Nitrate Removal Characteristics of Nitrate-reducing Bacteria *Pseudomonas* sp. XS-18 at Low Carbon-to-nitrogen Ratio

L. L. Yan,* M. Y. Yin, C. X. Wang, M. Y. Yang, L. T. Sun, and Y. Jiao
College of Resource and Environment,
Northeast Agricultural University, Harbin 150030 China

doi: https://doi.org/10.15255/CABEQ.2023.2179

Original scientific paper
Received: January 15, 2023
Accepted: September 8, 2023

Low carbon-to-nitrogen (C/N) ratios are the main characteristic of domestic wastewater. In this study, we evaluated the nitrate removal characteristics of *Pseudomonas* sp. XS-18 at low C/N ratios (4.0 and 6.0) and pH 11.0. We also analyzed the causes of the nitrate removal at low C/N ratios in this strain. At pH 11.0 and a C/N ratio of 4.0, the strain was effective in removing nitrate. Additionally, the total organic carbon content decreased over time at low C/N ratios, requiring more energy to complete vital activities. Furthermore, low C/N conditions resulted in less strain secretion compared to high C/N settings, and the polysaccharide content degraded more quickly than protein over time. To provide a carbon source for the nitrate reduction process, strains with low C/N ratio and high pH secreted more soluble microbial by-products. This strain is able to metabolize its own secreted extracellular polymeric substances as a carbon source to enhance nitrate removal.

Keywords
aerobic nitrate reduction, low C/N ratio, high pH, extracellular polymeric substances, nitrate assimilation process

Introduction

Water scarcity has become a significant issue due to the rapid development of society, and water pollution is a significant contributor to this problem.[1] Water pollution not only exacerbates the water shortage in China but also harms the natural world and jeopardizes public health.[2]

The extensive use of chemical fertilizers in the agricultural system, and the discharge of point-source pollutants, such as domestic sewage and industrial wastewater, have increased nitrate levels in river water in recent years. This pollution has affected the aquatic ecosystems and contaminated sources of drinking water, posing a threat to human health and disrupting ecological equilibrium.[3–5] Therefore, the removal of nitrogen from wastewater presents a serious challenge to the public.

Nitrate is one of the main contaminants in water bodies, and measures should be taken to address nitrate pollution.[6] According to a previous study,[7] in China, the nitrate content in 7.83 % drinking water (45 mg L^{-1}) exceeds acceptable levels. The concentration of nitrate in rivers such as Mudanjiang (Linkou), Haihe (Beijing), and the Yangtze River estuary (Shanghai), is twice the national drinking water standard. In the Yellow River Delta and some rapidly urbanizing areas in China, groundwater nitrate concentrations greatly exceed the legal standard (10 mg L^{-1}).[8–10] Thus, the removal of nitrate from water has attracted significant attention.[6]

It has been reported that the typical influent carbon-to-nitrogen (C/N) ratio of municipal wastewater treatment plants worldwide falls between 10.5 and 12.6.[11] However, in some Chinese cities, this ratio can drop below 5 due to the discharge of industrial wastewater into domestic wastewater treatment plants.[12,13] Meanwhile, the C/N ratio of the secondary effluent from wastewater treatment plants is generally low.[14] Although domestic wastewater treatment facilities frequently rely on biological treatment methods,[15] classic biological denitrification methods are now severely hindered by the lack of carbon sources. Due to an insufficient carbon source, the ability of microorganisms to reduce nitrate is inhibited, resulting in residual nitrate levels in the reactor effluent of up to 25 mg L^{-1}.[16] The addition of organic matter and carbon sources can improve the efficiency of denitrification; however, this could lead to higher operating costs, increased sludge production, and potential secondary contamination of effluent water.[16]

*Corresponding author: Tel.: +86 451 5519 0825;
fax +86 451 5519 1170. E-mail address: yanll98@163.com

Current research on biological decarbonization indicates that both traditional biological denitrification techniques and anaerobic ammonia oxidation are employed to purify water by releasing the nitrogen exclusively as a gas into the atmosphere,[17,18] thus improving water quality. Simultaneously, to enhance the treatment effectiveness of low carbon-to-nitrogen ratio wastewater, nitrate-reducing strains, such as *Bacillus thuringiensis* strain WXN-23,[19] *Trichomonas* strain YSF15, [20] and *Bacillus colorless* strain JL9,[21] which are suitable for low carbon-to-nitrogen ratios, have been continuously screened from the environment. Their removal effects, influencing factors, and potential nitrogen removal mechanism have been studied under low C/N ratio conditions. In general, the genera suitable for nitrate removal at low carbon-to-nitrogen ratios are still relatively limited, and these genera primarily convert nitrate into nitrogen gas. While this method can purify wastewater, it inevitably results in nitrogen loss or disruption of nitrogen cycle pathways. Additionally, it leads to increased greenhouse gas emissions of N_2O as a by-product, accounting for roughly 1.3 percent of all N_2O emissions.[22] In contrast, the conventional nitrate isomerization reduction process can convert nitrate to ammonia nitrogen, which subsequently enters the nitrification process. This can remove nitrates, but does not achieve the goal of water purification.[23] The nitrate assimilation process converts nitrogen in wastewater into microbial cell components.[24,25] At the same time, it has the potential to recover nitrogen while purifying wastewater and shortening the nitrogen cycle pathway. Therefore, selecting a strain with efficient nitrate reduction at a low C/N ratio is crucial.

The traditional denitrification process requires a sufficient carbon source. However, a common water quality characteristic of these facilities is a low carbon-to-nitrogen (C/N) ratio. Due to the lack of carbon sources, the ability of microorganisms to reduce nitrate is inhibited, leading to residual nitrate in the reactor's effluent.[16] Addition of organic matter can enhance denitrification efficiency,[26] but it can also increase operating expenses and pose a risk of contaminating the quality of the secondary effluent.[16] Hence, it is important to screen for strains that are capable of reducing nitrate under low C/N ratio conditions and recovering nitrogen from wastewater.

Building upon the aforementioned, this experiment screened high-efficiency nitrate-reducing bacteria (NRB) and discussed the viability of *Pseudomonas* sp. XS-18 in nitrate removal and nitrogen recovery at low C/N. This analysis was based on early-stage assessments of nitrate removal and alkali-tolerant mechanisms.[27] Simultaneously, the composition and fluorescence properties of extracellular polymeric substances (EPSs) were described to investigate the nitrogen removal process of *Pseudomonas* sp. XS-18 in the absence of an adequate carbon supply. This work provides a theoretical foundation and a reference point for the utilization of NRB in wastewater with low C/N ratio.

Materials and methods

Bacterial cultivation

Pseudomonas sp. XS-18 was isolated from aerobic granular sludge in the lab's sequencing batch reactor (SBR) for the experiment.[27] The specific operational metrics of the reactor were as described in the literature.[27]

In this experiment, enrichment medium (EM) and nitrate medium (NM) were used, consistent with previous experiments.[27] The carbon source was sodium citrate, while the nitrogen source was potassium nitrate. The initial nitrogen concentration of the NM medium was 50 mg L^{-1}, and the organic carbon concentration varied with different C/N. The pH of the medium was adjusted using 1 mol L^{-1} HCl and 1 mol L^{-1} NaOH.

Determination of nitrate-reducing capability

Pseudomonas sp. XS-18 was received into EM, and incubated at 160 rpm for 24 h at 25 °C. The enriched cultures were centrifuged at 8000 rcf for 5 min, and the OD_{600} values were adjusted to 2.0 with sterilized distilled water to obtain bacterial suspensions. Subsequently, 5 mL of the bacterial suspension was added to conical flasks containing 95 mL of NM for incubation, where the C/N values of the NM varied (4.0 and 6.0). The optimal C/N=13.0 medium obtained from previous experiments was used as a control.[27] To examine the dynamic changes in nitrogen concentration and pH over time, several sampling intervals (30, 60, 90, 120, 150, 180, 210, 240, 300, and 360 min) were set.

