Effect of adding microorganism and carbon source to substrate on nitrogen removal treating the drainage of WWTP

Han Wei, Li Ting-mei, Cheng Lu-lu, Liu Lu, Yu Lu-ji and Peng Zhao-xu

ABSTRACT

Developing substrate with carbon release properties is helpful to enhance nitrogen removal in low C/N ratio wastewater treatment. In this study, substrates with and without adding carbon source and microorganism were prepared to treat the drainage effluent from a wastewater treatment plant (WWTP), the difference in nitrogen removal were investigated. The results showed that adding a carbon source and microorganism to substrates could not only increase the amount of chemical oxygen demand (COD) released, but also enhance the adsorption capacities of NH$_4^+$-N and NO$_3^-$-N. The carbon release process followed the first-order kinetic equation. A nitrogen removal model consisting of four phases of diffusion, adsorption, assimilation and transformation was proposed. In the short term, nitrogen was mainly removed by adsorption; adding microorganism contributed to enhance nitrification and denitrification. In the long-term, nitrogen removal performances were similar whether microorganism was added or not, and microbial species on the surface of substrates were similar. This work suggested when using substrate to treat wastewater for nitrogen removal, preparing a substrate with excellent property for biofilm formation was the most important factor.

Key words | adsorption, carbon release, nitrogen removal, slow-release carbon source, substrate

NOMENCLATURE

C the solute concentration, mg/L, Equation (5); below Equation (6)
k the constant of carbon release rate, Equations (1)–(3)
k$_a$ the adsorption rate constant, g/(mg·min), Equation (4)
K$_F$ Freundlich constant, Equation (6)
K$_L$ the Langmuir constant of adsorption equilibrium, L/mg, Equation (5)
M$_\infty$ the ultimate amount of released carbon, mg/L, Equations (1)–(3)
M$_t$ the amount of released carbon at t, mg/L, Equations (1)–(3)
N the carbon release index, Equation (3)
n intensity factor, Equation (6)
q the adsorbed amount at t, mg/g, Equations (4)–(6)
q$_m$ the maximum adsorption capacity, mg/g, Equations (4) and (5)
t time, Equation (1); below Equations (2)–(4)

INDEX

A$^2$O anaerobic-anoxic-oxic
AS activated sludge
CC corn cob
COD chemical oxygen demand
CWs constructed wetlands
DFs denitrification filters
MLSS mixed liquor suspended solids
PCL polycaprolactone
PHBV 3-hydroxybutyrate-co-3-hydroxyvalerate
PLA polyacetic acid
PVA polyvinyl alcohol
R$^2$ correlation coefficients
SA sodium alginate
SEM scanning electron microscope
WWTP wastewater treatment plant

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INTRODUCTION

The effluent from wastewater treatment plants (WWTPs) contains many nitrogenous substances such as ammonia and nitrate, which can easily cause eutrophication. So further treatment for nitrogen removal is necessary before discharge, such as through constructed wetlands (CWs) or denitrification filters (DFs). For the effluent from WWTPs, total nitrogen (TN) is mainly composed of nitrate (Pan et al. 2016). Substrates, also known as media, are one of the major components in CWs and DFs, where biofilm is formed to degrade pollutants (Yang et al. 2018). Traditionally, the main pathway to reduce nitrate is denitrification, where denitrifying bacteria use a carbon source as an electron donor to reduce nitrate. However, the carbon source is usually deficient. So exploiting substrate with excellent properties of carbon release and biofilm formation is one of the key technical issues for nitrate removal.

