

Review

Plant Carbon Sources for Denitrification Enhancement and Its Mechanism in Constructed Wetlands: A Review

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Abstract: Nitrogen pollution in water bodies is a serious environmental problem worldwide. Plant carbon source (PCS) enhanced denitrification in constructed wetlands (CWs) for wastewater with low chemical oxygen demand to total nitrogen (COD/N) has been one of the most exciting research topics. This paper summarized the related studies with VOSviewer software and found that the major interests were denitrification performance and mechanism in CWs. This article mainly focused on the PCSs' characteristics, denitrification rate, the influences of key environmental and operational parameters, surface morphology variation, microbial community structure, and denitrification genes. Engineering prospects and existing problems were also introduced. PCSs' degradation consumes DO and creates favorable conditions for denitrification. The COD/N of wastewater should be maintained at 4–5 by adding PCSs, thus improving denitrification performance and reducing nitrous oxide emission. Aerobic degradation, anaerobic fermentation, dissimilatory nitrate reduction to ammonium, and sulfate reduction processes may consume the carbon released by PCSs depending on the influent quality and environmental conditions. More attention should be paid to the reduction of greenhouse gases and emerging pollutants in CWs with PCSs.

Keywords: constructed wetlands; plant carbon sources; denitrification mechanism; nitrogen removal; denitrification functional genes



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1. Introduction

Constructed wetlands (CWs), as a kind of green treatment technology by simulating natural wetlands, have been widely used to treat municipal wastewater treatment effluent, agricultural runoff, groundwater, sludge supernatant, industrial wastewater, aged landfill leachate, urban stormwater, and micro-polluted water sources [1–5]. CWs have the unique advantages of low management and operational cost, eco-friendly characteristics, etc., compared to conventional activated sludge systems, membrane bioreactors, etc. [6–8].

CWs could effectively remove organic matter, nitrogen, phosphorous, etc., through interactions among plants, media, wastewater, and microorganisms [1]. Wastewater with residual nitrogen is one of the main pollution sources for waterbody eutrophication and threats to water sources and human health [9,10]. Nitrogen removal carried out in CWs is mainly through biological (microbial nitrification, denitrification, plant absorption, biomass assimilation, and dissimilatory nitrate reduction), physicochemical (ammonia volatilization and absorption), and other paths (partial nitrification-denitrification, anaerobic ammonium oxidation, and simultaneous partial nitrification and anammox processes) [1]. It has been

reported that microbial denitrification is the main nitrogen removal mechanism in CWs, accounting for 87.00–96.00% of the total nitrogen (TN) removal efficiency [5,11].

Heterotrophic denitrifying bacteria are the main denitrifying microorganisms; they use carbon as electron donors to reduce nitrate in anaerobic or anoxic conditions [1]. However, wastewater with low chemical oxygen demand (COD) to total nitrogen (COD/N), especially municipal wastewater treatment effluent and agricultural runoff, limited their nitrogen removal efficiency [2]. Liquid carbon sources (methanol, dissolved sodium acetate, etc.) were added to enhance nitrogen removal performance in CWs [12,13], but they had the disadvantages of high cost, difficult dosage control, and frequent effluent quality deterioration [14]. Solid carbon sources have drawn the attention of researchers due to their long carbon release duration and favorable environment for microbial biofilm attachment [2], and plant carbon sources (PCSs), including macrophytes (*calamus*, *cattail*, reed straw, *Arundo donax*, *Pontederia cordata*, etc.), agricultural wastes (corn stubble, wheat straw, etc.), and forestry residues (woodchips, saw power, etc.), have significant advantages because of their low cost and wide availability [15–22]. PCSs are mainly composed of cellulose, hemicellulose, and lignin, which can be converted into soluble and micro-molecular substrates to some extent and then utilized by denitrifying bacteria [14]. The utilization of PCSs can reduce the amount of agricultural, forestry, and wetland plant solid wastes, which conforms to the principle of sustainable development [23]. Most studies are focused on the denitrification rate influenced by plant species and different operational parameters in CWs [24,25], and the mechanism of heterotrophic denitrification enhanced by PCSs through physicochemical and molecular biological processes has also been extensively studied [15,24,25].

In this paper, bibliometric assays are used to visualize research hotspots with VOSviewer software [26]. Then, we provide an overview of the denitrification rate enhanced by PCSs and its mechanism in CWs, including the PCSs' characteristics, the denitrification performance of PCSs under different operational conditions, and the influence of environmental and operational parameters on the denitrification rate. The mechanism is reviewed by the physicochemical and molecular biological characterization research results. The limitations and future perspectives are also summarized to provide the theoretical basis for the further application of PCSs in practical CWs.

2. Bibliometric Assay Based on Keywords

The search topics were “denitrification”, “plant carbon source (PCSs)”, and “constructed wetlands (CWs)”, and their exported data were from the Web of Science core collection. Subsequently, 127 references were obtained, and manual selection was performed to ensure that the references were related to the three topics. Finally, 75 articles selected were analyzed by VOSviewer software (version 1.6.18, Leiden University, Netherlands). The software calculated according to keyword co-occurrence, and the results are shown in Figure 1.

The minimum number of occurrences was 5 in VOSviewer, and 43 keywords participated in the overlay visualization. It can be seen in Figure 1 that the most frequently occurring items (largest circles) were denitrification, CWs, nitrogen removal, nitrate removal, performance, efficiency, functional genes, carbon sources, etc. The reports related to denitrification, CWs, and PCSs were divided into five clusters. The colors of the clusters are red, green, blue, yellow, and purple. The centers of the five clusters are denitrification, nitrate removal, performance, nitrogen removal, and efficiency, respectively. It should be noticed that some terms representing PCSs, such as solid carbon sources, aquatic plants, biomass, vegetation, and macrophytes, are distributed in the five clusters, which indicates that PCSs play important roles in denitrification in CWs. The terms in the red cluster represent denitrification and nitrification in CWs, and the closely related terms are denitrification, CWs, nitrification, and nitrous oxide. The green cluster is mainly related to nitrate removal and related terms, such as carbon source and aquatic plants. The blue cluster is mainly about microbial community, solid carbon sources, and functional genes. The yellow

cluster is mainly related to nitrogen removal, and closely related terms include carbon source, plant, and biofilm. The purple cluster is mainly about degradation, efficiency, and macrophytes. These five clusters generally represent the main interests of researchers in the denitrification process enhanced by PCSs in CWs, which mainly includes denitrification performance and its mechanism using different carbon sources.

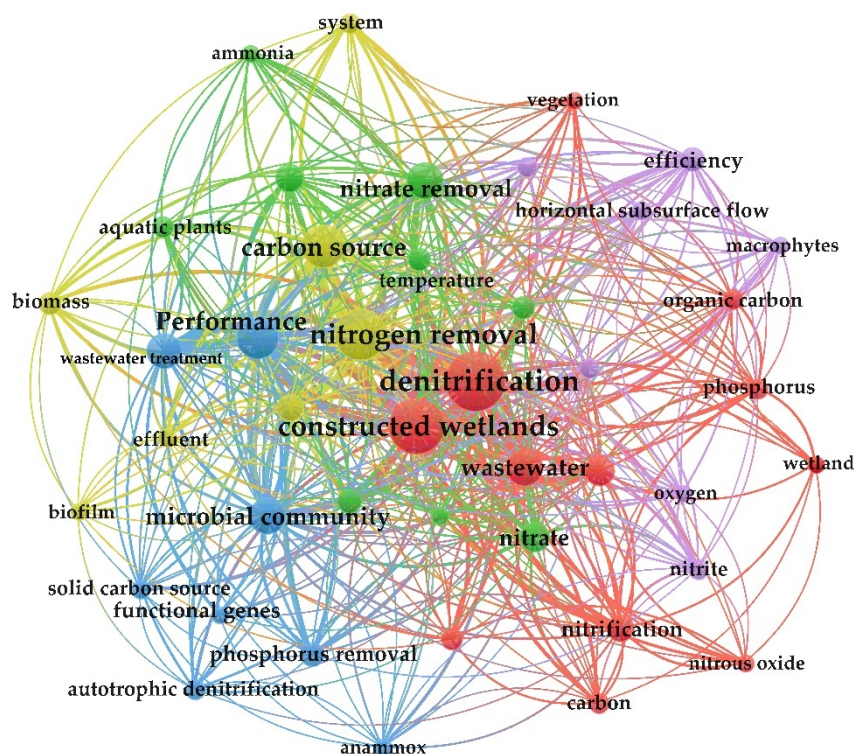


Figure 1. The bibliometric assay based on keywords generated from VOSviewer software. Note: The thickness of the lines refers to the correlation (thicker means higher correlation). The color of the lines is consistent with the color of the keywords. The similarity of the color of the keywords refers to the correlation, and high similarity means high correlation.

