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Abstract: The anaerobic-multistage anoxic/aerobic (A-MAO) process has shown good potential for advanced nitrogen removal in recent years, but its greenhouse gas emissions still need to be fully explored. The effects of the influent distribution and external carbon source sodium acetate on nutrient removal, greenhouse gas emissions, and the microbial community structure in a continuous plug-flow A-MAO reactor fed with real low C/N ratio domestic sewage were investigated. The results showed that altering the allocation of carbon source resulted in average chemical oxygen demand (COD) and total nitrogen (TN) concentration in effluent reduced to 26.10 ± 4.86 and 6.65 ± 1.73 mg/L, respectively. Both operations reduced the emission rate of greenhouse gas. While the addition of external carbon sources leaded to lower N₂O emission rates and higher CO₂ and CH₄ emission rates. The addition of sodium acetate facilitated nitrification and denitrification processes, thereby leading to a reduction in N₂O production. Meanwhile, it spurred the growth of methanogenic bacteria and heterotrophic microorganisms, thus boosting the production of CO₂ and CH₄. Influent distribution promoted the increase of Bacteroidota, Chloroflexi and Acidobacteriota of the reactor. The enrichment of typical hydrolytic bacteria and glycogen accumulating organisms (GAOs) increased the utilization efficiency of carbon sources in the system after the addition of sodium acetate. The significant increase of typical denitrifying bacteria (DNBs) Azospira reduced the N₂O emission during heterotrophic denitrification process, which was considered to be an important functional genus for increasing nitrogen loss in this system. The rational utilization of carbon source makes the difference in metabolism function. The study provides a valuable strategy for comprehensively evaluating the pollutant removal and greenhouse gas emission reduction from the A-MAO process.

Keywords: low C/N municipal wastewater; biological nutrient removal; GHG emission; microbial community

1. Introduction

With the rapid development of the global economy and living standards, the amount of treated wastewater discharged into surface water has increased. Municipal wastewater treatment plants (WWTPs) are, therefore, essential for preventing the eutrophication of surface water bodies [1,2]. In recent years, many countries and regions have introduced stricter discharge standards for WWTPs to improve the environmental water quality, especially the requirements for organic matter and total nitrogen (TN) indicators [3]. The high nitrogen removal efficiency depends on a higher influent carbon/nitrogen (C/N) ratio (C/N greater than 5) [4]. However, most municipal wastewater in China, especially in the southern region, has a low C/N ratio [5], which leads to the insufficient organic carbon used for denitrification in wastewater [6]. Therefore, how to achieve the stable removal of
nutrients in the case of limited carbon sources to meet the discharge standards of WWTPs still needs further research.

Meanwhile, the use of activated sludge for the biological treatment of domestic wastewater may lead to significant greenhouse gas (GHGs) emissions, such as CO₂, CH₄, and N₂O, thus exacerbating future climate change [7]. Greenhouse gas emissions from WWTPs are divided into direct greenhouse gas emissions and indirect greenhouse gas emissions [8]. Direct greenhouse gas emissions come from biological treatment processes. CO₂ is mainly released from microbial respiratory activities, N₂O is mainly released from the nitrification and denitrification stages, and CH₄ mainly comes from anaerobic digestion [9]. Indirect greenhouse gas emissions are mainly related to electricity or heat consumption. Currently, approximately 12% of anthropogenic CH₄ and 4% of anthropogenic N₂O are produced by wastewater treatment and discharge worldwide (Emissions Database for Global Atmospheric Research (EDGAR) v6.0, https://edgar.jrc.ec.europa.eu/ (accessed on 22 February 2024)). Therefore, it is necessary to explore mitigation strategies to reduce GHG emissions from wastewater treatment processes.

At present, the anaerobic-anoxic-aerobic (AAO) process is one of the most widely used biological nitrogen removal technologies in municipal WWTPs [10]. However, the nitrogen removal efficiency of most AAO systems is still in the low range of 50–65%, making it difficult for AAO systems to meet increasingly stringent TN emission standards without the addition of external carbon sources [11]. For low C/N ratio wastewater, one of the most effective methods is to add additional carbon sources [12]. However, this approach increases operating costs. Therefore, it is necessary to transform the conventional treatment process to fully exploit its treatment efficiency. In this study, the anaerobic-multistage anoxic/aerobic (A-MAO) process was put forward to remedy the defects of AAO process. Influent distribution was performed in the process: divides the influent in two parts: one part enters the anaerobic tank and the other enters the second anoxic tank, which can fully utilize the organic matter in influent to facilitate pollutant removal. Studies have shown the nutrient removal and microbial metabolism characteristics of the alkaline-fermented-sludge-enhanced treatment of low C/N ratio urban wastewater in the A-MAO process [13]. Nevertheless, the greenhouse gas emissions of the A-MAO process under the influent distribution and the addition of external carbon sources are still unclear.

Minimizing greenhouse gas emissions on the basis of a high nutrient removal efficiency is essential for the sustainable treatment of urban domestic sewage. This study established a continuous plug-flow A-MAO pilot system for the treatment of organic matter and nitrogen in low C/N ratio wastewater. The main objectives of this study were to: (a) evaluate the effects of the influent distribution and the addition of sodium acetate as an external carbon source on the nutrient removal efficiency and GHGs emission rates of the A-MAO process for treating low C/N ratio domestic wastewater over a 180-day experimental period; and (b) reveal the dynamic changes of key microorganisms during the operation of the A-MAO process and further reveal the mechanism of pollutant degradation.