Solid carbon sources are traditionally classified into natural material (such as corn cob (CC) and peanut shell) and artificial products (such as polyvinyl alcohol (PVA) and polyacrylate (PCL)) (Li et al. 2017). In practical application, they are often mixed with gravel, zeolite and sponge iron to improve strength and surface area. Wu used polyacetic acid (PLA) and 3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) to treat the effluent from wastewater treatment plants (WWTPs), TN reduced from over 10 mg/L to less than 0.15 mg/L (Wu et al. 2012). Bai added calcium carbonate into PVA and boric acid, which significantly improved mass transfer (Bai et al. 2010). In addition to solid carbon source, biofilms formed on substrate are also important for denitrification. Li used wood chips, slag and gravel to treat high concentration nitrogen wastewater. Rich microorganisms were found at the surface of the substrate, and the removal rate of TN reached 57.4–86% (Li et al. 2017). To increase microbial biomass, immobilization was often used to prepare the substrate (Qiao et al. 2010). However, microbial activity was difficult to guarantee. Creating sufficient space at micro level is an effective way to maintain activity; Kuma found that when PVA was crosslinked with sodium alginate (SA), the high-porosity carrier had good biological compatibility (Kumar et al. 2016).

From above, it can be seen that preparing a substrate with large porosity, high microbial activity and good carbon release helps to improve nitrogen removal in wastewater treatment. However, the extent of improvement from adding a porous carbon source and microorganism to substrate was still unclear. This study used PVA and SA as carriers, CC as the porous carbon source, and activated sludge (AS) as the microorganism to prepare the substrates. Through comparing the performances of nitrogen removal, adsorption and biofilm formation between each substrate, the nitrogen removal mechanism was investigated. It is also hoped to provide theoretical guidance and technical reference for actual application.

MATERIALS AND METHODS

Preparation of substrates

PVA (polymerization degree 1799, alcoholysis degree >99%) and SA (viscosity of 1.0% aqueous solution at 20 °C, 80–120 mPa-s) were purchased from Shenzhen Huixin Plastic Chemical Co. Ltd (China). CC was purchased from Puyang Jianshan Commercial Trading Company (China) with a particle size of 60–80 mesh, it was dried at 50 °C for 4 h and ground into granules with a particle size of 2–4 mm. AS was collected from the anoxic tank of an A2O WWTP in Zhengzhou (China) with an initial concentration of 2,800 mg/L, and was centrifuged at 200 r/min for 1 min.

The substrates were prepared as follows: 24 g PVA and 3 g SA were mixed in deionized water and diluted to 300 mL. The suspension was reacted at 95 °C for 3 h, and 0 g and 3 g CC were added to prepare mother liquor I and mother liquor II, respectively. For mother liquor I, 0 mL and 20 mL concentrated AS were added, respectively. After cooling to 25 °C, the mixture was extruded with an injector (inner diameter 270 μm) into a mixed aqueous solution of saturated H3BO3 solution and 4% CaCl2, and cross-linking reacted at 4 °C for 8 h. Finally, they were put into a solution of 0.8 mol/L Na2SO4 for 16 h cross-linking reaction to prepare the substrates of PVA-SA and PVA-SA-AS. For mother liquor II, the same operational procedure was carried out to prepare the substrates of PVA-SA-CC and PVA-SA-CC-AS.

Carbon release experiment

Each substrate weighed 2 g and was added to Erlenmeyer flasks, respectively, followed by the addition of deionized water (100 mL), and stirred (60 rpm) at 25 °C for 14 d. Samples for the concentration of chemical oxygen demand (COD) measurement were taken at the end of each day. After each sampling, the original solution was discharged and 100 mL of fresh deionized water was added. The
carbon release process was fitted by the kinetic equations of zero-order, first-order and Ritger-Peppas, respectively, as follows.

**Zero-order release equation:**

\[
\frac{M_t}{M_\infty} = kt
\]  

**First-order release equation:**

\[
\ln\left(1 - \frac{M_t}{M_\infty}\right) = -kt
\]  

**Ritger-Peppas (R-P) release equation:**

\[
\frac{M_t}{M_\infty} = kt^N
\]

where \(M_t\) is the amount of COD released at \(t\), mg/L; \(M_\infty\) is the ultimate amount of COD released (when daily COD released is less than 2 mg/L, the release process is considered to have finished), mg/L; \(k\) is the constant of carbon release rate; and \(N\) is the carbon release index (Ritger & Peppas 1987).

