococcaceae* was predominantly enriched in the attached biomass by almost 7 times compared to that in the suspended sludge.

In Fig. 5b, the log2-fold difference in the relative abundances of nitrifying genera between the conventional MBR and MBR with PAC and biofilm carriers is illustrated. This represents the relative enrichments of each genus in both suspended and attached sludge in PAC_Carriers_MBR compared to the suspended sludge in the conventional MBR. Notably, the AOB genera *Nitrosomonas* and *Nitrosococcaceae* were significantly enriched, likely attributed to the addition of biofilm carriers, as discussed above. In terms of NOB, although *Nitrospira* showed a high relative abundance, the difference in enrichment between the two reactors was not substantial. Despite the initial low relative abundances of *Nitrobacter* and *Nitrosomonas* in both reactors, the introduction of PAC and biofilm carriers led to a significant increase in their abundances. This enhancement suggests the feasibility of achieving higher nitrification rates and lower HRT within the same reactor volume, indicating the potential for more compact and highly efficient MBR system for source-separated urine nitrification.

In conclusion, the integrated application of PAC and biofilm carriers in MBR system synergistically enhanced key functional microorganisms, leading to improved nitrification and organics removal. This achievement aligns with the primary objective of producing a safe and odourless liquid fertiliser within a reduced HRT. Firstly, the substantial adsorption capacity of PAC contributed to a significant increase in the removal of organic matter. The subsequent aggregation of microorganisms fostered the formation of biologically activated carbon (BAC), facilitating further degradation of organics (Sohn et al., 2021). Microbial composition analysis revealed that PAC-incorporated suspended sludge exhibited an approximately 3 % higher abundance of functional bacteria involved in organic degradation. However, regarding nitrifying bacteria, biofilm carriers showed a remarkable seven-fold enrichment solely in AOB compared to suspended sludge, while PAC addition enriched both AOB and NOB by approximately 1 %. Consequently, this combined enrichment strategy within the MBR system significantly enhanced both AOB and NOB, contributing to improved nitrification rates and a reduction in HRT. In alignment with the main goal of this MBR system, which is the production of effective NPK liquid fertiliser with a minimized footprint and energy consumption, the incorporation of PAC and biofilm carriers played a pivotal role, manifesting synergistic effects to attain this objective. In particular, this study demonstrated a substantial enrichment of nitrifying bacteria compared to the inoculum derived from a conventional wastewater treatment plant. These findings illuminate the promising prospect of establishing an infrastructure for urine

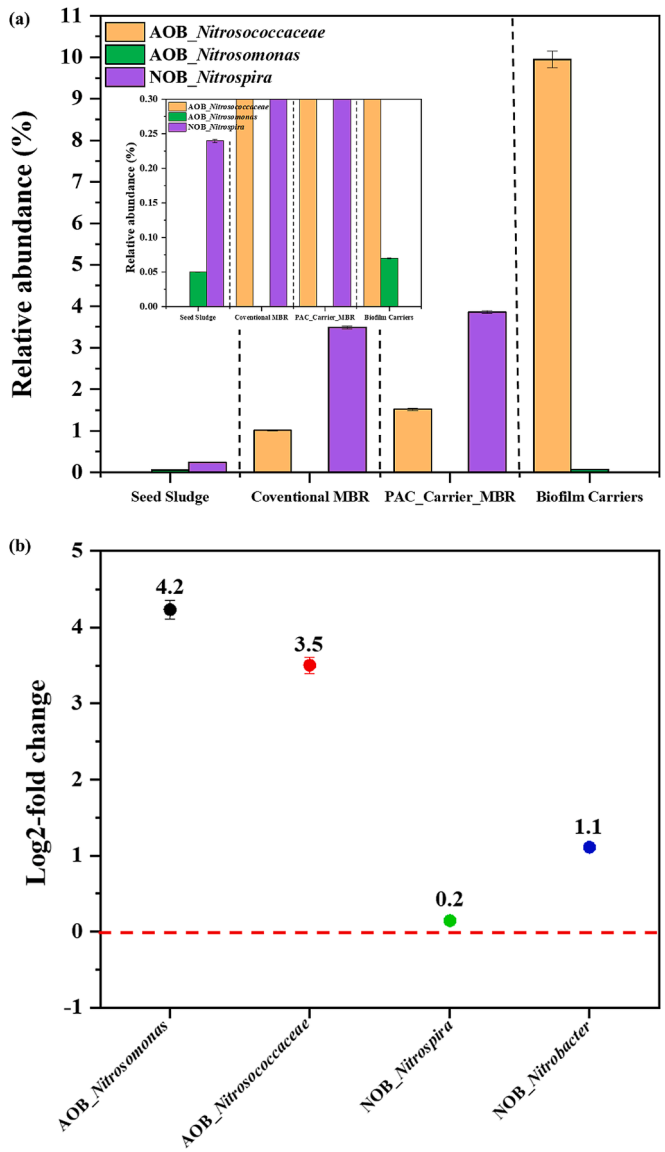

Fig. 5. (a) The relative abundance of ammonia-oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) genera; and (b) log2-fold change in the relative abundances in the incorporated MBR vs Conventional MBR.

source-separation and on-site nutrient recovery, utilizing this efficient and compact MBR system. These advancements represent a step towards a nutrient circular economy.

4. Conclusion

A comprehensive analysis of the microbial communities in the incorporated MBR, compared to the conventional MBR, was conducted in this study to demonstrate the abundance and distribution of key functional genera in achieving efficient MBR performance treating source-separated urine. The findings highlighted the importance of understanding the association between suspended and attached growth in the incorporated MBR, as these different forms of growth exhibited varying microbial dynamics. The enriched nitrifying bacteria played a vital role in enhancing the nitrification performance in the incorporated MBR, presenting promising prospects for the development of compact and efficient MBR systems for source-separated urine treatment.

CRediT authorship contribution statement

Weonjung Sohn: Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jiaxi Jiang:** Writing – review & editing. **Zicheng Su:** Data curation, Formal analysis, Writing – review & editing. **Min Zheng:** Writing – review & editing, Validation, Data curation. **Qilin Wang:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Sherub Phuntsho:** Writing – review & editing, Formal analysis, Data curation. **Ho Kyong Shon:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2024.130462.

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