Nitrogen balance pathway analysis

A nitrogen balance analysis of the nitrate reduction process was performed to better understand the processes of nitrogen migration and transformation in the nitrate reduction of *Pseudomonas* sp. XS-18. The specific steps were as follows:[27] To prevent exogenous nitrogen from interfering with the test, the *Pseudomonas* sp. XS-18 was cultured in EM for 24 hours, centrifuged for 5 minutes at 5000 rpm, washed three times with sterile water, adjusted OD_{600} to about 2, and added 5 mL of the bacterial suspensions to 95 mL of pH 7.0 with C/N 4.0, and

6.0 with a pH of 11.0 with a C/N 4.0 already adjusted NM. To ensure system stability, bacterial solution and NM were added to sterilized serum bottles and sealed after passing through pure oxygen. As a blank control, no bacterial solution was added. The cells were crushed with an ultrasonic cell crusher for 360 min at 25 °C and 160 rpm. Subsequently, a nitrogen balance analysis was conducted using the method described by Guo *et al.*[28]

Intracellular nitrogen = total nitrogen after fragmentation – total dissolved nitrogen

Dissolved organic nitrogen = total dissolved nitrogen – nitrate – nitrite-ammonia nitrogen

Extraction of EPSs

EPSs were extracted using the centrifugation method.[29] A 5 mL bacterial solution with an adjusted OD_{600} = 2.0 was inoculated into 95 mL of NM medium, and incubated for specific durations (30, 60, 90, 120, 180, 240, and 360 min). The bacterial culture was centrifuged at 8000 rpm for 10 min at 4 °C, and the filtrate obtained after filtering the supernatant through a 0.22 μm aqueous membrane was referred to as EPSs. To determine the cells' dry weight, the suspensions were freeze-dried.

Analytical and statistical methods

Standard methods were employed to quantify ammonia, nitrite, nitrate, OD_{600}, and pH.[27] The total organic carbon (TOC) was determined using a TOC analyzer (Multi N/C 2100S Analytikjena, Germany). Total inorganic nitrogen (TIN) was calculated as the sum of NH_4^+-N, NO_3^--N and NO_2^--N. Anthrone colorimetric and a modified Lowry method were respectively used to determine the anthrone protein (PN) and polysaccharide (PS) in EPSs.[30] The EPSs were analyzed using three-dimensional fluorescence spectra (Excitation Emission Matrix Spectra, EEMs) (F-7000 Hitachi, Japan), according to the method described in the literature.[31]

Other instruments used in the experiment were centrifuge (Microfuge 22R BECKMAN COULTER, America), constant temperature incubator (BSP-250, Shanghai Boxun, China), ultra-clean bench (BJ-CD, Shandong Boke, China), electric steam pressure sterilizer (DSX-280B Shanghai Shenan, China), pH meter (pHS-25 Shanghai Lei magnetic, China), cell crusher (Scienttz-IIIE Shanghai Deyang Yibang, China), and UV spectrophotometer (TU-1810 Puxi, China). The chemicals used in this experiment primarily included: KNO_3, KH_2PO_4, $MgSO_4$, $FeCl_2{\cdot}6H_2O$, $CaCl_2{\cdot}7H_2O$, sodium citrate, HCl, NaOH, and $NaHCO_3$. These chemicals were purchased from Tianjin Kermel Chemical Reagent Company, China. Three replicates of each group and control group were run for each statistical analysis. Tukey's HSD was employed to evaluate the results of the one-way ANOVA ($p < 0.05$), and Origin 2021 was used for data plotting.

Results and discussion

Nitrate-reducing performance of *Pseudomonas* sp. XS-18

Nitrate-reducing performance of the strain under low C/N conditions

The variation of TIN concentration over time during nitrate removal by *Pseudomonas* sp. XS-18 was tested at C/N 6.0 and 4.0 (Fig. 1). At a C/N of 6.0, 40.10 mg L^{-1} of nitrate was removed in 210 minutes, and the nitrate concentration remained around 16.61 mg L^{-1}, with 70.70 % nitrate and 65.49 % TIN removed (Fig. 1a). This nitrate removal efficiency was 11.62 % lower compared to the condition with a C/N of 13.0.[27] The total amount of nitrite accumulated was less than 5.0 mg L^{-1}, indicating that the low C/N ratio condition was able to reduce nitrite accumulation. In contrast, Zhang *et al.*[32] observed a higher tendency for nitrite accumulation under low C/N conditions, which was attributed to differences in the metabolic properties of different strains. The OD_{600} value of *Pseudomonas* sp. XS-18 steadily increased to 0.53 (Fig. 1b) although growth and reproduction rate slowed down due to a lack of nutrients, with an average rate of 0.08 h^{-1}. This rate was much lower than that observed under the C/N=13.0 condition (0.12 h^{-1}). The pH value of the solution steadily rose from 7.0 to 8.5 before stabilizing, similar to the previous results observed under C/N 13.0 conditions.[27]

When C/N was reduced to 4.0, 25.33 mg L^{-1} nitrate was removed, and nitrite buildup remained at around 1.50 mg L^{-1} for 360 min (Fig. 1c). The removal percentages for nitrate and TIN were 47.57 % and 44.85 %, respectively. This represented a decrease of 23.13 % and 20.64 % from the condition of C/N=6.0. The pH value of the solution gradually increased from 7.0 to 8.5 during this time, which was consistent with the findings observed under C/N ratios 13.0 [27] and 6.0. The OD_{600} of the strain gradually increased to 0.45 with an average rate of 0.04 h^{-1} during this time.

Further comparative analysis revealed that the nitrate removal rate by *Pseudomonas* sp. XS-18 at a C/N of 6.0 (5.06 mg L^{-1} h^{-1}) was greater than that at a C/N of 4.0 (4.22 mg L^{-1} h^{-1}). The rates of growth, reproduction, and metabolic activity of *Pseudomonas* sp. XS-18 slowed down when there was an insufficient carbon source, consistent with previous findings.[5]

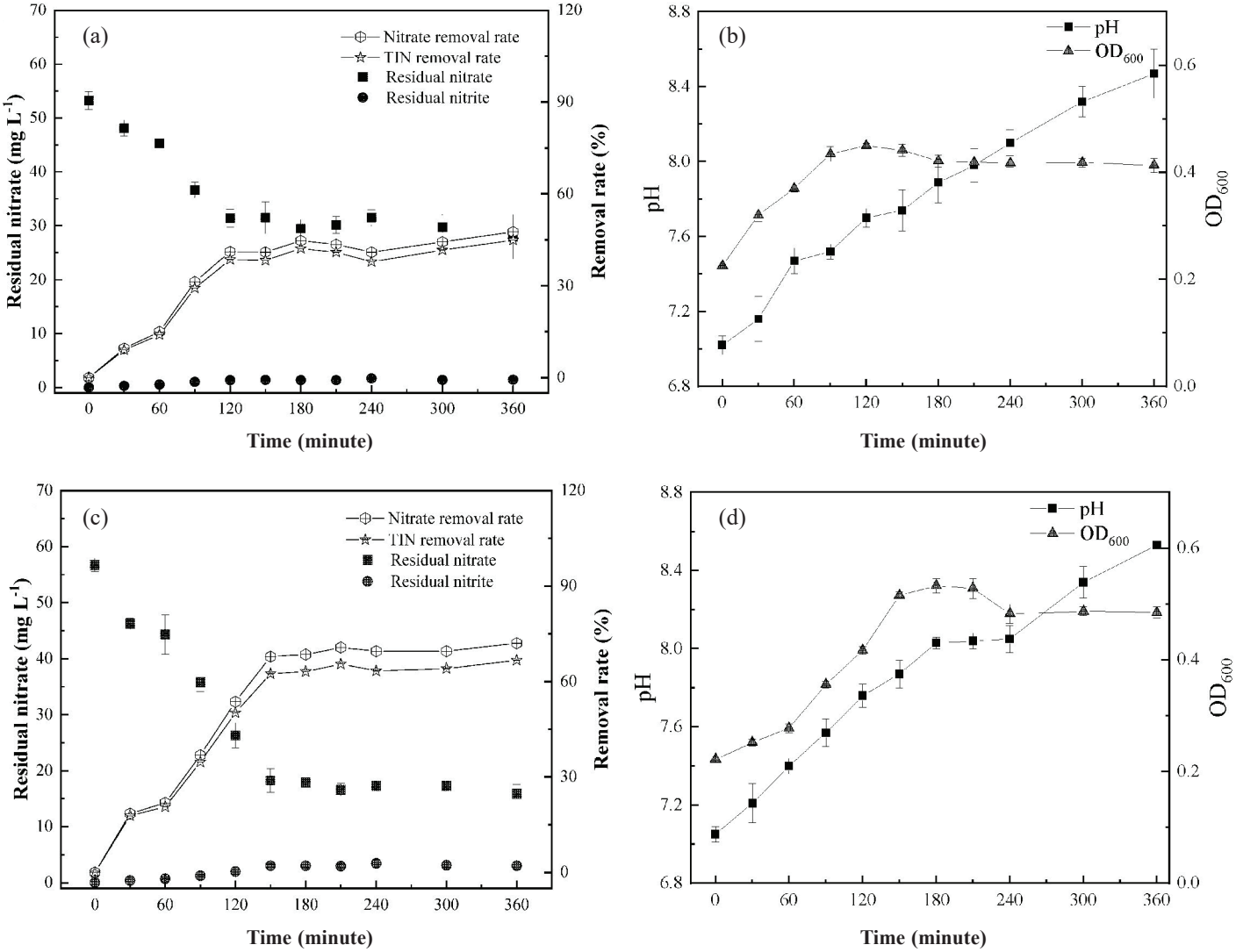


Fig. 1 – *Nitrogen concentration and OD and pH of Pseudomonas sp. XS-18 under C/N 4.0 and 6.0: (a) nitrogen concentration of C/N 4; (b) OD and pH of C/N 4.0; (c) nitrogen concentration of C/N 6.0; (d) OD and pH of C/N 6.0*

Nitrate-reducing performance of the strain under alkaline and low C/N conditions

In a previous study, *Pseudomonas* sp. XS-18 was found to exhibit high alkalinity tolerance. Notably, certain wastewaters from the paper, textile, and petroleum sectors also have high alkalinity and low C/N ratios.[27] Therefore, studying *Pseudomonas* sp. XS-18's nitrate-reducing capabilities in alkaline and low C/N environments is of great practical significance.

