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Deciphering the effect of salinity on the performance of submerged membrane bioreactor for aquaculture of bacterial community

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HIGHLIGHTS

- Performance of MBR in different salinity.
- SND occurs when salinity was 21 $g \cdot L^{-1}$ and 28 $g \cdot L^{-1}$.
- Present the roles of bacteria for short-cut nitrification and denitrification.

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ABSTRACT

The study tended to disclose the evolutionary process of bacterial community that affected the performance of submerged membrane bioreactor (MBR) for aquaculture wastewater treatment via PCR amplification of 16S rRNA genes combined with denaturing gradient gel electrophoresis (DGGE) analysis. The selection pressure of gradually-increased salinity would worsen the treatment performance of MBR. COD_{Mn} removal rate slightly decreased from ca. 98.39% to 95.82%. Short-cut nitrification and denitrification (SND) phenomenon took place when salinity was ca. 21 $g \cdot L^{-1}$ and 28 $g \cdot L^{-1}$, respectively. Effluent NO_2^-–N concentrations increased from 0.05 $mg \cdot L^{-1}$ to 1.05 $mg \cdot L^{-1}$ and reached to 2.66 $mg \cdot L^{-1}$ when salt concentration was 28 $g \cdot L^{-1}$. TN removal efficiency increased due to the SND process taking place in MBR. As the selection pressure of salt stress changed characteristics of community structures in the MBR, salt-tolerant microbe tended to gradually dominate in the population. Community-structure analysis indicated that *Thiothrix eikelboomii*, *Pedomicrobium australicum*, and *Paracoccus bengalensis* may hold crucial roles in the community ecology. Comparative PCR-DGGE analysis upon the mixed consortia in suspended activated sludge and cell-immobilized membrane revealed that there was no significant change of microbial structure taking place in the community.

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1. Introduction

For applications in water recycling and reuses, membrane bioreactors (MBRs) have recently emerged as a promising mode of operation for wastewater treatment [1,2]. In fact, the advantages of membrane bioreactor (MBR) systems over the conventional activated sludge process (CAS) have been widely mentioned in literature, including a higher biomass concentration, reduced footprint, less sludge production, and highly effective degradation of pollutant(s) [3–6].

As a matter of fact, saline wastewaters produced from food, petroleum, textile and leather industry could be treated by modified MBRs prior to myriads of applications [7]. However, the salinity expressed adverse impact on the performance of a typical activated sludge system [8–10]. As saline wastewater usually expressed high salt stress to bacterial cells, the efficiency of the biological treatment process (e.g., BOD or COD removal) would be inevitably attenuated. Apparently, bacteria had to defense against high gradient gaps in osmotic pressure due to the loss of cellular water (plasmolysis) or recession of the cytoplasm [11,12]. Moreover, Reid et al. [13] also observed the negative effect of salt shocks (e.g., Na^+ and K^+) on sludge filterability, mainly due to the release of polysaccharides. Thus, MBRs were popularly reported to have highly-tolerant characteristics of microbes to salinity and have a promising pollutant removal efficiency [14–18].

To decipher how and why highly efficient microbes for pollutant degradation, PCR-DGGE would be used as a rapid, culture-independent detection technique for microorganism populations. It has been successfully used in myriad fields of microbial ecology to assess the species diversity of microbial communities and to determine its transient dynamics responding to environmental variations [19–22]. Recently, studies considering bacterial diversity in wastewaters using a DGGE-based approach have been extended to uncover the performance of reactors systems associated to species evolution. In fact, PCR-DGGE is very likely the most cost-effective technique for assessment upon biodiversity of

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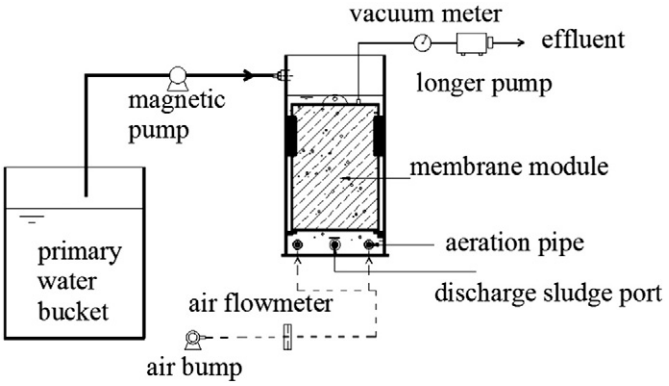

Fig. 1. Schematic diagram of the experimental setup.

bioreactor samples [23–26], since DGGE protocols were relatively simple and straightforward to obtain results in a relatively reasonable period of time.

This study primarily focused on evaluation of the operating performance of sequencing MBR for COD and microbial community, nitrogen removal in response to changes in the community under different saline stresses. Furthermore, samples analyzed via PCR-DGGE and cloning and sequencing of the 16S rRNA genes clearly uncovered how species evolution was taking place to influence operation performance in MBRs under saline stress.

