



# Comparison of Swine Wastewater Treatment by Microalgae and Heterotrophic Nitrifiers: Focusing on Nitrogen Removal Mechanism Revealed by Microbiological Correlation Analysis

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**Abstract** Swine wastewater (SW) poses a great threat to the environment due to its high-nutrient profiles if not properly managed. Advanced biological treatment method is an efficient method to treat SW by screening potent microalgae or bacterial strains. In this study, activated sludge, three locally isolated heterotrophic nitrification bacteria and one microalgal strain (*Chlorella*) were used as inoculums in treating a local SW. Their treatment efficiencies were compared, while the nitrogen removal mechanisms and microbiome profile were explored in detail. It was found that certain heterotrophic nitrification strains had a slight advantage in removing chemical oxygen demand and phosphorus from SW, with the highest removal efficiencies of 83.9% and 76.2%, respectively. The removal efficiencies of ammonia

nitrogen and total nitrogen in wastewater by microalgae reached 80.9% and 66.0% respectively, which were far higher than all the heterotrophic nitrification strains. Biological assimilation was the main pathway of nitrogen conversion by microalgae and heterotrophic nitrifying bacteria; especially microalgae showed excellent biological assimilation performance. Correlation analysis showed that *Comamonas* was highly positively correlated with nitrogen assimilation, while *Acidovorax* was closely correlated with simultaneous nitrification and denitrification. This study gives a comparison of microalgae and heterotrophic nitrifying bacteria on the nitrogen transfer and transformation pathways.

**Keywords** Bioaugmentation · Swine wastewater · Nitrogen removal mechanism · *Chlorella* · Heterotrophic nitrification

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## 1 Introduction

China is the world's largest producer and consumer of pork. According to the National Statistics Bureau of China, the number of domestic pigs raised in 2018 was about 428.17 million, about twice the number of 1970. The urine, excrement and flushing water produced in the process of raising pigs lead to the discharge of a large amount of highly polluted swine wastewater. Swine wastewater usually contains chemical oxygen demand (COD) of 5000–30,000 mg/L, total nitrogen (TN) of 800–6000 mg/L and total phosphorus (TP) of 100–1400 mg/L (Ding et al., 2017). The direct discharge of swine wastewater will cause severe environmental problems such as eutrophication, odor and hypoxia (Cao et al., 2019).

Traditional biological treatment methods used in municipal wastewater treatment could not be adapted to treat high-strength swine wastewater, due to the high operational costs and complicated processes featured by multiple steps of anoxic-aerobic combinations in order to deal with high concentrations of COD, TN and TP. Therefore, the strategy of locating some highly efficient microorganisms (such as microalgae or heterotrophic nitrifying bacteria) to treat swine wastewater while realizing a simultaneously nutrient recovery has received extensive attention in recent years (Chen et al., 2018; Wang et al., 2015; Wen et al., 2017). Microalgae could play an important role in the high-strength swine wastewater remediation evidenced by a high member of published works. Microalgae species, especially *Chlorella*, are highly capable of absorbing nitrogen and phosphorus from swine wastewater (Ledda et al., 2015; Wang et al., 2015; Wen et al., 2017). The study done by Wen et al. found that the removal efficiencies of TN and TP can reach 90.51% and 91.54%, respectively, by using *Chlorella vulgaris* isolated from swine wastewater to treat undiluted swine slurry with a relatively low nutrient profile (COD  $824.53 \pm 31.58$  mg/L, TN  $547.78 \pm 44.53$  mg/L, TP  $81.72 \pm 14.25$  mg/L) (Wen et al., 2017). Ledda et al. (2015) applied a wild *Chlorella* strain isolated from a fecal storage tank to purify swine manure digestate. The removal efficiencies for  $\text{NH}_4^+\text{-N}$ , TP and COD reached 95%, 85% and 73%, respectively, for starting concentrations of  $60.00 \pm 0.61$  mg  $\text{NH}_4^+\text{-N}$  /L,  $18.11 \pm 0.06$  mg TP /L and  $1099.07 \pm 6.83$  mg COD /L. The analysis of nitrogen mass balance showed that only 30% of the

total nitrogen was absorbed into microalgae, while most of the nitrogen was lost to the atmosphere due to stripping caused by aeration and high pH. Certain heterotrophic nitrifying strains as well as microalgae could play important roles in the remediation process. In addition, some studies were carried out to screen heterotrophic nitrifiers in order to realize the intensive treatment of high-nutrient wastewater (Chen et al., 2012, 2018; Qing et al., 2018). Chen et al. (2018) isolated *Acinetobacter* sp. T1 from pig farm sewage treatment facilities and found that the strain had capabilities of heterotrophic nitrification and aerobic denitrification. It was found that when strain T1 was used to treat swine wastewater (COD 918.29 mg/L,  $\text{NH}_4^+\text{-N}$  508.36 mg/L, TN 656 mg/L), the removal efficiency of COD,  $\text{NH}_4^+\text{-N}$  and TN reached 64%, 42% and 28%, respectively, higher than 30%, 15% and 16% of traditional activated sludge. Qing et al. (2018) isolated *Pseudomonas* AD-1 from activated sludge, which can not only perform aerobic denitrification, but also absorb large amounts of  $\text{NO}_3^-\text{-N}$ ,  $\text{NO}_2^-\text{-N}$  and  $\text{NH}_4^+\text{-N}$  for growth through cell assimilation. By nitrogen balance calculation, it was found that about 78% of  $\text{NO}_3^-\text{-N}$  was absorbed by strain AD-1 and transformed into intracellular nitrogen. Meanwhile, about 18% of  $\text{NO}_3^-\text{-N}$  was converted into gas, and a small part was converted into organic nitrogen and ammonia nitrogen. *Rhodococcus* sp. CPZ24 isolated from swine wastewater showed different denitrification performance (Chen et al., 2012). It was found that in a 50-L bioreactor, about 85% of ammonia nitrogen was removed and converted into nitrification products ( $\text{NO}_3^-\text{-N}$  and  $\text{NO}_2^-\text{-N}$ ) (13%) and gas (48%) respectively, while only 24% was assimilated into intracellular nitrogen. Therefore, strains and reaction conditions may be the influencing factors of different nitrogen transformation pathways. The above studies have used the screened strains or microalgae to enhance the removal efficiency of swine wastewater, and some studies have also measured nitrogen conversion pathways. However, few studies have compared the effects and mechanisms of the selected microalgae and strains with the same swine wastewater and experimental environment, while there is poor data on the analysis of the correlation between bacterial species and nitrogen conversion pathways in mixed bacteria culture.

