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Microbial community structure analyses and cultivable denitrifier isolation of *Myriophyllum aquaticum* constructed wetland under low C/N ratio

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ABSTRACT

With the rapid expansion of livestock production, the amount of livestock wastewater accumulated rapidly. Lack of biodegradable organic matter makes denitrification of livestock wastewater after anaerobic digestion more difficult. In this study, *Myriophyllum aquaticum* constructed wetlands (CWs) with efficient nitrogen removal performance were established under different carbon/nitrogen (C/N) ratios. Analysis of community composition reveals the change of *M. aquaticum* CWs in microbial community structure with C/N ratios. The proportion of Proteobacteria which is one of the dominant phyla among denitrifier communities increased significantly under low C/N ratio conditions. Besides, to obtain cultivable denitrifier that could be added into CWs in situ, 33 strains belonging to phylum Proteobacteria were isolated from efficient *M. aquaticum* CWs, while the best-performing denitrification strain M3-1 was identified as *Bacillus velezensis* JT3-1 (GenBank No. CP032506.1). Redundancy analysis and quadratic models showed that C/N ratio had significant effects on disposal of nitrate (NO_3^--N) and the strains isolated could perform well in denitrification when C/N ratio is relatively low. In addition, they have relatively wide ranges of carbon sources, temperature and a high NO_3^- removal rate of 9.12 mg/(L·hr) at elevated concentrations of 800 mg/L nitrate. Thus, strains isolated from *M. aquaticum* CWs with low C/N ratio have a practical application value in the treatment of nitrate-containing wastewater. These denitrifying bacteria could be added to CWs to enhance nitrogen removal efficiency of CWs for livestock wastewater with low C/N ratio in the future.

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Introduction

Worldwide, the amount of wastewater from livestock has built up rapidly with the rapid expansion of livestock production (Burka et al., 2021; Xu et al., 2004). Large-scale industrial livestock production is increasingly becoming a significant source of agricultural sewage that seriously pollutes the rural water environment in China (Ongley et al., 2010; Zhang et al., 2021). Excess nitrogen from livestock wastewater has the potential to pollute the environment (Ma et al., 2020), causing algal proliferation (Shakya et al., 2017), disrupting fishing and tourism (Mohajerani et al., 2017; Yang et al., 2012), as well as threats to aquatic safety (Paerl et al., 2016; Yang et al., 2013). Furthermore, ammonium-N has been converted to nitrate and nitrite, causing serious human and animal health problems (Zhai et al., 2017). At the moment, effective solutions to mitigate nitrogen in livestock wastewater are critical and urgent (Li et al., 2021).

Most farms in rural China are solely equipped with anaerobic digesters (Sui et al., 2014). Anaerobic digestion makes it possible to recycle organic waste utilizing anaerobic microorganisms (Surendra et al., 2014). However, effluent released from the anaerobic digester contained high levels of nitrogen and was deficient in biodegradable organic matter (Kim et al., 2016). It was reported by Zheng et al. (2019) that the concentration range of chemical oxygen demand (COD) and total nitrogen (TN) of livestock wastewater after treated was 320–919, 49–140 mg/L (carbon/nitrogen (C/N) ratio: 6.5). Zhou et al. (2021) indicated that the wastewater after anaerobic digestion had a low C/N ratio ranging from 1.07 to 1.53, which is in line with many swine wastewater (Chen et al., 2009). As such, this wastewater is a low C/N ratio type of wastewater with low microbial degradation capacity (De Los Reyes et al., 2014), which increases the difficulty of self-cleaning of the recipient water. Post-remedial strategies were therefore still needed. Faced with such serious issues, constructed wetlands (CWs) were widely used to treat livestock wastewater after anaerobic digestion (Vymazal, 2011; Wu et al., 2014). There are many studies about wetlands constructed with various plants, such as the wetland planted with *Phragmites australis* which can remove 60.74% TN and 93.07% NH_4^+-N in four months (Wu et al., 2011); In the wetland planted with *Typha Orientalis*, the TN and NH_4^+-N removal efficiencies were 60.94% and 88.27%, respectively (Weragoda et al., 2012); Comparatively, the NO_3^--N, and NH_4^+-N removal efficiencies of *Scirpus grossus* were 52.1% and 59.4%, respectively (Jinadasa et al., 2008).

Recently, Liu et al. found high nitrification and denitrification rates in surface flow constructed wetlands (SFCWs) vegetated with *Myriophyllum aquaticum*, which facilitates the rapid removal of N from livestock wastewater (Liu et al., 2016; Luo et al., 2018). These performances were stable, even with the high average COD and TN removal efficiencies of 92.3% and 97.9%, respectively, in winter with temperature about 10°C (Sun et al., 2017). Our previous study has explored the mechanisms underlying the high and stable treatment performance of nitrogen therapy during a long-term operation in *M. aquaticum* CWs (Sun et al., 2017). It has been reported that the rate of nitrogen removal by microorganisms in CWs may exceed 60% (Faulwetter et al., 2009), and plants are capable of eliminating approximately 30% (Gottschall et al., 2007). The results revealed *M. aquaticum* could affect the microbial community by reducing TN and NH_4^+-N (Philippot et al., 2013). It could absorb nutrients and promote the coupling between nitrification and denitrification by amplification of DO gradients (Vila-Costa et al., 2016), and particularly radial oxygen loss and radicular respiration of *M. aquaticum* can create many aerobic/anoxic sedimentary zones (Li et al., 2019b). These modifications could alter the abundance and structure of nitrifying and denitrifying communities in sediments (Vila-Costa et al., 2016).