2. Materials and methods

2.1. Experimental set-up and procedure

The one MBR was self-made with acrylic, as shown in the schematic diagram of the experimental set-up in Fig. 1 (the operating volume of MBR was ca. 10 L), where the membrane was a submerged flat-sheet membrane (polyvinylidene fluoride membrane) with the pore size of 0.1 μm and the effective filtration area of 0.085 m^2 (purchased from SINAP Membrane Tech Co., Ltd China). The MBR was operated in a model of submerge reactor. A coarse bubble diffused aeration system was provided for oxygen supply to the growth of microbes and also cleanup of the membrane surface. Dissolved oxygen (DO) was ranged 1.0–2.0 $mg \cdot L^{-1}$, hydraulic retention time (HRT) was set at ca. 6.0 h. To maintain biologically activated sludge for this study, sludge discharge process was performed manually, the sludge retention time (SRT) was ca. 45 d. The system was conducted under constant membrane flux. When the transmembrane pressure (TMP) increased to 40 KPa, the membrane module would be washed with tap water manually to recover the membrane flux. The MBR was subsequently operated under steady-state conditions for at least 50 d prior to this treatability study. MBR was then continuously operated for 180 d. During continuous operation, salt concentration was step-increase from 0 to 35 $g \cdot L^{-1}$ for 6 stages, the increased salinity gradient at ca. 7 $g \cdot L^{-1}$ per stage and 30 d operation time of each stage. Activated sludge samples were collected from MBR sewage treatment system with different saline conditions preserved in the freezer at −20 °C. The sludge was transferred by using a 1.5–2.0 ml Eppendorf tube and centrifuged at 12,000 ×*g* for 10 min. Then, the supernatant was removed, and the pellet was stored at −20 °C for in-need analysis afterwards.

2.2. Wastewater composition and chemical analysis

Aquaculture wastewater used in this study contained glucose, soluble starch, NH_4Cl, KH_2PO_4 and $NaHCO_3$. Typical composition of the aquaculture wastewater was COD_{Mn} (chemical oxygen demand) = 150 $mg \cdot L^{-1}$, TN (total nitrogen) = 14.8 $mg \cdot L^{-1}$, NH_4^+–N = 14.8 $mg \cdot L^{-1}$, TP (total phosphorus) = 2.5 $mg \cdot L^{-1}$, (time series profile of influent and effluent concentrations of COD_{Mn}, TN, NH_4^+–N as shown in Figs. 2 and 3). Influent $NaHCO_3$ was amply provided as buffering solutions for nitrification process, the pH level of the wastewater was ca. 7.0 ± 0.5. The bioreactor was filled with the aquaculture wastewater, inoculated with activated sludge from an oxidation ditch in a municipal wastewater treatment plant. The activated sludge was cultivated in aquaculture wastewater through a fill-and-draw mode of operation for approx one month to guarantee stability of microbial activities. The operation parameters for monitoring the performance of treatment included the influent and effluent concentrations of COD_{Mn} (potassium permanganate digestion method), TN (ultraviolet spectrophotometry), NH_4^+–N (hypobromite oxidation method), NO_2^-–N (n-(1-naphthyl) ethylenediamine spectrophotometry method) and NO_3^-–N (ultraviolet spectrophotometry). They were measured via Chinese NEPA Standard Methods (Chinese NEPB, 2002) [27].

Extraction and measurement of Extracellular Polymeric Substances (EPS) were conducted as described by Chang's methods. The mixed liquor of activated sludge was centrifuged in order to remove the bulk solution (3200 rpm, 30 min). After discarding the supernatant, the remaining pellet was washed and resuspended with saline water (0.9% NaCl solution). The extracted solution was obtained from heat treatment (100 °C, l h) of this resuspended solution. The extracted solution was centrifuged again under the same operating condition, the EPS content was measured by analyzing volatile solids (VS) [28].

2.3. DNA extraction

The pellets of activated sludge samples were washed three times via centrifugation at 12,000 ×*g* via sterile high-purity water for 5 min. DNA extraction was then performed using an Aqua-SPIN Bacteria gDNA Isolation Mini Kit (Hua Shun, China) according to the manufacturer's protocol.

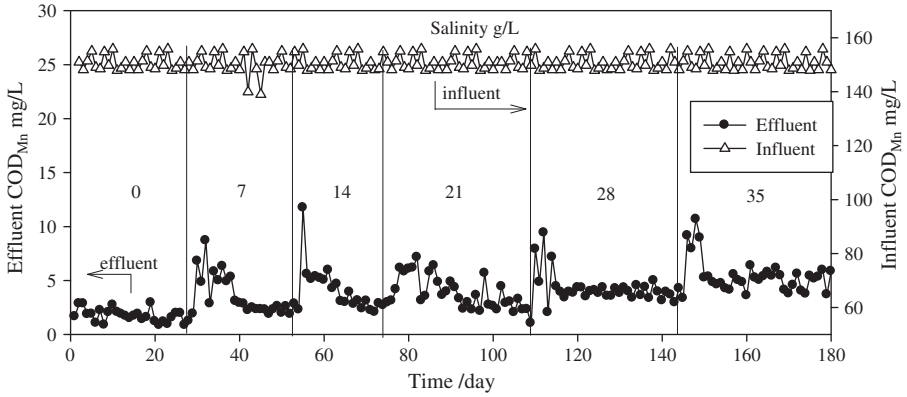

Fig. 2. Variation of COD_{Mn} influent and effluent concentrations under different salinity, where numbers in the box denoted salinity level in $g \cdot L^{-1}$.