3. PCS Characteristics

3.1. Classification of PCSs and Available Substances Released by PCSs in CWs

PCSs for CW denitrification are mainly divided into the exogenous type and endogenous type [27]. The exogenous PCSs include adscititious plant litters, plant leachates, agricultural wastes, forestry residues, and anaerobic fermentation broths, while the endogenous PCSs include the organic carbon decomposed by plant litters during the senescent period and plant root exudates in CWs [15–21]. Figure 2 depicts the system diagram of CWs with PCS supplement. Exogenous PCSs were added to the influent or mixed with matrix to release available substance for denitrification enhancement. Endogenous PCSs, such as macrophytes, in CWs mainly play roles in the senescent period.

Lignocellulose, which is generally used to describe the main component of PCSs, is composed of cellulose, hemicellulose, lignin, other organic substances (such as protein, fat, pectin), ash, salt, and minerals [28,29]. It was reported that cellulose accounts for 38.00–50.00%, hemicellulose for 15.00–35.00%, and lignin for 15.00–25.00% of the total plant biomass [30,31]. The main components of PCSs, i.e., cellulose, hemicellulose, and lignin, strongly affect the availability of carbon sources for denitrification and then influence the denitrification rate. The minor components (such as protein, fat, amino acids, and phenols) originate from soaking and anaerobic fermentation of exogenous PCSs, and root secretions of endogenous PCSs can also be utilized as carbon sources for denitrifying microorganisms. Exogenous PCSs are generally added to CWs in a raw or pretreated (water soaking, acid

soaking, alkali soaking, or anaerobic fermentation) way. Different pretreatment of plants leads to different available carbon source components for denitrifying bacteria in CWs. Raw solid PCSs can act as biofilm carriers for denitrifying bacteria and fungi [32]. The plant litters, agricultural wastes, and forestry residues are mixed with the substrate in which are hydrolyzed by the extracellular enzymes released from microbes attached to them and then decompose into small and soluble molecular substances, such as reducing sugar, protein, and volatile fatty acids (VFAs), for the utilization of denitrifying bacteria [14]. Since the concentrations of reducing sugar, protein, and VFAs are determined by the balance between substance release and degradation of PCSs, they can hardly be analyzed quantitatively, and few quantitative research works have been reported. The main components of plant leachate are hemicellulose, total sugar, and humic acids. The total sugar and humic acid-like compounds account for 96.00–98.00% of the total organic carbon (TOC) in *Iris pseudacofurus* leachate [33]. No cellulose or lignin appear in the plant leachate for their stable molecular structures, which are difficult to degrade without promotion measures [34]. The lignocellulose can be hydrolyzed into reducing sugars, and then the monosaccharides can be converted to VFAs through anaerobic microbial metabolism, and so the available substances of anaerobic fermentation broths are mainly VFAs such as acetic and propionic acids, soluble protein, and reducing sugar [35]. VFAs are easily biodegraded by denitrifying bacteria, and acetic acid is the fastest electron donor for denitrifying bacteria [36]. The acetic acid in VFAs reaches 62.30% when anaerobic fermentation broths of *Typha latifolia* litter are used, and the calculated COD conversion factor of plant fermentation broth is about $0.25 \text{ g COD g}^{-1}$ dry mass plant litter [37].

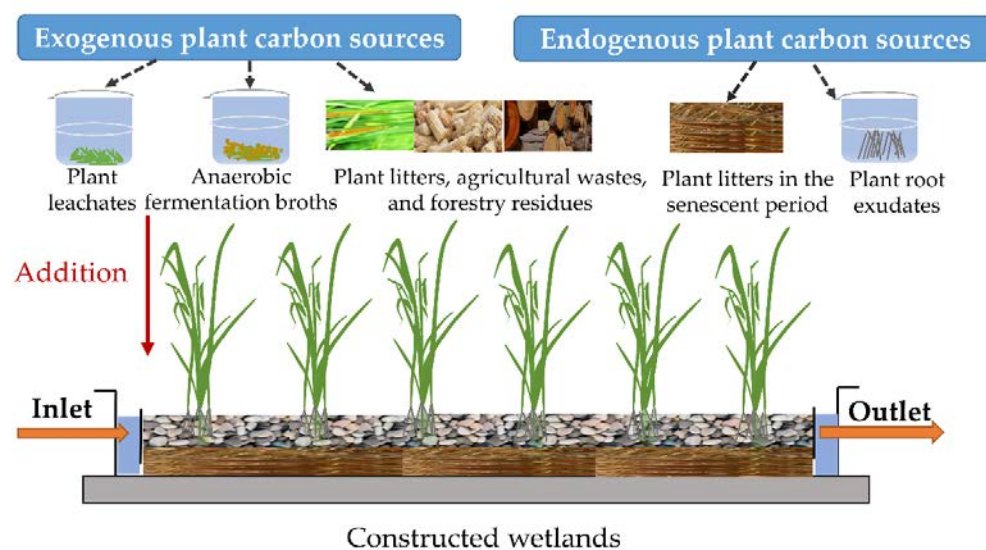


Figure 2. System diagram of CWs with PCS supplement.

Endogenous PCSs include aged plant litters and plant root exudates in CWs. The plant litters in the senescent period are similar to the exogenous solid PCSs; both of them can act as biofilm carriers and release carbon sources. Aquatic macrophyte root secretes small molecular substances such as sucrose, glucose, and low-molecular-weight organic acids [38]. A total of 5.00–25.00% of photosynthetically fixed carbon can be transferred to the rhizosphere and then released through the root, which induces the growth of specific bacterial groups and creates well-defined bacterial communities around the root rhizosphere [39]. The investigation of three aquatic plants (*Phragmites australis*, *Typha angustifolia*, and *Cyperus alternifolius*) showed that the root released an average TOC ranging from 0.48 to 0.92 mg g^{-1} root dry mass d^{-1} in summer [27].

Exogenous PCSs, such as plant leachate, plant litter, and anaerobic fermentation broths, have higher denitrification capacities than endogenous PCSs in the early stage of CWs. Exogenous solid PCSs such as plant litters, agricultural wastes, and forestry residues could

act as biofilm carriers for microorganisms which could regulate and balance the amount of available carbon sources released. Thus, the secondary pollution risk of solid PCSs is lower compared to endogenous PCSs such as aged plant litters in the senescent period, which need to be harvested regularly.

3.2. Comparison of Raw and Pretreated PCSs

The ideal PCSs should have a low lignin content, high hemicellulose and cellulose content, high carbon content, low carbon release rate, and low nitrogen and phosphorus content to reduce the risk of secondary pollution [40]. The covalent bonds between lignin and cellulose enhance the strength of the cell wall and prevent the exposure of carbohydrates to enzymatic hydrolysis [41], and the irreversible adsorption of lignin onto cellulose reduces its activity [28]. Thus, lignin removal is necessary to improve the utilization of PCSs. Lignin can be removed from the cell wall by high temperature, acid, alkalis, and oxidants, which cause the cracking of the ester and ether bonds [28]. PCSs used in CWs are usually herbaceous plants. The delignification efficiency of alkali pretreatment of herbaceous biomass is higher than that of woody biomass, due to its high lignin content and high molecular weight [42]. Table 1 illustrates the variation of carbon to nitrogen (C/N), cellulose, hemicellulose, and lignin before and after the pretreatment of PCSs, from which it can be seen that the lignin content is reduced and the C/N is increased after pretreatment. Wen et al. pretreated *cattail* litter (*Typha latifolia*) with 2% NaOH solution and found that the CWs with alkali-pretreated litter addition were more efficient than those with raw plant material in the initial stage (1–25 d) [21]. The widely used acid and alkali for PCS pretreatment are low concentration H₂SO₄ (<5%) or NaOH (1–2%) solution [21,43,44]. Anaerobic fermentation is an effective method to improve the hydrolysis of lignocellulosic materials, and the main component of anaerobic fermentation broths is VFAs, which is beneficial for denitrification [37,45].

Table 1. The main compositional variation between the raw and pretreated PCSs.

PCSs	Pretreatment Method	Raw PCSs				Pretreated PCSs				Ref
		Cellulose	Hemicellulose	Lignin	C/N	Cellulose	Hemicellulose	Lignin	C/N	
Mixed <i>Cattail</i> , canna, and rice straw	Water extracted					37.20 ^a	29.38 ^a	13.12 ^a	NA	[44]
	2% H ₂ SO ₄ extracted	34.08 ^a	26.19 ^a	15.80 ^a	NA	38.87 ^a	31.92 ^a	11.02 ^a	NA	
	5% H ₂ SO ₄ extracted					41.81 ^a	33.95 ^a	9.53 ^a	NA	
<i>Cattail</i> litter	2% NaOH extracted	29.10 ^a	11.10 ^a	12.40 ^a	60	38.01 ^a	13.43 ^a	10.04 ^a	669	[21]
<i>Cattail</i> litter (<i>Typha latifolia</i>)	Anaerobic fermentation	28.60 ^a	13.70 ^a	9.30 ^a	NA	Mainly composed of VFAs				[37]

PCSs, Plant carbon sources; C/N, carbon/nitrogen; VFAs, volatile fatty acids; ^a mean value of references; NA, no data available.