Previous results also indicated that influent C/N ratio was essential in coupled nitrification and heterotrophic denitrification (Saeed and Sun, 2012). Unfortunately, the lack of biodegradable organic matter makes denitrification of livestock sewage more difficult, particularly after solid-liquid separation and anaerobic digestion (Chen et al., 2009). Based on that, the effect of different C/N ratios on the nitrogen removal and microbial community structure of *M. aquaticum* CWs is to be further explored in this study. Analysis of community composition can reveal the change in microbial community structure with the C/N ratios in CWs and dominant denitrifier community in *M. aquaticum* CWs with low C/N ratio. Besides, although adjusting influencing C/N ratio by adding external carbohydrates could effectively enhance denitrification performance in CWs (Cheng et al., 2019; Lu et al., 2009; Zhu et al., 2014), it may lead to increased operating costs and the risk of organic carbon contaminate of effluent. Correspondingly, other technologies including addition of microorganisms, have been adopted to enhance the efficiency of TN removal by CWs with relatively low C/N ratio (Wu et al., 2014, 2015; Zhang et al., 2017). Adding efficient denitrifiers isolated from *M. aquaticum* CWs in situ is a feasible strategy. The potential for artificial cultivation of denitrification strains in CWs under low C/N ratio is a key to improving the denitrification of CWs. Thus, this study will isolate cultivable denitrifying microorganisms with a high nitrate conversion rate from *M. aquaticum* CWs that operated under low C/N ratio conditions. Furthermore, explore the optimal conditions of denitrifiers to guide the practical application.

Denitrifiers have been isolated in many environments such as soil, lakes, and aquaculture wastewater. NO_3^--N removal rate of *Rhodococcus* sp. and *Klebsiella* sp. isolated from swine and domestic wastewater can reach 0.93 mg/(L·hr) (Chen et al., 2012) and 2.2 mg/(L·hr) (Padhi et al., 2013), respectively. *Pseudomonas stutzeri* YG-24 from swine sewage has a relatively high removal rate of 7.73 mg/(L·hr) for NO_3^--N (Chen et al., 2012). Our study indicated *M. aquaticum* may supple the facultative anaerobic denitrifying agents in the rhizoplane to obtain denitrification in the CW (Sun et al., 2019), and genes from facultative anaerobic denitrifiers, such as Rhodocyclaceae, Aeromonas, and Pseudomonas have been considerably enriched in the water and rhizoplane (Sun et al., 2019). Therefore, isolating denitrifiers from the sediment of *M. aquaticum* CWs is possible. Traditionally, the denitrification process is primarily conducted by anaerobic denitrifiers (Morris et al., 2013) and denitrifying activity for most denitrifiers is eliminated when anaerobic is inadequate (Takaya et al., 2003; Wittorf et al.,

2016), thus denitrifiers will be isolated under anaerobic conditions in this study.

As a whole, this study aims to reveal the change of microbial community structure with the C/N ratios and the dominant phyla among the denitrifier community in *M. aquaticum* CWs with low C/N ratio. Besides, cultivable denitrifier which has the potential to enhance the denitrification of CWs by being added into CWs in situ will be isolated from efficient *M. aquaticum* CWs exploited with low C/N ratio. On this basis, molecular identification and response surface models would be used to identify the strains and explore the effects of environmental conditions on the disposal of NO_3^--N by these strains obtained, respectively. This study has a practical application value in the treatment of nitrate-containing wastewater. In the future, denitrifying bacteria obtained could be added to CWs in situ to enhance the nitrogen removal efficiency of CWs for livestock wastewater with low C/N ratio.

1. Materials and methods

1.1. Wetlands construction and sample collection

Three *M. aquaticum* CWs (length, 15 m; width, 2 m; and water depth, 0.2 m) were constructed in Hunan province, China, which has a subtropical monsoon climate with annual precipitation and an average temperature of 1330 mm and 17.5°C, respectively. The density of *M. aquaticum* planted is 3 kg/m^2, the hydraulic retention time is 11 days, and the daily flow rate is 0.18 m^3/day. Two different C/N ratios (Low C/N treatment, LCN: 3.7:1; High C/N treatment, HCN: 10:1) were established to investigate the changes of microbial communities under different C/N ratios. The cell unplugged with *M. aquaticum* was installed as control (CK). The influent of CWs is from tanks located in front of the CWs, and the effluents were applied to the soil. In this experiment, livestock wastewater after anaerobic digestion was used, and C/N ratio of the wastewater was determined to be stable at about 3.7. The influent of low C/N ratio constructed wetlands (LCN: 3.7:1, COD: 546.23 ± 13.42 mg/L, TN: 147.8 ± 7.86 mg/L) was this wastewater after subtle variation. The influent of high C/N ratio constructed wetlands (HCN: 10:1, COD: 1473.78 ± 21.51 mg/L, TN: 147.6 ± 6.51 mg/L) was the livestock wastewater with artificially added carbon source, and the carbon source used in this experiment was sodium citrate. The chemical characteristics of different livestock wastewater influents were listed in Appendix A Table S1. The dissolved oxygen (DO) and pH were measured using a multiparameter water quality monitoring instrument (HACH, HQ30D, USA).

Water samples were collected 3 times monthly and immediately analyzed in the laboratory. The water samples were filtered through a 0.22 mm cellulosic membrane. COD was measured using a standardized potassium dichromate assay (Federation and Association, 2005). NH_4^+, NO_3^-, NO_2^-, and TN concentrations were measured with a continuous flow analyzer (AA3, Seal Analytical, Norderstedt, Germany). Sediment samples (5-10 cm depth) were obtained from the root of *M. aquaticum* (3 replicates). They are sampled using the 5-point sampling method and carefully blended, then sealed in a thermostat and transported to the lab. Part of the sediment samples are lyophilized, then crushed, and sieved. Samples for DNA extraction are refrigerated at -80°C.

1.2. Total DNA extraction and MiSeq sequencing

0.5 g of each mixed sediment sample were used with the FastDNA®SPIN Kit for Soil (MP Biomedical, Santa Ana, CA, USA) approximately to extract genomic DNA. The V4 region of the bacterial 16S rRNA gene was amplified using the primers 515F (50-GTGCCAGCGCCGCGGTAA-30) and 806R(50-GGACTACGGGTTCTAAT-30) (Caporaso et al., 2012). Each pair of primers had a 12-base barcode on the forward and reverse primers. The PCR conditions were executed in a PCR instrument (Eastwin, China). The amplification procedures included an initial denaturation step at 94°C for 1 min, followed by 35 cycles at 94°C for 30 sec, 57°C for 20 sec and 72°C for 30 sec, and a final extension step at 72°C for 10 min. Subsequently, the PCR product was purified with the Gel Extraction Kit (D2500-02, Omega BioTek, Norcross, GA, USA) and DNA concentrations were quantified by NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies, Wilmington, DE, USA). A DNA library was obtained according to the MiSeq Reagent Kit Preparation Guide (Illumina, San Diego, CA, USA), and then the DNA library was sequenced using the Illumina MiSeq platform by Shanghai Majorbio Bio-pharm Technology Co., Ltd., China. All the raw sequences have been saved in NCBI Sequence Read Archive with accession no. SRP218539.

