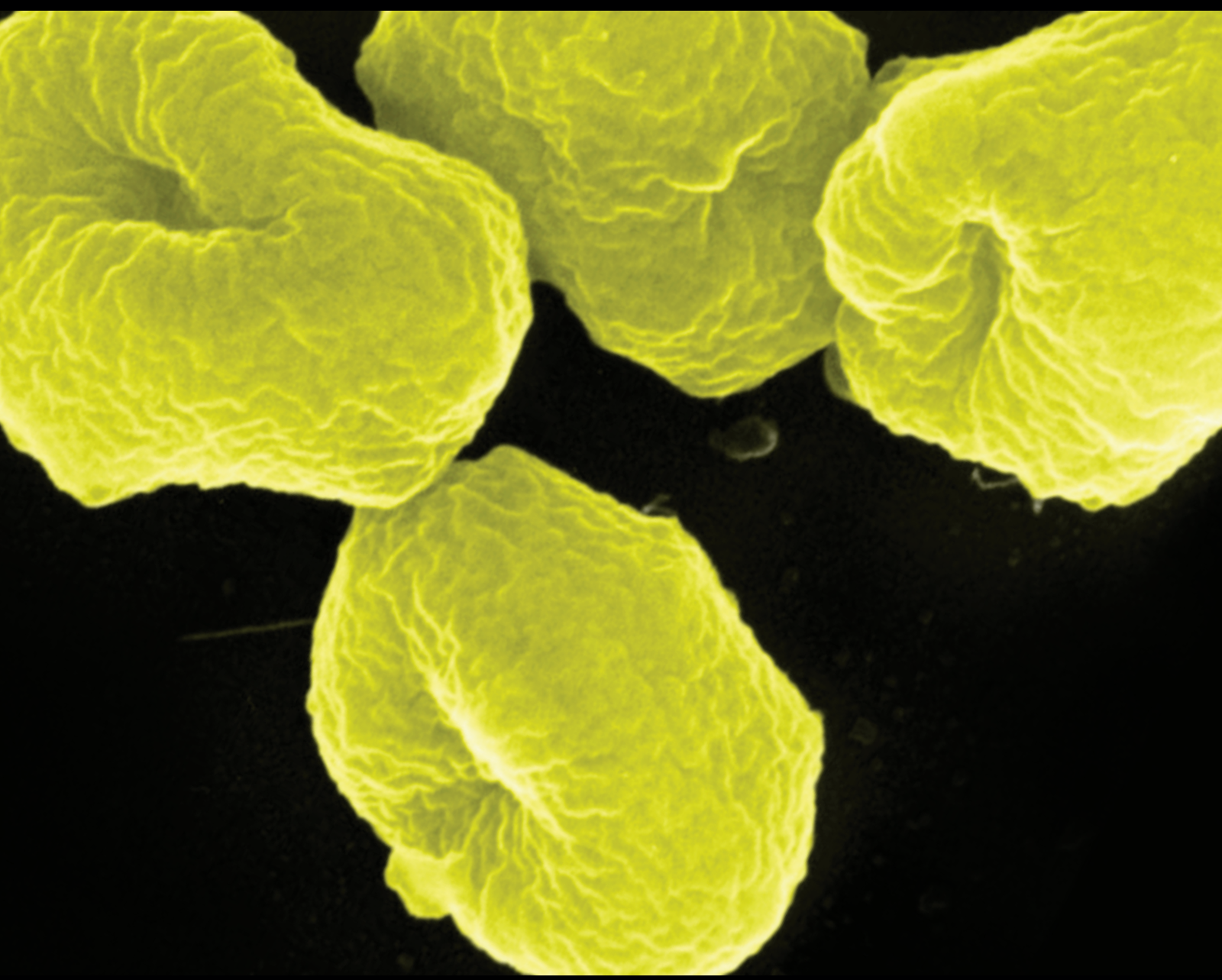
Archaea

Archaea in Wastewater Treatment: Current Research and Emerging Technology

Lead Guest Editor: Jin Li
Guest Editors: Rutao Liu, Yu Tao, and Guangbin Li



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Editorial Board

Contents

Hindawi
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Editorial

Archaea in Wastewater Treatment: Current Research and Emerging Technology

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Wastewater treatment is quite imperative for sustainable development and is critical for an ecosystem and for human health. Typically, wastewater is purified through multiple processes of microbial metabolisms. As a result, organic matters, ammonia, sulfate, and phosphate are either removed or transformed into other forms with lower harm to receiving aqua. We could not see a critical contribution of Archaea to wastewater treatment until recently when we learned that they are responsible for methane production, carbon mineralization, nitrification, and denitrification. Furthermore, a newly discovered denitrifying anaerobic methane oxidation process, through which methane is oxidized anaerobically, challenges the cliché concept of archaea involvement in wastewater treatment. To date, thousands of wastewater treatment facilities are confirmed to have an ecological and functional contribution by Archaea, which also benefits pollutant removal with low chemical and/or energy input.

Archaea-involved technology is essential for wastewater treatment by integrating energy production and resource recovery into a process for producing clean water. Archaea play important roles in converting pollutants into environmentally friendly materials. However, compared with bacteria that are widely studied in wastewater treatment systems, the characteristics and contributions of archaea are still not well known. For instance, ecological patterns of archaea in a complex wastewater microbiome are not fully understood, as well as the metabolisms of certain key archaea. For these reasons, we organized a special issue with a specific topic on why a comprehensive understanding of the identity, physiology, ecology, and population dynamics of archaea is urgently needed to improve wastewater treatment efficiency and process stability. It will be possible to find selective principles for regulating certain populations and managing a microbial community. It is entirely necessary to further study archaea in wastewater treatment. Such investigations can not only optimize the current wastewater treatment process but also innovate emerging technology.

Twelve papers, including both reviews and research articles, are selected in this Special Issue, covering the topics about the distributions and contributions of Archaea in wetlands, oilfield soil, and wastewater-treating bioreactors. Three review articles are highlighted in this Special Issue. One of the review papers focuses on the critical role of Archaea in bioremediation from halophilic hydrocarbon degradation to acidophilic hydrocarbon degradation in various environments such as oceans, soils, and acid mine drainage. Another article reviewed the characteristics and treatment of leachate, and more importantly, pointed out future directions for leachate research and development. The third review paper overviews the current knowledge on ammonium-oxidizing archaea and ammonium-oxidizing bacteria that are involved in wastewater treatment systems.

A wetland is an excellent combination of natural and engineered forms of wastewater treatment. Two research articles address bacterial and archaeal microbial community

structures in constructed wetlands, with one focusing on microbiome differences between sediments and water, while another one focuses on microbial interactions in a pilot-scale wetland that treated saline wastewater from a land-based Atlantic salmon plant.

A microbial world in oilfield soil is unveiled in this Special Issue by two research articles, one focusing on physicochemical properties, contents of primary pollutants, and fungal diversity of an aged oil sludge-contaminated soil, while another one focuses on soil bacterial community diversity around an aging oil sludge in the Yellow River Delta of China.

Bioreactor microbiomes are the most popular topics in this Special Issue, including four research articles. An integrated biofilm-membrane bioreactor treating mustard tuber wastewater was reported with a particular focus on microbial mechanisms leading to membrane fouling. Another study focused on microbial community and the performance of an autohydrogenotrophic membrane biofilm reactor for removing nitrate from the wastewater with high sulfate concentrations. Anaerobic digestion reactors are also addressed in two research articles, with one focusing on a transitional role of hydrogen-producing acetogens and its application in bioaugmentation, and another one discussing the bacterial and archaeal roles in an integrated anaerobic fluidized-bed membrane bioreactor treating synthetic high-strength benzothiazole wastewater.

With rapid growth in biomonitoring tools, we have learned more details of archaea-involved bioprocesses than at any time in history. Following the publication of this current Special Issue, we expect more exciting breakthroughs of Archaea studies in wastewater treatment from theory improvement to technology innovation.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Yu Tao
Guangbin Li

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Research Article

The Occurrence of Putative Nitric Oxide Dismutase (Nod) in an Alpine Wetland with a New Dominant Subcluster and the Potential Ability for a Methane Sink

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Recently, a new oxygenic pathway has been proposed based on the disproportionation of NO with putative NO dismutase (Nod). In addition to a new process in nitrogen cycling, this process provides ecological advantages for the degradation of substrates in anaerobic conditions, which is of great significance for wastewater treatment. However, the Nod distribution in aquatic environments is rarely investigated. In this study, we obtained the *nod* genes with an abundance of $2.38 \pm 0.96 \times 10^5$ copies per gram of dry soil from the Zoige wetland and aligned the molecular characteristics in the corresponding Nod sequences. These Nod sequences were not only found existing in NC10 bacteria, but were also found forming some other clusters with Nod sequences from a WWTP reactor or contaminated aquifers. Moreover, a new subcluster in the aquifer-similar cluster was even dominant in the Zoige wetland and was named the Z-aquifer subcluster. Additionally, soils from the Zoige wetland showed a high potential rate (10.97 ± 1.42 nmol of CO_2 per gram of dry soil per day) for nitrite-dependent anaerobic methane oxidation (N-DAMO) with low abundance of NC10 bacteria, which may suggest a potential activity of Nod in other clusters when considering the dominance of the Z-aquifer subcluster Nod. In conclusion, we verified the occurrence of Nod in an alpine wetland for the first time and found a new subcluster to be dominant in the Zoige wetland. Moreover, this new subcluster of Nod may even be active in the N-DAMO process in this alpine wetland, which needs further study to confirm.

1. Introduction

Photosynthesis is a widely known biological pathway that produces oxygen, but it is not the only pathway. In recent years, in addition to chlorate respiration [1] and detoxification of reactive oxygen species [2], a new oxygen-forming pathway has been proposed. The new pathway has the ability to produce oxygen in anaerobic conditions with nitrogen oxides (except N_2O) as substrates [3], which is very favorable for the degradation and oxidation of organic matter in anaerobic environments rich in nitrogen oxides, such as wetlands and wastewaters.

The critical step in this pathway is the proposed disproportionation of NO with putative NO dismutase (Nod) [3–6]. Nod was first proposed in enrichment cultures dominated by "*Candidatus* Methylomirabilis oxyfera," a representative species in the NC10 phylum [7, 8]. Currently, the NC10 bacteria have been reported to exist in various kinds of environments, such as lakes [9, 10], rivers [11], paddy fields [12], marine environments [13], and especially wetlands [14–16]. However, there are few reports of environmental Nod sequences. In a nitrite-dependent anaerobic methane oxidation (N-DAMO) reactor inoculated with river sediments, *nod* genes are first detected with specific primers [17]. Then, *nod* genes are reported to be abundant in contaminated aquifers and wastewater treatment systems [18]. In addition to these, *nod* transcripts in marine oxygen minimum zone water bodies were also reported [13].

Table 1: Physiochemical properties of soils from the Zoige wetland.

Samples	T (°C)	pH	Water content (%)	SO_4^{2-} (mg/l)	NH_4^+ (mg/l)	NO_3^- (μg/l)
Site1	13.0	7.42 ± 0.03	0.71 ± 0.02	5.69 ± 0.43	5.52 ± 0.94	13.33 ± 3.56
Site2	13.5	7.28 ± 0.02	0.73 ± 0.01	3.17 ± 0.68	4.08 ± 0.14	17.56 ± 0.58
Site3	13.5	7.31 ± 0.02	0.71 ± 0.01	2.26 ± 0.26	4.51 ± 0.97	27.67 ± 4.59

In addition to NC10 bacteria, Nod is also speculated to exist in a facultatively denitrifying γ-proteobacterium strain HdN1, which can grow on alkanes from C6 to C30 (except hexadecane) with nitrate or nitrite in anaerobic conditions [5, 6, 19]. This means Nod exists not only in NC10 phylum but also in other microbes that can use oxygen for substrate activation [6]. The direct investigation of Nod will be more valuable than just the investigation of NC10, especially in understanding the environmental significance of this oxygenic pathway.

With the oxygen produced by Nod, NC10 bacteria have the ability to aerobically oxidize methane in anaerobic conditions with nitrite as the electron acceptor [3]. NC10 and "*Candidatus* Methanoperedens nitroreducens," an archaeal group which oxides methane with nitrate as the electron acceptor, together perform the complete denitrifying anaerobic methane oxidation (DAMO). The DAMO process not only provides the unique link between the nitrogen and carbon cycles [20], but also has been considered as a solution to the sustainable operation of WWTP [21]. Therefore, the study of the key enzyme Nod in aquatic environments will not only promote the understanding of the metabolism of substances in the biogeochemical cycle, but also likely present better solutions for wastewater treatment operation.

Located on the Tibetan Plateau, the Zoige wetland is a typical alpine wetland and has large emissions of methane resulting from a huge carbon stock [22]. Although there is a prevalence of NC10 bacteria in wetlands [14–16], the presence and ecological significance of Nod in wetlands is still lacking. Here, we propose the following hypotheses: (1) Nod exists in this alpine wetland, and (2) microbes containing Nod may play a role in the methane sink of a natural wetland.

2. Materials and Methods

2.1. Sampling Methods. The samples used in this study were from the Zoige wetland, which is located on the Tibetan Plateau. Three sampling sites across the Zoige wetland were set in this study. For each sampling site, a five-point sampling method was used and sampling depths were 10 to 20 cm below the soil surface. For all the sampling sites, a depth of about 5 to 15 cm of standing water remained during the sampling period. The fresh soils were transported at 4°C to the laboratory. All experiments in this study were conducted in triplicates.

2.2. DNA Isolation. DNA was isolated from soils using a method described before [23] with slight modifications. The Lysing Matrix E tubes from MP Biomedicals were used with FastPrep-24 from the same company for the lysing step. Before the total nucleic acids were precipitated, RNase stock solution was added with a final concentration of 10 μg/ml. After being incubated at 37°C for at least one hour, the added enzyme was removed by repeated mixing of chloroform-isoamyl alcohol (24 : 1) and repeated centrifugation. The quality of the obtained DNA solutions was checked by agarose gel analysis, and the concentration was measured with a Nanodrop® ND-1000 UV-Vis spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

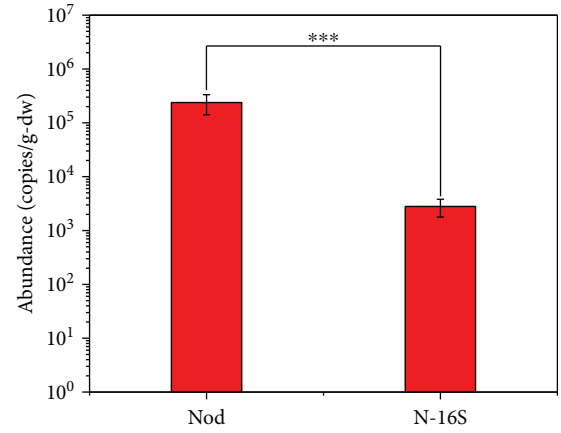

Figure 1: Abundance of NC10 16S rRNA and *nod* genes in soil from the Zoige wetland.

2.3. PCR and qPCR. DNA samples diluted 5- or 10-fold were used as templates for PCR and qPCR analyses. Primer pairs used for PCR and qPCR of the *nod* gene were nod684Fv2/nod1706Rv2 and nod1446F/nod1706Rv2, respectively [18]. The primer pair used for qPCR of NC10 16S rRNA gene was qP2F/qP2R [8]. The PCR reactions were performed using a volume of 26 μl with the following composition: 22 μl of Golden Star T6 Super PCR Mix (1.1x) (Beijing TsingKe Biotech Co. Ltd.), 1 μl of each primer (10 μM), and 2 μl of template DNA. The program was as follows: 98°C for 2 min, followed by 37 cycles of 98°C for 15 s, 57°C for 20 s, and 72°C for 30 s. Then, there was a final 5 min extension at 72°C. The sequences from qPCR products were verified by cloning and sequencing in pLB vector and were then used as standards for *nod* gene and NC10 16S rRNA gene. The standard curve concentrations for *nod* gene were from 2.580×10^3 to 2.580×10^{10} copies per gram of soil. The standard curve concentrations for NC10 16S rRNA gene were from 2.183×10^3 to 2.183×10^{10} copies per gram of soil. A Bio-Rad CFX Connect™ Real-Time PCR Detection System and SYBR® Premix Ex Taq™ (Tli RNaseH Plus) were employed in the qPCR reactions. The qPCR reactions were

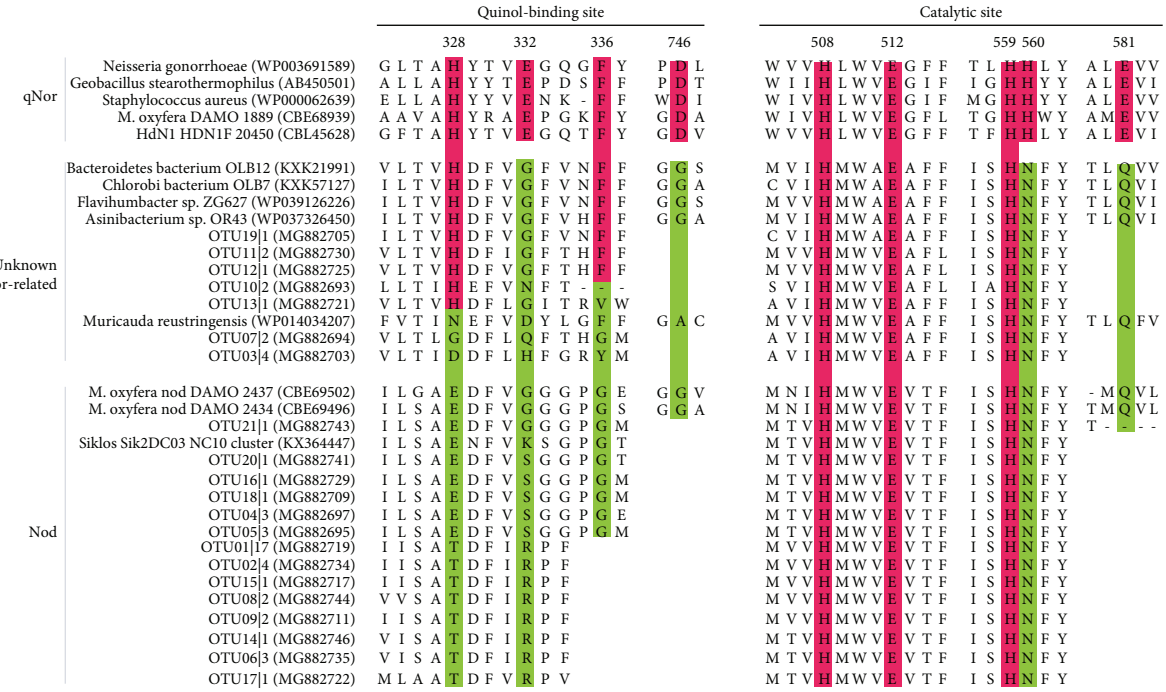

FIGURE 2: Alignment of the quinol-binding site and the catalytic site in Nod sequences and qNor sequences. The conserved residuals in qNor are highlighted in red, and the substitutions in putative Nod and putative Nor are shown in green.

performed using a volume of 25 μl with the following composition: 12.5 μl of SYBR Premix Ex Taq (Tli RNaseH Plus) (2x), 0.5 μl of each primer (10 μM), 2 μl of the DNA template, and 9.5 μl of sterile distilled water. The qPCR program was as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 30 s. Then, a melt curve was performed with 95°C for 5 s and 60°C to 95°C increasing at a rate of 0.5°C/5 s. The standards and samples were quantified in triplicate, and the analysis was performed with an efficiency of 100 ± 10%.

2.4. Cloning, Sequencing, and Phylogenetic Analysis. PCR products of the *nod* gene were purified with a TIANgel Midi Purification Kit (TIANGEN, Beijing) according to the manufacturer's protocol. Purified PCR products were cloned using a Lethal Based Fast Cloning Kit (TIANGEN, Beijing). The colonies were detected by PCR and agarose gel analysis for positive colonies, and then the PCR products of positive colonies were sent to sequencing. The high-quality sequences obtained were assigned to the same operational taxonomic units (OTUs) by Mothur based on a cutoff of 0.03. Also, the rarefaction curve was also calculated with Mothur. Then, the representative sequences of each OTU were translated to amino acids and aligned with selected qNor, cNor, and some published Nod sequences using a previously published method [18]. Then, based on the alignment file, a phylogenetic tree was constructed with MEGA7 using the neighbor-joining method.

2.5. Incubation Experiments. In an anaerobic box, soils were mixed with sterile anaerobic distilled water in a volume ratio of 1 : 4, and the roots in the slurry were removed. Then, the slurry was split into 120 ml glass vials with 10 ml of slurry in each bottle. After being sealed with butyl rubber stoppers and aluminum caps, these bottles were taken out of the anaerobic box. Then, the bottles were vacuumed and flushed with high-purity argon for 5 min in five cycles. After the final step of the flush, the pressure in the headspace gas was balanced by a syringe. Then, these bottles were preincubated at 14°C for 116 days. After preincubation, $NaNO_2$ was added with a final concentration of 200 μM in triplicate bottles, and sterile anaerobic distilled water was used for the control. After the headspaces of these bottles were all vacuumed and flushed like before, 5 ml of gas of the headspace was replaced by an equal volume of $^{13}CH_4$ (99.9% purity, 99.8% atom% ^{13}C). Samples were incubated at 14°C. The production of $^{13}CO_2$ was measured by a gas chromatograph mass spectrometer (GCMS-QP2010 Ultra, Shimadzu). The rates were calculated by a linear regression of the produced $^{13}CO_2$ over time.

2.6. Statistical Analyses. All statistical analyses in this article were conducted using SPSS software (PASW Statistics 18, IBM, USA). The significance of the difference between the abundance of NC10 16S rRNA gene and *nod* gene was performed by a nonparametric test. The significance of difference between rates in different treatments was calculated by a general linear model (univariate).

3. Results and Discussion

The physiochemical properties of soils from the Zoige wetland are shown in Table 1. The results of the quantification showed that we obtained the amplification products of *nod* genes in the Zoige wetland with an abundance of $2.38 \pm 0.96 \times 10^5$ copies per gram of dry soil (Figure 1).

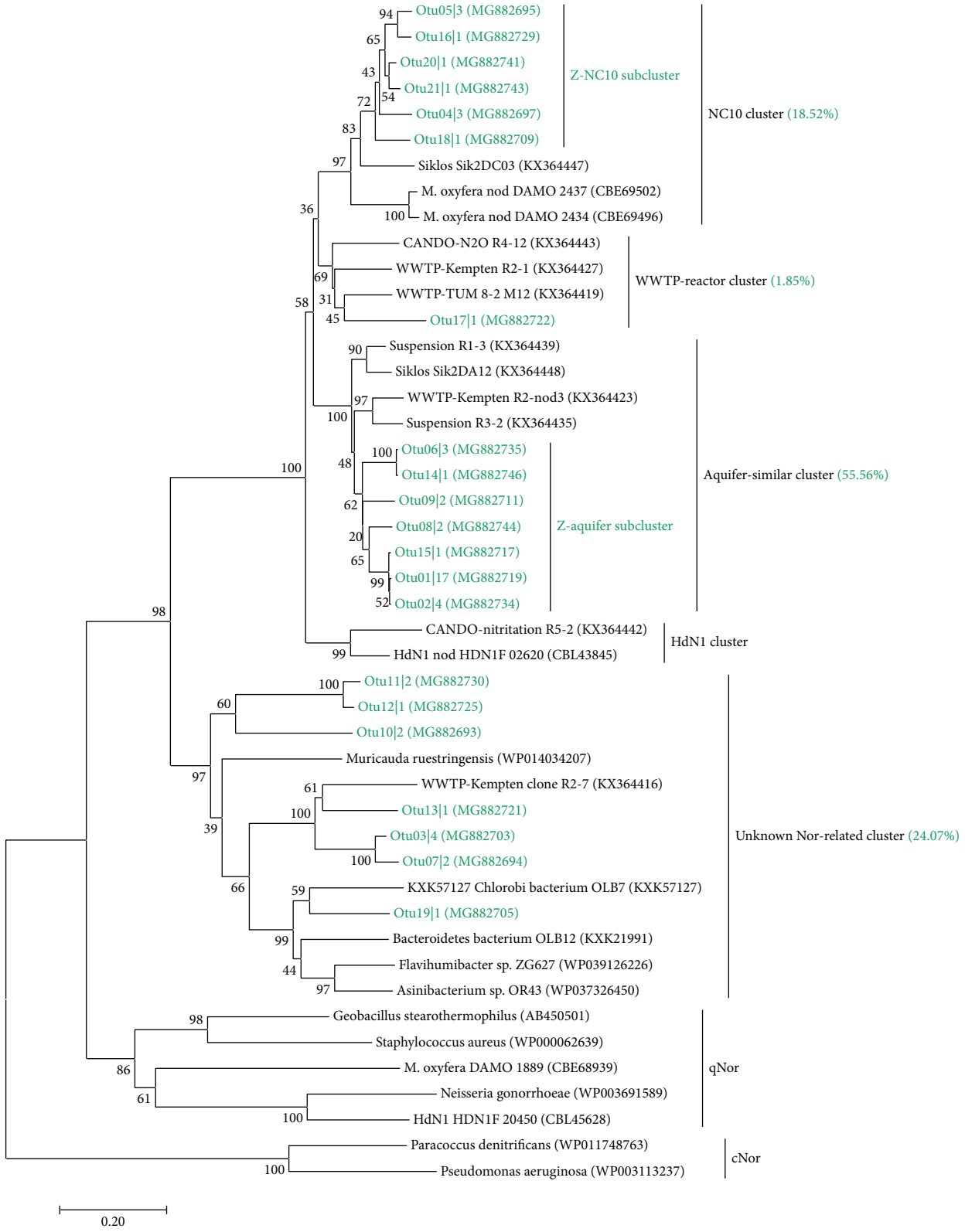

Figure 3: Phylogenetic tree of putative Nod obtained in this study with selected qNor, cNor, and Nod sequences. The accession numbers of the reference sequences and representative *nod* sequences obtained in this study are shown in parentheses. The Nod sequences obtained in this study are shown in green. The relative abundance of the four clusters obtained in this study are shown in parentheses after the names of the clusters.

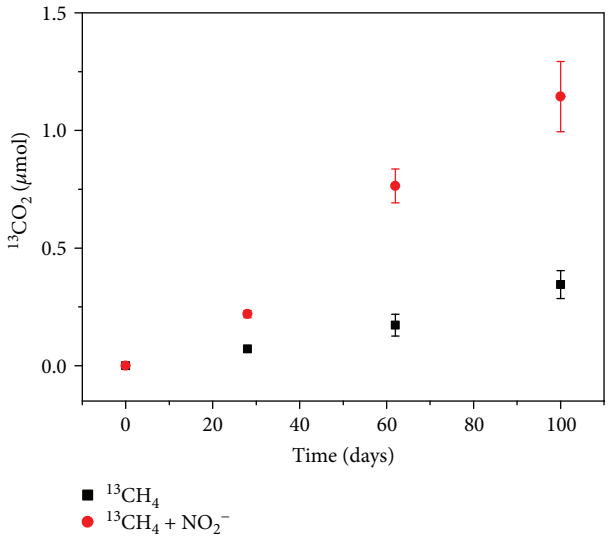

FIGURE 4: The production rate of $^{13}CO_2$ from $^{13}CH_4$ in the incubation of soils from the Zoige wetland.

Since Nod is proposed based on quinol-dependent NO reductase (qNor) paralogs, it has a close phylogenetic distance with canonical qNor, and the characteristics for Nod are several amino acid substitutions in the quinol-binding sites and catalytic sites, which are essential for the canonical qNor [6, 24]. The Nod sequences (translated from *nod* sequences) recovered in the Zoige wetland also had substitutions in these key amino acid sites, which are similar to those in *M. oxyfera* (Figure 2). These results suggest the actual occurrence of Nod in the Zoige wetland.

The results of the quantification also verified the existence of NC10 bacteria with an abundance of $2.80 \pm 1.02 \times 10^3$ copies per gram of dry soil, which is significantly lower than that of *nod* genes (Figure 1). This may be the result of more *nod* gene copy numbers than 16S rRNA in one single cell or the *nod* genes may exist in microbes other than NC10 bacteria. After phylogenetic analysis of the obtained 54 high-quantity Nod sequences (21 OTUs), we found that there are partial sequences that have large distances with the published *M. oxyfera* Nod sequences (Figure 3). All the sequences obtained from the Zoige wetland formed three clusters with published Nod sequences and one cluster with some unknown Nor-related sequences, which were all distinct from qNor and cNor (Figure 3). The three clusters of Nod were named after the closely related published Nod sequences [18], namely, the NC10 cluster, the aquifer-similar cluster, and the WWTP-reactor cluster. In addition, Nod sequences from the Zoige wetland in the NC10 cluster and the aquifer-similar cluster even formed subclusters, which were named as Z-NC10 subcluster and Z-aquifer subcluster, respectively. The sequences in the aquifer-similar cluster and the WWTP-reactor cluster were not only distinct from the NC10 Nod clusters but also had distances with the HdN1 Nod cluster. Moreover, in the alignment with qNor sequences, substitutions of the His328 and Glu332 sites in the Nod sequences of the aquifer-similar cluster and the WWTP-reactor cluster were different from those in the NC10 Nod sequences (Figure 2). These results suggest that the Nod sequences in the aquifer-similar cluster and the WWTP-reactor cluster are from microbes other than NC10 and HdN1. In addition, these unknown microbes were more abundant than NC10 in the Zoige wetland according to the relative abundance of each cluster (Figure 3).

With the activity of Nod, NC10 has the ability to oxide methane in anaerobic conditions with nitrite as the electron acceptor. To test the N-DAMO activity of soils from the Zoige wetland, $^{13}CH_4$ was added to trace the methane oxidation process. The result showed that the methane oxidation rate with nitrite as the electron acceptor was 15.39 ± 1.29 nmol of CO_2 per gram of dry soil per day ($R^2 = 0.97$), which is significantly higher ($P \leq 0.01$) than the methane oxidation rate in the control (4.43 ± 0.43 nmol of CO_2 per gram of dry soil per day ($R^2 = 0.97$)) (Figure 4). This suggests a significant N-DAMO activity in soils from the Zoige wetland. After calculation, the net oxidation rate of methane was 10.97 ± 1.42 nmol of CO_2 per gram of dry soil per day ($R^2 = 0.95$). According to a previous report [25], the mean methane flux in the Zoige wetland was approximately 2.43 mg m^{-2} h^{-1}. The density of soils from the Zoige wetland was measured to be 0.31 g/ml in the current study. Assuming that the active layer of N-DAMO was only in the depth of 10–20 cm, which is the sampling depth in the current study, the net N-DAMO was about 0.23 mg m^{-2} h^{-1}, which is about 9.5% of the reported methane fluxes. In addition, the N-DAMO rate in our study is similar to that of a minerotrophic peatland [14] and even higher than the rate in an urban wetland [26] and some other wetlands [15]. However, the abundance of NC10 in the Zoige wetland ($2.80 \pm 1.02 \times 10^3$) is much lower than its abundance in all these wetlands, which is approximately 10^6–10^7 copies per gram of soil. Moreover, in these previous reports [15, 26], N-DAMO activity was usually not detected when the abundance of NC10 declined close to 10^5 copies per gram of soil. Therefore, the high N-DAMO rate in the current study may not only be performed by the NC10 bacteria, especially considering the dominance of new subclusters in the Zoige wetland (Figure 3). This means that the Nod in some unknown microbes may also be active in utilizing nitrous oxides and may even play a role in coupling carbon and nitrogen cycling. This speculation needs further studies to confirm, such as the analysis of the ^{13}C-labeled DNA in the N-DAMO process.

4. Conclusion

This study revealed the occurrence of *nod* gene in an alpine wetland for the first time with an abundance of $2.38 \pm 0.96 \times 10^5$ copies per gram of dry soil. In addition to the reported Nod in NC10 bacteria, there were some different Nod sequences and one of a subcluster (Z-aquifer subcluster) that was even dominant in the Zoige wetland. Moreover, soils from the Zoige wetland exhibited a high N-DAMO rate with a low abundance of NC10 bacteria, which may mean a potential ability of Nod in other clusters to oxide methane in an anaerobic condition. However, this speculation needs further work to confirm.

Data Availability

The representative nod sequences and nor-related sequences obtained in this study were deposited at NCBI under the accession numbers MG882693~MG882746. It is also available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

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Supplementary Materials

Figure S1: rarefaction curve for the nod gene library in this study. *(Supplementary Materials)*

References

[1] G. B. Rikken, A. G. M. Kroon, and C. G. van Ginkel, "Transformation of (per) chlorate into chloride by a newly isolated bacterium: reduction and dismutation," *Applied Microbiology and Biotechnology*, vol. 45, no. 3, pp. 420–426, 1996.

[2] K. Apel and H. Hirt, "Reactive oxygen species: metabolism, oxidative stress, and signal transduction," *Annual Review of Plant Biology*, vol. 55, no. 1, pp. 373–399, 2004.

[3] K. F. Ettwig, M. K. Butler, D. le Paslier et al., "Nitrite-driven anaerobic methane oxidation by oxygenic bacteria," *Nature*, vol. 464, no. 7288, pp. 543–548, 2010.

[4] M. L. Wu, K. F. Ettwig, M. S. M. Jetten, M. Strous, J. T. Keltjens, and L. Niftrik, "A new intra-aerobic metabolism in the nitrite-dependent anaerobic methane-oxidizing bacterium *Candidatus* "Methylomirabilis oxyfera"," *Biochemical Society Transactions*, vol. 39, no. 1, pp. 243–248, 2011.

[5] J. Zedelius, R. Rabus, O. Grundmann et al., "Alkane degradation under anoxic conditions by a nitrate-reducing bacterium with possible involvement of the electron acceptor in substrate activation," *Environmental Microbiology Reports*, vol. 3, no. 1, pp. 125–135, 2011.

[6] K. F. Ettwig, D. R. Speth, J. Reimann, M. L. Wu, M. S. M. Jetten, and J. T. Keltjens, "Bacterial oxygen production in the dark," *Frontiers in Microbiology*, vol. 3, p. 273, 2012.

[7] K. F. Ettwig, S. Shima, K. T. van de Pas-Schoonen et al., "Denitrifying bacteria anaerobically oxidize methane in the absence of *Archaea*," *Environmental Microbiology*, vol. 10, no. 11, pp. 3164–3173, 2008.

[8] K. F. Ettwig, T. van Alen, K. T. van de Pas-Schoonen, M. S. M. Jetten, and M. Strous, "Enrichment and molecular detection of denitrifying methanotrophic bacteria of the NC10 phylum," *Applied and Environmental Microbiology*, vol. 75, no. 11, pp. 3656–3662, 2009.

[9] J. S. Deutzmann, P. Stief, J. Brandes, and B. Schink, "Anaerobic methane oxidation coupled to denitrification is the dominant methane sink in a deep lake," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 51, pp. 18273–18278, 2014.

[10] H. Kojima, M. Tsutsumi, K. Ishikawa, T. Iwata, M. Mußmann, and M. Fukui, "Distribution of putative denitrifying methane oxidizing bacteria in sediment of a freshwater lake, Lake Biwa," *Systematic and Applied Microbiology*, vol. 35, no. 4, pp. 233–238, 2012.

[11] L.-d. Shen, S. Liu, Q. Zhu et al., "Distribution and diversity of nitrite-dependent anaerobic methane-oxidising bacteria in the sediments of the Qiantang River," *Microbial Ecology*, vol. 67, no. 2, pp. 341–349, 2014.

[12] L.-d. Shen, S. Liu, Q. Huang et al., "Evidence for the cooccurrence of nitrite-dependent anaerobic ammonium and methane oxidation processes in a flooded paddy field," *Applied and Environmental Microbiology*, vol. 80, no. 24, pp. 7611–7619, 2014.

[13] C. C. Padilla, L. A. Bristow, N. Sarode et al., "NC10 bacteria in marine oxygen minimum zones," *The ISME Journal*, vol. 10, no. 8, pp. 2067–2071, 2016.

[14] B. Zhu, G. van Dijk, C. Fritz et al., "Anaerobic oxidization of methane in a minerotrophic peatland: enrichment of nitrite-dependent methane-oxidizing bacteria," *Applied and Environmental Microbiology*, vol. 78, no. 24, pp. 8657–8665, 2012.

[15] B. L. Hu, L. D. Shen, X. Lian et al., "Evidence for nitrite-dependent anaerobic methane oxidation as a previously overlooked microbial methane sink in wetlands," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 12, pp. 4495–4500, 2014.

[16] G. Zhu, L. Zhou, Y. Wang et al., "Biogeographical distribution of denitrifying anaerobic methane oxidizing bacteria in Chinese wetland ecosystems," *Environmental Microbiology Reports*, vol. 7, no. 1, pp. 128–138, 2015.

[17] A. S. Bhattacharjee, A. M. Motlagh, M. S. M. Jetten, and R. Goel, "Methane dependent denitrification—from ecosystem to laboratory-scale enrichment for engineering applications," *Water Research*, vol. 99, pp. 244–252, 2016.

[18] B. Zhu, L. Bradford, S. Huang et al., "Unexpected diversity and high abundance of putative nitric oxide dismutase (Nod) genes in contaminated aquifers and wastewater treatment systems," *Applied and Environmental Microbiology*, vol. 83, no. 4, pp. e02750–e02716, 2017.

[19] P. Ehrenreich, A. Behrends, J. Harder, and F. Widdel, "Anaerobic oxidation of alkanes by newly isolated denitrifying bacteria," *Archives of Microbiology*, vol. 173, no. 1, pp. 58–64, 2000.

[20] Y. Wang, D. Wang, Q. Yang, G. Zeng, and X. Li, "Wastewater opportunities for denitrifying anaerobic methane oxidation," *Trends in Biotechnology*, vol. 35, no. 9, pp. 799–802, 2017.

[21] D. Wang, Y. Wang, Y. Liu et al., "Is denitrifying anaerobic methane oxidation-centered technologies a solution for the sustainable operation of wastewater treatment plants?," *Bioresource Technology*, vol. 234, pp. 456–465, 2017.

[22] H. Chen, N. Wu, Y. Wang, Y. Gao, and C. Peng, "Methane fluxes from alpine wetlands of Zoige Plateau in relation to water regime and vegetation under two scales," *Water, Air, & Soil Pollution*, vol. 217, no. 1–4, pp. 173–183, 2011.

[23] R. I. Griffiths, A. S. Whiteley, A. G. O'Donnell, and M. J. Bailey, "Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-

based microbial community composition," *Applied and Environmental Microbiology*, vol. 66, no. 12, pp. 5488–5491, 2000.

[24] J. Reimann, M. S. M. Jetten, and J. T. Keltjens, "Metal enzymes in "impossible" microorganisms catalyzing the anaerobic oxidation of ammonium and methane," in *Sustaining Life on Planet Earth: Metalloenzymes Mastering Dioxygen and Other Chewy Gases*, P. M. H. Kroneck and M. E. Sosa Torres, Eds., pp. 257–313, Springer International Publishing, Cham, 2015.

[25] D. X. Wang, "Emission fluxes of carbon dioxide, methane and nitrous oxide from peat marsh in Zoige Plateau," *Wetland Science*, vol. 8, no. 3, pp. 220–224, 2010.

[26] L.-d. Shen, S. Liu, Z. F. He et al., "Depth-specific distribution and importance of nitrite-dependent anaerobic ammonium and methane-oxidising bacteria in an urban wetland," *Soil Biology and Biochemistry*, vol. 83, pp. 43–51, 2015.

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Research Article

Variation of Bacterial and Archaeal Community Structures in a Full-Scale Constructed Wetlands for Wastewater Treatment

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Microorganisms play important roles in the reduction of organic and inorganic pollutants in constructed wetlands used for the treatment of wastewater. However, the diversity and structure of microbial community in constructed wetland system remain poorly known. In this study, the Illumina MiSeq Sequencing of 16S rDNA was used to analyze the bacterial and archaeal microbial community structures of soil and water in a free surface flow constructed wetland, and the differences of bacterial communities and archaeal compositions between soil and water were compared. The results showed that the Proteobacteria were the dominant bacteria, making up 35.38%~48.66% relative abundance. Euryarchaeotic were the absolute dominant archaea in the influent sample with the relative abundance of 93.29%, while Thaumarchaeota showed dominance in the other three samples, making up 50.58%~75.70%. The relative abundances of different species showed great changes in bacteria and archaea, and the number of dominant species in bacteria was much higher than that in archaea. Compared to archaea, the community compositions of bacteria were more abundant and the changes were more significant. Meanwhile, bacteria and archaea had large differences in compositions between water and soil. The microbial richness in water was significantly higher than that in soil. Simultaneously, soil had a significant enrichment effect on some microbial flora.

1. Introduction

As a new type of sewage treatment system, constructed wetlands have gradually entered the field of vision. Constructed wetlands for wastewater treatment were widely used in developed countries, such as the United States and Germany, because of its low costs, good removal rates for organic substances and also for nutrients (N, P), and higher surface water quality [1]. Shandong Province had built many constructed wetlands which occupied 7.6% of the land [2] and mainly distributed in Nansi Lake and Dongping Lake [3]. The constructed wetlands could remove pollutants through providing habitats for microbes to stimulate their activities [4]; therefore, microorganisms were particularly important in the reduction of organic and inorganic pollutants in constructed wetlands. Due to the uncertainty and variability of the distribution of microbial community structure in constructed wetlands, it had aroused the interest and attention of scholars.

At present, extensive researches have been conducted on microbial community structure of sewage treatment systems [5–7]. Recently, with the development of high-throughput sequencing technology, it has also been widely used in environmental samples, such as the bacterial community structures in airborne [8] and water [9] and the archaeal community structures in soil [10], even in the sludge of wastewater treatment [11]. However, the above studies have rarely analyzed the bacterial and archaeal community structures of the same samples at the same time. Similar studies also show significant differences due to environmental differences in the study sites.

Therefore, in this study, the water and soil samples, collected from a free surface flow constructed wetland, were assessed by Illumina MiSeq high-throughput method, the objective was to investigate the microbial community structures and compare the microbial abundance differences between water and soil, including bacteria and archaea.

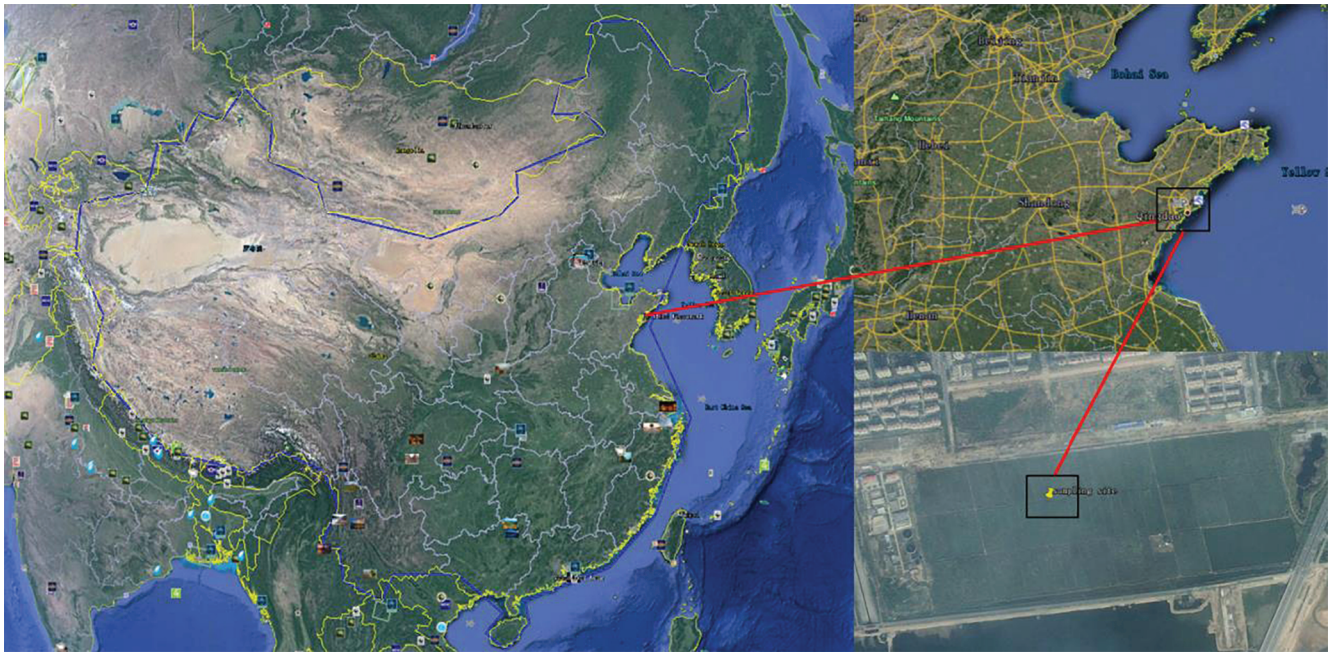

Figure 1: Map showing the location of the sampling sites in constructed wetland.

2. Methods

2.1. Sampling Sites. The free water surface constructed wetland, located in the interior of Huangdao District (Qingdao City, Shandong Province, China), at a latitude of 35°35′ to 36°08′ north and a longitude of 119°30′ to 120°11′ east, is a part of an integrated sewage purification system. This region has a warm temperate continental monsoon with a mean annual temperature of 12.0°C and a mean annual precipitation of 794 mm. The constructed wetland wastewater treatment system had a total area of 76.7 hm^2 and a treatment capability of 3.0×10^4 $m^3 \cdot d^{-1}$ and was surrounded by the Yellow Sea on east and south. It consisted of 99 treatment beds and received secondary unchlorinated wastewater from Jiaonan Municipal Wastewater Treatment Facility with A^2O as the secondary treatment. All beds were planted with common reed (*Phragmites australis*) and a number of naturally germinated wetland plants (*Typha orientalis*, *Scirpus validus*, *Lemna minor*, etc.). To facilitate the harvest progress of above-ground biomass, sewage did not enter the constructed wetland bed from December to March of next year. In this study, two different constructed wetland treatment units with and without sewage water were selected, wet soil and dry soil from each unit, and influent and effluent from unit with sewage water were sampled in May 2017. Detailed geographic information of the sampling sites is shown in Figure 1.

2.2. Sampling Methods. 50 g of soil sample and 10 L of water sample were collected from each sample site by sterile sealed bags and sterile bottles, respectively. After removing the fine roots in soil samples, the water and soil samples were transferred to the laboratory immediately. After dewatered by centrifugation, a fraction of the soil samples were stored at −20°C for molecular analysis. A part of water samples was filtered by a vacuum pump with 45-mm-diameter microporous membrane, then using douching and centrifugation method carefully transferred into 2 mL sterile centrifuge tubes and stored at −20°C until DNA extraction; meanwhile, the other part was stored at 4°C for chemical analysis.

2.3. DNA Extraction. Soil DNA and water DNA were extracted from 500 mg of frozen soil and 500 mg of filter residue, respectively, using a Soil DNA Kit (OMEGA, China) according to the manufacturer's instructions. The extracted DNA was checked using the UV/nucleic acid protein detector (IMPLEN, Germany).

2.4. Illumina MiSeq Sequencing. Targeting target sequences reflects the compositions and diversities of microbes, designing corresponding primers according to the conserved regions in the sequences and adding sample-specific barcode sequences to further amplify the variable region of the rRNA gene (single or continuous) or specific gene fragments for PCR amplification. PCR amplification products were detected by 2% agarose gel electrophoresis, and the target fragment was excised from the gel. PCR products were recovered for fluorescence quantification, according to the needs of each sample sequencing volume, and the samples were mixed in the appropriate ratio. Sequencing libraries were prepared using Illumina's TruSeq Nano DNA LT Library Prep Kit and on the machine for high-throughput sequencing.

2.5. Sequence Data Analyses. In order to integrate the original double-end sequencing data, the two-terminal sequence of FASTQ format was first screened by sliding window. The size of the window is 10 bp and the step size is 1 bp. Starting from the first base position on the 5′ end, the average base mass in the window is ≥Q20 (i.e., the base average measurement

Table 1: Physical and chemical characteristics in samples.

Samples	Total phosphorus (g/kg)	Total nitrogen (g/kg)	Organic matter (g/kg)	Samples	Dissolved oxygen (mg/L)	Ammonia nitrogen (mg/L)	Nitrite nitrogen (mg/L)	pH
Dry soil	2.66	22.12	391.61	Influent	8.93	6.05	1.06	6.97
Wet soil	0.38	7.56	26.75	Effluent	11.53	1.30	0.36	6.95

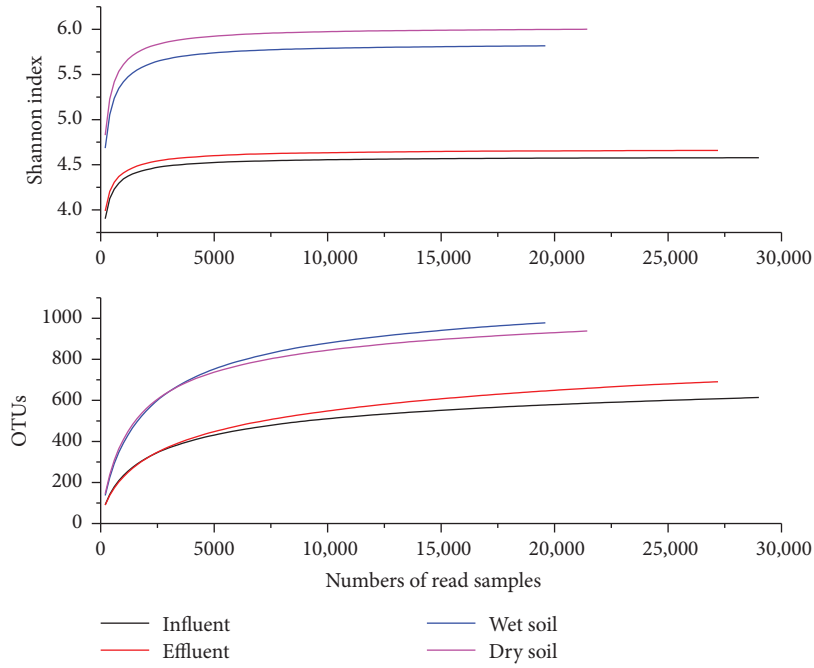

Figure 2: Bacterial rarefaction curves and Shannon diversity index curves.

accuracy is ≥99%). From the first truncated sequences at windows, with average mass values below Q20, we requir a truncated sequence ≥150 bp in length with no ambiguous base N allowed. Subsequently, the FLASH software [12](v1.2.7, http://ccb.jhu.edu/software/FLASH/) was used to pair the double-stranded sequences that passed the quality screening according to overlapping bases. It is required that the overlapping base length of two sequences of read 1 and read 2 be ≥10 bp and the base mismatch is not allowed. Finally, based on the index information (i.e., barcode sequence, for the beginning of the sequence used to identify a small base sequence) corresponding to each sample, the connected sequence identification is assigned to the corresponding sample (requires index sequence exactly match), to obtain a valid sequence for each sample.

3. Results

3.1. Physical and Chemical Characteristics of Soil and Water in Constructed Wetlands. The results of soil and water basic properties were listed in Table 1. All the chemical indicators of wet soil were far below the dry soil, especially content of organic matter, and dry soil was about 15 times more than wet soil. The constructed wetlands had a very good purification effect; ammonia nitrogen and nitrite nitrogen in effluent decreased obviously. Simultaneously, the content of dissolved oxygen also improved.

3.2. Bacterial Community Structures of Soil and Water in Constructed Wetlands

3.2.1. Bacterial Alpha Diversity Analysis. Rarefaction curves of the four samples were shown in Figure 2. The rarefaction curves and Shannon diversity index curves clearly revealed that the bacterial community structures of soil samples were considerably higher than those in water samples. Two kinds of curves tended to be gentle, suggesting that the sequencing results had been enough to reflect the diversity of the current sample, and increasing the depth of sequencing could not detect more new OUTs. The sequencing results could basically reflect the microbial community structures of four samples.

A total of 29,000, 27,204, 19,597, and 21,439 trimmed reads for samples influent, effluent, wet soil, and dry soil were obtained, respectively, after the removal of unqualified reads (Table 2). ACE estimator [13] and Chao1 estimator [14] were used to estimate the number of species actually present in the community. The greater Chao1 estimator, the higher richness of the community, and so was the ACE estimator. The community richness in soil samples was much higher than that in water samples, showing microbes were more likely

Table 2: Bacterial alpha diversity indices of four samples.

Samples	Reads	OTUs	ACE	Chao1	Shannon	Simpson
Influent	29,000	614	676.31	707.02	4.58	0.05
Effluent	27,204	691	792.39	783.76	4.66	0.03
Wet soil	19,597	978	1051.89	1080.43	5.82	0.01
Dry soil	21,439	938	1002.35	1019.01	6.00	0.01

to attach to solid particles. Shannon diversity index [15] and Simpson index [16] were both the commonly used index for evaluating community diversity; the higher Shannon index and the lower Simpson diversity index could explain the higher community diversity. The community diversity in dry soil sample was the highest in this study, while that in influent sample was the lowest. Simpson index was more sensitive to uniformity and dominant OTUs in the community, and it demonstrated a high degree of uniformity in four samples.

3.2.2. Bacterial Community Structures of Soil and Water in Constructed Wetlands. Bacterial sequences in the four samples were classified into taxonomic classes using the default settings of the mothur platform. A total of 29 bacterial phyla were found in this study. The total phylum numbers in influent, effluent, wet soil, and dry soil were 22, 23, 23, and 23, respectively. Four samples were similar in the number of phyla levels, but quite different in compositions, and the detailed relative abundances were shown in Figure 3. In the four samples, Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Cyanobacteria, and Chloroflexi were the most common bacterial phyla with a high relative abundance, while the proportion of the other phyla were very low. Proteobacteria were the most dominant phylum in the four samples with the relative abundance of 35.38%~48.66%. Firmicutes in influent sample (30.12%) and Bacteroides in effluent (30.03%) and wet soil (20.05%) samples also showed in absolute superiority. Different from the other three samples, the proportion of Chloroflexi in dry soil was high, accounting for 18.96%. The community structures of the two water samples were more similar, and so were the two soil samples. Most bacterial phyla were found in all 4 samples, such as Verrucomicrobia, Planctomycetes, and Ignavibacteriae. However, Aquificae, Lentisphaerae, and Synergistetes were emerged only in the water samples, while Thermotogae, Deferribacteres, Calditrichaeota, and Armatimonadetes existed only in the soil samples. Tenericutes and Fusobacteria were detected in all samples except dry soil, while Balneolaeota were emerged only in the effluent with a very low relative abundance. It is worth noting that Euryarchaeota, which belonged to archaea, were also detected in this bacterial sequencing.

The distribution characteristics of classes were analyzed, and the results were shown in Figure 4. A total of 68 bacterial classes were found in this study. The total class numbers in influent, effluent, wet soil, and dry soil were 44, 47, 55, and 53, respectively. The abundance distributions of Alphaproteobacteria, Sphingobacteriia, and Gammaproteobacteria in the four samples were relative average, while the relative

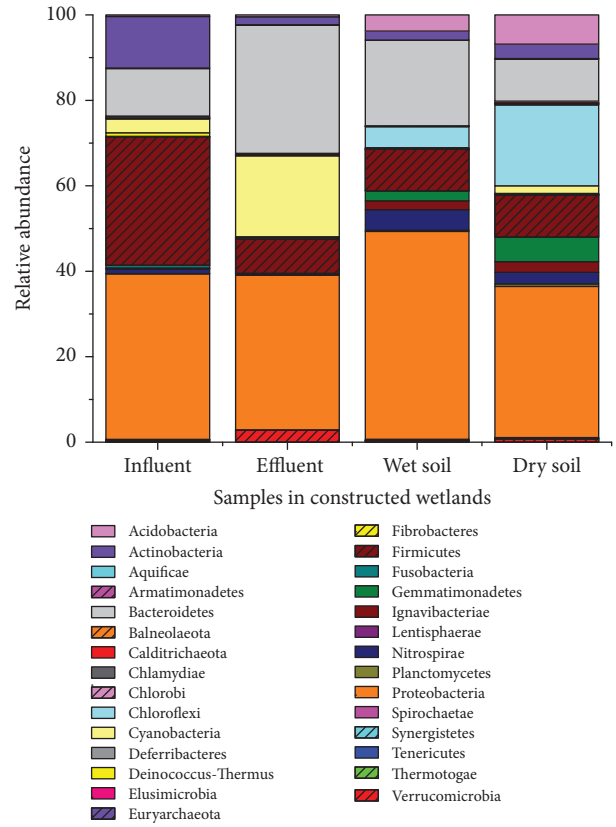

Figure 3: Bacterial relative abundance of four samples in phyla in constructed wetlands.

abundances of the other classes were quite different. Clostridia, Actinobacteria, and Epsilonproteobacteria were the dominant classes in the influent with the relative abundance of 26.91%, 11.96%, and 7.76%, respectively, while they did not exceed 3% in the other three samples; however, the relative abundances of Fusobacteriia were obviously higher in influent than the other three samples. The relative abundances of Fimbriimonadia and Fibrobacteria in wet soil and Ignavibacteria in dry soil were much higher than the other three samples. Cyanobacteria and Flavobacteriia were most frequently detected in effluent accounting for 18.84% and 18.02%, respectively, while they showed a lower relative abundance in the other three samples. The relative abundances of Betaproteobacteria were less than 10% in the dry soil, while it showed advantage in the other three samples accounting for 18.15%~20.9%. Coriobacteriia, Chloroflexia, etc., a total of 19 classes, were only detected in the soil samples, and among them, there were 3 classes only in wet soil and 5 classes in dry soil. Deltaproteobacteria, Erysipelotrichia, etc., a total of 8 classes, were only detected in the water samples and 3 classes emerged only in effluent.

Due to the huge amount of data, the dominant genera, with relative abundances over 1%, were listed in Table 3. A total of 40 bacterial genera were found. The total genus numbers in influent, effluent, wet soil, and dry soil were 28, 33, 28, and 22, respectively. In influent sample, in addition to *Mycobacterium* and *Rhodoferax*, the other 10 dominant genera in the other three samples, the relative abundances were all less

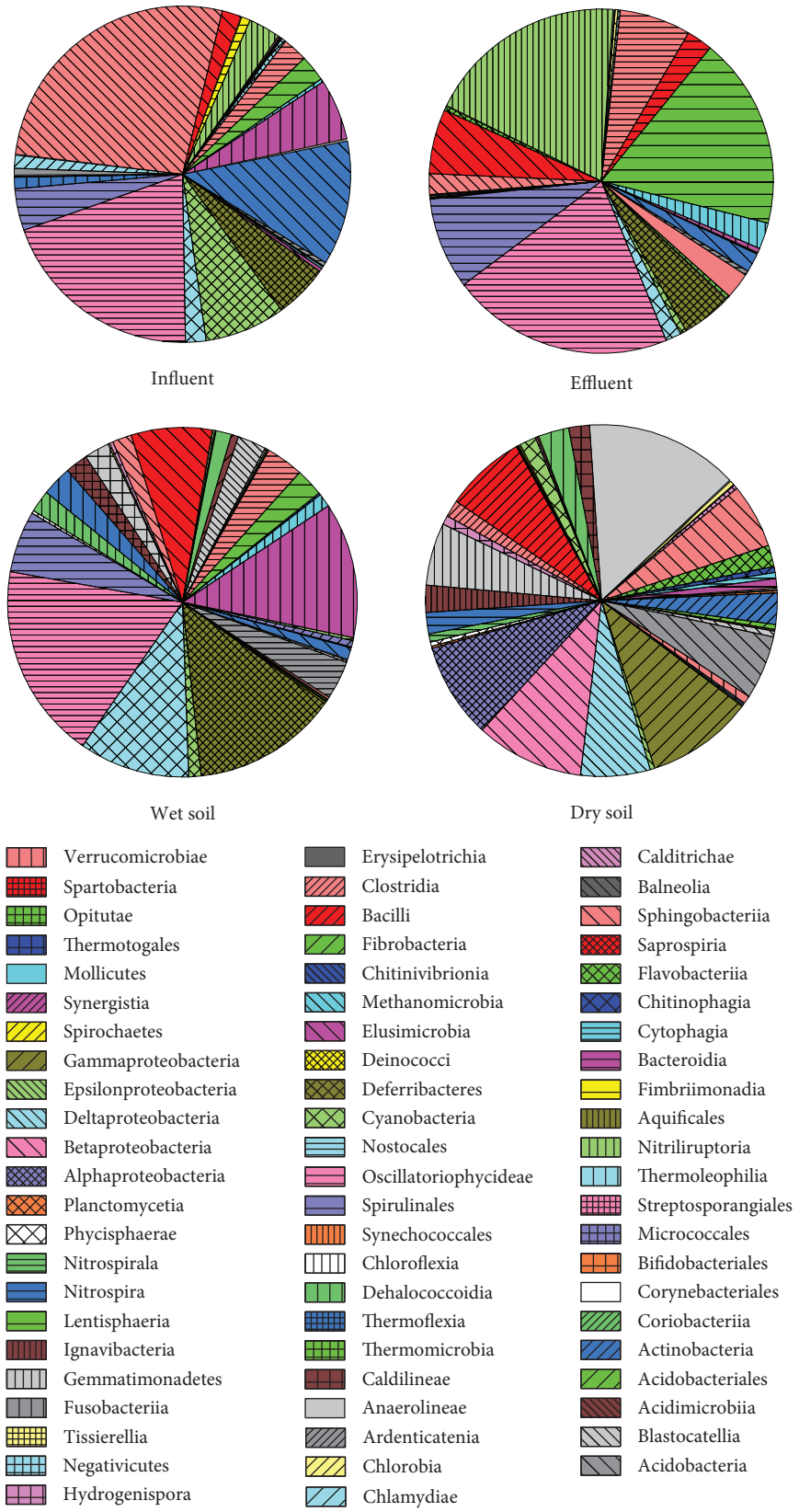

Figure 4: Bacterial relative abundance of four samples in classes in constructed wetlands.

Table 3: The bacterial dominant genera in four samples in constructed wetlands.

Name of similar genera	Influent (%)	Effluent (%)	Wet soil (%)	Dry soil (%)
Aliterella	—	1.39	0.01	—
Alkaliphilus	20.67	0.36	0.01	—
Arcobacter	4.74	0.24	0.34	—
Bacillus	0.12	1.46	1.60	1.69
Calothrix	0.71	12.92	0.01	0.49
Curvibacter	1.33	0.21	0.01	0.01
Dechloromonas	3.03	0.13	0.29	0.05
Desulfobulbus	—	—	1.05	0.07
Dysgonomonas	1.08	0.05	—	—
Enterococcus	0.30	1.61	1.58	1.48
Flavobacterium	0.09	10.93	0.13	0.54
Fluviicola	0.29	1.53	0.04	—
Gemmatimonas	0.12	0.01	1.10	2.41
Gemmobacter	0.10	1.18	0.03	—
Haliscomenobacter	—	2.40	—	—
Herminiimonas	4.82	0.33	—	—
Hydrogenophaga	0.21	2.58	0.18	0.04
Janthinobacterium	0.17	1.85	—	—
Lactococcus	0.23	2.26	3.22	3.11
Limnohabitans	0.07	6.83	—	—
Mycobacterium	8.33	1.35	0.10	0.11
Nordella	1.09	0.21	0.04	0.03
Owenweeksia	—	1.20	—	—
Polaribacter	—	1.30	—	—
Prevotella	1.56	0.15	—	—
Pseudomonas	0.22	0.08	0.32	1.40
Rhodoferax	2.97	4.08	0.96	0.08
Sediminibacterium	0.33	1.03	—	—
Steroidobacter	0.03	—	1.06	0.36
Sulfuricaulis	—	—	0.61	2.00
Synechococcus	—	1.60	—	—
Tabrizicola	0.07	1.03	0.09	0.01
Taibaiella	—	1.21	0.02	0.04
Tangfeifania	—	—	6.71	—
Thermanaerothrix	—	—	0.01	1.07
Thermodesulfovibrio	—	—	1.19	0.50
Thiobacillus	0.06	0.01	3.61	1.96
Thiohalobacter	—	—	1.35	0.03
Trichococcus	1.10	0.03	—	—
Vogesella	1.12	0.03	—	—

than 1%. *Haliscomenobacter*, *Synechococcus*, *Polaribacter*, and *Owenweeksia* were emerged only in the effluent, simultaneously, and *Herminiimonas*, *Prevotella*, *Vogesella*, *Trichococcus*, and *Dysgonomonas* were detected only in the water samples. The quantities of dominant genera in the soil samples were lower than those of the water samples, obviously. *Tangfeifania* were emerged only in the wet soil, while *Sulfuricaulis*, *Thermanaerothrix*, *Thermodesulfovibrio*, *Desulfobulbus*, and *Thiohalobacter* were detected only in the water samples. Interestingly, the relative abundance of *Alkaliphilus* in influent was as high as 20.67%, while the sum of all dominant genera in the dry soil was 15.12%.

3.3. Archaeal Community Structures in Constructed Wetlands

3.3.1. Archaeal Alpha Diversity Analysis. Rarefaction curves of the four samples were shown in the Figure 5. The rarefaction curves and Shannon diversity index curves of four samples clearly revealed that the archaeal community structures of soil samples were considerably higher than those of water samples. Two kinds of curves tended to be gentle, suggesting that the sequencing results had been enough to reflect the diversity of the current sample, and increasing the depth of sequencing could not detect more new OUTs. The sequencing results could basically reflect the microbial community structures of four samples. The trend changes of rarefaction curves and Shannon diversity index curve between archaea and bacteria were exactly the same.

A total of 56,140, 32,879, 61,599, and 28,301 trimmed reads for samples influent, effluent, wet soil, and dry soil were obtained, respectively, after the removal of unqualified reads (Table 4). The community richness in wet soil sample was much higher than that in the other three samples, suggesting that archaea became active under wet anoxic conditions [17]. The community diversity of archaea in wet soil sample was the highest in this study, while that in effluent sample was the lowest. The Shannon index was more sensitive to the abundance of the community and the rare OTUs, indicating that there were more unidentified species in the archaeal community. In addition to the ACE estimator and Chao1 estimator in effluent and dry soil, the other alpha diversity indices were all higher than the bacterial community structures.

3.3.2. Archaeal Community Structures of Soil and Water in Constructed Wetlands. Archaeal sequences in the four samples were classified into taxonomic classes using the default settings of the Qiime platform. Unlike bacteria, the result of archaea is quite simple and the number of phyla was very low. A total of 3 archaeal phyla were found existing in all four samples, but quite different in compositions, and the detailed relative abundances were shown in Figure 6. Euryarchaeotic were the absolute dominant phylum in the influent sample with the relative abundance of 93.29%, while it was no more than 15% in the other three samples. Thaumarchaeota showed dominance in the other three samples (50.58%~75.70%) but accounted for only 1.28% in the influent. Crenarchaeota were one of the common archaeal phyla in the soil samples with a high relative abundance (20.86% and 33.61%), while the proportion was very low in the water samples (0.34% and 0.61%). Simultaneously, some archaeal phyla and no blast hit sequences were also found in the samples which were classified into others. The community structures of the two soil samples were more similar, while the structures of two water samples were quite different.

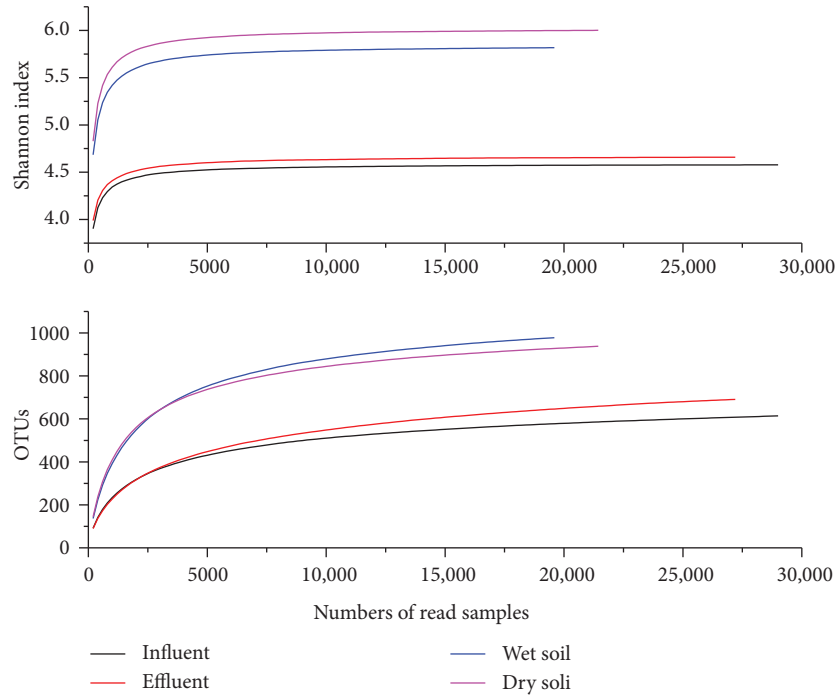

Figure 5: Archaeal rarefaction curves and Shannon diversity index curves.

Table 4: Archaeal alpha diversity indices of four samples.

Samples	Reads	OTUs	ACE	Chao1	Shannon	Simpson
Influent	56,140	2185	850.94	817.04	6.23	0.96
Effluent	32,879	1752	727.75	714.25	5.61	0.92
Wet soil	61,599	3994	1510.08	1454.2	7.54	0.98
Dry soil	28,301	1546	773.00	773.00	6.49	0.97

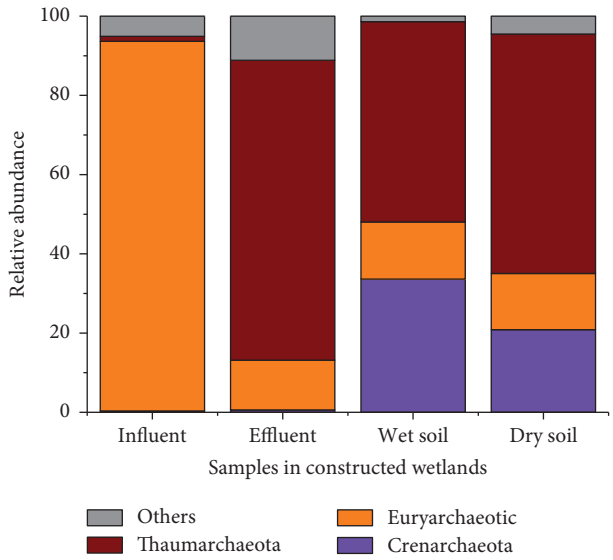

Figure 6: Archaeal relative abundance of four samples in phyla in constructed wetlands.

The distribution characteristics of classes were shown in Figure 7. A total of 11 archaeal classes were found, and the total class numbers in influent, effluent, wet soil, and dry soil all were 10. The relative abundances of different classes were quite different. Methanomicrobia and Thermoprotei were the dominant classes in the influent and wet soil, with the relative abundances of 81.58% and 33.61%, while Nitrosopumilales showed advantage in the effluent and dry soil, accounting for 75.12% and 51.51%. It was worth pointing out that the relative abundances of Nitrosopumilales were high not only in the soil samples but also in the effluent (30.44%), while it was very low in the influent, indicating that Nitrosopumilales was nonexistent in the sewage and mainly existed in the wetland matrix. Methanomicrobia had a low concentration in the other three samples, except in the influent, guessing the main source of it was the sewage treatment process. The relative abundances of Thermoprotei, Thermoplasmata, and Nitrososphaeria in soil samples were much higher than those in water samples.

Due to the huge amount of data, the dominant genera, with relative abundances over 1%, were listed in Table 5. A total of 13 archaeal genera were found in this study. The archaeal dominant genera in four samples accounted for 86.83%~95.95% in archaeal microbial communities. In influent sample, *Methanosaeta* and *Methanocorpusculum* were the dominant genera, but they had very low relative abundances in the other three samples. Except for them, the other 11 dominant genera were all less than 7%. The relative abundances of *Nitrososphaera*, *Ignisphaera*, *Staphylothermus*, *Thermodiscus*, and *Methanomassiliicoccus* in soil samples were much higher than those in water samples. It was worth pointing out that *Nitrosopumilus* all had very high relative

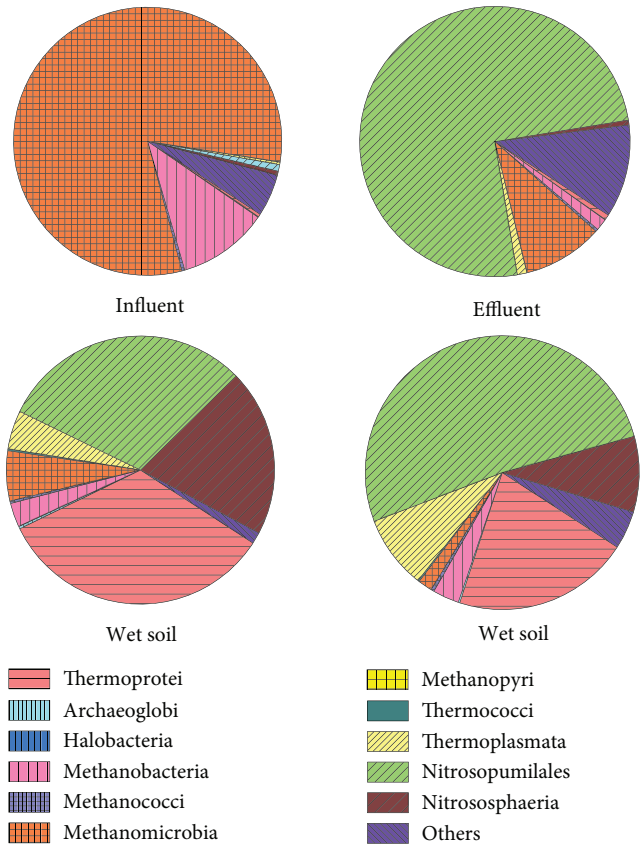

Figure 7: Archaeal relative abundance of four samples in classes in constructed wetlands.

Table 5: The archaeal dominant genera in four samples in constructed wetlands.

Name of similar genera	Influent (%)	Effluent (%)	Wet soil (%)	Dry soil (%)
Nitrososphaera	0.49	0.57	20.14	8.93
Nitrosopumilus	0.79	75.12	30.44	51.51
Methanomassiliicoccus	0.24	0.36	4.48	8.61
Methanosarcina	2.39	0.02	2.68	0.37
Methanomethylovorans	1.13	0.33	0.03	—
Methanosaeta	42.44	3.04	2.16	0.14
Methanocorpusculum	34.42	5.60	0.12	—
Methanobrevibacter	6.29	0.75	0.44	0.45
Methanobacterium	3.95	0.41	1.89	2.53
Thermodiscus	0.24	0.50	25.53	14.08
Staphylothermus	0.06	0.05	1.73	1.89
Ignisphaera	0.02	0.04	4.58	3.17
Desulfurococcus	0.02	0.02	1.77	1.71

abundances in effluent, wet soil, and dry soil samples, but less than 1% in influent sample.

4. Discussion

4.1. Bacterial Diversity and Community of Soil and Water in Constructed Wetlands. To date, little is known about bacterial community structures in the free water surface constructed wetlands. Proteobacteria was the dominant bacteria of all the water and soil samples, with the relative abundance all over the 35% in phyla. The same conclusions had been confirmed in previous coastal water [18], airborne [19], and soil [20] studies, which could prove that Proteobacteria were the dominant phylum in almost all environmental samples. Microorganisms in Proteobacteria were gram-negative bacteria, and a large number of nitrogen-related microorganisms were distributed in Proteobacteria [21, 22]; these may explain why the relative abundances of Proteobacteria in constructed wetlands were higher than those in natural wetlands [23–25]. Bacteroidetes and Firmicutes both belonged to the gut microorganisms [26, 27], and the high relative abundances in this study may be due to the relatively open characteristics of the constructed wetlands, and there were a large number of birds and insects inhabiting the surrounding area, at the same time, Firmicutes were able to degrade a variety of organic pollutants [28], and the sewage treatment systems were their main source, which may explain why the relative abundances of Firmicutes in influent were higher than those in other three samples. Chloroflexi was proved to be a common phylum in various wastewaters from constructed wetland systems [29, 30]; however, in this study, the relative abundances of Chloroflexi in soil were significantly higher than those in water, so this was speculated that some microorganisms would be enriched in the soil, perhaps the same conclusions could be also summarized in Acidobacteria, Ignavibacteriae, Gemmatimonadetes, and Nitrospirae. Currently, numerous studies had found that toxigenic Cyanobacteria [31, 32] and the high content of Cyanobacteria in the effluent should cause the attention of the monitoring department. In this study, three bacterial phyla were emerged only in the water, while four existed only in the soil, indicating that even if the sampling locations were similar, different environmental sample sources would still cause different bacterial community structures.

The dominant bacteria found in study were basically consistent with previous studies. A small amount of *Steroidobacter*, a microcystin-degrading Gammaproteobacterium isolated from soil [33], was found in influent in this study, guessing it might come from the sewage treatment process. *Taibaiella* was the dominant genera in the biofilms [34] and soil [35]; interestingly, it was not found in influent. *Sulfuricaulis* was mainly isolated from sediment of a lake in Japan [36]; however, it was only exited in soil in this study. *Desulfobulbus* was isolated from marine sediment [37] and also only in soil, fit in with the characteristics of the sampling location geographical environment, adjacent to the ocean. In recent years, there had been very little related research on *Alkaliphilus*, but its abundance in influent was as high as 20.67%, which needed our more attentions. *Limnohabitans*, novel planktonic Betaproteobacteria, isolated from a freshwater reservoir, could prove that the quality of water across constructed wetlands had improved significantly. *Calothrix* is the dominant flora in natural water [38], and the relative abundances increased significantly after purification.

This study found that the soil community diversities were lower than those of the water, while the dry soil bacterial

structures were simpler than wet soil. *Mycobacterium* is an important global threat to individuals with cystic fibrosis [39], and the relative abundances in water were much higher than those in soil in this study, reduced substantially through the water treatment, which had been confirmed to be correlated with turbidity [40]. *Enterococcus* showed high cholesterol removal ability [41] and were capable of hydrogen production [42], which could lay the foundation for researches on new energy. *Dysgonomonas* could cause liver abscesses [43] and played a major role in the mechanism for electricity generation [44], which were found only in water. *Flavobacterium* caused devastating mortality in various freshwater fish species globally [45] and were isolated from the China No. 1 glacier, as a kind of psychrophilic bacteria [46]. *Pseudomonas* was responsible for chronic infection [47] and was the most common bacteria in the soil [48], and this may explain why the relative abundances of *Pseudomonas* in dry soil sample were higher than those in water and wet soil samples. *Janthinobacterium* may cause a soft rot disease of Agaricus bisporus [49] and were isolated from both water [50] and soil [51]; however, there were no such genus in the soil samples of this study.