Pretreatment as dilute acid, dilute alkali, or anaerobic fermentation always reduces the content of lignin. New pretreatment methods need to be developed to further improve the lignin removal efficiency. Methods such as the utilization of lignocellulose for the production of bioethanol, biogas, organic acids, enzymes, and biological adsorbents could be referenced for PCS pretreatment [45–49], which aims at the enhancement of lignin removal efficiency. Many pretreatment methods for plant biomass, including mechanical (milling, grinding, and slicing), chemical (acid, alkali, and ionic liquid pretreatment), and biological (lignin hydrolase system such as laccase, high-redox-potential peroxidase, and oxidase) processes, have been reported in different research fields [28], which provides helpful information for the pretreatment of PCSs in CWs.

4. Enhanced Denitrification by PCSs in CWs

4.1. Denitrification Performance Enhanced by PCSs in CWs

PCSs, which act as electron donors, influence denitrification performance. The nitrate removal efficiency and denitrification rate of different PCSs in CWs under different environmental and operational conditions are listed in Table 2. The nitrate removal efficiency equals the influent and effluent nitrate concentration difference divided by the influent concentration, and the denitrification rate is the influent and effluent nitrate-N concentration difference divided by hydraulic retention time (HRT).

The denitrification rate of CWs with PCS addition is generally higher than that of CWs without PCSs. When 2.80 g L^{-1} cattail litter was added to subsurface-flow CWs (SSF CWs) for synthetic wastewater treatment, the denitrification rate reached $15.66 \text{ g N m}^{-3} \text{ d}^{-1}$, which was 2.62 times higher than that without addition [21]. Different exogenous PCSs lead to different denitrification rates of CWs. Anaerobic fermentation broths from three kinds of PCSs, i.e., *Arundo donax*, *Pontederia cordata*, and mixed *Arundo donax* and *Pontederia cordata*, were added to adjust the influent COD/N to 9, and the highest denitrification rate ($12.22 \text{ g N m}^{-3} \text{ d}^{-1}$) and nitrate removal efficiency (94.50%) were achieved in the CWs with mixed *Arundo donax* and *Pontederia cordata* broths; the denitrification rate and nitrate removal efficiency of the CWs with *Arundo donax* broth addition were $11.02 \text{ g N m}^{-3} \text{ d}^{-1}$ and 70.40%, while those of the CWs with *Pontederia cordata* broth addition were $10.87 \text{ g N m}^{-3} \text{ d}^{-1}$ and 81.00% [16]. The difference in the denitrification rates of CWs with different PCS additions mainly depends on their type and the biodegradability of their released carbon [52]. Among these PCSs, herbaceous plants, especially cattail litter, originating from CWs and with high carbon release capacities are widely used as supplementary PCSs for CWs [18,37,53].

Exogenous and endogenous PCSs result in different denitrification rates. Zhang et al. studied four continuous-flow SSF CWs for wastewater treatment plant effluent in Shanghai [37]. Influent COD/N was adjusted from 2.20 to 4.79 by the addition of anaerobic fermentation broth of *Typha latifolia*, and the denitrification rate was changed from 0.95 to $3.72 \text{ g N m}^{-3} \text{ d}^{-1}$. When 20 plants m^{-2} *Typha latifolia* was planted, the denitrification rate increased from 0.24 of the unplanted CWs to $0.33 \text{ g N m}^{-3} \text{ d}^{-1}$. The enhancement effect of root exudates of macrophytes for denitrification was usually lower than that of exogenous PCS addition. Fan et al. found that the mean TN removal efficiency (61.80%) with 0.1 g L^{-1} calamus addition in surface-flow CWs (SF CWs) was approximately triple that (20.20%) without calamus addition [15]. Zhao et al. added 2 g dried leaf and stem from *Potamogeton crispus* litter to simulate the natural environment of the senescent period [50], and the average nitrate removal efficiency increased from 41.6 to 68.6% after the PCS addition. Endogenous PCSs as root exudates can hardly meet the needs of denitrifying bacteria in CWs, and exogenous PCSs should be supplemented to achieve a higher denitrification rate.

When the carbon source was insufficient in CWs, denitrifying bacteria would provide electrons by endogenous metabolism, thus resulting in cytoplasmic reduction and ammonia production [54]. Theoretically, it takes 2.86 g COD to convert 1 g nitrate-N into nitrogen. Considering the heterotrophic yield and endogenous respiratory loss of microorganisms and the consumption of other microorganisms (aerobic heterotrophic bacteria, sulfate-reducing bacteria, methanogenic bacteria, etc.) [37,52], a higher COD/N than the theoretical one is needed for complete denitrification in CWs. Because of the different PCS bioavailability and the different influent COD/N, it is difficult to calculate the PCS dosage for nitrate-rich wastewater treatment. It has been reported that a high nitrate removal efficiency and denitrification rate can be achieved at 4–5 influent COD/N adjusted by PCS addition [37,55,56].

Table 2. Nitrate removal efficiency and denitrification rate of CWs with PCSs under different environmental and operational conditions.

PCSs	Influent	Plant Dosage (g L ⁻¹)	Influent Nitrate (mg L ⁻¹)	Influent COD/N	HRT (d)	Temperature (°C)	Nitrate Removal Efficiency (%)	Denitrification Rate (g N m ⁻³ d ⁻¹)	Note	Ref.
<i>Iris pseudacorus</i>	SIS	0.50–1.00 ^b	10 ^b	0 ^b	2 ^b	25–29 ^b	45.00–95.00 ^b	2.25–4.75 ^b	PL	[33]
Control Cattail	SIS	0 ^b 2.80 ^b	50.00 ^b	0.37 ^b	5 ^b	greenhouse	29.90–100.00 ^b 56.30–100 ^b	0.98–5.97 1.81–29.13		[21]
Microalgal	SIS	0 ^b 0.10 ^b	10 ^a	4 ^b	6 ^b	Summer	51.20 ^a 65.30–79.10 ^a	0.28 ^b 0.36–0.43 ^b	PL	[43]
<i>Arundo donax</i> <i>Pontederia cordata</i> Mixed <i>Arundo donax</i> and <i>Pontederia cordata</i>	SIS	influent COD/N = 9	10 ^a	1.5 ^b	0.83 ^b	27 ^a	70.40 ^b 81.00 ^b 94.50 ^b	10.87 ^b 11.02 ^b 12.20 ^b	AFbs	[16]
Control <i>Typha latifolia</i>	SW	NA 20 ^d 0.93–3.68 ^{b,c} 20 ^d and 0.93–3.68 ^{b,c}	15 ^b	1.22 ^b	4 ^b	25 ^a	6.60 ^b 8.60 ^b 25.70–98.70 ^b 30.50–98.70 ^b	0.24 ^b 0.33 ^b 0.95–3.72 ^b 1.17–3.74 ^b	Ps Afbs Ps and Afbs	[37]
<i>Calamus</i>	SIS	0 ^b 0.10 ^b	13.10 ^b TN	TOC/TN = 0.62–0.72 ^b	1 ^b	23.6–31.5 ^b Winter–Summer	20.20 ^b TN 27.50–61.80 ^b TN	2.65 ^b TN 3.60–8.10 ^b TN		[15]
<i>Potamogeton crispus</i>	SW	0 ^b 0.63 ^b	5.27 ^a	2.50	5 ^b	23.6 ^b	41.6% ^b 68.6% ^b	0.62 ^b 0.85 ^b		[50]
Cattail litter, leaves, and stems	SIS	0 ^b 1.00 ^b	10.00 ^b	TOC/TN = 0.5 ^b	1 ^b	19.7–26.6 ^b Winter–Summer	20.50 ^b TN 43.20 ^b TN 24.40–42.20 ^b TN	2.63 ^b TN 5.50 ^b TN 3.50–5.88 ^b TN		[18]
Corn stubble	SWB	0 ^b 6.00–8.00 ^b	18.00 ^b 21.00–22.00 ^b	NA	2.13 ^b 0.83h ^b	16.0 ^b 15.8–24.6 ^b	38.50 ^b ≥90.00 ^b	3.29 ^b 20.80–22.40 ^b	0–7d	[19]
Reed straw	SIS	0 ^b 65.71 ^b	16.40 ^a	0.80 ^b	2–4 ^b	greenhouse	14.40–26.80 ^b 62.10–87.40 ^b	0.60–2.25 ^b 3.67–5.22 ^b		[17]

Table 2. Cont.

