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Enhanced Long-Term Nitrogen Removal and Its Quantitative Molecular Mechanism in Tidal Flow Constructed Wetlands

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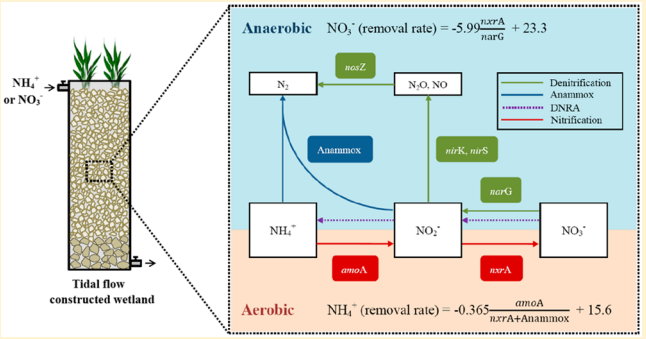
Supporting Information

ABSTRACT: Tidal flow constructed wetlands (TF CWs) have recently been studied as a sustainable technology to achieve enhanced nitrogen removal; however, the underlying mechanisms responsible for removing ammonium (NH_4^+) and nitrate (NO_3^-) have not been compared and quantified at the molecular level (genes) in controlled TF CWs. In this study, two TF CWs T1 (treating NH_4^+ wastewater) and T2 (treating NO_3^- wastewater) achieved high removal efficiencies for chemical oxygen demand (COD, 92 ± 2.7% and 95 ± 2.4%, respectively), NH_4^+/NO_3^- (76 ± 3.9% and 97 ± 2.2%, respectively), and total nitrogen (TN, 81 ± 3.5% and 93 ± 2.3%, respectively). Combined analyses revealed that the presence of simultaneous nitrification, anammox, and denitrification processes and the coupling of dissimilatory nitrate reduction to ammonium, ammonia oxidation, and anammox were the primary reason accounted for the robust treatment performance in T1 and T2, respectively. Results from stepwise regression analysis suggested that the NH_4^+ removal rate in T1 was collectively controlled by *amo*A, *nxr*A, and anammox, while the NO_3^- removal rate in T2 was governed by *nxr*A and *nar*G gene.



INTRODUCTION

Constructed wetlands (CWs) have been constructed and intensively studied as a sustainable technology that harnesses the power of physical, chemical, and biological processes for wastewater treatment and nutrient management. Given their technical feasibility, economic advantage, and ecological benefits, these engineered systems are primarily designed and used for nitrogen removal from domestic wastewater.[1−3] However, many operating CWs exhibit limited and fluctuating reductions in nitrogen levels, with removal efficiencies less than 50%,[4] which highlights the urgent need to enhance their treatment performance. Ever-increasing pollution discharges and more stringent nitrogen discharge limits further drive intensive efforts toward improved engineering design and application of CWs.

Horizontal flow constructed wetlands (HF CWs) provide suitable anoxic or anaerobic conditions for denitrification, but generally suffer from a lack of sufficient oxygen for nitrification.[2] Vertical flow constructed wetlands (VF CWs), however, are more aerobic for nitrification due to the enhanced oxygen diffusion into the beds.[5] Other studies have demonstrated that adequate total nitrogen (TN) removal could not be achieved in a single-stage CW due to its inability to simultaneously provide both nitrification and denitrification conditions.[5,6] Therefore, CW-centered hybrid systems with multiple treatment units have been developed to meet the increasing need for enhanced TN removal. The most commonly configured hybrid systems are VF-HF and HF-VF CWs, with TN removal efficiencies between 52% and 79%, depending on the type and composition of the wastewater.[2,7,8] Other complex hybrid systems that integrate more than two treatment units of VF and HF CWs have also been frequently implemented in the field, with typical TN removal efficiencies ranging from 53% to 83%.[2,9,10] Various types of hybrid systems that couple CWs with other treatment systems have also been recently developed,[11] and the electrolysis system[12] and the microbial fuel cell (MFC) system[13] have been demonstrated to intensify nitrogen removal from wastewater. However, the extra capital investment and complex operating conditions required by these CW-centered hybrid systems inevitably create

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economic and technical barriers for increasing field application at a large scale.

Tidal flow constructed wetlands (TF CWs) have been proposed as a compact and efficient modification to enhance removal of nitrogen,[14−16] including ammonium (NH_4^+) and TN. TF CWs generate a rhythmic sequential cycle of a "filled/wet" phase and a "drained/dry" phase that increases both nitrification and denitrification in a single reactor, thus minimizing land occupation and initial capital cost. Zhi and Ji achieved high removal efficiencies of NH_4^+ (63−80%) and TN (50−82%) with a single-stage TF CW under varying loading rates of chemical oxygen demand (COD) without costly aeration.[17] Hu et al. reported that TN was reduced by 85% through use of a TF CW.[18] To date, only a few studies focusing on operation parameters and treatment performance have been published, and very little is known about the nitrogen removal pathway and the underlying mechanism that governs the nitrogen transformation process in TF CWs. The lack of a quantitative link between macro-scale nitrogen processes and microscale functional genes limits our ability to optimize treatment processes and to reliably predict long-term effluent quality. TF CWs offer a higher degree of operational flexibility with adjustable parameters, such as flooded/drained time to balance aerobic and anaerobic conditions for maximum nitrogen removal. This feature also enables TF CWs to handle varying loading rates of wastewater associated with unexpected events and/or seasonal fluctuation. This inspires us to investigate the technical viability of TF CWs to process different forms of nitrogen, NH_4^+, and nitrate (NO_3^-), which require different treatment conditions.