2. Materials and Methods
2.1. Bioreactors and Experimental Arrangement

The lab-scale continuous plug-flow reactor was established, including an A-MAO reactor with 177.5 L working volume and a sedimentation tank with 30 L working volume. The A-MAO reactor (as shown in Figure 1) was made of transparent acrylic plates and divided into anaerobic zone (Ana, 26.25 L)/first anoxic zone (Ano1, 29.4 L)/first aerobic zone (Aer1, 58.8 L)/second anoxic zone (Ano2, 31.5 L)/second aerobic zone (Aer2, 31.5 L). The entire experimental procedure was carried out at natural temperature, maintaining a hydraulic retention time (HRT) of 17.7 h. The wastewater treated by the reactor was discharged from the sedimentation tank as effluent. The sludge in the sedimentation tank returned to the anaerobic zone with 100% reflux ratio. The mixed liquid suspended solids (MLSS) concentrations was controlled at 3–4 g/L. Double mixed liquor reflux was applied in this study, in which the first return mixed liquor (R1) from Aer1 was pumped into the
start of Ano1 and the second return mixed liquor (R2) from Aer2 was pumped into the Ano2, with both mixed liquor reflux ratios of 100%.

Figure 1. Diagram of A-MAO process reactor.

The reactor was operated for 180 days and divided into three phases. In Phase I (1–40 d), the influent was only pumped into anaerobic tank. Mechanical stirrers were installed in the anaerobic tank and two anaerobic tanks to ensure that the sludge and water were fully mixed. The bottom of the two aerobic tanks was equipped with microporous aeration disc, and the aeration intensity was adjusted via a gas flow meter to maintain the dissolved oxygen (DO) at approximately 2–4 mg/L. In Phase II (41–100 d), the influent was pumped into the Ana and Ano2 at 70% and 30% distribution ratios, respectively. The main operating parameters remained unchanged. In Phase III (101–180 d), different amounts of sodium acetate were added. To enhance carbon source utilization, Sodium acetate was added to the Ano1 and Ano2 zones through peristaltic pumps, with a distribution ratio of 70% and 30%, respectively. The dosage was 40, 80, 120, and 160 mg/L COD for 20 consecutive days for a total of 80 days. During the entire operation, with the exception of sampling and discharge of water containing a minimal quantity of suspended solids (SS), no excess sludge was discharged, thereby the sludge retention time (SRT) exceeded 30 days.

2.2. Inoculated Sludge and Wastewater Source

The inoculated sludge was collected from the aerobic tank of the AAO reactor in the municipal wastewater treatment plant (Yancheng, China), which has good nutrient removal performance and can accelerate the enrichment of bacteria. This experiment was conducted in the WWTP. The wastewater source was collected from the plant’s primary sedimentation tank to a large storage tank, and then pumped into the A-MAO reactor as influent. The main characteristics of influent wastewater were provided in Table 1.

2.3. Chemical Analysis Methods

Samples were collected from the different compartments of the reactor once two days. All samples were left for 30 minutes and analyzed after filtrating through 0.45 μm filter paper. According to the Examination of Water and Wastewater published by the American Public Health (APHA, 2005), the levels of COD, TN, NH₄⁺-N, NO₃⁻-N, and nitrite nitrogen (NO₂⁻-N) were measured using DR6000 ultraviolet spectrophotometer (Hach, DR6000, Loveland, CO, USA). Gravimetric method was used to measure MLSS and mixed liquid
volatile suspended matter (MLVSS). A portable dissolved oxygen meter (HQ30d, Hach) and pH meter (HQ1110, Hach) were used to determine DO and pH.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range</th>
<th>Average</th>
</tr>
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<tbody>
<tr>
<td>COD (mg/L)</td>
<td>168–217</td>
<td>191.8</td>
</tr>
<tr>
<td>NH₄⁺-N (mg/L)</td>
<td>34.6–48.7</td>
<td>41.5</td>
</tr>
<tr>
<td>NO₃⁻-N (mg/L)</td>
<td>0–4.2</td>
<td>0.6</td>
</tr>
<tr>
<td>TN (mg/L)</td>
<td>35.7–58</td>
<td>46.6</td>
</tr>
<tr>
<td>pH</td>
<td>6.7–8.3</td>
<td>7.2</td>
</tr>
<tr>
<td>T/°C</td>
<td>11–25</td>
<td>17</td>
</tr>
</tbody>
</table>

**Abbreviations:** COD, chemical oxygen demand; NH₄⁺-N, ammonia nitrogen; NO₃⁻-N, nitrate nitrogen.

2.4. Greenhouse Gas Collection and Measurement

Due to sufficient stirring of the reactor mixture, the dissolved gas content was relatively low, so the concentration of dissolved greenhouse gases was not measured in this study [14]. Set a sampling point in each cell, a total of five. A self-made greenhouse gas collection device (as shown in Figure 2) was used to collect the gas above the reactor liquid level and measured the concentrations of CH₄, N₂O, and CO₂. During each flux measurement, the gas collection device was placed on the reactor for 0.5 h in total, with five 50-mL headspace air samples being collected at 6-min intervals. Greenhouse gas collection was performed on the last day of each phase. The concentrations of CH₄, N₂O, and CO₂ in the samples were measured by a gas chromatograph (7890A, Agilent, CA, USA) after collection. The emission rates of CH₄, N₂O, and CO₂ (µg/min/g MLSS) were determined using the linear regression formula for greenhouse gas concentrations in the sample over time [15]. The direct emissions of N₂O, CH₄, and CO₂ were calculated through the following equation:

\[
W_1 = V_2 \cdot (w_{\text{air},n} - w_{\text{air},n-1}) \cdot M \cdot P / (T \cdot R \cdot V_1 \cdot MLSS \cdot \Delta t)
\]

where \(W_1\) is the direct emission rate of N₂O, CH₄, and CO₂ (µg/min/g MLSS) from the tanks; \(V_2\) is the volume of the upper-water space of each tank; \(w_{\text{air},n}\) is the concentration of N₂O, CH₄, and CO₂ at point \(n\) (v/v); \(M\) is the molecular weight of N₂O, CH₄, and CO₂ (44.02, 16, and 44 g/mol); \(P\) is the atmospheric pressure (1 atm); \(T\) is the temperature (293 K); \(R\) is the gas constant (0.082 L atm K⁻¹ mol⁻¹); \(V_1\) is the volume of the each tank; MLSS is the mixed liquor suspended solid (g/L); and \(\Delta t\) is the time interval between each sampling point (0.5 h).

2.5. High-Throughput Sequencing Analysis

To reveal the variation in the microbial communities and typical function microorganism during the 180-day experiment of the A-MAO process, the suspended activated sludge samples were gathered from the corresponding tank on days 1, 40, 100, and 180 for 16S rRNA gene amplicon sequencing. Primers 341F (5’- CCTAYGGRGBGCASCAAG-3’) and 806R (5’- GGACTACNNGGTATCTAAT-3’) were used for PCR amplification. Subsequently, all amplicon sequencing of the samples was performed on the Illumina-MiSeq platform (Shanghai BiozeroBio-Pharm Technology Co., Ltd., Shanghai, China).
Figure 2. Diagram of the self-made greenhouse gas collection device ((a): air collecting port; (b): fan; and (c): atmospheric pressure gauge).

3. Results and Discussion
3.1. Nutrient Removal Performance of A-MAO Process

3.1.1. Overall Performance of Organics and Nitrogen Removal

The duration of the continuous experiment of lab-scale A-MAO system was 180 days. During the operational period, the variations in key operating parameters, including COD, NH$_4^+$-N, and TN concentrations, are illustrated in Figure 3. The start-up process took about 40 days to reach steady. In phase I (1–40 d), the concentrations of influent COD, TN, and NH$_4^+$-N were 186.43 ± 10.7 mg/L, 45.47 ± 4.6 mg/L, and 41.5 ± 3.3 mg/L, respectively. The nutrient removal efficiency gradually increased with the experiment, and the concentrations of effluent COD, TN, and NH$_4^+$-N reached 53.8 ± 6.62 mg/L, 17.54 ± 3.2 mg/L, and 4.11 ± 2.78 mg/L. The removal rates of COD, NH$_4^+$-N, and TN were 70.97 ± 4.24%, 90.11 ± 6.4%, and 61.27 ± 6.41%, respectively.

In Phase II (41–100 d), the distribution of influent flow was adjusted to achieve efficient utilization of the carbon source. While maintaining a constant influent flow rate and concentration, the influence of influent distribution on effluent quality was investigated. The influent C/N ratio remained at 4.2, which was too low to meet the denitrification requirements of the system. After the influent distribution in the system, the effluent concentrations of COD, TN, and NH$_4^+$-N further decreased to 34.27 ± 12.13 mg/L, 14.53 ± 2.76 mg/L, and 1.69 ± 1.84 mg/L. The removal rates of COD, NH$_4^+$-N, and TN were 81.39 ± 6.78%, 95.94 ± 4.62%, and 68.57 ± 7.04%. 

3.2. Nitrogen Removal Efficiency of A-MAO Process
Figure 3. Long-term nutrient removal performance at different phases ((a): chemical oxygen demand (COD); (b): ammonia nitrogen (NH$_4^+$-N); and (c): total nitrogen (TN)).

In Phase III (101–180 d), sodium acetate (40–160 mg/L COD) was gradually added to the system on the basis of the influent distribution. The results revealed that, under the condition of a constant nitrogen load on the influent, the addition of external carbon sources had the opposite effect on the removal of organic matter and nitrogen in the system. As the dosage increased, the removal efficiency of NH$_4^+$-N exhibited a decline trend, subsequently followed by an upward. The addition of external carbon sources may
have increased the content of heterotrophic bacteria in the system. While the content of autotrophic nitrifying bacteria, which play a major role in the nitrification process, was relatively reduced, resulting in a decrease in the nitrification effect of the system and an increase in the effluent NH$_4^+$-N concentration. However, the continuous increase of sodium acetate may also lead to the enrichment of heterotrophic nitrification bacteria in the system, thereby enhancing the nitrification effect. Previous research has shown that there are heterotrophic nitrifying bacteria in the sewage treatment system [16]. The COD removal rate of the system showed a trend of first increasing and then decreasing, which may be due to the addition of sodium acetate stimulating the growth of activated sludge. However, with the further increase in sodium acetate, the upper limit of COD removal in the system was reached, and the effluent COD concentration gradually increased. The effluent TN concentration decreased with the increase in the carbon source dosage. When the carbon source dosage was 120 mg/L COD, the effluent TN decreased to $6.72 \pm 2.42$ mg/L, stably reaching integrated wastewater discharge standard (class A discharge standards, GB18918-2002, China) which were lower than 15.0 mg/L. With the further increase of dosage, the effluent TN concentration decreased to $6.65 \pm 1.73$ mg/L, and the removal efficiency hardly increased, indicating that the addition of the carbon source should be controlled within a reasonable range.