1.3. Isolation of anaerobic denitrifiers

Four types of media which are Luria-Bertani (LB) (Sezonov et al., 2007) , enrichment (EM) (Sun et al., 2015), denitrification (DM) (Zhu et al., 2012), and screening medium (GN) (Zhang et al., 2015) were used to separate and purify anaerobic denitrifiers. The water used for the preparation of the medium is distilled water, and the solid medium is obtained by adding 2% agar to the liquid medium and autoclaving in an autoclave at 121°C for 20 min. All the following operating steps are performed on the anaerobic workbench. The serum bottle used for enriched strains is hermetic and filled with argon to provide anaerobic conditions for the survival of anaerobic denitrifying microorganisms.

Sediment samples were collected from the roots of *M. aquaticum*. Firstly, 10 g precipitate was added to a 250 mL anaerobic serum bottle containing 100 mL of sterile 1 × phosphate buffer solution (PBS) and a small number of glass beads in the ultra-clean workbench. Subsequently, the serum vial was then agitated in a constant temperature agitator at a temperature and speed of 30°C and 150 r/min for 30 min. Then 10 mL sediment suspension was transferred to a 250 mL anaerobic serum bottle containing 100 mL EM and incubated on a shaker at 30°C (150 r/min) for 28 days. During the enrichment stage, 10 mL of the mixed solution containing EM and microorganisms was vacuumed and transferred to fresh EM every 2 days. After 28 days of incubation in EM on a shaker, a series of 10-fold dilutions of 0.1 mL bacterial suspension was inoculated on a DM solid plate, coated with a sterile spreading rod. They were then put into cultivation at a constant temperature of 30°C. The cultivated colonies with different appearances and shapes were separated on the DM solid plate by

making streaks with an inoculation loop to obtain a unique colony. Then the single colony was purified on a solid DM medium for 3 generations. Finally, the purified microorganisms were inoculated on a solid GN medium and cultured at 30°C, the strains changed GN medium from green to blue were re-screened strains.

A scanning electron microscope (SEM) was used to observe the morphological features of denitrifiers obtained by re-screening after 24 hr incubation. Firstly, the strains were fixed with 2.5% glutaraldehyde and washed with 0.1 mol/L PBS (pH 6.8). Then different concentrations of ethanol were used for dehydration. Finally, they were replaced with pure isoamyl acetate. After replacement, the sample was placed in the refrigerator at 4°C and freeze-dried for 12 hr, then sprayed with gold, and observed in a scanning electron microscope (JSM-5800, Hitachi, Japan) at an accelerating voltage of 18 kV.

Strains obtained after separation, purification, and refocusing were used for 16s rRNA extraction. The corresponding operations are identical to those above (1.2 Total DNA extraction and MiSeq sequencing). The sequencing results were uploaded into the Genbank database for comparison. On this basis, the phylogenetic tree of strains was constructed by MEGA X using the neighbor-joining method (Zhang et al., 2008).

1.4. *Growth and nitrogen removal of strains*

The 12 mL of seed activation solution was added into a 250 mL anaerobic serum bottle containing 120 mL of LB and incubated at 30°C at a speed of 150 r/min for 72 hr with sampling at regular intervals. A UV-1240 spectrophotometer (Shimadzu Kyoto, Japan) was used to measure the optical density (OD_{600}) of the bacterial suspension at 600 nm.

The single colony obtained as a result of retesting was collected and transferred to 2 mL of sterile distilled water. It was placed in a sterilized LB liquid medium serum bottle with a sterile syringe for 12 hr, and culture solutions of it were collected in the logarithmic growth stage. After centrifugation, washing, and puffing, inoculate suspended bacterial solution into DM at a rate of 150 r/min, 30°C shaker culture for 3 days, each strain is set in three parallels to determine denitrification performance. The initial nitrate concentration in the DM medium was 28 mg/L. After inoculating the strains, water samples were collected in the serum vials every four hours to determine the concentrations of TN, NH_4^+-N, NO_3^--N, and NO_2^--N.

The response surface methodology was used to investigate the impacts of carbon source, C/N ratio, temperature, pH, and inoculum size on denitrification. Many other studies also use this methodology to explore the influence of independent factors, such as the response surface methodology model was used to investigate the interactive effects of pH, temperature, C/N ratio, and shaking speed on TN removal (Ye et al., 2016). The denitrification medium contained 28 mg/L NO_3^--N. K_2HPO_4 and KH_2PO_4 were used to adjust the pH and $C_6H_5Na_3O_7$ was used to adjust the C/N ratio in denitrification medium. A four-factor-five-level experiment was defined using the Box-Behnken design (**Appendix A Table S2**). According to the Box-Behnken design, 29 experiments were performed in 250 mL shaker flasks including 120 mL medium and enrichment culture. Both experimental samples were collected at 0 and 24 hr to detect nitrate change and calculate the nitrate removal rate. Design-Expert program (Version 10.0.3, Stat-Ease, Inc., USA) was applied to analyse the experimental data. Then a quadratic model and the coefficients were obtained. The quality of the fit of the model was evaluated using the analysis of variance (ANOVA).

Conditions were optimized for the maximum nitrate elimination rate by response surface methodology. Then the experiment was conducted under the optimal conditions. Samples were still collected at 0 and 24 hr to calculate the nitrate removal rate. Finally, the experimental data were compared against predicted values based on the mean square error (RMSE) to validate the model.

1.5. *Data analysis*

One-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test was used to check for quantitative differences between treatments using Statistics for Windows version 20.0 (IBM Corp., Armonk, NY, USA).

The removal efficiency of nitrate, nitrite, and TN is calculated by Eq. (1):

$$R = (S_0 - S_1) / S_0 \times 100\% \quad (1)$$

Among them, R (%) is nitrogen removal rate; S_0 (mg/L) and S_1 (mg/L) represent initial and final concentrations of nitrate, nitrite, and TN, respectively. All results are statistically analyzed by Excel and the graph is drawn by Origin 8.