4.2. Archaeal Diversity and Community of Soil and Water in Constructed Wetlands. The distributions of archaeal abundance in water and soil in constructed wetlands were poorly understood, which increased difficulty in the analysis of this study. Among them, 1.03%~9.07% of the sequences could not find its chimera, and 0.14%~2.07% was identified as bacteria. Three archaeal phyla was found in this study, but the gap between water and soil was very large. Euryarchaeotic, accounting for 93.29%, were the dominant phylum in influent, which is involved in methane production [52]. At present, in the constructed wetland system, there were only a few related researches which revealed that Euryarchaeotic was an advantage phylum [53, 54]. In this study, it had reached as much as 93.29%, which should arouse our attention. Most previous studies suggested that Euryarchaeotic was a major archaeal group in constructed wetland system [55, 56], but the influential factors, which affect the relative abundance of Euryarchaeotic, were not yet clear [57]. Thaumarchaeota was a marine archaea and abundant ammonia-oxidizers [58], which ensured the purification efficiency of constructed wetlands and had been widely reported before [59, 60]. This study also found a small amount of Crenarchaeota, which had a high abundance in a temperate acidic forest soil [61], and this conclusion was also consistent with the water quality of the constructed wetland.

Previous researches had reported *Nitrososphaera* and *Nitrosopumilus* [62, 63] belonged to ammonia-oxidation archaea, their large amount of existence could guarantee the purification effect of the constructed wetland system. The relative abundances of *Nitrosopumilus* increased suddenly after passing through the constructed wetlands, from 0.79% to 75.12%, which may be mainly related to the concentration of dissolved oxygen. *Methanomassiliicoccus*, *Methanosarcina*, *Methanomethylovorans*, *Methanocorpusculum*, *Methanobrevibacter*, and *Methanobacterium* were all classified as methanogenic archaea had great potentials for different industrial uses [64]. The methanogenic archaeon *Methanomassiliicoccus* was isolated from human feces [65], and the discovery of it filled the blank of the natural coal-based methanogen group records. *Methanosarcina* played an important role in the long-term bioremediation of uranium-contaminated aquifers and had the potential to influence uranium geochemistry in a diversity of anaerobic sedimentary environments [66]. *Methanosaeta* had only been reported once in the past three years [67], and its research should be strengthened later. *Methanomethylovorans* was also a methylotrophic archaea and had a great potential as additional inoculum for bioreactors to carry out biogas production and other related processes [68]. *Desulfurococcus* was an anaerobic, hyperthermophilic crenarchaeon and able to use a variety of different carbon sources [69]. In addition to the genera mentioned above, this study could not find the previous studies on *Thermodiscus*, *Staphylothermus*, and *Ignisphaera*, which should be emphasized in later studies because of their high relative abundances in soil samples.

5. Conclusion

Taken together, the present study, using the Illumina MiSeq high-throughput sequencing method, provided a detailed picture of bacterial and archaeal community variations on phylum, classes, and genus level under the full-scale constructed wetlands. Sequencing results and alpha diversity indices indicated that the total bacterial OTUs could be assigned into 29 different phyla, while archaeal OTUs were only 3. Among them, Proteobacteria were the most dominant bacterial phyla with the relative abundance of 35.38%~48.66%. Euryarchaeotic and Thaumarchaeota were the dominant archaeal phyla. The diversity of bacterial community structure was significantly higher than that of archaea simultaneously, and the community structures of soil microorganisms were obviously different from the water microorganisms. At genus level, nine bacterial genera had close relation with animal or plant diseases, which could be used for microbial risk assessment simultaneously, and archaeal genera were mainly concentrated in methanogens or anaerobic archaea, which might provide some useful microbial information for the bioremediation. It is worth noting that the lack of researches in archaea had brought great difficulties to this study, which should be emphasized in later studies.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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References

[1] Y. C. Zheng, X. C. Wang, Y. Ge, M. Dzakpasu, J. Q. Xiong, and Y. Q. Zhao, "Effect of plant harvest on nutrients removal by surface-flow constructed wetland for water quality improvement," in *14th IWA International Conference on Wetland System for Pollution Control*, Shanghai, China, 2014.

[2] L. V. Bao-Ping, D. W. Jia, W. X. Tian, T. XB, and J. FH, "Analysis on types and environmental and geological problems of wetlands in Shandong Province," *Land and Resources in Shandong Province*, vol. 4, p. 23, 2008.

[3] Y. W. Feng, K. Hou, H. Y. Pei, W. R. Hu, Y. Ren, and J. L. Wei, "Desmid community characteristics and its relationship with environmental variables in Nansi Lake and Dongping Lake," *Chinese Journal of Ecology*, vol. 34, no. 6, pp. 1738–1746, 2015.

[4] Z. Yang, Q. Wang, J. Zhang, H. Xie, and S. Feng, "Effect of plant harvesting on the performance of constructed wetlands during summer," *Water*, vol. 8, no. 1, p. 24, 2016.

[5] S. Q. Wu, J. J. Chang, Y. Dai, Z. B. Wu, and W. Liang, "Treatment performance and microorganism community structure of integrated vertical-flow constructed wetland plots for domestic wastewater," *Environmental Science and Pollution Research*, vol. 20, no. 6, pp. 3789–3798, 2013.

[6] J. B. Ramond, P. J. Welz, D. A. Cowan, and S. G. Burton, "Microbial community structure stability, a key parameter in monitoring the development of constructed wetland mesocosms during start-up," *Research in Microbiology*, vol. 163, no. 1, pp. 28–35, 2012.

[7] K. Song, S. H. Lee, and H. Kang, "Denitrification rates and community structure of denitrifying bacteria in newly constructed wetland," *European Journal of Soil Biology*, vol. 47, no. 1, pp. 24–29, 2011.

[8] G. Xu, Y. Han, L. Li, and J. Liu, "Characterization and source analysis of indoor/outdoor culturable airborne bacteria in a municipal wastewater treatment plant," *Journal of Environmental Sciences*, 2018.

[9] Y. Wan, X. Ruan, Y. Zhang, and R. Li, "Illumina sequencing-based analysis of sediment bacteria community in different trophic status freshwater lakes," *Microbiologyopen*, vol. 6, no. 4, article e00450, 2017.

[10] C. Hong, Y. Si, Y. Xing, and Y. Li, "Illumina MiSeq sequencing investigation on the contrasting soil bacterial community structures in different iron mining areas," *Environmental Science and Pollution Research International*, vol. 22, no. 14, pp. 10788–10799, 2015.

[11] K. Kuroda, M. Hatamoto, N. Nakahara et al., "Community composition of known and uncultured archaeal lineages in anaerobic or anoxic wastewater treatment sludge," *Microbial Ecology*, vol. 69, no. 3, pp. 586–596, 2015.

[12] T. Magoč and S. L. Salzberg, "FLASH: fast length adjustment of short reads to improve genome assemblies," *Bioinformatics*, vol. 27, no. 21, pp. 2957–2963, 2011.

[13] A. Chao and M. C. K. Yang, "Stopping rules and estimation for recapture debugging with unequal failure rates," *Biometrika*, vol. 80, no. 1, pp. 193–201, 1993.

[14] A. Chao, "Nonparametric estimation of the number of classes in a population," *Scandinavian Journal of Statistics*, vol. 11, no. 4, pp. 265–270, 1984.

[15] C. E. Shannon, "A mathematical theory of communication: the bell system technical journal," *Journal of the Franklin Institute*, vol. 196, no. 4, pp. 519-520, 1938.

[16] E. H. Simpson, "Measurement of diversity," *Nature*, vol. 163, no. 4148, p. 688, 1949.

[17] R. Angel, P. Claus, and R. Conrad, "Methanogenic archaea are globally ubiquitous in aerated soils and become active under wet anoxic conditions," *ISME Journal*, vol. 6, no. 4, pp. 847–862, 2012.

[18] S. Rampadarath, K. Bandhoa, D. Puchooa, R. Jeewon, and S. Bal, "Early bacterial biofilm colonizers in the coastal waters of Mauritius," *Electronic Journal of Biotechnology*, vol. 29, pp. 13–21, 2017.

[19] R. M. Bowers, I. B. McCubbin, A. G. Hallar, and N. Fierer, "Seasonal variability in airborne bacterial communities at a high-elevation site," *Atmospheric Environment*, vol. 50, pp. 41–49, 2012.

[20] R. Sun, J. Jin, G. Sun, Y. Liu, and Z. Liu, "Screening and degrading characteristics and community structure of a high molecular weight polycyclic aromatic hydrocarbon-degrading bacterial consortium from contaminated soil," *Journal of Environmental Sciences*, vol. 22, no. 10, pp. 1576–1585, 2010.

[21] O. V. Tsoy, D. A. Ravcheev, J. Čuklina, and M. S. Gelfand, "Nitrogen fixation and molecular oxygen: comparative genomic reconstruction of transcription regulation in *Alphaproteobacteria*," *Frontiers in Microbiology*, vol. 7, no. 201, p. 1343, 2016.

[22] A. Vaksmaa, T. A. van Alen, K. F. Ettwig et al., "Stratification of diversity and activity of methanogenic and methanotrophic microorganisms in a nitrogen-fertilized Italian paddy soil," *Frontiers in Microbiology*, vol. 8, p. 2127, 2017.

[23] Q. Cao, H. Wang, and X. Chen, "Composition and distribution of microbial communities in natural river wetlands and corresponding constructed wetlands," *Ecological Engineering*, vol. 98, pp. 40–48, 2017.

[24] J. Zhang, Q. Wang, J. Fan et al., "Comparisons of microbial abundance and community among different plant species in constructed wetlands in summer," *Ecological Engineering*, vol. 82, pp. 376–380, 2015.

[25] Y. Guo, H. Gong, and X. Guo, "Rhizosphere bacterial community of *Typha angustifolia* L. and water quality in a river wetland supplied with reclaimed water," *Applied Microbiology and Biotechnology*, vol. 99, no. 6, pp. 2883–2893, 2015.

[26] F. Thomas, J. H. Hehemann, E. Rebuffet, M. Czjzek, and G. Michel, "Environmental and gut *Bacteroidetes*: the food connection," *Frontiers in Microbiology*, vol. 2, p. 93, 2011.

[27] G. Ramachandran, A. Miguel-Arribas, D. Abia et al., "Discovery of a new family of relaxases in Firmicutes bacteria," *PloS Genetics*, vol. 13, no. 2, article e1006586, 2017.

[28] B. Nogales, E. R. B. Moore, W. R. Abraham, and K. N. Timmis, "Identification of the metabolically active members of a bacterial community in a polychlorinated biphenyl-polluted moorland soil," *Environmental Microbiology*, vol. 1, no. 3, pp. 199–212, 1999.

[29] L. Yang and C.C. Hu, "Treatments of oil-refinery and steel-mill wastewaters by mesocosm constructed wetland systems," *Water Science and Technology*, vol. 51, no. 9, p. 157, 2005.

[30] G. Ansola, P. Arroyo, and L. E. Sáenz de Miera, "Characterisation of the soil bacterial community structure and composition of natural and constructed wetlands," *Science of the Total Environment*, vol. 473-474, no. 3, pp. 63–71, 2014.

[31] B. A. Neilan, L. A. Pearson, M. C. Moffitt et al., "The genetics and genomics of cyanobacterial toxicity," in *Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs*, pp. 477–486, Springer New York, 2017.

[32] S. I. Sidelev, L. G. Korneva, V. V. Solovyeva, A. A. Zubishina, and D. N. Pligin, "Molecular genetic identification and seasonal succession of toxigenic cyanobacteria in phytoplankton of the Rybinsk Reservoir (Russia)," *Inland Water Biology*, vol. 9, no. 4, pp. 368–374, 2016.

[33] Z. L. Gong, C. F. Zhang, R. Jin, and Y. Q. Zhang, "*Steroidobacter flavus* sp. nov., a microcystin-degrading Gammaproteobacterium isolated from soil," *Antonie Van Leeuwenhoek*, vol. 109, no. 8, pp. 1073–1079, 2016.

[34] M. el-Fadel, F. Sleem, J. Hashisho, P. E. Saikaly, I. Alameddine, and S. Ghanimeh, "Impact of SRT on the performance of MBRs for the treatment of high strength landfill leachate," *Waste Management*, vol. 73, pp. 165–180, 2018.

[35] S. G. Szerdahelyi, Á. Szu˝cs, H. G. Daood et al., "*Taibaiella coffeisoli* sp. nov. isolated from the soil of a coffee plantation," *International Journal of Systematic and Evolutionary Microbiology*, vol. 66, no. 4, pp. 1627–1632, 2016.

[36] T. Watanabe, H. Kojima, and M. Fukui, "*Sulfuricaulis limicola* gen. nov., sp. nov. a sulfur oxidizer isolated from a lake," *International Journal of Systematic and Evolutionary Microbiology*, vol. 66, no. 1, pp. 266–270, 2016.

[37] H. Kharrat, F. Karray, M. Bartoli et al., "*Desulfobulbus aggregans*, sp. nov. a novel sulfate reducing bacterium isolated from marine sediment from the Gulf of Gabes," *Current Microbiology*, vol. 74, no. 4, pp. 449–454, 2017.

[38] E. Berrendero, E. Perona, and P. Mateo, "Genetic and morphological characterization of *Rivularia* and *Calothrix* (Nostocales, Cyanobacteria) from running water," *International Journal of Systematic and Evolutionary Microbiology*, vol. 58, no. 2, pp. 447–460, 2008.

[39] J. M. Bryant, D. M. Grogono, D. Rodriguez-Rincon et al., "Emergence and spread of a human-transmissible multidrug-resistant nontuberculous mycobacterium," *Science*, vol. 354, no. 6313, pp. 751–757, 2016.

[40] J. O. Falkinham, C. D. Norton, and M. W. Lechevallier, "Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other mycobacteria in drinking water distribution systems," *Applied and Environmental Microbiology*, vol. 67, no. 3, pp. 1225–1231, 2001.

[41] L. Guo, T. Li, Y. Tang, L. Yang, and G. Huo, "Probiotic properties of *Enterococcus* strains isolated from traditional naturally fermented cream in China," *Microbial Biotechnology*, vol. 9, no. 6, pp. 737–745, 2016.

[42] G. Stary, "Paul Pelliot (1878-1945). His life and works - a bibliography. (Indiana University oriental series, vol. IX) by Hartmut Walravens; Denis Sinor," *Applied & Environmental Microbiology*, vol. 70, no. 9, pp. 5373–5382, 2004.

[43] C. H. Chen, S. S. Wu, and R. H. Hsiu, "*Dysgonomonas capnocytophagoides* bacteremia due to liver abscesses after radiofrequency ablation in a patient with hepatocellular carcinoma," *Journal of the Formosan Medical Association*, vol. 115, no. 10, pp. 889-890, 2016.

[44] K. Watanabe, M. Miyahara, T. Shimoyama, and K. Hashimoto, "Population dynamics and current-generation mechanisms in cassette-electrode microbial fuel cells," *Applied Microbiology and Biotechnology*, vol. 92, no. 6, pp. 1307–1314, 2011.

[45] P. Kayansamruaj, H. T. Dong, I. Hirono, H. Kondo, S. Senapin, and C. Rodkhum, "Comparative genome analysis of fish pathogen *Flavobacterium columnare* reveals extensive sequence diversity within the species," *Infection, Genetics and Evolution*, vol. 54, pp. 7–17, 2017.

[46] F. Zhu, S. Wang, and P. Zhou, "*Flavobacterium xinjiangense* sp. nov. and *Flavobacterium omnivorum* sp. nov., novel psychrophiles from the China No. 1 glacier," *International Journal of Systematic & Evolutionary Microbiology*, vol. 53, no. 3, pp. 853–857, 2003.

[47] D. Araújo, M. Shteinberg, S. Aliberti et al., "The independent contribution of *Pseudomonas aeruginosa* infection to long-term clinical outcomes in bronchiectasis," *European Respiratory Journal*, vol. 51, no. 2, article 1701953, 2018.

[48] R. E. Ingham, J. A. Trofymow, E. R. Ingham, and D. C. Coleman, "Interactions of bacteria, fungi, and their nematode grazers: effects on nutrient cycling and plant growth," *Ecological Monographs*, vol. 55, no. 1, pp. 119–140, 1985.

[49] S. P. Lincoln, T. R. Fermor, and B. J. Tindall, "*Janthinobacterium agaricidamnosum* sp. nov., a soft rot pathogen of *Agaricus bisporus*," *International Journal of Systematic Bacteriology*, vol. 49, no. 4, pp. 1577–1589, 1999.

[50] X. Gong, S. Skrivergaard, B. S. Korsgaard et al., "High quality draft genome sequence of *Janthinobacterium psychrotolerans* sp. nov. isolated from a frozen freshwater pond," *Standards in Genomic Sciences*, vol. 12, no. 1, p. 8, 2017.

[51] P. D. Schloss, H. K. Allen, A. K. Klimowicz et al., "Psychrotrophic strain of *Janthinobacterium lividum* from a cold Alaskan soil produces prodigiosin," *DNA and Cell Biology*, vol. 29, no. 9, pp. 533–541, 2010.

[52] A. Hu, L. Hou, and C. P. Yu, "Biogeography of planktonic and benthic archaeal communities in a subtropical eutrophic estuary of China," *Microbial Ecology*, vol. 70, no. 2, pp. 322–335, 2015.

[53] T. He, W. Guan, Z. Luan, and S. Xie, "Spatiotemporal variation of bacterial and archaeal communities in a pilot-scale constructed wetland for surface water treatment," *Applied Microbiology and Biotechnology*, vol. 100, no. 3, pp. 1479–1488, 2016.

[54] Y. Liu, H. Li, Q. F. Liu, and Y. H. Li, "Archaeal communities associated with roots of the common reed (*Phragmites australis*) in Beijing Cuihu wetland," *World Journal of Microbiology and Biotechnology*, vol. 31, no. 5, pp. 823–832, 2015.

[55] M. Bouali, I. Zrafi, A. Bakhrouf, S. Chaussonnerie, and A. Sghir, "Bacterial structure and spatiotemporal distribution in a horizontal subsurface flow constructed wetland," *Science of the Total Environment*, vol. 98, no. 7, pp. 3191–3203, 2014.

[56] M. Bouali, E. Pelletier, S. Chaussonnerie, D. le Paslier, A. Bakhrouf, and A. Sghir, "Characterization of rhizosphere prokaryotic diversity in a horizontal subsurface flow constructed wetland using a PCR cloning-sequencing based approach," *Applied Microbiology and Biotechnology*, vol. 97, no. 9, pp. 4221–4231, 2013.

[57] Y. Long, H. Yi, S. Chen et al., "Influences of plant type on bacterial and archaeal communities in constructed wetland

treating polluted river water," *Environmental Science & Pollution Research*, vol. 23, no. 19, pp. 19570–19579, 2016.

[58] N. A. Ahlgren, Y. Chen, D. M. Needham et al., "Genome and epigenome of a novel marine Thaumarchaeota strain suggest viral infection, phosphorothioation DNA modification, and multiple restriction systems," *Environmental Microbiology*, vol. 19, no. 6, pp. 2434–2452, 2017.

[59] Y. Zou, G. Zhu, X. Feng, C. Xia, and L. Zhou, "Enhanced ammonium-n removal in a constructed wetland and the microbial mechanisms of ammonia-oxidization at low temperature," *Acta Scientiae Circumstantiae*, vol. 34, no. 4, pp. 864–871, 2014.

[60] Y. Liu, J. Zhang, L. Zhao, Y. Li, Y. Dai, and S. Xie, "Distribution of sediment ammonia-oxidizing microorganisms in plateau freshwater lakes," *Applied Microbiology and Biotechnology*, vol. 99, no. 10, pp. 4435–4444, 2015.

[61] J. E. Lima-Perim, E. M. Romagnoli, F. Dini-Andreote, A. Durrer, A. C. F. Dias, and F. D. Andreote, "Linking the composition of bacterial and archaeal communities to characteristics of soil and flora composition in the Atlantic rainforest," *PLoS One*, vol. 11, no. 1, article e0146566, 2016.

[62] G. Feng, W. Sun, F. Zhang, L. Karthik, and Z. Li, "Inhabitancy of active *Nitrosopumilus*-like ammonia-oxidizing archaea and *Nitrospira* nitrite-oxidizing bacteria in the sponge *Theonella swinhoei*," *Scientific Reports*, vol. 6, no. 1, article 24966, 2016.

[63] D. Gang, W. Qi, H. Liu, and J. Qu, "The impact of water level change on the abundance and diversity of ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) in water-fluctuating zone," *Huanjing Kexue Xuebao*, vol. 37, no. 5, pp. 1615–1622, 2017.

[64] Y. Q. Wei, R. J. Long, H. Yang et al., "Fiber degradation potential of natural co-cultures of *Neocallimastix frontalis* and *Methanobrevibacter ruminantium* isolated from yaks (*Bos grunniens*) grazing on the Qinghai Tibetan Plateau," *Anaerobe*, vol. 39, pp. 158–164, 2016.

[65] C. Bang, T. Vierbuchen, T. Gutsmann, H. Heine, and R. A. Schmitz, "Immunogenic properties of the human gut-associated archaeon *Methanomassiliicoccus luminyensis* and its susceptibility to antimicrobial peptides," *PLoS One*, vol. 12, no. 10, article e0185919, 2017.

[66] D. E. Holmes, R. Orelana, L. Giloteaux et al., "Potential for *Methanosarcina* to contribute to uranium reduction during acetate-promoted groundwater bioremediation," *Microbial Ecology*, 2018.

[67] T. Koo, S. G. Shin, J. Lee et al., "Identifying methanogen community structures and their correlations with performance parameters in four full-scale anaerobic sludge digesters," *Bioresource Technology*, vol. 228, pp. 368–373, 2017.

[68] M. Cerrillo, L. Morey, M. Viñas, and A. Bonmatí, "Assessment of active methanogenic archaea in a methanol-fed upflow anaerobic sludge blanket reactor," *Applied Microbiology and Biotechnology*, vol. 100, no. 23, pp. 10137–10146, 2016.

[69] D. Susanti, E. F. Johnson, A. Lapidus et al., "Permanent draft genome sequence of *Desulfurococcus mobilis* type strain DSM 2161, a thermoacidophilic sulfur-reducing crenarchaeon isolated from acidic hot springs of Hveravellir, Iceland," *Standards in Genomic Sciences*, vol. 11, no. 1, p. 3, 2016.

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Review Article

Ammonia-Oxidizing Archaea (AOA) Play with Ammonia-Oxidizing Bacteria (AOB) in Nitrogen Removal from Wastewater

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An increase in the number of publications in recent years indicates that besides ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA) may play an important role in nitrogen removal from wastewater, gaining wide attention in the wastewater engineering field. This paper reviews the current knowledge on AOA and AOB involved in wastewater treatment systems and summarises the environmental factors affecting AOA and AOB. Current findings reveal that AOA have stronger environmental adaptability compared with AOB under extreme environmental conditions (such as low temperature and low oxygen level). However, there is still little information on the cooperation and competition relationship between AOA and AOB, and other microbes related to nitrogen removal, which needs further exploration. Furthermore, future studies are proposed to develop novel nitrogen removal processes dominated by AOA by parameter optimization.

1. Introduction

Nitrogen-containing pollutants are considered one of the most common environmental pollutants in various types of wastewater, and they are an important pollution factor that causes eutrophication. The conventional biological system for nitrogen removal from wastewater is usually through the biological oxidation of ammonia and organic nitrogen (nitrification) and the biological reduction of the oxidation products, that is, nitrate (denitrification). From the viewpoint of microbial transformation of nitrogen, the nitrification process includes ammonia oxidation (NH_3-N $\rightarrow$ NO_2^--N) and nitrite oxidation (NO_2^--N $\rightarrow$ NO_3^--N). As the rate-limiting step of the nitrification, ammonia oxidation is the key process for biological nitrogen removal from wastewater, thus attracting wide attention from researchers.

In the past 100 years, ammonia-oxidizing bacteria (AOB) were considered as the dominant microorganism in the ammonia oxidation process [1]. With the development of molecular biology techniques in recent years, it had been found that the *amoA* gene, a kind of indicative gene of ammonia oxidation, exists in large numbers of archaea distributed in the marine environment, proving that archaea also have the capacity of ammonia oxidation at the physiological metabolic level [2]. Hereafter, ammonia oxidations conducted by archaea were widely found in hot springs, soils, oceans, sediments, and wetlands and these archaea were formally known as ammonia-oxidizing archaea (AOA) in subsequent studies [3–5]. In addition, a large number of studies have reported that the AOA abundance and the archaeal *amoA* gene abundance are significantly higher than that of AOB in farmland soils, river sediments, and oceans [6], indicating that AOA are the main driver of ammonia oxidation in these habitats and play a more important role in the global nitrogen cycle.

2. Cell Structure and Metabolism Physiology of AOA

The cell volumes of most AOA are 10 to 100 times smaller than those of known AOB. This has implicated that the ammonia oxidation rates per cell for *Nitrosopumilus*

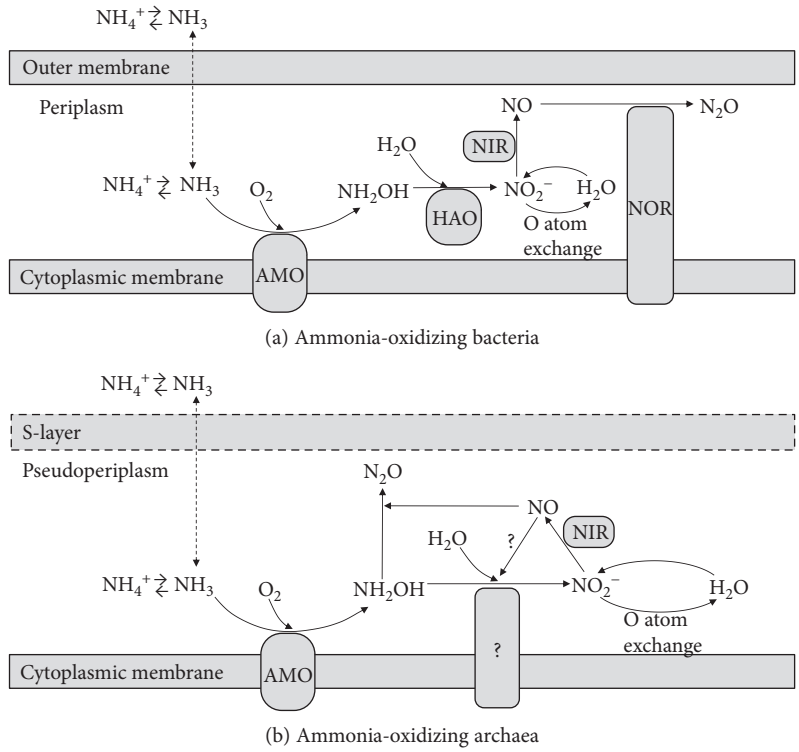

Figure 1: Schematic illustration of ammonia oxidation pathways in ammonia-oxidizing bacteria (a) and archaea (b). The figure is reproduced from Kozlowski et al. and Nishizawa et al. [66, 67]. Abbreviations: HAO, hydroxylamine dehydrogenase; NIR, nitrite reductase; NOR, nitric oxide reductase.

maritimus SCM1 (AOA) were reported to be 10-fold lower than those of AOB [7]. Thus, the individual contributions of AOA and AOB to ammonia oxidation should be identified by considering not only the relative abundance of cell counting but also activity-correlated analyses [8]. The tetraether lipid-based membranes of AOA cells make it less permeable to ion than AOB membranes, thus resulting in the reduction in the amount of futile ion cycling and lower levels of maintenance energy relative to AOB, offering the advantages of their adaption to extreme environments [9]. In addition, according to cryoelectron tomography data, the cells of *Nitrosopumilus maritimus* SCM1 in exponential growth harbor ~1000 ribosomes per ~0.023 μm^3 cell volume [10]. The high numbers of ribosomes of AOA offer organisms the ability to respond quickly to changing environmental conditions (e.g., fluctuating ammonia levels). This is consistent with the observations that most archaea, in contrast to bacteria, are highly adapted to energy-stressed environments [9]. Available data on the stability of mRNAs, ammonia monooxygenase (AMO), and ribosomal proteins of AOA are still lacking but could be essential in understanding the ecological adaptations of AOA compared to AOB.

It is generally accepted that not NH_4^+ but NH_3 is the substrate for bacterial AMO [11], while the true substrate for archaeal AMO remains to be elucidated. As shown in Figure 1, in AOB, the membrane-associated AMO catalyzes the aerobic oxidation of NH_3 to hydroxylamine (NH_2OH) which is subsequently oxidized to NO_2^- by the periplasmic hydroxylamine oxidoreductase (HAO) [12]. Without the discovery of the HAO homologue, enzymes for the detoxification of NH_2OH, or cytochrome *c* in any AOA genome, it is unclear whether archaeal AMO catalyzes the same reaction as AOB [13, 14]. Either archaeal AMO reaction or unidentified enzyme substitutes for HAO in AOA might yield a different product [14]. It was suggested that nitroxyl hydride (HNO) might be generated by archaeal AMO, which could be subsequently oxidized to NO_2^- via nitroxyl oxidoreductase (NxOR) [14]. The activation of O_2 for the monooxygenase reaction could also be achieved by nitric oxide (NO), the reaction product of nitrite reductase (NIR), which would result in N_2 gas production [2]. It was also reported that archaeal *nirK* (encoding copper-dependent NIR) genes are expressed under aerobic conditions [15, 16], suggesting a different behaviour of these enzymes in AOA compared to the bacterial counterparts. Furthermore, the lack of cytochrome *c* proteins and the existence of numerous genes encoding copper-containing proteins (multicopper oxidases and plastocyanin-like domain proteins) in AOA suggest a different electron transport mechanism [14] from that of the highly iron-heme-dependent AOB [17, 18]. A copper-based biochemistry would help to explain the ecological success of marine AOA (compared to AOB), because dissolved copper concentrations are generally an order of magnitude higher than those of iron in seawater [10].

3. The Discovery of AOA in Wastewater Treatment System

The first report on AOA in wastewater treatment systems was reported in 2006. Park et al. [19] detected the archaeal

amoA gene from the activated sludge in nitrification tanks of five wastewater treatment plants in the United States through the polymerase chain reaction (PCR) method. However, due to limited technical means at that time, it was difficult to obtain quantitative data of the absolute abundance of AOA. In 2009, Wells et al. [20] used quantitative PCR to detect AOA in a wastewater treatment system for the first time. Since then, the researchers focused their attention on the comparison of AOA and AOB abundance in the wastewater system for nitrogen removal, as shown in Table 1. Some researchers found that the abundance of AOA was higher than that of AOB in domestic wastewater treatment systems [21–25], whereas the situation was reversed in the systems for industrial wastewater treatment [23–25]. However, Gao et al. [26, 27] found that the abundance of AOB was approximately 3 orders of magnitude higher than that of AOA in the investigation of 8 wastewater treatment systems (including industrial wastewater and domestic wastewater) in Beijing. Muβmann et al. [28] found high abundance of AOA in four industrial wastewater treatment systems, and even the abundance of AOA in one of the systems was 4 orders of magnitude higher than AOB. Zhang et al. [29] showed that high concentrations of spiramycin caused a significant increase in the relative abundance of AOA in pharmaceutical wastewater treatment systems.

In recent years, AOA have been successfully cultivated and enriched in pure medium [30–33], but there is still no information on the enrichment of AOA in the actual wastewater nitrogen removal system. Using inorganic medium, Sonthiphand and Limpiyakorn [34] had attempted to enrich ammonia-oxidizing microorganisms in activated sludge which contained a nearly equal number of archaeal *amoA* genes to bacterial *amoA* genes, but AOA gradually disappeared from the ammonia-oxidizing consortiums in all reactors with the prolongation of cultivation time. Compared with suspended floc activated sludge, stable ecological conditions of attached biofilm provide a habitat for more microbes especially with long generation. Roy et al. [35] found that AOA outnumber AOB and contribute to ammonia oxidation in the biofilm samples of trickling filter and moving bed bioreactor treating municipal wastewater, with the abundance of the archaeal *amoA* gene 2-3 orders of magnitude higher than that of the bacterial *amoA* gene. Chen et al. [36] also had the same observation in the biofilm in biological aerated filters for municipal wastewater treatment, and a single AOA strain was enriched from the filtering materials using synthetic medium [37].

Based on the reviewed literature, the distribution of AOA and AOB in different wastewater treatment systems is still unclear, and the differences in the research results may be affected by the characteristics of treated wastewater (ammonia level, organic loading) and process operating parameters (temperature, dissolved oxygen (DO) concentration) [38].

4. Environmental Factors Affecting AOA and AOB

4.1. Ammonia Level. As a common substrate (nitrogen source) of AOA and AOB, the concentration of ammonia in the environment significantly influences the growth of these two kinds of ammonia-oxidizing microorganisms. AOA have a higher affinity for ammonia than AOB [7, 21], resulting in lower inhibitory concentration for AOA. Exposed in a higher ammonia concentration, AOA might face the suppressed situation earlier than AOB. Sauder et al. [39] demonstrated that the amount of AOA *amoA* gene was reduced with the increase in the ammonia concentration in the rotating biological contactors of a municipal wastewater treatment plant, indicating that AOA were suitable for low ammonia level. According to Gao et al. [40], AOB were more competitive than AOA under high concentrations of ammonia, and the higher the ammonia concentration was, the higher the AOB abundance was [28]. There was also no big difference in the abundance of AOA at different ammonia nitrogen levels (14, 56, and 140 mg N/L) [28]. Ye and Zhang [41] observed that in the nitrification tank for salty wastewater treatment, when the concentration of ammonia increased from 200 mg/L to 300 mg/L, the abundance of AOA was considerably reduced but the abundance of AOB remained stable. In addition, in the landfill leachate treatment system with a high ammonia concentration (2180 ± 611 mg N/L), the ammonia oxidation process was dominated by AOB [42]. It could be concluded that the level of ammonia which was affected by the types of wastewater could result in the differences in the microbial community structure of AOA and AOB.

4.2. Organic Loading. Organic matter objectively affects the growth of ammonia-oxidizing microorganisms. AOB are recognized as autotrophic microorganisms, while it is not clear whether AOA are strictly autotrophic or mixotrophic. Some studies have reported that the presence of organic substances had a significant inhibitory effect on the growth of some certain AOA strains such as *Nitrosopumilus maritimus* SCM1 and *Nitrosocaldus yellowstonii* [43, 44]. The latest study found that the addition of organic substances could promote the growth of AOA strains PS0 and HCA1, showing their characteristics of mixotrophic growth [45]. It had also been proved using genome sequencing that some AOA strains had two different carbon utilization mechanisms: 3-hydroxypropionic acid/4-hydroxybutyric acid cycle (autotrophic metabolism) and tricarboxylic acid cycle (heterotrophic metabolism), indicating that these AOA strains had the potential for autotrophic and heterotrophic metabolism [30, 46]. Compared with AOB, AOA may have more complex metabolic pathways and may show different metabolic characteristics under different carbon source conditions, resulting in changes in ammonia oxidation capacity of AOA and AOB.

4.3. Temperature. The effect of temperature on ammonia-oxidizing microorganisms is mainly manifested in the effect on the activity of ammonia monooxygenase [47]. The currently found AOB belong to mesophiles, while the range of adaptation temperature of AOA is very large. It could be observed that active ammonia oxidations by AOA occur at 0.2°C in the deep water region of the North Japan Sea and at 74°C in the hot spring in Yellowstone National Park

Table 1: Comparison of AOA and AOB in different wastewater treatment systems.

Biomass samples	Wastewater type	Influent Ammonia level (mg N/L)	COD (mg/L)	Process parameter		AOA *amoA* gene abundance	AOB *amoA* gene abundance	AOA?AOB	Reference
				Temperature (°C)	DO (mg O_2/L)				
AS[a]	Municipal wastewater	14–33	116–233	18.2–25.4	3.08–4.50	8×10^1–2×10^3 copies mL^{-1} sludge	1.2×10^6–4.1×10^6 copies mL^{-1} sludge	AOA < AOB	[20]
AS	Municipal wastewater	5.4–38.6	24.8–152.0	N/A	0.5–3.25	$3.28 \times 10^4 \pm 1.74 \times 10^4$–$2.23 \times 10^8 \pm 1.92 \times 10^8$ copies mL^{-1} sludge	$8.05 \times 10^3 \pm 5.20 \times 10^3$–$5.72 \times 10^6 \pm 5.69 \times 10^5$ copies mL^{-1} sludge	AOA > AOB	[22]
AS	Municipal wastewater	16.3–76.6	115–580	N/A	N/A	6.3×10^5–4.5×10^6 copies g^{-1} sludge	7.2×10^3–1.7×10^5 copies g^{-1} sludge	AOA > AOB	[23]
AS	Municipal wastewater	5.6–11.0	23.4–68.0	N/A	N/A	$1.05 \times 10^5 \pm 6.74 \times 10^4$–$7.48 \times 10^8 \pm 2.08 \times 10^8$ copies mL^{-1} sludge	$3.73 \times 10^5 \pm 3.07 \times 10^5$–$9.05 \times 10^7 \pm 2.77 \times 10^7$ copies mL^{-1} sludge	AOA > AOB	[24]
AS	Municipal wastewater	14–58	154–603	N/A	0.2-3.5	$9.38 \times 10^2 \pm 4.74 \times 10^1$–$1.11 \times 10^6 \pm 1.46 \times 10^6$ copies g^{-1} sludge	$1.50 \times 10^5 \pm 6.90 \times 10^4$–$3.32 \times 10^8 \pm 6.10 \times 10^7$ copies g^{-1} sludge	AOA < AOB	[26]
AS	Municipal wastewater	15.9	174	30	1.2	$1.11 \times 10^3 \pm 3.02 \times 10^1$–$2.35 \times 10^3 \pm 7.34 \times 10^1$ copies ng^{-1} DNA	$6.35 \times 10^1 \pm 2.3$–$1.76 \times 10^2 \pm 1.56 \times 10^1$ copies ng^{-1} DNA	AOA > AOB	[38]
AS	Municipal wastewater	35.8	336	16	1.7	<LOD[b]	$1.36 \times 10^3 \pm 3.68 \times 10^1$–$2.71 \times 10^4 \pm 1.35 \times 10^4$ copies ng^{-1} DNA	AOA < AOB	[38]
AS	Municipal wastewater	15.9	110	22	1.4	<LOD	$3.69 \times 10^4 \pm 1.5 \times 10^3$ copies ng^{-1} DNA	AOA < AOB	[38]
AS	Municipal wastewater	18.3	100	N/A	N/A	23–39 copies ng^{-1} DNA	16–220 copies ng^{-1} DNA	AOA < AOB	[64]
AS	Municipal wastewater	N/A	N/A	13–23	N/A	1.6×10^2–1.9×10^2 copies ng^{-1} DNA	1.1×10^3 copies ng^{-1} DNA	AOA < AOB	[69]
AS	Municipal wastewater	N/A	N/A	10–18	N/A	1.0×10^2–4.0×10^2 copies ng^{-1} DNA	1.1×10^3–1.3×10^3 copies ng^{-1} DNA	AOA < AOB	[69]
AS	Municipal/ industrial wastewater	20.5–474.8	365.2–2508.7	N/A	1.5–7.5	<LOD-1.9×10^7 copies g^{-1} sludge	4.625×10^4–9.99×10^9 copies g^{-1} sludge	AOA < AOB	[27]
AS	Industrial wastewater	35.2–262.0	524–2730	N/A	N/A	5.7×10^3–9.9×10^3 copies g^{-1} sludge	2.6×10^7–3.6×10^9 copies g^{-1} sludge	AOA < AOB	[23]
AS	Industrial wastewater	36.1–422.3	192–1410	N/A	N/A	<LOD	$2.78 \times 10^6 \pm 1.32 \times 10^6$–$4.25 \times 10^7 \pm 9.65 \times 10^6$ copies mL^{-1} sludge	AOA < AOB	[24]

Table 1: Continued.

Biomass samples	Wastewater type	Influent Ammonia level (mg N/L)	COD (mg/L)	Process parameter		AOA *amoA* gene abundance	AOB *amoA* gene abundance	AOA?AOB	Reference
				Temperature (°C)	DO (mg O_2/L)				
AS	Spiramycin production wastewater	249	4575	N/A	N/A	$1.72 \times 10^5 \pm 3.02 \times 10^5$ copies ng^{-1} DNA	$3.25 \times 10^4 \pm 3.17 \times 10^2$ copies ng^{-1} DNA	AOA > AOB	[29]
AS	Oxytetracycline production wastewater	164	3200	22	N/A	$3.6 \times 10^1 \pm 3.0 \times 10^1$ copies ng^{-1} DNA	$3.9 \times 10^4 \pm 1.94 \times 10^3$ copies ng^{-1} DNA	AOA < AOB	[29]
AS	Landfill leachates	2180 ± 611	5565 ± 3397	N/A	0.3–2.5	<LOD-$1.1 \times 10^4 \pm 2.0 \times 10^2$ cells in extracted DNA	$2.1 \times 10^3 \pm 4.0 \times 10^1$–$1.3 \times 10^5 \pm 1.0 \times 10^3$ cells in extracted DNA	AOA < AOB	[42]
Biofilm	Municipal wastewater	9.8	104	N/A	N/A	6.0×10^5 copies g^{-1} sludge	3.6×10^4 copies g^{-1} sludge	AOA > AOB	[23]
Biofilm	Municipal wastewater	0.3–7.2	N/A	10–22	2–5	2.2 ± 0.3–7.8 ± 0.9 copies μL^{-1} DNA	9.2 ± 0.7–128.0 ± 4.0 copies μL^{-1} DNA	AOA < AOB	[35]
Biofilm	Municipal wastewater	N/A	N/A	10–22	2–5	$4.5 \times 10^5 \pm 0.1 \times 10^5$–$1.9 \times 10^6 \pm 0.3 \times 10^6$ copies μL^{-1} DNA	$4.5 \times 10^3 \pm 0.1 \times 10^3$–$1.1 \times 10^4 \pm 0.1 \times 10^4$ copies μL^{-1} DNA	AOA > AOB	[35]
Biofilm	Municipal wastewater	2.7–11.7	43–121	10–22	5	$2.2 \times 10^6 \pm 0.1 \times 10^6$–$1.0 \times 10^7 \pm 0.1 \times 10^7$ copies μL^{-1} DNA	$3.4 \times 10^4 \pm 0.3 \times 10^4$–$1.0 \times 10^5 \pm 0.3 \times 10^5$ copies μL^{-1} DNA	AOA > AOB	[35]
Biofilm	Municipal wastewater	10.6	38	23.6–24.0	0.9–4.6	6.32×10^3–3.8×10^4 copies ng^{-1} DNA	20.6–105.2 copies ng^{-1} DNA	AOA > AOB	[36, 37]
Wetland soil	Effluent from WWTP	20–30	45–70	5.5–24	N/A	$2.1 \times 10^6 \pm 0.2 \times 10^6$–$1.8 \times 10^7 \pm 0.2 \times 10^7$ copies g^{-1} soil	$1.2 \times 10^5 \pm 0.2 \times 10^5$–$5.2 \times 10^7 \pm 0.2 \times 10^7$ copies g^{-1} soil	AOA > AOB	[51]

[a]AS, activated sludge. [b]LOD, limit of detection.

[44, 48]. He et al. [49] found that the dominant ammonia oxidation microorganisms in the sediments near the Rushan Bay of Shandong Peninsula were AOB during the summer (water temperature = 21–25 °C) while AOA in the winter (water temperature = 3-4 °C). Niu et al. [50] found that in the biological activated carbon filtration system for drinking water purification, the AOB *aomA* gene abundance decreased significantly in winter (water temperature = 4.6–5.5 °C) compared with that in summer (water temperature = 17.7–28.6 °C), while the AOA gene abundance changed little. Sims et al. [51] also observed that AOB were more sensitive to low temperatures than AOA in the constructed wetland system for wastewater treatment. The adaptation ability of AOA to temperature changes is inseparable with the special structure of glycerol ether in the cell membrane, thus making the activity of ammonia monooxygenase relatively less affected by temperature and endowing AOA with a competitive advantage under extreme temperature conditions.

4.4. Oxygen. Oxygen is a necessary reaction substrate of the nitrification process. Due to the difference in the affinity of nitrifying microbes for oxygen (AOA > AOB > NOB (nitrite-oxidizing bacteria)), the oxygen concentration will affect the nitrification process. High oxygen affinity makes AOA more competitive than AOB in hypoxic environments such as deep oceans, deep soils, and sediments [7, 52]. Park et al. [19] detected large amounts of AOA with low dissolved oxygen level (<0.2 mg/L) in the outer ditch of an Orbal oxidation ditch, and found that simultaneous nitrification and denitrification occurred in the outer ditch at the same time [53]. Li et al. [54] also predicted that AOA and heterotrophic denitrifying bacteria could be coupled in a single reactor by reducing the aeration pressure to inhibit the activity of NOB, and nitrogen could be removed by shortcut simultaneous nitrification and denitrification. In addition, using real-time quantitative PCR, Yapsakli et al. [42] detected the coexistence of AOB, NOB, AOA, and anaerobic ammonium oxidation (anammox) bacteria at low dissolved oxygen (DO = 0.3–1.5 mg/L) in the system for landfill leachate treatment. Establishing a mathematical model, Liu et al. [55, 56] predicted that in a wide ammonia nitrogen concentration range (30–500 mg/L), with less oxygen consumption and stronger inhibitory effect on NOB activity, autotrophic nitrogen removal by coupled AOA nitritation with anammox was more effective than coupled AOB with anammox. Nitrogen removal by the cooperative AOA, AOB, and denitrifying bacteria or anammox bacteria could be achieved through the regulation of dissolved oxygen level to optimize the community structure. It is also expected to provide new ideas for the development of wastewater nitrogen removal process with high efficiency and low consumption [36, 57].

4.5. pH. It was reported that the pH range of AOA strain SAT1 enriched from activated sludge was 5.0 to 7.0, with the optimum pH at 6.0, indicating that the strain SAT1 was neutrophilic [31]. The ammonia bioavailability can be reduced by the protonation of ammonia when pH decreases, which might be more favourable for the growth of AOA from the perspective of substrate utilization. Recent studies had provided evidence that ammonia oxidation in acidic soils was dominated by AOA, whereas AOB had difficulty surviving at low pH values and were mainly responsible for nitrification in alkaline soils [58–62]. However, it was also reported that alkaline soil was also suitable for the growth of *Candidatus* Nitrosotalea devanaterra (AOA) [63] which showed strong adaptability to pH variation. Until now, the differences in the relative contributions of these two groups of ammonia oxidation microorganisms affected by environmental pH remain a topic of debate. There is also little information concerning the effects of pH on the distribution of AOA and AOB in wastewater treatment systems. However, the AOA strain with strong adaptability to pH changes provides the possibility of its application in wastewater treatment systems with acidic influent.

Based on the literature review above, AOA/AOB in response to the varying environmental factors including ammonia, organic loading, oxygen level, and temperature is proposed in Figure 2. AOA would be dominant over AOB in low ammonium and/or low DO and/or low organic loading environments. AOA would also be more active than AOB when they are exposed to extreme high/low temperatures. In addition, compared with AOB, AOA would be dominant in salinity-containing wastewater [64, 65].

5. Recommendations for Further Study Associated with AOA

Since the discovery of AOA in wastewater treatment plant bioreactors in 2006 [19], AOA have been recognized as potential ammonia oxidizers involved in nitrogen removal from wastewater. The current available information indicates that knowledge of these microorganisms in engineered systems is still at a primary stage. Challenges for practical application include the complexity of wastewater, the uncertainty of operational parameters affecting the activity and functions of AOA, and the limitations of the techniques available. Combined microbiological and engineering points of view are required in the future study. According to the latest literature reviewed, the following further studies were recommended:

(1) Compared with AOB, AOA behaved more active in extreme environments. Therefore, AOA are expected to be effectively enriched and cultured under low temperature conditions or low dissolved oxygen level in wastewater treatment systems (probably in biofilm systems), thus solving the problem of poor nitrification that often happens in wastewater treatment plants in cold regions and providing a new breakthrough for an effective nitrification process.

(2) Although the prediction results of a mathematical model increase the possibility of the development of novel nitrogen removal processes dominated by AOA coupled with denitrifying bacteria or anammox bacteria [55, 56], the structure of the ammonia oxidation functional microbes still needs to be further

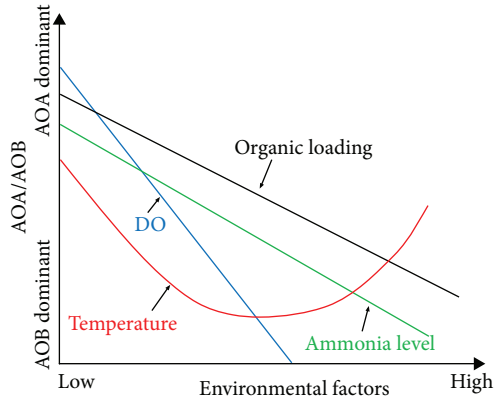

Figure 2: The proposed AOA/AOB in response to the varying environmental factors (ammonia, organic loading, oxygen level, and temperature) (based in part on Guo et al. [68]).

studied in the actual wastewater nitrogen removal system. The optimization of process parameters is also necessary to achieve effective nitrogen removal.

(3) The variations in the population structure of microorganisms (AOA, AOB, NOB, anammox bacteria, and denitrifying bacteria) and their contributions to the nitrogen removal process in actual wastewater treatment systems under different environmental conditions need to be investigated to explain the coexistence, coordination, and competition mechanisms among the microbes associated with the nitrogen removal function.

6. Conclusions

The discovery of AOA breaks the traditional view for the past 100 years that ammonia oxidation is only conducted by AOB, improving the knowledge of the global nitrogen cycle. AOA also appear to play an important role in nitrogen removal from wastewater. Hence, the nitrogen cycle in a wastewater treatment system needs reevaluation. The collaborative, competitive, and inhibitive relationships in microbial communities need further exploration in actual wastewater nitrogen removal systems. The ammonia-oxidizing microorganisms are affected by various environmental conditions, and AOA have stronger environmental adaptability than AOB, which provides the possibility for the development of novel nitrogen removal processes with ammonia oxidation dominated by AOA under extreme environmental conditions (such as low temperature and low oxygen level).

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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References

[1] I. Ferrera and O. Sánchez, "Insights into microbial diversity in wastewater treatment systems: how far have we come?," *Biotechnology Advances*, vol. 34, no. 5, pp. 790–802, 2016.

[2] C. Schleper and G. W. Nicol, "Ammonia-oxidising archaea—physiology, ecology and evolution," *Advances in Microbial Physiology*, vol. 57, pp. 1–41, 2010.

[3] A. Y. Hu and N. Z. Jiao, "Ammonia-oxidizing archaea—a hotspot of environmental microbial ecology," *Progress in Natural Science*, vol. 19, no. 4, pp. 370–379, 2009.

[4] E. W. Vissers, F. S. Anselmetti, P. L. E. Bodelier et al., "Temporal and spatial coexistence of archaeal and bacterial amoA genes and gene transcripts in Lake Lucerne," *Archaea*, vol. 2013, Article ID 289478, 11 pages, 2013.

[5] M. Coci, N. Odermatt, M. M. Salcher, J. Pernthaler, and G. Corno, "Ecology and distribution of Thaumarchaea in the deep hypolimnion of Lake Maggiore," *Archaea*, vol. 2015, Article ID 590434, 11 pages, 2015.

[6] J. You, A. Das, E. M. Dolan, and Z. Hu, "Ammonia-oxidizing archaea involved in nitrogen removal," *Water Research*, vol. 43, no. 7, pp. 1801–1809, 2009.

[7] W. Martens-Habbena, P. M. Berube, H. Urakawa, J. R. de la Torre, and D. A. Stahl, "Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria," *Nature*, vol. 461, no. 7266, pp. 976–979, 2009.

[8] R. Hatzenpichler, "Diversity, physiology and niche differentiation of ammonia-oxidizing archaea," *Applied and Environmental Microbiology*, vol. 78, no. 21, pp. 7501–7510, 2012.

[9] D. L. Valentine, "Adaptations to energy stress dictate the ecology and evolution of the archaea," *Nature Reviews Microbiology*, vol. 5, no. 4, pp. 316–323, 2007.

[10] H. Urakawa, W. Martens-Habbena, and D. A. Stahl, "Physiology and genomics of ammonia-oxidizing Archaea," in *Nitrification*, pp. 117–155, American Society of Microbiology Press, Washington, DC, USA, 2011.

[11] I. Suzuki, U. Dular, and S. C. Kwok, "Ammonia or ammonium ion as substrate for oxidation by *Nitrosomonas europaea* cells and extracts," *Journal of Bacteriology*, vol. 120, no. 1, pp. 556–558, 1974.

[12] E. Bock and M. Wagner, "Oxidation of inorganic nitrogen compounds as an energy source," in *The prokaryotes*, pp. 457–495, Springer, New York, NY, USA, 2006.

[13] J. Simon and M. G. Klotz, "Diversity and evolution of bioenergetic systems involved in microbial nitrogen compound transformations," *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, vol. 1827, no. 2, pp. 114–135, 2013.

[14] C. B. Walker, J. R. de la Torre, M. G. Klotz et al., "*Nitrosopumilus maritimus* genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 19, pp. 8818–8823, 2010.

[15] R. Bartossek, G. W. Nicol, A. Lanzen, H. P. Klenk, and C. Schleper, "Homologues of nitrite reductases in ammonia-oxidizing archaea: diversity and genomic context," *Environmental Microbiology*, vol. 12, no. 4, pp. 1075–1088, 2010.

[16] M. B. Lund, J. M. Smith, and C. A. Francis, "Diversity, abundance and expression of nitrite reductase (nirK)-like genes in marine thaumarchaea," *The ISME Journal*, vol. 6, no. 10, pp. 1966–1977, 2012.

[17] J. B. Glass and V. J. Orphan, "Trace metal requirements for microbial enzymes involved in the production and consumption of methane and nitrous oxide," *Frontiers in Microbiology*, vol. 3, 2012.

[18] L. V. Godfrey and J. B. Glass, "The geochemical record of the ancient nitrogen cycle, nitrogen isotopes, and metal cofactors," in *Methods in enzymology*, pp. 483–506, Academic Press, 2011.

[19] H. D. Park, G. F. Wells, H. Bae, C. S. Criddle, and C. A. Francis, "Occurrence of ammonia-oxidizing archaea in wastewater treatment plant bioreactors," *Applied and Environmental Microbiology*, vol. 72, no. 8, pp. 5643–5647, 2006.

[20] G. F. Wells, H. D. Park, C. H. Yeung, B. Eggleston, C. A. Francis, and C. S. Criddle, "Ammonia-oxidizing communities in a highly aerated full-scale activated sludge bioreactor: betaproteobacterial dynamics and low relative abundance of Crenarchaea," *Environmental Microbiology*, vol. 11, no. 9, pp. 2310–2328, 2009.

[21] T. Limpiyakorn, M. Fürhacker, R. Haberl, T. Chodanon, P. Srithep, and P. Sonthiphand, "amoA-encoding archaea in wastewater treatment plants: a review," *Applied Microbiology and Biotechnology*, vol. 97, no. 4, pp. 1425–1439, 2013.

[22] P. Kayee, P. Sonthiphand, C. Rongsayamanont, and T. Limpiyakorn, "Archaeal amoA genes outnumber bacterial amoA genes in municipal wastewater treatment plants in Bangkok," *Microbial Ecology*, vol. 62, no. 4, pp. 776–788, 2011.

[23] Y. Bai, Q. Sun, D. Wen, and X. Tang, "Abundance of ammonia-oxidizing bacteria and archaea in industrial and domestic wastewater treatment systems," *FEMS Microbiology Ecology*, vol. 80, no. 2, pp. 323–330, 2012.

[24] T. Limpiyakorn, P. Sonthiphand, C. Rongsayamanont, and C. Polprasert, "Abundance of amoA genes of ammonia-oxidizing archaea and bacteria in activated sludge of full-scale wastewater treatment plants," *Bioresource Technology*, vol. 102, no. 4, pp. 3694–3701, 2011.

[25] P. Srithep, P. Pornkulwat, and T. Limpiyakorn, "Contribution of ammonia-oxidizing archaea and ammonia-oxidizing bacteria to ammonia oxidation in two nitrifying reactors," *Environmental Science and Pollution Research*, vol. 25, no. 9, pp. 8676–8687, 2018.

[26] J. F. Gao, X. Luo, G. X. Wu, T. Li, and Y. Z. Peng, "Quantitative analyses of the composition and abundance of ammonia-oxidizing archaea and ammonia-oxidizing bacteria in eight full-scale biological wastewater treatment plants," *Bioresource Technology*, vol. 138, no. 6, pp. 285–296, 2013.

[27] J. Gao, X. Luo, G. Wu, T. Li, and Y. Peng, "Abundance and diversity based on amoA genes of ammonia-oxidizing archaea and bacteria in ten wastewater treatment systems," *Applied Microbiology and Biotechnology*, vol. 98, no. 7, pp. 3339–3354, 2014.

[28] M. Mußmann, I. Brito, A. Pitcher et al., "Thaumarchaeotes abundant in refinery nitrifying sludges express amoA but are not obligate autotrophic ammonia oxidizers," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 40, pp. 16771–16776, 2011.

[29] Y. Zhang, Z. Tian, M. Liu et al., "High concentrations of the antibiotic spiramycin in wastewater lead to high abundance of ammonia-oxidizing archaea in nitrifying populations," *Environmental Science & Technology*, vol. 49, no. 15, pp. 9124–9132, 2015.

[30] L. A. Sauder, M. Albertsen, K. Engel et al., "Cultivation and characterization of *Candidatus* Nitrosocosmicus exaquare, an ammonia-oxidizing archaeon from a municipal wastewater treatment system," *The ISME Journal*, vol. 11, no. 5, pp. 1142–1157, 2017.

[31] Y. Li, K. Ding, X. Wen, B. Zhang, B. Shen, and Y. Yang, "A novel ammonia-oxidizing archaeon from wastewater treatment plant: its enrichment, physiological and genomic characteristics," *Scientific Reports*, vol. 6, no. 1, p. 23747, 2016.

[32] W. B. Jin, Z. X. Li, Y. Y. Yue, and Z. Y. Liang, "Ammonia oxidizing archaea enrichment culture of a sewage treatment system," 2013, CN 201310041633 (in Chinese).

[33] Y. Li, X. Wen, K. Ding, B. Zhang, and B. Shen, "Cultivation and characterization of an ammonia oxidizing archaeon enriched from wastewater treatment plant," *Acta Microbiologica Sinica*, vol. 55, no. 7, pp. 882–891, 2015.

[34] P. Sonthiphand and T. Limpiyakorn, "Change in ammonia-oxidizing microorganisms in enriched nitrifying activated sludge," *Applied Microbiology and Biotechnology*, vol. 89, no. 3, pp. 843–853, 2011.

[35] D. Roy, J. McEvoy, M. Blonigen, M. Amundson, and E. Khan, "Seasonal variation and ex-situ nitrification activity of ammonia oxidizing archaea in biofilm based wastewater treatment processes," *Bioresource Technology*, vol. 244, Part 1, pp. 850–859, 2017.

[36] H. Chen, W. Jin, Z. Liang et al., "Abundance and diversity of ammonia-oxidizing archaea in a biological aerated filter process," *Annals of Microbiology*, vol. 67, no. 6, pp. 405–416, 2017.

[37] H. Chen, Y. Yue, W. Jin et al., "Enrichment and characteristics of ammonia-oxidizing archaea in wastewater treatment process," *Chemical Engineering Journal*, vol. 323, pp. 465–472, 2017.

[38] N. Sinthusith, A. Terada, M. Hahn, P. (. L.). Noophan, J. Munakata-Marr, and L. A. Figueroa, "Identification and quantification of bacteria and archaea responsible for ammonia oxidation in different activated sludge of full-scale wastewater treatment plants," *Journal of Environmental Science and Health. Part A, Toxic/Hazardous Substances & Environmental Engineering*, vol. 50, no. 2, pp. 169–175, 2015.

[39] L. A. Sauder, F. Peterse, S. Schouten, and J. D. Neufeld, "Low-ammonia niche of ammonia-oxidizing archaea in rotating biological contactors of a municipal wastewater treatment plant," *Environmental Microbiology*, vol. 14, no. 9, pp. 2589–2600, 2012.

[40] J. Gao, X. Fan, G. Wu, T. Li, and K. Pan, "Changes of abundance and diversity of ammonia-oxidizing archaea (AOA) and bacteria (AOB) in three nitrifying bioreactors with different ammonia concentrations," *Desalination and Water Treatment*, vol. 57, no. 45, pp. 21463–21475, 2016.

[41] L. Ye and T. Zhang, "Ammonia-oxidizing bacteria dominates over ammonia-oxidizing archaea in a saline nitrification reactor under low DO and high nitrogen loading," *Biotechnology and Bioengineering*, vol. 108, no. 11, pp. 2544–2552, 2011.

[42] K. Yapsakli, C. Aliyazicioglu, and B. Mertoglu, "Identification and quantitative evaluation of nitrogen-converting organisms in a full-scale leachate treatment plant," *Journal of Environmental Management*, vol. 92, no. 3, pp. 714–723, 2011.

[43] M. Könneke, A. E. Bernhard, J. R. de la Torre, C. B. Walker, J. B. Waterbury, and D. A. Stahl, "Isolation of an autotrophic ammonia-oxidizing marine archaeon," *Nature*, vol. 437, no. 7058, pp. 543–546, 2005.

[44] J. R. De la Torre, C. B. Walker, A. E. Ingalls, M. Könneke, and D. A. Stahl, "Cultivation of a thermophilic ammonia oxidizing

archaeon synthesizing crenarchaeol," *Environmental Microbiology*, vol. 10, no. 3, pp. 810–818, 2008.

[45] W. Qin, S. A. Amin, W. Martens-Habbena et al., "Marine ammonia-oxidizing archaeal isolates display obligate mixotrophy and wide ecotypic variation," *Proceedings of the National Academy of Sciences*, vol. 111, no. 34, pp. 12504–12509, 2014.

[46] S. J. Hallam, T. J. Mincer, C. Schleper et al., "Pathways of carbon assimilation and ammonia oxidation suggested by environmental genomic analyses of marine *Crenarchaeota*," *PLoS Biology*, vol. 4, no. 4, article e95, 2006.

[47] C. D. Khangembam, "Effect of temperature on nitrifying microbes, emphasizing on ammonia oxidizing archaea and bacteria," *Journal of Biological Sciences and Medicine*, vol. 2, no. 2, pp. 7–14, 2016.

[48] T. Nakagawa, K. Mori, C. Kato, R. Takahashi, and T. Tokuyama, "Distribution of cold-adapted ammonia-oxidizing microorganisms in the deep-ocean of the northeastern Japan Sea," *Microbes and Environments*, vol. 22, no. 4, pp. 365–372, 2007.

[49] H. He, Y. Zhen, T. Mi, L. Fu, and Z. Yu, "Ammonia-oxidizing Archaea and Bacteria differentially contribute to ammonia oxidation in sediments from adjacent waters of Rushan Bay, China," *Frontiers in Microbiology*, vol. 9, p. 116, 2018.

[50] J. Niu, I. Kasuga, F. Kurisu, H. Furumai, T. Shigeeda, and K. Takahashi, "Abundance and diversity of ammonia-oxidizing archaea and bacteria on granular activated carbon and their fates during drinking water purification process," *Applied Microbiology and Biotechnology*, vol. 100, no. 2, pp. 729–742, 2016.

[51] A. Sims, S. Gajaraj, and Z. Hu, "Seasonal population changes of ammonia-oxidizing organisms and their relationship to water quality in a constructed wetland," *Ecological Engineering*, vol. 40, no. 3, pp. 100–107, 2012.

[52] B. J. Park, S. J. Park, D. N. Yoon, S. Schouten, J. S. Sinninghe Damste, and S. K. Rhee, "Cultivation of autotrophic ammonia-oxidizing archaea from marine sediments in coculture with sulfur-oxidizing bacteria," *Applied and Environmental Microbiology*, vol. 76, no. 22, pp. 7575–7587, 2010.

[53] Y. Lian, M. Xu, Y. Zhong, Y. Yang, F. Chen, and J. Guo, "Ammonia oxidizers in a pilot-scale multilayer rapid infiltration system for domestic wastewater treatment," *PLoS One*, vol. 9, no. 12, article e114723, 2014.

[54] M. Li, C. du, J. Liu, X. Quan, M. Lan, and B. Li, "Mathematical modeling on the nitrogen removal inside the membrane-aerated biofilm dominated by ammonia-oxidizing archaea (AOA): effects of temperature, aeration pressure and COD/N ratio," *Chemical Engineering Journal*, vol. 338, pp. 680–687, 2018.

[55] Y. Liu, H. H. Ngo, W. Guo et al., "Autotrophic nitrogen removal in membrane-aerated biofilms: archaeal ammonia oxidation versus bacterial ammonia oxidation," *Chemical Engineering Journal*, vol. 302, pp. 535–544, 2016.

[56] Y. Pan, B. J. Ni, Y. Liu, and J. Guo, "Modeling of the interaction among aerobic ammonium-oxidizing archaea/bacteria and anaerobic ammonium-oxidizing bacteria," *Chemical Engineering Science*, vol. 150, pp. 35–40, 2016.

[57] J. H. Guo and Y. Z. Peng, "Heterotrophic nitrification, anaerobic ammonia oxidation and archaeal ammonia oxidation in a new nitrogen cycle," *Acta Scientiae Circumstantiae*, vol. 28, no. 8, pp. 1489–1498, 2008.

[58] L. E. Lehtovirta-Morley, K. Stoecker, A. Vilcinskas, J. I. Prosser, and G. W. Nicol, "Cultivation of an obligate acidophilic ammonia oxidizer from a nitrifying acid soil," *Proceedings of the National Academy of Sciences*, vol. 108, no. 38, pp. 15892–15897, 2011.

[59] H. Yao, Y. Gao, G. W. Nicol et al., "Links between ammonia oxidizer community structure, abundance and nitrification potential in acidic soils," *Applied and Environmental Microbiology*, vol. 77, no. 13, pp. 4618–4625, 2011.

[60] J. Z. He, J. P. Shen, L. M. Zhang et al., "Quantitative analyses of the abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea of a Chinese upland red soil under long-term fertilization practices," *Environmental Microbiology*, vol. 9, no. 9, pp. 2364–2374, 2007.

[61] X. Jiang, X. Hou, X. Zhou, X. Xin, A. Wright, and Z. Jia, "pH regulates key players of nitrification in paddy soils," *Soil Biology and Biochemistry*, vol. 81, pp. 9–16, 2015.

[62] H. Xiao, D. A. Schaefer, and X. Yang, "pH drives ammonia oxidizing bacteria rather than archaea thereby stimulate nitrification under *Ageratina adenophora* colonization," *Soil Biology and Biochemistry*, vol. 114, pp. 12–19, 2017.

[63] C. Gubry-Rangin, B. Hai, C. Quince et al., "Niche specialization of terrestrial archaeal ammonia oxidizers," *Proceedings of the National Academy of Sciences*, vol. 108, no. 52, pp. 21206–21211, 2011.

[64] Y. J. Wu, L. M. Whang, T. Fukushima, and S. H. Chang, "Responses of ammonia-oxidizing archaeal and betaproteobacterial populations to wastewater salinity in a full-scale municipal wastewater treatment plant," *Journal of Bioscience and Bioengineering*, vol. 115, no. 4, pp. 424–432, 2013.

[65] T. Zhang, T. Jin, Q. Yan et al., "Occurrence of ammonia-oxidizing Archaea in activated sludges of a laboratory scale reactor and two wastewater treatment plants," *Journal of Applied Microbiology*, vol. 107, no. 3, pp. 970–977, 2009.

[66] J. A. Kozlowski, M. Stieglmeier, C. Schleper, M. G. Klotz, and L. Y. Stein, "Pathways and key intermediates required for obligate aerobic ammonia-dependent chemolithotrophy in bacteria and Thaumarchaeota," *The ISME Journal*, vol. 10, no. 8, pp. 1836–1845, 2016.

[67] M. Nishizawa, S. Sakai, U. Konno et al., "Nitrogen and oxygen isotope effects of ammonia oxidation by thermophilic Thaumarchaeota from a geothermal water stream," *Applied and Environmental Microbiology*, vol. 82, no. 15, pp. 4492–4504, 2016.

[68] J. Guo, Y. Peng, S. Wang et al., "Pathways and organisms involved in ammonia oxidation and nitrous oxide emission," *Critical Reviews in Environmental Science and Technology*, vol. 43, no. 21, pp. 2213–2296, 2013.

[69] A. Rodriguez-Caballero, S. Hallin, C. Påhlson, M. Odlare, and E. Dahlquist, "Ammonia oxidizing bacterial community composition and process performance in wastewater treatment plants under low temperature conditions," *Water Science and Technology*, vol. 65, no. 2, pp. 197–204, 2012.

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Research Article

Effects of Aged Oil Sludge on Soil Physicochemical Properties and Fungal Diversity Revealed by High-Throughput Sequencing Analysis

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The oilfield soil was contaminated for years by large quantities of aged oil sludge generated in the petroleum industry. In this study, physicochemical properties, contents of main pollutants, and fungal diversity of the aged oil sludge-contaminated soil were analyzed. Results revealed that aged oil sludge significantly changed physical and chemical properties of the receiving soil and increased the contents of main pollutants (petroleum hydrocarbons and heavy metals) in soil. Meanwhile, the internal transcribed spacer (ITS) sequencing by Illumina Miseq platform at each taxonomic level demonstrated that the toxicological effect of oil pollutants obviously influenced the fungal diversity and community structure in soil. Moreover, it was found that the presence of three genera (*Cephalotheca*, *Lecanicillium*, and *Septoriella*) appeared in aged oil sludge-contaminated soil. And oil pollutants promoted the growth of certain genera in *Ascomycota* (70.83%) and *Basidiomycota* (10.78%), such as *Venturia*, *Alternaria*, and *Piloderma*. Nevertheless, the growth of *Mortierella* (9.16%), *Emericella* (6.02%), and *Bjerkandera* (0.00%) was intensively limited. This study would aid thorough understanding of microbial diversity in oil-contaminated soil and thus provide new point of view to soil bioremediation.

1. Introduction

Oil sludge is one of the most significant hazardous solid wastes generated in oil industry in China [1]. It was mainly generated from the process of drilling, exploitation, transportation, refining, and storage of crude oil. A large amount of oil sludge consisted of many hazardous chemicals, such as petroleum hydrocarbons (PHCs) and heavy metals (HMs), which are of great concern for the potential toxicity to human, have been emitted into the local environment [2–5]. On the receiving soils, the toxic components in oil sludge may cause nutrient deficiency or limit the growth of seed and plants [6]. Oil sludge was stacked in the open air with continually decreasing of volatile components (mainly some light oil components) and moisture content, generating quantities of aged oil sludge (AOS) [7]. As time passed, the hazardous chemicals in AOS were continuously emitted into the local environment, resulting in chronic pollution effects on the receiving soil. Compared with oil sludge, AOS has the characteristics of higher heavy oil content, longer pollution period, and less comprehensive utilization [7].

In previous studies, much attention has been paid to the proper disposal and sufficient treatment of the stacking oil sludge [7, 8]. The spilled oil sludge in oil-producing regions, especially around the oil wells, was ignored for years or even decades, forming lots of AOS-contaminated sites on soil. Until now, as the most deleterious components in AOS [9], the contents of total petroleum hydrocarbons (TPHs) and

HMs as well as fungal diversity of the long-time oil-polluted soil are still unknown and rarely referred and reported globally.

In recent decades, within the various biological techniques, high-throughput sequencing technology with better capacity for detecting rare species [10] is identified as a highly efficient tool for researching the entire profile of microorganism community [11]. It has been proved that soil microorganisms are far more sensitive to pollution than soil, animals, or plants [12–14], with the evidence that the microorganisms in oil-contaminated soil are obviously different from those in background soil [15, 16]. Some domain bacteria phyla like *proteobacteria* are found in different contaminated receptors, such as soil [17] and activated sludge [11]. However, as a good indicator of pollution, fungal diversity in contaminated soil is rarely reported.

This is the first study that took fungi as the typical indicators of pollution to evaluate the microbial variations in soil caused by AOS. In this study, an AOS site of 4 years on soil was selected as the point source pollution of sampling, in which the fungal diversity and community structure in soil were explored with the method of high-throughput sequencing technique. This study is aimed at evaluating the influence from AOS on physicochemical properties, fungal community structure, and diversity in soil, as well as screening dominant or core oil-resistant fungal genera for potential use in soil bioremediation. The results and related findings would aid in thorough understanding of microorganism structure in oil-contaminated soil and provide new point of view to soil bioremediation.

2. Materials and Methods

2.1. Sampling Site. The samples of aged oil sludge were obtained from Gudao oil factory, the largest output plant of crude oil in Shengli oilfield. Gudao lies in semiarid warm temperate monsoon climate zone at latitudes 37°47′ N to 37°84′ N and longitudes 118°39′ E to 119°8′ E. The soil in Gudao is saline-alkali and raises reeds as the major vegetation. Gudao locates inside the region of Yellow River Delta, in which there is a National Nature Reserve with hundreds of protected animals and plants distributed over 4500 km^2 of wetlands. For the reason of oil exploration, there are many oil wells in Gudao and lots of spilled oil sludge sites on soil, resulting in relatively small and decentralized AOS sites around oil wells.

2.2. Experimental Setup. An AOS spot around an oil well drilled 4 years ago with the approximate diameter of 40 cm was selected. Two soil samples at the horizontal center of AOS spot were collected from 0 cm and 20 cm vertically below the surface of soil, respectively (labeled as S1 and S2). Then, another soil sample obtained from the surface soil and 120 m away from the AOS center without oily sludge surroundings was chosen as blank (labeled as S3, uncontaminated soil sample) (Figure 1). All the samples were stored in an ice chest with stones and plant residues removed, carried backed to the laboratory within 4 hours. Those soil samples were divided into two parts. One part was used for the determination of physicochemical properties, HMs and TPHs (air-dried and processed with a 100-mesh sieve). The other part was analyzed by high-throughput sequencing.

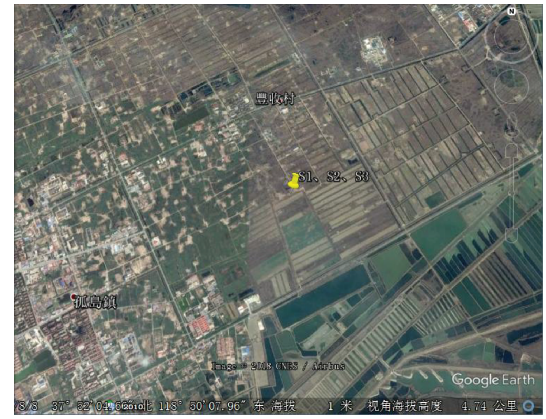

Figure 1: Sampling sites.

2.3. Analytical Methods

2.3.1. Determination of Soil Physicochemical Properties. The pH was tested in deionized water at a soil/water solution ratio of 1 : 2.5 using a pH meter (Mettler-Toledo Instruments, Shanghai, China) [18]. Moisture content was determined with gravimetric method by weighing samples before and after oven-drying at 105°C for 24 h [19]. Salinity was determined by the difference in weight of the solid after the process of washing, filtration, oxidation with H_2O_2, and drying at 100~105°C to a constant weight. The Walkley-Black method was used in this study to determine the dry mass of organic carbons in soil samples [17].

Contents of heavy metals (copper, zinc, and chromium) were determined by atomic absorption spectrophotometer (AAS7000, SHIMADZU, Japan), with pretreatment of digesting by nitric acid, hydrofluoric acid, and hydrogen peroxide system (5 : 2 : 1 by volume), respectively, in microwave digestion instrument [20]. Contents of TPHs were measured using a Purge & Trap Sample Concentrator (Eclipse 4660, OI, USA) combined with GC (7890A with FID detector, Agilent Technologies) [21].

2.3.2. Extraction of Genomic DNA and PCR Amplification. Genomic DNA of the three soil samples were isolated and extracted using the soil DNA kit (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's protocols. The DNA extracts were stored at −20°C for the PCR amplification (95°C for 5 min, followed by 27 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s and a final extension at 72°C for 5 min) which was performed in an ABI GeneAmp 9700 (USA). The fungal rDNA-ITS region was amplified using universal primers ITS1F (5′-CTTGGTCATTTAGAGG AAGTAA-3′) and ITS2R (5′-GCTGCGTTCTTCATCG ATGC-3′) where barcode is an eight-base sequence unique to each sample. PCR reactions were performed in triplicate 20 μL mixture containing 4 μL of 5x FastPfu buffer, 2 μL of

Table 1: Physicochemical properties of aged oil sludge-contaminated soil.

Sample	pH	Salinity (%)	MC[a] (%)	TOC[b] (%)	HMs[c] (mg•kg^{-1})			TPHs[d] (mg•kg^{-1})
					Cu	Zn	Cr	
S1	8.44	0.27	21.05	0.41	76.60	131.63	74.55	15.2
S2	8.56	0.36	22.55	0.35	47.93	93.81	111.46	13.6
S3	8.11	1.45	13.95	0.22	12.20	15.68	34.07	<5

[a]MC: moisture content; [b]TOC: total organic carbon; [c]HMs: heavy metals; [d]TPHs: total petroleum hydrocarbons.

2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu polymerase, and 10 ng of template DNA.

2.3.3. Illumina Miseq PE2500 Sequencing. Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions and quantified using QuantiFluor: trademark: -ST (Promega, USA). Purified amplicons were pooled in equimolar and paired-end sequenced (2×250) on an Illumina Miseq platform according to the standard protocols.

2.3.4. Data Analysis. Raw data must be processed to remove the low-quality data [22] for further analysis. After removing the adaptors, primers and low-quality reads, the pair-end reads were overlapped to assemble the final sequences using the FLASH software. The criterions of overlapping were that the overlapping lengths were >10 bp and the default threshold values were ≤0.2. Chimera tags were further filtered out using the Gold database by UCHIME (version 4.2.40), and finally the effective tags were generated. The operational taxonomic unit (OTU) analysis was performed using the Uparse package (version 7.0.1001) with a 97% sequence identity. Each OTU was taxonomically assigned to the UNITE database using the ribosomal database project (RDP) classifier. OTUs were processed by removing chloroplast sequences, chondriosome sequences, and unclassified sequences. Finally, the OTUs with relative abundance of above 1% were retained.