3.1.2. Nutrient Removal Efficiency in Different Units

For fully understanding the influence of operational variables on organic and nutrient removal mechanism in the A-MAO system, pollutant concentrations and utilization performance were tested in five functional units at different stages (as shown in Figure 4). In Phase I (1–40 d), the removal efficiency of COD and TN in Ano1 and Aer1 zones were 30.48% and 15.63%, which were significantly higher than 6.42% and 9.97% in Ano2 and Aer2 zones. Clearly, the first A/O group played an important role in the removal of pollutants throughout the process. This was related to the metabolism of aerobic microorganisms in Aer1 and the facultative microorganisms in Ano1 using influent nutrients as carbon sources for denitrification. Meanwhile, due to the low influent C/N ratio, the denitrification of the first A/O group exhausted the easily biodegradable COD, causing the microorganisms in the second A/O group to lack sufficient nutrients for rapid reproduction, resulting in the removal of residual pollutants is not ideal.

In Phase II (41–100 d), after partially feeding the influent into Ano2, the COD concentration and sludge concentration of the second A/O group were increased. The average effluent NH$_4^+$-N concentration was consistently less than 2.00 mg/L, probably due to the long hydraulic retention time of the aerobic tank leading to complete nitrification. There were 19.27% COD and 12.92% TN removed from the second A/O group, respectively, indicating that the influent carbon source was more effectively utilized after influent distribution. Despite the enhanced denitrification effect of the process, the effluent under the condition of a low C/N ratio still failed to meet the emission standard. Therefore, the first A/O group made a contribution to the removal of COD and nitrogen, while the second A/O group may also be a key supplement for improving the removal of pollutants.

In Phase III (101–180 d), the effect of sodium acetate on nutrient removal in the A-MAO process is shown in Figure 4c. The variation of nutrients in different treatment units was similar to that in influent distribution, but the addition of sodium acetate did not cause significant effect on TN removal. The concentration of COD in the anaerobic chamber dropped from 161.45 mg/L to 54.70 mg/L. Throughout the entire experiment, the anaerobic zone exhibited the highest pollutant removal efficiency, attributable to hydrolytic acidification. The addition of carbon source did not cause significant changes in the effluent COD concentration, which may be caused by the long hydraulic residence time of the system. The easily decomposable components of the influent and external carbon source were fully absorbed and utilized by microorganisms, while organic matter that was difficult to degrade or unable to be metabolized was discharged through the effluent. The concentration of NH$_4^+$-N in the anaerobic zone decreased from 37.3 mg/L to 16.9 mg/L, and then decreased to
8.6 mg/L and 1.72 mg/L after the first A/O group, respectively. The concentration change in other chambers were in small increments, which had appeared in previous studies [17]. The concentration of TN in Ana, Ano1, and Ano2 declined rapidly, and the changes were not obvious in the other compartments. Compared with the influent distributed condition (effluent TN concentration was 17.08 mg/L), the effluent TN concentration decreased to 8.5 mg/L. Thus, sodium acetate, as an external carbon source, increased the organic matter content of the Ano1 and Ano2 zones and promoted the denitrification process.

Figure 4. Change of nitrogen and COD in different units during (a) Phase I, (b) Phase II, and (c) Phase III.

3.2. Greenhouse Gas Emissions

3.2.1. N₂O Emission

In municipal WWTPs, most of the N₂O is emitted during the biological nitrogen removal process, including nitrification and denitrification reactions. The N₂O emission
rates in the A-MAO bioreactor system at different stages is shown in Figure 5a. The N$_2$O emission fluxes in anaerobic and anoxic zones made a small contribution to the total emission, while the emission in the two aerobic zones was significantly high throughout the entire experiment. The main reasons for the high release rate in the aerobic tank may be as follows: (a) an incomplete nitrification reaction leads to the formation of N$_2$O; (b) lack of sufficient denitrifying bacteria resulting in the production of intermediates in the denitrification process; and (c) the turbulent flow caused by intense aeration maximizes the release of dissolved N$_2$O [18].

![Figure 5](image)

**Figure 5.** Greenhouse gas emission rate of each functional unit at different stages (a): N$_2$O; (b): CH$_4$; and (c): CO$_2$.