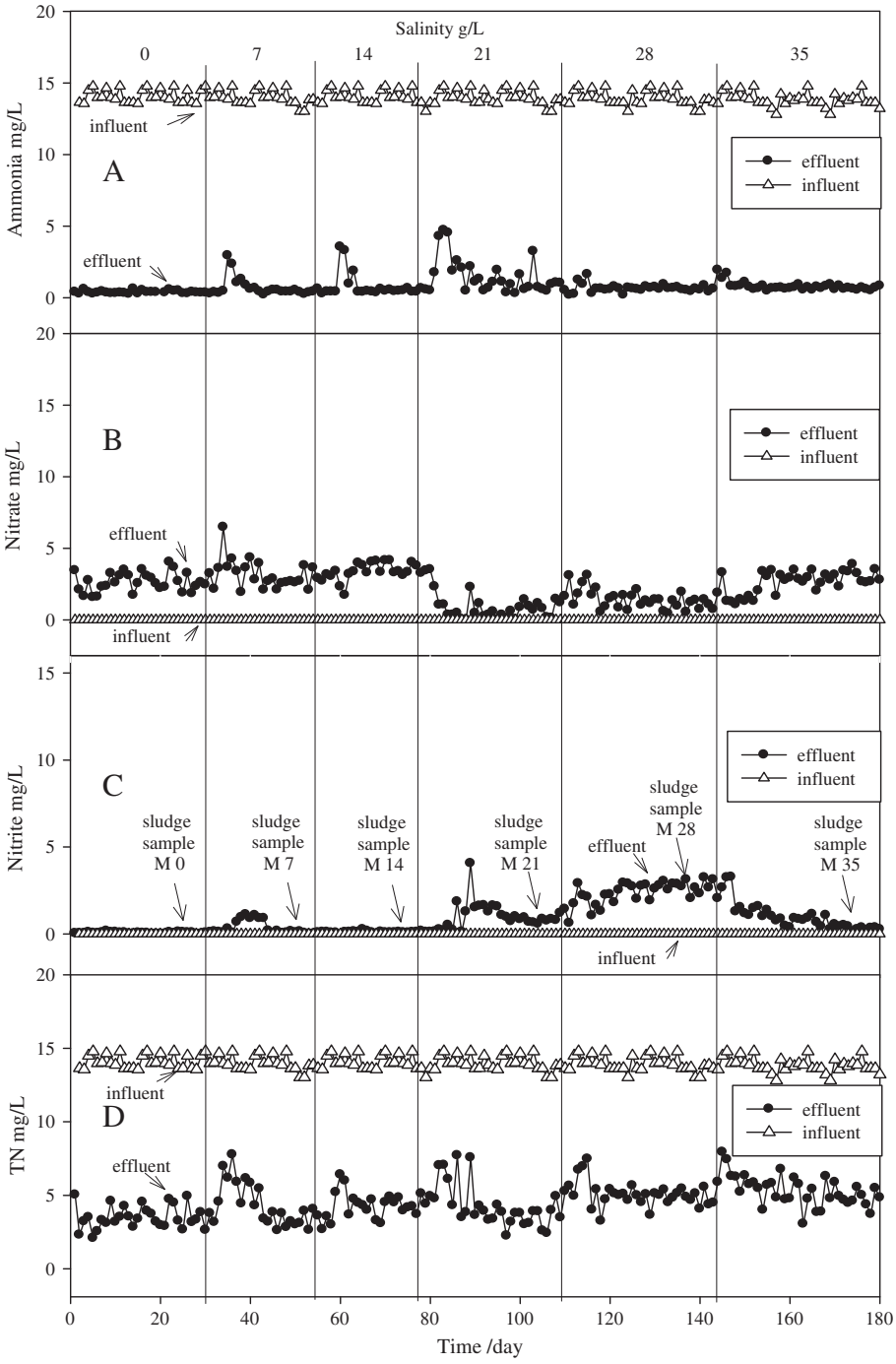

Fig. 3. Time-courses of nitrogen concentrations of influent and effluent streams under different salinity (0, 7, 14, 21, 28, and 35 denoted salinity level in $g \cdot L^{-1}$, respectively; sludge samples M0, M7, M14, M21, M28, and M35 denoted sludge sampling for PCR-DGGE analysis at different salinity level; A — NH_4^+–N; B — Nitrate; C — Nitrite; and D — TN.).