**Nitrogen removal experiment**

Synthetic wastewater was prepared for the adsorption experiment and nitrogen removal experiment, its composition was 0.0236 g (NH₄)₂SO₄, 0.003 g FeSO₄·7H₂O, 0.003 g MgSO₄·7H₂O, 0.025 g K₂HPO₄, 0.36 g NaHCO₃, 0.03 g NaCl, 0.047 g C₆H₁₂O₆ and 0.0361 g KNO₃ per litre, the correlative water quality is shown in Table 1. Each substrate weighed 10 g and was added into an Erlenmeyer flask, followed by the addition of 100 mL synthetic wastewater to react at 25°C for 28 days. Samples were taken at the end of each day. After each sampling, the original solution was discharged and 100 mL of fresh synthetic wastewater was added.

**Adsorption experiment**

Each substrate weighed 10 g and was added to Erlenmeyer flasks, followed by the addition of synthetic waste water (100 mL) and immersed at 25°C for 24 h. The pseudo-second order model was used to illustrate the adsorption process, which can be expressed as follows.

**Pseudo-second order equation:**

\[
\frac{t}{q} = \frac{1}{(k_bq_m^2)} + \frac{t}{q}
\]  

The adsorption isotherms were investigated by the Langmuir and Freundlich models.

**Langmuir adsorption equation:**

\[
q = q_mK_LC/(1 + K_LC)
\]  

**Freundlich adsorption equation:**

\[
q = K_FC^{1/n}
\]

where \(q\) is the adsorbed amount at \(t\), mg/g; \(q_m\) is the maximum adsorption capacity, mg/g; \(k_b\) is the adsorption rate constant, g/(mg·min); \(C\) is the solute concentration, mg/L; \(K_L\) is the Langmuir constant of adsorption equilibrium, L/mg; \(K_F\) and \(n\) are the Freundlich constant and intensity factor, respectively.

**Monitor and analysis methods**

COD was measured by a water quality analyzer (Model DR/2500 Laboratory Spectrophotometer, Hach Co., USA). NH₄⁺-N, NO₂⁻-N, NO₃⁻-N, and TN were determined by national standard method (Gilcreas 1996). The morphology of substrates was observed through scanning electron microscope (SEM) (Model ULTRA 55, Caise Co., Germany). Fourier transform infrared (FTIR) spectra were collected using a FTIR spectrometer (Model FTIR-650, Tianjin Gangdong Co., China). Each FTIR spectrum was recorded as an average of 40 scans in the range of 370–4,000 cm⁻¹, samples before and after the adsorption experiment were scanned at a rate of 16 scans/min. DO and pH were monitored by WTW 340i dissolved oxygen meter. Pyrosequencing analysis was carried out to investigate microbial species and abundance by the Bio-engineering Co., Ltd (Shanghai). Microbial samples were taken from initial AS and four substrates after the nitrogen removal experiment, which were numbered as Y₀,Y₁,Y₂,Y₃ and Y₄. The genome was

**Table 1** | Water quality of synthetic wastewater

<table>
<thead>
<tr>
<th>Water quality</th>
<th>COD</th>
<th>BOD₅</th>
<th>NH₄⁺-N</th>
<th>NO₂⁻-N</th>
<th>NO₃⁻-N</th>
<th>PO₄³⁻-P</th>
<th>Alkalinity (CaCO₃)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (mg·L⁻¹)</td>
<td>50.00</td>
<td>33.00</td>
<td>5.00</td>
<td>5.00</td>
<td>4.45</td>
<td>380</td>
<td></td>
</tr>
</tbody>
</table>
extracted by the OMEGA soil extraction kit (E. Z. N. A. Soil DNA Kit). Primers used for PCR were 341F (CCCTACAC GACGCTCTTCCGATCTG (barcode) CCTACGGGNGGCW GCAG) and 805R (GACTGGAGTTCCTTGGCACCCGAAATTCCAG ACTACHVGGGTATCTA ATCC). The platform was MiSeq.