At pH 11.0 and C/N 4.0 (Fig. 2), the nitrate removal reached 31.54 mg L^{-1} at 360 min, and the nitrite accumulation remained at around 2.00 mg L^{-1}. The removal efficiencies for nitrate and TIN at the same C/N were 54.26 % and 50.93 %, respectively, which was better than under neutral conditions. This showed that *Pseudomonas* sp. XS-18 is an alkalophilic bacterium, because it demonstrated better nitrogen removal under alkaline conditions (pH 11.0) and exhibited a higher nitrate removal rate.[30] Meanwhile, the pH value of the solution slowly decreased from 11.0 to 8.6, consistent with a previous study,[30] and the OD_{600} of *Pseudomonas* sp. XS-18 steadily increased to 0.46 with an average rate of 0.04 h^{-1} (Fig. 2b).

Most of the strains that have been identified so far, such as *Acinetobacter junii* YB,[33] *Pseudomonas stutzeri methylobacterium gregans* DC-1,[3] *Vibrio diabolicus* SF16,[34] *Acinetobacter* sp. ND7,[35] and *Ochrobactrum anthropic* LJ81,[16] are capable of surviving or functioning in neutral or mildly alkaline conditions. Recently, some aerobic denitrifying bacteria that are acid-base tolerant have also been discovered.[5,36,37] However, there is knowledge about aerobic nitrate-assimilating/-isomerizing bacteria that are highly acid-base tolerant, especially under conditions of lower C/N. *Pseudomonas* sp. XS-18 exhibited better nitrate nitrogen removal under alkaline conditions than under neutral conditions, ex-

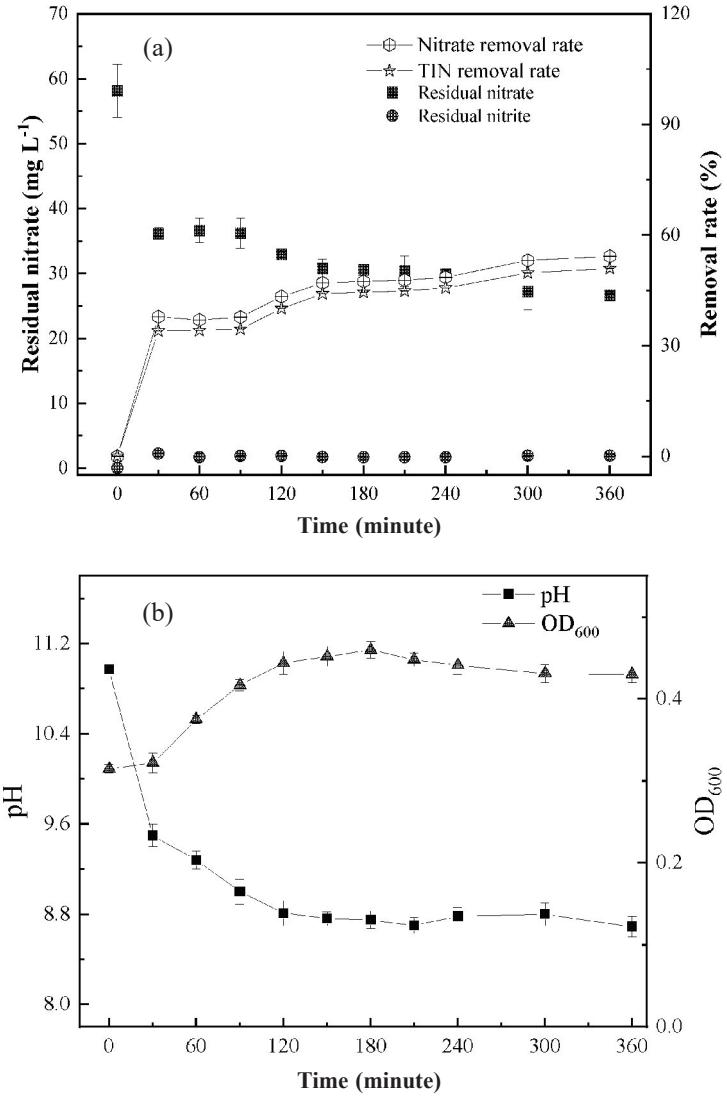


Fig. 2 – *(a) Nitrogen concentration of Pseudomonas sp. XS-18 under pH 11.0 and C/N 4.0; (b) OD and pH of Pseudomonas sp. XS-18 under pH 11.0 and C/N 4.0*

cellent alkaline resistance, and the ability to remove nitrogen from specific industrial wastes, including those from the paper, textile, and petroleum industries. This eliminates the necessity for acid-base adjustment processes and reduces operating costs.[38] Simultaneously, the nitrogen assimilated into the organism also facilitates the creation of conditions for the next stage of resource recovery. Consequently, *Pseudomonas* sp. XS-18 holds significant application potential.

Nitrogen balance analysis

Nitrogen balance analyses were conducted on *Pseudomonas* sp. XS-18 under C/N 4.0 and 6.0, and pH 7.0 conditions. Nitrate was completely eliminated within 360 minutes with a sufficient carbon source available (C/N 13.0).[27] In contrast, nitrate nitrogen reduced by 30.69±0.63 and 41.07±0.65 mg L^{-1}, respectively, at C/N ratios of 4.0 and 6.0 due to carbon source deficiency (Table 1). Compared to C/N of 13.0 conditions, the nitrite buildup increased by 0.95±0.20 and 19.8±0.15 mg L^{-1}, respectively, and no ammonia nitrogen was detected. At low C/N 4.0 and 6.0, *Pseudomonas* sp. XS-18 grew slower, and the OD_{600} was slightly lower than at C/N=13.0, by 0.452 and 0.427, respectively. The rates of nitrogen removal by *Pseudomonas* sp. XS-18 were 10.95, 13.91, and 10.47 mg L^{-1} h^{-1} OD_{600}^{-1} at C/N of 4.0, 6.0, and 13.0, respectively. These results indicated that *Pseudomonas* sp. XS-18 demonstrated strong nitrate-reducing performance even under conditions of insufficient carbon sources. Therefore, the reduced nitrogen removal efficiency of *Pseudomonas* sp. XS-18 at low C/N was attributed to its slower growth rate compared to a C/N ratio of 13. Meanwhile, the amount of organic nitrogen decreased by 31.30 % and 40.40 %, and the amount of nitrogen in the cells decreased to 4.81±0.45 and 6.33±0.54 mg L^{-1}. These results suggest that the low C/N affected the growth and reproduction rate, and nitrate-to-nitrogen conversion pathway of *Pseudomonas* sp. XS-18.

The strains isolated so far mainly convert nitrate nitrogen to nitrogen gas. Even though a small number of strains can convert nitrate nitrogen to organic nitrogen, the conversion ratio remains low. For instance, *Bacillus thuringiensis* strain WXN-23 exhibited only 17.9 % initial N assimilation at a low C/N ratio.[19] *Comamonas* sp. YSF15 has almost no assimilation effect,[20] which was significantly lower than the assimilation effect of *Pseudomonas* sp. XS-18. The strain retained 31.30 % and 40.40 % of nitrogen in the form of organic nitrogen in the cells at a C/N of 4.0 and 6.0, respectively. Therefore, *Pseudomonas* sp. XS-18 has great potential to recover nitrogen nitrate from wastewater.

Given the high alkaline tolerance of *Pseudomonas* sp. XS-18, a nitrogen balance analysis of the nitrogen removal process was conducted at C/N 4.0, pH 11.0, and pH 7.0 as the control group to determine the feasibility of treating low C/N wastewater under high alkaline conditions. The reduction in organic and bacterial nitrogen content affected the removal of nitrate nitrogen. The nitrate was reduced by only 32.84 mg L^{-1} and nitrite accumulated by 1.93±0.09 mg L^{-1} within 360 min (Table 1). Compared to the neutral conditions, the bacterial nitrogen and intracellular organic nitrogen were both significantly lower, consistent with previous studies.[27] *Pseudomonas* sp. XS-18 exhibited a higher nitrate removal rate at pH 11.0, suggesting its potential use in treating low C/N strongly alkaline wastewater, because the OD_{600} also reduced by 0.037 compared to the pH 7.0 condition.