Under low-C/N-ratio influent conditions, the N$_2$O emission of each treatment unit of the A-MAO process was significantly higher, especially in the aerobic zone. Due to insufficient nutrition in the system, nitrification and denitrification using endogenous carbon source were difficult to complete, resulting in increased N$_2$O formation, accumulation and release. High N$_2$O emission rates under low C/N ratio influent conditions had also been reported [19]. Therefore, the total N$_2$O emission of the system decreased significantly when sodium acetate was added. This indicated that the addition of external carbon sources can reduce the N$_2$O conversion rate. In Figure 3a, the emission rate of N$_2$O in the anaerobic zone was greater than that in the anoxic. This may be due to the presence of recalcitrant pollutants in the influent, which prevents denitrifying bacteria from utilizing exogenous carbon as an electron donor and can forces them to rely only on endogenous carbon as an electron donor. Additionally, due to the high concentrations of NO$_3^-$-N and NO$_2^-$-N in the effluent, a considerable amount of NO$_3^-$-N and NO$_2^-$-N was carried into the anaerobic tank during sludge reflux. Some fermenting bacteria used them as electron acceptors to perform denitrification reaction, thereby inhibiting the acid production from organic matter
fermentation. This might even lead to some denitrifying phosphorus-accumulating bacteria using endogenous products for the denitrification reaction. Beun J.J. et al. believed that the consumption of PHB (poly-\(\beta\)-hydroxybutyrate) was the rate-limiting step in the process of the microbial utilization of endogenous carbon [20]. This leads to competition among denitrifying reductases, making the reduction rate of NO less than that of \(\text{N}_2\text{O}\), resulting in the release of \(\text{N}_2\text{O}\). This also explains the increase of \(\text{N}_2\text{O}\) emission in Ano2 after the influent distribution. In brief, this study indicates that denitrification is the main source of \(\text{N}_2\text{O}\) emissions in the A-MAO process at a low influent C/N ratio. The distribution of the influent can improve the utilization efficiency of the carbon source in the system, resulting in a slight decrease in total \(\text{N}_2\text{O}\) emissions. The addition of external carbon sources is the key operation for the reduction of \(\text{N}_2\text{O}\) emission.

3.2.2. CH\(_4\) Emission

The CH\(_4\) emission rates of each unit in the A-MAO process under different operating conditions are shown in Figure 5b. Under the three different operating conditions, the CH\(_4\) emission of A-MAO bioreactor system was mainly from Aer1, followed by Ana and Ano1. Theoretically, the CH\(_4\) produced by wastewater treatment mainly comes from the anaerobic respiration of microorganisms, and the organic pollutants are converted into small carbon molecules and easily degraded products, which are utilized by methanogenic bacteria, resulting in the generation of CH\(_4\). However, an interesting finding in this study was that the CH\(_4\) emission rates of Ano1 and Aer1 were significantly high. This is because the pyrogenic bacteria mainly use acetic acid or \(\text{H}_2\) to produce CH\(_4\), and acetic acid and \(\text{H}_2\) are produced after hydrolysis fermentation bacteria and hydrogen-producing acid-producing bacteria decompose macromolecular organic matter. The degradation of macromolecular organic matter mainly occurred in the anaerobic zone, so the generation of CH\(_4\) was completed in the anoxic zones, resulting in more CH\(_4\) produced per unit time. In addition, the following three reasons may explain the high CH\(_4\) emission rate of Aer1: firstly, the high aeration rate will strip most of the dissolved CH\(_4\) in the wastewater into the atmosphere [21]; secondly, most of the dissolved CH\(_4\) in the aerobic region was oxidized [22]; and, thirdly, CH\(_4\) is not produced under aerobic conditions. Since there was almost no CH\(_4\) in Aer1 after adequate mechanical aeration, and the subsequent aerobic conditions were not suitable for methanogenic bacteria growth, the CH\(_4\) flux in Ano2 and Aer2 was at the lowest level.

In Phase II (day 100), the CH\(_4\) emission flux in the Ano2 region increased as shown in Figure 5b. This is because the anaerobic environment in the sewage network causes the influent to contain a considerable amount of dissolved CH\(_4\). When the influent was distributed to Ano2, the dissolved CH\(_4\) was released due to water flow fluctuations caused by mechanical agitation. With the addition of sodium acetate as an external carbon source, the CH\(_4\) emission rate of the anaerobic zone and anoxic zone increased significantly, while there was little change in the aerobic zone. Methanogenic bacteria are strictly anaerobic bacteria, which are believed to be generated only in the anaerobic environment, and basically not generated in the aerobic treatment process [23]. Therefore, the change in the influent load has little impact on the CH\(_4\) emission of the aerobic pool. After studying the release of CH\(_4\) in wetlands, Wang et al. concluded that methanogens may need a quick-available carbon source for activation [24]. The addition of external carbon sources such as acetate and glucose can increase the CH\(_4\) production rate of wetland soil. Zhou et al. added exogenous carbon substrates to carbon-poor hydroponic wastewater [25], which greatly increased the carbon availability of microbial activities, thus stimulating the production and release of CH\(_4\) under anaerobic conditions.

3.2.3. CO\(_2\) Emission

The CO\(_2\) emission rate of the A-MAO bioreactor system under different operating conditions has been investigated. The result is illustrated in Figure 5c. Each treatment unit of the A-MAO reactor can produce CO\(_2\), of which the CO\(_2\) emission rate in the aerobic zone
was much higher than that in the anaerobic and anoxic pools. The generation of CO$_2$ in the wastewater treatment process stems from the respiration of activated sludge mineralization to remove organic matter and microorganisms, which produces CO$_2$ and completes the synthesis of its own cellular substances, thereby purifying the sewage [7]. The direct CO$_2$ emission in this study was related to the amount and removal efficiency of influent COD. Moreover, within the same wastewater treatment system, the amount of CO$_2$ generated corresponding to the similar influent was positively correlated with the removal efficiency of COD. For the aerobic treatment stage of sewage, the oxidation removal of COD results in a large amount of CO$_2$ emission. Therefore, the decrease in the total CO$_2$ emission flux after the influent distribution is related to the increase in the COD removal rate. During the entire experimental period, the addition of sodium acetate (Day 180) significantly increased the average CO$_2$ emission rate compared to Day 40 and Day 100. This is reasonable because the addition of foreign carbon can stimulate microbial respiration, resulting in a large amount of carbon dioxide production.