The present study aimed to compare the enhanced effects of heterotrophic nitrification strains and

microalgae (*Chlorella*) on the nutrient removal efficiencies during swine wastewater treatment. To this end, firstly, certain heterotrophic nitrification strains were screened from aerobic activated sludge. Then, the nutrient removal performances using either heterotrophic nitrification strains or microalgae as inoculums were investigated with a diluted local swine wastewater. Finally, the mechanisms were analyzed in terms of nitrogen transformation pathways, microbial communities and correlation analysis between nutrient removal and given bacterial species.

## 2 Methods

### 2.1 Microbial Screening and Culture

The microalgae used in this experiment were *Chlorella micrococcus*. The algae specie was prepared for use in Tris–Acetate–Phosphate Medium (TAP). The activated sludge was taken from an aerobic activated sludge Sequencing Batch Reactor (SBR) operating in the laboratory. The heterotrophic nitrification bacteria were isolated and screened by phenol red vickers salt medium. The composition of phenol red vickers salt medium was as follows:  $(\text{NH}_4)_2\text{SO}_4$  2.4 g/L, glycerin 16 g/L, phenol red 0.5 mg/L, vickers salt 50 mL/L, agar 20 g/L. The composition of vickers salt is as follows (g/L):  $\text{K}_2\text{HPO}_4$  5,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  2.5, NaCl 2.5,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.05,  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  0.05. The solid screening medium was prepared by adding 20 g/L agar into vickers salt liquid medium. The activated sludge in the aerobic activated sludge SBR reactor was centrifuged (5000 r/min, 5 min) and resuspended for further use. Then, appropriate

amount of bacterial suspension was applied to the solid culture medium of phenol red vickers salt and incubated for 3 days under constant temperature of  $25 \pm 2$  °C, and afterwards the yellow colonies were chosen to inoculate the liquid medium for the preparation of the cultures. After 3–5 days of culture, 9 heterotrophic nitrification strains were screened and inoculated into simulated swine wastewater. The selected heterotrophic nitrification strains were named from S1 to S9 according to the sequence. Three nitrifying heterotrophic strains (S1, S2 and S4) with strong nutrient removal ability were selected and compared with microalgae.

### 2.2 The Collection and Analysis of Swine Wastewater

The swine wastewater was collected from an initial sink of a pig farm in Chongming county, Shanghai. The original wastewater was diluted with water 10 times, mixed, centrifuged, while the supernatant was filtered further through a 0.45- $\mu\text{m}$  filter to remove insoluble solids. The pH value of the filtrate, nutrient concentration (COD, TP, TN,  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_3^-\text{-N}$ ,  $\text{NO}_2^-\text{-N}$ ) and the detected metal and non-metal element concentrations are shown in Table 1.

### 2.3 Tests of the Effects of Different Inoculums on Swine Wastewater Treatment

Microalgae, heterotrophic nitrifying strains and activated sludge, all being in their logarithmic growth stage were taken and centrifuged at 5000 g for 10 min to remove the supernatant and then inoculated respectively into 100 mL swine wastewater at a

**Table 1** The composition of wastewater

Parameters (mg/L)	Initial swine wastewater	Experimental swine wastewater	Parameters (mg/L)	Experimental swine wastewater
pH	7.26	7.60	Zn	0.10
COD	8900	3040	Mg	37.08
TP	390	62	Na	61.29
$\text{NH}_4^+\text{-N}$	2660	218.49	S	33.42
TN	6820	338	B	0.17
$\text{NO}_3^-\text{-N}$	0.002	18.73	Mn	0.53
$\text{NO}_2^-\text{-N}$	0.2	0.001	Ca	42.21
Cu	\	0.08	Si	2.02
Ni	\	0.05		

concentration of 0.025 g/L and cultured in a thermostatic oscillating incubator. The culture environment had a temperature of  $25 \pm 2$  °C, an oscillation speed of 150 rpm and a light intensity of  $285 \pm 20$  lx. External experimental group of microalgae with strong light intensity ( $7791 \pm 300$  lx) was set up with the same temperature, oscillation speed and reaction mode as the above experimental groups. The growth of microorganisms and the removal of nutrients from wastewater were observed for 3 consecutive days when the nutrient concentration maintained relatively stable. In this study, the experimental group of microalgae under low light intensity ( $285 \pm 20$  lx) was named A1, and the experimental group of microalgae under strong light intensity ( $7791 \pm 300$  lx) was named A2. The activated sludge experimental group was named SL. Three nitrifying heterotrophic strains (S1, S2 and S4) with strong nutrient removal ability were selected and compared with microalgae.

## 2.4 Physiochemical Analytical Methods

The nutrient concentration of wastewater before and after microbial culture was centrifuged and filtered to detect the nutrient concentration. The COD, TP, TN and  $\text{NH}_4^+\text{-N}$  in sample were determined by spectrophotometry (DR2800, Hach, USA). The biological dry weight, nitrate nitrogen ( $\text{NO}_3^-\text{-N}$ ) and nitrite nitrogen ( $\text{NO}_2^-\text{-N}$ ) in the samples were determined by standard method (APHA, 2005). The content of metallic and nonmetallic elements in the sample was determined by inductively coupled plasma spectrometer (Prodigy-ICP, USA). The nitrogen content in biomass was determined by elemental analyzer (VarioELIII, Elementar, Germany). The pH value of the sample was determined by pH meter (PB-10, Sartorius, Germany).

