Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)

Postprint of: W. Liu, Md. Hasibur Rahaman, J. Mąkinia, J. Zhai, Coupling transformation of carbon, nitrogen and sulfur in a long-term operated full-scale constructed wetland, Science of The Total Environment (2021), 146016, DOI: 10.1016/j.scitotenv.2021.146016

Highlights:

- Both autotrophic and heterotrophic denitrification were present in CW
- Heterotrophic denitrification was the main nitrogen removal pathway
- Heterotrophic denitrification did not inhibit autotrophic denitrification
- Increasing S^{2-} will promote autotrophic denitrification

Coupling transformation of carbon, nitrogen and sulfur in a long-term operated full-scale constructed wetland

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1. Introduction

Constructed wetlands (CWs) are well-established, nature-based, and robust wastewater treatment technology, which have been applied worldwide in the last decades (Carvalho et al., 2017; Ilyas and Masih, 2017). The physical, chemical, and biological processes in CWs can efficiently remove different kinds of pollutants, including organic matter, suspended solids, metals, and even emerging contaminants (Almeida et al., 2017; He et al., 2018; Jóźwiakowski et al., 2019). Among these pollutants, nitrogen (N) removal efficiency in previous studies are ambiguous (Ilyas and Masih, 2017; Vymazal, 2013). It has been reported that the total nitrogen (TN) removal in several investigated CWs in Poland, Brazil, and China ranged from lower than 10% to 80% (Jóźwiakowski et al., 2019; Li et al., 2018; Machado et al., 2017). These CWs may fail to comply with the increasingly stringent effluent TN standards. A combination of different types of CWs, namely hybrid CWs, is more efficient in N removal than a single CW (Vymazal, 2013). A previous study shows that a full-scale VBFW-HSFW (Vertical Baffled Flow Wetland – Horizontal Subsurface Flow Wetland) system can achieve 83% of NH_4^+ removal and 77% of total nitrogen (TN) removal (Zhai et al., 2016). The high nitrogen removal efficiency in the hybrid CWs is probably attributed to the presence of novel nitrogen removal pathway like sulfur-based autotrophic denitrification, anaerobic ammonia oxidation and dissimilatory nitrate reduction to ammonimum (Zhai et al., 2016).

In the wetlands, the most commonly known N transformation processes are nitrification and denitrification. However, recent studies reported other N transformation processes, including anaerobic ammonia oxidation (anammox), autotrophic denitrification, and dissimilatory nitrate reduction to ammonia (DNRA), are also found in the CWs (Ma et al., 2020; Nizzoli et al., 2010; Valipour and Ahn, 2017; Zhai et al., 2016). The different C/N ratios affect C and N removal pathways in CWs. During anammox, NH_4^+ can be oxidized by NO_2^- into N_2. As reported previously, over 40% of NH_4^+ could be removed attributing to anammox in a plant-bed/ditch system of a constructed wetland (Wang et al., 2018). Instead of NO_2^-, SO_4^{2-} can also be used to oxidize NH_4^+ in anammox, namely SO_4^{2-}-dependent anammox process (Rios-Del Toro et al., 2018). In this process, NH_4^+ is oxidized to N_2 coupled to the reduction of SO_4^{2-} to S^0. Autotrophic denitrification is another important N transformation pathway. The autotrophic denitrification uses sulfur-compounds, such as elemental sulfur (S^0), $S_2O_3^-$, and S^{2-} or FeS, as the electron donor to convert NO_3^- or NO_2^- into N_2. Other electron donors like hydrogen (H_2) or reduced metal can also be used in the autotrophic denitrification (Guo et al., 2020; Tang et al., 2020). It was reported in an anaerobic digester that the contribution of autotrophic denitrification to TN removal can reach 90% without organic supplementation (Qiu et al., 2020). This result showed that autotrophic denitrification has great potential for TN removal in CWs when organic supply was limited. However, autotrophic denitrification may cause NO_2^- accumulation, limiting TN removal efficiency (Chen et al., 2018; Qiu et al., 2020). The dissimilatory nitrate reduction to ammonia (DNRA) is an alternative NO_3^- reduction pathway observed in the constructed wetland (Zhang et al., 2021). Previous studies showed that the DNRA could outcompete denitrification in natural system like subtropical pasture soils, freshwater sediment and marine sediment under certain conditions like high C/N ratios, high concentrations of sulfide, low concentrations of iron, or high temperature (Friedl et al., 2018; Holmes et al., 2019). The N removal in the CWs is coupled with C and S compound transformation. The sulfate reduction using SO_4^{2-} to oxidize organic carbon shows the coupling transformation of C and S compounds in the CWs (Guo et al., 2020). Thus, the carbon, nitrogen and sulfur transformation in CWs is intertwined with one another (Baldwin, 2016; Zhou, 2007). Table 1 summarized the biochemical reactions involved in coupling carbon, nitrogen, and sulfur transformation commonly observed in the CWs. However, experiments on coupling carbon, nitrogen, and sulfur transformation were primarily conducted in lab-scale CWs, and studies on full-scale systems are limited. Investigation on carbon, nitrogen, and sulfur transformation in full-scale systems, especially in long-term operated CWs, is required.

Table 1 Potential biochemical reactions involved in coupling carbon, nitrogen, and sulfur transformation in the CWs [a]

Biochemical reactions	No.	Type [b]	E acceptor	E donor	Functional Microorganisms	Ref.
Methanogenesis						
$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	(1)	H	CH_3COO^-	CH_3COO^-	Methanogens	(Fenchel et al., 2012)
Nitrification						
$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 4H^+ + 2H_2O$	(2)	A	O_2	NH_4^+	Ammonia-oxidizing bacteria	(Saeed and Sun, 2012)
$2NO_2^- + O_2 \rightarrow 2NO_3^-$	(3)	A	O_2	NO_2^-	Nitrifying bacteria	(Saeed and Sun, 2012)
Anaerobic ammonia oxidation (Anammox)						
$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$	(4)	A	NO_2^-	NH_4^+	Anammox bacteria	(Holmes et al., 2019)
Sulfate-reducing anaerobic ammonia oxidation						
$8NH_4^+ + 3SO_4^{2-} \rightarrow 4N_2 + 3HS^- + 12H_2O + 5H^+$	(5)	A	SO_4^{2-}	NH_4^+	Anammox bacteria	(Rikmann et al., 2012)
Heterotrophic denitrification						
$4NO_3^- + CH_3COO^- \rightarrow 4NO_2^- + 2HCO_3^- + H^+$	(6)	H	NO_3^-	CH_3COO^-	Nitrate-reducing bacteria	(Castro-Barros et al., 2017)
$8NO_3^- + 5CH_3COO^- + 3H^+ \rightarrow 4N_2 + 10HCO_3^- + 4H_2O$	(7)	H	NO_3^-	CH_3COO^-	Nitrate-reducing bacteria	(Castro-Barros et al., 2017)
$8NO_2^- + 3CH_3COO^- + 5H^+ \rightarrow 4N_2 + 6HCO_3^- + 4H_2O$	(8)	H	NO_2^-	CH_3COO^-	Nitrite-reducing bacteria	(Castro-Barros et al., 2017)
Autotrophic denitrification						
$HS^- + 4NO_3^- \rightarrow 4NO_2^- + SO_4^{2-} + H^+$	(9)	A	NO_3^-	HS^-	Sulfide-oxidizing bacteria	(Ma et al., 2020)
$5HS^- + 8NO_3^- + 3H^+ \rightarrow 4N_2 + 5SO_4^{2-} + 4H_2O$	(10)	A	NO_3^-	HS^-	Sulfide-oxidizing bacteria	(Ma et al., 2020)
$3HS^- + 8NO_2^- + 5H^+ \rightarrow 4N_2 + 3SO_4^{2-} + 4H_2O$	(11)	A	NO_2^-	HS^-	Sulfide-oxidizing bacteria	(Ma et al., 2020)
Dissimilatory nitrate reduction to ammonium (DNRA)						
$NO_3^- + CH_3COO^- + H^+ + H_2O \rightarrow NH_4^+ + 2HCO_3^-$	(12)	H	NO_3^-	CH_3COO^-	Sulfide-oxidizing bacteria	(Castro-Barros et al., 2017)
Dissimilatory sulfate reduction						
$SO_4^{2-} + CH_3COO^- \rightarrow HS^- + 2HCO_3^-$	(13)	H	SO_4^{2-}	CH_3COO^-	Sulfate-reducing bacteria	(Schreier et al., 2010)

a. The acetate (CH_3COO^-) was used as a model organic carbon source in reaction
b. A=Autotrophic process; H=Heterotrophic process

In this study, sediment collected from a full-scale hybrid CW were incubated for 48h in the laboratory to reveal the coupling transformation of carbon, nitrogen and sulfur compounds by varying initial dosages of key compounds, including organic carbon (acetate), NH_4^+, NO_2^-, NO_3^-, S^{2-}, and SO_4^{2-}. Besides, microbial high-throughput sequencing analysis was to identify key microbes responsible for transforming carbon, nitrogen, and sulfur compounds. The outcomes will lead to a better understanding of coupling carbon, nitrogen, and sulfur transformation and key nitrogen removal pathways in long-term operated CWs.