To fill the knowledge gap in TF CWs, this study presents the first attempt to explore their operational adaptability and feasibility for treating two types of nitrogen (ammonium and nitrate) under the same operating conditions, as well as to quantitatively understand their underlying molecular mechanisms. The following three specific objectives were pursued: (1) to evaluate the long-term treatment performance of NH_4^+ and NO_3^- removal; (2) to quantify the absolute abundance of the functional genes involved in nitrogen removal and to determine the quantitative link between the nitrogen transformation processes and functional genes; and (3) to discern the primary nitrogen removal pathway and to identify the key functional genes that shape the nitrogen removal rates.

MATERIALS AND METHODS

Tidal Flow Constructed Wetlands. Two lab-scale vertical CWs (termed T1 and T2) with working volumes of 40 L ($L \times W \times H = 40 \times 20 \times 120$ cm^3) were built. The CW beds consisted of two functional layers (Supporting Information, SI, Figure S1), the water distribution layer (0−20 cm, filled with gravel of 10−20 mm in diameter) and the treatment layer (20−100 cm, filled with lava rock of 8−10 mm in diameter). *Iris pseudacorus* plants were planted on the top surface at an initial density of 125 plants/m^2. For each of these two CWs, two perforated PVC pipes ($L \times D = 100 \times 5$ cm^2) harboring a total of eight columns (filled with the same bed material of CWs) that wrapped up by highly permeable nylon mesh were vertically preburied in the center of CW bed for microbial sampling (SI Figure S2). Eighty holes were evenly drilled into each PVC pipe to allow for the same wastewater/air contact and hydraulic conditions (wetting and drying cycle) for the overall wetland bed and the substrate in the columns.

T1 and T2 were fed with NH_4^+ and NO_3^- wastewater, respectively, to investigate the effects of nitrogen species on the long-term treatment performance and nitrogen removal pathway. Synthetic wastewater (SI Table S1) was made from tap water (Beijing groundwater) and contained the following (per liter): 0.19 g glucose, 0.089 g NH_4Cl (or 0.049 g KNO_3), and 0.0043 g KH_2PO_4, resulting in concentrations of 200 mg/L COD, 30 mg/L NH_4^+ (or NO_3^-), and 3.0 mg/L PO_4^{3-}. The synthetic wastewater was prepared daily in a feeding tank and then pumped into the beds though flumes in the distribution layer.

The experiment began on December 5, 2011 and lasted a total of 245 days. The experiment involved a start-up stage ending on February 21, 2012 (70 d) and an operational stage extending from February 22, 2012 to August 16, 2012 (175 d). During this experimental period, T1 and T2 were operated under the same tidal flow conditions, and their influents and effluents ranged in temperature from 15.1 to 27.0 °C. The flood-and-drain cycle, generated by a metering pump (ProMinent, Dalian, Liaoning, China) and a programmable timer, rhythmically repeated every 36 h, providing 24 h of wastewater-bed contact during a "wet" phase and 12 h of reoxygenation during a "dry" phase. The "wet" phase began with a 10 min complete filling of influent into CW bed, and the "dry" phase began with a 10 min complete drainage of effluent from the CW bed to the effluent tank. As the treated wastewater retreated during the 10 min drainage, air was pulled into the porous CW bed, providing aeration. After the drainage (in the "dry" phase), oxygen transferred from the atmosphere to the CW bed continued, contributing to reoxygenation via natural diffusion. Given such operation strategy, the hydraulic loading rates for T1 and T2 were maintained at 0.33 m^3/m^2·d.

Sample Collection and Determination. Water samples were collected from T1 and T2 two or three times each month and were analyzed immediately at the Key Laboratory of Water and Sediment Sciences, Peking University. The dissolved oxygen (DO) was measured by a DO200 dissolved oxygen meter (YSI, Yellow Springs, Ohio, U.S.A.). COD was determined with a HACH DR2800 (HACH, Loveland, Colorado, USA),[19] and NH_4^+, nitrite (NO_2^-), NO_3^-, and TN were measured by a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). All variables were analyzed according to standard analytical procedures.[20] Microbial samples from T1 and T2 were collected for functional gene analysis at the end of weeks 3, 5, 9, 15, 20, 25, 30, and 35. During each microbial sampling, one column (as shown in SI Figure S2) was extracted at the above designed date and then thoroughly mixed to obtain one homogeneous sample for the best representation of the whole wetland bed. Then the sample was placed in an ice incubator and prepared for subsequent DNA extraction.