## 2.5 Microbial Diversity Detection Methods

Sampling and identification of microbial composition after repeated batches of domestication. The 16S rRNA high-throughput sequencing and species diversity analysis were performed after the supernatant was removed from wastewater mixed with microorganisms by centrifugation. The processes of DNA extraction, PCR and data processing in 16S rRNA high-throughput sequencing are described in detail in the supporting information (SI).

## 2.6 Calculation Methods

### 2.6.1 Nitrogen Conversion Calculation

In this study, the part of nitrogen absorbed and assimilated (Assimilation) by microorganisms in wastewater was calculated by measuring nitrogen content in the harvested freeze-dried biomass. Nitrogen removed by simultaneous nitrification and denitrification (SND) was obtained by subtracting biomass nitrogen content from total nitrogen content ( $\Delta\text{TN}$ ) (Fig. 4).

$$\Delta\text{TN} = \text{TN}_{\text{influent}} (\text{mg/L}) - \text{TN}_{\text{effluent}} (\text{mg/L});$$

$$\text{Assimilation} (\text{mg/L}) = \text{N\%} * \text{Dry weight} (\text{mg/L});$$

$$\text{SND} (\text{mg/L}) = \Delta\text{TN} (\text{mg/L}) - \text{Assimilation} (\text{mg/L}).$$

### 2.6.2 Correlation Analysis

The iSanger cloud platform provided by Majorbio BioPharm Technology Co. Ltd. (Shanghai, China) was used to analyze the heatmap of the correlation between the calculated results of nitrogen transformation pathway in Section 2.6.1 (nitrification, denitrification and biological assimilation denitrification) and the abundance of each flora in the bacterial experimental groups (SL, S1, S2, S4, S5, S9,  $n=6$ ). The calculated numerical matrix was visualized by the heatmap diagram by calculating the Spearman rank correlation coefficient between the environmental factor and the selected species. The color information reflected the data information in the two-dimensional matrix or table, and the color depth indicated the size of the data value. The analysis result is shown in Fig. 5.

## 3 Results and Discussions

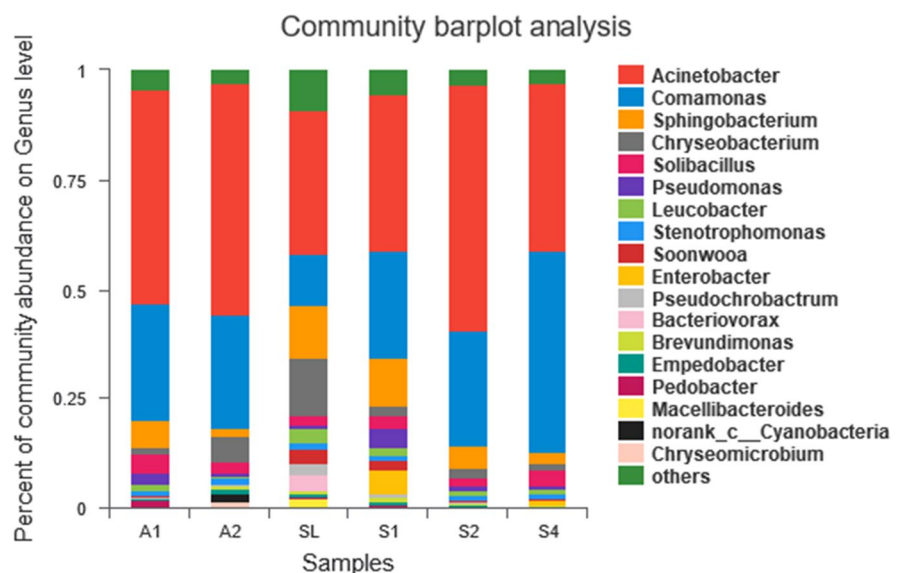
### 3.1 Microalgae and Heterotrophic Nitrifying Bacteria on the Community Composition and Diversity

Microorganisms are the main body of nutrient removal and transformation in wastewater. The addition of heterotrophic nitrifying bacteria or

microalgae to swine wastewater may have different effects on the composition and structure of microorganisms. The identification results of microbial community composition in the experimental group S1, S2, S4, SL, A1 and A2 are shown in Table S1 and Fig. 1. Table S1 shows the Shannon index of microbial community diversity in each experimental group. Shannon index is usually used to reflect the diversity of microbial community. It can be clearly observed that the Shannon index of swine wastewater treated with activated sludge (SL) is the highest among all samples. This also indicates that the microbial species in the experimental group SL is far more than that in the other experimental groups. As shown in Fig. 1, *Acinetobacter*, *Comamonas*, *Sphingobacterium*, *Chryseobacterium* and *Solibacillus* were the dominant species in all samples at the genus level (species with abundance below 0.01 are classified in the term “others”). It can be observed that in the samples of different experimental groups, the species composition is basically similar, but the abundance distribution characteristics are completely different. *Acinetobacter* occupied a relatively high abundance in each sample, among which the highest abundance in group S2 was 0.563, followed by 0.530 in group A2, and the lowest abundance in group SL was 0.326. For another genus with a relatively high proportion, the abundance of *Comamonas* in S4 was significantly higher than that in other samples, reaching 0.463, while