2. Materials and Methods

2.1 Study site

The sediments used in this study were collected from a full-scale hybrid CW in Chongqing, China which is operating since January 2011. This hybrid CW consists of pre-treatment stages, a vertical-baffled flow wetland (VBFW, first stage), a horizontal subsurface flow wetland (HSFW, second stage) and a clean water pond for post-treatment (Zhai et al., 2016) (Figure S1). The influent of the CW was mainly composed of the municipal wastewater and partial agriculture wastewater at the flow rate of 500-600 m^3/d. The studied HSFW contained 3-5 sections of HSFW bed (Figure S1) with a water depth of 0.4-0.6m (Zhai et al., 2016). The HSFW was operated under a combination of anoxic and aerobic conditions (DO < 0.5mg/L, 100mV < ORP < 150 mV) with effective TN removal (~77%) (Zhai et al., 2016).

2.2 Sediment sampling

The sediment samples collected from the HSFW from December 2017 to June 2018 were used for incubation in batch experiments (Figure S1, red circle indicated sampling locations), while all the sediment samples were used for microbial analysis. The temperature and pH of the samples were measured on-site using a multi-detector (Waters, USA).

During each sampling period, composite sediment samples were collected from 6 locations by opending the HSFW bed at the depth of approximately 10cm below the sediment surface (50-70cm below the water surface). Therefore, the anaerobic sediment samples were taken. The collected sediment samples were immediately transferred into the containers which were flushed by pure N_2 and sealed before use. The collected sediment samples from 6 location will be mixed homogenously after pre-treatment. The pre-treatment of the samples included two steps: (1) the sediment was sieved using a 100-mesh sieve to remove small stones, grass, and other waste; and (2) the sieved samples were further mixed and settled for solid-liquid separation. The liquid portion was discarded, and the solid portion was used as inoculum in the batch experiments and subsequent microbial investigations. All the sediment samples were stored in an icebox and kept at -4 ºC during the transport to the laboratory. Based on the temperature during the sampling campaign, the whole experiment was broadly divided into the high-temperature season (HT), from May to October with an average temperature of 30±2ºC, and the low-temperature season (LT), from November to April next year with an average temperature of 16±2 ºC.

2.3. Batch experiments

The collected sediments were used as inoculum in the batch experiments to investigate coupling transformation of carbon, nitrogen, and sulfur compounds by using various initial concentrations. Approximately 100 mL pre-treated sediment samples were added into a 1000 mL bottle containing 500 mL medium (the recipe is shown in Table S1). All the bottles were flushed with helium for 20 min to ensure anaerobic condition. The bottles were cultivated at the temperature which was same as the water temperature of the water layer above the sediment. The temperature was maintained by the air bath for 48h.

During the experiments, sodium acetate (CH_3COONa), ammonium chloride (NH_4Cl), sodium nitrite ($NaNO_2$), sodium nitrate ($NaNO_3$), sodium sulfide (Na_2S) and potassium sulfate (K_2SO_4) were added at three different initial concentrations (Low, Medium, and High). The low concentration groups did not add target compounds while the medium concentration group added sufficient amount of target compounds. In the high concentration groups, the supplied amounts of target compounds was doubled. These concentrations were selected to reveal what biochemical process was present in the CW for the transformation of carbon, nitrogen and sulfur, and also reveal the potential maximum capacity of transformation. Using the same approach, the blank control with autoclaved sediments was carried out in parallel with the other experiments. In total, 33 experiments in six trials were carried out to investigate the key carbon, nitrogen, and sulfur transformation processes (Table 2).

Table 2 Feed composition in the batch test trials

Test trial	Batch No.	CH_3COONa	NH_4Cl	$NaNO_2$	$NaNO_3$	Na_2S	K_2SO_4
		(mg C/L)	(mg N/L)			(mg S/L)	
Blank control (Autoclaved)		0	15	5	17.5	2.5	10
Batch 1 (organic carbon transformation)	C1	0	15	5	17.5	2.5	10
	C2	25	15	5	17.5	2.5	10
	C3	50	15	5	17.5	2.5	10
Batch 2 (NH_4^+ transformation)	N1	0	0	5	17.5	2.5	10
	N2	0	15	5	17.5	2.5	10
	N3	0	30	5	17.5	2.5	10
	N4	25	0	5	17.5	2.5	10
	N5	25	15	5	17.5	2.5	10
	N6	25	30	5	17.5	2.5	10
Batch 3 (NO_2^- transformation)	N7	0	15	0	17.5	2.5	10
	N8	0	15	5	17.5	2.5	10
	N9	0	15	10	17.5	2.5	10
	N10	25	15	0	17.5	2.5	10
	N11	25	15	5	17.5	2.5	10
	N12	25	15	10	17.5	2.5	10
Batch 4 (NO_3^- transformation)	N13	0	15	5	0	2.5	10
	N14	0	15	5	17.5	2.5	10
	N15	0	15	5	35	2.5	10
	N16	25	15	5	0	2.5	10
	N17	25	15	5	17.5	2.5	10
	N18	25	15	5	35	2.5	10
Batch 5 (S^{2-} transformation)	S1	0	15	5	17.5	0	10
	S2	0	15	5	17.5	2.5	10
	S3	0	15	5	17.5	5	10
	S4	25	15	5	17.5	0	10
	S5	25	15	5	17.5	2.5	10
	S6	25	15	5	17.5	5	10
Batch 6 (SO_4^{2-} transformation)	S7	0	15	5	17.5	2.5	0
	S8	0	15	5	17.5	2.5	10
	S9	0	15	5	17.5	2.5	20
	S10	25	15	5	17.5	2.5	0
	S11	25	15	5	17.5	2.5	10
	S12	25	15	5	17.5	2.5	20

During the batch experiments, samples were collected at 0, 24 and 48 h. The bottles were mixed gently and settled for at least 30 min. Then, 50 mL water samples were taken using a syringe, filtrated immediately on a 0.45µm pore size membrane filter, and analyzed for total organic carbon (TOC), dissolved inorganic carbon (DIC), total carbon (TC), NH_4^+, NO_2^-, NO_3^-, total dissolved inorganic nitrogen

(TDIN), dissolved S^{2-} and SO_4^{2-}. The samples were stored at 4 ºC before the analysis within 2 h. To avoid rapid oxidation of S^{2-}, the samples for S^{2-} analysis was measured immediately after sampling. Gas samples from the batch bottles were collected with a micro-syringe and immediately analyzed for methane (CH_4) and carbon dioxide (CO_2).

2.4 Microbiological investigations

The sediments were centrifuged and washed by sterilized Mili Q water for 2 times at 5000 rpm for 15min. The liquid parts after centrifugation were collected and mixed, and the mixture was centrifuged at 8000 rpm for 5 min at 4 ºC. Then, the solid part was used for DNA extraction in the microbial analysis. The DNA extraction was performed with an EZNA® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instructions.