The Shannon-Weaver diversity index (H) and Simpson index (D) were used to express the diversity of the soil fungal community, which are calculated as follows:

$$H_{\text{Shannon}} = -\sum_{i=1}^{S_{\text{obs}}} \frac{n_i}{N} \ln \frac{n_i}{N},$$
$$D_{\text{Simpson}} = \frac{\sum_{i=1}^{S_{\text{obs}}} n_i(n_i - 1)}{N(N-1)}, \quad (1)$$

where S_{obs} is the number of OTUs, n_i is the number of sequence in OTU$_i$, and N is the sum of all the sequences in OTUs.

All the determinations were performed at least in triplicate. Statistical significance was determined at the confidence levels of 0.05.

3. Results and Discussion

3.1. Overview of Changes in Soil Physicochemical Properties. As a result of its high viscosity, aged oil sludge can be fixed in soil pores or adsorbed onto the surface of soil mineral constituents, causing reduction of water retention capacity and hydraulic conductivity of the soil [1, 23, 24]. It would lead to ultimate change in physical and chemical properties of oil receiving soil. The main physicochemical properties of the samples were determined and listed in Table 1. It was observed that the pH values of the three soil samples ranged from 8.11 to 8.56. The salinity of S1 (0.27%) and S2 (0.36%) was much lower than that of S3 (1.45%) which indicated a higher biomass in the AOS-contaminated soil [15]. The total organic carbon (TOC) in S1 and S2 was much higher than that in S3 due to the oil input. The moisture contents in S1 (21.05%) and S2 (22.55%) were generally higher compared with S3 (13.95%) which was inconsistent with previous studies [1, 7]. It is probably because that hydrophobic crusts formed by the heavy oil components in AOS limited the evaporation of water and water/air exchange of soil [25]. Therefore, the soil around AOS discharged was arid and saline-alkali soil; meanwhile, its physical and chemical properties were significantly changed by AOS. It has been demonstrated that differences in edaphic properties, such as pH and moisture, are often associated with differences in soil fungal communities, not only in richness but also in composition and structure [17, 26, 27].

Along with the emission of oil sludge to the receiving soil, the contents of TPHs and HMs were intensively increased. The concentrations of Cu, Zn, and Cr in S1 were approximately the same with those in S2, while 6.3, 8.4, and 2.2 times of those in S3, respectively (Table 1). Most heavy metals had a cumulative effect and were of particular hazard to ecological receptors and humans [1]. The fungal communities were reported strongly sensitive to the presence of HMs [28] and have higher toleration to metal pollutants than bacteria [20]. On the other side, the contents of TPHs of S1 and S2 were 15.2 mg/kg and 13.6 mg/kg, respectively. While in S3, there was no (or under detection limit of method) TPHs detected. Higher concentrations of TPHs have been reported showing stronger toxic effects on the activity of soil enzymes and microorganisms [23]. In particular, the polycyclic aromatic hydrocarbons (PAHs) in AOS were of great concern for genotoxicity to human and could migrate to groundwater through soil profile [13, 29]. Furthermore, it was found that there was joint toxic effect between PAHs and HMs in AOS-contaminated soil, which was studied by our research group.

3.2. Miseq Sequencing Results and Fungal Community Structures. By amplifying the ITS region of fungi, Illumina high-throughput sequencing which adopted a sequencing-

Table 2: Sequence information and fungal diversity indexes of samples.

Sample ID	Reads	0.97 (the similarity threshold of OTUs)					
		OTUs	Ace	Chao1	Coverage	Shannon-Weaver	Simpson
S1	31,118	475	476	478	0.999734	4.36	0.0467
S2	31,938	557	558	558	0.999812	4.61	0.0355
S3	33,275	565	567	566	0.999730	4.44	0.0414

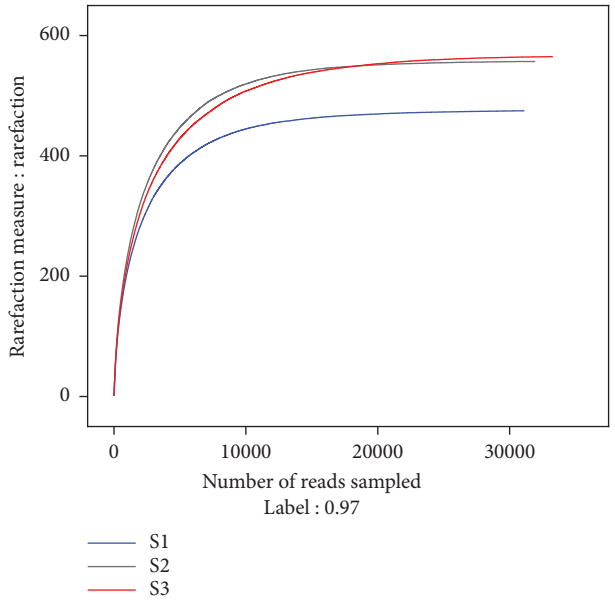

Figure 2: Rarefaction curves based on the 18s rRNA gene sequencing.

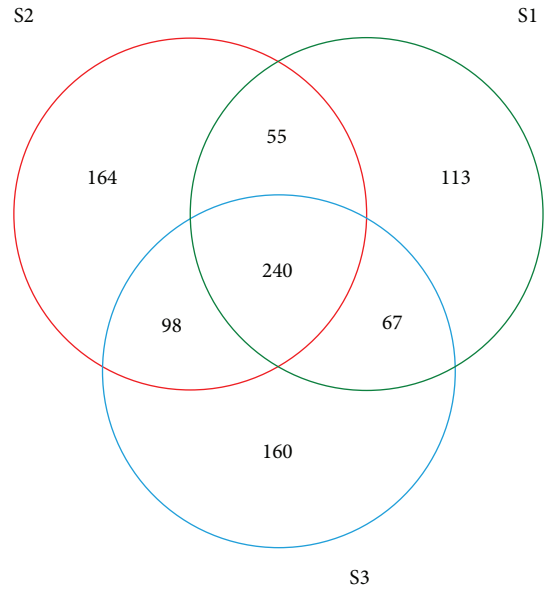

Figure 3: OTU venn analysis in different samples.

by-synthesis approach [30] enabled thorough identification of fungal community structures, including those that could not be cultured or detected in traditional approaches [31–34]. Totally, 96,331 valid sequences of the ITS gene were obtained with an average length of 241 bp. RDP classifier was used in hierarchical clustering analysis at the similarity threshold of 97%. The sequence information and fungal diversity indexes are listed in Table 2. The numbers of OTUs, Ace, Chao 1, the Shannon-Weaver indexes, and Simpson indexes of the three samples showed obvious change after AOS exposure. The numbers of OTUs, Ace, and Chao1 indexes reduced from S3 to S2 and S1, indicating less OTUs and species richness in contaminated soils [35]. S1 had the lowest Shannon-Weaver index (4.36) and the highest Simpson index (0.0467), suggested smallest fungal community diversity [35]. While the Shannon-Weaver index (4.61) and Simpson index (0.0355) of S2 reflected the biggest fungal community diversity. The Good's coverage estimator was used to assess the sampling completeness, obtaining the results of above 0.999, indicating an appropriate reveal of most fungal diversity in all samples [36, 37].

The rarefaction analysis was used to verify whether the volume or the depth of sampling was sufficient to capture the existing OTUs [22, 36]. As shown in Figure 2, the three soil samples generally had the same patterns of rarefaction curves that showed a trend to level off, indicating a sufficient sampling of the fungal species. The Venn diagram was used to analyze the species composition by evaluating the distribution of fungal community. It could provide direct expression of the similarity and overlapping numbers of OTUs between different samples. As shown in Figure 3, the numbers of OTUs in S1, S2, and S3 were 475, 557, and 565, separately, among which 240 OTUs accounted for 73.09% of the total sequences were shared, and 168 OUTs in S1 and 219 OTUs in S2 were unique, respectively, after AOS exposure. Meanwhile, comparing to S3, 160 OTUs disappeared in the contaminated soils. Therefore, AOS affected the richness of fungal community and changed fungal diversity to a certain extent.

3.3. Taxonomic Complexity of Fungal Community. The fungal community compositions of the three soil samples reflected similar diversities but different abundances. Figure 4 provided the fungal community information in phylum level. Among the total 6 identified phyla, *Ascomycota* was the most abundant phylum followed by *Basidiomycota* in all samples. *Ascomycota* has been reported as the largest phylum by far with over 64,000 identified species in almost 6400 genera [38] and the most typical dominant phylum in soil [39]. In this study, *Ascomycota* accounted for 63.67% of total DNA sequences in the noncontaminated soil (S3). While in the contaminated soil samples, the percentage of *Ascomycota* increased to 71.58% in S1 and 70.08% in S2, indicating that oily sludge was beneficial to the existence of

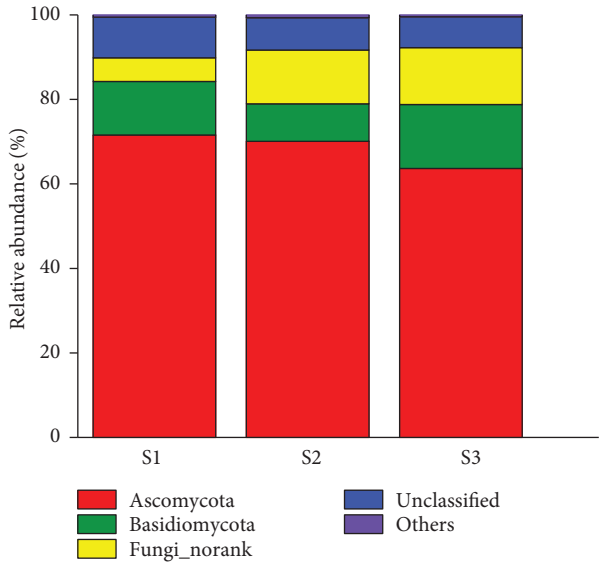

Figure 4: Histogram of fungal community structure at phylum level.

Ascomycota. According to Aranda [40], PAH-polluted soils were found to be colonized mostly by *Ascomycota* and indigenous ascomycete was able to transform or remove PAHs. PAHs are the main components of the hydrocarbons in AOS. Therefore, it might explain the enrichment of *Ascomycota* in soils after long-term AOS exposure. The *Basidiomycota* has been recognized as prominent tool for the degradation of recalcitrant pollutants due to their rich supply of laccase, tyrosinase, and soluble extracellular enzymes, such as lignin-modifying enzyme (LME) [40, 41]. In this study, we observed a decrease of the percentage of *Basidiomycota* (by −2.46% in S1 and −6.26% in S2) in contaminated soils compared with S3 (15.13%). Although the growth was limited, *Basidiomycota* was still the second largest phyla in the contaminated soils.

Further analysis was carried out to analyze the fungal community composition in family levels. In general, 91.83% of the total sequences were assigned and there were 28 identified families with the relative abundance of above 1%. As shown in Table 3, S1 and S2 had similar family structures and diversities. In the noncontaminated soil (S3), the top three abundant families were Mortierellaceae (13.46%), Aspergi laceae (11.32%), and Meruliaceae (9.33%). While in contaminated soils, the growth of the above families was powerfully limited and their percentages in S1 reduced to 5.58%, 10.76%, and even 0.00%, respectively. In contrast, the proportion of certain families was significantly increased in contaminated soils compared with S3, such as Cephalothecaceae, Cordycipitaceae, Pleosporaceae, and Thelephoraceae. Especially the family of Cephalothecaceae, as the largest family among all the identified families, it was a new-appeared fungal family in contaminated soils with the abundance of 16.76% in S1 and 12.63% in S2. The family Cephalothecaceae in Sordariomycetes (Ascomycota) was incertae sedis [42] because of uncertain phylogenetic placement and differing morphology [43]. In addition, Cordycipitaceae and Pleosporaceae are members of Ascomycota, while Thelephoraceae belongs in Basidiomycota.

Table 3: The fungal community structures and diversities at family level.

OTU ID	S1	S2	S3
Cephalothecaceae	16.76%	12.63%	0.00%
Aspergillaceae	10.76%	2.80%	11.32%
Unclassified	9.61%	7.63%	7.28%
Mortierellaceae	5.58%	12.74%	13.46%
Thelephoraceae	5.36%	2.18%	0.58%
Nectriaceae	4.22%	5.08%	5.24%
Cordycipitaceae	4.06%	3.09%	1.43%
Hypocreaceae	3.52%	1.11%	4.22%
Trichocomaceae	2.72%	2.36%	3.17%
Pleosporaceae	2.46%	5.44%	0.19%
Lasiosphaeriaceae	2.39%	6.26%	5.50%
Hypocreales_norank	2.27%	3.01%	3.85%
Pseudeurotiaceae	2.15%	0.91%	2.03%
Didymellaceae	1.91%	1.42%	0.80%
Chaetomiaceae	1.67%	2.97%	3.62%
Venturiaceae	1.60%	0.40%	0.08%
Stachybotriaceae	1.48%	0.33%	1.47%
Sporormiaceae	1.28%	0.35%	1.09%
Atheliaceae	1.21%	0.37%	0.10%
Sebacinaceae	1.17%	0.26%	0.16%
Botryosphaeriaceae	1.15%	3.09%	4.46%
Helotiales_norank	1.12%	0.92%	0.55%
Hyaloscyphaceae	0.86%	2.09%	2.01%
Cystofilobasidiaceae	0.82%	1.49%	1.38%
Herpotrichiellaceae	0.57%	0.59%	1.15%
Polyporales_norank	0.47%	1.04%	0.94%
Clavicipitaceae	0.42%	1.21%	0.72%
Phaeosphaeriaceae	0.34%	1.74%	0.06%
Meruliaceae	0.00%	0.00%	9.33%

The relative fungal abundance at genus level was also analyzed. Totally, 39 genera with the abundance of above 1% were classified in the samples. It was observed that the growth of most identified genera was limited, with the possible reason that AOS discharged TPHs into the receiving soil. TPHs comprised hydrogen and carbon, but lack of nitrogen, sulfur, and phosphorus essential for microbial growth [44]. As the dominant fungi in S3, the growth of *Mortierella* (13.46%), *Emericella* (10.77%), and *Bjerkandera* (9.33%) presented a decreased dynamic to 12.74%, 2.08%, and 0.00% in S1, respectively (Figure 5(a)), while *Alternaria*, *Cephalotheca*, and *Lecanicillium* with the abundances of 5.31%, 12.63%, and 1.74% in S1 was found oil-tolerant (the relative abundances in S3 were 0.05%, 0.00%, and 0.00%) (Figure 5(b)). In general, there were three new genera (*Cephalotheca*, *Lecanicillium*, and *Septoriella*) which did not exist in noncontaminated soil (0.00%), and another three genera (*Venturia*, *Alternaria*, and *Piloderma*) almost not found in S3 of which the percentage was below 0.1% of the total genera.

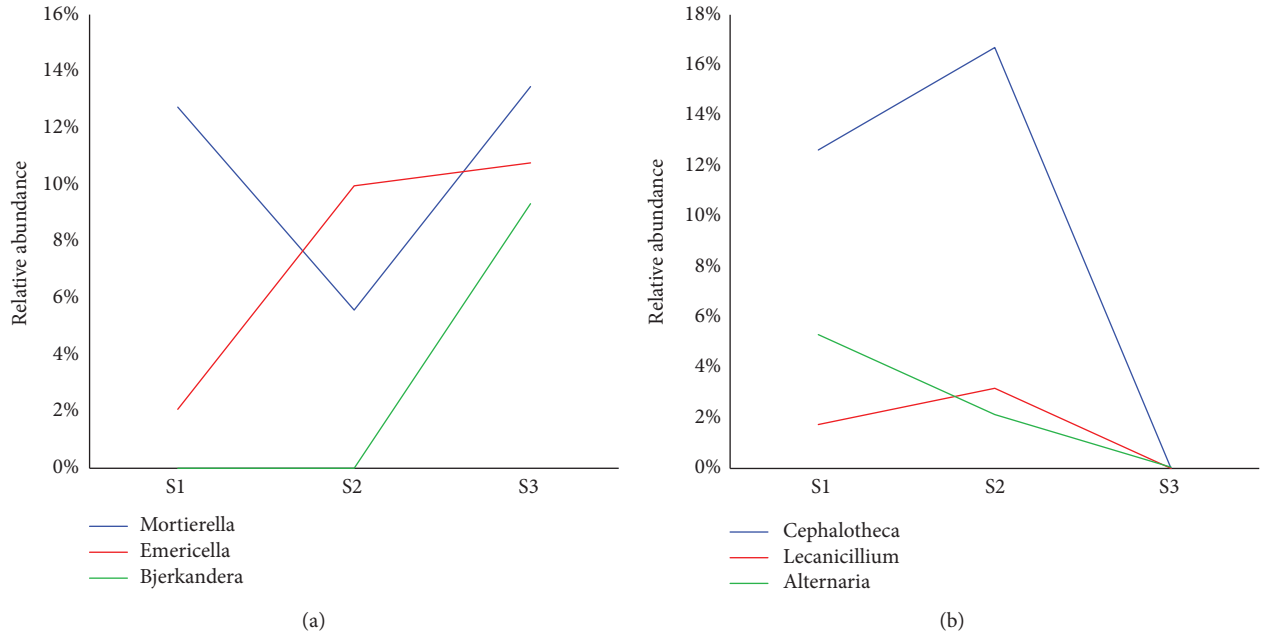

Figure 5: Relative abundance of the three (a) limited genera and (b) oil-resistant genera in samples.

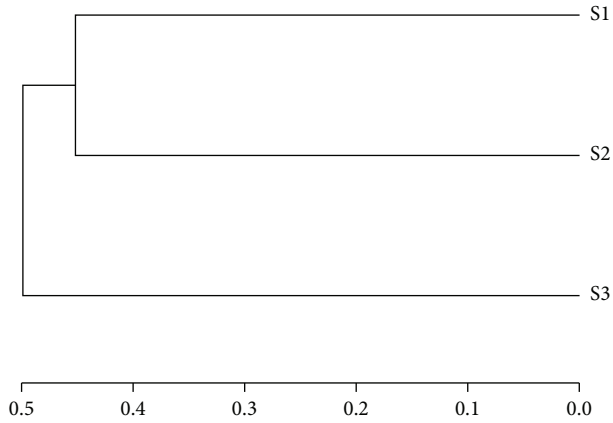

Figure 6: Multiple sample cluster tree.

Cephalotheca was the genus of fungi in the *Cephalothecaceae* family of the *Ascomycota*. It was the core genus with high abundance of 12.63% (in S1) and 16.70% (in S2) in AOS-contaminated soils. *Lecanicillium* is well known as entomopathogenic fungal species. Temperature between 10 and 25°C and higher moisture are benefit to conidial germination of *Lecanicillium* spp. [45]. Considering that the average temperature of Shengli oilfield was 12.9°C, and the AOS-contaminated soils had higher moisture contents than noncontaminated soil (Table 1), it might be the reason for the appearance of *Lecanicillium*. Furthermore, *Septoriella*, *Venturia*, and *Alternaria* were the genera of fungi in *Ascomycota*, and *Piloderma* was the genus of fungi in *Basidiomycota* that could adapt oily circumstance. They are the promising fungi genera that could be used in bioremediation of aged oil contamination in the future.

The analysis of multiple samples shown as cluster tree (Figure 6) demonstrated that the three samples were divided into two clusters. S1 and S2 were clustered together, indicating a more similar fungi community structures and diversities. While as noncontaminated sample, S3 was divided into a separated group, implying distinguishing structures and diversities of fungi communities from the contaminated soils. Furthermore, the two clusters were well separated, which suggested a clear distinction of the fungi community structures and diversities between the two clusters [17].

In the present study, combined with the results of the high-throughput sequencing, further study should be carried out in the future to explore more microorganisms like bacteria and archaea with powerful features of oil resistance before strict screening, culturing, and domesticating of the dominant fungi genera.

4. Conclusions

This is the first study that evaluated the significant effects on physicochemical properties and fungal diversities of soil caused by AOS contamination. The results revealed that longtime oil exposure made the receiving soil arid, saline-alkali, and unsuitable for agriculture. The contents of both TPHs and HMs in the contaminated soils were apparently increased compared with noncontaminated soil. High-throughput sequencing results by Miseq platform showed significant changes in fungi community compositions and diversities. It was observed that oily circumstance could limit the growth of most genera and meanwhile promote the growth of certain oil-resistant fungi like *Venturia* (*Ascomycota*), *Alternaria* (*Ascomycota*), and *Piloderma* (*Basidiomycota*). In particular, there were three new-appeared genera in aged oil sludge-contaminated soils, among which *Cephalotheca* was identified as the core genus with the highest abundance of 16.76% in all the retained genera. The results could present a thorough understanding of microbial

diversity in oil-contaminated soil and a better insight into the soil bioremediation.

Abbreviations

ITS: International transcribed spacer
PHCs: Petroleum hydrocarbons
HMs: Heavy metals
AOS: Aged oil sludge
TPHs: Total petroleum hydrocarbons
OUT: Operational taxonomic unit
RDP: Ribosomal database project
TOC: Total organic carbon
PAHs: Polycyclic aromatic hydrocarbons
LME: Lignin-modifying enzymes
MC: Moisture content.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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References

[1] G. Hu, J. Li, and G. Zeng, "Recent development in the treatment of oily sludge from petroleum industry: a review," *Journal of Hazardous Materials*, vol. 261, pp. 470–490, 2013.

[2] S. Deng, X. Wang, H. Tan et al., "Experimental and modeling study of the long cylindrical oily sludge drying process," *Applied Thermal Engineering*, vol. 91, pp. 354–362, 2015.

[3] G. Hu, J. Li, and H. Hou, "A combination of solvent extraction and freeze thaw for oil recovery from petroleum refinery wastewater treatment pond sludge," *Journal of Hazardous Materials*, vol. 283, pp. 832–840, 2015.

[4] J. Jasmine and S. Mukherji, "Characterization of oily sludge from a refinery and biodegradability assessment using various hydrocarbon degrading strains and reconstituted consortia," *Journal of Environmental Management*, vol. 149, pp. 118–125, 2015.

[5] S. Wang, X. Wang, C. Zhang, F. Li, and G. Guo, "Bioremediation of oil sludge contaminated soil by landfarming with added cotton stalks," *International Biodeterioration & Biodegradation*, vol. 106, pp. 150–156, 2016.

[6] N. Al-Mutairi, A. Bufarsan, and F. Al-Rukaibi, "Ecorisk evaluation and treatability potential of soils contaminated with petroleum hydrocarbon-based fuels," *Chemosphere*, vol. 74, no. 1, pp. 142–148, 2008.

[7] X. Wang, Q. Wang, S. Wang, F. Li, and G. Guo, "Effect of biostimulation on community level physiological profiles of microorganisms in field-scale biopiles composed of aged oil sludge," *Bioresource Technology*, vol. 111, pp. 308–315, 2012.

[8] S. J. Wang, X. Wang, G. L. Lu, Q. H. Wang, F. S. Li, and G. L. Guo, "Bioremediation of petroleum hydrocarbon-contaminated soils by cold-adapted microorganisms: research advance," *Chinese Journal of Applied Ecology*, vol. 22, no. 4, pp. 1082–1088, 2011.

[9] R. A. Tahhan and R. Y. Abu-Ateih, "Biodegradation of petroleum industry oily-sludge using Jordanian oil refinery contaminated soil," *International Biodeterioration & Biodegradation*, vol. 63, no. 8, pp. 1054–1060, 2009.

[10] T. Rämä, M. L. Davey, J. Nordén et al., "Fungi sailing the Arctic Ocean: speciose communities in North Atlantic driftwood as revealed by high-throughput amplicon sequencing," *Microbial Ecology*, vol. 72, no. 2, pp. 295–304, 2016.

[11] Q. Ma, Y. Qu, W. Shen et al., "Bacterial community compositions of coking wastewater treatment plants in steel industry revealed by Illumina high-throughput sequencing," *Bioresource Technology*, vol. 179, pp. 436–443, 2015.

[12] L. Cao, G. Shen, and Y. Lu, "Combined effects of heavy metal and polycyclic aromatic hydrocarbon on soil microorganism communities," *Environmental Geology*, vol. 54, no. 7, pp. 1531–1536, 2008.

[13] N. M. E. J. Leys, A. Ryngaert, L. Bastiaens, W. Verstraete, E. M. Top, and D. Springael, "Occurrence and phylogenetic diversity of Sphingomonas strains in soils contaminated with polycyclic aromatic hydrocarbons," *Applied and Environmental Microbiology*, vol. 70, no. 4, pp. 1944–1955, 2004.

[14] R. Turpeinen, T. Kairesalo, and M. M. Häggblom, "Microbial community structure and activity in arsenic, chromium and copper contaminated soils," *FEMS Microbiology Ecology*, vol. 47, no. 1, pp. 39–50, 2004.

[15] Y. C. Gao, J. N. Wang, S. H. Guo et al., "Effects of salinization and crude oil contamination on soil bacterial community structure in the Yellow River Delta region, China," *Applied Soil Ecology*, vol. 86, pp. 165–173, 2015.

[16] Q. Helmy, R. Laksmono, and E. Kardena, "Bioremediation of aged petroleum oil contaminated soil: from laboratory scale to full scale application," *Procedia Chemistry*, vol. 14, pp. 326–333, 2015.

[17] W. Zhang, L. Chen, R. Zhang, and K. Lin, "High throughput sequencing analysis of the joint effects of BDE209-Pb on soil bacterial community structure," *Journal of Hazardous Materials*, vol. 301, pp. 1–7, 2016.

[18] Q. Yao, J. Liu, Z. Yu et al., "Changes of bacterial community compositions after three years of biochar application in a black soil of northeast China," *Applied Soil Ecology*, vol. 113, pp. 11–21, 2017.

[19] C. Zhang, S. Nie, J. Liang et al., "Effects of heavy metals and soil physicochemical properties on wetland soil microbial biomass and bacterial community structure," *Science of the Total Environment*, vol. 557-558, pp. 785–790, 2016.

[20] C. A. Macdonald, I. M. Clark, F.-J. Zhao, P. R. Hirsch, B. K. Singh, and S. P. McGrath, "Long-term impacts of zinc and copper enriched sewage sludge additions on bacterial, archaeal and fungal communities in arable and grassland soils," *Soil Biology and Biochemistry*, vol. 43, no. 5, pp. 932–941, 2011.

[21] China EPA Method 350, "Standard of soil quality assessment for exhibition sites," 2007.

[22] L. Zhang, M. Kang, Y. Huang, and L. Yang, "Fungal communities from the calcareous deep-sea sediments in the Southwest

India Ridge revealed by Illumina sequencing technology," *World Journal of Microbiology and Biotechnology*, vol. 32, no. 5, p. 78, 2016.

[23] R. R. Suleimanov, I. M. Gabbasova, and R. N. Sitdikov, "Changes in the properties of oily gray forest soil during biological reclamation," *Biology Bulletin*, vol. 32, no. 1, pp. 93–99, 2005.

[24] S. Y. Trofimov and M. S. Rozanova, "Transformation of soil properties under the impact of oil pollution," *Eurasian Soil Science*, vol. 36, pp. S82–S87, 2003.

[25] J. Tang, X. Lu, Q. Sun, and W. Zhu, "Aging effect of petroleum hydrocarbons in soil under different attenuation conditions," *Agriculture, Ecosystems & Environment*, vol. 149, pp. 109–117, 2012.

[26] Z. He, Y. Piceno, Y. Deng et al., "The phylogenetic composition and structure of soil microbial communities shifts in response to elevated carbon dioxide," *ISME Journal*, vol. 6, no. 2, pp. 259–272, 2012.

[27] Z. He, M. Xu, Y. Deng et al., "Metagenomic analysis reveals a marked divergence in the structure of belowground microbial communities at elevated CO_2," *Ecology Letters*, vol. 13, no. 5, pp. 564–575, 2010.

[28] M. op de Beeck, J. Ruytinx, M. M. Smits, J. Vangronsveld, J. V. Colpaert, and F. Rineau, "Belowground fungal communities in pioneer Scots pine stands growing on heavy metal polluted and non-polluted soils," *Soil Biology and Biochemistry*, vol. 86, pp. 58–66, 2015.

[29] H. Wake, "Oil refineries: a review of their ecological impacts on the aquatic environment," *Estuarine, Coastal and Shelf Science*, vol. 62, no. 1-2, pp. 131–140, 2005.

[30] M. Quail, M. E. Smith, P. Coupland et al., "A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers," *BMC Genomics*, vol. 13, no. 1, p. 341, 2012.

[31] L. E. Sáenz de Miera, P. Arroyo, E. de Luis Calabuig, J. Falagán, and G. Ansola, "High-throughput sequencing of 16S RNA genes of soil bacterial communities from a naturally occurring CO_2 gas vent," *International Journal of Greenhouse Gas Control*, vol. 29, pp. 176–184, 2014.

[32] R. Kjøller and S. Rosendahl, "Cultivated and fallow fields harbor distinct communities of Basidiomycota," *Fungal Ecology*, vol. 9, pp. 43–51, 2014.

[33] C. Liu, N. Ding, Q. Fu et al., "The influence of soil properties on the size and structure of bacterial and fungal communities along a paddy soil chronosequence," *European Journal of Soil Biology*, vol. 76, pp. 9–18, 2016.

[34] J. Lou, Y. Liu, and Y. Li, "Review of high-throughput sequencing technologies in studies of soil microbial diversity," *Chinese Agriculture Science Bulletin*, vol. 30, pp. 256–260, 2014.

[35] D. Zhu, S.-H. Tanabe, C. Yang, W. Zhang, and J. Sun, "Bacterial community composition of South China Sea sediments through pyrosequencing-based analysis of 16S rRNA genes," *PLoS One*, vol. 8, no. 10, article e78501, 2013.

[36] A. Dalmasso, M. de los Dolores Soto del Rio, T. Civera, D. Pattono, B. Cardazzo, and M. T. Bottero, "Characterization of microbiota in Plaisentif cheese by high-throughput sequencing," *LWT - Food Science and Technology*, vol. 69, pp. 490–496, 2016.

[37] M. del Carmen Portillo and A. Mas, "Analysis of microbial diversity and dynamics during wine fermentation of Grenache grape variety by high-throughput barcoding sequencing," *LWT - Food Science and Technology*, vol. 72, pp. 317–321, 2016.

[38] C. Beimforde, K. Feldberg, S. Nylinder et al., "Estimating the Phanerozoic history of the Ascomycota lineages: combining fossil and molecular data," *Molecular Phylogenetics and Evolution*, vol. 78, pp. 386–398, 2014.

[39] P. Lienhard, S. Terrat, N. C. Prévost-Bouré et al., "Pyrosequencing evidences the impact of cropping on soil bacterial and fungal diversity in Laos tropical grassland," *Agronomy for Sustainable Development*, vol. 34, no. 2, pp. 525–533, 2014.

[40] E. Aranda, "Promising approaches towards biotransformation of polycyclic aromatic hydrocarbons with Ascomycota fungi," *Current Opinion in Biotechnology*, vol. 38, pp. 1–8, 2016.

[41] L. Martínková, M. Kotik, E. Marková, and L. Homolka, "Biodegradation of phenolic compounds by Basidiomycota and its phenol oxidases: a review," *Chemosphere*, vol. 149, pp. 373–382, 2016.

[42] S. S. N. Maharachchikumbura, K. D. Hyde, E. B. Gareth Jones et al., "Families of Sordariomycetes," *Fungal Diversity*, vol. 79, no. 1, pp. 1–317, 2016.

[43] S. S. N. Maharachchikumbura, K. D. Hyde, E. B. Gareth Jones et al., "Towards a natural classification and backbone tree for Sordariomycetes," *Fungal Diversity*, vol. 72, no. 1, pp. 199–301, 2015.

[44] S. S. Cameotra and P. Singh, "Bioremediation of oil sludge using crude biosurfactants," *International Biodeterioration & Biodegradation*, vol. 62, no. 3, pp. 274–280, 2008.

[45] H. H. Kope, R. I. Alfaro, and R. Lavallée, "Effects of temperature and water activity on Lecanicillium spp. conidia germination and growth, and mycosis of Pissodes strobi," *BioControl*, vol. 53, no. 3, pp. 489–500, 2008.

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Review Article

Treatment of Landfill Leachate Using Activated Sludge Technology: A Review

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Landfill leachate contains a large amount of organic matter and ammoniacal nitrogen. As such, it has become a complex and difficult issue within the water treatment industry. The activated sludge process has been found to be a good solution with low processing costs and is now therefore the core process for leachate treatment, especially for nitrogen removal. This paper describes the characteristics and treatment of leachate. Treatment of leachate using the activated sludge process includes the removal of organic matter, ammoniacal nitrogen, and total nitrogen (TN). The core method for the removal of organic matter involves anaerobic treatment supplemented with an aerobic process. Ammoniacal nitrogen is commonly removed using a conventional aerobic treatment, and advanced TN removal is achieved using endogenous denitrification or an anaerobic ammonium oxidation (ANAMMOX) process. Since biological processes are the most economical method for TN removal, a key issue is how to tap the full potential of the activated sludge process and improve TN removal from leachate. This complex issue has been identified as the focus of current scholars, as well as an important future direction for leachate research and development.

1. Introduction

Solid waste sanitary landfills have been the most common and most important way of dealing with garbage across the world. To take China as an example, the country's total solid waste reached 1.73 billion kilograms in 2013 and 80% of this output was processed through sanitary landfills because of the low costs associated with this method [1].

Leachate is a by-product of sanitary landfills, and, due to its large concentration of pollutants, it must be properly treated before being discharged. The total amount of leachate generated by solid waste sanitary landfills has reached 30 million tons per year. Because the waste composition is very complex, with high organic, ammoniacal nitrogen, and salt content, landfill leachate is considered to be a special wastewater [2–4]. The amount of pollutants in one ton of landfill leachate is equivalent to the amount of pollutants found in 100 tons of urban wastewater. Directly discharging leachate into the surrounding environment would cause irreversible harm, especially to groundwater systems.

Conventional sewage treatment leaves behind high concentrations of ammoniacal nitrogen in landfill leachate which can cause the eutrophication of water bodies. Although biochemical treatments are used to reduce the ammoniacal nitrogen concentration to agreed levels, nitrite concentration in leachate can remain high. Nitrite is a recognized carcinogen; if attention is only given to the control of ammoniacal nitrogen and TN is neglected, the detrimental effects of leachate on the environment could be substantial. Implementing more stringent emission standards for TN in leachate is therefore imperative for countries that want to protect their local environment. In 2008, China revised and implemented new leachate emission standards (GB16889-2008). The new standard increased regulations on the discharge of TN, total phosphorus, and six heavy metal indicators. The requirements of these regulations, especially in relation to TN emissions, are both a challenge and an opportunity for leachate treatment. The

Table 1: Characteristics of landfill leachate with different periods.

Leachate type	Early	Medium-term	Old
Landfill useful life (years)	<5	5–10	>10
pH (–)	6.5–7.5 (7.0)	7.0–8.0 (7.5)	7.5–8.5 (8)
COD (g/L)	10–30 (15)	3–10 (5)	<3 (2)
BOD/COD (–)	0.5–0.7 (0.6)	0.3–0.5 (0.4)	<0.3 (0.2)
NH_4^+-N (mg/L)	500–1000 (700)	800–2000 (1000)	1000–3000 (2000)
COD/NH_4^+-N	5–10 (6)	3-4 (3)	<3 (1.5)

*The values in parentheses are typical values.

challenge is that they are more stringent, increasing the difficulty associated with leachate disposal. However, on the other hand, the new standards will accelerate the development and promotion of new technologies.

2. The Characteristics of Landfill Leachate

Landfill leachate is a foul-smelling black or brown liquid. It contains large amounts of organic and inorganic material, including a number of refractory organics such as aromatic compounds and humus; inorganic salts such as ammoniacal nitrogen, carbonate, and sulfate; and metal ions such as chromium, lead, and copper [5, 6]. Because of the complex composition of the waste, a characteristic of leachate water quality is that it contains high levels of contaminants and, often, biological toxicity.

As a result, chemical oxygen demand (COD) in leachate is typically above 20000 mg/L. Besides toxic aromatic compounds, leachate is also rich in organic macromolecules such as humus and humic acid. Ammoniacal nitrogen concentration above 2000 mg/L is often achieved. This toxic organic matter and these high ammoniacal nitrogen levels cause difficulty during processing, especially for biological treatments. Even in the absence of toxicity, organisms cannot achieve effective microbial degradation because of the large molecular weight and insufficient chemical stability. Therefore, an activated sludge process cannot achieve effective reduction of COD and an advanced treatment process must therefore be developed.

Another feature of leachate is the variance in the quality and quantity of wastewater from different landfills; location has a significant impact. Relatively speaking, the concentration of leachate pollutants in the United States and Europe is much lower than in Asian countries. For example, ammoniacal nitrogen in leachate from European and American countries is generally below 1000 mg/L, while it is generally above 1000 mg/L in Asian countries [7–12]. These differences may relate to different cultures and behaviors in the various regions. In addition, leachate quality can differ in the same place at different times and can be divided into early (less than five years old), medium-term (5–10 years old), and old landfill leachate (more than 10 years old) [13]. Leachate characteristics identified at different times are presented in Table 1 where the values in parentheses represent typical levels.

As shown in Table 1, the features of early leachate are high organic content, strong biodegradability, and relatively low ammoniacal nitrogen concentration. The features of old leachate are high ammoniacal nitrogen content, little biodegradability, and poor COD/NH_4^+-N (or carbon-to-nitrogen ratio). The quality of medium-term leachate water is somewhere between that of the early and old leachates [14, 15]. Meanwhile, the quantity of leachate in the same area is larger in the rainy season and contains higher organic content. The amount of leachate is much reduced in the dry season and it contains high ammoniacal nitrogen concentrations. The third characteristic of leachate water quality is nutritional imbalance; organic matter, ammoniacal nitrogen, and heavy metal concentrations are very high but phosphorus content is very low. Low phosphorus content and concentrated heavy metals increase the difficulty of developing an effective biological treatment for leachate.

3. Treatment of Landfill Leachate and Difficulty

3.1. Treating Landfill Leachate. Since leachate contains large amounts of organic matter and ammoniacal nitrogen, general disposal methods have included physical and chemical pretreatment followed by aerobic and anaerobic biochemical processes, concluding with further physical and chemical methods for final in-depth treatment.

The main functions of pretreatment are to remove suspended solids, degrade some of the organic matter and ammoniacal nitrogen, reduce toxicity, and improve the overall biodegradability of the leachate. This is achieved through coagulating and stripping the ammoniacal nitrogen from the leachate. The function of the subsequent biochemical stage is to remove the biodegradable organic matter and ammoniacal nitrogen. The core technologies in these biochemical processes are, for example, the upflow anaerobic sludge blanket (UASB), membrane bioreactors (MBR), the anoxic-oxic (A/O) process, and sequencing batch reactors (SBR). The later deep treatment of leachate further removes organic matter and TN and can include Fenton oxidation, electrochemical processes, activated carbon adsorption, and membrane treatment processes.

This largely biochemical disposal method results in most biodegradable organic compounds and ammoniacal nitrogen being removed, along with a portion of TN. However, the leachate water still contains a large amount of refractory organic compounds and some TN. In order to achieve current discharge standards, double membranes using nanofiltration and reverse osmosis are employed as safeguards.

3.2. Difficulties in Treating Landfill Leachate. At present, the main difficulties in leachate treatment are as follows:

(1) Leachate has high organic content and a large amount of toxic and organic molecules. Discharge standards cannot be achieved using a single biochemical or physicochemical process; a combination of physicochemical and biochemical processing is required. Choosing a reasonable, economical, and efficient combined process is the first challenge

(2) Ammoniacal nitrogen levels are high, and identifying an effective and complete nitrogen removal process for leachate is difficult. Traditional biological treatment processes can effectively remove ammoniacal nitrogen, but it is not ideal for TN removal. Improving the efficiency of TN removal by biological treatment process is the second key difficulty

(3) The significant changes in water quality and quantity increase the difficulty of identifying a stable standard discharge method. In different seasons, leachate water quality and quantity can be very different which challenges both the selection and the operation of a suitable treatment process. Identifying a suitable combination of available technologies and how to use them to ensure a stable operation are the third challenge in leachate treatment

(4) The treatment process is complex and the costs are very high. In order to achieve discharge standards, leachate treatment plants often use nanofiltration and reverse osmosis which makes treatment costs high. Reducing costs in leachate treatment is the fourth main difficulty

To summarize, it is necessary to identify the appropriate combination of biochemical and physicochemical treatments to dispose of landfill leachate. In addition, maximizing the potential of the biochemical treatment process, improving the TN removal rate, and reducing the total costs are the main challenges associated with developing leachate treatment processes.

4. The Removal of Organic Matter by Activated Sludge

Leachate contains a considerable amount of both biodegradable and nonbiodegradable organic matters. Activated sludge processes can effectively remove biodegradable organic material by completely converting it to carbon dioxide and water. The process can involve anaerobically or aerobically activated sludge. The advantages of an anaerobic process are that it has low energy consumption and can produce energy itself. The disadvantages are that effluent COD is high and retains some biodegradable organic matter. Alternatively, the advantages of an aerobic process are high biodegradation and organic removal rate, as well as good water quality. A disadvantage is high energy consumption throughout the process.

Table 2: The organic treatment efficiency of landfill leachate by the anaerobic activated sludge process.

Processes	COD of leachate	Removal rate	Reference
UASB	5400 mg/L–20000 mg/l	80%	[16]
UASB	8550 mg/L–12500 mg/L	80%	[17]
UASB	12350 mg/L–47800 mg/L	80%	[18]
UASB	3500 mg/L–4200 mg/L	76%	[19]
Anaerobic MBR	2800 mg/L–5000 mg/L	95%	[20]
Anaerobic MBR	13000 mg/L	62%	[21]
EGSB	33000 mg/L	85%–90%	[22]

4.1. Anaerobic Activated Sludge Process. The anaerobic activated sludge process for treating landfill leachate can include upflow anaerobic sludge blanket (UASB), anaerobic membrane bioreactor (MBR), and expanded granular sludge blanket reactor (EGSB). The efficiency of organic removal by anaerobic activated sludge processes is shown in Table 2.

The UASB process results in high removal efficiency and large volumetric loading. This method is therefore often used to treat leachate with high organic content. Agdag and Sponza report on the use of a UASB to dispose of landfill leachate [16]. The hydraulic retention time (HRT) was 1.25 days and the COD removal rate was 80%. Peng et al. combined two processes to process leachate, using a UASB alongside the anoxic-oxic (A/O) process, which resulted in COD of 8550 mg/L–12500 mg/L [17]. The total volumetric loading of the UASB reached 21 kg COD/m^3·d and COD was reduced by more than 80%.

Callia et al. also used a UASB to dispose of leachate observing COD levels of 12350–47800 mg/L [18]. The volumetric loading of their UASB reached 23.5 kg COD/m^3·d and the COD removal rate was 80%. Bohdziewicz and Kwarciak used UASB to dispose of leachate, which saw the COD at 3500 mg/L–4200 mg/L [19]. The influent included 20% wastewater. The final HRT was two days and the volumetric loading was 2 kg COD/m^3·d. The final removal of organic waste was above 76%. Compared to the results of Callia et al., the low volumetric loading of Bohdziewicz's UASB was due to the lower levels of organic matter in the influent.

Anaerobic MBR contain a high concentration of sludge and the effluent quality from this process is good. Bohdziewicz et al. used an anaerobic MBR to process influent that comprised 20% leachate and 80% wastewater [20]. COD was observed at 2800–5000 mg/L and COD was reduced by up to 95%. The HRT and organic loading rate of the MBR were two days and 2.5 kg COD/m^3·d, respectively. Xie et al. also used an anaerobic MBR to dispose of leachate, observing a COD level at 13000 mg/L and an ammoniacal nitrogen level at 3000 mg/L [21]. The average COD removal rate was 62% while the volumetric loading was 4.87 kg COD/m^3·d. Furthermore, the results demonstrated that *Alkaliphilus*, *Petrimonas*, *Fastidiosipila*, and vadin BC27 were the abundant fermentation bacteria found in the bacterial communities.

Table 3: The organic treatment efficiency of landfill leachate by aerobic activated sludge process.

Processes	COD of leachate	Removal rate	Reference
SBR	1348 mg/l	COD 80%–85% BOD 99%	[23]
SBR	1040 mg/L–4870 mg/L	COD 70%	[24]
MBR	2200 mg/L	COD 60%	[25]
MBR	5445 mg/L	COD 98.1%–99.25%	[26]
AO	2000 mg/L–3000 mg/L	COD 40%	[17]

The EGSB, a third-generation anaerobic reactor, has the characteristic of high volumetric loading. Liu et al. studied the effects of ammoniacal nitrogen concentration on organic matter removal efficiency when using an EGSB to dispose of landfill leachate [22]. The results showed that the influent's average COD was around 33000 mg/L and the EGSB's maximum volumetric loading was 64 kg COD/m^3·d. The COD removal rate was 85%–90%. When the concentration of ammoniacal nitrogen was under 1500 mg/L, the removal rate of COD was slightly affected.

4.2. Aerobic Activated Sludge Process. The aerobic activated sludge process for treating leachate includes sequencing batch reactors (SBR), aerobic membrane bioreactors (MBR), the A/O process, and biofilm reactors. The efficiency of aerobic activated sludge processes in removing organic matter from landfill leachate is shown in Table 3.

SBR are the predominant technology used in landfill leachate treatment because of their simple structure and large capacity. Klimiuk et al. used an SBR to process leachate which saw COD at 1348 mg/L [23]. With an HRT of 12 hours, COD was reduced by 80%–85% and a 5-day biochemical oxygen demand (BOD_5) was reduced by more than 99%. By increasing the filler in an SBR, its handling capacity can also be increased. Lim et al. used rice husks as filler in an SBR, thereby observing COD at 1040–4870 mg/L and a COD removal rate of over 70% [24].

MBR are often used to treat leachate because of their high sludge concentration and good effluent water quality. Zolfaghari et al. used an MBR that saw COD at 2200 mg/L and COD removal rate was stabilized at 90% [25]. A high concentration of activated sludge and rich microbial populations provide a good foundation for the reduction of COD. Sanguanpaka et al. studied the treatment efficiency of an MBR using water with different pH levels [26]. The average COD of the influent leachate was 5445 mg/L when initial pH levels ranged between 5.66 and 8.79. Changes to the COD removal rate were small with levels maintained at 98.1%–99.25%.

The A/O process is often used to treat leachate because of its strong nitrogen removal; the rate of COD reduction is also very good when A/O is used to process landfill leachate. Peng et al. used a UASB and the A/O process (UASB + A/O) whereby effluent from the UASB enters an A/O system to further reduce COD [17]. The A/O influent's COD was 2000–3000 mg/L and the effluent's COD was around 1500 mg/L; COD was therefore reduced by more than 40%. The combined UASB + A/O system delivered COD and BOD_5 removal rate of 80%–92% and 99%, respectively.

At present, the aerobic activated sludge process is used to remove ammonium from leachate. However, the efficacy of COD reduction is also very important. Once aerobic treatment of leachate is complete, biological organisms can be almost completely removed. Consequently, threats to the environment caused by landfill leachate are significantly reduced.

4.3. Summary of Activated Sludge Processes. The removal of organic matter by an activated sludge process is considered the most effective and economical way of achieving the desired outcome. The low energy consumption of the anaerobic process combined with the efficiency of the aerobic method could greatly reduce the environmental harm caused by leachate. However, due to the complex composition of leachate, large amounts of organic matter remain in the final effluent discharged after these biological treatments and this makes it difficult to reach regulatory standards. Further development and more effective disposal methods are yet required.

5. The Removal of Ammoniacal Nitrogen by Activated Sludge

Ammoniacal nitrogen found in leachate typically exceeds 1000 mg/L, although some leachates contain even higher levels of up to 3000 mg/L. Significant discharge of ammoniacal nitrogen directly into the surroundings can cause great harm to the environment and especially local groundwater systems. Many developed countries have devised strict emission standards in regard to landfill leachate. In 1997, China implemented one such set of regulations (GB16889-1997). The standards stipulate specific permitted discharge levels of suspended solids, BOD_5, COD, ammoniacal nitrogen, and *E. coli*. As a result, the cost-effective removal of ammoniacal nitrogen from landfill leachate has been a significant challenge for the water treatment industry. Accordingly, activated sludge processes have been key methods in ammoniacal nitrogen disposal because of the low associated costs and low secondary pollution.

5.1. Ammoniacal Nitrogen Removal by SBR. SBR are the preferred process used for landfill leachate ammoniacal nitrogen disposal. Lo used SBR to dispose of landfill leachate and the ammonia nitrogen removal rate was 99% [27]. Similarly, Spagni and Marsili-Libelli used an SBR to process leachate and observed an average COD of 2055 mg/L [28]. The average level of ammoniacal nitrogen was 1200 mg/L, and the

shortcut nitrification and ammoniacal nitrogen removal rates reached 98% and 99%, respectively. Because of a significant imbalance in the carbon-to-nitrogen (C/N) ratio in the leachate, the test used an additional carbon source to achieve denitrification. The TN removal efficiency was more than 95%.

Aziz et al. employed two different SBR to process landfill leachate—one with powdered activated carbon (PAC) and one without [29]. The average COD and average ammoniacal nitrogen in the leachate were 1396 mg/L and 579 mg/L, respectively. Without PAC, the ammoniacal nitrogen removal rate of the SBR was 85.5%. This increased to 89.4% with the addition of PAC; the energy-saving effect is evident.

Sun et al. investigated an SBR's capacity for removing ammoniacal nitrogen at low temperatures [30]. In their study, the leachate's average COD and ammoniacal nitrogen levels were 665 mg/L and 155 mg/L, respectively. The results showed that even at low temperatures of between 13 and 17.6°C, the system achieved rapid shortcut nitrification and ammoniacal nitrogen was removed at a rate of more than 99%. The TN removal rate reached 90% with the addition of a carbon source. Sun et al. studied the effect of using a combination of a UASB and an SBR to treat leachate [31]. The influent COD and ammoniacal nitrogen were 1237–13500 mg/L and 738–2400 mg/L, respectively. The results showed that the system's ammoniacal nitrogen removal rate reached 99.5% and, after adding external carbon to the SBR, the TN removal rate exceeded 99.1%.

Granular sludge sequencing batch reactors (GSBR) provide a new process with high rates of nitrogen removal. Ren et al. report on the use of a GSBR in leachate treatment, resulting in ammoniacal nitrogen levels of 498 mg/L at a removal rate of more than 99% [32]. The microenvironment of the granular sludge was found to achieve good simultaneous nitrification and denitrification, with the GSBR's TN removal rate reaching 50%–60%.

5.2. Ammoniacal Nitrogen Removal by MBR. Canziani et al. used an MBR to dispose of leachate which averaged COD and ammoniacal nitrogen levels at 6361 mg/L and 1497 mg/L, respectively [33]. Ammoniacal nitrogen was removed at a rate of 95% and a stable shortcut nitrification rate of 90% was achieved. Zolfaghari et al. used a sequencing batch MBR in their study [34]. COD in the leachate was 1550 mg/L–2122 mg/L and ammoniacal nitrogen was 288 mg/L–434 mg/L. The results showed that the COD and ammoniacal nitrogen removal rates in summer were 63.4% and 98.2%, respectively. The COD and ammoniacal nitrogen removal rates in the winter were 53.2% and 99.2%, respectively. Zhang et al. used a combination of an MBR with Fenton oxidation and reverse osmosis to process leachate [35]. COD of the MBR influent was around 1500 mg/L, and the ammoniacal nitrogen level was between 600 mg/L and 700 mg/L. The COD removal rate of MBR was more than 95% and the ammoniacal nitrogen removal rate was more than 80%.

Additionally, Remmas et al. studied MBR leachate treatment, observing an average COD level of 1600 mg/L and an average ammoniacal nitrogen level at 600 mg/L [36]. In order to ensure the success of the tests, the researchers used diluted leachate at the beginning of the study. When the process was considered stable, the proportion of leachate was gradually increased until the influent was composed entirely of leachate. The rates of reduction in COD and ammoniacal nitrogen were more than 50% and 95%, respectively, when ammoniacal nitrogen levels were below 600 mg/L. When ammoniacal nitrogen in the influent was above 800 mg/L, the removal rate clearly declined, indicating that high ammoniacal nitrogen levels impact the stability of the system. After denitrification through the addition of carbon, the TN removal rate was 80%–90%.

5.3. Other Processes to Remove Ammoniacal Nitrogen. There are many activated sludge methods used to dispose of leachate in addition to SBR and MBR. These include the conventional continuous flow and A/O processes, the use of rotating biological contactors (RBC), and sequencing batch biofilter granular reactors (SBBGR), as well as combinations of activated sludge processes.

The continuous flow process has a simple construction and a high rate of ammoniacal nitrogen removal. Yusof et al. employed a continuous flow process to process leachate, and they report average COD and ammoniacal nitrogen levels of 2897 mg/L and 1452 mg/L, respectively [37]. The final ammoniacal nitrogen volumetric loading was 3 kg N-NH_4^+/m^3·d and the removal rate was 99%. Effluent nitrate was maintained at around 1200 mg/L. Elsewhere, Halim et al. used a fixed-bed column process achieving average COD and ammoniacal nitrogen levels of 2580 mg/L and 1030 mg/L, respectively [38]. The reduction in COD and ammoniacal nitrogen reached rates of 92.6% and 86.4%, respectively. After system regeneration, the rates of reduction in COD and ammoniacal nitrogen increased to 93.7% and 90.0%, respectively.

Because the A/O process has both nitrification and denitrification applications, it can remove not only ammoniacal nitrogen but also TN by using a reflux nitrification liquid. As previously outlined, Peng et al. used a UASB + AO process to treat landfill leachate [17]. The ammoniacal nitrogen level after A/O process was 1100–2000 mg/L, and the ammoniacal nitrogen removal rate was 99%. The maximum ammonia nitrogen removal volumetric loading was 0.68 kg N-NH_4^+/m^3·d. Through the denitrification process of UASB + AO, the TN removal rate was 91–93%. Wu et al. also used UASB + AO to dispose of leachate which averaged COD and ammoniacal nitrogen levels at 9500 mg/L and 2000 mg/L, respectively, and the rate of ammoniacal nitrogen removal was over 97% [39]. By using a denitrification process in the anoxic zone of the A/O process, a TN removal rate of 80–85% was achieved.

Chen et al. modify the A/O process to process the leachate in their study; an anoxic tank was added after the aerobic tank for denitrification [40]. The average COD and ammoniacal nitrogen levels of the leachate were 3144 mg/L and 1425 mg/L, respectively. The ammoniacal nitrogen and TN removal rates of this system were 95% and 66.4%, respectively. The shortcut nitrification rate was maintained at 90%.

RBC is easily managed and has low consumption. Kulikowska et al. used two RBC processes to treat leachate which averaged the ammoniacal nitrogen concentration level at

834 mg/L [41]. The single-stage RBC could achieve good nitrification when ammoniacal nitrogen volumetric loading was at 1.92 g N-NH_4^+/m^2d and the rate of ammoniacal nitrogen removal exceeded 99%. When the ammoniacal nitrogen volumetric loading was at 3.6 g N-NH_4^+/m^2d, two RBC processes were required to achieve complete nitrification, and when the ammoniacal nitrogen volumetric loading was at 4.79 g N-NH_4^+/m^2d and 6.63 g N-NH_4^+/m^2d, the removal rate decreased to 74.4% and 71.6%, respectively [41].

The SBBGR is a new type of activated sludge process which is characterized by a high concentration of sludge and effective leachate treatment. Iaconi et al. employed an SBBGR to dispose of leachate and observed COD and ammoniacal nitrogen levels at 2200–3200 mg/L and 1500–2000 mg/L, respectively [42]. The ammoniacal nitrogen removal rate of the reactor reached over 99%, and the TN removal rate reached more than 99% by the addition of an external carbon source.

Because of the large amount of contaminants in leachate and their complex components, some studies use a combination of processes to ensure the effectiveness of the treatment. For example, Liu et al. studied a two-stage A/O and MBR process to treat leachate; the MBR replaced the secondary sedimentation tank in the standard A/O process [43]. This ensures not only that the sludge concentration of the system is maintained but also that the removal of COD and ammoniacal nitrogen is optimized. In this study, COD and ammoniacal nitrogen were at 4000–20000 mg/L and 1450–2100 mg/L, respectively. The ammoniacal nitrogen and TN removal rates reached 99.04% and 74.87%, respectively. High-throughput sequencing analysis indicated that Proteobacteria (44.57–50.36%), Bacteroidetes (22.09–27.25%), Planctomycetes (6.94–8.47%), Firmicutes (3.31–4.53%), and Chloroflexi (3.13–4.80%) were the dominating phyla in the system's bacterial community.

5.4. Summary of Ammoniacal Nitrogen Removal by Different Activated Sludge Processes. Since ammoniacal nitrogen has strong chemical stability, it is very difficult to remove it through standard physical or chemical methods. Activated sludge processes are therefore the main technologies used for the removal of ammoniacal nitrogen today. Whichever process is used, most ammoniacal nitrogen found in leachate can be effectively removed through acclimated nitrification of or nitrifying bacteria. Because high levels of ammonia nitrogen have high toxicity, in an actual application, the influent ammonia nitrogen load is very important to understand and control. A high ammonia nitrogen load may poison microbes and reduce the removal rate of the system.

6. The Removal of TN by Activated Sludge

Conventional activated sludge processes might achieve the ammoniacal nitrogen emission standards for landfill leachate. However, leachate organic matter is depleted during the nitrification process which poses significant challenges for traditional denitrification processes. In order to solve the problem of TN removal, a number of researchers identified that adding a further carbon source could initiate advanced denitrification. However, the cost of this approach was considered too high and not applicable in engineering scenarios. In order to reduce processing costs for TN removal, researchers have used more advanced treatment processes in recent years such as endogenous denitrification (ED) and the anaerobic ammonium oxidation process (ANAMMOX). These processes can not only meet the requirements of leachate TN removal but also have low associated costs. This is of great significance to meet the needs of the industry and to promote further development of landfill leachate treatments.

6.1. TN Removal by Endogenous Denitrification. Denitrifying bacteria are able to maintain a carbon source during leachate treatment. When sewage has no external source of carbon on which it might draw, this kind of bacteria uses internal carbon sources from within itself for denitrification. If this characteristic could be successfully enhanced, advanced denitrification could be achieved for landfill leachate without the addition of an external carbon source.

Zhu et al. used an aerobic sequencing batch reactor (ASBR) and an SBR to treat early landfill leachate [44]. Influent COD and ammoniacal nitrogen levels were at 8528 mg/L and 1154 mg/L, respectively. The primary role of the ASBR was to regulate the leachate's C/N ratio. The SBR influent C/N ratio was around four to one. After the first filling, the SBR was stirred and an aeration nitrification process was generated. After the last aeration, agitation continued until the system had completely removed the TN. The main purpose of premixing the raw water was to maintain a carbon source for denitrification, and the last agitation was performed to utilize the internal carbon source. The system achieved COD and TN removal rates of 89.61–96.73% and 97.03–98.87%, respectively, without any external source of carbon required.

Wang et al. also used an ASBR and SBR system to treat early leachate with COD and ammoniacal nitrogen levels of 6000 mg/L and 1100 mg/L, respectively [45]. Similar to the study of Zhu et al. above, the primary role of the ASBR here was to regulate the leachate's C/N ratio. However, in contrast to that study of Zhu et al., the SBR in this study was operated in an influent-stirring-aeration-stirring-sedimentation-draining process. After stirring the leachate, denitrifying bacteria would absorb the carbon and convert it into an internal form such as PHB. When nitrification was complete, the denitrifying bacteria used this stored carbon to realize ED. The system's COD and TN removal rates were 90% and 95%, respectively.

In conclusion, advanced nitrogen removal can be achieved through endogenous denitrification. The disadvantage of this process is that it can only treat early and medium-term leachates with a C/N ratio that is greater than four. If the C/N ratio is below four, the technology cannot be used.

6.2. TN Removal by Anaerobic Ammonium Oxidation. ANAMMOX is an advanced autotrophic denitrification process. Its biggest advantages are that it requires no carbon source and that TN removal efficiency is high. The main difficulty related to this process is the source of nitrite.

ANAMMOX currently used for treating landfill leachate has two major categories: one-stage ANAMMOX and two-stage ANAMMOX. One-stage ANAMMOX achieves autotrophic denitrification in one reactor; having a small number of reactors is an advantage, but control is difficult. Two-stage ANAMMOX, which involves short nitrification and anaerobic ammonium oxidation, is performed in two reactors with two functions. The first reactor realizes semishortcut nitrification and the second reactor enables ANAMMOX itself; shortcut nitrification occurs in the first reactor and the effluent is mixed with raw water to become the influent of the ANAMMOX reactor. The advantage of two-stage ANAMMOX is that bacteria are highly enriched enabling higher nitrogen removal efficiency. Its complexity is its disadvantage.

6.2.1. Semishortcut Nitrification by Activated Sludge. SBR are especially conducive to realizing semishortcut nitrification in landfill leachate. Ganigué et al. used an SBR to process leachate and observed ammoniacal nitrogen levels of 1623 mg/L [46]. By controlling the alkalinity of the leachate, the ammonia nitrogen volumetric loading was kept at 1–1.5 kg $N\text{-}NH_4^+/m^3\cdot d$. The effluent supported stable semishortcut nitrification, and the ratio of nitrite nitrogen and ammoniacal nitrogen was 6 : 4. Meanwhile, the nitrate concentration was very low due to the high water temperatures and dissolved oxygen, which was less than 5% of water TN. In 2012, Ganigué's research group published a report in *Bioresource Technology* about the semishortcut nitrification of leachate [14]. At 6000 mg/L, the ammoniacal nitrogen level of the leachate in this study was higher than that in previous research. The results showed that, at 25°C and 35°C, stable semishortcut nitrification was enabled by controlling the ratio of alkalinity to ammoniacal nitrogen. The final effluent's ammoniacal nitrogen to nitrite ratio could be controlled at 4 : 3 which provided a good basis for ANAMMOX. Li et al. also used an SBR to treat leachate [47]. In contrast to Ganigué et al., Li et al. identified that semishortcut nitrification was mainly controlled by the amount of aeration in the SBR and the pH level of the effluent. The average ammoniacal nitrogen of the leachate in Li et al.'s study was 1748 mg/L. When aeration at $19.6 \pm 171\ m^3\cdot air/m^3\cdot h$ was applied, the volumetric loading of ammoniacal nitrogen reached 0.71 ± 0.14 kg $N\text{-}NH_4^+/m^3\cdot d$. To achieve stable semishortcut nitrification, the effluent pH range was adjusted according to the different ammoniacal nitrogen volumetric loads. This was generally between 8.18 and 8.39.

Thus, there are two ways to realize semishortcut nitrification–adjust the alkalinity of the leachate or the pH level of the effluent. Due to significant differences in water quality of leachate, it is difficult to maintain stable semishortcut nitrification by only controlling the pH and alkalinity of the leachate. How to realize stable semishortcut nitrification requires further exploration and innovation.

6.2.2. One-Stage and Two-Stage ANAMMOX. Wen et al. used a one-stage sequencing batch biofilm reactor (SBBR) ANAMMOX process to process leachate [48]. The study investigated the TN removal capacity of the SBBR under different dissolved oxygen conditions. The results showed that when the dissolved oxygen was controlled at 2.7 mg/L, the TN removal rate was at its highest and stabilized at 90%. Thus, dissolved oxygen is very important in one-stage ANAMMOX.

To inhibit the effects of dissolved oxygen on ANAMMOX bacteria, Xu et al. used an intermittent aeration one-stage SBR to treat old leachate [49]. Short nitrification occurred when the SBR was aerated and ANAMMOX occurred when the SBR was stirred. The dissolved oxygen was controlled at 1.0–1.5 mg/L during the aeration process. Ultimately, the TN removal efficiency of the SBR exceeded 90%. The TN effluent mainly included nitrate. The activities of aerobic ammonium oxidization, anaerobic ammonium oxidization, and denitrification reached 2.83 kg NH_4^+-N/kg_{dw}/day, 0.65 kg NH_4^+-N/kg_{dw}/day, and 0.11 kg NO_3^--N/kg_{dw}/day, respectively.

Similarly, Zhang et al. used a one-stage intermittent aeration SBR process to treat leachate in their study and observed COD and ammoniacal nitrogen levels at 1900 ± 200 mg/L and 1950 ± 250 mg/L, respectively [50]. An ammonium conversion efficiency of $99.3 \pm 0.3\%$ and a TN removal efficiency of $99 \pm 0.1\%$ were subsequently obtained. Based on the nitrogen balance, the nitrogen removal contribution was 77.1% for ANAMMOX and 15.6% for denitrification. Thus, intermittent aeration could resolve disturbances from dissolved oxygen on the ANAMMOX bacteria but manipulation is very complex.

The two-stage ANAMMOX is more complicated than the one-stage version but removal efficiency is much higher. Miao et al. used three SBR in the treatment of leachate which saw COD and ammoniacal nitrogen levels at 2200 ± 200 mg/L and 2000 ± 200 mg/L, respectively [51]. The system included a carbon removal SBR, a shortcut nitrification SBR, and an ANAMMOX SBR. The carbon removal SBR uses simple aeration to remove organic matter and therefore ensure anaerobic ammonium oxidation activity which would be otherwise inhibited. The function of the shortcut nitrification SBR is to provide nitrite for the ANAMMOX SBR via a shortcut nitrification process, and the ANAMMOX SBR completes the process with final denitrification by ANAMMOX. The TN removal of the system was 90% and the ammoniacal nitrogen volumetric loading and ammoniacal nitrogen removal volumetric loading were 0.81 kg $N\text{-}NH_4^+/m^3\cdot d$ and 0.76 kg $N\text{-}NH_4^+/m^3\cdot d$, respectively. In 2016, Miao used a two-stage SBR and SBBR process which measured COD and ammoniacal nitrogen levels at 3000 ± 100 mg/L [52]. The SBR served to remove organic matter and realize shortcut nitrification, and the function of the SBBR was achieved using ANAMMOX, changing the traditional mode of operation. It took five hours to fill the system; the aim of prolonging the filling time was to avoid the inhibition of nitrite for the ANAMMOX bacterium. By changing the mode of operation, the TN removal rate exceeded 95% and the effluent TN was below 20 mg/L. Adding fillers significantly improved the efficiency of the nitrogen removal in the system.

Li et al. used an SBR plus UASB process, achieving stable semishortcut nitrification in the SBR by adjusting the pH of the effluent water [53]. ANAMMOX stability was then achieved in the UASB, and, ultimately, the ammoniacal nitrogen volumetric loading was 1 kg $N\text{-}NH_4^+/m^3{\cdot}d$ and the TN removal rate was 85 ± 1%. The ammonia-oxidizing bacteria (AOB) in the partial nitration SBR was mainly affiliated with *Nitrosomonas* sp. IWT514 and *Nitrosomonas eutropha*. The anaerobic AOB in the ANAMMOX reactor were mainly affiliated with *Kuenenia stuttgartiensis* [53].

Wang et al. used an A/O + UASB system to process leachate which saw COD and ammoniacal nitrogen levels of 2305 mg/L and 1240 mg/L [54]. The function of the A/O process was anoxic denitrification and shortcut nitrification. The A/O effluent entered an intermediate tank and then entered the UASB along with raw water. The COD and TN removal rates were 62% and 94%, respectively. In quantitative PCR reactions, the proportions occupied by AOB, nitrite-oxidizing bacteria, and ANAMMOX in the A/O were 11.39%, 1.76%, and 0.05%, respectively, and the proportions in the UASB were 0.35%, 4.01%, and 7.78%, respectively.

Wu et al. used a more complex UASB + AO + UASB system and observed COD and ammoniacal nitrogen levels of 2500–3000 mg/L and 1900-2000 mg/L, respectively [55]. The function of the first UASB was denitrification using carbon from within the raw water, the A/O process served to initiate shortcut nitrification, and the function of the second UASB was to realize nitrogen removal. The system's final effluent presented COD, ammoniacal nitrogen, and TN levels of 70 mg/L, 11.3 mg/L, and 39 mg/L, respectively. The denitrification contribution rates by the three reactors were 24.6%, 49.6%, and 16.1%, respectively.

Phan et al. used a two-stage reactor to treat old leachate in which internal circulation ANAMMOX was implemented [56]. The influent's ammoniacal nitrogen and nitrite concentrations were 235–655 mg/L and 261–858 mg/L, respectively. Due to the excellent performance of the internal circulation system, the ammoniacal nitrogen volumetric loading rate exceeded 10 kg $N\text{-}NH_4^+/m^3{\cdot}d$. A high TN removal rate of 9.52 ± 1.11 kg $N\text{-}NH_4^+/m^3{\cdot}d$ was observed when the TN concentration of the influent was 1500 mg/L. The specific ANAMMOX activity was found to be 0.598 ± 0.026 g N_2-Ng/VSS·d. DNA analysis showed that *Candidatus Kuenenia stuttgartiensis* was the dominant species in the reactor at 37.45%.

6.3. Summary of TN Removal by Activated Sludge Processes. TN removal has been a problem in all previous research and activities associated with leachate treatment. As new technologies, ED and ANAMMOX have positive and negative characteristics. The biggest advantage of ED is that no external carbon source is needed to obtain high TN removal and operation is simple. A disadvantage of this process, however, is that it can only process the leachate when it contains enough carbon and this limits its application.

ANAMMOX is a hot water treatment technology, and its advantages are low costs, high TN removal rates, and not needing an external carbon source. However, the drawbacks of ANAMMOX are that it is a complicated process and management of the system is difficult. Moreover, ANAMMOX bacteria are difficult to obtain and slow to grow. The domestication of the system is problematic.

7. Summary

In summary, due to low costs and good results, activated sludge processes are the preferred technology for landfill leachate treatment. Discharge that meets the required standards would be easy to realize if the problems of organic matter and TN could be solved. TN removal from leachate is particularly difficult; conventional disposal processes are currently low in efficiency or high in cost, and this makes it difficult to apply them practically to leachate treatment. New treatment processes, such as ED and ANAMMOX have significant advantages. The design of these processes and their parameters should be pursued and optimized to aid the water treatment industry; future research should explore and focus on these core leachate processes.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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References

[1] C. Amor, E. D. Torres-Socías, J. A. Peres et al., "Mature landfill leachate treatment by coagulation/flocculation combined with Fenton and solar photo-Fenton processes," *Journal of Hazardous Materials*, vol. 286, pp. 261–268, 2015.

[2] S. Sri Shalini and K. Joseph, "Nitrogen management in landfill leachate: application of SHARON, ANAMMOX and combined SHARON-ANAMMOX process," *Waste Management*, vol. 32, no. 12, pp. 2385–2400, 2012.

[3] S. Renou, J. G. Givaudan, S. Poulain, F. Dirassouyan, and P. Moulin, "Landfill leachate treatment: review and opportunity," *Journal of Hazardous Materials*, vol. 150, no. 3, pp. 468–493, 2008.

[4] N. J. Horan, H. Gohar, and B. Hill, "Application of a granular activated carbon-biological fluidised bed for the treatment of landfill leachates containing high concentrations of ammonia," *Water Science and Technology*, vol. 36, no. 2-3, pp. 369–375, 1997.

[5] T. H. Christensen, P. Kjeldsen, P. L. Bjerg et al., "Biogeochemistry of landfill leachate plumes," *Applied Geochemistry*, vol. 16, no. 7-8, pp. 659–718, 2001.

[6] A. Pivato and L. Gaspari, "Acute toxicity test of leachates from traditional and sustainable landfills using luminescent

bacteria," *Waste Management*, vol. 26, no. 10, pp. 1148–1155, 2006.

[7] J. G. Henry, D. Prasad, and H. Young, "Removal of organics from leachates by anaerobic filter," *Water Research*, vol. 21, no. 11, pp. 1395–1399, 1987.

[8] I. W. C. Lau, P. Wang, and H. H. P. Fang, "Organic removal of anaerobically treated leachate by Fenton coagulation," *Journal of Environmental Engineering and Science*, vol. 127, no. 7, pp. 666–669, 2001.

[9] G. Baumgarten and C. F. Seyfried, "Experiences and new developments in biological pretreatment and physical post-treatment of landfill leachate," *Water Science and Technology*, vol. 34, no. 7-8, pp. 445–453, 1996.

[10] J. J. Wu, C. C. Wu, H. W. Ma, and C. C. Chang, "Treatment of landfill leachate by ozone-based advanced oxidation processes," *Chemosphere*, vol. 54, no. 7, pp. 997–1003, 2004.

[11] D. Trebouet, J. P. Schlumpf, P. Jaouen, J. P. Maleriat, and F. Quemeneur, "Effect of operating conditions on the nanofiltration of landfill leachates: pilot-scale studies," *Environmental Technology*, vol. 20, no. 6, pp. 587–596, 1999.

[12] A. Uygur and F. Kargi, "Biological nutrient removal from pretreated landfill leachate in a sequencing batch reactor," *Journal of Environmental Management*, vol. 71, no. 1, pp. 9–14, 2004.

[13] S. Baig, I. Coulomb, P. Courant, and P. Liechti, "Treatment of landfill leachates: Lapeyrouse and Satrod case studies," *Ozone: Science & Engineering*, vol. 21, no. 1, pp. 1–22, 1999.

[14] J. Gabarró, R. Ganigué, F. Gich, M. Ruscalleda, M. D. Balaguer, and J. Colprim, "Effect of temperature on AOB activity of a partial nitritation SBR treating landfill leachate with extremely high nitrogen concentration," *Bioresource Technology*, vol. 126, pp. 283–289, 2012.

[15] E. S. K. Chian and F. B. DeWalle, "Sanitary landfill leachates and their treatment," *Journal of the Environmental Engineering Division*, vol. 102, pp. 411–431, 1976.

[16] O. N. Agdag and D. T. Sponza, "Anaerobic/aerobic treatment of municipal landfill leachate in sequential two-stage up-flow anaerobic sludge blanket reactor (UASB)/completely stirred tank reactor (CSTR) systems," *Process Biochemistry*, vol. 40, no. 2, pp. 895–902, 2005.

[17] Y. Peng, S. Zhang, W. Zeng, S. Zheng, T. Mino, and H. Satoh, "Organic removal by denitritation and methanogenesis and nitrogen removal by nitritation from landfill leachate," *Water Research*, vol. 42, no. 4-5, pp. 883–892, 2008.

[18] B. Callia, B. Mertoglu, K. Roest, and B. Inanc, "Comparison of long-term performances and final microbial compositions of anaerobic reactors treating landfill leachate," *Bioresource Technology*, vol. 97, no. 4, pp. 641–647, 2006.

[19] J. Bohdziewicz and A. Kwarciak, "The application of hybrid system UASB reactor-RO in landfill leachate treatment," *Desalination*, vol. 222, no. 1-3, pp. 128–134, 2008.

[20] J. Bohdziewicz, E. Neczaj, and A. Kwarciak, "Landfill leachate treatment by means of anaerobic membrane bioreactor," *Desalination*, vol. 221, no. 1-3, pp. 559–565, 2008.

[21] Z. Xie, Z. Wang, Q. Wang, C. Zhu, and Z. Wu, "An anaerobic dynamic membrane bioreactor (AnDMBR) for landfill leachate treatment: performance and microbial community identification," *Bioresource Technology*, vol. 161, pp. 29–39, 2014.

[22] J. Liu, J. Luo, J. Zhou, Q. Liu, G. Qian, and Z. P. Xu, "Inhibitory effect of high-strength ammonia nitrogen on bio-treatment of landfill leachate using EGSB reactor under mesophilic and atmospheric conditions," *Bioresource Technology*, vol. 113, pp. 239–243, 2012.

[23] E. Klimiuk and D. Kulikowska, "Organics removal from landfill leachate and activated sludge production in SBR reactors," *Waste Management*, vol. 26, no. 10, pp. 1140–1147, 2006.

[24] P. E. Lim, S. P. Lim, C. E. Seng, and A. M. Noor, "Treatment of landfill leachate in sequencing batch reactor supplemented with activated rice husk as adsorbent," *Chemical Engineering Journal*, vol. 159, no. 1-3, pp. 123–128, 2010.

[25] M. Zolfaghari, P. Droguia, S. K. Brar, G. Buelna, and R. Dubé, "Effect of bioavailability on the fate of hydrophobic organic compounds and metal in treatment of young landfill leachate by membrane bioreactor," *Chemosphere*, vol. 161, pp. 390–399, 2016.

[26] S. Sanguanpaka, C. Chiemchaisri, W. Chiemchaisri, and K. Yamamoto, "Influence of operating pH on biodegradation performance and fouling propensity in membrane bioreactors for landfill leachate treatment," *International Biodeterioration & Biodegradation*, vol. 102, pp. 64–72, 2015.

[27] I. M.-C. Lo, "Characteristics and treatment of leachates from domestic landfills," *Environment International*, vol. 22, no. 4, pp. 433–442, 1996.

[28] A. Spagni and S. Marsili-Libelli, "Nitrogen removal via nitrite in a sequencing batch reactor treating sanitary landfill leachate," *Bioresource Technology*, vol. 100, no. 2, pp. 609–614, 2009.

[29] S. Q. Aziz, H. A. Aziz, and M. S. Yusoff, "Powdered activated carbon augmented double react-settle sequencing batch reactor process for treatment of landfill leachate," *Desalination*, vol. 277, no. 1-3, pp. 313–320, 2011.

[30] H. Sun, Y. Peng, S. Wang, and J. Ma, "Achieving nitritation at low temperatures using free ammonia inhibition on Nitrobacter and real-time control in an SBR treating landfill leachate," *Journal of Environmental Sciences*, vol. 30, pp. 157–163, 2015.

[31] H. Sun, Y. Peng, and X. Shi, "Advanced treatment of landfill leachate using anaerobic-aerobic process: organic removal by simultaneous denitritation and methanogenesis and nitrogen removal via nitrite," *Bioresource Technology*, vol. 177, pp. 337–345, 2015.

[32] Y. Ren, F. Ferraz, M. Lashkarizadeh, and Q. Yuan, "Comparing young landfill leachate treatment efficiency and process stability using aerobic granular sludge and suspended growth activated sludge," *Journal of Water Process Engineering*, vol. 17, pp. 161–167, 2017.

[33] R. Canziani, V. Emondi, M. Garavaglia, F. Malpei, E. Pasinetti, and G. Buttiglieri, "Effect of oxygen concentration on biological nitrification and microbial kinetics in a cross-flow membrane bioreactor (MBR) and moving-bed biofilm reactor (MBBR) treating old landfill leachate," *Journal of Membrane Science*, vol. 286, no. 1-2, pp. 202–212, 2006.