Quantitative Polymerase Chain Reaction (qPCR). Total genomic DNA from microbial samples was first extracted and purified using Soil DNA kits D5625−01 (Omega Bio-Tek, Norcross, Georgia, U.S.A.) and then detected with 1% agarose gel electrophoresis and stored at −20 °C until use. The qPCR was employed to investigate the "key players" in the nitrogen transformation to understand their population dynamics.[21] The absolute abundance of bacterial 16S rRNA, archaeal 16S rRNA, anammox bacterial 16S rRNA, ammonia monooxygenase (*amo*A), nitrite oxidoreductase (*nxr*A), membrane-bound nitrate reductase (*nar*G), copper-containing nitrite reductase (*nir*K), cd1-containing nitrite reductase (*nir*S), and nitrous oxide reductase (*nos*Z) were quantified three times for each

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sample with a MyiQ2 Real-Time PCR Detection System (Bio-Rad, Hercules, California, U.S.A.) using previously described primers and protocols.[17,22,23]

Data Analysis. The influent and effluent concentrations of COD, NH_4^+, NO_2^-, NO_3^-, and TN along with hydraulic retention time (HRT = 36 h) and surface area (A = 0.4 × 0.2 m^2) were used to calculate the removal efficiencies (%), mass removal rates (g/m^2·d), and nitrogen transformation rates (mg/L·d; SI Figure S3). Standard deviations (S.D.) of gene abundance data were calculated using three replicates measured in qPCR and plotted as error bars for assessing data variations and measurement errors. The absolute abundances of seven functional genes (i.e., *amo*A, *nxr*A, anammox 16S rRNA, *nar*G, *nir*K, *nir*S, and *nos*Z) were averaged and then used as basic candidate variables in stepwise regression analyses (SPSS 20, U.S.A.) for correlations with nitrogen transformation rates.

RESULTS AND DISCUSSION

Treatment Performance. During the operation (105−245 d; Figure 1), T1 and T2 achieved COD removal efficiencies of 92 ± 2.7% and 95 ± 2.4% (mean ± S.D.), respectively. To our knowledge, these COD removal efficiencies are higher than those of HF and VF CWs, which are commonly used to treat domestic wastewater with typical removal efficiencies ranging from 64% to 82% and from 77% to 83%, respectively.[24−27] Our single-stage TF CWs achieved COD removal rate at 62 g/m^2·d (averaged by T1 and T2), higher than the COD removal rates reported by other studies, such as 9.8 g/m^2·d by Albuquerque et al. (2009),[28] 17 g/m^2·d by Avsar et al. (2007),[29] 20 g/m^2·d by Konnerup et al. (2009),[30] and 57 g/m^2·d by Fan et al. (2013).[31] This enhanced treatment performance in terms of COD removal efficiency and removal rate was largely attributed to the intensified oxygen supply generated by the tidal flow operation.[32] T2 achieved a higher COD removal than T1 with less operational time (d) because the abundantly available NO_3^- in T2 could directly lead to more denitrification with simultaneous utilization of COD as a required organic source. However, the first and major nitrogen transformation that occurred in T1 was nitrification ($NH_4^+ \rightarrow NO_3^-$), which did not contribute to COD consumption. Nitrification was also the rate-limiting step for providing NO_3^- for denitrification and COD consumption.

Whereas the NH_4^+ removal efficiency in T1 stabilized at 76 ± 3.9% after 105 d, the NO_3^- removal efficiency in T2 exceeded 90% after 42 d. T1 and T2 achieved TN removal efficiencies (105−245 d; Figure 1c) of 81 ± 3.5% and 93 ± 2.3%, respectively. Ju et al. reported 76−90% removal of TN using a TF CW coupled with an electrolysis system.[33] In two other hybrid CW systems involving an HF−HF−VF configuration and a VF−HF−VF configuration,[9,34] TN was reduced by 73% and 45%, respectively. Compared to those hybrid CW systems using multiple treatment units, T1 and T2 achieved equivalent or even higher TN removal efficiencies. The highest TN removal rate in T1 and T2 was achieved at 10.3 and 11.3 g/m^2·d, respectively. Liu et al. (2013) reported TN removal rates of 0.69−1.2 g/m^2·d for nonaerated VF CWs.[35] Maltais-Landry et al. (2009) and Zhang et al. (2010) achieved averaged TN removal rates in aerated CWs at 0.8 and 5.0 g/m^2·d, respectively.[36,37] Other similar studies reported typical TN removal rates ranging from 0.9 to 4.6 g/m^2·d.[28,38,39] Thus, our single-stage TF CWs exhibited promising performance and operational flexibility for nitrogen removal (both NH_4^+ and NO_3^-), allowing TF CWs to complement and even replace hybrid CW systems in field applications, while providing more efficient space utilization and lower capital costs.

