Activated sludge diffusion for efficient simultaneous treatment of municipal wastewater and odor in a membrane bioreactor

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Abstract: Although activated sludge (AS) diffusion is promising for odor control, disagreement still exists on its treatment efficiency of varying odorants, and its microbiological mechanisms remain largely unknown. Herein, we investigated the deodorization efficiency, wastewater treatment performance, and microbiological and ecological effects of AS diffusion in two in situ membrane bioreactors (MBRs). AS diffusion removed ≥ 94.7% of H₂S and 100% of NH₃, while other odorants were released at low concentrations. The odor-diffused system also achieved appreciable wastewater treatment performance (effluent phosphate at 0.13 ± 0.12 mg/L, COD at 12.1 ± 1.8 mg/L, and total nitrogen at 5.9 ± 1.8 mg-N/L), while membrane fouling was mitigated. Notably, influent wastewater substrates and nutrients, other than MBR system settings, significantly affected system performance, main functional taxa and bacterial community dynamics. Ecological null model and network analysis revealed that odor diffusion strengthened the niche-based deterministic processes (i.e., environmental selection) and caused more negative and intense microbial interactions. Organic competition was enhanced, and some hydrolytic bacteria correspondingly became keystone taxa in the odor-diffused system. The findings provide valuable guidance in establishing efficient AS systems for integrated wastewater and odor treatment.

Response to Reviewers: Dear Prof. Li,

We would like to submit our updated manuscript entitled “Activated sludge diffusion for efficient simultaneous treatment of municipal wastewater and odor in a membrane bioreactor” for publication as a research article in Chemical Engineering Journal. We thank the Editorial Board of Chemical Engineering Journal for providing a revision opportunity to improve the quality of our manuscript (ID: CEJ-D-20-16556). We also highly appreciate the valuable comments provided by the Editor and three Reviewers. All the comments and suggestions have been carefully considered and addressed point-by-point in the revised manuscript. Our revisions are highlighted in yellow in the manuscript. The revisions improved the manuscript readability and highlighted the importance and the discovery of activated sludge diffusion in odor control. Please kindly find the details of the revisions in our response. The comments from the reviewers are in black, responses from the authors are in blue, and revisions to the manuscript are in red.

We hope these modifications will meet your and the reviewers’ requirements. Also, we look forward to hearing from you in due time regarding our submission and to respond to any further questions and comments you may have.
Activated sludge diffusion for efficient simultaneous treatment of municipal wastewater and odor in a membrane bioreactor

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Abstract

Although activated sludge (AS) diffusion is promising for odor control, disagreement still exists on its treatment efficiency of varying odorants, and its microbiological mechanisms remain largely unknown. Herein, we investigated the deodorization efficiency, wastewater treatment performance, and microbiological and ecological effects of AS diffusion in two in situ membrane bioreactors (MBRs). AS diffusion removed ≥ 94.7% of H₂S and 100% of NH₃, while other odorants were released at low concentrations. The odor-diffused system also achieved appreciable wastewater treatment performance (effluent phosphate at 0.13 ± 0.12 mg/L, COD at 12.1 ± 1.8 mg/L, and total nitrogen at 5.9 ± 1.8 mg-N/L), while membrane fouling was mitigated. Notably, influent wastewater substrates and nutrients, other than MBR system settings, significantly affected system performance, main functional taxa and bacterial community dynamics. Ecological null model and network analysis revealed that odor diffusion strengthened the niche-based deterministic processes (i.e., environmental selection) and caused more negative and intense microbial interactions. Organic competition was enhanced, and some hydrolytic bacteria correspondingly became keystone taxa in the odor-diffused system. The findings provide valuable guidance in establishing efficient AS systems for integrated wastewater and odor treatment.

Keywords: Deodorization, Wastewater treatment, Microbial community structure, Keystone species, Ecological mechanism
1. Introduction

Due to the negative impacts on the environment and quality of life in neighboring communities, odors released from municipal wastewater treatment plants (WWTPs) emerge as a growing problem, both ecologically and socially [1-3]. As a group of compounds, unpleasant odors are regarded as harmful atmospheric pollutants, which feature low olfactory thresholds but nuisance and adverse health effects [4]. Volatile odors, such as hydrogen sulfide, are corrosion culprits of pipes and other wastewater processing facilities [5]. Therefore, stringent odor-based environmental regulations have become more widely adopted.

To meet environmental regulations, a range of physical-chemical and biological techniques have been developed to control odor emissions in WWTPs. Biotechnologies are more widely adopted because of their robust, economic, and environmentally friendly properties, of which the most commonly implemented technologies are biofilters and biotrickling filters [6, 7]. However, long-term applications of media-based biofilters and biotrickling filters cannot avoid their limitations such as packing media compaction, accumulation of toxic metabolites, as well as sophisticated pH and moisture control [4, 8, 9].

In particular, emerging odor treatment technologies such as liquid-based activated sludge (AS) diffusion, which can avoid these problems, are gaining increasing attention. The malodorous emissions are directly sparged into the sludge of aeration tank where they can be adsorbed or
absorbed and subsequently biodegraded. This technology uses existing facilities and equipment and harbors obvious merits in capital expenditure, maintenance and operation [7, 10].