Table 1 – *Nitrogen balance analysis under different C/N and pH conditions*

Medium condition	Initial nitric nitrogen	Nitrogen balance (mg L^{-1})				Cell nitrogen	OD_{600}
		Nitrate	Nitrite	Ammonia nitrogen	Intracellular organic nitrogen		
C/N 4.0, pH 7.0	58.60±1.14	27.91±0.51	1.45±0.21	0	18.34±0.40	4.81±0.45	0.467
C/N 6.0, pH 7.0	56.93±1.07	15.86±0.42	3.02±0.16	0	23.00±0.57	6.33±0.54	0.492
C/N 4.0, pH 11.0	59.42 ±1.90	26.58±0.38	1.93±0.09	0	16.07±0.68	3.87±0.75	0.430

Variation of TOC under different conditions

The TOC content gradually reduced, indicating that TOC was used for the growth and nitrogen removal of *Pseudomonas* sp. XS-18 under the four conditions (Fig. 3). The TOC content decreased significantly under neutral and alkaline conditions, decreasing from 343.02 mg L^{-1} and 295.80 mg L^{-1} to 13.21 mg L^{-1} and 12.54 mg L^{-1} within 360 min. When the C/N was 4.0, the TOC concentration rapidly decreased from 136.59 and 127.71 mg L^{-1} to 10.58 (pH 7.0) and 10.43 (pH 11.0) mg L^{-1}, respectively.

In recent years, the screening of strains, such as the strain *Achromobacter* sp. JL9 screened by Liang *et al.*,[21] which is also capable of functioning under low C/N conditions, has laid the foundation for low C/N wastewater treatment. However, research on high alkalinity and low C/N remains limited. Additionally, the rate of change in TOC content (19.55 and 47.21 (mg L^{-1} h^{-1})) was lower under alkaline conditions than in neutral conditions (21.00 and 54.97 (mg L^{-1} h^{-1})) (C/N 4.0 or 13.0). This suggest that *Pseudomonas* sp. XS-18 requires more energy in an alkaline environment to perform vital processes like EPSs secretion.

Further analysis found that 4.96 and 3.72 mg TOC/mg NO_3^--N were consumed, respectively, for each mg of nitrate nitrogen removed at C/N of 4.0 and pH 7 and 11.0. However, 6.87 and 6.74 mg TOC/mg NO_3^--N were required to be consumed, respectively, at a C/N of 11.0 and pH of 7.0 and 11.0.[27] This indicated that the amount of TOC consumed for the removal of nitrate nitrogen was lower when the carbon source was insufficient. Specifically, the amount of TOC consumed for nitrate nitrogen removal was less under alkaline conditions than under neutral conditions. It is hypothesized that *Pseudomonas* XS-18 requires additional carbon sources, such as EPSs, to complete nitrate nitrogen removal.

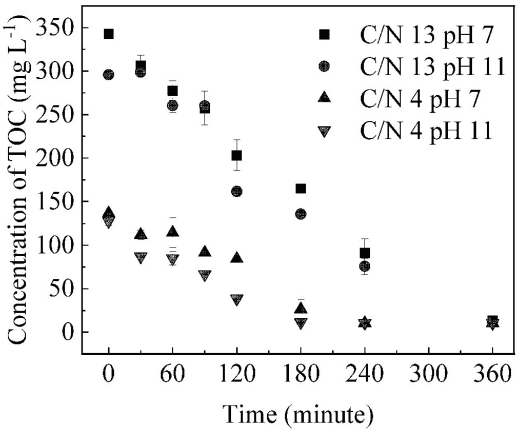


Fig. 3 – *TOC concentration variation under different C/N and pH conditions*

Analysis of EPSs components

The low C/N ratio is one of the main challenges in current wastewater treatment today due to energy shortages. Microbes in the actual wastewater or the laboratory-selected target strains face difficulty in performing their intended roles in wastewater treatment when there is a lack of energy. Bacteria decompose their cells and consume organic matter, producing EPSs, which are a complex mixture of macromolecules.[39] When exposed to various types of pollution, EPSs manifest differently, and the mechanisms behind these variations are complex.

Under the conditions of C/N 13.0 and neutral, the EPSs started at 528.55 mg g^{-1} DCW. As the incubation time increased to 360 min, the EPSs slowly decreased to 289.33 mg g^{-1} DCW, with PS consistently higher than PN (Fig. 4a). This is consistent with previous results.[20] In neutral and alkaline settings, the overall trend of EPSs was the same (Fig. 4b), with a reduction from 448.90 mg g^{-1} DCW to 150.70 mg g^{-1} DCW. Furthermore, the PS content under pH 11.0 conditions was much higher than that under neutral conditions before 240 minutes, especially before 90 minutes, despite the overall PN content being lower than that under neutral conditions. This indicates that *Pseudomonas* sp. XS-18 released more PS to counteract the effects of the high pH under pH 11.0 conditions and prevent toxicity, aligning with what has been observed in the literature.[40]

Additionally, Fig. 5a illustrates the dynamics of *Pseudomonas* sp. XS-18 EPSs at C/N 4.0 and two distinct pH levels (7.0 and 11.0). The PS content remained higher than the PN level, but the EPSs content was only 106.72 mg g^{-1} DCW due to insuf-

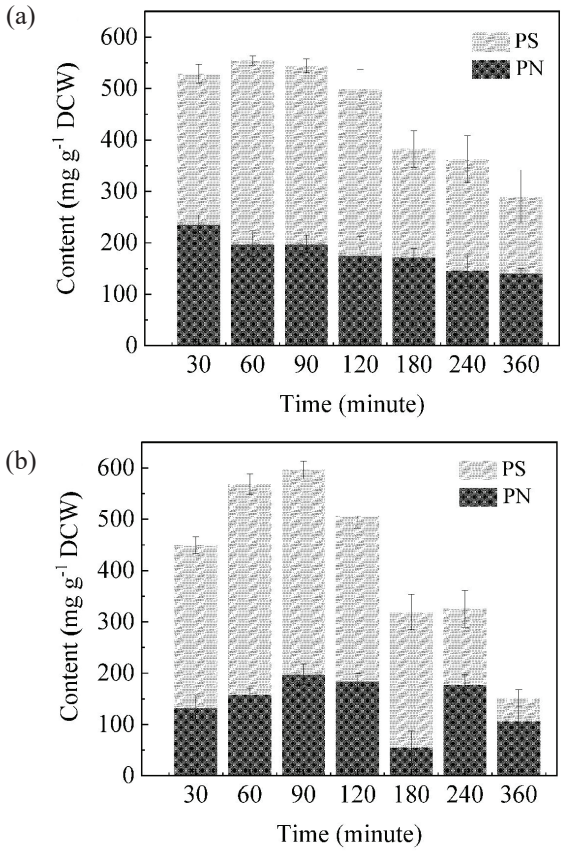


Fig. 4 – *PN and PS of Pseudomonas sp. XS-18 changed in EPSs at different time intervals within 360 min under C/N 13.0: (a) pH 7.0; (b) pH 11.0*

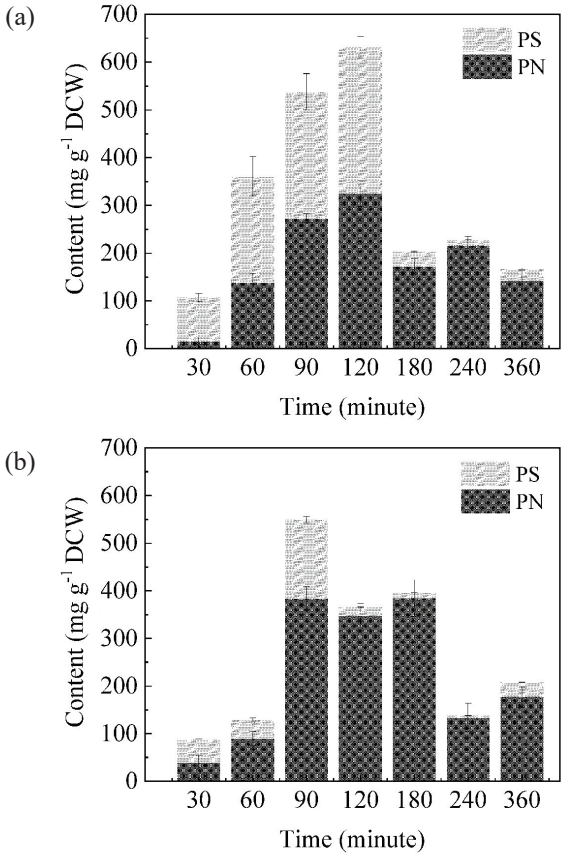


Fig. 5 – *PN and PS contents of strain Pseudomonas sp. XS-18 changed in EPSs at different time intervals within 360 min under C/N 4.0: (a) pH 7.0; (b) pH 11.0*

ficient carbon source. However, *Pseudomonas* sp. XS-18 released more EPSs as a backup carbon source for growth and nitrate reduction after 30 min when the response time started to run out. Similar results were found by Sheng *et al.,*[39] who showed that EPSs can protect cells in challenging situations and act as a backup carbon source for starving cells. The EPSs, which served as temporary storage as the organic matter in the medium was consumed were gradually depleted, especially the PS content, which significantly reduced from 305.71 mg g^{-1} DCW to 24.28 mg g^{-1} DCW, while the PN decreased by 181.88 mg g^{-1} DCW. This indicates that PS was the primary component utilized when *Pseudomonas* sp. XS-18 lacked carbon sources.