RESULTS

Carbon release performance

Each substrate exhibited the same release behavior, with an initial fast stage and later slow stage (shown in Figure 1). The cumulative released COD of PVA-SA, PVA-SA-CC, PVA-SA-AS and PVA-SA-CC-AS was 9.87, 12.04, 11.49 and 13.12 mg/g, respectively. With the addition of CC and AS, the amount of COD released increased by 21.99% and 16.41%, respectively. The carbon release process was fitted by kinetic Equations (1)–(3), the results are shown in Table 2. The equation with the maximum correlation coefficients (R²) was selected as the release equation. It was found for each substrate the first-order release equation was basically followed (R² > 0.99), which indicated the release rate was proportional to the mass of substrate.

Adsorption performance

The adsorption of NH₄⁺-N and NO₃⁻-N by each substrate followed the pseudo-second-order kinetic equation quite well (shown in Table 3). The addition of CC could increase the maximum adsorption capacity of NH₄⁺-N and NO₃⁻-N by 17.20% and 8.23%, respectively. This was because not only did CC have a larger specific surface area (Gilcreas 1996), but also the chemical bonds of C-C, Si-O, C-O, C-H and O-H could provide positions for adsorption (Liang et al. 2009; Zhang & Ye 2014) (shown in Figure 2). It can be seen that after the adsorption experiment, the absorption of the above chemical bonds all decreased, indicating that these positions were occupied by adsorbates. While adding AS could improve adsorption capacity even more, the adsorption of AS was so excellent that the ‘adsorption-regeneration process’ was explored to treat wastewater (Wu et al. 2014). However, when CC and AS were added together, they occupied each other’s surfaces and pores, and adsorption capacity remained almost unchanged. The corresponding values of Langmuir and Freundlich equations were calculated (shown in Table 3). It was observed that the Freundlich equation better described the adsorption processes, with a higher R², which indicated the adsorbed NH₄⁺-N and NO₃⁻-N formed multilayer coverage on the surface of the substrates.

Nitrogen removal performance

The TN in synthetic wastewater was composed of ammonia and nitrate. Each day after adding synthetic wastewater, the mixture changed from an aerobic condition to an anoxic condition with DO decreasing from 7–8 mg/L to below 0.5 mg/L. The addition of CC and AS could both improve the removal of NH₄⁺-N and NO₃⁻-N. The average effluent

Table 2 | Fitting results of carbon release process

<table>
<thead>
<tr>
<th>No.</th>
<th>Zero-order</th>
<th>First order</th>
<th>Ritger-Peppas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fitting equation</td>
<td>R²</td>
<td>Fitting equation</td>
</tr>
<tr>
<td>PVA-SA</td>
<td>y = 0.0012x + 0.6945</td>
<td>0.8426</td>
<td>y = -0.0104x – 0.8148</td>
</tr>
<tr>
<td>PVA-SA-CC</td>
<td>y = 0.0014x + 0.6416</td>
<td>0.8674</td>
<td>y = -0.0105x – 0.6187</td>
</tr>
<tr>
<td>PVA-SA-AS</td>
<td>y = 0.0013x + 0.6375</td>
<td>0.8818</td>
<td>y = -0.0096x – 0.6546</td>
</tr>
<tr>
<td>PVA-SA-CC-AS</td>
<td>y = 0.0014x + 0.6201</td>
<td>0.8737</td>
<td>y = -0.0092x – 0.6357</td>
</tr>
</tbody>
</table>

Figure 1 | COD release curve of different water purification bases.
concentrations of NH₄⁺-N and NO₃⁻-N treated by PVA-SA, PVA-SA-CC, PVA-SA-AS and PVA-SA-CC-AS were 2.52, 2.02, 1.88, 1.65 (shown in Figure 3), and 2.68, 0.33, 0.82 and 0.02 mg/L (shown in Figure 4), respectively. When they were added together, the best removal performance could be reached. It should be noted that when treating with PVA-SA and PVA-SA-AS, obvious NO₂⁻-N was found in the effluent (1–2 mg/L), while for substrates with CC added, little NO₂⁻-N appeared. This was because CC could not only increase the amount of released carbon, but also helped the released carbon source to be transported through the porous structure.