AS diffusion utilizes diverse sludge microorganisms for deodorization, and the robustness and efficiency of odor abatement biotechnology have been verified [11, 12]. Lebrero et al conducted a comparative deodorization assessment of lab-scale biofiltration and AS diffusion bioreactors and confirmed the robustness and efficiency of AS diffusion for odor treatment [9, 11]. Microbial community based and deodorization enhancement based studies regarding AS diffusion applications were also reported in recent years [8, 13]. However, due to the improper application of AS diffusion and insufficient mechanism investigations, its broad application is still limited. Issues such as the insufficient treatment of odorous volatile organic compounds (VOCs) [11], the disagreement regarding their effects on field existing wastewater treatment [12], and the very limited understanding of microbial mechanisms (e.g., microbial community structure, interaction patterns, and assembly processes) governing odor removal [8, 13], still exist. Membrane bioreactor (MBR) systems feature high biomass and diverse functional microorganisms [14, 15], which can strengthen the AS adsorption and degradation capability of odorants. Thus, AS diffusion coupled with MBR systems is promising for efficient simultaneous treatment of wastewater and odor. Recently, statistical tools, such as null models, were employed to decipher the assembly mechanisms of AS communities [16, 17], while ecological network analysis was reported to illustrate the possible species interactions in
various systems [18, 19]. Currently, very limited studies investigated the diversity and
dynamics of microbial communities governing the *in situ* simultaneous odor and wastewater
treatment in an MBR system [8, 9, 13]. Studies tackling the complex and dynamic ecological
mechanisms in deodorization-involved MBR systems, such as the bacterial assembly patterns
and the roles of keystone species in microbial succession, are more rarely documented.

This study aimed to comparatively evaluate the impact of AS diffusion on the
simultaneous treatment of odor and wastewater from both engineering, microbiological, and
ecological perspectives. Two MBRs with and without odor involvement were established in an
*in situ* WWTP to reveal the real odor and wastewater treatment conditions. The effectiveness
of odor removal and the effect of odor diffusion on wastewater treatment, microbial community
diversity and dynamics, along with the associated ecological parameters, during the operation
were evaluated. Specifically, the deterministic and/or stochastic assembly process in shaping
AS communities, the interplay patterns and the ecological roles of coexisting species were
unraveled using diverse statistical approaches to elucidate microbiological and ecological
mechanisms during the simultaneous odor and wastewater treatment.
2. Materials and methods

2.1 Reactor configuration and operation

Two identical anoxic-oxic membrane bioreactors (AO-MBRs) were used in this study (24 L each), in which the anoxic tank was separated into two tanks A1 and A2. Thus, the volume ratio of tanks A1, A2, and O was 1:1:2. For comparison purposes, one system is used as a blank control (MBR-B) only for treating wastewater, and the other is used for simultaneous odor and wastewater treatment (MBR-E). The integrated system was thoroughly mixed, and sludge from tank O was recycled to tank A1 with a return ratio of 200%. The municipal wastewater from Xinhua WWTP (Guangzhou, China) was used as the influent at a flow rate of 50mL/min. Odors from the unit of fine filter in WWTP were collected into Tedlar PVF bags (500 L, Dalian Hede Technologies LTD., Dalian, China) for short-term storage and then diffused into the reactor MBR-E. Odors were diffused into tank A2 of MBR-E at flow rates of 0, 30, 90, and 150 mL/min at different phases (the 4 phases lasted 24, 34, 34, and 38 days, respectively). The odor/wastewater ratio was determined from the treatment capacity of wastewater in the plant (100,000 m³/d) and the elimination capacity of No. 2 biofilter (6000 m³/h), which was operated for abatement of odors collected from the filters, grit chambers and sludge processing units. Tank A2 in both systems was sealed to favor the sampling of treated air samples by a Tedlar PVF bag (5 L) and the air sampling was conducted at least every other day. The two reactors were operated for 130 days with 20 days and 8 hours as the solids retention time (SRT) and
hydraulic retention time (HRT), respectively. The temperature in two MBR systems was identical under real operational conditions (20.4~29.0°C). Dissolved oxygen (DO) concentrations were kept in the range of 0.5 to 2 mg/L despite the influent fluctuations. Other operational parameters, such as transmembrane pressure (TMP) and flux of membrane module, are presented on page S2 of the SI.

2.2 Physical and chemical analyses

The redox potential (Eh) was measured using a waterproof ORP probe with a platinum band electrode (ORP30, Clean L’eau, USA). The DO, temperature, and pH of the bulk liquid were measured through a DO meter (Oxi 3310, WTW, Germany) and a multimeter (HQ440D Benchtop multimeter, HACH, USA). The concentrations of sulfide, total phosphorus (TP), total nitrogen (TN), ammonium (NH$_4$-N), nitrate (NO$_3$-N), nitrite (NO$_2$-N), chemical oxygen demand (COD), mixed liquor suspended solids (MLSS) and volatile suspended solids (MLVSS), coupled with the indicator of sludge volume index (SVI), were determined following the Standard Methods [20]. The NH$_3$ and H$_2$S concentrations of MBR-E and MBR-B (as a blank control) in air samples were measured by an ammonia analyzer (GT-903-NH$_3$, Shenzhen Korno Electronic Technology Co., Ltd., China) and a multi-gas analyzer (BIOGAS 5000, Geotechnical Instruments Ltd., UK), respectively. The biological nutrient removal (BNR) activities, including specific nitrification rates (SNRs), specific denitrification rates (SDNRs),
specific phosphorus uptake rates (SPURs) and specific phosphorus release rates (SPRRs), were determined using batch tests as previously reported [21].

The odor concentration was determined using the triangle odor bag method [22]. Carbon disulfide, methanethiol, dimethyl sulfide, dimethyl disulfide, trimethylamine, styrene, and 34 other VOCs in gas samples were pre-concentrated with a three-stage cold trap concentrator (TD-100-xr) and then determined following a gas chromatography-mass spectrometry (GC-MS) (ISQ7000-TRACE1300, Thermo Fisher Scientific, USA) profiling protocol (the operating conditions were available in SI) [23]. Polysaccharides (PS) and proteins (PN) in the soluble microbial product (SMP) and extracellular polymeric substance (EPS) were determined by the phenol-sulfuric acid method [24] and Lowry method [25], respectively. All analyses were run at least in duplicate, and the results of measurements agreed to within 95% confidence.