3.3. Microbial Community Structure Analysis
3.3.1. Microbial Diversity and Community Composition

The bacterial communities of each compartment were analyzed through high-throughput 16S rRNA gene amplicon sequencing. The dynamic changes in the microbial community structure among functional units are shown in Figure 6. Based on the relative abundance of bacteria at the phylum and genus level, significant changes in the community composition were found in five units.

(a)

Figure 6. Cont.
which had the highest abundance, are anoxic DNBs, indicating that the system mainly used anoxic denitrification for nitrogen removal. Denitratisoma, Zoogloea, Flavobacterium, and Comamonas with aerobic denitrification function can adapt to the treatment units with a high degree of similarity in the Ana, Ano1, Ano2, Aer1, and Aer2 samples, respectively. In addition, Dechloromonas, Azospira, Saprospiraceaeuncultured, and Sulfuritalea had a significantly higher abundance in oxygen treatment units with dissolved oxygen. Thauera (3.03%) belongs to the category of facultative denitrifying bacteria, which can perform both aerobic and anoxic denitrification.

At the phylum level (Figure 6a), more than 40 phyla were detected in each sample, with the top 10 dominant phyla (relative abundance greater than 1%) accounting for more than 90% of the total community. The relative abundance of Proteobacteria ranges from 47.35% (Ana) to 52.69% (Aer1). Previous studies have shown that Proteobacteria were generally abundant in WWTPs [17], including most facultative or obligate anaerobic microorganisms, as well as many nitrifying and denitrifying bacteria, which played a crucial role in nutrient uptake and utilization [26]. Bacteroidetes (16.1~22.99%), Chlorobacteria (3.37~13.12%), and Firmicutes (5.71~7.86%) were the most dominant phyla after Proteobacteria. Other phyla, such as Acidobacteriota, Nitrospirola, Patescibacteria, Myxococcota, and Gemmatimonadota, were detected in more than 1% of the five units. At the genus level, there were apparent differences in microbial diversity among the samples of five treatment units. The six most dominant genera (Dechloromonas, Azospira, Saprospiraceaeuncultured, Sulfuritalea, Hydrogenophilaceaeuncultured, and Denitratisoma) in each treatment unit accounted for over 35%. Interestingly, the dominant genera retained a high degree of similarity in the Ana, Ano1, Ano2, Aer1, and Aer2 samples, respectively. In addition, Dechloromonas, Azospira, Saprospiraceaeuncultured, and Sulfuritalea had a significantly higher abundance in oxygen-rich environments.

The nitrifying and denitrifying bacterial communities that played a key role in nitrogen removal in the A-MAO process were stable. Nitrosomonas was the main ammonia-oxidizing bacteria (AOB) genus, with a relative abundance ranging from 0.33 to 0.76% (Figure 6b), and no members of the AOB genus were detected during the whole experiment. Previous studies have found that Nitrospira, Nitrospine, Nitrobacter, and Nitrococcus were the core bacteria of nitrite-oxidizing bacteria (NOB) [27]. However, in this system, except Nitrospira (1.04~2.73%), the abundance of other genera was below 0.1%. Thus, it can be concluded that Nitrosomonas and Nitrospira occupied a dominant position in AOB and NOB communities in the A-MAO process, essential for maintaining stable nitrification [28]. This conclusion is consistent with the reported data [29]. Denitrifying bacteria (DNBs) use electron donors to reduce NO$_3^-$ or NO$_2^-$ to $N_2$. There were three types of denitrifying bacteria in the system, including aerobic denitrifying bacteria, anoxic denitrifying bacteria, and facultative denitrifying bacteria. Dechloromonas, Azospira, and Balitalea, which had the highest abundance, are anoxic DNBs, indicating that the system mainly used anoxic denitrification for nitrogen removal. Denitratisoma, Zoogloea, Flavobacterium, and Comamonas with aerobic denitrification function can adapt to the treatment units with a
high level of dissolved oxygen. Thauera (3.03%) belongs to the category of facultative denitrifying bacteria, which can perform both aerobic and anoxic denitrification [16]. Hence, multiple denitrifying microorganisms jointly completed the nitrification and denitrification process in this system.

3.3.2. Dynamic Change of Functional Microorganism

To further explore the mechanism of pollutant removal in the A-MAO process, microbial community structure fluctuations of the A-MAO bioreactor on Days 1, 40, 100, and 180 are showed in Figure 7. At the phylum level, Proteobacteria (49.6~72.49%), Bacteroidota (7.95~18.9%), and Chloroflexi (2%~8.15%) dominated the A-MAO bioreactor during the operation. The relative abundance of Proteobacteria decreased from 72.49% on Day 1 to 49.6% on Day 180 gradually, but it still held an obvious advantage. As the experiment progressed, the relative abundance of Bacteroidota, Chloroflexi, and Acidobacteriota gradually increased, indicating that the environmental conditions and operating modes of A-MAO were conducive to the enrichment and growth of these microorganisms. It has been reported that Bacteroidetes and Chloroflexi also possess the ability to decompose macromolecular substances (such as lignin and cellulose), thereby improving their biodegradability [30]. Notably, after the addition of sodium acetate, the abundance of Firmicutes increased greatly from 0.44% to 6.43%. Firmicutes can perform metabolic activities by degrading macromolecular organic matter such as cellulose, protein, and polysaccharide [31]. This indicated that the addition of sodium acetate improved the utilization of refractory organic compounds in the system.