At pH 11.0, the PS content was slightly higher than PN at 30 min, and the overall EPSs content (88.38 mg g^{-1} DCW) was lower than at pH 7.0 (Fig. 5b). Additionally, it was observed that PN was consistently the primary component of EPSs, with its content higher than that under neutral conditions except during the first 90 minutes. This suggests that, as PS was primarily required to maintain metabolic activity for the growth and reproduction of *Pseudomonas* sp. XS-18, the strain could not produce more PS in the absence of a carbon supply, but instead secreted more PN to counteract the negative effects of an alkaline environment. After 90 minutes, the amount of PS reduced significantly, indicating that PS was mainly used as a backup carbon source.[39,40]

Recent research has shown that bacteria can utilize EPSs as a carbon source in nutrient-poor environments. For instance, the *Comamonas* sp. YSF15 strain that Su *et al.*[20] used in their screening was capable of nitrate reduction while also producing its own EPSs, with PS content in EPSs consistently higher than PN. In this study, due to adaptation to the new environment's low C/N ratio, *Pseudomonas* sp. XS-18 initially produced fewer EPSs under low C/N conditions. As the strain adapted to the new environment, the amount of EPS produced by the strain increased. The PS content gradually decreased over the incubation time because it served as a backup carbon supply and consumed organic materials in the reaction system.[20]

EEM analysis under different conditions

To gain a deeper understanding of *Pseudomonas* sp. XS-18's ability to use its own EPSs to reduce nitrate, the fluorescent fractions in the EPSs were tested at C/N = 4.0, 13.0, and pH 7.0 and 11.0. Five distinct types of fluorescent peaks were identi-

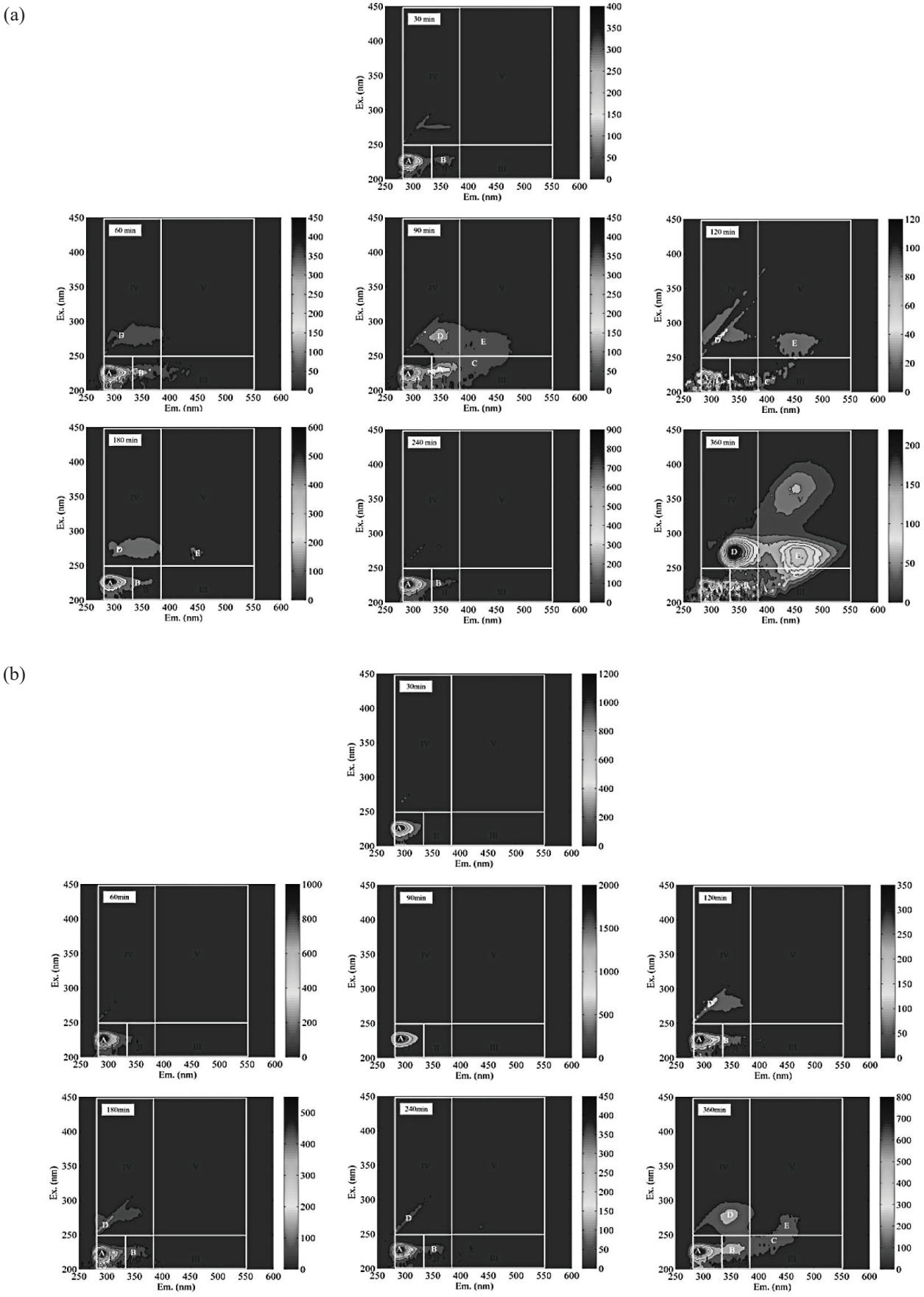
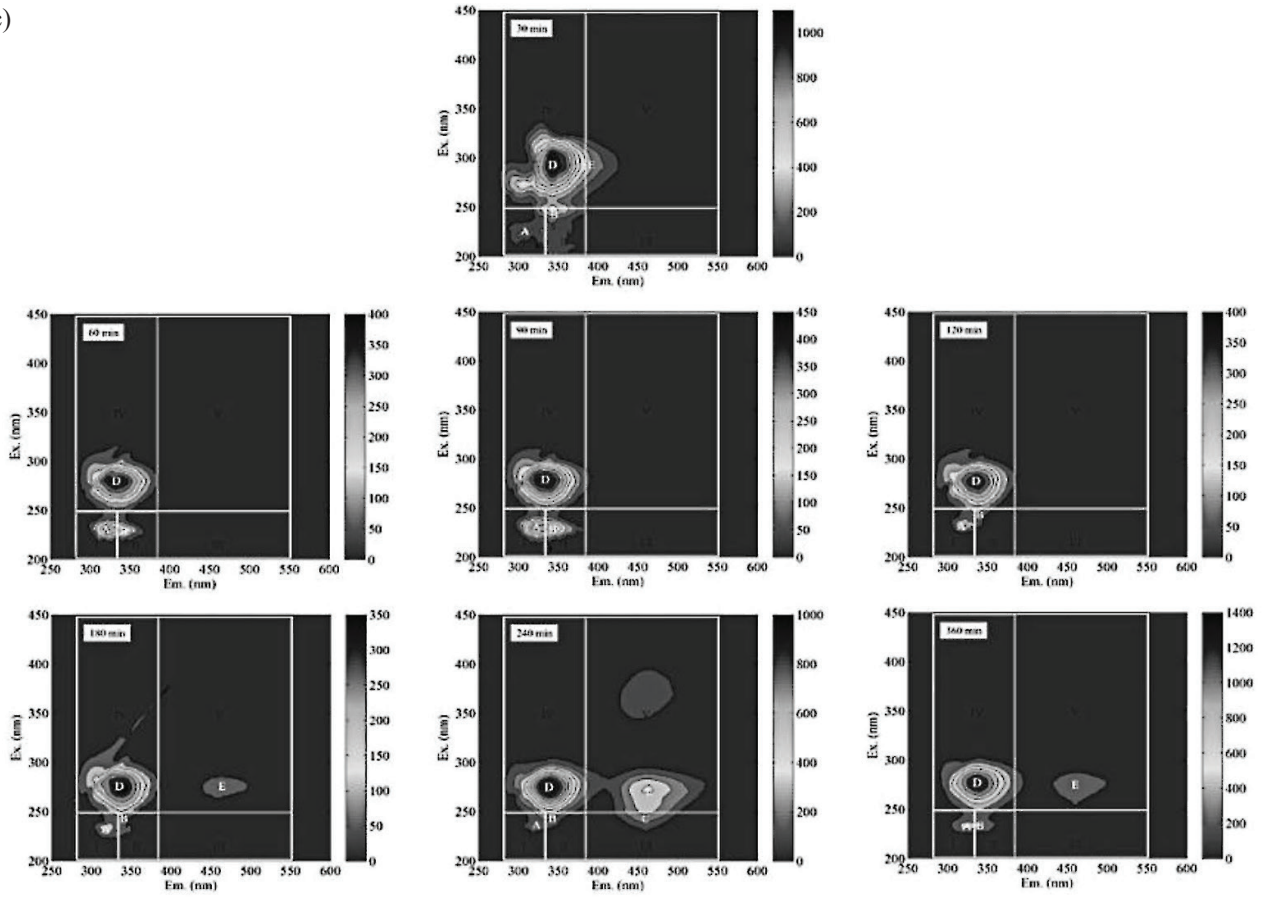