The extracted DNA was amplified with a pair of primers 338F (5'-AC TCC TAC GGG AGG CAG A-3') and 806R (5'-GG ACT ACH VGG GTW TCT AAT-3'). Reaction mixtures (20μL) for PCR contained: 10 ng of template DNA, 0.8 μL of each primer (5 μM), 4μL of 5-fold Fast*Pfu* Buffer, 2 μL of dNTPs (2.5 mM), 0.4 μL of Fast *Pfu* Polymerase and ultrapure H_2O. The PCR procedure was: initial denaturation at 95 °C for 3 min, followed by 28 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s; final extension at 72 °C for 10 min. The PCR products were extracted, purified with an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), quantified with QuantiFluor™ -ST (Promega, USA), and then sequenced on the Illumina MiSeq platform (Shanghai Majirbio Technology Co., Ltd., China). The operational taxonomic units (OTU) of the identified 16S rRNA gene sequences were analyzed by the Majorbio I-Sanger Cloud online platform (www.i-sanger.com), using RDP classifier Bayesian Algorithms (3% difference of the sequence as classification standards).

2.5 Analytical methods

Analysis of gas samples for CH_4 and CO_2 was performed by a gas chromatograph (7820A, Agilent, USA) equipped with a thermal conductivity detector (TCD), a hydrogen flame ionization detector (HFID) and packed columns of MolSieve 5A, Heyesep Q. Pure helium was supplied as the carrier gas during the measurement. The Ideal-Gas Equation was used to convert the percentage of CH_4 and CO_2 into the concentration of the gaseous C (mg C/L).

Analysis of total organic carbon (TOC) and dissolved inorganic carbon (DIC) was performed with a TOC analyzer (TOC-L, SHIMADZU, Japan). The analysis of anions in the liquid phase including NO_2^-, NO_3^-, and SO_4^{2-} were performed by an ion chromatograph equipped with anion self-regenerating suppressor (Dionex ASRS 600, 4 mm)， an IonPac AS19 separation column (Dionex AS19, 4×250 mm) and an IonPac AG19 anion guard column (Dionex AG19, 4×50 mm) according to the standard methods (Rice et al., 2012). The details can be found in supplementary materials (Text S1). The S^{2-} analysis was performed by methylene blue colorimetric method based on the standard methods (Rice et al., 2012).

2.6. Data analysis

Differences in batch experiment outcomes and abundance of microorganisms at HT and LT were determined by one-way analysis of variance (ANOVA) using Origin Pro 2018 (OriginLab co., USA). Spearman's correlation coefficient (ρ) was used in this study to quantify the strength of the relationship among the microbial abundance, the carbon, nitrogen, and sulfur transformation capacity, and temperature (Xie et al., 2016). During the experiments, the initial concentrations of carbon, nitrogen, and sulfur compound all varied, as well as the temperature. The relationship between carbon, nitrogen, and sulfur transformation in the batch experiment and microbial association was done by canonical correlation analysis (CCA) using Canoco 4.5 software (Zhimiao et al., 2016). All the other figures were produced via Origin Pro 2018 (OriginLab co., USA).

3. Results and Discussion

3.1 Effects of organic carbon on N and S transformation

The effects of organic carbon (TOC) on N and S transformation (Batch 1) were shown in Figure 1. In the low temperature (LT) season, increasing initial TOC dosages led to decrease NO_2^- generation and obtained slight removal (34.05% of generation to 10.98% of removal, p=0.12), and higher NO_3^- removal (0.26% to 30.06%, p=0.41). The SO_4^{2-} generation was declined from 18.85% to 10.81% (p=0.73) at high TOC dosage. However, increasing TOC dosages had no effects on the removal of NH_4^+ (1.10% to 1.66%, p=0.72) and S^{2-} (51.21% to 59.60%, p=0.95). The results also showed that the differences of N and S compound transformation at different TOC levels in LT seasons were insignificant (p> 0.05).

Similar to the LT, in high temperature (HT) season, increasing initial TOC dosages had no effects on NH_4^+ removal (5.63% to 5.92%, p=0.66) and S^{2-} removal (51.05% to 58.45%, p=0.52). But increasing TOC dosages significantly promote both NO_2^- transformation from 77.33% of generation to 87.23% of removal (p=0.009<0.01), and NO_3^- removal increased from 31.06% to 92.36% (p=0.008<0.01). Besides, the generation of SO_4^{2-} was lowered at higher TOC dosages (40.97% to 27.64%), though the difference was insignificant (p=0.29).

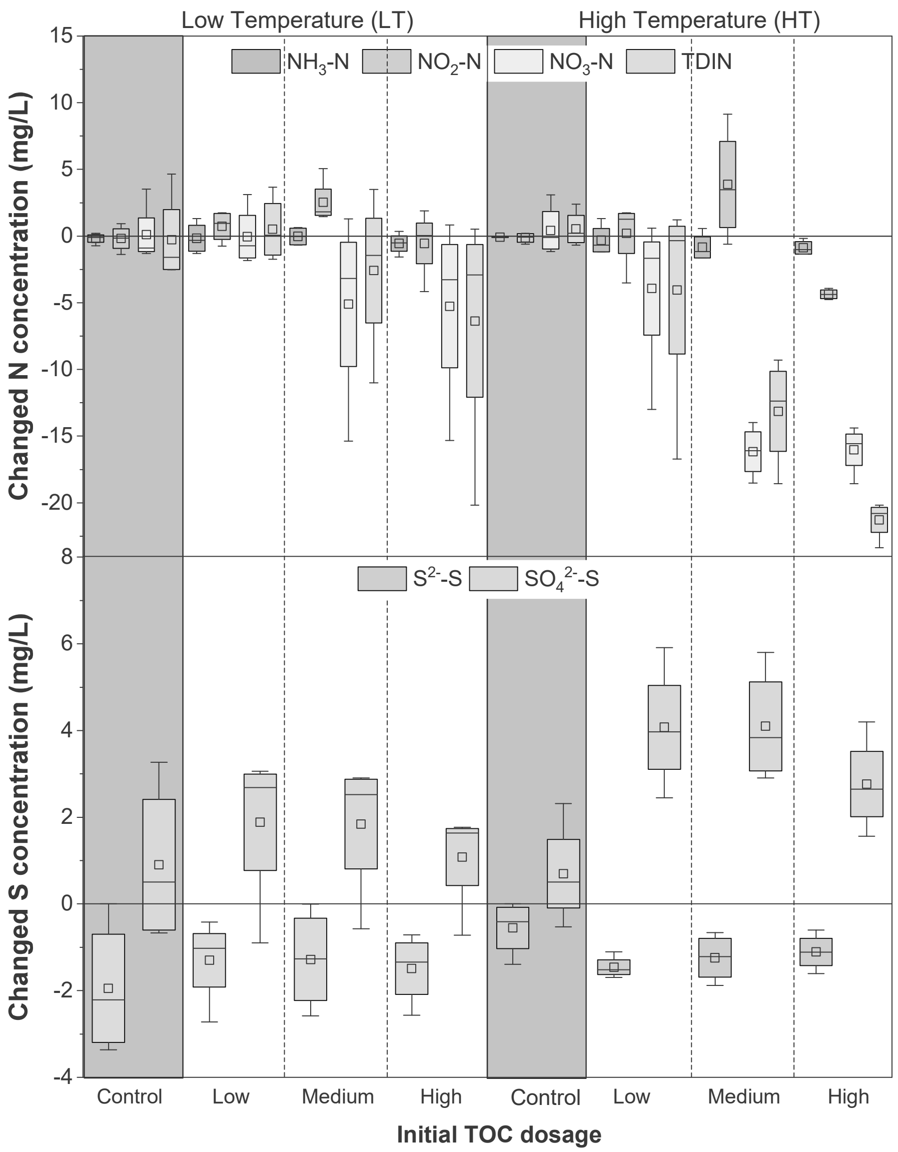


Figure 1. Transformation of N and S compounds at different initial TOC dosage. Control: Abiotic control group; Low: $[TOC]_0$=0 mgC/L; Medium: $[TOC]_0$=25 mgC/L; High: $[TOC]_0$=50 mgC/L. The positive values represent generation and the negative values represent removal. The small square represents the average value.

3.2 Effects of N compounds on carbon, nitrogen, and sulfur transformation

(1) NH_4^+

Effects of NH_4^+ on N and S transformation with and without organic carbon (Batch 2) was shown in Figure 2. Without organic carbon, increasing NH_4^+ dosages had no significant effects on the NH_4^+ removal (LT:1.63% to 2.74%, p=0.96; HT: 1.97% to 2.96%, p=0.96), NO_3^- removal (LT: 3.99% to 7.07%, p=0.94; HT:15.79% to 22.43%, p=0.89), S^{2-} removal (LT: 40.65% to 44.52%, p=0.97; HT:43.59% to 55.29%, p=0.38), and SO_4^{2-} generation (LT: 2.27% to 8.39%, p=0.81; HT:36.34% to 40.74%, p=0.92) in both LT and HT season. The NO_2^- generation in LT season was stable (15.35% to 19.84%, p=0.51) but insignificant decrease from 11.13% to 3.89% (p=0.92) in the HT season.