Figure 7. The relative abundance of bacterial communities in different phase at the phylum level. Typical AOB&NOB, DNBs, GAOs, and hydrolytic bacteria were identified at the genus level (Figure 8). As mentioned above, Nitrosomonas and Nitrospira were the dominant AOB and NOB in A-MAO reactors [32], which co-convert NH$_4^+$-N to NO$_3^-$-N during
nitrification. After the allocation of influent, the relative abundance of Nitrosomonas and Nitrospira increased from 0.33% and 1.04% to 0.76% and 2.35%, respectively, especially in the two aerobic zones. The relative abundance of Nitrospira increased slightly after the addition of sodium acetate, while Nitrosomonas remained almost unchanged, indicating that the addition of external carbon source did not significantly improve the nitrification capacity of the system. The Dechloromonas, Azospira, sulfitalea, Denitratisoma, and Thauera in this system are typical heterotrophic DNs, widely existing in various wastewater treatment reactors. Nevertheless, the relative abundance of Dechloromonas, Thiitalea, Denitratisoma, and Thauera decreased significantly, and even Thauera almost disappeared, indicating that the system was not suitable for their survival. Meanwhile, the relative abundances of Azospira, Terrimonas, and JGI 0001001-H03 increased significantly from 3.5%, 0.55%, and 0.2% on Day 40 to 16.43%, 0.71%, and 0.69% on Day 180. As a nitrate-dependent ferrous oxide bacterium widely present in natural water bodies and sediments, Azospira can utilize nitrate and chlorate as electron acceptors of ferrous oxide under hypoxic conditions [33]. Some species in Azospira have been reported to have a higher N₂O-reducing capacity than other known N₂O-reducing agents [34], and their increased abundance may reduce emissions of the potent greenhouse gas N₂O during heterotrophic denitrification. Therefore, Azospira increased significantly after the addition of sodium acetate to the system (Figure 8), which is considered to be an important functional genus for increasing the nitrogen loss in wastewater treatment systems.

![Chart](image.png)

**Figure 8.** The relative abundance of functional bacterial community at genus level. (GAOs: glycogen-accumulating organisms; AOB: ammonia oxidation bacteria; NOB: nitrite oxidation bacteria; and DNs: denitrifying bacteria).

For typical GAOs, such as Candidatus Competibacter, Comamonadaceae_Unclassified, Comamonadaceae, and Defluviicoccus, the total relative abundance increased from 0.24% and 0.62% on Day 1 to 3.57% and 0.9% on Day 100, respectively. Typical GAOs can form intracellular carbon sources in anaerobic environments and perform endogenous denitrification (END) in anoxic environments [35]. This indicates that the alternating anoxic and aerobic environmental conditions of A-MAO are conducive to the enrichment of key microorganisms in the END, thus reducing NO₂⁻⁻N and NO₃⁻⁻N. The relative abundance of typical hydrolytic microorganisms (Saprospiraceae_uncultured, Hydrogenophilaceae_uncultured, and Saccharimonadales_norank) increased from 4.22%, 3.53%, and 0.23% on Day 1 to 8.6%, 4.17% and 0.44% on Day 180. Saprospiraceae_uncultured and
hydrogenophilic aceae_uncultured are the primary microorganisms in hydrolysis and fermentation systems. They can degrade macromolecular organic compounds such as carbohydrates and proteins in suspended sludge systems, and play a crucial role in improving the production rate of VFAs in sludge.

3.3.3. Significant Differences of Microbial Communities and Metabolic Functions

Principal co-ordinate analysis (PCoA) mainly uses Unifrac, Bray-Curtis, and other sample distance information for calculation and dimensional reduction graphic display. A closer distance indicates that the microbial community composition of the two samples is similar [36]. To further confirm that microbial communities in the activated sludge were significantly different under different operating conditions, PCoA analysis was conducted on wastewater treatment units at each stage (as shown in Figure 9). PCoA clusters microbial communities based on time, separating different stages along Axis 1, which explains 62% of the variation. The samples from the same stage were tightly clustered together, showing that changes in operating conditions such as the influent distribution and external-carbon-source dosing caused changes in the bacterial community structure.

Figure 9. Comparison of microbial communities by principal coordinate analysis (PCoA).

In addition, we assessed the presence of different bacterial genera using Linear discriminant analysis Effect Size (LEfSe) methods. By comparing microbial communities at different stages, LEfSe revealed biomarker bacteria (BmB) under different operating conditions [37]. The phylogenetic tree diagram of BmB (Figure 10a) shows the evolutionary branching of systemically dominant species at different stages, indicating significant differences in dominant species at different phases. Applying LEfSe to microbial community data of 20 samples in three phases, 26 taxonomic clades with diverse LDA scores greater than 4.0 were found (Figure 10b).
Figure 10. LEfSe analysis of microbial abundance at different times. (a) is the cladogram of microbial communities. (b) is LDA score, and identified the size of differentiation with a threshold value of 4.