Fig. 6 – *Three-dimensional fluorescence spectra of EPSs: (a) pH 7.0 C/N 13; (b) pH 11.0 C/N 13; (c) pH 7.0 C/N 4; (d) pH 11.0 C/N 4*

(c)



(d)

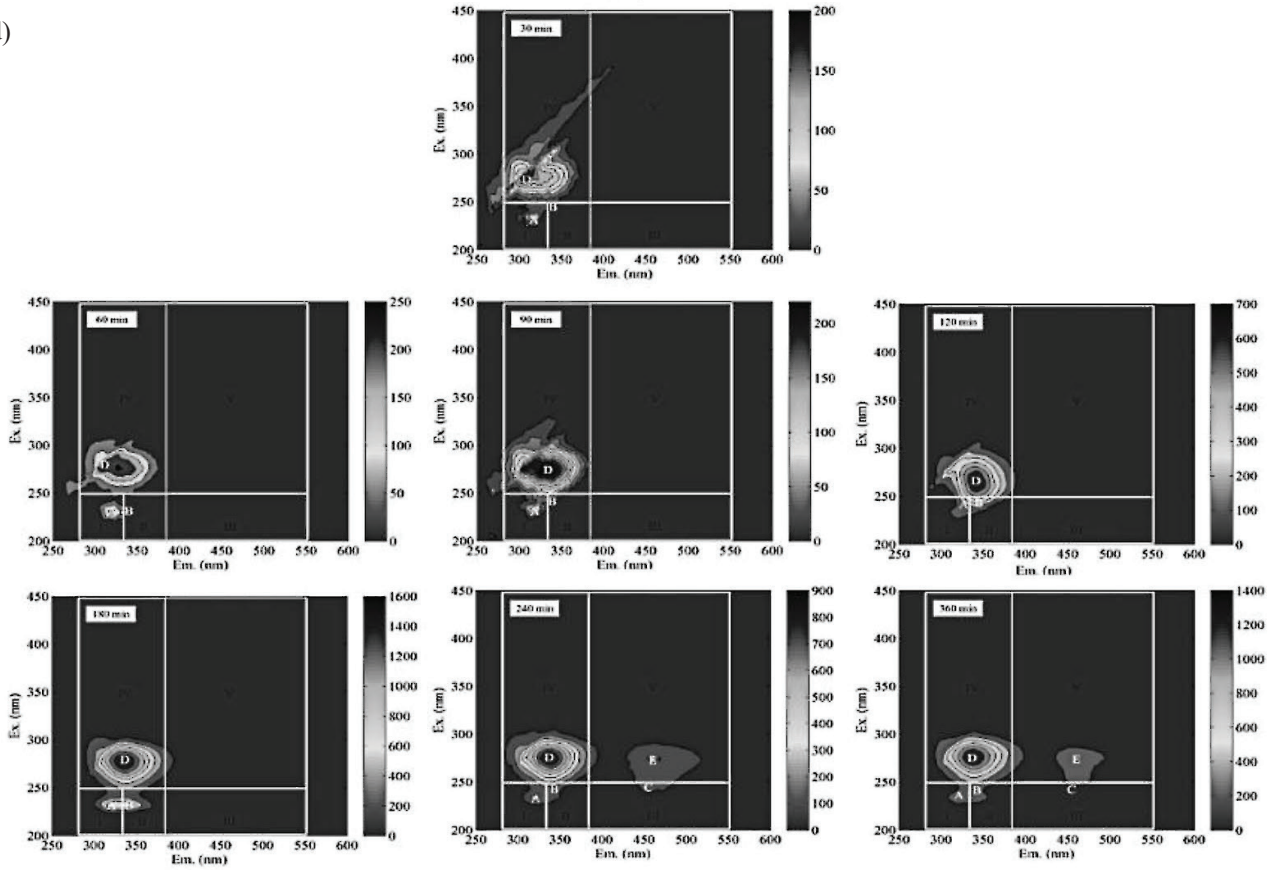


Fig. 6 – *Continued*

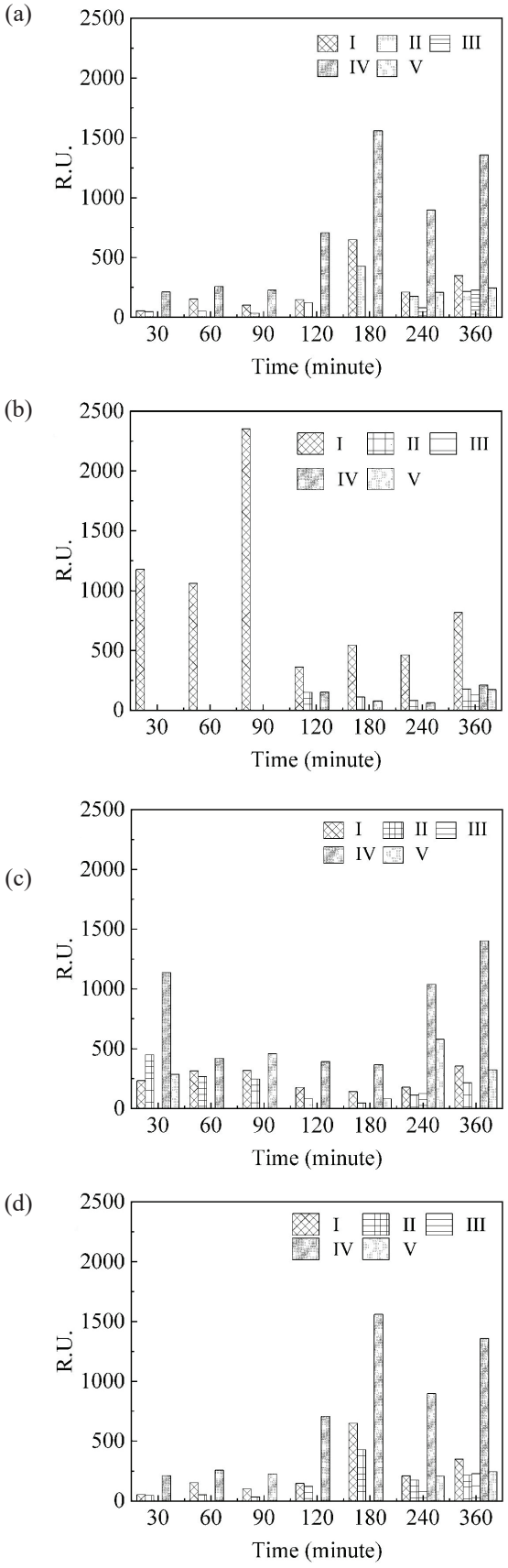


Fig. 7 – *Changes in fluorescence components in EPSs over incubation time within 360 min: (a) C/N 13.0 pH 7.0; (b) C/N 13.0 pH 11.0; (c) C/N 4.0 pH 7.0; (d) C/N 4.0 pH 11.0*

fied in the EPSs of each treatment broth (Fig. 6), namely, tyrosine-like (region I, peak A), tryptophan-like (region II, peak B), fulvic acid-like (region III, peak C), soluble microbial by-products (SMPs) (region IV, peak D), and humic acid-like (region V, peak E).[41] The content of EPSs at pH 11.0 was significantly higher than that of pH 7.0 (Fig. 7), and their content fluctuations exhibited a pattern of initially increasing and subsequently decreasing. Tryptophan-like components and three other substances emerged as the process advanced, and remained at low concentrations. Thus, it is suggested that tyrosine-like components play a significant role in *Pseudomonas* sp. XS-18's adaptation to alkaline conditions.