2.4. PCR amplification and purification

Bacterial 16S rRNA gene fragments were amplified using the forward primer 341 F (5′-TACGGGAGGCAGCAG-3′) and the reverse primer 517R (5′-ATTACCGCGGCTGCTGG-3′). A GC clamp (CGCCCGCCGCGCGCGGCG GGCGGGGCGGGGGCACGGGGGGCC) was added to the forward primer [29]. The length of the expected amplified fragment was ca. 200 bp. All PCR reactions with a final volume of 50 μL contained 5 μL 10× PCR buffer (TaKaRa, Japan), 1 μL deoxynucleotide triphosphate (dNTP) mix (10 mM, TaKaRa, Japan), 2.5 U TaKaRa Taq™ enzyme (5 U/μL, TaKaRa, Japan), 5 μL of each primer(10 μmol/L, TaKaRa, Japan), 5 μL BSA (1 mg/mL, TaKaRa, Japan) and approximately 100 ng template DNA. PCR was performed in a thermocycler (MJ Research, USA) under the following conditions: initial denaturing step of 5 min at 94 °C followed by 30 cycles of denaturing (94 °C for 1 min), annealing (55 °C for 45 s), and elongation (72 °C for 1 min). PCR products were analyzed on 1.0% (w/v) agarose gels to confirm the product size.

2.5. DGGE analysis

DGGE was performed with the Dcode Universal Mutation Detection System (Bio-Rad, USA), according to manufacturer's instructions. Equal amounts of PCR products were loaded onto 8% (w/v) polyacrylamide gels in 1×TAE (40 mM Tris, 20 mM acetate, 1.0 mM Na_2-EDTA) with denaturant-gradient of 40–60% (100% denaturant was 7 M urea and 40% (w/v) deionized formamide). The gel electrophoresis was

performed at a constant voltage of 110 V, 60 °C for 5.0 h. After electrophoresis, DGGE gels were stained with a routine silver staining protocol as described elsewhere [30].

2.6. Cloning and sequencing

The target bands were excised from DGGE gels, washed twice in Tris-EDTA buffer and incubated in 50 μL of the same buffer overnight at 4 °C. The eluted DNA was re-amplified with primers 341 F-517R under the same conditions as aforementioned. Purified PCR products were ligated into pMD19-T cloning vectors (TaKaRa, Japan) and transformed into competent *Escherichia coli* DH5α cells (TaKaRa, Japan) according to the manufacturer's suggestions. Transformants were selected by ampicillin resistance, and blue–white screening was performed to identify clones with inserts. Eighteen white colonies were specifically selected and cultivated. The colonies products were outsourced for sequencing (Shanghai Sangon Biological Engineering Technology, Shanghai, China). All sequences were submitted to GenBank and subjected to a Basic Local Alignment Search Tool (BLAST) search on the national center for biotechnology information (NCBI) website to identify sequences with the highest similarity. Highly similar sequences and some reference sequences of dominant groups were added to the data set for CLUSTAL X multiple sequence alignment and the phylogenetic tree was constructed in MEGA 4 by applying the neighbor-joining method with 1000 bootstrap replicates.

3. Results and discussion

3.1. The performance of MBR

3.1.1. Effect of salinity on removal rate of COD_{Mn}

As depicted in time courses of COD_{Mn} in the MBR effluent at different salinity (Fig. 2.), COD_{Mn} level appeared to be different, effluent COD_{Mn} concentration slightly increased from 1.93 to 5.01 $mg \cdot L^{-1}$ when salt concentration increased from 0 to 35 $g \cdot L^{-1}$. Meanwhile, COD_{Mn} removal rate decreased from ca. 98.39% to 95.82%. Apparently, gradually-increased salt concentrations only resulted in slight decreases in COD_{Mn} removal efficiency. It is generally known that salts are detrimental to biological treatment systems by causing plasmolysis and/or loss of cell activities, thereby lowering the system performance [31]. The inhibition effect of salt on mixed cultures resulting in loss of metabolic activity and plasmolysis caused release of intracellular constituents and soluble microbial products decreased in organic removal efficiency [14,32]. However, for this MBR system a slightly decreased efficiency of treatment was found when salinity was changed. This was very likely due to cellular adaptation to environmental changes. Once cultured cells adapted to such all environment, the operation efficiency would be stabilized in a short-period of time. Compared to other aerobic treatment for highly saline wastewater process, MBR presented high organic removal efficiency (e.g., SBR: 87.9% at salinity 10 $g \cdot L^{-1}$ [33]; SBR: 89% at salinity 5 $g \cdot L^{-1}$, 92% at salinity 15 $g \cdot L^{-1}$, 98% at salinity 28 $g \cdot L^{-1}$, 98% at salinity 40 $g \cdot L^{-1}$ [34]; activated sludge: 76% at 15 $g \cdot L^{-1}$NaCl, 68% at 30 $g \cdot L^{-1}$ NaCl [35]; Fed-batch reactor: 80% at salinity 20 $g \cdot L^{-1}$ [36]).