The Eq. (2) of the removal rate is as follows:

$$Q = (S_0 - S_e) / (t_e - t_0) \quad (2)$$

where, Q (mg/(L·hr)) is the removal efficiency; S_0 (mg/L), S_e (mg/L) is the concentration of nitrogen in the medium at t_0 , t_e, respectively; t_0 (hr) is the initial time of the experiment, t_e (hr) is the time for the nitrogen concentration to stabilize and change.

2. Results and discussion

2.1. *Effects of C/N on* M. aquaticum *CWs*

Compared with wetland unvegetated, both *M. aquaticum* CWs operating under high and low C/N ratio conditions had more significant removal effects on nitrogen and COD in influent water. The average TN concentration of (147.7 ± 6.51) mg/L in the wastewater inflow was reduced to an average effluent level of (33.4 ± 4.64) mg/L, which achieved a removal rate of 77.85% ± 1.46% by *M. aquaticum* CWs with relatively high C/N ratio at 10:1. Among them, the average removal rate of NH_4^+-N, which accounted for more than 70% of TN inputs of the *M. aquaticum* CWs, was 79.13% ± 5.46%. Besides, the CWs under high C/N ratio achieved a COD removal rate of 87.58% ± 5.38% under the average inflow concentration of (1473.78 ± 21.51) mg/L. These performances were stable during the operation periods, even in winter. However, wetland unvegetated had relatively poor treatment effects on TN, NH_4^+-N, and COD, with the removal rate 26.78% ± 3.32%, 28.23% ± 6.63%, and

Table 1 – Statistics of the main physical and chemical indicators of water quality.

	HCN (10:1)	LCN (3.7:1)	CK
pH	7.39 ± 0.34	7.77 ± 0.25	7.36 ± 0.34
DO (mg/L)	0.08 ± 0.03	0.07 ± 0.01	0.23 ± 0.15
TEMP (°C)	22.1 ± 1.32	21.6 ± 1.78	22.1 ± 2.03
TN removal efficiency (%)	77.85 ± 1.46	55.85 ± 4.35	26.78 ± 3.32
NH_4^+-N removal efficiency (%)	79.13 ± 5.46	58.92 ± 8.95	28.23 ± 6. 63
COD removal efficiency (%)	87.58 ± 5.38	92.21 ± 6.36	43.24 ± 7. 31

HCN: the *M. aquaticum* constructed wetlands operated under high C/N ratio (10:1); LCN: the *M. aquaticum* constructed wetlands operated under low C/N ratio (3.7:1); CK: The constructed wetlands unplugged with *M. aquaticum*; DO: dissolved oxygen; TEMP: temperature; TN: total of nitrogen; COD: chemical oxygen demand.

43.24% ± 7.31%, respectively. These results which are consistent with previous reports confirm that *M. aquaticum* CWs could treat livestock wastewater with nitrogen pollution efficiently. The change of TN concentrations in the effluent samples reflected the effect of C/N ratio influents on nitrogen removal performance (Table 1). Relative to the 3.7:1 C/N ratio, lower effluent TN concentrations were observed at 10:1 C/N ratio throughout the experimental period. The phenomenon corresponded to the theoretical result that high C/N ratio conditions lead to more optimal denitrification rates, and therefore more effective N removal (Li et al., 2019a). In the novel tidal flow CWs, satisfactory TN removal (66%) was observed only when C/N ratio exceeded 6:1, with the highest TN removal efficiency (82%) occurring at a C/N ratio of 12:1 (Zhi and Ji, 2014).

However, it is interesting to find that nitrogen removal efficiency corresponding to the consumption of per unit carbon source under the condition of low C/N ratio was much higher than that both of *M. aquaticum* CWs with high C/N ratio and wetlands without *M. aquaticum*. LCN wetland could remove 16.38% ± 2.51% of TN per unit carbon source, while this value of HCN and CK wetland is only 8.89% ± 4.27% and 13.59% ± 2.52%, respectively. The influents of most livestock sewage treatment systems have a relatively lower C/N ratio (Zhou et al., 2021). The lack of biodegradable organic matter makes it more difficult to denitrify livestock wastewater, particularly after solid-liquid separation and anaerobic digestion (Chen et al., 2009). Therefore, in terms of the full utilization capacity of limited carbon sources, LCN wetland has a better treatment capacity and is more suitable for treating livestock wastewater with low C/N ratio. Moreover, efficiently denitrifying bacteria which is different from traditional heterotrophic denitrifying bacteria that require a large number of carbon sources are more likely to exist in LCN. And these unique denitrifying strains adapt to the conditions of low C/N ratio are a good source of in-situ addition to CWs with low C/N ratio. Therefore, subsequent analysis of community structure and isolating of strains mainly focused on microorganisms in the sediment of LCN wetland.

Nitrogen removal is attributable to N-transforming microorganisms, and the influent C/N ratio typically plays a critical role in the distribution of ammonia oxidants and other microorganisms that transform N (Wu et al., 2012). A phylum-level bacterial taxonomy of the study samples revealed that Firmicutes, Chloroflexi, and Proteobacteria, accounting for 72.99%–79.86% of all bacterial sequences, were the three dominant phyla among microbial communities (Fig. 1). Zhou et al. (2020) indicated that due to their thick cell wall structures, Proteobacteria, Bacteroidetes, and Firmicutes have become dominant communities under harsh environments. Relative abundance of Firmicutes increased under C/N ratio influences from 3.7:1 to 10:1. Additionally, the relative abundances of the phyla Chloroflexi, and Bacteroidetes ranged from 15.3% to 18.0% and 6.1% to 7.2%, respectively, following varying influent C/N ratios. These results are corresponding with other studies, such as Proteobacteria (10.0%–60.4%), Firmicutes (8.8%–51.9%), and Chloroflexi (7.9%–23.0%) were the three most prevalent phylum in the *M. aquaticum* CWs, and Proteobacteria were highly diversified and demonstrated to process good denitrification capability at the class level (Xu et al., 2020).