[34] M. Zolfaghari, K. Jardak, P. Drogui, S. K. Brar, G. Buelna, and R. Dubé, "Landfill leachate treatment by sequential membrane bioreactor and electro-oxidation processes," *Journal of Environmental Management*, vol. 184, Part 2, pp. 318–326, 2016.

[35] G. Zhang, L. Qin, Q. Meng, Z. Fan, and D. Wu, "Aerobic SMBR/reverse osmosis system enhanced by Fenton oxidation for advanced treatment of old municipal landfill leachate," *Bioresource Technology*, vol. 142, pp. 261–268, 2013.

[36] N. Remmas, P. Melidis, I. Zerva et al., "Dominance of candidate Saccharibacteria in a membrane bioreactor treating medium age landfill leachate: effects of organic load on

microbial communities, Hydrolytic potential and extracellular polymeric substances," *Bioresource Technology*, vol. 238, pp. 48–56, 2017.

[37] N. Yusof, M. A. Hassan, L. Y. Phang et al., "Nitrification of ammonium-rich sanitary landfill leachate," *Waste Management*, vol. 30, no. 1, pp. 100–109, 2010.

[38] A. A. Halim, H. A. Aziz, M. A. M. Johari, K. S. Ariffin, and M. N. Adlan, "Ammoniacal nitrogen and COD removal from semi-aerobic landfill leachate using a composite adsorbent: fixed bed column adsorption performance," *Journal of Hazardous Materials*, vol. 175, no. 1-3, pp. 960–964, 2010.

[39] L. Wu, C. Peng, S. Zhang, and Y. Peng, "Nitrogen removal via nitrite from municipal landfill leachate," *Journal of Environmental Sciences*, vol. 21, no. 11, pp. 1480–1485, 2009.

[40] Z. Chen, X. Wang, Y. Y. Yang, M. W. Mirino Jr., and Y. Yuan, "Partial nitrification and denitrification of mature landfill leachate using a pilot-scale continuous activated sludge process at low dissolved oxygen," *Bioresource Technology*, vol. 218, pp. 580–588, 2016.

[41] D. Kulikowska, T. Jóźwiak, P. Kowal, and S. Ciesielski, "Municipal landfill leachate nitrification in RBC biofilm-process efficiency and molecular analysis of microbial structure," *Bioresource Technology*, vol. 101, no. 10, pp. 3400–3405, 2010.

[42] C. di Iaconi, M. de Sanctis, S. Rossetti, and A. Mancini, "Biochemical treatment of medium-age sanitary landfill leachates in a high synergy system," *Process Biochemistry*, vol. 46, no. 12, pp. 2322–2329, 2011.

[43] J. Liu, H. Zhang, P. Zhang et al., "Two-stage anoxic/oxic combined membrane bioreactor system for landfill leachate treatment: pollutant removal performances and microbial community," *Bioresource Technology*, vol. 243, pp. 738–746, 2017.

[44] R. Zhu, S. Wang, J. Li et al., "Biological nitrogen removal from landfill leachate using anaerobic–aerobic process: denitritation via organics in raw leachate and intracellular storage polymers of microorganisms," *Bioresource Technology*, vol. 128, pp. 401–408, 2013.

[45] K. Wang, S. Wang, R. Zhu, L. Miao, and Y. Peng, "Advanced nitrogen removal from landfill leachate without addition of external carbon using a novel system coupling ASBR and modified SBR," *Bioresource Technology*, vol. 134, pp. 212–218, 2013.

[46] R. Ganigué, H. López, M. D. Balaguer, and J. Colprim, "Partial ammonium oxidation to nitrite of high ammonium content urban landfill leachates," *Water Research*, vol. 41, no. 15, pp. 3317–3326, 2007.

[47] H. Li, S. Zhou, G. Huang, and B. Xu, "Partial nitritation of landfill leachate with varying influent composition under intermittent aeration conditions," *Process Safety and Environmental Protection*, vol. 91, no. 4, pp. 285–294, 2013.

[48] X. Wen, J. Zhou, J. Wang, X. Qing, and Q. He, "Effects of dissolved oxygen on microbial community of single-stage autotrophic nitrogen removal system treating simulating mature landfill leachate," *Bioresource Technology*, vol. 218, pp. 962–968, 2016.

[49] Z. Y. Xu, G. M. Zeng, Z. H. Yang et al., "Biological treatment of landfill leachate with the integration of partial nitrification, anaerobic ammonium oxidation and heterotrophic denitrification," *Bioresource Technology*, vol. 101, no. 1, pp. 79–86, 2010.

[50] F. Z. Zhang, Y. Peng, L. Miao, Z. Wang, S. Wang, and B. Li, "A novel simultaneous partial nitrification Anammox and denitrification (SNAD) with intermittent aeration for cost-effective nitrogen removal from mature landfill leachate," *Chemical Engineering Journal*, vol. 313, pp. 619–628, 2017.

[51] L. Miao, K. Wang, S. Wang et al., "Advanced nitrogen removal from landfill leachate using real-time controlled three-stage sequence batch reactor (SBR) system," *Bioresource Technology*, vol. 159, pp. 258–265, 2014.

[52] L. Miao, S. Wang, T. Cao, Y. Peng, M. Zhang, and Z. Liu, "Advanced nitrogen removal from landfill leachate via Anammox system based on sequencing biofilm batch reactor (SBBR): effective protection of biofilm," *Bioresource Technology*, vol. 220, pp. 8–16, 2016.

[53] H. Li, S. Zhou, W. Ma et al., "Long-term performance and microbial ecology of a two-stage PN–ANAMMOX process treating mature landfill leachate," *Bioresource Technology*, vol. 159, pp. 404–411, 2014.

[54] Z. Wang, Y. Peng, L. Miao et al., "Continuous-flow combined process of nitritation and ANAMMOX for treatment of landfill leachate," *Bioresource Technology*, vol. 214, pp. 514–519, 2016.

[55] L. N. Wu, D. W. Liang, Y. Y. Xu, T. Liu, Y. Z. Peng, and J. Zhang, "A robust and cost-effective integrated process for nitrogen and bio-refractory organics removal from landfill leachate via short-cut nitrification, anaerobic ammonium oxidation in tandem with electrochemical oxidation," *Bioresource Technology*, vol. 212, pp. 296–301, 2016.

[56] T. N. Phan, T. T. van Truong, N. B. Ha et al., "High rate nitrogen removal by ANAMMOX internal circulation reactor (IC) for old landfill leachate treatment," *Bioresource Technology*, vol. 234, pp. 281–288, 2017.

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Review Article

Diversity and Niche of Archaea in Bioremediation

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Bioremediation is the use of microorganisms for the degradation or removal of contaminants. Most bioremediation research has focused on processes performed by the domain *Bacteria*; however, *Archaea* are known to play important roles in many situations. In extreme conditions, such as halophilic or acidophilic environments, *Archaea* are well suited for bioremediation. In other conditions, *Archaea* collaboratively work alongside *Bacteria* during biodegradation. In this review, the various roles that *Archaea* have in bioremediation is covered, including halophilic hydrocarbon degradation, acidophilic hydrocarbon degradation, hydrocarbon degradation in nonextreme environments such as soils and oceans, metal remediation, acid mine drainage, and dehalogenation. Research needs are addressed in these areas. Beyond bioremediation, these processes are important for wastewater treatment (particularly industrial wastewater treatment) and help in the understanding of the natural microbial ecology of several *Archaea* genera.

1. Introduction

The contamination of soil, sediment, and water from industrial and other human inputs is widespread and poses a threat to human and ecological health. Bioremediation is the use of microbes for the beneficial removal of contaminants of concern [1]. The microbial processes involved in bioremediation are normally natural components of respiration or adaptation, often a component of carbon cycling or metal redox cycling. Thus, bioremediation often occurs without direct intervention; however, biostimulation (the addition of nutrients or adjustment of conditions) and bioaugmentation (the addition of microbes capable of bioremediation) are often important for the complete removal of contaminants within an economical timeframe. The field of bioremediation research has traditionally focused heavily on processes from the domain *Bacteria*, which has a large diversity of bioremediation applications. In many applications where *Bacteria* are the key players in bioremediation, however, *Archaea* are often involved as well. In "extreme" environments, archaeal processes are of particular interest for bioremediation. Many *Archaea* are extremophiles, capable of living in environments considered uninhabitable by most other organisms, and many extreme environments become contaminated and are in need of remediation. Furthermore, many industrial wastewaters have hypersaline, hyperthermal, metallic, and/or an acidic or alkaline pH, where extremophilic *Archaea* have the potential to play key functions for contaminant removal.

This manuscript aims at providing an overview of the various roles that *Archaea* have in bioremediation. This review is meant to be comprehensive but with a particular focus on recent contributions. Both pure culture and mixed community studies are included in the review. The review does not cover nutrient cycling. Nor does it explicitly cover wastewater treatment or provide any explicit review of the environmental microbiology of *Archaea*; however, bioremediation is heavily interconnected to these areas. The review summarizes major findings and suggests future areas of research needed to strengthen our understanding of the contributions of *Archaea* in bioremediation. Though many chapters and reviews exist that encompasses pieces of the

topics below, as of the submission of this article, the authors have not uncovered any other comprehensive review that focuses purely on *Archaea* in the bioremediation area.

2. *Archaea* in the Degradation of Organics in Hypersaline Environments

Perhaps, the most developed research area that connects *Archaea* to bioremediation lies within the degradation of organics in hypersaline environments. Natural hypersaline environments include salterns, salt lakes, salt marshes, salt flats (sabkhas), and oil and gas production wastewaters. The contamination of these environments with crude oil is common, and about 5% of the chemical, pharmaceutical, and oil industries have highly saline wastewater effluents in need of treatment [2]. Members of both *Bacteria* and *Archaea* are known to inhabit such environments and these are often referred to as "halobacteria" and "haloarchaea," respectively. Recent reviews have focused on hydrocarbon degradation by halobacteria and haloarchaea [3–5], the biotechnological potential of the hydrolytic enzyme [6], the biodiversity of microbial communities in halophilic environments [7, 8], the potential of haloarchaea in bioremediation processes [9], and the growing rate of research of haloarchaea in bioremediation [10]. Recently, a new database—called HaloDom—has compiled all isolated halophilic species into a single online resource [11]. Many *Bacteria* can degrade at salinities of up to 15% such as strains of the genera *Ralstonia*, *Halomonas*, *Dietzia*, and *Alcanivorax* [12, 13]. Here, an overview of the haloarchaeal strains isolated on the ability to degrade hydrocarbons, such as crude oil, is provided.

The haloarchaea cluster into a single class (the class *Halobacteria*) within the phylum *Euryarchaeota*. They are typically cultured at neutral pH and temperatures of 30-45°C, and they require high salinities of 1.8–5.0 M NaCl [14–17]. Many strains have been traditionally isolated on a standard nutrient media that contains heterotrophic carbon and energy sources [15]. Table 1 lists the strains associated with hydrocarbon degradation and their degradative abilities. Additionally, a phylogenetic tree of many of these strains (where nearly full-length 16S rRNA gene sequences were available), as well as other strains and phylogenetic groups discussed in this manuscript, is shown in Figure 1. The metabolic capabilities of haloarchaea for hydrocarbon degradation appear vast, and these *Archaea* all inhabit a close phylogenetic association.

The connection between the haloarchaea and the degradation of crude oil and xenobiotic pollutants extends past three decades. A haloarchaea strain named EH4, later determined to be closely related to *Haloarcula vallismortis* [18], was isolated in 1990 from a salt marsh in France and found able to degrade various aliphatic and aromatic hydrocarbons [14]. The discovery of hydrocarbon-degrading haloarchaea was independently confirmed with a manuscript published in 1991 reporting the isolation of a *Halobacterium* strain from a hypersaline wastewater in Russia that degrades alkanes [19]. *Haloferax volcanii* strain D1227 was then isolated from a saline oil brine from Michigan (USA) on monoaromatic carboxylic acids as sole carbon and energy sources [20] and later found to degrade 3-phenylpropionate [21]. *Haloferax mediterranei* st. M-11 was isolated from the brine of the Kalamkass oil field (Mangyshlak, Kazakhstan) [22]. *Haloarcula* st. D1 was then isolated and capable of aerobically degrading 4-hydroxybenzoic acid which is a pollutant in certain industrial wastewaters [23]. The degradation pathway consisted of a gentisate-1,2-dioxygenase pathway which was found key in the degradation pathways for *Haloferax volcanii* st. D1227 as well [24, 25]. A sampling of hypersaline lakes in Turkey resulted in 33 isolates of *Halobacteriaceae* across 9 genera [26]. Though these isolates were not directly tested for degradation of crude oil or related hydrocarbons, all 33 isolates tested positive for catalase and oxidase activity and 15 tested positive for Tween 80 hydrolysis [26]. A recent manuscript reported the isolation of four further *Halobacteriaceae* that could also hydrolyze Tween 20 and Tween 80 [27]. Though the Tween 80 and Tween 20 tests are used as a standardized physiological lipase test for microbes [28], it is potentially of particular interest in bioremediation because Tween 80 and related compounds are used as surfactants in oil spill remediation and in hydraulic fracturing mixtures [29, 30].

The study of haloarchaea in bioremediation has gained significant traction in recent years. Four heptadecane-degrading halophilic archaeal strains were isolated from an uncontaminated salt crystallization pond in Camargue, France (*Haloarcula* st. MSNC 2, *Haloferax* st. MSNC 2, *Haloferax* st. MSNC 14, and *Haloferax* st. MSNC 16) [18]. *Haloferax* st. MSNC 14 also grew on phenanthrene while the other three isolates could not [18]. Later research found that *Haloferax* st. MSNC 14 produced surfactants during growth on *n*-heptadecane, pristane, and phenanthrene, but not during growth on acetate [31]. Thus, it was able to increase the bioavailability of low-solubility hydrocarbons during their degradation [31]. Four strains were also isolated from soil and water in a hypersaline coastal area of the Arabian Gulf (*Haloferax* st. HA-1, *Haloferax* st. HA-2, *Halobacterium* st. HA-3, and *Halococcus* st. HA-4) with a multitude of alkane and aromatic degradation abilities [16]. Ten strains of *Haloarchaea* closely related to *Haloferax* were isolated from salt marshes, salterns, crystallizer ponds, salt flats, and the Dead Sea and were found to degrade a mixture of polycyclic aromatic hydrocarbons and crude oil [17]. This study also found that *Haloferax volcanii* st. DS2 could degrade these polycyclic aromatic compounds [32]. This strain, which was isolated from the Dead Sea on glycine and yeast autolysate [33], has just prior had its genome sequenced [34]. *Haloterrigena mahii* sp. H13, collected from a saltern pond in San Diego, CA, USA, also had its genome sequenced and contains genes that may be involved in the degradation of 1,2-dichloroethane, naphthalene/anthracene, γ-hexachlorocyclohexane, 1-/2-methylnapthalene, and benzoate [17, 35]. A literature search has not uncovered any research that directly tested the aforementioned biodegradation capabilities with this pure culture.

The diversity of haloarchaea-degrading hydrocarbons, and of xenobiotics that they can degrade, has been expanding. A strain of *Halobacteriaceae* (named L1) was isolated from the Dead Sea and could grow on benzoic acid [36].

Table 1: The strains of hydrocarbon-degrading halophilic *Archaea*.

Strains	Hydrocarbons degraded	Citation
Haloarcula st. EH4	Tetradecane, hexadecane, eicosane, heneicosane, pristane, acenaphthene, phenanthrene, anthracene, and 9-methyl anthracene	[14]
Haloferax sp. D1227	Benzoate, p-hydroxybenzoate, cinnamate, and phenylpropionate	[20, 21]
Haloferax mediterranei st. M-11	Oil	[22]
Haloarcula st. D1	4-Hydroxybenzoic acid	[23]
Haloferax st. MSNC 4 and MSNC 16 *Haloarcula* sp. st. MSNC 2	Heptadecane	[18]
Haloferax st. MSNC 14	Heptadecane, phenanthrene, and pristane	[18, 31]
Haloferax sp. HA-1 *Haloferax* sp. HA-2 *Halobacterium* sp. st. HA-3 *Halococcus* sp. st. HA-4	Crude oil, C8-C34 n-alkanes, benzene, toluene, phenanthrene, biphenyl, and/or naphthalene	[16]
Haloferax alexandrinus st. B03, B06, AA31, and AA35 *Haloferax* sp. SC1-9 st. B07, MM17, AA41, and PR13 *Haloferax* sp. HSC4 st. MM27 *Haloferax sulfurifontix* st. CL47	Naphthalene, anthracene, phenanthrene, pyrene, and/or benz[a]anthracene	[32]
Haloferax volcanii st. DS2	Anthracene	[32]
*Haloterrigena ma*hii sp. H13	Putatively: 1,2-dichloroethane, naphthalene/anthracene, γ-hexachlorocyclohexane, 1-/2-methylnapthalene, and benzoate	[35]
Halobacteriaceae st. L1	Benzoic and p-hydroxybenzoic acid	[36]
Natrialba sp. st. C21	Phenol, naphthalene, and pyrene	[37]
Haloferax sp. C-24 and C-27, *Halobacterium piscisalsi* st. C-37, *Halobacterium salinarum* st. C-51, *Halorubrum ezzemoulense* st. C-41 and C-46, *Halorubrum* sp. st. C-43, and *Halobacteriaceae* st. C-50 and C-52	Naphthalene, phenanthrene, pyrene, and/or p-hydroxybenzoate	[39]
Haloferax lucentense st. A01 *Halobacterium salinarum* st. A02 *Halobacterium* piscisalsi st. A03 *Haloferax mucosum* st. A04 *Halobacterium sulfurifontis* st. A05	Crude oil, Tween 80, n-octadecane, and phenanthrene	[49, 50]
Haloferax elongans st. M4 *Halobacterium salinarum* st. M5	Crude oil, n-hexadecane, and phenanthrene as part of a biofilm	[52]
Halobacterium noricense st. SA1 *Haloferax larsenii* st. SA2, WA3 *Haloferax elongans* st. SA3, WA1 *Halobacterium* sp. st. SA4 *Halobacterium noricense* st. WA2 *Halobacterium salinarum* st. WA4	Oil, alkanes (C9-C40), benzene, biphenyl, anthracene, naphthalene, and/or phenanthrene	[54]
Haloferax elongans st. SA3 *Halobacterium salinarum* st. YS06_13_22	Crude oil	[55]

Natrialba sp. st. C21 has also been isolated from oil-contaminated saline water in Ain Salah, Algeria [37]. This strain can degrade phenol, naphthalene, and pyrene through an ortho-cleavage pathway and exhibits catalase, oxidase, and Tween 80 esterase activity [37]. Acikgoz and Ozcan [38] found eight *Halobacteriaceae* out of a screening library of 103 isolates that could degrade and tolerate above 200 ppm phenol. The fastest phenol-degrading strain was identified as a *Haloarcula* sp., but more detailed phylogenetic characterization was not provided [38]. In another study, nine isolates were found that can use aromatic hydrocarbons for carbon and energy sources [39]. These isolates were identified as members of *Haloferax* sp. (isolates C-24 and C-27), *Halobacterium piscisalsi* (st. C-37), *Halobacterium salinarum* (st. C-51), *Halorubrum ezzemoulense* (st. C-41 and C-46), and *Halorubrum* sp. (st. C-43), and two strains (C-50 and

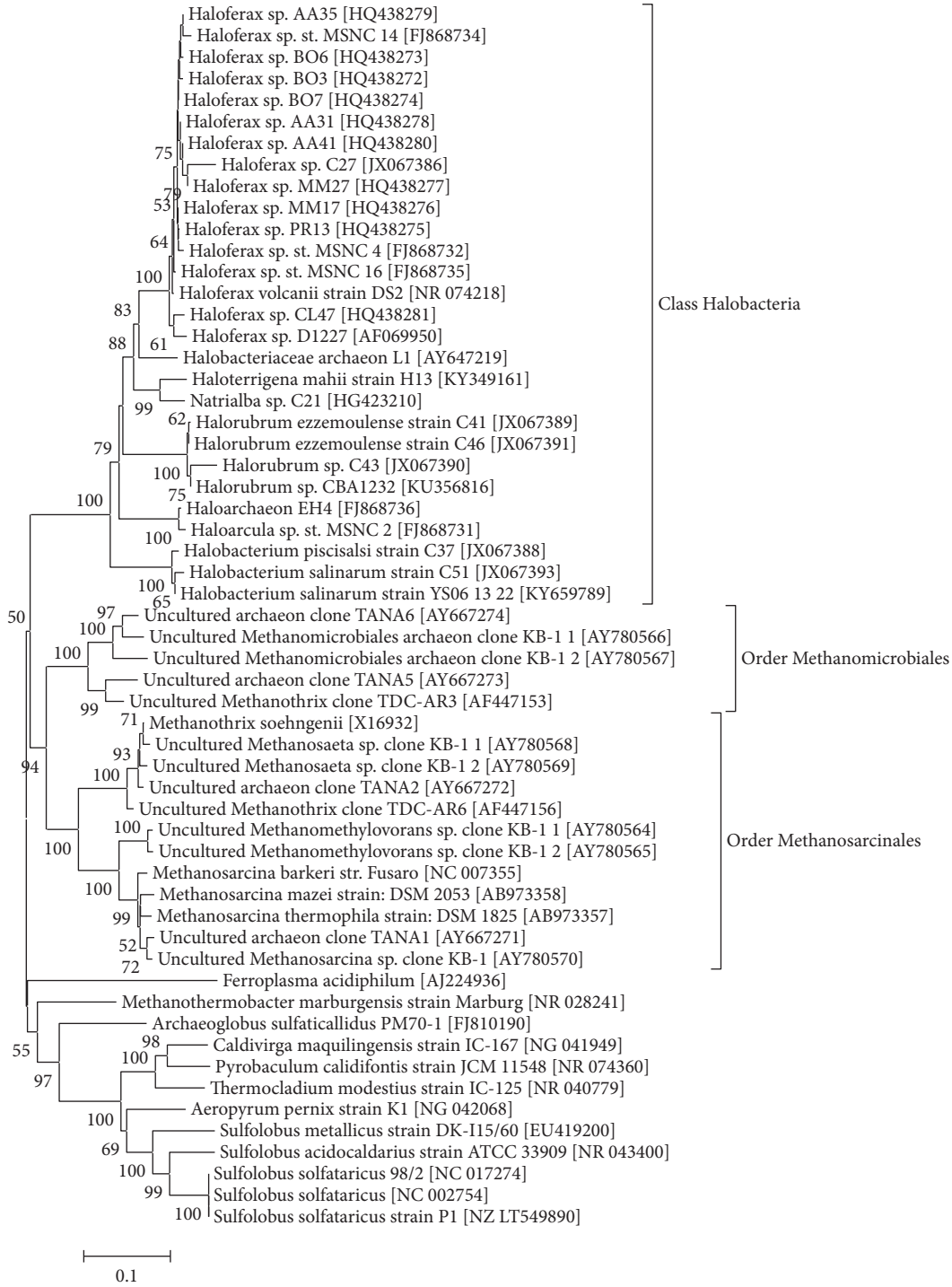

Figure 1: Phylogenetic analysis of strains, or related strains, of the *Archaea* discussed in this manuscript. Alignment and tree analysis was performed in MEGA 6.0 [167]. Sequences were imported from GenBank, alignment was performed with MUSCLE, tree was built with neighbor-joining method with 1000 bootstraps, and evolutionary distances were inferred with maximum composite likelihood method.

C-52) reported with less than 93% 16S rRNA gene identity to any isolated strains [39]. Upon inspection of the deposited sequences in NCBI's GenBank, the sequence for strain C-50 appears to have poor sequence quality; a BLAST search of the first 280 bp recovered zero alignments to sequences in GenBank. Strain C-52 has 99% identity along the more recently deposited 16S rRNA gene of *Halorubrum trapanicum* CBA1232, which has a deposited genome (NCBI BioProject PRJDB4921); however, no publications are associated with this genome [40]. All nine strains degraded naphthalene, phenanthrene, and pyrene, and all but strain C-37 and C-51 degraded p-hydroxybenzoate [39]. Degradation in all cases

was through ortho-oxidation through a catechol 1,2-dioxygenase or a protocatechuate 3,4-dioxygenase pathway [39]. A microbial community enriched from the Great Salt Lake (Utah, USA) consisted of several genera entirely of the class *Halobacteria*, with 91% belonging to the genera *Halopenitus* as determined by 454 sequencing of 16S rRNA genes [41]. This community could grow on 4-hydroxybenzoate but not the other carbon sources tested, and the degradative pathways and genes were analyzed with PCR approaches of functional genes [41].

Though the isolation of haloarchaeal strains from contaminated sites is successful and haloarchaea are often found in natural environments (i.e., [42, 43]), the understanding of the microbial ecology of these strains on oil contamination under in situ conditions is not well developed. A few studies investigating the distribution of the haloarchaea have been done. The archaeal community in a saline-alkali soil in the Dagang Oilfield (China) differed significantly along a petroleum contamination gradient, with four groups of *Archaea*, including *Haloferax* and *Natronomonas*, being abundant in the contaminated soils while five different groups of *Archaea* were dominant in noncontaminated soils [44]. Other studies have profiled further diversity of haloarchaeal groups in oilfield sites, including the genera *Halalkalicoccus*, *Natronomonas*, *Haloterrigena*, and *Natrinema*, suggesting that varied haloarchaea are widely present in these contaminated environments [45]. Though *Haloferax* has a number of isolates known to degrade aromatics, *Natronomonas* is not as well established to oil degradation, though it does contain fatty acid degradation pathways and is thus putatively able to degrade alkanes [46]. Thus, these genera are likely degrading the organics in situ. In contrast, in a hypersaline-produced water from the Campos Basin (Brazil) contaminated with phenol and aromatics, the archaeal community consisted of no detected haloarchaea in situ but was rather dominated by methanogens (59% *Methanosaeta* and 37% *Methanoplanus*) [47]. Methanogens have a role in the final degradation of hydrocarbons in coculture with hydrocarbon-degrading *Bacteria* (see below); the presence of methanogens and the lack of haloarchaea suggest a highly reduced environment. Hydrocarbon-degrading halophilic bacteria (specifically, *Halomonas*) were isolated from these waters and could degrade these contaminants, especially with biostimulation [48]. The contaminants in this production water were also degraded more significantly in a previous study with the bioaugmentation of haloarchaea strains [32]. The bacteria *Halomonas* and haloarchaea survive in similar salinities and contain similar degradative capabilities [4]; however, it is not known what drives the competitive advantage of one over the other.

Recently, further studies have progressed towards evaluating bioremediation techniques with haloarchaeal communities. A recent study focused on how vitamin amendments may stimulate crude oil degradation [49]. Vitamin B_{12} enhanced the degradation of crude oil from five *Archaea* strains tested (*Haloferax lucentense* st. AO1, *Halobacterium salinarum* st. AO2, *Halobacterium piscisalsi* st. AO3, *Haloferax mucosum* st. AO4, and *Halobacterium sulfurifontis* st. AO5) [49]. Pyridoxine enhanced the biodegradation of oil by four of these strains (A01, A02, A04, and A05), riboflavin enhanced the degradation by three strains (A01, A02, and A05), folic acid enhanced the degradation by three strains (A01, A03, and A05), and thiamin enhanced the degradation by one strain (AO5), but biotin did not enhance oil degradation significantly by any of the five strains [49]. The biostimulation with vitamins is not surprising, as earlier work has shown that a nutritional yeast extract amendment significantly increases hydrocarbon degradation [32]. The strains were found to also degrade Tween 80, *n*-octadecane, and phenanthrene and were also enhanced with 0.75 M KCl and 2.25 M $MgSO_4$ [49, 50]. In another study, continuous illumination and casamino acids were found to increase oil biodegradation by mixed cultures dominated by *Haloferax* sp. and by four isolates (two identified as *Haloferax*, one as a *Halobacterium*, and one as a *Halococcus*) [51]. *Haloferax elongans* st. M4 and *Halobacterium salinarum* st. M5 were found capable of being cultured onto a *Bacteria-Archaea* biofilm community for the degradation of crude oil, *n*-hexadecane, and phenanthrene [52]. Such biofilm communities have advantages in bioremediation technologies. There too, vitamins stimulated crude oil degradation in the biofilm [52]. In yet another study with a mixed community of *Bacteria* and *Archaea*, the addition of casamino acids and citrate was required for oil degradation and the microbial community dynamics were observed [53]. After adding crude oil to the culture, biotic degradation could not occur and the archaeal community shifted away from what was previously high levels of *Haloquandratum*, to one in which only *Natronomonas* spp. remained, while the bacterium *Salinibacter* was selected [53]. With the additional amendment of casamino acids and citrate, the community could degrade oil with an archaeal enrichment of *Haloarcula*, *Haloterrigena*, and *Halorhabdus* [53]. A recent study investigated the biostimulation of oil-degrading cultures derived from a hypersaline sabkha and found that Fe^{+3}, Ca^{+2}, Mg^{+2}, K^{+}, animal blood, and commercial yeast all had a stimulatory effect towards oil degradation [54]. Haloarchaeal communities were dominated by *Haloferax* spp. and *Halobacterium* spp., and eight strains were isolated (two associated with *Halobacterium noricense*, two with *Haloferax larsenii*, a *Halobacterium salinarium*, and a *Halobacterium* sp.) [54]. These strains could grow on a variety of alkanes and aromatics and degraded between 22 and 36% of amended crude oil over 2 weeks [54].

Cocontamination of different types of pollutants often complicates bioremediation, and a recent study has investigated the effect of heavy metal cocontamination with hydrocarbon degradation in hypersaline systems [55]. Strains of both *Archaea* (a strain of *Haloferax elongans* and a *Halobacterium salinarum*) and *Bacteria* (a strain each of *Arhodomonas*, *Marinobacter*, and *Halomonas*) were inhibited with elevated levels of Hg, Pb, Cu, Cd, and As and were more sensitive to these metals in the presence of crude oil [55]. Overall, the archaeal strains had less tolerance for heavy metals than three halophilic/halotolerant *Bacteria* tested, though the bacterial genus *Kocuria* had similar levels of sensitivity to heavy metal toxicity [55]. For the *Haloferax elongans*, Fe^{III} amendment lessened the toxicity of Hg, Pb, Cu, and Cd, while for the *Halobacterium salinarum*, Fe^{III}

amendment lessened the toxicity of Cu, Cd, and As and proline lessened the toxicity limit of Cd [55]. For the *Halobacterium salinarum*, the rate of crude oil consumption was tested under heavy metal stress with and without Fe^{III} or proline amendment. The crude oil degradation rate increased significantly under Hg or Pb stresses with Fe^{III} or proline amendment, while the enhancement of oil consumption rates in Cu-, Cd-, and As-stressed cultures were more nuanced [55]. At low-salt concentrations (<1.5 M), many of these heavy metals, to a certain concentration, increased cell growth presumably from affecting cytoplasmic osmolality [55]. In previous research, the strain *Haloferax* sp. st. BBK2 was affected by 0.5 mM concentrations of Cd but was resistant to Cd toxicity up to 4 mM levels and it accumulated Cd intracellularly [56].

The progress within this area from simple discovery to in-depth biostimulation analysis over the last decade is tremendous despite the relatively few investigators that have been steadily producing significant findings in this area. The diversity of strains and isolates within the haloarchaea is large, but not exhaustive [41, 57]. The study of haloarchaea benefits from moderate growth rates (doubling times of ~24–32 hr), fruitful isolation attempts, and easy culturing conditions (aerobic, diverse organic substrates, etc.) [14–17]; however, more molecular-based research to monitor and detect in situ degradation is needed to better understand these archaeal biodegradation processes in contaminated hypersaline environments. Though they have relatively warm temperature preferences (generally greater than 30°C) and have vitamin needs [14–17, 32, 49], the broad distribution of haloarchaea in hypersaline environments, the broad metabolic capabilities found on xenobiotics and crude oil, and the relatively quick degradation rates all provide promise that if properly stimulated, bioremediation of hydrocarbons in hypersaline environments should proceed quickly.

3. Degradation of Organics with Thermophilic *Sulfolobus solfataricus*

A few strains of thermophilic and acidophilic *Archaea* have been found capable of pollutant degradation. Such biodegradation capabilities are of interest, as many industrial wastewater streams are hot. Genomic sequencing of *Sulfolobus solfataricus* st. P2 found genes for aromatic degradation and it was found to be able to degrade phenol aerobically through *meta*-ring cleavage [58]. A strain of the closely related thermophilic *Sulfolobus solfataricus* st. 98/2 was later found to be able to degrade phenol at 80°C and 3.2 pH [59, 60] through *meta*-ring cleavage also [61]. A dienelactone hydrolase from *Sulfolobus solfataricus* st. P1 was also identified and characterized [62]. This enzyme is important for chloroaromatic degradation, such as 2,4-dichlorophenoxyacetic acid [63], though direct testing of this enzyme on chloroaromatics was not reported. To our findings, this seems to be the extent of current research on *Sulfolobus* in terms of bioremediation applications, but a review of *Sulfolobus* in broader biotechnology applications has recently been published [64]. This research field is still developing and there are likely more thermophilic hydrocarbon degraders; however, culturing thermophilic strains is difficult due to maintaining high temperatures for cellular growth, the increased volatility of the hydrocarbons at high temperatures, and for aerobes, the low oxygen solubility at high temperatures.

4. Degradation of Hydrocarbons in Soils with *Archaea*

In nonextreme environments, *Bacteria* are better known to perform the degradation of hydrocarbons; however, *Archaea*, particularly the methanogens, are often a component of the degradation process. Hydrogenotrophic and acetoclastic methanogens convert hydrogen and acetate, respectively, to methane gas in anaerobic conditions [65]. In degradative processes where hydrogen or acetate are waste products, these methanogens can thus increase the thermodynamic favorability by reducing hydrogen and acetate concentrations and in effect drive the degradative process forward [66]. This forms a syntrophic relationship between *Bacteria* that degrades the compound of interest and the methanogenic *Archaea* that removes the waste products of that degradation [67]. Acetoclastic methanogens are found in the order *Methanosarcinales*, notably the genera *Methanosaeta* and *Methanosarcina*, while hydrogenotrophic methanogens are found in the orders *Methanococcales*, *Methanobacteriales*, *Methanosarcinales*, *Methanomicrobiales*, *Methanopyrales*, and *Methanocellales* [68]. Here, we review the key roles of *Archaea* in soils and freshwater systems contaminated with hydrocarbons. A recent review was published that more broadly covers microbial community responses to petroleum contamination [69].

Two decades ago, an analysis of the microbial communities in a jet fuel and chlorinated solvent-contaminated aquifer found that *Methanosaeta* spp. dominated the archaeal community and it was proposed that it performs the terminal step in hydrocarbon degradation in methanogenic zones [70]. Soon thereafter, enrichment cultures showed that long-chain alkanes can be degraded anaerobically to methane with a culture of *Syntrophus* spp. (including one closely related to a sequence recovered from the jet fuel/chlorinated solvent-contaminated aquifer in [69]) and both acetoclastic (*Methanosaeta* sp.) and hydrogenotrophic (*Methanoculleus* sp. and *Methanospirillum* sp.) methanogens [71]. Since then, many field studies with in situ hydrocarbon degradation have investigated for the presence of methanogenic *Archaea*. Soil contaminated with petroleum and undergoing remediation was found enriched significantly for *Methanosarcinales* strains with a denaturing gradient gel electrophoresis (DGGE) method [72]. *Methanomicrobiales*, *Methanosarcinales*, *Methanobacteriales*, and *Thermoplasmatales* were all found in other soil samples contaminated with petroleum hydrocarbons [73]. High abundances of *Methanosaeta* were observed in a diesel-contaminated soil—up to 30% of all 16S rRNA genes in some of the samples [74]. This compares to normal abundances of 2% *Archaea* in natural soils, which are also typically dominated by *Crenarchaeota* and not the *Euryarchaeota* of which the methanogens belong [75]. Processed oil sands were also found to contain archaeal communities dominated by the acetoclastic *Methanosaeta* spp.

[76]. A coculture of *Anaerolineae* and *Methanosaeta* was found to predominate in an alkane degradation culture over 1300 days with similar 16S rRNA gene concentrations of each, presumably with *Anaerolineae* breaking down alkane chains through acetate and *Methanosaeta* fermenting acetate into methane [77]. Another study found that the genus *Methanoculleus* was the more abundant methanogen in an anaerobic alkane degrading culture containing the bacteria *Thermodesulfovibrio* and *Anaerolineaceae* [78].

Often, the diversity of *Archaea* detected in hydrocarbon degrading cultures is low but the diversity of *Archaea* in one heavy crude oil-contaminated soil was found to be higher than the diversity of *Archaea* in a pristine soil [79]. Clone libraries indicated that the contaminated soil contained many members of deeply branching *Methanomicrobiales*, *Halobacteriales*, *Methanosarcinales*, and many *Euryarchaeota* and *Crenarchaeota* of uncultured genera, while the pristine soil only contained *Natronomonas*-like sequences among the *Archaea* [79]. In a hydrocarbon-contaminated sludge from an oil storage facility, *β-Proteobacteria* was found in coculture with a diverse archaeal community consisting of *Thermoprotei* (54%), *Methanocellales* (33%), and then *Methanosarcinales*/*Methanosaetacaea* (8%) [80].

The study of syntrophic hydrocarbon degradation has advanced to studying systems under biostimulation conditions. The anaerobic degradation of benzene is oftentimes slow or nonexistent [4]. In a field-based study comparing the natural attenuation of B20 biodiesel blend and a biostimulation with an ammonium acetate injection, it was found that *Archaea* populations significantly increased from less than 10^3 to 3.7×10^8 16S rRNA genes·g^{-1} under the biostimulation conditions commensurately with enhanced BTEX degradation [81]. Conversely, in a recent study of an Alpine Petroleum-contaminated site, the archaeal community was mostly found unchanged on the phyla level (based on read depth analysis of a 16S rRNA gene amplification) and overall archaeal abundance (measured with qPCR) decreased during fertilization biostimulation or increased temperature [82]. The only archaeal enrichment appeared to be *Woesearchaeota* which became more abundant compared to other archaeal phyla with a temperature increase to 20°C [82]. This study did not report data on finer phylogenetic scales.

The syntrophic relationship between hydrocarbon-degrading *Bacteria* and methanogenic *Archaea* is not always present in degradation cultures. *Euryarchaeota* and *Thaumarchaeota* completely disappeared in one set of microcosms amended with spent motor oil [83]. Similarly, Illumina sequencing of a 16S rRNA gene amplification did not widely detect *Archaea* in one petroleum enrichment culture [84]. A GeoChip analysis of the archaeal community in a different study found that archaeal abundance was negatively impacted by oil contamination in an aerobic soil with numbers reduced to 10% of the archaeal abundance in noncontaminated soil [85]. A DGGE-based community profile of an Antarctic soil contaminated with diesel under various remediation conditions found no substantial differences in the archaeal community during bioremediation [86]. Another study found that *Archaea* were scarce (<1% of the population) in an aquifer above a coal-tar DNAPL with only a low abundance of methanogens [87]. Other than the reduced redox conditions required for methanogenesis, it is not clear why *Archaea* respond strongly to oil contamination in certain environments and not others.

A diverse and varying dominance of archaeal members (as well as bacterial members) exists in soils and groundwater during hydrocarbon bioremediation. Controlled experiments in which physicochemical conditions (such as redox, salinity, temperature, and trace element availability) are varied in hydrocarbon-contaminated soils may help determine the role that these factors play in selecting the specific archaeal communities (if any at all) that are stimulated. The research in this area also uses a variety of methodologies to study the *Archaea*, and similar methodologies (clone libraries) still often use different primer sets. Studies in which these methodologies are compared for the same sample would help elucidate the extent that the varying results above are a function of the chosen methodology.

5. *Archaea* in the Degradation of Oil in Oceans and Marine Sediments

The role of *Archaea* in the degradation of oil in marine systems is oftentimes unclear as well. It is believed that *Bacteria* play the dominant role in oil biodegradation in oceans [88], but the role of *Archaea* in oil degradation in oceans is not fully understood. *Archaea* in many studies have been found to be sensitive to oil compounds. In a lab-based study of beach sediment microcosms, *Archaea* 16S rRNA genes became difficult to amplify with a PCR method after incubation with oil, suggesting a large decrease in archaeal populations [89]. That study however only detected two tight clusters of *Archaea* in its analysis, a group of Marine Group II *Euryarchaeota* and a group of *Crenarchaeota* [89]. A later study found that the nitrifying *Nitrosopumilus maritimus*, a member of the Marine Group I *Archaea*, was also very sensitive to crude oil presence [90]. In another study, the oil degrading bacteria that were found to grow were heavily dependent on temperature but the archaeal community structure was minimally affected [91]. The study also observed few *Archaea* groups—predominately a tight phylogenetic group of Marine Group II *Archaea* and eight other OTUs related to *Euryarchaeota* and *Thaumarchaeota* [91]. The isolation of hydrocarbon-degrading strains in coastal sediment contaminated with petroleum off of the coast of Sicily (Italy) recovered only isolates from the domain *Bacteria* [92]. The natural diversity of archaeal communities were determined with DGGE and was found to consist of uncultured *Crenarchaeota* and *Thaumarchaeota* which did not significantly change in crude oil-amended microcosms [92]. Though members of *Thaumarchaeota* are hypothesized to be able to aerobically degrade crude oil [93], no direct evidence with cultured strains yet exists.

Other studies have detected shifts in archaeal communities that suggest that some *Archaea* may at times play a role in degradation. One study tested the change in the archaeal community before and after adding either heptadecane, naphthalene, or crude oil in seawater and marine sediment

at two locations near Rio de Janeiro (Brazil) [94]. While no *Archaea* could be identified in the water samples, the archaeal community in the marine sediment uniquely changed for each of the hydrocarbons that were added [94]. The method detected primarily uncultured *Archaea*, which were mostly *Euryarchaeota* [94]. In a field study, a DGGE analysis of archaeal 16S rRNA genes indicated that oil contamination in mangrove sediments differed compared to a pristine site [95]; again, the method predominately detected uncultured groups of *Archaea*. In a recent survey of Atlantic and Mediterranean coastal sediments around Europe, the presence and abundance of the Miscellaneous Crenarchaeotic Group (MCG) were also found to correlate to oil-contaminated sediments [96]. These findings suggest that some uncultured groups of *Archaea* may have roles in oil degradation in marine systems.

Methanogens have been connected to hydrocarbon degradation in some marine systems as well. Methanogenesis increased commensurately with hydrocarbon degradation in microcosms seeded with contaminated sediments taken from Halic Bay (Turkey) and stimulated with phosphorus and/or nitrogen [97]. A research study also found that adding methanol or acetate could stimulate degradation of petroleum hydrocarbons in marine sediment [98]. The acetoclastic methanogenic *Methanosarcinales* increased in the sediment with acetate stimulation and temporarily with methanol stimulation [98]. *Methanomicrobiales*, which are hydrogenotrophic methanogens, increased with methanol stimulation as well, but not with acetate stimulation [98].

Though haloarchaea contain many strains that require high levels of NaCl, recent evidence suggests that marine systems have phylogenetically related strains as well. Samples taken from the Amazon equatorial ocean basin and amended with oil droplets had significant variation in the community composition of the *Archaea* domain upon oil biodegradation as detected with metagenomic techniques, including a relative enrichment of the *Halobacteriaceae* [99]. In a mesocosm study of archaeal and bacterial diversity from oil contamination in mangrove sediments, bacterial diversity was more significantly affected from oil contamination than archaeal diversity [100]. The genus *Nitrosopumilus*, common in marine systems, was inhibited with oil degradation, but the read depth for the family *Halobacteriaceae* was stimulated from combined oil and nitrate additions, of which members related to *Haloferax* increased marginally with oil additions [100]. *Archaea* was not found to be affected by oil contamination in the coastal water of the Gulf of Finland, but they were impacted in the coastal sediments [101]. The *Halobacteriaceae* was significantly more abundant where the sediment was contaminated with oil [101]. Archaeal cytochrome 450 and retinol metabolism pathways were enhanced where oil was also present which signifies active oil degradation [101]. Altogether, these results indicate that some haloarchaea likely have roles in oil biodegradation at least in sediments. Degradation of oils in sediments is important, as coastal systems are oftentimes more contaminated with oil than open oceans.

In many of the studies above, a limited diversity of *Archaea* was measured, typically with methods relying on a PCR amplification with universal primers followed by an analysis. Interpreting results from these studies should be done cautiously because amplification-dependent methodologies may miss clades of *Archaea* due to primer mismatching and/or PCR biases [102]. With modern metagenomic sequences, it may be worthwhile to reexamine old assumptions based on these results. Indeed, recent metagenomic-based methods are elucidating much greater diversity of *Archaea* in marine systems than the earlier studies using methods dependent on PCR amplification were detecting (i.e., [99]).

6. Archaea in Heavy Metal Remediation

Bioremediation of metals can take many forms [103]. Oftentimes, it involves the redox cycling of the metals for the conversion of toxic redox states to nontoxic redox states. Alternatively, redox cycling may convert soluble metal redox states to insoluble redox states, or vice versa, and the effect of which is precipitation or mobilization of the metal. Additionally, metals may be removed through reactions that permit volatilization of heavy metals or through sorption into biomass. These processes are also important for radioactive metals [104], but *Archaea* are poorly studied in this area despite some archaeal strains having high tolerance of radioactivity [105]. A recent review over the bioremediation of heavy metals was published, but did not address *Archaea* [106]. A comprehensive review of metal-tolerant thermophiles has been published recently including significant information regarding *Archaea* and the significant context in terms of bioremediation [107]; thus, here, we do not cover thermophiles and metal bioremediation in as much detail.

Arsenite (As^{III}) is a toxic form of arsenic, but it can be oxidized to less toxic arsenate (As^{V}). In a study of an acidic, sulfuric thermal spring in Yellowstone National Park (USA), arsenite oxidation coincided with the appearance of unisolated *Crenarchaeota* and *Euryarchaeota* and it was thus hypothesized that *Archaea* could oxidize arsenite [108]. In earlier work, the *Sulfolobus acidocaldarius* st. BC was indeed confirmed to oxidize arsenite to arsenate [109]. From reviews of the deposited genomic sequences in GenBank, the *Archaea* strains *Aeropyrum pernix* st. K1, *Pyrobaculum calidifontis* st. JCM 11548, and *Sulfolobus tokodaii* st. 7 are found to contain arsenite oxidase genes [110, 111]. A recent metagenomic study of Diamante Lake (Argentina) found a large abundance of arsenate reduction and arsenite oxidation genes and haloarchaea [112]. Fourteen isolates of the genus *Halorubrum* were found to contain arsenite oxidation genes and one strain was confirmed capable of arsenite oxidation [112]. Arsenate reduction by *Archaea* is also common which in turn would increase arsenic toxicity (i.e., [113]).

Mercuric mercury (Hg^{II}) is highly toxic and one method of removal is via biological reduction to volatile zero-valent mercury (Hg^{0}). This is carried about by enzymes encoded by mercury reductase genes which have been identified in several diverse *Crenarchaeota* and *Euryarchaeota* [114]. A study of a mercury-containing hot spring in Yellowstone National Park (USA) found novel and deeply rooted mercury reductase genes associated with *Archaea* [115]. Mercury

reductase was found upregulated in *Sulfolobus solfataricus* and was needed for mercury resistance [116], and mercury volatilization was also measured from *Halococcus*, *Halobacterium*, and, to a lesser extent, *Haloferax* [117]. Direct study of zero-valent mercury volatilization from *Archaea* is otherwise rather scarce. Conversely, mercury methylation by methanogens, which increases toxicity, is well documented [118].

The precipitation of uranium by the reduction of U^{VI} to U^{IV} is one mechanism for the immobilization of uranium in environments where it may impact ground and surface waters [119]. *Pyrobaculum* sp., which are hyperthermophiles, are capable of uranium reduction [120]. These *Archaea* have large redox capabilities for other metals (i.e., [121]) and thus may be beneficial in many types of metal-contaminated hyperthermic waste streams.

Another way in which metals may be bioremediated is via intracellular or extracellular binding or sorption. *Methanobacterium bryantii* was found to excrete extracellular proteins to chelate copper [122]. *Sulfolobus acidocaldarius* was found to bind U^{VI} into organophosphate groups [123]. Halophilic microbes are often able to absorb heavy metals, as well [124]. *Halobacterium* sp. GUSF was found to be able to absorb manganese at high rates and high concentrations [125]. *Halobacterium noricense* was found to adsorb cadmium [126]. As noted above, *Haloferax* st. BBK2 was found to accumulate cadmium intracellularly [56]. The archaeon *Halobacterium noricense* DSM15987 was found to accumulate U^{IV} with phosphoryl and carboxylate groups compared to a direct biosorption process with the bacterium *Brachybacterium* sp. G1 [127, 128]. These results show promise that the haloarchaea can be used in the treatment of hypersaline environments and wastewaters for heavy metal removals.

7. *Archaea* in Acid Mine Drainage

Acid mine drainage is a major contributor to water pollution by introducing a highly acidic effluent with toxic metals in solution. Acid mine drainage occurs when oxygen, introduced due to mining activities, reacts with metal sulfide minerals (such as FeS_2) resulting in the production of sulfuric acid and lower pH; this reaction is often aided by aerobic iron- and sulfur-oxidizing microbes [129]. Many microorganisms including many *Archaea* tolerate and thrive in the acidic and metal dense environments found in acid mine drainage. *Ferroplasma* spp. are acidophilic metal oxidizers with preferences of very low pH (<1.5) and are major players in the production of acid mine drainage and the biogeochemical cycling of sulfur [130, 131]. At Iron Mountain (CA) which has acid mine drainage, *Archaea* are the major proportion of the prokaryotes and *Ferroplasma* dominates (85% of *Archaea*) [130]. Many other *Archaea* are involved in similar ways. For example, *Sulfolobusmetallicus*, which is also acidophilic, thermophilic, and chemolithoautotrophic, can oxidize elemental sulfur and sulfidic ores, producing sulfuric acid and causing the leaching of uranium, zinc, and copper [132]. Exploiting these *Archaea* may be important for mining of metals and biocatalysis under extreme conditions (i.e., [133]) but may not be helpful in an acid mine bioremediation context where increased toxic metal mobility and acidification is typically not a favorable outcome. However, the diversity of the *Archaea* in the order *Thermoplasmatales* and their resistance to toxic metal resistance [134] may prove useful for other metal remediation purposes.

The biological treatment applying sulfate-reducing bacteria is an attractive option to treat acid mine drainage and to recover metals [135]. The process produces alkalinity, neutralizing the acid mine drainage simultaneously. There are two lineages of archaeal sulfate reducers: the *Archaeoglobus*, within the *Euryarchaeota*, and *Thermocladium* and *Caldivirga* within the *Crenarchaeota* [136]. *Archaeoglobus* are thermophilic but not acidophilic [137]. *Thermocladium* and *Caldivirga* are moderately acidophilic and can tolerate pH down to about 2.3 but are still thermophilic and thus are not suitable for acid mine drainage [138, 139].

8. Archaea in Reductive Dehalogenation

Reductive dehalogenation removes halides from organic compounds resulting in lower halogenated or nonhalogenated products and is important in bioremediation. This field has been largely focused on the organohalide-respiring *Bacteria* that can use organohalides as terminal electron acceptors. However, the ability of methanogens to dehalogenate has been long established. Many papers were published in the 1980s and 1990s discovering the various substrates subject to dechlorination by methanogens. Various strains of *Methanosarcina* were found to dehalogenate pentachlorophenol [140], perchloroethylene [141], trichloroethene [142], chloroform [143], and trichlorofluoromethane [144]. *Methanobacterium ivanovii* strain T1N was able to degrade pentachlorophenol [140]. Cell suspensions of *Methanosarcina barkeri* (DSM 2948), *Methanosarcina mazei* (DSM 2053) (which was incorrectly referred to as *Methanococcus mazei* despite reclassification 8 years prior [145]), *Methanobacterium thermoautotrophicum* st. Marburg (DSM 2133) (which has since been reclassified as *Methanothermobacter marburgensis* [146]), and *Methanothrix soehngenii* (DSM 2139) dechlorinate 1,2-dichloroethane through dihaloelimination to the product ethylene and through hydrogenolysis to chloroethane [147]. The ability to dehalogenate is likely from the high concentrations of corrinoids, such as cobalamin, in methanogens which are needed for methanogenesis [148, 149]. Corrinoids are able to dehalogenate organics abiotically [150, 151].

Archaea are also commonly reported as a part of microbial communities dechlorinating chloroethenes (Table 2). *Methanobacterium congolense* was found in the well-studied chloroethene-dechlorinating ANAS culture [152]. Inhibition of the methanogens with 2-bromoethanesulphonate (BES) was reported to not affect the "ability to dechlorinate trichloroethene completely"; however, further information was not provided [152]. *Methanothrix*, *Methanomethylovorans*, and an unclassified *Archaea* were present in a column treating perchloroethene [153]. At a site undergoing remediation from trichloroethene to ethene, *Methanosaeta* sp., *Methanospirillum* sp., *Methanosarcina*, and an unclassified

Table 2: The methanogens present in chloroethene-dechlorinating cultures.

Methanogenic strains	Culture notes	Citation
Methanosarcina st. KB-1, *Methanomethylovorans* st. KB-1 1 and st. KB-1 2, *Methanomicrobiales* st. KB-1 2, and *Methanosaeta* st. KB-1 1 and st. KB-1 2	*Dehalococcoides*-dominated KB-1 enrichment culture	[155]
Uncultured *Methanobacterium congolense*	*Dehalococcoides*-, *Desulfovibrio*-, and *Clostridia*-dominated ANAS enrichment culture	[152]
Methanothrix st. TDC-AR3, *Archaea* st. TDC-AR4, *Methanomethylovorans* st. TDC-AR5, and *Methanothrix* sp. st. TDC-AR6	*Dehalococcoides*- and *Acetobacterium*- containing culture	[153]
Uncultured *Methanosaeta* st. TANA2, uncultured *Methanospirillum* st. TANA5, uncultured *Methanosarcina* st. TANA1, and uncultured *Methanomicrobiales* st. TANA6	Trichloroethene-contaminated aquifer undergoing bioremediation to ethene with a diverse bacterial community	[154]

Methanomicrobiales were found present [154]. *Methanosarcina*, *Methanomethylovorans*, *Methanomicrobiales*, and *Methanosaeta* were reported as significant components of the well-studied and highly enriched KB-1 organochloride-dechlorinating culture [155]. *Methanosarcina* was found to be important for the dechlorination of vinyl chloride in an enriched *Dehalococcoides*-containing culture, while *Methanosaeta* had no impact [156]. It was hypothesized that the *Methanosarcina* were producing H_2 from acetate oxidation for the *Dehalococcoides* in these cultures [156]. Hydrogenotrophic methanogens in other cultures are conversely likely competing for H_2 substrate with the organohalide-respiring bacteria [157, 158]. Many dechlorinators, such as the versatile *Dehalococcoides*, lack the ability to synthesize needed corrinoids for reductive dehalogenation and instead have genes for corrinoid scavenging and import [159, 160]. Methanogens in these cultures may provide these key corrinoids for the organohalide-respiring bacteria in these communities, though this role may be fulfilled by other corrinoid-producing bacteria [158]. A recent review on cobalamin synthesis in the context of dehalogenation has been published [161]. The ability of methanogens to dechlorinate suggests that these *Archaea* may contribute to dechlorination activities even in systems dominated by organohalide respirers. The roles and antagonism of *Archaea* in reductive dechlorination systems are likely complex. Recent research has started investigating the natural cycling of organohalides but has only thus far focused on *Bacteria* [162–164].

9. Research Needs

A primary hurdle in the study of *Archaea* in bioremediation systems is methodological. Many studies on bioremediation do not study archaeal community members explicitly nor have methods that would allow for the discovery of archaeal diversity or activities. Additionally, many methodologies that have been used to study *Archaea* are prone to biases, which may cloud our understanding. A varied number of archaeal and "universal" amplification primer pairs are known and are used to study archaeal diversity [32, 37, 82, 83, 86]. Interpreting results from these methods should be done carefully. PCR amplifications of entire prokaryotes or entire domains are prone to biases, which can underrepresent and overrepresent various microbial community members [102]. Analyses that are based on a high phylogenetic level (i.e., phylum-based analyses) can also hide trends on the finer phylogenetic levels (i.e., genus). Recent publications above often rely on "relative read depth" analysis of the high throughput sequencing of a 16S rRNA gene amplification product to provide quantitative measurement of specific *Archaea* taxonomic groups; however, these methods are still exposed to PCR biases. For analysis of mixed cultures, metagenomic sequencing of unamplified DNA and more quantitative PCR (qPCR) methods should also be used. QPCR has a high sensitivity, can be designed for high specificity, and can be quality controlled [165] and thus makes a superior quantitative method to "relative read depth" analysis which lacks these characteristics. In a recent publication, read depth analysis from an Illumina-sequenced PCR product was able to identify enriched taxonomic groups, but the read depth analysis agreed poorly with the actual quantification with qPCR [164]. Some qPCR methods have been developed for certain *Archaea* (i.e., [155]); however, more methods need to be developed to further extend the study of *Archaea* in mixed microbial communities.

An additional hurdle in studying *Archaea* in bioremediation again is methodological. Dose growth response analysis is often used to measure community members that outcompete others at a given physicochemical condition on a given substrate. One hypothesis of *Archaea* evolution suggests that *Archaea*'s niche and advantage in the environment is operating under energy stress, and thus, dose growth response methods provide conditions where *Archaea* may easily be outcompeted [166]. In the environment, biodegradation activity often occurs in heterogeneous environments with microniches, energy stresses, and complex microbial communities where *Archaea* are thus theoretically more heavily involved than what will be found using many traditional microcosm/enrichment culture methodologies.

Though this field has made significant advances in the last several years, it is still developing and all forms of research will continue to advance the field. The potential of *Archaea* to serve in bioremediation applications (outside of hypersaline environments) is not well understood. The extremophilic nature of many *Archaea* make them uniquely suitable for biodegradation of "extreme" environments and

waste streams, yet many of these possibilities are not yet tested. Future research in bioremediation should be conscious of the potential roles of *Archaea* in bioremediation processes, and thus, methods should be more routinely used to analyze the *Archaea*.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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References

[1] J. T. Cookson Jr, *Bioremediation Engineering: Design and Applications*, McGraw-Hill Education, 1st edition, 1994.

[2] O. Lefebvre and R. Moletta, "Treatment of organic pollution in industrial saline wastewater: a literature review," *Water Research*, vol. 40, no. 20, pp. 3671–3682, 2006.

[3] S. Le Borgne, D. Paniagua, and R. Vazquez-Duhalt, "Biodegradation of organic pollutants by halophilic Bacteria and Archaea," *Journal of Molecular Microbiology and Biotechnology*, vol. 15, no. 2-3, pp. 74–92, 2008.

[4] B. Z. Fathepure, "Recent studies in microbial degradation of petroleum hydrocarbons in hypersaline environments," *Frontiers in Microbiology*, vol. 5, article 173, 2014.

[5] L. C. Castillo-Carvajal, J. L. Sanz-Martín, and B. E. Barragán-Huerta, "Biodegradation of organic pollutants in saline wastewater by halophilic microorganisms: a review," *Environmental Science and Pollution Research*, vol. 21, no. 16, pp. 9578–9588, 2014.

[6] M. A. Amoozegar, M. Siroosi, S. Atashgahi, H. Smidt, and A. Ventosa, "Systematics of haloarchaea and biotechnological potential of their hydrolytic enzymes," *Microbiology*, vol. 163, no. 5, pp. 623–645, 2017.

[7] A. Oren, "Halophilic microbial communities and their environments," *Current Opinion in Biotechnology*, vol. 33, pp. 119–124, 2015.

[8] D. K. Maheshwari and M. Saraf, *Halophiles: Biodiversity and Sustainable Exploitation, Volume 6 in the Sustainable Development and Biodiversity Series*, K. G. Ramawat, Ed., Springer, 2015.

[9] M. J. Bonete, V. Bautista, J. Esclapez et al., "New uses of Haloarchaeal species in bioremediation processes," in *Advances in Bioremediation of Wastewater and Polluted Soil*, InTech, 2015.

[10] S. Aracil-Gisbert, J. Torregrosa-Crespo, and R. M. Martínez-Espinosa, "Recent trend on bioremediation of polluted salty soils and waters using Haloarchaea," in *Advances in Bioremediation and Phytoremediation, Chapter 4*, pp. 63–77, InTech, 2018.

[11] A. Loukas, I. Kappas, and T. J. Abatzopoulos, "HaloDom: a new database of halophiles across all life domains," *Journal of Biological Research-Thessaloniki*, vol. 25, no. 1, p. 2, 2018.

[12] S. Kleinsteuber, V. Riis, I. Fetzer, H. Harms, and S. Müller, "Population dynamics within a microbial consortium during growth on diesel fuel in saline environments," *Applied and Environmental Microbiology*, vol. 72, no. 5, pp. 3531–3542, 2006.

[13] M. T. García, E. Mellado, J. C. Ostos, and A. Ventosa, "*Halomonas organivorans* sp. nov., a moderate halophile able to degrade aromatic compounds," *International Journal of Systematic and Evolutionary Microbiology*, vol. 54, no. 5, pp. 1723–1728, 2004.

[14] J. C. Bertrand, M. Almallah, M. Acquaviva, and G. Mille, "Biodegradation of hydrocarbons by an extremely halophilic archaebacterium," *Letters in Applied Microbiology*, vol. 11, no. 5, pp. 260–263, 1990.

[15] E. B. M. Denner, T. J. McGenity, J.-J. Busse, W. D. Grant, G. Wanner, and H. Stan-Lotter, "*Halococcus salifodinae* sp. nov., an archaeal isolate from an Austrian salt mine," *International Journal of Systematic and Evolutionary Microbiology*, vol. 44, no. 4, pp. 774–780, 1994.

[16] D. M. Al-Mailem, N. A. Sorkhoh, H. Al-Awadhi, M. Eliyas, and S. S. Radwan, "Biodegradation of crude oil and pure hydrocarbons by extreme halophilic archaea from hypersaline coasts of the Arabian Gulf," *Extremophiles*, vol. 14, no. 3, pp. 321–328, 2010.

[17] J.-Y. Ding, S.-C. Chen, M.-C. Lai, and T.-L. Liao, "Haloterrigena mahii sp. nov., an extremely halophilic archaeon from a solar saltern," *International Journal of Systematic and Evolutionary Microbiology*, vol. 67, no. 5, pp. 1333–1338, 2017.

[18] Y. H. Tapilatu, V. Grossi, M. Acquaviva, C. Militon, J.-C. Bertrand, and P. Cuny, "Isolation of hydrocarbon-degrading extremely halophilic archaea from an uncontaminated hypersaline pond (Camargue, France)," *Extremophiles*, vol. 14, no. 2, pp. 225–231, 2010.

[19] I. S. Kulichevskaya, E. I. Milekhina, I. A. Borzenkov, I. S. Zvyagintseva, and S. S. Belyaev, "Oxidation of petroleum hydrocarbons by extremely halophilic archeobacteria," *Mikrobiologiya*, vol. 60, no. 5, pp. 596–601, 1991.

[20] D. Emerson, S. Chauhan, P. Oriel, and J. A. Breznak, "*Haloferax* sp. D1227, a halophilic Archaeon capable of growth on aromatic compounds," *Archives of Microbiology*, vol. 161, no. 6, pp. 445–452, 1994.

[21] W. Fu and P. Oriel, "Degradation of 3-phenylpropionic acid by *Haloferax* sp. D1227," *Extremophiles*, vol. 3, no. 1, pp. 45–53, 1999.

[22] I. Zvyagintseva, S. Belyaev, I. Borzenkov, N. Kostrikina, E. Milekhina, and M. Ivanov, "Halophilic archaebacteria from the Kalamkass oil field," *Mikrobiologiya*, vol. 64, no. 1, pp. 83–87, 1995.

[23] D. J. Fairley, D. R. Boyd, N. D. Sharma, C. C. R. Allen, P. Morgan, and M. J. Larkin, "Aerobic metabolism of 4-hydroxybenzoic acid in Archaea via an unusual pathway involving an intramolecular migration (NIH shift)," *Applied and Environmental Microbiology*, vol. 68, no. 12, pp. 6246–6255, 2002.

[24] D. J. Fairley, G. Wang, C. Rensing, I. L. Pepper, and M. J. Larkin, "Expression of gentisate 1, 2-dioxygenase (*gdoA*) genes involved in aromatic degradation in two haloarchaeal genera," *Applied Microbiology and Biotechnology*, vol. 73, no. 3, pp. 691–695, 2006.

[25] W. Fu and P. Oriel, "Gentisate 1,2-dioxygenase from *Haloferax* sp. D1227," *Extremophiles*, vol. 2, no. 4, pp. 439–446, 1998.

[26] B. Ozcan, G. Ozcengiz, A. Coleri, and C. Cokmus, "Diversity of halophilic Archaea from six hypersaline environments in

Turkey," *Journal of Microbiology and Biotechnology*, vol. 17, no. 6, pp. 985–992, 2007.

[27] S. Mazguene, M. Rossi, M. Gogliettino et al., "Isolation and characterization from solar salterns of North Algeria of a haloarchaeon producing a new halocin," *Extremophiles*, vol. 22, no. 2, pp. 259–270, 2018.

[28] L. G. Wayne, H. C. Engbaek, H. W. B. Engel et al., "Highly reproducible techniques for use in systematic bacteriology in the genus *Mycobacterium*: tests for pigment, urease, resistance to sodium chloride, hydrolysis of Tween 80, and β-galactosidase," *International Journal of Systematic and Evolutionary Microbiology*, vol. 24, no. 4, pp. 412–419, 1974.

[29] J. D. Rogers, T. L. Burke, S. G. Osborn, and J. N. Ryan, "A framework for identifying organic compounds of concern in hydraulic fracturing fluids based on their mobility and persistence in groundwater," *Environmental Science and Technology Letters*, vol. 2, no. 6, pp. 158–164, 2015.

[30] E. Nyankson, D. Rodene, and R. B. Gupta, "Advancements in crude oil spill remediation research after the Deepwater Horizon oil spill," *Water, Air, and Soil Pollution*, vol. 227, no. 1, p. 29, 2016.

[31] I. Djeridi, C. Militon, V. Grossi, and P. Cuny, "Evidence for surfactant production by the haloarchaeon *Haloferax* sp. MSNC14 in hydrocarbon-containing media," *Extremophiles*, vol. 17, no. 4, pp. 669–675, 2013.

[32] M. R. L. Bonfá, M. J. Grossman, E. Mellado, and L. R. Durrant, "Biodegradation of aromatic hydrocarbons by Haloarchaea and their use for the reduction of the chemical oxygen demand of hypersaline petroleum produced water," *Chemosphere*, vol. 84, no. 11, pp. 1671–1676, 2011.

[33] M. F. Mullakhanbhai and H. Larsen, "*Halobacterium volcanii* spec. nov. a Dead Sea halobacterium with a moderate salt requirement," *Archives of Microbiology*, vol. 104, no. 1, pp. 207–214, 1975.

[34] A. L. Hartman, C. Norais, J. H. Badger et al., "The complete genome sequence of *Haloferax volcanii* DS2, a model archaeon," *PLoS One*, vol. 5, no. 3, article e9605, 2010.

[35] J.-Y. Ding and M.-C. Lai, "The biotechnological potential of the extreme halophilic archaea *Haloterrigena* sp. H13 in xenobiotic metabolism using a comparative genomics approach," *Environmental Toxicology*, vol. 31, no. 8-9, pp. 905–914, 2010.

[36] S. Cuadros-Orellana, M. Pohlschröder, M. J. Grossman, and L. R. Durrant, "Biodegradation of aromatic compounds by a halophilic archaeon isolated from the dead sea," *Chemical Engineering Transactions*, vol. 27, pp. 13–18, 2012.

[37] S. Khemili-Talbi, S. Kebbouche-Gana, S. Akmoussi-Toumi, Y. Angar, and M. L. Gana, "Isolation of an extremely halophilic arhaeon *Natrialba sp*. C21 able to degrade aromatic compounds and to produce stable biosurfactant at high salinity," *Extremophiles*, vol. 19, no. 6, pp. 1109–1120, 2015.

[38] E. Acikgoz and B. Ozcan, "Phenol biodegradation by halophilic archaea," *International Biodeterioration and Biodegradation*, vol. 107, pp. 140–146, 2016.

[39] S. F. Erdoğmuş, B. Mutlu, S. E. Korcan, K. Güven, and M. Konuk, "Aromatic hydrocarbon degradation by halophilic archaea isolated from Çamaltı Saltern, Turkey," *Water, Air and Soil Pollution*, vol. 224, no. 3, article 1449, 2013.

[40] S. W. Roh and H. S. Song, "Complete genome sequence of Halorubrum trapanicum CBA1232," Submission to NCBI GENBANK, 01-Jul-2016, Bioproject PRJDB4921, Accession number AP017569, accessed 02-May-2018.

[41] S. Dalvi, N. H. Youssef, and B. Z. Fathepure, "Microbial community structure analysis of a benzoate-degrading halophilic archaeal enrichment," *Extremophiles*, vol. 20, no. 3, pp. 311–321, 2016.

[42] M. S. Elshahed, F. Z. Najar, B. A. Roe, A. Oren, T. A. Dewers, and L. R. Krumholz, "Survey of archaeal diversity reveals an abundance of halophilic *Archaea* in a low-salt, sulfide- and sulfur-rich spring," *Applied and Environmental Microbiology*, vol. 70, no. 4, pp. 2230–2239, 2004.

[43] A. K. Borsodi, T. Felföldi, I. Máthé et al., "Phylogenetic diversity of bacterial and archaeal communities inhabiting the saline Lake Red located in Sovata, Romania," *Extremophiles*, vol. 17, no. 1, pp. 87–98, 2013.

[44] X. Wang, Z. Han, Z. Bai et al., "Archaeal community structure along a gradient of petroleum contamination in saline-alkali soil," *Journal of Environmental Sciences*, vol. 23, no. 11, pp. 1858–1864, 2011.

[45] W. Sun, J. Li, L. Jiang, Z. Sun, M. Fu, and X. Peng, "Profiling microbial community structures across six large oilfields in China and the potential role of dominant microorganisms in bioremediation," *Applied Microbiology and Biotechnology*, vol. 99, no. 20, pp. 8751–8764, 2015.

[46] K. Konstantinidis, A. Tebbe, C. Klein et al., "Genome-wide proteomics of *Natronomonas pharaonis*," *Journal of Proteome Research*, vol. 6, no. 1, pp. 185–193, 2007.

[47] F. Piubeli, M. J. Grossman, F. Fantinatti-Garboggini, and L. R. Durrant, "Phylogenetic analysis of the microbial community in hypersaline petroleum produced water from the Campos Basin," *Environmental Science and Pollution Research*, vol. 21, no. 20, pp. 12006–12016, 2014.

[48] F. Piubeli, M. J. Grossman, F. Fantinatti-Garboggini, and L. R. Durrant, "Enhanced reduction of COD and aromatics in petroleum-produced water using indigenous microorganisms and nutrient addition," *International Biodeterioration and Biodegradation*, vol. 68, pp. 78–84, 2012.

[49] D. M. Al-Mailem, M. Eliyas, and S. Radwan, "Enhanced bioremediation of oil-polluted, hypersaline, coastal areas in Kuwait via vitamin-fertilization," *Environmental Science and Pollution Research*, vol. 21, no. 5, pp. 3386–3394, 2014.

[50] D. M. Al-Mailem, M. Eliyas, and S. S. Radwan, "Bioremediation of oily hypersaline soil and water via potassium and magnesium amendment," *Canadian Journal of Microbiology*, vol. 59, no. 12, pp. 837–844, 2013.

[51] D. M. Al-Mailem, M. Eliyas, and S. S. Radwan, "Enhanced haloarchaeal oil removal in hypersaline environments via organic nitrogen fertilization and illumination," *Extremophiles*, vol. 16, no. 5, pp. 751–758, 2012.

[52] D. M. Al-Mailem, M. Eliyas, M. Khanafer, and S. S. Radwan, "Biofilms constructed for the removal of hydrocarbon pollutants from hypersaline liquids," *Extremophiles*, vol. 19, no. 1, pp. 189–196, 2015.

[53] Y. Y. Corsellis, M. M. Krasovec, L. L. Sylvi, P. P. Cuny, and C. C. Militon, "Oil removal and effects of spilled oil on active microbial communities in close to salt-saturation brines," *Extremophiles*, vol. 20, no. 3, pp. 235–250, 2016.

[54] D. M. Al-Mailem, M. Al-Deieg, M. Eliyas, and S. S. Radwan, "Biostimulation of indigenous microorganisms for bioremediation of oily hypersaline microcosms from the Arabian

Gulf Kuwaiti coasts," *Journal of Environmental Management*, vol. 193, pp. 576–583, 2017.

[55] D. M. Al-Mailem, M. Eliyas, and S. S. Radwan, "Ferric sulfate and proline enhance heavy-metal tolerance of halophilic/halotolerant soil microorganisms and their bioremediation potential for spilled-oil under multiple stresses," *Frontiers in Microbiology*, vol. 9, article 394, 2018.

[56] D. Das, B. B. Salgaonkar, K. Mani, and J. M. Braganca, "Cadmium resistance in extremely halophilic archaeon *Haloferax* strain BBK2," *Chemosphere*, vol. 112, pp. 385–392, 2014.

[57] N. H. Youssef, K. N. Ashlock-Savage, and M. S. Elshahed, "Phylogenetic diversities and community structure of members of the extremely halophilic archaea (order Halobacteriales) in multiple saline sediment habitats," *Applied and Environmental Microbiology*, vol. 78, no. 5, pp. 1332–1344, 2012.

[58] V. Izzo, E. Notomista, A. Picardi, F. Pennacchio, and A. di Donato, "The thermophilic archaeon *Sulfolobus solfataricus* is able to grow on phenol," *Research in Microbiology*, vol. 156, no. 5-6, pp. 677–689, 2005.

[59] P. Christen, A. Vega, L. Casalot, G. Simon, and R. Auria, "Kinetics of aerobic phenol biodegradation by the acidophilic and hyperthermophilic archaeon *Sulfolobus solfataricus* 98/2," *Biochemical Engineering Journal*, vol. 62, pp. 56–61, 2012.

[60] P. Christen, S. Davidson, Y. Combet-Blanc, and R. Auria, "Phenol biodegradation by the thermoacidophilic archaeon *Sulfolobus solfataricus* 98/2 in a fed-batch bioreactor," *Biodegradation*, vol. 22, no. 3, pp. 475–484, 2011.

[61] A. Comte, P. Christen, S. Davidson et al., "Biochemical, transcriptional and translational evidences of the phenol-*meta*-degradation pathway by the hyperthermophilic *Sulfolobus solfataricus* 98/2," *PLoS One*, vol. 8, no. 12, article e82397, 2013.

[62] Y.-J. Park, S.-J. Yoon, and H.-B. Lee, "A novel dienelactone hydrolase from the thermoacidophilic archaeon *Sulfolobus solfataricus* P1: Purification, characterization, and expression," *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1800, no. 11, pp. 1164–1172, 2010.

[63] A. Kumar, N. Trefault, and A. O. Olaniran, "Microbial degradation of 2,4-dichlorophenoxyacetic acid: insight into the enzymes and catabolic genes involved, their regulation and biotechnological implications," *Critical Reviews in Microbiology*, vol. 42, no. 2, pp. 1–15, 2016.

[64] J. Quehenberger, L. Shen, S.-V. Albers, B. Siebers, and O. Spadiut, "Sulfolobus-a potential key organism in future biotechnology," *Frontiers in Microbiology*, vol. 8, article 2474, 2017.

[65] W. B. Whitman, T. L. Bowen, and D. R. Boone, "The methanogenic bacteria," in *The Prokaryotes, vol. 1*, A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K. H. Schleifer, Eds., Springer-Verlag, New York, NY, USA, 1992.

[66] M. J. McInerney, C. G. Struchtemeyer, J. Sieber et al., "Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism," *Annals of the New York Academy of Sciences*, vol. 1125, no. 1, pp. 58–72, 2008.

[67] B. Tan, X. Dong, C. W. Sensen, and J. Foght, "Metagenomic analysis of an anaerobic alkane-degrading microbial culture: potential hydrocarbon-activating pathways and inferred roles of community members," *Genome*, vol. 56, no. 10, pp. 599–611, 2013.

[68] F. Enzmann, F. Mayer, M. Rother, and D. Holtmann, "Methanogens: biochemical background and biotechnological applications," *AMB Express*, vol. 8, no. 1, p. 1, 2018.

[69] A. Mukherjee and D. Chattopadhyay, "Exploring environmental systems and processes through next-generation sequencing technologies: insights into microbial response to petroleum contamination in key environments," *The Nucleus*, vol. 60, no. 2, pp. 175–186, 2017.

[70] M. A. Dojka, P. Hugenholtz, S. K. Haack, and N. R. Pace, "Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation," *Applied and Environmental Microbiology*, vol. 64, no. 10, pp. 3869–3877, 1998.

[71] K. Zengler, H. H. Richnow, R. Rossello-Mora, W. Michaelis, and F. Widdel, "Methane formation from long-chain alkanes by anaerobic microorganisms," *Nature*, vol. 401, no. 6750, pp. 266–269, 1999.

[72] Y. Kasai, Y. Takahata, T. Hoaki, and K. Watanabe, "Physiological and molecular characterization of a microbial community established in unsaturated, petroleum-contaminated soil," *Environmental Microbiology*, vol. 7, no. 6, pp. 806–818, 2005.

[73] D.-C. Zhang, C. Mörtelmaier, and R. Margesin, "Characterization of the bacterial archaeal diversity in hydrocarbon-contaminated soil," *Science of the Total Environment*, vol. 421-422, pp. 184–196, 2012.

[74] N. B. Sutton, F. Maphosa, J. A. Morillo et al., "Impact of long-term diesel contamination on soil microbial community structure," *Applied and Environmental Microbiology*, vol. 79, no. 2, pp. 619–630, 2012.

[75] S. T. Bates, D. Berg-Lyons, J. G. Caporaso, W. A. Walters, R. Knight, and N. Fierer, "Examining the global distribution of dominant archaeal populations in soil," *ISME Journal*, vol. 5, no. 5, pp. 908–917, 2011.

[76] T. J. Penner and J. M. Foght, "Mature fine tailings from oil sands processing harbour diverse methanogenic communities," *Canadian Journal of Microbiology*, vol. 56, no. 6, pp. 459–470, 2010.