PCSs	Influent	Plant Dosage (g L ⁻¹)	Influent Nitrate (mg L ⁻¹)	Influent COD/N	HRT (d)	Temperature (°C)	Nitrate Removal Efficiency (%)	Denitrification Rate (g N m ⁻³ d ⁻¹)	Note	Ref.
Control Wheat straw Cotton	SIS	NA	43.00 ^b	2.44–2.52 ^b	1 ^b	12.5–24.6 ^b	13.95–26.47 ^b 49.16–98.32 ^b 53.89–70.86 ^b	6.00–9.00 ^b 21.13–42.28 ^b 23.17–30.46 ^b		[24]
Woodchip	SIS	NA	47.42–99.25 ^b	1.87–0.98 ^b	2 ^b	20.0–29.0 ^b	94.50–80.00 ^b	22.41–39.70 ^b		[51]
Only aerated without PCSs Wheat straw Apricot pit Walnut shell	SIS	0 ^b Carbon:substrate = 1:1 ^b	41.79–44.24 ^b TN	0.5–5 ^b	3 ^b	25.0 ^b	3.98–32.60 ^b TN 59.20–96.90 ^b TN 19.40–46.30 ^b TN 46.30–62.90 ^b TN	0.59–4.62 ^b TN 8.72–14.28 ^b TN 2.86–6.55 ^b TN 6.55–9.27 ^b TN	High ammonia concentration	[20]
<i>Oenanthe javanica</i>	SW	NA 26 ^d	25.00 ^b TN	0.6 ^b	10 ^b	<10 ^b	8.00 ^b TN 43.00 ^b TN	0.20 ^b 1.08 ^b		[25]

COD/N, chemical oxygen demand to total nitrogen; HRT, hydraulic retention time; SIS, simulated sewage; PL, plant leachate; Afbs, anaerobic fermented broths; Ps, plants; SWB, subsurface water body; SW, secondary wastewater; NA, no data available. ^a Mean value; ^b the value inferred or calculated based on the data given in the reference; ^c COD_{add}/N; ^d plant density: Plants m⁻²; TOC/TN, total organic carbon to total nitrogen.

4.2. Secondary Pollutant Release

The utilization of plant biomass is generally divided into the process of dissolving neutral detergent solution and hydrolysis of cellulose and hemicellulose. Nitrogen and phosphorus will inevitably be released in the process of dissolution and hydrolysis of PCSs. When the biomass dosage is in excess, COD accumulation may occur. Gu et al. measured the TN release from *Iris pseudacorus* leachate with different dosages (0.5–1.5 g L⁻¹) and found that TN was only detected on the first day [33]. TN released accounted for 0.31–0.37% of the *Iris pseudacorus* total mass, in which ammonia-N and organic nitrogen accounted for about 5% and 95%, respectively [33]. The addition of PCSs is not conducive to the removal of ammonia-N, because PCSs can not only release ammonia-N but also consume the DO of CWs, thus reducing the nitrification of ammonia-N [16]. The ammonia-N in the effluent may slightly increase, but the TN concentration is generally low and can meet the first-class A discharge standard of pollutants for municipal wastewater treatment plants (GB18918-2002, China) or surface water quality standards (Class V, China) in CWs [21,33]. Zhong et al. observed that fast release rates of nitrogen and phosphate occurred on the first day in microalgal powder leachate, and then they achieved a stable period [43].

A significant positive correlation between the plant biomass dosage and TOC released could be observed in the *Iris pseudacorus* leachate [33]. Researchers found that plant fermentation broths could improve nitrate removal efficiency, and the effluent COD did not cause secondary pollution in the stable period in horizontal SSF CWs [37,43]. Secondary pollutant release mainly occurred in the early rapid release stage. Ling et al. found that the carbon releases of agricultural wastes such as rice straw, wheat straw, corn stalk, corncob, soybean stalk, and soybean hull are fast in the first 2 days [23]. Optimization of the amount and frequency of the plant biomass dosage could achieve sufficient carbon sources and minimize the nitrogen and phosphate released. It is recommended to add the leachates of plants soaked for 3 days to the CWs in batches and to add the plants soaked for 3 days to CWs, thus keeping the available carbon sources relatively stable and reducing the risk of secondary pollution.

5. Influencing Factors of Denitrification Rate

5.1. Temperature

The microbial activity, community structure, and release rate of PCSs are highly related to temperature [24,27,57], which influences the catabolic path and denitrification processes caused by the microbes [58]. In CWs with only endogenous PCSs for denitrification, temperature has a great influence on the growth and withering of plants. Previous studies demonstrated that the organic carbon release rate of cellulose materials was positively correlated with temperature, and, thus, the denitrification rate was improved with the increase in temperature [24,59]. Si et al. found that more sufficient electron donors could be obtained at high temperature (24.55 ± 2.35 °C) than that achieved at low temperature (12.5 ± 4.0 °C) with wheat straw addition, which achieved a higher denitrification rate ($42.28 \text{ g N m}^{-3} \text{ d}^{-1}$) at high temperature compared with that ($21.13 \text{ g N m}^{-3} \text{ d}^{-1}$) at low temperature [24]. Wu et al. reported that the denitrification rate ($42.20 \text{ g N m}^{-3} \text{ d}^{-1}$) in summer was significantly higher than that ($24.40 \text{ g N m}^{-3} \text{ d}^{-1}$) in winter when *Typha latifolia* litter was added to SF CWs for synthetic wastewater treatment [18].

Temperatures of 25–30 °C promote microorganic activities and then improve denitrification performance. Some heat preservation measures (such as covering plastic films and building greenhouses) have been used in CWs to raise the temperature in winter [60,61]. However, the plastic films are easily broken and impede oxygen mass transfer, and greenhouse building increases the infrastructure investment. Cultivation of cold-resistant plants such as *Lolium perenne* and *Carex aquatilis* and optimization of plant allocation are recommended for CWs [62]. Zhao et al. interplanted cold-season and warm-season macrophytes (i.e., *O. decumbens* and *H. verticillata*) in CWs to enhance the nitrogen removal efficiency

in the early cold season, and successful results were observed [63]. Moreover, the risk of secondary pollution caused by litter decomposition of macrophytes was reduced.

5.2. HRT

HRT is related to the contact time between denitrifying bacteria and nitrogen pollutants in CWs. Previous studies showed that the nitrogen removal efficiency increased with the increase in HRT from 2 to 10 days in CWs [1]. Liu et al. also reported that the nitrate and TN removal efficiency increased with the HRT increase from 2 to 4 days when *Iris pseudacorus* was planted in SSF CWs for aquaculture wastewater treatment [57], and the mean nitrate removal efficiency and denitrification rate (78.70% and $0.17 \text{ g N m}^{-3} \text{ d}^{-1}$) at 4 days HRT (i.e., 37.80 L d^{-1} flux and $0.06 \text{ m}^3 \text{ m}^{-2} \text{ d}^{-1}$ hydraulic loading) were higher than those (30.60% and $0.13 \text{ g N m}^{-3} \text{ d}^{-1}$) at 2 days HRT (i.e., 75.60 L d^{-1} flux and $0.13 \text{ m}^3 \text{ m}^{-2} \text{ d}^{-1}$ hydraulic loading). Wang et al. found a significant positive correlation between TN removal efficiency and HRT when raw reed straw was added to lab-scale CWs for stimulated typical local agricultural runoff treatment [17], and the TN removal efficiency under 2, 3, and 4 days HRT was 53.05, 64.35, and 74.16%, respectively.

A continuous increase in HRT is not appropriate when a relatively high nitrate removal efficiency is achieved, because a HRT that is too long often leads to a lower denitrification rate. For example, when raw reed straw was added to CWs, the nitrate removal efficiency at 4 days HRT (87.46%) was higher than that at 2 days HRT (62.12%), but the denitrification rate at 4 days HRT ($3.59 \text{ g N m}^{-3} \text{ d}^{-1}$) was lower than that at 2 days HRT ($5.09 \text{ g N m}^{-3} \text{ d}^{-1}$) [17]. Appropriate HRT is beneficial for the reduction in effluent color and the decrease in CW construction cost, which can also avoid secondary pollution caused by the high effluent dissolved organic carbon (DOC) released from PCSs. The optimal HRT of CWs for nitrogen removal usually varies between 1 and 6 days according to the influent quality; this can lead to a high denitrification rate [15,20,37,43,50].

5.3. Influent Dissolved Oxygen (DO)

Most denitrifiers are heterotrophic facultative anaerobes, and heterotrophic denitrification mainly occurs under anaerobic conditions [64]. Some heterotrophic denitrifying bacteria use both oxygen and nitrate as terminal electron acceptors, and the utilization of oxygen is easier than nitrate [65–67]. When solid PCSs are added to CWs, inner anoxic zones can be formed on the PCS surface because of influent DO diffusion, which is beneficial to the adhered microorganisms in carrying out denitrification [51]. Denitrification occurs when the oxidation reduction potential (ORP) is less than 350 MV, and low ORP enhances denitrification performance [68]. The degradation of PCSs can consume DO and reduce the ORP of CWs. Yuan et al. found that woodchips reduced the ORP to less than 170 MV and created favorable conditions for denitrification in SSF CWs [51].