**Mechanism of nitrogen removal**

According to the nitrogen removal performance, various approaches such as adsorption, nitrification, denitrification and assimilation happened. Based on this phenomenon, a nitrogen removal model was proposed. In this model, nitrogen removal process consisted of four phases (shown in Figure 5). (1) Diffusion phase: organic matter, NH₄⁺-N and NO₃⁻-N diffuses from solution to substrate, while in

![Figure 2](https://iwaponline.com/wst/article-pdf/79/10/1947/591411/wst079101947.pdf)

**Figure 2** | FTIR spectra of the CC powder surface.

![Figure 3](https://iwaponline.com/wst/article-pdf/79/10/1947/591411/wst079101947.pdf)

**Figure 3** | NH₄⁺-N removal performances.

![Figure 4](https://iwaponline.com/wst/article-pdf/79/10/1947/591411/wst079101947.pdf)

**Figure 4** | NO₃⁻-N removal performances.

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**Table 3 | Fitting results of adsorption kinetics and thermodynamics**

<table>
<thead>
<tr>
<th>No.</th>
<th>Pseudo-second-order</th>
<th></th>
<th>Langmuir adsorption</th>
<th>Freundlich adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kₕ g·mg⁻¹·min⁻¹</td>
<td>qᵣ mg·g⁻¹</td>
<td>R²</td>
<td>Kₐ L·mg⁻¹</td>
</tr>
<tr>
<td>PVA-SA NH₄⁺-N</td>
<td>0.1156</td>
<td>0.0343</td>
<td>0.9452</td>
<td>136.5</td>
</tr>
<tr>
<td>PVA-SA NO₃⁻-N</td>
<td>0.7626</td>
<td>0.0474</td>
<td>0.997</td>
<td>47.16</td>
</tr>
<tr>
<td>PVA-SA-CC NH₄⁺-N</td>
<td>0.1158</td>
<td>0.0402</td>
<td>0.9421</td>
<td>282.5</td>
</tr>
<tr>
<td>PVA-SA-CC NO₃⁻-N</td>
<td>0.1604</td>
<td>0.0513</td>
<td>0.9823</td>
<td>*</td>
</tr>
<tr>
<td>PVA-SA-AS NH₄⁺-N</td>
<td>0.1077</td>
<td>0.0444</td>
<td>0.9732</td>
<td>99.59</td>
</tr>
<tr>
<td>PVA-SA-AS NO₃⁻-N</td>
<td>0.0203</td>
<td>0.0683</td>
<td>0.9574</td>
<td>193.00</td>
</tr>
<tr>
<td>PVA-SA-CC-AS NH₄⁺-N</td>
<td>0.1575</td>
<td>0.0353</td>
<td>0.9376</td>
<td>242.47</td>
</tr>
<tr>
<td>PVA-SA-CC-AS NO₃⁻-N</td>
<td>0.3925</td>
<td>0.0485</td>
<td>0.9911</td>
<td>*</td>
</tr>
</tbody>
</table>

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**Note:** * denotes that the parameter is fixed in the fitting process.
Figure 5 | Model of nitrogen removal by substrate in wastewater treatment. (a) Diffusion phase; (b) adsorption phase; (c) assimilation phase; (d) transformation phase.
substrate the released organic matter diffuses from the inside to the surface; (2) adsorption phase: organic matter, NH$_4^+$-N and NO$_3^-$-N are adsorbed on the surface of the substrate; (3) assimilation phase: microorganisms use the adsorbed substances to form biofilm; (4) transformation phase: the fresh biofilm nitrifies NH$_4^+$-N and denitrifies NO$_3^-$-N, the aged biofilm falls from the substrate.