The EPSs still exhibited the five types of fluorescence peaks under low C/N conditions (Fig. 7c and d), but their primary components differed from those under high C/N with SMPs component being notably more prominent. After 30 minutes of incubation, the SMPs content increased to 54.02 % of all components under neutral conditions. This indicated that the absence of a carbon source accelerated the secretion of large amounts of SMPs. *Pseudomonas* sp. XS-18 can utilize SMPs as a backup carbon source when nutrients are limited.[32] In contrast, the content of the SMPs fraction increased considerably at 240 minutes, possibly as a result of *Pseudomonas* sp. XS-18 breaking down and releasing substantial amounts of SMPs, or it could be the initial secretion of insufficient SMPs for *Pseudomonas* sp. XS-18 to sustain normal essential activities.[32] Tyrosine-like content was significantly lower at C/N 4.0 than at C/N 13.0 under alkaline conditions, indicating that low C/N leads to a reduction in tyrosine-like content. The content of SMPs initially increased and then decreased. By 360 minutes, the content of other components was similar, except for the content of quasi-fulvic acid (Fig. 7c). Further research is needed on this topic as changes in fluorescence components and the regulation of *Pseudomonas* sp. XS-18 EPSs are complicated due the effects of both high pH and low C/N.

The investigation of pure bacterial metabolites has received limited attention, despite EEM being frequently utilized to analyze the levels of fluorescent fractions in waste and soil. Su *et al.*[20] showed that the primary fluorescent substances were tyrosine-like and tryptophan-like, although variations in the fluorescence characteristics of strain EPSs were observed under different culture conditions. Additionally, tryptophan is a crucial component of PN, and tyrosinase has the ability to oxidize amino acids resembling tyrosine into PS. In this study, EPSs fractions were examined using EEM to demonstrate how the number of luminous fractions varied with respect to pH and C/N. Similar to the study of Su *et*

al.,[20] the primary component in this study was tyrosine-like at high C/N and high pH, whereas tryptophan-like was present only in trace levels. The fact that SMPs exhibited the highest fluorescence when compared to other fractions at low C/N suggests that *Pseudomonas* sp. XS-18 secretes more SMPs to store nutrients when conditions are nutrient-poor. Similar to what this study has discovered, bacteria can induce the degradation of SMPs when the carbon source is insufficient to obtain energy. The quantity of SMPs increased as the incubation time progressed. This occurred because the breakdown of cells released more SMPs.[32]

Conclusion

Pseudomonas sp. XS-18 demonstrated superior nitrate reduction capabilities and a significant potential for nitrate nitrogen recovery from wastewater, both at high pH and low C/N. At C/N 4.0 and 6.0, 31.30 % and 40.40 % of the nitrogen were retained in the cells as organic nitrogen, respectively. At pH 11.0 and C/N 4.0, it removed 54.26 % of nitrate within 360 minutes. The specific rates of change in TOC content (19.55 and 47.21 mg L^{-1} h^{-1}) were lower in pH 11.0 than in pH 7.0 conditions (21.00 and 54.97 mg L^{-1} h^{-1}), irrespective of the C/N status (4.0 and 13.0). In contrast, *Pseudomonas* sp. XS-18 secreted fewer EPSs under C/N=4.0, and the PS content decreased more rapidly with time than PN. Overall, the PS content exceeded that of PN. Furthermore, under high C/N and high pH conditions, the strain had the highest tyrosine content, while under low C/N and high pH conditions, the strain secreted more SMPs to serve as an alternative carbon source.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (No. 52270060).

References

1. *Xiong, Y., Ran, Y., Zhao, S., Zhao, H., Tian, Q.,* Remotely assessing and monitoring coastal and inland water quality in China: Progress, challenges and outlook, Crit. Rev. Environ. Sci. Technol. **50** (2020) 1266. doi: https://doi.org/10.1080/10643389.2019.1656511
2. *Tang, W., Pei, Y., Zheng, H., Zhao, Y., Shu, L., Zhang, H.,* Twenty years of China's water pollution control: Experiences and challenges, Chemosphere **295** (2022) 133875. doi: https://doi.org/10.1016/j.chemosphere.2022.133875
3. *Wang, H., Wang, T., Yang, S., Liu, X., Kou, L., Huang, T., Wen, G.,* Nitrogen removal in oligotrophic reservoir water by a mixed aerobic denitrifying consortium: Influencing factors and immobilization effects, Int. J. Environ. Res. Public Health **16** (2019) 583. doi: https://doi.org/10.3390/ijerph16040583
4. *Wang, Z., He, S., Huang, J., Zhou, W., Chen, W.,* Comparison of heterotrophic and autotrophic denitrification processes for nitrate removal from phosphorus-limited surface water, Environ. Pollut. **238** (2018) 562. doi: https://doi.org/10.1016/j.envpol.2018.03.080
5. *Yang, J. R., Wang, Y., Chen, H., Lyu, Y. K.,* Ammonium removal characteristics of an acid-resistant bacterium *Acinetobacter* sp. JR1 from pharmaceutical wastewater capable of heterotrophic nitrification-aerobic denitrification, Bioresource Technol. **274** (2019) 56. doi: https://doi.org/10.1016/j.biortech.2018.10.052
6. *Zhang, H., Yan, M., Huang, T., Huang, X., Yang, S., Li, N., Wang, N.,* Water-lifting aerator reduces algal growth in stratified drinking water reservoir: Novel insights into algal metabolic profiling and engineering applications, Environ. Pollut. **266** (2020) 115384. doi: https://doi.org/10.1016/j.envpol.2020.115384
7. *Zhang, X., Zhang, Y., Shi, P., Bi, Z., Shan, Z., Ren, L.,* The deep challenge of nitrate pollution in river water of China, Sci. Total Environ. **770** (2021) 144674. doi: https://doi.org/10.1016/j.scitotenv.2020.144674
8. *Long, D., Tang, X., Cai, K., Chen, G., Chen, L., Duan, D., Zhu, J., Chen, Y.,* Cr(VI) reduction by a potent novel alkaliphilic halotolerant strain *Pseudochrobactrum saccharolyticum* LY10, J. Hazard. Mater. **24** (2013) 256. doi: https://doi.org/10.1016/j.jhazmat.2013.04.020
9. *Zhang, Q., Sun, J., Liu, J., Huang, G., Lu, C., Zhang, Y.,* Driving mechanism and sources of groundwater nitrate contamination in the rapidly urbanized region of south China, J. Community Health **182** (2015) 221. doi: https://doi.org/10.1016/j.jconhyd.2015.09.009
10. *Zhou, W., Liu, X., Dong, X., Wang, Z., Yuan, Y., Wang, H., He, S.,* Sulfur-based autotrophic denitrification from the micro-polluted water, J. Environ. Sci. **44** (2016) 180. doi: https://doi.org/10.1016/j.jes.2016.01.002
11. *Zhang, Z., Yu, Z., Dong, J., Wang, Z., Ma, K., Xu, X., Alvarezc, P., Zhu, L.,* Stability of aerobic granular sludge under condition of low influent C/N ratio: Correlation of sludge property and functional microorganism, Bioresource Technol. **270** (2018) 391. doi: https://doi.org/10.1016/j.biortech.2018.09.045
12. *Hao, L., Liss, S., Liao, B.,* Influence of COD:N ratio on sludge properties and their role in membrane fouling of a submerged membrane bioreactor, Water Res. **89** (2016) 132. doi: https://doi.org/10.1016/j.watres.2015.11.052
13. *Liu, Y., Fang, H. H. P.,* Performance of aerobic granular sludge in a sequencing batch bioreactor for slaughterhouse wastewater treatment, Bioresource Technol. **190** (2015) 487. doi: https://doi.org/10.1080/10643380390814479
14. *Zhang, Q., Ji, F., Xu, X.,* Optimization of nitrate removal from wastewater with a low C/N ratio using solid-phase denitrification, Environ. Sci. Pollut. R. **23** (2016) 698. doi: https://doi.org/10.1016/j.jconhyd.2015.09.009
15. *Samarpita, R., Nirakar, P., Yong, N. H., Stefan, W.,* Denitrification kinetics indicates nitrous oxide uptake is unaffected by electron competition in Accumulibacter (preprint), Bioengineering Water Res. **2020** (2020) 189. doi: https://doi.org/10.1101/2020.05.14.092429
16. *Gao, L., Han, F., Zhang, X., Liu, B., Fan, D., Sun, X., Zhang, Y., Yan, L., Wei, D.,* Simultaneous nitrate and dissolved organic matter removal from wastewater treatment plant effluent in a solid-phase denitrification biofilm reactor, Bioresource Technol. **314** (2020) 123714. doi: https://doi.org/10.1016/j.biortech.2020.123714