Several studies have focused on denitrification performance under different DO conditions of CWs with PCS addition. Si et al. observed high nitrate removal efficiency (98.32%) under 7.69 mg L^{-1} influent DO in lab-scale vertical-flow CWs (VF CWs) with wheat straw addition [24], and the effluent DO (0.84 mg L^{-1}) was low due to consumption by PCSs and diffusion. Wu et al. reported that DO decreased from 3.70 mg L^{-1} of influent to 0.98 mg L^{-1} of effluent because of the *cattail* litter biodegradation in SF CWs [18], and the low DO was favorable to the denitrification process. When plants were used as carbon sources and biofilm carriers in CWs, inner anoxic zones formed on the plant surfaces, and 5 mg L^{-1} influent DO or even less had little influence on the denitrification process. However, a too high influent DO is not conducive to the growth of denitrifying bacteria, and it will consume the available substances released by PCSs, which is not conducive to the denitrification process [52].

Nitrogen removal efficiency enhanced by PCSs is closely related to PCSs' DOC release [69]. Chen et al. found that more DOC could be produced in an anaerobic environment ($0 \pm 0.05 \text{ mg L}^{-1}$ DO) than that in aerobic conditions ($3 \pm 0.05 \text{ mg L}^{-1}$ DO) for SSF CWs with *cattail* litter supplement [67]; the anaerobic environment enhanced the extracellular

enzyme activity of cellulose and hemicellulose, and the denitrification rate in anaerobic conditions was 7.40–12.60 times as much as that in aerobic circumstances. Anaerobic conditions should be designed in CWs to promote the activity of denitrifying microbes and the accumulation of available carbon sources for denitrification. In practical engineering applications, horizontal SSF CWs include both aerobic and anaerobic zones, and PCSs should be added at the bottom of the inlet anaerobic zone to improve denitrification performance.

5.4. pH

The optimum pH for denitrification ranges from 7.0 to 7.5 [70]. Yu et al. investigated the nitrate removal efficiency of SSF CWs at different pH (6.5, 7.0, 7.5, and 8.0) and found the highest denitrification rate achieved at pH 7.5 [54]. It has been reported that the denitrification process is significantly inhibited at low pH (2.5–5.8) conditions [71], and nitrite accumulation occurs when the pH is higher than 8.0 [72]. The expression levels of nitrate reductase and gaseous nitric oxide reductase, which are relatively insensitive to pH in the range of 6 to 8, decrease significantly at pH 5 [73]. The organic acids released from PCSs lead to a decrease in pH, and the decrease rate is positively correlated with PCS dosage during the early decomposition stage, which is attributed to the rapid leaching of organic acids from PCSs; subsequently, the carbon release arrives at a stable stage, and the organic acid utilization by heterotrophic microbes results in a pH increase in the later decomposition stage [74]. Organic acids released from PCSs and alkalinity produced by denitrification keep the pH balance in CWs [15]. Although PCS addition in CWs leads to a pH decrease at the early stage, their influence on pH and operation in the long term can be neglected [24].

6. Denitrification Mechanism of PCSs

6.1. Physicochemical Mechanism

Some exogenous solid PCSs (wheat straw, cotton, woodchips, etc.) not only release carbon for utilization by denitrifying bacteria but also provide surfaces for microorganism attachment. Figure 3 shows the surface morphology of the raw and used plants, and a certain damage of the PCSs' surfaces occurs after their carbon release and utilization [32]. Serious surface damage to wheat straw (Figure 3a₂) and broken cotton (Figure 3b₂) is observed after their carbon release [24]. Xiong et al. found that a large number of macropores were formed on the surface of corncob (Figure 3c₂) and on peanut shell (Figure 3d₂) surface during their carbon release processes [75]. The surface morphological variation of PCSs indicates that they could be degraded during carbon utilization, and the degree of damage reflects their biodegradability to some extent. The high degree of surface damage to PCSs represents a high capacity to release available carbon substances for denitrifying bacteria, which is beneficial to the denitrification process.

Endogenous PCSs mainly include plant root exudates and macrophyte litters at the senescent stage. The main nitrate removal in CWs results from denitrification by denitrifying bacteria, and plant absorption, sedimentation burial, and ammonia reduction can also remove nitrate to some extent [63]. Chen et al. reported that denitrification, sedimentation burial [76], and plant uptake contributed to 54.00–94.00%, 1.00–46.00%, and 7.50–14.30% of the nitrate removal in sequencing batch SF CWs, respectively.

The denitrification mechanism should be investigated further by physicochemical methods. SEM–energy dispersion spectroscopy can be used to analyze the distribution and carbon content on the PCSs' surfaces qualitatively and semi-quantitatively, and Fourier transform infrared spectrometry can be applied to exhibit the variation in PCS composition and structure through the absorption of infrared band data [75].

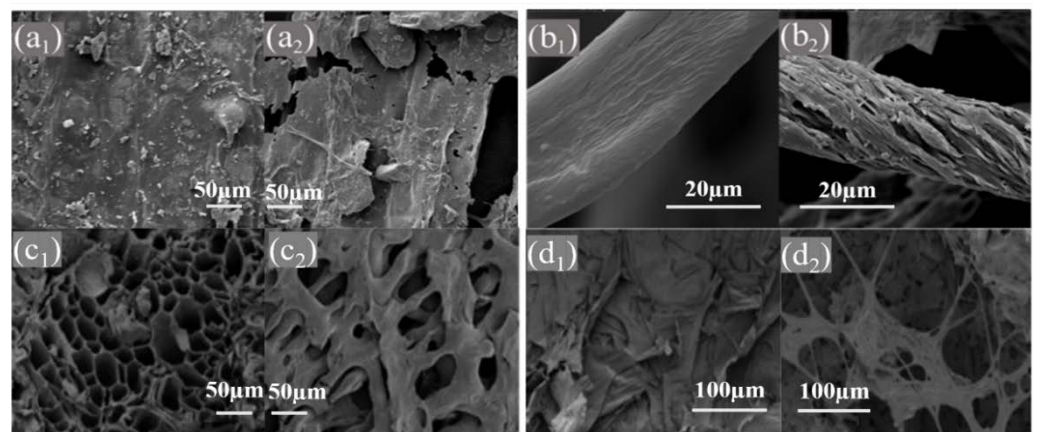


Figure 3. SEM images of the PCSs (a₁) raw wheat straw, (a₂) used wheat straw, (b₁) raw cotton, (b₂) used cotton, (c₁) raw corncob, (c₂) used corncob, (d₁) raw peanut shell, (d₂) used peanut shell. (a₁,a₂,b₁,b₂) was reprinted/adapted with permission from Si, Z.; Song, X.; Wang, Y.; Cao, X.; Zhao, Y.; Wang, B.; Chen, Y.; Arefe, A, Intensified heterotrophic denitrification in constructed wetlands using four solid carbon sources: Denitrification efficiency and bacterial community structure; published by Elsevier, 2018; (c₁,c₂,d₁,d₂) was reprinted/adapted with permission from Xiong, R.; Yu, X.X.; Zhang, Y.G.; Peng, Z.X.; Yu, L.J.; Cheng, L.L.; Li, T.M. Comparison of agricultural wastes and synthetic macromolecules as solid carbon source in treating low carbon nitrogen wastewater; published by Elsevier, 2018.

6.2. Molecular Biology Mechanism

Bacterial communities participate in the biogeochemical cycles of the substrate, and their activities are crucial to energy flow and nutrient transformation in CWs [27]. Nitrogen removal is an essential process in CWs dominated by bacteria which carry out nitrification and denitrification through the enzymes encoded by functional genes [77]. Functional genes are often used as biomarkers due to their being highly related to the targeting microorganisms [78]. It is necessary to analyze the variations in microbial functional genes and microbial community structure to further understand the molecular biology mechanism.

6.2.1. Denitrification Functional Genes

The nitrogen-transforming process in CWs mainly involves nitrification, denitrification, dissimilatory reduction, assimilation, nitrogen fixation, etc. Nitrate is mainly removed through the heterotrophic denitrification process. Figure 4 shows the major nitrogen removal routes, the related denitrification genes, and the reactions in different denitrification steps in CWs. Firstly, nitrate is reduced to nitrite by relevant bacteria under the catalysis of nitrate reductases (encoded by *narG*, *napA*, etc. genes) in the denitrification process. Secondly, nitrite is reduced to nitric oxide under the catalysis of nitrite reductase (encoded by *nirS*, *nirK*, etc. genes), and the *nirS* gene encodes cd₁-containing nitrite reductase, while the *nirK* gene encodes copper-containing nitrite reductase. Thirdly, nitric oxide is reduced to nitrous oxide by the nitric oxide reductases (encoded by *qnorB*, *cnorB*, etc. genes). Finally, the nitrous oxide is reduced to nitrogen gas by nitrous oxide reductase (encoded by *nosZ* and *nosR* genes). The *narG* gene encoding membrane-bound nitrate reductase and *napA* gene encoding periplasmic nitrate reductase are usually used as nitrate reduction marker genes in the study of denitrifying bacterium communities [79], and they have been widely studied in CW denitrification processes [80]. The *nirS* and *nirK* genes are considered functional markers of the nitrite to nitric oxide step in denitrification, and the *nosZ* gene is considered the marker gene of complete denitrification [81].