3.1.2. Effect of salinity on nitrogen removal

Time-courses of nitrogen concentrations (NH_4^+–N, NO_3^-–N, NO_2^-–N, TN) of influent and effluent streams under different salinity was shown in Fig. 3. The effluent and removal rate of NH_4^+–N and TN seemed not to be significantly affected by the selection pressure of salinity. There might be some halobacteria which still survive in MBR in saline wastewater. As shown in time courses of influence and effluent NH_4^+–N concentration at different salinity (Fig. 3A), NH_4^+–N removal efficiency decreased from 96.92% to 91.08% when salinity increased from 0 to 35 $g \cdot L^{-1}$. Compared to other treatment of aquaculture wastewater process, MBR also presented high ammonia removal efficiency (e.g.,

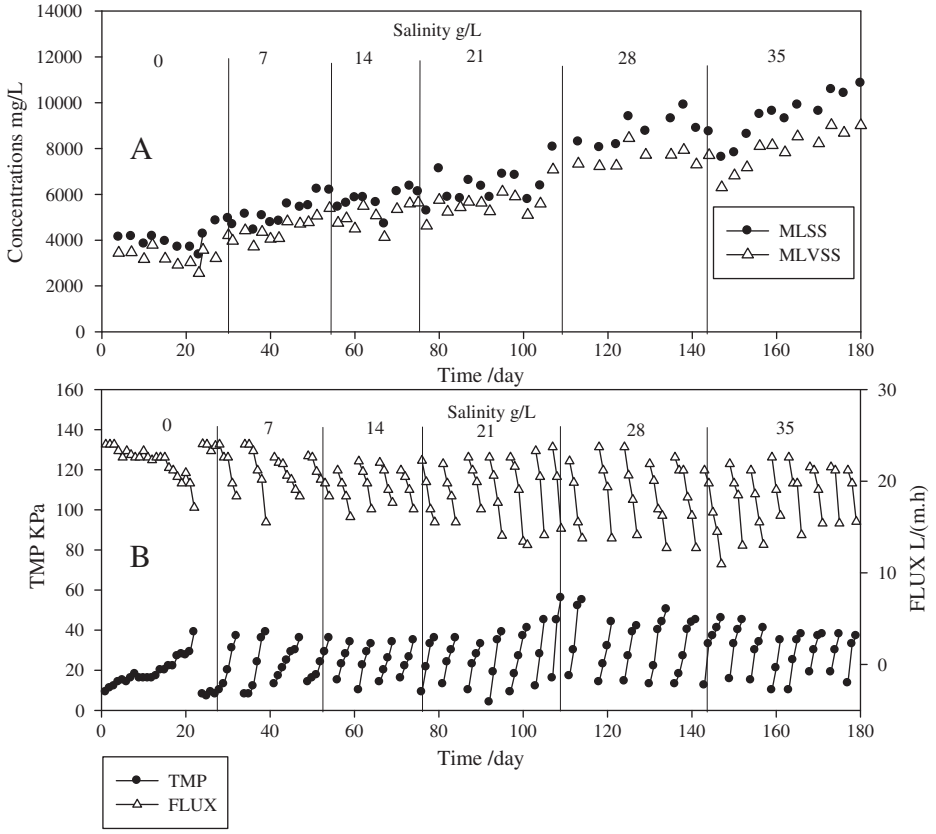

Fig. 4. (A) MLSS and MLVSS of MBR and (B) TMP and membrane flux of MBR.

activated sludge: 87% [37], 91.07% [35]; halophyte filter beds: 50%–91% [38]; wetlands system: 86%–98% [39]).

Time courses of effluent NO_2^-–N and NO_3^-–N concentrations with salt concentration were also shown in Fig. 3B, C. Initially, effluent NO_2^-–N and NO_3^-–N concentrations was 0.05 mg·L^{-1} and 3.1 mg·L^{-1} respectively. When salt concentration increased, both effluent concentrations of NO_2^-–N and NO_3^-–N also increased in gradually. However, when the salt concentration increased to 21 g·L^{-1}, the effluent NO_2^-–N concentration increased from 1.05 mg·L^{-1} to 2.66 mg·L^{-1}. As salinity was ca. 28 g·L^{-1}, nitrate concentration was 1.04 mg·L^{-1}, 1.48 mg·L^{-1} respectively. This phenomenon indicated that the biological nitrogen removal pathway was mainly switched to SND, nitrite accumulation rate (NO_2^-–N/NO_x^-–N) was over 50%. However, when salt concentration reached 35 g·L^{-1}, the SND capability of biological nitrogen removal disappeared. This was perhaps due to that bacterial species associated with SND were not capable to adapt in such high salt conditions, and other species seemed to dominate in MBR.

In addition, variations of TN influence and effluent concentration under different salt concentration are elucidated in Fig. 3D. Initially, effluent TN concentration was 3.58 mg·L^{-1}, when the salinity reached 14 g·L^{-1}, effluent TN concentration was 4.28 mg·L^{-1}, TN removal efficiency decreased from 72.1% to 66.5%. When the salinity reached 21 g·L^{-1}, TN removal efficiency would increase due to such a SND process (removal rate 72.8%). However, SND phenomenon was not taking place when salinity was 35 g·L^{-1}, at this period, effluent TN concentration was ca. 5.06 mg·L^{-1} and removal efficiency decreased to only 60.1%.

Thus, with MBR a technically-efficient waste removal efficiency would be maintained at different salinity, suggesting that some microbial species associated with SND tended to be dominant in the MBR when salinity was 21 g·L^{-1} and 28 g·L^{-1}; however, such a phenomenon was turned off once salinity reached over 35 g·L^{-1}.