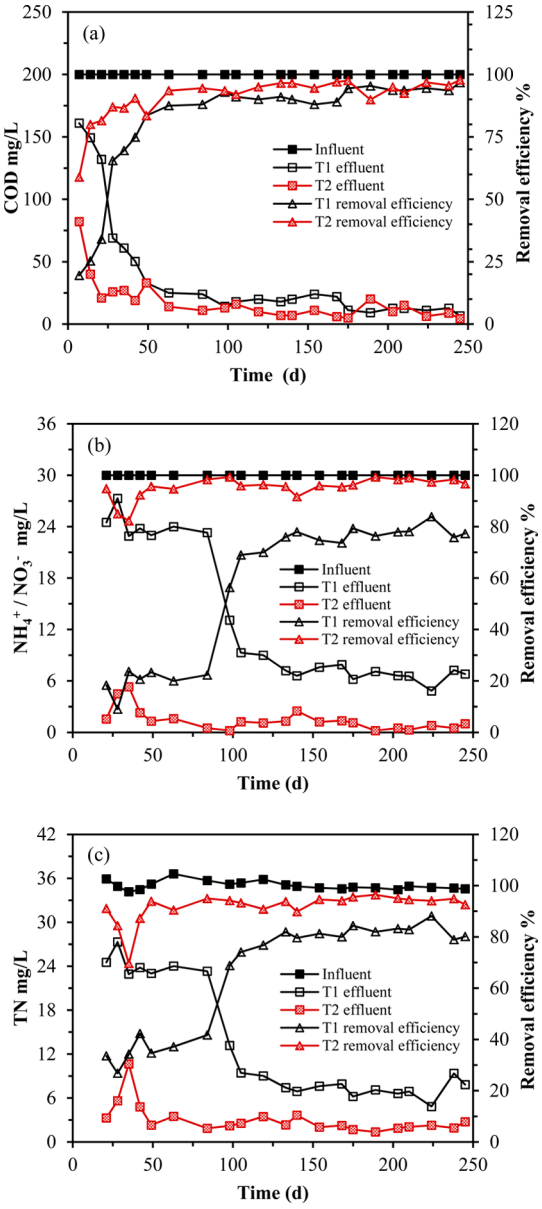

Figure 1. Treatment performances in tidal flow constructed wetlands: (a) COD; (b) NH_4^+ and NO_3^-; and (c) TN.

Nitrogen Transformation. A comparison between T1 and T2 on nitrogen transformation is shown in Figure 2. The NO_3^- concentration in our synthetic wastewater varied from 1.2 to 5.3 mg/L throughout our study (245 d), leading to a slight fluctuation in the influent TN. Starting in the winter (21−63 d), T1 reduced NH_4^+ and TN by 19 ± 5.4% and 35 ± 5.1%, respectively. The negligible nitrification observed in Figure 2a suggests that the biological NH_4^+ transformation contributes little to NH_4^+ removal due to the negative impact of the low influent temperature (15.1−16.2 °C) on nitrifying bacteria, which are slow-growing chemoautotrophic organisms with optimal growing and functioning conditions above 30 °C.[40−42] Therefore, the physicochemical NH_4^+ adsorption by porous lava rock in the T1 bed was the main NH_4^+ removal pathway in the start-up period. The 16% discrepancy between the NH_4^+

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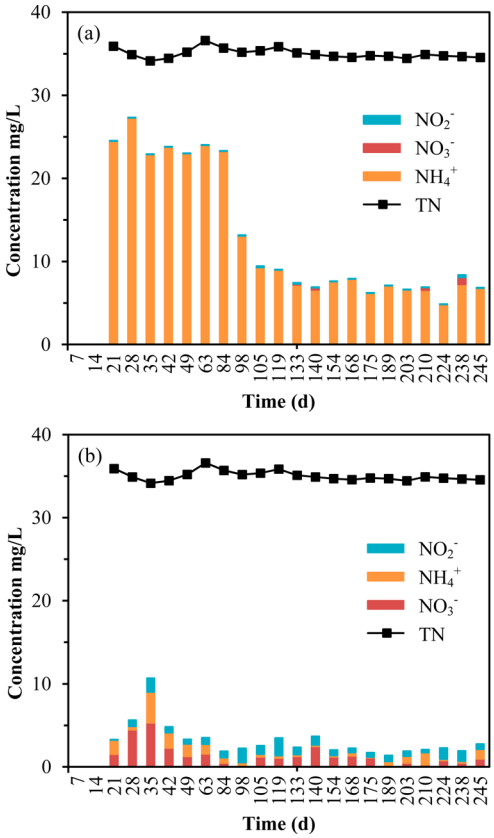

Figure 2. Long-term dynamic transformations of nitrogen in tidal flow constructed wetlands: (a) T1 and (b) T2.