the lowest abundance still occurred in group SL at 0.116. *Acinetobacter* is considered to be a critical polyphosphate-accumulating organisms (PAOs) (Lu et al., 2014) and can be used for heterotrophic nitrification and aerobic denitrification with ammonia, nitrite and nitrate as substrates (Su et al., 2015). *Comamonas* is considered as a microbiome closely related to anaerobic denitrification (Sayess et al., 2013). Among the other four samples, the abundance of *Comamonas* was similar between 0.246 and 0.268. The above two species were not only abundant but also had the function of participating in nitrogen and phosphorus migration and transformation. *Chryseobacterium* has also been reported as a nitrifying and denitrifying bacterium (González et al., 2017). It can be found that the highest abundance of this genus is 0.130 in SL group, followed by 0.057 in A2 group, while the abundance value in other groups is lower than 0.024. Meanwhile, a variety of bacteria closely related to denitrification were detected in the samples, such as *Pseudomonas*, *Leucobacter*, *Brevundimonas* and *Pseudochrobactrum* (Srinandan et al., 2011; Sui et al., 2018; Weon et al., 2012; Zhang et al., 2018). The distribution difference of these bacteria must be the main factor of the diverse nutrient removal rates in swine wastewater, especially nitrogen removal. Moreover, the activity of a variety of anaerobic denitrifying bacteria is one of the factors leading to the pH change of wastewater (Youneng et al., 2011). In addition,

**Fig. 1** Bacterial community diversity (relative abundance > 1%) analysis of all samples at genus level



*Sphingobacterium* has certain biological flocculation effect and can degrade fatty acids and complex organic compounds (Lee et al., 2013). *Solibacillus* is associated with fermentation of complex carbohydrates (Jonge et al., 2017). These two species were present in all experimental samples and played an important role in the removal of organic matter from wastewater. *Macellibacteroides* and *Bacteriovorax* were only found in SL samples, which were also believed to have the function of degrading nutrient. *Macellibacteroides* is a major carbohydrate degrading bacteria in sludge (Chen & Chang, 2017). *Bacteriovorax* is mainly found in activated sludge and participates in the waste treatment process (Reddy & Mohan, 2012). It is speculated that the screening process is the main reason why these two species were not found in the experimental group of heterotrophic nitrifying bacteria (e.g. S1, S2 and S4). Some pathogens from feces have also been identified, such as *Stenotrophomonas* and *Enterococcus*. *Enterococcus* is a typical nosocomial pathogenic bacterium, which is commonly found in the digestive tract of humans and breeding animals and mainly enters the environment with feces (Chajęcka-Wierzchowska et al., 2017). *Stenotrophomonas* had similar abundance in each sample, while *Enterobacter* had higher abundance in S1 (0.055), followed by S4 (0.012), while the abundance in other samples was almost negligible. *Cyanobacteria* were detected only in A1 and A2, and a higher abundance of 0.016 was found in A1. This could be explained because in general *Cyanobacteria* are sensitive to light and prefer relatively low light intensities (Häder, 1987). In addition, some species have been found to perform a variety of functions in the consortium, such as being associated with sludge settling properties and adaptability to nutrient loads. *Chryseobacterium* and *Acinetobacter* were found to converge and secrete protein to enhance this aggregation and adapt to higher COD load (Adav et al., 2010). *Leucobacter* is a highly hydrophobic microorganism with a strong negative surface charge, which is related to the high flocculation index and thus has a certain impact on the sedimentation performance of sludge (Gonzalez-Martinez et al., 2017).

In summary, a variety of functional species were identified and found in the different experimental samples; particularly, 8 of the identified bacterial species are closely related to nitrogen transformation

(Table 2). The abundance of these bacteria in each experimental group was  $0.850 \pm 0.033$  (A1, A2),  $0.822 \pm 0.081$  (S1, S2 and S4) and 0.652 (SL), respectively.

### 3.2 Comparison of the Removal Efficiencies of Nutrients from Wastewater by Microalgae and Heterotrophic Nitrifying Bacteria

The removal of nutrients from wastewater in the experimental groups was observed, and the nutrient concentration maintained relatively stable after 3 days of reaction. As shown in Fig. S1, when the heterotrophic nitrifying strains (S1, S2 and S4), *Chlorella* (A1, A2) and activated sludge (SL) were used to the swine wastewater treatment, the pH of the wastewater increased after the process. The pH of wastewater in the group A2 increased from 7.60 to 10.03, which was the highest among all groups. This is mainly related to the absorption of  $\text{CO}_2$  by microalgae during photosynthesis that is taken by the dissolved inorganic carbon  $\text{HCO}_3^-$  and the releasing of  $\text{OH}^-$  ions ( $\text{HCO}_3^- + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3 + \text{OH}^-$ ,  $\text{H}_2\text{CO}_3 \rightleftharpoons \text{CO}_2 + \text{H}_2\text{O}$ ), leading to an increase in pH (Darvehei et al., 2018). Compared with this group, the pH of the wastewater in the group A1 was only 8.75 after the reaction, which further showed that the different growth rates and the higher amounts of  $\text{CO}_2$  absorbed by microalgae induced by increased light intensity were the main factors leading to the huge differences in the pH of wastewater. Moreover, it is hypothesized that the different degrees of denitrification in A1 and A2 may also cause diverse pH changes, which however requires further detailed identification and verification. The increase of the wastewater pH in the group SL, S1, S2 and S4 was closely related to the denitrification of microorganisms.