Discussion

According to the model, various pathways contributed to nitrogen removal. In the first 5 days, adsorption was the dominant pathway, few microorganisms were observed on the surface of the substrate (shown in Figure 5(a) and 5(b)). With the growth of biofilm (shown in Figure 5(c)), denitrification occurred. Synthetic wastewater only contained inorganic nitrogen, and microbial assimilation converted adsorbed inorganic nitrogen into organic nitrogen (Hao et al. 2014). When the aged biofilm fell from the substrates (shown in Figure 5(d)), organic nitrogen appeared in the effluent. For substrates with CC addition, organic nitrogen was observed from the 6th day. While for substrates without CC addition, organic nitrogen was not observed until the 10th day (shown in Figure 6). The addition of CC increased the ratio of organic nitrogen to TN in the effluent. The average ratios of PVA-SA, PVA-SA-CC, PVA-SA-AS and PVA-SA-CC-AS were 58.97%, 72.16%, 47.47% and 66.79%, respectively. This indicated the porous structure of CC contributed to biofilm formation, which well demonstrated the proposed model.

The addition of AS could significantly enhance the denitrification process, especially in the first 3 days (shown in Figure 7). Sometimes the denitrified NO$_3^-$-N was even greater than 5 mg/L. While for substrates without AS addition, the denitrification process also improved gradually. By the 21th day, the denitrifying capacity of all substrates was almost the same. Pyrosequencing analysis was conducted to analyze the microbial community structure. The Shannon index, evaluating the diversity of a microbial community, obtained a value of 7.87, 6.11, 6.84, 7.18 and 7.34 in Y0, Y1, Y2, Y3 and Y4, respectively. This indicated the microbial community diversity on the surface of PVA-SA was lowest. The main genera, with relative abundances greater than 0.3%, are shown in Figure 8. More genera were discovered in Y0, which was consistent with the higher Shannon index. In each sample, *Pseudomonas* was the dominant genus, which was followed by *Blastocatella*. *Blastocatella* was commonly found in biofilms (Wang et al. 2018), its abundance indicated biofilm was well formed on the substrates. *Pseudomonas* contained denitrifying bacteria (Feng et al. 2018), its abundance indicated that biofilm had good denitrification capacity. It should be noted that Y0 showed good nitrification capacity with a relative abundance of *Nitrospira* as high as 14.3% (Ushiki et al. 2011). However, organic matter released from substrate inhibited the nitrification process, and the relative abundance of *Nitrospira* gradually dropped below 1.00%. Microbial species of different substrates tended to be the same, which was consistent with nitrogen removal performance.

According to the above discussion, adding a porous carbon source could increase the amount of released carbon, and significant carbon release occurred within 14 days (350 h). However, the amount of released carbon in the initial 24 h was so large that it was likely to cause secondary pollution, which should be avoided in practical applications.
application. Adding microorganisms could contribute to nitrogen removal in the short term, while preparing substrate with excellent property of biofilm formation was more important.

**CONCLUSION**

The effect on nitrogen removal of adding a carbon source and microorganism to substrate was investigated. It was found that multiple approaches such as adsorption, nitrification, denitrification and assimilation contribute to nitrogen removal. The adsorption process followed the Freundlich equation; adding CC and AS both significantly increased the adsorption of NH4⁺-N and NO3⁻-N. In the short term, adding AS could provide rich Nitrospira and Pseudomonas to nitrify and denitrify, while in the long term microbial species on different substrates were similar, and nitrogen removal performances between each substrate were almost the same.

**ACKNOWLEDGEMENTS**

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