3.1.3. Operation performance of MBR

As Fig. 4(A) illustrated for the course of the activated sludge concentrations, TMP, and membrane flux of MBR during operation, MLSS of MBR increased from initially ca. 4220 mg·L^{-1} to ca. 10095 mg·L^{-1} when salinity increased from 0 to 35 g·L^{-1}, revealing that MBR could result in high activated sludge concentrations when the inhibitory pressure of salt existed. As shown in TMP and membrane flux of MBR (Fig. 4B), filterability of the sludge decreased largely, feasible time of operation decreased from ca. 18 d to 4 d as salinity increased and thus the efficiency of filtration performance of MBR decreased largely. High salt concentration was known to drastically decrease the filterability of the sludge [16]. When salinity of MBR was 0 g·L^{-1} and 35 g·L^{-1}, the EPS was 13.79 mg·(gMLSS)$^{-1}$ and 39.98 mg·(gMLSS)$^{-1}$, correspondingly.

Generally, the EPS concentration of MBR was ca. 20–30 mg/gMLSS, low food to biomass ratio also could limit the production of EPS [40]. In addition, membrane fouling occurred at low SRT due to increased EPS level [40–42]. In this study, the food to biomass ratio (0.12) was relatively low compared to traditional wastewater treatment process (e.g., 0.16–0.47 [40]). Moreover, SRT was ca. 45 d, thus it should have resulted in a low level of EPS. However, EPS concentration increased from 13.79 mg·(gMLSS)$^{-1}$ to 39.98 mg·(gMLSS)$^{-1}$, higher than 30 mg·(gMLSS)$^{-1}$. The results indicated that salt inhibit inhibition resulted in the increasing of EPS concentration. The formation of EPS might be the reason of a drastically decrease in the filterability for MBR. Chang and Lee [28] considered that in any physiological status of activated sludge, a higher content of EPS in the activated sludge had a higher risk of membrane fouling. In fact, as high monovalent cations concentrations might cause weaker intrapolymer bridges, the flocs are more sensitive to shear stress under these conditions [16]. Under these conditions, it was anticipated that more EPS would be released from the sludge flocs to the bulk liquid (i.e., membrane foulants) [43]. This would cause a significant reduction of sludge filterability [13].

3.2. Analysis of microbial community diversity using PCR-DGGE

To disclose the knowledge gaps behind differences of operation performance inspected, the change in the diversity of microbial community in the bioreactor was inspected via PCR-DGGE analysis.

3.2.1. DGGE fingerprint

DGGE analysis was used to investigate evolution of bacterial community, total DNAs were extracted from samples and V3-16S rRNAs were then amplified. As shown in the bacterial community structure during MBR operation (Fig. 5), total 33 predominant bands were detected in 40–60% denaturant gradient gels. Moreover, the activated sludge in MBR changed with the variation of salinity, some bacterial population became either dominant or extinct; for instance, band 5, 6 disappeared when salinity reached 21 g·L^{-1}; band 16 only showed up when the salinity reached over 35 g·L^{-1}.

It is evident that some specific species could be propagated in MBR under some appropriate conditions and their competitors not favorable to such conditions would die out in the MBR. Bacterial population increased with the increased salinity, and the microbial community could express different degree of tolerance to such an altered stresses of salinity. For instance, when the salinity reached 35 g·L^{-1}, the dominate

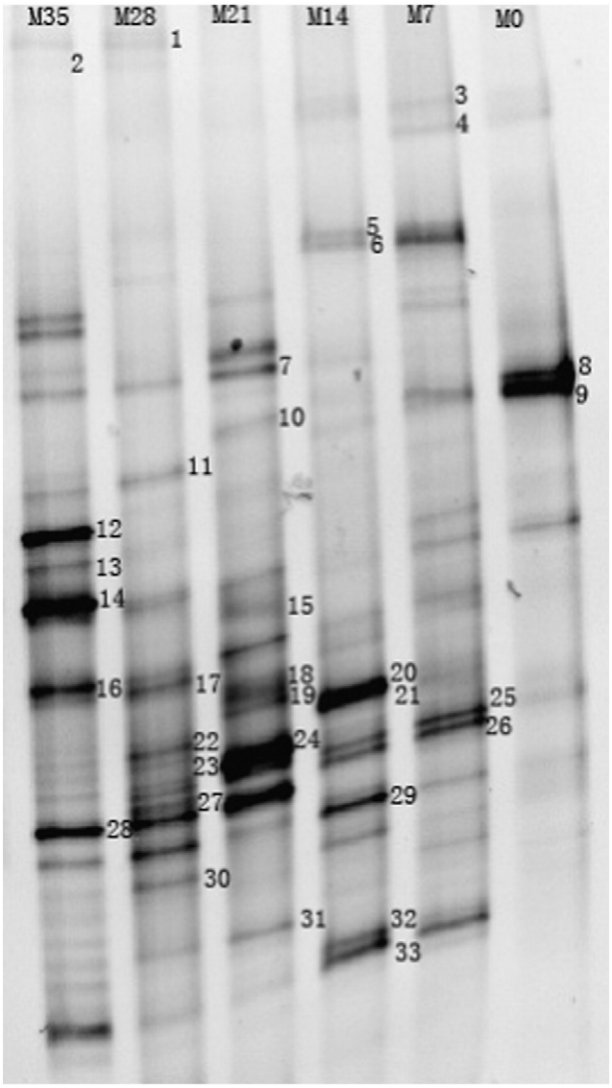

Fig. 5. DGGE of PCR-amplified 16S rRNA extracted from the microbial communities in MBR (M0, M7, M14, M21, M28, and M35 was suspended sludge samples at salinity of 0, 7, 14, 21, 28, and 35 g·L^{-1}, respectively).