[77] B. Liang, L.-Y. Wang, S. M. Mbadinga et al., "*Anaerolineaceae* and *Methanosaeta* turned to be the dominant microorganisms in alkanes-dependent methanogenic culture after long-term of incubation," *AMB Express*, vol. 5, no. 1, p. 37, 2015.

[78] B. Liang, L.-Y. Wang, Z. Zhou et al., "High frequency of *Thermodesulfovibrio* spp. and *Anaerolineaceae* in association with *Methanoculleus* spp. in a long-term incubation of *n*-alkanes-degrading methanogenic enrichment culture," *Frontiers in Microbiology*, vol. 7, article 1431, 2016.

[79] R. Liu, Y. Zhang, R. Ding, D. Li, Y. Gao, and M. Yang, "Comparison of archaeal and bacterial community structures in heavily oil-contaminated and pristine soils," *Journal of Bioscience and Bioengineering*, vol. 108, no. 5, pp. 400–407, 2009.

[80] R. Das and S. K. Kazy, "Microbial diversity, community composition and metabolic potential in hydrocarbon contaminated oily sludge: prospects for in situ bioremediation," *Environmental Science and Pollution Research*, vol. 21, no. 12, pp. 7369–7389, 2014.

[81] D. T. Ramos, M. L. B. da Silva, H. S. Chiaranda, P. J. J. Alvarez, and H. X. Corseuil, "Biostimulation of anaerobic BTEX biodegradation under fermentative methanogenic conditions at source-zone groundwater contaminated with a biodiesel blend (B20)," *Biodegradation*, vol. 24, no. 3, pp. 333–341, 2013.

[82] J. A. Siles and R. Margesin, "Insights into microbial communities mediating the bioremediation of hydrocarbon-contaminated soil from an Alpine former military site," *Applied Microbiology and Biotechnology*, vol. 102, no. 10, pp. 4409–4421, 2018.

[83] L. B. Salam, S. O. Obayori, F. O. Nwaokorie, A. Suleiman, and R. Mustapha, "Metagenomic insights into effects of spent engine oil perturbation on the microbial community composition and function in a tropical agricultural soil," *Environmental Science and Pollution Research*, vol. 24, no. 8, pp. 7139–7159, 2017.

[84] S. Fuentes, B. Barra, J. G. Caporaso, and M. Seeger, "From rare to dominant: a fine-tuned soil bacterial bloom during petroleum hydrocarbon bioremediation," *Applied and Environmental Microbiology*, vol. 82, no. 3, pp. 888–896, 2016.

[85] Y. Liang, G. Li, J. D. van Nostrand et al., "Microarray-based analysis of microbial functional diversity along an oil contamination gradient in oil field," *FEMS Microbiology Ecology*, vol. 70, no. 2, pp. 324–333, 2009.

[86] H. E. de Jesus, R. S. Peixoto, J. C. Cury, J. D. van Elsas, and A. S. Rosado, "Evaluation of soil bioremediation techniques in an aged diesel spill at the Antarctic Peninsula," *Applied Microbiology and Biotechnology*, vol. 99, no. 24, pp. 10815–10827, 2015.

[87] K. E. Scherr, D. Backes, A. G. Scarlett, W. Lantschbauer, and M. Nahold, "Biogeochemical gradients above a coal tar DNAPL," *Science of the Total Environment*, vol. 563-564, pp. 741–754, 2016.

[88] T. C. Hazen, R. C. Prince, and N. Mahmoudi, "Marine oil biodegradation," *Environmental Science and Technology*, vol. 50, no. 5, pp. 2121–2129, 2016.

[89] W. F. M. Röling, I. R. Couto de Brito, R. P. J. Swannell, and I. M. Head, "Response of Archaeal communities in beach sediments to spilled oil and bioremediation," *Applied and Environmental Microbiology*, vol. 70, no. 5, pp. 2614–2620, 2004.

[90] H. Urakawa, J. C. Garcia, P. D. Barreto, G. A. Molina, and J. C. Barreto, "A sensitive crude oil bioassay indicates that oil spills potentially induce a change of major nitrifying prokaryotes from the Archaea to the Bacteria," *Environmental Pollution*, vol. 164, pp. 42–45, 2012.

[91] M. C. Redmond and D. L. Valentine, "Natural gas and temperature structured a microbial community response to the Deepwater Horizon oil spill," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 50, pp. 20292–20297, 2012.

[92] V. Catania, S. Cappello, V. Di Giorgi et al., "Microbial communities of polluted sub-surface marine sediments," *Marine Pollution Bulletin*, vol. 131, part A, pp. 396–406, 2018.

[93] A. Mikkonen, M. Santalahti, K. Lappi, A.-M. Pulkkinen, L. Montonen, and L. Suominen, "Bacterial and archaeal communities in long-term contaminated surface and subsurface soil evaluated through coextracted RNA and DNA," *FEMS Microbiology Ecology*, vol. 90, no. 1, pp. 103–114, 2014.

[94] D. Jurelevicius, C. R. de Almeida Couto, V. M. Alvarez, R. E. Vollú, F. de Almeida Dias, and L. Seldin, "Response of the archaeal community to simulated petroleum hydrocarbon contamination in marine and hypersaline ecosystems," *Water, Air & Soil Pollution*, vol. 225, no. 2, article 1871, 2014.

[95] A. C. F. Dias, F. Dini-Andreote, R. G. Taketani et al., "Archaeal communities in the sediments of three contrasting mangroves," *Journal of Soils and Sediments*, vol. 11, no. 8, pp. 1466–1476, 2011.

[96] M. Jeanbille, J. Gury, R. Duran et al., "Chronic polyaromatic hydrocarbon (PAH) contamination is a marginal driver for community diversity and prokaryotic predicted functioning in coastal sediments," *Frontiers in Microbiology*, vol. 7, article 1303, 2016.

[97] M. Kolukirik, O. Ince, and B. K. Ince, "Increment in anaerobic hydrocarbon degradation activity of Halic Bay sediments via nutrient amendment," *Microbial Ecology*, vol. 61, no. 4, pp. 871–884, 2011.

[98] Z. Zhang and I. M. C. Lo, "Biostimulation of petroleum-hydrocarbon-contaminated marine sediment with co-substrate: involved metabolic process and microbial community," *Applied Microbiology and Biotechnology*, vol. 99, no. 13, pp. 5683–5696, 2015.

[99] M. E. Campeão, L. Reis, L. Leomil et al., "The deep-sea microbial community from the Amazonian Basin associated with oil degradation," *Frontiers in Microbiology*, vol. 8, article 1019, 2017.

[100] L. Wang, X. Huang, and T.-L. Zheng, "Responses of bacterial and archaeal communities to nitrate stimulation after oil pollution in mangrove sediment revealed by Illumina sequencing," *Marine Pollution Bulletin*, vol. 109, no. 1, pp. 281–289, 2016.

[101] L. Yan, D. Yu, N. Hui et al., "Distribution of Archaeal communities along the coast of the Gulf of Finland and their response to oil contamination," *Frontiers in Microbiology*, vol. 9, p. 15, 2018.

[102] A. J. Pinto and L. Raskin, "PCR biases distort Bacterial and Archaeal community structure in pyrosequencing datasets," *PLoS One*, vol. 7, no. 8, article e43093, 2012.

[103] M. Singh, P. K. Srivastava, V. K. Jaiswal, and R. N. Kharwar, "Biotechnological applications of microbes for the remediation of environmental pollution," in *Biotechnology: Trends and Applications*, R. Singh and M. Trivedi, Eds., pp. 179–214, Stadium Press LLC, USA, 2016.

[104] D. Prakash, P. Gabani, A. K. Chandel, Z. Ronen, and O. V. Singh, "Bioremediation: a genuine technology to remediate radionuclides from the environment," *Microbial Biotechnology*, vol. 6, no. 4, pp. 349–360, 2013.

[105] Y. Zivanovic, J. Armengaud, A. Lagorce et al., "Genome analysis and genome-wide proteomics of *Thermococcus gammatolerans*, the most radioresistant organism known amongst the Archaea," *Genome Biology*, vol. 10, no. 6, article R70, 2009.

[106] M. Choudhary, R. Kumar, A. Datta, V. Nehra, and N. Greg, "Bioremediation of heavy metals by microbes," in *Bioremediation of Salt Affected Soils: An Indian Perspective*, S. Arora, A. Singh, and Y. Singh, Eds., pp. 233–255, Springer, Cham, 2017.

[107] P. Ranawat and S. Rawat, "Metal-tolerant thermophiles: metals as electron donors and acceptors, toxicity, tolerance and industrial applications," *Environmental Science and Pollution Research*, vol. 25, no. 5, pp. 4105–4133, 2018.

[108] C. R. Jackson, H. W. Langner, J. Donahoe-Christiansen, W. P. Inskeep, and T. R. McDermott, "Molecular analysis of microbial community structure in an arsenite-oxidizing acidic thermal spring," *Environmental Microbiology*, vol. 3, no. 8, pp. 532–542, 2001.

[109] H. M. Sehline and E. B. Linström, "Oxidation and reduction of arsenic by *Sulfolobus acidocaldarius* strain BC," *FEMS Microbiology Letters*, vol. 93, no. 1, pp. 87–92, 1992.

[110] E. Lebrun, M. Brugna, F. Baymann et al., "Arsenite oxidase, an ancient bioenergetic enzyme," *Molecular Biology and Evolution*, vol. 20, no. 5, pp. 686–693, 2003.

[111] A. Heinrich-Salmeron, A. Cordi, C. Brochier-Armanet et al., "Unsuspected diversity of arsenite-oxidizing bacteria as revealed by widespread distribution of the *aoxB* gene in prokaryotes," *Applied and Environmental Microbiology*, vol. 77, no. 13, pp. 4685–4692, 2011.

[112] O. F. Ordoñez, M. C. Rasuk, M. N. Soria, M. Contreras, and M. E. Farías, "Haloarchaea from the Andean Puna: biological role in the energy metabolism of arsenic," *Microbial Ecology*, vol. 76, 2018.

[113] R. Huber, M. Sacher, A. Vollmann, H. Huber, and D. Rose, "Respiration of arsenate and selenate by hyperthermophilic Archaea," *Systematic and Applied Microbiology*, vol. 23, no. 3, pp. 305–314, 2000.

[114] E. S. Boyd and T. Barkay, "The mercury resistance operon: from an origin in a geothermal environment to an efficient detoxification machine," *Frontiers in Microbiology*, vol. 3, p. 349, 2012.

[115] Y. Wang, E. Boyd, S. Crane et al., "Environmental conditions constrain the distribution and diversity of Archaeal *merA* in Yellowstone National Park, Wyoming, USA," *Microbial Ecology*, vol. 62, no. 4, pp. 739–752, 2011.

[116] J. Schelert, V. Dixit, V. Hoang, J. Simbahan, M. Drozda, and P. Blum, "Occurrence and characterization of mercury resistance in the hyperthermophilic Archaeon *Sulfolobus solfataricus* by use of gene disruption," *Journal of Bacteriology*, vol. 186, no. 2, pp. 427–437, 2003.

[117] D. M. Al-Mailem, H. Al-Awadhi, N. A. Sorkhoh, M. Eliyas, and S. S. Radwan, "Mercury resistance and volatilization by oil utilizing haloarchaea under hypersaline conditions," *Extremophiles*, vol. 15, no. 1, pp. 39–44, 2011.

[118] C. C. Gilmour, M. Podar, A. L. Bullock et al., "Mercury methylation by novel microorganisms from new environments," *Environmental Science and Technology*, vol. 47, no. 20, pp. 11810–11820, 2013.

[119] L. Newsome, K. Morris, and J. R. Lloyd, "The biogeochemistry and bioremediation of uranium and other priority radionuclides," *Chemical Geology*, vol. 363, pp. 164–184, 2014.

[120] K. Kashefi, B. M. Moskowitz, and D. R. Lovley, "Characterization of extracellular minerals produced during dissimilatory Fe(III) and U(VI) reduction at 100°C by *Pyrobaculum islandicum*," *Geobiology*, vol. 6, no. 2, pp. 147–154, 2008.

[121] K. Kashefi and D. R. Lovley, "Reduction of Fe(III), Mn(IV), and toxic metals at 100°C by *Pyrobaculum islandicum*," *Applied and Environmental Microbiology*, vol. 66, no. 3, pp. 1050–1056, 2000.

[122] B. K. Kim, T. D. Pihl, J. N. Reeve, and L. Daniels, "Purification of the copper response extracellular proteins secreted by the copper-resistant methanogen *Methanobacterium bryantii* BKYH and cloning, sequencing, and transcription of the gene encoding these proteins," *Journal of Bacteriology*, vol. 177, no. 24, pp. 7178–7185, 1995.

[123] T. Reitz, M. L. Merroun, and S. Selenska-Pobell, "Interactions of *Paenibacillus* sp. and *Sulfolobus acidocaldarius* strains with U(VI)," in *Uranium, Mining, and Hydrogeology*, B. J. Merkel and A. Hasche-Berger, Eds., Springer, Berlin, Heidelberg, 2008.

[124] X. Zhuang, Z. Han, Z. Bai, G. Zhuang, and H. Shim, "Progress in decontamination by halophilic microorganisms in saline wastewater and soil," *Environmental Pollution*, vol. 158, no. 5, pp. 1119–1126, 2010.

[125] S. Naik and I. Furtado, "Equilibrium and kinetics of adsorption of Mn^{+2} by Haloarchaeon *Halobacterium* sp. GUSF (MTCC3265)," *Geomicrobiology Journal*, vol. 31, no. 8, pp. 708–715, 2014.

[126] A. R. Showalter, J. E. S. Szymanowski, J. B. Fein, and B. A. Bunker, "An x-ray absorption spectroscopy study of Cd binding onto a halophilic archaeon," *Journal of Physics: Conference Series*, vol. 712, article 012079, 2016.

[127] M. Bader, K. Müller, H. Foerstendorf et al., "Comparative analysis of uranium bioassociation with halophilic bacteria and archaea," *PLoS One*, vol. 13, no. 1, article e0190953, 2018.

[128] M. Bader, K. Müller, H. Foerstendorf et al., "Multistage bioassociation of uranium onto an extremely halophilic archaeon revealed by a unique combination of spectroscopic and microscopic techniques," *Journal of Hazardous Materials*, vol. 327, pp. 225–232, 2017.

[129] D. K. Nordstrom and G. Southham, "Geomicrobiology:interactions between microbes and minerals," in *Reviews in Mineralogy, vol. 35*, J. F. Banfield and K. H. Nealson, Eds., pp. 361–390, Mineralogical Society of America, Washington, DC, USA, 1997.

[130] K. J. Edwards, P. L. Bond, T. M. Gihring, and J. F. Banfield, "An Archaeal iron-oxidizing extreme acidophile important in acid mine drainage," *Science*, vol. 287, no. 5459, pp. 1796–1799, 2000.

[131] O. V. Golyshina and K. N. Timmis, "Ferroplasma and relatives, recently discovered cell wall-lacking archaea making a living in extremely acid, heavy metal-rich environments," *Environmental Microbiology*, vol. 7, no. 9, pp. 1277–1288, 2005.

[132] G. Huber and K. O. Stetter, "*Sulfolobus metallicus*, sp. nov., a novel strictly chemolithoautotrophic thermophilic archaeal species of metal-mobilizers," *Systematic and Applied Microbiology*, vol. 14, no. 4, pp. 372–378, 1991.

[133] D. E. Rawlings, "Heavy metal mining using microbes," *Annual Review of Microbiology*, vol. 56, no. 1, pp. 65–91, 2002.

[134] A. P. Yelton, L. R. Comolli, N. B. Justice et al., "Comparative genomics in acid mine drainage biofilm communities reveals metabolic and structural differentiation of co-occurring archaea," *BMC Genomics*, vol. 14, no. 1, p. 485, 2013.

[135] K. B. Hallberg, "New perspectives in acid mine drainage microbiology," *Hydrometallurgy*, vol. 104, no. 3-4, pp. 448–453, 2010.

[136] G. Muyzer and A. J. M. Stams, "The ecology and biotechnology of sulphate-reducing bacteria," *Nature Reviews Microbiology*, vol. 6, no. 6, pp. 441–454, 2008.

[137] J. Beeder, R. K. Nilsen, J. T. Rosnes, T. Torsvik, and T. Lien, "*Archaeoglobus fulgidus* isolated from hot north sea oil field waters," *Applied and Environmental Microbiology*, vol. 60, no. 4, pp. 1227–1231, 1994.

[138] T. Itoh, K.-I. Suzuki, P. C. Sanchez, and T. Nakase, "*Caldivirga maquilingensis* gen. nov., sp. nov., a new genus of rod-shaped crenarchaeote isolated from a hot spring in the Philippines," *International Journal of Systemic and Evolutionary Microbiology*, vol. 49, no. 3, pp. 1157–1163, 1999.

[139] T. Itoh, K.-I. Suzuki, and T. Nakase, "*Thermocladium modestius* gen. nov., sp. nov., a new genus of rod-shaped, extremely

thermophilic crenarchaeote," *International Journal of Systemic and Evolutionary Microbiology*, vol. 48, pp. 879–887, 1998.

[140] L. Bhatnagar, S. P. Li, M. K. Jain, and J. G. Zeikus, "Growth of methanogenic and acidogenic bacteria with pentachlorophenol as a co-substrate," in *Biotechnology applications in hazardous waste treatment*, pp. 383–393, Engineering Foundation, New York, NY, USA, 1989.

[141] B. Z. Fathepure, J. P. Nengu, and S. A. Boyd, "Anaerobic bacteria that dechlorinate perchloroethene," *Applied and Environmental Microbiology*, vol. 53, no. 11, pp. 2671–2674, 1987.

[142] P. E. Jablonski and J. G. Ferry, "Reductive dechlorination of trichloroethylene by the CO-reduced CO dehydrogenase enzyme complex from *Methanosarcina thermophila*," *FEMS Microbiology Letters*, vol. 96, no. 1, pp. 55–59, 1992.

[143] M. D. Mikesell and S. A. Boyd, "Dechlorination of chloroform by *Methanosarcina* strains," *Applied and Environmental Microbiology*, vol. 56, no. 4, pp. 1198–1201, 1990.

[144] U. E. Krone and R. K. Thauer, "Dehalogenation of trichlorofluoromethane (CFC-11) by *Methanosarcina barkeri*," *FEMS Microbiology Letters*, vol. 90, no. 2, pp. 201–204, 1992.

[145] R. A. Mah and D. A. Kuhn, "Transfer of the type species of the genus *Methanococcus* to the genus *Methanosarcina*, naming it *Methanosarcina mazei* (Barker 1936) comb. nov. et emend. and conservation of the genus *Methanococcus* (Approved Lists 1980) with *Methanococcus vannielii* (Approved Lists 1980) as the type species," *International Journal of Systematic and Evolutionary Microbiology*, vol. 34, no. 2, pp. 263–265, 1984.

[146] A. Wasserfallen, J. Nölling, P. Pfister, J. Reeve, and E. Conway de Macario, "Phylogenetic analysis of 18 thermophilic *Methanobacterium* isolates supports the proposals to create a new genus, *Methanothermobacter* gen. nov., and to reclassify several isolates in three species, *Methanothermobacter thermautotrophicus* comb. nov., *Methanothermobacter* wolfeii comb. nov., and *Methanothermobacter marburgensis* sp. nov," *International Journal of Systemic and Evolutionary Microbiology*, vol. 50, pp. 43–53, 2000.

[147] C. Holliger, G. Schraa, A. J. M. Stams, and A. J. B. Zehnder, "Reductive dechlorination of 1,2-dichloroethane and chloroethane by cell suspensions of methanogenic bacteria," *Biodegradation*, vol. 1, no. 4, pp. 253–261, 1990.

[148] J. R. Roth, J. G. Lawrence, and T. A. Bobik, "COBALAMIN (COENZYME B_{12}): synthesis and biological significance," *Annual Review of Microbiology*, vol. 50, no. 1, pp. 137–181, 1996.

[149] D. Jan, "Thermodynamic considerations for dehalogenation," in *Dehalogenation: Microbial Processes and Environmental Applications*, M. M. Häggblom and I. D. Bossert, Eds., Kluwer Academic Publishers, Boston, MA, USA, 2003.

[150] U. E. Krone, R. K. Thauer, and H. P. C. Hogenkamp, "Reductive dehalogenation of chlorinated C1-hydrocarbons mediated by corrinoids," *Biochemistry*, vol. 28, no. 11, pp. 4908–4914, 1989.

[151] B. Heckel, K. McNeill, and M. Elsner, "Chlorinated ethene reactivity with vitamin B_{12} is governed by cobalamin chloroethylcarbanions as crossroads of competing pathways," *ACS Catalysis*, vol. 8, no. 4, pp. 3054–3066, 2018.

[152] R. E. Richardson, V. K. Bhupathiraju, D. L. Song, T. A. Goulet, and L. Alvarez-Cohen, "Phylogenetic characterization of microbial communities that reductively dechlorinate TCE based upon a combination of molecular techniques," *Environmental Science and Technology*, vol. 36, no. 12, pp. 2652–2662, 2002.

[153] P. C. Dennis, B. E. Sleep, R. R. Fulthorpe, and S. N. Liss, "Phylogenetic analysis of bacterial populations in an anaerobic microbial consortium capable of degrading saturation concentrations of tetrachloroethylene," *Canadian Journal of Microbiology*, vol. 49, no. 1, pp. 15–27, 2003.

[154] T. W. Macbeth, D. E. Cummings, S. Spring, L. M. Petzke, and K. S. Sorenson Jr., "Molecular characterization of a dechlorinating community resulting from in situ biostimulation in a trichloroethene-contaminated deep, fractured basalt aquifer and comparison to a derivative laboratory culture," *Applied and Environmental Microbiology*, vol. 70, no. 12, pp. 7329–7341, 2004.

[155] M. Duhamel and E. A. Edwards, "Microbial composition of chlorinated ethene-degrading cultures dominated by Dehalococcoides," *FEMS Microbiology Ecology*, vol. 58, no. 3, pp. 538–549, 2006.

[156] A. C. Heimann, D. J. Batstone, and R. Jakobsen, "*Methanosarcina* spp. drive vinyl chloride dechlorination via interspecies hydrogen transfer," *Applied and Environmental Microbiology*, vol. 72, no. 4, pp. 2942–2949, 2006.

[157] D. E. Fennell and J. M. Gossett, "Modeling the production of and competition for hydrogen in a dechlorinating culture," *Environmental Science and Technology*, vol. 32, no. 16, pp. 2450–2460, 1998.

[158] Y. Men, H. Feil, N. C. VerBerkmoes et al., "Sustainable syntrophic growth of *Dehalococcoides ethenogenes* strain 195 with *Desulfovibrio vulgaris* Hildenborough and *Methanobacterium congolense*: global transcriptomic and proteomic analyses," *The ISME Journal*, vol. 6, no. 2, pp. 410–421, 2012.

[159] S. Yi, E. C. Seth, Y.-J. Men et al., "Versatility in corrinoid salvaging and remodeling pathways supports corrinoid-dependent metabolism in *Dehalococcoides mccartyi*," *Applied and Environmental Microbiology*, vol. 78, no. 21, pp. 7745–7752, 2012.

[160] M. Fincker and A. M. Spormann, "Biochemistry of catabolic reductive dehalogenation," *Annual Review of Biochemistry*, vol. 86, no. 1, pp. 357–386, 2017.

[161] M. Guo and Y. Chen, "Coenzyme cobalamin: biosynthesis, overproduction and its application in dehalogenation – a review," *Reviews in Environmental Science and Biotechnology*, vol. 17, no. 2, pp. 259–284, 2018.

[162] M. J. Krzmarzick, B. B. Crary, J. J. Harding et al., "Natural niche for organohalide-respiring *Chloroflexi*," *Applied and Environmental Microbiology*, vol. 78, no. 2, pp. 393–401, 2011.

[163] M. J. Krzmarzick, P. J. McNamara, B. B. Crary, and P. J. Novak, "Abundance and diversity of organohalide-respiring bacteria in lake sediments across a geographical sulfur gradient," *FEMS Microbiology Ecology*, vol. 84, no. 2, pp. 248–258, 2013.

[164] M. L. Lim, M. D. Brooks, M. A. Boothe, and M. J. Krzmarzick, "Novel bacterial diversity is enriched with chloroperoxidase-reacted organic matter under anaerobic conditions," *FEMS Microbiology Ecology*, vol. 94, no. 5, article fiy050, 2018.

[165] C. J. Smith and A. M. Osborn, "Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology," *FEMS Microbiology Ecology*, vol. 67, no. 1, pp. 6–20, 2009.

[166] D. L. Valentine, "Adaptations to energy stress dictate the ecology and evolution of the Archaea," *Nature Reviews Microbiology*, vol. 5, no. 4, pp. 316–323, 2007.

[167] K. Tamura, G. Stecher, D. Peterson, A. Filipski, and S. Kumar, "MEGA6: molecular evolutionary genetics analysis version 6.0," *Molecular Biology and Evolution*, vol. 30, no. 12, pp. 2725–2729, 2013.

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Research Article

Soil Microbial Community Structure and Diversity around the Aging Oil Sludge in Yellow River Delta as Determined by High-Throughput Sequencing

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Microorganisms are sensitive indicators of edaphic environmental variation. The Illumina MiSeq sequencing technology was used to analyze soil bacterial community diversity around an aging oil sludge in the Yellow River Delta. The alpha diversity index of soil bacterial community results (Ace, Chao, Shannon, and Simpson) determined that bacterial community diversity sampling within the scope of a 20 cm radius from the center of an aging oil sludge spot showed the most abundant diversity. The level of diversity distributed symmetrically with radial direction from the center of the aging oil sludge spot. Over the distance of 100 m from the center, bacterial community diversity tends to be monotonous, with small differences especially in the horizontal direction underground. The alpha-diversity indicators also showed that the bacterial diversity of samples were close under the aging oil sludge. In addition, the aging oil sludge inhibited the growth of bacteria compared with the referenced unpolluted soil sample and also increased the diversities of soil bacteria. At the phylum level, the *Proteobacteria*, *Chloroflexi*, and *Actinobacteria* existing in the aging oil sludge-contaminated wetland soil constituted a larger proportion of the community, while the proportion of *Firmicute* was relatively less. On the contrary, *Firmicute* showed the highest content of 63.8% in the referenced soil. Under the genus level and family level, the corresponding strains that resisted the aging oil sludge were selected. According to the bacterial diversity analysis, the basic structure of the bacterial community which could be used for remediation of aging oil sludge-contaminated soil was also developed.

1. Introduction

Microorganisms play an important role in the soil environment. The variation of the microbial populations often indicates the change of the soil environment. Environmental pollution may cause a transformation in microbial community composition and activity [1, 2]. Moreover, there is a dependent relation between microbial diversity and soil contamination [1, 3]. The stability of microbial diversity represents the status of a microbial community, which could be used to predict the transformation trend of the environmental quality and soil nutrient conditions. Thus, it is considered to be one of the most sensitive biological indicators. Soil microorganisms occupy an important position in the cycle of the biological geochemical system. Simultaneously, it plays an important role in soil self-purification, toxic compound transition, and transformation of the soil environment. Soil microorganisms are far more sensitive to contaminants than soil animals and plants [4], which is an indicator of the changes in the physical and chemical properties of the soil and environmental quality.

With the increasing demand of petroleum, oil production is increasing sharply, leading to incidents of serious soil pollution. The Shengli Oil Field, located in the Yellow River

Delta, is the second largest oilfield in China. However, there is a National Nature Reserve in the Yellow River Delta with hundreds of animals and plants that need to be protected. It also has wetlands that cover an area of over 4500 km^2. Obviously, the oil production process has a negative effect on the protection of endangered organisms and wetlands. Especially, the aging oil sludge (long-term untreated oil spots scattering around oil wells), with the potential risk of long-time and rock-ribbed pollution to the surroundings, presents a thorny problem of treatment and disposal. Meanwhile, a lot of toxic substances are included in the oil sludge. For example, the soil around a crude oil storage site in the Jianghan Oil Field of China was severely contaminated with polycyclic aromatic hydrocarbons (PAHs). The soil around the oil sludge or in the oil exploration area was similarly contaminated with PAHs [5–7].

However, much attention has been paid to the stacking oil sludge in the open air [8–10]. The scattered oil sludge in oil-producing regions, especially around the oil wells, were ignored for years, forming a lot of aging oil sludge-contaminated sites on the soil. Until now, the quantities of the most deleterious components in the aging oil sludge [11], such as total petroleum hydrocarbons (TPHs) and heavy metals, as well as bacterial diversity of the long-time oil-polluted soil, are still unknown and rarely referred to and reported globally [12].

Currently, high-throughput sequencing is recognized as a powerful method to analyze a bacterial community. Its specific primers are also known as accurately providing the diversity of bacterial groups at a fine scale [13, 14]. The MiSeq sequencing system adopted the mature TruSeq with synthetic sequencing technology simultaneously, which integrated amplification, sequencing, and data analysis in one machine. Illumina technology was commonly used as the foundation with the method of the reversible termination reagent, which simultaneously detects millions of pieces at a large-scale. To add each dNTP, the terminator is imaged with fluorescent tags and subsequently cut. Because four kinds of reversible termination were combined with dNTPs, natural competition with minimum deviation detects the base sequence directly by virtue of each cycle of a fluorescence signal. Now, the high-throughput sequencing method is widely used in the process of microbial diversity analysis, since it has the characteristics of no culture, high sensitivity, and low detection limit [15, 16].

It was reported that the types and quantities of microorganisms were closely associated with the contents of soil moisture, organic carbon, soil clay, and soil microorganisms [17]. Researchers analyzed the characteristics of microbial diversity in the Yellow River Delta wetland. The results showed that the number of microorganisms and the diversity of cultured microorganisms decreased with the increase of inorganic salt [18, 19]. Moreover, the geochemical parameters such as pH, Eh, As, sulfate, and water temperature also had significant effects on the indigenous microbial community [20].

This work mainly researched on the transformation of the bacterial community structure caused by the aging oil sludge using high-throughput sequencing technology [2, 9, 10, 21]. The 16S rRNA gene analysis and taxonomical analysis were performed with a clone library. The results and related findings would aid in a thorough understanding of the microorganism structure in aging oil sludge-contaminated soil and thus provide a new point of view to soil bioremediation.

2. Materials and Methods

2.1. Experimental Setup. The soil samples in this experiment were collected from the Shengli Oil Field in Shandong Province, China. Meanwhile, the test soil was obtained from three separate sites across the oil field. Three points in various directions around the aging oil sludge were selected, 0 and 20 cm below the earth of the aging oil sludge, and over 100 m in horizontal direction from the aging oil sludge; the latter was used as the corresponding reference soil.

2.1.1. Sampling and Processing. 1 kg each of the three soil samples was collected, removing the stones and plant debris with the tube labeled for sampling time, sampling site, sampling code, and the surrounding geographical environment outside. Soil samples were preserved at a temperature of 4°C after being transported to the laboratory. A four-point method was used to remove excess soil and the soil finally remained as representative samples. Soil samples were dried naturally and were passed through 100-mesh sieves to determine their physical and chemical properties.

2.1.2. Microbial Diversity Experiment. A sterilized shovel was used to collect soil samples around the aging oil sludge while removing the stones and debris. Finally, 50 g of soil was collected as a representative sample. Meanwhile, there was a need to change to a new sterilized shovel for each soil sample to prevent cross-contamination. The representative samples were preserved in sterile tubes and marked for sampling time, sampling site, sampling code, and surrounding geographical environment outside the tubes. Soil samples were transported with carbon dioxide, and then stored with liquid nitrogen under the temperature of −80°C immediately.

2.2. Analytical Methods

2.2.1. Determination of Soil Physical and Chemical Properties. Moisture content was determined by weighing samples before and after oven drying at 105°C for 24 h. The pH was measured with a pH meter (Mettle-Toledo Instruments, Shanghai, China). The determination of the soil organic carbon was referred to GB 9834-1988. Heavy metals, including copper, zinc, and chromium, were measured by an atomic absorption spectrophotometer (GFA-7000, Japan). The lights of the microwave digestion method were used and the acid system was nitric acid, hydrofluoric acid, and hydrogen peroxide. Experimental steps were as follows: first, 0.25 g of soil sample was accurately put in a microwave digestion jar. Then, 5 ml of nitric acid, 2 ml of hydrofluoric acid, and 1 ml of hydrogen peroxide were added successively. Then, the appropriate temperature and time were set to cool down the soil samples. In addition, the digestion liquids were transferred to a 50 ml beaker for digesting. After a period of

cooling and dissolving the residue, the solution was transferred to a 50 ml volumetric flask with 5 ml of 5% La $(NO_3)_3$ (Cu/Zn) or 5 ml of 10% NH_4Cl (Cr). Besides, the determination of total petroleum hydrocarbons (TPH) was referred to HJ/T 350-2007, China.

2.2.2. DNA Extraction, 16S rRNA Gene Amplification, and High Throughput Sequencing. Soil microbial metagenomic DNA was isolated with a Soil DNA Kit (Omega Bio-tek, Norcross, GA, US) according to the manufacturer's instruction. The DNA extracts were stored at −20°C for the following PCR amplification. The universal 16S rRNA gene primers were the 515F (5′-GTGCCAGCMGCCG CGG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The barcode and adapter were incorporated between the adapter and the forward primers. The PCR used was the TransStart FastPfu DNA Polymerase, a 20 μL reaction system. The PCR mixture contained 10 ng of DNA template, 2 μL of 2.5 mM dNTPs, 0.8 μL of both primers, 0.4 μL of FastPfu Polymerase, 4 μL of 5x FastPfu Buffer, and sterile double-distilled H_2O in a total volume of 20 μL of PCR amplification which was performed in a ABI GeneAmp 9700 (USA). The PCR process consisted of an initial 5 min denaturation at 95°C, followed by 27 cycles of denaturing at 95°C for 30 s, and annealing at 55°C for 30 s, with an extension at 72°C for 45 s. The same sample was mixed with the PCR product with 2% agarose gel electrophoresis detection. By virtue of the AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, CA, US) PCR products were cut, eluted with Tris-HCl, and subjected to 2% agarose electrophoresis detection at the same time. The QuantiFluor™-ST (Promega, US) blue fluorescence quantitative system for PCR products was used for detection. The next step was building the MiSeq library. We chose the TruSeq™ DNA Sample Prep Kit and cBot TruSeq PE Cluster Kit v3-cBot-HS. Specific steps were as follows: the P7 and P5 joints were connected; the magnetic bead filter was used to remove the irregular fragments; PCR amplification was used to enrich the library template; and alkali degeneration was used to produce single-stranded DNA fragments. The cBot Truseq PE Cluster Kit v3-cBot-HS was the cluster-generation kit for bridge amplification.

The treated samples in the MiSeq PE300 platform were sequenced for about 65 hours. The end DNA fragments complemented with the primer base and were fixed on a chip, while the other end complemented randomly with the other primers, and was then fixed and formed a "bridge." PCR amplification and DNA clusters were produced at the same time. Subsequently, DNA linearization was performed while producing a single DNA strand. The modified DNA polymerase and four kinds of fluorescence-labeled dNTP were added while each cycle used only a synthetic base. The polymerization nucleotide species in each template was obtained by scanning the plate reaction surface with a laser. The chemical cutting of the "fluorescent groups" and "end groups" was carried out, and the viscosity ending of 3′ was restored. Meanwhile, the second nucleotide was aggregated and the results of fluorescent signal in each round were calculated and collected, while the template DNA sequence was obtained.

2.2.3. Sequencing Data Processing. The overlap relationship was used to obtain the PE reads while carrying out quality control and filtering the quality of the sequence at the same time. The OTU taxonomists' analysis, cluster analysis, and diversity index analysis were carried out after distinguishing the samples. Meanwhile, we conducted various diversity index analyses. The detection of sequencing depth was also conducted on the basis of OTU clustering analysis. The community structure analysis in each classification level was conducted by the taxonomy information. On the basis of the analysis above, the study was carried out on a serial analysis of community structure, system development, and visualization.

According to the similarity levels, all the sequences were taken using OTU division. Meanwhile, by dint of the OTU biological information, under a 97% similarity level, the statistical analysis was carried out. Analysis steps were as follows: extracting the nonrepeated sequence from an optimized sequence to reduce the redundant computation in the analysis course (http://drive5.com/usearch/manual/dereplication.html); removing the nonrepeated single sequence (http://drive5.com/usearch/manual/singletons.html); and conducting OTU cluster on nonrepeated sequences and removing the chimera in the process of clustering while obtaining a representative sequence of OTU under a similarity of 97%. All the optimized sequences were mapped to the representative OTU sequence, and the sequences which own the similarity level by more than 97% were selected while generating the OTU form.

To acquire the information of the corresponding species classification for each OTU, the RDP classifier Bayesian algorithm analyzed representative sequences of OTU under the 97% similarity level and calculated the community composition in each classification level. The databases were as follows: Silva (release 119 http://www.arb-silva.de) and RDP (release with the 11.1 http://rdp.cme.msu.edu/). The rarefaction curve was obtained from the sequencing depth of the sample. Rarefaction analysis was conducted with 97% similarity OTU, using mothur and R language tools to make a graph. Bacterial diversity indices were as follows: Chao—the Chao1 estimator (http://www.mothur.org/wiki/Chao); Ace—the ACE estimator (http://www.mothur.org/wiki/Ace); Shannon—the Shannon index (http://www.mothur.org/wiki/Shannon); Simpson—the Simpson index (http://www.mothur.org/wiki/Simpson); and the index of the sequencing depth coverage—the Good's coverage (http://www.mothur.org/wiki/Coverage). According to the analysis of beta diversity for a hierarchical clustering distance matrix, the group used the method of UPGMA (unweighted pair group method with arithmetic mean) to construct a tree structure.

2.3. Statistical Analysis. Statistical analysis was carried out with SPSS 19.0 software and Origin 8.0.

Table 1: The soil physical and chemical properties.

Sample ID	pH	Moi (%)	TOC (%)	Cu (mg/kg)	Zn (mg/kg)	Cr (mg/kg)	TPH (mg/kg)
Number 1	8.55	26.6	0.05	47.93	93.81	111.46	15.2
Number 2	8.44	21.1	0.41	76.60	131.63	74.55	<5
Number 3	8.11	14.0	0.22	12.20	15.68	34.07	<5

3. Results

3.1. Physical and Chemical Characteristics. The physical and chemical properties of the soil were summarized in Table 1. Results demonstrated that the contaminated soils were slightly alkaline and generally had a higher pH value than the corresponding reference soils. According to the soil and environmental quality standard, the heavy metal contents including copper, zinc, and chromium in aging oil sludge-contaminated soils were not fit for planting. Meanwhile, the heavy metal content could cause serious damage and pollution to the soil. Similarly, the contaminated soil showed an elevated level of organic matter (organic carbon) with a range of 0.05%–0.41%. Compared with the reference value, the content of total organic carbon was moderate. Moreover, the results of TPH demonstrated that petroleum hydrocarbons were detected in the soil which was closest to the aging oil sludge.

3.2. MiSeq-Pyrosequencing Results and Microbial Structures. 16S rRNA sequencing had provided a detailed view on the composition. The analysis of the single sample diversity (alpha diversity) could reflect the richness and the diversity of the microbial community. The study aimed at analyzing the bacterial diversity of the soil contaminated with the aging oil sludge. The soil sample obtained from 20 cm below the earth of the aging oil sludge was marked as number 1. Sample number 2 was fetched below the aging oil sludge directly. Sample number 3 was the plain soil more than 100 m distant from the aging oil sludge. Meanwhile, number 3 was used as the corresponding reference soil. The sequence information and microbial diversity index of the samples are listed in Table 2. OTU numbers of the three samples were 1452, 1280, and 747, respectively. Among the entire diversity index, Ace estimated the OTU number in the community, while Chao estimated the OTU number in the soil sample. In other words, Ace and Chao indicated the community richness. In addition, the value of Shannon estimated the microbial diversity, positively associated with microbial diversity. On the contrary, Simpson was used to estimate microbial diversity, the value of which was negatively correlated with microbial diversity. Meanwhile, coverage was the probability of the measured sample sequence.

Index results showed that the numbers of Ace, Chao, Shannon, and Simpson had no significant differences between sample numbers 1 and 2. It was clear that the Ace, Chao, and Shannon were higher in the aging oil sludge-contaminated soil than in the corresponding reference soil, while the Simpson was lower than that of the corresponding reference soil. It was concluded that soil samples under the aging oil sludge showed the highest content on bacterial diversity. Similarly, bacterial diversity was lower in soil which was directly under the aging oil sludge. The numerical value also showed that sample number 3, obtained at a location furthest away from the aging oil sludge, showed the lowest bacterial diversity. It was obvious that samples from the furthest distance to the aging oil sludge presented the lowest microbial diversity. Moreover, the alpha diversity also indicated that the aging oil sludge inhibited microbial diversity to a certain extent, as we found out from the soil 0 cm under the aging oil sludge, while we could also see that the aging oil sludge might produce an increase of bacterial diversity from the soil 20 cm under the aging oil sludge. This explains why different distances from the aging oil sludge leads to soil microbial populations with different microbial diversity.

Table 2: Different bacterial diversity indices in different samples.

Sample ID	Ace	Chao	Shannon	Simpson	Coverage
Number 1	1577	1563	5.52	0.0185	0.993
Number 2	1348	1349	5.21	0.0237	0.996
Number 3	760	757	3.43	0.1780	0.999

A rarefaction curve was built with the numbers of selected individuals from a sample with certain quantity and species numbers counted from the individuals represented. The rarefaction curve was used to indicate whether the sequencing quantity was enough. It was commonly used to compare the sequencing data volume of species richness in different samples and to indicate whether the quantity of sequencing data were reasonable. A smooth curve meant reasonable sequencing data, and much more data only produced a little new OTU. As illustrated in Figure 1(a), the result in the study demonstrated that all the aging oil sludge-contaminated soil had a similar pattern, which was different from the corresponding reference soil. Within a certain range, the curves with a sharp-rise trend indicate that a lot of species had been found in the community. A flattened curve meant that the species would not significantly increase with the increase of sample size in the environment. Meanwhile, the result also showed that the sequencing number was easier to reach in the corresponding reference soil. The study similarly illustrated that the corresponding reference soil had the lowest richness, and a little sequencing number could also perfectly reflect bacterial diversity in the samples.

Venn analysis was used for counting the number of common and unique OTU in different samples with the similar level of 97%. Figure 1(b) shows that the OTU numbers were 1452, 1280, and 747 in the three samples. The proportion of unique OTU was 10.5% in the number 1 soil and 9.3% in the number 2 soil. Moreover, the

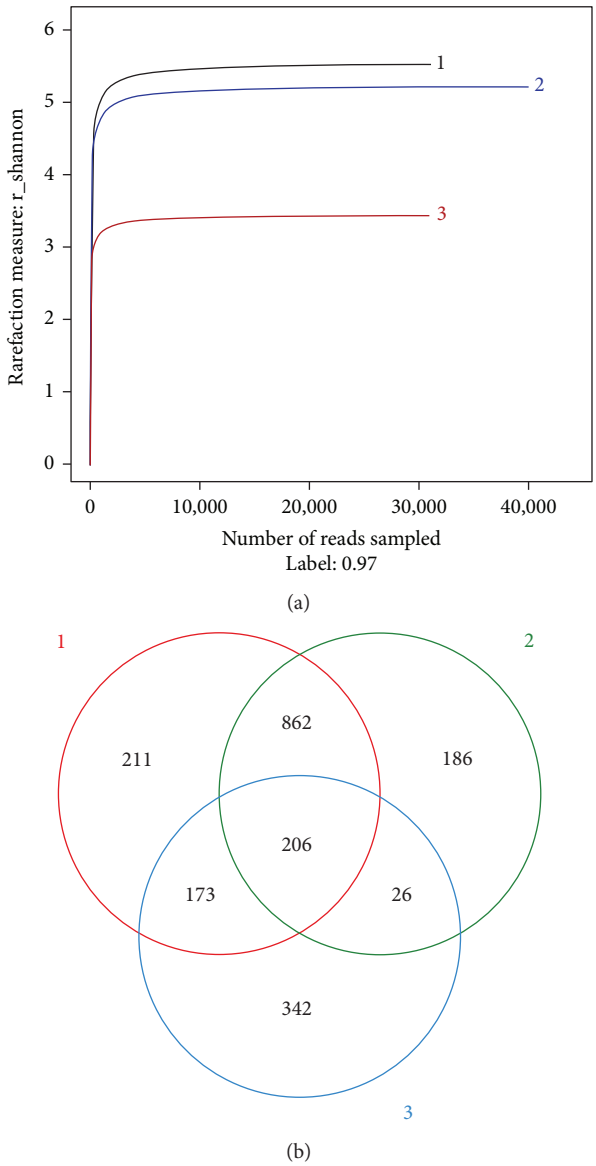

Figure 1: Rarefaction curves and OTU Venn analysis in different samples.

proportion of unique OTU was 17% in the corresponding reference soil. In addition, the common OTU in aging oil sludge-contaminated soil was 53.2%, while the proportion of the common OTU was 10.3% in three samples.

3.3. Taxonomic Complexity of Bacterial Community. The richness of the bacterial community in different levels was illustrated in Figure 2. As shown in Figure 2(a), the dominant phyla in aging oil sludge-contaminated soil were mainly *Proteobacteria*, *Chloroflexi*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, and *Firmicute*, with a higher content in samples under the aging oil sludge. *Proteobacteria*, *Firmicute*, *Actinobacteria*, *Cyanobacteria*, and *Bacteroidetes* were the main bacteria in the corresponding reference soil. *Proteobacteria* was the highest phylum in the aging oil sludge-contaminated soil, with the relative content of 40.30% and 57.60% in aging oil sludge-contaminated soil, respectively, while it was 8.89% in the corresponding reference soil. Moreover, the highest phylum in the corresponding reference soil was *Firmicute*, with a relative content of 63.81%; however, it was reduced sharply in the aging oil sludge-contaminated soil. In addition, *Chloroflexi* was 12.05% and 8.70% in soil sample number 1 and number 2, respectively, while the content was 0.56% in the corresponding reference soil. The change trend of *Acidobacteria* is the same with *Chloroflexi* in the three samples. In general, these results reflected the differences and relationships of phylum diversity in the three samples. The richness of the bacterial community at the family level was illustrated in Figure 2(b). From the picture, we could see that *Pseudomonadace*, *Anaerolineaceae*, *Oceanospirillaceae*, *Flavobacteriaceae*, and *Pseudomonadaceae* are the main families in the aging oil sludge-contaminated soil. The contents of *Streptococcacea* were 9.16% and 1.34% respectively in soil 20 cm and 0 cm below the earth of the aging oil sludge, which indicated that the aging oil sludge might have different effects on bacterial diversity. *Streptococcaceae*, *SubsectionI_Family I*, *Lactobacillaceae*, and *Halomonadaceae* were the dominant species in the corresponding reference soil. *Oceanospirillaceae* showed a higher content in the aging oil sludge-contaminated soil but there was no *Oceanospirillaceae* in the corresponding reference soil. Similarly, *Streptococcaceae* was the highest family in the reference soil and the percentage of *Streptococcaceae* was 52.35%. The level of genus bacterial diversity (Figure 2(c)) reflected that *Pseudomonas*, *Anaerolineaceae_uncultured*, *Marinobacterium*, and *Sphingorhabdus zeaxanthinibacter* were the dominant genera in the aging oil sludge-contaminated soil. It was obvious that *Lactococcus* and *Synechococcus* are the main genera in the reference soil. The content of *Lactococcus* was 50.32% in the corresponding reference soil and 8.80% and 1.29% in soil 20 cm and 0 cm below the earth of the aging oil sludge. The results demonstrated that the percentage of *Zeaxanthinibacter* was 4.56% and 2.28% in the aging oil sludge-contaminated soil, but there was none in the corresponding reference soil. In addition, the clustering analysis method was used in the study and the branch length represented the distance in different samples. The short range of branches meant that the species composition of the sample was much more similar. Similarly, the branch structure was used to describe and compare similarities and differences between multiple samples. As illustrated in Figure 3, the aging oil sludge-contaminated soil was classified together and the corresponding reference soil was classified separately.

High-richness and low-richness species were partitioned and gathered together with a heat map. It reflected the similarities as well as the differences of the sample community composition at the phylum classification level in virtue of the color gradient and similar degree. Figure 4 shows the bacterial community analyzed under the level of phylum. The number of phyla which hardly existed, respectively, was 4, 7, and 13 among all the soil samples, respectively. The three samples, having no phylum, were 0. Some communities, such as *Omnitrophica*, *Marinimicrobia*, and *Bacteroidetes*, were few in all the samples. After our analysis and statistics, *Proteobacteria*, *Firmicutes*, *Chloroflexi*,

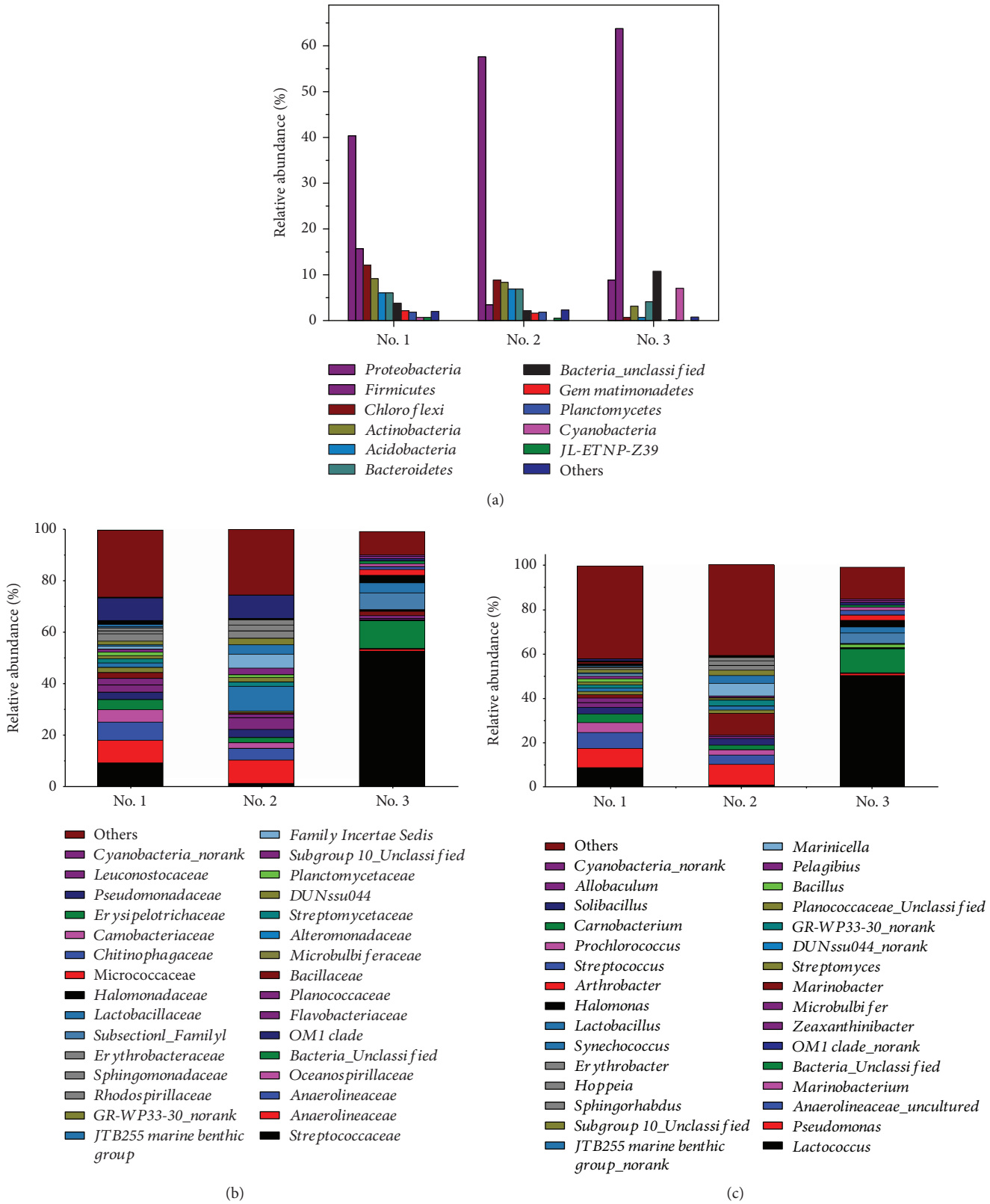

Figure 2: The bacterial histogram of different samples.

Actinobacteria, *Acidobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, and *Planctomycetes* were high (>1%) in 20 cm soil contaminated with the aging oil sludge. Furthermore, the contents of *Proteobacteria*, *Chloroflexi*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Firmicutes*, *Gemmatimonadetes*, and *Planctomycetes* were all over 1% in the 0 cm soil

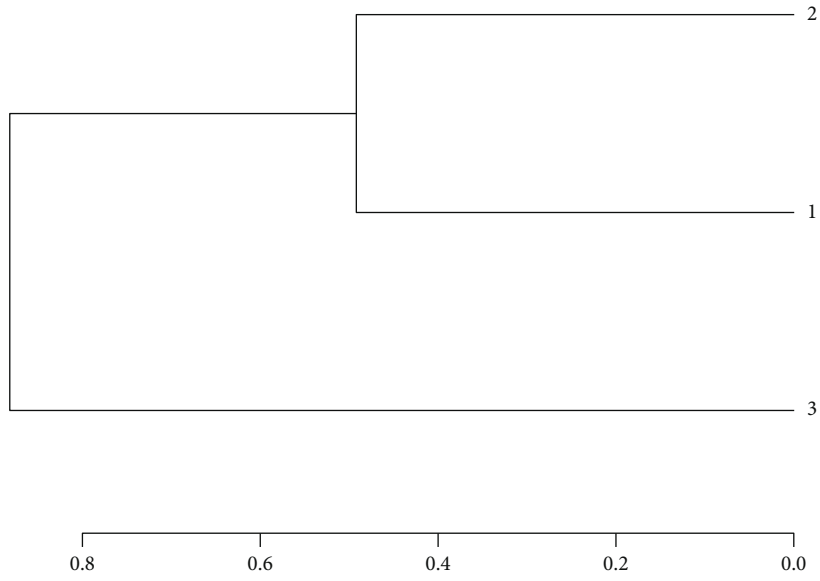

Figure 3: Multiple sample similarity tree.

contaminated with the aging oil sludge. *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Cyanobacteria* were more than 1% in the corresponding reference soil. The content of *Chloroflexi*, *Acidobacteria*, *Gemmatimonadetes*, and *Planctomycetes* in the reference soil was lower compared with the aging oil sludge-contaminated soil. In addition, the content of *Proteobacteria* in the aging oil sludge-contaminated soil was high while the content reduced sharply in the corresponding reference soil. Inversely, the content of *Firmicute* plummeted in the aging oil sludge-contaminated soil, but it was the highest phylum in the corresponding reference soil.

4. Discussion

The community structure of soil microorganisms was always related to the soil physical and chemical properties. He et al. showed that soil properties could significantly change the richness, composition, and structure of microbial species, which might improve and modify ecosystem function [22, 23]. The results of physical and chemical properties showed that the soil was alkaline. pH must be taken into account for its effect in the growth of bacteria. A decreasing pH and increased metal contamination showed a negative effect on bacterial growth [24]. It may be for this reason that sample 1 has the largest bacterial diversity. Moisture could affect microbial activity, transcription, and composition [25]. The moisture contents in sample 1 (26.6%) and sample 2 (21.1%) were generally higher compared with sample 3 (14.0%) which was inconsistent with previous studies [9, 10, 26]. It is probably the hydrophobic crusts formed by the heavy oil components in AOS which limited the evaporation of water and the water/air exchange of soil [27]. The results of the soil's physical and chemical properties indicated that the aging oil sludge had little effect on the receiving soils. In general, the high pH indicated that the aging oil sludge-contaminated soil was not suitable for planting; the soil should be cultivated with alkali plants. It could not only improve soil pH, but could also maintain soil moisture. In addition, this method could also improve soil physical and chemical properties and make the soil suited for farming.

Bacterial richness and diversity were usually considered as biological indicators of the origin for soil aggregates [28]. MiSeq sequencing revealed significant differences in the microbial taxonomic composition between the contaminated soil and corresponding reference soil. In total, 9 phyla were identified and *Frimicute* and *Proteobacteria* were widely recognized as the predominant phyla in the soil. In the background soil sample, *Frimicute* was the dominant phylum. While in the contaminated soil samples, *Frimicute* decreased sharply and *Proteobacteria* and *Chloroflexi* became the dominant phyla. In other words, *Firmicute* was sensitive to the aging oil sludge. Without the influence of the aging oil sludge, the richness of *Firmicute* decreased dramatically. The aging oil sludge had no adverse effects on *Proteobacteria* and *Chloroflexi*, which increased the relative richness of the two phyla. Namely, the two phyla might be the functional bacterium for oil degradation.

Further analysis was made to present the richness and diversity of the bacterial community at the family level. *Streptococcaceae* was the dominant bacteria in the corresponding reference soil and maintained a little content in the aging oil sludge-contaminated soil. Besides, *Pseudomonadaceae* showed a high content in the aging oil sludge-contaminated soil, while it decreased dramatically in the corresponding reference soil. The number was 20 in the aging oil sludge-contaminated soil. The community richness of *Streptococcaceae* decreased significantly in the three soils. However, the richness of *Pseudomonas* and *Alteromonadaceae* increased, which meant that *Streptococcaceae* was sensitive to the aging oil sludge. Moreover, the richness of *Streptococcaceae* reduced rapidly in the aging oil sludge-contaminated soil, while the relative abundance of

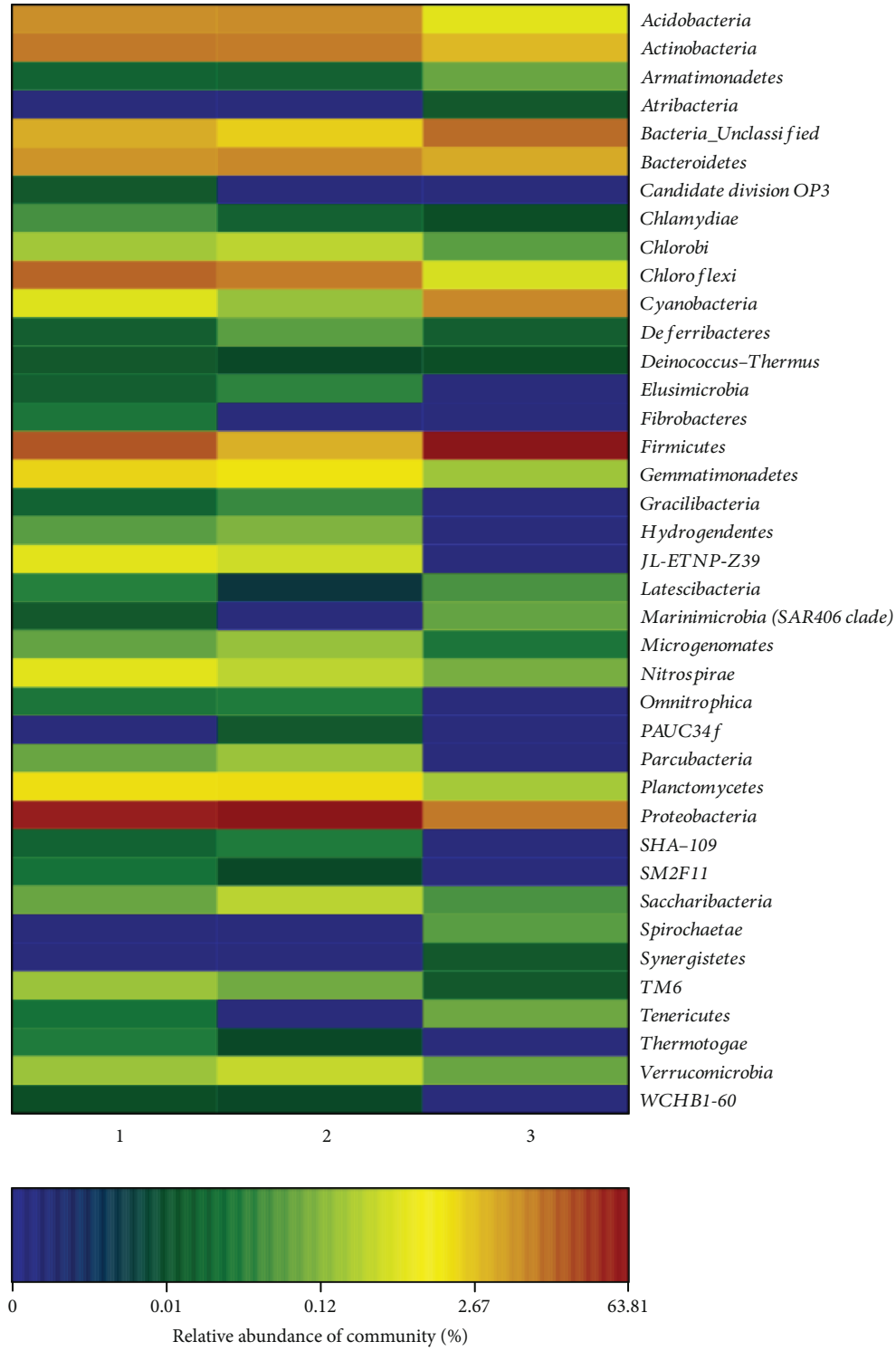

Figure 4: Bacterial community heat map analysis.

Pseudomonas and *Alteromonadaceae* increased. Analysis results indicated that *Pseudomonas* and *Alteromonadaceae* have better resistance in the aging oil sludge. The shrinking of *Streptococcaceae* provided greater living space to *Pseudomonas* and *Alteromonadaceae*. Moreover, these two strains of microorganisms existed to degrade the contaminant in the aging oil sludge. Previous studies repeatedly demonstrated that *Acinetobacter* and *Pseudomonas* could degrade toxic organic compounds [29–31], which was of great significance to the microbial remediation of the aging oil sludge.

Under the level of genus, *Lactococcus* was the dominant bacteria in the corresponding reference soil and

Pseudomonas showed a high content in the aging oil sludge-contaminated soil. The study showed that bacterial diversity was richer in the aging oil sludge-contaminated soil, from which we could choose bacteria to resist the aging oil sludge. Bacteria which resisted the aging oil sludge could lay a foundation for bioremediation in the aging oil sludge-contaminated soil.

Soil used in the study was obtained from the Shengli Oil Field in China, which had a long history of contamination with petroleum hydrocarbons [32]. High-throughput sequencing had enabled in-depth exploration of microbial diversity in the environment. In addition, the Ace and Chao were the indicators which indicated microbial richness, while the Shannon-Weaver and Simpson indices reflected microbial diversity [33]. The bacterial diversity index showed that the aging oil sludge significantly affected the microbial diversity. The contrast analysis of all the soil samples demonstrated that microbial species were richer in the aging oil sludge-contaminated soil. As a result, the soil at a 20 cm vertical distance under the aging oil sludge showed higher community diversity than the surface of the earth and the corresponding reference soil. Different soils contaminated with different degrees of the aging oil sludge indicated that the aging oil sludge not only could promote bacteria microbial diversity but could inhibit bacteria microbial diversity. The study also found that the aging oil sludge could significantly influence bacterial diversity among different pollution degrees. Moreover, we also selected the dominant microorganisms in soil contaminated with the aging oil sludge. In a subsequent study, the researchers planned to use the microorganisms which resisted the aging oil sludge to restore contaminated soil and especially decrease the content of petroleum hydrocarbons.

5. Conclusions

The study investigated soil bacterial diversity around an aging oil sludge and analyzed the community structure and richness of bacteria. The research results showed that the aging oil sludge could significantly affect the growth of soil bacteria and inhibit the growth of bacteria. Under different pollution degrees of the aging oil sludge, the results showed different bacterial diversity. At the phylum level, the *Proteobacteria*, *Chloroflexi*, and *Actinobacteria* existing in the aging oil sludge-contaminated wetland soil constituted a larger proportion of the community, while the proportion of *Firmicute* was relatively less. On the contrary, *Firmicute* showed the highest content of 63.8% in the referenced soil. Under the genus and family levels, the corresponding strains that resisted the aging oil sludge were selected. According to the bacterial diversity analysis, the resistance bacteria laid a foundation for the subsequent soil bioremediation.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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References

[1] C. Zhang, S. Nie, J. Liang et al., "Effects of heavy metals and soil physicochemical properties on wetland soil microbial biomass and bacterial community structure," *Science of the Total Environment*, vol. 557-558, pp. 785–790, 2016.

[2] Q. Helmy, R. Laksmono, and E. Kardena, "Bioremediation of aged petroleum oil contaminated soil: from laboratory scale to full scale application," *Procedia Chemistry*, vol. 14, pp. 326–333, 2015.

[3] Y. C. Gao, J. N. Wang, S. H. Guo et al., "Effects of salinization and crude oil contamination on soil bacterial community structure in the Yellow River Delta region, China," *Applied Soil Ecology*, vol. 86, pp. 165–173, 2015.

[4] L. Cao, G. Shen, and Y. Lu, "Combined effects of heavy metal and polycyclic aromatic hydrocarbon on soil microorganism communities," *Environmental Geology*, vol. 54, no. 7, pp. 1531–1536, 2008.

[5] S. Kuang, Z. Wu, and L. Zhao, "Accumulation and risk assessment of polycyclic aromatic hydrocarbons (PAHs) in soils around oil sludge in Zhongyuan oil field, China," *Environmental Earth Sciences*, vol. 64, no. 5, pp. 1353–1362, 2011.

[6] E. Winquist, K. Björklöf, E. Schultz et al., "Bioremediation of PAH-contaminated soil with fungi—from laboratory to field scale," *International Biodeterioration & Biodegradation*, vol. 86, pp. 238–247, 2014.

[7] Z.-F. Zhou, M. X. Wang, X. H. Zuo, and Y. H. Yao, "Comparative investigation of bacterial, fungal, and archaeal community structures in soils in a typical oilfield in Jianghan, China," *Archives of Environmental Contamination and Toxicology*, vol. 72, no. 1, pp. 65–77, 2017.

[8] S. J. Wang, X. Wang, G. L. Lu, Q. H. Wang, F. S. Li, and G. L. Guo, "Bioremediation of petroleum hydrocarbon-contaminated soils by cold-adapted microorganisms: research advance," *Chinese Journal of Applied Ecology*, vol. 22, no. 4, pp. 1082–1088, 2011.

[9] X. Wang, C. Zhang, S. J. Wang, Q. H. Wang, F. S. Li, and G. L. Guo, "Dynamic properties of microbial communities during the treatment of aged oil sludge by field-scale biopiles," *Research of Environmental Sciences*, vol. 25, pp. 998–1004, 2012.

[10] X. Wang, Q. Wang, S. Wang, F. Li, and G. Guo, "Effect of biostimulation on community level physiological profiles of microorganisms in field-scale biopiles composed of aged oil sludge," *Bioresource Technology*, vol. 111, pp. 308–315, 2012.

[11] R. A. Tahhan and R. Y. Abu-Ateih, "Biodegradation of petroleum industry oily-sludge using Jordanian oil refinery contaminated soil," *International Biodeterioration & Biodegradation*, vol. 63, no. 8, pp. 1054–1060, 2009.

[12] G. D. Sun, Y. Xu, J. H. Jin et al., "Pilot scale ex-situ bioremediation of heavily PAHs-contaminated soil by indigenous microorganisms and bioaugmentation by a PAHs-degrading and bioemulsifier-producing strain," *Journal of Hazardous Materials*, vol. 233-234, pp. 72–78, 2012.

[13] K. Tago, H. Itoh, Y. Kikuchi et al., "A fine-scale phylogenetic analysis of free-living *Burkholderia* species in sugarcane field soil," *Microbes and Environments*, vol. 29, no. 4, pp. 434–437, 2014.

[14] P. A. Schmidt, M. Balint, B. Greshake, C. Bandow, J. Rombke, and I. Schmitt, "Illumina meta-barcoding of a soil fungal community," *Soil Biology and Biochemistry*, vol. 65, pp. 128–132, 2013.

[15] P. Czechowski, L. J. Clarke, J. Breen, A. Cooper, and M. I. Stevens, "Antarctic eukaryotic soil diversity of the Prince Charles Mountains revealed by high-throughput sequencing," *Soil Biology and Biochemistry*, vol. 95, pp. 112–121, 2016.

[16] L. Yuan, A. Qi, Y. Cheng, G. Sagen, Y. Qu, and B. Liu, "Fecal microbiota of three Bactrian camels (*Camelus ferus* and *Camelus bactrianus*) in China by high throughput sequencing of the V3-V4 region of the 16S rRNA gene," *Journal of Arid Land*, vol. 9, no. 1, pp. 153–159, 2017.

[17] M. Naveed, L. Herath, P. Moldrup et al., "Spatial variability of microbial richness and diversity and relationships with soil organic carbon, texture and structure across an agricultural field," *Applied Soil Ecology*, vol. 103, pp. 44–55, 2016.

[18] J. Li, Y. T. Li, X. D. Yang, J. J. Zhang, Z. A. Lin, and B. Q. Zhao, "Microbial community structure and functional metabolic diversity are associated with organic carbon availability in an agricultural soil," *Journal of Integrative Agriculture*, vol. 14, no. 12, pp. 2500–2511, 2015.

[19] Z.-Y. Wang, Y.-Z. Xin, D.-M. Gao, F.-M. Li, J. Morgan, and B.-S. Xing, "Microbial community characteristics in a degraded wetland of the Yellow River Delta," *Pedosphere*, vol. 20, no. 4, pp. 466–478, 2010.

[20] W. Sun, E. Xiao, Y. Dong et al., "Profiling microbial community in a watershed heavily contaminated by an active antimony (Sb) mine in Southwest China," *Science of the Total Environment*, vol. 550, pp. 297–308, 2016.

[21] Z. He, Y. Piceno, Y. Deng et al., "The phylogenetic composition and structure of soil microbial communities shifts in response to elevated carbon dioxide," *The ISME Journal*, vol. 6, no. 2, pp. 259–272, 2012.

[22] S. Banerjee, B. Helgason, L. Wang, T. Winsley, B. C. Ferrari, and S. D. Siciliano, "Legacy effects of soil moisture on microbial community structure and N_2O emissions," *Soil Biology and Biochemistry*, vol. 95, pp. 40–50, 2016.

[23] Z. He, M. Xu, Y. Deng et al., "Metagenomic analysis reveals a marked divergence in the structure of belowground microbial communities at elevated CO_2," *Ecology Letters*, vol. 13, no. 5, pp. 564–575, 2010.

[24] W. Zhang, L. Chen, R. Zhang, and K. Lin, "High throughput sequencing analysis of the joint effects of BDE209-Pb on soil bacterial community structure," *Journal of Hazardous Materials*, vol. 301, pp. 1–7, 2016.

[25] N. Gartzia-Bengoetxea, E. Kandeler, I. Martínez de Arano, and A. Arias-González, "Soil microbial functional activity is governed by a combination of tree species composition and soil properties in temperate forests," *Applied Soil Ecology*, vol. 100, pp. 57–64, 2016.

[26] G. Hu, J. Li, and G. Zeng, "Recent development in the treatment of oily sludge from petroleum industry: a review," *Journal of Hazardous Materials*, vol. 261, pp. 470–490, 2013.

[27] J. Tang, X. Lu, Q. Sun, and W. Zhu, "Aging effect of petroleum hydrocarbons in soil under different attenuation conditions," *Agriculture, Ecosystems & Environment*, vol. 149, pp. 109–117, 2012.

[28] S. Zhang, X. Xia, N. Xia, S. Wu, F. Gao, and W. Zhou, "Identification and biodegradation efficiency of a newly isolated 2, 2′,4, 4′-tetrabromodiphenyl ether (BDE-47) aerobic degrading bacterial strain," *International Biodeterioration & Biodegradation*, vol. 76, pp. 24–31, 2013.

[29] J. A. Field and R. Sierra-Alvarez, "Microbial transformation and degradation of polychlorinated biphenyls," *Environmental Pollution*, vol. 155, no. 1, pp. 1–12, 2008.

[30] K. Taira, N. Hayase, N. Arimura, S. Yamashita, T. Miyazaki, and K. Furukawa, "Cloning and nucleotide sequence of the 2,3-dihydroxybiphenyl dioxygenase gene from the PCB-degrading strain of *Pseudomonas paucimobilis* Q1," *Biochemistry*, vol. 27, no. 11, pp. 3990–3996, 1988.

[31] R. Mazzoli, E. Pessione, M. G. Giuffrida et al., "Degradation of aromatic compounds by *Acinetobacter radioresistens* S13: growth characteristics on single substrates and mixtures," *Archives of Microbiology*, vol. 188, no. 1, pp. 55–68, 2007.

[32] G.-Z. Wu, F. Coulon, Y. W. Yang, H. Li, and H. Sui, "Combining solvent extraction and bioremediation for removing weathered petroleum from contaminated soil," *Pedosphere*, vol. 23, no. 4, pp. 455–463, 2013.

[33] D. Zhu, S.-H. Tanabe, C. Yang, W. Zhang, and J. Sun, "Bacterial community composition of South China Sea sediments through pyrosequencing-based analysis of 16S rRNA genes," *PLoS One*, vol. 8, no. 10, article e78501, 2013.

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Research Article

Enhancement of Organic Matter Removal in an Integrated Biofilm-Membrane Bioreactor Treating High-Salinity Wastewater

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High salinity can strongly inhibit microbial activity and decrease the sedimentation ability of activated sludge. The combination of biofilm and membrane bioreactor is a practical approach towards effective removal of pollutants and low fouling rate. An integrated biofilm-membrane bioreactor (BMBR) treating mustard tuber wastewater was investigated. An average COD removal efficiency of 94.81% and ammonium removal efficiency of 96.84% were achieved at an organic load of 0.5 kg COD/(m^3·d). However, the reactor showed a relatively low efficiency in total nitrogen and soluble phosphorus removal due to the lack of anaerobic environment. The increase of influent organic load resulted in a performance degradation because a balance between the degradation ability and pollution has been reached. Images of scanning electron microscopy revealed that halophilic bacteria were the dominant microbe in the system that leads to a loose sludge structure and declined settling properties. It was found that membrane fouling was the consequence of the interaction of microbial activities and NaCl crystallization.

1. Introduction

There are a number of mustard tuber pickling plants in the Three Gorges reservoir watershed, which is one of the most important fresh water resources in China. These pickling plants play an important role in the local economic development. However, serious environmental pollution has emerged due to illegal discharge of the mustard tuber wastewater, which is characterized by high salinity, high nitrogen and phosphorus level, and high organic load. Direct discharge of this type of wastewater has a detrimental impact on the ecosystem, e.g., eutrophication, dehydration and death of biological cells, and changes in biodiversity [1].

Currently, treatment of high-salinity wastewater mainly includes two methods: biological treatment and physicochemical treatment. Compared with biological treatment, the physicochemical method cannot effectively remove dissolved organic matter and requires a high level of pretreatment [2]. The operational cost is so high that the wide application of the physicochemical method is prohibited. Therefore, further researches were undertaken to seek for an efficient biological process to treat high-salinity wastewater [3, 4].

It has been reported that high salinity can strongly inhibit microbial activity [5] and decrease the sedimentation ability of activated sludge [6]. Hence, it challenges the system stability and results in a low treatment efficiency. In order to overcome these difficulties, application of membrane biological reactor (MBR) to treat high-salinity wastewater has been investigated. With the advantage of membrane filtration, MBR allows more biomass to be maintained in the reactor and could achieve a complete separation of hydraulic retention time and sludge retention time [7]. Gaetano et al. [8] reported that membrane bioreactor showed high removal efficiencies under the condition of normal salinity. However, the increase of salinity significantly promoted the soluble microbial products leading to membrane fouling. Other studies showed similar results [9–11]. Membrane fouling still represents one of the major drawbacks for MBRs [12, 13]. This problem is further aggravated when they are used to treat high-salinity wastewater because microbial community characteristics play an important role in biofouling [14].

The moving bed biofilm reactor-membrane bioreactor (MBBR-MBR), proposed by Leiknes and Ødegaard [15], has been considered to be an effective biological process to mitigate the biofouling in MBR systems. Biofilm can immobilize microbes and increase the biomass concentration while the membrane separates the suspended solids and sludge. Comparative studies of the performance between MBBR-MBR and MBR have been conducted. It has been proved that the degree of membrane fouling for MBBR-MBR was far lower than that for MBR [16, 17]. Daniele et al. [18] tested the impacts of salinity on the performance of MBBR-MBR. Results indicated that the gradual salinity increase helped the acclimation of biomass, but biofilm detachments from carriers led to the irreversible cake deposition. To our knowledge, there are few studies using MBBR-MBR to treat wastewater containing high-concentration salinity, organic matter, and nutrient. The mechanism of biofouling in MBBR-MBR when treating such wastewater is still unclear.

In this context, an integrated biofilm-membrane biological reactor (BMBR) was established to treat mustard tuber wastewater in our study. The objective of the study includes (i) evaluate the performance of BMBR treating high-salinity wastewater; (ii) investigate the effect of organic load on the removal efficiency of BMBR; and (iii) explore the role of halophilic bacteria in membrane fouling.

2. Materials and Methods

2.1. Reactor Set-Up and Operation. The BMBR used in the study was made of steel plates with dimensions of 1.08 m × 0.75 m × 0.6 m resulting in a working volume of 400 L. The reactor was divided into a biofilm zone and a membrane zone by a baffle (Figure 1). Semisoft media were assembled in the biofilm zone with a density of 30%. Membrane zone was equipped with hollow fiber membrane module, controlled by a special valve. The influent fully contacted with semisoft media in the upward flow, then overflow into the membrane area. Perforated aeration pipes with a diameter of 20 mm were installed at the bottom of the reactor. The perforated pipes were connected with an air pump, through which the air was aerated into the wastewater. During the experiments, the reactor was operated in continuous influent-intermittent effluent way. Effluent from a mustard WWTP was collected in the regulating tank and then pumped into the reactor. The raw water consisted of 2~3% salinity, 770~1240 mg/L COD, 103~191 mg/L NH_4^+-N, 207~409 mg/L TN, 21~48 mg/L phosphate, and 237~525 mg/L SS. The drainage pump worked in an intermittent mode and controlled by a PLC automatic system. The operating cycle of drainage pump was set to be 13 min in total including a 10 min uptake time and a 3 min off time. The membrane flux was measured by a liquid flowmeter. The pressure difference between inside and outside membrane was measured by a negative pressure meter.