In Phase I (Day 40), Dechloromonas, Thiothrix (member of Thiotrichales), and Thauera were significantly enriched and the microbial community structure of the system was altered compared to inoculated sludge samples (Day 1). In Phase II (Day 100), Methylotenera (members of Methylophilaceae) are known to degrade molecules such as antibiotics through the cleavage or splitting of C-N, S-N, and N-N bonds [38]. This confirms an
improvement in the system’s ability to degrade complex substances. Azospira, Candidatus Competibacter, and CLB13 were abundant at the genus level in Phase III, which was consistent with the previous microbial community analysis. This explains the obvious improvement of the nitrogen removal effect and the decrease in N₂O in this stage.

FAPROTAX was used for the functional prediction of microbial communities, and the functional classification of significant differences was performed by a STAMP difference analysis (as shown in Figure 11). In each stage of the experiment, microorganisms mainly engaged in the carbon (C) and nitrogen (N) element cycling metabolism and organic matter decomposition. Chemo heterotrophic and aerobic chemoheterotrophic microorganisms associated with carbon cycle metabolism dominated among them. Nitrogen-metabolism-related microorganisms are mainly characterized by nitrate reduction, nitrogen respiration, nitrate respiration, and nitrite respiration, nitrogen fixation, and nitrate denitrification and nitrite denitrification. The microbial decomposition of organic matter mainly includes methanol oxidation, methylation, and the dark oxidation of sulfur compounds. Pathogenic microorganisms are not analyzed here.

Figure 11. Comparison of microbial community functions at different stages. (a) is the comparison between Phase I and Phase II; and (b) is the comparison between Phase II and Phase III.

Comparing the carbon metabolism process of bacteria and microorganisms during the experiment, the relative abundance of microorganisms playing a chemoheterotrophic role decreased significantly, while aerobic chemoheterotrophic microorganisms were relatively stable in each stage of the experiment. The relative abundance of microorganisms involved in nitrate reduction, nitrogen respiration, nitrate respiration, and nitrite respiration decreased first, and then increased during the comparative experiment. And the microorganisms that engage in nitrogen fixation, nitrate denitrification, and nitrite denitrification have significantly increased in Phase III, while, in Phase II and III, the number of these microorganisms is almost zero. The results indicated that the addition of a carbon source significantly promoted the nitrogen removal function of the system. Comparing the organic
matter decomposition process of microorganisms in the experiment, methanol oxidation and methylothrophy were significantly increased in Phase II, indicating that the influent distribution improved the microbial decomposition ability of organic matter in the system. Overall, the improvement of the decomposition capacity of carbon- and sulfur-containing organic matter in Phase II indicates that the operation of the influent distribution has a certain potential in the treatment of high-load organic wastewater and sulfur-containing wastewater. The increasing abundance of nitrogen fixation and denitrification in Phase III indicates that the addition of sodium acetate as an external carbon source has a good effect on the treatment of wastewater with a high nitrogen load.

4. Conclusions

The effects of the influent distribution and the addition of sodium acetate as an external carbon source on nutrient removal and direct greenhouse gas emissions in low-C/N-ratio domestic wastewater treatment were investigated through the long-term operation of the A-MAO process in this study. The influent distribution and external-carbon-source addition reduced effluent COD to $34.27 \pm 12.13$ and $30.2 \pm 4.34$ mg/L, and effluent TN to $14.53 \pm 2.76$ and $6.65 \pm 1.73$ mg/L, respectively. The TN removal efficiency can be improved by increasing external carbon sources within a certain range, but it is ineffective if an excess of carbon sources is used. The total $\text{N}_2\text{O}$, $\text{CH}_4$, and $\text{CO}_2$ emissions decreased after influent distribution, which was related to the increased COD and TN removal rates of the system. The addition of external carbon sources resulted in a significant decrease in the $\text{N}_2\text{O}$ emission rate in the system, while the emission rates of $\text{CO}_2$ and $\text{CH}_4$ increased notably. This showed that the addition of sodium acetate promoted the complete nitrification and denitrification of the system, thus reducing the production of the intermediate $\text{N}_2\text{O}$, and promoting the growth of methanogens and heterotrophic microorganisms in the system, resulting in the increased production and emission of $\text{CO}_2$ and $\text{CH}_4$. High-throughput sequencing further revealed that the influent distribution promoted the increase in Bacteroidota, Chloroflexi, and Acidobacteriota in each functional unit of the reactor, indicating that the decomposition capacity of organic matter in the system had improved. The relative abundance of typical GAOs (Candidatus Competibacter), NOBs (Nitrospira), DNBs (Azospira), and hydrolytic bacteria (Saprospiraceae_uncultured) in the system increases significantly after the addition of sodium acetate. Among them, the significant increase in Azospira can also reduce the emission of $\text{N}_2\text{O}$ in the process of heterotrophic denitrification, which was considered to be an important functional genus for increasing the nitrogen loss in this system. The influent distribution enhanced the carbon metabolism and organic decomposition function of the system, while the addition of sodium acetate as an external carbon source improved the nitrogen metabolism function of the system. In conclusion, the strategies investigated provide valuable insights for achieving advanced pollutant removal and minimizing carbon emissions. They pave the way for the design of more efficient and sustainable wastewater treatment plants, thereby advancing practical environmental technologies.

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