and TN removal efficiencies was attributed to the denitrification of influent NO_3^- from groundwater rather than from NH_4^+ transformation. In the operational stage (after 105 d), the residual NH_4^+ in the effluent significantly declined to 7.2 ± 1.2 mg/L ($P = 9.5 \times 10^{-13} < 0.05$). This significant increase in NH_4^+ removal was attributed to nitrification ($NH_4^+ \rightarrow NO_2^-/NO_3^-$) because the NH_4^+ adsorption by porous lava rock was considered to be transient due to the limited adsorption capacity of T1's bed.[17] Throughout this study, little NO_2^- and NO_3^- were accumulated in T1, suggesting that denitrification at a C/N ratio of six was sufficient for nitrification to completely remove nitrogen from the wastewater.[17,43] In other words, the nitrification in our TF CW was to a certain extent still limited by oxygen transfer (effluent DO ranging from 0.51 to 0.82 mg/L) under the current tidal flow operation strategy (24 h flooded and 12 h drained). This result highlights the need for a longer "dry" phase for more aeration to balance the nitrification and denitrification to achieve maximum NH_4^+ and TN removal in TF CWs.

T2 achieved an NO_3^- removal efficiency of 91 ± 5.7% in the start-up stage, increasing to 97 ± 2.2% in the operational stage. Compared to the lagging NH_4^+ removal in T1, the NO_3^- removal in T2 was more rapid and efficient. This was because the shortcut nitrogen removal pathway in T2, the denitrification process, was unrestricted to the rate-limiting nitrification step. The rapid denitrification in T2 also suggested that the heterotrophic denitrifiers were better able to survive and function at low temperatures.[44] An unexpected NH_4^+ concentration of 0.82 ± 0.87 mg/L (Figure 2b) was probably originated from a competing nitrate reduction process (to denitrification) that has been known as dissimilatory nitrate reduction to ammonium (DNRA) for more than 25 years.[45,46] Although the DNRA process has been described in many systems, including tropical forest soils,[47] freshwater sediments,[48] marine environments,[49,50] and coastal ecosystems,[51,52] few studies on DNRA in CWs have been reported, and thus little is known about the fate of nitrogen after its transformation from NO_3^- to NH_4^+ via DNRA. On the basis of the complete denitrification that occurred in T1, it is highly possible that the accumulated NO_2^- (0.85 ± 0.49 mg/L) observed in T2 was derived from the NH_4^+ produced via nitrification rather than from denitrified NO_3^-. Therefore, the combined amount of NH_4^+ and NO_2^- (1.7 ± 0.98 mg/L), accounting for 5.1 ± 3.4% of the removed NO_3^-, was likely to be attributed to the DNRA process in T2. In summary, although the NO_3^- removal in T2 was largely attributed to denitrification, the less-studied DNRA was an existing but previously underestimated pathway contributing to nitrate removal.

Nitrogen Removal Mechanisms. The absolute abundances of bacterial 16S rRNA, archaeal 16S rRNA, anammox bacterial 16S rRNA, *amo*A, *nxr*A, *nar*G, *nir*K, *nir*S, and *nos*Z were quantified eight times during the experimental period to determine their dynamic populations (Figure 3), assisting in our study of the development of nitrogen removal pathways in T1 and T2. Small error bars in Figure 3 suggest that the measured three replicates are close to the mean value, justifying the use of mean values of the three replicates in the following analysis for minimizing potential measurement errors. The absolute abundances of bacterial 16S rRNA in T1 and T2 showed rapid increases of 53-fold and 52-fold, respectively, in the start-up stage. These increases directly promoted COD consumption, leading to 2.6-fold and 1.2-fold increases in the COD removal efficiencies of T1 and T2, respectively. Although archaeal 16S rRNA showed a much lower abundance compared to bacterial 16S rRNA, these minor members of the microbial community may play important roles in nitrogen cycling and greenhouse gas emissions.[53,54]

The NH_4^+ transformation communities harboring *amo*A, *nxr*A, and anammox bacteria 16S rRNA are summarized in Figure 3c,d. During the first 105 d, the *amo*A gene in T1 promptly increased by 69-fold, thereby promoting nitrification as a major NH_4^+ removal pathway (SI Figure S4) in addition to NH_4^+ adsorption. However, the abundance of the *amo*A gene gradually declined over the remaining 140 d, whereas anammox bacteria 16S rRNA continued to increase and ultimately exceeded the abundance of the *amo*A gene by a factor of 244. This result indicates that anammox was markedly enhanced after the midpoint of the operational stage and eventually replaced nitrification as the leading NH_4^+ elimination pathway in T1, consistent with our previous study.[17] One possible explanation is that DO was primarily consumed in the oxidation of substantial organic matter (92 ± 2.7% COD removal) after 105 d, which created anoxic conditions favorable for the enrichment of anammox bacteria as the dominant driver of NH_4^+ and NO_2^- removal. Due to the DNRA process, the occurrence of nitrification and the existence of the *amo*A and *nxr*A genes were expected in T2. However, the abundances of the nitrifying genes *amo*A and *nxr*A in T2 were only 13% and 23% of those in T1, respectively. These lower abundances were attributed to the lower level of NH_4^+ in T2, which was only, on average, 6.3% of that in T1 (Figure 2). The anammox 16S rRNA increased in T2 along with the *amo*A gene because anammox was dependent on the NO_2^- produced from ammonia oxidation performed by the *amo*A gene as a required