During the growth process of microorganisms in the wastewater, various organic and inorganic nutrients are consumed and therefore removed. As shown in Fig. 2a, the removal efficiency of COD in the experimental group ranged from 75.99 to 83.88%. It can still be observed that the COD removal efficiencies of the two microalgae groups (A1, A2) were slightly lower than that of the other experimental groups. Moreover, the COD removal efficiency of the group A2 was also lower than that of the group A1, while the microalgae concentration of A2 was higher. This phenomenon is closely related to the fact that

**Table 2** The main characteristics of each species

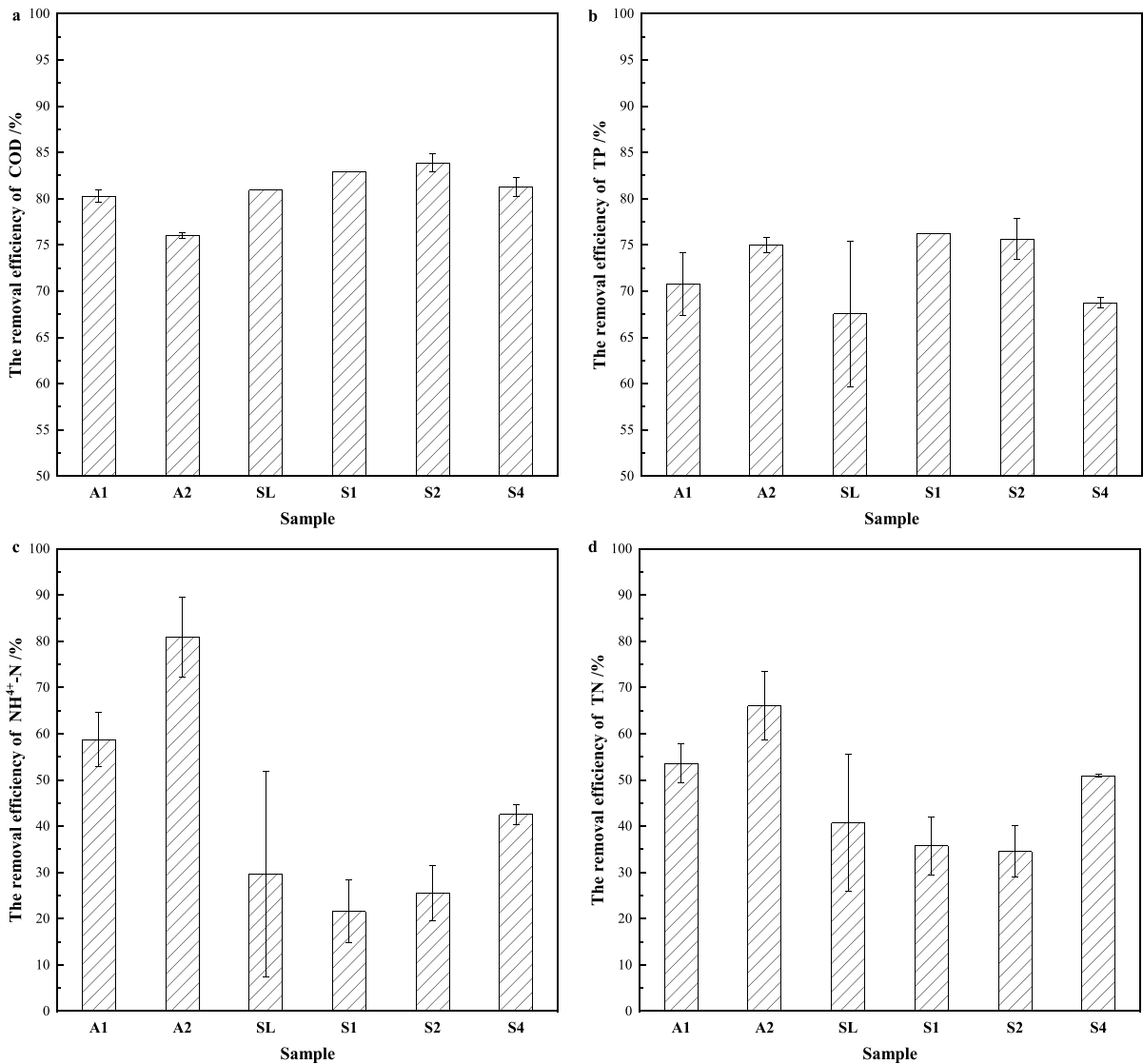
Species	The main characteristics	Reference
<i>Acinetobacter</i>	PAOs, heterotrophic nitrification, aerobic denitrification, converge and secrete protein to enhance this aggregation and adapt to higher COD load	Lu et al., 2014, Su et al., 2015, Adav et al., 2010
<i>Comamonas</i>	Anaerobic denitrification, nitrogen assimilation and alienation	Sayess et al., 2013, Moura et al., 2018
<i>Sphingobacterium</i>	Biological flocculation effect, degrade fatty acids and complex organic compounds	Lee et al., 2013
<i>Chryseobacterium</i>	A nitrifying and denitrifying bacterium	González et al., 2017
<i>Solibacillus</i>	Fermentation of complex carbohydrates	Jonge et al., 2017
<i>Pseudomonas</i>	Denitrification	Srinandan et al., 2011
<i>Leucobacter</i>	Denitrification	Sui et al., 2018
<i>Brevundimonas</i>	Denitrification	Weon et al., 2012
<i>Pseudochrobactrum</i>	Denitrification	Zhang et al., 2018
<i>Macellibacteroides</i>	A major carbohydrate degrading bacteria in sludge	Chen & Chang, 2017
<i>Bacteriovorax</i>	Mainly found in activated sludge and participates in the waste treatment process	Reddy & Mohan, 2012
<i>Stenotrophomonas</i>	Pathogens	/
<i>Enterococcus</i>	A typical Nosocomial pathogenic bacterium	Chajęcka-Wierzchowska et al., 2017
<i>Cyanobacteria</i>	Sensitive to light and prefers low light intensity	Häder, 1987
<i>Chryseobacterium</i>	Converge and secrete protein to enhance this aggregation and adapt to higher COD load	Adav et al., 2010
<i>Leucobacter</i>	Related to the high flocculation index	Gonzalez-Martinez et al., 2017

the microalgae being phototrophs do not remove efficiently organic matter, while in contrast they might release organics into the surrounding water (Hulatt & Thomas, 2010; Vandamme et al., 2012) leading sometimes also to the increase of total COD (Markou et al., 2016).