2.3 DNA extraction, PCR amplification, sequencing, and data processing

Based on the comparative microbiological characterization needs, 1, 5, 4, 4, and 1, 6, 5, 5 sludge samples at different stages (flow rates of 0, 30, 90, and 150 mL/min) were collected periodically from the MBR-B (n=14) and MBR-E (n=17) systems, respectively. Microbial DNA was extracted using an E.Z.N.A. soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) and further quantitatively determined. The V3-V4 hypervariable regions of the 16S rRNA gene were amplified with primers 338F and 806R [26]. PCRs were performed as described in a previous study [27]. The purified amplicons were pooled in equimolar and paired-end (2 ×300)
sequencing on an Illumina MiSeq platform (Illumina, San Diego, USA) at Shanghai Personal Biotechnology Co., Ltd. The raw reads were available under accession PRJNA624976 in the NCBI Sequence Read Archive database. Raw fastq files were demultiplexed, quality-filtered by Trimmomatic and merged by FLASH following the guidelines described by Peng et al [28]. The process resulted in a total of 1,363,843 sequences for 31 AS samples, and the minimum sequencing depth of 31,048 was adopted for normalization. Other data processing procedures also followed the protocols of Peng et al [28].

2.4 Statistical analysis

A paired t-test was used to compare the differences of paired physico-chemical measurements in the MBRs. The indexes Chao, ACE, Shannon, and Simpson values were adopted in the α-diversity analysis. At the operational taxonomic unit (OTU) level, principal coordinate analysis (PCoA) based on the Bray-Curtis distance metric was conducted on QIIME 1.9.1 to examine the sample clustered patterns [26]. Meanwhile, permutational multivariate analysis of variance using distance matrices (Adonis) and analysis of similarity (ANOSIM) were performed to reflect the variations in bacterial community structures. Canoco 5 was used for principal component analysis (PCA) to illustrate the correlations of environmental and operating factors with system performance. Redundancy analysis (RDA) was conducted to examine the influence of reactor settings on the bacterial community structures using the vegan package of R 3.6.2.
2.5 Null model and molecular ecological network analysis

A phylogenetic β-diversity framework was adopted to quantify the relative influence of deterministic and stochastic processes (i.e., environmental selection, homogenizing dispersal, dispersal limitation, and ecological drift) on microbial community assemblage [29]. The relative proportion of four ecological processes in shaping MBR-B (14 samples) and MBR-E (17 samples) sludge communities was assessed by calculating the abundance-weighted β-nearest taxon distance (βNTI) and the Raup–Crick metric (RC_{Bray}). The phylogenetic molecular ecological network (pMEN) based on random matrix theory (RMT) was constructed through the online MENA pipeline (http://ieg4.rccc.ou.edu/mena/). Two networks using the OTU tables of MBR-B and MBR-E bioreactors were constructed according to previous studies [18]. Absolute correlation values greater than 0.89 and 0.90 ($P < 0.05$) for each pair of nodes were considered significant in the MBR-B and MBR-E communities, respectively. The networks were visualized by Gephi (WebAtlas, Paris, France) as described before [30]. To assess the statistical significance of network indexes, a total of 100 randomized networks were generated for comparison with the topology of the real network. Two network indicators were used to illustrate the topological role of each node (OTU): within-module degree $Z$ and among-module connectivity $P$. For assorting species (i.e., peripherals, connectors, module hubs, and network hubs), the values of 2.5 and 0.62 were adopted as the $Z$ and $P$ thresholds, respectively [31].
3. Results and discussion

3.1 Odor removal performance

The concentrations of two representative odors, NH₃ and H₂S, were measured regularly. As shown in Fig. 1a, 100% NH₃ was removed under all experimental conditions regardless of the inlet concentrations (215~2051 ppm) and odor flow rates. Similarly, the removal efficiencies of H₂S were all over 94.7% and occasionally reached 100%. H₂S was either completely removed or released below 1.5 ppm at inlet concentrations of 12~135 ppm. Additionally, no aqueous sulfide was detected in any tank of the MBRs. Previous studies of AS diffusion also found similar H₂S removal efficiencies (>94% or approximately 100%), while a low outlet H₂S concentration was maintained [12, 32]. For instance, the results from on-site investigation are comparable with the results of Rodríguez et al [13] in an AS diffusion bioreactor treating a synthetic malodorous emission containing H₂S (16.9~23.8 mg/m³) and other VOCs.

(Insert Figure 1 here)

To comprehensively evaluate the AS performance in odor abatement, the odor samples in MBR-E before and after system treatment were collected and analyzed on Day 102 and Day 126 (during the stage at a flow rate of 150 mL/min). As revealed by Table 1 and Table S1, the major odors entering the MBR were carbon disulfide, styrene, toluene, ethylbenzene, xylene
isomers, tetrachloroethylene, trichloromethane, and other VOCs. The removal efficiencies of odors determined by the triangle odor bag method were over 80%, depending on inlet odor concentrations and AS properties (Table 1). As the dominating odor component, carbon disulfide (sweet chloroform-like odor) was removed at high efficiencies (>98%). The removal efficiencies of VOCs varied (Fig. 1b), whereas the outlet VOCs maintained relatively low concentrations. For instance, the typical VOC in WWTPs, i.e., styrene, was observed in trace levels (≤0.0012 mg/m³) after the AS treatment. Similar results were obtained for benzene, toluene, ethylbenzene and the three xylene isomers (BTEX) in Table S1. In particular, the concentrations of some odor compounds, such as benzene, 1,2-dichloropropane and tetrachloroethylene, increased slightly (all ≤0.004 mg/m³) after AS diffusion (Fig. 1b and Table S1). They are believed to be the degradation intermediates of other odorants or organics (such as benzene from other BTEX species) during the short odor retention time.