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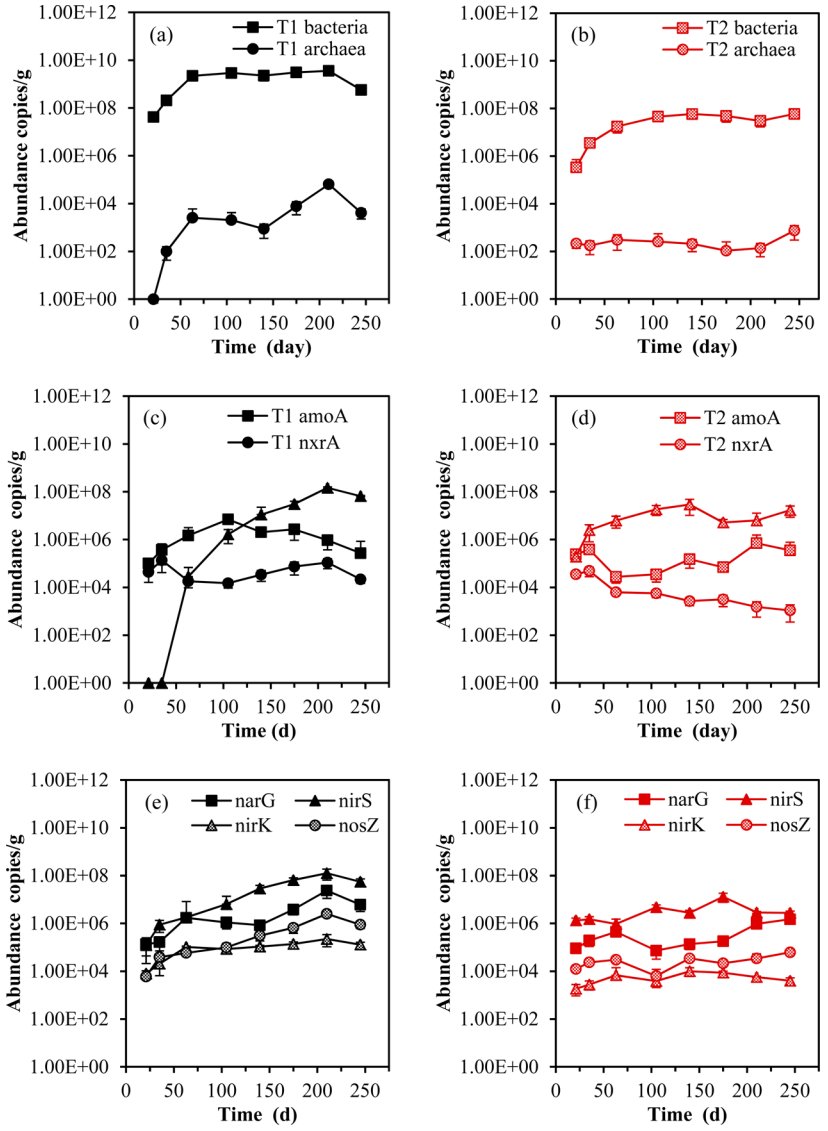

Figure 3. Dynamic populations of nitrogen functional genes in T1 (black color) and T2 (red color): (a, b) bacterial and archaeal 16S rRNA; (c, d) *amo*A, *nxr*A, and anammox 16S rRNA; and (e, f) *nar*G, *nir*S, *nxr*K, and *nir*Z. The absolute abundances (copies/g) are shown on log10 scale (*Y*-axis). The abundances of archaeal and anammox 16S rRNA below detection limits are reported as zero in parts a and c, respectively. Standard deviations of three replicates are indicated by error bars. Invisible error bars indicate that the standard deviations are smaller than the marker size.

substrate. Due to the direct substrate competition for NO_2^- with anammox bacteria, the *nxr*A gene in T2 declined in abundance throughout the entire period. Therefore, anammox coupled with DNRA and ammonia oxidation was determined to be an important pathway contributing to nitrate removal, similar to a recent study that first reported that co-occurring anammox and DNRA were responsible for the intensive nitrogen loss in the ocean.[52] Our results highlight the need to further examine the effects of coupled nitrogen transformations on nitrate removal, which have so far been ignored in CW studies, yet may rival denitrification in importance and contribution to nitrogen balance.