Regarding phosphorus removal efficiency, there were some differences between the different experimental groups. As shown in Fig. 2b, the experimental groups S1, S2 and A2 showed the highest total phosphorus removal efficiencies and reached 76.2%, 75.6% and 75.0%, respectively, while the total phosphorus removal efficiency of the experimental groups A1, SL and S4 were lower. This observation could be explained by taking into account different factors, such as the type and activity of the microorganisms and the final pH of the wastewater. The types and activities of microorganisms in different experimental groups have certain influence on the utilization and removal of phosphorus from wastewater. In an indirect way, when pH of the wastewater is lowered, then it is not conducive to PAOs, and thus phosphorus removal is reduced (Filipe et al., 2001). On the other

hand, higher pH could induce the removal of phosphorus through precipitation. In some experimental groups, it was observed that when pH was risen a significant reduction in calcium ions was simultaneously occurred. After 3 days, the calcium content in the wastewater decreased from an initial concentration of 42.21 mg/L to 26.04 ± 1.02 mg/L on average. The lowest concentration observed was 14.23 mg/L in the group A2 where the final pH was the highest among the groups. The decrease in calcium ion concentration accompanying the increase in pH suggests that a portion of soluble phosphorus was removed by precipitation (Diaz et al., 1994). Therefore, the activity of microorganisms and the subsequent pH change caused are the main factors of phosphorus removal from swine wastewater in this experiment.

The removal efficiency of nitrogen from swine wastewater by microorganisms in each experimental group is shown in Fig. 2c (NH<sub>4</sub><sup>+</sup>-N) and Fig. 2d (TN). It is clearly observed that the microalgae groups (A2 and A1) displayed the highest nitrogen removal. Among them, the group A2 showed the highest removal efficiency of ammonia nitrogen and



**Fig. 2** The removal efficiency of nutrients from wastewater. (a) COD, (b) TP, (c) NH<sub>4</sub><sup>+</sup>-N, (d) TN

total nitrogen, reaching 80.90% and 66.04% respectively. The removal efficiency of ammonia nitrogen and total nitrogen in S4 was the highest among the screened heterotrophic nitrification bacteria, reaching 42.53% and 50.89%, respectively. This indicates that the nitrogen removal performance of some bacterial strains is higher than others, but it is still lower than that of microalgae. It is noteworthy that the total nitrogen removal efficiency of microalgae was lower than that of ammonia nitrogen removal, while the activated sludge and heterotrophic nitrifying bacteria showed completely different behavior. This suggests

that bacteria and microalgae have completely different nitrogen removal pathways. These results suggest that microalgae preferably take up the inorganic form (either as ammonia or as nitrates), while bacteria because they degrade organic complexes are more capable to remove total nitrogen more efficiently. In addition, microalgal (*Chlorella*) biomass is known to have a high protein content (45–65%) (Becker, 2003), which also shows that they require high nitrogen amounts (60–100 mg-N per g of dry biomass) in order to synthesize proteins. It is noteworthy that for the experimental groups with low community



diversity (see Section 3.1), such as A2, S4, etc., the removal of nutrients, especially nitrogen, was more efficient. Therefore, it is considered that these results suggest that some bacteria had a relatively smaller contribution to denitrification and dephosphorization in the inoculated activated sludge. It is further hypothesized that screening the microorganisms used in swine wastewater treatment is an effective strategy to improve the efficiency of nutrient removal or recovery.

### 3.3 Comparison of Microalgae and Heterotrophic Nitrifying Bacteria on the Nitrogen Transfer and Transformation Pathways

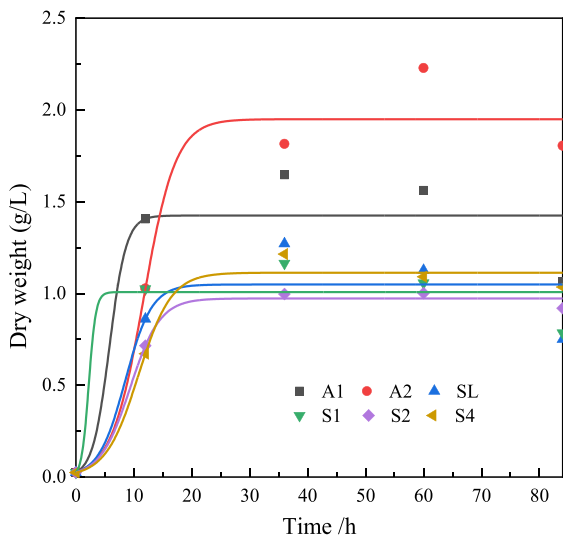
The Logistic function was used to fit the dry weight curve of microorganism, and good results were obtained. The fitting curve and its function information are shown in Fig. 3 and Table 3 respectively. In the whole reaction process, the growth of microorganism can be divided into growth period and relatively stable period. It can be seen that the growth rate of some screened heterotrophic nitrifying bacteria was faster than that of microalgae, but microalgae can generally accumulate more biomass than bacteria. The screening process can change the growth rate and bioaccumulation of strains to some extent. In addition, it was observed that the growth rate of