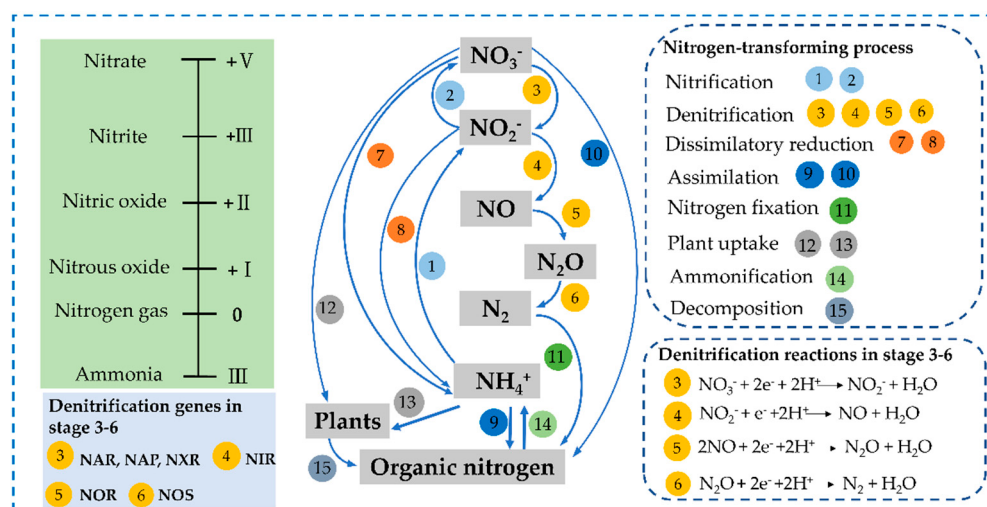


Figure 4. The major nitrogen removal routes, related denitrification genes, and different denitrification reactions in CWs. Note: NAR, NAP, NXR, nitrate reductase; NIR, nitrite reductase; NOR, Nitric oxide reductase; NOS, Nitrous-oxide reductase.

The number of *nirS*, *nirK*, and *nosZ* genes is 10^6 – 10^{10} , 10^5 – 10^8 , and 10^4 – 10^9 copies g^{-1} in CWs, respectively [27,65,82,83]. Hallin et al. suggested that gene copy numbers (especially the *nirS* gene) were related to the genetic potential for denitrification [84], and *nirS* gene copy numbers were generally higher than that of the *nirK* gene. The *nirS* gene is widely distributed, and the *nirK* gene is only found in about 30.00% of all known denitrifying bacteria; the *nirS* and *nirK* genes are mutually exclusive among denitrifying bacteria [85]. About two-thirds of the denitrifying bacteria lack nitrous oxide reductase, which is encoded by the *nosZ* gene, and so the *nosZ* gene copy number is lower than that of the *nirS* or *nirK* gene [82,86].

PCS addition improves the denitrification gene copies and, thus, promotes denitrification. The *nirS* and *nosZ* genes in lab-scale SSF CWs with 200 g *Typha angustifolia* litter addition showed 10 times the copies of those without litter addition, and the *nirK* gene copies were also increased; all of the *nirK*, *nirS*, and *nosZ* gene copies with 200 g *Typha angustifolia* litter addition were higher than those with 100 g *Typha angustifolia* litter addition [82]. Pilot-scale SSF CWs planted with *Oenanthe javanica* were developed to treat the wastewater treatment plant effluent under low temperature ($<10\text{ }^{\circ}\text{C}$) conditions in eastern China; the *nirS*, *nirK*, and *nosZ* gene copies were significantly higher than those in the non-planted microcosm, and the *nosZ* gene (1.716×10^6 copies g^{-1} sand) in the planted CWs was 4.4 times the number of that (3.895×10^5 copies g^{-1} sand) in the non-planted CWs [25]. Similar results were observed in the root soils of *Phragmites australis*, *Typha angustifolia*, and *Cyperus alternifolius* in practical CWs [27].

Exogenous PCS addition always has a more significant impact on the copy numbers of denitrification function genes than endogenous PCSs, which is consistent with their denitrification rates. The *nirS* and *nosZ* genes with *cattail* litter addition were 10 times the copies of those without PCS addition, but the *nirS* and *nosZ* gene copies were not significantly changed between the *Typha latifolia* planted and non-planted CWs [82]. Chen et al. reported that the planted macrophytes had little effect on the copy numbers of denitrification genes [87], which resulted from the increase in oxygen concentration and the subsequent nitrification promotion in the rhizosphere.

Research on the denitrification mechanism by denitrifying functional genes is mostly focused on the relationship between denitrification performance and a single functional gene (such as the *nirS*, *nirK*, or *nosZ* gene), which cannot elaborate on denitrification performance precisely. Functional genomes directly or indirectly affects the nitrate transformation or accumulation at the molecular level. Luo et al. pointed out that functional genomes involved in the denitrification process (*napA/nirS*, (*napA* + *narG*)/*nirS*, (*napA* + *narG*)/

(*nirS* + *qnorB*), (*napA* + *narG*)/(*nirS* + *qnorB* + *nosZ*), etc.) had stronger interactions with the denitrification processes [79]. Different denitrification stages occur at the same time and influence each other [88], and so the absolute copy number of a single functional gene is not of great value for characterizing the dynamic changes in nitrogen. Zhi et al. found that nitrate removal efficiency was influenced by *nxrA* (encoding nitrite oxidoreductase) and *narG* [80]. Chen et al. reported a significant positive correlation between the copies of the nitrite reductase gene $\Sigma(nirS + nirK)$ and the denitrification rate of the microcosm with *Typha latifolia* litter addition [82], and its addition directly changed DO and DOC in CWs and indirectly affected denitrification gene copies. Plant litters promote the growth of bacteria containing *nirS* and *nirK* genes by DO reduction and DOC increment and improve the abundance of bacteria containing the *nosZ* gene by DO reduction. Further studies concerning the impact of functional genomes on nitrogen removal and how PCSs affect functional genes should be strengthened to clearly understand the mechanism of enhanced denitrification by PCSs.

6.2.2. Community Structure and Abundance of Microorganisms

There are many ecological relationships and interactions among microbial communities involved in nitrogen transformation [89]. The common bacteria in CWs, which can be divided into *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Planctomycetia*, *Acidobacteria*, and *Actinobacteria* at the phylum level, account for more than 80.00% of the total bacterial population [24,25,27,90,91]. At the phylum level, denitrifying bacteria are mainly classified as *Proteobacteria*, *Actinomycetes*, *Aquificaceae*, *Bacteroides*, and *Firmicutes*, which include at least one strain with denitrification ability [92,93].

The species and abundance of microorganisms on the surface biofilms of macrophytes or substrates are highly related to their denitrification performance [24,25,94]. The denitrifying bacteria and the lignocellulose degradation functional bacteria at genus levels and their percentage in total bacterial reads after PCS addition are shown in Table 3, from which it can be seen that PCS addition increases the abundance of denitrifying bacteria, carbohydrate degradation bacteria, and organic acid synthesis bacteria. Si et al. found that the abundance of denitrifying bacteria (*Dechloromonas*, *Thauera*, *Paludibacter*, etc.) in CWs with wheat straw addition were higher than that without PCS addition [24]. The abundance of bacteria related to denitrification and biodegradation (*Paludibacter*), lignocellulose degradation (*Dechloromonas* and *Thauera*), and carbohydrate fermentation and organic acid synthesis (*Levilinea* and *Saccharofermentans*) in CW substrate is also increased with wheat straw addition. The Chao1 index and Shannon index represent the abundance and diversity of microorganisms, respectively [95]. Both the Chao1 index and Shannon index of CWs with wheat straw addition are higher than those without wheat straw addition, which indicates that wheat straw improves the richness and diversity of species; a large number of denitrifying bacteria and biodegradable bacteria are consistent with the higher TN removal efficiency (90.82%) of CWs with wheat straw addition compared with that (17.55%) of the control group [24]. Fan et al. reported that the bacteria related to heterotrophic denitrification increased from 1.70% (1.00% *Flavobacterium*, 0.14% *Dechloromonas*, etc.) before *calamus* litter addition to 15.51% (8.19% *Flavobacterium*, 4.96% *Bacillus*, etc.) after its dosage [15]. A relatively low DO concentration occurs for PCS consumption, which is beneficial for denitrifying bacteria and decreases the abundance of nitrifying bacteria [15,18]. The abundance of *Nitrospira*, which is related to nitrification, decreased with *calamus* addition in SF CWs [15]. In short, by adding PCSs, functional microorganisms such as denitrifying bacteria and lignocellulose degradation functional bacteria were enriched in CWs, which significantly improved the denitrification rate.

Table 3. Major and related bacteria after PCS addition and their proportion of the total bacterial reads at genus levels in CWs.