Four denitrifying genes *nar*G, *nir*K, *nir*S, and *nos*Z are summarized in Figure 3e,f. All four genes exhibited increases in T1 over the entire period, leading to enhanced denitrifying activity responsible for eliminating NO_3^- and NO_2^- (Figure 2a). The coenrichment of these four denitrifying genes in T1 was attributed to their similar environmental adaptations to anoxic conditions, as well as to their related yet distinct ecological niches. Specifically, the *nar*G gene that performed nitrate reduction provided a necessary substrate (NO_2^-) to the *nir*K and *nir*S genes for nitrite reduction (NO_2^- to NO), followed by the *nos*Z gene that required N_2O to be converted from NO for the final denitrification step. Although the denitrifying genes in T2 showed a smaller increase in absolute abundance, they were very likely to be stimulated by the presence of abundant NO_3^- with higher level of gene expression. Absolute abundance measured in this study is useful for quantifying the growth (rate) of denitrifying bacteria, while gene expression (using mRNA) is an indicator useful for characterizing metabolically active denitrifiers, which are more relevant to denitrifying activity. Thus, the increase in gene expression rather than in absolute abundance is more likely to be the reason accounting for the enhanced denitrifying activity in T2. The *nir*S gene had a significantly higher abundance than the counterpart *nir*K gene in both T1 and T2 ($P = 0.0298$ and $0.0155 < 0.05$, respectively), similar to other studies reporting that the *nir*S gene was environmentally more abundant than the

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*nir*K gene.[55] Another common phenomenon shared by T1 and T2 was that the *nos*Z gene abundance increased in T1 and T2 by 148-fold and 5-fold, respectively. This increase in the *nos*Z gene during long-term operation enhanced the last step in the denitrification pathway, leading to complete denitrification and a potential reduction in the emission of the greenhouse gas N_2O (not measured in this study).

Quantitative Response Relationships. Although the single nitrogen transformation process and the underlying functional genes that drive the cycling are well understood, our knowledge of the roles of these functional genes in nitrogen removal is still descriptive. Therefore, quantitative response relationships were developed to link macro-scale nitrogen processes and microscale functional genes and to advance our quantitative understanding of the key genes that govern the nitrogen removal processes.

For T1, four equations for NH_4^+, NO_2^-, NO_3^-, and TN were established with R^2 values ranging from 0.985 to 1.00 (Table 1). The NH_4^+ transformation rate was negatively correlated with *amo*A/(*nxr*A + anammox). This relationship exists because the ammonia-oxidizing bacteria (AOB) responsible for the NH_4^+ transformation are inhibited by the presence and/or accumulation of their own metabolic product, NO_2^-.[56,57] The level of NO_2^- accumulation is denoted by the ratio of *amo*A/(*nxr*A + anammox) because *amo*A was directly involved in the production of NO_2^-, whereas *nxr*A and anammox were responsible for NO_2^- consumption (SI Figure S4). This result suggests the involvement of the *nxr*A gene and anammox 16S rRNA in the process of ammonia oxidation, which is traditionally believed to be driven and influenced only by the *amo*A gene. The NO_2^- accumulation rate was numerically determined by both the *nir*S and *nir*K genes. The variables (*nir*S + *nir*K)/bacteria and *nir*K were directly involved in NO_2^- transformation in the denitrification pathway and were therefore negatively correlated with the NO_2^- accumulation rate. Thus, the increasing *nir*S and *nir*K genes were the key factors responsible for eliminating NO_2^- accumulation. Although denitrification accounted for the NO_3^- removal, the equation of the NO_3^- transformation rate indicates that the nitrification process denoted by *amo*A and *amo*A/(*nxr*A + anammox) was the major regulator for the NO_3^- transformation rate. One possible explanation is that the greater quantity of available NO_2^- derived from NH_4^+ results in more NO_3^-, which could be completely removed by denitrification in T1. The TN transformation related to the nitrification, anammox, and denitrification processes was collectively determined by *amo*A/(*nxr*A + anammox), *nir*S/*nir*K, and (*nir*S + *nir*K)/bacteria. On the molecular level, these results indicate the existence of simultaneous nitrification, anammox, and denitrification (SNAD) processes can assist in the simultaneous and robust removal of nitrogen and COD in a single system without a sequential chain of treatments.[58]

Table 1. Quantitative Response Relationships between Nitrogen Transformation Rates (mg/L·d) and Functional Gene Abundance (Copies/g) in T1 (NH_4^+ Treatment)

stepwise regression models (equations)	R^2	*P* value
$NH_4^+ = -0.365(amoA/(nxrA + Anammox)) + 15.6$	0.998	0.001
$NO_2^- = -7.54((nirS + nirK)/bacteria) - 2.14 \times 10^{-6}$ *nir*K + 0.221	0.985	0.002
$NO_3^- = 0.044(amoA/(nxrA + Anammox)) + 7.72 \times 10^{-8}$ *amo*A + 2.85	1.000	0.000
$TN = 0.001(nirS/nirK) - 0.322(amoA/(nxrA + Anammox)) - 12.7((nirS + nirK)/bacteria) + 18.6$	1.000	0.000