Interestingly, the proportion of Proteobacteria (18.05% ± 0.04%) in the *M. aquaticum* CWs operated under low C/N ratio increased significantly ($p < 0.05$) compared with it (10.17% ± 1.70%) in the CWs unplugged. And compared with the proportion in the *M. aquaticum* CWs with high C/N ratio (10.07% ± 2.85%), Proteobacteria in the *M. aquaticum* CWs under low C/N ratio has a tendency to increase. Proteobacteria is the most abundant epiphytic and planktonic bacterium in freshwater (Huang et al., 2011; Xie et al., 2015; Yan et al., 2019). It is easy to attach to the surface of the carriers through bacterial lipopolysaccharides (Huang et al., 2011; Tang et al., 2018). This characteristic explains that Proteobacteria is more abundant in *M. aquaticum* wetlands that provide sufficient attachment surfaces for it. Moreover, Proteobacteria could survive under anaerobic conditions and may use nitrate for denitrification (Zhao et al., 2019). It is ubiquitously found in wetland substrate media and capable of high resistance to environmental insults due to its ability to form spores (Zhang et al., 2016). Therefore, Proteobacteria may be more adaptable to relatively unfavorable environment such as insufficient carbon source and thus occupy a dominant position in *M. aquaticum* CWs with low C/N ratio. From these results, it is reasonable to speculate that the strains belonging to Proteobacteria existed in LCN may be the ideal strains that could adapt to low C/N ratio and efficiently utilize both carbon and nitrogen sources meanwhile.

Heatmap (**Appendix A Fig. S1**) is used to explain the functional microorganisms involved in N-removal in the *M. aquaticum* CWs. The 50 most common types of sequences

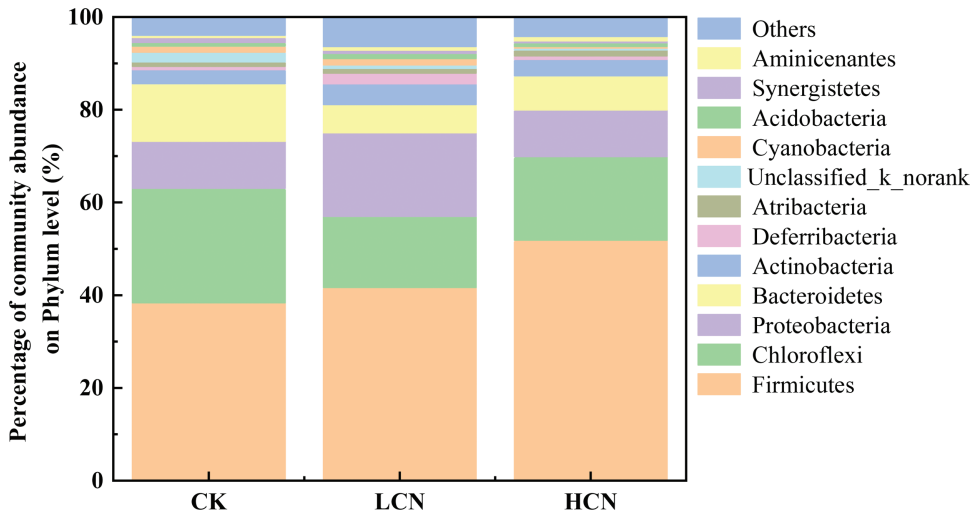

Fig. 1 – Phylum-level taxonomy of relative abundances of bacterial communities in the *M. aquaticum* CWs.

identified were variable among the different C/N ratios. The most dominant genus at a C/N ratio of 3.7:1 was *Clostridium_sensu_stricto_1* (16.34% ± 0.25%), followed by *Terrisporobacter* (10.10% ± 0.06%) and *norank_f__Anaerolineaceae* (6.98% ± 0.36%). *Clostridium_sensu_stricto_1* (23.74% ± 1.85%), *Terrisporobacter* (14.56% ± 2.86%) and *norank_c__SJA-15* (3.986% ± 0.76%) were the dominant genera at a C/N ratio of 10:1. Some genera with denitrification capabilities, such as *Thiocapsa* were more prevalent at low C/N ratio (3.05% ± 0.02%) compared to higher ratio (0.12% ± 0.01%). The majority of denitrifiers detected were from Proteobacteria, which was also in agreement with other studies (Fu et al., 2019). In addition, *Thiocapsa* (3.05% ± 0.02%), *Terrisporobacter* (14.56% ± 2.86%), and *Turicibacter* (2.15% ± 0.02%) accounted for a considerable portion of the *M. aquaticum* CWs operated under low C/N ratio in comparison with these (0%, 7.29% ± 0.03%, 0.86% ± 0.10%) in non-vegetated CWs. Thus, these CWs with low C/N ratio also contributed to the abundance of some species of denitrifiers.

The relationship between environmental variables and the composition of the microbial community has been clarified by the redundancy analysis (RDA) (Fig. 2). The first RDA dimension accounted for 96.72% of the variation in the diversity and distribution of bacterial communities, and the second 1.54%. RDAs results, a significant model with a confidence level of $p < 0.001$, indicated that C/N ratio was a significant environmental factor controlling the microbial community structure. Other environmental variables such as DO content and pH, also contributed to the relationship between the bacterial community and the environment.

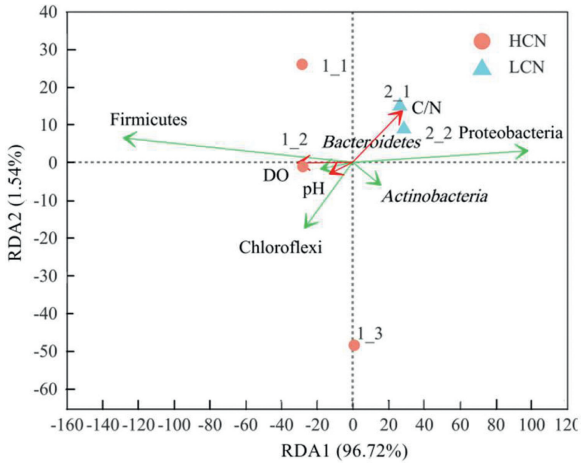

Fig. 2 – Redundancy analysis (RDA) biplot for the first two dimensions shows the relationship between the bacterial communities (symbols) and environmental factors (arrows).

2.2. *Isolation and identification of strains*

In total, 33 strains with denitrification characteristics were isolated and purified. These strains were added to the medium of 28 mg/L nitrates as the sole source of nitrogen for preliminary verification of denitrification efficiency. The 24 hr removal rate of nitrate was shown in Fig. 3. All 33 bacteria demonstrated good nitrate removal capacity which was distributed in intervals of 55%–73%. The most effective nitrate removal effect of 33 strains is M3-1 with a removal rate of 86.48% ± 1.53%, followed by L3-2 and C1-3 with NO_3^--N disposal rates of 84.85% ± 1.23% and 84.28% ± 0.68%, respectively.