17. *Zhang, H., Zhao, Z., Kang, P., Wang, Y., Feng, J., Jia, J., Zhang, Z.,* Biological nitrogen removal and metabolic characteristics of a novel aerobic denitrifying fungus *Hanseniaspora uvarum* strain KPL108, Bioresource Technol. **267** (2018) 569.
doi: https://doi.org/10.1016/j.biortech.2018.07.073
18. *Zheng, Z., Zhang, D., Li, W., Qin, W., Huang, X., Lv, L.,* Substrates removal and growth kinetic characteristics of a heterotrophic nitrifying-aerobic denitrifying bacterium, *Acinetobacter harbinensis* HITLi7T at 2 °C, Bioresource Technol. **259** (2018) 286.
doi: https://doi.org/10.1016/j.biortech.2018.03.065
19. *Xu, N., Liao, M., Liang, Y., Guo, J., Zhang, Y., Xie, X., Fan, Q., Zhu, Y.,* Biological nitrogen removal capability and pathways analysis of a novel low C/N ratio heterotrophic nitrifying and aerobic denitrifying bacterium (*Bacillus thuringiensis* strain WXN-23), Environ. Res. **195** (2021) 110797.
doi: https://doi.org/10.1016/j.envres.2021.110797
20. *Su, J. F., Yang, S., Huang, T. L., Li, M., Liu, J. R., Yao, Y. X.,* Enhancement of the denitrification in low C/N condition and its mechanism by a novel isolated *Comamonas* sp. YSF15, Environ. Pollut. **256** (2020) 113294.
doi: https://doi.org/10.1016/j.envpol.2019.113294
21. *Liang, D., Hu, Y., Cheng, J., Chen, Y.,* Simultaneous sulfamethoxazole biodegradation and nitrogen conversion in low C/N ratio pharmaceutical wastewater by *Achromobacter* sp. JL9, Sci. Total Environ. **703** (2020) 135586.
doi: https://doi.org/10.1016/j.scitotenv.2019.135586
22. *Cooper, R. N., Houghton, J. T., McCarthy, J. J., Metz, B.,* Climate change 2001: The scientific basis, Foreign Affairs **81** (2002) 208.
doi: https://doi.org/10.2307/20033020
23. *Wang, S., Pi, Y., Jiang, Y., Pan, H., Wang, X., Wang, X., Zhou, J., Zhu, G.,* Nitrate reduction in the reed rhizosphere of a riparian zone: From functional genes to activity and contribution, Environ. Res. **180** (2020) 108867.
doi: https://doi.org/10.1016/j.envres.2019.108867
24. *Huang, X., Jiang, D., Ni, J., Xie, D., Li, Z.,* Removal of ammonium and nitrate by the hypothermia bacterium *Pseudomonas putida* Y-9 mainly through assimilation, Environ. Technol. Innov. **22** (2021) 101458.
doi: https://doi.org/10.1016/j.eti.2021.101458
25. *Li, C., Yang, J., Wang, X., Wang, E., Li, B., He, R., Yuan, H.,* Removal of nitrogen by heterotrophic nitrification–aerobic denitrification of a phosphate accumulating bacterium *Pseudomonas stutzeri* YG-24, Bioresource Technol. **182** (2015) 18.
doi: https://doi.org/10.1016/j.biortech.2015.01.100
26. *Maqbool, T., Cho, J., Hur, J.,* Importance of nutrient availability for soluble microbial products formation during a famine period of activated sludge: Evidence from multiple analyses, J. Environ. Sci. **84** (2019) 112.
doi: https://doi.org/10.1016/j.jes.2019.04.021
27. *Yan, L., Wang, C., Jiang, J., Liu, S., Zheng, Y., Yang, M., Zhang, Y.,* Nitrate removal by alkali-resistant *Pseudomonas* sp. XS-18 under aerobic conditions: Performance and mechanism, Bioresource Technol. **344** (2022) 126175.
doi: https://doi.org/10.1016/j.biortech.2021.126175
28. *Guo, L. J., Zhao, B., An, Q., Tian, M.,* Characteristics of a novel aerobic denitrifying bacterium, *Enterobacter cloacae* strain HNR, Appl. Biochem. Biotechnol. **178** (2016) 947.
doi: https://doi.org/10.1007/s12010-015-1920-8
29. *Tan, Z., Chen, J., Liu, Y., Chen, L., Xu, Y., Zou, Y., Li, Y., Gong, B.,* The survival and removal mechanism of *Sphingobacterium changzhouense* TC931 under tetracycline stress and its' ecological safety after application, Bioresource Technol. **333** (2021) 125067. S
doi: https://doi.org/10.1016/j.biortech.2021.125067
30. *Yang, M., Yin, M., Zheng, Y., Jiang, J., Wang, C., Liu, S., Yan, L.,* Performance and mechanism of tetracycline removal by the aerobic nitrate-reducing strain *Pseudomonas* sp. XS-18 with auto-aggregation, Bioresource Technol. **359** (2022) 127442.
doi: https://doi.org/10.1016/j.biortech.2022.127442
31. *Yan, L., Liu, C., Zhang, Y., Liu, S., Zhang, Y.,* Effects of C/N ratio variation in swine biogas slurry on soil dissolved organic matter: Content and fluorescence characteristics, Ecotox. Environ. Safe. **209** (2021) 111804. z
doi: https://doi.org/10.1016/j.ecoenv.2020.111804
32. *Zhang, S., Ali, A., Su, J., Huang, T., Zheng, Z., Wang, Y., Li, M.,* Lower C/N ratio induces prior utilization of soluble microbial products with more dramatic variability and higher biodegradability in denitrification by strain YSF15, Bioresource Technol. **335** (2021) 125281.
doi: https://doi.org/10.1016/j.biortech.2021.125281
33. *Yang, L., Ren, Y. X., Liang, X., Zhao, S. Q., Wang, J., Xia, Z. H.,* Nitrogen removal characteristics of a heterotrophic nitrifier *Acinetobacter junii* YB and its potential application for the treatment of high-strength nitrogenous wastewater, Bioresour. Technol. **193** (2015) 227.
doi: https://doi.org/10.1016/j.biortech.2015.05.075
34. *Hu, B., Wang, T., Ye, J., Zhao, J., Yang, L., Wu, P., Duan, J., Ye, G.,* Effects of carbon sources and operation modes on the performances of aerobic denitrification process and its microbial community shifts, J. Environ. Manage. **239** (2019) 299.
doi: https://doi.org/10.1016/j.jenvman.2019.03.063
35. *Xia, L., Li, X., Fan, W., Wang, J.,* Heterotrophic nitrification and aerobic denitrification by a novel *Acinetobacter* sp. ND7 isolated from municipal activated sludge, Bioresource Technol. **301** (2020) 122749.
doi: https://doi.org/10.1016/j.biortech.2020.122749
36. *Chen, M., Wang, W., Feng, Y., Zhu, X., Zhou, H., Tan, Z., Li, X.,* Impact resistance of different factors on ammonia removal by heterotrophic nitrification–aerobic denitrification bacterium *Aeromonas* sp. HN-02, Bioresource Technol. **167** (2014) 456.
doi: https://doi.org/10.1016/j.biortech.2014.06.001
37. *Hayatsu, M., Tago, K., Uchiyama, I., Toyoda, A., Wang, Y., Shimomura, Y., Okubo, T., Kurisu, F., Hirono, Y., Nonaka, K., Akiyama, H., Itoh, T., Takami, H.,* An acid-tolerant ammonia-oxidizing γ-proteobacterium from soil, ISME J. **11** (2017) 1130.
doi: https://doi.org/10.1038/ismej.2016.191
38. *Jain, R. M., Mody, K. H., Keshri, J., Jha, B.,* Biological neutralization and biosorption of dyes of alkaline textile industry wastewater, Mar. Pollut. Bull. **84** (2014) 83.
doi: https://doi.org/10.1016/j.marpolbul.2014.05.033
39. *Sheng, G. P., Yu, H. Q., Li, X. Y.,* Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review, Biotechnol. Adv. **28** (2010) 882.
doi: https://doi.org/10.1016/j.biotechadv.2010.08.001
40. *Lu, H., Wang, Z., Xu, C., Li, L., Yang, C.,* Multiomics analysis provides insights into alkali stress tolerance of sunflower (*Helianthus annuus* L.), Plant Physiol. Biochem. **166** (2021) 66.
doi: https://doi.org/10.1016/j.plaphy.2021.05.032
41. *Xi, M., Zi, Y., Wang, Q., Wang, S., Cui, G., Kong, F.,* Assessment of the content, structure, and source of soil dissolved organic matter in the coastal wetlands of Jiaozhou Bay, China, Phys. Chem. Earth, Parts A/B/C **103** (2018) 35.
doi: https://doi.org/10.1016/j.pce.2017.03.004