microorganism seemed to be *Salinimicrobium xinjiangense* (i.e. band 12, 13, 14)which was reported to be halophilic [44]. Band 16, 17 was *Rhodobacter maris*, a phototrophic α-proteobacterium, capable to tolerate a 3% NaCl concentration [45]. In addition, there were some other salt-resistant microorganisms dominated at different salinity in MBR. For example, *Thiothrix flexilis* (band 15 in M21) was slightly suppressed when the NaCl concentration was 2% (w/v), and it was capable to reduce nitrate [46]. Moreover, *Thiothrix eikelboomii* (i.e., band 19, 23 in M21; band 19, 23 in M28), a heterotrophic bacteria, that could utilize ammonia, nitrate and nitrite as N source; in particular, nitrate could be reduced to nitrite [47]. *Paracoccus bengalensis* (i.e. band 20, 29, 30 in M14; band 20, 29 in M21; and band 29, 30 in M28), was chemolithoautotroph bacteria that could be isolated in brine, and could utilize ammonia nitrate as N source and sulfide as electron donor [48]. *Pelagicoccus albus* (i.e. band 25 in M7, M14, M21, M28), a chemoheterotrophic bacterim, apparently required NaCl for cell growth and was capable to tolerate up to 7% (w/v) NaCl [49]. *Pedomicrobium australicum* (band 27 in M21, M28), which was popularly known to tolerate in 1–2.5% (w/v) NaCl, could utilize ammonia salt as N source, and conducted nitrate reduction to nitrite [50]. *Alicycliphilus denitrificans* (band 11 in M28), a facultative anaerobic bacterium, could also use oxygen, nitrate and nitrite as electron acceptor [51].

In summary, the SND and nitrite accumulation phenomena taking place in M21 and M28 may be strongly associated with the *T. eikelboomii*, *P. australicum*, and *P. bengalensis*. These bacteria could utilize ammonia, nitrate as N source, reduce nitrate to nitrite, and nitrate and nitrite as the electron acceptor for cellular metabolism, showing the effective nitrogen removal efficiency at high salt concentrations. Apparently, these species could be seen in M21 and M28, but not in M35. This might explain the disappearance of dominated strains of SND in M35 samples.

Moreover, as there existed some sulfur-oxidizing bacteria (e.g., *P. bengalensis*, *Rhodobacter blasticus*), this might explain why the mariculture also accumulated a significant amount of inorganic sulfate.

3.2.2. Analysis of the 16S rRNA clone library

Fig. 6 showed the phylogenetic relationships among DGGE fragments and 16S rRNA of isolated bacteria. The predominant members in the microbial community were related to Proteobacteria (mainly α-proteobacteria, second rank γ-proteobacteria). The second dominant group seemed to be Bacteroidetes, the next frequently detected phylum was Bacteroidetes. Other minor lineage detected was Actinobacteria and Firmicutes.

3.2.3. Microorganism analysis of bulk activated sludge and membrane

To investigate the difference of microbial population between bulk activated sludge and immobilized microbes in MBR, DGGE-PCR profiles were also conducted (Fig. 7). As DGGE band patterns for microorganism from suspended bulk sludge and immobilized cells on the membrane surface were similar, evidently there were no apparent difference taking place in microbial community.

4. Conclusion

This first-attempt study explored the effect of salinity on wastewater treatment and succession of bacterial community of activated sludge in a MBR system. The results showed that when salt concentration gradually increased, COD_{Mn}, NH_4^+–N and TN removal