When organic carbon added, increasing NH_4^+ dosages led to stable removal of TOC (LT: 24.65% to 31.64%, p=0.96; HT: 76.62% to 84.76%, p=0.19). Similarly, removal of NH_4^+ (LT: 2.66% to 4.89%, p=0.85; HT:5.26% to 6.05%, p=0.44), NO_3^- (LT:37.31% to 37.69%, p=0.99; HT: 98.89% to 99.39%, p=0.82), S^{2-} (LT: 31.56% to 39.65%, p=0.91; HT: 47.19% to 57.44%, p=0.80), and generation of NO_2^- (LT: 68.59% to 74.19%, p=0.14; HT: 262.82% to 323.25%, p=0.23) and SO_4^{2-} (LT: 12.94% to 18.64%,p=0.91; HT:24.98% to 35.49%, p=0.51) at different NH_4^+ dosages had no significant differences.

(2) NO_2^-

Effects of NO_2^- on N and S transformation with and without organic carbon (Batch 3) was shown in Figure 2. Without organic carbon, increasing initial NO_2^- dosages did not affect NH_4^+ removal (LT: p=0.80; HT: p=0.08). Besides, the NO_2^- accumulation (LT: p= 0.78; HT: p=0.29) and NO_3^- removal (LT: p=0.83; HT: p=0.70) were stable, as well as the S^{2-} removal (LT: p=0.79; HT: p=0.40) and SO_4^{2-} generation (LT: p=0.63; HT: p=0.65) were also stable at various initial NO_2^- dosages.

Adding organic carbon to TOC= 25mgC/L led to higher NO_3^- removal and NO_2^- accumulation (Figure 2, S3). However, increasing initial NO_2^- in presence of TOC still had no effects on TOC removal (LT: 19.80% to 20.54%, p= 0.86; HT: 77.96% to 82.61%, p=0.75), NH_4^+ (LT: p= 0.91; HT: p=0.12) and NO_3^- removal (LT: p= 0.96; HT: p=0.91) and NO_2^- generation (LT: p= 0.53; HT: p=0.89) transformation, and

S^{2-} removal (LT: p= 0.98; HT: p=0.57) and SO_4^{2-} transformation (LT: p= 0.36; HT: p=0.79).

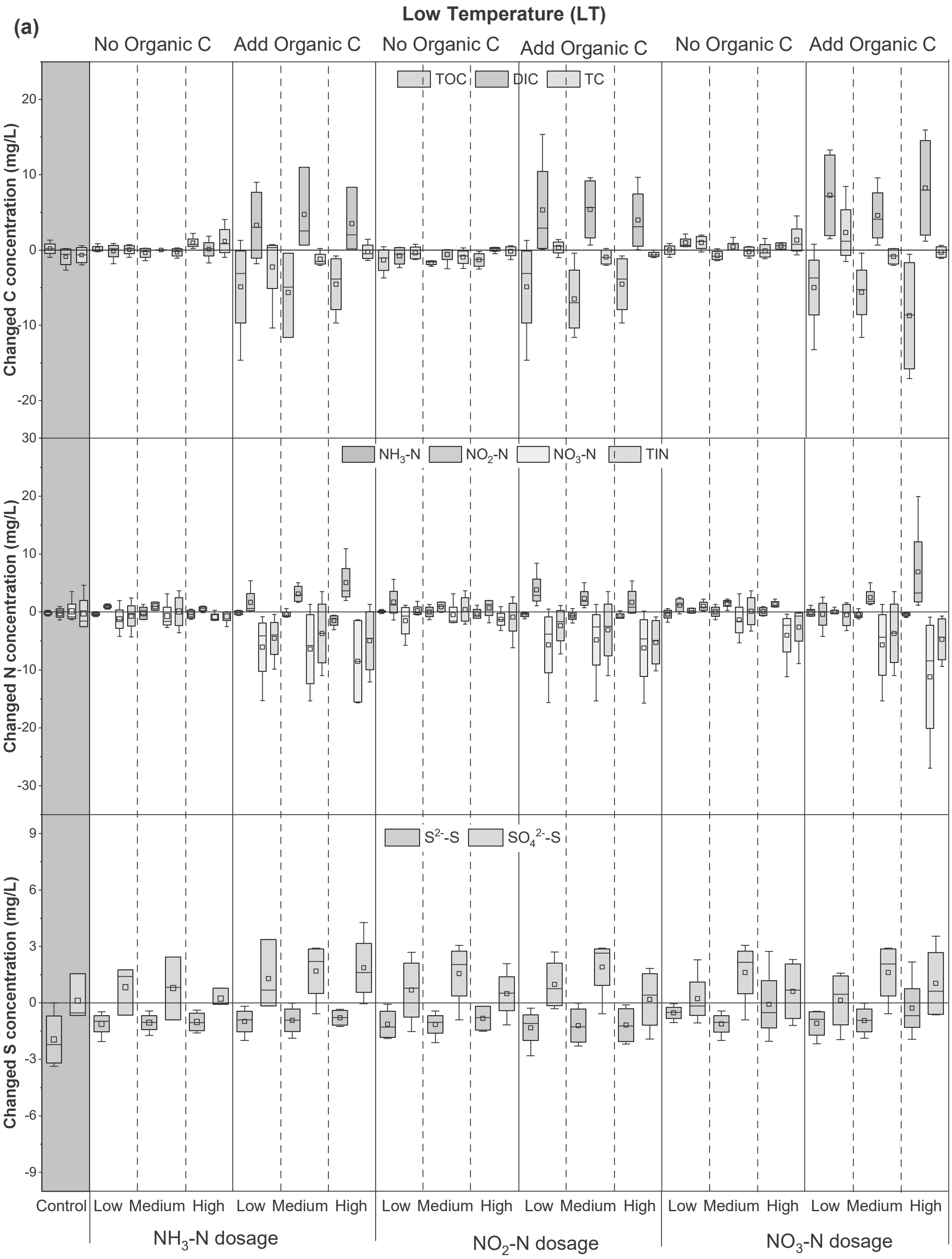
(3) NO_3^-

In Batch 4, different dosages of NO_3^- (0, 10, 20 mgN/L) were added. When no organic carbon was added, increasing initial NO_3^- dosages had no significant influence on the transformation of carbon, nitrogen, and sulfur compounds in both LT and HT seasons (Figure 2, S3, P > 0.10, Table S2). When organic carbon was added (TOC=25 mgC/L), increasing initial NO_3^- dosages will promote TOC removal from 20.79% to 37.48% (p=0.68) in LT season and from 32.15% to 81.55% (p=0.004 < 0.01) in the HT season. Besides, differences of NO_3^- removal (LT: p=0.19, HT: $p=1.35\times10^{-6}$) and NO_2^- accumulation (LT: p=0.21, HT: p=0.008) at different NO_3^- dosages was also significant, especially in the HT season. The S^{2-} removal and SO_4^{2-} generation was not significantly different at various NO_3^- dosages (p> 0.52). Overall, current results indicate that the variation in initial N compounds had little or no significant influences on carbon, nitrogen, and sulfur transformation throughout the batch experimental periods.

At neutral pH used in our experiment, the main form of ammonia nitrogen was NH_4^+. A high concentration of NH_4^+ was regarded as an inhibitor in anaerobic digestion but the ammonium toxicity was very reversible (Yenigün and Demirel, 2013). The high NH_4^+ concentration is also toxic to the bacteria. The high NH_4^+ concentration will lead to the active transport of this compound across the cytoplasmic membrane. As a result, a harmful energy-wasting futile cycling occurred to move the accumulated NH_4^+ back into the medium (Müller et al., 2006). In our experiment, increasing NH_4^+ to 30 mgN/L had no inhibiting effects on carbon, nitrogen, and sulfur transformation.