PCSs	Major and Related Bacteria after PCS Addition and Their Proportion	Ref.
Control	<i>Dechloromonas</i> ^a (NA), <i>Thauera</i> ^a (NA), <i>Paludibacter</i> ^{a,b} (0.23%), <i>Levilinea</i> ^c (NA), <i>Saccharofermentans</i> ^d (<0.10%)	
Wheat straw	<i>Dechloromonas</i> ^a (1.97%), <i>Thauera</i> ^a (1.02%), <i>Paludibacter</i> ^{a,b} (2.69%), <i>Levilinea</i> ^c (0.91%), <i>Saccharofermentans</i> ^d (3.14%)	[24]
Cotton	<i>Dechloromonas</i> ^a (3.92%), <i>Thauera</i> ^a (2.35%), <i>Paludibacter</i> ^{a,b} (NA), <i>Levilinea</i> ^c (NA), <i>Saccharofermentans</i> ^d (<0.10%)	
Control	<i>Halomonas</i> ^a (18.68%), <i>Flavobacterium</i> ^a (1.19%), <i>Bacillus</i> ^a (0.29%), <i>Acidovorax</i> ^a (0.11%), <i>Azoarcus</i> ^a (1.26%), <i>Azospira</i> ^a (0.16%), <i>Azospirillum</i> ^a (0.03%), <i>Bradyrhizobium</i> ^a (0.07%), <i>Dechloromonas</i> ^a (0.23%), <i>Hyphomicrobium</i> ^a (0.15%), <i>Mesorhizobium</i> ^a (0.07%), <i>Pseudomonas</i> ^a (9.64%)	[25]
<i>Oenanthе javanica</i>	<i>Halomonas</i> ^a (14.49%), <i>Flavobacterium</i> ^a (2.79%), <i>Bacillus</i> ^a (0.27%), <i>Paenibacillus</i> ^a (0.01%), <i>Acidovorax</i> ^a (0.03%), <i>Azoarcus</i> ^a (1.15%), <i>Azospira</i> ^a (0.16%), <i>Azospirillum</i> ^a (0.15%), <i>Bradyrhizobium</i> ^a (0.09%), <i>Dechloromonas</i> ^a (0.31%), <i>Hyphomicrobium</i> ^a (0.09%), <i>Mesorhizobium</i> ^a (0.15%), <i>Pseudomonas</i> ^a (5.19%)	
Control	<i>Bacillus</i> ^a (0.23%), <i>Flavobacterium</i> ^a (1.00%), <i>Hyphomicrobium</i> ^a (0.12%), <i>Rhodobacter</i> ^a (0.01%), <i>Comamonas</i> ^a (0.06%), <i>Hydrogenophaga</i> ^a (0.05%), <i>Azospira</i> ^a (0.01%), <i>Dechloromonas</i> ^a (0.14%), <i>Thauera</i> ^a (0.08%)	[15]
<i>Calamus</i> litter	<i>Bacillus</i> ^a (4.96%), <i>Flavobacterium</i> ^a (8.19%), <i>Hyphomicrobium</i> ^a (0.06%), <i>Rhodobacter</i> ^a (0.18%), <i>Comamonas</i> ^a (0.04%), <i>Hydrogenophaga</i> ^a (0.38%), <i>Azospira</i> ^a (0.04%), <i>Dechloromonas</i> ^a (1.63%), <i>Thauera</i> ^a (0.03%)	
Control	<i>Dechloromonas</i> ^a (1.03%), <i>Rhodobacter</i> ^a (NA), <i>Thiobacillus</i> ^a (NA)	[18]
<i>Cattail</i> litter	<i>Dechloromonas</i> ^a (6.67%), <i>Rhodobacter</i> ^a (NA), <i>Thiobacillus</i> ^a (NA)	
Control	<i>Halomonas</i> ^a (27.81%)	
<i>Iris pseudacorus</i>	<i>Flavobacterium</i> ^a (18.28%), <i>Halomonas</i> ^a (2.91%), <i>Aeromonas</i> ^a (1.81%), <i>Pseudomonas</i> ^a (1.68%), <i>Dechloromonas</i> ^a (1.29%), <i>Azospira</i> ^a (0.67%), <i>Shewanella</i> ^a (0.56%), <i>Arcobacter</i> ^a (0.24%)	[91]
<i>Typha orientalis</i>	<i>Flavobacterium</i> ^a (19.47%), <i>Halomonas</i> ^a (12.21%)	
starch/ PCL	<i>Bacillus</i> ^a (24.25%), <i>Thauera</i> ^a (9.36%), <i>Acidovorax</i> ^a (5.37%), <i>Chlorobium</i> ^e (7.06%), <i>Desulfobacter</i> ^f (6.15%), <i>Desulfobulbus</i> ^g (6%), <i>Desulfovibrio</i> ^g (5.26%)	[93]
Control	<i>Thauera</i> ^a (5.70%), <i>Dechloromonas</i> ^a (2.20%), <i>Flexibacter</i> ^a (1.90%), <i>Thiobacillus</i> ^a (1.50%), <i>Anaerolineaceae</i> ^a (1.30%), <i>Rhodobacter</i> ^a (1.20%), <i>Haliangium</i> ^a (1.20%)	
<i>Typha latifolia</i>	<i>Paracoccus</i> ^a (1.90%), <i>Hyphomicrobium</i> ^b (1.80%), <i>Novosphingobium</i> ^b (1.70%), <i>Nocardioides</i> ^a (1.60%), <i>Pseudonocardia</i> ^a (1.50%), <i>Sinobacteraceae</i> ^a (1.40%), <i>Flexibacter</i> ^a (1.30%), <i>Nocardioides</i> ^a (1.60%), <i>Pseudonocardia</i> ^a (1.50%), <i>Sinobacteraceae</i> ^a (1.40%), <i>Flexibacter</i> ^a (1.30%)	[87]
<i>Cattail</i> litter	<i>Thauera</i> ^a (6.20%), <i>Dechloromonas</i> ^a (4.40%)	

^a Denitrifying bacteria; ^b biological degradation; ^c carbohydrate metabolism and organic acid synthesis; ^d anaerobic carbohydrate fermentation process; ^e green sulfur bacteria; ^f sulfate-reducing bacteria; ^g sulfate-reducing bacteria and bacteria which can reduce nitrate or nitrite to ammonia; NA, no data available.

The carbon released from PCSs is utilized not only by denitrifying bacteria but also by other heterotrophic microorganisms. Aerobic heterotrophic bacteria oxidize organics and release carbon dioxide and other chemical compounds which use oxygen as the final electron acceptor, and anaerobic heterotrophic bacteria play critical roles in the fermentation and methanogenesis processes [96]. Acid-forming bacteria hydrolytic acidulate PCSs to monomers (such as organic acids, monosaccharides, alcohols, and carbon dioxide), and then methane-forming bacteria (methane-oxidizing bacteria and methanogenic archaea) change primary fermentation products to methane and carbon dioxide [96]. Sulfate-reducing bacteria (*Desulfobulbus* (6%) and *Desulfovibrio* (5.26%)) and some bacteria involved in the process of dissimilatory nitrate reduction to ammonium (DNRA) are found in CWs, which indicates that the PCSs are simultaneously used by denitrification, DNRA, and sulfate-reduction processes [93,97]. The schematic diagram of PCS utilization by denitrifying and related bacteria is illustrated in Figure 5. It could be concluded that when the PCSs were added to the CWs in the form of a liquid (i.e., suspension, anaerobic fermentation broths, and root exudates) or solid (i.e., mixed with matrix), small micro-molecular substances (such as VFAs, sugar, etc.) were released. According to the influent water quality, environment, and operating conditions, these released substances would be supplied to denitrification, DNRA, anaerobic fermentation, and sulfate-reduction processes. Denitrification is the main process of PCS consumption in CWs.

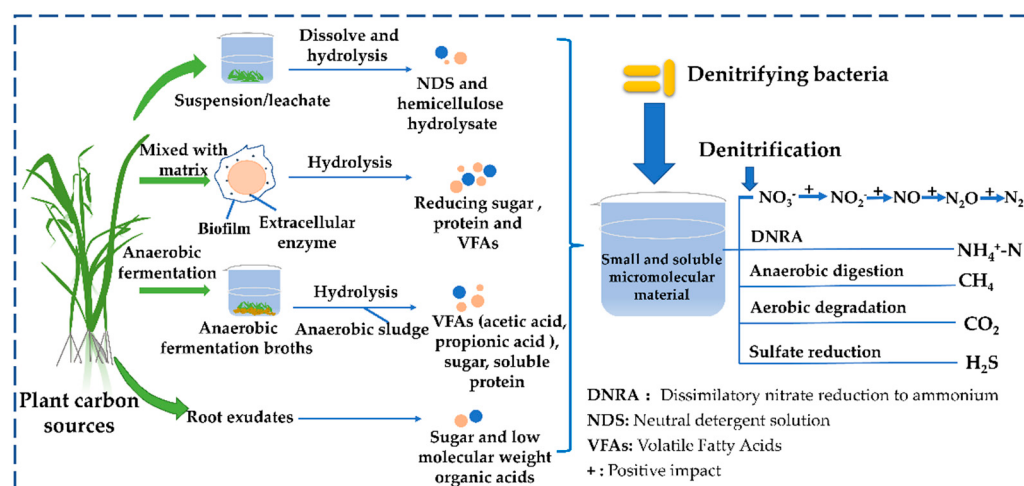


Figure 5. Schematic diagram of PCS utilization by denitrifying and related bacteria.