**Table 3** Fitting curve formula and fitting degree

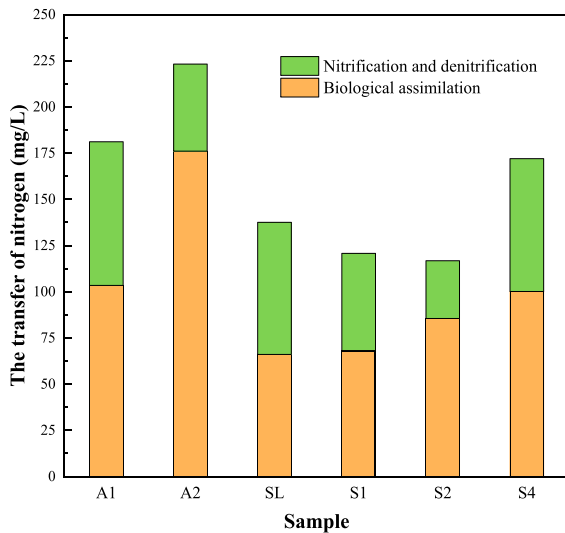
Sample	Fitting equation	<i>a</i>	<i>xc</i>	<i>k</i>	<i>r</i> <sup>2</sup>
A1	$y = a / (1 + \exp(-k * (x - xc)))$	1.42	5.80	0.69	0.78
A2	$y = a / (1 + \exp(-k * (x - xc)))$	1.95	11.70	0.36	0.92
SL	$y = a / (1 + \exp(-k * (x - xc)))$	1.05	8.52	0.44	0.69
S1	$y = a / (1 + \exp(-k * (x - xc)))$	1.01	2.22	1.65	0.82
S2	$y = a / (1 + \exp(-k * (x - xc)))$	0.97	9.37	0.39	0.99
S4	$y = a / (1 + \exp(-k * (x - xc)))$	1.11	10.82	0.35	0.96

microalgae under higher light was slower but higher biomass could be obtained. This means that although the screening process can increase the maximum biomass and growth rate of some strains, it is difficult to catch up with the huge advantages of microalgae in terms of proliferation rate and biomass accumulation.

The net pollutant removal in these systems is basically the additive effect of their assimilation by the microalgae (Su et al., 2011), biological processes (nitrification/denitrification) and stripping phenomena such as ammonia volatilization. The latter is potentially brought about by the high pH levels induced through photosynthetic microalgal growth (Li et al., 2011). Ammonia volatilization is favored by pH values higher than 10 and also depends on the gas–liquid equilibrium of ammonia (Delgadillo-Mirquez et al., 2016). In this experiment, the pH values were monitored and increased from 7.60 to 10.03 in the group A2, while all other groups were below 9. Thus, the nitrogen transfer and transformation pathways in swine wastewater were mainly divided into two parts, namely (i) biological assimilation and (ii) nitrification and denitrification removal. As shown in Fig. 4, there are huge differences in nitrogen transfer and transformation pathways in different experimental groups. It was found that the removal of nitrogen by microalgae under two kinds of light (strong and weak) culture conditions was higher than that of bacteria groups. After calculation, nitrogen taken up by organisms and finally transformed into biomass reached 103.43 mg/L (57.08%) (A1) and 176.13 mg/L (78.91%) (A2), respectively. The two microalgae groups showed a nitrogen transfer pathway dominated by biological assimilation. In addition, the increase of light intensity in A2 group promoted a stronger proliferation of microalgae, which enhanced biological assimilation of nitrogen but possibly inhibited nitrification and denitrification. In order to get as close



**Fig. 3** Fitting curve of dry weight of microorganisms over time ( $p < 0.05$ )



**Fig. 4** Nitrogen transfer and transformation pathways

as possible to the real environment, the swine waste water in this experiment was not sterilized, and the experimental environment was not completely sterile, which resulted in nitrification and denitrification due to the presence of bacteria originated in the wastewater and the removal of some nitrogen. A portion of nitrogen could be also removed through ammonia stripping, especially at the higher pH, where free ammonia dominates over the ionized ammonium form. During the 3 days' operation, the pH of A2 was below 9 until 48 h later; thus, the ammonia stripping pathway was not explored by abiotic test as described in published research (Delgado-Mirquez et al., 2016). They reported that the 17% of ammonia was lost in the abiotic assay at pH 10 (192 h). Comparing the nitrogen transfer pathways of bacteria and microalgae, it can be found that biological assimilation is still the main nitrogen removal pathway except the SL group, although all bacterial groups have poor nitrogen removal ability. It was also found that some enhanced strains could be obtained by screening activated-sludge bacteria, such as S4, which not only maintained high nitrification and denitrification performance (71.81 mg/L), but also enhanced biological assimilation performance (100.19 mg/L). However, the nitrogen removed by nitrification and denitrification and biological assimilation in the original activated sludge (SL) was 71.52 mg/L and 66.08 mg/L, respectively. These phenomena may be closely related

to the species and composition of microorganisms in each experimental group, so further in-depth analysis was conducted at the level of biodiversity. These results suggest that microalgae are advantageous compared to bacteria in terms of biomass accumulation and phosphorus and nitrogen transformation into biomass and hence are more appropriate to recycle the nutrients (especially nitrogen) contained in wastewater through biomass reuse.

The differences in species composition between the activated sludge group (SL) and the heterotrophic nitrifying bacteria groups (S1, S2, S4) indicate that the screening effectively eliminated some species, thus achieving higher nitrogen and phosphorus removal efficiency. It is hypothesized that the addition of heterotrophic nitrifying strains changed the abundance of some functional bacteria in the original swine wastewater, while microalgae itself had a relatively slight effect on the original bacteria in the wastewater, but its own metabolic action changed the nutrient removal performance of the system. Secondly, it was found that the abundance of some pathogenic bacteria in different samples was completely different, indicating that the enhancement of microorganisms helped to suppress the pathogenic bacteria. It was noteworthy that high concentration of microalgae had an obvious inhibitory effect on *Enterobacter*. Finally, some species in the sample were not only associated with nutrient removal, but may also play an important role in other activities, such as changes in pH of the wastewater, sludge settling properties and nutrient load adaptability.