The NO_2^- can also be used as an electron acceptor in both heterotrophic denitrification and sulfide-based autotrophic denitrification (Mahmood et al., 2007; Sun and Nemati, 2012), but high concentrations of NO_2^- were also toxic to microbial communities, leading to inhibition of both denitrification processes

(Glass et al., 1997; Tang et al., 2020). In our experiment, increasing NO_2^- dosage showed no obvious inhibiting effects on microbial carbon, nitrogen, and sulfur transformation.



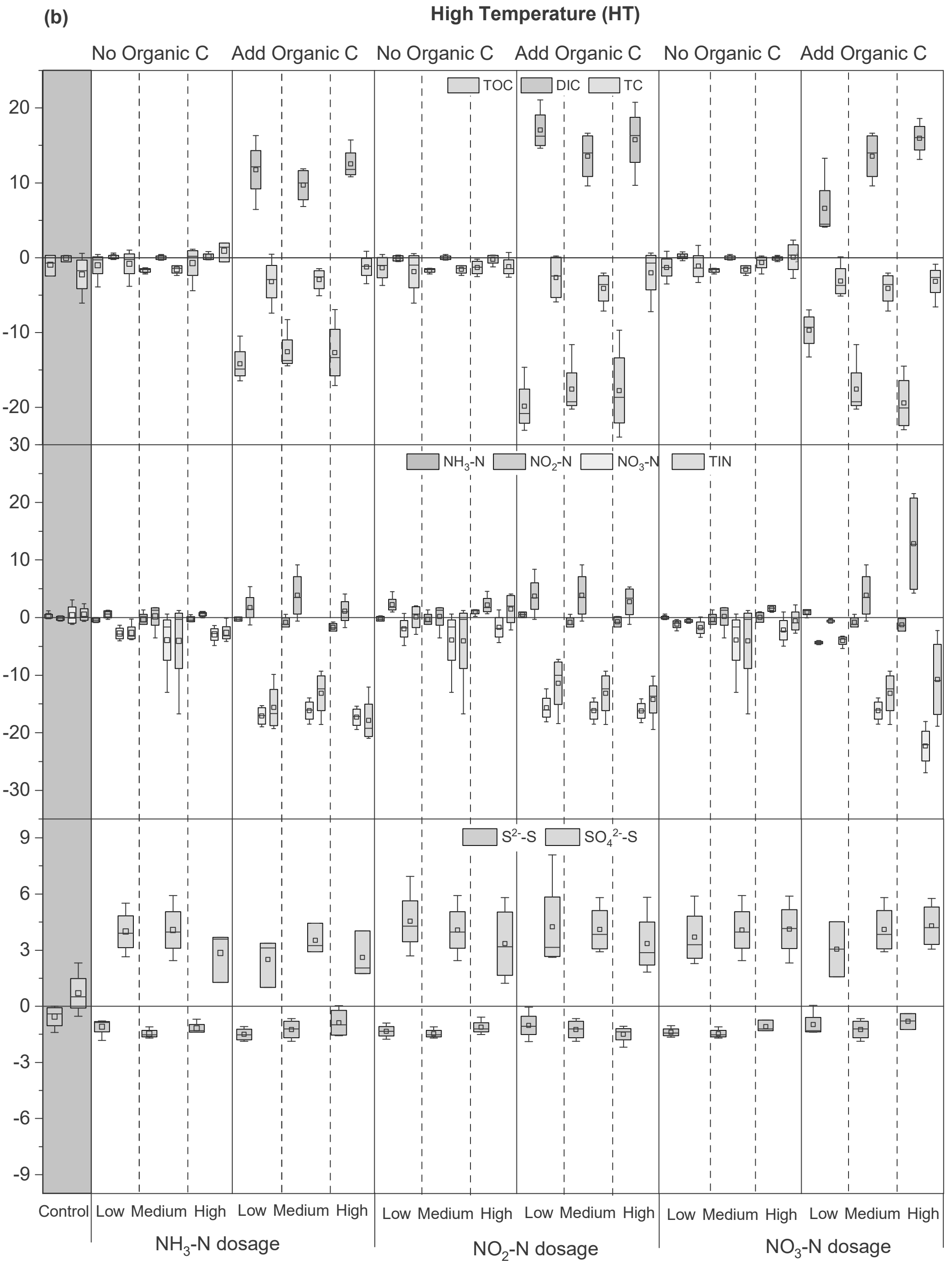


Figure 2. Transformation of Carbon (C), Nitrogen (N) and Sulfur (S) compounds at different initial dosage of N compounds. (a) in the low temperature seasons; (b) in the high temperature seasons. Control: Abiotic control group; Low: $[NH_4^+]_0$=0 mgN/L, $[NO_2^-]_0$=0 mgN/L, $[NO_3^-]_0$=0 mgN/L; Medium: $[NH_4^+]_0$=15 mgN/L, $[NO_2^-]_0$=5 mgN/L, $[NO_3^-]_0$=10 mgN/L; High: $[NH_4^+]_0$=30

mgN/L, $[NO_2^-]_0$=10 mgN/L, $[NO_3^-]_0$=20 mgN/L. The positive values represent generation and the negative values represent removal. The small square represents for the average value.