Chen et al. revealed that *cattail* litter addition increased the carbon content and sediment pH [87], thus indirectly affecting the operational taxonomic units of bacteria, and planting *Typha latifolia* in CWs mainly decreased the pH and then indirectly influenced the bacterial community structure. The mechanism of PCS influence on the bacterial community needs to be further explored.

7. Engineering Prospects

PCSs originating from plant litter, agricultural wastes, macrophytes, and forestry residues are easily available all around the world. The application of PCSs to denitrification is a green treatment technology in CWs [75]. The productivity of plant litters which originate from the abundant carbon sink in CWs was about $500\text{--}2000\text{ g carbon m}^{-2}\text{ year}^{-1}$ in matured wetlands [98]. Raw plant litters could remove $55.00\text{ mg N g}^{-1}\text{ dry mass}$, and the carbon content of PCSs was about 50% [37,99]. The potential nitrate removal efficiency of CWs was about $36.50\text{--}394.20\text{ g N m}^{-2}\text{ year}^{-1}$. Wu et al. found that macrophytes released $0.17\text{--}0.92\text{ mg organic carbon g}^{-1}\text{ root dry mass d}^{-1}$ [27], which could support a denitrification rate of $15.60\text{--}84.10\text{ g N m}^{-2}\text{ year}^{-1}$ theoretically. Therefore, the denitrification rate supported by plant litters and root exudates can reach $52.10\text{--}478.30\text{ g N m}^{-2}\text{ year}^{-1}$. The Na-

tional Bureau of Statistics showed that the total area of CWs in China was $6.75 \times 10^{10} \text{ m}^2$ in 2013 [100], and so the estimated nitrate removal potential by CWs could attain 3.52×10^{12} – $3.23 \times 10^{13} \text{ g N year}^{-1}$ in China. The major treatment object of CWs is wastewater treatment plant effluent [101]. The effluent TN limitation value of first-class A is 15 mg L^{-1} (Discharge standard of pollutants for municipal wastewater treatment plant, GB18918-2002, China), and the effluent nitrate-N is about 12 mg L^{-1} , because it accounts for 80% of the TN in the effluent [102]. Assuming that the 12 mg L^{-1} nitrate-N is completely removed, the PCSs of CWs in China could treat about 1.90×10^{11} – $1.58 \times 10^{12} \text{ m}^3$ of effluent annually.

The annual production of agricultural straw, which is considered the most abundant lignocellulosic source, reached approximately 860 million tons from 2015 to 2018 in China, and the top three are corn straw (30.31%), rice straw (25.07%), and wheat straw (17.89%); about 25% of the agricultural straw is not comprehensively utilized, which causes a serious waste of resources [103]. Yang et al. found that the denitrification potential rate of rice straw and corncob was 105.30 – $140.10 \text{ mg N g}^{-1}$ [104]. Nitrogen removal could reach about $10^{14} \text{ g N year}^{-1}$ if all of the agricultural straw were utilized. There are also rich forestry residues (such as tree branches and saw power), and local materials are recommended to reduce transportation costs. Therefore, using PCSs is an economical way to decrease the advanced wastewater treatment cost and reduce the waste of plant biomass.

8. Existing Problems

A problem restricting the engineering applications of PCSs is their renewal cycle. In lab-scale CWs, corn stubble maintained a high denitrification rate for 7.5 months; however, in a practical engineering application in the Lerma area, CWs with corn stubble addition worked effectively only for 3 months, i.e., effluent compliance could not be met thereafter [19]. Sequential batch PCSs are needed to solve the problem of the renewal cycle. A COD/N of 4–5 achieved by PCS addition in wastewater kept a high nitrate removal efficiency and reduced the release of greenhouse gases such as nitrous oxide and methane [105]. Anaerobic fermentation of macrophytes after collection could achieve 98.68% nitrate removal efficiency in SSF CWs for wastewater treatment plant effluent, which was conducive to the realization of nitrate removal in CWs without extra PCS addition [37]. Macrophytes should be harvested to avoid the risk of secondary pollution caused by the decay of plant residues, and anaerobic fermentation broths of macrophyte addition are needed, which is beneficial to the establishment of self-sufficient CWs and the reduction in PCS transportation costs.

Another issue of concern is the release of greenhouse gases such as nitrous oxide, methane, and carbon dioxide. Nitrous oxide and methane have a global warming potential of 296 and 23 times that of carbon dioxide, respectively [106]. Therefore, all of the nitrate is expected to convert into nitrogen gas during the denitrification process. Incomplete denitrification leads to nitrous oxide production under the condition of low temperature or high DO [107]. When sucrose was used as the carbon source in SSF CWs for the treatment of simulated wastewater treatment plant effluent with 12 COD/N, the nitrous oxide emissions ($8.2 \text{ mg m}^{-2} \text{ d}^{-1}$) were relatively low, accounting for 1.44% of the TN removal amount [108]. Wu et al. reported that low nitrous oxide emissions ($0.0011 \text{ kg N}_2\text{O per kg of N input}$) and high nitrogen removal efficiency (higher than 94.59%) could be achieved during the operation of SF CWs with 5 COD/N maintained by sucrose addition [109]. The greenhouse gas emissions of CWs with PCS addition have hardly been studied in recent years.

Complex microbial interactions and a wide range of redox conditions in CWs promote the removal of emerging pollutants such as antibiotics and pesticides [110]. Ramprasad and Philip studied the removal of sodium dodecyl sulphate, propylene glycol [111], and trimethylamine in pilot SSF CWs and VF CWs for greywater treatment, and the removal efficiency of these emerging pollutants ranged from 85.00 to 98.00%. The removal and transformation of emerging pollutants in CWs vary with the differences in their molecular weight, octanol–water partition coefficient, and functional groups. The mechanisms of

emerging pollutant removal in CWs, such as microorganism degradation, PCS adsorption, and the formation of toxic intermediate products, have not been explicitly elaborated, and more research about the behavior of these pollutants in CWs should be carried out.

9. Conclusions

The application of PCSs to CWs is a promising technology to enhance the denitrification of low COD/N wastewater. The composition of PCSs and their pretreatment methods and operational conditions affect the denitrification rate of CWs. The ideal PCSs, which must be easily degraded into micro-molecular substances for utilization by denitrifying bacteria, should have low lignin, high hemicellulose and cellulose, low nitrogen and phosphorus, and high carbon contents. The cattail originating from CWs is widely used as supplementary PCSs for CWs, and local materials are recommended to reduce transportation costs. In addition, anaerobic fermentation of PCSs in the bottom inlet of anaerobic zones in horizontal SSF CWs is recommended. A supplemental dose of PCSs should be maintained at 4–5 COD/N in wastewater; this could achieve a high nitrate removal efficiency and reduce nitrous oxide emissions. Adding PCSs could increase the denitrifying functional gene copies, improve the community richness and diversity of microorganisms, and increase the abundance of denitrifying bacteria and the bacteria related to lignocellulose and organic matter degradation, thus enhancing the denitrification process. Further studies should be carried out in the following fields:

1. Acid or alkali combined with biological pretreatment (laccase, high-redox-potential peroxidase, and oxidase) of PCSs, aiming to make full use of the lignocellulose.
2. Denitrification rates reflected in denitrification functional genomes and the influence of PCSs on denitrification functional genes and bacterial community structure in order to better understand the mechanism of denitrification enhancement by PCSs.
3. Greenhouse gas (nitrous oxide and methane) emission reduction during the denitrification process.
4. Synergistic removal of nitrogen and emerging pollutants such as pesticides and pharmaceutical and personal care products in CWs.

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Abbreviations

PCSs	Plant carbon sources
CWs	Constructed wetlands
COD	Chemical oxygen demand
COD/N	Chemical oxygen demand to total nitrogen
TN	Total nitrogen
VFAs	Volatile fatty acids
TOC	Total organic carbon
C/N	Carbon to nitrogen

HRT	Hydraulic retention time
SSF CWs	Subsurface-flow CWs
SF CWs	Surface-flow CWs
DOC	Dissolved organic carbon
DO	Dissolved oxygen
VF CWs	Vertical-flow CWs
<i>NapA</i>	Periplasmic nitrate reductase
<i>NarG</i>	Membrane-bound nitrate reductase
<i>NirS</i>	Cd ₁ -containing nitrite reductase
<i>NirK</i>	Copper-containing nitrite reductase
<i>QnorB/cnorB</i>	Nitric oxide reductase
<i>NosZ/nosR</i>	Nitrous oxide reductase

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