MiSeq sequencing was required to identify those strains. After comparison with NCBI, it was determined that the strains belonged to phylum Proteobacteria, γ-Proteobacteria. Phylogenetic tree (**Appendix A Fig. S2**) constructed based on the 16S rDNA sequence of strains show that species of these strains belong to *Bacillus* sp., *Acinetobacter* sp., *Klebsiella* sp., *Pseudomonas* sp., *Pantoea* sp., and *Enterobacter*, respectively. The isolated bacteria varieties were consistent with the findings from the community analysis. Particularly, from community analysis, only the proportion of Proteobacteria was observed to increase significantly in low C/N ratio operations compared with CW under high C/N ratio conditions. And the strains obtained belong to this phylum after molecular identification. Therefore strains isolated in this study are likely to be the

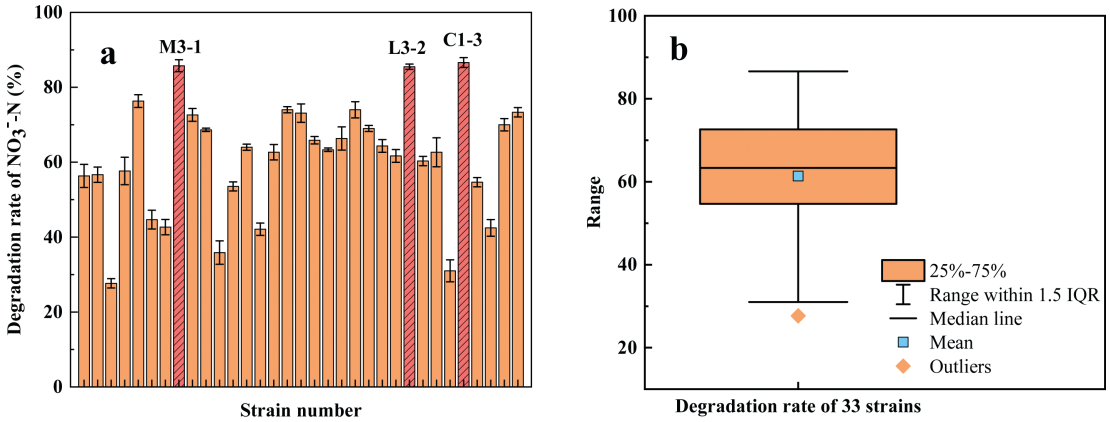

Fig. 3 – Degradation rate of strains isolated to NO_3^--N. (a) the removal rate; (b) the distribution of rate and key values.

dominant denitrifying bacteria in the *M. aquaticum* CW under operating conditions of low C/N ratio, and they may have great potential advantages of enhancing denitrification of CWs by in-situ addition.

According to 16S rDNA sequence and BLAST comparison analysis, denitrifying strains M3-1, L3-2, and C1-3 have the highest similarity with *Bacillus velezensis* JT3-1 (99%) (GenBank No. CP032506.1), *Bacillus thuringiensis* QZL38 (99%) (GenBank No. CP032608.1) and *Enterobacter asburiae* M4-VN (99%) (GenBank No. LC4156 12.1), respectively. Phenotypic observation indicates that the colony morphology of the three strains is similar in form, texture, and transparency. Furthermore, all three strains were short stem-shaped by scanning electron microscopy (**Appendix A Fig. S3**). The apparent information conforms to the phenotypic characteristics of the corresponding strains, proving once again the species of these three strains.

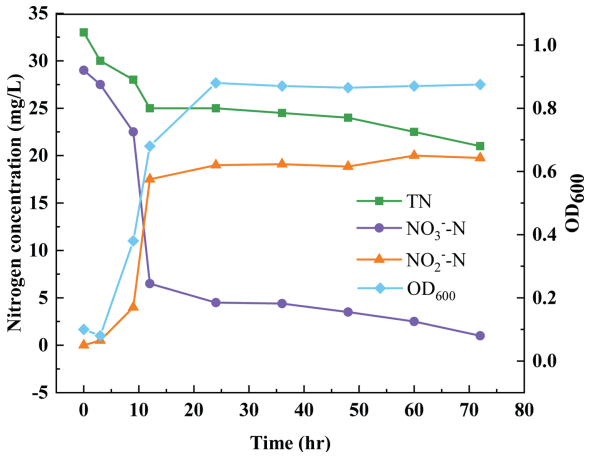

Fig. 4 – Growth and denitrification curve of strain M3-1.

2.3. *Evolution of bacterial growth and nitrate amount*

Growth and denitrification performance of isolated denitrifying strains was investigated by selecting M3-1 as a representative. The growth curve and NO_3^--N removal effect of M3-1 are shown in Fig. 4. M3-1 enters the logarithmic growth phase with almost no latency period, and then reaches a steady phase within 24 hr and continues through the detection time. The growth curve was adjusted with Slogistic 1 Model ($R^2 = 0.988$), and maximum OD_{600} and growth rate were 0.87 and 0.37 OD_{600}/hr, respectively.

$$y = 0.87 / (1 + \exp[-0.37 \times (x - 9.25)]) \quad (3)$$

where, x (hr) is the time, and y is the OD_{600} at that time.

The concentration of NO_3^--N decreased rapidly in 12 hr, with a maximum degradation rate of 83.25%. At 72 hr, the strain could completely degrade NO_3^--N at a concentration of approximately 28 mg/L, with a 100% elimination rate. The removal of NO_3^--N by M3-1 complies with the Logistic model ($R^2 = 0.98$, $p < 0.01$).

$$y = 3.158 + (27.5 - 3.15) / (1 + (x / 10.10) / 10.65) \quad (4)$$

The model shows that M3-1 could remove 50% of NO_3^--N in 7.16 hr, and the removal efficiency of NO_3^--N is 1.955 mg/(L·hr). It can also be seen that NO_2^--N content increased rapidly as NO_3^--N rapidly degraded. After 72 hr, NO_2^--N increased to 18.71 mg/L. This phenomenon led to the 72 hr TN withdrawal rate of 37.3%.