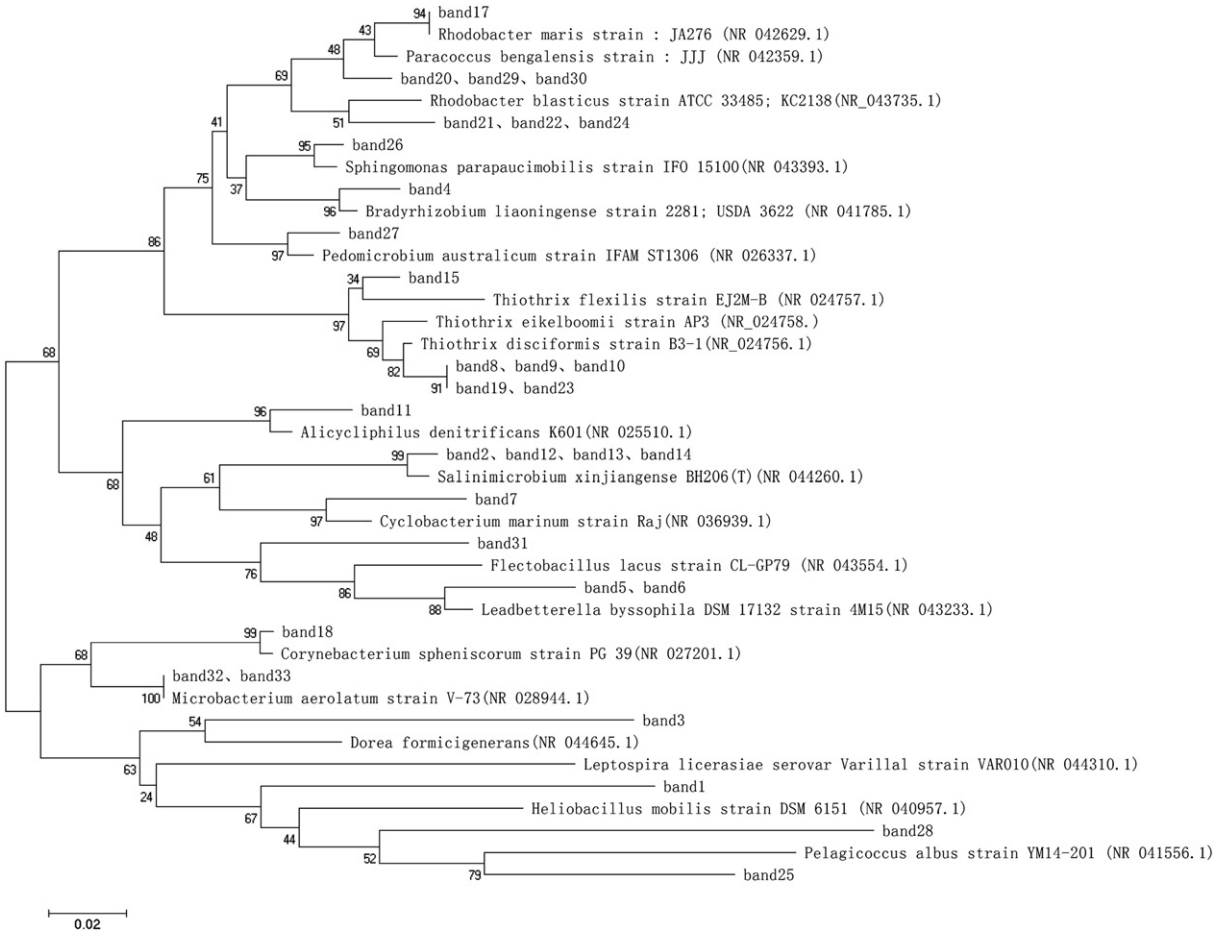

Fig. 6. Phylogenetic tree of bacteria based on the results of BLAST.

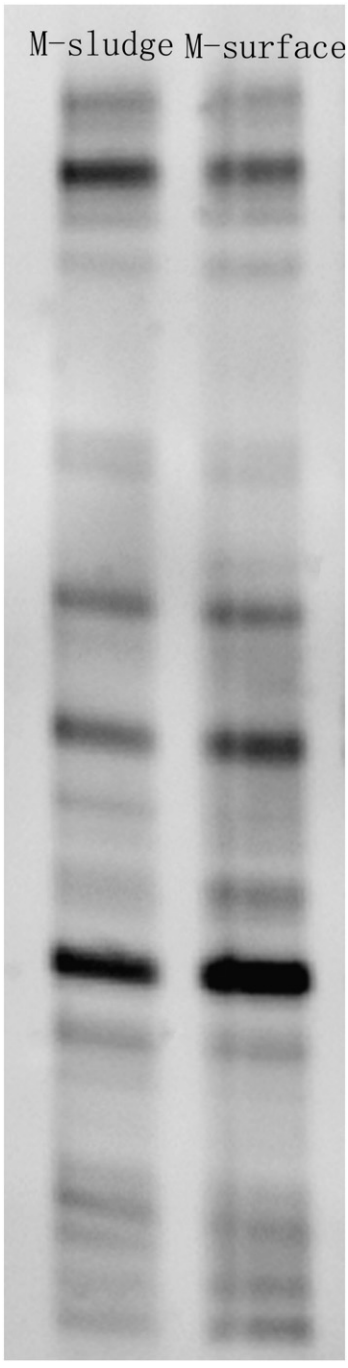

Fig. 7. DGGE fingerprints of microorganism from bulk activated sludge and membrane (sludge and surface denoted sludge samples of MBR suspended sludge and immobilized on the membrane).

efficiencies decreased in parallel. SND apparently controlled the performance of treatment; in particular, when salinity was ca. 21 $g \cdot L^{-1}$ and 28 $g \cdot L^{-1}$. EPS increased largely from salinity 0 $g \cdot L^{-1}$ to 35 $g \cdot L^{-1}$, resulting in significant membrane fouling. PCR-DGGE analysis revealed salt-tolerant microorganism present in MBR, the predominance of *T. eikelboomii, P. australicum*, and *P. bengalensis* might play crucial roles for this shortcut nitrification denitrification and nitrite accumulation.

Acknowledgments

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