3.3 Effects of S compounds on carbon, nitrogen, and sulfur transformation

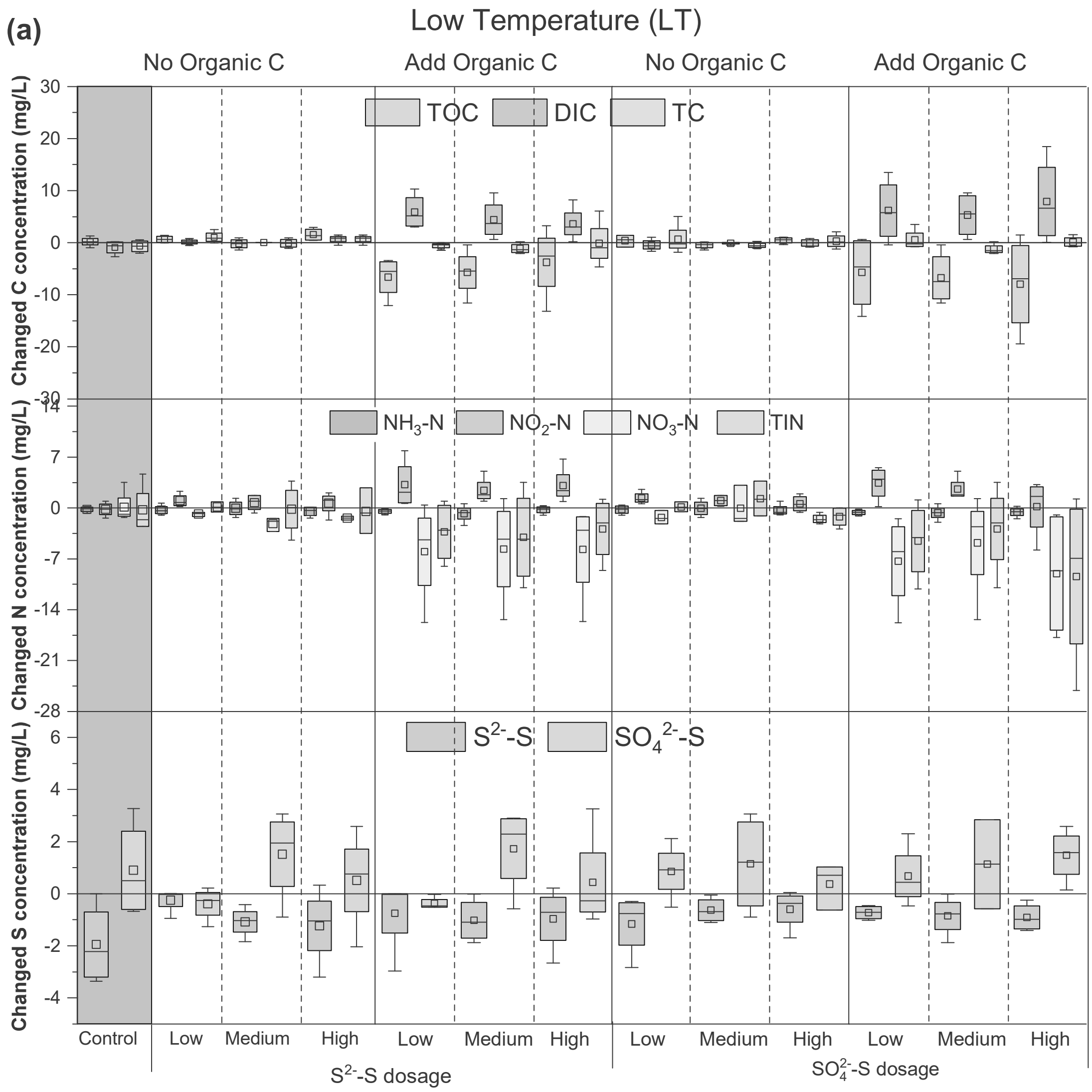
(1) S^{2-}

In Batch 5, various initial S^{2-} dosages (0, 2.5, and 5 mgS/L) were added into the batches. When there was no organic carbon added, increasing initial S^{2-} dosages led to higher NO_3^- removal and NO_2^- generation (Figure 3, S4) but the differences were not significant. The removal efficiency of NO_3^- increased from 2.8% to 11.9% in LT season ($p=0.98$) and from 7.1% to 25.2% in HT season ($p=0.53$). Meanwhile, the S^{2-} removal (LT: $p=0.19$, HT: $p=0.19$) increased but SO_4^{2-}generation decreased (LT: $p=0.14$, HT: $p=0.09$). Adding organic carbon improved NO_3^- removal and NO_2^- generation (Figure 3, S4), but increasing initial S^{2-} dosages resulted in no significant differences in NO_3^- removal (LT: $p=0.99$, HT: $p=0.95$) and NO_2^- generation (LT: $p=0.90$, HT: $p=0.96$). As to the S compounds, higher initial S^{2-} dosages promoted S^{2-} removal (LT: $p=0.81$, HT: $p=0.02$) but led to less SO_4^{2-} generation (LT: $p=0.07$, HT: $p=0.27$).

(2) SO_4^{2-}

In the Batch 6, initial SO_4^{2-} dosages were varied in the experiment (0, 10, 20 mgS/L). When no organic carbon was added, increasing initial SO_4^{2-} dosages had no influence on carbon, nitrogen, and sulfur transformation ($p>0.16$, Figure 3, S4, Table S2). When organic carbon was added to 25 mgC/L (TOC), increasing initial SO_4^{2-} dosages had no influence on TOC removal (LT: $p=0.90$, HT: $p=0.77$), NO_3^- removal (LT: $p=0.98$, HT: $p=0.95$) and NO_2^- generation (LT: $p=0.75$, HT: $p=0.98$). Even though the S^{2-} removal was stable (LT: $p=0.89$, HT: $p=0.47$), higher SO_4^{2-} dosages seems lead to less SO_4^{2-} generation (LT: $p=0.69$, HT: $p=0.95$).

It is notable that the amount of S forming SO_4^{2-} was higher than the detected S^{2-} converted. This is because S^{2-} was easily form precipitations like FeS and retained in solid form, leading the total amount of S^{2-} is higher than the amount in the liquid phase. In our experiments, the detected S^{2-} initial concentration was 1.31±0.25 mg S/L, lower than the 2.5 mg S/L supplied by adding Na_2S (Table 2). The solid S^{2-} was also feasible electron donors for sulfide-based autotrophic denitrification (Eq. 12) (Hu et al., 2020; Wei et al., 2017) and converted into SO_4^{2-}. Generally, varying S compounds will affect the transformation of N but the differences were insignificant.



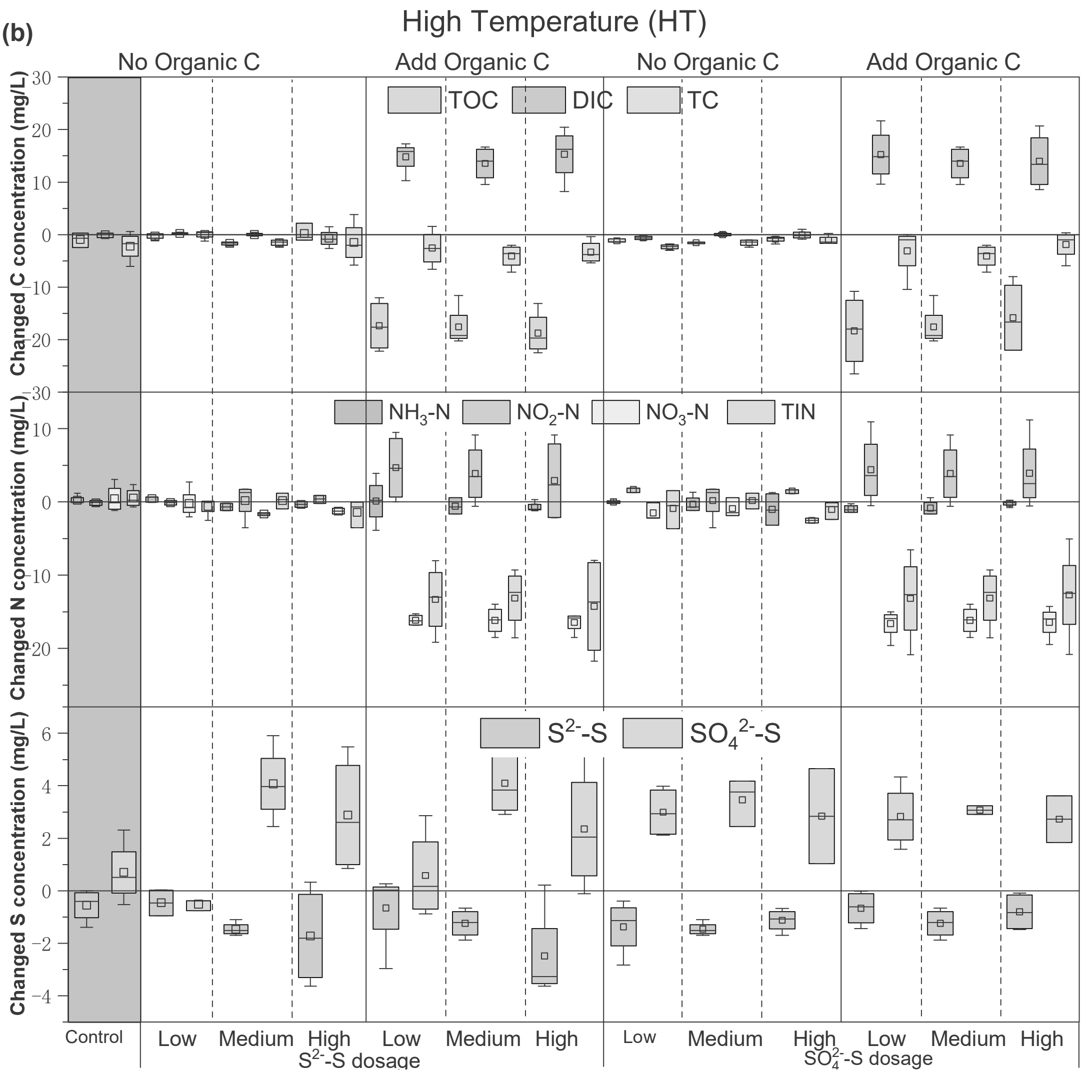


Figure 3. Transformation of Carbon (C), Nitrogen (N) and Sulfur (S) compounds at different initial dosage of S compounds. (a) in the low temperature seasons; (b) in the high temperature seasons. Control: Abiotic control group; Low: $[S^{2-}]_0$=0 mgS/L, $[SO_4^{2-}]_0$=0 mgS/L; Medium: $[S^{2-}]_0$=2.5 mgS/L, $[SO_4^{2-}]_0$=10 mgS/L; High: $[S^{2-}]_0$=5 mgS/L, $[SO_4^{2-}]_0$=20 mgS/L. The positive values represent generation and the negative values represent removal. The small square represents for the average value.

3.4 Electron balance analysis and potential biochemical processes.

The electron balance analysis was performed to help identify the potential biochemical processes that occurred in the batch experiments. When there was no organic carbon added, the electron donation was mainly from S^{2-} oxidation to SO_4^{2-}. Even though NH_4^+ was also an electron donor in processes like anammox, the electrons donated from NH_4^+ oxidation could be neglectable because the transformation of NH_4^+ was low (< 8%) and insignificant in all batches (Figure 1-3, S3, Table S2). The theoretical electron requirement for 1mol NO_2^- and 1mol NO_3^- converting into N_2 was 3mol and 5mol, respectively.