The ultimate objective of the denitrification process is to achieve the final conversion of NO_3^--N into green gas nitrogen. However, the strain isolated in this study could rapidly degrade NO_3^- but cause NO_2^- accumulation meanwhile. Generally, the denitrification process includes the steps of $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. The entire denitrification process is coordinated by several enzymes such as the *Nar*G enzyme that converted NO_3^- to NO_2^-, the *Nir*S enzyme converted NO_2^- to NO. It is difficult for microorganisms to complete the process themselves. Numerous strains may rapidly eliminate NO_3^- and accumulate NO_2^- in previous studies, for example, *Pseudomonas stutzeri* C3 has been shown to accumulate 25.6 mg/L NO_2^- during the denitrification process (Ji et al., 2015a). Nitrite accumulation may occur for various reasons, some of which are inadequate carbon sources and the deletion or weak expression of *nir*S and *nos*Z genes in the strain. It was found that the abundance of the *nar*G gene in the sed-

iment of *M. aquaticum* CWs was about two orders of magnitude greater than the abundance of *nosZ* (Li et al., 2018). This interesting phenomenon favors the implementation of partial denitrification, which is one of the real applications of strains.

2.4. *Effects of different factors on the denitrification*

Single-factor experiments were conducted to explore optional conditions of M3-1 to achieve the best NO_3^--N removal efficiency. By changing conditions such as C/N ratio, temperature, pH, and inoculum size, a quadratic model reflecting the correlation between the four environmental factors and NO_3^--N removal of M3-1 was established:

$$Y = 81.77 - 38.81 \times A + 29.99 \times B - 6.37 \times C - 5.43 \times D - 3.65 \times A \times B$$

$$-52.57 \times A \times C + 21.98 \times A \times D + 44.41 \times B \times C + 10.58 \times B \times D - 2.79 \times C \times D$$

$$-1.36 \times A^2 - 7.75 \times B^2 + 4.24 \times C^2 - 12.76 \times D^2 \quad (5)$$

where, *Y* (%) is nitrate removal efficiency; *A* (°C), *B*, *C*, and *D* (%) are the coded values of temperature, C/N ratio, pH, and inoculum size, respectively. The quadratic models showed that temperature had a significant effect on NO_3^--N removal ($p < 0.05$) (**Appendix A Table S3**). The response surface methodology model was used to investigate interactive effects that were visually marked by response surfaces and corresponding contours (Fig. 5).

C/N ratio has a significant impact on the TN removal rate ($p < 0.1$). Unsurprising, denitrifiers isolated in this study could still perform well in denitrification with relatively low C/N ratio. There is a trend to a NO_3^--N removal rate of nearly 100% when C/N ratio is below 10. When C/N ratio is relatively low in the range of 1 to 3.7, denitrifiers could still perform well in denitrification under appropriate conditions. Perhaps the low demand for carbon sources has made this strain one of the dominant species and plays the corresponding denitrification role in the *M. aquaticum* CWs under low C/N ratio operation. Consequently, it is promising to add them *in situ* into wetlands to enhance the nitrogen removal efficiency in a low C/N ratio environment.

It is interesting to note that the importance of environmental factors in NO_3^--N removal efficiency was: Temperature > C/N ratio > pH > inoculum size. As shown in Fig. 5, the effect of temperature on the NO_3^--N removal rate was significant ($p < 0.05$). Many findings indicated that temperature rather than the availability of carbon sources was more effective in the microbial transformation of nitrogen. At low temperatures, NO_3^--N accumulation was recognized as the primary factor responsible for the reduction in TN removal efficiency, which recovered until temperatures increased (Pang et al., 2015). Significant nitrate accumulation was observed at temperatures < 10°C, while nitrate concentration was approximately zero at temperatures > 20°C (Chen et al., 2021; Ilies and Mavinic, 2001; Wang et al., 2015). However, the results of the response surface methodology model showed that strain isolated in this study has a better nitrate removal effect at medium temperature. At temperatures in the range of 20–35°C, M3-1 could degrade nitrate efficiently. Most denitrification strains are mesophilic with an appropriate temperature of about 30°C (Amatya et al., 2009). Studies of A13 isolated from aquaculture water have found that denitrification is most effective at 32.5°C (Zeng, 2008). Comparatively, M3-1 has a better nitrate removal effect at medium temperature (20–25°C), which corresponds to the average temperature in subtropical China, indicating that the strain is appropriate for most wetlands in rural areas of southern China and the addition of these strains that adapt to low temperatures is of great benefit in improving the nitrogen removal performance of wetlands in winter.

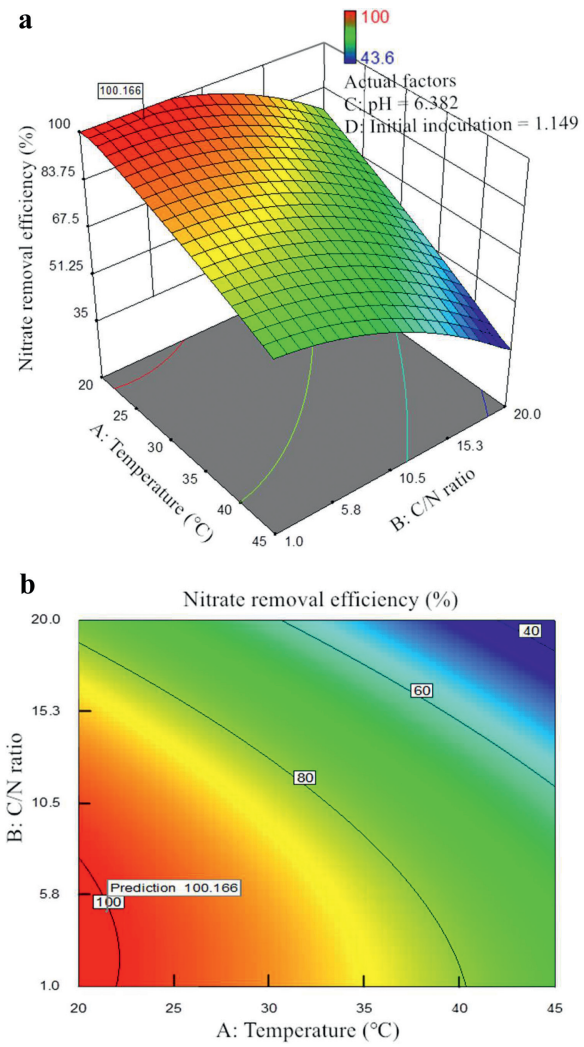

Fig. 5 – (a) Response surface (3D) and (b) corresponding contour (2D) plots for NO_3^--N removal by strain M3-1.