(Insert Table 1 here)

Overall, high removal efficiencies of the major odorants NH₃, H₂S, and carbon disulfide were constantly achieved, while other outlet VOCs maintained relatively low concentrations after AS diffusion. The efficiency and robustness of the technology were consistent with previous lab or field investigations [7, 11, 12, 33]. Odorants are first adsorbed by AS, and the dissolved or suspended contaminants are then converted into cell mass, carbon dioxide, energy and other byproducts through microorganisms [6, 34]. The mass transfer and biodegradation
are two main limiting steps for odor removal. Although some odorants (e.g., carbon disulfide and other VOCs) are only slightly water-soluble, the high MLSS concentrations in the MBR systems improve the AS viscosity [35] and very possibly enhance the adsorption ability of odorants. The odorants adsorbed by sludge will be either digested anaerobically (e.g., by yeast) or aerobically (with oxygen) by the diverse microorganisms in tank A2 or tank O. Furthermore, the diverse and abundant microorganisms in MBR can result in appreciable odor degradation capability and high resistance towards in situ fluctuations [13, 36]. For instance, sulfide-oxidizing bacteria (SOB) such as Thauera, Thiothrix and Thiobacillus found in sludge communities (presented in Section 3.3) were functional for H$_2$S removal [37-39]. These are the two main reasons why the MBR system can reach appreciable VOCs removal results when compared with other air diffusion reactors for odor abatement [8, 13].

3.2 Reactor operation performance

3.2.1 Organics and nutrients removal

The effect of odor involvement on reactor performance was evaluated. As shown in Fig. 2, the influent ammonium and TN concentrations of the WWTP fluctuated in the range of 4.6~33.8 mg-N/L and 11.6~35.2 mg-N/L, respectively, during the study period. The variations in local weather conditions (e.g., heavy rain and temperature variations) and diversity of sewage sources were the main causes. However, both MBRs achieved high ammonium removal of 98~100% under normal operating conditions (Fig. 2a). The average effluent nitrate
concentrations of MBR-E and MBR-B were 5.2 ± 1.4 and 4.6 ± 1.4 mg-N/L ($P < 0.05$), while the TN values were 5.6 ± 1.8 and 5.9 ± 1.8 mg-N/L ($P > 0.05$), respectively. Considering the nitrogen introduction induced by odor diffusion (NH$_3$) in MBR-E, the corresponding TN removal rates were 73.2% and 72.6% ($P > 0.05$), respectively. In addition, both MBR-B and MBR-E maintained stable and highly efficient phosphorus removal performance (0.13 ± 0.12 and 0.18 ± 0.18 mg/L in the effluent, respectively), although influent TP fluctuated from 1.4 to 4.3 mg/L (Fig. 2d). PCA results showed that influent substrates were positively associated with the amounts of nitrogen species but negatively linked to phosphate levels in the effluent (Fig. S1).

As seen in Fig. S2, COD was also efficiently removed in MBR-B and MBR-E (13.1 ± 2.2 and 12.1 ± 1.8 mg/L, respectively) when the influent levels ranged from 87.2 to 353.5 mg/L. Although the settling ability of sludge gradually increased, no significant difference was found in the SVI, nitrite, MLSS, and MLVSS concentrations ($P > 0.05$) between the two MBRs (Fig. S2). As indicated by the nutrient distribution patterns in different tanks (Fig. S3), marginally higher levels of nitrite but lower levels of nitrate, ammonium, and TP were observed in MBR effluent compared to the AS supernatant. Denitrification and phosphorus release mainly occurred in tank A1 of the two MBRs. In tank A2 of MBR-E, partial phosphorus uptake and nitrification occurred (Figs. S1 and S3). The results indicated that adverse effects such as
deterioration of sludge settleability [32] and interrupted nitrification by odor diffusion [40] were not observed.

### 3.2.2 Specific activities of microorganisms responsible for BNR

BNR activities were also evaluated by a series of batch tests using bulk sludge from both MBRs. As revealed by Fig. S4, both the phosphorus release and uptake rates increased with time (Fig. S4a), in which the difference in SPRRs was insignificant. The released phosphorus due to acetate addition in the anaerobic period was assimilated in the following aeration period (Fig. S4e). The SPURs of MBR-B were marginally higher than those of MBR-E, which was consistent with the finding of relatively lower effluent TP levels in MBR-B (Fig. 2d). As expected, the bulk sludge of the two MBRs harbored similar nitrification and denitrification activities. Thus, the relatively elevated nitrate concentrations in MBR-E effluent (Fig. 2b) were linked to the shorter denitrifying time compared to MBR-B.