In T2, three equations of NO_3^-, NO_2^-, and TN were constructed with R^2 values ranging from 0.891 to 0.999 (Table 2). The NO_3^- transformation rate was negatively correlated with *nxr*A/*nar*G because the ratio of *nxr*A/*nar*G represents the accumulation level of NO_3^- (increased), which is the opposite of NO_3^- transformation (decreased) in terms of reaction direction. This result suggests that, even in T2 (the treatment system dominated by denitrification), the nitrifying gene *nxr*A may play an underlying, but previously unrecognized, role in the denitrification process and nitrogen removal. This functional interaction between the nitrifying and denitrifying communities may alter our traditional perspective that the nitrification and denitrification process, which requires different conditions, are functionally independent and separate.[59] The NO_2^- accumulation rate was collectively determined by the *nir*S, *nir*K, and *nos*Z genes. The second variable (*nir*S + *nir*K)/*nos*Z in the equation denotes the accumulation levels of NO and N_2O, and this variable's positive correlation with the NO_2^- accumulation rate is in agreement with previous studies that reported the inhibitory effect of NO_2^- on N_2O reduction in denitrification.[60] With respect to TN removal, the first variable (*amo*A + anammox)/bacteria partially supports our previous analysis that the coupling of DNRA, ammonia oxidation, and anammox is a noteworthy pathway contributing to TN removal. Both the archaeal nitrifier *Nitrosopumilus maritimus* sp. and the archaeal denitrifier *Pyrobaculum aerophilum* sp. have been isolated and are reported to play roles in nitrogen transformation,[61,62] accounting for the involvement of archaea in the TN equation. The third variable *nos*Z/(*nir*K + *nir*S) represents the level of nitrogen removed via the N_2 pathway, leading to its positive correlation with the TN transformation rate. Given our evidence for the involvement of the DNRA process in nitrate removal, we believe that the lack of DNRA gene data in the NO_3^- equation is the reason for the relatively lower R^2 value (0.891), as all other established equations in Tables 1 and 2 have R^2 values higher than 0.985.

Table 2. Quantitative Response Relationships between Nitrogen Transformation Rates (mg/L·d) and Functional Gene Abundance (Copies/g) in T2 (NO_3^- Treatment)

stepwise regression models (equations)	R^2	*P* value
$NO_3^- = -5.99(nxrA/narG) + 23.3$	0.891	0.005
$NO_2^- = 0.79((nirS + nirK)/bacteria) + 3.19 \times 10^{-5}$ $((nirS + nirK)/nosZ) + 0.113$	0.999	0.000
$TN = 0.22((amoA + Anammox)/bacteria) -$ 0.001archaea +0.79($nosZ/(nirK + nirS)$) + 23.4	0.999	0.001

Environmental Implications and Significance. CWs have been intensively studied and used as a promising technology to achieve sustainable wastewater treatment and nutrient management. However, most CWs operating in North America and Europe exhibit fluctuating nitrogen removal efficiencies of less than 50%, even with optimized designs.[4,63] During our operation (105−245 d), the TF CWs demonstrated robust removal of NH_4^+ (T1:76 ± 3.9%), NO_3^- (T2:97 ± 2.2%), and TN (T1 and T2:81 ± 3.5% and 93 ± 2.3%, respectively). More importantly, the higher removal efficiency and the flexibility to treat different types of nitrogen were achieved in a single-stage treatment unit of the TF CW without the need for costly operation (aeration) or extra capital

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investment (multiple treatment systems). Therefore, the combined advantages of technical viability, operational flexibility, and economic sustainability offer major benefits for the use of TF CWs in field applications, such as economically replacing CW-centered hybrid systems that require multistage treatment units of various types (i.e., surface flow, horizontal subsurface flow, vertical subsurface flow, and other treatment systems) or the easy incorporation into existing treatment systems as a deep-treatment step to further enhance nitrogen removal and polish effluent.

For a long time, denitrification was believed to be the only significant mechanism in nitrate removal.[43,64] However, our results suggest that the DNRA process, which has so far been ignored in the field of CWs, maybe an important pathway for nitrate removal. This study therefore highlights the need for more research efforts to study the importance of DNRA in the nitrogen balance. These efforts should include using stable isotope techniques (^{15}N) to accurately evaluate the contribution of DNRA to nitrogen removal. The presence of SNAD processes in T1 and the coupling of DNRA, ammonia oxidation, anammox, and denitrification in T2, which accounted for the simultaneous robust removal of nitrogen (NH_4^+ and/or NO_3^-) and COD, demonstrate that the nitrogen transformation communities were functionally dependent. This coupled multipath interactions and the presence of *nos*Z gene are believed to favor complete nitrogen removal and control of the greenhouse gas N_2O.

Although effluent nitrogen can be easily measured and continuously monitored, these data alone are not sufficient for the long-term accurate prediction of dynamic variation in water quality without insights into the underlying mechanisms that govern the removal processes. Our equations with functional gene data (Tables 1 and 2) are critical for an in-depth quantitative understanding of these underlying mechanisms, and the insights gained from these equations are therefore valuable to guide and facilitate engineering applications by incorporating multiple parameters of functional genes into existing "black-box" empirical models for the design of reliable treatment facilities and the optimization of this delicate process.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic wastewater composition and operation strategy (Table S1), schematic diagram of experimental setup (Figure S1), photo of microbial sampling columns (Figure S2), nitrogen transformation rates (Figure S3), and nitrogen transformation pathways (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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