The BMBR was firstly inoculated with the sludge from aerobic reactor in the Fuling WWTP and keep the mixed liquor suspended solids (MLSS) above 5 g/L. The reactor was operated continuously under different organic load. The operation of BMBR can be divided into three periods

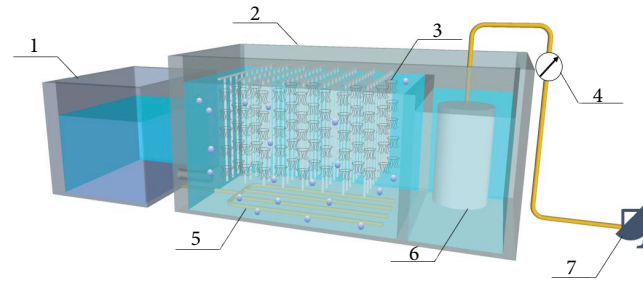


Figure 1: Schematic of the biofilm-membrane bioreactor. 1: regulating tank; 2: biofilm-membrane bioreactor; 3: biofilm carrier; 4: pressure meter; 5: perforated aeration pipes; 6: membrane module; 7: drainage pump.

with a corresponding organic load of 0.5 kg COD/(m^3·d), 1.0 kg COD/(m^3·d), and 1.5 kg COD/(m^3·d). During a 110 days operation time period, the aeration intensity was kept at 0.8m^3/h, and a transmembrane pressure difference (TMP) was remained at 15Kpa. Membranes were cleaned chemically or physically in a way described below. The operating condition and organic load are summarized in Table 1.

2.2. Analytical Methods. Samples of influent and effluent were collected from the reactor and analyzed immediately. The following parameters including chemical oxygen demand (COD), suspended solid (SS), ammonium, total nitrogen, and dissolved phosphate were measured according to APHA Standard Methods. DO and pH were measured by a DO detector (HACH, HQ30d, USA) and a pH detector (HACH, sension2, USA), respectively.

2.3. Membrane Fouling Analysis and Cleaning. The membrane module was firstly taken out of the reactor and then was scrubbed softly with a sponge under tap water. Physical cleaning was performed to restore the membrane flux by removing the cake layer from the membrane surface. After that, chemical cleaning was carried out to further improve the membrane flux. The membrane module was soaked in NaClO solution (0.5%, m/m) for 24 h and then soaked in tap water for 2 h.

The attachment of membrane was determined by scanning electron microscopy (SEM; Hitachi S-3400N, Hitachinaka, Japan) to get an additional visual insight into the deposition on the surface of membrane.

3. Results and Discussion

3.1. Reactor Performance

3.1.1. COD Removal Efficiency. The COD of influent and effluent over the 110-day operation time period is shown in Figure 2. In stage I, the average COD removal efficiency was greater than 94% with an average COD value of 48.18 mg/L. In stage II, when the influent organic load was 1.0 kg COD/(m^3·d), the average COD removal rate decreased from 94.81% to 89.35%. In stage III, with the increase of organic load, the average COD removal rate furtherly decrease to 84.90% with average COD of 155.46 mg/L. The existence of a short adaptation period in the beginning of

Table 1: Summary of the tested schemes.

Operating condition	Organic load (COD/(m^3·d))	Average influent concentration (mg/L)				
		COD	NH_4^+	TN	PO_4^{3-}	SS
Stage I	0.5	962.44	137.51	284.23	34.92	402.26
Stage II	1.0	959.97	142.92	317.98	30.64	355.90
Stage III	1.5	1054.29	133.64	293.64	33.58	428.46

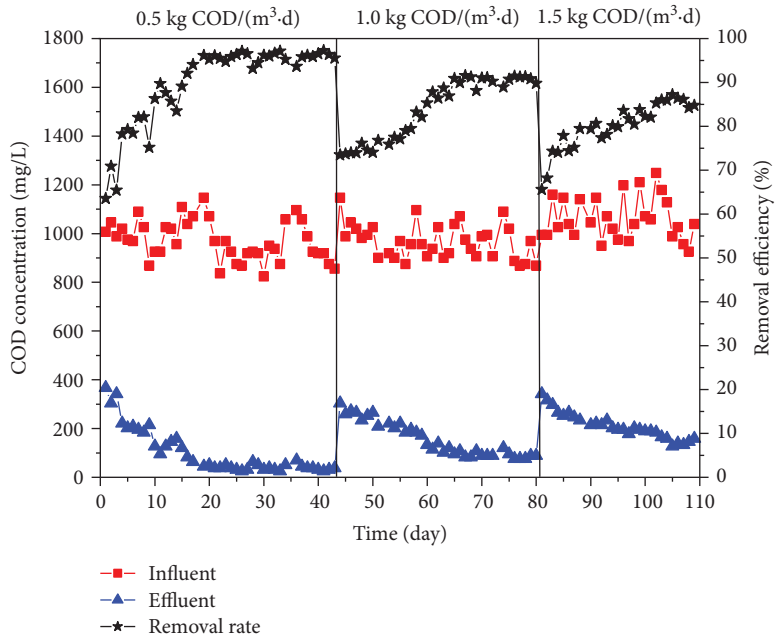


Figure 2: COD concentration variations in influent and effluent.

each stage was observed, indicating that the high salinity and organic load had a negative impact on microbes' growth [19]. Due to the application of biofilm and membrane process, the sludge was retained and immobilized in the reactor so that the biomass increased quickly in a short period. After the adaptation period, the COD removal efficiency stayed stable at a high level (84.90%~94.81%). Such a result confirmed the effectiveness and robustness of the biofilm-membrane bioreactor system even in a high organic pollution and salinity level [18]. However, with the increase of organic load from 0.5 kg COD/(m^3·d) to 1.5 kg COD/(m^3·d), the trend of COD removal rate started to decline. One possible explanation is that the balance between the microbial degradation ability and pollution loading has been reached when the organic load was below 1.0 kg COD/(m^3·d). Additionally, the deficiency of dissolved oxygen may hinder the reactivity and growth of microbes because the aeration intensity was kept constant at all stages. Hence, a greater aeration intensity is needed to improve the COD removal efficiency at such a high organic load. Although the COD removal efficiency dropped with the increase of organic load, BMBR still exhibited a great performance and salinity tolerance comparing with the conventional MBRs [20]. Mannina et al. reported that when the feeding salt rate up to 20 g/L, the total COD removal rate decreased from 96% to 75% at an influent COD concentration of 350 mg/L [21].

3.1.2. NH_4^+ Removal Efficiency. Membrane played an important role in the NH_4^+ removal. From Figure 3, the performance of NH_4^+ removal was achieved at high level, with a mean removal rate of 96.84% in stage I and 91.26% in stage II. Since nitrifying bacteria are autotrophic bacteria, a longer sludge retention time (SRT) is required for them to reproduce. The function of membrane filtration makes the SRT as long as possible, in which way the nitrifying bacteria accumulated and nitrification enhanced. It should be noted that the NH_4^+ removal efficiency has not been influenced greatly when organic load increased from 0.5 to 1.0 kg COD/(m^3·d). This reflects biofilm in BMBR can improve the impact resistance of the system [22]. However, when the organic load increased from 0.5 to 1.5 kg COD/(m^3·d), the NH_4^+ removal rate sharply dropped by 13.72%. Oxygen availability is one of the most important factors in the nitrification process for nitrifying bacteria. Under the condition that influent COD concentration was up to 1054.29 mg/L, nitrifying bacteria were inferior to other heterotrophic bacteria in the competition for dissolved oxygen, resulting in the reduction of NH_4^+ removal efficiency. On the other hand,

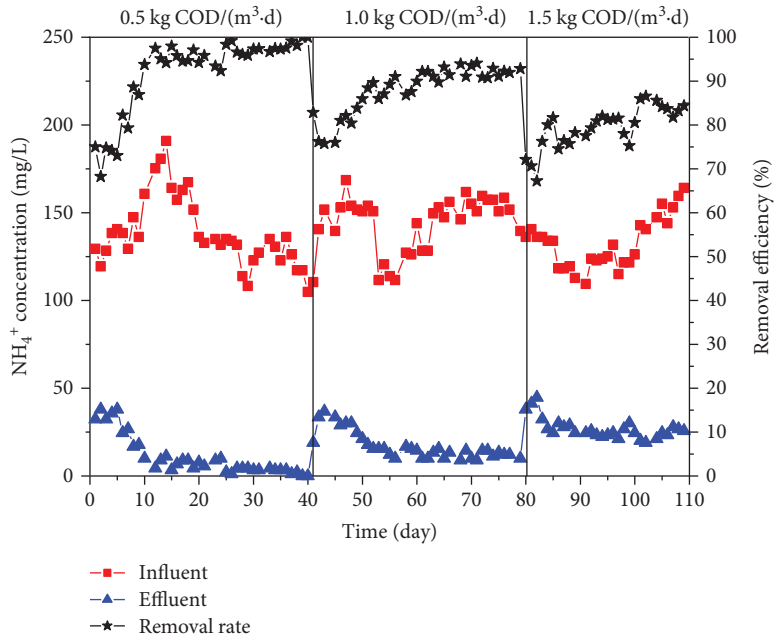


Figure 3: NH_4^+-N concentration variations in influent and effluent.

high salinity may exert inhibition on the nitrification process [23]. Previous studies have confirmed that high salinity negatively affected the transport of nutrient from medium to the cell, consequently modifying and reducing cell metabolism that lead to cell lysis [24]. Zhao et al. discovered that when salt concentration was above 20 g/L, NH_4^+ removal efficiency decreased, and the bioreactor collapsed [5].

3.1.3. TN Removal Efficiency. Fluctuations in TN removal efficiency were observed (Figure 4). The overall TN removal efficiency was relatively low comparing to previous studies. The main reason for the poor TN removal rate was the lack of an anoxic environment for denitrification [25]. Excessive dissolved oxygen made denitrifying bacteria switch from anaerobic to aerobic metabolism so that denitrification was inhibited. There was a general trend of decreasing TN removal as organic load increased from 0.5 to 1.5 kg COD/(m^3·d). This decrease may attribute to the incomplete nitrification. It has been proved that nitrification is crucial to stimulate TN removal because nitrification can provide nitrate or nitrite needed in denitrification. Although there are multiple novel nitrogen removal paths, e.g., partial nitrification-denitrification, ammonium oxidation [26], nitrification is the first step in nitrogen removal. Therefore, with the decrease of NH_4^+ removal efficiency, TN removal rate declined accordingly. Apart from oxygen and nitrification, another important factor that influenced denitrification was salinity. Denitrifying bacteria are more sensitive to toxic substance than nitrifying bacteria [27]. It is detrimental for the growth of denitrifying bacteria in high-salinity environment.

3.1.4. Soluble PO_4^{3-} Removal Efficiency. Suspended solids and particle-associated phosphorus could be captured via membrane filtration. In this study, focus was put on the removal efficiency of soluble phosphorus in the BMBR. During the experiment, the general PO_4^{3-} removal efficiency was poor with significant fluctuations (Figure 5), ranging from 19.23% to 53.89%, which reflected similar results when comparing with other studies [28, 29]. Biological phosphorus removal includes two steps: anaerobic phosphorus release and aerobic phosphorus uptake. However, there was no anaerobic environment available in BMBR. Phosphorus removal mainly depended on biological assimilation. Moreover, phosphorus-rich sludge cannot discharge the reactor in time, leading to the low PO_4^{3-} removal efficiency. The high PO_4^{3-} removal that occurred in the beginning of each stage was observed. This was because the chemical cleaning of the membrane module was performed before the working condition changed so that the membrane module can work under the same condition. Phosphorus-rich sludge adhered to the surface of the membrane was cleaned out, and the microbial biomass suddenly decreased. Consequently, more microorganism proliferated, and phosphorus was stored in microbial cells, in which way PO_4^{3-} removal efficiency increased temporarily.

3.2. The Role of Halophilic Bacteria on Membrane Fouling. Halophilic bacteria are special microbes that only grow in saline environment. Halophilic can metabolize organic matter and nutrient in the wastewater to gain energy. It is promising to treat high-salinity wastewater using halophilic bacteria [30]. To determine the substance causing membrane fouling, SEM was utilized to analyze the microscopic structure of the membrane pollution (Figure 6). With the increase of influent organic load, halophilic bacteria gradually predominate by succession, characterized by abundance of bacillus and coccus in the reactor. The stabilization of microbial community structure has a beneficial effect on removal efficiency [31, 32]. High salinity also changed the structure and property of sludge [28]. When there is no salt or a low-

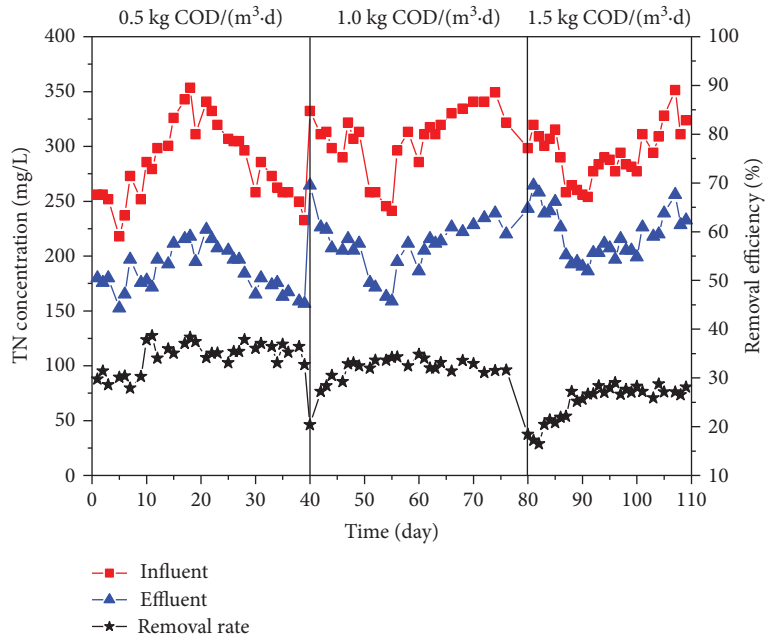


Figure 4: TN concentration variations in influent and effluent.

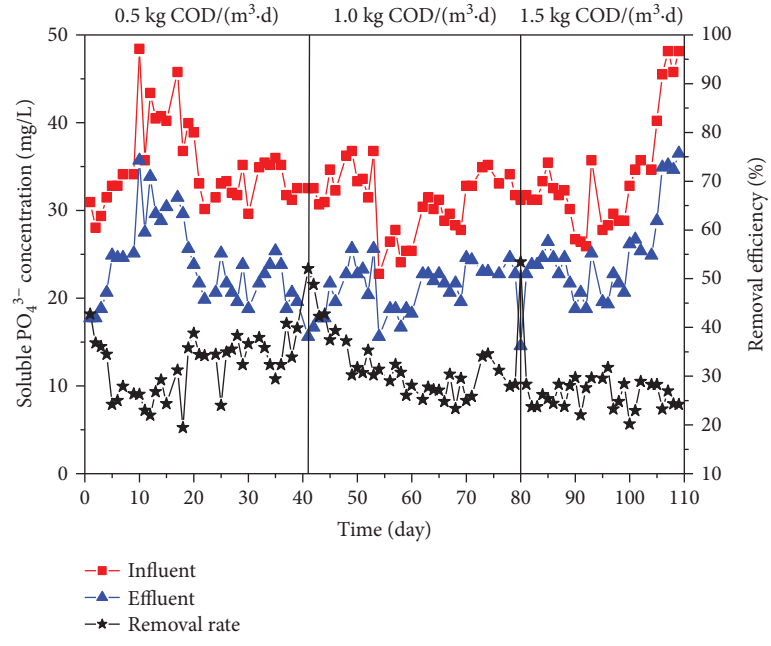
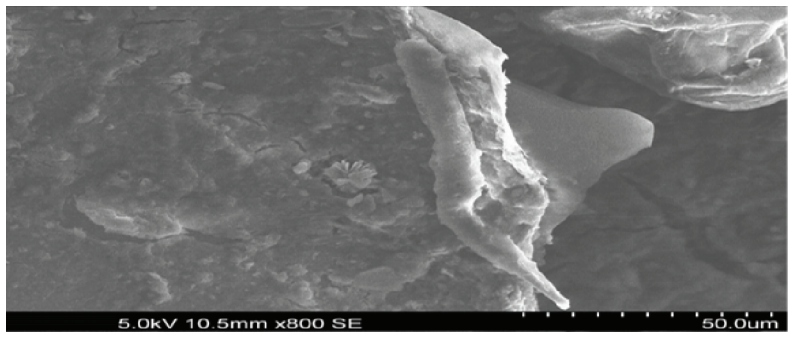
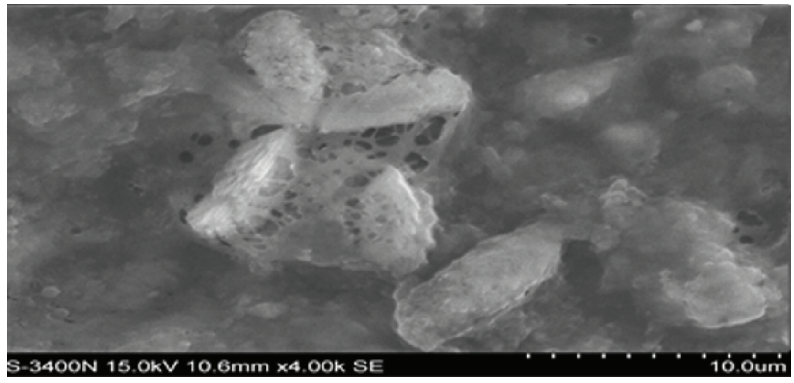


Figure 5: PO_4^{3-} concentration variations in influent and effluent.

concentration salt exists, the size of sludge floc is large. However, the sludge floc mainly composed of halophilic bacteria was small and loose [33], which can block the membrane pore and cause irreversible contamination. Some kind of sludge floc attached to the surface of the membrane and formed a gel layer which contained different kinds of extracellular polymeric substance (EPS). Sludge microorganisms secreted EPS to resist adverse saline environment. Hong et al. [34] reported that increasing salt concentration resulted in the rise of EPS concentration. The soluble portion of EPS as well as bound EPS facilitated the formation of the gel layer on the membrane surface [35], which cannot be readily removed by physical cleaning [36]. On the other hand, when the water temperature was below 10°C, the soluble salt recrystallized (Figure 7), contributing to the sharp decrease of membrane flux. Summarily, the membrane fouling was the consequence of the interaction of microbial activities and NaCl crystallization. If the goal is to mitigate the membrane fouling when treating high-salinity wastewater, a low operating temperature should be avoided.



(a)



(b)

Figure 6: SEM images of membrane fouling. (a) The microorganisms on cake layer; (b) the EPS on gel layer.

Figure 7: The photograph of NaCl crystallization on the surface of the membrane.

4. Conclusion

The biological treatment of mustard tuber wastewater presents to be a great challenge due to the high concentration of organic carbon, nutrient, and salinity that can strongly inhibit microbial activity and damage the settling ability of activated sludge. A novel technology combined with biofilm and membrane bioreactor was developed to treat mustard tuber wastewater. In detail, the microbial biomass can increase quickly in BMBR system because the immobilized biofilm enhances the growth of bacteria. A high removal efficiency of organic carbon and ammonium was achieved indicating that heterotrophic bacteria and nitrifying bacteria maintained high reactivity in the saline environment. However, the removal of total nitrogen and soluble phosphorus was relatively low due to the lack of anaerobic environment. With the increase of influent organic load, the performance of the BMBR degenerated when the organic load exceeded the microbial degradation ability. Halophilic bacteria played a key role in pollutant removal as well as in the biofouling process. Under a low-temperature operation, membrane fouling was the consequent of the interaction of microbial activities and NaCl crystallization. Finally, BMBR system showed a high potentiality in treating high-concentration or high-salinity wastewater.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no competing interests.

Acknowledgments

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References

[1] J. Wang, B. Gong, W. Huang, Y. Wang, and J. Zhou, "Bacterial community structure in simultaneous nitrification, denitrification and organic matter removal process treating saline mustard tuber wastewater as revealed by 16S rRNA sequencing," *Bioresource Technology*, vol. 228, pp. 31–38, 2017.

[2] O. Lefebvre and R. Moletta, "Treatment of organic pollution in industrial saline wastewater: a literature review," *Water Research*, vol. 40, no. 20, pp. 3671–3682, 2006.

[3] X. Shi, O. Lefebvre, K. K. Ng, and H. Y. Ng, "Sequential anaerobic-aerobic treatment of pharmaceutical wastewater with high salinity," *Bioresource Technology*, vol. 153, pp. 79–86, 2014.

[4] R. Boopathy, C. Bonvillain, Q. Fontenot, and M. Kilgen, "Biological treatment of low-salinity shrimp aquaculture wastewater using sequencing batch reactor," *International Biodeterioration & Biodegradation*, vol. 59, no. 1, pp. 16–19, 2007.

[5] Y. Zhao, H. D. Park, J. H. Park et al., "Effect of different salinity adaptation on the performance and microbial community in a sequencing batch reactor," *Bioresource Technology*, vol. 216, pp. 808–816, 2016.

[6] M. M. Amin, M. H. Khiadani (Hajian), A. Fatehizadeh, and E. Taheri, "Validation of linear and non-linear kinetic modeling of saline wastewater treatment by sequencing batch reactor with adapted and non-adapted consortiums," *Desalination*, vol. 344, pp. 228–235, 2014.

[7] F. Meng, S. R. Chae, A. Drews, M. Kraume, H. S. Shin, and F. Yang, "Recent advances in membrane bioreactors (MBRs): membrane fouling and membrane material," *Water Research*, vol. 43, no. 6, pp. 1489–1512, 2009.

[8] G. Di Bella, D. Di Trapani, M. Torregrossa, and G. Viviani, "Performance of a MBR pilot plant treating high strength wastewater subject to salinity increase: analysis of biomass activity and fouling behaviour," *Bioresource Technology*, vol. 147, pp. 614–618, 2013.

[9] S. Lim, S. Kim, K.-M. Yeon, B. I. Sang, J. Chun, and C. H. Lee, "Correlation between microbial community structure and biofouling in a laboratory scale membrane bioreactor with synthetic wastewater," *Desalination*, vol. 287, pp. 209–215, 2012.

[10] J. Nakajima and I. Mishima, "Measurement of foam quality of activated sludge in MBR process," *Acta Hydrochimica et Hydrobiologica*, vol. 33, no. 3, pp. 232–239, 2005.

[11] G. P. Sheng, H. Q. Yu, and X. Y. Li, "Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: a review," *Biotechnology Advances*, vol. 28, no. 6, pp. 882–894, 2010.

[12] A. Gonzalez-Martinez, J. C. Leyva-Díaz, A. Rodriguez-Sanchez et al., "Isolation and metagenomic characterization of bacteria associated with calcium carbonate and struvite precipitation in a pure moving bed biofilm reactor-membrane bioreactor," *Biofouling*, vol. 31, no. 4, pp. 333–348, 2015.

[13] W. Guo, H. H. Ngo, and J. Li, "A mini-review on membrane fouling," *Bioresource Technology*, vol. 122, pp. 27–34, 2012.

[14] D. W. Gao, Z. D. Wen, B. Li, and H. Liang, "Microbial community structure characteristics associated membrane fouling in A/O-MBR system," *Bioresource Technology*, vol. 154, pp. 87–93, 2014.

[15] T. Leiknes and H. Ødegaard, "The development of a biofilm membrane bioreactor," *Desalination*, vol. 202, no. 1-3, pp. 135–143, 2007.

[16] Q. Yang, J. Chen, and F. Zhang, "Membrane fouling control in a submerged membrane bioreactor with porous, flexible suspended carriers," *Desalination*, vol. 189, no. 1–3, pp. 292–302, 2006.

[17] F. Yang, Y. Wang, A. Bick et al., "Performance of different configurations of hybrid growth membrane bioreactor (HG-MBR) for treatment of mixed wastewater," *Desalination*, vol. 284, pp. 261–268, 2012.

[18] D. Di Trapani, G. Di Bella, G. Mannina, M. Torregrossa, and G. Viviani, "Comparison between moving bed-membrane bioreactor (MB-MBR) and membrane bioreactor (MBR) systems: influence of wastewater salinity variation," *Bioresource Technology*, vol. 162, pp. 60–69, 2014.

[19] M. A. H. Johir, S. Vigneswaran, J. Kandasamy, R. BenAim, and A. Grasmick, "Effect of salt concentration on membrane bioreactor (MBR) performances: detailed organic characterization," *Desalination*, vol. 322, pp. 13–20, 2013.

[20] X. Wang, T. Yang, B. Lin, and Y. Tang, "Effects of salinity on the performance, microbial community, and functional proteins in an aerobic granular sludge system," *Chemosphere*, vol. 184, pp. 1241–1249, 2017.

[21] G. Mannina, A. Cosenza, D. Di Trapani, M. Capodici, and G. Viviani, "Membrane bioreactors for treatment of saline wastewater contaminated by hydrocarbons (diesel fuel): an experimental pilot plant case study," *Chemical Engineering Journal*, vol. 291, pp. 269–278, 2016.

[22] J. P. Bassin, M. Dezotti, and G. L. Sant'Anna Jr, "Nitrification of industrial and domestic saline wastewaters in moving bed biofilm reactor and sequencing batch reactor," *Journal of Hazardous Materials*, vol. 185, no. 1, pp. 242–248, 2011.

[23] D. Jang, Y. Hwang, H. Shin, and W. Lee, "Effects of salinity on the characteristics of biomass and membrane fouling in membrane bioreactors," *Bioresource Technology*, vol. 141, pp. 50–56, 2013.

[24] B. Dalmacija, E. Karlovic, Z. Tamas, and D. Miskovic, "Purification of high-salinity wastewater by activated sludge process," *Water Research*, vol. 30, no. 2, pp. 295–298, 1996.

[25] K. K. Ng, X. Shi, S. L. Ong, C. F. Lin, and H. Y. Ng, "An innovative of aerobic bio-entrapped salt marsh sediment membrane reactor for the treatment of high-saline pharmaceutical wastewater," *Chemical Engineering Journal*, vol. 295, pp. 317–325, 2016.

[26] A. V. del Rio, A. Pichel, N. Fernandez-Gonzalez et al., "Performance and microbial features of the partial nitritation-anammox process treating fish canning wastewater with variable salt concentrations," *Journal of Environmental Management*, vol. 208, pp. 112–121, 2018.

[27] A. R. Dinçer and F. Kargi, "Salt inhibition of nitrification and denitrification in saline wastewater," *Environmental Technology*, vol. 20, no. 11, pp. 1147–1153, 2010.

[28] A. Uygur and F. Kargı, "Salt inhibition on biological nutrient removal from saline wastewater in a sequencing batch reactor," *Enzyme and Microbial Technology*, vol. 34, no. 3-4, pp. 313–318, 2004.

[29] F. Kargi and A. Uygur, "Improved nutrient removal from saline wastewater in an SBR by Halobacter supplemented activated sludge," *Environmental Engineering Science*, vol. 22, no. 2, pp. 170–176, 2005.

[30] O. Lefebvre, N. Vasudevan, M. Torrijos, K. Thanasekaran, and R. Moletta, "Halophilic biological treatment of tannery soak

liquor in a sequencing batch reactor," *Water Research*, vol. 39, no. 8, pp. 1471–1480, 2005.

[31] T. P. H. van den Brand, K. Roest, G.-H. Chen, D. Brdjanovic, and M. C. M. van Loosdrecht, "Long-term effect of seawater on sulfate reduction in wastewater treatment," *Environmental Engineering Science*, vol. 32, no. 7, pp. 622–630, 2015.

[32] E. Ferrer-Polonio, J. A. Mendoza-Roca, A. Iborra-Clar, J. L. Alonso-Molina, and L. Pastor-Alcañiz, "Comparison of two strategies for the start-up of a biological reactor for the treatment of hypersaline effluents from a table olive packaging industry," *Chemical Engineering Journal*, vol. 273, pp. 595–602, 2015.

[33] Y. Cui, H. Su, Y. Chen, Y. Chen, and Y. Peng, "Mechanism of activated sludge floc disintegration induced by excess addition of NaCl," *CLEAN - Soil, Air, Water*, vol. 43, no. 8, pp. 1197–1206, 2015.

[34] J. Hong, W. Li, B. Lin, M. Zhan, C. Liu, and B. Y. Chen, "Deciphering the effect of salinity on the performance of submerged membrane bioreactor for aquaculture of bacterial community," *Desalination*, vol. 316, pp. 23–30, 2013.

[35] A. Ramesh, D. J. Lee, and J. Y. Lai, "Membrane biofouling by extracellular polymeric substances or soluble microbial products from membrane bioreactor sludge," *Applied Microbiology and Biotechnology*, vol. 74, no. 3, pp. 699–707, 2007.

[36] A. Drews, "Membrane fouling in membrane bioreactors—characterisation, contradictions, cause and cures," *Journal of Membrane Science*, vol. 363, no. 1-2, pp. 1–28, 2010.

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Research Article

Autohydrogenotrophic Denitrification Using the Membrane Biofilm Reactor for Removing Nitrate from High Sulfate Concentration of Water

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This study investigated the performance of an autohydrogenotrophic membrane biofilm reactor (MBfR) to remove nitrate from water with high sulfate concentrations. The results of simulated running showed that TN removal could be over than 98.8% with the maximum denitrification rate of 134.6 g N/m^3 d under the conditions of the influent sulfate concentrations of 300 mg SO_4^{2-}/l. The distribution ratio of H_2 electron donor for nitrate and sulfate was 70.0 : 26.9 at the high influent loading ratio of sulfate/nitrate of 853.3 g SO_4^{2-}/m^3 d : 140.5 g N/m^3 d, which indicated that denitrification bacteria (DB) were normally dominated to complete H_2 electron with sulfate bacteria (SRB). The results of molecular microbiology analysis showed that the dominated DB were *Rhodocyclus* and *Hydrogenophaga*, and the dominated SRB was *Desulfohalobium*, under the high influent sulfate concentrations.

1. Introduction

Nitrate-contaminated river or groundwater occurred everywhere in the world because the fertilizers were utilized extensively and part of the wastewater from industries was discharged randomly, especially in developing countries [1, 2]. The high concentrations of nitrate in drinking water (>10 mg N/l) would have a high risk to produce nitrosamines and cause methemoglobinemia, which was harmful to people's health [3, 4]. Therefore, a lot of methods to reduce nitrate from water sources have been reported [5, 6].

The effective methods to reduce nitrate include ion exchange [7] and reverse osmosis [8–10]. Due to the high cost of physiochemical technologies, their applications are limited in some extent [11]. The two normal types of the biological treatment are heterotrophic denitrification and autotrophic denitrification [12, 13]. The cost of the heterotrophic denitrification is high because the organic materials need often to add the carbon source for bacteria in the process which are low in groundwater [14, 15]. There are lots of advantages of autohydrogenotrophic technology, such as clear with hydrogen, low cost, and without secondary pollution [16, 17].

Recently, a new technology of hydrogen- (H_2-) based membrane biofilm reactor (MBfR) has developed and got a good effect, which used autohydrotrophic bacteria in the denitrification processes [16, 18, 19]. The oxidized pollutants, such as SO_4^{2-}, CrO_4^{2-}, AsO_3^-, TCE, ClO_4^-, BrO_3^-, and SeO_4^{2-}, could be reduced by MBfR using H_2 as electron donors [20–23]. While NO_3^- and SO_4^{2-} are chemical oxyanions that normally coexist in a variety of waters. There are many reasons caused NO_3^- and SO_4^{2-} coexisting in

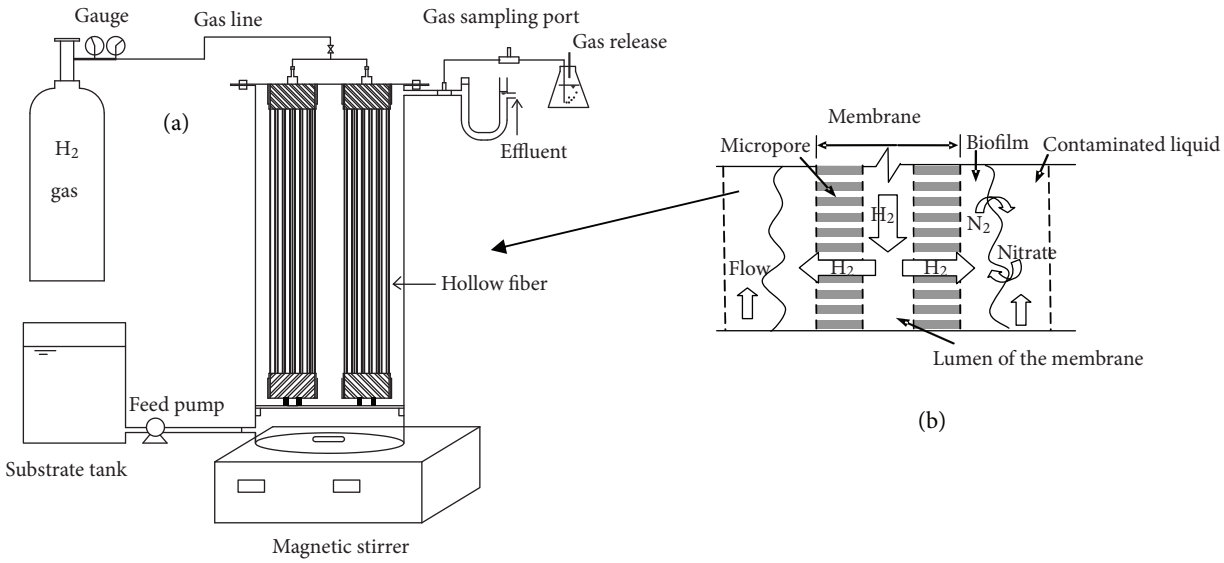

Figure 1: MBfR in the experiment (a) and theoretical views of MBfR (b).

water, such as anthropogenic activities related to overusing of fertilizers and wastewater discharges, natural mineralogy related to SO_4^{2-} minerals, and atmospheric deposition of NO_3^- and SO_4^{2-} [24]. On the other hand, in MBfR, the autohydrogenotropic bacteria could utilize NO_3^- and SO_4^{2-} as electron acceptors to generate energy for their growth [25], and several sulfate-reducing bacteria (SRB) are able to use alternative terminal electron acceptors to reduce sulfate such as nitrate [26].

The following equations could describe the stoichiometry of hydrogenotrophic denitrification and sulfur-reducing:

$$2NO_3^- + 2H^+ + 5H_2 \rightarrow N_2 + 6H_2O$$
$$4H_2 + SO_4^{2-} \rightarrow H_2S + 2H_2O + 2OH^- \quad (1)$$

While in some sites in the world (e.g., natural mineralogy), the contents of sulfate could be as high as hundreds or thousands micrograms per liter in the groundwater, which is used as a drinking water. Because SO_4^{2-} is not normally considered a health concern, and no MCL has been established for SO_4^{2-}, so many references of autohydrogenotrophic denitrification could concern about sulfate reduction, but the concentrations of SO_4^{2-} were relatively lower in the influents for research [27].

The aim of this study was to investigate the performance of autohydrogenotrophic denitrification under the high concentrations of sulfate by a hollow fiber membrane bioreactor with polyvinyl chloride (PVC) membrane.

2. Materials and Methods

2.1. Reactor in the Study. The theory of denitrification using hydrogenotrophic bacteria is shown in Figure 1(b); the denitrification attached on the outside surface of membrane would utilize the H_2 transferred from the lumen of the membrane at some extent of pressure to accomplish the denitrification. For the reactor, we use a transparent plastic cylinder to hold two membrane modules, and the influent fluid was flowed from upper side to the lower outlet, and the flow rate was controlled by a peristaltic pump (longer BT50-1J, Baoding, PRC), and the membrane made of polyvinyl chloride membrane with hydrophobicity alloy fiber was used in the study. The detailed schematic of the reactor could be seen in Figure 1(a). Also, the parameters of the membrane and the reactor are listed in Table 1.

Table 1: The parameters of the reactor.

Parameters	Unit	Value
Numbers of fiber module		2
Outer diameter of fiber	cm	0.15
Inner diameter of fiber	cm	0.085
Fiber number in the reactor		96
Length of fiber	mm	140
Volume of fibers	cm^3	23.74
Available surface area	cm^2	633.34
Available volume of reactor	cm^3	560
Void ratio	%	95.76
Specific surface area	m^2/m^3	113.10
Height	cm	22.0
Section area of reactor	cm^2	28.26
Diameter of reactor	cm	6.0
Available volume of reactor	cm^3	560

2.2. Influent Water Source and Experimental Conditions. In the study, the influent water was taken from the sulfate- and nitrate-contaminated groundwater in the vegetable land at the suburb of Qingzhou (Weifang, China), where a lot of fertilizer had been used in the lands. The shallow groundwater around the vegetable land had been contaminated by nitrate and sulfate, and the water quality is shown in Table 2.

Table 2: Water quality parameters of the groundwater.

Total dissolved solids (mg/l)	pH	Alkalinity (mg/l as $CaCO_3$)	Hardness (mg/l as $CaCO_3$)	DO	Nitrate (mg N/l)	Nitrite (mg N/l)	Sulfate (mg/l)
300–400	7.2~7.5	320~500	400~650	6.0–6.4	35~60	ND	250~450

ND: not detected.

Table 3: Experimental design of the reactor running.

	Start-up	Run I	Run II	Run III
Running time (day)	3	1–40	41–80	81–155
H_2 pressure in the fiber (MPa)	0.02	0.03	0.04	0.05
Nitrate concentration in the influent (mg N/l)	10.0 ± 2.0	20.0 ± 2.0	40.0 ± 4.0	50.0 ± 4.0
Sulfate concentration in the influent (mg/l)	100 ± 10.0	200 ± 10.0	250 ± 10.0	300 ± 10.0
Flow rate (ml/min)			1.1	
HRT (h)			8.5	

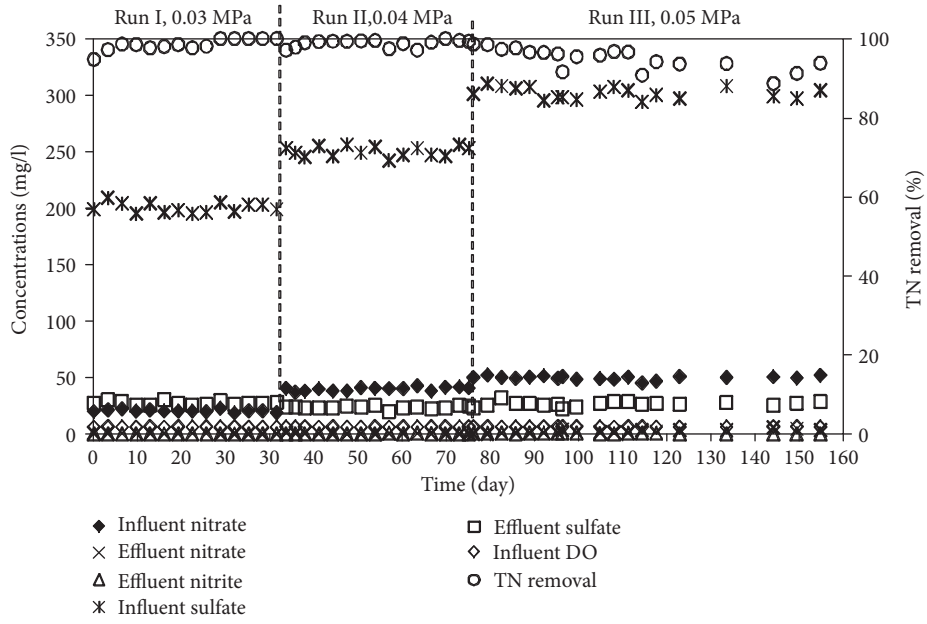

Figure 2: The water quality in the influent and effluent and TN removal.

We stated up the reactor by inoculating the biofilm microorganisms from other MBfRs running for hydrogenotrophic denitrification for years in our lab. For simulating the different concentrations of sulfate in the influent water, some dosage of $FeSO_4 \cdot 7H_2O$ was fed in the influent pumped from the actual groundwater. The detailed experimental design of the reactor running could be seen in Table 3.

All the fluid samples collected in the experiments were kept at 4°C until the samples were analyzed. The NO_3^--N, NO_2^--N, and SO_4^{2-} were measured by the ion chromatography (Dionex ICS 3000). The H_2 unutilized by the denitrifiers would go into the headspace of the reactor. A GC 14-B equipped with a TCD detector (Shimadzu Co.) was used to test the H_2 gas concentration in the headspace in the reactor by pumping gas from the gas port by a syringe, and the hydrogen content in the liquid could be calculated by Henry's law.

2.3. Sampling for Biofilm and the Analysis of Microbiology. In the experiments, at different running periods for the reactor, the biofilm would be sampled to analyze the changes of the microbial communities. For our study, when the water quality in the effluent was steady, that is, at day 40, day 80, and day 150, the biofilm samples were collected. According to our previous research, DNA extractions, PCR, and DGGE were done; see the detailed methods in [28]. As for the nucleotide sequencing, the reamplified DNA products were analyzed by Sangon Company (Shanghai, China). Shannon-Wiener index was used to analyze the diversity changes of microbial communities in different running periods of the reactor. The relation and the dendrogram generation among

Table 4: The influent loadings and volume reductions for nitrate and sulfate under different influent sulfate concentrations.

Influent sulfate contents (mg/l)	Influent sulfate loading (g/m^3 d)	Volume sulfate reduction (g/m^3 d)	Nitrate loading ($g\,N/m^3$ d)	Volume denitrification rate ($g\,N/m^3$ d)	Sulfate in effluent (mg/l)	Nitrate in effluent (mg N/l)	References
200	566.3	155.4	57.8	55.7	145.3	0.7	This study
250	707.3	166.3	112.5	111.6	191.3	0.3	This study
300	853.3	226.7	140.5	134.6	221.5	2.1	This study
42	118.5	50.7	56.5	55.5	24	0.3	[29]
92	262.6	109.6	139.5	133.8	54	2	[29]
78	216.8	85.3	141.7	136	46.5	2	[30]

Table 5: Distributions of hydrogen electron in electron acceptors at different influent sulfate contents.

Influent sulfate (mg/l)	Influent nitrate (mg N/l)	Nitrate (%)	Sulfate (%)	Oxygen (%)	Cr (VI) (%)	References
200	20	57.9	36.1	6.0		This study
250	40	71.8	24.4	3.8		This study
300	50	70.0	26.9	3.1		This study
42	20	76.0	15.9	8.1		[29]
92	50	81.2	15.2	3.6		[29]
78	50	87.5	12.5			[30]
78	10	69.9	29.2		0.9	[33]
78	5	55.7	42.8		1.5	[33]

the biofilm bacteria in different running periods were calculated and analyzed by cluster analysis through the NTSYS-pc (2.10, Exeter Software, USA).

3. Results and Discussion

3.1. Operation and Effluent Quality of MBfR. In the beginning of the experiment, the biofilm established on the out surface of the fiber was only taken 3 days just because of the inoculation of bacteria from the reactors running over than years. Then, the reactor was operated over 155 days to evaluate the performance of MBfR under different conditions. The performance of MBfR over the operation periods was illustrated in Figure 2.

As shown in Figure 2, the influent concentrations of nitrate and sulfate ranged from 10–50 mg N/l and 100–300 mg SO_4^{2-}/l through the experiments, respectively. In the whole experiment period, the averages of TN removal were 96.4 ± 2.3%, 98.8 ± 1.0%, and 94.9 ± 2.8% in the Run I, Run II, and Run III, respectively. As for the water quality in the effluent, the averages of nitrate concentrations in the effluents were 0.7, 0.3, and 2.1 NO_3^--N mg/l, for Run I, Run II, and Run III, respectively. And for nitrite in the effluent, the contents of nitrite in Run I are not detected, but were 0.2 and 0.4 NO_2^--N mg/l, in Run II and Run III, respectively. It suggested that the high concentrations of sulfate have some extent inhabitation to denitrification in MBfR processes.

3.2. Performance of MBfR under High Concentration of Sulfate. In this experiment, the high sulfate concentrations up to 300 mg/l in the influent were used to investigate the performance of MBfR. Under the conditions of the different contents of sulfate in the influent, the denitrification loadings and sulfate loadings could be seen in Table 4.

The volumetric denitrification rates were changed from 55.7 $g\,N/m^3$ to 134.6 $g\,N/m^3$ with a good TN removal over than 94.9%, which was mainly caused by increasing the influent nitrate loadings. The sulfate reduction rate was changed from 155.4 to 266.7 $g\,SO_4^{2-}/m^3$, which was not mainly controlled by the influent sulfate loading of 566.3–853.3 $g\,SO_4^{2-}/m^3$, and the average sulfate removals were about 23.5–27.4%. It indicated that the nitrate would be utilized preferentially by denitrification bacteria (DB) than sulfate utilized by SRB in completion with H_2 in MBfR, and nitrate respiration is energetically more favorable than sulfate respiration [31].

In the autohydrogenotrophic denitrification in MBfR, the SRB also utilized hydrogen as electron donor to reduce sulfate to sulfide; therefore, there would be a competition for hydrogen between the reductions of nitrate, sulfate, and other electron acceptors. The distributions of hydrogen electron in electron acceptors at different influent sulfate contents in this study and references are shown in Table 5. The calculations of the hydrogen electron's distributions in MBfR were according to our previous research [30]. Distributions of hydrogen electron were not only dependent on the concentrations of electron acceptors but also on the types of electron acceptors. But the distribution ratio of H_2 on sulfate would be high as its concentration increases at the same conditions. As for sulfate in this study, even the influent sulfate loading increased

Table 6: The H_2 utility in the MBfR.

	Sum of H_2 utility (%)	H_2 utility for nitrate (%)	H_2 utility for sulfate (%)	H_2 utility for O_2 (%)
Run I	97.7	61.1	36.6	9.3
Run II	99.4	75.2	24.2	6.0
Run III	99.5	73.0	26.6	4.9

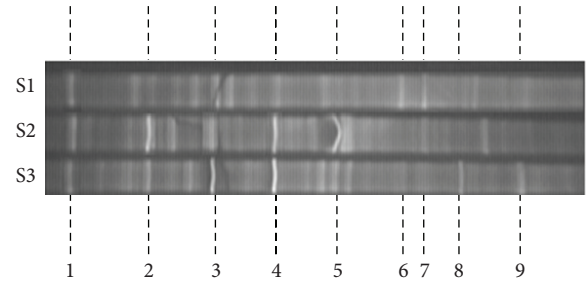

Figure 3: DGGE and on the day 40 (S1), day 80 (S2), and day 150 (S3) (the Arabic numerals meant the different dominated bands in the operation of MBfR).

gradually, the sulfate removal was contained at steady figure of about 25%, while the TN removal was almost over 95%, which can be seen from the distribution of electron-equivalent fluxes that the ratio of nitrate : sulfate was 70.0% : 26.9% (Run III). It indicated that DB could get more H_2 than SRB whatever of the acceptor influent loading changes. While Table 5 also indicated that the high influent sulfate concentrations or high ratio of influent sulfate concentration to influent nitrate concentration would lead SRB to get more power in the competition for hydrogen among the electron acceptors, which could be used to select the special bacteria in MBfR operations for minimizing sulfate reduction [32].

3.3. H_2 Utility. The effluent H_2 concentrations in Runs I–III were very low, from 0.10 to 0.52 mg/l, which indicated that the H_2 could be transferred well without bubble from the PVC membrane and be used sufficiently by DB and SRB; meanwhile, the system got an effective removal of nitrate.

The % unutilized hydrogen was calculated according to (2), that is, the part of H_2 leaving out of reactor: the part utilized by bacteria. The H_2 utility in the reactor is shown in Table 6.

$$\%H_2\text{unutilized} = 100\% \times \frac{S_{H,o}}{0.143(S_{3,i} - S_{3,o}) + 0.214(S_{3,i} - S_{3,o} - S_{2,o}) + 0.083(S_{4,i} - S_{4,o}) + 0.125(S_{5,i} - S_{5,o}) + S_{H,o}}, \quad (2)$$

where the detailed meanings of $S_{3,i}$, $S_{3,o}$, $S_{2,o}$, $S_{5,i}$, $S_{5,o}$, and $S_{H,o}$ could be seen in [30].

As shown in Table 6, the sum of hydrogen utilization efficiency over the 3 periods was 97.7–99.5%; the remains may go into the effluent or out of the water. Among the sum of the H_2 utility, nitrate got much more quota than that of sulfate and oxygen.

3.4. Analyses of Microbial Community. The microbial communities in each running period of the reactor could be seen in the analyses of the DGGE (Figure 3). The DGGE indicted the dominant bands. Even the operation period was long in each running stage with different concentrations of sulfate in the influent, while the autohydrogenotrophic bacteria growth was very slow and the change of microbial community was considerately slow. In the beginning period of Run I, the bands were not clear and complicated, which indicated that the biofilm needs acclimation furthermore. While several bands, which were clear and simple, could be seen in Run II and Run III. The special bands with number 2, 3, and 4 in DGGE which were dominated were cut and sent to be sequenced. The results indicated that the bacteria in bands 2, 3, and 4 were similar to *Rhodocyclus*, *Hydrogenophaga*, and *Desulfohalobium*, with the similarity of 99%, 98%, and 99%, respectively. The *Rhodocyclus* and *Hydrogenophaga* were normal autotrophic bacteria, belonging to beta divisions within the Proteobacteria. This is consistent with our previous study [28]. The *Desulfohalobium* was found in Runs II and III, which is a Gram negative, anaerobic, sulfate-reducing, moderately halophilic, and rod-shaped bacterial genus from the family of Desulfovibrionaceae. This indicated that the SRB could be abundant with the influent concentration increasing and could enhance its strength of competition with nitrate for H_2 [31].

4. Conclusion

The study investigated the performance of MBfR to remove nitrate companied with high influent concentrations of sulfate over 155 days. The results indicated that even in high concentration of sulfate in influent, the MBfR also could get a good denitrification effect with nitrate and nitrite under the US standard. The analysis of the molecular microbiology showed that microbial community structures of Runs II and III were similar, simple, and stable. The bacteria species of Betaproteobacteria which include *Rhodocyclales* and *Hydrogenophaga* were dominant DB for nitrate removal. The *Desulfohalobium* was found to be a dominant SRB in Runs II and III under the high concentrations of sulfate. The results would give some directions on the actual application of MBfR to remove nitrate or other oxidations in the drinking water.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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References

[1] B. Gu, Y. Ge, S. X. Chang, W. Luo, and J. Chang, "Nitrate in groundwater of China: sources and driving forces," *Global Environmental Change*, vol. 23, no. 5, pp. 1112–1121, 2013.

[2] A. Menció, J. Mas-Pla, N. Otero et al., "Nitrate pollution of groundwater; all right…, but nothing else?," *Science of the Total Environment*, vol. 539, pp. 241–251, 2016.

[3] D. C. Bouchard, M. K. Williams, and R. Y. Surampalli, "Nitrate contamination of groundwater: sources and potential health effects," *Journal - American Water Works Association*, vol. 84, no. 9, pp. 85–90, 1992.

[4] Q. Wang, C. Feng, Y. Zhao, and C. Hao, "Denitrification of nitrate contaminated groundwater with a fiber-based biofilm reactor," *Bioresource Technology*, vol. 100, no. 7, pp. 2223–2227, 2009.

[5] D. Ucar, E. U. Cokgor, and E. Sahinkaya, "Heterotrophic–autotrophic sequential system for reductive nitrate and perchlorate removal," *Environmental Technology*, vol. 37, no. 2, pp. 183–191, 2015.

[6] D. Ucar, E. U. Cokgor, E. Sahinkaya et al., "Simultaneous nitrate and perchlorate removal from groundwater by heterotrophic-autotrophic sequential system," *International Biodeterioration and Biodegradation*, vol. 116, pp. 83–90, 2017.

[7] S. Samatya, N. Kabay, Ü. Yüksel, M. Arda, and M. Yüksel, "Removal of nitrate from aqueous solution by nitrate selective ion exchange resins," *Reactive and Functional Polymers*, vol. 66, no. 11, pp. 1206–1214, 2006.

[8] J. J. Schoeman, "Nitrate-nitrogen removal with small-scale reverse osmosis, electrodialysis and ion-exchange units in rural areas," *Water SA*, vol. 35, no. 5, 2009.

[9] J. Bi, C. Peng, H. Xu, and A.-S. Ahmed, "Removal of nitrate from groundwater using the technology of electrodialysis and electrodeionization," *Desalination and Water Treatment*, vol. 34, no. 1-3, pp. 394–401, 2011.

[10] Y. Y. Birdja, J. Yang, and M. T. M. Koper, "Electrocatalytic reduction of nitrate on tin-modified palladium electrodes," *Electrochimica Acta*, vol. 140, pp. 518–524, 2014.

[11] M. Pirsaheb, T. Khosravi, K. Sharafi, and M. Mouradi, "Comparing operational cost and performance evaluation of electrodialysis and reverse osmosis systems in nitrate removal from drinking water in Golshahr, Mashhad," *Desalination and Water Treatment*, vol. 57, no. 12, pp. 5391–5397, 2015.

[12] A. X. Zhou, X. S. Su, J. Y. Dang, and Y. L. Zhang, "Relationships between microbial compositions and hydrochemical factors in nitrate contaminated groundwater of Hun River alluvial proluvial fan, China," *Journal of Agricultural Science and Technology*, vol. 17, pp. 735–746, 2015.

[13] S. Demirel and I. Bayhan, "Nitrate and bromate removal by autotrophic and heterotrophic denitrification processes: batch experiments," *Journal of Environmental Health Science and Engineering*, vol. 11, no. 1, pp. 27–27, 2013.

[14] M. A. Gomez, E. Hontoria, and J. Gonzalez-Lopez, "Effect of dissolved oxygen concentration on nitrate removal from groundwater using a denitrifying submerged filter," *Journal of Hazardous Materials*, vol. 90, no. 3, pp. 267–278, 2002.

[15] G. Huang, Y. Huang, H. Hu, F. Liu, Y. Zhang, and R. Deng, "Remediation of nitrate–nitrogen contaminated groundwater using a pilot-scale two-layer heterotrophic–autotrophic denitrification permeable reactive barrier with spongy iron/pine bark," *Chemosphere*, vol. 130, pp. 8–16, 2015.

[16] K. C. Lee and B. E. Rittmann, "A novel hollow-fibre membrane biofilm reactor for autohydrogenotrophic denitrification of drinking water," *Water Science and Technology*, vol. 41, no. 4-5, pp. 219–226, 2000.

[17] H. I. Park, D. K. Kim, Y. J. Choi, and D. Pak, "Nitrate reduction using an electrode as direct electron donor in a biofilm-electrode reactor," *Process Biochemistry*, vol. 40, no. 10, pp. 3383–3388, 2005.

[18] R. Nerenberg and B. E. Rittmann, "Perchlorate as a secondary substrate in a denitrifying, hollow-fiber membrane biofilm reactor," *Water Science and Technology: Water Supply*, vol. 2, no. 2, pp. 259–265, 2002.

[19] Y. Tang, C. Zhou, S. W. Van Ginkel, A. Ontiveros-Valencia, J. Shin, and B. E. Rittmann, "Hydrogen permeability of the hollow fibers used in H_2-based membrane biofilm reactors," *Journal of Membrane Science*, vol. 407-408, pp. 176–183, 2012.

[20] S. W. Van Ginkel, Z. Yang, B.-o. Kim, M. Sholin, and B. E. Rittmann, "Effect of pH on nitrate and selenate reduction in flue gas desulfurization brine using the H_2-based membrane biofilm reactor (MBfR)," *Water Science & Technology*, vol. 63, no. 12, pp. 2923–2928, 2011.

[21] S. W. van Ginkel, R. Lamendella, W. P. Kovacik Jr, J. W. Santo Domingo, and B. E. Rittmann, "Microbial community structure during nitrate and perchlorate reduction in ion-exchange brine using the hydrogen-based membrane biofilm reactor (MBfR)," *Bioresource Technology*, vol. 101, no. 10, pp. 3747–3750, 2010.

[22] M. C. Ziv-El and B. E. Rittmann, "Systematic evaluation of nitrate and perchlorate bioreduction kinetics in groundwater using a hydrogen-based membrane biofilm reactor," *Water Research*, vol. 43, no. 1, pp. 173–181, 2009.

[23] S. Velizarov, J. G. Crespo, and M. A. Reis, "Removal of inorganic anions from drinking water supplies by membrane bio/processes," *Reviews in Environmental Science and Bio/Technology*, vol. 3, no. 4, pp. 361–380, 2004.

[24] N. van Breemen and H. F. G. van Dijk, "Ecosystem effects of atmospheric deposition of nitrogen in the Netherlands," *Environmental Pollution*, vol. 54, no. 3-4, pp. 249–274, 1988.

[25] H. Liu, W. Jiang, D. Wan, and J. Qu, "Study of a combined heterotrophic and sulfur autotrophic denitrification technology

for removal of nitrate in water," *Journal of Hazardous Materials*, vol. 169, no. 1-3, pp. 23–28, 2009.

[26] A. Marietou, "Nitrate reduction in sulfate-reducing bacteria," *FEMS Microbiology Letters*, vol. 363, no. 15, 2016.

[27] B. E. Rittmann, R. Nerenberg, K. C. Lee et al., "Hydrogen-based hollow-fiber membrane biofilm reactor (MBfR) for removing oxidized contaminants," *Water Science and Technology: Water Supply*, vol. 4, no. 1, pp. 127–133, 2004.

[28] Y. Zhang, F. Zhong, S. Xia, X. Wang, and J. Li, "Autohydrogenotrophic denitrification of drinking water using a polyvinyl chloride hollow fiber membrane biofilm reactor," *Journal of Hazardous Materials*, vol. 170, no. 1, pp. 203–209, 2009.

[29] Y. Zhang, L. Huang, Z. Zhang, C. Sun, and J. Li, "Actual application of a H_2-based polyvinyl chloride hollow fiber membrane biofilm reactor to remove nitrate from groundwater," *Journal of Chemistry*, vol. 2015, 7 pages, 2015.

[30] S. Xia, Y. H. Zhang, and F. H. Zhong, "A continuous stirred hydrogen-based polyvinyl chloride membrane biofilm reactor for the treatment of nitrate contaminated drinking water," *Bioresource Technology*, vol. 100, no. 24, pp. 6223–6228, 2009.

[31] Y. Tang, A. Ontiveros-Valencia, L. Feng, C. Zhou, R. Krajmalnik-Brown, and B. E. Rittmann, "A biofilm model to understand the onset of sulfate reduction in denitrifying membrane biofilm reactors," *Biotechnology and Bioengineering*, vol. 110, no. 3, pp. 763–772, 2013.

[32] J. G. de Lomas, A. Corzo, J. M. Gonzalez, J. A. Andrades, E. Iglesias, and M. J. Montero, "Nitrate promotes biological oxidation of sulfide in wastewaters: experiment at plant-scale," *Biotechnology and Bioengineering*, vol. 93, no. 4, pp. 801–811, 2006.

[33] J. Chung, R. Nerenberg, and B. E. Rittmann, "Bio-reduction of soluble chromate using a hydrogen-based membrane biofilm reactor," *Water Research*, vol. 40, no. 8, pp. 1634–1642, 2006.

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Research Article

Bioaugmentation with Mixed Hydrogen-Producing Acetogen Cultures Enhances Methane Production in Molasses Wastewater Treatment

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Hydrogen-producing acetogens (HPA) have a transitional role in anaerobic wastewater treatment. Thus, bioaugmentation with HPA cultures can enhance the chemical oxygen demand (COD) removal efficiency and CH_4 yield of anaerobic wastewater treatment. Cultures with high degradation capacities for propionic acid and butyric acid were obtained through continuous subculture in enrichment medium and were designated as Z08 and Z12. Bioaugmentation with Z08 and Z12 increased CH_4 production by glucose removal to 1.58. Bioaugmentation with Z08 and Z12 increased the COD removal rate in molasses wastewater from 71.60% to 85.84%. The specific H_2 and CH_4 yields from COD removal increased by factors of 1.54 and 1.63, respectively. Results show that bioaugmentation with HPA-dominated cultures can improve CH_4 production from COD removal. Furthermore, hydrogen-producing acetogenesis was identified as the rate-limiting step in anaerobic wastewater treatment.

1. Introduction

High-strength organic wastewater and municipal sludge can be efficiently treated through anaerobic processes, which produce CH_4 as the main product [1]. The microbial cultures used in anaerobic wastewater treatment are highly complex and include fermentative bacteria, hydrogen-producing acetogens (HPA), and methanogenic bacteria (MB) [2, 3]. HPA species are applied in anaerobic wastewater treatment as an alternative to MB, sulfate-reducing bacteria, and other hydrogen-consuming bacteria [4]. However, only a few strains of HPA have been isolated and purified because the species are obligate or facultative anaerobe. HPA mainly converts volatile fatty acids (VFAs) and ethanol into acetic acid, H_2, and CO_2 [5, 6]. The metabolic products of HPA, in turn, promote CH_4 production by MB [7].

Propionate acid tends to accumulate in high-strength organic wastewater, and the COD removal efficiency from wastewater decreases with increasing influent COD [8]. Previous studies attributed this phenomenon to methanogenesis because MB has a slow growth rate, narrow ecological niche, and stringent requirements for living conditions [9, 10]. In addition, VFA degradation is the rate-limiting step in anaerobic wastewater treatment because it is subject to the acetic acid degradation pathway and can decelerate and decrease acetic acid conversion [10, 11]. The degradation of propionate and butyrate acids by HPA cannot proceed spontaneously under normal conditions because it requires energy consumption [6]. By contrast, the terminal product CH_4 can be spontaneously produced under normal conditions when acetic acid, H_2, and CO_2 are present in sufficient amounts [6]. This phenomenon indicates that the substrate

conversion capacity of MB is higher than that of HPA. Therefore, hydrogen-producing acetogenesis likely exerts considerable influence on the effectiveness of anaerobic wastewater treatment. The growth rate of HPA is as typically as slow as that of MB [12, 13]. HPA, however, requires more rigorous living conditions than MB [14]. Thus, HPA could potentially become the rate-limiting factor in anaerobic wastewater treatment under certain conditions.

HPA is a strictly anaerobic eubacteria, and most HPA species are mutualists [15, 16]. The latter characteristic implies that the growth and metabolism of HPA completely depend on the presence of other microorganisms, such as methanogens [17]. McInerney and Bryant [4] and McInerney et al. [12] isolated four HPA strains that can degrade butyrate; comprehensively analyzed the growth, metabolism, phosphatidic acid composition, and nutrition of the isolates; and established the Syntrophomonadaceae family through 16S rRNA sequencing analysis [18]. Medium-temperature propionic acid-oxidizing bacteria [19] have been recently obtained in fumarate culture medium. These bacteria exhibited remarkable activity in propionate oxidation associated with sulfate reduction. *Syntrophobotulus glycolicus*, *Syntrophothermus lipocalidus*, *Sporomusa sphaeroides*, and *Moorella thermoacetica* have been subsequently isolated [20–23]. However, given that pure HPA cultures are difficult to obtain, the ability of a HPA-dominated coculture of anaerobic microbes to enhance CH_4 production and contaminant removal should be investigated [10, 24].

The effectiveness of anaerobic wastewater treatment depends mainly on the enrichment of functional microorganisms [25, 26]. The performance of anaerobic wastewater treatment can be improved through bioaugmentation, which involves the addition of specific strains or dominated flora to the reaction system [27]. Bioaugmentation accelerates the start-up and maintains the stability of bioreactors and enhances the conversion rate of complex substrates. The methane production increased at least 38% [26, 27] and has increased total biogas and CH_4 yields through COD removal [13, 14]. In addition, the ability of propionate-oxidizing and butyrate-oxidizing HPA to enhance CH_4 production has been investigated.

In this work, cultures dominated by propionate-oxidizing and butyrate-oxidizing HPA were obtained from anaerobic sludge through enrichment culture. The organic substrate degradation capability of the propionate-oxidizing and butyrate-oxidizing microflora was investigated through batch cultures. The enhancement in CH_4 production and COD removal rates by bioaugmentation with the mixed HPA culture was evaluated.

2. Materials and Methods

2.1. Seed Sludge and Enrichment Medium. The original anaerobic activated sludge used to screen for HPA-dominated cultures was collected from an anaerobic baffled reactor [28]. The enrichment medium, micronutrient solution, and vitamin solution were prepared as described by Liu et al. [13] and Wang et al. [14]. 10 mL of anaerobic sludge sampled and inoculated to 300 mL serum bottles, and each bottle contained 100 mL propionic acid or butyric acid enrichment medium. The serum bottles were purged with nitrogen gas for 20 min and then cultivated under shaking at 130 r/min and 35°C. Only when the consumption of propionic acid or butyric acid was up to 85% that 10 mL of bacterial suspension was extracted and injected as inocula for the subsequent batch cultures. The successful enrichment of HPA-dominated cultures (Z08 for HPA-dominated culture that oxidized propionic acid; Z12 for HPA-dominated culture that oxidized butyric acid) depended on the rate of CH4 production from propionic acid and butyric acid [13, 14].

2.2. Glucose and Molasses Wastewater. Glucose wastewater contained 5000 mg/L of glucose and was modified with 1000 mg/L of NH_4Cl, 600 mg/L of NaCl, 200 mg/L of $FeCl_2$, 300 mg/L of KH_2PO_4, and 300 mg/L of K_2HPO_4. The COD of molasses wastewater was 8000 mg/L. To maintain the bioactivity of the anaerobic activated sludge, NH_4Cl and K_2HPO_4 were added at a COD : N : P ratio of 500 : 8 : 1. $NaHCO_3$ was used to adjust the initial pH value of the wastewater to 7.8–8.0.

2.3. Bioaugmentation Batch Test. Bioaugmentation batch tests were conducted to evaluate the effect of HPA-dominated microflora. Four serum bottles (500 mL) were used for glucose degradation. Each serum bottle contained 300 mL of glucose wastewater and 30 mL of anaerobic activated sludge. The original mixed liquor volatile suspended solids (MLVSS) of anaerobic sludge, Z08, and Z12 was 12400 mg/L, 2500 mg/L, and 3600 mg/L, respectively; in particular, the MLVSS of anaerobic sludge, Z08, and Z12 were uniformly diluted to 350 mg/L to maintain the initial MLVSS which was equal in each sample. Each serum bottle contained biomass at the rate of 40 mg MLVSS/L. The experimental scheme for bioaugmented glucose wastewater treatment was designed as follows: FH1 (30 mL of anaerobic activated sludge), FH2 (27 mL of anaerobic activated sludge and 3 mL of Z08), FH3 (26 mL of anaerobic activated sludge and 4 mL of Z12), and FH4 (27 mL of anaerobic activated sludge, 1.8 mL of Z08, and 1.2 mL of Z12). Four serum bottles (500 mL) were utilized for normal molasses wastewater treatment. Each serum bottle contained 240 mL of normal molasses wastewater and 40 mL of anaerobic activated sludge. Each serum bottle contained biomass in the form of MLVSS at the rate of 50 mg MLVSS/L. The experimental scheme for bioaugmented molasses wastewater treatment was designed as follows: QJ1 (40 mL of anaerobic activated sludge), QJ2 (36 mL of anaerobic activated sludge and 4 mL of Z08), QJ3 (35 mL of anaerobic activated sludge and 5 mL of Z12), and QJ4 (36 mL of anaerobic activated sludge, 2.0 mL of Z08, and 2.0 mL of Z12). All serum bottles were cultivated under shaking at 130 r/min and 35°C.

2.4. Iodonitrotetrazolium Chloride–Dehydrogenase. Dehydrogenase is an organic macromolecule that is secreted by microorganisms. It is used as an index for the evaluation of the bioactivity of anaerobic activated sludge [29]. Iodonitrotetrazolium chloride (INT) has low redox potential (+90 mV). This characteristic indicates that INT has high

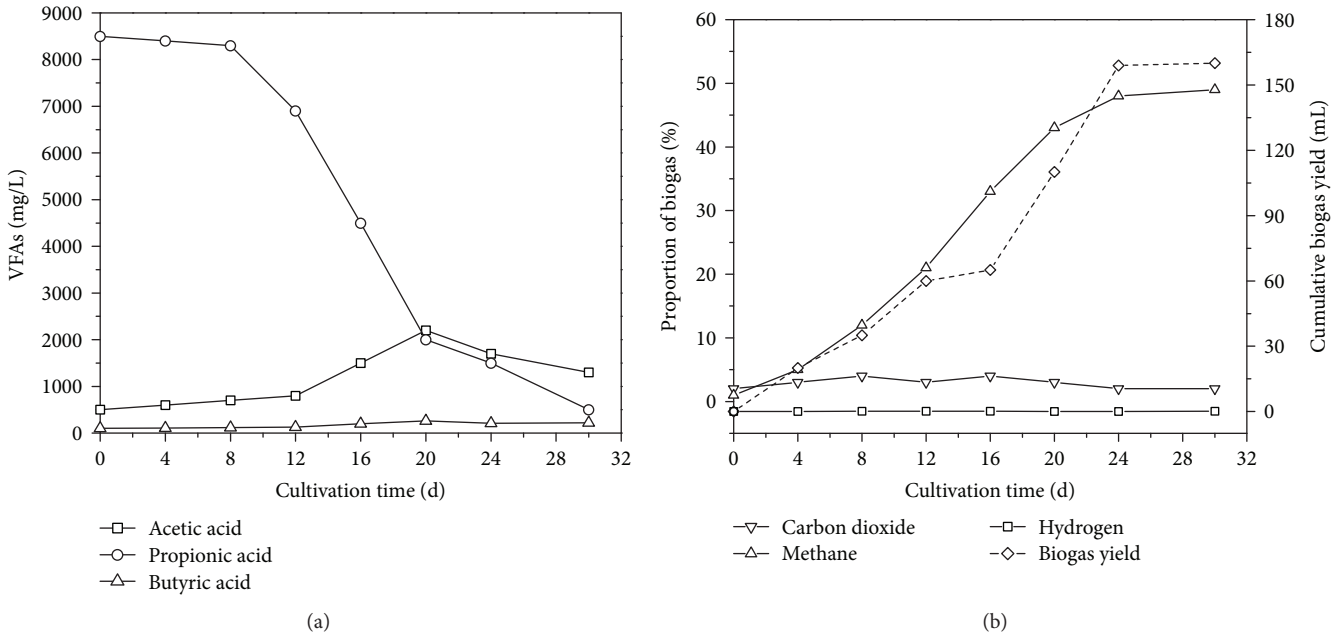

Figure 1: Performance of Z08 through propionic acid degradation (a) and methane production (b).

electron affinity [30] and suggests that dehydrogenase activity can be measured on the basis of INT activity. Dehydrogenase activity (UI) can be calculated using

$$UI = 15.15 \cdot \frac{A}{W}, \quad (1)$$

where UI is the dehydrogenase activity (μmol INT/g·min), A denotes the absorbance of the extract liquor, and W represents biomass content (MLVSS, mg).

2.5. Analytical Methods. COD and MLVSS values were measured in accordance with standard methods [31]. Glucose was measured through the phenol–sulfuric acid method [32]. The biogas yield in each bottle was measured periodically using 5 and 50 mL syringes, and biogas constituents (H_2, CO_2, and CH_4) were characterized through gas chromatography (Lunan SC-7, China). The components of ethanol and VFAs (acetic acid, propionic acid, and butyric acid from the liquid phase of the reaction system) were analyzed through gas chromatography (AAC GC-112, China). The experiment was run in triplicate.

3. Results and Discussion

3.1. Enrichment of HPA

3.1.1. Propionate-Oxidizing HPA. Z08, a mixed culture dominated by propionate-oxidizing HPA, was successfully obtained after ten generations of continuous subculture. As listed in Supplementary Table 1, the acetic acid yield and accumulative H_2 yield was 1007.9 mg/L and 49.2 mL, respectively, indicating that the propionate-oxidizing HPA performed well in propionic acid degradation and supplied sufficient substances for methane production. The conversion rate of propionic acid was 18.5 mmol/gMLVSS·d, and the rate of methane production from propionic acid was 0.49. As shown in Figure 1(a), bioaugmentation with Z08 rapidly decreased propionic acid concentration from 8436.71 mg/L to 8083.74 mg/L and increased acetic acid concentration from 524.61 mg to 701.43 mg. This result indicates that Z08 has good adaptation performance. After 9 days of inoculation with Z08, propionic acid concentration significantly decreased from 8083.74 mg/L to 2008.91 mg/L, whereas acetic acid concentration increased from 701.43 mg/L to 2251.49 mg/L. The H_2 and CO_2 contents of the biogas increased from 0.06% to 0.09% and from 11.53% to 18.76% (Figure 1(b)), respectively, whereas CH_4 content sharply increased to 45.42%. However, the degradation of propionic acid slowed down and decreased to 351.14 mg/L after 30 days of subculture. The accumulated acetic acid concentration was 1203.53 mg/L. The cumulative biogas yield was 161 mL, and H_2, CH_4, and CO_2 contents were 0.12%, 49.14%, and 11.27%, respectively. In addition, the terminal pH value of the entire reaction system stabilized at 7.30–7.40. This pH range is suitable for enhanced propionic acid removal and CH_4 production [33]. The average degradation rate of propionic acid under bioaugmentation with Z08 was 269.5 mg/L·d. The conversion rate of propionic acid was 22.1 mmol/gMLVSS·d, and the rate of CH_4 production from propionic acid was 0.41.

Propionic acid degradation can be divided into three stages on the basis of two distinct turning points. The first stage is the adaptation stage and occurred from days 0 to 8 of degradation. During this stage, propionic acid degradation was low. The second stage occurred from days 9 to 20 of degradation. During this stage, the microorganisms in Z08 adapted to the new living conditions and actively degraded propionic acid. Most of propionic acid was consumed through the synergistic action of MB [13, 34]. The third stage occurred from days 21 to 30 of degradation. As the propionic

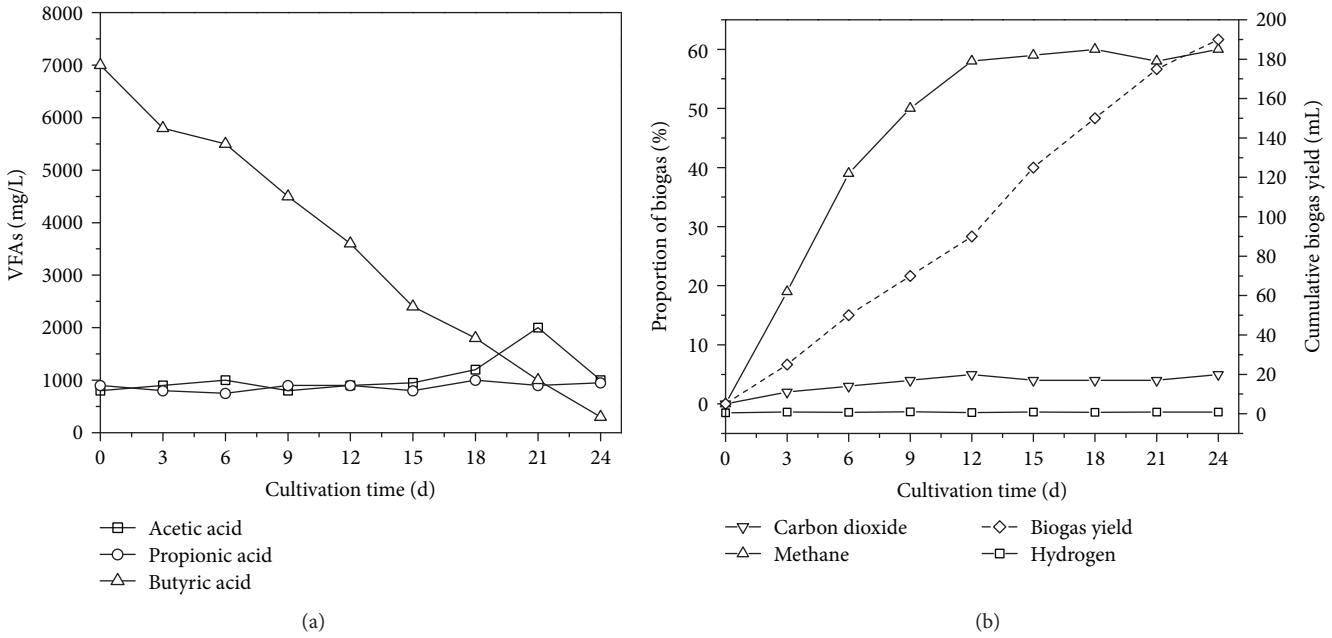

Figure 2: Performance of Z12 through butyric acid degradation (a) and methane production (b).

acid content of the culture medium decreased, microbial activity was reduced because the microorganisms in Z08 competed with one another. In addition, excessive acetic acid generation during stage 2 triggered feedback inhibition as shown by (2). Feedback inhibition then decelerated propionic acid degradation [35]. However, the bioactivity of MB in Z08 was not inhibited, and propionic acid concentration decreased again when acetic acid was converted to CH_4 by MB.

$$CH_3CH_2COOH + H_2O \longrightarrow 2CH_3COOH + 3H_2 + CO_2 \triangle G^{0\prime} = +76.1\,\text{kJ/mol} \quad (2)$$

3.1.2. Butyrate-Oxidizing HPA. Z12, a mixed culture dominated by butyrate-oxidizing HPA, was successfully obtained after seven generations of continuous subculture. As listed in Supplementary Table 2, the acetic acid yield and accumulative H_2 yield was 900.7 mg/L and 51.6 mL, respectively, indicating that the butyrate-oxidizing HPA presented good capacity in butyric acid degradation and provided sufficient substances for methane production. The conversion rate of butyric acid was 15.5 mmol/gMLVSS·d, and the rate of methane production from butyric acid was 0.75. As illustrated in Figure 2(a), over 3 days of inoculation with Z12, butyric acid concentration decreased from 7063.64 mg/L to 5727.3 mg/L, and acetic acid concentration increased from 659.88 mg/L to 788.59 mg/L. H_2, CH_4, and CO_2 concentrations in biogas increased by 0.06%, 18.68%, and 4.64% (Figure 2(b)), respectively, indicating that HPA in Z12 had begun to degrade butyric acid into acetic acid, H_2, and CO_2 to provide substrates for MB in Z12. However, butyric acid degradation slowed down from days 4 to 6 along with the treatment process, and butyric acid content remained at 5500 mg/L. Subsequently, butyric acid concentration sharply decreased from 5457.28 mg/L to 776.29 mg/L, and the cumulative acetic acid concentration peaked at 1762.43 mg/L. The H_2 content of the biogas peaked on day 9, and CH_4 and CO_2 content also rapidly increased. Butyric acid concentration decreased to 211.83 mg/L on day 24, whereas acetic acid concentration gradually decreased on day 21. Moreover, the H_2 content of the biogas also declined. The terminal concentrations of butyric acid and acetic acid were 211.83 and 827.65 mg/L, respectively. The cumulative biogas yield was 191 mL, and CH_4 and CO_2 contents reached as high as 60.76% and 16.45%, respectively. The final pH value of the whole reaction system stabilized at 7.40–7.50, which is desirable for good butyric acid removal and CH_4 production. The average degradation rate of butyric acid under bioaugmentation with Z12 was 285.5 mg/L·d. The conversion rate of butyric acid was 15.8 mmol/gMLVSS·d, and the rate of CH_4 production from butyric acid was 0.74.