### 3.2.3 Membrane fouling characteristics

The membrane fouling performance was indicated by the TMP development over the study period. As revealed by Fig. S5, similar fouling behavior was observed in both MBRs during the early two rounds of the fouling period. Afterwards, with the stabilization of reactor performance, the fouling rates of MBR-B became slower (fouling interval of 56 days), while no further membrane fouling of MBR-E occurred. The prolonged fouling interval was believed to be linked to the operating conditions (e.g., membrane flux, aeration condition, and
temperature), sludge properties and influent wastewater characteristics [21, 41]. Notably, the odor diffusion in MBR-E (especially at flow rates of 90 and 150 mL/min) was one of the main causes. As revealed by the PS and PN concentrations at different treatment stages (Fig. S6), both the PS and PN contents of SMP exhibited a decreasing trend, although the PS and PN contents of EPS remained relatively stable. Notably, both PS and PN concentrations of SMP and EPS in MBR-E were generally lower than those of MBR-B, especially after reactor start-up. This trend is consistent with the relatively lower COD concentrations in MBR-E system. EPS contents are directly linked with the properties (such as structure and porosity) of the bio-fouling layer developed on the membrane [42], which are very possibly reduced owing to the changes of microbial community structure (e.g. more functional organic degrading bacteria) induced by odor diffusion.

### 3.3 Characterization of microbial community structure

The sludge system in wastewater treatment process will enrich selected functional microorganisms, thus accelerating microbial activities in the sludge community and enabling the removal of diverse and complex contaminants in the sludge system. Accordingly, the microbial community structures were characterized in different aspects.

#### 3.3.1 Bacterial community diversity and composition

Bacterial community richness, evenness and temporal changes in the two MBR systems were revealed through α- and β-diversity indexes. During the early three stages, the α-diversity
indexes Chao, ACE, and Shannon in both MBRs exhibited an increasing trend and then decreased at the last stage (Fig. S7). MBR-E harbored higher ACE and Chao indexes than MBR-B in each stage (Figs. S7a and S7b), while the other α-diversity indexes were not significantly differentiated (Figs. S7c and S7d). The PCoA results in Fig. 3a showed that the bacterial groups from MBR-B and MBR-E at different phases were clearly separated, whereas the temporal community variations were higher than those between the MBR-B and MBR-E groupings. At the OTU level, the MBR-B and MBR-E communities were mainly differentiated in OTUs 2849, 3193, and 2858, which were affiliated with Burkholderiaceae, OLB8 (Saprospiraceae) and Agitococcus (Fig. 3b), respectively. The multivariate analysis including Adonis and ANOSIM also indicated no significant difference between MBR-B and MBR-E communities ($P > 0.05$), suggesting that influent characteristics, other than the reactor configuration, exerted a greater influence on the AS community (Table S2). Thus, odor diffusion in MBR-E may result in slightly higher bacterial richness that MBR-B, but its effect on the community diversities was not significant. The relatively higher bacterial richness in MBR-E may result from the increment of odor-degrading bacteria or organic degrading bacteria in consideration of its relatively lower COD concentration. It is very possible that some anoxic species increased as odor microaeration in tank A2 led to a DO concentration ranging from 0.03 to 0.25 mg/L.

(Insert Figure 3 here)
Fig. 4 illustrates the cluster-specific core microbial populations controlling system performance. The most abundant phylum was Proteobacteria (Fig. 4a), accounting for 44.1% and 48.8% of MBR-B and MBR-E community sequences, respectively. Notably, nitrogen metabolism-associated Nitrospirae [43] was also identified as a core phylum. According to the results of Welch’s t-test (Fig. S8), the core phyla Proteobacteria and Patescibacteria were significantly differentiated between the MBR-B and MBR-E communities.

(Insert Figure 4 here)

At the genus level, more than 1071 bacterial taxa were detected. The six most abundant genera in the sludge samples included Dechloromonas, Nitrospira, Haliangium, Azospira, and two other genera belonging to Sapropiraceae and Hydrogenophilaceae (Fig. 4b). The genera Dechloromonas and Azospira were common denitrifying bacteria, while Nitrospira was the key nitrite oxidizer in both MBRs [44, 45]. Notably, the abundance of the non-motile heterotrophic genus Streptococcus, i.e., facultative anaerobes involved in the manufacture of certain fermented products [46, 47], increased progressively (Figs. 4b and S9). In comparison, the abundance of the strictly aerobic non-motile genus Terrimonas exhibited a decreasing trend (Figs. 4b and S9). Overall, most of the dominating genera in the two reactors showed similar temporal dynamics.
3.3.2 Core functional communities

The temporal dynamic changes of potential functional bacteria are highly related to wastewater performance. The relatively abundant genus *Nitrospira* (3.51% vs. 3.53% for MBR-B and MBR-E, Fig. 4c) was detected as the only nitrite-oxidizing bacteria (NOB) [48]. As described in Fig. 4c and Table S3, the reported ammonium oxidizing bacteria (AOB) mainly consisted of *Nitrosospira*, *Nitrosomonas*, and 8 other genera in the family *Nitrosomonadaceae* [49, 50]. The abundances of AOB were relatively low (0.04%~0.67%), and *Nitrosomonadaceae Ellin6067* (0.67%) and *Nitrosomonas* (0.23%) were the major components. As expected, the denitrifying bacteria were diverse, of which the genera *Dechloromonas*, *Azospira*, *Candidatus Accumulibacter*, *Thauera*, *Novosphingobium*, *Hyphomicrobium*, *Zoogloea*, *Denitratisoma*, *Halomonas*, and *Thiobacillus* dominated. In addition, three phosphate-accumulating organisms (PAOs) were identified as *Candidatus_Accumulibacter*, *Thiothrix*, and *Tetrasphaera*.