In the experiment without adding organic carbon, the donated electron calculated based on the SO_4^{2-} generation was close to the accepted electrons calculated by NO_3^- and NO_2^- reduction (Details in Text S1). The results showed that the sulfide-based autotrophic denitrification was present in the studied CW. In our experiment, both increasing NO_2^- dosages (Batch 3) and increasing NO_3^- dosages (Batch 4) had no effects on S^{2-} removal or SO_4^{2-} generation, but increasing initial S^{2-} dosages (Batch 5) led to higher NO_3^- removal and NO_2^- generation. The result was different from previous studies, in which increasing NO_3^- could promote sulfide-based autotrophic denitrification (Sun and Nemati, 2012). In our experiment, the limiting factor for sulfide-based denitrification was the concentration of S^{2-} instead of concentrations of electron acceptors. The NO_2^- accumulation was also observed (Figure 2,3) as reported previously in sulfur/sulfide-based autotrophic denitrification (Ge et al., 2012; Liu et al., 2017). That was because nitrate reductase (Nar) is more competitive to obtain electrons comparing with nitrite reductase (Nir) (Ge et al., 2012), leading to NO_3^- a preferable electron acceptor in sulfide-based autotrophic denitrification.

When organic carbon (CH_3COO^- in the experiment) was added, TOC was also an important electron donor. In theory, oxidizing 1 mol TOC into CO_2 could donate 4 mol electron. Electron balance analysis showed that the heterotrophic denitrification occurred when organic carbon was added (Text S1). The S^{2-} removal (LT: $p>0.76$; HT: $p>0.41$) and SO_4^{2-} generation (LT: $p>0.19$; HT: $p> 0.25$) with and without organic carbon was almost the same in all batches (Table S2), indicating that the sulfide-based autotrophic denitrification was co-existed with heterotrophic denitrification. Simultaneous sulfur/sulfide-based autotrophic and heterotrophic denitrification has been reported in CWs and other anaerobic bioreactors (Guerrero et al., 2016; Ma et al., 2020; Oh et al., 2001). Initial TOC dosage had no effects on S^{2-} removal (LT: p=0.94; HT: p=0.52) and the slight decrease of SO_4^{2-} generation (LT: p=0.73; HT: p=0.29), showing that promote heterotrophic denitrification will not inhibit sulfide-based autotrophic denitrification.

Increasing TOC addition (Batch 1) could reduce NO_2^- accumulation and promote NO_3^- removal, leading to better heterotrophic denitrification, while increasing NO_3^- (Batch 4) also promoted TOC removal, similar to other studies (Sun and Nemati, 2012). However, increasing NO_2^- (Batch 3) had no effects on TOC removal. In denitrification, NO_2^- and NO_3^- will compete for organic carbon as electron donor, and NO_3^- was the prior to use because of the higher electron-obtain capacity of Nar than Nir. Therefore, the organic carbon became the limiting factor in heterotrophic denitrification using NO_2^- in this experiment. Thus, whether the dissimilatory SO_4^{2-} reduction (Eq.13) (Qian et al., 2019) was present in this study was unclear. The insignificant reduction of SO_4^{2-} generation at higher TOC was probably via dissimilatory SO_4^{2-} reduction (Eq. 13).

In Batch 2, increasing NH_4^+ dosages insignificantly decreased NO_2^- generation without organic carbon in the HT season, but under the same conditions in Batch 3, increasing NO_2^- concentrations did not influence NH_4^+ removal. Based on the result, it is hard to exclude anammox in the system but the evidence of anammox was unclear. Since increasing initial SO_4^{2-} dosages did not influence NH_4^+ transformation, the contribution of SO_4^{2-} -dependent anammox in the CW was negligible. Besides, since no CH_4 was detected during the experiment, methanogenesis was not observed.

4. Role of microorganisms in carbon, nitrogen, and sulfur transformation

As shown in Figure 4, the dominant bacteria (proportion > 1% in the genus level) were categorized based on their function in carbon, nitrogen, and sulfur transformation processes. The complete list of different functional bacteria was listed in Table S3. The dominant genus in the community was *Thiobacillus* in both HT season (16.5%) and LT season (12.1%). This genus was associated with sulfide oxidation and the *T. denitrificans* was a well-known bacterium mediated sulfide-based autotrophic denitrification (Lens, 2009; Ma et al., 2020). Previous studies showed that the *Thiobacillus* genus would be enriched in sulfide(FeS)-based autotrophic denitrification (Yang et al., 2017). In HT season, *Anaerolineaceae_uncultured* (9.8 %) and *Bacillus* (7.1%) were both dominant genera. The *Anaerolineaceae_uncultured* and *Thiobacillus* were both ubiquitous and abundant in both autotrophic and heterotrophic denitrification (Huang et al., 2021). This genus could consume organic carbon, creating C-limiting conditions for autotrophic denitrification, and it could convert the complex organic compounds into small molecular organic matters, promoting heterotrophic denitrification. The *Bacillus* contained strains could mediate denitrification (Lu et al., 2012) and sulfur oxidation (Ryu et al., 2009). In the LT season, *Methylophilus* (11.8%) was the dominant genus followed by Bacteroidetes_vadinHA17_norank (10.2%). The *Methylophilus* was a heterotrophic bacterium whose role was expected as *Anaerolineaceae_uncultured* to create C-limiting conditions for autotrophic denitrification and to provide small organic compounds for heterotrophic denitrification. The Bacteroidetes_vadinHA17_norank was also heterotrophic bacteria commonly detected in anaerobic systems, which was also found in the denitrification system (Zhang et al., 2018). The presence of norank_f__Nitrosomonadaceae (AOB), *Nitrospira* (NOB), Acidobacteria_norank (SRB) showed the potential presence of ammonia oxidation (Prosser et al., 2014), nitrification (Daims et al., 2015), and dissimilatory sulfate reduction (Anantharaman et al., 2018) in the experiment, even though they are not observed. Similarly, the genus mediating anammox, *Candidatus Brocadia*, was observed at 0.06% in the microbial community. The genus *Desulfovibrio*, probably mediating DNRA, was also found in the microbial community (Su et al., 2020).

The abundance of heterotrophic NRB (LT: 21.59%; HT: 23.50%) and SOB (LT: 21.45%; HT: 25.86%) were higher than other functional groups. Besides, all the SOB genus listed in Figure 4 could mediate sulfide/sulfur-based autotrophic denitrification (Ma et al., 2020; Xing et al., 2017). This was in accordance with results from the batch experiments in which heterotrophic denitrification and sulfide-based autotrophic denitrification were observed, but other processes contributed little to carbon, nitrogen, and sulfur transformation.