Although the process of butyric acid degradation can also be divided into three phases, it differed from that of propionic acid degradation. Stage I, the acclimation period of Z12, occurred during days 1 to 3 of degradation and was shorter than the acclimation period of Z08. During this stage, Z12 rapidly degraded butyric acid, and acetic acid content increased. During stage II (days 4–6), the degradation rate of butyric acid declined (Figure 2(a)). In contrast to HPA, MB displayed good bioactivity in the reaction system because the methane production rate kept increasing during this stage. The slight accumulation of acetic acid indicated that hydrogenotrophic methanogen was dominant in MB and the community structure of Z12 thus facilitated H2 consumption, which further enhanced butyric acid degradation by HPA [36]. During stage III (days 7–24), HPA efficiently converted butyric acid to acetic acid and H_2, and the acetic acid and H_2 contents of the reaction system increased

Table 1: Biogas yields and methane production performance of FH1 to FH4.

	FH1	FH2	FH3	FH4
Glucose conversion (%)	96	99	99	99
Biogas yield (mL)	140	198.9	205.7	259.9
Maximum specific methane production rate (mmol/gMLVSS·d)	0.89	1.27	1.56	2.26
Rate of methane production from glucose (mol/mol)	1.32	1.60	1.79	2.32
Enhanced ratio of methane production (%)	—	125	224	262

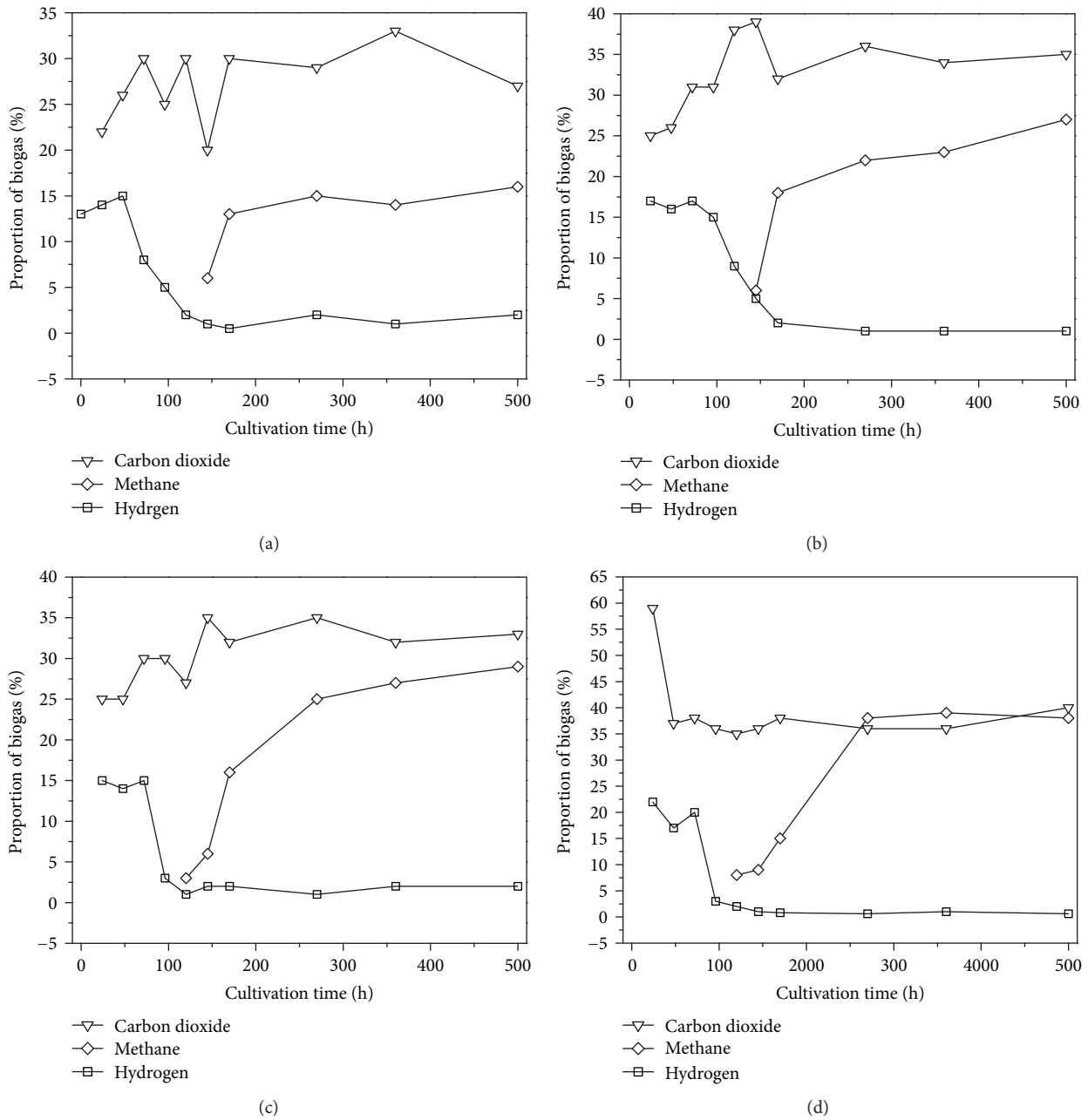

Figure 3: Biogas yields and component variation of QJ1 (a), QJ2 (b), QJ3 (c), and QJ4 (d).

temporarily (Figure 2(b)). By contrast, acetic acid concentration remained low because of the good substrate conversion efficiency of MB.

3.1.3. Rate-Limiting Step of Anaerobic Wastewater Treatment. In general, acetic acid degradation by MB is an energy-reducing reaction that can occur spontaneously under

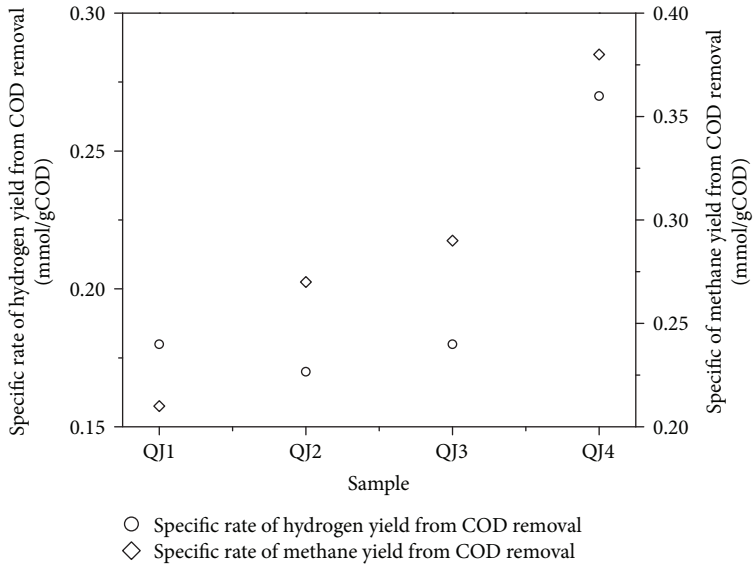

Figure 4: Specific hydrogen and methane production ratio by COD removal.

standard conditions. By contrast, as shown by (3), butyric acid degradation by HPA cannot occur spontaneously under standard conditions [35]. This behavior implies that the degradation of acetic acid by MB is easier than that of butyric acid by HPA.

$$CH_3CH_2CH_2COOH + 2H_2O \longrightarrow 2CH_3CH_2OH + 2H_2 \triangle G^{0\prime} = +48.1 \quad (3)$$

Although hydrogenotrophic methanogens could not deplete H_2 in time and decrease pH_2, acetogenic methanogens converted acetic acid into CH_4 in the culture medium [37]. Therefore, the reduction in acetic acid concentration could promote the degradation of butyric acid.

Similarly, propionate degradation by HPA cannot proceed spontaneously under normal conditions because this reaction requires energy consumption [6]. Nevertheless, propionic acid degradation could be enhanced by decreasing H_2 concentration. Furthermore, propionic acid degradation requires a low system pH_2 given its high standard Gibbs-free energy [38, 39]. In accordance with hydrogen partial pressure theory, propionic acid was rapidly degraded when pH_2 was low, and propionic acid degradation slowed down when H_2 accumulated (Figure 1).

Moreover, the acetic acid concentration of the culture medium was maintained at approximately 1000 mg/L throughout the reaction (Figures 1(a) and 2(a)) because of the presence of MB, which could release feedback inhibition on propionic and butyric acid accumulation. Although the degradation of butyric acid in stage II was less and thus resulted in the accumulation of acetic acid (Figures 2(a) and 2(b)), methane production still increased, emphasizing that the rate-limiting step was not methanogenesis. The high CH_4 yield implied the good bioactivity of MB and that the rate-limiting step of propionic acid and butyric acid degradation can be attributed to HPA [8, 40].

3.2. Performance of Mixed HPA Culture in Glucose Degradation. The biogas yield, maximum specific CH_4 production rate, and CH_4 production rate from glucose in FH4 were higher than those in FH1, FH2, and FH3 (Table 1). The contents of terminal VFAs (acetic acid, propionic acid, and butyric acid) in FH4 (139, 109, and 297 mg/L) were markedly lower than those in FH3 (189, 149, and 433 mg/L). These results indicated that the mixed HPA culture and the anaerobic activated sludge exhibit high glucose conversion rates. Bioaugmentation enhanced the rate of CH_4 production from glucose, and the variation in pH corresponded to the variation in glucose degradation by the dominant microflora. The initial pH was maintained at 8.0. The acidification ratio reached 42.3% as glucose degradation proceeded [41], causing the pH value to decrease to 5.7. This pH value is unfavorable for MB [33]. Thereafter, the pH value recovered to 7.1 through the synergy of HPA and MB. This effect was particularly pronounced under high acetic acid conversion rates. The two types of dominant bacteria (Z08 and Z12) grew independently and performed specific microbial activities. The promoting effects of these activities on high-strength organic wastewater treatment require further study.

3.3. Performance of Mixed HPA Culture in Normal Molasses Wastewater Treatment

3.3.1. Biogas Components and Yields. As shown in Figure 3, the majority of the substrates in molasses wastewater were converted to H_2, CO_2, and CH_4. These results indicate that bioaugmentation improves resource recovery. All reaction systems provided high H_2 yields during the initial stages of treatment, and QJ4 provided the highest H_2 yield (23.76%) among all reaction systems. H_2 content remained as high as 15% for the first 72 h of treatment and subsequently declined. By contrast, CH_4 was not detected, indicating that homoacetogenic bacteria in the

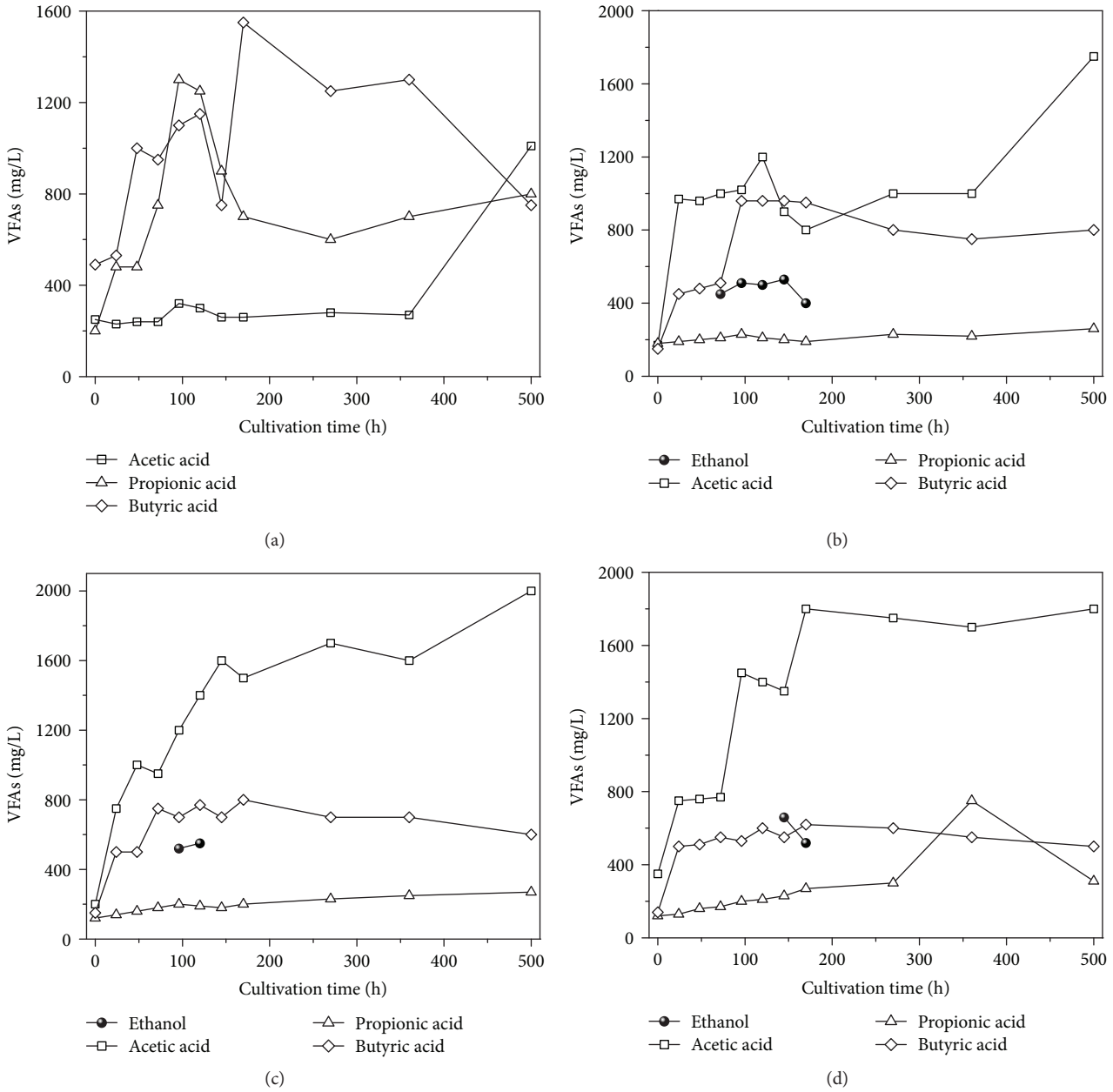

Figure 5: Terminal soluble products through normal molasses conversion of QJ1 (a), QJ2 (b), QJ3 (c), and QJ4 (d).

reaction system utilized H_2 and CO_2 to produce acetic acid [42]. CH_4 was detected after 120 h in QJ3 and QJ4 and after 145 h in QJ1 and QJ2. These results imply that the bioactivity of the butyric-oxidizing HPA is higher than that of the propionic-oxidizing HPA [35]. The CH_4 contents of the QJ2, QJ3, and QJ4 systems remained above 25% during acetogenesis, and the CH_4 content of QJ4 reached as high as 37%. However, the CH_4 content of QJ1 was only approximately 15% because HPA has low acetic acid, H_2, and CO_2 conversion capacities. The anaerobic activated sludge modified with the mixed HPA culture could produce sufficient substrates for MB because ethanol, propionic acid, and butyric acid, as indicated by the quick and efficient conversion of the substrates into acetic acid, H_2, and CO_2.

The biogas yields of QJ2, QJ3, and QJ4 were 183, 226, and 252 mL, respectively, and were moderately higher than that of QJ1. The cumulative H_2 yields of QJ1, QJ2, QJ3, and QJ4 were 48.93, 51.21, 56.27, and 89.43 mL, respectively. The cumulative CH_4 yields of QJ1, QJ2, QJ3, and QJ4 were 32.33, 45.97, 49.14, and 61.91 mL, respectively. These results collectively imply that HPA bioaugmentation increases H_2 and CH_4 production and improves molasses conversion. As shown in Figure 4, the specific rates of H_2 and CH_4 yields from COD removal under bioaugmentation with the mixed HPA culture in QJ4 had increased by a factor of 1.54 and 1.63 compared with those in QJ1. The experimental results show that bioaugmentation has a detectable effect and that it can effectively improve the efficiency of anaerobic wastewater treatment.

Table 2: Correlation of specific dehydrogenase activity and COD removal.

	Related parameters	Measurement time of parameters				
		48 h	96 h	270 h	360 h	500 h
QJ1	Specific dehydrogenase activity (μmol INT/g·min)	12.12	6.27	3.52	3.77	5.61
	COD removal (%)	40.90	7.87	7.08	5.51	10.24
	Correlation coefficients			0.9609		
QJ2	Specific dehydrogenase activity (μmol INT/g·min)	16.16	7.36	5.13	4.86	5.73
	COD removal (%)	45.70	13.39	3.94	5.51	11.81
	Correlation coefficients			0.9924		
QJ3	Specific dehydrogenase activity (μmol INT/g·min)	15.15	7.79	5.13	4.55	6.45
	COD removal (%)	39.40	19.69	4.72	5.51	14.17
	Correlation coefficients			0.9841		
QJ4	Specific dehydrogenase activity (μmol INT/g·min)	22.22	9.52	6.16	5.83	7.00
	COD removal (%)	38.60	17.32	13.39	5.51	11.02
	Correlation coefficients			0.9776		

3.3.2. Terminal Soluble Products. As illustrated in Figure 5, the degradation of molasses wastewater by anaerobic activated sludge and HPA was inconsistent with that of glucose wastewater. Ethanol could be detected after 6 h of glucose degradation and after 72 h of molasses degradation. These results indicate that the mixed HPA culture can effectively convert ethanol into acetic acid, H_2, and CO_2 [43]. In addition, ethanol was not detected in QJ, suggesting that bioaugmentation with Z08 and Z12 promotes ethanol conversion from molasses and thereby decreases the possibility of propionic acid and butyric acid conversion from molasses. The conversion of ethanol into acetic acid is a spontaneous reaction [35]. Therefore, the substrate conversion rate increased in QJ2, QJ3, and QJ4 under relatively high ethanol content (500 mg/L). HPA-dominated microflora has a transitional role in anaerobic wastewater treatment [34, 44], thus enhancing resource recovery (Figure 4).

In QJ1, no characteristics of VFA degradation were observed, and the terminal acetic acid, propionic acid, and butyric acid contents were 1000, 780, and 770 mg/L, respectively, after 500 h of degradation. By contrast, in QJ2, QJ3, and QJ4, propionic and butyric acid degradation showed clear trends and improved as acetic acid content increased. The terminal acetic acid, propionic acid, and butyric acid contents were 1751, 230, and 847 mg/L in QJ2, respectively; 2047, 220, and 590 mg/L in QJ3, respectively; and 1841, 375, and 580 mg/L in QJ4, respectively. The propionic acid and butyric acid contents in QJ2, QJ3, and QJ4 were significantly lower than those in QJ1 because propionic acid and butyric acid could be effectively degraded by the HPA-dominated culture, and microbial metabolic products could be utilized by MB. Moreover, at 72–120 h of the reaction, acetic acid content considerably increased, H_2 content decreased, and CH_4 was not detected in QJ4 (Figures 3(d) and 5(d)). These results imply that homoacetogenesis has occurred in the reaction system. The initial pH value of the reaction system was 8.10, which then sharply decreased to 4.50 within the first 48 h of the reaction because a large amount of VFAs were produced through acidogenesis [6, 41]. Correspondingly, H_2 conversion increased. Thereafter, given the synergism of HPA and MB, propionic acid, butyric acid, acetic acid, H_2, and CO_2 were successively utilized, and the pH value of the reaction system was maintained at approximately 7.00.

3.3.3. Correlation of Specific Dehydrogenase Activity and COD Removal. The COD removal efficiencies in QJ1, QJ2, QJ3, and QJ4 were 71.7%, 80.3%, 83.5%, and 85.8%, respectively, after 500 h of anaerobic treatment. In QJ4, bioaugmentation with the mixed HPA culture increased substrate degradation and CH_4 production. In addition, specific dehydrogenase activity was measured on the basis of INT throughout the process of molasses wastewater treatment. The specific dehydrogenase activity in QJ4 was significantly higher than that in QJ1, indicating that bioaugmentation with Z08 and Z12 improves microbial activity. The correlation coefficients between specific dehydrogenase activity and COD removal in the four systems were 0.9609, 0.9924, 0.9841, and 0.9776, as calculated by the CORREL function (Table 2). The experimental results demonstrate that the INT-specific dehydrogenase activity of anaerobic activated sludge is highly correlated with COD removal rate. Thus, the bioactivity of anaerobic activated sludge can be objectively and accurately reflected by INT-specific dehydrogenase activity [45].

4. Conclusion

Mixed cultures dominated by propionic- and butyric-oxidizing HPA were obtained through more than seven generations of continuous subculture. The rate of CH_4 production from propionic acid and butyric acid were 0.41 and 0.74, respectively. Hydrogen-producing acetogenesis was identified as the rate-limiting factor of anaerobic wastewater treatment. Inoculation with the mixed cultures of Z08 and Z12 increased the biogas yield, maximum specific CH_4 production rate, and CH_4 production rate of glucose and molasses wastewater treatment, as well as increased the

specific rates of H_2 and CH_4 yield from COD removal by a factor of 1.54 and 1.63, respectively. The INT-specific dehydrogenase activity of anaerobic activated sludge was highly correlated with COD removal efficiency.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Table S1: metabolic characteristics of the subcultured microflora that oxidize propionic acid. Table S2: metabolic characteristics of the subcultured microflora that oxidize butyric acid. *(Supplementary Materials)*

References

[1] A. T. W. M. Hendriks, J. B. van Lier, and M. K. de Kreuk, "Growth media in anaerobic fermentative processes: the underestimated potential of thermophilic fermentation and anaerobic digestion," *Biotechnology Advances*, vol. 36, no. 1, pp. 1–13, 2018.

[2] P. Antwi, J. Li, P. Opoku Boadi et al., "Functional bacterial and archaeal diversity revealed by 16S rRNA gene pyrosequencing during potato starch processing wastewater treatment in an UASB," *Bioresource Technology*, vol. 235, pp. 348–357, 2017.

[3] C. S. He, P. P. He, H. Y. Yang et al., "Impact of zero-valent iron nanoparticles on the activity of anaerobic granular sludge: from macroscopic to microcosmic investigation," *Water Research*, vol. 127, pp. 32–40, 2017.

[4] M. J. McInerney and M. P. Bryant, "Anaerobic degradation of lactate by syntrophic associations of *Methanosarcina barkeri* and *Desulfovibrio* species and effect of H_2 on acetate degradation," *Applied and Environmental Microbiology*, vol. 41, no. 2, pp. 346–354, 1981.

[5] I. Valdez-Vazquez, A. L. Morales, and A. E. Escalante, "History of adaptation determines short-term shifts in performance and community structure of hydrogen-producing microbial communities degrading wheat straw," *Microbial Biotechnology*, vol. 10, no. 6, pp. 1569–1580, 2017.

[6] J. Li, G. Zheng, J. He, S. Chang, and Z. Qin, "Hydrogen-producing capability of anaerobic activated sludge in three types of fermentations in a continuous stirred-tank reactor," *Biotechnology Advances*, vol. 27, no. 5, pp. 573–577, 2009.

[7] Z. Zhang, P. Gao, J. Cheng, G. Liu, X. Zhang, and Y. Feng, "Enhancing anaerobic digestion and methane production of tetracycline wastewater in EGSB reactor with GAC/NZVI mediator," *Water Research*, vol. 136, pp. 54–63, 2018.

[8] L. Wang, Q. Zhou, and F. Li, "Avoiding propionic acid accumulation in the anaerobic process for biohydrogen production," *Biomass and Bioenergy*, vol. 30, no. 2, pp. 177–182, 2006.

[9] C. Y. Lin, K. Sato, T. Noike, and J. Matsumoto, "Methanogenic digestion using mixed substrate of acetic, propionic and butyric acids," *Water Research*, vol. 20, no. 3, pp. 385–394, 1986.

[10] M. Lange and B. K. Ahring, "A comprehensive study into the molecular methodology and molecular biology of methanogenic Archaea," *FEMS Microbiology Reviews*, vol. 25, no. 5, pp. 553–571, 2001.

[11] J. L. Garcia, B. K. C. Patel, and B. Ollivier, "Taxonomic, phylogenetic, and ecological diversity of methanogenic *Archaea*," *Anaerobe*, vol. 6, no. 4, pp. 205–226, 2000.

[12] M. J. McInerney, M. P. Bryant, and N. Pfennig, "Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens," *Archives of Microbiology*, vol. 122, no. 2, pp. 129–135, 1979.

[13] C. Liu, J. Li, S. Wang, and L. Nies, "A syntrophic propionate-oxidizing microflora and its bioaugmentation on anaerobic wastewater treatment for enhancing methane production and COD removal," *Frontiers of Environmental Science & Engineering*, vol. 10, no. 4, 2016.

[14] S. Wang, J. Li, C. Liu, L. Nies, and J. Li, "Enhanced methane production through bioaugmentation of butyrate-oxidizing hydrogen-producing acetogens in anaerobic wastewater treatment," *Environmental Progress & Sustainable Energy*, vol. 37, no. 1, pp. 367–374, 2018.

[15] M. P. Bryant, E. A. Wolin, M. J. Wolin, and R. S. Wolfe, "*Methanobacillus omelianskii*, a symbiotic association of two species of bacteria," *Archiv für Mikrobiologie*, vol. 59, no. 1–3, pp. 20–31, 1967.

[16] J. Luo, Y. Chen, and L. Feng, "Polycyclic aromatic hydrocarbon affects acetic acid production during anaerobic fermentation of waste activated sludge by altering activity and viability of acetogen," *Environmental Science & Technology*, vol. 50, no. 13, pp. 6921–6929, 2016.

[17] H. Junicke, H. Feldman, M. C. M. van Loosdrecht, and R. Kleerebezem, "Limitation of syntrophic coculture growth by the acetogen," *Biotechnology and Bioengineering*, vol. 113, no. 3, pp. 560–567, 2016.

[18] H. Zhao, D. Yang, C. R. Woese, and M. P. Bryant, "Assignment of fatty acid-β-oxidizing syntrophic bacteria to *Syntrophomonadaceae* fam. nov. on the basis of 16s rRNA sequence analyses," *International Journal of Systematic Bacteriology*, vol. 43, no. 3, 1993.

[19] A. J. Stams, J. Van Dijk, C. Dijkema, and C. M. Plugge, "Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria," *Applied and Environmental Microbiology*, vol. 59, no. 4, pp. 1114–1119, 1993.

[20] M. Friedrich, N. Springer, W. Ludwig, and B. Schink, "Phylogenetic positions of *Desulfofustis glycolicus* gen. nov., sp. nov. and *Syntrophobotulus glycolicus* gen. nov., sp. nov., two new strict anaerobes growing with glycolic acid," *International Journal of Systematic Bacteriology*, vol. 46, no. 4, pp. 1065–1069, 1996.

[21] Y. Sekiguchi, Y. Kamagata, K. Nakamura, A. Ohashi, and H. Harada, "Syntrophothermus lipocalidus gen. nov., sp. nov., a novel thermophilic, syntrophic, fatty-acid-oxidizing anaerobe which utilizes isobutyrate," *International Journal of Systematic and Evolutionary Microbiology*, vol. 50, no. 2, pp. 771–779, 2000.

[22] S. Kato, I. Yumoto, and Y. Kamagata, "Isolation of acetogenic bacteria that induce biocorrosion by utilizing metallic iron as the sole electron donor," *Applied and Environmental Microbiology*, vol. 81, no. 1, pp. 67–73, 2015.

[23] A. Kita, Y. Iwasaki, S. Yano, Y. Nakashimada, T. Hoshino, and K. Murakami, "Isolation of thermophilic acetogens and transformation of them with the *pyrF* and *kan*^{r} genes," *Bioscience, Biotechnology, and Biochemistry*, vol. 77, no. 2, pp. 301–306, 2013.

[24] J. Li, B. Li, G. Zhu, N. Ren, L. Bo, and J. He, "Hydrogen production from diluted molasses by anaerobic hydrogen producing bacteria in an anaerobic baffled reactor (ABR)," *International Journal of Hydrogen Energy*, vol. 32, no. 15, pp. 3274–3283, 2007.

[25] G. Llorens-Blanch, E. Parlade, M. Martinez-Alonso, N. Gaju, G. Caminal, and P. Blanquez, "A comparison between biostimulation and bioaugmentation in a solid treatment of anaerobic sludge: drug content and microbial evaluation," *Waste Management*, vol. 72, pp. 206–217, 2018.

[26] B. E. Öner, Ç. Akyol, M. Bozan, O. Ince, S. Aydin, and B. Ince, "Bioaugmentation with *Clostridium thermocellum* to enhance the anaerobic biodegradation of lignocellulosic agricultural residues," *Bioresource Technology*, vol. 249, pp. 620–625, 2018.

[27] S. Aydin, "Enhancement of microbial diversity and methane yield by bacterial bioaugmentation through the anaerobic digestion of *Haematococcus pluvialis*," *Applied Microbiology and Biotechnology*, vol. 100, no. 12, pp. 5631–5637, 2016.

[28] G. F. Zhu, J. Z. Li, P. Wu, H. Z. Jin, and Z. Wang, "The performance and phase separated characteristics of an anaerobic baffled reactor treating soybean protein processing wastewater," *Bioresource Technology*, vol. 99, no. 17, pp. 8027–8033, 2008.

[29] C. Debnar-Daumler, A. Seubert, G. Schmitt, and J. Heider, "Simultaneous involvement of a tungsten-containing aldehyde:ferredoxin oxidoreductase and a phenylacetaldehyde dehydrogenase in anaerobic phenylalanine metabolism," *Journal of Bacteriology*, vol. 196, no. 2, pp. 483–492, 2014.

[30] W. von mersi and F. Schinner, "An improved and accurate method for determining the dehydrogenase-activity of soils with iodonitrotetrazolium chloride," *Biology and Fertility of Soils*, vol. 11, no. 3, pp. 216–220, 1991.

[31] APHA, *Standard Methods for the Examination of Water and Wastewater*, American Public Health Association, American Water Works Association, Water Environmental Federation, Washington, DC, USA, 21st edition, 2005.

[32] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, "Colorimetric method for determination of sugars and related substances," *Analytical Chemistry*, vol. 28, no. 3, pp. 350–356, 1956.

[33] M. S. Barredo and L. M. Evison, "Effect of propionate toxicity on methanogen-enriched sludge, Methanobrevibacter smithii, and Methanospirillum hungatii at different pH values," *Applied and Environmental Microbiology*, vol. 57, no. 6, pp. 1764–1769, 1991.

[34] G. Zheng, J. Li, F. Zhao et al., "Effect of illumination on the hydrogen-production capability of anaerobic activated sludge," *Frontiers of Environmental Science & Engineering*, vol. 6, no. 1, pp. 125–130, 2012.

[35] Y. Liu and W. B. Whitman, "Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea," *Annals of the New York Academy of Sciences*, vol. 1125, no. 1, pp. 171–189, 2008.

[36] T. Amani, M. Nosrati, and S. M. Mousavi, "Using enriched cultures for elevation of anaerobic syntrophic interactions between acetogens and methanogens in a high-load continuous digester," *Bioresource Technology*, vol. 102, no. 4, pp. 3716–3723, 2011.

[37] F. Chaucheyras, G. Fonty, G. Bertin, and P. Gouet, "In vitro H2 utilization by a ruminal acetogenic bacterium cultivated alone or in association with an archaea methanogen is stimulated by a probiotic strain of saccharomyces cerevisiae," *Applied and Environmental Microbiology*, vol. 61, no. 9, pp. 3466-3467, 1995.

[38] S. de Kok, J. Meijer, M. C. M. van Loosdrecht, and R. Kleerebezem, "Impact of dissolved hydrogen partial pressure on mixed culture fermentations," *Applied Microbiology and Biotechnology*, vol. 97, no. 6, pp. 2617–2625, 2013.

[39] O. Sarkar, S. K. Butti, and S. Venkata Mohan, "Acidogenesis driven by hydrogen partial pressure towards bioethanol production through fatty acids reduction," *Energy*, vol. 118, pp. 425–434, 2017.

[40] C. Gallert and J. Winter, "Propionic acid accumulation and degradation during restart of a full-scale anaerobic biowaste digester," *Bioresource Technology*, vol. 99, no. 1, pp. 170–178, 2008.

[41] H. H. P. Fang and H. Yu, "Mesophilic acidification of gelatinaceous wastewater," *Journal of Biotechnology*, vol. 93, no. 2, pp. 99–108, 2002.

[42] C. Liu, J. Li, Y. Zhang et al., "Influence of glucose fermentation on CO_2 assimilation to acetate in homoacetogen *Blautia coccoides* GA-1," *Journal of Industrial Microbiology & Biotechnology*, vol. 42, no. 9, pp. 1217–1224, 2015.

[43] X. Yang, K. Wang, H. Wang, J. Zhang, L. Tang, and Z. Mao, "Control of pH by acetic acid and its effect on ethanol fermentation in an integrated ethanol–methane fermentation process," *RSC Advances*, vol. 6, no. 63, pp. 57902–57909, 2016.

[44] H. Rajhi, D. Puyol, M. C. Martinez, E. E. Diaz, and J. L. Sanz, "Vacuum promotes metabolic shifts and increases biogenic hydrogen production in dark fermentation systems," *Frontiers of Environmental Science & Engineering*, vol. 10, no. 3, pp. 513–521, 2016.

[45] J. K. Friedel, K. Mölter, and W. R. Fischer, "Comparison and improvement of methods for determining soil dehydrogenase activity by using triphenyltetrazolium chloride and iodonitrotetrazolium chloride," *Biology and Fertility of Soils*, vol. 18, no. 4, pp. 291–296, 1994.

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Research Article

Dynamics of Archaeal and Bacterial Communities in Response to Variations of Hydraulic Retention Time in an Integrated Anaerobic Fluidized-Bed Membrane Bioreactor Treating Benzothiazole Wastewater

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An integrated anaerobic fluidized-bed membrane bioreactor (IAFMBR) was investigated to treat synthetic high-strength benzothiazole wastewater (50 mg/L) at a hydraulic retention time (HRT) of 24, 18, and 12 h. The chemical oxygen demand (COD) removal efficiency (from 93.6% to 90.9%), the methane percentage (from 70.9% to 69.27%), and the methane yield (from 0.309 m^3 CH_4/kg·$COD_{removed}$ to 0.316 m^3 CH_4/kg·$COD_{removed}$) were not affected by decreasing HRTs. However, it had an adverse effect on membrane fouling (decreasing service period from 5.3 d to 3.2 d) and benzothiazole removal efficiency (reducing it from 97.5% to 82.3%). Three sludge samples that were collected on day 185, day 240, and day 297 were analyzed using an Illumina® MiSeq platform. It is striking that the dominant genus of archaea was always *Methanosaeta* despite of HRTs. The proportions of *Methanosaeta* were 80.6% (HRT 24), 91.9% (HRT 18), and 91.2% (HRT 12). The dominant bacterial genera were *Clostridium* in proportions of 23.9% (HRT 24), 16.4% (HRT 18), and 15.3% (HRT 12), respectively.

1. Introduction

The widespread use of antibiotics has generated large volumes of contaminated antibiotic wastewater. Antibiotics have not been degraded entirely even after passing through the processing of conventional wastewater treatment plants (WWTP) [1, 2]. They gradually enter the water environment when wastewater is discharged. Therefore, antibiotics have been detected in surface water [3, 4], groundwater, and soils, generating worldwide attention. The occurrence and release of antibiotics have adversely affected bioreactor treatment in decreasing COD removal efficiency because of their bacterial toxicity [5]. Furthermore, antibiotics are considered to be pollutants because antibiotics and their transformation products may lead to spread/transfer of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) when microbes are exposed to antibiotics in the long term.

Among the processes used for wastewater treatment, anaerobic treatment has some technical advantages, such as the production of methane, lower energy costs, and lower excess sludge production [6]. Anaerobic bioreactors have been used for high-strength organic wastewater treatment, including treatment of contaminated antibiotic wastewater [5, 7]. However, anaerobic bioreactors alone cannot fulfill the demands of stringent effluent standards. To improve effluent quality, some researchers have combined anaerobic and membrane reactors [8–10]. A two-stage anaerobic fluidized-membrane bioreactor reportedly was used to treat municipal wastewater containing 20 pharmaceuticals, achieving pharmaceutical removal efficiencies of 78%–100% [11]. Also, membrane bioreactors could have an advantage in the release of antibiotic-resistant bacteria (genes). Munir et al. have researched the effluent and biosolids of five wastewater utilities in Michigan [12]. They found that membrane

bioreactor has the least release of antibiotic-resistant bacteria (genes) compared to the four other types of wastewater treatment utilities.

Parameters such as hydraulic retention time (HRT), temperature, and solid retention time (SRT) have a significant effect on the performance and running life of a bioreactor. A large number of different combinations of operation conditions have been reported, such as SRT from a few days [13] to about a year [14], temperature from psychrophilic [15] to thermophilic, and HRT from a few hours [16] to a few days [17]. HRT is one of the essential operating conditions, which has a direct influence on the performance of the bioreactor [17]. In the light of different chemical compositions of antibiotic wastewater, it is important to select the corresponding HRT. The change of performance inevitably affects membrane fouling development in AnMBR. It has been reported that a decrease in HRT enhanced accumulation of soluble microbial products (SMP), which accelerated membrane fouling [18]. Our previous research showed the feasibility of an integrated anaerobic fluidized-bed membrane bioreactor treating synthetic benzothiazole wastewater [10]. However, little information is available about the influence of HRT on IAFMBR treating high-strength benzothiazole wastewater.

This study investigated the feasibility of an integrated anaerobic fluidized-bed membrane bioreactor (IAFMBR) to treat high-strength wastewater containing benzothiazole. This research was focused on the impact of hydraulic retention time (HRT) on the performance of the IAFMBR and the succession of microbial community structures.

2. Materials and Methods

2.1. Integrated Anaerobic Fluidized-Bed Membrane Bioreactor. The integrated anaerobic fluidized-bed membrane bioreactor (IAFMBR) was made of 10 mm Plexiglas with a total volume of 8.9 L (effective volume of 6.1 L) [10]. The reactor consisted of an outer tube, a middle tube, an inner tube, a three-phase separator, and a membrane module (Figure 1). The outer tube was filled with anaerobic granular sludge. A hollow fiber membrane (Mitsubishi Rayon Co., Ltd., Tokyo, Japan) was equipped in the inner zone with a total surface membrane area of 0.21 m^2 and a pore diameter of 0.4 μm. The designed membrane flux was 11.3 L/m^2 h.

The IAFMBR consisted of an AFBR (anaerobic fluidized-bed reactor) and an AnMBR (anaerobic membrane bioreactor). The AFBR effluent was treated by anaerobic granular sludge. The IAFMBR effluent was a membrane permeate which was treated by anaerobic granular sludge and membrane.

2.2. Reactor Operation Conditions. The reactor had stably operated for 151 d, including a start-up phase (1–58 d) and an adaptation phase (59–151) (Table 1). In this study, the reactor was operated under HRT of 24 (152–185 d), 18 (186–240 d), and 12 h (241–297 d) (Table 1). Continuous membrane filtration was performed. During the whole experiment, the reactor was wrapped with an electrothermal wire to keep the temperature at 35°C. The SRT was 35 d, and the concentration of benzothiazole was 50 mg/L.

2.3. Inoculation and Feed Composition. The reactor was inoculated with 1.2 L anaerobic granular sludge that was taken from an anaerobic reactor treating wastewater from an alcohol-producing plant in Daqing, China. The MLVSS was 4850 mg/L, and the MLVSS/MLSS was 0.67.

Synthetic wastewater was fed according to the characteristics of antibiotic production wastewater coming from a pharmaceutical factory in Harbin, China. The concentration of benzothiazole was 50 mg/L. Glucose and acetate were used to maintain the COD (2961–3337 mg/L). The other compositions of wastewater were as follows (mg/L): NH_4Cl, 140; urea, 40; KH_2PO4, 45; $MgSO_4$, 55; and $CaCl_2$, 15. The inorganic nutrient composed is according to the previous study [8].

2.4. Sample Analysis. The COD was measured according to standard methods [19]. Biogas production was measured using a wet gas meter. Biogas production was detected using a gas chromatograph (Agilent GC 7890A, USA) with a thermal conductivity detector. The sample of VFAs was filtrated with a 0.45 μm Millipore filter, and it was determined by a gas chromatograph (Agilent GC 7890, USA) equipped with a flame ionization detector. Benzothiazole concentration was detected by a high-performance liquid chromatography instrument (Waters e2695, USA) under ultraviolet detection set at 254 nm with a C18 column (SB-C18, 250 mm × 4.6 mm, Agilent Co., Ltd., USA). The mobile phase, flow rate, and temperature were as described previously[10].

The samples of mixed liquor were taken from the reactor. The sample of cake layer was taken from the membrane by flushing the membrane surface with a certain amount of deionized water. The extraction of EPS and SMP was based on [20]. Both SMP and EPS were quantified through a measurement of protein and polysaccharides. The concentration of proteins was detected by the modified BCA kit (Sangon Biotech Ltd., Shanghai, China) following the manufacturer's protocols. The concentration of polysaccharides was determined by the phenol sulphuric acid method [21].

2.5. Microbial Community Analysis

2.5.1. Sample Collection and DNA Extraction. The sludge samples (HRT 24, 18, and 12 collected on days 185, 240, and 297, resp.) were taken from the AFBR reactor. Total DNA analysis was performed by extraction using a FastDNA SPIN Kit for Soil (MP Biomedicals (Shanghai) Ltd., China) following the manufacturer's protocols.

2.5.2. PCR Amplification and Illumina® MiSeq Method. PCR amplification, production purification, and quantification were afforded by a sequencing company (Sangon Biotech Ltd., Shanghai, China). The extracted DNA was amplified using a set of bar-coded primers 341F and 805R for bacteria. The amplification of archaea DNA was used using nested PCR (two sets of primers). One set of primers was 340F and 1000R, and the other set of primers was 349F and 806R. The composition of different primers was reported in the previous study [10].

The thermocycling steps were as follows: 94°C for 3 min, followed by 5 cycles at 94°C for 30 s, 45°C for 20 s, and 65°C for 30 s; 20 cycles at 94°C for 20 s, 55°C

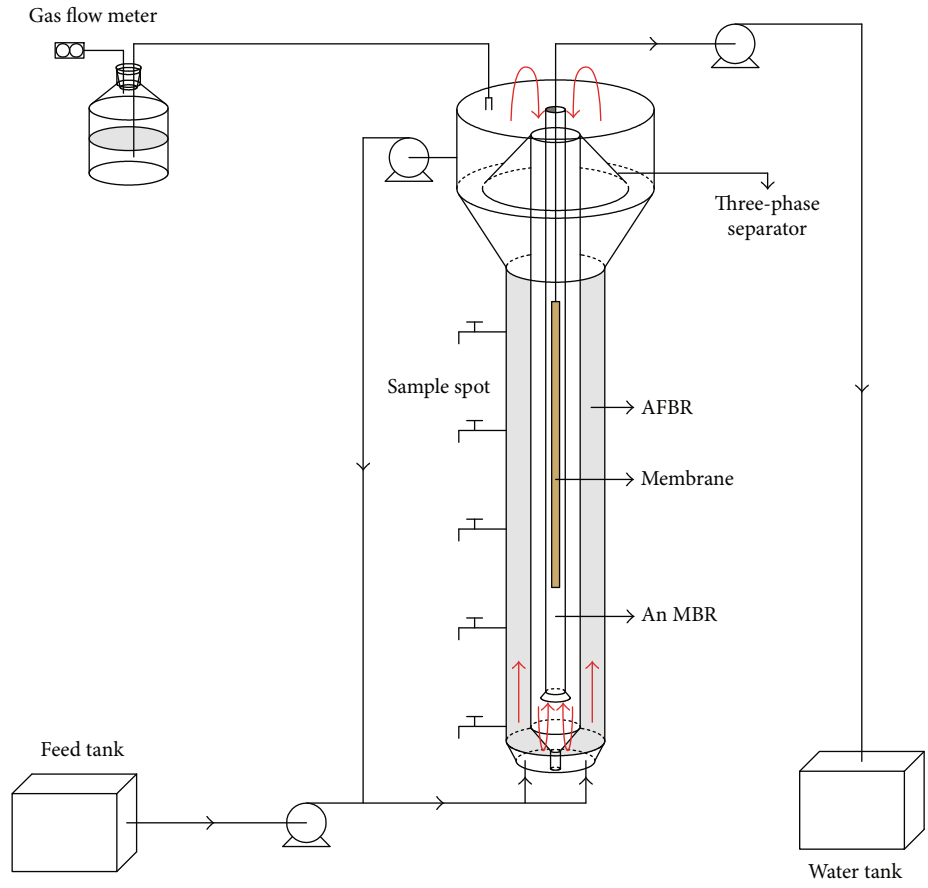

Figure 1: The schematic diagram of the IAFMBR.

Table 1: The summary of operating conditions of IAFMBR system.

Phase	Start-up	Adaptation	HRT 24	HRT 18	HRT 12
Days (d)	1–58 d (58 d)	59–151 d (93 d)	152–185 d (34 d)	186–240 d (55 d)	241–297 d (57)
Benzothiazole (mg/L)	0	1–50	50	50	50
HRT (h)	24	24	24	18	12
Temperature (°C)	35	35	35	35	35
OLR (kg COD/m^3·d)	3.33	3.26	3.13	4.64	6.36

for 20 s, and 72°C for 30 s; and a final extension step at 72°C for 5 min. The PCR productions were sequenced by an Illumina MiSeq high-throughput platform (Sangon Biotech Ltd., Shanghai, China).

2.5.3. Biodiversity Analysis and Phylogenetic Classification. The raw reads were demultiplexed. The adapters, barcode, and primers in all reads were trimmed. Sequences shorter than 200 bp were removed with the PRINSEQ software. The UCHIME software was used to detect chimera sequences [10].

Operational taxonomic units (OTUs) were clustered by 97% similarity (3% dissimilarity level) using the UCLUST algorithm (http://www.drive5.com/uclust/downloads1_1-579.html). The Shannon index and Chao1 index were calculated to compare the diversity and richness of microbial structures [22].

3. Results and Discussion

3.1. Performance of IAFMBR

3.1.1. COD Removal. The variations of COD were investigated during the three phases (Figure 2). In general, the COD removal efficiency of IAFMBR was relatively stable, and the numerical values were 93.6 ± 0.6%, 91.2 ± 1.7, and 90.9 ± 0.9% at the HRT of 24 h, 18 h, and 12 h. For AFBR, the COD removal efficiency was slightly impacted by the HRT. The COD removal efficiency was maintained at about 87.3 ± 0.6% at the HRT of 24 h, and the effluent COD was 398 mg/L. When the HRT was reduced to 18 h, the effluent COD was increased to 828 mg/L at the beginning and then decreased to 467 ± 57 mg/L at a stable period, corresponding to an efficiency of 84.9 ± 2.1%. However, as the HRT was

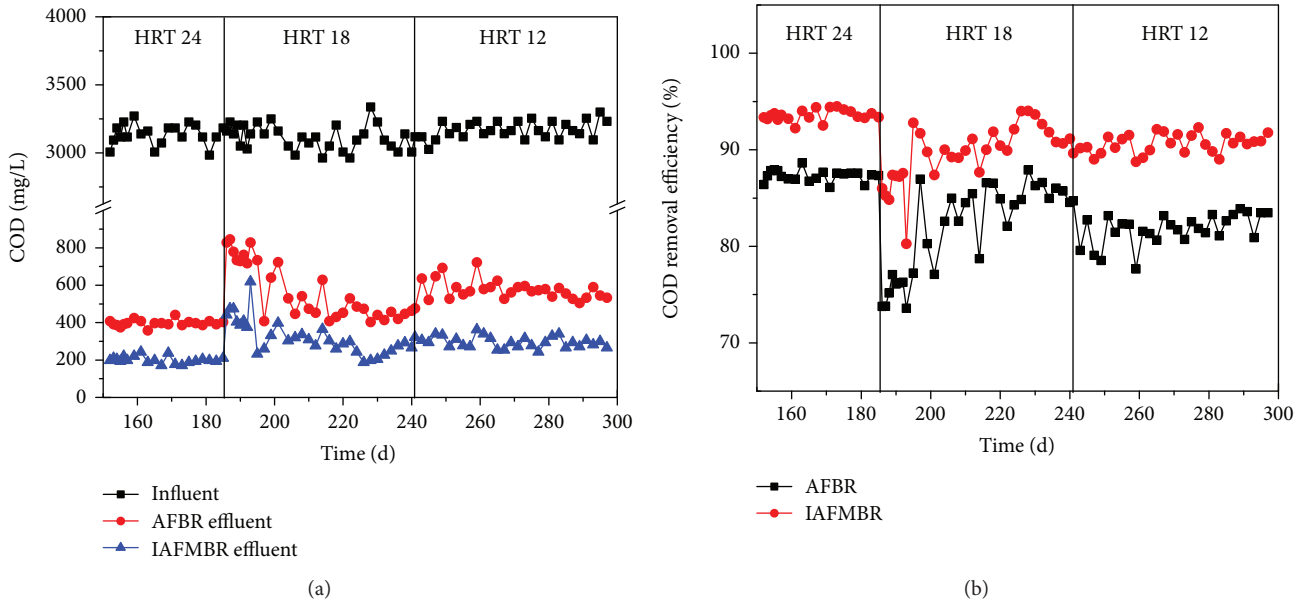

Figure 2: COD removal performance at different HRT. (a) Variations of COD concentration and (b) variations of COD removal efficiency.

reduced to 12 h, the effluent COD was 557 ± 28 mg/L, and the COD efficiency was 82.5 ± 1.1%.

The impact of HRT on the performance has been researched in some studies. The antibiotic wastewater that contained amoxicillin (AMX) was treated by an expanded granular sludge bed (EGSB) at an HRT of 8–20 h, and the COD removal efficiency dropped from 85% to 36.5% [23]. Gao et al. used IAFMBR treating domestic wastewater [8]. They found that the COD removal efficiency obviously decreased from 63.6 ± 2.5% (HRT 8) to 48.4 ± 2.6% (HRT 4). Compared to those studies that were previously mentioned, HRT variations did not obviously affect the COD removal efficiency in this study. This is because the synthetic feed (the main carbon sources were glucose and acetate) is easy to biodegrade by microorganisms.

3.1.2. Benzothiazole Removal. Benzothiazole removal efficiency decreased with the stepwise drop of HRT (Figure 3). The average AFBR (IAFMBR) effluent benzothiazole concentrations were 2.03 ± 0.24 mg/L (1.23 ± 0.27 mg/L), 9.60 ± 1.36 mg/L (7.28 ± 1.36 mg/L), and 12.02 ± 1.71 mg/L (8.99 ± 1.89 mg/L) at the HRT of 24, 18, and 12 h. The benzothiazole removal efficiency of AFBR (IAFMBR) was 96.0 ± 0.5% (97.6 ± 0.5%), 81.1 ± 1.9% (85.7 ± 2.6%), and 76.4 ± 3.4% (82.3 ± 3.7%) at the HRT of 24, 18, and 12 h.

HRT is one of the critical factors that affect the degradation of antibiotics. It has been reported that the main removal pathway of benzothiazole was biodegradation [10]. For biodegradation, the contact time between biodegraded material and sludge was important which affects the treatment efficiency. For AFBR, a lower HRT applied may cause the washout of the functional bacteria that is required for the biodegradation of antibiotics [24]. For IAFMBR, the functional microbe could wash out and into the inner tube. However, the membrane fouling cycle was relatively short resulting in frequent membrane cleaning. The functional microbe could not enrich in the inner tube.

3.1.3. VFA Accumulation. The accumulation and composition of the volatile fatty acids (VFAs) were supervised in different HRT (Figure 4). Acetate was the major component of VFAs in the AFBR effluent, which increased with the change of HRT, and its concentrations in the AFBR effluent were 88.44 ± 11.84 mg/L (HRT 24 h), 206.93 ± 15.58 mg/L (HRT 18 h), and 242.82 ± 9.55 mg/L (HRT 12 h), being accounted as about 73.31%, 69.98%, and 68.26% of total VFAS, respectively. The same phenomenon of acetate accumulation was also indicated in previous studies [8, 10]. Acetate is the substrate for acetotrophic methanogens which play an important role in CH_4 production and for homoacetogenic bacteria, transforming acetate to hydrogen and CO_2 [25].

The increment of propionate increased slightly, and its concentrations were 15.86 ± 3.31 mg/L, 18.84 ± 5.75 mg/L, and 23.01 ± 0.79 mg/L at the HRT of 24, 18, and 12 h in the AFBR effluent. The concentration of butyrate increased from 16.33 ± 3.07 mg/L (HRT 24 h) to 69.93 ± 9.10 mg/L (HRT 18 h) to 89.91 ± 4.14 mg/L (HRT 12 h) in the AFBR effluent, being accounted as about 13.54%, 23.65%, and 25.27% of total VFAs, respectively. It was reported that antibiotics had an adverse effect on butyrate-oxidizing bacteria [25]. In this study, the residual concentration of benzothiazole increased with a decreased HRT (Figure 3), which could inhibit butyrate degradation. A similar inhibition of butyrate degradation was found in other antibiotics [26]. Valerate was not detected during all periods.

In general, tVFA accumulation increased with the declining HRT. The tVFAs in IAFMBR effluent was lower than those in AFBR, which were 57.83 mg/L ± 13.81 mg/L, 154.66 ± 18.50 mg/L, and 171.04 ± 10.88 mg/L at the HRT of 24 h, 18 h, and 12 h.

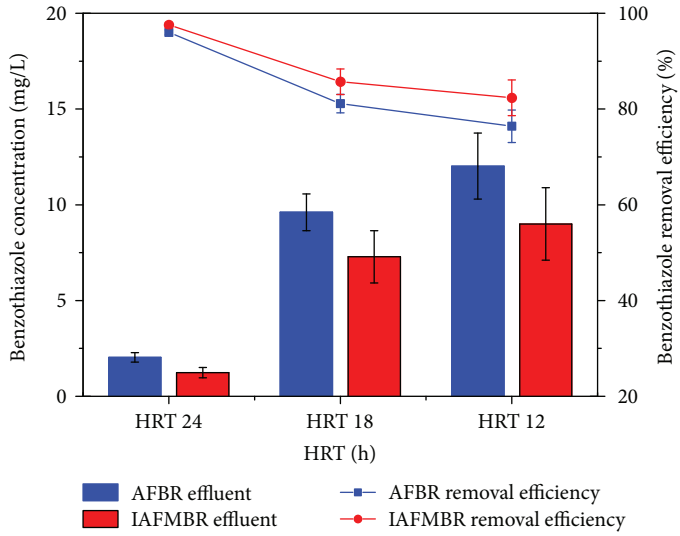

Figure 3: The variations of benzothiazole removal performance at different HRT.

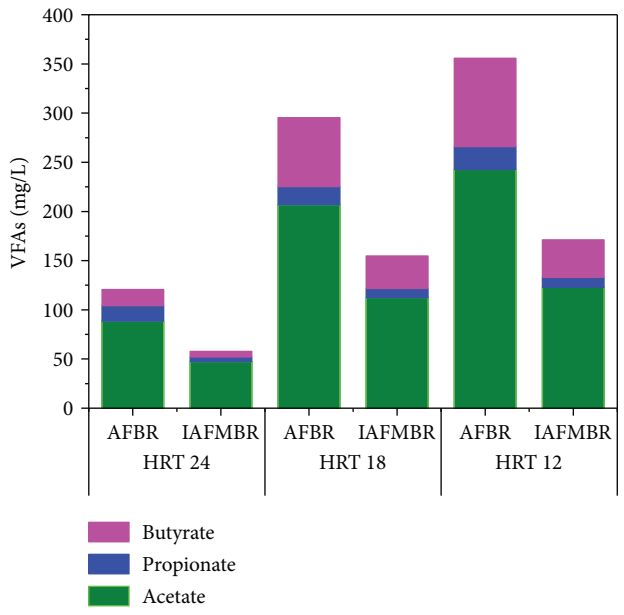

Figure 4: VFA accumulation during different HRT (average value) in AFBR and IAFMBR.

3.1.4. Biogas Production. Biogas production was monitored throughout the three phases of reactor operation (Table 2), particularly for the evaluation of methanogenic activity. The biogas production volume was greatest at HRT of 12 h (21.49 ± 0.39 L/d) compared with that of HRT of 18 (14.00 ± 0.78 L/d) and 24 h (10.74 ± 0.39 L/d). Methane production apparently increased from 7.60 ± 0.26 L/d to 10.29 ± 0.57 L/d to 14.88 ± 1.57 L/d at HRT of 24, 18, and 12 h. These data showed that methane production augmented with an increase in the organic loading rate, which was similar to previous studies [8].

However, the methane percentage was slightly affected by the change of HRT (70.9 ± 0.3%, 73.5 ± 2.1%, and 69.3 ± 1.6% at HRT of 24, 18, and 12 h). About 70% methane content was similar to previous studies [25]. The methane yield is a useful parameter to evaluate the performance of an anaerobic reactor [5]. The methane yield was relatively stable, and the values were 0.309 ± 0.014 m^3 CH_4/kg $COD_{removed}$ (HRT24), 0.327 ± 0.028 m^3 CH_4/kg $COD_{removed}$ (HRT18), and 0.316 ± 0.022 m^3 CH_4/kg $COD_{removed}$ (HRT12), respectively. There are two possible reasons. On the one hand, methanogens were in the anaerobic granular sludge. This structure protected the activity of methanogens. On the other hand, the effect of BTH on the methanogens was not significant.

Table 2: The biogas production at different HRTs (average concentrations at steady-states).

HRT	Biogas production (L/d)	Methane production (L/d)	Methane percentage (%)	Methane yield (m^3 CH_4/kg·$COD_{removed}$)
24	10.74 ± 0.39	7.60 ± 0.26	70.9 ± 0.3	0.309 ± 0.014
18	14.00 ± 0.78	10.29 ± 0.57	73.5 ± 2.1	0.327 ± 0.028
12	21.49 ± 1.26	14.88 ± 1.57	69.3 ± 1.6	0.316 ± 0.022

In order to show the carbon flow, a mass balance (based on COD) was illustrated (Figure 5(a)). About 70% carbons were converted to methane at different HRT. The data of mass balance and methane yield (Table 2) was similar, which showed that the production of methane was not affected by the reducing HRT.

3.2. Membrane Fouling

3.2.1. TMP Fraction. The change of transmembrane pressure (TMP) was used as an indicator of membrane fouling. Clean-up or backflushing was not applied in order to detect the one-time operational duration of membrane fouling. In this experiment, the TMP was collected at the stable period of different HRTs, and the value of TMP reached 16 kPa as membrane fouling.

In general, the trends of the membrane fouling cycle were similar at different HRT (Figure 5(b)). The membrane fouling cycle was 5.3, 3.7, and 3.2 d at HRT of 24, 18, and 12 h.

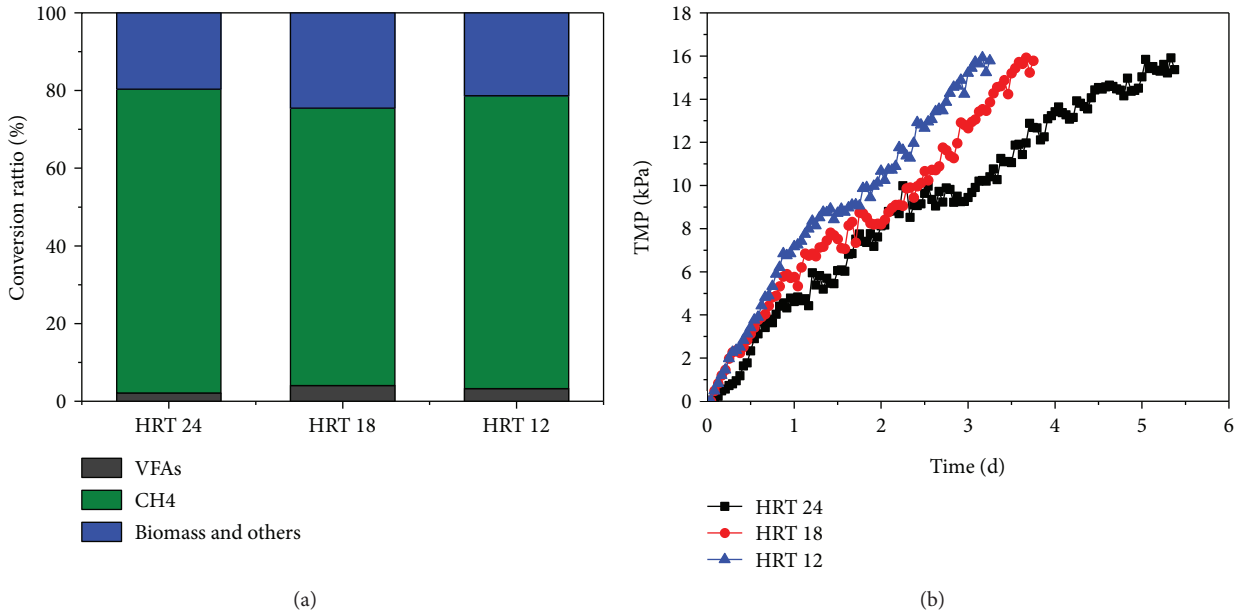

Figure 5: Mass balance and transmembrane pressure (TMP). (a) Mass balance at different HRT and (b) TMP profile at different HRT.

When HRT was 24 h, the TMP rapidly increased to 9 kPa on day 2.4 in a linear manner and then had transient platform fluctuations. Finally, TMP was close to 16 kPa on day 5.3 in a linear manner again.

Generally, with the shortened HRT, the influent COD of AnMBR was increased (from 398 mg/L to 557 mg/L) (Figure 2(a)), which led to the decrease in the membrane fouling cycle. These results were similar to previous studies [8]. Gao et al. have researched the control of membrane fouling by addition of granular-activated carbon (GAC) at HRT 4, 6, and 8 h in an anaerobic membrane bioreactor. They found that the membrane fouling cycle at HRT 4 h (about 15 d) was almost two times of that at HRT 8 h (about 31 d) when 40 g GAC was added. The membrane fouling cycle of this study was obviously short. The possible reasons are as follows: (1) no addition of GAC. The fluidization of GAC could evidently reduce TMP [16]. However, maintenance of the fluidization of GAC demands a lot of energy to consume. (2) The feed had high COD (2961–3337 mg/L) and antibiotic (50 mg/L benzothiazole), which resulted in aggravation membrane fouling.

3.2.2. EPS and SMP Fraction. The variations of EPS and SMP, from both mixed liquor and cake layer, in different TMPs were detected (Figure 6). In the mixed liquor, there was no significant difference in SMP under different HRT. For instance, when the HRT was 24 h, the SMP were 43.00 mg/L, 48.19 mg/L, and 47.88 mg/L at TMP of 5 kPa, 10 kPa, and 15 kPa, respectively (Figure 6(a)). The concentrations of EPS and SMP were different at HRT 24, 18, and 12 h in the mixed liquor, but the trends were similar. EPS and SMP were not affected by TMP variations in each certain HRT with stable performance (Figures 6(a) and 6(c)). The possible reason was that the mixed liquor and microbiology communities were relatively stable, which did not change with TMP.

However, for cake layer, the concentrations of EPS and SMP increased with rising TMP in each certain HRT (Figures 6(b) and 6(d)). For instance, when the HRT was 24 h, the SMP were 22.28 mg/L, 34.74 mg/L, and 50.73 mg/L at TMP of 5 kPa, 10 kPa, and 15 kPa, respectively. Those EPS and SMP in the cake layer came from the biomass growth with rising TMP on the membrane surface. Sludge cake formation on the membrane surface is viewed as the major cause of membrane fouling [27]. It has been reported that cake sludge deposited on the membrane surface has much higher specific filtration resistance than that of bulk sludge liquor [28].

The concentrations of EPS and SMP in mixed liquor and the cake layer increased with decreasing HRT, which was due first to the faster growth of anaerobic sludge with shorter HRT [18]; secondly, more undegraded substrates were present in the mixed liquor. It has been reported that SMP occurs in response to environmental stress, such as that caused by toxic compounds [29]. In this study, the concentration of benzothiazole was increased (from 1.23 ± 0.27 mg/L to 12.02 ± 1.71 mg/L) with the change of HRT, which could explain the increasing SMP. The major fraction of SMP was the soluble phase of EPS, and SMP consistently varied with EPS in the aerobic MBR [30].

The concentration of protein was much higher than that of polysaccharide either in mixed liquor or cake layer, in EPS or SMP, which was consistent with previous studies[8]. Meng et al. found that proteins are more hydrophobic, adhere more easily to the membrane surface, and induce membrane fouling [31]. In addition, our group has reported that protein had a negative impact on membrane fouling compared to polysaccharide [32]. This conclusion explained the cause of serious membrane fouling in another aspect.

3.3. Microbial Community Structure. Normally, bacteria play a dominant role in antibiotic wastewater treatment systems:

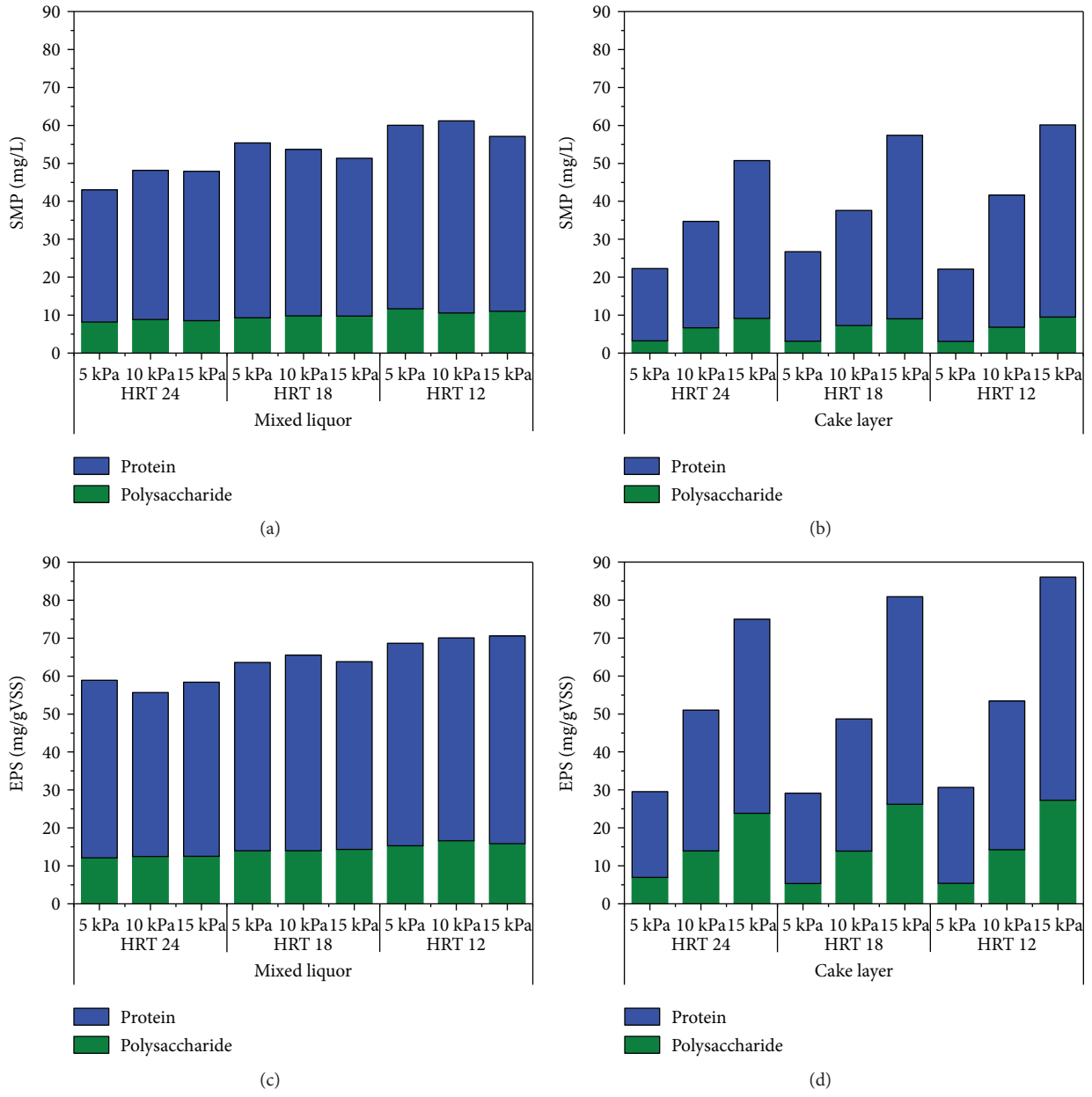

Figure 6: The variation of extracellular polymeric substances (EPS) and soluble microbial products (SMP) in the mixed liquor and cake layer. (a) The SMP in the mixed liquor, (b) the SMP in the cake layer, (c) the EPS in the mixed liquor, and (d) the EPS in the cake layer.

bacteria carbon transformation functions may be disturbed. Meanwhile, bacteria possessing antibiotic resistance could survive in this condition [33]. That is why it is important to understand the microbial community structure.

3.3.1. Bacterial Community Analysis. The Illumina MiSeq high-throughput platform was used to determine three microbial samples (HRT 24, 18, and 12 collected on days 185, 240, and 297, resp.), which were taken from the AFBR. The qualified sequencing reads were in the range of 24,182 to 42,241, which were clustered in more than 1500 OTUs based on a threshold of 97%.

The relative abundance of phylum, class, and genus levels was described in order to understand the communities better. More than 20 types of bacterial phyla were recovered altogether, and the main phyla were Firmicutes (27.7%–41.4%), Proteobacteria (8.9%–21.6%), Chloroflexi (12.4%–25.3%), and Bacteroidetes (8.3%–9.4%) (Figure 7(a)). These phyla were found to be significant microbial groups in other anaerobic bioreactors treating antibiotic wastewater [7, 9, 10].

At the class level, two of the most important classes were Clostridia (21.2%–30.2%) and Anaerolineae (10.9%–23.2%), (Figure 7(b)). The sample of HRT 24 was dominated by Clostridia (30.2%), Anaerolineae (10.9%), and δ-Proteobacteria (9.6%). The HRT 18 community was dominated by Clostridia (30.2%), followed by γ-Proteobacteria (18.4%) and Anaerolineae (17.6%). The HRT 12 community was dominated by Anaerolineae (23.2%) and Clostridia (21.2%).

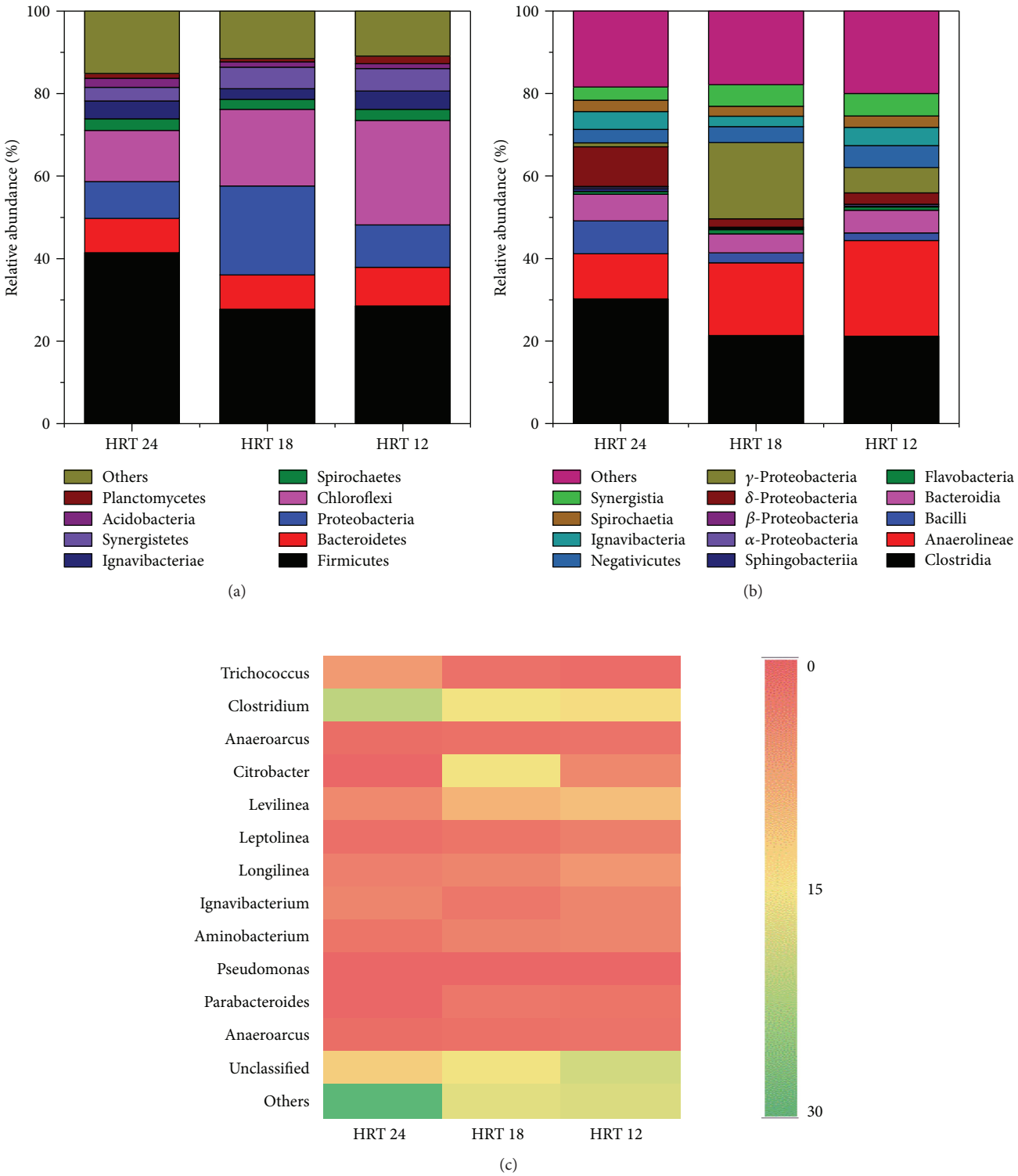

Figure 7: Continued.

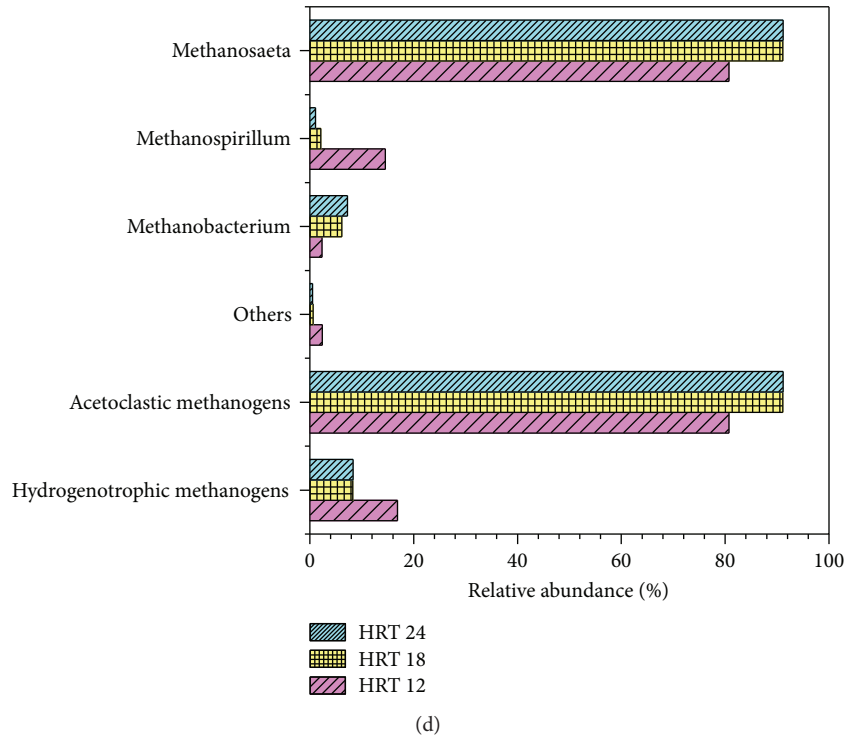

Figure 7: Taxonomic classification of bacteria and archaea form different HRT (collected on day 185, 240, and 297): (a) bacteria phylum, (b) bacteria class, (c) bacteria genus, and (d) archaea genus.

Clostridia had many carbon-degrading functions, which played a main role in COD removal. Some Clostridia were able to cleave aromatic rings and utilize the methyl group of aromatic methyl ethers as carbon source [34, 35]. Clostridia and δ-Proteobacteria were the major classes associated with antibiotic environments [33, 36]. Moreover, Anaerolineae was found in an anaerobic bioreactor [37].

In general, microbes from two samples showed similar diversities but different abundance. The sample of HRT 24 was dominant by *Clostridium* (23.9%), followed by *Trichococcus* (6.9%), and *Levilinea* (4.8%). The major community in a sample of HRT 18 was *Clostridium* (16.4%), followed by *Citrobacter* (16.3%) and *Levilinea* (10.0%). The sample of HRT 18 was dominant by *Clostridium*, *Levilinea*, and *Longilinea* in the proportion of 15.3%, 11.6%, and 6.3% (Figure 7(c)).

In this study, no matter how the condition changes, *Clostridium* was the dominant genus, which was a common genus of dominant bacteria in anaerobic bioreactors [38, 39]. *Clostridium* belonging to phyla of Firmicutes with hard cell walls can produce endospores. *Clostridium* spp. were reported to have the ability of degrading complex organic matters from acid by producing or secreting hydrolases, such as protease and α-amylase[40]. The relative abundance of some genera was increased in response to HRT, such as *Levilinea*, *Leptolinea*, and *Longilinea*. *Levilinea*, *Leptolinea*, and *Longilinea* are Gram-negative, belonging to the class of Anaerolineae and phyla of Chloroflexi with flexible filaments [41, 42]. Meanwhile, the decreasing HRT led to the increasing residual concentration of benzothiazole. This phenomenon indicated that the three genera could be inclined to develop in residual benzothiazole.