Most of the functional taxa maintained relatively stable abundance (e.g., *Nitrospira*, *Nitrosomonas*, *Azospira*, and *Thiothrix*), and no significant difference was observed between the two MBRs (*P* > 0.05). In particular, the denitrifying *Dechloromonas* [27, 51] gradually increased over the study period, while another nitrate reducer, *Hydrogenophaga* [52, 53], exhibited a decreasing trend. This is possibly related to their differentiated organic utilization patterns. For PAOs, MBR-B harbored a marginally higher abundance of functional PAOs.
Tetrasphaera (0.11% vs. 0.08%) but a lower amount of Candidatus_Accumulibacter (0.67% vs. 0.92%) than MBR-E (Fig. 4c). This is consistent with the fact that MBR-B exhibited marginally better TP removal performance (Fig. 2). Notably, the genus Tetrasphaera occupied a slightly different ecological niche and preferred lower environmental redox conditions compared with Candidatus_Accumulibacter [54, 55]. Under more reducing conditions when ORP is as low as -300 mV, Tetrasphaera can ferment complex organic molecules (e.g., carbohydrates and amino acids) and supply volatile fatty acids (VFAs) for other PAOs [54]. Thus, MBR-B without odor diffusion in A2 achieved a more reducing environment and facilitated the growth of more functional Tetrasphaera [56] and slightly better TP removal.

3.3.3 Factors affecting microbial community structure

RDA was employed to visualize the main factors influencing the microbial community structure. The influence of odor involvement on the bacterial community dynamics was identified, especially during the stages with flow rates of 90 and 150 mL/min. This implies that higher odor diffusion exerted a higher influence on the MBR-E community structure. In particular, the indicators ammonium, TN, TP, and COD of influent wastewater were positively correlated (Fig. S10), which were similar to their effects on reactor performance (Fig. S1). The influent substrate showed a positive relationship with the SS of MBRs, suggesting their direct effect on system biomass development. PICRUSt analysis predicted the functional genes of AS microorganisms for nitrogen metabolism (Fig. S11) and found that they were positively linked.
to influent ammonium and TN concentrations. Thus, the influent wastewater substrates and
nutrients significantly affected the treatment system performance, biomass development,
functioning of keystone species, and bacterial community dynamics.

In contrast to laboratory synthetic wastewater, real municipal wastewater features
fluctuations in the concentrations of substrates and nutrients and the introduction of exogenous
microorganisms into the AS system. The influent characteristics resulted in varying microbial
growth and metabolism [57, 58], which significantly influenced the system performance, such
as reactor parameters (DO, SVI and SS), effluent phosphate and nitrogen contents (Fig. S1),
microbial community structure (Figs. 4b, S9, S10, and S11), diversity (Fig. 4a and S7) and
keystone species (Table S4) (i.e., substrate selection). Recently, Lee et al [58] found that 4.3–
9.3% of the OTUs detected in AS of four full-scale WWTPs were shared with influent
wastewater. The upstream influent microorganisms proved to work as microbial reservoirs to
partially supplement sludge microbiota and potentially improve the diversity and system
stability of the AS system [57]. Moreover, influent microbiota immigration is expected to play
a major role in microbial assembly mechanisms [16]. In our study, the observed functional
genera *Thiobacillus* and *Thiothrix* (for denitrification, sulfide oxidation, or phosphate removal)
in the sludge community were common bacteria in sewer systems [59, 60]. The continuous
inoculation of AS bioreactors with sewer microbiota was at least partially beneficial since it
supplements functional populations (e.g., hydrolytic bacteria, PAOs and denitrifiers), enabling
the system to be more resistant to operational failure [16]. Accordingly, influent microorganisms, especially the immigrated rare and functional taxa, deserve to be systematically investigated, thus revealing their explicit roles in system functioning and AS community succession.

3.4 Bacterial assembly and species interactions

Of the four ecological processes illustrated in the null model test, environmental selection dominated community structuring and explained 58.2% and 80.9% of the MBR-B and MBR-E community assembly, respectively (Fig. 5). Thus, the microbial community under both conditions assembled/developed in a deterministic (i.e., selection) manner, which was largely determined by the operating conditions, influent substrate selection, and biotic interactions among microorganisms [61, 62]. For instance, the dedicated reactor configuration (24 L) under well-controlled conditions provided a selecting environment. Additionally, as an important operating parameter in environmental filtering, odor diffusion partially strengthened the niche-based deterministic processes in MBR-E [17, 62]. Homogenizing dispersal, denoting a very strong dispersal among species, occupied the rest of the ecological process in both MBRs. The observed high dispersal rate without dispersal limitation indicated low turnover in the community composition [29]. Specifically, the vigorous and continuous AS mixing in MBRs promoted microbial immigration from the influent, which possibly contributed to the stochastic bacterial assembly process (i.e., homogenizing dispersal). Overall, selection-based bacterial
assembly pattern revealed that the influent substrate not only largely affected the bacterial community structures but also determined the community assembly. This discovery is related to the appreciable odor removal performance in the MBR system as all the odorous compounds are derived from the influent substrate and could be degraded by the MBR bacterial communities.

(Insert Figure 5 here)

The RMT-based network approach was used to explore the co-occurrence (i.e., positive) and co-exclusion (i.e., negative) patterns of bacterial taxa in MBRs (Fig. 6). The topological parameters of the MBR networks were significantly different from those of the corresponding random networks (Table S5). Ecological networks of the MBR-B and MBR-E communities consisted of 554/1220 and 347/777 nodes/edges, respectively. The two networks harbored similar average connectivity (avg. K), whereas MBR-B possessed slightly higher average clustering coefficient (avg. CC) and modularity indexes. This suggested that the network of the MBR-B community exhibited a greater extent of niche differentiation and was more pronounced to have a modular structure than MBR-E [63]. In particular, the average path length in the network of the MBR-E community indicated its slightly higher information or mass transport efficiency than MBR-B (5.842 vs. 5.441). Intriguingly, positive interactions, which indicated the preferred conditions or cooperative behaviors (e.g., cross-feeding and
syntrophic/mutualistic interactions) [64], were more pronounced in the MBR-B network than in the MBR-E network (91.3% vs. 80.4%).