### 3.4 Correlation Analysis

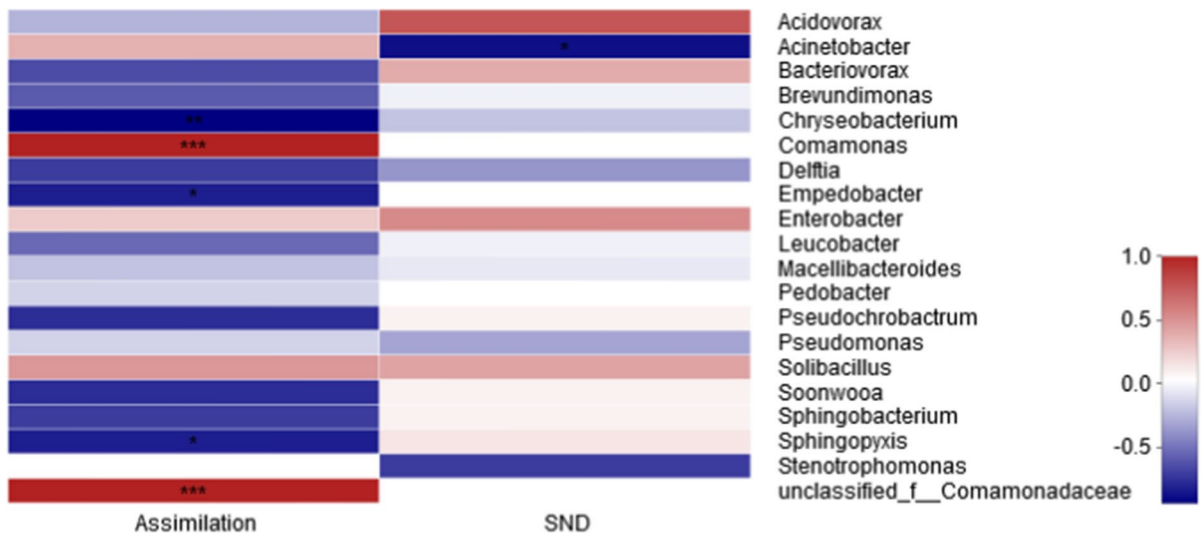
Correlation analysis was conducted between nitrogen migration pathway and species abundance at the genus level of swine wastewater in bacterial experimental groups (SL, S1, S2, S4, S5, S9,  $n=6$ ). The calculation method of nitrogen transfer pathway (Fig. 4) is divided into biological assimilation denitrification (Assimilation) and simultaneous nitrification and denitrification (SND). Spearman correlation analysis and heat mapping were performed using the cloud computing platform provided by bio-sequencing company, as shown in Fig. 5. The analysis revealed that *Comamonas* and *Comamonadaceae* had a significant positive correlation with the biological assimilation of nitrogen, and the correlation coefficient was relatively high. It

indicated that these two kinds of bacteria played an important role in the biological assimilation of nitrogen, that is, a large amount of nitrogen was taken up and assimilated by these bacteria. Studies have also found that *Comamonas* plays an important role in nitrogen assimilation and alienation (Moura et al., 2018). *Comamonadaceae* has also been found to have multiple nitrogen assimilation genes (Gil-Pulido et al., 2018). Species *Chryseobacterium*, *Empedobacter* and *Sphingopyxis* had a certain negative correlation with the biological assimilation of nitrogen, and the correlation coefficient  $r^2$  reached  $-0.943, -0.829, -0.829$ , respectively. The results indicated that these three species might release some nitrogen-containing compounds and might only participate in part of the nitrogen conversion process but not be able to remove nitrogen from wastewater independently. For example, *Empedobacter* has a good nitrification effect (Kumar & Lin, 2010), and *Sphingopyxis* was found to be positive for nitrate reduction (Zhao et al., 2014). *Acidovorax* species has a positive correlation with synchronous nitrification and denitrification (SND), with the correlation coefficient  $r^2$  reaching 0.771, and the bacteria has been found to have a certain degree of synchronous nitrification and denitrification (Moura et al., 2018). *Acinetobacter* has a certain negative influence on the simultaneous nitrification and denitrification, with a correlation coefficient of  $-0.886$ ; although the bacteria

has been found to be generally good at denitrification (Yang et al., 2018), it is also closely related to the degradation of organic matter (Vijayalayan et al., 2014). However, in this experiment, *Acinetobacter* showed the phenomenon of inhibiting the simultaneous nitrification and denitrification. It is speculated that the bacteria may have a certain relationship with the carbon source competition with other simultaneous nitrification and denitrifying bacteria. It is also worth noting that the abundance of *Comamonas* in microalgae groups A1 and A2 is basically close to or even lower than that in certain heterotrophic nitrifying bacteria groups. However, the very high nitrogen assimilation values in the A2 group indicated that microalgae and heterotrophic nitrifying bacteria had significantly different nitrogen conversion modes.

### 4 Conclusions

Screening heterotrophic nitrifying bacteria and microalgae for the removal and recovery of nutrients in swine wastewater is an effective strategy. The removal of COD and TP by bacteria was more efficient, while the removal efficiency of nitrogen ( $\text{NH}_4^+\text{-N}$  and TN) by microalgae was better. Biological assimilation was the main pathway of nitrogen conversion by microalgae and bacteria, especially microalgae showed excellent



**Fig. 5** Heatmap map of relevance. (The  $R$  value is shown in different colors in the figure. If the  $p$  value is less than 0.05, it is marked with \*. The figure on the right is the color interval of different  $R$  values. \*  $0.01 < p \leq 0.05$ , \*\*  $0.001 < p \leq 0.01$ , \*\*\*  $p \leq 0.001$ )

biological assimilation performance. The screening and inoculation of strains can improve the abundance of some functional bacteria, especially related to nitrogen, and *Comamonas* and *Acidovorax* are highly correlated with nitrogen assimilation and simultaneous nitrification and denitrification, respectively. In the future, the screened bacteria-microalgae system has the potential to provide an integrated approach for nitrogen and phosphorous cycling and recovery during swine wastewater treatment. However, the success of large-scale application at specific pig farm would require further systematic studies including the mixed ratio of microalgae and screened bacteria, the operation conditions and the photobioreactor design.

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**Data Availability** The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Competing Interests** The authors declare no competing interests.

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