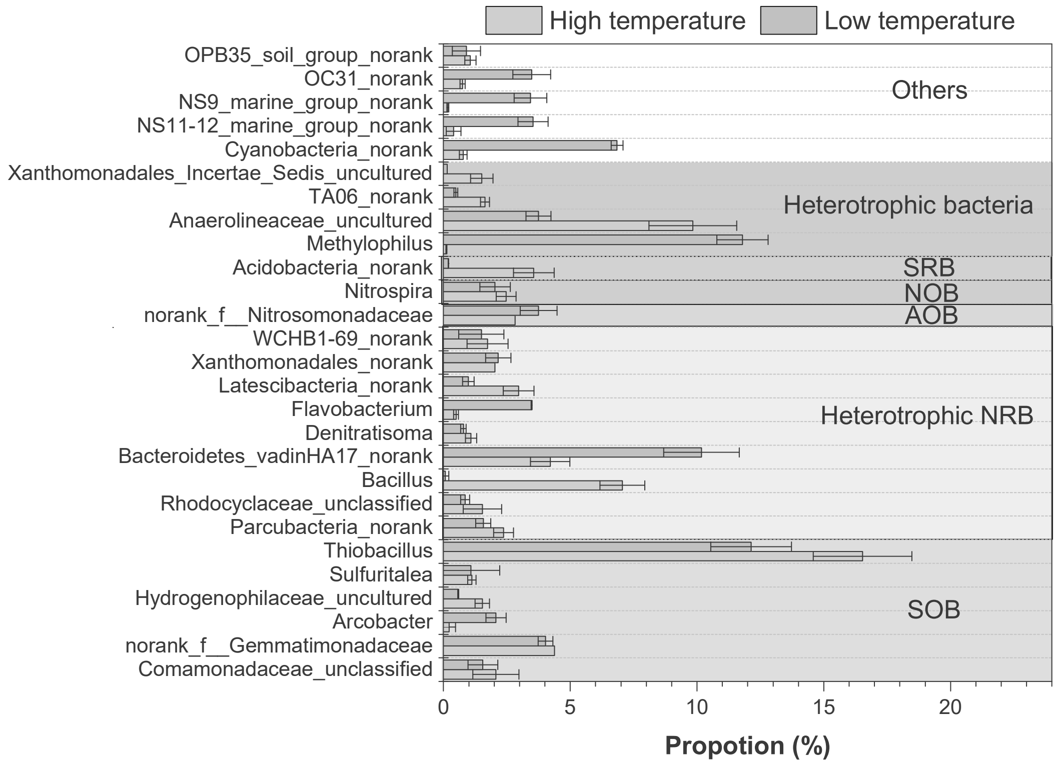


Figure 4. The relative abundance of dominant bacteria (>1%) in the microbial community at the Genus level. AOB=Ammonia-oxidizing bacteria, NOB=Nitrifying bacteria, NRB= denitrification bacteria (Nitrate-reducing bacteria), SOB= Sulfide-oxidizing bacteria, SRB=Sulfate-reducing bacteria. The "heterotrophic bacteria" referred to the bacteria mediated organic carbon degradation but not involved in

heterotrophic denitrification or dissimilatory SO_4^{2-} reduction. The bacteria in the square could mediate sulfide-based autotrophic denitrification.

The correlation between functional bacteria and the carbon, nitrogen, and sulfur transformation was further revealed by the Spearman's correlation coefficient (ρ, Table S4) and canonical correlation analysis (CCA, Figure 5). The results clearly showed that NO_3^- removal was closely related to TOC removal (ρ=0.786, p=0.04) and SO_4^{2-} generation (ρ=-0.643, p=0.12), and strongly correlated with NRB (ρ = 0.571, p=0.18). The correlation coefficient and CCA further confirmed that the presence of heterotrophic denitrification and sulfide-based autotrophic denitrification in the studied CW.

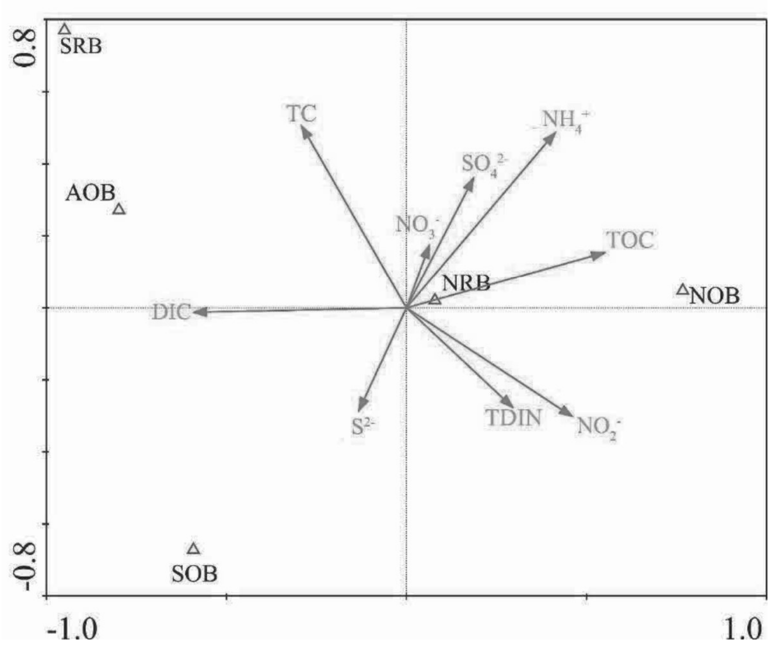


Figure 5. Canonical correlation analysis (CCA) plot of bacteria responding to the removal efficiency of different carbon, nitrogen, and sulfur compounds. The arrow is the transformation of carbon, nitrogen, and sulfur compounds. The length of the arrow represents the impact of the transformation on the system; the angles between arrows represent the correlation between the transformations; the vertical distance between the arrow and triangle is the correlation between

microorganisms and the transformation efficiency of carbon, nitrogen, and sulfur compounds.

5. Coupling transformation of carbon, nitrogen, and sulfur compounds in the long-term operated CW

Based on the results, the coupling transformation of carbon, nitrogen, and sulfur compounds in the long-term operated HSFW was shown in Figure 6. The batch experiment, electron balance analysis, as well as the microbial community analysis all showed that the main N removal pathway in the system was heterotrophic denitrification, coupling NO_3^- removal and TOC removal. At the same time, sulfur-based autotrophic denitrification was also present. The contribution of sulfur-based autotrophic denitrification to TN removal was about 15.2%, similar to the result from other studies ranging from 7% to 16% (Chen et al., 2016). This process coupled the reduction of NO_3^- to NO_2^-, and further to N_2, with the oxidation of S^{2-} to SO_4^{2-}. The bacteria mediating ammonia oxidation, nitrification, anammox, dissimilatory DNRA, and dissimilatory sulfate reduction were also found in the studied CW. However, the results of the batch experiment showed that these processes did not significantly contribute to carbon, nitrogen, and sulfur transformation.

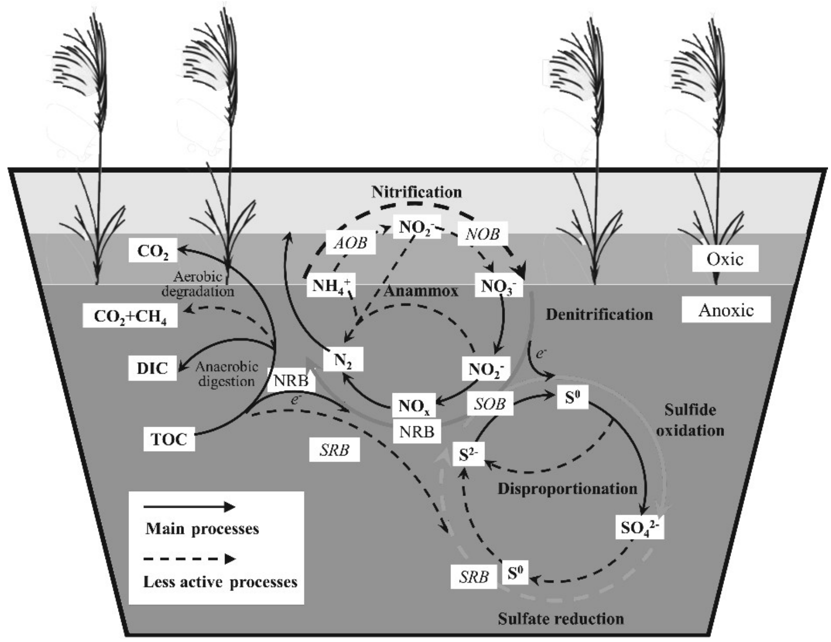


Figure 6: Key processes of carbon, nitrogen, and sulfur transformation in CW. The solid line represents the main processes for carbon, nitrogen, and sulfur transformation, the dashed line represents the processes that had little effects

6. Conclusion

In conclusion, the key processes of carbon, nitrogen, and sulfur transformation in the HFSW of the studied hybrid CW were denitrification. Based on the electron balance analysis, both heterotrophic denitrification and autotrophic denitrification were observed concurrently in the HFSW. The autotrophic denitrification used NO_3^- as the electron acceptor and S^{2-} as the electron donor. Increasing initial S^{2-} could promote the sulfur-based autotrophic denitrification. The heterotrophic denitrification was the main pathway for N removal, while the sulfur-based autotrophic denitrification was not inhibited by the heterotrophic denitrification. This research improved the understanding of microbial mechanisms behind the pollutant removal in the CWs in practice. The results of this study could help to understand the high N removal efficiency in CWs, and promote the understanding of potential carbon, nitrogen, and sulfur coupling transformation.

Acknowledgment

This research was financially supported by National Natural Science Foundation of China (No. 51878093). The authors appreciated the help from the staff in the wastewater treatment plants during the sampling.

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