(Insert Figure 6 here)

Overall, 13 and 16 keystone OTUs were identified in the MBR-B and MBR-E networks, respectively (Fig. 6 and Table S4). The nodes in both networks were peripherals, and no network hub was found. The two networks shared no common keystone OTUs, whereas CL500-29_marine group (OTUs 1629 and 646) and Gemmatimonadaceae (OTUs 2983 and 493) were identified as the shared lowest taxa (Table S4). The average abundance of keystone OTUs ranged from 0.003% to 0.806%. In addition, the function of keystone species was diverse but closely associated with the co-assimilation of nitrogen and organics (e.g., polysaccharides and proteins) and/or resistance to environmental fluctuations (Table S4).

As mentioned above, MBR-E achieved marginally better COD removal efficiency than MBR-B. Odor diffusion mitigated membrane fouling and reduced PS/PN contents in SMPs of MBR-E. The phenomena were linked with the functional community and species-species interaction patterns in MBR-E. Odor microaeration in tank A2 (DO ranged from 0.03 to 0.25 mg/L) introduced oxygen and promoted organic consumption in AS, thus enhancing the organic degradation potential of the AS community. For example, ecological network analysis revealed that the characteristic OTUs 3134 and 2026, potentially capable of breakdown/hydrolysis of complex organic compounds (e.g., polysaccharides) or related to
sludge reduction [65-67], became the keystone taxa in the MBR-E community (Table S4). The differentiated biomacromolecule metabolism indicated greater organics competition in the odor-diffused system. According to network analysis, the MBR-E community harbored relatively higher mass transport efficiency, fewer positive links, and more keystone species (Fig. 6), which further verified its intensified microbial relationship [16]. It is assumed that although odor diffusion caused greater organics competition in the system, some microbes may need to cooperate in tighter connections to enhance substrate availability [68].

3.5 Environmental implications

Our results found that AS diffusion under in situ conditions proved to be a robust deodorization technology without compromising wastewater treatment performance. Both MBRs showed similar performance in sludge properties, effluent TN, community diversity, and abundance of AOB and NOB. In addition, other meaningful findings, such as the enhanced competition for organics, more hydrolytic keystone taxa and intensified microbial interactions in MBR-E, along with the underestimated influence of influent wastewater, all extended our understanding of in situ AS diffusion-based deodorization in diverse aspects.

Notably, MBR-E harbored remarkably higher bacterial richness than MBR-B. This suggested that more rare species existed in the MBR-E system, while some were even identified as keynote taxa (e.g., OTUs 2786, 5200, and 2026 accounted for 0.004%, 0.007%, and 0.003% abundance in Table S4, respectively). The rare species were reported to be mostly involved in
positive associations, forming small network modules [69]. They potentially made metabolic contributions in enhanced biological phosphorus removal [70], substrate hydrolysis [71] or other environmentally important chemicals such as endocrine-disrupting compounds (EDCs) in WWTPs [72]. Further investigation on the roles and sources of rare taxa can thus help better elucidate WWTP functions. Based on our results, although 100% NH$_3$ was removed and the outlet concentrations of dominating odor contributor carbon disulfide were much lower than the factory boundary concentration limit (2 mg/m$^3$) of WWTPs. However, not all the odor indexes were continuously lower than the limit. We are unsure whether the overall odor discharge will meet the factory boundary concentration limit due to the disparity of odor concentrations from tank A2, overall biological treating units, and the boundary concentration of WWTPs. Nevertheless, under proper operation, AS diffusion is highly worth considering as partial or overall odor control strategies in WWTPs. Future directions could also be placed on optimization of MBR configuration (e.g. odor residence time), the impact of inlet odor concentrations and the deodorization mechanisms (e.g., the adsorption/biodegradation behavior and metabolic mechanisms of characteristic odorants).

**4. Conclusions**

The main conclusions of this study are drawn as follows:
(1) AS diffusion proved to be a robust deodorization technology without compromising wastewater treatment performance. High removal rates of NH₃, H₂S and carbon disulfide were achieved while other odorants (e.g. VOCs) maintained low outlet concentrations.

(2) Odor diffusion in tank A2 of MBR-E enhanced organics competition and caused intense microbial interactions. The odor involvement resulted in lower SMP and EPS production, decreased effluent COD concentrations and mitigated membrane fouling.

(3) Odor diffusion strengthened the deterministic process in bacterial assemblage and enabled hydrolytic bacteria capable of assimilating complex organics to become keystone taxa in MBR-E community.

(4) The influent wastewater substrates and nutrients significantly affected the treatment system performance, biomass development, keystone species functioning and bacterial community dynamics.