3.3.2. Archaeal Community Analysis. Over 30,000 qualified sequences were produced by an Illumina MiSeq high-throughput platform. The dominant genus of the archaeal community was *Methanosaeta* in proportions of 80.8% (HRT 24), 91.1% (HRT 18), and 91.2% (HRT 12) followed by *Methanospirillum* (14.5%, 2.1%, and 1.1% in HRT 24, 18, and 12, resp.) and *Methanobacterium* (2.3%, 6.1%, and 7.3% in HRT 24, 18, and 12, resp.) (Figure 7(d)). The proportion of acetotrophic methanogens (*Methanosaeta*) increased from 80.8% to 91.2%, and the proportion of hydrogenotrophic methanogens (*Methanospirillum* and *Methanobacterium*) decreased from 16.9% to 8.3%. Overall, the dominant participant was always *Methanosaeta* (acetotrophic methanogens), no matter how the HRT changes.

Methanosaeta was an important archaea in anaerobic bioreactors [43, 44]. *Methanosaeta* belongs to acetotrophic methanogens which can convert acetic acid to methane and CO_2, and this process produces 70% of methane [45]. Wang et al. treated brewery wastewater using a continuous stirred microbial electrochemical reactor (CSMER) [37]. The CSMER comprised a complete mixing zone (CMZ) and microbial electrochemical zone (MEZ), and the anaerobic sludge was inoculated in CMZ. They found that *Methanosaeta* (40.3%) was the predominant archaea in $CSMER_{CMZ}$ and *Methanosaeta* existed in each sample. *Methanosaeta* have been found to have high methane yield so that the higher relative abundance of *Methanosaeta* manifested a favorable condition for methane yield [46]. And this finding was in line with the higher methane yield in HRT 18 (0.327 m^3 CH_4/kg $COD_{removal}$) and 12 (0.327 m^3 CH_4/kg $COD_{removal}$) compared with HRT 24 (0.315 m^3 CH_4/kg $COD_{removal}$).

4. Conclusions

This study indicated the feasibility of an IAFMBR to the treatment of high concentration wastewater containing antibiotics at different HRT. The COD removal efficiency, the methane percentage, and the methane yield were not affected by HRT decreasing from 24 h to 12 h. The decreased HRT had an adverse effect on membrane fouling and benzothiazole removal efficiency. For bacteria, the dominant phyla, class, and genera were Firmicutes, Clostridia, and Clostridium. For archaea, the dominant genera were *Methanosaeta*. With the decreased HRT, the acetotrophic methanogens increased while that of hydrogenotrophic methanogens decreased. The best performance was obtained at HRT of 24 h.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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References

[1] S. Castiglioni, R. Bagnati, R. Fanelli, F. Pomati, D. Calamari, and E. Zuccato, "Removal of pharmaceuticals in sewage treatment plants in Italy," *Environmental Science & Technology*, vol. 40, no. 1, pp. 357–363, 2006.

[2] A. Y.-C. Lin, T.-H. Yu, and S. K. Lateef, "Removal of pharmaceuticals in secondary wastewater treatment processes in Taiwan," *Journal of Hazardous Materials*, vol. 167, no. 1–3, pp. 1163–1169, 2009.

[3] B. Halling-Sørensen, S. Nors Nielsen, P. F. Lanzky, F. Ingerslev, H. C. Holten Lützhøft, and S. E. Jørgensen, "Occurrence, fate and effects of pharmaceutical substances in the environment—a review," *Chemosphere*, vol. 36, no. 2, pp. 357–393, 1998.

[4] K. Kümmerer, *Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks*, Springer Science and Business Media, Berlin/Heidelberg, Germany, 2008.

[5] S. Chelliapan, T. Wilby, and P. J. Sallis, "Performance of an upflow anaerobic stage reactor (UASR) in the treatment of pharmaceutical wastewater containing macrolide antibiotics," *Water Research*, vol. 40, no. 3, pp. 507–516, 2006.

[6] B. Lew, S. Tarre, M. Beliavski, C. Dosoretz, and M. Green, "Anaerobic membrane bioreactor (AnMBR) for domestic wastewater treatment," *Desalination*, vol. 243, no. 1–3, pp. 251–257, 2009.

[7] L. W. Meng, X. K. Li, K. Wang, K. L. Ma, and J. Zhang, "Influence of the amoxicillin concentration on organics removal and microbial community structure in an anaerobic EGSB reactor treating with antibiotic wastewater," *Chemical Engineering Journal*, vol. 274, no. 15, pp. 94–101, 2015.

[8] D.-W. Gao, Q. Hu, C. Yao, N.-Q. Ren, and W.-M. Wu, "Integrated anaerobic fluidized-bed membrane bioreactor for domestic wastewater treatment," *Chemical Engineering Journal*, vol. 240, pp. 362–368, 2014.

[9] G. Qiu, Y.-h. Song, P. Zeng, L. Duan, and S. Xiao, "Characterization of bacterial communities in hybrid upflow anaerobic sludge blanket (UASB)–membrane bioreactor (MBR) process for berberine antibiotic wastewater treatment," *Bioresource Technology*, vol. 142, pp. 52–62, 2013.

[10] Y. Li, Q. Hu, C.-H. Chen, X.-L. Wang, and D.-W. Gao, "Performance and microbial community structure in an integrated anaerobic fluidized-bed membrane bioreactor treating synthetic benzothiazole contaminated wastewater," *Bioresource Technology*, vol. 236, pp. 1–10, 2017.

[11] K. Dutta, M.-Y. Lee, W. W.-P. Lai et al., "Removal of pharmaceuticals and organic matter from municipal wastewater using two-stage anaerobic fluidized membrane bioreactor," *Bioresource Technology*, vol. 165, pp. 42–49, 2014.

[12] M. Munir, K. Wong, and I. Xagoraraki, "Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan," *Water Research*, vol. 45, no. 2, pp. 681–693, 2011.

[13] Z. Huang, S. L. Ong, and H. Y. Ng, "Feasibility of submerged anaerobic membrane bioreactor (SAMBR) for treatment of low-strength wastewater," *Water Science & Technology*, vol. 58, no. 10, pp. 1925–1931, 2008.

[14] A. P. Trzcinski and D. C. Stuckey, "Anaerobic digestion of the organic fraction of municipal solid waste in a two-stage membrane process," *Water Science & Technology*, vol. 60, no. 8, pp. 1965–1978, 2009.

[15] P. H. Wu, K. K. Ng, P. K. A. Hong, P. Y. Yang, and C. F. Lin, "Treatment of low-strength wastewater at mesophilic and psychrophilic conditions using immobilized anaerobic biomass," *Chemical Engineering Journal*, vol. 311, no. 1, pp. 46–54, 2017.

[16] J. Kim, K. Kim, H. Ye et al., "Anaerobic fluidized bed membrane bioreactor for wastewater treatment," *Environmental Science & Technology*, vol. 45, no. 2, pp. 576–581, 2011.

[17] E. Jeong, H.-W. Kim, J.-Y. Nam, and H.-S. Shin, "Enhancement of bioenergy production and effluent quality by integrating optimized acidification with submerged anaerobic membrane bioreactor," *Bioresource Technology*, vol. 101, no. 1, pp. S7–S12, 2010.

[18] Z. Huang, S. L. Ong, and H. Y. Ng, "Submerged anaerobic membrane bioreactor for low-strength wastewater treatment: Effect of HRT and SRT on treatment performance and membrane fouling," *Water Research*, vol. 45, no. 2, pp. 705–713, 2011.

[19] APHA, *WEF (1998) Standard methods for the examination of water and wastewater*, American Public Health Association, Washington, DC, 1998.

[20] S. Malamis and A. Andreadakis, "Fractionation of proteins and carbohydrates of extracellular polymeric substances in a membrane bioreactor system," *Bioresource Technology*, vol. 100, no. 13, pp. 3350–3357, 2009.

[21] M. DuBois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, "Colorimetric method for determination of sugars and related substances," *Analytical Chemistry*, vol. 28, no. 3, pp. 350–356, 1956.

[22] J. Ma, Z. Wang, Y. Yang, X. Mei, and Z. Wu, "Correlating microbial community structure and composition with aeration intensity in submerged membrane bioreactors by 454 high-throughput pyrosequencing," *Water Research*, vol. 47, no. 2, pp. 859–869, 2013.

[23] L.-W. Meng, X.-K. Li, K. Wang, K.-L. Ma, and J. Zhang, "Pretreating amoxicillin contained wastewater with an anaerobic

expanded granular sludge bed (EGSB)," *Desalination and Water Treatment*, vol. 57, no. 34, pp. 16008–16014, 2016.

[24] R. K. Dereli, M. E. Ersahin, H. Ozgun et al., "Potentials of anaerobic membrane bioreactors to overcome treatment limitations induced by industrial wastewaters," *Bioresource Technology*, vol. 122, pp. 160–170, 2012.

[25] S. Aydin, Z. Cetecioglu, O. Arikan, B. Ince, E. G. Ozbayram, and O. Ince, "Inhibitory effects of antibiotic combinations on syntrophic bacteria, homoacetogens and methanogens," *Chemosphere*, vol. 120, pp. 515–520, 2015.

[26] M. M. Amin, J. L. Zilles, J. Greiner, S. Charbonneau, L. Raskin, and E. Morgenroth, "Influence of the antibiotic erythromycin on anaerobic treatment of a pharmaceutical wastewater," *Environmental Science & Technology*, vol. 40, no. 12, pp. 3971–3977, 2006.

[27] H. J. Lin, K. Xie, B. Mahendran et al., "Sludge properties and their effects on membrane fouling in submerged anaerobic membrane bioreactors (SAnMBRs)," *Water Research*, vol. 43, no. 15, pp. 3827–3837, 2009.

[28] H. Lin, B.-Q. Liao, J. Chen et al., "New insights into membrane fouling in a submerged anaerobic membrane bioreactor based on characterization of cake sludge and bulk sludge," *Bioresource Technology*, vol. 102, no. 3, pp. 2373–2379, 2011.

[29] W. C. Kuo and G. F. Parkin, "Characterization of soluble microbial products from anaerobic treatment by molecular weight distribution and nickel-chelating properties," *Water Research*, vol. 30, no. 4, pp. 915–922, 1996.

[30] K. Yamamoto, M. Hiasa, T. Mahmood, and T. Matsuo, "Direct solid-liquid separation using hollow fiber membrane in an activated sludge aeration tank," *Water Science and Technology*, vol. 21, no. 4-5, pp. 43–54, 1988.

[31] F. Meng, H. Zhang, F. Yang, Y. Li, J. Xiao, and X. Zhang, "Effect of filamentous bacteria on membrane fouling in submerged membrane bioreactor," *Journal of Membrane Science*, vol. 272, no. 1-2, pp. 161–168, 2006.

[32] D.-W. Gao, T. Zhang, C.-Y. Y. Tang et al., "Membrane fouling in an anaerobic membrane bioreactor: differences in relative abundance of bacterial species in the membrane foulant layer and in suspension," *Journal of Membrane Science*, vol. 364, no. 1-2, pp. 331–338, 2010.

[33] Y. Zhang, J. Xie, M. Liu et al., "Microbial community functional structure in response to antibiotics in pharmaceutical wastewater treatment systems," *Water Research*, vol. 47, no. 16, pp. 6298–6308, 2013.

[34] A. El Kasmi, S. Rajasekharan, and S. W. Ragsdale, "Anaerobic pathway for conversion of the methyl group of aromatic methyl ethers to acetic acid by Clostridium thermoaceticum," *Biochemistry*, vol. 33, no. 37, pp. 11217–11224, 2002.

[35] J. Winter, M. R. Popoff, P. Grimont, and V. D. Bokkenheuser, "*Clostridium orbiscindens* sp. nov., a human intestinal bacterium capable of cleaving the flavonoid C-ring," *International Journal of Systematic Bacteriology*, vol. 41, no. 3, pp. 355–357, 1991.

[36] Y. Deng, Y. Zhang, Y. Gao et al., "Microbial community compositional analysis for series reactors treating high level antibiotic wastewater," *Environmental Science & Technology*, vol. 46, no. 2, pp. 795–801, 2011.

[37] H. Wang, Y. Qu, D. Li et al., "Cascade degradation of organic matters in brewery wastewater using a continuous stirred microbial electrochemical reactor and analysis of microbial communities," *Scientific Reports*, vol. 6, no. 1, p. 27023, 2016.

[38] H. Ozgun, Y. Tao, M. E. Ersahin et al., "Impact of temperature on feed-flow characteristics and filtration performance of an upflow anaerobic sludge blanket coupled ultrafiltration membrane treating municipal wastewater," *Water Research*, vol. 83, no. 15, pp. 71–83, 2015.

[39] H. Wang, Y. Tao, D. Gao et al., "Microbial population dynamics in response to increasing loadings of pre-hydrolyzed pig manure in an expanded granular sludge bed," *Water Research*, vol. 87, no. 15, pp. 29–37, 2015.

[40] X. Zheng, Y. Su, X. Li, N. Xiao, D. Wang, and Y. Chen, "Pyrosequencing reveals the key microorganisms involved in sludge alkaline fermentation for efficient short-chain fatty acids production," *Environmental Science & Technology*, vol. 47, no. 9, pp. 4262–4268, 2013.

[41] T. Yamada, H. Imachi, A. Ohashi et al., "*Bellilinea caldifistulae* gen. nov., sp. nov. and *Longilinea arvoryzae* gen. nov., sp. nov., strictly anaerobic, filamentous bacteria of the phylum Chloroflexi isolated from methanogenic propionate-degrading consortia," *International Journal of Systematic and Evolutionary Microbiology*, vol. 57, no. 10, pp. 2299–2306, 2007.

[42] T. Yamada, Y. Sekiguchi, S. Hanada et al., "*Anaerolinea thermolimosa* sp. nov., *Levilinea saccharolytica* gen. nov., sp. nov. and Leptolinea tardivitalis gen. nov., sp. nov., novel filamentous anaerobes, and description of the new classes *Anaerolineae* classis nov. and *Caldilineae* classis nov. in the bacterial phylum *Chloroflexi*," *International Journal of Systematic and Evolutionary Microbiology*, vol. 56, no. 6, pp. 1331–1340, 2006.

[43] D. S. M. Ghasimi, Y. Tao, M. de Kreuk, B. Abbas, M. H. Zandvoort, and J. B. van Lier, "Digester performance and microbial community changes in thermophilic and mesophilic sequencing batch reactors fed with the fine sieved fraction of municipal sewage," *Water Research*, vol. 87, no. 15, pp. 483–493, 2015.

[44] D. S. M. Ghasimi, Y. Tao, M. de Kreuk, M. H. Zandvoort, and J. B. van Lier, "Microbial population dynamics during long-term sludge adaptation of thermophilic and mesophilic sequencing batch digesters treating sewage fine sieved fraction at varying organic loading rates," *Biotechnology for Biofuels*, vol. 8, no. 1, p. 171, 2015.

[45] W. Gujer and A. J. Zehnder, "Conversion processes in anaerobic digestion," *Water Science and Technology*, vol. 15, pp. 127–167, 1983.

[46] V. Razaviarani and I. D. Buchanan, "Reactor performance and microbial community dynamics during anaerobic co-digestion of municipal wastewater sludge with restaurant grease waste at steady state and overloading stages," *Bioresource Technology*, vol. 172, pp. 232–240, 2014.

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Research Article

Characterization of Microbial Communities in Pilot-Scale Constructed Wetlands with *Salicornia* for Treatment of Marine Aquaculture Effluents

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Microorganisms play an essential role in the performance of constructed wetlands (CWs) for wastewater treatment. However, there has been limited discussion on the characteristics of microbial communities in CWs for treatment of effluents from marine recirculating aquaculture systems (RAS). This study is aimed at characterizing the microbial communities of pilot-scale CWs with *Salicornia bigelovii* for treatment of saline wastewater from a land-based Atlantic salmon RAS plant located in Northern China. Illumina high-throughput sequencing was employed to identify the profile of microbial communities of three CWs receiving wastewater under different total ammonia nitrogen (TAN) concentrations. Results of this study showed remarkable spatial variations in diversity and composition of microbial communities between roots and substrates in three CWs, with distinct response to different TAN concentrations. In particular, Proteobacteria, Firmicutes, Cyanobacteria, and Bacteroidetes were predominant in roots, while Cyanobacteria, Proteobacteria, Firmicutes, Verrucomicrobia, and Bacteroidetes were prevalent in substrates. Moreover, redundancy analysis indicated that specific functional genera, such as *Nitrosopumilus*, *Vibrio*, *Pseudoalteromonas*, *Nitrospina*, and *Planctomyces*, played key roles in the removal of nitrogen/phosphorus pollutants and growth of wetland plants. From a microorganism perspective, the findings of this study could contribute to better understanding of contaminants' removal mechanism and improved management of CWs for treatment of effluents from land-based marine aquaculture.

1. Introduction

Development of environment-friendly and efficient aquaculture effluent treatment system is crucial for sustainable intensification of aquaculture, including recirculating aquaculture systems (RAS). Due to large volumes of wastewater with high salinity, it remains a challenge for treatment of effluents from land-based marine aquaculture. A number of physical (e.g., mechanical filtration [1]), chemical (e.g., catalytic reduction [2]), and biological (e.g., periphyton biofilters [3]) methods, used in conventional wastewater treatment, have been applied for treating mariculture wastewater, while they are costly in terms of capital investment, energy demand, and system maintenance [4]. Alternatively, constructed wetlands (CWs) act as a natural biofilter and can remove considerable amounts of nutrients, organic matter, and suspended solids from wastewater [5, 6]. Owing to low capital, operating costs, and low energy consumption,

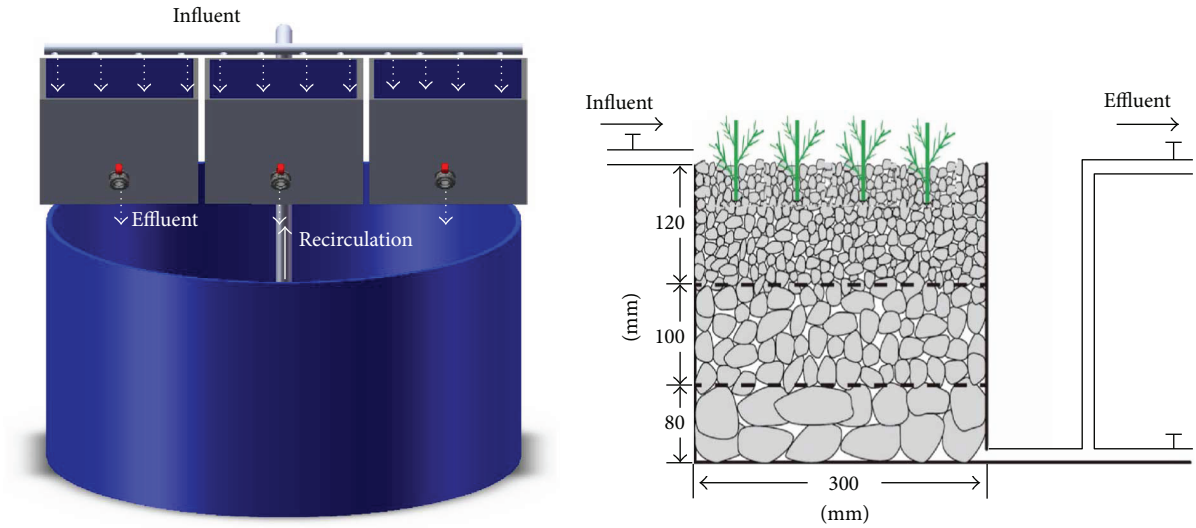

Figure 1: The pilot CW system (a) and a single CW unit (b). (Figure (b) was adapted from Li et al. (unpublished data) [70]).

CWs are becoming a promising technique to treat aquaculture effluents before discharge.

The performance of CWs largely depends on the interaction of wetland substrates, plants, and their associated microorganisms [7]. In particular, microorganisms within the biofilm on the surface of filter media and plant roots are widely considered to play a key role in the removal of many organic and inorganic pollutants [8, 9]. In recent years, a growing body of literature has examined the response of microbial community in CWs to wastewater quality characteristics [9], substrate type [10], plant diversity [11], pH variation [12], operational time [13] and so on. In a generic context, a better understanding of microbial communities in CWs and their influential parameters could aid in optimization and management of CWs toward further efficiency enhancement [14, 15]. Until now, only a few published studies have focused on CWs for treatment of saline wastewater from offshore and coastal marine aquaculture [16], while the characteristics of microbial communities in CWs for mariculture wastewater treatment have not yet been dealt with in depth.

A number of methods are available for the assay of environmental microbial characteristics, for example, plate count method, machine learning-based measurements, and molecular technologies [7, 17]. High-throughput sequencing technology is a highly efficient molecular biology method to profile complicated microbial populations of CWs [10, 18, 19], which provides an opportunity to investigate the links between the microbial communities and operational environment of CWs in particular [15, 20]. Recently, Urakawa and Bernhard [21] emphasized further research on high-throughput sequencing of wetland microbial communities to support the potential use of microorganisms as effective biological indicators for wetland management. To date, there are few published studies on the characteristics of microbial communities in CWs treating mariculture effluents, based on the high-throughput sequencing technology.

The aim of this study was to characterize the diversity and structure of microbial communities attached to substrate surface and plant roots in CWs with *Salicornia* spp. for treatment of mariculture wastewater under three different total ammonia nitrogen (TAN) concentrations, using Illumina high-throughput sequencing method. Moreover, the contributing microorganisms and core genera to the removal of nitrogen and phosphorus from wastewater were identified, and the relationships between nutrients' removal efficiency and corresponding functional genera were investigated.

2. Materials and Methods

2.1. Experimental Wetland System. Three pilot-scale recirculating horizontal subsurface flow CW systems (Figure 1) were constructed to treat simulated wastewater from a land-based intensive Atlantic salmon (*Salmo salar*) farm, located in Shandong Province, Northern China. Each CW system had one cylindrical barrel (diameter (Ø), 900 mm; height, 670 mm) and three respective CW tanks (300 mm × 300 mm × 300 mm, W × L × H). Each CW tank was filled with graded smooth cobblestone (Ø, 30–50 mm; height, 80 mm) as the bottom layer, haydite (Ø, 5–8 mm; height, 100 mm) as the middle layer, and smaller haydite (Ø, 3–5 mm; height, 120 mm) as the top layer. A total of 12 *Salicornia* plants (fresh weight, 2.0 ± 0.1 g/plant) were planted in each tank. Before the experiment, the *Salicornia* plants were, first, subjected to salt acclimation for 30 days for adaptation to the salinity of seawater used in this farm and then moved to the CWs and fed with seawater in batches for 60 days.

Fermented with Atlantic salmon residual excrement bait [22], the simulated wastewater was diluted to different TAN concentrations and classified into three groups, namely, low-concentration group (L, 0.75 ± 0.01 mg/L), middle-concentration group (M, 2.31 ± 0.09 mg/L), and high-concentration group (H, 7.23 ± 0.18 mg/L), representing

Table 1: Characteristics of the influent and effluent of CWs treating mariculture wastewater under different TAN concentrations.

Parameters	TAN (mg/L)	NO_2^--N (mg/L)	NO_3^--N (mg/L)	PO_4^{3-}-P (mg/L)	pH	T (°C)
Final effluent						
L	0.020 ± 0.001^a	0.008 ± 0.002^a	1.348 ± 0.331^a	0.420 ± 0.005^a	7.88	19.9
M	0.773 ± 0.178^b	0.006 ± 0.003^a	1.145 ± 0.074^a	0.398 ± 0.008^a	7.72	19.9
H	3.510 ± 0.479^c	0.013 ± 0.002^b	0.675 ± 0.035^b	0.356 ± 0.026^b	7.62	19.8
Final variation						
L	-0.028 ± 0.001^a	-0.187 ± 0.002^a	-1.462 ± 0.331^a	-0.008 ± 0.005	+0.04	+0.3
M	-0.323 ± 0.178^b	-0.008 ± 0.003^b	-1.210 ± 0.074^a	-0.033 ± 0.008	+0.03	+0.5
H	-1.247 ± 0.479^c	-0.005 ± 0.002^b	-0.285 ± 0.035^b	-0.026 ± 0.026	+0.10	+0.4
Removal rate (%)						
L	58.51 ± 2.13^a	96.07 ± 1.04^a	52.03 ± 11.77^a	1.87 ± 0.012		
M	29.46 ± 16.21^b	59.26 ± 19.25^b	51.38 ± 3.13^a	8.20 ± 0.019		
H	26.21 ± 10.07^b	27.93 ± 10.92^c	29.69 ± 3.65^b	6.88 ± 0.068		

Final variation = effluent concentration − influent concentration; removal rate = ((effluent concentration − influent concentration)/influent concentration) × 100%. L: low influent TAN concentration group (0.75 mg/L); M: middle influent TAN concentration group (2.31 mg/L); H: high influent TAN concentration group (7.23 mg/L). Differences in the final effluent and removal rate among the groups were tested using one-way ANOVA. Different characters indicate significant differences ($p < 0.05$) (means ± SD, $n = 3$).

the range of observed TAN concentrations in actual wastewater from the salmon farm under study. The simulated wastewater was stored in the barrel and then pumped by peristaltic pumps to the CW system (each with three parallel CW units). The outflows of the CWs went back to the barrel by gravity. Wastewater in the barrel was completely replaced every 18 days. During the experimental period, wastewater flowed into the CWs at a rate of 100 mL/min. Before sample collection, all the three CWs were in operation continuously for 72 days. At the end of the experiment, sample collection and monitoring of influent and effluent wastewater quality were performed. Table 1 presented the effluent wastewater characteristics and removal performance of the CWs, including TAN, nitrite (NO_2^--N), nitrate (NO_3^--N), phosphate (PO_4^{3-}-P), temperature (T), and pH. The removal performance was expressed by final variation and removal rate. By the end of the experiment, the fresh weight of the harvested *Salicornia* plants (g/plant) in the three CWs was 10.0 ± 1.4 (low-concentration group), 12.8 ± 3.6 (middle-concentration group), and 9.8 ± 3.9 (high-concentration group).

2.2. Sample Collection and DNA Extraction. In order to achieve the maximum recovery rate and representative information on microbial populations, samples were collected both from the plant roots (R-samples) and the substrate (S-samples) surface on several selected spots of each experimental wetland system. In total, nine R-samples ($1\,g{\cdot}ind^{-1}$) were collected, including three from the L group (L-R, replicate samples marked as L1-R, L2-R, and L3-R), three from the M group (M-R, replicate samples marked as M1-R, M2-R, and M3-R), and three from the H group (H-R, replicate samples marked as H1-R, H2-R, and H3-R). Similarly, nine S-samples ($10\,g{\cdot}ind^{-1}$) were collected from the top layer of the CWs, including three from the L group (L-S, replicate samples marked as L1-S, L2-S, and L3-S), three from the M group (M-S, replicate samples marked as M1-S, M2-S, and M3-S), and three from the H group (H-S, replicate samples marked as H1-S, H2-S, and H3-S).

The attached biofilms on the R- and S-samples were extracted by means of shaking each sample in 100 mL of sterile physiological saline with 100 μL of Tween 80 detergent solution using a vortex mixer for 10 min. Then, the solution was filtered through a 0.22 μm polycarbonate filter (Millipore, MA, USA) to collect the microorganisms. All the processed samples were stored at −80°C until microbial DNA extraction. The total DNA on the filter paper was extracted with E.Z.N.A.® Water DNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's protocol. Thereafter, the extracted DNA was subjected to electrophoresis using 1.0% agarose gel at 150 V for 20 min to examine the quality of DNA. DNA purity and quantity were determined using a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). The extracted DNAs were stored at −80°C before being subjected to high-throughput sequencing.

2.3. High-Throughput Sequencing. Deep sequencing of the 16S rRNA gene amplicons from the 18 samples was performed using Illumina MiSeq paired-end sequencing platform (Illumina, San Diego, CA, USA). First, polymerase chain reaction (PCR) was carried out using 25 μL of reaction mixture containing 1x PCR buffer, 10 ng of genomic DNA, 0.5 U of Ex Taq (Takara, Dalian, China), 1.5 mmol/L $MgCl_2$, 0.4 μmol/L deoxynucleoside triphosphate (dNTP), and 1.0 μmol/L each primer. The primer pair used for PCR was 515F (5′-GTGYCAGCMGCCGCGGTA-3′) and 909R (5′-CCCCGYCAATTCMTTTRAGT-3′), targeting the V4-V5 hypervariable region of bacterial 16S rRNA gene [23, 24]. The PCR profile consisted of initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 56°C for 60 s, elongation at 72°C for 60 s, and final extension at 72°C for 10 min. Each sample was amplified in duplicate and then combined together. The PCR products were separated by electrophoresis on 1.2% agarose gel and purified using SanPrep DNA Gel Extraction

Table 2: Diversity estimation of the 16S rRNA gene libraries for the R- and S-samples.

Sample	OTU number		Chao 1 index		Shannon index		Simpson index	
	R	S	R	S	R	S	R	S
L	2007 ± 275	1492 ± 274	8704 ± 582	6947 ± 1464	7.41 ± 0.78	5.51 ± 1.20	0.96 ± 0.02	0.86 ± 0.08
M	1986 ± 268	1618 ± 246	9759 ± 1750*	6557 ± 685**	7.34 ± 0.85	5.70 ± 0.90	0.96 ± 0.03	0.86 ± 0.07
H	2089 ± 202	1698 ± 251	8338 ± 407	7234 ± 859	7.85 ± 0.71	6.50 ± 1.03	0.97 ± 0.02	0.91 ± 0.04

R: root samples; S: substrate samples; L: low influent TAN concentration group (0.75 mg/L); M: middle influent TAN concentration group (2.31 mg/L); H: high influent TAN concentration group (7.23 mg/L). Differences among the L, M, and H groups were tested using one-way ANOVA. Different characters indicate significant differences ($p < 0.05$). Differences between the R- and S-samples of each group were determined using Student's t-test. "$*$" and "$**$" indicate significant differences ($p < 0.05$) (means ± SD, $n = 3$).

Kit (Sangon Biotech, Shanghai, China). The DNAs in the PCR products were quantified with NanoDrop, and amplicons from each sample were pooled at equimolar ratios based on the DNA concentration. The purified mixtures were finally sequenced on the Illumina MiSeq platform.

2.4. Statistical Analyses. The raw data obtained from the Illumina MiSeq paired-end sequencing platform were merged with FLASH [25]. After quality filtering, the merged sequences were screened and filtered for quality and length using QIIME 1.9.0 [26]. Clean sequences (length> 300 bp, without ambiguous base "N" and average base quality score > 30) were checked and filtered using UCHIME program to remove chimeric sequences [27], and effect sequences without chimera were clustered into operational taxonomic units (OTUs) at 97% similarity. Representative sequences processed with QIIME 1.9.0 were used for taxonomic assignments based on Ribosomal Database Project classifier [28] and Greengenes database [29]. In order to compare the microbial communities of the collected samples, alpha diversity indices were obtained using QIIME 1.9.0 package, including Chao 1 richness estimator, Shannon index, and Simpson index. While visualizing the differences in the microbial community structure among the samples, a hierarchical cluster heatmap was generated and principal coordinate analysis (PCoA) on weighted and unweighted UniFrac distances of the 16S rRNA genes was performed with the R package vegan. Moreover, redundancy analysis (RDA) was conducted, using Canoco version 5.0, to explore all possible correlations between functional genera and nutrients' removal effect in the CWs. All other statistical analyses were made using SPSS version 13.0 along with Student's t-test and one-way analysis of variance (ANOVA), with significant difference set at $p < 0.05$.

3. Results and Discussion

3.1. Analysis of Sequence Data and Alpha Diversity. In this study, a total of 306,489 high-quality 16S rRNA gene sequence reads were obtained from the 18 samples subjected to Illumina MiSeq sequencing. Each library contained 9989–39,691 reads that were normalized to 9989 for comparison of microbial community diversity. The alpha diversity indices (OTU number, Chao 1 index, Shannon index, and Simpson index) were calculated for comparison of the microbial community richness and diversity between the R- and S-samples collected from the three CWs (Table 2).

Results of this alpha diversity analysis showed that the microbial population on the plant roots had higher community richness and diversity compared to the substrate surface. As seen in Table 2, all of the four alpha diversity indices for the R-samples, especially the Chao 1 index of the R-samples from the M group ($p < 0.05$), were higher than those of the S-samples from the three CWs. The OTU number and Chao 1 index [30] were used to analyze the microbial community richness of the R- and S-samples from the three CWs. In total, 32,670 OTUs were generated with a threshold of 0.97. Based on the OTU results (ranging from 1492 ± 274 to 2089 ± 202 (mean ± standard deviation)), the samples were ranked as L-S < M-S < H-S < M-R < L-R < H-R. Based on the Chao 1 index (average, varying from 6557 ± 685 to 9759 ± 1750), the samples were ranked as M-S < L-S < H-S < H-R < L-R < M-R. Furthermore, Shannon and Simpson indices were employed to analyze diversity and evenness of microbial species [31, 32]. The results of Shannon index were as follows: L-S (5.51 ± 1.20) < M-S (5.70 ± 0.90) < H-S (6.50 ± 1.03) < M-R (7.34 ± 0.85) < L-R (7.41 ± 0.78) < H-R (7.85 ± 0.71), which were similar to those of Simpson index (ranging between 0.86 ± 0.08 and 0.97 ± 0.02). Moreover, the relatively small standard deviation of the alpha diversity results within each treatment group indicated a good reproducibility of our experiments.

Results of this study indicated a remarkable spatial variation in the microbial community richness and diversity in the CWs. These results reinforce previous findings in the literature on spatial diversity of microbial communities. For example, Urakawa et al. [33] demonstrated that rhizosphere attracts microbial cells and maintains larger microbial diversity indices than the biofilm on substrate in a floating treatment wetland. Differently, results of the alpha diversity indices obtained in the present study are slightly higher than those reported in previous studies [10, 20], probably owing to varying operational factors of CWs (e.g., plant species, hydraulic loading rates, and wastewater characteristics) in those studies.

3.2. Comparison of Microbial Community Structures. Results of hierarchical cluster heatmap analysis of the microbial communities at genus level (Figure S1, Supplementary Material) and PCoA based on weighted and unweighted UniFrac distances (Figure 2) served as a basis for analysis of the relationships of microbial communities among the three different CWs. As seen from the heatmap and PCoA, good reproducibility of our experiments could be

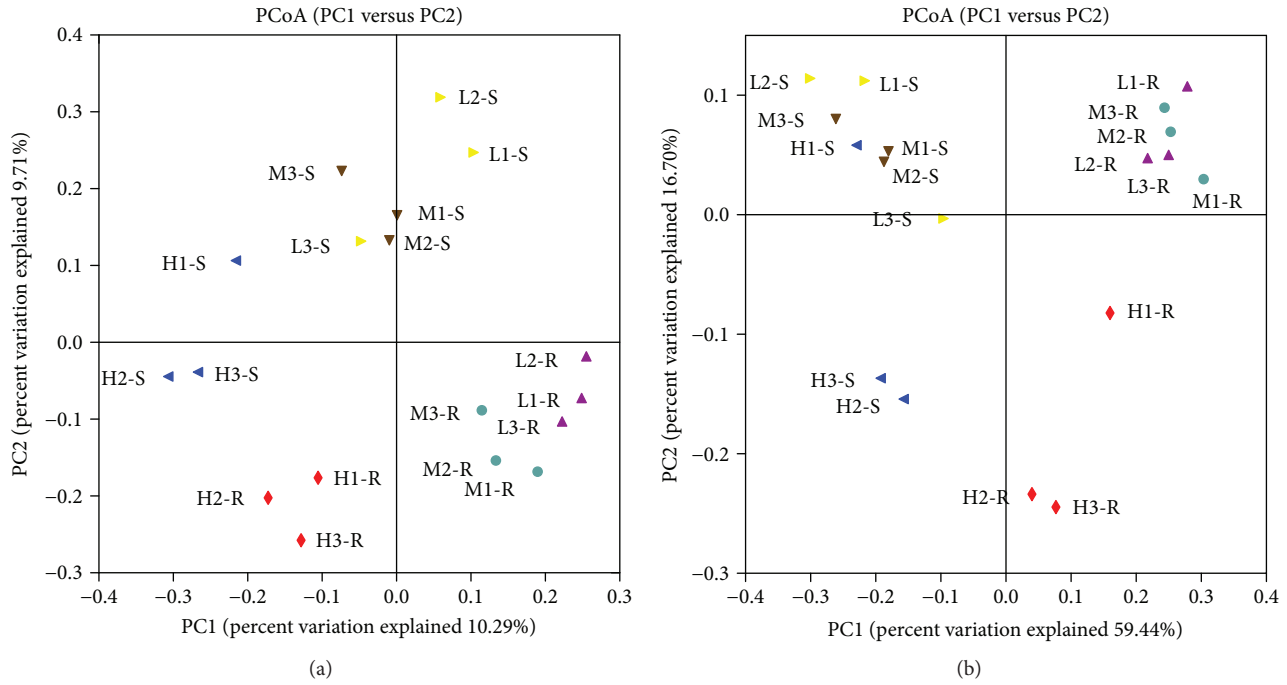

FIGURE 2: Unweighted (a) and weighted (b) principal coordinate analysis (PCoA) of the R- and S-samples from the three CW groups treating mariculture wastewater with different TAN concentrations.

speculated from the result that three parallel samples in every treatment group were clustered together. In specific, all the R-samples were clustered in the left subgroup and all S-samples were clustered in the right subgroup, which indicated different microbial communities and a remarkable spatial variation between the plant roots and substrate surface. Furthermore, all the R-samples were gathered into three distinctive clusters according to different TAN concentrations, showing that they harbored different microbial communities. For the S-samples, most of them were tightly clustered by TAN concentrations, though they were not well grouped. This result demonstrated that microbial communities both on plant roots and substrate were influenced by the TAN concentrations of the CW inflows. The principal component axes PC1 and PC2 accounted for 59.44% and 16.70% of the total changes in the bacterial community structure, respectively.

This study revealed a spatial variation in the microbial communities on the roots and substrate, which might be attributed to oxygen diffusions and secretions from root. It is interesting to note that the oxygen concentration differs between the root and substrate areas because of root respiration and plant mechanisms for transporting oxygen to the rhizosphere [33]. For instance, Ansola et al. [20] reported that the microbial community gradient from flooded areas (lagoon) to dry-wet areas (zones with plant) was different and possibly related to oxygen concentration (from oxygen-poor flooded areas to dry areas with higher oxygen diffusivity). Haichar et al. [34] suggested that nutrient compounds and/or allelochemicals as root exudate could control microbial populations.

Results of this study, as mentioned above, showed that TAN concentrations of the CW inflow affected the microbial communities both on plant roots and substrate. This result was consistent with previous findings on the impact of TAN concentrations on microbial community, especially on ammonia-oxidizing prokaryote community [35, 36]. For instance, Shen et al. [35] found that nitrogen inputs significantly altered ammonia-oxidizing prokaryote community, with the influence varying among different systems. According to Urakawa et al. [36], ammonia availability is a major factor that determines the distribution of ammonia-oxidizing prokaryotes in coastal water.

3.3. Composition of Dominant Microbial Population. Microbial phylum with a detection frequency of >0.5% in one or more samples was defined as a dominant phylum in this study. A total of 12 phyla (11 bacterial phyla and 1 archaeal phylum) were identified among the 18 samples (Figure 3), including Proteobacteria, Firmicutes, Cyanobacteria, Bacteroidetes, Planctomycetes, Thaumarchaeota (archaea), Acidobacteria, Actinobacteria, Verrucomicrobia, Chloroflexi, WS3, and Chlorobi. Only a small proportion of sequences (0.88–1.87%) retrieved from the three CWs could not be affiliated with known bacterial phyla.

In all R-samples from the three CWs, the most abundant phylum was Proteobacteria (average abundance: 63.69–72.52% of total effective sequences), followed by Firmicutes (4.14–11.35%), Cyanobacteria (7.46–11.62%), and Bacteroidetes (3.15–12.15%). Regarding the S-samples, the most abundant phylum was Cyanobacteria (35.65% and 40.98%), followed by Proteobacteria (36.39% and 37.63%), Firmicutes (9.06% and 7.65%), and Verrucomicrobia (7.76% and 5.28%) in CWs treating wastewater with low and middle TAN concentrations; however, those were Proteobacteria (36.44%), Firmicutes (10.49%), and Bacteroidetes (9.39%) dominant

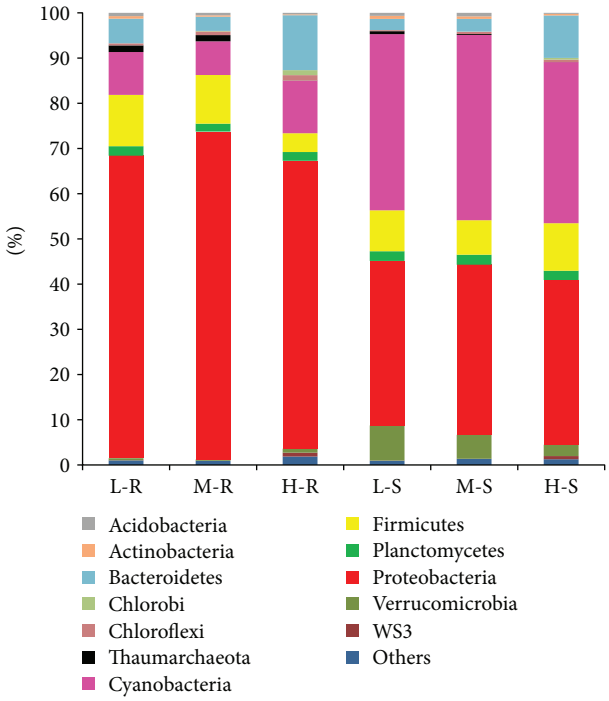

Figure 3: Relative abundance of microorganisms at the phylum level. "Others" refers to the sum of rare taxa each < 0.5% of the total.

in CWs treating wastewater with high TAN concentration. Furthermore, some dominant phyla exhibited statistical differences (Table S1, Supplementary Material). With regard to the R- and S-samples, statistical differences were noted among Proteobacteria, Cyanobacteria, Actinobacteria, and Verrucomicrobia (Student's t-test, $p < 0.05$). Regarding CWs with influents under different TAN concentrations, statistical differences were observed among Bacteroidetes, Thaumarchaeota, Verrucomicrobia, WS3, and Chlorobi (one-way ANOVA, $p > 0.05$). These results supported the abovementioned findings on spatial variation in microbial communities and the influence of TAN concentrations.

Since Proteobacteria is a functionally and phylogenetically diverse phylum, it was further analyzed by class (Figure 4). In total, six well-recognized classes (Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria, and Zetaproteobacteria) were observed, among which Gammaproteobacteria (31.70–42.23%) in the R-samples and Alphaproteobacteria (22.13–25.40%) in the S-samples were the top two most abundant classes.

Most of the phyla identified in this study have been discussed in the literature on their contribution to pollutant degradation [10, 20]. For example, Firmicutes, Bacteroidetes, and Actinobacteria have been reported to be the ubiquitous phyla in CWs and wastewater treatment processes, which are critical for the decomposition of contaminants [37, 38]. Verrucomicrobia are almost pervasive in soil [39], which explains its higher enrichment in the substrate than in the roots (Table S1, Supplementary Material). As a common wastewater treatment filamentous bacterium, the high relative abundance of Chloroflexi indicates its potential role in organic decomposition [40]. Moreover, previous studies have reported that many Planctomycetes can perform "anammox" metabolism [41]. Wang et al. [42] have concluded that high enrichment of Cyanobacteria is beneficial for maintaining high removal efficiency during summertime. In the present study, Cyanobacteria accounted for the largest proportion of the microbial communities in the S-samples, and its photosynthetic activity could produce oxygen (a key electron acceptor for pollutant-degrading bacteria) and organic exudates (key carbon source for heterotrophic bacteria) [43].

Proteobacteria are regarded as dominant in CWs treating wastewater [10, 20, 44] and in various rhizosophere systems [45, 46]. Microorganisms belonging to the phylum Proteobacteria are involved in the biodegradation of numerous pollutants, such as organic matter, nitrogen, and phosphorus [33, 47]. In the present study, Proteobacteria was the most abundant phylum in the R-samples and second largest phylum in the S-samples. At the class level, this study showed that Gammaproteobacteria dominated Proteobacteria in the R-samples, and Alphaproteobacteria was the most abundant class of Proteobacteria in the S-samples. For comparison, Urakawa et al. [33] reported that Alphaproteobacteria in plant rhizospheres and Gammaproteobacteria in substrate biofilms were the most abundant classes of Proteobacteria in a floating treatment wetland. Those inconsistent results between this study and the literature indicated that microbial communities could be affected by a number of factors, such as plant diversity [11], operation time [13], and wastewater quality characteristics [9].

The significant roles of archaea in water treatment have attracted intense attention in the literature, especially on their roles in nitrogen transformation [48]. In the present study, it was interesting to note that Thaumarchaeota, as the only detected archaea phylum, tended to be significantly richer in the CWs treating wastewater with low TAN concentrations ($p < 0.05$) (Table S1). This archaea group includes currently known ammonia-oxidizing archaea (AOA), such as *Nitrosopumilus* and *Nitrososphaera*, which play an important role in nitrogen removal, especially the ammonia oxidation process [49]. When ammonia is a limiting resource for microbial growth, AOA were reported generally higher numbers in low ammonia environments as they are not limited by ammonia [50] concentrations in the low range.

3.4. Functional Genera and Their Relationships with Nutrient Removal. Analysis at the genus level allowed further verification of microbial diversity and relative abundance of genera in the R- and S-samples from CWs treating mariculture wastewater with different TAN concentrations (Figure S1). Microbial genus with a detection frequency of >1% in one or more samples was defined as a dominant genus. A total of 67 dominant genera (66 bacterial genera and 1 archaeal genus) were identified among the 18 samples, of which specific functional genera have been reported in the literature to play important roles in the key processes of CWs for the removal of various pollutants, especially marine nitrogen (Figure 5). The relative abundances of the functional genera in the R- and S-samples from CW receiving inflows with different concentrations of TAN were presented in Table 3.

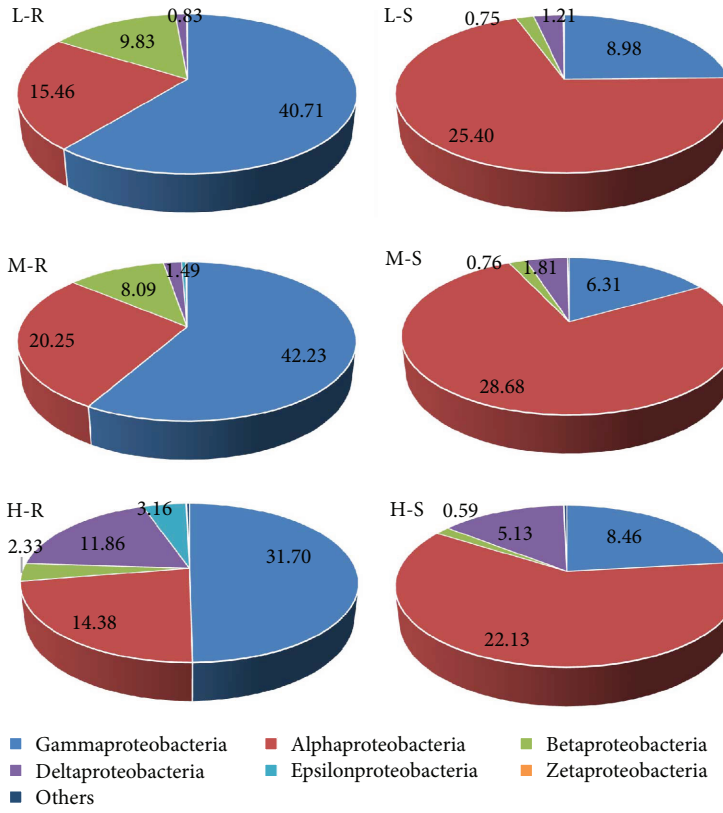

Figure 4: Relative abundance of Proteobacteria at the class level. Other Proteobacteria with relative abundance of <0.01% in each sample are included as "Others".

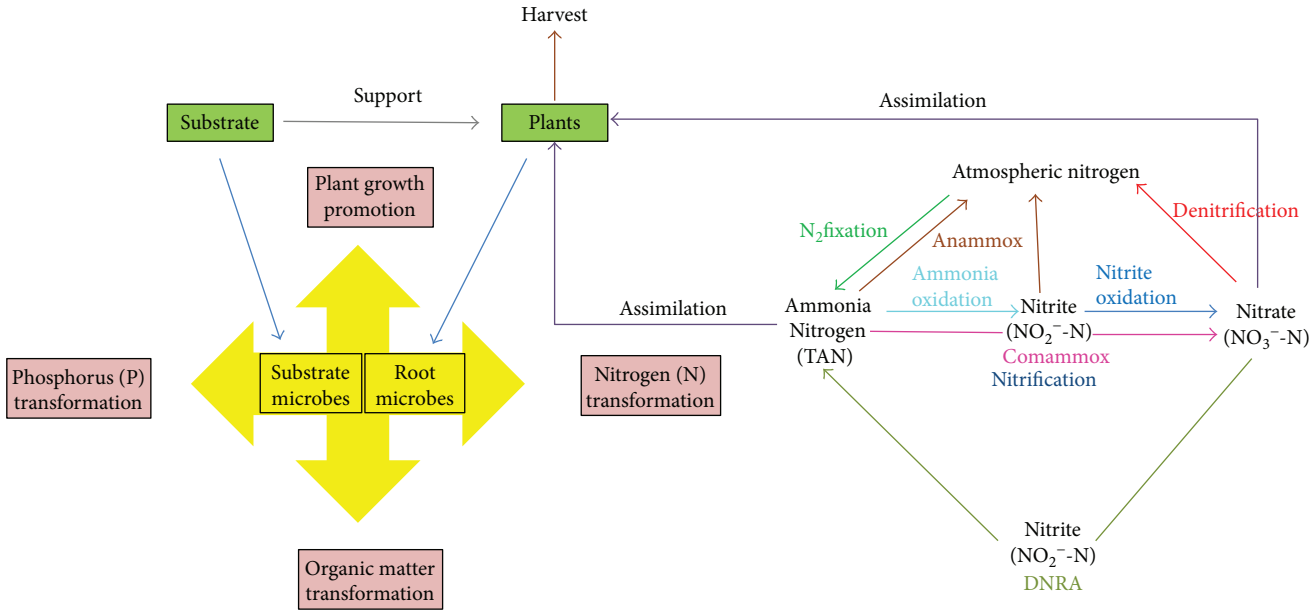

Figure 5: Key processes of recirculating CWs involved in the removal of various pollutants, especially marine nitrogen.

In order to determine efficient microbial indicator, the relationships between the functional genera, nutrients' (TAN, NO_2^--N, NO_3^--N, and PO_4^{3-}-P) variation and removal rates, and even plant growth were evaluated by RDA biplot (Figure 6). As shown in Figure 6, the first and second axes explained 71.17% and 28.83% variation in the removal rates, respectively, which was consistent with all the other nutrients' variation, except for TAN. *Acinetobacter*, *Nisaea*, *Nitrosopumilus*, *Comamonas*, *Bacillus*, *Pseudomonas*, *Vibrio*, *Stenotrophomonas*, *Pseudoalteromonas*, and genus of

Table 3: Relative abundances of some functional genera in the R- and S-samples from CWs treating mariculture wastewater with different TAN concentrations.

Microorganism	Function	Sample	L		M		H	
			Mean	SD	Mean	SD	Mean	SD
Pseudoalteromonas	Biofilm formation	R	0.0258	0.0390	0.0022	0.0007	0.0033^{*}	0.0019
		S	0.0007^{ab}	0.0006	0.0020^{a}	0.0010	0.0002^{**b}	0.0002
Acinetobacter	Denitrification, nitrification	R	0.0765	0.0519	0.1593	0.1105	0.0204	0.0225
		S	0.0003	0.0002	0.0001	0.0002	0.0001	0.0001
Bacillus	Plant growth promotion, denitrification, nitrification	R	0.0199	0.0074	0.0202	0.0116	0.0070	0.005
		S	0.0167	0.0131	0.0133	0.0028	0.0186	0.0261
Pseudomonas	Denitrification, plant growth promotion	R	0.0115^{*a}	0.0055	0.0090^{*ab}	0.0031	0.0034^{b}	0.0019
		S	0.0019^{**}	0.0017	0.0018^{**}	0.0009	0.0024	0.0032
Vibrio	Plant growth promotion, nitrification	R	0.0167	0.0091	0.0216	0.0148	0.0080	0.0053
		S	0.0115	0.0184	0.0019	0.0013	0.0096	0.0007
Stenotrophomonas	Denitrification	R	0.0150^{*}	0.0104	0.0069^{*}	0.0050	0.0034^{*}	0.0050
		S	0^{**}	0	0^{**}	0	0^{**}	0
Comamonas	Denitrification	R	0.0123^{*a}	0.0034	0.0075^{ab}	0.0048	0.0022^{b}	0.0022
		S	0.0017^{**}	0.001	0.0032	0.0020	0.0016	0.0011
Nisaea	Denitrification, nitrite oxidation	R	0.0019	0.0011	0.0016	0.0013	0.0007^{*}	0.0002
		S	0.0002	0.0002	0.0003	0.0003	0^{**}	0
Nitrospina	Nitrite oxidation	R	0.0005	0.0002	0.0012	0.0005	0.0011	0.0008
		S	0.0009	0.0004	0.0016	0.0015	0.0002	0.0001
Genus of Nitrosomonadaceae	Ammonia oxidation	R	0.0001^{a}	0.0001	0.0011^{b}	0.0004	0.0016^{b}	0.0006
		S	0.0051	0.0069	0.0022	0.0013	0.0024	0.0016
Nitrosopumilus	Ammonia oxidation	R	0.0145^{a}	0.0020	0.0135^{a}	0.0220	0.0003^{b}	0.0003
		S	0.0063	0.0069	0.0020	0.0029	0	0
Planctomyces	Anammox, P solubilization	R	0.0045	0.0026	0.0058	0.0025	0.0071	0.0042
		S	0.0060	0.0042	0.0081	0.0018	0.0064	0.0053

SD: standard deviation; R: root samples; S: substrate samples; L: low influent TAN concentration group (0.75 mg/L); M: middle influent TAN concentration group (2.31 mg/L); H: high influent TAN concentration group (7.23 mg/L). Differences among the L, M, and H groups were tested using one-way ANOVA. Different characters indicate significant differences ($p < 0.05$). Differences between the R- and S-samples of each group were determined using Student's t-test. "$*$" and "$**$" indicate significant differences ($p < 0.05$).

Nitrosomonadaceae were positively correlated with the removal of nitrogen (Figure 6). Among them, *Vibrio* contributed most to the removal rate of NO_2^--N, and *Pseudoalteromonas* were most related to the variation in NO_2^--N. With regard to plant growth, *Nitrospina* had maximum effect, followed by *Acinetobacter*, *Pseudomonas*, and *Vibrio*. Besides, *Planctomyces* had a significant impact on phosphorus removal and variation in TAN.

While microbial communities have been proven to be influenced by different nutrient concentrations (such as TAN [36]), the established microbial communities, especially some functional microorganisms, can in turn affect nutrients' removal (Figure 6). Some microbial genera have been reported to directly participate in the nitrogen removal by ammonia oxidation, nitrification, and denitrification. For instance, *Acinetobacter* could transform nitrogen by heterotrophic nitrification and aerobic denitrification [51, 52]. And *Nisaea*, comprising two species, namely, the type species *Nisaea denitrificans* and *Nisaea nitritireducens* [53], can participate in denitrification and NO_2^--N oxidation in nitrification, reducing NO_3^--N and NO_2^--N. Furthermore, *Nitrosopumilus* spp. and genus of Nitrosomonadaceae can oxidize ammonia [54–56] and contributed to ammonia removal in CWs. Besides, *Stenotrophomonas*, *Comamonas*, *Bacillus*, *Vibrio*, and *Pseudomonas* have also been reported to participate in the transformation of nitrogen [57–60]. *Pseudoalteromonas* has been shown to influence biofilm formation in various marine econiches [61–63] and could indirectly affect the removal of various pollutants such as NO_2^--N. Similarly, in the present study, *Pseudoalteromonas* was noted to contribute most to the variation in NO_2^--N. In fact, nitrite reduction is a challenging topic that researchers are dedicated to finding new solutions such as catalytic treatment [64]. The application of nitrite reduction bacteria *Pseudoalteromonas* could be a promising alternative.

Although rhizosphere is known to solubilize phosphorus through the chemical activity of root exudates and biological activity of rhizosphere bacteria, the underlying mechanisms are not yet clear enough. This study showed that *Planctomyces* had a significant impact on phosphorus removal. Similarly, Wu et al. [65] demonstrated that *Planctomyces* are positively correlated with available phosphorus content.

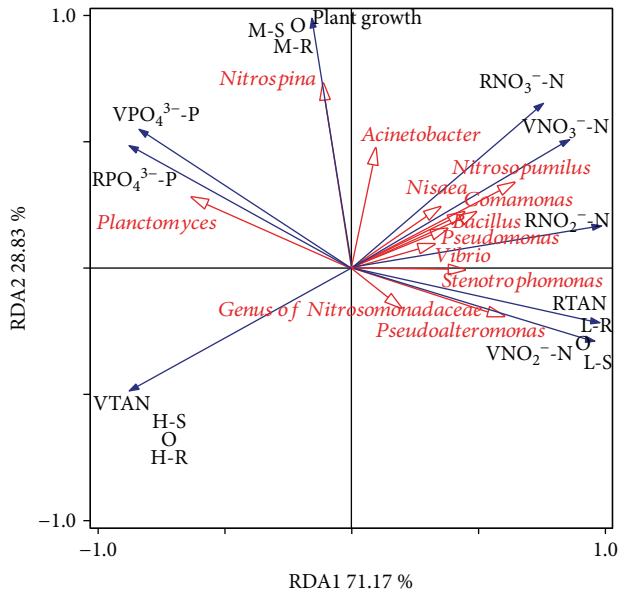

Figure 6: Redundancy analysis (RDA) biplot showing the relationship among functional genera in the sequencing data, nutrients' variation and removal rates, and plant growth. The first axis is horizontal, and the second axis is vertical. RTAN, RNO_2^--N, RNO_3^--N, and RPO_4^{3-}-P represent the removal rates of TAN, NO_2^--N, NO_3^--N, and PO_4^{3-}-P, respectively. VTAN, VNO_2^--N, VNO_3^--N, and VPO_4^{3-}-P denote the variation in TAN, NO_2^--N, NO_3^--N, and PO_4^{3-}-P after CW treatment, respectively.

Furthermore, *Planctomyces* has been reported to anaerobically oxidize ammonium (anammox) [66], which supports the finding of the present study that *Planctomyces* had a positive correction with the variation in TAN. *Nitrospina* are NO_2^--N-oxidizing bacteria, which could transform NO_2^--N to NO_3^--N that can be easily taken up by plants [67, 68]. Interestingly, *Nitrospina* was noted to have maximum effect on plant growth in the present study. In consistent with the RDA results (Figure 6) in this study, Jha et al. [69] reported that *Pseudomonas* and *Vibrio* are *Salicornia* plant growth-promoting rhizobacteria, which can directly and indirectly improve the extent or quality of plant growth.

4. Conclusions

This study characterized the profile of microbial communities of three pilot-scale CWs treating mariculture wastewater under different TAN concentrations. The Illumina high-throughput sequencing results revealed a remarkable spatial variation in the diversity and composition of microbial communities between root and substrate in the CWs, which differed with the varying TAN concentrations in the mariculture wastewater. In particular, functional genera, such as *Nitrosopumilus* (archaea), *Vibrio*, *Pseudoalteromonas*, *Nitrospina*, and *Planctomyces*, were found to contribute to plant growth and effective removal of nitrogen and phosphorus from wastewater. The findings of this study could broaden the knowledge of the removal mechanism of contaminants in CWs and serve as a basis for the potential use of microorganisms as a biological indicator in CW management.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Supplementary Materials

Table S1: relative abundances of the major phyla in the R- and S-samples from recirculating CWs treating mariculture wastewater with different TAN concentrations. The values given are percentages of relative abundance. Figure S1: heatmap of microbial genera in the R- and S-samples from CWs treating mariculture wastewater with different TAN concentrations (dominant genus of relative abundance > 1%). *(Supplementary Materials)*

References

[1] C. Steicke, V. Jegatheesan, and C. Zeng, "Mechanical mode floating medium filters for recirculating systems in aquaculture for higher solids retention and lower freshwater usage," *Bioresource Technology*, vol. 98, no. 17, pp. 3375–3383, 2007.

[2] Z. Zhang, W. Shi, W. Wang et al., "Interfacial electronic effects of palladium nanocatalysts on the by-product ammonia selectivity during nitrite catalytic reduction," *Environmental Science: Nano*, vol. 5, no. 2, pp. 338–349, 2018.

[3] A. Levy, A. Milstein, A. Neori, S. Harpaz, M. Shpigel, and L. Guttman, "Marine periphyton biofilters in mariculture effluents: nutrient uptake and biomass development," *Aquaculture*, vol. 473, pp. 513–520, 2017.

[4] A. Turcios and J. Papenbrock, "Sustainable treatment of aquaculture effluents—what can we learn from the past for the future?," *Sustainability*, vol. 6, no. 2, pp. 836–856, 2014.

[5] X. Wang, L. M. Olsen, K. I. Reitan, and Y. Olsen, "Discharge of nutrient wastes from salmon farms: environmental effects, and potential for integrated multi-trophic aquaculture," *Aquaculture Environment Interactions*, vol. 2, no. 3, pp. 267–283, 2012.

[6] J. Vymazal, "Constructed wetlands for treatment of industrial wastewaters: a review," *Ecological Engineering*, vol. 73, pp. 724–751, 2014.

[7] J. L. Faulwetter, V. Gagnon, C. Sundberg et al., "Microbial processes influencing performance of treatment wetlands: a review," *Ecological Engineering*, vol. 35, no. 6, pp. 987–1004, 2009.

[8] J. B. Ramond, P. J. Welz, D. A. Cowan, and S. G. Burton, "Microbial community structure stability, a key parameter in monitoring the development of constructed wetland

mesocosms during start-up," *Research in Microbiology*, vol. 163, no. 1, pp. 28–35, 2012.

[9] J. J. Chang, S. Q. Wu, K. Liang, Z. Wu, and W. Liang, "Comparative study of microbial community structure in integrated vertical-flow constructed wetlands for treatment of domestic and nitrified wastewaters," *Environmental Science and Pollution Research*, vol. 22, no. 5, pp. 3518–3527, 2015.

[10] W. Guan, M. Yin, T. He, and S. Xie, "Influence of substrate type on microbial community structure in vertical-flow constructed wetland treating polluted river water," *Environmental Science and Pollution Research International*, vol. 22, no. 20, pp. 16202–16209, 2015.

[11] C. B. Zhang, J. Wang, W. L. Liu et al., "Effects of plant diversity on microbial biomass and community metabolic profiles in a full-scale constructed wetland," *Ecological Engineering*, vol. 36, no. 1, pp. 62–68, 2010.

[12] Y. Zhang, M. Hu, P. Li, X. Wang, and Q. Meng, "Analysis of trichloroethylene removal and bacterial community function based on pH-adjusted in an upflow anaerobic sludge blanket reactor," *Applied Microbiology and Biotechnology*, vol. 99, no. 21, pp. 9289–9297, 2015.

[13] M. Bouali, I. Zrafi, A. Bakhrouf, S. Chaussonnerie, and A. Sghir, "Bacterial structure and spatiotemporal distribution in a horizontal subsurface flow constructed wetland," *Applied Microbiology and Biotechnology*, vol. 98, no. 7, pp. 3191–3203, 2014.

[14] T. He, W. Guan, Z. Luan, and S. Xie, "Spatiotemporal variation of bacterial and archaeal communities in a pilot-scale constructed wetland for surface water treatment," *Applied Microbiology and Biotechnology*, vol. 100, no. 3, pp. 1479–1488, 2016.

[15] F. Zhong, J. Wu, Y. Dai et al., "Bacterial community analysis by PCR-DGGE and 454-pyrosequencing of horizontal subsurface flow constructed wetlands with front aeration," *Applied Microbiology and Biotechnology*, vol. 99, no. 3, pp. 1499–1512, 2015.

[16] Y. Liang, H. Zhu, G. Bañuelos et al., "Constructed wetlands for saline wastewater treatment: a review," *Ecological Engineering*, vol. 98, pp. 275–285, 2017.

[17] Z. Liu, H. Li, and G. Cao, "Quick estimation model for the concentration of indoor airborne culturable bacteria: an application of machine learning," *International Journal of Environmental Research and Public Health*, vol. 14, no. 8, p. 857, 2017.

[18] T. Ligi, K. Oopkaup, M. Truu et al., "Characterization of bacterial communities in soil and sediment of a created riverine wetland complex using high-throughput 16S rRNA amplicon sequencing," *Ecological Engineering*, vol. 72, pp. 56–66, 2014.

[19] M. Xu, W. Liu, C. Li et al., "Evaluation of the treatment performance and microbial communities of a combined constructed wetland used to treat industrial park wastewater," *Environmental Science and Pollution Research*, vol. 23, no. 11, pp. 10990–11001, 2016.

[20] G. Ansola, P. Arroyo, and L. E. Sáenz de Miera, "Characterisation of the soil bacterial community structure and composition of natural and constructed wetlands," *Science of The Total Environment*, vol. 473-474, pp. 63–71, 2014.

[21] H. Urakawa and A. E. Bernhard, "Wetland management using microbial indicators," *Ecological Engineering*, vol. 108, pp. 456–476, 2017.

[22] Y. Zhang, J. Chen, J. Shen, S. Hou, Y. Liu, and Y. Liu, "A study on transformation of main pollutants along the wastewater flow in biological aerated filter," *China Environmental Science*, vol. 31, pp. 1808–1814, 2011.

[23] H. Li, J. Qu, T. Li, J. Li, Q. Lin, and X. Li, "Pika population density is associated with the composition and diversity of gut microbiota," *Frontiers in Microbiology*, vol. 7, p. 758, 2016.

[24] H. Tamaki, C. L. Wright, X. Li et al., "Analysis of 16S rRNA amplicon sequencing options on the Roche/454 next-generation titanium sequencing platform," *PLoS One*, vol. 6, no. 9, article e25263, 2011.

[25] T. Magoc and S. L. Salzberg, "FLASH: fast length adjustment of short reads to improve genome assemblies," *Bioinformatics*, vol. 27, no. 21, pp. 2957–2963, 2011.

[26] J. G. Caporaso, J. Kuczynski, J. Stombaugh et al., "QIIME allows analysis of high-throughput community sequencing data," *Nature Methods*, vol. 7, no. 5, pp. 335-336, 2010.

[27] R. C. Edgar, B. J. Haas, J. C. Clemente, C. Quince, and R. Knight, "UCHIME improves sensitivity and speed of chimera detection," *Bioinformatics*, vol. 27, no. 16, pp. 2194–2200, 2011.

[28] J. R. Cole, B. Chai, T. L. Marsh et al., "The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy," *Nucleic Acids Research*, vol. 31, no. 1, pp. 442-443, 2003.

[29] T. Z. DeSantis, P. Hugenholtz, N. Larsen et al., "Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB," *Applied and Environmental Microbiology*, vol. 72, no. 7, pp. 5069–5072, 2006.

[30] A. Chao, "Estimating the population size for capture-recapture data with unequal catchability," *Biometrics*, vol. 43, no. 4, pp. 783–791, 1987.

[31] E. M. Bik, P. B. Eckburg, S. R. Gill et al., "Molecular analysis of the bacterial microbiota in the human stomach," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 3, pp. 732–737, 2006.

[32] J. Luo, H. Liang, L. Yan, J. Ma, Y. Yang, and G. Li, "Microbial community structures in a closed raw water distribution system biofilm as revealed by 454-pyrosequencing analysis and the effect of microbial biofilm communities on raw water quality," *Bioresource Technology*, vol. 148, pp. 189–195, 2013.

[33] H. Urakawa, D. L. Dettmar, and S. Thomas, "The uniqueness and biogeochemical cycling of plant root microbial communities in a floating treatment wetland," *Ecological Engineering*, vol. 108, pp. 573–580, 2017.

[34] F. e. Z. Haichar, C. Marol, O. Berge et al., "Plant host habitat and root exudates shape soil bacterial community structure," *The ISME Journal*, vol. 2, no. 12, pp. 1221–1230, 2008.

[35] X. Y. Shen, L. M. Zhang, J. P. Shen, L. H. Li, C. L. Yuan, and J. Z. He, "Nitrogen loading levels affect abundance and composition of soil ammonia oxidizing prokaryotes in semiarid temperate grassland," *Journal of Soils and Sediments*, vol. 11, no. 7, pp. 1243–1252, 2011.

[36] H. Urakawa, W. Martens-Habbena, C. Huguet et al., "Ammonia availability shapes the seasonal distribution and activity of archaeal and bacterial ammonia oxidizers in the Puget Sound Estuary," *Limnology and Oceanography*, vol. 59, no. 4, pp. 1321–1335, 2014.

[37] C. Ahn, P. M. Gillevet, and M. Sikaroodi, "Molecular characterization of microbial communities in treatment microcosm wetlands as influenced by macrophytes and phosphorus loading," *Ecological Indicators*, vol. 7, no. 4, pp. 852–863, 2007.

[38] L. Ye, M. F. Shao, T. Zhang, A. H. Y. Tong, and S. Lok, "Analysis of the bacterial community in a laboratory-scale nitrification reactor and a wastewater treatment plant by 454-pyrosequencing," *Water Research*, vol. 45, no. 15, pp. 4390–4398, 2011.

[39] L. Zhang and Z. Xu, "Assessing bacterial diversity in soil," *Journal of Soils and Sediments*, vol. 8, no. 6, pp. 379–388, 2008.

[40] Y. Chen, Y. Wen, J. Zhou, Q. Zhou, J. Vymazal, and P. Kuschk, "Transformation of chloroform in model treatment wetlands: from mass balance to microbial analysis," *Environmental Science & Technology*, vol. 49, no. 10, pp. 6198–6205, 2015.

[41] G. Zhu, S. Wang, X. Feng, G. Fan, M. S. M. Jetten, and C. Yin, "Anammox bacterial abundance, biodiversity and activity in a constructed wetland," *Environmental Science & Technology*, vol. 45, no. 23, pp. 9951–9958, 2011.

[42] Q. Wang, H. Xie, H. H. Ngo et al., "Microbial abundance and community in subsurface flow constructed wetland microcosms: role of plant presence," *Environmental Science and Pollution Research*, vol. 23, no. 5, pp. 4036–4045, 2016.

[43] R. M. M. Abed, "Interaction between cyanobacteria and aerobic heterotrophic bacteria in the degradation of hydrocarbons," *International Biodeterioration & Biodegradation*, vol. 64, no. 1, pp. 58–64, 2010.

[44] X. Huang, C. Liu, C. Gao et al., "Comparison of nutrient removal and bacterial communities between natural zeolite-based and volcanic rock-based vertical flow constructed wetlands treating piggery wastewater," *Desalination and Water Treatment*, vol. 51, no. 22–24, pp. 4379–4389, 2013.

[45] Y. Tanaka, H. Tamaki, H. Matsuzawa, M. Nigaya, K. Mori, and Y. Kamagata, "Microbial community analysis in the roots of aquatic plants and isolation of novel microbes including an organism of the candidate phylum OP10," *Microbes and Environments*, vol. 27, no. 2, pp. 149–157, 2012.

[46] S. Uroz, M. Buée, C. Murat, P. Frey-Klett, and F. Martin, "Pyrosequencing reveals a contrasted bacterial diversity between oak rhizosphere and surrounding soil," *Environmental Microbiology Reports*, vol. 2, no. 2, pp. 281–288, 2010.

[47] X. B. Liao, C. Chen, Z. Wang et al., "Changes of biomass and bacterial communities in biological activated carbon filters for drinking water treatment," *Process Biochemistry*, vol. 48, no. 2, pp. 312–316, 2013.

[48] J. You, A. Das, E. M. Dolan, and Z. Hu, "Ammonia-oxidizing archaea involved in nitrogen removal," *Water Research*, vol. 43, no. 7, pp. 1801–1809, 2009.

[49] G. W. Nicol and C. Schleper, "Ammonia-oxidising Crenarchaeota: important players in the nitrogen cycle?," *Trends in Microbiology*, vol. 14, no. 5, pp. 207–212, 2006.

[50] T. Nakagawa and D. A. Stahl, "Transcriptional response of the archaeal ammonia oxidizer *Nitrosopumilus maritimus* to low and environmentally relevant ammonia concentrations," *Applied and Environmental Microbiology*, vol. 79, no. 22, pp. 6911–6916, 2013.

[51] S. Yao, J. Ni, T. Ma, and C. Li, "Heterotrophic nitrification and aerobic denitrification at low temperature by a newly isolated bacterium, *Acinetobacter* sp. HA2," *Bioresource Technology*, vol. 139, pp. 80–86, 2013.

[52] S. M. Taylor, Y. He, B. Zhao, and J. Huang, "Heterotrophic ammonium removal characteristics of an aerobic heterotrophic nitrifying-denitrifying bacterium, *Providencia rettgeri* YL," *Journal of Environmental Sciences*, vol. 21, no. 10, pp. 1336–1341, 2009.

[53] L. Urios, V. Michotey, L. Intertaglia, F. Lesongeur, and P. Lebaron, "*Nisaea denitrificans* gen. nov., sp. nov. and *Nisaea nitritireducens* sp. nov., two novel members of the class Alphaproteobacteria from the Mediterranean Sea," *International Journal of Systematic and Evolutionary Microbiology*, vol. 58, no. 10, pp. 2336–2341, 2008.

[54] A. Hu, H. Wang, X. Yang et al., "Seasonal and spatial variations of prokaryoplankton communities in a salinity-influenced watershed, China," *FEMS Microbiology Ecology*, vol. 93, no. 8, 2017.

[55] A. E. Ingalls, S. R. Shah, R. L. Hansman et al., "Quantifying archaeal community autotrophy in the mesopelagic ocean using natural radiocarbon," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 17, pp. 6442–6447, 2006.

[56] A. C. Mosier, E. E. Allen, M. Kim, S. Ferriera, and C. A. Francis, "Genome sequence of "*Candidatus Nitrosopumilus salaria*" BD31, an ammonia-oxidizing archaeon from the San Francisco Bay estuary," *Journal of Bacteriology*, vol. 194, no. 8, pp. 2121-2122, 2012.

[57] M. I. Bellini, L. Gutiérrez, S. Tarlera, and A. F. Scavino, "Isolation and functional analysis of denitrifiers in an aquifer with high potential for denitrification," *Systematic and Applied Microbiology*, vol. 36, no. 7, pp. 505–516, 2013.

[58] S. Itoi, N. Ebihara, S. Washio, and H. Sugita, "Nitrite-oxidizing bacteria, *Nitrospira*, distribution in the outer layer of the biofilm from filter materials of a recirculating water system for the goldfish *Carassius auratus*," *Aquaculture*, vol. 264, no. 1–4, pp. 297–308, 2007.

[59] J. K. Kim, K. J. Park, K. S. Cho, S. W. Nam, T. J. Park, and R. Bajpai, "Aerobic nitrification–denitrification by heterotrophic *Bacillus* strains," *Bioresource Technology*, vol. 96, no. 17, pp. 1897–1906, 2005.

[60] Y. Li, Y. Wang, L. Fu, Y. Gao, H. Zhao, and W. Zhou, "Aerobic-heterotrophic nitrogen removal through nitrate reduction and ammonium assimilation by marine bacterium *Vibrio* sp. Y1-5," *Bioresource Technology*, vol. 230, pp. 103–111, 2017.

[61] M. Button, J. Nivala, K. P. Weber, T. Aubron, and R. A. Müller, "Microbial community metabolic function in subsurface flow constructed wetlands of different designs," *Ecological Engineering*, vol. 80, pp. 162–171, 2015.

[62] J. P. Bowman, "Bioactive compound synthetic capacity and ecological significance of marine bacterial genus *Pseudoalteromonas*," *Marine Drugs*, vol. 5, no. 4, pp. 220–241, 2007.

[63] D. Rao, J. S. Webb, and S. Kjelleberg, "Competitive interactions in mixed-species biofilms containing the marine bacterium *Pseudoalteromonas tunicata*," *Applied and Environmental Microbiology*, vol. 71, no. 4, pp. 1729–1736, 2005.

[64] S. Seraj, P. Kunal, H. Li, G. Henkelman, S. M. Humphrey, and C. J. Werth, "PdAu alloy nanoparticle catalysts: effective candidates for nitrite reduction in water," *ACS Catalysis*, vol. 7, no. 5, pp. 3268–3276, 2017.

[65] W. Wu, J. Wu, X. Liu, X. Chen, Y. Wu, and S. Yu, "Inorganic phosphorus fertilizer ameliorates maize growth by reducing metal uptake, improving soil enzyme activity and microbial community structure," *Ecotoxicology and Environmental Safety*, vol. 143, pp. 322–329, 2017.

[66] O. Lahav, I. B. Massada, D. Yackoubov et al., "Quantification of anammox activity in a denitrification reactor for a recirculating aquaculture system," *Aquaculture*, vol. 288, no. 1-2, pp. 76–82, 2009.

[67] H. A. Levipan, V. Molina, and C. Fernandez, "*Nitrospina*-like bacteria are the main drivers of nitrite oxidation in the seasonal upwelling area of the Eastern South Pacific (Central Chile ~36°S)," *Environmental Microbiology Reports*, vol. 6, no. 6, pp. 565–573, 2014.

[68] E. Spieck and E. Bock, "*Nitrospina*," *Bergey's Manual of Systematics of Archaea and Bacteria*, pp. 1–7, 2015.

[69] B. Jha, I. Gontia, and A. Hartmann, "The roots of the halophyte *Salicornia brachiata* are a source of new halotolerant diazotrophic bacteria with plant growth-promoting potential," *Plant and Soil*, vol. 356, no. 1-2, pp. 265–277, 2012.

[70] M. Li, Z. Liang, M. D. Callier et al., "Nutrients removal and substrate enzyme activities in vertical subsurface flow constructed wetlands for mariculture wastewater treatment: Effects of ammonia nitrogen loading rates and salinity levels," *Marine Pollution Bulletin*, vol. 131, pp. 142–150, 2018.