As well, the interactions of pH and inoculum size with nitrate removal were insignificant. This may be explained by M3-1 having relatively good adaptability to environmental conditions. The most suitable pH for denitrifiers is neutral and alkaline (Šimek et al., 2002), which also corresponds to strain M3-1. The predicted results indicated that the optimal conditions for nitrate removal by M3-1 were 21.5°C, C/N ratio = 4.9, pH = 6.4, and inoculum size of 1.2%. The simulation NO_3^--N

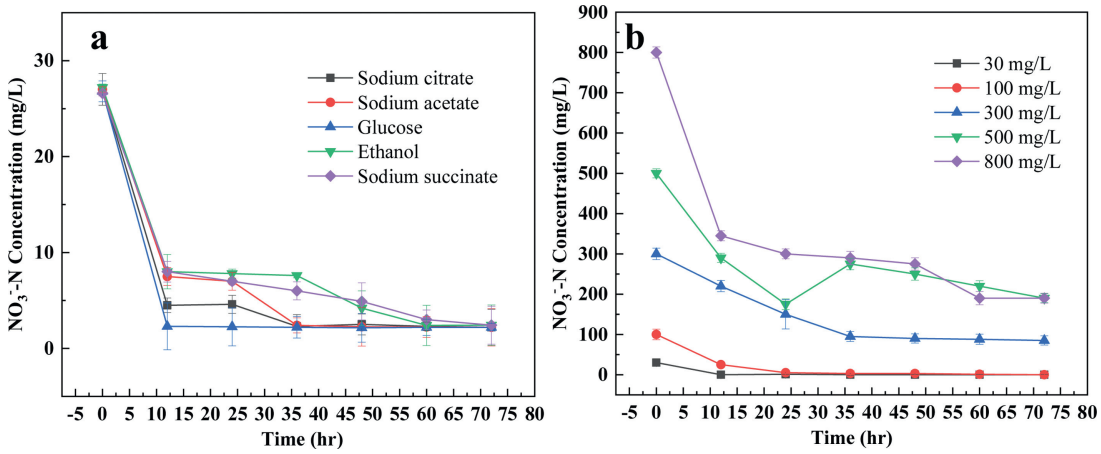

Fig. 6 – NO_3^--N removal performance of M3-1 in the presence of (a) different carbon sources and (b) different nitrate concentrations.

removal efficiency was 100% under these conditions. Confirmation experiments were also conducted under these conditions. The NO_3^--N removal efficiency in the verification test was 98.7%. The fact that the experimental results did not completely agree with the simulation results may be due to deviations in nitrate concentration measurement, but the fact that they were so approximate indicates that the model was suitable for experimental data.

Different carbon sources may greatly influence stain denitrification. Studies have reported that *Pseudomonas* ADN-42 has the greatest denitrification effect when the carbon source is sodium citrate (Ji et al., 2015b), and *Klebsiella* HY3-2 in glycerol has the greatest ability to denitrify (Chen et al., 2011). From Fig. 6a when M3-1 uses glucose as a carbon source (21.5°C, C/N ratio = 4.9, pH = 6.4, inoculum size of 1.2%, and initial nitrate concentration of 28 mg/L), the highest NO_3^--N elimination rate in 12 hr is 93.74% ± 3.52%. In general, however, M3-1 has a fairly wide spectrum of carbon sources. Besides, it is worthwhile studying the upper bound for nitrate wastewater treatment by the strain isolated. These experiments are conducted by changing the initial nitrate concentration while maintaining other conditions as 21.5°C, C/N ratio = 4.9, pH = 6.4, inoculum size of 1.2%, and sodium citrate as carbon source. As shown in Fig. 6b, the utilization rate of NO_3^--N by M3-1 at a concentration of 800 mg/L within 72 hr still exceeds 80%. M3-1 is tolerant to high concentrations of nitrate and the removal rate is as high as 9.12 mg/(L·hr), far exceeding other denitrifiers, such as *Enterobacter cloacae* with a removal rate of for NO_3^--N at 4.58 mg/(L·hr) (Guo et al., 2016).

3. Conclusions

This study explored the effect of different C/N ratios on the nitrogen removal and microbial community structure of *M. aquaticum* CWs. Meanwhile, cultivable denitrifying microorganisms with a high nitrate conversion rate from *M. aquaticum* CWs that operated under low C/N ratio conditions were isolated. On the basis of the experiments, the following conclusions were obtained.

(1) High C/N ratio conditions (C/N ratio:10) resulting in more efficient N removal (77.85% ± 1.46%) of *M. aquaticum* CWs. But *M. aquaticum* CWs with low C/N ratio have better treatment capacity (16.38% ± 2.51% of TN removal for per unit carbon source) in terms of full utilization of limited carbon sources.
(2) Microbial community structure analyses reveal that *M. aquaticum* contributed to the abundance of some denitrifiers. Firmicutes, Chloroflexi, and Proteobacteria (accounting for 72.99%–79.86%) were the three dominant phyla while the proportion of Proteobacteria increased (HCN ratio: 10.07%, LCN ratio: 18.05%) in the *M. aquaticum* CWs under low C/N ratio conditions.
(3) 33 strains belonged to *Bacillus* sp., *Acinetobacter* sp., and *Enterobacter* with denitrification characteristics were isolated and purified. They demonstrated good nitrate removal capacity, while the best is M3-1 which is identified as *Bacillus velezensis* JT3-1 with a removal rate of 86.48% ± 1.53%.
(4) Quadratic models showed that C/N ratio and temperature obviously affected NO_3^--N removal of M3-1. Denitrifiers isolated could perform well in denitrification when C/N ratio is relatively low (C/N ratio: 1-3.7). The addition of the strains in situ is promising to improve the denitrification efficiency of CWs with low C/N ratio.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A Supplementary data

Supplementary data associated with this article can be found, in the online version at doi:10.1016/j.jes.2022.04.010.

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