Acknowledgments

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Symbols and abbreviations

List of symbols and abbreviations used in this paper
AO-MBRs: anoxic-oxic membrane bioreactors

ANOSIM: analysis of similarity

AS: activated sludge

βNTI: β-nearest taxon distance

BNR: biological nutrient removal

BTEX: benzene, toluene, ethylbenzene and xylene

COD: chemical oxygen demand

EDCs: endocrine-disrupting compounds

Eh: redox potential

EPS: extracellular polymeric substance

GC-MS: gas chromatography-mass spectrometry

HRT: hydraulic retention time

MBR: membrane bioreactor

MBR-B: blank control of membrane bioreactor

MBR-E: experimental membrane bioreactors

MLSS: mixed liquor suspended solids

MLVSS: mixed liquor volatile suspended solids

OTU: operational taxonomic unit

PAOs: phosphate-accumulating organisms

PCA: principal component analysis

PCoA: principal coordinate analysis

pMEN: phylogenetic molecular ecological network

PN: proteins

PS: polysaccharides

RC_{Bray}: Raup–Crick metric

RDA: redundancy analysis

RMT: random matrix theory

SDNRs: specific denitrification rates

SMP: soluble microbial product

SNRs: specific nitrification rates

SOB: sulfide-oxidizing bacteria

SPRRs: specific phosphorus release rates

SPURs: specific phosphorus uptake rates

SRT: solids retention time

SVI: sludge volume index

TMP: transmembrane pressure

TN: total nitrogen

TP: total phosphorus

VFAs: volatile fatty acids

VOCs: volatile organic compounds

WWTPs: wastewater treatment plants

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Table captions

Table 1. The inlet and outlet concentrations of different odor species under a flow rate of 150 mL/min in tank A2 of MBR-E.

Figure captions

Fig. 1. Removal performance of different odors: (a) time course of the inlet and outlet concentrations of H₂S and NH₃ and their removal efficiencies and (b) removal efficiencies of different odors under a flow rate of 150 mL/min in MBR-E. Error bars were added as the concentrations of some outlet odorants are beyond detection limits.

Fig. 2. Reactor performance of organic and nutrient removal: (a) ammonium; (b) nitrate; (c) TN; (d) TP.

Fig. 3. Variations in bacterial community composition in AS samples at the OTU level. Principal coordinate analysis (PCoA) showing the difference in whole bacterial community composition of the AS samples at different stages (a). PC1 covered 35.25% of the total variation, and PC2 covered 12.01% of the total variation. The pairwise comparison of MBE-B and MBR-E was compared by Welch’s t test and presented by bar plot (b).

Fig. 4. Relative abundance of major phyla (a), top 30 genera (b) and functional groups (c) in MBR-B and MBR-E sludge samples. The samples were named to indicate their stages and the sampling dates during the MBR operation.

Fig. 5. Bacterial assembly of the MBR-B (a) and MBR-E (b) sludge communities with/without odor involvement. The relative proportion of four ecological processes (i.e., environmental selection,
dispersal limitation, homogenizing dispersal and ecological drift) in shaping sludge microbiota was assessed by calculating the abundance-weighted β-nearest taxon distance (βNTI) and the Raup–Crick metric (RCBray).

**Fig. 6. Phylogenetic molecular ecological networks (pMENs) and Zi-Pi plots of OTUs for blank and odor-involved experimental bacterial communities.** In networks of blank (a) and odor-involved experimental (b) bacterial communities, the node colors represent the modularity class of OTUs, and the node sizes represent their connectivity values (i.e., node degree). Light blue and red edges indicate positive and negative connections, respectively. The edge thickness is proportional to the absolute value of the correlation coefficient. Zi-Pi plots for blank (c) and odor-involved experimental (d) bacterial communities are displayed on the basis of their topological roles. Each dot denotes an OTU. The module hubs and connectors are colored pink and dark blue, respectively.
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### Table 1. The inlet and outlet concentrations of different odor species under a flow rate of 150 mL/min in tank A2 of MBR-E.

<table>
<thead>
<tr>
<th>Odorant</th>
<th>Odor concentration Unit</th>
<th>Carbon disulfide (CS₂) mg/m³</th>
<th>Trimethylamine (N(CH₃)₃) mg/m³</th>
<th>Styrene (C₈H₈) mg/m³</th>
<th>Methanethiol (CH₃S) mg/m³</th>
<th>Dimethyl sulfide (C₂H₆S) mg/m³</th>
<th>Dimethyl disulfide (C₂H₆S₂) mg/m³</th>
<th>Total VOCs (34 species) mg/m³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1 Inlet</td>
<td></td>
<td>7244</td>
<td>3.5 (ND (&lt;0.0025)</td>
<td>0.0043</td>
<td>ND (&lt;0.001)</td>
<td>ND (&lt;0.001)</td>
<td>ND (&lt;0.001)</td>
<td>0.133</td>
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<tr>
<td>Sample 1 Outlet</td>
<td></td>
<td>417</td>
<td>0.05 (ND (&lt;0.0025)</td>
<td>0.0012</td>
<td>ND (&lt;0.001)</td>
<td>ND (&lt;0.001)</td>
<td>ND (&lt;0.001)</td>
<td>0.0836</td>
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<tr>
<td>Sample 1 Removal</td>
<td></td>
<td></td>
<td></td>
<td>94.2%</td>
<td>98.6%</td>
<td>-</td>
<td>-</td>
<td>37.1%</td>
</tr>
<tr>
<td>Sample 2 Inlet</td>
<td></td>
<td>5495</td>
<td>2.57 (ND (&lt;0.0025)</td>
<td>0.0175</td>
<td>ND (&lt;0.001)</td>
<td>ND (&lt;0.001)</td>
<td>ND (&lt;0.001)</td>
<td>0.672</td>
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<tr>
<td>Sample 2 Outlet</td>
<td></td>
<td>977</td>
<td>&lt;0.03 (ND (&lt;0.0025)</td>
<td>0.0009</td>
<td>ND (&lt;0.001)</td>
<td>ND (&lt;0.001)</td>
<td>ND (&lt;0.001)</td>
<td>0.137</td>
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<tr>
<td>Sample 2 Removal</td>
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<td></td>
<td></td>
<td>82.2%</td>
<td>&gt;98.8%</td>
<td>94.9%</td>
<td>-</td>
<td>79.6%</td>
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